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TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

D I P L O M A R B E I T

„Entwicklung und Testung genetisch kodierter NO-Sensoren zur Messung von NO-Konzentrationsveränderungen in einzelnen Zellen an hochauflösenden Fluoreszenzmikroskopen“

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Wien, im Januar. 2014

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Acknowledgement

I am using this opportunity to express my gratitude to everyone who supported me through this exciting project.

First I would like to thank Professor Roland Malli who not only served as my supervisor but also encouraged and challenged me throughout my project work for the last 14 month, who guided me through this process, sharing his truthful and illuminating views on a number of issues related to the project.

I am thankful for Dr. Markus Waldeck-Weiermaier for his friendly advice, constructive criticism, great support in the lab, and for his spent of countless hours to listening to me and talk about my research.

This project would have been impossible without the support of Prof. Dr. Wolfgang Graier, who never accepting less than my best efforts and who funded this project at hard times.

I would like to thank Dr. Seth Hallström for his extraordinary support in this project.

I am sincerely grateful to Benjamin Gottschalk, René Rost, Sandra Blass who supported me throughout the course of this work.

I would also like to thank my teacher and project external guide Prof. Dr. Robert L. Mach for his friendly support.

But most of all, I thank my wife Halime who cared, loved and supported me for all the late nights, early mornings and weekends when I spent my time in the lab, my deepest gratitude. Your help when the times got rough is invaluable.

Above all, thank you Rabbul Alemin.

Dedication

I dedicate all my work
to my children
Sarah, Dilara, Kerem and Muaz

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Abstract

Nitric oxide (NO) is a short-lived radical with a wide range of biological effects that are involved in multiple physiological and pathological processes. Given the importance of NO in biology, the measurement of NO dynamics in living cells is obvious. However, direct NO imaging emerged as a difficult task. We have generated a novel class of genetically encoded fluorescent NO probes, the geNOps, by fusing fluorescent proteins of different spectral properties to the NO-binding domain of norR, a bacterial transcription factor. This approach resulted in cyan, green, yellow and orange fluorescent constructs that directly, specifically and reversibly respond to NO by a significant reduction of the fluorescence intensity and allow bio-imaging of cellular NO dynamics in real time. In endothelial cells geNOps were used to study the dynamics of Ca^{2+}-induced NO biosynthesis. This class of probes opens a new era of NO bioimaging.

Kurzfassung

Stickstoffmonoxid radikale (NO) sind kurzlebige Moleküle die in vielen physiologischen und pathophysiologischen Prozessen eine bedeutende Rolle spielen. Für das Verständnis der Funktionen von NO als zellulärer Botenstoff ist es wichtig, die dynamische Regulierung von NO in lebenden Zellen messen zu können. Leider haben sich die bisherigen beschriebenen Methoden des NO Nachweises als wenig tauglich für die Messung von NO Konzentrationsveränderungen in einzelnen Zellen erwiesen. Deshalb gibt es trotz jahrzehnter langer Forschung in diesem Feld noch immer Kontroversen über die Dynamik der NO Homöostase in einzelnen Zellen. In dieser Arbeit wird eine neue Klasse von fluoreszenzprotein-basierenden genetisch kodierten NO Sensoren vorgestellt, die es erlauben NO auf direktem Wege in einzelnen Zellen zu messen. Die Fusion eines bekannten NO sensitiven bakteriellen Transkriptions-Faktors norR, oder norR Domänen mit verschiedenen fluoreszierenden Eiweißmolekülen ergab funktionelle NO-Sensoren, welche als sogenannten geNOps bezeichnet wurden. Diese geNOps stehen nun als cyan, grün, gelb und orange fluoreszierende NO Sensoren zur Verfügung, welche hochsensitiv, selektiv und in reversibler Form Echtzeitmessungen von NO-Signalen in einzelnen Zellen erlauben. Erste Ergebnisse zeigen, dass sich die Sensoren für physiologische Messungen in Endothelzellen ausgezeichnet eignen. In dieser Arbeit wurden Ca^{2+}-induzierte NO Signale mit Hilfe der geNOps-Technologie in Endothelzellen visualisiert. Die Zukunft wird zeigen, inwieweit die Einführung von geNOps eine neue Ära des NO-Bio-Imagings ermöglichen wird.

1. Introduction

1.1. Background and aims of the study

Nitric oxide is a free radical molecule with an unpaired electron. However, throughout this work NO instead of NO• is used as an abbreviation for the nitric oxide radical. For more than three decades, the biological importance of nitric oxide is known. Nowadays, there are already a number of methods available to determine NO. Especially approaches based on fluorescent microscopy are powerful techniques to monitor intracellular processes with high spatial and temporal resolution. Unfortunately, the known methods based on fluorescence for NO measurements are limited and are not capable to perform credible measurements of NO in single cells (Hunter et al. 2013). An applicable way is long overdue. That is why we have set ourselves the goal to construct a fluorescent NO probe to image the NO-dynamics in single living cells.

At the beginning of this exciting path we were first looking for a protein which is able to bind NO. The specificity and wide variety of bacterial ligand binding proteins, which undergoes ligand specific conformational changes, are commonly used templates for the generation of fluorescent biosensors (de Lorimier, Robert M et al. 2002). In fact, until now the only regulatory protein in enteric bacteria known to serve exclusively as an NO-responsive transcription factor was the enhancer binding protein norR. (D'Autréaux et al. 2005). At a first glance it seems possible to generate a working genetically encoded NO sensor based on the described structure and function of this bacterial protein. However, as a NO-induced conformational change of norR most likely requires an interaction with specific sites of bacterial DNA (D'Autréaux et al. 2005), the generation of such a norR-based genetically encoded fluorescent NO probe for eukaryotic cells might be challenging. Thus, the aims of the present study were to design, generate and test if fusions of fluorescent proteins to norR or the NO-binding GAF-domain of norR results in simple genetically encoded fluorescent NO sensors that also work in higher cell types.

1.2. NO is one of the most studied molecules

NO is a small free radical and plays an important role in the chemical industry, in environmental ecology and above all in biology (Wang et al. 2002). In 1988 it was shown by Palmer et al. (Palmer et al. 1988) that NO is not just an environmental contaminant (reviewed in Osorio and Recchia 2000). Based on these findings NO has become one of

the most studied molecules in the last decades. In comparison to other bioinorganic molecules, NO is one of the latest molecules which has been taken into consideration in cell biology, initiated by the paper which was published 27 years ago (Ignarro et al. 1987). To date more than 31,000 papers have been published headed with NO and more than 65,000 correlate with NO in some way. (Moncada, Higgs 2006). It is estimated that yearly approximately 3,000 scientific articles are published on the biological roles of NO. Arginine derived NO synthesis has been identified in various organisms (Liu, Gross 1996). NO is known as a ubiquitous cellular messenger that plays multiple physiological roles in plants, bacteria and mammalian, including the regulation of vascular tone, neurotransmitter function and mediation of cellular defence (Moncada, Higgs 2006). Furthermore, NO interacts with organelles such as mitochondria in mammals to control mitochondrial biogenesis (Nisoli et al. 2003). NO acts also in plants-microbe interactions as a response to abiotic stress (Mur, Luis A J et al. 2013). NO generated by bacterial nitric oxide synthase (bNOS) protects bacteria to be resistant against a broad spectrum of antibiotics (Gusarov et al. 2009) and is essential to the virulence of *Bacillus anthracis* (Shatalin et al. 2008) or enables *Streptomyces turgidiscabies* to infect plants (Johnson et al. 2008). Given the broad spectrum of NO effects in biology, it is obvious why the development of useful methods for detecting NO in biological systems is very important.

1.3. NO in mammalian physiology

NO is a small signalling molecule which is formed by three isoforms of NO synthase, eNOS, iNOS and nNOS (NOS; EC 1.14.13.39). All three forms utilize l-arginine and molecular oxygen as substrates and require the cofactors reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydrobiopterin (BH4) (Förstermann, Sessa 2012). In the NOS catalysed reaction l-arginine is converted to l-citrulline and the NO radical.

1.3.1. NO the pressure regulator

The membrane associated endothelial nitric oxide synthase (eNOS) is dynamically regulated at the transcriptional, posttranscriptional, and posttranslational levels (Förstermann, Sessa 2012) and catalyzes the reaction:

$$\text{L-arginine} + \frac{3}{2}\,\text{NADPH} + \text{H}^+ + 2\text{O}_2 \rightarrow \text{Citrulline} + \text{Nitric Oxide} + \frac{3}{2}\,\text{NADP}^+$$

In endothelial cells eNOS derived NO, accounts for the vascular relaxation which is either constitutive or induced by lipopolysaccharides, acetylcholine, histamine, ATP and other endothelium-dependent agonists (Hamid et al. 1993). eNOS derived NO regulates vascular tone primarily by an activation of the soluble guanylat cyclase (sGC), which is the primary NO receptor (Stasch et al. 2001). In addition, NO can modulate cellular functions by a nitrosation of proteins (Sessa 2004). However, the main role of NO in the vascular system is to regulate the blood pressure (Umans, Levi 1995). Smooth muscle cells, which surround the vessels, are strongly influenced by NO. Vasodilation and decreasing blood flow is the result of the NO-induced, sGC and protein kinas G (PKG) dependent signalling in smooth muscle cells (Sessa 2004).

1.3.2. NO the sensitive

Besides its effects on the vascular tone NO is also an important modulator of neuronal function. 1988 Garthwaite et al. observed that the excitatory amino acid glutamate, by acting on N-methyl-D-aspartate receptors (NMDA) induces the release of an agent with similar properties to endothelium derived relaxing factor (EDRF) (Garthwaite et al. 1988). This led suggest that EDRF is a messenger molecule within the central nerve system. Few years later it was demonstrated for the first time that NO modulates the neuronal release of neurotransmitter under in vitro (Hanbauer et al. 1992) and in vivo (Prast, Philippu 1992) conditions whereby it was shown, that neurotransmission is controlled by NO (Prast, Philippu 2001). NO synthesis by nNOS in neurons is also stimulated by Ca^{2+}-influx which can be induced by activation of glutamate receptors. However, NO produced by non-neuronal cells in the brain is also influencing neurons (Prast, Philippu 2001).

1.3.3. NO the guardian

During the past two decades, NO has been recognized as one of the most important players in the immune system. It is involved in pathogenesis and control of infectious diseases, tumors and autoimmune processes. Because of its variety of reaction partners, its widespread production and the fact that its activity is strongly influenced by its concentration, NO continues to amaze immunologists (Bogdan 2001). Hitherto the production of NO in immune cells such as macrophages has remained unclear due to the deficiency of sensitive reagents and methods to detect NO directly (Jung et al. 2013).

Expression of inducible NOS (iNOS) in macrophages is controlled by cytokines and microbial products like lipopolysaccharides, primarily by transcriptional induction and in comparison to eNOS and nNOS, iNOS is Ca^{2+}-independent (Tariq 2008). Sustained NO production of macrophages protects the host against viruses, bacteria, fungi, protozoa, helminths, and tumor cells (MacMicking et al. 1997). In comparison to endothelial cells and neuronal cells, immune cells lack the NO synthase under basal conditions. However, activation of iNOS expression can be fired by a variety of extracellular stimuli. Bacterial lipopolysaccharides (LPS) are strong activators of this pathway. LPS interacts with LPS-binding protein (LPB) which delivers LPS to CD14 receptor, whereby the activation of the Toll-like receptor in conjunction with the small extracellular protein MD-2 occurs. Now the intracellular signalling cascade is activated that includes a variety of signal proteins to activate further pathways comprising the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor κB (NF-κB) pathway. These pathways are actually responsible for an activation of iNOS transcription in macrophages (Lowenstein, Padalko 2004).

1.3.4. NO the ugly

A number of cardiovascular diseases are associated with a limited generation of NO in endothelial cells. Vasoconstriction and elevated blood pressure, thrombus formation are some of this pathologies which are caused by reduced basal NO synthesis (Recchia et al. 1998). However, given the reactivity of NO, a logical conclusion would be that wherever NO is formed physiologically pathological processes can be triggered. An overproduction of NO was indeed shown to lead to cardiovascular problems (Moncada, Higgs 2006). Moreover, there are multiple mechanisms by which NO can regulate tumor metabolism (Chang et al. 2014). Inducible iNOS has been implicated as a component in many aggressive tumor phenotypes like breast cancer (Timoshenko et al. 2004).Since NO is a modulator of cellular pathways, in many ways similar to the alteration of cellular metabolism observed in aggressive tumors (Chang et al. 2014,). Excessive formation of NO emerges as an important neurotoxin in a variety of disorders (Bal-Price, Brown 2001). For example, recently it was shown, that inflammatory neuro-degeneration, caused by NO which is formed by activated glia-inhibiting neuronal respiration, seems to be the reason for neuronal death, bred by inhibition of mitochondrial respiration (Boje, Arora 1992). It was also shown that NO derived from activated astrocytes leads to an

fast and strong inhibition of neuronal respiration (Bal-Price, Brown 2001). That NO is not always good, was shown quite early by demonstrating the cytotoxicity and mutagenicity of this molecule. Direct addition of NO to the medium in cell cultures leads to substantial concentration dependent decrease in relative survival and concentration dependent increase in mutant-fraction at particular loci in human lymphoblasts (T. Nyugen 1992).

1.4. How to make this tiny molecule visible

Although already very much is known about the physiological role of NO, there is still much more that is unknown caused by the limitations of the detection methods, which will be discussed subsequently. Before that, we want briefly consider the chemical properties of NO. NO oxidize stepwise to nitrite and nitrate (Yoshida et al. 1983). In plasma or other physiological fluids or buffers NO is oxidized almost completely to nitrite. The new formed NO_2^- remains stable for several hours (Kelm 1999). The oxidation of NO by molecular oxygen is second order with respect to NO (Bryan 2007). Starting from this metabolism, the NO- level hitherto has been mainly detected by the determination of by-products of NO. In general, NO measurement techniques can be classified as direct and indirect methods, whereby most of the techniques described to date sense NO by quantifying by-products such as NOS activities, GC-derived cGMP, RSNOs or nitrite and nitrate. Analytical tools for the detection of these by-products are electrochemical or spectroscopic methods including colorimetric, fluorometry, and electron spin resonance spectroscopy (ESR) (Hetrick, Schoenfisch 2009).

