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Polarity-dependent Photophysical Properties of Hemicyanine Dyes and Their Application in 2-Photon Microscopy Biological Imaging

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Two fluorescent hemicyanine type compounds, **1** and **2** have been developed for membrane staining, and their photophysical properties were investigated in various solvents. The demonstrated 2-photon-microscopy-biological-imaging application indicates the usefulness of **1** as a fluorescent probe.

Aminostyryl benzimidazolium (Hemicyanine) dyes have been used as fluorescent probes in bioimaging[1–4] and have also found application in lasers,[5] nonlinear optical devices,[6] materials for second harmonic generation (SHG),[7,8] and SHG probes for imaging.[9–11] They consist of a positively charged nitrogen-containing heteroaromatic moiety that serves as an electron acceptor, and another nitrogen conjugated by a polymethine unit that acts as an electron donor. Hemicyanine exhibits charge transfer in both the ground and excited states associated with these electronic donor–acceptor properties. The charge-transfer character relates to the polarity dependence of the absorption and fluorescence spectra and fluorescence efficiency.[12–14] The fluorescence of hemicyanine is usually very weak in aqueous solution but is enhanced in less polar media.[15] In addition, many hemicyanines have a rod-shaped amphiphilic structure that contributes to their affinity for micelles or biomembranes, and thus these compounds have been used for membrane staining. Particularly, FM dyes (Molecular Probes), originally reported by Mao,[16] are widely used for live cell imaging because of their high affinity for the cell membrane, nontoxic nature, stability, ease of use, and availability.[2,3,17–19] However the basic photochemistry of FM dyes in conventional organic solvents is still a relatively undeveloped area that is worthy of study.

In the present paper we briefly describe the photochemical and photophysical behaviors of **FM1-43** (4-[4-(dibutylamino)-styryl]-1-[3-(triethylammonio)propyl]pyridinium dibromide) and the new hemicyanine dyes (Figure 1) (*E*)-1-[3-(triethylammonio)propyl]-4-{2-[9,9-diethyl-7-(dihexylamino)-9*H*-fluoren-2-yl]vinyl}pyridinium dibromide (**1**) and (*E*)-1-[3-(triethylammonio)propyl]-4-{2-[7-(diethylamino)-9,9-dimethyl-9*H*-fluoren-2-yl]vinyl}pyridinium dibromide (**2**) and demonstrate their use as a 2-photon absorption (TPA) fluorophore to perform non-invasive membrane staining. Although **FM1-43** is a known compound,[16] its basic photophysical features in various conventional solvents is still of interest because it is one of the most widely used FM dyes for bioimaging.[20–24]

Aminofluorene skeleton was employed for new dyes **1** and **2**, because of its preferable photophysical properties including 2-photon absorption.[25,26] Due to a longer donor–acceptor distance, **1** and **2** are expected to have larger dipole moments than that of **FM1-43**, which may lead to significant solvatochromic and nonlinear optical effects.

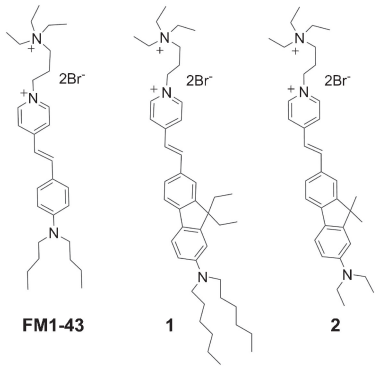

Figure 1. Chemical structures of **FM1-43**, **1**, and **2**.

The syntheses of **FM1-43**, **1**, and **2** are depicted in Supporting Information.[36]

It should first be noted that hemicyanine dyes can easily form aggregates and confined specific regions (such as monolayers) in some solvents.[27] The dyes **1** and **2** also exhibited aggregate behavior and, therefore, the exact molar extinction coefficient values cannot be obtained in any conventional solvents we used. However, we could choose some solvents which can dissolve the dyes in this experiments as "good" solvents (Figures 2a–2c): a less polar aprotic solvent (chloroform), a highly polar aprotic solvent (DMSO), and protic solvents (MeOH and phosphate buffer at pH 7.4), of which the polarities are in the following order: phosphate buffer > DMSO > MeOH > chloroform.

