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Probing the Molecular Mechanism of Human Soluble Guanylate Cyclase Activation by NO *in vitro* and *in vivo*

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Soluble guanylate cyclase (sGC) is a heme-containing metalloprotein in NO-sGC-cGMP signaling. NO binds to the heme of sGC to catalyze the synthesis of the second messenger cGMP, which plays a critical role in several physiological processes. However, the molecular mechanism for sGC to mediate the NO signaling remains unclear. Here fluorophore FIAsh-EDT₂ and fluorescent proteins were employed to study the NO-induced sGC activation. FIAsh-EDT₂ labeling study revealed that NO binding to the H-NOX domain of sGC increased the distance between H-NOX and PAS domain and the separation between H-NOX and coiled-coil domain. The heme pocket conformation changed from “closed” to “open” upon NO binding. In addition, the NO-induced conformational change of sGC was firstly investigated *in vivo* through fluorescence lifetime imaging microscopy. The results both *in vitro* and *in vivo* indicated the conformational change of the catalytic domain of sGC from “open” to “closed” upon NO binding. NO binding to the heme of H-NOX domain caused breaking of Fe-N coordination bond, initiated the domain moving and conformational change, induced the allosteric effect of sGC to trigger the NO-signaling from H-NOX *via* PAS & coiled-coil to the catalytic domain, and ultimately stimulates the cyclase activity of sGC.

The diatomic gas nitric oxide (NO) is an essential signaling molecule in biology. NO signaling controls several physiological processes including vasodilation, neurotransmission and platelet aggregation^{1–5}. Soluble guanylate cyclase (sGC) is a primary receptor of NO⁶. When activated, sGC catalyzes the conversion of substrate GTP to cGMP. cGMP, as a second messenger, triggers the downstream signaling cascades, including ion-gated channels, phosphodiesterases (PDEs) and cGMP-dependent protein kinases. Impairment of the NO-sGC-cGMP signaling has been linked to heart disease, hypertension, stroke, neurodegeneration and erectile dysfunction^{7,8}. Therefore, sGC is a therapeutic drug target for treating diseases by improving the NO-sGC-cGMP signaling^{9,10}.

The isoform $\alpha 1\beta 1$ is ubiquitously distributed in cytosolic fractions of tissues, while $\alpha 2\beta 1$ is mainly in brain. The most commonly studied and predominant sGC isoform is a Heme-containing heterodimeric enzyme composed of $\alpha 1$ and $\beta 1$ subunits. Each subunit contains four domains: an N-terminal heme-NO/O₂-binding (H-NOX) domain, a Per/Arnt/Sim (PAS) domain, a helical (CC) domain and a C-terminal catalytic domain¹. However, the crystal structure of the human sGC holo-enzyme remains unknown, although one human sGC domain structure (catalytic domain) and homologies of other domains have been characterized by crystallography^{11–15}. The H-NOX domain of $\beta 1$ subunit binds heme through His105 and senses NO^{11,16}, but the corresponding domain (pseudo-H-NOX) of $\alpha 1$ subunit does not bind heme and its function remains unclear. Both the PAS and CC domains of $\alpha 1$ and $\beta 1$ subunits are involved in the heterodimer formation, cyclase activation regulation and signaling transmission^{13,15,17,18}. The C-terminal catalytic domain constitutes the active site formed at the interface between $\alpha 1$ and $\beta 1$ subunits^{14,19}. Recently, the higher-order domain architecture of the sGC heterodimer have been reconstructed through diverse approaches, including small-angle X-ray scattering (SAXS), Hydrogen/deuterium exchange mass spectrometry (HDX-MS), and single-particle electron microscopy^{20–22}. These studies

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have revealed that the inter-domain interactions are responsible for the NO-signaling from the H-NOX domain to the catalytic domain. Besides, the heme-binding H-NOX domain interacts directly with PAS domain, which allows small-scale changes in the H-NOX domain to be quickly sensed by the abutting PAS domain. The flexible PAS-helical linker and the helical-catalytic domain linker are involved in the NO-signaling transmitting. Behrends *et al.* confirmed that the close proximity of H-NOX domains and the catalytic domain using the fluorescent fusion proteins by FRET analysis²³. Besides, Marletta *et al.* also indicated the similar results through HDX-MS studies²¹.

The molecular mechanism for NO-induced sGC activation remains a central question in NO-sGC-cGMP signaling. NO binding to the ferrous heme of sGC leads to the formation of a penta-coordinated Fe-NO complex through the dissociation of the proximal histidine H105, initiates a largely conformational change and ultimately stimulates the cyclase activity. Several models have been proposed to clarify the mechanism of NO-induced sGC activation using a variety of techniques, including mutational and truncation studies, FRET and HDX-MS *in vitro*^{21,23–26}. Both these models revealed the inter-domain interactions and the conformational change upon NO binding. The heme-associated α F helix is the trigger and the PAS domain is the mediator in the NO-signaling transduction. The flexible PAS-helical linker transmits the signals from H-NOX domain to catalytic domain through helical domain. However, the detailed molecular mechanism underlying NO activation of sGC is largely unknown, and the mechanism of NO-induced sGC activation *in vivo* remains unclear. Carbon oxide (CO), as another weak activator of sGC, can also stimulate sGC upon binding to the heme cofactor, but stimulation is much weaker than that of NO, which may be linked to the differences of conformational change^{11,27,28}.

In this study, firstly a fluorophore FLAsH-EDT₂, as a conformational change indicator, was introduced into the truncated and full-length sGC β 1 subunits to study the NO-induced conformational change of sGC *in vitro*, which revealed that NO binding could increase the distance between the H-NOX and PAS domain and also the distance between H-NOX and coiled-coil domain. These results reflected the NO-induced allosteric effect of sGC. Secondly, FRET analysis of fluorescent proteins, which were fused to the N-/C- terminal of sGC α 1 and β 1 subunits, showed the conformational change from “open” to “closed” of catalytic domain. Finally, the conformational change upon NO binding was also studied *in vivo* by fluorescence lifetime imaging microscopy (FLIM)-based FRET. These results indicated the conformational change of the catalytic domain upon NO binding, which was line with the results obtained *in vitro*. The conformational allosteric effect induced the NO-signaling from H-NOX domain to the catalytic domain to ultimately stimulate the cyclase activity of sGC.

Results

NO-induced sGC Conformational Change *in vitro*. *Site-specific Labeling of the sGC β 1 with FLAsH-EDT₂ and Monitoring the Distance Change by FRET.* The biarsenical dye FLAsH-EDT₂ with rather small in size can bind tightly to a small tetracysteine (TC; CCPGCC) motif and can be readily introduced by mutagenesis^{29,30}. For the emission spectrum of FLAsH-EDT₂ has good overlap with α and β absorbance bands of the heme, the FLAsH-EDT₂ is a good probe to study the conformational change induced by NO binding based on the energy transfer between heme and FLAsH-EDT₂. Previous work has been confirmed that the replacement of the residues (TC) and the FLAsH-EDT₂ would not affect the heme microenvironment and heme binding in the sGC β 1 variants [sGC β 1(1-385)-³⁸⁶TC³⁹¹, sGC β 1(1-385)-²⁴³TC²⁴⁸ and sGC β 1(1-619)-²⁴³TC²⁴⁸_{insect}]³¹.

To monitor the domain movement relative to the heme upon NO binding, the FLAsH-labeled sGC β 1(1-385) truncations were anaerobically mixed with NO donor, DEA/NO, and the fluorescence change was detected as shown in Fig. 1a and b. Both the sGC β 1(1-385)-²⁴³TC²⁴⁸ and sGC β 1(1-385)-³⁸⁶TC³⁹¹ showed an increased fluorescence. However, the conformational change degree of the sGC β 1(1-385)-³⁸⁶TC³⁹¹ with FLAsH-EDT₂ labeled at the helical domain was much larger than that of the sGC β 1(1-385)-²⁴³TC²⁴⁸ with FLAsH-EDT₂ labeled at PAS domain, indicating that the distance of the helical domain moving away from the heme was much larger than the PAS domain.

