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Fluorescent Biomembrane Probe for Ratiometric Detection of Apoptosis

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Abstract: Herein, we developed the first ratiometric fluorescent probe for apoptosis detection. This probe incorporates selectively into the outer leaflet of the cell plasma membrane and senses the loss of the plasma membrane asymmetry occurring during the early steps of apoptosis. The high specificity to the plasma membranes was achieved by introduction into the probe of a membrane anchor, composed of a zwitterionic group and a long (dodecyl) hydrophobic tail. The fluorescence reporter of this probe is 4′-(diethylamino)-3-hydroxyflavone, which exhibits excited-state intramolecular proton transfer (ESIPT), resulting in two-band emission highly sensitive to the lipid composition of the biomembranes. Fluorescence spectroscopy, flow cytometry, and microscopy measurements show that the ratio of the two emission bands of the probe changes dramatically in response to apoptosis. This response reflects the changes in the lipid composition of the outer leaflet of the cell plasma membrane because of the exposure of the anionic phospholipids from the inner leaflet at the early steps of apoptosis. Being ratiometric, the response of the new probe can be easily quantified on an absolute scale. This allows monitoring by laser scanning confocal microscopy the degree and spatial distribution of the apoptotic changes at the cell plasma membranes, a feature that can be hardly achieved with the commonly used fluorescently labeled annexin V assay.

Normal cells exhibit a remarkable asymmetry of the lipid distribution between inner and outer leaflets of cell membranes,[1−3] which is lost during the early steps of apoptosis and necrosis.[4] Most characteristic in this change is the exposure to cell surface of amine-containing phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS).[4−6] This exposure is functionally important because it provides the signal for recognition and elimination of apoptotic cells by macrophages.[7] These changes in the plasma membrane are also used to detect apoptosis. In this respect, the most common detection method relies on the use of fluorescently labeled annexin V, which interacts with PS exposed on the membrane surface.[8,9] This interaction requires the presence of Ca^{2+} ions (up to 2.5 mM). Moreover, annexin V can associate with membrane surfaces containing negatively charged byproducts of lipid peroxidation,[10] and its lipid binding specificity can be affected by detergents in the medium.[11] In addition, routinely used cell-harvesting techniques for adhering cells, such as trypsinization, can also produce false positive results in application of this method.[9] These disadvantages of annexin V as a PS-sensing reagent stimulate the search for alternative means to detect the changes of the cell plasma membranes during apoptosis.[12−15]

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In previous studies,[16−20] we focused on the unique properties of 3-hydroxyflavone (3HF) derivatives as environment-sensing probes with two-color ratiometric response. 3HF dyes exhibit an excited-state intramolecular proton transfer (ESIPT) reaction giving a dual fluorescence with two emission bands, corresponding to the initially excited normal (N*) and the ESIPT reaction product, tautomer (T*) forms.[21] Recently, functional derivatives of 3HFs have been developed to provide a strong two-color response to changes of the composition of lipid membranes.[22,23] The most significant response was induced by variation of the surface charge as observed by comparing neutral and negatively charged phospholipid bilayers.[22,24,25] Since apoptosis modifies the surface charge of the outer leaflet of the cell plasma membranes, we utilized 3HF fluorophore to develop a new fluorescent probe capable to detect apoptosis. Our results show that the new probe reports on apoptosis by changing the relative intensities of its two emission bands. This ratiometric response constitutes an essential advantage of the new probe compared to the reference annexin V-based assay, allowing quantitative monitoring of the apoptotic changes at the cell plasma membranes both in single cells and in cell suspensions.

Experimental Section

Materials. Actinomycin D; propidium iodide (PI); type I-A RNAse A; egg yolk phospholipids phosphatidyl-choline, -glycerol, and -ethanolamine (EYPC, PG, and PE); and bovine brain phosphatidylserine (BBPS) were from Sigma. Hank's balanced salt solution (HBSS) was from Gibco and Annexin V−FITC Kit was from Immunotech.

