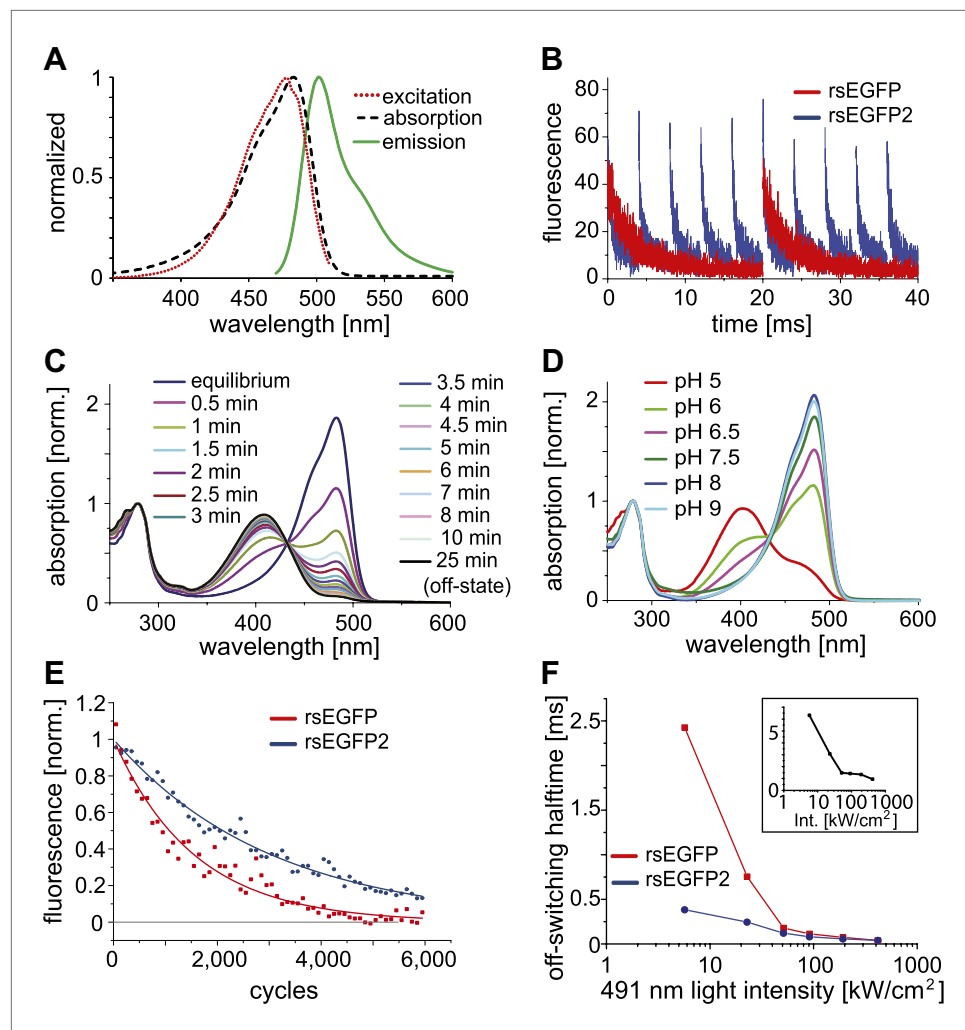


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## Figures and figure supplements

