Supplementary Figures and Tables

A reversibly photoswitchable GFP-like protein with fluorescence excitation decoupled from switching

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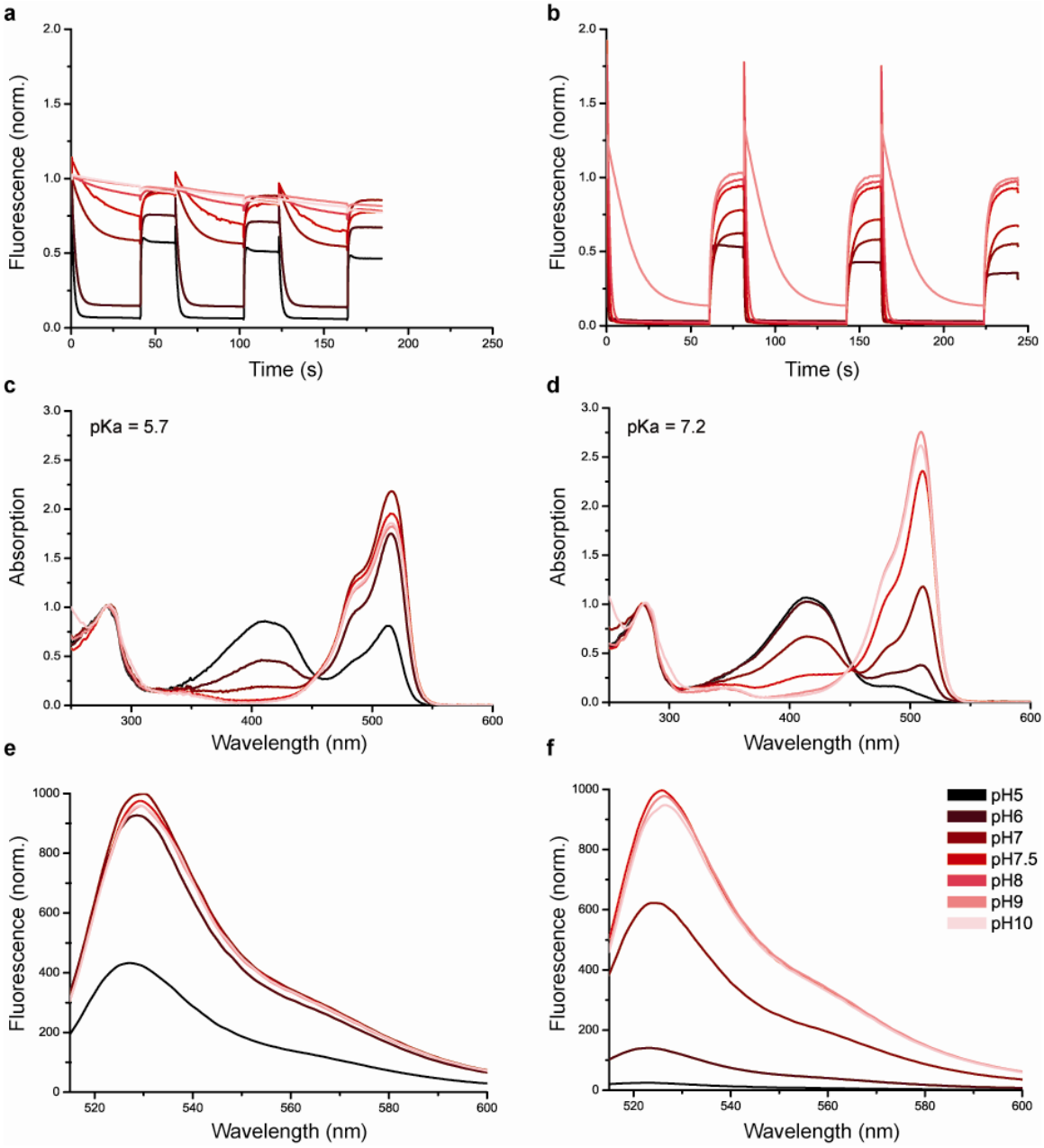

Supplementary Fig. 1 pH influence on Dreiklang and Citrine. (**a,c,e**) Citrine. (**b,d**,**f**) Dreiklang. (**a,b**) Influence of the pH on the light driven switching. Switching was performed as given in **Supplementary Table 1**. Note that the initial increase in fluorescence signal due to irradiation with 405 nm is likely caused by excited state proton transfer, as commonly observed in RSFPs. (**c,d**) Absorption spectra of the equilibrium states at the indicated pH values. (**e,f**) Fluorescence emission spectra at different pH values. The absorption and the emission spectra were normalized to the absorption at 280 nm, which corresponds to the protein concentration. Note, that switching at pH 5.0 is not shown in (b), because the detected fluorescence signal was too low and that a pH of 7.5 was only used in (**a,b**).

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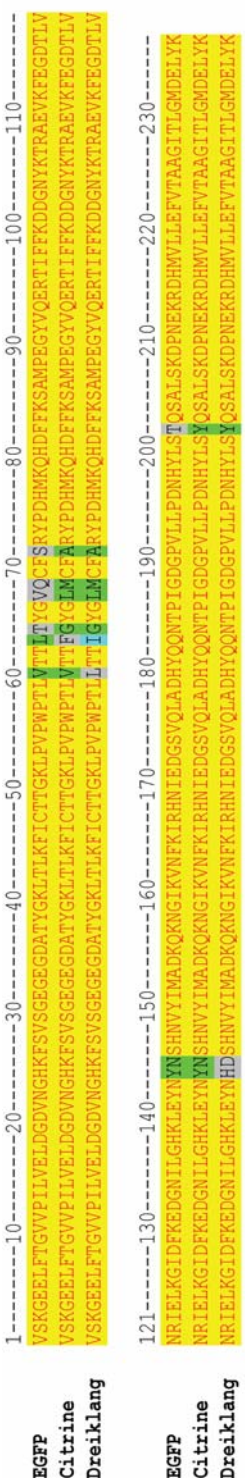

Supplementary Fig. 2 Amino acid alignment of EGFP (Clontech, Madison, WI), Citrine and Dreiklang. Identical amino acid residues are depicted in yellow; differences are highlighted in grey or green.

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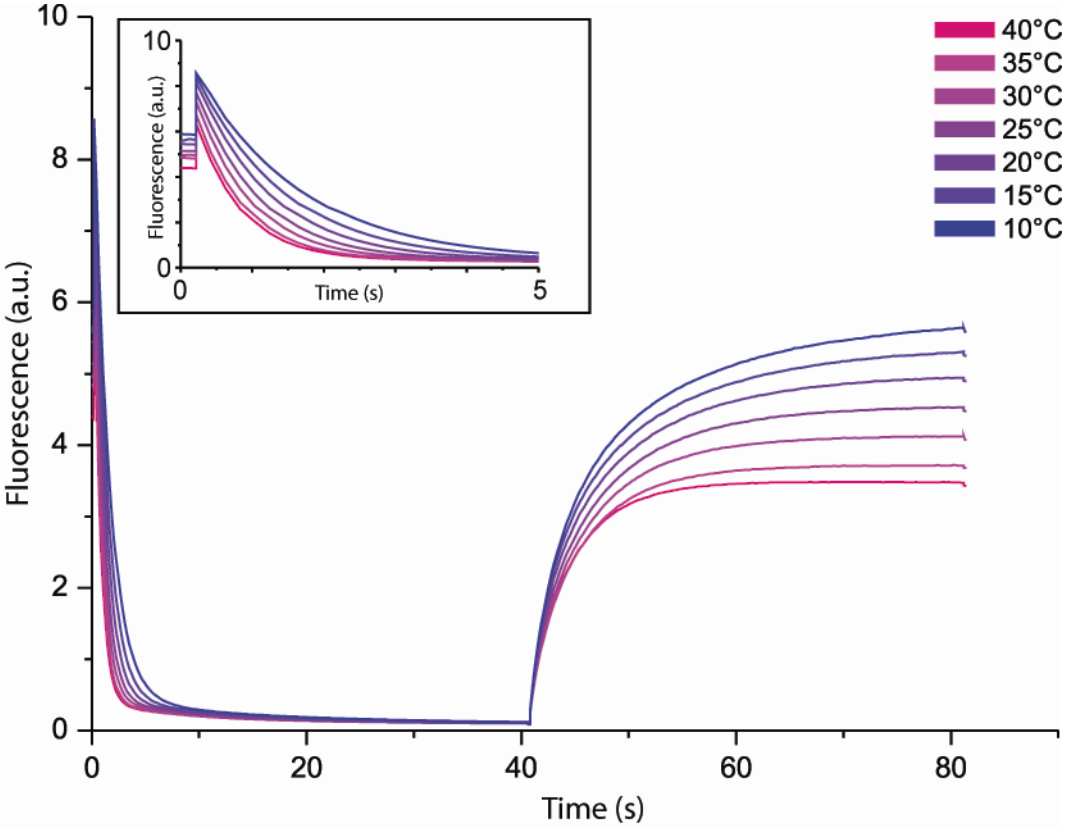

Supplementary Fig. 3 Influence of the temperature on the switching of Dreiklang. First a purified Dreiklang solution (in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) was probed by irradiation with 515/10 nm (0.2 s, 13 W/cm^2). Subsequently it was switched into the off-state by irradiation with light of 405/10 nm (40 s, 70 W/cm^2) and then switched into the on-state by irradiation with 365/25 nm (40 s, 0.7 W/cm^2). Fluorescence was continuously recorded by irradiation with light of 515/10 nm (13 W/cm^2). Inset: magnification of the first 5 seconds.

