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Fluorescence intensity and lifetime measurement of free and particle-bound fluorophore in a sample stream by phase-sensitive flow cytometry

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We report a novel method to quantify fluorescence intensity and lifetime of free (solution) and particle-bound fluorophore in a flow stream. The technique combines flow cytometry and frequency-domain lifetime spectroscopy principles to make unique fluorescence measurements on free fluorophore and fluorophore-labeled particles. Fluorophore-labeled microspheres suspended in a fluorophore solution are analyzed as they flow through a chamber and pass across an intensity-modulated laser beam consisting of a continuous-wave (cw) direct-current (dc) and high-frequency (sine wave) excitation component. Fluorescence emission signals consisting of a dc-offset steady-state sinusoidal signal (fluorophore solution) and a sinusoidally modulated Gaussian-shaped signal pulse (fluorophore-labeled particles) are processed electronically to quantify intensities and lifetimes. The cw-excited, particle-associated fluorophore (pulse) and steady-state (dc) solution fluorescence intensity signals are measured using low-pass filtering to remove the high-frequency signal components and an ac-coupled and a gated dc amplifier to process the respective particle-bound and free fluorophore signals. The high-frequency excited, particle-bound fluorophore and free fluorophore lifetimes are individually measured using two pairs of phase-sensitive detectors to provide signals proportional to the sine and cosine of the respective phase shifts, which are ratioed to determine the respective lifetimes. The fluorescence signal intensity and lifetime detection channel outputs are displayed as frequency distribution histograms using a computer-based data acquisition system. [S0034-6748(99)00612-7]

I. INTRODUCTION

Flow cytometry (FCM) instruments rapidly measure biochemical, functional, and physical properties of individual cells and subcellular components, e.g., chromosomes. They are used for clinical diagnostic medicine and in biological and biomedical research applications. These applications are primarily based on labeling cells with multiple color fluorochromes for correlated analysis of biomolecules, such as, DNA, RNA, protein, enzymes, calcium, lipids, and cell-surface receptors (human lymphocyte subsets). In addition to utilizing the color and intensity properties of the spectral emission from fluorophore-labeled cells, excited-state lifetimes provide a means to discriminate among fluorescent markers[1–3] and can serve as spectroscopic probes to study the interaction of the markers with their targets, each other, and the surrounding microenvironment.[4–8]

The direct measurement of excited-state lifetimes is important because it provides information about fluorophore/cell interactions at the molecular level. An advantage of lifetime measurements is that, in some instances, lifetimes can be considered as absolute quantities. However, the lifetimes of fluorophores bound to macromolecules can be influenced by physical and chemical factors near the binding site, such as solvent polarity, cations, *p*H, energy transfer, and excited-state reactions. Static spectroscopic fluorescence measurements of excited-state lifetimes are made in the time domain by time-correlated, single-photon counting, or in the frequency domain by determining the frequency response (phase and modulation) of the fluorescence emission to a set of continuous intensity-modulated excitation frequencies.[9,10] Time-domain methods have been employed to measure the lifetime of fluorophores bound to cells in fluorescence microscopy[11,12] and more recently in flow cytometry.[13] In the frequency-domain method, the fluorescence emission signal has the same frequency content as the excitation, but is shifted in phase and the depth of modulation is decreased due to the finite lifetimes of the excited states. There has been remarkable progress in frequency-domain spectrofluorometric developments during the past several years.[14–20] These developments have been applied to microscope-based cellular imaging systems[21–25] and to flow cytometers for measuring lifetimes of fluorophores bound to cells using real time analog[26,27] and digital signal[28–30] processing methods.

In this report we describe the theory and the instrumentation used to measure fluorescence intensity and lifetime of free and particle-bound fluorophores as they pass through a flow chamber and are excited using a single-frequency, sinusoidally modulated laser beam. Polystyrene latex microspheres labeled with the fluorophores fluorescein isothiocyanate (FITC) and propidium iodide (PI) and suspended in a solution of their respective fluorescent dyes were selected for these studies. Future work will involve viable and fixed cells labeled with fluorescent probes as biochemical and func-

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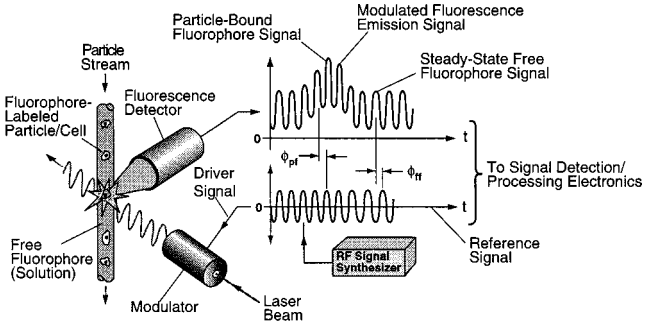

FIG. 1. Conceptual diagram illustrating the modulated laser excitation beam, particle sample stream consisting of free fluorophore and fluorophore-labeled particles, particle-stream laser-beam intersection point in the flow chamber (flow chamber not shown), fluorescence detector, modulated fluorescence signal consisting of particle-bound fluorophore and steady-state free fluorophore superimposed signals, reference signal for the phase-sensitive detectors, and rf signal synthesizer.

tional markers in biological applications involving cell labeling and free fluorophore interactions.

