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Fluorescence Lifetime-Based Discrimination and Quantification of Cellular DNA and RNA With Phase-Sensitive Flow Cytometry

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Background: Simultaneous measurement of cellular DNA and RNA content provides information for determination of the functional status of cells and, clinically, for the diagnosis and grading assessment of various tumors. Most current flow cytometric methods are based on resolving the fluorescence emission spectra of dyes that bind preferentially to either type of nucleic acid. However, several monochromatic nucleic acid–binding fluorochromes display resolvable differences in fluorescence lifetime when bound to DNA or RNA. The differences in the lifetime of one fluorescent probe provide an alternate means to distinguish the binding of one probe to these cellular macromolecules and to simultaneously measure their cellular contents.

Methods: Three nucleic acid intercalating dyes, propidium iodide, ethidium bromide, and ethidium homodimer 1, were selected to study differences in fluorescence lifetimes when bound to cellular DNA and RNA. Fixed HL-60 cells were treated with specific nucleases to initially determine the lifetime values of each dye when bound to the cellular DNA, RNA, or both. The lifetime values were then used as the signatures to resolve the cellular DNA and RNA contents in untreated cells.

Results: All three dyes showed fluorescence lifetime differences when bound to RNase-treated, DNase-treated, or untreated cells. With these lifetime values, the fluorescence emissions from DNA, RNA, or DNA/RNA were resolved from untreated cells with the use of phase-sensitive detection. The lifetime differences resulting from the binding to either type of nucleic acid depended on the dye, the staining concentration, and the analysis condition.

Conclusions: The lifetimes of the nucleic acid–binding fluorochromes were altered when binding to different macromolecules under different conditions. Phase-sensitive flow cytometry provided a unique means for simultaneous discrimination and quantification of subcellular macromolecules with one fluorescent probe. The data demonstrated the capabilities for resolving relative cellular DNA and RNA contents based on fluorescence lifetime. Cytometry Part A 52A:46–55, 2003.
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Key terms: flow cytometry; DNA; RNA; propidium iodide; ethidium bromide; ethidium homodimer 1; fluorescence lifetime; phase-sensitive detection; phase-resolved separation

We previously demonstrated by flow cytometry (FCM) that fluorescent nucleic acid intercalating probes have different lifetime values when bound to DNA and RNA (1,2). These studies were confirmed recently by two-photon excitation lifetime imaging analysis of viable cells labeled with SYTO13 (3). Those studies allowed for the sequential and repetitive assessment of the staining patterns of DNA and RNA in Chinese hamster ovary cells. Our studies initially employed enzymatic treatment of cells to verify the lifetime values of the fluorochromes bound to DNA and RNA in cells. These studies were performed with a unique flow cytometer that measures fluorescence lifetime and the conventional FCM signals simultaneously, thereby allowing for correlated analysis of lifetime with multiple cellular parameters.

Measurements of cellular DNA and RNA content have broad applications in basic cell biology and clinical research. Simultaneous measurement of both types of nu-

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cleic acid provides approaches to analyze proliferation and differentiation potentials of cells and to localize cells in different cell cycle stages (4). Clinical research has demonstrated that cellular contents of DNA and RNA and their ratios are useful for assessment of various types of cancers, such as breast carcinoma (5) and bone sarcomas (6). FCM methodology has been explored and developed since the late 1970s to pursue the simultaneous detection in single cells. One conventional method was to use metachromatic fluorochromes whose absorption and emission spectra shift when bound to different substrates. Acridine orange is one of the most commonly used metachromatic dyes, which binds differentially to cellular DNA and RNA under different treatments and has different emission spectra (7). This technique requires certain stringent staining conditions, and the sensitivity is reduced due to the filtration required to separate overlapping fluorescence emissions. A combination of Hoechst 33342 and pyronin Y also has been widely used (8). The detection requires a dual-excitation flow cytometer for two fluorochromes with different excitation wavelengths. The three-laser excitation FCM system was used to measure DNA, RNA, and protein in cells by using a staining combination of Hoechst 33342, pyronin, and fluorescein isothiocyanate (9).

In addition to the spectral properties of fluorescent probes (color and intensity), fluorescence lifetime is an important feature of fluorescent molecules and is defined as the average period a fluorescent molecule remains in the excited state after excitation. The distinct chemical structure of a fluorescent dye provides a characteristic value of the lifetime that is sometimes considered to be an absolute measurement (10). However, the interaction between a fluorescent probe and its target under a certain environmental condition will generate fluorescence lifetime changes that often reflect the structural alterations caused by the probe–target interaction. A previous study with two-photon time-resolved fluorescent microscopy showed that fluorescent probes have different lifetimes when incorporated into different cellular organelles and cytoplasm (11).

The development of phase-sensitive FCM (PS-FCM) (12–15) has advanced the time-resolved fluorescence measurement so as to allow rapid detection and quantitation at the single-cell level with large cell populations. The PS-FCM system uses a frequency-domain method of measuring time-resolved fluorescence from cells and particles labeled with fluorescent probes. It measures lifetimes from phase shift between intensity-modulated excitation and the corresponding emission by using real-time analog phase-sensitive detection and can separate signals from simultaneous fluorescence emissions in cells labeled with fluorochromes having different lifetimes. The phase-sensitive detection of lifetime also can be used to resolve the emissions of multiple fluorochrome labels with overlapping emission spectra that cannot be resolved by conventional FCM methodology (16). Combining phase-sensitive detection with FCM provides the capability of multiparameter measurements that allows the correlation of lifetime with many other cellular measurements and thus provides additional information about structural changes during normal or abnormal cellular processes. Previous studies with PS-FCM have demonstrated the usefulness of fluorescence lifetime to monitor DNA damage, such as that induced by apoptosis (1,17) and the maturation of testicular cells (1), and have shown the different lifetimes of one probe when bound to cellular double-stranded DNA and RNA (1,2).

