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Title: FLOW CYTOMETRIC FLUORESCENCE LIFETIME ANALYSIS OF DNA BINDING FLUOROCHROMES

Author(s): Harry A. Crissman, 079117 B-2
Hong Helen Cui, 175226 B-2
John A. Steinkamp, 077743 B-2

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Flow Cytometric Fluorescence Lifetime Analysis of DNA Binding Fluorochromes

Harry A. Crissman, H. Helen Cui and John A. Steinkamp
Bioscience Division, Los Alamos National Laboratory
Los Alamos, NM USA

Introduction

Most flow cytometry (FCM) applications monitor fluorescence intensity to quantitate the various cellular parameters; however, the fluorescence emission also contains information relative to the fluorescence lifetime. Recent developments in FCM (Pinsky et al., 1993; Steinkamp & Crissman, 1993; Steinkamp et al., 1993), provide for the measurement of fluorescence lifetime which is also commonly referred to as fluorescence decay, or the time interval in which a fluorochrome remains in the excited state. Many unbound fluorochromes have characteristic lifetime values that are determined by their molecular structure; however, when the probe becomes bound, the lifetime value is influenced by a number of factors that affect the probe interaction with a target molecule. Monitoring the changes in the lifetime of the probe yields information relating to the molecular conformation, the functional state or activity of the molecular target. In addition, the lifetime values can be used as signatures to resolve the emissions of multiple fluorochrome labels with overlapping emission spectra that cannot be resolved by conventional FCM methodology. Such strategies can increase the number of fluorochrome combinations used in a flow cytometer with a single excitation source.

Our studies demonstrate various applications of lifetime measurements for the analysis of the binding of different fluorochromes to DNA in single cells. Data presented in this session will show the utility of lifetime measurements for monitoring changes in chromatin structure associated with cell cycle progression, cellular differentiation, or DNA damage, such as induced during apoptosis. Several studies show that dyes with specificity for nucleic acids display different lifetime values when bound to DNA or to dsRNA. The Phase Sensitive Flow Cytometer is a multiparameter instrument, capable of performing lifetime measurements in conjunction with all the conventional FCM measurements. Future modifications of this instrumentation will provide the capability for simultaneously measuring multiple lifetimes, thereby significantly enhancing the subcellular resolution of the multiple complexes of fluorescent compounds, such as chemotherapeutic agents, in single cells (Sailer et al., 1997b).

Instrumentation

Fluorescence lifetime measurements were performed on the Phase-Sensitive Flow Cytometer developed at the Los Alamos National Laboratory (Steinkamp et al. 1993, Steinkamp & Crissman, 1993). Briefly, an argon ion laser with a modulated beam (sine wave) produced by an electro-optic modulator is used to excite stained cells. The modulated fluorescence signals from the cells are detected by a photomultiplier tube, and processed by analog phase-sensitive detection electronics to obtain the phase shift and lifetime at the set modulation frequency. Coulter DNA Check alignment fluorospheres (7 ns lifetime) are used to calibrate the flow cytometer.

Results and Discussion

Fluorescence Lifetime Determinations of DNA-bound Dyes by Flow Cytometry:

Studies utilizing combinations of fluorochromes with overlapping emission spectra and different DNA base-pair specificity, such as the G-C binding 7-aminoactinomycin D (7-AAD) and the A-T binding ethidium homodimer II (EthD II), can be used to monitor cell cycle or functionally related chromatin changes. We have performed lifetime analysis on ethanol fixed, RNase treated Chinese hamster ovary (CHO) cells stained with a number of DNA binding fluorochromes (Sailer et al., 1996; 1997a) to evaluate the accuracy of our FCM lifetime measurements, and to determine the potential for separating fluorochromes with overlapping emissions by lifetime. FCM analysis determined the lifetime values for the probes as: 2.7 nanoseconds (ns) for DAPI, 2.25 ns for Hoechst 33342 (HO), 1.5 ns for mithramycin (MI), 0.77 ns for 7-AAD, 10.5 ns for EthD II, 2.4 ns for TOTO (Molecular Probes, Inc.), 2.25 ns for YOYO (Molecular Probes, Inc), 15.0 ns for propidium iodide (PI), and 19.5 ns for ethidium bromide (EB). Values obtained were comparable to those previously reported from spectroscopic or microscopic and show the accuracy of the flow cytometric methodology. Furthermore, these data demonstrate that the combination of some fluorescent probes with overlapping emission spectra, such as PI, EB and EthD II, is possible since fluorescence emissions can be resolved based on the differences in the lifetime values.

