UnaG, A Photoswitchable Fluorogen-binding Protein for STORM

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ABSTRACT STORM (stochastic optical reconstruction microscopy), which offers an order of magnitude improvement in resolution, is based on high-precision single-molecule localization of photoswitchable fluorophores. The performance of STORM depends on photophysical properties of photoswitching fluorophores. Conventional fluorescent proteins used in STORM suffer from fast photobleaching, which results in limited number of independent snapshots in live-cell super-resolution imaging. We aim to develop a new STORM fluorophore for virtually no photobleaching. UnaG, a fluorogen-binding protein derived from the Japanese freshwater eel, fluoresces upon binding of nonfluorescent bilirubin, an endogenous metabolite. We discovered photoswitching behavior of UnaG: UnaG fluorescence turns off upon 488-nm illumination; UnaG in the dark state comes back to the fluorescence state upon 405-nm illumination. The photoswitching of UnaG can be repeated about hundreds of switching cycle. Most notably, we found that UnaG fluorescence recovers after photobleaching when exogenous bilirubin is added into the solution. We have applied UnaG to STORM imaging of the subcellular ultrastructure.

Introduction

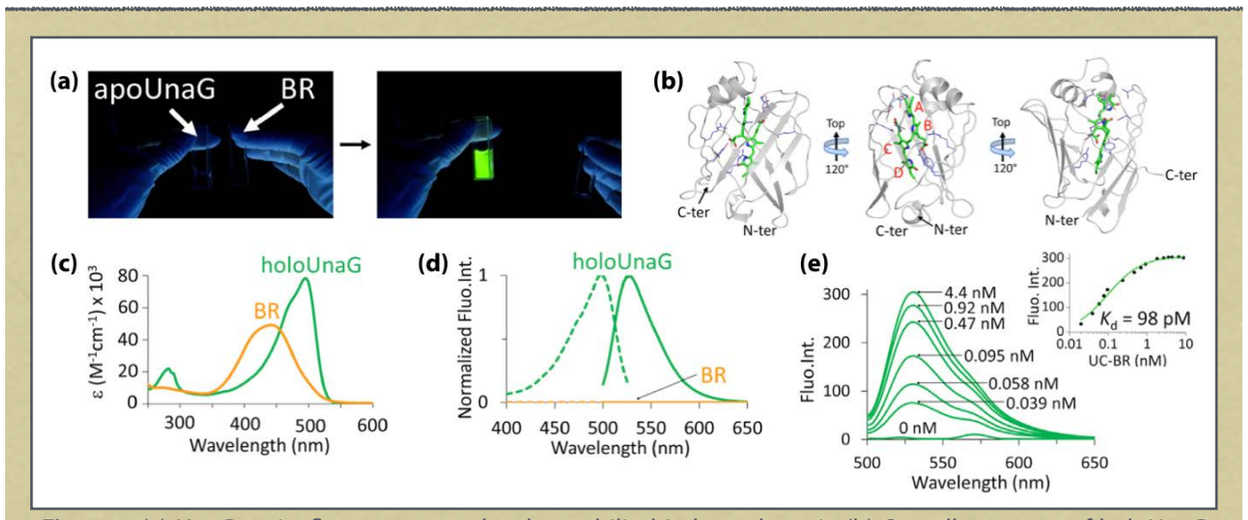

Figure 1 (a) UnaG emits fluorescence only when a bilirubin bounds on it. (b) Overall structure of holoUnaG represented in three different angles. (c) Absorption spectrum of holoUnaG (green) and bilirubin (orange). (d) Excitation (dashed lines) and emission (solid lines) spectrum of holoUnaG (green) and bilirubin (orange). (e) Titration of apoUnaG (5 nM) with unconjugated bilirubin.

- ☑ UnaG is a protein derived from Japanese eel, and can bind to a fluorogen, bilirubin, to emit fluorescence.

