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A highly sensitive fluorescent viscosity sensor

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ABSTRACT

Variation at the 3' position of fluorescein via Suzuki–Miyaura cross-coupling with aryl and heteroaryl moieties gave a family of anthofluoresceins whose spectroscopic properties were studied. The 1-methylindole derivative gave the highest quantum yield and was observed to behave as a molecular rotor, displaying marked variations in fluorescent intensities with viscosity and offering possible application in cellular sensing and fluorescent polarisation assays.

Fluorophores are a central component of many laboratory techniques, with applications ranging from labels for antibodies and protease substrates to fluorescent sensors for metal ions and reactive oxygen species.[1–3] Typically, organic dyes are rapidly responsive and sensitive and are the tools of choice for labelling a wide range of biomolecules and probing biochemical processes.[1–4] Ever increasingly sophisticated in vitro and in vivo studies demand ever more complex fluorescent reporters, such as probes for specific intracellular processes,[4,5] cellular tracking,[6,7] pH measurments,[8–10] ion sensing,[11–13] and in cellulo synthesis.[14,15] As such the demands and expectations placed on specific dyes and chemosensors continues to increase. This includes growing interests in the development of environmentally-sensitive dyes; so-called molecular rotors; whose changes in fluorescence can be 'translated' into viscosity data.[16–18] Such a phenomenon can also be applied in place of techniques such as fluorescence lifetime imaging and fluorescent polarisation.[19,20]

Xanthene dyes (with fluorescein, **1**, the parent of the family, Fig. 1) remain the most broadly utilized family of dyes.[1,2,5,8–16] These versatile dyes can be readily functionalized to allow biomolecule labeling,[21] while the introduction of electron-withdrawing/donating groups on the xanthene core has been used to modify the phenol's p*K*a, thereby tuning its deprotonation to physiologically relevant pH's.[22–25]

We recently developed anthofluorescein, **2**, a fluorescein-derived dye whose absorption and emission spectra display high sensitivity to pH changes between 7 and 10 (ranging from 460–530 nm to 530–580 nm respectively).[26] These optical properties arise due to a combination of factors (see Fig. 1). Firstly tautomer II contains a 4-oxo-2,5-cyclohexadien-1-ylidene group that expands the dye's π-electron system (conjugation expander), leading to a red-shifted E_x/E_m spectra relative to fluorescein; secondly, the *p*-hydroxyphenyl group of tautomer *I* has freedom to rotate, forming a twisted intramolecular charge transfer (TICT) state upon photoexcitation and thus exhibiting two competing relaxation pathways fluorescence emission and non-radiative de-excitation by internal rotation. Consequently, the ratio of these tautomers and their non-ionic forms in different solvents and pH's governs the overall optical properties of anthofluorescein resulting in a multi-functional and highly-sensitive probe.

Encouraged by the unique optical properties of anthofluorescein and the potential to generate a sensitive viscosity sensor, a 20-member library of anthofluorescein derivatives was synthesized in order to study the influence of aryl and heteroaryl moieties decorated with electron-donating/withdrawing groups on the system's fluorescent properties.

A variety of anthofluorescein derivatives were synthesised by mono-triflation of fluorescein, **1**, with *N*,*N*-bis(trifluoromethylsulfonyl)aniline followed by cross-coupling with a series of aryl and heteroaryl boronic acids containing electron donating/withdrawing groups and/or rings. The synthetic procedure is outlined in Scheme 1 (with yields between 63 and 88%) with the list of probes synthesized given in Table 1.

