

Genetically Encoded Fluorescent Redox Biosensors

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In the most general sense of this term, a genetically encoded fluorescent redox biosensor is a kind of genetic system that changes a fluorescence in response to a redox event. Most often, such a tool is a chimeric protein consisting of a sensory domain that responds to a redox stimulus, and a domain whose fluorescence changes depending on alterations occurring with the sensory domain. It can also be a single-domain fluorescent protein, the structure of which changes when exposed to a redox stimulus, and the change in the structure leads to a change in fluorescence. In addition, there are transcriptional reporters, the principle of which is based on the alteration of the transcription of the fluorescent protein gene upon exposure to a redox stimulus. This article will focus on protein genetically encoded fluorescent redox biosensors that are currently used in *in vivo* research.

fluorescent proteins

genetically encoded biosensors

redox biology

1. roGFP type biosensors

Today, the most widely used *in vivo* redox probes belong to the redox-sensitive green FP (roGFP) and HyPer families. The roGFP family includes redox-sensitive proteins, more or less selective to E_{GSH} ^{[1][2][3][4]} and probes that are relatively specific to other thiols^{[5][6][7]} or H_2O_2 ^{[8][9]}, which were developed by fusing roGFPs with different adapter proteins. The first roGFP variants were developed in 2004 by introducing redox-active cysteine residues to the GFP β -barrel surface in spatial proximity to the chromophore^[1] (a similar design was applied for the first time to yellow FP (YFP) by Winther et al. in 2001, giving the redox-sensitive YFP (rxYFP) probe^[10]). Thus, a number of roGFPs were developed, and roGFP1 and roGFP2 gained the most widespread popularity in further research. roGFP1 and roGFP2 demonstrate a ratiometric signal of relatively high amplitude, moreover, the roGFP1 signal is not pH-dependent^[1].

Two special improvements in the roGFP1 have subsequently been reported, namely, the roGFP1-Rx^[2] and roGFP1-iX^[3] groups, with the former group consisting of variants with improved response rate, and the latter group demonstrating less negative midpoint redox potentials, allowing for the use of roGFP1-iN in relatively oxidizing subcellular compartments, such as the ER and Golgi apparatus. In 2008, an important optimization of roGFP2 was published: a fusion protein consisting of human glutaredoxin 1 (Grx1) and roGFP2 showed a significantly higher response rate and selectivity to glutathione^[4]. The idea behind the probe was as follows: human Grx1 specifically catalyzes the transfer of oxidative equivalents between the glutathione redox pair and roGFP2, thus, the spatial proximity of the enzyme and the sensor molecule leads to a more rapid and specific equilibration. The obtained

probe, Grx1-roGFP2, allowed ratiometric pH-independent (pH 5.5–8.5) measurements and became one of the commonly applied *in vivo* redox biosensors. In a similar way to Grx1-roGFP2, probes to assess the redox potential of *Mycobacterium tuberculosis* mycothiol (Mrx1-roGFP2^[5]), *Staphylococcus aureus* bacillithiol (Brx-roGFP2^[6]), and trypanosome trypanothione (Tpx-roGFP2^[7]), were developed.

The roGFP-based probes specific to H₂O₂, roGFP2-Orp1^[8] and roGFP2-Tsa2ΔC_R (and the ΔC_RΔC_P variant)^[9], have been described and used *in vivo*. RoGFP2-Orp1^[8] was designed as a fusion protein consisting of roGFP2 and Orp1 peroxiredoxin. Orp1 was found to efficiently mediate electron flow between H₂O₂ and roGFP2, creating a special redox relay when placed in close proximity to the target protein. The indicator allows ratiometric measurements and is widely used in different model organisms for H₂O₂ registration. Recently, an ultrasensitive probe with a similar design idea has been reported^[9]. The yeast peroxiredoxin Tsa2 was fused to roGFP2 to mediate the oxidation of the latter. The fundamental difference is that Tsa2 is a 2-Cys peroxiredoxin, which are the most efficient thiol peroxidases and react with H₂O₂ 100 times faster than Orp1. The indicators roGFP2-Tsa2ΔC_R and ΔC_RΔC_P variant, enable the detection of endogenous basal H₂O₂ levels, which may give new insights into H₂O₂ biology. However, since the probes were developed based on the yeast platform, some optimization may be needed for their use in other model organisms.

