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Sustainable bioimaging using a fluorescent protein with unprecedented photostability

The jellyfish-derived green fluorescent protein StayGold is bright and hardly fades, contributing to improving spatiotemporal resolution and dramatically extending the observation period. To fully benefit from the rich photon budget, we tried some unusual illumination modalities for sustainable, quantitative live-cell or volumetric imaging.

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The problem

Thanks to improvements in gene transfer and protein-targeting techniques, it is now possible to label and thereby efficiently observe subcellular components such as organelles and the cytoskeleton using fluorescent proteins. Furthermore, recent temporally well-resolved observations of subcellular components revealed rapid motion of their fine structures[1,2]. In comparison with chemical dyes, however, fluorescent proteins are notorious for their rapid photobleaching, which means that researchers must use brightly labeled cells, minimize illumination power, and use time-lapse imaging. However, there is concern that brightly labeled cells may produce artifactual signals because of overexpression, low illumination power may result in a poor photon budget that impedes the use of most super-resolution imaging techniques, and temporal downsampling (time lapse) may miss transient but important signals. Therefore, highly photostable fluorescent proteins are needed to ameliorate these undesirable situations.

The solution

One solution to this problem was uncovered through our molecular cloning and mutagenesis studies on a wild-type fluorescent protein from the jellyfish *Cytaeis uchidae* (Fig. 1a). The engineered fluorescent protein called StayGold is over an order of magnitude more photostable than any currently available fluorescent proteins (Fig. 1b). This enhanced photostability is intriguing because developing fluorescent proteins seems to involve a trade-off between brightness and photostability. Molecular oxygen (O_2) is often a double-edged sword when using fluorescent proteins. On the one hand, maturation of fluorescent protein chromophores requires oxidation reaction(s), and high accessibility to O_2 contributes to the increase in practical brightness. On the other hand, decomposition of fluorescent protein chromophores is caused by O_2 while fluorescent protein chromophores are in the singlet or triplet excited state, and thus enhanced O_2 accessibility should decrease photostability.

With StayGold, we preferentially selected moderately bright cells to perform cell-wide, fast, continuous super-resolution imaging for extended times. We mostly employed three-dimensional structured illumination microscopy (3D-SIM), which creates a 3D interference pattern using three-beam illumination[3]. We imaged endoplasmic reticulum (ER) network dynamics in multiple cells for 6 min without deterioration of super-resolution image quality. The 6-min observation period was long enough for successive administration of two drugs, one to initiate Ca^{2+} mobilization and the other to shut it down. We discovered that the overall rearrangement of the ER network was attenuated upon Ca^{2+} mobilization (Fig. 1c). However, once samples are fixed, photocytotoxicity is no longer a concern and it is possible to greatly increase signal intensity by using intense, long illumination for excitation. After fixation of SARS-CoV-2–infected cells, we achieved fine intracellular mapping of the viral spike protein by super-resolution volumetric imaging with nanobody–StayGold fusions.

Future directions

Photostability of fluorescent proteins is one of the most important hurdles in modern bioimaging, and we believe that StayGold demonstrates performance that makes sustainable bioimaging possible without being limited by significant photobleaching.

We are still further developing StayGold technology. As StayGold is a dimer, the development of monomeric StayGold (mStayGold) for protein fusion applications is under way. In the meantime, we created a tandem dimer StayGold (tdStayGold) by fusing two copies of the fluorescent protein construct, which we successfully used to visualize the dynamics of a microtubule-associated protein complex and an excitatory postsynaptic density protein (Fig. 1d). We are also developing a StayGold variant that is completely fusion-tolerant by further modifying its amino and carboxyl termini. Solving the crystal structure, which is under way, will be helpful for the directed evolution of StayGold by designed mutagenesis. It is important to recognize that all wild-type fluorescent proteins have been extensively engineered to improve their performance in fluorescence imaging, as seen in the development of the mFruit series[4]. Integration of tdStayGold (or later, mStayGold) at specific loci using genome-editing techniques will make it possible to quantitatively visualize fluorescent protein–tagged proteins at low copy number and to track such proteins in cells over extended time periods.

We have circumvented the brightness–photostability trade-off to develop StayGold, which has outstanding photostability and excellent brightness, and these features prompt us to consider how StayGold interacts with O_2 for chromophore maturation and decomposition. Once we obtain an atomic-level understanding of the mechanism underlying the unique photostability of StayGold, it will be interesting to determine whether the mechanism of photostability is transferrable to other popular fluorescent proteins.

