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Extending roGFP Emission via FRET Relay Enables Simultaneous Dual Compartment Ratiometric Redox Imaging in Live Cells

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Abstract

Reactive oxygen species (ROS) mediate both intercellular and intraorganellar signaling, and ROS propagate oxidative stress between cellular compartments such as mitochondria and the cytosol. Each cellular compartment contains its own sources of ROS as well as antioxidant mechanisms, which contribute to dynamic fluctuations in ROS levels that occur during signaling, metabolism, and stress. However, the coupling of redox dynamics between cellular compartments has not been well studied because of the lack of available sensors to simultaneously measure more than one subcellular compartment in the same cell. Currently, the redox-sensitive green fluorescent protein, roGFP, has been used extensively to study compartment-specific redox dynamics because it provides a quantitative ratiometric readout and it is amenable to subcellular targeting as a genetically-encoded sensor. Here, we report a new family of genetically-encoded fluorescent protein sensors that extend the fluorescence emission of roGFP via Förster-type resonance energy transfer to an acceptor red fluorescent protein for dual-color live-cell microscopy. We characterize the redox and optical properties of the sensor proteins, and we demonstrate that they can be used to simultaneously measure cytosolic and mitochondrial ROS in living cells. Furthermore, we use these sensors to reveal cell-to-cell heterogeneity in redox coupling between the cytosol and mitochondria when neuroblastoma cells are exposed to reductive and metabolic stresses.

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Author Contributions

All authors contributed to the design of the experiments. S.N., K.T., J.S., S.D., K.T. performed the experiments, and all authors contributed to data analysis and interpretation. The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

Supporting Information Available: The following files are available free of charge. Supporting Information.pdf. Supporting information includes extended methods, spectral cross-talk data, steady-state spectra for all constructs, confocal images of mitochondrial localization, control experiments for live-cell imaging, NAC and mitochondrial inhibition experiments for mRuby2-roGFP1 and mCherry-roGFP2 constructs, additional discussion on menadione as an oxidizing agent, and protein sequences for all constructs.

Graphical Abstract

Keywords

Fluorescent protein; redox; reactive oxygen species; roGFP; FRET; ratiometric; live-cell imaging; mitochondria

Reduction-oxidation (redox) reactions must be kept in a careful balance in order to maintain healthy cell growth and function^{1–3}. Loss of redox balance can lead to both reductive and oxidative stresses associated with aging, cancer, cardiovascular disease, and Parkinson's disease^{4–7}. For example, reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•−}), can oxidize protein-bound cysteines and other molecular species during normal metabolism and signaling⁸. However, excess ROS can cause oxidative stress and damage that leads to loss of function or cell death. To prevent such pathologies, redox enzymes and cellular redox buffers such as glutathione (GSH) are critical for maintaining proper redox balance. Together, both enzymatic and non-enzymatic components make up a “redox network”¹ that contributes to homeostasis in the face of changing intracellular and environmental conditions faced by prokaryotes and eukaryotes.

Like metabolic and signaling networks, the redox network is spatially organized within a cell, and compartments such as the cytosol and mitochondria contain distinct sources of ROS as well as distinct antioxidant mechanisms involving redox enzymes and redox buffers¹. As a result, compartment-specific redox and ROS dynamics exist with varying degrees of cross-compartment coupling^{9–12}. Redox coupling across compartments is a critical aspect of network response. For example, the production of mitochondrially-derived cytosolic ROS plays an integral role in retrograde mitonuclear communication and stress response^{13,14}. However, compartment-specific ROS dynamics and redox signaling between organelles has been poorly studied because of the lack of spectrally-compatible redox probes that are available to simultaneously quantify redox in multiple compartments within the same living cell.

Currently, the redox-sensitive green fluorescent protein (roGFP) sensors are widely used to study redox biology across model species, including yeast, plants, and animals^{15–21}. The roGFP sensors were originally developed by engineering two solvent-facing cysteines on the β -barrel of GFP¹⁸. Upon oxidation, the cysteines form a disulfide bond, causing a structural

change that alters the protonation state of the internal chromophore. Redox state is reported as the ratio of the two peaks in the fluorescence excitation spectrum near 400 nm and 485 nm, in which oxidation causes an increase in the 400 nm peak and a decrease in the 485 nm peak. There are two versions of the sensor: the roGFP1 sensor is based on wildtype GFP in which the 400 nm peak is greater in absolute magnitude, and the roGFP2 sensor is based on GFP(S65T) in which the 485 nm peak is greater in absolute magnitude. The roGFP sensors are particularly useful because they can be genetically targeted to specific cell types and subcellular locations, including the cytosol and mitochondria. Furthermore, they provide ratiometric readouts that are independent of expression level and enable quantitative measurements that can be compared between independent experiments.