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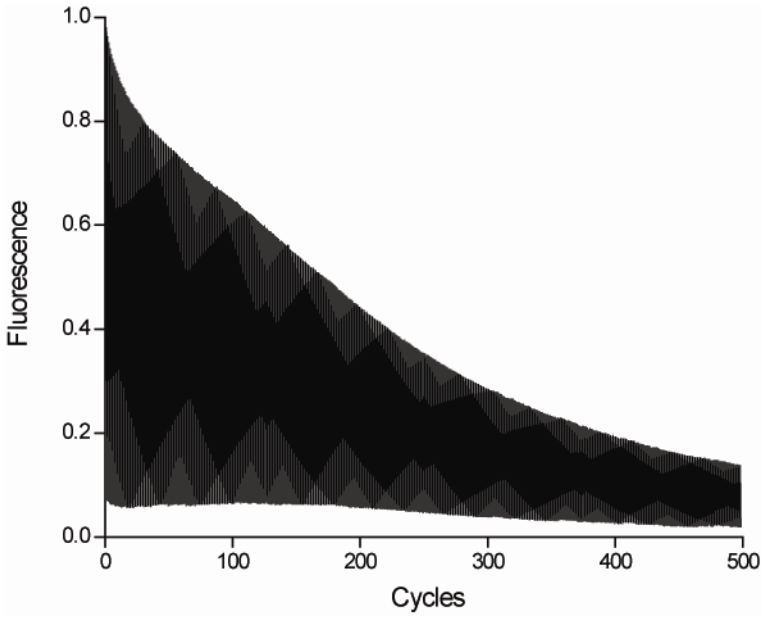

Supplementary Fig. 4 Switching of Dreiklang fluorescence recorded on colonies of living *E. coli* cells expressing Dreiklang. Care was taken to ensure full switching cycles (i.e. the fluorescence signal reached the minimum and the maximum in each individual cycle). The irradiation sequence was as follows: (1) 0.1 s, 515 nm (0.82 W/cm^2); (2) 7 s, 405 nm (110 W/cm^2); (3) 0.1 s, 515 nm (0.82 W/cm^2); (4) 3 s, 365 nm (1.4 W/cm^2); (5) 0.1 s, 515 nm (0.82 W/cm^2). Plotted is the fluorescence signal that was recorded during steps (1), (3), and (5).

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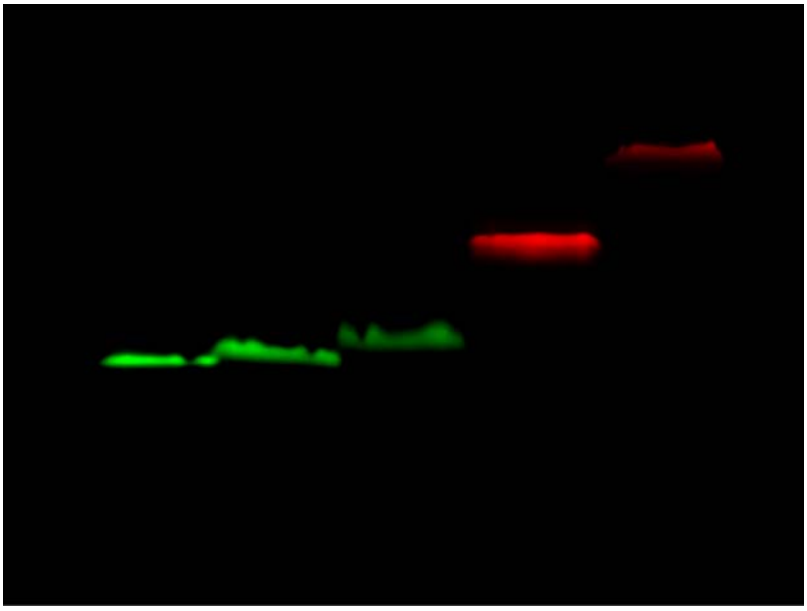

Supplementary Fig. 5 Semi-native PAGE gel electrophoresis. From left to right: EGFP, Citrine, Dreiklang, dTomato (dimer), DsRed (tetramer). Green fluorescence (EGFP, Citrine and Dreiklang) was excited by irradiation with 470/5 nm and detected at 525/30 nm, whereas red fluorescence (dTomato and DsRed) was excited by irradiation with 545/10 nm and detected at 617/37 nm. Both images were overlaid and are represented in false colours.

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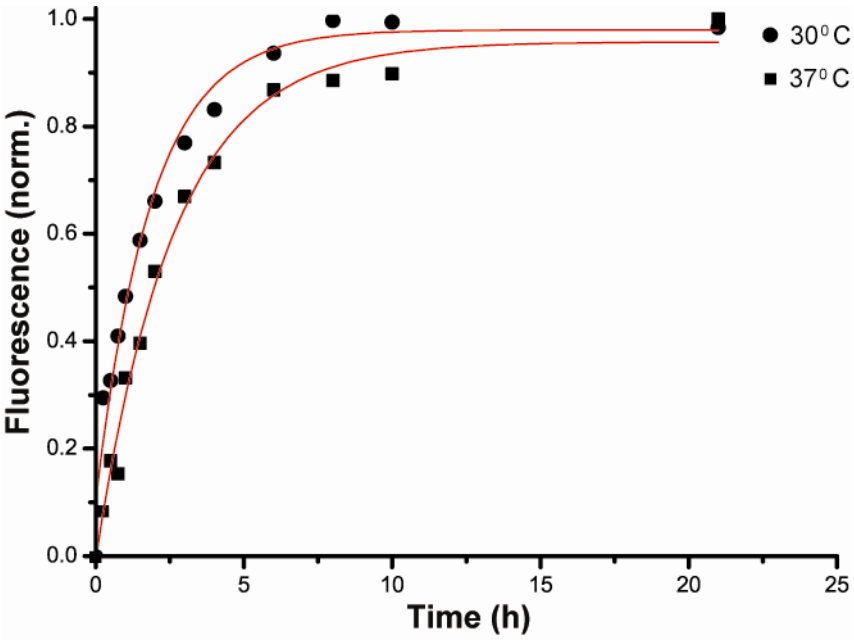

Supplementary Fig. 6 Maturation of Dreiklang at 30 °C and 37 °C. Plotted is the fluorescence signal at the indicated time points after purification of the proteins. Red line: fit to the data points.

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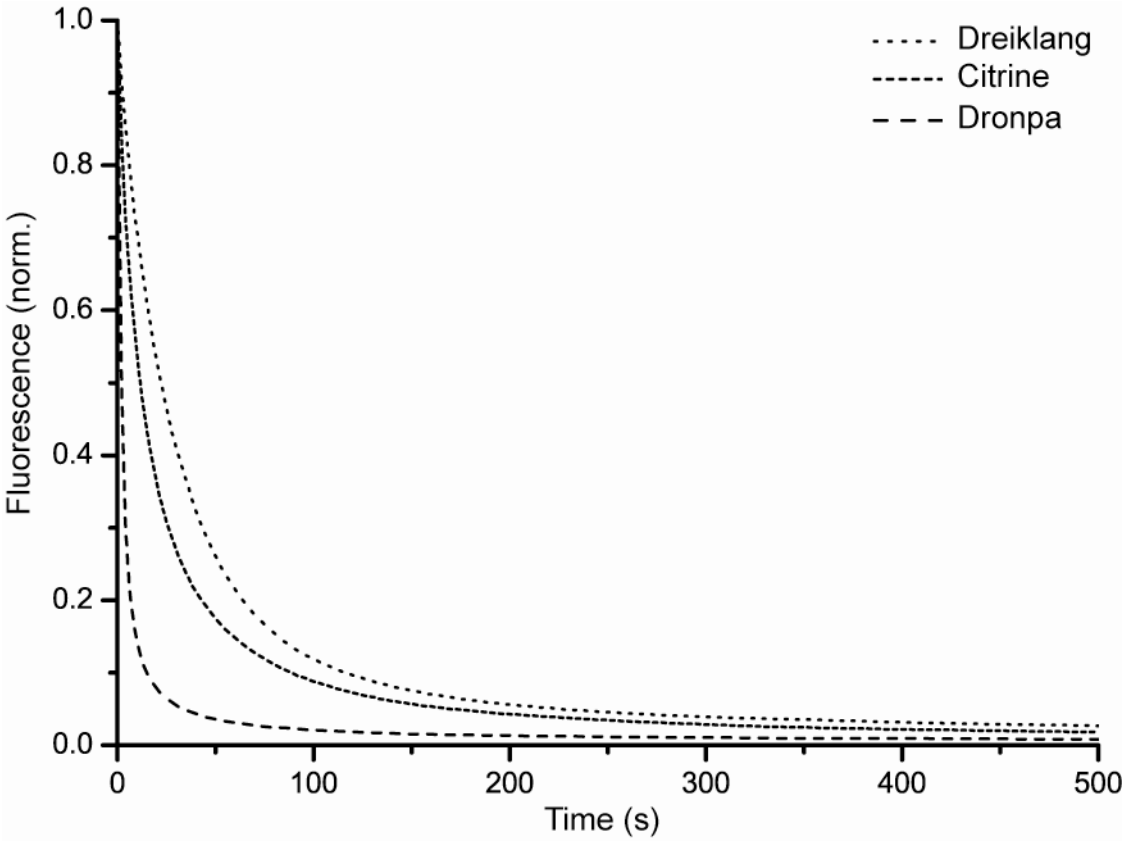

Supplementary Fig. 7 Photobleaching of Dreiklang, Citrine and Dronpa. For Dreiklang and Citrine, the decay of the fluorescence signal was recorded on *E. coli* colonies expressing the respective protein using constant irradiation with 491 nm (188 kW/cm^2). For Dronpa expressing *E. coli* cells, constant irradiation of 491 nm (188 kW/cm^2) and 405 nm (105 kW/cm^2) (to maintain the protein in the on-state) was used. The measured bleaching half-times under these light intensities are: $t_{1/2\ Dreiklang} \sim= 21$ s, $t_{1/2\ Citrine} \sim 10$ s and $t_{1/2\ Dronpa} \sim 2$ s.