Ethidium bromide (EB) and propidium iodide (PI) are commonly used phenanthridine dyes that bind to double helical nucleic acid structures. Various ethidium derivatives, including homo- and heterodimers such as ethidium homodimer (EthD-1), have been developed to enhance the intensity and/or binding affinity (18). These nucleic acid intercalating dyes bind to the double helical structures of DNA and RNA, so hereafter we use the terms *DNA* and *RNA* to refer to the double-stranded structures. Assaying the fluorescence lifetimes of dyes that bind to cellular DNA and RNA provides insight for selection of the dyes with significant differences in lifetime and allow resolution of cellular DNA and RNA content simultaneously based on the lifetime difference with phase-sensitive detection. As an expansion of our previous studies, the current studies with PI, EB, and EthD-1 were performed to determine the influence of a number of factors that could affect the fluorescence lifetime. These factors include the differences in dye structures, interaction of dye with cellular DNA and RNA, the staining concentration, and the analyzing conditions. Four sets of experimental data are presented: (a) multiparameter measurement capability of PS-FCM and the correlation of fluorescence lifetime with other descriptors; (b) the specificity of sample analyses in dye-containing staining solution and dye-free phosphate-buffered-saline (PBS); (c) the fluorescence lifetime values of PI, EB, and EthD-1 when complexed with cellular DNA, RNA, or both; and (d) the correlation of fluorescence lifetime and intensity of DNA-bound dye analyzed at various nucleotide-to-dye ratios. The bivariate distribution obtained with PS-FCM demonstrated the accuracy to measure and quantitate cellular DNA and RNA contents simultaneously.

MATERIALS AND METHODS

Cell Culture

Human promyelocytic leukemia (HL-60) cells were maintained in a continuous suspension culture of RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1.25 mM of L-glutamine at 37°C in T-175 tissue culture flasks. The population doubling time was 17–19 h when cell densities were maintained at 50,000 – 400,000/ml.

Ethanol Fixation and Sample Staining

HL-60 cells were harvested from suspension culture by centrifugation at 200*g* for 5 min at 5°C and fixed in 80% ethanol/PBS at a density of 1×10^6 cells/ml for at least

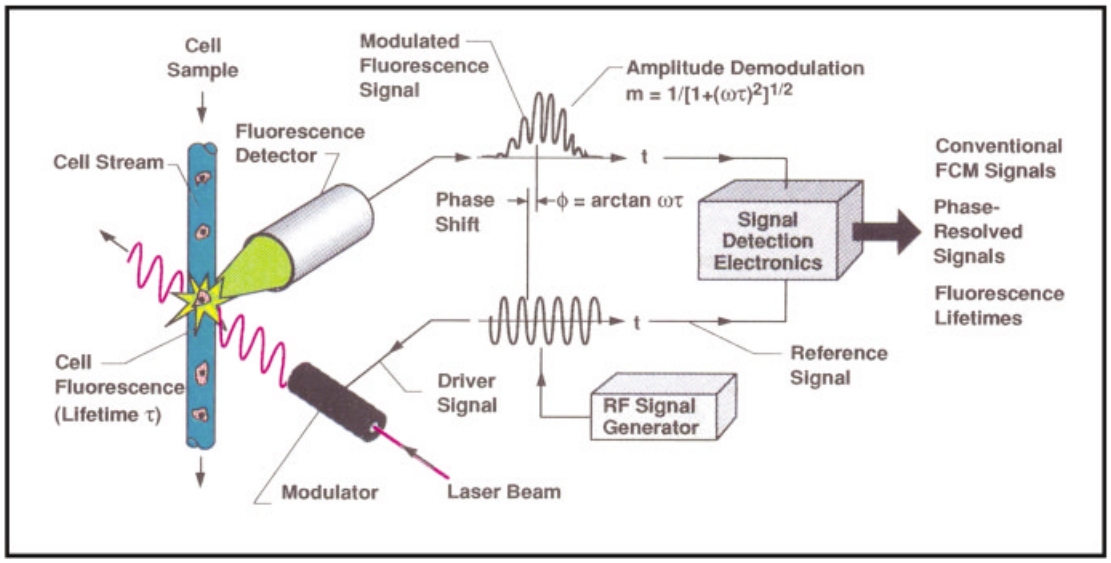

FIG. 1. Conceptual diagram of the phase-sensitive flow cytometer that measures conventional flow cytometry (FCM), phase-resolved fluorescence, and fluorescence lifetime signals. The stained cells intersect with a modulated laser beam and emit modulated fluorescence signals. The phase shift of the signal with respect to the reference signal provides information for determining absolute lifetime values and the phase-resolved signals. Conventional FCM measurements also are obtained simultaneously for multiparameter analysis. RF, radiofrequency.

24 h at −20°C before additional processes. Before staining, cells were rinsed from the fixative and treated with 1 mg/ml of RNase (Sigma, St. Louis, MO) at 37°C for 1 h for DNA analysis or with 1 mg/ml of DNase I (Sigma) in the presence of 5 mM $MgCl_2$ at 37°C for 1 h for RNA analysis. Untreated samples were incubated with 5 mM of $MgCl_2$ in PBS at 37°C for 1 h for subsequent DNA and RNA analysis, thus resembling the consistent condition that cells encounter. After enzyme digestion, cells were stained at a density of 1×10^6 cells/ml for 2 h at room temperature with one of the three fluorochromes, EB (Sigma), PI (Molecular Probes, Eugene, OR), or EthD-1 (Molecular Probes), at the concentrations indicated in the Results and figure captions. For experiments testing the effects of different concentrations on fluorescence lifetime, the concentrations of each fluorophore were chosen so that all the dyes were tested and compared at equal molar concentrations. Cell samples, as indicated in the Results, were analyzed in the original staining solution or centrifuged, washed, and resuspended in PBS for analysis in the dye-free solution. For EthD-1 staining, all samples were incubated at 37°C for 2 h before analysis by PS-FCM (19).