However, the roGFP-based sensors are solely green fluorescent, which makes accurate measurement of redox dynamics in multiple compartments within the same cell difficult or impossible. Recently, red fluorescent redox sensors, HyPerRed²² and rxRFP²³, were engineered and can be used for dual-compartment, dual-color live-cell microscopy²⁴, but these sensors are not ratiometric, which can render quantitative analysis more challenging.

Therefore, in this work we describe the design, development, characterization, and validation of a first-generation family of roGFP-based sensors that exhibit long-wavelength emission via Förster-type resonance energy transfer (FRET) from a roGFP donor to a red fluorescent protein (RFP) acceptor. Importantly, we show that the redox properties of the parent roGFP donor are maintained when measuring the red emission of the roGFP-RFP sensors. We also report proof-of-principle studies that demonstrate that using multicolor imaging we can measure redox dynamics in the cytosol and mitochondria simultaneously within the same cell.

Results and Discussion

Sensor Design

Our sensor design employs a FRET “relay” strategy in which the roGFP serves as a redox-sensing donor that is fused to a RFP acceptor (Figure 1)^{25–28}. Excitation of the roGFP at any wavelength in its excitation spectrum will result in red fluorescence from the RFP while preserving both redox sensing properties and a ratiometric readout. In principle, the roGFP-RFP red emission will be spectrally distinct and enable dual color imaging by co-expressing both roGFP and roGFP-RFP in the same cell. This FRET relay strategy has been demonstrated with both CFP-YFP as well as GFP-RFP fusions engineered for high efficiency FRET, and this relay strategy has been used to overcome autofluorescence in live-cell imaging^{25–28}. It is important to note that FRET between the roGFP donor and RFP acceptor is designed to be constant in this strategy, and the ratiometric redox sensing originates solely from the change in the roGFP excitation spectrum. Thus, our design is fundamentally different from sensors that report redox changes by a change in FRET^{29,30}.

To implement our design, we first generated a library of twelve roGFP-RFP fusions to identify constructs that provide red emission via our FRET relay strategy. FRET efficiency depends on the distance, orientation, and spectral overlap between the donor and acceptor fluorescent proteins³¹. Therefore, we considered four main variables in our library design:

the roGFP choice, the fusion linker, the RFP choice, and the orientation of the fusion. We included both roGFP1 and roGFP2 as possible donors in our library because they have similar redox sensing capabilities and provide ratiometric readouts¹⁸. Although the roGFP2 sensor is more commonly used, the roGFP1 sensor can be beneficial for live-cell imaging because of its greater brightness when excited at 400 nm. For the fusion linker, we used a short seven amino acid linker (GGSGGRS) that has previously been used for high efficiency FRET between fluorescent proteins³². For the acceptor, we included the RFPs mRuby2, mApple, and mCherry (Figure 1)^{33–35}. The mRuby2 acceptor provides the greatest spectral overlap between the roGFP green emission and its absorbance, and it has been used as a FRET acceptor for the GFP Clover³³. The mCherry acceptor provides the greatest spectral separation between the green and red emission profiles, and it has been used in FRET pairs with GFPs both *in vitro* and *in vivo*³⁶. We included mApple as an acceptor with intermediate spectral profile and high brightness³⁴. Finally, we also included constructs in which the RFP acceptor was fused to either the N- or C-terminus of the roGFP donor (Figure 1).

Library screen and characterization of constructs as purified protein in solution

In order to select the best performing fusion constructs, we screened our library using steady-state and time-resolved fluorescence measurements of the purified proteins in solution. We first qualitatively screened our twelve constructs by measuring emission spectra and fluorescence anisotropy. We discovered that all twelve roGFP-RFP fusion constructs exhibit a clear red fluorescence FRET emission peak upon donor excitation (Figures S-1, S-2). Although there is substantial residual donor green fluorescence, the red emission peak is distinct and well above background (Table S-1). Furthermore, FRET is expected to cause depolarization of the red emission and thus a decrease in anisotropy. Indeed, all constructs also exhibit a large decrease in fluorescence anisotropy in the red emission channel upon donor excitation, despite the increase in overall protein size (Table 1). Thus, significant FRET occurs in all twelve constructs.

