Fluorescent proteins such as eGFP lead to catalytic oxidative stress in cells

Douglas Ganini, Fabian Leinischi, Ashutosh Kumar, JinJie Jianga, Erik J. Tokarb, Christine C. Malone, Robert M. Petrovich, Ronald P. Mason

A Free Radical Biology, Immunity, Inflammation & Disease Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA
b Stem Cell Toxicology Group, National Toxicology Program Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA
c Protein Expression Core Facility, Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

ARTICLE INFO

Keywords:
GFP
H₂O₂ (hydrogen peroxide)
O₂•⁻ (superoxide free radical anion)
Free radicals
Oxidative stress
Redox biology

ABSTRACT

Fluorescent proteins are an important tool that has become omnipresent in life sciences research. They are frequently used for localization of proteins and monitoring of cells [1,2]. Green fluorescent protein (GFP) was the first and has been the most used fluorescent protein. Enhanced GFP (eGFP) was optimized from wild-type GFP for increased fluorescence yield and improved expression in mammalian systems [3]. Many GFP-like fluorescent proteins have been discovered, optimized or created, such as the red fluorescent protein TagRFP [4].

Fluorescent proteins are expressed colorless and immature and, for eGFP, the conversion to the fluorescent form, mature, is known to produce one equivalent of hydrogen peroxide (H₂O₂) per molecule of chromophore [5,6]. Even though it has been proposed that this process is non-catalytic and generates nontoxic levels of H₂O₂ [6], this study investigates the role of fluorescent proteins in generating free radicals and inducing oxidative stress in biological systems. Immature eGFP and TagRFP catalytically generate the free radical superoxide anion (O₂•⁻) and H₂O₂ in the presence of NADH. Generation of the free radical O₂•⁻ and H₂O₂ by eGFP in the presence of NADH affects the gene expression of cells. Many biological pathways are altered, such as a decrease in HIF1α stabilization and activity. The biological pathways altered by eGFP are known to be implicated in the pathophysiology of many diseases associated with oxidative stress; therefore, it is critical that such experiments using fluorescent proteins are validated with alternative methodologies and the results are carefully interpreted.

Since cells inevitably experience oxidative stress when fluorescent proteins are expressed, the use of this tool for cell labeling and in vivo cell tracing also requires validation using alternative methodologies.

1. Introduction

Fluorescent proteins are commonly used in biology for protein labeling and cell tracing [1,2]. Green fluorescent protein (GFP) was the first and has been the most used fluorescent protein. Drs. Shimomura, Chalfie and Tsien were awarded a Nobel Prize in chemistry “for the discovery and development of the green fluorescent protein, GFP,” in 2008 [7]. Enhanced GFP (eGFP) was developed for increasing the fluorescence yield and improving its expression in mammalian systems [3]. More recently, many optimized GFP-like fluorescent proteins have been discovered, developed or created, such as the red fluorescent protein TagRFP [4].

The fluorescent chromophore of fluorescent proteins is formed through an intramolecular reaction between the side chains of certain amino acids localized inside the barrel structure of the protein [5,8]. Even though it is known that chromophore formation requires cyclization and air-mediated oxidation, proposed mechanisms for this complex reaction remain to be proved. It has been calculated that one molecule of hydrogen peroxide (H₂O₂) is generated independently of NAD(P)H during the maturation of each chromophore of eGFP. This non-catalytic H₂O₂ generation is within the cellular baseline level and, consequently, should not be toxic to cells or organisms [6]. Maturation...
of the fluorescent chromophore of TagRFP has been proposed to generate two molecules of H$_2$O$_2$ independently of NAD(P)H [9], which is believed to be low and nontoxic to cells. However, many reports show that eGFP expression is cytotoxic [10–14] and, even though it is believed that it does not induce lethality in animals, other GFP-like fluorescent proteins and eGFP are shown to induce abnormalities in skeletal muscle [15] and heart [16]. Since chemical fluorescent probes such as DCFH are known to catalyze the formation of superoxide anion free radical (O$_2^-$) and H$_2$O$_2$ in biological systems by a variety of mechanisms [17], we have searched for the formation of these reactive oxygen species by fluorescent proteins.

