



T.C.

BİRÜNİ UNIVERSITY

INSTITUTE OF GRADUATE EDUCATION

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MOLECULAR AND MEDICAL GENETICS GRADUATE PROGRAM

**REAL-TIME IMAGING OF NITRIC OXIDE (NO) SIGNALS
DERIVED FROM DIFFERENTIALLY TARGETED
ENDOTHELIAL NITRIC OXIDE SYNTHASES (eNOS) USING
GENETICALLY ENCODED BIOSENSORS**

Mete Emir ÖZGÜRSES

ADVISOR

Asst. Prof. Dr. Emrah EROĞLU

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DECLARATION

I declare that I have designed and performed all experiments in the current study entitled “Real-time imaging of nitric oxide (NO) signals derived from differentially targeted endothelial nitric oxide synthases (eNOS) using genetically encoded biosensors” according to good scientific practices. I obtained all the information contained in this thesis under academic and ethical rules. All the information I have used from secondary literature has been respectively referenced. I also declare that have not violated any patents and copyrights during the preparation and writing of this thesis.



Mete Emir Ozgurses



To all human being,

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to everyone who has supported me through this thesis.

Firstly, I would like to thank Asst. Prof. Emrah Eroğlu for allowing me to work in his lab. He has guided me throughout the project both by challenging me to be more competent in science and by sharing his instructive ideas. His experience and dedication to science have opened me new perspectives on both the psychological and emotional levels, which has pushed me more to always strive for a better.

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ABBREVIATIONS

B-GECO:	Blue variant of Genetically Encoded Ca^{2+} Indicators for Optimal imaging
BFP:	Blue Fluorescent Protein
Ca^{2+} :	Calcium
CaM:	Calmodulin
Cav-1:	Caveolin-1
CFP:	Cyan Fluorescent Protein
cGMP:	Cyclic Guanosine Monophosphate
cpEGFP:	Circularly Permuted Enhanced Green Fluorescent Protein
cpFPs:	Circularly Permuted Fluorescent Protein
DAF:	Diamino Fluorescein
deGFP:	Dual-Emission Green Fluorescent Protein
DEPC water:	Diethylpyrocarbonate treated water
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	Dimethylsulphoxide
EDFR:	Endothelial-Derived Releasing Factor
eNOS:	Endothelial Nitric Oxide Synthase
ER:	Endoplasmic Reticulum
FAD:	Flavin Adenine Dinucleotide
FMN:	Flavin Mononucleotide
FP:	Fluorescent Protein
FRET:	Fluorescent Resonance Energy Transfer
FRET-MT:	FRET-based biosensor containing hMTIIa
GABA:	Gamma-Aminobutyric Acid
GAF:	sGC Activating Factor
GEB:	Genetically Encoded Biosensors
GECO:	Genetically Encoded Ca^{2+} Indicators for Optimal imaging
geNOps:	Genetically Encoded NO probes
GFP:	Green Fluorescent Protein
hMTIIa:	Human Type IIa Metallothionein
iNOS:	Inducible Nitric Oxide Synthase
IP_3 :	Inositol 1,4,5-Triphosphate

LB agar:	Lysogeny Broth agar
LED:	Light-Emitting Diodes
MNNG:	N-methyl-N'-nitro-N-nitrosoguanidine
NADPH:	Nicotinamide adenine dinucleotide phosphate
NasS:	Nitrate Assimilation System gene, S subunit
NasT:	Nitrate Assimilation System gene, T subunit
Nitrate:	NO_3^-
Nitrite:	NO_2^-
nNOS:	Neuronal Nitric Oxide Synthase
NO:	Nitric Oxide
NOA-1:	A Fluorescence Indicator for NO with a Signal Amplifier
Noc-7:	3-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine
NorR:	NO Reductase Transcription Regulator
NOS:	Nitric Oxide Synthase
NOSIP:	Nitric Oxide Synthase Interacting Protein
O-geNOP-NES:	Orange variant of geNOPs tagged with Nuclear Excision Sequence
OFP:	Orange Fluorescent Protein
Peroxynitrite:	ONOO^-
R-GECO:	Red variant of Genetically Encoded Ca^{2+} indicators for optimal imaging
R-GECO-UT:	Red variant of Genetically Encoded Ca^{2+} indicators for optimal imaging- untargeted
RFP:	Red Fluorescent Protein
sGC:	soluble guanylyl cyclase
sNOOpy:	Sensor for $\text{NO}_3^-/\text{NO}_2^-$ in physiology
WT-eNOS:	Wild-Type endothelial Nitric Oxide Synthase
YFP:	Yellow Fluorescent Protein
Zn^{2+} :	Zinc

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TURKISH ABSTRACT and KEYWORDS

Nitrik oksit (NO), makrofaj, nöron ve endotel hücreleri dahil birçok farklı hücre tiplerinde üretilen gaz halindeki bir moleküldür. Endotelial hücrelerde NO, düz kas hücrelerinin gevşemesini ve vasküler tonunun düzenlediği bilinen kalsiyum bağımlı endotelial nitrik oksit sentaz (eNOS) tarafından üretilir. Fizyolojik koşullarda, eNOS hücre zarındaki kaveolada lokalizedir genellikle protein-protein etkileşimleri nedeniyle aktif değildir. eNOS'un hücre içi farklı bölmeleri arasındaki translokasyonun NO üretimini etkileyebileceği de gösterilmiştir. Bununla birlikte, literatürdeki ilgili çalışmaların çoğunda, uygun araçların bulunmaması nedeniyle lokal eNOS kaynaklı NO biyoyararlanımı araştırılamamıştır. Bu diploma tezinde NO ve Ca^{2+} için yeni multispektral görüntüleme tekniklerinden yararlanılarak bu konu gözden geçirilmiştir. Bu amaçla, yüksek mekân-zamansal çözünürlük ve yüksek seçicilik ile canlı hücrelerde NO tespiti sağlayan yeni genetik olarak kodlanmış NO sensörü (geNOps) kullanılmıştır. Lokal Ca^{2+} sinyallerinin görselleştirilebilmesi için GECCO'ların kırmızı ve farklı hedefli varyantları kullanılmıştır. Farklı hücrelerel lokallerde eNOS aktivitesini incelemek için HEK293 hücreleri model sistem olarak kullanılarak yabani tip (wild type)-eNOS ile nükleer hedefli eNOS karşılaştırılmıştır. Her iki bölmede de eNOS'un aktivasyonu için gerekli olan Ca^{2+} sinyalleri tespit edilmiştir. Bu yaklaşım, çekirdekdeki eNOS aktivitesinin, aynı deney koşulları altında yabani tip-eNOS'a kıyasla önemli ölçüde azaldığını, ancak yine de aktif olduğunu açıkça gösterilmiştir. Bu tezde, farklı hücre içi lokallerdeki eNOS aktivitesi farklılıklarını ortaya çıkarmak amacıyla multispektral görüntüleme tekniklerinden yararlanılmıştır ve lokal Ca^{2+} ortamının eNOS aktivitesindeki farklılıkları etkilemediği ilk kez, doğrudan kanıtlanmıştır.

Anahtar Kelimeler: Endotel nitrik oksit sentaz, Genetik olarak kodlanmış biyosensörler, Nitrik Oksit

ABSTRACT and KEYWORDS

Nitric oxide (NO) is a gaseous molecule produced in many different cell types, including macrophages, neurons, and endothelial cells. In endothelial cells, NO is produced by the calcium-dependent endothelial nitric oxide synthase (eNOS), which is known to regulate smooth muscle cell relaxation and vascular tone. Under physiological conditions, eNOS is localized to the caveolae in the cell membrane and is generally inactive due to protein-protein interactions. It has also been shown that translocation between different compartments of eNOS inside the cell can affect NO production. However, in most of these studies the local eNOS derived NO bioavailability could not be studied due to the lack of proper tools. In this diploma, issue by exploiting novel multispectral imaging techniques for NO and Ca^{2+} was revisited. For this purpose, the novel genetically encoded NO sensor (geNOPS), which provides NO detection in living cells with high-spatial and temporal resolution and high selectivity, was used. To visualize local Ca^{2+} signals the well-established red-shifted and differentially targeted variants of the GECOs was exploited. To study eNOS activity in different cellular locales, HEK293 cells was used as a model system and compared wild type-eNOS versus nuclear-targeted eNOS. In both compartments, Ca^{2+} signals required for eNOS activation was detected upon stimulation. This approach clearly showed that eNOS activity in the nucleus is significantly reduced but still active compared to the wild type-eNOS under the same experimental conditions. In this master thesis, multispectral imaging techniques was used to unveil differences in eNOS activity in different subcellular locales. This master thesis provided for the first-time direct evidence that the local Ca^{2+} environment does not dictate differences in endothelial nitric oxide synthase activity.

Keywords: Endothelial nitric oxide synthase, Genetically encoded biosensors, Nitric Oxide

INTRODUCTION and PURPOSE

NO is one of the most studied molecules in biology (Hukkanen et al., 1998), documented by approximately 7000 published papers per year according to the PubMed database. NO can be found in all three domains of life and is probably the most versatile molecule in nature. In mammalian cells, NO can be synthesized by specialized enzymes called nitric oxide synthases (NOS). Three isoforms belong to the NOS family, which are found in different tissues. These are the endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Tegeder, 2019). All isoforms are Ca^{2+} -dependent, however, due to the high affinity of iNOS to the Ca^{2+} /Calmodulin complex, this isozyme seems to be constitutively active. In the current study, we focused in particular on the function of the endothelial NOS isoform, which is predominantly located in caveolar regions of the cell membrane (W. Su et al., 2016). eNOS is tightly regulated by different cofactors, proteins, and post-transcriptional modifications to prevent its activation upon stimulation with molecules other than Ca^{2+} . Interacting proteins such as Caveolin-1 (Cav-1) (Lei, Song, Wu, Xing, & Sun, 2016) and NOS interacting protein (NOSIP) (Nakamura, Zimmer, Friedmacher, & Puri, 2019) control deactivation of eNOS and reduces the NO production. Other proteins such as calmodulin (CaM) (Porter, Jang, Haque, Stuehr, & Mehl, 2020) and heat shock protein (Hsp90) (Averna et al., 2008), initiates a conformational change in eNOS, thus activate NO production. However, due to the extremely short half-life of NO, which is less than a second under ambient conditions (Thomas, Liu, Kantrow, & Lancaster, 2001), it has been a challenge to real-time detect NO concentration changes on cellular levels. Exploiting the physical and chemical properties of NO, many different techniques have been developed for NO detection (Hogg, Zielonka, & Kalyanaraman, 2017). One of the most prominent and still widely used techniques is the Griess assay which permits the concentration determination of stable byproducts of NO (Schmölz, Wallert, & Lorkowski, 2017), the enzymatic based citrulline assay (Combet, Balligand, Lameire, Goffin, & Devuyst, 2000), and fluorescent-based diamino fluorescein (DAF) dyes (Zhou & He, 2011). However, all these golden standard techniques bear serious limitations such as indirect NO measurement, low sensitivity and selectivity, toxicity, and the limitation of NO measurement in whole cell populations. Only the DAF imaging method permits single-

cell imaging in real-time, however, due to the less selectivity to NO, irreversibility, and non-targetability, these chemical probes turned to be less suitable for subcellular NO imaging in living cells. Thus, owing to the lack of proper imaging tools, the direct NO production derived from eNOS in sub localizations has not been studied yet. In a recent advance in the field of genetically encoded NO biosensors, a novel NO sensor has been developed, termed as geNOps, permit for the first time real-time imaging of NO levels in subcellular levels in a simultaneous manner (Eroglu et al., 2018).

In this current study, we addressed this timely issue: exploiting this powerful imaging tools we are investigating for the first time the complex biochemistry of differentially targeted eNOS. For this purpose, we particularly studied the functionality of eNOS in the nucleus by specifically targeting the enzyme to this locale and compare it NO synthesis capacity in comparison to the WT-eNOS. Thus, we test the hypothesis that differential targeting of the eNOS affects the NO formation activity of the enzyme.

GENERAL INFORMATION

2.1 The Glorious History of Nitric Oxide

Nitric Oxide (NO) was identified as a hydrophobic, colorless gas, and was named as “Nitrous Air” by Joseph Priestly in 1772 (Smith, 1965). Not earlier than four decades ago, Murad et al. showed that different NO donors, such as nitroprusside and nitroglycerin, increase the levels of the secondary messenger cyclic GMP (cGMP) in rat liver and bovine tracheal smooth muscle (Katsuki, Arnold, Mittal, & Murad, 1977). Gruetter et al. treated bovine coronary artery with nitroprusside, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and NO, and observed a relaxation effect of the vascular smooth muscle cells, which again gave reason to believe that NO might be a vasodilator (Gruetter et al., 1979). However, still the direct function of NO in living organisms was not yet identified (Yetik-Anacak & Catravas, 2006). Furchgott and Zawadzki also studied the vasorelaxation effect of NO in 1980. They treated vessels with acetylcholine and observed the production of an unstable, diffusible compound which they referred to as an endothelium-derived relaxing factor (EDRF). Without being aware that the EDRF they have discovered, which causes the relaxation of vascular smooth muscle cells is NO (Furchgott & Zawadzki, 1980). A few years later, in 1987, finally, EDRF was proven to be NO, simultaneously by two research groups; Ignarro et al., from UCLA and Palmer et al. from Wellcome Research Laboratories in the UK (Bruckdorfer, 2005). These findings opened new avenues in the field of NO biochemistry and the importance of NO in the medical fields. For their discoveries, in 1998, Murad, Furchgott, and Ignarro were awarded the Nobel Prize in physiology and medicine. It is worth mentioning that Salvador Moncada, who also independently discovered NO these days was not honored with the Nobel Prize due to his origin and political reasons (Palmer, Ferrige, & Moncada, 1987). On the other hand, other groups were studying the same mechanism in different tissues. In 1977, Takeo Deguchi from the Tokyo metropolitan institute of neurosciences found soluble-guanylate cyclase (sGC), which is a direct receptor of NO (Martin, Berka, Tsai, & Murad, 2005), activity in the rat brain's mitochondria. Further, in 1982, Deguchi and Yoshioka have also identified L-arginine as the endogenous activator of sGC (Deguchi, 1977; Deguchi & Yoshioka, 1982). After identifying the L-arginine as an endogenous activator of sGC, in 1988, Garthwaite et al. have found glutamate increases cGMP and created a similar

profile as EDNR acting on N-methyl D-aspartate (NMDA) receptors (Garthwaite, Charles, & Chess-Williams, 1988). Later, it was found that the NMDA receptor stimulates NO formation from L-arginine, thus specifying the presence of NO in brain tissue (Garthwaite, Garthwaite, Palmer, & Moncada, 1989).

In 1985, Stuehr et al. found that T-lymphocyte mediated activation of macrophages were able to produce huge amounts of nitrate/nitrite and concluded that NO might have cytotoxic functions in these cell types. In 1987, Hibbs et al. have found the activated CAMs are dependent on the L-arginine deiminase, which results in oxidation of NO to nitrite (Hibbs, Taintor, & Vavrin, 1987). Later in 1988, Hibbs team have intraperitoneally infected C₃H/HeN mice with Bacillus Calmette Guerin, and stimulated macrophages were found to produce NO upon exposure to the virus. (Hibbs, Taintor, Vavrin, & Rachlin, 1988). These findings have documented the baseline for NO studies on the molecular level.

