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TITLE RESOLUTION OF HETEROGENEOUS FLUORESCENCE EMISSION SIGNALS AND DECAY LIFETIME MEASUREMENT ON FLOUROCHROME-LABELED CELLS BY PHASE-SENSITIVE FCM

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Resolution of heterogeneous fluorescence emission signals and decay lifetime measurement on fluorochrome-labeled cells by phase-sensitive FCM

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ABSTRACT

A phase-sensitive flow cytometer has been developed to resolve signals from heterogeneous fluorescence emission spectra and quantify fluorescence decay times on cells labeled with fluorescent dyes. This instrument combines flow cytometry (FCM) and fluorescence spectroscopy measurement principles to provide unique capabilities for making phase-resolved measurements on single cells in flow, while preserving conventional FCM measurement capabilities. Stained cells are analyzed as they pass through an intensity-modulated (sinusoid) laser excitation beam. Fluorescence is measured orthogonally using a collecting lens, a longpass barrier filter to block scattered laser excitation light, and a photomultiplier tube detector. The modulated detector output signals, which are shifted in phase from a reference signal and amplitude demodulated, are processed by phase-sensitive detection electronics to resolve signals from heterogeneous emissions and quantify decay lifetimes directly. The output signals are displayed as frequency distribution histograms and bivariate diagrams using a computer-based data acquisition system. Results have demonstrated: 1) signal phase shift, amplitude demodulation, and average measurement of fluorescence lifetimes on stained cells; 2) a detection limit threshold of 300 to 500 fluorescein isothiocyanate (FITC) molecules equivalence for excitation frequencies 1 to 30 MHz; 3) fluorescence measurement precision of 1.3% on alignment fluorospheres and 3.4% on propidium iodide (PI)-stained cells; 4) the resolution of PI and FITC signals from cells stained in combination with PI and FITC, based on differences in their decay lifetimes; and 5) the ability to measure single decay times by the two-phase, phase comparator, method.

1. INTRODUCTION

Flow cytometry (FCM) is an important diagnostic tool for use in clinical immunology, hematology, and oncology, including its application to basic biomedical research. Clinical tests and biological experiments often require the labeling of cells with multiple fluorochromes for correlated analysis of cellular properties. The major limitation of these procedures is the availability of fluorescent dyes with a common excitation region, i.e., requiring only one excitation source, and emission spectra that are sufficiently separated to permit measurement by conventional multicolor detection methods that employ employ dichroic and bandpass filters (1). If fluorochromes have emission spectra that cannot be resolved by optical filtration, but have separated excitation spectra, multiple excitation sources of different wavelengths can be employed to sequentially excite fluorochrome-labeled cells and thus resolve the fluorescence emission signals (2). This approach has increased the number of fluorochromes available for multicolor labeling studies, but the instrumentation has become increasingly complex.

We have developed a new FCM approach (3) based on phase-resolved fluorescence spectroscopy (4) to separate fluorescence signals from multiple overlapping emissions on fluorochrome-labeled cells. Our technique will resolve signals based on differences in fluorescence decay lifetimes that are observed as phase shifts in the fluorescence emission signals by phase-sensitive detection (5) as cells pass across a modulated excitation source. By separating the fluorescence emission signal components on the basis of decay time, rather than spectroscopically with optical filters, the entire spectrum of each component is utilized so that light loss due to filtering is minimized. In addition, electronics have been developed for quantifying fluorescence decay time in cells labeled with fluorophores having single component decay times. The

measurement of fluorescence lifetime is of considerable importance because it potentially provides additional information about fluorochrome/cell interactions that are influenced by local environmental factors near the binding site, such as solvent polarity, energy transfer, excited-state reactions, and quenching. Structural and functional information also may be obtainable by analyses of fluorescence lifetimes. For example, this technique gives the possibility of using lifetime measurements to probe for changes that occur in DNA and chromatin structure during cell activation, differentiation, and division.

2. THEORY

Stained cells suspended in normal saline are analyzed as they intersect an intensity-modulated (sinusoid) laser excitation beam (see Fig. 1). Orthogonal fluorescence is measured using a detector consisting of a collection lens, longpass barrier filter to block scattered laser excitation light, and a photomultiplier tube. The detector output signals, which are shifted in phase (ϕ) from the excitation or a reference signal by an amount:

$$\phi = \arctan \omega t \tag{1}$$

and demodulated by a factor (m) (6), where

$$m = (1 + (\omega t)^2)^{-1/2}, \tag{2}$$

are processed by phase-sensitive detection electronics to resolve signals from heterogeneous fluorescence emissions and quantify decay lifetimes directly. The detector outputs are amplified and input to a computer for display as frequency distribution histograms and bivariate contour diagrams. Conventional FCM signals are made available by low-pass filtering as described below.

