

Expanding discriminative dimensions for analysis and imaging

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Contents of the Supporting Information

- Reduction of photo-(physical)chemical mechanisms to a two-state exchange;
- Kinetic analysis of the two-state model;
- Retrieval of concentrations from the observables;
- Improvement of the spatial resolution.

S1 Reduction of photo(physical)chemical mechanisms to a two-state exchange

In the following subsections, we show that various mechanisms involving light absorption can be reduced to the two-state model (1) at an appropriate time scale.

S1.1 A photoswitchable probe

In a first step, we examine the behaviour of a photoswitchable probe. Light absorption is supposed to drive the exchange between two ground singlet states of a probe denoted $S_{0,i}$ ($i=1$ or 2). In the considered scheme, the state $S_{0,1}$ is supposed to be thermodynamically more stable than the state $S_{0,2}$. Mechanistically, this exchange involves light absorption leading each ground state $S_{0,i}$ to its corresponding first singlet excited state $S_{1,i}$. The latter is then assumed to relax either by leading back to the $S_{0,i}$ state (for instance by emission of a photon when the probe is a photoswitchable fluorophore) or by photoisomerization (yielding the other ground state). The ground state $S_{0,2}$ can also notably relax thermally toward the more stable $S_{0,1}$ state. The overall scheme is displayed in Figure S1.

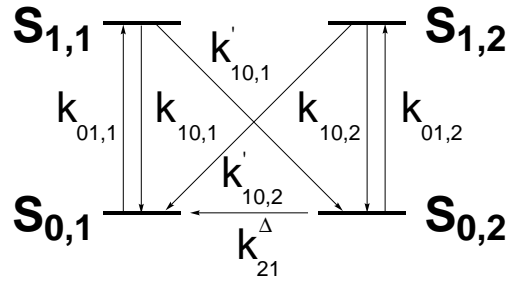


Figure S1: Mechanism accounting for the behaviour of a photoswitchable probe upon illuminating. The arrows and the associated rate constants refer to the exchange processes. See Text.

Relying on the mechanism displayed in Figure S1, we write Eqs.(S1–S4) to describe the concentration evolutions:

$$\frac{dS_{0,1}}{dt} = -k_{01,1}S_{0,1} + k_{10,1}S_{1,1} + k_{21}^{\Delta}S_{0,2} + k'_{10,2}S_{1,2} \quad (\text{S1})$$

$$\frac{dS_{0,2}}{dt} = k'_{10,1}S_{1,1} - (k_{01,2} + k_{21}^{\Delta})S_{0,2} + k_{10,2}S_{1,2} \quad (\text{S2})$$

$$\frac{dS_{1,1}}{dt} = k_{01,1}S_{0,1} - (k_{10,1} + k'_{10,1})S_{1,1} \quad (\text{S3})$$

$$\frac{dS_{1,2}}{dt} = k_{01,2}S_{0,2} - (k_{10,2} + k'_{10,2})S_{1,2}. \quad (\text{S4})$$

In Figure S1, the rate constants $k_{10,i}$ and $k'_{10,i}$ are notably much larger than the rate constants $k_{01,i}$ and k_{21}^{Δ} . $k_{10,i}$ and $k'_{10,i}$ are typically larger than 10^9 s^{-1} .^[1] In contrast, $k_{01,i}$ is in the 10 s^{-1} range under typical illumination conditions.* Moreover k_{21}^{Δ} is at most in the 10^6 s^{-1} range. Thus it is possible to apply the steady-state approximation to $S_{1,1}$ and $S_{1,2}$ beyond the nanosecond time scale. Under such conditions it is meaningful

*Using $k_{01,i} = \sigma_{01,i}I^0$ by considering that the typical photon flux at the sample of an illumination setup is $I^0 = 10^{21} \text{ photons.m}^{-2}.\text{s}^{-1}$ and that the molecular cross section for light absorption $\sigma_{01,i} = 2.3\epsilon_i/N_A$ (N_A is the Avogadro number) is in the $10^{-20} \text{ m}^2/\text{molecule}$ range for the considered probes.

to consider that the states $\mathbf{S}_{0,i}$ and $\mathbf{S}_{1,i}$ are in a fast exchange so as to introduce an average species \mathbf{i} for which $\mathbf{S}_{0,i}$ and $\mathbf{S}_{1,i}$ contribute in $p_{\mathbf{S}_{0,i}}$ and $p_{\mathbf{S}_{1,i}}$ respective proportions where

$$p_{\mathbf{S}_{0,i}} = \frac{1}{1 + K_{01,i}} \quad (\text{S5})$$

$$p_{\mathbf{S}_{1,i}} = \frac{K_{01,i}}{1 + K_{01,i}} \quad (\text{S6})$$

with

$$K_{01,i} = \frac{k_{01,i}}{k_{10,i} + k'_{10,i}}. \quad (\text{S7})$$

Under the experimental conditions used in this study, the relative proportions in the excited and ground states are typically $p_{\mathbf{S}_{1,i}} \sim 10^{-8}$ and $p_{\mathbf{S}_{0,i}} \sim 1$.

Considering that the concentration in \mathbf{i} , i , is equal to $S_{0,i} + S_{1,i}$, Eqs.(S1–S4) reduce to Eq.(S8) beyond the time scale at which the steady-state approximation is valid.

$$\frac{d2}{dt} = -\frac{d1}{dt} = \left(\frac{K_{01,1}}{1 + K_{01,1}} k'_{10,1} \right) 1 - \left(\frac{K_{01,2}}{1 + K_{01,2}} k'_{10,2} + \frac{1}{1 + K_{01,2}} k_{21}^{\Delta} \right) 2. \quad (\text{S8})$$

Considering that, far from saturation, $K_{01,i}$ is proportional to the photon flux I^0 and much lower than 1, one can write:

$$\frac{K_{01,1}}{1 + K_{01,1}} k'_{10,1} \simeq K_{01,1} k'_{10,1} = \frac{k'_{10,1}}{k_{10,1} + k'_{10,1}} \sigma_{01,1} I^0 = k_{12}^{h\nu} \quad (\text{S9})$$

$$\frac{K_{01,2}}{1 + K_{01,2}} k'_{10,2} \simeq K_{01,2} k'_{10,2} = \frac{k'_{10,2}}{k_{10,2} + k'_{10,2}} \sigma_{01,2} I^0 = k_{21}^{h\nu} \quad (\text{S10})$$

$$\frac{1}{1 + K_{01,2}} k_{21}^{\Delta} \simeq k_{21}^{\Delta}. \quad (\text{S11})$$

Hence Eq.(S8) can be alternatively written

$$\frac{d2}{dt} = -\frac{d1}{dt} = k_{12}^{h\nu} 1 - \left(k_{21}^{h\nu} + k_{21}^{\Delta} \right) 2 \quad (\text{S12})$$

in which we introduced the notations $k_{12}^{h\nu}$ and $k_{21}^{h\nu}$ to express that the corresponding terms are proportional to the incident photon flux. Eq.(S12) is dynamically identical to Eqs.(S42–S43).

S1.2 A three-state electronic model

As a second relevant situation, we analyze the behavior of a chromophore, which light excitation yields its first excited singlet state \mathbf{S}_1 from its ground singlet state \mathbf{S}_0 . \mathbf{S}_1 can subsequently relax to the ground state \mathbf{S}_0 (for instance by fluorescence emission) or be converted to its first triplet state \mathbf{T}_1 . \mathbf{T}_1 then deexcites to give back the ground state \mathbf{S}_0 . The overall scheme is displayed in Figure S2.

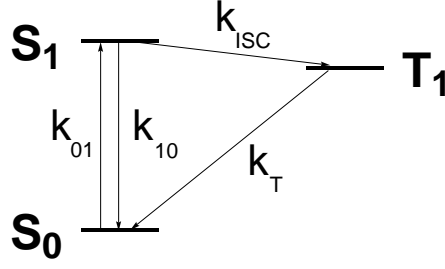


Figure S2: Mechanism accounting for the behaviour of a chromophore yielding its first singlet and triplet excited states upon illuminating. The arrows and the associated rate constants refer to the exchange processes. See Text.

Relying on the mechanism displayed in Figure S2, we write Eqs.(S13–S15) to describe the concentration evolutions:

$$\frac{dS_0}{dt} = -k_{01}S_0 + k_{10}S_1 + k_T T_1 \quad (\text{S13})$$

$$\frac{dS_1}{dt} = k_{01}S_0 - (k_{10} + k_{ISC}) S_1 \quad (\text{S14})$$

$$\frac{dT_1}{dt} = k_{ISC}S_1 - k_T T_1. \quad (\text{S15})$$

In Figure S2, the rate constant k_{10} (typically larger than 10^9s^{-1} ,^[1]) is notably much larger than the other rate constants. Again $k_{01,i}$ would be in the 10s^{-1} under typical illumination conditions. In addition k_{ISC} and k_T are usually in the 10^6 and 10^3 – 10^6s^{-1} range.^[1] Thus we can apply the steady-state approximation to S_1 beyond the nanosecond time scale. Under such conditions it is meaningful to consider that the states S_0 and S_1 are in a fast exchange so as to introduce an average species **1** for which S_0 and S_1 contribute in p_{S_0} and p_{S_1} respective proportions where

$$p_{S_0} = \frac{1}{1 + K_{01}} \quad (\text{S16})$$

$$p_{S_1} = \frac{K_{01}}{1 + K_{01}} \quad (\text{S17})$$

with

$$K_{01} = \frac{k_{01}}{k_{10}}. \quad (\text{S18})$$

Under typical experimental conditions, the relative proportions in the excited and ground states are typically $p_{S_1} \sim 10^{-8}$ and $p_{S_0} \sim 1$.

