CHEMISTRY OF LIGHT INTERACTION

CHEMPHOTOCHEM

ACROSS THE WHOLE SPECTRUM

Accepted Article

Title: Fluorescent flavoprotein heterodimers: combining photostability with singlet oxygen generation

Authors: Alberto Rodríguez-Pulido, Joaquim Torra, Sara H. Mejías, Aitziber L. Cortajarena, Rubén Ruiz-González, Santi Nonell, and Cristina Flors

To be cited as: *ChemPhotoChem* 10.1002/cptc.201800002

Link to VoR: http://dx.doi.org/10.1002/cptc.201800002

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chemphotochem.org

Fluorescent flavoprotein heterodimers: combining photostability with singlet oxygen generation

Alberto Rodríguez-Pulido,[a] Joaquim Torra,[b] Sara H. Mejías,[a] Aitziber L. Cortajarena,[a, c, d] Rubén Ruiz-González,[b] Santi Nonell,[b] and Cristina Flors*[a]

Abstract: Flavoproteins that combine stable fluorescence emission with the ability to photosensitize reactive oxygen species (ROS) have great potential for advanced imaging techniques such as correlative light and electron microscopy (CLEM). However, ROS photosensitization results in substantial fluorescence photobleaching in flavoproteins, limiting their application as genetically-encoded microscopy tags. To address this issue, we have engineered two novel flavoprotein heterodimers from monomers with complementary properties, and show that a good compromise between photostability and singlet oxygen photosensitization can be achieved. The potential of the heterodimers as tags for CLEM is also investigated and discussed. Since the size of the tandem heterodimers is still small, similar to that of a single green fluorescent protein, these novel tags are a useful addition to the flavoprotein toolbox for a growing number of advanced imaging techniques.

Flavoproteins (or flavin-based fluorescent proteins, FbFPs) have recently emerged as promising genetically encoded fluorescent tags for modern imaging techologies.[1] While FbFPs were initially developed as an alternative to GFP-based proteins for imaging in low-oxygen conditions,[2] they also show other advantages as their smaller size (~12 kDa) and stable fluorescence over wider pH range.[1a, 1b, 3] FbFPs are derived from blue light photoreceptors known as light, oxygen, and voltage (LOV) domains that are naturally expressed in fungi, algae and bacteria.[4] LOV domains typically host flavin mononucleotide (FMN) into a scaffold of two α-helixes with an interposed five-stranded β-sheet. Upon blue light illumination, the FMN chromophore covalently binds to a cysteine of the LOV domain, setting up a very weak fluorescent complex that generally reverts to its initial photocyclic state by thermal relaxation.[5] To prevent the formation of the covalent adduct and enhance fluorescence, the cysteine residue can be mutated, providing a framework to engineer FbFPs.[1]

While many FbFPs have been developed as merely fluorescent tags, others combine fluorescence with the ability to generate reactive oxygen species (ROS) with high spatial precision,[6] which is relevant to a number of advanced imaging and optogenetic techniques.[7] The first variant of this novel family was miniSOG (for “mini Singlet Oxygen Generator”), which was developed as a genetically-encoded tag for photooxidation-based correlative light and electron microscopy (CLEM).[6a] This method relies on the local photooxidation of 3,3’-diaminobenzidine (DAB) by a photosensitizer (in this case miniSOG) to form an insoluble osmiophilic polymer, which provides contrast in EM after staining with osmium tetroxide.[8] Although the photooxidation reaction was assumed to be induced by singlet oxygen ($^1O_2$), we have recently reported that the ability to polymerize DAB does not clearly correlate with the efficiency of the photosensitizers to generate $^1O_2$.[6d] An improved mutant, miniSOG-Q103V, was developed with 10-fold higher efficiency of $^1O_2$ production and a 1.3-fold higher ability to polymerized DAB than miniSOG.[6d] However, miniSOG and variants suffer from relatively high photo-degradation,[9] most likely due to their ability to photosensitize ROS, which restricts their use for light microscopy and thus for CLEM. It is reasonable to think that the engineering of photostability and ROS photosensitization go in the opposite direction, and therefore these two properties are difficult to combine in the same protein.

