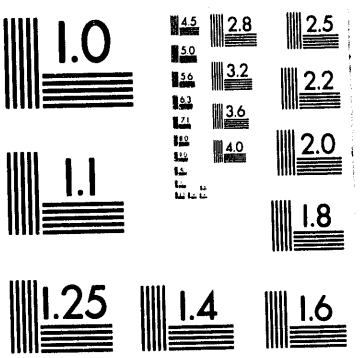
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Phase-Sensitive Flow Cytometry: New Technology For Analyzing Biochemical, Functional, and Structural Features in Fluorochrome-Labeled Cells/Particles

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ABSTRACT

A phase-sensitive flow cytometer has been developed that provides unique capabilities for making laser-excited, phase-resolved measurements on fluorochrome-labeled cells and particles.

Phase-Sensitive Flow Cytometry: New Technology For Analyzing Biochemical, Functional, and Structural Features in Fluorochrome-Labeled Cells/Particles.

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SUMMARY

Flow cytometry (FCM) instruments rapidly measure biochemical, functional, and cytological properties of individual cells and macromolecular components, e.g., chromosomes, for clinical diagnostic medicine and biomedical and environmental research applications. These measurements are based on labeling cells with multiple fluorochromes for correlated analysis of macromolecules, such as, DNA, RNA, protein, and cell-surface receptors. In addition to utilizing the spectral emission properties of fluorescent markers, i.e., different colors/intensities, to measure specific cellular features, the excited state lifetimes also can provide a means to discriminate among the different fluorochromes. A new FCM approach, based on phase-resolved fluorescence lifetime spectroscopy methods (Vesoelova *et al*, 1970, Lakowicz and Cherek, 1981), recently has been developed to provide unique capabilities for separating signals from multiple overlapping emissions in fluorochrome-labeled cells as they pass across a modulated laser excitation source (Steinkamp and Crissman, 1993). In addition, the measurement of fluorescence lifetime (Pinsky *et al*, 1993, Steinkamp *et al*, 1993) also is of importance because it provides information about fluorophore/cell interactions. An important advantage of lifetime measurements is that lifetimes in some case can be considered as absolute quantities. However, the lifetime of fluorophores bound to cellular macromolecules can be influenced by physical and chemical factors near the binding site, such as solvent polarity, cations, pH, energy transfer, excited-state reactions, and quenching. Thus, lifetime measurements can be used to probe the cellular environment, possibly including chemical and structure changes that occur in DNA and chromatin. Table I lists the lifetimes of some typical fluorochromes that are used to quantify cellular DNA, total protein, and antibody-labeling to cellular antigens.

The phase-sensitive flow cytometer is a novel instrument that combines flow cytometry and fluorescence lifetime frequency-domain spectroscopy principles to resolve emission signals from multiple fluorescent probes based on differences in their lifetimes and to measure lifetimes directly. Fluorochrome-labeled cells are analyzed as they intersect an intensity-modulated laser excitation beam consisting of a dc and a high-frequency (sinusoidal) excitation component (see Fig. 1). Fluorescence is measured orthogonal to the laser-beam cell-stream intersection point using a single detector consisting of a collection lens, a longpass barrier filter, and a photomultiplier tube. The fluorescence signals which are shifted in phase (ϕ) relative to the excitation frequency and amplitude

TABLE I

FLUORESCENT DYE/COMPOUND	EXCITATION WAVELENGTH (nm)	FLUORESCENCE LIFETIME (ns)	PHASE SHIFT* AT 10 MHz (degrees)
Hoechst 33258	uv	3.5	12.4
DAPI (DNA)	uv	~4.0	14.0
Mithramycin	420	3.0	10.7
Propidium Iodide	515	1.2	4.3
Propidium Iodide (Cells)	515	13.0	39.2
Ethidium Bromide	515	1.8	6.5
Ethidium Bromide (Cells)	515	19.0	50.0
Ethidium Bromide (DNA)	515	22.5	54.6
Acridine Orange (Cells)	480	3.0 (Green)	10.7
Acridine Orange (Cells)	480	13.0 (Red)	39.2
Fluorescein	480	4.7	16.4
FITC	480	3.6	12.5
Texas Red-Avidin	530	4.6	16.1
Phycoerythrin-Avidin	530	3.5	12.6

*Phase shift equals arctan $\omega\tau$, where $\omega = 2\pi f$ the angular frequency and τ = fluorescence lifetime