Considering that the concentration in **1**, 1 , is equal to $S_0 + S_1$ and denoting T_1 by **2** in the following, Eqs.(S13–S15) reduce to Eq.(S19) beyond the time scale at which the steady-state approximation is valid.

$$\frac{d2}{dt} = -\frac{d1}{dt} = \left(\frac{K_{01}}{1 + K_{01}} k_{ISC} \right) 1 - k_T 2. \quad (\text{S19})$$

Considering that, far from saturation, K_{01} is proportional to the photon flux I^0 and much lower than 1, one

can write:

$$\frac{K_{01}}{1 + K_{01}} k_{ISC} \simeq K_{01} k_{ISC} = \frac{1}{k_{10}} \sigma_{01} I^0 k_{ISC} = k_{12}^{h\nu} \quad (\text{S20})$$

$$k_{21}^{\Delta} = k_T. \quad (\text{S21})$$

Hence Eq.(S19) can be alternatively written

$$\frac{d2}{dt} = -\frac{d1}{dt} = k_{12}^{h\nu} 1 - k_{21}^{\Delta} 2 \quad (\text{S22})$$

in which we introduced the notations $k_{12}^{h\nu}$ to express that the corresponding term is proportional to the incident photon flux. Eq.(S22) is dynamically identical to Eqs.(S42–S43) upon noting that $k_{21}^{h\nu} = 0$.

S1.3 A four-state model associated to titration of an analyte **A**

In this last subsection, we reduce the dynamic behavior associated to a photoswitchable sensor. The generic dynamic model is a four-state mechanism consisting of the two exchanging free states of a photoswitchable probe, **1F** and **2F**, which can both bind an analyte **A** to yield the corresponding bound states, **1B** and **2B**. This situation is relevant of a photoswitchable pH-indicator for instance. As in the previous subsections, **1** and **2** states interconvert by photoisomerization in both the bound and free states while only **2** to **1** conversion may occur by thermally-driven exchange. The associated rate constants are presented in Figure S3, where the superscripts $h\nu$ and Δ respectively denote photochemical and thermal contributions.

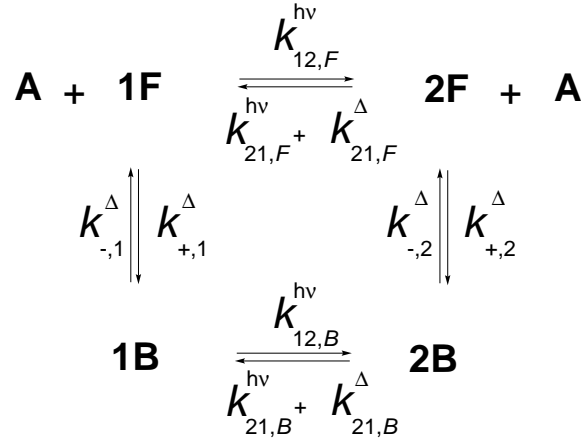


Figure S3: Four-state mechanism accounting for the photochemical and probing behavior of a photoswitchable sensor in the presence of an analyte **A**.

The theoretical framework closely mirrors that described in a previous work by Emond et al.^[2] The con-

centration profiles within the four state model are governed by the equations:

$$\frac{d1B}{dt} = -(k_{1B \rightarrow 1F} + k_{1B \rightarrow 2B}) 1B + k_{2B \rightarrow 1B} 2B + k_{1F \rightarrow 1B} A 1F \quad (S23)$$

$$\frac{d2B}{dt} = k_{1B \rightarrow 2B} 1B - (k_{2B \rightarrow 2F} + k_{2B \rightarrow 1B}) 2B + k_{2F \rightarrow 2B} A 2F \quad (S24)$$

$$\frac{d1F}{dt} = k_{1B \rightarrow 1F} 1B - (k_{1F \rightarrow 1B} A + k_{1F \rightarrow 2F}) 1F + k_{2F \rightarrow 1F} 2F \quad (S25)$$

$$\frac{d2F}{dt} = k_{2B \rightarrow 2F} 2B + k_{1F \rightarrow 2F} 1F - (k_{2F \rightarrow 2B} A + k_{2F \rightarrow 1F}) 2F \quad (S26)$$

The temporal dependence of concentrations cannot be obtained in the most general case. However, it can be analyzed in asymptotic situations according to the nature of the rate-limiting steps, which are associated to either the photochemical reactions or the complexation reactions.[†] In this account, we are interested to examine the case in which the photochemical steps are rate limiting. Indeed it should be relevant in most sensing situations.[‡]

In the corresponding “low”-illumination regime, it is meaningful to introduce the average species **1** and **2** (concentrations $1 = 1F + 1B$ and $2 = 2F + 2B$). The “instantaneous” concentrations in **1F**, **1B**, **2F** and **2B** then follow,

$$1F = \frac{1}{1 + K_1^\Delta A} 1 \quad (S27)$$

$$1B = \frac{K_1^\Delta A}{1 + K_1^\Delta A} 1 \quad (S28)$$

$$2F = \frac{1}{1 + K_2^\Delta A} 2 \quad (S29)$$

$$2B = \frac{K_2^\Delta A}{1 + K_2^\Delta A} 2 \quad (S30)$$

where

$$K_1^\Delta = \frac{k_{+,1}^\Delta}{k_{-,1}^\Delta} \quad (S31)$$

$$K_2^\Delta = \frac{k_{+,2}^\Delta}{k_{-,2}^\Delta}. \quad (S32)$$

Thus Eqs.(S23–S26) transform into Eq.(S33):

$$\frac{d1}{dt} = -\frac{d2}{dt} = -k_{12} 1 + k_{21} 2 \quad (S33)$$

[†]Crossing between both kinetic regimes typically occurs when the relaxation times associated to the photoisomerization and the complexation reactions are equal.

[‡]The rate constants associated to the photochemical steps adopt the expression $k_{ij,S}^{h\nu} = \sigma_{ij,S} \phi_{ij,S} I^0$ (i and j are equal to 1 or 2 with $i \neq j$, $S = F$ or B). By considering that the typical photon flux at the sample of an illumination setup is $I^0 = 10^{21}$ photons.m⁻².s⁻¹ and that the molecular cross section for light absorption $\sigma_{01,i} = 2.3\epsilon_i/N_A$ (N_A is the Avogadro number) and the quantum yield $\phi_{ij,S}$ associated to photoisomerization are typically in the 10⁻²⁰ m²/molecule and 0.1 ranges respectively for the considered probes, we derive $k_{ij,S}^{h\nu} \simeq 1$ s⁻¹. Hence the relaxation times associated to photoisomerizations would be typically in the 1 s range. Such a range is above the order of magnitude of the relaxation times associated to titration reactions. For instance, diffusion-limited reactions (e. g. most proton exchanges^[3]) are associated with rate constants in the 10¹⁰ M⁻¹s⁻¹ range. They would give rise to relaxation times below the millisecond for titrating reagents with $10^3 < K_i^\Delta < 10^7$ upon assuming K_i^Δ ($i=1$ or 2) to be tailored such that the concentration in analyte **A** is in the range of $1/K_i^\Delta$ to get the largest sensitivity.

with

$$k_{12} = \frac{k_{12,F}^{h\nu} + k_{12,B}^{h\nu} K_1^\Delta A}{1 + K_1^\Delta A} \quad (\text{S34})$$

$$k_{21} = \frac{\left(k_{21,F}^{h\nu} + k_{21,F}^\Delta\right) + \left(k_{21,B}^{h\nu} + k_{21,B}^\Delta\right) K_2^\Delta A}{1 + K_2^\Delta A}. \quad (\text{S35})$$

In the most general case, Eq.(S33) has no analytical solution, because k_{12} and k_{21} are time-dependent as a result of the $A(t)$ term. However, a tractable temporal dependence of the concentrations upon light excitation can be obtained in the most important sensing situation when the total concentration of the analyte (A_{tot}) is much larger than that of the photoswitchable sensor (P_{tot}): $A_{tot} \gg P_{tot}$.[§] Introducing

$$k_{12} = \frac{k_{12,F}^{h\nu} + k_{12,B}^{h\nu} K_1^\Delta A_{tot}}{1 + K_1^\Delta A_{tot}} \quad (\text{S36})$$

$$k_{21}^{h\nu} = \frac{k_{21,F}^{h\nu} + k_{21,B}^{h\nu} K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}} \quad (\text{S37})$$

$$k_{21}^\Delta = \frac{k_{21,F}^\Delta + k_{21,B}^\Delta K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}}, \quad (\text{S38})$$

Eq.(S33) can be alternatively written

$$\frac{d2}{dt} = -\frac{d1}{dt} = k_{12}^{h\nu} 1 - \left(k_{21}^{h\nu} + k_{21}^\Delta\right) 2 \quad (\text{S39})$$

in which we introduced the notations $k_{12}^{h\nu}$ and $k_{21}^{h\nu}$ to express that the corresponding terms are proportional to the incident photon flux. Eq.(S39) is dynamically identical to Eqs.(S42–S43).[¶]

[§]The most general case is made complex by the existence of the product of concentrations of the reactants in the dynamic law. In principle, this law could be linearized around chemical equilibrium when light modulation is of small amplitude but it would yield intricate resonance conditions. Moreover, to subsequently implement experimentally these resonance conditions would generally necessitate the knowledge of the overall concentrations of both the probe and the analyte. Whereas to have a guess about the concentration of the analyte is classical in titration protocols (and our final protocol precisely integrates such a guess), we estimated that it would be too demanding prior the titration experiment to have another guess about the concentration of the probe. In particular we had in mind biological applications for which intra-cellular or intra-organism partition of an externally added molecule can be hardly predicted. Therefore we deliberately favored the case where the concentration of the probe is much smaller than the one of the analyte. Classically encountered for titrations, this situation can be easily fulfilled when a sensitive observable such as fluorescence is experimentally implemented.

