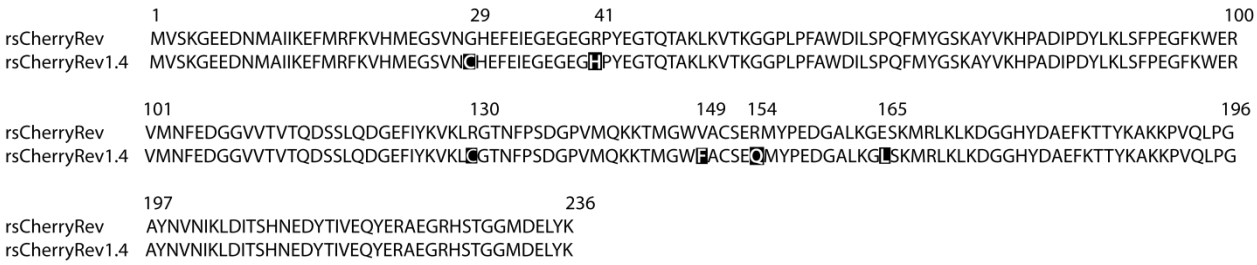
CHEMPHYSCHEM

Supporting Information

Two-Color RESOLFT Nanoscopy with Green and Red Fluorescent Photochromic Proteins**

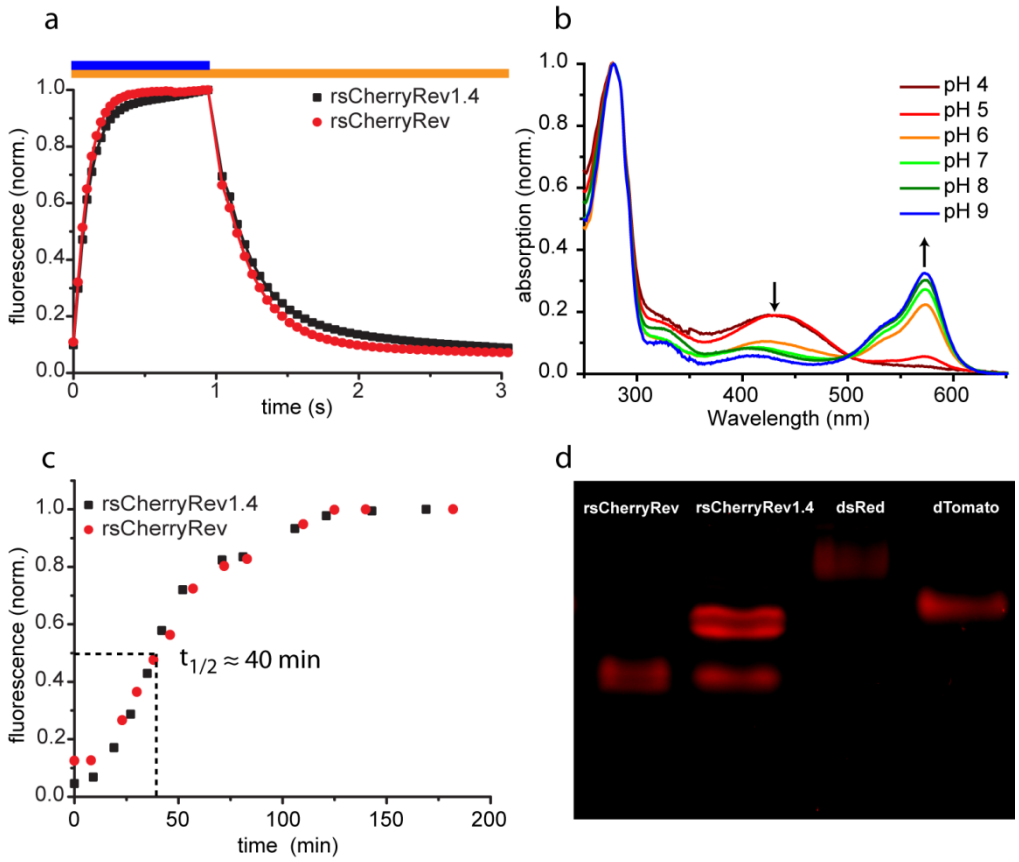
Flavie Lavoie-Cardinal,[a] Nickels A. Jensen,[a] Volker Westphal,[a] Andre C. Stiel,[a] Andriy Chmyrov,[a] Jakob Bierwagen,[a] Ilaria Testa,[a] Stefan Jakobs,*[a, b] and Stefan W. Hell*[a]