rsEGFP2 enables fast RESOLFT nanoscopy of living cells

**Tim Grotjohann, et al.**

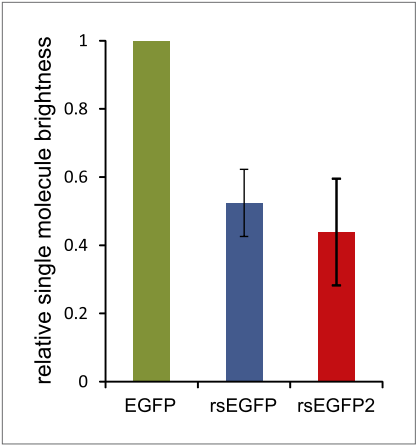


**Figure 1.** Characteristics of rsEGFP2. **(A)** Absorption (black dashed line), excitation (red dotted line), and emission (green solid line) spectra of rsEGFP2 in its equilibrium state at pH 7.5. **(B)** Switching curves of rsEGFP2 (blue) and rsEGFP (red). Switching was performed on purified proteins immobilized in a PAA-layer (pH ~6.5) by alternating irradiation with 491 nm (~2 kW/cm<sup>2</sup>) and 405 nm light (~2 kW/cm<sup>2</sup>, 40  $\mu$ s). Fluorescence was recorded only during irradiation with light of 491 nm. Each curve is an average over 10 switching cycles. **(C)** Changes in the absorption spectrum of rsEGFP2 upon switching with light of 488 nm from the equilibrium to the off-state. The spectra were taken at the indicated time points and recorded on purified rsEGFP2 at pH 7.5 **(D)** Absorption spectra of equilibrium-state rsEGFP2 at different pH values. The absorption bands at 408 nm and 503 nm presumably correspond to the protonated and the de-protonated cis-chromophore, respectively. **(E)** Switching fatigue of rsEGFP2 (blue) and rsEGFP (red). Switching was performed on living PtK2 cells expressing Vimentin-rsEGFP or Vimentin-rsEGFP2 by alternate irradiation with 405 nm (2 kW/cm<sup>2</sup>) and 491 nm (5.7 kW/cm<sup>2</sup>) light. Illumination times were chosen so that the fluorescence was fully switched to the minimum or maximum, respectively, in each cycle. Each plotted data point is the average (over 100 cycles) of the maximum fluorescence intensity in each cycle. The data points were fitted by a mono-exponential function, and the resulting curve was baseline corrected and normalized to 1. **(F)** Comparison of the ensemble off-switching halftimes (defined as the time after which the fluorescence reached 50% of its initial value) of rsEGFP (red) and rsEGFP2 (blue) at different 491 nm light intensities. On-switching 405 nm light was kept constant (3 kW/cm<sup>2</sup>). Data were collected on living PtK2 cells expressing Vimentin-rsEGFP or Vimentin-rsEGFP2, respectively. Inset: Graph showing the ratio (R) of the off-switching half-time of rsEGFP divided by the off-switching half-time of rsEGFP2 against the 491 nm light intensity.

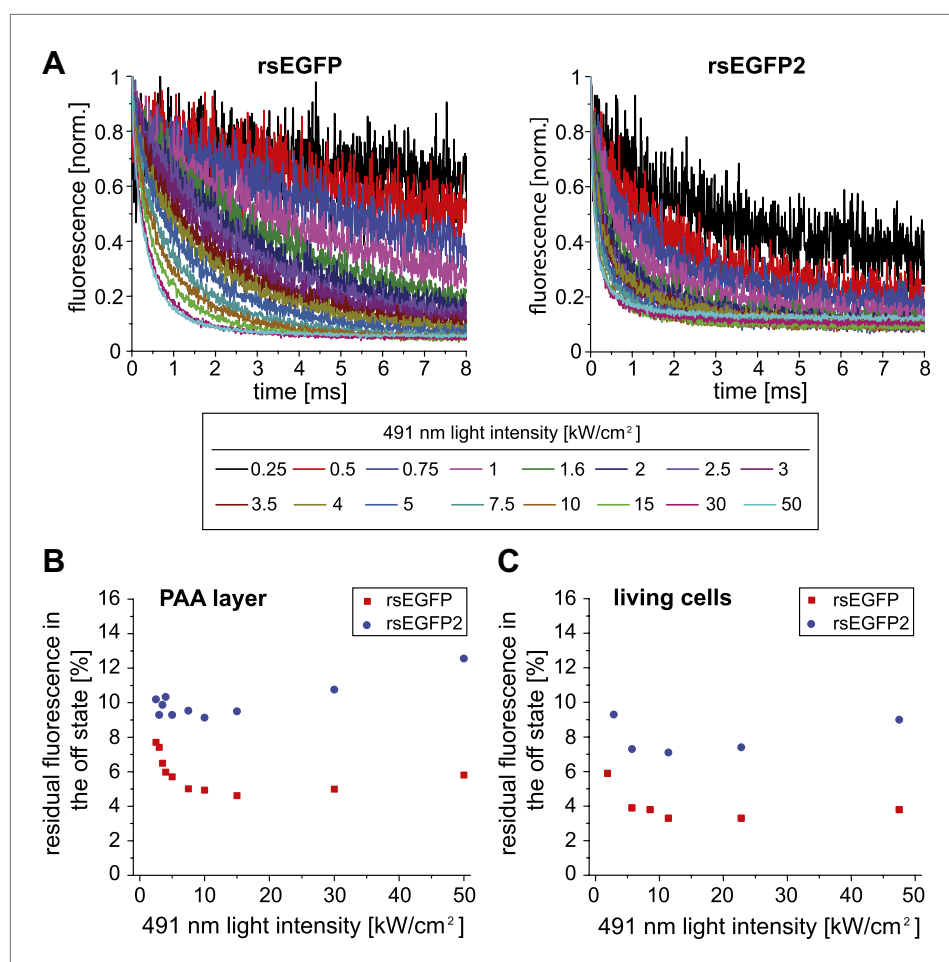
DOI: [10.7554/eLife.00248.003](https://doi.org/10.7554/eLife.00248.003)

