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Counterion Exchange Enhances the Brightness and Photostability of a Fluorous Cyanine Dye

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Abstract

Fluorofluorophores are a unique class of fluorophores that can be solubilized in perfluorocarbons (PFCs) and used to study biological systems. However, because of the low dielectric constant and high oxygen solubility in the fluorous phase, the brightness and photostability of the fluorofluorophores are significantly diminished. Here, we leveraged the tight ion pairing in the fluorous phase to improve the photophysical properties of a fluorous soluble pentamethine dye (FCy5) via counterion exchange. We found that larger, softer, fluorinated, aryl borate counterions promote the ideal polymethine state where charge delocalization across the polymethine chain increases the brightness (6-fold) and photostability (55-fold) of FCy5.

Keywords

Counterion; Fluorescent probes; Fluorofluorophore; Perfluorocarbon; Photobleaching; Photophysics

Introduction

Perfluorocarbons (PFCs) are highly fluorinated compounds poised for many unique applications due to their inertness, high gas solubilities, and phase separation to form a fluorous phase.[1,2] PFCs have found widespread utility as components of medical devices, drug delivery vehicles, and sensors.[3–7] As the use of PFCs has grown, so has the array of fluorophores that can be employed to label the fluorous phase. We have deemed these

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Conflict of Interests
The authors declare no conflict of interest.

Supporting Information Summary
The authors have cited additional references within the Supporting Information.[41,42]

Supporting information for this article is available on the WWW under https://doi.org/10.1002/chem.202402514 fluorophores “fluorofluorophores” where the first “fluoro” refers to fluorine and the second refers to fluorescence.[8]

The development of fluorofluorophores is challenging due to the non-polarizability of PFCs.[2] Fluorofluorophores, particularly red-shifted ones, contain large polarizable **π** systems which resist solubilization in the extremely non-polar and non-interacting fluorous phase. In previous work, we successfully appended multiple fluorous tags onto cyanine and chromenylium polymethine fluorophores to establish a spectrum of fluorofluorophores.[9,10] These fluorofluorophores have enabled tracking of PFCs in cells, zebrafish, and mice.[9,10] However, challenges in brightness and photobleaching are particularly apparent when fluorofluorophores are dissolved in PFCs. These limitations are derived from the low dielectric constants and high gas solubilities of PFCs.[1,2,11] Here, we report a simple counterion exchange strategy to enhance both the brightness and photostability of a cyanine pentamethine fluorofluorophore (FCy5, **1**, Figure 1A).

The counterion of a polymethine dye is often an ill-considered aspect of the fluorophore. Previous works on the role of the counterion have largely centered on altering solubility and aggregation properties.[12,13] However, work by Maury and coworkers has shown that in the solid state or in non-polar solvents, the counterion can affect the photophysical properties by modulating ground state desymmetrization.[14,15] Ground state desymmetrization occurs when the polymethine dye adopts polyene character with the charge localized on one heterocycle rather than complete charge delocalization throughout the **π** system (*i. e.* ideal polymethine state, IPS) (Figure 1B).[14,15] The polyene state displays broadened absorption, decreased absorption coefficients (**ε**), and decreased radiative rates as compared to the IPS.[14–17] Lastly, there are reports suggesting that the counterion can modulate the photostability of polymethine dyes, although this mechanism is less established.[18,19]

We envisioned that counterion effects would be particularly significant on polymethine fluorofluorophores due to the extremely low dielectric constants (ε_o) of PFCs.[20] Bühlman and coworkers have characterized ion pairing to be 10^6 times stronger in PFCs than organic solvents.[21] With this hypothesis, we exchanged the chloride counterion on our previously reported FCy5 **(1)** to different borate counterions ranging in weight-percent fluorine (wt% F) and size (Figure 2, Table S1) and characterized the solubility, photophysical properties and photobleaching of each dye-counterion pairing.

Results and Discussion

Unlike standard structural modifications via synthesis, counterion modification is simple and straightforward. Counterions were exchanged by sonicating a mixture of FCy5 chloride **(1•Cl)** in dichloromethane (DCM) and the applicable counterion in water.[22] The exchanged dye was isolated from the DCM layer and purified via column chromatography (Figure 2A). The chloride anion of **1** was exchanged with tetrafluoroborate ($^{-}BF_4$), tetrakis[3,5-bis(tri-fluoromethyl)phenyl]borate ($^{-}B(ArF_6)_4$), tetrakis [3,5-bis(1,1,1,3,3,3-hexafluoro-2-methoxy-2-propyl)phenyl]borate, ($^{-}B(ArF_{12})_4$) and tetrakis[3,5-bis(perfluorohexyl)phenyl]borate ($^{-}B(ArF_{26})_4$) (Figure 2B). All dyes were isolated in 89% yield or greater and complete counterion exchange was confirmed by ^{19}F-NMR.

