



Methods in Free Radical Biology and Medicine

Measuring E_{GSH} and H_2O_2 with roGFP2-based redox probesBruce Morgan¹, Mirko C. Sobotta¹, Tobias P. Dick^{*}

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ABSTRACT

Redox biochemistry plays an important role in a wide range of cellular events. However, investigation of cellular redox processes is complicated by the large number of cellular redox couples, which are often not in equilibrium with one another and can vary significantly between subcellular compartments and cell types. Further, it is becoming increasingly clear that different redox systems convey different biological information; thus it makes little sense to talk of an overall “cellular redox state”. To gain a more differentiated understanding of cellular redox biology, quantitative, redox couple-specific, *in vivo* measurements are necessary. Unfortunately our ability to investigate specific redox couples or redox-reactive molecules with the necessary degree of spatiotemporal resolution is very limited. The development of genetically encoded redox biosensors offers a promising new way to investigate redox biology. Recently developed redox-sensitive green fluorescent proteins (roGFPs), genetically fused to redox-active proteins, allow rapid equilibration of the roGFP moiety with a specific redox couple. Two probes based on this principle are now available: Grx1-roGFP2 for the measurement of glutathione redox potential (E_{GSH}) and roGFP2-Orp1 for measuring changes in H_2O_2 concentration. Here we provide a detailed protocol for the use of these probes in both yeast and mammalian systems using either plate-reader- or microscopy-based measurements.

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Introduction

Cellular redox changes are increasingly realized to be associated with a wide range of physiological and pathophysiological conditions. Redox changes correlate with many cell fate decisions including proliferation, differentiation, and apoptosis [1], and increased production of reactive oxygen species (ROS) is observed in a large number of diseases, including many cancers [2–4]. However, understanding the causal relationship between ROS and disease biology is incredibly complex; for example, it remains unclear if increased ROS levels are a cause or a result of tumor progression, or both [5].

The investigation of cellular redox processes is a technically challenging area of research. The various cellular redox couples are not in thermodynamic equilibrium in the cell, but rather are kinetically separated [1,6]. As well as varying significantly between different subcellular compartments, cellular redox couples can also respond rapidly to both endogenous and exogenous stimuli. Such spatiotemporal segregation makes investigation difficult; measurement of one redox couple offers little or no information relating to the state of other cellular redox couples or indeed the same redox couple in other subcellular compartments. The measurement of dynamic changes in redox couples presents still further challenges. These challenges are reflected in the literature by the frequent use of poorly defined terminology such as “cellular redox state,”

which, without further definition, refers to all redox couples as though they are one entity and further does not account for differences between various subcellular compartments. The development of more advanced and appropriate investigative tools and techniques is crucial to obtain a deeper spatial and temporal understanding of cellular redox processes.

Glutathione is the major cellular redox buffer, because of its low standard redox potential (−240 mV) and high cellular concentration (1–13 mM) [7–9]. There is a large published literature relating to glutathione homeostasis in many model systems. Despite this, many aspects of glutathione homeostasis are still not well understood at a fundamental level. Measurements of glutathione are conventionally performed on whole-cell extracts using techniques including reverse-phase high-performance liquid chromatography or spectrophotometric and fluorescence-based assays, for example, the 5,5'-dithiobis(2-nitrobenzoic acid) recycling assay [10,11]. These assays measure the total concentrations of reduced (GSH) and oxidized (GSSG) glutathione present in cellular extracts and can attain a high degree of redox-couple specificity, sensitivity, and reproducibility. However, their applicability to measure rapid dynamic processes is at best limited. Further, whereas these assays are undoubtedly useful in some contexts, such whole-cell measurements will crucially destroy all subcellular compartment-specific information, leading to mixing of the separate compartmental glutathione pools, and may also be susceptible to post-cell lysis artifacts. Whole-cell assays therefore cannot and should not be used to make claims about individual compartments. Despite these considerations, whole-cell measurements are still often regarded in the literature as largely representative of the glutathione redox state in the cytosol.

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Hydrogen peroxide (H_2O_2) is one of the most important ROS molecules in the cell. It can be produced as a by-product of a number of cellular processes, for example, respiratory chain activity and β -oxidation of fatty acids, and has traditionally been viewed as an unwanted, damaging molecule [12–15]. However, H_2O_2 is now increasingly recognized to have important roles in cellular signaling [16], and enzymes dedicated to H_2O_2 production have been discovered [17,18]. Measurements of H_2O_2 have traditionally suffered from the problem of specificity, in addition to a lack of high temporal and spatial resolution. Conventional redox-sensitive dyes lack specificity, for example, the commonly employed fluorescent dye 2',7'-dihydrodichlorofluorescein has been shown to react readily with a number of other cellular species, including cytochrome c, metals, and peroxidases [19]. Further, these probes react irreversibly and thus preclude dynamic measurements. A new generation of chemical probes is currently being developed, which appears to be H_2O_2 specific and may have some potential for subcellular targeting [20–23]. However, at present these probes still suffer from low reaction rates and measurements are based on absolute fluorescence intensity changes. Therefore, comparison between samples may be complicated by factors including differential probe uptake. In the future it is likely that these chemical probes will be complementary to the approaches detailed in this article.

Many of the problems of traditional E_{GSH} and H_2O_2 measurements as outlined above can be overcome using novel genetically encoded fluorescent probes. In this article we describe methods and considerations for the use of two fluorescence-based, genetically encoded probes that enable real-time, nondisruptive, and subcellular compartment-specific measurement of the redox potential of the GSH:GSSG redox couple (E_{GSH}) and changes in H_2O_2 concentration. These probes are fusion proteins consisting of redox-active green fluorescent protein 2 (roGFP2) genetically fused to the redox enzymes human glutaredoxin-1 (Grx1), for measurement of E_{GSH} , and Orp1 from *Saccharomyces cerevisiae*, for measurement of H_2O_2 as described previously [24,25].

Our measurements of the cytosolic E_{GSH} in *S. cerevisiae* using the Grx1-roGFP2 probe typically give values of between -310 and -320 mV ([26] and unpublished data), which is consistent with a number of other studies employing genetically encoded fluorescent probes [7,8,27–29]. Assuming a total glutathione concentration of 10 mM this would imply a GSH:GSSG of between 20,000:1 and 40,000:1. Thus, GSSG appears to be present only in nanomolar amounts in the cytosol, suggesting that original estimates of the GSH:GSSG in this compartment are wrong by 2 to 3 orders of magnitude.