	1	47
EGFP	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT	LKFI
rsEGFP	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT	LKFI
rsEGFP2	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT	LKFI
	48	65 69 95
EGFP	CTTGKLPVPWPTLVTTLT	YGVQCFSRYPDHMKQHDFFKSAMPEGYVQE
rsEGFP	CTTGKLPVPWPTLVTTLT	YGVLCFSRYPDHMKQHDFFKSAMPEGYVQE
rsEGFP2	CTTGKLPVPWPTLVTTLT	AYGVLCFSRYPDHMKQHDFFKSAMPEGYVQE
	96	143
EGFP	RTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY	
rsEGFP	RTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY	
rsEGFP2	RTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEY	
	144 150 163 191	
EGFP	NYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDG	
rsEGFP	NYNSHNVYIMADKQKNGIKSNFKIRHNIEDGSVQLADHYQQNTPIGDG	
rsEGFP2	NYNSHNVYIMADKQKNGIKSNFKIRHNIEDGSVQLADHYQQNTPIGDG	
	192 205 206 238	
EGFP	PVLLPDNHYLSTQSALSKDPNEKRDHMLLEFVTAAGITLGMDELYK	
rsEGFP	PVLLPDNHYLSTQNLKSKDPNEKRDHMLLEFVTAAGITLGMDELYK	
rsEGFP2	PVLLPDNHYLSTQSKLSDPNEKRDHMLLEFVTAAGITLGMDELYK	

**Figure 1—figure supplement 1.** Alignment of the amino acid sequences of EGFP (GenBank Accession #U55762), rsEGFP (GenBank Accession #JQ969017), and rsEGFP2. Differences are highlighted.  
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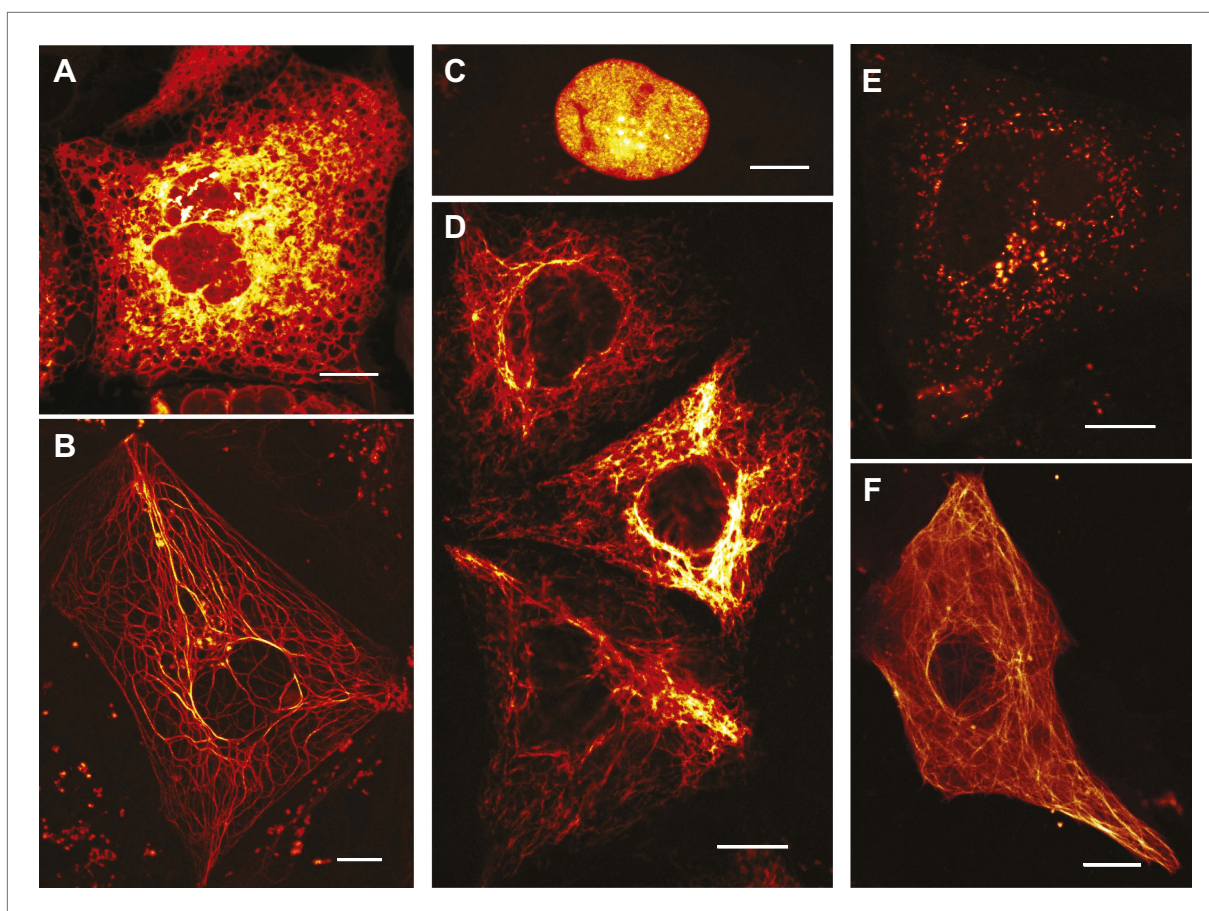


