Antioxidants and Redox Signaling

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Forum Review

Transgenic organisms meet redox bioimaging: one step closer to physiology

Lija Swain[1], Maithily Nanadikar[2], Sergej Borowik[3], Anke Zieseniss[4], Dörthe M. Katschinski[5]

[1] Boston University School of Medicine, Boston University, USA, lswain@bu.edu

[2] Institute of Cardiovascular Physiology, University Medical Center Göttingen, Georg-August University of Göttingen, Germany
Humboldtallee 23, D-37073 Göttingen, Germany. Tel: +49 551 399778, Fax: +49 551 395896,
maithily.nanadikar@med.uni-goettingen.de

[3] Institute of Cardiovascular Physiology, University Medical Center Göttingen, Georg-August University of Göttingen, Germany
Humboldtallee 23, D-37073 Göttingen, Germany. Tel: +49 551 399778, Fax: +49 551 395896,
sergej.borowik@stud.uni-goettingen.de

[4] Institute of Cardiovascular Physiology, University Medical Center Göttingen, Georg-August University of Göttingen, Germany
Humboldtallee 23, D-37073 Göttingen, Germany. Tel: +49 551 399778, Fax: +49 551 395896,
anke.zieseniss@med.uni-goettingen.de

[5] Institute of Cardiovascular Physiology, University Medical Center Göttingen, Georg-August University of Göttingen, Germany
Humboldtallee 23, D-37073 Göttingen, Germany. Tel: +49 551 399778, Fax: +49 551 395896,
doerthe.katschinski@med.uni-goettingen.de

Corresponding author, e-mail: doerthe.katschinski@med.uni-goettingen.de

Dörthe Katschinski, Institute of Cardiovascular Physiology, University Medical Center Göttingen, Humboldtallee 23, 37073 Göttingen, Germany

doerthe.katschinski@med.uni-goettingen.de

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Abstract

Significance: Redox signaling is a common mechanism in the cellular response towards a variety of stimuli. For analyzing redox-dependent specific alterations in a cell, genetically encoded biosensors were highly instrumental in the past. To advance the knowledge about the importance of this signaling mechanism *in vivo*, models, which are as close as possible to physiology are needed.

Recent advances: The development of transgenic redox biosensor animal models has enhanced the knowledge of redox signaling under patho(physio)logical conditions. So far commonly used small animal models, i.e. *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio* and genetically modified mice were employed for redox biosensor transgenesis. However, especially the available mouse models are still limited.

Critical issues: The analysis of redox biosensor responses *in vivo* at the tissue level, especially for internal organs, is hampered by the detection limit of the available redox biosensors and microscopy techniques. Recent technical developments like redox histology and the analysis of cell-type-specific biosensor responses need to be further refined and followed up in a systematic manner.

Future directions: The usage of transgenic animal models in the field of redox signaling has helped to answer open questions. Application of the already established models and consequent development of more defined transgenic models will enable this research area to define the role of redox signaling in (patho)physiology in further depth.

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Introduction

Redox signaling occurs when cells respond to a change in the level of reactive oxygen species (ROS) or in consequence to a shift in the redox state of a responsive group such as a dithiol group (24). It involves the production of a specific ROS or specific dithiol couple modifications in target proteins at defined time points and locations. In addition to target proteins ROS modify also other biomolecules, e.g., lipids. For some time the specificity of this response was questioned, however it is now clear that ROS can indeed act as specific messengers both in the extracellular environment and within cells. This nuanced view of redox alterations as eminent key signaling events has at least in part benefited from recent technical developments in terms of genetically encoded redox biosensors, which allow measuring defined redox-signaling events. Although genetically encoded redox biosensors do not permit exact quantification of the signals, the semi-quantitative measurements have stimulated an improved insight into redox biology (28). Additionally, spatiotemporal analyses and dynamic real-time measurements of defined redox processes with subcellular compartment resolution can be achieved (13).

Genetically encoded biosensors have been applied for *in vitro* and *in vivo* studies. Cell lines are simple and powerful tools to study general cellular principles in the laboratory routine. Serial passaging of cell lines can cause genotypic and phenotypic variations over time. Therefore researchers try to analyze primary cells whenever possible (21). As a matter of fact, data generated from either stable cell lines or primary cell culture might however lack important information closer to the *in vivo* situation. Intercellular communication between different cell types, biophysical properties, local changes in the tissue environment like pH, pO_2 etc. are just some arguments for the need to study cells within the tissue context to reflect the physiological importance of the data analyzed. To this end introducing genetically encoded biosensors as transgene into a living organism is a new technical avenue in the field of redox biology.