We next assessed whether the constructs preserve redox sensing by measuring the steady-state fluorescence excitation spectra in the presence of excess reduced or oxidized dithiothreitol (10 mM DTT). Purified proteins of all twelve constructs exhibit a redox-dependent ratiometric change in the excitation spectrum when collecting either direct donor roGFP green fluorescence emission or FRET acceptor red fluorescence emission (Figures S-1, S-2). From the excitation spectra, we quantified the excitation ratios for the FRET acceptor red fluorescence emission channel ($\text{Ratio} = F_{400\text{nm}}/F_{485\text{nm}}$) in the oxidized and reduced states, and we measured the dynamic range as the maximal fold change in ratio signal upon oxidation ($\text{dynamic range} = \text{Ratio}_{\text{oxidized}}/\text{Ratio}_{\text{reduced}}$) (Table 1). Compared to the roGFP1 and roGFP2 parent sensors, the roGFP-RFP fusion constructs exhibit on average a 40% and 50% reduction in dynamic ranges. The attenuation in the dynamic ranges is a consequence of spectral cross-talk, and the primary contribution comes from the direct excitation of the RFP acceptor when exciting at 490 nm (Table S-1). Despite the attenuation in dynamic range, all constructs exhibit clear preservation of redox sensing as well as a ratiometric response of the same magnitude as other sensors that have proven useful in live-cell imaging^{19,20,33}.

In order to quantitatively compare FRET efficiencies of the twelve constructs, we measured donor fluorescence lifetimes, which decrease with increasing FRET efficiency. As expected, the purified proteins of all of the roGFP-RFP constructs exhibit reduced donor fluorescence lifetimes relative to the roGFP1 and roGFP2 parent sensors, indicating FRET efficiencies from 25–32% (Table 1). We therefore selected three constructs with the highest FRET efficiencies, roGFP1-mApple, mRuby2-roGFP1, and mCherry-roGFP2, for further characterization of their redox sensing properties (Figure 2).

To determine if the redox properties of the parent roGFP are preserved in these three selected fusion constructs, we carried out redox titrations against increasing ratios of oxidized-to-reduced DTT. We determined that the midpoint potentials of roGFP1-mApple (-287.1 ± 0.4 mV) and mRuby2-roGFP1 (-288.7 ± 0.8 mV), measured in both direct donor green fluorescence and FRET acceptor red fluorescence channels, are in agreement with the midpoint potential of the parent roGFP1 measured in this work (-287.4 ± 0.7 mV) and as originally reported by Hanson *et al.* using the same method (-288 mV)^{15,18}. Likewise, we also determined that the midpoint potential of mCherry-roGFP2 (-272 ± 1 mV) is in agreement with the midpoint potential of the parent roGFP2 measured in this work (-274.4 ± 0.5 mV) and as originally reported (-272 mV)^{15,18}. Thus, our results confirm that our FRET relay constructs preserve the original redox properties of the parent roGFP and provide an excitation ratiometric response when measuring the FRET acceptor red fluorescence emission. (n=3, mean $\pm$ stdev)

With ~30% FRET efficiency, the roGFP-RFP constructs generate significant red fluorescence signal, but there is still a substantial amount of residual green fluorescence from the donor. The remaining spectral overlap precludes the use of both the parent roGFP and these first-generation roGFP-RFP sensors within the same cellular compartment. However, despite the residual green donor emission, we hypothesized that the roGFP-RFP fluorescence could be spatially separated from the roGFP fluorescence by targeting the roGFP-RFP sensors to a subcellular location. Thus, in order to validate the function of the roGFP1-mApple, mRuby2-roGFP1, and mCherry-roGFP2 constructs for dual-color imaging, we next measured mitochondrial and cytosolic redox potentials simultaneously within the same cells.