Differences in Fluorescence Lifetime of PI and EB when bound to DNA or dsRNA:

Previous microscopic studies showed differences in the lifetime of EB when bound to nuclei, nucleoli, or cytoplasmic RNA. Our FCM lifetime studies provided the distinction of DNA and dsRNA from the observed differences in the fluorescence lifetime of either PI or EB when bound to either nucleic acid (Cui et al, 2001; Sailer et al., 1998). Data obtained revealed a lifetime value of 15.6 ns for DNA-bound PI in ethanol-fixed HL-60 cells treated with RNase prior to staining and analysis, while a value of 17.2 ns was noted for dsRNA-bound PI in cells treated with DNase. An intermediate lifetime value of 16.4 ns was obtained for PI bound to untreated samples. Lifetime differences of a similar magnitude were noted when EB was used as the probe in similar studies. Results obtained from the untreated sample represent the average lifetime value for both DNA and dsRNA so an intermediate lifetime value is to be expected. These differences in lifetime relate to the differences in the structure of the nucleic acid complexes, and reflect the dissimilarities in the intercalation of these fluorochromes into DNA or dsRNA.

Lifetime Discrimination of Apoptotic Cells:

The induction of apoptosis results in the activation of cellular endonucleases, which cleave DNA at nucleosomal linker regions in chromatin. Fluorescence lifetime of EB bound to apoptotic sub-populations was noted to be significantly reduced compared to the non-apoptotic portion of the population. A bivariate profile of fluorescence lifetime versus DNA content was obtained from analysis of EB stained, HL-60 cell populations induced to undergo apoptosis following 3h treatment with camptothecin. These data showed a significant reduction in both DNA content and EB lifetime (18.0 ns) for the apoptotic cells compared to the non-apoptotic portion of the population (22.0 ns, Sailer et al., 1996). Similar results have been obtained with samples stained with PI or EthD II. When cells were labeled with non-intercalating DNA fluorochromes, such as HO or DAPI, the apoptotic subpopulation showed only decreased fluorescence intensity, but not the dramatic decrease in lifetime values noted for the intercalating dyes (Sailer et al, 1997b). It would appear that the binding to the A-T regions on DNA by HO and DAPI is less sensitive to chromatin degradation in apoptotic cells, as reflected by modifications in lifetime.

Cell Cycle Related Changes in Fluorescence Lifetime:

Progression of cells through the various phases of the cell cycle involves significant transitions in chromatin organization. Using bivariate DNA content and lifetime analysis, we recently detected and characterized a unique subpopulation of cells with prominent reduced EB-lifetime, located at very early S phase in cultures of primary human skin fibroblast (HSF) cells, human lung fibroblasts and human bronchial epithelial cells. At 1 to 2 h following release of synchronized cells from G1/S phase, the subpopulation of cells with reduced EB lifetime enters S phase with lifetime values slightly elevated above the G1 phase cells in the population. At 3.0 h another set of cells has progressed through G1 and appears at the position of the initial subpopulation. There is a continued increase in EB lifetime as cells proceed to the G2/M phase at 6 h. Multiparameter DNA content, EB lifetime and immunofluorescent antibody analysis of cyclin D and cyclin E levels were performed on asynchronous cells. Gated EB lifetime analysis was used to provide data demonstrating that some of the cells in the subpopulation contain elevated levels of cyclin D, but the majority of the cells have elevated cyclin E, characteristic of cells at the G1/S boundary.