cphc_201301016_sm_miscellaneous_information.pdf



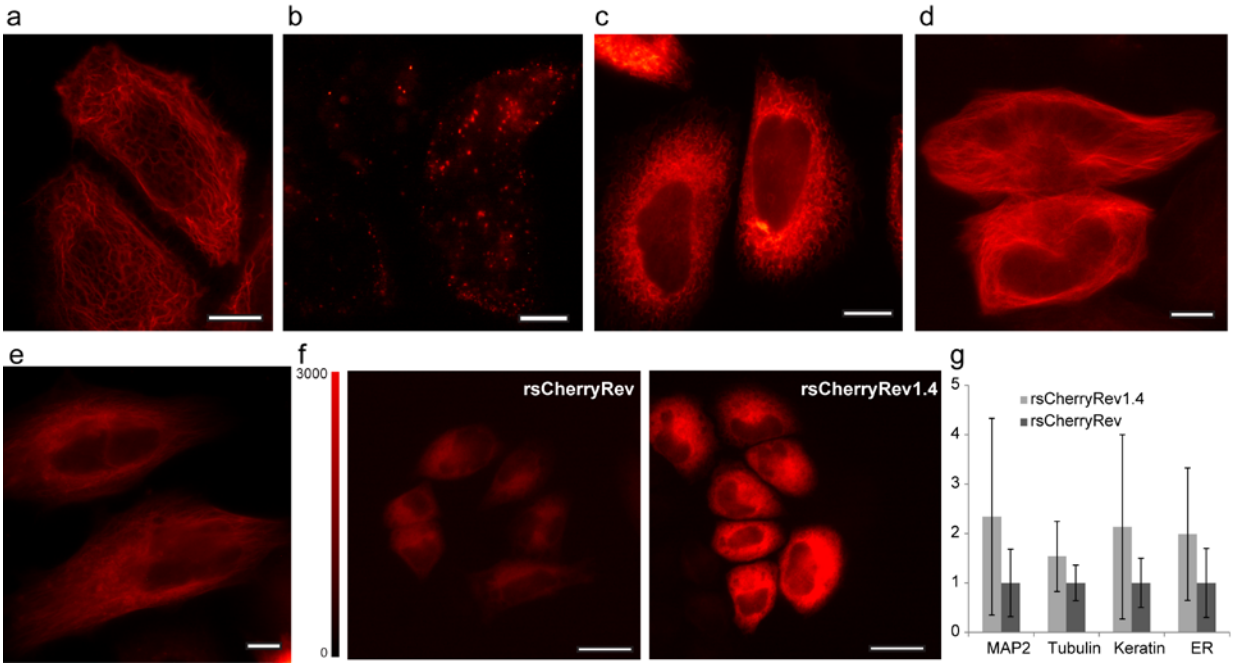
Supplementary figure 1 – Alignment of the amino acid sequences of rsCherryRev and rsCherryRev1.4

The mutations of rsCherryRev1.4 are highlighted with a black background.



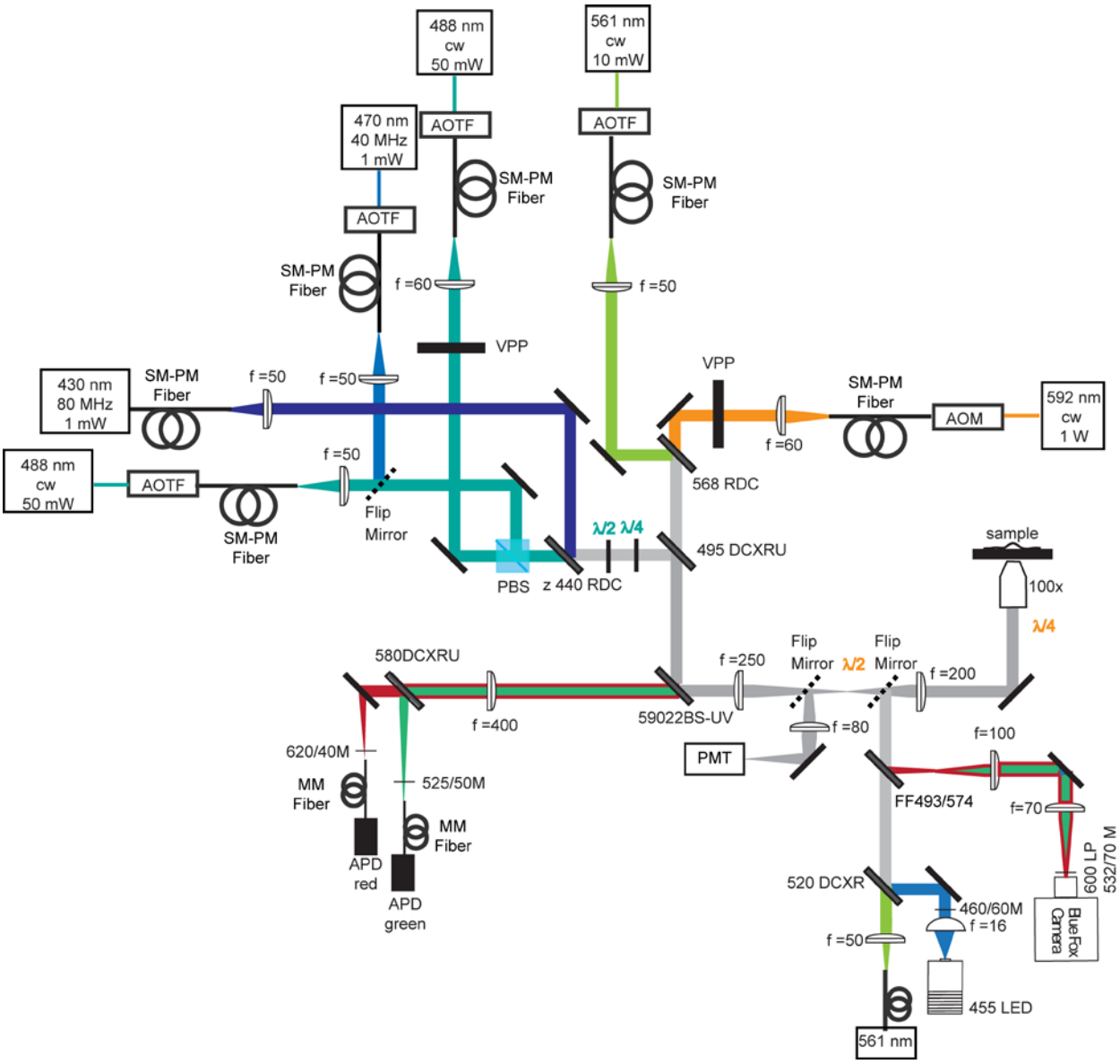
Supplementary figure 2 - Properties of rsCherryRev1.4