Principles

The group of James Remington has previously created redox-sensitive fluorescent probes based on GFP engineered to contain two cysteine residues capable of forming a disulfide bond [30]. Several variants of these probes have been developed with a range of redox potentials [31,32] and have been used to perform measurements of compartment-specific glutathione redox potential *in vivo* [30,33,34].

A major advantage of roGFPs over other fluorescent probes such as rYFP is their ratiometric fluorescence excitation behavior (Fig. 1). When measuring fluorescence emission at 510 nm, roGFP2 exhibits excitation maxima at both 400 and 490 nm. The amplitude of both excitation maxima depends upon the oxidation state of the disulfide bond in the roGFP2 molecule; oxidation increases the intensity of the 400 nm peak while decreasing the intensity of the 490 nm peak. Such ratiometric measurements are much less dependent upon factors such as probe expression level and photobleaching, thus simplifying comparison between samples. Moreover, although both rYFP and roGFP2 suffer from pH-dependent changes in total fluorescence emission intensity, the ratio of the intensity of the two fluorescence excitation maxima in roGFP2 is unaffected by pH changes within the physiological range (pH 5.5–8.0). Thus, roGFP2-based ratio measurements are effectively pH-independent.

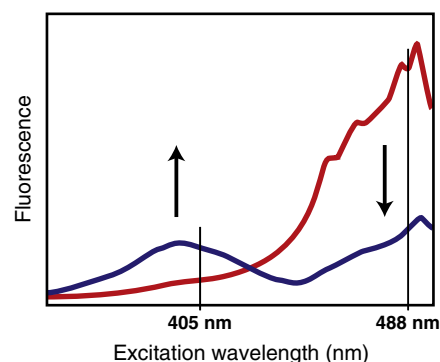


Fig. 1. Fluorescence excitation spectra of roGFP2 in the fully reduced (red line) or fully oxidized (blue line) state. Emission is followed at 510 nm.

Although they offer a significant improvement over previous methodologies, conventional roGFPs still have some limitations. Although they are known to equilibrate predominantly with the glutathione redox couple through mediation by endogenous glutaredoxins [7,35], it is not known to what extent other redox enzymes or processes can influence the probes *in vivo* and therefore it is unclear whether absolute specificity can be ensured. Indeed the probes developed in our laboratory and described in this article clearly demonstrate that certain peroxidases can also drive roGFP2 redox changes [24]. Second, the roGFP response kinetics are still relatively slow, taking from several minutes to tens of minutes for the roGFP to fully equilibrate with a new glutathione redox state [25,30,31]; thus rapid cellular redox events may be missed by these probes. Third, the dependence of the roGFP2 response on the activity of endogenous glutaredoxins implies the possibility that changes in glutaredoxin expression or activity could simulate changes in the glutathione redox potential that do not really exist.

The kinetic limitations of roGFPs can be overcome by genetic fusion to specific redox enzymes, which facilitates the rapid equilibration of the roGFP2 moiety with a specific cellular redox couple. This is achieved because covalent fusion increases the local concentration of redox enzyme available to interact with the attached roGFP2 by at least 3 orders of magnitude compared to the endogenous redox enzyme concentration [28]. This also helps to ensure that the probe response kinetics will be the same in all tissue types, irrespective of endogenous redox enzyme activity and expression level [28]. The reaction mechanism for both Grx1-roGFP2 and roGFP2-Orp1 is illustrated in Fig. 2 and described in detail elsewhere [24,25,28].

It is important to be aware of exactly what is and what is not measured by the probes described here. The Grx1-roGFP2 probe measures the redox potential of the glutathione redox couple (E_{GSH}), which is a function of the total glutathione concentration and the GSH:GSSG ratio. This means that the probe cannot be used to determine GSH:GSSG without further information relating to absolute glutathione concentration. The calculation of absolute redox potentials also depends upon the compartmental pH, which can be determined using a pH probe, such as pHluorin, targeted to the specific subcellular compartment, as described before [26].

Orp1 is well characterized as a highly sensitive thiol peroxidase that is oxidized by H_2O_2 [36]. The fusion of roGFP2 to Orp1 creates a redox relay in which oxidizing equivalents from H_2O_2 are very efficiently passed via Orp1 to roGFP2 [24]. *In vitro*, roGFP2-Orp1 responds efficiently to H_2O_2 but very inefficiently to a range of other oxidants, including GSSG, cystine, and hydroxyethyl disulfide [24]. Thus roGFP2-Orp1 responds rapidly, highly efficiently and specifically to H_2O_2 and can thus report relative differences in H_2O_2 concentration between different conditions or after various treatments. It is important to be aware that roGFP2-Orp1 cannot yield information relating to absolute H_2O_2 concentrations, and it is not possible to determine absolute rates of H_2O_2 production.

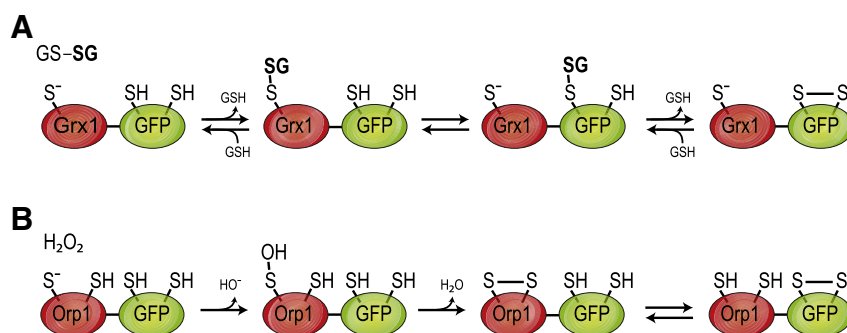


Fig. 2. (A) The nucleophilic cysteine residue of Grx1 reacts with GSSG to form an S-glutathionylated Grx1 intermediate. This is attacked by one of the roGFP2 cysteine residues leading to formation of an S-glutathionylated roGFP2, which rearranges to form the intramolecular disulfide bond. The whole series of reactions is fully reversible. (B) The nucleophilic cysteine residue of Orp1 attacks H₂O₂, leading to the formation of a sulfenic acid group, which condenses with the resolving cysteine of Orp1, leading to formation of an intramolecular disulfide bond. This disulfide is then transferred to roGFP2 by thiol–disulfide exchange.