Each counterion exchange introduced more fluorine atoms into the FCy5 fluorophore, and we first evaluated these effects on fluorous solubility. We characterized the fluorous partition coefficient ($\log_F$) for each FCy5 dye.[9] All the dyes had positive $\log_F$ values, which indicates preference for the fluorous phase (Figure S1). All values were greater than or equal to the starting **1•Cl**, with statistically significant differences only observed for the largest, most fluorous $^{-}$B(ArF$_{26}$)$_4$ anion. Additionally, we did not observe a significant change to the solubility limit between **1•Cl** and **1•B(ArF$_6$)$_4$** (Table S2). These data suggest that the fluorous solubility is not significantly modulated by the counterion exchange and remains ~0.6 mM for the FCy5 dyes. This value is well above the concentrations used for characterization of photophysical properties, suggesting that any changes observed are not a result of aggregation. This conclusion is further corroborated by concentration-dependent absorption and excitation spectra (Figure S2).

We proceeded to investigate the photophysical properties of the FCy5 dyes in three fluorous solvents: perfluorooctylbromide (PFOB), perfluorohexanes (PFH) and HFE-7700. These solvents all have relatively low dielectric constants (1.57–6.7), high oxygen solubilities (>0.1 mL$_{oxygen}$/mL$_{PFC}$) and have previously been used with fluorofluorophores in biological and materials applications.[1,23–26] For reference, we also measured the photophysical properties in acetone where counterion effects are significantly diminished due to the solvent's high dielectric constant (ε_o=20.7). Differences in the absorption spectra upon counterion exchange were immediately apparent (Figure 3, S3). Dyes **1•Cl** and **1•BF$_4$** displayed broadened spectra, while **1•B(ArF$_6$)$_4$**, **1•B(ArF$_{12}$)$_4$**, and **1•B(ArF$_{26}$)$_4$** displayed characteristic cyanine dye spectra with a narrow absorption peak and vibronic shoulder. Notably, these differences were not observed in acetone (Figure 3). We quantified these absorbance changes by full width half maximum (FWHM, Table 1). These data are consistent with counterion effects on ground state desymmetrization where the small, hard counterions $^{-}$Cl and $^{-}$BF$_4$ promote the polyene state. Meanwhile, the narrow absorption traces of **1•B(ArF$_6$)$_4$**, **1•B(ArF$_{12}$)$_4$**, and **1•B(ArF$_{26}$)$_4$** are indicative of the larger, softer counterions promoting the IPS.[14,15]

Next, we evaluated the counterion effects on ε and fluorescence quantum yield (Φ_F), which together constitute a fluorophore's brightness (brightness=$\varepsilon * \Phi_F$). We measured the ε of FCy5 in PFOB, PFH, HFE-7700 and acetone (Table 1). We found that **1•Cl** consistently had lower ε than **1•B(ArF$_6$)$_4$**, **1•B(ArF$_{12}$)$_4$**, and **1•B(ArF$_{26}$)$_4$**, which can be explained by the greater polyene character in **1•Cl**. However, **1•BF$_4$** did not follow this trend, suggesting other factors are also influencing ε. Then, we found that the Φ_F of FCy5 associated with larger counterions were higher than **1•Cl** across all fluorous solvents (Table 1). Again, we observed variability in **1•BF$_4$** depending on the solvent. Dyes **1•B(ArF$_6$)$_4$**, **1•B(ArF$_{12}$)$_4$**, and **1•B(ArF$_{26}$)$_4$** displayed a 1.5 to 6-fold increase in brightness compared to **1•Cl**. In acetone, the brightness of the exchanged dyes was diminished possibly from decreased preference for organic solvents (Table 1). To gain insight into the observed trends in brightness, we measured the fluorescence lifetimes of the FCy5 fluorofluorophores by time-correlated single-photon counting (TCSPC) in PFOB (Figure S4). From these data, we calculated the radiative (k_r) and nonradiative decay constants (k_{nr}) (Table S3). We observed an increase in k_r and decrease in k_{nr} with **1$^\bullet$B(ArF$_6$)$_4$**, **1$^\bullet$B(ArF$_{12}$)$_4$**, and **1$^\bullet$B(ArF$_{26}$)$_4$** indicative of higher fluorescence energy conversion while stabilized in the IPS.

