

# FRET-based visualization of cGMP dynamics in glomerular endothelial cells and podocytes

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## List of abbreviations

ACE	Angiotensin-converting enzyme
AKS	Acute kidney slices
ANGII	Angiotensin II
ANP	Atrial natriuretic peptide
ARBs	Angiotensin II receptor blockers
ARNI	Angiotensin receptor neprilysin inhibitor
ASN	American Society of Nephrology
AT1	Angiotensin II type-1 receptor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BNP	Brain natriuretic peptide
bp	Base pair
BP	Blood pressure
CaCl <sub>2</sub> x 2H <sub>2</sub> O	Calcium chloride dihydrate
CFP	Cyan fluorescent protein
cGMP	Guanosine 3',5'-cyclic monophosphate
CKD	Chronic kidney disease
CNG	cGMP-gated ion channel
CNP	C-type natriuretic peptide
D(+)-Glucose x H <sub>2</sub> O	D(+)-Glucose monohydrate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOCA	Deoxycorticosterone acetate
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked immunoassay
eNOS	Endothelial nitric oxide synthase
F:	forward
FBS	Fetal Bovine Serum
FPs	Foot processes
FRET	Förster Resonance Energy Transfer
GBM	Glomerular basement barrier
GC	Guanylyl cyclase
GC-A	Natriuretic peptide receptor A
GEC	Glomerular endothelial cells
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
GTP	Guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
HCl	Hydrogen chloride
HEK 293T	Human embryonic kidney cells
HF	Heart failure
hm	homozygous
ht	heterozygous
IBMX	3-Isobutyl-1-methylxanthine
IFIB	Interfaculty Institute of Biochemistry
iNOS	Inducible nitric oxide synthase
IR	Ischemia reperfusion
IVC	Individually ventilated cage
IVI	Intravital imaging



ivRF	<i>in vivo</i> Research Facility
KCl	Potassium chloride
KH <sub>2</sub> PO <sub>4</sub>	Potassium dihydrogen phosphate
KHB	Krebs-Henseleit buffer
KO	Knock-out
LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen
LMA	Low Melt Agarose
MgCl <sub>2</sub> x 6H <sub>2</sub> O	Magnesium chloride hexahydrate
MgSO <sub>4</sub> x 7H <sub>2</sub> O	Magnesium sulphate heptahydrate
Na <sub>2</sub> EDTA x 2H <sub>2</sub> O	EDTA, Disodium Salt dihydrate
Na <sub>2</sub> HPO <sub>4</sub> x 7H <sub>2</sub> O	di-Sodium hydrogen phosphate heptahydrate
NaCl	Sodium chloride
NaHCO <sub>3</sub>	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NEP	Nepilysin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Natriuretic peptide
NS	Nephrotic syndrome
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PFA	Paraformaldehyde
pGC	Particulate guanylyl cyclase
PH	Pulmonary hypertension
PIC	Protease Inhibitor Cocktail
PKC	Protein kinase C
PKG	Protein kinase G
PPAR-γ	Peroxisome proliferator-activated receptor γ
R:	reverse
RAAS	Renin-angiotensin-aldosterone-system
RBF	Renal blood flow
RIA	Radioimmunoassay
ROI	Region of Interest
ROS	Reactive oxygen species
rSAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulfate
sGC	Soluble guanylyl cyclase
SNAP	S-nitroso-N-acetylpenicillamine
TEA/HCl	Triethanolamine hydrochloride
tg	transgenic
Tris-HCl	Trizma® hydrochloride
TRPC	Transient receptor potential channel
v/v	volume per volume
VEGF	Vascular endothelial growth factor
VEGFR1/2	VEGF receptors 1 and 2
w/o	without
w/v	weight per volume
WR	Working reagent
wt	wildtype
YFP	Yellow fluorescent protein

## Abstract

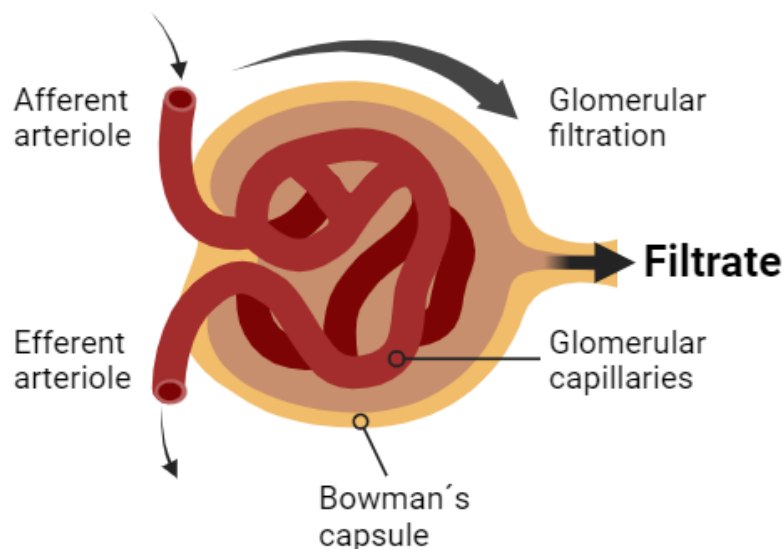
Podocytes play a critical role in maintaining the integrity of the glomerular filtration barrier, a trilaminar structure that prevents the loss of macromolecules into the urine. These cells extend prominent cellular protrusions, termed foot processes, which contain an actin-based contractile system as a distinctive cellular structure. Podocytes can adapt to renal hemodynamic changes by modifying the foot process actin cytoskeleton, while they are exposed to significant physical forces resulting from pressure and fluid flow across the filtration slit. However, due to their limited capacity for self-renewal, podocyte loss is a hallmark in the progression of glomerular kidney diseases. Vasoactive factors, including angiotensin II (ANGII), are synthesized by glomerular cells upon stimulation and influence glomerular filtration. Among these factors, the role of the second messenger cyclic guanosine 5' monophosphate (cGMP) in podocytes and glomerular endothelial cells (GECs) remains elusive. Additionally, the presumed protective role of restored cGMP levels in various cardiovascular and renal diseases has sparked interest in exploring novel therapeutic strategies. This work aimed to evidence cGMP signaling in podocytes and GECs, and to elucidate cell-specific differences in cGMP dynamics. Using a stepwise approach, we analyzed isolated glomeruli and acute kidney slices from transgenic mice expressing the FRET-based ratiometric cGMP sensor (cGi500) either in podocytes or in endothelial cells. We stimulated both cGMP-synthesizing guanylyl cyclase systems (soluble (sGC) or particulate/membrane-bound (pGC)) independently or simultaneously. Both cell types responded with an increase in intracellular cGMP, while showing differences in both the intensity and duration of the response. Podocytes of cGi500 biosensor mice responded with a prolonged pGC-mediated cGMP signaling, emphasizing a predominant role for this pathway. GECs of cGi500 biosensor mice answered with a transient cGMP response after stimulation. Co-stimulation in podocytes did not result in the expected additive effect on cGMP levels, as observed in GECs. Moreover, increased cGMP levels were triggered by stimuli inhibiting cGMP degradation, cGMP export, or indirectly through calcium influx-related mechanisms in podocytes. Cell-specific differences in cGMP degradation were evident, with podocytes exhibiting comparatively lower baseline activity of the tested phosphodiesterase (PDE) isoforms. Notably, the most active isoforms, PDE3 and PDE5, had a preference towards the pGC/cGMP signaling pathway. On the contrary, in GECs, the basal activity of PDE2a was the highest of all isoforms tested, and had an equivalent impact on both cGMP pathways.

In summary, our data provided unprecedented insights into cell-specific dynamics of cGMP signaling in glomerular cells. These findings contribute to a deeper understanding of cGMP signaling in renal physiology and reveal potential targets for therapeutic interventions.

# 1 Introduction

## 1.1 Glomerular filtration barrier

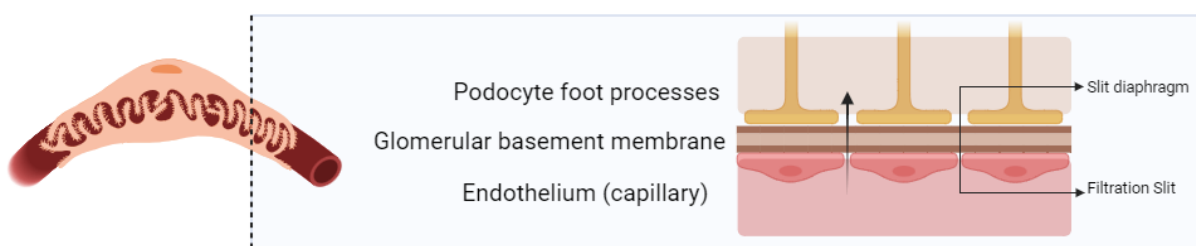
One of the main tasks of the kidney is to filter fluids and solutes according to their molecular size and charge, aiming to preserve essential plasma proteins (e.g. albumin) within the bloodstream. The nephron, which is a fundamental structural unit of the kidney, includes the renal corpuscle connected to the tubular system. This corpuscle consists of a glomerulus surrounded by the Bowman's capsule, forming the filtration unit of the nephrons. The glomerulus plays a crucial role in blood filtration and the generation of primary urine.<sup>1</sup> Comprised of a complex network of capillaries, the glomerulus is positioned between two resistance vessels: the blood arrives via the afferent arteriole and exits through the efferent arteriole. These pre-and postglomerular arterioles contribute to renal blood flow (RBF) autoregulation by altering their diameter through vasodilation or vasoconstriction (**Figure 1**).<sup>2,3</sup>



**Figure 1: Schematic illustration of the filter unit of the kidney, the glomerulus**

Blood entering through the afferent arteriole undergoes filtration across the glomerular capillary walls, producing the filtrate (primary urine) in the Bowman's capsule. Subsequently, this filtrate enters the tubule system at the urinary pole. The blood that has undergone filtration exits the glomerulus through the efferent arteriole. *Created with BioRender.com.*

The trilaminar structure of the glomerular capillary wall creates the glomerular filtration barrier (GFB), which incorporates the glycocalyx-covered fenestrated endothelium (proximal component layer), the glomerular basement membrane (GBM) and the visceral epithelial cells, known as podocytes (distal layer) (**Figure 2**).<sup>4</sup> Podocytes are terminally differentiated cells and possess a complex cellular structure consisting of a cell body and major processes that give rise to interdigitating actin-based foot processes (FPs) enwrapping the glomerular capillaries.<sup>5</sup> Adjacent FP are connected by cell-cell junctions called slit diaphragms, bridging the filtration slit between neighboring podocyte FPs.<sup>6,7</sup> An emerging field of research focuses on cell-cell communication through soluble mediators, in particular the crosstalk between endothelium and podocytes.<sup>8,9</sup> Prominent example is the expression of vascular endothelial growth factor (VEGF) in podocytes, functioning in an autocrine and paracrine manner to regulate the structure and function of GECs through ligand-receptor binding to VEGF receptors 1 (VEGFR1) and 2 (VEGFR2).<sup>10-13</sup>



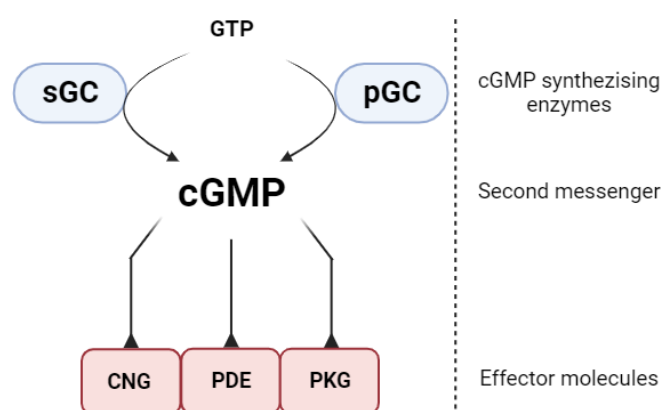
**Figure 2: Trilaminar structure of the glomerular filtration barrier**

Visual representation of a glomerular capillary surrounded by two podocytes and a sectional view of the glomerular filtration barrier (GFB), showcasing the fenestrated endothelium connected to the basement membrane and the foot processes of podocytes. Arrow indicates the filtration direction. *Created with BioRender.com.*

Elevated protein excretion (proteinuria) in the urine reflects impaired permselectivity/ increased permeability for proteins, indicating a possible dysfunction in one or more layers of the glomerular filtration barrier. Changes in the density or diameter of GECs fenestrations, characterized as transcellular holes through the peripheral cytoplasm, and glycocalyx disruptions can disturb the proximal filtration layer.<sup>14-16</sup> Changes in the slit diaphragm, cell adhesion molecules, and disturbances in actin organization, referred to as foot process effacement, can compromise structural integrity of the distal filtration layer. This can result in detachment from the GBM and consequently to podocyte cell loss.<sup>17-19</sup>

## 1.2 cGMP signaling in the kidney

In 1963, researchers first discovered the presence of cGMP in the urine of rats,<sup>20</sup> and since then, its role in controlling various cell functions in health and disease has been studied extensively.<sup>21</sup> The cGMP-dependent signal transduction induces specific biological responses that regulate renal physiological function (e.g. natriuresis, diuresis, glomerular and tubular function).<sup>22</sup> External stimuli (e.g. shear stress, release of specific neurotransmitters, vasoactive substances, hormones) initiate internal signaling cascades that culminate in the generation of the cyclic nucleotide cGMP. This leads to the activation of downstream effector molecules such as cGMP-gated ion channels (CNG), cGMP-regulated phosphodiesterase (PDE), and cGMP-dependent protein kinase G (PKG). The regulated widening of blood vessels, known as vasodilation, is an essential physiological process guided by intricate signaling cascades and can be cited as an example of cGMP-mediated signal transduction. Endothelial cells, lining the inner vascular walls, respond to stimuli by releasing nitric oxide (NO). NO diffuses into smooth muscle cells, activates the soluble guanylate cyclase and increases cGMP levels. Elevated intracellular cGMP leads to the activation of PKG, which phosphorylates various proteins, opens potassium channels and closes calcium channels. The subsequent dephosphorylation of myosin, a contractile protein, facilitates smooth muscle relaxation.<sup>23,24</sup> GTP-specific succinyl-CoA synthetase, a component of the citric acid cycle, generates guanosine triphosphate (GTP), which is enzymatically converted into cGMP by guanylyl cyclases (GC) (**Figure 3**).<sup>25,26</sup> These enzymes are located either in the cytosol (sGC) or exist as membrane-bound intracellular catalytic domains (pGC) associated with an extracellular peptide receptor domain.<sup>26–28</sup>



**Figure 3: Guanylyl cyclase/cGMP signaling cascade**

The second messenger cGMP is synthesized by two different guanylyl cyclase systems (sGC and pGC) from GTP. The effector molecules (cGMP-gated ion channels (CNG), cGMP-regulated phosphodiesterase (PDE), and cGMP-dependent protein kinase G (PKG)) transduce the signal and mediate physiological responses. *Created with BioRender.com.*

In the kidney, sGC is abundantly expressed as  $\alpha 1\beta 1$  heterodimer,<sup>29,30</sup> and is activated by NO binding to the ferrous heme of sGC $\beta$ .<sup>31–34</sup> The main source of endogenous NO is generated as a by-product during the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) enzymes. The nitrate–nitrite–NO pathway offers an alternate route where endogenous or dietary inorganic nitrate and nitrite undergo serial reduction to produce NO, thereby complementing the NOS system.<sup>35–38</sup> The termination of the NO signaling pathway can occur through various mechanisms, including sGC desensitization via heme oxidation, heme loss, heme nitrosylation by a second NO molecule, S-nitrosation of cysteine residues, or heterodimer dissociation.<sup>39,40</sup> Moreover, the accessibility of NO to sGC can be compromised by potent NO scavenger, thereby influencing the extent of its functional impact. Included among these scavengers are partially reduced molecular oxygen products ( $O_2$ ), known as reactive oxygen species (ROS), or hemoglobin present in erythrocytes.<sup>40,41</sup> The NO-dependent cGMP signaling cascade impacts various physiological functions of the kidney, including the increase of renal blood flow or the stimulation of renin secretion.<sup>42–44</sup> The primary source of renin production and secretion occurs in juxtaglomerular epithelioid cells situated within the walls of renal afferent arterioles.<sup>45</sup> Renin serves as a crucial regulator of the renin-angiotensin-aldosterone-system (RAAS), as its proteolytic enzyme activity results in the cleavage of angiotensinogen into angiotensin I, which is subsequently processed to form angiotensin II (ANGII).<sup>46</sup> The metabolism of angiotensin peptides takes place in mesangial cells, podocytes, and endothelial cells, whereas ANGI (acting through ANGI type-1 (AT1) receptor) is the main angiotensin peptide that regulates glomerular hemodynamics.<sup>47</sup> ANGI-induced formation of ROS participates in pro- and antioxidant pathways in the kidney.<sup>48</sup>

Different classes of mammalian pGC (GC-A to GC-G) have been identified.<sup>28,49</sup> The first three react to specific peptide ligands (GC-A: atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP); GC-B: C-type natriuretic peptide (CNP); GC-C: the heat stable enterotoxins of *Escherichia coli* and guanylin) and are expressed in the kidney.<sup>50–52</sup> The remaining receptors (GC-D found in the olfactory epithelium; GC-E and GC-F in the retina; GC-G in the lung, intestine, and skeletal muscle) are referred to as 'orphan' receptors due to the absence of identified activating ligands.<sup>53</sup> ANP and BNP are predominantly secreted by cardiomyocytes (ANP: atrium and BNP: ventricle) and their binding to GC-A mediates e.g. inhibition of renin secretion, an increase in the glomerular filtration rate (GFR) by dilating the afferent arterioles and constricting the efferent arterioles.<sup>54–56</sup> The ligand-receptor binding of GC-B lacks the natriuretic and diuretic effects seen with ANP/BNP binding to the GC-A receptor, and its specific physiological role in the kidney is not yet fully understood.<sup>57,58</sup> CNP has a strong binding affinity to GC-B, and accumulating data indicate that urinary CNP may play a role in the early detection of renal dysfunction.<sup>59</sup> Urinary CNP excretion was detected in individuals with nephrotic syndrome (NS),<sup>60</sup> and CNP mitigated ischemia–reperfusion (IR)-induced acute

kidney injury by inhibiting apoptotic and oxidative stress pathways.<sup>61</sup> Clearance of natriuretic peptides occurs either by receptor-mediated internalization or by degradation via NPR-C (clearance receptor), by proteolysis via neprilysin (NEP) or insulysin (insulin degrading enzyme), or by excretion.<sup>59,62</sup>

### 1.2.1 cGMP signaling in the glomerulus (endothelial cell/podocytes)

There is a scarcity of information regarding cGMP signaling within glomerular cell layers, which reflects a substantial lack of research into this signaling cascade and its impact on the filtration unit of the kidney. The existence of both cGMP synthesizing GC systems in GECs is unknown and available literature on podocytes contains conflicting data. Recent findings demonstrated strong mRNA expression of GC-A and NPR-C in podocytes, while the expression level of GC-B was low.<sup>52,63–68</sup> In GECs, on the other hand, expression of GC-A and GC-B was also observed, although to a lesser extent.<sup>52</sup> Others presented additional proof of mRNA expression of GC-B and CNP in immortalized podocytes.<sup>69</sup> Furthermore, a strong detection of ANP mRNA and NEP was found in podocytes.<sup>70–72</sup> NEP is a neutral endopeptidase and capable of degrading all three natriuretic peptides. The expression pattern of binding and degradation enzymes highlights a significant impact of natriuretic peptides on podocytes, specifically emphasizing the paracrine influence of circulating ANP. The systemic antihypertensive effect of circulating ANP was demonstrated by the appearance of salt-sensitive hypertension caused by genetic deletion of the proANP gene in mice (ANP-KO).<sup>73</sup> While salt-resistant hypertension was present in global GC-A-KO mice and endothelial cell-specific GC-A-KO mice,<sup>74,75</sup> podocyte cell-specific GC-A-KO mice showed no basal phenotype related to blood pressure (BP), GFR, or natriuresis.<sup>63</sup> However, following deoxycorticosterone acetate (DOCA) salt treatment, the mice displayed albuminuria, decreased GFR, along with heightened expression of the canonical transient receptor potential 6 channel (TRPC6) and an increase in ATP-triggered calcium influx within podocytes. The TRPC6 is a glomerular slit diaphragm-associated channel and essential for maintaining regular podocyte function and structure.<sup>76</sup> It has also been functionally linked to the podocyte actin cytoskeleton.<sup>77</sup> Genetic and developed abnormalities affecting this channel are linked to proteinuric kidney diseases,<sup>77</sup> whereas excessive intracellular calcium is linked to various types of cell death in renal cells.<sup>78</sup> Augmented TRPC6-mediated  $\text{Ca}^{2+}$  influx causes podocyte injury and damage, while the utilization of cGMP-elevating substances was shown to counteract this effect, demonstrating the protective nature of the cGMP signaling pathway.<sup>79–81</sup>

The discovery of a contractile architecture in the foot processes sensitive to vasoactive substances suggests a regulatory influence on glomerular filtration by modulating the permeability.<sup>82–85</sup> Furthermore, a link between natriuretic peptide related signaling and

mechanical stress was demonstrated *in vitro*, highlighting the influence of cGMP on the mechanobiology of podocytes.<sup>69</sup> The natriuretic peptide (NP) system is designated as an antagonist to RAAS. ANGII functions as a natural suppressor of (GC-A)-mediated cGMP synthesis through an ANGII type-1 (AT1) receptor/protein kinase C (PKC) dependent mechanism.<sup>86</sup> Nonetheless, ANGII has also been known as a stimulant for NO production in podocytes.<sup>87,88</sup> Evidence showed that the ANGII-induced podocyte phenotype shifts from being dynamically stable to adaptively migratory with a persistent activation of the RAAS.<sup>85</sup> Maintaining a balance between the RAAS and cGMP-producing systems appears pivotal for enabling podocytes to adapt to the continuous fluctuations in cardiorenal hemodynamics, emphasizing the fundamental importance of understanding these mechanisms in living animals.

The majority of available data on ANP-induced cGMP synthesis in podocytes is based on cell culture studies and indicates a consensus regarding the existence of a NP/pGC/cGMP signaling pathway in this cell type, while the presence of an active NO/sGC/cGMP signaling pathway has been questioned.<sup>29,68,72</sup> Members of the NOS family - neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) - are essential for initiating the activation of the NO/sGC/cGMP signaling pathway by producing NO. mRNA for eNOS and iNOS was detected in both human glomerular endothelial cells and podocytes.<sup>80</sup> Although there was minimal protein expression of eNOS in podocytes, it was abundant in glomerular endothelial cells. Moreover, the presence of sGC was confirmed in both cell types using human renal tissue,<sup>90</sup> and the subunits were identified in cultured human cell lines (podocytes: sGC $\alpha$ 1, sGC $\alpha$ 2, sGC $\beta$ 1 and sGC $\beta$ 2; glomerular endothelial cells: sGC $\alpha$ 2 and sGC $\beta$ 2).<sup>80</sup> Despite deviations in species-dependent sGC detection (especially in murine samples), a NO-induced increase in intracellular cGMP was observed in immortalized podocyte cell lines,<sup>68,72,80</sup> podocytes of isolated glomeruli<sup>88,91</sup> as well as in glomeruli of kidney slices.<sup>92</sup> In eNOS-KO mice, the deficiency of the NO-producing enzyme alone led to the occurrence of mild glomerular disease, characterized by podocyte injury and low-grade albuminuria.<sup>93</sup> In young and aged eNOS-KO mice, the podocyte number was significantly diminished in comparison with wild type mice.<sup>94</sup> Furthermore, Hart et al. showed that NO deficiency leads to TRPC6 overexpression upon adriamycin-induced nephropathy in eNOS-KO mice and postulated the presence of a paracrine (e)NOS/NO/sGC signaling between glomerular endothelial cells and podocytes.<sup>80</sup> The impact of autocrine/paracrine nitric oxide (NO) production on podocytes remains largely unexplored.



### 1.3 Pharmacological manipulation of cGMP signaling

Multiple medications addressing cGMP deficiency in disease states have been approved and established as treatment options, primarily for pulmonary and cardiovascular diseases.<sup>95–97</sup> Accumulating evidence emphasizes renoprotective properties linked to reestablished cGMP levels in kidney disease, positioning the cGMP signaling pathway as a potential therapeutic target and underlining the need for basic research on cell-specific differences in renal cell types.<sup>22,98–100</sup> The novel medications seek to counteract the pathologically decreased cGMP synthesis through various mechanisms: activating sGC NO-independently with/without heme, inducing specific subtypes of guanylyl cyclases with native and designer natriuretic peptides, inhibiting degradation by specific PDE isoform inhibitors, blocking NPR-C or NEP activity and targeting the NO pathway by supplementation diets.<sup>101–108</sup> PDE5, 6 and PDE9 are cGMP-specific PDEs and are essential for the hydrolysis of sGC- and pGC-synthesized cGMP pools. The other identified PDE isoforms are either cAMP-specific PDEs (PDE4, 7, and 8) or exhibit dual-specificity (PDE1, 2, 3, 10, and 11).<sup>109</sup> Sildenafil, a well-studied PDE inhibitor, effectively blocks PDE5 activity, exhibits vasorelaxant properties and is used in clinical practice to treat erectile dysfunction and pulmonary hypertension (PH).<sup>110–113</sup> Multiple studies have linked PDE5 inhibition with favorable renal effects.<sup>114–117</sup> In addition, there is increasing evidence that podocytes respond to PDE5 inhibition, emphasizing the significance of interactions among cGMP, PKG-I, Peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ), and TRPC6 signaling pathways in glomerular diseases.<sup>79–81,118</sup> Another promising approach is the inhibition of PDE9a, which is highly expressed in the kidney,<sup>119</sup> and augments ANP-mediated cGMP synthesis, while showing no response to NO/cGMP activation.<sup>120,121</sup> In diseases where NO-dependent cGMP synthesis is suppressed, PDE9a inhibition could potentially be utilized as a therapeutic strategy. The cGMP-synthesizing guanylyl cyclase system and RAAS function as opponents, are tightly regulated and prone to imbalance in disease states. ANGII has vasoconstrictive properties and was shown to upregulate PDE5, which enhanced hydrolysis of NP-mediated cGMP synthesis.<sup>122</sup> Inhibiting the conversion of angiotensin I to ANGII by angiotensin-converting enzyme (ACE) or receiving angiotensin II receptor blockers (ARBs) protects the kidneys of patients with chronic kidney disease (CKD).<sup>123,124</sup> Furthermore, combination therapy with the angiotensin receptor neprilysin inhibitor (ARNI) reduced hospitalization for heart failure (HF) patients and all-cause mortality in a landmark clinical trial (PARADIGM-HF).<sup>125</sup> Overall, targeted treatment of deficiency and/or impairment of the cGMP system in kidney disease is once again the focus of research, while novel medications are being developed.

## 1.4 Fluorescence resonance energy transfer (FRET) microscopy

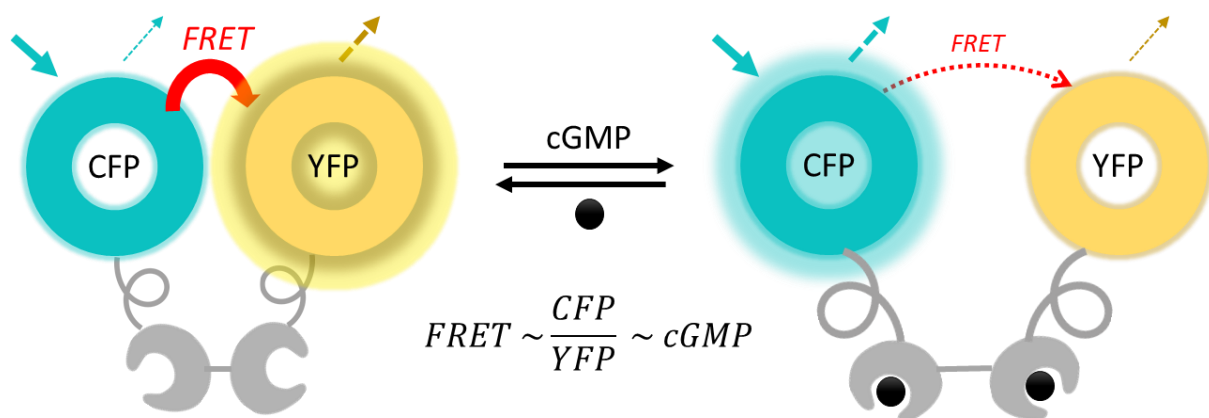
Fluorescence resonance energy transfer (FRET) is a distance-dependent physical phenomenon where energy is transferred nonradiatively from an excited donor fluorophore to the acceptor fluorophore through intermolecular long-range dipole–dipole coupling. FRET serves as a precise measurement of molecular proximity within angstrom distances (10-100 Å) and is efficient when the donor and acceptor are positioned within the Förster radius. The Förster radius is the distance at which half the excitation energy of the donor is transferred to the acceptor, typically falling within the range of 3–6 nm.<sup>126</sup> Basic requirements for FRET are:<sup>127,128</sup>

- (i) the emission spectrum of the donor fluorophore and the absorbance spectrum of the acceptor molecules must overlap (spectral overlap integral)
- (ii) the donor and acceptor fluorophores must be positioned within a specific range, typically between 1 and 10 nanometers.
- (iii) the dipoles of the donor and acceptor molecules should be oriented in a favorable manner relative to each other.
- (iv) Controlling environmental factors like temperature, pH, and ionic strength is crucial as they can impact the efficiency of FRET during experiments.

### 1.4.1 FRET-based cGMP Imaging

Conventional biochemical techniques to measure cGMP levels involve radioactive cGMP tracer (Radioimmunoassay (RIA)) or non-radioactive methods (Enzyme-linked immunoassay (ELISA)) that bind to anti-cGMP antibodies.<sup>129–132</sup> An alternative approach includes indirect evidence through a traditional Western blot, which detects the phosphorylation state of downstream targets resulting from the activation of cGMP-PKG kinase activity by using phospho-specific antibodies.<sup>133</sup> All of these techniques share a common necessity: the cells/tissue need to undergo lysis to liberate the cyclic nucleotide in quantities sufficient to meet the detection limit of the assays. Moreover, it is standard practice to include non-specific PDE inhibitors like isobutylmethylxanthine (IBMX) to prevent the degradation of cGMP during preparation. As a result, the ability to effectively monitor fluctuating intracellular cGMP levels with a spatiotemporal resolution is compromised. To overcome these obstacles, genetically encoded fluorescent cGMP biosensors have been developed that enable real-time imaging of this second messenger.<sup>130,134</sup> The cGMP detection can involve either FRET-based (e.g. Red cGES-DE5)<sup>135</sup> or non-FRET-based (e.g. FlincG)<sup>136</sup> fluorescent indicators and rely on either intensimetric (e.g. Green cGull)<sup>137</sup> or ratiometric (e.g. PfPKG)<sup>138</sup> fluorescence principles. Biosensors have the capability to move freely within the cell or can be directed to specific subcellular microdomains by attaching them to particular proteins or short peptide sequences.

