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THE CHANGE OF CELLULAR MEMBRANES ON APOPTOSIS: FLUORESCENCE DETECTION

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The strong plasma membrane asymmetry existing in living cells is lost on apoptosis, and it is commonly detected with the probes interacting strongly and specifically with phosphatidylserine (PS). This phospholipid becomes exposed to the cell surface, and the labeled annexin V is used for its detection. The requirement for early and Ca^{2+}-independent detection of apoptosis in the formats of spectroscopy of cell suspensions, flow cytometry, microarray technology and confocal or two-photon microscopy stimulated efforts for the development of new methods. Since the PS exposure must produce integrated changes of electrostatic potential and hydration in the outer leaflet of cell membrane, its detection can be provided by direct response of smart fluorescence probes. This review is focused on basic mechanisms underlying the loss of membrane asymmetry during apoptosis and the principles lying in the background of new methods that demonstrate essential advantages over the annexin V-binding assay. The convenient wavelength-ratiometric technique based on fluorescent probe F2N12S is described in detail. It incorporates spontaneously into outer leaflet of cell membrane and the color change of its fluorescent emission associated with apoptosis can be easily detected. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

Key Words: **phosphatidylserine, programmed cell death, apoptosis, membrane asymmetry, flow cytometry, fluorescence microscopy, 3-hydroxyflavones, F2N12S.**

INTRODUCTION

Detection of apoptosis is of great importance in many areas of biological and medical research. In cancer, the balance between cell proliferation and apoptosis shifts toward cell proliferation, and therefore there is an active search for the most efficient and safe anticancer treatments that induce apoptosis [1]. The ability to monitor apoptosis using noninvasive sensing and imaging techniques would markedly stimulate the studies of this phenomenon and enhance early assessment and continuous evaluation of the efficacy of anticancer drugs [2]. Apoptosis is involved in a wide variety of physiological and pathological processes [3, 4], and exposure of phosphatidylserine (PS) on the extracellular face of the plasma membrane is an important index of apoptotic cell transformation [5, 6]. The exposed PS on cell surface functions as a tag for such specific recognition of apoptotic cells producing a specific "eat-me" signal for their subsequent detaining and degradation by macrophages [7].

Until present, the most efforts were directed on detection of such exposure by the dye-labeled PS-binding proteins, such as annexin V. This methodology is attractive, since PS provides extracellular binding targets that can be detected in a non-perturbing manner, without the need for fluorescence reporter to penetrate the cell. Being not toxic or destructive in cellular research, this method allows extension to the tissue and whole-body level. Meantime, its important disadvantages and limitations that will be discussed below stimulate the search for alternative methodologies. In this Review it will be shown that it is not only the externalization of PS but also the integrated properties of membranes change dramatically on apoptosis, and this suggests new methods of its detection. In more detail we will present the new method based on application of smart fluorescent dye F2N12S developed for detecting these changes and discuss its applicability.

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Abbreviations used: F2N12S – N-[[2-[4-Diethylaminophenyl]-3-hydroxychromon-6-yl]-methyl]-N-methyl-N-(3-sulfopropyl)-1-dodecanaminium; PI – propidium iodide; PS – phosphatidylserine.

AMINOPHOSPHOLIPID ASYMMETRY OF PLASMA MEMBRANE AND ITS APOPTOTIC CHANGES

Plasma membrane of a living cell is a highly organized three-dimensional system composed of lipids, proteins and glycans, so that the two leaflets forming it are of quite different composition. The major part of electrically neutral phosphatidylcholine (PC) and sphyngomielin (SM) is located in its outer leaflet, whereas most of phosphatidylethanolamine (PE) and practically all anionic lipids PS and phosphatidic acid (PA) face the cytosolic milieu [8, 9]. Such asymmetry creates a strong distribution of electrostatic charge between two membrane surfaces that is maintained due to the action of specific lipid-translocating proteins (Fig. 1). Its maintaining is of great biological importance allowing correct assembly of membrane proteins and interaction with intracellular structures [10, 11].

