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Multiphoton-evoked color change of DsRed as an optical highlighter for cellular and subcellular labeling

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DsRed, a recently cloned red fluorescent protein, has attracted great interest as an expression tracer and fusion partner for multicolor imaging. We report that three-photon excitation (λ <760 nm) rapidly changes the fluorescence of DsRed from red to green when viewed subsequently by conventional (one-photon) epifluorescence. Mechanistically, three-photon excitation (λ <760 nm) selectively bleaches the mature, red-emitting form of DsRed, thereby enhancing emission from the immature green form through reduction of fluorescence resonance energy transfer (FRET). The "greening" effect occurs in live mammalian cells at the cellular and subcellular levels, and the resultant color change persists for >30 h without affecting cell viability. This technique allows individual cells, organelles, and fusion proteins to be optically marked and has potential utility for studying cell lineage, organelle dynamics, and protein trafficking, as well as for selective retrieval of cells from a population. We describe optimal parameters to induce the color change of DsRed, and demonstrate applications that show the potential of this optical highlighter.

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The introduction of green fluorescent protein (GFP) as an *in vivo* reporter for subcellular localization and protein expression has revolutionized the study of cell biology[1]. However, despite the availability of red-shifted mutants, GFPs encompass only a relatively small spectral range and cannot be well separated for multilabeling studies. Recently, a red fluorescent protein (drFP583) was cloned from tropical corals[2] and is commercially available as DsRed. DsRed has an emission maximum (583 nm) well separable from that of GFP and has therefore attracted great interest as an expression marker and fusion partner for multicolor cellular imaging[2–5].

During experiments to explore the use of DsRed for multiphoton confocal imaging[6], we found that exposure to a femtosecond-pulsed laser beam caused a color change in DsRed fluorescence from red to green. Cells expressing DsRed fluoresced bright red with femtosecond excitation, not only at wavelengths (>900 nm) roughly twice that required for one-photon excitation[7], but also at short wavelengths (<760 nm). Red fluorescence excited by short wavelengths faded rapidly, but cells bleached in this way then appeared green when viewed by conventional (one-photon) epifluorescence. This vivid color change persisted for hours or days without apparent deleterious effects on cell viability. Whereas long-wavelength femtosecond excitation is preferable for multiphoton confocal imaging of DsRed, the red–green color change induced by short-wavelength femtosecond irradiation provides a powerful tool to optically highlight individual cells, subcellular organelles, and fusion proteins. Here we describe optimal parameters for the multiphoton imaging and "greening" of DsRed, and show examples that illustrate the technique's utility for cell isolation and tracking applications as well as for studies of cellular protein dynamics.

Results

Multiphoton imaging of DsRed. We used a femtosecond infrared laser and a video-rate scanning confocal microscope for multiphoton imaging of mammalian cell lines transiently transfected with DsRed 24–72 h before observation. As the laser was tuned to wavelengths between 960 and 710 nm, a strong red fluorescence was observed at λ >900 nm (Fig. 1A), corresponding to roughly twice the wavelength required for one-photon excitation[4,7,8]. Fluorescence declined between 880 and 810 nm, but increased strongly again at λ <780 nm. At both short (750 nm) and long (950 nm) wavelengths, fluorescence emission increased as about the second power of laser intensity, consistent with two-photon excitation (Fig. 1B), although the power function was slightly steeper at the longer wavelength (1.96 ± 0.08 at 950 nm, 10 cells; 1.50 ± 0.04 at 750 nm, 18 cells).

DsRed fluorescence bleached more rapidly with two-photon excitation at 750 nm than at 950 nm (Fig. 1C). Therefore, the longer wavelength is more appropriate for multiphoton imaging of DsRed. During 750 nm excitation, the red fluorescence decayed exponentially (Fig. 1D, inset), with a rate constant that increased as a third-power function of laser intensity (2.98 ± 0.10, 134 cells; Fig. 1D). Thus, whereas fluorescence excitation at 750 nm is a two-photon process, bleaching involves three-photon absorption.

