Feb.
2007
No. 22

CONFOCAL APPLICATION LETTER

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Using photoactivatable fluorescent proteins Dendra2 and PS-CFP2 to track protein movement with Leica TCS SP5

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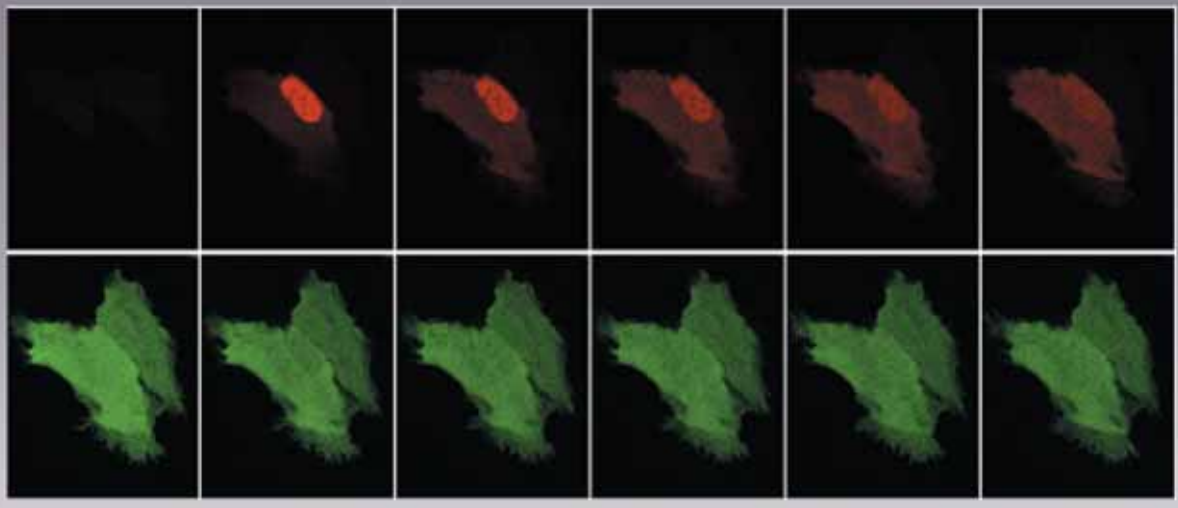

LAS AF: FRAP Wizard – Live Data Mode
Photoconversion

Leica
MICROSYSTEMS

Using photoactivatable fluorescent proteins Dendra2 and PS-CFP2 to track protein movement with Leica TCS SP5 confocal system

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Photoactivatable fluorescent proteins (PAFPs) are GFP-like proteins capable of pronounced changes in fluorescence characteristics in response to relatively intense irradiation at a specific wavelength. Today they are becoming widely popular as unique tools allowing precise photolabeling and tracking of moving objects in living systems[1]. They also open novel perspectives for high resolution imaging techniques[2, 3]. A whole palette of these proteins is available with various spectral characteristics, photobehaviour and biochemical properties (Table 1).

To label and track cells and cell organelles *in vivo*, one can potentially work with all proteins listed in Table 1. However, the choice of the photoactivatable protein for the labeling and tracking of the protein of interest is restricted to the monomeric PAFPs available (**Table 1, part a**). Indeed, the tendency to form homo-dimers and -tetramers is a particular problem of many GFP-like proteins, especially those derived from Anthozoa species, which makes them generally unsuitable for native *in vivo* protein labeling[4].

Recently two monomeric photoactivatable proteins, Dendra[5] and PS-CFP[6], which are highly suitable for generation of fusion protein constructs were developed. Both proteins are dual color, i.e. they are initially fluorescent, but switch to the spectrally different form upon photoactivation. This feature allows monitoring protein of interest redistribution in a ratiometric manner, providing maximum information. Here we report some technical recommendations and examples, based on our experience with photoactivation and tracking of enhanced versions of these proteins (Dendra2 and PS-CFP2) on the novel confocal system Leica TCS SP5.

Table 1: Photoactivatable fluorescent proteins

a. Monomeric

Protein	Activating light	Activation	Fluorescence changes	Contrast, fold	Reference
PA-GFP	UV-Violet	Irreversible	None to Green	100	7
PS-CFP2	UV-Violet	Irreversible	Cyan to Green	> 2000	6
mEosFP	UV-Violet	Irreversible	Green to Red	?	8
Dendra2	UV-Violet or Blue	Irreversible	Green to Red	> 4000	5
Dronpa	UV-Violet	Reversible	None to Green	> 100	9

b. Tetrameric.

Protein	Activating light	Activation	Fluorescence changes	Contrast, fold	Reference
Kaede	UV-Violet	Irreversible	Green to Red	2000	10
KikGR	UV-Violet	Irreversible	Green to Red	?	11
asulCP (asCP, asFP595)	Green	Reversible	None to Red	70a	12
asulCP-148G	Green	Reversible	None to Red	70a	13
KFP-Red	Green	Reversible, Irreversible	None to Red	35b	14

[a]Contrast is given for the reversibly activated form.
[b]Contrast is given for the irreversibly activated form.

Dendra2

General properties

Dendra2 is a dual-color monomeric photoswitchable fluorescent protein, which can be irreversibly photoconverted from a green to a red fluorescent form using intense violet or blue light irradiation. Upon Dendra2 photoconversion, green fluorescence decreases 10-15 fold and the red fluorescence increases 150-300 fold. The total increase in the red-to-green fluorescence ratio results in more than a 4000-fold contrast (**Figure 1**, **Table 1 and 2**). Considerable decrease of the green fluorescence simultaneously allows both tracking the movement of the activated protein and its replacement with the non-activated form. Dendra2 efficiently matures both at 20ºC and 37ºC, and thus can be applied both to common cultured mammalian cell systems and to experiments in cold-blooded animals.

