A feasible method for comparing the power dependent photostability of fluorescent proteins

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A feasible method of combining the concept of fluorescence half-life and the power dependent photobleaching rate for characterizing the practical photostability of fluorescent proteins (FPs) was introduced. Furthermore, by using a fluorescent photostability standard, a relative comparison of the photostabilty of FPs from different research groups was proposed, which would be of great benefit for developing novel FPs with optimized emission wavelength, better brightness, and improved photostability. We used rhodamine B as an example to verify this method and evaluate the practical photostability of a far-red FP, mKate-S158C. Experimental results indicated good potential of this method for further study.

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Biological research is being profoundly influenced by the application of fluorescent proteins (FPs) as fusion tags to track protein behavior in living cells. The recent usage of FPs as an invasive tool for studying protein kinetics and activities has been stimulating the protein engineering[1]. This results in an explosion of the diversity of available FPs, which promises a wide variety of new FP markers for biological imaging. The established strategies for optimizing FPs have primarily focused on color and brightness; however, recently the photostability of FPs is realized as a third primary consideration. Therefore, choosing the most photostable protein is believed as a critical factor to success in long-term biological imaging due to the fact that different FP variants demonstrate distinct diversities of their photostabilities[2,3]. Unfortunately, compared with the other two characteristics of protein, the photostability characterization undergoes the incomprehension and complexity of the photobleaching process as well as numerous systematic uncertainties, hence relative photostabilities reported within a single paper should be more reliable than the absolute values[4−6]. Until now a unified method of quantifying FPs' photostability has been lacking in the scientific literature[6].

Recently, through precisely accounting for the detailed information of the experimental components and manipulating the initial photon emission rate, Shaner *et al.* proposed a parameter of fluorescence half-life, $t_{1/2}$, indicating the time for the FP to be photobleached from 1000 down to 500 emitted photons per second (referred to as $t_{1/2}$ in the subsequent paragraphs)[6]. This has for the first time suggested as a solid parameter for the absolute photostability characterization. However, the required precise figuring of the whole experimental system may bring extra complexity. Moreover, in practical biological imaging experiments, little or no guidance could be obtained from the $t_{1/2}$ value, as the initial photon emission rate (1000 photons/s) has no obvious connection to practical experimental configurations. On the other side, Patterson *et al.* suggested a well-accepted method which used another definition, power dependent photobleaching rate which is obtained by exponential fitting of corresponding photobleaching curves, for defining the photostability of FPs[7]. This method requires no absolute control of the experimental condition for the required intensity of initial fluorescence emission. Thus it is of comparable simplicity for practical experiments. Nevertheless, the photobleaching curves of some FP variants have complex profiles probably due to complex chromophore dynamics, which cannot be exponentially fitted[8].

In this letter, by combining the advantages of the above two well-accepted methods for the photostability characterization of FPs, we introduce a new parameter, power dependent fluorescence half-life, $t^P_{1/2}$, for characterizing the practical photostability of FPs. In addition, we apply the method from Demas *et al.* for the quantum yield measurements[9] to the comparison of photostabilities from different groups.

In the method demonstrated by Patterson *et al.*[7], an exponential model is applied to fit the photobleaching curves of the FP, $I = I_0 \cdot \exp(-k \cdot t)$, where I is the normalized fluorescent intensity, I_0 is the initial intensity of the fluorescence, t represents the time, k is defined as the photobleaching rate of a FP. According to the idea of absolute $t_{1/2}$ by Shaner *et al.*, we define $t^P_{1/2}$ as the power dependent fluorescence half-life of the FP to the extent of the duration between which the initial fluorescent intensity decreases from 1 to 0.5 from the normalized curve, regardless of the initial fluorescent photon flux. Thus when $I = I_0/2$, $t^P_{1/2}$ is calculated as $t^P_{1/2} = \ln 2/k$. Here, according to the power dependence of the photobleaching rate to practical environments[7], corresponding relation exists between the fluorescence half-life and the excitation power. This indicates: (1) in ideal case where the photobleaching curves follow exponential decay, by knowing the excitation power for the 1000 photons/s initial fluorescence emission situation under practical systems, the absolute fluorescence half-

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life $t_{1/2}$ could be calculated from the photobleaching rate measured from the method proposed by Patterson *et al.* (2) For practical experiments where excitation intensity needs to be optimized, instead of using $t_{1/2}$, one needs to use power dependent fluorescence half-life $t^P_{1/2}$ as a parameter for characterizing the photostability of FPs. More importantly, $t^P_{1/2}$ is a more feasible and practical reference which could be used as a robust evidence for characterizing new FP variants with uncertain photostability behaviors.