In the last decades, NO has found to be an essential, versatile signaling molecule in cancer progression (Muntané & De la Mata, 2010). Production of NO in cancer cells possess both anti-tumor and pro-tumorigenic effect (Zou et al., 2020). NO concentrations slightly above the physiological condition promote metastasis and tumor progression, while much higher levels of NO suppresses the metastasis (Vahora, Khan, Alalami, & Hussain, 2016). Due to the similarity between the cancer microenvironment and local inflammation (Q. Lin et al., 2018), both conditions increase the NO production by migrated immune cells. An inevitable increase in the production of NO creates a favorable environment for tumor initiation and progression by increasing angiogenesis through arterial vasodilation (Tran, Porrini, & Ramarao, 2020). In contrast, many studies have also shown the overproduction of NO can reduce cancer cell proliferation in vivo and in vitro (Burke, Sullivan, Giles, & Glynn, 2013), thus suggesting the phenomenon of 'NO the Doubled-Edged Sword' in cancer (Xu, Liu, Loizidou, Ahmed, & Charles, 2002).

The use and production of NO are not confined to animal species. The ability to engage with the atmospheric NO has been known for both plants and bacteria (Berger, Boscarì, Frendo, Brouquisse, & Foyer, 2019). The presence of cGMP in plants was first shown by mass spectroscopy and further NO treatment experiments

have also revealed that NO can induce cGMP in plants (Brown & Newton, 1992). Administration of NO to tobacco plant triggered the production of defense genes like pathogenesis related-1 (Hausladen & Stamler, 1998) and depletion of NO has decreased the growth of tobacco plant (Nejamkin et al., 2020). Furthermore, NO was also found to trigger cell death in plants (Locato, Paradiso, Sabetta, De Gara, & de Pinto, 2016). In bacteria, NO has been known to have both protective and metabolic adaptation effects. NO produced from *Bacillus anthracis* provides resistance for oxidative stress generated from macrophages (Shatalin et al., 2008), and NO from *Staphylococcus aureus* contributes to survival against neutrophils (Vaish & Singh, 2013). Also, *S. aureus* increases the production of NO under oxygen-deprived conditions and shifts the metabolism from aerobic to nitrogen-based respiration, providing adaptation (Kinkel et al., 2016). Overall, original studies since the discovery of NO back in the late 80s taught us that NO can be found in all organisms as a different actor. It is also hard to find a disease, which is not related to NO alterations. The understanding of the complex biochemistry of NO is still an ongoing process and mysteries related to this tiny radical are by far not discovered yet.

2.2 Biosynthesis of NO

NO can be produced in many cells throughout the body, such as macrophages, Kupffer cells, astrocytes, neutrophils, monocytes, neurons, endothelial cells, and cancer cells (Ignarro, 2009). Each cell type can synthesize NO by different NOS isoenzyme family (Lidington, Li, & Tyml, 2007). To date, there have been three types of NOS identified, depending on the type and function in the cells (Govers & Rabelink, 2001). The neuronal NOS (nNOS or NOS1) occurs predominantly in the neurosystem, in skeletal muscles, and permits synaptic plasticity (Kovacs, Cai, & Larson, 2001). The endothelial NOS (eNOS or NOS3) is expressed in endothelial cells and produces NO as a vasodilator to relax the vascular smooth muscle cells (Hong et al., 2019). Both NOS3 and NOS1 are constitutively active and calcium/calmodulin-dependent (Tousoulis, Kampoli, Tentolouris Nikolaos Papageorgiou, & Stefanadis, 2012). The inducible NOS (iNOS or NOS2), however, is mainly expressed in immune cells and its constantly active (Du & Geller, 2010).

NO has an important effect on wound healing, development of immune response against infection, neurotransmission, and relaxation of smooth muscle cells

(Vallance & Collier, 1994). Usually, endothelial cells produce NO for vasodilation of smooth muscle cells. Upon scar formation, NO is eliminated by the excessive reactive oxygen species produced at the wound area, thus, NO from the other endothelial cells creates a favorable environment for guiding the platelets to the wound area and initiates the wound healing process (Malone-Povolny, Maloney, & Schoenfisch, 2019). In the inflammation phase of wound healing, NO has been found to increase to its peak one day after the injury, and decrease as the healing process continues (Han et al., 2012). NO is also linked with the protection against pathogens in mammals. Upon viral infection, iNOS is found to be activated due to interferon- γ leading to NO overproduction (Akaike & Maeda, 2000). Furthermore, in different studies, it has been shown that the mice lack the iNOS gene is more susceptible to viral infections, indicating the importance of both normal immunity and fighting against viral and bacterial pathogens (Barros et al., 2019) (Akaike, 2000).

In the nervous system, nNOS has been found in nervous related tissues including, hypothalamus, pineal gland, brain, and spinal cord and NO is a crucial messenger in the central nervous system (Džoljić, Grabatinić, & Kostić, 2015). nNOS is dependent to the NMDA receptor to function (Dagdeviren, 2017). Glutamate that is released from the pre-synaptic neuron increases the influx of Ca^{2+} in post-synaptic neurons and leads to the direct activation of nNOS, which is anchored to NMDA with post-synaptic density protein, and initiates NO production (Calabrese et al., 2007; Ghasemi, Claunch, & Niu, 2019). The produced NO can diffuse back to the pre-synaptic neuron to activate the sGC (Reis, Albuquerque, Maron-Gutierrez, Silva, & Neto, 2017). The activated sGC converts GTP to cGMP in the pre-synaptic neuron, and functions in different mechanisms. For example, cGMP inhibits the activity of gamma-aminobutyric acid-mediated transmission and activation of a synaptic vesicle glycoprotein that functions in the fusion of vesicles released from pre-synaptic neuron (Bidmon et al., 2006; Wang, Mergia, Koesling, & Mittmann, 2017).

In the endovascular system, NO is produced by eNOS. The functional state of eNOS is a homodimer located at caveolae regions on the cell cytoplasm and interacts with a well-known eNOS inhibitor Cav-1 protein. Each monomer of eNOS contains N-terminal oxygenase and C-terminal reductase regions. When ligands such as Ca^{2+} agonists, histamine and ATP, binds to the G-protein coupled receptor (GPCR) (Eroglu,

Saeedi Saravi, Sorrentino, Steinhorn, & Michel, 2019; Sriram, Laughlin, Rangamani, & Tartakovsky, 2016), it activates Phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate and generates inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (Billups, Billups, Challiss, & Nahorski, 2006). The IP₃ further binds to the IP₃ receptor located at the surface of the endoplasmic reticulum (ER) and initiates the Ca²⁺ release from ER (Nakayama et al., 2010). Released Ca²⁺ activates calmodulin (CaM), which is an important protein to start NO production. Activated CaM binds to its target on eNOS with heat shock protein 90 (Hsp90) (**Figure 2.1**) (Z. Chen et al., 2018; Y. Su, 2014), disassociates Cav-1, leading to the alignment of the oxygenase and reductase regions of eNOS (Shu et al., 2015).

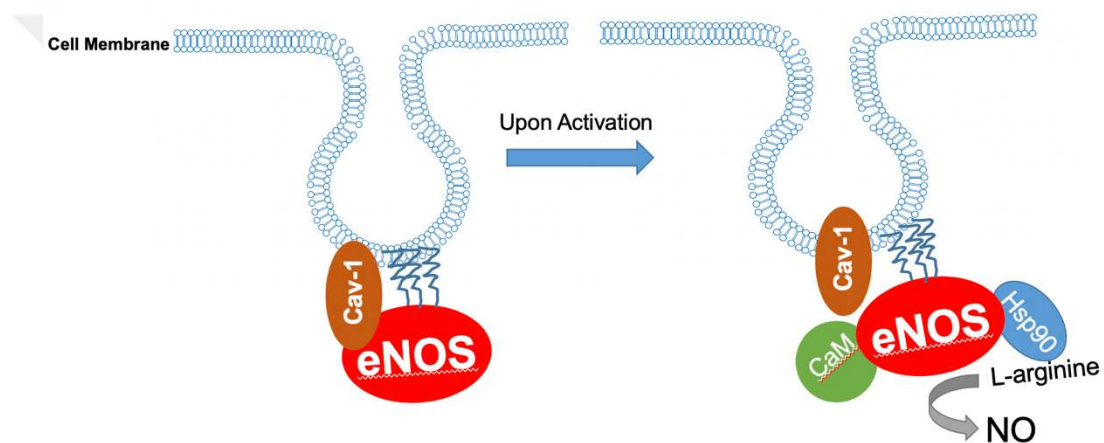


Figure 2.1: Schematic representation of eNOS in physiological conditions. The inactive state of eNOS is co-localized with Cav-1 protein at caveolae. Upon activation, eNOS interacts with the active state of CaM and Hsp90 Proteins and produces NO from L-arginine.

The conformational change permits the electron transfer donated from nicotinamide adenine dinucleotide phosphate (NADPH) flow to flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and finally to Heme group (Gliozzi et al., 2019). Oxidized heme group, triggers NO production from L-Arginine and oxygen (**Figure 2.2**). (Rafikov et al., 2011). NO produced from the endothelial cell can easily diffuse into smooth muscle cells and stimulates sGC (Melichar et al., 2004), which initiates the production of cGMP. cGMP in smooth muscle cells activates vasodilation and inhibits inflammation, apoptosis, remodeling, and platelet formation (Stasch, Pacher, & Evgenov, 2011).

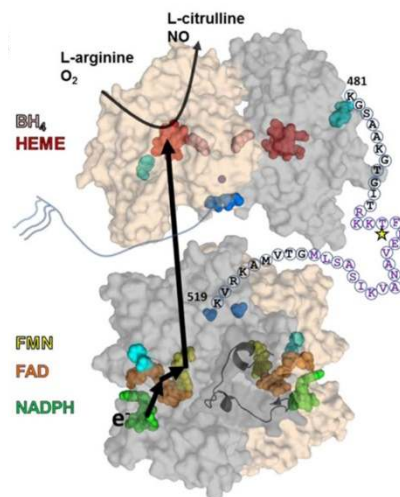


Figure 2.2: 3D representation of homodimer eNOS with its interacting proteins and co-factors (Shu et al., 2015). Upon stimulation with Ca^{2+} , the eNOS homodimer is able to accept an electron from NADPH. The received electron shifts through FAD and FMN regions until the heme group. When the heme group is reduced with the electron, it leads to NO production from L-Arginine.

Besides, post-translational modifications also occur on eNOS to maintain its inactive state and for correct localization. The N-terminus of eNOS has three acylation regions, G2, C15, and G26 amino acid residues. G2 provides localization of eNOS to caveolae, specifically to the Cav-1 protein by a process called myristoylation. C15 and C26 are covalently bound to fatty acids, with a process called palmitoylation, which increases the hydrophobicity of the protein and provides subcellular trafficking (Rocks et al., 2005; Yeh, Duncan, Yamashita, & Michel, 1999)

2.3 Genetically Encoded Fluorescent Biosensors

The field of genetically encoded biosensors (GEB), roots to the discovery of the fluorescent protein (FP) described in the 1960s from jellyfish, *Aequorea Victoria* (Shimomura, Johnson, & Saiga, 1962). *A. Victoria* was found to emit green light due to two closely associated proteins; green fluorescent protein (GFP) and aequorin. At first, GFP was discovered as aequorin associated, a 240 amino acid protein that contains a chromophore formed by the three amino acids Serine-Tyrosine-Glycine between the residues 65-67 (**Figure 2.3**) (Tsien, 1998).

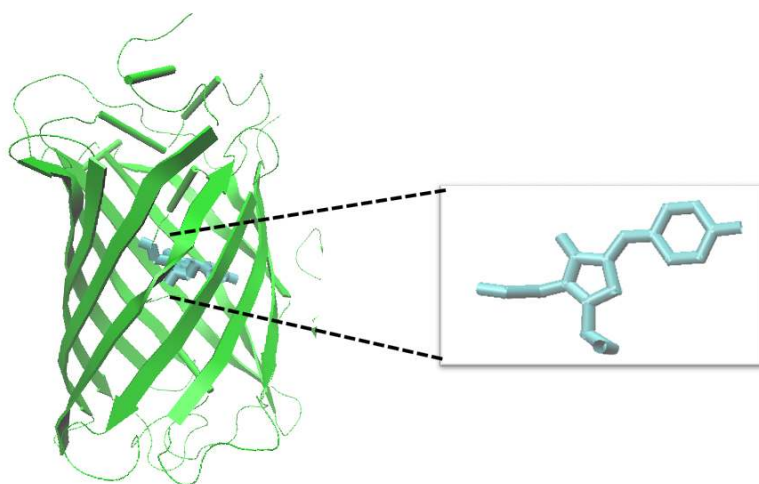


Figure 2.3: 3D representation of the Green Fluorescent Protein (GFP). GFP is a protein with a barrel structure which has the ability to emit green light upon stimulation. The green light is generated due to the chromophore, which is composed of Serine-Tyrosine-Glycine as shown in the figure (F. Yang, Moss, & Phillips, 1996).

Aequorin, however, is a 189 amino acid bioluminescent calcium-binding protein that contains Ca^{2+} binding sites (Dikici et al., 2009). When Ca^{2+} is available and binds to the specific residues on aequorin, the protein undergoes a conformational change, leading to the oxidation of chromophore, emitting blue light (470 nm) (Baubet et al., 2000). Due to the proximity of aequorin and GFP under natural conditions, the emission light generated by aequorin is transferred to GFP, by a phenomenon called Fluorescent resonance energy transfer (FRET) (Tsuji, 2010). The principle of the FRET activated GFP is shown in **Figure 2.4**.

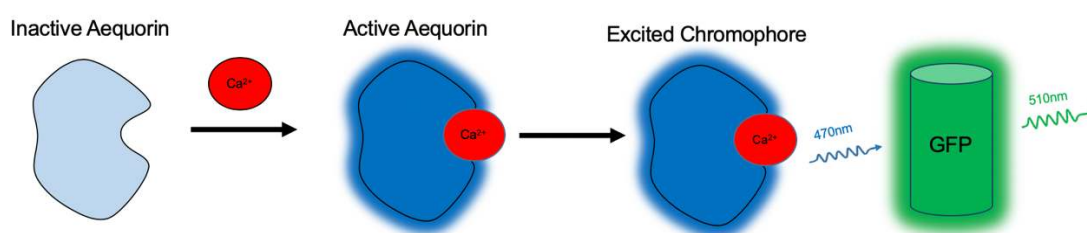


Figure 2.4: Schematic representation of the mechanism of light emission by aequorin upon activation with Ca^{2+} . Upon activation of aequorin with Ca^{2+} , aequorin emits blue light (470nm) and due to the proximity to the GFP, the emitted energy excites the GFP, which in turn emits green light (510nm).

The discovery of these two proteins was revolutionary in the field of fluorescent microscopy, protein engineering, and GEBs. Roger Tsien, Martin Chalfie and Osamu Shimamura were awarded the Nobel Prize in the field of chemistry in 2008 for the discovery and development of GFP. Since the identification of GFP, in the 1990s, Roger Tsien has expanded his studies into engineering novel GEBs (Tsien, 1980). First, his team has focused on native GFP, and exploited random mutagenesis experiments to generate various FP variants with different spectral properties that all were derived from the wild-type GFP. Early studies yielded new blue-shifted FPs such as the cyan-colored CFP that contained a Y66W mutation, a blue FP with a Y66H mutation in the chromophore (Heim, Prasher, & Tsien, 1994), and a yellow FP variant (YFP) that was generated by introducing a mutation at position Q69K (Tsien, 1998). Furthermore, his team has generated an enhanced GFP (EGFP), by changing the Serine, S65, to a Threonine. This approach yielded a brighter response over the native GFP, which became the widely used FP in the field of fluorescent proteins (Heim, Cubitt, & Tsien, 1995).

