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Photoelectrocyclization Turn-On Probe for Organelle Specific Spatiotemporally Defined Live Cell Imaging

Mai N. Tran and David M. Chenoweth

Department of Chemistry, University of Pennsylvania 231 South 34th Street, Philadelphia, PA 19104 (USA)

David M. Chenoweth: dcheno@sas.upenn.edu

Abstract

Photoactivatable fluorophores are useful tools in live cell imaging due to their potential for precise spatial and temporal control. In this report, we describe a new photoactivatable organelle specific live cell imaging probe based on a 6π -electrocyclization/oxidation mechanism. We show that this new probe is water soluble, non-cytotoxic, cell permeable and useful for mitochondrial imaging. The probe displays large Stokes shifts in both pre-activated and activated forms, allowing simultaneous use with common dyes and fluorescent proteins. Sequential single cell activation experiments in dense cellular environments demonstrate high spatial precision and utility in single or multi cell labeling experiments.

Keywords

photoactivatable fluorescent probes; imaging agents; photochemistry; photoelectrocyclization

Photoactivatable fluorescent probes are powerful tools for studying biological system due to the high spatial and temporal control afforded by light. Photoactivatable probes can be genetically encoded proteins^[1] or small molecules.^[2] Each of these technologies comes with its own benefits and limitations and may be thought of as complimentary to one another depending on the application. The protein “Kaede” represents the first discovery of a photoactivatable fluorescent protein and much progress has been made in recent years with similar genetically encoded approaches.^[3] Small molecule photoactivatable probes have also found widespread use and they generally rely on a small number of photoactivation mechanisms such as photoisomerization,^[4] photo-uncaging,^[5] photodecomposition of azides,^[6] or photoclick reactions,^[7] to name a few. These strategies primarily depend on the conversion from an initial non-fluorescent state to a fluorescent state.

Here, we present a new intracellular imaging probe utilizing a photoactivation mechanism based on a photochemically allowed 6π -electrocyclization/oxidation. This mechanism expands intracellular photoactivatable probe modalities for conversion of one fluorescent probe state to another, allowing for tracking of both pre- and post-activated forms. To

Correspondence to: David M. Chenoweth, dcheno@sas.upenn.edu.

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demonstrate this strategy, we introduce a water soluble, noncytotoxic, and readily cell permeable photoactivatable fluorescent probe for mitochondrial specific live cell imaging. We compare the cytotoxicity this probe to a commonly used and commercially available mitochondrial dye.^[8] We also demonstrate its utility in spatiotemporally defined live-cell imaging of mitochondria in dense cellular populations through sequential single cell activation experiments.

We developed a rapid and efficient synthesis of the desired photoactivatable probe starting with commercially available 2-cyanopyridine (Scheme S1). Following a similar route reported in our previous studies,^[9] we elaborated 2-cyanopyridine to thioketone **1**. Homocoupling of **1**, using copper powder in toluene under refluxing conditions, yielded 1,1'-diazaxanthilidene **2** as an interconverting mixture of *E* and *Z* isomers in 84% yield (Scheme 1). Methylation of **2** with dimethyl sulfate afforded the desired monomethylated product **3-E/3-Z** in 96% yield. The overall yield of the probe from commercial starting materials after 6 steps was 24% (Scheme S1, Scheme 1). Single crystals of **2-E** and **3-Z** were obtained by slow evaporation from chloroform:methanol (1:1) and water, respectively. Both structures revealed *anti*-folded conformations despite the increased steric influence of methylation in **3-Z** (Scheme 1). Variable temperature ¹H-NMR^[10] of **2-E/2-Z** in deuterated *N,N*-dimethylformamide confirmed the dynamic interconversion of the *E* and *Z* isomers at room temperature, consistent with our previous studies (Figure S2).^[9] At low temperature, separate sets of sharp and well-resolved signals were observed for the *E* and *Z* isomers of **3**. Incremental heating resulted in significant signal broadening followed by sharpening and resolved coupling at high temperature. This result indicated rapid exchange. Coalescence was observed at 45 °C, and the activation energy was determined to be $G^\ddagger = 15.7$ kcal/mol (Figure S1).