The appearance of anionic PS at the cell surface is the most remarkable feature in apoptosis because of abundance of this phospholipid, its negative charge and the ability to change interactions with other lipids [6] that probably disrupts lipid rafts [12]. It should be noted, however, that the specificity of PS externalization is not absolute for apoptosis and may indicate different pathological conditions [11, 13, 14]. It was reported about occurrence of cell surface expression of PS in living endothelial cells of tumor vasculature and stressed tumor cells [15, 16]. Exposed PS is ob-

served on platelet activation, where it is a part of blood clotting process. Anionic lipids expressed on activated platelets are important for the coordinated assembly of coagulation factors [17]. Activated macrophages that are engaged to engulf dying cells also expose PS on their surface, and inhibition of their PS exposure greatly impairs phagocytic capacity [18]. Therefore externalization of PS and associated changes of surface potential cannot be considered as highly specific indicators of apoptosis. Their relation with other processes that characterize this phenomenon (caspase cascade activation, release of mitochondrial factors) is not clear. But being the most efficient practically, the methods indicating these changes are the most popular.

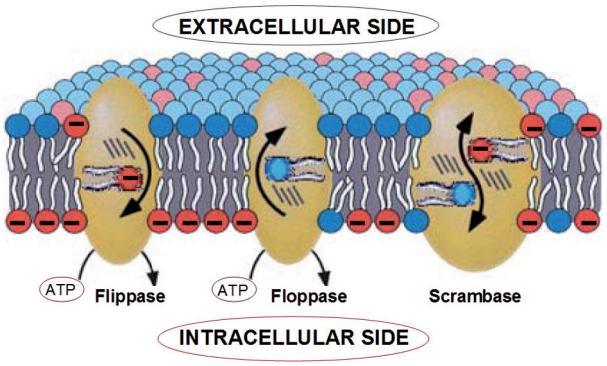

Fig. 1. Transporter-controlled exchange of phospholipids between intracellular and extracellular leaflets of the cell membrane creating strong charge asymmetry. Reproduced with modifications from ref. [11]

ANNEXIN V AND OTHER ASSAYS BASED ON THE BINDING TO EXTERNALIZED PS

The labeled annexin V is currently the most frequently used probe to visualize the early-stage apoptosis in microscopy and to characterize the apoptotic cell populations by flow cytometry [5, 19, 20]. The principle of annexin V assay is presented in Fig. 2.

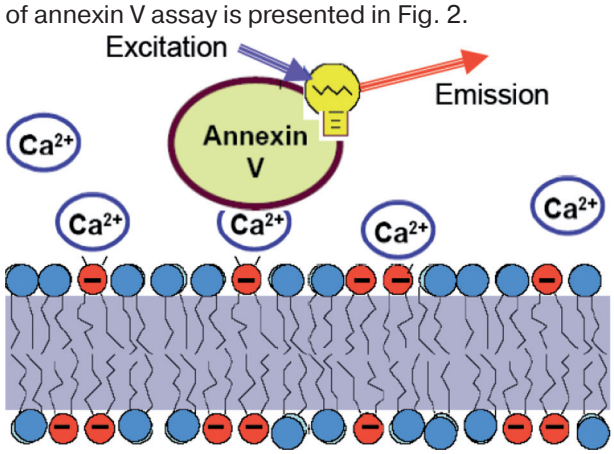

Fig. 2. The principle of apoptosis detection method based on annexin V. With the aid of Ca^{2+} ions, this protein interacts with high affinity with PS heads exposed on the membrane surface. Annexin V can be labeled with fluorescent dye that allows visualization of cell with exposed PS

Operating in physiological conditions, this 36-kDa calcium-binding protein binds with relatively high affinity (K_d ~10^{-7}–10^{-8} M) to the surface of cells that expose negatively charged PS, and due to attached fluorescent dye these cells become labeled. The methods of its conjugation to FITC and similar fluorophores are well described. Labeling with NIR dyes extend applications towards tissue tomography [21]. Fluorescent semiconductor nanoparticles (Quantum Dots) can be also used for annexin V labeling with the advantage of higher brightness and photostability [22]. Interacting with vital or apoptotic cells, the annexin V molecule is unable to penetrate through the phospholipid bilayer and label inner leaflet. However, in dead cells, since the integrity of the plasma membrane is lost, the membrane inner leaflet is available for labeling. Double labeling with annexin V and cell penetrating dye propidium iodide (PI) allows discriminating apoptotic and dead cells.

The years of successful application of annexin V binding assay still allowed revealing its important disadvantages.