It was Roger Tsien again who recognized the potential of FPs and generated the first GEB for Ca^{2+} , referred to as Chameleons (Miyawaki et al., 1997). In the last two decades, scientists advanced different types and classes of GEBs to image different targets such as; metabolites including malonyl-CoA, lactate, and pyruvate, neurotransmitters such as dopamine, gamma-aminobutyric acid (GABA), glutamate (Leopold, Shcherbakova, & Verkhusha, 2019; Marvin et al., 2019). Besides, GEBs has been also developed to measure intracellular pH fluctuations (Hanson et al., 2002), cellular signals (W. Lin, Mehta, & Zhang, 2019), ions like Ca^{2+} (Miyawaki et al., 1997), H_2O_2 (Pak et al., 2020), NO (Eroglu et al., 2016), and cell cycle proteins (Sakaue-Sawano et al., 2017). GEBs can be classified in 6 different classes as shown in **Table 2.1**.

Table 2.1: List of 6 different classes of GEBs

1.	FRET-based biosensors which undergo a conformational change
2.	FRET-based biosensors for enzymatic activity
3.	FRET-based biosensors for enzymatic cleavage
4.	Translocation based probes
5.	Single FP based intensimetric probes
6.	Single FP based ratiometric probes

FRET-based biosensors are composed of two fluorophores with an at least 30% overlapping excitation-emission spectrum (Zadran et al., 2012). One of these proteins referred to as the FRET donor, which is excited in the first phase, and the second protein referred to as the FRET acceptor, which emits light from the energy transferred from the donor. To generate FRET, the distance between the two FPs must be between 2-8 nm (Niekamp et al., 2019). A typical FRET-based biosensor is made of CFP/YFP, or BFP/GFP, or GFP/OFP FRET pair. Between the two fluorophores, the sensory domain is sandwiched, which undergoes conformational rearrangement upon activation and bring two FPs close, optimally in a concentration-dependent and reversible manner. (**Figure 2.5, A**). (Bajar, Wang, Zhang, Lin, & Chu, 2016) FRET-based biosensors for enzymatic activity are composed of a single protein structure containing sensory protein with double FP. When the substrate binds to the sensory protein, it undergoes a conformational change, thus bringing two FPs close for FRET (**Figure 2.5, B**) (Vogel, Van der Meer, & Blank, 2014). FRET-based biosensors for enzymatic cleavage sensors are composed of a sensory protein fused with double FP. Upon treatment with an enzyme, the sensory protein degrades and reduces the FRET signal (**Figure 2.5, C**) (Faccio & Salentinig, 2017).

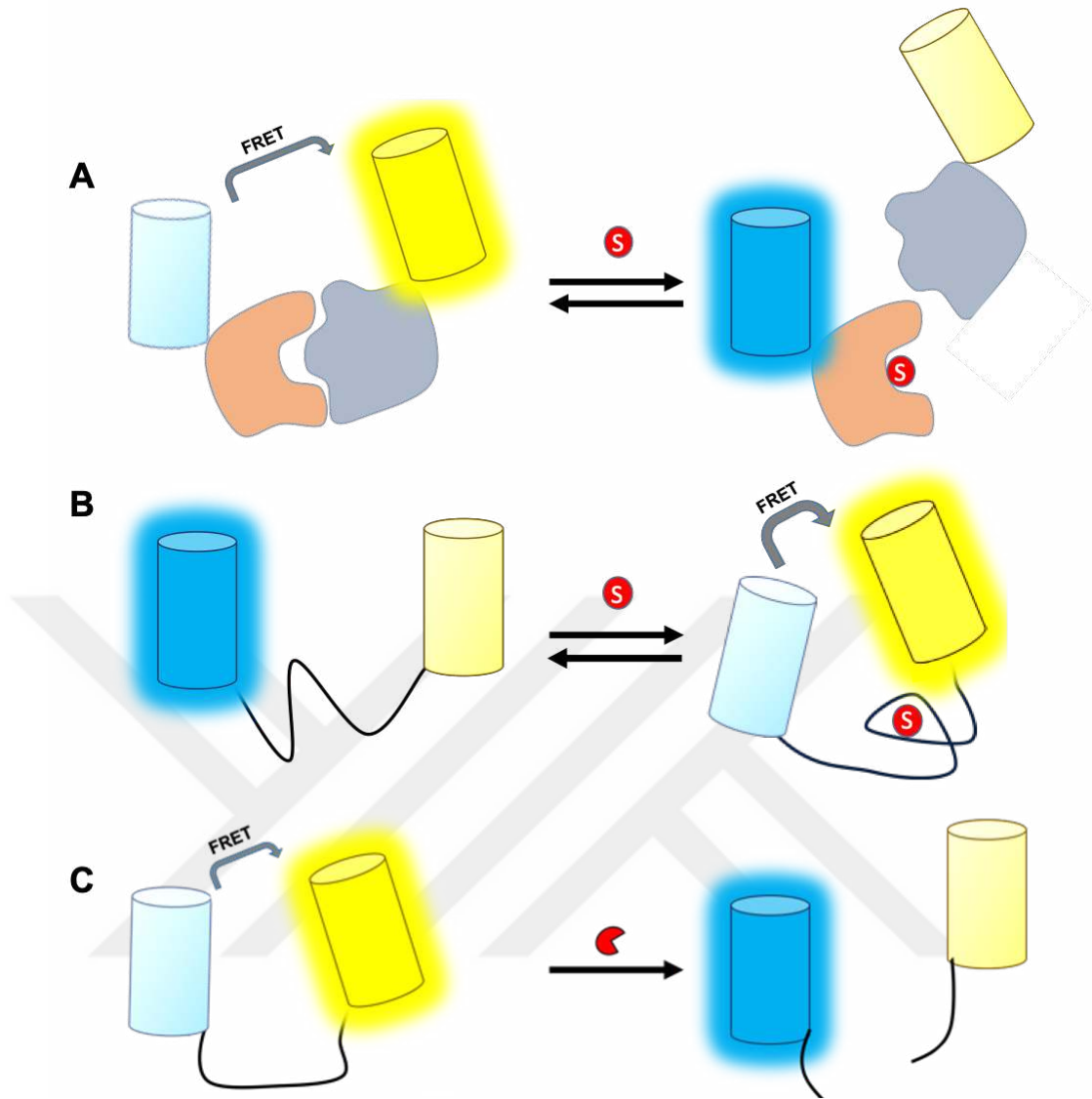


Figure 2.5: Schematic representation of FRET-based sensors: (A) Substrate binding to the sensory domain between the two FPs leads to a conformational change, separating two FPs, thus reducing the FRET signal. (B) FRET increases upon activation with the substrate and (C) FRET is reduced if the sensor is enzymatically cleaved.

Single FP-based sensors are fused to one, two, or more sensory domains (Tainaka et al., 2010). This principle can be exploited to visualize translocation events by generating FP containing chimera targeted to a specific locale (Snapp, 2005). Single FP based biosensors have also been designed that permit intensimetric and ratiometric readouts in real-time (Newman & Zhang, 2014). Location-specific biosensors are simply FP tagged versions of the target protein (Figure 2.6) (Keppler, Pick, Arrivoli, Vogel, & Johnsson, 2004).

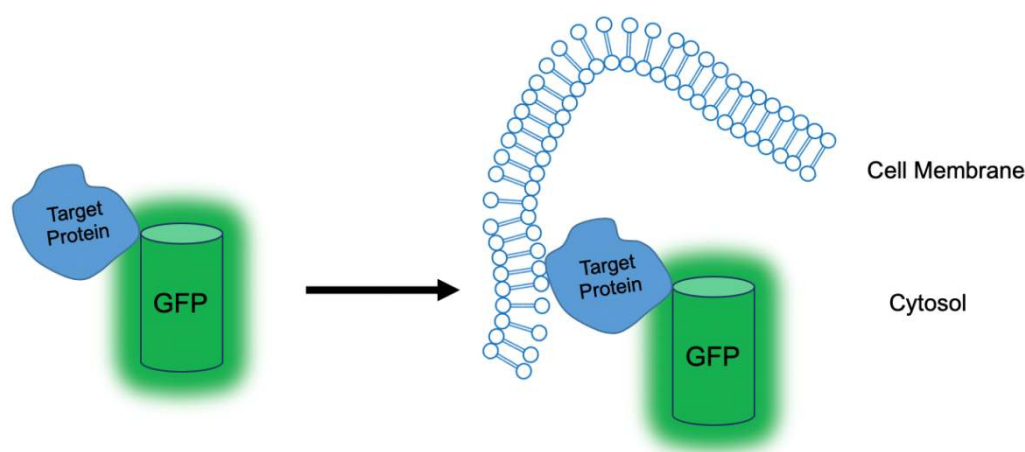


Figure 2.6: Schematic representation of translocation based single FP probes. GFP fused with the target protein leads GFP into a desired location and provides location-specific information.

Intensiometric biosensors can be further divided into two groups. This kind of probes provide an emission in only one single wavelength, that can be either lead to gain of intensity or lead to loss of intensity upon activation by the analyte. The great advantage of this principle is that several spectrally distinct biosensors can be used for multichromatic imaging. However, the disadvantage compared to double FP based FRET biosensors is that a single wavelength readout bears the danger of artificial effects, such as cell movement, cell contraction, local pH fluctuations, and other disruptions caused by optically interfering parameters. (**Figure 2.7, A**). To overcome these limitations, new approaches such as circularly permuted FPs (cpFPs) were developed (Baird, Zacharias, & Tsien, 1999). cpFPs are rearranged versions of normal FPs by changing the locations of C terminus and N terminus and connected with the desired sensory protein (**Figure 2.7, B**) (Kostyuk, Demidovich, Kotova, Belousov, & Bilan, 2019). This approach enables the sensory protein to act as a switch, thus increasing the intensity of emitted light from the FP. (**Figure 2.7, B**) (Tebo et al., 2018). A significant drawback of intensiometric cpFP-based biosensors is that they only provide information by the change in the intensity of the biosensor. Therefore, it becomes difficult to determine if the signals changed due to analyte concentration, photobleaching, or biosensor concentration in a single cell. Ratiometric cpFP-based biosensors, however, have two different excitation or emission maxima and provides opposing fluorescent signals upon activation with an analyte. Hanson et al. generated

dual-emission GFP (deGFPs) variants by creating mutations in specific chromophore locations. S65T provides the dual emission property to the biosensor, due to changing the proton locations. deGFPs contain two absorption bands as wild type GFP. However, the natural and excited FP intensity ratio was highly dependent on the microenvironmental factors such as pH. at lower pH levels, the biosensor has a maximum emission at 400nm, creating a blue signal and at higher pH levels, creating a green signal with maxima at 500nm (Hanson et al., 2002). Ratiometric cpFP signals thus offer a more precise, unbiased results and eliminate the distortions of unnecessary data caused by the single-cell movement, photobleaching, and nonuniform concentration of the biosensor in each cell.

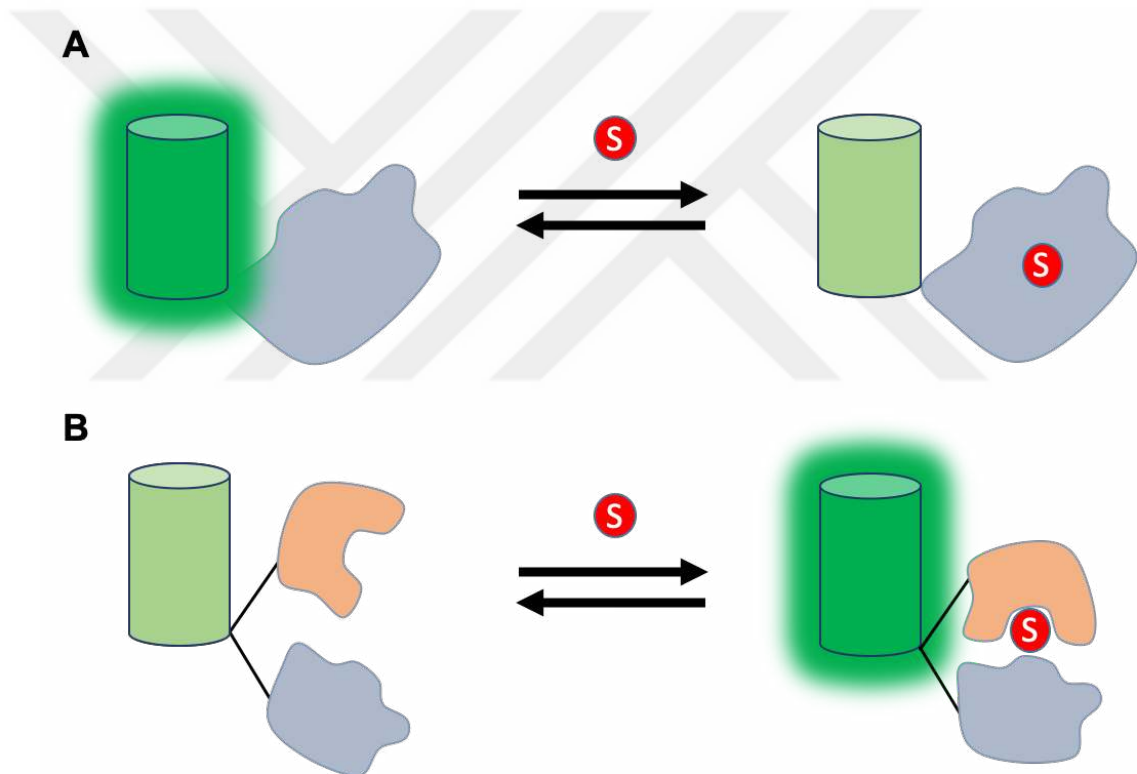


Figure 2.7: Schematic representation of Intensiometric and ratiometric single FP biosensors. A) Single FP based intensiometric sensors decrease the intensity of the FP upon substrate binding to the sensory domain of the sensor **B)** cpFPs contain a sensory protein which increases the intensity of the FP upon substrate binding.