1.4.1. Direct methods to estimate NO

1.4.1.1. Electron paramagnetic resonance spectroscopy

This method utilizes the paramagnetic nature of NO, whereby the main advantage of EPR in comparison to other NO detection methods is that it only detects paramagnetic molecules. Since the EPR spectrum of NO is a fingerprint, it is easily distinguishable (Csonka et al. 2014). For detection a commercially available spectrometer can be used. The magnetic field is usually set on near 220mT, with a microwave power of 8-10 mW frequency of 9 GHz, and modulation amplitude of 0.1 mT. Experiments need to be performed in darkness to prevent photolysis of the spin traps. When NO interacts with

the nitroso compounds, a triplet signal is formed that can be characterized by its line width an hyperfine coupling constant (Zweier et al. 1995). Major advantage of this method is the high specificity. Disadvantages are that the method is expensive, not commonly used, and that the handling requires expertise.

1.4.1.2. Electrochemical assays using microelectrodes specific for NO.

This method integrates the advantage of electrochemistry. The measurement needs a small amount of a sample, allows fast measurements and no chemical contamination of the samples is observed. Amperometric detection of NO for the direct measurement is the choice of methods to detect relevant concentrations in real-time and in vivo (Yap et al. 2013). Major advantage of such techniques is to observe fast responses and high sensitivity. Critical steps to get reliable results are the preparation of standard solutions to calibrate the electrode which is not trivial. Oxidation of the standard needs to be prevented and NO can be formed by chemical reactions. Temperature may affect sensitivity to the electrode by influencing the sensor components, whereby a careful temperature control is needed. A number of external electrical noise sources may impose undesirable signals on the output record (Csonka et al. 2014).

1.4.1.3. Diaminonaphthalene Assay

Fluorescence assays are sensitive techniques for NO detection (Planchet, Kaiser 2006). Basic principle of such systems is based on the interaction of NO with non-fluorescent dyes forming a fluorescent product. Diaminonaphthalene Assay uses the aromatic diamino compound 2,3-diaminonaphthalene (DAN) as an indicator of NO formation (Miles et al. 1996). The principle of this method is based on the rapid interaction of N_2O_3 which is formed by NO after reacting with oxygen, to yield the highly fluorescent product 2,3-naphthotriazole. Limit of detection overlies about 10-30 nM. In living cells formed nitrite decomposition does not interfere with naphthotriazole, this allows NO detection of biolocically relevant concentrations (Bryan 2007).

1.4.1.4. Diaminofluoroscein-2 Assay

In Further studies it was demonstrated that diaminofluoroscein-2 (DAF-2) may be used to determine the presence of NO in vitro and in situ (Kojima et al. 1998). The basic principle of DAF-2 is like DAN, a fluorescent triazole is formed. The advantage of this

compound is to be easily adapted to detect NO in vitro and in vivo. But major disadvantages according to recent investigations which suggests that oxidative nitrosylation may represent an artificial response (Espey et al. 2002). Since, DAF-2 can be oxidized by one electron species such as NO_2, whereby a subsequent rearrangement leads to the fluorescent triazole. In biological conditions the triazole can be formed either nitrosative or oxidatively. Recently it has been shown that nitroxyl (HNO) reacts with DAF-2 giving even higher yields of triazole than NO (Espey et al. 2002). Another important limitation of DAF-2 is when it is used to determine basal levels of NO in tissues (Rodriguez et al. 2005). The assay allows no compartmentalization which is in biological measurements essential. Non-specific interactions with mercuric chloride cause spectral changes that may be misinterpreted as NO signals (Bryan 2007). Under right conditions DAF-2 can be applied using excitation and emission wavelengths of 485 and 538 nm, respectively to monitor the fluorescence. At neutral pH the detection limit for NO is 2-5 nM (Nakatsubo et al. 1998).

1.4.2. Indirect methods to estimate NO

NO is due to its short half-life (<1s) extremely challenging to measure directly in biological conditions. Indirect NO measuring methods are based on the determination of nitrate and nitrite. The determination of the end products NO_x is simpler. For that purpose a number of analytical techniques are available including colorimetric assays, chemiluminescence, and electrochemical detection, chromatographic methods including GC-MS, capillary electrophoresis and HPLC. Most of these techniques are expensive and error prone. Environmental conditions such as pollution in chemical reagents and in cell culture media may interfere with nitrite and nitrate determination (Csonka et al. 2014).

1.4.2.1. Chemilumineszent probes

For an indirect determination of NO by NO_2^- a sensitive method is available which is based on chemiluminescence. If NO reacts with ozone it gives an excited state of NO_2^- which emits light when its return to ground state. For the detection of the emitted light a photomultiplier tube is needed. This method was first described by Samoilov and Zweier (reviwed in Bryan 2007).

1.4.2.2. Citrulline assay

Another interesting method to determine NO is the enzymatic based citrulline assay. In this case, the turnover of substrate composition serves as an indicator for the determination of formed NO. This enzymatic assay is one of the most used methods to determine NOS activity to conclude for the formed NO concentration. It is based on the biochemical conversion of L-arginine to L-citrulline by NOS. NOS activity is measured by monitoring of the conversion of arginine to citrulline. Radioactive [3H]-arginine or [14C]-arginine is used to ensure the sensitivity as well as the specificity of the assay for the direct enzymatic conversion of arginine to citrulline. Neutrally charged citrulline from positively charged arginine allows an easy separation by removal of unreacted l-arginine (Kameya, Asano 2014). Formed NO is simply calculated by the radioactivity of the endproduct l-citrulline which is proportional to NOS activity. It is obvious, that such techniques which indicates the amount of enzymatically active NOS protein in a biological system under optimized in vitro condition, does not necessarily reflect the actual NO production in living cells. Furthermore, non-enzymatic NO formation is not at all detected by this method (Csonka et al. 2014).

1.4.2.3. cGMP assay

In biological systems, NO interacts mostly with sGC. sGC is responsible to form cyclic guanosine monophosphate (cGMP). As a most known target of NO, sGC activity can be used to determine NO levels. cGMP is simple used as a surrogate for NO because of the close connection between NO and cGMP (Tsikas 2008). There are several methods available to determine cGMP, but radioimmunassay (RIA) is the choice of method (Steiner 1974). The principle of this assay is based on the competitive binding of cGMP in a given sample with a radio-iodinated derivate of cGMP$^{[125]}$ to high specific antibodies. After removing of antibody-bound cGMP from free cGMP, the labelled cGMP is determined by a gamma counter. Nowadays immune detection approaches are preferred instead of radioactive reagents such as enzyme-linked immunosorbent assay (ELISA). Alternatively for quantification, liquid chromatography coupled to tandem mass spectrometry (LC-MS) would be a good alternative (Lorenzetti et al. 2007). Such assays harbour the disadvantage to be expensive, but principally indirect and not usable to determine the actual NO level in single living cells.

1.4.2.4. Genetically encoded sensor for cGMP for indirect NO• measurements

As mentioned in the previous chapter, sGC is the most known target for NO in mammalian cells. Several years ago Sato et al developed a genetically encoded fluorescent indicator, where they used the ability of sGC to bind NO (Sato, 2005). They generated two subunits, sGCα and sGCβ which are fused to a cGMP sensitive Förtser resonance energy transfer (FRET) sensor (CGY). NO binding to the haeme containing sGCα unit results in a spontaneous dimerization with sGCβ. This interaction activates the enzyme to produce cGMP. Subsequently cGMP is sensed by CGY, which is a protein kinase G alpha- (PKGα) dependent cGMP probe. Rapidly formed cGMP interacts with CGY to give a FRET signal. This FRET-based probe was referred to as NOA-1 and used to visualize NO in living cells in the low nM level. Although NOA-1 exhibits a high sensitivity within the nano molar range of NO (detection limit of 0.1 nM), (Sato, 2005) the major bottleneck of this sensor is that it actually respond to cGMP, which can be formed in living cells NO-independently.

1.5. Fluorescent proteins as tools for the development of genetically encoded probes

In previous paragraphs we discussed about a number of chemical dyes which are used extensively to label biological specimens. The major disadvantage of such dyes is that they must be added exogenously, which can be incompatible with living systems. More suitable tools would be genetically encoded fluorescent proteins based probes. (Chudakov et al. 2005). 1994 when the green fluorescent protein GFP was cloned the first time it attracted attention for cell biologists. During the past few years GFP and its mutants soon became popular tools for molecular biology. Now a panel of fluorescent proteins is available that covers the whole visible spectrum. The development of various sophisticated FPs has opened up novel approaches for in vivo labelling (Chudakov et al. 2005). Fluorescence microscopy and the development of new genetically encoded fluorescent probes with the increasing ability of computer software for image acquirement and calculation, have been enabled more sophisticated studies of cellular functions and processes ranging from gene expression (Sekar, Periasamy 2003) to intercellular signalling (Roessel and Brand, 2002). When the first FRET based cAMP sensor was developed 23 years ago by Adams et al (Adams et al. 1991) a new era in this field was opened. Genetically encoded biosensors became important tools for real-time

measurements of the distribution of ions and secondary metabolites in living cells. The main advantage of such a FRET based sensor is the ability to be ratiometric, since they can compensate signal variations caused by cellular fluorophore concentrations and excitation laser intensities (Merkx et al. 2013). These sensors report the concentration change by recruiting one or a few ion(s) to their substrate specific sensing domain, inducing a conformational change leading to an altered FRET efficiency between the FRET donor, in most cases a cyan fluorescent protein (CFP), and the FRET acceptor, e.g. a yellow fluorescent protein (YFP).

1.5.1.1. FRET-based probes

Förster resonance energy transfer (FRET) is a process of non-radiative energy transfer between donor (e.g. CFP) and acceptor (e.g. YFP) fluorophores (Jares-Erijman, Jovin 2003). A FRET-based genetically encoded sensor is composed of two fluorescent proteins keeping apart by a substrate specific binding domain. Interaction of the substrate specific binding protein with his substrate leads to a conformational change and brings the fluorophores closer together. By this event, when the fluorophores are closer than 10nm apart, a so called FRET occurs, whereby the donor channel signal is quenched and the acceptor channel signal is increased (Herman, 1998). This process depends on the suitable spectral overlap of the donor emission maximum and optimal acceptor excitation. FRET biosensors have been increasingly used to visualize the activities of cellular signalling messengers such as Ca^{2+} (Mank et al. 2006), phospholipids (Cicchetti et al. 2004), small GTPases (Komatsu et al. 2011b), protein Kinases(Harvey et al. 2008), ATP (Imamura et al. 2009), pyruvate (San Martín et al. 2014) .The genetically encoded FRET sensors are classified into two groups; unimolecular and bimolecular FRET biosensors (Miyawaki 2003). The first mentioned contains both, the donor and acceptor FPs within the a single biosensor, whereas the latter consist of a pair of molecules conjugated with a donor FP and an acceptor FP, respectively (Komatsu et al. 2011a). To visualize this mechanism, FRET microscopes are used which are based on the capability to detect fluorescent signals from dynamic interactions of labelled molecules in single living cells. With FRET microscopic imaging, not only co-localization of the donor and acceptor-labelled probes can be seen, but molecular associations such as specific interactions of proteins at close distances can be

identified (Sekar, Periasamy 2003). Because of the importance of this issue, fluorescent microscopy will be discussed separately more in detail below.

1.5.1.2. Non-FRET-based genetically encoded sensors

Non-FRET-based genetically encoded sensors are so called pericams for example, which are based on single fluorescent proteins (Whitaker 2010). The N-terminal and C-terminal section of the GFP had been interchanged and reconnected by a short linker between the original ends. This technique is known as circularly permutation. By the fusion of calmoduline (CaM) and myosin light chain kinase (M13) to the original termini of GFP, a novel sensor was developed which changes the spectral properties reversibly with the amount of Ca^{2+}. These sensors were called pericams (Nagai et al. 2001). The mechanism is not clear, but the interaction of the calcium specific binding domains may lead to an alteration of the environmental surrounding the chromophore. So far three types of pericam were generated by mutating several amino acids adjacent to the chromophore. Of these, flash pericam, inverse pericam and ratiometric-pericam. The latter mentioned possess the exception, that the excitation wavelength changes calcium dependent manner, which allows ratiometric measurements (Nagai et al. 2001). Follower of pericam are the so called GECOs. Genetically encoded calcium sensors for optical imaging are also single FP based sensors containing the same substrate specific binding domains as the pericam. Different coloured FPs were engineered by directed protein evolution to obtain improved brightness or altered hue (Zhao et al. 2011).

The palette of genetically encoded biosensor is huge including specific sensors from arabinose (Kaper et al. 2008) to zinc (Qiao et al. 2006) almost for every metabolite and ion a specific biosensor is available (Lindenburg, Merkx 2014). Nowadays, even a list of gene-specific RNA-biosensors is obtainable (Schubert et al. 2004). However, to our knowledge a genetically encoded nitric oxide sensor, which directly responds to NO dynamics within cells, is not available so far.

1.6. Fluorescence microscopy

1852 George G. Stokes coined the term fluorescence, when he described as first the phenomenon of the absorption and subsequent re-radiation of light by fluorophores. The emission of light through the fluorescence process is closely simultaneous with the absorption of the excitation light, ranging less than 1 µs (Johnson 2007). Because the use

of multiple fluorescence labelling, different probes can be simultaneously identified. However, a variety of specimens exhibit autofluorescence, without the application of fluorochromes when they are irradiated, a phenomenon that has been well observed in biology which makes studies of animal tissues and pathogens often complicated (Neumann, Gabel 2002). The extensive growth in the use of fluorescence microscopy is closely associated with the development of new synthetic fluorophores with known intensity profiles

The basic function of a fluorescence microscope is to irradiate the fluorophore with distinct wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. In an accurately configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark background. In a fluorescence illuminator, light of a specific wavelength often in the visible spectrum, is produced by passing multispectral light from a lamp source through a wavelength selective excitation filter. Wavelengths passed by the excitation filter reflect from the surface of a dichroic mirror or beam-splitter, through the microscope objective to excite the specimen with intense light. If the specimen fluoresces, the emission light gathered by the objective passes back through the dichromatic mirror and is subsequently filtered by emission-filter, which blocks the unwanted excitation wavelengths. Under ideal conditions, it is often possible to detect the fluorescence emission from a single molecule, on condition that the background and detector noise are sufficiently low. A single fluorescein molecule could emit as many as 300,000 photons before it is destroyed by photobleaching. Using suitable detection cameras for such experiments, it is possible to monitor the behaviour of single molecule, provided that an adequate suppression of the optical background noise is given (Silva et al. 2014).