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Genetically encoded redox biosensors

Early optical redox sensors relied mainly on small molecules changing their fluorescence behavior by reacting with the target of interest (27). The further development of imaging-based sensor techniques benefited from the cloning and recombinant production of fluorescent proteins. The green fluorescent protein (GFP) is composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range (38). GFP was first isolated from the jellyfish *Aequorea victoria*. The fluorescence characteristic of GFP is without need of any co-factor, which makes it a suitable reporter protein. The groundbreaking importance for the biosciences made by the achievements regarding the characterization of GFP was honored by awarding the Nobel prize in chemistry in 2008 "*for the discovery and development of the green fluorescent protein, GFP*" to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien (11). Many mutations have been engineered in the GFP sequence, including some which affect color, pH stability etc.. The establishment of the reduction-oxidation sensitive (ro) GFP was highly instrumental for the redox biology field (19). roGFP is a GFP variant in which surface-exposed cysteines are added to the molecule (Fig. 1a). roGFP, like wild-type GFP, has two excitation peaks (at ~400 nm and ~480 nm) and one emission peak at ~510 nm. Oxidation of the cysteine residues in roGFP results in an increase in excitation at ~400 nm (Fig. 1b). Reduction of those cysteines favors excitation at ~480 nm. Two major roGFP variants, i.e. roGFP1 and roGFP2 are commonly used. roGFP1 is derived from the wild-type chromophore with the amino acid S65 in the chromophore. It displays a dominant excitation peak in the UV range. The roGFP2 variant derived from enhanced GFP (EGFP) contains an S65T mutation and has a main excitation peak at 490 nm (19). The roGFP alone has a slow response to redox changes. To improve the specificity of the signal and the reaction rate, glutaredoxin-1 (Grx1) was fused to roGFP, which results in a rapid equilibrium between the glutathione (GSH)/glutathione disulfide (GSSG) couple and thus reflects the glutathione redox potential (E_{GSH}) (16). Other genetically encoded fluorescence protein-based biosensors were designed to sense specifically hydrogen peroxide (H_2O_2), which is one of the main ROS generated by cells. The fusion of the yeast peroxidase (Orp1) to roGFP2 and the Hydrogen peroxide sensor (HyPer) are examples for H_2O_2 sensing probes. HyPer is a fusion protein of the circularly permutated yellow fluorescent protein

(cpYFP) and the H_2O_2 sensitive bacterial protein OxyR (4,5,12). Its fluorescence is highly pH sensitive and measurements with HyPer should therefore always include simultaneous pH recordings. A more detailed overview about the available genetically encoded redox biosensors including benefits and limitations is given in a separate manuscript in the same special issue.

Genetically modified *in vivo* redox biosensor models

Genetically modified plant or animal models represent the most efficacious method of functional gene analysis. For overexpression of a gene in plants, nematodes, fish and mice, transgenesis is commonly applied. These model organisms were also used to generate transgenic (tg) redox biosensor models in the past (Fig. 2). In this review, we will summarize the results of the so far established redox biosensor models with a major emphasis on the mouse models described.

Redox imaging in plants, insects, nematodes and fishes

Plant (Arabidopsis thaliana)

Plants can frequently encounter different forms of environmental stress. Maintenance of the redox balance is vital for successfully responding to a change in the environment (30). Tg expression of roGFP1 and roGFP2 in the model plant *Arabidopsis thaliana* proved to be a powerful tool to monitor redox changes *in vivo* in different subcellular localizations in real time (22,29). Most interestingly the average redox potential determined with roGFP either targeted to the mitochondria or expressed in the cytosol was found to be distinct in the roots demonstrating that plants maintain a subcellular-specific redox homeostasis (22,40). Moreover the elongation zone of the Arabidopsis root was found to be more oxidized than either the root cap or meristem (22). Compartment and subcellular specific redox characteristics were further extended to organelle-specific oxidation by analyzing roGFP targeted to chloroplasts, mitochondria, peroxisomes and endoplasmic reticulum (7). The established methodology in *Arabidopsis* was subsequently used for understanding compartment and organelle-specific responses to different stress forms. It allowed analyzing the response towards nitrogen, drought, water, extended

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darkness, and salt stress (7,25,36,37,40). Employing redox biosensor *Arabidopsis* models thus helped to uncover compartment and subcellular redox potentials.

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Insect (Drosophila melanogaster)

Tg *Drosophila* flies were established employing the glutathione (Grx1-roGFP2 or the inverted domain order roGFP-Grx1) and H_2O_2 (Orp1-roGFP2) biosensors with either cytosolic or mitochondrial localization under the control of the tubulin promoter. Living larvae can be imaged *in vivo* based on their transparency during development. For imaging adult flies, preparations of the multicellular non-transparent tissues need to be performed. Albrecht et al. could elegantly demonstrate that during tissue preparations a non-specific oxidation of GFP based biosensors can occur (1). This can be prevented by adding N-ethyl maleimide (NEM) during sample preparation. NEM is a thiol blocking agent, which shields thiol groups from oxidation. Having solved the problem of non-specific oxidation, the authors went on to image the different roGFP tg fly models. They demonstrate both tissue-specific and cellular compartment-specific changes in the glutathione redox state and H_2O_2 levels as well as the involvement of redox alterations for aging processes (1,2). Comparing the results obtained with the glutathione specific Grx1-roGFP and the H_2O_2 specific Orp1-roGFP biosensors revealed that changes in the glutathione redox state and changes of H_2O_2 levels are not necessarily coupled (1). This is important evidence, which demonstrates that the glutathione pool can be regulated independently from altered H_2O_2 production in living organism.

Nematode (Caenorhabditis elegans)

HyPer and Grx1-roGFP2 tg *C. elegans* under the control of the large ribosomal subunit L17 promoter were one of the first redox biosensor nematode models, which allowed to detect distinct redox properties *in vivo* (3). The successful establishment of these models resulted in uncovering regions with distinct H_2O_2 production and GSSG/2GSH ratios as well as the characterization of redox mechanisms during development and aging (26). Subsequently, a mitochondria targeted roGFP1 *C. elegans* strain as well as roGFP1 expressing strains with a restricted expression in pharyngeal muscle (myo-2 promoter),

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intestine (sur-5 promoter) or mechanosensory neurons (mec-4 promoter) were developed and permitted the analysis of subcellular compartments or compartments of organs (23,35). These studies demonstrated a spatial organization of the E_{GSH} at the tissue and sub-tissue level.