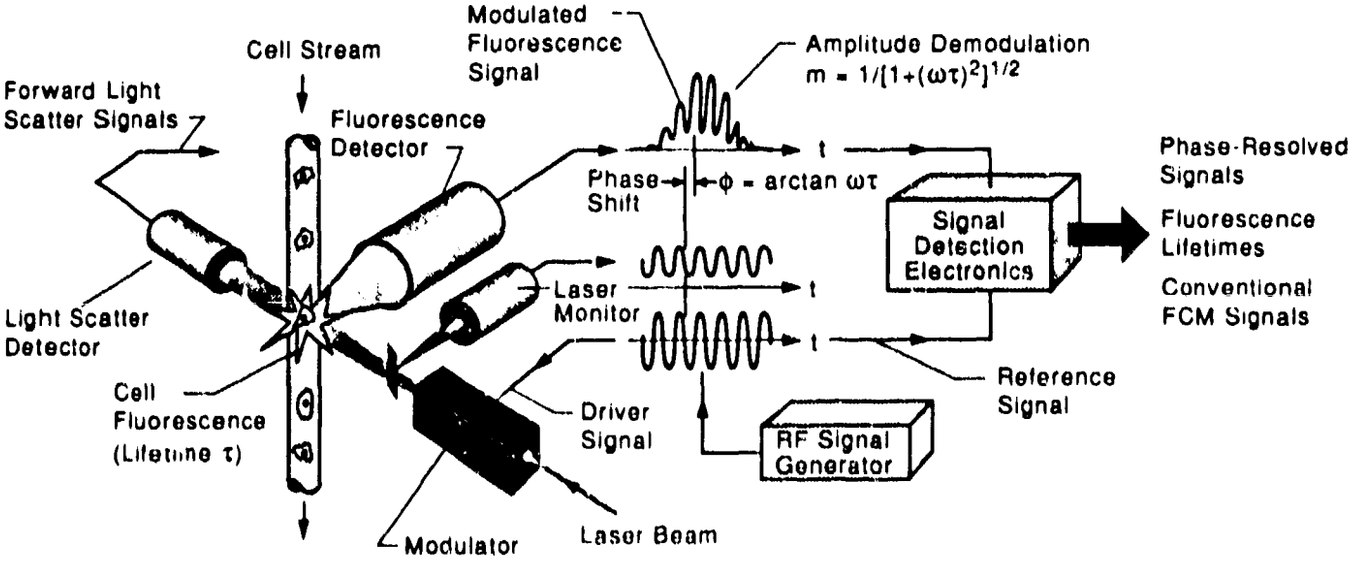

Figure 1. Conceptual diagram of the flow cytometer for phase resolved measurement of fluorescence lifetimes.

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

$$v(t) = V[1 + m\cos(\omega t - \phi)] \cdot e^{-a^2(t-t_0)^2} \qquad (3)$$

where V is the fluorescence signal intensity, ω is the angular excitation frequency, ϕ and m are the signal phase shift and demodulation terms associated with a single fluorescence decay time, t is time, and a is a term related to the flow velocity of a cell across the laser beam at time t_0 (7) (see Fig. 2A). 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 excitation depth-of-modulation factor (m_{ex}) which will reduce the high-frequency signal component intensity. The cw-excited dc signal component is extracted using a low-pass filter to give conventional fluorescence intensity information (see Fig. 2A). The high-frequency signal

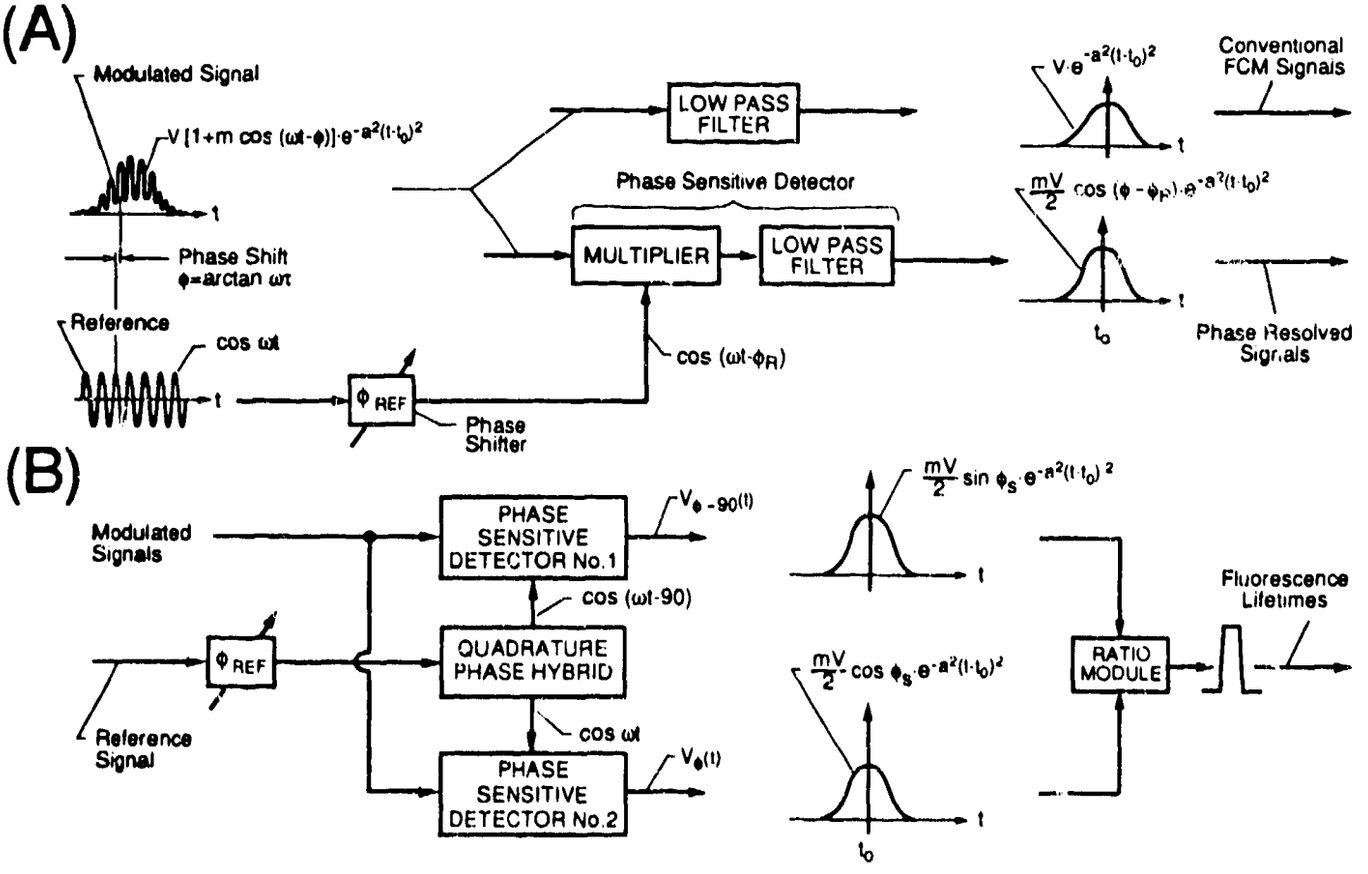

Figure 2. Conceptual diagrams of time-dependent modulated fluorescence and reference signals, corresponding amplitude spectra (frequency domain), signal detection electronics, detector signal output amplitude spectra, and corresponding time-dependent output signals (A) and two-phase detector (phase comparator) for quantifying fluorescence lifetime , where $\tau = 1/\omega\,[V(\phi - 90)/V(\phi)]$ (B).