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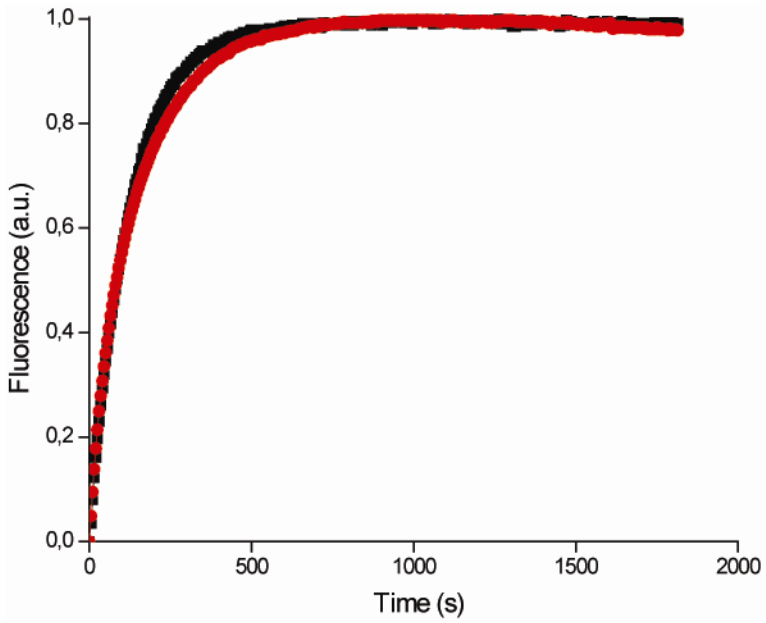

Supplementary Fig. 8 The thermal relaxation of the fluorescence signal from the light-driven off-state to the equilibrium state is very similar in Dreiklang protein crystals and Dreiklang in solution. Dreiklang in solution (pH 4.6) or protein crystals in crystal reservoir solution (pH 4.6) were irradiated with light of 405 nm (110 W/cm^2) until the fluorescence signal reached a minimum. Subsequently, the recovery of the fluorescence signal was monitored by short pulses of 515 nm light (8.2 W/cm^2). Red line: Dreiklang protein crystal, black line: Dreiklang protein in solution.

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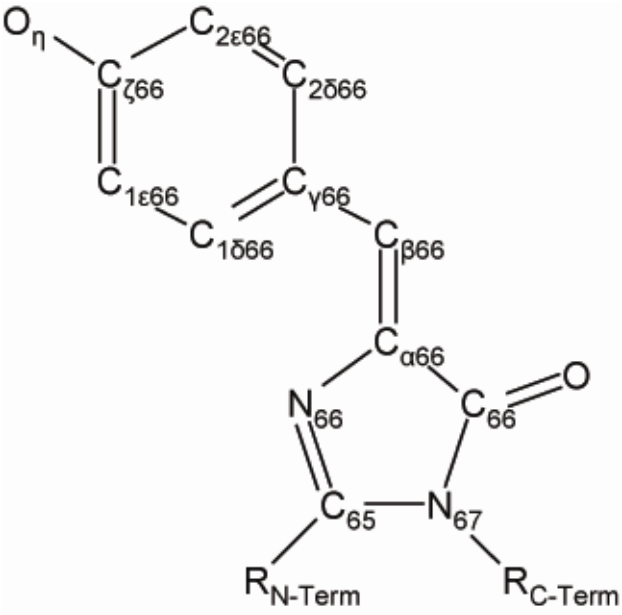

Supplementary Fig. 9 Nomenclature of the atoms in the Dreiklang chromophore. Depicted is the on-state chromophore.

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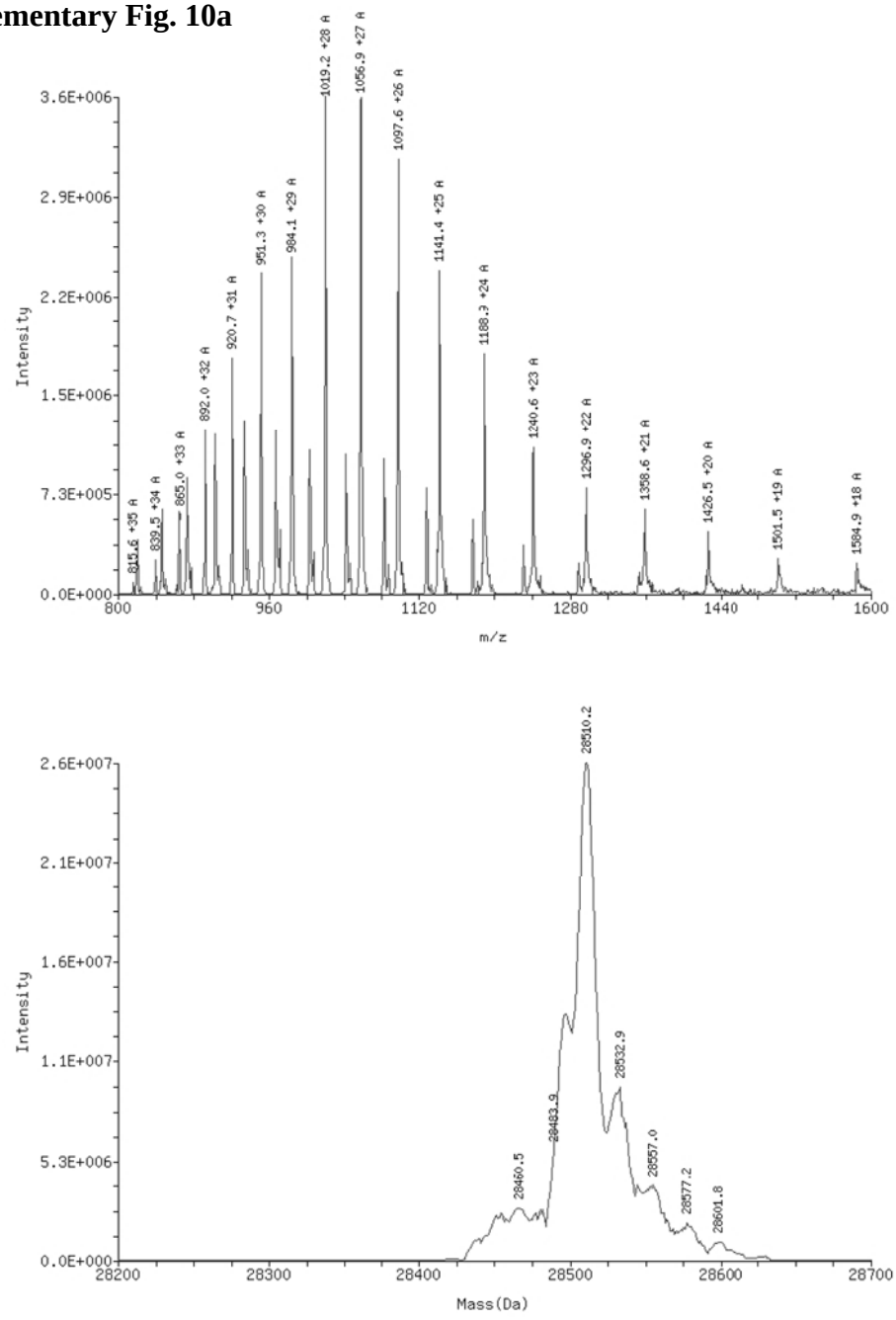
Supplementary Fig. 10a



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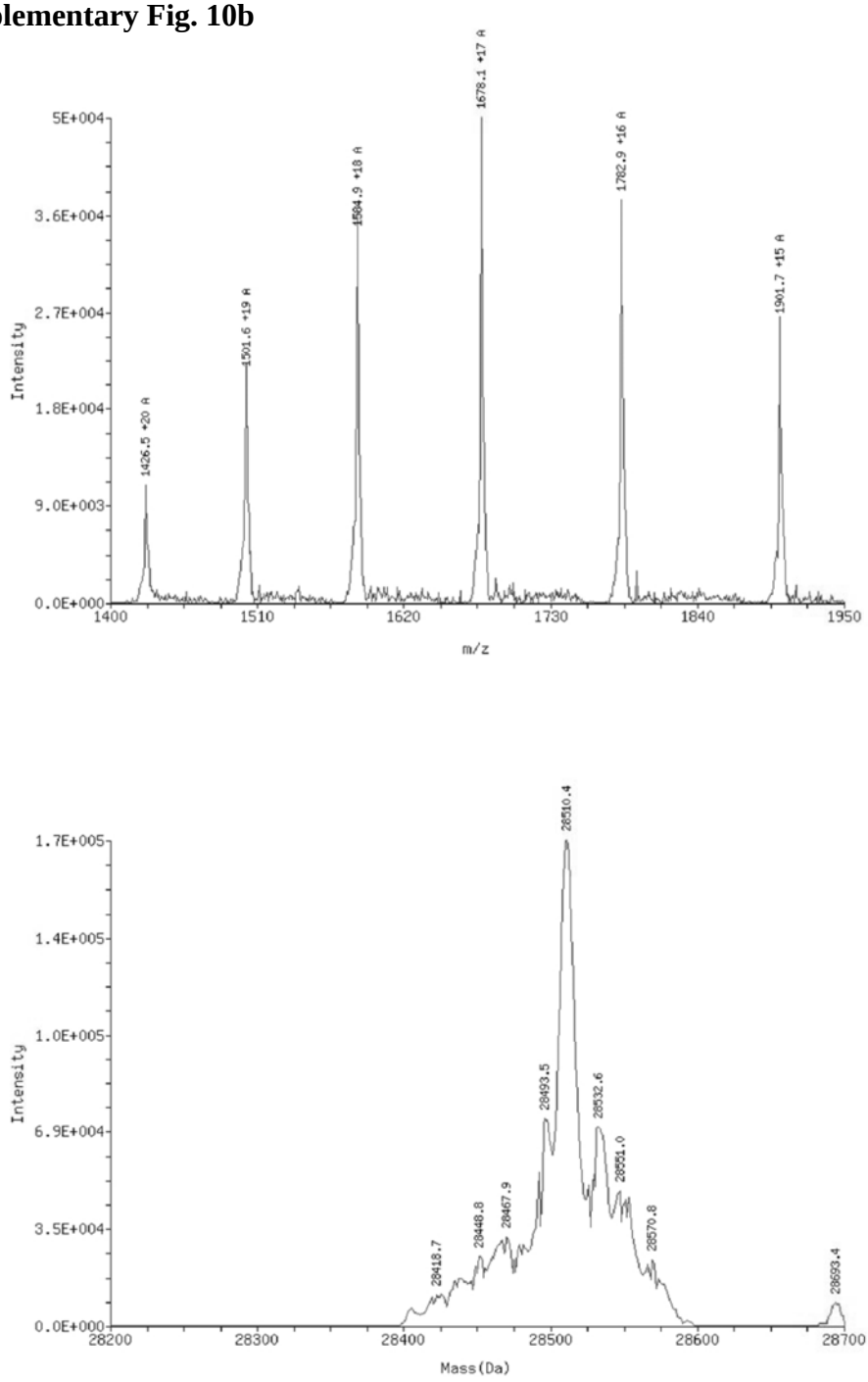
Supplementary Fig. 10b



