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Push-pull dioxaborine as fluorescent molecular rotor: far-red fluorogenic probe for ligand-receptor interactions

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Abstract

Fluorescent solvatochromic dyes and molecular rotors increase their popularity as fluorogenic probes for background-free detection of biomolecules *in cellulo* in no-wash conditions. Here, we introduce a push-pull boron-containing (dioxaborine) dye that presents unique spectroscopic behavior combining solvatochromism and molecular rotor properties. Indeed, in organic solvents, it shows strong red shifts in the absorption and fluorescence spectra upon increase in solvent polarity, typical for push-pull dyes. On the other hand, in polar solvents, where it probably undergoes Twisted Intramolecular Charge Transfer (TICT), the dye displays strong dependence of its quantum yield on solvent viscosity, in accordance to Förster-Hoffmann equation. In comparison to solvatochromic and molecular rotor dyes, dioxaborine derivative shows exceptional extinction coefficient ($120,000 \text{ M}^{-1} \text{ cm}^{-1}$), high fluorescence quantum yields and red/far-red operating spectral range. It also displays much higher photostability in apolar media as compared to Nile Red, a fluorogenic dye of similar color. Its reactive carboxy derivative has been successfully grafted to carbetocin, a ligand of the oxytocin G protein-coupled receptor. This conjugate exhibits >1000-fold turn on between apolar 1,4-dioxane and water. It targets specifically the oxytocin receptor at the cell surface, which enables receptor imaging with excellent signal-to-background ratio (>130). We believe that presented push-pull dioxaborine dye opens a new page in the development of fluorogenic probes for bioimaging applications.

1 Introduction

Fluorogenic dyes that turn on their fluorescence in response to the changes of their environment gain increasing attention because they offer the possibility of imaging of target

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biomolecules with minimal background.^{1–5} Fluorogenic dyes have already been used as probes for ligand-receptor binding^{6, 7} and protein interactions,^{4, 8} guest fluorophores that turn on fluorescence with target RNA aptamer,^{9, 10} labels of biomolecules,¹ micro-viscosity probes¹¹ as well as cell plasma membrane probes.^{12, 13} Variety of mechanisms have been exploited to achieve the turn on response, such as charge transfer,^{14, 15} dye dimerization,^{7, 16} chemical reaction (e.g. reversible spirolactone formation),¹⁷ bond twisting (molecular rotors),¹⁸ etc. The most common mechanism is intramolecular charge transfer in solvatochromic dyes, where changes in solvent polarity produce strong shifts of the emission maximum and thus drastic changes in the intensity at a given wavelength.^{10, 11} Moreover, solvatochromic dyes display strong variation of their fluorescence quantum yields upon changes in solvent polarity, so that they are usually quenched in polar solvents, especially water.⁴ This property is extensively used for sensing biomolecular interactions and imaging lipid membranes.^{5, 19} The typical example is Nile Red, which was used to design fluorogenic membrane probe sensitive to lipid order,²⁰ as well as to create fluorogenic label to monitor interaction of carbetocin ligand with its target oxytocin receptor⁶ and turn on probe for SNAP-tag labeling.²¹ Another rapidly expanding approach is to use bond twisting in molecular rotors.¹⁸ Molecular rotors are poorly fluorescent in water, but drastically increase their fluorescence when exposed to highly viscous media. They were successfully used to study microviscosity in the whole cell¹¹ and in the lipid membranes.^{22, 23} Important emerging applications of molecular rotors are turn-on probes for oligonucleotide aptamers (e.g. synthetic GFP fluorophore),^{9, 10} and protein aggregation (e.g. thiophlavin B).^{24–26} However, molecular rotors usually operate in the green and blue spectral regions, while examples of red emissive rotors either exhibit limited quantum yields (like malachite green),²⁷ or moderate sensitivity to viscosity (like cyanines or squaraines).^{28, 29} Moreover, the use of molecular rotors to monitor ligand-receptor binding has never been shown so far. Here, we present a new class of molecular rotor dyes based on dioxaborine push-pull dye (DXB Red, Fig. 1). In dioxaborines, enol and ketone oxygens are bridged by boron, which improves spectroscopic properties of these dyes,^{30–32} similarly to BODIPY and related systems.³³ Previous report showed that DXB Red exhibits strong variation of its quantum yield between polar acetonitrile and apolar chloroform,³⁴ although neither solvatochromism nor molecular rotor properties of this dye were evaluated. Our current studies in organic solvents revealed that DXB Red behaves like polarity sensitive dye, showing strong red shifts of its absorption and emission spectra on increase in the polarity. Moreover, it showed drastic drop of its quantum yield in polar solvents and practically zero emission in water. Remarkably, in polar solvents its quantum yield shows clear dependence on viscosity, which results in nearly 30-fold increase in fluorescence between methanol and glycerol. This dye, grafted to carbetocin ligand, showed strong fluorescence after binding to the target receptor oxytocin, which enabled imaging of the latter at the cellular surface with excellent signal to background ratio.

