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An NIR-emitting cyanine dye with pyridinium groups: the impact of regio-bond connection on the photophysical properties[†]

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Two ESIPT-based isomeric cyanines were synthesized with significant bathochromic shift in the optical absorption λ_{abs} and emission λ_{em} , along with a very large Stokes shift. Probe 2 exhibited a longer conjugation and better photostability. Both compounds exhibited good selectivity for labeling the plasma membrane of prokaryotic cells and the hair cells of zebrafish.

Hearing disorders or deafness are common diseases that can be age-related, or noise- or certain ototoxic drug-induced.^{1,2} In the U.S., about 1 in every 8 individuals in the working population have hearing difficulty.³ Most hearing loss is related to the irreversible damage of sensory hair cells in the inner ear,^{4,5} which is often induced by exposure to loud noises⁶ or certain chemicals/drugs that can kill the hair cells in the inner ear. Management of hearing loss requires identification of methods that can stimulate hair cell regeneration in mammals. Due to the limited ability for mammals to regenerate sensory hair cells,⁷ there is significant interest in studying the sensory hair cells of non-mammalian vertebrates such as zebrafish, whose hair cells can be readily regenerated after damage.⁸⁻¹⁴ Recently, zebrafish have been recognized as an excellent animal model to study hair cell development and regeneration, as the zebrafish lateral line opens the gate to identifying the complex signaling events triggered by injury and regeneration.^{4,5,7,15–17}

Zebrafish lateral line hair cells are located in the center of a mechanosensory organ known as the neuromast, where the hair cells are surrounded by inner support cells and an outer ring of mantle cells^{4,5,18} (Fig. 1). Neuromasts can be marked by specific fluorescent dyes such as 4-Di-2-ASP.^{19–21} Development of improved imaging methods is desirable to aid these studies.

Excited state intramolecular proton transfer (ESIPT) has emerged to be a powerful strategy in designing fluorescent molecular probes, as they often exhibit unusually large Stokes shifts ($\Delta \lambda \ge 150$ nm) and dual emission for ratiometric sensing. However, the majority of known ESIPT-based sensors give greenred emission, with very few giving near-infrared emission (NIR-I, 650-950 nm) that is desirable for imaging applications (especially for in vivo). Despite recent progress, it remains a challenge to tune the emission of this class of materials to well above 700 nm. In an effort to search for new dyes with improved optical characteristics, we recently reported that probe 1 with a pyridinium terminal group can label neuromast hair cells on wild type zebrafish.^{22,23} Due to the presence of excited state intramolecular proton transfer (ESIPT), probe 1 exhibits a large Stokes shift ($\Delta\lambda \sim 260$ nm) and a good quantum yield. In comparison with the styryl dye 4-Di-2-ASP ($\lambda_{\rm em} \approx 590-610$ nm, depending on the solvents), the emission of probe 1 occurs at a longer wavelength ($\lambda_{em} \approx 684 \text{ nm in CH}_2\text{Cl}_2$).^{22,23} We now report an improved version by synthesis of 2 and 3 that includes a furan group in the styryl pyridinium segment for extended π conjugation (Scheme 1). Inclusion of the furan group effectively tunes the emission towards an even longer wavelength (to well above 700 nm) while retaining their cellular selectivity, making them even more attractive for imaging applications (e.g. neuromast labeling and staining of E. coli membranes).

Synthesis. Compounds 2 and 3 were synthesized via Suzuki coupling reaction in good yields. The products were characterized



Fig. 1 Zebrafish lateral line neuromasts. (a) Schematic depicting a larval zebrafish. Red patches indicate the location of neuromasts in the lateral line (LL) system. Orange patches represent the location of the anterior (ALL) and posterior (PLL) LL ganglia. (b) A side view of a single LL neuromast structure.

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Scheme 1 The structures of commercial styryl dye and ESIPT compounds, along with their absorption and fluorescence

by ¹H-NMR and ¹³C-NMR spectroscopy, and ESI-MS (see ESI,† Fig. S1-S9 for details).

Photophysical properties. In comparison with the styryl dye (4-Di-2-ASP) that has a small Stokes shift ($\Delta\lambda \sim 21$ nm in CH₂Cl₂), the ESIPT dyes (1-3) revealed a quite large Stokes shift $(\Delta \lambda > 250 \text{ nm in CH}_2\text{Cl}_2)$ (Table 1).^{22–24} As a consequence of including the furan ring in the structure, the absorption and emission of 2 ($\lambda_{\rm max} \sim 490$ nm, $\lambda_{\rm em} \sim 750$ nm in CH₂Cl₂) exhibited a significant bathochromic shift (by \sim 70 nm) from 1 $(\lambda_{\text{max}} \sim 421 \text{ nm}, \lambda_{\text{em}} \sim 686 \text{ nm in CH}_2\text{Cl}_2)$. Interestingly, both 2 and 3 maintained reasonably high fluorescence (e.g. $\phi_{\rm fl} \sim 0.26$ for 1 in CH_2Cl_2), which makes them suitable for imaging. In addition, the fluorescence quantum yield of 2 decreased dramatically in an aqueous medium (e.g., $\phi_{\rm fl} \sim 0.003$ for 2), showing a large solvent effect (ESI,† Fig. S10-S13). Drastic fluorescence quenching in H₂O would be useful for minimizing fluorescence background.