Synthesis of F2N12S. Primarily, 0.1 g of 6-bromomethyl-4′-*N*,*N*-diethylamino-3-hydroxyflavone[22] was reacted with 0.2 mL of dodecylmethylamine in 3 mL of dry THF at room temperature for 3 h. After rotor evaporation, the crude product (sufficiently pure according to thin layer chromatography) was dissolved in 2 mL of toluene and 50 μL of dimethylformamide followed by addition of 45 μL of propansultone. The reaction mixture was refluxed for 20 h. After cooling, the mixture was diluted with hexane and the product **F2N12S** was filtered. Purification of **F2N12S** was done on silica gel column chromatography (dichloromethane/methanol, 4/1, v/v). Yield 40%; UV max in acetonitrile 416 nm, $\epsilon = 34\,000$ L × mol^{-1} × cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86 (t, $J = 5.80$ Hz, 3H), 1.16 (t, $J = 6.8$ Hz, 6H), 1.2−1.3 (m, 20H), 1.75 (m, 2H), 2.13 (m, 2H), 2.94 (s, 3H), 3.10−3.30 (m, 2H), 3.40−3.60 (m, 6H), 4.70 (s, 2H), 6.84 (d, $J = 8.1$ Hz, 2H), 7.84 (d, $J = 8.4$ Hz, 1H), 7.91 (d, $J = 8.4$, 1H), 8.13 (d, $J = 8.1$ Hz, 2H), 8.33 (s, 1H); MS-FAB $C_{37}H_{56}N_2O_6S$ (*m/z*): 642.4 (M^+).

Lipid Vesicles. Large unilamellar vesicles (LUV) of 1 mM lipid concentration were obtained in 15 mM phosphate buffer, pH 7.4, by the classical extrusion method.[26] LUVs were labeled by adding an aliquot of probe stock solution in DMSO to a final concentration of 0.1 μM. The fluorescence spectrum was recorded immediately after addition of the probe, since a very rapid binding kinetics was determined.

Cell Preparation and Staining. Human lymphoid CEM T cells were cultured in X-vivo 15 serum-free medium (Cambrex, France) at 37 °C in humidified 5% CO_2 atmosphere. Cell viability was checked by trypan blue exclusion. Cells were seeded at 5×10^5 cells/mL in the presence or absence of actinomycin D (0.5 μg/mL) for 18 h. Cells were pelleted by centrifugation at 1500 rpm for 5 min and were resuspended at 10^6 cells/mL in X-vivo 15 for experiments.

For all the measurements, the cells were fluorescently labeled using the same procedure. They were washed twice by HBSS before each experiment. In sorting and control experiments, cells were resuspended in the binding buffer provided with the annexin V−FITC kit (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). For fluorescence measurements with **F2N12S**, the cells were resuspended in HBSS containing 0.1−0.2 μM of **F2N12S**. Since the solubility of **F2N12S** is limited in water, the buffer containing the probe was prepared by addition of an aliquot of 0.2 mM stock solution of **F2N12S** in DMSO to the HBSS buffer (i.e., DMSO final concentration was ≤0.1%) and was used immediately after preparation. When the probe was kept in the buffer for more than 10 min, an efficient cell staining was not achieved, probably because of aggregation of the probe. The cell suspension was then incubated with the probe in the dark at room temperature for 5 min before measurements. The staining pattern of the cells was checked at room temperature with a Confocal Imaging System MRC-1024 (BioRad) to ascertain that fluorescence originates from the plasma membrane.

Flow Cytometry. Cells treated by actinomycin D were sorted into living, apoptotic, and cells dead by apoptosis using FITC-labeled annexinV and propidium iodide (PI) with an FACStar + cell sorter (BD Biosciences, CA) equipped with an argon laser (488 nm) and emission filters DF30 and DF22 centered at 530 and 630 nm, respectively. Populations of sorted cells were then used for fluorescent analysis with the new probe.

For the validation of **F2N12S** in cell cytometry, we used an FACSAria Cell Sorter (BD Biosciences, CA) equipped with an argon laser (488 nm) and a diode laser (405 nm) and emission filters DF30, DF42, and DF20 centered at 530, 585, and 610 nm, respectively. The optical pathways corresponding to the argon laser and the diode laser are independent. Data were analyzed using the FACSDiva Software (BD Biosciences). The granulation, size, and fluorescence intensity were recorded at a rate of ∼1000 cells per second.

Toxicity Test. The toxicity of **F2N12S** was checked by flow cytometry using PI (19 μM). CEM T cells were washed twice in HBSS and were separated in nine batches: three batches for control measurements (without probe), three batches with 0.1 μM **F2N12S**, and three batches with 1 μM **F2N12S**. The procedure of cell treatment was the same as for fluorescence measurements in suspension. The batches were incubated (37 °C incubator) for 0.5, 1, and 4 h, respectively. The 488-nm line of an argon laser was used as the excitation light source. Emission was collected by using the DF22 filter centered at 630 nm.

