

Mitochondria-targeted cpYFP: pH or superoxide sensor?

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The *JGP* series Perspectives on: SGP Symposium on Mitochondrial Physiology and Medicine includes articles by Wei and Dirksen (2012) and by Santo-Domingo and Demarex (2012), which highlight two fields of research in mitochondrial physiology experiencing renewed interest thanks to the development of new genetically encoded probes: mt-cpYFP (a mitochondria-targeted superoxide sensor) and mitoSypHer (a mitochondria-targeted pH probe). Interestingly, both articles discuss the detection of spontaneous spatiotemporal restricted elevations in the fluorescence intensity of the sensors (“flashes”). In one case, flashes are attributed to a quantal production of superoxide (O_2^-) (Wei and Dirksen, 2012) and in the other one to spontaneous elevations in mitochondrial pH (Santo-Domingo and Demarex, 2012). In the following paragraphs, we discuss the possible nature of the flashes in the light of the properties of each sensor.

Discovery and generation of the mt-cpYFP and mitoSypHer

It is amusing to consider that both probes have been serendipitously discovered while running experiments on “old sensors.” Mt-cpYFP is derived from the mitochondria-targeted calcium (Ca^{2+}) indicator ratiometric pericam, described by Nagai et al. in 2001. It was used as the core of this Ca^{2+} probe before its sensitivity for O_2^- was proposed by Wang et al. in 2008. cpYFP was generated by circular permutation and point mutation of a YFP variant, EYFP(V68L/Q69K), with the two original termini being connected by the linker VDGSGGGTG (Nagai et al., 2001).

SypHer is a mutated form of Hyper, a genetically encoded sensor for hydrogen peroxide, developed by Belousov et al. in 2006. Hyper is composed of a cpYFP inserted into Oxy-RD, the regulatory domain of *Escherichia coli* OxyR, which is specifically sensitive to H_2O_2 . cpYFP was obtained by circular permutation of the EYFP sequence from the plasmid pEYFP-N1 (Takara Bio Inc.). The linker VDGSGGGTG was also used between the two termini. Several mutations were introduced to optimize folding and chromophore maturation.

SypHer was developed by Poburko et al. (2011) by mutating one of the two H_2O_2 -sensing cysteine residues of the OxyRD domain of Hyper (C199S), following Belousov et al. (2006). This single mutation rendered SypHer unresponsive to H_2O_2 while preserving its pH sensitivity.

cpYFP: A pH or superoxide sensor?

MitoSypHer as a superoxide sensor? Even if the two cpYFPs constituting the cores of mt-cpYFP and mitoSypHer have been engineered by two different groups, they only differ by seven residues. Strikingly, none of these residues should be involved in O_2^- sensing. According to Wang et al. (2008), two cysteines are involved in mt-cpYFP O_2^- sensing: C171 and C193. These two residues are present in the cpYFP contained in the Hyper/SypHer. Surprisingly, although the O_2^- sensitivity of mt-cpYFP has been repeatedly challenged, the reciprocal hypothesis that Hyper/SypHer might also detect O_2^- has been overlooked. The only article (Belousov et al., 2006) investigating the sensitivity of Hyper to O_2^- shows no increase in its fluorescence upon the addition of 30 μ M xanthine and 25 mU xanthine oxidase in vitro. However, the in vitro characterizations of Hyper and mt-cpYFP by Belousov et al. (2006) and Wang et al. (2008), respectively, have been conducted under different experimental conditions. Hyper isolation procedures were performed in a buffer containing 5 mM 2-mercaptoethanol, and characterization of Hyper specificity was performed in a buffer containing 0.5 mM 2-mercaptoethanol. The calibration solution of Wang et al. (2008) contained HEPES, KCl, and EDTA, but the 10 mM of reduced dithiothreitol used in previous steps was removed from the calibration solution. It has been shown that the presence of oxidants or of 2-mercaptoethanol in the recording solution affects the photobehavior of diverse fluorescent proteins (Bogdanov et al., 2009). The proposed mechanism involves the formation of radical intermediates of the chromophore. It is thus possible that, in the study of Belousov et al. (2006), the 2-mercaptoethanol present in the calibration medium affects

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the reactivity of the fluorescent protein toward O_2^- . Interestingly, Bogdanov et al. (2009) reported variable photobehaviors of the GFP probes among different cell lines and in a same line among different cellular compartments. They attributed this phenomenon to differences in redox environment.