We report herein a different strategy to bring together photostability and ROS photosensitization based on engineering flavoprotein heterodimers. FbFPs are ideal candidates for tandem oligomerization since their resulting size is still sufficiently small to avoid possible cell function disruptions. While the expression of multiple miniSOG units (trimers)[9b] or heterodimers of miniSOG fused to a GFP-like protein has been

[a] Dr. A. Rodríguez-Pulido, Dr. S. H. Mejías, Dr. A. L. Cortajarena, Dr. C. Flors
Madrid Institute for Advanced Studies in Nanoscience (IMDEA Nanoscience) and Nanobiotechnology Unit Associated to the National Center for Biotechnology (CNB-CSIC)
C/ Faraday 9, 28049 Madrid, Spain
E-mail: cristina.flors@imdea.org
[b] Mr. J. Torra, Dr. R. Ruiz-González, Prof. S. Nonell
Institut Químic de Sarrià, Universitat Ramon Llull
Via Augusta 390, 08017 Barcelona, Spain
[c] Dr. A. L. Cortajarena
CIC biomaGUNE, Parque Tecnológico de San Sebastián, Paseo de Miramón 182, 20014 Donostia-San Sebastián, Spain
[d] Dr. A. L. Cortajarena
Ikerbasque, Basque Foundation for Science, Mª Díaz de Haro 3, 48013 Bilbao, Spain

Supporting information for this article is given via a link at the end of the document.((Please delete this text if not appropriate))

used as a strategy to aid in its visualization,[10] our approach is to combine two different monomeric subunits with complementary properties for CLEM, i.e. efficient DAB photo-oxidation and resistance to photodegradation. MiniSOG and miniSOG-Q103V were chosen as moieties to endow efficient DAB photo-oxidation.[6d] To provide photostability we have incorporated phiLOV2.1 (for "photostable improved LOV"), whose resistance to photobleaching is thought to be a consequence of the constraint of the FMN chromophore in the protein pocket.[11] We have engineered two tandem flavoprotein heterodimers: (i) phiSOG (for "photostable improved Singlet Oxygen Generator"), which has been constructed by fusion of miniSOG and phiLOV2.1 monomers; and (ii) phiSOG-Q103V, which has been constructed by fusion of miniSOG-Q103V with phiLOV2.1. We study their photophysical properties, photostability and screen their ability to photoxidize DAB in order to predict their performance in CLEM.

To obtain the flavoprotein heterodimers phiSOG and phiSOG-Q103V we fused the phiLOV2.1 domain with either miniSOG or miniSOG-Q103V domains, respectively (Figure 1a). We introduced a flexible linker (Gly-Ser) between domains in order to avoid possible interactions between fused domains and preserve their structural integrity. The size of the flavoprotein heterodimers was determined by polyacrylamide gel electrophoresis and mass spectrometry (see Materials and Supplementary Figure S1). PhiSOG and PhiSOG-Q103V heterodimers showed a size corresponding to that expected by the addition of their respective monomers (~26 kDa), similar to the size of a single GFP protein.