Furthermore, CO-induced conformational change of FLAsH-labeled sGC β 1(1-385) truncations was also monitored, the result was shown in Fig. 1c and d. The results showed no obvious change in fluorescence of FLAsH-labeled sGC β 1(1-385)-²⁴³TC²⁴⁸ and sGC β 1(1-385)-³⁸⁶TC³⁹¹ upon CO binding, indicating that CO binding of sGC heme could not induce the conformational change. Previous work has shown that CO-bound sGC formed a six-coordinated heme and the axial His-Fe coordination bond did not break, but NO binding formed a five-coordinated heme and ruptured the Fe-N coordination bond^{11,27,28,32}. Besides, CO has a much lower affinity and stimulates sGC activity only four-fold compared with 200-fold by NO^{27,28,33,34}. Recently, several approaches has been applied to study the NO activation of sGC, indicating that it is the allosteric effect that activates the cyclase activity and NO-induced conformational change is responsible to transmit the NO-signaling from H-NOX to the catalytic domain^{23,25,26}. Our results could explain why the CO stimulated activity was much lower than NO. Since CO binding of heme would not obviously affect sGC conformational change, thus it can be considered as a control to study the structural bases of the activation mechanism of sGC by NO. In addition, we used the mutant sGC β 1(1-385)H105A-²⁴³TC²⁴⁸ and β 1(1-385)H105A-³⁸⁶TC³⁹¹ as another controls (see Supplementary Fig. S1), the results showed no obvious fluorescence change upon NO and CO binding. Since the sGC β 1(1-385)H105A-²⁴³TC²⁴⁸ and β 1(1-385)H105A-³⁸⁶TC³⁹¹ could not bind heme, NO/CO binding could not induce the conformational change of sGC, which also on the other hand reflected that the small molecular NO and CO could not affect the fluorescence of the FLAsH-EDT₂²⁵.

To rule out the possibility that the fluorophore FLAsH-EDT₂ fluorescence change was caused by the change in the mobility or orientation of the fluorophore, the anisotropy of the FLAsH-EDT₂ labeled sGC β 1(1-385) truncations was determined before or after treatment with CO or DEA/NO, shown in Fig. 2. The anisotropy of FLAsH-EDT₂ bound sGC β 1(1-385)-²⁴³TC²⁴⁸ and sGC β 1(1-385)-³⁸⁶TC³⁹¹ showed no significant change upon DEA/NO or CO treatment.

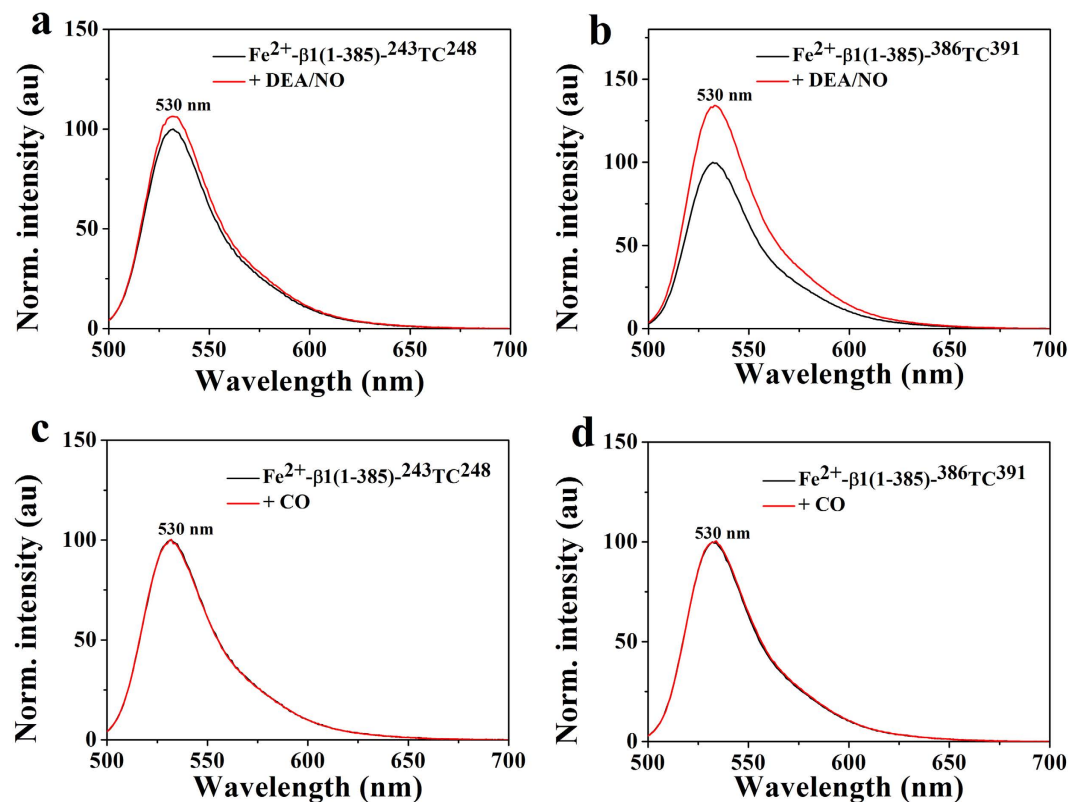


Figure 1. Conformational change of FIAsH-labeled sGC $\beta 1(1-385)$ - $^{243}\text{TC}^{248}$, and sGC $\beta 1(1-385)$ - $^{386}\text{TC}^{391}$ upon NO binding (a and b) or CO binding (c and d). The heme concentration was $2\ \mu\text{M}$ in 20 mM HEPES, 150 mM KCl, pH 7.4.

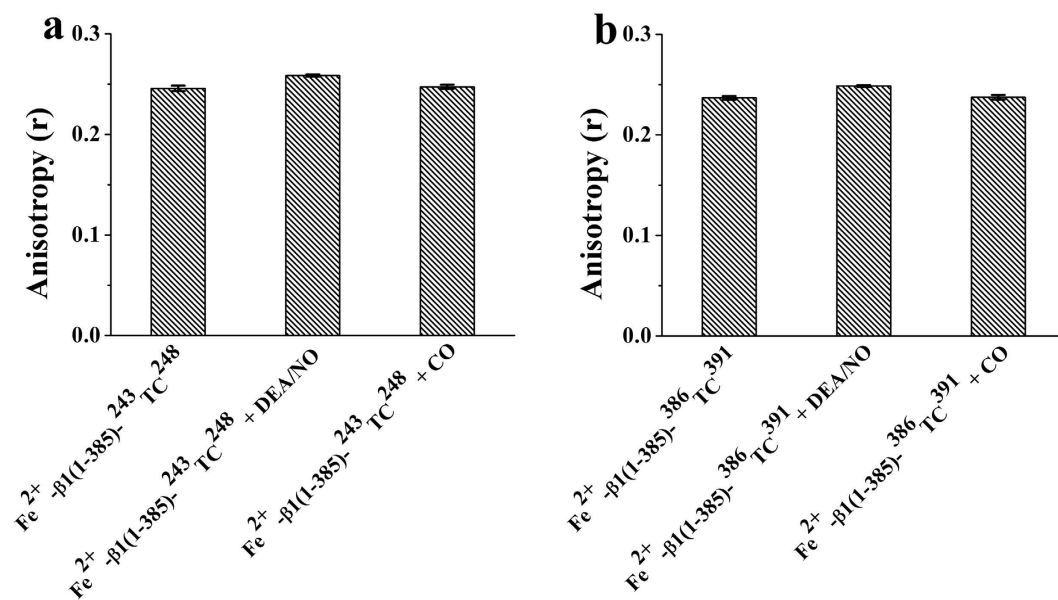


Figure 2. Anisotropy of FIAsH-labeled sGC $\beta 1(1-385)$ - $^{243}\text{TC}^{248}$ and sGC $\beta 1(1-385)$ - $^{386}\text{TC}^{391}$ upon NO binding (a) or CO binding (b). The heme concentration was $3\ \mu\text{M}$ in 20 mM HEPES, 150 mM KCl, pH 7.4. Anisotropy data are the mean \pm S.E. of two independent experiments performed in triplicate.

Furthermore, the full-length sGC $\beta 1(1-619)$ - $^{243}\text{TC}^{248}$ and $\beta 1(1-619)$ - $^{386}\text{TC}^{391}$ with FIAsH-EDT₂ labeled at the PAS domain and coiled-coil domain were also applied to study the NO-induced conformational change (Fig. 3). Addition of DEA/NO to the FIAsH-labeled sGC $\beta 1(1-619)$ - $^{243}\text{TC}^{248}$ and $\beta 1(1-619)$ - $^{386}\text{TC}^{391}$, the fluorescence

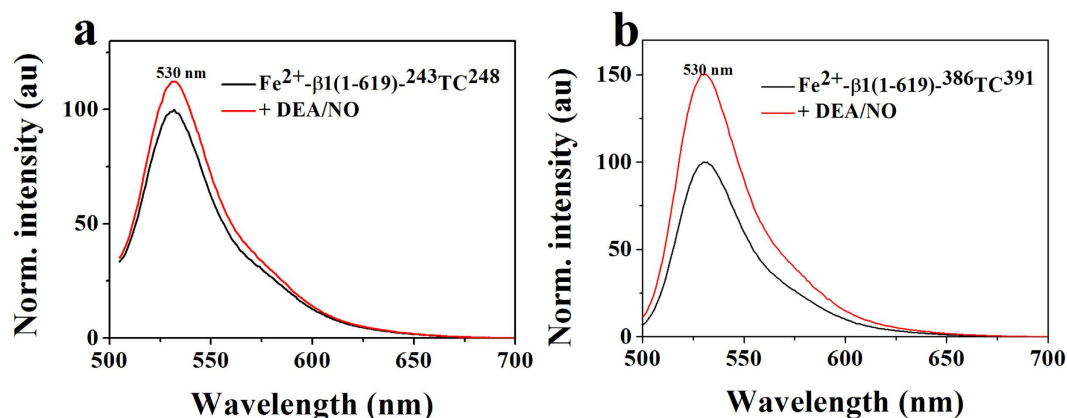


Figure 3. Conformational change of FLAsH-EDT₂-labeled sGC $\beta 1(1-619)$ -²⁴³TC²⁴⁸ (a) and sGC $\beta 1(1-619)$ -³⁸⁶TC³⁹¹ (b) upon NO binding. The heme concentration was 3 μ M in 20 mM HEPES, 150 mM KCl, pH 7.4.