(a) Fluorescence switching kinetics of rsCherryRev1.4 (black) and rsCherryRev (red) measured at low switching light intensities at room temperature on *E. coli* colonies; on-switching at 450/40 nm wavelength at 10 W/cm^2, off-switching at 577/10 nm and 40 W/cm^2. The on-switching step was performed with simultaneous excitation at 577/10 nm, which also switches the protein to the off-state, thereby delaying the on-switching. The fluorescence intensity is normalized to the maximal intensity for each protein; in absolute numbers, the fluorescence intensity of rsCherryRev1.4 expressed in *E. coli* was two to three times higher than the fluorescence intensity of rsCherryRev. The switching speed of both proteins was very similar, whereby the time limiting step was the off-switching step in both cases. (b) pH-dependence of the absorption spectrum of rsCherryRev1.4. (c) Maturation time of rsCherryRev and rsCherryRev1.4 measured at 37°C. Both show a maturation half-time of about 40 min. (d) Semi-native polyacrylamide gel electrophoresis of rsCherryRev (monomer), rsCherryRev1.4, dsRed (tetramer) and dTomato (dimer).



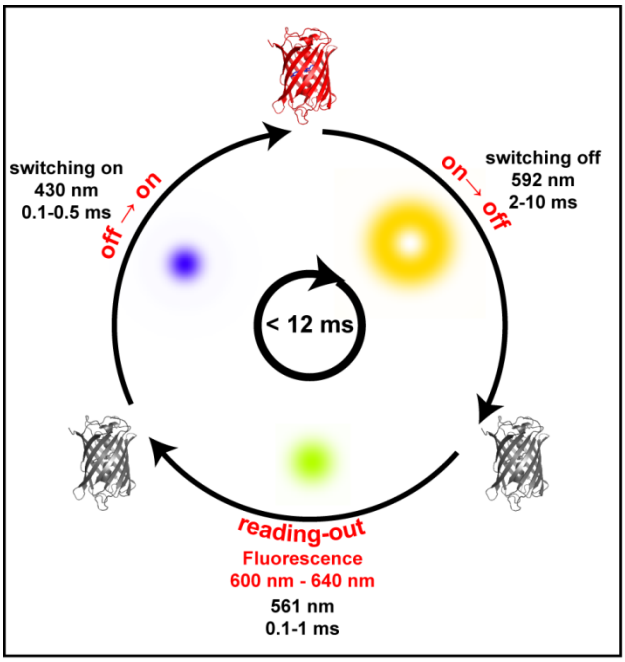
Supplementary figure 3 – Expression of various rsCherryRev1.4 fusion proteins in mammalian cells

Epifluorescence images of living HeLa cells expressing various fusion proteins. (a) keratin19-rsCherryRev1.4. (b) caveolin1- rsCherryRev1.4. (c) rsCherryRev1.4-ER. (d) rsCherryRev1.4-MAP2. (e) rsCherryRev1.4-α-tubulin. (f) HeLa cells expressing rsCherryRev-ER (left) or rsCherryRev1.4-ER (right). (g) Analysis of the fluorescence intensity of HeLa cells (n>100 each) expressing rsCherryRev or rsCherryRev1.4 fused with different proteins. The error bars represent the standard deviations of the mean cellular fluorescence intensity. rsCherryRev1.4 expressing cells were on average 1.5 to 2.5 times brighter than cells expressing rsCherryRev. Scale bars: 10 µm (a-e) and 25 µm (f).