By transfecting mouse skeletal muscle fibers with either mitoHyper or its C199 mutated version (mitoSypHer), we were able to detect transient increases in fluorescence (Fig. 1), which resemble the superoxide flashes that we already described in skeletal muscle (Pouvreau, 2010). Like mt-cpYFP flashes, mitoHyper/SypHer transients are accompanied by a depolarization of the mitochondria. Moreover, the mitoHyper/SypHer

transients are simultaneous with increases in fluorescence intensity of MitoSOX, a mitochondrial targeted synthetic probe for O_2^- . These results are in accordance with Azarias and Chatton (2011), who reported flashes in mitoSypHer fluorescence accompanied by increases in MitoSOX fluorescence in astrocytes. However, because mitoSypHer was initially described as a pH sensor, Azarias and Chatton interpreted the spontaneous fluorescence flashes as pH transients. MitoSOX sensitivity to O_2^- is well established, even if its specificity for O_2^- over other reactive oxygen species (ROS) has been questioned. This limitation of the sensor is of minor importance here, as cpYFP emission is unchanged upon the addition of various ROS (Wang et al., 2008). The least

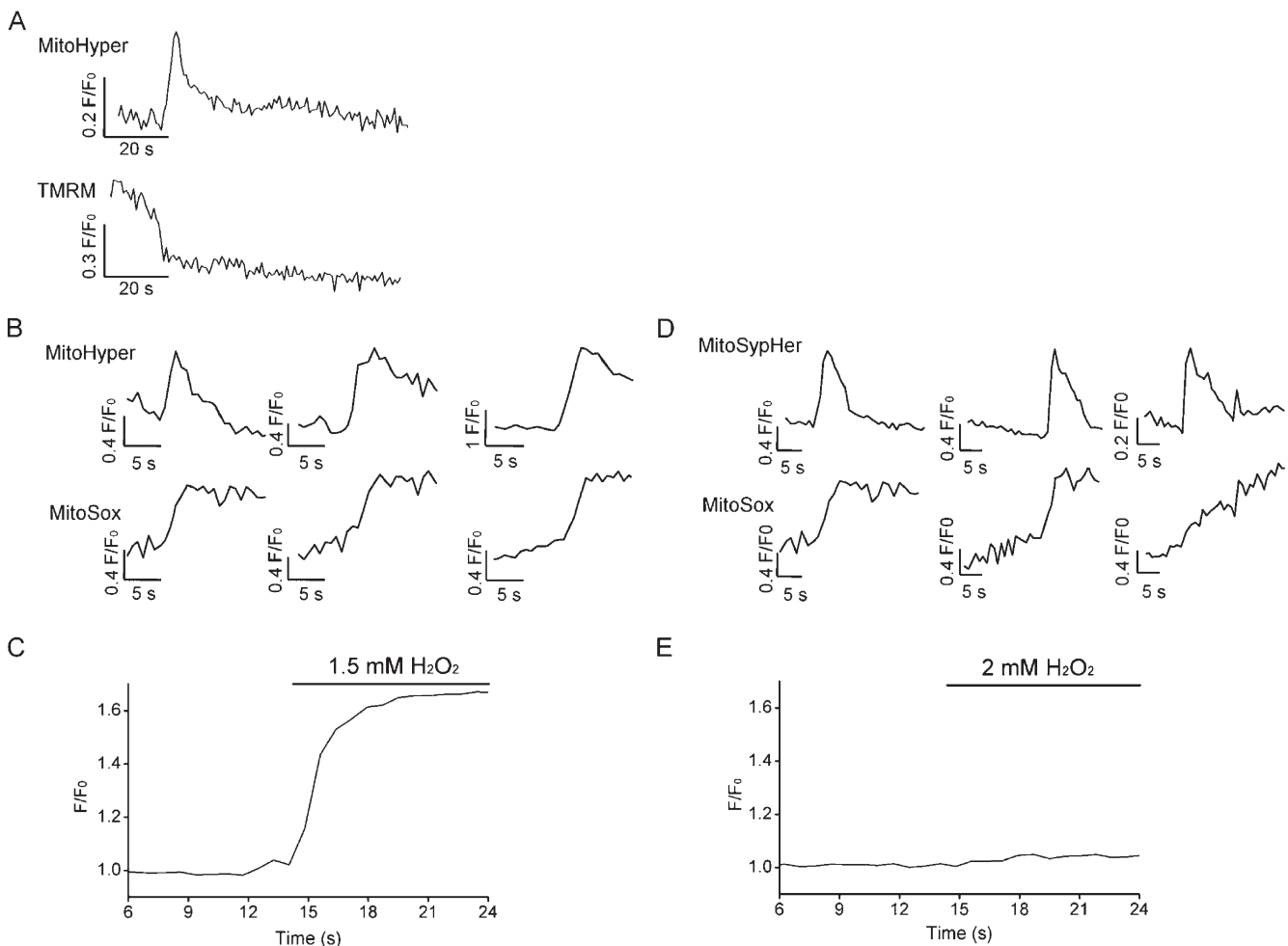


Figure 1. Mitochondrial flashes are detected with mitoHyper and mitoSypHer. Experiments were performed on 5–8-wk-old male OF1 mice (Charles River). In vivo transfection of mitoHyper or mitoSypHer within the flexor digitorum brevis of the animals, single fiber isolation, TMRM, and MitoSOX loading were performed as described in Pouvreau (2010). Bi-wavelength imaging of mitoHyper/mitoSypHer and MitoSOX was performed by line-interleaving excitation at 488 and 543 nm, and emission was collected at 505–545 and >560 nm, respectively. Regions of interest (ROI) were detected as described previously (Pouvreau, 2010) and correspond to areas of flashing mitochondria. All experiments and procedures were performed in accordance with the guidelines of the French Ministry of Agriculture (87/848), the European Community (86/609/EEC). (A) Time course of changes in normalized fluorescence of mitoHyper (top) and TMRM (bottom) within one ROI. (B and D) Time course of changes in normalized fluorescence of mitoHyper/mitoSypHer (top) and MitoSOX (bottom) within three ROI. (C and E) Fluorescence intensity of mitoHyper (C) or mitoSypHer (E) in a cell incubated with 1.5 or 2 mM of H₂O₂, respectively.

we can conclude from this set of experiments is that an increase in ROS production is simultaneous to the flashes. Thus, in light of these data, further work appears necessary to reevaluate the potential superoxide sensitivity of mitoSypHer.