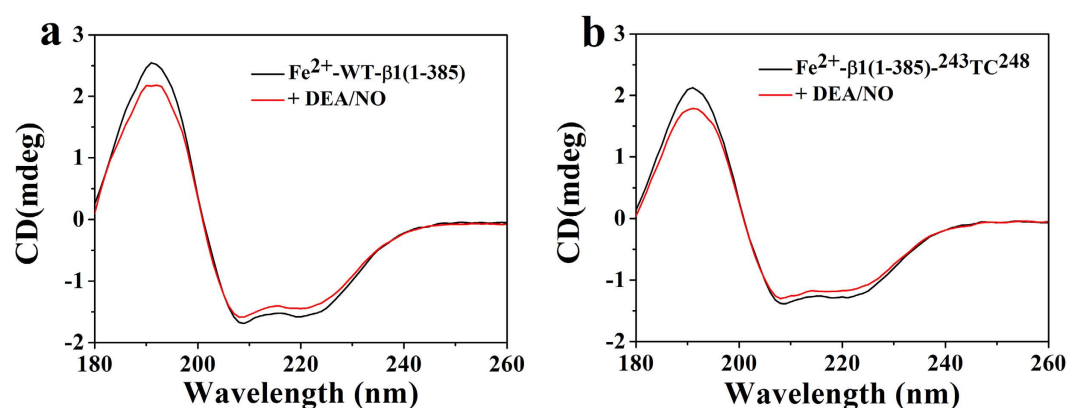


Figure 4. SRCD spectra in the far-UV region of sGC $\beta 1(1-385)$ (a) and sGC $\beta 1(1-385)$ -²⁴³TC²⁴⁸ (b) with and without DEA/NO. The heme concentration of sGC $\beta 1(1-385)$ and sGC $\beta 1(1-385)$ -²⁴³TC²⁴⁸ was 50 μ M and 45 μ M, respectively, in 10 mM HEPES, pH 7.4.

increased, suggesting an increased distance between heme and FLAsH-EDT₂. As shown in Fig. 3, the conformational change trend of full-length sGC $\beta 1(1-619)$ -²⁴³TC²⁴⁸ and $\beta 1(1-619)$ -³⁸⁶TC³⁹¹ was the same as that of the truncated sGC $\beta 1(1-385)$ -²⁴³TC²⁴⁸ and $\beta 1(1-385)$ -³⁸⁶TC³⁹¹, but the degree was larger than that of the truncated sGC $\beta 1(1-385)$ -²⁴³TC²⁴⁸ and $\beta 1(1-385)$ -³⁸⁶TC³⁹¹. It may be because that the full-length sGC contains the catalytic domain. In addition, we used the mutant sGC $\beta 1(1-619)$ H105A-²⁴³TC²⁴⁸ and $\beta 1(1-619)$ H105A-³⁸⁶TC³⁹¹ as another controls (see Supplementary Fig. S2), the results showed no obvious fluorescence change upon NO and CO binding, which was the same as that of the truncated sGC $\beta 1(1-385)$ mutant.

Study on the Secondary Structure Change upon NO Binding using SRCD. CD spectroscopy was widely used to study protein secondary structure. Synchrotron radiation circular dichroism (SRCD) had advantages relative to the conventional CD. SRCD spectroscopy with the intense light of a synchrotron beam was used to investigate the integrity of secondary structure, which had a greater sensitivity than conventional UV-CD³⁵⁻³⁷. Herein, we used SRCD to explore whether the secondary structure of sGC $\beta 1(1-385)$ changed upon DEA/NO binding or after the introduction of the TC motif. As shown in Fig. 4, no matter the introduction of the TC motif or the addition of the DEA/NO, the secondary structure of the sGC $\beta 1(1-385)$ did not be greatly disturbed (Table 1). The results indicated that NO activation of sGC did not induce the secondary structure change of sGC, which further suggested that the conformational change motioned above were indeed caused by the domain motion.

FRET study of fluorescent-tagged sGC variants. Two fluorescent tagged sGC heterodimers were constructed and expressed in sf9 cells (sGC $\alpha 1(1-690)$ -CFP_{insect} & $\beta 1(1-619)$ -YFP_{insect} and sGC CFP- $\alpha 1(1-690)$ _{insect} & $\beta 1(1-619)$ -YFP_{insect}). For sGC $\alpha 1(1-690)$ -CFP_{insect} & $\beta 1(1-619)$ -YFP_{insect}, the CFP and YFP were fused to the C-terminus of the $\alpha 1(1-690)$ and $\beta 1(1-619)$, separately. For sGC CFP- $\alpha 1(1-690)$ _{insect} & $\beta 1(1-619)$ -YFP_{insect}, the CFP was fused to the N-terminus of the $\alpha 1(1-690)$ and YFP was fused to the C-terminus of $\beta 1(1-619)$. Electronic absorption spectra of the two fluorescent-tagged sGC heterodimers indicated that the fused CFP or YFP did not alter the heme microenvironment and heme binding, as shown in Fig. 5. The ferrous form and NO-bound form fluorescent-tagged sGC heterodimer exhibited the similar Soret band absorbance as the wide type sGC, but the

	α -Helix	β -sheet	Turns	Unordered
sGC β 1(1-385)	0.42	0.29	0.07	0.22
sGC β 1(1-385) + DEA/NO	0.43	0.27	0.08	0.22
sGC β 1(1-385)- ²⁴³ TC ²⁴⁸	0.41	0.30	0.07	0.22
sGC β 1(1-385)- ²⁴³ TC ²⁴⁸ + DEA/NO	0.39	0.30	0.07	0.23

Table 1. The calculated secondary structure composition of sGC β 1(1-385) and sGC β 1(1-385)-²⁴³TC²⁴⁸ with and without DEA/NO.

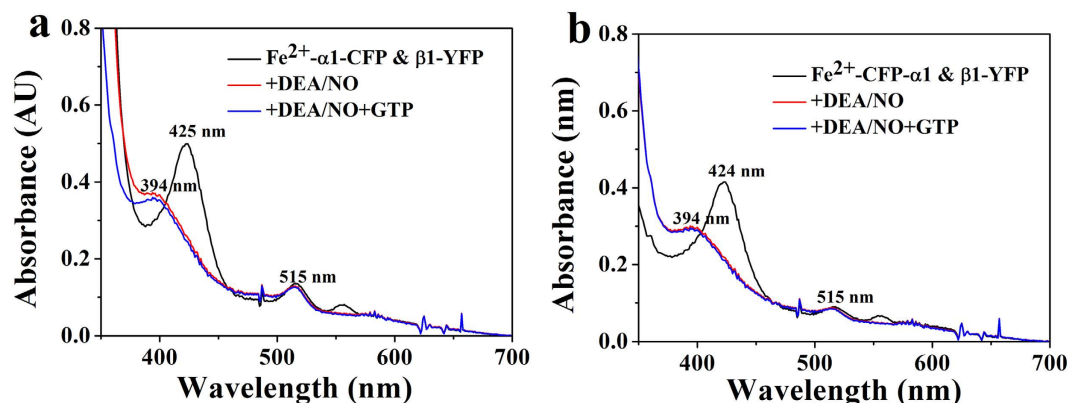


Figure 5. Electronic absorption spectra of sGC α 1(1-690)-CFP_{insect} & β 1(1-619)-YFP_{insect} (a) and sGC CFP- α 1(1-690)_{insect} & β 1(1-619)-YFP_{insect} (b) before and after addition of DEA/NO and the mixture of DEA/NO and substrate GTP.

