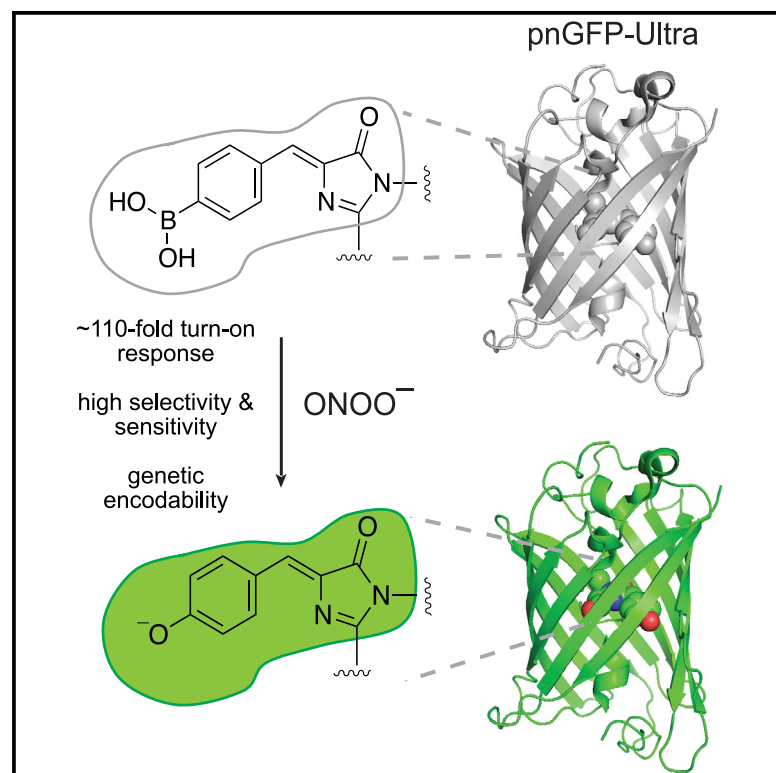


Cell Chemical Biology

A high-performance genetically encoded fluorescent biosensor for imaging physiological peroxynitrite

Graphical Abstract



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In Brief

Advanced molecular tools that permit the selective, sensitive, and noninvasive detection of peroxynitrite are essential for understanding its pathophysiological functions. Chen et al. report on pnGFP-Ultra, a high-performance fluorescent turn-on biosensor for minimally invasive and selective imaging of peroxynitrite production in live cells.

Highlights

- pnGFP-Ultra is a genetically encoded biosensor with an ~110-fold turn-on response
- pnGFP-Ultra exhibits high selectivity and sensitivity toward peroxynitrite
- An optimized plasmid-based system increases noncanonical amino acid incorporation
- pnGFP-Ultra robustly detects peroxynitrite in macrophages and primary glial cells

Article

A high-performance genetically encoded fluorescent biosensor for imaging physiological peroxynitrite

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SUMMARY

Peroxynitrite is a reactive nitrogen species (RNS) that plays critical roles in signal transduction, stress response, and numerous human diseases. Advanced molecular tools that permit the selective, sensitive, and noninvasive detection of peroxynitrite are essential for understanding its pathophysiological functions. Here, we present pnGFP-Ultra, a high-performance, reaction-based, genetically encodable biosensor for imaging peroxynitrite in live cells. pnGFP-Ultra features a *p*-boronophenylalanine-modified chromophore as the sensing moiety and exhibits a remarkable ~110-fold fluorescence turn-on response toward peroxynitrite while displaying virtually no cross-reaction with other reactive oxygen/nitrogen species. To facilitate the expression of pnGFP-Ultra in mammalian cells, we engineered an efficient noncanonical amino acid (ncAA) expression system that is broadly applicable to the mammalian expression of ncAA-containing proteins. pnGFP-Ultra robustly detected peroxynitrite production in activated macrophages and primary glial cells. pnGFP-Ultra fills an important technical gap and represents a valuable addition to the molecular toolbox for probing RNS biology.

INTRODUCTION

Reactive oxygen species (ROS) have been studied intensively for decades, but our understanding of their cousins, reactive nitrogen species (RNS), lagged far behind. Peroxynitrite (ONOO⁻) is an RNS formed from a diffusion-controlled reaction between nitric oxide (•NO) and superoxide anion (O₂^{•-}) (Ischiropoulos et al., 1992; Radi, 2013a), and has been implicated in numerous pathophysiological processes, including cardiac (Mungrue et al., 2002; Ronson et al., 1999), vascular (Förstermann and Münzel, 2006), circulatory (Szabó, 1996), inflammatory (Van Der Veen et al., 1997), diabetic (Zou et al., 2004), and neurodegenerative (Smith et al., 1997; Torreilles et al., 1999) diseases. Although no enzyme dedicated to direct ONOO⁻ production has been identified, enzymes responsible for the biogenesis of the parental species of peroxynitrite are widely known: •NO (Beckman and Koppenol, 1996) and O₂^{•-} (Fridovich, 1997) can be generated by nitric oxide synthetase and NADPH oxidase (Brewer et al., 2015), respectively (Figure 1A). In addition, the mitochondrial electron transport chain has been recognized as

an important source of O₂^{•-} (Brand, 2016). These primary species present limited cellular toxicity but can react with one another and/or transition metals to produce highly reactive secondary species (e.g., hypochlorous acid, hydroxyl radical, peroxynitrite) that could have deleterious consequences. For example, the direct toxicity of •NO is modest but can be greatly enhanced by reacting with O₂^{•-} to produce ONOO⁻. As such, many nitrosative stresses historically attributed to •NO have been gradually reassigned to ONOO⁻ (Pacher et al., 2007), a far more potent RNS capable of mediating radical (•OH, CO₃^{•-}, and •NO₂) generation, DNA damage, lipid peroxidation, protein nitration, and cell death (apoptosis or necrosis) (Szabó et al., 2007). In particular, ONOO⁻-mediated post-translational protein tyrosine nitration plays important roles in cell signal transduction (Liaudet et al., 2009). In macrophages, ONOO⁻ protects cells against invading pathogens by acting as a strong oxidant (Allen et al., 2012). Thus, like many reactive oxygen/nitrogen species (ROS/RNS), peroxynitrite displays a sophisticated signal/stress dichotomy that is inextricably linked to its complex biology (Pacher et al., 2007).

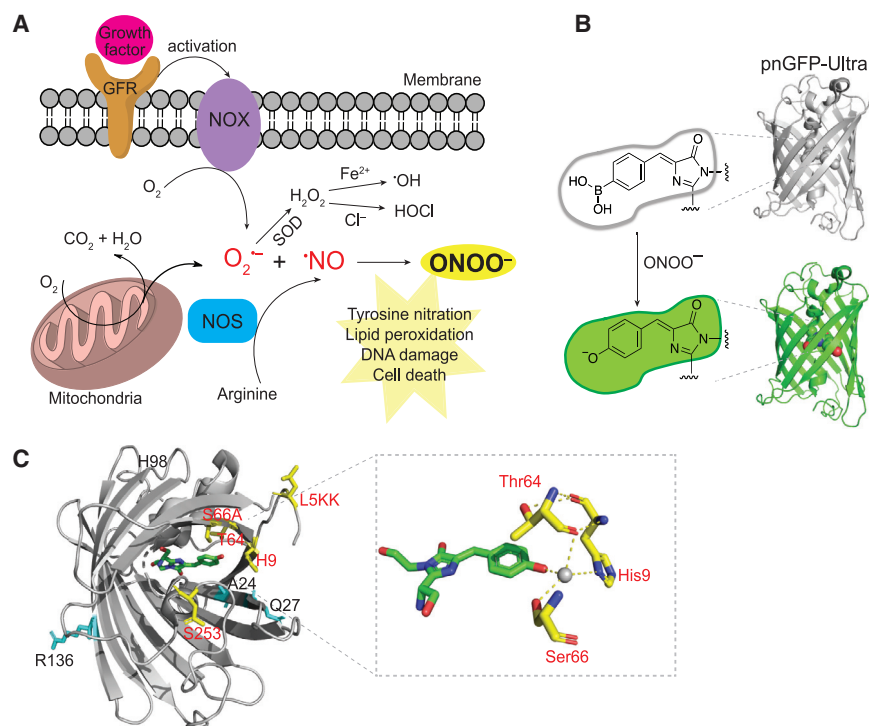


Figure 1. Peroxynitrite biogenesis and sensing mechanism of pnGFP-Ultra

(A) Illustration of the major peroxynitrite (ONOO^-) biogenesis pathway. ONOO^- is generated from the diffusion-controlled reaction between superoxide ($\text{O}_2^{\cdot -}$) and nitric oxide (NO). $\text{O}_2^{\cdot -}$ could be produced during mitochondrial respiration (oxidative phosphorylation) or by activated NADPH oxidase (NOX). NOX can be activated during cell signaling such as a growth factor binding to a growth factor receptor (GFR). Superoxide dismutase (SOD) can convert $\text{O}_2^{\cdot -}$ to hydrogen peroxide (H_2O_2), which can mediate secondary radical (e.g., $\text{OH}^\cdot$ or $\text{OCl}^\cdot$) production. NO is produced by nitric oxide synthetase (NOS), which is coupled to arginine metabolism. ONOO^- is a highly reactive nitrogen species that plays crucial roles in tyrosine nitration, lipid peroxidation, DNA damage, and cell death.

(B) Sensing mechanism of pnGFP-Ultra. pnGFP-Ultra has a boronic acid-modified chromophore that can be converted to a phenolate chromophore upon reaction with ONOO^- , resulting in a large fluorescence turn-on response.

(C) Cartoon representation of pnGFP1.5-Y.Cro (PDB: 5F9G) and key residues targeted during pnGFP-Ultra development. Residues identified during directed evolution that increase the brightness and folding of cpsGFP are highlighted

as cyan sticks. Residues that tune the chemoselectivity of pnGFP-Ultra are highlighted as yellow sticks. The chromophore is shown in green. The inset on the right shows the chromophore and its interacting residues. The gray sphere denotes a water molecule. The structure was prepared using PyMOL.

