Photoactivatable Fluorescent Dyes with Hydrophilic Caging Groups and Their Use in Multicolor Nanoscopy

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ABSTRACT: We propose a series of fluorescent dyes with hydrophilic carbamate caging groups that undergo rapid photoactivation under UV (≤400 nm) irradiation but do not undergo spurious two-photon activation with high-intensity (visible or infrared) light of about twice the wavelength. The caged fluorescent dyes and labels derived therefrom display high water solubility and convert upon photoactivation into validated super-resolution and live-cell-compatible fluorophores. In combination with popular fluorescent markers, multiple (up to six)-color images can be obtained with stimulated emission depletion nanoscopy. Moreover, individual fluorophores can be localized with precision <3 nm (standard deviation) using MINSTED and MINFLUX techniques.

Recently, sulfonated rhodamine- and silicon-rhodamine-derived probes, internalizing upon conjugation to their molecular targets, have been described as cell-membrane-impermeant fluorescent substrates for SNAP-tag- and HaloTag-fused cell surface proteins. Here, following an entirely different approach, we propose the introduction of polar sulfonate groups onto the lipophilic 2-nitrobenzyl carbamate protecting groups, rendering the caged dyes highly water-soluble and allowing photocontrol over their membrane permeability. Following a preliminary screening of the substitution pattern of the hydrophilic caging groups, the molecules HCage 520 (4aa), HCage 580 (4ba), HCage 620 (4ca), and HCage 680 (4cb) (Figure 1) have been selected for the optimal combination of stability against two-photon activation with 595 and 775 nm STED light pulses and solubility in aqueous media without any addition of organic cosolvents. These target compounds have been prepared from known 6′-(tert-butoxycarbonyl)fluorescein, -carbofluorescein, and -silicofluorescein triflates11 1a–1c and the corresponding carbamates 2a and 2b via a double Buchwald–Hartwig amidation catalyzed with a Pd-JackiePhos system12 under anhydrous conditions. The polar SO₃H groups, anionic under physiological conditions and imparting solubility in water, were introduced into the intermediates 3aa–3cb via basic hydrolysis of the esters and peptide coupling with taurine. The final deprotection of the 6′-carboxylate group offered the target caged dyes 4aa–4cb suitable for conjugation.

Upon photoactivation with UV light (400 nm LED, 355 or 405 nm laser sources), the caged dyes are cleanly converted...
Figure 1. Synthesis of the photoactivatable triarylmethane dyes with hydrophilic caging groups (HCage dyes).
fluorescence intensity peaking after 60 s (four confocal frames with 900 μm² imaging area) due to diffusion of the uncaged 620SiR-Halo, and was followed by slow bleaching of the labeled structure (Figure S7). The dynamics of vimentin filaments were also observed (Video S1). A similar observation was made for 4ba-Halo (500 nM; Figure S8 and Video S2) and confocal UV activation, revealing complete labeling of the target structure within 5−6 min. The labeled structure could be imaged with sub-diffraction resolution (Figure S9). The specificity of the staining was confirmed by an in situ uncaging experiment with fixed U2OS cells stably expressing vimentin-HaloTag labeled with HCage 620 (as 4ca-Halo) and indirect immunostaining of vimentin (Figure S10).

To demonstrate the potential of spatially controlled activation of caged fluorogenic labels directly under microscopic conditions, U2OS cells transfected with Tomm20-HT7-T2A-EGFP plasmid and mounted in a live imaging chamber in medium containing 500 nM of 4ba-Halo ligand were irradiated at several 4 μm² sized loci in close proximity to the cell membrane, and the development of target labeling was monitored over multiple frames (Figure S11 and Video S3).

High fluorogenicity of the 4ba-Halo uncaging product 580CP-Halo, along with its rapid binding kinetics with HaloTag protein (with $k_{app}$ estimated at 4.01 ± 0.31 × 10⁷ M⁻¹ s⁻¹ for HT7 version, see Figure S12), provides a realistic background-free dynamic visualization of live-cell labeling. We can therefore recommend this caged HaloTag substrate for real-time observation experiments such as comparing the cellular uptake of the fluorescent probe under varying conditions, or for tagging small molecules of biological relevance and targeting them to the HaloTag-fused proteins of interest within the living cells. The required spatial and temporal control over the generation of a cell-permeant label can be conveniently achieved with brief focused UV irradiation of moderate (~12 MW/cm²) intensity.