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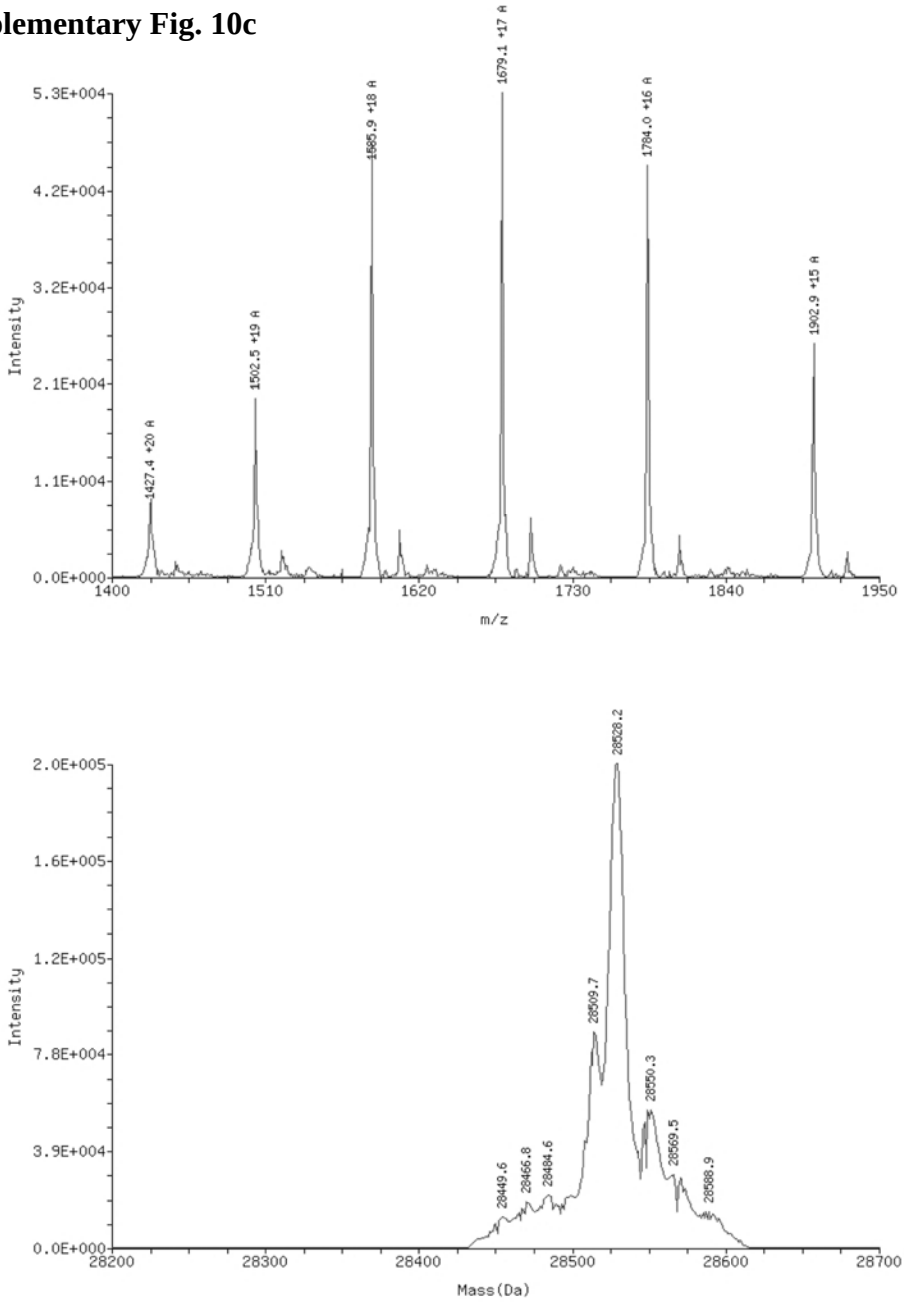
Supplementary Fig. 10c



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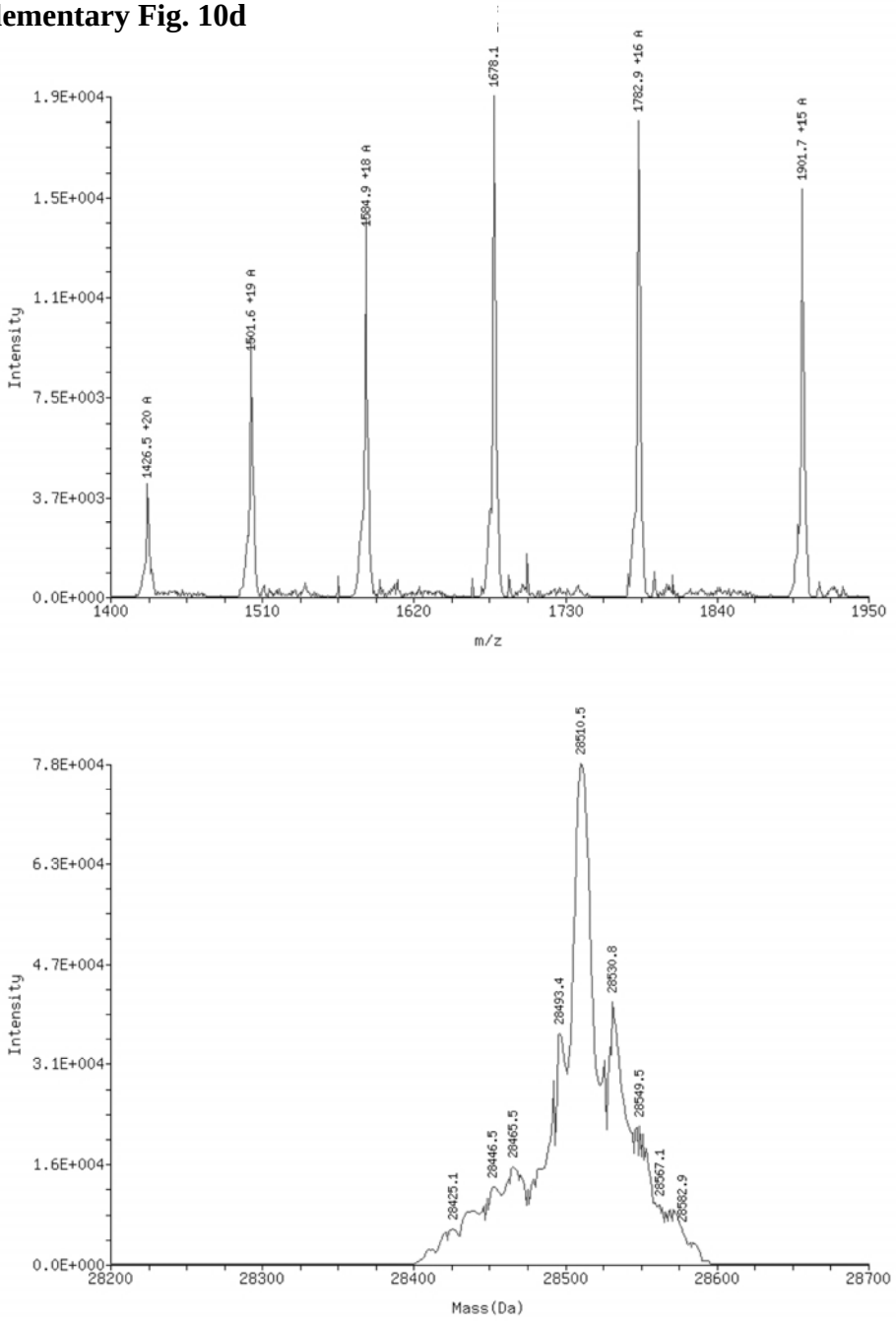
Supplementary Fig. 10d



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Supplementary Fig. 10 ESI-MS analysis of Dreiklang in the various (light switched) states. **(a-d)** Shown are the ESI-MS raw spectra recorded on an Orbitrap MS (top) and the deconvoluted MS spectra (bottom). **(a)** Spectra of equilibrium-state Dreiklang recorded under denaturing conditions (i.e. in the presence of 50 % (v/v) acetonitrile, 0.1 % formic acid). **(b)** Spectra of equilibrium-state Dreiklang recorded under native conditions (10 mM NH_4OAc in 18 % (v/v) acetonitrile, pH 6.9). **(c)** Spectra of off-state Dreiklang recorded under native conditions. **(d)** Spectra of on-state Dreiklang recorded under native conditions.
Note that the determined absolute masses were always approximately 13 Da higher than the calculated masses, irrespective of whether the proteins were analysed on an Orbitrap or Q-ToF MS either in the denatured or in the native state. These extra masses were also found in case of several other fluorescent proteins (amongst others Citrine, EGFP and Dronpa) that were expressed and purified under the same experimental conditions. We conclude that this extra mass of ~ 13 Da is due to an unknown modification of the intact protein that does not affect the switching mechanism. To confirm that this modification does not take place at the chromophore of Dreiklang, we performed MALDI-ToF/ToF analysis of tryptic peptides of the protein (see **Supplementary Fig. 11**).