II. METHODS

A. Theory

Free fluorophore and particles labeled with fluorophore are analyzed as they intersect an optically focused cw laser beam which is intensity modulated using a high-frequency (ω) sine wave (see Fig. 1). The modulated laser excitation intensity $[E(t)]$ is expressed as

$$E(t)=E_0[1+m_{\mathrm{ex}}\sin(\omega t)], \tag{1}$$

where E_0 is the cw laser excitation intensity, m_{ex} is the excitation depth of modulation term, and t is time. The time-dependent fluorescence emission signal $[\nu(t)]$ consists of a steady-state sinusoidally modulated cw signal (free fluorophore in the sample stream), plus a Gaussian-shaped sinusoidally modulated signal pulse which results from the passage of the fluorophore-labeled particle across the laser beam. The modulated fluorescence emission signal can be expressed in an approximate form as

$$\nu(t)=V_{\mathrm{ff}}[1+m_{\mathrm{ff}}\sin(\omega t-\phi_{\mathrm{ff}})]+V_{\mathrm{pf}}[1+m_{\mathrm{pf}}\sin(\omega t-\phi_{\mathrm{pf}})]e^{-a^2(t-t_0)^2}, \tag{2}$$

where V_{ff}, V_{pf}, ϕ_{ff}, ϕ_{pf} and m_{ff}, m_{pf} are the respective free (ff) and particle-bound (pf) fluorophore signal intensities (V), phase shifts (ϕ) associated with single fluorescence decay times τ_{ff} and τ_{pf}, and corresponding relative fluorescence modulation terms (m) defined as m_{ex} times the demodulation factor due to the lifetime of the respective fluorophore,[31] and a is a term related to the laser beam height and to the velocity of a particle crossing the laser beam at time t_0.

1. Measurement of fluorescence signal intensities

The particle-bound fluorescence signal intensity $[\nu_{\mathrm{pf}}(t)]$ is obtained from the modulated fluorescence emission signal $\nu(t)$ of Eq. (2) by first inputting it to a low-pass filter to remove the high-frequency sinusoidal modulation components and pass the steady-state, cw-excited free fluorophore (dc) and particle-bound fluorophore (pulse) terms as illustrated in Fig. 2(a). The low-pass filter output $[\nu_{\mathrm{lp}}(t)]$ is expressed as

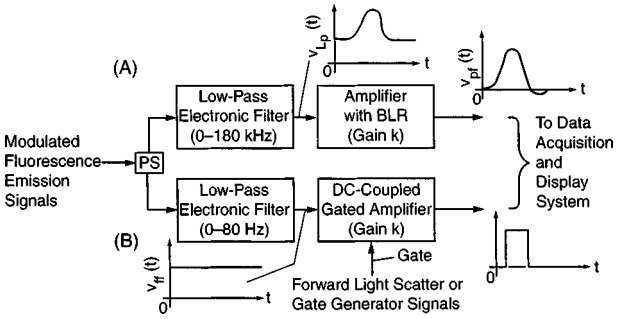

FIG. 2. Block diagram of the electrical signal processing electronics for detecting fluorescence intensities of particle-bound (A) and free (B) fluorophore.

$$\nu_{\mathrm{lp}}(t)=V_{\mathrm{ff}}+V_{\mathrm{pf}}\,e^{-a^2(t-t_0)^2}, \tag{3}$$

which is input to an ac-coupled amplifier to remove the free fluorophore dc component term V_{ff} and amplify (gain k) the particle-related fluorescence term given as

$$\nu_{\mathrm{pf}}(t)=kV_{\mathrm{pf}}\,e^{-a^2(t-t_0)^2}. \tag{4}$$

In a similar manner, the free fluorophore dc signal intensity in the sample stream is quantified by inputting the modulated fluorescence emission signal to a low-pass filter to remove the high-frequency sinusoidal modulation components and cw-excited particle-bound fluorophore (pulse) term of Eq. (2) and pass the steady-state, cw-excited free fluorophore dc term as illustrated in Fig. 2(b). The low-pass filtered steady-state free fluorophore output is expressed as

$$\nu_{\mathrm{ff}}(t)=V_{\mathrm{ff}}. \tag{5}$$

This signal is input to a dc-coupled gated amplifier (gain k), the output being a signal pulse whose amplitude is proportional to the free fluorophore fluorescence signal intensity.

2. Measurement of fluorescence lifetimes

The modulated fluorescence emission signal is input to a band-pass filter (center frequency $f=\omega/2\pi$) shown in Fig. 3. The band-pass filtered output is expressed as

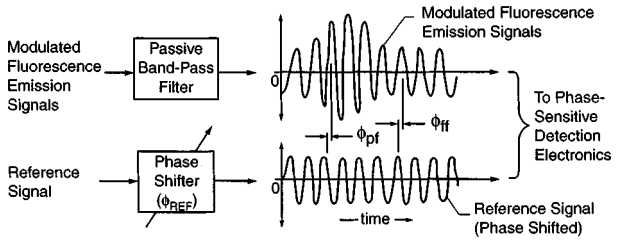

FIG. 3. Electrical diagram illustrating the band-pass filtering of modulated fluorescence emission signals and phase shifter used to shift the phase of the reference signal with respect to the band-pass filtered modulated fluorescence emission signal.

