**Supporting Text**

**Autocorrelation Analysis.** A autocorrelation function $g^{(2)}(t')$ is determined as the probability of detecting pairs of photons separate in time by an interval $t'$.

$$g^{(2)}(t') = \frac{\langle I(t) I(t+t') \rangle}{\langle I(t) \rangle^2}. \qquad [1]$$

$I(t)$ is the fluorescence intensity measured in the integration interval centered at time $t$ (angle brackets denote average). The resulting autocorrelation functions were fitted by an exponential model,

$$g^{(2)}(t') = 1 + A \exp(-t'/\tau_{ac}), \qquad [2]$$

where $\tau_{ac}$ denotes the autocorrelation time, and $A$ is the amplitude of the component. The average on- and off-times can be expressed as (1)

$$\frac{1}{\tau_{ac}} = \frac{1}{\tau_{off}} + \frac{1}{\tau_{on}}, \qquad [3]$$

$$A \cong \frac{\tau_{off}}{\tau_{on}}. \qquad [4]$$

**Population of the Protonated Form.** As discussed in the main text, the protonated form that is in the acid–base equilibrium ($A_1$) does not contribute to the photoswitching. Under physiological conditions (pH 7.4), only 0.4% of the Dronpa molecules have the $A_1$ form, which demonstrates that almost all of the molecules can be switched by irradiation.

**Detailed Analysis of the Time Evolution of the Absorption Spectrum on Irradiation.** As discussed in the main text, the photoswitched protonated form ($A_2$) is not

interconvertible with the protonated form, which is in the acid–base equilibrium ($A_1$) with the deprotonated form (B). This observation suggests that the population ratio between B and $A_1$ changes accompanied by the switching between B and $A_2$. Thus, the time evolution of the absorption spectrum on irradiation should be analyzed, taking into account the equilibrium to obtain the change in the population ratio between B and $A_2$ on irradiation.

The absorbance of B, $A_1$, and $A_2$ are expressed as

$$\mathrm{Abs}_B = \varepsilon_B [C_B] l , \qquad [5]$$

$$\mathrm{Abs}_{A1} = \varepsilon_{A1} [C_{A1}] l , \qquad [6]$$

$$\mathrm{Abs}_{A2} = \varepsilon_{A2} [C_{A2}] l , \qquad [7]$$

where $\varepsilon_B$, $\varepsilon_{A1}$, and $\varepsilon_{A2}$ are the extinction coefficient of B, $A_1$, and $A_2$, respectively; $[C_B]$, $[C_{A1}]$, and $[C_{A2}]$ are the concentration of B, A1, and A2, respectively; and $l$ is the optical path length. In our measurements, $l$ is 1 cm. The concentration of B is described as

$$[C_B] = \mathrm{Abs}_B / \varepsilon_B , \qquad [8]$$

which suggests the linear dependence of $[C_B]$ on the absorbance of the deprotonated form.

Because the absorption spectrum of $A_1$ and $A_2$ are almost identical, the absorbance of the protonated forms ($\mathrm{Abs}_A$) is expressed as

$$\mathrm{Abs}_A = \mathrm{Abs}_{A1} + \mathrm{Abs}_{A2} = \varepsilon_{A1} [C_{A1}] + \varepsilon_{A2} [C_{A2}], \qquad [9]$$

where $A_1$ is in equilibrium with B. The concentration of B and $A_1$ is expressed as follow

$$\frac{[C_{\mathrm{B}}]}{[C_{\mathrm{A1}}]} = 10^{(\mathrm{pH}-\mathrm{p}K_{\mathrm{a}})}, \qquad [10]$$

where pH and p$K_{\mathrm{a}}$ are the solution pH (7.4 or 5.0) and p$K_{\mathrm{a}}$ of the chromophore of Dronpa (5.0). $\mathrm{Abs_A}$ is then rewritten with Eqs. **9** and **10**.

$$\mathrm{Abs_A} = \frac{1}{10^{(\mathrm{pH}-\mathrm{p}K_{\mathrm{a}})}} \varepsilon_{\mathrm{A1}}[C_{\mathrm{B}}] + \varepsilon_{\mathrm{A2}}[C_{\mathrm{A2}}]. \qquad [11]$$

At pH 7.4, $\mathrm{Abs_A}$ is expressed as

$$\mathrm{Abs_A} = \frac{1}{10^{2.4}} \varepsilon_{\mathrm{A1}}[C_{\mathrm{B}}] + \varepsilon_{\mathrm{A2}}[C_{\mathrm{A2}}] \approx \varepsilon_{\mathrm{A2}}[C_{\mathrm{A2}}]. \qquad [12]$$

The concentration of $A_2$ is described as

$$[C_{\mathrm{A2}}] \approx Abs_{\mathrm{A}} / \varepsilon_{\mathrm{A2}}, \qquad [13]$$

which suggests that the contribution of $A_2$ is dominant for the absorption of the protonated forms. Eqs. **8** and **13** suggest that, at pH 7.4, the change in the absorption of the deprotonated and protonated forms directly reflects the change in the population ratio between B and $A_2$.