2.3.1 Genetically Encoded Calcium Indicators

The field of Ca^{2+} imaging has emerged with Roger Tsien and his colleagues' technological advances in the field of genetically encoded biosensors. Other than the first natural Ca^{2+} sensor, aequorin, Tsien's team has successfully generated chemical biosensor that is a selective fluorescent indicator for Ca^{2+} termed as Fura (2-[6-[Bis[2-

[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-2-benzofuranyl]-5-oxazolecarboxylic acid (acetyloxy)methyl ester). Notably, Fura and its derivatives are still one of the most commonly used Ca^{2+} indicators utilized in life-sciences (Grynkiewicz, Poenie, & Tsien, 1985). Despite many advantages such as providing ratiometric read-outs, high selectivity and sensitivity, Fura and similar chemical indicators are often less suitable for subcellular imaging because of their non-targetability. In 1999, Tsien and his team designed, developed, and generated a novel class of genetically encoded Ca^{2+} sensors which they termed Cameleons. Cameleons are the first genetically encoded FRET-based biosensors composed of either a CFP or blue fluorescent protein (BFP) as a FRET donor fused to a CaM with CaM-binding domain of myosin light chain kinase (M13) proteins as sensory protein and a yellow fluorescent protein (YFP) as a FRET acceptor. In the presence of Ca^{2+} , CaM and M13 undergo a conformational change, leading to the donor FP to come close to acceptor YFP, generating FRET (Miyawaki et al., 1997). Later in 1999, the team had studied the circularly permuted versions of GFP (cpGFP) and found that adding the CaM protein between the cpGFP, the sensor was able to generate a fluorescent signal in the presence of Ca^{2+} (Baird et al., 1999). The developed sensors had inadequate signal intensity, which made it harder to measure Ca^{2+} . To eliminate this low single-to-noise ratio, Nakai et al. generated a new single FP biosensor with cp enhanced GFP (cpEGFP) and CaM, referred to as GCaMP (Nakai, Ohkura, & Imoto, 2001). Lastly, in 2011, to accelerate the development of GCaMPs, Zhao et al. used directed protein evolution techniques with different screening technologies to identify new GCaMP-type indicators produced by *Escherichia coli* colonies. The team has produced a variety of different colored GCaMPs and named as Genetically Encoded Ca^{2+} indicators for optimal imaging (GECO) with Blue (B-GECO) and Red (R-GECO) variants (**Figure 2.8**). These biosensors turned out to be more selective to Ca^{2+} , and have provided an expanded pallet of different colored Ca^{2+} sensors that permitted scientists to image Ca^{2+} in different spectral properties for multispectral imaging (Zhao et al., 2011)

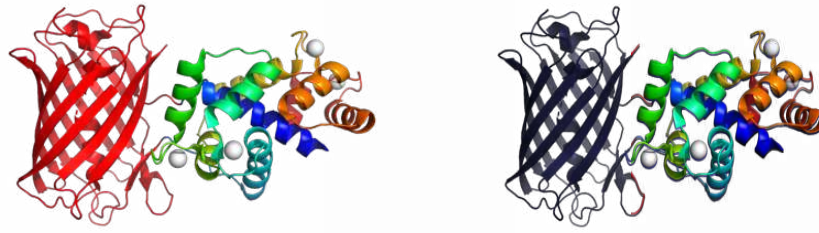


Figure 2.8: 3D representation of R-GECO and B-GECO. GECO sensors are composed of mutated versions of cpEGFP, linked with CaM and CaM binding domain of myosin light chain kinase (M13). The left panel shows the 3D structure of the Red variant of GECO and the right panel shows the 3D image of the Blue variant of GECO. White spheres indicate the Ca^{2+} ions.

2.3.2 *Genetically Encoded Nitric Oxide Biosensors*

Pearce and his colleagues generated the first-ever genetically encoded NO biosensor with CFP and YFP tagged human type IIa metallothionein (hMTIIa) (Pearce et al., 2000). Metallothioneins are a cysteine-rich metal-binding protein (Rigby Duncan & Stillman, 2006), which is suggested to be highly crucial for zinc (Zn^{2+}) homeostasis, as it can interact with seven Zn^{2+} molecules (St Croix et al., 2002). NO, however, has been shown to interact with MTs and trigger the release of bound metal ions (Khatai, Goessler, Lorencova, & Zangger, 2004). Due to these features of MTs, the authors generated a novel FRET-based biosensor containing hMTIIa, which they termed FRET-MT. According to their studies, the addition of NO led to a decrease of the FRET signal, by increasing the distance between two FPs (Pearce et al., 2000). Later, NO was successfully detected with FRET-MT in cultured sheep pulmonary endothelial cells (St Croix et al., 2002)

Chronologically, the second-ever genetically encoded NO biosensor was developed by Sato et al. termed as A Fluorescence indicator for NO with a signal amplifier (NOA-1) (Moritoshi Sato, Hida, & Umezawa, 2005). Since sGC is the primary receptor for NO (Montfort, Wales, & Weichsel, 2017), the team had generated NOA-1 with both, the alpha and beta subdomain of sGC and fused it with a known FRET-based cGMP biosensor (CGY) (M. Sato, Hida, Ozawa, & Umezawa, 2000). Upon NO binding to the heme group in the beta subunit of sGC activates the enzyme, thus increases the production of cGMP from GTP. Because the sensor is fused to CGY, which is a FRET-biosensor, the produced cGMP can be simultaneously detected upon formation. This approach allowed to indirectly measure NO concentrations in single cells by exploiting the reaction cascade caused by NO. The authors claim that NOA-1

can detect NO levels in a low nanomolar range down to 0.1nM in a reversible manner. However, NOA-1 sensors are two large fusion proteins that must be co-expressed in each cell. To generate a correct signal, equal amounts of each protein must be present (Moritoshi Sato et al., 2005). To overcome this situation, Sato et al. have generated the cell-based sensor named Piccel (Moritoshi Sato, Nakajima, Goto, & Umezawa, 2006). Piccels are generated by pig-kidney delivered cell line (PK15) because these cells endogenously express sGC (Ye, Rubakhin, & Sweedler, 2008), therefore to create a cell-based indicator, the team only transfected cells with CGY. The authors claim that Piccels were capable to detect NO in a picomolar level. Furthermore, the team has also tried to detect NO produced in vascular endothelial cells. For that, the Piccel sheet was prepared on a coverslip and transferred into the endothelial cells, which were grown in a glass dish. Upon stimulation with ATP, NO produced from the endothelial cells diffused into the Piccel sheet, generating a signal. However, the team found that if the distance between the Piccel sheet and endothelial cell was greater than 60 μ m, the response obtained from the Piccel sheet has disappeared (Moritoshi Sato et al., 2006). Both NOA-1 and Piccel turned to be capable to measure NO indirectly.

In 2013, a group at the University of California came up with an idea of generating a single FP-based biosensor, which is selectively sensitive to the unstable peroxynitrite (ONOO⁻) (Z. J. Chen, Ren, Wright, & Ai, 2013). ONOO⁻ is generated by superoxide anions and NO, and it is considered as an essential molecule in redox biology functioning in different physiological and pathological conditions including inflammation, neurogenerative disorders, and ischemia (Khan, Alam, Zafaryab, & Rizvi, 2017; Pacher, Beckman, & Liaudet, 2007). It has been known that boronic acid organic dyes selectively reacts with ONOO⁻, generating phenolic constructs with high fluorescent property (Sikora, Zielonka, Lopez, Joseph, & Kalyanaraman, 2009). By using this approach, the team has location specifically introduced a boronic acid moiety to cpGFP, called pnGFP. In the presence of ONOO⁻, it generates phenolic products that yield a strong fluorescent signal allowing to image ONOO⁻. However, one of the major drawbacks of this sensor is the irreversible response to ONOO⁻. Since ONOO⁻ is produced by the reaction of NO with superoxide anions (Beckman & Koppenol, 1996), the biosensor has been exploited to visualize NO indirectly.

In 2016, a new genetically encoded FRET-based biosensor, named sNOOpy (Sensor for NO_3^- / NO_2^- in physiology), allowed the visualization of nitrate and nitrite in single living cells. sNOOpy is composed of two microorganismal nitrate assimilation system genes, NasS and NasT, fused with Venus and CFP, respectively (Hidaka et al., 2016). These two subunits are known to function in NO_3^- absorption and digestion in the root nodule bacterium *Bradyrhizobium japonicum* (Luque-Almagro et al., 2017). It was found that these two genes create a stable structure in the absence of the NO_3^- , giving the possibility of creating a biosensor (Sánchez et al., 2014). In the absence of the NO_3^- , NasS-Venus and CFP-NasT form a stable complex generating FRET. Upon exposure to NO_3^- or NO_2^- FRET decreases due to the NO_3^- / NO_2^- binding to NasS-Venus. With this generated biosensor, the authors showed in HeLa cells NO_3^- and NO_2^- uptake mechanisms with exogenously applied NO_3^- / NO_2^- and proved that the sensor can also be used for real-time cell imaging (Hidaka et al., 2016). Notably, NO_3^- and NO_2^- are stable byproducts of NO and can be used as a surrogate for real-time NO imaging.

In the same year, a new class of single FP-based genetically encoded biosensors were developed that permit direct detection of NO with high spatial and temporal resolution. The authors termed these tools as geNOps, which stands for genetically encoded nitric oxide probe. To generate geNOps, the team has first studied the NO binding proteins of different organisms, to find the suitable NO binding proteins and also can change the fluorescence intensity. In geNOps, the cyclic guanylyl cyclase-activating factor (GAF) domain of an E. coli, which is an anaerobic NO reductase transcription regulator (NorR) gene was fused with a FP (**Figure 2.9**). Generating differently colored FPs to the GAF domain yielded a colorful palette of new biosensors that permit multichromatic imaging in a given cell. Because geNOps are single FP based, the spectral read-out of the probes is intensimetric. As discussed before, intensimetric probes bear the risk to yield false-negative results due to the optical interference of local pH changes.

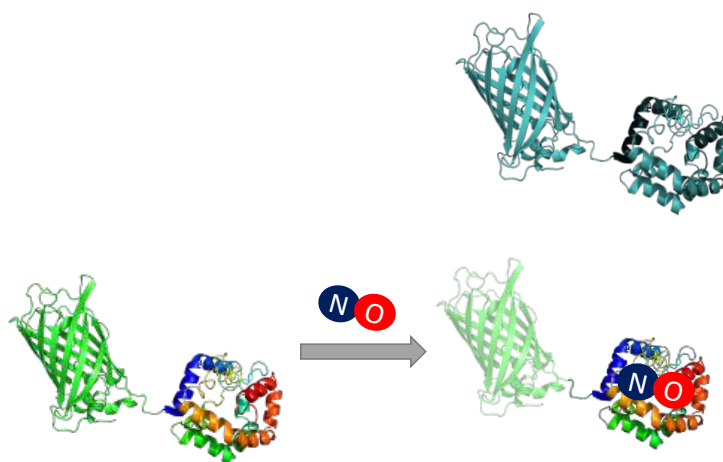


Figure 2.9: 3D protein structure of Intensimetric geNOps. The upper panel shows the enhanced Green fluorescent and Cyan variants of geNOps. Lower panel Indicates the working mechanism of geNOps. When NO gets in contact with the GAF domain, it decreases the fluorescent intensity, thus providing a tool for NO imaging. All images were generated with I-TASSER, a server for protein structure and function predictions (Roy, Kucukural, & Zhang, 2010; J. Yang et al., 2015; Zhang, 2008).

Ratiometric geNOps, however, contains two FP's, GAF domain, plus a linker sequence (**Figure 2.10**). The linker sequence provides one FP to be in a distant location, preventing the quenching of the FP when NO binds to the GAF domain. At the resting state, the ratiometric geNOps provides an equal response from the two FPs. Upon activation with NO, the proximity FP quenches, but not the distant one, thus provide a ratiometric signal (Eroglu et al., 2016).

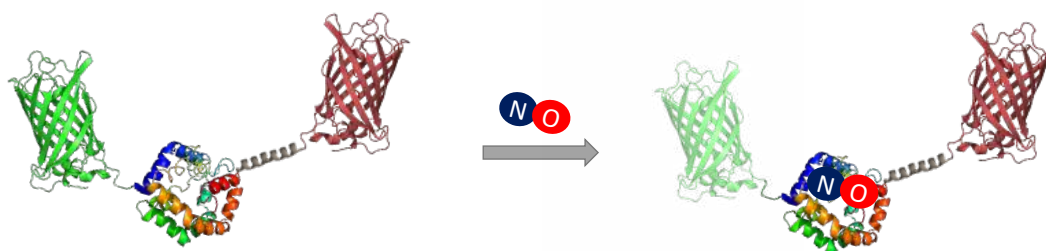


Figure 2.10: 3D protein structure of ratiometric geNOps. Ratiometric geNOps are composed of 2 FP's, GAF domain, and a linker. Upon activation with NO, there is a decrease in the closer, rather the other one still emits fluorescent as before, thus providing a ratiometric result.

MATERIALS and METHODS

3.1 Molecular Cloning

Each construct used in these studies including the NLS-eNOS-RFP (**Figure 3.1, Right panel**), WT-eNOS-RFP (**Figure 3.1, Left panel**), G-geNOP, O-geNOP-NES, R-GECO-NLS (**Figure 3.2, Right Panel**) and R-GECO-UT (**Figure 3.2, Left Panel**) were received on a filter paper. All constructs were cut around the indicated border on the filter paper, placed in different 1,5 ml Eppendorf tubes, and incubated in 30 μ l of DEPC water to dissolve the plasmids from the filter paper. Tubes were then centrifuged at 13,000 rpm for 1 minute and subsequently preserved at 4 °C. At least 16 hours later, plasmids were transformed into competent *E. Coli* (NEB Order C2987I) using a heat shock procedure according to the manufacturer's protocol. 100 ng of plasmid DNA were used from each construct and 50 μ l of competent bacteria was placed on ice for 30 minutes. The transformation was done by heat shock at 42°C for 30 seconds. Cells were then placed on ice for an additional 5 minutes. 900 μ l SOC medium was added and incubated at 37°C in an orbital shaker for 1 hour. Cells were then plated onto a selection plate of LB agar supplemented with 100 μ g/mL ampicillin and incubated at 37°C overnight. Individual colonies were inoculated with LB broth supplemented with ampicillin. For the constructs which were received from Addgene in form of bacterial agar stabs, single bacterial colonies were obtained using an inoculation loop and overnight incubation on a selection plate. Single bacterial colonies were then picked and inoculated for 16 hours at 37°C in an orbital shaker. After incubation, bacteria were centrifuged at 4700 rpm for 15 minutes at 4°C. The supernatant was removed, and plasmids were isolated according to Qiagen plus MIDI prep kit (12943; Qiagen). Isolated plasmid DNA was eluted with 30 μ L nuclease-free water and purity was confirmed spectroscopically by determining the ratio at 260nm and 280nm. Samples were considered as pure DNA when the ratio $A_{230:260:280}$ yielded a value of 1.85. Correct size insertion was determined by enzymatic digestion using the enzymes, HindIII, NotI, XbaI, and BamHI, followed by gel electrophoresis on a 1.2% agarose gel. G-geNOPs that were digested with XbaI and NotI expected to have an insert size of 1250 bp. WT-eNOS-RFP digested with HindIII and BamHI yielded an insert size of 4448 bp.



Figure 3.1: Vector maps of red tagged eNOS variants: The right panel shows the map of nuclear targeted version of red tagged eNOS and the left panel shows the wild type version of red tagged eNOS (Iwakiri et al., 2006).

