Transient light-induced intracellular oxidation revealed by redox biosensor

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ABSTRACT

We have implemented a ratiometric, genetically encoded redox-sensitive green fluorescent protein fused to human glutaredoxin (Grx1-roGFP2) to monitor real time intracellular glutathione redox potentials of mammalian cells. This probe enabled detection of media-dependent oxidation of the cytosol triggered by short wavelength excitation. The transient nature of light-induced oxidation was revealed by time-lapse live cell imaging when time intervals of less than 30 s were implemented. In contrast, transient ROS generation was not observed with the parental roGFP2 probe without Grx1, which exhibits slower thiol-disulfide exchange. These data demonstrate that the enhanced sensitivity of the Grx1-roGFP2 fusion protein enables the detection of short-lived ROS in living cells. The superior sensitivity of Grx1-roGFP2, however, also enhances responsiveness to environmental cues introducing a greater likelihood of false positive results during image acquisition.

1. Introduction

The concentration of glutathione in mammalian cells ranges between 1 and 14 mM, with the reduced form of glutathione (GSH) over 100 fold more prevalent than the oxidized form (GSSG). An abundance of data indicates that the GSH/GSSG redox couple is the most important determinant of the intracellular redox environment [1–3]. Modest alterations in the GSH/GSSG ratio influence vital cell functions, including proliferation, apoptosis, and death. Therefore, the quantitative measurement of GSH/GSSG alterations with high spatiotemporal resolution provides a necessary tool linking the glutathione redox potential with intricate redox regulated processes.

Recent progress in the development of several variants of redox-sensitive green fluorescent proteins (roGFPs) probes enabled studies of intracellular redox conditions [4–6]. The roGFPs conveniently exhibit two distinct excitation peaks responding reciprocally to redox changes, thereby enabling ratiometric analyses. The excitation ratio from these two wavelengths (400 and

490 nm for roGFP2) is much less dependent upon probe expression level and varying fluorescence output due to photobleaching, thus simplifying comparison between samples [6,7]. The fluorescence ratio indicates the extent of probe oxidation, and can be used for quantification of glutathione redox potential after additional calibration by exposing cells to strong reducing and oxidizing agents at the conclusion of an experiment [4,6]. RoGFP2-based measurements are effectively pH-independent since the ratio of the intensity of the two fluorescence excitation maxima in roGFP2 is unaffected by pH changes within physiological range (pH 5.5–8.0) [8–10]. Furthermore, technical protocols for monitoring GFP-based probes using epifluorescence microscopes and plate readers are currently available [8,10–13].

Because the redox equilibrium of roGFP2 occurs through interaction with glutaredoxins, the roGFP2 probe was recently fused to human glutaredoxin 1 (Grx1) to improve specificity and rate of thiol-disulfide exchange with intracellular GSH/GSSG couple [6,8]. However, the extent to which Grx1 affects the read-out of the roGFP2 (Grx1-roGFP2) sensor is not fully documented. It was essential to verify the exogenous moderators of Grx1-roGFP2 as recent reports have indicated influence of environmental growth conditions on the performance of roGFP2 [6,10,14]. Due to its rapid response to glutathione poise perturbations, the performance of Grx1-roGFP2 may be influenced to an even greater extent by

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experimental conditions that affect redox status, such as the source of excitation light, cell growth conditions, cell-cycle phase, and cell density [6,15–17]. These factors affecting live cell microscopy have been thoroughly discussed elsewhere [8,18].

Our aim was to further characterize Grx1-roGFP2 and identify factors affecting performance of the probe during live cell imaging. First, we demonstrate the occurrence of rapid light-induced generation of ROS. Second, we show that Grx1 attached to roGFP2 enables sensing of transient light-induced cytosolic ROS, which was dependent on the type of imaging media. Third, detection of transient ROS by Grx1-roGFP2 depends on time intervals between images. Fourth, the parental roGFP2 probe without Grx1 is incapable of detecting short-lived ROS. Finally, we demonstrate that media-dependent ROS is triggered by near UV light.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma (St. Louis, MO, USA) unless specified otherwise. Enzymes for the modification of DNA as well as the transformation reagent Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA). Chinese hamster ovary (CHO) K1 fibroblasts were from ATCC (Manassas, VA, USA). The oligonucleotides used for molecular cloning were obtained from Integrated DNA Technologies (Coralville, IA, USA). Plasmid pIRESpuro3 was purchased from Clontech (Mountain View, CA, USA). QIAprep spin miniprep and QIAquick PCR purification kits were from Qiagen (Valencia, CA, USA). RoGFP2 in pEGFP-N1 and Grx1-roGFP2 in pQE60 were kind gifts from Dr. James Remington (University of Oregon, OR, USA) and Dr. Tobias Dick (Cancer Research Center. Heidelberg, Germany), respectively.