Upon irradiation at 365 nm using a Rayonet photoreactor, the **3-E/3-Z** mixture was found to undergo photocyclization, followed by oxidation to yield photoproduct **4** (Figure 1A). To promote the photocyclization step of **3-E/3-Z**, both the *anti*-folded conformation and dynamic interconversion are crucial. According to Woodward-Hoffmann rules, electrocyclization of the 1,3,5-hexatriene moiety of **3-Z** (4n+2 electron system) is thermally allowed in a disrotatory manner and photochemically allowed in a conrotatory manner.^[11] The *anti*-folded conformation of **3-Z** is preorganized for conrotatory cyclization, satisfying the requirement for photochemical reaction. This conclusion was further confirmed by TD-DFT calculation^[12] of **3-Z**. Conrotatory cyclization of the LUMO led to constructive interaction (photochemically favored) (Figure 1B). The structure of **3-E** did not permit such cyclization but *E-Z* isomerization allowed **3-Z** to be produced, promoting photoreaction. As a result, the dihydrophenanthrene intermediate was attained, followed by oxidation to give photoproduct **4**. The dihydrophenanthrene intermediate was not isolable from the reaction mixture.

Compound **3-E/3-Z** is fluorescent with a large Stokes shift (135 nm). Photoproduct **4** is red-shifted in both absorbance and emission by approximately 100 nm while retaining a large Stokes shift of 108 nm (Figure 1C). Live cell imaging studies of **3-E/3-Z** were performed in HeLa cells, resulting in specific sub-cellular localization, consistent with mitochondrial

uptake. An engineered HeLa cell line expressing GFP labeled proteins specifically localized to the outer mitochondria membrane (mito-GFP cell line), as introduced in one of our previous studies,^[5b] was used as a control to confirm mitochondrial localization for **3-E/3-Z** in this report. The localization statistics were also compared to that of a commonly used and commercially available MitoTracker dye.^[8] Our control cell line consisted of a population of HeLa cells expressing a GFP-fusion protein localized specifically to the outer mitochondrial membrane (mito-GFP cell line) in addition to a population of non-GFP expressing HeLa cells, which serve as an internal control for compound localization in the absence of GFP signal. Two channels 405/635 and 488/525 were used to detect **3-E/3-Z** and GFP, respectively, with no bleed-through observed. All images were kept at the same brightness and contrast settings. Incubating mito-GFP cells with **3-E/3-Z** allowed colocalization to be assessed (Figure 2A and Figure S3). Colocalization statistics calculated over multiple frames for a total of 80 cells showed significant overlap (Pearson's coefficient: 0.81 ± 0.02 ; Manders coefficients: 0.98 ± 0.01 and 1.00 ± 0.00 ; Spearman correlation: 0.88 ± 0.02). High Manders coefficients point to near exclusive mitochondrial localization of **3-E/3-Z** in GFP positive cells. Variation in intensity between localized **3-E/3-Z** and GFP resulted in a slightly lower Pearson's coefficient. This small deviation may be attributed to the difference between internal mitochondrial localization of **3-E/3-Z** versus external mitochondrial membrane localization of GFP. Intensity profiles across multiple cells are shown in Figure 2B. Additional colocalization studies were carried out using commercially available Mitotracker Red^[8] and similar results were observed when comparing colocalization to GFP labeled mitochondria (Figure S4). High signal to noise was observed in the intensity line plots for both GFP and **3-E/3-Z** (Figure 2B). The stability of 1 mM aqueous solution of **3-E/3-Z** was assessed under ambient light conditions at 25 °C for 10 days. HPLC analysis of the resulting solution revealed no evidence for decomposition or photoconversion compared to a standard sample of photoproduct **4** (Figure 2C). Cytotoxicity studies on HeLa cells over 24, 48, and 72 hours confirmed that **3-E/3-Z** had very low cytotoxicity. For comparison, MitoTracker Red FM, one of the most commonly used mitochondria imaging probes, showed significant cytotoxicity over the same time course with a 5 fold lower concentration (Figure 2D).