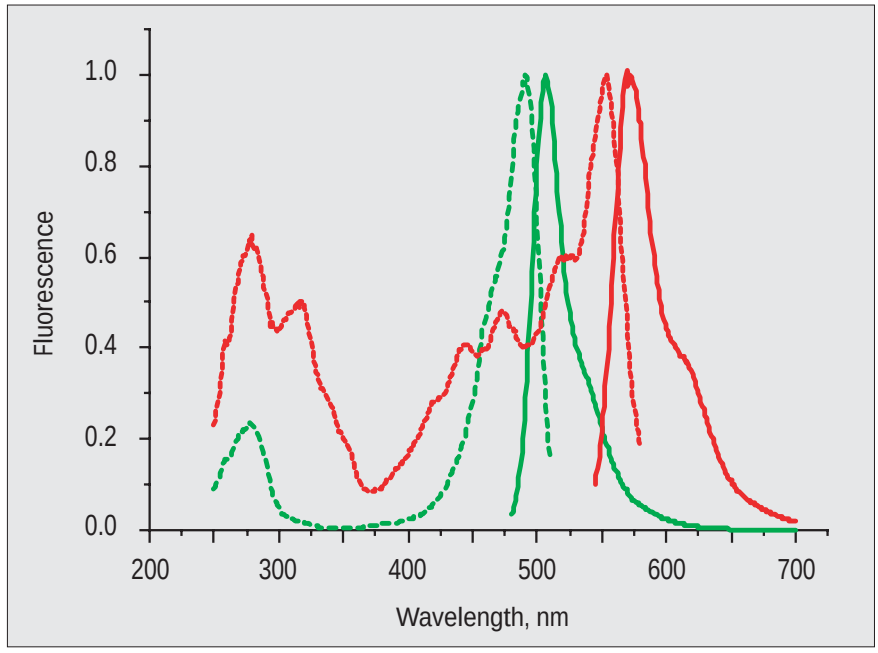


Figure 1: Excitation (dashed lines) and emission (solid lines) spectra for non-activated (green lines) and activated (red lines) Dendra2 forms.

Imaging prior to photoactivation

Since the initial spectral characteristics of Dendra2 are quite close to those of common green fluorescent proteins, it can also be visualized in the same GFP or FITC filter sets. However, importantly, excitation with common arc lamp easily photoconverts Dendra2 to the activated, red fluorescent variant. Therefore, it can be generally recommended to avoid prolonged and/or bright arc lamp irradiation for preliminary visualization of Dendra2 expressing cells.

The most convenient way to find the focus position is to use white light in a bright field mode. Later one can switch to the confocal mode and employ excitation with 488 nm laser line of low or moderate intensity in a fast scanning mode, emission collected at 500-550 nm, to look for the appropriate cells, to check protein localization and to choose the region of interest for the photoactivation. Such irradiation doesn't cause noticeable photobleaching or photoactivation of Dendra2.

Table 2: Dendra2 Properties

Characteristic	before activation	after activation
Fluorescence color	green	red
Excitation maximum	490 nm	553 nm
Emission maximum	507 nm	573 nm
Quantum yield	0.50	0.55
Extinction coefficient, M-1cm-1	45,000	35,000
Brightness*	0.68	0.58
pKa	6.6	6.9
Structure	monomer	
Activating light	UV-violet (e.g. 405 nm) or blue (e.g., 488 nm)	
Contrast, fold	>4000	

*Comparing to EGFP.

Photoactivation

Violet (as 405 nm laser line) light irradiation causes fast and effective photoconversion of Dendra2 (see **Examples**). Adopting the irradiation conditions for the photoactivation, one should keep in mind that the real irradiation dose depends on many parameters, including percentage of laser intensity set by AOTF, scanning frequency, resolution format (voxel resolution) and zoom factor. Obviously, while insufficient irradiation will result in low efficiency of photoactivation, too high levels can lead to overwhelming photobleaching effects, as well as to definitely undesirable phototoxic effects which can be caused by the violet 405 nm laser line[15].

In contrast to other irreversibly photoactivatable proteins, Dendra2 can be activated not only by a violet, but also by a blue light. This characteristic is a beneficial property, since blue light is less harmful for the cells. However, photoactivation by the blue light is less effective and strictly requires continuous irradiation. For example, activation using common GFP or FITC excitation filters, blue light of the arc lamp is effective. In the confocal mode, 488 nm laser lines can be used for the photoactivation as well, but requires long pixel dwell times, which can be achieved by point bleaching or by using the slowest available scanning rate.

