Electronic Supplementary Material (ESI) for Photochemical & Photobiological Science

Spectroscopic characterization of Venus at the single molecule level

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Supplementary Information

S1) pH dependence of the absorption spectrum of Venus

To calculate the pKa value of Venus, the maximum absorption peak of anionic (circles) and neutral (squares) states were measured between pH 4 and 10 and the titration was fitted to a Henderson-Hasselbalch relation (sigmoid curves). The pKa value of Venus was determined at 5.5 (6.0[1]).

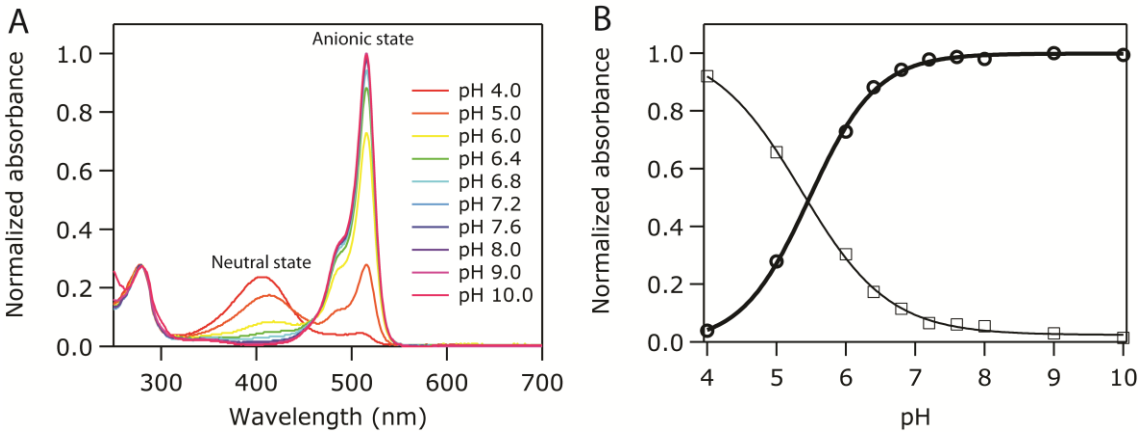

Fig. S1 (A) pH dependence of the absorption spectrum of Venus. (B) Normalized absorbance of Venus at 405 nm (squares) and at 516 nm (circles).