[¶]In the corresponding regime, one can also derive the average brightnesses of the average states **1** and **2**. Denoting Q_i for the individual brightness of the species **1F**, **2F**, **1B**, and **2B**, one has

$$Q_2 = \frac{Q_{2F} + Q_{2B} K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}} \quad (\text{S40})$$

$$Q_1 = \frac{Q_{1F} + Q_{1B} K_1^\Delta A_{tot}}{1 + K_1^\Delta A_{tot}}. \quad (\text{S41})$$

S2 Kinetic analysis of the two-state model

S2.1 The model

We consider relaxation mechanisms which can be reduced to the two-state exchange displayed in Figure S4.

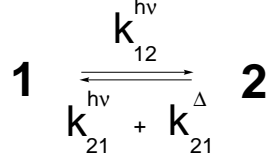


Figure S4: Reduced mechanism accounting for the photochemical behavior of a photoswitchable probe. The most stable state of the probe is here assumed to be **1**, which can switch to the less stable state **2** by photoisomerization. Back reaction from **2** to **1** can occur either by photoisomerization or thermally-driven exchange.

We assume that the system is either homogeneously illuminated or that it can be considered homogeneous at any time of its evolution. Then we rely on the mechanism displayed in Figure S4 to write Eqs.(S42–S43) describing the concentration evolutions:

$$\frac{d1}{dt} = -(k_{1\rightarrow 2}) 1 + k_{2\rightarrow 1} 2 \quad (\text{S42})$$

$$\frac{d2}{dt} = k_{1\rightarrow 2} 1 - (k_{2\rightarrow 1}) 2 \quad (\text{S43})$$

where we make explicit the photochemical and thermal contributions to the rate constants by writing

$$k_{1\rightarrow 2} = k_{12}^{h\nu} \quad (\text{S44})$$

$$k_{2\rightarrow 1} = k_{21}^{h\nu} + k_{21}^{\Delta} \quad (\text{S45})$$

where the exponent indicates the nature of the contribution.

In the following, we are interested to derive the theoretical expressions of (i) the concentrations in **1** and **2** (1 and 2 respectively), (ii) the overall observable originating from the photoswitchable probe, and (iii) the convolution of the preceding overall observable with the light excitation (we chose fluorescence emission as a representative example), upon applying either a jump or a periodic modulation of the illumination.

S2.2 Light-jump experiments

We here consider that the system is “suddenly”^{||} illuminated, so that the incident surfacic photon flux varies from 0 to I^0 :

$$I(t) = I^0 \quad (\text{S46})$$

for $t \geq 0$.

^{||}Note that “suddenly” here refers to a time interval such that Eqs.(S42,S43) can be considered valid.

S2.2.1 Expression of the concentrations

Denoting σ_{12} and σ_{21} the excitation cross sections associated to the transitions from the state **1** to the state **2**, and from the state **2** to the state **1** respectively, the apparent rate constants $k_{12} = k_{12}^{h\nu} = \sigma_{12}I^0$ and $k_{21} = k_{21}^{h\nu} + k_{21}^{\Delta} = \sigma_{21}I^0 + k_{21}^{\Delta}$ can be considered constant and equal to k_{12}^0 and k_{21}^0 . Upon introducing the total concentration in photoswitchable probe **P**, $P_{tot} = 1 + 2$, Eqs.(S42,S43) yield:

$$-\frac{d(2 - 2^0)}{dt} = (k_{12}^0 + k_{21}^0)(2 - 2^0) \quad (\text{S47})$$

from which we derive

$$2 - 2^0 = 1^0 - 1 = -2^0 \exp\left(-\frac{t}{\tau_{12}^0}\right) \quad (\text{S48})$$

where

$$2^0 = \frac{K_{12}^0}{1 + K_{12}^0} P_{tot} \quad (\text{S49})$$

$$1^0 = \frac{1}{1 + K_{12}^0} P_{tot} \quad (\text{S50})$$

$$\tau_{12}^0 = \frac{1}{k_{12}^0 + k_{21}^0} \quad (\text{S51})$$

$$K_{12}^0 = \frac{k_{12}^0}{k_{21}^0} \quad (\text{S52})$$

In Eqs.(S48–S52), 2^0 , 1^0 , τ_{12}^0 , and K_{12}^0 respectively denote the steady-state value of the concentrations in **2** and **1**, the relaxation time associated to the exchange between the states **1** and **2**, and the apparent photoisomerization constant of the photoswitchable fluorophore **P**, in the presence of light at constant light flux I^0 .

S2.2.2 Expression of the overall observable from the photoswitchable probe

The overall observable O from the photoswitchable probe results from the individual contributions of the states **1** and **2**. Denoting Q_i for the molecular brightness, one has

$$O(t) = Q_1 1(t) + Q_2 2(t). \quad (\text{S53})$$

Upon applying a light jump from 0 to I^0 , the overall observable evolves as

$$O(t) = O^0 + (Q_1 - Q_2) 2^0 \exp\left(-\frac{t}{\tau_{12}^0}\right) \quad (\text{S54})$$

where

$$O^0 = Q_1 1^0 + Q_2 2^0. \quad (\text{S55})$$

S2.2.3 Expression of the fluorescence intensity

We now consider the probe to be fluorescent. Fluorescence intensity $I_F(t)$ can be written

$$I_F(t) = O(t)I(t) = [Q_1 1(t) + Q_2 2(t)] I(t) \quad (\text{S56})$$

where the expressions of $I(t)$, 1 and 2 can be retrieved from Eqs.(S46,S48). Then one has:

$$I_F = \left[O^0 + (Q_1 - Q_2) 2^0 \exp\left(-\frac{t}{\tau_{12}^0}\right) \right] I^0. \quad (\text{S57})$$

S2.3 Light modulation experiments

In the following, we successively consider two different types of periodically modulated illumination: (i) a sinusoidal modulation (Figure S5a); (ii) a square wave modulation modelled by its expansion as a Fourier series (Figure S5b; *vide infra*). For each type of modulation, the corresponding cases of small and large modulation are successively considered.

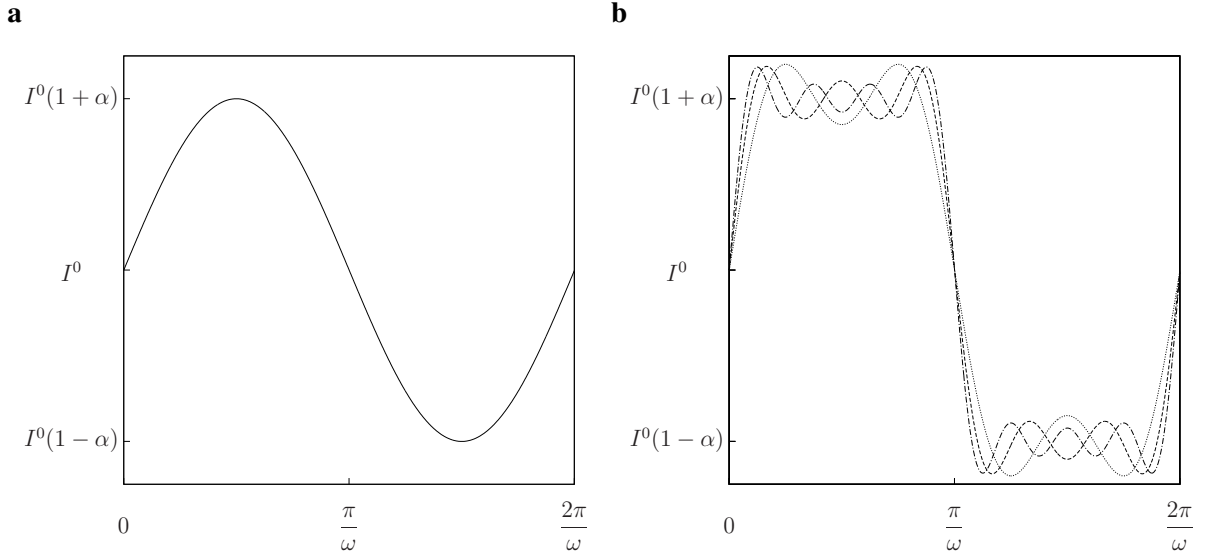


Figure S5: Periodically modulated illuminations, which have been considered in the present study. **a**: Sinusoidal modulation of amplitude α around the average value I^0 : $I(t) = I^0 [1 + \alpha \sin(\omega t)]$; **b**: Square wave modulation of amplitude α : $I(t) = I^0 \left\{ 1 + \frac{4\alpha}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin[(2p+1)\omega t] \right\}$ at first- ($p=1$, dotted line), second- ($p=2$, dashed line) and third- ($p=3$, dashed-dotted line) orders of the Fourier series expansion.

