Nitric oxide (NO\textsuperscript*) is a free radical with a wide range of biological effects, but practically impossible to visualize in single cells. Here we report the development of novel multicoloured fluorescent quenching-based NO\textsuperscript* probes by fusing a bacteria-derived NO\textsuperscript*-binding domain close to distinct fluorescent protein variants. These genetically encoded NO\textsuperscript* probes, referred to as geNOps, provide a selective, specific and real-time read-out of cellular NO\textsuperscript* dynamics and, hence, open a new era of NO\textsuperscript* bioimaging. The combination of geNOps with a Ca\textsuperscript{2+} sensor allowed us to visualize NO\textsuperscript* and Ca\textsuperscript{2+} signals simultaneously in single endothelial cells. Moreover, targeting of the NO\textsuperscript* probes was used to detect NO\textsuperscript* signals within mitochondria. The geNOps are useful new tools to further investigate and understand the complex patterns of NO\textsuperscript* signalling on the single (sub)cellular level.
The nitric oxide radical \( (\text{NO})^\bullet \) is one of the most studied molecules\(^1\). The interest in \( \text{NO}^\bullet \) is based on the important roles this radical plays in the chemical industry, in environmental ecology and, above all, in biology, where it represents one of the most versatile mediators in the (cardio-)vascular, nervous and immune systems\(^2\). Recent studies indicate that \( \text{NO}^\bullet \) is also a crucial messenger in tumour cell signalling\(^3\), plant–microbe interactions\(^4\) and the development of resistance of bacteria against antibiotics\(^5\). The wide range of physiological and pathological effects of \( \text{NO}^\bullet \) are partially induced by the reactivity of the molecule, which is able to modify biomolecules including proteins, lipids and nucleic acids\(^6\).

In addition, \( \text{NO}^\bullet \) works as a signalling molecule via binding to metalloproteins with specific iron(II) or zinc(II)-containing domains\(^7\). These domains, \( \text{NO}^\bullet \) reversibly interacts with the metal ion and thereby modulates the conformation and activity of the whole signalling protein\(^8\). Although the fundamental roles of \( \text{NO}^\bullet \) in biology have been established undoubtedly, many questions remain unanswered, because of limitations of the methods available to detect \( \text{NO}^\bullet \) in biological samples\(^9\). Multiple methods to determine \( \text{NO}^\bullet \) concentrations including organ assays\(^9\), cell assays\(^10\), enzymatic assays\(^11\), electrochemical microelectrodes\(^12\), spectroscopic measurements\(^13\) and fluorescent probes\(^14,15\) have been developed. However, despite the availability of such a broad range of \( \text{NO}^\bullet \) detection techniques, research activities designed to investigate the complex metabolism and signalling patterns of \( \text{NO}^\bullet \) in physiology and pathology suffer from the lack of practicable methods for intracellular, single-cell \( \text{NO}^\bullet \) detection\(^6\).

To overcome this limitation, we aimed to develop genetically encoded fluorescent probes that specifically and directly respond to \( \text{NO}^\bullet \), thus providing a quantifiable and real-time readout of cellular \( \text{NO}^\bullet \) dynamics. Therefore, we designed, produced and characterized various genetically encoded \( \text{NO}^\bullet \) probes (geNOps) by selecting a suitable \( \text{NO}^\bullet \)-binding domain that was conjugated with differently coloured fluorescent protein (FP) variants. We assumed that specific \( \text{NO}^\bullet \) binding close to FP in such constructs considerably influences the fluorescence signal by affecting the electron density within certain amino acids forming the chromophore. In this study, we demonstrate that such fluorescent chimeras, referred to as geNOps, represent a completely novel class of \( \text{NO}^\bullet \) indicators that allow direct imaging of (sub)cellular \( \text{NO}^\bullet \) dynamics in real time.