S2) Time resolved ensemble spectroscopy of Venus

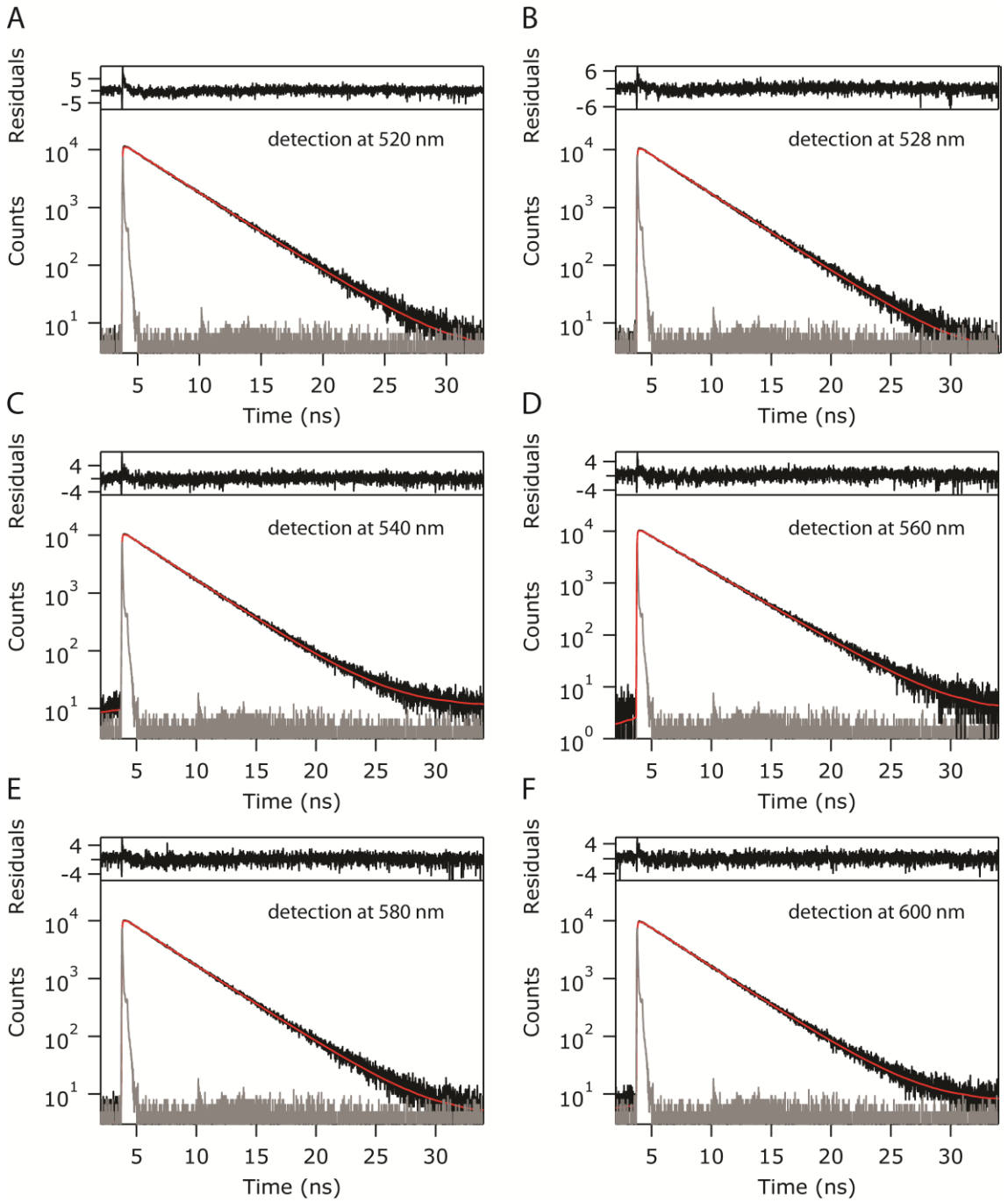

Fig. S2 Ensemble fluorescence spectroscopy of Venus. Fluorescence decay of Venus (pH 7.4) excited at 514 nm and detected at 520 nm (A), 528 nm (B), 540 nm (C), 560 nm (D), 580 nm (E) and 600 nm (F). The lower black graph represents the experimental data. The gray line represents the time resolved signal of scattered excitation light. The red line represents the fit of the experimental data with a single exponential with a time constant of 3.2 ns. The upper graph represents the residuals of the fit to the experimental data. Residuals are a measure for goodness-of-fit.

S3) Autocorrelation method

Using the autocorrelation method, the fast on-off blinking due to triplet state formation was analyzed[2]. An autocorrelation function is determined as the probability of detecting pairs of photons separated in time by a time interval t_0:

$$g^{(2)}(t') = \frac{\langle F(\tau)F(\tau+t')\rangle}{\langle F(\tau)\rangle^2} \qquad (1)$$

$F(\tau)$ is the fluorescence intensity measured in the integration interval centered at time τ. The resulting autocorrelation functions can be fitted by a single exponential model:

$$g^{(2)}(t') = 1 + Ae^{\left(-\frac{t'}{\tau_{AC}}\right)} \qquad (2)$$

or by a double exponential model:

$$g^{(2)}(t') = 1 + A_1 e^{\left(-\frac{t'}{\tau_{AC1}}\right)} + A_2 e^{\left(-\frac{t'}{\tau_{AC2}}\right)} \qquad (3)$$

where τ_{ACi} denotes the autocorrelation time and A_i is the amplitude of the i-th component. The average on- and off- times can be expressed as:

$$\frac{1}{\tau_{AC}} = \frac{1}{\tau_{off}} + \frac{1}{\tau_{on}} \qquad (4)$$

$$A \cong \frac{\tau_{off}}{\tau_{on}} \qquad (5)$$

If the autocorrelation function (eq. 1) of a single molecule fluorescence intensity trace could be fitted with one exponential (eq. 2); the amplitude A and the autocorrelation time τ_{AC} were used to calculate the short off-time and the on-time of the molecule (eq. 4 and 5). The determined on-time thus includes the short on/off blinking due to triplet formation.

If the autocorrelation function (eq. 1) of a single molecule fluorescence intensity trace could be fitted with the double exponential decay function (eq. 3); the amplitude A_1 and the autocorrelation time τ_{AC1} on the shortest time scale were used to calculate the short off-time and the on-time of the molecule (eq. 4 and 5) as described above. The amplitude A_2 and the autocorrelation time τ_{AC2} on the longest time scale were used to calculate the medium-long off-time.