Absorption (Figure 1b) and fluorescence spectra (Figure 1c) of the new heterodimers and their monomeric components were recorded in phosphate buffered saline (PBS) at pH 7.4. The absorption and emission spectra of miniSOG and phiLOV2.1 are almost identical, which therefore results in phiSOG also showing similar spectrum. On the other hand, miniSOG-Q103V presents a 7-9 nm blue-shifted maximum in both absorption and fluorescence compared to the other two monomeric flavoproteins (Table 1). The absorption spectrum of phiSOG-Q103V corresponds to the spectral addition of its monomeric components, with a maximum at 444 nm and a less pronounced vibronic structure. Interestingly, the fluorescence spectrum of phiSOG-Q103V is closer to that of phiLOV2.1, with maxima at 497 and 498 nm, respectively, which may reflect resonant energy transfer from blue-shifted miniSOG-Q103V to phiLOV2.1. Fluorescence quantum yields (Φ_F) were determined using FMN as standard ($\Phi_{F,FMN}$ = 0.25 ± 0.01) (Table 1 and Supplementary Figure S2).[9a] Heterodimeric phiSOG shows a value of Φ_F which is the exact average between that of its monomers, as expected from their overlapping fluorescence spectra. On the other hand, the Φ_F value of phiSOG-Q103V is closer to that of phiLOV2.1, consistent with (partial) energy transfer from miniSOG-Q103V to phiLOV 2.1.

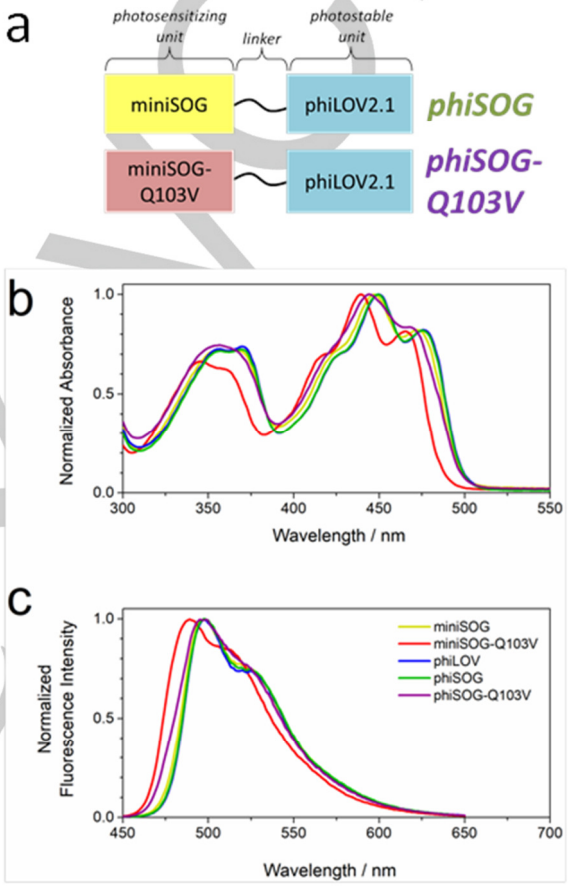


Figure 1. (a) Scheme of heterodimeric tandem flavoproteins: phiSOG and phiSOG-Q103V. (b) Absorption and (c) fluorescence emission spectra of miniSOG (yellow), miniSOG-Q103V (red), phiLOV2.1 (blue), heterodimeric phiSOG (green) and heterodimeric phiSOG-Q103V (purple). Fluorescence spectra were recorded by excitation at the corresponding absorption maximum wavelength of each flavoprotein.

We also determined the $^1O_2$ generation of all the flavoproteins by detecting directly its phosphorescence in the NIR upon illumination at 473 nm (Supplementary Figure S3). Time-resolved phosphorescence curves were fitted to one growth and one decay components in order to determine the quantum yield of $^1O_2$ generation (Φ_Δ) (Table 1). We find that phiLOV2.1 produced a lower amount of $^1O_2$ (Φ_Δ = 0.01), compared to the other two miniSOG monomeric variants. We

recall here that phiLOV2.1 was developed as a photostable variant,[11] and it is therefore interesting to note that its Φ_Δ value is still significant, and higher than those measured for GFP-like proteins.[12] The heterodimer phiSOG shows approximately a Φ_Δ averaged between its monomers, miniSOG and phiLOV2.1. On the other hand, the Φ_Δ of the heterodimer phiSOG-Q103V is smaller than the average of its monomers and closer to the Φ_Δ of phiLOV2.1. This result reinforces the hypothesis of energy transfer from miniSOG-Q103V to phiLOV2.1 which may (partially) compete with intersystem crossing to miniSOG-Q103V triplet state and in turn energy transfer to molecular oxygen to generate $^1O_2$ by the latter moiety.

