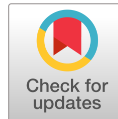

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A Highly Photostable Near-infrared Labeling Agent Based on a Phospha-rhodamine for Long-term and Deep Imaging

Marek Grzybowski,[a] Masayasu Taki,*[a,b] Kieko Senda,[c] Yoshikatsu Sato,[a] Tetsuro Ariyoshi,[d] Yasushi Okada,[d,e] Ryosuke Kawakami,[f] Takeshi Imamura,[f] and Shigehiro Yamaguchi*[a,c]

Dedication ((optional))

Abstract: Various fluorescence microscopy techniques require bright NIR-emitting fluorophores with high chemical and photostability. Herein, the significant performance improvement of phosphorus-substituted rhodamine dyes (PORs) upon substitution at the 9-position with a 2,6-dimethoxyphenyl group is reported. The thus obtained dye PREX 710 was used to stain mitochondria in living cells, which allowed long-term and three-color imaging in the vis-NIR range. Moreover, the high fluorescence longevity of PREX 710 allows tracking a dye-labeled biomolecule by single-molecule microscopy under physiological conditions. Deep imaging of blood vessels in mice brain has also been achieved using the bright NIR-emitting PREX 710-dextran conjugate.

Recent advances in optical microscopy techniques, *e.g.* multi-photon, super-resolution, and single-molecule microscopy, have revolutionized our understanding of the structures and functions of biomolecules at the sub-cellular and molecular level.[1] In parallel, various fluorescence-labeling methods as well as valuable fluorescent chemical probes and proteins have been developed.[2] In particular, dyes that absorb and emit light in the near-infrared (NIR) region are ideal to minimize interference from autofluorescence, which is a major contributor to the background noise in cells. However, most NIR-emitting dyes currently used in bioimaging, especially cyanine-based fluorophores, suffer from inherently low chemical and photostability.[3] This significantly limits the utility of these dyes in long-term fluorescence imaging, which requires exposure to light in multimolecular cellular environment.

To improve the photostability of organic fluorophores, various strategies, such as the introduction of a triplet quencher significantly[4] or the incorporation of a four-membered azetidinyl group into rhodamine fluorophores[5] have been proposed. As another approach, we have recently proposed structurally reinforced π-conjugated skeletons that contain an electron-withdrawing phosphine oxide (P=O) moiety.[6] Such P=O-substituted fluorophores enable the acquisition of repeated images in stimulated emission depletion (STED) microscopy, where the fluorophores are exposed to an extremely intense depletion laser field.[7]

The incorporation of a P=O group also leads to bathochromic shifts of the absorption and emission maxima. Indeed, the replacement of the endocyclic oxygen atom in xanthene scaffolds with a P=O moiety generates the corresponding fluorescein (POF),[8] rhodol (PORL),[9] and rhodamine (POR)[10] derivatives (Figure 1a), which emit fluorescence in the far-red to NIR region in PBS (pH = 7.4).

We have previously reported that POFs and PORLs exhibit remarkable photostability, surpassing that of their oxygen- and silicon-substituted counterparts.[8a,11] As for POR dyes, two groups have independently reported dyes that contain P(=O)Me[10a] and P(=O)OH or P(=O)OR moieties,[10b] and their utility as NIR-emissive probes in fluorescence imaging. In the present study, we synthesized a series of tetraethyl rhodamine PORs that bear different aryl groups at the 9-position (Figure 1b). We evaluated the substituent effects on the chemical and photophysical properties and discovered that POR dye **1c** exhibited outstanding performances in multi-facet applications, including not only multicolor imaging in the vis-NIR region, but also long-term time-lapse data acquisition, single-molecule analysis, and deep imaging *in vivo*.

POR dyes **1a**–**c** were synthesized by the treatment of the common xanthone precursor **4** with the corresponding aryllithium (**1a**,**c**) or Grignard (**1b**) reagents (Scheme S1). The structure of **1b** was determined by single- crystal X-ray diffraction analysis (Figure S1). Unlike the oxygen-bridged parent rhodamine, the xanthene skeleton of **1b** is slightly bent due to the larger diameter of the phosphorus atom (Figure S1c). The dihedral angle between the mean planes of the two benzene rings is 16.1(1)°. The analysis of the bond lengths in the xanthene skeleton revealed a typical quinoidal character, which is indicative of an efficient delocalization of the positive charge over the entire π-conjugated skeleton (Figure S1b).