Visualization after photoactivation

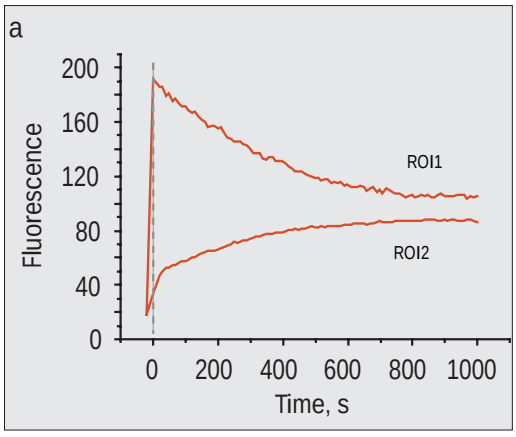
The photoconverted Dendra2 type is red fluorescent with excitation-emission maxima at 553 and 573 nm (**Figure 1**), respectively. Common TRITC filter sets or similar can be used for the activated Dendra2 visualization. With a confocal microscope, the red fluorescent signal can be acquired using 532 nm, 543 nm or 561 nm excitation laser lines and detected at 560-700 nm (or 570-700 nm for the 561 nm excitation laser line). Importantly, photoconverted Dendra2 is characterized with rather high photostability (more than 3 times higher comparing to DsRed). This property allows visualizing long time series and/or to track protein movement with a high scan frequency (see **Examples 1 and 2**). In general, high contrast and photostability of the red photoactivated signal allow monitoring of protein movement events precisely in various time scales.

While tracking of the activated red signal redistribution gives direct information concerning protein of interest movement, replacement of the photoconverted Dendra2 with the non-activated green variant is also informative, and can be monitored analogous to the commonly used FRAP technique. Indeed, upon photoconversion, green fluorescence of the initial Dendra2 decreases multi-fold. This green form can be continuously visualized both before and after photoactivation using excitation with 488 nm laser line of low intensity, emission collected at 500-550 nm.

Importantly, while photoactivation of even a minor portion of the dual color photoswitchable protein can provide bright and contrasting red fluorescent signal, one has to activate in a chosen region a significant portion of the protein pool to allow informative tracking of this pool replenishment, similar to the photobleaching recovery measurement. In Example 1, only a minor portion of protein was photoconverted, and therefore only minor changes in green fluorescence are observed (**Figure 2b**). Contrary to this, significant portions of protein were photoconverted in the **Examples 2 and 4**, and thus the replenishment of the pool can be tracked (**Figure 3d, Figure 6a,b**).

Sequential or simultaneous imaging of the two Dendra2 forms

As can be concluded from the spectra (**Figure 1**), green fluorescence of the initial Dendra2 form gives minor emission crosstalk with red fluorescence of the photoactivated Dendra2. This can be overcome by using sequential excitation scanning modes for the accurate separation of the two signals. However, dual channel sequential scanning requires twice as much image acquisition time compared to simultaneous scan. Therefore, for the faster dual color tracking of Dendra2, one can neglect this minor crosstalk and employ simultaneous excitation with 488 nm and 561 nm laser lines to visualize both protein forms redistribution, as shown in examples.