Fluorescence Spectroscopy and Microscopy. Fluorescence spectra were recorded on a FluoroMax-3 (JobinYvon Horiba) spectrofluorometer at room temperature. To obtain selectively the fluorescence spectrum of **F2N12S**, an excitation wavelength at 400 nm, where the absorption of FITC is minimal, was used and the residual emission of FITC was subtracted. All spectra were corrected for lamp intensity variations and fluorescence from the blank. For ratiometric imaging of cells, an inverted laser scanning confocal microscope Meta 510 (Zeiss) was used. Cells were prepared at 3×10^5 cells per mL in Ludin chamber by following the protocol described above. The microscopy experiments were done at 37 °C. The 405-nm line of an LED and the 488-nm line of an argon laser were used as excitation light sources for **F2N12S** and annexin V−FITC/PI, respectively. In the case of **F2N12S**, emission was collected by using two filters: 500/50 BP for the N* band and

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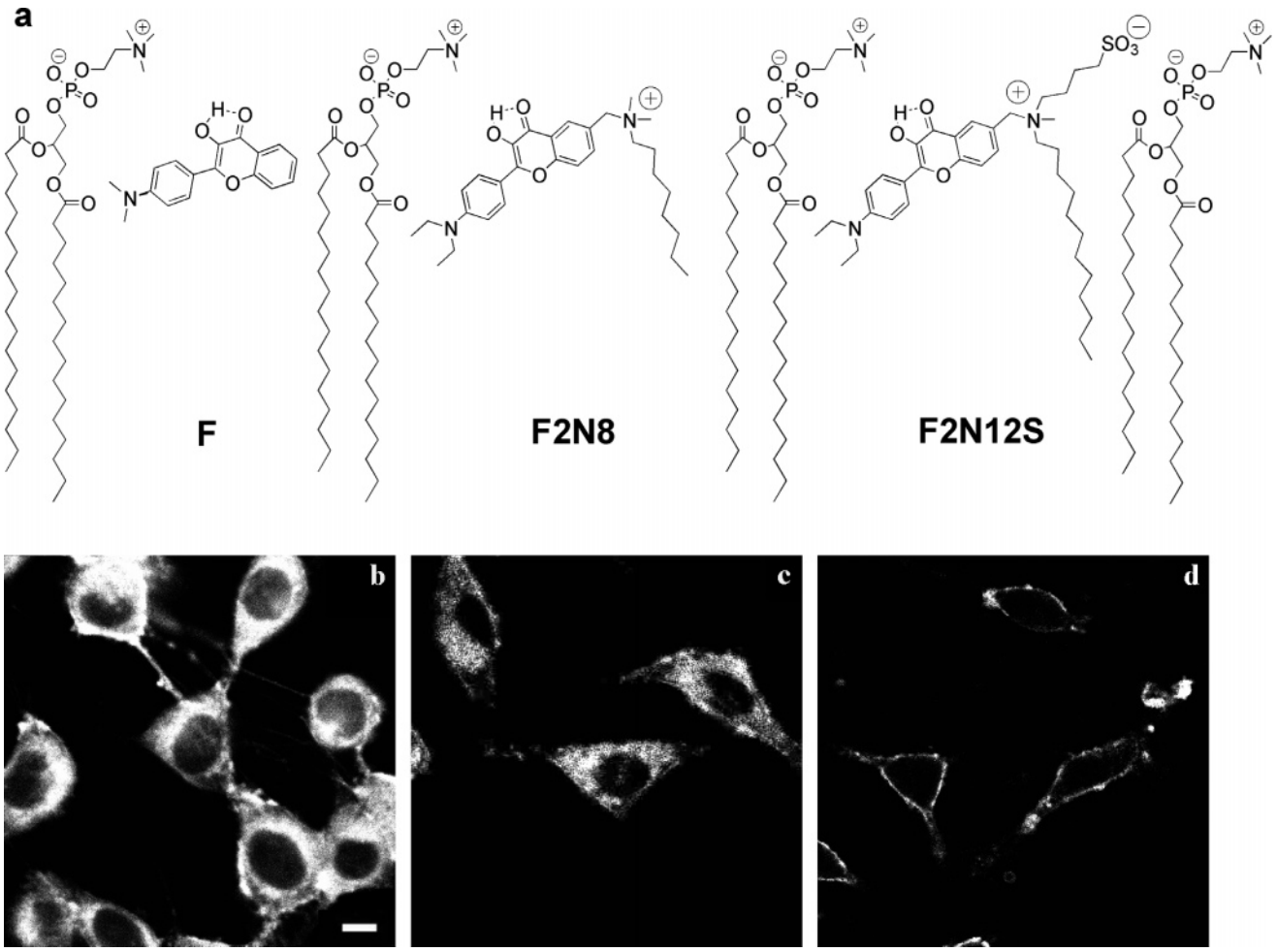