The photophysical properties of **1a**–**c** were measured in 10 mM PBS at pH = 7.4 (Table S1, Figures S3–S5). These compounds show absorption (λ_{abs}) and emission (λ_{em}) maxima in the NIR region with large molar absorption coefficients

[a] Dr. M. Grzybowski, Prof. Dr. M. Taki, Prof. Dr. Y. Sato, Prof. Dr. S. Yamaguchi
Institute of Transformative Bio-Molecules (WPI-ITbM)
Nagoya University, Furo, Chikusa, Nagoya 464-8601 (Japan)
E-mail: taki@itbm.nagoya-u.ac.jp, yamaguchi@chem.nagoya-u.ac.jp
[b] Prof. Dr. M. Taki
PRESTO, Japan Science and Technology Agency (JST)
4-1-8 Honcho, Kawaguchi, Saitama 332-0012 (Japan)
[c] Ms. K. Senda, Prof. Dr. S. Yamaguchi
Department of Chemistry, Graduate School of Science, and Integrated Research Consortium on Chemical Sciences (IRCCS), Nagoya University, Furo, Chikusa, Nagoya 464-8602 (Japan)
[d] Dr. T. Ariyoshi, Prof. Dr. Y. Okada
Laboratory for Cell Polarity Regulation, Center for Biosystems Dynamics Research (BDR), RIKEN, Suita, Osaka 565-0874 (Japan)
[e] Prof. Dr. Y. Okada
Department of Physics and Universal Biology Institute (UBI), Graduate School of Science, and International Research Center for Neurointelligence (WPI-IRCN), The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
[f] Prof. Dr. R. Kawakami, Prof. Dr. T. Imamura
Department of Molecular Medicine for Pathogenesis, Graduate School of Medicine
Ehime University, Shitsukawa, Toon-city, Ehime, 791-0295 (Japan)

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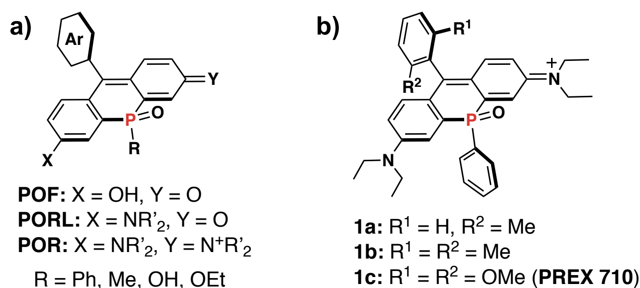

Figure 1. Molecular structures of P=O-containing xanthene dyes. a) POF: phospha-fluorescein; PORL: phospha-rhodol; POR: phospha-rhodamine. b) PORs used in this study (**1a**-**c**).

(ε ~1 × 10^5 M^{-1} cm^{-1}) and sufficient fluorescence quantum yields (Φ_F ~0.13). Their longer $\lambda_{abs}/\lambda_{em}$ compared to those of PORs involving P(=O)Me[10a] and P(=O)OEt[10b] moieties were ascribed to the electron-withdrawing inductive effect of the phenyl group on the phosphorus atom. Compound **1c**, which bears a 2,6-dimethoxyphenyl group, exhibited λ_{abs} and λ_{em} values of 712 nm and 740 nm, respectively (Figure S5), which are slightly longer wavelengths than those of **1a** and **1b**, and hence indicative of an electronic perturbation by the aryl group at the 9-position. The brightness ($\Phi_F \times \varepsilon$) is comparable to the existing NIR-fluorescent silicon-rhodamine dye SiR700.[12,13]

To assess the potential utility of **1a**-**c** as labeling reagents, we initially evaluated the chemical stability of these dyes against nucleophiles. The absorption changes of their aqueous solutions were monitored at pH = 4.2, 7.4, and 10.3. Compound **1a**, which contains a relatively unhindered *o*-tolyl group at the 9-position, exhibited a gradual decrease to 40% of the initial value after 8 h at pH = 10.3 (Figure S8). This degradation is probably due to the nucleophilic addition of a hydroxide at the 9 position to form the corresponding carbinol.[9] The pK_R^+ value of **1a** (pK_R^+ = 8.8, Figure S11), which is an index for the thermodynamic stability of carbocations, is much lower than that of silicon-rhodamine dye SiR650 (pK_R^+ = 10.8, Figure S12). This difference is due to the strong electron-withdrawing effect of the phosphine-oxide moiety, resulting in an enhanced electrophilic character of the positively charged xanthene skeleton.