**Figure 1—figure supplement 2.** Single-molecule brightness values of EGFP, rsEGFP, and rsEGFP2 measured in PBS buffer (pH 7.5). Average and standard deviation of >30 FCS measurements at various laser intensities between 5 and 100 kW/cm<sup>2</sup>. Values normalized to EGFP. The error bars represent the error in the FCS experiments with the value for EGFP normalized to 1 in each individual experiment.  
DOI: 10.7554/eLife.00248.005



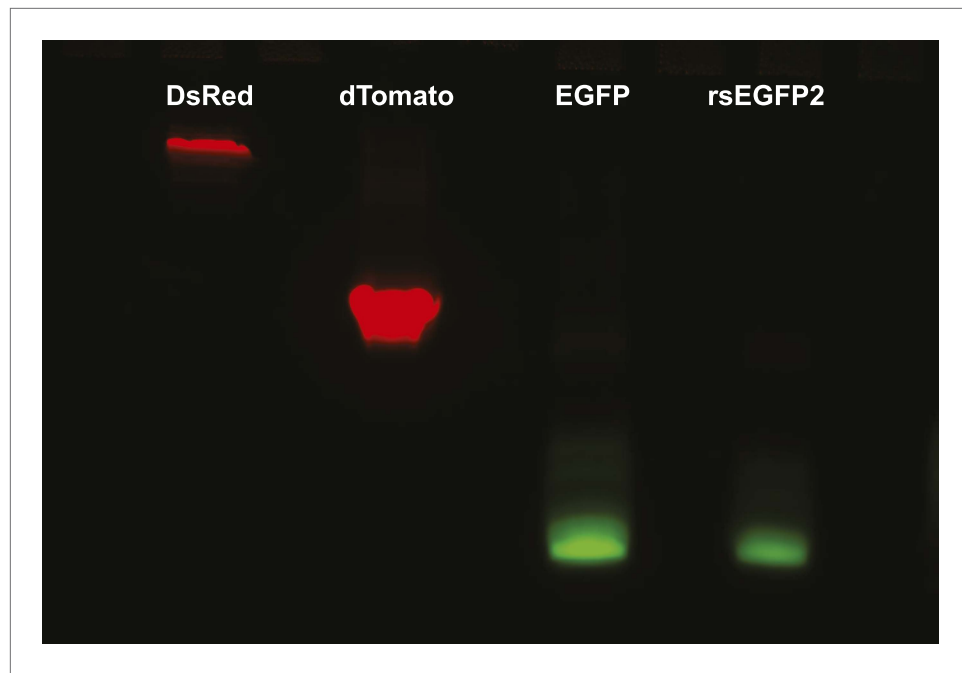
**Figure 1—figure supplement 3.** Off-switching speed of rsEGFP and rsEGFP2. **(A)** Off-switching kinetics of rsEGFP and rsEGFP2 embedded in a PAA layer (pH ~6.5) determined at different intensities of the 491 nm off-switching light. Each curve is an average of 100 measurements. In each measurement the proteins were switched into the on-state for 40  $\mu$ s with 405 nm light (2 kW/cm<sup>2</sup>) and subsequently the decay of fluorescence was recorded over time at the indicated 491 nm light intensities. **(B)** Dependence of the residual fluorescence (off-state fluorescence) in the ensemble off-state as a function of the off-switching light intensity. **(C)** Dependence of the residual fluorescence (off-state fluorescence) in the off-state of Vimentin-rsEGFP or Vimentin-rsEGFP2 in living Ptk2 cells as a function of the off-switching light intensity.

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**Figure 2.** Expression of various functional rsEGFP2 fusion proteins in mammalian cells. **(A)** rsEGFP2-KDEL (targeting to the ER), **(B)** Keratin19-rsEGFP2, **(C)** Histone H2B-rsEGFP2, **(D)** Vimentin-rsEGFP2, **(E)** Pex16-rsEGFP2, and **(F)** rsEGFP2- $\alpha$ -tubulin. Shown are single confocal sections (**C**, **F**) and maximum intensity projections of confocal images (**A**, **B**, **D**, **E**) recorded on living cells. Fluorescence was excited by simultaneous irradiation with light of 488 nm and 405 nm. **(A–E)**: PtK2 cells; **(F)**: Vero cell. Scale bars: 10  $\mu$ m.