1. The test requires the presence of millimolar (usually ~2.5 mM) concentrations of Ca^{2+} ions, since only with their complexation the protein binds to PS with high affinity. This is not always desirable or even tolerable. For instance, the isolation of blood cells requires adding Ca^{2+} chelators for suppressing blood clotting, and after that the chelators have to be washed-out and Ca^{2+} ions added again for apoptosis assay. Also, false positive results may appear because most animal cells have a Ca^{2+}-dependent scramblase that can move PS to the cell surface.

2. The complete annexin V binding requires incubation times from tens of minutes up to one-two hours. This makes its application problematic for kinetic analysis of the process that develops on the same scale of hours and this does not allow catching its earliest steps of PS exposure occurring in minutes [23]. The annexin V binding is limited by its slow diffusion and slow formation of its high-affinity complexes.

3. The false positive results may result due to annexin V binding to negatively charged aldehyde adducts that may appear on cell surface in stress conditions [24] and in some apoptosis-independent pathologies, such as Barth syndrome [25]. Therefore an increased binding of annexin-V (whatever its ligand is) does not necessarily reflect an apoptotic state of the cell [26].

4. As a protein, annexin V can itself be a subject of enzyme degradation. This limits its application *in vivo* and also requires controlled suppressing of protease activity if the cells from solid tissues are prepared by separation with the aid of proteases [19]. Manipulation with detergents may also be of concern influencing its lipid binding specificity [27].

5. The labeled recombinant annexin V is expensive and moderately unstable. Therefore the assay with its use is not convenient for high throughput screening needed e.g. in drug discovery.

With the purpose of finding PS-binding probes that are alternatives to annexin V, investigators have examined other proteins such as annexin VI, lactadhedrin and synaptotagmin-I [28, 29]. Their PS-binding recombinant fragments were expressed that allowed site-specific binding of fluorophores, and their smaller size may be an advantage in some applications.

The development of low-molecular-weight high-affinity PS chelators use the same approach — the recognition of PS on the surface of apoptotic cells, thus mimicking the recognition mechanism of annexin V [20]. Among suggested compounds are the synthetic zinc(II)-di-2-picolylamine (Zn^{2+}-DPA) coordination complexes [30]. Similarly to Ca^{2+} ions in annexin V assay, the Zn^{2+} ions mediate cooperative association of the probe molecule to the anionic head groups of the membrane-bound PS, so that selective recognition of anionic membranes over the near-neutral charge membranes of healthy cells needs a three-component assembly process. In initially suggested compound, two DPA subunits were attached to an anthracene scaffold absorbing light at 380 nm, and this compound was called PSS-380 [30]. Two of Zn^{2+}-DPA molecules can be also attached to side groups of fluorescein-labeled short peptides for increasing the strength of membrane binding [31]. Substitution of fluorescein by a near-IR fluorescence reporter allowed extending the imaging possibilities [32]. Thus, a probe, named PSS-794, was assessed for its ability to detect cell death in animal models. The whole-body and *ex vivo* imaging showed the effect on membranes of the radiation treatment of tumor. PSVue®, the commercial product of Molecular Targeting Technologies, Inc., is based on these developments.

Thus we observe that cited above PS detecting methods did not exclude the problem of ion dependence addressed to annexin V assay but allowed broader flexibility in use for *ex vivo* and *in vivo* studies. Multivalent display of Zn^{2+}-DPA units binding PS would further enhance the probe affinity and could provide new synthetic nanomaterials with tunable characteristics. With this goal, the attempts to use nanoparticles as the scaffolds for these units have been made [33]. Diaminopropionic acid (Dpr), was selected as the building unit of the short PS-binding peptide. On each peptide, four DPA units were appended to the side chain of Dpr residues to increase the binding affinity and the labeling with fluorescein was performed. A good correlation with annexin V staining of apoptotic cells was observed.

The species with high PS binding properties can be selected from the peptide libraries. Thus, the peptide with a sequence CLSYYPSYC was selected, labeled with fluorescein and applied for *in vivo* imaging [34]. Nanomolar binding affinity towards PS was achieved with a 14-residue peptide [35]. Promising in applications are the designed cyclic peptides that mimic the recognition site of the natural PS binding protein lactadherin [36]. Such binding does not need any metal cation cofactors.

The other recent development is based on a class of "polarity-sensitive" annexin labels, so that on Ca^{2+}-dependent membrane interactions their environment changes from a polar (aqueous solution) to a nonpolar (lipid membrane). On this transition the fluorescence is dramatically enhanced reporting on PS exposure [37]. Known under the name pSIVA this technique marks an important step forward in annexin V based methodology.