Interestingly, compounds 2 and 3 exhibit a minor fluorescence peak at 570-590 nm, especially in CH₂Cl₂ (Fig. 2), which is not present in compound 1.22,24 In addition, the relative intensity of the minor peak has no significant change over a wide concentration range (2-10 µM) (ESI,† Fig. S14 and S15). This minor



		DCM	DMSO	MeOH	H_2O	MeCN
1	$\lambda_{ m abs}/ m nm \ arepsilon/ m M^{-1} \ m cm^{-1} \ \lambda_{ m em}/ m nm \ \lambda_{ m em}/ m nm \ \Phi_{ m fl}$	421 28 133 686 0.34	395 35 249 701 0.25	401 32 984 695 0.18	392 29 683 697 0.06	397 26 103 690 0.19
2	$\lambda_{ m abs}/ m nm \ \epsilon/M^{-1} \ m cm^{-1} \ \lambda_{ m em}/ m nm \ \mu_{ m fl}$	490 42 957 750 0.266	447 39 128 790 0.115	457 65 417 770 0.109	450 15 410 770 0.003	448 41 851 770 0.115
3	$\lambda_{ m abs}/ m nm \ arepsilon/ m M^{-1} \ m cm^{-1} \ \lambda_{ m em}/ m nm \ \lambda_{ m em}/ m nm \ arPsilon_{ m fl}$	474 35 445 725 0.183	437 36 679 759 0.180	445 41 811 743 0.165	440 25 075 770 0.009	436 38 521 738 0.172



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fluorescence peak was not associated with H-aggregation, as its content was not increased in less concentrated solution.

In order to understand the origin of the observed minor fluorescence peaks, time-dependent density functional theory (TD-DFT) was used to calculate their UV-Vis and fluorescence spectra in CH₂Cl₂ (ESI,[†] Fig. S16–S19). The calculated absorption peaks from the enol forms of 2 and 3 were at 490 nm and 474 nm (ESI,† Fig. S16 and S18), respectively, which matched well with their experimental values (λ_{abs} = 490 nm and 483 nm). The calculation also showed that the fluorescence of the enol forms of 2 and 3 were at 588 and 583 nm, respectively. The results thus pointed to the fact that the minor emission peaks observed at 570-590 nm (Fig. 2) could be attributed to the enol tautomer. The major emission peaks at \sim 700 nm could be attributed to the keto form, in agreement with the ESIPT mechanism.^{23,25} The fluorescence spectra (Fig. 2) thus suggested that ESIPT remained to be a predominant process for new probes 2 and 3, giving major emission from their keto tautomer while having minor emission from the enol forms.

It should be noticed that the emission wavelength is dependent on the extent of intramolecular charge transfer (ICT) in their respective keto tautomer, which is enabled by ESIPT (Scheme 2).^{23,24} Thus, the emission of 2 would occur at a longer



Scheme 2 Schematic illustration of the formation of the keto form via excited state intramolecular proton transfer (ESIPT). And the intramolecular charge transfer

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wavelength than that of its isomer 3, as the keto tautomer of the former (2') had a more extended ICT interaction (involving two C=C bonds in the pyridinium ring, Scheme 2). This was further supported by a computational study, which revealed that 2 had a lower HOMO-LUMO energy gap than 3 (ESI,† Fig. S20), consistent with the experimental observation.

Compounds 2 and 3 generated a new peak at 550–580 nm and became non-fluorescent in the NIR range when pH > 7 (ESI,[†] Fig. S21–S25), as the deprotonation occurred to give aromatic anion Ar–OH \rightarrow Ar–O⁻. With Boltzmann's fitting, the pK_a values of 2 and 3 were determined to be similar at 6.6 and 6.5, respectively (ESI,[†] Fig. S23 and S26), which is notably different from that of 1 ($pK_a = 8.26$). The phenolic proton in 2 and 3 becomes more acidic, induced by the furan group, which is in good agreement with the observed ¹H NMR ($\delta = 13.31$ ppm for the phenolic proton in DMSO).

In order to evaluate the photostability, the solution of 2 and 3 in CH_2Cl_2 was irradiated with a blue LED light (455–465 nm, 7000–8000 mcd), while the absorption spectrum was monitored at different time intervals. Interestingly, compound 2 exhibited significantly better stability than its isomer 3 (Fig. 3), under the same conditions. This result showed a large impact of *regio*bond connection (ESI,† Fig. S27 and S28).