The optical properties of the new derivatives demonstrated a broad and slightly red-shifted emission compared to unmodified

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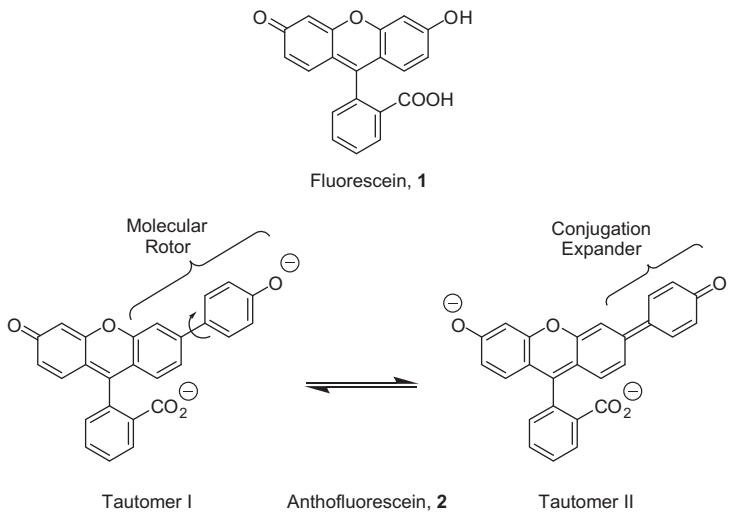
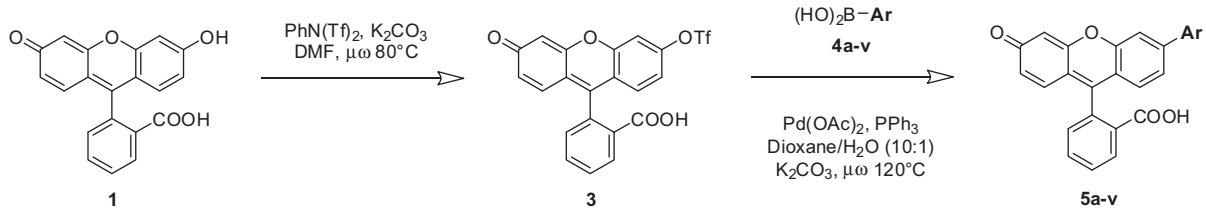


Figure 1. Structures of fluorescein, **1**, and two tautomeric forms of the deprotonated anthofluorescein, **2**.



Scheme 1. Suzuki cross-coupling between fluorescein-monotriflate (**1**) and boronic acids (**4a–v**).

fluorescein. Compounds containing electron rich heterocycles (**5a–i**) typically gave quantum yields (Φ) in methanol ranging from 0.40 to 0.56, superior to anthofluorescein (0.33). On the contrary, compounds containing substituted phenyl derivatives produced dyes with lower Φ than anthofluorescein, with some dyes containing electron withdrawing groups falling below 0.2 (**5q**, **5r** and **5t**).

According to the Föster–Hoffmann equation, there is a direct relationship between viscosity and quantum yield (see equation below, where Φ is the quantum yield; η is the viscosity, C is a system-dependant constant; x is a dye dependant-constant).[27]

$$\log \Phi = C + x \log \eta$$

While for most small molecule dyes viscosity changes lead to small variations of Φ, these variations are highly prominent in the case of fluorescent molecular rotors.[16,17,27] Due to their inherent potential to dissipate energy by intramolecular rotation, photoactivated molecules may relax by non-radiative decay processes. Viscous environments reduce the loss of energy via this mechanism, thus increasing the relative proportion of relaxation via the fluorescent excited state via photon emission.[17]

From the library generated, the probe with a 1-methylindole moiety (compound **5e**) had the highest quantum yield (0.56) in methanol and maximum emission at 537 nm. To investigate which members of the library were more sensitive to viscosity changes, we determined their corresponding Φ in ethylene glycol (low viscous solvent) and glycerol (highly viscous solvent) (see Table 1). This study showed that compound **5e** showed the highest dynamic range, with Φ = 0.20 in ethylene glycol and Φ = 0.67 in glycerol.

