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A single fluorescent probe enables clearly discriminating and simultaneously imaging liquid-ordered and liquid-disordered microdomains in plasma membrane of living cells

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ABSTRACT

Liquid-ordered (Lo) and liquid-disordered (Ld) microdomains in plasma membrane play different yet essential roles in various bioactivities. However, discrimination of the two microdomains in living cells is difficult, due to the similarity in their constituents and structures. Up to now, polarity sensitive probes are the only tool for imaging the two microdomains, but their small difference between emission spectra in the two microdomains (less than 50 nm) limited their application in living cells. In this work, we first presented an aggregation/monomer type of fluorescent probe (**2,7-9*E*-BHVC12**) with much larger separation in emission wavelength (up to 100 nm), for dual-color visualizing the two membrane microdomains in living cells. The probe can form red-emissive aggregates and yellow-emissive monomers when induced by Lo and Ld microdomains, respectively, and thus enables clear visualization of the two membrane microdomains in living cells with dual colors, and thus high-fidelity images of substructures of plasma membrane have been obtained. According to the images of three kinds of normal cells and three kinds of cancer cells stained with **2,7-9*E*-BHVC12,** significant difference in plasma membrane microstructure of cancer cells was found. In terms of **2,7-9*E*-BHVC12**, normal cells were mainly consisted of either Lo or Ld microdomains all over their membranes, while cancer cells all clearly display coexistence of Lo and Ld membrane microdomains. Therefore, **2,7-9*E*-BHVC12** can serve as a powerful tool for studies of membrane microdomains, and the different results of normal and cancer cells would also deepen our understanding in cancer science.

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1. Introduction

Plasma membrane displays a tremendous complexity of constituents including various lipids, carbohydrates, and proteins to accurately coordinate its diverse biological functions [1–3]. According to recent studies, not only these components are indispensable [4], but also different microdomains assembled by membrane substances carry out crucial functions that cannot be solely achieved by any of the constituents [5,6]. Plenty of biological works reveal that sphingolipids and saturated phospholipids constitute closely packed liquid-ordered (Lo) microdomains (also called lipid rafts), while various unsaturated phospholipids together can form loosely packed liquid-disordered (Ld) microdomains [7]. Actually, sphingolipids and saturated phospholipids exhibit very similar chemical structures and amphiphilic properties with unsaturated phospholipids, with only nuance in the side-chains (saturated or unsaturated), which results in similar

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chemical and physical properties of Lo and Ld microdomains. However, the two kinds of microdomains play different yet important roles in various bioactivities. Lo microdomains are related to the formation of proteins clusters, signal transduction, apoptosis, cell adhesion and migration, synaptic transmission, organization of the cytoskeleton, protein sorting during both exocytosis and endocytosis, and virus invasion [8–15]. On the other hand, Ld microdomains, due to its fluidity and loosely packed state, are important for membrane fusion processes and maintaining the lipids homeostasis in plasma membrane [16]. Particularly, elevated levels of unsaturated lipids and loosely packed Ld microdomains would suppress the secretion and efflux of cholesterol, which would increase the risk of atherosclerotic cardiovascular disease [17,18]. Therefore, observation and investigation of Lo and Ld membrane microdomains are of fundamental importance for biology and pathology [19]. Up to now, membrane microdomains have been widely investigated by diverse methods, including dynamic simulations, detergent resistance experiments, mass spectrometry analysis, and atom force microscopy, etc [20–24]. As is well-known, fluorescent imaging exhibits unprecedented advantages, such as *in situ* observation, real time visualization [25–27], and low injury to living biosamples [28,29], which is an ideal method for the studies on Lo and Ld membrane microdomains. Currently, a single fluorescent probe that enables simultaneous and clear visualization of the two microdomains is in great demand for biological researches [30].

As mentioned above, Lo and Ld microdomains with only nuance in constituents and structures possess similar chemical and physical properties. Thus, clearly discriminating and simultaneously imaging the two microdomains in living cells is a very challenging task. To achieve this task, the minor difference between two membrane microdomains must be utilized as far as possible. One of their differences is that Ld microdomains display stronger hydration than Lo ones, and therefore exhibit higher polarity [30]. Based on this fact, solvatochromic fluorescent dyes sensitive to environmental polarity have been explored. A polarity sensitive probe, Laurdan, was first used to dual-color image the two membrane microdomains [31] and has achieved some success in giant unilamellar vesicles (GUVs): it shows blue emission peaked at 440 nm in Lo phase GUVs, while displays green fluorescence peaked at 490 nm in Ld phase ones. However, two kinds of microdomains in living cells cannot be clearly discriminated and imaged [31,32]. The biggest disadvantage of laurdan is the minor difference of its emission wavelength in two microdomains (only 50 nm in GUVs), and this difference becomes further smaller in living cells [33]. To overcome this drawback, various new polarity probes have been devoted in recent years, such as di-4-ANEDHPPQ, F2N12S, NR12S, and their derivatives [33–37], for simultaneous visualization of the two membrane microdomains. Unfortunately, color separation of all these probes in Lo and Ld phase GUVs was no more than 50 nm. Consequently, their applications in living cells were still in limitation [30], although these probes can clearly image the two microdomains with dual colors in GUVs [33–37]. This minor color separation is mainly originated from the confined polarity difference of Lo and Ld membrane microdomains, and this polarity difference in living cells is smaller than in GUVs [33,38,39], which results in application limitation of polarity probes in living cells. To break this bottleneck, exploring probes ultrasensitive to environmental polarity is a possible way. But comparatively, it is more feasible and necessary to exploit novel fluorescent probes based on new mechanisms.