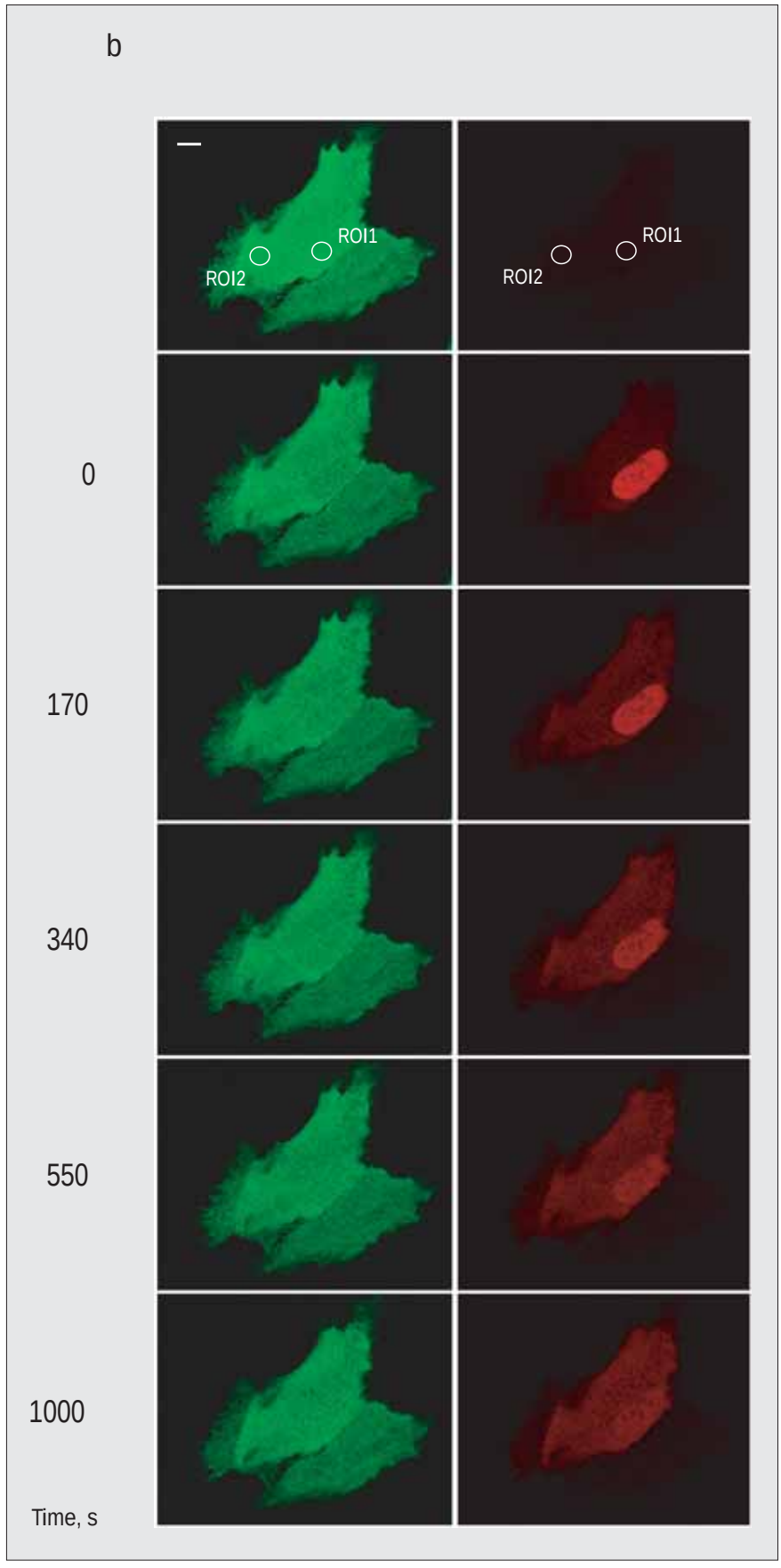


Figure 2:
Tracking redistribution of the photoactivated Dendra2 between the nucleus and cytosol.
a. Time course of the red fluorescent signal redistribution. Time zero is set at the commencement of photoactivation. Scale bar, 10 µm.
b. selected snapshots of the tracking series in both channels. Time, in seconds, is shown on the left.

Example 1:

Tracking Dendra2 redistribution between the nucleus and cytosol of HeLa cell.

HeLa cells transiently transfected with pDendra2-N vector (Evrogen) were monitored 18 h after transfection with an HCX PL APO 63x/1.4 objective. Fast confocal scanning (1% of 488 nm laser line intensity, emission collected at 495-553 nm) was used for preliminary visualization of green fluorescence of Dendra2.

Photoactivation was performed in a FRAP wizard, with 48% of 405 nm diode laser line intensity, 1 scan in "zoom to ROI" mode (zoom 17.5). A further time series (1 frame per 10 s) was captured using simultaneous imaging in two channels, to monitor the redistribution of both signals (**Figure 2**).

The parameters were adopted for the tracking of slow Dendra2 redistribution.

Example 2:

Tracking Dendra2-fibrillarin fusion redistribution within and between nucleoli of HeLa cell nucleus.

HeLa cells transiently transfected with pDendra2-Fibrillarin vector (Evrogen) were monitored 18 h after transfection, with an HCX PL APO 63x/1.4 objective. Fast confocal scanning (1% of 488 nm laser line intensity, emission collected at 495-553 nm) was used for preliminary visualization of green fluorescence of Dendra2.

Photoactivation was performed in a FRAP wizard, with 15% of 405 nm diode laser line intensity, 2 scans 3x frame average, 6 scans totally in bi-directional scan mode, approximately 3 s). "Zoom to ROI" mode (zoom 64x). A further time series (1 frame per 4 s) was captured using simultaneous imaging in two channels, to monitor the redistribution of both signals (**Figure 3**).

The parameters were adopted for the medium speed acquisition.

Example 1:

	First channel	Second channel
Scan format	1024 x 1024 pixels	1024 x 1024 pixels
Zoom	1.8x	1.8x
Pixel size	133.9 x 133.9 nm	133.9 x 133.9 nm
Pinhole size	1 Airy unit	1 Airy unit
Scan speed	400 Hz, bidirectional scan	400 Hz, bidirectional scan
Number of average	2 x frame, 3 x line	2 x frame, 3 x line
Laserline / AOTF	488 nm / 1%	561 nm / 7%
Gain / offset	1131 / 0 Volt	1250 / 0 Volt
Detection range	495-553 nm.	570-760 nm

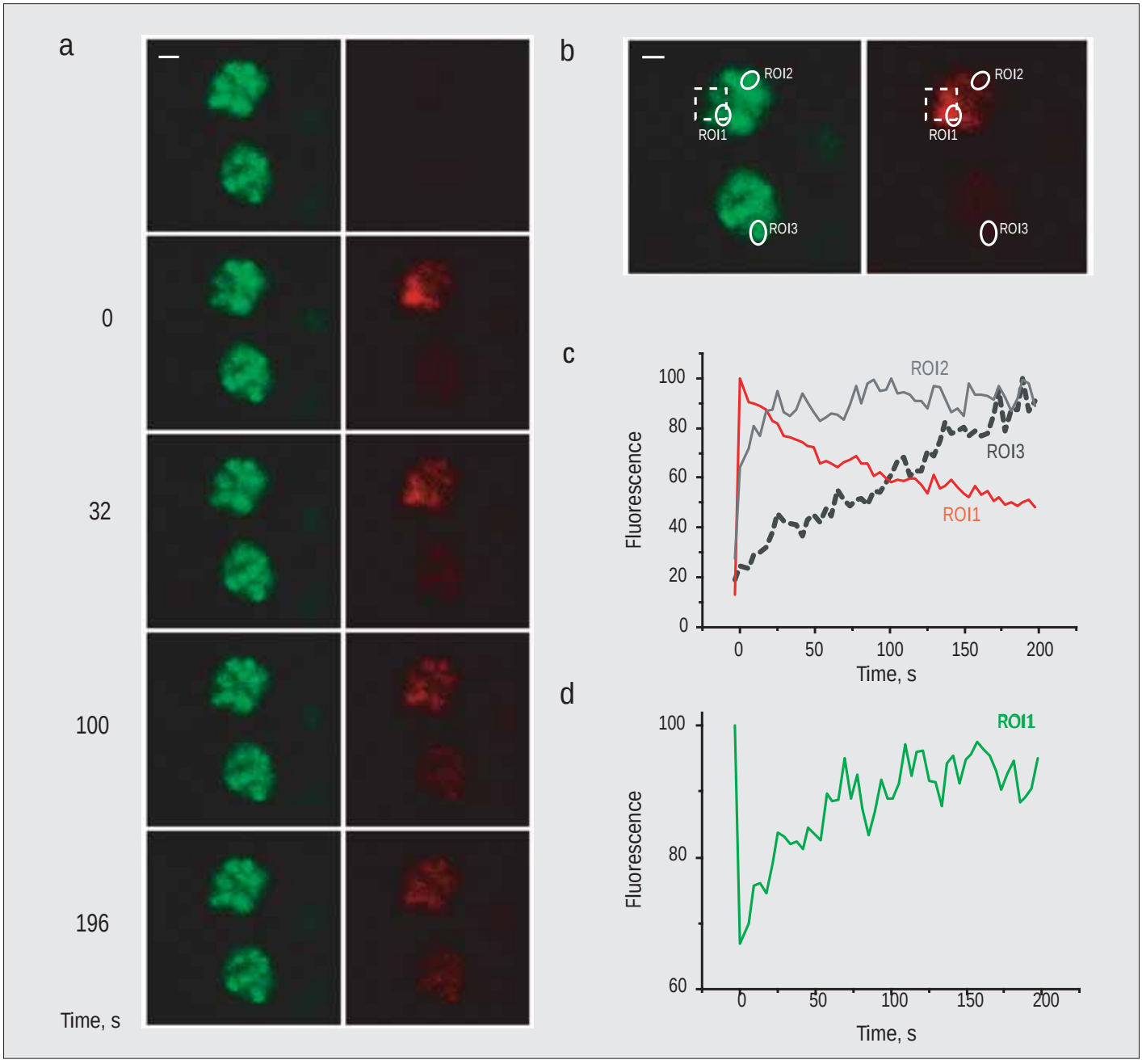
Example 2:

	First channel	Second channel
Scan format	512 x 512 pixels	512 x 512 pixels
Zoom	19x	19x
Pixel size	25.2 nm x 25.2 nm	25.2 nm x 25.2 nm
Pinhole size	1.67 Airy unit	1.67 Airy unit
Scan speed	400 Hz, bidirectional scan	400 Hz, bidirectional scan
Number of average	3 x frame	2 x frame
Laserline / AOTF	488 nm / 1%	561 nm / 4%
Gain / offset	1100 / 0 Volt	1250 / 0 Volt
Detection range	495-555 nm	575-760 nm

Figure 3:
Dendra2-fibrillarin fusion redistribution within and between nucleoli of HeLa cell nucleus.
a. Selected snapshots of the tracking series in both channels. Two nucleoli of a single HeLa cell nucleus are shown. Time, in seconds, is shown on the left. Scale bar, 1 µm.
b. Regions of interest tracked: ROI1 – region within the photoactivated field; ROI2 – intact region in the same nucleoli; ROI3 – intact region in the neighboring nucleoli. ROIs were selected to include the whole nucleoli substructures, taking into account their minor movement during the experiment. Photoactivated field is shown with a dashed square.
c,d. Normalized time course of the red (c) and green (d) fluorescent signals redistribution. Time zero is set at the commencement of photoactivation. Scale bar, 1 µm.

Availability

Vectors encoding Dendra2 are commercially available from Evrogen (http://www.evrogen.com/Dendra2.shtml)



PS-CFP2

General properties

PS-CFP2 is a high-contrasting monomeric dual-color photoactivatable fluorescent protein, which can be irreversibly photoconverted from a cyan to a green fluorescent variant by violet light (as 405 nm laser line) irradiation. Photoconversion results in a 5.5 fold decrease of cyan fluorescence and more than 400-fold growth of the 490 nm excited green fluorescence with emission maximum at 511 nm. Therefore, total increase in the green-to-cyan fluorescence ratio reaches more than 2000-fold. Comparing to the most famous photoactivatable protein, PA-GFP, PS-CFP2 is characterized with essentially higher contrast (Table 3). As well as Dendra2, ratiometric fluorescent changes allow to track both the movement of the photoactivated protein and its replacement with the non-activated variant.

Fluorescent characteristics of the initial cyan PS-CFP2 variant are completely different to those of cyan fluorescent proteins as ECFP or Cerulean, for which the blue shift is determined by Y66W mutation in the chromophore. In contrast, PS-CFP2 carries a common GFP chromophore, though characterized with fully protonated state and slowed excited state proton transfer[16]. This results in excitation/emission peaks of PS-CFP2 located at unique 400/468 nm. Therefore, initial PS-CFP2 cyan fluorescence can be easily and perfectly separated from 490 nm-excited green fluorescence of its photoactivated signal, as well as the fluorescence from common green fluorescent proteins (Figure 4).

Imaging prior to photoactivation

Remarkably, for PS-CFP2 it was shown that illumination by the moderate intensity violet light from the arc lamp causes neither significant protein photoactivation, nor photobleaching. Moreover, PS-CFP2 was recommended as a photostable blue fluorescent label, which is easy to separate with common green fluorescent proteins [17].

Therefore, PS-CFP2 does not restrict the scientist in the preliminary visualization of the protein localization, using appropriate filter sets. Based on the excitation spectra of PS-CFP2 and keeping in mind the undesirable crosstalk with excitation spectra of green fluorescent proteins (as well as photoactivated PS-CFP2 variant), it can be recommended to employ an excitation range from 350-410 nm, which includes 365 and 405 nm peaks of the mercury lamp spectra and to collect emission from 430 nm to 500 nm (or even up to 550 nm to gain the full PS-CFP2 brightness). In general, filter sets that are used for DAPI visualization can be recommended for PS-CFP2 (Table 4).

