SUPPLEMENTARY INFORMATION

1. MATERIALS AND METHODS

Citrate/phosphate buffer preparation Buffer solutions were prepared with potassium gluconate (120 mM), sodium gluconate (40 mM), sodium phosphate (10 mM), sodium citrate (2 mM), calcium chloride (0.5 mM) and magnesium sulfate (0.5 mM), and pH was adjusted using either sodium hydroxide 1 M or sulfuric acid 1 M. All substances were purchased from Sigma-Aldrich.

HEPES buffer preparation Buffer solutions were prepared with potassium gluconate (120 mM), sodium gluconate (40 mM), HEPES (20 mM), calcium chloride (0.5 mM) and magnesium sulfate (0.5 mM), and pH was adjusted using either sodium hydroxide 1 M or sulfuric acid 1 M. All substances were purchased from Sigma-Aldrich.

Absorption measurements Absorption spectra of E^2GFP solutions (citrate/phosphate buffer) were recorded on a Jasco V550 UV/Vis spectrophotometer (JASCO, Easton, MD) by setting the monochromator slits to 2 nm, the scanning speed to 200 nm/min, the data resolution to 1 nm, and the time collection average on each wavelength interval to 0.25 s. The absorption spectrum of each sample was corrected for background and normalized to the measured optical density at 278 nm.

Cell culture Chinese Hamster Ovary (CHO) K1 cells and were grown in Dulbecco's modified Eagle medium (DMEM) with F-12 nutrient mix (DMEM/F-12, Invitrogen, Carlsbad, CA) supplemented by 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen). HeLa cells were grown in DMEM (Invitrogen) supplemented by 10% fetal bovine serum, 2mM glutamine, 10 U/mL penicillin and 10 µg/mL streptomycin. NIH 3T3 cells were grown in DMEM supplemented by 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen) Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Protein expression constructs pcDNA3.1+ expression vector for E^2GFP was prepared as reported in ref: 1; E^2GFP fused with the subunit VIII of human cytochrome c oxidase (COX8) for mitochondrial expression was prepared by site-directed mutagenesis of pcDNA3.1 expressing COX8-EGFP generously provided by Prof. A. S. Verkman.[1]

Cell transfection The transfections of E^2GFP and COX8-E^2GFP were carried out using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. For live imaging, cells were plated 24 hours before transfection onto a 35-mm glass-bottom dish (WillCo-dish GWSt-3522) at 60-70% confluence.

Fluorescence Lifetime analysis of solutions Lifetime measurements were performed on 1-10 μM E^2GFP solutions in citrate/phosphate or PBS buffer. Protein fluorescence was measured by single (SPE) or two-photon excitation (TPE). SPE measurements were performed using a Leica TCS SP5 inverted confocal microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with an external pulsed Ar laser for excitation at 405 nm. Laser repetition rate was set to 40 Hz. Image size was 64x64 pixels and scan speed was usually set to 400 Hz (lines per second). The pinhole aperture was set to 1.0 Airy. Solution pH was checked after each measurement. E^2GFP solutions were placed into glass bottom cuvettes and imaged using a 63.0x1.20 numerical aperture water immersion objective (Leica Microsystems). Emission was monitored in the 500–600 nm range using the built-in acousto-optical beam splitter (AOBS) detection system of the confocal microscope. Acquisition lasted until about 100-200 photons per pixel were collected, at photon counting rate of 100-500 kHz. Lifetime images were binned to a single pixel and elaborated using Picoquant SymPhoTime and FluoFit softwares for conventional FLIM analysis, and with Globals for Images software (available at www.lfd.uci.edu) for the phasor transformation.

TPE measurements were performed using a Zeiss 710 microscope equipped with a Ti:Sapphire laser system (Spectra-Physics Mai Tai) and a ISS A320 FastFLIM card. A 40x1.2 numerical aperture water immersion objective (Zeiss Korr C-Apochromat) was used. Image size was 256x256 pixels with 25 μs pixel dwell time. A primary dichroic filter with a wavelength of 690 nm was used to separate the fluorescence signal from the laser light and the fluorescence coming from the sample. The emission beam was further split by a long pass filter with a wavelength of 495 nm and detected by two photomultipliers (H7422P-40, Hamamatsu), one coupled to a short pass filter with a wavelength of 460-480 nm and one to a filter with a wavelength of 540-550 nm.