A limitation across all fluorescence imaging platforms is the photostability of the fluorophore. The photobleaching mechanism of polymethine dyes has been well studied: excited fluorophores can undergo intersystem crossing (ISC) to the triplet state and sensitize oxygen, which then undergoes a [2+2] cycloaddition with a vinylene on the polymethine chain (Figure 4A).[27,28] Unfortunately, because PFCs can dissolve more oxygen (ca. 84-fold) than water, and because singlet oxygen ($^1O_2$) has a much longer lifetime in PFCs than in water ($\tau \sim 10^{-2}$ s in PFC vs 10^{-5} s in water),[24,29] photobleaching of fluorofluorophores is a significant problem. Approaches to prevent photobleaching include making covalent modifications to the chromophore scaffold or using photostabilizing additives.[30–32] Structural modifications require additional synthetic steps and optimization, and additives require high concentrations to be effective. Therefore, counterion exchange would be a more facile method to enhance the photostability.

To examine the photostability of FCy5, we exposed the fluorophores to a 660 nm LED light and monitored their absorption loss over time. We observed dramatic differences in the photostabilities of **1$^\bullet$B(ArF$_6$)$_4$**, **1$^\bullet$B(ArF$_{12}$)$_4$**, and **1$^\bullet$B(ArF$_{26}$)$_4$** compared to **1$^\bullet$Cl** and **1$^\bullet$BF$_4$** (Figures 4B and C). In PFOB, **1$^\bullet$Cl** loses 70% of its original absorbance within 30 minutes while **1$^\bullet$B(ArF$_6$)$_4$** and **1$^\bullet$B(ArF$_{26}$)$_4$** take 30 and 50 hours, respectively, to undergo the same amount of degradation (Figure 4B). Quantifying the photobleaching rates ($k_{photobleach}$) indicated that in PFOB, **1$^\bullet$Cl** and **1$^\bullet$BF$_4$** were up to 55-fold less stable than **1$^\bullet$B(ArF$_6$)$_4$**, **1$^\bullet$B(ArF$_{12}$)$_4$**, and **1$^\bullet$B(ArF$_{26}$)$_4$** (Figure 4B). Photobleaching in PFH and HFE-7700 yielded similar trends (Figure S5). Within the polymethine photobleaching pathway there are numerous possibilities for the role of the counterion in reducing photobleaching rates including: (a) decreasing the rate of ISC, (b) decreasing the ability to sensitize oxygen, (c) decreasing the ground state reactivity of the fluorophore towards $^1O_2$ (Figure 4A).

Starting with possibility (c), we studied counterion effects on the ground state reactivity of **1$^\bullet$Cl**, **1$^\bullet$B(ArF$_6$)$_4$**, and **1$^\bullet$B(ArF$_{26}$)$_4$**. The rationale for differences in the ground state reactivity is that if the larger counterions are stabilizing FCy5 in its IPS, we expect the dye to have less double bond character across the polymethine chain, and therefore decreased reactivity towards $^1O_2$. Alternatively, larger counterions centered above the polymethine chain in the IPS state (vs. associated with a heterocycle in the polyene state) could sterically protect the polymethine chain. To evaluate the reactivity of FCy5 to $^1O_2$, we chemically generated $^1O_2$ from H_2O_2 and NaOCl and exposed it to **1$^\bullet$Cl**, **1$^\bullet$B(ArF$_6$)$_4$**, and **1$^\bullet$B(ArF$_{26}$)$_4$** in the dark. We measured the absorbance after 15 minutes and correlated the loss of absorption with $^1O_2$ reactivity. We found that **1$^\bullet$Cl** displayed significantly more absorption loss than **1$^\bullet$B(ArF$_6$)$_4$**, and **1$^\bullet$B(ArF$_{26}$)$_4$** (Figure 4C), suggesting that the larger counterions stabilize the ground state. However, we did not observe a significant difference in absorption loss between **1$^\bullet$B(ArF$_6$)$_4$** and the 2.5-fold larger **1$^\bullet$B(ArF$_{26}$)$_4$**, suggesting that **1**'s reactivity towards $^1O_2$ is mediated primarily by the counterion's electronic effects rather than steric protection. We performed a similar experiment with perfluorodecanethiol which also displayed decreased reactivity towards **1$^\bullet$B(ArF$_6$)$_4$** (Figure S6). While it appears ground state reactivity contributes to the differences in photobleaching, the fold-changes in these experiments were lower compared to those observed in the overall photobleaching studies (7-fold vs. 55-fold, Figure 4C). This suggests the counterion is also affecting other steps within the photobleaching pathway.

