

## Real-Time Monitoring the Spatiotemporal Dynamics of Intracellular cGMP in Vascular Smooth Muscle Cells

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### Abstract

Real-time and noninvasive imaging of intracellular second messengers in mammalian cells, while preserving their in vivo phenotype, requires biosensors of exquisite constitution. Here we provide the methodology for utilizing the single wavelength cGMP-biosensor  $\delta$ -FlnG in aortic vascular smooth muscle cells.

**Key words** cGMP, Biosensors, Nitric oxide, Vascular smooth muscle, Live-cell imaging

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### 1 Introduction

The intracellular second messenger, cyclic guanosine-3',5'-monophosphate (cGMP), is a critical modulator of vascular smooth muscle (VSM) in the regulation of arterial vasodilation, essential for the maintenance of blood flow. cGMP is synthesized through activation of soluble guanylyl cyclases (sGC) and particulate guanylyl cyclases (pGC) by nitric oxide (NO) and natriuretic peptides (NPs), respectively [1, 2], and degraded by phosphodiesterases (PDEs) [3]. cGMP interacts with three main classes of downstream proteins: cyclic nucleotide-gated cation channels (CNG) whose main functions are in photoreceptors and olfactory neurons, cGMP-dependent kinases (PKG), and PDEs [4, 5]. PKGs represent a small subfamily of the AGC (PKA, PKG, and PKC)-type serine/threonine kinases that are activated specifically by cGMP [6, 7]. PKG's interactome consists of a limited number of targets, including the large conductance calcium-sensitive potassium ( $BK_{Ca}$ ) channel, G-substrate, PDE type 5, phospholamban, RhoA, Telokin, vasodilator-stimulated phosphoprotein (VASP), vimentin and myosin-binding subunit (MBS), MYPT1, regulator of G-protein 2 (RGS2), and  $IP_3$  receptor type I-associated cGMP kinase substrate (IRAG) [8–13]. In VSM, the actions of PKG activation ultimately lead to dilation of the vessel through many mechanisms mainly

involving the reduction of intracellular calcium. Several mechanisms of note are through IRAG and phospholamban on the sarcoplasmic reticulum, induction of membrane hyperpolarization through the BK<sub>Ca</sub>-induced hyperpolarization, and L-type voltage-dependent Ca<sup>2+</sup> channel (L-VDCC), and prevention of myosin contraction through myosin light chain phosphatase (MLCP) activation and RhoA/ROCK inhibition to induce increased dephosphorylation of myosin light chain (MLC) [9, 12–17].

Phosphodiesterase types 1, 2, 3, 5, 6, 9, 10, and 11 all have tissue-specific cGMP degradation activities. VSM mainly expresses PDE5, but has also been shown to contain PDE1 [18, 19]. PDE5 is unique in that it can be phosphorylated to enhance and prolong its activity [3, 19–25]. The level of phosphorylation has been shown to be essential for cGMP dynamics in VSM cells [26], and less critical in platelets, astrocytes, and sGC-transfected HEK cells [27–29]. This differential in the level of involvement in regulating [cGMP]<sub>i</sub> seems to be cell- and tissue-type specific. Therefore, the maintenance and monitoring of [cGMP]<sub>i</sub> is imperative for the study of vasomotor reactivity.

Accurate, kinetic measurements of [cGMP]<sub>i</sub> have proven difficult in the past. A common methodology used is the cGMP radioimmunoassay, where I<sup>125</sup>-labelled cGMP is incubated with cell lysates and a specific cGMP antibody is used to extract the label [30, 31]. This method only allows fixed time points to be analyzed, which can make extrapolating fine kinetic details tenuous, and the assay measures total rather than free cGMP, and often requires a PDE inhibitor to increase sensitivity. This method is also indirect, where the quantity of antibody binding is measured rather than cGMP itself.

