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moxDendra2: an inert photoswitchable protein for oxidizing environments[†]

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Abstract

Fluorescent proteins (FPs) that can be optically highlighted, enable PALM super-resolution microscopy and pulse-chase experiments of cellular molecules. Most FPs were evolved in and for the cytoplasm and, therefore, may fold incorrectly in subcellular organelles. Here, we describe the first monomeric photoswitchable from green to red bright FP adapted for oxidizing environments.

Graphical Abstract

The authors describe the engineering of the first monomeric photoswitchable fluorescent protein for use in oxidizing cellular environments.

Photoswitchable fluorescent proteins (PSFPs) have important applications in cell biology. These fluorescent protein (FP) variants mature with characteristic excitation and emission spectra that can be optically highlighted resulting in longer wavelength spectra, i.e. a green FP is irradiated with a 405 nm laser that converts the protein into a red FP.^{1, 2} A cell, organelle or population of PSFP-tagged fusion proteins can be optically highlighted with pixel precision using a laser scanning confocal microscope to perform a pulse-chase experiment.^{3, 4} PSFPs also enable PALM modes of super resolution microscopy.⁵ To fully

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exploit the potential of PSFPs, these proteins must i) correctly fold in cells (to enable chromophore formation) and ii) be inert and not alter the localization, function or behaviour of a tagged fusion protein. For example, an ideal fusion protein tag should be monomeric to avoid artifactual oligomerization of a fusion protein.^{6–8} Nearly all FPs and PSFPs are inherently cytoplasmic proteins or were engineered in the cytoplasm of cells. As a result, these FPs were optimized for the cytoplasmic environment. However, the same FPs can misfold and/or be post-translationally modified in the different luminal environments of subcellular organelles. Within the endoplasmic reticulum, the oxidizing environment and protein disulphide isomerase chaperones can promote the formation of non-native disulphide bonds within and between FPs that contain cysteines.^{9–11} Similarly, the N-linked glycosylation machinery in the ER can append large sugar groups to asparagines that are followed by a consensus glycosylation sequence (N-X-S/T).^{12, 13} We and others have previously shown such modifications can generate misfolded populations of dark FPs^{9, 10}, enlarge their size¹³, and incorrectly localize fusion proteins.¹¹ To avoid such problems, we have identified and optimized multiple monomeric FPs for use in oxidizing environments including the ER, the eukaryotic secretory pathway, the mitochondrial inner membrane space, the chloroplast inner membrane space, and the periplasm of gram-negative bacteria.^{10, 11, 13} Here, we turned our attention to optimization of a PSFP for use in oxidizing environments.

We began by focusing our efforts on PSFPs already established as *bona fide* monomeric proteins. mEos3.2, Dendra2, PAGFP with an A206K mutation, and mMaple3 meet this essential criterion.^{14, 15} Based on our success with the creation of moxFPs¹¹, we initially attempted to incorporate superfolder GFP (sfGFP)¹⁶ mutations into PAGFP, with a monomerizing A206K mutation. However, the superfolder mutations altered the photoactivation properties of the PAGFP and resulted in a protein with a substantial population of already green (the photoconverted form) species in the absence of any attempts to photoconvert the protein (data not shown). Due to the unexpected and substantial decrease in signal to noise, we abandoned further attempts to modify PAGFP.

Next, we considered mEos3.2 and the closely related Dendra2. These proteins are not related to the *A. victoria* GFP and thus, we could not use our previous strategy of incorporating sfGFP mutations to promote tolerance of mutagenesis of any cysteines. Both PSFPs contained 3 cysteines (Fig. 1). The efforts of Jain et al. and our experience with engineering of secBFP2 made us wary of mutating cysteines, which might produce a dark FP.^{9, 13} We felt it would also be important to make as few mutations as possible to the protein to avoid accidentally altering protein photophysics. Therefore, we decided to re-engineer mEos3.2 as the cysteines were the only mutations necessary to render the protein inert in secretory pathway. Dendra2 contains two potential N-linked glycosylation consensus sequences, which would further complicate mutagenesis efforts.