2. Material and methods

2.1. Chemicals and buffer

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Chelex-100 resin was purchased from Bio-Rad (Hercules, CA). DMPO was purchased from Dojindo (Rockville, MD). Ampex Red was purchased from Invitrogen (Life Technologies, Grand Island, NY). To suppress trace metal contamination, phosphate buffer was Chelex-100-treated and contained 25 $\mu$M diethylene triamine pentaacetic acid (DTPA).

2.2. eGFP and TagRFP preparation in E. coli

Gateway® technology (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) was used to generate the protein-expression vectors. The pDEST vectors with eGFP and TagRFP genes were transformed by heat shock into Rosetta 2 DE3 plasmid competent cells. Ampicillin- and chloramphenicol-resistant colonies were grown in LB with the selecting agents. After cultures of OD 0.05 reached OD 0.6–0.8 (2 h, 220 rpm at 37°C), protein expression was induced by the addition of 0.1 mM isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG); and then cultures were incubated for 4 h at RT (220 rpm). eGFP and TagRFP were purified using an ÄKTAL-PLC system (GE Healthcare Life Science, Pittsburgh, PA). Concentrations of the fluorescent proteins were determined based on protein content using the BCA Protein Assay Kit from Pierce (Rockford, IL) following the manufacturer’s instructions. The molecular mass of eGFP was adopted as 27,333.8 M$\text{d}_\text{a}$/mol and that of TagRFP was adopted as 26,732.5 M$\text{d}_\text{a}$/mol. No difference was detected when eGFP or TagRFP concentrations were calculated using protein content or absorbance at 280 nm ($\varepsilon$ for eGFP=21,890 M$^{-1}$ cm$^{-1}$ and $\varepsilon$ for TagRFP=27,640 M$^{-1}$ cm$^{-1}$). eGFP was expressed and purified 7 independent times.

2.3. Quantification of H$_2$O$_2$ in vitro

Samples containing eGFP and NADH were kept under continuous agitation (700 rpm) at room temperature, and H$_2$O$_2$ was quantified using the FOX1 assay [18] adapted to a microplate format. Briefly, 5 $\mu$L of samples or H$_2$O$_2$ standards (1–100 $\mu$L, $\varepsilon$ at 240 nm=43.6 M$^{-1}$ cm$^{-1}$) were added to 200 $\mu$L of FOX assay reagent (100 mM xylol orange, 250 $\mu$L ammonium iron$^{3+}$ sulfate, 100 $\mu$L sorbitol in 25 mM H$_2$SO$_4$) in wells of 96-well microplates. After 30 min, microplates were read for absorbance at 560 nm. The concentration of H$_2$O$_2$ was calculated after subtracting the background absorbance of wells containing 200 $\mu$L FOX1 assay reagent where 5 $\mu$L buffer blank was added.

2.4. eGFP preparation in HEK 293 cells

We prepared a pDEST26-eGFP plasmid using Gateway® technology from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA). This plasmid makes an N-terminal 6xHis tagged eGFP under the control of the cytomegalovirus (CMV) promoter. FreestyleTM293-F cells (Thermo Fisher Scientific, Carlsbad, CA) were grown in FreestyleTM293 expres-
according to the manufacturer’s instructions. Purified RNA was reverse transcribed to complementary DNA with the use of random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase from Applied Biosystems (Foster City, CA). Gene-specific primers (Supplementary Data Table S2) were designed using the NCBI/Primer BLAST Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The ABsolute SYBR Green ROX Mix from Abgene (Rockford, IL) was used for amplifications. Conditions for amplification were as follows: 15 min at 95 °C, followed by 40 cycles of 95 °C for 1 min and 60 °C for 1 min. Cycle time (Ct) values for the selected genes were normalized to values for 16 S rRNA (n=3 in pseudo triplicates). Ct values for all controls were set at 100%.

2.8. Mammalian cell culture

The HeLa cell line stably expressing eGFP (HeLa/eGFP) was purchased from Cell Biolabs, Inc. (San Diego, CA) and grown in parallel with wild-type HeLa cells. Those cell lines were certified to be free of mycoplasma. HeLa.tet and HeLa.tet.eGFP were purchased from GenTarget Inc. (San Diego, CA) and were also certified to be free of mycoplasma. Cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium, supplemented with 10% heat-inactivated fetal bovine serum (v/v), 50 μg/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine and 0.1 mM MEM nonessential amino acids (NEAA). Cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and cultured in T-75 Falcon tissue culture flasks at an initial density of 1×10⁶ cells/flask. The culture medium was replaced every 2 days and passaged when confluence was reached. Three independent cell cultures were prepared. Cultures of HeLa.tet and HeLa.tet.eGFP at 70% confluence were exposed to 1 μg/mL doxycycline for 48 h before collection. Confluent cells were collected for RNA isolation by trypsinization, washed with PBS and stored at −80 °C.

