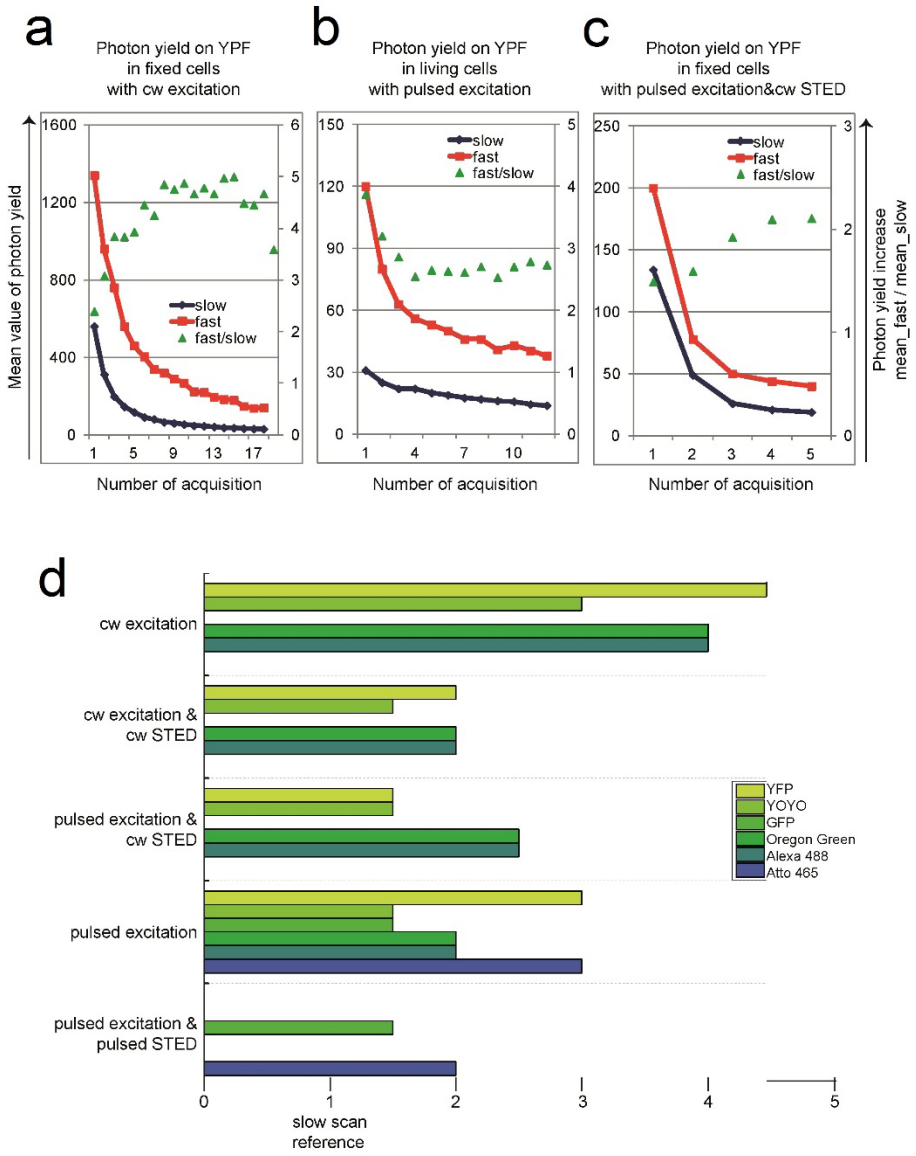