Based on our experience with secBFP2, we anticipated that the cysteine mutations most likely to be tolerated would be small hydrophobic amino acids. Each cysteine of mEos3.2 was mutated to alanine, valine or methionine. No fluorescence was observed for methionine substitutions and valine substitutions were extremely dim. Only alanine mutations maintained significant unactivated mEos3.2 green fluorescence and 405 nm light

photoswitched to a red state. Next, all three cysteines (101, 171, and 195) were mutated to alanines. The resulting protein was clearly fluorescent and readily photoswitched when expressed in the ER (Fig. S1A ESI[†]). The new “mox” (monomeric oxidation protected) PSFP was now resistant to inappropriate disulphide bond formation when expressed in the ER, while the parent “wt” mEos3.2 formed a ladder of covalent oligomers, with noticeably very little protein in the monomeric state (Fig. S1B ESI[†]). We also confirmed that the alanine mutations did not alter the monomericity of mEos3.2 by performing a CytERM assay for FP oligomerization (Fig. S1C ESI[†]). Despite these successful outcomes, we noticed that moxEos3.2 was visually much dimmer in the green and red states in comparably transfected and expressing cells. Furthermore, this mox form bleached substantially faster than the wt parent in both the green and red states (Fig. S1D ESI[†]). Taken together, the optimization of mEos3.2 for the secretory pathway had impaired the performance of the protein.

Therefore, we sought to re-engineer yet another PSFP to be more inert in cells. We chose Dendra2, which shares considerable sequence identity with mEos3.2, despite derivation from different organisms (*Dendrophthya* sp. and *Lobophyllia hemprichii*, respectively). Dendra2 had more residues to mutate than mEos3.2, but Dendra2 has reportedly higher brightness values than mEos3.2 in green or red states. Based on the mutagenesis results with mEos3.2, we converted each cysteine (residues 106 and 176) to either an alanine or (residue 118) a threonine (as in mEos3.2) and N-glycosylation asparagines (residues 45 and 205) to glutamines and synthesized a moxDendra2.

The mutations minimally impacted the spectra for absorbance and emission for the green and red forms of moxDendra2 (Fig. 2A–D). The green form of moxDendra2 photobleached to a greater degree than the parent, and the red form photobleached more rapidly, but to a similar degree relative to the parent (Fig. 2E, F). There was little difference in the rate and extent of photoconversion for Dendra2 and moxDendra2 (Fig. 2G, H). These minor decreases in performance were at least partially offset by substantial improvements in extinction coefficients for both the green and red forms of moxDendra2, which led to increased brightness (26% for the green form and 17% for the red form) (Table. S1 ESI[†]). The increased brightness had a noticeable performance impact when Dendra2 and moxDendra2 were expressed in the oxidizing environment of the gram-negative *E. coli* periplasm (Fig. 2I). First, the bacteria were noticeably brighter. This likely reflects both increased brightness and improved folding in the periplasm. That is, cysteine-containing Dendra2 has a high probability of misfolding into a dark molecule in the oxidizing environment. Second, the periplasmic moxDendra2 appears to correctly localize in a cell outline distribution. In contrast, periplasmic Dendra2 appears in large bright clumps, possibly misfolded aggregates. It is worth noting that this differs from what was observed with misfolded periplasmic EGFP, where no fluorescence was detected.¹⁰ There are two potential explanations for this difference. First, Dendra2 contains a cysteine on the outside of the β -barrel, while EGFP does not. This means that the Dendra2 could still form a barrel and the surface cysteine could form a dimerizing disulphide bond. Whether this would lead to

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aggregate formation is unclear. A second possibility is that the periplasmic Dendra2 reporter enters the periplasm poorly and accumulates in a misfolded or aggregated form in the cytoplasm. This was observed for EGFP and for some other co-translationally translocated proteins.^{10, 17}

Next, to assess performance in mammalian cells, we asked whether any of the mutations had adversely impacted the monomericity of the protein. Dendra2 is robustly monomeric, which was a major reason for focusing on this protein for these studies.¹⁴ To test this, we fused moxDendra2 to the monomericity reporter, CytERM, expressed the reporter in HeLa cells and imaged cells for evidence of membrane accumulations driven by ER membrane adhesion by oligomeric proteins. No whorls or karmellae were observed (Fig. S2 ESI[†]) and we concluded that the moxDendra2 is robustly monomeric.⁷