Elucidating the various roles ONOO^- plays, whether in the context of normal cell signaling or pathogenesis, is pivotal for developing targeted therapeutics (Beckman, 2009; Szabó et al., 2007). Efforts to study ONOO^- encounter hurdles similar to those in the study of other ROS/RNS, such as a very short half-life, permeability through and reactions within membranes, multiplex reaction pathways, and signal/stress dichotomy (Ferrer-Sueta and Radi, 2009; Hardy et al., 2018). Traditionally, detection of ONOO^- relied primarily on the immunostaining of 3-nitrotyrosine footprints, but this method suffers from poor antibody specificity, a requisite for cell lysis, and interference from other nitration sources (Radi, 2013b). More recently, fluorescent probes for ONOO^- (Chen et al., 2013; Li et al., 2015; Lin et al., 2013; Peng and Yang, 2010; Peng et al., 2014; Sikora et al., 2009, 2020; Sun et al., 2009, 2014, 2015; Tian et al., 2011; Ueno et al., 2006; Xu et al., 2011; Yang et al., 2006; Yu et al., 2011, 2013; Zhang et al., 2012; Zielonka et al., 2010) have emerged as powerful tools for ONOO^- detection, due to their high responsiveness, signal-to-background ratio, spatial-temporal resolution, and compatibility with well-established fluorescence microscopy platforms (Nadler and Schultz, 2013; Ueno and Nagano, 2011). Deployment of these tools in pathophysiologically relevant settings, such as in activated macrophage cells (Weber et al., 2020), in live smooth muscles from a mouse model of atherosclerosis (Peng et al., 2014), and in the cerebral vasculature of live mice (Li et al., 2015), has revealed unprecedentedly rich information on peroxynitrite production and pathogenesis. Despite the progress, developing probes with both high responsiveness and high selectivity for ONOO^- remains a fundamental challenge (Chen et al., 2016a; Hardy et al., 2018). Of all the peroxynitrite probes reported thus far, none have shown absolute

selectivity toward ONOO^- over other ROS/RNS such as H_2O_2 , $\text{OH}^\cdot$, and ClO^- . For example, HKGreen-4, one of the most advanced probe for ONOO^- , shows reactivity toward $\text{OH}^\cdot$ and ClO^- at 1 equivalent of the probe concentration (1 μM) (Peng et al., 2014); NP3, a probe with high ONOO^- reactivity cross-reacts (albeit mildly) with H_2O_2 and other species at 2 equivalents of the probe concentration (10 μM) (Li et al., 2015); and the probe 1-D-fructose shows substantial cross-reaction with ClO^- (Sun et al., 2014). In particular, H_2O_2 , a common cellular ROS that can be generated at markedly higher concentrations in certain pathophysiological conditions (Brewer et al., 2015), represents a major species competing for probe reactivity in a complex cellular milieu, confounding the unambiguous detection of ONOO^- . Finally, existing probes—predominately in the form of small-molecule dye derivatives—often require organic synthesis and lack the capability to be genetically encoded, a feature that would greatly lower the barrier to the widespread adoption of these probes by other laboratories. A genetically encodable sensor would also facilitate cell- or tissue-specific expression for *in vivo* studies, as exemplified by the popular genetically encoded calcium sensors (e.g., GCaMP series) (Chen et al., 2017; Lin and Schnitzer, 2016).

We previously developed pnGFP (Chen et al., 2013), a genetically encoded fluorescent probe for the selective detection of peroxynitrite, by site-specific incorporation of *p*-boronophenylalanine (pBoF) into the chromophore of a circularly permuted superfolder green fluorescent protein (cpsGFP). This is achieved via genetic code expansion (Chin, 2017; Liu and Schultz, 2010), a revolutionary technique that uses an orthogonal aminoacyl-tRNA synthetase (aaRS)-tRNA pair to direct the incorporation of a noncanonical amino acid (ncAA) into proteins in response to a

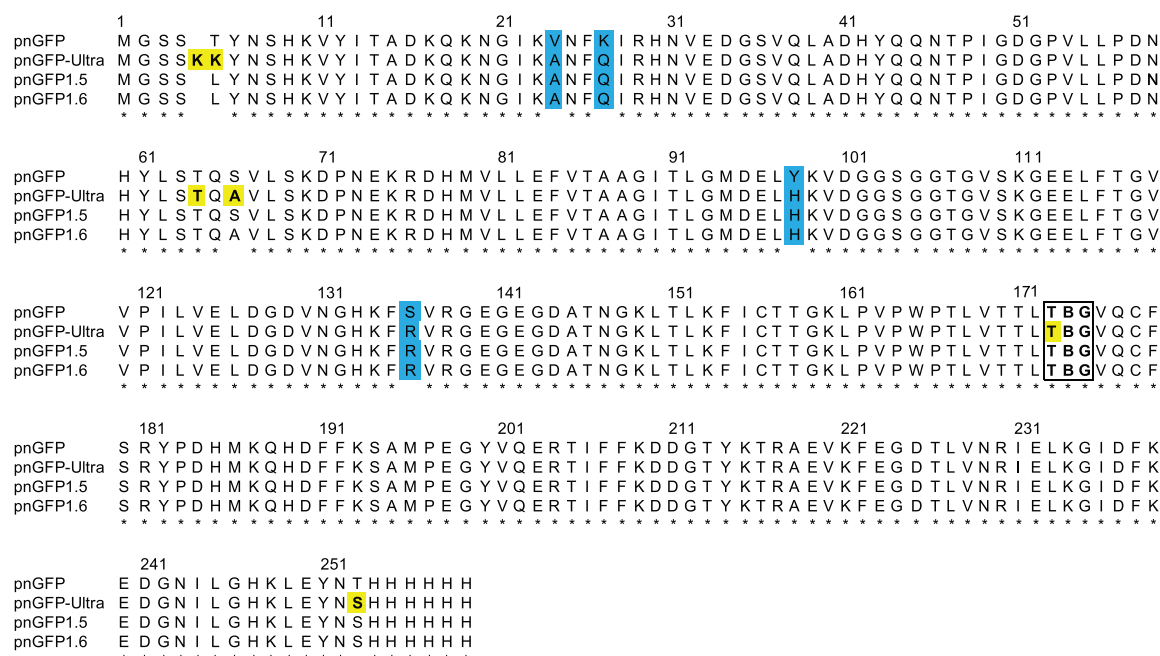


Figure 2. Sequence alignment of pnGFP-Ultra and related proteins

Mutations identified during directed evolution are highlighted in cyan. Residues subjected to rational mutagenesis are highlighted in yellow. The chromophore-forming residues (B denoting pBoF) are highlighted in a box. Residues are numbered according to the numbering of pnGFP1.5-Y.Cro (PDB: 5F9G).

nonsense codon (often the amber UAG codon). Interestingly, we serendipitously discovered that the boronic acid-based chromophore of pnGFP shows unusually high selectivity for ONOO^- : essentially nonresponsive to even 1 mM H_2O_2 (>2,000 equivalents of the probe concentration). Mechanistic studies using site-directed mutagenesis, X-ray crystallography, ^{11}B NMR, and computational simulation collectively revealed that the boron atom in pnGFP is converted to an sp^3 -hybridized form by a nearby histidine residue via a polarized water bridge (Chen et al., 2016b). The unique pathway by which pnGFP gains high selectivity suggests that the protein scaffold of genetically encoded probes can be engineered to tune chemoselectivity, which remains challenging to obtain from small-molecule probes.

Here, using directed evolution, rational mutagenesis, and targeted reactivity screening guided by the crystal structure of pnGFP1.5-Y.Cro (Chen et al., 2016b), we engineered a high-performance peroxynitrite probe, pnGFP-Ultra, which shows a low detection limit and ultra-high responsiveness and selectivity for ONOO^- . Compared with pnGFP, pnGFP-Ultra has better chromophore maturation, brighter fluorescence, and a 6-fold enhancement on both reactivity and selectivity, making it the most advanced genetically encoded biosensor for peroxynitrite. To facilitate the use of pnGFP-Ultra in mammalian cells, we systematically optimized the plasmid-based expression system for ncAA incorporation, leading to a 13.3-fold higher pBoF-incorporated protein expression in HEK 293T cells. We demonstrate that pnGFP-Ultra can be used to robustly detect physiological peroxynitrite production in activated macrophages and primary mouse glia. Together, these developments make pnGFP-Ultra a powerful tool for imaging peroxynitrite, especially in experiments where genetic encodability and high selectivity are of primary interest.

RESULTS

Design and engineering of pnGFP1.5

pnGFP was engineered by replacing the chromophore tyrosine residue of a cpsGFP with an ncAA, pBoF (Chen et al., 2013). Upon reaction with ONOO^- , the boronic acid-based chromophore (dark) is converted into its phenolate form (bright), leading to a potent fluorescence turn-on response (Figure 1B). Despite the conceptual success, the broad utility of pnGFP is stymied by several of its drawbacks, including poor protein folding/chromophore maturation, low mammalian cell expression, and dim fluorescence before and after ONOO^- conversion. Given the sensing mechanism of pnGFP, we aimed to first improve the folding, expression, and brightness of cpsGFP (Chen and Ai, 2014), the parental template of pnGFP that has a tyrosine-derived chromophore (Figure S1). We carried out multiple rounds of directed protein evolution followed by colony-based screening in *Escherichia coli* cells (Figures S1A), leading to a brighter mutant, cpsGFP2 (cpsGFP-V24A K27Q Y98H S136R) (Figure S1B). Next, we substituted the chromophore tyrosine residue of cpsGFP2 with pBoF (Y174B, where B stands for pBoF) through genetic code expansion. To screen for mutants with high reactivity and selectivity toward ONOO^- , we performed saturation mutagenesis on threonine 5 (T5) and threonine 253 (T253), two terminal residues that have previously been shown to influence the reactivity of ncAA-containing fluorescent biosensors (Chen and Ai, 2014; Chen et al., 2012, 2013, 2016b). Library screening coupled with fluorescence-based *in vitro* assays (Figure S1) resulted in pnGFP1.5 (cpsGFP2-T5L Y174B T253S) (Figure 2), which exhibited a remarkable 132-fold fluorescence turn-on response to 100 μM ONOO^- (Figure 3A). pnGFP1.5 has a higher

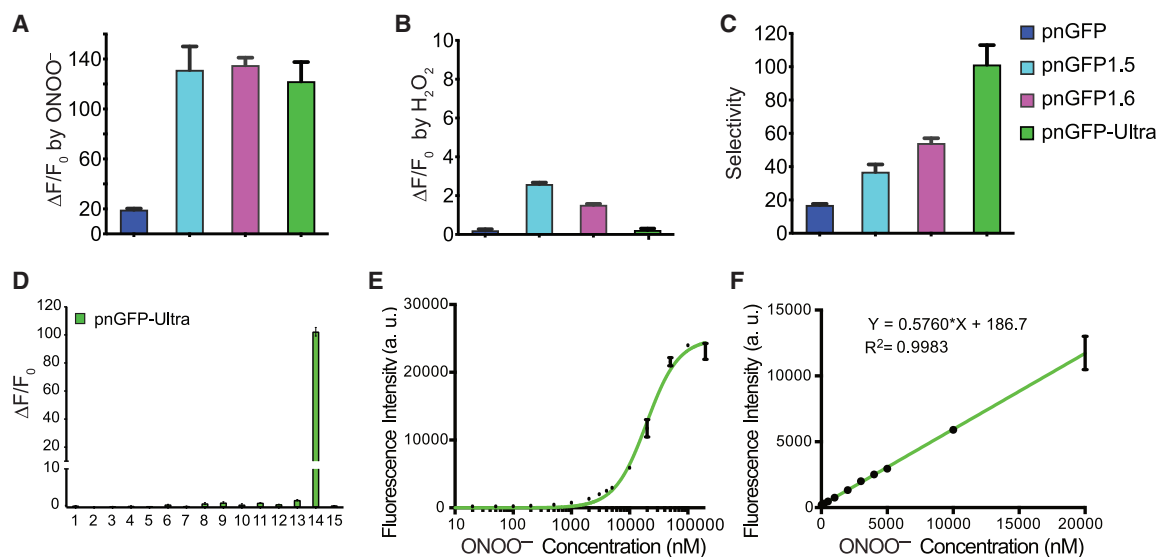


Figure 3. *In vitro* characterization of pnGFP-Ultra

(A) Dynamic range (expressed as $\Delta F/F_0$) of pnGFP-Ultra and relevant mutants toward ONOO^- . F_0 is the initial fluorescence intensity. ΔF is the final fluorescence intensity (after treatment) minus F_0 . Samples were treated with 100 μM ONOO^- for 1 h.