Next, we probed counterion contribution towards oxygen sensitization. We established oxygen dependency by photobleaching $\mathbf{1^{\bullet}Cl}$ in the absence of oxygen. The $k_{photobleach}$ of deoxygenated $\mathbf{1^{\bullet}Cl}$ is substantially reduced, similar to the $k_{photobleach}$ of oxygenated and deoxygenated $\mathbf{1^{\bullet}B(ArF_6)_4}$, supporting an oxygen-dependent photobleaching mechanism (Figure 4C). To probe $^1O_2$ formation, we measured $^1O_2$ phosphorescence detected at 1270 nm in PFOB and observed a 2-fold decrease in $^1O_2$ phosphorescence from $\mathbf{1^{\bullet}Cl}$ compared to $\mathbf{1^{\bullet}B(ArF_6)_4}$ and $\mathbf{1^{\bullet}B(ArF_{26})_4}$ (Figures 4D, S7). These experiments indicate that the counterion identity also affects the sensitization efficiency of the fluorophore to generate $^1O_2$.

The above results could be ascribed to changes in ISC rates (possibility a) or energy transfer to oxygen (possibility b). Energy transfer to oxygen occurs through a Dexter mechanism that requires close contact and at least 22 kcal/mol between the triplet and ground states.[33] The increased steric profile of the larger counterions could prevent the close contact required for energy transfer. However, we do not favor this mechanism as we did not observe significant steric effects when we photobleached **1** with chemically generated $^1O_2$ and did not observe significant differences in $k_{photobleach}$ between $\mathbf{1^{\bullet}B(ArF_6)_4}$ and $\mathbf{1^{\bullet}B(ArF_{26})_4}$ (Figure 4C). Another possibility for the distinct differences in $^1O_2$ formation between $\mathbf{1^{\bullet}Cl}$ and $\mathbf{1^{\bullet}B(ArF_6)_4/1^{\bullet}B(ArF_{26})_4}$ could be derived from reducing the energy gap between the triplet state and ground state to below the energy needed to sensitize oxygen. The large fluorinated counterions that promote the IPS also decrease the HOMO and LUMO energies due to their electron withdrawing nature.[34,35] The stabilization of the ground state and singlet excited state appear proportional to each other as we observed minimal changes in $\lambda_{max,abs}$ upon counterion exchange. The triplet energies of pentamethine cyanine dyes are generally 35–37 kcal/mol above the ground state, far exceeding the energy necessary to sensitize oxygen.[36,37] While we cannot rule out that the counterions stabilize the triplet state ~13 kcal/mol more than the ground state, we believe it is more likely that smaller changes to the energy of the triplet state modulate ISC (possibility a) rather than preventing energy transfer (possibility b).

Favoring possibility (a), we looked to prepare two additional variants of **1** to change the ISC rates and analyze the resulting photobleaching and $^1O_2$ phosphorescence. Increasing ISC rates has been widely studied for applications in photodynamic therapy, where the most common approaches include decreasing the energy difference between the singlet and triplet excited states and enhancing the spin-orbit coupling.[38–40] With the hypothesis that the fluorinated counterions stabilize the triplet state disproportionately to the ground and singlet excited states, we looked to test the non-fluorinated aryl borate counterion $^{-}BPh_4$, which has significantly different electronics than the fluorinated aryl borate anions.[17] We found that $\mathbf{1^{\bullet}BPh_4}$ displayed more polyene character than $\mathbf{1^{\bullet}B(ArF_6)_4}$ and $\mathbf{1^{\bullet}B(ArF_{26})_4}$, and had photobleaching rates and $^1O_2$ phosphorescence values similar to $\mathbf{1^{\bullet}Cl}$ and $\mathbf{1^{\bullet}BF_4}$ (Figure 4D, 4E, S8, S9, Table S4). These results are consistent with the hypothesis that the electron withdrawing nature of the aryl borate counterions decreases the photobleaching rates by increasing the energy gap between the singlet and triplet state, which lowers the ISC rate. To compare the counterion effects we have observed through the hypothesized modulation of triplet energy to the more-established enhanced spin-orbit coupling mechanism, we prepared **1•I** and measured its photophysical properties (Figures S8, S9, Table S4). For the case of **1•I**, we observed 3-fold increase in ${}^1O_2$ phosphorescence compared to **1•Cl** and a decrease in Φ_F (Figures 4D and E, Table S4). Generally, increased ISC rates lead to an increase in ${}^1O_2$ quantum yield (Φ_Δ) at the expense of Φ_F.[40] While it is difficult to determine Φ_Δ values in the fluorous phase as there are limited reference dyes, performing comparative Φ_Δ analysis using relative integration of ${}^1O_2$ phosphorescence (Figures 4E, S10), we find that as the Φ_Δ increases the Φ_F decreases, which also supports that we are modulating the ISC rates. Ultimately, further studies directly probing the triplet state in the fluorous phase will enhance our understanding of these counterion effects on photobleaching.

