A Novel Violet-Laser Excitable Ratiometric Probe for the Detection of Membrane Asymmetry Breakdown during Apoptosis

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ABSTRACT

Apoptosis is carefully regulated and essential part of normal tissue development and homeostasis. Regulatory changes in the apoptotic pathway have been implicated in many diseases; most notable are many types of cancers and autoimmune disorders. Normal cells exhibit a remarkable asymmetry in lipid distribution between the outer and inner cell membranes characterized by phosphatidyl-serine (PS) and phosphatidylethanolamine (PE) normally located on the inner leaflet of the cell membrane. During apoptosis translocation of PE and PS to the external cellular environment facilitates recognition and elimination of these cells by macrophages. The Violet Ratiometric Membrane Asymmetry Probe, 4'-N,N-diethylamino-6-(N-dodecyl-N-methyl-N-(3-sulfopropyl))ammoniomethyl- 3-hydroxyflavone (**F2N12S**), is a novel violet excitable dye for the detection of membrane asymmetry changes during apoptosis. The dye exhibits an excited-state intramolecular proton transfer (ESIPT) reaction resulting in a dual fluorescence with two emission bands corresponding to 530 nm and 585 nm, producing a two-color ratiometric response to variations in surface charge. This ratiometric probe is a self-calibrating absolute parameter of apoptotic transformation, which is independent of probe concentration, cell size, and instrument variation. The two-color ratiometric response of the F2N12S reagent will detect apoptotic cells in samples that vary in cell concentration by as much as 100 fold. The Violet Ratiometric Membrane Asymmetry Probe rapidly targets the plasma membrane requiring only a 5 minute room temperature incubation without additional buffers or wash steps reducing the chance of cell death that can occur from prolonged sample processing. The Violet Ratiometric Membrane Asymmetry Probe will work in both suspension and adherent cell lines and its efficacy is confirmed by caspase and mitochondrial apoptosis markers. Annexin V staining produced a larger population of apoptotic cells during early time points in both suspension and adherent cell lines that do not correlate with caspase and mitochondrial apoptosis markers. The violet 405 nm excitation of F2N12S expands the capability of multi-parameter apoptosis assays to study the apoptotic process while reducing or eliminating the need to create complex compensation controls by taking advantage of the time and spectral separation a violet-excitable reagent provides.

Figure 1. F2N12S Dual Fluorescence Ratiometric Response

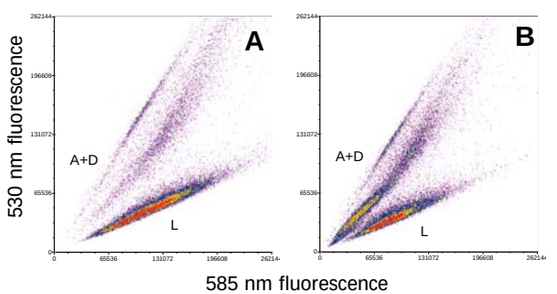

Figure 1. Control (A) or camptothecin treated (B) Jurkat cells stained with 200 nM F2N12S (405 nm excitation 530/30, 585/42 bandpass). All data was collected on a BD™ LSR II with 405, 488, and 633 nm excitation sources and analyzed using FCS Express analysis software. Live cells (L) can be distinguished from apoptotic and dead (A+D) cells by the relative intensity of the two emissions bands of F2N12S (585 nm or 530 nm), indicating a strong two color response to changes in the lipid membrane.

Figure 2. Rapid detection of apoptosis

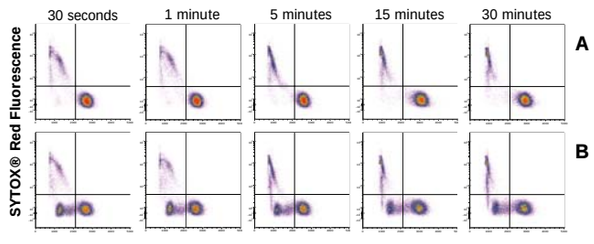

Figure 2. Control (A) or camptothecin treated (B) Jurkat cells were incubated with 200 nM F2N12S from 30 seconds up to 30 minutes before analysis by flow cytometry. Samples were co-stained with 1 µM SYTOX® Red dead cell stain (633 nm excitation 660/20 nm bandpass) 5 minutes prior to addition of F2N12S to discriminate dead cells from the apoptotic population. Populations of live and apoptotic cells can be resolved in as little as 30 seconds after addition of F2N12S allowing rapid analysis after treatment, thereby reducing the number of cells that continue from apoptosis to death after stimulation.