A. Kumagai et al., *Cell*, **153**, 1602 (2013)

Background

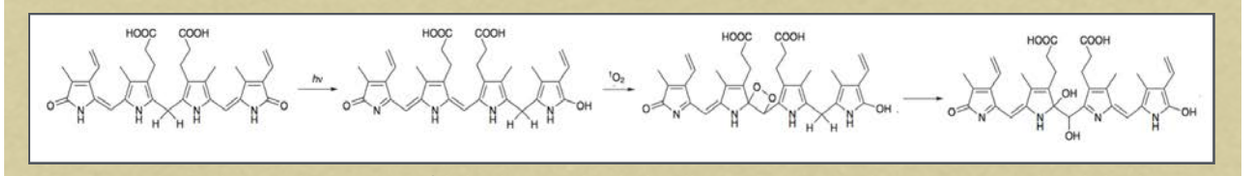

Figure 2 Photochemical reaction of bilirubin under light illumination. Singlet oxygen oxidize bilirubin to biliverdin, which proceeds internal molecular transition.

- ☑ Bilirubin is an endogenous metabolite that can bind to the UnaG to form holoUnaG. Although free bilirubin do not fluoresce, the holoUnaG emit strong fluorescence by absorbing blue light. Singlet oxygen can be added to the bilirubin to form biliverdin, and it results the photobleaching of holoUnaG

C. S. Berry et al., *Biochem. Biophys. Res. Comm.*, **49**, 1366 (1972)

Photoswitching of holoUnaG

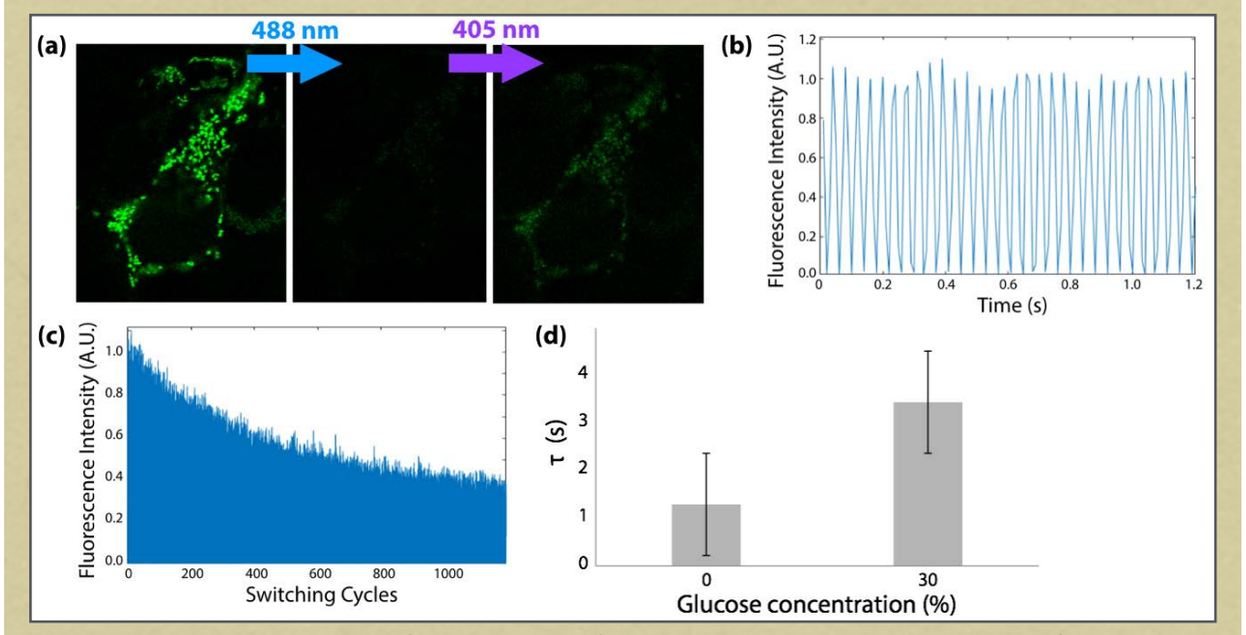

Figure 3 (a) Fluorescence image of Mito-UnaG transfected HEK293T cells. Strong illumination of 488-nm laser turns off UnaG fluorescence, where it recovered with 405-nm laser illumination. (b,c) Fluorescence intensity under alternative illumination of 488- and 405-nm lasers. >50 % of molecules can survive after 600 switching cycles. (d) Concentration of dissolved oxygen influences to the photoswitching rate of UnaG.