Conclusions

In summary, we discovered that a simple counterion exchange can enhance the brightness of an FCy5 dye by 6-fold and photostability by 55-fold, overcoming brightness and photostability challenges that often plague fluorofluorophores. Specifically, we found that when FCy5 is associated with large, soft counterions, the brightness and photostability is considerably increased compared to small, hard counterions. We attribute the increase in brightness to improvement of the ε and $\Phi_{F,}$ where larger counterions increase the cyanine character and radiative rates of FCy5. Through a series of systematic photobleaching experiments, we ascribe the dramatic increase in photostability observed to be primarily a result of electronic modulation rather than steric protection of the polymethine chain. Counterion effects in organic solvent show modest photophysical changes compared to those of FCy5 in fluorous solvents, a good indication that dielectric constants and ion pairing play significant roles in counterion effects. The work described herein will expand the utility of fluorofluorophores in biological imaging and beyond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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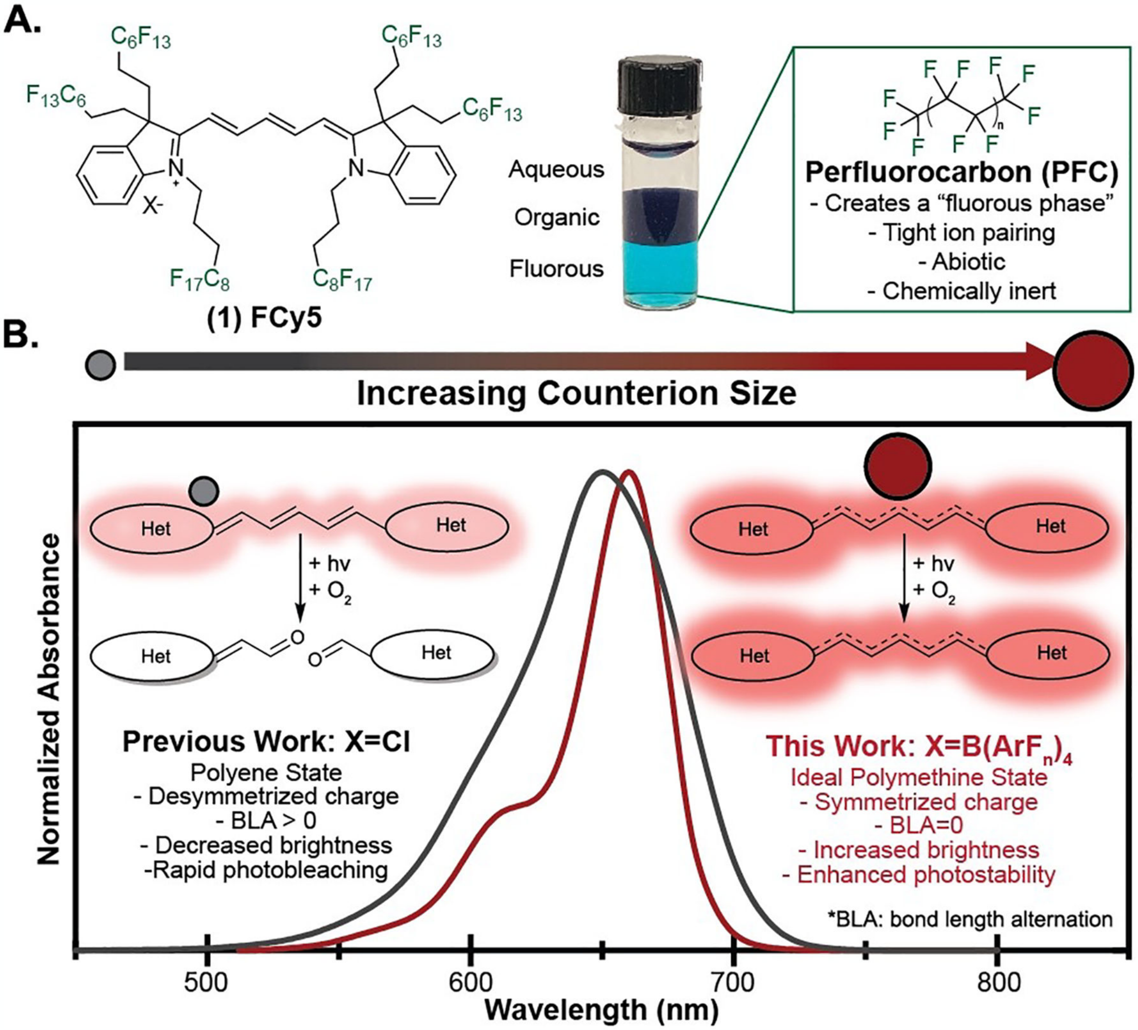

Figure 1.