S4) Photoswitching of Venus at the ensemble level

Reversible photoswitching of Venus was characterized at the ensemble level using a dedicated home-made setup. Absorption spectra were measured as a function of time using a UV-Vis-NIR light source (DT-MINI-2-GS, Ocean Optics, Duiven, The Netherlands) and a CCD spectrometer (USB4000-UV-VIS, Ocean Optics). The sample was irradiated by 488 nm laser light and 405 nm laser light (Coherent, Santa Clara, CA) controlled by internal and external shutters. Shutters and acquisition were controlled by home-written software based on Igor Pro (Wave Metrics Inc., Lake Oswego, Oregon, USA). Data analysis was performed in Igor Pro.

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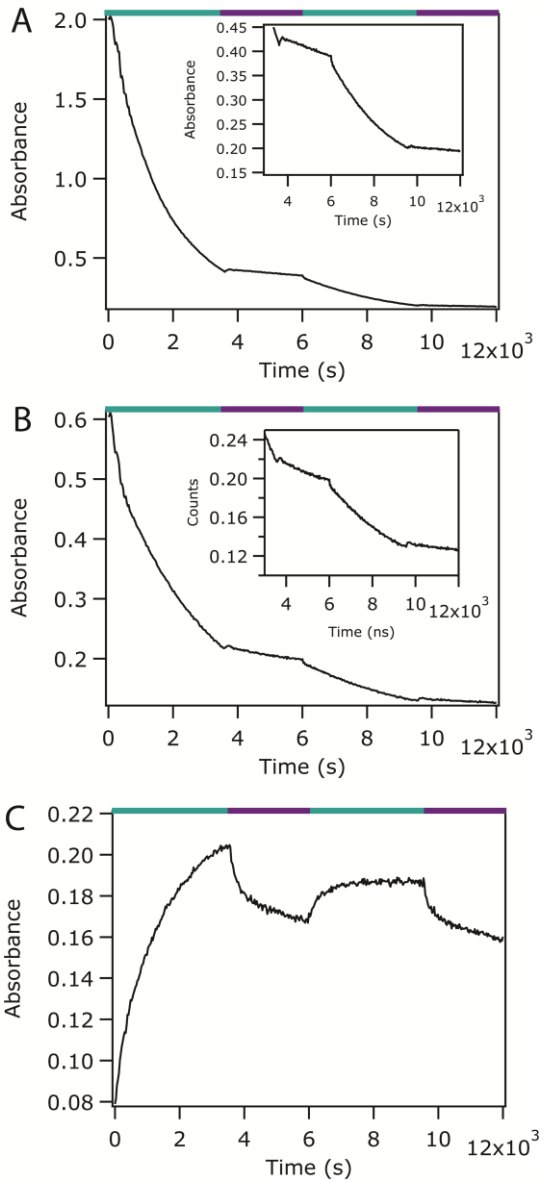

Fig. S4 Photoswitching of Venus on the ensemble level. The evolution of the absorbance of Venus is shown at 514 nm (A), 488 nm (B) and 405 nm (C). The sample was alternately illuminated with 488 nm laser light (light blue bar) and 405 nm laser light (purple bar).

The photobleaching of Venus using 488 nm laser light generates species with an absorption band in the UV region. This observation may be similar to the mechanism described in YFP, where CFP-like species are formed during photobleaching[3-5].

S5) Supplementary movie

References

1. T. Nagai, K. Ibata, E. S. Park, M. Kubota, K. Mikoshiba and A. Miyawaki, A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications, *Nat Biotechnol*, 2002, **20**, 87-90.
2. S. Habuchi, R. Ando, P. Dedecker, W. Verheijen, H. Mizuno, A. Miyawaki and J. Hofkens, Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa, *Proc Natl Acad Sci U S A*, 2005, **102**, 9511-9516.
3. T. B. McAnaney, W. Zeng, C. F. Doe, N. Bhanji, S. Wakelin, D. S. Pearson, P. Abbyad, X. Shi, S. G. Boxer and C. R. Bagshaw, Protonation, photobleaching, and photoactivation of yellow fluorescent protein (YFP 10C): a unifying mechanism, *Biochemistry*, 2005, **44**, 5510-5524.
4. G. Valentin, C. Verheggen, T. Piolot, H. Neel, M. Coppey-Moisan and E. Bertrand, Photoconversion of YFP into a CFP-like species during acceptor photobleaching FRET experiments, *Nat Methods*, 2005, **2**, 801.
5. M. T. Kirber, K. Chen and J. F. Keaney, Jr., YFP photoconversion revisited: confirmation of the CFP-like species, *Nat Methods*, 2007, **4**, 767-768.