# The 'mitoflash' probe cpYFP does not respond to superoxide

ARISING FROM E.-Z. Shen *et al. Nature* **508,** 128–132 (2014); doi:10.1038/nature13012

Ageing and lifespan of organisms are determined by complicated interactions between their genetics and the environment, but the cellular mechanisms remain controversial; several studies suggest that cellular energy metabolism and free radical dynamics affect lifespan, implicating mitochondrial function. Recently, Shen *et al.*[1] provided apparent mechanistic insight by reporting that mitochondrial oscillations of 'free radical production', called 'mitoflashes', in the pharynx of three-day old *Caenorhabditis elegans* correlated inversely with lifespan. The interpretation of mitoflashes as 'bursts of superoxide radicals' assumes that circularly permuted yellow fluorescent protein (cpYFP) is a reliable indicator of mitochondrial superoxide[2], but this interpretation has been criticized because experiments and theoretical considerations both show that changes in cpYFP fluorescence are due to alterations in pH, not superoxide[3–7]. Here we show that purified cpYFP is completely unresponsive to superoxide, and that mitoflashes do not reflect superoxide generation or provide a link between mitochondrial free radical dynamics and lifespan. There is

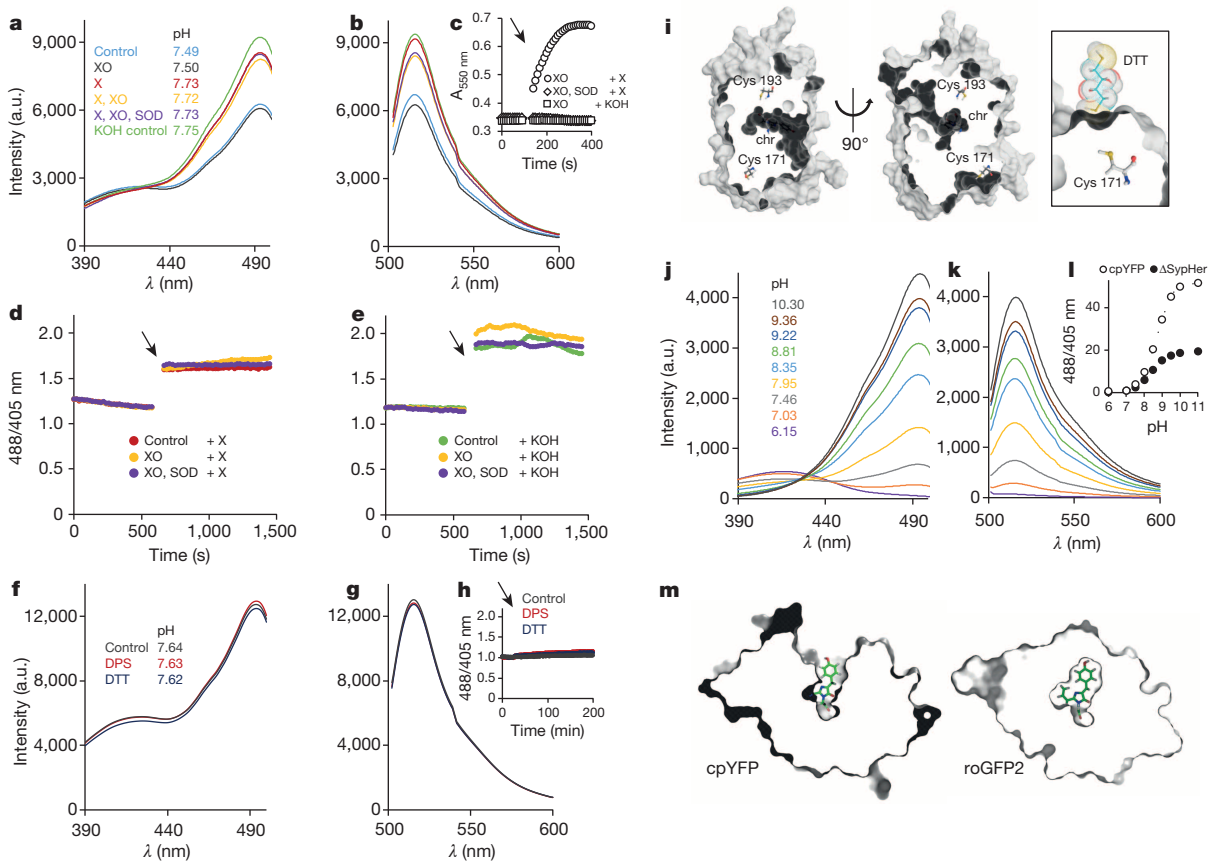


**Figure 1 | Spectroscopic and structural analysis of the 'mitoflash' probe cpYFP.** **a**, **b**, cpYFP fluorescence excitation spectra (emission at 515 nm) (**a**) and emission spectra (excitation at 488 nm) (**b**) after addition of xanthine (X; 2 mM), xanthine oxidase (XO; 100 mU ml$^{-1}$), bovine Cu/Zn superoxide dismutase (SOD; 600 U ml$^{-1}$) and KOH (solvent control for xanthine; note pH increase), and the *in situ* pH of the assayed 200 µl reaction mix. a.u., arbitrary units. **c**, Cytochrome *c* reduction detected by absorption at 550 nm to measure superoxide generation in response to the