2. Materials and Methods

2.1. Cloning of geNOps

Briefly, cloning was performed according to standard procedures and all products were verified by sequencing. Genomic DNA of E.Coli DH10α was isolated by DNA extraction protocol using Phenol/Chlorophorm extraction followed by ethanol precipitation and then resolved in 30µL deionized water. The DNA was used as a template to isolate the GAF-subunit of the norR transcription factor in a PCR with following primers: Gaf_Cla1_for 5'-GGCATCGATATGAGTTTTTCCGTTGATGTGC-3' which adds a Cla1 restriction site and Gaf_EcoR1_rev 5'-GCCGAATTCATCCTTCAATCCCAGACGTTT-3' and then purified by electrophoresis on a 1,5 % agarose gel followed by gel extraction. To obtain an FRET-like genetically encoded Nitric oxide Sensor (further called as double-geNOp) the PCR product was ligated between the restriction sites Cla1 and EcoR1 of a common FRET pair sECFP and cpV instead of the ε-subunit of an ATP sensor [1] in a mammalian expression vector pcDNA3.1(-) (Invitrogen, Austria).The ligation reaction was used for transformation of Top 10 chemically competent E.Coli cells by heat shock at 42°C for 60 s. Cells were plated on LB-Agar plates supplemented with ampicillin. Individual clones were selected for inoculation of 5 ml of LB liquid medium supplemented with ampicillin and were incubated over night at 37°C in a shaker. Then a STET mini preparation was performed to isolate the plasmid DNA. To verify the insert the following sequencing primers were used: pcDNA3.1(-)_for 5'-CACTGCTTACTGGCTTATCG-3' and pcDNA3.1(-)_rev 5'-CAACAGATGGCTGGCAACTA-3'. For large scale plasmid purification, stock cultures of the verified clones were used to inoculate 200 ml LB-liquid medium supplemented with ampicillin and were shaken for approximately 16 h at 37°C at 200 rpm. Plasmids from large scale were purified using the Maxiprep System PureYield™ Plasmid (Promega, Germany) with nuclease-free water and stored for cell transfection.

For cloning of single FP-based geNOps to obtain C-terminal tagged probes, the same PCR-product was subcloned using the GAF Cla for primer and a GAF rev primer including a Stop codon and a HindIII-site, GGCAAGCTTAAGGGGACAAGCCAATCATCT, in frame with the appropriate fluorescent Protein, such as eGFP, Gem-GECO, cpV or mOrange.

To construct the mutated probe, so called ΔgeNOp a two-step PCR protocol was performed using the additional primers GAFmutR75AR81A_P2_for 5'-A**GCG**CTGGAAGCGATTGCC**GCC**G-3' and GAFmutR75AR81A_P1_rev

5’-CC**GGC**GGCGGCAATCGCTTCCAG**CGC**T-3’ which carries at particular sites two single nucleotide exchanges to obtain a substitution of R75G and R81G of the GAF domain. In the first PCR step the n-terminal and c-terminal part of the GAF domain were amplified by using the primer pairs Gaf_Cla1_for/GAFmutR75AR81A_P1_rev and Gaf_EcoR1_rev/ GAFmutR75AR81A_P2_for. The PCR-products were purified by gel electrophoresis as described previously. Both PCR-products were designed to form an overlapping region which was used in the second PCR-step. Equal amounts of both products were mixed together with the primers Gaf_EcoR1_rev and Gaf_Cla1_for to obtain full length GAF-domain carrying the site specific substitutions at position 75 and 81. The backbone of double-geNOp as well as the mutated PCR-Product were thendigested with Cla1 and EcoR1 and purified by gel electrophoresis. Insert and backbone were ligated and further preceeded as described previously to obtain large scale plasmid.

2.2. (Bio)Chemicals and buffer solutions:

Cell culture materials were obtained from PAA laboratories (Pasching, Austria). Histamine, Iron(II)fumarate, BHQ and EGTA were purchased from Sigma Aldrich (Vienna, Austria). NOC7, NOC12 and NOC5 were form Santa Cruz (San Diego, USA). Potassium ferrocyanide(II), Potassium ferricyanide(III) and Iron(II)sulphate were obtained from Roth (Graz, Austria). Sodium nitroprusside was purchased from Gatt-Koller (Absam, Austria). Reagents for protein purification were obtained from Qiagen (Hilden, Germany). Prior to experiments, cells were washed and maintained for 20minutes in a HEPES-buffered solution containing 138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM HEPES, 2.6 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 10 mM d-glucose, 0.1% vitamins, 0.2% essential amino acids and 1% penicillin-streptomycin, the pH was adjusted to 7.4 with NaOH. Further preincubation shortly before imaging was done with a HEPES-buffered solution containing additionally 1 mM Iron(II)fumarate and 1 mM ascorbic acid. During the experiments, cells were perfused in a physiological Ca^{2+}-containing buffer, which consisted of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM d-glucose and 1 mM HEPES, the pH was adjusted to 7.4 with NaOH. During experiments Nitric oxide solution was applied to the cells corresponding to the amount of NOC7 that was added to the physiological buffer.

2.3. Cell Culture and transfection

HeLa (Callaway 2013) and raw264.7 (Saxena et al. 2003) cells were grown in Dulbeccos's Modified Eagle Medium (Sigma Aldrich) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and they were plated on 30-mm glass coverslips. Culture medium of EA.hy926 (Ahn et al. 1995) cells contained additionally HAT. At 60–80% confluence, cells were transfected with 1.5 µg (per 30-mm well) of plasmid DNA encoding the appropriate geNOp using TransFast™ transfection reagent at 3µg/well (Promega, Madison, W) in 1ml of serum- and antibiotic-free medium. Cells were maintained in a humidified incubator (37°C, 5% CO_2, 95% air) for 16–20 hours prior to changing back to the respective culture medium. All experiments were performed either 24 hours or 48 hours after transfection.

2.4. Recombinant expression of geNOps and protein purification

For ex vivo investigations the double geNOp was used as a template to isolate the full length of the probe in a PCR with following primers: double_geNOp_Nco1_for 5'-ATCCCATGGTGAGCAAGGGCGAGGAG-3' which adds an Nco1 restriction site and double_geNOp_Kpn1_rev 5'-CCCGGTACCTTACTCGATGTTGTGGCGGAT-3' which adds a Kpn1 restriction site. The PCR product, composed of ECFP-GAF-cpVenus was subcloned into a bacterial expression vector pETM-11 by using the restriction sites Kpn1 and Nco1. After large scale plasmid purification the expression vectors were transformed to an E.Coli expression strain BL21 and cells were plated on a LB-agar supplemented with Kanamycin. To look for presence of the desired proteins a test expression was performed by picking individual colonies which were grown overnight for small preculture. 50 ml LB-kanamycin was inoculated with 5 ml of the preculture and monitored until an optical density (OD600) of 0.6 was observed. Induction of the flask-culture was conducted with 0.4 mM IPTG and incubated with constant shaking at 37°C for 3 hours at 200 rpm. Pre and post induction samples were taken and resuspended in PBS. Each sample was sonicated for 30 sec and centrifuged for 10 min at 14,000 rpm. Supernatant and pellet was separated and 50µL of each was resuspended with 1x sample buffer. 5 µL of the supernatant and pellet for both pre and post induced samples respectively were run on SDS-PAGE after boiling for 5 min. For large scale purification the same protocol was applied with appropriate amounts according to a 1 L flask culture. After induction and incubation for 16 h at 18°C cells were harvested by

centrifugation at 4,000 rpm and 4°C. The supernatant was discarded and pellets were resuspended with Lysis Buffer (50 mM NaH_2PO_4, 5 mM Imidazol pH(8.0), 150 mM NaCl, 2mM BME). A 15 min sonication step on ice was applied to break up the cells. To pellet the cell debris the suspension was centrifuged again as previously. The supernatant was loaded on a 1 cm diameter column containing 4 ml Ni-NTA agarose beads which were equilibrated with an equilibration buffer containing 50 mM NaH_2PO_4,5 mM Imidazol pH (8.0), 150 mM NaCl. The column was washed 4 times using a Salt gradient. Proteins were eluted by increasing the Imidazol concentration to 400 mM. Isolated proteins were pressure dialyzed against deionized water. In a further step, the proteins were highly purified by size exclusion chromatography. For determination of Protein concentrations Lowry assay and photometric determination according to Warburg Christian was applied.

2.5. Fluorescence measurements of NO concentrations with geNOps

Characterization of the probes was performed in transient transfected single HeLa cells using different concentrations of a NO-Donor as described in the Results section. Measurements were mainly performed on two different microscopes: For physiological measurements an iMIC inverted and advanced fluorescent Microscope motorized sample stage (©Till Photonics, Graefling, Germany) was used with following setups: Zeiss Tubus-lens f=164.5, Polychrome 5000 (1109-0-693; ©Till Photonics), Filter-set observed from AHF Analysentechnik; GFP/OFP Emission filter ET Fitc/Tritc dual emitter, Excitation filter GFP/OFP E500spuv, Dichroic 560dcxr, Dichroic 495dcxru. FRET based probes geNOp4.0 was excited at 430 nm and emission was collected simultaneously at 480 nm and 535 nm using an optical beam-splitter (Dichroic 560dcxr for GFP/OFP, Dichroic 495dcxru for CFP/YFP). Camera Allied Vision Technologies. For Control and acquisition the software *Live acquisition 2* (©Till Photonics) was used.

Experiments for characterization of geNOps were performed on a Nikon eclipse TE300 inverted microscope (Tokio, Japan) using the setups as followed: Nikon tubus-lens plan flour 40x/1.30 Oil DicH ∞/0.17 WD 0.2. Light source, Xenon-lamp Optiquip Model 770. Shutter observed from LEP 093586 (Ludl electronic products). Camera; Spot pursuit CCD Camera. FRET based probe geNOp4.0 was excited at 430nm and Emission was collected using a beamsplitter O1-05-Em 505 dcxr D480/30m D535/40m. Simultaneous measurements with OFP derived o-geNOp and fura-2 was excited at 550nm and 400 nm,

respectively. Emissions were collected using the dichroic filter XF56 (Omega Opticals). For Control and acquisition the software *Visiview*® (Visitron Systems GmbH, Germany) were used. Depiction-forms of figures are given accordingly.

2.6. 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). This software uses a profile-profile alignment algorithm to predict the 3-dim structure of the desired protein by entering the sequence of the protein of interest. The alignment is based on hidden Markov models via HHsearch (Söding 2005) to significantly improve accuracy of alignment and detection rate. Analysis of the predicted proteins was performed with the software DeepView / Swiss Pdb viewer V4.1.0 observed from expasy.

2.7. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). Each experiment was at least performed four times. Analysis of variance (ANOVA) and t-test were used for evaluation of the statistical significance. $p < 0.05$ was defined to be significant

3. Results & Discussion

3.1. A novel rational strategy for the design of genetically encoded NO-sensors

We attempted to develop a functional FRET-Sensor using a wild-type protein backbone structure derived from *E.coli* which is capable to sense nitric oxide. Based on a literature search the bacterial transcription factor norR was found as a promising candidate (D'Autréaux et al. 2005). The norR protein is composed of three domains: the N-terminal NO-binding GAF domain, the central ATPase domain, and the C-terminal DNA binding domain with a helix-turn-helix (HTH) motif (Fig. 1A and C). The physiological role of norR is to activate the transcription of enzymes, which convert the toxic NO to N_2O. This detoxification of NO allows bacterial cells to survive macrophages induced NO stress (D'Autréaux et al. 2005). It has been shown that NO binding to norR induces a reversible structural rearrangement (D'Autréaux et al. 2005) during which the GAF domain detaches from the AAA+ domain (Fig 1A).

We hypothesized that this conformational change can be used to visualize NO dynamics also in mammalian cells. Hence, we generated a putative FRET-based NO probe, which consisted of the whole norR transcription factor sandwiched by two fluorescent proteins, the super enhanced cyan fluorescent protein (seCFP) and the circularly permuted venus (CPV), respectively (Fig. 1B). Notably, eCFP is frequently used as a FRET donor and CPV as a FRET acceptor in genetically encoded probes (Tramier et al. 2006). This construct was named geNOp1.0. Based on recent data, which indicate that NO binding to norR releases the GAF domain from the AAA+ domain (D'Autréaux et al. 2005), we expected to observe a decrease of the FRET ratio signal upon addition of NO donors to cells expressing geNOp1.0. We observed a small increase of the FRET-ratio signal, when cells were exposed to 10 μM of the NO-donor NOC-7 (Fig.1D). Considering the single wavelength of the donor (seCFP) and FRET channel, respectively, we recognized that both fluorescence signals were quenched in response to the NO donor (Fig. 1E). As the seCFP signal was, however, quenched stronger than the fluorescence of the FRET channel, the FRET ratio signal (FRET/seCFP) increased (Fig. 1E).

