Mitochondrial ‘flashes’: a radical concept repHined

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Mitochondrial free radicals and redox poise are central to metabolism and cell fate. Their measurement in living cells remains a major challenge and their in vivo dynamics are poorly understood. Reports of ‘superoxide flashes’ in single mitochondria have therefore been perceived as a major breakthrough: single mitochondria expressing the genetically encoded sensor circularly permuted yellow fluorescent protein (cpYFP) display spontaneous flashes of fluorescence that are responsive to metabolic changes and stressors. We critically review the evidence that underpins the interpretation of mitochondrial cpYFP flashes as bursts of superoxide production and conclude that flashes do not represent superoxide bursts but instead are caused by transient alkalisation of the mitochondrial matrix. We provide a revised framework that will help to clarify the interpretation of mitochondrial flashes.

Mitochondrial free radical dynamics in living cells
Mitochondrial bioenergetic and redox status are central to energy metabolism and the determination of cell fate, and mitochondrial dysfunction is implicated in a wide range of pathologies such as cancer and cardiovascular and neurodegenerative diseases [1]. Despite its fundamental importance, mitochondrial biochemistry continues to be difficult to characterize in living tissues. However, state-of-the-art imaging approaches are, literally and metaphorically, bringing new light to the subject. Key hallmarks of mitochondrial function such as membrane energisation, redox poise, calcium load, and the generation of free radicals have become experimentally accessible due to technical advances in the design of fluorescent sensors [2–7]. Mitochondria are a major intracellular source of free radicals and other reactive oxygen species (ROS). ROS generation causes mitochondrial oxidative damage and is critical for mitochondria’s central role in cell death and senescence.

Glossary
Circularly permuted yellow fluorescent protein (cpYFP): a yellow fluorescent protein that has been circularly permuted in its sequence (i.e., the N and C termini are fused via a linker and new termini are generated by cleavage in a central area of the protein). It is the basis for several genetic sensors that were constructed by inserting protein sequences between the termini that can bind a substrate and alter spectrophotometric properties in response [8,23]. In the absence of additional substrate-binding domains, cpYFP was proposed to act as a superoxide sensor [8].
Enhanced yellow fluorescent protein (EYFP): derived from green fluorescent protein (GFP) by mutagenesis that causes increased brightness and a shift in excitation and emission towards longer wavelengths.
Mitochondrial electron transport chain (mtETC): the large protein complexes located in the inner mitochondrial membrane that couple oxidation of substrates to ATP synthesis via the generation of mtPMF. Complexes I–IV facilitate electron flux from reduced substrates to oxygen while driving proton translocation across the membrane. Complex V harnesses the generated mtPMF for ATP synthesis.
Mitochondrial permeability transition pore (mtPTP): a large, nonspecific pore in the inner mitochondrial membrane that can open under various conditions including calcium overload and oxidative stress, leading to equilibration of molecules of less than 1.5 kDa between the matrix and the cytosol. mtPTP opening is often associated with cell death.
Mitochondrial proton-motive force (mtPMF): the proton electrochemical potential gradient that is established by mtETC-dependent proton pumping across the inner mitochondrial membrane. It comprises an electrical component, \( ΔΨ_m \), and a chemical component, the pH gradient (\( ΔpH \)). Under normal conditions, \( ΔΨ_m \) is the major contributor to the mtPMF.
Nigericin: an ionophore that acts as a specific potassium–proton exchanger in biological membranes. In the presence of high potassium concentrations, nigericin can be used to collapse \( ΔΨ_m \) and clamp the inner to outer proton concentration ratio. Because potassium–proton exchange is charge neutral, the membrane potential (\( ΔΨ_m \)) is preserved.
Reactive oxygen species (ROS): collective name for various chemical compounds with diverse properties that contain oxygen in an intermediate reduction state making them highly reactive. Biologically prominent ROS include the superoxide radical, hydrogen peroxide, the hydroxyl radical, and singlet oxygen.
Superoxide radical (\( O_2^- \)): produced by a single electron reduction of molecular oxygen. In the cell, single electron transfers can arise from reduced protein metal redox centres that are accessible to oxygen; including those in complex I and III of the mtETC. Superoxide reacts readily with transition metal compounds and other radicals. Two superoxide molecules spontaneously dismutate to generate hydrogen peroxide and oxygen. This is catalysed by SOD in mitochondria, limiting half-life to a few microseconds and diffusion distance to <1 μm [22].
However, ROS have been particularly difficult to measure in vivo almost by definition, given their short half-lives and broad reactivity. With this in mind there appeared to be a major serendipitous breakthrough when, in 2008, a mitochondria-targeted cpYFP (mt-cpYFP) (see Glossary) was reported to be a fluorescent sensor of superoxide [8].