X/XO system in the presence and absence of SOD, and in response to KOH as a solvent control for X. Cytochrome *c* 100 µM; arrow indicates X or KOH addition. **d**, The response of cpYFP excitation ratio (488/405 nm) to superoxide generation. Arrow indicates introduction of X to constitute the X/XO system. Controls contained either SOD addition or no XO. **e**, The same assays were performed with KOH introduction as solvent control for X. **f**, **g**, cpYFP fluorescence excitation spectra (**f**) and emission spectra (**g**) after 24 h incubation with dithiothreitol (DTT; 10 mM) and 2,2'-dipyridyl disulphide (DPS; 1 mM), and the *in situ* pH of the assayed 200 µl reaction mix. **h**, cpYFP fluorescence ratio after DTT and DPS addition over 3 h; arrow indicates DTT or DPS addition. **i**, Cross-sections through a surface model of cpYFP (left and middle). Chromophore (chr) and cysteine residues are represented as ball-and-stick models. The Cys 171 thiol is relatively close to the protein surface, but unlikely to be accessible to solutes as indicated by the docking of a DTT molecule to the protein surface (right). **j**, **k**, cpYFP fluorescence excitation spectra (emission at 515 nm) (**j**) and emission spectra (excitation at 488 nm) (**k**) in response to pH as determined *in situ* after the measurements. **l**, pH dependence of cpYFP excitation ratio (488/405 nm; normalized to pH 7.0) as compared to the cpYFP part of the pH sensor SypHer (ΔSypHer). **m**, Sectional views through volume models of cpYFP and redox-sensitive GFP (roGFP2), with the clipping plane parallel to and just above the chromophore phenoxy ring. The chromophore is represented as a ball-and-stick model. Data in **a**–**h** and **j**–**l** are background corrected, and experiments were repeated at least five times with consistent results.

a Reply to this Brief Communication Arising by Cheng, H. *et al.* *Nature* **514,** http://dx.doi.org/10.1038/nature13859 (2014).