The response of 2 and 3 to anionic and cationic species was also examined. Common metal cations such as Na⁺, K⁺, Co³⁺, Al³⁺, Ag⁺, Hg⁺, Pd²⁺, Zn²⁺, Mg²⁺, and Ni²⁺ exhibited basically no interference in both absorption and emission (ESI,[†] Fig. S29, S30, S33, S34). Biologically important anions, such as ATP, ADP, AMP, Br⁻, I⁻, HPO₄²⁻ and citrate, were found not to interfere with the emission of probe 2. Although basic anions (*e.g.* PO₄³⁻, CO₃²⁻ and acetate (OAc⁻)) were found to cause a significant change in absorption and fluorescence spectra (ESI,[†] Fig. S31 and S32), due to deprotonation of phenol, and they would have little impact on the potential imaging applications due to their low abundance in biological systems.

Bioimaging study. The presence of similar terminal groups as **1** encouraged us to examine new probes **2** and **3** for potential labeling of the *E. coli* cell membrane and hair cells on zebrafish (see ESI† for details, All animal related procedures were



Fig. 3 Comparison of photostability for 2 and 3 in DCM (10 $\mu M),$ by monitoring the decay of $\lambda_{abs}.$



Fig. 4 Fluorescent confocal microscope images of *E. coli* cells stained with 1 μ M compound **2** (a)–(c) and **3** (d)–(f) in TD (a) and (d), excitation/emission = 488/700 nm (b) and (e) and overlap (c) and (f) under magnification of 100× and digitally enhanced by 10 times.

approved by the Institutional Animal Care and Use Committee at The University of Akron (D16-00501)).^{22,24} Thus, these new probes were used to stain *E. coli* cells. Clear fluorescence signals could be observed with low concentration of dye (1 μ M) (Fig. 4(b) and (e)), showing that both probes exhibited good selectivity to stain the plasma membrane (Fig. 4(c) and (f)). In order to compare their relative stability, the stained *E. coli* cells (with 1 μ M of 2 and 3) were continuously irradiated for two minutes (ESI,† Fig. S37). Interestingly, the cells stained with 2 retained 77% average fluorescence intensity, while those stained with 3 only have 56% intensity remaining. The observation indicated that 2 had better photostability than 3, which was consistently observed in CH₂Cl₂ (Fig. 3) and in H₂O (ESI,† Fig. S39).

Compounds 2 and 3 were further used to investigate their potential imaging applications in zebrafish. When the probes were used to stain zebrafish embryos, nonuniform fluorescence signals were observed on the fish's body, indicating selective labeling (Fig. 5 and ESI,† Fig. S38). The labeling pattern by using 2 or 3 was comparable to that using 4-Di-2-ASP (Fig. 5(a)-(d) and ESI,† Fig. S38(a), (d)), showing selective labeling of neuromasts. Although the new probes were used in a lower concentration (10 µM) in comparison with 4-Di-2-ASP (50 µM), they gave comparable fluorescence intensity during the imaging study (Fig. 5 and ESI,† Fig. S38).²⁶ Both probes labeled the central region of the neuromasts where hair cells are located. In summary, both 2 and 3 labeling were nearly identical with the commercial dye 4-Di-2-ASP (Fig. 5(d)-(f)). Probes 2 and 3 would be an attractive alternative to the commercial 4-Di-2-ASP for hair cell labeling, due to their attractive photophysical properties (e.g. large Stokes shift and NIR emission).

Two NIR-emitting (>700 nm) ESIPT dyes 2 and 3 were synthesized in high yield, to examine the impact of a furan group in the styryl pyridinium segment. Inclusion of a furan ring in the styryl segment is found to extend π -conjugation effectively, while retaining the desired cellular selectivity for zebrafish neuromasts and *E. coli* membrane. The attractive optical properties of 2 ($\lambda_{abs} \approx 490$ nm, $\lambda_{em} \approx 770$ nm) make it a rare example of a NIR-emitting fluorophore that exhibits



Fig. 5 Confocal images of zebrafish embryos (72 hpf) labeled with 4-Di-2-ASP (b) and (e) and probe **2** (c) and (f), merged (a) and (d) under $10\times$ (first row) and $100\times$ (second raw) magnification. Excitation/emission = 488/595 nm for 4-Di-2-ASP, and excitation/emission = 488/700 nm for probe **2**.

very large Stokes shift and high fluorescence ($\phi_{\rm fl} \approx 0.26$ in CH₂Cl₂). Optical comparison between **2** and **3** reveals a moderate difference in the absorption and emission wavelengths, attributed to the impact of *regio*-bond connection on the pyridinium ring. This finding is further supported by the TD-DFT calculation.

Molecular imaging studies show that both probes 2 and 3 are useful for labeling the plasma membrane of prokaryotic cells and hair cells of zebrafish with good selectivity, although the former is found to be more photolytically stable. The study thus successfully demonstrates that new probe 2 could be an attractive candidate for imaging hair cells, due to its good cellular selectivity, NIR emission ($\lambda_{\rm em} \approx 770$ nm) with large Stokes shift, and improved photostability. Such an imaging tool could play an important role to aid the study of the regeneration of hair cells and screening/evaluation of *ototoxic* drugs.

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Conflicts of interest

There are no conflicts to declare.

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