Fluorescence Lifetime Imaging of living cells E^2GFP-transfected cells were placed in glass bottom WillCo dishes and mounted in a thermostated chamber at 37 °C

(Leica Microsystems) and humidified with 5% CO_2 atmosphere, and images were taken either with the Leica TCS SP5 microscope or with the Zeiss 710 microscope described above. Acquisition parameters were the same as for E^2GFP solutions except for the dimension of lifetime images, which was 512x512 pixels in the Leica system.

Cell lifetime images were elaborated using Picoquant SymPhoTime and FluoFit softwares for conventional FLIM analysis, and Globals for Images for the phasor transformation.

Lifetime measurements of pH-clamped living cells were carried out by perfusing E^2GFP-transfected cells with 1 ml of HEPES buffer adjusted to the desired pH. After perfusion, 10 μM nigericin was added. Images were taken 10 minutes after treating with the ionophore. Solution pH was checked after each measurement and data were analyzed as described above.

Lifetime measurements of living cells undergoing oxidative stress were performed by perfusing E^2GFP-transfected cells with 1.0-1.5% H_2O_2. Images were taken 5 minutes after treating with H_2O_2 and data were analyzed as described above.

2. THEORY OF PHASOR DEPENDENCE ON pH

According to the phasor transformation originally conceived by Gratton et al.,[2] the $g_{i,j}$ and $s_{i,j}$ coordinates in the phasor plot corresponding to a fluorescence decay $I_{i,j}(t)$ collected from pixel i,j are expressed by:

$$g_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\cos(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt} \quad \text{(S1a)}$$

$$s_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\sin(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt} \quad \text{(S1b)}$$

where ω is the laser repetition angular frequency (or the angular frequency of light modulation). Let us assume that two states, 1 and 2, each one characterized by a multiexponential decay, contribute to $I_{i,j}(t)$, we can write:

$$I_{i,j}(t) = C_1 \cdot \left[\sum_k \alpha_k \exp\left(-t/\tau_k\right)\right]_1 + C_2 \cdot \left[\sum_r \alpha_r \exp\left(-t/\tau_r\right)\right]_2 \quad \text{(S2)}$$

where:

- C_1 and C_2 are the concentrations of states 1 and 2, respectively;
- k and r indexes run over exponential components of states 1 and 2, respectively;
- α and τ denote the molar amplitude and time constant of each decay, respectively.

Applying transformations S1a and S1b to S2, we obtain:

$$g_{i,j}(\omega) = x_1 \cdot \left[\sum_k \frac{f_k}{1+\left(\omega\tau_k\right)}\right]_1 + x_2 \cdot \left[\sum_k \frac{f_r}{1+\left(\omega\tau_r\right)}\right]_2 \quad \text{(S3a)}$$

$$s_{i,j}(\omega) = x_1 \cdot \left[\sum_k \frac{f_k\omega\tau_k}{1+\left(\omega\tau_k\right)}\right]_1 + x_2 \cdot \left[\sum_k \frac{f_r\omega\tau_r}{1+\left(\omega\tau_r\right)}\right]_2 \quad \text{(S3b)}$$

where:

- x_1 and x_2 are the molar fractions of states 1 and 2, respectively;
- $f_k = \frac{\alpha_k\tau_k}{\sum_k \alpha_k\tau_k}$ and $f_r = \frac{\alpha_r\tau_r}{\sum_r \alpha_r\tau_r}$.

It is now worth noting that eq. S3a,b can be rewritten:

$$g_{i,j}(\omega) = x_1 \cdot g_{i,j}(\omega)_1 + x_2 \cdot g_{i,j}(\omega)_2 \quad \text{(S4a)}$$

$$s_{i,j}(\omega) = x_1 \cdot s_{i,j}(\omega)_1 + x_2 \cdot s_{i,j}(\omega)_2 \quad \text{(S4b)}$$

where each sum has been replaced by the proper phasor coordinate of either state 1 and 2. Eq. S4a,b represents the addition principle of phasors for two distinguishable optical states, which can be graphically expressed by a molar fraction-weighted sum of phasors **considered as vectors** in the *g*,*s* plane. Note that this vector addition rule can be extended to any number of states, as the Fourier transform (Eq. S1a,b) is a linear operator.