2 Experimental

2.1 Material

All chemicals and solvents for synthesis were from Sigma-Aldrich, TCI Europe or Alfa Aesar. Synthesis of DXB Red (8-(Diethylamino)-4-(4-(diethylamino)styryl)-2,2-difluoro-5-

oxo-(5H)-chromeno[4,3-d]-1,3,2-(2H)-dioxaborine) was described elsewhere.³⁴ Analytical RP-HPLC separations were performed on a C18 Ascentis Express column (2.7 μm , 4.6 mm $\times$ 75 mm) using a linear gradient (5% to 100% of solvent B in solvent A in 7.4 min, flow rate of 1.6 mL $\cdot\text{min}^{-1}$, detection at 220 nm, solvent A: 0.1% TFA in H_2O , v/v, solvent B: 0.1% TFA in CH_3CN , v/v). Semi-preparative RP-HPLC separations were performed on a SunFire C18 column (5 μm , 19 $\times$ 150 mm). NMR spectra were recorded at 400 MHz on a Bruker Advance spectrometer. Chemical shifts are reported in parts per million (ppm), coupling constants (J) are reported in hertz (Hz). HRMS were acquired on a Bruker MicroTof mass spectrometer, using ESI and a TOF analyzer.

2.2 Synthesis of DXB-COOH

A mixture of dioxaborine **1** (8-(diethylamino)-2,2-difluoro-4-methyl-5-oxo-(5H)-chromeno[4,3-d]-1,3,2-(2H)-dioxaborine) **34** (1.5 mmol, 0.48 g) and aldehyde **2** (3-((4-formylphenyl)(methyl)amino)propanoic acid) **35** (1.5 mmol, 0.31 g) in acetic anhydride (2 mL) was heated at 100°C for 1 h. The resulting mixture was poured into iced water and stirred at r.t. for 1 h. The precipitate was filtered and washed with water. The crude dye was refluxed with benzene (25 mL) overnight. The precipitate was filtered, washed with benzene and dried in high vacuum to give DXB-COOH as a violet solid (0.37 g, 75%). M.p. 228-230 °C. ^1H NMR (CDCl_3): δ 1.24 (t, $J=6.4$ Hz, 6H, NCH_2CH_3), 2.67 (t, $J=6.2$ Hz, 2H, NCH_2CH_2), 3.10 (s, 3H, NCH_3), 3.46 (q, $J=6.4$ Hz, 4H, NCH_2CH_3), 3.78 (t, $J=6.2$ Hz, 2H, NCH_2CH_2), 6.35 (s, 1H, *HetH*), 6.60 (d, $J=8.8$ Hz, 1H, *HetH*), 6.70 (d, $J=7.6$ Hz, 2H, *ArH*), 7.65 (d, $J=7.6$ Hz, 2H, *ArH*), 7.92 (d, $J=8.8$ Hz, 1H, *HetH*), 8.21 (d, $J=15.2$ Hz, 1H, *CH*), 8.29 (d, $J=15.2$ Hz, 1H, *CH*).