component is processed by a phase-sensitive detector (PSD) consisting of a multiplier and a low-pass filter (8) as illustrated in Fig. 2A. 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 v(t). The PSD output is a Gaussian-shaped

signal that is proportional to fluorescence intensity (V), the demodulation factor, and the cos (ϕ - ϕ_R) expressed as:

$$v_0(t) = V\, m / 2 \cos(\phi - \phi_R)\, e^{-a^2(t-t_0)^2} \tag{4}$$

The principle of phase suppression as applied to flow for separating two fluorescence signals $v_1(t)$ and $v_2(t)$, based on differences in their decay lifetime τ_1 and τ_2, by phase-sensitive detection is shown in Fig. 3.

- Let $v_1(t) = V_1(1+m_1 \cos(\omega t-\phi_1)) \cdot e^{-a^2(t-t_0)^2}$ and $v_2(t) = V_2(1+m_2 \cos(\omega t-\phi_2)) \cdot e^{-a^2(t-t_0)^2}$

- Output of Phase Sensitive Detector:

$$v_0(t) = \frac{V_1 m_1}{2} \cos(\phi_1-\phi_R) \cdot e^{-a^2(t-t_0)^2} + \frac{V_2 m_2}{2} \cos(\phi_2-\phi_R) \cdot e^{-a^2(t-t_0)^2}$$

(a) Let $\phi_R = \phi_2 - \pi/2$, then $v_0(t) = \frac{V_1 m_1}{2} \sin(\phi_2-\phi_1) \cdot e^{-a^2(t-t_0)^2}$

(b) Let $\phi_R = \phi_1 + \pi/2$, then $v_0(t) = \frac{V_2 m_2}{2} \sin(\phi_2-\phi_1) \cdot e^{-a^2(t-t_0)^2}$

Figure 3. Theory for resolving signals from simultaneous fluorescence emissions, 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.

By shifting the phase of the reference signal by ϕ_2 - $\pi/2$ or ϕ_1 + $\pi/2$ degrees, $v_1(t)$ and $v_2(t)$ are respectively resolved at the output of the phase sensitive detector, but with a loss in amplitude by a factor sin (ϕ_2 - ϕ_1).

The measurement of fluorescence decay time by the two phase, i.e., phase comparator, method (9) is illustrated in Fig. 2B. This technique employs a quadrature phase hybrid circuit to generate reference signals that are 90° out of phase with each other which are input to two phase-sensitive detectors. This results in two detector output signals expressed as:

$$v_{\phi\cdot 90}(t) = V\, m / 2 \sin(\phi_s)\, e^{-a^2(t-t_0)^2} \text{ and} \tag{5}$$

$$v_{\phi}(t) = V\, m / 2 \cos(\phi_s)\, e^{-a^2(t-t_0)^2}, \tag{6}$$

ϕ_S is signal phase shift (see Equation 1). The ratio of these two signals is directly proportional to the tangent of the signal phase shift (ϕ_S). The fluorescence decay time is expressed as:

$$\tau = 1/\omega \tan \phi_S, \tag{7}$$

using Equation 1.

3. MATERIALS AND METHODS

3.1. Instrumentation

A Spectra Physics Model 2025-05 5W argon laser operating at 488 nm wavelength (0.6 W) is used as the excitation source and the modulator is a Conoptics Model 50 electro-optical dc-50 MHz bandwidth unit (see Fig. 1). A Hewlett Packard Model 3335A signal synthesizer is used as the frequency source to drive the modulator and supply the reference phase shifter with a sinewave signal. The laser beam (Gaussian intensity profile) is focused by a pair of crossed cylindrical lenses of focal length 30 and 5.4 cm into an elliptical shape onto the cell stream in a "Biosense" flow chamber (Coulter Corp.) (not shown in Fig. 1). A beamsplitter located between the modulator and the beam-shaping optics is used to direct modulated laser light to the laser monitor. The fluorescence detector consists of a f/0.95 CCTV lens to collect and collimate the modulated fluorescence emission to a second CCTV lens which focuses the emitted light onto a 100 μm diameter pinhole spatial filter located in front of a photomultiplier tube (PMT). A Corning 3-69 or Schott OG 515 colored glass filter located between the two lenses, functions as a longpass barrier filter to block scattered laser light and pass emitted fluorescence. Forward light scatter is focused using a 10-cm focal-length lens (not shown in Fig. 1) placed 20 cm from the laser beam-cell stream intersection point in the flow chamber on the beam axis onto the photocathode of a PMT (light scatter detector) located 20 cm from the collection lens. An obscuration bar located in front of the collection lens blocks incident laser light from the detector and neutral density filter placed in front of the PMT detector limits the current. A Burle Industries Model 4526 dormer window 10-stage PMT, with the anode connected to a Comlinear Model 401 operational amplifier configured in the transimpedance mode , serves as the photodetector in the laser monitor and the fluorescence and light scatter detectors (1). The light scatter and fluorescence detector and reference signals are routed to the signal detection/processing electronics.