Cytosolic and mitochondrial redox potential

When Neuro2A mouse neuroblastoma cells were co-transfected pairwise with a mitochondrially-targeted roGFP-RFP fusion and its respective parent roGFP for cytosolic expression, we found that the red and green fluorescence signals were spectrally and spatially separated as hypothesized. The mito-roGFP-RFP fusions were targeted to the mitochondrial matrix by appending the signal sequence from cytochrome c oxidase subunit VIII (Cox8), which we and others have previously employed, and we observed excellent subcellular localization to mitochondria using confocal microscopy, as expected (Figure S-3)³⁷. Next, ratiometric imaging was carried out using widefield microscopy with sequential collection of green and red emission, in which the red emission was localized to mitochondria (Figure 3). The red emission was also used to generate a mitochondrial mask during image analysis in order to isolate mitochondrial and cytosolic signals, minimizing

mixing of the residual roGFP-RFP donor emission with the cytosolic roGFP signal. In order to measure redox potentials, we carried out baseline ratio measurements followed by a sensor calibration as previously described^{15,18,20}. In the calibration, sensors were fully oxidized by the addition of 1 mM H₂O₂ to the imaging solution followed by full reduction with 10 mM DTT, and the calibration values were used to calculate the percent oxidation of the respective sensors (Figure 3, Figure S-4)^{15,18,20}. As expected from previous reports, the mitochondrially targeted sensors on average are more oxidized than the cytosolic sensors because of the alkaline pH of the mitochondrial matrix^{15,18,20}. Taking compartment-specific pH into account (assuming cytosolic pH = 7.2 and mitochondrial matrix pH = 8.0)^{38,39}, our average measurements of the cytosolic and mitochondrial redox potential, -298 ± 6 mV and -338 ± 5 mV (mean $\pm$ stdev) respectively, agree well with previously reported values (Figure 3)^{15,18,20}. Importantly, our approach enables the direct comparison of the average mitochondrial and cytosolic redox potentials within the same cell. We discovered that mitochondrial redox potential is set -40 mV relative to cytosolic redox potential, which was highly consistent across independent cells and sensor pairings (Figure 3). Thus, our roGFP-RFP FRET relay redox sensors enable steady-state differences in redox potential between subcellular compartments to be quantified. We next tested whether our new sensors could also quantify differences in subcellular redox dynamics.

Heterogeneous responses to cytosolic reductive stress

We found that our dual-compartment imaging approach was able to reveal cell-to-cell population heterogeneity in compartment-specific responses to cytosolic reductive stress induced by exposure to excess N-acetylcysteine (NAC). In our initial redox studies (Figure 3), we observed that the Neuro2A cells exhibited an oxidative rebound following DTT addition during the final calibration phase, which indicates that Neuro2A cells respond to reductive stress. It has previously been observed that reductive stress causes a paradoxical oxidative response in HEK293, H9c2, and other cell types⁴⁰⁻⁴². NAC is a cell-permeant reductant that increases the levels of cytosolic reduced glutathione, but NAC is mitochondrial-lyimpermeant^{40,42}. Interestingly, NAC-induced reductive stress causes an oxidative response in mitochondria but not the cytosol^{40,42}. However, the compartment-specific difference in redox dynamics was determined by comparing population averages from cells separately transfected with cytosolic or mitochondrial roGFP-based probes, and therefore open questions remain regarding whether a difference in compartment-specific responses actually exists within a single cell and whether there is variability from cell-to-cell.

To answer this question, we used our dual-compartment imaging approach. After a baseline measurement period, Neuro2A cells expressing mitochondrial roGFP1-mApple and cytosolic roGFP1 were exposed to 6 mM NAC^{40,42} followed by cell-by-cell sensor calibration with H₂O₂ and DTT after every experiment (Figure 4). At the level of the population average, we observed that both the cytosol and mitochondrial matrix experienced an initial reduction in redox potential upon addition of NAC; however, a small oxidative rebound was observed in the mitochondria after several minutes, which was absent in the cytosol (Figure 4). Our population measurement is in agreement with the previous population measurements, and the smaller magnitude of the oxidative rebound likely reflects

cell-type differences^{40,42}. Furthermore, our measurements also reveal a reductant-specific difference in the stress response because, while NAC-induced stress causes an oxidative rebound in mitochondria only, DTT-induced stress causes an oxidative rebound in both the cytosol and the mitochondria. Importantly, our dual-compartment imaging approach revealed heterogeneous responses at the single-cell level. That is, some cells showed no change, some cells showed a reduction, and some cells showed an oxidation of the mitochondria relative to the cytosol in response to NAC-induced stress (Figure 4). In order to quantify the cell-to-cell heterogeneity, we scored the difference in compartment-specific responses by measuring the ratio of the mito-roGFP1-mApple percent sensor oxidation to the cytosolic roGFP1 percent sensor oxidation, which reflects the change in mitochondrial redox relative to cytosolic redox upon NAC treatment (Figure 4). The heterogeneity reflects cell-to-cell differences in compartmentalized redox buffering capacity as well as cross-compartment redox coupling^{9,40–42}. Thus, our roGFP-RFP FRET relay redox sensors enable differential compartment-specific dynamics to be quantified, showing that cytosolic stress can affect mitochondrial redox dynamics.

