Dyes/Pigments

Hydroxylated Fluorescent Dyes for Live-Cell Labeling: Synthesis, Spectra and Super-Resolution STED** Microscopy

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Abstract: Hydroxylated rhodamines, carbopyronines, silico- and germanorhodamines with absorption maxima in the range of 530–640 nm were prepared and applied in specific labeling of living cells. The direct and high-yielding entry to germ-a- and silaxanthones tolerates the presence of protected heteroatoms and may be considered for the syntheses of various sila- and germafluorescins, as well as -rhodols. Application in stimulated emission depletion (STED) fluorescence microscopy revealed a resolution of 50–75 nm in one- and two-color imaging of vimentin-HaloTag fused protein and native tubulin. The established structure–property relationships allow for prediction of the spectral properties and the positions of spirolactone/zwitterion equilibria for the new analogues of rhodamines, carbo-, silico-, and germanorhodamines using simple additive schemes.

Among the multitude of fluorophores reported so far, only rhodamines,[1] carbopyronines,[14] and silicon-rhodamines (SiR)[2] bearing a carboxyl in the ortho-position of the pendant aromatic ring provide specific vital labeling and perform well in super-resolution fluorescence microscopy. These dyes exist in equilibrium between zwitterionic (fluorescent) and spirolactone (non-fluorescent) forms. Many cationic lipophilic triaryl methane dyes bind non-specifically and stain membrane structures.[3] Numerous anionic fluorescent labels, commercially available as sulfonates or phosphates, are hydrophilic and highly water soluble but do not penetrate the plasma membrane and are therefore used nearly exclusively in immunostaining of fixed cells; alternative strategies of membrane-impermeant label delivery employ cell-penetrating peptide conjugates[4] and reversible permeabilization.[5]

On the contrary, several rhodamine-type dyes specifically stain intact cells when applied as conjugates with small molecule recognition units.[1a,2a,c,6] The most widely used recognition units are BG-NH2, BC-NH2 and HaloTag amine (covalent ligands of the SNAP-tag,[7] CLIP-tag,[8] and HaloTag proteins).[9] Several non-covalent ligands (such as docetaxel, jasplakinolide, or pepstatin A, binding to native β-tubulin,[2b] F-actin,[2b] or aspartyl proteases in lysosomes,[2b] respectively) have been successfully used as conjugates with fluorescent dyes (see Figure S1, Supporting Information). However, the affinity and staining specificity of a given marker depends on the cell line, the nature of the ligand and the dye, the length of the linker between them, and the cell staining buffer composition.[2b] Moreover, the spectral variety of photostable dyes suitable for live cell super-resolution microscopy still remains limited.

To address some of the above-stated limitations, we have recently proposed the introduction of hydroxyl groups into the fluorescent dye molecules as a method for increasing polarity, improving solubility in water and preventing unspecific binding. In our previous study, we reported the dye 580R,[1a,10] a live-cell two-color STED imaging marker), a rhodamine with two hydroxyl groups in aliphic positions. In 580R and its predecessor Atto 590,[16] the conjugated alkene bonds in dihydroquinoline fragments provide the bathochromic and bathofluoric shifts.[1a,10] However, these fragments are prone to photo-oxidation and may negatively affect the photostability.[11] Aimed at

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developing methods for preventing unspecific binding and improving water solubility of fluorescent dyes for living cells, we designed fluorophores with additional hydroxy groups (530RH and 575RH, Figure 1) in non-allylic and non-benzylic positions.

Substitution of an oxygen atom in pyrimines with a group 14 element atom (X = Si, Ge, Sn) leads to significant bathochromic shifts in the absorption and emission spectra.[1, 2] The effect is due to lower LUMO energy of these fluorophores explained by conjugation between the π* orbitals of exocyclic X–R bonds and the π*-system of the X-containing tricyclic fragment.[1, 2] The extent of the red shift falls in the order Si > Ge > Sn (β-C), as the efficiency of π*–π* overlap decreases with the increasing atomic radius of X and C–X bond length. The corresponding Sn-pyrimine dyes are unstable; however, GeR dye, the direct Ge analogue of the widely used fluorogenic dye SiR[6a] and its bis-azetidinyl analogue (Ge analogue of the dye JF646[6b]) remain unknown[13] (Supporting Information Figure S2). To investigate the properties of the new fluorophores and the influence of hydroxylation on staining specificity, we prepared dyes listed in Table 1 (see also Figure S3 in the Supporting Information).