2.2. Cell culture, transfection and cell sorting

Tissue culture supplies were from Falcon (Franklin Lakes, NJ, USA) and Corning (Corning, NY, USA). Cells were maintained at 37 °C in 5% CO$_2$ in Dulbecco's Modified Eagle Media (DMEM) (Cell Media Facility, University of Illinois, Urbana, IL, USA) containing phenol red, 1.5 g/L bicarbonate, 4.5 g/L glucose, 1 mM pyruvate, NEAA, and 10 mM HEPES buffer. Media were supplemented with 10% fetal bovine serum (Tissue Culture Biologicals, Tulare, CA, USA), streptomycin (50 mg/L), penicillin (50,000 U/L), and fungizone (0.25 mg/L). Streptomycin, penicillin, and fungizone were purchased from GIBCO (Grand Island, NY, USA), and Dulbecco's phosphate buffered saline (DPBS) from Lonza (Walkersville, MD, USA), HBSS and trypsin from Mediatech (Manassas, VA, USA). Trypan blue and DMSO, used respectively in cell counting and cryogen storage, were from MP Biomedicals (Solon, OH, USA), and other tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Cells were transfected using Lipofectamine 2000 (Invitrogen) to derive stable CHO cell lines, and cell sorting was performed as described elsewhere [19].

2.3. Genetic constructs

The PCR product of Grx1-roGFP2 sensor obtained from pQE-60 with the oligonucleotide primer set Cyto-For 5′-GCTAGCCATGGCT-CAAGAGTTTGTGAACTGCAA-3′ and Cyto-Rev 5′-GGATCCTTACTTGTACAGCTCGTCCATGCCGAGAGT-3′ was cloned into NheI/BamHI sites of pIRES-puro3. All PCR products were initially ligated into pCR2.1 (TOPO kit, Invitrogen, USA) and confirmed by automated sequencing.

2.4. Fluorescence microscopy

Image acquisition of live cells expressing roGFP2 or Grx1-roGFP2 constructs were performed in μ-Slide 8 well, ibiTreat microscopy chambers (Ibidi, Munich, Germany) and cultured in phenol red-free DMEM medium containing 3.7 g/L bicarbonate, 1 mM pyruvate, NEAA, 10% FBS, and 15 mM HEPES was used to incubate cells in imaging slides for 48 h. The adherent cells in the wells were washed twice with DPBS supplemented with 5% FBS and 10 mM glucose just prior to data acquisition. Cells were imaged on an inverted microscope (Zeiss Axiovert 200 M, Carl Zeiss AG, Switzerland) using a Zeiss Plan-Apochromat 63× oil immersion objective (1.4NA). The fluorescence microscope was also equipped with an environmental chamber with temperature and CO$_2$ controlled to 37 °C and 5%, respectively. Images were taken with a cooled CCD camera (cascade 512b, Photometrics, Tucson, AZ) controlled by AxioVision 4.8 software (Zeiss, Thornwood, NY, USA). Dual excitation ratio imaging used two cubes purchased as filter sets contained excitation filters 395/11 and 494/20 nm. A 510 nm dichroic mirror, and an emission filter at 527/20 nm were used for both cubes (Semrock, Rochester, NY, USA). The excitation light was provided by an EXFO X-Cite 120 metal halide lamp operated at 12% iris to reduce photobleaching. Exposure times were set at 100–400 ms, and images were taken every 15 s or as indicated further. Fluorescence images were background-corrected by manual selection of cell-free regions and were analyzed using AxioVision 4.8 Software (Zeiss).