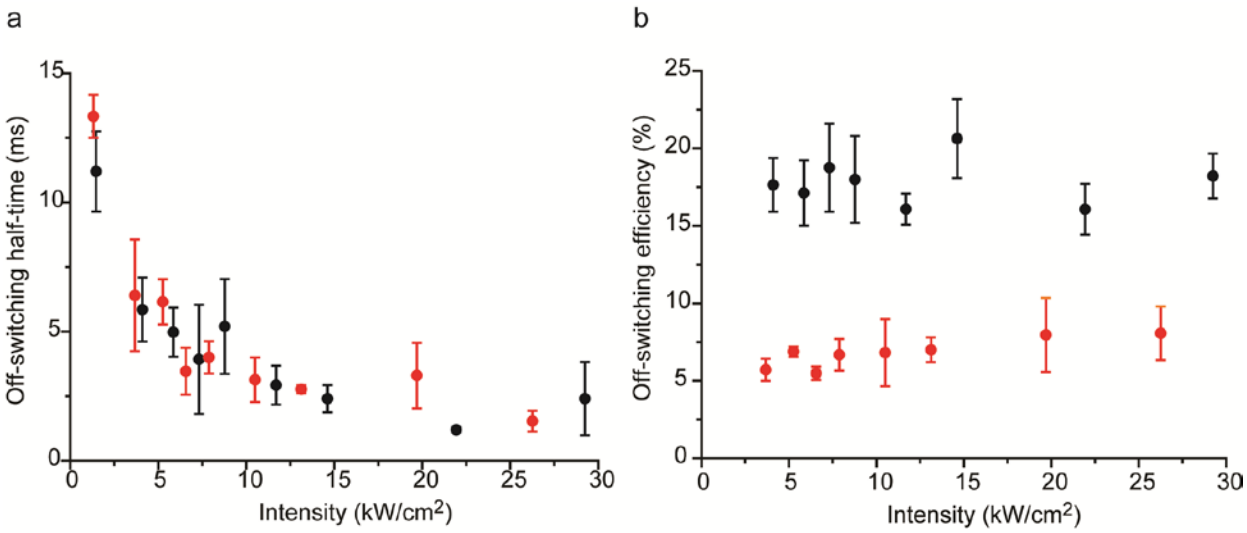
Supplementary figure 4 - Scheme of the home-built point-scanning RESOLFT microscope.

APD : Avalanche Photodiode, PMT : Photomultiplier tube, AOTF : Acousto-optic tunable filter, AOM : Acousto-optic modulator, VPP : vortex phase plate, LED : light emitting diode, cw : continuous wave, SM-PM Fiber : single mode polarization maintaining fiber, MM Fiber : multimode fiber with 62.5 µm core diameter, PBS : polarization beam splitter, λ/2- λ/4 : retarding plates, 568RDC – 495DCXRU – z440RDC – 59022BS-UV – 520DCXR – 580DCXRU : dichroic mirrors, 620/40M – 525/50M – FF493/574 – 460/60M – 5252/70M – 600LP : fluorescence filters. All lenses were achromatic lenses with the specified focal length in mm.



Supplementary figure 5 – Switching scheme for RESOLFT imaging with rsCherryRev1.4.

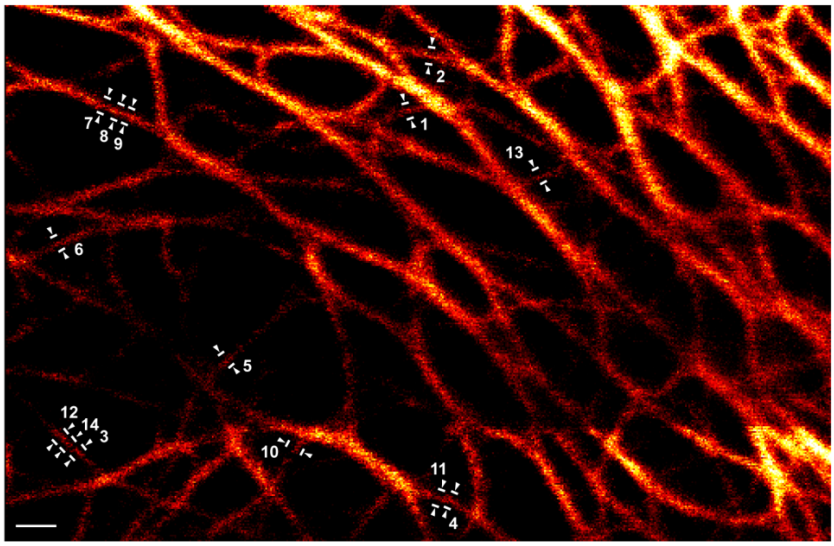
The on-switching step is carried out with a regularly focused beam of 430 nm wavelength at low intensity (0.5-2 kW/cm^2) in less than 500 µs. The proteins are switched off with a doughnut shaped beam of 592 nm at light intensities between 25 kW/cm^2 and 70 kW/cm^2. The off-switching time step is the time limiting step and takes between 2 ms and 10 ms, depending on the used laser intensity. Finally, the fluorescence of the proteins that were not switched off by the doughnut shaped beam (in the center) is read out with a focused beam of 561 nm. The time and the light intensity needed for this step depend on the brightness of the imaged structures. The reading-out step is carried out for 0.1 ms to 1 ms with light intensities between 1 kW/cm^2 and 15 kW/cm^2 and the fluorescence emitted between 600 nm and 640 nm is detected with a counting avalanche photodiode.