Bis-hydroxylated dye 530RH with 6′-carboxy-Q-rhodamine (Rh6-CO2H)[13] core was prepared according to Scheme 1a. Although the parent Rh6-CO2H is cell-permeant and its conjugate with HaloTag amine provided specific staining in living cells,[13] the dye is poorly soluble in aqueous media and its secondary amino groups are prone to acylation, which hinders NHS ester unblocking. To circumvent these drawbacks, a new dye 530RH was designed, bearing two 2,2,2-trifluoroethoxy groups to block its nitrogen atoms from acylation and two hydroxyl groups to offset the hydrophobic properties of N-trifluoroethoxy substituents. These substituents impart only slight bathochromic shifts in the absorption and emission spectra, but shift the equilibrium between the zwiterionic and spiranolate forms significantly towards the latter,[14] facilitating cell membrane penetration. Following the same logic, the dye 560CP (Supporting Information Figure S4) and its hydroxylated analogue 570CPH were designed as useful derivatives of the N,N-unsaturated carbopyrione, which itself has not yet found any application in fluorescent labeling.

The synthesis of hydroxylated ROX dyes—6′-carboxy derivatives of X-rhodamine (Rhodamine 101), known to have high fluorescence quantum yields both in organic and aqueous solutions—is shown in Scheme 1b. The scrambling condensation between acetate 9 and benzophenone 12a afforded two other dyes (13b,c) besides the expected 575RH (13a) due to two consecutive acid-catalyzed reactions: retro-Friedel–Crafts dissociation of 12a to compounds 10 and 11 (or trimesitic acid), followed by Friedel–Crafts acylation of 9 with 11. As a result, a new benzophenone with a 2-hydroxy- or 2-acetoxylolridine fragment was formed, leading to dihydroxylated dye 13b. 6-ROX (13c) arose similarly from the retro-Friedel–Crafts byproduct 10.

The hydroxylated analogues of the unknown bis-(N-azetidinyl)-GeR and JF646[6c]—dyes 630GeRH and 640SiRH, respectively—were prepared following the general route on Scheme 2. The method involves a regioselective bromination of di-O-TIPS-protected bis(3-hydroxyphenyl)silanes or -germanes 16b,c (TIPS group is required for selectivity) and a double lithium–halogen exchange on dibromides 16 followed by a reaction with dimethylcarbamoyl chloride to yield germa- and siloxanethenes 17. The intermediates 17 are general precursors to the variety of sila- and germafluoresceins and -rhodols, and the proposed approach offers a significant improvement with regard to the number of steps,[6, 15] yield[16] and functional group tolerance as compared to earlier preparations.

To evaluate the response of our dyes to the polarity of the media, a series of absorption spectra were recorded in aqueous dioxane solutions with varying water content. The spiranolate–zwiterion equilibrium is shifted in favor of the colored and fluorescent zwiterionic form as the water content increases (Figure 2). For each dye, the DLS parameter[17] interpolating the interfacial constant of the dioxane–water mixture, at which the normalized absorption A / Amax (or extinction ε / ε max) of this dye equals one half of the maximal value observed across the entire dioxane–water gradient, was determined (Table 1).

Table 1. Spectral properties of cell-permeant dyes in aqueous PBS buffer (pH 7.4) at room temperature (STED at 775 nm, unless noted otherwise).

