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Improved Photostable FRET-Competent Biarsenical−Tetracysteine Probes Based on Fluorinated Fluoresceins

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Biarsenical probes are membrane-permeable fluorogenic dyes, which form highly stable complexes with tetracysteine motifs engineered into a target protein of interest.[1] The probes and targets are small compared to visible fluorescent proteins, thereby reducing potential stereochemical interference while providing (i) controllable times of delivery in pulse chase experiments,[2] and (ii) special reactivities enabling chromophore-assisted light inactivation experiments (CALI) and nonfluorescent readouts.[2] Furthermore, the combination of biarsenical dyes with visible fluorescence proteins (VFPs) as Förster resonance energy transfer[3] donor−acceptor (DA) pairs constitutes a very attractive technology for the assessment of conformational changes and molecular interactions in living cells. Due to the inverse 6th power distance dependence of FRET, the range of separations that can be determined with confidence about the Förster critical distance for 50% FRET efficiency (R_o) is very narrow.[4,5] The generation of DA pairs with large R_o is essential for extending the range over which FRET is operative.

Although optimization of the biarsenical binding motif has led to significant improvements in affinity and signal levels,[6] the limited photostability and pH sensitivity of fluorescein derivatives in the physiological range constitute inherent limitations that still preclude their widespread application.

Here we introduce fluoro-substituted versions, F2FlAsH and F4FlAsH (Figure 1), exhibiting significant improvements in important properties over the original fluorescein derivative FlAsH.[7] Compared to FlAsH, F2FlAsH has higher absorbance, larger Stokes shift, higher quantum yield, higher photostability, and reduced pH dependence. The emission of F4FlAsH lies in a region intermediate to that of FlAsH and ReAsH (a resorufin biarsenical),[7] providing a new color and excellent luminosity. In addition, the two new probes form a new FRET pair with a substantially larger R_o value than any obtained with these dyes (see below).

Syntheses of the tetrafluorinated (F4FlAsH−EDT_2) and difluorinated FlAsH (F2FlAsH−EDT_2) derivatives (Figure 1) were accomplished from the parent halogenated fluoresceins according to published procedures[7] (Supporting Information).

FlAsH-EDT_2, F2FlAsH-EDT_2, and F4FlAsH-EDT_2 are practically nonfluorescent. Upon formation of a complex of F2FlAsH with a 12-mer peptidic sequence (FLNCCPGCCMEP, P12) as a model target,[6] a striking increase in fluorescence is observed with an emission peak at 522 nm. The absorption maximum of F2FlAsH−P12 is shifted 11 nm to the blue, compared to FlAsH−P12, whereas the maximum of F4FlAsH−P12 is displaced 17 nm to the red (Table 1). Thus, the Stokes shift is 22 nm, 7 nm greater than that of the FlAsH complex. The fluorescence intensity of the peptide adduct (λ_{exc} = 490 nm, λ_{em} = 525 nm) is 4-fold brighter than that of the complex with the parent FlAsH probe (Figure 2). This enhancement is attributable to a larger extinction coefficient at 490 nm (2×) and a greater emission quantum yield (2×). The radiative lifetime of F2FlASH−P12 (4.78 ns) is similar to that of the corresponding FlAsH complex (4.88 ns, Table 1).

The emission peak of F4FlAsH−P12 at 544 nm expands the spectral range of the biarsenical dyes. In addition, the fluorescence lifetime increases to a value of 5.2 ns. The two fluorinated derivatives provide new combinations with FRET donors and acceptors within and outside of the biarsenical family.

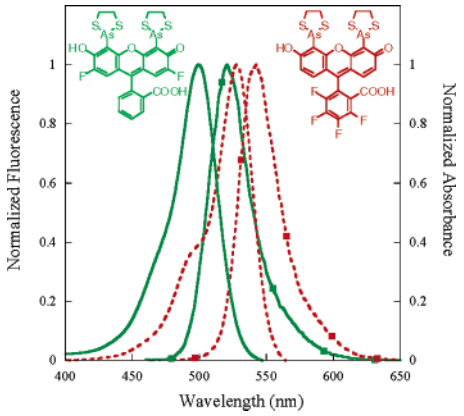

Figure 1. Dye structures and absorption and emission (□) spectra of F2FlAsH−P12 (green) and F4FlAsH−P12 (red).