The initial PS-CFP2 variant can be visualized in a confocal mode as well, using 405 nm laser line excitation, emission colleted at 420-500 nm or up to 420-550 nm for the maximum brightness. However, it is recommended to minimize 405 nm laser light intensity and use high scanning speed and low resolution scanning to avoid undesirable photoconversion during visualization.

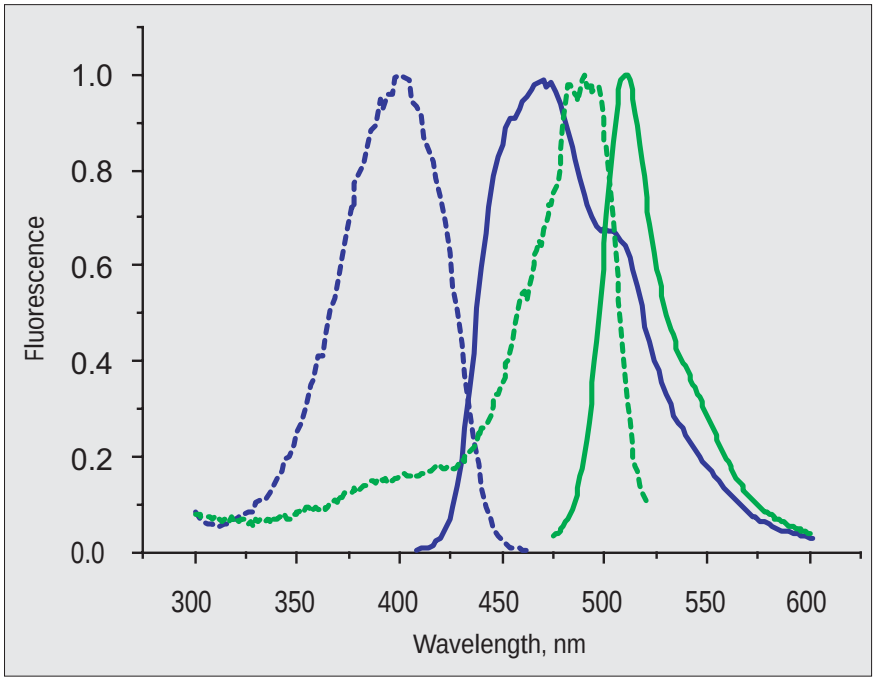


Figure 4: Excitation (dashed lines) and emission (solid lines) spectra for non-activated (cyan lines) and activated (green lines) PS-CFP2 variants.

Table 3: PS-CFP2 properties in comparison with PA-GFP

Characteristic	PS-CFP2		PA-GFP	
	before activation	after activation	before activation	after activation
Fluorescence color	cyan	green	green	green
Excitation maximum	400 nm	490 nm	400 nm	504 nm
Emission maximum	468 nm	511 nm	515 nm	517 nm
Quantum yield	0.20	0.23	0.13	0.79
Extinction coefficient, M-1cm-1	43,000	47,000	20,700	17,400
Brightness*	0.26	0.33	0.08	0.42
pKa	4.3	6.1	4.5	–
Structure	monomer			
Activating light	UV-violet (405 nm laser line)			
Contrast, fold	>2000		>100	

*Compared to EGFP

Table 4: Filter sets recommended for cyan PS-CFP2 variant visualization:

Company	Optimal filter sets	Suitable filter sets
Omega Optical:	XF119-2, XF131	XF06, XF03, XF11, XF129-2, XF05-2
Semrock:	DAPI-5060B	DAPI-1160A
Chroma:	31037, 31041	31016, 31021, 31000v2, 31009v2, 31013v2, 11005v2, 31047

Photoactivation

PS-CFP2 can be effectively photoactivated by 405 nm laser line irradiation. In general, all considerations described for the Dendra2 photoactivation above are equally applicable to the photoactivation of PS-CFP2. The final decision on the laser intensity used for the photoactivation will depend on the frequency of scanning, pixel resolution and zoom factor (see **Examples 3, 4**).

Tracking

Due to the emission crosstalk, simultaneous imaging of the initial cyan and photoactivated green variants of PS-CFP2 is inapplicable (see **Figure 4**). Therefore, to visualize both protein variants (see **Figure 5**) without the cross-talk, and to get the full advantages of the dual color ratiometric imaging of the photoswitchable protein redistribution, sequential excitation by 405 and 488 nm laser lines in a sequential scanning mode should be applied, as shown in **Example 3**. (It is fair to say that similar dual channel tracking was also quite possible for PA-GFP in our experience. Visualization of initial 405 nm excited PA-GFP variant in a confocal mode with minimized violet laser intensity didn't cause significant protein photoactivation. The brightness of the non-activated cyan PS-CFP2 variant was notice-ably higher in equal conditions.)

Alternatively, for the faster image acquisition, single tracking in the 488 nm excited green fluorescence channel can be employed, after the preliminary visualization of cyan fluorescent variant, as shown in **Example 4** (see **Figure 6**).

For the visualization of the photoactivated PS-CFP2 variant using arc lamp excitation, common filter sets used for GFPs or FITC visualization can be recommended. Importantly, excitation wavelengths must not be below 450 nm to avoid cross-excitation of non-photoactivated PS-CFP2.