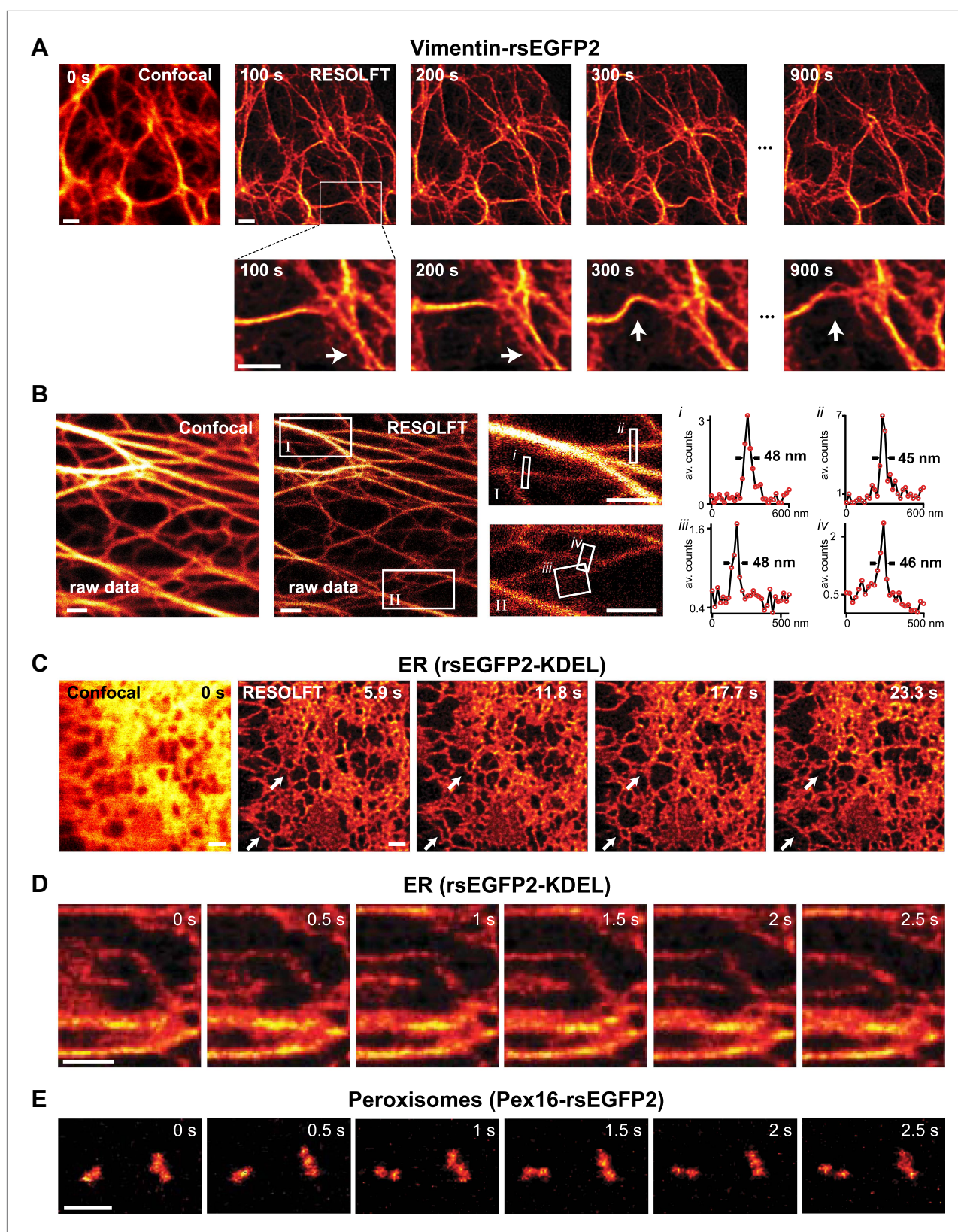
DOI: [10.7554/eLife.00248.008](https://doi.org/10.7554/eLife.00248.008)



**Figure 2—figure supplement 1.** Semi-native polyacrylamide gel electrophoresis of rsEGFP2. Purified monomeric EGFP, dimeric dTomato, tetrameric DsRed, and rsEGFP2 were separated on a semi-native gel (a two-phase polyacrylamide gel) consisting out of a 12.5% separation gel (6.3 ml H<sub>2</sub>O, 5 ml 1.5 M Tris-HCl pH 8.8, 8.3 ml Rotiphorese Gel 30 solution [Roth, Karlsruhe, Germany], 200  $\mu$ l 10% [wt/vol] sodiumdodecyl sulphate [SDS], 200  $\mu$ l 10% [wt/vol] ammonium persulfate (APS), 20  $\mu$ l Tetramethylethylenediamin [TEMED]) and a 5% loading gel (5.6 ml H<sub>2</sub>O, 2.5 ml 1.5 M Tris-HCl pH 6.8, 1.7 ml Rotiphorese Gel 30 solution, 100  $\mu$ l 10% [wt/vol] SDS, 100  $\mu$ l 10% [wt/vol] APS, 10  $\mu$ l TEMED). Images were taken with a custom-built gel documentation system. To detect green fluorescence (EGFP and rsEGFP2) the gel was irradiated with light of  $470 \pm 5$  nm and fluorescence was detected at  $525 \pm 30$  nm. To detect red fluorescence (dTomato and DsRed) the gel was irradiated with light of  $545 \pm 10$  nm and fluorescence was recorded at  $617 \pm 37$ . Both images were overlaid and are represented in false colors.