By combining the observed photoreaction (Figure 1A) and the ability to selectively label mitochondria for live cell imaging (Figure 2A), intracellular photoactivation of **3-E/3-Z** was attempted. The ~100 nm red-shifted absorbance and emission spectra of **4** compared to **3-E/3-Z** allowed for selective excitation of individual species. Alternating use of the two channels (i.e., 444/525 (for **3-E/3-Z**) and 488/632 (for **4**)) resulted in photoconversion of **3-E/3-Z** to **4** in live cells. A significant increase in the intensity of **4** was achieved after 30 seconds, using 200 total scans (Figure 3A, 3B). To further investigate the spatial selectivity, a targeted 405 nm laser (CrystaLaser, DL405-050-O) was used to target smaller areas of the cells. Confocal images were captured using a 488 nm excitation laser and a 632 nm emission filter. Immediately after the first activation, a bright signal was detected at the targeted region. The tubular morphology of the mitochondria was observed and the remaining portions of the mitochondria remained dim for an additional 10 seconds using the 488 nm excitation laser.

As a final application we show that individual cells can be photoactivated in extremely crowded confluent cellular environments. Figure 3C shows a normalized intensity plot for sequential photoactivation of two adjacent cells. A standard 405 nm laser, commonly used for photobleaching experiments, was used for sequential photoactivation with excellent spatial control over individual cells in crowded environments (Figure 3D). Using 50 20-second pulses, the mitochondrial probe **3** was photoconverted to **4**. Two individual cells out of 20 cells were sequentially photoactivated (cell 7 and cell 19, Figure 3D).

In conclusion, we have developed a photoactivatable probe based on a 6π -electrocyclization/oxidation mechanism that is water-soluble, cell permeable, non-cytotoxic and can selectively stain mitochondria. More studies are underway to utilize this new photoactivation process for the development of cellular imaging probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) *E-Z* interconversion and photocyclization/oxidation reaction of **3-E/3-Z**. (B) Idealized and calculated LUMO of **3-Z** (method: TD-DFT B3LYP, basis set = 6-311+G(2d,p)). Note the orbital preorganization for photochemically favored conrotatory cyclization. (C) Absorbance and emission spectra of **3-E/3-Z** (Abs λ_{max} = 408 nm, Em λ_{max} = 543 nm, ϵ = 11040 M⁻¹cm⁻¹, ϕ = 0.021, τ = 0.97 ns) and the **4** (Abs λ_{max} = 504 nm, Em λ_{max} = 612 nm, ϵ = 11496 M⁻¹cm⁻¹, ϕ = 0.100, τ = 4.77 ns) in water.

Figure 2.

(A) Confocal images of mito-GFP cells (right panel), HeLa cells stained with 3-E/3-Z (middle panel), and mito-GFP cells stained with 3-E/3-Z (left panel). Compound 3-E/3-Z was observed at 405/635 while GFP was observed at 488/525. All images were kept at the same contrast/brightness and bleed-through was not detected between channels. Scale bar = 10 μm . (B) Line plot of fluorescence intensity across multiple cells showing high colocalization and signal to noise ratio for both GFP and 3-E/3-Z. The colocalization plot in the bottom right of panel A designates the nuclear regions in adjacent cells with “n”.

Pearson's colocalization coefficient was 0.87 ± 0.01 . Manders' overlap coefficients were 0.98 ± 0.01 and 1.00 ± 0.00 . (C) HPLC chromatogram of **3-E/3-Z** (1 mM solution in water) after 10 days under ambient light (top chromatogram) and HPLC chromatogram of **4** (bottom chromatogram). No decomposition or photoproduct of **3-E/3-Z** was detected. (D) Cell viability was determined using HeLa cells in the presence of **3-E/3-Z** and MitoTracker Red FM at 5 μ M for **3-E/3-Z** and 1 μ M for MitoTracker Red FM.

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Figure 3.

(A) Confocal microscopy images of HeLa cells stained with **3-E/3-Z**, observed at 444/525 and 488/632 over 30 seconds with 200 alternating 150 ms pulses. (B) Average intensity of channels 444/525 and 488/632 after 200 alternating pulses. The data from each of the two channels were fitted to a one phase exponential curve and the obtained rate constant and half-lives were 0.060 s^{-1} and 11.61 s for the 444/525 channel and 0.030 s^{-1} and 23.05 s for the 488/632 channel. (C) Normalized intensity plot for sequential photoactivation of two cells. (D) Selective sequential photoactivation of two cells (7 and 19) among a field of view

containing 20 confluent cells. Each cell was activated by 50 20-second pulses of a normal 405 nm FRAP laser. Dashed lines indicate cells boundary. Scale bar = 10 μm .

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Scheme 1.
Synthesis of 3-E/3-Z and crystal structures of 2-E and 3-Z.