One of the extensively used cGMP biosensors is cGi500, classified as a monomolecular ratiometric FRET-based biosensor.<sup>139,140</sup> The probe consists of tandem cGMP-binding sites of PKG-I, positioned between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), both of which are derivatives of green fluorescent protein (GFP)(**Figure 4**). The interaction with cGMP induces a change in conformation that modifies the distance between the donor and acceptor fluorophores, consequently decreasing the FRET efficiency. The CFP/YFP emission ratio reflects changes in intracellular cGMP concentrations. The expression of cGi500 in transgenic mice was achieved by a knock-in strategy into the Rosa26 locus along with Cre/lox-activatable expression of the sensor, driven by the cytomegalovirus early enhancer/chicken  $\beta$ -actin/ $\beta$ -globin promoter. Mating with Cre mice enables cell-specific biosensor expression, facilitating real-time FRET/cGi500 imaging in selected cell types across a presumed physiological range of 100 nM to 3  $\mu$ M.<sup>140</sup> The sensor has previously been used in the isolation of cells, tissues, organs, and *in vivo* experiments.<sup>92,140–143</sup>



**Figure 4: Working principle of the FRET-based biosensor cGi500**

In the absence of cGMP, FRET takes place from the excited CFP to YFP, resulting in light emission from YFP. Upon the binding of cGMP, a conformational change occurs, causing a decrease in FRET efficiency. As a result, the light emission from YFP is diminished, while the emission from CFP is increased. Monitored FRET response (CFP/YFP emission ratio) reflects changes in intracellular cGMP concentration.

## **2 Aim of the thesis**

Therapeutic modulation of the cGMP pathway has only recently entered clinical practice and demonstrated that its broad spectrum of beneficial effects extends beyond its protective function in cardiovascular diseases. The lack of consistent and relevant literature on the presence of cGMP synthesizing systems in glomerular cell layers hampers our understanding of associated renal physiological functions. Most of the available data relies on methods that involve cell destruction and, as a result, cannot comprehensively capture the spatial and temporal dynamics of cGMP-related systems. The objective of this thesis was to gain a deeper insight into the spatial cGMP dynamics of podocytes and glomerular endothelial cells in their healthy state. Expression of the FRET-based cGMP biosensor in two different model organisms, HEK 293T cells and transgenic mice, allowed the visualization of cGMP dynamics in real time in intact cells while preserving the physiological environment. This approach enabled us to test agonists and antagonists of cGMP signaling in a close-to-native scenario.

## 3 Material and methods

### 3.1 Material

**Table 1: List of materials**

Material	Catalog Number	Company
μ-Dish 35 mm, high Glass Bottom	81158	Ibidi
μ-Slide 8 Well, ibiTreat	80826	Ibidi
8-Lid chain, flat	65.989.002	Sarstedt
Blades	121-6	Ted Pella
Cell lifter	3008	Corning
Cell Strainer, 100 μm	83.3945.100	Sarstedt
CELLSTAR™ 96 Well Cell Culture Plate	655180	Greiner BioOne
CELLSTAR™ tubes 15 ml	188271-N	Greiner BioOne
CELLSTAR™ tubes 50 ml	227261	Greiner BioOne
Corning® 100 mm TC-treated Culture Dish	430167	Corning
Corning® 150 mm TC-treated Culture Dish	430599	Corning
Corning® 35 mm TC-treated Culture Dish	430165	Corning
Coverslip (18 x 18 mm # 1.5)	LH22.1	Roth
Coverslip Ø 18 mm # 1.5	0117580	Marienfeld
Coverslip Ø12 mm # 1.5	11911998	Gerhard Menzel
Discardit™ 5 ml Syringe	309050	Becton Dickinson
Dynabeads M-450 Tosyl-activated	14013	Thermo Fisher
Female Luer Lock	LF31	Drifton A/S
Instant Adhesive	Best-CA 221	Best Klebstoffe
Masterflex® silicone hose	96400-13	Cole-Parmer
Micro tubes 1.5 ml	72.690.001	Sarstedt
Microfuge Tube Polypropylene (1.5 ml)	357448	Beckman Coulter
Multiply®-μStrip 0.2 ml chain	72.985.002	Sarstedt
Original Perfusor® Line	8723060	Braun SE
Parafilm™	PM-996	Bemis
Peel-A-Way® S-22	E6032-1CS	Sigma
Petri dish, 92 x 16 mm (Agar plates)	82.1473	Sarstedt
Plastipak™ 1 ml Syringe	303172	Becton Dickinson

*Continued on next page*

Polyethylene tubing (PE10)	B-PE-10-100FT	Braintree Scientific
Rotilabo®-syringe filter, 0.22 µm	P666.1	Roth
SafeSeal tube 1.5 ml, brown	72.706.001	Sarstedt
Scalpel No.21	E72042-21	Feather
Sealing foam tape, 10mm(W)x1mm(T)x16.4Ft.(L)		SourcingMap
Serological pipette 10 ml	86.1254.001	Sarstedt
Serological pipette 25 ml	86.1685.001	Sarstedt
Serological pipette 5 ml	86.1253.001	Sarstedt
Sterican® Nr.20 27G x ¾	260092	Braun
Surgical suture (5-0-USP)	H2F	Resorba
Suture material (6-0 USP)	88153	Resorba
TipOne® Pipette Tip 10 µl, refill	S1111-3700	Starlab
TipOne® Pipette Tip 1000 µl, refill	S1111-6700	Starlab
TipOne® Pipette Tip 200 µl, refill	S1111-1700	Starlab

### 3.1.1 Technical equipment

**Table 2: List of technical equipment**

Equipment	Provider
Avanti® J-26S XPI	Beckman Coulter
Bioruptor®Pico	Diagenode
Buffer Puffer™ Gel systems	OWL
Centrifuge 5424	Eppendorf
CO <sub>2</sub> Incubator BD 115	Binder
Corning® LSE™ Mini Microcentrifuge	Corning
Digital Heatblock II	VWR
DynaMag™-2 Magnet	Thermo Fisher
Electrophoresis Power Supply EPS-601	Amersham Pharmacia
EnSpire® Multimode Plate Reader	PerkinElmer
FiveEasy F20	Mettler Toledo
Gel iX 20 Imager	Intas Science Imaging
Hair Trimmer Contura (HS61)	Wella
Heating system (water) (KS 92-1)	Medres
IKA®-Schüttler MTS 4	IKA-Labortechnik
IKAMAG® RET	IKA-Labortechnik

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Isoflurane Vapor	Dräger
Kidney Holder (custom made)	Medres
Knock out box	Medres
LOG200 TC 5005-0204	Dostmann Electronic
LSM Meta 710 Axio Observer	Zeiss
Masterflex® L/S® Easy-Load® II	Cole-Parmer
Masterflex® L/S® peristaltic pump	Cole-Parmer
Microscope Stage Chamber MS4	Digitimer
Minichiller 300	Huber
Multifuge® 4 KR	Heraeus
Multitron Pro	Infors HT
NanoDrop® 1000 Spectrophotometer	Thermo Fisher
Objective heating collar OBJ-COLLAR-3342	Okolab
S1000™ Thermal Cycler	Bio-Rad
Slice anchor SHD-42/10	Multi Channel Systems
Stereo Microscope M80	Leica Microsystems
TCS SP8 gSTED 3X microscope	Leica Microsystems
TCS SP8 MP-OPO microscope	Leica Microsystems
Temperature controller H401-T-CONTROLLER	Okolab
Truelife Invio BW Double	Mercateo
Ultracentrifuge Optima MAX-XP	Beranek
Vibratome Leica VT 1200S	Leica Microsystems
Vortexer REAX2000	Heidolph
Waterbath WNB 22	Memmert

### 3.1.2 Software

**Table 3: List of software**

Software	Company
EnSpireManager	PerkinElmer
Galene 2.2.0	Sean C. Warren
Graphpad 10.1.0 (316)	GraphPad Software Inc.
ImageJ/Fiji 1.54f	Wayne Rasband
INTAS GelDoc	Intas
LAS X	Leica Microsystems

*Continued on next page*

Nanodrop100  
ZEN 2009

Thermo Fisher  
Zeiss

### 3.1.3 Chemical - Reagents

**Table 4: List of chemicals and reagents**

Chemicals/Reagents	Catalog Number/Company
0.1% (w/v) Poly-L-lysine solution	P8920/Sigma
1% (w/v) Ethidium bromide solution	2218.2/Roth
1-thioglycerol	M1753/Sigma
3-Isobutyl-1-methylxanthine (IBMX)	I5879/Sigma
6X Loading dye	R0611/Thermo Fisher
70-kDa Texas Red dextran	D1830/Invitrogen
Acetic acid	1.00056.1000/Merck
Adenosine-5'-triphosphate disodium salt hydrate (ATP)	A2383-25G/Sigma
Agarose	A9539/Sigma
Ampicillin	K029.2/Roth
Atrial natriuretic factor (1-28) trifluoroacetate (ANP)	01-4030380/Bachem
Avanafil	A12619/Hölzel
BAY 60-7550	Cay10011135/Biomol
BNP-45 (mouse) trifluoroacetate salt (BNP)	4095506.0500/BACHEM
Boric acid	B0252/Sigma
Bradykinin	J63131.MA/Alfa Aeser
Calcium chloride dihydrate (CaCl <sub>2</sub> x 2H <sub>2</sub> O)	HN04.2/Roth
Cilostamide	A13596/Hölzel
Collagenase from Clostridium histolyticum, Type IA	C9891/Sigma
cOmplete™ EDTA-free Protease Inhibitor (PIC w/o EDTA)	4693132001/Roche
D(+)-Glucose monohydrate (D(+)-Glucose x H <sub>2</sub> O)	K23604142/Merck
D(+)-Glucose	G7021/Sigma
D-Fructose	F0127/Sigma
Dimethyl sulfoxide (DMSO)	1.02952.1000/Merck
di-Sodium hydrogen phosphate heptahydrate (Na <sub>2</sub> HPO <sub>4</sub> x 7H <sub>2</sub> O)	X987.2/Roth
Dithiothreitol (DTT)	A2948.0005/Applichem
EDTA, Disodium Salt dihydrate (Na <sub>2</sub> EDTA x 2H <sub>2</sub> O)	EDTAD250/Quantum
Ethanol	9065.3/Roth

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Ethylenediamine-tetraacetic acid (EDTA)	EDS/Sigma
Evans blue	E2129/Sigma
GeneRuler 1 kb DNA ladder	SM0311/Thermo Fisher
Gibco™ 0.05% Trypsin-EDTA (1X)	25300-054/Thermo Fisher
Gibco™ DMEM - high glucose	31966-021/Thermo Fisher
Gibco™ DMEM - high glucose	D1145/Sigma
Gibco™ Fetal Bovine Serum (FBS)	10270-106/Thermo Fisher
Gibco™ GlutaMAX-I (100X)	35050-061/Thermo Fisher
Guanosine 3',5'-cyclic monophosphate (cGMP)	G7504/Sigma
HEPES	HN78.3/Roth
Hydrogen chloride (HCl)	T134/Roth
Isoflurane	103058/Piramal Healthcare
Jedi2	SML2532-5MG/Sigma
LB-Agar	X965.1/Roth
LB-Medium	X964.2/Roth
Low-melting Agarose	BS 20.47.025/Bio&Sell
Magnesium chloride hexahydrate (MgCl <sub>2</sub> x 6H <sub>2</sub> O)	2189.2/Roth
Magnesium sulphate heptahydrate (MgSO <sub>4</sub> x 7H <sub>2</sub> O)	P027.2/Roth
Nitroglycerin/glycerol trinitrate 1mg/ml	Carinopharm
Normal Saline (0.9%)	Rev.03/Fresenius Kabi
Paraformaldehyde (PFA)	P6148/Sigma
PF-04449613	PZ0349/Sigma
Phosphate buffered saline (PBS)	D5652/Sigma
Potassium chloride (KCl)	6781.1/Roth
Potassium dihydrogen phosphate (KH <sub>2</sub> PO <sub>4</sub> )	3904.1/Roth
Probenecid	Cay14981-5/Cayman
REDTaq® ReadyMix™	R2523/Sigma
Roflumilast	A10804/Hözel
S-nitroso-N-acetylpenicillamine (SNAP)	Cay82250/Biomol
Sodium chloride (NaCl)	3957.1/Roth
Sodium dodecyl sulfate (SDS)	L3771/Sigma
Sodium hydrogen carbonate (NaHCO <sub>3</sub> )	6885.2/Roth
Sodium hydroxide (NaOH)	98108.290/VWR
Sodium pyruvate solution	S8636/Sigma
Tamgesic® 1 ml	Indivior
Triethanolamine hydrochloride (TEA/HCl)	T1502/Sigma

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Tris 1 M pH8.0 RNasefree	AM9855G/Ambion
Triton® X-100	A4975/AppliChem
TriTrack DNA Loading Dye (6X)	R1161/Thermo Fisher
Trizma® base	T1503/Sigma
Trizma® hydrochloride (Tris-HCl)	T3253/Sigma
Water	W1754-1VL/Sigma

### 3.1.4 Solutions - Buffer - Gels - Cell culture media

**Table 5: List of solutions**

Solution/Buffer	Composition										
<u>LB-Agar plate with Ampicillin</u>	Dissolve 32.0 g of LB-Agar in 1.0 L ddH <sub>2</sub> O Sterilize by autoclaving Cool to 50°C in a temperature-controlled water bath Add 1 ml of 100 mg/ml Ampicillin (Sterile filtered) Homogenize for 2 min on a magnetic stirrer Pour into plates										
<u>Liquid LB-Medium</u>	Dissolve 20.0 g LB-Medium in 1.0 L ddH <sub>2</sub> O Sterilize by autoclaving Cool to RT and store at 4°C → 100 ml with Ampicillin Add 100 µl of 100 mg/ml Ampicillin (Sterile filtered)										
<u>Agarose Gel</u>	→ TAE-Buffer 25X <table> <tr> <td>Trizma Base</td> <td>121.0 g</td> </tr> <tr> <td>Acetic acid</td> <td>28.5 ml</td> </tr> <tr> <td>Na<sub>2</sub>EDTA x 2H<sub>2</sub>O</td> <td>18.6 g</td> </tr> </table> Dissolve in 1.0 L ddH <sub>2</sub> O → TAE-Buffer 1X <table> <tr> <td>TAE-Buffer 25X</td> <td>80.0 ml</td> </tr> </table> Add 3.6 L ddH <sub>2</sub> O Add 200 µl 1% (w/v) Ethidium bromide solution → 1%/ 2% (w/v) Agarose Gel <table> <tr> <td>Dissolve 4.0 g/8.0 g Agarose in 400 ml TAE-Buffer 1X</td> <td></td> </tr> </table> Boil in the microwave and store at 60°C	Trizma Base	121.0 g	Acetic acid	28.5 ml	Na <sub>2</sub> EDTA x 2H <sub>2</sub> O	18.6 g	TAE-Buffer 25X	80.0 ml	Dissolve 4.0 g/8.0 g Agarose in 400 ml TAE-Buffer 1X	
Trizma Base	121.0 g										
Acetic acid	28.5 ml										
Na <sub>2</sub> EDTA x 2H <sub>2</sub> O	18.6 g										
TAE-Buffer 25X	80.0 ml										
Dissolve 4.0 g/8.0 g Agarose in 400 ml TAE-Buffer 1X											

DMEM/10% (v/v) FBS

Add 50.0 ml FBS to 500 ml DMEM (Cat. No. 31966-021)

Phenol red free DMEM

Add 50.0 ml FBS to 500 ml DMEM (Cat. No. D1145)

GlutaMAX-I 5.0 ml

Sodium pyruvate solution 5.0 ml

Transfection Reagent

→ 0.25 M CaCl<sub>2</sub>

Dissolve 18.4 g CaCl<sub>2</sub> x 2H<sub>2</sub>O in 500 ml ddH<sub>2</sub>O

Filter sterile with 0.22 µm (bottle top filter)

→ 2X HEBS (Brian's Mix)

HEPES Acid 10.0 g

NaCl 16.0 g

KCl 0.7 g

Na<sub>2</sub>HPO<sub>4</sub> x 7H<sub>2</sub>O 0.3 g

D(+)-Glucose 2.0 g

10X PBS 10.0 ml

Dissolve in 900 ml ddH<sub>2</sub>O

Adjust pH to 7.09 (with approx. 3 ml 5N NaOH, then with 1 N NaOH exactly to pH 7.09)

Fill up to 1.0 L with ddH<sub>2</sub>O and filter sterile with 22 µm (bottle top filter)

Homogenization buffer (50.0 ml)

(50 mM) NaCl 146.1 mg

(1 mM) EDTA 18.6 mg

(50 mM) TEA/HCl 464.1 mg

Adjust pH to 7.4 with NaOH

Fill up to 50.0 ml with ddH<sub>2</sub>O and store at RT

→ ready-to-use

For 12 dishes, prepared on the day of harvest

Homogenization buffer 4.2 ml

(2 mM) 1 M DTT 8.4 µl

PIC w/o EDTA 1.7 µl

Buffer A (50.0 ml)

(50 mM)	TEA/HCl	464.1 mg
	Adjust pH to 7.4 with NaOH	
	Fill up to 50.0 ml with ddH <sub>2</sub> O and store at RT	
→ ready-to-use	Prepared on the day of measurement	
	Buffer A	3.7 ml
(2 mM)	1 M DTT	7.4 µl
(10 mM)	1 M MgCl <sub>2</sub>	36.8 µl

Base Solution 50X (50.0 ml)

	5 N NaOH	12.5 ml
	0.5 M EDTA	1.0 ml
	ddH <sub>2</sub> O	36.5 ml
→ ready-to-use (1X)	Stock solution 50X	0.5 ml
	ddH <sub>2</sub> O	24.5 ml
	pH 12 (self-adjusting)	

Neutralization Solution 50X (50.0 ml)

	Tris-HCl	15.8 g
	Fill up to 50.0 ml with ddH <sub>2</sub> O	
→ ready-to-use (1X)	Stock solution 50X	0.5 ml
	ddH <sub>2</sub> O	24.5 ml
	pH 5 (self-adjusting)	

HBSS Solution A 10X (1.0 L)

	KCl	4.0 g
	Na <sub>2</sub> HPO <sub>4</sub> x 7H <sub>2</sub> O	0.9 g
	KH <sub>2</sub> PO <sub>4</sub>	0.6 g
	NaHCO <sub>3</sub>	3.5 g
	NaCl	80.0 g
	D(+)-Glucose	10.0 g
	Add ddH <sub>2</sub> O to 1.0 L and adjust pH to 7.4	
	Filter sterile and store at 4°C	

HBSS Solution B 10X (1.0 L)

CaCl <sub>2</sub> x 2H <sub>2</sub> O	1.9 g
MgCl <sub>2</sub> x 6H <sub>2</sub> O	1.0 g
MgSO <sub>4</sub> x 7H <sub>2</sub> O	1.0 g
Fill up to 1.0 L with ddH <sub>2</sub> O and store at 4°C	

HBSS Buffer 1X (ready-to-use)

1 ml Solution A + 1 ml Solution B  
Add 8 ml ddH<sub>2</sub>O and store at 4°C

Imaging buffer (pH 7.4) (1.0 L)

140 mM NaCl	8.2 g
5 mM KCl	0.4 g
1.2 mM MgSO <sub>4</sub> x 7H <sub>2</sub> O	0.3 g
2 mM CaCl <sub>2</sub> x 2H <sub>2</sub> O	0.3 g
5 mM HEPES	1.2 g
Fill up to 1.0 L with ddH <sub>2</sub> O	
Adjust to pH 7.4 with NaOH	
→ 1 M D(+)-Glucose x H <sub>2</sub> O	Dissolve 9.9 g in 50.0 ml ddH <sub>2</sub> O, Store at 4°C
→ ready-to-use	Prepared on the day of measurement
(5 mM)	Add 1 M D(+)-Glucose x H <sub>2</sub> O 5.0 ml

10X KHB stock solution (2.0 L)

→ Solution A (1.0 L)	CaCl <sub>2</sub> x 2H <sub>2</sub> O	7.34 g
	Fill up to 1.0 L with ddH <sub>2</sub> O	
→ Solution B (1.0 L)	KCl	7.46 g
	NaCl	138 g
	MgSO <sub>4</sub> x 7H <sub>2</sub> O	5.42 g
	KH <sub>2</sub> PO <sub>4</sub>	3.26 g
	Fill up to 1.0 L with ddH <sub>2</sub> O	
	Mix Solution A+B for the final stock solution	
	Store at 4°C for about 6 months	
→ ready-to-use (1X) (1.0 L)	Prepared on the day of slicing	
	10X KHB	100 ml
	NaHCO <sub>3</sub>	2.1 g
	D(+)-Glucose x H <sub>2</sub> O	5.0 g
	HEPES	2.4 g
	Fill up to 1.0 L with ice-cold ddH <sub>2</sub> O	

Clearing solution (1.0 L)

(200 mM)	Boric acid	12.36 g
(4% w/v)	SDS	40.0 g
	Fill up to 1.0 L with ddH <sub>2</sub> O	
	Adjust to pH 8.5 with NaOH	

Saturated fructose solution (20 ml)

(80.2%, w/w)	D-Fructose	20.25 g
	Add 5 ml ddH <sub>2</sub> O	
	Heat to 60-70°C (1-4 h completely dissolved)	
(0.5%, v/v)	1-Thiglycerol	100 µl
	Fill up to 20 ml with ddH <sub>2</sub> O	

**3.1.5 Testkits****Table 6: List of testkits**

Kit	Company	Catalog Number/LOT
GeneJET Plasmid Miniprep Kit	Thermo Scientific	K0503/00667076
NucleoBond® Xtra Midi Kit	Macherey-Nagel	740410.100/2206-004
Pierce™ BCA Protein Assay Kit	Thermo Scientific	23225/4F285907

**3.1.6 Genotyping primer****Table 7: List of genotyping primer**

Primer	Orientation:Sequence (5' to 3')	Amplicon size (bp)
Beta globin	F: TGCTCACACAGGATAGAGAGGGCAGG R: GGCTGTCCAAGTGATTCAGGCCATCG	494 → wt
Cre	F: ACCCGACGGTCTTTAGGG R: GCAAACGGACAGAAGCATTT	494 + 269 → tg/wt
Cdh5.cre	F: GCTCGGCCCTGGACAGAC R: GTGAAACAGCATTGCTGTCACTT	494 + 363 → tg/wt
mTmG wt	F: CTCTGCTGCCTCCTGGCTTCT R: CGAGGCGGATCACAAGCAATA	330 → wt 330 + 250 → ht
mTmG tg	R: TCAATGGGCGGGGGTTCGTT	250 → hm

wt\* wildtype, tg\* transgenic, ht\* heterozygous, hm\* homozygous

### 3.1.7 Restriction enzyme and buffer

**Table 8: List of restriction enzymes and buffer**

Enzyme/Cut Site (5' to 3')	Company	Catalog Number/LOT
EcoRI (20.000 U/ml)/(G/AATTC)	BioLabs	R0101S/0331305
NEBuffer™ EcoRI/SspI	BioLabs	B0101S/0704
NEBuffer™ 2.1	BioLabs	B7202S/10029698

### 3.1.8 Online tools/Primer for Sanger Sequencing

Primer design for sequencing: <https://benchling.com> (Benchling, Inc., San Francisco, US)

Oligo synthesis: <https://idtdna.com> (Integrated DNA Technologies, Inc., Coralville, US)

**Table 9: List of primer for Sanger sequencing**

Primer	Orientation:Sequence (5' to 3')
CFP fsp	F:GGCTAACTAGAGAACCCACTGCTTACTGGC
cGMP-BD fsp	F:AGGAGATGTGGGGTCACTGGTGTATGT
YFP Rsp	R:CACCAGCCACCACCTTCTGATAGGC

### 3.1.9 Antibodies

#### Primary:

$\alpha$ -nephrin

→ 1:100, guinea pig, 20R-NP002, Fitzgerald Industries International, Inc., Acton, US

CD31/PECAM-1

→ 1:200, goat, AF3628, R&D Systems, Inc., Minneapolis, US

GFP-Alexa647

→ 1:100, rabbit, A-31852, Thermo Fisher Scientific Inc., Massachusetts, US

#### Secondary:

donkey anti-guinea pig Atto594

→ 1:100, coupled in house

donkey anti-goat Atto594

→ 1:100, coupled in house

## 3.2 Methods

### 3.2.1 *In vitro* characterization of cGi500

The plasmid DNA MH20\_pCMV\_cGi500 (7767 bp) encoding the single-chain cGMP biosensor cGi500 (**Supplemental Figure 1**) was kindly provided by Prof. Dr. Robert Feil from the Interfaculty Institute of Biochemistry (IFIB), University of Tübingen, Germany. To elute the filter paper-spotted plasmid (5 µg) the marked area was excised and 50 µl of Tris (pH 8.0) was added to the 1.5 ml tube. Plasmid release from the filter was provoked by incubation at RT for 1 h followed by storage at 4°C. Verification of the recombinant plasmid construction was completed by Sanger sequencing (Microsynth AG, Balgach, CH, <https://srvweb.microsynth.ch/>).

#### 3.2.1.1 Bacterial transformation with Mini- & Midi preparation

Production of a sufficient quantity of cGi500 for subsequent applications required bacterial transformation with chemically competent cells. DH10B T1 served as the bacterial strain of choice with well-proven transformation efficiency and susceptibility to chemical treatment. After thawing the cells on ice, 50 µl of bacteria were added to 5 µl of plasmid DNA and mixed gently. The mixture was incubated on ice for 30 min and then subjected to a heat shock interval of 45 seconds at 42°C in a water bath. Briefly, after 2 minutes on ice, the transformation was spread onto a 10 cm LB-Agar plate containing Ampicillin (decontaminate). The dried plate was incubated at 37°C overnight. Only cells harboring the plasmid form single colonies and were picked in the afternoon for inoculation of 3 ml liquid LB-Medium with Ampicillin for a starter culture ("mini culture"). Incubation at 37°C for approximately 12-16 h hours with shaking at 250-300 rpm maintained bacterial growth with enriched cell number and high yields of plasmid DNA. The plate was sealed with parafilm and stored with the lid down at 4°C. Plasmid harvest and purification was performed using the GeneJET Plasmid Miniprep Kit according to the manufacturer's instructions with elution in 50-100 µl elution buffer (10 mM Tris-HCl, pH 8.5). Purified plasmid DNA was quantified using the NanoDrop 1000 Spectrophotometer with subsequent storage at 4°C.

A mini digest was prepared to confirm the vector construction prior to sequencing. The restriction enzyme digest was composed of 10 µl miniprepped plasmid DNA (111.2 ng/µl) along with 1 µl enzyme EcoRI + 1.5 µl NEBuffer 2.1 (10X) and filled up to 15 µl with ddH<sub>2</sub>O. The digest mixture was incubated for 30 min at 37°C in the heating block and then centrifuged for a few seconds. Virtually digested and experimentally confirmed by a 1% (w/v) agarose gel, DNA Ladder GeneRuler 1 kb, 2 µl Loading dye and electrophoresis (80 V, 400 mA, 45 min), the result consists of two DNA fragments with a length of 5433 bp and 2334 bp.



Following confirmatory Sanger sequencing with the designed primer named CFP fsp, cGMP-BD fsp, YFP Rsp, the residue of the mini culture was used to increase the yield of isolated plasmid DNA. For this purpose, 4 g LB-Medium was dissolved in 200 ml ddH<sub>2</sub>O, 200 µl of 100 mg/ml Ampicillin was added and the solution was inoculated in the afternoon. The Midi preparation was incubated in a sterile 500 ml Erlenmeyer flask overnight at 37°C with 90-120 rpm in a shaking incubator. The application of the NucleoBond™ Xtra Midi Kit according to the manufacturer's instructions completed the purification of the plasmid. Purified plasmid DNA was quantified using the NanoDrop 1000 Spectrophotometer with subsequent storage at 4°C. Eluates were diluted if the value exceeded the upper measurement range of 3000 ng/ul.

### **3.2.1.2 HEK 293T cell transient transfection and harvest**

HEK 293T cells cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 31966-021) with 10% fetal bovine serum (FBS) were splitted 1:6 in 12 x 10-cm dishes the day before transfection. On the day of transfection, 500 µl of CaCl<sub>2</sub> was added to a 1.5 ml tube for each experimental point together with 5 µg cGi500 DNA (Midi prep). While vortexing, 500 µl of 2X HEBS was added dropwise into the diluted DNA with additional 5 sec of vortexing. For each dish, 980 µl of DNA solution was added dropwise onto the cells, then the plate was moved from top to bottom and from right to left to distribute the DNA solution evenly. The cells were incubated at 37°C in a CO<sub>2</sub> incubator starting from 10 am with replacing the present medium after 7 h by 8 ml of phenol red free DMEM (Cat. No. D1145) supplemented with 10% FBS, 1% GlutaMAX-I and 1% Sodium pyruvate solution.

Two days after transfection: Dishes showing a cell confluency of 80-95% and a transfection rate of 90-95% were further processed according to the protocol published by Russwurm et al.<sup>139</sup> Transfected cells from a 10-cm dish (1x10<sup>7</sup> cells) were harvested and lysed. All preparation steps were performed on ice. After the dishes were washed twice with ice-cold 5 ml of sterile PBS, 300 µl of freshly prepared homogenization buffer (pH 7.4, ice-cold) was added. Following cell scraping, the lysate (approximately 600 µl) was transferred to sterile 1.5 ml tubes and subsequently sonicated (5 sec ON, 30 sec OFF, 1 cycle) and ultracentrifuged (100,000 g for 30 min at 4°C). The supernatant was transferred to brown 1.5 ml tubes and stored at - 20°C.

### **3.2.1.3 Bicinchoninic acid (BCA) assay**

The quantitative protein determination was performed with the Pierce™ BCA Protein Assay Kit. Components of the homogenization buffer (50 mM TEA/HCl, 2 mM DTT) are listed as interfering factors in the compatibility table for the protein assay and influence the determined protein concentration due to their metal chelating properties. Consequently, the ratio of BCA

reagent A to B was adjusted from 50:1 to 50:5 in the preparation of the BCA working reagent (WR). According to the manufacturer's instructions, the microplate procedure involved the addition of 200  $\mu$ l of WR (50:5/A:B) to 10  $\mu$ l of sample replicate (cGi500 cytosolic fractions) in a 96-well F-bottom plate, followed by 30 sec on a plate shaker and 30 min of incubation at 37°C. For another 15 min the plate was kept at RT followed by absorbance measurement at 562 nm on the EnSpire Multimode Plate Reader.

#### **3.2.1.4 Live-cell imaging of cGi500-transfected HEK 293T cells**

HEK 293T cells cultured in DMEM/10% (v/v) FBS were splitted 1:6 in 10-cm dishes the day before the cells were washed with 1X PBS and trypsinized with 1 ml for 5 min at 37°C. Trypsin inactivation was achieved by adding 9 ml of pre-warmed supplemented phenol red free DMEM. The cell suspension was diluted (1 ml + 4 ml supplemented phenol red free DMEM) and sub-cultivated in  $\mu$ -Slide 8 Well (ibiTreat) chambers. The next day, the cells were transfected as described in chapter 3.2.1.2 using 150 ng cGi500 DNA with media exchange after 6 h. One day later, the experiment was performed by exchanging the medium with freshly prepared imaging buffer (RT) and incubating the cells for 20 min. Administration of isobutylmethylxanthine (IBMX, stock: 10 mM dissolved in DMSO), a cGMP-enhancing compound, was performed by pipetting 50  $\mu$ l of a 600  $\mu$ M predilution directly into the imaging-buffer filled IbiWell (250  $\mu$ l). Time-lapse images were captured every 2 sec with a confocal microscope (LSM Meta 710, Inverse, Axio Observer, Carl Zeiss AG) equipped with a Plan-Apochromat 20x/0.8 M27 objective (Zeiss) in conjunction with ZEN 2009 (Zeiss). Donor fluorophore (CFP) excitation was performed at 405 nm together with a dichroic beam splitter (MBS-405) and simultaneous detection of CFP and YFP emission at  $480 \pm 25$  nm and  $535 \pm 20$  nm. Intensity changes in the 512 x 512 x 16-bit emission images (mean fluorescence intensity) were offline analyzed to calculate the baseline-normalized CFP/YFP emission ratio ( $\Delta R/R_0$ ) reflecting cGMP concentration changes.