"Greening" of DsRed. DsRed-expressing cells that were bleached by three-photon absorption displayed a vivid green fluorescence when subsequently viewed by conventional (one-photon) epifluorescence microscopy. We obtained images of two adjacent fibroblasts expressing cytosolic DsRed, using a standard fluorescein isothiocyanate (FITC) filter cube (420–480 nm excitation; Fig. 2A). Both cells initially appeared orange-red, but selective exposure of the upper cell to femtosecond laser light (λ = 750 nm) changed it to a distinctive green. Emission spectra of DsRed expressed in a single mammalian cell revealed that "greening" involved both a diminution of red emission (λ = 560–650 nm) to ~15%, and an enhancement of pre-existing green fluorescence (λ = 510–550 nm) to ~225% (Fig. 2B). By restricting the area of the laser scan, cell-specific greening could be accomplished even within a confluent monolayer, providing a means to mark selected cells for subsequent identification (Fig. 2C). Laser exposure resulted in discrete populations of red and

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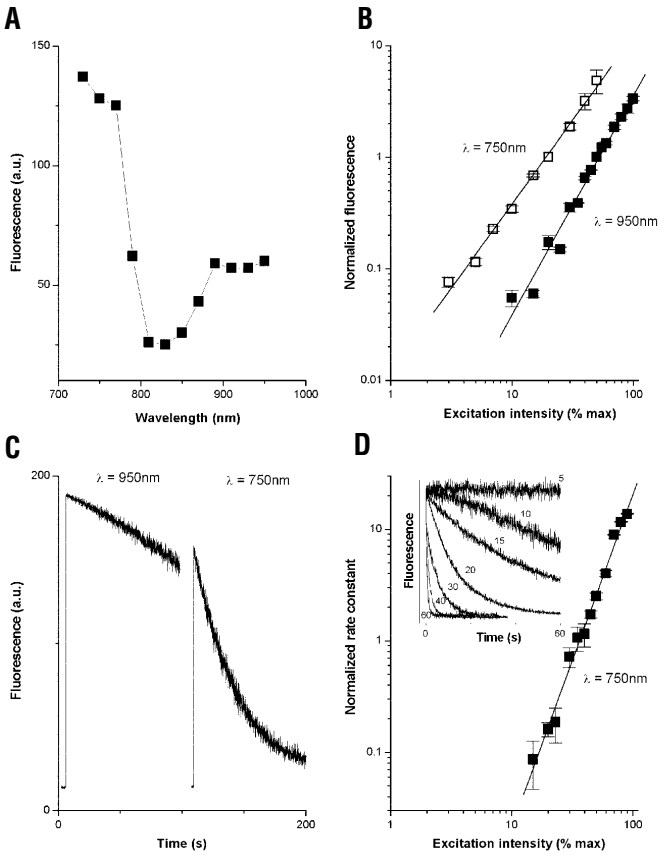

Figure 1. Multiphoton excitation and bleaching of red fluorescence of DsRed. (A) Red fluorescence emission (560–650 nm) from a DsRed-expressing CHO cell as a function of femtosecond laser excitation wavelength (laser irradiance of 16 μW/μm^2 throughout). Curve representative from n = 3 cells. (B) Power-law dependence of normalized red fluorescence emission on relative laser intensity at wavelengths of 750 and 950 nm (n ≥10 cells). The slopes of the regression lines on double-logarithmic coordinates correspond to the power function ($y \alpha x^a$) by which fluorescence increases with laser intensity. (C) DsRed red fluorescence bleaches slowly with long-wavelength (950 nm) multiphoton excitation, but rapidly at short wavelengths (760 nm). The laser power was reduced to 16% after tuning the laser to 750 nm, to excite comparable fluorescence at each wavelength. (D) Inset, superimposed traces of normalized red fluorescence from different CHO cells during continued exposure to the indicated laser powers (760 nm; in microwatts per square micron). Double-logarithmic plot shows the normalized rate constant of fluorescence decay, derived from single-exponential fits to individual traces like those in the inset (n = 134 cells).