The forward light scatter signals are demodulated and amplified using a Canberra Model 1415 RC Amplifier. Fluorescence signals are similarly processed to obtain the cw-excited signal component using the low-pass section (0-160 KHz) of a Krohn-Hite Model 3201 electronic filter and amplified and integrated. The phase-sensitive detection electronics consists of a Mini-Circuits Model ZRPD-1 phase detector, i.e., multiplier, and a Krohn-Hite Model 3201 electronic filter (see Fig. 2A). Allen Avionics Models V127050 (0-127 nsec) and VAR011 (0-11 nsec) switchable delay lines having 1.0 and 0.5 nsec time resolution, i.e., 3.6 and 1.8 degree phase resolution at 10 MHz, respectively, are used to shift the phase of the reference signal with respect to the modulated fluorescence signals. The PSD detector output signals are inverted and amplified/integrated (linear) or amplified (logarithmic).

The two-phase ratio detector circuit for making direct single fluorescence decay time measurements on cells consists of an Anzac Model JH-6-4, 2-32 MHz, quadrature phase hybrid module functions to output two reference signals that are 90° out of phase with each other to two phase-sensitive detector circuits for generating outputs $V(\phi\text{-}90)$ and $V(\phi)$, the ratio of which is equal to the tan ϕ_S and directly proportional to the decay lifetime (see Fig. 2B). A reference phase-shifter, consisting of Allen Avionics switchable delay lines as described above, is used to initialize the two-phase measurement network for zero lifetime signals, i.e., set $\phi_R = \phi_S = 0°$ using nonfluorescent microspheres. The phase-sensitive detector circuits consist of double balanced mixer/multipliers, low pass electronic filters, inverting amplifiers, and amplifier/ integrators as described above. The ratio module (10) consists of gated peak sense and hold circuits to acquire the numerator $V(\phi\text{-}90)$ and demoninator $V(\phi)$ signals followed by a high-speed analog divider.

The above signals are recorded (List Mode) for display as frequency distribution histograms and bivariate contour diagrams using the LACEL data acquisition system (11).

3.2. Cell preparation and staining

Human skin fibroblast (HSF) cells arrested in G_0/G_1 growth phase by growth (monolayer) in low serum were harvested, fixed in 70% ethanol (1hr), centrifuged, and the ethanol was removed by aspiration prior to staining (12). Fixed cells were stained for total cellular DNA content in a PBS solution containing 7.5 µg/ml of propidium iodide (PI) (Polysciences, Warrington, PA) and 50 µg/ml RNase (Worthington, Freehold, NJ, Code R) for 30 min at 37°C. Chinese hamster cells (line CHO) maintained in exponential growth phase in suspension culture were harvested and fixed as described above. Fixed cells were stained for DNA content in a PBS solution containing 15.0 µg/ml (PI) and 50 µg/ml RNase for 30 min at 37°C. For combined PI and fluorescein isothiocyanate (FITC) staining of DNA and protein, fixed CHO cells were treated in a solution containing 1.5 µg/ml PI, 0.5 µg/ml FITC isomer 1 (BBL, Cockeysville, MD), and 50 µg/ml RNase, for 30 min at 37°C. The cell density was maintained at 2.5 x 10^6 cells/ml in all stain solutions. Stained cell samples were analyzed by phase-sensitive FCM at room temperature within 1-2 hr after staining.

4. RESULTS

To verify fluorescence signal phase shift and amplitude demodulation on fluorochrome-labeled cells in flow, we used human skin fibroblast (HSF) cells arrested in the G_0/G_1 phase of the cell cycle that were stained with PI and photographed the fluorescence detector preamplifier output and reference signals at a 16 MHz modulation frequency (see Fig. 4A). Since the modulation frequency is very high, compared to the velocity of the cells across the laser beam, i.e., fluorescence signal pulse width, the signals appear as a Gaussian-shaped white blurred region when viewed and photographed with an oscilloscope (2 µsec sweep speed) (see Fig. 4A). To photograph the phase shift of the high-frequency component of the modulated fluorescence signal, a Chesterfield Products 16 MHz bandpass filter was placed between the preamplifier output and the oscilloscope input and the oscilloscope sweep speed was increased to 20 nsec/div. Using 7 µm diameter, uniform-size, nonfluorescent, polystyrene latex particles, the longpass barrier filter was removed from the fluorescence detector and the reference signal was aligned to be in phase ($\Delta\phi = 0°$) with the orthogonal light scatter signals using a switchable delay line in the reference signal line to the oscilloscope. The barrier filter was then replaced and the fluorescence and reference signals were photographed (see Fig. 4B). Neutral density (ND) filters were used in the fluorescence detector channel when the light scatter signals were photographed to equalize the light scatter signal amplitude with respect to the fluorescence signals without having to change the PMT gain (high voltage). The measured fluorescence phase shift (lag) with respect to the reference was approximately 54°, which corresponds to a fluorescence decay lifetime of 14 nsec, as determined using Equation (1).

The initial experiments to determine if fluorescence lifetime could be quantified in flow by phase shift and amplitude demodulation measurement were made on PI-stained (15.0 µg/ml) CHO cells analyzed at 10 MHz. Using uniform-size, nonfluorescent, alignment microspheres as test particles, the barrier filter was removed from the fluorescence detector and the preamplifier output signals were aligned to be -90° out of phase with the reference by adjusting the delay line (see Fig. 2A). ND filters were used to limit the orthogonal light scatter signal intensities to the same value as the corresponding fluorescence signals, both recorded at the same PMT gain. When the light scatter and reference signals were aligned at -90° phase with respect to each other, the output of the phase-sensitive detector was nulled/zeroed and the switchable delay line setting of the reference (ϕ_R(LS)) was recorded. PI-stained cells were then analyzed by replacing the barrier filter and removing the ND filters in the fluorescence detector and determining the phase shift (ϕ_R(FL)) required to null the PSD output. The phase shift was determined from the relationship: $\phi = \phi_R(LS) - \phi_R(FL)$ and the lifetime (12.5 nsec) was calculated using Equation 3. When CHO cells stained with PI at 1.5 µg/ml were analyzed, the measured lifetime was 15.0 nsec. The fluorescence lifetimes of PI-stained