Conventional Flow Cytometry

Nucleic acid content histogram analysis of PI-stained HL-60 cells in staining solution or in dye-free PBS was performed on a FACS Calibur flow cytometer (Becton Dickson, San Jose, CA) using CELLQuest software for data acquisition and analysis. Stained cells were excited at 488 nm and the fluorescence emission was measured at a wavelength of 564–606 nm.

Phase-Sensitive Flow Cytometry

Fluorescence lifetime and phase-resolved fluorescence measurements were performed on the Los Alamos phase-sensitive flow cytometer, as previously described (13, 20) and illustrated in Figure 1. Stained cells were excited with an argon ion laser (Model 2025-05, Spectra Physics, Palo Alto, CA) with a sinusoidally modulated intensity at 10 MHz. Excitation wavelengths used for the various fluorochromes were 488 nm for EB and PI and 514 nm for EthD-1. The intensity modulation was accomplished by using a Model 350 electro-optic modulator (Conoptics, Danbury, CT). The fluorescence emission from the cells was detected, and the modulated fluorescence signal was processed by analog phase-sensitive signal detection electronics to obtain conventional FCM signals, phase-resolved fluorescence, and fluorescence lifetime signals based on the phase shift. Fluorescence lifetime measurements on RNase and DNase enzymatically treated and untreated cells labeled with PI, EB, and EthD-1 were made by using a dual-channel phase-sensitive detector arrangement of the signal processing electronics shown in Figure 1 for generating the ratio of the sine and cosine phase-shift signals, i.e., fluorescence lifetime (20). DNA check alignment fluorespheres (lifetime, 7 ns; Beckman-Coulter, Miami, FL) were used to calibrate the PS-FCM. Similarly, phase-resolved fluorescence measurements were made by using a slightly different dual-channel phase-sensitive detector arrangement (13) to resolve relative DNA and RNA contents in non-enzymatically treated EthD-1–labeled cells. RNase- and DNase-treated cells labeled with EthD-1 were used as controls to adjust the phase shifts (null points) of the dual-channel phase-sensitive detector chan-

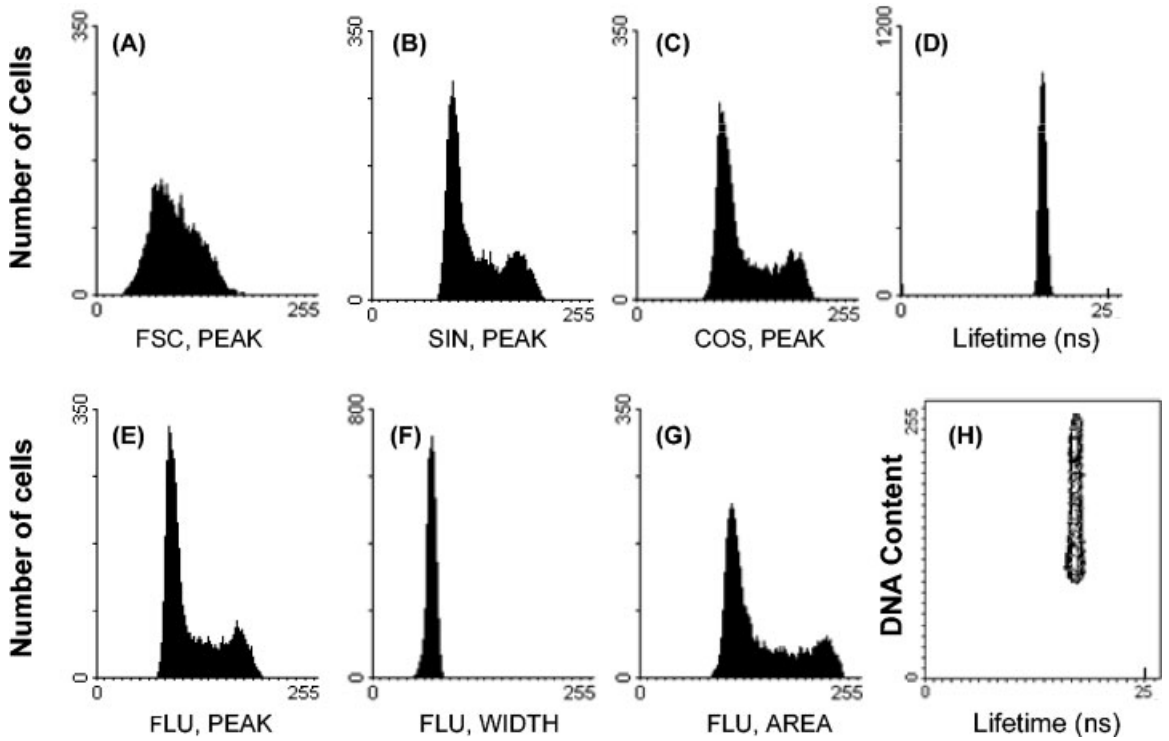

FIG. 2. Cell number frequency histograms simultaneously obtained from multiparameter analyses by phase-sensitive flow cytometry. HL-60 cells were treated with RNase before propidium iodide (PI) staining and analyzed in staining buffer. **A:** Forward light scatter (FSC). **B:** Sine signal (SIN). **C:** Cosine signal (COS). **D:** Lifetime. **E–G:** Histograms of the fluorescence peak (FLU, PEAK), the fluorescence width (FLU, WIDTH), and the fluorescence area (FLU, AREA), as derived by DiDAC signal processing from the fluorescence intensity signals of the DNA-bound PI in stained cells. **H:** Bivariate contour profile of DNA content and fluorescence lifetime.

nels before quantifying DNA and RNA contents of the non-enzymatically untreated EthD-1–labeled sample. The Digital Data Acquisition and Control (DiDAC) system, developed by the National Flow Cytometry Center at Los Alamos National Laboratory, was used to collect the data and convert the analog signals to digital information to derive the desired parameters. In the DiDAC system, amplified sensor signals are digitized continuously at high (20 MHz) rates with 10-bit accuracy. When data collection is completed, all data are stored as permanent files in FCS 3.0 format for subsequent processing and display (21).