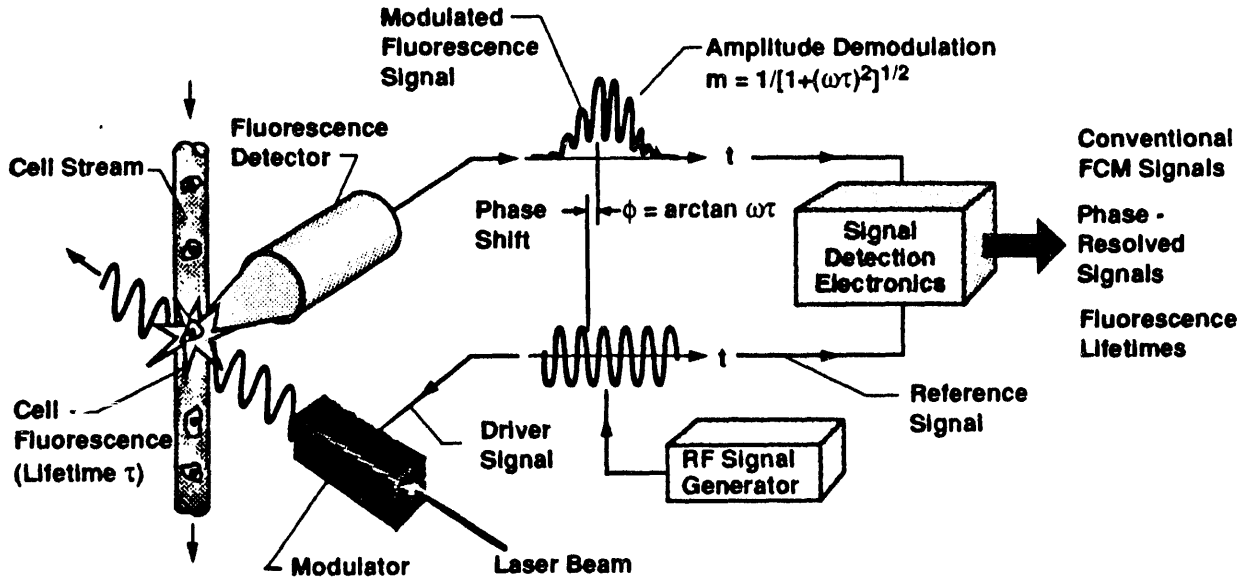

Figure 1. Conceptual diagram of the phase-sensitive flow cytometer illustrating the laser excitation source, modulator, RF signal generator, laser beam-cell stream intersection point in flow chamber (flow chamber not shown), fluorescence detector, and signal detection/processing electronics. The fluorescence signal phase shift (ϕ) with respect to the reference signal is equal to the arctan $\omega\tau$, where ω is the angular frequency ($2\pi\phi$) and τ is the fluorescence lifetime.

demodulated, are processed by phase-sensitive signal-detection electronics to resolve signals from heterogeneous fluorescence emissions resulting from differences in their lifetimes and quantify fluorescence lifetimes directly.

The time-dependent fluorescence emission signal [v(t)] is a high-frequency, Gaussian-shaped, modulated pulse that results from the passage of the cell across the focused laser beam and it can be expressed as:

$$v(t) = V[1 + m\cos(\omega t - \phi)] \cdot e^{-a^2(t-t_0)^2}, \qquad (1)$$

where V is the signal intensity, ω is the angular excitation frequency, ϕ and m are the respective signal phase shift and demodulation terms associated with a single fluorescence decay time (t), t is time, and a is a term related to the velocity of a cell crossing the laser beam at time t_0. This equation is derived for cells that are excited by an excitation source with a 100% depth of modulation. A more general expression will take into account the laser excitation depth of modulation factor (m_{ex}) which will reduce the high-frequency signal amplitude term. The cw-excited dc signal component is extracted using a low-pass filter to give conventional fluorescence intensity information. The high-frequency signal component, which is shifted in phase (ϕ) by an amount

$$\phi = \arctan \omega\tau \qquad (2)$$

relative to the excitation frequency and demodulated by a factor (m), where

$$m = 1/(1 + (\omega\tau)^2)^{1/2}, \qquad (3)$$

is processed by a phase-sensitive detector (PSD) consisting of a multiplier and a low-pass filter. A phase shifter is used to shift the phase (ϕ_R) of the reference signal input to the multiplier with respect to modulated fluorescence signal. The PSD output is a Gaussian-shaped signal that is proportional to fluorescence intensity, the demodulation factor, and the cos ($\phi - \phi_R$) expressed as:

$$v_o(t) = \frac{1}{2} mV\cos(\phi - \phi_R) \cdot e^{-a^2(t-t_0)^2}. \qquad (4)$$

The output intensity can thus be made to vary from both positive and negative values depending upon the sign of the $\phi - \phi_R$ phase shift term.