The high electrophilicity of **1a** also manifests in its reactivity toward a reduced form of glutathione (GSH), which is the most abundant low-molecular-weight thiol in living cells. The absorption and emission bands of **1a** decreased to 9% of the initial value when 10 mM GSH was added to a solution of **1a** in PBS (pH = 7.4) (Figure S13). The dissociation constant toward GSH (K_d = 1.34 ± 0.05 mM, Figure S16) is much lower than that of SiR650 (K_d > 100 mM), which carries the same *o*-tolyl group.[14] Although, in general, it is considered that decolorization of **1a** caused by GSH is a fatal drawback as a labeling reagent in fluorescence live-cell imaging, **1a** with K_d = 1.34 mM for GSH could serve as a promising NIR probe to understand GSH homeostasis (0.5 – 10 mM) in living cells.

In contrast to the high reactivity of **1a**, sterically protected **1b** and **1c** with a 2,6-disubstituted phenyl group showed high resistance toward nucleophiles. Negligible spectral changes were observed for **1b** and **1c** both under weakly basic conditions and in a solution containing 10 mM GSH. Moreover, **1b** and **1c** are stable at pH = 4–10, *i.e.*, in the biologically relevant range. Only under strongly basic conditions (pH > 11), **1b** and **1c** undergo hydrolysis to the corresponding phospha-rhodols and -fluoresceins, which we have already reported.[9a] Furthermore, **1b** and **1c** exhibited high stability in fetal bovine serum (FBS) (Figure S17), suggesting that sterically protected PORs should be suitable for practical applications in various biological systems including in cells and blood.

The steric bulk of the aryl group at the 9-position also plays a crucial role for the improvement of the photostability. Among **1a**-**c**, compound **1c** exhibited the highest photostability, *i.e.*, negligible photobleaching (99.5% of the dye remained intact) was observed even after a solution of **1c** was exposed for 2 h under a Xe lamp irradiation (435 mW/cm^2), whereas a more significant decrease was observed for **1a** (17%) and **1b** (13%) under the same conditions (Figure S19). Although the exact role of the methoxy groups in **1c** for the suppression of the photobleaching is not yet fully understood, these may affect the kinetics of the dark states (triplet and radical anion) in the photobleaching.[15] Overall, the examination of the photophysical properties, chemical stability, and photoresistance revealed an excellent potential for **1c** as an NIR-emissive fluorophore. We therefore named this dye PREX 710, where PREX has a meaning of Photo-REsistant Xanthene dye.

We next prepared a PREX 710 derivative that bears an NHS ester (PREX 710 NHS, Scheme S1) for labeling amino groups of biomolecules, and then evaluated the performance of PREX 710 in fluorescence imaging. The high photostability of PREX 710 is retained even in conjugation with an antibody. The PREX 710-conjugated IgG displayed much higher resistance to photobleaching in PBS buffer than fluorescent antibodies labeled with commercially available NIR dyes such as Cy5.5, Alexa Fluor 680, Alexa Fluor 700, and STELLA Fluor 700 (Figure S21). The photostability in the immunocytochemistry application was further examined by repetitive imaging of HeLa cells after staining microtubules with these dyes. Almost 80% of the initial fluorescence intensity of PREX 710 was retained even after continuous irradiation with an excitation laser at 670 nm for 10 min (174 images in total), whereas the emission faded completely when using Cy5.5 under the same imaging conditions (Figure 2a). Superior photostability of PREX 710 was also observed in the comparison with STELLA Fluor 700 (Figures S31 and S32).

The photostability of PREX 710 in single-molecule imaging was then assessed in comparison with Alexa Fluor 647, a widely used cyanine dye.[16] Both dyes were conjugated to NeutrAvidin to immobilize on a biotinylated coverslip. Fluorescence signals were recorded by total internal reflection fluorescence microscopy (Movie S1). After 2 min of observation (1200 frames in total) at 60 W/cm^2 irradiance, more than 80% of the initial fluorescence intensity of PREX 710 remained (Figure 2b), whereas the signal rapidly decayed within 30 s for Alexa Fluor 647 (Figure 2c). Under these imaging conditions, repetitive blinking events was often observed with PREX 710 (Figure S23). The single-step blinking behavior would reflect the transition to and from the triplet state, which is a good indicator to guarantee that signals from each single molecule are actually recorded. The signals from single PREX 710 molecules were strong