Superoxide flashes in single mitochondria?
Using this probe, brief flashes of fluorescence from mt-cpYFP in single mitochondria were reported in vivo. These were interpreted as short bursts of increased superoxide release and named ‘superoxide flashes’. Although highly variable in amplitude and duration, flashes appeared as an approximately 40% increase in fluorescence signal on average and lasted for tens of seconds (Figure 1). Although initially observed in cardiac myocytes, superoxide flashes were also reported from many other tissues [8–10]. The occurrence of flashes was correlated with stimulation of metabolism or stress such as reperfusion after anoxia. The mechanism behind these flashes was suggested to involve transient opening of the mitochondrial permeability transition pore (mtPTP), thereby triggering a burst of superoxide generation by the mitochondrial electron transport chain (mtETC). These observations seem fundamental for the understanding of mitochondrial function and there have been several follow-up reports using the sensor and dissecting the characteristics of superoxide flashes [9–21]. However, we suggest that the phenomenon of superoxide flashes requires reappraisal to ensure accuracy and relevance to future research efforts focused on understanding mitochondrial function in living tissues.

The cpYFP sensor is unlikely to respond to superoxide
The interpretation of fluorescence flashes from the cpYFP sensor as superoxide bursts would necessitate that the cpYFP probe has a high degree of specificity to superoxide. Superoxide sensitivity requires an underlying molecular interaction between cpYFP and superoxide; reversible ‘binding’ of superoxide appears unlikely due to the lack of potential receptors on cpYFP. More probably, superoxide would need to react with cpYFP to modify its structure and change its fluorescence properties. Moreover, the reaction between superoxide and cpYFP would need to be highly favourable due to competition from superoxide dismuta- tion, which is an extremely efficient reaction in the mitochondrial matrix due to the presence of superoxide dismutase (SOD) (second order rate constant \(10^{9} \text{M}^{-1} \text{s}^{-1}\)) [22]. However, in contrast to these expectations, the cpYFP chromophore environment does not exhibit any chemical features that lend themselves to any currently known specific modification by superoxide. Although superoxide readily undergoes single electron transfer reactions, either with radical compounds [such as itself or nitric oxide (NO)] or with redox active metals [such as iron–sulfur clusters or the manganese centre of mitochondrial SOD], its reactivity with most organic compounds, including proteins, is thought to be low.