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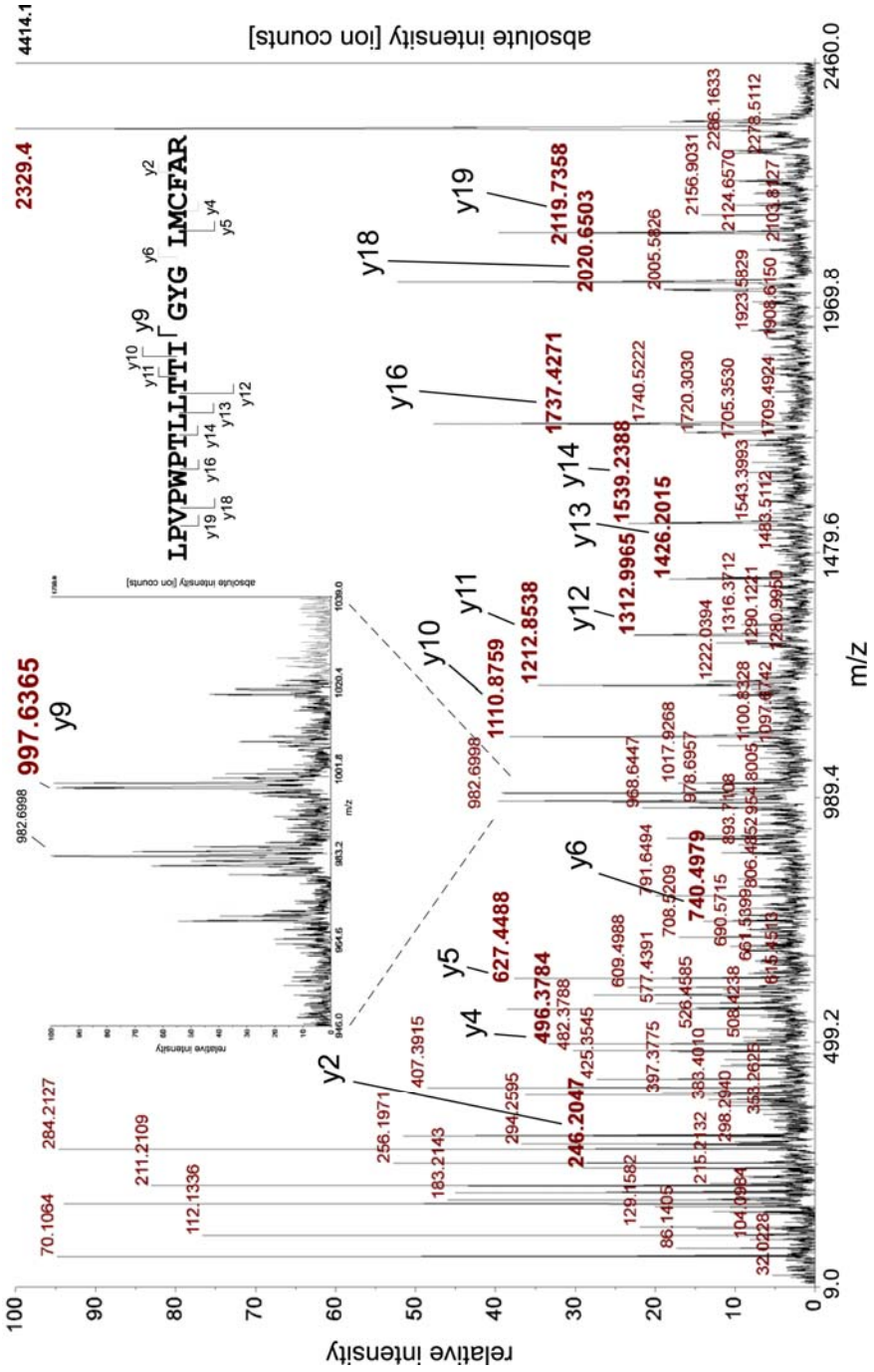

Supplementary Fig. 11 MALDI-ToF/ToF sequence analysis of a tryptic peptide derived from equilibrium-state Dreiklang. The monoisotopic precursor mass ($[M+H]^+$) of the tryptic peptide is 2329.4 Da. This corresponds exactly to the calculated monoisotopic mass of the peptide ($[M+H]^+_{cal}$ = 2349.3 Da) minus 20 Da (i. e. loss of one water molecule (18 Da) and two protons (2 Da)), due to chromophore maturation [16]. The sequence of the peptide was confirmed by the almost complete y-type ion series. Importantly, the y9 fragment ion proves the existence of a mature chromophore without further modifications in equilibrium-state Dreiklang.

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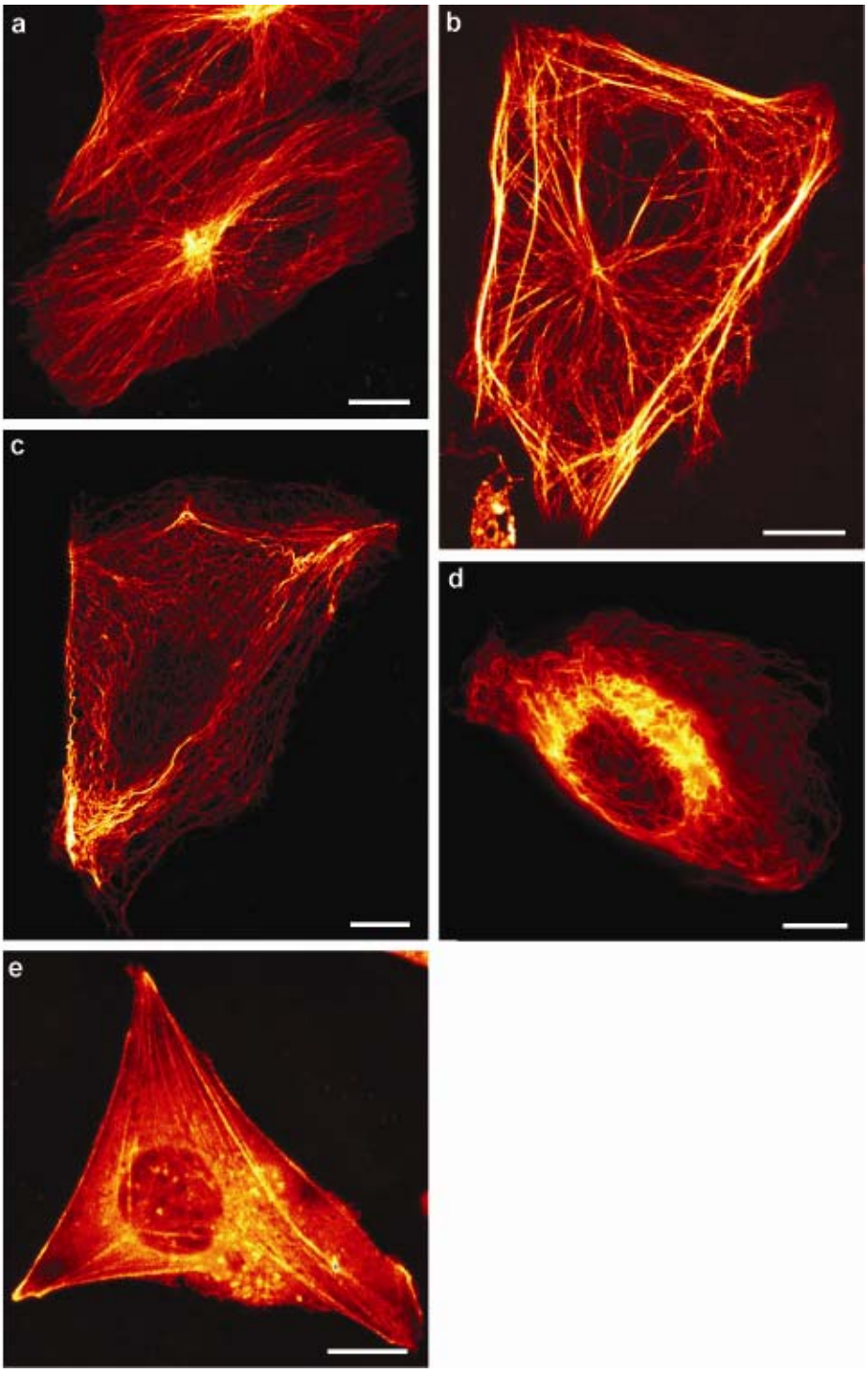
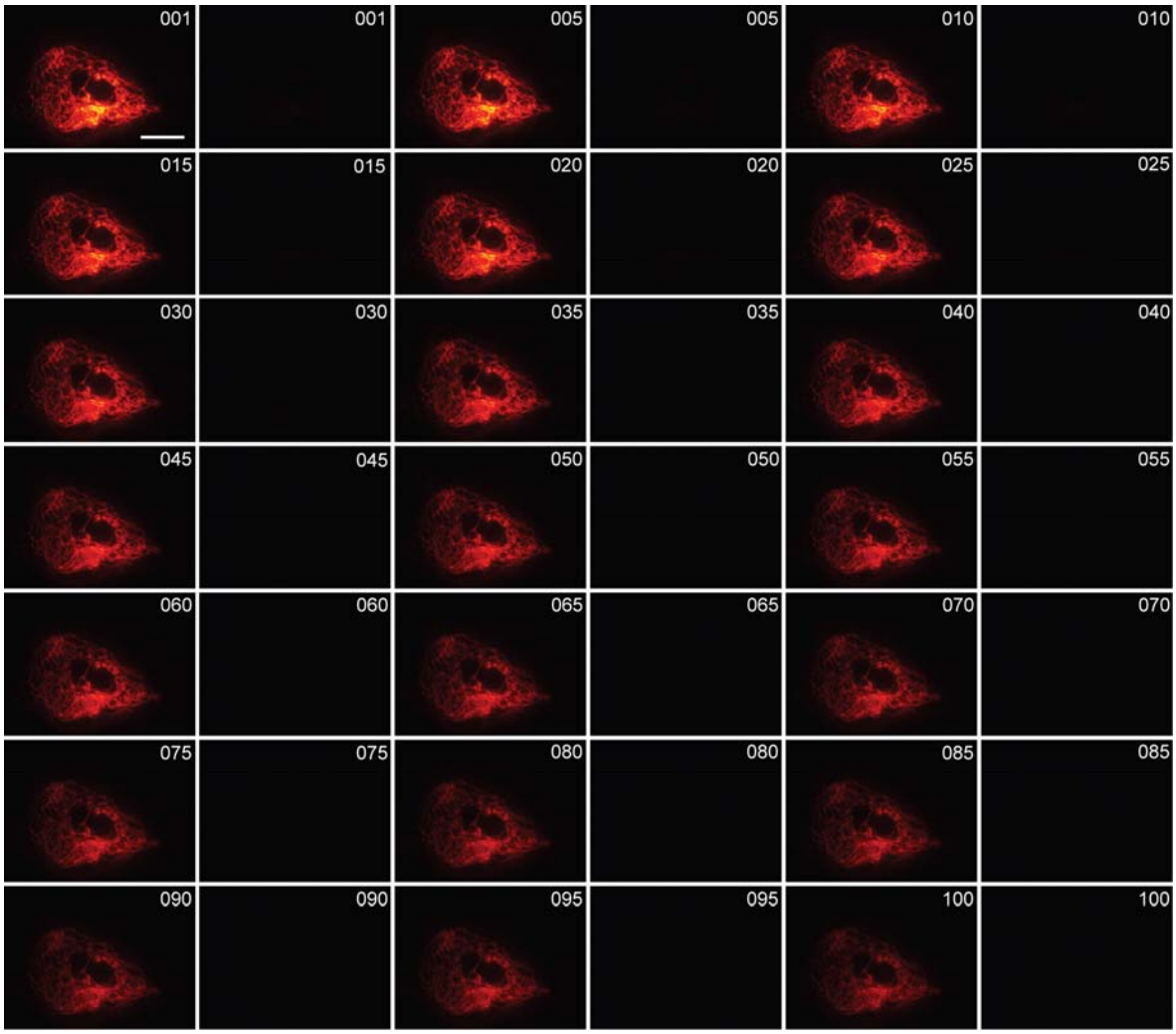