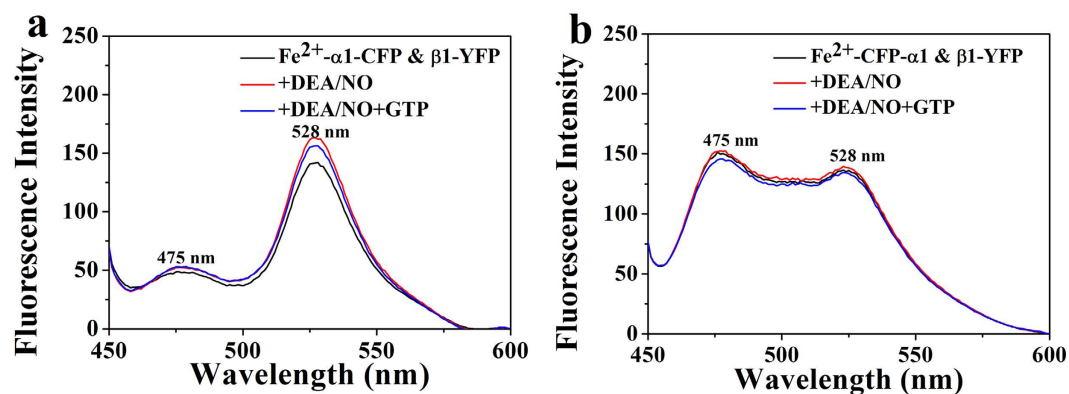


Figure 6. FRET analysis of purified sGC heterodimer. Fluorescent spectra of sGC α 1(1-690)-CFP_{insect} & β 1(1-619)-YFP_{insect} (a) and sGC CFP- α 1(1-690)_{insect} & β 1(1-619)-YFP_{insect} (b) at excitation wavelength of 432 nm before and after addition of DEA/NO and the mixture of DEA/NO and substrate GTP.

α/β band was not observed for the absorbance of YFP at about 515 nm^{23,38}. Besides, addition of DEA/NO and the substrate GTP to the ferrous form sGC, the Soret band absorbance was similar to that of the NO-bound form sGC shown in Fig. 5. Previous work has identified that fusing the CFP and YFP to the C-terminus of sGC α or β subunit, or fusing the CFP and YFP to the N-terminus of sGC α or β subunit displayed NO and NO/YC-1 stimulated sGC activity similar to the non-tagged sGC heterodimer²³. Thus, we used sGC α 1(1-690)-CFP_{insect} & β 1(1-619)-YFP_{insect} and sGC CFP- α 1(1-690)_{insect} & β 1(1-619)-YFP_{insect} to study the conformational change of sGC terminus upon NO activation.

To examine the conformational change of the C-terminal catalytic domain of sGC under NO activation, the FRET of the sGC α 1(1-690)-CFP_{insect} & β 1(1-619)-YFP_{insect} was detected upon NO binding. The results shown in Fig. 6a indicated that the NO binding resulted in the increase of the FRET efficiency compared with the ferrous form sGC, suggesting that the distance of the C-terminus of sGC α 1 and β 1 subunit decreased. However, upon both addition of NO and the substrate GTP to sGC, the FRET efficiency showed a little decrease relative to the NO-bound sGC, but a little increase relative to the ferrous form sGC. This may be because that the GTP occupied

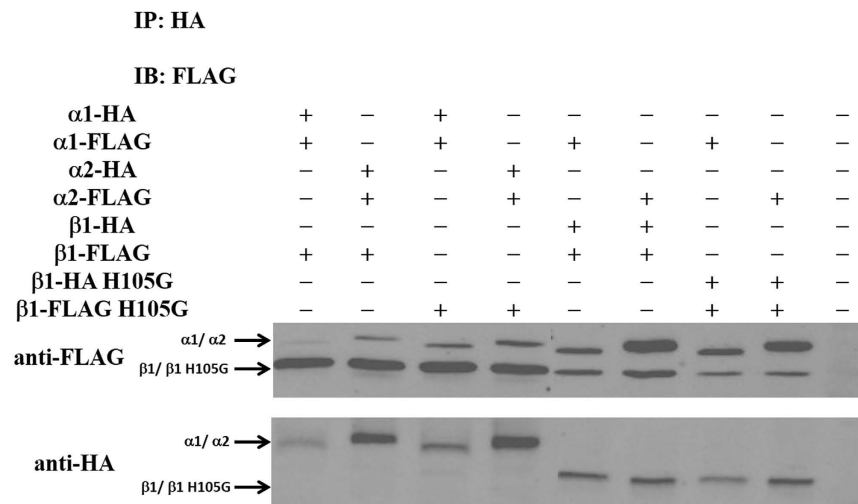


Figure 7. Co-immunoprecipitation analysis using transiently transfected SH-SY5Y cells expressing the wide type sGC α and $\beta 1$ subunit or the sGC α and $\beta 1$ mutant. Cells were co-transfected with HA- and FLAG-tagged constructs, and the HA-tagged constructs were precipitated using anti-HA antibody. Co-precipitated FLAG-tagged constructs were detected by SDS-PAGE/immunoblotting using anti-FLAG antibody (top). As a control, precipitated HA-tagged constructs were detected using anti-HA antibody (bottom). The gels were cropped and the full-length gels were shown in Supplementary Fig. S3.

the space of the active site in the catalytic domain and could induce the decrease of the conformational scale compared with that without GTP. Previous work has shown that NO activation could lead to conformational change of the catalytic domain from “open” state to the “closed” state^{20,21,26}, which was consistent with our results.

However, the FRET study of the sGC CFP- $\alpha 1(1-690)_{\text{insect}}$ & $\beta 1(1-619)$ -YFP_{insect} indicated that NO binding did not show obvious FRET efficiency change shown in Fig. 6b. Furthermore, both the NO and GTP binding also did not show obvious change in FRET efficiency. These indicated that the distance between N-terminus of $\alpha 1$ subunit and C-terminus of $\beta 1$ subunit did not change, reflected that the N-terminus of $\alpha 1$ subunit did not involve in the conformational change upon NO binding and its exact role in NO activation of sGC was unclear^{24,26}.

The result *in vitro* suggested that the conformation of discrete region changed upon NO binding, and the domain motion transmitted the NO-signaling from H-NOX domain to the catalytic domain to activate the cyclase activity. However, how the conformational change *in vivo* was still unknown. The following work focused on studying the conformational change *in vivo* using FLIM-FRET.

NO-induced Conformational Change *in vivo*. *Co-immunoprecipitation Confirmed the Heterodimeric formation of sGC $\alpha 1\beta 1$ and sGC $\alpha 2\beta 1$ *in vivo*.* Previous research has identified that only the sGC heterodimer can catalyze the conversion of GTP to cGMP and can be activated by NO. Thus, we first confirmed that the co-transfected sGC $\alpha 1$ or $\alpha 2$ and $\beta 1$ constructs form the heterodimer in SH-SY5Y cells. To this end, SH-SY5Y cells were transiently co-transfected with the HA- and FLAG-tagged constructs according to the following combination: 1. $\alpha 1$ -HA, $\alpha 1$ -FLAG, $\beta 1$ -FLAG; 2. $\alpha 2$ -HA, $\alpha 2$ -FLAG, $\beta 1$ -FLAG; 3. $\alpha 1$ -HA, $\alpha 1$ -FLAG, $\beta 1$ -FLAG H105G; 4. $\alpha 2$ -HA, $\alpha 2$ -FLAG, $\beta 1$ -FLAG H105G; 5. $\alpha 1$ -FLAG, $\beta 1$ -HA, $\beta 1$ -FLAG; 6. $\alpha 2$ -FLAG, $\beta 1$ -HA, $\beta 1$ -FLAG; 7. $\alpha 1$ -FLAG, $\beta 1$ -HA, $\beta 1$ -FLAG H105G; 8. $\alpha 2$ -FLAG, $\beta 1$ -HA, $\beta 1$ -FLAG H105G. The HA-tagged constructs were precipitated using anti-HA antibodies and detected by SDS-PAGE/immunoblotting using anti-HA antibodies (Fig. 7, bottom). Co-precipitated FLAG-tagged constructs were detected using anti-FLAG antibodies (Fig. 7, top). Co-immunoprecipitation of the FLAG-tagged constructs were detectable in the case of the $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 1$ H105G, indicating that the heterodimer $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 1\beta 1$ H105G and $\alpha 2\beta 1$ H105G were the main existing forms in SH-SY5Y cells. However, there were also a small amount of homodimer $\alpha 1\alpha 1$, $\alpha 2\alpha 2$, $\beta 1\beta 1$ and $\beta 1$ H105G $\beta 1$ H105G. Furthermore, the H105 mutant did not affect the formation of the heterodimer, which further identified that the H105 was not the key factor to mediate the dimerization. Previous work has shown that the PAS and helical domain play an important role in heterodimer function^{13,15,17}. The Co-immunoprecipitation further indicated that sGC α and β subunits can be co-expressed well and form the heterodimer in SH-SY5Y cells, which could be used in the FLIM-FRET study.