GLYCOPROTEINS AS THE CELL SURFACE MARKERS

Recently, it was found that apoptosis is accompanied by not only the loss of plasma membrane asymmetry detected by annexin V, but also by changes in cell surface glycoconjugates. These novel markers of apoptosis are α-d-mannose and β-d-galactose-specific plasma membrane glycoproteins whose expression is substantially increased after induction of apoptosis. The carbohydrate recognizing proteins lectins can label the cell by binding to these saccharide residues [38]. Apoptosis detection methods were developed that use not only fluorophore-labeled lectins as the single-site binders. Exploring the polyvalency of lectins, the detection could be based also on agglutination of apoptotic cells. Moreover, the application of lectins allows isolation of these cells from mixed populations. It is still not clear, on what step of apoptosis the increased expression of alpha-D-mannose and/or beta-D-galactose containing glycoproteins occurs.

As a new development, the fluorescent nanocomposites with lectins attached to their surface were designed. They are luminescent GaN:Eu^{3+}-doped nanoparticles with wide green emission and very narrow red emission and fluorescent green-emitting fluorescein-containing polystyrene nanoparticles. Possessing high brightness and low level of photobleaching they interact efficiently with dying cells, thus discriminating them from the intact ones [39].

FLUORESCENCE METHODS TARGETING CELL SURFACE CHARGE

When the negatively charged PS re-locates to cell surface, it substitutes the neutral lipids and generates change in the surface charge. Since this charge becomes more negative, this favors absorption on the cell surface of cationic molecules. Those could be the dyes with positive charge. One of these tested dyes was merocyanine 540 [40]. With its application the difference in fluorescence response was demonstrated on binding to vital and apoptotic cells. Small affinity of dyes of this type to negatively charged membranes and their weak response to the binding as the change of fluorescence intensity plus their toxicity did not favour further developments and applications.

The situation becomes different with the application of polycationic species that could provide multipoint binding to apoptotic cell membrane. Thus, liposomes formed of artificial cationic lipids bind selectively to apoptotic cells [41]. Meantime, their low stability is not attractive for practical use. The most interesting and practically attractive in this respect is the application of conjugated polymers with side cationic groups that combine multipoint binding with strong fluorescence. In a recent study [42] it was shown that poly(p-phenylene vinylene) derivative demonstrates remarkable ability to discriminate apoptotic from normal cells. This method offers a strong advantage over annexin V in terms of cost and stability. However, the incubation time to reach the equilibrium binding

conditions with the cells is even longer than for annexin V, which strongly limits its application.

THE METHOD BASED ON F2N12S PROBE

Fluorescence probing is the traditional method of studying the cellular membranes for a number of years with a developed methodology for characterizing their structure, dynamics and interactions in terms of local polarity, viscosity and hydration [43]. The surface potential of outer leaflet must be changed with the increase of its negative charge [44]. In addition, apoptosis decreases its lipid order [45] and increases the level of lipid oxidation [46], which must lead to an increase of the polarity and hydration of the membrane. Therefore the problem was to develop the method that could respond to these changes in the most sensitive and characteristic way.

On this pathway, several problems have to be solved. One is selecting the format of fluorescence detection that can fit the broad range of applications and all kinds of inexpensive instrumentation (fluorimeters, different types of fluorescent microscopes, flow cytometers, plate readers). For that, the response should be self-referenced on molecular level and instrumentation-independent. The only fluorescence format that satisfies these requirements in all these applications is the wavelength-ratiometry [47] that allows detecting fluorescence signal at two wavelength channels and obtain their ratio (Fig. 3).

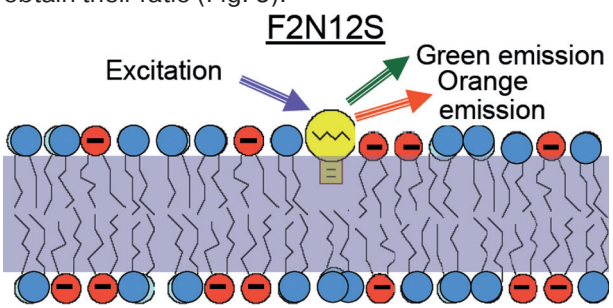

Fig. 3. The principle of apoptosis detection method based on F2N12S dye. Due to high affinity, the dye is incorporated into phospholipid moiety of the outer leaflet of the membrane and reports on the changes in its properties by the change of color of its emission. On apoptosis the relative intensity of "green" emission increases