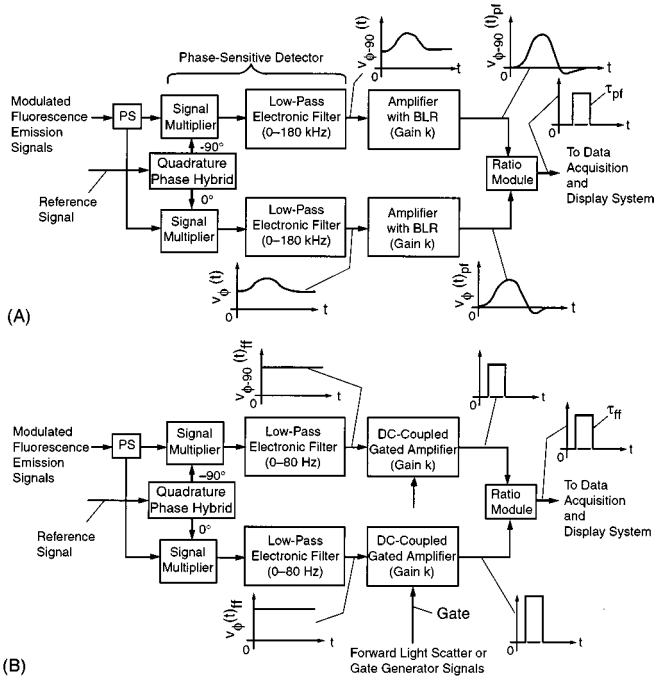

FIG. 4. Block diagram of the phase-sensitive detection signal processing electronics for quantifying fluorescence lifetimes of particle-bound (A) and free (B) fluorophore.

$$\nu_{\rm bp}(t)=V_{\rm ff}m_{\rm ff}\sin(\omega t-\phi_{\rm ff})+V_{\rm pf}m_{\rm pf}\sin(\omega t-\phi_{\rm pf})e^{-a^2(t-t_0)^2}. \tag{6}$$

The free and particle-bound fluorophore high-frequency signal components are shifted in phase by an amount

$$\phi_{\rm ff}=\arctan\omega\tau_{\rm ff} \quad \text{and} \quad \phi_{\rm pf}=\arctan\omega\tau_{\rm pf}, \tag{7}$$

and are processed by phase-sensitive detectors (PSDs)[32,33] consisting of analog multipliers and low-pass filters to quantify fluorescence lifetimes as illustrated in Fig. 4. A quadrature phase hybrid circuit is used to form two reference signals which are 90° out of phase with each other. The $-90°/0°$ reference signals are input to the PSDs along with the modulated fluorescence signal. A phase shifter is used to shift the phase (ϕ_R) of the reference signal with respect to the modulated fluorescence emission signal (see Fig. 3). The outputs of the PSDs for quantifying fluorescence lifetime of the particle-bound fluorophore are

$$\nu_{\phi-90}(t)=\tfrac{1}{2}m_{\rm ff}V_{\rm ff}\sin\phi_{\rm ff}+\tfrac{1}{2}m_{\rm pf}V_{\rm pf}\sin\phi_{\rm pf}e^{-a^2(t-t_0)^2}, \tag{8}$$

$$\nu_{\phi}(t)=\tfrac{1}{2}m_{\rm ff}V_{\rm ff}\cos\phi_{\rm ff}+\tfrac{1}{2}m_{\rm pf}V_{\rm pf}\cos\phi_{\rm pf}e^{-a^2(t-t_0)^2}, \tag{9}$$

as illustrated in Fig. 4(A). The pass-band of the low-pass filters is set just high enough to pass the cw-excited free (dc) and particle-bound fluorophore signal terms (pulse). The ac-coupled baseline restorer (BLR) in the amplifiers (gain k) removes the free fluorophore dc signal terms, leaving the particle-bound fluorophore terms of Eqs. (8) and (9) expressed as

$$\nu_{\phi-90}(t)_{\rm pf}=\frac{k}{2}m_{\rm pf}V_{\rm pf}\sin\phi_{\rm pf}e^{-a^2(t-t_0)^2},$$

$$\nu_{\phi}(t)_{\rm pf}=\frac{k}{2}m_{\rm pf}V_{\rm pf}\cos\phi_{\rm pf}e^{-a^2(t-t_0)^2}, \tag{10}$$

where $\phi_{\rm pf}$ is the particle-labeled fluorophore signal phase shift [see Eq. (7)]. These signals are input to a ratio module, the output $[\nu_{\phi-90}(t)_{\rm pf}/\nu_{\phi}(t)_{\rm pf}]$ which results in the $\tan\phi_{\rm pf}$[33] which is directly proportional to the fluorescence decay time of the particle-bound fluorophore expressed as

$$\tau_{\rm pf}=\frac{1}{\omega}\tan\phi_{\rm pf}=\frac{1}{\omega}[V(\phi-90)_{\rm pf}/V(\phi)_{\rm pf}]. \tag{11}$$