(B) Dynamic range ($\Delta F/F_0$) of pnGFP-Ultra and relevant mutants toward H_2O_2 . Samples were treated with 100 μM H_2O_2 for 1 h.

(C) Selectivity of pnGFP-Ultra and its mutants. Arbitrarily defined selectivity is calculated as the fold of fluorescence enhancement by ONOO^- /fold of fluorescence enhancement by H_2O_2 .

(D) Response of pnGFP-Ultra post 1-h incubation with a panel of redox-active chemicals: (1) $\bullet\text{OtBu}$ (1 mM Fe^{2+} and 100 μM HOtBu), (2) 100 μM HOtBu , (3) $\text{OH}^\bullet$ (1 mM Fe^{2+} and 100 μM H_2O_2), (4) 5 mM oxidized glutathione, (5) 100 μM $\text{O}_2^{\bullet-}$, (6) 100 μM NOC-7 (NO donor), (7) 100 μM HOCl , (8) 100 μM NaHS (H_2S donor), (9) 5 mM L-cysteine, (10) 1 mM DL-homocysteine, (11) 1 mM vitamin C, (12) 100 μM H_2O_2 , (13) 1 mM H_2O_2 , (14) 100 μM ONOO^- , (15) Tris buffer.

(E) Fluorescence response of pnGFP-Ultra (0.2 μM) to nanomolar to high-micromolar concentrations of ONOO^- . The EC_{50} was determined to be 19.5 μM .

(F) Linear fluorescence response of pnGFP-Ultra (0.2 μM) to nanomolar to low-micromolar concentrations of ONOO^- . The limit of detection was determined to be 122 nM with a signal-to-noise ratio of 3.

Data in (A–F) represent the mean $\pm$ SD of triplicates.

protein expression level in *E. coli* compared with pnGFP, presumably because of enhanced protein folding and chromophore maturation.

Structure-guided engineering and *in vitro* characterization of pnGFP-Ultra

Compared with pnGFP, pnGFP1.5 showed a 6-fold enhancement in the dynamic range toward ONOO^- (Figure 3A), but also an undesirable ~ 3 -fold increase in the dynamic range toward H_2O_2 (Figure 3B). The overall selectivity (arbitrarily defined as fold of fluorescence enhancement by ONOO^- /fold of fluorescence enhancement by H_2O_2) of pnGFP1.5 remained higher than that of pnGFP (Figure 3C). We next sought to tune the selectivity of pnGFP1.5 by rational mutagenesis. In our previous attempt to decipher the unusual chemoselectivity of pnGFP, we solved the X-ray crystal structure of pnGFP1.5-Y.Cro (pnGFP1.5-B174Y, PDB: 5F9G), which contains a tyrosine-derived chromophore (Chen et al., 2016b). The structure indicates that three residues—namely Thr64, Ser66, and His9—are in close proximity to the phenolate oxygen of the chromophore (Figure 1C). We speculate that these residues may interact with the pBoF-derived chromophore in pnGFP1.5 to fine-tune the chromophore environment (electrostatics, hydrophobicity, and solvent accessibility). In addition, the first chromophore-forming residue (Thr173) can also affect electron distribution within the conjugate chromophore structure. Thus,

these four residues were next subjected to site-directed mutagenesis (Figures 2 and S1). Based on our prior experiences with pnGFP, we created a panel of pnGFP1.5 mutants and assayed their selectivity *in vitro* (Figure S2). From this screening, we identified pnGFP1.6 (pnGFP1.5-S66A), which showed much lower reactivity toward H_2O_2 , but maintained the same amplitude of response toward ONOO^- . All other mutants exhibited lower selectivity than pnGFP1.5, due to either low fluorescence intensity after ONOO^- conversion or high reactivity toward H_2O_2 (Figure S2). We next focused on improving pnGFP1.6 (Figure S1B).

pnGFP gains selectivity, in part, by leveraging the nucleophilic attack efficiency differences between ONOO^- and HOO^- (Chen et al., 2016b). Due to pK_a differences (11.6 for H_2O_2 and 6.8 for ONOOH), the portion of HOO^- at neutral pH is much lower than that of ONOO^- , thereby disfavoring the reaction of pnGFP with H_2O_2 . However, this difference alone is not sufficient to explain the remarkable selectivity of pnGFP, suggesting that the protein scaffold may further modulate reactivity and specificity. To this end, we substituted threonine 5—which gates an opening toward the chromophore—with two randomized residues by using degenerate NNK codons. Screening of the resultant library against both H_2O_2 and ONOO^- led to pnGFP-Ultra (pnGFP1.6-T5KK, Figure 2 and S1B), which showed virtually no response to 100 μM H_2O_2 even after 1-h incubation (Figure 3B). Although this came at the price of a slightly reduced reactivity to ONOO^-

(Figure 3A), the selectivity of pnGFP-Ultra is 1.9- and 6-fold that of pnGFP1.6 and pnGFP, respectively (Figure 3C). Interestingly, in pnGFP-Ultra, threonine 5 is replaced by two consecutive lysine residues (Figure 2), which possibly abolish the reactivity of pnGFP-Ultra toward H_2O_2 by channeling a positively charged entry route that diminishes local availability of H_2O_2 near the pBoF-derived chromophore. This unexpected screening result further highlights the unique scaffolding effect of protein-based sensors, which could be harnessed to achieve unusual chemoselectivity.

We tested *in vitro*-purified pnGFP-Ultra against a panel of redox-active molecules, including ROS/RNS and thiols that are commonly present in a complex cellular milieu (Figure 3D). Except for ONOO^- , none of the species surveyed triggered appreciable fluorescence changes, confirming that pnGFP-Ultra is highly selective toward ONOO^- (Figure 3D). Immediately (less than 10 s) upon incubation with 100 μM ONOO^- , pnGFP-Ultra displayed a robust ~ 110 -fold fluorescence enhancement. By contrast, 1-h incubation of pnGFP-Ultra with 100 μM and 1 mM H_2O_2 triggered only 0.31- and 1.46-fold fluorescence increase ($\Delta F/F_0$), respectively (Figure 3D). Given the low basal fluorescence intensity of pnGFP-Ultra (essentially dark), these small fluorescence increases are negligible in most applications. pnGFP-Ultra responded to high-nanomolar to high-micromolar concentrations of ONOO^- and the half-maximal response concentration (EC_{50}) was determined to be 19.5 μM (Figure 3E). Furthermore, the fluorescence intensity of 0.2 μM pnGFP-Ultra increased linearly from high-nanomolar to ~ 20 μM ONOO^- concentrations, with the limit of detection (LOD) determined to be 122 nM (Figure 3F). Note that the EC_{50} and LOD determined from these assays depend on sensor concentrations. Indeed, when a higher sensor concentration (1 μM) was used, the EC_{50} and LOD increased (Figures S3A and S3B). Compared with pnGFP, pnGFP-Ultra exhibits an overall 6-fold improvement in both the dynamic range and the selectivity (Figures 3A and 3C). We further compared the time-lapse responses of pnGFP-Ultra with those of coumarin boronic acid (CBA), a small-molecule boronate-based probe (Zielonka et al., 2010, 2012a). The fluorescence of CBA increased drastically in response to both H_2O_2 and SIN-1 (a ONOO^- donor), while pnGFP-Ultra showed excellent selectivity, although the response magnitude was lower and kinetics were slower than those of CBA (Figure S3C). This phenomenon is expected given the solvent-exposed boronic acid group (i.e., high accessibility to both H_2O_2 and ONOO^-) in CBA. Taken together, these results strongly demonstrate that pnGFP-Ultra is a high-performance RNS biosensor that is highly responsive to and selective for peroxynitrite.

Development of an enhanced ncAA incorporation system in mammalian cells

Efficient co-translational ncAA incorporation in response to the amber TAG codon is crucial for the success of pnGFP-Ultra and other applications that require ncAA incorporation. Indeed, one of the fundamental limitations of pnGFP and other ncAA-based biosensors is their inadequate expression in mammalian cells. Whereas ncAA incorporation in *E. coli* has been thoroughly optimized to an extent that ncAA incorporation can reach a level comparable to that of canonical amino acids,

mammalian incorporation of ncAA has proven to be far more challenging, presumably due to a more complicated ribosomal translation machinery.

Existing plasmid-based systems for ncAA incorporation (Chen et al., 2009; Hino et al., 2012; Liu et al., 2007; Schmied et al., 2014; Xiao et al., 2013) vary in the type and copy number of the engineered orthogonal tRNA-aaRS pair and the choices of vectors and promoters, making it difficult to cross-compare the efficiencies of these systems. Several strategies for enhancing amber codon suppression efficiency, such as increased copy number of tRNAs (Liu et al., 2007), use of type-3 RNA polymerase III promoters to express the engineered tRNAs (Wang et al., 2007), and ectopic expression of a dominant-negative eukaryotic release factor 1 mutant (eRF1-E55D) (Schmied et al., 2014), have proven to be generalizable. To systematically optimize ncAA incorporation into proteins in mammalian cells, we constructed a series of expression plasmids (plasmids a-e, Figure 4A) and tested ncAA incorporation in HEK 293T cells using an EGFP_{TAG} fluorescent reporter gene that bears an amber TAG codon at position 39 of EGFP. Incorporation of an ncAA in response to the amber TAG codon (amber suppression) would generate the full-length EGFP, while failure to do so would result in a truncated EGFP (nonfluorescent). Previously, pBoF was incorporated into pnGFP by co-transfecting the CMV promoter-driven pnGFP reporter plasmid with the suppressor plasmid, which harbors one copy of CMV promoter-driven poly-specific aaRS (Poly-aaRS) and four copies of U6/H1 promoter-driven suppressor tRNAs (Figure 5A). Since orthogonal tRNA expression is a limiting factor in ncAA incorporation efficiency, we cloned four copies of tRNAs on the reporter plasmid c as well (Figure 4A). Compared with our previous expression method using plasmids a + b, addition of extra copies of tRNA on the reporter plasmid doubled the incorporation efficiency of pBoF (Figure 4B) into the EGFP_{TAG} reporter (Figure 4C, plasmids c + b). To test whether ectopic expression of eRF1-E55D further enhances ncAA incorporation efficiency, eRF1-E55D was bicistronically expressed with Poly-aaRS via a self-cleaving T2A peptide linker (Figure 4A, plasmid d). Co-expression of eRF1-E55D-containing suppressor plasmid d, in lieu of b, with the original reporter plasmid a resulted in a 10.9-fold enhancement in pBoF incorporation (Figure 4C). Remarkably, we observed further improvements in incorporation efficiency using plasmids c + d, suggesting that the benefit from the expression of extra copies of tRNA and the eRF1-E55D mutant are synergistic (Figure 4C). To simplify the expression system, we integrated all genetic components necessary for ncAA incorporation—including the reporter gene, Poly-aaRS, and orthogonal tRNAs—into a single plasmid (Figure 4A, plasmid e). Expression of plasmid e alone gave rise to incorporation efficiency comparable to that of using a + d. Co-transfection of plasmid e with other plasmids led to various degrees of expression efficiency. Nevertheless, all strategies tested surpassed our original method of using plasmids a + b for ncAA incorporation, with plasmids c + d and c + e having the highest incorporation efficiency for pBoF (13.3-fold enhancement) (Figure 4C). We achieved similar enhancement in incorporation efficiency for p-azido-phenylalanine (pAzF) (Figure S4), suggesting that this plasmid-based system is quite general and can be used to incorporate other ncAAs.