Figure 1. Structure and expected location of probes **F**, **F2N8**, and **F2N12S** in phospholipid membranes and cells. (a) Structure of the first-generation probes **F** and **F2N8** and of the newly synthesized probe **F2N12S** and their expected location in phospholipid membranes.[22] Confocal fluorescence images of L 929 cells stained with probes **F** (b), **F2N8** (c), and **F2N12S** (d). Images were obtained by collecting fluorescence in the 585−700 nm wavelength range, 10 min after probe addition. Scale bar, 10 μm (b).

560LP for the T* band. For collecting emission of annexin V−FITC and PI, 517/24 BP and 610LP filters were used, respectively. Collected images were analyzed by the MetaMorph software.

Results

Development of a New Probe with High Selectivity to Cell Plasma Membranes. It has been established that probes **F** and **F2N8** (Figure 1a) are highly sensitive to the electrostatic surface potential in model membrane systems.[22,24,25] However, confocal images of adherent L 929 cells reveal a rapid penetration of these probes inside the cells (Figure 1b, c). To overcome this essential drawback, we designed a new molecule **F2N12S** (Figure 1a), which is an improved analogue of **F2N8**. Probe **F2N12S** possesses a zwitterionic group, which should strongly interact with the polar heads of the lipids, and a long hydrocarbon chain, which should increase the hydrophobic interactions with the fatty acid chains. These modifications are thought to improve the affinity of the probe to the cell plasma membrane and to decrease the penetration rates of the probe through the bilayer.

In accordance with our expectations, confocal images of adherent L 929 cells stained with probe **F2N12S** (Figure 1d) demonstrate emission exclusively from the plasma membranes. Moreover, because of the very low fluorescence of this probe in water, the background fluorescence is negligibly low even when the cells are not washed after staining. Importantly, the probes stay in the membrane during the whole observation time under the microscope, which is limited by the lifetime of the cells in the HBBS buffer outside of the incubator (approximately 1 h). We also observe some inhomogeneity in the distribution of the fluorescence intensity at the plasma membrane. This is probably connected with the heterogeneous lipid/protein distribution in the bilayer that affects the probe distribution.

Next, the cytotoxicity of **F2N12S** was evaluated on the human lymphoid cell line CEM T. **F2N12S** was added at 0.1 μM and 1 μM and was incubated for different times (0.5, 1, and 4 h). No considerable cytotoxicity was observed with 0.1 μM **F2N12S** for a 4-h incubation time: the fraction of dead cells in the treated and nontreated (control) cells was 2.4 and 1.6%, respectively. Even at 1 μM concentration, **F2N12S** cytotoxicity was very limited, since the percentage of dead cells for 4-h incubation did not exceed 4%.

Effect of Surface Potential on F2N12S Fluorescence. The surface potential of lipid membranes, which spans between the membrane interface and the bulk solution, is created by the charged head groups of lipids. To model the increase in the negative surface charge at the outer leaflet of the plasma

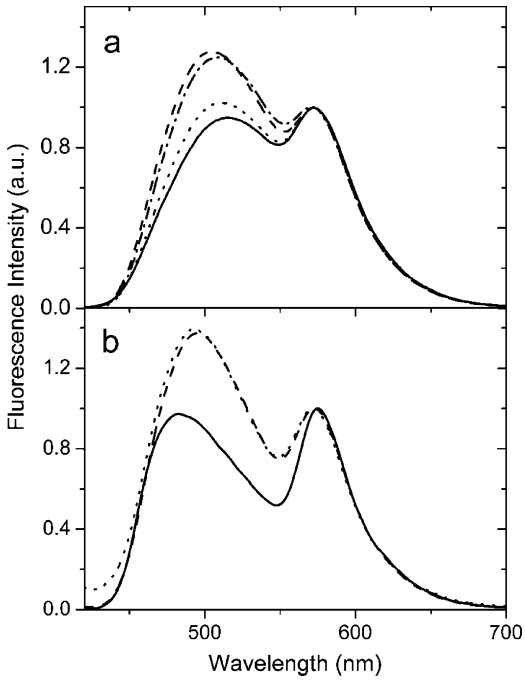

Figure 2. Fluorescence spectra of **F2N12S** in lipid vesicles (a) and in cells (b). In a, the lipid vesicles are composed of EYPC (solid), EYPE (dot), EYPG (dash dot), or PS (dash). In b, the spectrum of **F2N12S** was recorded in normal (solid) or in apoptotic CEM cells either in the absence (dash) or in the presence of 2 mM Ca^{2+} (dot). Excitation wavelength was 400 nm. Final probe concentration was 0.1 μM.