Table 1. Absorption and emission maxima, fluorescence and $^1O_2$ quantum yields, relative fluorescence photobleaching and DAB polymerization efficiency for all studied flavoproteins.

Flavoprotein	Protein construct	$\lambda_{abs,\,max}$ (nm)	$\lambda_{fluo,\,max}$ (nm)	Φ_F[a]	Φ_Δ[f]	Relative rate of fluorescence photobleaching	Relative ability for DAB polymerization
miniSOG	monomer	447	498	0.44, (0.37-0.41)[b,c,d]	0.04, 0.03[g]	1	1
miniSOG-Q103V	monomer	440	489	0.49	0.39[h], 0.36[i]	2.6 ± 0.1	1.2 ± 0.1, 1.3 ± 0.2[i]
phiLOV2.1	monomer	449	498	0.28, 0.20[d,e]	0.010	0.12 ± 0.01	0.27 ± 0.02
phiSOG	heterodimer	449	498	0.36	0.02	0.36 ± 0.01	0.51 ± 0.03
phiSOG-Q103V	heterodimer	444	497	0.35	0.15	0.52 ± 0.04	0.51 ± 0.03

[a] Uncertainties are ± 0.03 for all the fluorescence quantum yields. [b] Ref. [13]. [c] Ref. [6a]. [d] Ref. [9a]. [e] Ref. [1b]. [f] In deuterated PBS. Uncertainties are ± 0.01 for all the flavoproteins, except for phiLOV2.1 that is ± 0.005. [g] Ref. [12b]. [h] Ref. [6d]. [i] Ref. [6e].

Regarding fluorescence photobleaching (Figure 2, Table 1), phiLOV2.1 exhibits the lowest photodegradation with almost no effect in its spectrum upon 60 min of 45.0 mW·cm^{-2} blue laser irradiation (Supplementary Figure S4), as expected for a flavoprotein engineered for photostability. The other two monomers miniSOG and miniSOG-Q103V undergo considerably higher photobleaching than phiLOV2.1, being the Q103V variant the least photostable flavoprotein, as previously reported.[6d],† On the other hand, both heterodimers show relatively good photostability, with values not too far from that of phiLOV2.1. The improvement in the heterodimer compared to the monomer is most evident in phiSOG-Q103V, again pointing to energy transfer processes to phiLOV2.1 and considering that this moiety will be the more resilient of the two. Similar trends of photobleaching have been observed in the absorption spectra of the flavoproteins herein studied (Supplementary Figure S5).The results show that the heterodimerization of either miniSOG or miniSOG-Q103V with phiLOV2.1 improves their photostability to a significant extent.

To further assess the suitability of heterodimeric tandem flavoproteins for their application in CLEM, we have studied their ability to photo-oxidize DAB (Figure 3 and Table 1). This can be determined by monitoring the increase in optical density at 440 nm corresponding to the product of DAB photo-polymerization.[6d, 8a] As previously shown, and in contrast to their very different values of Φ_Δ, miniSOG-Q103V only photo-oxidizes DAB about 20% more efficiently than miniSOG upon irradiation with a blue light-emitting diode (LED, 3.0 mW·cm^{-2}).[6d] As a consequence, both heterodimers behave similarly in terms of DAB photo-oxidation, i.e. are half as efficient compared to miniSOG and twice more efficient compared to phiLOV2.1. In addition, these results confirm that the ability to polymerize DAB does not clearly correlate with the efficiency of the photosensitizers to generate $^1O_2$, and that other processes such as the participation of radicals or the direct reaction between the photosensitizer and DAB may occur, as we have previously discussed[6d]