As introduced previously, although the relative photostability in a single research is more reliable than the absolute values, the photostability comparison among different researches is of much more importance for the screening and developing of FP markers in fluorescent imaging[2,3]. However, the photostability characterization was effected by numerous systematic uncertainties, for example, nonuniform illumination and wavelength responses of optical components (excitation and emission monochromator, filters, and detectors)[6], so $t^P_{1/2}$ could not be easily characterized. To minimize these effects, we applied the method from Demas *et al.*[9] for the quantum yield measurements to the quantification of photostabilities from different groups. In practice, commercial and well-defined fluorescence dyes could be selected and used as photostability standards, since fluorescence dyes are cheaper and easier to be acquired than FPs. By comparing $t^P_{1/2}$ of photostability standards and FPs measured under the same conditions, such as the proportion between two values, which we will demonstrate later, the various diversities of experimental environments can be minimized and the evidence can be provided for a fair comparison of photostability of FPs in different investigations.

For experimental verification of this method, we characterized the photostability of a well-known fluorescence standard, rhodamine B (RhB), and the monomeric version of a far-red FP, mKate-S158C[8]. In our experiment, RhB is selected as the photostability standard by two considerations. Firstly, it is commercially available. Secondly, the excitation and emission spectra of RhB[10] have favorable overlap with that of the mKate-S158C (Fig. 1), which is a more important criterion for the photostability standard selection.

In the photobleaching experiments, RhB (Sinopharm Chemical Reagent Co.) was firstly solved in ethanol and then diluted by 70% sucrose solution to obtain a concentration of about 10^{-5} mol/L. The purified mKate-S158C was mixed with mineral oil[11]. Microdroplets with dimension of $5-30$ μm for photobleaching were prepared as previously published[7,12] and used as samples, which allow the photobleaching in a volume small enough to avoid diffusion problems. Photobleaching by mercury lamp were performed on an inverted fluorescent microscope (IX71, Olympus). A fluorescent filter cube (U-MWIY2, EX: BP 545-580, DM: 600 LP, EM: BA 610IF, Olympus) was used according to the excitation and emission spectra of mKate-S158C. The excitation light from mercury lamp was focused by a 40× objective (LUCPlan FLN, numerical aperture (NA) = 0.6, Olympus) into the sample. The excitation power at the sample was adjusted with four neutral density filters and measured with a power meter (NOVA, Ophir). The fluorescent image of the microdroplet under the wide-field illumination was continually recorded by a charge-coupled device (CCD) camera (Retige Exi, Qimaging) with an interval of 0.5 min. The regions of interest encompassing the interior of the microdroplet were determined by comparison with the mean value of the selected background area. The value of fluorescence intensity was calculated as the mean value of the distinguished region of interest. All the photobleaching curves were normalized.

The photobleaching curves of RhB and mKate-S158C were provided in Figs. 2 and 3. It can be seen that, with the increase of excitation intensities, the measured photobleaching curves tend to decay with significantly shorter times. The fluorescence intensity of RhB

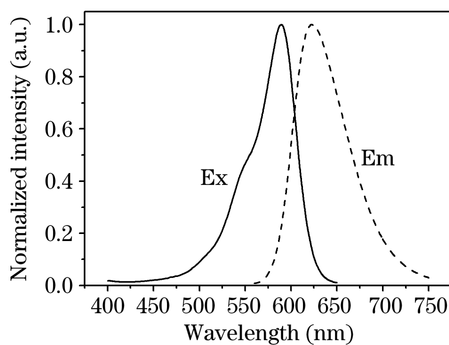

Fig. 1. Excitation (Ex) and emission (Em) spectra of fluorescent protein mKate-S158C.