2.9. Amplex Red assay for determination of H₂O₂ production by HeLa cells

Mammalian cells were trypsinized and 1×10⁵ cells were seeded to a black 96-well plate, incubated overnight and washed with PBS. Wells with cells (n=8 for each cell line) were filled with 100 μL of 50 μM Amplex Red, 0.2 U/mL HRP in PBS and 0.5% DMSO (v/v) from the Amplex Red stock solution. Catalase was added to control wells (1 μL/well, 1 kU/mL). Exposure to light was avoided by preparing the assay in a dark room [19]. A microplate reader was prewarmed and kept at 37 °C, and fluorescence intensities (filter: λ_ex=530 nm, λ_em=590 nm) were measured from the top of each well every 10 min for 120 min. E. coli was also used for this assay, but Luria-Brentani broth medium was used at 1.0 of OD at 600 nm/mL of bacterial cells. Experiments were repeated three independent times with mammalian cells and two independent times with bacterial cells.

2.10. RNA isolation from HeLa cells, microarray and GSEA analyses

Total RNA was isolated from three independent triplicates using the QIAshredder and RNeasy Midi+ Kit (QIAGEN, Valencia, CA) with in-column DNase treatment (QIAGEN, Valencia, CA). Gene expression analysis was conducted using Agilent Whole Human Genome 4×44 Multiplex Arrays (014850, Agilent Technologies, CA). Total RNA samples were labeled with Cy3 according to the manufacturer’s protocol. Data were obtained using the Agilent Feature Extraction Software (v. 12). Data was further processed using R (version 3.1.3) and RStudio (version 0.98.1103). Raw microarray data was normalized and the background subtracted using the limma package (version 3.22.6). Annotation was made using the hgu4112a database. Heat maps were prepared in R using the gplot package (version 2.16.0).

Two different 2-contrast GSEA analyses [20] were conducted: 1. HeLa/eGFP versus HeLa and 2. the differences in the log2-transformed Fig. 1. Reaction of NADH with eGFP leads to the formation of superoxide free radical anion. (a) Spin trapping of superoxide free radical anion (O₂⁻) generated by eGFP in the presence of NADH. Samples containing 50 μM GPP, 500 μM NADH and 100 mM DMPO showed a multiline-signal, trace (1). The effect of incubation time and eGFP or NADH was also studied. SOD was added to samples at 500 U/mL and catalase at 1 kU/mL. (b)
3. Results

3.1. Generation of O₂⁻ and H₂O₂ by eGFP and TagRFP in the presence of NAD(P)H

Initially, we used purified recombinant eGFP expressed in E. coli. As shown in Fig. 1a, we detected for the first time the generation of the free radical O₂⁻ in samples of eGFP and NADH using ESR spin trapping with 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The signal was a superimposition of the adducts DMPO/•OH and DMPO/•OOH (Fig. 1b; analysis using spectral simulation [21]), was present for over 30 min and was totally dependent on eGFP and NADH. DMPO/•OOH is known to decay to DMPO/•OH by a number of pathways. The signal was sensitive to superoxide dismutase (SOD), but not to catalase, as expected. We demonstrated that molecular oxygen is required for O₂⁻ formation (Fig. 1c) since the consumption of NADH did not occur in the absence of oxygen (Fig. 1d). No change in the light absorption for the chromophore of mature eGFP was detectable in samples either aerobically or anaerobically prepared (Fig. 1d). The reaction restarted as soon as the sample was oxygenated.