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enough and were traced for more than a few minutes. It should be noted that the single-molecule fluorescence of conventional dyes can be traced only for a few seconds under the same conditions (Figure S24), and can only be extended to 10-30 s even in a special buffer with additives and/or oxygen removal to prevent bleaching.[4] In addition to the comparison in practical conditions, we made a quantitative comparison by compensating for the difference in the absorption coefficients of these dyes at 640 nm. Namely, five times stronger laser power was used for the excitation of PREX 710 than for Alexa Fluor 647 (Figure S25; Movies S2 and S3). The duration of the signals from single molecules was statistically analyzed for PREX 710 (N = 278) and Alexa Fluor 647 (N = 274). The average time constant for photobleaching was estimated by fitting the frequency distribution of the longevity to a single exponential distribution (Figures S26 and S27). The time constant for PREX 710 was 34.6 s, more than 10 times longer than Alexa Fluor 647 (3.0 s). The data were better fitted with a mixture of two exponential distributions [PREX 710: τ_1 = 12.4 s (49.2%) and τ_2 = 52.9 s (50.8%); Alexa Fluor 647: τ_1 = 1.7 s (85.7%) and τ_2 = 8.8 s (14.3%)]. The longer time constant component would reflect the process of bleaching after multiple rounds of blinking that was not detected with the current imaging conditions (200-1000 ms exposure). The higher mixing proportion (50.8%) for PREX 710 is consistent with the suppression of photobleaching from the dark states as discussed previously. More practically, three times more photons were detected from a single PREX 710 molecule than Alexa Fluor 647 before bleaching (photon budget), even with our system adjusted to the spectrum of Alexa Fluor 647. These results collectively indicate that PREX 710 is far more superior to conventional fluorescent dyes used as the probe for the single fluorescent molecule imaging. Each single molecule can be imaged and traced for much longer time or much more frames without bleaching even in an environment like in the living cells, where oxygen depletion or supplementation with anti-fading reagents is difficult to apply.

PREX 710 itself is membrane-permeable and predominantly localized in the mitochondria of living cells (Figure S34). Taking advantage of this feature and the other characteristics of PREX 710, we wanted to demonstrate its high utility in practical fluorescence imaging. Firstly, the NIR excitation and emission properties of PREX 710 effectively reduce spectral cross-talk with other dyes, which allows conducting multi-color imaging beyond 500 nm. In fact, fluorescence signals observed from the plasma membrane, nucleus, and mitochondria, which were stained with DiI, SiR-DNA, and PREX 710, respectively, could be clearly separated using a simple wide-field microscope equipped with filter sets for commercially available dyes (Figure 3a). Moreover, we demonstrated the utility of PREX 710 as a labeling reagent to track cell movement. For that purpose, HeLa cells were stained with PREX 710 and monitored at intervals of 30 s (exposure time: 500 ms). It should be noted that the movement of the mitochondria and acidic organelles in the cell cycle including the cell division was successfully observed for more than 7 h thanks to the low concentration of PREX 710 (0.2 μM) and the reduced phototoxicity of the irradiation conditions using NIR light (Movie S4).[17] Although further evaluations of the potential effects of PREX 710 on the cell behavior and

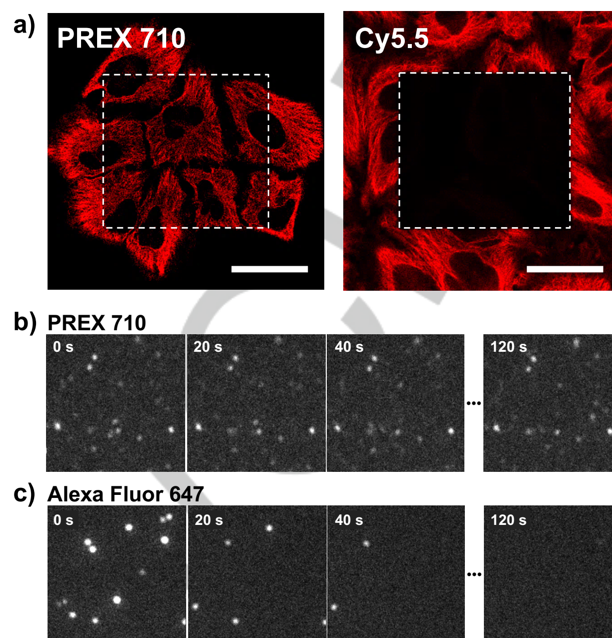

Figure 2. A comparison of the photostability. a) Confocal images of microtubule immunolabeled with PREX 710 (left) and Cy5.5 (right) after the rectangular area marked by dashed lines was repeatedly scanned for 10 min with excitation light at 670 nm. Scale bar: 50 *μ*m. Single-molecule images of (b) PREX 710 and (c) Alexa Fluor 647 immobilized on a glass surface. Images were acquired continuously at 10 frames per second for 120 s with excitation light at 640 nm (60 W/cm^2) in PBS at pH = 7.4.

metabolism are still required, PREX 710 and its derivatives should be useful tools for the investigation of the dynamics of living organisms, tissues, cells, and molecules.