The greatest advance in cGMP detection has come from the use of fluorescent indicators based on green fluorescent protein (GFP). Two of the most commonly used GFP variants are cyan and yellow (CFP and YFP, respectively). The excitation and emission wavelengths of these two fluorophores make an ideal pair for fluorescence-resonance electron transfer, or FRET [32, 33]. FRET occurs when one fluorophore, the donor, is excited and its emission causes the excitation of the second fluorophore, the acceptor, if the two are in close proximity to one another (20–60 Å) [32, 34, 35]. The first cGMP indicators were termed cyclic GMP *indicators* using *energy transfer* or “cygnets” and consisted of a fragment of PKGIα sandwiched between ECFP and EYFP or the pH-stable citrine [36, 37]. Cygnets were shown to be capable of monitoring both spatial and temporal changes in cGMP [36, 38]. Another, more recent, FRET-based cGMP indicator was created using the cGMP-binding GAF domains of PDE2 and PDE5 [39]. Although FRET-based indicators were pioneering developments in their ability to monitor intracellular second messengers, they do not have the capacity to monitor small spatial changes as would be required

with high-speed confocal microscopy. Serendipitously, circular permuted EGFP (cpEGFP) provided a unique opportunity to create a single wavelength biosensor, although cpEGFPs have a decreased total fluorescence intensity [40]. The  $\text{Ca}^{2+}$  indicator, G-CaMP, was the first of this single fluorophore-based indicator where the fluorophore was sandwiched between a calmodulin and an M13 domain [41]. A further advance of this principal design was the developments of *fluorescent indicators of cGMP* (FlnG). FlnGs include fragments of the PKGI $\alpha$  regulatory domain containing the two cGMP-binding domains fused to cpEGFP on the N-terminus [42]. Particularly, the variant  $\delta$ -FlnG exhibited high cGMP specificity, rapid binding, and dissociation kinetics and the capacity for confocal imaging, providing the first evidence for distinct spatial localization of cGMP signals in VSM cells [26, 42].  $\delta$ -FlnG has provided vertical progress by granting the most accurate method for cGMP detection applicable to many different cell types [26, 28, 42–47]. FlnG-type biosensors have helped to overcome the two major roadblocks in determining VSM signaling: (1) smooth muscle cells lose the expression of many smooth muscle-specific markers with culturing [38], and (2) nitric oxide has an extremely short biological half-life [48–50], which creates an experimental dilemma whereby small signaling events may be missed with traditional immunoblotting or kinase assays. Using FlnGs have allowed us to elicit the separate localization of the cytosolic NO- and membrane-restricted natriuretic peptide (NP)-induced cGMP in VSM cells, as well as create cells capable of detecting picomolar concentrations of cGMP produced by sGC [28, 42]. We have also determined the temporal kinetic relationship of NO and NO-induced cGMP, and which PDEs are critical in its maintenance in VSM [26].

A critical aspect to our careful analysis using FlnGs has been the cellular model with which we have chosen to study. VSM cells have a tendency to lose many smooth muscle-specific markers (PKG, PDE5, sGC, myosin heavy chain) over time in culture [38]. To ensure our cultured VSM cells are as close to *in vivo* cells as possible, we have developed an isolation assay to maintain their integrity. In combination with the adenovirus-transfected FlnG, we have developed an incredibly sensitive readout system for a major intracellular signaling molecule in vessel musculature.

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## 2 Materials

### 2.1 Tools

1. Two forceps (Dumont #5 or #55 works well), small spring scissors, and large dissection scissors (mouse) or large spring scissors and large chicken bone kitchen shears (rat).
2. 0.22  $\mu\text{M}$  syringe filters, 5 mL luer-lock syringes, 60 mm tissue culture dishes, 9" glass Pasteur pipettes, automatic pipettor.

3. Bioptechs Delta T4 culture dishes with clear glass bottoms. These dishes work with the Bioptechs Delta T4 temperature control system mounted on the microscope stage (*see Note 1*).

## **2.2 Microscope Setup**

1. Whether using epifluorescence or confocal microscopy, the stage should be equipped with a dish warmer, such as the Delta T4 culture system. Maintaining 37 °C is critical for proper enzymatic and biochemical kinetics.
2. Since  $\delta$ -FlnG can be used as a single-excitation biosensor, epifluorescence imaging requires a mercury-halide lamp (X-CITE 120, EXFO Photonics, Toronto, ON) coupled with a single 480 nm excitation filter and 535 nm emission filter. Confocal imaging can be performed with a 488 nm laser, collecting the emission above 510 nm.