#### **3.2.2 Mouse lines**

Animals were housed according to standardized specific pathogen-free conditions in CECAD's *in vivo* research facility (ivRF). Kept in individually ventilated cage systems (IVC cages type II long) with centrally monitored light periodicity (light/dark cycle 12h/12h,  $55 \pm 10\%$  humidity,  $22 \pm 2^\circ\text{C}$  room temperature), they had access to standard rodent chow and tap water *ad libitum*. The experimental procedures were evaluated and approved by the State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV NRW) VSG 81-02.04.2018.A351 and 4.18.023).

The 'floxed' cGi500 sensor line Gt(ROSA)26Sor<sup>tm1(CAG-ECFP/EYFP\*)</sup>Feil (mixed background 129 SV, C57BL/6N, C57BL6/NRj >70%) was provided by Prof. Dr. Robert Feil from the Interfaculty Institute of Biochemistry (IFIB), University of Tübingen, Germany and generation was described previously.<sup>140</sup> Cell-specific Cre-driver mouse lines were leveraged for crossbreeding to achieve cytosolic cGi500 biosensor expression in podocytes (Pod:Cre line<sup>144</sup> resulting in Pod:Cre/cGi500) or endothelial cells (Tie2:Cre line<sup>145</sup> or Cdh5:Cre line<sup>146</sup> resulting in Tie2:Cre/cGi500 or Cdh5:Cre/cGi500). Experiments were performed with Pod:Cre/cGi500 mice carrying two cGi500 alleles, whereas Tie2:Cre/cGi500 or Cdh5:Cre/cGi500 mice carried one cGi500 allele. Reported nonspecific recombination in the F2 generation of Tie2:Cre mice restricted its use to the F1 generation.<sup>147,148</sup> To exclude gender-specific variations, mice of both sexes were used at 8-27 weeks of age.

### **3.2.2.1 Genotyping**

#### **3.2.2.1.1 HotSHOT DNA preparation**

The ear puncture of the laboratory mice *Mus musculus* served as identification and tissue sampling for the preparation of genomic DNA using HotSHOT.<sup>149</sup> The biopsy material stored at -20°C was mixed with 75 µl base solution (alkaline lysis reagent) and incubated at 95°C for 30 min. Cooled to 4°C-10°C, the same amount of neutralization solution was added with final vortexing.

#### **3.2.2.1.2 Setup of polymerase chain reaction (PCR)**

To prepare the PCR reaction, the REDTaq® ReadyMix™ PCR reaction mix was used according to the manufacturer's instructions with the addition of the specific primers listed in section 3.1.6. Gel electrophoresis at 120 V, 400 mA for 45 min in combination with a 2% (w/v) agarose gel was used to separate PCR products (12 µl/sample) according to their fragment length in base pairs.

**Table 10: PCR and cycling conditions**

Reaction composition	Cycling conditions
<p><b>ROSA26</b></p> <p>5 µl ddH<sub>2</sub>O</p> <p>1 µl mTmG wt_F (10 µM)</p> <p>1 µl mTmG wt_R (10 µM)</p> <p>1 µl mTmG tg_R (10 µM)</p> <p>10 µl REDTaq®</p> <p>2 µl DNA</p>	<p><b>ROSA26</b></p> <p>94°C 3 min</p> <p>94°C 30 sec</p> <p>61°C 60 sec</p> <p>72°C 60 sec</p> <p>goto step 2,</p> <p>repeat 35 times</p> <p>72°C 120 sec</p> <p>10°C for ever</p>
<p><b>CRE</b></p> <p>5 µl ddH<sub>2</sub>O</p> <p>1 µl Beta globin_F (10 µM)</p> <p>1 µl Beta globin_R (10 µM)</p> <p>1 µl Cre_F (10 µM)</p> <p>1 µl Cre_R (10 µM)</p> <p>10 µl REDTaq®</p> <p>2 µl DNA</p>	<p><b>CRE</b></p> <p>94°C 3 min</p> <p>94°C 45 sec</p> <p>59°C 60 sec</p> <p>72°C 45 sec</p> <p>goto step 2,</p> <p>repeat 30 times</p> <p>72°C 10 min</p> <p>10°C for ever</p>
<p><b>Cdh5</b></p> <p>5 µl ddH<sub>2</sub>O</p> <p>1 µl Beta globin_F (10 µM)</p> <p>1 µl Beta globin_R (10 µM)</p> <p>1 µl Cdh5.cre_F (10 µM)</p> <p>1 µl Cdh5.cre_R (10 µM)</p> <p>10 µl REDTaq®</p> <p>2 µl DNA</p>	<p><b>Cdh5</b></p> <p>94°C 3 min</p> <p>94°C 30 sec</p> <p>58°C 30 sec</p> <p>72°C 45 sec</p> <p>goto step 2,</p> <p>repeat 35 times</p> <p>72°C 5 min</p> <p>15°C for ever</p>

### 3.2.2.2 Isolation of mouse glomeruli

Isolation of mouse glomeruli was prepared according to the protocol of Takemoto et al.<sup>150</sup> Modifications started with euthanizing the animals directly by cervical dislocation without prior anesthesia. The kidneys were removed from the abdomen with all connected vessels including the aorta and stored intermediately in ice-cold HBSS (Hank's Balanced Salt Solution) buffer. After removal of muscle tissue and fat, the aorta was longitudinally incised to allow insertion of a 27G needle into the renal artery. This enabled perfusion with Dynabeads M-450 tosyl-activated, bead size  $\phi$  4.5 µm (20 µl/ml HBSS) followed by removal of the kidney capsule. Cutting the kidney into small pieces preceded digestion with Collagenase IA (1 mg/ml) at 37°C for 20 min with gentle shaking. After digestion, the tissue-solution mixture was homogenized using a 1000 µl pipette tip (tip cut off) and transferred to a 100 µm filter (sitting on top of a 50 ml Falcon on ice). The tissue was gently pushed through the filter with the cone of a 5-ml syringe, interspersed with several washes with 3-5 ml of ice-cold HBSS buffer. This procedure was repeated with a second 100-µm filter before the flow-through was loaded into several 1.5-ml tubes placed in the DynaMag™-2 Magnet. The magnetic beads in the capillary loops of the glomeruli cause adhesion to the magnet so that the remaining eluate can be removed. After

removal of the magnet, the isolated glomeruli are pooled in 1 ml HBSS buffer in a 1.5 ml tube and stored on ice under exclusion of light for further use. In order to keep the quality of the isolation constant and to identify possible sources of error, the time of sacrifice, the end of isolation as well as the quality of perfusion were noted.

#### **3.2.2.2.1 Poly-L-lysine coating**

Immobilization of isolated glomeruli for real-time imaging was performed by coating coverslips ( $\emptyset$  18 mm, #1.5) with a 0.1% (w/v) poly-L-lysine solution (dropwise applied) for 15 min in a large petri dish. After incubation, the coverslips were washed three times with ddH<sub>2</sub>O, placed on a tissue paper to dry, and sterilized under UV light (15 min). After transferring to the large petri dish and sealing with the lid, the coated coverslips were stored at RT.

#### **3.2.2.2.2 Custom-built imaging chamber and perfusion system**

Real-time imaging requirements were met by fabricating an imaging chamber that allows insertion of coverslips ( $\emptyset$  18 mm, #1.5) with an interfaced perfusion system. The construction of the imaging chamber and the microscope stage insert (**Figure 5**) was realized in cooperation with the Zentrale Wissenschaftliche Werkstatt of the Medical Faculty, University of Cologne. The complete superfusion system is shown in **Figure 5D**, demonstrating where the side ports of the microscope stage insert will be connected:

- (1) Original Perfusor® Line, Type: IV Standard-PE
- (2) Masterflex®, Peroxide-Cured Silicone Tubing; L/S 13
- (3) Masterflex® L/S® peristaltic pump (Cat. No. 07555-15) with Masterflex® L/S® Easy-Load® II
- (4) The tubing end of the Original Perfusor® Line inserted in 1 ml Stripette®

The setup allowed image acquisition while the submerged glomeruli were continuously rinsed with buffer or stimulants/inhibitors. Restriction of flow to one spot in the center of the coverslip resulted in consistent exposure of chemicals. Therefore, immobilization of the glomeruli on the poly-L-lysine coated coverslips was a critical necessity.



**Figure 5: Custom-built imaging chamber and perfusion system for real-time imaging of isolated glomeruli**

(A) Closed imaging chamber. (B) Upper ring (left) and lower ring with coverslip cavity (right). (C) Microscope stage insert (pink) with yellow inlet and outlet needle functioning as side ports for fluid exchange. (D) Assembled superfusion system.

### 3.2.2.2.3 Real-time imaging of isolated glomeruli

Prior to FRET measurements, the tubing of the assembled superfusion system was flushed with 70% EtOH and freshly prepared imaging buffer (RT). Isolated glomeruli (10  $\mu$ l) from Pod:Cre/cGi500 mice were positioned centrally onto the submerged coated coverslip, allowing them to stick onto the surface for about 5 min. To obtain a stable baseline, the cGi500-expressing glomeruli were superfused for 5 min with imaging buffer (flow rate: 1 ml/min). Images were captured every 2 sec, recording 40 frames for baseline, followed by drug exposure (frame 40 to 305) and wash-out with imaging buffer (RT) until 570 frames were acquired. FRET/cGi500 imaging was performed with a confocal microscope (LSM Meta 710, Inverse, Axio Observer, Carl Zeiss AG) equipped with a Plan-Apochromat 20x/0.8 M27 objective (Zeiss) in conjunction with ZEN 2009 (Zeiss). Donor fluorophore (CFP) excitation was performed at 405 nm together with a dichroic beam splitter (MBS-405) and simultaneous

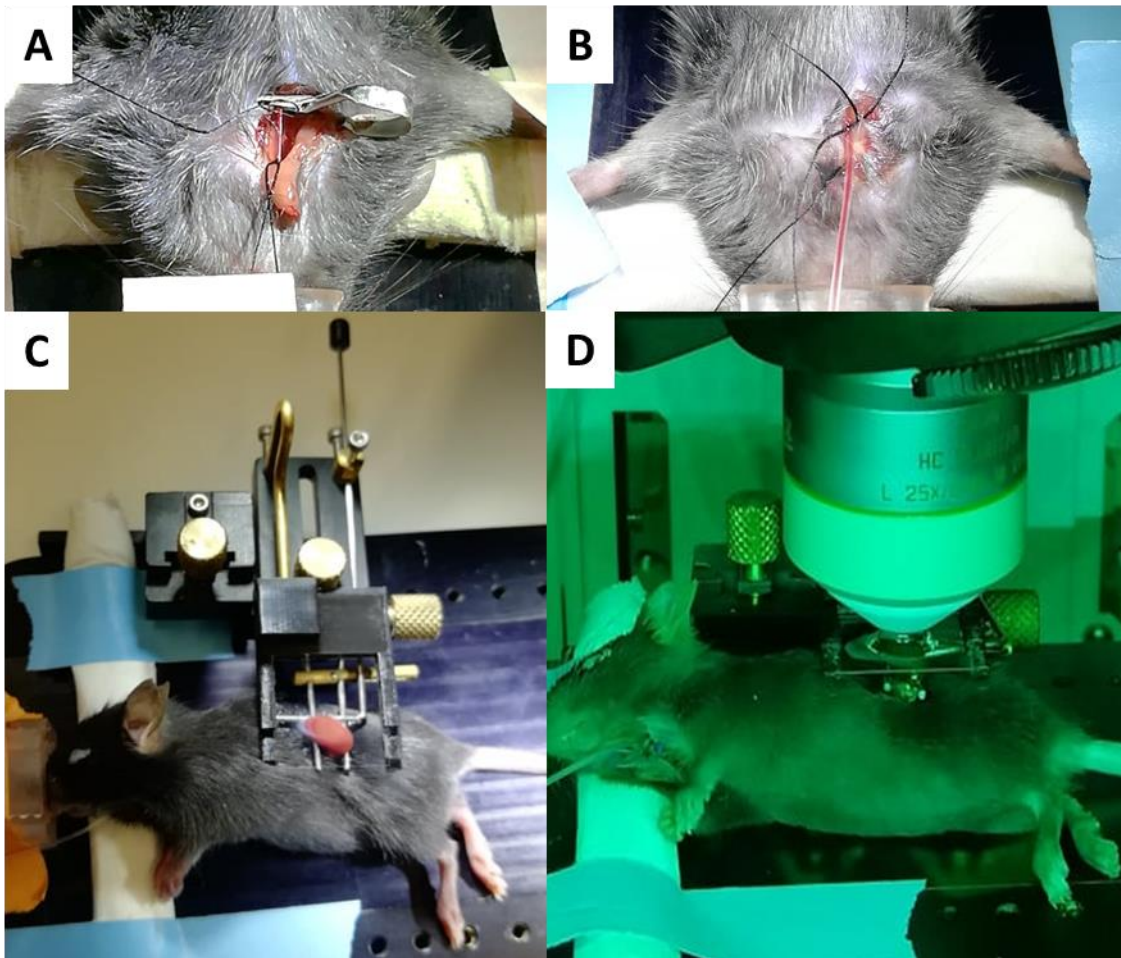
detection of CFP and YFP emission at  $480 \pm 25$  nm and  $535 \pm 20$  nm. Intensity changes in the 512 x 512 x 16-bit emission images (mean fluorescence intensity) were offline analyzed to calculate the baseline-normalized CFP/YFP emission ratio ( $\Delta R/R_0$ ) reflecting cGMP concentration changes.

Real-time imaging of acute kidney slices is described in detail in **chapter 5.2.6**.

### **3.2.2.3 Intravital imaging (IVI)**

Pod:Cre/cGi500 mice (3-4 weeks old) were prepared for intravital imaging according to the procedure published by Binz-Lotter et al.<sup>151</sup> Anesthetized/analgesized with isoflurane/buprenorphine (0.05 mg/kg), the mouse was fixed on the temperature-controlled surgery stage and underwent catheterization (Polyethylene tubing (PE10), Braintree Scientific, Cat. No. B-PE-10-100FT) of the right carotid artery through a longitudinal incision (**Figure 6**). The mouse was repositioned, shaved at the incision site to exteriorize the kidney and subsequently immobilized in a custom-build kidney holder (Medres, medical research company GmbH) for imaging. Intravital imaging was performed using an upright multiphoton microscope (TCS SP8 MP-OPO, DM 6000 CFS, Leica Microsystems) with an IR Apo L25x/0.95 W objective (Leica) in conjunction with LAS X (Leica software). Biosensor excitation was performed at 860 nm together with a YFP/DsRed (BP 535/30) filter and simultaneous detection of CFP and YFP emission at 467-499 nm and 500-550 nm (HyD-RLD detector) with following settings: 1000 Hz, Bidirectional ON, Line Average 2. Blood circulation was labeled with 70 kDa Texas Red dextran dissolved in 0.9% NaCl.

Intensity changes in the 512 x 512 x 16-bit emission images (mean fluorescence intensity) were offline analyzed to calculate the baseline-normalized CFP/YFP emission ratio ( $\Delta R/R_0$ ) reflecting cGMP concentration changes.



**Figure 6: Surgery procedure for intravital imaging**

(A) Isolation of the right carotid artery with caudal occlusion (clamp) and cranial blockage by a knot. (B) Successful catheterization was evidenced by the inflow of blood into the PE hose filled with saline solution. The catheter was secured by an additional knot around the artery. (C) Immobilized kidney clamped in the custom-build holder with positioned coverslip. (D) Mouse placed under the upright microscope awaiting injection of the test substance through the inserted catheter.

### 3.3 Analysis pipeline

Data analysis was conducted using ImageJ/Fiji (NIH) and Excel (Microsoft Corporation).

#### 3.3.1 Selection

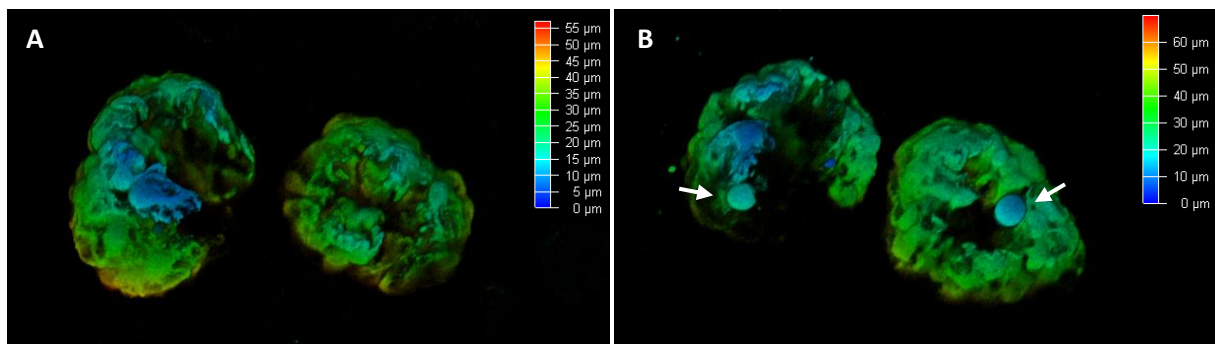
**HEK 293T cells:** Analyzed cells of the FRET/cGi500/HEK 293T imaging data were selected based on cell morphology, sensor localization (homogeneously distributed, no bright spots) and fluorescence brightness (avoidance of dim and very bright cells).

**Isolated glomeruli:** Due to the separation process, isolated glomeruli are subjected to mechanical forces that lead to cell detachment. Only glomeruli with preserved integrity and



minor structural changes were analyzed. The possibility of damaged cells within this cell cluster cannot be excluded.

**Acute kidney slices (AKS):** Glomeruli damaged by the cutting process of AKS display noticeable morphological changes, which are evident in the form of swollen and rounded cellular structures (**Figure 7**). ROIs (whole glomeruli) exhibiting increased intensity in the Evans Blue detection channel were excluded from the analysis due to the azo dye's ability to permeate through ruptured membranes, resulting in cellular staining.



**Figure 7: Identification of damaged cells in glomeruli close to the cutting surface**

Representative images of glomeruli identified as undamaged (**A**) or damaged (**B**, white arrows) by their morphology using Z-stack depth coding.

**IVI:** Intravital imaging was performed on superficial glomeruli due to the limitations associated with multiphoton microscopy in terms of tissue penetration depth caused by high cellular heterogeneity of the kidney.

### 3.3.2 Line scan analysis – Intensity profile plot

Evidence of pixel-precise alignment of the acquired CFP and YFP images is particularly important for ratiometric time-lapse FRET analysis and indicates colocalization of the two fluorophores, functional basis of the intramolecular FRET biosensor.

- ImageJ commands

Load raw data>Select line width tool \*Straight\*>Select line width>Add line to ROI Manager

Image>Color>Split Channels

Analyze>Plot Profile for CFP and YFP channel>Data>Save data (Excel)>Normalize to highest value

### 3.3.3 CFP/YFP Ratio images

The changing FRET ratio (CFP/YFP) served as an index of the cGMP increase in HEK 293T cells after IBMX administration and was visualized according to the protocol published by Kardash et al. with minor modifications.<sup>152</sup>

- ImageJ commands

Load raw data>ROI selection for Crop>Crop both channels: Image>Crop>Image>Color>Split Channels

32-Bit conversion (necessary for thresholding): For both channels: Image>Type>32 bit

Threshold (Only for YFP image): Image>Adjust>Threshold>Select Default, B&W, Dark Background>Press Apply>Set to NaN

Ratio CFP/YFP: Plugins>Ratio Plus>CFP image for image 1, YFP image for image 2

Pseudo-color (Lookup Tables): Click on the new Ratio image: Image>Lookup table>Green Fire Blue>Analyze>Tools>Calibration bar

Scale bar: Analyze>Tools>Scale bar

### 3.3.4 Movement correction (Galene)

Raw imaging data from real-time measurements of AKS and IVI were processed with the open-source package Galene.<sup>153</sup> Sample motion was corrected with realignment mode: Warp, points: 10.

### 3.3.5 Image segmentation

The image segmentation approach published by Thunemann et al. was applied to IVI data to visualize FRET changes evoked by the binding of cGMP to cGi500 *in vivo*.<sup>142</sup>

### 3.3.6 Calculation of baseline-normalized CFP/YFP ratio ( $\Delta R/R_0$ )

For FRET/cGi500 imaging data selected ROIs (region of interest) defined a whole cell (HEK 293T) or a single glomerulus (isolated glomeruli, AKS, IVI). The  $CFP_{ROI(t)}$  and  $YFP_{ROI(t)}$  emission intensities from the time-laps recordings were extracted and saved (Excel) followed by baseline normalization (HEK 293T, AKS, IVI: 0-20 frames, Isolated glomeruli: 0-40 frames) leading to  $\Delta F/F_0$  values (formula not shown). Prior to baseline normalization, division of  $CFP_{ROI(t)}$  by  $YFP_{ROI(t)}$  resulted in CFP/YFP emission intensity ratios reflecting cGMP concentration changes as  $\Delta R/R_0$  (**Formula 1-3**).

CFP/YFP emission intensity ratio  $R(t) = \frac{CFP_{ROI(t)}}{YFP_{ROI(t)}} \quad (1)$

Baseline normalization  $R_0 = \frac{1}{n} \sum_{t_0}^{t_1} R(t) \quad (2)$

%Single change relative to baseline  $\frac{\Delta R}{R_0} = \frac{R(t) - R_0}{R_0} \times 100\% \quad (3)$

(t) Time dependency

(n) Obtained baseline frames: 20 or 40

(t<sub>0</sub> to t<sub>1</sub>) Ratio values during the baseline period (20 or 40 frames)

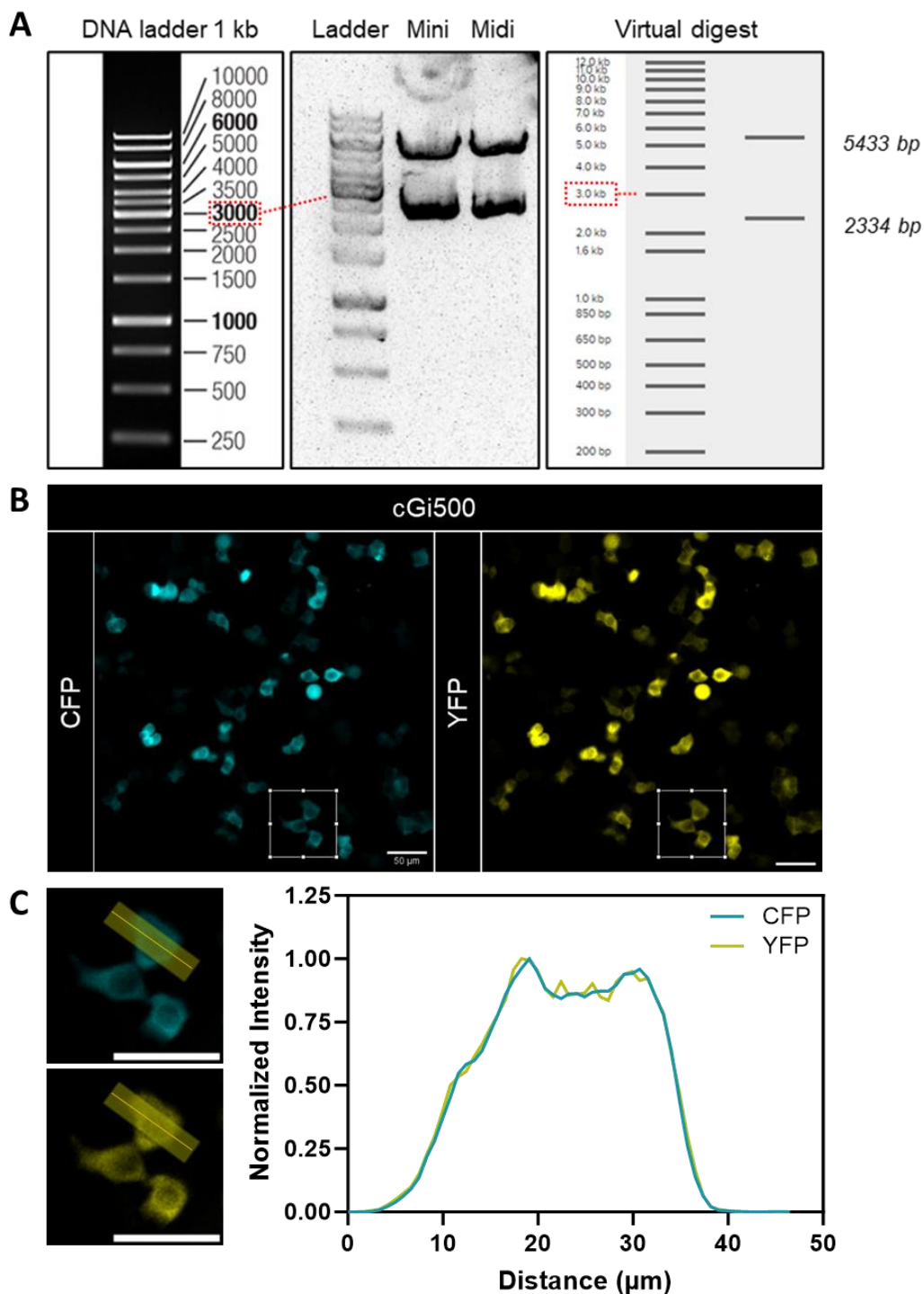
### 3.4 Statistics

Statistics were performed using GraphPad Prism 10.1.0 (Graphpad Software, Inc.) utilizing a paired, two-tailed Student's t-test or One-way ANOVA (Tukey's post hoc test or Dunnett T3, Šídák's multiple comparisons test) to assess differences between groups. Differences between groups were considered statistically significant if  $p < 0.05$  (P value style "GP"). Results are reported as mean  $\pm$  standard error of the mean (SEM). Figure legends indicate the number of animals used.

## 4 Results

### 4.1 Functional cGMP biosensor (cGi500) expression in HEK 293T cells

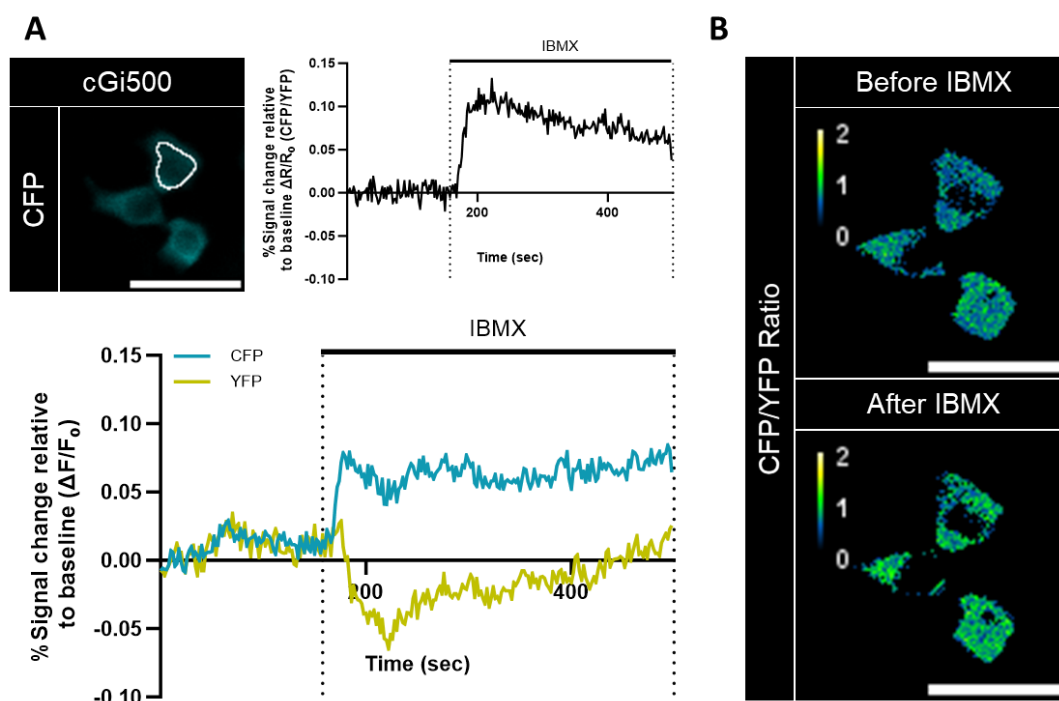
For the systematic characterization of the FRET-based ratiometric biosensor, HEK 293T cells were used as a cellular tool. Based on horizontal gene transfer, the plasmid MH20\_pCMV\_cGi500 (7767 bp) encoding the single-chain cGMP biosensor cGi500 was successfully transfected into the host cells, leading to recombinant protein expression. Sanger sequencing and restriction enzyme digestion verified the recombinant plasmid sequence and matched the expected results of the virtual EcoRI digest, resulting in 2334 bp and 5433 bp long fragments (**Figure 8A**). Transfected adherent HEK 293T cells exhibited homogenous expression of the cGMP biosensor, primarily within the cytosolic region. Following donor fluorophore (CFP) excitation of the FRET construct at 405 nm, the cGMP equilibrium was visualized by simultaneous emission detection of CFP and YFP at  $480 \pm 25$  nm and  $535 \pm 20$  nm (**Figure 8B**). For the subsequent functional validation of the sensor, pixel-precise alignment of the emission FRET images was a fundamental component, as deviations can lead to a weaker FRET response based on the calculated %signal change from CFP/YFP emission ratios. The line scan analysis involved averaging pixels along a designated line (width: 15 pixels), which extended diagonally across the cell periphery through the soma. The intensity profiles of CFP and YFP were normalized to the highest value and were superimposed, confirming the absence of misalignment (**Figure 8C**).



**Figure 8: cGi500 biosensor expression in HEK 293T cells**

(A) EcoRI restriction enzyme digest of mini- & midiprep plasmid DNA encoding cGi500 and virtual digest performed with the online tool Benchling. DNA ladder GeneRuler 1 kb (Figure origin: reference <sup>154</sup>). (B) Representative low-magnification confocal fluorescence images of cGi500-expressing HEK 293T cells. The CFP donor fluorophore is depicted in cyan, while the YFP acceptor fluorophore is represented in yellow. (C) Zoomed inset with diagonally positioned scan line across the cell and corresponding intensity profile plots of CFP and YFP (data was normalized to the highest value). Scale bar: 50  $\mu$ m.

The working principle of the FRET construct was tested through the administration of IBMX, a nonspecific phosphodiesterase (PDE) inhibitor. This substance inhibits endogenous PDE isoforms responsible for cGMP hydrolysis, which results in the accumulation of intracellular cGMP. Time-lapse recordings for live cell imaging of cGi500-transfected HEK 293T cells included acquisition of a stable baseline followed by IBMX exposure, resulting in antiparallel changes in emission intensity of the donor and acceptor fluorophore in response to cGMP accumulation (**Figure 9A**). In particular, the interaction of cGMP with the indicator protein of the sensor (tandem cGMP-binding sites from bovine cGMP-dependent protein kinase type I) positioned between CFP and YFP prompts a conformational change, resulting in a reduction in FRET efficiency. This manifests as an increase in CFP emission and a concurrent decrease in YFP emission. The baseline-normalized CFP/YFP emission ratios ( $\Delta R/R_0$ ) reflect changes in cGMP concentration. Upon the direct addition of IBMX to the imaging buffer-filled well, a rapid increase in cGMP levels was initially observed. However, by the end of the measurement, the response flattened due to the distribution of the added substance (**Figure 9A**). CFP/YFP ratio images visualized changes in subcellular cGMP dynamics after IBMX administration (**Figure 9B**).