The fluorescent probe should satisfy many additional requirements. It should be nontoxic and uncharged for not disturbing the membrane. Being excited at a single wavelength it should present switching between well resolved fluorescence bands in response to surface charge, polarity, and/or hydration. In our knowledge and previous experience only 4'-(diethylamino)-3-hydroxyflavones may satisfy these requirements [43, 44]. Among them we can select the dye producing simultaneously with initially excited "green" N* fluorescence emission band (at 515–545 nm) the "orange" T* band (at 564–606 nm), which is the product of intramolecular excited-state reaction. These two emissive forms are in dynamic equilibrium that is established prior to emission, so the factors changing the fluorescence lifetime (temperature, dynamic quenchers) do not change the response in ratio of two intensities. The "blue" excitation (with the maximum at about 420 nm) allows avoiding light-scattering artefacts.

The high specificity of binding to outer leaflet of cell membrane must be achieved, without the probe move to inner leaflet. This was accomplished by synthesizing the derivative containing a membrane anchor, composed of two groups of opposite charge interacting with phospholipid heads and a long (dodecyl) hydrophobic tail. As a consequence a developed probe F2N12S (Fig. 4) satisfied all the requirements, including the last one. It exhibits only a limited flip-flop in the membrane of normal cells during the time course of typical experiments (0.5–1 h), as shown by the absence of time-dependent changes in its emission spectrum on this time scale [48].

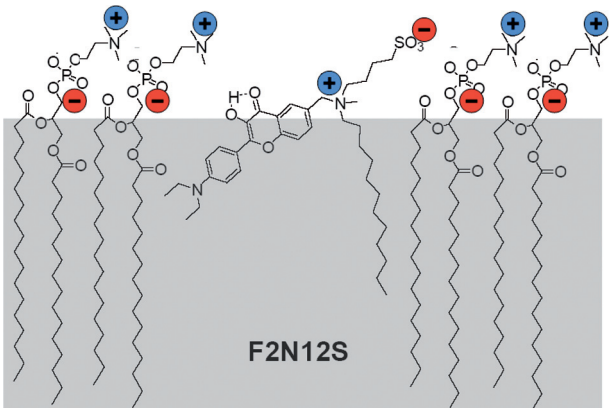

Fig. 4. Location of probe F2N12S in outer leaflet of biological membrane

This probe was first tested on neutral and negatively charged membrane models and showed dramatic difference in response between them. Then expected differences were observed on cells in culture in three formats — spectroscopy, fluorescence microscopy and flow cytometry. In all these studies it was shown that the ratio of intensities of the two emission bands of the probe changed dramatically in response to apoptosis.

Fig. 5 demonstrates the results obtained with flow cytometry. They show that regardless of the difference of probe uptake by the cells in population leading to dramatic difference in fluorescence intensity of both bands (leading to increase of intensity along both N* and T* coordinates in Fig. 5, *a*), the ratio of these intensities does not change being characteristic for the steps of apoptosis and cell depth. In Fig. 5, *a* we clearly observe two populations, one of them (shown in green) demonstrates low N*/T* intensity ratio and we assign it in accordance with our previously presented data on spectroscopic and microscopic analysis to viable cells. Another population is characterized by high N*/T* intensity ratio and has to be assigned to apoptotic and dead cells. In the histograms of Fig. 5, *b* we observe unexpectedly strong separation between these populations, which was never achieved with annexin V–PI assay. In our case PI can also be used in simultaneous experiment to separate apoptotic and dead cells. Since with the development of apoptosis larger amounts of probe are integrated into cellular membrane it becomes possible to discriminate between early and late apoptotic cells [48].

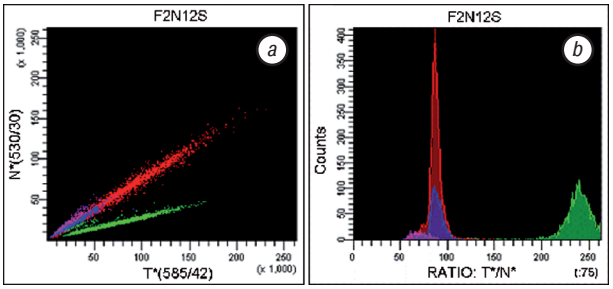

Fig. 5. Flow cytometry with F2N12S. (*a*) Two-dimensional fluorescence intensity profiles demonstrating the distribution of treated cells stained by F2N12S between their living (green), dead (red), early apoptotic (blue) and late apoptotic (violet) populations. The wavelengths of maximal transmission and bandwidths of used filters are indicated in brackets. (*b*) Cell histograms built on ratiometric fluorescence response of F2N12S in these cells