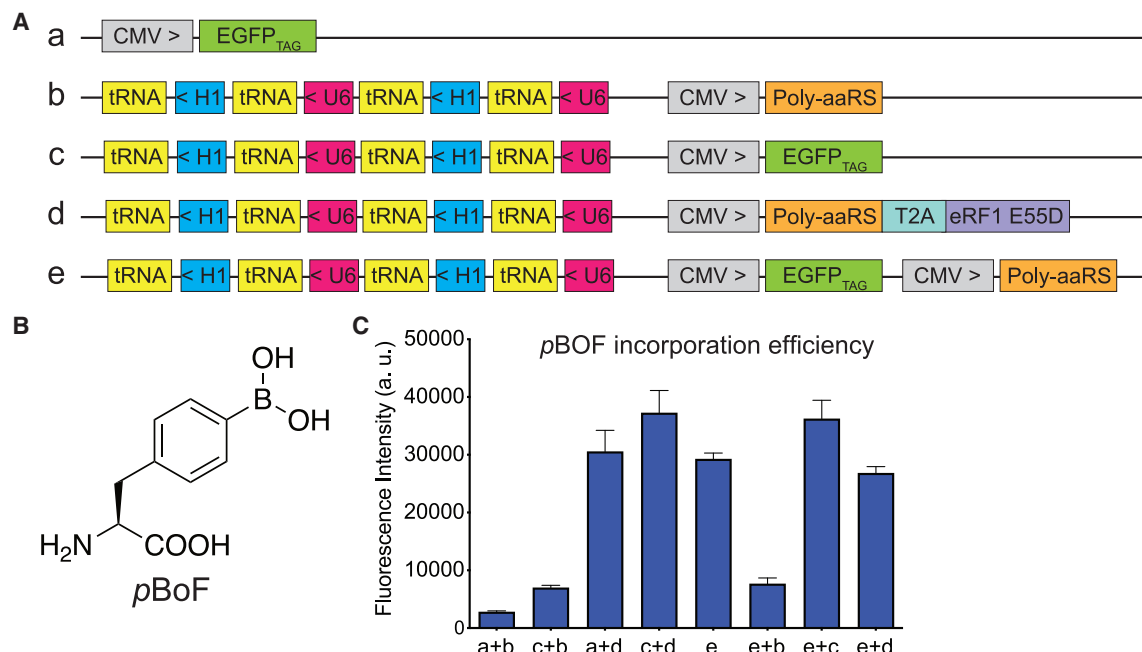


Figure 4. Engineering of a highly efficient plasmid-based ncAA incorporation system in mammalian cells

(A) Schematics of various expression plasmids. Poly-aaRS is the poly-specific aminoacyl tRNA synthetase, eRF1 E55D is the mutant eRF1 gene, T2A is a self-cleaving 2A peptide, tRNA is the orthogonal tRNA, U6 indicates the U6 promoter, H1 indicates the H1 promoter, CMV is the CMV promoter, and EGFP_{TAG} is the EGFP reporter gene with a TAG codon at position 39. > or < denotes the direction of the gene cassette.

(B) Chemical structure of *p*-boronophenylalanine (*p*BoF).

(C) Quantification of *p*BoF (1 mM) incorporation into the EGFP_{TAG} reporter gene measured in a cell-based fluorescence assay. The indicated constructs were transiently expressed in HEK 293T cells and the cell lysate green fluorescence was quantified in a plate reader at 515 nm emission with excitation at 490 nm. Data represent the mean $\pm$ SD of triplicates.

Imaging of peroxynitrite in live cells using pnGFP-Ultra

Next, we constructed a mammalian expression plasmid by replacing the EGFP_{TAG} gene with the pnGFP-Ultra gene in plasmid *c* (Figure 5A). We co-expressed the resultant plasmid with plasmid *d*, as this combination gave rise to the highest *p*BoF incorporation efficiency in the above EGFP_{TAG} reporter assay (Figure 4C). To compare with the first-generation biosensor, we expressed pnGFP in parallel by utilizing our previous method (Figure 5A). After treatment with SIN-1, the fluorescence intensities from pnGFP-Ultra-expressing HEK 293T cells were much higher than those from pnGFP-expressing cells (Figure 5B). It is also evident (from the number of fluorescent cells) that the expression efficiency of pnGFP-Ultra is much higher than that of pnGFP. Both pnGFP and pnGFP-Ultra are selective toward ONOO[−], with no obvious response to H₂O₂ (1 mM) or HOCl (100 μ M) (Figures S5A and S5B). We further expressed pnGFP-Ultra-Y.Cro, which contains a tyrosine-derived chromophore, in HEK 293T cells. Treating these cells with SIN-1 did not change the fluorescence (Figures S5C and S5D), confirming that the *p*BoF-derived chromophore in pnGFP-Ultra is responsible for ONOO[−] sensing. Moreover, in the presence of 100 μ M urate or minocycline, both of which are well-established ONOO[−] scavengers (Schildknecht et al., 2011), the fluorescence response of pnGFP-Ultra in HEK 293T cells to SIN-1 was largely attenuated (Figures S6A and S6B). Because ONOO[−] was not enzymatically synthesized under this treatment condition, 1400W,

a selective inhibitor of inducible nitric oxide synthase (iNOS) (Garvey et al., 1997), was unable to attenuate the fluorescence turn-on caused by SIN-1 (Figures S6A and S6B). Furthermore, the SIN-1 treatment significantly reduced the viability of HEK 293T cells, while urate or minocycline, but not 1400W, rescued SIN-1-induced cell death (Figure S6C). Taken together, pnGFP-Ultra is a high-performance biosensor for imaging ONOO[−] in mammalian cells. We attribute the superior performance of pnGFP-Ultra to both its large dynamic range (~110-fold fluorescence enhancement) and the substantially improved *p*BoF incorporation efficiency.

Visualization of peroxynitrite production in activated macrophages

To further demonstrate the utility of pnGFP-Ultra in imaging physiological ONOO[−], we expressed pnGFP-Ultra in mouse RAW264.7 macrophage immune cells, which are known to produce ONOO[−] from nitric oxide and superoxide under immune stimulation (Allen et al., 2012; Ischiropoulos et al., 1992; Peng et al., 2014; Xia and Zweier, 1997). We first pre-stimulated the cells for 10 h using the bacterial endotoxin lipopolysaccharide (LPS) and the pro-inflammatory cytokine interferon- γ (IFN- γ) to increase iNOS expression, and next, incubated the cells with phorbol myristate acetate (PMA) for 1 h to activate NADPH oxidase (Zielonka et al., 2012b). We observed strong fluorescence signal in pnGFP-Ultra expressing cells (Figure 6A). The mean fluorescence of cells in the treated group was ~7.1-fold higher

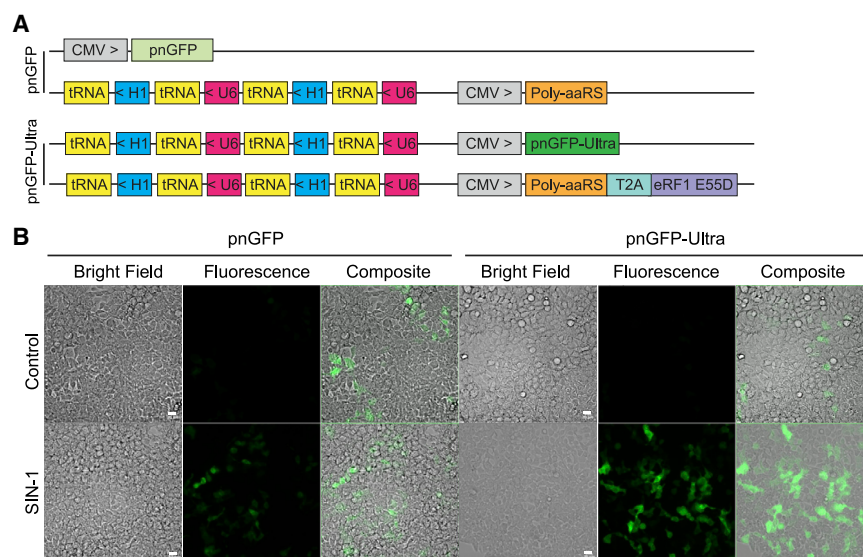


Figure 5. Live-cell imaging of peroxynitrite in HEK 293T cells

(A) Constructs used for expressing pnGFP (top two plasmids) and pnGFP-Ultra (bottom two plasmids).

(B) Green fluorescence and bright-field images of cells expressing pnGFP (left) or pnGFP-Ultra (right). In the bottom row, HEK 293T cells in HBSS were treated with 100 μ M SIN-1 for 90 min before imaging. Experiments were repeated three times with independent cultures and similar results were obtained. Scale bars, 20 μ m.

than that of untreated cells (Figure 6B), while IFN- γ and LPS, or PMA alone, only minimally affected the fluorescence. Furthermore, in the presence of ONOO $^-$ scavengers (100 μ M urate or minocycline) or the iNOS inhibitor (100 μ M 1400W), the fluorescence increase of pnGFP-Ultra was attenuated (Figure 6B). We next evaluated the impact of these conditions on the viability of RAW264.7 cells. As expected, treatment with LPS, IFN- γ , and PMA induced cell death; either urate, or minocycline, or 1400W partially rescued cell viability (Figure 6C). The observed fluorescence and cell viability changes are aligned with our further validation, which used SIN-1 to deliver ONOO $^-$ to RAW264.7 macrophage cells. SIN-1 activated the fluorescence of pnGFP-Ultra and induced the death of RAW264.7, both of which can be largely reverted by supplementing the ONOO $^-$ scavengers (Figure S7). As a control, we expressed pnGFP-Ultra-Y.Cro in RAW264.7 cells, and no fluorescence change was observed post-treatment of cells with LPS, IFN- γ , and PMA (Figures 6D and 6E). Collectively, these results demonstrate that pnGFP-Ultra can reliably detect physiological ONOO $^-$ production.