Similarly, the fluorescence lifetime of the free fluorophore is quantified as illustrated in Fig. 4(B). The pass-band of the low pass filters in the PSDs is set low enough to completely block the particle-bound fluorophore (pulse) terms and pass the steady-state dc terms associated with the free fluorophore of Eqs. (8) and (9), which can be expressed as

$$\nu_{\phi-90}(t)_{\rm ff}=\frac{1}{2}m_{\rm ff}V_{\rm ff}\sin\phi_{\rm ff}, \quad \nu_{\phi}(t)_{\rm ff}=\frac{1}{2}m_{\rm ff}V_{\rm ff}\cos\phi_{\rm ff}, \tag{12}$$

where $\phi_{\rm ff}$ is the signal phase shift [see Eq. (7)]. The PSD outputs are amplified using gated amplifiers and input to a ratio module. The $\nu_{\phi-90}(t)_{\rm ff}/\nu_{\phi}(t)_{\rm ff}$ ratio expression results in the $\tan\phi_{\rm ff}$ which is directly proportional to the fluorescence decay time of the free fluorophore expressed as

$$\tau_{\rm ff}=\frac{1}{\omega}\tan\phi_{\rm ff}=\frac{1}{\omega}[V_{\rm ff}(\phi-90)/V_{\rm ff}(\phi)]. \tag{13}$$

The above lifetime equations are derived for a single-component exponential decay of fluorescence from homogeneous emitting fluorophore populations. Measurement by time-resolved[13] or by multifrequency[34,35] methods, i.e., over a wide range of frequencies, is required to accurately characterize multiexponential decays.

B. Instrumentation

A Spectra Physics model 2025 (5W all lines visible) argon laser operating at 488 nm wavelength (800 mW) served as the excitation source and the modulation system was a Conoptics model 50 dc 50 MHz bandwidth driver unit with a model 350 KDP* electro-optic modulator as previously described[27] and illustrated in Fig. 1. A Hewlett Packard model 3335A RF signal synthesizer was used as the sine wave generator for the modulator drive electronics and as the reference frequency source for homodyne signal detection.[32] The modulation frequency was set at 29 and 10 MHz, respectively, for FITC and PI measurements described below. The modulation frequencies were selected based on FITC and PI phase shifts to approximate 45° for maximum measurement sensitivity and the availability of band-pass filters. Since a 29 MHz band-pass filter was the highest available, 29 MHz was selected as the modulation frequency for FITC measurements. The modulated laser beam was focused by a pair of crossed cylindrical lenses of focal length 30 and 5.4

cm into an elliptical shape onto the sample stream in a Beckman–Coulter Corp. Biosense flow chamber having a 200-μm-sq. (internal cross section) flow channel. Fluorophore-labeled particles suspended in phosphate buffered saline (PBS) or in PBS containing fluorophore solution were passed through the chamber at rates of a few hundred per second. The velocity of particles crossing the focused laser beam resulted in Gaussian-shaped light scatter and fluorescence signals 12–15 μs in duration, which corresponds to an approximate measured bandwidth of 160–170 kHz. Therefore, the low-pass filters used in the intensity and lifetime measurements below were set at 180 kHz bandwidth to pass signals with minimal distortion. If the particle flow velocity were to be increased, i.e., shorter signal pulse widths, then the passband of the low-pass filtering will in turn have to be increased. The sample stream diameter was 20–40 μm and it was surrounded by a particle-free PBS sheath fluid as they both passed through the chamber.

The fluorescence detector consisted of an f/0.95 CCTV lens which collected and collimated the modulated fluorescence emission to a second f/0.95 lens which focused the emitted light onto a 100-μm-diam pinhole spatial filter located in front of a photomultiplier tube (PMT). A long-pass optical (barrier) filter located between the two lenses functioned to pass fluorescence emission and block 488 nm scattered laser excitation light. A Melles Griot OG-515 and RG-610 long-pass filter were used in the fluorescence detector for FITC and PI fluorescence intensity and lifetime measurements, respectively, as described below. A Burle Industries model 4526 PMT/Comlinear model 401 high-speed operational amplifier configured in the transimpedance mode served as the photodetector. The modulated fluorescence emission preamplifier signals were electronically buffered and input to a signal splitters for low-pass filtering (two channels) to measure the fluorescence signal intensities of the particle-bound and free fluorophore (see Fig. 2). Forward scattered light from particles was focused and directed by lens onto the photocathode of a PMT detector (not shown in Fig. 1). The light scatter signals were demodulated using a Krohn–Hite model 3202 low-pass electronic filter set at 180 kHz to obtain the cw-excited laser excitation light scatter signal, which was used to trigger computer data acquisition of fluorophore-labeled particles suspended in PBS or fluorophore solution. Data acquisition was triggered by an external pulse (gate) generator for acquiring histogram data on free fluorophore alone in solution.

The particle-bound fluorophore signal intensity was obtained by inputting the modulated fluorescence emission signal to Krohn–Hite model 3202 low-pass electronic filter set at 180 kHz [see Fig. 2(A)], which rejects the high-frequency signal components of Eq. (2) and passes the low-frequency free (dc) and particle-bound fluorophore superimposed signals of Eq. (3). The superimposed free and particle-bound fluorophore signals are input to an amplifier which contains an ac-coupled BLR circuit[36] to remove/block the free fluorophore dc signal component and amplify (gain k) the particle-bound fluorophore signal as shown in Fig. 2(A). In a similar manner, the free fluorophore signal intensity in the particle stream was quantified by inputting the modulated fluorescence emission signal to a Krohn–Hite model 3202 low-pass electronic filter set at 80 Hz [see Fig. 2(B)], which effectively removes both the high-frequency signal component and the particle-bound fluorophore signal pulse and leaves only the free fluorophore dc signal. This in turn greatly improves the dc measurement precision by reducing the effects of small fluctuations in the sample stream and random background noise. The free dc fluorophore signal output of the low-pass filter is input to a dc-coupled gated amplifier (gain k), the output being a rectangular pulse whose amplitude was proportional to the free fluorophore signal intensity. Both the particle-bound and free fluorophore rectangular signal pulses were input to a computerized data acquisition system[37] for storing as list mode data and displaying as frequency distribution histograms.