Supplementary figure 6 – Comparison between 561 nm and 592 nm for the off-switching of rsCherryRev1.4.

Intensity dependence of (a) the off-switching half-time and (b) the off-switching efficiency with 561 nm (black) and 592 nm (red) measured on vimentin-rsCherryRev1.4 filaments in living HeLa cells. As can be seen in (a) the switching speed is not affected by the wavelength; however, the efficiency is improved by a factor of three when using 592 nm instead of 561 nm light. The off-switching half-time is defined as the time needed to reach 50 % of the initial measured fluorescence. The off-switching efficiency was measured after 100 ms, when the off-switching process was completed for all light intensities used. The on-switching step was kept constant (0.5 ms, 0.7 kW/cm^2). The values shown are averaged values with their standard deviation for n = 3-5.

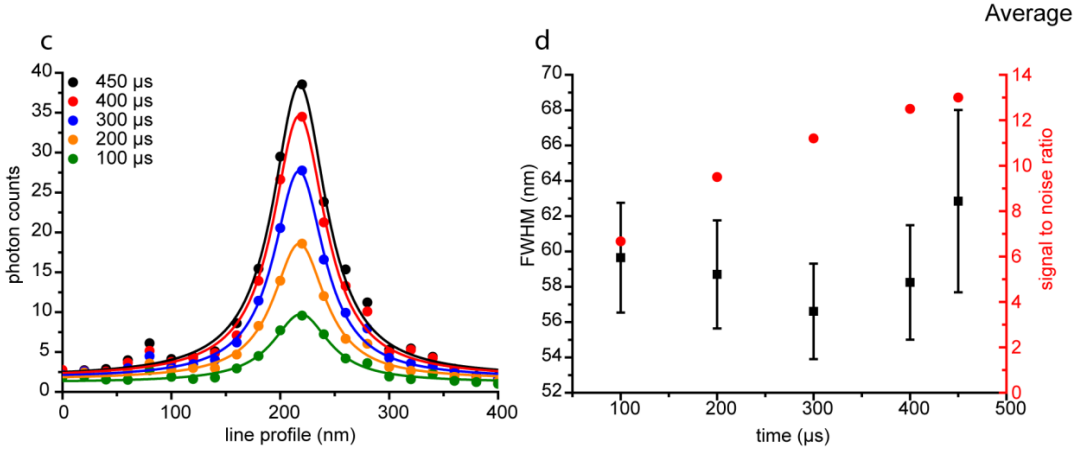
a



b

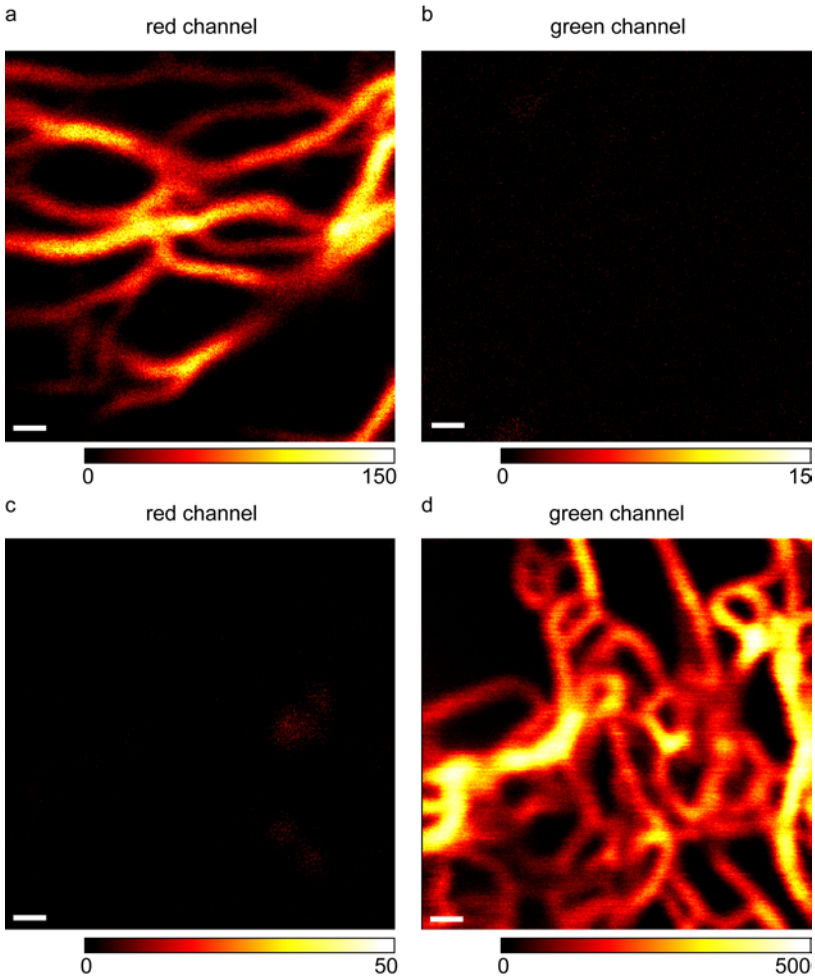
Profile #	FWHM (nm)
1	55
2	49
3	46
4	64
5	61
6	53
7	68
8	55
9	63
10	61
11	66
12	64
13	50