Finally, we used PREX 710 for the *in vivo* imaging of blood vessels in the deep region of mouse brain. For that purpose, Aminodextran (70 kDa, Thermo Fisher) was labeled with PREX 710 NHS to provide the dextran-conjugate with a DOL value of 3.5, which was subsequently injected into the bloodstream via the tail vein (7.0 mg/mL in PBS; injection volume: 0.1 mL). Confocal images were recorded at an excitation wavelength of 638 nm and the 3-D image was reconstructed using 41 images acquired at 10 μm Z-steps (depth: 400 μm, Figure 3b). Intense fluorescence signals were observed up to a depth of ~0.3 mm from the surface, despite the fact that the excitation efficiency of PREX 710 at 638 nm is only approximately one-third of the maximum, indicating sufficiently high brightness of the conjugate. As the detection range for PREX 710 extends to much longer wavelengths relative to those of other commonly used fluorescent proteins for *in vivo* imaging such as YFP and mCherry, PREX 710 should also be useful for multi-color deep imaging with a two-photon microscope.[18]

In summary, we have developed the photostable, water-soluble NIR fluorophore PREX 710 for multi-facet and high-performance bioimaging applications. We have successfully demonstrated that PREX 710 can be used for single-molecule, deep-tissue, and long-term imaging, as well as multicolor-fluorescence imaging in the vis-NIR region. PREX 710 selectively binds to mitochondria, while its NHS ester can be

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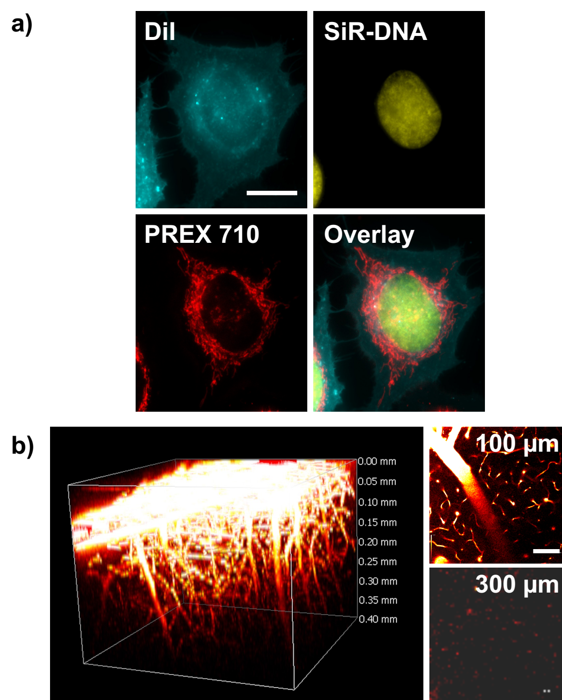

Figure 3. a) Three-color imaging of cell membrane (cyan), nucleus (yellow), and mitochondria (red) in living HeLa cells stained with DiI, SiR-DNA, and PREX 710; scale bar: 20 μm. b) 3-D image of blood vessels in mouse brain stained with PREX 710-dextran conjugate. Maximum intensity projections of the 3-D stacks were obtained using a CW laser at 638 nm. Single xy frames from the z-stack at 100 μm and 300 μm depth are shown on the right; scale bar: 100 μm.

conjugated to multiple targets, thus serving as a universal NIR fluorescence label. In all the tested applications, PREX 710 exhibited excellent photostability, outperforming commercially available state-of-the-art fluorescence dyes. Moreover, we discovered that the sterically less hindered POR **1a** exhibits an appropriate dissociation constant for GSH, rendering it a promising candidate for monitoring the GSH level in living cells and tissues in the NIR region. The development of NIR fluorescence probes based on phosphorus-bridged rhodamine dyes is currently in progress in our laboratories.

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Keywords: near-infrared • labeling agent • photoresistance • phospha-rhodamine • optical imaging

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Layout 1:

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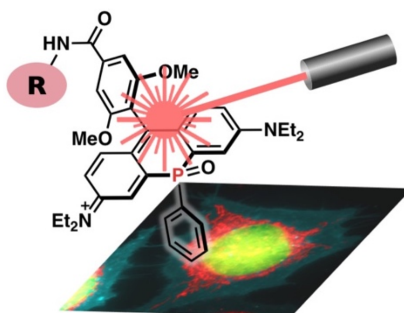
Photostable NIR dye: PREX 710 is a P=O-containing rhodamine dye that is substituted with a 2,6-dimethoxyphenyl group at the C9-position. The NIR-emission and high fluorescence longevity of PREX 710 allow multi-color imaging, cell-tracking, and long-term single-molecule tracking under physiological conditions as well as deep-tissue imaging *in vivo.*



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