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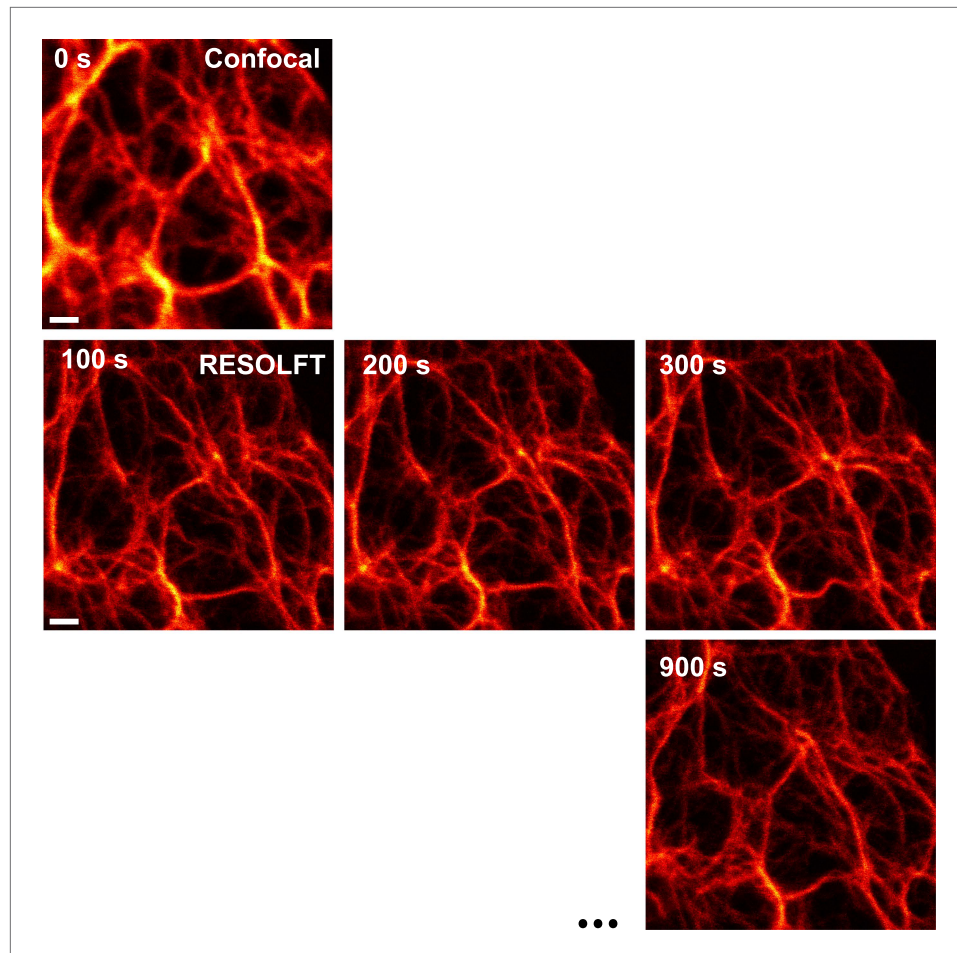


**Figure 3.** RESOLFT time lapse imaging using rsEGFP2 in living mammalian PtK2 cells. **(A)** Cells expressing Vimentin-rsEGFP2: initial confocal (left) and subsequent RESOLFT images taken every 100 s. Lower row: magnifications of the indicated areas. **(B)** Lateral resolution measurement: raw images of Figure 3. Continued on next page