(A) FCy5 and the unique properties of perfluorocarbons. (B) Counterion effects on ground state desymmetrization of polymethine dyes.

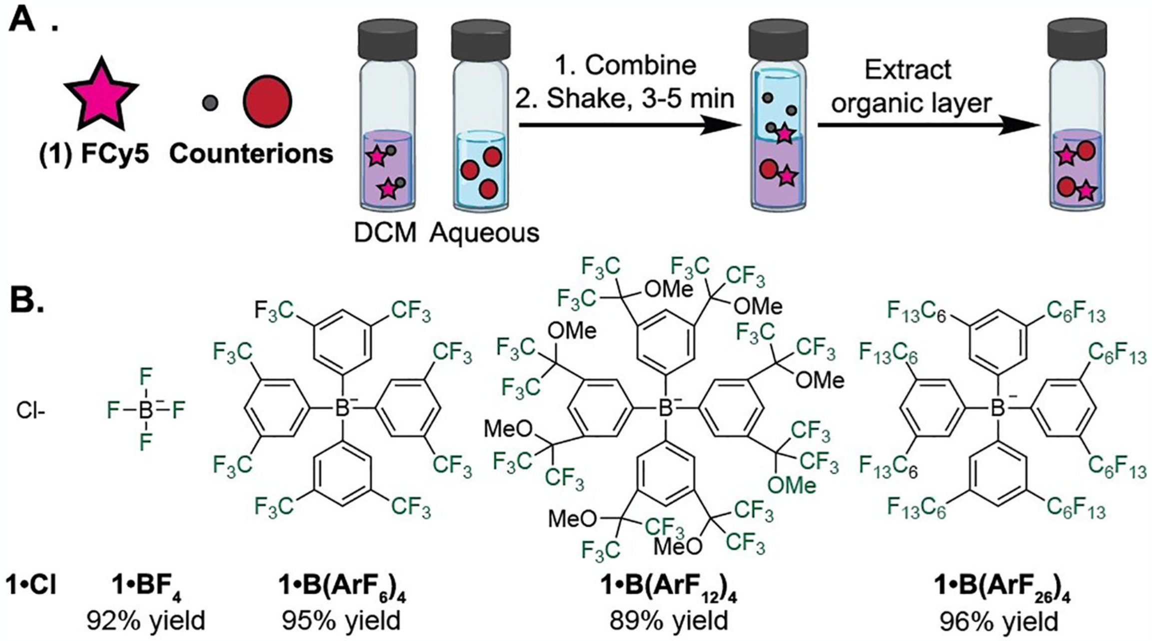

Figure 2.

(A) Depiction of the counterion exchange strategy. (B) The counterions used for this study.