Eventually, we identify states 1 and 2 with protonated and deprotonated chromophore of indicator E^{2}GFP (actually we may consider *any* pH indicator characterized by two lifetime-distinguishable protonation states). In this case, the molar fractions of 1 and 2 are connected to pH via the Henderson-Hasselbach equation:[3]

$$\frac{x_2}{x_1} = 10^{(pH - pK_a)} \quad \text{(S5)}$$

Use of S5 in S4a,b, together with mass balance x_1=1-x_2, gives:

$$g_{i,j}(\omega) = \frac{g_{i,j}(\omega)_1 + g_{i,j}(\omega)_2 \cdot 10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}} \quad \text{(S6a)}$$

$$s_{i,j}(\omega) = \frac{s_{i,j}(\omega)_1 + s_{i,j}(\omega)_2 \cdot 10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}} \quad \text{(S6b)}$$

3. MULTIEXPONENTIAL FITTING OF LIFETIME DECAYS COLLECTED FOR CALIBRATION SOLUTIONS

The fluorescence lifetime decays collected for pH calibration solution by single photon excitation at 405 nm (see materials and methods for acquisition parameters) were fitted to a triexponential equation of the form:

$$I(t) = A_1 \exp\left(-t/\tau_1\right) + A_2 \exp\left(-t/\tau_2\right) + A_3 \exp\left(-t/\tau_3\right) \quad \text{(S7)}$$

where $A_{1\text{-}3}$ and $\tau_{1\text{-}3}$ represent the amplitude and time constant of each decay component, respectively. The use of a triexponential decay law represents the minimum model for interpreting the photophysical properties of E^2GFP chromophore, which is known to be associated with 3 pH-dependent states; significantly, triexponential fitting always returned $\chi^2 \leq 1.5$. We then calculated the intensity-averaged decay time $<\tau>$ as:

$$<\tau> = \frac{\sum_{k=1}^{3} A_k \tau_k^2}{\sum_{k=1}^{3} A_k \tau_k} \quad \text{(S8)}$$

Finally, the $<\tau>$ *vs*. pH plot (Fig. S1) was fitted to the Henderson-Hasselbach equation according to:[4]

$$<\tau> = a + b\left[1 + 10^{(pK_a - pH)}\right]^{-1} \quad \text{(S9)}$$

where a and b are the offset and dynamic range of lifetime span, respectively. We found $pK_a = 6.85 \pm 0.06$, in excellent agreement with both the thermodynamic pK_a[5] and the value obtained from phasor analysis (see main text).

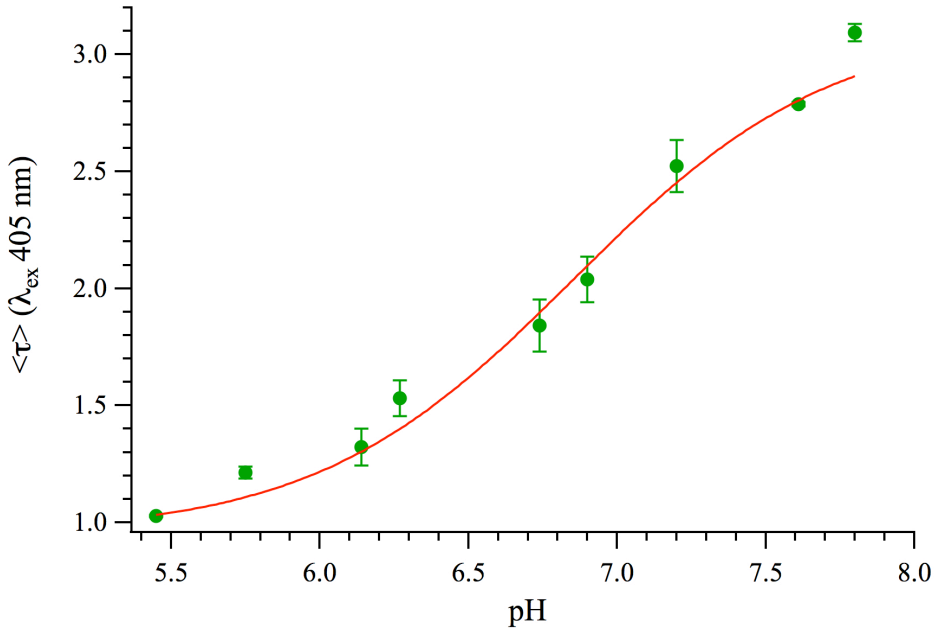

Fig. S1 Full green circles: plot of $<\tau>$ of E^2GFP *vs*. pH determined for the calibration solution (single photon excitation, λ_{ex}=405 nm 40 MHz); for each solution, $<\tau>$ was obtained by triexponential fit of the fluorescence decay (eq. S7 and S8). Red line: fit of $<\tau>$ *vs*. pH to eq. S9, expressing the Henderson-Hasselbach equation.