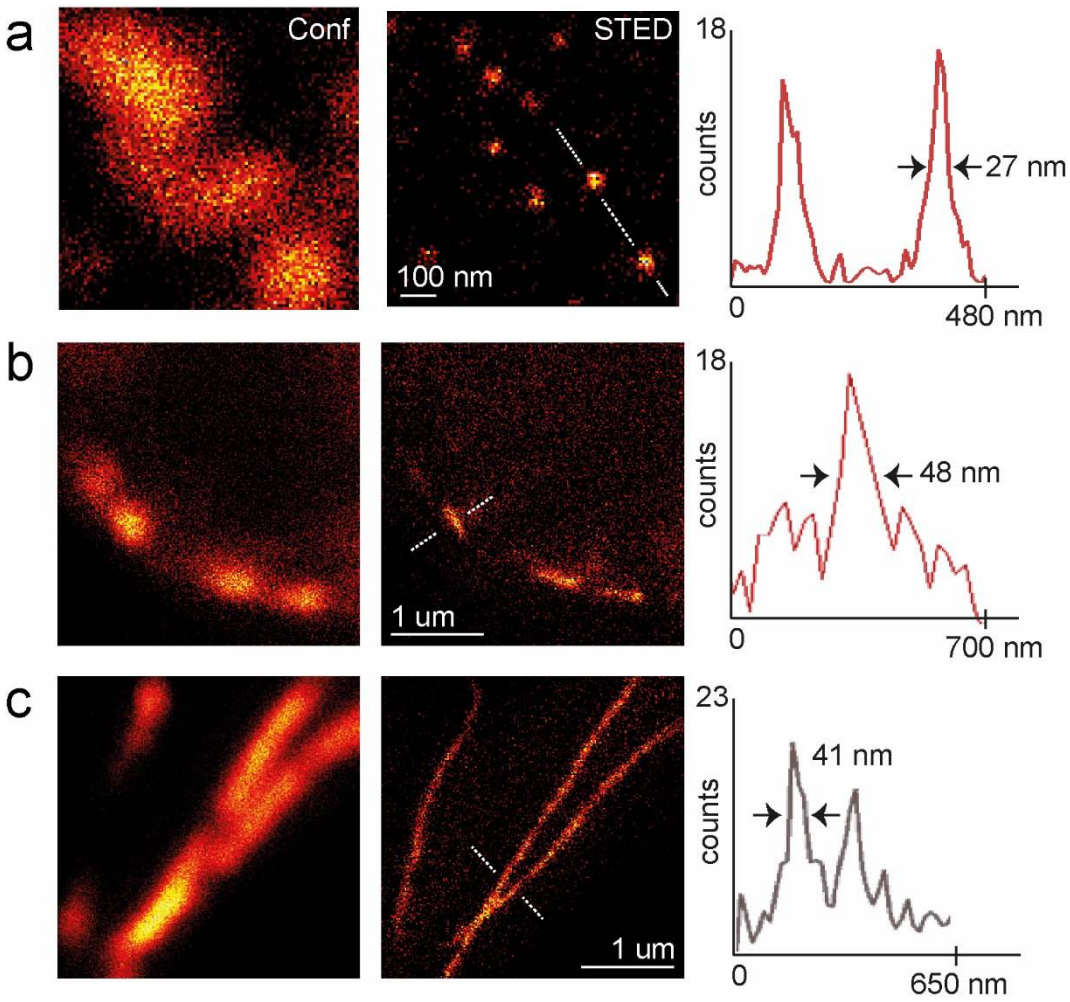