2.3 Synthesis of dye-peptide conjugate DXB-CBT

To a stirred solution of DXB-COOH (0.98 μmol , 0.5 mg) and Lys8-CBT6 (1.07 μmol , 1.2 mg) in dry DMF (0.5 mL) PyBOP (2.44 μmol , 1.27 mg) was added followed by DIEA (7.81 μmol , 1.29 μL). The reaction mixture was stirred at r.t. for 15 min (the completion of the reaction was monitored by analytical RP-HPLC). The resulting mixture was purified by semi-preparative HPLC eluted with $\text{CH}_3\text{CN}/(\text{H}_2\text{O} + 0.1\% \text{ TFA})$: 20 to 80% in 30 min, to give DXB-CBT as a violet solid (402 nmol; 41% yield determined by UV absorbance in 1,4-dioxane). RP-HPLC purity: >98%. HRMS (ESI): calcd for $\text{C}_{71}\text{H}_{96}\text{BF}_2\text{N}_{14}\text{O}_{17}\text{S}$ ($[\text{M} + \text{H}]^+$) 1497.6859, found 1497.6852.

2.4 Instrumentation

Absorption spectra were recorded on a Cary 4000 spectrophotometer (Varian) and fluorescence spectra on a FluoroMax 4 (Jobin Yvon, Horiba) spectrofluorometer. Unless specified, fluorescence spectra were recorded at 540 nm excitation wavelength at 20°C. All spectra were corrected for instrumental effects. Conversion of wavelengths to wavenumbers was performed using the equation $I(\nu) = \lambda^2 I(\lambda)$.³⁶ Fluorescence quantum yields were determined according to following equation: $\Phi_{\text{Fs}} = \Phi_{\text{Fr}} \times (A_{\text{r}}/A_{\text{s}}) \times (S_{\text{s}}/S_{\text{r}}) \times (n_{\text{s}}/n_{\text{r}})$, where Φ_{F} , A , S , and n denote fluorescence quantum yield, absorbance at the excitation wavelength (540 nm), area under the emission peak, and refractive index of solvent, respectively.

Inferior letters, “r” and “s”, denote reference and sample, respectively. The Φ_{Fr} value of Nile Red in dioxane ($\Phi_{\text{Fr}} = 0.91$) was used as a reference.²⁰

In photodegradation assays, 200 nM solution of a given dye in toluene in a closed quartz micro-cuvette (50 μM volume) was illuminated by a 525 nm light of Xenon lamp of a FluoroLog spectrofluorometer (slits were open to 8 nm). The excitation wavelength was selected to ensure that the extinction coefficients of DXB Red and Nile Red were close. The illumination power density was $\sim 1 \text{ mW cm}^{-2}$. During the time of illumination (1 h), the fluorescence at 600 nm was recorded as a function of time.

Fluorescence confocal microscopy experiments were performed on a Leica TCS SPE-II microscope with a HXC PL APO 63x/1.40 OIL CS objective. Excitation was performed with a 561 nm 10 mW laser (30% intensity), emission was collected at 575-750 nm interval.

2.5 Cell lines, culture conditions, and treatment

HEK293T cells stably expressing the wild-type oxytocin receptor (wtOTR) were cultured in Eagle's minimal essential medium (MEM, Invitrogen 21090) with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 $\mu\text{g/mL}$ of streptomycin, 2 mM of glutamine and 500 $\mu\text{g/mL}$ of G418 at 37 °C in a humidified 5% CO_2 atmosphere. 70-80% cell confluence was maintained by removal of a portion of the culture and replacement with fresh medium twice a week.

For microscopy studies, cells were seeded onto a chambered cover glass (IBiDi) at a density of 5×10^4 cells/IBiDi 24 h prior to the experiment. Cells were washed two times by gentle rinsing with PBS, then solutions of fluorescent ligands in 1 mL of Leibovitz's L-15 medium (no phenol red) were added. Cells were incubated with fluorescent ligand at room temperature for 5 min before imaging.