The oxidation of the roGFP2–Orp1 probe is fully reversible *in vivo*. In yeast cells the natural reductant of Orp1 is the thioredoxin system [36], and thus the thioredoxin system is the most likely candidate for reducing the roGFP2–Orp1 probe, although a contribution by endogenous glutaredoxins cannot be strictly ruled out [24,28].

Evidently roGFP-based probes can be expressed in a variety of systems, from single-celled to transgenic multicellular model organisms and mammalian tissue culture and they can be targeted to a variety of subcellular compartments. Measurements at the single-cell level are based on microscopy, whereas population behavior can be studied by flow cytometry or microplate readers. For additional theoretical background, previous applications, and a comparison with other genetically encoded probes, the interested reader is referred to our recent review [28]. In the current review we focus specifically on the experimental details of two exemplary applications: the use of a plate-reader technique for the cytosolic Grx1–roGFP2 probe in yeast cells and of microscopy analysis of the cytosolic roGFP2–Orp1 probe in mammalian cell tissue culture.

However, the reader should be aware that the protocols described in this article are more broadly applicable, for example, the microscopy protocol can be used for either probe in both mammalian and yeast cells. The plate-reader protocol can also be used for the Orp1 probe in yeast. However, it may be worth noting that we have so far found it difficult to make reliable mammalian cell measurements with the plate reader.

Measurement of cytosolic E_{GSH} in yeast

Materials

- 100 mM Mes/Tris buffer, pH 6.0
- Dithiothreitol (DTT; AppliChem, Darmstadt, Germany, Product A2948)
 - 1 M stock in 100 mM Mes/Tris, pH 6.0
- Diamide (Sigma–Aldrich Chemie GmbH, Munich, Germany, Product D3648)
 - 0.2 M stock in 100 mM Mes/Tris, pH 6.0
- Hydrogen peroxide (30% v/v) (Merck, Darmstadt, Germany, Product 107210)
 - Stock solution of 14.33× the desired final experimental H₂O₂ concentration
- YPD Media
 - Bacto™ Yeast extract (1% w/v) (BD–Diagnostic Systems, Heidelberg, Germany, Product 212750)
 - Bacto™ Peptone (2% w/v) (BD–Diagnostic Systems, Product 211677)
 - 2% glucose

- YPD agar plates—as for YPD with the addition of 1.5% (w/v) agar (Invitrogen GmbH, Darmstadt, Germany, Product 30391–023)
- Yeast One-Step-Transformation Buffer
 - 0.2 M lithium acetate, pH 5.0
 - PEG 3350 (40% w/v) (Sigma–Aldrich, Product P4338)
 - 100 mM DTT
- Hartwell's Complete (HC) medium (minus leucine), as developed in the lab of Lee Hartwell—widely used and complete recipe available online (all components from Sigma–Aldrich)
- Hartwell's Complete medium agar plates, as for HC medium, with the addition of 1.5% (w/v) agar
- All published yeast probe expression constructs are available from our lab after signing a Material Transfer Agreement.

Instrumentation

- Fluorescence plate reader equipped with 390 and 480 nm excitation filters and a 510 nm emission filter. We use a FLUOstar Omega (BMG–Labtech, Offenburg, Germany), with Omega Control software and MARS data analysis software (provided with plate reader).
- Flat-bottomed, black, 96-well imaging plates (BD Falcon, Heidelberg, Germany, Product 353219).
- Centrifuge capable of taking 96-well plates.
- Spectrophotometer.

Protocol

The yeast *S. cerevisiae* is particularly amenable to measurement in 96-well plate-reader format. Here we describe one particular methodology used in our laboratory for employing the probes in the yeast system. In principle there are many steps in the protocol for which alternative options could be considered. We will attempt to highlight important considerations and possible alternatives throughout the protocol.

Introduction of probe into yeast cells

Typically in our lab roGFP2-based redox probes are introduced into and expressed in yeast cells on an extrachromosomal plasmid, p415TEF [37,38], using a standard transformation protocol. We use yeast strains based on the BY-deletion series [39,40]. It is also important to transform cells separately with an empty vector to use for background subtraction. Expression of the probes from an extrachromosomal plasmid is particularly convenient for rapid screening of many strains; however, the probes can be chromosomally integrated if desired.

1. Inoculate 5 ml YPD medium with a colony of yeast from a YPD agar plate and grow overnight at 25 °C, with shaking at 150 rpm, until midlogarithmic phase.

- Remove 500 μ l culture, centrifuge at 4000 g for 1 min at room temperature. Resuspend in 1 ml Milli-Q H₂O (MQH₂O), and centrifuge again for 1 min at 4000 g; remove liquid and resuspend cells in 100 μ l One-Step-Transformation Buffer.
- Add 5 μ l 10 mg/ml salmon sperm carrier DNA (preheated to 95 °C for 5 min, followed by 1 min cooling on ice). Add 100 ng plasmid DNA, briefly vortex, and incubate at 45 °C for 30 min.
- Plate out transformation mixture onto selective medium (HC minus leucine agar plates). After 2 days of growth pick a colony from the transformation plate and streak out onto a fresh HC minus leucine agar plate.
- To ensure consistent results freshly transformed cells should be prepared every 2 to 3 weeks. After this period we observe increased resistance to oxidant stress, which is reflected by the Grx1-roGFP2 and roGFP2-Orp1 probe response to oxidant treatment.

Growth of cells

- Inoculate separate aliquots of 5 ml HC minus leucine with a colony of either empty vector- or probe-expressing cells from the agar plate.
- Grow for 24 h at 25 °C to allow the cells to reach stationary phase. Inoculate fresh HC medium 1:50 with precultures and grow for 16 h at 25 °C. For any given strain this will yield cells at consistently similar optical densities, mid- to late exponential phase (OD_{600} 3.5–4) for wild-type cells.