## Figure 3. Continued

cells expressing Keratin19-rsEGFP2 recorded with a RESOLFT Quad P microscope (Abberior Instruments GmbH, Göttingen, Germany) with similar imaging conditions as in (A) (on: 405 nm, 5 kW/cm<sup>2</sup>, 20 μs; off: 488 nm, 34 kW/cm<sup>2</sup>, 360 μs; read-out: 488 nm, 76 kW/cm<sup>2</sup>, 20 μs). From left to right: confocal raw image and corresponding raw RESOLFT image. Magnifications of the boxed areas in the RESOLFT image. The graphs show averaged line profiles across the indicated filaments (*i–iv*) within the respective boxes. The line profiles used for averaging were taken equidistant (20 nm) along the whole respective indicated area. (C),(D) rsEGFP2 targeted to the ER (rsEGFP2-KDEL): (C) 10 μm × 10 μm initial confocal (left) and subsequent RESOLFT images recorded every 5.9 s, and (D) 2.8 μm × 3.2 μm RESOLFT image-series imaged at 2 Hz. (E) RESOLFT imaging of peroxisomes labeled by Pex16-rsEGFP2 fusion proteins. Pixel step sizes: 20 nm (A, B) and 40 nm (C–E). Pixel dwell times: 380 μs (A), 400 μs (B), 75 μs (C, D), and 120 μs (E). In (D) and (E) pixels were interpolated to a size of 20 nm × 20 nm. The arrows indicate moving structures. Richardson Lucy restoration was used for all RESOLFT images except (B). Scale bars: 1 μm.

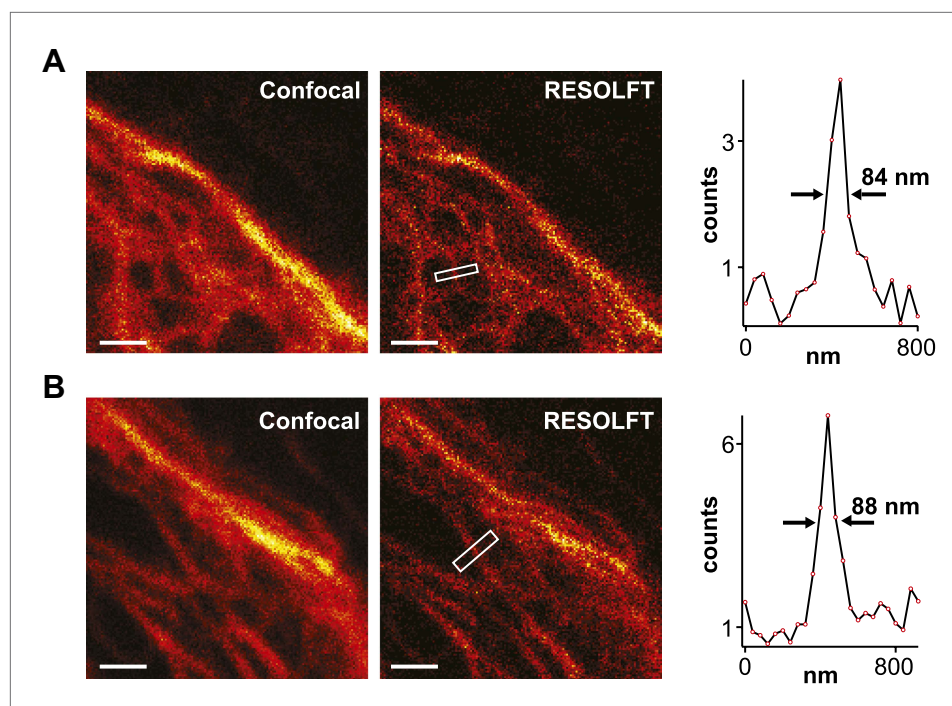
DOI: [10.7554/eLife.00248.010](https://doi.org/10.7554/eLife.00248.010)



**Figure 3—figure supplement 1.** Raw RESOLFT images of **Figure 3A**. No image processing was applied. Shown are PtK2 cells expressing Vimentin-rsEGFP2: initial confocal (left) and subsequent RESOLFT images taken every 100 s. Scale bar: 1 μm.