From this experiment one can derive the most important advantage of F2N12S compared to annexin V and related probes. This ratiometric response provides a self-calibrating absolute parameter of apoptotic transformation. Moreover, since F2N12S is nonfluorescent in water, no washing step to remove the unbound probe is required. 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. In addition, since F2N12S is a relatively small organic molecule, it is more stable than a protein-based reagent, diffuses rapidly and is easy to handle. It can be readily synthesized from inexpensive compounds, and the kits for analysis are produced and distributed by Invitrogen. Thus, the advantages of new technology can be summarized as follows:

- Due to slow molecular size, the binding kinetics is fast and occupies only several minutes. This allows observing apoptosis process in development starting from very early times.
- Incorporation of probe with high affinity occurs to all types of cells (healthy, apoptotic or dead), which allows detecting these cells on comparable scale of intensity analyzing only their distinguishing ratiometric signal.
- Background probe signal is undetected not only due to complete incorporation to any type of cells but also due to the absence of fluorescence of unbound probe in water or in aqueous buffers. So, any washing steps are not required.
- Binding of probe is independent of Ca2+ or any other type of ions. Apoptosis can be detected under a broad variety of conditions (temperature, pH, etc,).
- Proteases cannot modify the probe and, therefore, fluorescence response.
- Being ratiometric, the response of the new probe can be easily quantified on an absolute scale, providing a dramatic color change.
- Since ratiometric signal is generated in a single dye, no double labeling is needed, and excitation can be provided by a single light source.
- Self-calibration of apoptotic signal on molecular scale allows resolving the spatial distribution of the apoptotic changes over the cell plasma membrane that is limited only by resolution of the instrument. The studying of generation and propagation of apoptotic signal over the membranes of individual cells becomes possible.

This technology is compatible with many current sensing and imaging techniques, including different versions of fluorescent microscopy and flow cytometry. It is essential that combination of such attractive features cannot be achieved by any 'labeling' technology aimed at detection of particular compounds.

CONCLUSIONS

In view of central importance of apoptosis to many areas of biological research there is a strong request for methods that permit continuous monitoring of cell viability or apoptotic changes in a nontoxic and noninvasive manner. Cell surface is easily accessible and interactions on this surface usually do not produce its damage. Detection of structural changes does not need to compromise the cell integrity or to provide penetration of odd compounds into its interior. These changes occur and can be detected on early steps of apoptosis when the cell integrity is not disrupted. Furthermore, such strategy of probing the cell surface opens pathways for the applications for site-specific *in vivo* imaging of apoptotic tissues that would be useful in the treatment of various diseases such as cancer and cardiovascular disease.

Two major methodologies can be applied for detecting and characterizing these changes. One is focused on detection of particular molecules, and the other on characterizing integral changes in the studied structure on larger scale. PS exposed to cell surface is the most efficiently used apoptosis marker, although its specificity to apoptosis is not absolute. The recently developed alternative methodology is based on small-size fluorescent organic molecule F2N12S that can be rapidly incorporated into cell membrane. Its design allows high-affinity labeling of only outer membrane leaflet, and its two-wavelength ratiometric response allows adapting to any fluorescence sensing formats, such as cuvette-based spectrofluorimetry, flow cytometry, microscopy, microarrays, etc [49]. Combining high spatial/temporal resolution, sensitivity, and ease of use, this methodology is open for further development.

REFERENCES

1. **Coppola JM, Ross BD, Rehemtulla A.** Noninvasive imaging of apoptosis and its application in cancer therapeutics. Clin Cancer Res 2008; **14**: 2492–501.

2. **Kim R, Tanabe K, Uchida Y, *et al.*** Current status of the molecular mechanisms of anticancer drug-induced apoptosis. The contribution of molecular-level analysis to cancer chemotherapy. Cancer Chemother Pharmacol 2002; **50**: 343–52.

3. **Ulukaya E, Acilan C, Yilmaz Y.** Apoptosis: Why and how does it occur in biology? Cell Biochem Funct 2011; **29**: 468–80.