Setting up the plate reader

- Using the Omega Control software create a new measurement protocol or select a previous protocol from the “Test Protocols” menu. Set up a method based on the “Fluorescence Intensity” option.
- In the “Basic Parameters” submenu set positioning delay to 0.2 s, set number of flashes per well to 10, and select “bottom optic” measurements. Select also the desired number of measurement cycles and time per cycle. Ensure that the number of multichromatics is set to 2 and that the correct excitation (410 and 480 nm) and emission (510 nm) filters have been selected.
- In the “Layout” submenu select the appropriate blank and sample wells.
- A basic measurement is achieved with the above settings; for more specialized procedures one should refer to the software manual.
- Set gain adjustment to 80% maximum and perform an automatic gain adjustment for each wavelength immediately before measurement.

Steady-state measurements

- Measure the OD_{600} of both the empty vector- and probe-expressing yeast cultures. For wild-type cells this should be 3.5–4.
- Harvest 1.5 OD_{600} units ($\sim 1.5 \times 10^7$ cells) of both empty vector- and probe-expressing cells per well of the 96-well plate to be used. More or fewer cells can be used depending upon probe expression level or other experimental requirements. Remember to harvest enough cells for the fully reduced and fully oxidized control wells.
- A separate empty vector control for background subtraction should be prepared for oxidized and reduced control cells as well as for each different yeast strain or measurement condition. Fully oxidized and fully reduced controls should also be prepared for each different condition, for example, if samples are to be measured in the presence of a factor X, it is good practice to have fully reduced and fully oxidized controls also in the presence of factor X, as well as empty vector controls for all conditions. Fig. 3 highlights an idealized plate layout that may be used to test the effect of a compound X on the probe readout, including all appropriate background and control samples that should be included.
- Centrifuge the yeast cells at 1000 g for 3 min at 25 °C, and resuspend in 1 ml MQH₂O.
- Repeat the centrifugation step and resuspend the cells in 200 μ l 100 mM Mes/Tris, pH 6.0, per 1.5 OD_{600} yeast cells. In principle any buffer, including growth medium (providing it does not absorb

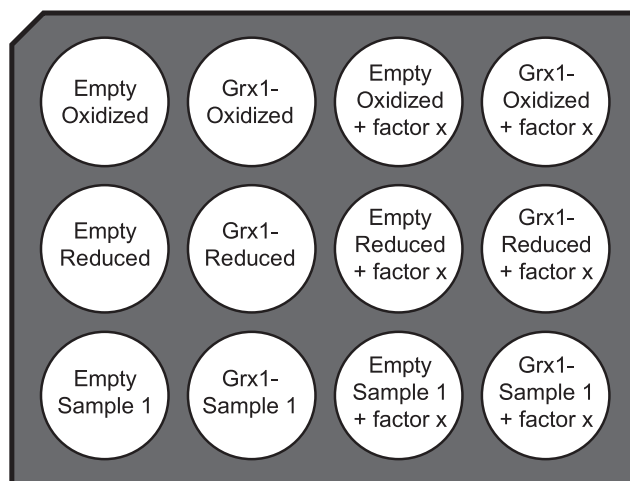


Fig. 3. Exemplary layout of a 96-well imaging plate for plate-reader-based measurements. It is important to have fully oxidized and fully reduced controls as well as empty vector-containing cells for background subtraction at each condition. Addition of a novel compound or treatment (+ factor X) should always be accompanied by oxidized and reduced controls as well as empty vector-transformed cells all in the presence of the novel treatment.

significantly at 400 or 490 nm), can be used for steady-state measurements. However, we have noticed significant differences between probe responses associated with resuspension in different buffers or growth media. This is particularly pronounced when observing dynamic probe responses (described below). The reasons for this are still under investigation in our lab, but all the data indicate a biological effect rather than any direct influence on probe behavior. Therefore care should be taken to ensure consistency of cell suspension buffer.

- Prepare a flat-bottom 96-well plate. For oxidized and reduced controls add 20 μ l 0.2 M diamide or 20 μ l 1 M DTT, respectively, to each well. Add 20 μ l 100 mM Mes/Tris, pH 6.0, to all sample wells to ensure equal dilution of all samples.
- Add 200 μ l (1.5 OD_{600}) of empty vector- or probe-expressing yeast cells to each well.
- Centrifuge the plate in a centrifuge that can hold 96-well plates for 5 min at 20 g and 25 °C. This will form a loose pellet of yeast cells at the bottom of each well. After centrifugation it is important to check that the cells are evenly spread across the bottom of the wells.
- Measurement of the plate should be made as quickly as possible.

Dynamic measurements

A strain deleted for glutathione reductase ($\Delta glr1$), the major enzyme responsible for reduction of oxidized glutathione, is well known to have a much greater cellular concentration of GSSG and increased sensitivity to oxidant treatment concomitant with GSSG accumulation [9,27]. Thus this strain provides a good control to test the function of the Grx1-roGFP2 probe. Interestingly, despite whole-cell GSSG levels 10–50 times higher than in wild type, at steady state the degree of roGFP2 oxidation (Ox_{roGFP2}) increases from ~5% in a wild-type strain to only ~35% in the $\Delta glr1$, which translates to a difference of ~20 mV at pH 7.0 (Fig. 4A), consistent with previously published data [7]. However, upon treatment with H₂O₂ the dynamic response of the Grx1-roGFP2 is clearly and dramatically different from that of the wild type. Consistent with the above observations, we find that steady-state cytosolic E_{GSH} is robustly maintained and relatively unaffected by either the composition of the cell resuspension buffer or the deletion of a range of other redox enzymes. However, in many cases significant differences in glutathione homeostasis can be revealed by observing the dynamic response of cytosolic E_{GSH} to an oxidative challenge, as illustrated in Fig. 4B for the case of glucose availability in the cell resuspension buffer.