The cysteine residues of cpYFP (Cys171 and Cys193) were proposed to interact with superoxide because cysteine-null variants showed no fluorescence changes on oxidation by aldrithiol [8]. However, Cys193 (corresponding to Cys70 in enhanced yellow fluorescent protein (EYFP) and enhanced green fluorescent protein (EGFP) [23]) is essential for fluorescent protein expression in the first place [5], explaining both the lack of fluorescence response and the very low baseline fluorescence reported for the cysteine-null variants in cardiac myocytes. Although a reaction between superoxide and a cysteine thiol is chemically possible, such reactions are unlikely to be important in cellular environments because their rate is \(10^6\) to \(10^8\)-fold slower than that of superoxide with SOD [24]. Assuming the theoretical possibility that a reaction between a thiol on cpYFP and superoxide could occur to form a thiol radical or a sulfenic acid, protein fluorescence could be modified as a result. A burst of superoxide generation might then be expected to cause a step-like change in the fluorescence of a sensor. To explain the observed reversible response of cpYFP, a hypothetical superoxide-induced modification would also need to be fully reversible. Cysteine modifications such as those mentioned above could in principle be reversed by intramitochondrial thiols. However, although this scenario could be invoked to explain the observed reversible response in mitochondria and cells, such hypothetical superoxide-induced modifications have not been found. More fundamentally, the cpYFP fluorescence increase that has been reported in response to a superoxide-producing system is reversible in vitro in the absence of any thiol reducing system [8], suggesting that reversible oxidation of protein thiols by superoxide is not involved in the changes of fluorescence. Our opinion is that, based on our current knowledge, it is chemically implausible that superoxide is responsible for the fluorescence flashes observed using cpYFP in single mitochondria.

![Figure 1. Mitochondrial 'flashes'. The fluorescent protein sensor circularly permuted yellow fluorescent protein (cpYFP) expressed in the mitochondrial matrix undergoes spontaneous and transient increases in fluorescence in single mitochondria [8]. These flashes are commonly interpreted as bursts of mitochondrial superoxide generation.](image-url)
The experimental evidence for the sensitivity of cpYFP to superoxide is based largely on an *in vitro* assay in which purified cpYFP responded to a superoxide-generating system with an approximately fourfold increase in fluorescence [8]. Assay fluorescence increased on addition of xanthine and xanthine oxidase, which generates superoxide, hydrogen peroxide, and uric acid. This was partially reversed by subsequent addition of SOD. As discussed above, the reversibility of this response suggests that factors other than superoxide are likely to be responsible for the changes in fluorescence. These may include spectroscopic effects of the xanthine–xanthine oxidase system with cpYFP or pH changes caused by the addition of substrates in different buffers. For these reasons, this experiment provides only weak evidence that cpYFP is sensitive to superoxide *in vitro*; more carefully controlled experiments with the purified sensor protein would be required. We do not exclude the possibility that cpYFP could be modified by superoxide, but proof that this occurs is lacking and the mechanism by which it could occur would be chemically interesting.

Although the pH dependence of the sensor has been noted, any contribution from changing matrix pH to the observed cpYFP dynamics in single mitochondria in living tissues was excluded based on a control assay using mitochondrial-targeted EYFP that did not show similar dynamics to cpYFP. Unfortunately, because the $pK_a$ values of cpYFP (~8.6 [25]) and EYFP (~7.1 [26]) are very different, this control was inappropriate. Given a usual resting mitochondrial matrix pH of approximately 7.6–8.0 [27], EYFP is too insensitive to increases in pH, whereas cpYFP will be highly responsive in a fully reversible fashion (Figure 2).

We believe that there is a lack of adequate theoretical and experimental evidence to support the conclusion that cpYFP functions as a superoxide sensor.

The pH sensitivity of cpYFP explains mitochondrial flashes

The pH sensitivity of cpYFP has been reported as a confounding factor in the interpretation of mitochondrial flashes in several publications [2,16,25,28–31] (Figure 2 and Box 1). Crucially, clamping matrix pH of isolated respiring mitochondria with nigericin, increasing matrix pH buffering by phosphate loading, or limiting proton pumping capacity by the mtETC all suppressed these flash events (Figure 3), strongly suggesting that the mitochondrial cpYFP flashes in fact report transient changes in mitochondrial matrix pH [25,31]. The mitochondria-targeted pH sensor SypHer which, in contrast to EYFP, has a $pK_a$ similar to that of cpYFP [25,32], displayed similar flashes in astrocytes and HeLa cells, independently confirming the occurrence of mitochondrial pH transients [33,34]. It is worth noting that the fluorescent protein part of the SypHer sensor is a cpYFP, making it likely that similarity in the critical parts of the probe structure accounts for the similar pH properties of the two sensors [6,32]. The theoretical possibility of artefactual superoxide sensitivity of SypHer, although improbable for the reasons stated above, can in principle be tested by further characterisation of the sensor as well as controlled for by using a chemically distinct probe for mitochondrial pH.