**Results**

**Generation of differently coloured geNOps.** Out of a limited number of known \( \text{NO}^\bullet \)-binding domains, we selected the GAF domain of the enhancer-binding protein NorR, a transcription factor of the enteric bacterium *Escherichia coli*\(^16,17\), for the development of fluorescent geNOps. Being bacteria-derived, the GAF domain of NorR was assumed not to interfere with signalling pathways in higher cells. In addition, the bacterial GAF domain is a small, simply built and specific \( \text{NO}^\bullet \)-binding domain with a non-haem iron(II) centre\(^17\), which appears to be highly stable sensors that enable the recording of extensive \( \text{NO}^\bullet \) fluctuations over long time. The consecutive addition and removal of different concentrations of NOC-7 (1–100 \( \mu \)M) revealed that the differently coloured geNOps respond in a concentration-dependent manner (Fig. 1e,f). The effector concentration for half-maximum response of NOC-7 to induce fluorescence quenching of geNOps was found to be between 50 and 94 \( \mu \)M (Fig. 1f, Supplementary Table 1). Considering the short half-time of NOC-7 (ref. 24) and \( \text{NO}^\bullet \) (ref. 25), these results indicate that geNOps are suitable to recording cellular \( \text{NO}^\bullet \) concentrations in the low physiological \( \text{nM} \) range. However, oxidation of \( \text{Fe}^{2+} \) to \( \text{Fe}^{3+} \) by hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) (Supplementary Fig. 5) or a suboptimal supply of geNOps with \( \text{Fe}^{2+} \) (Supplementary Fig. 6) significantly reduced the response to the \( \text{NO}^\bullet \) donor. These findings support the idea that nitrosylation of \( \text{Fe}^{2+} \) of the non-haem iron(II) centre within the GAF domain is essential to induce fluorescence quenching of the attached FP. We further confirmed the \( \text{Fe}^{2+} \)-dependent \( \text{NO}^\bullet \)-sensing mechanism of geNOps by generating a mutant lacking the arginines at position 75 (deletion) and 81 (R81G), which are
essential for the coordinative binding of Fe²⁺ in the non-haem iron(II) centre\(^{16,17}\) (Supplementary Fig. 7). In contrast to functional geNOps, the fluorescence signal of this mutated construct remained unaffected by the addition of high concentrations of the NO* donor to cells expressing the AIP probe (Fig. 1g). In line with these findings, increasing the NO* concentration in cells expressing the same FP variants alone or fused to either Ca²⁺ - or ATP-binding domains did not impact any of these fluorescence signals (Supplementary Fig. 8). This indicates that the NO* radical, even at high concentrations, does not directly affect the fluorescence of FPs. Consistent with this assumption, the addition of NOC-7 did not affect the fluorescence of HyPer, a genetically encoded H₂O₂ probe\(^{25}\), which showed a clear reduction of fluorescence upon cell treatment with 50 μM H₂O₂ (Supplementary Fig. 9). Contrariwise, the fluorescence of C-geNOpmut was considerably quenched by adding NOC-7 but remained unaffected by administration of H₂O₂, showing that geNOps do not respond to cellular H₂O₂ fluctuations (Supplementary Fig. 9). To further examine the selectivity of geNOps, compounds chemically related to NO*, including carbon monoxide, superoxide and peroxynitrite, were tested. While the used compounds have been shown to at least partially diffuse across the plasma membrane of cells\(^{27-29}\), none of these compounds affected the geNOp fluorescence signal in HeLa cells, demonstrating the high selectivity of the sensor in its exclusive response to intracellular NO* fluctuations (Fig. 1h). As superoxide anions as well as peroxynitrite might not fully penetrate into cells, we also generated a glycosylphosphatidylinositol (GPI)-anchored C-geNOpmut (GPI-C-geNOpmut), which localized at the outer surface of the cell membrane (Supplementary Fig. 10a,b). GPI-C-geNOpmut strongly responded to the addition of NO* donors (Supplementary Fig. 10c), indicating that the probe remains functional upon targeting to the outer surface of the plasma membrane. Addition of either superoxide anions or peroxynitrite significantly affected the fluorescence of GPI-C-geNOpmut (Supplementary Fig. 10c), confirming the high NO* selectivity of geNOps. Moreover, the responsiveness of geNOps to NO* remained at different intracellular pH values (Supplementary Fig. 11). Due to the general pH sensitivity of FPs\(^{19}\), the fluorescence of geNOps was altered upon changes of the intracellular proton concentration.
(Supplementary Fig. 12). O-geNOp containing mKbO (ref. 21) showed the highest pH stability between pH 7 and 9 (Supplementary Fig. 12). Expectedly, the pH-dependent effects on the fluorescence intensity of functional C-geNOp and G-geNOp were equal to that of respective NO*-insensitive mutated constructs (Supplementary Fig. 13). Thus, we assume that a clear discrimination between real cellular NO* and pH fluctuations is possible by comparing measurements using on the one hand functional NO* probes and on the other hand mutated geNOps (geNOps\textsuperscript{mut}) under the same experimental conditions.

**Generation of mitochondria-targeted geNOps.** Several studies point to a particular role of NO* within mitochondria\textsuperscript{3,30}. However, real-time detection of NO* signals within mitochondria in intact cells has not been accomplished so far. Accordingly, we tested whether mitochondria-targeted geNOps (mt-geNOps) allow to overcome this limitation. For this purpose, we constructed mtC-geNOp and mtG-geNOp by fusing a mitochondria-targeting sequence to the N terminus of the fluorescence of mt-geNOps was again boosted by Fe\(^{2+}\)/C15 generation of mitochondria-targeted geNOps. Both mtC-geNOp and mtG-geNOp co-localized with MitoTrackerRed, confirming correct targeting of the NO* probes to mitochondria (Supplementary Fig. 14). To test the functionality of mitochondria-targeted geNOps, cells expressing these probes were treated with NOC-7. Similar to the non-targeted probes, addition of the NO* donor instantly and significantly reduced the fluorescence intensity of mtC-geNOp and mtG-geNOp (Fig. 2b), demonstrating the efficiency of mitochondria-targeted geNOps. The NO* induced quenching of the fluorescence of mt-geNOps was again boosted by Fe\(^{2+}\)/C15 supplementation (Fig. 2b). Mitochondria targeting did not affect the quality of geNOps to detect consecutive pulses of NO* over a long period of time (Fig. 2c). In addition, both mtC-geNOp and mtG-geNOp showed similar sensitivities and responsiveness to different concentrations of NOC-7 compared with the respective non-targeted NO* probes (Fig. 2d). These data prove that mitochondriatargeted geNOps can be used for live-cell imaging of NO* signals within these cellular organelles.

