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Regular Article

Determination of diffusion coefficients in live cells using fluorescence recovery after photobleaching with wide-field fluorescence microscopy

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Fluorescence recovery after photobleaching (FRAP) enables characterization of quantitative dynamic properties such as diffusion coefficients of fluorescent molecules in living cells by analyzing the recovery of fluorescence intensity after photobleaching in a specific cellular compartment or area. To quantitatively determine high intracellular diffusion coefficients, a suitable optical system as well as an appropriate model for fast diffusion analysis is necessary. Here, we propose a procedure to quantify the diffusion coefficient of rapidly-diffusing fluorescent molecules that makes use of an epi-fluorescence microscope with a photobleaching laser in combination with established models for diffusion analysis. Analysis for the diffusion coefficients of tandemly oligomerized green fluorescent proteins (GFPs) in living cells when changing the photobleaching times showed that photobleaching with shorter times than the diffusion speed indicated not the only way to obtain appropriate diffusion coefficients of fast-moving molecules. Our results also showed that the apparent spreading of the effective radius of the photobleached area works as a correction factor for determining appropriate diffusion coefficients of fast-moving molecules like monomeric GFPs. Our procedure provides a useful approach for quantitative measurement of diffusion coefficients in living cells. This procedure is relevant for characterizing dynamic molecular interactions, especially of fast-moving molecules, and is relevant for studies in many biological fields.

Key words: fluorescence recovery after photobleaching, diffusion coefficient, green fluorescent protein, fluorescence microscopy

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Movement of molecules in and out of cellular compartments or structures is a basic physical phenomenon that is important for many biological processes including transcription, molecular transport, signal transduction, and accumulation of molecules [1–4]. A variety of methods enabling molecular dynamics to be characterized in biological samples have been recently developed and widely used in molecular and cellular biology [5–8]. Fluorescence recovery after photobleaching (FRAP) is widely used for analysis of molecular dynamics [9,10]. FRAP allows quantitative determination of

◀ ***Significance*** ▶

Fluorescence recovery after photobleaching (FRAP), that is widely used for analysis of molecular dynamics, allows quantitative determination of static and dynamic properties of molecules of interest. To know diffusion states of rapidly-diffusing fluorescent molecules such as green fluorescent protein (GFP) monomers in living cells, we proposed a straightforward procedure for measuring diffusion coefficients using FRAP with an epi-fluorescence microscope. In this procedure, the apparent spreading of the effective radius of the photobleached area works as a correction factor. This procedure can be used to measure the diffusion coefficients of almost any fluorescently-labeled fusion protein in living cells without using any specialized microscopy.

static and dynamic properties of molecules of interest by measuring recovery of fluorescence intensity after photobleaching in a specific cellular compartment or structure. FRAP can determine the diffusive and/or dynamic properties of a molecule of interest even if a subset of the molecule is immobile. By combining FRAP analysis with other approaches, functional correlates of molecular dynamics have been elucidated [11,12].

The diffusion coefficient (also termed diffusivity) is a quantitative measure of a molecule's movement in and out of a cell as well as in solution. The diffusion coefficient is a constant expressing the relationship between flux due to molecular diffusion and the concentration gradient of the molecule of interest [13]. Higher diffusion coefficients indicate faster rates of molecular diffusion. Typically, the diffusion coefficient of green fluorescent protein (GFP) monomers in cells is higher than that of GFP-tagged proteins [8,10,14,15]. Measuring high diffusion coefficients ($>10\ \mu m^2 \cdot s^{-1}$) within a cell, such as those associated with GFP monomers, requires the use of a suitable microscopy system as well as an appropriate model for diffusion analysis. Several models for determining diffusion coefficients using FRAP have been proposed [16–19]. Quantitative measurement of a high diffusion coefficient using a conventional laser scanning microscope (LSM), as is typically used for FRAP analysis, is difficult because the scanning speed of the microscope is slow relative to the diffusing speed of the molecule. As a result, the molecule of interest diffuses during FRAP photobleaching [17]. One factor contributing to erroneous diffusion coefficients measured using LSMs arises from analysis models that do not take into consideration scanning effects. Use of an improved analysis model enables measurement of high diffusion coefficients ($\sim 80\ \mu m^2 \cdot s^{-1}$) using FRAP with a LSM [17].