Figure 1

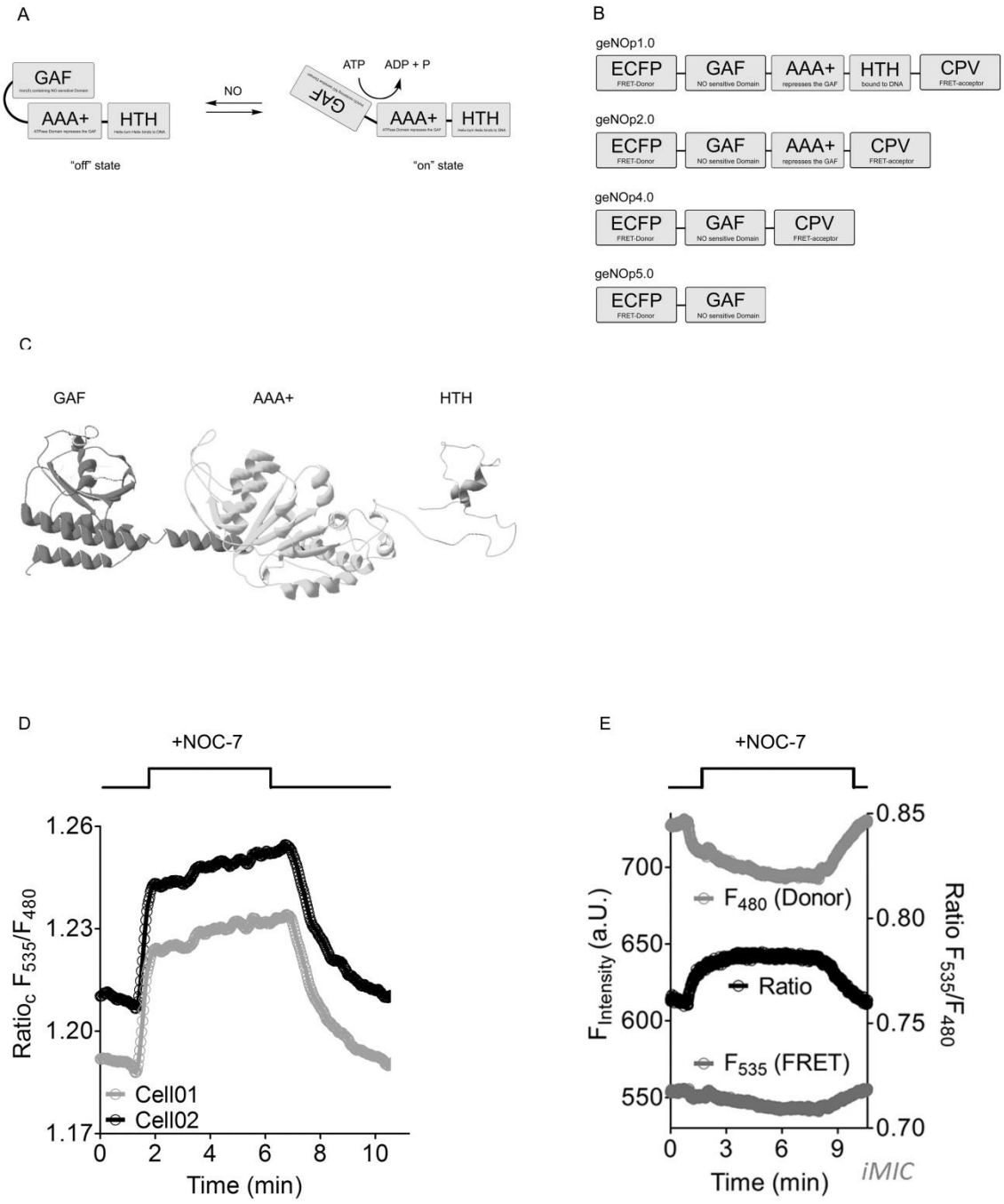

Fig. 1: ***A bacterial EBP derived fluorescent probe for visualizing NO in single living cells*****. (A)** The transcription factor norR is represented in the inactivated state and activated state when the iron in the GAF domain is nitrosylated . **(B)** Schematic composition of the various geNOps. Full length norR and truncated variants are sandwiched between two fluorescent proteins. **(C)** Predicted 3-dimensional structure of bacterial enhancer binding protein norR, which is composed of the iron binding domain GAF, central ATPase (AAA+) domain and the DNA binding domain HTH. **(D)** FRET ratio signals (F_{535}/F_{480}) of geNOp1.0 in response to 10µM NOC-7. As indicated the NO donor NOC-7 was added for 4 minutes. **(E)** Representative curves showing the change in fluorescence intensity over time of the FRET donor (F_{480}), the FRET signal (F_{535}) and the respective FRET ratio signal (black curve in the middle) in response to 10 µM NOC-7.

Next we tested if shortening the construct by removing the C-terminal HTH domain (Fig.1B; geNOp2.0) or both the HTH and the central AAA+ domain (Fig. 1B; geNOp4.0) results in constructs with higher basal FRET ratio signals and NO-induced FRET ratio changes. Using geNOp2.0, which comprises just the NO-sensitive GAF-domain and the ATPase domain, showed a slight increase in the basal FRET ratio signal, while the NO-induced increase of the FRET ratio was comparable with that of geNOp1.0 (data not shown).

The next surprise brought the shorter geNOp4.0 which is composed of CFP, the GAF domain and CPV (Fig.1B). Although the basal FRET ratio signal of geNOp4.0 was significantly higher compared to that of geNOp1.0 (Fig. 2A), the FRET ratio was not influenced by addition of the NO-donor NOC-7 (Fig. 2B). However, the stable FRET ratio signal was due to an equal NO-induced quenching of both the FRET donor and acceptor signals (Fig. 2C). These experiments indicate that a NO-induced conformational change cannot be visualized measuring FRET signals from the norR-derived constructs.

Figure 2

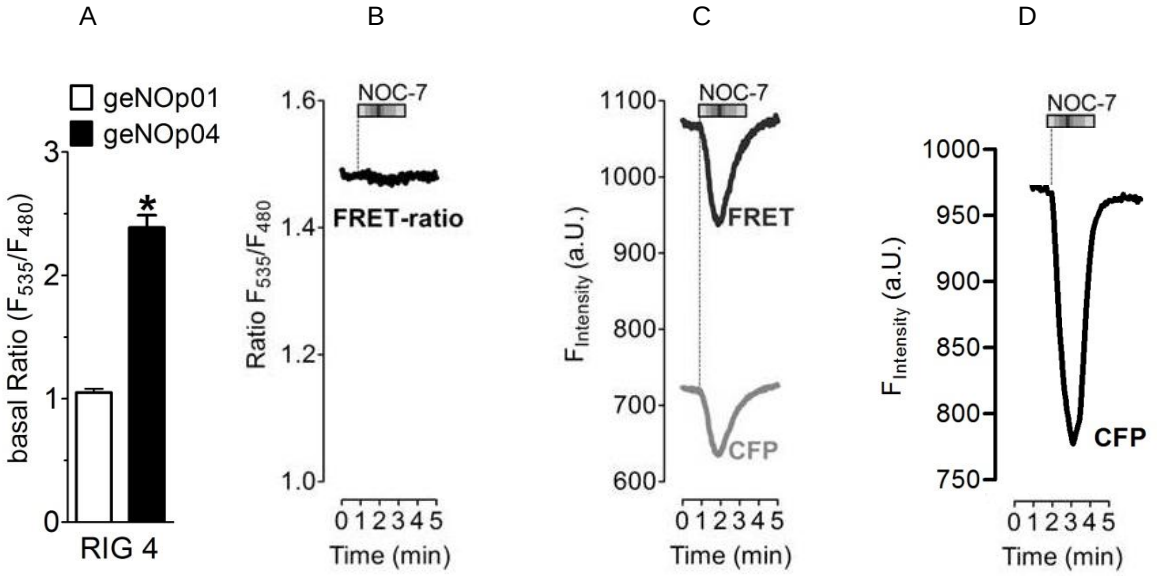

Fig. 2. ***NO induced fluorescent quenching in geNOp4.0*****. (A)** Average basal FRET-ratio signals of geNOp4.0 (black bar, n=7) and geNOp1.0 (white bar, n=9). geNOps were expressed in HeLa cells. **(B)** Representative curve showing changes in the FRET/seCFP emission ratio of geNOp4.0 upon 10μM NOC-7 addition. **(C)** Equal NO induced quenching of both, the FRET (black line) channel and donor (CFP) channel of geNOp4.0 in response to 10μM NOC-7 (grey line). **(D)** Changes in the fluorescence intensity of CFP geNOp5. upon 10μM NOC-7 stimulation.

However, a considerable and reversible quenching of the fluorescence intensity of the attached FPs was observed in response to the addition of NO-donors. If NO binding in the vicinity of a FP indeed specifically quenches the fluorescence intensity, then the same phenomenon should be observed with a single FP fused to the NO-sensitive GAF domain. So we constructed geNOp5.0, a single eCFP-based sensor with a single substrate binding domain (Fig. 1B). Cells expressing geNOp5.0 were used to study the impact of NO addition on the eCFP fluorescence. Indeed, addition of 10 µM NOC-7 reversible reduced the eCFP signal (Fig 2D). In fact, these experiments confirmed our hypothesis that NO-binding in the vicinity of an FP induces a significant decrease of its fluorescence intensity. This hypothesis is in line with a described physical phenomenon. Eiserich and colleagues reported about a relation between metalloproteins and radicalized of certain amino acids by NO. Interestingly, exactly the amino acids tryptophan, tyrosine and glycine, which are mainly involved in forming the chromophore of FPs, have been shown to be influenced by NO. In their study it was mentioned also, that NO would be capable of direct and rapid scavenging of Trp and Tyr radicals in proteins. During this process NO effectively interferes with electron transfer processes between tryptophan and tyrosine residues in proteins (Eiserich et al. 1995). Considering this report it would be very likely that NO binding on the GAF domain leads to an electron-shift within the chromophore of the adjacent FP, which consequently results in a loss of fluorescent intensity. Nevertheless, our preliminary findings and such considerations were used as a basis for the development of a novel class of genetically encoded NO probes. Before we have started with the optimization of this kind of quenching-based fluorescent sensors, we clarified an important question: Is the quenching effect indeed NO specific?

3.2. Ensuring the specificity of geNOps

A non-heme iron center within the GAF domain is essential for NO binding to norR. In the presence of NO, the iron is nitrosylated. Once the iron in the GAF domain is nitrosylated by NO, a conformational rearrangement in the transcription factor norR occurs and transcription of the corresponding genes is released (D'Autréaux et al. 2005).. In biological systems inorganic compounds including metals are found as coordinative complexes surrounded by distinct amino acid residues (Karlin 1993). So far several putative amino acids were reported which are most likely responsible to complex the central iron in the non-heme iron center of the GAF-domain (D'Autréaux et al. 2005). These amino acid residues are placed at the positions: arginine 81, asparagine 96, asparagine 99, cysteine 113 and asparagine 131 (Fig. 3). An iron atom is capable to complexes six coordination sites. In the GAF domain one of these coordination sites is free to interact with NO. A hypothetical 3-dimensional figure of the non-heme iron binding pocket is shown in figure 3. This model of the 3-D structure of the non-heme iron center was obatined by using online protein prediction tools (Phyre2: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and the analysis software DeepView - Swiss-PdbViewer V4.1 (see also Materials & Methods).

Figure 3

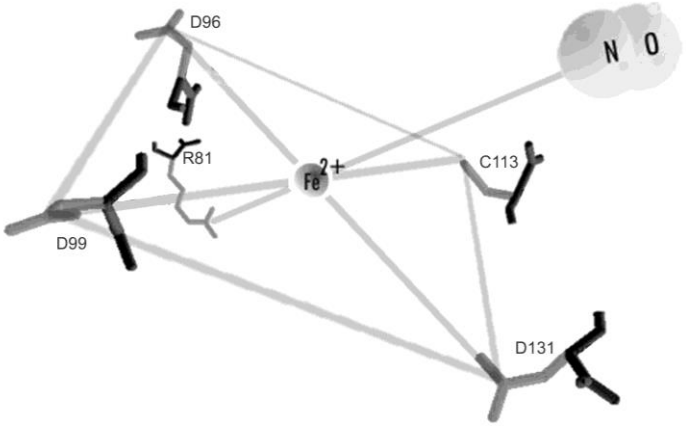

Fig. 3. ***Predicted 3-dim structure of the iron centre of the GAF domain*****.** Iron binding site of norR containing the amino acids R81, D96, D99, C113 and D131. One coordination site is free to interact with NO.

Based on this model it should be possible to lose the non-heme iron centre by mutating one of the essential amino acids. Such a mutated construct would be incapable to bind NO and, hence, fluorescence intensity of the fused FP should be insensitive to NO.
Accordingly, we mutated geNOp4.0 by a knock-out of the arginine at position R81. This construct was named ΔgeNOp4.0. The mutation was introduced by a two-step PCR-method as described in materials and methods. HeLa cells transfected with ΔgeNOp4.0 were measured with the same standard protocol as applied for the characterization for the other geNOps. Adding of 10µM NOC-7 does not give a loss of intensity of the FPs in ΔgeNOp4.0 (Fig. 4A). In fact, this experiment confirmed our hypothesis that NO-binding to the GAF-domain is essential for quenching of the fused FP.

Figure 4

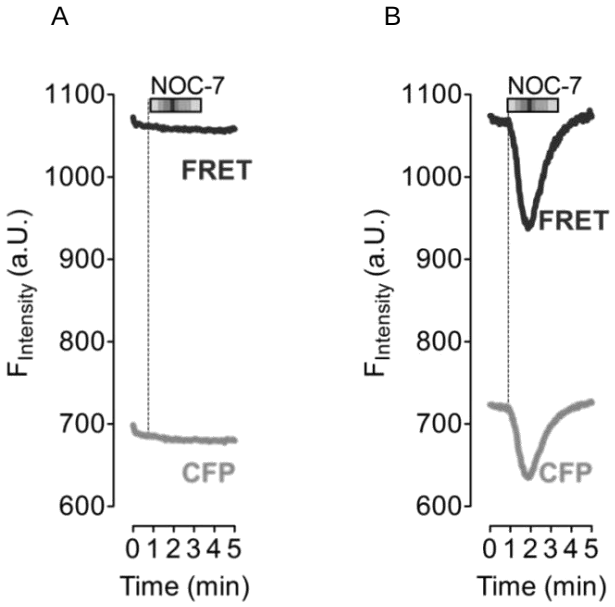

Fig. 4. ***Effect on fluorescence response by a single amino acid mutation of the iron centre.*** Representative curves showing the impact of 10 MM NOC-7 on the fluorescence intensity of the FRET channel (black curves) and the CFP donor channel (grey curves) of **(A)** ΔgeNOp4.0 and **(B)** wild-type geNOp4.0 using HeLa cells expressing the constructs, respectively.

Additionally, this finding confirms that the amino acid arginine at position 81 of the non-heam iron centre of the GAF domain is essential for NO binding. In view of the fact that the central iron of the GAF domain plays an important role for the functioning and probably the efficiency of geNOps, further efforts for an optimization of this part of the probes have been implemented (see chapter 3.3).

3.3. Optimization of geNOps by iron supplementation

The iron metabolism in mammalian cells is not a trivial matter. Both an excess and a deficiency leads to pathologies. The cellular iron homeostasis is mainly regulated by ferroportin, which exports iron from cells, and hepcidin, which blocks the activity of ferroportin (Hentze et al. 2010). However, it is not clear to which extend iron is integrated in the bacterial GAF domain when mammalian cells overexpress the protein. Hence, it is possible that only part of geNOps actually contain iron in the form of Fe^{2+} in mammalian cells. Lack of iron within the GAF-domain, however, would have a serious impact on the responsiveness of the geNOps to NO. Hence an iron supplementation probably improves significantly the NO-induced FP-quenching of geNOps in mammalian cells. Accordingly, we supplemented different iron compounds including iron(II) chloride, iron(II) sulphate, potassium ferrocyanid, potassium ferricyanid, sodium nitroprusside or iron(II) fumarate in the cell storage medium. Cells expressing geNOps were incubated for 10 minutes prior to measurements with the respective iron (1mM) supplemented media. Only sodium nitroprusside and iron(II)fumarate, most likely due to their plasma membrane permeability, indeed highly improved the NO-induced quenching of geNOps in HeLa cells as shown in figure 5A and B. In fact, these experiments fulfilled our expectations that iron(II) supplementation improves the responsiveness of geNOps in mammalian cells.

However, these results do not entirely clarify whether there is indeed an iron(II) deficiency or if most of the iron within geNOps is oxidized to iron(III) in mammalian cells. Notably, in a recent study, it was confirmed, that also the GAF domain requires iron(II) and not iron (III) to bind NO (Baptista et al. 2012). Accordingly we tested if addition of ascorbic acid, which as a reducing agent can restore iron(II) from iron (III), improves the NO-induced FP-quenching of geNOps. For this purpose HeLa cells expressing the eCFP containing geNOp5.0 were pre-treated with 1mM ascorbic acid for 10 minutes prior to fluorescence microscopy. Ascorbic acid pre-treatment did not significantly impact on the NO-induced fluorescence signal of geNOp5.0 (Fig. 6A and B), indicating that the GAF-domain of geNOps in mammalian cells is most likely not occupied by iron(III).