3. Results and discussion

3.1. Glutaredoxin 1 enables monitoring media-dependent light-induced oxidation

To keep cells healthy throughout the duration of live cell imaging, we and others use standard culture medium without the pH indicator phenol-red as imaging media [10,14,20]. After selecting an area of interest in bright field, cells are briefly exposed to excitation light to determine the appropriate exposure time and adjust the focal plane for a clear view of the fluorescent signal. During image acquisitions in phenol-free DMEM, we noticed unanticipated variation in the probe baseline. Remarkably, all cells in a single field of view had similarly elevated 395/494 nm ratios as compared to the basal excitation ratio observed with unamended roGFP2 indicating that Grx1-roGFP2 was more oxidized at $t = 0$. To investigate the role of imaging media on Grx1-roGFP2 performance, both roGFP2 and Grx1-roGFP2 probes were targeted to cytosol of fibroblast CHO cells (Fig. 1). Fluorescence images of the probes were acquired at 530/10 nm after two sequential flashes of 395/12 nm and 494/10 nm every 15 s. Ratiometric analysis of time-lapse acquisition is expressed through the time resolved changes in excitation ratio of two channels (395/494 nm) depicted on Y-axis (Fig. 1A and A′). Excitation ratio is directly linked to extent of sensor oxidation, with a higher ratio corresponding to greater oxidation. Rapid light-induced oxidation of the cytosolic Grx1-roGFP2 sensor was observed in CHO cells imaged in clear DMEM. Conversely, the 395/494 nm ratio of roGFP2 sensor was not altered at steady state in the same medium and was subsequently increased 4 fold after oxidative challenge with diamide (Fig. 1B and B′). The oxidation state of Grx1-roGFP2 notably surged during the first time points and quickly plateaued within the measurable range of the sensor. Further application of diamide (after 6 time points) mediated full probe oxidation (Fig. 1A′). Thus, the redox state of Grx1-roGFP2 rapidly responded to perturbations in cytosolic redox within <15 s between consecutive acquisition time points until redox equilibrium was reached. To determine if the light-induced
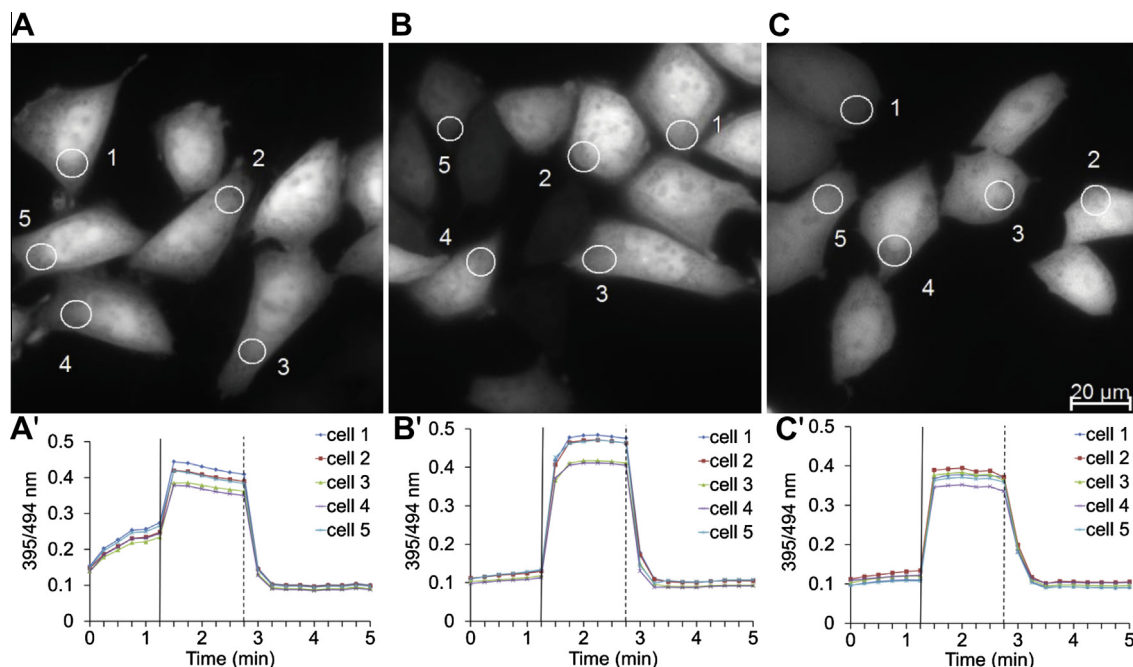
Fig. 1. Medium-dependent light-induced oxidation alters resting cytosolic glutathione poise. Time-lapse response of the (A, C) Grx1-roGFP2 and (B) roGFP2 sensors expressed in CHO cells and visualized in (A, B) DMEM and (C) DPBS to sequential treatment with exogenous oxidant and reductant. Each trace within panels (A′–C′) indicates a separate cell. Diamide (vertical solid line) and DTT (vertical dashed line) were added to cells at concentrations of 1 and 10 mM, respectively. The data are representative of 3 experiments.