Table 1. Photophysical Data for the Biarsenical−P12 Complexes

	λ_{abs} (nm)	λ_{em} (nm)	ϵ_{max} ($M^{-1}cm^{-1}$)	τ (ns)	k_{bl} (s^{-1})	pb[a] (%)
FlAsH−P12	511	527	52000	4.88	3.2×10^4	87
F2FlAsH−P12	500	522	65500	4.78	6.2×10^2	32
F4FlAsH−P12	528	544	35100	5.18	9.1×10^3	89

[a] Photobleaching; loss of fluorescence after 120 min of irradiation.

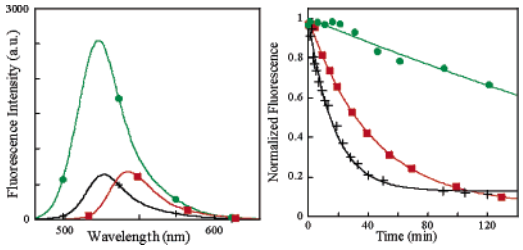

Figure 2. Fluorescence emission spectra (left panel, 0.1 μM) and photobleaching (right panel) of F2FlAsH−P12 (●, green), F4FlAsH−P12 (■, red) and FlAsH−P12 (+). In b, full lines correspond to exponential fits; complexes of the three dyes (10 μM) were irradiated with a mercury arc lamp through a 490−560 nm filter with an irradiance of 70 mW/cm^2.

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Table 2. Critical Förster Distances

	Acceptor			
	ReAsH		F4FlAsH	
donor	$10^{14}\cdot J$	R_o Å (Φ_d)[a]	$10^{13}\cdot J$	R_o Å (Φ_d)
FlAsH	5.03	39 (0.4)[b]	1.48	47 (0.4)
F2FlAsH	3.44	41 (0.8)	1.58	54 (0.8)
F4FlAsH	6.45	41 (0.4)	1.17	45 (0.4)
	F2FlAsH		FlAsH	
	$10^{13}\cdot J$	R_o Å (Φ_d)	$10^{14}\cdot J$	R_o Å (Φ_d)
F2FlAsH	1.37	52 (0.8)	6.41	41 (0.4)

[a] Emission quantum yield. [b] Approximate value from ref 7.

It has been reported that fluorination of fluorescein[8] leads to greater resistance to photobleaching to a degree that depends on the number of fluorine atoms. We observed a 50-fold increase in photostability of the 2′,7′-difluoro derivative, whereas for the tetrafluoro derivative, the photostability is similar to that of the parent dye complex (Figure 2, Table 1). The fluorine atoms in positions 2′ and 7′ presumably lead to a reduction in lifetime of the triplet state that serves as an intermediate in the photobleaching process.[9]

To evaluate the sensitivity to pH, the probes were dissolved in phosphate buffers ranging in pH from 7.8 to 5.6. FlAsH−P12 displayed a 50% decrease at the absorption peak of the dianion responsible for fluorescence, whereas the absorption of F2FlAsH−P12 and F4FlAsH−P12 only decreased by 16% (Figure S2, Supporting Information). The complexes of the fluorinated dyes exhibited a corresponding brighter emission at lower pH. These results were expected in view of the lower p*K*'s of the dianion and monoanion forms of the parent fluorinated fluoresceins.[8]

The R_o values of F2FlAsH, F4FlAsH, FlAsH, and ReAsH in different donor−acceptor combinations are given in Table 2. F2FlAsH and F4FlAsH have a very favorable spectral overlap, leading to a large J (the overlap integral) and thus an R_o of 5.4 nm, greater than any of the values obtained with the other combinations of donors and acceptors based on biarsenical dyes. For example the FlAsH−ReAsH pair has an R_o of 3.9 nm. Thus, the new F2FlAsH−F4FlAsH probes constitute a new donor−acceptor pair extending the dynamic range for heteroFRET in studies of living cells by 40%. They also provide new possibilities in combination with other FRET donors and acceptors within and outside the biarsenical family.

The F2FlAsH−F4FlAsH DA pair was employed in a titration of a F2FlAsH complex of biotin-P12 bound to streptavidin, with the same peptide bearing F4FlAsH (Figure 3). Upon saturation of the free sites remaining on the tetrameric streptavidin, the FRET efficiency was 0.34, corresponding to an apparent mean computed transfer distance of 5.0 nm.