Klymchenko and co-workers have reported two polarity-sensitive probes **F2N12S** and **F2N8** with similar chemical structures [40]. Amongst, **F2N12S** has been successfully applied to dual-color image Lo and Ld domains, while **F2N8** exclusively partitioned into Ld phase. These experimental facts are very helpful, because they provided a possible way to design fluorescent probes directly sensing the packing states of membrane microdomains. According to Klymchenko's work, loosely packed Ld microdomains in plasma membranes can accommodate the probe that refused by closely packed Lo ones. This is mainly because the size of the probe is small enough to insert into inter-lipid space of Ld domains, but too large to partition into Lo ones. Therefore, decorating two long chains with proper size on fluorogens could create such a probe, which can wholly insert into Ld domains and just partition two long chains into inter-lipid spaces of Lo ones, as shown in Scheme 1a and 1b. Thus, Ld microdomains can accommodate the probe as monomers, and Lo ones may induce its special H-aggregation or J-aggregation on membrane surface. If the aggregates of the probe just can exhibit different emission colors from its monomers, it would be an aggregation/monomer type probe potential to directly and in-site image Lo and Ld microdomains with dual colors.

Recently, plenty of fluorogens with different emission colors in their aggregation and monomer states have been reported [41–43]. In our previous work, *N*-ethyl-2,7-bis(4-vinylpyridinium)-carbazole diiodide (**2,7-9*E*-BHVC**) and its isomer *N*-ethyl-3,6-bis(4-vinylpyridinium)-carbazole diiodide (**3,6-9*E*-BHVC**) both exhibited red-shifted emission in aggregation state [44,45]. Based on works above, herein, two isomers, 9-(2-ethoxyethyl)-2,7-bis(1-dodecyl-4-vinyl-pyridinium)-carbazole di-iodide (**2,7-9*E*-BHVC12**) and 9-(2-ethoxyethyl)-2,7-bis(1-dodecyl-4-vinyl-pyridinium)-carbazole di-iodide (**3,6-9*E*-BHVC12**) (chemical structures in Scheme 1c and 1d) were firstly designed and synthesized by means of the synthetic routine in Scheme 2. According to experimental results, both the two probes can dual-color and *in situ* image Lo and Ld membrane microdomains in GUVs. Delightfully, the emission color separation of **2,7-9*E*-BHVC12** between the two microdomains was up to 100 nm in both GUVs and living cells, much larger than polarity probes (no more than 50 nm), although the separation in emission spectra of **3,6-9*E*-BHVC12** in the two microdomains is only 30 nm in GUVs, and it cannot image the two membrane microdomains in living cells. Furthermore, high-fidelity images of membrane substructures have been captured with **2,7-9*E*-BHVC12**, and the Lo and Ld membrane microdomains of living cancer cells can be clearly visualized with dual colors. At the same time, the *in situ* emission spectra of **2,7-9*E*-BHVC12** in Lo and Ld microdomains in plasma membrane of living cells and the GUVs were shown respectively, in comparison with polarity probes reported whose spectra were only available in the GUVs. As cancer science is a significant and hot area currently, three kinds of cancer cells together with three kinds of normal cells were investigated with probe **2,7-9*E*-BHVC12**. The results demonstrated that normal cells mainly exhibit dominated Lo or Ld microdomains, while the cancer cells all clearly display coexistence of Lo and Ld membrane microdomains.

2. Materials and methods

2.1. Synthesis of *2,7-9E-BHVC12* and *3,6-9E-BHVC12*

2.1.1. Synthesis routine of *2,7-9E-BHVC12*

Probe **2,7-9*E*-BHVC12** was synthesized following the synthetic routine in Scheme 2a. The ^{1}HNMR, ^{13}CNMR, HRMS, and IR spectra were placed in Supporting Information.

4,4′-Dibromo-2-nitrobiphenyl (1): 4,4′-Dibromobiphenyl 10 g (32 mmol) was dissolved in glacial acetic acid (120 mL), and the mixture was stirred and heated to 100 °C. Then, fuming concentrated nitric acid (95%, 40 mL) was added and the resulting mixture was allowed to react for another 30 min. After the reaction solution was cooled to room temperature, the crude product was filtered.

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