Zebrafish (Danio rerio)

The transparency of tg zebrafish makes this model highly useful for bioimaging. In zebrafish mainly different versions of HyPer (6,18,31) and Grx1-roGFP2 (41) were used for transgenesis and helped to elucidate mechanisms of leukocyte recruitment to a site of sterile inflammation as well as heart regeneration. These results were reviewed recently elsewere (10).

Redox biosensor transgenic mouse models

Generation and application of tg redox biosensor expressing mice have been developed as a powerful technical tool to analyze redox signaling in the last years. They allow semi-quantitative analysis in complex tissue preparations (Table 1). Analyzing biosensor signals in intact mice is challenged by the necessity to apply suitable imaging techniques including confocal, 2-photon or wide field microscopy imaging techniques. Based on the size of a mouse and the wavelengths of the biosensors involved it is however still difficult to image internal organs *in situ*. Isolation of cells, tissue slices or isolated and *ex vivo* perfused organs are alternatives, which are as close as possible to the *in vivo* situation. To this end it is understandable that brain slices, isolated neurons or cardiomyocytes, Langendorff-perfused hearts and the skin are the most frequently analyzed cells/tissues applying genetically modified redox biosensor mice up to now.

A recently developed redox histology protocol, in which the *in situ* redox state is fixed by applying NEM on cryosections and is quantified by calibration with diamide and dithiothreitol (DTT), is a further important milestone in the usage of mice as “redox” model organism (14). Redox histology in combination with confocal microscopy allows imaging of the biosensor in histological sections. Adjacent cryosections of a snap frozen tissue are either left untreated, or are treated with NEM, DTT or diamide. DTT and diamide treated sections allow visualization of the biosensor in its fully reduced and oxidized form,

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respectively. The DTT and diamide response should be carefully titrated to ensure the full oxidation and reduction response. The NEM treated cryosection makes an insight into the actual redox status of the biosensor within the tissue possible. Comparison of the ratios of the fluorescence intensities obtained from the NEM treated cryosection with the data obtained from the DTT and diamide treated samples allows semi-quantitative insights into redox signaling at the tissue levels. The protocol was developed as an endpoint experiment. Analyses at different time points after adding DTT or diamide within the same sample are therefore not possible. The redox histology protocol was successfully applied to healthy, non-diseased and malignant tissue samples. The biosensors tested with this protocol include roGFP2-Orp1 as well as Grx1-roGFP2. Whereas the Grx1-roGFP2 was driven by the neuronal Thy1 promoter, roGFP2-Orp1 was driven by the artificial CAG promoter in the first description of the newly established protocol (14). The CAG promoter is a strong synthetic promoter containing the CMV early enhancer element, the first exon and intron of the chicken α-actin gene and the splice acceptor of the rabbit β-globin gene, which is frequently used to drive high levels of gene expression in mammalian expression vectors. Cells with a typical histological signature are easily detectable in tissue cryosections. An example of a redox-histology staining using cardiomyocyte-specific expression of Grx1-roGFP2 located to the mitochondrial matrix is shown in Fig. 3a and b. The mitochondrial signals in the tg cardiomyocytes are visible. The fluorescence is restricted to one cell type due to the cell-type specific promoter. Within the organ level however several cell types exist. This explains the necessity to further develop a possibility to distinguish between different cell types for example by immunohistochemistry techniques in combination with the redox histology protocol. This would enable researchers to unequivocally detect the response of a specific cell type when using a ubiquitously active non-cell type restricted promoter.

In the medical sciences the refined development of tg biosensor mouse models and respective imaging techniques is of utmost importance since the mouse is still an important model organism for many relevant diseases. Genetically modified mice and experimental interventions mimicking relevant diseases have been developed. The combination of tg redox biosensor mouse models crossed to a wide range of available

knock out mice or applied to disease models is thus the next logical step. This could help to advance the knowledge about the involvement of specific redox signaling pathways in physiology and pathology within the next years. The already established tg biosensor mouse models using either a restricted cell type specific or a ubiquitous promoter, prove that they are a sound basis for studying the importance of redox signaling in health and disease.

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Neuron-specific roGFP mice

Various neuron-specific roGFP-based mouse models have been established. They differ in the biosensor protein used, the promoter-driven specific cellular expression as well as the subcellular compartment analyzed.