3 Results and discussion

3.1 Photophysical properties of DXB Red

We investigated the photophysical properties of the new dye in organic solvents of different polarity and viscosity in order to understand its solvatochromic and fluorogenic character. As demonstrated in Fig. 2 and Table 1, absorption and fluorescence spectra of DXB Red displayed high sensitivity to solvent polarity, with the pronounced shift to the red upon the increase in solvent polarity. The red shift in the absorption indicates that DXB Red possess significant dipole moment in the ground state, which further increases on electronic excitation to the Frank-Condon state.¹⁵ Remarkably, DXB Red exhibited high extinction coefficients (e.g., $\epsilon = 120,000 \text{ M}^{-1} \text{ cm}^{-1}$ in ethanol). To the best of our knowledge, such a high extinction coefficient has never been reported for existing solvatochromic and molecular rotor dyes. For example, BODIPY rotor, ROBOD, shows $71,000 \text{ M}^{-1} \text{ cm}^{-1}$,^{37, 38} while for DCDHF and analogues fluorophores the extinction coefficient is between 30,000 and $71,000 \text{ M}^{-1} \text{ cm}^{-1}$.³⁹ In addition, DXB Red exhibits extremely high photostability even in apolar solvent like toluene where typical push-pull dyes become less photostable (Fig. 3).⁴⁰ In fact, Nile Red, operating in a similar spectral range and being considered as highly photostable among solvatochromic dyes, lost $\sim 30\%$ of initial fluorescence intensity after 1

hour of continuous excitation, whereas in the same conditions the photodegradation of DXB Red was practically not observed (Fig. 3). Such high photostability of DXB Red would be ascribed to the dioxaborine structure, where non-bonding electron pairs (n electrons) on a carbonyl group are involved in the complexation with boron. High energetic n electrons in carbonyl containing push-pull dyes play an important role in generating excited triplet state of the dye and following photodegradation,⁴¹ so that hydrogen bonding that excludes n electrons from the excitation makes these dyes highly photostable in protic solvents.⁴⁰ In the case of DXB Red, complexation with boron may produce similar stabilizing effect on the carbonyl n electrons. Excellent photostability observed for this dye presents a strong advantage of the use of DXB Red for probing apolar biological environment, e.g. cell plasma membrane and hydrophobic pockets of receptors. Furthermore, the absorption and fluorescence maxima of DXB Red varied between yellow (~550 nm) and far-red (>650 nm) region depending on the environment (Table 1), which would be also beneficial for cellular imaging to decrease the autofluorescence from endogenous fluorophores.

3.2 Fluorescence solvatochromism

Next, we investigated the correlation between the fluorescence band position and polarity index, $E_T(30)$ (Fig. 4a). In aprotic solvents, a well-fitted linear correlation was observed, indicating that the excited state of DXB Red undergoes intramolecular charge transfer (ICT). In fact, this behavior is common for the molecules bearing electron donor and acceptor moieties, so-called dipolar or push-pull dyes showing fluorescent solvatochromism.^{40, 44–46} In addition, we compared the sensitivity to the environment polarity of DXB Red and Nile Red, a commonly used solvatochromic dye.^{20, 47} In the linear fit, DXB Red displayed similar slope as Nile Red, indicating comparable sensitivity of DXB Red dye to the solvent polarity (Fig. 4a). This also implies that DXB Red can monitor polarity changes in the immediate local environment similarly to popular Nile Red and its derivatives, being used in material science and biology.^{6, 20, 21, 48} In non-viscous protic solvents such as methanol and ethanol, the emission maxima were deviated to the shorter wavelengths compared to the solvents of similar $E_T(30)$ index (Table 1). This indicates that in these protic solvents, the H-bonding interactions do not induce additional red shift of the spectra, in contrast to $E_T(30)$ index that is a function of both dielectric constant and H-bond donor ability.¹⁵ We can hypothesize that in DXB Red the boron atom withdraws electron density from the carbonyl, which may minimize its H-bonding interactions with protic solvents, thus producing the deviation with respect to $E_T(30)$ index.