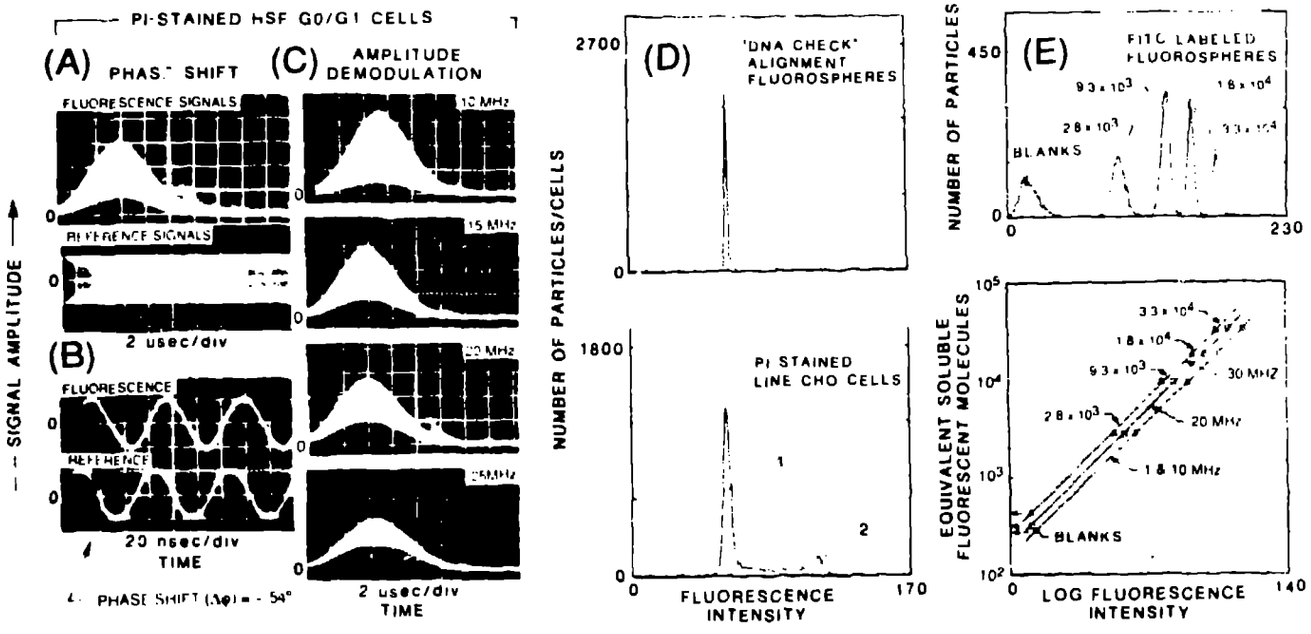

Figure 4. Oscilloscope photographs of: (A) modulated fluorescence preamplifier output signals from PI-stained HSF G_0/G_1 cells excited at 488 nm wavelength using a 10 MHz modulation and reference frequency signal recorded at 2µsec/div sweep speed; (B) the corresponding phase shift (lag, 54°) of fluorescence signals with respect to reference signals recorded at 20 nsec/div; and (C) fluorescence preamplifier output signals recorded (2 µsec/div) from PI-stained HSF cells at 10, 15, 20, and 25 MHz using 488 nm laser excitation. Frequency distribution histograms recorded on Coulter DNA-Check fluorospheres and PI-stained (15 µg/ml) CHO cells that were excited using 488 nm wavelength modulated at 10 MHz (D) and a log fluorescence intensity frequency distribution histogram recorded on a four component FITC-labeled fluorosphere mixture (plus blanks) at a 10 MHz excitation frequency and sensitivity plots of the fluorescence signal levels in equivalent soluble fluorescent molecules using FITC-labeled (and blank) fluorospheres ranging from 2.8 x 10^3 to 3.3 x 10^4 fluorescein equivalence molecules that were excited at a 1, 10, 20 and 30 MHz modulation frequency using 488 nm wavelength (E).

HSF G_0/G_1 cells were measured by amplitude demodulation. Figure 4C illustrates the demodulation effects recorded on stained HSF cells by photographing the fluorescence detector output at excitation frequencies ranging from 10 to 25 MHz. These results graphically show the effect of modulation frequency on fluorochrome-labeled cells. Based on the oscilloscope recordings of fluorescence and laser monitor signals, we calculated the decay lifetimes at different excitation frequencies using the relationships as previously described (13). The results (12.6 +/- 1.3 nsec) compare favorably with independent measurements (14) and will serve as a basis for future direct lifetime measurement by amplitude demodulation.

In fluorescence measurement precision tests on Coulter Corp. alignment fluorospheres (DNA Check) and PI-stained (15.0 µg/ml) CHO cells we recorded signals and displayed the frequency distribution histograms using 10 MHz modulation and phase-sensitive detection (see Fig. 4D). The reference signal was adjusted to be in phase with the high-frequency component of the pulse-modulated fluorescence signal ($\phi_R = \phi_{PI}$) to maximize the PSD output. The recorded histograms compared favorably with those obtained without modulating the laser beam (12). Peak 1 of the DNA distribution represents 2C diploid DNA content cells in

G_1/G_0 phase, prior to DNA replication, and peak 2, the 4C DNA content cells in G_2 and M phase following DNA replication. The cells contained between the two peaks are in S phase. The peak 2 to peak 1 modal fluorescence intensity ratio is 1.93 and the coefficient of variation (CV) (standard deviation divided by the mean) is 1.3 % for the alignment fluorospheres and 3.4 % for peak 1 of the DNA content histogram.