**Imaging of cellular NO* signals in response to NO* donors.** We next applied different NO* donors to visualize and compare NO* dynamics on the single cell level (Fig. 3). For this purpose, we used low-molecular-weight NO* donors and S-nitrosated human serum albumin (S-NO-HSA) with a high capacity to stably release NO* over time, due to its long half-life\textsuperscript{31}. While 30 s perfusion of HeLa cells with NOC-7 and SNP evoked almost identical cellular NO* signals, PROLI NONOate, a more instable compound\textsuperscript{32}, led to a more transient NO* increase, with the highest peak under these conditions (Fig. 3a). In HeLa cells, addition of NOC-7 to the image medium induced clear variances of the strength of NO* signals within cells on the same dish, while the average responses among different dishes were nearly homogeneous (Fig. 3b). These findings might point to cell-to-cell heterogeneities in the NO* scavenging capacity of HeLa cells. Addition of S-NO-HSA induced a distinctly slower increase of cellular NO* levels compared with the fast NO* liberating low-molecular-weight NO* donors (Fig. 3c; Supplementary Fig. 15, left panel). SNP, which is known to liberate NO* by reacting with biomolecules in the cell\textsuperscript{33}, increased cellular NO* levels only at high concentrations (≥1 mM; Supplementary Fig. 15, right panel), pointing to a weak capacity of this compound to release NO*. These experiments demonstrate that geNOps enable the precise characterization of highly diverse NO* donors by providing a

**Figure 2 | The properties of geNOps remain unaffected upon mitochondria targeting.** (a) Confocal images of HeLa cells expressing either mtC-geNOp (left image) or mtG-geNOp (right image). Scale bar, 10 μm. (b) Normalized average curves ± s.e.m. of mtC-geNOp (left panel) and mtG-geNOp (right panel) signals with (n = 4 for mtC-geNOp; n = 7 for mtG-geNOp) and without (n = 5 for mtC-geNOp; n = 4 for mtG-geNOp) iron(II)/vitamin C pretreatment. Experiments were performed using HeLa cells. (c) Representative original curve showing fluorescence over time of mtC-geNOp expressed in HeLa cells in response to consecutive applications of 3 mM SNP (n = 3). (d) Concentration response curves showing the effects of different NOC-7 concentrations on fluorescence intensities of either mtC-geNOp (left panel, cyan curve, n = 4) versus C-geNOp (left panel, grey curve, for n see Fig. 1f) or mtG-geNOp (right panel, green curve, n = 6) versus G-geNOp (right panel grey curve, for n see Fig. 1f). Experiments were performed using HeLa cells. Points represent average values ± s.e.m.
reliable, real-time readout of the actual NO* dynamics on the single-cell level in response to these compounds. Such information is valuable for an efficient testing of newly developed, NO*-releasing and NO*-scavenging drugs. On the basis of the capacity of S-NO-HSA to stably release constant amounts of NO*, this compound was further used to estimate the concentration reflected by geNOps signals. For this purpose, the free NO* concentrations released by different concentrations of S-NO-HSA were determined using a highly sensitive NO*/C15 porphyrinic nanosensor (Supplementary Fig. 16) and plotted against respective geNOps responses (Fig. 3d). This analysis was further used to estimate the physiological NO* concentration in single endothelial cells. Moreover, the approach was used to estimate the on and off kinetics of C-geNOps to respond to NO* (Supplementary Note 2).

Correlations of NO* signals with cell functions. To further demonstrate the applicability of geNOps in other cell types, the probes were expressed in primary embryonic ventricular cardiomyocytes. By measuring geNOps signals, we could show that the addition of nitric oxide donors allowed us to evoke controllable cellular NO* elevations in this cell type (Supplementary Fig. 17). Hence, we further used this approach to mimic and investigate the paracrine effect of exogenously generated NO* on spontaneous Ca2+ signals in single cardiomyocytes. Elevation of NO* did not prevent Ca2+ transients but temporally correlated with a moderate increase of the frequency of Ca2+ oscillations (Fig. 4a), confirming that NO* is a regulator of myocardial function. In an additional set of experiments, we used the geNOps technology to relate elevated cellular NO* levels with the motility of individual glioblastoma cells (Fig. 4b–d). Short treatment of the cells with a mixture of PROLI NONOate and NOC-7 highly increased the cellular NO* concentration (Fig. 4b). This procedure did not affect the overall cell motility (Fig. 4c) but markedly reduced the radius of cell movements (Fig. 4d), indicating that high NO* pulses might impair the metastatic spread of glioblastoma cells.