membrane during apoptosis, we used large unilamellar vesicles (LUV) composed either of neutral (egg yolk phosphatidylcholine, PC, and ethanolamine, PE) or anionic (egg yolk phosphatidylglycerol, PG, and bovine brain phosphatidylserine, PS) phospholipids. The **F2N12S** probe is nonfluorescent in water, while on binding to lipid vesicles it exhibits strong two-color fluorescence (Figure 2). According to our previous studies of parent compounds **F** and **F2N8**, these bands can be assigned to the emission of the normal (N*, short-wavelength)[27] and tautomer (T*, long-wavelength) forms.[22,28,29] The observed two-band emission spectrum is independent of the probe concentration, indicating that the staining of the lipid vesicles is homogeneous. Variation of the surface potential in LUV results in significant changes in the dual emission of the new probe **F2N12S**, similar to those observed previously with **F** and **F2N8**.[22] Indeed, in anionic vesicles (PG and PS), this probe shows a higher relative intensity of the short-wavelength (N*) band as compared to neutral PC and PE vesicles (Figure 2). Since cell apoptosis results in the appearance of anionic lipids (PS) in the outer leaflet, probe **F2N12S** should respond to apoptosis by an increase of the relative intensity of its short-wavelength band.

Response of F2N12S to Apoptosis in Cell Suspensions. Apoptotic CEM T cells were obtained by treatment[30] with actinomycin D and were sorted by flow cytometry. The cells were stained with **F2N12S** by resuspension in a buffer containing the probe. Apoptosis changes dramatically the fluorescence spectrum of **F2N12S**, since the relative intensity of the short-wavelength band is nearly 1.5-fold higher than in normal cells (Figure 2b). This result is in line with our model experiments on the surface charge effect in lipid vesicles (Figure 2a). However, the separation of the two bands of **F2N12S** is significantly larger in living cells than in lipid vesicles. According to our previous data on the parent probe **F2N8** in LUV, this increase in the band separation may be connected with a decrease in the bilayer hydration.[29] Therefore, we speculate that in living cells, the hydration of the outer leaflet is significantly lower than in phospholipid vesicles. This is likely related to the presence in the outer leaflet of cholesterol and sphingomyelin (SM)[4] which form strongly dehydrated structures.[31] In contrast, apoptosis decreases the band separation (Figure 2b) in comparison to living cells, suggesting an increase of the hydration in the outer leaflet. This hydration increase is probably due to the substitution of SM with PS and PG, which form more hydrated structures. Thus, **F2N12S** probe allows a fast and easy detection of apoptosis in cell suspensions by spectrofluorometry, which cannot easily be realized with the common annexin V-based assay, since it would require additional steps for separating nonbound fluorescent probe molecules.

Moreover, the response of **F2N12S** to apoptosis is independent of Ca^{2+} in the concentration range 0−2 mM (Figure 2b). This result is an additional important advantage over the annexin V-based assay, which requires the presence of ca. 2 mM Ca^{2+} in the medium for getting an optimal response.

F2N12S versus Annexin V−FITC in Flow Cytometry. Since fluorescently labeled annexin V in association with propidium iodide (PI) constitutes a major tool for separating apoptotic, necrotic, and living cells by flow cytometry, our next step was to validate **F2N12S** as an alternative in this application. To this end, T lymphoblastoid cells were treated with actinomycin D and were labeled simultaneously with FITC−annexin V, propidium iodide (PI), and **F2N12S**. Selective excitation of FITC and PI was obtained at 488 nm, a wavelength where **F2N12S** does not absorb. Since PI enters only in the dead cells, the bi-parametric representation of the fluorescence intensities (PI versus FITC) shows three distinct populations, (1) viable cells, which exhibit low FITC and PI signals, (2) apoptotic cells, which exhibit high FITC and low PI signals, and (3) dead cells, which exhibit both high FITC and PI signals (Figure 3a). The percentage of living, apoptotic, and dead cells were 33, 18, and 49%, respectively. Selective excitation of **F2N12S** in the same cells was achieved with a second laser source at 405 nm. The bi-parametric representation of **F2N12S** data (N* band versus T* band intensities at 530 and 585 nm, respectively) (Figure 3b) clearly shows two populations. The first one exhibits a high T*/N* intensity ratio and can be assigned to living cells according to our spectroscopic data. The second one is characterized by a low T*/N* intensity ratio and can be assigned to apoptotic and dead cells. Noticeably, when probe **F2N12S** was used without annexin V−FITC and PI, the T*/N* ratios were very close to those observed in the triple staining procedure (not shown), indicating that in our experiments, the fluorescence