Figure 2a shows the absorption spectra of **FM1-43** in good solvents where **FM1-43** should exist as a monomeric structure. It is well known that the strong S_0–S_1 absorption band of hemicyanine, which originates from the intramolecular charge-transfer (ICT) transition between donor (aniline site) and acceptor (pyridinium cation site) appears around 500 nm. Like other hemicyanines,[13] **FM1-43** exhibits negative solvatochromism; the absorption peak shifts to shorter wavelengths as the solvent polarity increases, indicating a larger dipole moment in the ground state than in the excited singlet state (Franck–Condon region).[12,28,29]

The negative solvatochromic shift is more prominent in **1** (Figure 2b). In chloroform, the absorption peak appeared at 545 nm, which is a longer wavelength than that for **FM1-43** (530 nm), probably due to the effects of π-extension. In an aqueous phosphate buffer at pH 7.4, however, the peak wave-

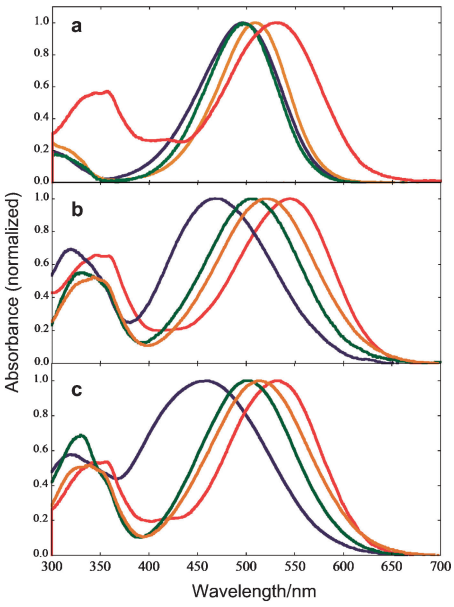

Figure 2. Absorption spectra of **FM1-43** (a), **1** (b), and **2** (c) in chloroform (red line), methanol (orange line), DMSO (green line), and phosphate buffer at pH 7.4 (blue line).

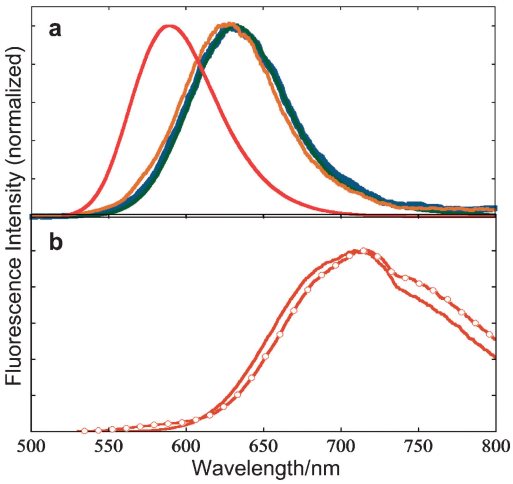

Figure 3. a) Fluorescence spectra of **FM1-43** in chloroform (red line), methanol (orange line), DMSO (green line), and phosphate buffer at pH 7.4 (blue line). The spectra in DMSO and phosphate buffer at pH 7.4 are overlapped. b) Fluorescence spectra of **1** (open circle) and **2** (solid line) in chloroform. No fluorescence was detected in methanol, DMSO, or phosphate buffer at pH 7.4.

length underwent a greater blue shift to 468 nm, an even shorter wavelength than that for **FM1-43** (495 nm). These results suggest that the ground state of **1** is stabilized by solvent molecules more significantly than that of **FM1-43**, probably due to a larger ground state dipole moment and more pronounced localization of the HOMO electrons in **1** (Figure S1[36]). The ground state dipole moments were calculated by B3LYP/6-31G* method to be 7.45 and 14.1 D for the simple model of **FM1-43** and **1**, respectively.[30]

Despite having the same hemicyanine skeleton, the absorption bands of **2** appeared in a shorter wavelength region than those of **1**. The spectral difference is not caused by the electronic effect of different alkyl groups, but rather is believed to be due to the different nature of the solvent–solute interaction resulting from the different steric bulk of the alkyl substituents.[14] The HOMO of this chromophore is located on the amine side and the LUMO on the pyridinium side, as shown in Figure S1.[31,36] The *N*-alkyl chain length in **2** is shorter than that in **1**, which can permit the interaction between the anilino nitrogen and the polar solvent molecules, resulting in stabilization of the ground state and the corresponding larger blue shift. This is consistent with the reported effect of the *N*-alkyl chain length on the absorption band of the other hemicyanines.[14]