Tyrosine hydroxylase promoter driven roGFP2 tg mice

Calcium entry can affect the mitochondrial oxidation status in autonomous pacemaking cells. This is of special importance for the disease progression of Parkinson`s disease (PD), which is a neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the substantia nigra. A decreased viability in the dopamine producing cells is associated with increased mitochondrial ROS and energy deficiency (34). Guzman et al generated a tyrosine hydroxylase promoter driven tg mouse model as well as control mice, in which the tg expression of roGFP2 is driven by the CMV promoter (17). The roGFP2 biosensor is targeted to the mitochondrial matrix in both models. Whereas the tyrosine hydroxylase promoter results in a restricted expression of the roGFP2 in dopamine producing cells, the CMV promoter allows ubiquitous expression. Oxidation of the biosensor in neurons was analyzed by two photon laser scanning microscopy employing midbrain slices. Relative oxidation of the sensor was analyzed after fully reducing or oxidizing the cells with DTT and aldrithiol, respectively. This strategy allowed comparing the relative sensor oxidation in dopaminergic neurons in the substantia nigra compacta and in the ventral tegmental area as well as cholinergic neurons in the dorsal motor nucleus of the N. vagus. These cells are affected during the onset and progression of PD. The results of the roGFP2 analyses revealed that autonomous pacemaking cells in the PD related areas share a functional relationship between membrane depolarization, spike activity, calcium entry and low calcium buffering capacity (15). Calcium must be extruded

from the pacemaking cells under consumption of ATP, which is produced during oxidative phosphorylation in the mitochondria. Using the above described mouse models the authors were able to convincingly show that calcium entry in the cells indeed affected the oxidation status of the mitochondrial matrix. This effect was further enhanced in a loss-of function mutation of DJ-1 a gene associated with early onset form of PD, which points to a functional involvement in the pathogenesis of this disease (17). In contrast to Grx1-roGFP2, non-coupled roGFP2 is in equilibrium with various other redox couples and thus is not suitable for example to measure specific ROS or the E_{GSH}. It is important to note that the redox status of roGFP thus is not necessarily equivalent to the increased production of ROS. Nonetheless the CMV-roGFP2 expressing mouse model was used in a later study to analyze the importance of mitochondria-derived ROS for antigen-specific T-cell activation (42).

Thy-1 promoter driven roGFP tg mice

Two independent groups generated thymocyte differentiation antigen/CD90 (Thy-1.2) promoter driven roGFP based tg mouse models (9,44). Whereas Breckwoldt et al used Grx1-roGFP2 targeted to the mitochondrial matrix, Wagener et al employed the uncoupled roGFP1 either located to the cytoplasm or targeted to the mitochondrial matrix. Since Grx1-roGFP2 and roGFP1 pick up the specific glutathione redox couple versus the more general cellular redox balance, the analyses are not directly comparable. Breckwoldt et al used their mouse model for analyzing the mitochondrial redox potential in different neurological diseases including spinal cord injury and amyotrophic lateral sclerosis. Upon injury they observed an influx of extracellular Ca^{2+}, which initiated a permanent mitochondrial glutathione oxidation that spread from the injury site along the affected axons. This was followed by mitochondrial shortening and fragmentation. Spontaneous redox signals in neurons at the single mitochondrion level where transients of glutathione oxidation go along with shortening and re-elongation of the organelle were also observed in healthy mitochondria, which further increased upon stimulating electrical activity (8).

Wagener et al focused in their study on mapping redox conditions at the subcellular level by concentrating on comparing the roGFP1 signals either expressed in the cytosol or the mitochondrial matrix (44). They calibrated the sensor response in both

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compartments by treating hippocampal slice with 10 mM DTT or 5 mM H_2O_2. Based on this calibration they determined the relative oxidation of the roGFP and quantified the redox potential. This revealed a significantly more oxidized E_{roGFP1} in the mitochondrial matrix compared to the cytosol in various brain regions. A striking difference was additionally detected by comparing the soma and the dendrites with a more oxidized roGFP1 in the soma (Table 1).

Erythrocyte-specific roGFP2

Although oxygenated hemoglobin [Hb(FeII)O_2] is considered to be a relatively stable molecule, it can physiologically auto-oxidize to methemoglobin [Hb(FeIII)]. Auto-oxidation is almost entirely responsible for ROS generation inside red blood cells. Erythrocytes are exposed to a wide variety of different pO_2 levels in the venous and arterial blood, which renders the erythrocytes prone to redox modifications. Xu et al developed tg mice, which express roGFP2 driven by a β-globin mini-promoter (47). Around 50% of peripheral red blood cells expressed roGFP2 in this mouse line. In unperturbed tg red blood cells a E_{roGFP2} of -318.5 ± 8.07 mV was calculated based on the degree of roGFP2 oxidation after treatment with 1 µM H_2O_2 and 10 mM DTT. The pro-hemolytic compound phenylhydrazine resulted in a dose-dependent increase of roGFP oxidation. A similar effect was observed as part of red blood cell aging, implying that oxidative alterations contribute to red blood cell senescence and damage.

Polypeptide chain elongation factor 1a promoter-driven roGFP

The polypeptide chain elongation factor 1a (EF1α) promoter in combination with the roGFP1 biosensor was used in tg mice for monitoring oxidative stress *in vivo* (46). RoGFP expression driven by the EF1α promoter was found in various organs. In the skin the expression was mainly restricted to keratinocytes demonstrating that the roGFP1 oxidation status can be tested in the skin of this tg mouse model as fluorescence of these cells is optically accessible in freely moving mice. The established mouse model is thus one of the rare examples for monitoring roGFP *in vivo* non-invasively. To this end the authors applied three light-emitting diodes turned on sequentially and synchronized to a charge coupled device camera. The contribution of autofluorescence however was significant

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when using C57BL/6J mice in this setup and demonstrates one current drawback of applying genetically encoded biosensors *in vivo*. The authors were able to reduce the autofluorescence problem in part by using albino hairless mice decreasing the autofluorescence generated by the fur and melanin.