The principle of phase suppression, as applied to flow, for separating two fluorescence signals having different emission decay times, i.e., phase shifts, by phase-sensitive detection, is illustrated

below. The output of the PSD is expressed (by superposition) as:

$$v_o(t) = \frac{1}{2} m_1 V_1 \cos(\phi_1 - \phi_R) \cdot e^{-a^2(t-t_0)^2} + \frac{1}{2} m_2 V_2 \cos(\phi_2 - \phi_R) \cdot e^{-a^2(t-t_0)^2}, \quad (5)$$

where V_1 and V_2 are the signal intensities, m_1 and m_2 are the demodulation factors, and ϕ_1 and ϕ_2 are the phase shifts that result when a cell stained with two fluorochromes, each having a different lifetime τ_1 and τ_2, is excited with a modulated excitation source. To resolve either of the two signals the reference phase is shifted by an amount $\pi/2 + \phi_1$ or $-\pi/2 + \phi_2$ degrees. This results in one signal being passed and the other being nulled. For example, if the reference phase is adjusted to equal $-\pi/2 + \phi_2$ degrees, the detector output is expressed as:

$$v_o(t) = \frac{1}{2} m_1 V_1 \sin(\phi_2 - \phi_1) \cdot e^{-a^2(t-t_0)^2}. \quad (6)$$

Similarly, if the reference phase is adjusted to equal $\pi/2 + \phi_1$ degrees, the output is expressed as:

$$v_o(t) = \frac{1}{2} m_2 V_2 \sin(\phi_2 - \phi_1) \cdot e^{-a^2(t-t_0)^2}. \quad (7)$$

Both signals are resolved, but with a loss in amplitude [sin $(\phi_2 - \phi_1)$].

Fluorescence lifetime is quantified directly by the two-phase method. A quadrature phase hybrid circuit is used to form two reference signals that are 90 degrees out of phase with each other. These signals are input as references to two PSDs, the outputs which are expressed as:

$$v_{\phi-90}(t) = \frac{1}{2} mV \sin\phi \cdot e^{-a^2(t-t_0)^2} \text{ and } v_{\phi}(t) = \frac{1}{2} mV \cos\phi \cdot e^{-a^2(t-t_0)^2}, \quad (8)$$

where ϕ is the signal phase shift (see Equation 2). The $v_{\phi - 90}(t) / v_{\phi}(t)$ ratio expression results in the tan ϕ which is directly proportional to the fluorescence decay time expressed as:

$$\tau = \frac{1}{\omega} \tan\phi = \frac{1}{\omega} [V(\phi - 90) / V(\phi)]. \quad (9)$$

The signal detection electronics outputs are amplified, integrated, and transferred to a computerized data-acquisition system for display as frequency distribution histograms or as bivariate dot and contour diagrams.

The initial results show signal phase shift and amplitude demodulation recorded on fluorospheres and cells stained for total DNA content using propidium iodide (PI) and ethidium bromide that were excited in a 1 to 30 MHz frequency range; a signal detection limit threshold range of 300 to 500 fluoresein molecules equivalence, compared to 250 molecules equivalence for cw/dc laser excitation; a measurement precision (coefficient of variation measured on frequency distribution histograms) of 1.8% on alignment fluorospheres and 3.6% on cells stained with PI (DNA content); the resolution of fluorescence signals from cells stained in combination with PI and fluorescein isothiocyanate (FITC), based only on differences in their decay lifetimes expressed as phase shifts [see Fig. 2, parts (A) - (E)]; and the direct measurement of nanosecond fluorescence lifetimes on fluorospheres and cells stained with PI and FITC [see Fig. 2(F)]. Of particular significance in the data recorded in Fig. 2(F) is the lifetime measurement linearity (compared to conventional static time-resolved measurements, see Table I) and the uniformity of the fluorescence lifetime histograms compared to the broader integrated fluorescence intensity measurements of total cellular DNA and protein [see Fig. 2(A) and 2(B)].

Like conventional flow cytometers, the phase-sensitive cytometer can analyze fluorochrome-labeled cells or subcellular components for clinical, biomedical, and environmental research applications. However, because this cytometer can electronically separate the signals from two different fluorochrome emissions based on their lifetimes and also make conventional measurements, it has a wide range of technical applications. Of particular importance are fluorescent probes in which the lifetime changes between the bound and unbound (free) state, e.g., calcium indicators. Also of interest are probes, such as, the Hoeschst DNA-binding fluorochromes, in which the lifetime changes as function of energy transfer/quenching by surrounding agents (DNA-incorporated BrdU). Phase-sensitive detection methods also have the potential to reduce background interferences, e.g., cellular autofluorescence and Raman and Rayleigh scatter, that cause decreased measurement sensitivity and precision. In addition, fluorescence lifetime can be used as a spectroscopic probe to study the interaction of fluorochromes with their targets, each other, and the surrounding environment. Alternative methods to resolve multicomponent emissions/lifetimes will require that multifrequency excitation be employed (Jameson *et al*, 1984).