The band-pass filtered modulated fluorescence emission and sine wave reference signals (see Fig. 3) were routed to the signal detection/processing electronics for quantifying fluorescence lifetimes (see Fig. 4). The two-phase ratio detector circuit (phase comparator) for making particle-bound fluorophore fluorescence lifetime measurements is shown in Fig. 4(A). A signal power splitter is used to divide the band-pass filtered, high-frequency fluorescence emission signals for input to two PSD circuits consisting of Mini-Circuits model ZRPD-1 multipliers and Krohn–Hite model 3202 low-pass filters set at 180 kHz. An Allen Avionics model V127050 (0–127 ns) switchable delay line is used to shift the phase of the reference signal with respect to the input fluorescence signals (see Fig. 3). The phase-shifted reference signal is input to an Anzac model JH-6-4 (2–32 MHz) quadrature phase hybrid module to supply two reference signals that are 90° out of phase with each other to the PSD circuits for generating sine and cosine outputs $\nu_{\phi-90}(t)_{\mathrm{pf}}$ and $\nu_{\phi}(t)_{\mathrm{pf}}$, respectively. The PSD outputs are amplified with an amplifier (gain k) having a BLR and input to an analog ratio module for determining particle-bound fluorophore lifetimes.

In a similar manner, the fluorescence lifetime of the free fluorophore in the sample stream was measured by inputting the band-pass filtered modulated fluorescence emission signals to two PSD circuits consisting of Mini-Circuits ZRPD-1 multipliers and Krohn–Hite 3202 low-pass filters set at 80 Hz [see Fig. 4(B)]. A model JH-6-4 Anzac quadrature phase hybrid module is used to supply the multipliers with two phase-shifted sine wave reference signals which are 90° out of phase with each other as described above. The two PSD dc level sine and cosine signals are each input to dc-coupled gated amplifiers (gain k), the outputs which are input to a ratio module for determining the lifetime of the fluorophore in solution. The particle-bound and free fluorophore lifetime signals were recorded as list mode data for display as frequency distribution histograms using the computer-based data acquisition system.

The longest lifetime which can presently be measured depends on the lowest usable excitation frequency, which is about 0.5 MHz. This corresponds to a 318 ns lifetime calculated at a 45° phase shift, but in practice will be somewhat higher in value. For modulation frequencies lower than 0.5 MHz, the cw-excited, low-frequency signal component interferes with the 0.5 MHz high-frequency signal. The shortest

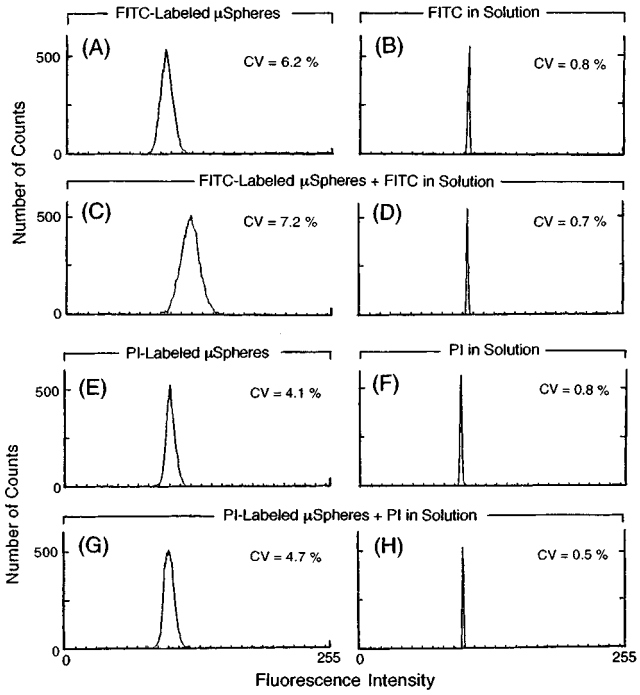

FIG. 5. Fluorescence intensity frequency distribution histograms recorded on FITC-labeled microspheres (μSpheres) suspended in PBS (A); FITC in solution (PBS) (B); FITC-labeled μSpheres suspended in FITC solution (C); FITC in solution containing μSpheres (D); PI-labeled μSpheres suspended in PBS (E); PI in solution (F); PI-labeled μSpheres suspended in PI solution (G); and PI in solution containing μSpheres (H).

measurable lifetime depends on the maximum highest modulation frequency usable and the bandwidth of the signal detection/processing electronics and data acquisition system. Lifetime measurement capabilities by flow cytometry of a few tenths of nanoseconds have been reported.[26,27]

C. Fluorescence lifetime calibration

Fluorescence lifetime measurements of particle-bound and free fluorophore were made by first removing the long-pass barrier filter in the fluorescence detector and adjusting the phase shift of the reference signal (ϕ_R) to null (zero) the $\nu_{\phi-90}(t)$ PSD sine output signal using 7.0-μm-diam nonfluorescent microspheres.[27] The long-pass filter was then replaced and Beckman–Coulter Corp. DNA Check alignment fluorospheres (lifetime 7 ns) were analyzed at the same reference signal phase shift and PMT/amplifier gain settings as used on the nonfluorescent microphores. The gain of ratio module was adjusted to align the modal channel histogram values in channels 70 and 140 for calibration of the horizontal axis of the histogram channel number, which corresponds to 7 ns, for the analysis of particle-bound and free PI and FITC, respectively.