(27) According to our previous studies, the short-wavelength band is a superposition of the emission of hydrated and nonhydrated normal forms of the dye. See refs 28 and 29.
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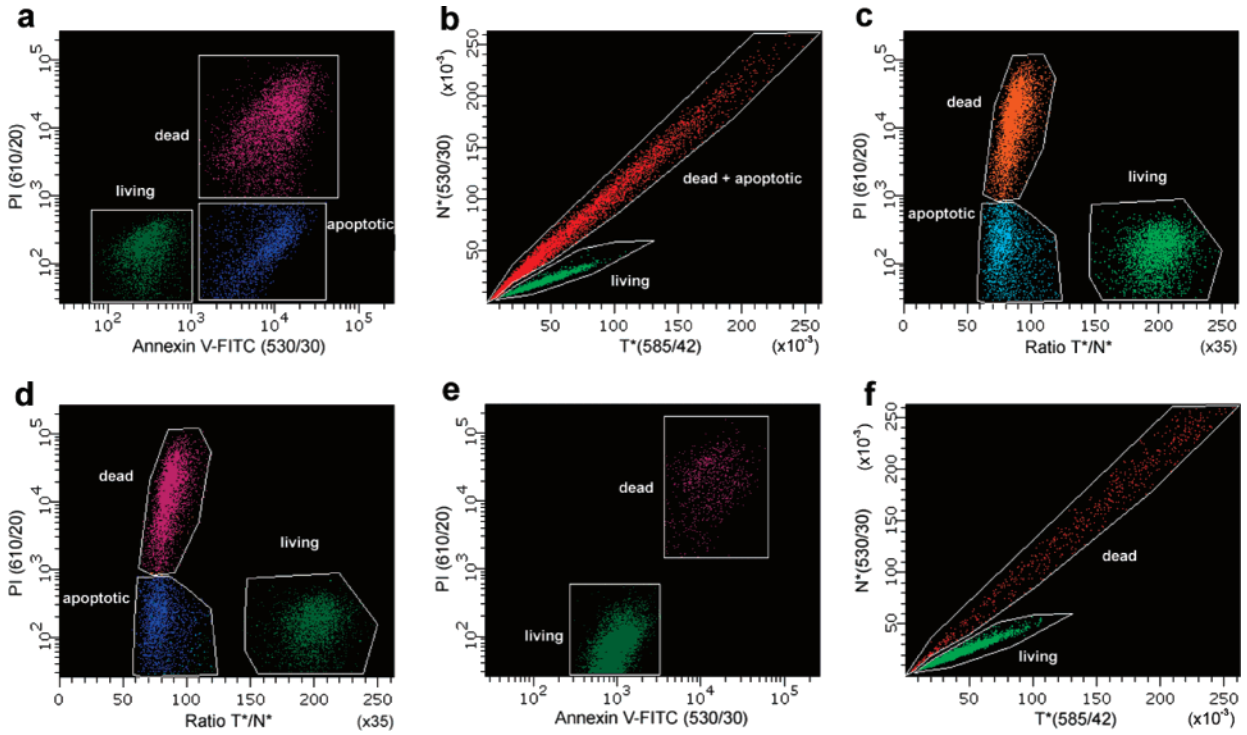

Figure 3. Flow cytometry of T lymphoblastoid cells treated by actinomycin D. Cells were stained by annexin V−FITC, PI, and **F2N12S**. In (a), annexin V−FITC and PI were selectively excited at 488 nm and their fluorescence at 530 and 610 nm, respectively, was collected simultaneously. Cells are sorted into living (dark green), dead (purple), and apoptotic (blue) cell populations by the absolute intensities of the two probes. In (b), **F2N12S** is selectively excited at 407 nm and its fluorescence was collected simultaneously at 530 and 585 nm. Living cells (green) are discriminated from apoptotic and dead cells (red) by the relative intensities of their two emission bands. In (c), the PI fluorescence intensities are plotted versus the ratio of the two emission bands (T*/N*, 585/530) of **F2N12S**. Living cells are in green, apoptotic cells are in cyan, and dead cells are in orange. In (d), the populations sorted using the **F2N12S**/PI couple were colored according to their response with the annexin V−FITC/PI couple. Comparison with c shows that the populations sorted by the two couples of probes are fully matching. Wavelengths of maximal transmission and bandwidths of used filters are indicated in brackets. (e and f) Comparison of annexin V−FITC and **F2N12S** responses on nontreated T lymphoblastoid cells. Cells were sorted into living (green) and dead (red) cell populations.

signal of **F2N12S** is not significantly contaminated by the fluorescence of the other two probes.