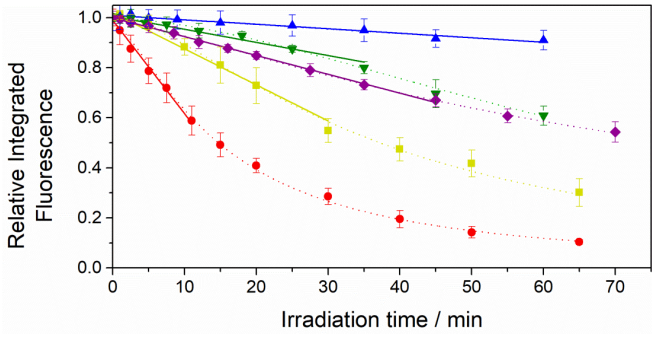


Figure 2. Relative fluorescence photobleaching of miniSOG (yellow), miniSOG-Q103V (red), phiLOV2.1 (blue), heterodimeric phiSOG (green) and heterodimeric phiSOG-Q103V (purple), upon blue laser illumination (45.0 mW cm^{-2}). Solid lines correspond to the linear fits of the experimental data.

In summary, we have engineered two fluorescent flavoprotein heterodimers, phiSOG and phiSOG-Q103V, from monomers with complementary properties. Both heterodimers endure photodegradation significantly, approaching the photostability of phiLOV2.1, the most photostable flavoprotein known to date.[9a] In addition, both heterodimers preserve high levels of $^1O_2$ photosensitization (specially the Q103V variant) and an adequate ability to photo-oxidize DAB. Interestingly, we have observed a process of energy transfer in phiSOG-Q103V from the photosensitizing to the photostable moiety. This results in a fluorescence spectrum close to that of phiLOV2.1, a partial loss of the $^1O_2$ generation and an increase in the expected photostability. Moreover, the heterodimers reported here are still relatively small (~26kDa) and comparable to GFP-like proteins, which is beneficial to avoid the disruption of protein function upon labelling. Therefore, these heterodimers are a useful addition to the fluorescent flavoprotein toolbox for an expanding number of imaging technologies,[7] in which the combination of photostable fluorescence, ROS photosensitization and/or DAB photo-oxidation is required.

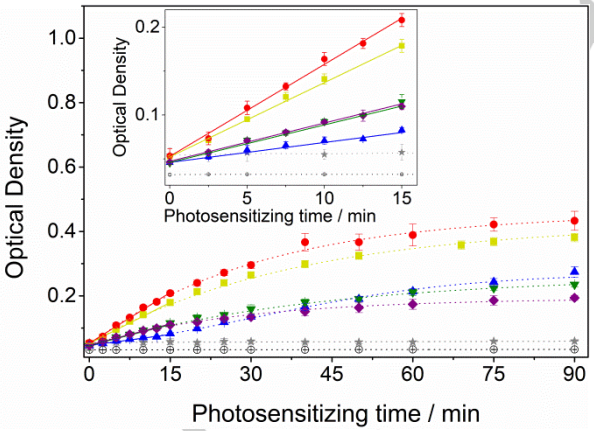


Figure 3. Photosensitized DAB polymerization. Optical density at 440 nm upon DAB polymerization photosensitized by miniSOG (yellow), miniSOG-Q103V (red), phiLOV2.1 (blue), heterodimeric phiSOG (green) and heterodimeric phiSOG-Q103V (purple). Controls: EGFP (grey) and absence of any photosensitizer (open circles). The inset shows the linear region and fits of the OD_{440} curves. All the samples were irradiated with a blue LED (3.0 mW cm^{-2}).

Experimental Section

Experimental details can be found in the Supporting Information.

Acknowledgements

We thank the Tsien and Shu labs at the University of California for plasmids encoding miniSOG. This work was supported by the Spanish *Ministerio de Economía y Competitividad* (CTQ2015-71896-REDT, MAT2015-66605-P, CTQ2016-78454-C2-1-R, BIO2016-77367-C2-1-R) and the Fundació la Marató de TV3 (grant No. 20133133). IMDEA Nanociencia acknowledges support from the 'Severo Ochoa' Programme for Centres of Excellence in R&D (MINECO, Grant SEV-2016-0686).