## **2.3 Enzyme Digestion Solutions**

1. Digestion solution #1: Dissolve 175 U/mL Collagenase Type 2 (Worthington Biochemical) and 1.25 U/mL Elastase (Worthington Biochemical) into 5 mL Hank's Balanced Salt Solution (with calcium and magnesium, without phenol red, HBSS, Cellgro, #21-023-CV) for digesting rat aortae. For digesting up to 8 mouse aortae, use 2.5 mL total solution. Sterile filter solution using a 0.2  $\mu$ m syringe filter. Keep on ice until use.
2. Digestion solution #2: Prepare on second day. Dissolve 175 U/mL collagenase and 2.5 U/mL elastase into 5 mL HBSS. Sterile filter. Keep on ice. Again, for digesting mouse aortae, use 2.5 mL total solution.

## **2.4 Anesthetics**

1. For rat euthanasia, expose to 100 % CO<sub>2</sub> for 5 min, then inject with 1 mL pentobarbital sodium (50 mg/mL) for rats 250–350 g.
2. For mouse euthanasia, inject a 25 g mouse with 100  $\mu$ L ketamine/xylazine in 0.9 % sterile saline solution (100 mg/kg ketamine, 10 mg/kg xylazine).

## **2.5 VSM Cell Culture Medium**

1. Rat VSM cell culture: Dulbecco's Modified Eagle Medium (DMEM) with high glucose, L-glutamine, and no sodium pyruvate (Invitrogen #11965-092) supplemented with 10 % bovine growth serum (BGS) and 1 $\times$  penicillin/streptomycin (100 U pen/0.1 mg strep). Rat cells do not require fetal bovine serum (FBS) (*see Note 2*).
2. Mouse VSM cell culture: DMEM supplemented with 10 % FBS (low grade is sufficient) and 1 $\times$  pen/strep. Sterile filtering is optional, but recommended.

## 2.6 $\delta$ -FlnG Adenovirus

1. Adenoviral- $\delta$ -FlnG was prepared using the ViraPower™ Adenoviral Expression System (Invitrogen). Viral supernatant ( $10^7$ – $10^9$  per mL titer), and not purified virus, was kept in frozen aliquots at  $-80^\circ\text{C}$ . Freeze–thaw cycles should be avoided to maintain viral integrity.

## 2.7 Preparation of Imaging Buffer

1. HBSS was supplemented with 10 mM 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES, pH 7.4) and 1 g/liter D-glucose and kept on ice. Before imaging, equilibrate to  $37^\circ\text{C}$ . 10 mM HEPES (pH 7.4) can also be used in place of TES buffer.

## 2.8 Imaging Compounds

1. NONOate family of NO donors (PROLI/NO, MAHMA/NO, DEA/NO, Spermine/NO, DETA/NO). These should be bought in 10 mg aliquots and stored at  $-80^\circ\text{C}$ , and kept on ice until dissolved in ice cold 10 mM NaOH to a stock of 50 mM.
2. CPTIO (2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide), an NO scavenger, should be dissolved in DMSO to a stock of 50 mM. It is deep blue in color, and can be stored at  $-20^\circ\text{C}$ . CPTIO is generally used at 50 or 60  $\mu\text{M}$  working solution.
3. ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), an inhibitor of sGC, should be dissolved fresh on the day of use in DMSO, and kept on ice as a 10 mM stock. ODQ can be used in a working solution at 10  $\mu\text{M}$ .
4. 8-Br-cGMP, a cGMP analog, can be dissolved in water and stored at  $-20^\circ\text{C}$  as a 100 mM stock, and used at 50  $\mu\text{M}$  for the working solution.