| Dye          | Absorption λmax[18] (nm, ε M−1 cm−1) | Emission λem [19] (nm) | Brightness rel. to SiR[6] | Fluor. Lifetime t , ns |
|--------------|-------------------------------------|------------------------|---------------------------|------------------------|
| Rh6-CO2H[13] | 540 (70/000) 561 (0.79)             | 1.45                   | <5.6 4.0                  |                        |
| 530RH       | 532 (56/000) 553 (0.89)             | 1.31                   | 29.6 4.0                  |                        |
| 560CP       | 561 (61/000) 588 (0.76)             | 1.22                   | 71.0 4.2                  |                        |
| 570CPH      | 571 (79/000) 600 (0.71)             | 1.47                   | 58.5 4.0                  |                        |
| 6-ROX       | 575 (82/000) 602 (0.76)             | 1.63                   | <5.6 4.3                  |                        |
| 575RH       | 574 (55/000) 597 (0.74)             | 1.07                   | <5.6 4.3                  |                        |
| GeR         | 634 (97/000) 655 (0.43)             | 1.09                   | 65.2 2.7                  |                        |
| 630GeRH     | 631 (61/000) 651 (0.60)             | 0.96                   | 72.2 3.2                  |                        |
| SiR[6]      | 645 (93/000) 661 (0.41)             | 1 (ref.)               | 64.5 2.7                  |                        |
| 640SiRH     | 641 (51/000) 662 (0.42)             | 0.56                   | 72.4 3.2                  |                        |

H denotes hydroxylated fluorophores. [a] absolute values; [b] relative brightness expressed as ε × εmax/ (ε × εmax); [c] see ref.[1a] for the definition of DLS; [d] pulsed STED at 631 nm; [e] CW gated STED at 660 nm (LEICA microsystems); [f] -COOH-X-Rhodamine.
As expected, the colorless spirolactones of GeR, fluorinated rhodamine 530RH and carbopyronine 560CP undergo ring opening to the colored and fluorescent zwitterionic forms in systems with high water content, whereas electron-rich ROX derivatives remain unresponsive, existing predominantly in the zwitterionic form (Supporting Information Figure S5). Hydroxylation of the N-alkyl substituents shifts the position of the equilibrium towards the spirolactone form as can be expected from the weak $\beta$-hydroxyl effect of $\beta$-hydroxy substituents (the increase in $D_{0.5}$ observed upon transition from SiR to 640SiRH and from GeR to 630GeRH is 7–8 units). The hydroxylated dyes display very similar photophysical properties to the parent fluoro-

Scheme 1. a) Synthesis of the bis-hydroxylated rhodamine dye 530RH (an analog of Rh$_2$CO$_2$H). b) Synthesis of hydroxylated ROX dyes (6-COOH-X-Rhodamines) 575RH (13a) and 13b. Alternative schemes indicate better yielding sequences. PPSE = trimethylsilyl polyphosphate.

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Scheme 2. Synthesis of GeR and SiR dyes. a) nBuLi, $-78{\degree}C$, then Me$_2$GeCl$_2$ or Me$_2$SiCl$_2$; b) NBS; c) nBuLi or nBuLi, $-78{\degree}C$, then Me$_2$NCOC$_i$; d) 20, nBuLi (2 equiv), $-78{\degree}C$ to RT, then HCl; e) 21, nBuLi, THF–pentane, $-100{\degree}C$ to RT; f) TBAF, then T$_2$O, pyridine; g) 3-(tert-butyloxysilyloxy)azetidine, cat. Pd$_2$(dba)$_3$/XPhos, K$_2$CO$_3$, dioxane, $100{\degree}C$, then TBAF, then TFA.

Figure 2. Normalized extinction $c/\lambda_{max}$ at $\lambda_{max}$ of the dyes from Table 1 versus dielectric constant $D$ of dioxane–water mixtures (575RH and 6-ROX are unresponsive and are not included). The $D_{0.5}$ values correspond to the intersection of interpolated graphs with $c/\lambda_{max}=0.5$ line.104.
phores, demonstrating up to 0.5 ns longer fluorescence lifetimes and, in the case of 630GeRH, an improved fluorescence quantum yield.

In agreement with our earlier observations,[14] HaloTag(O2) amine ligands derived from silico- and germanorhodamines, as well as from fluorinated carbopyronines 560CP and 570CPH, demonstrated significant fluorogenic response (increase in fluorescence intensity upon covalent binding to HaloTag protein) in the presence of serum proteins background (Supporting Information Figure S6). The magnitude of the response was consistently smaller for hydroxylated dyes, suggesting a decreased binding affinity of hydroxylated ligands. We can therefore conclude that moderate or high values of $D_{0.5}$ seem to be required, but not sufficient for the desirable fluorogenic behavior of triarylmethane fluorescent labels.[1a, 2c]