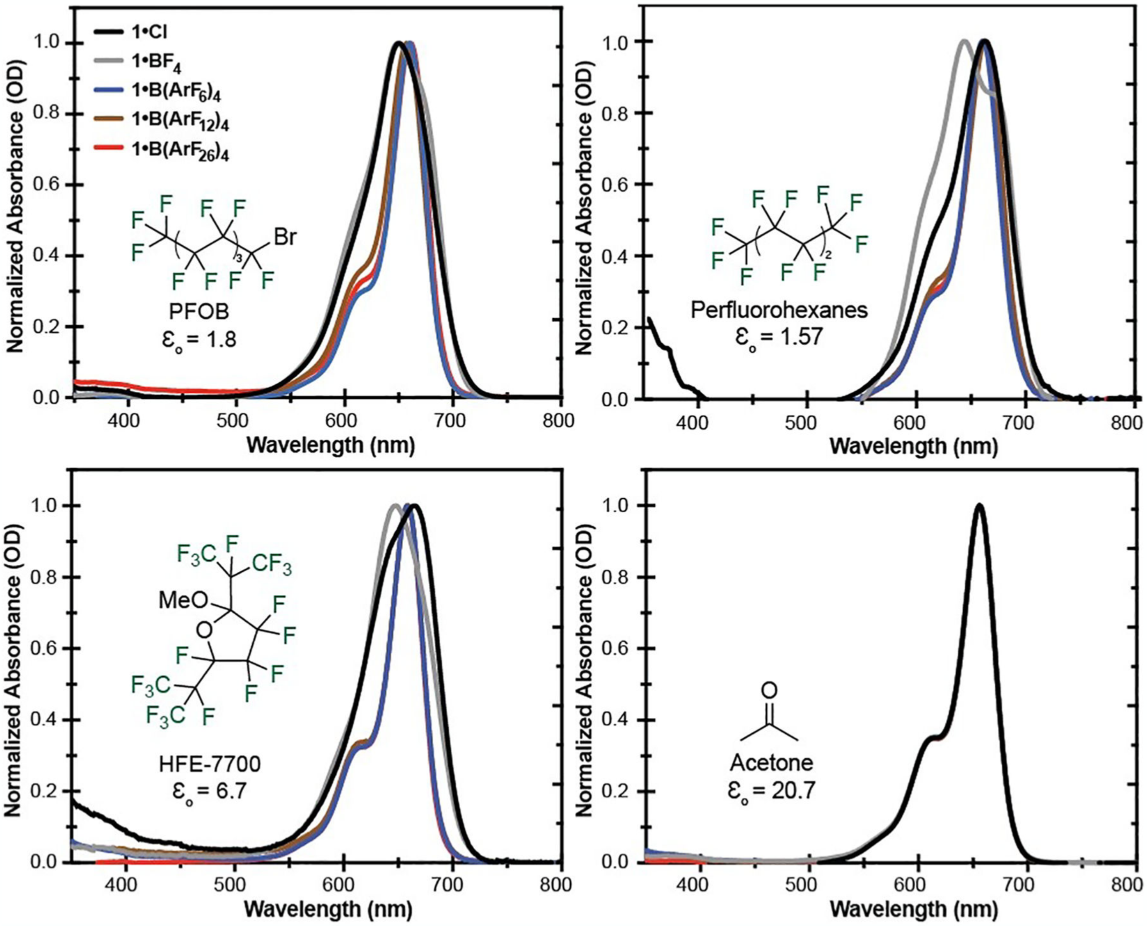

Figure 3.

Absorption spectra of FCy5 dyes in PFOB, perfluorohexanes, HFE-7700, and acetone (40 μM).