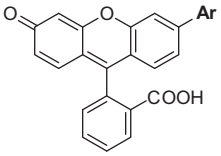
Thus the optical properties of **5e** were measured in mixtures of ethylene glycol and glycerol; a pair of miscible solvents which allow the formation of solvent mixtures of different viscosity with minimal changes in polarity.[17,27] As observed in Figure 2, fluorescent analysis showed that fluorescent intensity was highly influenced by the viscosity of the environment: the higher the viscosity of the mixture the greater the fluorescence emission.

Plots of log $I_{\mathbf{5e}}$ against the log η as shown in Figure 3, give a linear relationship as predicted.[27–29]

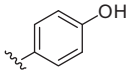
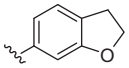
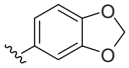
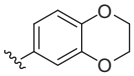
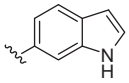
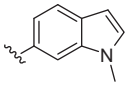
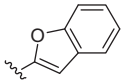
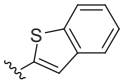
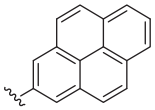
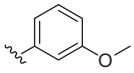
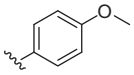
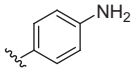
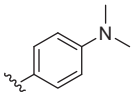
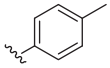
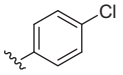
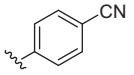
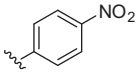
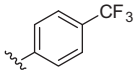
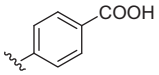
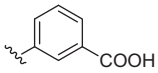
Incubation of **5e** with HeLa cells showed that the dye was rapidly up taken and was clearly visible (results not shown). This is supposed to be due to the high viscosity of the cellular cytoplasm which in terms of viscosity is more akin to glycerol than water[29] confirming the previous viscosity observations. To facilitate future measurements of intracellular viscosity, it would be desirable that the probe would be retained inside the cells after fixation. To verify this, cells were incubated with compound **5e** for 2 h, fixed with paraformaldehyde, washed with PBS and imaged by fluorescent microscopy, showing clear fluorescence signal coming from the cells while eliminating all extracellular background (Fig. 4).

In conclusion a series of anthofluorescein derivatives were synthesised via Suzuki cross-coupling chemistry and their spectroscopic properties studied. Emission spectra showed all derivatives had a broad and slightly red-shift emission compared with fluorescein. Dye **5e** containing a 1-methyl-1*H*-indol-5-yl moiety showed significant variations of its fluorescent properties with viscosity, which followed the Förster–Hoffmann equation. These results strongly suggest **5e** behaves as a molecular rotor where its emission intensity is highly influenced by the environment viscosity. Preliminary in vitro assays showed **5e** was cell permeable and successfully labelled HeLa cells. This probe could be used as a microviscosity sensor, making it a valuable tool for biological studies including a new angle on fluorescent polarisation type assays where binding events cause changes in rotational rates.

Table 1
The anthofluorescein derivatives synthesised and their fluorescent properties (20 μM in methanol, ethylene glycol and glycerol)



5a-v

Probe	Ar	E_x/E_m (nm)	Φ In methanol	Φ In ethylene glycol	Φ In glycerol	Probe	Ar	E_x/E_m (nm)	Φ In methanol	Φ In ethylene glycol	Φ In glycerol
2		478/538	0.33	0.19	0.40	**5l**		478/533	0.22	0.24	0.35
5a		482/528	0.49	0.29	0.45	**5m**		470/532	0.23	0.20	0.26
5b		480/532	0.51	0.33	0.50	**5n**		476/532	0.26	0.23	0.27
5c		480/536	0.50	0.28	0.49	**5o**		475/530	0.30	0.28	0.30
5d		480/531	0.47	0.25	0.54	**5p**		478/529	0.27	0.21	0.27
5e		480/537	0.56	0.20	0.67	**5q**		470/535	0.13	0.16	0.24
5f		482/533	0.45	0.29	0.41	**5r**		480/534	0.18	0.20	0.20
5g		477/528	0.46	0.33	0.36	**5s**		482/531	0.21	0.18	0.25
5h		480/536	0.42	0.27	0.40	**5t**		480/533	0.19	0.20	0.23
5i		479/538	0.40	0.29	0.37	**5u**		480/532	0.29	0.22	0.27
5j		470/535	0.30	0.28	0.32	**5v**		480/530	0.29	0.19	0.32
5k		477/525	0.32	0.26	0.28						