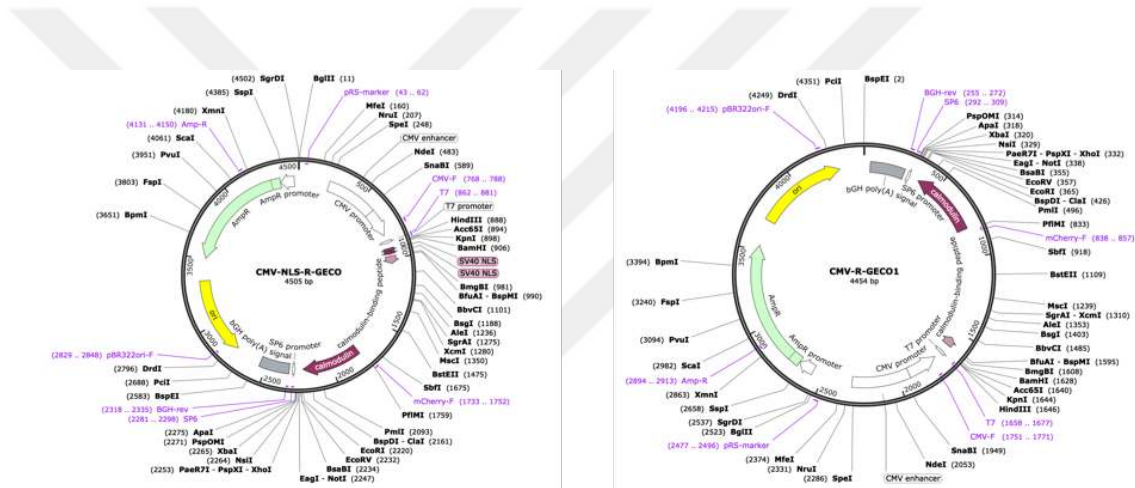


Figure 3.2: Vector maps of R-GECOs: The right panel shows the map of nuclear targeted version of R-GECO and the left panel shows the cytosolic version of R-GECO (Zhao et al., 2011).

3.2 Cell Culture

Preserved HEK293 cells (ATCC® CRL-1573™) at the vapor phase of liquid nitrogen were placed in a water bath until completely dissolved. To dilute the DMSO, 9 ml of fresh DMEM, supplemented with 10% of Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (complete medium) was added to HEK293 cell in freezing medium (complete medium supplemented with 10% DMSO). Cells were then seeded on a 10 mm cell culture dish and placed in a humidified growth chamber supplemented with 5% CO₂ at 37°C. Confluent cells were passaged to maintain their progressive growth. Cells were washed with phosphate buffer saline (PBS), and then treated with 1x Trypsin and incubated at 37°C for 3 to 5 minutes. Trypsin was deactivated by

adding 5 ml of complete medium. Cells were counted with a hemocytometer and the approximate cell number was found with the formula; the number of cells counted from the 4 corners / $4 \times 10,000 \times$ dilution factor. 500,000 cells were seeded in a new 10 mm cell culture dish. The old medium was replaced by a fresh full medium every second day. Before transfection, HEK293 cells were maintained in complete medium at 5% CO₂ and 37°C until 60% confluency was reached. For imaging experiments, 30,000 HEK293 cells were seeded onto a 6-well plate one day before the transfection on a 30 mm diameter and 0,1 thick cover glass (Asistent, Germany). 1-hour before transfection, the old complete medium was replaced with a fresh complete medium. PolyJet™ *in vitro* DNA transfection reagent (SL100688; SignaGen) was used for the transfection of HEK293 cells with the purified plasmids. For each well, 1 µg of plasmid DNA needed with 3 µl PolyJet reagent in a 100 µl serum, antibiotic, and phenol-free high-glucose (4.5 mM) medium. To mix the plasmid DNA with the Polyjet reagent mix for efficient transfection, two Eppendorf tubes were prepared. One Eppendorf tube contained 3 µg of plasmid DNA with 150 µl serum-, antibiotic-, and phenol-free high-glucose (4.5 mM) medium, and other tube contained 3 µl Polyjet reagent with the same medium. Immediately after adding the Polyjet to the second tube, all mix was pipetted in the second tube and directly added onto the plasmid DNA containing tube. The transfection mix was incubated at room temperature for 15 minutes. After 15 minutes of incubation, the transfection mix was dropwise added to the cells in complete medium and incubated in a humidified growth chamber supplemented with 5% CO₂ at 37°C for 3 hours. After incubation, the medium is aspirated, and fresh complete medium was added. Cells were placed in a humidified growth chamber supplemented with 5% CO₂ at 37°C until imaging experiments typically 24 to 48 hours.

3.3 Buffer Solutions

All chemicals for preparing the buffers were obtained from NeoFroxx GmbH-Labochem International, Germany. A HEPES-based loading buffer was prepared to preserve cells outside of the humidified incubator without changing their physiological conditions. To prepare a HEPES based loading buffer, 10 mM HEPES, 2 mM CaCl₂, 138 mM NaCl, 1 mM MgCl₂, 5 mM KCl, 2.6 mM NaHCO₃, 0.44 mM KH₂PO₄, 0.34

mM Na₂HPO₄, 10 mM Glucose, 1% penicillin/streptomycin, 0.1% of amino acids, 0.1% vitamins in ddH₂O was used and pH was adjusted to 7.42 by adding NaOH.

During the experiments, cells were perfused either containing or lacking Ca²⁺ buffers. Ca²⁺ containing buffer was composed of 10 mM HEPES, 2 mM CaCl₂, 138 mM NaCl, 1 mM MgCl₂, 10 mM D-Glucose, 5 mM KCl, pH adjusted to 7.42 with NaOH. Ca²⁺ lacking buffer is composed of 10 mM HEPES, 10 mM EGTA, 138 mM NaCl, 1 mM MgCl₂, 10 mM D-Glucose, 5 mM KCl, pH adjusted to 7.42 with NaOH.

Additionally, geNOps are iron(II) ion-dependent due to their non-heme iron domain that functions as the active site for NO binding. For this purpose, we added 1 mM iron (II) fumarate and 1 mM ascorbic acid to a Ca²⁺ containing buffer and vigorously stirred on a magnetic stirrer in dark for at exactly 2 hours until the iron (II) fumarate and ascorbic acid completely dissolved and the color changed to yellowish. To retard the unsolved iron(II) fumarate, the buffer was filtered with a 0.22 µm syringe filter and refrigerated in a dark place for up to one week. Before imaging experiments, geNOps transfected cells were first incubated in a prewarmed iron(II) buffer for exactly 20 minutes, following two wash steps with a HEPES-based loading buffer. Cells were further incubated for two hours in the same buffer until the imaging experiment to allow the cells to equilibrate to the ambient temperature and conditions. In all of the experiments, cells were stimulated with either 100 µM ATP (Sigma-Aldrich, A6419-5G) or 10 µM 3-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine (NOC-7) (Santa Cruz Biotechnology, 146724-84-7) prepared in the either containing or lacking Ca²⁺ buffers according to the experiment.

3.4 Fluorescence Imaging and Image Analysis

Transfected HEK293 cells were placed into a perfusion chamber, which was connected to a homemade and gravity-based superfusion system. The chamber was connected from one end to the perfusion system to provide different buffers containing various agonists, small compounds, and inhibitors. The other end of the perfusion chamber was connected to a peristaltic pump to remove the perfusion buffer (**Figure, 3.3**).

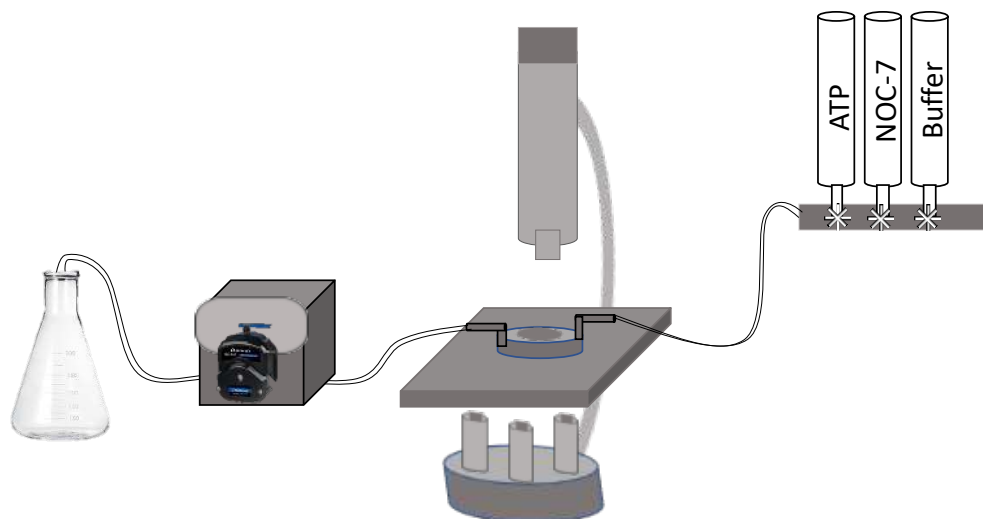


Figure 3.11: Schematic representation of gravity-based perfusion system: the perfusion system was composed of gravity-based perfusion system connected to perfusion chamber located on the microscope. The other end of the perfusion chamber connected to the waste with the peristaltic pump for the removal of the buffer. ATP: Adenosine triphosphate.

Imaging experiments were performed on a Zeiss Axiovert A1 inverted microscope with Axiovert charged-coupled device (CCD) camera, equipped with a Colibri 2 LED light source. For the green G-geNOps imaging experiments, the filter set 38 HE, containing a dichroic mirror with 495 LP, excitation filter (470/40 BP), and emission filter (525/50 BP) was used. G-geNOps were excited at 477 nm wavelength. For calcium imaging experiments using the red FP based R-GECOs the filter set 43 with a dichroic 570 LP, excitation filter (545/25 BP), and emission filter (605/70 BP) was used. R-GECO's were excited with 575 nm wavelength. In all experiments, the following set up was used; 10% intensity used for the excitation light, camera binning was set to 4x4 to reduce phototoxicity. Time-laps were recorded every 3 seconds. For high-resolution images, camera binning was set to 1x1. Exposure was set between 50 ms to 800 ms, this was dependent on the expression efficiency of the respective biosensors. After live-cell imaging experiments, each cell was selected as a region of interest (ROI) with Zeiss's Zen Pro program (Carl Zeiss, Germany) with an additional ROI covering a cell-free region to record the background signal. Collected data were exported to an Excel sheet for data processing. Background values were subtracted from the signals obtained from each cell. Baseline values were selected to obtain a one-phase decay plot. The model of one-phase-decay expressed as $Y = (Y_0 - \text{Plateau}) * \exp(-K * X) + \text{Plateau}$. Y_0 value referred to as the value at the beginning of the experiment. X is the independent variable (time), the plateau is the Y value in infinite

time point and K is the rate constant, which determines the sharpness of the curve. After obtaining the one-phase decay curve according to the baseline values, it was used as the baseline for every time point in the experiment. Data values obtained from the experiment were analyzed with $1-Y/Y_0$ formula to normalize the NO signals.

3.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). Live-cell imaging results were analyzed using open-access image analysis software, Fiji, and Zeiss's Zen Pro program. All experiments were performed in 3 technical replicates. Maximum NO signals from each individual cell were found for Student's t-test. For all analyses, $p < 0.05$ was considered as significant.

RESULTS

4.1 Characterization of G-geNOps and O-geNOps in Responses to Exogenously Applied NO in Single Human Embryonic Kidney Cells

geNOps are genetically encoded NO probes for real-time imaging of NO for single cells and subcellular locales. Here we have characterized for the first time an exclusively cytosolic-targeted O-geNOp along with the well-established G-geNOp, which is a non-targeted variant. For the characterization and testing of the functionality of the new geNOps variant, we decided to use the human embryonic kidney cell line HEK293 (**Figure 4.1, left panel**). This cell-line is a well-characterized, robust, and easy-to-transfect mammalian cell-line, which turned to be excellent for the characterization of genetically encoded biosensors. Moreover, these cells lack an enzyme - such as NOS - that can synthesize NO, which is an important feature that allows studying NO signals of differentially targeted eNOS as shown later. To test the functionality of the O-geNOp-NES we compared the new biosensor with the well-established G-geNOps. Consistent with former studies (Eroglu et al., 2016), G-geNOps showed robust fluorescence in HEK293 cells 24 hours after transfection (**Figure 4.1, middle panel**). As expected, the exclusively cytosolic-targeted O-geNOp-NES showed correct localization to the cytosol and a similar fluorescence intensity compared to G-geNOp (**Figure 4.1, right panel**).

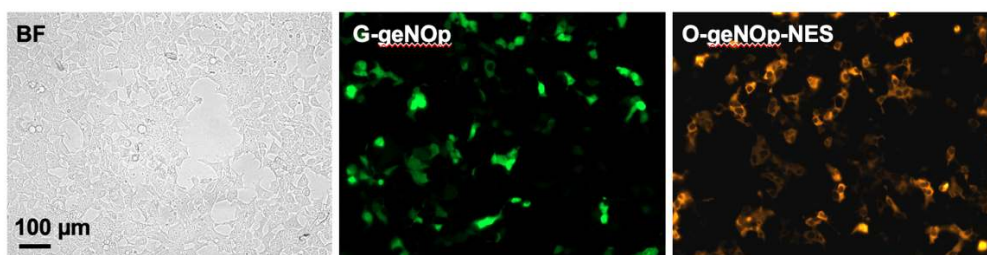


Figure 12.1: Transfection efficiency of cytosolic targeted and untargeted geNOps. Representative wide-field images of HEK293 cells in the bright field mode (left panel) or expressing the green variant of geNOps (G-geNOps, middle panel), or expressing the cytosolic targeted orange geNOps variant (O-geNOps-NES, right panel). Expression profiles were determined on different days with different passages of HEK293 cells expressing the respective geNOp variants.

Next, we tested the fluorescence signal changes of G-geNOp and O-geNOp-NES in response to an exogenously applied NO-donor, termed NOC-7. For this purpose, we used an in-house-made and gravity-based superfusion system to apply and withdraw the compounds using a perfusion chamber connected to a vacuum pump. As

shown in **Figure 4.2**, the administration of NOC-7 evoked an instant and strong geNOps response within the first minute. Withdrawing the compound led to a total recovery of the signal back to the baseline. As expected, cytosolic targeting of O-geNOps did not affect the NO responsiveness of the biosensor (**Figure 4.2**). Moreover, the O-geNOps biosensor showed the same NO profile as G-geNOps. However, to our surprise, the G-geNOps amplitude evoked by 10 μ M NOC-7 was not significantly different compared to the O-geNOps. This observation is in contrast to the initial results published in the first article describing geNOps (Eroglu et al., 2016). In the first paper published in 2016, the maximum response of G-geNOps was shown to be almost 100% stronger as the O-geNOps variant. Our data, however, clearly show no significant difference between the two variants, indicating that either targeting the biosensors to the cytosol may alter the performance of the sensor.

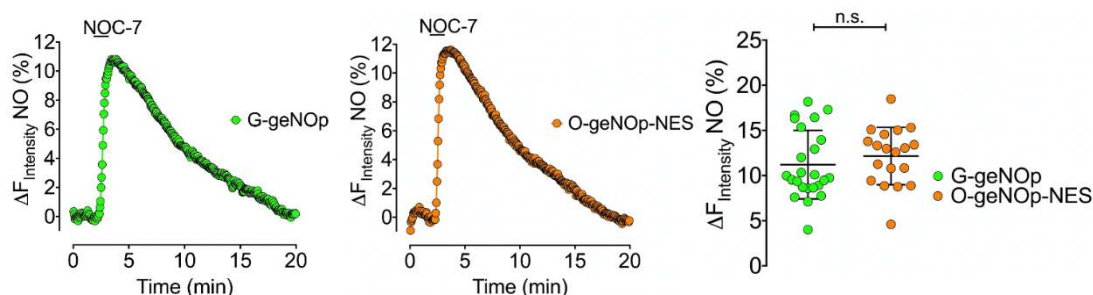


Figure 13: Representative time course of exogenously applied NOC-7 signals in HEK293 cells. The left panel shows NO signals of HEK293 cells expressing G-geNOp in response to 10 μ M NOC-7. The middle panel shows the same experimental condition, using the orange and cytosolic variant of geNOps. The right panel shows scatter dot-blots representing the statistical comparison between the maximum response to exogenously applied NOC-7 of G-geNOps (n=3/29) and O-geNOps (n=3/19). Student's t-test was performed, $p = 0,3861$.