In 2017, some limitations for *in vivo* application of the Grx1-roGFP2 and roGFP2-Orp1 probes were observed by Müller et al.^[11]. It was found that micromolar amounts of hypochlorous acid and polysulfides contribute to roGFP2-based probe responses *in vitro*. Peroxynitrite may also influence the probes redox state, especially for roGFP2-Orp1. The required concentrations of HOCl, for instance, can be produced during the phagocyte respiratory burst. These facts imply that these indicators should be used carefully in physiological conditions, pathological conditions, or in specific cell types, where high amounts of the aforementioned oxidants are generated^[11].

In addition to roGFP family probes, there are indicators constructed in a similar way. Among these are oxidation-sensitive Oba-Q reporters^[12], the fluorescence of which is drastically quenched upon oxidation, as reported by Sugiura et al. The Oba-Q indicators are based on blue-shifted FPs, so multiparameter imaging is possible using Oba-Q in combination with biosensors of different colors. Two red probes, redox sensitive red FP (rxRFP)^[13] and Grx1-roCherry^[14] have been applied *in vivo*. RxRFP was based on circularly permuted (cp) red FP cp-mApple with cysteine residues introduced on its mutated N- and C-termini^[13]. Grx1-roCherry was constructed as a fusion protein consisting of human Grx1 and the redox-sensitive red FP roCherry^[14]. Both probes are redox-sensitive, and Grx1-roCherry demonstrates a sufficient response rate and relative specificity to glutathione redox potential due to the Grx1 domain. Red probes are preferable in some conditions because, firstly, their excitation and emission light is less toxic for living objects, gives less autofluorescence, and penetrates tissues better, and secondly, red indicators allow for multiparameter imaging when used in combination with blue and green indicators.

A novel redox-sensitive probe, which was based on the Japanese eel FP UnaG^[15] has been reported by Hu et al.^[16]. Like other redox indicators, redox-sensitive UnaG (roUnaG) contains a pair of cysteines on the β-barrel surface, which form a disulfide bond and change the chromophore spectral properties. Interestingly, UnaG emits light only upon binding of bilirubin, which acts as an external chromophore. This makes it possible to use roUnaG

to monitor the redox state in hypoxic conditions, which may be a good alternative to GFP-based proteins because the GFP chromophore requires the molecular oxygen for maturation. However, there may be some limitations due to the necessity of bilirubin introduction.

2. HyPer type biosensors

The HyPer family is a group of H_2O_2 sensing probes^{[17][18][19][20][21]}. The first HyPer was designed as a chimera consisting of a regulatory domain of the *Escherichia coli* H_2O_2 sensing transcriptional factor OxyR (OxyR-RD) and cpYFP, integrated into a flexible region of the former^[18]. In the primary structure of OxyR-RD there are two key cysteine residues, which form a disulfide bond under H_2O_2 -mediated oxidation, with consequent dramatic conformational changes in the protein structure. The conformational rearrangement triggers a change in the cpYFP chromophore microenvironment and, accordingly, fluorescence intensity. Two upgrades of HyPer, namely HyPer-2^[19] and HyPer-3^[17], with improved maximum response amplitude and kinetic properties were subsequently reported. In 2020, an ultrasensitive pH-stable probe HyPer7, characterized by significantly enhanced brightness, kinetics, and sensitivity was described by Pak et al.^[20]. The fundamental difference of HyPer7 from previous HyPers is that its sensory domain is represented by *Neisseria meningitidis* OxyR-RD, which presumably has a higher sensitivity to H_2O_2 than its *E. coli* homolog, although this fact has not been known a priori. The red probe HyPerRed^[21], which is based on cp-mApple, also has also been applied *in vivo*. Except for HyPer7, all HyPers respond to pH alterations, so experiments with the H_2O_2 -insensitive HyPers-C199S, or SypHers, are needed to control for possible artifacts. In HyPers-C199S one of the two key cysteines is replaced with serine, which makes the probe unresponsive to H_2O_2 , retaining the same pH dependency of fluorescence ratio. HyPer-3, HyPer7, and HyPerRed could be used in combinations to study several redox processes simultaneously. While the application of HyPerRed with either green variant allows real-time imaging in different organelles, HyPer7 and HyPer-3 allow H_2O_2 dynamics to be monitored in conditions where different sensitivity of the indicator is needed.