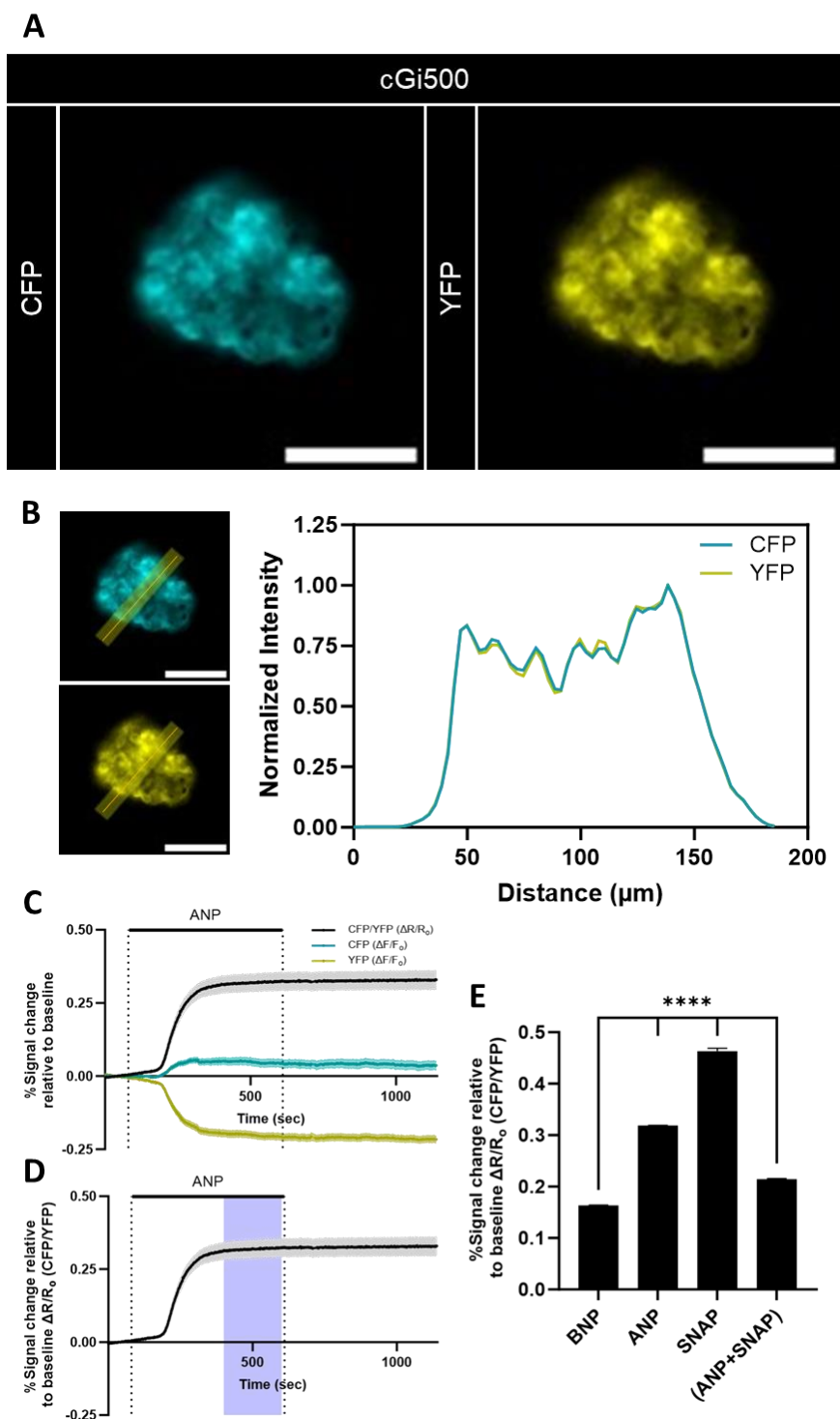
**Figure 9: IBMX-induced cGMP accumulation in cGi500-transfected HEK 293T cell**

(A) Representative CFP emission image of cGi500-expressing HEK 293T cells with superimposed ROI (white). Time-lapse recordings represent baseline-normalized emission intensity changes of the individual CFP (cyan) and YFP (yellow) traces ( $\Delta F/F_0$ ) or the CFP/YFP ratio (black,  $\Delta R/R_0$ ) upon IBMX administration (50  $\mu$ l of 600  $\mu$ M IBMX stock = final concentration 100  $\mu$ M) starting at 160 sec. (B)

CFP/YFP ratio image at frame 5 (10 sec, before IBMX) and frame 120 (240 sec, after IBMX) visualizes an increased FRET response after IBMX administration reflecting intracellular cGMP accumulation. Scale bar: 50  $\mu$ M.

## 4.2 Targeting the NO/sGC/cGMP and NP/pGC/cGMP axis

Transgenic mice expressing the cGMP biosensor exclusively in the cytosol of podocytes (Pod:Cre/cGi500) were euthanized to prepare isolated glomeruli (**Figure 10A**). Line scan analysis across the glomerulus, the branched capillary system, demonstrated the colocalization of the donor and acceptor fluorophore with pixel-precise alignment (**Figure 10B**). A superfusion system facilitated the introduction and removal of test substances, enabling real-time observation of their impact on cGMP signaling due to changes in FRET signal intensity. The isolated glomeruli were superfused with imaging buffer for an 80-second baseline recording followed by the stimulation with test compounds (530 sec) and subsequent wash-out. Regarding the NP/pGC/cGMP axis, the natriuretic peptides ANP and BNP served as ligands for the natriuretic peptide receptors GC-A or GC-B. To stimulate the NO/sGC/cGMP axis, the widely used NO donor SNAP was administered. Its NO-releasing property was induced 10 minutes before the start of measurements by adding the stock solution directly into the imaging buffer (pH 7.4, ambient temperature). All test substances provoked an increase in intracellular cGMP concentration, suggesting the presence of both guanylyl cyclase systems (pGC and sGC) in podocytes (**Figure 10E**). However, ANP induced a stronger response compared to BNP and was chosen for further experiments. The cGMP synthesis induced by SNAP exhibited the most pronounced FRET change in comparison to all other tested substances. Co-stimulation of ANP+SNAP did not demonstrate an additive effect in podocytes, indicating a reciprocal interaction between cGMP-synthesizing systems.



**Figure 10: NP/pGC and NO/sGC induced cGMP synthesis in isolated glomeruli**

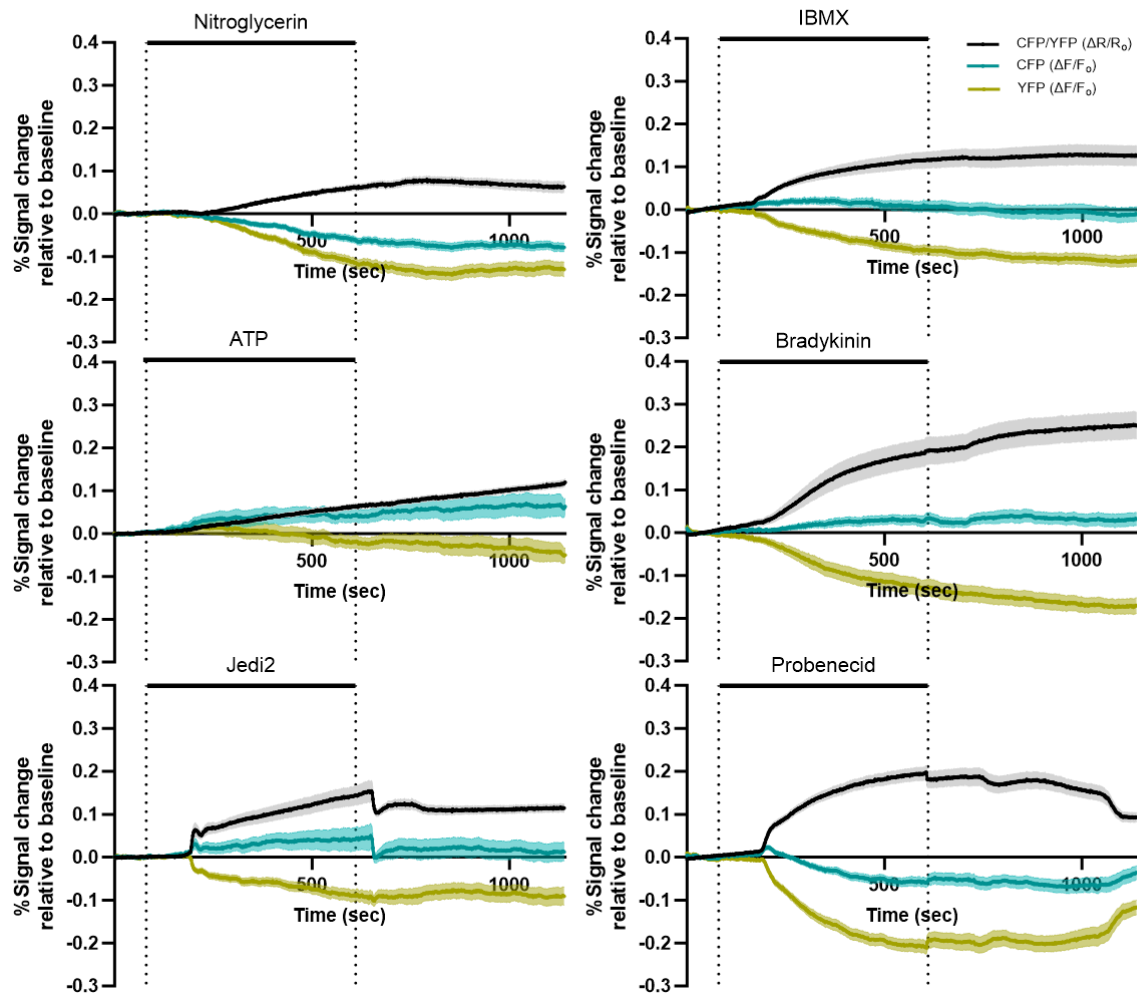
(A) Representative confocal fluorescence images of a glomerulus isolated from a transgenic mouse kidney expressing the cGMP biosensor in podocytes (Pod:Cre/cGi500). The CFP donor fluorophore is depicted in a cyan color, while the YFP acceptor fluorophore is represented in yellow. (B) Depiction of the diagonally positioned scan line across the whole glomerulus and corresponding intensity profile plots of CFP and YFP (data was normalized to the highest value). Scale bar: 100  $\mu\text{m}$ . (C) Time-lapse recordings represent baseline-normalized emission intensity changes of the individual CFP (cyan) and YFP (yellow) traces ( $\Delta F/F_0$ ) and the CFP/YFP ratio (black,  $\Delta R/R_0$ ) upon administration of 500 nM ANP



from 80 sec to 610 sec (dotted line). **(D)** Analysis interval (400-600 sec) used in **(E)** is shown in color. **(E)** Bar graphs display the averaged FRET response ( $\Delta R/R_0$ ) for the analysis interval (purple) shown in **(D)**. Data represent mean  $\pm$  SEM after administration of 500 nM BNP (n=21 of 5 mice), 500 nM ANP (n=20 of 3 mice), 250  $\mu$ M SNAP (n=20 of 5 mice) and (500 nM ANP+ 250  $\mu$ M SNAP) (n=28 of 3 mice). One-way ANOVA (Šídák's multiple comparisons test). \*P < 0.05

### 4.3 Investigation of cellular biokinetics of cGMP from synthesis to export

The presence of sGC in podocytes is debated due to inconsistencies in data from different species (mouse,<sup>29</sup> rat,<sup>155</sup> human<sup>80,90</sup>). To ensure that SNAP-induced cGMP synthesis, mediated by NO release and sGC activation in podocytes, is not an artifact introduced by SNAP, another NO donor was tested. Nitroglycerin mobilizes the free radical NO through enzymatic bioactivation,<sup>156</sup> and elicited a cGMP increase in isolated glomeruli from Pod:Cre/cGi500 mice (**Figure 11**). The use of IBMX results in the inhibition of PDE-mediated cGMP degradation, leading to an observable accumulation of the cyclic nucleotide. The relationship between cGMP signaling and calcium signaling was investigated using substances that promote Ca<sup>2+</sup> influx, such as Bradykinin, ATP and Jedi2 (activator for the mechanosensitive ion channel Piezo1). Each of these substances elicited a FRET response. The administration of Probenecid, an inhibitor of organic anion transporter, resulted also in increased cGMP levels, suggesting the existence of a cGMP efflux system in podocytes with a potential contribution to the regulation of cGMP dynamics. A general observation throughout all measurements was that despite the wash-out phase with imaging buffer after each test substance, the FRET response did not return to its baseline level. This observation may be attributed to the damage caused by the isolation process of the glomeruli. Therefore, in the next step, we prepared AKS to preserve the physiological environment and additionally excluded damaged glomeruli from analysis (**see chapter 5**).



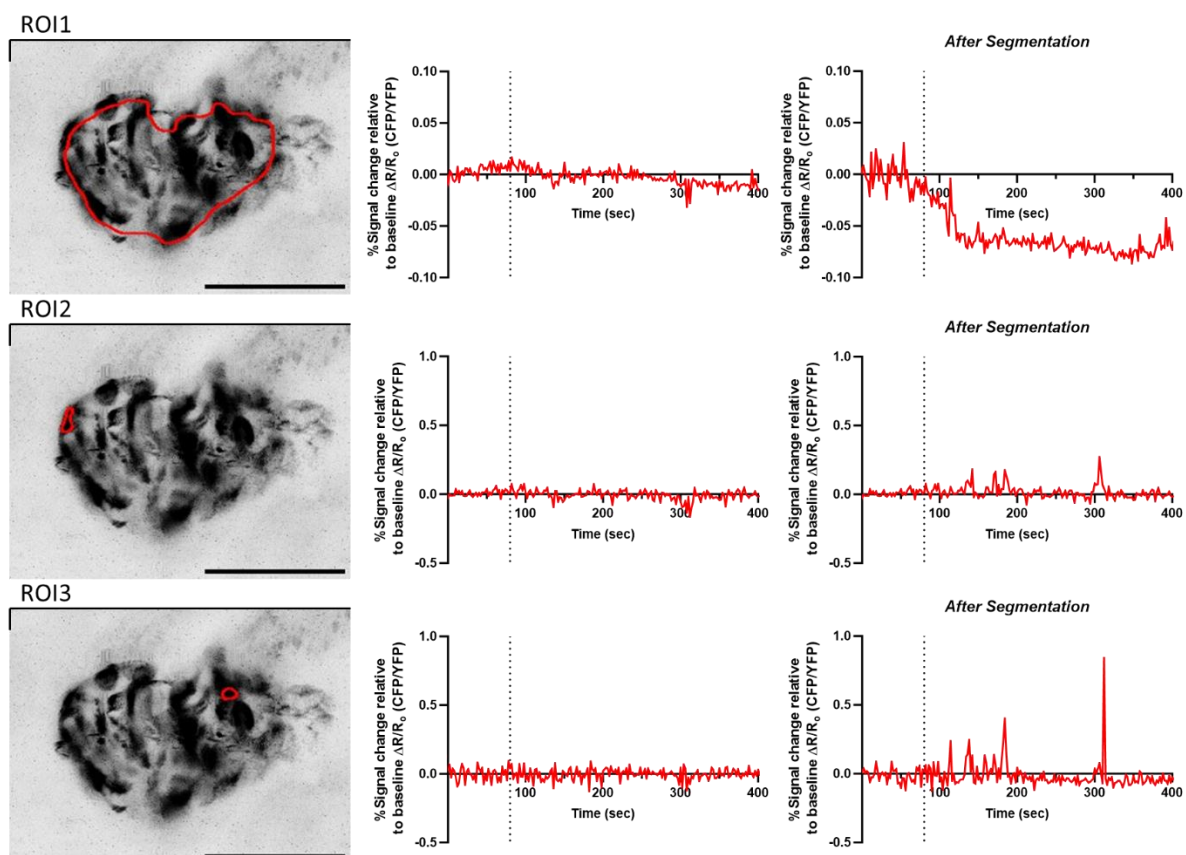
**Figure 11: Elevation of intracellular cGMP following various stimuli in isolated glomeruli**

Time-lapse recordings represent baseline-normalized emission intensity changes of the individual CFP (cyan) and YFP (yellow) traces ( $\Delta F/F_0$ ) and the CFP/YFP ratio (black,  $\Delta R/R_0$ ) upon administration of 750  $\mu\text{M}$  Nitroglycerin ( $n=21$  of 3 mice), 200  $\mu\text{M}$  IBMX ( $n=24$  of 5 mice), 500  $\mu\text{M}$  ATP ( $n=17$  of 3 mice), 25  $\mu\text{M}$  Bradykinin ( $n=11$  of 3 mice), 500  $\mu\text{M}$  Jedi2 ( $n=14$  of 3 mice) and 2 mM Probenecid ( $n=24$  of 4 mice) from 80 sec to 610 sec (dotted line). Data represent mean  $\pm$  SEM.

#### 4.4 Intravital imaging of cGi500-expressing glomeruli failed to demonstrate cGMP responses

The final aim of this thesis was to characterize the two known guanylyl cyclase systems (pGC and sGC) in podocytes and GECs in healthy living animals. Homozygosity of the sensor coincides with higher cGi500 signal intensity in Pod:Cre/cGi500 mice compared to Tie2:Cre/cGi500. Therefore, intravital imaging (IVI) was performed with Pod:Cre/cGi500 mice by administering various concentrated cGMP-elevating agents via bolus injection. Limitations of substance applicability was based on the solvent (e.g. ANP in ddH<sub>2</sub>O, SNAP in 0.5 M HCl, IBMX in DMSO) and the concentration. In addition, targeted stimulation of the cGMP axes led

to vasodilatory effects that caused a significant drop in blood pressure, ultimately resulting in the death of the animal. Higher concentrations of these substances would have been necessary to trigger a sufficient measurable signal-to-noise ratio, as discussed in **chapter 6.6**. Undesired acceptor fluorophore excitation while using two photon-excitation, as well as respiratory artifacts contributed to the difficulties in analyzing the FRET/cGi500 changes. Even a previously described image segmentation approach for FRET/cGi500-mediated cGMP changes in live tissue acquired with a multiphoton microscope could not unmask convincing cGMP responses (**Figure 12**),<sup>142</sup> which is why further IVI attempts were abandoned and open questions were answered *ex vivo* through AKS.



**Figure 12: Imaging of cGi500-expressing podocytes *in vivo* did not show convincing cGMP responses**

Evaluation of *in vivo* FRET/cGMP recordings from cGi500-expressing podocytes (Pod:Cre/cGi500 mouse). Representative image of biosensor expression in podocytes as maximum Z-projection in inverted grayscale with superimposed ROI's covering either the whole glomerulus (ROI1) or distinct podocytes (ROI2+3). Scale bar: 50  $\mu\text{m}$ . Time-lapse recordings represent baseline-normalized emission intensity changes of the CFP/YFP ratio (red,  $\Delta R/R_0$ ) upon bolus injection of 0.03 mg/kg Nitroglycerin (Dashed line: start bolus injection at 80 sec) without applying image segmentation (left) and after image segmentation (right).

## 5 Manuscript

### **Real-time imaging of cGMP signaling shows pronounced differences between glomerular endothelial cells and podocytes**

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EW - performed immunolabeling and STED imaging

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NR and MH - wrote the manuscript

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NR, EW, JBL, SF, RF, MHK - review & editing of the manuscript

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*Supplementary Material is provided as Supporting information SI 1. (Appendix)*

This manuscript contributes significantly to our understanding of cyclic guanosine monophosphate (cGMP) signaling within the kidney, particularly in glomerular endothelial cells (GECs) and podocytes. Utilizing the genetically encoded fluorescent biosensor cGi500 expressed in acute kidney slices from transgenic mice, the authors conducted real-time measurements of cGMP dynamics in these specific cell types. Their findings elucidate distinct cGMP responses in GECs and podocytes upon stimulation with the vasoactive substances atrial natriuretic peptide (ANP) or nitric oxide (NO). Furthermore, the discovery of distinct phosphodiesterase (PDE) activities, responsible for cGMP degradation, in each cell type highlights the intricate nature of cGMP regulation within the glomerulus. This comprehensive characterization of cell-specific cGMP signaling pathways paves the way for novel therapeutic strategies targeting kidney diseases, offering potential avenues for more effective interventions.

## **Real-time imaging of cGMP signaling shows pronounced differences between glomerular endothelial cells and podocytes**

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### **Key points**

1. ANP/pGC and NO/sGC pathways produce cGMP in podocytes and glomerular endothelial cells.
2. ANP induces a long-lasting cGMP elevation in podocytes; endothelial cells show transient responses.
3. Glomerular endothelial cells exhibit broader PDE activity compared to podocytes.

## Abstract

### Background

In recent years, successful clinical trials of drugs elevating cyclic guanosine monophosphate (cGMP) signaling for cardiovascular diseases have renewed interest in cGMP biology within the kidney. However, the role of cGMP signaling in glomerular cell types, especially in endothelial cells and podocytes, remains largely unexplored. The majority of available data relies on cell culture studies and cGMP detection after cell lysis precludes continuous measurements.

### Methods

Acute kidney slices from mice, in which the genetically encoded fluorescent FRET-based cGMP biosensor cGi500 was exclusively expressed in endothelial cells or podocytes, enabled real-time visualization of cGMP. Slices were stimulated with the atrial natriuretic peptide (ANP) or/and SNAP (NO donor) without or with several phosphodiesterase (PDE) inhibitors.

### Results

Application of ANP or SNAP led to an elevation of intracellular cGMP in glomerular endothelial cells (GECs) and podocytes. While GECs exhibited a transient cGMP response upon activation of the soluble or particulate guanylyl cyclase (sGC or pGC) pathway, the cGMP response in podocytes reached a plateau following ANP administration. Simultaneous stimulation with ANP and SNAP led to an additive response in GECs, whereas cGMP levels in podocytes were lower compared to ANP stimulation alone. Administration of PDE inhibitors revealed a broader basal PDE activity in GECs dominated by PDE2a. In podocytes, basal PDE activity was mainly restricted to PDE3 and PDE5 activity.

### Conclusions

Our data demonstrate the existence of the ANP/pGC/cGMP and NO/sGC/cGMP pathways in GECs and podocytes with cell-specific differences regarding cGMP synthesis and degradation. Further insights into cell-specific cGMP signaling could provide new therapeutic options for treating kidney diseases.

## 5.1 Introduction

Recent clinical trials have demonstrated renoprotective effects of enhancing cGMP signaling in patients,<sup>1,2</sup> and ongoing studies are investigating further substances showing renoprotective effects in animal models.<sup>3,4</sup> The second messenger cGMP mediates signal transduction by activation of downstream effector molecules such as protein kinase G (PKG), cyclic nucleotide-gated (CNG) channels or phosphodiesterases (PDE), which degrade cGMP. Two types of guanylyl cyclases catalyze the conversion of guanosine-5'-triphosphate (GTP) to cGMP. These are particulate/membrane-bound receptors with an intracellular guanylyl cyclase domain (pGC-A/B/C) and soluble guanylyl cyclases (sGC) located in the cytosol.<sup>5</sup> Natriuretic peptides, such as atrial natriuretic peptide (ANP), activate the particulate guanylyl cyclase (pGC) signaling cascade via ligand-receptor binding, causing natriuresis, diuresis, suppression of the renin-angiotensin-aldosterone system (RAAS), and an increase in the glomerular filtration rate (GFR).<sup>6-8</sup> The NO/sGC-mediated cGMP synthesis involves nitric oxide synthases (NOS), which generate NO through the conversion of L-arginine to L-citrulline. NO diffuses across cell membranes and activates sGC present in target cells.<sup>5</sup> The NO/sGC/cGMP pathway influences glomerular function by dilating both pre- and post-glomerular arterioles and counteracts the effects of reactive oxygen species (ROS).<sup>9-11</sup> In disease states, cGMP signaling is diminished, suggesting a therapeutic benefit of its restoration. Several approved drugs elevating cGMP signaling became available in recent years.<sup>12,13</sup> Sacubitril inhibits the degradation of ANP,<sup>14,15</sup> whereas riociguat and vericiguat activate sGC and sildenafil and tadalafil inhibit PDE5.<sup>16-19</sup> To date, the role of GECs and podocytes in the renoprotective effect of cGMP signaling remains unclear. Measurements of cGMP levels in podocytes mostly rely on cell culture work, utilizing conventional biochemical techniques such as immunohistochemistry, radioimmunoassays (RIA), and enzyme-linked immunoassays (ELISA).<sup>20-24</sup> However, these techniques require cell lysis and often involve the addition of unspecific PDE inhibitors to enhance sensitivity. This inhibition disrupts cGMP dynamics and precludes a detailed analysis of signaling processes. The development of Förster resonance energy transfer (FRET)-based cGMP biosensors, such as cGi500,<sup>25,26</sup> enables the real-time monitoring of cGMP fluctuations within living cells. Binding of cGMP leads to a conformational change of the indicator protein cGi500, resulting in increased CFP fluorescence (donor) and decreased YFP fluorescence (acceptor). This sensor is capable of visualizing cGMP changes in intact tissue in high spatial and temporal resolution, overcoming some of the hurdles that hampered cGMP research in previous years.<sup>27-29</sup>

Utilizing the cGMP biosensor cGi500 with cell-specific expression, we performed a comparative analysis on GECs and podocytes to investigate cGMP signaling in vital mouse kidney tissue.

## 5.2 Methods

### 5.2.1 *In vitro* characterization of cGi500

The plasmid DNA MH20\_pCMV\_cGi500 (7767 bp, Supplemental Figure 1) encoding the single-chain cGMP biosensor cGi500 was provided by the Feil lab based on the sequence designed<sup>26</sup> by Michael Russwurm.

HEK 293T cells maintained in DMEM (Thermo Fisher Scientific, 31966-021) supplemented with 10% FBS (Thermo Fisher Scientific, 10270106) were transiently transfected by calcium-phosphate mediated DNA transfer. Cells were incubated at 37°C with replacement of the present medium after 7 h with supplemented phenol red free DMEM (Sigma-Aldrich, D1145) using 10% FBS, 1% GlutaMAX™ (Thermo Fisher Scientific, 35050-061) and 1% sodium pyruvate solution (Sigma-Aldrich, S8636). Transfected cells from a 10-cm dish (1x10<sup>7</sup> cells) were harvested and lysed according to a previously published protocol,<sup>26</sup> with minor modifications: Considering temperature and light sensitivity, all preparation steps were performed on ice with avoidance of strong light exposure. Cells were washed twice with 5 ml of ice-cold sterile PBS, followed by 300 µl of freshly prepared ice-cold homogenization buffer (pH 7.4). The adherent cells were scraped off and the lysate was transferred to sterile tubes. A cytosolic fraction was obtained by sonication (5 sec ON, 30 sec OFF, 1 cycle), followed by ultracentrifugation at 100000 g for 30 min at 4°C (Optima MAX-XP, Beckman Coulter, Rotor TLA-55). The volume for 25 µg protein (BCA assay, Thermo Fisher Scientific, 23225) was added to freshly prepared buffer A to achieve a final volume of 300 µl. An ascending cGMP (Sigma-Aldrich, G7504) dilution series (0.01 µM to 100 µM) was then added (2.1 µl) and incubated for 5 min. Fluorescence of the biosensor was recorded using a confocal microscope (LSM Meta 710 Axio Observer, Zeiss) equipped with an EC Plan-Neofluar 10x/0.3 objective and ZEN 2009 software (Zeiss). Intensity changes in the 512 x 512 x 16-bit emission images (mean fluorescence intensity) were analyzed using ImageJ (NIH) to calculate the CFP/YFP emission ratio (FRET ratio). The performed concentration-response curve analysis reflects the concentration-dependent cGMP binding properties of the sensor.

### 5.2.2 Mouse lines

Cell-specific cytosolic expression of the FRET-based biosensor cGi500 was accomplished by crossing the 'floxed' cGi500 sensor line<sup>25</sup> Gt(ROSA)26Sor<sup>tm1(CAG-ECFP/EYFP\*)Feil</sup> (mixed background 129 SV, C57BL/6N, (C57BL6/NRj > 70%)) with Pod:Cre mice<sup>30</sup> to achieve sensor expression in podocytes, and Tie2:Cre mice<sup>31</sup> or Cdh5:Cre mice<sup>32</sup> for expression in GECs. Experiments with Pod:Cre/cGi500 mice were performed with homozygous cGi500 alleles. Experiments with Tie2:Cre/cGi500 mice were performed with the first filial generation of offspring (carrying only one cGi500 allele) due to a reported nonspecific recombination in the



F2 generation.<sup>33,34</sup> Experiments with *Cdh5:Cre* mice were also conducted using the first-generation of offspring. Mice of both sexes, aged 8-27 weeks, were used. Animals were housed under standardized, specific pathogen-free conditions and kept in individually ventilated cage systems (IVC cages type II long) with centrally monitored light periodicity (light/dark cycle 12 h/12 h,  $55 \pm 10\%$  humidity,  $22 \pm 2^\circ\text{C}$  room temperature). Experimental procedures were evaluated and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW (VSG 81-02.04.2018.A351.)

### 5.2.3 Poly-L-lysine coating

To immobilize kidney slices for real-time imaging, coverslips ( $\varnothing$  12 mm, #1.5, Gerhard Menzel) were coated with approximately 700  $\mu\text{l}$  of a 0.1% poly-L-lysine solution (Sigma-Aldrich, P8920) for 1 hour.

### 5.2.4 Acute kidney slices (AKS)

Kidney slices were prepared according to the protocol of Poosti et al.,<sup>35</sup> with the following modifications: On the day of the experiment, 1 L of 1X Krebs-Henseleit buffer (KHB) was prepared, kept cold, and gassed with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  for 30 min using a gas dispersion tube with a fritted disc. Subsequently, the solution was titrated to achieve a pH of 7.42. Mice were euthanized by cervical dislocation and the kidneys were directly transferred to ice-cold 1X KHB, followed by removal of the renal capsule and adherent vessels. The kidneys were embedded in 4% low melt agarose (Bio&SELL, BS20.47.100) and then cut into 250  $\mu\text{m}$  thick tissue sections using a Leica VT1200S vibratome (Leica Microsystems), amplitude: 1.0 mm and cutting speed: 0.20 mm/sec. Protected from light, slices were stored in ice-cold 1X KHB for up to 6 h until imaging.

### 5.2.5 Custom-built perfusion system

The perfusion system included the microscope stage chamber MS4 (Digitimer) connected through the female luer lock (Drifton, LF31) to the tubing Original Perfusor<sup>®</sup> line (B. Braun, 8723060). The respective end of the Perfusor<sup>®</sup> line tubing was inserted into a 1 ml Stripette<sup>®</sup> (Corning, 4011) to provide a stable support for the inlet and outlet. The system was connected to the Masterflex<sup>®</sup> silicone hose (Cole-Parmer, 96400-13) and clamped into the pump head with 2 channels (Masterflex<sup>®</sup> L/S<sup>®</sup> Easy-Load<sup>®</sup> II, Cole-Parmer, 77202-50) of the Masterflex<sup>®</sup> L/S<sup>®</sup> peristaltic pump (Cole-Parmer, 07555-15). The temperature of the buffer was kept constant at 37°C with the Truelife Invio BW Double (Mercateo, 615-Y791972) and was monitored with an external temperature probe LOG200 TC 5005-0204 (Dostmann Electronic). The Perfusor<sup>®</sup> line tubing was wrapped with sealing foam tape (SourcingMap) to avoid temperature loss of the heated buffer.