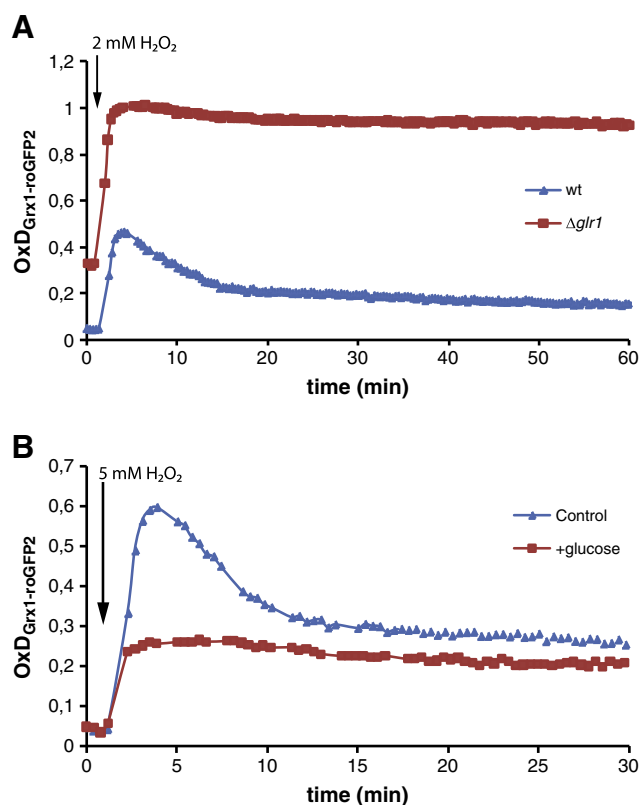


Fig. 4. Assessment of Grx1-roGFP2 response to H_2O_2 treatment. (A) BY4742 wild-type (wt) or BY4742 Δglr1 cells were treated with 2 mM H_2O_2 , and Grx1-roGFP2 response was followed for 60 min. (B) BY4742 wt cells in 100 mM Mes/Tris, pH 6.0, with (+ glucose) or without (control) the addition of 2% glucose were treated with 5 mM H_2O_2 . $\text{OxD}_{\text{Grx1-roGFP2}}$ was calculated according to Eq. (1).

When adding chemical compounds to cells, care should be taken with respect to the choice of solvent for water-insoluble compounds. In particular, we observe considerably different cytosolic glutathione response, as reported by the Grx1-roGFP2 probe, when yeast cells are exposed to dimethyl sulfoxide, even at relatively low (<0.5%) concentrations. Therefore, it is important to perform further controls if applying a compound solubilized in a previously untested solvent. We would recommend testing the effect of a known E_{GSH} -perturbing agent, for example, H_2O_2 , on cells in the presence of solvent alone. In the case of a significant difference between cells in the presence and absence of the solvent we would recommend testing alternative solvents, for example, we have observed no effect of ethanol ($\leq 2\%$) on the glutathione response reported by the Grx1-roGFP2 probe. After measurement, data can be exported to Microsoft Excel, using the data export button in the “Table View” window of the MARS data analysis software.

Calculation of $\text{OxD}_{\text{roGFP2}}$ and E_{GSH}

For each yeast strain and condition to be analyzed six separate sets of values must be taken into consideration. These are the background corrected fluorescence emission intensities of the fully oxidized and fully reduced controls and the sample after excitation at both 390 and 480 nm. These six values can be used to determine $\text{OxD}_{\text{roGFP2}}$, according to Eq. (1) [28]. Assuming the probe and the glutathione redox couple are in equilibrium, then E_{GSH} is equal to the calculated probe redox potential according to Eq. (2), where $E_{\text{roGFP2}} = -280 \text{ mV}$ [30]:

$$\text{OxD}_{\text{roGFP2}} = \frac{I_{390} \times I_{480_{\text{red}}} - I_{390_{\text{red}}} \times I_{480}}{I_{390} \times I_{480_{\text{red}}} - I_{390} \times I_{480_{\text{ox}}} + I_{390_{\text{ox}}} \times I_{480} - I_{390_{\text{red}}} \times I_{480}} \quad (1)$$

$$E_{\text{GSH}} = E_{\text{roGFP2}} = E_{\text{roGFP2}}' - \frac{RT}{2F} \ln \left(\frac{1 - \text{OxD}_{\text{roGFP2}}}{\text{OxD}_{\text{roGFP2}}} \right) \quad (2)$$

For accurate determination of absolute E_{GSH} values it is necessary to control temperature and measure subcompartment pH. A determination of subcellular compartment-specific pH can be achieved using subcompartment-targeted GFP-based pHluorin pH probes as described previously [26]. A pH-corrected E_{GSH} can then be calculated according to Eq. (3):

$$E_{\text{GSH}}^{\text{pH}} \approx E_{\text{roGFP2}}^{\text{pH}} = E_{\text{roGFP2}} - 59.1 \text{ mV} \times (\text{pH} - 7) \quad (3)$$

Because the pK_a values of glutathione and roGFP2 are both ~ 9 [1,41], a change in pH in the physiological range (pH 6–8) will affect E_{GSH} and E_{roGFP2} equally and Eq. (3) remains valid.

Measurement of H_2O_2 in mammalian cell culture

Mammalian cells expressing genetically encoded roGFP-based sensors allow live imaging with high spatiotemporal resolution on the single-cell level. In principle, live imaging can be applied to any mammalian system into which a sensor can be introduced, including cultured cell lines, primary cells, and tissue samples from sensor-transgenic animals. Imaging established cell lines is the simplest possibility and suits the purpose of testing compounds or gene depletions for their effect on defined cellular redox couples or oxidant levels. In the following section we describe a basic protocol for live redox imaging of cell lines. Whereas the yeast section of this article describes the use of the Grx1-roGFP2 sensor, the mammalian part will focus on the H_2O_2 sensor roGFP2-Orp1.

Materials

- Dulbecco's modified Eagle medium (DMEM; GIBCO, Darmstadt, Germany, Product 41965)
- Trypsin–EDTA (0.05%) (GIBCO, Product 25300)
- DMEM high glucose for imaging, without phenol red, 25 mM Hepes (GIBCO, Product 21063)
- Dulbecco's phosphate buffered saline (PBS; GIBCO, Product 14190)
- Polyethyleneimine (PEI), linear, MW $\sim 25,000$ (Polysciences, Eppelheim, Germany, Product 23966)
 - 1 $\mu\text{g}/\mu\text{l}$ stock solution in TBS
- FluoroDish (World Precision Instruments, Berlin, Germany, Product FD35-100)
- Chambered coverglass (Lab-Tek, Langensfeld, Germany, Product 155411)
- Gel-loading pipette tips
- Dithiothreitol DTT
 - 1 M DTT stock solution in PBS
- Diamide
 - 0.2 M diamide stock solution in PBS
- Hydrogen peroxide 30% (v/v)
- Biological samples: cell lines, stably or transiently transfected with sensor construct
 - HEK293
- All published mammalian probe expression constructs are available from our lab after signing a Material Transfer Agreement.