Figure 5

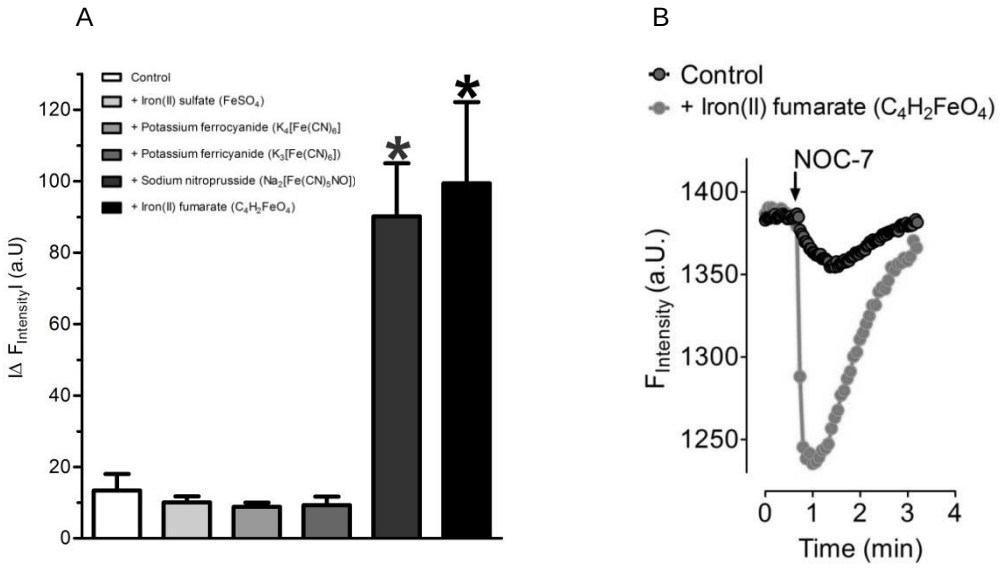

Fig. 5. ***Iron-supplementation improves the signal to noise ratio*****. (A)** Columns represent average maximal changes of the fluorescence intensity of the FP in geNOps upon addition of 10 µM NOC-7. Cells expressing the geNOp were pre-incubated for 10 minutes with iron-free storage buffer (control, white bar, n=4), 1 mM iron(II)sulfate (light grey bar, n=6), 1 mM potassium ferrocyanide (middle grey bar, n=7), 1mM potassium ferricyanide (grey bar, n=6), 1mM sodium nitroprusside (dark grey bar, n=7), and iron(II) fumarate (black bar, n=6). *P < 0.05 vs. control **(B)** Representative time course showing FP changes in response to 10µM NOC-7 of cells expressing geNOps under control conditions (darker curve) and after pre-incubation with 1 mM iron(II) fumarate (grey curve). geNOps were expressed in HeLa-cells.

Figure 6

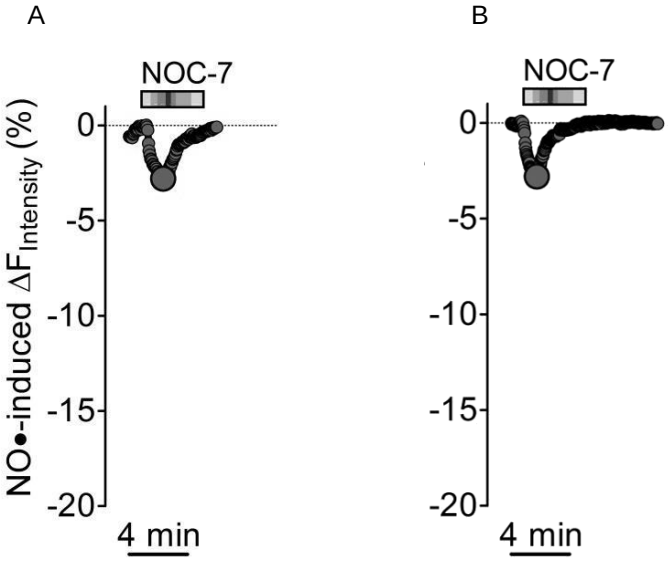

Fig. 6. ***Impact of Ascorbic acid on NO-induced changes of the FP-intensiy in geNOps*****. (A)** Average control response to 10µM NOC-7 of non-treated HeLa cells expressing geNOp5.0 (n=7) in comparison to **(B)** cells pre-treated for 10 min with 1mM ascorbic acid (n=9).

To verify if iron(II) of the GAF domain is essential for the NO-induced quenching of the fluorescence intensity of geNOps, hydrogen peroxide (H_2O_2), an oxidizing agent, was used. Cells expressing geNOps were first kept in a buffer containing 1 mM iron(II)fumarate and then 1mM H_2O_2 was added for 10 minutes to oxidize iron(II) to iron(III) within the GAF-domain. Notably, addition of 10 µM H_2O_2 did not affect the fluorescence intensity of geNOps (Fig 7C). However, pre-treatment with H_2O_2 considerably reduced the NO-induced fluorescence quench (Fig 7B). These experiments demonstrated that oxidizing iron(II) to iron (III) results in a loss of function of geNOps and that H_2O_2 is not sensed by geNOps.

Figure 7

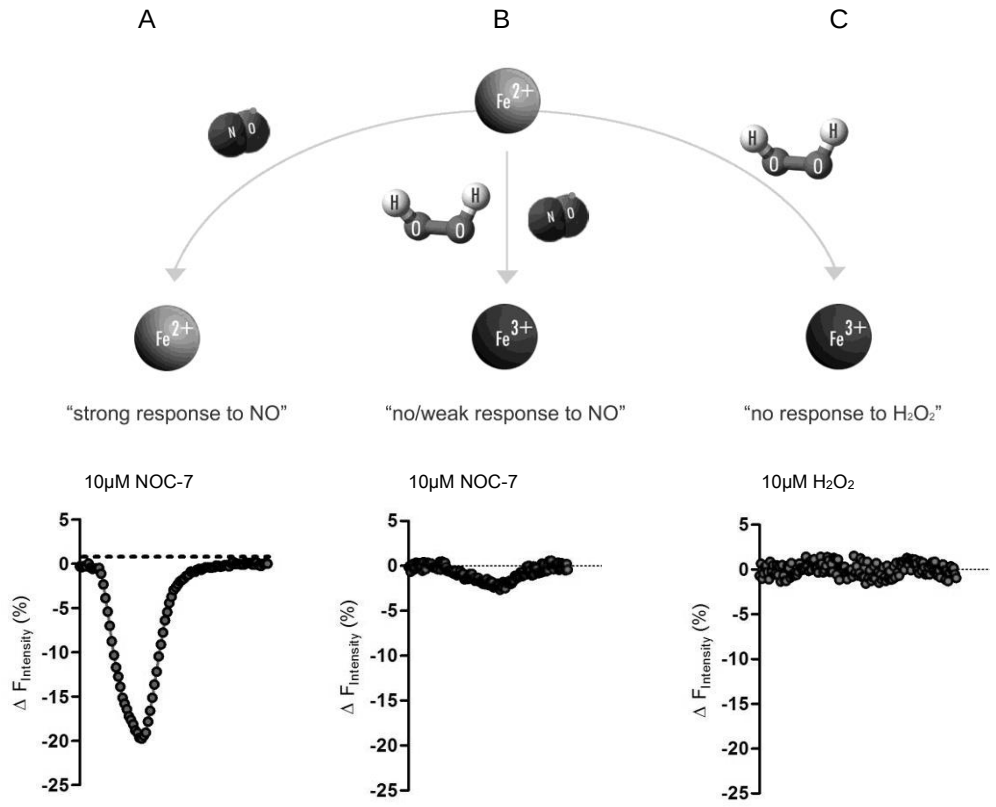

Fig. 7. ***The importance of iron supplementation. Upper panel:*** Schematic illustration of the oxidation status of the iron in the GAF domain under control conditions (A, all the GAFdomains are occupied by iron (II) and able to bind NO), under oxidizing conditions (B, e.g in the presence of the oxidizing agent H_2O_2, where parts of the GAF domains contain iron (III) instead of iron (II) in the presence of NO), and in the absence of NO, but in the presence of H_2O_2 (C). ***Lower panel:*** **(A)** Representative response of a control measurement for 10µM NOC-7 with iron(II) fumarate supplemented HeLa cells. **(B)** Pre-treatment with 1mM iron(II) fumarate for 10 min and subsequent pre-treatment with 1mM H_2O_2 for 10 min prior to measurement. **(C)** Addition of 10µM H_2O_2 on iron(II) fumarate treated cells.

Figure 8

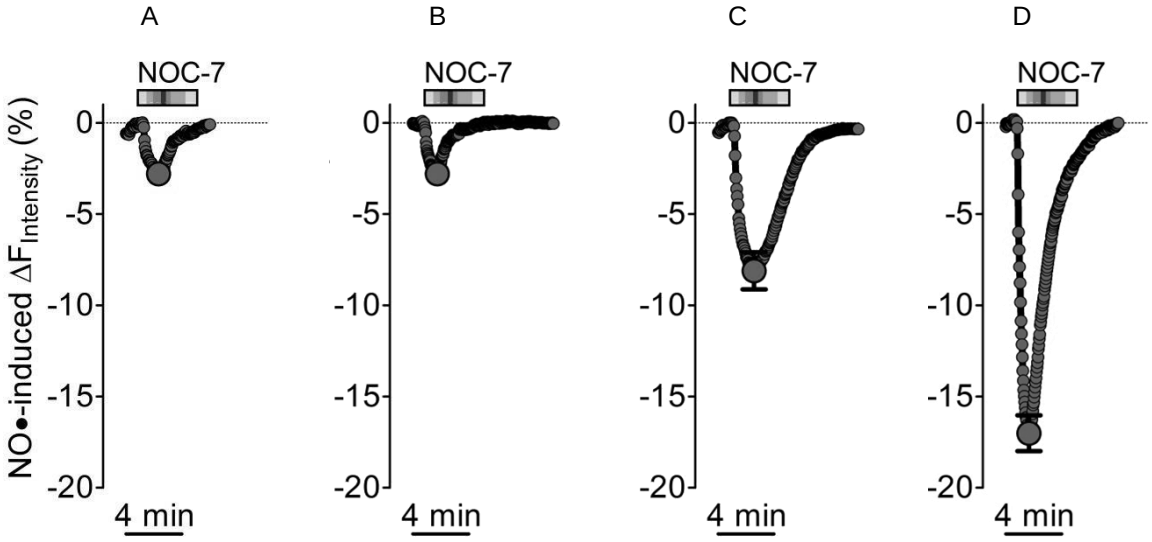

Fig. 8 ***Benefit of iron supplementation*****:** Average curves of the time course of geNOps responses to 10µM NOC-7 addition of untreated control cells **(A, n=7)**, or with 1mM ascorbic acid treated **(B, n=11)**, or with 1mM sodium nitroprosside treated **(C, n=15)**, or with 1mM iron(II)fumarate and ascorbic acid **(D, n=12)**, respectively, treated HeLa cells.

In summary these findings indicate that iron(II) within the GAF-domain of geNOps is essential. Hence, for all further experiments cells were stored in a medium containing 1mM ascorbic acid and 1mM iron(II)fumarate at least for 10 minutes prior to measurements. Figure 8 illustrates the benefit of such a procedure compared to control conditions (cells were measured directly from the culture medium) and cells pre-treated with either ascorbic acid or SNP alone. Already a short pre-treatment with the right enhancer solution led to a strong fluorescence response as a quenching up to 18% upon addition of 10 µM NOC-7.

3.4. Development of additional single FP-based NO-sensors painting a colourful way of a new era of NO bio-imaging

Nowadays a vast amount of fluorescent proteins are available to cover the whole visible spectrum from blue to deep-red and beyond even far-red FPs are obtainable (Chudakov et al. 2010). So far the revealed results in our current work were mainly performed with the double FP-based (eCFP & CPV) geNOp4.0 or the single FP-based (CFP) geNOp5.0. One of the most interesting questions for us was to clarify, whether or not other coloured fluorescent proteins fused to the GAF domain also respond to NO-binding by a significant loss of their fluorescence intensity. Accordingly, we designed and engineered more than 20 different variants of constructs that contained blue, cyan, green, yellow, orange and red fluorescent proteins (Table 1).

This approach resulted in several functional geNOps of different spectral properties which were further characterised. Surprisingly, when we measured all the listed constructs almost every FP fused to the GAF domain resulted in a functional geNOp except the constructs containing EBFP and tdTomato, respectively. Additional research is necessary to understand why exactly the most far blue and most far red fluorescent proteins in geNOp8.0 and geNOp16.0 are not quenched by NO addition.

Figure 9

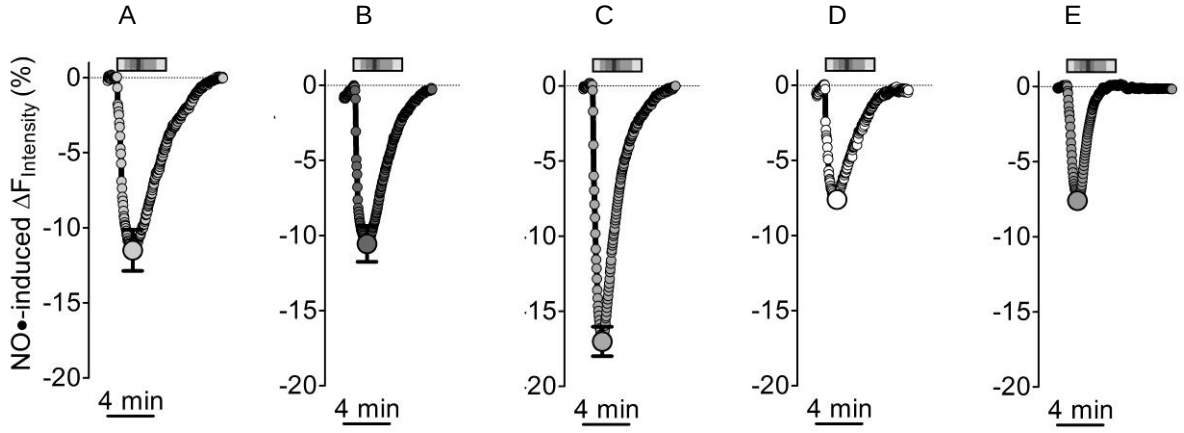

Fig. 9. ***Colour range of functional single FP-based geNOps.*** Average response upon 10µM NOC-7 treatment of cell expressing **(A, n=10)** geNOp5.0, or **(B, n=11)** geNOp9.0, or **(C, n=12)** geNOp.11.0, or **(D, n=13)** geNOp 6.0, or **(E, n=9)** geNOp14.0.