### 5.2.6 Real-time imaging of AKS

AKS were transferred onto a poly-L-lysine coated coverslip, positioned centrally in the imaging chamber, and secured with the slice anchor SHD-42/10 (Multi Channel Systems MCS, SKU:641419). Thereafter, the slice was allowed to adapt to the pre-tempered 1X KHB (37°C) for 10 min at a continuous flow rate of 1 ml/min using the custom-build perfusion system. Stimulants tested on AKS were: Atrial natriuretic factor (1-28) trifluoroacetate (ANP, Bachem, 01-4030380); Diethylammonium(Z)-1-(N,N-diethylamino)diazene-1-ium-1,2-diolate (DEA NONOate, Biomol, Cay82100-100); S-nitroso-N-acetylpenicillamine (SNAP, Biomol, Cay82250). Prior to DEA NONOate administration, NO release was induced by diluting the stock solution in 1X KHB for 5 min. Prior to SNAP administration, NO release was induced by diluting the stock solution in 1X KHB for 10 min. PDE inhibitors tested were: Avanafil (Hözel Diagnostika, A12619); BAY 60-7550 (Biomol, Cay10011135); Cilostamide (Hözel Diagnostika, A13596); Roflumilast (Hözel Diagnostika, A10804); PF-04449613 (Sigma-Aldrich, PZ0349); 3-Isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, I5879). Drug exposure and vitality of the tissue was monitored by the delivery and uptake of 1  $\mu$ M Evans Blue (Sigma-Aldrich, E2129) from the administered solution. Real-time cGMP imaging was performed using the TCS SP8 MP-OPO microscope (Leica Microsystems) and an IR Apo L25x/0.95 W objective in conjunction with the LAS X software (Leica). The objective was equipped with an objective heating collar (OBJ-COLLAR-3342, Okolab) adjusted to 37°C, monitored and controlled by a temperature controller (H401-T-CONTROLLER, Okolab). Biosensor excitation was conducted with a diode laser at 448 nm together with a sequential excitation of Evans Blue with the OPSP laser at 552 nm in combination with the TD beam splitter 448/514/552. Due to cGi500 biosensor heterozygosity, real-time imaging of Tie2:Cre/cGi500 and Cdh5:Cre/cGi500 mice was performed with higher laser power compared to homozygous Pod:Cre/cGi500 mice. Simultaneous detection of emission changes was performed at  $480 \pm 25$  nm (CFP),  $535 \pm 20$  nm (YFP) and at  $685 \pm 35$  nm (Evans Blue) with a bidirectional scan at 600 Hz and a line average of 4. Time series were recorded with one frame every 10 sec for 150 frames, starting with a baseline acquisition of 20 frames.

### 5.2.7 FRET imaging data analysis

Sample motion was corrected through the open-source package Galene (realignment mode: warp, points: 10).<sup>36</sup> The reassembled data was further processed using ImageJ (NIH) by superimposing a region of interest (ROI) over the whole glomerulus. Glomeruli showing morphological abnormalities and positive Evans Blue staining, indicating damage, were excluded from analysis (Supplemental Figure 2/3). Baseline normalization (averaged baseline signal: frame 0-20) was applied either to the CFP and YFP emission intensities ( $\Delta F/F_0$ ) or to CFP/YFP emission ratios ( $\Delta R/R_0$ ) (Supplemental Figure 4/5). Relative signal changes in  $\Delta R/R_0$

(%) reflect changes in intracellular cGMP concentration. The depicted curves represent the mean of baseline-normalized CFP/YFP emission ratios (FRET ratio) of all analyzed glomeruli. Scatter plots/Scattered bar plots display the maximal  $\Delta R/R_0$  response (maximal %signal change relative to baseline) in a selected time period of all analyzed glomeruli. Introduced delay in the cGMP/FRET response (lag time) ensued post substance administration, with dependency on the length of the assembled superfusion system hose (inlet). Lag time correction (stimulation intervall + 60 sec) was applied uniformly to all displayed time-lapse recordings with specification of the exact stimulation intervall (w/o correction) in the figure legend.

### 5.2.8 Immunostaining in optically cleared kidney slices

Immunolabeling of cleared kidney slices was based on an adapted protocol by Unnersjö-Jess et al.<sup>37</sup> Freshly prepared AKS of biosensor mice were fixed overnight in 4% PFA (Sigma-Aldrich, P6148) in 1X PBS. The solution was replaced with 1X PBS. Optical clearing was accomplished by immersing the AKS in 1 ml clearing solution (200 mM boric acid (Sigma-Aldrich, B0252), 4% SDS (Sigma-Aldrich, L3771), pH 8.5) at 50°C for 24 h, followed by a washing step with PBS-T for 5 min (1X PBS with 0.1% Triton X-100 (AppliChem, A4975)). Primary antibodies: GFP-Alexa647 (1:100, rabbit, Thermo Fisher Scientific, A-31852),  $\alpha$ -nephrin (1:100, guinea pig, Fitzgerald Industries International, 20R-NP002), CD31/PECAM-1 (1:200, goat, R&D Systems, AF3628). Secondary antibodies: donkey anti-guinea pig Atto594 or donkey anti-goat Atto594 (1:100, coupled in house). Immunolabeling was carried out at 37°C overnight with a PBS-T washing step in between and at the end of antibody incubation. Afterwards, AKS were embedded in a saturated fructose solution (80.2% D-fructose (Sigma-Aldrich, F0127), 0.5% 1-thioglycerol (Sigma-Aldrich, M1753)) and placed in glass-bottom dishes with a cover slip on top for mounting. Samples were imaged using a Leica TCS SP8 gSTED 3X (Leica Microsystems).

### 5.2.9 Statistics analyses

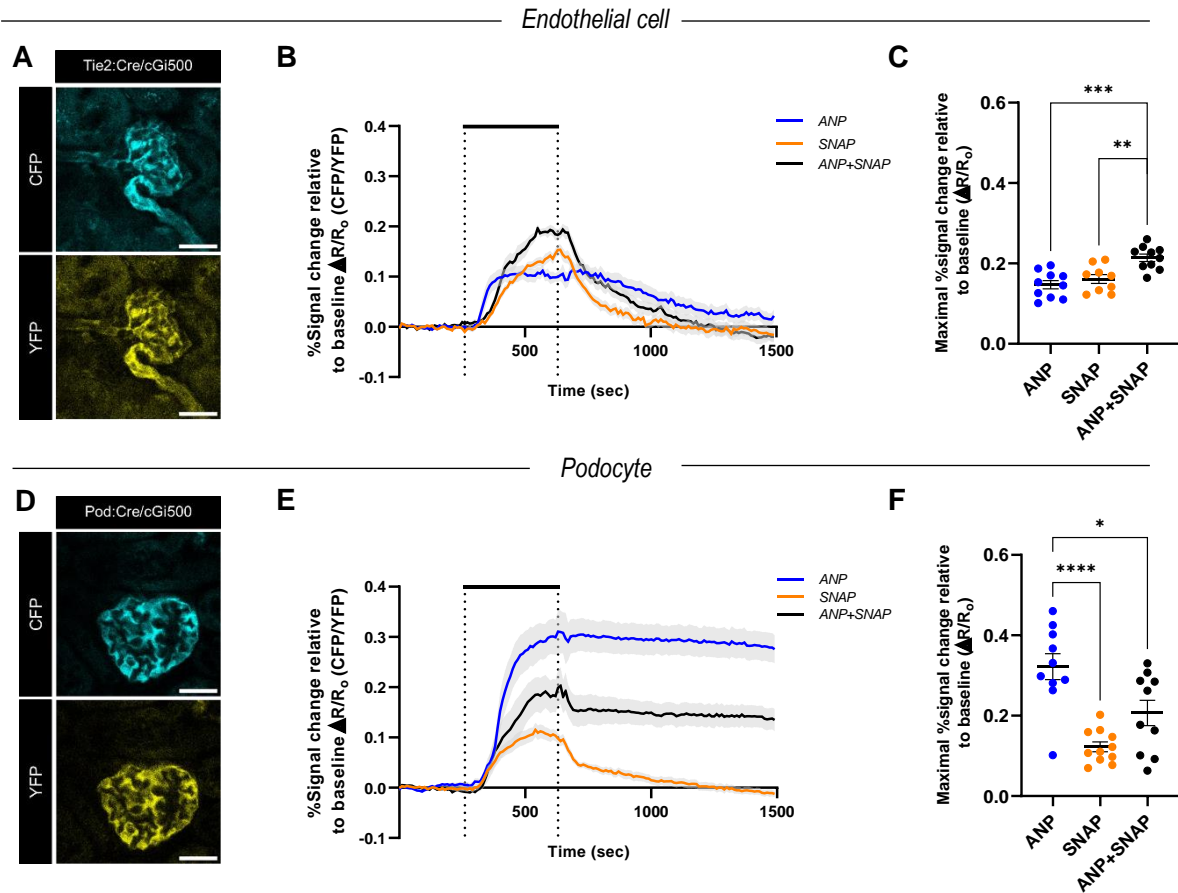
Statistical analysis was performed with GraphPad Prism 10.1.0 (Graphpad Software) utilizing a paired, two-tailed Student's t-test or One-way ANOVA (Tukey's post hoc test or Dunnett T3 post hoc test) to assess differences between groups. Differences between groups were considered statistically significant if  $p < 0.05$  (P value style "GP"). Results are reported as mean  $\pm$  standard error of the mean (SEM).

## 5.3 Results

### 5.3.1 Differential regulation of cGMP pathways in GECs and podocytes

Cell-specific cGi500 expression in GECs and podocytes was confirmed by optical tissue clearing and immunofluorescence labeling of AKS (Supplemental Figure 6). Tie2:Cre/cGi500 and Pod:Cre/cGi500 mice were used to visualize cell-specific changes in the cGMP/FRET response ( $\Delta R/R_0$ ) following the activation of the ANP/pGC/cGMP and the NO/sGC/cGMP pathways in glomeruli of freshly prepared kidney slices (Figure 1). Binding of cGMP to the FRET-based biosensor results in antiparallel intensity changes of the CFP/YFP FRET pair, thereby reporting changes in intracellular cGMP concentration. GECs of Tie2:Cre/cGi500 mice showed a similar maximal cGMP/FRET response after stimulation with 1  $\mu$ M ANP or 1 mM SNAP (NO donor). Compared to the ANP stimulation, SNAP stimulation reached the maximal cGMP/FRET response later and declined earlier (Figure 1B). SNAP was chosen for its longer half-life compared to other NO donors, making it well-suited for image acquisition over longer periods of time. However, high concentrations of SNAP (1 mM) are required to elicit a cGMP increase in tissue slices (Supplemental Figure 7A).<sup>38,39</sup> To address potential unspecific effects of SNAP attributable to its concentration, we applied another NO donor, DEA NONOate, which triggered a transient signal of comparable strength in GECs (Supplemental Figure 8). For Tie2:Cre mouse lines, non-endothelial Cre recombinase activity has been reported.<sup>33,34</sup> To demonstrate endothelial specificity, we repeated the experiments with ANP or/and SNAP and DEA NONOate in Cdh5:Cre/cGi500 mice and obtained similar results (Supplemental Figure 9). Podocytes of Pod:Cre/cGi500 mice showed an increase in cGMP levels after administration of 1 mM SNAP with similar kinetics and a maximal cGMP/FRET response compared to GECs. ANP-triggered cGMP synthesis in podocytes resulted in the highest cGMP/FRET response of all experiments and remained consistently high throughout the measurement period (Figure 1E). This long-lasting plateau was also present with half of the ANP concentration (Supplemental Figure 7B) and receded only after a total recording time of 4250 sec (~ 70 min), as demonstrated in a long-term measurement (Supplemental Figure 10). In GECs, co-stimulation (ANP+SNAP) resulted in an additional elevation of the maximal cGMP/FRET response compared to the response induced by each substance alone (Figure 1C). The time course of signal increase and decrease was comparable to SNAP stimulation (Figure 1B). In podocytes, co-stimulation failed to elicit a synergistic additive effect. The maximal cGMP/FRET response was even lower compared to the stimulation with ANP alone (Figure 1F). After a transient peak, most likely due to the NO release from SNAP, the curve displayed a plateau formation similar to the long-lasting cGMP response after ANP administration alone (Figure 1E).

Overall, our experiments demonstrate clear differences in the activity of the ANP/pGC/cGMP and NO/sGC/cGMP pathways between GECs and podocytes, and indicate a predominant role of the ANP/pGC/cGMP signaling pathway with sustained cGMP elevation in podocytes.

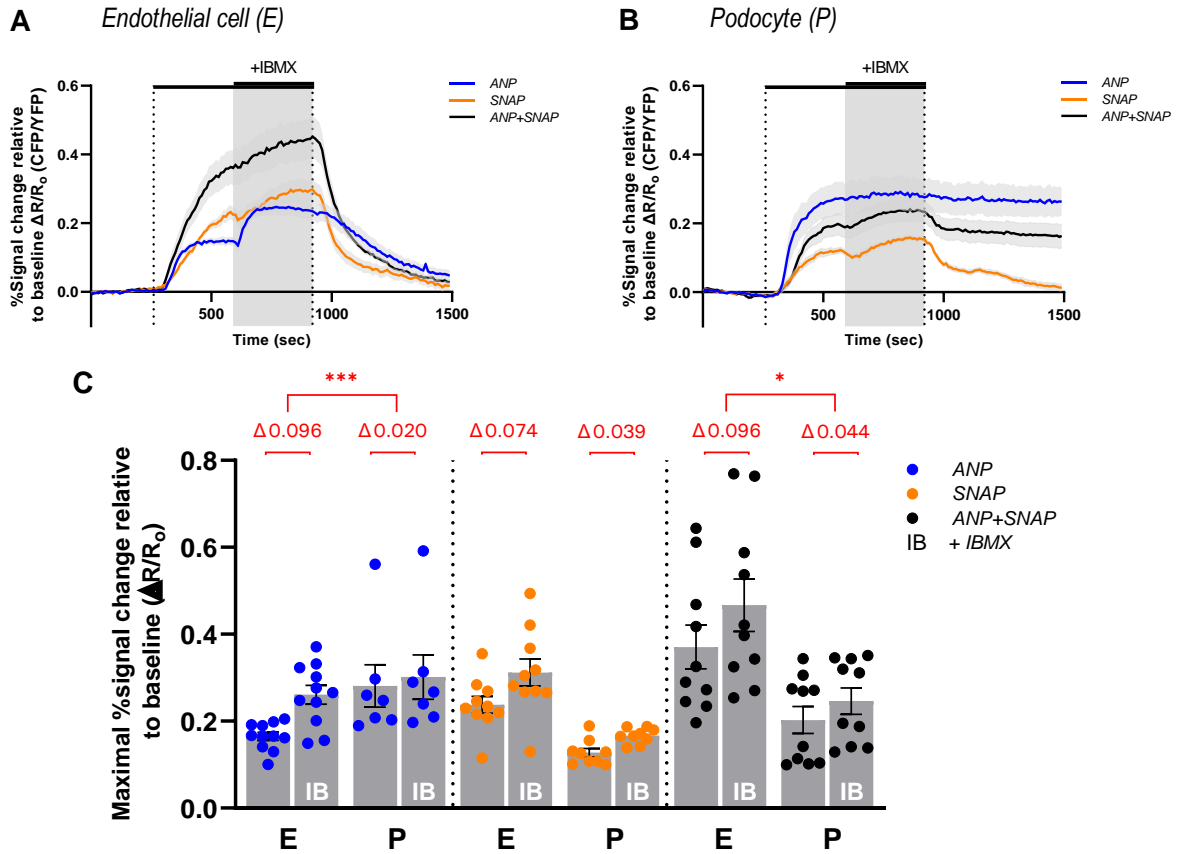


**Figure 1: cGMP response upon stimulation of the ANP/pGC/cGMP and NO/sGC/cGMP pathway in GECs and podocytes**

Acute kidney slices obtained from (A) Tie2:Cre/cGi500 mice or (D) Pod:Cre/cGi500 mice demonstrate cell-specific cGi500 biosensor expression with overlapping CFP/YFP fluorescence patterns in GECs and podocytes. (B, E) AKS were superfused (dotted lines) from 200 sec to 570 sec with 1  $\mu$ M ANP (blue trace), 1 mM SNAP (orange trace), or the combination of both (black trace). Stimulation was followed by a washout phase with 1X KHB (37°C). Time-lapse recordings for each cell type represent the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli. Relative signal changes in  $\Delta R/R_0$  (%) reflect changes in intracellular cGMP concentration. (C, F) Scatter dot plots display the maximal  $\Delta R/R_0$  response for each glomerulus derived from the indicated stimulation of (B) Tie2:Cre/cGi500 mice or (E) Pod:Cre/cGi500 mice during the entire measurement period of 1500 sec. Data represent mean  $\pm$  SEM of all analyzed glomeruli of six Tie2:Cre/cGi500 mice ( $n = 10$  for ANP,  $n = 9$  for SNAP,  $n = 10$  for ANP+SNAP) and five Pod:Cre/cGi500 mice ( $n = 10$  for ANP,  $n = 11$  for SNAP,  $n = 10$  for ANP+SNAP). One-way ANOVA, Tukey's post hoc test, \* $P < 0.05$ . Scale bar 25  $\mu$ M.

### **5.3.2 cGMP levels are controlled by a higher PDE activity in GECs compared to podocytes**

The balance of intracellular cGMP concentration within cells or tissues is maintained by the coordinated interplay between cGMP synthesis and its degradation, primarily mediated by phosphodiesterases (PDEs). Therefore, we added the broad-spectrum PDE inhibitor IBMX to the ongoing stimulation with ANP or/and SNAP. In GECs of Tie2:Cre/cGi500 mice, IBMX administration to an ongoing ANP or ANP+SNAP stimulation resulted in a higher additional cGMP/FRET response than the IBMX administration to an ongoing SNAP stimulation (Figure 2A). In contrast, in podocytes of Pod:Cre/cGi500 mice, the addition of IBMX to an ongoing ANP stimulation led only to a minimal additional increase in the cGMP/FRET response (Figure 2B), while the addition of IBMX to SNAP or to ANP+SNAP stimulation resulted in a further increase in cGMP levels. However, in all tested combinations, the additional cGMP/FRET response elicited by IBMX was consistently lower in podocytes compared to GECs, which reached statistical significance for ANP and ANP+SNAP (Figure 2C). This suggests a less pronounced PDE activity in podocytes compared to GECs.

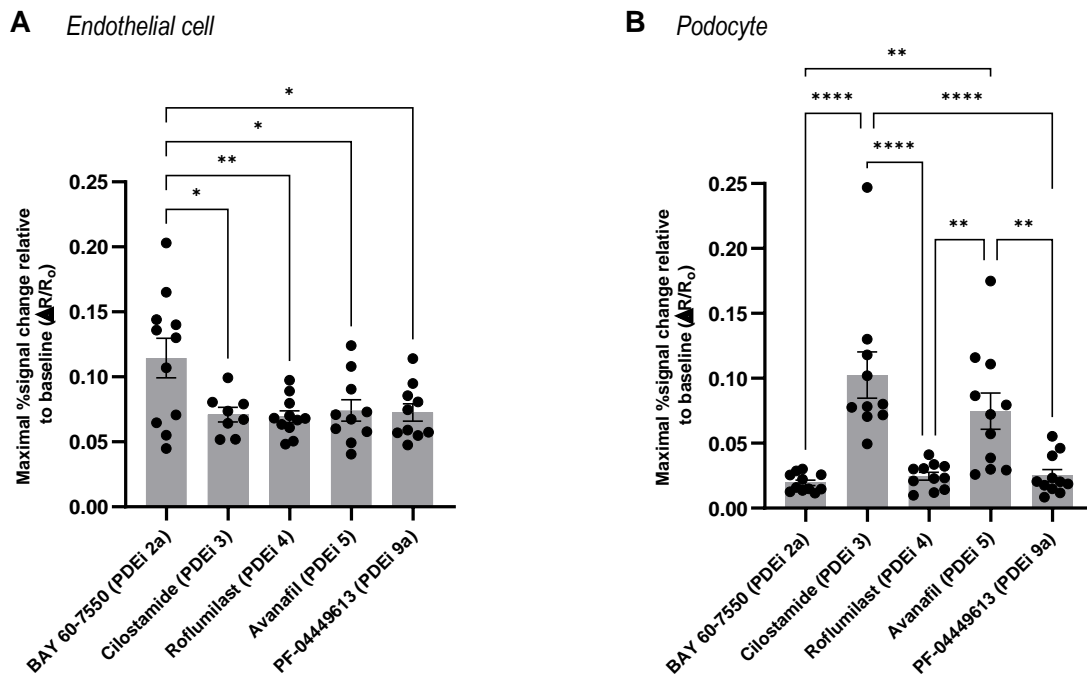


**Figure 2: Addition of the PDE inhibitor IBMX reveals higher PDE activity in GECs compared to podocytes**

Acute kidney slices from (A) Tie2:Cre/cGi500 mice or (B) Pod:Cre/cGi500 mice were superfused (outer dotted lines) from 200 sec to 860 sec with 1  $\mu$ M ANP (blue trace), 1 mM SNAP (orange trace) or the combination of both (black trace). In addition to the ongoing stimulation, 500  $\mu$ M IBMX was administered from 530 sec to 860 sec (gray area). Time-lapse recordings for each cell type represent the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli. (C) Comparison of the additional cGMP/FRET response after IBMX administration between GECs and podocytes. Scattered bar plot displays the maximal  $\Delta R/R_0$  response for each glomerulus in the respective measurement interval (w/o IBMX: 260-590 sec; with IBMX: 600-1500 sec). The delta (mean difference, shown in red) between the two time intervals was compared between GECs (E) and podocytes (P). Data represent mean  $\pm$  SEM of all analyzed glomeruli of three Tie2:Cre/cGi500 mice ( $n = 11$  for ANP,  $n = 10$  for SNAP,  $n = 10$  for ANP+SNAP) and four Pod:Cre/cGi500 mice ( $n = 7$  for ANP,  $n = 9$  for SNAP,  $n = 10$  for ANP+SNAP). One-way ANOVA (Brown Forsythe ( $p < 0.0001$ ), Welch test ( $p < 0.0001$ )), Dunnett T3 post hoc test, \* $P < 0.05$ .

### 5.3.3 Predominant PDE2a activity in GECs and PDE3 & PDE5 activity in podocytes

To further analyze PDE activity in GECs and podocytes, we inhibited several PDE isoforms and investigated their effect on cGMP levels without ANP or/and SNAP stimulation. Suitable PDE candidates were identified in proteomics datasets of podocytes<sup>40</sup> and respective PDE inhibitors were applied on AKS of Tie2:Cre/cGi500 mice (Figure 3A) and Pod:Cre/cGi500 mice (Figure 3B). In GECs, inhibition of PDE2a by BAY 60-7550 alone resulted in the highest maximal cGMP/FRET response of all tested substances. The inhibition of all other PDE isoforms tested (PDE3, 4, 5, 9a) also elicited a robust cGMP/FRET response. In contrast, in podocytes PDE3 inhibition by Cilostamide induced the highest cGMP/FRET response of all tested substances, followed by inhibition of PDE5 with Avanafil. The other PDE inhibitors elicited only a minor cGMP/FRET response. This suggests a broader PDE activity in GECs compared to podocytes.



**Figure 3: PDE inhibition reveals predominant PDE2a activity in GECs and PDE3 & PDE5 activity in podocytes**

Acute kidney slices from (A) Tie2:Cre/cGi500 mice or (B) Pod:Cre/cGi500 mice were superfused for 370 sec with 50  $\mu$ M BAY 60-7550, 250  $\mu$ M Cilostamide, 250  $\mu$ M Roflumilast, 250  $\mu$ M Avanafil or 100  $\mu$ M PF-04449613. The primarily inhibited PDE isoform is stated in brackets. Baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli were calculated. Scattered bar plots display the maximal  $\Delta R/R_0$  response for each compound during the entire measurement period of 1500 sec. Data

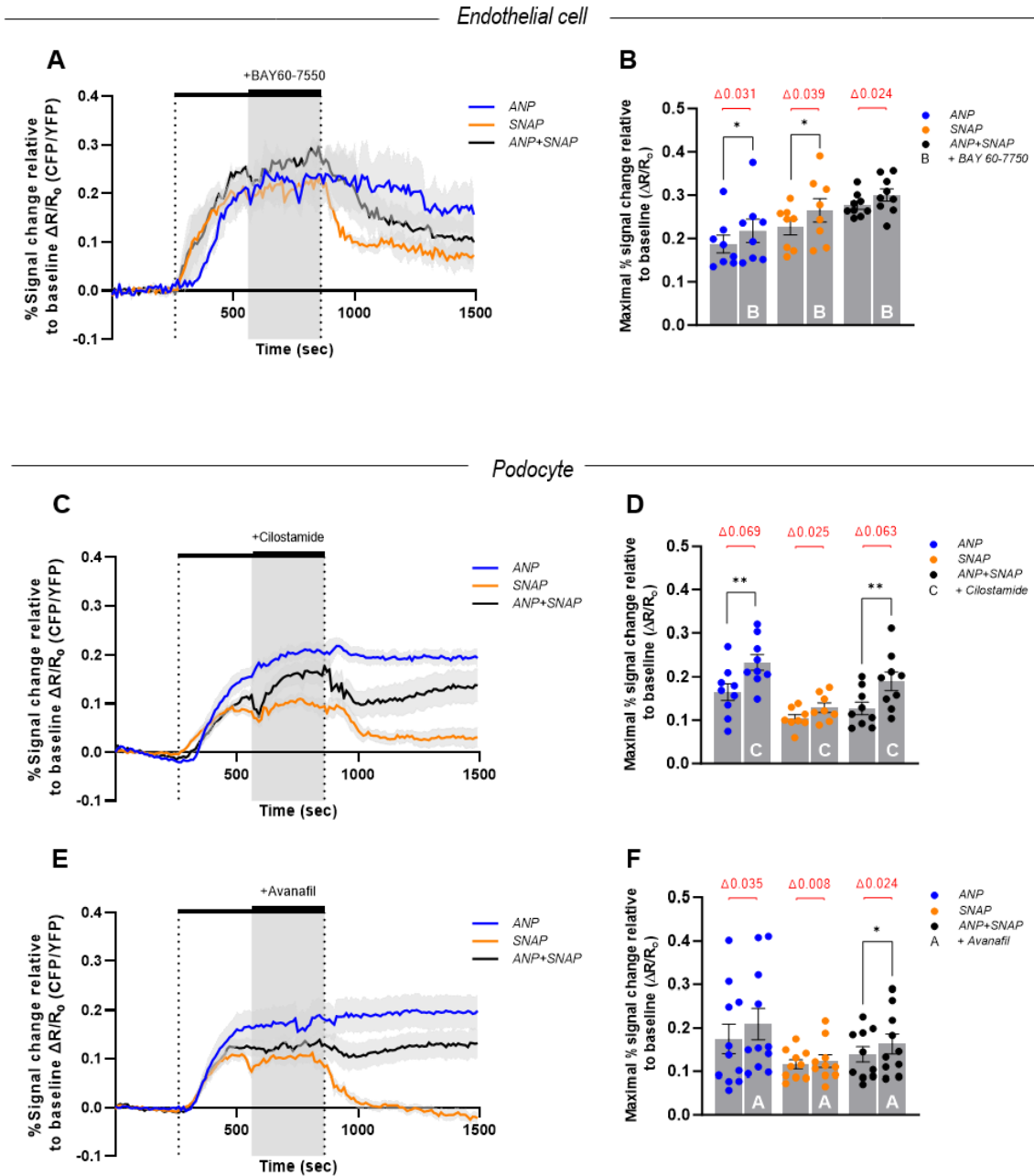


represent mean  $\pm$  SEM of all analyzed glomeruli of nine Tie2:Cre/cGi500 mice ( $n = 11$  for BAY 60-7550,  $n = 8$  for Cilostamide,  $n = 11$  for Roflumilast,  $n = 10$  for Avanafil,  $n = 10$  for PF-04449613) and of six Pod:Cre/cGi500 mice ( $n = 11$  for BAY 60-7550,  $n = 10$  for Cilostamide,  $n = 11$  for Roflumilast,  $n = 11$  for Avanafil,  $n = 11$  for PF-04449613). One-way ANOVA, Tukey's post hoc test, \* $P < 0.05$ .

#### **5.3.4 PDE2a inhibition prevents cGMP degradation in GECs, whereas PDE3 & PDE5 inhibition augment the ANP/pGC/cGMP pathway in podocytes**

To investigate the effect of PDE inhibition on the ANP/pGC/cGMP and NO/sGC/cGMP pathways, we applied inhibitors of specific PDE isoforms to an ongoing stimulation with ANP or/and SNAP on AKS of Tie2:Cre/cGi500 mice or Pod:Cre/cGi500 mice. In GECs of Tie2:Cre/cGi500 mice, the administration of BAY 60-7550 (PDE2a inhibitor) resulted only in a minor additional cGMP/FRET response during ANP or/and SNAP stimulation (Figure 4B). However, the increased cGMP levels lasted considerably longer than the cGMP/FRET response after ANP or/and SNAP stimulation without PDE inhibition, as none of the FRET ratios returned back to baseline within the measurement period (Figure 4A). To study the effect of inhibition of specific PDE isoforms in podocytes, we applied Cilostamide (PDE3 inhibitor) or Avanafil (PDE5 inhibitor) on AKS of Pod:Cre/cGi500 mice. Both substances showed a pronounced effect on the ANP and ANP+SNAP stimulation (Figure 4C-F). However, the additional cGMP/FRET response after SNAP stimulation was small for Cilostamide (Figure 4D) and almost absent for Avanafil (Figure 4F). The plateau-like cGMP response seen after ANP and ANP+SNAP stimulation was unaffected by inhibition of PDE3 or PDE5.

Taken together, we were able to demonstrate clear pathway-dependent differences in the activity of PDE isoforms between GECs and podocytes.



**Figure 4: PDE2a inhibition prevents cGMP degradation in GECs, whereas PDE3 & PDE5 inhibition augment the ANP/pGC/cGMP pathway in podocytes**

Acute kidney slices from (A) Tie2:Cre/cGi500 mice or (C, E) Pod:Cre/cGi500 mice were superfused from 200 sec to 800 sec (outer dotted lines) with 1  $\mu$ M ANP (blue trace), 1 mM SNAP (orange trace) or the combination of both (black trace). In addition to the ongoing stimulation, Tie2:Cre/cGi500 slices were superfused with 50  $\mu$ M BAY 60-7550 and Pod:Cre/cGi500 slices were superfused with 250  $\mu$ M Cilostamide or 250  $\mu$ M Avanafil in the time period from 500 sec to 800 sec (gray area). Time-lapse recordings represent the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli. (B, D, F) Scattered bar plots display the maximal  $\Delta R/R_0$  response for each glomerulus in the respective measurement interval (w/o PDE inhibitor: 260-570 sec; with PDE inhibitor: 580-1000 sec) for (B) BAY 60-7550, (D) Cilostamide or (F) Avanafil. The delta (mean difference) between the two time

periods is shown in red. Data represent mean  $\pm$  SEM of all analyzed glomeruli of six Tie2:Cre/cGi500 mice after administration of BAY 60-7550 ( $n = 8$  for ANP,  $n = 8$  for SNAP,  $n = 9$  for ANP+SNAP), eight Pod:Cre/cGi500 mice for Cilostamide ( $n = 9$  for ANP,  $n = 8$  for SNAP,  $n = 9$  for ANP+SNAP) and nine Pod:Cre/cGi500 mice for Avanafil ( $n = 11$  for ANP,  $n = 10$  for SNAP,  $n = 10$  for ANP+SNAP glomeruli of nine mice). Two-tailed paired t-test. \* $P < 0.05$ .