3.3 Quantum yield variation

Next, the dependency of the fluorescence quantum yield (Φ_F) values on solvent polarity was investigated (Fig. 4b and Table 1). DXB Red showed high Φ_F values in apolar solvents ($\Phi_F = 0.46, 0.93$ and 0.72 in cyclohexane, toluene, and dioxane, respectively). On the other hand, the Φ_F values were largely decreased in solvents of higher polarity, starting from ethyl acetate ($\Phi_F = 0.16$) and decreased further in more polar solvents ($\Phi_F < 0.05$) except glycerol. Moreover, in water DXB Red was practically non-fluorescent. In a dioxane-water mixture, Φ_F decreased rapidly with addition of water, reaching ~10-fold lower values already at 20% of water (Fig. 4c). The decrease in the Φ_F value in polar solvents is expected to be derived from twisted intramolecular charge transfer (TICT) state where the molecule in

excited state forms twisted structure.¹⁴ TICT species are effectively generated in polar solvents, and then strongly stabilized due to their increased electronic charge separation state. This large stabilization effect leads to internal conversion according to *Energy gap rule*,⁴⁹ followed by thermal deactivation (lower Φ_F value). Such TICT formation is often observed in push-pull dyes possessing vinyl group in the conjugation system, typified by DCVJ,^{50, 51} DCDHF,³⁹ and chalcone analogs.⁵² As an evidence that DXB Red forms TICT, the Φ_F value of DXB Red in highly polar but viscous glycerol was strongly enhanced ($\Phi_F = 0.36$). This is a typical behavior for the dyes undergoing TICT mechanism because the above-mentioned twisting becomes kinetically less favorable in viscous media, thus reducing the contribution to internal conversion pathway and increasing Φ_F value.

Due to the above-mentioned characteristics, TICT has been used as a key fluorescence mechanism for molecular rotor dyes. Herein, the dependence of fluorescence quantum yield of DXB Red on viscosity of the media was studied using methanol and glycerol binary mixtures. As expected, Φ_F values increased gradually with the increase in the ratio of glycerol/methanol, i.e. viscosity of the mixture (Fig. 5 and Table 2). Moreover, we found an excellent correlation of Φ_F values *versus* viscosity expressed by Förster-Hoffmann equation in wide viscosity range from 30 to 1300 cP, indicating the Φ_F value of DXB Red in polar media is purely dependent on their viscosity (Fig. 5b). Moreover, the value of slope in that correlation is estimated about 0.6, which is expected value for the dyes in the class of molecular rotor,⁵³ and suggests that DXB Red will work as a viscosity indicator at least in polar environments.

3.4 Bioconjugation and fluorogenic property

Due to the highly reduced fluorescence in aqueous solutions, fluorogenic dyes offer a possibility of no-wash fluorescence imaging of biological structures and processes in living cell. To demonstrate the potential of DXB Red to visualize membrane receptors in no-wash experiments, we derivatized the dye with the carboxyl function and grafted the obtained fluorescent label DXB-COOH onto carbetocin which is a peptide ligand of the oxytocin G protein-coupled receptor (OTR) (Scheme 1). Acetic anhydride-driven condensation of **1** with benzaldehyde **2**, prepared according to previous report,³⁵ afforded the functionalized dioxaborine DXB-COOH in 75% yield. Coupling of DXB-COOH with the Lys8 derivative of carbetocin⁶ was carried out under the PyBOP *in situ* activation, and allowed to obtain the target fluorescent OTR ligand DXB-CBT in 41% yield. The identity and the purity of DXB-CBT were confirmed by LC-HRMS.

To verify the fluorogenic behavior of DXB-CBT conjugate, we measured its absorption and fluorescence spectra in apolar (1,4-dioxane) and polar (water) solvents. In water, DXB-CBT showed a broad absorption band with a maximum around 560 nm (Fig. 6a). This broadening could be related to the direct solvatochromic effect and/or partial aggregation of the dye in highly polar environment. In 1,4-dioxane, the absorption spectrum of DXB-CBT was narrow, similar to that of DXB Red in the same solvent (Figs. 6a and 2a). Importantly, DXB-CBT is practically non-fluorescent in water. The residual fluorescence was strongly red shifted with a maximum ~660 nm (Fig. 6b). This negligible red-shifted emission is clearly a result of combined effects of high polarity and relatively low viscosity of water that quench

efficiently the dye fluorescence in aqueous media. In sharp contrast, DXB-CBT was strongly fluorescent in 1,4-dioxane (Fig. 6b). The intensity at the maximum of this conjugate in dioxane (614 nm) was >1200 higher compared to that for DXB-CBT in water at the same wavelength, showing strong fluorogenic response of the dye to the environment.