Heterogeneous response to mitochondrial inhibition

Lastly, we tested whether our dual-compartment imaging approach could reveal cell-to-cell population heterogeneity in compartment-specific responses to mitochondrial inhibition. The mitochondrial electron transport chain is a major source of both mitochondrial and cytosolic ROS. Inhibition of electron transport is expected to cause a decrease in ROS production, but low dose treatment with transport inhibitors or proton un-couplers can also cause an increase in ROS^{40,43,44}.

However, there remains an open question to what extent mitochondrial and cytosolic redox dynamics are coupled in the face of mitochondrial inhibition. To answer this question Neuroa2A cells expressing roGFP1-mApple in mitochondria and roGFP1 in the cytosol were grown in low glucose media in order to increase mitochondrial respiration prior to imaging⁴⁵. During imaging, mitochondrial inhibition was induced with the complex I inhibitor rotenone and the uncoupling protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Figure 5). At the level of the population average, we observed a trend suggesting that mitochondrial inhibition causes cytosolic reduction and mitochondrial oxidation (Figure 5). Importantly, our dual compartment imaging approached revealed cell-to-cell variation in the magnitude of mitochondrial oxidation relative to cytosolic reduction, and it also revealed that at the single-cell level complex compartment-specific dynamics can occur in response to mitochondrial inhibition (Figure 5). The heterogeneity may reflect cell-specific differences in respiratory efficiency as well as variability in redox buffer capacity in the face of metabolic inhibition. Overall, these results demonstrate that our roGFP-RFP FRET relay redox sensors can quantify how mitochondrial stress alters cytosolic redox dynamics at the single-cell level.

Conclusion

In this work, we developed a first-generation family of genetically-encoded redox sensors that exhibit an excitation-ratiometric red fluorescence readout, and we demonstrated their

utility in dual-color, dual-compartment live-cell imaging. These roGFP-RFP sensors utilize a FRET relay strategy to extend the emission spectrum of the donor roGFP out to red fluorescence wavelengths, and they preserve the redox sensing properties of the donor roGFP sensors. However, these first-generation sensors exhibit modest FRET efficiencies of approximately 30%, and they suffer from residual donor green fluorescence. Future optimization of the linker length could improve the FRET efficiency, but currently the residual donor emission leads to a spectral mixing problem that precludes the use of the roGFP-RFP sensors with other green fluorescence sensors if they are expressed in the same compartment. To circumvent this problem, we targeted the roGFP-RFP sensors to mitochondria and roGFP sensors to the cytosol, and spatial localization allowed us to measure cytosolic and mitochondrial redox potentials simultaneously within the same cell for the first time to our knowledge. Thus, these sensors are advantageous for quantifying subcellular redox potentials because they can be targeted to organelles, they exhibit an emission profile that is spectrally distinct from green fluorescent redox sensors, and they preserve the redox sensing properties of the roGFP sensors. Our results also demonstrate that our FRET relay strategy can in principle be used to extend the fluorescence emission of other green fluorescent sensors of redox or other analytes into red wavelengths for multiparameter imaging studies.

Methods

Materials

Chemicals and cell culture reagents were purchased from Sigma, Formedium, Qiagen, and ThermoFisher Scientific.

Molecular Biology and Protein Expression

Standard molecular biology, protein expression, and nickel affinity purification procedures were used. Polyhistidine-tagged proteins were expressed using the pRSETB bacterial expression vector. Protein lacking the polyhistidine tag was expressed in mammalian cells using the GW1 mammalian expression vector with four copies of the COX8 mitochondrial signal sequence for mitochondrial localization or without any signal sequence for cytosolic expression³⁷. Plasmid constructs are distributed via Addgene.