In sensitivity tests using Flow Cytometry Standards, Inc., calibrated microspheres, we determined the fluorescence detection threshold level by first recording the log fluorescence frequency distribution histograms at different excitation frequencies from a mixture of fluorospheres labeled with known equivalent amounts of FITC (plus blanks) as illustrated in Fig. 4E. The mean log fluorescence intensities were then plotted as a function of the number of fluorescein equivalence molecules at 1, 10, 20 and 30 MHz excitation frequencies and the minimum detection level was estimated from the intensity of the nonfluorescent blanks. A detection threshold of 200-300 equivalent molecules of FITC was measured using a 1 and 10 MHz modulation frequency, compared to 250 equivalent molecules by conventional FCM (data not shown) (see Fig. 4E). From 20 to 30 MHz the fluorescence detection level was between 300 and 500 FITC equivalence molecules. These preliminary results demonstrate the potential for analyzing weak signals from FITC-labeled immunofluorescence properties of cells by phase-sensitive detection.

The resolution of signals from fluorescence emissions having different lifetimes by phase sensitive detection is illustrated in Fig. 5. In this experiment CHO cells were stained with FITC alone, PI alone, and PI/FITC in combination and analyzed at 10 MHz. The PSD output signals were photographed and the frequency distribution histograms were displayed. PI- and FITC-stained cells were first analyzed separately to determine the maximum PSD output signals as a function of the reference signal phase (ϕ_R) with respect to the modulated signal input to the phase detector. ϕ_R was adjusted to - 43.2° and - 12.6°, respectively, to maximize the PSD output for PI- and FITC-stained cells and the corresponding histograms were displayed (see Fig. 5A). Next, ϕ_R was adjusted to null the PSD output while FITC-stained cells were being analyzed (see Fig. 5B). No histogram was recorded. PI alone-stained cells were then analyzed at the same reference phase setting and the DNA content histogram was displayed (see Fig. 5B). The G_0/G_1 signal amplitude (peak 1) was reduced in value compared to the data recorded in Fig. 5A. This reduction in signal amplitude is due to the sin $(\phi_2 - \phi_1)$ relationship expressed in Fig. 3, where ϕ_1 and ϕ_2 are the -12.6 and -43.2 degree FITC and PI signal phase shifts, respectively. PI-stained cells were next analyzed by determining the ϕ_R phase shift needed to null the PSD output (see Fig. 5C). FITC-stained cells were then analyzed at the same reference signal phase shift setting and the PSD output signals were photographed and the corresponding histogram was displayed. CHO cells stained in combination with PI and FITC were analyzed by PSD to illustrate the resolution of fluorescence signals from cells labeled with of two fluorochromes having different lifetimes (see Fig. 5D). The reference signal phase was first adjusted to - 100.8° $(-\pi/2 + \phi_{FITC})$ and the PSD output signals were photographed and the histogram was displayed. The reference phase was then adjusted to 50.4° $(\pi/2 + \phi_{PI})$ and the PSD outputs were recorded. This data graphically illustrates the capability to resolve fluorescence signals based on differences in their lifetimes.

The ability to quantify fluorescence decay time on particles and cells labeled with fluorescent dyes by direct measurement in flow is illustrated in Fig. 6. In this example we recorded the histogram outputs of phase-sensitive detector No. 1 (proportional to $mV_S \sin \phi_S$), phase-sensitive detector No. 2 (proportional to $mV_S \cos \phi_S$), the ratio module (proportional to decay time), where V_S and ϕ_S are the signal intensity and phase shift (see Equation 1), respectively. The outputs of PSD Nos. 1 and 2 were initialized by zeroing and maximizing, respectively, using nonfluorescent microspheres prior to analyzing fluorospheres and stained cells by removing the longpass barrier filter and adjusting ϕ_R to zero and maximize the PSD outputs using nonfluorescent microspheres. The barrier filter was then replaced and the fluorospheres and stained cells