3.5 Live-cell microscopy studies of the oxytocin receptor

Confocal microscopy studies of the fluorescent ligand DXB-CBT were performed on living HEK293T cells expressing the full-length OTR at the cell surface. Clear membrane staining of cells was obtained already with 10 nM solution of DXB-CBT without any washing steps (Fig. 7a). To prove the receptor specificity of the OTR ligand, we performed the competition experiment with the non-fluorescent competitor CBT at 2 μ M, which demonstrated the significant decrease in the membrane fluorescence comparing to the experiment without competitor (Fig. 7b). The quantitative analysis of the panel (a) of Fig. 7 revealed the 130-fold difference between the membrane and the background fluorescence intensity (Fig. 7d), which is among the highest ratio detected so far for the membrane receptor detection in no-wash conditions. Importantly, when 10 times higher concentration (100 nM) of the fluorophore was added to cells, signal to background ratio remained relatively high (~20), confirming the excellent fluorogenicity of the dioxaborine dye (Figs. 7c and d). For comparison, in similar conditions, CBT labeled with non-fluorogenic lissamine rhodamine B showed signal to background ratio of ~2, because of strong fluorescence of non-bound fluorescent ligand. Moreover, the average membrane fluorescence intensity DXB-CBT at 100 nM remained similar to that in the experiment with 10 nM of the ligand (Fig. 7d), suggesting that all available receptors were already saturated with 10 nM of DXB-CBT, and, more importantly, the dye did not bind non-specifically to cell plasma membrane.

Taking together, the microscopy data demonstrate the excellent fluorogenic properties of dioxaborine molecular rotor and its high potential in detecting membrane receptors in no-wash conditions.

4 Conclusions

In the present work, we demonstrated that dioxaborine push-pull dye combines properties of solvatochromic and molecular rotor dye. Our studies in organic solvents revealed that it behaves like polarity-sensitive dye, showing strong red shifts of its absorption and emission spectra on increase in the polarity. Moreover, it shows drastic drop of its quantum yield in polar solvents and it is non-fluorescent in water. Remarkably, in polar solvents its quantum yield displays clear dependence on viscosity, which results in nearly 30-fold increase in fluorescence between methanol and glycerol. In comparison to other solvatochromic and molecular rotor dyes, dioxaborine derivative exhibits exceptional extinction coefficient ($120,000 \text{ M}^{-1} \text{ cm}^{-1}$). Moreover, it is one of the first dyes of this class having absorption and emission reaching far-red region. This dye, grafted to the oxytocin receptor ligand, showed strong fluorescence after specific binding to the target receptor, which enabled receptor imaging at the cellular surface in no-wash conditions with excellent signal to background ratio.

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Fig. 1. Structure and rotor properties of dioxaborine molecular rotors. (a) Structure of the molecular rotor DXB Red. (b) Fluorescence response of the molecular rotor DXB Red to the change of the environment from fluid (MeOH, black) to viscous (glycerol, red). (c) Structure of the carbetocin conjugate DXB-CBT.

Fig. 2. Normalized absorption (a) and fluorescence (b) spectra of DXB Red (1 μM) in the solvents of different polarity. Excitation wavelength was 540 nm.

Fig. 3. Photostability of DXB Red and of Nile Red in toluene. Excitation wavelength was 525 nm; emission was detected at 600 nm; illumination power density was $\sim 1 \text{ mW cm}^{-2}$; concentration of dyes was 200 nM.

Fig. 4.

Solvatochromic and fluorogenic properties of DXB Red. (a) Position of emission maxima of DXB Red and Nile Red versus $E_T(30)$ in aprotic solvents. The slope of the linear fits for DXB Red and Nile Red are 156 and 119 cm^{-1} , respectively, and r^2 value (goodness of fits) are 0.85 and 0.93 , respectively. (b) Dependency of fluorescence quantum yield on solvent polarity. Red circle and blue triangle represent ethylene glycol and glycerol, respectively. (c) Fluorescence quantum yield as a function of water content in dioxane.

