Electronic Supplementary Material (ESI) for Photochemical & Photobiological Science

Supporting information

A photochromic bacterial photoreceptor with potentialfor super-resolution microscopy

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Experimental Methods

His_6-tag furnished YtvA was expressed in E. coli BL21 cells from the pET28a vector (Novagen-Merck, Darmstadt, Germany) as previously described. [1] The protein was purified by affinity chromatography on a Talon resin (Qiagen, Hilden, Germany) and finally concentrated in Na-phosphate buffer 10 mM, NaCl 10 mM, pH = 8. Solutions were prepared in a 10 mM phosphate buffer at pH = 7. Absorption spectra were recorded using a JASCO 7850 spectrophotometer. Fluorescence emission and excitation spectra were collected using a Perkin Elmer LS50. Both instruments were thermostated using a circulating water bath.

LEDs used in the experiments had emission peaks at 356 nm (LED356), 405 nm (LED405), and 465 (LED465) and FWHM of ~ 25 nm (Roithner Lasertechnik GmbH, Vienna, Austria). The emission curves are reported as Figure S1 in Supporting Information. In photoconversion experiments, the LED emission covered the whole volume of the liquid inside the cuvette.

Nanosecond laser flash photolysis

The laser flash photolysis setup was partly described elsewhere. [2] Excitation at 355 nm is achieved with the output of a Q-switched nanosecond Nd:YAG laser (Spectron Laser).

Absorbance changes are monitored using a monochromatic cw output of a 150 W Xe arc lamp coupled to a 0.25-m monochromator (AMKO GmbH). After passing through the sample cuvette, the monochromatic probe beam is focussed onto the entrance slit of a second, 0.125-m monochromator (77250, LOT-Oriel) and then is finally detected by a 5 stages photomultiplier (Applied Photophysics). The voltage signal is digitized by a digital oscilloscope (LeCroy Waverunner 104-Xi, 5 GS/s; 1 GHz). A shutter (Vincent Associates, Uniblitz VS35 controlled by the driver VMM-T1) is positioned between the output of the first monochromator and the sample holder. The LED465 is placed on top of the cuvette in order to keep the sample in the YtvAL state. A second shutter is used to pick laser pulses at a repetition rate of 1 shot per minute to allow for full recovery of the YtvAL state between subsequent laser shots. The synchronization of the laser and the shutters is controlled by a digital delay generator (Berkeley Nucleonics). The sample holder is accurately temperature controlled with a Peltier element, allowing a temperature stability of better than 0.1 °C (Flash100, Quantum Northwest).

Time resolved photoacoustics

The time resolved photoacoustics setup was described earlier. The pump laser was the same as the one described above. The cuvette holder FLASH 100 (Quantum Northwest, Spokane, WA, USA)

was temperature controlled to ± 0.02 °C. The signal was detected by a V103-RM transducer and fed into a 5662 preamplifier (Panametrics Inc., Waltham, MA, USA). The pulse fluence was varied with a neutral density filter and measured with a pyroelectric energy meter (RJP735 head connected to a meter RJ7620 from Laser Precision Corp.). The beam was shaped by a 1 × 12 mm slit, allowing a time resolution of ~50 ns by using deconvolution techniques. The experiments were performed in the linear regime of amplitude versus laser fluence and the total incident energy normally used was ~40 μJ/pulse. The LED465 was placed on top of the cuvette in order to keep the sample in the YtvAL state, averaging then 100 shots. For detecting photoacoustic signals originating from YtvAD, we averaged 10 shots, stirring the sample to avoid appreciable accumulation of YtvAL. At the end of the experiments less than 5% of YtvAD was photoconverted, as proven by recording absorption spectra.

New coccine was used as photocalorimetric reference compound. [3] The deconvolution analysis of the photoacoustic waveforms was performed according to previously described methods. [4] Fractions of absorbed energy released as heat and volume changes associated with each kinetic step were retrieved using a two-temperature method. [5]

Individual molecule localization set-up and FPALM analysis:

PALM imaging was performed on an inverted fluorescence microscope (NIKON TiE/B). An activation laser (Coherent CUBE 405-100mW) and a readout laser (Coherent Sapphire OPSL 488nm or Coherent Sapphire OPSL 561nm-200mW) lasers are coupled into a collinear path by a dichroic filter (Chroma Tech. Z488RDC). Imaging is performed using a 100x 1.4 NA Nikon objective lens on a back-illuminated electron-multiplying charge-coupled device (CCD) camera (Andor Ixon DU–897E–CS0BV) running at approximately 25 Hz (45 ms exposure time). Dichroic mirrors (Chroma, T505LP) and band-pass dichroic filters allowed selection of the emitted signal (Semrock BLP01-488R-25). Analysis of localized molecules position and rendering of PALM images are performed using MATLAB (The Mathworks). The molecules position is found by means of a gaussian fitting procedure [6] and the localization precision is calculated according to Mortensen et al. 2010[7]. Such a model provides an estimation of the localization precision taking into account also for the excess noise introduced by electron multiplying process

of the EMCCD. Before the identification of single molecule events, a roll-ball background subtraction is performed to minimize the effects due to unspecific signal and autofluorescence. The rendering of the super-resolution image is obtained plotting the position of each single event as a gaussian spot with standard deviation corresponding to the calculated localization precision. Before

the rendering of the final image, a filter on brightness and molecule dimension is applied and unsuitable events are rejected.

E.Coli cells were placed on a sterilized and Poly-L-Lysine-coated glass slide. The bacteria were immobilized using 1% agarose gel as mounting medium.

FLIM analysis

Lifetime maps were collected under two-photon excitation by means of a time-correlated single-photon counting (TCSPC) approach using a SPC-730 module (Becker & Hickl, Berlin, Germany). Two-photon excitation was performed using the infrared port of the Leica TCS STED-CW microscope directly coupled, by means of a custom-made optical pathway, to a fs-pulsed Ti:sapphire laser Chameleon Ultra II (Coherent, Santa Clara, CA, USA) operating at 770nm nm and at 80 MHz of repetition rate.

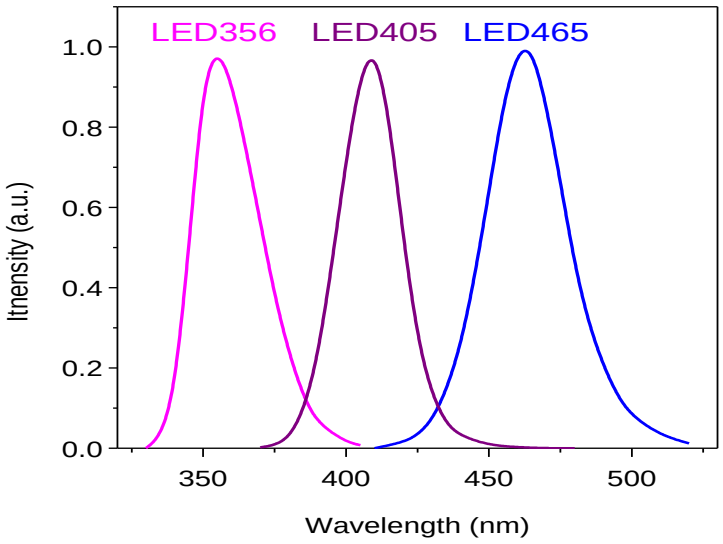

Figure S1. Emission spectra of LEDs used in the bulk photoconversion experiments. Magenta, LED356; purple, LED405; blue, LED465.

SO(YtvAD) and *SO(YtvAL)* are defined as the spectral overlaps between the LED emission and the absorption spectra of YtvAL and YtvAD, respectively and are represented as the shaded areas in Figure S2. These quantities represent the efficiencies of the molecular species in absorbing incoming photons.