NO Binding Induced sGC Conformational Change Monitored by FLIM-FRET. To further investigate the NO-induced conformational change of sGC $\alpha 1\beta 1$ heterodimer *in vivo*, a FLIM-based FRET approach was applied, which allowed us to observe the interaction in live SH-SY5Y cells. The lifetime of the donor (CFP) decreased when the tagged proteins are interacting. Representative images obtained for the $\alpha 1$ -CFP alone, negative control and experimental groups in SH-SY5Y cells were shown in Fig. 8, indicating that both sGC $\alpha 1$ and $\beta 1$ subunits were expressed in cytoplasm, not in cell nucleus. The FLIM-FRET efficiency was calculated using CFP lifetime values and the distance between donor and acceptor was also calculated according to the equation ($r = R_0 (1/E - 1)^{1/6}$, $R_{0\text{CFP/YFP}} = 4.9$ nm), shown in Fig. 8h and l, and Table 2. Figure 8b was the negative control

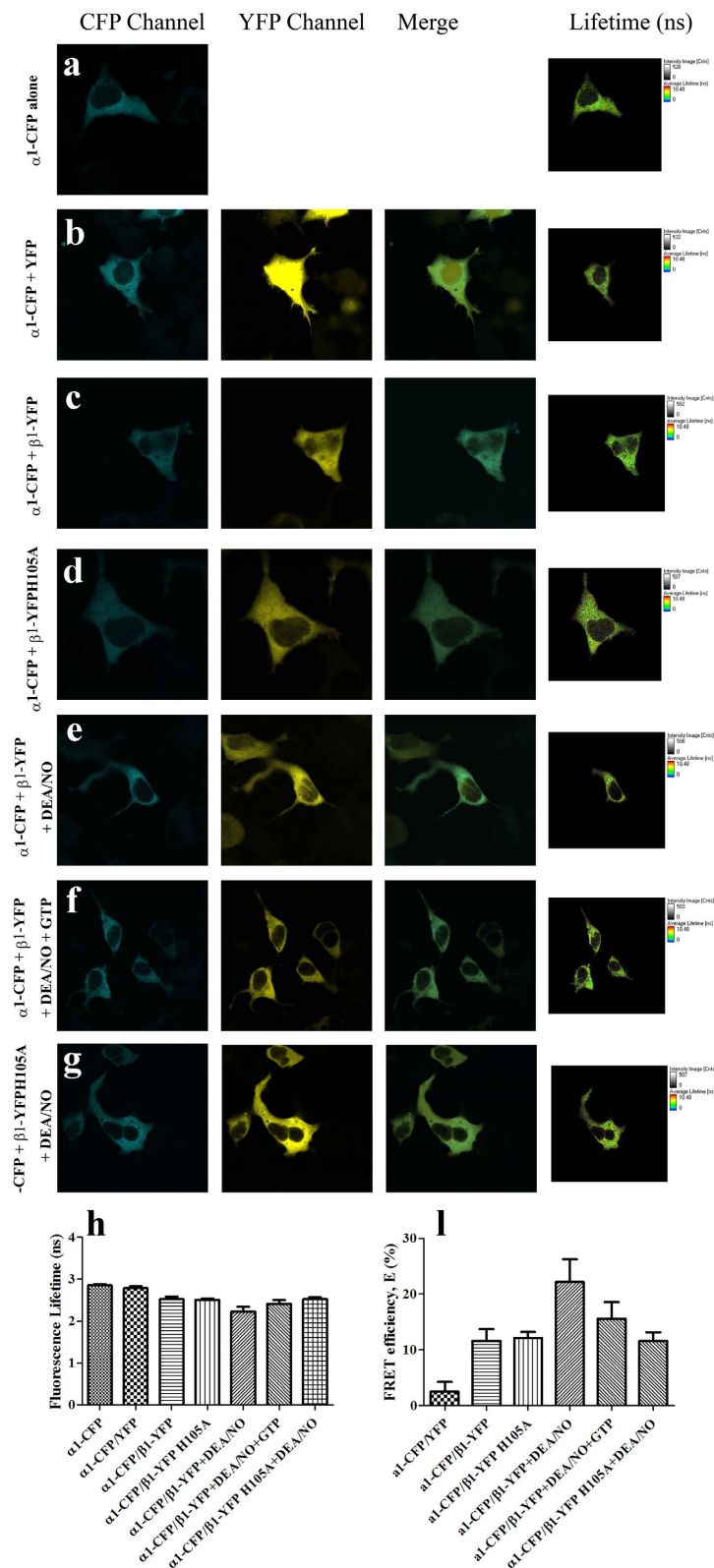


Figure 8. FLIM-FRET analysis in total cells. The images were taken with SH-SY5Y cells transfected with sGC $\beta 1$ -CFP alone (**a**), sGC $\beta 1$ -CFP and YFP (**b**), sGC $\beta 1$ -CFP and sGC $\beta 1$ -YFP (**c**), sGC $\beta 1$ -CFP and sGC $\beta 1$ -YFP H105A (**d**), sGC $\beta 1$ -CFP and sGC $\beta 1$ -YFP after addition of DEA/NO (**e**), sGC $\beta 1$ -CFP and sGC $\beta 1$ -YFP after addition of DEA/NO and GTP (**f**), and sGC $\beta 1$ -CFP and sGC $\beta 1$ -YFP H105A after addition of DEA/NO (**g**). Confocal images show the cellular localization of α and β subunits. The rightmost images show the lifetime images. (**h**) The lifetime values were the average lifetime through fitting the decay curve to the double exponential function from FLIM images. (**i**) FLIM-FRET efficiency (**E**) values (mean \pm S.E.) were calculated based on CFP lifetime values and obtained over 5–6 cells from 4 to 5 different samples.

	Efficiency (%)	Distance (Å)
sGC α 1-CFP/YFP	2.5 \pm 1.4	93.8 \pm 10.4
sGC α 1-CFP& β 1-YFP	11.6 \pm 1.8	68.9 \pm 2.2
sGC α 1-CFP& β 1-YFP H105A	12.0 \pm 1.0	68.3 \pm 1.2
sGC α 1-CFP& β 1-YFP + DEA/NO	22.2 \pm 3.5	60.5 \pm 2.1
sGC α 1-CFP& β 1-YFP + DEA/NO + GTP	15.6 \pm 2.4	65.1 \pm 2.1
sGC α 1-CFP& β 1-YFP H105A + DEA/NO	11.6 \pm 1.3	68.8 \pm 1.5

Table 2. FLIM-FRET efficiency and distance distribution of CFP and YFP-tagged sGC heterodimers co-expressed in SH-SY5Y cells.

with co-expression of α 1-CFP and YFP in the co-transfected cells, showing no obviously change in the average mean lifetime (τ) relative to the expression of α 1-CFP alone in the cells. Co-expression of α 1-CFP and β 1-YFP in the SH-SY5Y cells resulted in the decreased fluorescence lifetime compared with the α 1-CFP alone, indicating that the sGC α 1 interacted with sGC β 1. Besides, the lifetime of α 1-CFP co-transfected with the mutant β 1-YFP H105A into the SH-SY5Y cells also decreased, identical to the wide-type with the same FLIM-FRET efficiency (Fig. 8c,d,h and l). The results indicated that the mutant H105A affect neither the interaction between sGC α 1 and β 1, nor the distance between C-terminus of the sGC α 1 and β 1. However, addition of DEA/NO to live SH-SY5Y cells with the co-expressed α 1-CFP and β 1-YFP, the lifetime of the donor α 1-CFP decreased relative to that without DEA/NO. The results indicated that NO binding increased the FLIM-FRET efficiency and decreased the distance between C-terminus of the sGC α 1 and β 1 about 8.4 Å shown in Fig. 8(c,e,h and l). Moreover, upon adding both NO and GTP to sGC in the co-expressed α 1-CFP and β 1-YFP in live SH-SY5Y cells, the lifetime of the donor α 1-CFP increased compared with that only with DEA/NO, but also decreased compared with that without DEA/NO and GTP. The distances decreased about 3.8 Å relative to that in the cells only co-transfected with α 1-CFP and β 1-YFP, as shown in Fig. 8(c,f,h and l). The results also indicated the conformational change of the catalytic domain upon activation by NO *in vivo*, which further was identified with the results *in vitro* as shown in Fig. 6a. Besides, addition of DEA/NO to live SH-SY5Y cells with the co-expressed α 1-CFP and β 1-YFP H105A, the lifetime did not change obviously compared with that without adding DEA/NO shown in Fig. 8(d,g,h and l), indicating that the mutant H105A could not induced the conformational change of the catalytic domain, and also reflecting that the small molecular NO could not affect the lifetime of the fluorescent proteins *in vivo*. The results were consistent with that obtained *in vitro*.

To further identify that the NO binding really causes the conformational change of the C-terminus of the sGC catalytic domain, the FLIM-FRET experiments in the live SH-SY5Y cells co-transfected with sGC α 2-CFP and sGC β 1-YFP were also performed and the results are shown in Fig. 9. The negative control with co-expression with α 2-CFP and YFP showed no obviously change in the lifetime of the donor α 2-CFP. The addition of DEA/NO to the co-expressed α 2-CFP and β 1-YFP live SH-SY5Y cells, the lifetime of the donor α 2-CFP also decreased relative to that without DEA/NO. This indicated that NO binding increased the FLIM-FRET efficiency and decreased the distance between C-terminus of the sGC α 2 and β 1 about 6.8 Å, as shown in Fig. 9 and Table 3. The results were identical to that of the cells co-transfected with α 1-CFP and β 1-YFP. Thus, we could speculate that no matter the heterodimer α 1 β 1 or the α 2 β 1, the NO-induced conformational change was the similar in which the decreased distance between C-terminus of sGC catalytic domain was about 7–8 Å *in vivo*. These results are consistent with what previous reported that the catalytic domain of the sGC can change from “open” state to the “closed” state^{20,21,26}.