Detection of peroxynitrite production in amyloid β -activated primary mouse glia

Despite the continuous improvement in life expectancy, Alzheimer's disease (AD) and other neurodegenerative disorders have become a global health and social challenge (Alzheimer's Association, 2020). ONOO $^-$ is known to play important roles in numerous neurodegenerative diseases. For example, ONOO $^-$ -mediated damage has been broadly observed in AD (Smith et al., 1997), and the amyloid β peptide, whose accumulation has been considered to be the hallmark of AD, is known to induce ONOO $^-$ production in glial cells (González-Reyes et al., 2017; Xie et al., 2002) and subsequently mediate neurotoxicity (Xie et al., 2002). To test whether pnGFP-Ultra can detect ONOO $^-$ production in neurological systems, we expressed pnGFP-Ultra in cultured primary mouse glia cells. Indeed, we observed dramatically enhanced fluorescence signal ($\sim$ 3.9-fold compared with untreated cells) from glia activated with amyloid β (Figures 7A and 7B). Either the ONOO $^-$ scav-

enger, minocycline, or the iNOS inhibitor, 1400W, attenuated the fluorescence increase of pnGFP-Ultra (Figures 7A and 7B). In addition, treating glia cells expressing pnGFP-Ultra-Y.Cro with amyloid β caused no fluorescence enhancement (Figures 7C and 7D). Thus, pnGFP-Ultra represents a valuable tool enabling the imaging of ONOO $^-$ production in neurological systems.

DISCUSSION

In summary, we have developed pnGFP-Ultra—a highly responsive and selective fluorescent ONOO $^-$ biosensor—by directed evolution, structure-guided rational design, and reactivity-based library screening. Compared with the first-generation ONOO $^-$ probe pnGFP, pnGFP-Ultra has much better chromophore maturation and a 6-fold larger ONOO $^-$ -induced fluorescence response, while retaining the high chemoselectivity against H $_2$ O $_2$ and other ROS/RNS. An apparent hurdle of applying ncAA-based biosensors to cell imaging studies has been the inefficiency with which ncAA can be incorporated into proteins in mammalian cells. Here, we systematically optimized and increased pBoF incorporation efficiency in HEK 293T cells by 13.3-fold. These developments make pnGFP-Ultra a high-performance biosensor capable of imaging physiological peroxynitrite, as we have demonstrated in activated macrophages and primary glial cells. pnGFP-Ultra thus represents an important advancement enabling the practical implementation of a genetically encoded biosensor for live-cell detection of peroxynitrite. We expect pnGFP-Ultra to find broad utility in probing RNS biology in diverse pathophysiological settings.

pnGFP-Ultra, in the purified form and at the concentration of 0.2 μ M, detected as low as 122 nM ONOO $^-$, and responded to up to $\sim$ 100 μ M ONOO $^-$ with a linear response range below 20 μ M ONOO $^-$. We applied a bolus addition of ONOO $^-$ in these measurements, and because ONOO $^-$ degrades in neutral aqueous solutions and biological systems within seconds and milliseconds, respectively (Radi, 2018; Szabó et al., 2007), it is difficult to directly translate the *in vitro* responses of pnGFP-Ultra to its performance in live cells. Nevertheless, given the irreversibility and 1:1 stoichiometry of the reaction, we estimate that only a small portion of ONOO $^-$ molecules reacted with pnGFP-Ultra, presumably due to the highly unstable and transient nature of ONOO $^-$ and its relatively restricted accessibility to the

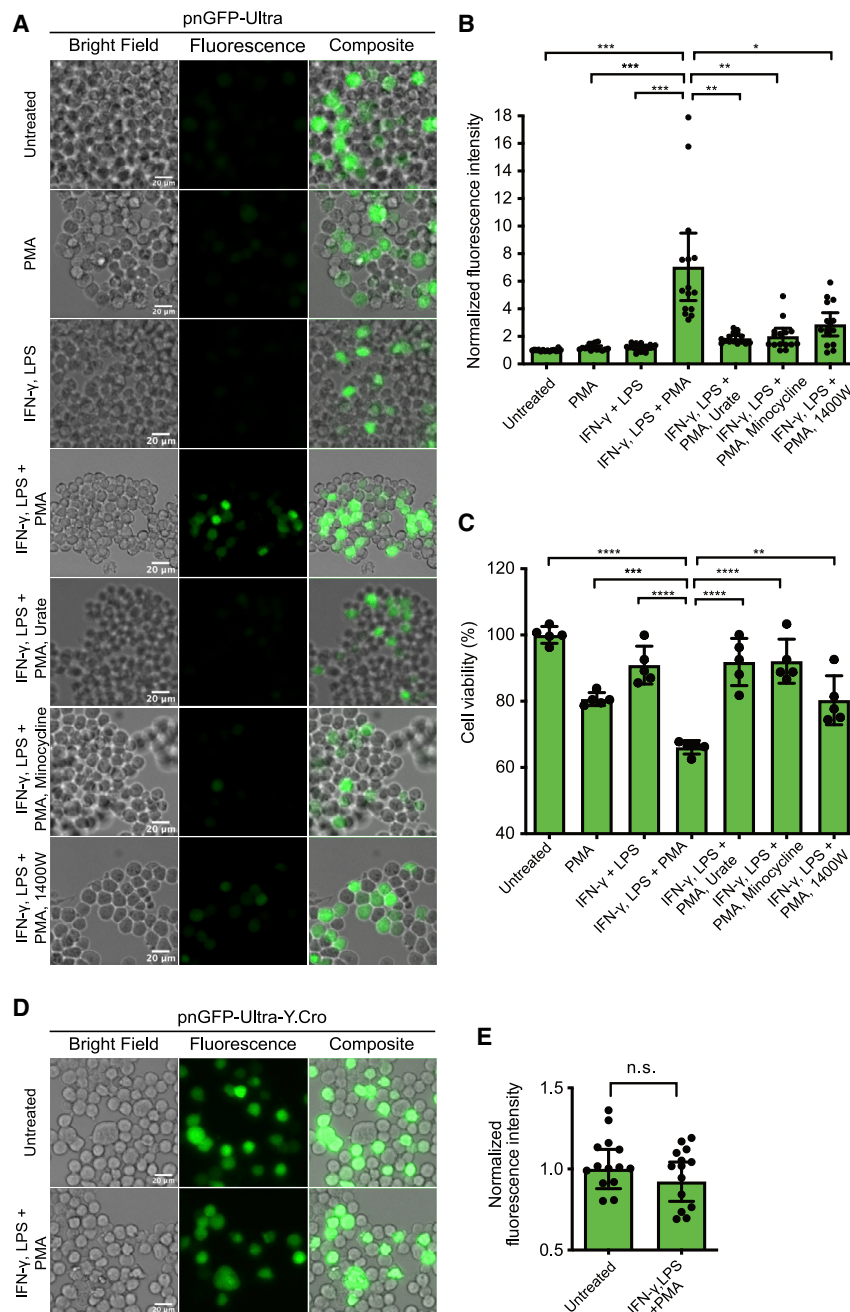


Figure 6. Live-cell imaging of peroxynitrite production in activated macrophages

(A) Mouse RAW264.7 cells were transfected with pnGFP-Ultra. Cells were next pre-treated with IFN- γ (50 U/mL) and LPS (0.5 μ g/mL) for 10 h. After LPS and IFN- γ were removed, the cells were further treated with PMA (200 ng/mL) in HBSS for 1 h before imaging. Several control groups were used. In the “untreated” group were cells untreated with either LPS, IFN- γ , or PMA. In the “PMA” group were cells without pre-treatment but directly treated with PMA for 1 h before imaging. In the “IFN- γ , LPS” group were cells pre-treated with LPS and IFN- γ but without additional PMA treatment. In addition, cells in other control groups were co-treated with ONOO⁻ scavengers (urate or minocycline) or an iNOS inhibitor (1400W) when the PMA treatment was implemented. Representative bright-field (left), fluorescence (middle), and overlay (right) images of each group are shown. Experiments were repeated three times with independent cultures and similar results were obtained. Scale bars, 20 μ m.

(B) Quantification of fluorescence intensity of cells in each treatment group in (A). Data are presented as the mean $\pm$ 95% confidential interval and black dots indicate fluorescence intensity of single cells (n = 15 individual cells from three independent replicates in each group). Statistical test was performed using Brown-Forsythe and Welch’s ANOVA followed by Dunnett’s T3 multiple comparisons test (** p < 0.001, ** p < 0.01, and * p < 0.05).

(C) MTT cell viability assay of RAW264.7 cells in each treatment group. Raw data were normalized to pnGFP-Ultra-expressing cells untreated with either LPS, IFN- γ , or PMA. Data are presented as the mean $\pm$ SD and black dots indicate the cell viability of individual replicates (n = 5 wells for each group). Statistical test was performed using ordinary one-way ANOVA followed by Dunnett’s multiple comparisons test (**** p < 0.0001, *** p < 0.001, and ** p < 0.01).

(D) Mouse RAW264.7 cells transiently expressing pnGFP-Ultra-Y.Cro (which contains a tyrosine-derived chromophore) were untreated or treated with LPS, IFN- γ , and PMA. Experiments were repeated three times with independent cultures and similar results were obtained. Scale bars, 20 μ m.

(E) Quantification of fluorescence intensity of cells in each group in (D). Data are presented as the mean $\pm$ 95% confidential interval and black dots indicate fluorescence intensity of single cells (n = 15 individual cells from three independent replicates in each group). Comparison was made using an unpaired two-tailed t test (n.s., not significant, p > 0.05).

chromophore. These results should further minimize concerns of perturbing and scavenging of endogenous ONOO⁻ when the biosensor is expressed in cells.

Previous studies estimated that the steady-state concentrations of ONOO⁻ in biological systems are likely in the nanomolar range, but its formation rate could be as high as $\sim$ 5 μ M $\cdot$ s⁻¹ (Radi, 2018; Szabó et al., 2007). Owing to the facts that cellular production of ONOO⁻ is often sustained and that pnGFP-Ultra is an irreversible biosensor, we indeed successfully used pnGFP-Ultra to detect physiological ONOO⁻ in both activated

macrophages and primary glial cells. Since pnGFP-Ultra was responsive to a large concentration range of ONOO⁻, it may be compatible with diverse cell types and microenvironments. Moreover, the large intensimetric response of pnGFP-Ultra will facilitate its future conversion into a genetically encoded ratiometric biosensor for *in situ* measurement and calibration of ONOO⁻ concentrations by fusing pnGFP-Ultra to an FRET donor (such as EBFP2, Ai et al., 2007), as we have recently demonstrated with a hydrogen sulfide biosensor (Youssef et al., 2019).