Two important conditions should be satisfied in order to measure rapid molecular movement using FRAP. First, strong laser power is required for efficient photobleaching of fluorescent molecules in a short time, in combination with efficient (but weak) excitation power that avoids photobleaching during image acquisition. Second, rapid acquisition of images immediately after FRAP photobleaching is necessary. In this study, we employed an epi-fluorescence microscope with a mercury lamp as a light source. This microscope included both a highly sensitive camera capable of capturing images with high temporal resolution and a high-power laser for photobleaching. We propose a procedure using this system for quantitatively determining diffusion coefficients of rapidly-diffusing molecules in living cells, such as GFP monomers.

Materials and Methods

Cell culture and transfection

Mouse neuroblastoma Neuro2A cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; #D5796; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; GE Healthcare, Logan, UT), 100 U/mL penicillin G (Sigma-Aldrich), and 100 µg/mL streptomycin (Sigma-Aldrich) at 37°C and 5% CO_2. Plasmids previously developed for expression of GFP oligomers (1-mer to 4-mer) [20], and a plasmid for expression of histone H2B tagged with GFP (H2B-GFP) [21] were used. Plasmid DNA (1.0 µg) and Lipofectamine 2000 (2.5 µl; Thermo Fisher Scientific, Waltham, MA) were used for transfection, as reported previously [10,11].

FRAP measurement in living cells

FRAP experiments were performed on a Leica inverted microscope DMi8 through a HC PL APO 100×/1.40NA oil immersion objective (Leica, Wetzler, Germany) using Leica Application Suite X (LAS X) software. GFP in Neuro2A cells was excited using a mercury lamp ($3.02\ mW/cm^2$) through a filter cube customized for FRAP analysis (FRAP450; Leica) that included an excitation filter (BP 460/50), a 495 nm dichroic mirror, and an emission suppression filter (BP 525/50). For photobleaching of GFP, a 450 nm ($0.08\ MW/cm^2$) diode laser was used. GFP fluorescence was collected using a cooled charge-coupled device (CCD) camera (DFC 360FX; Leica). Each frame was acquired with a 19 ms exposure using 8×8 pixel binning (the dimensions of each image were 172 pixels [x-axis] × 130 pixels [y-axis]). After acquisition of 50 images prior to photobleaching, 200 images were acquired after photobleaching to measure recovery of fluorescence. FRAP photobleaching times of 1, 2, 4, 8, or 16 ms were used for GFP monomers measurements. For measurement of oligomeric GFPs, the FRAP photobleaching time was fixed at 8 ms. Relative fluorescence intensity (RFI) at time t was calculated using Eq. 1.

$$\mathrm{RFI} = \frac{I_{\mathrm{BL}}(t)/I'_{\mathrm{BL}}}{I_{\mathrm{Ref}}(t)/I'_{\mathrm{Ref}}} \tag{1}$$

In Eq. 1, $I_{BL}(t)$ and $I_{Ref}(t)$ are the intensity measured at time t in the photobleached region and the reference region, respectively. I'_{BL} and I'_{Ref} are the intensities measured before photobleaching. The recovery curve of RFI was fitted with Eq. 2 [18] using Origin 2016 software (OriginLab Corp., Northampton, MA).

$$F(t) = Ae^{-2\tau_D/t}\left[\boldsymbol{I}_0\left(\frac{2\tau_D}{t}\right) + \boldsymbol{I}_1\left(\frac{2\tau_D}{t}\right)\right] + b \tag{2}$$

In Eq. 2, $F(t)$ is the intensity at time t, $\boldsymbol{I}_0$ and $\boldsymbol{I}_1$ are modified Bessel functions, A is the maximum recovery proportion, b is the baseline intensity, and τ_D is the characteristic diffusion time. τ_D in this equation is defined as $\omega^2/4D$, where ω is the radius of the FRAP photobleached area and D is the diffusion coefficient.