RESULTS

Multiparameter Measurement Capacity of PS-FCM

One main advantage of PS-FCM over microscope-based fluorescence lifetime measurement is the speed and sensitivity for performing simultaneous multiparameter analysis, which allows the correlation of fluorescence lifetime analysis to other parameters routinely obtained by conventional FCM. RNase treated HL-60 cells stained with 7.5 μM of PI were analyzed first, and the cell number frequency histograms for various parameters obtained from PS-FCM analyses are shown in Figure 2A to 2D, respectively: the forward angle light scatter for linear distribution of cell size for the cell population; the peak value of sine signal; the peak value of the cosine signal; and the fluorescence decay time (lifetime) in nanoseconds, which is the tangent of the phase shift (ϕ) value calculated from electronic ratio analysis of the sine and cosine signals. The DNA-bound PI fluorescence lifetime obtained in this study was 16.77 ns (Fig. 2D), consistent with established values from spectroscopic studies (1,18). The small histogram coefficient of variation (CV) of G_0/G_1 cells indicates the uniformity of lifetime of PI over the cell population. Figure 2E through 2G, respectively, shows the histograms of the fluorescence peak, the fluorescence width, and the fluorescence area, as derived by DiDAC signal processing from the fluorescence intensity signals of the DNA-bound PI in stained cells. Figure 2H is a bivariate display of DNA content and fluorescence lifetime, showing the relation of DNA-bound PI fluorochrome lifetime to cell cycle distribution in HL-60 cells.

Comparison of Nucleic Acid Content Histograms Obtained From Samples Analyzed in the Staining and Dye-Free Solutions

Most FCM DNA content analyses are performed in the staining solution under equilibrium staining conditions that produce greater fluorescence intensity and lower CV values. Under this condition, high- and low-affinity bindings are available for dye–nucleic acid interactions. Removing dye molecules from the surrounding environment

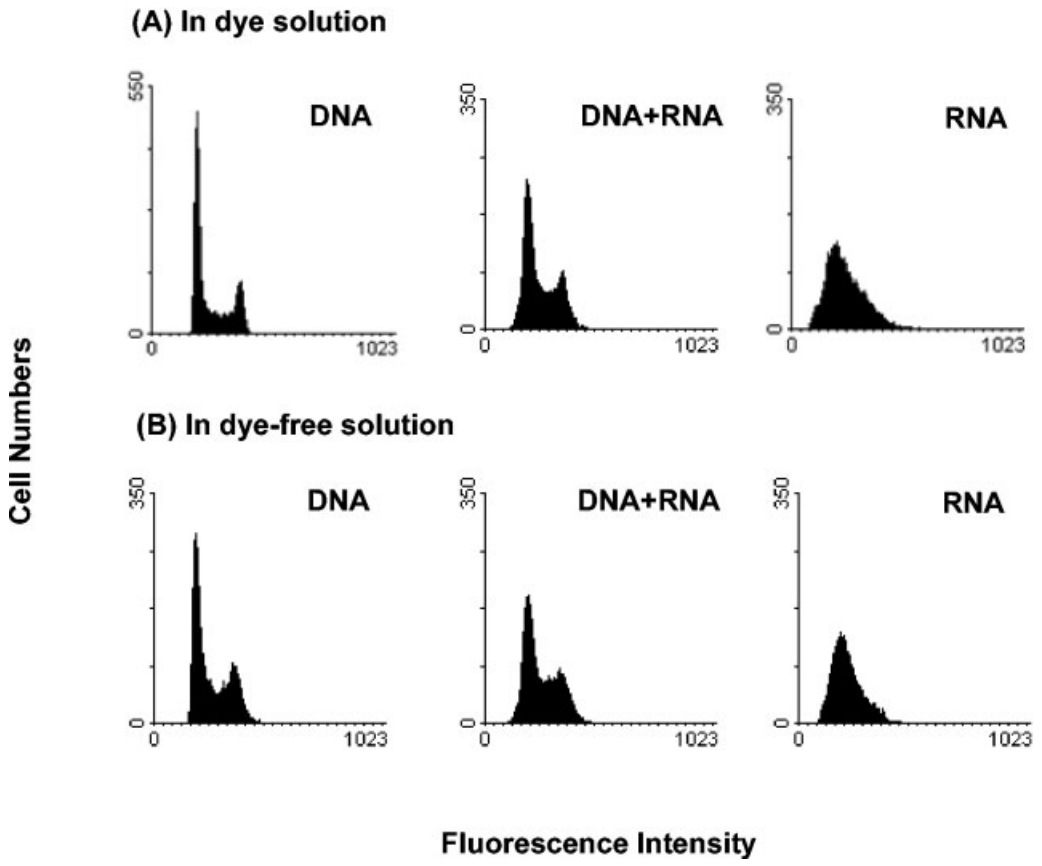

FIG. 3. Nucleic acid content histograms obtained in dye solution (**A**) or dye-free phosphate buffered saline (**B**). HL-60 cells were treated with RNase, no enzyme, or DNase before staining with 7.5 μm of propidium iodide.

removes low-affinity binding after the equilibrium binding is established. Analyzing samples in dye-free solution thus may affect the fluorescence intensity and/or lifetime and the resolution reflected from the CV values of fluorescent measurements. The data presented in Figure 3 were obtained from the analysis performed in dye-free solution after the initial staining, because improved resolution was found under this condition. To evaluate the specificity of the fluorochrome in dye-free solution, we compared the nucleic acid content profiles of samples analyzed in staining solution or rinsed and then analyzed in dye-free PBS. RNase-treated, untreated, or DNase-treated HL-60 cells were stained with 7.5 μM of PI and analyzed in dye (Fig. 3A) or dye-free (Fig. 3B) solution. DNA histogram profiles from either analyzing condition showed a typical cell cycle distribution, with distinguishable G_0/G_1, S, and G_2/M peaks. The RNA histograms displayed an expected increase in RNA content that correlated with cell size changes during the cell cycle. The histograms obtained from non–enzyme-treated cell samples resulted from the binding of PI to cellular DNA and RNA. The profiles of these histograms more closely resembled DNA histogram features, indicating the higher binding affinity of PI to DNA. The corresponding histograms for DNA, DNA and RNA, and RNA were similar for analyses from both conditions. Although the CV value for G_1-phase cell DNA content in dye-free sample (4.2%) was greater than the value obtained for analysis in dye-containing solution (2.1%), the structures of the DNA histograms were very similar, indicating that the specificity of dye–DNA interaction was conserved.