Mt-cpYFP as a pH sensor? In a letter to the editor in the journal *Free Radical Biology and Medicine*, published a few months after the seminal article of Wang et al. (2008), Muller questioned the validity of mt-cpYFP as a superoxide probe, mostly on the basis of the peculiar pharmacology of the flashes (Muller, 2009), and proposed that mt-cpYFP detects ATP. The different points of this letter have been answered (Huang et al., 2011), and the hypothesis of the sensitivity of mt-cpYFP to ATP was discarded. We do believe nonetheless that the main question raised by Muller (2009), the unexpected effect of complex III inhibitor antimycin A on superoxide flash frequency, requires further investigations. Such a work might also provide new insights into the mechanisms underlying ROS production by the electron transport chain.

A second critical evaluation of the superoxide nature of the flashes was published by Schwarzländer et al. (2011). In their article, the authors claimed that the mt-cpYFP flashes are not superoxide flashes but pH flashes. The authors based their conclusion on (a) the absence of changes in global fluorescence intensity of mt-cpYFP upon manipulation of mitochondria O_2^- levels in their experimental model (isolated mitochondria preparation from the plant *Arabidopsis*), (b) the sensitivity of cpYFP to pH, and (c) the fact that manipulating mitochondrial pH affects the properties of superoxide flashes. With cpYFP being, just like several fluorescent proteins, sensitive to pH under physiological conditions (Nagai et al., 2001; Belousov et al., 2006; Wang et al., 2008), the real matter of discussion is whether mt-cpYFP is also detecting O_2^- . We and others demonstrated in mammalian cells that changes in the O_2^- content affect the properties of the flashes: the addition of antioxidants decreases the frequency of flashes (Wang et al., 2008; Pouvreau, 2010; Huang et al., 2011), whereas menadione, which mediates O_2^- release in mitochondria, and knocking down superoxide dismutase 2 increase flash frequency (Huang et al., 2011). Furthermore, Tiron treatment increased significantly the time to peak of mt-cpYFP flashes, which would be expected if flashes reflected transient increases in O_2^- (Pouvreau, 2010). Results relying on manipulation of mitochondrial pH should be carefully interpreted, as O_2^- production has been shown to be dependent on the pH gradient across the mitochondrial inner membrane (Lambert and Brand, 2004). Interestingly, no pH flashes have been reported by several groups studying mitochondrial pH using other probes: mt-EYFP (pKa = 7; see, for instance, Wang et al., 2008) and mitoAlpHi

(pKa = 8.5; see, for instance, Abad et al., 2004). Importantly, mt-cpYFP pKa is similar to that of mitoAlpHi (Nagai et al., 2001). Given the sensitivity of cpYFP to pH, it is possible that at least part of the increase in the mt-cpYFP fluorescence during a flash is caused by detection of changes in pH. However, further experiments are required to validate this hypothesis. Measurement of flashes simultaneously with cpYFP and a red-shifted chemically distinct pH-sensitive probe would be particularly informative.