Measuring the radius of the FRAP photobleached area

After the image that included the photobleached area was cropped (to 9×9 pixels), the cropped images were rescaled

(to 36×36 pixels) using a bilinear interpolating method in ImageJ 1.50i (National Institute of Health, Bethesda, MD). Fluorescence intensity of the FRAP photobleached region in the rescaled image was measured using Radial Profile Plot, an ImageJ plug-in. Intensity in the FRAP photobleaching region was fitted with Eq. 3 [16] using Origin 2016 software.

$$F(r) = F_0 \exp\left[-K \exp\left(-2\frac{r^2}{\omega^2}\right)\right] \quad (3)$$

In Eq. 3, $F(r)$ is the intensity at position r, F_0 is the intensity of unbleached area, ω is the radius of the FRAP photobleached area, and K is the photobleaching efficiency. For the measurement of immobilized fluorescent molecules, cells expressing H2B-GFP were fixed in 4% paraformaldehyde in 100 mM HEPES/KOH (pH 7.5) solution at 37°C for at least 1 h. After cells were washed in Tris-buffered saline (TBS) three times, FRAP measurements were performed.

Generation of graphs, movie, and statistics

Student's *t*-test was used to determine statistical significance using Microsoft Excel 2016. Graphs were drawn using Origin 2016. Movie of image series of GFP monomers-expressing cells during FRAP experiment was created using ImageJ 1.50i.

Results

FRAP measurements made with an epi-fluorescence microscope combined with a photobleaching laser

After photobleaching a circular region (radius of 1.8 μm) for 16 ms in the nucleus of live GFP monomers-expressing Neuro2A cells, fluorescence images were acquired at a frame rate of 19 ms. Photobleaching resulted in decreased fluorescence intensity in the target region (arrow in Fig. 1A; panels a and b). Fluorescence intensity gradually increased and recovered to pre-bleaching levels 1.8 s after FRAP photobleaching (Fig. 1A; panels c–g, and Supplementary Movie), indicating that FRAP can detect fast diffusion states such as those exhibited by GFP monomers in living cells. When successively shorter photobleaching times were used (8, 4, 2, and 1 ms), the recovery rate of RFI immediately after FRAP photobleaching became faster, and the magnitude of RFI shortly after photobleaching became higher (Fig. 1B). These results indicate that the diffusion time for GFP monomers to pass through the FRAP photobleached spot decreased as the duration of the photobleaching time fell. By contrast, as the duration of the photobleaching time increased, the calculated radius of the photobleached area increased (Fig. 1C). As has previously been established [16,17,19,22,23], this is due to molecular movement within the target area during FRAP photobleaching.

We calculated diffusion coefficients, which can be treated as a constant for the diffusion state of the molecule, using the diffusion time and the spot radius obtained for independent cells from images collected just after FRAP photobleaching. The calculated diffusion coefficient of GFP monomers in the nucleus was consistent with the value reported in a previous study that used fluorescence correlation spectroscopy (FCS), a method that allows quantitative determination of high diffusion coefficients [20]. Measurements made with different photobleaching times resulted in calculation of identical mean diffusion coefficients of GFP monomers (dark gray bars in Fig. 1D), suggesting that increases in diffusion time within the photobleached spot and apparently spreading radius due to diffusion of molecules canceled each other out. Next, we compared these values to diffusion coefficients of GFP monomers calculated using the mean intensity profile of the photobleached spot radius (light gray bars; Fig. 1D). Diffusion coefficients calculated using these two approaches were not significantly different. However, the diffusion coefficients showed a tendency toward smaller values when the mean photobleached spot radius was used and photobleaching times were 1 or 2 ms (Fig. 1D). Thus, it is preferable to calculate the diffusion coefficient using the photobleached spot radius determined in individual cells.

Next, to verify which photobleaching time provided the most accurate measurement for the diffusion coefficient, the coefficient of variation (CV; also known as relative standard deviation [RSD]) of the diffusion coefficient was calculated. The CV values of both the diffusion coefficient and the FRAP photobleaching radius became smaller as photobleaching time increased, suggesting that the diffusion coefficient calculated after measurements made with long photobleaching times had smaller error rates (Fig. 1E and 1F). Although longer FRAP photobleaching times (20 and 32 ms) showed lower CV values, diffusion coefficients calculated using these photobleaching times showed dramatically increased (Supplementary Fig. 1A and 1B). A possible reason may be due to dramatic increase of FRAP photobleaching radius (Supplementary Fig. 1C and 1D). Moreover, diffusion coefficients calculated using 6, 8, and 12 ms of FRAP photobleaching times showed 20–25 $\mu m^2/s$, and CV values of the diffusion coefficients were similar (0.452–0.489; Supplementary Fig. 1B). We therefore concluded that photobleaching times of 6–12 ms were appropriate for determining high diffusion coefficients associated with GFP monomers in living cells when using FRAP in this system. In the subsequent analysis, 8 ms of photobleaching was used.