Keywords: flavoprotein • fluorescent protein • photosensitization • reactive oxygen species • singlet oxygen

† Although showing similar trends, the results of these fluorescence photobleaching studies slightly differ from those obtained in our previous work[6d] due to the use of different sources of irradiation.

[1] a) A. Mukherjee, C. M. Schroeder, *Curr. Opin. Biotechnol.* **2015**, *31*, 16-23; b) A. M. Buckley, J. Petersen, A. J. Roe, G. R. Douce, J. M. Christie, *Curr. Opin. Chem. Biol.* **2015**, *27*, 39-45; c) D. M. Shcherbakova, A. A. Shemetov, A. A. Kaberniuk, V. V. Verkhusha, in *Annual Review of Biochemistry, Vol 84, Vol. 84* (Ed.: R. D. Kornberg), Annual Reviews, Palo Alto, **2015**, pp. 519-550.

[2] T. Drepper, T. Eggert, F. Circolone, A. Heck, U. Krauss, J. K. Guterl, M. Wendorff, A. Losi, W. Gartner, K. E. Jaeger, *Nat. Biotechnol.* **2007**, *25*, 443-445.

[3] A. Mukherjee, J. Walker, K. B. Weyant, C. M. Schroeder, *PLoS One* **2013**, *8*, 15.

[4] a) J. Herrou, S. Crosson, *Nat. Rev. Microbiol.* **2011**, *9*, 713-723; b) J. M. Christie, J. Gawthorne, G. Young, N. J. Fraser, A. J. Roe, *Mol. Plant.* **2012**, *5*, 533-544.

[5] a) T. E. Swartz, S. B. Corchnoy, J. M. Christie, J. W. Lewis, I. Szundi, W. R. Briggs, R. A. Bogomolni, *J. Biol. Chem.* **2001**, *276*, 36493-36500; b) A. Losi, *Photochem. Photobiol.* **2007**, *83*, 1283-1300.

[6] a) X. Shu, V. Lev-Ram, T. J. Deerinck, Y. Qi, E. B. Ramko, M. W. Davidson, Y. Jin, M. H. Ellisman, R. Y. Tsien, *PLoS Biol.* **2011**, *9*, e1001041; b) J. Torra, A. Burgos-Caminal, S. Endres, M. Wingen, T. Drepper, T. Gensch, R. Ruiz-González, S. Nonell, *Photochem. Photobiol. Sci.* **2015**, *14*, 280-287; c) M. Westberg, L. Holmegaard, F. M. Pimenta, M. Etzerodt, P. R. Ogilby, *J. Am. Chem. Soc.* **2015**, *137*, 1632-1642; d) A. Rodríguez-Pulido, A. L. Cortajarena, J. Torra, R. Ruiz-González, S. Nonell, C. Flors, *Chem. Comm.* **2016**, *52*, 8405-8408; e) M. Westberg, M. Bregnhøj, M. Etzerodt, P. R. Ogilby, *J. Phys. Chem. B* **2017**; f) K. Makhijani, T. L. To, R. Ruiz-Gonzalez, C. Lafaye, A. Royant, X. K. Shu, *Cell Chem. Biol.* **2017**, *24*, 110-119.

[7] a) T. L. To, M. J. Fadul, X. Shu, *Nat. Commun.* **2014**, *5*, 4072; b) Y. Sano, W. Watanabe, S. Matsunaga, *J. Cell Sci.* **2014**, *127*, 1621-1629; c) A. P. Wojtovich, T. H. Foster, *Redox Biol.* **2014**, *2*, 368-376; d) M. J. C. Long, J. R. Poganik, S. Ghosh, Y. Aye, *ACS Chem. Biol.* **2017**, *12*, 586-600; e) H. N. Jiang, Y. Li, Z. J. Cui, *Front. Physiol.* **2017**, *8*, 13; f) E. A. Souslova, K. E. Mironova, S. M. Deyev, *J. Biophotonics* **2017**, *10*, 338-352.