Differences in the Fluorescence Lifetimes of Selected Phenanthridinium Dye Bound to Cellular DNA, RNA, or DNA/RNA in Dye-Free PBS

The previous study using PS-FCM showed a difference in PI fluorescence lifetime when bound to cellular DNA and RNA at 15.6 and 17.2 ns, respectively (1). In this study, three structurally similar phenanthridine dyes, PI, EB, and EthD-1, were chosen for the fluorescence lifetime differences. Fixed HL-60 cell samples were treated initially with DNase, RNase, or control PBS to allow selective staining of cellular RNA, DNA, or both. PS-FCM analysis was carried out under the dye-free condition for the lifetime measurement. The data presented in Figure 4 represent the results of at least three independent experiments. At an equal molar concentration of 7.5 μM of dye, the fluorescence lifetime differences for DNA and RNA were evident with all three dyes. The lifetime values obtained for untreated stained samples were between the values

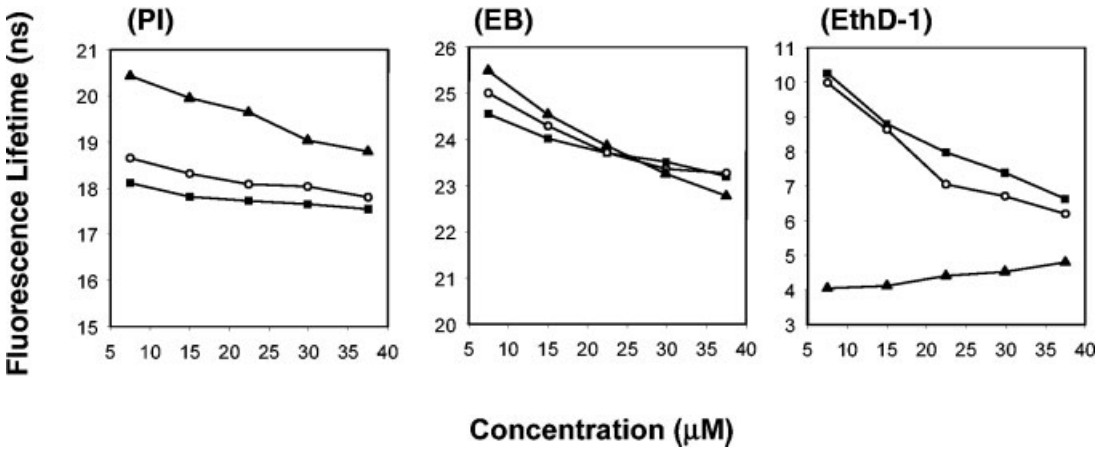

FIG. 4. Comparison of fluorescence lifetimes of selected ethidium derivatives bound to cellular DNA (solid square), RNA (solid triangle), or DNA and RNA (open circle) in dye-free phosphate buffered saline. HL-60 cells were treated with nucleases as described in Materials and Methods to allow each fluorochrome to bind to cellular DNA, RNA, or both at the indicated dye concentrations. Cells were then stained with propidium iodide (PI), ethidium bromide (EB), and ethidium homodimer (EthD-1) at the indicated concentrations (μM).

from DNA- and RNA-bound dyes but were always closer to the DNA-bound dye value, presumably due to the greater binding affinity of the dye to DNA. However, due to the difference in the chemical structure of the dyes, the lifetime values appeared to decrease with increase in the dye concentration. With 7.5 μM of PI, RNA-bound dye showed the longest lifetime (20.43 ns) compared with the values obtained for DNA-bound dye (18.11 ns) or untreated (18.65 ns) samples. At the same molar concentration, EB behaved similarly to PI by producing the longest lifetime with RNA-bound dye (25.49 ns), the shortest lifetime with DNA-bound dye (24.55 ns), and an intermediate lifetime in untreated cells (25.00 ns). A significant contrast was noted with the dimeric dye molecule, EthD-1. The lifetimes from all conditions were much shorter than those from the monomers. Further, the DNA–dye complex yielded the logest lifetime (10.26 ns), and the RNA–dye complex yielded the shortest lifetime (4.04 ns). The EthD-1–stained untreated cells yielded a lifetime value very close to the DNA-bound samples (9.98 ns). This result may indicate the dominant binding of dye to DNA, which is consistent with the design of this dimeric fluorescent molecule.

The effect of dye concentration on fluorescence lifetime was also analyzed. The HL-60 cell samples were stained with increasing concentrations at equal molarity for all three dyes, ranging from 7.5 to 37.5 μM, and analyzed in dye-free PBS solution (Fig. 4). In general, the lifetime was reduced with the increase of dye concentration. The fluorescence lifetime of PI bound to RNA appeared consistently longer than that of DNA-bound dye across the entire range examined, with the values of untreated samples were between those values but closer to the DNA-bound values. EB showed longest lifetimes when complexed with either nucleic acid compared with the PI and EthD-1. At lower concentrations, the RNA–EB complex had a longer lifetime than did the DNA–EB complex, and the lifetime decreased when the dye concentrations increased, similar to the trend noted with PI. However, the magnitude of decrease was more apparent with RNA-bound dye, resulting in a crossover of two curves and a higher value from the DNA–dye complex than from RNA–dye complex at high concentration. EthD-1 also showed a different pattern of lifetime change in response to the increase of dye concentration. The lifetimes of DNA–dye and DNA/RNA–dye complexes were reduced considerably with increasing dye concentration, but the RNA-bound dye showed only a minimal increase. The dimerization in EthD-1 appeared to induce a different binding affinity for DNA and RNA that also resulted in a reversal in fluorescence lifetime values in DNA and RNA versus EB.