Errors in calculated diffusion coefficients due to changes in the radius of the FRAP photobleached area

As shown in Figure 1D, the apparent radius of the photobleached spot spread due to diffusion of molecules during FRAP photobleaching may be important to obtain reasonable diffusion coefficient. However, the model used to calculate the diffusion coefficient in this analysis assumes molecules are immobile during FRAP photobleaching [18]. We thus checked whether the calculated diffusion coefficients differed when using the radius measured with GFP-tagged histone H2B (H2B-GFP), an immobile nuclear protein [7].

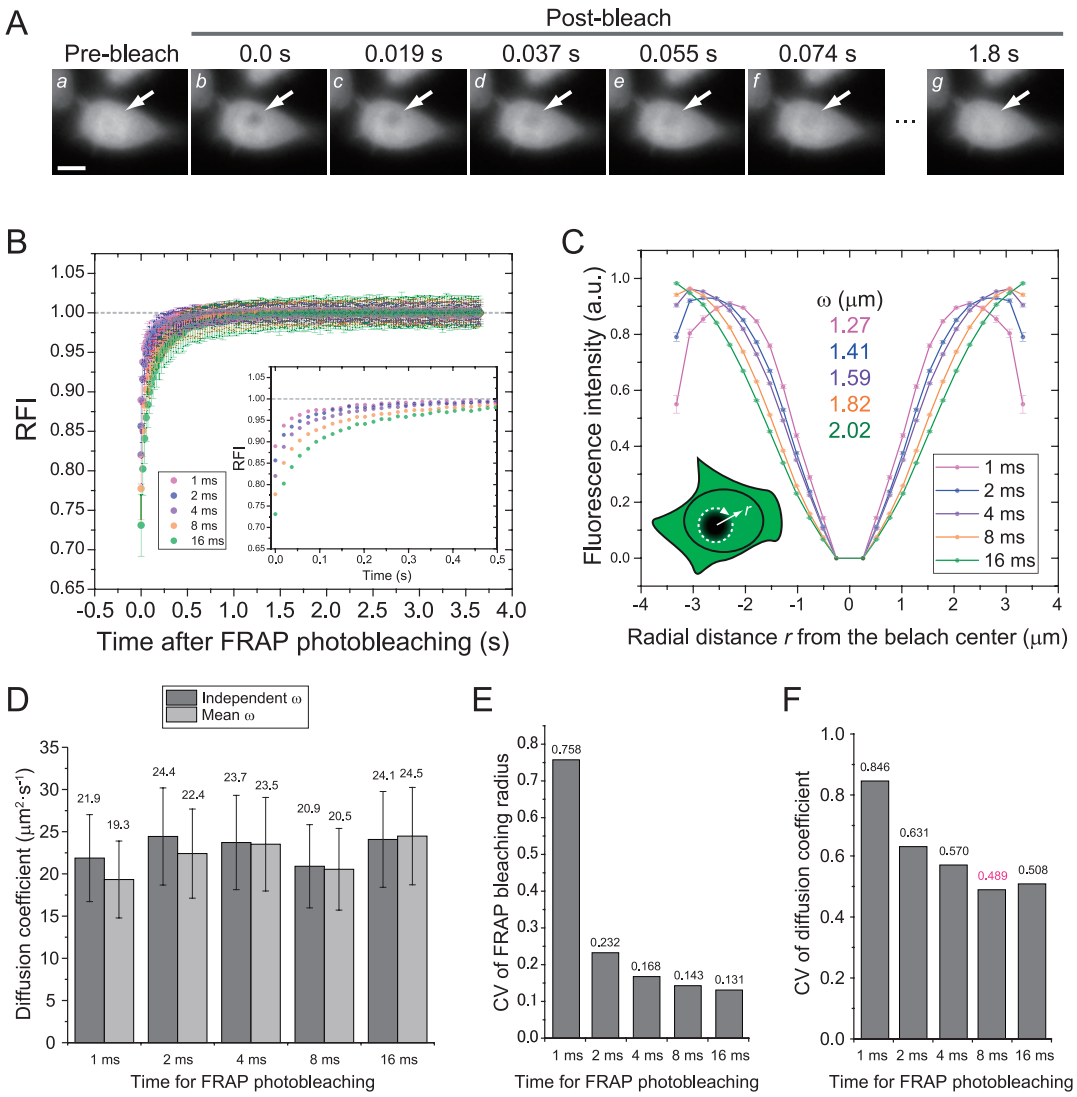

Figure 1 Comparison of diffusion coefficients of GFP monomers obtained with different FRAP photobleaching times
(A) Representative image series of fluorescence intensity of GFP monomers in living Neuro2A cells during the FRAP experiment. Times after 16 ms of photobleaching are indicated above the images. The white arrow indicates the FRAP photobleached spot in the nucleus. The scale bar in the left image indicates 10 μm. (B) Time course of RFI (mean±SD) after FRAP photobleaching. The dashed gray line indicates an RFI value of 1.0 when molecules are entirely mobile. Inset: Enlarged view of the recovery curve from 0 to 0.5 s after FRAP photobleaching. Dots indicates mean RFI at each time point. (C) Normalized rolling average of fluorescence intensity just after FRAP photobleaching in the photobleached spot. Bars indicate mean±SEM (n=18). Left inset cartoon: schematic of FRAP photobleached spot profile within cells. In the schematic, *r* indicates the linear distance of the white arrow. The dotted arrow indicates the direction in which rolling averaging was carried out. Colored values (top middle) indicate the calculated effective radii (ω) of the FRAP photobleached spot for each duration of photobleaching. (D) Comparison of diffusion coefficients of GFP monomers in the nucleus of live Neuro2A cells for different FRAP photobleaching times (1, 2, 4, 8, and 16 ms). Dark and light gray bars