Two recent studies have also shown that mitochondrial flashes coincide with cation influx into the mitochondrion [31,33]. This causes a transient decrease in membrane potential ($\Delta V_m$) and an increase in the rate of electron flux through the mtETC and associated proton pumping, driving transient matrix alkalinisation. Such a situation, referred to as ‘respiratory state 6’ in the early days of mitochondrial bioenergetics [35], provides a compelling explanation for how transient matrix alkalinisation events may occur and be conditionally modulated by matrix buffering and mtETC function (Figure 4).

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**Figure 2.** The pH response of circularly permuted yellow fluorescent protein (cpYFP) and enhanced yellow fluorescent protein (EYFP) in mitochondria prevents detection of pH transients by EYFP. (a) Fluorescence changes in cpYFP ($pK_a = 8.6$, black dot) and EYFP ($pK_a = 7.1$, black dot) in response to pH. In the pH range of the mitochondrial matrix (highlighted in grey), alkalinisation (highlighted in blue) causes a strong increase in the fluorescence of cpYFP but not of EYFP. Broken horizontal lines indicate the baseline fluorescence of EYFP (top) and cpYFP (bottom) at matrix pH 7.9 [26,27]. On average, a ‘flash’ as reported in the literature [8] causes a 40% increase in the cpYFP signal, which is schematically represented in (b). Such a response can be triggered by a pH increase of <0.2, considering the pH behaviour of cpYFP at a matrix pH of 7.9. The same pH shift causes a 5% increase in the EYFP signal, which is likely to be lost in noise in an experiment, depicted by overlaying the graphs from (b) with simulated noise (d). For the brightest 10% of flashes, a 100% increase of cpYFP fluorescence was found [8], which is represented schematically in (d). This can be triggered by a pH increase of <0.4, which is equivalent to a 5% increase in the EYFP signal, which is still unlikely to be discriminated from noise (e) under experimental conditions. Noise is represented as a Box–Muller transform of uniformly distributed random numbers.
Box 1. The effect of pH on fluorescent proteins

Sensitivity to pH is an intrinsic property of fluorescent proteins; fluorescence intensity changes depend on the protonation status of the chromophore. The impact of pH on fluorescence is particularly close to the pKₐ of the chromophore, whereas pH changes have little effect in pH ranges distant from the pKₐ. When expressed in the cell, the fluorescence of the protein is determined by its pKₐ in relation to the local pH of the surrounding medium. This can lead to quenching of common GFP variants (pKₐ ~5-6) in acidic compartments, whereas red fluorescent protein (RFP) fluorescence can be largely unaffected (pKₐ ~4). In neutral compartments, GFP is pH stable, whereas YFP (pKₐ ~7) reaches this stability in more alkaline ranges.

These properties become particularly critical when protein fluorescence intensity serves as a quantitative measure. The pH response of the different fluorescent proteins can be exploited to determine the subcellular pH of different subcellular compartments. Fluorescent protein probes designed to sense other physiological parameters require careful characterisation of their pH properties, however, because pH-dependent fluorescence changes can be many times stronger than the specific dynamic response range of the sensor [2,6,23]. Ideally, the readout of a protein sensor is largely pH insensitive (e.g., due to internal compensation for pH effects [38,40] or the application of the sensor in a pH environment distant from the pKₐ). However, if measurements in the pH dynamic range of the pKₐ are required, several different strategies have been used to separate out pH sensitivity, including the use of independent pH sensors for correction [3,41], the attachment of the sensor to a pH probe [42], or even the removal of pH sensitivity by a mutagenesis screen [43]. For the hydrogen peroxide sensor HyPer [6], an elegant approach to generate a pH control probe of matching pKₐ [32] involved mutating a single cysteine residue to abolish its H₂O₂ response and lock the protein in a state similar to the reduced form of HyPer, while largely retaining its pH sensitivity. Consequently, changes in the fluorescence of SyPHer with pH will be similar to those caused by pH alone in the response of HyPer.