Next, we investigated performance of moxDendra2 in the oxidizing environment of the mammalian secretory pathway, specifically in the lumen of the Golgi complex using the common Golgi complex targeting sequence of the human galactosyltransferase signal anchor.¹⁸ When this reporter was fused to EGFP family members, we observed a large ER pool of dark misfolded proteins that were revealed only through immunofluorescence microscopy.¹¹ Given that Dendra2 contains 3 cysteines relative to EGFP's 2 cysteines, we hypothesized that we could observe Golgi complex localization of the correctly folded fluorescent form of the reporter and ER accumulation of the misfolded dark form. Surprisingly, we observed a very different phenotype, mobile vesicles with little resemblance to the stack of perinuclear membranes characteristic of the Golgi complex (Fig. S3 ESI[†]). We previously observed Golgi-complex targeted red fluorescent proteins in a vesicular pattern.¹¹ Co-localization experiments revealed those vesicles to be lysosomes. Because Dendra2 has a pK_a of 6.6¹⁹, it is extremely unlikely that the luminal Dendra2 would be fluorescent at pH 4 of a lysosome. The origin of these vesicles is currently unclear. We confirmed this aberrant phenotype in a second cell type, HeLa cells (Fig. 3). In contrast, in both U-2 OS and HeLa cells, we observed well defined Golgi complex structures for GalT-moxDendra2. Golgi complex localization for GalT-moxDendra2, but not GalT-Dendra2 was confirmed by co-expressing the validated Golgi complex marker, GalT-oxBFP in HeLa cells (Fig. 4).¹¹

Finally, we tested whether moxDendra2 photoswitches in the high calcium oxidizing environment of the Golgi complex. HeLa cells expressing GalT-moxDendra2 were imaged in red and green channels before and after photoswitching by exposure to high intensity 405 nm irradiation (Fig. 5). As we observed in the bacterial periplasm, 405 nm illumination led to photoswitching of Golgi complex-localized moxDendra2 into the red form. Taken together, the removal of cysteines and N-linked sugars yields a green-to-red PSFP suitable for use in the mammalian secretory pathway.

In summary, we have created the first monomeric PSFP that can be used robustly in oxidizing cellular environments including gram-negative bacteria periplasm and the mammalian secretory pathway. The new moxDendra2 is brighter and exhibits spectra and activation kinetics comparable to the parental Dendra2. There are other cysteineless photoconvertible proteins, such as PAmCherry.²⁰ However, that protein is initially dark and

then photoactivated to become red, which makes initial identification of a pool of protein to image more challenging.

Going forward, our efforts now establish the feasibility of re-engineering different families of FPs and PSFPs for use in a range of different cellular environments. Our studies suggest that researchers developing new FPs can consider a parallel evolution workflow to evolve a cysteineless version of an FP for optimal performance. The failure of pools of organelle-targeted proteins to fold or localize correctly highlights the critical need for inert FPs. These reagents are essential to decrease artefacts in cell biology studies and future efforts to tag endogenous genes with FPs using CRISPR/Cas9 technology. Given that approximately one third of the human genome encodes secretory or membrane proteins, it will be essential to use inert FPs and PSFPs. moxDendra2 represents a significant improvement for laboratories studying fusion proteins and reporters in the chemically distinct noncytoplasmic cell environments