We carried out experiments with purified recombinant cpYFP sensor protein to test whether it responds to superoxide (Fig. 1a–e). Exposure of cpYFP to a superoxide-generating system (xanthine (X) and xanthine oxidase (XO)) slightly changed the excitation and emission spectra. However, the same change occurred when cpYFP was incubated with the individual assay constituents in the absence of superoxide production, or when Cu/Zn superoxide dismutase (SOD) was added to degrade superoxide (Fig. 1a, b). The cytochrome *c* reduction assay confirmed that superoxide is produced by the X/XO system, and is abolished by SOD (Fig. 1c). Xanthine is dissolved in potassium hydroxide, causing a small increase in pH after addition. There was an excellent correlation between spectral changes and resulting assay pH after xanthine (that is, potassium hydroxide) addition (Fig. 1a, b). In time course assays in which superoxide generation was started by the addition of xanthine (Fig. 1d), the addition of potassium hydroxide as the solvent control for xanthine (Fig. 1e) gave the same increase in fluorescence ratio. Extended reductive or oxidative treatment with thiol redox agents (the reducing agent dithiothreitol (DTT) and oxidizing agent 2,2'-dipyridyl disulphide (DPS)) did not alter the spectral behaviour (Fig. 1f–h), consistent with structural information suggesting that both Cys residues are buried inside the mature protein and are unlikely to be accessible for thiol redox chemistry (Fig. 1i). Likewise, reductive pre-treatment with DTT under inert atmosphere, followed by DTT removal, did not affect the outcome of the superoxide assays. Further variation of experimental variables, including pre-incubation conditions, pH buffer systems and a 100-fold range of sensor concentrations, did not lead to any rapid, reversible change in cpYFP sensor signal required for superoxide-related mitoflashes, as long as the pH and halide ion concentrations were kept constant.

Mitoflashes can be fully explained by the extraordinary pH sensitivity of cpYFP, which has a p$K_a$ value of ~8.7 (determined by measuring fluorescence after excitation at 488 nm, the wavelength at which flashes are observed) and shows a >50-fold change in fluorescence ratio between pH 7 and 10, similar to the structurally related ratiometric pH-sensor SypHer[8] (Fig. 1j–l). In the mitochondrial matrix, a resting pH (~7.9) close to sensor p$K_a$ and a limited pH buffering capacity mean that even minor perturbations will elicit a pronounced sensor response (Fig. 1a, b, d, e). The cpYFP pH sensitivity is due to the structural perturbation caused by the circular permutation. A large cleft in the β-barrel exposes the pH-active phenoxy group of the chromophore (Fig. 1m, left), which is concealed in non-permuted green fluorescent protein (GFP)-based biosensors (Fig. 1m, right).

On the basis of this evidence using purified cpYFP and earlier studies in cells and isolated mitochondria[5,6,9], the mitoflash phenomenon cannot be attributed to bursts of mitochondrial superoxide. In accordance with the pH responsiveness of the probe, recent work with different sensors suggests that mitoflash events indicate brief periods of alkalinisation in individual mitochondria, possibly as a result of acceleration in proton pumping, triggered by mitochondrial fusion initiation and/or a change in ion homeostasis[6,9,10].

The debate about the nature of mitoflashes has focused on *in situ* evidence that has left space for interpretation on both sides. Critics have pointed out the implausibility of 'superoxide flashes' on the basis of mitochondrial energetics[3,5,9], the absence of a plausible chemical mechanism for the reversible interaction between cpYFP and superoxide[4,7], and the fact that the pH sensor SypHer also detects mitoflashes[6,10]. These arguments have been countered by data suggesting a correlation of mitoflashes with the response of chemical probes for reactive oxygen species[11–13], the notion that the pH probe SypHer may also respond to superoxide[14], and the suggestion that a mitoflash represents a mixture of superoxide burst and pH transient[11,13]. Ultimate resolution of the debate has been hampered by the use of different biological systems and the complexity of mitochondrial physiology, where matrix pH and free radical release are connected by the electron transport chain and linked to several other parameters such as availability of respiratory substrates, membrane potential, redox and ion homeostasis, and mitochondrial morphology[2,5–7,10,15–17]. Here we resolve the controversy by a thorough analysis of the fundamental properties of the mitoflash sensor cpYFP. Previous work already excluded the suggestion that the pH probe SypHer responds to superoxide[6]. We now provide definitive evidence that cpYFP itself does not respond to superoxide and that flashes recorded by cpYFP do not represent superoxide bursts. Of course, sudden changes in mitochondrial physiology may still include altered free radical levels. Although the mitoflash phenomenon may reflect an important feature of mitochondrial function that deserves further mechanistic analysis, the interpretation of the events by Shen *et al.* lacks a biophysical foundation and mitoflashes cannot serve as evidence for free radical involvement in determining lifespan.