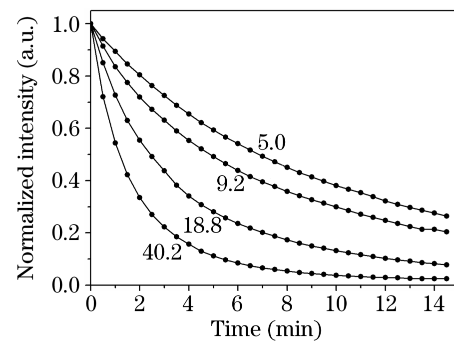

Fig. 2. Photobleaching curves of RhB in sucrose (10^{-5} mol/L, pH 7.3) at four different excitation intensities (in mW). The initial intensity is normalized and images are collected at 0.5-min time interval.

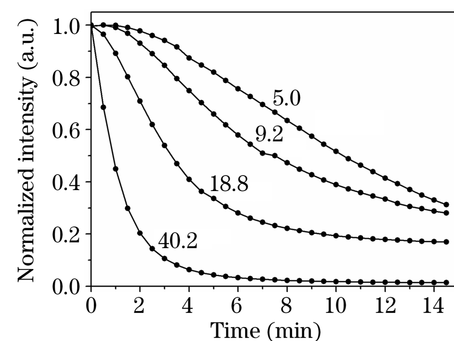

Fig. 3. Photobleaching curves of purified mKate-S158C, prepared in mineral oil (pH 7.4) at four different excitation intensities (in mW). The initial intensity is normalized and images are collected at 0.5-min time interval.

Table 1. Power Dependent Photostability Parameters of RhB and mKate-S158C

P (mW)[a]	k (min^{-1})[b]	$t^P_{1/2,\mathrm{RhB}}$ (min)[c]	$t^P_{1/2,\mathrm{mKate}}$ (min)[d]
40.2	10.6	1.2	1.5
18.8	8.5	2.4	3.3
9.2	4.5	4.9	7.4
5.0	2.1	6.9	10.3

[a]Excitation power; [b]Photobleaching rate of RhB; [c,d] Obtained values of the power dependent fluorescence half-life of RhB and mKate-S158C at four different power levels.

decreases exponentially while mKate-S158C presents a complicated variance which firstly remains and then decreases. For comparison, Table 1 summarizes the power dependent photostability parameters of RhB and mKate-S158C under four different excitation powers. The $t^P_{1/2}$ values of RhB and the protein indicate a good linear dependence to the excitation power, evidencing expected behaviors under one photon excitation. Deviation to the linear dependence at 5.0 mW may result from insufficient illumination power. An approximate photostability relation between the two samples under mercury lamp illumination can be found: $t^P_{1/2}\,(m\mathrm{Kate}-\mathrm{S158C}) \approx 1.4 \cdot t^P_{1/2}\,(\mathrm{RhB})$. This relation may be an evidence for other researchers to estimate the photostability of their FPs with mKate-S158C. By using photostability standards (RhB in this case) according to different classes of FPs, the comparison can be applied in large variety of FPs from different research groups.

To fully evaluate the potential of our method, significant complemental work has to be carried out, for example, careful considerations on the experimental details, comparisons of various FPs with other photostability standards, applying this method to different imaging modalities, and photostability characterization under laser scanning confocal microscopy or two-photon fluorescent imaging[13]. To this extent, in this letter only a preliminary demonstration is presented and more experiments need to be done in the future.

In summary, a simple and feasible method for comparing the practical photostability of FPs in wide-field microscopy was introduced, which would help to improve the relative reliability within a single research paper and provide a fair comparison to the photostability of FPs from various research groups. To verify this method, a commercially available well-known fluorescent dye, RhB, is selected as an example to characterize the photostability of a far-red fluorescent protein, mKate-S158C, under wide-field illumination. Experimental data and analysis indicate good potential of our method for evaluating the practical photostablity of FPs.

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