S2.3.1 Sinusoidal modulation of small amplitude

Expression of the concentrations We first consider that the system is submitted to a weak sinusoidal modulation of illumination around the averaged value I^0 . We correspondingly adopt

$$I(t) = I^0 [1 + \varepsilon \sin(\omega t)] \quad (\text{S58})$$

with $\varepsilon \ll 1$. One has

$$k_{12}^{h\nu}(t) = k_{12}^{h\nu,0} [1 + \varepsilon \sin(\omega t)] \quad (\text{S59})$$

$$k_{21}^{h\nu}(t) = k_{21}^{h\nu,0} [1 + \varepsilon \sin(\omega t)]. \quad (\text{S60})$$

The equations (S42,S43) are solved at first order of the light perturbation upon writing:

$$2 = 2^0 + \varepsilon 2^1(t) = 2^0 - \varepsilon 1^1(t) \quad (\text{S61})$$

$$1 = 1^0 - \varepsilon 2^1(t) = 1^0 + \varepsilon 1^1(t) \quad (\text{S62})$$

$$k_{12}(t) = k_{12}^{h\nu,0} [1 + \varepsilon \sin(\omega t)] \quad (\text{S63})$$

$$k_{21}(t) = k_{21}^{h\nu,0} [1 + \varepsilon \sin(\omega t)] + k_{21}^{\Delta} \quad (\text{S64})$$

to yield

$$\frac{d2^1}{dt} + \frac{1}{\tau_{12}^0} 2^1 = \rho_{12}^0 p_{21}^{\Delta} \sin(\omega t) \quad (\text{S65})$$

where

$$\rho_{12}^0 = k_{12}^0 1^0 = k_{21}^0 2^0 \quad (\text{S66})$$

$$p_{21}^{\Delta} = \frac{k_{21}^{\Delta}}{k_{21}^{h\nu,0} + k_{21}^{\Delta}}. \quad (\text{S67})$$

ρ_{12}^0 and p_{21}^{Δ} designate the steady-state rate of reaction (1) and the relative thermal contribution to **2** relaxation upon illuminating at I^0 .

Beyond the relaxation time τ_{12}^0 , one enters into a permanent regime in which the respective contributions of the in- and out-phase terms $2^{1,in}$ and $2^{1,out}$ such that $2^1(t) = 2^{1,in} \sin(\omega t) + 2^{1,out} \cos(\omega t)$ respectively obey**

$$2^{1,in} = -1^{1,in} = \rho_{12}^0 \tau_{12}^0 p_{21}^{\Delta} \frac{1}{1 + (\omega \tau_{12}^0)^2} \quad (\text{S71})$$

$$2^{1,out} = -1^{1,out} = -\rho_{12}^0 \tau_{12}^0 p_{21}^{\Delta} \frac{\omega \tau_{12}^0}{1 + (\omega \tau_{12}^0)^2}. \quad (\text{S72})$$

Upon noting that

$$\rho_{12}^0 \tau_{12}^0 = \frac{K_{12}^0}{(1 + K_{12}^0)^2} P_{tot}, \quad (\text{S73})$$

**Alternatively, one has

$$2^1(t) = \frac{\rho_{12}^0 \tau_{12}^0 p_{21}^{\Delta}}{\sqrt{1 + (\omega \tau_{12}^0)^2}} \sin(\omega t - \phi_{12}) \quad (\text{S68})$$

with

$$\cos \phi_{12} = \frac{1}{\sqrt{1 + (\omega \tau_{12}^0)^2}} \quad (\text{S69})$$

$$\sin \phi_{12} = \frac{\omega \tau_{12}^0}{\sqrt{1 + (\omega \tau_{12}^0)^2}}. \quad (\text{S70})$$

Eqs.(S71,S72) become

$$2^{1,in} = -1^{1,in} = p_{21}^{\Delta} \frac{K_{12}^0}{(1 + K_{12}^0)^2} \frac{1}{1 + (\omega\tau_{12}^0)^2} P_{tot} \quad (S74)$$

$$2^{1,out} = -1^{1,out} = -p_{21}^{\Delta} \frac{K_{12}^0}{(1 + K_{12}^0)^2} \frac{\omega\tau_{12}^0}{1 + (\omega\tau_{12}^0)^2} P_{tot} \quad (S75)$$

Expression of the overall observable from the photoswitchable probe In the case of a sinusoidal modulation of light intensity obeying Eq.(S58), the temporal dependence of the overall observable $O(t)$ originates from the temporal dependence of $1^1(t)$ and $2^1(t)$ (given in Eqs.(S74,S75))

$$O(t) = Q_1 1 + Q_2 2 = O^0 + O^{1,in} \sin(\omega t) + O^{1,out} \cos(\omega t) \quad (S76)$$

with

$$O^{1,in} = (Q_2 - Q_1) 2^{1,in} \varepsilon \quad (S77)$$

$$O^{1,out} = (Q_2 - Q_1) 2^{1,out} \varepsilon. \quad (S78)$$

Expression of the fluorescence intensity Fluorescence emission $I_F(t)$ is extracted from Eq.(S56) by using the temporal dependence (S58) of the exciting light source and the expressions of 1 and 2 from Eqs.(S49,S50) and (S61,S62). Retaining only the terms up to the first order in the perturbation in the expression

$$I_F(t) = [O^0 + O^{1,in} \sin(\omega t) + O^{1,out} \cos(\omega t)] I^0 [1 + \varepsilon \sin(\omega t)] \quad (S79)$$

yields^{††}

$$I_F(t) = I_F^0 + I_F^{1,in} \sin(\omega t) + I_F^{1,out} \cos(\omega t) \quad (S83)$$

with

$$I_F^0 = (Q_1 1^0 + Q_2 2^0) I^0 \quad (S84)$$

$$I_F^{1,in} = \varepsilon [(Q_1 1^0 + Q_2 2^0) + (Q_2 - Q_1) 2^{1,in}] I^0 = \varepsilon [I_F^0 + (Q_2 - Q_1) 2^{1,in} I^0] \quad (S85)$$

$$I_F^{1,out} = \varepsilon (Q_2 - Q_1) 2^{1,out} I^0. \quad (S86)$$

^{††} Similarly, using Eq.(S68), Eq.(S76) can be rewritten to explicit the phase delay between the fluorescence emission and the exciting light :

$$Q_1 1 + Q_2 2 = O^0 + \varepsilon (Q_2 - Q_1) \frac{\rho_{12}^0 \tau_{12}^0 p_{21}^{\Delta}}{\sqrt{1 + (\omega\tau_{12}^0)^2}} \sin(\omega t - \phi_{12}) \quad (S80)$$

Therefore, the temporal dependence of the fluorescence emission given in Eq.(S83) becomes :

$$I_F(t) = I_F^0 [1 + \varepsilon \sin(\omega t) + \varepsilon R_{12}^0 \sin(\omega t - \phi_{12})] \quad (S81)$$

with

$$R_{12}^0 = \frac{Q_2 - Q_1}{Q_1 1^0 + Q_2 2^0} \frac{\rho_{12}^0 \tau_{12}^0 p_{21}^{\Delta}}{\sqrt{1 + (\omega\tau_{12}^0)^2}} \quad (S82)$$

S2.3.2 Square wave modulation of small amplitude

Expression of the concentrations We now consider that the system is submitted to a weak square wave modulation of illumination around the averaged value I^0 . We correspondingly adopt

$$I(t) = I^0 \left\{ 1 + \frac{4\varepsilon}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t] \right\} \quad (\text{S87})$$

by choosing the starting time ($t = 0$) in this expansion halfway through the first pulse with $\varepsilon \ll 1$. The equations (S42,S43) are then solved at first order of the light perturbation upon introducing

$$k_{12}(t) = k_{12}^{h\nu,0} \left\{ 1 + \frac{4\varepsilon}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t] \right\} \quad (\text{S88})$$

$$k_{21}(t) = k_{21}^{h\nu,0} \left\{ 1 + \frac{4\varepsilon}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t] \right\} + k_{21}^{\Delta} \quad (\text{S89})$$

$$2 = 2^0 + \varepsilon \sum_{p=0}^{\infty} 2_{(2p+1)}^1 \quad (\text{S90})$$

$$1 = 1^0 - \varepsilon \sum_{p=0}^{\infty} 2_{(2p+1)}^1 \quad (\text{S91})$$

to yield

$$\sum_{p=0}^{\infty} \frac{d2_{(2p+1)}^1}{dt} + \frac{1}{\tau_{12}^0} \sum_{p=0}^{\infty} 2_{(2p+1)}^1 = \rho_{12}^0 p_{21}^{\Delta} \frac{4}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t]. \quad (\text{S92})$$

Beyond the relaxation time τ_{12}^0 , one enters into a permanent regime in which the respective contributions of the in- and out-phase terms $2_{(2p+1)}^{1,in}$ and $2_{(2p+1)}^{1,out}$ such that $2_{(2p+1)}^1(t) = 2_{(2p+1)}^{1,in} \sin [(2p+1)\omega t] + 2_{(2p+1)}^{1,out} \cos [(2p+1)\omega t]$ respectively obey

$$2_{(2p+1)}^{1,in} = -1_{(2p+1)}^{1,in} = \rho_{12}^0 \tau_{12}^0 p_{21}^{\Delta} \frac{4}{\pi} \frac{1}{2p+1} \frac{1}{1 + (2p+1)^2 (\omega \tau_{12}^0)^2} \quad (\text{S93})$$

$$2_{(2p+1)}^{1,out} = -1_{(2p+1)}^{1,out} = -\rho_{12}^0 \tau_{12}^0 p_{21}^{\Delta} \frac{4}{\pi} \frac{1}{2p+1} \frac{(2p+1)\omega \tau_{12}^0}{1 + (2p+1)^2 (\omega \tau_{12}^0)^2}. \quad (\text{S94})$$