Average = 58 ± 7 nm



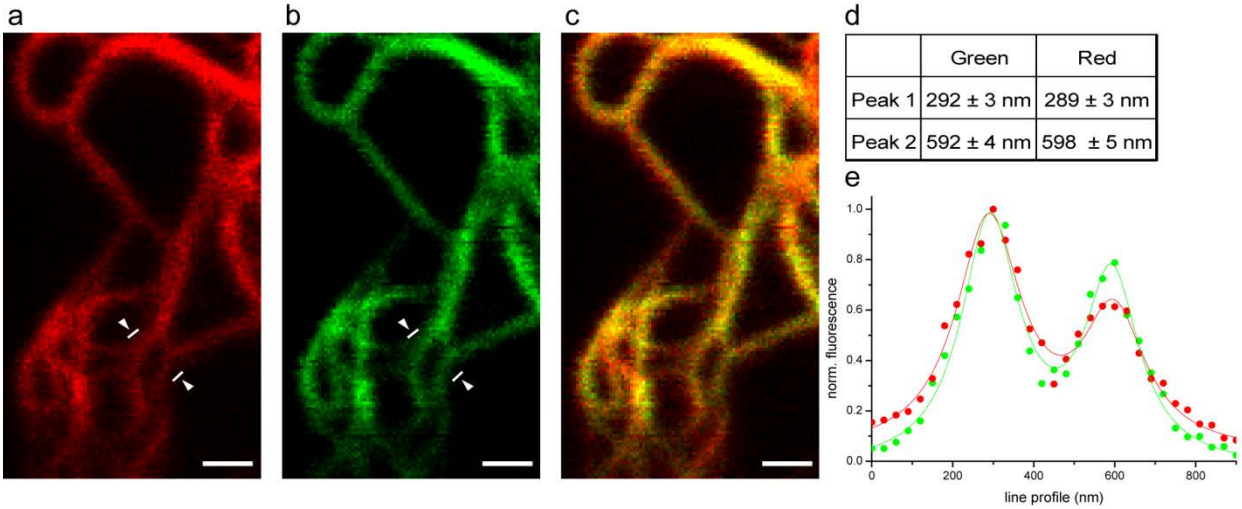
Supplementary figure 7 – Resolution of the single-beam point-scanning RESOLFT microscope with rsCherryRev1.4

(a-b) The resolution was determined by measuring the averaged line profile of 5 neighboring pixels (100 nm) across the regions marked between the arrows on a small filament of keratin19-rsCherryRev1.4. Thirteen different filaments were taken into account, fitted with a Lorentzian function; the average of the thirteen fits was calculated. As a result we determined a resolution of < 58 ± 7 nm (mean ± s.d.). (c) Influence of the recording time on the measured line profile for the filament 14 marked in image (a). At every pixel, the fluorescence signal was recorded for 450 µs with a time resolution of 50 µs. Hence subsequently the data could be analyzed for different recording times in order to optimize the signal to noise ratio (SNR) without degrading the obtained resolution. The resulting raw RESOLFT data (dots) were fitted with a Lorentzian function (solid line) and the FWHM was calculated for the different recording times analyzed. (d) FWHM (black) from the Lorentzian fit for the profiles shown in (c) and comparison with the SNR (red) measured for this filament. Increasing the recording time improves the SNR without strongly degrading the resolution. The recording time of 400 µs for image (a) was chosen, such that the SNR was > 12 for a resolution < 60 nm.