Are we all looking at the same events?

Mt-cpYFP and mitoSypHer are both ratiometric probes (excitation wavelengths being 405/488 or 491 nm in the studies with mt-cpYFP, and 420/490 nm with mitoSypHer), and their pKa values are comparable: ≈ 8.6 for mt-cpYFP (Schwarzländer et al., 2011; Wei and Dirksen, 2012) and 8.71 for mitoSypHer (Poburko et al., 2011). Unfortunately, currently available data do not allow a fine comparison of the respective spectral behavior of the two probes, nor of their spectral response to pH versus O_2^- , as the spectra provided by the different groups have been acquired under different conditions (Belousov et al., 2006; Wang et al., 2008; Poburko et al., 2011; Schwarzländer et al., 2011). Data showing spectral properties of mt-cpYFP and mitoSypHer, as well as spectral response of the two probes to pH and O_2^- under the same conditions, would be paramount.

Based on their 2011 study stating that mt-cpYFP was in fact a pH sensor, Schwarzländer et al. (2012) published an article looking at spontaneous membrane depolarizations (pulsing) in mitochondria isolated from *Arabidopsis*, which they claim are accompanied by pH transients mirroring the kinetics of the depolarization transients, as well as mitochondrial Ca^{2+} transients. The authors suggest that the pH flash is a compensatory mechanism to depolarization. Interestingly, pulsing is affected by changes in ROS level. The transient events reported in this study differ from the ones reported in mammalian cells using mt-cpYFP (Wang et al., 2008, Pouvreau, 2010) or mitoSypHer (Azarias and Chatton, 2011) in several aspects. First, Wang et al. (2008), Pouvreau (2010), and Azarias and Chatton (2011) showed no change or a decrease in mitochondrial Ca^{2+} level during cpYFP transients. This discrepancy might be caused by the difference in experimental models (mammalian cells vs. isolated mitochondria from plants). Second, membrane depolarizations reported by Wang et al. (2008) and Pouvreau (2010) do not systematically mirror the mt-cpYFP flashes: they can be square-shaped or long-lasting, or in other words, of longer duration than the flashes. Here, we also report a long-lasting depolarization accompanying a mitoHyper flash (Fig. 1 A). Furthermore, Pouvreau (2010) showed that one third of the spontaneous mitochondrial depolarizations are devoid of flashes. Hence, the hypothesis

that cpYFP flashes are alkalinization events that preserve the proton-motive force during spontaneous mitochondria depolarizations seems to be questionable in our system. However, in keeping with the chemiosmotic theory, variations of pH gradient are expected during drops in mitochondrial membrane potential. Hence, pH variation during mitochondrial depolarization should be investigated using different pH probes.

Unfortunately, most of the data collected with mt-cpYFP or mitoHyper/SypHer have been acquired by different groups in different systems. To our knowledge, the results reported here are the first performed with these probes under the same conditions. Our data suggest that the two probes are detecting the same events, and that these events are concomitant with MitoSOX steps. Needless to say, this constitutes only a small step in regard to the work required to answer the questions: Are mt-cpYFP and mitoSypHer flashes reporting the same events? Are the events reflecting changes in pH or in O_2^- level? Further tests conducted by different groups in their different systems are necessary.

Conclusion

Although the current available literature provides convincing evidences that cpYFP acts as a superoxide sensor, the hypothesis that the reported “superoxide flashes” are at least partially caused by pH detection cannot be refuted. Indeed, the pH sensitivity of cpYFP, as well as of many other GFP-based sensors, is well established. Further studies are required to clarify the remaining concerns on the superoxide nature of the flashes. Unraveling the chemical nature and mechanisms of superoxide sensing by cpYFP should definitely resolve the disagreement. Beyond this controversy, the discovery of mitoSypHer/mt-cpYFP fluorescence transients provides exciting insights into the temporal and spatial functioning of mitochondria. The triggering of mitoSypHer flashes by localized decreases in ATP concentration has been shown (Azarias and Chatton, 2011), and could explain the well-defined localization of flashes among a background of quiescent mitochondria. MitoSypHer and mt-cpYFP flashes are accompanied by transient depolarization, whose mechanism is not unraveled yet. Although an opening of the mitochondrial permeability transition pore can explain this depolarization in cardiac myocytes (Wang et al., 2008), such a mechanism was not observed in skeletal muscle (Pouvreau, 2010). All these exciting questions will undoubtedly be the subject of future research studies.