An important consideration related to quantitative FRET determinations based on VFPs is the restricted motion of the chromophore inside the β-barrel of the protein. To accurately estimate distances by FRET, one requires knowledge of the relative orientation of the dyes. The general assumption of the value 2/3 for the orientation factor κ^2 only applies if both donor and acceptor are in rapid, isotropic rotational motion. This is impossible for VFPs due to their mass (27 kDa); the rotational correlation times are much longer than the fluorescence lifetimes.

The fluorescence anisotropies determined for F2FlAsH−P12 biotin and F4FlAsH−P12 biotin (1 μM in 20 mM HEPES, pH 7.4) were 0.038 and 0.046, respectively, implying that one can apply

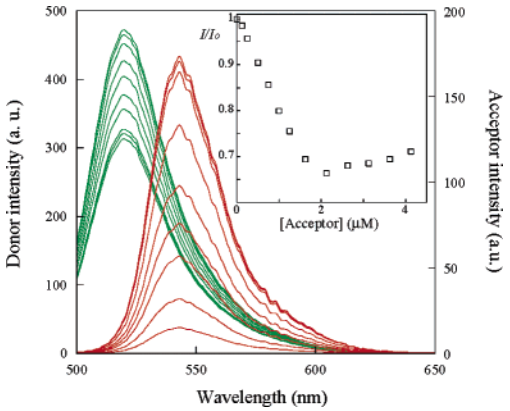

Figure 3. Titration of a complex of streptavidin (0.8 μM)-biotinylated F2FlAsH−P12 (0.8 μM), with biotinylated F4FlAsH−P12 (spectrally unmixed data; the contribution from direct excitation of the acceptor at 490 nm has been subtracted from the acceptor signal at every concentration). Inset: relative change in donor emission (at 520 nm).

the 2/3 κ^2 value with confidence in the case of small and/or mobile targets, thereby allowing accurate distance determinations by FRET. Rotational motion may be restricted in larger proteins, thereby enabling FRET measurements by homotransfer.[4] Both F2FlAsH and F4FlAsH bind the same peptide sequence P12. Evaluation of FRET in the cell requires explicit consideration of potentially concurrent homo- and heteroFRET processes. A quantitative treatment of this problem has been reported by Robia et al. for the evaluation of FRET using FlAsH and ReAsH.[10]

In conclusion, we present two new derivatives of the FlAsH family, one with a remarkably greater (50×) photostability, lower pH sensitivity, higher absorbance and quantum yield, and the second adding a new color to the palette of biarsenical dyes. In combination, the two compounds form an excellent FRET pair with a large critical distance. These properties are expected to facilitate improved structural and dynamic studies of proteins in living cells.

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Supporting Information Available: Experimental procedures and characterization data for all unknown compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269−272.
(2) Tour, O.; Meijer, R. M.; Zacharias, D. A.; Adams, S. R.; Tsien, R. Y. *Nat. Biotechnol.* **2003**, *21*, 1505−1508.
(3) Hoffmann, C.; Gaietta, G.; Bunemann, M.; Adams, S. R.; Oberdorff-Maass, S.; Behr, B.; Vilardaga, J. P.; Tsien, R. Y.; Eisman, M. H.; Lohse, M. J. *Nat. Methods* **2005**, *2*, 171−176.
(4) Jares-Erijman, E. A.; Jovin, T. M. *Nat. Biotechnol.* **2003**, *21*, 1387−1395.
(5) Selvin, P. R. *Annu. Rev. Biophys. Biomol. Struct.* **2002**, *31*, 275−302.
(6) Martin, B. R.; Giepmans, B. N. G.; Adams, S. R.; Tsien, R. Y. *Nat. Biotechnol.* **2005**, *23*, 1308−1314.
(7) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2002**, *124*, 6063−6076.
(8) Sun, W. C.; Gee, K. R.; Klaubert, D. H.; Haugland, R. P. *J. Org. Chem.* **1997**, *62*, 6469−6475.
(9) Lindqvist, L. *Ark. Kemi* **1961**, *16*, 79−138.
(10) Robia, S. L.; Flohr, N. C.; Thomas, D. D. *Biochemistry* **2005**, *44*, 4302−4311.

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