**Figure 3** | Imaging of cellular NO* dynamics with geNOps in response to different NO*-liberating molecules. (a) Representative single HeLa cell NO* dynamics in response to 1μM NOC-7, 1mM SNP or 1μM PROLI NONOate. Cells expressing C-geNOps were imaged. Inverted curves (1 – F/F0 in %) are shown. Average curves with s.e.m. are shown in Supplementary Fig. 15. (b) Scatter dot plot showing maximal single-cell C-geNOps signals in response to 10μM NOC-7 on different dishes. White column represents the normalized average ± s.e.m. C-geNOps signal of all single HeLa cells (n = 67). (c) Intracellular NO* dynamics of a single HeLa cell expressing C-geNOps in response to different concentrations of S-NO-HSA (curve is inverted). (d) Respective ΔF/Δt intensity mean values ± s.e.m. are blotted against NO* concentrations that are released by 1, 3, 10 and 30 μM S-NO-HSA (n = 6). NO* released by S-NO-HSA was quantified using a porphyrinic nanosensor (for details see Supplementary Fig. 16 and methods).

**Imaging of Ca2+-induced NO* formation in endothelial cells.** We tested the utility of geNOps in visualizing physiologically triggered, Ca2+-activated enzymatic NO* generation in the human umbilical vein cell line EA.hy926, which is known to solidly express the endothelial nitric oxide synthase (eNOS). Ca2+ mobilization with different concentrations of the physiological inositol 1,4,5-trisphosphate (IP3)-generating agonist histamine resulted in clear responses of functional (Fig. 5a), but not mutated geNOps (Supplementary Fig. 18), demonstrating endogenous Ca2+-triggered concentration-dependent NO* production in single endothelial cells. The NO* signals in endothelial cells were reduced in the absence of Ca2+ entry (Supplementary Fig. 19), confirming the importance of Ca2+ influx for sustained eNOS activity. Moreover, as expected the histamine-evoked NO* signals were strongly diminished in the presence of NOS inhibitors (Fig. 5b,c; Supplementary Fig. 20). While cell treatment either with the IP3-generating agonist histamine or ATP induced almost identical patterns of NO* elevations, the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) inhibitor thapsigargin evoked a clearly delayed, slower
and weaker NO* rise in endothelial cells (Fig. 5d). To correlate the temporal patterns of cytosolic NO* and Ca2+ dynamics in individual cells, red-shifted geNOps (either G-geNOp or O-geNOp) were co-imaged with fura-2, an ultraviolet excitable chemical Ca2+ indicator36 (Fig. 5e,f). This approach unveiled a temporal delay and slower kinetics of cellular NO* dynamics compared with respective cytosolic Ca2+ signals elicited by addition of either histamine (Fig. 5e; Supplementary Fig. 21) or the Ca2+ ionophore ionomycin (Fig. 5f). However, these experiments also highlighted a strict correlation between the enzymatic NO* production and cytosolic Ca2+ signals in single endothelial cells.

**Imaging of NO* within mitochondria of endothelial cells.** Next, we used endothelial cells expressing mitochondria-targeted G-geNOp to test whether endogenously generated NO* is detectable within these organelles. Cell treatment with ATP elicited clear mtG-geNOp signals, which were strongly reduced by the addition of L-NAME and recovered robustly in the presence of NOC-7 (Fig. 6a,c). The fluorescence of the NO*-insensitive mtG-geNOpmut did, however, not respond to any of these treatments under the same experimental conditions (Fig. 6a). Respective geNOps signals in endothelial cells expressing non-targeted cytosolic G-geNOp did not significantly differ from the mitochondrial responses (Fig. 6b,c). These data demonstrated that NOS activation upon Ca2+ mobilization with an IP3-generating agonist also yield a significant elevation of NO* within mitochondria in single endothelial cells. Next, we performed multichannel imaging of mitochondria-targeted and cytosolic geNOps in the same single cells to correlate NO* signals within both compartments. While the fluorescence of mtC-geNOp could be completely separated from the fluorescence of cytosolic G-geNOp using confocal microscopy (Fig. 6d), a spectral overlap between ECFP- and EGFP-based geNOps was observed using a wide-field imaging system (Supplementary Fig. 22). Hence, we applied spectral unmixing37, which eliminated the spectral crosstalk between mitochondria-targeted and cytosolic geNOps (Supplementary Fig. 22; Supplementary Note 3). To validate this procedure, endothelial cell co-expressing C-geNOpmut and mtG-geNOp were treated first with ATP and subsequently with NOC-7. Neither ATP nor NOC-7 significantly affected the fluorescence of the non-targeted cytosolic C-geNOpmut, while in the same cell the mitochondria-targeted mtG-geNOp showed clear responses, confirming complete separation of respective fluorescence channels (Supplementary Fig. 23). Co-imaging of mtC-geNOp and cytosolic G-geNOp revealed identical ATP-triggered NO* signals in both compartments of a single individual endothelial cell (Fig. 6e). The same result was obtained in cells expressing both mtG-geNOp and cytosolic C-geNOp (Supplementary Fig. 23). These data indicate that upon eNOS activation NO* instantly and efficiently increases both in the cytosol and within the mitochondrial matrix. In addition, our data demonstrate that upon removal of the agonist, NO* declines with the same kinetics in both compartments (Fig. 6e; Supplementary Fig. 23).