Expression of the overall observable from the photoswitchable probe In the case of a square wave modulation of light intensity obeying Eq.(S87), the temporal dependence of the overall observable $O(t)$ originates from the temporal dependence of $1_{(2p+1)}^1(t)$ and $2_{(2p+1)}^1(t)$ (given in Eqs.(S93,S94))

$$O(t) = Q_1 1 + Q_2 2 = O^0 + \sum_{p=0}^{\infty} \left\{ O_{(2p+1)}^{1,in} \sin [(2p+1)\omega t] + O_{(2p+1)}^{1,out} \cos [(2p+1)\omega t] \right\} \quad (\text{S95})$$

with

$$O_{(2p+1)}^{1,in} = (Q_2 - Q_1) 2_{(2p+1)}^{1,in} \varepsilon \quad (\text{S96})$$

$$O_{(2p+1)}^{1,out} = (Q_2 - Q_1) 2_{(2p+1)}^{1,out} \varepsilon. \quad (\text{S97})$$

Expression of the fluorescence intensity Fluorescence emission $I_F(t)$ is extracted from Eq.(S56) by using the temporal dependence (S87) of the exciting light source and the expressions of 1 and 2 from Eqs.(S49,S50) and (S90,S91). Retaining only the terms up to the first order in the perturbation in the expression

$$I_F(t) = I^0 \left[O^0 + \sum_{p=0}^{\infty} \left[O_{(2p+1)}^{1,in} \sin [(2p+1)\omega t] + O_{(2p+1)}^{1,out} \cos [(2p+1)\omega t] \right] \right] \times \left[1 + \frac{4\varepsilon}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t] \right] \quad (\text{S98})$$

yields

$$I_F(t) = I_F^0 + \sum_{p=0}^{\infty} \left[I_{F,(2p+1)}^{1,in} \sin [(2p+1)\omega t] + I_{F,(2p+1)}^{1,out} \cos [(2p+1)\omega t] \right] \quad (\text{S99})$$

with

$$I_{F,(2p+1)}^{1,in} = \varepsilon \left[\frac{4}{\pi} \frac{1}{2p+1} I_F^0 + (Q_2 - Q_1) 2_{(2p+1)}^{1,in} I^0 \right] \quad (\text{S100})$$

$$I_{F,(2p+1)}^{1,out} = \varepsilon (Q_2 - Q_1) 2_{(2p+1)}^{1,out} I^0. \quad (\text{S101})$$

S2.3.3 Sinusoidal modulation of large amplitude

Expression of the concentrations We now consider that the system is submitted to a large sinusoidal modulation of illumination around the averaged value I^0 . We adopt

$$I(t) = I^0 [1 + \alpha \sin(\omega t)]. \quad (\text{S102})$$

Then one has

$$k_{12}^{h\nu}(t) = k_{12}^{h\nu,0} [1 + \alpha \sin(\omega t)] \quad (\text{S103})$$

$$k_{21}^{h\nu}(t) = k_{21}^{h\nu,0} [1 + \alpha \sin(\omega t)]. \quad (\text{S104})$$

The equations (S42,S43) are solved upon introducing:

$$2 = 2^0 + \alpha f(t) \quad (\text{S105})$$

$$1 = 1^0 - \alpha f(t) \quad (\text{S106})$$

$$k_{12}(t) = k_{12}^{h\nu,0} [1 + \alpha \sin(\omega t)] \quad (\text{S107})$$

$$k_{21}(t) = k_{21}^{h\nu,0} [1 + \alpha \sin(\omega t)] + k_{21}^{\Delta} \quad (\text{S108})$$

to yield

$$\frac{df(t)}{dt} = -\frac{1}{\tau_{12}^0} f(t) + \rho_{12}^0 p_{21}^{\Delta} \sin(\omega t) - \alpha \left(k_{12}^{h\nu,0} + k_{21}^{h\nu,0} \right) f(t) \sin(\omega t). \quad (\text{S109})$$

Upon introducing

$$x = \frac{t}{\tau_{12}^0} \quad (\text{S110})$$

$$a = \rho_{12}^0 p_{21}^{\Delta} \tau_{12}^0 \quad (\text{S111})$$

$$b = \alpha \left(k_{12}^{h\nu,0} + k_{21}^{h\nu,0} \right) \tau_{12}^0 \quad (\text{S112})$$

$$\theta = \omega \tau_{12}^0 \quad (\text{S113})$$

Eq.(S109) becomes

$$\frac{df(\theta x)}{dx} = -f(\theta x) + [a - bf(\theta x)] \sin(\theta x). \quad (\text{S114})$$

Beyond the relaxation time τ_{12}^0 , one enters into a permanent regime in which $f(\theta x)$ is a continuous periodic function. Then $f(\theta x)$ can be expressed as a Fourier series

$$f(\theta x) = a_0 + \sum_{n=1}^{+\infty} [a_n \cos(n\theta x) + b_n \sin(n\theta x)] \quad (\text{S115})$$

where a_n and b_n designate the amplitudes of the n -th components of the Fourier series. The a_n and b_n terms can be extracted from Eq.(S114) upon identifying the amplitudes of the components of the same order. We derived

- Zeroth order:

$$a_0 = -b \frac{b_1}{2} \quad (\text{S116})$$

- First order:

$$-a_1 \theta = -b_1 + a - a_0 b + b \frac{a_2}{2} \quad (\text{S117})$$

$$b_1 \theta = -a_1 - b \frac{b_2}{2} \quad (\text{S118})$$

- Order $n > 1$:

$$a_n n \theta = b_n - \frac{b}{2} (a_{n+1} - a_{n-1}) \quad (\text{S119})$$

$$b_n n \theta = -a_n - \frac{b}{2} (b_{n+1} - b_{n-1}) \quad (\text{S120})$$

from which we obtained the following expressions of the concentrations in **1** and **2**:

$$2 = 2^0 + \alpha \left\{ a_0 + \sum_{n=1}^{+\infty} [a_n \cos(n\theta x) + b_n \sin(n\theta x)] \right\} \quad (\text{S121})$$

$$1 = 1^0 - \alpha \left\{ a_0 + \sum_{n=1}^{+\infty} [a_n \cos(n\theta x) + b_n \sin(n\theta x)] \right\}. \quad (\text{S122})$$

Hence, at steady-state, a large sinusoidal modulation of illumination causes modulation of the concentrations in **1** and **2** at an infinite number of radial frequencies.

Eqs.(S121,S122) can be transformed to explicit the amplitudes of the concentration modulations at all radial frequencies. Thus we write

$$2 = z^\circ + \alpha \sum_{n=1}^{+\infty} \left[z^{n,\text{in}} \sin(n\omega t) + z^{n,\text{out}} \cos(n\omega t) \right] \quad (\text{S123})$$

$$1 = \mathbf{1}^\circ - \alpha \sum_{n=1}^{+\infty} \left[z^{n,\text{in}} \sin(n\omega t) + z^{n,\text{out}} \cos(n\omega t) \right] \quad (\text{S124})$$

and conversely

$$2 = z^\circ - \alpha \sum_{n=1}^{+\infty} \left[\mathbf{1}^{n,\text{in}} \sin(n\omega t) + \mathbf{1}^{n,\text{out}} \cos(n\omega t) \right] \quad (\text{S125})$$

$$1 = \mathbf{1}^\circ + \alpha \sum_{n=1}^{+\infty} \left[\mathbf{1}^{n,\text{in}} \sin(n\omega t) + \mathbf{1}^{n,\text{out}} \cos(n\omega t) \right] \quad (\text{S126})$$

where

$$z^\circ = 2^0 + \alpha a_0 \quad (\text{S127})$$

$$\mathbf{1}^\circ = \mathbf{1}^0 - \alpha a_0 \quad (\text{S128})$$

$$z^{n,\text{in}} = -\mathbf{1}^{n,\text{in}} = b_n \quad (\text{S129})$$

$$z^{n,\text{out}} = -\mathbf{1}^{n,\text{out}} = a_n. \quad (\text{S130})$$

In the case of sinusoidal light modulation of small amplitude, $z^{1,\text{out}}$ is optimal when the resonance conditions (14,15) are fulfilled. In the absence of analytical expressions for $z^{1,\text{out}}$, such conclusions cannot be directly derived in the case of a sinusoidal modulation of large amplitude, so we evaluated their relevance by means of numerical calculations.

We first analyzed the dependence of $\mathbf{1}^{1,\text{out}} = -z^{1,\text{out}} = -a_1$ on the control parameters ω and I^0 . To do so, we analytically retrieved the $2n + 1$ unknown parameters (a_0, \dots, a_n, b_n) upon truncating the Fourier expansion (30) at increasing orders n . Figure S6 displays representative results, which have been obtained with $n = 5$.