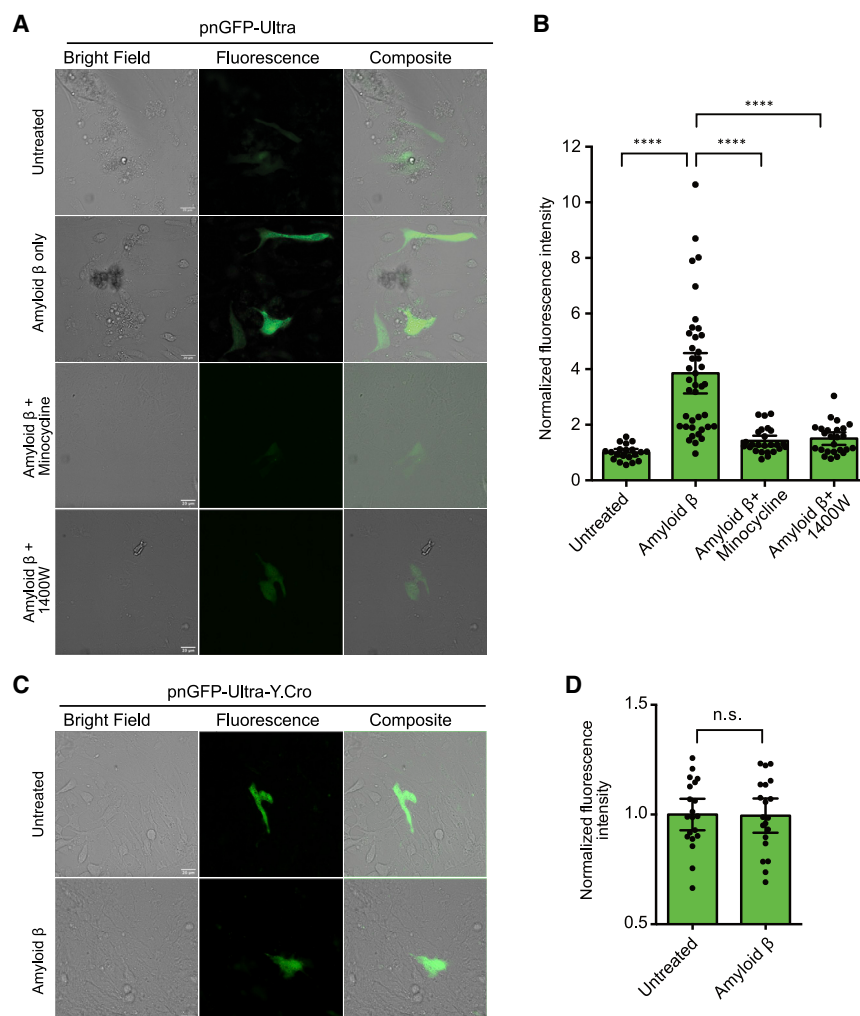


Figure 7. Live-cell imaging of amyloid β -induced peroxynitrite in primary glial cells

(A) Primary mouse glia cells were isolated and transfected with pnGFP-Ultra. After 24 h, the samples were untreated or treated with amyloid β (5 μ M) in the absence or presence of an ONOO[−] scavenger (minocycline) or an iNOS inhibitor (1400W) for 42 h before imaging. Representative bright-field (left), fluorescence (middle), and overlay (right) images of each group are shown. Experiments were repeated three times with independent cultures and similar results were obtained. Scale bars, 20 μ m.

(B) Quantification of fluorescence intensity of cells in each treatment group in (A). Data are presented as the mean $\pm$ 95% confidential interval and black dots indicate fluorescence intensity of single cells ($n = 20, 40, 25$, and 25 individual cells from three independent cultures for untreated, amyloid β -treated, amyloid β and minocycline-treated, and amyloid β and 1400W-treated groups, respectively). Statistical test was performed using Brown-Forsythe and Welch's ANOVA followed by Dunnett's T3 multiple comparisons test (**** $p < 0.0001$).

(C) Primary mouse glia cells expressing pnGFP-Ultra-Y.Cro (which contains a tyrosine-derived chromophore) untreated or treated with amyloid β (5 μ M) for 42 h before imaging. Experiments were repeated three times with independent cultures and similar results were obtained. Scale bars, 20 μ m.

(D) Quantification of fluorescence intensity in (C). Data are presented as the mean $\pm$ 95% confidential interval and black dots indicate fluorescence intensity of single cells ($n = 20$ individual cells from three cultures for each group). Comparison was made using an unpaired two-tailed t test (n.s., not significant, $p > 0.05$).

pnGFP-Ultra not only fills an important technological gap in the field of RNS, but also conveys innovations that will inspire the development of future biosensors for other bioanalytical targets. First, the chromophore (and other residues) of circularly permuted fluorescent proteins can be modified into a hub of chemical sensing, binding, reactivity, and catalysis, thereby greatly expanding the use of fluorescent proteins as reporters. Second, direct evolution and rational mutagenesis performed on ncAA-modified proteins can lead to new, and sometimes surprising, functions, as we have demonstrated here via the engineering of exquisite chemoselectivity of pnGFP-Ultra. While this paper was under review, the genetic introduction of thyronine (Thy), another ncAA, into sfGFP and cpsGFP was reported and the resultant proteins showed early promise for the detection of ONOO[−] (Li et al., 2020). Because aryloxyphenols also react with other highly reactive ROS such as hydroxyl radical ($\cdot$ OH) (Price et al., 2009), we envision that the method we describe here may be used to further engineer Thy-based biosensors specifically responsive to either ONOO[−] or other highly oxidizing species.

We compared the responses of pnGFP-Ultra with a synthetic boronate-based probe, CBA, using activity-based

assays. Although the selectivity is of course a function of the incubation time, pnGFP-Ultra is convincingly a much more selective ONOO[−] biosensor than CBA. The increased chemoselectivity of pnGFP-Ultra is accompanied by reduced reactivity, as the sensory moiety is shielded by the protein scaffold. To date, significant efforts have been devoted to modulating the chemoselectivity of various small-molecule ONOO[−] biosensors (Sikora et al., 2020), and our study provides a unique example that uses a protein scaffold to modulate reactivity and specificity. The engineered protein scaffold may directly scavenge highly reactive ROS, such as HOCl. More generally, the protein scaffold provides a special environment—which affects the reactivity and accessibility of various ROS and RNS—and can serve as a widely adaptable strategy for developing chemoselective biosensors. Furthermore, pnGFP-Ultra still has residual activity with H₂O₂. With extended incubation, it is difficult to completely rule out the contribution of H₂O₂ to the fluorescence increase of pnGFP-Ultra. We thus recommend the use of complementary tools, such as peroxynitrite scavengers, $\cdot$ NO synthesis inhibitors, and additional nitrotyrosine detection methods (Teixeira et al., 2016), for further confirmation.

Compared with existing small-molecule based ONOO[−] probes, pnGFP-Ultra has another advantage of being genetically encodable. This feature permits long-term *in vivo* imaging and organelle-, cell-, and tissue-specific targeting, making it possible to apply pnGFP-Ultra to a variety of biological and disease models. More importantly, because of the convenient fluorescence readout and robust off-on response of pnGFP-Ultra, cell lines stably expressing this reporter can be used in high-throughput drug screening assays. Such capability will aid the development of drugs that protect neurons or other cell types against ONOO[−]-mediated cell toxicity or death (Szabó et al., 2007). pnGFP-Ultra may also be used in genome-wide CRISPR screens (Shalem et al., 2015) to uncover new genes involved in NO, O₂[−], or ONOO[−] production pathways. Because pnGFP-Ultra can be widely distributed in the form of plasmids, it represents a valuable catalyst for future breakthroughs in the field of RNS.

In addition, our optimized plasmid system will greatly facilitate the site-specific incorporation of various ncAAs into proteins in mammalian cells. It can be adapted to overexpress other engineered orthogonal aaRS-tRNA pairs. Also, because the Poly-aaRS can cross-react with an array of *para*-substituted phenylalanine analogs, including *p*BoF, *p*AzF, *p*-acetylphenylalanine, *p*-iodophenylalanine, *p*-bromophenylalanine, *p*-cyanophenylalanine, *p*-nitrophenylalanine, *p*-benzoyl-phenylalanine, 4,4'-biphenylalanine, *O*-methyl-tyrosine, *O*-propargyl-tyrosine, and *O*-allyl-tyrosine (Chatterjee et al., 2013; Chen et al., 2013), the plasmids we describe here may be used directly to express proteins harboring these diverse functional groups, thereby easing and enabling many new studies.

SIGNIFICANCE

pnGFP-Ultra, a high-performance, reaction-based, genetically encodable biosensor for imaging ONOO[−], is presented. pnGFP-Ultra uses a *p*BoF-modified chromophore as the sensing moiety and an engineered circularly permuted fluorescent protein scaffold for further modulation of reactivity and specificity. pnGFP-Ultra exhibits a remarkable ~110-fold fluorescence turn-on response toward ONOO[−] while displaying virtually no cross-reaction with other reactive oxygen/nitrogen species. We developed an optimized plasmid system to facilitate the expression of pnGFP-Ultra in mammalian cells and demonstrated that pnGFP-Ultra could reliably detect physiological ONOO[−] in various cell culture models such as activated macrophages and primary glial cells. pnGFP-Ultra thus represents an important advancement enabling the practical implementation of a genetically encoded biosensor for live-cell detection of ONOO[−]. In addition to live-cell imaging, we envision high-throughput drug and genetic screening to be an important avenue of application where pnGFP-Ultra shall prove to be an enabling tool. Moreover, our unique sensor design and development strategy may be widely adaptable and will inspire the development of chemoselective biosensors for other bioanalytical targets. Furthermore, the optimized plasmid system presented here will facilitate the site-specific incorporation of diverse ncAAs into proteins in mammalian cells.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.chembiol.2021.01.013>.