Cardiomyocyte-specific roGFP based tg mice

αMHC promoter driven cyto and mito Grx1-roGFP2 mice

Various cardiovascular diseases have been strongly associated with an altered redox signaling (39). Especially the ROS-generating NADPH oxidase enzyme complexes 2 and 4 are regarded to be of importance for detrimental or protective effects during the course of heart failure development in consequence of ischemia, mechanical load etc. (48). The intracellular compartmentalization and organization of cardiomyocytes is dominated by the sarcomere units. Contractile activity depends on mitochondrial ATP production. Cellular metabolism, cellular redox signaling and excitation contraction coupling are thus inevitably linked. The recently established Grx1-roGFP2 tg mice, in which the transgene is driven by the cardiomyocyte-specific αMHC promoter, allowed gaining insight into the changes of the cellular E_{GSH} in resting cardiomyocytes and after stimulation (43). Since Grx1-roGFP2 was either located in the cytoplasm or targeted to the mitochondria the E_{GSH} was compared in these two subcellular compartments. Most interestingly the mitochondrial matrix was found to be more reduced (E_{GSH} -278.9 mV) compared to the cytoplasm (E_{GSH} -254.8 mV) (Table 1). This difference observed in isolated cardiomyocytes was further confirmed by analyzing Langendorff-perfused whole hearts. Intriguingly the difference between the E_{GSH} in the cytoplasm and mitochondrial matrix in cardiomyocytes is in sharp contrast to the redox potential as determined in the Thy-1 promoter driven roGFP1 tg mice described above. The discrepancy might be due to the different sensors, i.e. Grx1-roGFP2 versus roGFP1, applied or is due to the different cell types, i.e. neurons versus cardiomyocytes, analyzed. Stimulation of the cardiomyocytes isolated from the Grx1-roGFP2 tg mice with isoprenaline or angiotensin II demonstrated that the E_{GSH} in the cytoplasm and the mitochondrial matrix responds with a specific pattern in each compartment.

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The formation of cellular ROS and alterations in redox signaling are linked to ageing and age-related diseases (20). Because of conflicting data, the link between ROS and ageing however is not exactly clear. ROS are in some ageing studies found to decrease lifespan and in other studies found to act as a pro-survival factor (45). Regarding the E_{GSH} mostly a pro-oxidant shift accompanied by an elevation of protein-glutathione mixed disulfides have been previously reported in *Drosophila melanogaster* (32) and different tissues of mice (33). However, these studies lack information about the E_{GSH} in different subcellular compartments. Tg redox biosensor animal models make it possible to answer open questions like these by following the biosensor response in ageing mice. Mice develop with increasing age an impaired heart function. In case of the αMHC Grx1-roGFP2 mice either expressing the sensor in the cytoplasm or the mitochondrial matrix a significantly impaired fractional area shortening (FAS) and ejection fraction (EF) is visible at an age of over 80 weeks (Fig. 4a). The E_{GSH} in isolated cardiomyocytes from young versus old mice were analyzed by treating the cells with H_2O_2 or DTT. Based on the maximum oxidation and maximum reduction response of the Grx1-roGFP2 biosensor the E_{GSH} was calculated as described earlier (43). The E_{GSH} of cardiomyocytes isolated from the aged mice compared to young animals revealed significant differences. The E_{GSH} in the cytoplasm of the aged mice was significantly reduced compared to the young animals. Interestingly this was accompanied by no change in the mitochondrial matrix demonstrating that both compartments respond independently from each other. The strict difference of the E_{GSH} in the cytoplasm and the mitochondrial matrix as seen in the young animals was lost with ageing. This data set demonstrates how tg redox biosensor animal models can be used to define the role of redox signaling in (patho)physiology in further depth.

As outlined above multiple tg biosensor organisms have been developed and have been employed to explore spatial and temporal changes of redox signaling in multicellular organisms. More cell type-specific characteristics in redox signaling are worth of being analyzed at the tissue level in the future. These analyses will rely on the further development of more cell-specific tg biosensor mice and other model organisms. However, there are several technical issues, which still need to be solved. Redox biosensor responses in tg animal/plant models cannot be easily quantified. So far only redox potentials of GSH

(Grx1-roGFP) and roGFP1/2 have been quantified in mouse models. This is partly due to the fact that calibration of the fluorescence signal in complex tissue preparations is difficult. Careful titration of the concentration of H_2O_2 or diamide and DTT needs to be performed to determine the concentrations required to achieve full oxidation or reduction of the biosensor, respectively. Such experiments can`t be done for all tissues *in vivo*. Furthermore, in all experiments a strict control for autofluorescence needs to be done. Moreover, not all tissues can be easily accessed by the applicable imaging techniques *in vivo* based on insufficient penetration depth. If sample or tissue preparation is needed to image otherwise inaccessible parts, a non-specific artificial oxidation during sample preparation needs to be prevented. Regarding pH-sensitive redox biosensors like HyPer, possibilities to monitor pH changes or pH control biosensors simultaneously need to be considered.

Innovation

Application of redox bioimaging in the context of tg organisms has enhanced our understanding about the importance of redox signaling under physiological and pathological conditions. Several technical issues regarding quantification of redox signaling *in vivo* still needs to be solved. However, a consequent development of this field will help to gain insight into redox biology and the specific physiological function.