Figure 3. F2N12S Ratiometric parameter is not effected by cell concentration

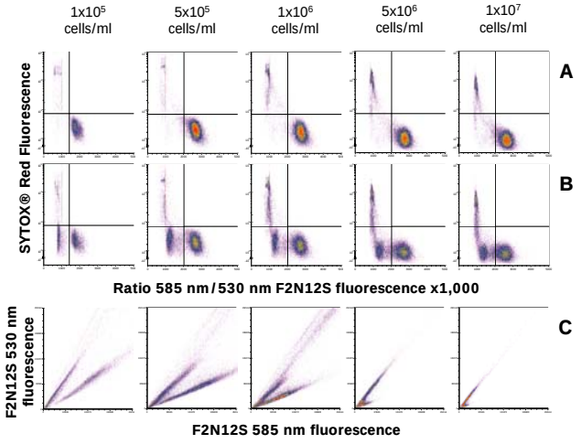

Figure 3. Control (A) and camptothecin treated (B) Jurkat cells were stained with 200 nM F2N12S and 1 µM SYTOX® Red dead cell stain across a 100 fold difference in cell concentration ($1x10^5$ to $1x10^7$ cells/ml). The F2N12S ratio parameter is not subject to probe depletion at high cell concentrations as traditional fluorescent probes are. As cell concentration increases the fluorescence intensity of both emission bands (530 nm and 585 nm) of F2N12S decrease (C) as would be expected when probe becomes limiting, but the ratio of 585/530 nm F2N12S fluorescence only begins to be effected around $1x10^7$ Cells/ml. Even at $1x10^7$ Cells/ml the apoptotic, live, and dead populations are still clearly discernable, providing a robust assay for apoptosis when cell concentration can not be accurately determined.

Figure 4. Apoptosis detection in suspension and adherent cell lines

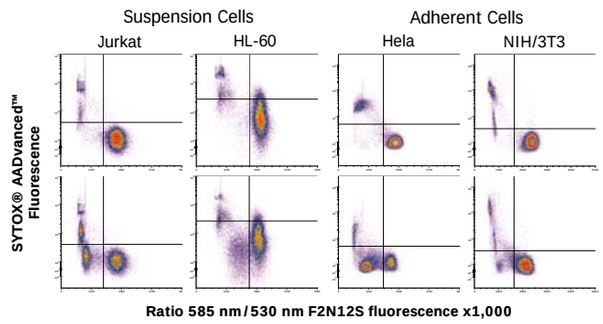

Figure 4. Suspension and adherent cell lines stained with 200 nM F2N12S and 1 µM SYTOX® AADvanced™ dead cell stain (488 nm excitation 695/40 bandpass). Top row of plots are unstimulated control cells. Bottom row of plots were stimulated for 4 hours at 37° C as follows: Jurkat and HL-60 suspension cells were stimulated with 10 µM camptothecin, Hela and NIH/3T3 adherent cells were stimulated with 1 µM and 10 µM staurosporine respectively. F2N12S ratio fluorescence is plotted as a derived parameter where 585 nm fluorescence is divided by 530 nm fluorescence and the product is multiplied by 1,000. Lower left quadrant (small 585/530 nm ratio) are apoptotic cells, lower right quadrant (high 585/530 nm ratio) are live cells, and upper left quadrant (small 585/530 nm ratio) and high SYTOX® AADvanced™ dead cell stain fluorescence are dead cells.