**Discussion**

Although the importance of NO* as a key regulator of diverse cell functions is well accepted, little is known about the actual dynamics of this radical within single cells and subcellular compartments. The lack of practicable techniques that provide a selective, direct and real-time readout of single (sub)cellular NO*
dynamics hampered investigations in this regard38, since NO\* has been discovered to function as an endothelium-derived relaxing factor in 1987 (ref 39). The differently coloured geNOps, we have introduced in this study, can be used for real-time tracking of NO\* in single cells and subcellular compartments such as mitochondria. The key feature of geNOps is that these probes selectively bind NO\*, which induces a significant quenching of the intensity of the FP within the probe. This concentration-dependent effect occurs immediately upon NO\* binding and is fully reversible and repeatable so that geNOps can be used to visualize (sub)cellular NO\* signals dynamically and over a long period of time.

Convincing measurements of single cell NO\* signals in real time with other small chemical fluorescent NO\* indicators such as 4,5-diaminofluorescein diacetate have not been accomplished so far. While such probes can be easily loaded into cells, NO\* and other reactive species irreversibly modify the chemical structure of these fluorescent indicators so that they do not provide a selective and actual readout of cellular NO\* signals40. Moreover, small chemical NO\* probes have been shown to be cytotoxic and can aggregate within certain cell compartments, both of which considerably limit their range of usability40,41. Hence, it is very important to develop novel improved NO\* probes that overcome these limitations. In contrast to small chemical indicators, genetically encoded fluorescent probes are usually not toxic for cells and can be efficiently localized to virtually any subcellular compartments42,43. The development of protein-based sensors is, however, challenging44. Usually, this requires fusion of proper sensing domains to one or more FPs in subcellular compartments 42,43. The development of functional fluorescent geNOps, Pearce et al. used metallothionein, a cysteine-rich small protein with unknown functions, to detect the production of NO\* in intact cells45. In their study, the authors could confirm that NO\* interacts with

![Image](https://example.com/image.png)

**Figure 5 | Live-cell imaging of Ca\(^{2+}\) -triggered NO\* production in signals endothelial cells.** (a) Single endothelial cell (EA.hy926 cells) NO\* responses upon cell treatment with different concentrations of histamine (right panel, 0.3 μM; 1.0 μM; 3.0 μM; 100 μM histamine, inverted curves are shown) in the absence of extracellular Ca\(^{2+}\). For the concentration response curve (right panel), cells expressing C-geNOp were stimulated with 0.1 μM (n = 6), 0.3 μM (n = 7), 1.0 μM (n = 7), 3.0 μM (n = 7), 10.0 μM (n = 7) or 100.0 μM (n = 12) histamine, yielding an effector concentration for half-maximum response of 1.4 (0.8–2.5) μM. Red points represent average values ± s.e.m. (b) Cellular NO\* dynamics of EA.hy926 cells expressing C-geNOp. Cells were stimulated with 100 μM histamine in Ca\(^{2+}\) containing buffer for 9 min under control conditions (black inverted curve, n = 4) or during stimulation, 1 mM L-NAME was added (red inverted curve, n = 9). (c) Columns represent maximal G-geNOps signals ± s.e.m. in response to either 10 (red columns) or 100 μM (black columns) histamine under control conditions (n = 5 for both histamine concentrations) and in the presence of the NOS inhibitor (1 mM; n = 10 for both histamine concentrations). *P < 0.05 versus control (10 μM histamine); #P < 0.05 versus control (100 μM histamine). P values were calculated using unpaired t-test. (d) Average NO\* curves over time (right panel) and statistics of the maximal cytosolic NO\* increase (columns representing average values ± s.e.m. in the left panel) in EA.hy926 cells in response to 30 μM histamine (black curve, black column, n = 16), 30 μM ATP (red curve and red column, n = 20) or 1 μM thapsigargin (blue curve, blue column, n = 15). Endothelial cells expressing C-geNOps were used *P < 0.05 versus histamine/ATP using unpaired t-test. (e) Curves represent simultaneous recordings of cellular Ca\(^{2+}\) (black ratio curve) and NO\* (red inverted curve) signals over time of a single fura-2/am-loaded endothelial cell expressing O-geNOp as shown in Supplementary Fig. 21. The cell was stimulated with 100 μM histamine in the presence of extracellular Ca\(^{2+}\). (f) Simultaneous recordings of cellular Ca\(^{2+}\) (black ratio curve) and NO\* (red inverted curve) signals over time of a single fura-2/am-loaded endothelial cell expressing G-geNOp. During imaging, the cell was treated with 1 μM ionomycin in the absence (1 mM EGTA) and presence of 2 mM Ca\(^{2+}\).
metallothionein, and that NO$^*$ binding affects the protein conformation, which results in increased Förster resonance energy transfer (FRET) between terminally located FPs. However, this FRET-based probe only provides a readout of a single NO$^*$ elevation, as it does not respond to NO$^*$ in a reversible manner. Moreover, the probe releases metal ions upon NO$^*$ binding that might impact cell functions. Actually, only few NO$^*$-binding proteins in mammals, plants and bacteria have been identified and characterized so far. Accordingly, the number of known putative NO$^*$-sensing domains for the development of protein-based NO$^*$ probes is quite limited. In mammals, the soluble guanylate cyclase (sGC) is the dominant NO$^*$ responsive target, which reversibly binds NO$^*$ via a haem iron centre. NO$^*$ binding to sGC stimulates the generation of cyclic GMP (cGMP), an intracellular second messenger that regulates multiple cell processes. While iron(II) supplementation did not cause any obvious problems when using the geNOps technology in cultured cells, this procedure might limit the applicability of geNOps. It might be challenging to increase the iron(II) amount of expressed geNOps when using this technology in vivo. On the other hand, the iron(II) homeostasis in living organisms might be anyway sufficient to supply expressed geNOps with iron(II) adequately. However, further experiments are necessary to investigate whether or not geNOps are useful tools to image NO$^*$ signals also in vivo.