4. **Pereira WO, Amarante-Mendes GP.** Apoptosis: A programme of cell death or cell disposal? Scand J Immunol 2011; **73**: 401–7.

5. **Boersma HH, Kietselaer BL, Stolk LM, *et al.*** Past, present, and future of annexin a5: From protein discovery to clinical applications. J Nucl Med 2005; **46**: 2035–50.

6. **Balasubramanian K, Schroit AJ.** Aminophospholipid asymmetry: A matter of life and death. Annu Rev Physiol 2003; **65**: 701–34.

7. **Ravichandran KS.** Find-me and eat-me signals in apoptotic cell clearance: Progress and conundrums. J Exp Med 2010; **207**: 1807–17.

8. **Kiessling V, Wan C, Tamm LK.** Domain coupling in asymmetric lipid bilayers. Biochim Biophys Acta 2009; **1788**: 64–71.

9. **Yamaji-Hasegawa A, Tsujimoto M.** Asymmetric distribution of phospholipids in biomembranes. Biol Pharm Bull 2006; **29**: 1547–53.

10. **van Meer G.** Cellular lipidomics. EMBO J 2005; **24**: 3159–65.

11. **Zwaal RF, Comfurius P, Bevers EM.** Surface exposure of phosphatidylserine in pathological cells. Cell Mol Life Sci 2005; **62**: 971–88.

12. **Oncul S, Klymchenko AS, Kucherak OA, *et al.*** Liquid ordered phase in cell membranes evidenced by a hydration-sensitive probe: Effects of cholesterol depletion and apoptosis. Biochim Biophys Acta 2010; **1798**: 1436–43.

13. **Schutters K, Reutelingsperger C.** Phosphatidylserine targeting for diagnosis and treatment of human diseases. Apoptosis 2010; **15**: 1072–82.

14. **Korngold EC, Jaffer FA, Weissleder R, *et al.*** Noninvasive imaging of apoptosis in cardiovascular disease. Heart Fail Rev 2008; **13**: 163–73.

15. **Ran S, Thorpe PE.** Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy. Int J Radiat Oncol Biol Phys 2002; **54**: 1479–84.

16. **Ran S, Downes A, Thorpe PE.** Increased exposure of anionic phospholipids on the surface of tumor blood vessels. Cancer Res 2002; **62**: 6132–40.

17. **Kerbiriou-Nabias D, Geldwerth-Feniger D.** Exposition membranaire de la phosphatidylsérine et activité procoagulante des plaquettes. Hematologie 2007; **13**: 94–109.

18. **Ravichandran KS, Lorenz U.** Engulfment of apoptotic cells: Signals for a good meal. Nat Rev Immunol 2007; **7**: 964–74.

19. **van Engeland M, Nieland LJ, Ramaekers FC, *et al.*** Annexin v-affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998; **31**: 1–9.

20. **Hanshaw RG, Smith BD.** New reagents for phosphatidylserine recognition and detection of apoptosis. Bioorg Med Chem 2005; **13**: 5035–42.

21. **Ntziachristos V, Schellenberger EA, Ripoll J, *et al.*** Visualization of antitumor treatment by means of fluorescence molecular tomography with an annexin v-cy5.5 conjugate. Proc Natl Acad Sci USA 2004; **101**: 12294–9.

22. **Le Gac S, Vermes I, van den Berg A.** Quantum dots based probes conjugated to annexin v for photostable apoptosis detection and imaging. Nano Lett 2006; **6**: 1863–9.

23. **Mourdjeva M, Kyurkchiev D, Mandinova A, *et al.*** Dynamics of membrane translocation of phosphatidylserine during apoptosis detected by a monoclonal antibody. Apoptosis 2005; **10**: 209–17.

24. **Balasubramanian K, Bevers EM, Willems GM, *et al.*** Binding of annexin v to membrane products of lipid peroxidation. Biochemistry 2001; **40**: 8672–6.

25. **Kuijpers TW, Maianski NA, Tool AT, *et al.*** Neutrophils in barth syndrome (bths) avidly bind annexin-v in the absence of apoptosis. Blood 2004; **103**: 3915–23.

26. **Dong HP, Holth A, Ruud MG, *et al.*** Measurement of apoptosis in cytological specimens by flow cytometry: Comparison of annexin V, caspase cleavage and dutp incorporation assays. Cytopathology 2011; **22**: 365–72.

27. **Meers P, Mealy T.** Calcium-dependent annexin v binding to phospholipids: Stoichiometry, specificity, and the role of negative charge. Biochemistry 1993; **32**: 11711–21.