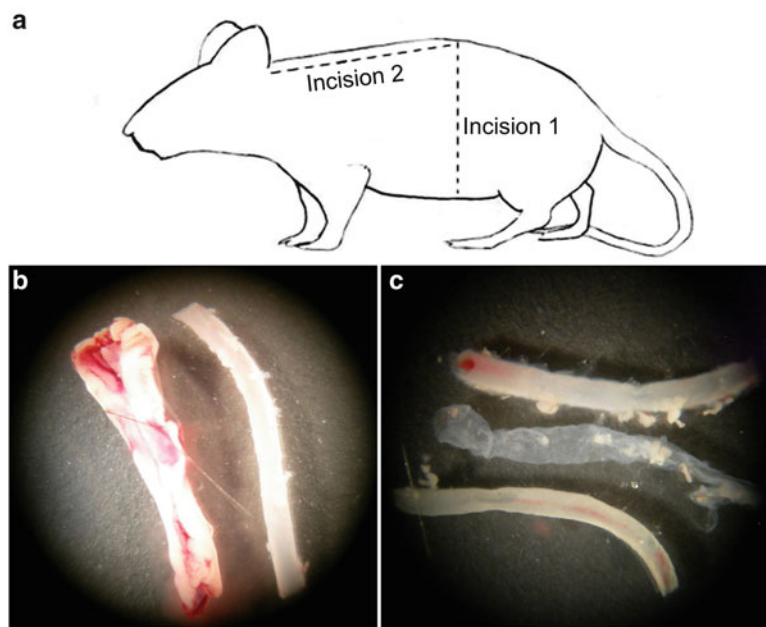
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## 3 Methods

The following protocols instruct the step-by-step isolation of aortic VSM cells from both rats and mice. Isolation of aortae is most successful from young adult animals (approximately 8 weeks).

### 3.1 Isolating VSM Cells

1. Day 1: Prepare digestion solution #1. Inject peritoneally a sub-lethal dose of anesthetic (pentobarbital or xylazine/ketamine) in accordance with animal welfare guidelines. Spray fur with 70 % ethanol. Laying the mouse/rat on its back, make an incision mid-torso, just below the diaphragm. Reposition the animal on its side, so the tail is to the right, and legs facing towards you (Fig. 1a). Enlarge the incision towards the spine. Locate the kidney, and sever the renal artery. Prop the animal up to exsanguinate (*see* Note 3).

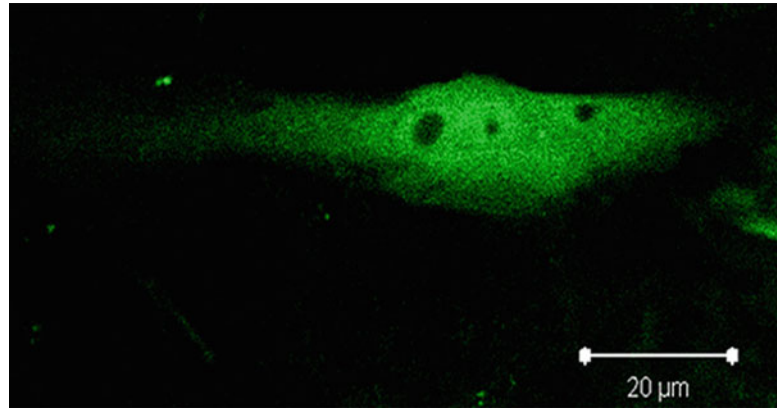


**Fig. 1** (a) Diagram of the lateral incisions for aortic dissection. The first incision runs from the mid-torso to the spine. The second incision follows the spine and dissects the rib cage from the vertebrae, up to the clavicle. (b) Stereoscope view of a freshly dissected rat aorta (*left*) and after removal of extraneous tissue (*right*). (c) Following the initial digestion step, the aorta will appear “fuzzy” (*upper vessel*). The adventitia can be teased apart and removed (*middle*), leaving the tunica media which consists mainly of smooth muscle cells (*bottom*)