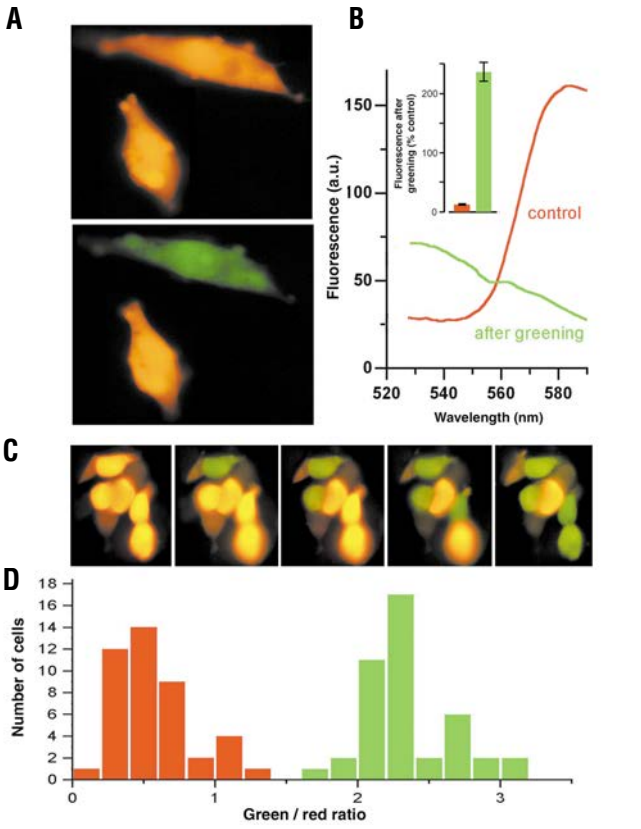

Figure 2. DsRed-expressing cells display vivid green fluorescence after three-photon photobleaching. (A) Top panel, control image of 3t3 fibroblasts expressing DsRed, photographed by conventional epifluorescence (FITC filter cube). Bottom panel, image obtained after exposing only the upper cell to femtosecond laser illumination for ~4 s (750 nm; 25 μW/μm^2). (B) "Greening" of DsRed arises both from reduced red fluorescence and enhanced green fluorescence. Plots of emission spectra (excitation 420–480 nm) from a single DsRed-expressing HEK cell before (red) and after (green) three-photon bleaching. The peak green emission is not fully resolved because of a 510 nm longpass filter in the fluorescence cube. Inset, magnitude of fluorescence changes after "greening". (C) Sequential greening of HEK cells by positioning the femtosecond laser scan in turn over individual cells. (D) "Greened" cells display a clearly resolvable fluorescence distribution, measured as the green/red fluorescence ratio before (red) and after (green) laser exposure.

"greened" cells with fluorescence distributions that could be unambiguously discriminated (Fig. 2D).

Mechanism of color change. *In vivo* and *in vitro*, the red fluorescence of DsRed takes several days to mature, passing through an obligatory green fluorescent intermediate[4,5,9]. Furthermore, DsRed appears to exist as an obligate tetramer[4,10], such that during maturation the protein consists of oligomers of both an immature, green-emitting form, and a mature, red-emitting form[4,5,9]. Therefore, with increasing time after transfection of cells with DsRed, red fluorescence increases considerably while green fluorescence shows far less of an increase (red squares, Fig. 3A). FRET occurs with high efficiency between these species[8], so that blue excitation (which excites both forms of the protein) normally results in strong red emission and weak green emission[4].