indicate diffusion coefficient calculated using independent effective radii obtained in distinct cells and the mean effective radius, respectively. Inset values indicate mean diffusion coefficients. Bars indicate mean±SEM (n=18). (E) Comparison of CVs of FRAP photobleaching radii in independent cells for different FRAP photobleaching time. Values above each bar indicate CV values. (F) Comparison of CV of diffusion coefficients as a function of different FRAP photobleaching times. Values above the bars indicate CV values. The minimum CV (0.489) is colored in magenta.

The FRAP photobleaching radius of H2B-GFP in living cells was not different from that measured in paraformaldehyde-fixed cells after 8 ms FRAP photobleaching (Fig. 2A), confirming that H2B-GFP in living cells is immobile. The FRAP photobleaching radius measured using H2B-GFP in living cells (1.32 μm) was significantly decreased relative to that measured using GFP monomers (1.82 μm). This result shows that the radius of the FRAP photobleached area increases when measured with a molecule that rapidly diffuses.

Next, diffusion coefficients of monomeric GFPs calculated with H2B-GFP using different photobleaching times were compared. The calculated diffusion coefficients monotonically decreased as bleaching time increased (Fig. 2B). The highest diffusion coefficient was obtained with a photo-

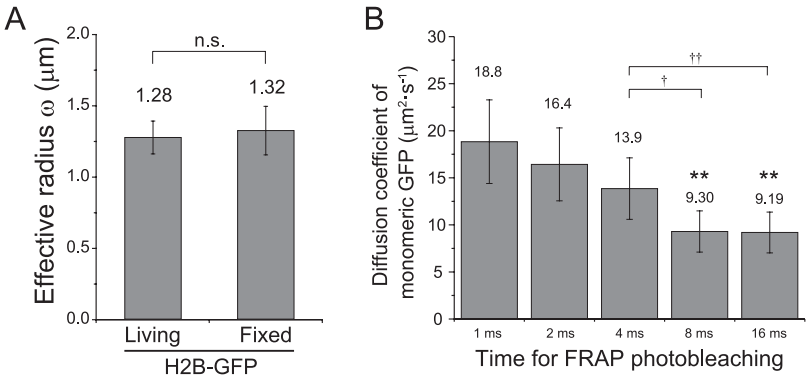

Figure 2 Diffusion coefficients of GFP monomers using photobleaching profiles obtained from immobile molecules
(A) Comparison of effective radii measured using living or paraformaldehyde-fixed Neuro2A cells expressing H2B-GFP. Values indicate mean effective radii. Bars indicate mean±S.E.M (n=15 for living cells and 20 for fixed cells, respectively). (B) Values indicate mean diffusion coefficients of monomeric GFP when using photobleaching radii calculated from H2B-GFP-expressing cells. Bars indicate mean±S.E.M (n=18). $^{**}p<0.01$ (vs photobleaching time for 1 or 2 ms; Student's *t*-test); $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$.