Conflict of interests:

There is no conflict of interest to declare

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Abbreviations

C. elegans, *Caenorhabditis elegans*; CMV, Cytomegalovirus; cpYFP, circularly permuted yellow fluorescent protein; DTT, dithiothreitol; EF, ejection fraction; EF1α, elongation factor 1α; EGFP, enhanced green fluorescence protein; E_{GSH}, glutathione redox potential; FAS, fractional area shortening; GFP, green fluorescence protein; Grx1, glutaredoxin1, GSH, glutathione, GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; HyPer, Hydrogenperoxide sensor; NEM, N-ethylmaleimide; PD, Parkinson`s disease; roGFP, reduction-oxidation sensitive GFP; ROS, reactive oxygen species; tg, transgene

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Table 1: Transgenic redox biosensor mouse models. Table gives an overview of the up to date developed redox biosensor mouse models, their application in terms of redox imaging and the observed phenotype.

Mouse line; (reference)	Promoter	Biosensor	Cellular compartment	Cell type specific expression	Imaging techniques	Redox potential	Phenotype
TH-mito-roGFP CMV-mito-roGFP; (Guzman et al., Nature, 2010; Goldberg et al., Nat Neurosci, 2012)	Tyrosine hydroxylase, CMV	roGFP2	mitochondrial matrix	dopamine producing cells, ubiquitous	2-photon laser scanning microscopy	-	activation of L-type Ca^{2+} channel induces changes in the redox status in the mitochondria in dopaminergic neurons
Thy1-mito-roGFP2; (Breckwoldt et al., Nature Med, 2014)	Thymocyte differentiation antigen	Grx1-roGFP2	mitochondrial matrix	neurons	excitation ratiometric wide field imaging, confocal and 2-photon imaging	-	axonal mitochondria undergo reversible redox changes, which are amplified by neuronal activity and neuronal insults

Thy1-mito-roGFP1 Thy-cyto-roGFP1; (Wagener et al., Antioxid Redox Signal, 2016)	Thymocyte differentiation antigen	roGFP1	mitochondrial matrix, cytoplasm	neurons	wide field ratiometric fluorescence life time imaging, ratiometric two photon imaging	E_{roGFP1} cytoplasm 305 ± 2 mV (CA1), -297.1 ± 4.2 mV (CA3)	mitochondria are more oxidized compared to the cytoplasm; subcellular differences in the oxidation between dendrites and soma
β-globin-roGFP2; (Xu et al., Blood, 2011)	β-globin mini promoter	roGFP2	cytoplasm	red blood cells	flow cytometry	E_{roGFP2} -318.5 ± 8.07 mV	hemolytic compounds and senescence increase oxidation response in red blood cells
EF1a-roGFP1 EF1a-PDHL-roGFP1; (Wolf et al., J Invest Derm, 2014)	elongation factor 1a	roGFP1	cytoplasm, mitochondria	keratinocytes, eyes, brain, pancreas, thymus and intestine	fluorescence LED-based imaging of the skin	-	UVA radiation results in an oxidation response in mitochondria but not cytosol of keratinocytes

Mito-roGFP2-Orp1; (Fujikawa et al., 2016)	CAG	roGFP2	mitochondria	ubiquitous	redox histology	-	
αMHC cyto-Grx1-roGFP2 αMHC mito-Grx1-roGFP2; (Swain et al., Circ Res, 2016)	αMHC	roGFP2	cytoplasm, mitochondria	cardiomyocytes	epifluorescence microscopy of isolated cardiomyocytes and Langendorff-perfused hearts	E_{GSH} cytoplasm -254.8 ± 0.8 mV E_{GSH} mitochondrial matrix -278.9 ± 0.4 mV	mitochondria are more reduced compared to the cytoplasm; subcellular differences after stimulation with isoprenaline or Angiotensin II