- ☑ Fluorescence of holoUnaG can be turned on (405 nm) and off (488 nm) by light illumination, and this switching process can be held more than 500 cycles.
- ☑ The off switching rate is considerably affected by the concentration of dissolved oxygen.

Fluorescence Recovery after Photobleaching

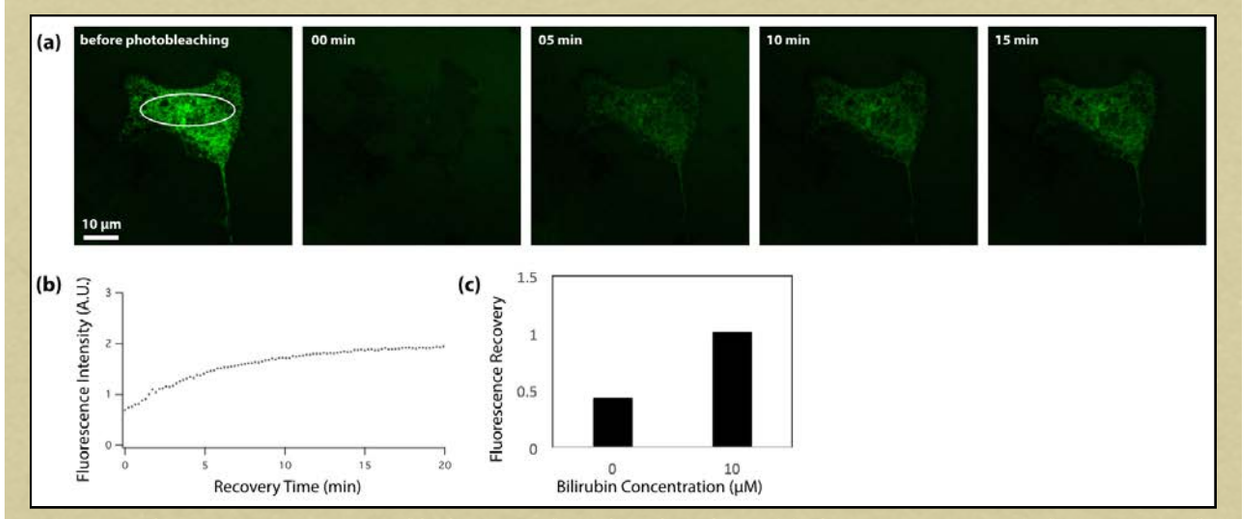

Figure 4 Fluorescence recovery of holoUnaG after photobeaching. (a) Fluorescence image of UnaG-Sec61ß transfected Cos7 cells. Strong illumination of 488-nm laser light induces photobleaching of holoUnaG. However, fluorescence of holoUnaG recovered gradually. (b) Fluorescence time trace of white marked region in (a). (c) The recovered fractions clearly depend on the concentration of bilirubin, suggesting that the damaged bilirubin is repeated to a fresh one to fluoresce again.

- ☑ Photobleached holoUnaG restores its fluorescence spontaneously.
- ☑ The fraction of recovered fluorescence depends on the concentration of dissolved bilirubin, indicating that exchanging reaction of bilirubin promotes the recovery.