III. RESULTS AND DISCUSSION

The fluorescence intensity measurement of particle-bound and free FITC and PI is illustrated in Fig. 5. Figure 5(A) shows the fluorescence frequency distribution histogram recorded on 7.4-μm-diam flow cytometry standards polystyrene latex microspheres labeled with 1.24×10^5 molecules equivalence of soluble FITC and suspended in PBS. The histogram is somewhat broad, having a coefficient of variation: standard deviation divided by the mean (CV) equal to 6.2%. The broadness is due to nonuniformity in microsphere size, FITC labeling variation and low-level photon measurement statistics. The minimum detection level is 300–400 molecules equivalence of FITC as measured by this type of flow cytometric instrumentation.[27] Figure 5(B) shows the fluorescence intensity histogram recorded on FITC alone in solution (20 ng/ml of FITC in PBS) flowing through the chamber with only a very slight intensity variation (CV $=$0.8%). The 20 ng/ml amount of FITC flowing in the sample stream is the level needed to approximately equal the FITC labeling intensity of the microspheres. The particle-bound FITC fluorescence intensity histogram recorded on FITC-labeled microspheres suspended in a FITC-PBS solution containing 20 ng/ml of FITC is shown in Fig. 5(C). The histogram CV of the FITC labeled microspheres is slightly broader (7.2%) than when the FITC-labeled microspheres were analyzed in PBS alone. The slight broadening of the CV and increase in intensity of the histogram is possibly due to additional nonuniform labeling of the microspheres surfaces by excess FITC molecules in solution. The fluorescence intensity histogram of FITC (20 ng/ml) in PBS containing FITC-labeled microspheres shown in Fig. 5(D) is nearly identical to the histogram of Fig. 5(B) (FITC alone in solution).

The fluorescence intensity histogram recorded on 8.8-μm-diam flow cytometry standards microspheres labeled with PI is fairly uniform with a CV of 4.1% [see Fig. 5(E)]. The fluorescence intensity histogram recorded on PI alone in solution (200 μg/ml of PI in PBS) flowing through the chamber shows only a slight variation in fluorescence intensity (CV$=$0.8%) as shown in Fig. 5(F) and is similar to the fluorescence intensity measurement for FITC in solution alone. The reason for the high concentration of free PI (200 μg/ml) is that PI in solution has a very low quantum efficiency, however, when PI is bound to microspheres or to DNA by intercalation, the quantum efficiency increases by more than 100 fold. The particle-bound PI fluorescence intensity histograms recorded on PI-labeled microspheres suspended in PBS containing PI (200 μg/ml) is shown in Fig. 5(G). The histogram CV of the PI-labeled microspheres (4.7%) is approximately the same as the PI-labeled microspheres analyzed in PBS alone [see Fig. 5(E)]. The fluorescence intensity histogram CV of 200 μg/ml of PI (0.5%) in a PBS solution containing PI-labeled microspheres shown in Fig. 5(H) is nearly identical to the histogram CV for PI alone in solution [see Fig. 5(F)].

The fluorescence lifetime measurement of particle-bound and free PI is illustrated in Fig. 6. Figure 6(A) shows the fluorescence lifetime frequency distribution histogram recorded on PI-labeled microspheres suspended in PBS, having a peak modal channel lifetime value of 12.0 nanoseconds (ns) and a CV of 2.3%. In Fig. 6(B), PI alone in solution (200 μg/ml) was analyzed at the same fluorescence intensity level as the PI-labeled microspheres with a peak modal channel lifetime value of 1.3 ns. The CV was somewhat higher (3.3%) than the PI-labeled microspheres alone. These lifetime values are in close agreement to that previously

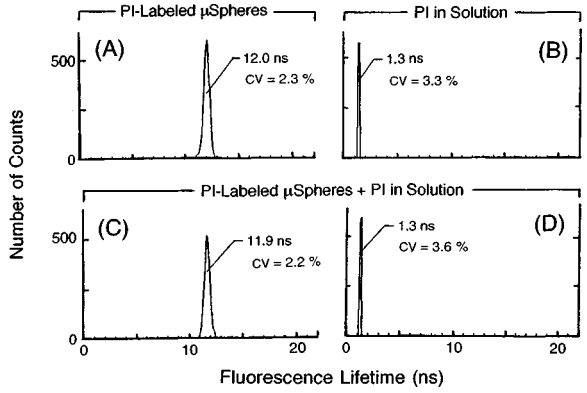

FIG. 6. Fluorescence lifetime frequency distribution histograms recorded on PI-labeled microspheres (μSpheres) suspended in PBS (A); PI in solution (PBS) (B); PI-labeled μSpheres suspended in PI solution (C); and PI in solution containing μSpheres (D).

reported[27] for free PI and PI bound to DNA in cells. The higher lifetime solution measurement CV is primarily due to the very low quantum efficiency of PI in solution. The lifetime measurement of PI-labeled microspheres suspended in a solution of PI is illustrated in Fig. 6(C). The lifetime of 11.9 ns and a CV of 2.2% are essentially the same as that recorded on PI-labeled microspheres alone suspended in PBS [see Fig. 6(A)]. Similarly, the lifetime of 1.3 ns and a CV of 3.6% measured on PI in solution containing PI-labeled microspheres [see Fig. 6(D)] is approximately the same as that recorded for PI in solution alone [see Fig. 6(B)].