[8] a) T. J. Deerinck, M. E. Martone, V. Levram, D. P. L. Green, R. Y. Tsien, D. L. Spector, S. Huang, M. H. Ellisman, *J. Cell Biol.* **1994**, *126*, 901-910; b) C. Meisslitzer-Ruppitsch, C. Rohrl, J. Neumuller, M. Pavelka, A. Ellinger, *J. Microsc.* **2009**, *235*, 322-335; c) H. D. Ou, T. J. Deerinck, E. Bushong, M. H. Ellisman, C. C. O'Shea,

Methods **2015**, *90*, 39-48; d) P. de Boer, J. P. Hoogenboom, B. N. G. Giepmans, *Nat. Methods* **2015**, *12*, 503-513.

[9] a) M. Wingen, J. Potzkei, S. Endres, G. Casini, C. Rupprecht, C. Fahlke, U. Krauss, K. E. Jaeger, T. Drepper, T. Gensch, *Photochem. Photobiol. Sci.* **2014**, *13*, 875-883; b) J. Ng, A. Browning, L. Lechner, M. Terada, G. Howard, G. Jefferis, *Sci. Rep.* **2016**, *6*, 14.

[10] a) J. Y. Lin, S. B. Sann, K. Zhou, S. Nabavi, C. D. Proulx, R. Malinow, Y. Jin, R. Y. Tsien, *Neuron* **2013**, *79*, 241-253; b) H. Strickfaden, Z. Z. Xu, M. J. Hendzel, *J. Vis. Exp.* **2015**, e52893.

[11] J. M. Christie, K. Hitomi, A. S. Arvai, K. A. Hartfield, M. Mettlen, A. J. Pratt, J. A. Tainer, E. D. Getzoff, *J. Biol. Chem.* **2012**, *287*, 22295-22304.

[12] a) A. Jiménez-Banzo, X. Ragàs, S. Abbruzzetti, C. Viappiani, B. Campanini, C. Flors, S. Nonell, *Photochem. Photobiol. Sci.* **2010**, *9*, 1336-1341; b) R. Ruiz-González, A. L. Cortajarena, S. H. Mejias, M. Agut, S. Nonell, C. Flors, *J. Am. Chem. Soc.* **2013**, *135*, 9564-9567; c) A. Jiménez-Banzo, S. Nonell, J. Hofkens, C. Flors, *Biophys. J.* **2008**, *94*, 168-172; d) X. Ragàs, L. P. Cooper, J. H. White, S. Nonell, C. Flors, *ChemPhysChem* **2011**, *12*, 161-165.

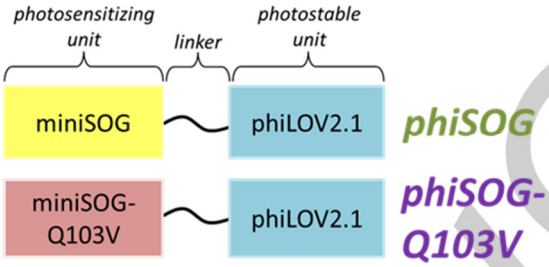
[13] F. M. Pimenta, R. L. Jensen, T. Breitenbach, M. Etzerodt, P. R. Ogilby, *Photochem. Photobiol.* **2013**, *89*, 1116-1126.

Entry for the Table of Contents

Layout 1:

COMMUNICATION

Best of both worlds. Heterodimeric flavoproteins incorporating moieties with complementary photophysical properties are a useful addition to the advanced fluorescence microscopy toolbox.



*Alberto Rodríguez-Pulido, Joaquim Torra, Sara H. Mejías, Aitziber L. Cortajarena, Rubén Ruiz-González, Santi Nonell, and Cristina Flors**

Page No. – Page No.

Fluorescent flavoprotein heterodimers: combining photostability with singlet oxygen generation