Mechanistically, the "greening" effect could result from selective bleaching of the red chromophore by three-photon excitation, thereby dequenching the green fluorescence[4], or from photoconversion of the red protein to either a new green species or back to the original, immature form. To distinguish these possibilities, we first compared the degree of "greening" in recently transfected cells (<15 h post transfection) with that in cells transfected four to five days previously. The percentage enhancement of green fluorescence following three-photon bleaching was closely similar in both populations (Fig. 3B, 152 ± 5% as compared to 159 ± 12%, respectively; n = 30 cells), despite the large increase in red fluorescence with increasing time after transfection (~35 fold, Fig. 3B)—a result that would not be expected from photoconversion. Second, we cultured DsRed-expressing bacteria to obtain sufficient protein for spectrofluorimetric analysis. Emission spectra of DsRed-expressing bacteria (red trace, Fig. 3C) showed a strong peak at ~583 nm and a weaker peak at ~495 nm. The emission and excitation spectra (Fig. 3C,D) correspond to the emission maxima of the immature and mature forms of DsRed (Fig. 3E,F). During the "greening" process, fluorescence emission progressively decreased at 583 nm, but increased at the 495 nm peak (gray traces, Fig. 3C), resulting in an ultimate ~2.4-fold enhancement of green emission and an ~6-fold decrease of red emis-

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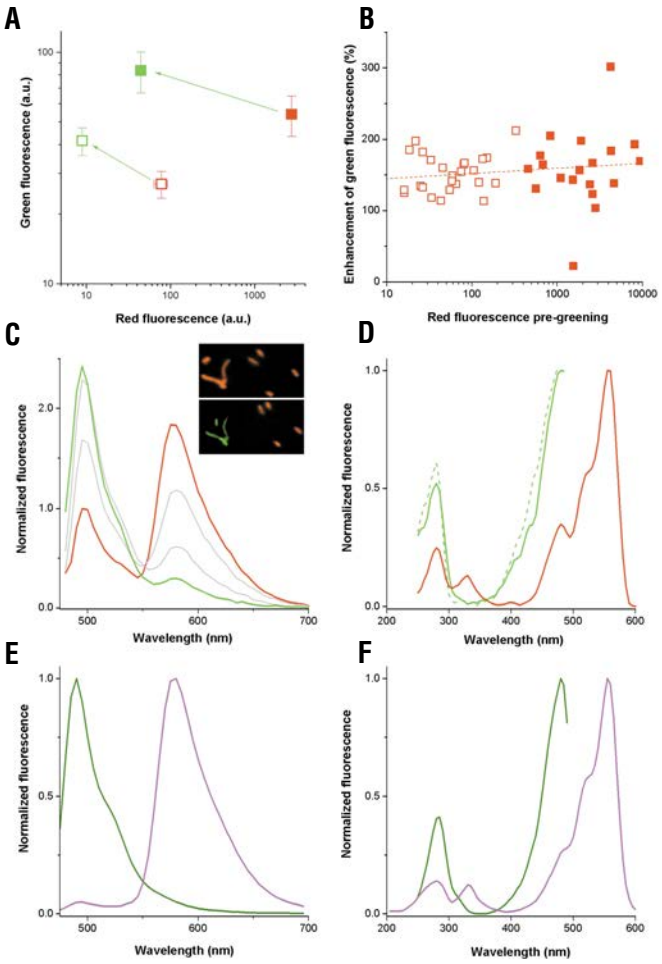

Figure 3. Mechanism of "greening". (A) Measurements of absolute red (560–650 nm) and absolute green (510–550 nm) fluorescent intensities before (red) and after "greening" (green) DsRed-expressing HEK cells transfected either <15 h (open symbols) or four to five days previously (filled symbols). Data are plotted on double-logarithmic coordinates to encompass the wide range of fluorescence values. (B) Percentage change in green emission after "greening" the two populations of cells illustrated in (A) (corresponding symbols). (C) Emission spectra depicting "greening" of DsRed. Spectra (460 nm excitation) were obtained from dilute bacterial suspensions expressing DsRed, shown normalized to the amplitudes of the highest peaks, before (red curve) and after 1 h of exposure to intense laser light (0.5 W, λ = 534 nm) to bleach the red fluorophore (green curve). Gray curves show intermediate spectra obtained at 20 min intervals during "greening". Green laser light was used because we were unable to bleach a sufficient volume for use in the spectrofluorimeter by three-photon excitation. Inset, Images of DsRed-expressing bacteria before (top panel) and after "greening" (below). (D) Excitation spectra corresponding to 620 nm emission (red curve) and 495 nm emission (dotted green curve) obtained using the unbleached sample of DsRed (red curve in part C). Solid green curve shows the excitation spectrum (495 nm emission) of the "greened" sample of DsRed (green curve in part C). (E) Emission spectra (460 nm excitation) obtained from a partially purified sample of DsRed (pink curve; predominantly the mature red form), and a diluted bacterial suspension expressing DsRed1-E5 arrested after 24 h of culture (green curve; predominantly the immature green form). (F) Corresponding excitation spectra from the immature DsRed1-E5 sample (green curve, 495 nm emission) and from the isolated DsRed protein (pink curve, 620 nm emission).