The basal fluorescence of geNOps was affected by pH changes as FPs are pH sensitive. However, the responsiveness of geNOps to NO$^*$ remained over a huge pH range, indicating that these probes can be also used in alkaline and acidic compartments such as mitochondria or endo- and lysosomes, respectively. Indeed, we demonstrated that mitochondria-targeted geNOps remain fully functional. Nevertheless, due to the pH sensitivity of FPs, acute pH changes within cells might complicate correct interpretation of geNOps signals. In this study, we, hence, performed key experiments using mutated probes that did not respond to NO$^*$, but kept their pH sensitivity. Using these probes under the same experimental conditions allowed us to estimate that the geNOps signals reflect real (sub)cellular NO$^*$ dynamics and were not due to acute pH changes. The development of novel optimized geNOps that contain other bright and pH-stable FPs would be a direct approach to circumvent this problem. Considering the high number of additionally available and responsive geNOps, we decided to further investigate the responsiveness of all the differently coloured probes. Iron(II) supplementation was essential to significantly increase the dynamic range of all geNOps in different cell types. We established a fast, simple and non-harmful method to supply geNOps-expressing cells with efficient amounts of iron(II), which under normal cell culture conditions is provided rather poorly.
newly developed FP variants with improved properties as well as novel techniques to generate and test whole libraries of altered probes, such efforts will certainly yield in advanced geNOps in near future. Due to the high signal-to-noise ratio of geNOps, we were able to study both the dynamics of (sub)cellular NO* signals in response to even low concentrations of different NO* donors and endogenously Ca2+-triggered NO* production in endothelial cells. Our experiments revealed that Ca2+ mobilization using the two different IP3-generating agonists histamine and ATP evoked identical NO* increases in endothelial cells, while the SERCA inhibitor thapsigargin was less effective to elevate NO* production. These results are consistent with other reports that show clear differences in the kinetics and amplitude of cytosolic Ca2+-signals in response to either IP3-generating agonists or SERCA inhibitors. The combination of fura-2 with red-shifted geNOps demonstrated that Ca2+-signals temporally correlate with respective NO* transients in endothelial cells. These findings point to a fast on and off kinetic of the Ca2+-regulated eNOS activity and displayed how tight this enzyme is under the control of the cytosolic Ca2+ concentration. Targeting NO* into the mitochondrial matrix in combination with cytosolic geNOps enabled us to simultaneously monitor NO* dynamics in both compartments in single individual endothelial cells. These experiments showed that Ca2+-triggered NO* signals are identical in both compartments, confirming the high capability of NO* to diffuse across biomembranes. It has been suggested that mitochondria are able to generate NO* autonomously under certain conditions. Moreover, the existence of NOS located in both mitochondrial compartments in single individual endothelial cells. These experiments showed that Ca2+-triggered NO* signals are identical in both compartments, confirming the high capability of NO* to diffuse across biomembranes. It has been suggested that mitochondria are able to generate NO* autonomously under certain conditions. Moreover, the existence of NOS located within mitochondria has been proposed, while the respective protein has not been identified explicitly so far. Our experiments shown in this manuscript neither confirm nor argue against a mitochondrial NO production, but the geNOps experiments shown in this manuscript neither confirm nor argue against a mitochondrial NO production, but the geNOps technology will be very useful to further investigate this and other remaining important question in the field of NO*-related cell biology.

In summary, we have generated differently fluorescent geNOps and have demonstrated their suitability to single-live-cell NO* imaging in different cell types. These novel tools will enhance the high-resolution investigation of intracellular NO* generation, degradation, as well as diffusion under physiological and pathological conditions. This, in turn, will improve our understanding of the complex cellular metabolism and signalling patterns of one of nature’s most reactive and versatile messengers.

Methods

Cloning of geNOps. Briefly, cloning was performed according to standard procedures and all products were verified by sequencing. Genomic DNA of E. Coli DH110v was isolated by a DNA extraction protocol using phenol/chloroform extraction followed by ethanol precipitation and subsequent solubilization in 30 μl deionized water. The bacterial DNA was used as a template to isolate the GAF subunit of the NorR transcription factor in a PCR with the following primers: forward 5′-GGCATCGATATGAGTTTTTCCGTTGATGTGC-3′ and reverse 5′-GGGAACTTAAAGGGACAACCGATATCCT-3′ including a stop codon and a HindIII site. To obtain various single FP-based geNOps, the PCR product of the GAF domain was C-terminally fused to a super ECFP, a blue-green emitting FP (EGM20), an ECFP, a circularly permuted Venus or a mKO, via CiaI and HindIII in a mammalian expression vector pcDNA3.1(-) (Invitrogen, Austria). To construct the NO*-insensitive probes (C-geNOpmut and G-geNOpmut), the two arginines at positions 75 and 81 of the GAF domain were mutated by a two-step PCR protocol using two additional primers forward 5′-AAGCGCTTAAAGGGACAACCGATATCCT-3′ and reverse 5′-CCGGGCGGGCCGCGCCGCGGCATGTTCGTCCGGCT-3′. For targeting geNOps into mitochondria, two COX VIII mitochondria-targeting sequences were added to the N terminus of respective constructs. To target C-geNOps to the outer surface of the plasma membrane, a membrane leading sequence of the human cadherin 13 (24 amino acids) was added to the N terminus and the GPI-anchor sequence of cadherin 13 (coding for 26 amino acids) were fused to the C terminus of C-geNOps, respectively.

