ANTIOXIDANTS & REDOX SIGNALING
Volume 25, Number 9, 2016
Mary Ann Liebert, Inc.
DOI: 10.1089/ars.2016.6819

LETTER TO THE EDITOR

Mitochondrial Flashes: Dump Superoxide and Dance with Protons Now

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Abstract

Transient changes in the physiology of individual mitochondria have recently drawn much interest. The use of a circular permuted yellow fluorescent protein (cpYFP) to monitor *mitochondrial flashes* and their interpretation as *superoxide bursts* has added confusion, however. Reviewing mitochondrial flashes in this Forum, Wang *et al.* again deem cpYFP to be a specific and reversible superoxide indicator, dismissing evidence that purified cpYFP is insensitive to superoxide. This interpretation lacks reproducible evidence and conflicts with the parsimony principle. We offer a constructive, transparent pathway to reach definitive clarification of contradictory reports. *Antioxid. Redox Signal*. 25, 550–551.

Dear Editor:

EXTRAORDINARY CLAIMS REQUIRE extraordinary evidence (1). Claiming that a fluorescent protein is selectively and reversibly modified by superoxide and reports "*superoxide flashes*" in living tissues as Wang *et al.* reiterate in this Forum (4), is extraordinary. Unfortunately the evidence is quite ordinary.

A molecular mechanism of superoxide sensitivity is lacking and chemically implausible given the absence of free-radical traps and supporting structural data. Direct experimental evidence for superoxide sensitivity is limited to a few fluorescence spectra of purified recombinant circular permuted yellow fluorescent protein (cpYFP), showing approximately twofold increase upon oxygenation and another approximately twofold after subsequent addition of a superoxide-generating system unless superoxide dismutase is present (3). The pH, pO_2, redox potential, and structural states of cpYFP before and after oxygenation and superoxide generation were not measured.

Scientific experiments must be reproducible. In no instance has the fluorescence response of purified cpYFP to superoxide been reproduced independently. In our hands, the spectroscopic properties of cpYFP are insensitive to oxidants regardless of pH, atmospheric composition, incubation time, and reducing pretreatment. Instead, cpYFP showed highly pH-sensitive, redox-resistant fluorescence (2). Wang *et al.* attribute this *disconnect* (5) to potential differences in cpYFP sequence and *Escherichia coli* strains, although sequence identity was verified and several strains consistently gave negative results. Their superoxide assay starts after oxygenation, yet they insist that *cpYFP must be fully reduced to reveal its response to oxidation and superoxide* (5), implying that a protein reversibly and repeatedly modified by superoxide in cells must be fully reduced to reveal its superoxide sensitivity when purified. The disturbing truth is that in >8 years, nobody has been able to obtain graded and reversible cpYFP responses to superoxide. The extraordinary evidence is lacking and the ordinary evidence is not reproducible.

Wang *et al.* initially dismissed any pH contribution to cpYFP flashes despite reporting ~10-fold cpYFP fluorescence increase between neutral and alkaline pH [see Fig. S1f in Ref. (3)]. They now reinterpret cpYFP flashes as *multifaceted signals including a superoxide or ROS signal and an alkalinisation signal* (5). This belatedly acknowledges flashes as pH events but adds new confusion by blending the invisible superoxide with undefined reactive oxygen species

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(ROS), despite their evidence that cpYFP fluorescence is unaffected by various reactive species (3). It is likely that pH flashes affect mitochondrial ROS dynamics, but this cannot be assessed with probes blind to reactive species.

Mitochondrial flashes are fascinating biological events. But to understand their origin we must know what we measure. The review by Wang *et al.* rehashes a controversy causing researchers to misuse the sensor and to misinterpret flashes. Although the burden of proof lies with the claimants of the extraordinary, we offer our constructive help to jointly test the explicit hypothesis that mature, purified cpYFP can respond to the acute generation of superoxide *in vitro* by a pronounced change of its fluorescence properties, in a rapid, rapidly reversible, specific, and reproducible manner. The yes/no outcome of these experiments, performed under each other's rigorous supervision, must be reported transparently.

References

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Date of first submission to ARS Central, July 9, 2016; date of acceptance, July 11, 2016.

Abbreviations Used

cpYFP = circular permuted yellow fluorescent protein
ROS = reactive oxygen species