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Figure legends

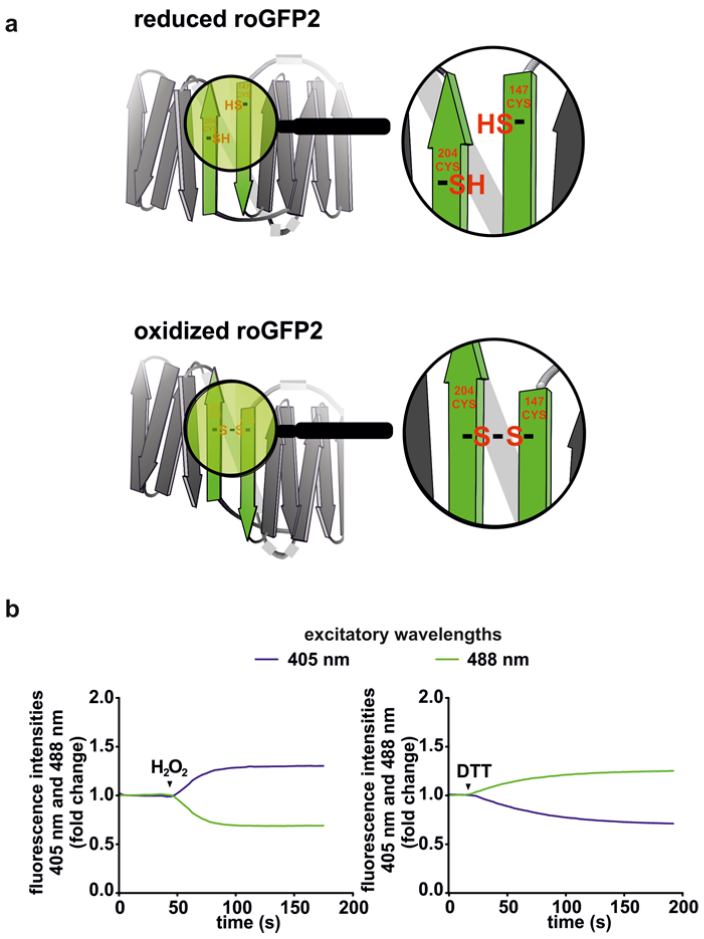

Figure 1: roGFP as a redox sensitive biosensor. (a) Schematic drawing of reduction oxidation (ro)GFP in its reduced and oxidized state. Two surface-exposed cysteines (Cys204 and Cys147) in the roGFP result in reversible disulfide formation on oxidation. Disulfide formation results in a structure-dependent shift of the chromophore, which is reflected in the fluorescence intensities at the excitatory wavelengths 405 and 488 nm. **(b)** Response of isolated αMHC promoter driven Grx1-roGFP2 transgenic (tg) cardiomyocytes to H_2O_2 and DTT. Fold changes of fluorescence intensities at the two excitatory wavelengths 405 and 488 nm were determined in cardiomyocytes. Cells were isolated from a tg mouse expressing Grx1-roGFP2 localized to the mitochondrial matrix. Fluorescence signals were recorded after a single bolus treatment with 100 µM H_2O_2 or 1 mM DTT. Experiments were performed as described earlier (43).

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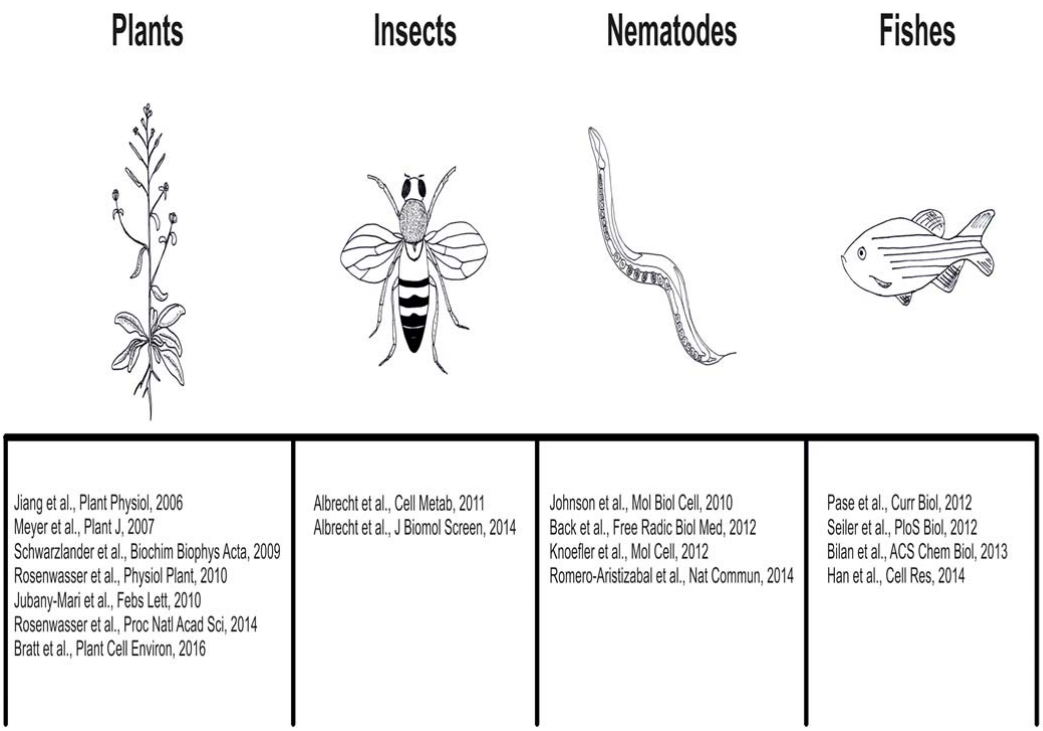

Figure 2: Applications of transgenic redox-biosensor plants and small animal models.

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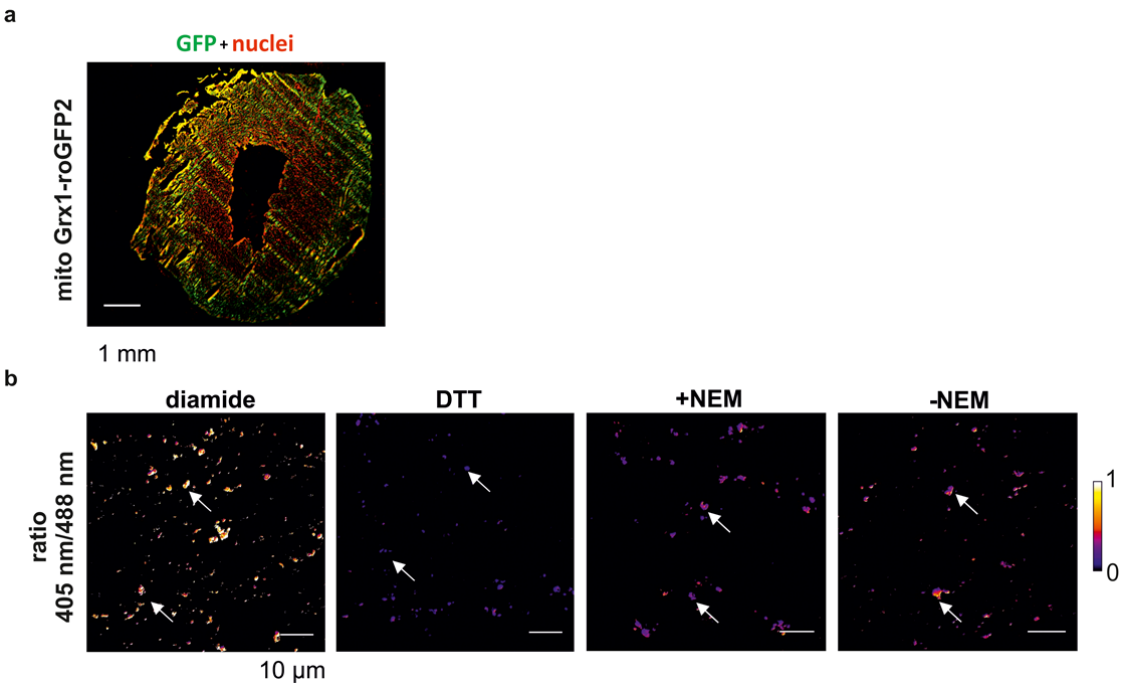