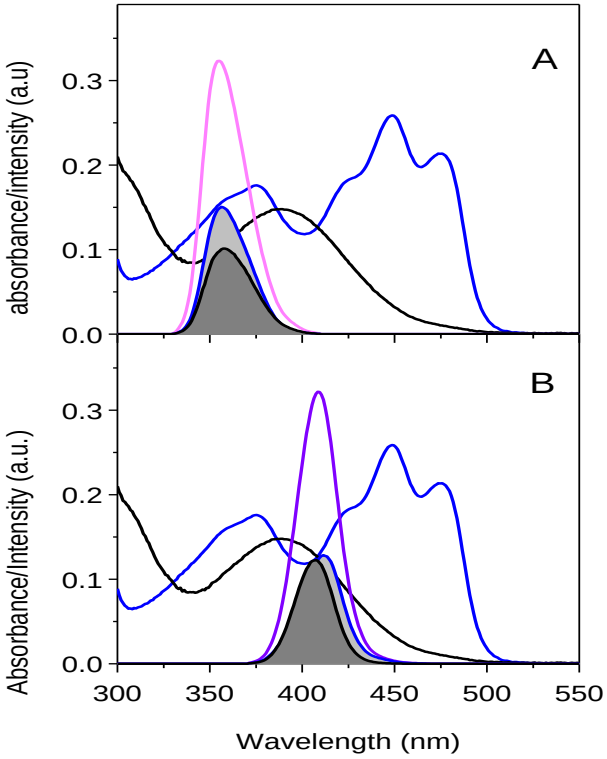

Figure S2. Spectral overlaps between the absorption spectra of YtvAD (blue), and YtvAL (black) and the 356 nm (A, light pink) and the 405 nm (B, purple) LEDs. The spectral overlaps are represented as shaded areas with the contour coloured as the corresponding absorption spectrum.

The spectra overlaps *SO(YtvAD)* and *SO(YtvAL)*, highlighted as the shaded areas in the plots, can be calculated as the integrals of the products between LED emissions and the corresponding absorption spectra.
The ratio *SO(YtvAD)*/*SO(YtvAL)* can then be calculated for LED356

$$\frac{SO(YtvAD)}{SO(YtvAL)} = 1.39$$

and for LED405

$$\frac{SO(YtvAD)}{SO(YtvAL)} = 0.913$$

Kinetic model for the establishment of the photoequilibrium

After collecting the kinetics at a given LED power, LED465 was applied for 5 minutes to ensure complete recovery of the YtvAL state before submitting the sample to the following illumination cycle with a different power of either LED356 or LED405. When the near UV or violet light is switched on, fluorescence emission is progressively recovered and, after some time, it saturates. The saturating level of fluorescence emission decreases as the LED power is increased, due to competition with the thermal relaxation of YtvAL to YtvAD. At high LED power the saturating level is independent of the LED power. The rate at which the saturating level is attained increases with the LED power. The above facts are consistent with bidirectional photoswitching between YtvAL and YtvAD induced by LED405 (LED356).

The kinetic model adopted to describe the establishment of the photoequilibrium is detailed below.[8, 9]

The concentrations of YtvAD and YtvAL are indicated as D = [*YtvAD*], L = [*YtvAL*], $YtvA_{tot}$ is a constant equal to [*YtvAD*]+[*YtvAL*].

The rate of change in D and L can be expressed as:

$$\begin{cases} \dfrac{dD}{dt} = \dfrac{S\Phi}{V}\left(-\dfrac{A_D}{A_D + A_L}\Phi_{DL} + \dfrac{A_L}{A_D + A_L}\Phi_{LD} \right) + k_{LD}^{d} L \\ \dfrac{dL}{dt} = \dfrac{S\Phi}{V}\left(\dfrac{A_D}{A_D + A_L}\Phi_{DL} - \dfrac{A_L}{A_D + A_L}\Phi_{LD} \right) - k_{LD}^{d} L \end{cases}$$

where Φ is the flux of incoming photons, V is the illuminated volume, A_D and A_L are the absorbances at the excitation wavelength (405 nm or 356 nm) of YtvAD and YtvAL, respectively, k_{LD}^{d} is the rate constant for the reverse dark reaction.

Since the following relations hold:

$$\frac{dL}{dt} = -\frac{dD}{dt}$$

$$L + D = YtvA_{tot} \Rightarrow L = YtvA_{tot} - D$$

we can solve only the rate equation for YtvAD.

Since the fluorescence quantum yields for YtvAD and YtvAL are:

$$\Phi_{F,D} = 0.22;\ \Phi_{F,L} = 0$$

Then the fluorescence emission from a solution containing a mixture of YtvAD and YtvAL only arises from molecules in the YtvAD state.

$$F = F_D = \Phi_{F,D}\left(1-10^{-A_D}\right)I_0 \approx \Phi_{F,D}\left(A_D \ln 10\right)I_0 = \Phi_{F,D}\left(\varepsilon_D D l \ln 10\right)I_0$$

Fluorescence intensity is fitted using the above expression, where D is obtained by integration of:

$$\frac{dD}{dt} = \frac{S\Phi}{V}\left(-\frac{A_D}{A_D + A_L}\Phi_{DL} + \frac{A_L}{A_D + A_L}\Phi_{LD}\right) + k_{LD}^{d} L$$