Generation of O₂⁻ by eGFP in the presence of NADH was also determined independently by the cytochrome c reduction assay (Fig. 2a). O₂⁻−mediated reduction of cytochrome c was followed optically. As expected, cytochrome c reduction was inhibited by SOD but was insensitive to catalase (Fig. 2a, red bars). Cytochrome c did not interfere with the rate of NADH consumption by eGFP (Fig. 2a, black bars). Formation of O₂⁻ was calculated over a range of NADH concentrations and was formed at approximately one third of the rate of NADH consumed (Fig. 2b). By using the same eGFP preparation, we also showed that the consumption of NADH was nearly equimolar to the formation of H₂O₂ (412 nM/min of H₂O₂ production, Fig. 2c). The catalytic activity of eGFP in this reaction was further tested in samples containing 25 μM eGFP and 1 μM NADH (Supplemental Data Fig S1a). The concentration of H₂O₂ was much greater than stoichiometric at the end of this reaction.

Fig. 2. Generation of superoxide free radical anion (O₂⁻) and H₂O₂ by eGFP in the presence of NADH. (a) Using eGFP expressed by E. coli, rates of NADH consumption and O₂⁻ formation were determined in samples containing 15 μM eGFP, 150 μM NADH and 20 μM cytochrome c. The effects of catalase (1 kU/mL) and SOD (250 U/mL) were also studied. O₂⁻ formation was determined by the cytochrome c reduction measured at 550 nm (ε₂₆₅₀ = 21,000 M⁻¹ cm⁻¹ [34]). (b) Rates of NADH consumption and O₂⁻ formation were studied in samples containing 15 μM eGFP, 20 μM cytochrome c and different concentrations of NADH. (c) H₂O₂ production was determined in samples containing 25 μM eGFP and 250 μM NADH. Every 5–6 min, an aliquot was withdrawn and H₂O₂ measured. Samples to which catalase (1 kU/mL) had been added were assayed in parallel. (d) Using eGFP expressed by the mammalian cell line HEK 293, the rate of O₂⁻ formation was determined in samples containing 15 μM eGFP, 150 μM NADH and 20 μM cytochrome c, as described in (a). The effect of SOD (250 U/mL) was also studied. * is used to show statistically significant differences between samples and controls.

Fig. 3. Oxidative stress response in E. coli follows the expression of eGFP. Oxidative stress response was determined by the quantification of mRNAs by real-time PCR of genes activated by the SoxR/S and OxyR regulons in E. coli. (a) eGFP expression (first three bars on the left) and quantification of genes that are targets for the activation of the SoxR/S region are shown. (b) Quantification of gene expression for different genes activated by OxyR. (c) Quantiﬁcation of mRNAs for MBP (maltose binding protein) and targets of SoxR/S and OxyR were determined independently by the cytochrome c reduction assay since the consumption of NADH did not occur in the absence of oxygen (Fig. 1d). No change in the light absorption for the chromophore of mature eGFP was detectable in samples either aerobically or anaerobically prepared (Fig. 1d). The reaction restarted as soon as the sample was oxygenated.

2.11. Statistical analyses

Experiments were routinely prepared in three independent replicates (n=3) and repeated on two different days. Mean and error bars as standard deviation are shown in graphs. ANOVA statistics and the post-hoc two-sided t-test were used for comparisons between groups. Comparisons with p-value lower than 0.05 were considered statistically significant.
D. Ganini et al.  
Redox Biology 12 (2017) 462–468

statistically signifi-

The contrasts were HeLa/eGFP versus HeLa, and Hela.tet.eGFP + doxycycline subtracted from HeLa.tet.eGFP versus HeLa.tet + doxycycline subtracted from HeLa.tet. The enriched gene sets with $p$-values lower than 0.001 were ranked, and matches between the two independent analyses are shown highlighted in yellow. The top genes contributing to the enrichment for the commonly enriched gene sets are depicted as heat maps in Supplementary Data Fig. S3.

(d) Intracellular levels of HIF1α for the commonly enriched gene sets are depicted as heat maps in Supplementary Data Fig. S3.

3.2. Cells expressing eGFP show oxidative stress and changes in many biological processes

In order to investigate how eGFP can alter gene expression associated with oxidative stress in living systems, we used first the bacterium E. coli to monitor the expression of genes under the control of the transcription factors SoxR and OxyR. SoxR is activated by direct reaction with superoxide anion free radical [22], whereas OxyR induces gene expression when key cytochromes are oxidized as a result of an oxidizing intracellular redox status [23]. Experiments were performed with E. coli expressing eGFP under low and high levels. All genes expressed upon activation of SoxR (Fig. 3a) and OxyR (Fig. 3b) were highly upregulated in these cells, proportionally to eGFP expression. A bacterium expressing maltose binding protein (MBP) was used as a control for the possible oxidative stress involved in overexpressing a protein. Notably, the high level of expression of MBP (Fig. 3c, first two columns) did not lead to changes in expression for any of the genes activated by SoxR or OxyR (Fig. 3c, left two panels). Even though the differences in gene expression among the bacteria expressing eGFP were striking, we did not detect differences in gene expression among the bacteria expressing eGFP.