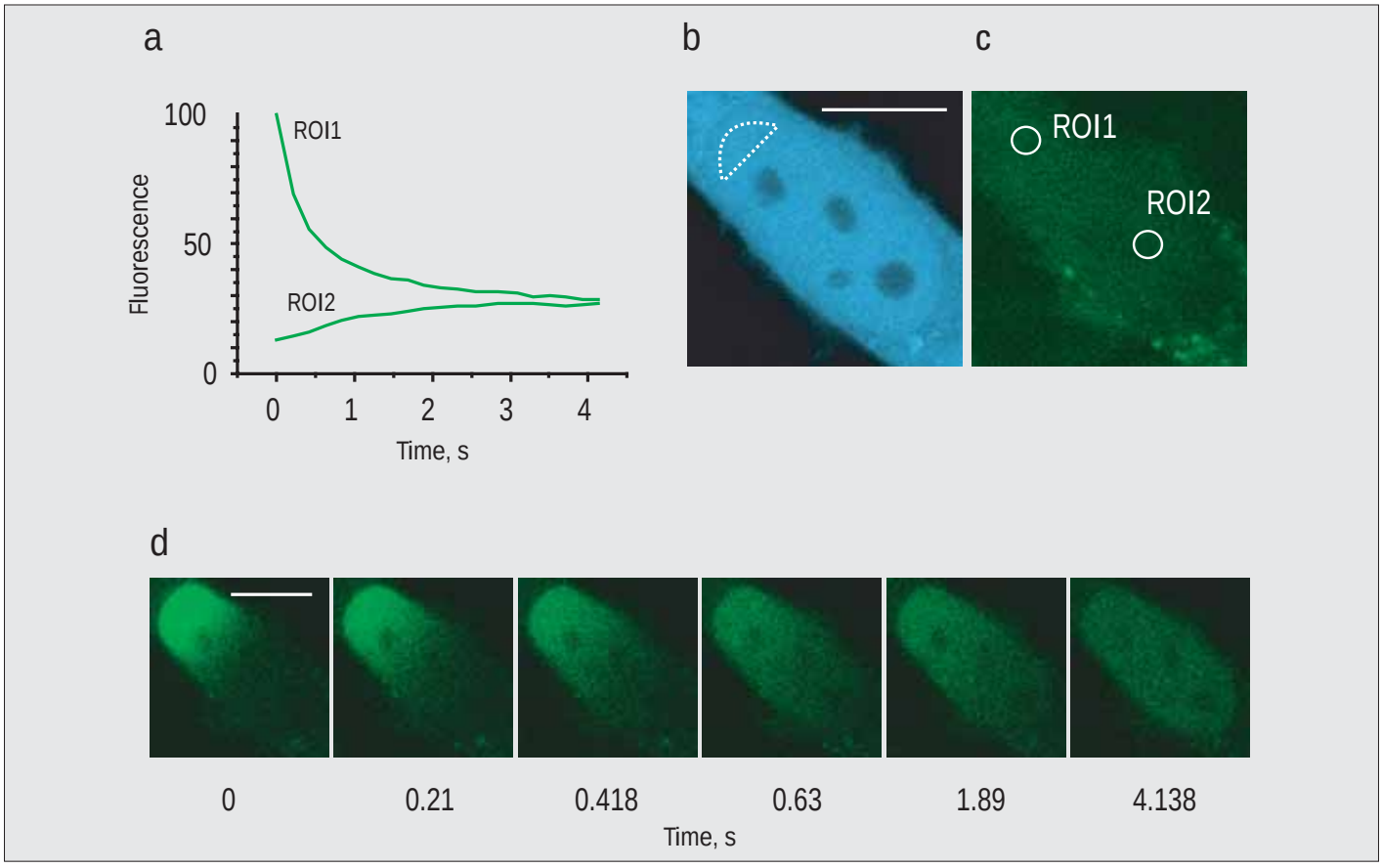
Example 3: Tracking PS-CFP2 redistribution within HeLa cell nucleus.

HeLa cells transiently transfected with pPS-CFP2-N vector (Evrogen) were monitored 18 h after transfection with an HCX PL APO 63x/1.40 objective. Preliminary visualization of cyan fluorescence of PS-CFP2 can be accomplished in two ways: With an UV lamp and a Leica filter cube A (excitation 340-380, emission 424LP) or by fast confocal scanning (3% of 405 nm laser line intensity, emission collected at 420-500 nm).

Photoactivation was performed with the FRAP wizard of the LAS-AF, with 100% 405 nm diode laser line intensity, single scan in "zoom to ROI" mode. A further time series was captured with 1 frame per 220 ms to monitor the redistribution of the photoactivated green signal. The following parameters were adopted for the fast protein movement tracking:

Scan format	256 x 256 pixels
Zoom	11,1x
Pixel size	86.6 x 86.6 nm
Pinhole size	1.41 Airy unit
Scan speed	700 Hz, bidirectional scan
Laserline / AOTF	488 nm / 4%
Gain / offset	1232 / 0 Volt
Detection range	500-550 nm

Figure 5:
Tracking redistribution of the photoactivated PS-CFP2 within the nucleus.
a. Time course of the redistribution of green fluorescence for ROI1 and ROI2 after photoactivation. Region of photoactivation is shown with dashed figure. Scale bar, 10 µm.
b. Cyan fluorescence before photoactivation.
c. Green fluorescence before photoactivation.
d. Selected snapshots of the tracking series. Time zero is set at the commencement of photoactivation. Scale bar, 10 µm.