Table 1 *Different genetically engineered constructs*

Construct name	Composition	Type	Colour
geNOp1.0	seCFP-GAF-AAA-HTH-CPV	N.C.* Double FP whole norR	Cyan/Yellow
geNOp2.0	seCFP-GAF-AAA-CPV	N.C. Double FP truncated	Cyan/Yellow
geNOp3.0	GAF-GEM-AAA	ce*. Single FP single GAF	Green EM.
geNOp4.0	seCFP-GAF-CPV	N.C. Double FP single GAF	Cyan/Yellow
geNOpΔ4.0	seCFP-GAF*-CPV	N.C. Double FP single GAF	Cyan/Yellow
geNOp5.0	seCFP-GAF	N.* Single FP single GAF	Cyan
geNOp6.0	CPV-GAF	N. Single FP single GAF	Yellow
geNOp6.1	GAF-CPV	C.* Single FP single GAF	Yellow
geNOp7.0	CPV-ECFP-GAF	N. Double FP single GAF	Yellow/Cyan
geNOp7.1	seCPF-CPV-GAF	N. Double FP single GAF	Cyan/Yellow
geNOp8.0	EBFP2-GAF	N. Single FP single GAF	Blue
geNOp8.1	EBFP2-EGFP-GAF	N. Double FP single GAF	Blue
geNOp9.0	EGFP-GAF	N. Single FP single GAF	Green
geNOp9.1	cpGFP-GAF	N. Single FP single GAF	Green
geNOp10.0	Clover-GAF	N. Single FP single GAF	Green
geNOp10.1	Clover-Clover-GAF	N. Double FP single GAF	Green
geNOp11.0	GEM-GAF	N. Single FP single GAF	Green Em.
geNOp12.0	Venus-GAF	N. Single FP single GAF	Yellow
geNOp13.0	cpYFP-GAF	N. Single FP single GAF	Yellow
geNOp14.0	mKOk-GAF	N. Single FP single GAF	Orange
geNOp15.0	mRuby-GAF	N. Single FP single GAF	Red
geNOp16.0	tdTomato-GAF	N. Single FP single GAF	Red
geNOp17.0	GAF-seCFP-GAF	ce. Single FP double GAF	Cyan

*Nomenclature: Different alignment of FPs in relation to the NO-binding transcription factor. The regarding fluorescent proteins are either amino-terminal and carboxy-terminal (**N.C.)**, or carboxyterminal **(C.)**, or amino-terminal **(N.)**, or central **(ce.)** fused to the corresponding domain of norR (only GAF, or GAF-AAA, or full length norR).

Nevertheless, the remaining functional single FP-based probes cover almost entirely the spectral range of visible light. These are the seCFP derived geNOp5.0, the EGFP derived geNOp9.0, the GEM derived geNOp11.0, the circular permuted Venus (CPV) derived

geNOp6.0 and the mKOk (Orange) derived geNOp14.0. All these probes react with a reversible loss of intensity in response to 10µM NOC7 (Fig. 9).

As these five FPs (seCFP, EGFP, GEM, CPV, and mKOk) are different in terms of their origin and the amino acid sequence (which form the chromophore), our results indicate that NO binding to GAF in the vicinity of FPs quenches fluorescence independently from the structure of the FP. The EGFP is closely related to the wild-type fluorescent protein, while the CPV is a circularly permuted construct. In the CPV the initial N-terminal and C-terminal section of Venus had been interchanged and reconnected between the original ends. The GEM FP for example is an engineered construct manufactured by directed protein evolution based on flash-pericams.

Moreover, we observed an unexpected phenomenon when we compared geNOp6.0 with geNOp6.1. The two constructs differ in the orientation of the GAF-domain related to the fused CPV (see table 1). Since the iron binding site of the GAF domain is closer to the N-terminus we assumed that the influence of NO on the fluorescence intensity is stronger when the FP is fused to this site of GAF. In fact, our results indicate that this is not the case. The quenching effect of NO on the fluorescent protein is independent from the site of the FP fusion to the GAF domain (Fig. 10A). This finding might indicate that the radius in which NO binding to the GAF domain influences attached FPs is approximately in the range of the diameter of the GAF domain (Fig. 10B)

Figure 10

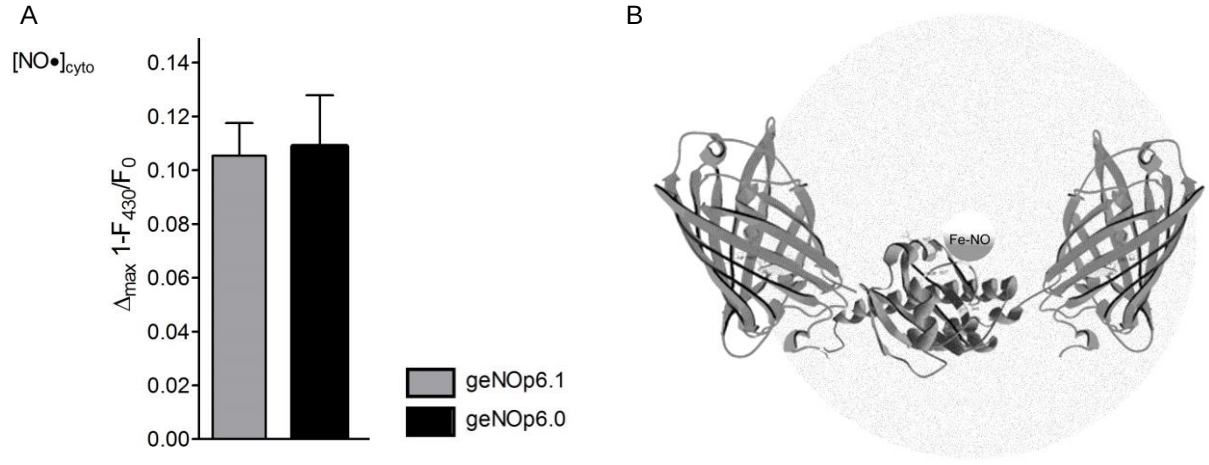

Fig. 10. ***Extensive effect of NO.*** **(A)** Comparison of the normalized fluorescence quenching effect of 10 NOC-7 between geNOp6.1 (CPV is c-terminal fused to GAF, grey column, n=7) and geNOp6.0 (CPV is n-terminal fused to GAF, black column, n=9). **(B)** Hypothetical radius in which NO impacts on FPs fused to the GAF-domain .

3.5. Determination of specificity and sensitivity

There are various secondary metabolites produced by cells including carbon monoxide (CO) hydrogen peroxide (H_2O_2), peroxy nitrite ($ONOO^-$) and superoxide (O_2^-) (Morita et al. 1995; Onda et al. 2011), which are potentially targets for the NO binding iron centre of the GAF domain within geNOps. It is well established that the described NOS isoforms are able to form O_2^- (Fleming 1999). An interaction of one of these compounds with the iron centre could theoretically influence the fluorescence intensity of the probes to yield a false positive signal or to prevent the binding of NO and, hence, the NO-induced quenching of FPs. We first studied the response of geNOp9.0 to CO. Transfected HeLa cells were stimulated with 100µM of the CO releasing compound CORM2 for 2 minutes. This harsh treatment did not change the fluorescent intensity of geNOp9.0 (Figure 11). In a similar manner we also examined the impact of H_2O_2, O_2^- and $ONOO^-$. When HeLa cells were stimulated with excess concentrations of these reactive compounds no significant response was detected (Figure 11).

Figure 11

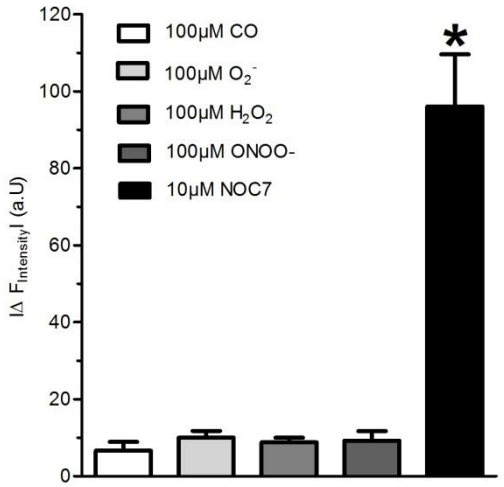

Fig. 11. ***Specificity and selectivity of geNOps.*** Changes in the fluorescent intensity of geNOp9.0 upon stimulation with 100µM CO (withe bar, n=11), superoxide (light gray bar, n=11), hydrogenperoxide (grey bar n=8), peroxinitrite (dark grey bar, n=9) respectively, and 10µM NOC-7 (black bar, n=10).

As neither CO, nor H_2O_2, nor O_2^- nor $ONOO^-$ affects the fluorescence of geNOps, these findings point to the high selectivity of our NO probes.

Next we studied the sensitivity of geNOps to respond to NOC-7. As shown in figure 10 the various geNOps respond with clearly different extent to the same NOC-7 concentration. While the geNOp11.0 (GEM) decreases by 18% when exposed to 10µM NOC-7, geNOp14.0 (OFP) shows a loss of intensity of just 8% in response to 10 µM NOC-7. The reason for these differences is not clear, but could reflect different sensitivities among the tested geNOps to respond to NO-binding. Therefore we determined the dissociation constant (K_d) values for geNOps by stimulating transfected HeLa cells with distinct concentrations of NOC7 (Fig. 12). This approach also demonstrated the limit of detection of the different geNOps. The following concentrations of NOC7 were used: 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, 1µM, 3µM, 10µM, 30µM and 100µM.

Figure 12

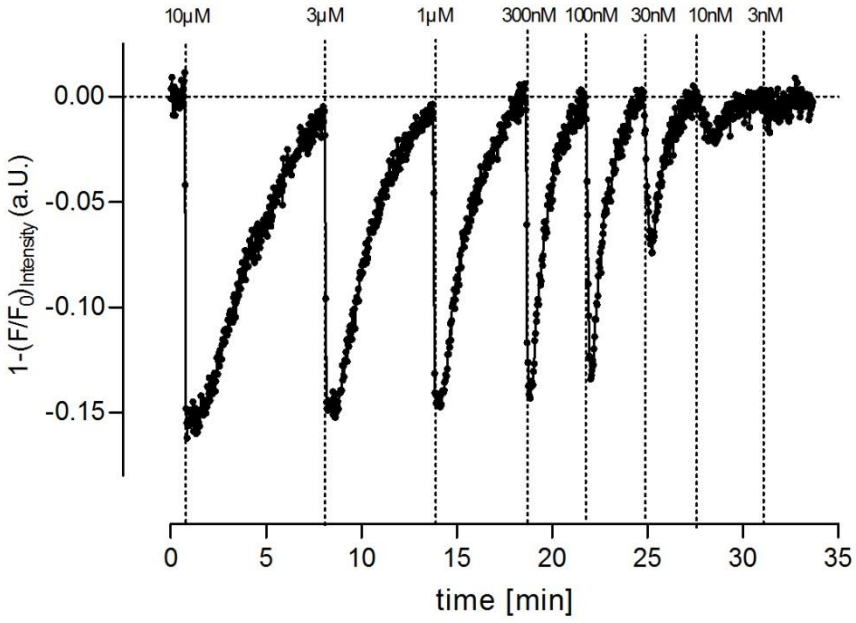

Fig. 12. ***Sensitivity of geNOps.*** Representative response of geNOp9.0 to consecutive additions of various concentrations of NOC-7 ranging from 10 µM to 3 nM, as indicated.

To determine the K_d with NOC-7, the maximum NO-induced slope were plotted against the respective NOC-7 concentrations (Fig. 13A & B). The EC_{50}-values were estimated using a sigmoidal dose-response curve equation *Y=Bottom + (Top-Bottom)/(1+10^((LogEC$_{50}$-X)*HillSlope)).* For all geNOps tested a similar K_d- and EC_{50}-value was obtained (Table 2). This indicates that the sensitivities of these probes with different FPs are in the same range. Considering that all probes have the same NO-binding domain, this observation was expectable.

Figure 13

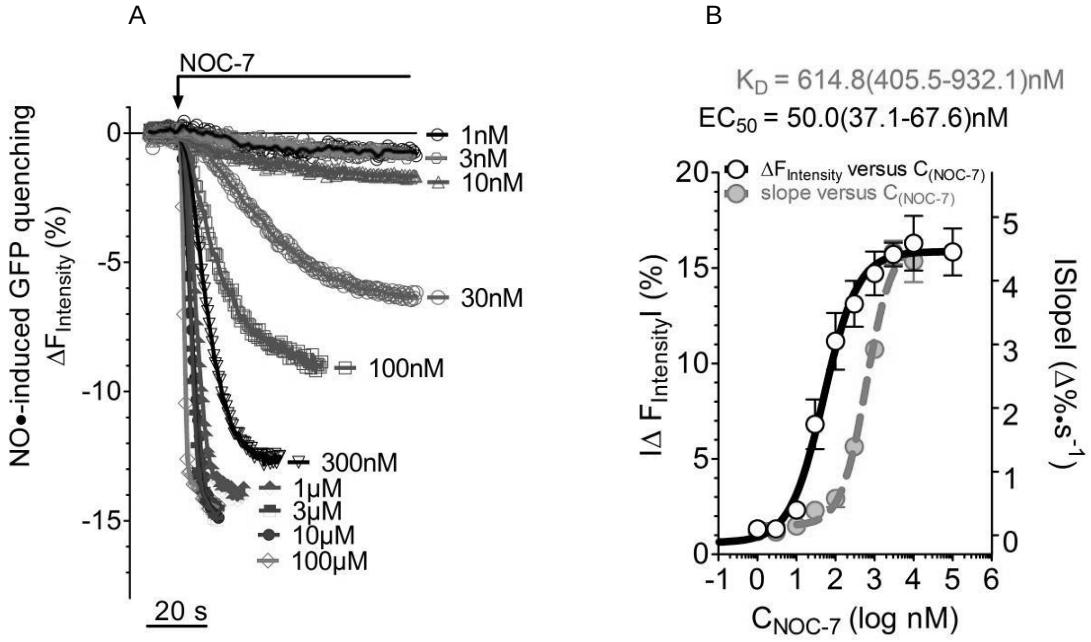

Fig. 13. ***Comparison of K_d and EC50*****. (A)** NO induced maximal quenching response of geNOp9.0 for various concentrations of NOC-7 as indicated. N=6 for all concentrations shown **(B)** Concentration response curves of geNOp9.0 in response to NOC-7 (n=6 for each concentration plotted). K_d and EC50-values of geNOp9.0. were calculated from the fits using prism-software.