We next used extensively purified eGFP expressed in the mammalian cell line HEK 293 (Fig. 2d). This protein was found approximately 85% in its chromophore-mature form. eGFP expressed in HEK 293 also generated O$_{2}^{•−}$ in the presence of NADH, as shown by the inhibition of cytochrome c reduction when SOD was present (first and second columns, Fig. 2d). Formation of O$_{2}^{•−}$ was dependent on NADH (third column, Fig. 2d).

We also tested the formation of O$_{2}^{•−}$ and consumption of NADH by a different fluorescent protein, the red fluorescent protein TagRFP. Highly purified TagRFP, produced in E. coli consumed NADH and generated O$_{2}^{•−}$ (Supplemental Data Fig. S1e). Purified TagRFP was approximately 90% in its chromophore-mature form ($ε$ at 555 nm=100,000 M$^{-1}$ cm$^{-1}$) [4]. The yield of O$_{2}^{•−}$ generation by TagRFP lies between the higher yields found for eGFP produced in E. coli and the lower yields for the eGFP produced in HEK 293.
Another six gene sets are downregulated in HeLa/eGFP compared to HeLa (Fig. 4d and e). It has been reported that under normoxia, the intracellular level of HIF-1α is decreased in response to the toxic effects of higher intracellular H₂O₂ and O₂⁻ formation [26,27], as such observed in HeLa/eGFP. This cell line secreted nearly 500 nM of H₂O₂ into the medium in the course of one hour (Fig. 4b).

Even though we do not have direct experimental evidence to support a definitive mechanism for this reaction, we speculate that the last immature intermediate in the proposed chromophore maturation process [28] reacts with NAD(P)H. This reaction would result in the regeneration of an earlier immature intermediate, which ultimately leads to the catalytical consumption of NADH with O₂⁻ and H₂O₂ formations. This is consistent with the finding that the mimic probe for the mature eGFP chromophore, 3,5'-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI, Lucerna, Inc., New York, NY), fails to consume NAD(P)H (data not shown).

More importantly, this chemistry likely occurs inside of bacteria and mammalian cells expressing eGFP. Our experiments were carried out with 50, 25 and 10 μM eGFP or 10 μM TagRFP, which is comparable to the concentration found in different cellular systems [29,30]. NAD(P)H is present at mM concentrations in biological systems; therefore, the chemistry shown here is likely to occur in most biological experiments, which, in turn, leads to oxidative stress and cellular adaptations in gene expression.

The level of extracellular H₂O₂ detected in HeLa cells stably expressing eGFP appears well in the range of concentration in which H₂O₂ acts as a global messenger. As a global messenger, H₂O₂ affects many biological processes beyond oxidative stress response, such as cell metabolism, cellular fate, immunity and homeostasis [31,32]. The mechanism shown here for the generation of O₂⁻ and H₂O₂ is slow since tertiary hydroperoxides are known to be highly stable, e.g., tert-butyl hydroperoxide.

Authors’ contributions

D.G. and F.L. designed experiments, performed experiments and wrote the paper. A.K. performed experiments with bacterial cells and wrote the paper. J.J. supervised the ESR experiments. E.T. supervised the experiments with bacterial cells. C.C.M. and R.M.P. expressed and

Red oxidation to levels that do not differ between cell lines (1.8 and 2.3 nM H₂O₂/min, respectively; black traces on Fig. 4b).