Chemicals and buffer solutions. Cell culture materials were obtained from PAA laboratories (Pasching, Austria). Histamine hydrochloride, Iron(II)omurate, 2,3-Di-t-butyl-1,4-benzohydroquinone, ethylene glycol tryacetate acid (EGTA), Tris-HCl, monensin, nigericin, CORM-3, L-NAME and potassium superoxide were purchased from Sigma Aldrich (Vienna, Austria). NONOate were from Cayman Chemical (Michigan, USA). SNP was purchased from Gatt-Koller (Absam, Austria). Ionomycin was obtained from Abcam (Cambridge, UK).

Before the experiments, cells were washed and maintained for 20 min in a HEPES-buffered solution (storage buffer) containing 138 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes and 1 mM HEPES, the pH was adjusted to 7.4 with NaOH. During the experiments, cells were perfused in a physiological Ca2+-containing buffer (Ca2+-buffer), which consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes and 1 mM HEPES, the pH was adjusted to 7.4 with NaOH. For Ca2+-free experiments, Ca2+-buffer was exchanged by a Ca2+-free buffer instead of 2 mM CaCl2. Preparation of iron(II) fumarate solution was performed in the Ca2+-buffer by adding 1 mM iron(II) fumarate and 1 mM ascorbic acid and stirring at room temperature in the dark. During the experiments, various NO donors or other pharmacological compounds were applied to the cells using a gravity-based perfusion system connected with a conventional vacuum pump (Chemistry diaphragm pump ME 1C, Vacuubrand, Wertheim, Germany).

Measurement of NO* release using a porphyric nanosensor. For estimation of NO* concentrations, release of NO* from S-NO-HSA dissolved in physiological saline was measured with a porphyric nanosensor in a tissue culture bath at identical concentrations as used for geNOp signal imaging. The nanosensor was assembled as a three-electrode system consisting of the sensor working electrode, a platinum wire (0.1 mm) counter electrode, and a standard calomel reference electrode. The current proportional to concentration was measured by the nanosensor operated in an amperometric mode at a constant potential of 0.65 V. The response time of the nanosensor was 0.1 ms. The NO* nanosensor was calibrated for the range 1 μmol l−1 using aliquots of a NO* standard-saturated aqueous solution (1.76 mmol l−1). The amperometric signals for NO* were recorded with a computer-based Gamry YF600 voltammetric analyser.

Equation for [NO*]cyto from respective changes in fluorescence intensities of C-geNOps (∆F) was obtained by plotting the respective NO* concentrations obtained with the porphyric nanosensor) against ∆Fmax, values and fitting the data with a saturation kinetic:

\[
\frac{[\text{NO*}]_{\text{cyto}}}{\Delta F} = \frac{\Delta F \times K}{\Delta F_{\text{max}}} 
\]

where K is the concentration of S-NO-HSA at half maximal response (4.50) and ∆Fmax is the maximal geNOp response (19.16).

Cell culture, transfection and fura-2/AM loading. Hela cells were grown in DMEM (Sigma Aldrich) containing 10% fetal bovine serum, 100 U ml−1 penicillin and 100 μg ml−1 streptomycin. Culture medium of EA.hy926 cells contained additionally 1% HAT (5 mM hypoxanthin, 20 μM aminopterin and 0.8 mM thymidine). Human glialblastoma U87-MG cells were cultured in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, 50 U ml−1 penicillin and 100 μg ml−1 streptomycin. At 60–80% confluence, cells in 30-mm imaging dishes were transfected with 1 ml of serum- and antibiotic-free medium containing 1.5 μg of the appropriate plasmid DNA and 3 μg of TransFast transfection reagent (Promega). Cells were maintained in a humidified incubator (37 °C, 5% CO2, 95% air) for 16–20 h before changing back to the respective culture medium. All experiments were performed either 24 or 48 h after transfection. For dual recordings using fura-2, cells were incubated in storage buffer containing 3.3 μM fura-2/AM for 40 min. Before the experiments, cells were incubated 10 min in the iron(II) fumarate solution.