Discussions

The molecular mechanism underlying NO activation of sGC is a central question in NO-sGC-cGMP signaling. To understand the NO-signaling molecular mechanism mediated by sGC, several studies *in vitro* have been reported. Behrends *et al.* identified the sGC domains organization using the fluorescent proteins through monitoring the FRET change *in vitro*, supporting the “trans” regulation of sGC activity by the H-NOX domains proposed by Marletta^{19,23}. Recently, Behrends and co-workers also studied the conformational change of sGC upon activation by NO through monitoring the energy transfer between the endogenous tryptophan residues and the substrate analogue 2'-Mant-3'-dGTP *in vitro*, suggesting that the respective domains act as a pair of tongs forcing the catalytic domain into the NO activated conformation²⁵. In addition, very recently Marletta *et al.* studied the NO-induced conformational change using HDX-MS and indicated the domains interaction upon NO binding²⁶. Although great progress has been made on the NO-induced conformational change of sGC *in vitro* as described above, the exact interaction of the inter-domains of sGC *in vitro* was unclear and the NO-induced conformational change in living cells remained unknown. Therefore, in this study, biarsenical fluorophore FAsH-EDT₂ and fluorescent proteins (CFP and YFP) were employed to study the NO-induced sGC activation mechanism *in vitro* and *in vivo*. FAsH-EDT₂ labeling experiments *in vitro* revealed that NO binding to the H-NOX domain of sGC increased the distance between H-NOX and PAS domain and the distance between H-NOX and coiled-coil domain. FRET analysis of fluorescent proteins *in vitro*, which were fused to the N-/C- terminal of the α 1 and β 1 subunits of sGC, exhibited the conformational change from “open” to “closed” state of sGC catalytic domain. In addition, the NO-induced conformational change of sGC was firstly investigated *in vivo* through fluorescence lifetime imaging microscopy FLIM-based FRET. The results also indicated the conformational change of the catalytic domain from the “open” to the “closed” state with the distance decreased about 8 Å upon NO binding, which was

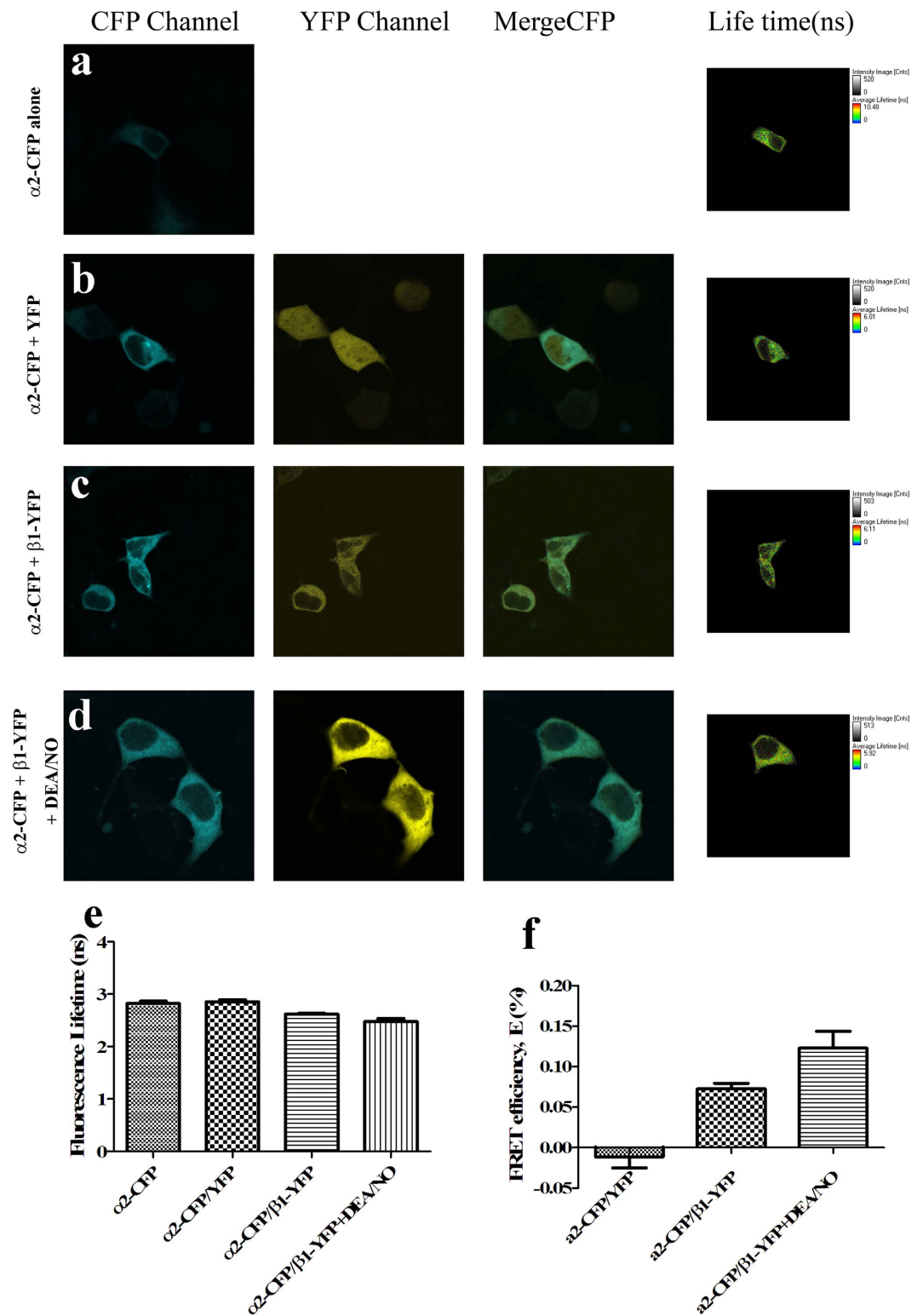


Figure 9. FLIM-FRET analysis in total cells. The images were taken with SH-SY5Y cells transfected with sGC $\alpha 2$ -CFP alone (a), sGC $\alpha 2$ -CFP and YFP (b), sGC $\alpha 2$ -CFP and sGC $\beta 1$ -YFP (c), and sGC $\alpha 2$ -CFP and sGC $\beta 1$ -YFP after addition of DEA/NO (d). Confocal images show the cellular localization of α and β subunits. The rightmost images show the lifetime images. (e) The lifetime values were the average lifetime through fitting the decay curve to the double exponential function from FLIM images. (f) FLIM-FRET efficiency (E) values (mean \pm S.E.) were calculated based on CFP lifetime values and obtained over 5–6 cells from 4 to 5 different samples.

	Efficiency (%)	Distance (Å)
sGC α 2-CFP/YFP	-1.1 ± 0.01	n/a
sGC α 2-CFP& β 1-YFP	7.3 ± 0.67	75.0 ± 1.1
sGC α 2-CFP& β 1-YFP + DEA/NO	13.6 ± 1.5	68.2 ± 2.2

Table 3. FLIM-FRET efficiency and distance distribution of CFP and YFP-tagged sGC heterodimers co-expressed in SH-SY5Y cells.

in line with the results *in vitro*. NO binding to H-NOX domain induced the allosteric effect of sGC to trigger the NO-signaling from H-NOX domain to the catalytic domain and ultimately stimulates the cyclase activity of sGC.

NO binding to the heme resulted in the rupture of the Fe^{2+} -N coordination and released the heme-associated α F helix^{24,39,40}. The displacement of the α F helix was also observed in *Shewanella oneidensis* H-NOX variant without the His ligand⁴¹. Besides, the replacement of the heme with the small molecular sGC activator BAY 58-2667 caused a rotation of the α F helix away from the heme pocket for removing the inhibitory of the His-heme coordination bond in the *Nostoc.sp. PCC 7120 H-NOX*⁴². Recent HDX-MS analysis has revealed the increased exchange rate in the heme-associated α F helix upon NO binding and implicated that the heme-associated α F helix was a focal point of the NO-induced conformational change²⁶.