probe oxidation was influenced by DMEM, cells were imaged in another medium. Fig. 1Ć shows changes of 395/494 nm excitation ratio of the Grx1-roGFP2 sensor over time in resting, oxidized and reduced cells imaged in DPBS. The intracellular oxidation of resting cells was blunted when stably transfected cells were visualized in DPBS. Therefore, excitation light during image acquisition oxidizes some component(s) of DMEM, which in turn affects cytosolic glutathione poise. The enhanced kinetics of Grx1-roGFP2 mediates detection of these redox perturbations. This finding mirrors the recent observation that ER-targeted roGFP-iL had an oxidative response to light excitation during time-lapses [21].

3.2. Time interval between images affects detection of light-induced transient oxidation

In contrast to Grx1-roGFP2, light-induced intracellular oxidation cannot be detected by roGFP2, which is characterized by its slow response to changes in redox potentials. This indicates that the oxidative event is rapidly reversed and detection of transient cytosolic oxidation may depend on the time interval between images of the time-lapse. Therefore, fluorescence images of the Grx1-roGFP2 sensor were taken with time intervals (between cycles) ranging from 10 to 60 s (Fig. 2). Additionally, each time-lapse was interrupted by a 5 min dark period. It became apparent that the level of sensor oxidation is strongly dependent on the time interval, indicating that light-induced oxidation is quickly reversed by the antioxidant capacity of a cell. In resting cells, the probe was most strongly oxidized with 10 s intervals, reaching a plateau after 1 min (Fig. 2A′). Cells were then provided with a 5 min dark period during which they fully recovered back to steady state, indicating that accumulative photooxidation is indeed reversible (Fig. 2A′ and B′). This recovery of the redox sensitive probe from oxidation confirms full integration of Grx1-roGFP into the cellular redox systems. Resuming image acquisition after a dark period caused a second oxidative event, validating the continued functionality of the probe and consistent occurrence of photooxidation. Furthermore,

extending the interval between images to 30 s enables cytosolic redox recovery from the preceding exposure, greatly reducing the level of oxidation observed (Fig. 2B′). Indeed, when images were taken every 1 min, the sensor remained at the basal state (Fig. 2C′). These data indicate the occurrence of rapid light-induced generation of ROS. The high antioxidant capacity of the cytosol is capable of quickly neutralizing these harmful species within tens of seconds. Therefore, even the rapid Grx1-roGFP2 could not detect media-dependent cytosolic oxidation when intervals were extended beyond 30 s. Alternatively, shorter time intervals enable visualization of light-induced transient oxidation because the cell is unable to restore glutathione poise between consecutive time points. As a result, during ten second intervals the sensor is strongly oxidized after the initial 3–4 cycles (Fig. 2A′). Thus, time intervals between images strongly affect the Grx1-roGFP2 probe read-out, which in turn may affect the accuracy of glutathione redox potential measurements.

3.3. Excitation at near-UV light triggers intracellular short-lived oxidation

In our experimental setup, each time point in live cell imaging is composed of two images collected from 395/11 nm and 494/20 nm excitation channels. To determine the contribution of individual channels to intracellular photooxidation, time lapse acquisition was collected with a single excitation wavelength (Fig. 3). Light-induced oxidation was observed only when resting cells were exposed to 395 nm excitation, while the probe remained steady at 494 nm excitation (Fig. 3A′ and B′). This observation was clearly in contrast to the redox-sensitive FRET-based CY-RL7 probe that responded independently of imaging media [20,22].