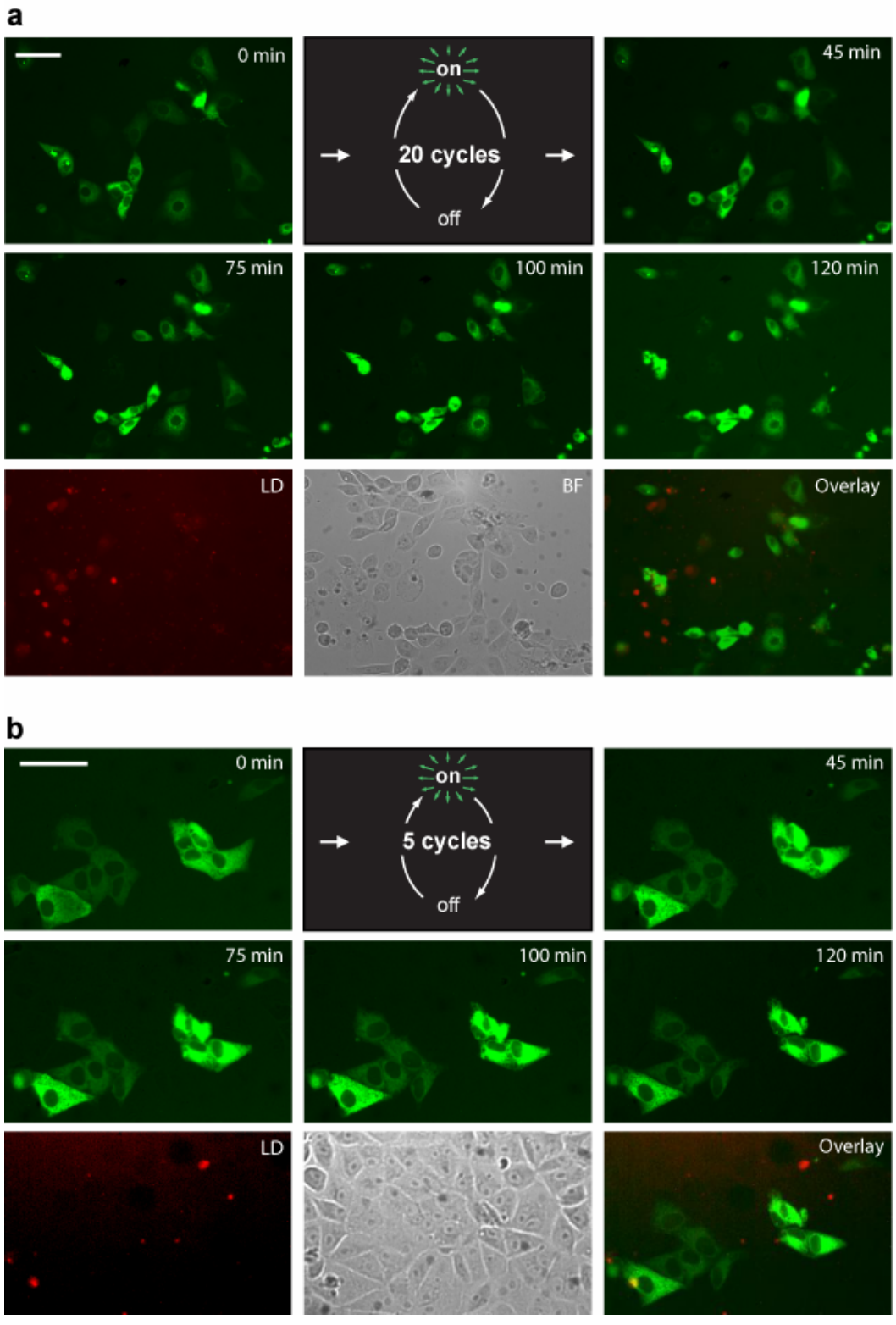
Supplementary Fig. 12 Representative confocal scanning images of living mammalian cells expressing various Dreiklang fusion proteins. Shown are add ups of several confocal sections. (**a**) Dreiklang-α-tubulin in Vero cells. (**b**) Dreiklang-Map2 in Vero cells. (**c**) keratin19-Dreiklang in PtK2 cells. (**d**) vimentin-Dreiklang in PtK2 cells. (**e**) beta-Actin-Dreiklang in PtK2 cells. Scale bars: 10 μm.

Nature BioTechnology: doi:10.1038/nbt.1952



Supplementary Fig. 13 100 consecutive switching cycles of vimentin-Dreiklang expressed in living PtK2 cells. Shown are images of every 5th cycle in the switched-on and switched-off states, respectively. Images were recorded with 495/15 nm (100 ms, 13 W/cm^2), switch-off: 420/30 nm (800 ms, 15 W/cm^2), switch-on: 360/40 nm (100 ms, 5 W/cm^2),. The cycle number is depicted in the upper right corner of each image. The whole imaging sequence took ~10 min. All 100 cycles are assembled in **Supplemental Movie 2**. Scale bar: 20 µm.