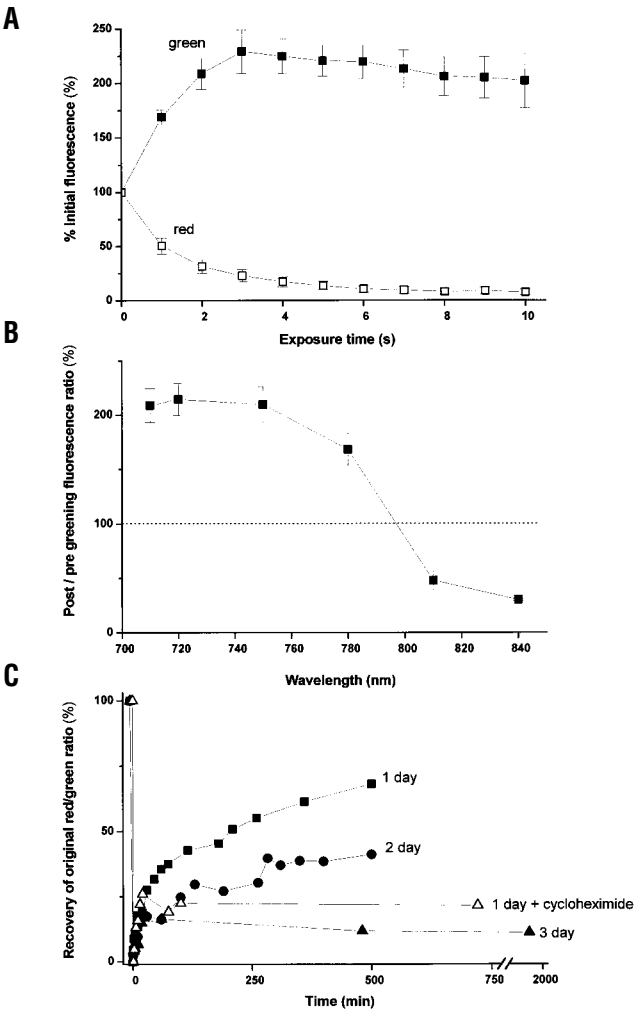

Figure 4. Optimal parameters for "greening". (A) Time course of color change. Measurements of red (560–650 nm) and green (510–550 nm) fluorescence emission (420–480 nm excitation) obtained after successive 1 s exposures to femtosecond laser illumination (750 nm; 25 μW/μm^2). Data are expressed as a percentage of values in individual CHO cells before laser exposure ($n \geq 3$ cells). (B) Maximal change in green fluorescence of DsRed after exposure of CHO cells to different wavelengths of femtosecond laser light. (C) Long-term stability of "greening". HEK cells had been transfected one day (squares), two days (circles) or three days (triangles) before. Open triangles represent one-day transfected cells, treated continuously with cycloheximide (20 μg/ml) to block protein translation beginning 4 h before imaging.

sion (green trace, Fig. 3C). The emission and excitation spectra of the enhanced green fluorescence after "greening" (green curves, Fig. 3C,D) were both similar to those of the immature form (Fig. 3E,F), further indicating that enhancement of green fluorescence arises through dequenching of a pre-existing green species rather than photoconversion of the red species.