Which is further simplified by using: $L=Ytva_{tot}-D$

$$\frac{dD}{dt} = \frac{S\Phi}{V}\left(-\frac{A_D}{A_D + A_L}\Phi_{DL} + \frac{A_L}{A_D + A_L}\Phi_{LD}\right) + k_{LD}^{d} L$$

and considering that:

$$\Phi = \frac{P}{S}\left(1-10^{-A}\right) \approx \frac{P}{S} A \ln 10 = \frac{P}{S}\left(A_D + A_L\right)\ln 10$$

where P is the light power, V is the illuminated volume, thus the differential equation which accounts for the time course of the concentration of YtvAD becomes:

$$\frac{dD}{dt} = \frac{P}{V}\ln 10\left(-A_D\Phi_{DL} + A_L\Phi_{LD}\right) + k_{LD}\left(YtvA_{tot} - D\right)$$

A similar relation holds for the concentration of YtvAL.

Saturation curves measured at different LED powers were globally analyzed and fitted to the solution of the above rate equation by optimizing parameter Φ_{DL}, the other parameters being known quantities.

The stability in spectral properties of the photoreceptor under steady state illumination is noticeably high, as confirmed by the data reported in Figure S3 where YtvA was repeatedly subjected to photoconversion cycles between YtvaD and YtvAL using LED465 and LED405, for about 3000 s. The change in the fluorescence emission (or absorption at 450 nm) after reactivation was very small indicating that irreversible bleaching was below 5 %.

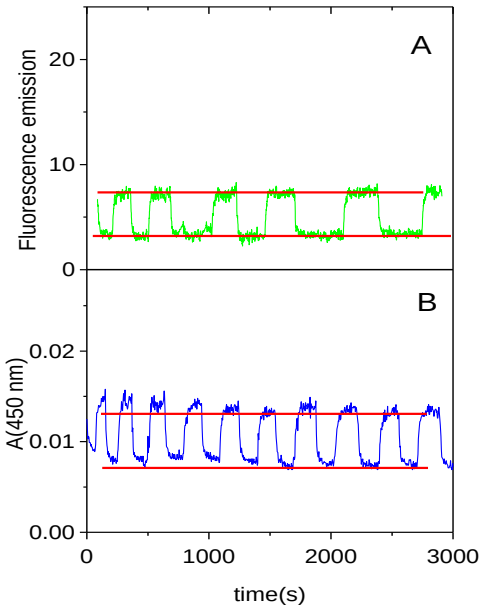

Figure S3. Fluorescence emission (A, excitation at 330 nm, detection at 500 nm) and absorbance at 450 nm (B) of an YtvA solution subjected to repeated bleaching/reactivation cycles between YtvAL and YtvAD. YtvAL was generated with LED465 (2.7 mW) while YtvAD was generated with LED405 (5.4 mW). Red solid lines are drawn to indicate the levels of the signals for the photoequilibrium mixture YtvAD/YtvAL (high fluorescence, high absorbance) and YtvAL (low fluorescence, low absorbance). The Fluorescence emission at t=0 represents the signal for the fully YtvAD sample. The drift in the absorption values is due to instrumental instability and not to bleaching of the chromophore, as indicated by the stability in the changes in absorbance between the D and L states.

Sub-diffraction localization of individual YtvA molecules

Collection and localization of individual fluorescent YtvA molecules is performed by imaging a layer of proteins. The layer was obtained by placing a drop (20 µl) of protein solution (100 nM) on a glass coverslip. The balancing between the fluorescent and non fluorescent population is obtained using a low-level 405nm activation laser and a 488nm readout laser continuously running (Figure S4). Localization of YtvA molecules can be performed with an average precision of 18 nm.