The fluorescence peaked at 589 nm in chloroform and was red-shifted to around 630 nm in the highly polar solvents DMSO, MeOH, and the pH 7.4 phosphate buffer (Figure 3a). The fluorescence red shift is caused by the solvent relaxation of the excited singlet state of **FM1-43** that has a strong intramolecular charge-transfer (ICT) character. The Stokes shift values were 1890, 3500, 4490, and 4300 cm^{-1} in chloroform, MeOH, DMSO, and phosphate buffer, respectively, almost increasing monotonically with increasing solvent polarity. The fluorescence quantum yield (Φ_f) of **FM1-43** was high in chloroform ($\Phi_f = 0.42$) and decreased in highly polar or protic solvents: 0.03, 0.005, and 0.002 in DMSO, MeOH, and phosphate buffer, respectively. The very low fluorescence quantum yield in aqueous solution is an important property for membrane staining.

The fluorescence spectra of **1** appeared at a much longer wavelength with a larger Stokes shift (4380 cm^{-1}) than that of **FM1-43**, even in chloroform (716 nm, Figure 3b). In addition, the fluorescence was weak in chloroform ($\Phi_f = 0.06$) and could not be detected in DMSO, MeOH, or pH 7.4 phosphate buffer. These results indicate that the solvent relaxation in the excited singlet state of **1** is more significant than in **FM1-43**. **2** exhibited a similar fluorescence spectra and Φ_f value in chloroform (709 nm, $\Phi_f = 0.08$), and was also nonfluorescent in DMSO, MeOH, and the phosphate buffer.

FM1-43 has already been reported to provide insights into endocytosis, exocytosis,[3] and vesicular release by single synaptic terminals,[32] and also has been used for 2-photon imaging.[23] Thus, we next tested the performance of **1** as a fluorescent dye for 2-photon excitation microscopy and compared the fluorescence signal with that from **FM1-43**.

Figure 4 shows the 2-photon fluorescence images of INS-1 cells that were immersed in solutions containing each of the three dyes at a concentration of 10 μM. The brightness of each dye was evaluated at two wavelengths (830 and 910 nm). Bright reticulation structures represent the plasma membranes of the cells within a cluster of INS-1 cells and are very similar to the β-cells in the islets in the previous studies using **FM1-43**.[33–35] We found that **1** successfully labeled the plasma membrane in the living INS-1 cells, as did **FM1-43**. Importantly, little fluorescence was found in the cytosol of the cells, indicating that **1** did not penetrate the plasma membrane, as is the case with **FM1-43**. At an excitation wavelength of 830 nm, **FM1-43** was brighter, whereas **1** exhibited brighter fluorescence at 910 nm. These findings suggest that the larger TPA cross-section of **1** at 910 nm offsets its lower fluorescence efficiency, whereas at 830 nm, **FM1-43** still have higher TPA efficiency.

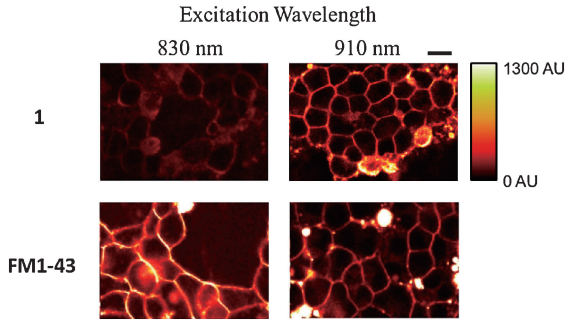

Figure 4. Two-photon excitation live images of clusters of INS-1 cells which were stained with either **1** or **FM1-43**. Each dye was applied from the extracellular medium at a concentration of 10 µM. The excitation wavelength was 830 or 910 nm, as indicated. The fluorescence intensities were represented by the pseudocolor code (inset), where AU stands for arbitrary unit. The mean AU values of the plasma membranes at 830 nm were 200 and 2600 AU for **1** and **FM1-43**, and those at 910 nm were 900 and 300 AU for **1** and **FM1-43**, respectively. The scale bar indicates 10 µm.

In conclusion, we have developed the new hemicyanine-type fluorescent dyes **1** and **2** and evaluated their photophysical properties. Compared to **FM1-43**, **1** and **2** exhibit a more significant solvatochromic shift in both their absorption and fluorescence spectra and a lower fluorescence quantum yield. The demonstrated 2-photon microscopy biological imaging application indicates the usefulness of **1** as a fluorescent probe, especially for 2-photon imaging in the longer wavelength region. More detailed studies of the structure–photophysical property relationships of the new dyes and their potential application for SHG imaging are under way.

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