2. Position the rat back in the side-lying position for a lateral thoracotomy. Using the large shears, cut through the ribcage along the spine up to the clavicle, 90° from the original incision. For a mouse, position on the belly, spine directly up, and make the same incision using the dissection scissors. Cut all the way through the clavicle.
3. Using the spring scissors, cut away the diaphragm and plural membrane. The aorta lies along the spine, sandwiched by a strip of fat above and below the vessel. Keeping the scissors parallel to the spine and using small incisions, cut the fat to release the aorta from the spine. The aorta will relax downward (*see Note 4*).
4. Cut across the aorta at the base of the diaphragm and just above the heart, at the aortic arch. Carefully pinching only the very end with the forceps, place the aorta in cold HBSS. Keep on ice (*see Note 5*).
5. Place the aorta in a 60 mm dish of cold HBSS. Refreshing the media with cold HBSS during the process helps to reduce the viscosity of the solution, making for an easier dissection.

Using a stereoscope and using small spring scissors, carefully trim away any fat attached to the aorta. The vessel will maintain a tube shape, aiding in the procedure (Fig. 1b). Try not to nick the aorta itself; this makes the digestion removal more difficult.

6. Transfer the aorta into a 60 mm dish with digestion solution #1 (35 mm dish for mouse aortae). Up to 2 rat aortae and 4 mouse aortae can be digested simultaneously. Place in a rocking 37 °C incubator for 25 min. The aorta should appear “fuzzy.” If not, keep incubating and monitor in 5 min intervals.
7. Transfer the aorta to HBSS. Under the stereoscope, gently tease one end of the aorta to separate the adventitia and media layers using two forceps. The adventitia appears as a white wispy covering, while the media emerges as a solid beige tube. Once the adventitia is loosened around the entire base of the media, hold the media with one set of forceps, and the adventitia in the other. Gently, in one smooth motion, gently remove the adventitia. The adventitia should peel off like a sock (Fig. 1c). Never pull on the media. It is paramount that during the entire procedure the media remains relaxed! If force is required, transfer the aorta back to the digestion solution and incubate for another 5 min. Repeat until the adventitia is completely removed.
8. Remove the media and place in a new dish with warm VSM culture medium. Let the tissue recover overnight at 37 °C, 5 % CO<sub>2</sub> in a humidified tissue culture incubator.
9. Day 2: Prepare digestion solution #2. Transfer the aortic media to a fresh dish of HBSS. Swirl to rinse. Transfer to digestion solution #2 with sterile forceps. Using small spring scissors, cut into 1–2 mm rings. Uniform sizes promote an even digestion.
10. Incubate at 37 °C, rocking for 2.5 h (45 min for mouse). The rings should appear very fuzzy and wispy.
11. Using a glass Pasteur pipette in an electric pipettor, gently triturate the solution four times. The shear forces should facilitate breaking apart the pieces of media and to dissociate individual cells. If there is little to no change, incubate for 5–10 min longer. Repeat trituration. Add 10 mL culture medium and transfer to a 15 mL conical tube.
12. Centrifuge at 130×g for 5 min. Aspirate the media, careful to leave the pellet undisturbed.
13. Add 1.5 mL BGS to the tube and resuspend the pellet by gentle trituration. Add 13.5 mL culture medium. Plate the cell suspension in 1 mL aliquots to 15 Delta T4 dishes that have been pre-rinsed with culture medium. For mouse cells,



**Fig. 2** Epifluorescent image of a  $\delta$ -FlnclG-transfected ( $100\ \mu\text{L}$   $10^7$  per mL titer adenovirus for 18 h), non-passaged VSM cell taken during live-cell imaging using a  $40\times$  objective, D480/20 m excitation filter, 505drr dichroic mirror, and D535/30 m emission with a mercury-halide lamp (X-CITE 120; EXFO Photonics, Toronto)

add  $600\ \mu\text{L}$  FBS for the pellet resuspension and add  $5.5\ \text{mL}$  culture medium. Plate on 6 Delta T4 dishes.

