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 germa- and silaxanthones tolerates the presence of protected heteroatoms and may be considered for the syntheses of various sila- and germafluoresceins, 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,[1a] 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 triarylmethanes provide specific vital labeling and perform well in live-cell super-resolution microscopy still remains limited. Moreover, the spectral variety of photostable dyes suitable for live-cell two-color STED imaging marker, a rhodamine with absorption maxima between 580 R and its prede-
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 pyromycin with a group 14 element atom (X = Si, Ge, Sn) leads to significant bathochromic shifts in the absorption and emission spectra.[12] The effect is due to lower LUMO energy of these fluoroalkyl groups.[2, 12] The effect is due to lower LUMO energy of these fluoroalkyl groups.

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 spirolactone–zwitterion equilibrium is shifted in favor of the colored and fluorescent zwitterionic form as the water content increases (Figure 2). For each dye, the D<sub>50</sub> parameter[14] an interpolated dielectric constant of the dioxane–water mixture at which the normalized absorption A/A<sub>max</sub> (or extinction ε/ε<sub>max</sub>) of this dye equals one half of the maximal value observed across the entire dioxane–water gradient, was determined (Table 1).

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 1a. In 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 trimellitic acid), followed by Friedel–Crafts acylation of 9 with 11. As a result, a new benzophenone with a 2-hydroxy- or 2-acetoxyloluidolide 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)-GeF and JF<sub>646</sub>—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 silaxanthones 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, yield and functional group tolerance as compared to earlier preparations.

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![Figure 1. Membrane-permeant and fluorescent rhodamines (R), carbopyronines (CP), silico- (SiR) or germanorhodamines (GeR) designed for STED microscopy of living cells (in free dyes, R = OH; in dye-ligand conjugates, R = NH-ligand or NH-linker-ligand). H in names stands for hydroxylated fluorophores.](image)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Absorption λ&lt;sub&gt;max&lt;/sub&gt; (nm, ε&lt;sub&gt;max&lt;/sub&gt; cm&lt;sup&gt;-1&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Emission λ&lt;sub&gt;max&lt;/sub&gt; (nm (ε&lt;sub&gt;max&lt;/sub&gt;))</th>
<th>Brightness rel. to SiR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fluor. lifetime τ&lt;sub&gt;n&lt;/sub&gt; (ns)</th>
</tr>
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<tr>
<td>R&lt;sub&gt;6&lt;/sub&gt;CO&lt;sub&gt;2&lt;/sub&gt;H&lt;sup&gt;c&lt;/sup&gt;</td>
<td>540 (70 000)</td>
<td>561 (0.79)</td>
<td>1.45</td>
<td>&lt;5.6 4.0</td>
</tr>
<tr>
<td>530RH</td>
<td>532 (60 000)</td>
<td>553 (0.89)</td>
<td>1.31</td>
<td>29.6 4.0</td>
</tr>
<tr>
<td>570CPH</td>
<td>571 (79 000)</td>
<td>600 (0.71)</td>
<td>1.47</td>
<td>58.5 4.0</td>
</tr>
<tr>
<td>6-ROX&lt;sup&gt;d&lt;/sup&gt;</td>
<td>575 (82 000)</td>
<td>602 (0.76)</td>
<td>1.63</td>
<td>&lt;5.6 4.3</td>
</tr>
<tr>
<td>575RH</td>
<td>574 (55 000)</td>
<td>597 (0.74)</td>
<td>1.07</td>
<td>&lt;5.6 4.3</td>
</tr>
<tr>
<td>GeR</td>
<td>634 (97 000)</td>
<td>655 (0.43)</td>
<td>1.09</td>
<td>65.2 2.7</td>
</tr>
<tr>
<td>630GeRH</td>
<td>631 (61 000)</td>
<td>651 (0.60)</td>
<td>0.96</td>
<td>72.2 3.2</td>
</tr>
<tr>
<td>SiR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>645 (93 000)</td>
<td>661 (0.41)</td>
<td>1 (ref.)</td>
<td>64.5 2.7</td>
</tr>
<tr>
<td>640SiRH</td>
<td>641 (51 000)</td>
<td>662 (0.42)</td>
<td>0.56</td>
<td>72.4 3.2</td>
</tr>
</tbody>
</table>

H denotes hydroxylated fluorophores. [a] absolute values; [b] relative brightness expressed as \( y \times \phi_{\text{max}}/ (x \times \phi_{\text{max}}) \); [c] see ref.[1a] for the definition of D<sub>50</sub>; [d] pulsed STED at 631 nm; [e] CW gated STED at 660 nm (LEICA microsystems); [f] 6-COOH-X-Rhodamine.