## Methods

cpYFP was purified from *Escherichia coli* Origami (DE3) and Rosetta 2 (DE3) 24 h after induction at 20 °C and assayed at 10, 25 and 1,000 μg ml$^{-1}$ using a Jasco spectrofluorimeter FP8300 and a BMG Labtech Clariostar plate reader. Detector gain was adjusted for individual experiments. Buffers contained 100 mM NaCl, 1 mM $Na_2EDTA$ and 100 mM Tris-HCl, pH 7.5 (for thiol redox and superoxide assays; degassed and under argon for thiol redox treatments) or 100 mM Tris-TES (for pH assays). All reagents were dissolved in assay buffer, except for xanthine (100× stock in 1 M KOH, base required for solubility) and xanthine oxidase (118× $(NH_4)_2SO_4$ suspension as delivered by Sigma). Protein structures (PDB entries 3O78 and 1JC1) were rendered using PyMOL.

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**Author Contributions** M.S., A.J.M., T.P.D. and M.P.M. conceived the project. M.S., S.W., Y.G.E. and V.V.B. performed spectroscopic experiments. S.W. and T.P.D. performed modelling of protein structures. All authors contributed to experimental design, discussed the results, and wrote the manuscript.

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# Cheng *et al.* reply

**REPLYING TO** M. Schwarzländer *et al. Nature* **514,** http://dx.doi.org/10.1038/nature13858 (2014)

In the accompanying Comment[1], Schwarzländer *et al.* challenged our recent study[2] because they failed to reproduce our previous finding that the fluorescence intensity of purified circularly permuted yellow fluorescent protein (cpYFP) increases in response to oxygen and superoxide anions produced by xanthine (X) plus xanthine oxidase (XO)[3]. Starting from a 'fully reduced' state (incubation with 10 mM dithiothreitol for >3 h) and in the presence of 75 mM HEPES, we demonstrated that cpYFP exhibits a twofold fluorescence increase after oxygenation, and an additional twofold increase after the subsequent addition of X plus XO, which could not be accounted for by solvent (potassium hydroxide)-induced alkalization. Furthermore, the xanthine plus xanthine oxidase-induced increase in cpYFP fluorescence was reversed by Cu/Zn superoxide dismutase (600 U $ml^{-1}$). We also found that the fluorescence intensity of fully reduced cpYFP increased >fourfold after incubation with 1 mM aldrithiol. Notably, recombinant cpYFP purified in the absence of dithiothreitol treatment exhibits a high fluorescence comparable to that of the fully oxidized state, indicating the high susceptibility of cpYFP to oxidation in non-reducing environments[3]. Therefore, ensuring a fully reduced state of cpYFP is essential for the probe to sense superoxide *in vitro*. This property is probably the reason that the probe functions readily as a reversible superoxide biosensor when targeted to the reduced environment of the mitochondrial matrix. Unfortunately, from the brief description of the methods and limited data provided by Schwarzländer *et al.*[1], it is not possible to determine whether cpYFP was fully reduced in their experiments, or whether sufficient precautions were taken to prevent oxidation of the probe. Moreover, in our experiments cpYFP was expressed in *Escherichia coli* BL21(DE3)LysS cells, whereas Schwarzländer *et al.*[1] used *E. coli* Origami, a *trxB* (thioredoxin reductase) mutant strain that also lacks glutathione reductase needed to fully limit cysteine oxidation[4], which could result in an increased oxidative status of their purified cpYFP rendering it non-responsive to superoxide.