Instrumentation

- Neubauer Improved counting chamber (BRAND GmbH, Wertheim, Germany, Product 717805)
- Zeiss LSM 710 ConfoCor 3 microscope
 - Objective EC “Plan-Neofluar” 40 \times /1.3 oil

- XL-LSM710 S1 Climate chamber, with CO₂ vent and humidifier
- 405-nm UV diode laser
- 488-nm Ar laser
- Software
 - Zen 5.5 with service pack 1
 - ImageJ (McMaster Biophotonics Facility version, www.macbiophotonics.ca)

Protocol

Transfection

In principle, any established transfection or transduction protocol may be used for introducing sensor constructs into mammalian cells. In our hands a standard chemical-based transfection [42] or retroviral transduction gives good results with HeLa and HEK293 cells. We routinely use the following transfection protocol for transient expression of probes in HEK293 and HeLa cells:

1. Trypsinize near-confluent cells and seed 8×10^5 HeLa or 4×10^6 HEK293 cells into a 75-cm² cell culture flask with 15 ml full medium. The cells should reach 50–80% confluence by the following day.
2. Twenty-four hours after seeding the cells mix 25 µg plasmid DNA with 1.5 ml TBS and add 75 µl 1 µg/µl PEI stock solution. Immediately vortex for 10 s and let stand for 30 min.
3. Add DNA precipitate to the cells. After 2–4 h wash the cells twice with warm PBS and add fresh full medium.
4. Cells can then be seeded for imaging the next day.

In many situations it is of advantage to use cells with stable and homogeneous probe expression. Stable probe-expressing cell lines may be generated by retroviral transduction and selected by fluorescence-activated cell sorting.

Cell maintenance and preparation for imaging

Redox processes in cultured cells can be influenced by a number of factors, such as the passage number, the degree of confluency before and after seeding, the composition of the growth medium, infection with mycoplasma or other microbes, trypsinization, and transfection. It is important to keep these factors as small or as constant as possible. As the pH indicator phenol red is fluorescent and may form cytotoxic adducts upon UV radiation, medium without this dye must be used. Use Hepes-buffered (25 mM) medium to maintain constant pH. In addition, a CO₂ atmosphere must be used for long-term measurements.

1. Trypsinize probe-expressing cells and resuspend in imaging medium (with 25 mM Hepes, without phenol red).
2. Count the cells and seed into Lab-Tek eight-chamber coverglasses or FluoroDishes according to Table 1.
3. Cells can be used for live imaging experiments the following day.

Choice of microscope

Although ratiometric imaging of roGFP-based sensors is also possible with wide-field epifluorescence microscopes, here we focus on confocal laser scanning microscopy.

Environmental control

Many aspects of mammalian cell redox homeostasis may be influenced by environmental factors. Thus it is crucial to ensure defined

and constant temperature, humidity, and atmosphere during live-cell imaging. Several environmental control systems are commercially available for microscopy, from small heated imaging chambers placed onto the microscope stage to closed-system chambers with temperature-controlled and humidified CO₂ atmosphere. The latter are compatible with various types of imaging dishes and minimize focus drift problems caused by temperature gradients. Cells should be allowed to recover after being mounted on the microscope. The recovery time also alleviates focus drift due to temperature changes. The continued viability of the cells must be monitored throughout the experiment.

Imaging chambers

Depending on the needs of the experiment, various imaging chambers may be used: dish-based imaging chambers (e.g., FluoroDish FD-35), slide-based microchambers (e.g., Lab-Tek), or miniperfusion chambers (e.g., ibidi, Martinsried, Germany). Dish-based imaging chambers are easy to handle but allow only one treatment at a time. Slide-based systems require more care but allow multiple parallel measurements.

Microscope settings

Based on the excitation maxima of roGFP2, the 405- and 488-nm laser lines are used together with an appropriate emission window, for example 500–530 nm. As laser light can induce artificial oxidation, it is important to minimize exposure while keeping the signal-to-noise ratio (SNR) as high as possible. The main parameters relevant to lowering light exposure are laser power and scanning time. However, these are linked to other microscope settings such as resolution, pixel dwell time, photomultiplier gain, and pinhole diameter. Although they help prevent light-induced oxidation, low laser power, high photomultiplier gain settings, or fast scanning speeds lead to a lower SNR. Thus, one has to find a compromise; for many applications spatial resolution, i.e., number of recorded pixels and depth of focus (wider pinhole), can be sacrificed for lower laser power or faster scanning. For applications in which high spatial resolution is important one needs to change and optimize the parameters accordingly. To reduce the risk of pixel drift between the two excitation frames the line-scanning mode should be used.

Protocol for imaging roGFP2-Orp1-expressing cells on a Zeiss LSM710

The following protocol offers a standard procedure to calibrate the laser settings of a Zeiss LSM710 to a roGFP-based sensor. The hardware settings of the lasers should always be checked before starting the measurement session, to ascertain that the same software settings will give comparable results. Table 2 lists the settings we typically use to image the roGFP2-Orp1 sensor in HEK293 cells.

Calibration

The microscope needs to be calibrated with both fully reduced and fully oxidized cells. We use separate dishes of cells for each control.

1. Set the climate chamber to 37 °C and wait for at least 4 h before you start the imaging session. Otherwise focus drift problems due to thermal variations may arise.
2. Set up the microscope according to Table 2. These settings should be considered a starting point and may need to be changed during calibration.
3. Mount a FluoroDish with probe-expressing cells onto the heated stage with the appropriate holder. Close the climate chamber.
4. Use the ocular and transmitted light with additional UV to find cells appropriate for imaging. Try to minimize the exposure of cells to UV radiation.
5. Use the “live” mode to find the right focus plane. Let the cells settle, take a snap picture every 2 min until no focus drift is observable. You may have to readjust the focus several times.
6. Set the microscope to “time series” mode with 20 cycles of 10 s.

Table 1
Number of cells for imaging.

	8-Chamber slide	FluoroDish
HeLa	15,000	200,000
HEK293	80,000	1,000,000
Volume	400 µl	2 ml

Table 2

Typical Zeiss LSM710 settings for imaging of roGFP2-Orp1-expressing HEK293 cells.