Cell Type Variations in Fluorescence Lifetime:

Cells from many species and lineage have notable differences in base sequences, base composition, DNA content and chromatin compaction. We used FCM to examine the fluorescence lifetime of EB bound to chromatin in four different cell lines; HL-60 cells, HSF cells, CHO cells, and HeLa cells. Cells were fixed in ethanol, treated with RNase, and stained prior to analysis. Cell numbers, dye concentration and analytical features were all kept constant, and experiments were repeated at least three different times. The fluorescence lifetime of EB bound to HL-60 cells was 21.13 ns, while in CHO cells the lifetime was 20.4 ns, 21.2 ns in HeLa cells, and 20.9 ns in HSF. These small differences in lifetime reflect some differences in chromatin organization and the EB binding characteristics in the various cell lines.

Fluorescence Lifetime Variation in Testicular Cell Subpopulations:

Fluorescence lifetime analyses were performed on PI-stained mouse testicular cell samples to determine the effects of changes in chromatin organization on the probe lifetime as cells traverse through different states of differentiation. Bivariate analysis identified the four major testicular sub-populations on the basis of DNA content and/or chromatin condensation and their corresponding PI-lifetime values. The DNA of the cells in both the 2C and 4C DNA content sub-populations is complexed with somatic histones, however the lifetime of PI bound to chromatin in the 2C compartment is reduced 1.5% to 15.71

ns compared to PI lifetime values for the 4C subpopulation of cells (15.95 ns). Following meiosis, the DNA in cells within the 1C compartment is associated with somatic histones, as well as other, testis-specific variants, and the lifetime of PI bound within this sub-population is further decreased 2.6% to 15.53 ns, compared to cells in the 4C sub-population. Further differentiation to testicular sperm cells involves the replacement of histones by species-specific protamines, which led to a 13.0% reduction in the lifetime of DNA-bound PI (13.88 ns) compared to the 4C sub-population. As sperm exit the testis and traverse the epididymis, further condensation and compaction occurs through the formation of disulfide links between and within protamine molecules, resulting in maximal chromatin condensation in the mature sperm in the vas deferens. The lifetime of DNA-bound PI in the mature sperm reached a minimum of 13.5 ns, a 15.4% reduction from the 4C sub-population. These data demonstrate the sensitivity of fluorescence lifetime measurements to detect differential interactions of the fluorochrome probe associated with alterations in DNA packaging during testicular differentiation and sperm cell maturation.

Decreased Lifetime of Hoechst 33342 Bound to DNA-Substituted BrdU:

In cell cycle studies we detected a decrease in Hoechst (HO) fluorescent lifetime when bound to BrdU incorporated DNA in cycling cells. These novel observations are the first to demonstrate that HO bound to DNA containing BrdU results in a decrease in HO lifetime, as predicted from spectroscopic studies. Bivariate profiles of HO lifetime and intensity demonstrated the detection of lifetime alterations in viable, synchronized CHO cells as early as 3.0 h following release from the G1/S phase boundary. By 6.0 h some of the labeled cells had divided and displayed both a further decreases in HO fluorescence and lifetime. These results indicate the bivariate profile of HO intensity and lifetime can be used to detect viable cycling and non-cycling cells with a single fluorochrome. Since lifetime can represent an absolute value, under a particular set of conditions, the levels of BrdU incorporation might be quantified from the level of reduction in HO lifetime.

Fluorescence Lifetime Analysis of Stained Chromosomes:

The Phase-Sensitive Flow Cytometer was used to analyze chromosomes isolated from Chinese hamster embryonic (line CCHE) cells stained with PI or EthD II. Using either stain, we are able to resolve all 10 CCHE chromosomes. PI bound to CCHE chromosomes had a fluorescence lifetime of 16.2 ns, while EthD II bound to the chromosomes had a lifetime of 13.8 ns. These fluorescence lifetime values are slightly higher than those obtained on fixed cells, and may reflect altered PI or EthD II intercalation in chromosomes compared to binding to nuclear DNA/chromatin. These results demonstrate the excellent resolution capabilities of the PSFCM and potential to provide for measurements of subcellular particles with the instrument.