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AUTHOR CONTRIBUTIONS

H.W., Z.C., and S.Z. conceived and planned the experiments; Z.C. and S.Z. carried out the experiments; X.L. isolated and cultured mouse glia cells. Z.C., S.Z., and H.W. performed data analysis and interpretation; Z.C. prepared figures and wrote the initial draft of the manuscript; Z.C., S.Z., and H.W. edited and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>Escherichia coli</i> DH10B	Thermo Fisher Scientific	Cat# 18297010
Chemicals, peptides, and recombinant proteins		
<i>p</i> -borono-DL-phenylalanine	Synthonix	Cat#: B5001; CAS:90580-64-6
<i>p</i> -azido-phenylalanine	Bachem	Cat#: F-4305; CAS:1241681-80-0
sodium peroxynitrite	Cayman Chemical	Cat#: 81565; CAS:14042-01-4
3-morpholinosydnonimine (SIN-1)	Sigma Aldrich	Cat#: M5793; CAS:16142-27-1
Urate	Alfa Aesar	Cat# A13346-14; CAS: 69-93-2
Minocycline	TCI	Cat# M2288; CAS: 13614-98-7
1400W	Enzo Life Sciences	Cat# ALX-270-073-M025; CAS: 214358-33-5
human Interferon- γ Protein, recombinant	Sigma Aldrich	Cat#: IF002
lipopolysaccharides (LPS) from <i>Salmonella typhosa</i>	Sigma Aldrich	Cat#: L7895
phorbol myristate acetate (PMA)	Sigma Aldrich	Cat#: P8139 CAS: 16561-29-8
coumarin boronic acid pinacolate ester (CBA)	Cayman Chemical	Cat#: 10818; CAS: 190788-61-5
L-tyrosine	Spectrum Chemical	Cat#: T1150; CAS: 60-18-4
β -amyloid (1-42), human	GenScript	Cat#: RP10017
TRI reagent	Thermo Fisher Scientific	Cat#: AM9738
β -mercaptoethanol	MP Biomedicals	Cat# 773648 CAS: 60-24-2
Viomer	ORIGENE	Cat# TT100312
lipofectamine 2000	Thermo Fisher Scientific	Cat# 11668027
SuperScript® IV Reverse Transcriptase	Thermo Fisher Scientific	Cat#: 18090010
RIPA Lysis and Extraction Buffer	Thermo Fisher Scientific	Cat# 89900
Critical commercial assays		
B-PER	Thermo Fisher Scientific	Cat#: 78243
Cell Proliferation Kit I (MTT)	Roche Diagnostics	Cat# 11465007001
Deposited data		
Structure of pnGFP1.5-Y.Cro (PDB ID: 5F9G)	(Chen et al., 2016b)	PDB ID: 5F9G
Experimental models: cell lines		
Human HEK 293T cells	ATCC	CRL-3216™
RAW264.7 Macrophage cells	ATCC	TIB-71™
Experimental models: organisms/strains		
BALB/C mice	The Jackson Laboratory	JAX: 000651
Oligonucleotides		
Primers for molecular cloning, see Table S1	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
Plasmid pEvol-BoF	(Young et al., 2010)	N/A
Plasmid pBAD-cpsGFP2	This paper	N/A
Plasmid pcDNA3-EGFP _{TAG}	This paper, plasmid a in Figure 4A	N/A
Plasmid pMAH-EGFP _{TAG}	This paper, plasmid c in Figure 4A	N/A
Plasmid pMAH-EGFP _{TAG} -POLY	This paper, plasmid e in Figure 4A	N/A
Plasmid pUltra	(Lou et al., 2012)	Addgene # 24129
Plasmid pMAH-POLY	(Chen et al., 2013), plasmid b in Figure 4A	Addgene # 64915
Plasmid pcDNA3-pnGFP	(Chen et al., 2013)	Addgene # 64913
Plasmid pBAD-pnGFP-Ultra	This paper	Addgene #157923
Plasmid pMAH-POLY-eRF1 _{E55D}	This paper, plasmid d in Figure 4A	Addgene #157925
Plasmid pMAH-pnGFP-Ultra	This paper	Addgene #157924
Software and algorithms		
Fiji	(Schindelin et al., 2012)	https://fiji.sc/
PyMOL	Schrödinger	https://pymol.org/2/
GraphPad Prism 8	GraphPad	https://www.graphpad.com/scientific-software/prism/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Huiwang Ai (huiwang.ai@virginia.edu).

Materials availability

All reagents created in this study (see Key Resources Table) are available on request. Key plasmids generated have been deposited to Addgene (http://www.addgene.org/Huiwang_Ai/) and the accession numbers for these plasmids are presented in the Key Resources Table.

Data and code availability

Raw source data are available upon reasonable request. The sequence information for key plasmids is available from Addgene and the accession numbers for these plasmids are presented in the Key Resources Table.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

HEK 293T cells (human, female, embryonic kidney) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS at 37°C and 5% CO₂ atmosphere. RAW264.7 cells (mouse, male, macrophage) were grown in DMEM supplemented with 10% FBS, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 58 µM β-mercaptoethanol at 37°C and 5% CO₂ atmosphere. Primary glial cells were isolated from the brain of newborn (0-2 days postnatal) BALB/C mice (both male and female) and cultured in DMEM (4.5 mg/L glucose) supplemented with 10% FBS, 5 mM glutamine and 100 U/ml penicillin and streptomycin at 37°C and 5% CO₂ atmosphere. Mice were maintained under standard housing conditions (12h/12h light-dark cycle, 22-25°C ambient temperature, and standard chow diet). All animal procedures were conducted in accordance with the protocol approved by the Animal Care and Use Committee at the University of Virginia.

METHODS DETAILS

Materials and general methods

Synthetic DNA oligonucleotides (Table S1) were purchased from Integrated DNA Technologies (San Diego, CA). Restriction endonucleases were purchased from Thermo Fisher Scientific (Waltham, MA). PCR and restriction digestion products were purified by gel electrophoresis and extracted using the Syd Labs Gel Extraction kit (Malden, MA). Plasmid DNA was purified using the Syd Labs Miniprep kit (Malden, MA). DNA sequencing was analyzed by Retrogen (San Diego, CA). Mammalian cell imaging was performed on a Leica DMI8 microscope equipped with a Photometrics Prime 95B sCMOS camera. Other materials and general procedures were acquired or performed as previously described (Chen et al., 2012, 2013, 2016b).

Engineering and screening of pnGFP1.5

To improve the brightness and folding of pnGFP, several rounds of error prone PCR (EP-PCR) based directed evolution were performed to improve its template—cpsGFP (Chen and Ai, 2014), which contains a tyrosine-derived chromophore. Briefly, oligos pBAD-FP and pBAD-RP were used to amplify the cpsGFP gene from the pBAD-cpsGFP plasmid in an EP-PCR reaction condition. The mutated PCR products were digested with XhoI and HindIII and ligated into a predigested compatible pBAD vector. The ligated product was used to transform *Escherichia coli* DH10B competent cells by electroporation. Cells were grown on Luria-Bertani (LB) broth agar plates supplemented with 100 μ g/mL ampicillin and 0.02% L-arabinose. LB agar plates were incubated at 37°C overnight. Bacterial colonies were illuminated under a laboratory-built colony fluorescence imaging system and examined using a pair of forensic yellow goggles with a cutoff wavelength of $\sim$ 490 nm. Plasmid DNA from the brightest colonies of each round were extracted and combined to serve as the template for the next round of EP-PCR. The fluorescence of the B-PER (Pierce, Rockford, IL) extracted cell lysate from the brightest colonies of the final round was quantitatively compared on the Synergy Mx Microplate Reader. The best mutant was sequenced and renamed as cpsGFP2.

To screen for pnGFP1.5, the codon for the chromophore tyrosine (Tyr174) of cpsGFP2 were mutated to TAG to allow for pBoF incorporation using the pEvol-BoF-based amber suppression system (Chen et al., 2013). Next, threonine 5 (Thr5) and threonine 253 (Thr253) of cpsGFP2-Tyr174TAG were randomized using NNK degenerate codons to construct a gene library. The library was used to screen for mutants with high reactivity to ONOO[−]. The best mutant from the library was sequenced and designated as pnGFP1.5.

Development and characterization of pnGFP-ultra

To create the various pnGFP1.5 mutants, site-directed mutagenesis of pnGFP1.5 was performed using an overlap extension PCR based strategy (Chen et al., 2016b), and the mutagenesis primers can be found in Table S1. Proteins for each mutant were purified, buffer-exchanged and concentrated as detailed below for pnGFP-Ultra. To test the reactivity and selectivity of the pnGFP1.5 mutants, 0.2 μ M purified protein was incubated with Tris buffer (150 mM Tris-HCl, 150 mM NaCl, pH 7.4), 100 μ M ONOO[−] or 100 μ M H₂O₂ for 1 hr at room temperature in Tris buffer. Fluorescence intensities at 510 nm with a 490 nm excitation were quantified and represented as means $\pm$ SD from three independent measurements. Fluorescence enhancements of pnGFP1.5 and relevant mutants towards ONOO[−] or H₂O₂ were calculated as “dynamic range” expressed as $\Delta F/F_0$, where F_0 is the initial fluorescence intensity (before treatment) and ΔF is the final fluorescent intensity (after treatment) minus F_0 . The selectivity of each mutant was defined as the ratio of the fluorescence enhancement from ONOO[−]-treated group over that from H₂O₂-treated group. The mutant with the highest selectivity, namely pnGFP1.5-S66A (pnGFP1.6), was chosen for further engineering. To screen for mutants with further enhanced selectivity, the threonine 5 (Thr5) codon of pnGFP1.6 was mutated into two consecutive degenerative codons (NNKNNK) using primers pnGFP1.6-F-NNK2 and pBAD-RP. The gene library was screened and constructed as described before. The mutant with the highest selectivity from this library was sequenced and designated as pnGFP-Ultra.

Characterization protocols for pnGFP-Ultra including the purification, reactivity measurements, selectivity tests over other reactive chemical species, and determination of the limits of detection (LOD) were the same as those for pnGFP (Chen et al., 2013), as detailed below.

To express pnGFP-Ultra, the pBAD plasmid harboring pnGFP-Ultra was used to co-transform DH10B *E. coli* cells with pEvol-pBoF plasmid. Cells were grown on LB agar plates containing 100 μ g/mL ampicillin and 50 μ g/mL chloramphenicol at 37°C overnight. A single colony from the co-transformation was grown overnight at 37°C in 5 ml LB media supplemented with appropriate antibiotics. Saturated overnight cultures were then diluted by 100-fold and grown in 2YT media to OD₆₀₀ = 0.6. L-arabinose (final conc. 0.2%) and pBoF (final conc. 1 mM) were added to express the full-length proteins. Growth continued with vigorous shaking at 37°C for 24 hours, and next, at room temperature for additional 24 hours. Cells were harvested and lysed by sonication. The His-tagged protein was purified using Ni-NTA agarose beads (Qiagen) according to the manufacturer’s instruction. The protein was next concentrated in Tris buffer (30 mM Tris-Cl, pH 7.4) using Amicon Ultra Centrifugal Filter Units (3,000 Da cutoff). Bradford Assays were performed to determined protein concentrations by comparing with a set of BSA standards.

Reactivity measurements were carried out on a monochromator-based Synergy Mx Microplate Reader (BioTek, Winooski, VT). Excitation at 490 nm and emission at 510 nm were used for single-point measurements, and a blank solution was used to subtract the background. Reactivity was reported as either arbitrary fluorescence emission intensity or dynamic range ($\Delta F/F_0$ = (final intensity – initial intensity)/initial intensity).

To test protein responses to various redox-active chemicals, concentrated protein stock solutions were diluted into 1x PBS to gain a final concentration of 200 nM (for data in Figure 3) or 1 μ M (for data in Figure S3). The following methods were utilized to supply individual redox-active species. They were added into proteins, followed with incubation at room temperature. Excitation at 490 nm and emission at 510 nm were used for measurement on a monochromator-based Synergy Mx Microplate Reader.

- Nitric oxide (NO[•]): 1-Hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) stock solution (100 mM) was prepared in 0.3 M NaOH. An appropriate amount of the stock solution was added to generate NO[•]. The final mixtures were confirmed to have no notably pH change from 7.4.
- Hydrogen sulfide (H₂S/HS[−]): Solid NaHS was dissolved in PBS to afford a 100 mM stock solution, whose pH was adjusted to 7.4. An appropriate amount of a freshly prepared stock solution was added. At physiological pH, H₂S and HS[−] are in equilibrium.