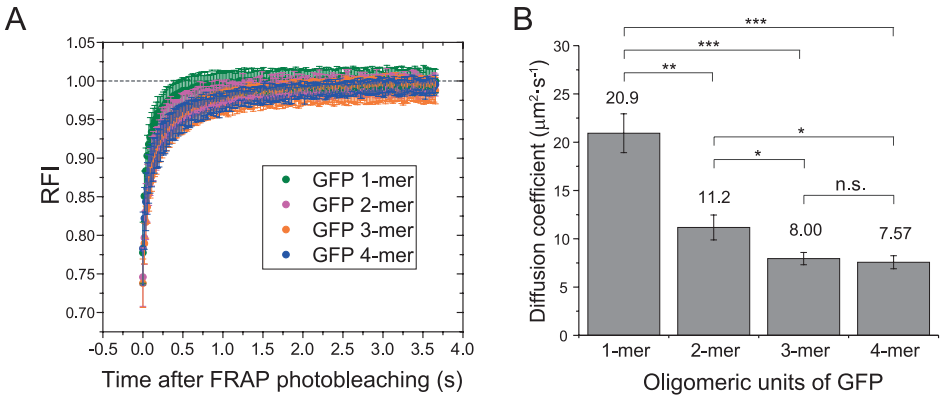

Figure 3 Diffusion coefficients of tandem GFP oligomers
(A) Time course of RFI after FRAP photobleaching (mean±SD; n=18). Dots indicates mean RFI for each time point after FRAP photobleaching. The dashed gray line indicates an RFI value of 1.0. (B) Bars indicate mean±S.E.M (n=18). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; n.s. no significant difference.

bleaching time of 1 ms (18.8 $\mu m^2 \cdot s^{-1}$); however, diffusion coefficients were lower overall than those calculated using rapidly-diffusing GFP monomers (compare Fig. 2B to Fig. 1D). These results suggest that calculation of an accurate diffusion coefficient requires either correction of the apparent photobleaching radius caused by molecular diffusion, or short photobleaching times that limit molecular diffusion during FRAP photobleaching.

Therefore, it is important to use the photobleached spot radius obtained from cells expressing the molecule of interest in an image acquired at short latency after FRAP photobleaching in order to obtain an accurate diffusion coefficient associated with a small error term.

Determination of diffusion coefficients of tandem GFP oligomers in living Neuro2A cells using FRAP

To determine if our results generalized to other molecules, we measured diffusion coefficients using tandem GFP oligomers (dimers, trimers, and tetramers) in living Neuro2A cells using FRAP as detailed in the preceding section. RFI recovery rates decreased as the number of oligomerized GFP units increased (Fig. 3A). Diffusion coefficients calculated using dimeric, trimeric, and tetrameric GFP oligomers were significantly lower than the value obtained using a GFP monomers (Fig. 3B). The diffusion coefficients calculated using GFP trimers and tetramers were significantly lower than the diffusion coefficient obtained using GFP dimers (Fig. 3B). Diffusion coefficients obtained using FRAP were slightly lower than but consistent with values previously reported in a study of tandem GFP oligomers using FCS [20]. A reason that the diffusion coefficients of tandem oligomers of GFPs were not inversely proportional to the increase of the oligomerized number may be due to a helical structure of the tandem oligomers because both N- and C-terminus of GFPs exists in the same direction as reported previously [20]. These results indicate that FRAP can be successfully

used to obtain diffusion coefficients using tandem GFP oligomers as well as GFP monomers.

There was no difference in diffusion coefficients calculated using trimeric vs. tetrameric GFP oligomers (Fig. 3B). In a previous report using FCS, trimeric and tetrameric GFP oligomers yielded diffusion coefficients that differed by ~1.3-fold [20]. The degree of molecular weight change of tandem GFP oligomers gradually decreases as the number of oligomerized unit is increased [20], such that differences in diffusion coefficients calculated with oligomers of increasing size become progressively smaller. Thus, to compare differences in diffusion coefficients calculated using measurements of trimers vs. tetramers, a large number of cells should be carried out. Even for measurements carried out using monomeric vs. dimeric GFP, demonstrating statistically significant differences in calculated diffusion coefficients required measurement of at least 10 independent cells (Supplementary Fig. 2). FRAP may not be sufficiently sensitive to detect small differences in diffusion coefficients; however, it can successfully detect significant differences in diffusion coefficients calculated from measurements made with monomers vs. dimers.