Figure 7 shows the fluorescence lifetime histograms measured particle-bound and free FITC in solution. The lifetime frequency distribution histogram on FITC-labeled microspheres suspended in PBS shown in Fig. 7(A) has a peak modal channel lifetime of 3.9 ns and a CV of 5.9%, and is in close agreement with previous measurements.[38] In Fig. 7(B), FITC in solution (20 ng/ml) was analyzed at the same fluorescence intensity level as the FITC-labeled microspheres. The value of the fluorescence lifetime (4.4 ns) was slightly greater than the value for the labeled microspheres (3.9 ns), however, the CV of the solution measurement (0.7%) was considerably less than that recorded for the labeled particles (5.9%). This was due to being able to electronically filter out random noise and intensity fluctuations in the phase-sensitive

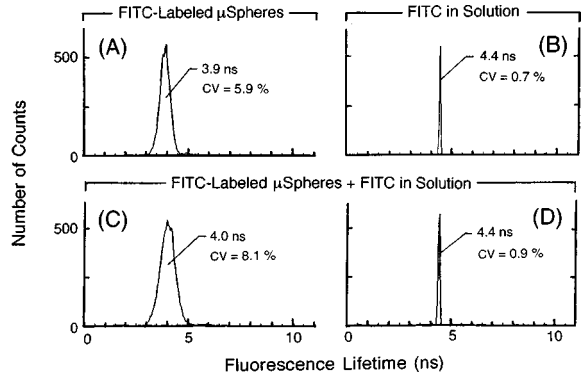

FIG. 7. Fluorescence lifetime frequency distribution histograms recorded on FITC-labeled microspheres (μSpheres) suspended in PBS (A); FITC in solution (PBS) (B); FITC-labeled μSpheres suspended in FITC solution (C); and FITC in solution containing μSpheres (D).

measurement by lowering the passband (80 Hz) of the low-pass filters in the PSDs, compared to the 180 kHz passband of the PSDs used in particle fluorescence lifetime measurements. The lifetime measurement of FITC-labeled microspheres in a solution containing FITC is illustrated in Fig. 7(C). The lifetime (4.0 ns) and CV (8.1%) are slightly larger than that recorded on FITC-labeled microspheres suspended in PBS alone [see Fig. 7(A)]. Similarly, the lifetime (4.4 ns) and CV (0.9%) measured on free FITC in a mixture of labeled particles in an FITC solution illustrated in Fig. 7(D) are essentially the same as that recorded on FITC in PBS with no particles present [see Fig. 7(B)].

The similarity of peak modal channel values and the broadness (CV) of the fluorescence lifetime histogram for the FITC-labeled microspheres suggests that it is not possible to discriminate free and bound FITC based on fluorescence lifetimes, whereas free and bound PI are readily separable based on their lifetime differences. However, in the case of the quenching of FITC labeling on the surfaces of microspheres[38] and on murine thymus cells labeled with antibody conjugated to FITC at different labeling dilutions and fluorescence-to-protein ratios,[4] free FITC in solution and that bound the microspheres and thymus cell surfaces may very well be discriminated based on lifetime differences. The sensitivity of the technology to discriminate the binding of fluorescent probes with respect to unbound fluorophore will depend upon measured average lifetime values (differences) and the broadness of the corresponding CVs.

In summary, we have developed a novel method to quantify fluorescence intensity and lifetime of free and particle-bound fluorophores in a sample stream. The technology, which combines flow cytometry and phase-sensitive measurement principles to provide unique features to make intensity and excited-state lifetime measurements of free and particle-bound fluorophore, adds a new dimension to the analysis of fluorophore-labeled particles and cells in a sample stream containing the same or a different fluorophore. In addition to measuring the fluorescence lifetime of particle-bound and free fluorophore by phase-sensitive detection, the corresponding signal intensities can be measured by taking the square root of the sum of the square of the $\nu_{\phi-90}(t)$ and $\nu_{\phi}(t)$ respective PSD outputs,[33] respectively, of Eqs. (10) and (12). Future studies are planned which will employ these and other fluorophores to study cell membrane and intracellular fluorophore labeling interactions with respect to the unbound fluorophore in the surrounding environment and the sample stream. Of particular interest is the potential capability of the lifetime-based sensing technology to monitor the uptake of cytotoxic anticancer drugs in cells[39] and blood[40] and thus be able to discriminate bound and unbound drugs. Additionally, antibody binding and ligand/membrane receptor interactions on the cell surface can be studied.