Optimal parameters for greening. Maximal enhancement of green fluorescence was achieved by exposing cells to femtosecond laser irradiation for a sufficient time to reduce the red fluorescence to about 20% (~4 s with a laser irradiance of 25 μW/μm^2), rather than by attempting to completely bleach all red fluorescence (Fig. 4A). "Greening" was hastened by repeatedly focusing the microscope throughout the thickness of the cell while scanning the laser beam. Alternatively, because cytosolic DsRed diffuses readily, cells could be "greened" within ~10 s of exposure to a stationary laser spot of equivalent power. Commercially available multiphoton confocal microscopes with slower scan rates should serve as well as our video-rate microscope[11]. The effectiveness of the greening depended critically on the wavelength of the femtosecond irradiation. The greatest

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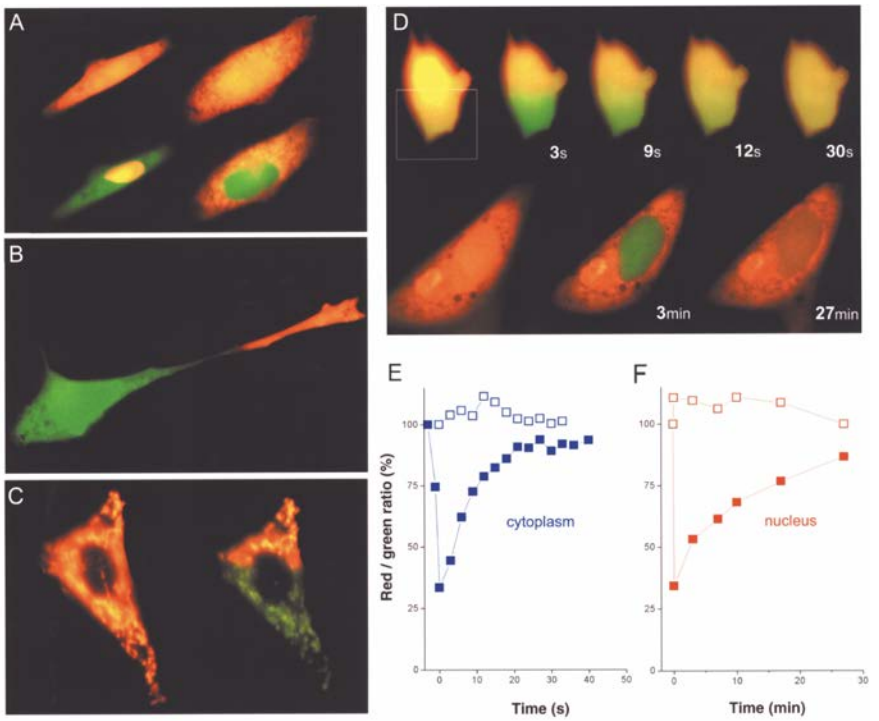

Figure 5. Applications of the greening technique for labeling subcellular regions. (A) Image pairs showing two DsRed-expressing HEK cells, before (upper) and after (lower) "greening" the cytoplasm (left) or the nucleus (right) by confining the laser scan within a 20 μm square region. (B) Slow equilibration of DsRed in cellular processes, demonstrated in a DsRed-expressing fibroblast 30 s after scanning the laser beam over the cell body but not the cellular process. (C) Subcellular "greening" of mitochondria within a 3t3 fibroblast expressing mitochondria-targeted DsRed. (D) Equilibration of DsRed within the cytoplasm (upper) and across the nuclear membrane (lower). The images show HEK cells before and at the indicated times after "greening" a defined cytoplasmic region (white box) or the nucleus. (E, F) Corresponding measurements of red/green fluorescence ratio from the "greened" regions of cytoplasm (E) and nucleus (F) (solid squares), compared to cellular regions untouched by the laser scan area (open squares).

enhancement of green fluorescence was observed with wavelengths of <760 nm, whereas wavelengths >790 nm actually reduced the green fluorescence (Fig. 4B).