Mode	Channel mode
<i>Imaging setup</i>	
Switch track every	"Line"
Track 1	405
Track 2	488 + T-PMT (transmission detector)
<i>Light path</i>	
Track 1 + 2	Detector range 500–530 nm
<i>Acquisition settings</i>	
Scan mode	Frame
Frame size	256 × 256
Scanning speed (pixel dwell)	"9" (3.15 μ s)
Averaging	"1" (off)
Bit depth	16 bit
Zoom	1.0
<i>Channel/laser settings</i>	
405-nm line	
Attenuator (transmission)	5%
Pinhole (section thickness)	3.78 (3 μ m)
Gain (V)	650
Digital gain	1
488-nm line	
Attenuator (transmission)	2%
Pinhole (section thickness)	3.78 (3 μ m)
Gain (V)	650
Digital gain	1

- Carefully remove 190 μ l of medium from the FluoroDish into a pre-warmed Eppendorf tube using a gel loading tip with a bent end and mix with 10 μ l of 1 M DTT. Take care not to touch the FluoroDish itself, to avoid disturbing the focus.
- Start the time series experiment and just before cycle 3 add the medium plus DTT back to the cells (5 mM final concentration): use a gel loading tip and add the full volume over a period of 3 s, evenly spreading the drops over the full cell culture area. Do not touch the stage or the dish itself, otherwise you will change position and focus.
- The cells should be fully reduced within 2 min. In the "split" view of the two wavelengths you may see a small increase in 488-nm fluorescence and a decrease in 405-nm fluorescence. Stop the time course when no further intensity change in either of the two channels takes place. Now the cells are fully reduced.
- Use a pseudocolor lookup table that highlights over- and under-runs in color but is otherwise grayscale (range indicator). Switch to "live" view and set the laser power/gain of the 488-nm line to a value at which the brightest cells are just below the saturation value. The laser power/gain of the 405-nm line should be set to a value at which cells with the lowest fluorescence are just above the underflow threshold. Take care not to bleach the cells, as this would lead to too-high settings. After finding a good setting, also look at different regions. Save your images and discard the cells.
- Repeat steps 3 to 6.
- Carefully transfer 190 μ l from the FluoroDish into a prewarmed Eppendorf tube using a gel loading tip with a bent end and mix with 5 μ l 0.2 M diamide.
- As in step 8, start the time series, then just before cycle 3, add back the medium containing diamide (final concentration 0.5 mM).
- The cells will become fully oxidized within 20 s. The brightness of the 488-nm line pictures should decrease considerably and the brightness of the 405-nm line should increase. Increasing the laser power/gain settings for the 405-nm line to a near-saturation value will improve the SNR but may also induce oxidative stress and/or photobleaching. If the setting of the 405- or 488-nm lines is changed, another DTT sample has to be measured to complete the calibration. Some cells are sensitive to diamide and lose viability within minutes—work as quickly as possible.
- Proceed with live imaging experiments.

Live imaging

The actual live imaging is similar to the calibration steps with DTT or diamide. In place of these chemicals apply the treatment of choice. The microscope settings must not be changed after calibration.

- Calibrate the microscope as described under Calibration.
- Use oil immersion objectives in preference to water immersion objectives because water evaporation leads to focus drift, especially during long-term experiments.
- Fix a FluoroDish or chambered coverglass onto the microscope and use transmitted light to find appropriate cells for imaging. Reduce the time using the mercury lamp to a minimum because this can induce cellular oxidation.
- For long-term measurements it is necessary to use a lid on the stage to control CO₂ and humidity. If you have to treat the cells during the measurement, make sure you do not disturb the focus while lifting or replacing the lid.
- Find the right focus plane using the "live" mode. Every time you mount new cells, let them settle; take a snap picture every 2 min until there is no focus drift observable.
- Once the cells are stable you can use the "positions" feature to add more regions of interest (FluoroDish) or additional wells of a chambered coverglass. This allows live imaging of cells with multiple treatments and imaging of different regions.
- As a control for UV laser-induced oxidation of your cells, run a time-series experiment of the expected experiment length with untreated cells. The 405:488 ratio should not change (see data analysis section below). It is necessary to sacrifice temporal resolution (number of cycles/time) for long-term experiments. Typically, we use short-term time series of 5 min with 20 cycles or long-term series of 4–6 h with images taken every 10 min, in both cases with no detectable UV-induced oxidation. This step does not have to be repeated every time but should be done before starting routine live imaging.
- For treating the cells take care to ensure good mixing of substances with the cell culture medium. A microliter-sized droplet of liquid will not mix properly with 2 ml medium. Therefore, remove 10 to 50% of the medium, mix it with the substance, and add it back to the cells. For pipetting inside the narrow climate chamber use a gel loading tip with the top bent at a right angle.

Data analysis using ImageJ

- On the LSM710 system save your data in the Zeiss .lsm format. Use the "save as" command to export the data to .tiff files. Each time point gives a single 16-bit .tiff file containing three channels: 405 nm, 488 nm, and transmitted light (TL). The file name consists of image and cycle number.
- Move all .tiff files of one time series into a new folder. Drag and drop this folder onto the ImageJ software and answer "yes" to the question "Open as a stack?"
- Convert to 32-bit image: Image—Type—32-bit.
- Separate channels: Plugins—Stacks—Shuffling—Deinterleave (3 channels). Picture 1 corresponds to the 405-nm line, picture 2 to the 488-nm line, and picture 3 to the TL.
- Set the lower and upper threshold for both channels (pictures 1 and 2): Image—Adjust—Threshold ... (dark background, press "Apply," "Set Background Pixels to NaN," answer "yes" to "Process all images"). Set the upper slider to a value at which the cells are red whereas the background remains largely black. If you had oversaturated pixels (for example, because of dead or rounded-up cells), move the lower slider slightly to the left to exclude those areas from the calculation. Beware that oversaturated pixels can also appear in later pictures of the time series. Use the same settings for all pictures you want to compare, make use of the presets. Only the highlighted area (red) will be considered for the analysis.