Among all the dyes examined, EthD-1 at 7.5 μM produced the largest separation of lifetime values of DNA- and RNA-bound dyes. This well-resolved separation provided extremely accurate resolution of cellular DNA and RNA contents when using phase-resolved detection.

Phase-Resolved Separation of Cellular DNA- and RNA-Bound Fluorescence Emissions

Conventional FCM measurements of structural and functional properties of individual cells are based primarily on labeling cells with multiple fluorochromes for correlated analysis of biomolecules such as DNA, RNA, and proteins. Multicolor detection methods using optical filters are used for measuring multiple fluorochromes emissions when spectra are sufficiently separated and only one excitation source is required. Partly overlapping spectral emissions are resolved by electronic compensation or computational methods. Multiple excitation sources can be employed sequentially for fluorochromes with separated excitation spectra to spatially resolve overlapping emission signals. However, these methods cannot resolve the signals from fluorescence emissions that greatly overlap.

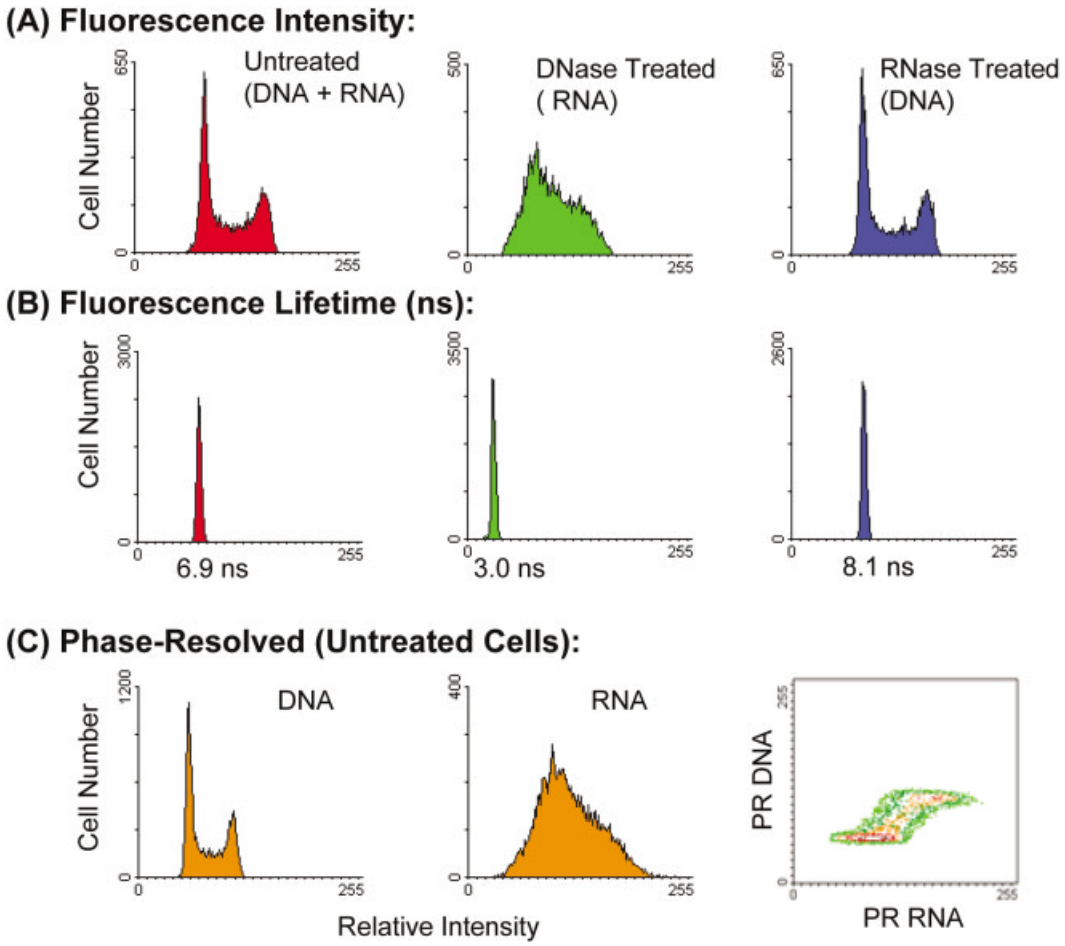

FIG. 5. Phase-resolved cellular DNA and RNA contents based on ethidium homodimer (EthD-1) fluorescence lifetime signatures. **A:** Nucleic acid histograms of EthD-1 staining in untreated, DNase-treated, and RNase-treated cells. **B:** EthD-1 lifetimes in nanoseconds obtained from cells with corresponding treatments. **C:** Phase-resolved (PR) cellular DNA and RNA contents obtained from untreated cells by using EthD-1 lifetimes as signatures and the bivariate display of the relative contents of cellular DNA and RNA.

Fluorescence lifetimes provide another means to discriminate among fluorescent markers. For example, the lifetime difference in EthD-1 with DNA and RNA provides distinct signatures for separating two fluorescence signals by phase-sensitive detection. In principle, phase suppression is applied for separating two fluorescence emission signals with different lifetimes by phase-sensitive detection (13). The output of phase-sensitive detection is a function of intensities of two signals, the modulation factors, and the two-phase shifts due to two lifetimes from one modulated source. This results in one signal being passed and the other being nulled. Both signals are therefore resolved. When fluorescence signals are processed by two phase-sensitive detectors operating in parallel, then the contributions to the total fluorescence signals are resolved by setting two detectors at angles different from the reference. By using phase-resolved separation of fluorescence emission signals, we were able to resolve the relative contents of cellular DNA and RNA bound to the dye EthD-1.