To examine the long-term stability of the color change, we measured the ratio of red/green fluorescence at various times after laser exposure (Fig. 4C). Cells transfected three days previously with the DsRed construct displayed little reversion of the color change for >30 h. In contrast, cells transfected more recently showed a progressively greater regression to a yellow or red color over several hours, probably because of ongoing translation or folding of red fluorescent protein. In agreement with this interpretation, a more stable color change was observed when recently transfected cells (one day) were treated with cycloheximide to block protein translation (Fig. 4C; open triangles). Therefore, there is a trade-off between the more vivid green fluorescence produced by "greening" recently transfected cells as opposed to the better stability of the color change in cells transfected several days earlier (Fig. 4C).

Finally, "greening" was without apparent deleterious effect on cell viability. We used trypan blue dye exclusion to assess viability in human embryonic kidney 293 (HEK 293), 3t3, and Chinese hamster ovary (CHO) cells. The survival of cells that had been "greened" at varying times beforehand (≤36 h) did not differ from control red cells or nonexpressing cells (<1% of each population showed staining; data not shown).

Applications for subcellular labeling. The "greening" technique can be used to optically mark entire cells expressing soluble DsRed (see Fig. 2A,C). Additionally, the tight three-dimensional localization of three-photon excitation allows "greening" to be restricted to defined subcellular regions, including the cytoplasm or nucleoplasm (Fig. 5A,D), specific regions of the cytoplasm (Fig. 5D), and selected cellular processes (Fig. 5B). Furthermore, the use of DsRed as a genetically encoded fluorescent tag broadens the applicability of this technique to studies of fusion proteins. For example, Figure 5C demonstrates spatially defined highlighting of localized mitochondria in a cell expressing DsRed fused to a mitochondrial import sequence.

Finally, we applied the "greening" technique to quantify equilibration rates of DsRed protein within the cytoplasm and across the nuclear membrane (Fig. 5D). Whereas selective "greening" of a restricted cytoplasmic region reversed quickly (half-time of ~9 s, Fig. 5E), equilibration of "greened" DsRed protein between the nucleus and cytoplasm was ~70-fold slower, with a half-time of ~10 min (Fig. 5F).

Discussion

We report that multiphoton excitation of cells expressing the red fluorescent protein DsRed induces a vivid red–green color shift when visualized by regular (one-photon) fluorescence microscopy. This color change probably arises because the fluorescence of the green species, normally quenched by FRET, is enhanced following selective bleaching of the red species by three-photon excitation. DsRed can therefore be utilized as an optical "highlighter", with specific advantages, including the readily visible and stable (≤30 h) nature of the color change; the rapidity and three-dimensional localization afforded by multiphoton excitation; and its applicability as a genetically encoded fluorescent label in live mammalian cells. This use of DsRed expands the repertoire of available biophotonic techniques and should be particularly valuable for studying organelle dynamics, protein trafficking, and cell lineage, as well as for selective retrieval of specifically marked cells from a population.

Optically induced changes in the emission of fluorescent proteins have been reported but have not provided a practicable means of photolabeling live mammalian cells. Photoconversion of GFPs from green to red emission was reported in bacteria and yeast[12,13], but required rigorously anaerobic conditions. Recently, Baird *et al.*[4] showed that the red fluorescence of DsRed could be bleached by intense orange or UV one-photon excitation, resulting in two- to fivefold enhanced green emission. However, the long (15–60 min) exposure times required render one-photon bleaching of DsRed impractical as a tool for cell labeling.

The key to our approach is the use of three-photon excitation to achieve a rapid (a few seconds), noninjurious change in fluorescence color. The precise mechanism by which three-photon excitation rapidly and selectively bleaches the red species remains to be elucidated, but may relate to the one-photon excitation peak at 330 nm displayed exclusively by the red, but not the green form of DsRed (Fig. 3D,F). Although three-photon excitation at 750 nm nominally corresponds to one-photon excitation at 250 nm, it is often the case that multiphoton excitation is considerably blue-shifted relative to the expected multiple of the one-photon excitation peak[14]. Direct excitation with UV light is unlikely to provide a useful alternative

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because of the long exposure time required[4], poor transmission by microscope optics, and cellular photodamage. Furthermore, the inherent localization of multiphoton excitation at the focused laser spot[6] ensures that greening of DsRed is restricted to within <1 μm in three dimensions. We illustrate this by selectively greening DsRed in the nucleus and mitochondria (Fig. 5), and anticipate further applications, such as marking cells at specific depths within a tissue.