Fig. 5.

(a) Fluorescence spectra of DXB Red in binary mixtures of methanol and glycerol of different ratio. (b) Correlation of the fluorescence quantum yield with the viscosity of the medium according to the equation $\text{Log } \Phi_F = C + x \log \eta$ (Förster-Hoffmann equation, where Φ_F is fluorescence quantum yield, η is solvent viscosity, x is dye-dependent constant and C is concentration and temperature dependent constant). The slope of the linear fits is 0.606, and r^2 value (goodness of fits) is 0.99.

Scheme 1.
Synthesis of DXB-CBT.

Fig. 6.

Fluorogenic behavior of DXB-CBT in solvents. Absorption (a) and fluorescence (b) spectra of DXB-CBT (1 μ M) in water and 1,4-dioxane. Inset shows the zoomed fluorescence spectrum of DXB-CBT in water of negligibly low intensity. Excitation wavelength was 560 nm for both solvents.

Fig. 7. Confocal microscopy studies. Confocal images of OTR cells with 10 nM of DXB-CBT (a), 10 nM of DXB-CBT and 2 μ M of CBT competitor (b), or 100 nM of DXB-CBT (c). Fluorogenic properties of DXB-CBT: average membrane and background fluorescence for all the images (d). DXB-CBT was incubated with cells 5 min before imaging. Laser excitation was done at 561 nm and the emission was collected at 575-750 nm interval.

Table 1 Spectroscopic parameters of DXB Red.^a

Solvent	$\lambda_{\text{abs,max}}$ [nm]	$\lambda_{\text{em,max}}$ [nm]	Φ_F	$E_T(30)$	ϵ_r	η [cP]
Cyclohexane	542	556	0.46	30.9	2.02	0.898
Toluene	567	597	0.93	33.9	2.37	0.59
1,4-Dioxane	569	610	0.72	36	2.21	1.3
Ethyl acetate	571	617	0.16	38.1	5.99	0.455
Chloroform	581	622	0.75	39.1	4.71	0.57
Dichloromethane	587	634	0.16	40.7	8.93	0.425
Acetone	584	636	0.021	42.2	20.5	0.316
DMSO	607	659	0.035	45.1	46.8	1.98
Acetonitrile	589	644	0.011	45.6	35.7	0.345
Ethanol	581	635	0.044	51.9	24.6	1.2
Methanol	582	637	0.016	55.4	32.6	0.6
Ethylene glycol	605	653	0.072	56.3	37.7	20
Glycerol	610	654	0.36	57	42.5	1317

^a $\lambda_{\text{abs,max}}$ and $\lambda_{\text{em,max}}$ are absorption and emission maxima; Φ_F is the fluorescence quantum yield. Nile Red in 1,4-dioxane was used as a reference.²⁰ $E_T(30)$ is polarity index; data from Ref.15 ϵ_r and η are dielectric constant and viscosity, respectively; data from Refs.42, 43

Table 2

Spectroscopic properties of DXB Red in binary mixtures of methanol and glycerol of different ratio.

Glycerol fraction (v/v)	η [cP]	$\lambda_{\text{abs,max}}$ [nm]	$\lambda_{\text{em,max}}$ [nm]	Φ_F
0	0.6	583	638	0.016
10	1.8	585	643	0.019
20	5.2	588	643	0.023
30	13.2	591	643	0.029
40	30.6	594	644	0.038
50	66.0	596	650	0.054
60	133.2	597	650	0.077
70	253.5	602	650	0.12
80	458.6	604	652	0.17
90	793.0	606	653	0.25
100	1317.0	610	653	0.36

^a η is viscosity of methanol/glycerol mixture; data from Ref.54 $\lambda_{\text{abs,max}}$ and $\lambda_{\text{em,max}}$ are absorption and emission maxima; Φ_F is the fluorescence quantum yield, measured using Nile Red in 1,4-dioxane as a reference.²⁰