Next, we attempted to image NO signals derived from differentially targeted endothelial nitric oxide synthase (eNOS). HEK293 cells are an excellent model system to study NOS derived NO profiles as shown in multiple studies before as these cells lack an enzyme that can generate NO. Thus, we co-transfected HEK293 cells with O-geNOps-NES and eNOS-GFP, which is a chimera of the wild-type eNOS fused to a green fluorescent protein (GFP). As shown in **Figure 4.3**, both constructs showed strong expression levels, indicating that cells tolerate the overexpression of both constructs. However, analysis of the excitation and emission profiles of the enhanced GFP (EFGP) and monomeric Kusabira Orange κ (mKO κ)- based FP showed significant overlap in the excitation and emission profile. This spectral overlap makes

the constructs eNOS-GFP and O-geNOp-NES less suitable for multispectral imaging because of the risk for spectral bleed through. A spectral crosstalk may lead to artificial or false-positive responses.

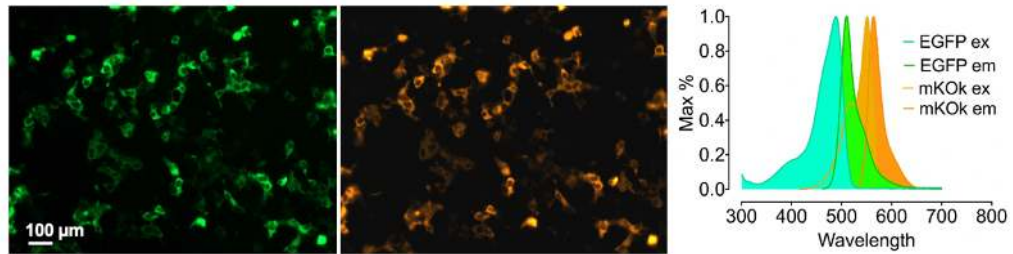


Figure 14: Co-imaging of eNOS-GFP and O-geNOp-NES. Representative widefield images showing HEK293 cells co-expressing eNOS-GFP (left panes) and O-geNOp-NES (middle panel). The right panel shows the excitation and emission spectra of EGFP with mKOκ fluorescent proteins. Cells were imaged with a 20x air objective.

To prevent spectral bleed-through, which corresponds to about 30% to each channel respectively for the GFP and OFP based construct (**Figure 4.3, right panel**), we next tested co-imaging of WT-eNOS-RFP and G-geNOp. As shown in **Figure 4.4**, both constructs showed a clear transfection efficiency. Besides, the spectral overlap between EGFP emission and mRFP1 was minimal due to the large distance between the excitation and emission maxima of the constructs (**Figure 4.4**). For the next multichromatic imaging studies we used henceforth, the RFP tagged eNOS and GFP based geNOp.

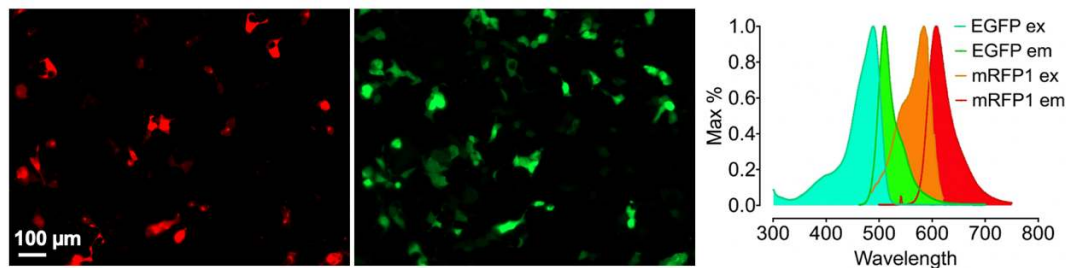


Figure 15: Co-imaging of eNOS and geNOp. Representative widefield images showing HEK293 cells co-expressing eNOS-RFP (left panel) and G-geNOp (middle panel). Excitation and emission spectra of used mRFP1 and EGFP. Cells were imaged with a 20x air objective.

4.2 Intracellular Calcium Imaging Using Genetically Encoded Calcium Indicators – The -GECOs

Under physiological conditions, intracellular Ca^{2+} modulates the endothelial and neuronal NOS (eNOS and nNOS). Thus, to study NO production derived from subcellular localized eNOS *in vitro*, we first characterized the intracellular Ca^{2+} profiles in HEK293 cells in response to pharmacological agonists. Among various agonists, we decided to use the well-characterized IP₃-generating agonist Adenosine triphosphate (ATP). For the visualization of intracellular Ca^{2+} , we exploited the RFP variants of the untargeted GECOs (R-GECO-UT). Notably, the R-GECOs are red-shifted intensimetric probes permitting co- imaging with the blue-shifted geNops (Eroglu et al., 2019). To characterize the intracellular Ca^{2+} profiles in HEK293 cells, we used two different imaging-buffers, containing or lacking Ca^{2+} . As shown in the micrographs in **Figure 4.5 A**, under both conditions, in the presence and absence of extracellular Ca^{2+} , the administration of ATP evoked a strong GECO-signal. Post-image-analysis unveiled that the initial Ca^{2+} increase was similar for both conditions, in the presence and absence of extracellular Ca^{2+} . However, after removal of the agonist from the imaging-media, the intracellular Ca^{2+} -signals in cells imaged in the absence of extracellular Ca^{2+} showed a significantly faster recovery of the GECO signal compared to the group imaged in the presence of extracellular Ca^{2+} (**Figure 4.5, B**). The faster recovery of the GECO signal may be caused by the lack of extracellular Ca^{2+} , which prevents the Ca^{2+} influx from extracellular space, leading to a faster recovery of GECO signal. Overall, no significant difference between the maximum responses in the presence or the absence of extracellular Ca^{2+} was observed (**Figure 4.5, C**). Notably, R-GECOs are intensimetric probes lacking the ability to quantitatively measure intracellular Ca^{2+} . Therefore, other than ratiometric biosensors, we can only relatively quantify and compare intracellular Ca^{2+} changes (**Figure 4.5, C**).

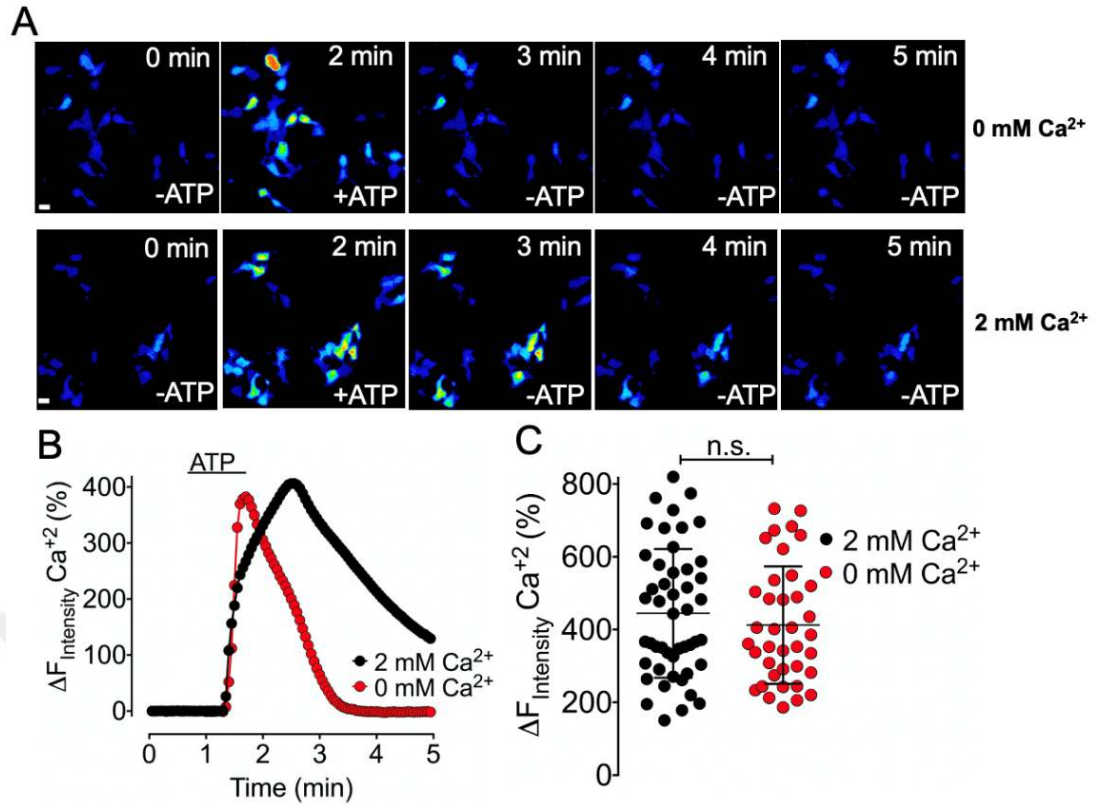


Figure 16: Calcium Imaging in HEK293 cells using R-GECOs. (A)

Representative pseudo-colored images of HEK293 cells expressing R-GECO-UT showing different time course in response to 100 μ M ATP. The upper row shows cells imaged in buffer containing 10 mM EGTA and 0 Ca²⁺. The lower row shows cells under the same experimental conditions using a physiological buffer containing 2 mM Ca²⁺. Scale bar represents 20 μ m. (B) Representative time course of intracellular Ca²⁺ signals in HEK293 cells expressing R-GECO-UT in response to 100 μ M ATP as indicated. Black curve shows Ca²⁺ signals in the presence of 2 mM Ca²⁺ and red curve shows Ca²⁺ signals in the absence of extracellular Ca²⁺ (C) Scatter dot blot represents the maximum response of HEK293 expressing R-GECO UT in response to 100 μ M ATP, (2mM Ca²⁺, n= 3/48; 0mM Ca²⁺, n=3/38) Student's t-test ($p = 0.3864$).

Taking advantage of the differential targetability of genetically encoded biosensors, we next tested the nucleus targeted R-GECO variant (R-GECO NLS), which the nuclear localization was previously confirmed by Zhao et al (Zhao et al., 2011). We have attempted to visualize Ca²⁺ profiles in the nucleus in response to Ca²⁺ mobilization. As expected, cell treatment with ATP induced a prominent GECO signal in the nucleus in the presence of extracellular Ca²⁺ (**Figure 4.6 A, lower row**). The R-GECO-NLS response in the absence of extracellular Ca²⁺, however, showed a weaker response to ATP (**Figure 4.6**). Upon withdrawal of the IP₃-generation agonist from the imaging media, lead to a full recovery of the GECO signal in the nucleus, while the cytosolic signal remained sustained. Our observations indicate that Ca²⁺

mobilization from the endoplasmic reticulum using IP3-generating agonists leads to fast diffusion of Ca^{2+} into the nucleus in HEK293 cells. This important phenomenon allows us to study the Ca^{2+} dependent eNOS functionality in the nucleus.

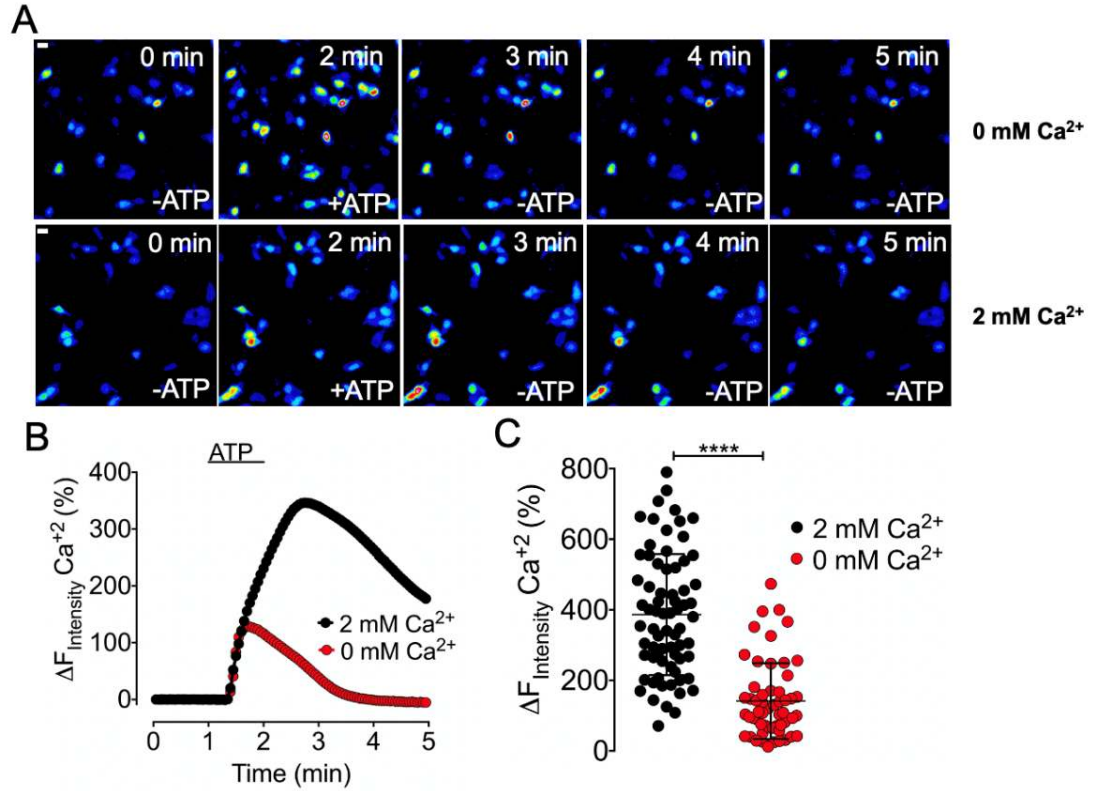


Figure 17: Comparison of nuclear Ca^{2+} signals. (A) Representative pseudo-colored images of HEK293 cells expressing R-GECO-NLS showing different time intervals. The upper row shows cells imaged in buffer containing 10 mM EGTA and 0 Ca^{2+} . The lower row shows cells under the same experimental conditions using a physiological buffer containing 2 mM Ca^{2+} . Scale bar represents 20 μm . (B) Representative time course of intracellular Ca^{2+} signals in HEK293 cells expressing R-GECO-NLS in response to 100 μM ATP. Black curve shows Ca^{2+} signals in the presence of 2 mM Ca^{2+} and red curve shows Ca^{2+} signals in the absence of extracellular Ca^{2+} (C) Scatter dot blot represents the maximum response of HEK293 expressing R-GECO-NLS in response to 100 μM ATP, (2mM Ca^{2+} , n= 3/71; 0mM Ca^{2+} , n=3/56), Student's t-test ($p < 0.0001$)

Next, we compared the Ca^{2+} signals between the cytosol and nucleus under the same experimental conditions. First, we analyzed the R-GECO UT and R-GECO-NLS signals in Ca^{2+} lacking or containing buffers individually. In the presence of extracellular Ca^{2+} , the response rate of both variants of R-GECO showed a similar profile with no significant difference (**Figure 4.7**). However, in the absence of extracellular Ca^{2+} cell treatment with ATP evoked a clear difference between the cytosol and nucleus (**Figure 4.8**). Our data indicate that intracellularly released Ca^{2+} quickly diffuses across the cytoplasm and nucleus when Ca^{2+} in the extracellular space is provided.