14. Incubate cells at  $37\ ^\circ\text{C}$ ,  $5\ \%$   $\text{CO}_2$  in a tissue culture incubator. After 24 h, change the media careful not to disturb the loosely attached cells, replacing with fresh  $10\ \%$  BGS culture medium. Cells will appear mostly rounded, but attached. Some will start to elongate.
15. Two days after plating, cells will appear elongated, with few processes. They have not yet begun to proliferate, which will ensure these VSM cells are as phenotypically close to their in vivo counterparts as possible.

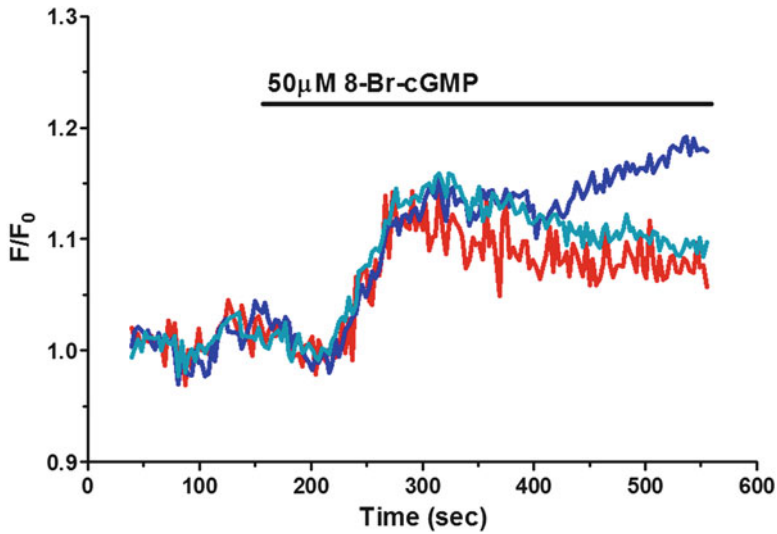
### 3.2 Adenoviral $\delta$ -FlnclG Infection

1. Thaw an aliquot of adenoviral supernatant on ice.
2. After 3 days in culture (2 days after plating), add  $1\ \text{mL}$  fresh VSM culture medium, adding  $100\ \mu\text{L}$  ( $10^7$  per mL titer) adenovirus. Incubate for 18 h at  $37\ ^\circ\text{C}$ .
3. Remove the adenovirus, and add fresh culture medium. Incubate for 2–3 h at  $37\ ^\circ\text{C}$ . Prepare imaging buffer (*see* **Note 6**).

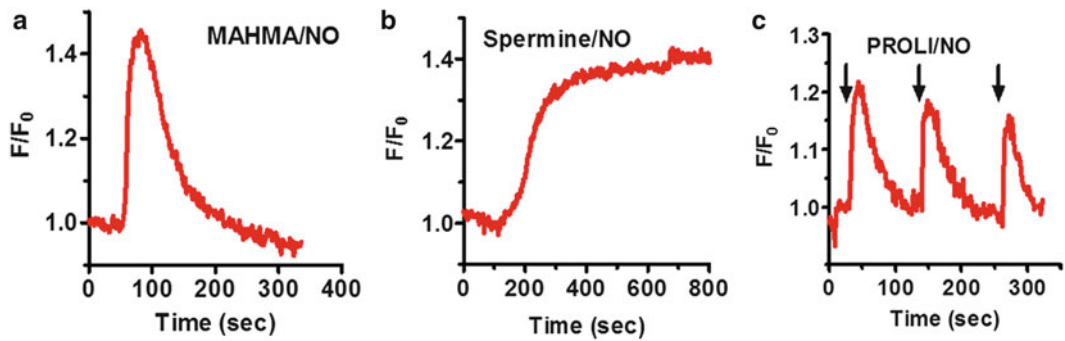
### 3.3 Epifluorescent Imaging

1. Keep the cells in a tissue culture incubator until imaging. For each dish, remove from the incubator and replace the culture medium with warm  $1\ \text{mL}$  imaging buffer just prior to imaging. At  $37\ ^\circ\text{C}$ , scan the plate for a group of well-transfected, healthy looking VSM cells (Fig. 2). These cells should have a smooth elongated appearance, with no processes and a mid-range of brightness. Overexpression of the indicator is usually toxic to the cells (rounding followed by apoptosis), while low-transfected cells are difficult to monitor and analyze.