For the evaluation of performance of our new dyes in super-resolution microscopy, living HeLa and U2OS cells expressing a vimentin-HaloTag fusion protein were incubated for 20 min with 1 μm solutions of 530RH, 570CPH, 575RH, 630GeRH, as well as non-hydroxylated GeR and 6-ROX (13 c), conjugated to HaloTag(O2) amine ligand. For labeling of tubulin filaments, HeLa cells were incubated with non-covalent β-tubulin ligands, prepared from GeR and 630GeRH and N-Boc-deprotected docetaxel, connected with an 8-aminoocetic acid linker[2b] (Supporting Information Figure S1). All dye conjugates mentioned above provided specific staining and good imaging performance in confocal and STED microscopy (Supporting Information Figures S7–S17), with hydroxylated dyes generally requiring higher concentrations (e.g., 4–5 μm for 630GeRH instead of 1 μm or below for GeR).

Isomerically pure 6-ROX dye is one of the “big four” dyes (FAM, JOE, TAMRA and ROX) dominating in the dye-terminator DNA sequencing, but has not yet been applied to live-cell imaging. We have demonstrated that the fluorescence of all 6-ROX dyes (13a–c) may be efficiently switched off by de-excitation at 775 nm, making them useful complementary partners in two-color STED nanoscopy with SiR or GeR labels. Figures 3, S16 and S17 demonstrate that HaloTag(O2) amine conjugate of 575RH in combination with GeR-tubulin or 630GeRH-tubulin ligand provide high quality two-color images in the most blue-shifted dye pair still applicable for the widely used 775 nm STED laser line.[17] Comparison of the Figures S9 and S10 confirms that hydroxylation of the rhodamine core in the dye 575RH improves image quality as related to the commercially available 6-ROX (Supporting Information Table S1).

In addition to the labeling of cytoskeleton proteins, the new dyes allow for equally specific staining of nuclear components. Living U2OS and Drosophila S2 cells expressing SNAP- and Halo-fusion constructs of different nuclear proteins (TRF2, PML, CID, CAL1) were incubated for 10–30 minutes with 0.5–1.0 μm of the chosen dye combination (610CP-BG & 640SiRH-Halo or 580R-Halo[19] and 640SiRH-BG; BG = SNAP-tag ligand) resulting in bright staining free from non-specific background (Supporting Information Figure S18). The excellent spectral separation of these combinations allowed for colocalization experiments without spectral unmixing, mapping the nuclear protein inter-
of the protein tags as well as the staining with our dye conjugates does not negatively affect cell viability.

In view of an expanding palette of live-cell compatible dyes and increasing fluorophore substitution diversity, we propose a general method relying on simple additive schemes to estimate the positions of absorption/emission maxima and $D_{0.5}$ values of the substitution pattern analogues of triarylmethane dyes. For example, using the data of our previous study,[14] the properties of dye 560CP in the same solvent (PBS, pH 7.4) have been accurately predicted before its synthesis (Scheme 3).

In conclusion, the proposed cell- and nucleus-permeant fluorescent dyes allow flexible single- and dual-color labeling in living cells. In STED imaging with de-excitation at 775 nm, several dye pairs have been validated (575RH and 635GeRH, 610CP and 640SRIRH, and 580R and 640SRIRH). The improved synthetic approach to silico- and germanorhodamines has been developed, and the new dyes 640SRIRH, GeR and 635GeRH as conjugates with docetaxel offer direct and specific visualization of native tubulin filaments in non-transfected cells. Hydroxylation of fluorophores, especially of lipophilic rhodamines, improves the staining quality at the cost of the necessity to use higher dye loadings. In sub-micromolar concentrations used for imaging, the dyes of the present study show no evidence of cytotoxicity. The design of future dye analogues can be streamlined with accumulation of photophysical data and estimation of properties of the new candidates using simple additive schemes. The degree of predictive precision achieved by using the measured values for the known structural analogues is higher than the accuracy provided by the present-day computational methods (DFT, TD-DFT), especially for the red-emitting fluorophores.[18] Further increasing of the spectral and structural variety of cell-permeant fluorophores will contribute to the design of new experiments in life sciences, including those with more sophisticated multiple color channel separation techniques, such as fluorescence lifetime imaging and hyperspectral detection.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: dyes/pigments · fluorescence · living cells · optical microscopy · rhodamines

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