Live-cell STORM imaging with UnaG

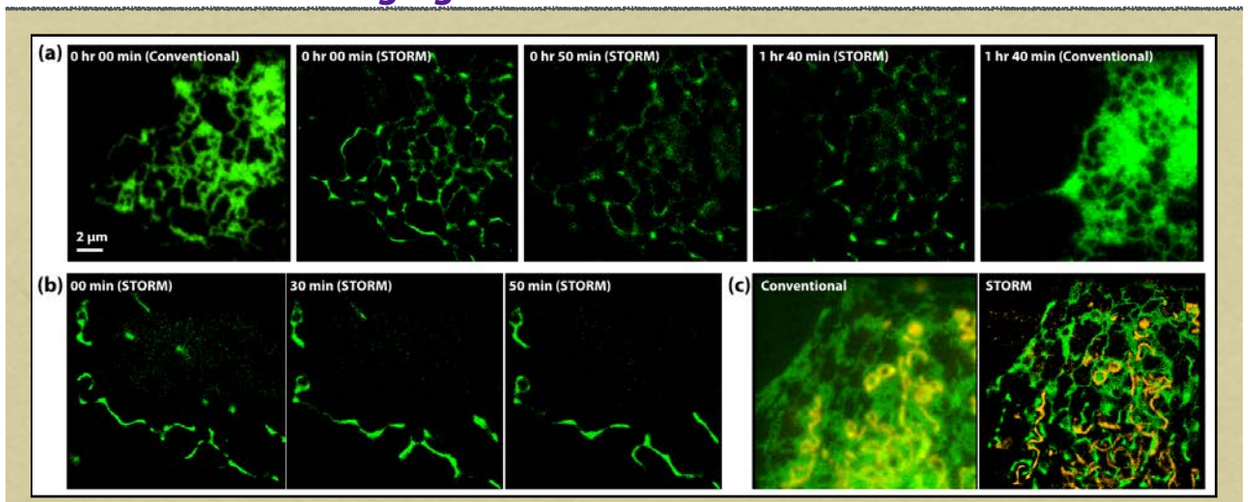

Figure 5 (a,b) STORM imaging of UnaG-Sec61ß (a, 10 ms/frame, 1000 frames) and ER-UnaG (b, 10 ms/frame, 5000 frames) expressed live Cos7 cells. (c) Two-color STORM imaging of live cells by using UnaG-Sec61 and Mitotracker Red.

- ☑ Two distinct photophysical features, the photoswitching and the fluorescence recovery, enable long-term STORM imaging more than 600 time points.
- ☑ Interestingly, UnaG shows different restoration capabilities in ER and mitochondria, probably due to the different local environments of subcellular structures.
- ☑ With Mitotracker Red, UnaG gives two-color STORM images for the live cells.

Conclusions and Future Works

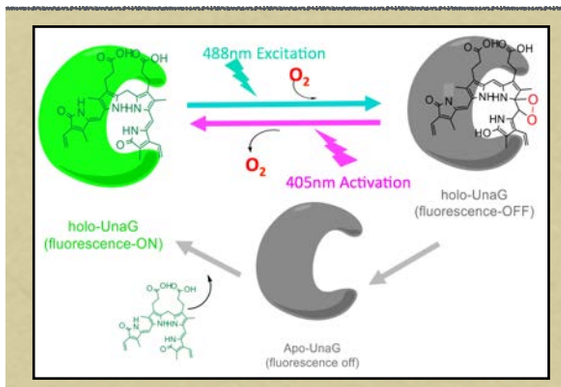

Figure 6 Suggested photophysical model of UnaG. Photoswitching and binding/unbinding processes occur simultaneously.

- ☑ UnaG emits fluorescence when a bilirubin binds on it, and its fluorescence can be switched by 405- and 488-nm lasers.
- ☑ Photobleached UnaG revives by replacing the damaged bilirubin.
- ☑ Restorable fluorescence of UnaG enables long-term super-resolution imaging without limited photobleaching.
- ☑ Further optimization of imaging conditions is required to improve the image quality and to make the cell lives longer.