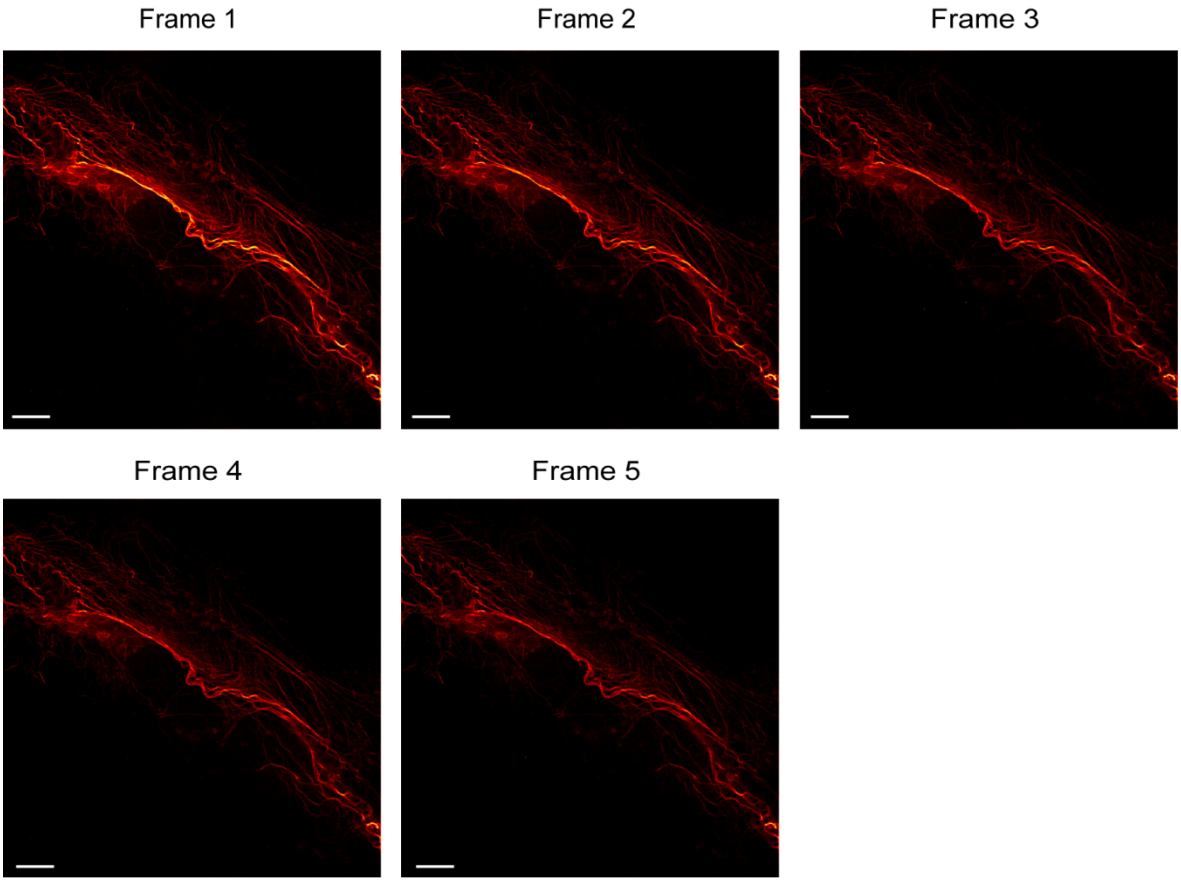
Supplementary figure 8– Fluorescence cross-talk for two-color point-scanning RESOLFT

(a-b) HeLa cells expressing vimentin-rsCherryRev1.4 imaged in the same measurement scheme as for two-color RESOLFT imaging, showing that no red fluorescence is recorded in the green detection channel. (c-d) HeLa cells expressing vimentin-DronpaM159T imaged in the same measurement scheme as for two-color RESOLFT images, showing that no green fluorescence is recorded in the red detection channel. All images were taken with typical laser intensities for fluorescence excitation (561 nm: 3.4 kW/cm^2 and 488 nm: 17 kW/cm^2) and with 50 µs recording time for each channel. Color scale**:** (a) 0 – 150**,** (b) 0-15**,** (c) 0-50**,** (d) 0-500 units. Scale bar 500 nm.