The low fluorescence background in samples labeled with caged dyes 4aa−4cb and their selective photoactivation with UV light prompted us to evaluate their performance in recently proposed advanced fluorescence nanoscopy methods, called MINFLUX¹⁶ and MINSTED.¹⁷ For benchmarking purposes, microtubules in glutaraldehyde-fixed U2OS cells were immunostained with Abberior Star RED (KK11418), a widely accepted photostable and highly water-soluble STED dye. The attainable resolution of both confocal and STED images (Figure 3a,b) could then be directly compared with the image consisting of overlaid single-molecule localizations of individual secondary antibodies labeled with HCage 620 (Figure 3c,d). The hollow tubular shape of an individual microtubule becomes evident in the y-integrated cross-section (x-z projection, Figure 3e) of the 3D MINFLUX image (for histograms including all localization, see Figure S13) and in the y-z-integrated cross-section (Figure 3f, including all localization).¹⁶b The combined use of HCage 620 dye and the MINFLUX method enabled a localization precision of 2.4 nm...
along the x-axis) and 2.7 nm (along the y-axis), estimated by a 2D-Gaussian fit on the spread of all localizations centered around their mean emitter positions (Figure 3g). Averaging the standard deviation of the localizations around their mean emitter position led to comparable results, with a median localization precision of 3.0, 4.0, and 3.1 nm along the x-, y-, and z-axes, respectively.

The high resistance of HCage 620-based probes against activation with STED laser and good photostability of 620SiR photoproduct allowed sparse activation of diverse caged ligands in MINSTED nanoscopy. \(^{17}\) We demonstrated effective MINSTED imaging of antibody-labeled caveolin clusters (Figure 4a, labeling with NHS ester), HaloTag- and SNAP-tag-labeled nucleoporins (Nup96 and Nup107, respectively, Figure 4b,c), and Nup107-mEGFP with anti-GFP nanobody (V\(_\text{H}\) heavy-chain IgG camelid antibody fragments) labeled with maleimide (Figure 4d). The localization precision for individual fluorophore emitters, singled out with MINSTED, was estimated at 2.6−3.5 nm (single standard deviation) for different ligands (Figure S15).

In conclusion, the proposed hydrophilic caged versions HCage S20, S80, 600, and 620 (4aa−4cb) of the established live-cell-compatible triaryl methane fluorophores S20R, S80CP, 600SiR, and 620SiR can be recommended for imaging in fixed (e.g., with immunostaining) and living cells (following on-demand uncaging to cell-permeant labels in the media). These caged dyes are applicable across most leading fluorescence nanoscopy modalities (STED, PALM, MINFLUX, and MINSTED). The precise spatiotemporal control over their photoactivation provides additional avenues for real-time monitoring of localized uptake of membrane-permeant fluorescent and fluorogenic ligands, such as S80CP-Halo\(^{11b}\) or 620SiR-SNAP,\(^{13}\) as well as fluorophore-tagged small molecules. In particular, the ability to sparsely activate and precisely localize individual labeled biomolecules in time and space brings us closer to the ultimate goal of understanding the biochemical processes inside a living cell on a molecular level.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c09999.

Synthetic procedures and characterizations of the compounds; description of sample preparation, labeling, imaging, and image processing, including Figures S1−S16 and Tables S1 and S2; and NMR spectra (PDF).
Supplementary Video S1: confocal and STED time-lapse video of living U2OS cells stably expressing vimentin-HaloTag fusion protein labeled with photoactivatable HCage 580-Halo (MP4)

Supplementary Video S2: photoactivation of HCage 580-Halo in the media and real-time observation of vimentin labeling in living U2OS cells stably expressing vimentin-HaloTag fusion protein (AVI)

Supplementary Video S3: localized uncaging of HCage 580-Halo and real-time observation of its uptake and Tomm20 labeling in living U2OS cells transfected with Tomm20-HT7-T2A-EGFP plasmid (AVI)

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Author Contributions

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Funding

This work has been supported by the Deutsche Forschungsgemeinschaft (DFG) (SFB1286/A07 to E.D. and S.W.H.) and German Federal Ministry of Education and Research (BMBF) (FKZ 13N14122 to S.W.H.). Open access funded by Max Planck Society.

Notes

The authors declare the following competing financial interest(s): A.N.B., M.W., and S.W.H. have filed patent applications on the caged fluorescent dyes of this work. The Max Planck Society holds patents on selected embodiments and procedures of MINFLUX and MINSTED, benefitting S.W.H. and M.W.

■ ACKNOWLEDGMENTS

We thank Jasmine Hubrich (Max Planck Institute for Medical Research), Dr. Ellen Rothermel and Tanja Koenen (Max Planck Institute for Biophysical Chemistry) for assistance with cell culture and labeling of cells, Dr. Jasmin K. Pape and Thea Moosmayer (MPI BPC) for providing additional expertise on MINFLUX data processing, Dr. Michelle Frei (MPI MR, Department of Chemical Biology) for the gift of Tomm20-HT7-T2A-EGFP plasmid, and the European Molecular Biology Laboratory (Heidelberg) for U-2 OS-CRISPR-NUP96-Halo, U-2 OS-CRISPR-NUP96-SNAP, and HK-2xZFN-mEGFP-NUP107 cells. We also thank Dr. Sebastian Fabritz and the staff of the MS core facility (MPI MR) for LC-HRMS measurements, and the Department of Chemical Biology (Prof. Kai Johnsson, MPI MR) for access to a Quantaurus-QY instrument. We appreciate the fruitful discussions with Dr. Marcel Leutenegger (MPI BPC) and Dr. Mariano L. Bossi (MPI MR).

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