## 5.4 Discussion

To our knowledge, our study is the first, which resolves a cell-specific cGMP response in glomerular cells in vital kidney tissue. For podocytes, we demonstrate an elevation of intracellular cGMP levels provoked by the stimulation of the ANP/pGC/cGMP-axis and NO/sGC/cGMP-axis. These findings contradict the work of Theilig et al. and Wang et al., who were unable to detect SNAP-induced cGMP synthesis in cultured podocytes,<sup>20,21</sup> but are in line with results from other authors.<sup>22,23,41</sup> However, limitations in conventional detection and quantification methods in these publications can hamper the assessment of cGMP dynamics.<sup>28</sup> Additionally, the severe differences in gene expression and morphology between cultured podocytes and podocytes *in vivo* have to be taken into account.<sup>42,43</sup> In agreement with our results, Mundel et al. and Jarry et al. localized sGC in podocytes of kidney tissue.<sup>44,45</sup>

Endothelial cells are known NO suppliers, but there is limited data on endogenous expression of guanylyl cyclases (sGC or pGC) and cGMP signaling in GECs of intact tissue.<sup>23,45-47</sup> Our data provide evidence for the existence of the ANP/pGC/cGMP and NO/sGC/cGMP pathways in GECs. We demonstrate that GECs possess an actively regulated cGMP signaling system, measured with high spatial and temporal resolution in glomeruli embedded in a complex kidney architecture. Compared to conventional assays that rely on a single cGMP endpoint determination, our experimental setup enabled real-time monitoring of cGMP dynamics throughout the study period.

We were able to demonstrate distinct differences in cGMP signaling between GECs and podocytes using the same cGMP biosensor expressed in these cell types. In podocytes, SNAP-induced cGMP synthesis resulted in a transient cGMP/FRET response, similar to ANP or/and SNAP stimulation in GECs. In contrast, the ANP-induced cGMP synthesis in podocytes resulted in a higher and far longer lasting cGMP increase than in GECs. The persistent ANP-triggered cGMP response in podocytes did not decline to baseline level during a total measurement time of 4250 sec ( $\sim 70$  min) (Supplemental Figure 10). Reducing the ANP concentration by 50% resulted in an identically sustained cGMP/FRET response, albeit at a lower level (Supplemental Figure 7B), which argues against a plateau formation due to oversaturation of the sensor. This observation could be explained by delayed internalization of

ligand-receptor complexes of GC-A,<sup>48,49</sup> as podocytes exhibit high expression levels of GC-A.<sup>46</sup> Alternatively, this phenomenon may arise from a positive feedback loop via PKG,<sup>50</sup> or a lower PDE activity, as demonstrated in our experiments. These results suggest an important role of ANP-mediated cGMP synthesis in podocytes, which has been demonstrated in disease states for a podocyte-specific GC-A knockout model. In these mice, a DOCA salt-induced hypertension led to development of proteinuria and foot process effacement.<sup>51</sup> In all of our experiments, the co-stimulation (ANP+SNAP) in podocytes resulted in a lower cGMP increase than the stimulation with ANP alone, whereas it had an additive effect in GECs. Similar results for podocytes regarding a possible mutual regulation of sGC and pGC activity have been published and suggest the avoidance of excessive cGMP concentrations in podocytes.<sup>52,53</sup>

Inhibition of PDE isoforms revealed substantial differences in their activity between GECs and podocytes. In GECs all tested PDE inhibitors (targeting PDE2a, 3, 4, 5 and 9a) increased basal cGMP levels, while inhibition of PDE2a had the strongest effect and delayed cGMP degradation following ANP or/and SNAP stimulation. In podocytes, however, only the PDE3 inhibitor Cilostamide and the PDE5 inhibitor Avanafil resulted in comparable increases of basal cGMP levels. Both substances predominantly augmented the ANP-induced cGMP synthesis without affecting the long-lasting cGMP/FRET response in the washout-phase. As a limitation, the applied PDE inhibitors predominantly inhibit one PDE isoform, but are not completely specific. Hence, it cannot be excluded that these inhibitors partially influence other PDE isoforms. Administration of the unspecific PDE inhibitor IBMX to podocytes resulted only in a small additional accumulation of cGMP levels, with a bigger effect on the NO/sGC/cGMP-pathway. The less pronounced effect of unspecific PDE isoform inhibition by IBMX on cGMP levels compared to specific PDE isoform inhibition might be explained by the unequally effective inhibition of different PDE isoforms by IBMX.<sup>54,55</sup> Taken together, this demonstrates that GECs and podocytes rely on distinct PDE isoforms and their impact varies between the two cGMP pathways, which mandates studying glomerular cGMP signaling with a high spatial and temporal resolution.

The FRET-based cGMP biosensor utilized here was capable of detecting small changes in intracellular cGMP concentrations (EC<sub>50</sub> ~ 532 nM, Supplemental Figure 1) and its functionality has been validated in other *ex vivo* settings.<sup>25,29,56</sup> Further improvement of the FRET sensor could help to increase the signal-to-noise ratio and facilitate cGMP measurements during *in vivo* imaging of the glomerulus. Due to the thickness of AKS and the necessary penetration, we had to apply higher doses of the substances than published for cell culture. Nevertheless, the reported differences between GECs and podocytes were measured, while all surrounding experimental conditions, e.g. the cutting process, the perfusion setup and the imaging protocol, remained unchanged.

Regarding the limitations of this study, our experiments were not designed to elucidate if stimulation of the ANP/pGC/cGMP and NO/sGC/cGMP pathways in GECs and podocytes occur by autocrine or paracrine signaling. Furthermore, we did not investigate downstream targets of the cGMP signaling pathways, which could explain the non-additive effect of ANP+SNAP stimulation or the long-lasting effect of ANP stimulation in podocytes. For these questions, further studies are required and partially ongoing.

Taken together, we demonstrated the presence of the ANP/pGC/cGMP as well as the NO/sGC/cGMP pathway in GECs and podocytes of healthy glomeruli. ANP stimulation resulted in higher and longer lasting cGMP production in podocytes compared to SNAP stimulation and the cGMP response triggered by ANP and/or SNAP in GECs. PDE activity in podocytes is dominated by PDE3 and PDE5, while in GECs all tested isoforms are active and inhibition of PDE2a had the strongest effect.

As cGMP signaling is downregulated in disease states and pharmacological manipulation intends to restore cGMP pathways, further studies with different disease models are warranted to increase our understanding of disease-associated changes in cGMP signaling in glomerular cells.

## Disclosures

All authors have nothing to disclose.

## Acknowledgments

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## Supporting information (SI 1)

Content: 10 Supplemental figures

Supplemental Figure 1: In vitro characterization of cGi500.

Supplemental Figure 2: Visualization of the cutting process-related damage to glomeruli.

Supplemental Figure 3: Evans Blue interferes at high concentrations with the FRET-based biosensor cGi500.

Supplemental Figure 4: Calculated FRET (CFP/YFP) ratio from CFP and YFP fluorescence traces demonstrates cGMP generation after activation of the ANP/pGC or/and NO/sGC pathway in GECs.

Supplemental Figure 5: Calculated FRET (CFP/YFP) ratio from CFP and YFP fluorescence traces demonstrates cGMP generation after activation of the ANP/pGC or/and NO/sGC pathway in podocytes.

Supplemental Figure 6: Cell-specific expression of the cGMP biosensor in optically cleared AKS.

Supplemental Figure 7: High doses of ANP and SNAP are required to elicit measurable cGi500-mediated cGMP signals.

Supplemental Figure 8: DEA NONOate triggers cGMP synthesis in Tie2:Cre/cGi500 mice.

Supplemental Figure 9: GECs of Cdh5:Cre/cGi500 mice respond similar to Tie2:Cre/cGi500 mice.

Supplemental Figure 10: Long-term measurement of the cGMP/FRET response in podocytes after ANP administration.

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## 6 Discussion

*The following discussion encompasses all compiled data, incorporating the results from the submitted manuscript (Chapter 5).*

### 6.1 cGMP signaling in podocytes

#### 6.1.1 Evidence for pGC-mediated cGMP synthesis in podocytes

Although podocytes may not exhibit fully developed foot processes or cell-specific markers, the use of conditionally immortalized podocyte cell lines from various species (mouse, rat, human) expressing the temperature-sensitive mutant of the SV40 T-antigen is common practice and formed the basis for cGMP research in podocytes.<sup>157,158</sup> Species-dependent differences manifest in the absence of essential key enzymes for the cGMP signaling cascade, such as sGC and its subunits, resulting in a lack of cGMP response upon stimulation.<sup>29,69,72</sup> As the data on the existence of guanylyl cyclase systems in this cell type are contradicting, we addressed this question by isolating glomeruli of transgenic mice (Pod:Cre/cGi500) expressing the cGMP biosensor exclusively in podocytes. The NP/pGC/cGMP pathway was stimulated by two different natriuretic peptides, ANP and BNP, which function as ligands for the natriuretic peptide receptors GC-A and GC-B. The interaction between ligands and receptor resulted in different cGMP responses, as evidenced by a stronger FRET/cGMP response elicited by 500 nM ANP compared to 500 nM BNP. In addition, AKS of Pod:Cre/cGi500 mice also demonstrated an ANP-evoked FRET/cGMP response (**Chapter 5, Fig. 1**). Previous studies conducted on cultured podocytes are in line with our results, demonstrating cGMP synthesis upon ANP stimulation.<sup>29,68,69,72,159,160</sup> The design of the cGi500 sensor did not allow for differentiation between cGMP pools synthesized by GC-A or GC-B activity. However, podocytes highly express GC-A mRNA and foot processes exhibit ANP-binding sites.<sup>52,65,67,161</sup> In addition, GC-A has shown a higher affinity towards ANP ( $\text{ANP} \geq \text{BNP}$ ), whereas GC-B preferentially binds CNP (not tested).<sup>162,163</sup> Therefore, we hypothesize, that GC-A is the receptor responsible for the cGMP synthesis after ANP and BNP administration. The importance of GC-A expression in renal tissue was demonstrated by a GC-A knockout model, in which GC-A protected against aldosterone-induced glomerular injury.<sup>164</sup> Furthermore, podocyte-specific knockout mice lacking GC-A (Podo-GC-A-KO) exhibited substantial albuminuria and glomerular damage following mineralocorticoid deoxycorticosterone acetate (DOCA) treatment or high salt-diet (6% NaCl diet and aldosterone).<sup>63,165</sup>

Isolated glomeruli and AKS of Pod:Cre/cGi500 mice showed a persistent elevated FRET/cGMP response following ANP administration throughout the entire measurement period. This observation was also made in AKS when using half the amount of ANP (**Supplemental Fig. 7**), which excludes sensor saturation as a potential cause for the prolonged signal. The signal declined slowly over a period of more than 1 hour, demonstrated in a long-term recording (**Supplemental Fig. 10**). As previously described in the **chapter 5.4**, the prolonged ANP-evoked cGMP synthesis could be maintained due to delayed internalization of ligand-receptor complexes of GC-A or delayed desensitization of the receptor by ligand-induced dephosphorylation or activation of PKC.<sup>166–169</sup> PKC is a downstream target of ANGII/AT1 signaling and plays a crucial role as mediator of the suppressive function on the GC-A receptor, emphasizing the counter-regulatory crosstalk between the RAAS and NP systems.<sup>86,159</sup> In addition, a positive feedback loop involving PKG or lower PDE activity, which would delay cGMP degradation, or a combination of the mentioned mechanism could be possible.<sup>170</sup> The plasma half-life for ANP/GC-A ligand-receptor binding ranges within 2 minutes,<sup>171,172</sup> prompting the hypothesis that signal termination occurs via circulating mediators in the plasma. Given that neither isolated glomeruli nor AKS are directly connected to the bloodstream, addressing this question would necessitate *in vivo* imaging. Due to the limitations preventing *in vivo* imaging with the biosensor cGi500 (discussed in **chapter 6.6**), the utilization of a more sensitive cGMP biosensor with enhanced signal-to-noise ratio could offer a potential solution.

To our knowledge, our data provides the first evidence of a long-lasting cGMP response following stimulation of the ANP/pGC/cGMP axis in podocytes. Conventional methods quantify cGMP levels after cell lysis using antibody-based techniques such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), which results in a loss of temporal resolution. The utilization of the FRET-based cGMP biosensor cGi500 demonstrates superiority over conventional biochemical methods in this context, enabling real-time monitoring of cGMP dynamics. This finding is particularly important in the context of reduced cGMP levels observed in disease, which are intended to be addressed through pharmacological treatment. A mutation in the ANP gene (mutant-ANP = MANP) was found to be more resistant to neprilysin-induced proteolytic degradation.<sup>173</sup> Pre-clinical results, including the first in-human study of MANP, suggest that this resistance may lead to prolonged effects in lowering blood pressure and suppressing aldosterone through GC-A activation.<sup>174,175</sup> Thus, a long-lasting ANP/GC-A/cGMP response in podocytes represents a potential target for novel therapeutic strategies and should be examined further for its clinical relevance in kidney diseases. However, our study was not designed to elucidate downstream effector molecules responsible for the prolonged response after ANP/pGC/cGMP axis stimulation in podocytes. Regarding the functional role of ANP stimulation, studies conducted with cultured rat podocytes suggest induced PKG $\alpha$

dimerization, cytoskeletal alterations, and increased albumin permeability.<sup>84,176</sup> This implies a potential influence on podocyte morphology, affecting foot processes and slit pore size, and ultimately glomerular permeability. However, this hypothesis has to be confirmed utilizing *ex vivo* or *in vivo* models.

### 6.1.2 Evidence for sGC-mediated cGMP synthesis in podocytes

To date, data on NO-related processes in isolated glomeruli have been limited to NO detection following stimulation with vasoactive substances such as ANGII,<sup>88,177</sup> while the localization of NO production remains speculative. Sharma et al. demonstrated glomerular cGMP production after BAY 41-2272 exposure, a NO-independent activator of sGC, but could not identify the specific cell type responsible.<sup>178</sup> Furthermore, data regarding the existence of a NO/sGC/cGMP pathway in podocytes is contradictory. Studies by Theilig et al. and Wang et al. failed to demonstrate a SNAP-mediated cGMP response in cultured podocytes.<sup>29,72</sup> To investigate whether podocytes from isolated glomeruli are capable of responding to stimulation of the NO/sGC/cGMP axis, SNAP was added through the perfusion system. SNAP, an NO donor, spontaneously releases the free radical NO under physiological conditions, triggered a robust FRET/cGMP response in cGi500-expressing podocytes, thereby indicating the existence of the NO/sGC/cGMP signaling pathway. To account for the possibility that SNAP stimulation exerts a paracrine effect on podocytes, another NO donor was used. Nitroglycerin generates NO through enzymatic bioactivation,<sup>179</sup> elicited also a FRET/cGMP response and provides additional evidence for NO/sGC-mediated cGMP synthesis in podocytes of isolated glomeruli. In addition, SNAP administration on AKS of Pod:Cre/cGi500 mice displayed a FRET/cGMP response, albeit to a lesser extent in terms of intensity (**Chapter 5, Fig. 1**). The signal profile was transient, which is in line with our expectations arising from the short half-life of NO in the range of a few milliseconds.<sup>180,181</sup> Without a connection to the blood stream, NO scavengers such as hemoglobin are not available to modulate fluctuating NO concentrations. As a result, the cGMP response observed in our experiments endures for an extended period and may potentially be terminated by desensitization of sGC.<sup>182</sup> Other studies are in agreement with our data, showing an increased cGMP concentration after SNAP administration.<sup>68,80,159,160</sup> Moreover, they also demonstrate that ANP consistently stimulates higher levels of cGMP production compared to cGMP synthesis elicited by SNAP. Increased cGMP levels following SNAP, as well as the sGC stimulator Riociguat, were able to decrease Adriamycin-induced TRPC6 expression and reduce podocyte injury, indicating a protective effect of this signaling pathway for podocytes.<sup>80</sup> Mundel et al. and Jarry et al. localized sGC in podocytes of kidney tissue and Hart et al. provided evidence for sGC subunit expression in a human podocyte cell line.<sup>80,90,155</sup> Our experimental setup did not allow for identification of the sGC subunit ( $\alpha 1$ ,  $\beta 1$ ,  $\alpha 2$ , and  $\beta 2$ ) configuration present in podocytes, additional experiments are required for this

purpose. The results obtained from antibody pull-down, Western blot analysis or immunolabeling, should be critically evaluated. The presence of sGC subunits does not necessarily imply functionality, and vice versa. Furthermore, evidence suggest a dynamic equilibrium between mature functional heterodimers and immature sGC forms.<sup>183</sup> During stress conditions, active sGC forms can transition to immature forms,<sup>184</sup> while sGC stimulators/activators hold therapeutic potential for restoring diminished cGMP responses resulting from this transition.<sup>185</sup> Thus, the relative levels of mature sGC heterodimer and immature sGC (sGC $\beta$ 1 subunit-Hsp90 complex) before and after stimulation should be assessed, as NO can promote the maturation process and can activate sGC $\beta$ 2 subunits without a second sGC subunit partner.<sup>183,186</sup>

Additionally, the administration of SNAP, a compound releasing NO spontaneously, can exert its influence on all cell types within isolated glomeruli and AKS. Spatial confinement of NO release can be achieved for single-cell studies using the photocaged NO donor (Npm-photocaged) NONOate, which has already been tested with cGi500-expressing vascular smooth muscle cells (VSMCs).<sup>187</sup> UV irradiation can induce cellular toxicity, whereas two-photon microscopy (2PE microscopy) offers advantages for the utilization of such NO donors. This method restricts excitation to a focal plane, unlike confocal microscopy where excitation occurs throughout the focus, requiring a pinhole for optical sectioning.<sup>188</sup> The photocaged NO donor Cupferron (CP) O-alkylated with anthracene (CPA), exhibited negligible photo-activated cell toxicity when coupled with 2PE microscopy,<sup>189</sup> thus presenting a promising option for future experiments. Our experiments were not designed to identify the NO-producing cell in the glomerulus, as both mesangial cells and glomerular endothelial cells possess NOS activity.<sup>80,190,191</sup> One way to test the influence of NO on podocytes is to use eNOS knockout mice (eNOS-KO mice). Endothelial NOS is mostly expressed in endothelial cells and deficiency of eNOS has been associated with podocyte loss and injury, emphasizing endothelial-podocyte interaction.<sup>93,94</sup>

In summary, our study provides evidence for the presence and functionality of both guanylyl cyclase pathways (sGC and pGC) in podocytes of isolated glomeruli and kidney slices from Pod:Cre/cGi500 mice, in which the synthesis of cGMP stimulated by ANP predominated.



### 6.1.3 Suppressed global cGMP levels after simultaneous activation of sGC & pGC-pathways in podocytes

After providing evidence for the existence of the sGC and pGC pathways in podocytes, we elucidated a potential reciprocal regulation between these two signaling pathways. Simultaneous co-stimulation with ANP+SNAP in isolated glomeruli from Pod:Cre/cGi500 mice resulted in a smaller FRET/cGMP response compared to the stimulation with ANP or SNAP alone. Co-stimulation in AKS confirmed this outcome, whereas only ANP stimulation exhibited a higher FRET/cGMP response in comparison (**Chapter 5, Fig. 1**). These findings align with the work of Lewko et al., but contradict the results observed in a later study (2015).<sup>68,160</sup> In the study carried out in 2015, co-stimulation in cultured mouse and rat podocytes resulted either in equivalent or even higher cGMP levels compared to ANP stimulation alone. However, in both studies, cGMP measurements were conducted using a radioimmunoassay after the addition of the unspecific PDE-inhibitor IBMX. In contrast, our experimental approach enabled us to monitor cGMP dynamics with temporal resolution and without inhibiting cGMP degradation. In AKS of Pod:Cre/cGi500 mice, the signal profile after co-stimulation revealed a direct inhibition of the global cGMP concentration that, as we hypothesize, is controlled by a potential negative feedback mechanism preferentially lowering ANP/pGC-controlled cGMP level (**Chapter 5, Fig. 1**). The reached FRET/cGMP response was higher compared to the response induced by SNAP, but lower than the response induced by ANP. The time course of the co-stimulation exhibited similarities with the individual stimulation by SNAP and ANP, presenting a progressively developing signal (peak) that then declined and transitioned into the characteristic prolonged plateau seen with ANP stimulation alone. This implies that both guanylyl cyclase (GC) systems operate concurrently, preventing excessively high cGMP levels even shortly after simultaneous stimulation of sGC and pGC. The absence of an additive effect is seen in all other test combinations with ANP+SNAP, suggesting that podocytes strictly regulate their cGMP homeostasis through intrinsic inhibition with a ceiling on the maximum intracellular cGMP level. However, sequential stimulation with ANP and SNAP, and vice versa, could elucidate which signaling pathway exerts a stronger influence on the other. Both Lewko et al. and Stepinski et al. discussed a mutual compensation between the two GC systems, where the suppressor effect of ANGII or the inhibition of the NOS system (also in a time-dependent manner) can exert influence.<sup>68,160,192,193</sup> Additionally, global cGMP levels can be diminished through elimination pathways like PDE-mediated hydrolysis or cGMP extrusion. Both options will be discussed in the subsequent sections. Besides PDE activity, our study was not designed to identify additional downstream effector molecules responsible for the hypothesized reciprocal interaction between sGC and pGC following co-stimulation with ANP+SNAP in podocytes.

## 6.2 Phosphodiesterase (PDE) activity in podocytes

The intracellular cGMP concentration is regulated by its synthesis and degradation. Degradation is mediated by PDEs, which cleave the 3', 5'-cyclic phosphate moiety of cGMP to produce the corresponding purine nucleotide GTP. Conventional techniques for assessing cGMP levels in isolated glomeruli involve the addition of the nonspecific PDE inhibitor IBMX as pretreatment prior to stimulation.<sup>194–196</sup> This approach stops dynamic cellular processes influenced by cGMP degradation, facilitating comparisons before and after stimulation of this pathway. We administered IBMX to elucidate the impact of PDE inhibition on basal cGMP levels in podocytes of isolated glomeruli from Pod:Cre/cGi500 mice, which resulted in a progressively increasing FRET/cGMP response. Subsequently, the impact of PDE inhibition on stimulated sGC or/and pGC signaling was tested. AKS of Pod:Cre/cGi500 mice were subjected to stimulation with ANP and/or SNAP, with the addition of IBMX during the course of stimulation (**Chapter 5, Fig. 2**). IBMX administration potentiated the FRET/cGMP response triggered by co-stimulation with ANP+SNAP the most, suggesting that the reduction in global cGMP concentration (discussed in **Chapter 6.1.3**) is partly influenced by enhanced PDE activity. IBMX administration on SNAP-evoked cGMP levels had a greater effect than on ANP-evoked cGMP level, indicating higher PDE activity after sGC activation. The prolonged cGMP response observed with ANP or ANP+SNAP stimulation was not influenced by PDE inhibition. These findings imply that cGMP pools synthesized by ANP/pGC exhibit less susceptibility to degradation by PDEs, which we hypothesize is based on the localization of the NP receptor family members (GC-A/B and NPR-C) on the cell body and/or foot processes. This hypothesis finds support in the detection of pGC activity in foot processes and the presence of ANP binding sites.<sup>197–199</sup> This suggests compartmentalization of cGMP pools, in addition to transmembrane-associated pGC activity and sGC localization in the cytosol. Furthermore, the spatial distribution of PDEs within the cell is a crucial factor to consider, as the majority of the PDEs would accumulate in the cell body to regulate the overall intracellular cGMP concentration. As a result, pGC/cGMP pools generated at the membrane, especially in the foot processes, may experience slower degradation. The lack of evidence on the abundance, localization, and activity or potential subcellular trafficking of PDEs following stimulation of the two cGMP signaling pathways in podocytes, leaves this hypothesis unanswered. However, it remains possible that the hydrolysis of ANP/pGC-synthesized cGMP pools would primarily depend on PDEs with a slow degradation rate. Different degradation rates are described for members of the PDE4 family and cAMP activity.<sup>200,201</sup> This would result in a prolonged cGMP response, indicating that enzymatic kinetics play a more significant role than the subcellular localization of PDEs with restricted cGMP accessibility. Of note, individual PDE isoforms are not equally effectively inhibited by IBMX,<sup>202,203</sup> highlighting the need to assess the inhibition of selected PDE isoforms on AKS.

### 6.2.1 PDE3 and PDE5 predominate in podocytes

Evidence regarding the profile of specific PDE isoforms within podocytes is primarily based on proteomics data. These datasets hint towards a subset of PDE isoforms that require further examination to elucidate their enzymatic activity. Building upon the findings of Rinschen et al. and corroborating literature on podocyte-specific and renal expression,<sup>66,72</sup> our selection included PDE2a, 3, 4, 5, and 9a. Commercially available inhibitors targeting these PDE isoforms were applied on AKS of Pod:Cre/cGi500 mice to assess their impact on cGMP signaling under basal conditions (**Chapter 5, Fig. 3**). Notably, the inhibition of PDE3 by Cilostamide elicited the highest FRET/cGMP response, followed by PDE5 inhibition with Avanafil. Avanafil was chosen as a potent PDE5 inhibitor due to its higher selectivity against other PDE isoforms compared to Sildenafil, a well-established PDE5 inhibitor.<sup>204</sup>

Subsequently, the impact of specific PDE inhibition on stimulated sGC or/and pGC signaling was tested. AKS of Pod:Cre/cGi500 mice were subjected to stimulation with ANP and/or SNAP, with the addition of Cilostamide (PDE3 inhibitor) or Avanafil (PDE5 inhibitor) during the course of stimulation (**Chapter 5, Fig. 4**). PDE3 inhibition potentiated the FRET/cGMP response triggered by ANP the most, followed by ANP+SNAP, indicating a preference for the ANP/pGC/cGMP axis. PDE3 hydrolyzes cAMP and cGMP: In the presence of elevated cGMP concentrations, cAMP hydrolysis by PDE3 is inhibited, leading to intracellular cAMP accumulation.<sup>205</sup> The interplay between cAMP and cGMP may function as a mechanistic regulator for cytoskeletal alterations, impacting foot processes and slit pore size, as postulated by Sharma et al.<sup>176</sup> Additionally, evidence suggests that cAMP promotes the assembly of cell-cell contacts among podocytes and prevents podocyte apoptosis.<sup>206–208</sup>

PDE5 inhibition during an ongoing stimulation with the trinary test set ANP or/and SNAP revealed a similar FRET/cGMP response to that observed with PDE3 inhibition, albeit to a lesser extent. Avanafil potentiated the synthesized cGMP pools following ANP and ANP+SNAP stimulation the most, while showing only a marginal impact on SNAP stimulation (**Chapter 5, Fig. 4**). PDE5 binds cGMP with high specificity. PDE5 inhibition in podocytes has demonstrated renoprotective effects in various kidney disease models.<sup>79,209–211</sup> Furthermore, research has linked PDE5 inhibition with podocyte motility,<sup>81</sup> and with the (e)NOS/NO/sGC/cGMP pathway that protects against podocyte damage.<sup>79,80</sup> Although PDE5 appears to be highly associated with the NO/sGC/cGMP axis, our data demonstrate a preference for pGC-synthesized cGMP pools. The possibility that the ANP/pGC/cGMP pathway may influence PDE5 activity is supported by the work of Polzin et al., where increased levels of PDE5 and PKG-II were observed in kidneys of corin knockout mice.<sup>71</sup> Corin is a transmembrane serine/threonine protease responsible for the conversion of pro-ANP to active

ANP.<sup>212</sup> Regarding the limitation of this study, we tested a subset of PDE isoforms using commercially available inhibitors, which results in a biased approach. These inhibitors are not completely specific, thus interference with other PDE isoforms cannot be excluded.

Taken together, PDE3 and PDE5 preferentially influence ANP/pGC-generated cGMP pools in podocytes. Specific PDE isoforms are targeted for the pharmacological elevation of diminished cGMP signaling in diseases, emphasizing the need to investigate their activity in podocytes.

### **6.3 Inhibition of cGMP extrusion increases intracellular cGMP levels in podocytes**

In addition to PDE-mediated hydrolysis, intracellular cGMP concentration can be regulated through cGMP efflux. Previous studies suggest the release of cGMP following stimulation of the ANP/pGC/cGMP axis in podocytes, as well as the extrusion of cGMP by ATP-binding cassette (ABC) transporters.<sup>160,213–217</sup> Moreover, probenecid, an inhibitor of the organic anion transporter OAT1, was able to induce cGMP accumulation in cultured rat podocytes,<sup>160</sup> providing evidence for an active transport by the organic anion transporter family. By administering probenecid to isolated glomeruli from Pod:Cre/cGi500 mice, we were able to demonstrate cGMP efflux at basal conditions. This was shown by an increase in the FRET/cGMP response in podocytes. A subsequent stimulation with ANP and/or SNAP could uncover potential differences in the degree of released cGMP and whether this elimination pathway partially accounts for the non-additive cGMP response after co-stimulation with ANP+SNAP seen in our experiments. Lewko et al. tested the effect of probenecid on ANP or/and SNAP stimulation in cultured rat podocytes, showing an increased cGMP accumulation after ANP+SNAP stimulation compared to ANP and SNAP alone.<sup>160</sup> These results are in line with our hypothesis, however, experiments utilizing isolated glomeruli or AKS should be conducted to further elucidate these findings.

Currently, there is no available data regarding the functional role of cGMP extrusion by podocytes, as well as the specific cell type targeted by this process. However, it is conceivable that cells downstream within the tubular system could be the next potential target and respond to extracellular cGMP as a component of a paracrine signaling cascade, which traverses the cell membrane. This assumption is supported by the fact that cultured renal proximal tubule (RPT) cells responded to the addition of the membrane-permeable analogue 8-Br-cGMP with an increase in intracellular cGMP levels.<sup>218</sup> Additionally, several studies confirm the presence of sGC in this cell type.<sup>29,105,219,220</sup> Furthermore, RPTs responded to the addition of 8-Br-cGMP, the NO donor sodium nitroprusside (SNP), or co-incubation with endothelial cells (stimuli-activated) with decreased Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and transcellular Na<sup>+</sup> transport.<sup>221</sup> *In vivo*

experiments involving the infusion of Texas red-conjugated cGMP into the renal cortical interstitium revealed that interstitial cGMP inhibits renal tubular Na<sup>+</sup> uptake without affecting hemodynamic parameters such as RBF and GFR. Infusion of conjugated-cGMP into the renal artery induced renal vasodilation and increased glomerular filtration, leading to a comparable level of natriuresis.<sup>222</sup> This data strongly suggests that cGMP release is involved in paracrine signaling, and the resulting effect depends on the local microenvironment.