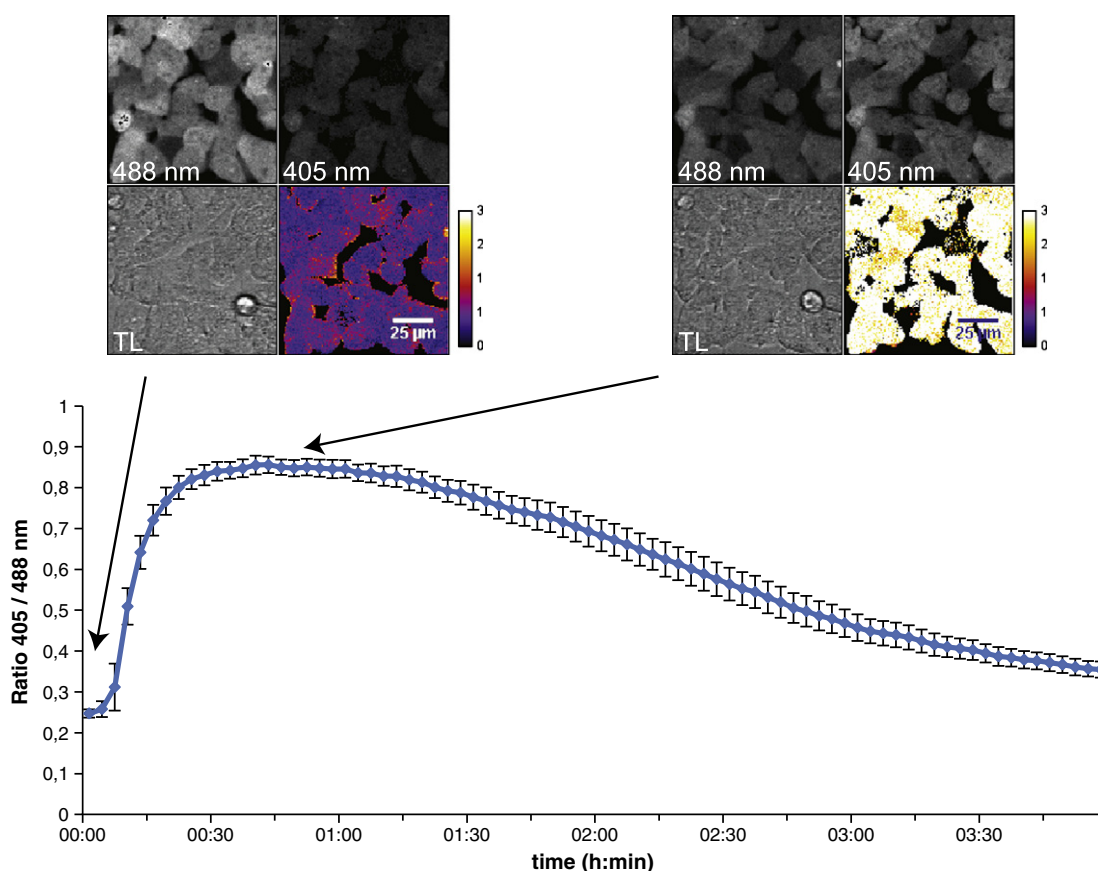


Fig. 5. Live imaging of HEK293 cells stably expressing roGFP2-Orp1. The day before live imaging 70,000 cells per well were seeded into a chambered eight-well coverglass. All eight wells were treated with 100 μM H_2O_2 after cycle 2 and imaged as described. The graph shows the mean ratio of all samples ($n=8$) and error bars representing the standard deviation. The top images show exemplary fluorescence (488 nm, 405 nm), transmitted light (TL), and ratiometric images.

6. Divide the 405-nm line stack by the 488-nm line stack: Process—Image Calculator ... Image #1 Divide by Image #2. Answer the question whether to process all pictures with “yes.”
7. The resulting new window shows the ratio of the 405-nm:488-nm lines in gray values. To visualize this in colors you can choose a different lookup table: Image—Lookup Tables—e.g., Fire.
8. To quantify the data first select an area (e.g., a single cell) with one of the sections tools or process the whole thresholded picture: Image—Stacks—Plot Z-axis Profile. The column “Mean” shows the mean ratio in the selected area at the various time points.

Data normalization

1. Analyze the data of the DTT and diamide treatments as shown above.
2. Dividing the highest ratio of the diamide time course (R_{ox}) by the lowest ratio of the DTT time course (R_{red}) gives the dynamic range of your setting. In our hands typical values for different sensors in different compartments range from 4 to 8.
3. To normalize the data, set the fully reduced state to an arbitrary value, e.g., 0.2. Then normalize the data using Eq. (4):

$$R_{\text{normalized}} = (0.2/R_{\text{red}}) \times \text{value.} \quad (4)$$

Expected results

Fig. 5 shows the results of a typical live imaging work flow as described. After calibration of the LSM710 microscope, roGFP2-Orp1-expressing HEK293 cells were challenged with a single 100 μM H_2O_2

bolus. Eight wells of a chambered coverglass were treated and imaged simultaneously. The data were analyzed using ImageJ and normalized to the fully reduced sample (the ratio of the DTT sample was set to 0.2). After addition of H_2O_2 the observed ratio increased, reaching a peak after 30 min. In this experiment, the cells did not decompose all the H_2O_2 within the time of the measurement. In general, the number of cells has a substantial influence on the kinetics of H_2O_2 elimination. In addition, the kinetics of H_2O_2 elimination also varies substantially between cell lines.

Future

There are many opportunities for the use of roGFP2-based probes, in particular through targeting to specific cellular locations. Genetically encoded redox probes have already been targeted to a range of organelles, including the endoplasmic reticulum, the mitochondrial matrix and intermembrane space, and the peroxisome [29,33,34,43,44]. However, many other possibilities exist, including targeting probes to sites of ROS generation, to specific membrane domains, or to the cytoskeleton, thus offering the potential to observe highly localized redox events.

Other exciting possibilities may include the development of redox-sensitive red-fluorescent proteins, which, should they become available, would allow dual-compartment or dual-redox-couple measurements within the same cell. This would enable study of the interplay between different redox couples or redox interactions between organelles on an individual cell basis. Red-shifted redox-sensitive fluorescent proteins would also overcome problems associated with excitation at 405 nm, e.g., autofluorescence issues. Alternatively, one may consider a fusion between a non-redox-active red fluorescent protein (RFP) and a roGFP2-based probe. In such a construct the RFP would provide

the reference against which the change in the 488-nm signal of roGFP2 is quantified. RFP-roGFP2 fusion proteins would suffer from a smaller dynamic range, but may offer advantages in situations in which background fluorescence at 405 nm is significant.

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