Interestingly, upon NO binding, both the PAS and coiled-coil domain of the truncated sGC β 1(1-385) shifted away from the heme through monitoring the fluorescence of the labeled FLAsH-EDT₂, which was shown in Fig. 1a and b. However, the degree of the conformational change of PAS domain was smaller than that of the coiled-coil domain. Previous work revealed the direct interaction between heme-associated α F helix and PAS domain, and the conformational change recognized by the abutting PAS domain^{21,26}. Besides, the PAS domain was a key mediator to transmit the conformational signals from the H-NOX domain to the catalytic domain. Thus, it is speculated that the heme-associated α F helix motion initiated the movement of the PAS domain (Fig. 1a). Recent single-particle EM results have shown that the high-order architecture was consisted of two rigid domains: the catalytic domain and the clustered H-NOX and PAS domain, connected by the coiled-coil domain at two flexible hinge points²⁰. The PAS-helix linker and the helix-catalytic linker played an important role in transducing the conformational signals²⁶. The PAS-helix linker might induce the helix domain movement away from the heme (Fig. 1b). Furthermore, prior work indicated that the direct interaction between the H-NOX domain and catalytic domain repress the sGC activity, which was supported by a FRET study that revealed close proximity between N-terminus and C-terminus of sGC^{19,23}. Recent HDX-MS analysis revealed the releasing inhibitory between H-NOX and catalytic domain caused by the motion of the PAS-helix junction²⁶. Thus, the movement of H-NOX domain away from the catalytic domain could explain why the conformational degree of coiled-coil domain was larger than the PAS domain relative to the heme (Fig. 3).

The FRET study using the fluorescent fusion proteins *in vitro* indicated that the distance between the C-terminus of the sGC α and β subunit decreased upon NO activation. Furthermore, the result obtained *in vivo* through FLIM-FRET study in live SH-SY5Y cells also showed the decreased distance between the C-terminus of the sGC α and β subunit. Although the change from the “open” to the “closed” was just the change of the conformation of the C-terminus of the sGC heterodimer, it can indirectly reflect the NO-signaling transduction from the H-NOX domain to the catalytic domain, which may involve the discrete domain movement of sGC. Recent HDX-MS study revealed that the catalytic domain changed from the inactive “open” state to the active “closed” state upon NO activation²⁶.

Based on the results obtained *in vitro* and *in vivo*, we proposed a most likely molecular mechanism underlying NO activation of sGC based on the high-order domain architecture from single-particle EM shown in Fig. 10, and various labeled proteins in relation to the sGC three dimensional model were shown in Supplementary Fig. S4. NO binding released the heme-associated α F and induced the PAS and coiled-coil domain moving away from the H-NOX domain, and ultimately led the catalytic domain change from the “open” state to the “closed” state to activate the cyclase activity of sGC. In addition, the GTP could occupy the space of the active site in the catalytic domain and induce the decrease of the conformational scale. Understanding the molecular mechanism underlying NO activation of sGC would be helpful to the development of therapeutics targeting cGMP-dependent physiological processes.

In conclusion, the NO-induced conformational change was studied using the fluorophore FLAsH-EDT₂ and fluorescent proteins *in vitro*, which indicated that the NO binding to the H-NOX domain of sGC increased the distance between H-NOX and PAS domain, and led to the conformation change of the catalytic domain from the “open” to “closed”. And most of all, we firstly studied the conformational change of sGC upon NO binding *in vivo* based on the lifetime imaging microscopy, which indicated the conformational change of the catalytic domain from “open” to “closed”. We expect that the NO-induced activation mechanism of sGC will promote the development of therapeutics targeting cGMP-dependent physiological processes.

Methods

Materials. FLAsH-EDT₂ was purchased from Toronto research chemicals Inc., and the transfection reagents LipofectamineTM 2000 were from Invitrogen. The monoclonal mouse anti-FLAG antibody was from Abcam and the monoclonal rabbit anti-HA was from Sigma. Pierce[®] Classic IP Kit (26146) was from Thermo Scientific. KOD-Plus-Mutagenesis Kit was from TOYOBO. The plasmid purification kit (12362), nickel nitrilotriacetic acid (Ni-NTA) resin and Sephadex G-25 resin were purchased from QIAGEN. DEAE SepharoseTM Fast Flow was purchased from GE Healthcare Bio-Science. SH-SY5Y cells were from the National Laboratory of Neurobiology of Fudan University. All other reagents were of analytic grade.

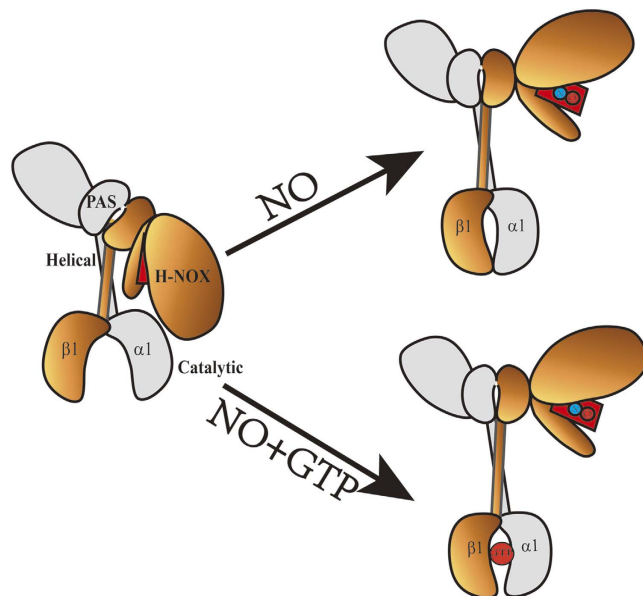


Figure 10. A proposed NO-Induced activation mechanism of sGC.

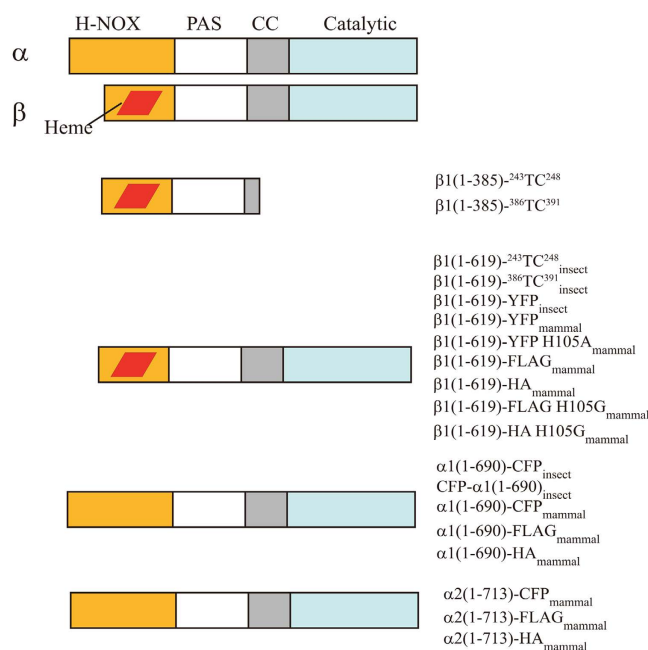


Figure 11. The domain architectures of sGC and schematic representations of sGC variants constructs used in this study.

Plasmid Constructs. The plasmid constructs used in this study were schematically shown in Fig. 11. The plasmids [sGC $\beta 1(1-385)-^{386}\text{TC}^{391}$, sGC $\beta 1(1-385)-^{243}\text{TC}^{248}$ and sGC $\beta 1(1-619)-^{243}\text{TC}^{248}$], which were introduced with a small tetracycline (TC: CCPGCC), have been constructed as described before³¹. The construction of the plasmid of sGC $\beta 1(1-619)-^{386}\text{TC}^{391}$ was the same as the sGC $\beta 1(1-619)-^{243}\text{TC}^{248}$. For the FRET experiment *in vitro*, the plasmids of sGC $\beta 1(1-619)$ -YFP_{insect}, sGC $\alpha 1(1-690)$ -CFP_{insect} and sGC CFP- $\alpha 1(1-690)$ _{insect} were constructed as described below. For the sGC $\beta 1(1-619)$ -YFP_{insect}, the YFP was C-terminally tagged to the pBAC-1-sGC $\beta 1(1-619)$ using restriction enzymes *NotI* and *XhoI*. For the sGC $\alpha 1(1-690)$ -CFP_{insect} and sGC CFP- $\alpha 1(1-690)$ _{insect}, the constructions contained a two-step procedure: firstly inserting the $\alpha 1(1-690)$ using the restriction enzymes *HindIII* and *NotI* of the vector pBAC-1, and secondly subcloning CFP in the C-terminus using the restriction enzymes *NotI* and *XhoI* or in the N-terminus using the restriction enzymes *BamHI* and *HindIII*. For the FLIM-FRET experiment *in vivo*, the plasmids were all constructed into the vector pcDNA3.1(-). Firstly, we constructed the $\beta 1(1-619)$, $\alpha 1(1-690)$ and $\alpha 2(1-713)$ into the vector pcDNA3.1(-), and secondly

YFP and CFP were C-terminally fused to the β 1(1-619), α 1(1-690) and α 2(1-713) [β 1(1-619)-YFP_{mammal}, α 1(1-690)-CFP_{mammal} and α 2(1-713)-CFP_{mammal}], respectively. In addition, the His105 in the β 1 subunit was mutated to alanine [β 1(1-619)-YFP H105A_{mammal}]. The pcDNA3.1(-)-CFP and pcDNA3.1(-)-YFP were used as a control. For the co-immunoprecipitation experiment, the FLAG/HA tag was C-terminally fused to the β 1(1-619), β 1(1-619) H105G, α 1(1-690) and α 2(1-713), respectively, as shown in Fig. 11.