Nature BioTechnology: doi:10.1038/nbt.1952



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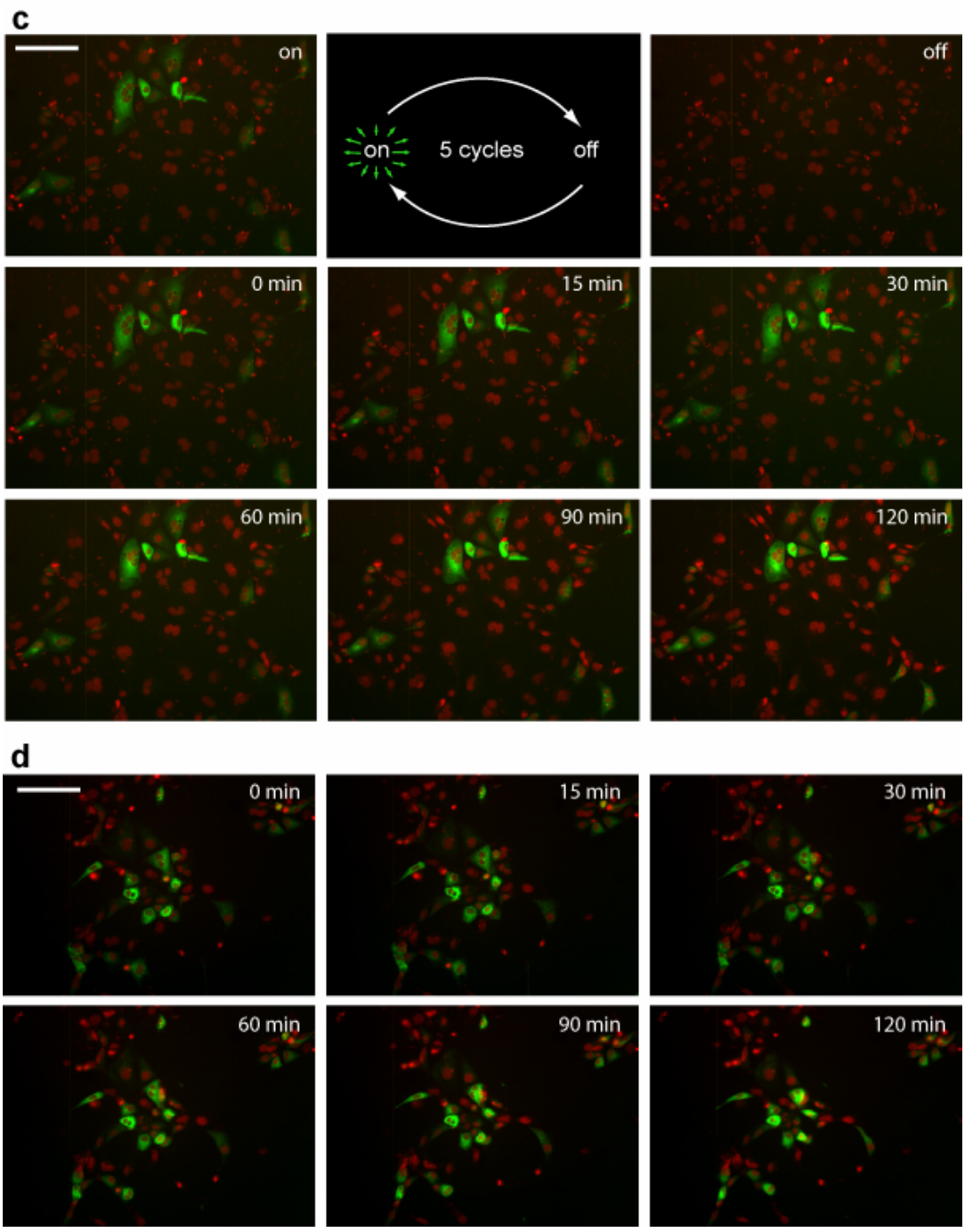
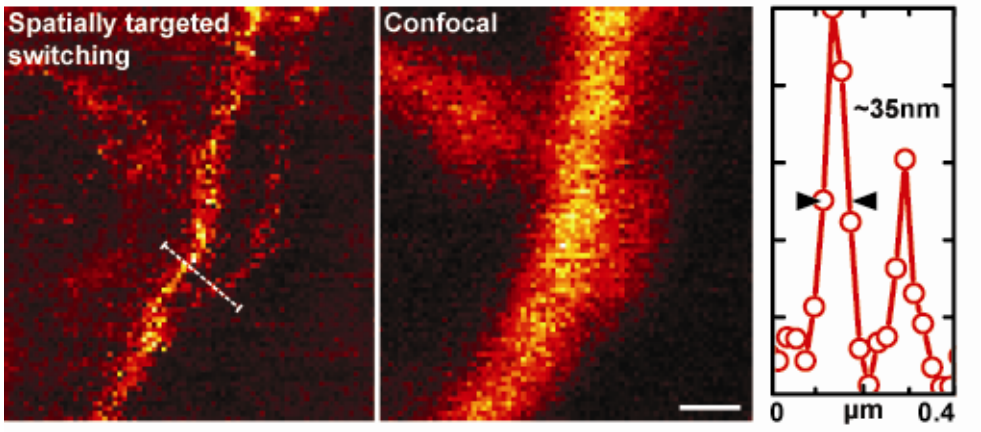


Supplementary Fig. 14 Influence of the irradiation on the cell viability. Dreiklang was targeted to the ER of PtK2 cells.
(**a, b**): The living cells were imaged at the indicated time points. After the first image, the fluorescence of Dreiklang was switched (**a**) 20 times or (**b**) 5 times completely between the fluorescent and the non-fluorescent states. Switching was performed by alternating

Nature BioTechnology: doi:10.1038/nbt.1952

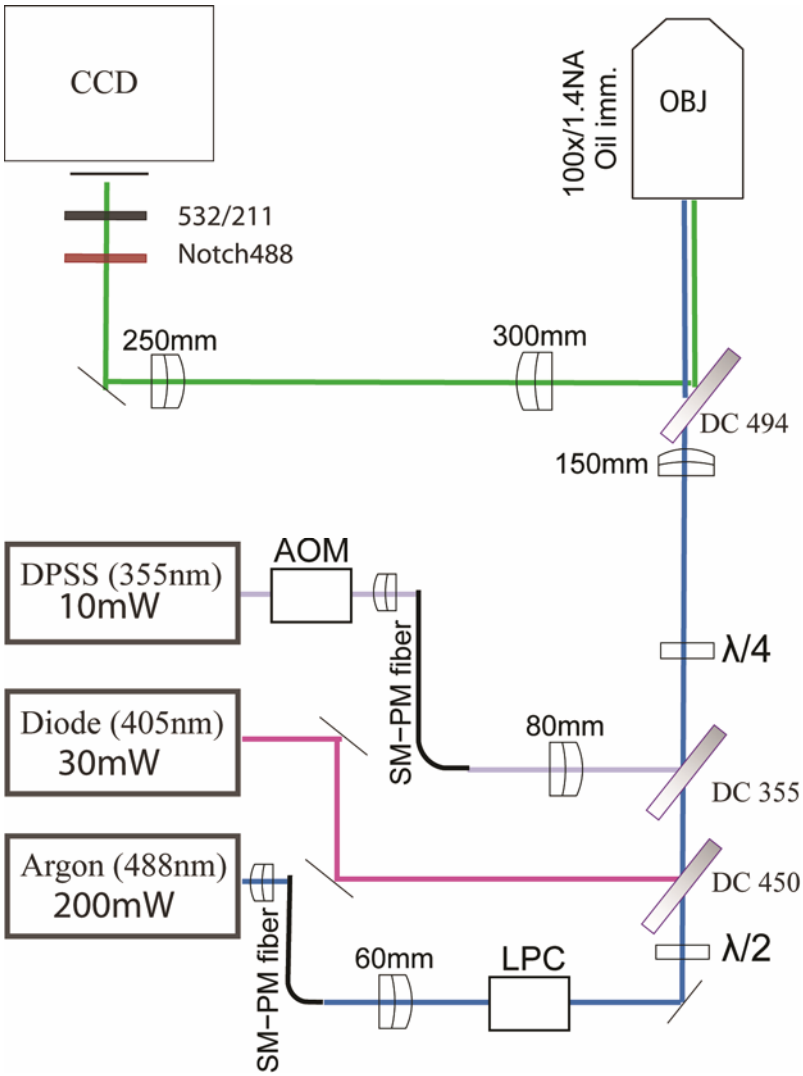
irradiation with near-UV light (4 s, 405/10 nm, 24 W/cm^2) and UV light (6 s, 365/25 nm, 1 W/cm^2). Images were recorded with light of 515/10 nm (1.3 W/cm^2) on a CCD camera. At t = 120 min the cells were stained with the indicator Sytox, which stains the nuclei of dead cell in red (fluorescence emission: 647 nm). BF: brightfield, LD: live-death (Sytox staining). Scale bar: 50µm.
(**c, d**): In a culture of PtK2 cells expressing Dreiklang targeted to the ER, the DNA was additionally labeled with the dye Hoechst33342 (HO342; Invitrogen, CA) to monitor the viability of the cells. Cells in (**c**) and (**d**) were treated equally, except that in (**c**) the Dreiklang fluorescence was switched 5 times completely between the on- and the off-state. Each switching cycle was performed by four irradiation steps, starting with near-UV light (11 s, 405/10 nm, 30 W/cm^2) followed by yellow light for 2 s (515/10 nm 6 W/cm^2), UV light for 11 s (365/25 nm, 0,5 W/cm^2) and again 2 s of yellow light (515/10 nm, 6 W/cm^2). Images were recorded at the indicated time points with a CCD camera, exciting Dreiklang with light of 515 nm and Hoechst33342 with 365 nm. The exposure time of the CCD camera was set to 600 ms. Scale bar: 100µm.
All imaging was performed at 37 °C.

Nature BioTechnology: doi:10.1038/nbt.1952



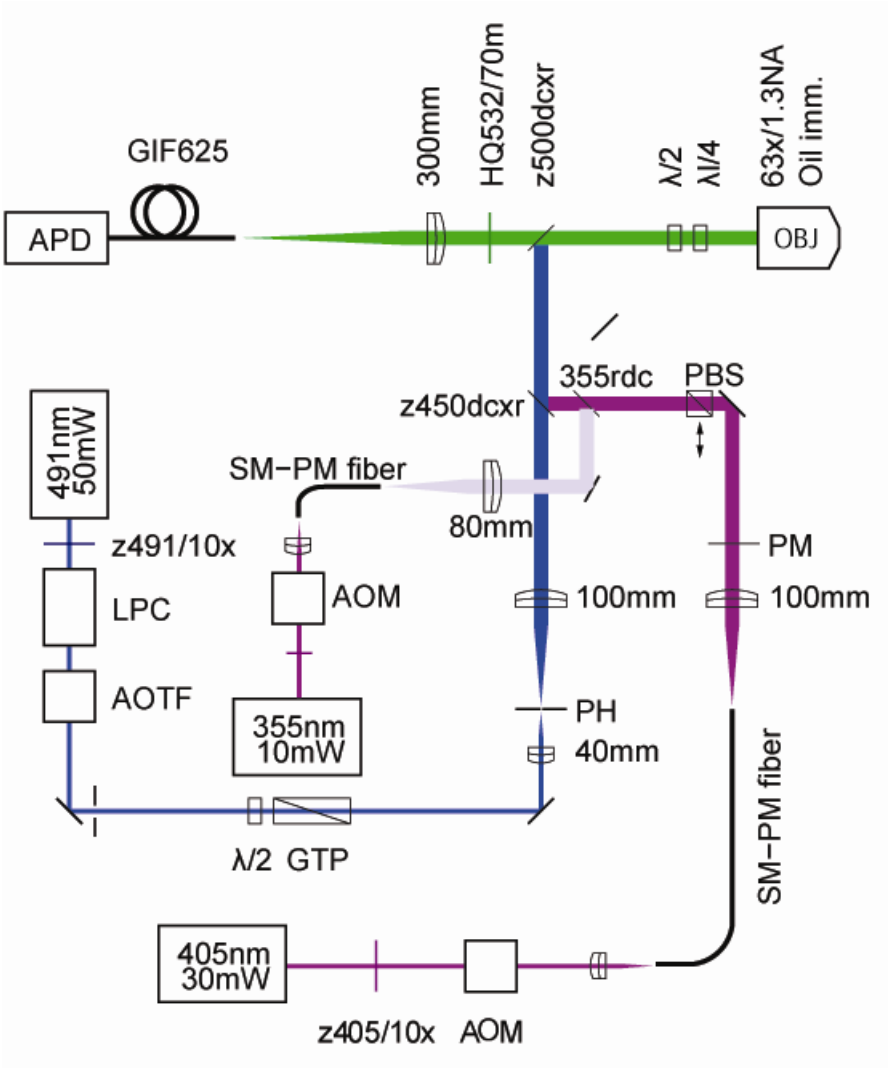
Supplementary Fig. 15 Super-resolution microscopy based on spatially targeted switching enables a spatial resolution of down to ~35 nm. Ensemble switching (left) and diffraction-limited (middle) recordings of a single vimentin-Dreiklang filament expressed in living PtK2 cells. Pixel size: 20 nm. Imaging conditions: 1 ms activation at 355nm (160 W/cm^2), 60 ms switching off at 405 nm (110 W/cm^2), 3 ms reading out at 491nm (2kW/cm^2). Scale bar: 250 nm.