- *tert*-butyl hydroperoxide (HOOtBu): 70% solution in H₂O was purchased from Sigma-Aldrich (St. Louis, MO). It was diluted with PBS to gain a 100 mM stock solution. An appropriate amount of a freshly prepared stock solution was used.
- Hydrogen Peroxide (H₂O₂): 30% solution in H₂O was purchased from Fisher (Hampton, NH). It was diluted with PBS to gain a 100 mM stock solution. An appropriate amount of a freshly prepared stock solution was added.
- Hyperchlorite (ClO[−]/HOCl): 5% NaOCl solution was purchased from Fisher (Hampton, NH). It was diluted with PBS to gain a 100 mM stock solution. The pH was adjusted to 7.4. An appropriate amount of a freshly prepared stock solution was added. At physiological pH, ClO[−] and HOCl are in equilibrium.
- Superoxide (O₂^{•−}): solid KO₂ was suspended in DMSO by sonication. An appropriate amount was directly added to a protein in aqueous buffer. Please note that superoxide decomposes quickly to form H₂O₂ in aqueous solution.
- Peroxynitrite (ONOO[−]/ONOOH): Purchased as a solution in 0.3 M NaOH (stored at −80°C). Concentration was determined before use by measuring absorbance at 302 nm. A 100 mM fresh stock solution was made by dilution using cold 0.3 M NaOH. An appropriate amount was utilized to provide ONOO[−]. The final mixtures were confirmed to have no notably pH change from 7.4. At physiological pH, ONOOH and ONOO[−] are in equilibrium.
- Hydroxy Radical (•OH): Generated by Fenton reaction between Fe²⁺ and H₂O₂. Fresh FeCl₂ and H₂O₂ stock solutions were prepared for dilution.
- *tert*-butoxy radical (•OtBu): Generated by Fenton reaction between Fe²⁺ and HOOtBu. Fresh FeCl₂ and HOOtBu stock solutions were prepared for dilution.

Construction of mammalian expression plasmids

Plasmids *a* and *b* (also known as pcDNA3-EGFP_{TAG} and pMAH-POLY, respectively) were previously reported (Chen et al., 2013). To construct plasmid *c* (pMAH-EGFP_{TAG}), the EGFP_{TAG} gene was amplified from plasmid *a* with oligos EGFP-F and EGFP-R. The PCR product was digested with HindIII and Apal, and ligated into plasmid *b*, which was predigested with the same enzymes. To construct plasmid *d* (pMAH-POLY-eRF1_{E55D}), the eRF1 gene was first cloned from the total cDNA of HEK 293T cells with oligos eRF1-F and eRF1-R. To prepare the cDNA, total mRNA from HEK293T cell was isolated with the TRI Reagent (Sigma, St. Louis, MO) and reverse-transcribed with the SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific, Carlsbad, CA). To introduce the E55D point mutation within eRF1, oligos eRF1-NheI-F and E55D-R, E55D-F and eRF1-EcoRI-R were used to amplify the two overlapping fragments flanking E55 position of eRF1, respectively. Next, the two fragments were joined by overlap extension PCR with oligos eRF1-NheI-F and eRF1-EcoRI-R, and sub-cloned into the pUltra vector (Addgene Plasmid #24129) between restriction sites NheI and EcoRI to generate pUltra-eRF1-E55D, which contains the T2A-eRF1-E55D-WPRE cassette. This cassette was amplified with oligos eRF1-GB-F and eRF1-GB-R and ligated into Apal-linearized plasmid *b* using Gibson assembly cloning (Gibson et al., 2009). To construct plasmid *e* (pMAH-EGFP_{TAG}-POLY), the CMV-EGFP_{TAG} cassette was amplified from plasmid *a* with oligos CMV-39TAG-XhoI-F and CMV-39TAG-XhoI-R. The PCR product was digested with XhoI and ligated into XhoI digested, DpnI dephosphorylated plasmid *b*. To construct the mammalian expression plasmid for pnGFP-Ultra, the pnGFP-Ultra gene was amplified from pBAD-pnGFP-Ultra with oligos pnGFP-Ultra-HindIII-F and pnGFP-Ultra-Apal-R, and sub-cloned into plasmid *c* between restriction sites Hind III and Apal.

Mammalian cell culture, transfection and imaging

Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37°C with 5% CO₂ in humidified air. To test the transfection and non-canonical amino acid (ncAA) incorporation efficiency of the various plasmids, cells were split and seeded into 24-well plates 16 hours before transfection. Cells were transfected with 600 ng DNA and 2 μg PEI (polyethylenimine, linear, M.W. 25 kDs) per well for three hours, and then cultured in complete medium supplemented with 2 mM DL-*p*BoF for 48 hours. Cells were lysed with 150 μL RIPA buffer and centrifuged at 13,000 rpm, 4°C for 5 mins. The fluorescence of the EGFP-containing supernatant (100 μL) was measured on the microplate reader with excitation and emission wavelength set at 490 nm and 515 nm, respectively. Transfections were triplicated and data were represented as mean ± SD.

To express pnGFP or pnGFP-Ultra, HEK 293T cells in each 35-mm plastic culture dish were co-transfected with 1.5 μg pnGFP-containing plasmid *a* and 1.5 μg plasmid *b* or 1.5 μg pnGFP-Ultra-containing plasmid *c* and 1.5 μg plasmid *d*, in the presence of 10 μg PEI. Other procedures were the same as those for pnGFP (Chen et al., 2013). Cells were imaged under a Leica DMI8 microscope equipped with a Photometrics Prime 95B sCMOS camera. A GFP filter cube with a 470/40 nm bandpass excitation filter and a 525/50 nm bandpass emission filter was used for cell imaging. To test the effect of ONOO[−] scavengers, transfected HEK 293T cells in Hank's Balanced Salt Solution (HBSS) were treated with 100 μM SIN-1 in the absence or presence of 100 μM urate or 100 μM minocycline. Incubation lasted for 90 min at 37°C under 5% CO₂ before cells were imaged. HEK 293T cells expressing pnGFP-Ultra-Y.Cro were prepared using the same procedure for pnGFP-Ultra, except for that 5 mM additional L-tyrosine (but no DL-*p*BoF) was supplemented into the complete culture medium.

RAW264.7 macrophage cell culture, transfection and activation

RAW264.7 cells were obtained from ATCC and grown at 37°C under 5% CO₂ in DMEM supplemented with 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 58 μM β-mercaptoethanol. Cells were passaged 18 h prior to transfection, and

next, transfected with 1.5 μ g pnGFP-Ultra-containing plasmid c and 1.5 μ g plasmid d in the presence of Viromer according to the manufacturer's instruction. After transfection, cells were cultured in the aforementioned complete medium supplemented with 2 mM DL-pBoF for 24 h. Next, the medium was replaced with fresh complete medium without DL-pBoF and cells were cultured for another 24 h. IFN- γ (50 U/ml) and LPS (0.5 μ g/mL) were added into the cell culture media, and the incubation lasted for another 10 h. After LPS and IFN- γ were removed, cells were rinsed with HBSS and further treated with PMA (200 ng/mL) in HBSS for 1 h before imaging. Additionally, cells in some groups were co-treated with 100 μ M urate, or 10 μ M minocycline, or 100 μ M 1400W when PMA (200 ng/mL) was used for stimulation. Cells were imaged under the same condition as used for imaging HEK 293T. In addition, the procedure to treat RAW264.7 cells with SIN-1 and ONOO⁻ scavengers is identical to that used for treating HEK 293T. RAW264.7 cells expressing pnGFP-Ultra-Y.Cro were prepared by supplementing 5 mM additional L-tyrosine into the complete culture medium.

Cell viability assay

MTT assays were performed using the Roche Cell Proliferation Kit I (MTT) according to the manufacturer's instruction. Briefly, HEK 293T or RAW264.7 cells that transiently expressing pnGFP-Ultra were cultured at a density of 1,000 cells/well in a 96-well glass-bottom plate (Cellvis, Cat. # P96-1.5H-N). Cells in individual wells were subjected to various chemical treatments. To determine cell viability, 10 μ L of the MTT labeling reagent was added into each well. Cells were incubated at 37°C and 5% CO₂ for additional 4 h. 100 μ L of the Solubilization solution was then added to the cells and incubated overnight at 37°C and 5% CO₂. Absorbance for each well was determined at 550 nm using a monochromator-based BioTek Synergy Mx plate reader. Baseline absorbance at 690 nm was also measured and used to subtract the absorbance at 550 nm. The absorbance differences ($A_{550\text{ nm}} - A_{690\text{ nm}}$) were normalized to the values of the control groups that were untreated by chemicals.

Isolation and culture of primary glial cells

Primary glial cells were isolated from the brain of newborn (0-2 days postnatal) wild-type BALB/C mice and cultured as previously described (Redlich et al., 2013; Seele et al., 2016). Briefly, meninges of mouse brains were removed and whole brains were cut into small pieces and treated with 0.25% trypsin (5 mL/brain) for 15 min at 37°C. After centrifugation (1000 $\times$ g, 1 min), trypsin was removed and DMEM (4.5 g/L glucose, 3 mL/brain) was added into the cell precipitation, which was further minced by pipetting up and down with a 1 mL pipette. After another centrifugation (750 $\times$ g, 8 min), the supernatant, which contained the needed cells, was filtered with a 40- μ m sterile cell strainer (Nylon mesh, Fisherbrand, Cat#: 22363547). Cells were next seeded onto 35 mm cell culture dishes with poly-D-lysine coated glass bottoms and cultured in DMEM (4.5 mg/L glucose) supplemented with 10% FBS, 5 mM glutamine and 100 U/ml penicillin and streptomycin at 37°C, 5% CO₂ for 8-10 days before transfection. This well-established procedure is known to derive mixed glial cells composed of astrocytes and microglia (Redlich et al., 2013; Seele et al., 2016).

Transfection and activation of primary glial cells

Primary glial cells were transfected with Lipofectamine 2000 by following the manufacturer's instruction. After transfection, cells were cultured in the complete culture media supplemented with 2 mM DL-pBoF. β -amyloid (1-42, human) was added at a final concentration of 5 μ M at 24 h post transfection. The incubation lasted for another 24 h and the culture media were replaced with fresh complete media supplemented with 5 μ M β -amyloid but without DL-pBoF. The treatment lasted for another 18 h before microscopic images were acquired under the same condition as used for imaging HEK 293T. Cells in the control groups were untreated with β -amyloid, or co-treated with 10 μ M minocycline or 100 μ M 1400W when 5 μ M β -amyloid was used for stimulation. To express pnGFP-Ultra-Y.Cro in primary glial cells, after transfection, cells were cultured in DMEM supplemented with 10% FBS and 5 mM additional L-tyrosine.

QUANTIFICATION AND STATISTICAL ANALYSIS

Microscopic images were analyzed with Fiji (ImageJ). Imaging background was subtracted by setting the rolling ball radius to 300 pixels, and the fluorescence intensities of each selected cells were next determined using Fiji (ImageJ). GraphPad Prism 8 was used for data and statistical analysis. Statistical methods used to determine p values are presented in the figure legends. Sample size, the number of replications for experiments, and dispersion and precision measures (individual data points, mean and SD or 95% confidential intervals) are also presented in figures or figure legends. For Students' t-tests, F-tests were pre-performed to compare the variability of the two samples. For AVOVA tests, Brown-Forsythe tests were pre-performed to compare the variability of the samples. For all comparisons, p values of less than 0.05 were considered statistically significant.