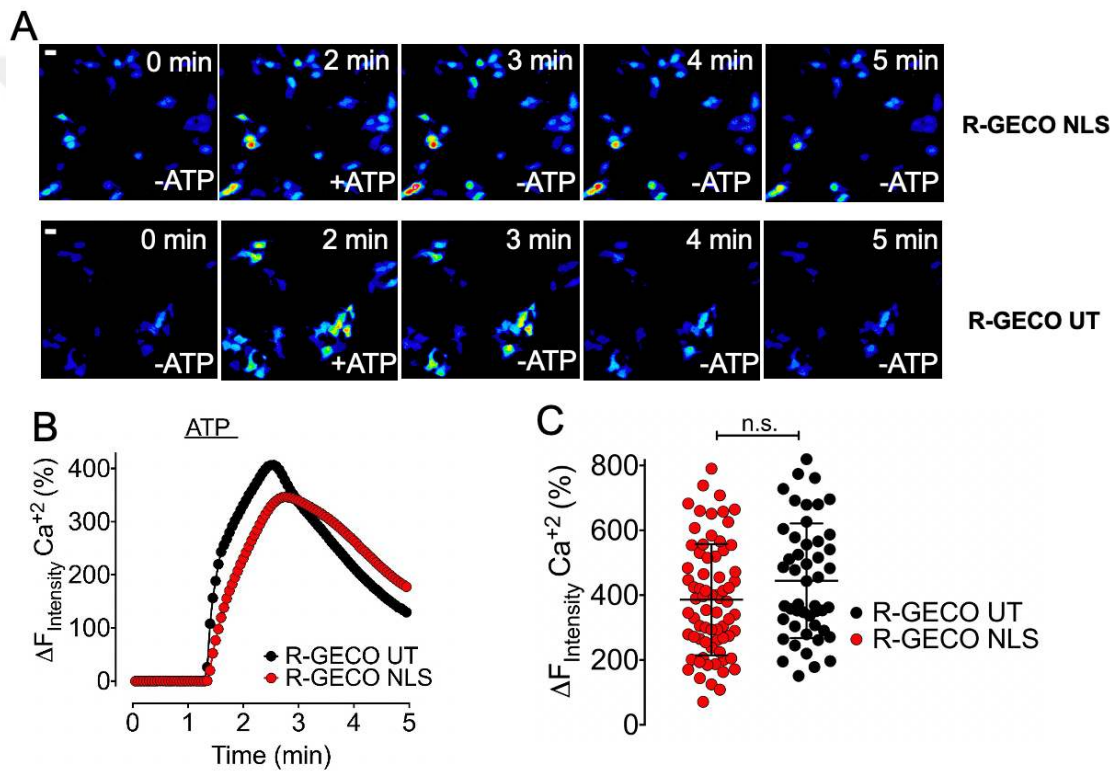


Figure 18: Calcium imaging in different subcellular locales with GECOs. (A) Representative pseudo-colored images of HEK293 cells expressing GECOs showing different time intervals in physiological buffer containing 2 mM Ca^{2+} . The upper row shows cells expressing R-GECO-NLS. The lower row shows cells expressing R-GECO UT. Scale bar represents 20 μm. (B) Representative time course of intracellular Ca^{2+} signals in HEK293 cells expressing GECOs in response to 100 μM ATP in physiological buffer containing 2 mM Ca^{2+} . The black curve shows Ca^{2+} signals obtained from R-GECO-UT and the red curve shows Ca^{2+} signals obtained from R-GECO-NLS. (C) Scatter dot blot represents the maximum response of HEK293 expressing GECOs in response to 100 μM ATP (R-GECO-NLS, n= 3/71; R-GECO UT, n=3/48) Student's t-test ($p = 0.0748$)

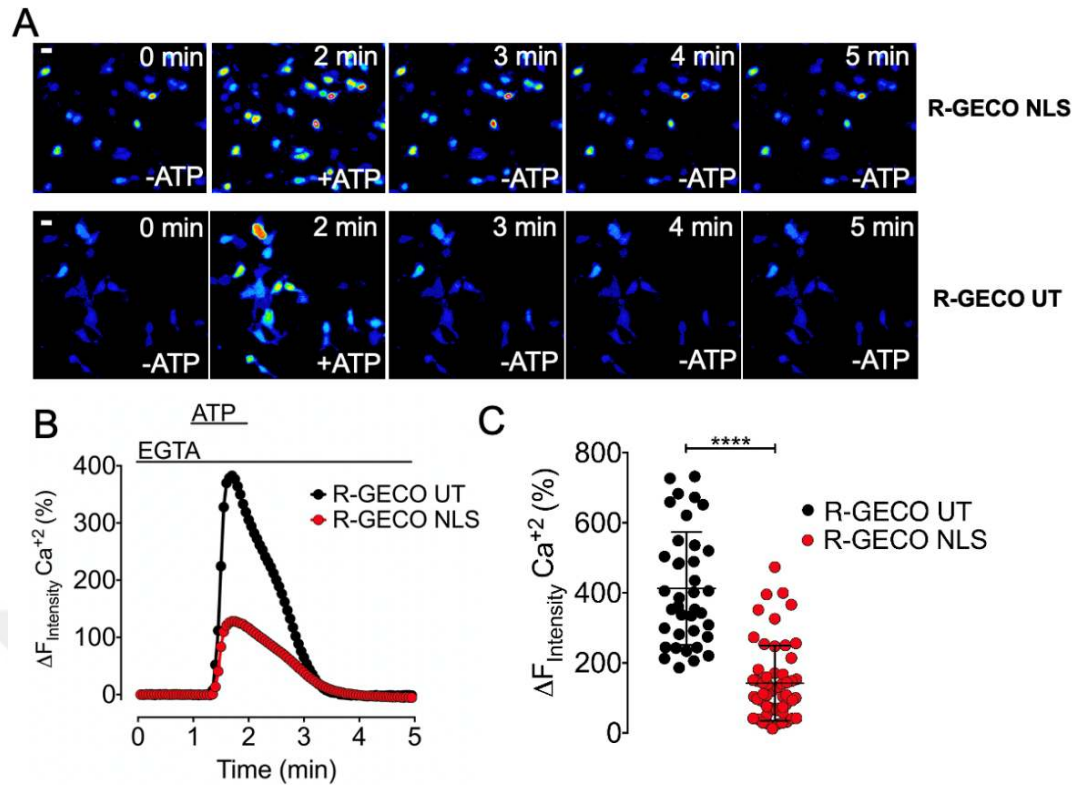


Figure 19: Calcium signals in the cytosol and nucleus upon intracellular Ca²⁺ mobilization. (A) Representative pseudo-colored images of HEK293 cells expressing GECOs showing different time intervals in physiological buffer containing 0 mM Ca²⁺. The upper row shows cells expressing R-GECO-NLS. The lower row shows cells expressing R-GECO UT. Scale bar represents 20 μm. (B) Representative time course of intracellular Ca²⁺ signals in HEK293 cells expressing GECOs in response to 100 μM ATP in buffer containing 0 mM Ca²⁺. The black curve shows Ca²⁺ signals obtained from R-GECO-UT and the red curve shows Ca²⁺ signals obtained from R-GECO-NLS (C) Scatter dot blot represents the maximum response of HEK293 expressing GECOs in response to 100 μM ATP (R-GECO-NLS, n= 3/56; R-GECO UT, n=3/38) Student's t-test ($p < 0.001$).

4.3 Visualizing Intracellular NO Production Derived from Differentially Localized eNOS

After characterizing the intracellular Ca²⁺ profiles, we next tested whether the NO bioavailability of differentially localized eNOS is affected. For this purpose, we co-transfected HEK293 cells with the WT-eNOS-RFP or RFP-eNOS-NLS, nuclear localization of eNOS was previously confirmed and studied by Iwakiri, Y et al. (Iwakiri, Y et al., 2006), along with G-geNOps. 24 hours after transfection, cells were efficiently co-transfected and correctly targeted to their specific locales (**Figure 4.9, right panel**). However, as shown in **Figure 4.9**, some cells were only transfected with

one of the constructs only; demonstrating that the co-transfection of different constructs is less efficient. To eliminate false-negative results we excluded cells expressing only one construct from image analysis.

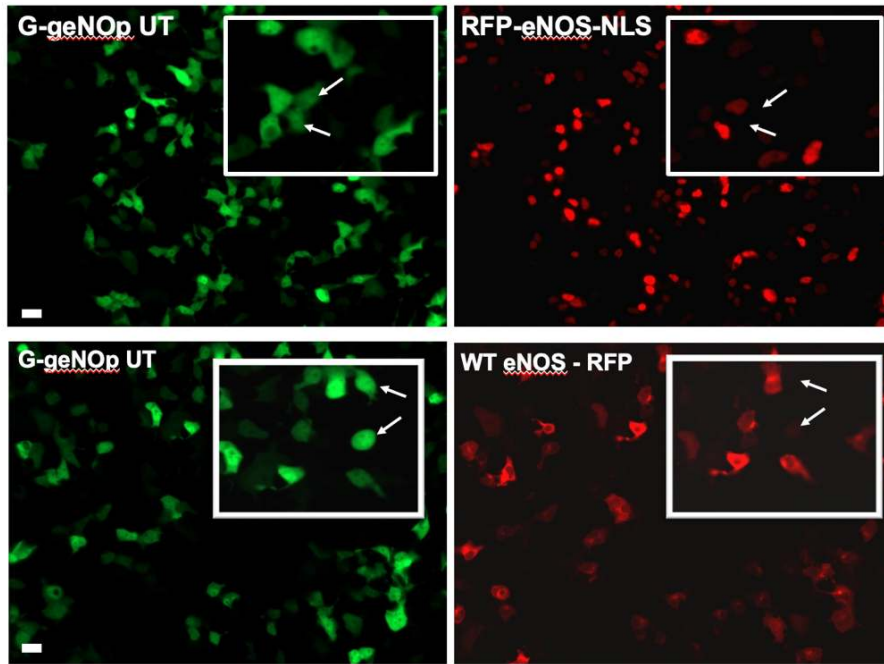


Figure 20: Co-transfection of HEK293 cells with G-geNOp and differentially targeted eNOS RFP variants. Representative fluorescent images of HEK293 cells transfected with G-geNOp UT and WT-eNOS (lower panels) or NLS-eNOS (upper panels). Arrows indicate cells are G-geNOp but not eNOS transfected. Scale bar represents 20 μm .

To visualize nuclear targeted and wild type (WT) eNOS derived NO signals in HEK293 cells we applied the same experimental conditions we had established for Ca^{2+} imaging as shown in the previous chapter. As expected, after exposing WT-eNOS expressing HEK293 cells to ATP in the presence of extracellular Ca^{2+} , the respective NO signal appeared smooth and longer-lasting as shown in **Figure 4.10, A**. This observation is in line with previous studies showing the NO profile in endothelial cells upon treatment with ATP (Eroglu et al., 2019). Nucleus targeted eNOS derived NO signals, however, showed reduced NO formation compared to WT (**Figure 4.10**). Moreover, after withdrawing ATP from the imaging media the NLS derived NO signals also lasted longer than the WT derived NO signal and did not return to the baseline, indicating that differential localization might affect the NO formation capacity of eNOS. (**Figure 4.10**).

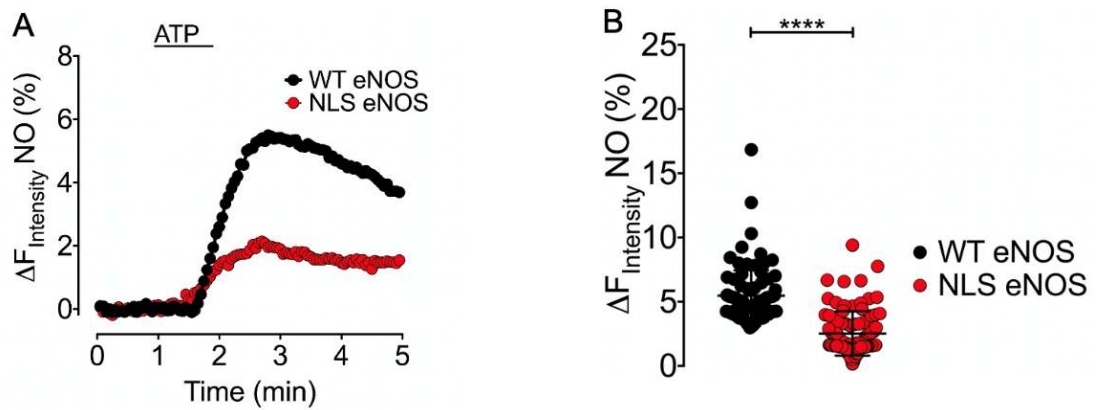


Figure 21: Co-imaging of NO with differentially targeted eNOS. (A) Representative time course of intracellular Ca^{2+} signals in HEK293 cells expressing G-geNOps in response to 100 μM ATP in buffer containing 2 mM Ca^{2+} . The black curve shows NO signals obtained from WT-eNOS-RFP and the red curve shows NO signals obtained from NLS-eNOS-RFP (B) Scatter dot blot represents the maximum response of HEK293 expressing eNOS variant in response to 100 μM ATP (WT-eNOS-RFP, $n = 3/38$; NLS-eNOS-RFP, $n = 3/59$) Student's t-test $p < 0.001$.

Next, we repeated the same experimental setup in an imaging buffer lacking Ca^{2+} . Cell treatment with ATP in the absence of extracellular Ca^{2+} in cells expressing the WT-eNOS yielded a similar NO profile compared to the ATP treated cells in the presence of Ca^{2+} . This observation indicates that the mobilization of intracellular Ca^{2+} is sufficient to activate the eNOS enzyme (**Figure 4.11**). As expected, NO production derived from cells expressing the NLS-eNOS-RFP was clearly lower than the WT-eNOS-RFP and quantitatively similar to the amount of NO production when stimulated in the presence of extracellular Ca^{2+} (**Figure 4.11**).