Supplementary Figure 1

Increased photon yield for several fluorophores by ultrafast scanning compared to the conventional slow-scanning mode.

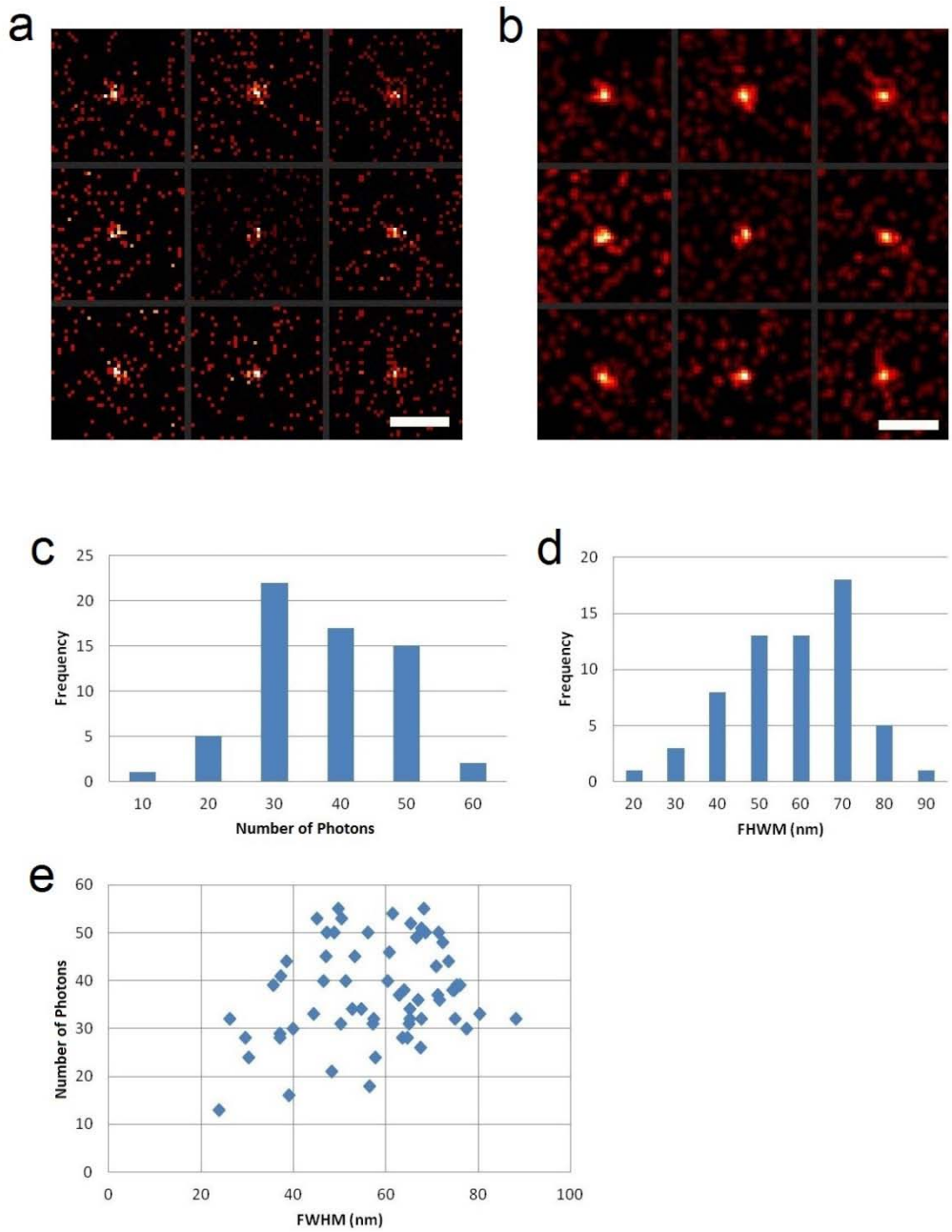
More than 40 comparative measurement series were performed in order to assess to which extent our fast scanner reduced bleaching. Samples with different labeling were studied with the different available laser configurations. For a single measurement, areas of the same size and of originally similar brightness within the same sample were scanned fast and slow respectively. The total image acquisition duration was kept the same for both cases to ensure the same light dose over the area. The pixel dwell time was 6.25 ns for the ultrafast scanning and 20 µs for the slow-scanning modality. Fluorophore molecules gradually bleached both in ultrafast- and slow-scanned areas, but the photon yield per applied light dose in ultrafast-scanned areas was clearly higher. (**a**-**c**) Examples of three measurement series on samples expressing yellow fluorescent protein (YFP): (**a**) labeled mitochondriae in fixed cells, subjected to continuous-wave (CW) excitation. (**b**) living neurons (cytosolic expression) in brain slices, subjected to pulsed excitation. (**c**) fixed neurons (cytosolic expression), subjected to pulsed excitation and CW-STED. The left axis shows the mean value of detected photons in the scanned areas after each consecutive scan. After each measurement series, the mean number of photons collected in the fast scanned area was divided by the mean number of photons in the equivalent slow-scanned area. High peak power of pulsed lasers presumably caused a significant amount of bleaching directly from the excited state before the molecules could cross to the triplet state. Thus, allowing triplet relaxation had a smaller effect. The obtained values are shown as green triangles (right axis of the graphs). These signal increase factors over many different analyzed samples are summarized in (**d**) with the photon yield in slow scanning given as 1.



Supplementary Figure 2

Resolution in ultrafast scanning STED nanoscopy.

Confocal and STED images of (**a**) yellow green fluorescent beads with a diameter of 24 nm, (**b**) MreB-rsEGFP in eisosomes in living *E. coli*, and (**c**) single-strand DNA labeled with the dye YOYO acquired by electro-optic temporally stochastic STED recordings. The line profiles indicate the resolution performance in the ultrafast scanning setup.



Supplementary Figure 3

Temporally stochastic STED imaging of single antibodies conjugated to Atto 647N.

(**a**) Examples of raw data and (**b**) data smoothed with a Gaussian filter (width of 2 pixels). Scale bars: 300 nm. (**c**) Histogram of numbers of detected signal photons per antibody for n=62 separate single-antibody scans. (**d**) Histogram of full-widths at half-maximum (FWHM) inferred by fitting symmetric Gaussian functions (with variable additive offset) to the smoothed data, for the same 62 single antibodies. (**e**) Number of signal photons vs. FWHM. The data show a mean resolution of ~58 nm for a mean of ~38 photons per antibody detected, at a background of ~0.06 photons per pixel (or ~0.6 photons per FHWM area). These data were recorded with fluorescence excitation at 640 nm (8 μW in back focal plane, 20 MHz) and STED at 775 nm (37.5 mW in back focal plane, 20 MHz) using a Leica 100× 1.46 NA oil immersion objective. (Pixel size: 17 nm, 30 000 frames of 5 ns dwell time, i.e. 150 μs effective pixel dwell time).