Figure S7 displays the dependence of the normalized amplitude, $|\mathbf{1}_{\text{norm}}^{1,\text{out}}| = |\mathbf{1}^{1,\text{out}}/P_{\text{tot}}|$, on the light flux I^0 and the adimensional radial frequency $\omega\tau_{12}^0$ when $\alpha = 1$. Truncation of the Fourier expansion of the $f(\theta x)$ function at the fifth order ($n = 5$) is definitively sufficient to observe convergence: the dependence of $|\mathbf{1}_{\text{norm}}^{1,\text{out}}|$ on I^0 and ω does not significantly evolve beyond $n = 3$. $|\mathbf{1}_{\text{norm}}^{1,\text{out}}|$ exhibits an optimum in the space (I^0, ω) , which position and amplitude are very close to those observed with a sinusoidal modulation of small amplitude (Table S1).

Note that the error done when taking the analytical expression of the resonance conditions, valid only for a modulation of small amplitude, is always less than 20 %, no matter which amplitude α is used. Such an error would be of the order of magnitude of the experimental errors done when fixing the average light intensity and radial frequency to their values at resonance, $I^{0,R}$ and ω^R .

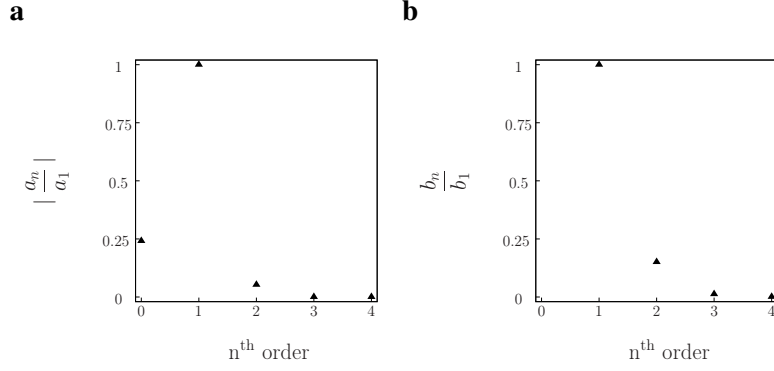


Figure S6: Computation of the amplitudes of the Fourier terms, $|a_n|$ (**a**) and b_n (**b**), from a solution of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to light harmonic forcing in the regime of large ($\alpha = 1$) amplitude modulation. The numerical computation has been performed upon truncating the Fourier f expansion at the fifth order ($n = 5$). $\sigma_{12} = 73 \text{ m}^2 \cdot \text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2 \cdot \text{mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$, $I^0 = 9.6 \times 10^{-5} \text{ ein} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ and $\omega\tau_{12}^0 = 1$.

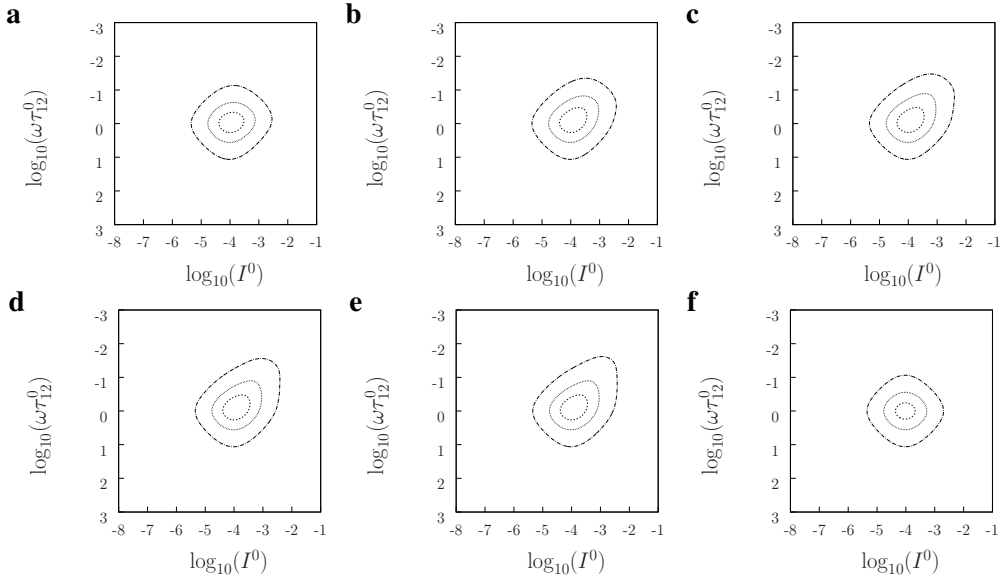


Figure S7: Theoretical dependence of the normalized amplitude of the out-of-phase oscillations in $\mathbf{1}$ concentration, $|1_{\text{norm}}^{1,\text{out}}| = |1^{1,\text{out}}/P_{\text{tot}}|$, of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to light harmonic forcing in the regime of large amplitude modulation ($\alpha = 1$) on the light flux I^0 (in $\text{ein} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) and the adimensional radial frequency $\omega\tau_{12}^0$. The numerical computation has been performed upon truncating the Fourier $f(\theta x)$ expansion at various orders n (**a**: 1, **b**: 2, **c**: 3, **d**: 4, **e**: 5). The dependence observed in a regime of small amplitude modulation, $|1_{\text{norm}}^{1,\text{out}}|$, is shown in **f**. $\sigma_{12} = 73 \text{ m}^2 \cdot \text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2 \cdot \text{mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$. The markers correspond to isodensity curves : 0.01 (dot dashed), 0.03 (dot) and 0.05 (dashed).

Expression of the overall observable from the photoswitchable probe In the case of a sinusoidal modulation of large amplitude obeying Eq.(S102), the temporal dependence of the overall observable $O(t)$ originates from the temporal dependence of $1^1(t)$ and $2^1(t)$ (given in Eqs.(S123,S124))

$$O(t) = Q_1 1 + Q_2 2 = \mathfrak{D}^o + \sum_{n=1}^{\infty} \left[\mathfrak{D}^{n,\text{in}} \sin(n\omega t) + \mathfrak{D}^{n,\text{out}} \cos(n\omega t) \right], \quad (\text{S131})$$

| Amplitude | n | $10^4 \times I^{0,R}$ ($\text{ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) | $10^2 \times \omega^R$ ($\text{rad}\cdot\text{s}^{-1}$) | $10^2 \times 1_{\text{norm}}^{1,\text{out}} $ |
|-----------|-----|---|--|--|
| Small | – | 0.96 | 3.02 | 5.81 |
| Large | 1 | 1.12 | 3.02 | 6.25 |
| Large | 2 | 1.19 | 2.82 | 6.38 |
| Large | 3 | 1.19 | 2.82 | 6.38 |
| Large | 4 | 1.19 | 2.82 | 6.38 |
| Large | 5 | 1.19 | 2.82 | 6.38 |

Table S1: Coordinates and amplitude of the $|1_{\text{norm}}^{1,\text{out}}|$ extremum from a photoswitchable fluorophore **1** \rightleftharpoons **2** submitted to light harmonic forcing in the regime of large amplitude modulation ($\alpha = 1$) as a function of the truncation order n of the Fourier expansion of the $f(\theta x)$ function. The Table also provides the coordinates and the amplitude of $|1_{\text{norm}}^{1,\text{out}}|$ extremum observed in a regime of small amplitude modulation.

with

$$\mathfrak{D}^{\circ} = Q_1 1^{\circ} + Q_2 2^{\circ} = Q_1 1^0 + Q_2 2^0 + (Q_2 - Q_1) \alpha a_0 \quad (\text{S132})$$

$$\mathfrak{D}^{\text{n,in}} = (Q_2 - Q_1) \alpha b_n \quad (\text{S133})$$

$$\mathfrak{D}^{\text{n,out}} = (Q_2 - Q_1) \alpha a_n \quad (\text{S134})$$

for $n > 0$.

Expression of the fluorescence intensity Fluorescence emission $I_F(t)$ is extracted from Eq.(S56) by using the temporal dependence (S102) of the exciting light source and the expression of $O(t)$ given in Eq.(S131).

Then writing

$$I_F(t) = \mathfrak{J}_{\mathfrak{F}}^{\circ} + \sum_{n=1}^{\infty} \left[\mathfrak{J}_{\mathfrak{F}}^{\text{n,in}} \sin(n\omega t) + \mathfrak{J}_{\mathfrak{F}}^{\text{n,out}} \cos(n\omega t) \right] \quad (\text{S135})$$

we derive

$$\mathfrak{J}_{\mathfrak{F}}^{\circ} = \left(\mathfrak{D}^{\circ} + \frac{1}{2} \alpha \mathfrak{D}^{1,\text{in}} \right) I^0 \quad (\text{S136})$$

$$\mathfrak{J}_{\mathfrak{F}}^{1,\text{in}} = \left(\alpha \mathfrak{D}^{\circ} + \mathfrak{D}^{1,\text{in}} - \frac{1}{2} \alpha \mathfrak{D}^{2,\text{out}} \right) I^0 \quad (\text{S137})$$

$$\mathfrak{J}_{\mathfrak{F}}^{1,\text{out}} = \left(\mathfrak{D}^{1,\text{out}} + \frac{1}{2} \alpha \mathfrak{D}^{2,\text{in}} \right) I^0 \quad (\text{S138})$$

$$\mathfrak{J}_{\mathfrak{F}}^{\text{n,in}} = \left[\mathfrak{D}^{\text{n,in}} + \frac{1}{2} \alpha \left(\mathfrak{D}^{\text{n-1,out}} - \mathfrak{D}^{\text{n+1,out}} \right) \right] I^0 \quad (\text{S139})$$

$$\mathfrak{J}_{\mathfrak{F}}^{\text{n,out}} = \left[\mathfrak{D}^{\text{n,out}} + \frac{1}{2} \alpha \left(\mathfrak{D}^{\text{n+1,in}} - \mathfrak{D}^{\text{n-1,in}} \right) \right] I^0 \quad (\text{S140})$$

for $n > 1$. As shown in Eqs.(S136–S140), a large sinusoidal modulation of illumination is expected to cause modulation of the fluorescence intensity at an infinite number of radial frequencies, which are integer multiples of ω .