Expression and Purification of sGC variants. The truncated sGC β 1(1-385) variants [sGC β 1(1-385)-³⁸⁶TC³⁹¹ and sGC β 1(1-385)-²⁴³TC²⁴⁸] were expressed in *E. coli* and purified as described before³¹. The protein concentration was estimated by the Soret peak using the pyridinehemochromagen assay. All the purified processes were performed in a glove-box under high pure nitrogen. The full-length sGC variants were all expressed in insect sf9 cells. The recombinant baculo-viruses of respective proteins [sGC β 1(1-619)-²⁴³TC²⁴⁸_{insect}, sGC β 1(1-619)-³⁸⁶TC³⁹¹_{insect}, sGC β 1(1-619)-YFP_{insect}, sGC α 1(1-690)-CFP_{insect} and sGC CFP- α 1(1-690)_{insect}] were generated according to the BAC-TO-BAC™ System. Sf9 cells were cultured in SF900II medium supplemented with 1% penicillin/streptomycin at 27 °C. To generate sGC β 1(1-619)-²⁴³TC²⁴⁸_{insect} or sGC β 1(1-619)-³⁸⁶TC³⁹¹_{insect} sf9 cells were transfected with sGC β 1(1-619)-²⁴³TC²⁴⁸_{insect} viruses or sGC β 1(1-619)-³⁸⁶TC³⁹¹_{insect} viruses. To generate recombinant sGC heterodimer carrying various fluorescent-tagged proteins, sf9 cells were co-transfected with α 1- and β 1-expression viruses. For example, to generate sGC α 1(1-690)-CFP_{insect} & β 1(1-619)-YFP_{insect}, Sf9 cells were co-transfected with sGC α 1(1-690)-CFP_{insect} and β 1(1-619)-YFP_{insect} viruses. The following purification processes were performed in a glove-box under high pure nitrogen as described before³¹. The recombinant proteins expressed in sf9 cells were obtained without heme group, and the heme reconstitution was performed as previously⁵. All the purification processes and heme reconstitution were performed in a glove-box under high pure nitrogen.

sGC Proteins Labeling with FIAsh-EDT₂ in vitro. The FIAsh-EDT₂, which can bind tightly to a small tetracysteine (TC: CCPGCC), was readily introduced into different position of sGC proteins. The procedure of the sGC variants [sGC β 1(1-385)-²⁴³TC²⁴⁸, sGC β 1(1-385)-³⁸⁶TC³⁹¹, the full-length sGC β 1(1-619)-²⁴³TC²⁴⁸_{insect} and sGC β 1(1-619)-³⁸⁶TC³⁹¹_{insect}] were labeled with the FIAsh-EDT₂ as described before³¹. In brief, the sGC proteins were reduced with 1 mM TCEP and then allowed to stand at room temperature for 2-3 h. The mixtures were diluted to 20 μ M to incubate with 1.5 equivalents of FIAsh-EDT₂ in buffer A (20 mM HEPES, 150 mM KCl, 1 mM TCEP, pH 7.4) for 3 h at room temperature in dark, and then applied to PD10 column to remove the excessive reagents in buffer B (20 mM HEPES, 150 mM KCl, pH 7.4). All of the processes were operated in a glove box under high pure nitrogen.

UV-visible Spectroscopy. Electronic Absorbent spectra of all the purified sGC variants in buffer B (20 mM HEPES, 150 mM KCl, pH 7.4), were recorded in anaerobic cuvette on an HP8453 UV-visible spectrophotometer. The preparation of the ferrous protein samples were as described before³¹. The heme concentration was 2 μ M or 3 μ M [$\epsilon_{431\text{nm}} = 120 \text{ mM}^{-1} \text{ cm}^{-1}$ for β 1(1-385), $\epsilon_{424\text{nm}} = 110 \text{ mM}^{-1} \text{ cm}^{-1}$ for β 1(1-619)]. The NO/CO-bound sGC proteins were produced by adding NO donator (DEA/NO) or bubbling with CO for 5 min.

FRET Measurements of sGC Proteins in vitro. The spectrofluorometric study was carried out in anaerobic cuvette with a Varian spectrofluorometer. The heme group can quench the FIAsh-EDT₂ through the Förster resonance energy transfer (FRET) for the good overlap with α and β absorbance bands of sGC heme group. Therefore, for the pair of FIAsh-EDT₂ and heme, the excitation was at 485 nm and emission spectra were recorded from 500 nm to 700 nm. In addition, for the pair of CFP and YFP, the excitation was at 432 nm and emission spectra were measured from 450 nm to 600 nm.

Fluorescence Anisotropy of sGC Proteins Labeled with FIAsh-EDT₂. The fluorescence anisotropy was measured as described³¹. Samples were excited at 485 nm. For determining the anisotropy of NO/CO-bound sGC proteins, samples were mixed and incubated for several minutes at room temperature.

Synchrotron Radiation Circular Dichroism Spectroscopy (SRCD). The SRCD spectra of the truncated type sGC β 1(1-385) and its variant β 1(1-385)-²⁴³TC²⁴⁸ were recorded from 190 nm to 270 nm at Beijing Synchrotron Radiation Facility (BSRF) beam line 4B8 in the Institute of High Energy Physics, Chinese Academy of Science. The cell chamber was 0.1 mm and filled with N₂. The temperature was kept at 4 °C. The protein buffer was buffer C (10 mM HEPES, pH 7.4).

Cell Culture and Transfection. The SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, at 37 °C under 5% CO₂. Transfection of the cells with Lipofectamine™ 2000 was carried out according to the supplier's recommendations 24 h after seeding of cells. Cells were used 44–45 h later after transfection.

Co-Immunoprecipitation. The FLAG and HA tagged plasmids were used for the Co-immunoprecipitation. Co-immunoprecipitation was carried out using Pierce® Classic IP Kit (26146) according to the supplier's protocol. In brief, SH-SY5Y cells (3×10^6) were grown on 100-mm dishes and transiently co-transfected with plasmids using Lipofectamine™ 2000 according to the supplier's recommendations. Cells were cultivated for 48 h, washed twice with PBS buffer (pH 7.4), and lysed for 5 min with 750 μ l ice cold IP Lysis/Wash Buffer. Insoluble debris was discarded by centrifugation ($\sim 13,000 \times g$, 10 min). The supernatant was supplemented with monoclonal rabbit anti-HA antibody and incubated overnight at 4 °C to form the immune complex. Then the antibody/lysate sample was supplemented with Protein A/G Plus Agarose and incubated with gentle end-over-end for 1 h. The resin was

washed three times with IP Lysis/Wash Buffer and once with 1× Conditioning Buffer, and then the FLAG-tagged constructs were precipitated. The immunoprecipitated proteins were analyzed *via* Western blot analysis.

Fluorescence Lifetime Image Microscopy (FLIM)-FRET. FLIM-FRET experiments were performed on Leica TCS SP8 equipped with SMD detector. SH-SY5Y cells transiently expressing the donors sGC $\alpha 1(1-690)$ -CFP_{mammal} or sGC $\alpha 2(1-713)$ -CFP_{mammal} alone or with YFP (as a control experiment), sGC $\beta 1(1-619)$ -YFP_{mammal} or sGC $\beta 1(1-619)$ -YFP H105A_{mammal} (acceptor) were grown on a Confocal Dish (Coverglass-Bottom Dish), and visualized using an upright fluorescence microscope, and observed through a 25× water immersion objective at room temperature. Channel pictures were taken prior to the recording of the FLIM pictures in order to estimate expression of the CFP- and YFP-tagged constructs as described above. The laser diode emitting at 440 nm were used for excitation and the emission was monitored using 450–490 nm detection filter. Lifetime images were analyzed by fitting a bi-exponential model equation to the fluorescence decay in every pixel of the image. To include all possible populations and conformations of the CFP donor (those that do and those that eventually do not do FRET), the fluorescence lifetime displayed in FLIM images or lifetime histograms corresponds to the average fluorescence lifetime calculated as the amplitude weighted mean value using the data from the bi-exponential fit. FRET was analyzed by monitoring the fluorescence lifetime of the donor in the presence and absence of acceptor and calculated according to formula $E = 1 - \tau_{\text{FRET}}/\tau_{\text{CFP}}$ ^{43,44}.

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Author Contributions

J.P. carried out most of the experiments and generated figures; H.Y. performed data analysis; X.Z. and H.Z. carried out sf9 cell culture; Q.X. performed sGC proteins purification from sf9; Y.Z. performed proteins characterization; L.T. and S.N. provide the instrument and the technique for Fluorescence Lifetime Image Microscopy; Z.H. reviewed and revised the manuscript; X.T. designed this study, supervised the project and wrote the manuscript.

Additional Information

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