#### **6.4 Calcium (Ca<sup>2+</sup>)-dependent mechanisms trigger cGMP release in podocytes**

The morphology, structure, and viability of podocytes are affected by calcium (Ca<sup>2+</sup>) signaling.<sup>223</sup> Despite being distinct cellular signaling pathways, calcium and cGMP signaling exhibit interactive relationships that mutually regulate each other through diverse mechanisms (e.g. cGMP hydrolysis by Ca<sup>2+</sup>/calmodulin-dependent PDE1).<sup>224</sup> Notably, GECs respond to Bradykinin or ATP by eliciting an increase in intracellular calcium levels ([Ca<sup>2+</sup>]<sub>i</sub>) and triggering both NO-in/dependent cGMP production in e.g. mesangial cells.<sup>225,226</sup> To assess the responsiveness of podocytes to stimuli that trigger calcium-dependent mechanisms, we tested the effect of Bradykinin and ATP on isolated glomeruli from Pod:Cre/cGi500 mice. Both compounds triggered a progressively evolving FRET/cGMP response that endured throughout the wash-out phase without returning to baseline levels. These data suggest that ligand-receptor binding may exert a direct effect on podocytes, thereby increasing cGMP levels. Alternatively, an indirect paracrine signaling, potentially involving eNOS/NO/sGC/cGMP signaling, could exert influence. Studies indicate the expression of bradykinin receptors and a bradykinin-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in cultured podocytes.<sup>227-230</sup> Furthermore, P2 receptors (comprising P2X and P2Y receptor subtypes) have been identified on both cultured podocytes and isolated glomeruli.<sup>231</sup> These receptors mediate an ATP-triggered Ca<sup>2+</sup> influx,<sup>232</sup> primarily through the metabotropic receptor subtypes P2Y1 and P2Y2.<sup>232,233</sup> A recent intravital multiphoton microscopy imaging study postulated a role for P2 receptors and transient receptor potential canonical 6 (TRPC6) channels in mechanical and metabolic sensing, emphasizing the significance of calcium-dependent mechanisms in podocytes.<sup>234</sup> In this context, both cGMP-synthesizing guanylyl cyclase systems (sGC and pGC) have been associated with a suppressive role on TRPC6 expression, in which Adriamycin-treated eNOS-KO mice and DOCA-treated podocyte-specific GC-A KO (Podo-GC-A-KO) mice showed an enhanced expression of glomerular TRPC6.<sup>63,80</sup> Furthermore, Sonneveld et al. postulated a connection between PDE5/cGMP/PKG-I signaling and the binding of PPAR-γ to the TRPC6 promoter, thereby reducing TRPC6-mediated Ca<sup>2+</sup> influx and ultimately mitigating podocyte injury.<sup>79</sup> Increased intracellular calcium levels in podocytes have been demonstrated to enhance cell motility,<sup>235,236</sup> albumin permeability, and are associated with podocyte injury,<sup>237</sup> accentuating

the need to investigate both calcium and cGMP signaling in health and disease. As damage to podocytes of isolated glomeruli cannot be avoided, we discontinued experiments with this method to differentiate whether NO-in/dependent mechanisms played a role in ATP-/bradykinin-evoked cGMP synthesis in podocytes. Instead, we plan to utilize whole-body eNOS-KO mice along with the calcium indicator GCaMP3 and the cGMP biosensor cGi500 used in this study, to resolve a potential endothelial-podocyte cross talk *ex vivo* and *in vivo*.

PIEZO1 is a mechanosensitive ion channel protein that plays a functional role in stretch-induced  $\text{Ca}^{2+}$  influx and ATP release.<sup>238</sup> Conditional endothelial cell-specific deletion of PIEZO1 has been demonstrated to influence eNOS expression in this cell type, suggesting the capability to detect and adjust to variations in shear stress induced by blood flow, consequently inducing vasodilation.<sup>239,240</sup> The presence of PIEZO channels has been confirmed on GECs as well as podocytes,<sup>241,242</sup> and mechanical stress has been shown to modulate cGMP production in conditionally immortalized mouse podocytes.<sup>69</sup> To investigate whether cGMP is a component of the PIEZO1-mediated mechanotransduction pathway in podocytes, isolated glomeruli were treated with Jedi2 (PIEZO1 activator), which elicited a cGMP response in podocytes from Pod:Cre/cGi500 mice. Further experiments are required to validate these findings and to elucidate the signaling cascade in greater detail.

In summary, our data suggest a connection between calcium-dependent mechanisms and cGMP signaling in podocytes, implicating their involvement in mechanosensation, and pointing towards a potential stimulus-driven endothelial-podocyte crosstalk.

## 6.5 cGMP signaling in glomerular endothelial cells (GECs)

### 6.5.1 Evidence for sGC-/pGC-mediated cGMP synthesis

Endothelial cells serve as a link between the circulatory system and tissues, regulating vasotonus through local mechanisms such as the secretion of nitric oxide via a calcium-calmodulin-mediated activation.<sup>243,244</sup> GECs exhibit a remarkably flattened morphology and transcellular fenestrations.<sup>245</sup> They express eNOS and respond to NOS agonists (such as L-arginine) by releasing nitric oxide (NO).<sup>80,246,247</sup> The production of NO plays a crucial role in renal hemodynamics, maintaining the integrity of the capillary protein permeability barrier, counteracting the generation of reactive oxygen species (ROS) through the NO/sGC/cGMP pathway.<sup>41,248</sup> However, little is known about whether GECs possess intrinsic cGMP dynamics. To address this question, kidney slices from Tie2:Cre/cGi500 mice expressing the cGMP biosensor exclusively in the cytosol of endothelial cells were exposed to cGMP-elevating agents. The NP/pGC/cGMP system was stimulated by ANP, while the NO/sGC/cGMP system was activated with the NO donors SNAP or DEA NONOate. Both stimulations resulted in an

increase in intracellular cGMP concentration with similar signal strength, and co-stimulation (ANP+SNAP) revealed an additive effect (**Chapter 5, Fig. 1, Supplemental Fig. 8**).

The cGMP response initiated in GECs after ANP stimulation is anticipated to result from the binding of the ligand to its receptor. Our findings are in line with the expression pattern of GC-A, GC-B and the NP-clearance receptor-C (NPR-C) on glomerular endothelial cells,<sup>52</sup> and with the work of Green et al. demonstrating a dose-dependent accumulation of cGMP following ANP administration in cultured human GECs.<sup>249</sup> The receptor selectivity of GC-A and GC-B for the natriuretic peptide family (ANP, BNP, CNP) in GECs remains uncertain, as does the hierarchy for cGMP production. The biological role of renal endothelial GC-A and GC-B is still unknown, but there are reports suggesting an influence of ANP on endothelial permeability and in renal inflammation.<sup>250–252</sup> Furthermore, mice with endothelial-restricted deletion of the GC-A gene proved that endothelial GC-A is indeed crucial for the ANP-induced acute increase in capillary permeability to macromolecules.<sup>75</sup> The design of the sensor precludes making conclusions regarding the specific NP receptor (GC-A or GC-B) involved in ANP-mediated cGMP synthesis in GECs. The NP-receptors GC-A and GC-B exhibit a high degree of structural similarity,<sup>253,254</sup> posing challenges for the targeted blockade of a specific receptor. The GC-A positive allosteric modulator (PAM), MUF-651, is a promising development, enhances ANP-mediated cGMP generation in HEK cells while demonstrating low affinity towards GC-B.<sup>255</sup> The utilization of this modulator has the potential to confirm the existence of an ANP/GC-A/pGC/cGMP signaling cascade within GECs.

In GECs of Tie2:Cre/cGi00 mice, stimulation with SNAP or DEA NONOate resulted in an elevation of intracellular cGMP levels, indicating that GECs are not only capable of producing NO but are also responsive to it. This implies that this cell type has the ability to induce cGMP synthesis in a sGC-dependent manner. The expression of two subunits of sGC (sGC $\alpha$ 2 and sGC $\beta$ 2) was shown in cultured human GECs.<sup>80</sup> Based on our knowledge, this study represents the first documentation of a functional NO/sGC/cGMP pathway in GECs. Our experimental setup lacks the capability to distinguish between the induced cGMP synthesis from different sGC subunit configurations. Studies examining sGC subunit expression in diverse cell types, including endothelial cells, have predominantly concentrated on systemic vasculature or non-renal tissues.<sup>29,30,256</sup> These investigations suggest that the sGC $\alpha$ 1 $\beta$ 1 heterodimer is the prevailing form. Further research is necessary to provide conclusive evidence regarding the sGC subunit expression present in glomerular endothelial cells and the function of NO/sGC/cGMP signaling in this cell type. Building upon Stehle et al.'s study, future experiments could integrate an sGC activator, such as BAY-543, known to induce cGMP generation under conditions of oxidative stress in glomeruli of kidney slices from cGi500 biosensor mice.<sup>92</sup> Moving away from real-time monitoring of cGMP dynamics *ex vivo*,

additional immunolabeling, Western blots or single cell proteomic analysis could provide a clearer picture about sGC expression in GECs. We investigated the impact of exogenous NO delivered to GECs through spontaneous release from NO donors. However, the study did not investigate whether endogenously produced NO via calcium-dependent or- independent activation of eNOS within the cell induces a different cGMP response or any response at all. This question could be addressed by using agents known to induce calcium-dependent eNOS activation, such as agonists like acetylcholine, bradykinin, or carbachol.

Evidence for an actively regulated cGMP system in GECs is almost non-existent in the literature, as even the presence of guanylyl cyclases is not well-documented. We claim to be the first to provide such data using real-time cGMP measurements in living kidney tissue. However, further investigation is required to elucidate the specific role of cGMP in GECs.

### **6.5.2 GECs exhibit high basal PDE activity, with PDE2a being the predominant isoform**

In our experiments, GECs exhibited a higher basal PDE activity compared to podocytes with a predominance of the PDE2a isoform (**Chapter 5, Fig. 3**). Stimulation with ANP and/or SNAP in addition with IBMX administration demonstrated a stronger PDE influence on the ANP/pGC-generated cGMP pools (**Chapter 5, Fig. 2**), while PDE2a showed no preference towards one cGMP signaling pathway (**Chapter 5, Fig. 4**). These findings align with the study conducted by Sadhu et al., who reported positive PDE2a immunostaining in capillary endothelial cells within the glomerulus of human renal tissue.<sup>257</sup> Expressed in GECs, PDE2 could potentially influence signaling pathways mediated by cAMP and cGMP, based on its capability to hydrolyze both. This assumption would support the work of Surapisitchat et al., suggesting a concentration-dependent influence of cGMP on endothelial permeability via PDE2 and PDE3 activity.<sup>258</sup> At lower concentrations, cGMP inhibits PDE3, which increases the local cAMP pool and decreases permeability. Conversely, at higher concentrations, cGMP activates PDE2, which leads to a decrease in the cAMP level and reverses the effect. Differential expression of PDE2 and PDE3 may serve as a regulatory mechanism between physiological and pathological cGMP levels, thereby fine-tuning endothelial permeability.<sup>259</sup> When interpreting data on endothelial cells, it should be noted that the relative expression levels of PDEs vary among different endothelial cell beds.<sup>260</sup> Additionally, the activity of PDE isoforms changes significantly in cultured endothelial cells between passages and in response to phenotypic alterations, as demonstrated in resting BAEC exhibiting cobblestone morphology compared to spindle-shaped proliferating BAEC.<sup>261</sup> This phenotypic heterogeneity of endothelial cells is rarely acknowledged in drug development.<sup>262,263</sup> Recent efforts utilizing single-cell mRNA sequencing aim to address this issue by identifying marker genes for GECs.<sup>264</sup> This could provide the



foundation for establishing knockout models specifically targeting intraglomerular endothelial cells or for the development of Cre mouse lines. However, the biosensor cGi500 was able to reveal a functional cGMP signaling in GECs, wherein PDE activity influenced the regulation of global cGMP levels.

## 6.6 Evaluating cGi500 biosensor performance for cGMP imaging: Strengths and Limitations

### 6.6.1 Comparison of cGi500 with other cGMP biosensors

As the majority of cGMP signaling-related studies are based on sample preparation from cultured cell lines, we first transfected HEK 293T cells with the MH20\_pCMV\_cGi500 plasmid encoding the monomolecular FRET-based cGMP biosensor cGi500 (**Supplemental Fig. 1**). Following the preparation of homogenates, in which the sensor was dissolved in solution, we performed a dose-response curve analysis by introducing ascending ligand concentrations ranging from 0.01  $\mu\text{M}$  to 100  $\mu\text{M}$  cGMP. The analysis revealed a half-maximal effective concentration (EC<sub>50</sub>) of  $\sim 532$  nM (**Supplemental Fig. 1**), which reflects the cGMP binding affinity of the sensor. This result closely corresponds to the postulated EC<sub>50</sub> value of 500 nM determined by Russwurm et al.<sup>139</sup> In comparison to other cGMP biosensors, cGi500 demonstrates superiority in terms of cGMP affinity, dynamic range and spatial distribution. The cGMP biosensor cGES-DE5 utilizes the same FRET pair as cGi500 (CFP/YFP), but exhibits a lower affinity for cGMP compared to cGi500, with an EC<sub>50</sub> of  $1.5 \pm 0.2$   $\mu\text{M}$ .<sup>138,265</sup> In contrast, the biosensor Yellow PfPKG exhibits a higher cGMP affinity (EC<sub>50</sub> of 22 nM) and displays stronger FRET ratio changes upon stimulation compared to cGi500.<sup>138</sup> However, Yellow PfPKG has a smaller dynamic range than cGi500, saturating at lower cGMP concentrations while cGi500 can detect higher concentrations. Despite these limitations, PfPKG displays a nonuniform distribution pattern, which restricts its function to certain subcellular areas. By replacing the FRET pair CFP/YFP, the new developed cGMP biosensors surpassed cGi500 in terms of sensitivity and selectivity (cAMP/cGMP), but they have not been fully evaluated in an *ex vivo* setting. Among these, the cGMP biosensor Cygnus stands out, allowing for triple-parameter fluorescence imaging of cAMP, cGMP, and  $\text{Ca}^{2+}$ , albeit with significant spectral bleed-through (a contaminant in FRET signaling).<sup>266</sup> Furthermore, attempts have been made to combine two FRET sensors (TagRFP/mPlum and ECFP/Venus) with fluorescence lifetime imaging (FLIM) to avoid spectral crosstalk.<sup>267</sup> Nevertheless, ratiometric FRET-based biosensors, such as cGi500, simultaneously capture two fluorescence intensities, rendering them less susceptible to motion artifacts in the Z-plane. This characteristic enables their utilization in both *ex vivo* and *in vivo* experiments.

### 6.6.2 Signal intensity

The functionality of the sensor was assessed *in vitro* by conducting real-time measurements on cGi500-transfected HEK 293T cells following IBMX stimulation. An accumulation of intracellular cGMP was observed, evident by the increase in donor (CFP) fluorescence and the decrease in acceptor fluorescence (YFP). The modest change in the CFP/YFP emission ratio (maximal  $\Delta R/R_0 \sim 0.10\%$ ), fell within the range documented for primary cultures of cerebellar granule neurons (CGNs) or vascular smooth muscle cells (VSMCs) derived from cGi500-indicator mice ( $\Delta R/R_0 \sim 0.60\%$ ).<sup>140,268</sup> However, during the transition from *in vitro* to *ex vivo* or *in vivo* settings, the intensity of cGi500-mediated cGMP responses (maximal  $\Delta R/R_0$ ) decreases,<sup>140,142</sup> a trend that was also noticeable in our study. An observation that is primarily attributed to the small FRET changes associated with the CFP/YFP fluorophore combination.<sup>269</sup> Although this FRET pair is widely used, its application comes with difficulties in detection due to a narrow FRET dynamic range (**Supplemental Fig. 11**) and a low signal-to-noise ratio. This aspect is particularly challenging in tissues with high background autofluorescence signals, as observed in the kidney.<sup>270,271</sup> Increasing the excitation laser power improves the distinction of the signal from the background, but it simultaneously induces sensor photobleaching. This fact poses a challenge in time-lapse recordings, as significant photobleaching was observed in cGi500 heterozygous Tie2:Cre/cGi500 mice (**Supplemental Fig. 4**). Furthermore, processes such as phototoxic CFP excitation or the conversion of YFP into cyan fluorescent protein-like species can play a role.<sup>272,273</sup>

Considering the accessibility of the substance to the target cell is another factor to take into account. While isolated glomeruli are superfused with solution from all angles, the formation of concentration gradients in AKS will affect the strength of FRET signals when glomeruli are detected in deeper tissue layers. In an effort to address challenges associated with substance delivery to the target cell, we tried to evaluate cGMP dynamics through an intravital imaging (IVI) approach using two-photon microscopy. In this context, diminished FRET/cGMP responses are expected due to the undesirable excitation of the acceptor fluorophore using two-photon excitation. Following the experimental guidelines for IVI by Thunemann et al. and conducting an excitation scan of cGi500-transfected HEK 293T homogenates (**Supplemental Fig. 11**),<sup>142</sup> we chose a two-photon excitation at 860 nm. This decision was motivated by the fact that renal cells exhibit lower autofluorescence at higher wavelengths.<sup>269,271</sup> In addition, it leads to decreased light scattering in biological tissue, ultimately resulting in an increased penetration depth. Unfortunately, we were unable to obtain reliable FRET/cGi500 signals through an image segmentation pipeline intended to enhance the signal-to-noise ratio. This challenge was compounded by difficulties in applying the substance in terms of concentration and its systematic effect on blood pressure. Furthermore, it is feasible that podocytes maintain

a very low cGMP level, which precludes the detection of high cGMP concentrations after stimulation.

Each FRET pair has its advantages and disadvantages, however, there is still no existing FRET system available for *in vivo* imaging of the kidney. Therefore, conducting *ex vivo* experiments with AKS represents the next best option for studying mechanisms under conditions that closely mimics the physiological environment.

### 6.6.3 Isolated glomeruli vers. AKS

Time-lapse recordings of isolated glomeruli from Pod:Cre/cGi500 mice displayed different signal profiles during the washout phase. However, none of these responses returned to baseline level during a measurement period of 19 min. In contrast, certain FRET/cGi500 responses in AKS returned to baseline level within 19 minutes (**Chapter 5, Figure 1 - 4**), and cGi500-mediated cGMP responses in cells or tissues typically display a transient pattern.<sup>140,143</sup> These differences could be attributed to the depth of the imaging chamber utilized for isolated glomeruli. Notably, the flow rate in experiments involving isolated glomeruli and AKS remained unchanged. Consequently, a deeper chamber recess might lead to prolonged washout times of the substances from the imaging chamber. Another explanation for the observations made with isolated glomeruli could be cell damage caused by the preparation process. Each step of glomeruli isolation (sieving, collagenase treatment, Dynabeads perfusion) contributes to podocyte damage, as demonstrated by Kindt et al. through staining with membrane-impermeable DNA-binding propidium iodide (PI).<sup>274</sup> In this context, it has been demonstrated that isolated glomeruli respond to ANGII stimulation with an increase in cytosolic calcium.<sup>275</sup> However, the proportion of reactive podocytes showing an ANGII-mediated calcium signal in *in vivo* experiments was remarkably low.<sup>151</sup> Emphasizing that podocytes behave differently due to induced damage from the isolation process compared with a more close-to-native scenario. Moreover, externally applied mechanical forces can lead to apoptotic cell death.<sup>276</sup> Cells undergoing apoptosis experience a phenomenon known as intracellular acidification, characterized by a significant decrease in intracellular pH.<sup>277</sup> The FRET acceptor YFP is highly pH-sensitive,<sup>278</sup> with acidic pH values causing changes in absorption and emission properties, potentially influencing FRET responses. The addition of HEPES to the imaging buffer aims to attenuate pH fluctuations caused by external sources. However, it cannot compensate for alterations in intracellular pH resulting from cell damage. In contrast to isolated glomeruli, experiments with AKS are more reliable as damaged glomeruli were excluded from analysis through vital staining with Evans Blue (**Supplemental Fig. 2 - 3**). The imaging setup also included real-time cGMP measurements performed at physiological temperature (37°C), which

was not implemented in experiments with isolated glomeruli. In summary, the utilization of AKS bridges the gap between *in vitro* and *in vivo* settings.

## 7 Conclusion and perspectives

Numerous hormones and vasoactive substances have the potential to influence cGMP-synthesizing systems, thereby affecting glomerular ultrafiltration.<sup>279</sup> The presence of an intrinsically regulated cGMP system, influenced by vasoactive substances, within cells of the glomerular filtration barrier remains uncertain. The work presented here, aims to resolve the discrepancies surrounding the presence of both cGMP signaling pathways in podocytes and GECs, utilizing the FRET-based cGMP biosensor cGi500 in a cell-specific approach. Real-time cGMP imaging on cGi500-transfected HEK 293T cells (sensor characterization) was followed by experiments involving isolated glomeruli and prepared AKS of transgenic mice. These experiments provided evidence for the existence of both guanylyl cyclase systems (sGC and pGC) in podocytes and GECs, including cell-specific differences. Podocytes showed a predominance of the ANP/pGC/cGMP pathway and a stringent regulation of global cGMP levels. To counteract excessively high cGMP peaks, this cell type may utilize mechanisms such as intrinsic inhibition of sGC/pGC, increased PDE activity, or cGMP efflux. Compared to GECs, basal PDE activity was lower in podocytes, wherein PDE3 & PDE5 were the most active isoforms. In contrast to podocytes, GECs showed no preference towards one specific cGMP pathway. However, the additive effect following co-stimulation was only observed in this cell type, suggesting an absence of a ceiling on the maximum intracellular cGMP concentration, as assumed for podocytes. GECs exhibited a high basal PDE activity, wherein PDE2a was the most active isoform. This result indicates a strong reliance on the elimination pathway mediated by cGMP-degrading PDEs to regulate global cGMP levels.

These cell-specific differences raise speculation that pathway-dependent cGMP synthesis could trigger diverse functions and, due to the close association of cGMP with the actin cytoskeleton, could impact cell morphology and, consequently, glomerular filtration. Furthermore, we hypothesize a cross-talk between podocytes and GECs, as the eNOS/NO/sGC/cGMP pathway is described to modulate vasotonus and podocytes express key molecules of a contractile apparatus. In this context, our data highlights mechanotransduction and calcium signaling as additional mechanisms to affect this intercellular communication. These aspects will be addressed in future experiments utilizing eNOS-KO mice, which express GCaMP3 (Ca<sup>2+</sup> sensor) or cGi500 (cGMP sensor) exclusively in podocytes or endothelial cells.

Taken together, our data provide new insights into cell-specific cGMP signaling in podocytes and GECs, laying a foundation for the development of cGMP-elevating drugs that target specific glomerular cell types. Ongoing research will continue to unravel the intricate details of cGMP signaling in renal physiology and pathology.

## Publications

Wiesner E, Binz-Lotter J, Hackl A, Unnersjö-Jess D, Rutkowski N, Benzing T, Hackl MJ: Correlation of calcium multiphoton with ultrastructural STED imaging of the slit diaphragm in the same glomerulus. bioRxiv [Preprint]. 2023 Sep. doi: <https://doi.org/10.1101/2023.09.27.559730>.

### Participation in conferences, congresses etc.

- 9th International Conference on cGMP: Generators, Effectors and Therapeutic Implications 2019, Mainz, DE

Poster\_\_Rutkowski N, Binz J, Benzing T, Hackl M: Hyperosmotic stress induces cGMP release in podocytes.

Meeting abstract published: No authors listed: Meeting abstracts from the 9th International Conference on cGMP: Generators, Effectors and Therapeutic Implications. J Transl Med. 2019 Aug 15;17(Suppl 2):254. doi: 10.1186/s12967-019-1994-0.

- CMMC SYMPOSIUM, 25 Years of Progress in Molecular Medicine: From Basic Research to Clinical Application 2021, Köln, DE

Poster\_\_Rutkowski N, Görlitz F, Höhne M, Benzing T, Hackl MJ: FRET-based visualization of compartmentalized cGMP signalling in podocytes.

- 13th International Podocyte Conference 2021, Manchester, UK

Presentation\_\_Rutkowski N, Görlitz F, Höhne M, Benzing T, Hackl M: FRET-based visualization of compartmentalized cGMP signalling in podocytes.

Meeting abstract published: Brix SR, Kanigicherla DAK, Wallace D, Desai T, Lennon R: The 13th International Podocyte Conference. Glomerular Dis. 2022 Apr; 2(Suppl 1): 1–78. doi: 10.1159/000525410.

- American Society of Nephrology (ASN) Kidney Week 2022, Orlando, US

Poster\_\_Rutkowski N, Görlitz F, Höhne M, Schermer B, Benzing T, Hackl M: FRET-based visualization of cGMP signaling in glomerular endothelial cells and podocytes.

- The 14th Biennial International Podocyte Conference 2023, Philadelphia, US

Poster\_\_Rutkowski N, Görlitz F, Höhne M, Schermer B, Benzing T, Hackl M: FRET-based visualization of cGMP signaling in glomerular endothelial cells and podocytes.

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# Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

## Teilpublikationen:

**Rutkowski N**, Görlitz F, Wiesner E, Binz-Lotter J, Feil S, Feil R, Benzing T, Hackl MJ: Real-time imaging of cGMP signaling shows pronounced differences between glomerular endothelial cells and podocytes.

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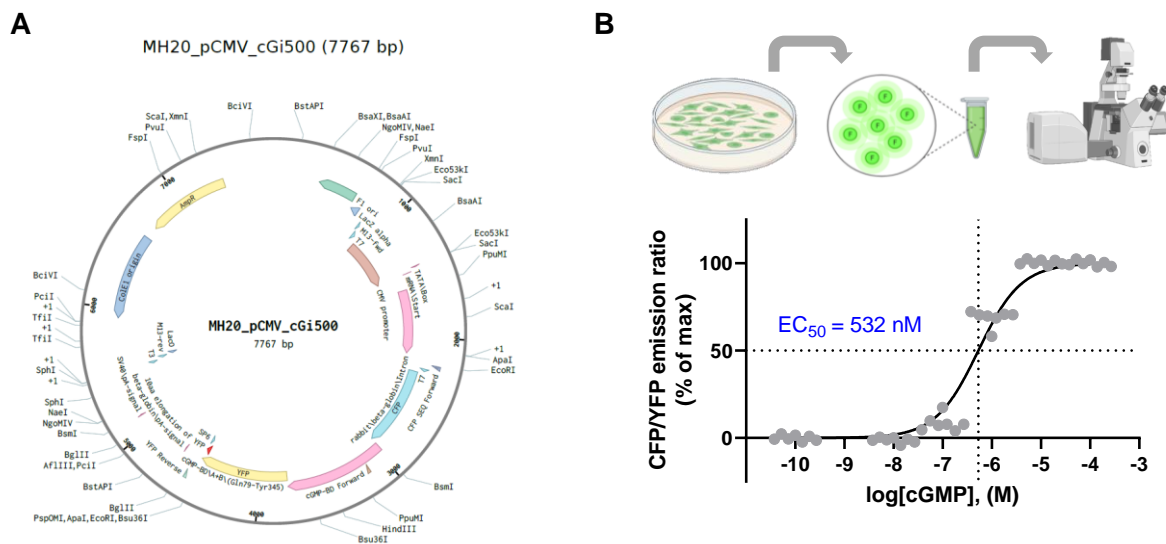
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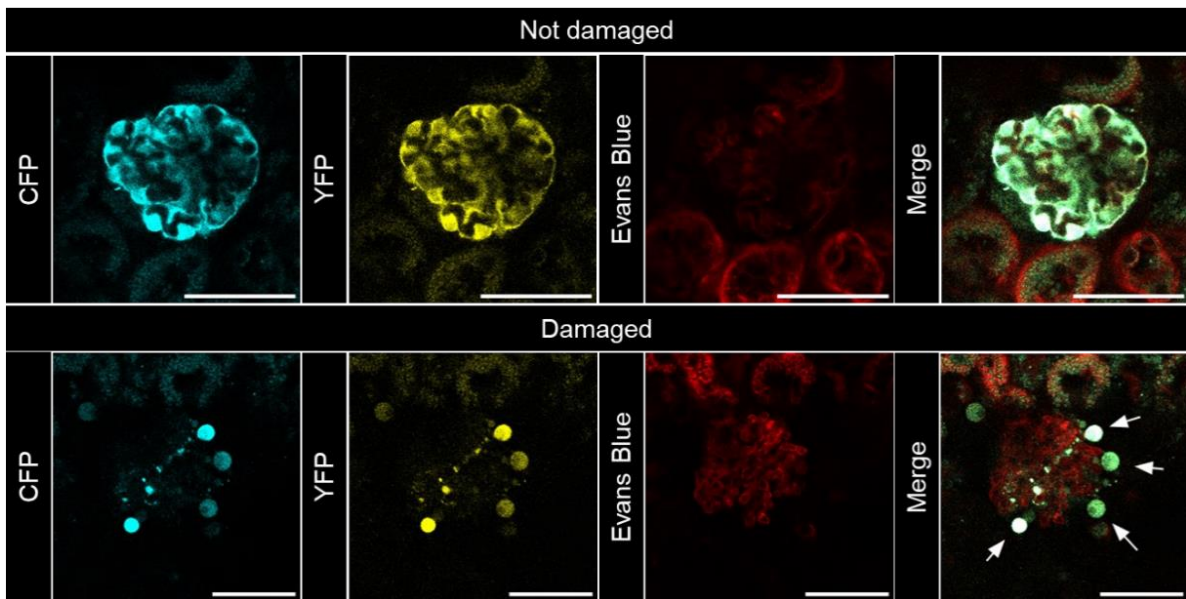
# Appendix

## Supporting information (SI 1)



### Supplemental Figure 1: *In vitro* characterization of cGi500

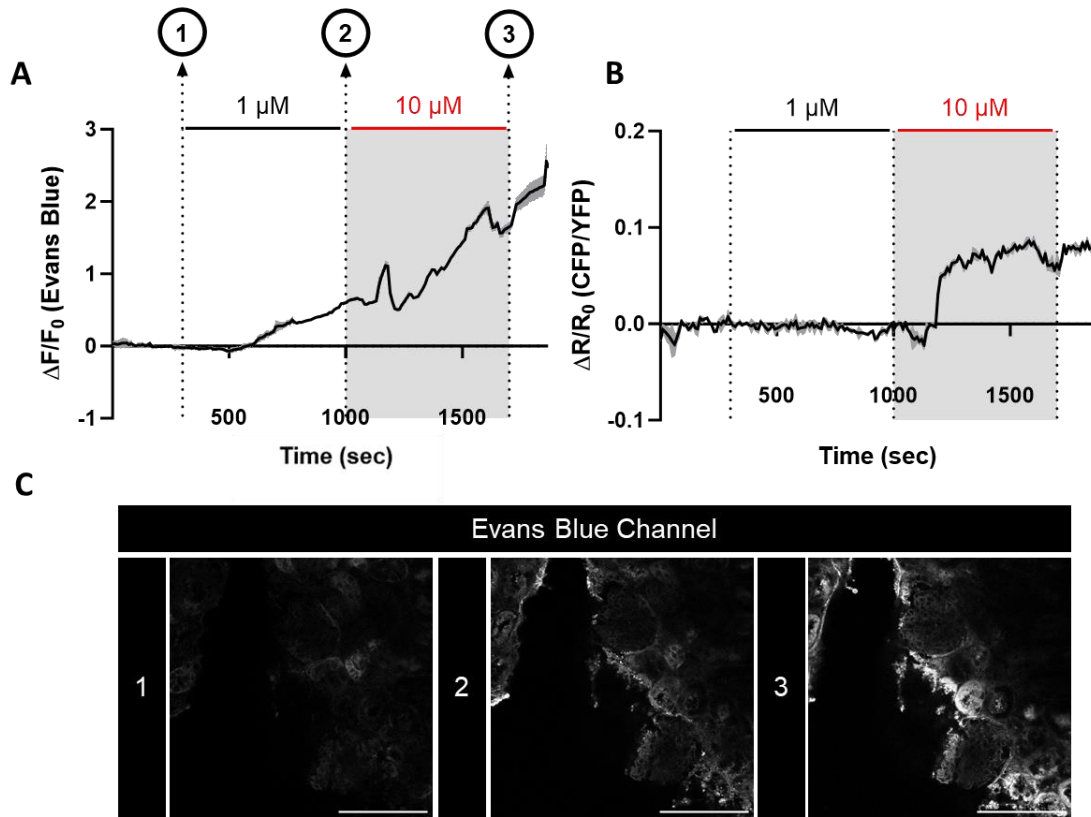
**(A)** Plasmid map of MH20\_pCMV\_cGi500 encoding the monomolecular FRET-based cGMP biosensor cGi500. **(B)** Concentration-response curve analysis of cGi500-transfected HEK 293T cell homogenates. Fluorescence measurements started with the highest cGMP concentration (100  $\mu\text{M}$  cGMP) and progressed to the lowest. Biosensor excitation was conducted with a diode laser at 405 nm together with a dichroic beam splitter (MBS-405) and simultaneous detection of CFP and YFP emission at  $480 \pm 25 \text{ nm}$  and  $535 \pm 20 \text{ nm}$ . The FRET (CFP/YFP) ratio changes were normalized to the first value (absence of cGMP) and the largest value (highest cGMP concentration) in the data set (Hill Slope = 1.0). Data are displayed for each replicate of seven independent experiments.