Steady-State Fluorescence Spectroscopy

For all spectroscopy measurements protein was diluted to a final concentration of 1 μ M in 75 mM HEPES, 125 mM KCl, 1 mM EDTA, pH 7.0 – 7.3. Solutions were degassed under vacuum and purged with argon gas, and protein solutions were equilibrated with 10 mM reduced DTT (1, 4-Dithiothreitol) or 10 mM oxidized DTT (trans-4,5-Dihydroxy-1,2-dithiane) for 1 hour before measurements. Fluorescence anisotropy was determined for samples oxidized with 10 mM ox DTT. Redox titrations carried out in solutions in which the total DTT concentration was held constant at 10 mM while the reduced DTT to oxidized DTT ratio was varied. Midpoint potentials were determined as previously described²⁰ and by fitting titration data to a Boltzmann equation.

Time-Resolved Fluorescence Spectroscopy

Fluorescence lifetimes of oxidized protein samples were measured on a custom-built microscope using a 447 nm pulsed diode laser with an instrument response function full-width at half maximum of 150 ps. roGFP donor emission was collected using a combination of 500 longpass and 550 shortpass filters. Empirical lifetimes were measured from the photon decays, and FRET efficiency was calculated as $1 - \tau_{\text{roGFP-RFP}}/\tau_{\text{roGFP}}$.

Live-Cell Imaging

Neuro2A cells were cultured according to ATCC recommendations and transfected with Effectene reagent (Qiagen) according to the manufacturer's instructions. Cells were imaged in glass-bottom 12-well plates at 6 minute intervals at room temperature on an Olympus IX83 microscope. DIC images were taken before and after experiments to verify cell health. Percent sensor oxidation and pH-adjusted midpoint potentials were calculated assuming cytosolic pH = 7.2 and mitochondrial matrix pH = 8.0 as previously described^{18,38,39}.

Data Analysis

Images were analyzed with ImageJ as previously described³⁷. Briefly, ratio signals were manually measured from ratio images obtained by dividing fluorescence images on a pixel-by-pixel basis. Fluorescence images were background subtracted and thresholded at three standard deviations above the mean background to reject background pixels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Design of the roGFP-RFP sensor library. (A) Diagram of the FRET relay from the roGFP donor to the RFP acceptor. (B) Fluorescence spectra showing roGFP (solid, green) emission and RFP excitation (dashed, left-to-right: mRuby2, mApple, mCherry). (C) Diagram of the N- versus C-terminal fusions tested in this work, in which L7 indicates the GGSGGRS linker.

Figure 2.

Characterization of steady-state and time-resolved fluorescence properties of the highest FRET efficiency roGFP-RFP constructs. (a–b) Redox-dependent ratiometric changes in the fluorescence excitation spectra when collecting (a) residual roGFP donor green emission (525 nm) and (b) RFP emission (mApple, 592 nm; mRuby2, 600 nm; mCherry 610 nm) via FRET. (c) Fluorescence emission spectra show the residual roGFP donor (green arrow) and RFP FRET acceptor emission peaks (red arrow). Emission spectra were collected with excitation at the isosbestic wavelength of 430 nm. (d) The roGFP-RFP constructs exhibit a decreased donor fluorescence lifetime (447 nm excitation), which is used to quantify FRET efficiency. (e) The roGFP-RFP constructs (circles, measured; dashed line, fitted) preserve redox sensing and exhibit similar DTT reduction potentials relative to the parent roGFP (triangles, measured).

Figure 3.

Simultaneous measurement of cytosolic and mitochondrial matrix redox potentials by the co-expression of cyto-roGFP and mito-roGFP-RFP in Neuro2A cells. (a) The mean response of the cell populations during the imaging time course (roGFP1-mApple, $n=12$ cells; mRuby2-roGFP1, $n=14$ cells; mCherry-roGFP2, $n=15$; errors are 95% confidence intervals). A baseline measurement period is followed by treatment with excess H_2O_2 and DTT (arrows) in order to calibrate the fully oxidized and fully reduced states of the sensor. The calibration is used to determine the sensor percent oxidation on a cell-by-cell basis for every experiment. (b) Single-cell analysis of compartment-specific redox potentials. Lines connect cytosolic and mitochondrial redox potentials for individual cells. Mean $\pm$ s.e.m. is shown for the population.

Figure 4.