In summary these experiments demonstrated that single FP-based geNOps allow the quantification of NO dynamics over a huge physiological relevant range (10 nM to 100 µM). However, it should be considered that the K_d of the NO probes were determined using NOC-7, a NO donor, and not NO directly. In addition NO is known to have a short half-life, which makes it difficult to maintain constant NO levels. Accordingly, we assume that the K_d's given in **table 2** are overestimated.

Table 2 *Estimated K_d' and EC50 values of various geNOps.*

Construct	***One site specific binding*** K_d*' for NOC7 [nM]*	***sigmoidal dose response*** **EC50** *for NOC7 [nM]*	***LOD* for NOC7 [nM]***
geNOp5.0	~660.4	~58.4	2.3
geNOp9.0	614.8	50.0	3.0
geNOp11.0	~650.7	~53.5	10.5
geNOp6.0	~720.4	~62.7	10.9
o-geNOp	~590.4	~48.5	1.1

* Equation for the calculation of the LOD:

LOD = $y_B + 3s_B$ (y_B:average blank value, s_B: standard deviation of blank value)

For ~ values further analysis is necessary to confirm the exact K_d' and/or EC_{50} values listed.

3.6. Characterization of different NO-Donors

So far all described experiments, were performed with NOC-7, a stable NO-amine complex that release NO under physiological conditions. NOC-7 has a half-life of approximately 10 minutes and induced clear effects on the fluorescence intensity of geNOps (Fig. 1-14). We next used the geNOp-technique in order to characterize different NO-donors on the single cell level. The NO-donors used and their features are listed in table 3.

Basically we used two classes of NO donors: i.) low molecular chemical compounds and ii.) nitrosylated albumin (table 3). All these donors are known pharmacological tools, which have been applied to study different aspects of NO signalling in e.g. cancer research (Huerta 1992) and cardiovascular physiology (Ignarro 2002).

Regarding to the described features from the literature of different NO-donors, we assumed, that it should be possible to image the different NO releasing kinetics of these compounds on the single cell level using geNOp4.0. As expected, every NO donor induced significantly different NO dynamics within HeLa cells (Fig. 14A and B), although the concentration and time period of application was equal.

Table 3 *Various NO-donors*

Donor	***Chemical notation***	***Kinetic***	***NO releasing***
NOC-7 Low molecular chemical compound	1-Hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene)	spontaneous fast release	2 mol NO, 10 min half life
ProliNONOate	1-(hydroxy-NNO-azoxy)-L-proline, disodium salt	spontaneous fast release	2 mol NO 1.8 s half life
Nitroprusside	Natriumpentacyano-nitrosylferrat(II)-Dihydrat	spontaneous fast release	Break down to NO after interacting with cellular compounds
NO-Albumine	S-Nitrosoalbumin (SNALB)	slow release	Liberation boosted by GSH

Figure 14

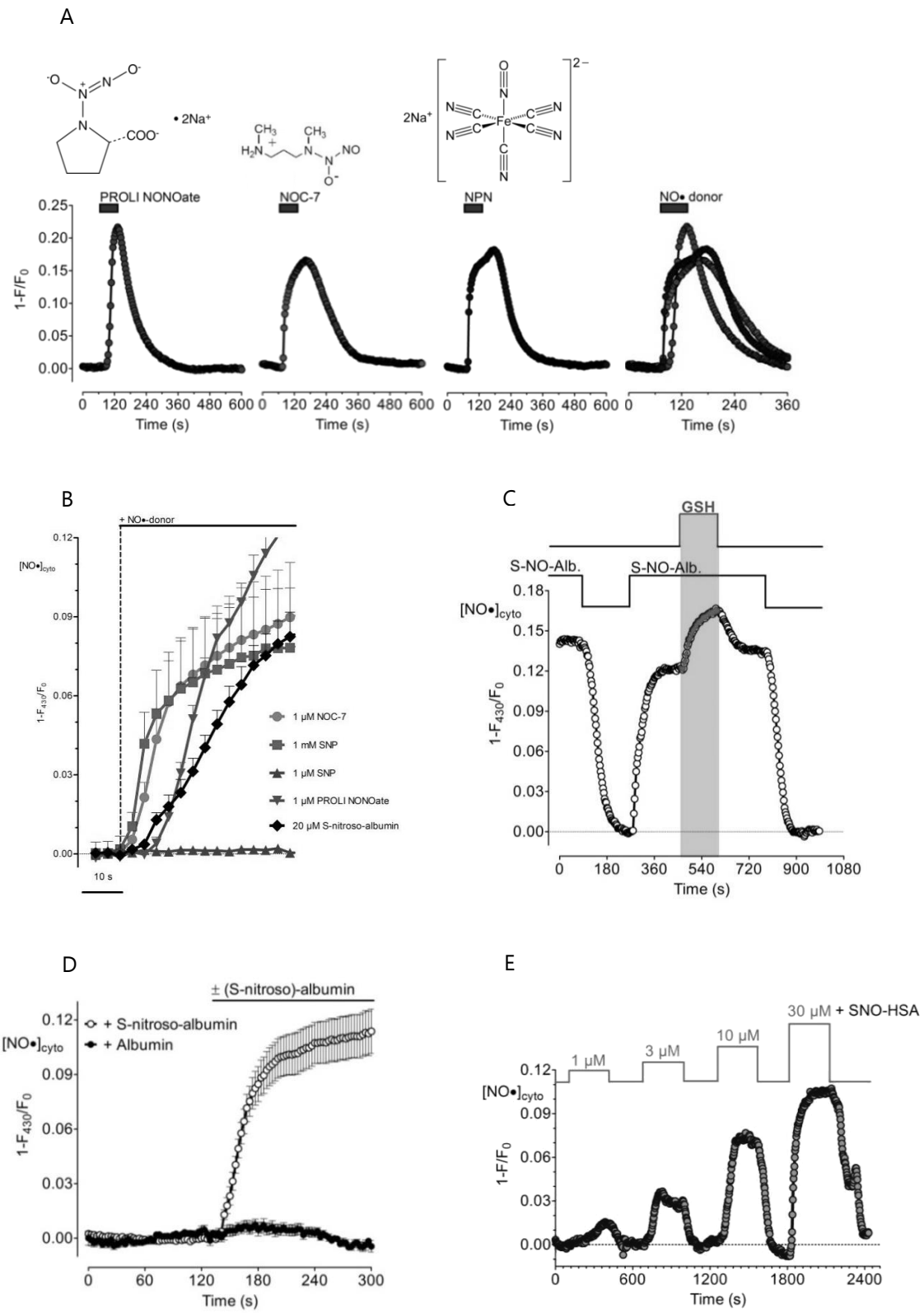

Fig. 14. ***Characterization of various NO-Donors.*** **(A)** and **(B)** Cellular NO dynamics in response to different NO donors were measured with HeLa cells expressing geNOp4.0. **(C)** Time course of cellular NO increases in response to 50 µM SNALP and the impact of 50 µM GSH. **(D)** Response of geNOp4.0 expressed in HeLa cells upon the addition of 30 µM SNALP (with dots, n=5) and 30 µM human serum albumin (black curve with black dots, n=3). **(E)** Dose response curve of geNOp4.0 for various concentrations of SNALP.

In line with the reported short half-life of PROLI NONOate (1.8 s) the respective cytosolic NO signal in response to this NO donor was transient compared to the more stable NOC-7 and SNP induced signals (Fig 14A). Interestingly, 10µM proli NONOate, which, such as NOC-7, releases 2 mol NO of 1 mol parent molecule, gave a stronger but delayed signal in comparison to NOC-7 (Fig. 14A and B). SNP did not increase NO levels within single HeLa cells at a concentration of 10µM (Fig 14B). This means that at these concentrations no or extremely less NO is released by SNP. While at a concentration of 1mM of SNP a fast and clear increase of cellular NO levels was observed (Fig. 14A and B). S-nitroso albumin (SNALP) is a known NO-donor with a very slow NO liberation kinetic *in vivo* (Rungatscher et al. 2014). Thus, 20µM of SNALP was necessary to observe a 10 µM NOC-7-comparable NO signal within HeLa (Fig. 14B). These results not only show the behaviour of different donors to study the releasing kinetics of such compounds, but in addition these experiments confirm the high sensitivity of geNOps to respond to NO. It is obvious that the developed probes allow to distinguishing very accurately the differences in the NO releasing kinetics of various NO-donors and furthermore allow making a quantitative comparison among different NO-donors.

SNALB is a special donor that's why we wanted to take a closer look on it. In previous studies it was shown, that the NO liberation of SNALB is boosted by glutathione (GSH) (Rungatscher et al. 2014). This effect was investigated on rats by determining the blood pressure. Using the geNOp-technology should allow to investigate the GSH effect on the single cell level. For this purpose cells were first stimulated with SNALB till a plateau was reached. The saturation level of NO within HeLa cells indicates the maximum NO-liberation capacity of SNALB, caused by the slow releasing of NO. When GSH was added at this phase, a further increase of cytosolic NO level was observed as long as GSH was present (Fig 14C). This experiment showed that we could indeed measure the increased NO liberation from SNALB upon the addition of GSH using geNOp4.0. To exclude artificial effects, SNALB were compared with non nitrosylated human serum albumin (HSA). As expected, HSA did not lead to a response (Fig. 14D). In these shown experiments, we used a 50% SNALB, which means, that 50% of the thiol-groups of albumin in the solution is occupied with NO. The remaining amount of SNALB is non nitrosylated i.e. normal albumin. We performed a dose response curve applying the concentrations 1µM, 3µM, 10µM and 30µM using 50% SNALB as shown in figure 14E. The maximum NO liberation capacity of a 30µM SNALB solution amounts to 130 nM free

NO (personal correspondence with Dr. Seth Hallström, who generated SNALP). This confirms the high sensitivity of geNOps for NO. In summary, we could demonstrate that geNOps are suitable sensors to study the characteristics of different NO-donors in single cells.

3.7. Heterologous expression and ex-vivo characterization of geNOps

For ex-vivo investigations, the geNOp4.0 was recombinant constructed as described in materials and methods. The underlying idea of this intension was to reproduce the observed fluorescence quenching effect in response to NO of geNOps *ex-vivo*. With the purified geNOp4.0 an emission scan from 400nm up to 600 nm by exiting the seCFP with 430 nm light was recorded. As shown in figure 15, two main peaks were observed. The peak maxima at 480nm and 535nm belong to the emission of seCFP and FRET between seCFP and CPV, respectively. If the purified geNOp4.0 solution was mixed with 10µM NOC7 a considerable quenching of the fluorescence intensity of the attached FPs was observed (Fig. 15).

In fact, we noted that as in living cell systems the FPs in geNOp4.0 are also quenched if the protein is exposed to a NO-donor ex-vivo. In addition, figure 15 shows that in the construct geNOp4.0, the ECFP and the respective FRET signal are quenched equally.

Figure 15

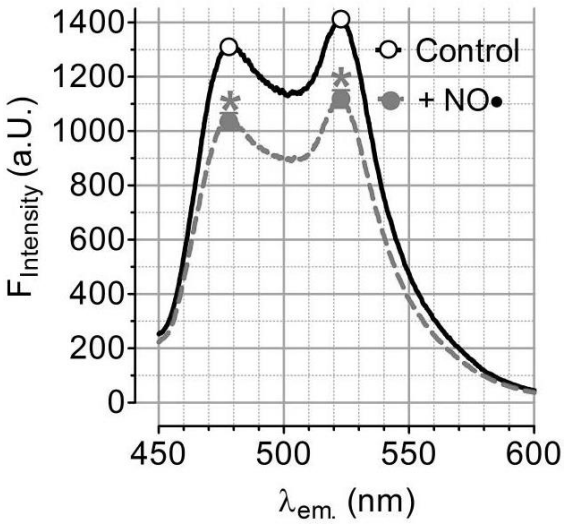

Fig. 15 ***Purified geNOp4.0 for ex vivo measurements*****.** Emission spectra of geNOp4.0. Control measurement (solid line) against with 10µM NOC-7 exposed proteins (dashed line)

3.8. First physiological applications of geNOps in mammalian endothelial cells

For the testing of geNOps in a physiological context, endothelial cells (EC) were used. The underlying connection between cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyto}$) and production of NO is a well-established mechanism. It is known, that NO production in EC is triggered by an increase of $[Ca^{2+}]_{cyto}$ (Busse, Mülsch 1990). In this current work, we, hence, investigated NO dynamics with geNOps expressed in endothelial cells applying different Ca^{2+} mobilizing agonists such as the IP_3.generating histamine (His) and ATP.
Cell stimulation with ATP induced a significant increase of cytosolic NO levels in an oscillatory manner (Fig 16). This is in line with other reports that show cytosolic Ca^{2+} oscillations in response to IP_3-generating agonists (Berridge 1991). The same protocol was performed with cells expressing the mutated ΔgeNOp4.0 as a negative control. As expected, no response was observed upon the addition of ATP to cells expressing ΔgeNOp4.0 (Fig 16).

Figure 16

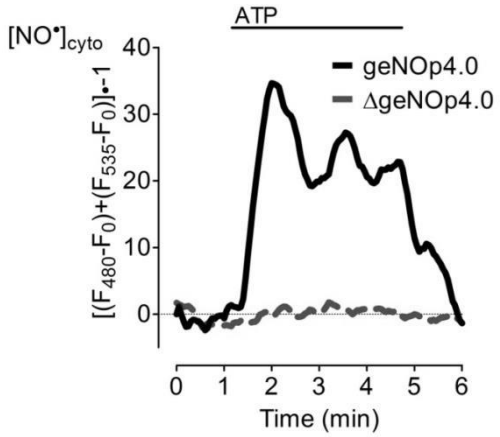

Fig. 16. ***Measurements of Ca^{2+}-induced cellular NO dynamics in EA.hy926 cells*** Curves represent representative responses of EA.hy926 cells expressing either geNOp4.0.(black continuous line) or ΔgeNOp4.0 (grey dasched line). Cells were stimulated with 100 µM ATP in the presence of 2 mM Ca^{2+} in the medium.

We next studied the cellular NO dynamics in response to different histamine concentrations including 300nM, 1µM, 3µM and 100µM (Fig. 17A). These experiments unveiled that even the low physiological concentration of 300 nM of histamine is sufficient to activate the eNOS activity and significantly increase cellular NO levels (Fig. 17A and B) The half maximal effective concentration (EC_{50}) of histamine to increase NO within EA.hy926 cells was found to be 1.4 µM (Fig. 17B).