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EXPERT OPINION

"StayGold overcomes the significant photobleaching seen in conventional fluorescent proteins, making it useful for visualizing tagged proteins at moderate to low expression levels and enabling avoidance of overexpression artifacts." **Jennifer Lippincott-Schwartz, Janelia Research Campus, Howard Hughes Medical Institute, VA, USA.**

FIGURE

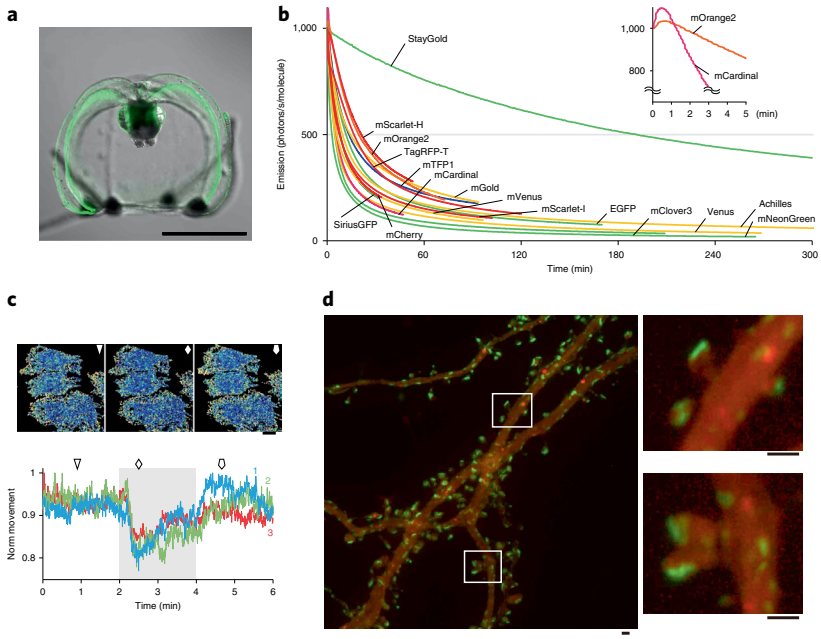

Fig. 1 | The benefits of the rich photon budget of StayGold. a, Natural fluorescence of *Cytaeis uchidae*, the source of StayGold. **b**, Photobleaching curves reveal that StayGold is more photostable than other fluorescent proteins. **c**, ER movement in three HeLa cells expressing StayGold as an ER marker, visualized with 3D-SIM. **d**, Postsynaptic densities in rat hippocampal neurons labeled with PSD95–tdoxStayGold (tandem cysteineless StayGold), visualized by spinning disk confocal super-resolution microscopy. Scale bars: 0.5 mm (**a**), 5 μm (**c**), 1 μm (**d**). © 2022, Hirano, M. et al., CCBY 4.0.

BEHIND THE PAPER

Cytaeis uchidae produces millimeter-sized free-swimming medusae that express green fluorescence in the epithelium of the ex-umbrella and sub-umbrella, as well as in the gonads (Fig. 1a). This jellyfish has been used as an educational tool to help school children in Sendai City, Japan, to learn about fluorescence. Although medusa fluorescence had been spectroscopically characterized, the molecular source remained unknown. The wild-type fluorescent protein that we cloned from *C. uchidae* seemed an impractical molecule, as it was poorly folded in both bacterial and mammalian expression systems, although, notably, the fraction that was folded was very photostable. It is therefore interesting to ask what kind of selection pressures this fluorescent protein was exposed to during the evolution of *C. uchidae*. Moreover, our discovery reveals that, beyond in vitro directed evolution, exploring nature has value for discovering fluorescent proteins of unprecedented photostability. **A.M.**

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FROM THE EDITOR

"Miyawaki et al. use the jellyfish *Cytaeis uchidae* to address the poor photostability of fluorescent proteins. They find that its main fluorescent protein is >10-fold more photostable than any fluorescent protein currently in use. By engineering a version with not only improved photostability but also a brightness comparable to that of other fluorescent proteins in use, they created StayGold, offering a powerful new tool for the imaging community." **Editorial Team, *Nature Biotechnology***