**Supplemental Figure 2: Visualization of the cutting process-related damage to glomeruli**

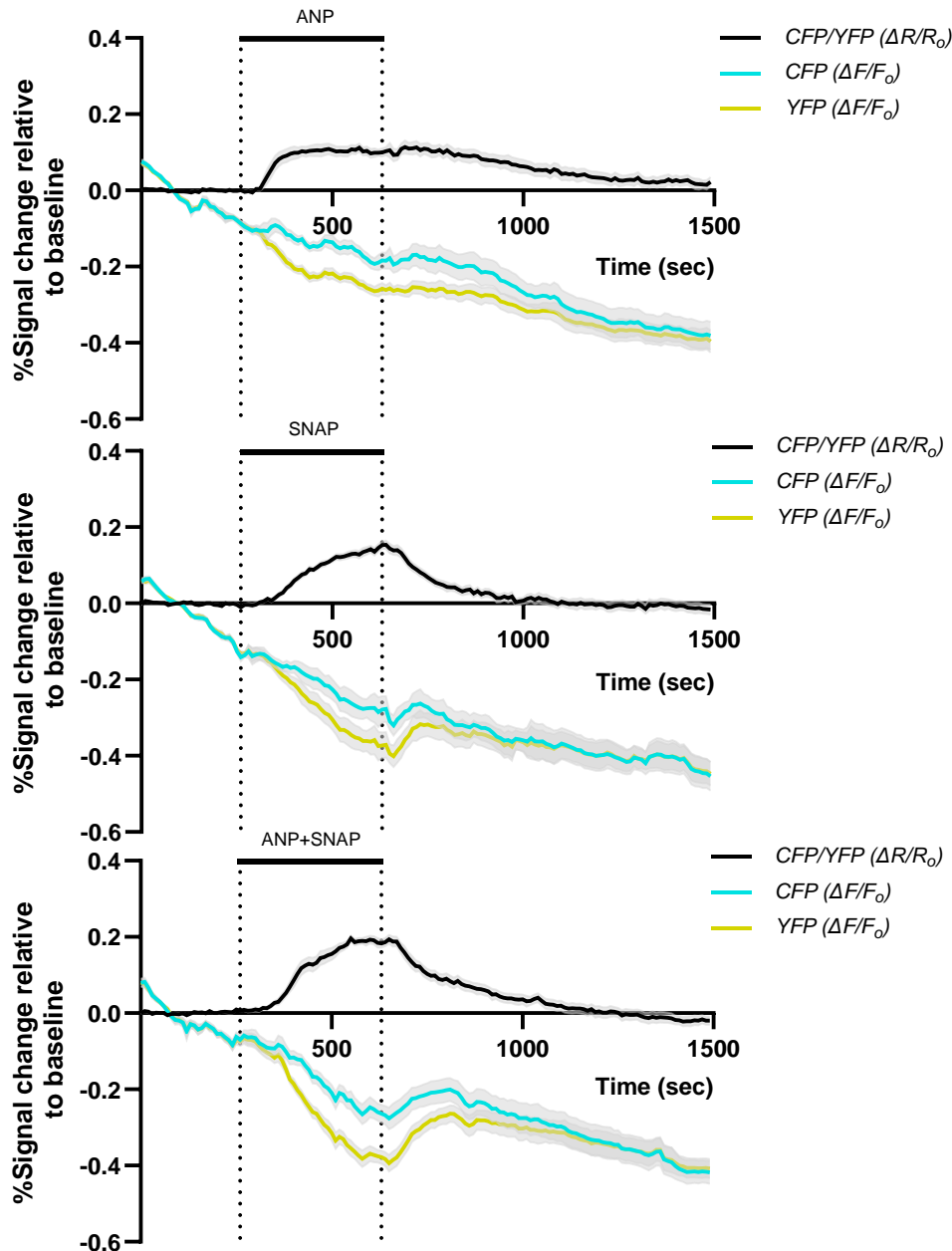
(**Top row**) Undamaged glomerulus shown in the CFP (cyan), YFP (yellow), Evans Blue (red) channel and the overlay of all channels (merge). The fluorescence in the Evans blue channel is due to expression of mTomato in all Cre-negative cells. (**Bottom row**) A glomerulus severely damaged by the cutting process with markedly deformed cells (white arrows). The uptake of Evans Blue into the glomerulus is visible in the red channel. Scale bar 50  $\mu$ M.





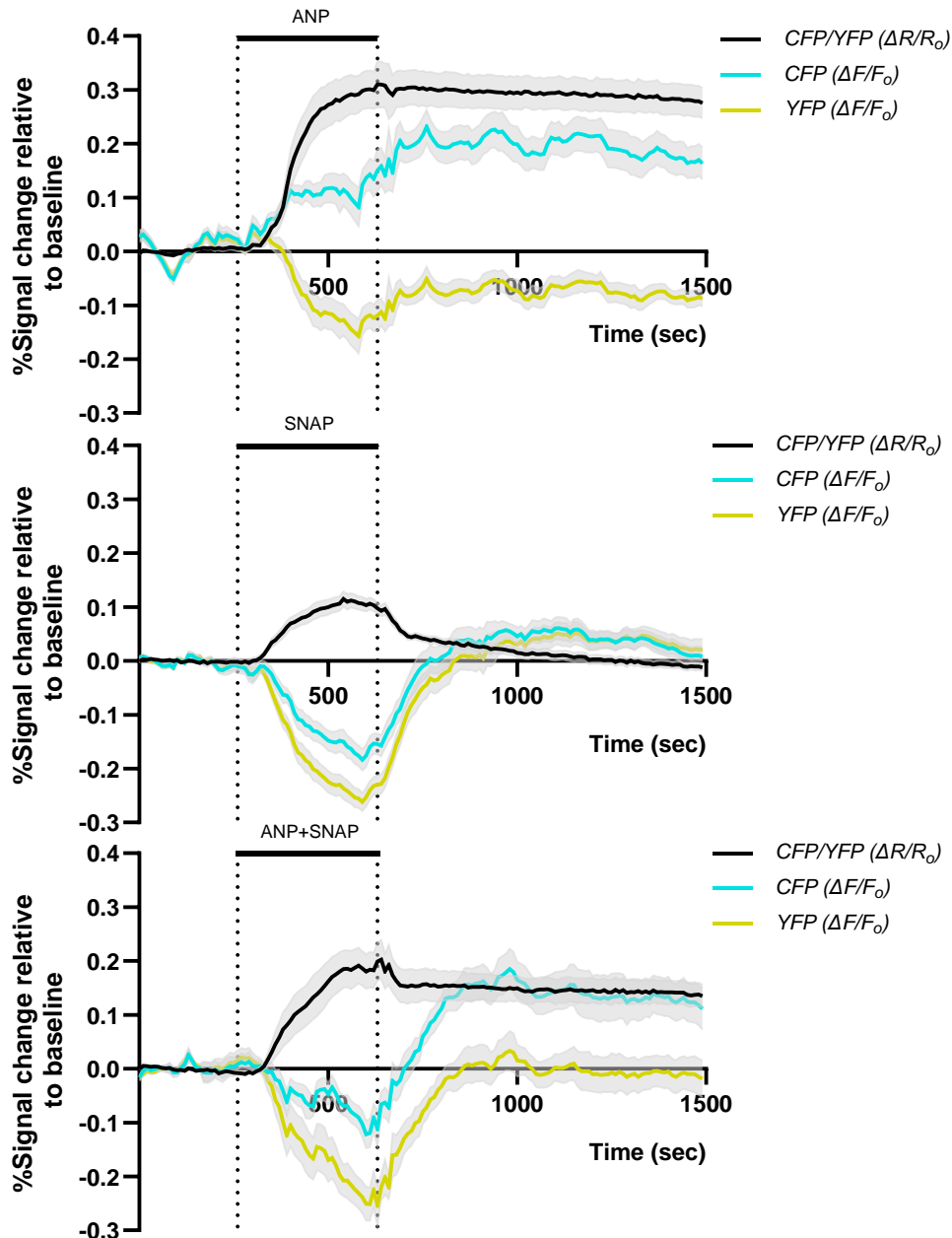
**Supplemental Figure 3: Evans Blue interferes at high concentrations with the FRET-based biosensor cGi500**

Acute kidney slice of a Pod:Cre/cGi500 mouse superfused with 1  $\mu\text{M}$  and 10  $\mu\text{M}$  Evans Blue solution (dotted lines) while recording time-lapse images. **(A)** While the fluorescence ( $\Delta F/F_0$ ) in the Evans Blue channel increases after superfusion with 1  $\mu\text{M}$  Evans Blue, a non-specific increase in the **(B)** FRET (CFP/YFP) ratio ( $\Delta R/R_0$ ) is only detectable after exposure to 10  $\mu\text{M}$  Evans Blue. **(C)** Images of the Evans blue channel at the time points 1 (300 sec), 2 (1000 sec) and 3 (1700 sec). All experiments shown in this manuscript were conducted with 1  $\mu\text{M}$  Evans Blue. Data represent mean  $\pm$  SEM. Scale bar 100  $\mu\text{M}$ .



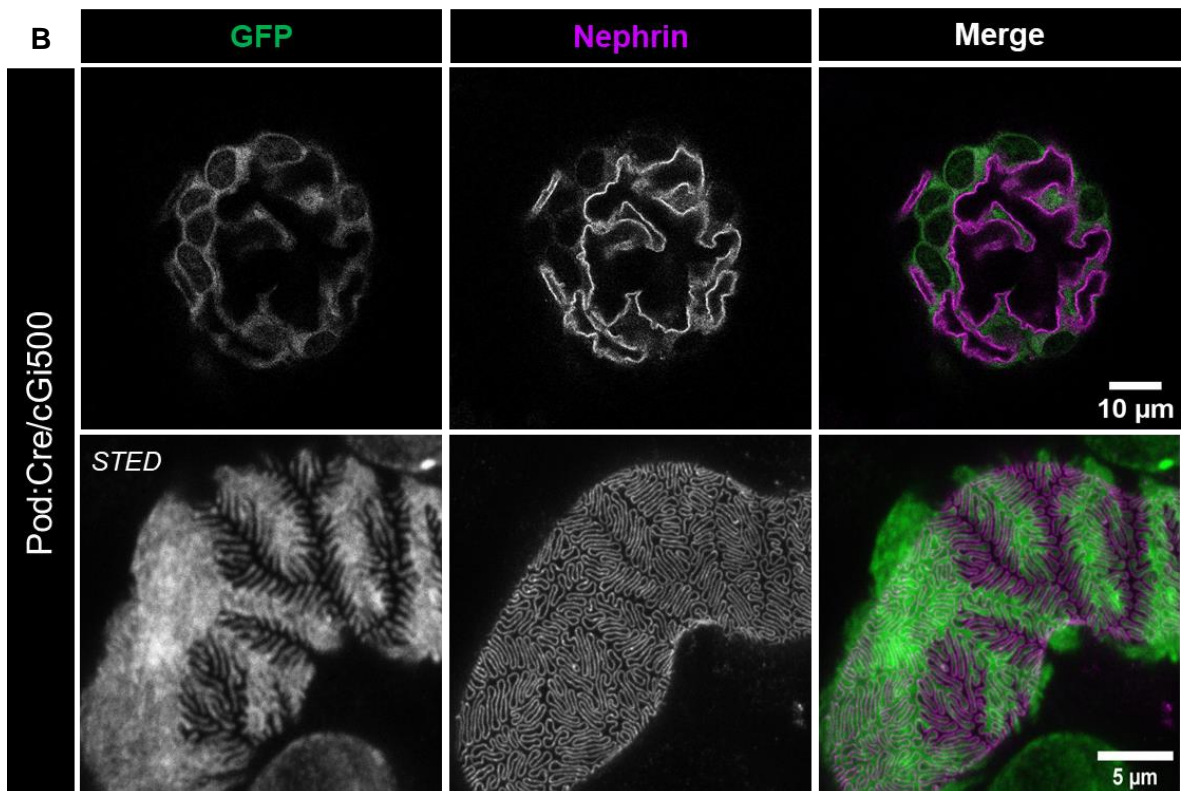
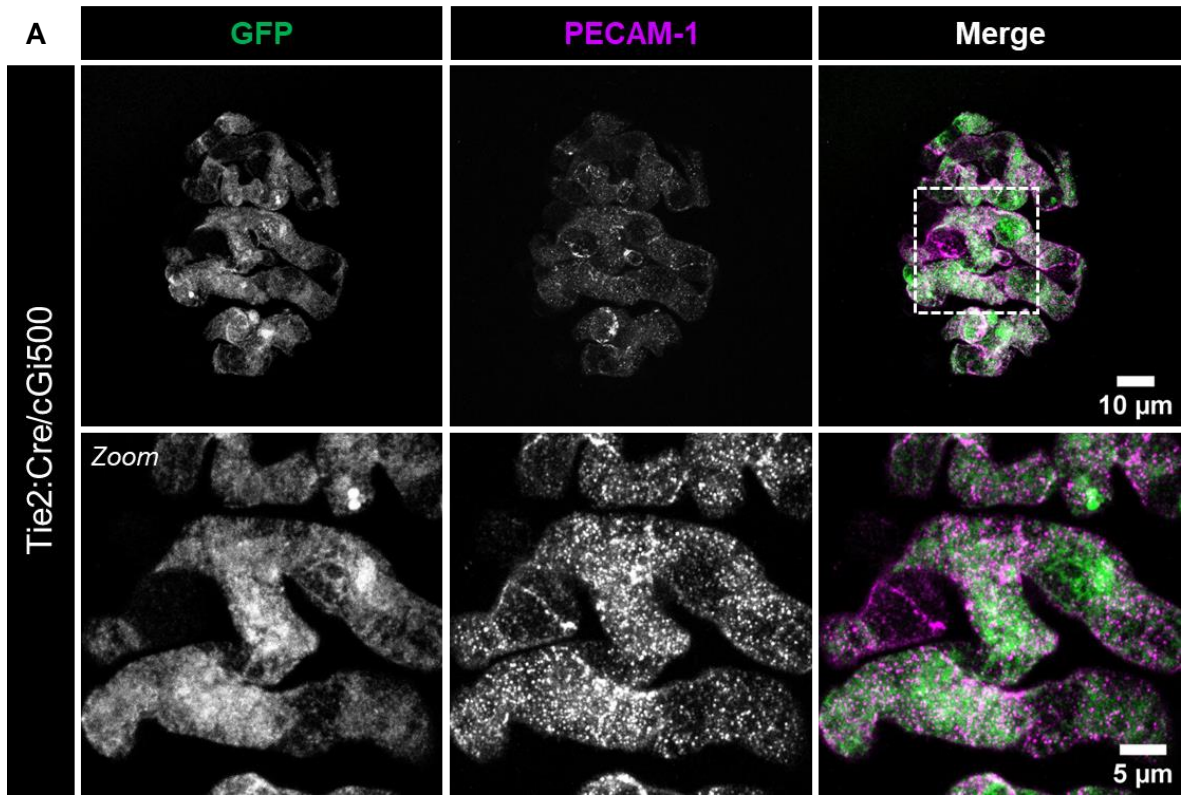
**Supplemental Figure 4: Calculated FRET (CFP/YFP) ratio from CFP and YFP fluorescence traces demonstrates cGMP generation after activation of the ANP/pGC or/and NO/sGC pathway in GECs**

Acute kidney slices from Tie2:Cre/cGi500 mice were subjected to stimulation with either 1  $\mu$ M ANP, 1 mM SNAP or a combination of both in the time period from 200 sec to 570 sec (dotted line). Displayed is the emission intensity change over a measurement period of 1500 sec with visualization of the individual CFP (cyan) and YFP (yellow) traces ( $\Delta F/F_0$ ) and the FRET (CFP/YFP) ratio (black,  $\Delta R/R_0$ ). Data represent mean  $\pm$  SEM of all analyzed glomeruli of six mice ( $n = 10$  for ANP,  $n = 9$  for SNAP,  $n = 10$  for ANP+SNAP).



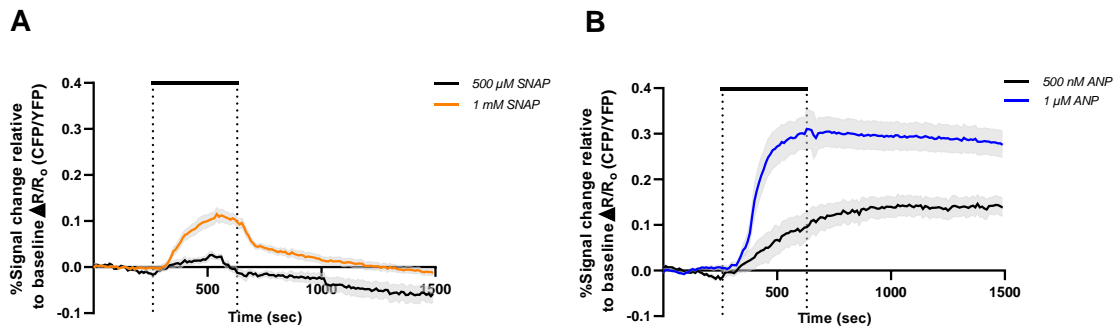
**Supplemental Figure 5: Calculated FRET (CFP/YFP) ratio from CFP and YFP fluorescence traces demonstrates cGMP generation after activation of the ANP/pGC or/and NO/sGC pathway in podocytes**

Acute kidney slices from Pod:Cre/cGi500 mice were subjected to stimulation with either 1  $\mu$ M ANP, 1 mM SNAP or a combination of both in the time period from 200 sec to 570 sec (dotted line). Displayed is the emission intensity change over a measurement period of 1500 sec with visualization of the individual CFP (cyan) and YFP (yellow) traces ( $\Delta F/F_0$ ) and the FRET (CFP/YFP) ratio (black,  $\Delta R/R_0$ ). Data represent mean  $\pm$  SEM of all analyzed glomeruli of five mice ( $n = 10$  for ANP,  $n = 11$  for SNAP,  $n = 10$  for ANP+SNAP).

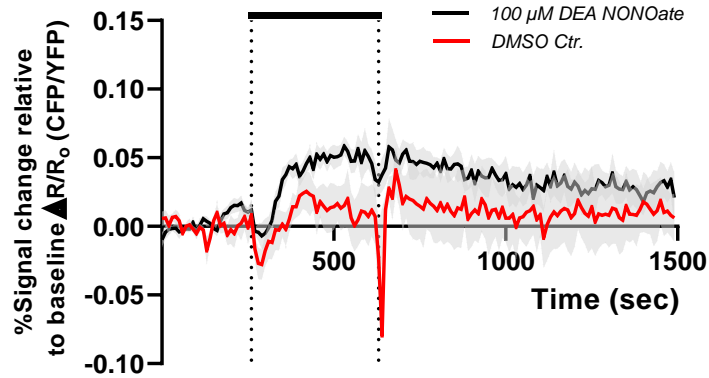


**Supplemental Figure 6: Cell-specific expression of the cGMP biosensor in optically cleared AKS**

(A) Tie2:Cre/cGi500: Confocal images show GFP staining (green) in GECs, thereby confirming cGi500 expression in this cell type. PECAM-1 staining (magenta) functions as a cell-specific marker for GECs and colocalizes with GFP-stained GECs. Bottom panels: High magnification of the area marked with a dashed rectangle in the right upper panel (Zoom). Both panels display a maximum intensity projection of a Z-stack. (B) Pod:Cre/cGi500: Confocal images demonstrate positive GFP staining (green) of podocytes, confirming the presence of biosensor cGi500 expression and overlapping nephrin staining (magenta), which labels the slit diaphragm. Higher magnification STED images (maximum intensity projection of Z-stacks) show a capillary area surrounded by podocytes (green) with characteristic slit diaphragm morphology (magenta).

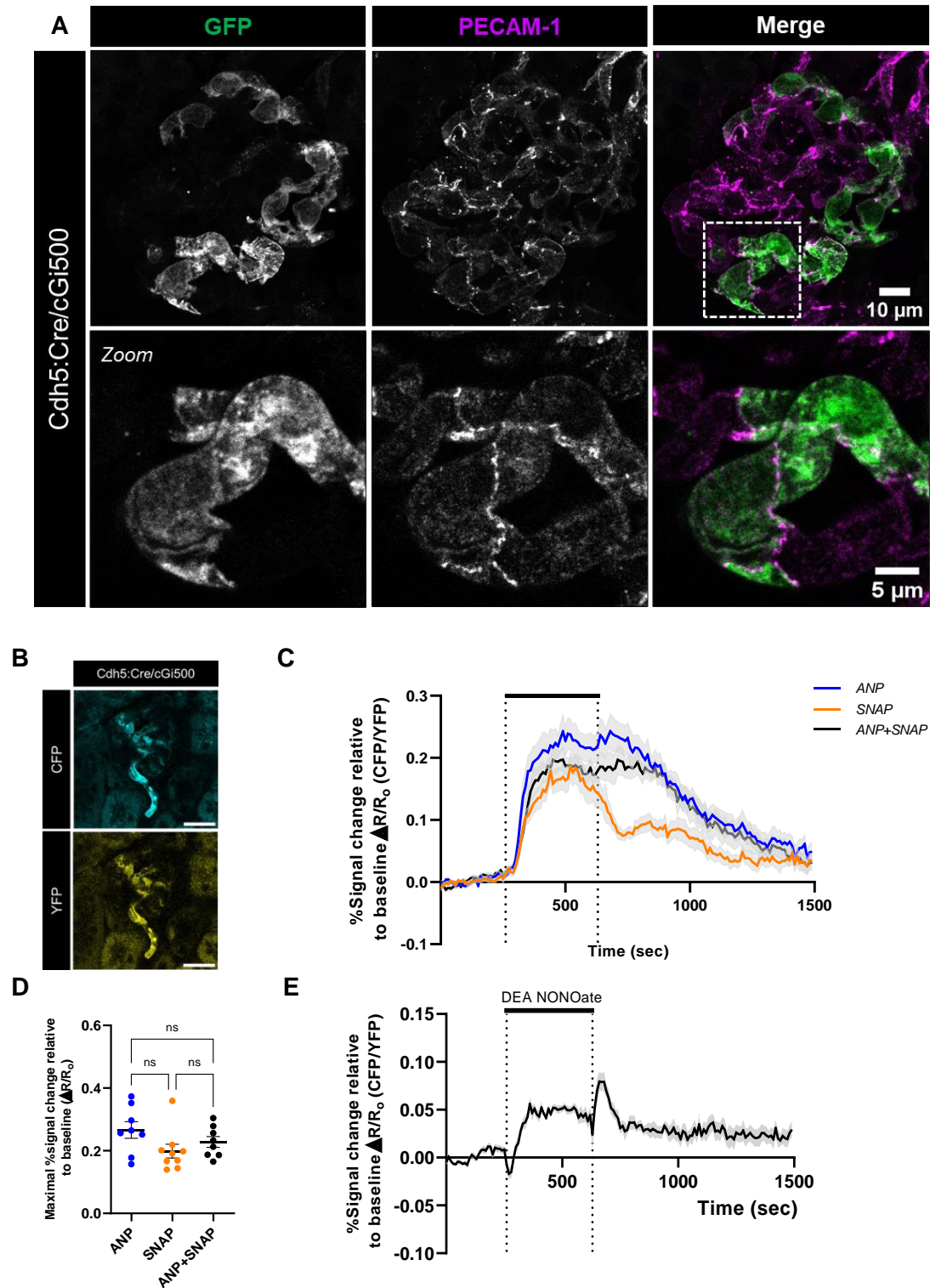
**Supplemental Figure 7: High doses of ANP and SNAP are required to elicit measurable cGi500-mediated cGMP signals**

Acute kidney slices from Pod:Cre/cGi500 mice were superfused (dotted lines) in the time period from 200 sec to 570 sec with (A) 1 mM SNAP (orange trace) and 500  $\mu$ M SNAP (black trace) or (B) 1  $\mu$ M ANP (blue trace) and 500 nM ANP (black trace). Stimulation was followed by pre-tempered 1X KHB (37°C), a time frame of 1500 sec was recorded. Graphs represent the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli. Data represent mean  $\pm$  SEM of all analyzed glomeruli of eight Pod:Cre/cGi500 mice ( $n = 6$  for 500 nM ANP,  $n = 10$  for 1  $\mu$ M ANP,  $n = 8$  for 500  $\mu$ M SNAP,  $n = 11$  for 1 mM SNAP).



### Supplemental Figure 8: DEA NONOate triggers cGMP synthesis in Tie2:Cre/cGi500 mice

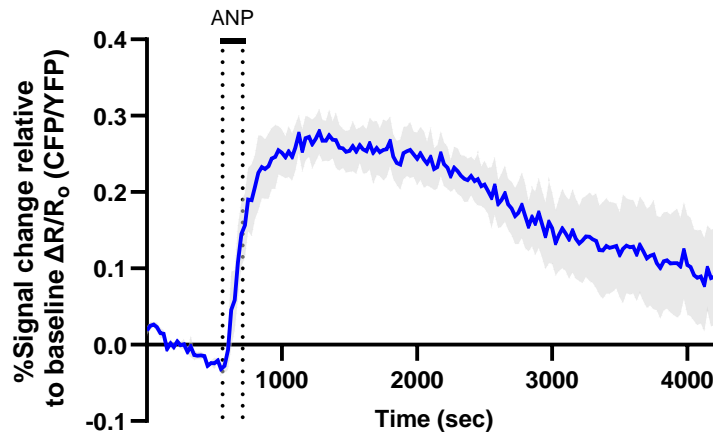
Acute kidney slices from Tie2:Cre/cGi500 mice were superfused (dotted lines) in the time period from 200 sec to 570 sec with 100 μM DEA NONOate (black trace) or DMSO (red trace). Stimulation was followed by pre-tempered 1X KHB (37°C), a total time frame of 1500 sec was recorded. Graph represent the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli. Data represent mean  $\pm$  SEM of all analyzed glomeruli of two Tie2:Cre/cGi500 mice ( $n = 7$  for DEA NONOate,  $n = 4$  for DMSO Ctr.).



**Supplemental Figure 9: GECs of *Cdh5:Cre/cGi500* mice respond similar to *Tie2:Cre/cGi500* mice**

(A) Immunostained kidney slice of a *Cdh5:Cre/cGi500* mouse. Confocal images show GFP staining (green) in GECs, thereby confirming cGi500 expression in this cell type. PECAM-1 staining (magenta) functions as a cell-specific marker for GECs and colocalizes with GFP-stained GECs. Bottom panels: High magnification of the area marked with a dashed rectangle in the right upper panel (Zoom). Acute kidney slices of *Cdh5:Cre/cGi500* mice (B, C) were superfused (dotted lines) in the time period from 200

sec to 570 sec with 1  $\mu$ M ANP (blue trace), 1 mM SNAP (orange trace), or the combination of both (black trace). Stimulation was followed by pre-tempered 1X KHB (37°C), a time frame of 1500 sec was recorded. Graphs represent the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli. (D) Scatter dot plot displays the maximal  $\Delta R/R_0$  response for each glomerulus derived from the indicated stimulation in (C) during the entire measurement period of 1500 sec. (E) Another NO donor (100  $\mu$ M DEA NONOate) was applied (dotted lines) in the time period from 200 sec to 570 sec, while a time series was recorded. Graph represents the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of all analyzed glomeruli. Data represent mean  $\pm$  SEM of all analyzed glomeruli of five Cdh5:Cre/cGi500 mice ( $n = 8$  for ANP,  $n = 9$  for SNAP,  $n = 8$  for ANP+SNAP,  $n = 7$  for DEA NONOate). One-way ANOVA, Tukey's post hoc test, \* $P < 0.05$ . Scale bar 25  $\mu$ M.

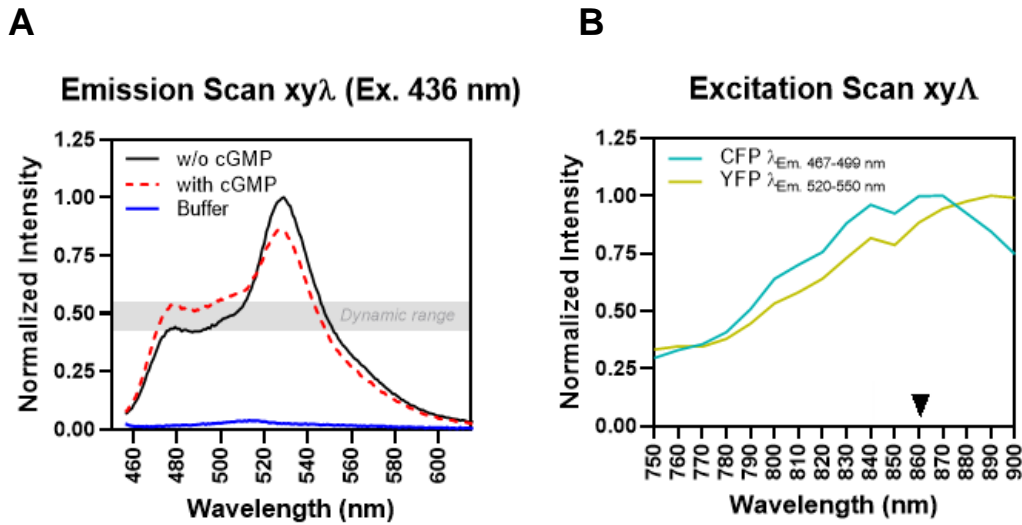


#### Supplemental Figure 10: Long-term measurement of the cGMP/FRET response in podocytes after ANP administration

Acute kidney slices from Pod:Cre/cGi500 mice were subjected to stimulation with 1  $\mu$ M ANP in the time period from 500-650 sec. Stimulation was followed by pre-tempered 1X KHB (37°C) for a total recording time of 4250 sec. The graph represents the mean of baseline-normalized FRET (CFP/YFP) ratios ( $\Delta R/R_0$ ) of 4 analyzed glomeruli.



## Supporting information (SI 2)



Supplemental Figure 11: Fluorescence spectra of cGi500

(A) Emission scan of cGi500 from transfected HEK 293T homogenates incubated without (black line) and with 100  $\mu$ M cGMP (red dashed line). Buffer A (without cGi500) served as control (blue line). The fluorescence was measured using the EnSpire® multimode plate reader. Data were normalized to maximum fluorescence in the absence of cGMP. (B) Excitation scan of cGi500 from transfected HEK 293T homogenates. Fluorescence in the CFP channel (cyan) and YFP channel (yellow) was measured using the multiphoton microscope TCS SP8 MP-OPO. Data were normalized to maximum fluorescence.