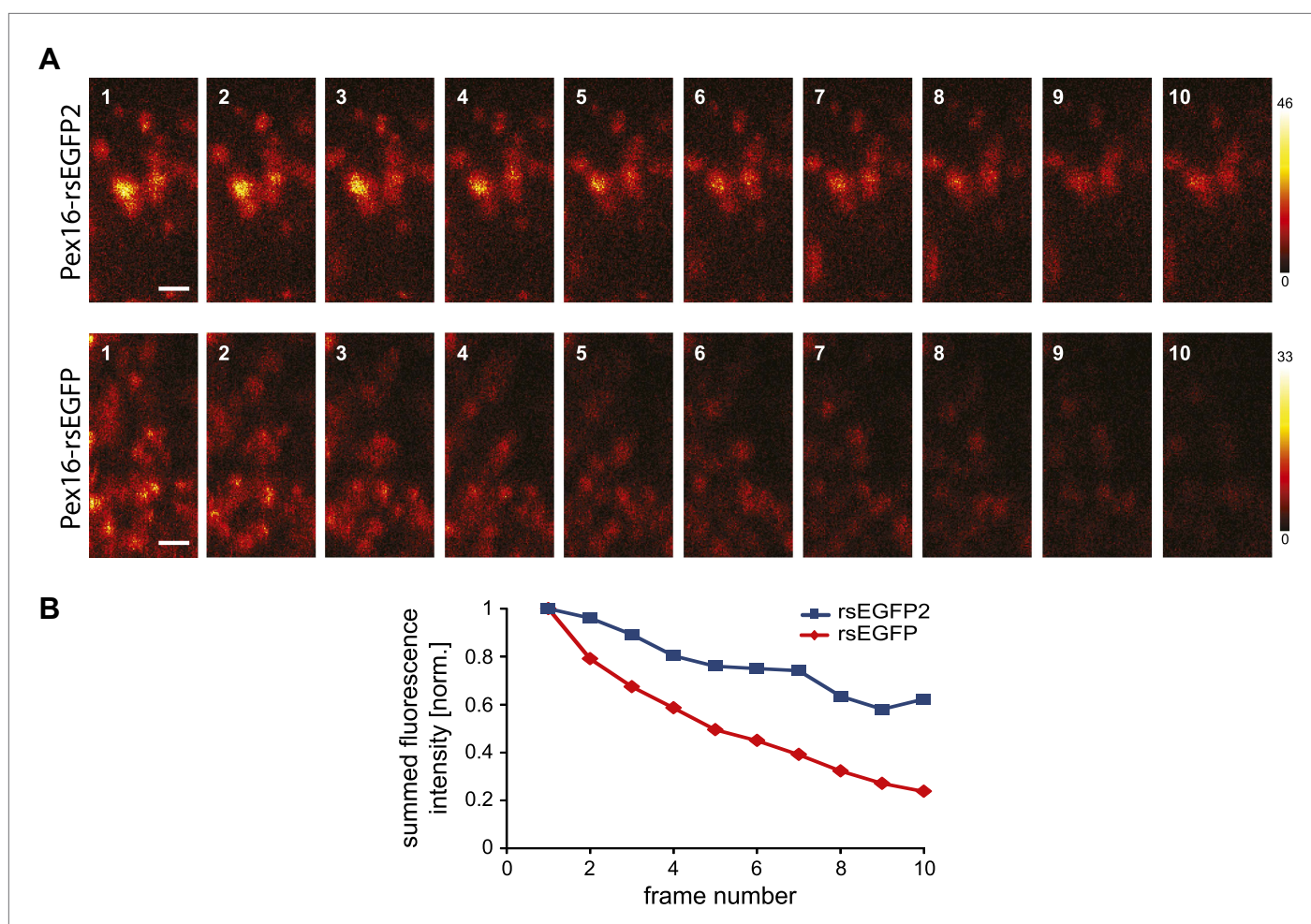
DOI: [10.7554/eLife.00248.011](https://doi.org/10.7554/eLife.00248.011)





**Figure 3—figure supplement 2.** Lateral resolution in fast RESOLFT imaging. (A),(B) Typical examples. Shown are raw images of cells expressing Keratin19-rsEGFP2 taken on a RESOLFT Quad P microscope (Abberior Instruments GmbH, Göttingen, Germany) with a pixel dwell time similar as in **Figure 3D,E**. Imaging conditions: (on: 405 nm, 8 kW/cm<sup>2</sup>, 2 μs; off: 488 nm, 68 kW/cm<sup>2</sup>, 61 μs; read-out: 488 nm, 200 kW/cm<sup>2</sup>, 7 μs; pixel size: 40 × 40 nm). From left to right: confocal raw image and corresponding raw RESOLFT image. The graphs show averaged line profiles across the indicated filaments within the respective boxes. The line profiles used for averaging were taken equidistant (40 nm) along the whole respective indicated area. Scale bar: 1 μm.

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**Figure 3—figure supplement 3.** Comparison of rsEGFP and rsEGFP2 at RESOLFT imaging conditions. **(A)** Repeated imaging of peroxisomes labeled by Pex16-rsEGFP or Pex16-EGFP2 fusion proteins. Imaging conditions were as in **Figure 3D**. Pixel step size: 40 nm; on: 405 nm, 4 kW/cm<sup>2</sup>, 20  $\mu$ s; off: 491 nm, 20 kW/cm<sup>2</sup>, 50  $\mu$ s; read-out: 491 nm, 76 kW/cm<sup>2</sup>, 5  $\mu$ s; pixel dwell time: 75  $\mu$ s. Images were taken every 5 s. Shown are raw data. **(B)** Decay of the summed fluorescence intensities of the images shown in **(A)**. Note that rsEGFP photobleaches faster than rsEGFP2. Scale bar: 1  $\mu$ m.

DOI: [10.7554/eLife.00248.013](https://doi.org/10.7554/eLife.00248.013)