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[1] J. A. Steinkamp and H. A. Crissman, Cytometry **14**, 210 (1993).
[2] J. F. Keij, C. Bell-Prince, and J. A. Steinkamp, Cytometry **35**, 48 (1999).
[3] J. A. Steinkamp, B. E. Lehnert, and N. M. Lehnert, Journal of Immunological Methods **226**, 59 (1999).
[4] C. Deka, B. E. Lehnert, N. M. Lehnert, G. M. Jones L. A. Sklar, and J. A. Steinkamp, Cytometry **25**, 271 (1996).
[5] B. L. Sailer, A. J. Nastasi, J. G. Valdez, J. A. Steinkamp, and H. A. Crissman, Cytometry **25**, 164 (1996).
[6] B. L. Sailer, A. J. Nastasi, J. G. Valdez, J. A. Steinkamp, and H. A. Crissman, J. Histochem. Cytochem. **45**, 165 (1997).
[7] B. L. Sailer, J. G. Valdez, J. A. Steinkamp, and H. A. Crissman, Cytometry **31**, 208 (1998).
[8] B. L. Sailer, J. A. Steinkamp, and H. A. Crissman, European Journal of Histochemistry **42**, 19 (1998).
[9] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy* (Plenum, New York, 1983).
[10] D. V. O'Connor and D. Phillips, *Time-Correlated Single Photon Counting* (Academic, New York, 1984).
[11] J. Vigo, J. M. Salmon, and P. Viallet, Rev. Sci. Instrum. **58**, 1433 (1987).
[12] S. M. Keating and T. G. Wensel, Biophys. J. **56**, 186 (1991).
[13] C. Deka and J. A. Steinkamp, Appl. Opt. **35**, 4481 (1996).
[14] E. Gratton and M. Limkeman, Biophys. J. **44**, 315 (1983).
[15] J. R. Lakowicz and B. P. Maliwal, Biophys. Chem. **21**, 61 (1985).
[16] E. Gratton, D. M. Jameson, N. Rosato, and G. Weber, Rev. Sci. Instrum. **55**, 486 (1984).
[17] G. Mitchell and K. Swift K, Proc. SPIE **1204**, 270 (1989).
[18] G. Laczko, I. Gryczynski, Z. Gryczynski, W. Wiczk, H. Malak, and J. R. Lakowicz, Rev. Sci. Instrum. **61**, 2331 (1990).
[19] K. W. Berndt, I. Gryczynski, and J. R. Lakowicz, Rev. Sci. Instrum. **61**, 1816 (1990).
[20] R. B. Thompson, J. K. Frisoli, and J. R. Lakowicz, Anal. Chem. **64**, 2075 (1992).
[21] J. R. Lakowicz, H. Szmacinski, K. K. Nowaczy, K. W. Berndt, and M. Johnson, Anal. Bio. Chem. **202**, 316 (1992).
[22] T. W. J. Gadella, T. M. Jovin, and R. M. Clegg, Biophys. Chem. **48**, 221 (1993).
[23] T. Oida, Y. Sako, and A. Kusmi, Biophys. J. **64**, 676 (1993).
[24] P. T. C. So, T. French, and E. Gratton, Proc. SPIE **2137**, 83 (1994).
[25] T. French, P. T. So, C. Y. Dong, K. M. Berland, and E. Gratton, Methods in Cell Biology **56**, 277 (1998).
[26] B. G. Pinsky, J. J. Ladasky, J. R. Lakowicz, K. Berndt, and R. A. Hoffman, Cytometry **14**, 123 (1993).
[27] J. A. Steinkamp, T. M. Yoshida, and J. C. Martin, Rev. Sci. Instrum. **64**, 3440 (1993).
[28] C. Deka, L. A. Sklar, and J. A. Steinkamp, Cytometry **17**, 94 (1994).
[29] W. Beisker and A. Klocke, Proc. SPIE **2982**, 4361 (1997).
[30] G. Durack, W. Yu, W. Mantulin, and E. Gratton, Cytometry **Suppl. 9**, 39 (1998).
[31] R. D. Spencer and G. Weber, Ann. (N.Y.) Acad. Sci. **158**, 361 (1969).
[32] D. P. Blair and P. H. Sydenham, J. Phys. E **8**, 621 (1975).
[33] M. L. Meade, J. Phys. E **15**, 395 (1982).
[34] D. M. Jameson, E. Gratton, and R. D. Hall, Appl. Spectrosc. Rev. **20**, 55 (1984).
[35] J. R. Lakowicz, G. Laczko, H. Cherek, E. Gratton, and M. Limkeman, Biophys. J. **46**, 463 (1984).
[36] L. B. Robinson, Rev. Sci. Instrum. **32**, 1057 (1961).
[37] R. D. Hiebert, J. H. Jett, and G. C. Salzman, Cytometry **1**, 337 (1981).
[38] J. A. Steinkamp, C. Deka, B. E. Lehnert, and H. A. Crissman, Proc. SPIE **2678**, 221 (1996).
[39] B. L. Sailer, J. G. Valdez, J. A. Steinkamp, Z. Darzynkiewicz, and H. A. Crissman, Exp. Cell Res. **236**, 259 (1997).
[40] T. G. Burke, H. Malak, I. Gryczynski, Z. Mi, and J. R. Lakowicz, Anal. Biochem. **242**, 266 (1996).