Alterations in the Fluorescence Lifetime of Differentiating HL-60 Cells:

Treatment of HL-60 cells with a variety of selected agents can induce terminal differentiation. Dimethyl sulfoxide (DMSO) induces a granulocyte-like differentiation and leads to decreased stainability of nuclear DNA with intercalating fluorochromes. Chromatin changes accompanying differentiation are associated with the loss of HMG1 and HMG2 proteins and a decrease in histone H2A, leading to a decrease in the stability of the DNA-protein complex. Lifetime analysis of DNA-bound EB or EthD II were performed following differentiation of HL-60 cells in culture with 1.6% DMSO for a five day period. The fluorescence lifetime of DNA-bound EB was reduced as the cells differentiated, from a value of 21.5 ns for non-differentiated cells, to 20.8 ns for differentiated cells. Using EthD II, the lifetime values were decreased from 8.4 ns for non-differentiated cells to 8.1 ns for differentiated HL-60 cells. The organizational alterations in chromatin that occur during differentiation are known to limit access of the DNA to the intercalating probe, and thereby reduce the fluorescence intensity in these cells. But, the altered interaction of the fluorochromes with the DNA also led to an unanticipated decrease in the lifetime of these intercalating probes.

Changes in DNA Accessibility and Lifetime with Selective Removal of Different Histone Fractions:

Incremental increases in ionic strength lead to the stepwise removal of specific histones and a concomitant change in the accessibility of fluorochromes to cellular DNA. The relaxation of double-stranded DNA following removal of histones with increasing NaCl concentrations leads to a loss of fluorescence intensity and an increase in lifetime for intercalating fluorochromes such as PI and EB. In recent studies, we examined the changes in relative fluorescence intensity (RFI) and lifetime following the selective removal of histones with NaCl. At a NaCl concentration of 0.5 M, histone H1 is selectively removed from the linker regions between nucleosomes and the RFI of PI and EB was significantly decreased by 11.8% and 8.0%, respectively. However, there was a corresponding significant increase in the lifetime of both PI and EB, by 13.5% and 8.2%, respectively. Histones H2A and H2B are removed from the octomer core at a NaCl concentration of 1.0 M, and the RFI for PI was further decreased (14.6% compared to distilled water), while the RFI for EB did not significantly change beyond 0.5 M

NaCl. The lifetime for the fluorochromes increases to a maximum value at the 1.0 M NaCl concentration (17% and 12%, PI and EB respectively). At higher NaCl concentrations, the RFI for PI only further decreases slightly (18.1% of distilled water), while no further increases in lifetime were noted for either fluorochrome. Increasing NaCl concentrations, in addition to producing changes in chromatin structure following the removal of histones, could potentially lead to stabilization of PI and EB binding to DNA, producing a shift to higher lifetime values.

Summary

A new dimension has been added to multiparameter flow cytometric analysis through the recent development of techniques for rapidly measuring the fluorescence lifetime of probes bound to single cells. The lifetime measurements are made by phase-sensitive detection techniques in a flow cytometer that also analyzes multicolor fluorescence intensities and other optical properties of stained cells. Studies presented show that lifetime assays provide the potential for elucidating the microenvironment of the interaction of fluorochrome probes and subcellular target molecules. Alterations in the lifetime of DNA probes have been observed in cells in different phases of the cell cycle, in different cell types, in differentiating cells, and in apoptotic cells with damaged chromatin. Lifetime differences that were also noted for intercalating dyes bound to DNA and dsRNA, indicate significant modifications in the modes of binding and allow for correlated analysis of both conformational states and nucleic acid metabolism. Future developments in the technology will provide multiple lifetime assays for use in studies designed to detect and quantitate selected subcellular probe-complexes based on differences lifetime signatures. These novel assays will expand the applications for quantitative studies on the binding of various chemical agents to DNA and other molecular targets in cells, and further improve methods for rapid screening of chemotherapeutic agents or environmentally toxic compounds in single cells.

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