To discriminate necrotic from apoptotic cells, we used **F2N12S** in combination with PI and displayed the fluorescence intensity of PI versus the T*/N* ratio of **F2N12S** (Figure 3c). Populations of apoptotic and dead cells are clearly discriminated by the differences in the PI intensities. Interestingly, the percentages of living, apoptotic, and dead cells (32.5, 19.5, and 48%, respectively) closely matched those obtained with annexin V−FITC/PI. This conclusion was further strengthened when the populations discriminated by the **F2N12S**/PI couple were colored according to their response with the annexin V−FITC/PI couple (Figure 3d). Comparison of Figure 3d with Figure 3c shows a very good overlap between the populations identified by the two couples of probes, validating the use of **F2N12S**/PI in cell sorting. Moreover, very similar **F2N12S** intensity ratio values were obtained when T lymphoblastoid cells were substituted by 3T3 fibroblasts or HeLa cells (data not shown), indicating that the **F2N12S** response is not specific to a given cell line.

Finally, the response of **F2N12S** (Figure 3f) was compared with the response of the annexin V−FITC/PI couple (Figure 3e) on nontreated T lymphoblastoid cells. Populations of living (green) and dead (red) cells could be easily discriminated in both cases with the same percentage of dead cells (5%), indicating that **F2N12S** alone can be used to control the percentage of dead cells.

Taken together, our data show that the **F2N12S** probe can substitute annexin V−FITC in flow cytometry applications.

Fluorescence Ratiometric Imaging of Normal and Apoptotic Cells. To further demonstrate the potency of **F2N12S**, two-color ratiometric images of **F2N12S**-labeled cells undergoing apoptosis were recorded by laser scanning confocal microscopy and were compared with intensity based images obtained using annexin V−FITC. Since annexin V−FITC labels only apoptotic and necrotic cells, it does not visualize normal cells in fluorescence images (Figure 4a). Moreover, the fluorescence intensity of apoptotic cells varies considerably from cell to cell. These differences are difficult to interpret since the intensity depends on the probe concentration as well as on the size and confluence of the cells. In contrast to annexin V−FITC, **F2N12S** stains both normal (Figure 4b) and apoptotic cells (Figure 4c) but in different colors. Normal cells stained with **F2N12S** exhibit intensity ratios between 4 and 6. Cells treated with actinomycin D exhibit more heterogeneous intensity ratios (Figure 4c) since cells with ratios close to 5, corresponding to normal cells, are observed together with cells that exhibit much lower T*/N* intensity ratios, between 1 and 2. According to our spectroscopy and flow cytometry data, the latter cells are probably apoptotic. Interestingly, cells with intermediate ratios between 2 and 4 are also observed and likely correspond to cells on the initial steps of apoptosis. This indicates that the new probe can not only identify apoptotic cells but can also quantify the level of their apoptotic transformation. Thus, unlike