Next, to rule out FBS as a possible mediator of light-induced intracellular oxidation, serum was excluded from imaging DMEM. However, serum deprivation did not affect photooxidation of the cytosol (not shown). As a result, we routinely supplemented DPBS with 5% FBS and 10 mM glucose in subsequent experiments. We
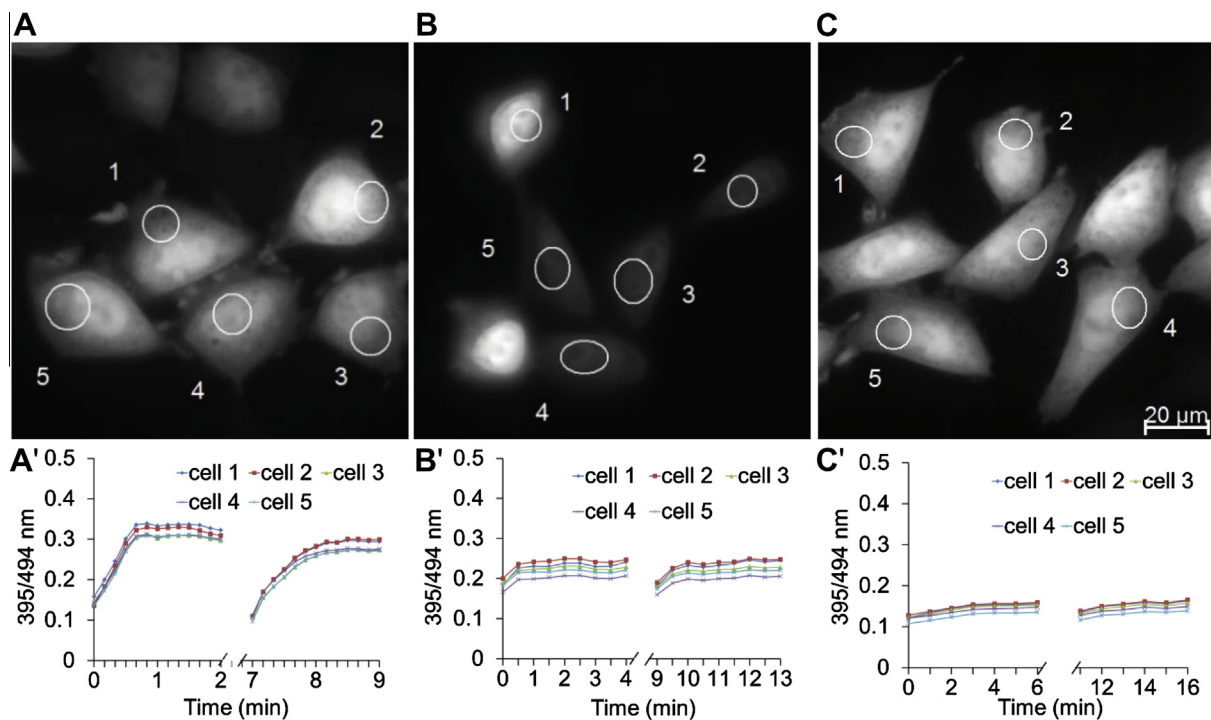
Fig. 2. Transient oxidation of cytosolic glutathione poise depends on time intervals between images. Time-lapse images of the Grx1-roGFP2 sensor targeted to cytosol of CHO cells were acquired in phenol-free DMEM with time intervals between images at (A) 10 s, (B) 30 s and (C) 60 s. Corresponding (A′–C′) ratiometric responses are shown. Each time lapse was interrupted by 5 min dark interval. The data are representative of 3 experiments.
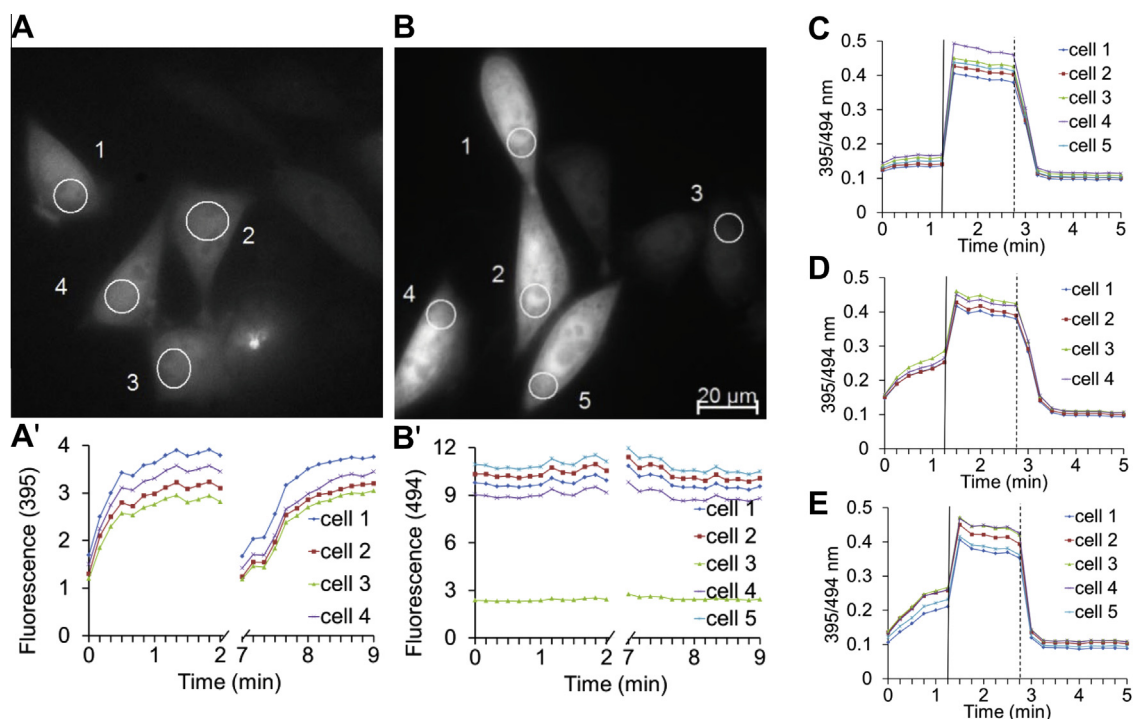
Fig. 3. Transient oxidation of cytosolic glutathione poise depends on excitation wavelength. Time-lapse images of the Grx1-roGFP2 sensor targeted to cytosol of CHO cells were acquired in phenol-free DMEM with a single wavelength excitation at (A) 395 nm and (B) 494 nm. Corresponding (A′–B′) ratiometric responses are shown. Ratiometric responses of the probe in DPBS supplemented with (C) amino acids, (D) vitamins and (E) riboflavin with amino acids mixtures. Diamide (vertical solid line) and DTT (vertical dashed line) were added to cells at concentrations of 1 and 10 m, respectively. The data are representative of 3 experiments.