Production of H₂O₂ was further investigated using a HeLa cell line engineered to express eGFP under the control of a tet-mediated promoter (HeLa.tet.eGFP). In this cell model, eGFP is expressed only when doxycycline is added to the cell culture medium (Supplemental Data Fig. S3a). Possible non-specific effects of tet and/or doxycycline were taken into account by analyzing the response of its isogenic parental cell line (HeLa.tet) exposed to doxycycline and found no detectable difference in the low rate of H₂O₂ generation in both cultures. The level of eGFP in the Hela.tet.eGFP exposed to doxycycline was approximately 13 times lower than the level in the stable eGFP-expressing cell line, HeLa/eGFP. However, when we compared the gene expression and GSEA (Gene Set Enrichment Analyses [20]) of the HeLa/eGFP versus the control HeLa cells, and the specific effects of eGFP expression in HeLa.tet.eGFP versus Hela.tet, we saw that both eGFP-expressing cell models showed significant gene set enrichment for nine biological processes (gene sets with p-values lower than 0.001, Fig. 4c). Three gene sets associated with pathway response and inflammation are upregulated and are related to interferon-α response, interferon-γ response, and TNF-α signalling via NFKB (Fig. 4c and Supplemental Data Fig. 3b–d), which are classically known to be activated when cells are producing intracellular H₂O₂ and suffering from oxidative stress [25]. Another six gene sets are downregulated in HeLa cells that express eGFP (Fig. 4c and Supplemental Data Fig. 3e–j), including hypoxia. In agreement with our analyses of global gene expression, we found three times lower levels of HIF-1α, the master regulator for hypoxia, in HeLa/eGFP compared to HeLa (Fig. 4d and e).

The mechanism shown here for the generation of O₂⁻ and H₂O₂ is slow since tertiary hydroperoxides are known to be highly stable, e.g., tert-butyl hydroperoxide.
purified eGFP and RFP. R.P.M. provided guidance and wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Materials and correspondence

Correspondence and materials requests should be addressed to Dr. Ronald P. Mason (mason4@niehs.nih.gov) and Dr. Douglas Ganini da Silva (ganinidasilvd@niehs.nih.gov).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.03.002.