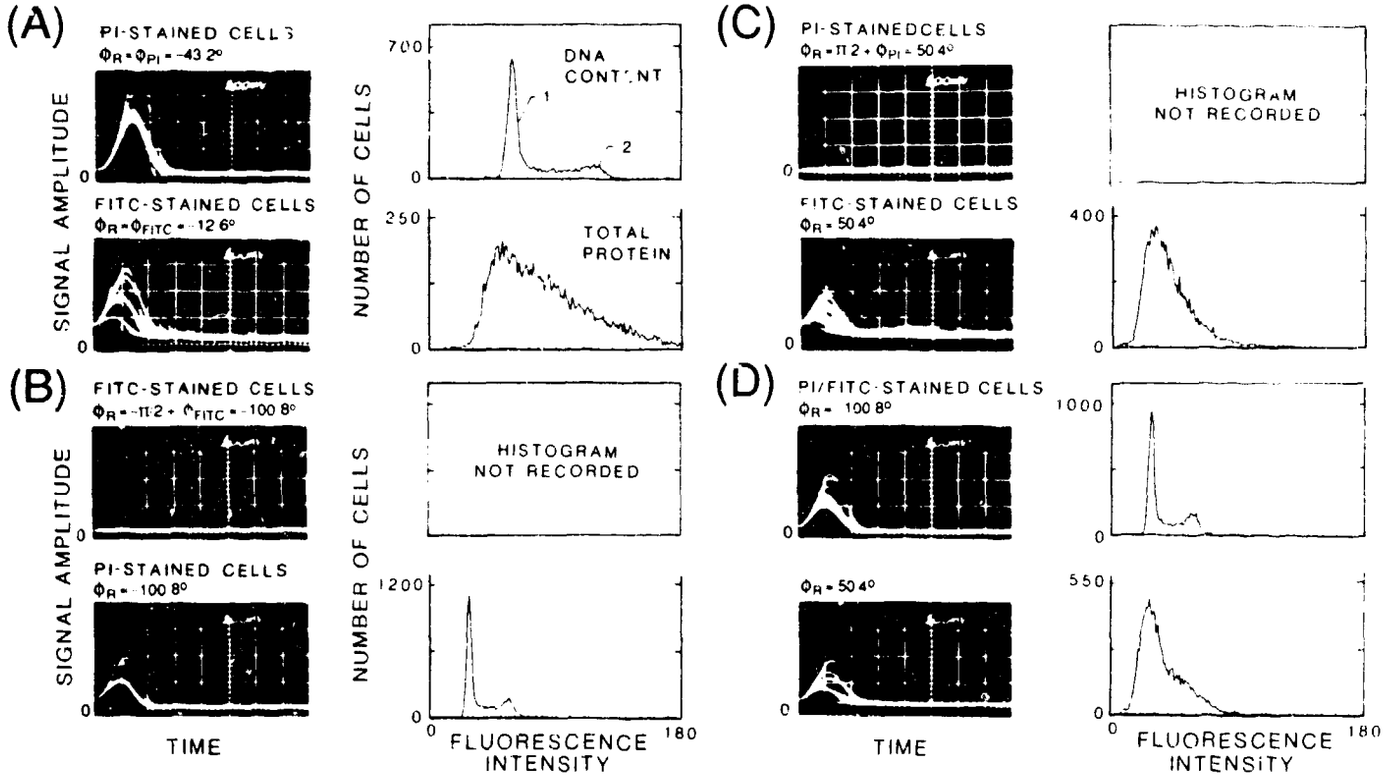

Figure 5. Photographs of the phase-sensitive detector output signals recorded at an oscilloscope sweep speed of 5 μsec/div and the corresponding cellular DNA content and total protein frequency distribution histograms individually recorded on CHO cell samples stained separately with PI (1.5 μg/ml) and FITC (0.5 μg/ml) and analyzed with: $\phi_R = \phi_{PI} = -43.2°$ and $\phi_R = \phi_{FITC} = -12.6°$, respectively (A); $\phi_R = -\pi/2 + \phi_{FITC} = -100.8°$ (FITC signals nulled) (B); and $\phi_R = \pi/2 + \phi_{PI} = 50.4°$ (PI signals nulled) (C). Phase-sensitive detector output signals and corresponding DNA content and total protein histograms individually recorded on CHO cells stained in combination with PI and FITC and analyzed at $\phi_R = -100.8°$ and $\phi_R = 50.4°$, respectively (D).

were analyzed at the same gain settings. Figure 6A shows the PSD No. 1, PSD No. 2, and ratio module output histograms recorded on DNA Check alignment fluorospheres (decay time 7 nsec). The CV of the lifetime ratio distribution is 0.8 %, which is less than the 1.3% recorded on the same fluorospheres based on fluorescence intensity measurement (see Fig. 4D). PI-stained CHO cells (15.0 μg/ml) were analyzed next at the same ratio module gain settings (see Fig. 6B). The histograms recorded at the PSD No. 1 and 2 outputs are similar to the DNA content distributions previously recorded in Fig. 4D. The fluorescence decay time histogram is unimodal with a CV of 1.0 %, which is less than the CV recorded on PI-stained cells (peak 1) based on intensity measurement. The modal value of the histogram is 14.2 nsec, which is in close agreement with decay lifetime measurements obtained by other methods (14). Figure 6C shows the PSD No. 1 and No. 2 and the ratio module output histograms recorded from Flow Cytometry Standards, Inc., FITC-labeled reference alignment fluorospheres. The fluorescence decay time distribution is again unimodal and centered about 3 nsec, which is slightly less than the reported lifetime for FITC (15).