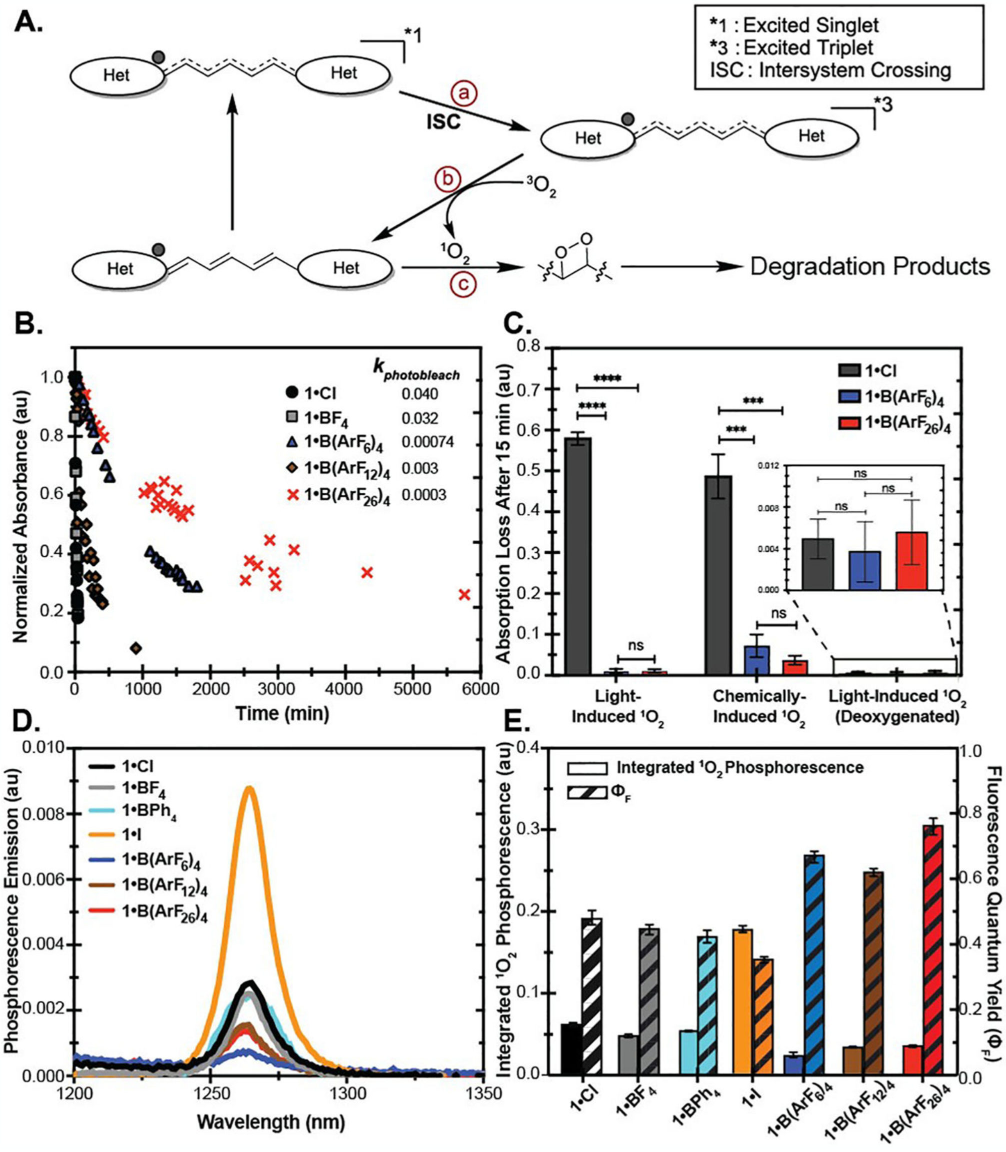

Figure 4.

(A) Scheme of polymethine dyes' photobleaching mechanism and pathways. (B) Absorption loss based on FCy5's counterion identity and photobleaching rates in PFOB derived from absorption loss. Measurements were done n=3–5 with the error bar representing ± SEM. One-way ANOVA was used to calculate p values where *** p=0.0001 and **** $p < 0.0001$. (C) Differences in reactivity of **$1^{\bullet}$Cl** and **$1^{\bullet}$B(ArF$_6$)$_4$** and 1$^{\bullet}$**B(ArF$_{26}$)$_4$** towards photochemically and chemically generated $^1O_2$. (D) $^1O_2$ phosphorescence in PFOB of FCy5 with different counterions. (E) Comparison of FCy5 integrated $^1O_2$ phosphorescence and Φ_F. All measurements were done in triplicate. The error bars represent ± SEM.

Table 1.

Photophysical properties of FCy5 with different counterions and in different solvents. FWHM is the full width at half maximum of the absorption spectra, ε is the absorption coefficient at $\lambda_{max, abs}$, Φ_F is the fluorescence quantum yield and $\varepsilon \times \Phi_F$ is the brightness.

Compound	Solvent	FWHM (nm)	ε (M^{-1} cm^{-1})	Φ_F	$\varepsilon \times \Phi_F$ (M^{-1} cm^{-1})
1·Cl	Perfluorooctylbromide	71	38000	0.19	7220
1·BF$_4$	Perfluorooctylbromide	80	100000	0.18	18000
1·B(ArF$_6$)$_4$	Perfluorooctylbromide	38	84000	0.27	22680
1·B(ArF$_{12}$)$_4$	Perfluorooctylbromide	43	100000	0.25	25000
1·B(ArF$_{26}$)$_4$	Perfluorooctylbromide	38	104000	0.30	31200
1·Cl	Perfluorohexanes	66	86000	0.15	12900
1·BF$_4$	Perfluorohexanes	86	19000	0.10	1900
1·B(ArF$_6$)$_4$	Perfluorohexanes	39	180000	0.41	73800
1·B(ArF$_{12}$)$_4$	Perfluorohexanes	41	137000	0.34	46580
1·B(ArF$_{26}$)$_4$	Perfluorohexanes	40	174000	0.40	69600
1·Cl	HFE-7700	58	48000	0.29	13920
1·BF$_4$	HFE-7700	43	98000	0.50	49000
1·B(ArF$_6$)$_4$	HFE-7700	34	94000	0.50	47000
1·B(ArF$_{12}$)$_4$	HFE-7700	35	160000	0.41	65600
1·B(ArF$_{26}$)$_4$	HFE-7700	34	183000	0.46	84180
1·Cl	Acetone	37	160000	0.35	54000
1·BF$_4$	Acetone	37	190000	0.36	68000
1·B(ArF$_6$)$_4$	Acetone	38	79000	0.39	31000
1·B(ArF$_{12}$)$_4$	Acetone	38	43000	0.38	16000
1·B(ArF$_{26}$)$_4$	Acetone	37	143000	0.16	23000