Culturing embryonic chicken ventricular cardiomyocytes. Ventricular myocytes were isolated from embryonic chick hearts. The hearts of 7-day embryos were removed, and the ventricles were chopped off, minced and transferred to a nominally Ca2+ 2− and Mg2+ 2− free Hanks’ balanced salt solution (HBBS; in mM: 137 NaCl, 5.4 KCl, 0.34 Na2HPO4, 0.44 KH2PO4, 4.2 NaHCO3 and 5 glucose, pH 7.4) containing 0.25% trypsin (bovine pancreas, Sigma-Aldrich). The suspension was transferred to a shaker bath at 37 °C for 7 min, afterwards cells were released with mechanical disruption (pipetting) and filtered through a 100-μm mesh. HBBS, supplemented with fetal calf serum (5% final concentration), was added to stop trypsin activity. The cell suspension was centrifuged at 100g for 5 min at 4 °C, the supernatant was discarded and the cell pellet was resuspended in fresh trypsin-free HBBS. The centrifugation and resuspension processes were then repeated. After the third times were resuspended in 1 ml of culture medium (Sigma-Aldrich, supplemented with 4% fetal calf serum, 2% horse serum and 0.7 mM glutamine, pH 7.4) to yield a density of 3.5 × 106 cells per ml.
Live-cell imaging of NO\(^+\) concentrations with geNOps. Measurements were performed on two different wide-field imaging systems: an inverted and advanced fluorescence microscope with a motorized sample stage (TILL Photonics, Graefelfing, Germany) was used. The probes were excited via a polychrome V (TILL Photonics), and emission was visualized using a \(\times 40\) objective (alpha Plan Fluor 40 ×, Zeiss, Gottingen, Germany) and a charge-coupled device camera (AVT Stringray F145B, Allied Vision Technologies, Stadtruda, Germany). C-geNOp and M-geNOp were excited at 430 nm, G-geNOp and Y-geNOp at 480 nm, and O-geNOp at 515 nm. Emitted light was collected with emission filters CFP emitter 482/18 nm, yellow fluorescent protein emitter 514/3 nm or orange fluorescent protein-emitting filter (560dxc), respectively. In addition, for simultaneous measurements of cytosolic Ca\(^2+\), Fura-2 was alternately excited at 340 and 380 nm, and emissions were captured at 515 nm (515dxc). For control and acquisition, the Live acquisition 2.0.12 software (TILL Photonics) was used.

Alternatively, geNOps were visualized on a Nikon eclipse TE300 inverted microscope (Tokyo, Japan) using a \(\times 40\) objective (Plan Fluor, Nikon, Vienna or Fluor, Zeiss, Jena, Germany) and fluorescence was recorded with a Spot pursuit charge-coupled device camera (Visitron Systems, Puchheim, Germany). Fura-2 and geNOps were excited as described above, and emissions were collected using emission filter 510WB40 or XF56 (Omega Opticals, Brattleboro, VT, USA). Data acquisition and control were done using the VisiView Premier Acquisition software (Visitron Systems).

Characterization of the pH sensitivity of geNOps. To characterize the pH sensitivity, HeLa cells expressing C-geNOp were treated using a series of buffers with various pH values ranging from 5 to 9. Cells were prepared with 10\(\mu\)M nigericin and 10\(\mu\)M monensin, and 20 mM MES (for pH 5–6.5), 20 mM HEPES (for pH 7–7.5) or 20 mM Tris-Cl (for pH 8–9) containing buffer. Cells were additionally stimulated with 10\(\mu\)M NOC-7 at respective pH values.

Construction of structural models of geNOps. Models of all geNOps were constructed with the online tool Phyre2 (Protein Homology/analogy Recognition Engine V 2.0). Analyses of the predicted proteins were performed with the software DeepView/Swiss Pdb viewer V4.1.0 observed from ExPASy.

Cell velocity measurements. Centre of mass was determined for cells over the whole stack after binarization with an Otzu auto threshold in ImageJ. To determine the cell velocity between consecutive positions, following equation was used:

\[
\nu = \sqrt{\left( \frac{x_{t+1} - x_{t}}{t_{t+1} - t_{t}} \right)^{2} + \left( \frac{y_{t+1} - y_{t}}{t_{t+1} - t_{t}} \right)^{2}}
\]

(\(x\) and \(y\) are the localization coordinates of the centre of mass at consecutive time points \(t_{t}\) and \(t_{t+1}\)).

Statistical analysis. Statistical analysis was performed using the GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). Analysis of variance and \(t\)-test were used for evaluation of the statistical significance. \(P<0.05\) was defined to be significant. At least three different experiments on different days have been performed for each experimental set-up.

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Author contributions

E.E. conceived the idea, designed the constructs, and performed the imaging experiments and data analysis. B.G., S.C. and S.B. performed the imaging and image analysis. H.B. generated the mitochondria-targeted gNOps, GPI-anchored C-gNOp and characterized the constructs. C.T.M.-S. tested cell viability and metabolic activity, R.R. organized cell culture, cell transfection and tested probes. B.P. isolated and cultured cardiomyocytes. W.S. and E.B. cultured and transfected glioblastoma cells. S.H. generated the constructs, and performed cloning and imaging experiments. W.F.G. together with E.E. and R.M. conceived and designed the study, interpreted the data and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: E.E., M.W.-W., R.M. and W.F.G. have filed a UK patent application (patent application number 1419073.0) that describe parts of the research in this manuscript. This does not alter the authors’ adherence to all of the Nature Communications policies on sharing data and materials presented in this manuscript. The remaining authors declare no competing financial interests.

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