Interestingly, cpYFP flashes coincided strictly with a decrease in ΔΨₘ and data from the literature [8-10] show an identical inverse correlation between mt-cpYFP intensity dynamics and ΔΨₘ readout (Figure 4a). However, an increase in superoxide production with decreased ΔΨₘ goes against current understanding of ROS production by the mtETC [36]. It was therefore postulated that flashes report a novel mode of mitochondrial ROS release, distinct from basal mitochondrial ROS release, that is dependent on active ATP synthesis and transient opening of the mtPTP [8]. This interpretation is an additional source of controversy; in isolated energized mitochondria, flashing was very active in the absence of ADP or phosphate (Pi) and therefore in the absence of ATP synthesis [25,31], showing the dispensability of ATP synthesis for the generation of flashes. Moreover, the evidence for the postulated involvement of the mtPTP, and its hypothesized transient opening under physiological conditions, remains unclear because observations using the mtPTP effectors cyclosporin A (or absence of cyclophilin D) and atracyloside were variable depending on tissue type [8,9,13]. In addition, two compounds used as mtPTP effectors (atracyslide and bongkrekic acid) inhibit the mitochondrial adenine nucleotide translocase [37] and thus are likely to affect the matrix ATP/ADP ratio, the proton-motive force (mtMPF), and matrix metabolite pools in vivo. Similar (although partly inverted) changes are expected on inhibition of ATP-synthase by oligomycin in vivo, but not in isolated mitochondria under state 2 respiration in the absence of an external ADP and phosphate supply [8,25]. Matrix alkalinisation does not require, and even excludes, transient opening of a nonspecific pore, because it needs active proton extrusion from the matrix. By contrast, a pore

Figure 3. Circularly permuted yellow fluorescent protein (cpYFP) ‘flashes’ can be quenched by manipulating matrix pH dynamics. In isolated respiring Arabidopsis mitochondria, cpYFP flashes are strongly suppressed by: (a) nigericin treatment in the presence of K⁺ clamping of matrix pH while maintaining membrane potential; (b) increasing matrix buffering capacity by phosphate loading; and (c) limiting electron transport and proton pumping capacity so it cannot further accelerate. For instance, under succinate-driven respiration, complex II as an entry point for electrons can be titrated with malonate as a competitive inhibitor to limit the capacity of the mitochondrial electron transport chain (mtETC) while maintaining the membrane potential [31]. Permeability of the outer membrane to small molecules due to porins is represented by holes.

Figure 4. Inversely correlated dynamics of circularly permuted yellow fluorescent protein (cpYFP) fluorescence and membrane potential (ΔΨₘ) in single mitochondria and a mechanistic model of transient matrix alkalinisation. (a) The overall dynamics of cpYFP fluorescence and ΔΨₘ are correlated for single mitochondria. cpYFP signal increases strictly coincide with decreases of membrane potential [8,10,31]. (b) Spontaneous cation influx into an actively respiring mitochondrion explains transient matrix alkalinisation events and cpYFP ‘flashes’. Cation influx (1) decreases ΔΨₘ (2) due to positive charge import. Lowered ΔΨₘ allows for acceleration of electron transport and proton pumping (3), lowering matrix proton concentration and increasing pH (4). This pH increase results in a flash-like increase in the fluorescence of the cpYFP sensor (5). Note that probe-specific properties will shape certain attributes of the probe response shown in (a), such as the amplitude and frequency of changes in probe fluorescence. The model shown in (b) predicts that correlation between the dynamic responses of the pH and ΔΨₘ probes will be affected by the ion exchange properties of the inner mitochondrial membrane, the pH buffering capacity of the matrix, and the capacity of the electron transport chain (ETC).
would lead to equilibration of proton concentration across the inner membrane.