Single-cell heterogeneity in compartment-specific redox dynamics is observed in response to reductive stress when Neuro2A cells are treated with 6 mM N-acetylcysteine (NAC). (a) The mean compartment-specific time-course responses of the cell populations (n=80 cells, errors are 95% confidence intervals.). On average, Neuro2A cells co-expressing mito-roGFP1-mApple (yellow) and cyto-roGFP1 (green) exhibit an oxidative rebound in the mitochondria (yellow arrow) that is not observed in the cytosol (green arrow). (b–d) Single-cell analysis reveals population heterogeneity in the compartment-specific response to NAC. (b) The ratio of mitochondrial sensor oxidation to cytosolic sensor oxidation quantifies the variability in the mitochondrial redox change relative to the cytosol at the single-cell level. Lines show responses for individual cells, and the population mean $\pm$ s.e.m. is shown. (c) Cell count histogram shows the heterogeneity in the magnitude of the single-cell responses. (d) Examples of single-cell responses.

Figure 5.

Single-cell heterogeneity in compartment-specific redox dynamics observed in response to metabolic stress when Neuro2A cells are treated with mitochondrial inhibitors (rot/FCCP: 2 μ M rotenone and 2 μ M FCCP) under low glucose conditions. (a) The mean compartment-specific time-course responses of the cell populations ($n=18$ cells, errors are 95% confidence intervals). On average, Neuro2A cells co-expressing mito-roGFP1-mApple (yellow) and cyto-roGFP1 (green) exhibit mitochondrial oxidation and cytosolic reduction in response to mitochondrial inhibition. (b–d) Single-cell analysis reveals significant heterogeneity in the extent to which mitochondria oxidize relative to the cytosol in individual cells. (b) The ratio of mitochondrial sensor oxidation to cytosolic sensor oxidation quantifies the variability in the mitochondrial redox change relative to the cytosol at the single-cell level. Lines show responses for individual cells, and the population mean $\pm$ s.e.m. is shown. (c) Cell count histogram shows the heterogeneity in the magnitude of the single-cell responses. (d) Examples of single-cell responses.

Table 1Steady-state and time-resolved fluorescence characterization of roGFP-RFP library (n=3, mean $\pm$ stdev.).

Construct	Anisotropy	Ratio(Ox) / Ratio(Red)	Donor Lifetime (ps)	FRET Efficiency
roGFP1	0.270 $\pm$ 0.001	2.61 $\pm$ 0.02	3145 $\pm$ 2	N/A
mApple-L7-roGFP1	0.047 $\pm$ 0.002	1.62 $\pm$ 0.04	2370 $\pm$ 10	0.247 $\pm$ 0.006
roGFP1-L7-mApple	-0.028 $\pm$ 0.001	1.51 $\pm$ 0.02	2175 $\pm$ 5	0.308 $\pm$ 0.003 *
mRuby2-L7-roGFP1	0.107 $\pm$ 0.001	1.63 $\pm$ 0.01	2140 $\pm$ 30	0.32 $\pm$ 0.01 *
roGFP1-L7-mRuby2	0.0660 $\pm$ 0.003	1.75 $\pm$ 0.01	2360 $\pm$ 20	0.249 $\pm$ 0.007
mCherry-L7-roGFP1	0.04 $\pm$ 0.02	1.44 $\pm$ 0.03	2280 $\pm$ 1	0.275 $\pm$ 0.001
roGFP1-L7-mCherry	0.03 $\pm$ 0.02	1.63 $\pm$ 0.04	2290 $\pm$ 20	0.273 $\pm$ 0.008
roGFP2	0.270 $\pm$ 0.001	8.07 $\pm$ 0.01	2905 $\pm$ 5	N/A
mApple-L7-roGFP2	0.022 $\pm$ 0.002	3.22 $\pm$ 0.03	2085 $\pm$ 2	0.282 $\pm$ 0.002
roGFP2-L7-mApple	-0.0132 $\pm$ 0.0008	3.66 $\pm$ 0.07	2074 $\pm$ 5	0.286 $\pm$ 0.003
mRuby2-L7-roGFP2	0.126 $\pm$ 0.001	4.86 $\pm$ 0.04	2130 $\pm$ 30	0.27 $\pm$ 0.01
roGFP2-L7-mRuby2	0.0698 $\pm$ 0.007	4.15 $\pm$ 0.07	2180 $\pm$ 20	0.248 $\pm$ 0.008
mCherry-L7-roGFP2	0.053 $\pm$ 0.002	2.92 $\pm$ 0.03	1986 $\pm$ 4	0.316 $\pm$ 0.003 *
roGFP2-L7-mCherry	0.018 $\pm$ 0.002	4.456 $\pm$ 0.008	2062 $\pm$ 2	0.290 $\pm$ 0.002

* Three highest FRET efficiency constructs chosen for in-depth characterization.