References

[1] N.C. Skaner, P.A. Steinbach, R.Y. Tsien, A guide to choosing fluorescent proteins, Nat. Methods 2 (2005) 905–909.
[2] J. Zhang, R.E. Campbell, A.Y. Ting, R.Y. Tsien, Creating new fluorescent probes for cell biology, Nat. Rev. Mol. Cell Biol. 3 (2002) 906–918.
[3] G. Zhang, V. Gurtu, S.K. Kain, An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells, Biochem. Biophys. Res. Commun. 227 (1996) 707–711.
[4] E.M. Merzlyak, et al., Bright monomeric red fluorescent protein with an extended fluorescence lifetime, Nat. Methods 4 (2007) 555–557.
[5] L. Zhang, H.N. Patel, J.W. Lappe, R.M. Wachter, Reaction progress of chromophore biosynthesis in green fluorescent protein, J. Am. Chem. Soc. 128 (2006) 4766–4772.
[6] C. Lu, C.R. Alhano, W.E. Bentley, G. Rao, Quantitative and kinetic study of oxidative stress regualns using green fluorescent protein, Biotechnol. Bioeng. 89 (2005) 574–587.
[7] R.Y. Tsien, Constructing and Exploiting the Fluorescent Protein Paintbox (Nobel Lecture), Angew. Chem.-Int. Ed. 48 (2009) 5612–5628.
[8] M. Ormo, et al., Crystal structure of the Aequorea victoria green fluorescent protein, Science 273 (1996) 1392–1395.
[9] O.M. Subach, et al., Structural characterization of acylmine-containing blue and red chromophores in mTagBFP and TagRFP fluorescent proteins, Chem. Biol. 17 (2010) 333–341.
[10] R.R. Taghipazdeh, J.L. Sherley, CFP and YFP, but not GFP, provide stable fluorescent marking of rat hepatic adult stem cells, J. Biomed. Biotechnol. 2008 (2008) 453590.
[11] A.M. Ansari, et al., Cellular GFP Toxicity and Immunogenicity: Potential Confounders in in Vivo Cell Tracking Experiments, Stem Cell Rev. 12 (2016) 553–559.
[12] M. Koike, Y. Yutoku, A. Koike, Kus80 attenuates cytotoxicity induced by green fluorescent protein transduction independently of non-homologous end joining, FEBS Open Bio. 3 (2013) 46–50.
[13] H.S. Lim, M.S. Jun, C.K. Chou, P.H. Chen, N.J. Ke, Is green fluorescent protein toxic to the living cells?, Biochem. Biophys. Res. Commun. 260 (1999) 712–717.
[14] H. Goto, et al., Transduction of green fluorescent protein increased oxidative stress and enhanced sensitivity to cytotoxic drugs in neuroblastoma cell lines, Mol. Cancer Ther. 2 (2003) 911–917.
[15] L.M. Wallace, A. Moreo, K.R. Clark, S.Q. Harper, Dose-dependent toxicity of humanized Renilla reniformis GFP (IreGFP) limits its utility as a reporter gene in mouse muscle, Molecular therapy, Nucleic Acids 2 (2013) e86.
[16] W.Y. Huang, J. Aramburu, P.S. Douglas, S. Izumo, Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy, Nat. Med 6 (2000) 482–483.
[17] P. Wardman, Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects, Free Radic. Biol. Med. 43 (2007) 995–1022.
[18] S.F. Wolff, Ferrous ion oxidation in presence of ferric ion indicator xylene orange for measurement of hydroperoxides, Methods Enzymol. 233 (1994) 182–189.
[19] F.A. Summers, B. Zhao, D. Ganini, R.P. Mason, Photooxidation of Amplex Red to resorufin: implications of exposing the Amplex Red assay to light, Methods Enzymol. 526 (2015) 1–17.
[20] A. Subramanian, et al., Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc. Natl. Acad. Sci. USA 102 (2005) 15545–15550.
[21] D.R. Duling, Simulation of multiple isotropic spin-trap EPR spectra, J. Magn. Reson. Ser. B 104 (1994) 105–110.
[22] M.S. Koo, et al., A reducing system of the superoxide sensor SoxR in Escherichia coli, EMBO J. 22 (2003) 2614–2622.
[23] C. Michan, M. Manchado, G. Dorado, C. Puyeo, In vivo transcription of the Escherichia coli oxyR regulon as a function of growth phase and in response to oxidative stress, J. Bacteriol. 181 (1999) 2759–2764.
[24] S. Dukan, D. Touati, Hypocholesterol acid stress in Escherichia coli: resistance, DNA damage, and comparison with hydrogen peroxide stress, J. Bacteriol. 178 (1996) 6145–6150.
[25] I. Dichi, Breganò, J.W., A.A.N. Colado Sima, R. Cecchini Role of oxidative stress in chronic diseases.
[26] H.F. Bunn, J. Gu, L.E. Huang, J.W. Park, H. Zhu, Erythropoietin: a model system for studying oxygen-dependent gene regulation, J. Exp. Biol. 201 (1998) 1197–1201.
[27] A.A. Quab, A.S. Popel, Reactive oxygen species regulate hypoxia-inducible factor Ialpha differentially in cancer and ischemia, Mol. Cell. Biol. 28 (2008) 5106–5119.
[28] D.P. Barondeau, C.J. Kassmann, J.A. Tainer, E.D. Getzoff, The case of the missing ring: radical cleavage of a carbon-carbon bond and implications for GFP chromophore biosynthesis, J. Am. Chem. Soc. 129 (2007) 3118–3126.
[29] K.D. Niewerder, S.M. Blackman, L. Rohde, M.A. Magnuson, D.W. Piston, Quantitative imaging of green fluorescent protein in cultured-cells - Comparison of microscopic techniques, use in fusion proteins and detection limits, J. Microsc.-Oxf. 180 (1995) 109–116.
[30] R.Y. Tsien, The green fluorescent protein, Annu. Rev. Biochem. 67 (1998) 509–544.
[31] J.R. Stone, S.P. Yang, Hydrogen peroxide: a signaling messenger, Antioxid. Redox Signal. 8 (2006) 243–270.
[32] G.A. Murrell, M.J. Francisc, L. Bromley, Modulation of fibroblast proliferation by oxygen free radicals, Biochem. J. 265 (1990) 659–665.
[33] E.A. Wood, Data for biochemical research (third edition) by R M C Dawson, D C Elliott, W H Elliott and K M Jones, pp 580. Oxford Science Publications, OUP, Oxford, 1986. ISBN 0-19-855558-7. Biochemical Education 15, 122, 1987.
[34] P.J. O’Brien, Superoxide dismutase, Methods Enzymol. 105 (1984) 370–378.