Figure 3: Redox histology of cryosections isolated from a transgenic Grx1-roGFP2 mouse. A heart was isolated from a transgenic (tg) αMHC promoter driven Grx1-roGFP2 mouse, in which the biosensor is localized to the mitochondrial matrix. After isolation the atria and right ventricle were removed. (**a**) Transverse cryosection of the left ventricle showing the GFP expressing cardiomyocytes (green) and TO-PRO-3 stained nuclei (red). The magnification applied is not sufficient to discriminate single mitochondria and shows some artefacts from the cryosection procedure. (**b**) Cryosections of the left ventricles were analyzed by the authors of this review article applying a redox histology protocol as described previously by others (14). Spots indicated as examples by the arrows represent the Grx1-roGFP expressed in the mitochondrial matrix. Dithiothreitol (DTT) and diamide were used to achieve full reduction or oxidation of the Grx1-roGFP, respectively. The values obtained from the fully reduced and oxidized sections were used for analyzing the cryosections, in which the redox status was either fixed with N-ethylmaleimide (NEM) or were left untreated (-NEM). The heat map was calculated based on the ratio (excitations at 405 nm/488 nm) of the fully oxidized state after treatment with diamide (=1) and the fully reduced state after treatment with DTT (=0).

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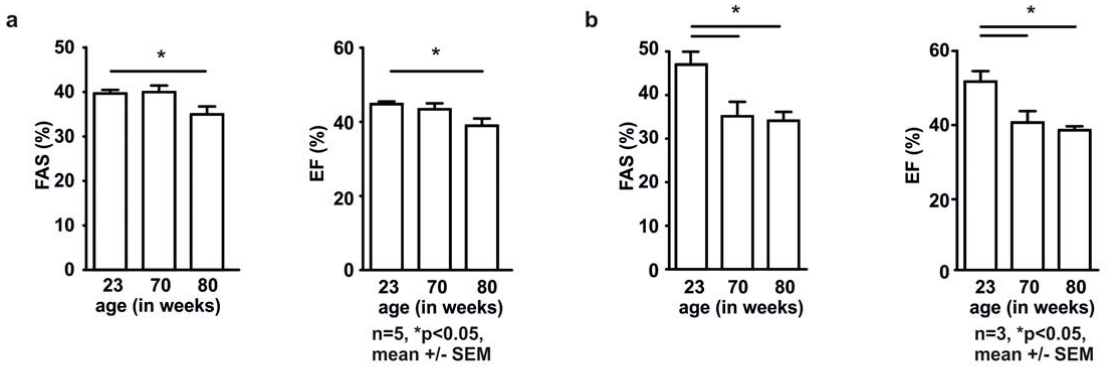

c

	Compartment			
	cyto age, 20 weeks	cyto age, >80 weeks	mito age, 20 weeks	mito age, >80 weeks
E_{GSH}	-262.3 ± 1.0 mV [a]	-278.1 ± 0.6 mV [b]	-276.1 ± 1.0 mV	-276.5 ± 0.8 mV

[a] p<0.05, cyto, age 20 weeks compared to mito, age 20 weeks
[b] p<0.05, cyto, age 20 weeks compared to cyto, age >80 weeks

Figure 4: Cardiac function and Glutathione redox potential (E_{GSH}) in the cytoplasm and the mitochondrial matrix of cardiomyocytes during ageing. Fractional area shortening (FAS) and ejection fraction (EF) were analyzed by echocardiography in mice of different age expressing Grx1-roGFP2 as a transgene under the control of the cardiomyocyte-specific promoter either in the cytoplasm (cyto) **(a)** or the mitochondrial matrix (mito) **(b)**. Intraventricular dimensions were analyzed by echocardiography. FAS (%) was determined by subtracting the end-diastolic area in the left ventricle minus the end-systolic area, divided by the end-diastolic area x 100. EF (%) was determined by dividing the stroke volume of the left ventricle by the end-diastolic volume x 100. **(c)** The E_{GSH} was determined in isolated cardiomyocytes in young (20 weeks old) and old (>80 weeks old) Grx1-roGFP2 mice as described earlier (44).