Most data that have hitherto been interpreted as changes in the production of mitochondrial superoxide can be explained by matrix pH fluctuations. This means that most reports characterizing cpYFP flashes in single mitochondria can be reinterpreted to draw valuable conclusions about mitochondrial pH dynamics. Thus, during anoxia, basal mt-cpYFP fluorescence declined. This was interpreted as a decrease in basal superoxide production [11]. However, although arrest of superoxide generation is expected during anoxia, so is cytosolic and mitochondrial acidification [32,38]. Because transient alkalinisation events by the respiratory chain require active proton pumping, which is strictly oxygen dependent, the observed anoxia-triggered suppression of flashes is expected. Absence of proton pumping due to either a lack of mtDNA or treatment with mtETC inhibitors, including antimycin A, also inhibited flashing [8]. Although these results have been interpreted as a novel respiration-dependent mechanism of superoxide release, lack of proton pumping also prevents matrix alkalinisation. That the response of cpYFP is actually due to a change in matrix pH also resolves the apparent contradiction that antimycin A, which is a robust inducer of superoxide production by the respiratory chain, inhibited cpYFP flashing and decreased basal mitochondrial cpYFP fluorescence [8,25,28]. Based on the observation that flashes in interconnected mitochondrial networks occur synchronously, an amplification mechanism for superoxide release as a potential mode of mitochondrial communication was postulated [10,15]. However, synchrony in matrix alkalinisation is an expected consequence because of the very rapid diffusion of protons through an interconnected mitochondrial network. Such an explanation does not require any amplification or communication and is instead an intrinsic property of the mitochondrial network within a cell. cpYFP flash activity in single mitochondria of myocytes was repressed by antioxidants and increased during re-oxygenation after hypoxia and when mitochondrial SOD was silenced [8,11]. Although this observation was interpreted as evidence of the superoxide specificity of cpYFP, it in fact only suggests that cpYFP flashes can be induced by downstream effects resulting from oxidative stress in mitochondria; the observation alone gives no clue to the chemical nature of the sensed transients. Consistent with this interpretation, mitochondrial oxidative stress was identified as a potential trigger of matrix alkalinisation events [31].

We conclude that mitochondrial cpYFP flashes do not report superoxide bursts. Instead, the pH sensitivity of cpYFP indicates that the probe is actually responding to mitochondrial matrix alkalinisation events that fully explain the observed flashes. Measurement of ROS in vivo is challenging and prone to artefacts that may lead to unintentional misinterpretation. As some of us have discussed earlier [39], it is vital to apply stringent criteria before accepting the results of any ROS probe at face value and to continually reassess and re-evaluate the methods used to infer roles for ROS in biological systems.

Concluding remarks: towards the physiological significance of mitochondrial flashes
The discovery of cpYFP flashes has revealed a fascinating phenomenon at the level of single mitochondria in living cells. However, the focus of further study should now be on determining the physiological significance of matrix pH transients and the term ‘superoxide flashes’ should be replaced by ‘cpYFP flashes’. A detailed body of data on mitochondrial cpYFP flashes is already available and valuable conclusions may be drawn by its critical re-interpretation. Suitable alternative protein sensors for pH are available and can be used in addition to cpYFP to avoid any further ambiguity. pH transients have the potential to act as signals in individual mitochondria and to be part of a dynamic remodelling of mitochondrial respiration and the composition of mtPMF in response to altered cellular status and stress. By taking a careful, considered, and evidential approach to future investigations, researchers will be able to unravel the true biological significance of mitochondrial flashes in physiology and pathology, while providing novel insights into mitochondrial heterogeneity, functional dynamics, and quality control in living tissues.

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