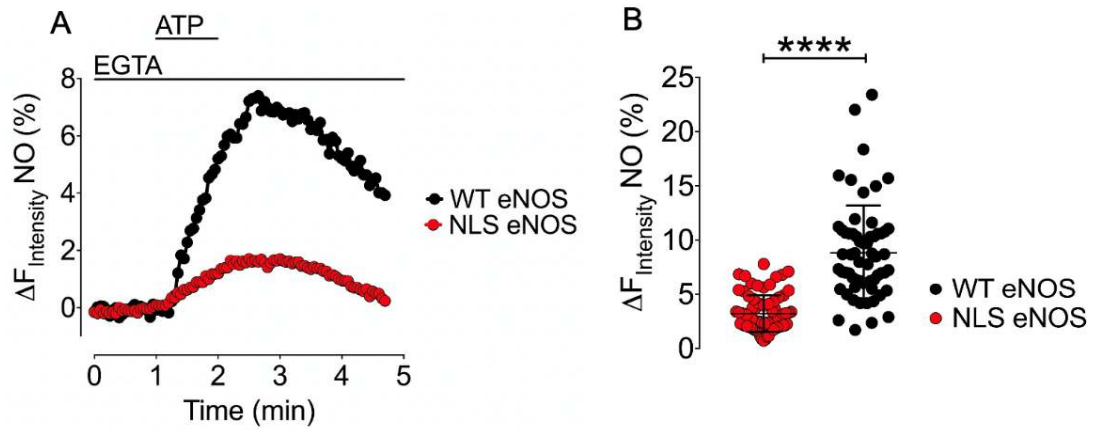


Figure 22: Co-imaging of NO with differentially targeted eNOS. (A) Representative time course of intracellular NO signals in HEK293 cells expressing G-geNOPS in response to 100 μM ATP in buffer containing 0 mM Ca^{2+} . The black curve shows NO signals obtained from WT-eNOS-RFP and the red curve shows NO signals obtained from NLS-eNOS-RFP (B) Scatter dot blot represents the maximum response of HEK293 expressing eNOS variant in response to 100 μM ATP (WT-eNOS-RFP, $n=3/62$; NLS-eNOS-RFP, $n=3/76$) Student's t-test $p < 0.001$.

To evaluate the extracellular Ca^{2+} dependencies of each subcellular localized eNOS, we have examined the same eNOS construct under different conditions. First, we have compared WT-eNOS-RFP in both Ca^{2+} containing and Ca^{2+} lacking buffers. As shown in **Figure 4.12, A**, WT-eNOS in Ca^{2+} lacking buffer showed significantly higher NO signals than the signal obtained from Ca^{2+} containing buffer. This observation might seem contradictory; however, we hypothesize that eNOS might be prestimulated in the presence of extracellular Ca^{2+} caused by Ca^{2+} overload of the cells that leads to a reduced geNOPS amplitude upon eNOS activation. (**Figure 4.12, B**).

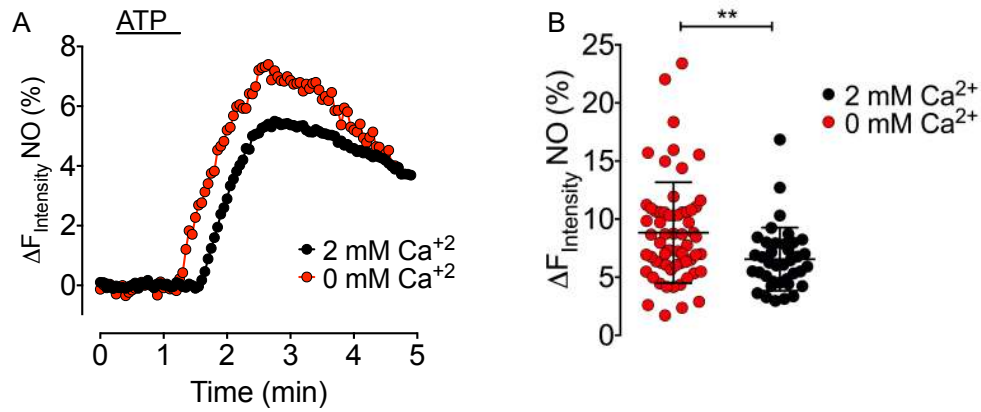


Figure 23: Co-imaging of NO with Wild Type eNOS. (A) Representative time course of intracellular NO signals in HEK293 cells expressing G-geNops in response to 100 μM ATP in buffer containing 2 mM Ca^{2+} and 0 mM Ca^{2+} . Black curve shows NO signals obtained from WT-eNOS-RFP in 2 mM Ca^{2+} buffer and red curve shows NO signals obtained from WT-eNOS-RFP in 0 mM Ca^{2+} (B) Scatter dot blot represents the maximum response of HEK293 expressing eNOS variant in response to 100 μM ATP (2 mM Ca^{2+} , $n= 3/38$; 0 mM Ca^{2+} , $n=3/62$) Student's t-test $p=0.0045$.

To confirm whether nucleus localized eNOS activity is affected, we compared the NO profiles derived from eNOS-NLS in the presence and absence of extracellular Ca^{2+} . As shown in **Figure 4.13**, cell treatment with ATP yielded under both conditions a comparable NO signal demonstrating that NLS localized eNOS is incapable to produce the amounts of NO compared to the wild type eNOS. Overall, our data demonstrate a significant decrease in the NO formation capacity of eNOS when localized to the cell nucleus compared to its wild-type form.

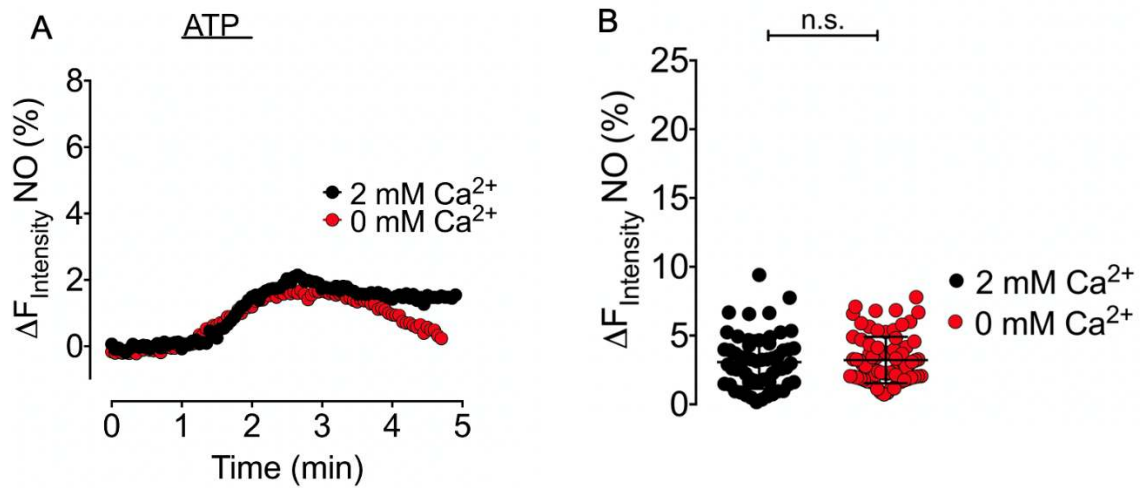


Figure 24: Co-imaging of NO with Nuclear Targeted eNOS. (A) Representative time course of intracellular NO signals in HEK293 cells expressing G-geNops in response to 100 μM ATP in buffer containing 2 mM Ca^{2+} and 0 mM Ca^{2+} . Black curve shows NO signals obtained from NLS-eNOS-RFP in 2 mM Ca^{2+} buffer and red curve shows NO signals obtained from NLS-eNOS-RFP in 0 mM Ca^{2+} (B) Scatter dot blot represents the maximum response of HEK293 expressing eNOS variant in response to 100 μM ATP (2 mM Ca^{2+} , $n=3/59$; 0 mM Ca^{2+} , $n=3/76$) Student's t-test $p=0.6494$.

DISCUSSION, CONCLUSION, and RECOMMENDATIONS

5.1 Discussion

The eNOS enzyme is an essential protein in endothelial cells to produce NO for vascular smooth muscle cell relaxation and the regulation of vascular tone (Zweier & Ilangovan, 2020). To date, mainly the NO production from wild type eNOS has been studied, with indirect approaches of NO detection and protein-based analysis (Figuerola et al., 2013; García-Ortiz et al., 2017). However, techniques used in most studies focusing on the biochemistry of eNOS do not permit direct imaging of NO in subcellular levels, but rather yield read-outs of secondary metabolites of an entire cell population. Due to the lack of proper tools, there are still less known about eNOS activity function and NO production capacity when in subcellular locales. To elucidate the NO production from differentially localized eNOS, we have used powerful imaging approaches, including genetically encoded biosensors for NO and Ca^{2+} , geNOps, and GECO respectively. First, we have identified the suitable geNOps variant for co-imaging of NO with eNOS by comparing the Orange and Green variants of the geNOps (**Figure 4.2**). Our data unveiled that both biosensors display a similar profile between each other in response to the NO donor NOC-7. The similarities found between green and orange variant of geNOps differs from the first article of geNOps published in 2016, where it was found that the green variant had a stronger response amplitude than the orange variant of geNOps (Eroglu et al., 2016). We think that this difference between the two variants may be due to the light source used in both experiments. The previous experiment held in 2016 have used a Xenon arc lamp, while we have used an LED as a light source. Since both light sources provide different characteristics, the monochromatic LED light source may have allowed a better performance of the biosensors. Arc lamps are known to be very unstable and show fast changes in the electron density that is generated by plasma oscillations and thermal runaway (Albeanu, Soucy, Sato, Meister, & Murthy, 2008). Moreover, Arc lamps cannot be regulated in intensity and require the use of diaphragms and neutral density filters to reduce the light intensity to prevent overexposure of the sample. LED emitter typically emits from a small spot or slit and have more complex emission pattern and potentially higher photon output flux. Moreover, very low light intensity – which can be regulated very precisely – is sufficient for live-cell imaging experiments, thus,

significantly reducing the phototoxicity (Albeanu et al., 2008; O'Toole & Diamond, 2008). These parameters may have a significant effect on the functionality of a biosensor and need to be further investigated to unveil such variabilities in the performance of the same biosensor but different instruments.

To visualize Ca^{2+} signals, we have exploited the well-established intensimetric variants of GECOs. ATP stimulation has evoked a similar response in both R-GECO-UT and R-GECO-NLS in the presence of extracellular Ca^{2+} (**Figure 4.7**). However, R-GECO-NLS had a significantly lower response in the absence of extracellular Ca^{2+} , thus providing the importance of extracellular Ca^{2+} to diffuse efficiently in intracellular compartments. In one study, it is shown that Ca^{2+} can freely pass the nuclear envelope in hippocampal neurons. Exchange of Ca^{2+} between nucleus and cytoplasm was investigated with laser-assisted photolysis of calcium compounds. Clearly, their data has shown that the Ca^{2+} ions can pass freely from nuclear pore complexes (Eder & Bading, 2007). These data are in line with our observations since we have also shown that Ca^{2+} can pass the nuclear envelope of HEK293 cells freely if there are sufficient extracellular Ca^{2+} (**Figure 4.7**).

Finally, we have studied the NO production from differentially targeted eNOS to the nucleus. Unexpectedly, we have found that in both the absence and presence of Ca^{2+} conditions, nuclear-targeted eNOS had significantly lower NO production than wild type eNOS. Furthermore, even though the NO production was lower in nuclear-targeted eNOS, its response was persistent, and there was no recovery observed as in the wild type geNOs. The targeting of eNOS to subcellular localizations was also studied by different research groups. One research group has identified that the eNOS shifts between Golgi and plasma membrane to regulate its activity. Fang et al. had studied the eNOS in nuclear fractions and found that when cells stimulated with vascular endothelial growth factor (VEGF), eNOS localized in the caveolae fraction has decreased and increased in the nuclear fractions after 30 to 60 minutes (Feng, Venema, Venema, Tsai, & Caldwell, 1999). In another study, overexpression of NOS interacting protein (NOSIP) has translocated eNOS to the nucleus and decreases eNOS activity specifically in the G2 phase of the cell cycle (Schleicher, Brundin, Gross, Müller-Esterl, & Oess, 2005). When the direct effects of NO in the cell cycle mechanism were studied, it was suggested that lower concentrations of NO might

increase the proliferation. In contrast, high concentrations of NO have shown to decrease vascular smooth muscle cell proliferation, especially in G1/S transition (Napoli et al., 2013). Our findings also showed that the nuclear-targeted eNOS does indeed produce NO in the nucleus yet not as much as the wild type form. According to the studies mentioned above, the mechanism of eNOS and NO is not very clear and needs to be elucidated.

5.2 Conclusion

eNOS in different subcellular localizations have been studied, and a decrease in the activity has been reported. In our study, we have performed direct real-time imaging of NO with geNOP genetically encoded biosensor to elucidate the activity of nuclear-targeted eNOS. We have found that the eNOS localized to the cell nucleus has a significant decrease in the NO formation capacity even when there is sufficient Ca^{2+} present in the nucleus.

5.3 Recommendations

NO experiments held in this study were accomplished using untargeted geNOPs, because it is known that NO can easily diffuse throughout the cell. However, it may still be thought that geNOPs specifically targeted to the nucleus can provide a different NO profile. In the future, we will generate and test a new geNOP variant specifically targeted to the nucleus to co-image with NLS-eNOS and compare it with the imaging results from UT-geNOPs. For this experiment, we will add a nuclear targeting sequence to the C-terminus end of untargeted G-geNOPs and co-transfect HEK293 cells with NLS-eNOS.

With the power of directly imaging NO with geNOPs, in the future, we would like to image NO with geNOPs in different phases of the cell cycle. For that, we will synchronize cultured HEK293 cells in different cell cycle phases, confirm their synchronization with a flow cytometer, and image the NO production capability. We believe that these further studies will elucidate the direct role of eNOS in each cell cycle phase, and direct effects of NO on the cell cycle. These studies have provided a different perspective in subcellular eNOS experiments, and it shows that direct imaging of generated NO from different localized eNOS provides deeper information.

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CURRICULUM VITAE

Name Surname: Mete Emir Özgürses

Date of Birth and Place of Birth: 20/12/1996 Istanbul

Education:

Degree	Institute Name / Department		Year of Graduation
Bachelor	Biruni University, Faculty of Engineering	Molecular Biology and Genetics	2019
Working Experience:			
Unvan	Görev Yeri	Yıl	

Publications

1. Taşdemir, M., **Özgürses, M.**, Darıcı, H. and ORDUERİ, N., 2020. Cytotoxic Effects Of N (4- *Acta Oncologica Turcica*, Chlorophenyl)-1H-Indole-2-Carboxamide On Bone Cancer Cells. 53(1), pp.173-180.
2. Pashaei E ,Guzel E ,**Ozgurses ME** ,Demirel G ,Aydin N ,Ozen M .AMeta-Analysis: Identification of Common Mir-145 Target Genes that have Similar Behavior in Different GEO Datasets. **PLoS One**. 2016 Sep 21;11(9):e0161491. (Research)
3. Esra Guzel , Omer Faruk Karatas , **Mete Emir Ozgurses** , Mustafa Ozen , New Insights into Laryngeal Squamous Cell Carcinoma: Cancer Stem-Like Cells, **Cancer Research Frontiers**. 2015 Apr; 1(2): 138-148. (Review)

Awards

1. **2015-2016: Dean (Merit-Based) Scholarship:** Highest GPA in Engineering and Natural Sciences Faculty (ranked as 1st)
2. **2016-2017: Dean (Merit-Based) Scholarship:** Highest GPA in Engineering and Natural Sciences Faculty (ranked as 1st)
3. **2017-2018: Dean (Merit-Based) Scholarship:** Highest GPA in Engineering and Natural Sciences Faculty (ranked as 1st)
4. **2019: Dean's List:** Graduated with the highest GPA in Engineering and Natural Sciences Faculty (ranked as 1st)
5. **2020: Fulbright Scholarship:** Nominated as Fulbright Scholar in the field of molecular biology and genetics for Ph.D. education in USA.

PLAGIARISM REPORT

REAL-TIME IMAGING OF NITRIC OXIDE (NO) SIGNALS
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