At pH 5.0, on the other hand, $\mathrm{Abs_A}$ is expressed as

$$\mathrm{Abs_A} = \varepsilon A([C_{\mathrm{B}}] + [C_{\mathrm{A2}}]), \qquad [14]$$

assuming the same extinction coefficient (ε) for $A_1$ and $A_2$. In this case, the concentration of $A_2$ is described as

$$[C_{\mathrm{A2}}] = \mathrm{Abs_A} / \varepsilon_{\mathrm{A}} - [C_{\mathrm{B}}], \qquad [15]$$

in which both $A_2$ and B contribute to the absorption of the protonated forms. Therefore, the analysis of the time evolution of the absorption spectrum at pH 5.0 was performed by using Eqs. **8** and **15** to obtain the change in the population ratio between B and $A_2$ on irradiation. The results are shown in Fig. 7.

**Calculation of the Survival Time of the Deprotonated Form.** The survival time of the deprotonated form, which corresponds to the on-time, can be calculated on the basis of the rate $k_{0,1}$ for excitation from the electronic ground-state $S_0$ to the first excited-state $S_1$ and the quantum yield of the photoswitching from the deprotonated to the photoswitched protonated form. $k_{0,1}$ is expressed as (2)

$$k_{0,1}(\lambda) = I\sigma(\lambda)\lambda / hc, \qquad [16]$$

where $I$, $\sigma$, $h$, and $c$ are the average laser power, the absorption cross-section at the wavelength $\lambda$, the Plank constant, and the velocity of light in vacuum, respectively. The average laser power at the focus point of an objective lens can be calculated as (3)

$$I = \frac{\pi}{2} P \left( \frac{n_r}{\lambda} \right)^2 (1-\cos\alpha)(3+\cos\alpha), \qquad [17]$$

$$\alpha = \sin^{-1}(NA / n_r), \qquad [18]$$

where $P$, $n_r$, and $NA$ are the input power, the refractive index of the immersion oil, and the numerical aperture of the objective, respectively. With a $P$ of 80, 220, and 600 nW, $n_r$ of 1.51, and $NA$ of 1.4, $I$ is calculated to be 250, 700, and 1,900 W/cm$^2$, respectively. $k_{0,1}$ is then calculated with $\sigma$ of $1.3 \times 10^{-16}$ cm$^2$ at 488 nm and $I$ to be $8.1 \times 10^4$, $2.2 \times 10^5$, and $6.1 \times 10^5$, respectively. Taking into account $\phi_{sw}^{BA}$ at the ensemble level, the rate of the switching from the deprotonated to the photoswitched protonated form is estimated to be $2.6 \times 10^1$, $7.1 \times 10^1$, and $1.9 \times 10^2$, respectively, which corresponds to a survival time (on-time) of 39, 14, and 5.0 ms for 80-, 220-, and 600-nW excitation, respectively.

**Dark-State Recovery of the Deprotonated Form.** The dark-state recovery of the deprotonated form takes place on the order of days in the ensemble measurement (see main text). The relatively fast recovery observed in the single-molecule measurement (11.0 s) has several potential reasons. First, the spontaneous recovery at the single-molecule level is biased to short times by the duration of the single-molecule experiment. The intensity trajectories of single molecules are usually followed for up to 5 min. Many traces show only one or two spikes of fluorescence during this period. So the very slow recovery events (>5 min) are just not taken into account. Second, in view of the limited number of long events that are measured, a bias can be created by a subpopulation of the protein that shows a somewhat faster/or more frequent recovery behavior. The difference between single-molecule and ensemble data can also be related to immobilization of the molecules in PVA. As discussed in the main text, the spectroscopic properties of the photoswitched protonated form ($A_2$) are very similar to those of the protonated form in the acid–base equilibrium with the deprotonated form ($A_1$). Most probably, $A_1$ and $A_2$ have a slightly different conformation including the hydrogen-bonding network. Immobilization in the PVA matrix could influence the conformation of the chromophore, which leads to faster dark state recovery. The influence of PVA immobilization has been observed for several GFP-like fluorescent proteins such as HcRed in which the fluorescence becomes brighter when immobilized in PVA (M. Cotlet, S.H., G. Dirix, J. Michiels, J. Vanderleyden, K. A. Lukyanov, F. C. De Schryver, and J.H., unpublished data). Finally, thermal-induced process also might play a role for the dark-state recovery.

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