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. Shcherbakova DM, Sengupta P, Lippincott-Schwartz J, Verkhusha VV. *Annu Rev Biophys.* 2014; 43:303–329. [PubMed: 24895855]
2. Shcherbakova DM, Verkhusha VV. *Curr Opin Chem Biol.* 2014; 20:60–68. [PubMed: 24819887]
3. Patterson GH, Lippincott-Schwartz J. *Science.* 2002; 297:1873–1877. [PubMed: 12228718]
4. Patterson GH. *Curr Protoc Cell Biol.* 2008; Chapter 21(Unit 21):26.
5. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF. *Science.* 2006; 313:1642–1645. [PubMed: 16902090]
6. Snapp EL, Hegde RS, Francolini M, Lombardo F, Colombo S, Pedrazzini E, Borgese N, Lippincott-Schwartz J. *J Cell Biol.* 2003; 163:257–269. [PubMed: 14581454]
7. Costantini LM, Fossati M, Francolini M, Snapp EL. *Traffic.* 2012; 13:643–649. [PubMed: 22289035]
8. Zacharias DA, Violin JD, Newton AC, Tsien RY. *Science.* 2002; 296:913–916. [PubMed: 11988576]
9. Jain RK, Joyce PB, Molinete M, Halban PA, Gorr SU. *Biochem J.* 2001; 360:645–649. [PubMed: 11736655]
10. Aronson DE, Costantini LM, Snapp EL. *Traffic.* 2011; 12:543–548. [PubMed: 21255213]
11. Costantini LM, Baloban M, Markwardt ML, Rizzo M, Guo F, Verkhusha VV, Snapp EL. *Nat Commun.* 2015; 6:7670. [PubMed: 26158227]
12. Costantini LM, Snapp EL. *DNA Cell Biol.* 2013; 32:622–627. [PubMed: 23971632]
13. Costantini LM, Subach OM, Jaureguiberry-Bravo M, Verkhusha VV, Snapp EL. *Biochem Biophys Res Commun.* 2013; 430:1114–1119. [PubMed: 23257162]
14. Wang S, Moffitt JR, Dempsey GT, Xie XS, Zhuang X. *Proc Natl Acad Sci U S A.* 2014; 111:8452–8457. [PubMed: 24912163]

15. Zhang M, Chang H, Zhang Y, Yu J, Wu L, Ji W, Chen J, Liu B, Lu J, Liu Y, Zhang J, Xu P, Xu T. *Nature methods*. 2012; 9:727–729. [PubMed: 22581370]
16. Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. *Nat Biotechnol*. 2006; 24:79–88. [PubMed: 16369541]
17. Tian P, Bernstein HD. *J Biol Chem*. 2009; 284:11396–11404. [PubMed: 19211555]
18. Cole NB, Smith CL, Sicaky N, Terasaki M, Edidin M, Lippincott-Schwartz J. *Science*. 1996; 273:797–801. [PubMed: 8670420]
19. Chudakov DM, Lukyanov S, Lukyanov KA. *Biotechniques*. 200; 42:553–555. 557 passim. [PubMed: 17515192]
20. Subach FV, Patterson GH, Manley S, Gillette JM, Lippincott-Schwartz J, Verkhusha VV. *Nature methods*. 2009; 6:153–159. [PubMed: 19169259]

Fig. 1.

Alignment of amino acids sequences for Dendra2 and mEos3.2. Residues buried inside of FPs' β -barrels are shaded. Amino acid numbering follows that of Dendra2. * indicates the residues that form a chromophore. Red font residues are Cys that can form disulphide bonds or Asp in consensus N-linked glycosylation sites.

Fig. 2.

Spectral and photochemical properties of Dendra2 and moxDendra2. Absorbance spectra of Dendra2 and moxDendra2 in green state (A) and after photoswitching (B). Excitation and emission spectra of Dendra2 and moxDendra2 in the green state (C) and after photoswitching (D). Photostability of Dendra2 and moxDendra2 in green state (E) and after photoswitching (F). Kinetics of Dendra2 (G) and moxDendra2 (H) photoswitching with 390/40 nm light. The error bars indicate the s.e.m. from 4 different experiments. (I) *E. coli* expressing periplasmic targeted Dendra2 or moxDendra2. Bacteria were imaged under identical conditions for the green channel and then identical conditions for the red channel. Upper left thumbnails in the unactivated green channel are enlarged about 4-fold.

Fig. 3. Localization of GalT-Dendra2 and GalT-moxDendra2 in HeLa cells. Cells were transiently transfected and imaged by fluorescence microscopy 16 h later. Scale bar = 5 μ m.

Fig. 4. Localization of GalT-Dendra2 and GalT-moxDendra2 in HeLa cells co-expressing the Golgi complex marker GalT-oxBFP. Cells were transiently transfected and imaged by fluorescence microscopy 24 h later. Scale bar = 5 μ m.

Fig. 5. Photoswitching of GalT-moxDendra2 in HeLa cells. Cells were transiently transfected and imaged by fluorescence microscopy 24 h later. moxDendra2 was photoswitched with 405 nm light for 45 s and then imaged again. The red form is clearly observed following photoswitching. Scale bar = 5 μ m.