![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).](image)
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). Hydroxyl-ation of the N-alkyl substituents shifts the position of the equilibrium towards the spirolactone form as can be expected from the weak −I effect of β-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.

Scheme 2. Synthesis of GeR and SiR dyes. a) nBuLi, −78 °C, then Me$_2$GeCl$_2$ or Me$_2$SiCl$_2$; b) NBS; c) nBuLi or tBuLi, −78 °C, then Me$_2$NCOCI; d) 20, tBuLi (2 equiv), −78 °C to RT, then HCl; e) 21, nBuLi, THF-pentane, −100 °C to RT; f) TBAF, then Tf$_2$O, pyridine; g) 3-(tert-butylsilyloxy)azetidine, cat. Pd$_2$(dba)$_3$/XPhos, K$_2$CO$_3$, dioxane, 100 °C, then TBAF, then TFA.

Figure 2. Normalized extinction $c/l_{max}$ at $l_{max}$ 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 $c/l_{max} = 0.5$ line.$^{11}$

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). Hydroxyl-
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,21]

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 330RH, 570CPH, 575RH, 630GeRH, as well as non-hydroxylated GeR and 6-ROX (13c), 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-aminooctanoic acid linker[26] (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 and 640SIRH-Halo or 580R-Halo[14] 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-action with sub-diffraction resolution (Supporting Information Figure S19).

Using so-called holographic microscopy, we have monitored cell morphology and proliferation of living S2 cells with the constant presence of 580R and 640SIRH in the media over 12–16 hours and observed normal cell division (Figure 4) with proper staining of the kinetochore proteins, verified with fluorescence microscopy. Therefore, we conclude that the presence

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Figure 3. Two-color STED image (raw data) of vimentin-HaloTag fusion protein (green; labeled with 1 μm 575RH-Halo) and endogeneous tubulin (magenta; labeled with 5 μm GeR-tubulin) in living HeLa cells, simultaneous incubation time 20 min, followed by 10 min washing. (a) STED image with confocal part in bottom left corner. (b) and (c): zoomed (confocal and STED) views of the region marked in (a) in separate colors. Scale bars 2 μm; pixel dwell time: 12 μs for both color channels; pixel size: 28 nm for STED and confocal image.
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 $560\text{CP}$ in the same solvent (PBS, pH 7.4) have been accurately predicted before its synthesis (Scheme 3).

![Scheme 3](image)

In conclusion, the proposed cell- and nucleus-permeant fluorophores 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 ($575\text{RH}$ and $635\text{GeRH}$, $610\text{CP}$ and $640\text{SiRH}$, and $580\text{R}$ and $640\text{SiRH}$). The improved synthetic approach to silico- and germanorhodamines has been developed, and the new dyes $640\text{SiRH}$, GeR and $635\text{GeRH}$ 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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*Figure 4.* An example of normal cell division (2 min between images) in the presence of $640\text{SiRH}$ bound to CID-SNAP and $580\text{R}$ bound to CAL1-Halo after incubation with 0.4 $\mu\text{M}$$\text{680-50-1501}$ to R.V. (4 h) followed by addition of 0.4 $\mu\text{M}$$640\text{SiRH}$-BG and imaging for 12 h. Cell viability in the presence of dyes in the centro-meres was verified by measuring cell morphology of Drosophila S2 cells using a holomographic microscope (Holomonitor M4). For the corresponding time-lapse movie, see supplementary Movie.
Conflict of interest

The authors declare no conflict of interest.

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


[17] AbbeIRROR Instruments GmbH offers STED microscopes with the pulsed 595 nm and 775 nm depletion lasers; Leica Microsystems–with 592 nm (gated; CW), 660 nm (gated; CW) and 775 nm (pulsed) depletion lasers; PicoQuant GmbH offers a STED microscope with a 765 nm depletion laser.

Modular design for portable dyes: Hydroxylated rhodamines, carbopyronines, and Si- and Ge-rhodamines absorbing at 530–640 nm showed specific labeling of living cells and provided optical resolution of 50–75 nm in one- and two-color STED microscopy. The established structure–property relationships allow property predictions of the new dye analogues.