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REFERENCES

- Abad, M.F.C., G. Di Benedetto, P.J. Magalhães, L. Filippin, and T. Pozzan. 2004. Mitochondrial pH monitored by a new engineered green fluorescent protein mutant. *J. Biol. Chem.* 279:11521–11529. <http://dx.doi.org/10.1074/jbc.M306766200>
- Azarias, G., and J.Y. Chatton. 2011. Selective ion changes during spontaneous mitochondrial transients in intact astrocytes. *PLoS ONE*. 6:e28505. <http://dx.doi.org/10.1371/journal.pone.0028505>
- Belousov, V.V., A.F. Fradkov, K.A. Lukyanov, D.B. Staroverov, K.S. Shakhbazov, A.V. Terskikh, and S. Lukyanov. 2006. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat. Methods*. 3:281–286. <http://dx.doi.org/10.1038/nmeth866>
- Bogdanov, A.M., A.S. Mishin, I.V. Yampolsky, V.V. Belousov, D.M. Chudakov, F.V. Subach, V.V. Verkhusha, S. Lukyanov, and K.A. Lukyanov. 2009. Green fluorescent proteins are light-induced electron donors. *Nat. Chem. Biol.* 5:459–461. <http://dx.doi.org/10.1038/nchembio.174>
- Huang, Z., W. Zhang, H. Fang, M. Zheng, X. Wang, J. Xu, H. Cheng, G. Gong, W. Wang, R.T. Dirksen, and S.S. Sheu. 2011. Response to “A critical evaluation of cpYFP as a probe for superoxide”. *Free Radic. Biol. Med.* 51:1937–1940. <http://dx.doi.org/10.1016/j.freeradbiomed.2011.08.024>
- Lambert, A.J., and M.D. Brand. 2004. Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem. J.* 382:511–517. <http://dx.doi.org/10.1042/BJ20040485>
- Muller, F.L. 2009. A critical evaluation of cpYFP as a probe for superoxide. *Free Radic. Biol. Med.* 47:1779–1780. <http://dx.doi.org/10.1016/j.freeradbiomed.2009.09.019>
- Nagai, T., A. Sawano, E.S. Park, and A. Miyawaki. 2001. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+} . *Proc. Natl. Acad. Sci. USA*. 98:3197–3202. <http://dx.doi.org/10.1073/pnas.051636098>
- Poburko, D., J. Santo-Domingo, and N. Demaurex. 2011. Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. *J. Biol. Chem.* 286:11672–11684. <http://dx.doi.org/10.1074/jbc.M110.159962>
- Pouvreau, S. 2010. Superoxide flashes in mouse skeletal muscle are produced by discrete arrays of active mitochondria operating coherently. *PLoS ONE*. 5:e13035. <http://dx.doi.org/10.1371/journal.pone.0013035>
- Santo-Domingo, J., and N. Demaurex. 2012. Perspectives on: SGP symposium on mitochondrial physiology and medicine. The renaissance of mitochondrial pH. *J. Gen. Physiol.* 139:415–423. <http://dx.doi.org/10.1085/jgp.201110767>
- Schwarzländer, M., D.C. Logan, M.D. Fricker, and L.J. Sweetlove. 2011. The circularly permuted yellow fluorescent protein cpYFP that has been used as a superoxide probe is highly responsive to pH but not superoxide in mitochondria: implications for the existence of superoxide ‘flashes’. *Biochem. J.* 437:381–387. <http://dx.doi.org/10.1042/BJ20110883>
- Schwarzländer, M., D.C. Logan, I.G. Johnston, N.S. Jones, A.J. Meyer, M.D. Fricker, and L.J. Sweetlove. 2012. Pulsing of membrane potential in individual mitochondria: a stress-induced mechanism to regulate respiratory bioenergetics in Arabidopsis. *Plant Cell*. 24:1188–1201. <http://dx.doi.org/10.1105/tpc.112.096438>
- Wang, W., H. Fang, L. Groom, A. Cheng, W. Zhang, J. Liu, X. Wang, K. Li, P. Han, M. Zheng, et al. 2008. Superoxide flashes in single mitochondria. *Cell*. 134:279–290. <http://dx.doi.org/10.1016/j.cell.2008.06.017>
- Wei, L., and R.T. Dirksen. 2012. Perspectives on: SGP symposium on mitochondrial physiology and medicine. Mitochondrial superoxide flashes: from discovery to new controversies. *J. Gen. Physiol.* 139:425–434. <http://dx.doi.org/10.1085/jgp.201210790>