The zeroth order term $\mathfrak{J}_{\mathfrak{F}}^{\circ}$ can be expressed using Eqs.(S84,S112,S116,S129,S133,S136). We obtained

$$\mathfrak{J}_{\mathfrak{F}}^{\circ} = I_F^0 + \frac{1}{2} \alpha^2 (Q_2 - Q_1) \left[1 - \left(k_{12}^{h\nu,0} + k_{21}^{h\nu,0} \right) \tau_{12}^0 \right] 2^{1,\text{in}} I^0. \quad (\text{S141})$$

In contrast to the out-of-phase first-order response, Eq.(S141) shows that the zeroth order response of the fluorescence emission upon sinusoidal light modulation of large amplitude significantly departs from I_F^0 , which is observed in the case of a same type of modulation but in a regime of small amplitude.

The first order terms can be expressed using Eqs.(S84, S129, S130, S133, S134, S137) and (S129, S130, S133, S134, S138) to write

$$\begin{aligned}\mathcal{J}_{\mathfrak{F}}^{1,\text{in}} &= \alpha I_F^0 + \alpha (Q_2 - Q_1) z^{1,\text{in}} I^0 \left[1 - \frac{1}{2} \alpha^2 (k_{12}^{h\nu,0} + k_{21}^{h\nu,0}) \tau_{12}^0 \right] - \frac{1}{2} \alpha^2 (Q_2 - Q_1) z^{2,\text{out}} I^0 \\ \mathcal{J}_{\mathfrak{F}}^{1,\text{out}} &= \alpha (Q_2 - Q_1) z^{1,\text{out}} I^0 + \frac{1}{2} \alpha^2 (Q_2 - Q_1) z^{2,\text{in}} I^0.\end{aligned}\quad (\text{S143})$$

In reference to the $g(z^{n,\text{in}}, z^{n,\text{out}})$ function introduced in the Main Text, one has thus

$$g(z^{n,\text{in}}, z^{n,\text{out}}) = \frac{1}{2} \alpha z^{2,\text{in}}. \quad (\text{S144})$$

To evaluate the possible interference originating from the second order term $g(z^{n,\text{in}}, z^{n,\text{out}})$, we used Eq.(40) to numerically compute the dependence of $\mathcal{J}_{\mathfrak{F}}^{1,\text{out}}$ on ω and I^0 . Figure S8 compares the absolute value of the normalized out-of-phase first-order amplitude $|\mathcal{J}_{\mathfrak{F},\text{norm}}^{1,\text{out}}| = |\mathcal{J}_{\mathfrak{F}}^{1,\text{out}} / (Q_2 - Q_1) I^0 \alpha P_{\text{tot}}|$ obtained in the case of the largest amplitude modulation ($\alpha = 1$) with that obtained for a modulation of small amplitude. The position and the amplitude of the optimum are similar in both cases: we found $I^{0,R} = 1.03 \times 10^{-4} \text{ ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, $\omega^R = 3.09 \times 10^{-2} \text{ rad}\cdot\text{s}^{-1}$, and $|\mathcal{J}_{\mathfrak{F},\text{norm}}^{1,\text{out}}| = 5.82 \times 10^{-2}$ in the case of a sinusoidal modulation of large amplitude, and $I^{0,R} = 0.96 \times 10^{-4} \text{ ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, $\omega^R = 3.02 \times 10^{-2} \text{ rad}\cdot\text{s}^{-1}$, and $|I_{F,\text{norm}}^{1,\text{out}}| = 5.81 \times 10^{-2}$ in the case of a sinusoidal modulation of small amplitude.

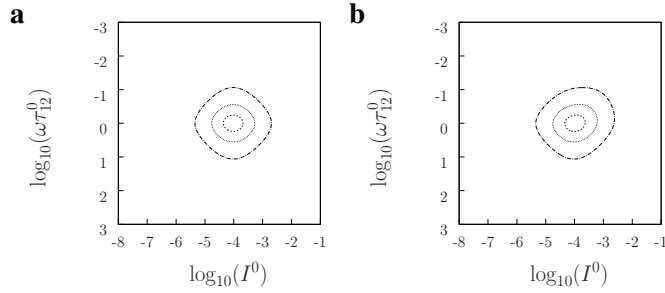


Figure S8: Theoretical dependence of the absolute value of the normalized amplitude of the out-of-phase oscillations of fluorescence intensity, $|\mathcal{J}_{\mathfrak{F},\text{norm}}^{1,\text{out}}| = |\mathcal{J}_{\mathfrak{F}}^{1,\text{out}} / (Q_2 - Q_1) I^0 \alpha P_{\text{tot}}|$, from a solution of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to light harmonic forcing in the regime of small (a) or large (b; $\alpha = 1$) amplitude modulation on the light flux I^0 (in $\text{ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) and the adimensional radial frequency $\omega\tau_{12}^0$. The numerical computation has been performed upon truncating the Fourier f expansion at the fifth order ($n = 5$). $\sigma_{12} = 73 \text{ m}^2\cdot\text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2\cdot\text{mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$. The markers correspond to isodensity curves : 0.01 (dot dashed), 0.03 (dot) and 0.05 (dashed).

S2.3.4 Square wave modulation of large amplitude

Expression of the concentrations We eventually consider that the system is submitted to a square wave modulation of illumination of large amplitude. We correspondingly adopt

$$I(t) = I^0 \left\{ 1 + \frac{4\alpha}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t] \right\} \quad (\text{S145})$$

by choosing the starting time ($t = 0$) in this expansion halfway through the first pulse.

The equations (S42,S43) are then solved upon introducing

$$2 = 2^0 + \alpha f(t) \quad (\text{S146})$$

$$1 = 1^0 - \alpha f(t) \quad (\text{S147})$$

$$k_{12}(t) = k_{12}^{h\nu,0} \left\{ 1 + \frac{4\alpha}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t] \right\} \quad (\text{S148})$$

$$k_{21}(t) = k_{21}^{h\nu,0} \left\{ 1 + \frac{4\alpha}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t] \right\} + k_{21}^{\Delta} \quad (\text{S149})$$

to yield

$$\frac{df(t)}{dt} = -\frac{f(t)}{\tau_{12}^0} + \left[\rho_{12}^0 p_{21}^{\Delta} - \alpha (k_{12}^{h\nu,0} + k_{21}^{h\nu,0}) f(t) \right] \frac{4}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\omega t]. \quad (\text{S150})$$

Upon introducing x , a , b , and θ given in Eqs.(S110–S113), Eq.(S150) becomes

$$\frac{df(\theta x)}{dx} = -f(\theta x) + [a - bf(\theta x)] \frac{4}{\pi} \sum_{p=0}^{\infty} \frac{1}{2p+1} \sin [(2p+1)\theta x] \quad (\text{S151})$$

Beyond the relaxation time τ_{12}^0 , one enters into a permanent regime in which $f(\theta x)$ is a continuous periodic function. Adopting again Eq.(S115) for its Fourier series, the a_n and b_n terms can be extracted from Eq.(S151) upon identifying the amplitudes of the components of the same order. We derived

- Zeroth order:

$$a_0 = -b \frac{2}{\pi} b_1 \quad (\text{S152})$$

- First order:

$$-a_1 \theta = -b_1 + \frac{4}{\pi} (a - a_0 b) + b \frac{2}{\pi} \sum_{p=1}^{\infty} \frac{a_{2p}}{2p-1} \quad (\text{S153})$$

$$b_1 \theta = -a_1 - b \frac{2}{\pi} \sum_{p=1}^{\infty} \frac{b_{2p}}{2p-1} \quad (\text{S154})$$

- Even order $n = 2k$ with $k \in \mathbb{N}^*$:

$$a_{2k} 2k\theta = b_{2k} + b \frac{2}{\pi} \left[\sum_{p=0}^{k-1} \frac{a_{2k-(2p+1)}}{2p+1} - \sum_{p=0}^{\infty} \frac{a_{2k+2p+1}}{2p+1} \right] \quad (\text{S155})$$

$$b_{2k} 2k\theta = -a_{2k} - b \frac{2}{\pi} \left[\sum_{p=0}^{\infty} \frac{b_{2k+2p+1}}{2p+1} - \sum_{p=0}^{k-1} \frac{b_{2k-(2p+1)}}{2p+1} \right] \quad (\text{S156})$$

- Odd order $n = 2k + 1$ with $k \in \mathbb{N}^*$:

$$a_{2k+1} (2k+1)\theta = b_{2k+1} + \frac{4}{\pi(2k+1)} (a_0 b - a) + b \frac{2}{\pi} \left[\sum_{p=0}^{k-1} \frac{a_{2(k-p)}}{2p+1} - \sum_{p=0}^{\infty} \frac{a_{2(k+p+1)}}{2p+1} \right] \quad (\text{S157})$$

$$b_{2k+1} (2k+1)\theta = -a_{2k+1} - b \frac{2}{\pi} \left[\sum_{p=0}^{\infty} \frac{b_{2(k+p+1)}}{2p+1} - \sum_{p=0}^{k-1} \frac{b_{2(k-p)}}{2p+1} \right] \quad (\text{S158})$$

from which we can retrieve the expressions of the concentrations in **1** and **2** using Eqs.(S121,S122). At steady-state, as for the sinusoidal modulation of large amplitude, square wave modulation causes modulation of the concentrations in **1** and **2** at an infinite number of radial frequencies. Eqs.(S121,S122) can be subsequently transformed to explicit the amplitudes of the concentration modulations at all radial frequencies using Eqs.(S123–S130).