then focused on the components of DMEM medium itself, which is supplemented with a complex mixture of amino acids and vitamins. First, DPBS supplemented with a commercially available mixture of the amino acids typically included in DMEM was used as imaging media. Regardless of time interval or amino acid concentration no oxidative effect was observed, so amino acids were eliminated as the causative component (Fig. 3C). Next, we implemented the same experimental design, but replaced the amino acid

mixture with a commercially available MEM vitamin mixture. Fig. 3D clearly demonstrates a pronounced effect of 8 vitamins (routinely included in commercial DMEM media), which closely resembled the oxidation observed on Fig. 1A'. Further screening of individual vitamins did not reveal any probe oxidation with the exception of riboflavin, but the response was very weak (not shown). However, when riboflavin was applied in conjunction with the aforementioned amino acid mixture the probe was strongly oxidized (Fig. 3E). Thus, intracellular oxidation upon exposure to blue light is the result of synergy between riboflavin either with vitamins or amino acids. Taken together, these data imply a DMEM-mediated biological effect rather than any direct influence on Grx1-roGFP2 behavior.

This study demonstrates that the Grx1-roGFP2 probe with improved rate of thiol-disulfide exchange over roGFP2 enables dynamic and rapid detection of changes in GSH poise in living cells that usually remained undetected because of high efficiency of scavenging systems. In turn, the sensitivity of Grx1-roGFP2 to oxidation at steady state (before any exogenous intervention) during set up of image acquisition became a major obstacle for determining basal glutathione redox potential. The results indicate that the choice of imaging media has profound effects on live cell oxidative stress assays, supporting previous observations on the potential influence of culture media in intracellular redox-regulated processes [10,23,24]. The present data clearly demonstrate that media-driven intracellular photooxidation is triggered exclusively by exposure to 400 nm excitation light. Extension of the interval between time points strongly diminishes the effect of imaging medium on the sensor response.

Acknowledgments

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