2. Using imaging software such as Metamorph, draw several regions, approximately 10 % of the visible cytosolic area in size, on each cell to collect data from. Multiple regions allow the determination of specific local or global events.
3. Collect at least 2 min of baseline. A good baseline should be relatively smooth, with little variation, with an  $F/F_0$  ratio of around 1.0. Most cells will have a slight decrease in their fluorescence within the first 30 s (*see* **Note 7**). Add compounds such as NO donors and cGMP analogs as 1:1,000 dilutions directly into the imaging buffer (*see* Subheading 2.7 for stock concentrations). Using a P1000 pipette, gently mix the buffer, careful not to touch the dish. Any additions should be mixed within 5–10 s to allow for accurate assessment of cGMP fluctuations.
4. To control for the transfection efficiency, and to assure cell viability and health, cGMP analogs, such as 8-Br-cGMP, should be employed. These analogs are cell permeable, and will give a delayed, slow, and steady rise in fluorescence. This increase is diffusion controlled, and maximal responses seen maybe lower than those seen with other, receptor-mediated ligands (*see* Fig. 3).
5. To determine the endogenous sGC activity, NO donors are ideal, because they release only one or two equivalents of nitric oxide and no other small molecules that can potentially harm cells. The NONOate family of donors (PROLI/NONOate, MAHMA/NONOate, DEA/NONOate, Spermine/NONOate, PAPA/NONOate, DETA/NONOate) have a variety of increasing NO release rates (1.8 s to 20 h; [51]). NONOates should be diluted in 10 mM NaOH to desired working concentrations and kept on ice to prevent NO release. Once added to the 37 °C, pH 7.4 imaging buffer, the donor will release NO, therefore, quick mixing is paramount. Exact concentrations of NO can be delivered if the NONOate donor is coupled with the NO scavenger, CPTIO [52, 53]. Mathematical software can be applied to calculate the concentrations required. For example, a 5 nM quick pulse of NO can be achieved with 50  $\mu$ M CPTIO pre-incubated for 3 min, and 200 nM MAHMA/NO (Fig. 4a). Likewise, a steady state application of 5nM NO is achieved by 60  $\mu$ M CPTIO 3 min pre-incubation and 8  $\mu$ M Spermine/NO (Fig. 4b), as we have reported recently [26].
6. Other pharmacological inhibitors of the sGC/cGMP/PKG pathway can be useful to assess cGMP dynamics. The sGC inhibitor, ODQ, is very effective at completely blocking basal and stimulated cGMP production by oxidizing the heme group



**Fig. 3**  $\delta$ -FlnG-detected cGMP from multiple regions within a single VSM cell (not shown). A single, non-passaged, FlnG-transfected VSM cell was imaged and then exposed to the cGMP analog, 8-Br-cGMP, resulting in a slow but steady rise in intracellular cGMP that remains elevated throughout analog application. The rise in cGMP is measured by FlnG fluorescence increase over baseline ( $F/F_0$ )



**Fig. 4** Average traces of common NO-mediated [cGMP]<sub>i</sub> responses. NONOate family donors were coupled with the NO scavenger CPTIO to control NO concentration. (a) Transient cGMP increases upon 5 nM pulsed NO utilized by 200 nM MAHMA/NO and 50  $\mu$ M CPTIO. (b) Sustained cGMP upon 5 nM clamped NO utilized by 8  $\mu$ M Spermine/NO and 60  $\mu$ M CPTIO. (c) Multiple, cGMP transients upon repeated 5 nM NO pulses utilized by 200 nM MAHMA/NO and 50  $\mu$ M CPTIO. Figures adapted from [26]

of sGC [54–56]. However, this compound must be dissolved in fresh DMSO the day of the experiment, and cannot be stored in solution form.