Nature BioTechnology: doi:10.1038/nbt.1952



Supplementary Fig. 16 Single Molecule Switching microscope set-up. LPC: laser power controller; AOM: acousto-optical modulator; SM-PM fiber: single-mode polarization-maintaining fiber; λ/2, λ/4: half- and quarter-wave plates; DC: dichroic mirrors with a cut-off at the given wavelengths; 532/211, Notch488: fluorescence and notch filters with the given spectral characteristics; 60, 80, … mm: achromatic lenses of given focal length; OBJ: objective lense; CCD: camera; DPSS: diode-pumped solid state.

Nature BioTechnology: doi:10.1038/nbt.1952



Supplementary Fig. 17 Set-up for ensemble switching. LPC: laser power controller; AOTF, AOM: acousto-optic modulators; GTP: Glan-Thompson polarizer; PBS: polarizing beam splitter; PH: pinhole (10 µm in diameter); PM: phase mask (vortex plate or phase disc); SM-PM fiber: single-mode polarization-maintaining fiber; 355rdc, z450dcxr, z500dcxr: dichroic mirrors with a cut-off at the given wavelengths; z405/10x, z491/10x: laser clean-up filters; HQ532/70m: fluorescence filter; 40, 80, … mm: achromatic lenses of given focal length; OBJ: objective lense; GIF625: 62.5µm core multi-mode fiber; APD: single-photon-counting avalanche photo-diode

Nature BioTechnology: doi:10.1038/nbt.1952

Supplementary Table 1 Irradiation schemes for the switching data shown in the manuscript.

Fig. 1c (1 cycle)

	Step 1	Step 2	Step 3	Step 4	Step 5
Time (s)	1	7	1	8	11
Irradiation (nm)	515 [a]	405 [b] and 515 [a]	515 [a]	365 [c] and 515 [a]	515 [a]

Fig. 1d (20 cycles)

	Step 1 (only first cycle)	Step 2 (not shown)	Step 3	Step 4 (not shown)	Step 5 (not shown)	Step 6
Time (s)	1	3	1	5	10	1
Irradiation (nm)	515 [d]	405 [b]	515 [d]	365 [c]	515 [d]	515 [d]

Fig. 1f (1 cycle)

continous **(black line)**

	Step 1 (not shown)	Step 2 (not shown)	Step 3
Time (s)	0,1	15	2400
Irradiation (nm)	515 [d]	405 [g]	515 [d]

pulsed **(red line)**

	Step 1 (not shown)	Step 2 (not shown)	Step 3 (pulse every 120 s)
Time (s)	0,1	15	2400
Irradiation (nm)	515 [d]	405 [g]	515 [d]

Fig. 1f (inset)

	Step 1 (not shown)	Step 2 (not shown)	Step 3 (pulse every 2 s)
Time (s)	0.2	20	8200
Irradiation (nm)	515 [f]	405 [g]	515 [f]

Supplementary Fig. 1a (3 cycles)

	Step 1	Step 2	Step 3	Step 4	Step 5
Time (s)	0,4	40	0,4	20	0,4
Irradiation (nm)	515 [a]	405 [b] and 515 [a]	515 [a]	365 [c] and 515 [a]	515 [a]

Supplementary Fig. 1b (3 cycles)

	Step 1	Step 2	Step 3	Step 4	Step 5
Time (s)	0,2	60	0,2	20	0,2
Irradiation (nm)	515 [a]	405 [b] and 515 [a]	515 [a]	365 [c] and 515 [a]	515 [a]

Nature BioTechnology: doi:10.1038/nbt.1952

Supplementary Fig. 3 (1 cycle)

	Step 1	Step 2	Step 3	Step 4	Step 5
Time (s)	0,2	40	0,2	40	0,2
Irradiation (nm)	515 [f]	405 [g] and 515 [f]	515 [f]	365 [h] and 515 [f]	515 [f]

Supplementary Fig. 4 (500 cycles)

	Step 1	Step 2 (not shown)	Step 3	Step 4 (not shown)	Step 5
Time (s)	0,1	7	0,1	3	0,1
Irradiation (nm)	515 [e]	405 [b]	515 [e]	365 [c]	515 [e]

Supplementary Fig. 8(1 cycle)

solution			
	Step 1 (not shown)	**Step 2 (not shown)**	**Step 3 (pulse every 5 s)**
Time (s)	0,3	20	1800
Irradiation (nm)	515 [i]	405 [g]	515 [i]
crystals			
	Step 1 (not shown)	**Step 2 (not shown)**	**Step 3 (pulse every 5 s)**
Time (s)	0,3	20	1800
Irradiation (nm)	515 [e]	405 [b]	515 [e]

[a] 515 ± 10 nm, 8.2 W/cm^2
[b] 405 ± 10 nm, 110 W/cm^2
[c] 365 ± 25 nm, 1.4 W/cm^2
[d] 515 ± 10 nm, 0.49 W/cm^2
[e] 515 ± 10 nm, 0.82 W/cm^2
[f] 515 ± 10 nm, 13 W/cm^2
[g] 405 ± 10 nm, 70 W/cm^2
[h] 365 ± 25 nm, 0.7 W/cm^2
[i] 515 ± 10 nm, 1.3 W/cm^2

Nature BioTechnology: doi:10.1038/nbt.1952

Supplementary Table 2 Crystallographic data and refinement.

Data Collection			
	Dreiklang-eq	**Dreiklang-off**	**Dreiklang-on**
Wavelength (Å)	0.91841	0.91841	0.91841
Temperature (K)	100	100	100
Space Group	$P2_12_12$	$P2_12_12$	$P2_12_12$
Unit Cell Parameters (Å, °)	68.057 136.213 81.247 90.000 90.000 90.000	68.030 135.340 81.100 90.000 90.000 90.000	68.253 136.033 81.252 90.000 90.000 90.000
Resolution (Å)	50-1.90 (1.92-1.90)[a]	50-1.70 (1.80-1.70)	50-2.00 (2.10-2.00)
Reflections			
Unique	59,298	82,517	51,771
Completeness (%)	98.4 (95.6)	99.4 (97.0)	99.8 (99.9)
Redundancy	3.55	4.01	4.07
I/σ(I)	12.75 (2.23)	14.38 (1.79)	16.71 (3.00)
R_{sym}(I)[b]	0.067 (0.523)	0.071 (0.740)	0.064 (0.495)
Refinement			
Resolution (Å)	41.42-1.90	41.29-1.70	41.44-2.00
Reflections			
Number	59,293	82,512	51,768
Completeness (%)	98.36	99.71	99.80
Test Set (%)	5.00	5.00	5.00
R_{work}[c]	0.158	0.159	0.161
R_{free}[c]	0.206	0.191	0.208
R_{all}[c]	0.161	0.161	0.164
ESU (Å)[d]	0.22	0.20	0.23
Contents of A.U.[f]			
Protein Molecul./Residues/Atoms	3/703/5960	3/707/5878	3/704/5816
Water Oxygens	665	733	504
Ligand Molecules/Atoms	14/70	13/65	7/35
Ramachandran Plot[g]			
Favored	98.4	98.4	98.4
Allowed	1.6	1.6	1.6
Outliers	0.00	0.00	0.00
Rmsd[h] **from Target Geometry**			
Bond Lengths (Å)	0.010	0.010	0.010
Bond Angles (°)	1.279	1.352	1.283
PDB ID	3ST2	3ST3	3ST4

[a] Data for the highest resolution shell in parentheses

[b] $R_{sym}(I) = \Sigma_{hkl}\Sigma_i |I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl}\Sigma_i |I_i(hkl)|$; for n independent reflections and i observations of a given reflection; $\langle I(hkl)\rangle$ – average intensity of the i observations

[c] $R = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$; R_{work} – $hkl \notin T$; R_{free} – $hkl \in T$; R_{all} – all reflections; T – test set

[d] ESU – estimated overall coordinate error based on maximum likelihood

[f] A.U. – asymmetric unit

[g] Calculated with MolProbity (http://molprobity.biochem.duke.edu/)

[h] Rmsd – root-mean-square deviation

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Supplementary Movie 1 Animated sequence of 33 individual images as shown in **Fig. 3a**. The images were written successively at the same position of a PVA-Dreiklang layer. Before writing each new frame, all molecules were photoswitched to the on-state. Side length of the image: 380 µm.

Supplementary Movie 2 Animated sequence of 100 consecutive switching cycles of vimentin-Dreiklang in PtK2 cells, as shown in **Supplementary Fig. 13**. Side length of each image: 87.5 µm.

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