Our data from intact cells demonstrate that, in addition to increasing mitoflash frequency, aldrithiol and menadione application also markedly increases basal cpYFP fluorescence intensity within the mitochondrial matrix[2,5]. In addition, nanomolar concentrations of nigericin, a $H^+/K^+$ antiporter, stimulates mitoflash activity[6]. These responses of cpYFP *in situ* are unlikely to be attributable to mitochondrial alkalization. We also found that the temporal profile of mitoflash events do not always mirror the change of the mitochondrial membrane potential in

cardiac and skeletal muscle cells[6,7], and this contradicts the suggestion that mitoflashes simply reflect increased proton pumping in response to membrane potential depolarization[8–10].

Until now, structural information about how cpYFP senses superoxide remains a mystery. In a unique class of enhanced green fluorescent protein (eGFP)-based calcium sensors, a reversible deprotonization of the chromophore occurs owing to calcium binding to a negatively charged site on the probe[11]. We are investigating whether a similar mechanism might underlie the reversible superoxide-sensing chemistry of cpYFP.

Despite the technical issue raised by Schwarzländer *et al.*[1], the existence of bursts of superoxide or reactive oxygen species (ROS) production in respiring mitochondria has been confirmed by several independent investigators using different probes. Two pH-insensitive, ROS probes, MitoSOX and 2′,7′-dichlorodihydrofluorescein diacetate, have validated ROS increases during cpYFP-reported 'flashes'[6,12,13]. When used individually to avoid possible fluorescence resonance energy transfer (FRET) effects and spectral cross-contamination, these pH-insensitive ROS sensors confirmed flash events of nearly identical frequency and spatiotemporal properties as that observed for cpYFP[13]. Quantification of the respective contributions of superoxide and pH to mitoflash events showed that a predominant superoxide component is coincident with a modest alkalization of the mitochondrial matrix in muscle cells[6]. Similarly, a previous report used MitoSOX to confirm bursts of superoxide during pH alkalization events in primary astrocytes transfected with the fluorescent pH-sensor mitoSypHer[14]. A recent report, which is co-authored by two authors of the accompanying Comment by Schwarzländer *et al.*[1], used roGFP2 to detect spontaneous, short-lived oxidative bursts that are accompanied by mitochondrial depolarization, transient matrix alkalization, and reversible mitochondrial 'contractions'[15], all of which we previously documented for cpYFP mitoflashes. Furthermore, in many cell types and tissues[3,5,12,13,16,17] and even in living animals[2], mitoflash activity is increased by oxidants (including menadione and paraquat) and reduced by antioxidants (including mitoTEMPO and SS31). Nevertheless, given the extreme diversity and plasticity of the mitochondria proteome[18], the relative contributions of superoxide and pH to cpYFP-reported mitoflash events may vary in a species-, cell-type- and context-dependent manner.

It has become increasingly appreciated that mitoflash activity is a complex phenomenon, comprising multifaceted and intertwined mitochondrial processes including quantal superoxide production, membrane depolarization, membrane permeability transition, NADH depletion, matrix alkalization and swelling that masquerades as mitochondrial contraction[3,6,14,15,17,19]. Ample evidence supports the notion that mitoflash activity serves as a novel and universal "frequency-coded optical readout reflecting free-radical production and energy metabolism at the single- mitochondrion level"[2]. The continuing debate on what drives, controls and contributes to these events does not change the fact that mitoflashes reflect a fundamental physiological phenomenon linked to energy metabolism and stress response, nor does it discount the significance of our finding that mitoflash frequency predicts lifespan at a very early age in *Caenorhabditis elegans*[2].

The Comment by Schwarzländer *et al.*[1] focuses exclusively on the controversy of cpYFP as a superoxide sensing probe, which was originally demonstrated in several publications by Wang, Dirksen, Sheu and Cheng, and therefore these authors were invited to respond to the Comment. M.-Q. Dong and 11 authors of the original paper[2] were not involved in the research that led to the discovery of cpYFP as a superoxide probe, so are not listed as authors (M.-Q. Dong was included in this Reply as a corresponding author).

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