Photostability of the photoactivated PS-CFP2 variant

Similarly to some YFPs, photobleaching of the photoactivated PS-CFP2 green fluorescent variant is characterized with two components. The first component, which is about 40% of total protein brightness, is characterized with relatively low photostability, but reversible nature (i.e. in the dark it comes back to the initial level of fluorescence brightness within seconds). Therefore, to gain maximum brightness it can be recommended to increase the interval between images obtained in time series. The remaining signal is characterized with much higher photostability, and allows protein visualization upon prolonged excitation. Still, it is preferable to minimize the frequency of images acquisition within a time series. Taking too many images in a large time series leads to undesirable photobleaching of the photoactivated protein variant.

Availability

Vectors encoding PS-CFP2 are commercially available from Evrogen
http://www.evrogen.com/PS_CFP2.shtml

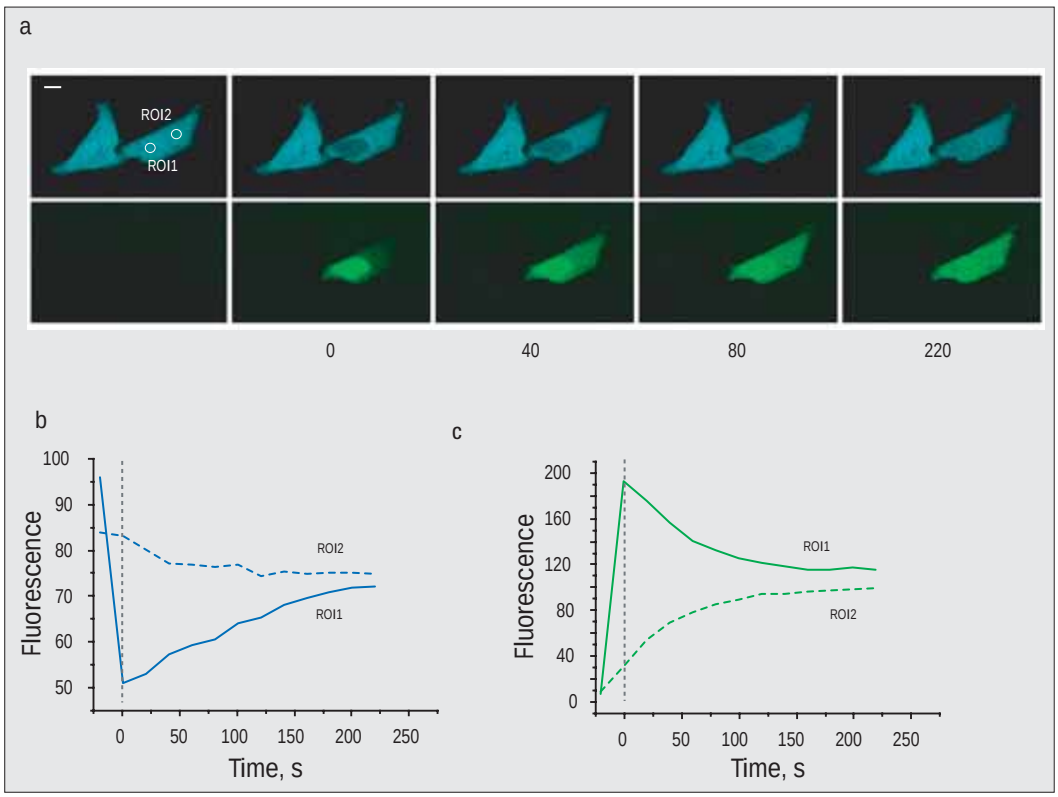
Example 4: Tracking PS-CFP2 redistribution between the nucleus and cytosol of HeLa cell.

Similarly, HeLa cells transiently transfected with pPS-CFP2-N vector (Evrogen) were monitored 18 h after transfection with an HCX PL APO 63x/1.40 objective. Preliminary visualization of cyan fluorescence of PS-CFP2 can be accomplished in two ways: With an UV lamp and a Leica filter cube A (excitation 340-380 nm, emission 424LP) or by fast confocal scanning (3% of 405 nm laser line intensity, emission collected at 420-500 nm).
Here photoactivation was performed using the Live Data Mode, with sequential scanning in time series. Photoactivation was performed with 30% 405 nm diode laser line intensity, 4 scans in "zoom to ROI" mode, programmed as Job1 and started in Live Data Mode.

A further time series (1 frame per 20 s) was captured using the following parameters:

	First channel	Second channel
Scan format	512 x 512 pixels	512 x 512 pixels
Zoom	1.7x	1.7x
Pixel size	282.7 nm x 282.7 nm	282.7 nm x 282.7 nm
Pinhole size	2.3 Airy unit	2.3 Airy unit
Scan speed	400 Hz, bidirectional scan	400 Hz, bidirectional scan
Number of average	2 x frame, 2 x line	2 x frame, 2 x line
Laserline / AOTF	405 nm / 3%	488 nm / 4%
Gain / offset	1100 / 0 Volt	1137 / 0 Volt
Detection range	420-500 nm	500-550 nm

Figure 6. Tracking redistribution of the photoactivated PS-CFP2 between the nucleus and cytosol.
a. Selected snapshots of the tracking series in both channels. Time, in seconds, is shown on the bottom.
b. Time course of the cyan fluorescent signal changes for ROI1 and ROI2.
c. Time course of the activated green fluorescent signal changes for ROI1 and ROI2.
Time zero is set at the commencement of photoactivation. Scale bar, 10 µm.



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