Nω-Nitro-L-arginine (LNNA) is an irreversible inhibitor of constitutive nitric oxide synthases and a reversible inhibitor of inducible nitric oxide synthases (Gyurko et al. 2000). EC cells were incubated 10 minutes in 100µM of a LNNA solution. Stimulation of ECs with 10µM or even with 100µM histamine did not result in increased NO levels under these conditions (Fig. 17C and D). This experiment indicated that geNOps indeed measure the Ca^{2+} induced NO formation in EA,hy926 cells.

Figure 17

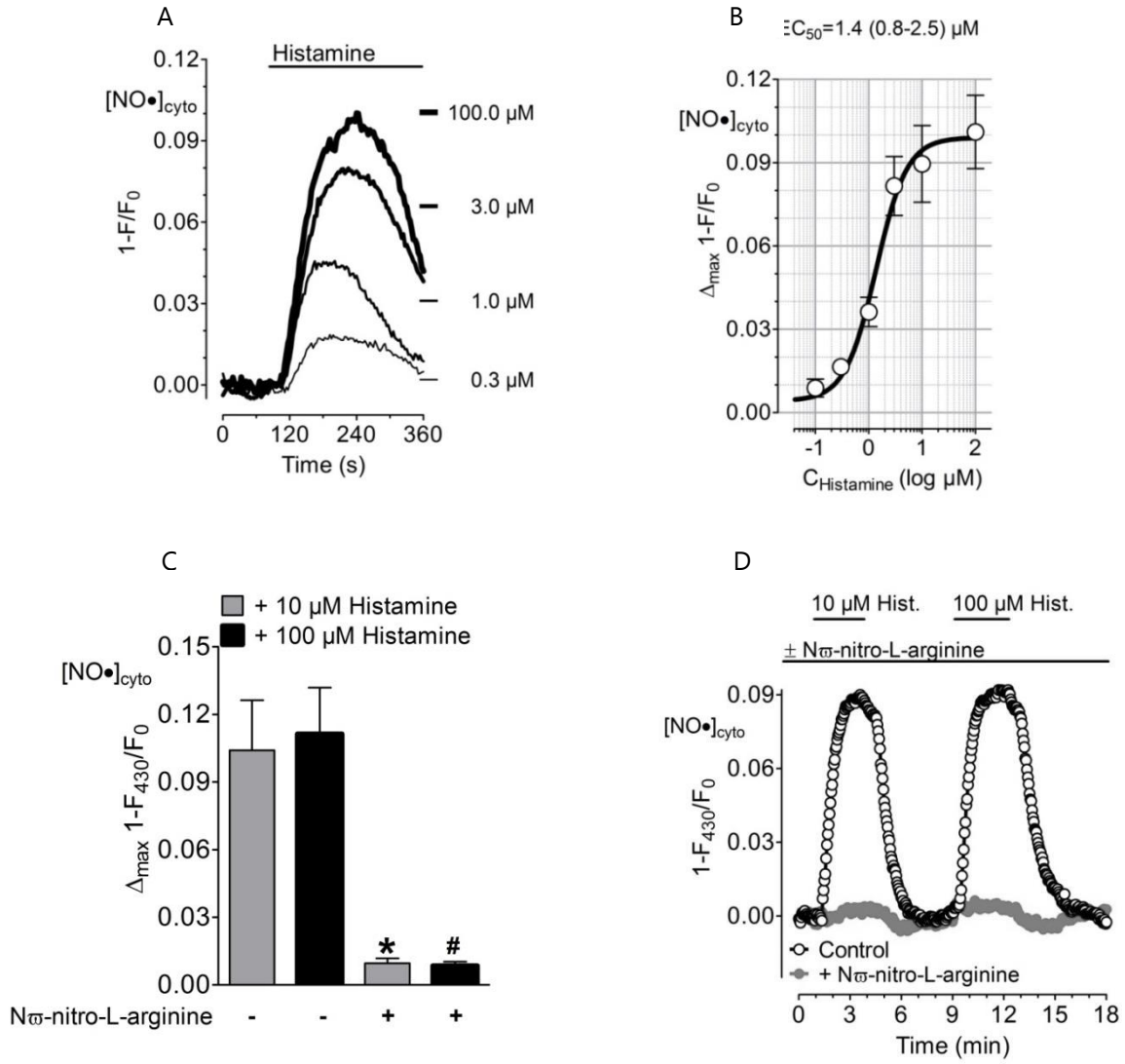

Fig. 17. ***Representative measurements of endogenic NO production*** **in** ***EA.hy926 cells*** **(A)** Representative cellular NO signals in response to different concentrations of histamine in Ea.hy926 cells expressing geNOp4.0. **(B)** EC_{50} value for Histamin was estimated as 1,4µM. **(C and D)** Impact of Nω-nitro-L-arginine treatment (n=10 for each histamine concentration) on EA.hy926 cells in comparison to non-treated cells (n=5 for each histamine concentration). *P < 0.05 vs. 10 µM Histamine without Nω-nitro-L-arginine; # < 0.05 vs. 100 µM Histamine without Nω-nitro-L- arginine.

4. Conclusion

Several publications reported about the difficulty of NO imaging in single living systems and the questionability of available NO imaging methods (Hunter et al. 2013). We were able to show that the fusion of a bacterial NO-sensing domain to different FPs results in a novel class of genetically encoded NO probes. This probes can be improved by iron(II) supplementation and used to visualize and quantify cellular NO dynamics also in mammalian cells. With the invention presented in this diploma work, new gates for an efficient NO bio-imaging with high spatial and temporal resolution have been opened. It is possible that these probes significantly stimulates the NO research field, as the usage of them is simple and allows answering of so far unresolved questions in cell physiology and pathology.

First experiments proofed that the probes also work ex-vivo. Further research is, however, necessary to develop NO sensing assays and devices that are constructed with these protein-based fluorescent NO sensors.

5. Appendix

5.1. Curriculum vitae

Personal Data

Name	Emrah Eroğlu
Place of birth	Windhaag bei Freistadt, (upper Austria)

Academic Education

2013-2014	Study of Medicine, Medical University of Graz
2012-2014	Study of Biotechnology and Bio-analytics, University of Technology, Vienna
2012-2012	Licensure as a pharmaceutical representative, Pharmig
2008-2012	Study of Molecular biology, Vienna University
2007-2008	University entrance exam, Vienna University

5.2. Patent specification

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27 October 2014

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Filing date(*)	27 October 2014
Earliest priority date (if any)	
Applicant(s) / contact point	Medizinische Universität Graz
Application fee paid	Yes
Description (number of pages or reference)	50
Certified copy of referenced application	Not applicable
If description not filed	Not applicable
Claims (number of pages)	5
Drawings (number of pages)	4
Abstract (number of pages)	1
Statement of inventorship (Form 7)	Yes
Request for search (Form 9A)	Yes
Request for examination (Form 10)	Yes
Priority Documents	None
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3.	Full name of the first applicant	**Medizinische Universität Graz**
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	Date:	**27 Oct 2014**
5.	Name, address and e-mail address of a contact point for PDAS	**Bühler, Dr. Dirk Johannes** **MAIWALD Patentanwalts GmbH** **Elisenstraße 3** **Elisenhof** **München 80335** **Germany** **Email: buehler@maiwald.eu**

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Application number GB1419073.0

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2.	Full name, address and postcode of the applicant or of each applicant Patents ADP number *(if you know it)*	**Medizinische Universität Graz** **Auenbruggerplatz 2./4. Stock** **Graz 8036** **Austria**
3.	Title of the invention	**Genetically encoded nitrogen monoxide sensors**
4.	Name of your agent *(if you have one)* "Address for service" to which all correspondence should be sent. This may be in the European Economic area or Channel Islands (see warning note below) *(including the postcode)* Patents ADP number *(if you know it)*	**Bühler, Dr. Dirk Johannes** **MAIWALD Patentanwalts GmbH** **Elisenstraße 3** **Elisenhof** **München 80335** **Germany**
5.	Priority declaration: Are you claiming priority from one or more earlier-filed patent applications? If so, please give details of the application(s)	

Country	Application number	Date of filing	PDAS Access Code

6.	Divisionals etc: Is this application a divisional application, or being made following resolution of an entitlement dispute about an earlier application. If so, please give the application number and filing date of the earlier application	Number of earlier UK application	Date of filing *(day / month / year)*
7.	Inventorship: (Inventors must be individuals not companies) Are all the applicants named above also inventors?	**No**	
8.	Are you paying the application fee with this form?	**Yes**	

Patents Form 1

9. Accompanying documents: please enter the number of pages of each item accompanying this form.

Continuation sheets of this form

Description:	**50**
Claim(s):	**5**
Abstract:	**1**
Drawing(s):	**4**

If you are not filing a description, please give details of the previous application you are going to rely upon

Country	Application number	Date of filing	PDAS Access Code

10. If you are also filing any of the following, state how many against each item.

Priority documents:	**0**
Statement of inventorship and right to grant of a patent (Patents Form 7):	**1**
Request for search (Patents Form 9A):	**1**
Request for substantive examination (Patents Form 10):	**1**
Any other documents (please specify):	**Sequence Listing** **PDAS Registration Form**

11. I/We request the grant of a patent on the basis of this application.

Date: **27 Oct 2014**

12. Name, e-mail address, telephone, fax and/or mobile number, if any, of a contact point for the applicant

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After an application for a patent has been filed, the Comptroller will consider whether publication or communication of the invention should be prohibited or restricted under section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you are resident in the United Kingdom and your application contains information which relates to military technology, or would be prejudicial to national security or the safety of the public, section 23 of the Patents Act 1977 prohibits you from applying for a patent abroad without first getting written permission from the Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked. Until such time or until the revocation of any direction, for any such application the address for service referred to at part 4 above must be in the United Kingdom.

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Intellectual Property Office

Patents Form 7
Patents Act 1977 *(Rule 10)*

Statement of inventorship and of right to grant of a patent

Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

1.	Your reference	**M 10878-GB DB.JvD.mma**
2.	Patent application number	**GB1419073.0**
3.	Full name of the or of each applicant	**Medizinische Universität Graz**
4.	Title of the invention	**Genetically encoded nitrogen monoxide sensors**
5.	State how the applicant*(s)* derived the right from the inventor*(s)* to be granted a patent	**The applicant Medizinische Universität Graz has derived the right to apply for a patent from inventors Graier, Malli, Eroglu and Waldeck-Weiermair by contract of employment.**
6.	How many, if any, additional Patents Forms 7 are attached to this form?	**1**
7.		I/We believe that the person*(s)* named over the page *(and on any extra copies of this form)* is/are the inventor*(s)* of the invention which the above patent application relates to. Date: **27 Oct 2014**
8.	Name, e-mail address, telephone, fax and/or mobile number, if any, of a contact point for the applicant	**Bühler, Dr. Dirk Johannes** **Email: buehler@maiwald.eu** **Telephone: +49897472660** **Fax: +4989776424**

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Country:	Austria

Intellectual Property Office

Patents Form 9A
Patents Act 1977 *(Rule 27)*

Request for search

Concept House
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South Wales
NP10 8QQ

1.	Your reference	**M 10878-GB DB.JvD.mma**
2.	Patent application number	**GB1419073.0**
3.	Full name of the applicant or of each applicant	**Medizinische Universität Graz**
4.	Is this request for: a) A search under Section 17(1) for an international application which has been searched in the international phase? b) A search under Section 17(1) for any other application?	**b**
5.	Do you want a copy of any patent documents cited in the search report?	**No**
6.	Are you paying the application fee with this form?	**No**
7.	Date:	**27 Oct 2014**
8.	Name, e-mail address, telephone, fax and/or mobile number, if any, of a contact point for the applicant	**Bühler, Dr. Dirk Johannes** **Email: buehler@maiwald.eu** **Telephone: +49897472660** **Fax: +4989776424**

Intellectual Property Office

Patents Form 10
Patents Act 1977 *(Rule 28)*

Request for a substantive examination

Concept House
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South Wales
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1.	Your reference	**M 10878-GB DB.JvD.mma**
2.	Patent application number	**GB1419073.0**
3.	Full name of the or of each applicant	**Medizinische Universität Graz**
4.		I/We request substantive examination of this application. Date: **27 Oct 2014**
5.	Name, e-mail address, telephone, fax and/or mobile number, if any, of a contact point for the applicant	**Bühler, Dr. Dirk Johannes** **Email: buehler@maiwald.eu** **Telephone: +49897472660** **Fax: +4989776424**

I/We wish to request a Combined Search and Examination

Intellectual Property Office

Fee Sheet Reference (see note 1) M 10878-GB DB.JvD.mma

Payment Method		Amount
a) Deposit Account	D05125	**£230.00**
b) Credit/Debit Card		

Fee Details

Form	Description of Fee	No. Filed	Fee Amount (£)
1	No Fee	1	0.00
	Application Fee	1	20.00
3	Request for late declaration of priority	0	0.00
7	No Fee	1	0.00
9A	Request for search	1	130.00
10	Substantive examination	1	80.00
23	This application as originally filed (sealed)	0	0.00
			£230.00

Date 27 Oct 2014

1. Your reference (if supplied) will be shown on fee sheet acknowledgements to enable you to link the transactions back to your supporting papers

5.3. Abbrevations

Table 4: *Abbrevations*

Acronym	Term
[…]	Concentration
µ	mykro (10^{-6})
ADP	Adenosin-5-diphosphate
ATP	Adenosin-5-triphosphate
BHQ	2.5-Di-tert-butylhydroquinone
BME	beta-mercapthoethanol
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
$[Ca^{2+}]_{cyto}$	Cytosolic Calcium concentration
seCFP	super enhanced Cyan fluorescing protein
cp	circular permuted
CPV	circular permuted Venus
DMEM	Dulbecco's modified Eagle's medium
EA.hy926	human umbilical vein cell line
EC(s)	Endothelial cells
eGFP	enhanced green fluorescent protein
eNOS	endothelial nitric oxide synthase
FCS	Fetal calf serum
FRET	Fluorescence resonance energy transfer
GFP	green fluorescent protein
HAT	Hypoxanthin, aminopterin, thymidine
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HSA	Human serum albumin
iNOS	inducible Nitric oxide synthase
IPTG	Isopropyl-β-D-thiogalactopyranosid
LNNA	Nω-Nitro-L-arginine
LPS	Lipopolysaccharide
nNOS	neuronal nitric oxide synthase

NO	Nitric oxide
NOC5	1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene
NOC7	3-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine
NOC12	3-Ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene
NOS	Nitric oxide synthase
OD_{600}	optical density at 600 nm
SDS-PAGE electrophoresis	Sodium dodecyl sulphate Polyacrylamide gel
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLC	phospholipase C
PTM	post translational modifications
Proli NONOate	Disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate
SNALB	S-nitroso albumin
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
sGC	soluble guanylat cyclase
YFP	Yellow fluorescing protein

5.4. Publication bibliography

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