Discussion

In the present study, we successfully used FRAP on an epi-fluorescence microscope to calculate diffusion coefficients of fast-diffusing GFP molecules in living cells. The radius of the photobleached spot used was obtained using methods established by Axelrod [16], and diffusion time within the photobleached spot was calculated using Soumpasis's procedure [18]. The Soumpasis model is based on the assumption that molecules are stationary during FRAP photobleaching, and that the shape of the photobleached spot is uniformly circular. We found that fluorescent molecules moved within the target area during FRAP photobleaching, resulting in apparent spreading of the photobleached spot. Diffusion coefficients obtained using FRAP were consistent with previously reported values obtained using FCS, even for rapidly-diffusing GFP monomers (Fig. 1D). The apparent spread of the photobleached spot may result in correction of calculated diffusion coefficients. Moreover, the diffusion coefficient calculated using immobile fluorescent molecules such as H2B-GFP was associated with a large error term relative to results reported using FCS (Fig. 2B), suggesting that short FRAP photobleaching times consistent with the Soumpasis model are likely necessary when using immobile molecules for calculation of the radius of the photobleached area. Therefore, use of a photobleached radius measured from an image acquired at short latency after FRAP photobleaching in each cell expressing molecule of interest is recommended to obtain an accurate diffusion coefficient value of a rapidly-diffusing molecule of interest in living cells. This approach can be used to obtain accurate diffusion coefficients without requiring a special microscopy system for carrying out FRAP photobleaching in a minimum amount of time.

FRAP can be used to measure mobility of various intracellular molecules using only a fluorescence microscope equipped with a highly sensitive detector such as CCD camera or photomultiplier tube (PMT) and a high-power laser for photobleaching. However, it is difficult to use FRAP to calculate the number of molecules in a target region and the fluorescence intensity per molecule, both of which can be calculated using FCS. These values are required to determine the absolute concentration of a molecule and to detect the presence of homo-oligomers. FCS, rather than FRAP, is generally considered to be more suitable for measurement of high diffusion coefficients, because FCS is a well-established method for obtaining diffusion coefficients, even for small fluorescent molecules (e.g., rhodamines and Alexa Fluors dyes, which typically have diffusion coefficients > 200 $\mu m^2 \cdot s^{-1}$). On the other hand, if photobleaching effects during measurement are minimized, FRAP and FCS provide similar diffusion coefficients for molecules such as transcription factors like the glucocorticoid receptor, with diffusion coefficients of about 2 or 3 $\mu m^2 \cdot s^{-1}$ in the presence of slowly moving binding components in living cells [8]. The FRAP method we propose in this study can be applied to analysis of fluorescent molecules that exhibit diffusion coefficients < 25 $\mu m^2 \cdot s^{-1}$. However, since several models for determining diffusion coefficients using FRAP analysis have been proposed (e.g., three-dimensional diffusion models and models that take into account the movement of molecules during photobleaching), checking the diffusion coefficient of the reference fluorescent molecule (such as tandem GFP oligomers) and comparing it with the value obtained using FCS is recommended.

Conclusion

We propose a straightforward procedure for measuring diffusion coefficients of rapidly-diffusing fluorescent molecules in living cells using FRAP with an epi-fluorescence microscope. This procedure can be used to measure the diffusion coefficients of almost any fluorescently-labeled fusion protein in living cells without using FCS. The procedure provides quantitative measurement of molecular dynamics and increased apparent molecular weight due to the interaction, and is especially useful for measurement of fast-moving molecules even when a subset of these molecules may be stationary or slowly-moving. We think this approach is likely to be of widespread interest for scientists working in a variety of fields, including molecular cell biology, developmental biology, and biophysics.

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Conflicts of interest

A. K. and M. K. declare that they have no conflict of interest.

Author Contributions

A. K. directed the project and performed all experiments including analysis. A. K. and M. K. composed the entire results and co-wrote the manuscript.

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