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Breaking up the StayGold dimer yields three photostable monomers

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The exceptionally photostable green fluorescent protein StayGold has been monomerized in different laboratories, which has generated three unique monomeric variants that will enable new imaging applications.

In the three decades since the cloning of the *Aequorea victoria* green fluorescent protein (*GFP*) gene[1], GFP has revolutionized cell biology by providing a fully genetically encoded fluorophore. Even though many improved fluorescent proteins have subsequently been engineered, the recent discovery of StayGold — a very bright green fluorescent protein with an exceptional photostability[2] — came as a big surprise. The photostability of StayGold is at least an order of magnitude higher than for other popular green fluorescent proteins, and it thereby enables almost unlimited live-cell imaging — a breakthrough for fluorescent protein imaging applications.

However, it turns out that StayGold is an obligate dimer, which is a common feature among naturally occurring fluorescent proteins. This dimerization complicates several applications, including protein tagging, biosensor engineering and the study of protein–protein interactions by fluorescence techniques (Fig. 1). To circumvent problems related to dimerization in protein tagging, a so-called tandem dimer can be created. Indeed, tandem-dimer StayGold constructs proved successful for prolonged live-cell imaging[2]. Yet this doubles the size of the tag and still precludes its use for single GFP-based biosensors.

Evolving true monomeric fluorescent proteins from dimerizing parents are challenging protein engineering projects, as these efforts are usually accompanied by a decline in other properties (for example, photostability or brightness), which requires multiparameter screens[3]. Now, three papers from three independent research teams have been published — two of which appear in this issue of *Nature Methods* — that report on a monomeric variant of StayGold[4–6], which underscores the importance of monomerization. For the names of the new, monomeric StayGold variants, we adhere to the names that are used at FPbase[7]: mStayGold, StayGold-E138D and mBaoJin (from 'stay gold' in Chinese).

Two teams first solved the crystal structure of StayGold, which revealed the dimerization interface[4,5]. These structures show that roughly 10 amino acids contribute to the interaction between the two β-barrels. Targeted mutagenesis of residues at the dimerization interface is a proven method to generate monomers. Remarkably, one of the papers uses a single mutation at the β-barrel interface — E138D (that is, effectively removing only one CH_2 unit in one amino acid side chain) — that completely disrupts dimerization[4]. However, disruption of the dimerization interface is often accompanied by a loss in brightness.

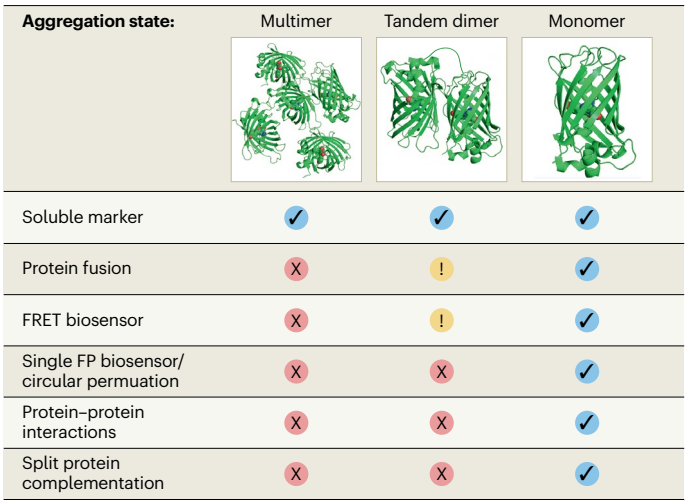

Fig. 1 | Compatibility of applications of fluorescent proteins with the aggregation state of the fluorescent protein that is used. Tick, compatible; cross, incompatible; exclamation mark, limited compatibility.

To retain all favorable properties, Miyawaki and colleagues first generated dozens of monomeric variants by targeting the dimer interface, and subsequently used multiple rounds of mutagenesis while screening for brightness, photostability and monomericity. The resulting mStayGold[5] has a brightness and photostability in cells that is equal to the parental StayGold.

A third endeavor by Subach and colleagues started by developing a screening method in bacteria that uses a transcription factor that, upon dimerization, drives the expression of a blue fluorescent protein[6]. After fusing StayGold to the transcription factor, this system can be used to detect homodimerization by observing blue fluorescence. Rewardingly, variants that displayed low blue fluorescence had a mobility that corresponded to monomers, as analyzed by liquid chromatography. Multiple rounds of mutagenesis were performed to yield a monomeric StayGold named mBaoJin, which is bright, photostable and tolerates the fixation and chemical treatments that are necessary for expansion microscopy.

The three monomeric StayGold variants were published almost simultaneously, and only the paper by Subach and colleagues reports a head-to-head comparison in cells. It turns out that all three variants are monomeric in the OSER assay. StayGold-E138D shows a twofold lower cellular brightness than mStayGold, but retains its high photostability. On the other hand, mBaoJin has twofold lower photostability than StayGold-E138D, mStayGold and the original StayGold. Intriguingly, a variant of mBaoJin in which one mutation is reverted to a serine that was present in StayGold has an improved photostability. The T55S variant

is not characterized in cells, but will be interesting to follow up on and may lead to mBaoJin2.

The head-to-head comparison on three critical parameters (brightness, photostability and monomericity) in standard mammalian cell lines suggests that mStayGold is the best suited of the three monomeric variants for most applications. But it should be noted that fluorescent proteins that are bright in mammalian cells may be suboptimal in other species[8]. In addition, Miyawaki and colleagues have shown that care is needed when making fusion proteins with monomerized or tandem Staygold variants. Although standard C-terminal tagging of proteins seems fine for mStaygold, N-terminal tagging requires a special linker sequence to maintain the superior spectral properties[5]. How this applies to mBaoJin or StayGold-E138D is not yet clear. Therefore, a critical assessment of new constructs is advisable before investing in elaborate biological experiments with these new GFPs.

Despite all the engineering efforts and new structural information, the molecular mechanism that explains the remarkable photostability of StayGold is still unclear. A notable feature of the crystal structure of StayGold is the presence of a chloride ion next to the chromophore. In other GFPs, this same cavity is usually occupied by water molecules. It is tempting to speculate that the chloride protects the GFP against photochemical destruction, possibly by hampering interaction of molecular oxygen with the chromophore and thereby preventing formation of superoxide radicals. Yet mNeonGreen also features a chloride ion next to the chromophore[9] and is much more susceptible to photobleaching. Regardless, it will be important to understand the mechanism that is responsible for the high photostability as it may enable the engineering of novel spectroscopically different GFP variants with a similar high photostability.

A recent preprint[10] reports that under high excitation-power regimes (such as confocal microscopy or in single molecule studies), the difference in photostability between StayGold and regular GFPs seems less prominent (although still improved). This suggests that the new monomeric StayGold variants may prove most versatile in prolonged live-cell imaging using widefield microscopy, spinning disk or fast-scanning confocal microscopy and light-sheet microscopy applications.

To conclude, monomeric StayGolds will stretch the limits of biological imaging and enable new applications (Fig. 1). As there is still lots to be learned about these exciting new probes, it is recommended that users do a critical evaluation of their own constructs, in their biological system and using the equipment to which they have access. Ideally, the results (and plasmids) will be shared rapidly for the benefit of the entire community.

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Competing interests

The authors declare no competing interests.