Although DsRed already provides a practical tool for live-cell photolabeling, several properties could be further optimized. One consideration is the protein's multimeric nature[4,10], which complicates applications where DsRed is fused to a host protein. A second is that the slow maturation of the green to red species[4,5] (recently utilized as the basis for a "fluorescent timer"[9]) contributes to variability in the color change resulting from "greening". It may be possible to engineer improved cellular probes that use the same principle to achieve an enhanced and more consistent color shift. For example, one could imagine a monomeric protein comprising both a rapidly maturing red fluorophore based on DsRed and a stable green fluorophore, such as a GFP or a nonmaturing DsRed mutant[4]. Such a protein would be characterized by efficient FRET between the two fluorophores, ready and selective bleaching of the acceptor (red) fluorophore by multiphoton excitation, and resistance of both fluorophores to bleaching by one-photon excitation.

Experimental protocol

Cell culture and expression of DsRed. HEK 293 cells, CHO cells, and 3t3 fibroblasts were grown (37°C, 5% CO_2) on poly-D-lysine coated Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Cells at 50–70% confluency were transfected with pDsRed1-N1 (cat. no. 6921-1; Clontech, Palo Alto, CA) or pDsRed1-Mito vector (cat no. 6928-1; Clontech) using Lipofectamine reagent (Life Technologies, Rockville, MD) and incubated for an additional 24–72 h. Then, before imaging at room temperature, cells were washed with HEPES-buffered medium (115 mM NaCl, 10 mM KCl, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25 mM HEPES, 15 mM glucose, pH 7.4 at 20°C). For measurements of cell viability, the vital stain trypan blue (0.1% solution) was used to assess plasma membrane integrity. For bacterial expression, plasmids encoding DsRed (pDsRed cat. no. 6923-1; Clontech) or DsRed1-E5 (pTimer, cat. no. 6941-1; Clontech), were transformed into chemically competent TOP10F′ *Escherichia coli* (Invitrogen, Carlsbad, CA) and positive transformants selected by plating onto ampicillin-supplemented agar.

Spectroscopic studies and cellular imaging. Fluorescence spectra were collected from aliquots of liquid cultures resuspended in PBS using a Fluorolog spectrometer with a slit width giving a resolution of 1 nm. DsRed spectra were compared with an early-stage (24 h) arrested DsRed1-E5 culture, and with a sample of DsRed protein, which was partially purified by hydrophobic affinity chromatography and exhibited predominantly red fluorescence. DsRed1-E5 is a DsRed mutant that exhibits a protracted maturation time[9] and therefore provides a convenient sample exhibiting predominantly green fluorescence.

For cellular imaging, experiments were done using an Olympus BX50 upright microscope, with a 40× water-immersion objective (numerical aperture 0.8) and standard epifluorescence system, with a 100 W mercury arc lamp and an FITC filter cube (excitation 420–480 nm; 500 nm dichroic; emission >510 nm). Color photographs were taken with a digital camera (Nikon Coolpix 990). Intensities of selected cells or defined cellular regions were derived using a photomultiplier and field aperture. Emitted fluorescence was monitored through red (560–650 nm) and green bandpass (530 ± 20 nm) filters. Fluorescence spectra from cells (Fig. 2B) were obtained using a custom prism spectrometer on the microscope photoport.

The microscope was also equipped with a custom-built video-rate multiphoton imaging system[11]. The beam from a femtosecond Ti-sapphire laser (80 fs pulses, 80 MHz repetition rate: Tsunami; Spectra-Physics, Mountain View, CA) was raster scanned at 30 frames/s, to uniformly irradiate a square (30 μm) in the microscope field. Laser power is stated as irradiance in microwatts per square micron at the specimen. All data are presented as mean ± s.e.m.

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