28. **Smith C, Mehta R, Gibson DF, *et al.*** Characterization of a recombinant form of annexin VI for detection of apoptosis. Bioconjug Chem 2010; **21**: 1554–8.

29. **Alam IS, Neves AA, Witney TH, *et al.*** Comparison of the c2a domain of synaptotagmin-i and annexin-v as probes for detecting cell death. Bioconjug Chem 2010; **21**: 884–91.

30. **Koulov AV, Hanshaw RG, Stucker KA, *et al.*** Biophysical studies of a synthetic mimic of the apoptosis-detecting protein annexin V. Israel J Chem 2005; **45**: 373–9.

31. **DiVittorio KM, Johnson JR, Johansson E, *et al.*** Synthetic peptides with selective affinity for apoptotic cells. Org Biomol Chem 2006; **4**: 1966–76.

32. **Smith B, Xiao S, Wolter W, *et al.*** *In vivo* targeting of cell death using a synthetic fluorescent molecular probe. Apoptosis 2011; **16**: 722–31.

33. **Quinti L, Weissleder R, Tung CH.** A fluorescent nanosensor for apoptotic cells. Nano Lett 2006; **6**: 488–90.

34. **Thapa N, Kim S, So IS, *et al.*** Discovery of a phosphatidylserine-recognizing peptide and its utility in molecular imaging of tumour apoptosis. J Cell Mol Med 2008; **12**: 1649–60.

35. **Xiong C, Brewer K, Song S, *et al.*** Peptide-based imaging agents targeting phosphatidylserine for the detection of apoptosis. J Med Chem 2011; **54**: 1825–35.

36. **Zheng H, Wang F, Wang Q, *et al.*** Cofactor-free detection of phosphatidylserine with cyclic peptides mimicking lactadherin. J Am Chem Soc 2011; **133**: 15280–3.

37. **Kim YE, Chen J, Chan JR, *et al.*** Engineering a polarity-sensitive biosensor for time-lapse imaging of apoptotic processes and degeneration. Nat Methods 2010; **7**: 67–73.

38. **Bilyy R, Stoika R.** Search for novel cell surface markers of apoptotic cells. Autoimmunity 2007; **40**: 249–53.

39. **Bilyy R, Tomyn A, Kit Y, *et al.*** Detection of dying cells using lectin-conjugated fluorescent and luminescent nanoparticles. Materialwissenschaft Werkstofftechnik 2009; **40**: 234–7.

40. **Laakko T, King L, Fraker P.** Versatility of merocyanine 540 for the flow cytometric detection of apoptosis in human and murine cells. J Immunol Methods 2002; **261**: 129–39.

41. **Bose S, Tuunainen I, Parry M, *et al.*** Binding of cationic liposomes to apoptotic cells. Anal Biochem 2004; **331**: 385–94.

42. **Zhu C, Yang Q, Liu L, *et al.*** A potent fluorescent probe for the detection of cell apoptosis. Chem Commun (Camb) 2011; **47**: 5524–6.

43. **Demchenko AP, Mely Y, Duportail G, *et al.*** Monitoring biophysical properties of lipid membranes by environment-sensitive fluorescent probes. Biophys J 2009; **96**: 3461–70.

44. **Demchenko AP, Yesylevskyy SO.** Nanoscopic description of biomembrane electrostatics: Results of molecular dynamics simulations and fluorescence probing. Chem Phys Lipids 2009; **160**: 63–84.

45. **Schlegel RA, Stevens M, Lumley-Sapanski K, *et al.*** Altered lipid packing identifies apoptotic thymocytes. Immunol Lett 1993; **36**: 283–8.

46. **Matsura T, Togawa A, Kai M, *et al.*** The presence of oxidized phosphatidylserine on Fas-mediated apoptotic cell surface. Biochim Biophys Acta 2005; **1736**: 181–8.

47. **Demchenko AP.** The concept of lambda-ratiometry in fluorescence sensing and imaging. J Fluoresc 2010; **20**: 1099–128.

48. **Shynkar VV, Klymchenko AS, Kunzelmann C, *et al.*** Fluorescent biomembrane probe for ratiometric detection of apoptosis. J Am Chem Soc 2007; **129**: 2187–93.

49. **Demchenko AP.** Introduction to fluorescence sensing. Amsterdam: Springer; 2009.