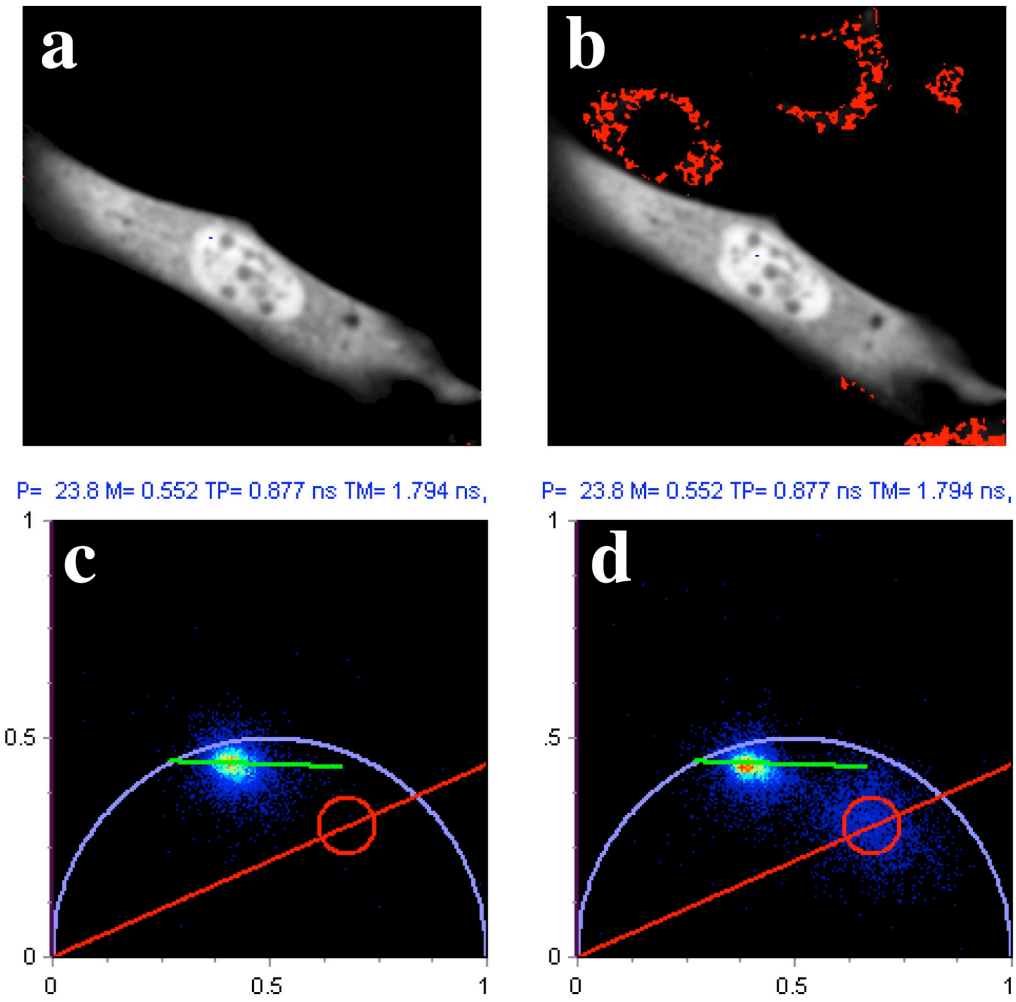

Fig. S2 A resting cell expressing E^{2}GFP is imaged by TPE at 800 nm (a) and 740 nm (b). The phasor plots of (a) and (b) are reported in panel (c) and (d), respectively. In both phasor plots the pixel clouds of E^{2}GFP are very intense and fall along the calibration line (green). In (d), however, a second, less-intense, phasor cloud falls off the calibration line: all pixels yielding phasors comprised in the red circle of panel (d) are highlighted in red in figure (b) and are attributable to the autofluorescence of cells not expressing E^{2}GFP. Notably, the same autofluorescence is minimally observed when excitation is at 800 nm (a,c).

REFERENCES

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