**3.4 Confocal Imaging**

1. Confocal imaging with a water-dipping objective provides the most spatially resolute traces and movies. As with epifluorescent imaging, replace the culture medium with pre-warmed imaging buffer, and insert into the 37 °C dish warmer on the

microscope stage. Focus, and scan for an ideally transfected VSM cell. Cell shape is important here as well. A cell with long processes tends to move out of the focal plane easily, skewing the data collected. Refocus just above the bottom membrane attached to the glass for optimal results.

2. Collect at least 45 s of baseline at an acquisition rate of 250 ms, and then add compounds in 1:1,000 mixed with a P100 pipette (*see* Subheading 2.7 for stock concentrations). A careful mixing is important, or the focus will be lost and the experiments are forfeited.

### 3.5 Data Analysis

1. The large series of images can then be imported into a commercially available analysis software such as Metamorph or custom-written software such as SparkAn (courtesy of A. Bonev and M. Nelson at UVM). Within these software packages, small regions can be drawn on each cell to collect the fluorescence intensity. Region placement is critical for accurate depiction of the cell's responses. For epifluorescence, regions should be well within the cytosol and not too close to the edges or nucleus. Confocal imaging allows the opportunity to distinguish between edge effects and the cytosolic events, so many regions in various locations should be analyzed. Cells tend to move during stimulation; therefore adjustment of the region location may be required to maintain the signal (*see* Note 7).
2. Once the fluorescence intensities for each region have been collected, the data can be normalized to the initial background, reported as  $F/F_0$ . These calculated values can be graphed with the baseline at 1 or, if preferred, as a percentile.
3. Changes in  $[cGMP]_i$  can be calculated simply by subtracting the total response intensity from the initial baseline, or the baseline just prior to the stimulus added. This generally gives a more accurate measurement, as the baseline may not be steady at 1 (or 0) for the duration of the experiment.
4. Transient changes in  $[cGMP]_i$  can be analyzed by several parameters. The lag time is described as the time from stimulus addition to the first 5 % of the total response elicited. The percent response is total fluorescence intensity achieved minus the baseline, divided by the baseline, multiplied by 100 %. The response time is the time from 5 to 95 % total response. The tau ( $\tau$ ) factor is the degradation parameter. This is described as the time from 95 % response to 5 % of the new baseline. The peak width ( $P_{1/2}$ ) is measured as the time difference at 50 % of the peak height. The area under the curve of each transient peak can also be an important measurement of total  $[cGMP]_i$  change (*see* Note 8).

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## 4 Notes

1. Alternatively, punch dishes with coverslip glass bottoms (MatTek or homemade) can be used. The drawback is a lack of temperature control and stability while imaging. The Bioptechs Delta T4 culture system provides a stable stage with heating capabilities to keep the cells in a more physiological state during the experiment. Maintenance of 37 °C is critical for the proper NO release kinetics of NO donors.
2. Culture medium should be used until a slight purple shade is noticed. Dispose and prepare fresh media. Old media yield poor growing conditions for VSM cells.
3. Proper exsanguination allows for an easier dissection of the thoracic aorta. Not only is the cavity empty of blood, but also the aorta contains fewer blood clots, which mitigates subsequent digestion.
4. During the initial excision, it is important not to stretch or pull on the aorta. Any stretch severely reduces the viability of the VSM cells after digestion.
5. Once removed from the animal, an aorta can be kept on ice for 1–2 h.
6. Primary VSM cells tend to only tolerate a single biosensor or dye added at one time. Co-transfections or additions of dyes such as Fura-2 or Fluo-4 for calcium detection or Daf-2 for NO measurements appear to be toxic to these cells (rapid apoptosis).
7. Sometimes imaging traces have a tendency to drift downwards over time. Realigning any response to the baseline just prior to adding each compound should be a general practice. This baseline should be relatively even to ensure a proper  $F/F_0$  ratio.
8. Data analysis is best done on an individual cell basis, and then averaged to give mean traces. Not all cells respond identically. Some cells may need to be excluded from the total analysis based on several factors: health of the animal, cell health, total fluorescence intensity, cell shape, movement, morphology changes, loss of focal plane, etc. However, extreme caution should be used when dismissing individual cells. Only strict guidelines and outlier statistical tests will assure proper and unbiased data analysis.

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