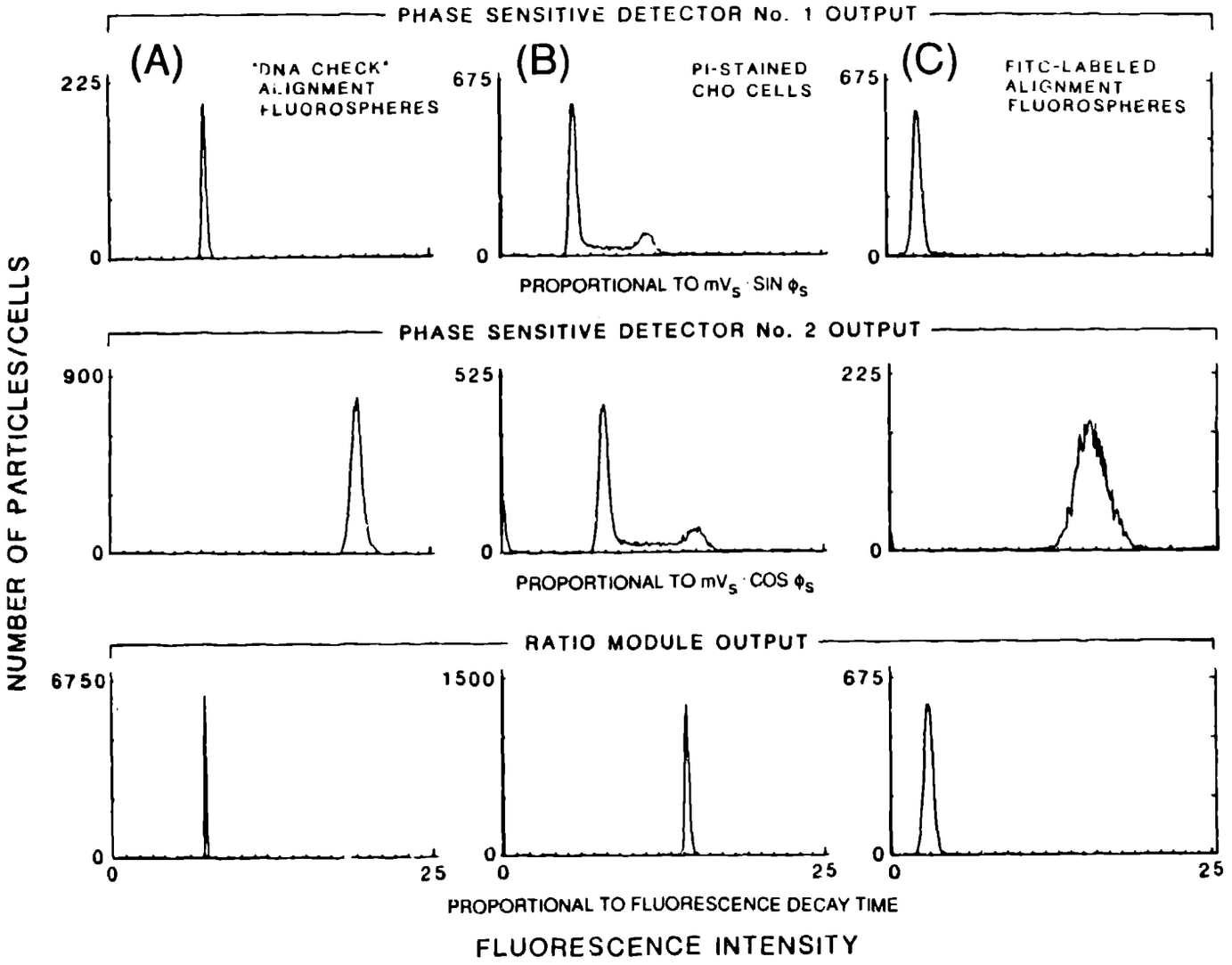

Figure 6. Frequency distributions histograms of phase-sensitive detector No. 1, phase-sensitive detec No. 2, and ratio module output (proportional to fluorescence decay time) for "DNA Check" alignm fluorospheres (A); CHO cells stained with PI (15.0 µg/ml) (B); and FITC-labeled reference alignment fluorospheres (C) that were excited and analyzed using 488 nm, modulated (10 MHz) laser excitation.

5. DISCUSSION

A phase-sensitive detection method to resolve signals from simultaneous fluorescence emissions in cells labeled with two fluorochromes, each having a different decay time, and quantify decay time of single component fluorophores has been described. This technology is readily adaptable to conventional flow cytometers by adding a frequency generator, optical modulator, high-speed PMT detector/preamplifier, and phase-sensitive detection electronics. In addition, other excitation sources also can be employed, e.g., synchronous pulsed laser (16). We intially selected a sinusoid modulated excitation source because of its simplicity in both excitation and signal demodulation. Conventional optical signals, e.g., axial light loss, forward and orthogonal light scatter, and multicolor fluorescence are also obtainable by low-pass electronic filtering.

The fluorescence measurement precision on alignment fluorospheres and the G_0/G_1 peak of PI-stained cells, along with measurement linearity expressed as $G_2 + M$ to G_0/G_1 DNA content ratio, as determined by phase-sensitive FCM, was similar to data previously reported by conventional flow cytometry (12). The fluorescence phase-sensitive detection thresold limit was 300 to 500 molecules equivalence for fluorescein-labeled fluorospheres excited at 1 to 30 MHz, compared to 250 molecules equivalence using nonmodulated excitation. The average fluorescence lifetime of PI bound to DNA in CHO cells at two stain concentrations was measured by determining the phase shift between labeled cells and nonfluorescent particles. The 15.0 nsec lifetime measured on cells stained with PI at a concentration of 1.5 µg/ml was slightly higher than the 13.0 nsec value previously reported by static, time-resolved, microscopic measurement (14). This was due primarily to an order of magnitude lower PI staining concentration. In phase shift measurements on cells stained with PI at 15.0 µg/ml, the decay time was 12.5 nsec. The average decay time measurment on HSF cells labeled with PI (7.5 µg/ml) and analyzed by amplitude demodulation at frequencies ranging from 10 to 25 MHz was 12.6 nsec.

The resolution of fluorescence signals from cells stained with fluorochromes having different decay times is dependent upon the individual signal amplitudes, the modulation indexes, the sin $(\phi_2 - \phi_2)$ relationship, and the excitation frequency. The phase-resolved measurement resolution is 1.8 degrees at 10 MHz as determined by the reference phase shifter (+/- 0.5 nsec). The resolution can be increased or decreased by lowering or raising the excitation frequency. We have been able to detect 0.5 to 1.0 nsec phase-related PSD output amplitudes using simulated test signals (17) of equal amplitude. Direct lifetime measurement on a cell-by-cell basis was demonstrated using the two-phase ratio method. The longest lifetimes that can be employed in the separation of fluorescence emission signals and quantified directly by phase-resolved measurement depends upon the lowest usable excitation frequency. In our system, we have experimentally determined the lowest excitation frequency to be about 500 kHz, which corresponds to a 300 nsec lifetime for a 45 degree phase shift (18). The shortest usable decay time depends upon the maximum usable excitation signal frequency, which is about 35 to 40 MHz (reference phase shifter 3 dB half-power point) and corresponds to about 1 nsec or less.

Future experiments are planned that will employ a variety of fluorochromes that have different lifetimes, including those with overlapping emission spectra. Also, metachromatic fluorochromes that have two distinct emission regions, each having a different lifetime, e.g., acridine orange, and fluorescent labels in which lifetime changes between the bound and unbound (free) state (19) will be tested. Phase-sensitive detection methods also will be tested to evaluate the efficacy of reducing background interferences, e.g., cellular autofluorescence (20) and Raman and Rayleigh scatter (18), that cause decreased measurement sensitivity and precision in the analysis of cells/particles by FCM. The significance of this new technology is that the number of fluorochromes usable in multilabeling experiments will be increased; background interferences, i.e., intrinsic cellular autofluorescence, unbound dye, nonspecific staining, and Raman and Rayleigh scatter, will be reduced or eliminated; and fluorescence lifetime can be quantified for application as a spectroscopic probe to study the interactions of fluorochrome binding

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