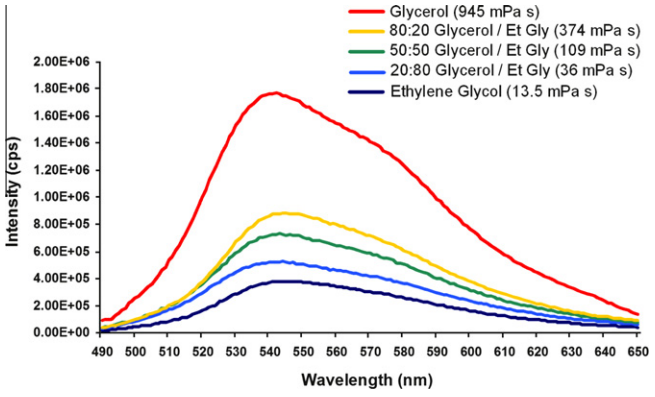


Figure 2. Effect of solvent viscosity on the fluorescent intensity of **5e**. Fluorescence excitation wavelength = 480 nm. Viscosity values of ethylene glycol and glycerol mixtures (as reported by Haidekker et al.[25]) are given between brackets. (Temperature at 25 °C, concentration at 945, 374, 109, 36 and 13.5 mPa s).

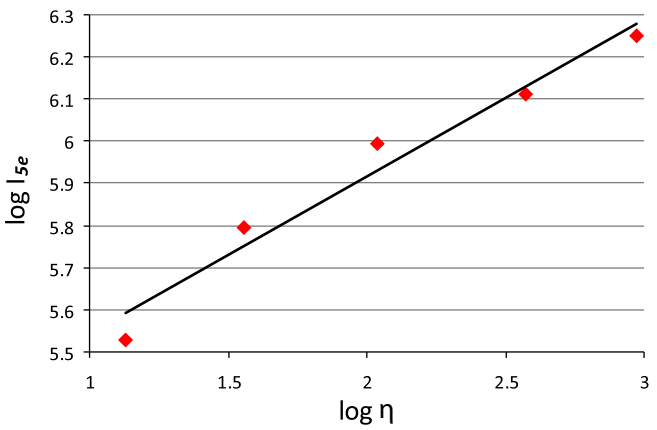


Figure 3. Plots of $\log I_{5e}$ vs $\log \eta$. I_{5e} = emission intensity of **5e** at 537 nm. η = viscosity values (mPa s) of ethylene glycol/glycerol mixtures.[25]

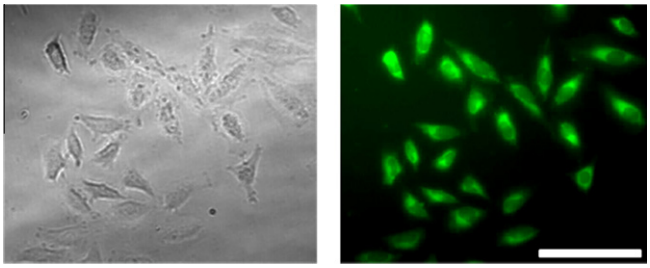


Figure 4. HeLa cells after incubation with a 5 μM solution of **5e** for 2 h at 37 °C. The cells were fixed and washed twice with PBS. HeLa cells were imaged using a ×20 objective (Leica fluorescence microscope) under brightlight (left) fluorescent image (right) with 488 nm excitation and no emission filter. Scale bar, 100 μm.

Acknowledgments

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Supplementary data

Supplementary data (general methods, experimental procedures and characterization) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.101.

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