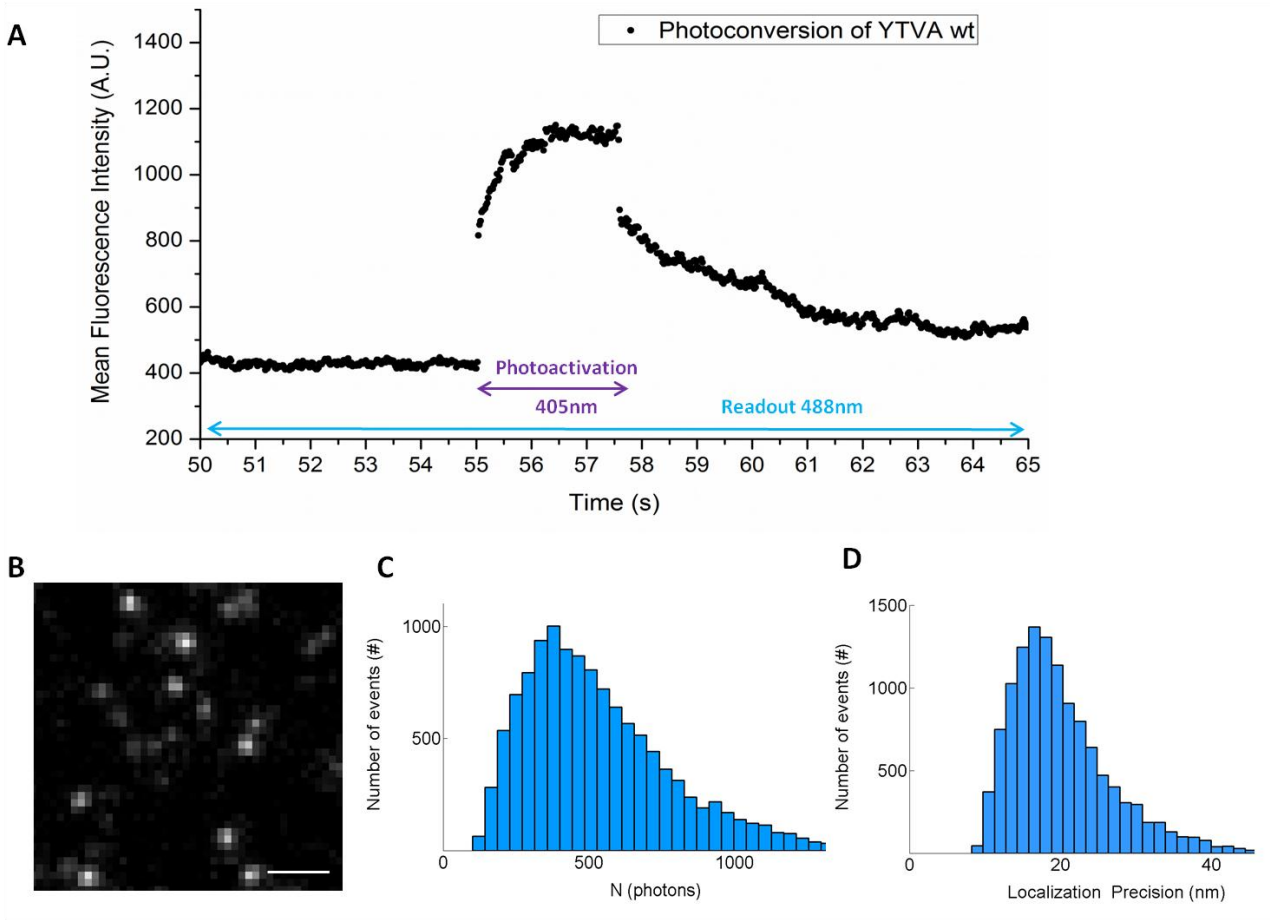

Figure S4. Single molecule collection and localization of YtvA. An immobile sample is obtained drying a thin layer of protein on a sterile glass slide and photoactivation experiments are performed using a PALM microscope. Panel A shows the behavior of the mean fluorescence intensity vs time when readout and activation laser are switched on. Single molecule regime has been obtained with a low-level 405 nm activation laser and a 488 nm readout laser continuously running. The activation and excitation laser powers were at 0.35 kW/cm^{-2} and1.7 kW/cm^{-2}, respectively. Fluorescence emission recovery after 405 nm photoactivation can be described by the kinetic model for photoequilibrium and fitting afforded the same photoconversion yield retrieved for bulk samples (data not shown).Panel B shows a representative image of single molecules of YtvA. The number of photons collected for each molecule and the localization precision are reported in panels C and D, respectively. The analysis is performed after the collection and localization of approximately 12,000 events. Images were acquired with a frame rate of 20 frames s^{-1}. Scale bar, 1 µm (B).

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