Using HyPer, HyPer-2, HyPer-3, and HyPer7 as an example, one can see how the gradual improvement of the biosensor makes it more and more optimal for its use *in vivo*. HyPer-2 differs from HyPer in its doubled response amplitude, which improves the target signal-to-noise ratio in living systems, especially at low H_2O_2 concentrations. HyPer-3 retained the high amplitude and showed faster oxidation/reduction dynamics, which made it possible to track changes in H_2O_2 levels more quickly. HyPer7 is rather brighter compared to its predecessors, which is an important improvement because it significantly facilitates its visualization in tissues. Moreover, the pH stability of the probe allows its use without pH-control, and this is rather important in animal models, because the more complex the organism, the less likely it is to make good control for the experiment since different individuals differ more from each other.

TriPer, a probe that reports H_2O_2 dynamics in the thiol-oxidizing environment of the ER, was developed by Melo et al.^[22]. The original HyPers are fully oxidized in such conditions, and the solution found by the authors was as follows: the third cysteine was introduced into the OxyR-RD moiety, which initially contains two redox active cysteines that form a disulfide bond under oxidation. The introduction of the third cysteine may permit rearrangement of the disulfide bonding pattern and preserve a fraction of the indicator in its reduced form even in

oxidizing conditions. Another H_2O_2 -sensing probe, NeonOxIrr, has been published recently^[23]. NeonOxIrr is characterized by a slow reduction rate and allows H_2O_2 registration *ex vivo* in fixed samples.

3. Biosensors that report NAD(H) redox state

Besides H_2O_2 and glutathione redox state sensing probes, sensors for NADH/NAD⁺ registration are commonly used, as this ratio serves as a metabolic and redox marker. Most representatives of this group are based on the bacterial repressor protein Rex. In the ligand binding site of Rex, competition between NAD⁺ and NADH occurs, and when the NADH/NAD⁺ ratio changes, the protein undergoes dramatic conformational rearrangement^[24]. Peredox was constructed by inserting the circularly permuted FP cpT-Sapphire between two *Thermus aquaticus* Rex (T-Rex) subunits with subsequent optimization and fusing mCherry to the construct for fluorescence signal normalization^[25]. The probe had remarkably high affinity to NADH. This may be beneficial as Peredox senses minimal changes in NADH levels, but in some conditions with initially high NADH levels it could limit the application of the probe due to saturation. This could happen when using the indicator in mitochondrial matrix^[25] or in bacteria^[26] for instance.