Figure 5. Multi-parameter apoptosis detection

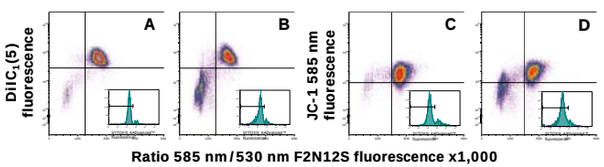

Figure 5. Control (A and C) and 4 hour camptothecin treated (B and D) Jurkat cells were stained with 1 µM SYTOX® AADvanced™ dead cell stain, 200 nM F2N12S, and either 50 nM MitoProbe™ $DiIC_1(5)$ (A and B; 633 nm excitation 660/20 bandpass) or 2 µM MitoProbe™ JC-1 (C and D; 488 nm excitation 585 nm bandpass). STYOX® AADvanced™ negative cells were first gated to exclude dead cells (inset histogram). Plots show 2 parameter apoptosis assay for mitochondrial membrane potential loss (decreased $DiIC_1(5)$ or JC-1 585 nm fluorescence) and breakdown of membrane asymmetry (smaller F2N12S 585/530 fluorescence ratio).

Figure 6. F2N12S correlates to other markers for apoptosis

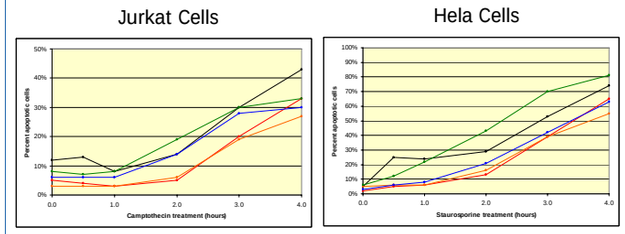

Figure 6. Jurkat and Hela cell lines assayed for apoptosis by treatment with 10 µM camptothecin or 1 µM staurosporine respectively for 30 minutes to 4 hours and assayed at each time point for apoptosis with the following: F2N12S (●), annexin V Alexa Fluor® 488 (●), Vybrant® FAM Caspase-3 and 7 (●; 488 nm excitation 530/30 nm bandpass), and two probes for mitochondrial membrane potential (MitoProbe™ $DiIC_1(5)$ (●), and MitoProbe™ JC-1 (●)). All assays included co-staining with SYTOX® AADvanced™ dead cell stain to exclude dead cells from the apoptotic population. In both suspension (Jurkat) and adherent (Hela) cell lines F2N12S correlates with mitochondrial membrane potential loss and Caspase 3 and 7 activity at 4 hours post stimulation. Mitochondrial membrane potential is lost first, followed by caspase 3 and 7 activity, then membrane asymmetry loss as assayed by F2N12S. Annexin V staining indicates a higher number of cells with membrane asymmetry loss before mitochondrial membrane potential loss, or caspase 3 and 7 activity. This more pronounced in the adherent cell line during 1st hour of drug treatment and might be an artifact of sample processing.

Summary

- The Violet Ratiometric Membrane Asymmetry Probe F2N12S provides a strong ratiometric response to changes in membrane asymmetry.
- A very rapid association with the plasma membrane allows for fast detection of apoptosis in 5 minutes, but can be detected in as little as 30 seconds after addition of F2N12S.
- The ratiometric parameter of F2N12S is not effected by differences in cell concentration up to 100 fold and can still discriminate live, apoptotic and dead cell populations.
- The Violet Ratiometric Membrane Asymmetry Probe F2N12S works in both suspension and adherent cell lines.
- The Violet Ratiometric Membrane Asymmetry Probe F2N12S correlates to other distinct markers for apoptosis, and does not produce the background signal seen with annexin V.
- The Violet Ratiometric Membrane Asymmetry Probe F2N12S is compatible with multiple dead cell stains and can be combined with other apoptosis markers to build a superior assay for apoptosis detection and analysis.

REFERENCES

1. van Engeland, M., et al., *Cytometry*, 1998,31, 1-9, 1998
2. Fadok, V. A., et al., *J. Immunol.*, 148, 2207-2216, 1992
3. Fadok, V. A., et al., *J. Immunol.*, 148, 4274-4285, 1993
4. Balasubramanian, K., et al., *Biochemistry*, 40, 8672-8676, 2001
5. Shynkar, V. V., et al., *J. Am. Chem. Soc.*, 129, 2187-2193, 2007