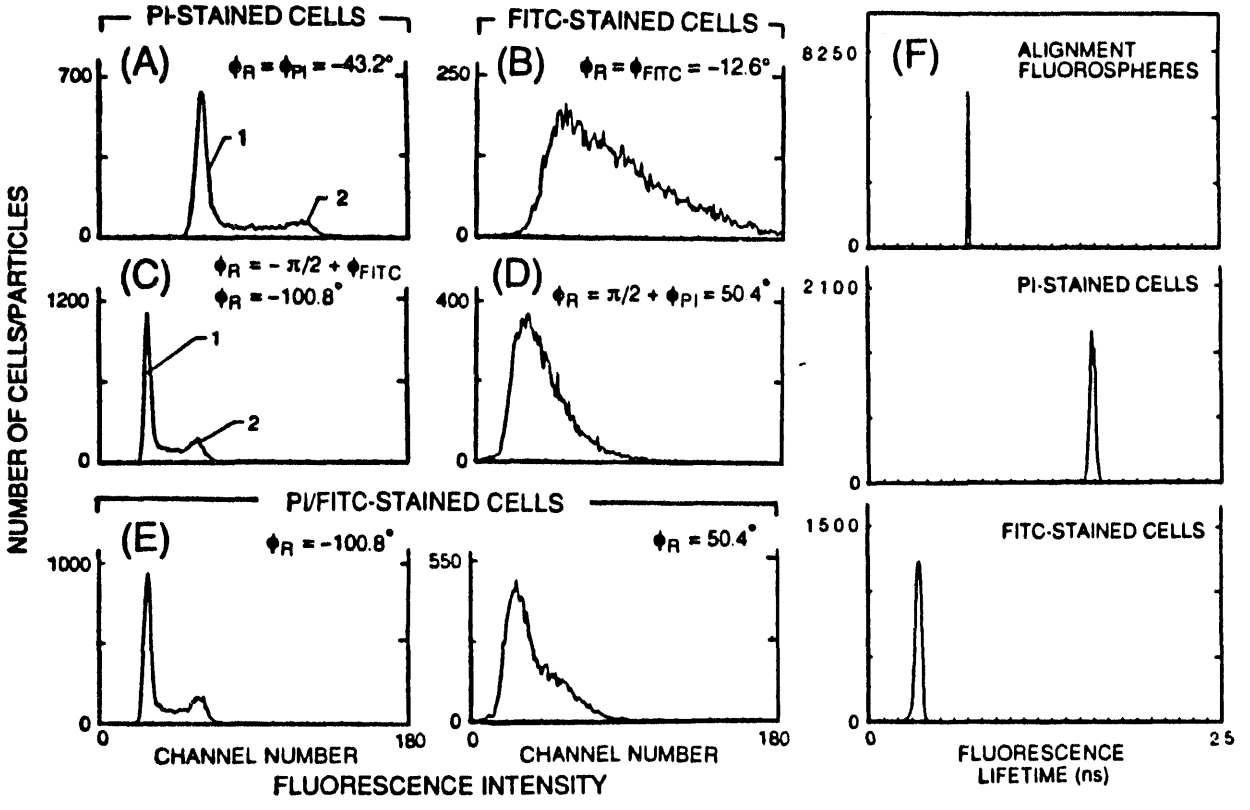

Figure 2, Parts (A) - (E). Frequency distribution histograms of the PSD output signals from line CHO cells stained separately with PI and FITC and in combination and analyzed using a 10 MHz modulation frequency and 488 nm wavelength laser excitation. In parts (A) and (B), the phase of the reference signal was adjusted to maximize the PSD output signals for PI-stained cells ($\phi_R = \phi_{PI} = -43.2°$) and for FITC-stained cells ($\phi_R = \phi_{FITC} = -12.6°$), respectively, and the corresponding histograms were recorded. In part (C), FITC-stained control cells were first analyzed and ϕ_R was adjusted ($\phi_R = -\pi/2 + \phi_{FITC} = -100.8°$) to null the PSD signals. PI-stained cells were then analyzed at the same phase shift and PMT/amplifier gains and the histogram was recorded. Similarly, in part (D), PI-stained cells were analyzed and ϕ_R adjusted ($\phi_R = \pi/2 + \phi_{PI} = 50.4°$) to null the PSD signals. FITC-stained cells were then analyzed at the same phase shift and gain settings and the corresponding histogram was recorded. In part (E), cells stained in combination with PI and FITC were analyzed by first recording the PSD output signal histograms with $\phi_R = -100.8°$ (PI signals resolved) and then with $\phi_R = 50.4°$ (FITC signals resolved). Figure 2, Part (F). Fluorescence lifetime frequency distribution histograms recorded on Coulter "DNA Check" alignment fluorospheres and on PI- and FITC-stained line CHO cells analyzed using a 10 MHz modulation frequency, 488 nm wavelength laser excitation, and a ratio module gain of 9.6X.

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