In a similar manner to Peredox, Frex and FrexH were developed by integration of cpYFP between one full-length and one truncated *Bacillus subtilis* Rex subunits, although, these probes are specific to NADH only^[27]. These indicators have an intensiometric response and are different in affinity to NADH and as well as in the direction and amplitude of response, so one could use either of them in appropriate experimental conditions. RexYFP is an intensiometric NADH/NAD⁺ reporter, which was based on a single T-Rex subunit with cpYFP integrated between DNA- and ligand-binding T-Rex domains^[28]. Thus, the RexYFP protein molecule is smaller than that of Peredox and Frex, which may facilitate its use when targeting to different subcellular compartments. The affinity of RexYFP to NADH ($K' = 180 \text{ nM}$) allows its implementation both in cytoplasm and mitochondria. The indicator also demonstrates low affinity to NADPH ($K' = 6.2 \text{ } \mu\text{M}$), which should not interfere in most experiments. However, since the NADP(H) pool is more reduced than NAD(H) pool in the cytoplasm, sensitivity to NADPH could alter the signal under certain conditions. Another probe, SoNar, was also constructed by inserting cpYFP into a T-Rex monomer but in this case the fluorescent core was placed in the surface loop of the nucleotide-binding domain, while the DNA-binding domain was truncated^[29]. The distinct feature of SoNar is the remarkably high amplitude of its ratiometric response (~15-fold) that makes its use *in vivo* rather preferable. Later, SoNar specificity was altered by rational mutagenesis and iNaps, which are NADPH sensors with different affinities, were reported^[30]. Like SoNar, iNaps are characterized by ratiometricity of the fluorescent signal and high response amplitudes. Recently, an NAD⁺/AXP reporter was developed by Zou et al.^[31] where AXP refers to the total pool of ATP and ADP. FiNad was obtained by optimizing one of the versions that preceded SoNar via random mutagenesis of short amino acid linkers between T-Rex and cpYFP. Then, mCherry was fused to the sensor to achieve a ratiometric fluorescence response. The authors suggest that FiNad actually reports NAD⁺ level shifts since the total physiological AXP pool is generally maintained in homeostasis.

4. Biosensors specific to other redox parameters

Along with redox-sensitive proteins and families of indicators specific to H_2O_2 and $\text{NAD}(\text{H})$, there are several probes that are specific to other redox parameters. Indicators for R- and S-diastereomers of methionine sulfoxide were reported by Tarrago et al.^[32]. Methionine sulfoxide is formed during oxidation of methionine by biological oxidants both under physiological and pathological conditions and may serve as redox biomarker. Each probe, MetSOx or MetROx, was constructed as a chimera consisting of *Saccharomyces cerevisiae* methionine sulfoxide reductase (MSRA or MSRB, specific to the diastereomer), its specific thioredoxin (Trx1 or Trx3) and cpYFP located between them. The following mechanism of action is expected for MetSOx and MetROx: under the influence of methionine sulfoxide, two redox active cysteines of MSR form a disulfide bond, which is subsequently reduced by a corresponding thioredoxin (Trx). During the process of reduction, a new disulfide is formed between Trx and methionine sulfoxide reductase, and this is accompanied by conformational changes that trigger a shift in the fluorescence signal. A probe that enables a visualization of organic hydroperoxides in living systems was developed by Simen Zhao et al.^[33]. In OHSer, cpVenus was placed in the most conformationally mobile region of the bacterial transcriptional factor OhrR, which is specific to organic hydroperoxides. Thus, the conformational change that occurs in the OhrR moiety after its interaction with organic hydroperoxides leads to a change in the spatial structure of cpVenus and the fluorescence signal.

5. FRET-based biosensors

Besides single FP-based biosensors, there are a number of Förster resonance energy transfer (FRET)-based redox-sensitive probes used *in vivo*. Redoxfluor, reported by Yano et al., has been used to monitor the redox states of peroxisomes^[34]. The biosensor molecule consists of Cerulean and Citrine FPs and a tandem repeat of part of the yeast transcriptional factor Yap1 between them. The Yap1 part is capable of undergoing conformational rearrangement during oxidation-reduction events represented by internal disulfide bond formation, and this results in a FRET response of the probe. A specific NADP^+ -sensing FRET-based probe, NADPsor^[35], was designed by inserting the NADP^+ -reporter element, ketopantoate reductase, between the FRET pair cyan FP and YFP. When binding to NADP^+ , ketopantoate reductase changes its conformation from relatively closed to open, so FPs move away from each other and the FRET between them weakens. A probe for measurements of the thioredoxin redox state in chloroplasts, CROST^[36], has been recently published by Sugiura et al. CROST contains the mTurquoise/cp-mVenus pair and partial sequence from CP12, a redox-sensitive regulatory and thioredoxin-targeted protein in chloroplasts, between FPs. As in other fluorescent biosensors, the reduction of internal cysteines in the CP12-derived domain by thioredoxin leads to conformational alterations in the molecule, and corresponding changes in the FRET ratio.

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