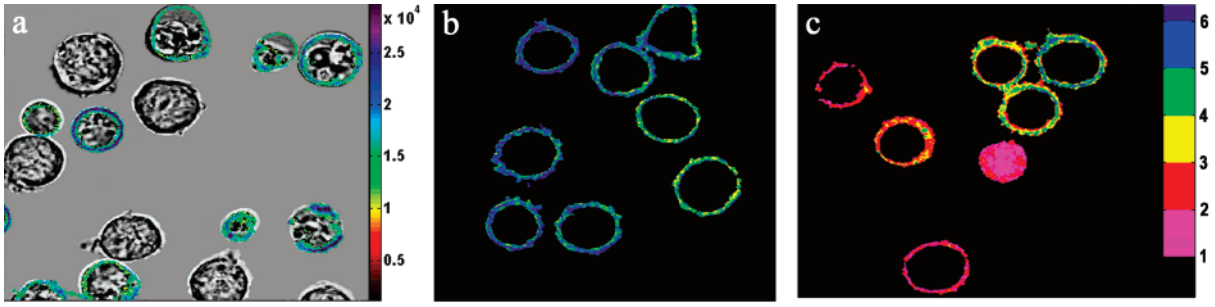

Figure 4. Laser scanning confocal imaging of T lymphocytes stained with annexin V−FITC (a) and **F2N12S** (b and c). (a) Combination of transmission and fluorescence images of cells treated with actinomycin D for 18 h. FITC intensity is displayed in pseudocolor by using the color code on the right scale. Because of their low binding to annexin V, normal cells are not visualized in the fluorescence images. (b and c) Ratiometric images of normal (b) and actinomycin D-treated (c) cells stained with 0.2 μM **F2N12S**. The ratios of intensities of the two bands T*/N* are displayed in pseudocolor by using the color code on the right scale. The size of the images is 60 × 73.1 μm.

annexin V−FITC, the new probe provides an absolute parameter, the intensity ratio, which can be calibrated, and can characterize the level of apoptosis independently of the probe/cell concentration and the excitation light intensity. In addition, we observe that in normal cells and cells on the initial steps of apoptosis, the T*/N* intensity ratio is not evenly distributed within the plasma membrane of a given cell. This may indicate an inhomogeneous lipid/protein composition of their plasma membranes that is likely related to the presence of lipid/protein domains.

Discussion

In the present work, we designed a new fluorescent probe, **F2N12S**, on the basis of 3-hydroxyflavone, to detect the changes in the lipid composition of the plasma membrane that occur in the early steps of apoptosis. A key problem was to develop a probe sensitive to lipid composition that could stay at the outer leaflet of the plasma membrane. This was achieved by coupling the 3HF fluorophore with a long hydrocarbon chain and a zwitterionic group which confers a high affinity to the polar interface of the membrane outer leaflet (Figure 1a). As a consequence, **F2N12S** was found to exhibit only a limited flip-flop in the membrane of normal cells during the time course of our experiments (0.5−1 h), as shown by the absence of time-dependent changes in its emission spectrum during this time scale. This is in line with the behavior of phospholipids that exhibit flip-flop kinetics on the time scale of hours.[32]

During apoptosis, the lipid asymmetry is lost so that in the outer membrane leaflet, PC and SM lipids are mixed with PS and PE. In addition to the increase in the net negative charge of the outer leaflet, apoptosis also decreases the lipid order[33] and increases the level of lipid oxidation,[34] leading to an increase of the polarity and hydration of the membrane. According to our studies in phospholipid bilayers, increases in the negative surface charge, polarity, and hydration additively increase the relative intensity of the N* band,[28] providing thus a dramatic color change.

The most important advantage of **F2N12S** compared to annexin V derived probes relies on its fluorescence ratiometric response to apoptosis. This ratiometric response provides a self-calibrating absolute parameter of apoptotic transformation, which does not depend on the probe concentration or on instrumental factors, such as fluctuations of light source intensity or sensitivity of the detector.[35,36] In this respect, the **F2N12S** probe is especially interesting for two-color ratiometric confocal imaging using a single excitation light source. Using this probe, the degree and the spatial distribution of the apoptotic changes over the cell plasma membranes can be sensitively monitored.

Additional important advantages of **F2N12S** probe could be stressed. While the common annexin V−FITC probe requires millimolar Ca^{2+} concentrations for ensuring optimal detection of PS,[8,9] the response of **F2N12S** is independent of the Ca^{2+} concentration. Moreover, no washing step is required since **F2N12S** is nonfluorescent in water. The latter simplifies significantly the staining protocol for experiments in cell suspensions, which makes the new probe attractive for high-throughput screening using reliable concentration-independent ratiometric detection technology.[36] In addition, since **F2N12S** is a relatively small organic molecule, it is more stable than a protein-based reagent, can be readily synthesized from inexpensive compounds, and is easier to handle. Noticeably, a fluorescent probe for apoptosis based on a small organic molecule has already been reported previously.[13] This probe works by a principle similar to the annexin V-based kit, so that it specifically interacts with PS lipids. However, since the PS recognition part of this molecule contains Zn^{2+} ions, the problem of ion dependence may remain for this probe as well.

A variety of applications of the new probe can be foreseen, because changes in transmembrane asymmetry are associated with a variety of important cellular processes, such as necrosis[37] and activation of thrombocytes.[38] Finally, since apoptosis is strongly related to cancer,[39,40] the **F2N12S** probe could be useful

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for monitoring the development of cancer tissues and evaluating the efficiency of anticancer drugs.[12,41−45]

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