Figure 5A shows the fluorescence intensity histograms when EthD-1 (15 μM) was bound to untreated, DNase-treated, and RNase-treated cells. Figure 5B shows the different lifetime values of EthD-1 when bound to corresponding cell samples. The lifetimes were 6.9, 3.0, and 8.1 ns, respectively. By using the lifetime values of RNase- and DNase-treated cells as the signatures and nulling the corresponding signals from these control cells, the contribution of DNA- or RNA-bound EthD-1 fluorescence signals was resolved (Fig. 5C). The histograms showed an anticipated distribution of both types of nucleic acid–bound dye. The DNA-bound dye showed a typical cycling cell DNA distribution, with distinct G_1/G_0, S, and G_2/M phases. The CV values for G_1-phase cell DNA content were 2.5–3.5%, depending on different experimental settings. The bivariate plot of DNA and RNA shows a typical correlation of the two types of nucleic acids in the exponentially growing cell population.

Fluorescence Lifetime and Intensity Change With Dye Concentration in Staining Solution and Dye-Free PBS

Fluorescence lifetime is not directly correlated with the fluorescence intensity. Although increasing the dye con-

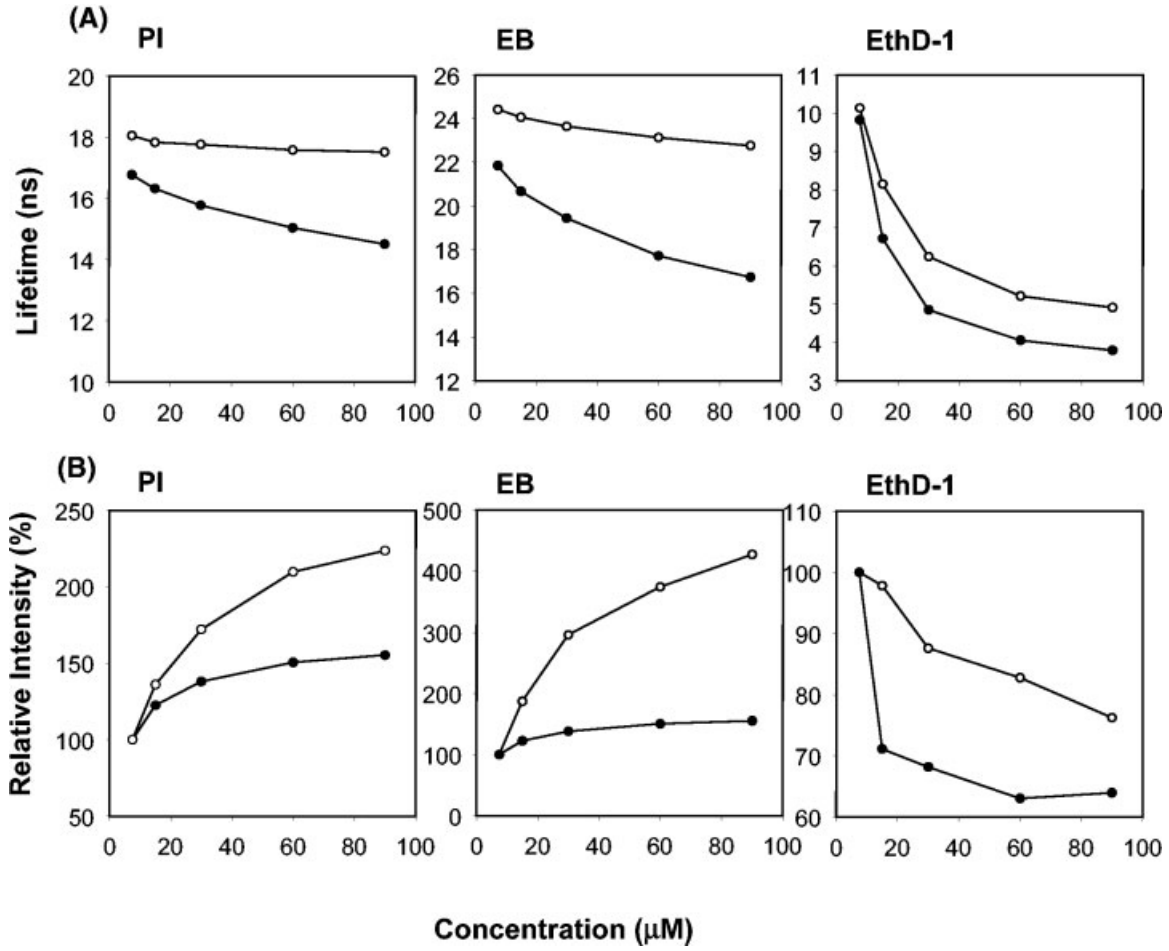

FIG. 6. Fluorescence lifetime (**A**) and intensity (**B**) composites for fixed and RNase-treated HL-60 cells stained with increasing concentrations of propidium iodide (PI), ethidium bromide (EB), and ethidium homodimer (EthD-1). Samples were analyzed in staining solution (solid circles) or dye-free phosphate buffered saline after rinsing from the dye solution (open circles).

centration does increase fluorescence intensity in some cases, there is a concomitant decrease in the fluorescence lifetime of a number of DNA-intercalating fluorochromes, including PI (1).