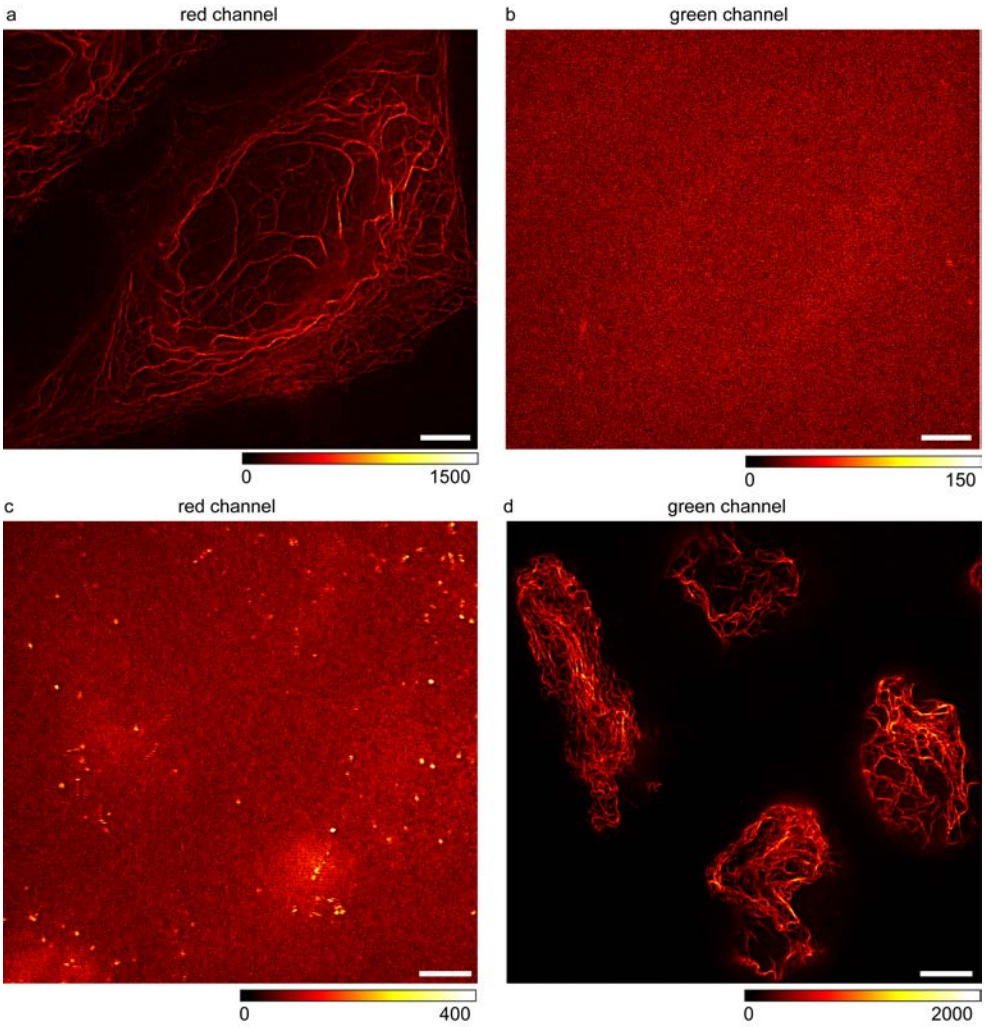
Supplementary figure 9 – Co-localization in the point-scanning two color RESOLFT setup.

RESOLFT image of a HeLa cell expressing vimentin-rsCherryRev1.4 (a) and vimentin-Dronpa-M159T (b) and overlay of both channels (c). RESOLFT images were taken according to the scheme shown in Fig. 4. (e) Line profiles corresponding to the region between the arrows in (a,b). The line profiles shown are the average over 5 neighboring pixels. The raw data are represented as dots and the Gaussian fits as solid lines for each channel. (d) Peak positions representing a measure for the co-localization were calculated from the Gaussian fits. Scale bar: 500 nm.



Supplementary figure 10 – Time lapse imaging with the parallelized RESOLFT Setup

Parallelized RESOLFT images of PtK2 cells expressing keratin19-rsCherryRev1.4. Five images were taken with a 10 s break between each image. The fluorescence decreases to ~ 60% of the original value after this time-lapse imaging, underscoring the photostability of rsCherryRev1.4. The field of view of 50 µm x 50 µm was reconstructed from 10 x 10 individual frames each. Frame dwell time: 112 ms. Imaging time for the whole field of view was approximately 14 s. Scale bars: 5 µm.



Supplementary figure 11 – Fluorescence cross-talk in the two-color parallelized RESOLFT microscope

(a -b) RESOLFT image of HeLa cells expressing keratin19-rsCherryRev1.4. No red fluorescence is recorded in the green detection channel. (c-d) RESOLFT image of HeLa cells expressing vimentin-Dronpa-M159T, imaged with the red and green detection channels, showing that no fluorescence is recorded in the red detection channel. Color scale: (a) 0 – 1500, (b) 0-150, (c) 0-400, (d) 0-2000 units. Scale bars: 5 µm.

Supplementary table 1 – Properties of rsCherryRev and rsCherryRev1.4

	rsCherryRev	rsCherryRev1.4
Absorption maximum off-state (nm)	572	572
Emission maximum (nm)	608	609
Off-switching half-time at 40W/cm^2 (ms)	200	220
Off-switching half-time at 17kW/cm^2 (ms)	12	5.7
Relative brightness in *E .coli* (norm. to rsCherryRev)	1	3

Supplementary table 2 – Imaging parameters (Fig. 3)

	on		off		reading out	
	Intensity (kW/cm^2)	**Time (ms)**	**Intensity (kW/cm^2)**	**Time (ms)**	**Intensity (kW/cm^2)**	**Time (ms)**
(a)	0.8	0.5	37	10	1.6	0.5
(b)	1.5	0.1	37	10	4.9	0.4
(c)	1.5	0.1	49	7	10	0.1
(d)	0.8	0.3	69	3	13.2	0.5

Supplementary table 3 – Imaging parameters (Fig. 6)

		on		off		reading out	
		Intensity (kW/cm^2)	**Time (ms)**	**Intensity (kW/cm^2)**	**Time (ms)**	**Intensity (kW/cm^2)**	**Time (ms)**
(a)	**red**	0.5	0.5	31	5	3	0.1
	green			5	0.5	12	0.1
(b)	**red**	0.5	0.5	26	7	6	1
	green			0.6	1.5	0.9	0.2