We examined the fluorescence lifetimes of the three dyes bound to cellular DNA at increasing staining concentrations and analyzed in the staining buffer or dye-free PBS solution. All three dyes displayed the common feature of the lifetime value obtained in dye-free solution being higher than that obtained in staining buffer (Fig. 6A). In PI, the initial lifetime values of 18.05 ns in dye-free solution and 16.77 ns in staining buffer solution were observed at the lowest concentration tested. Over the concentration range of 7.5–90 μM, PI lifetime decreased with increasing concentrations in both conditions. However, the magnitude of decrease was more pronounced when samples were analyzed in staining buffer. EB showed a greater decrease in lifetime under both conditions, and similar to PI, the decrease in the staining solution appeared more pronounced. Ethidium homodimers are designed to enhance DNA-binding affinity. The lifetimes of EthD-1 obtained from both conditions decreased considerably after increasing the staining concentrations and reached a plateau at high concentration (~90 μM). Although the lifetime values of samples analyzed in dye-free buffer remained consistently higher than those in staining solution, the reduction in both conditions showed a clear parallel pattern and the difference of the lifetime values obtained from both conditions remained small compared with PI or EB. EthD-1 retains a strong binding affinity for DNA during analysis in dye-free solution; therefore, the dye–DNA base-pair ratio remained similar in both analysis conditions. When EthD-2 was examined under similar conditions, the data showed a close resemblance to that obtained with EthD-1 (data not shown), indicating that structural similarity contributed to the fluorescence lifetime in both analyzing conditions.

Fluorescence intensities of dyes under the same conditions as above were analyzed with the FACS Calibur to establish the relation between the lifetime and intensity of these dyes. The fluorescence intensity was measured as FL-2 height on a log scale so that a large span of diverse

intensity could be compared directly. Figure 6B shows data plotted on a relative scale, with an arbitrary intensity setting at 100% for 7.5 μM for each dye. With PI and EB, the intensity increases with the increasing dye concentration; with EthD-1, the fluorescence intensity decreases over the same concentration range. At a concentration range of 0.75–7.5 μM, the DNA-bound EthD-1 intensity increased with increased dye concentrations (data not shown).

DISCUSSION

Cellular DNA and RNA content measurements and their ratios indicate the cell proliferation stage, the translational potential and phenotype of the differentiated cells, and clinical grading of various tumor types (4–6). PS-FCM provides a new approach for measuring cellular DNA and RNA contents simultaneously in individual cells based on fluorescence lifetimes of fluorophores.

Unbound dyes have characteristic fluorescence lifetimes depending on their structures. However, the lifetimes of target-bound probes often reflect the variations in fluorophore–target interactions resulting from changes in the structure of the fluorochromes and/or target molecules and environmental changes. The fluorescence lifetimes of three ethidium probes measured by PS-FCM were significantly different when bound to cellular DNA and RNA. The difference in lifetime offered a new means to discriminate the binding of probe to different intracellular macromolecules. Monochromic detection of cellular DNA and RNA contents provides an opportunity for additional labeling with fluorescent probes for specific proteins.

The differences in lifetime values depend on the staining concentration and analysis condition. For many fluorophores, intermolecular interactions and energy transfer between molecules in close proximity result in self-quenching. This self-quenching phenomenon was also observed in fluorochrome-conjugated antibodies to cell surface antigens, showing that the fluorescence lifetime diminishes as a function of antibody-labeling concentration (22). The fluorescence lifetimes of free EB and DNA-intercalated EB have been established in various studies to be 1.6–1.8 ns and 20–25 ns, respectively (23,24). In one study (25), a reduced DNA-bound EB lifetime of 10–15 ns was reported, and the investigators suggested that this reduced lifetime was due to a high ratio of dye to DNA base pair. They also implied that the spatial distribution of the intercalated ethidiums on the DNA lattice is the primary factor influencing the appearance of this complexed lifetime. The self-quenching of ethidium molecules intercalating at closely spaced sites could differ with increased stain concentrations. The increase in dye concentration would enhance the quenching caused by the higher ratio of dye molecules per nucleic acid base pair and thus reduce the fluorescence lifetimes.

In most FCM studies, DNA content analysis of stained cells is performed in the staining solution, which allows high- and low-affinity bindings of the dye to nucleic acid under equilibrium conditions. This analysis procedure is favored because it produces higher fluorescence intensities and lower CVs. When stained cell samples are analyzed in dye-free solution, only the high-affinity binding is maintained, and the dye per DNA base-pair ratio is presumably reduced. This reduced dye–DNA ratio, based on the observation by Hochstrasser and Millar (25), may significantly influence the fluorescence lifetime.

The capability of our frequency-domain detection was limited to single exponential decay of fluorescence intensity. When measured by single-photon counting, the lifetime analysis of DNase-treated cells stained with EthD-1 displayed two-exponential decay (data not shown), which may be due to heterogeneity of cellular RNA conformation and of the interaction between the dye and nucleic acids. However, this multiexponential decay was not displayed in our current study, and our results may be the overall output of the RNA-bound dye at different states.

Collectively, these experiments demonstrated that the fluorescence lifetime analyzed in the dye-free solution produced higher values due to the low dye–dye proximity. The structural difference of the dye molecules may play an important role in determining the fluorescence lifetime.

Fluorescence lifetime is influenced by a number of factors that can be used to probe the structure of DNA in cells and chromosomes under a variety of environmental conditions. Further lifetime analysis may provide a probe for chromatin structural changes during normal and abnormal cellular processes that would allow analysis for progressive changes in chromatin structure and individual chromosomes in selected biological systems. PS-FCM measurement of fluorescence lifetime and phase-resolved emission detection is a relatively new technique, and understanding the advantages and limitations of the methodology will expand its applications. The development of this new methodology will be beneficial to comprehend important relevant biological studies. Ethidium derivatives have binding properties analogous to many cancer chemotherapeutic agents; therefore, studying the behavior of these compounds interacting with cell nucleic acids can be useful to the study of the interaction of drug with cellular nucleic acids. For example, we found that the fluorescence lifetime of a chemotherapeutic agent, ellipticine, is altered when bound to cellular double-stranded DNA or RNA (26), indicating the potential for monitoring drug uptake or drug–target interactions with fluorescence lifetime changes.

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