We showed that, in the case of sinusoidal light modulation of small amplitude, $z^{1,\text{out}}$ is optimal when the resonance conditions (14,15) are fulfilled. In the absence of analytical expressions for $z^{1,\text{out}}$, such conclusions cannot be directly derived in the case of a square wave modulation of large amplitude, so we evaluated their relevance by means of numerical calculations. Compared to the sinusoidal modulation, square wave modulation introduces harmonics at higher frequencies, which could interfere with resonance of the radial frequency. We therefore analyzed the dependence of $\mathbf{1}^{1,\text{out}} = -z^{1,\text{out}} = -a_1$ on the control parameters, ω and I^0 by analytically retrieving the $2n + 1$ unknown parameters (a_0, \dots, a_n, b_n) upon truncating at the orders p and n in the expansions of the light excitation in Eq.(21) and the $f(\theta x)$ function in Eq.(30). Figure S9 displays representative results, which have been obtained upon truncating at the 3rd and 4th orders the expansions of the light excitation and the $f(\theta x)$ function.

Figure S10 displays the dependence of the normalized amplitude, $|\mathbf{1}_{\text{norm}}^{1,\text{out}}| = |\mathbf{1}^{1,\text{out}}/P_{\text{tot}}|$, on the light flux I^0 and the adimensional radial frequency $\omega\tau_{12}^0$ when $\alpha = 0.8$ (see also Figure S11 showing that $|\mathbf{1}_{\text{norm}}^{1,\text{out}}|$ is essentially independent on α).

Truncation in the expansions of the light excitation in Eq.(21) and the $f(\theta x)$ function in Eq.(30) at the 3rd and 4th orders respectively is sufficient to observe convergence: the dependence of $|\mathbf{1}_{\text{norm}}^{1,\text{out}}|$ on I^0 and ω does not significantly evolve beyond $p = 1$ and $n = 4$. As in the case of sinusoidal modulation of large amplitude, $|\mathbf{1}_{\text{norm}}^{1,\text{out}}|$ exhibits an optimum in the space (I^0, ω) , which position and amplitude are very close to those observed with a sinusoidal modulation of small amplitude (Table S2). Note that the amplitudes are respectively $|\mathbf{1}_{\text{norm}}^{1,\text{out}}| = 8.16 \times 10^{-2}$ for the square wave modulation of large amplitude and $\frac{4}{\pi} |\mathbf{1}_{\text{norm}}^{1,\text{out}}| = 7.40 \times 10^{-2}$ for the modulation of small amplitude ($\frac{4}{\pi}$ is a correcting term resulting from Eq.(21)) (Table S2).

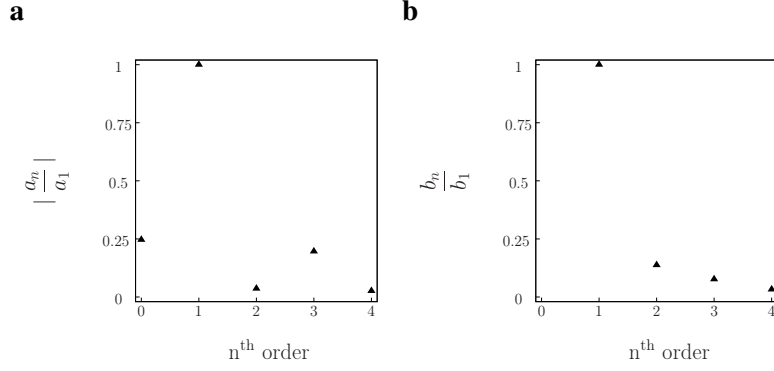


Figure S9: Computation of the normalized amplitude $|a_n|$ (**a**) and b_n (**b**) from a solution of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to square wave forcing ($\alpha = 0.8$). The numerical computation has been performed upon truncating at the 3rd and 4th truncation orders the expansions of the light excitation and the $f(x)$ function. $\sigma_{12} = 73 \text{ m}^2 \cdot \text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2 \cdot \text{mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$, $I^0 = 9.6 \times 10^{-5} \text{ ein} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ and $\omega\tau_{12}^0 = 1$.

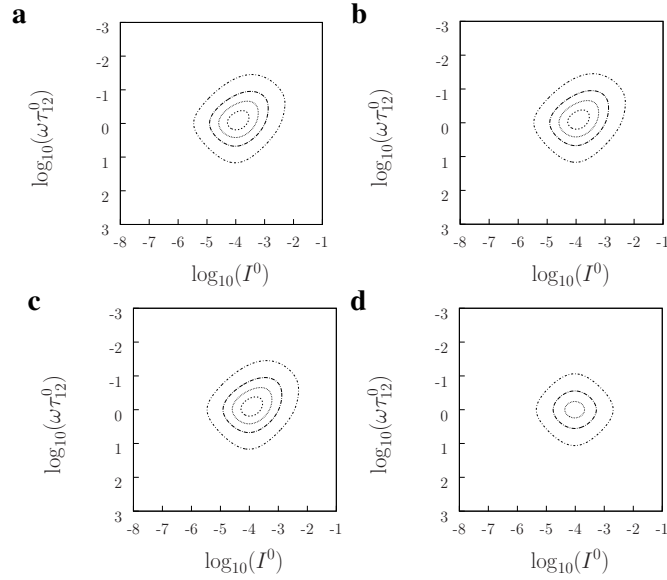


Figure S10: Theoretical dependence of the normalized amplitude of the out-of-phase oscillations in $\mathbf{1}$ concentration, $|1_{\text{norm}}^{1,\text{out}}| = |1^{1,\text{out}}/P_{\text{tot}}|$, of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to light square wave forcing ($\alpha = 0.8$) on the light flux I^0 (in $\text{ein} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) and the adimensional radial frequency $\omega\tau_{12}^0$. The numerical computation has been performed upon adopting various values of p and n for the truncation of the orders in the expansions of the light excitation in Eq.(21) and the $f(\theta x)$ function in Eq.(30). $p = 1$ and $n = 4$ (**a**); $p = 2$ and $n = 6$ (**b**); $p = 3$ and $n = 8$ (**c**). The corresponding theoretical dependence of the normalized amplitude of the out-of-phase oscillations of in $\mathbf{1}$ concentration from a solution of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to light harmonic forcing in the regime of small amplitude modulation is displayed in **d** as a reference. $\sigma_{12} = 73 \text{ m}^2 \cdot \text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2 \cdot \text{mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$. The markers correspond to isodensity curves : 0.01 (dot small dashed), 0.03 (dot large dashed), 0.05 (dot) and 0.07 (dashed).

Expression of the overall observable from the photoswitchable probe The expression of the overall observable in the case of a square wave modulation obeying Eq.(S145) is identical to the one derived for a sinusoidal modulation. Then Eqs.(S131,S132,S133,S134) are here also valid.

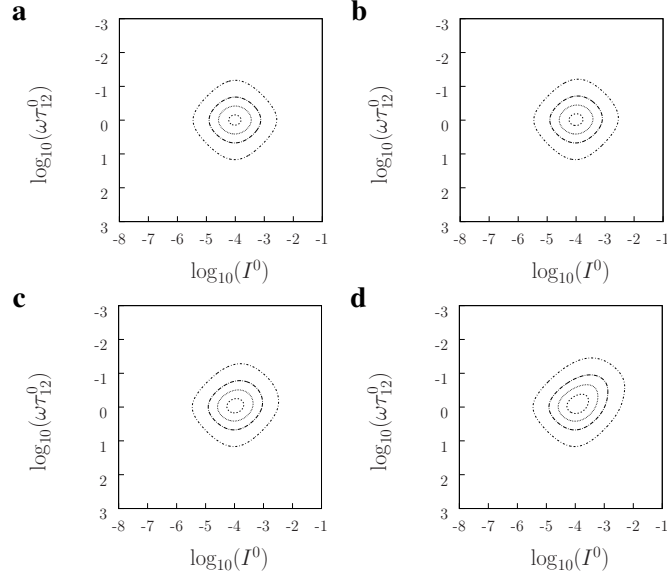


Figure S11: Theoretical dependence of the normalized amplitude of the out-of-phase oscillations in **1** concentration, $|\mathbf{1}_{\text{norm}}^{\mathbf{1},\text{out}}| = |\mathbf{1}^{\mathbf{1},\text{out}}/P_{\text{tot}}|$, of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to light square wave forcing on the light flux I^0 (in $\text{ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) and the adimensional radial frequency $\omega\tau_{12}^0$ for various values of the amplitude modulation α . The numerical computation has been performed upon adopting $p = 1$ and $n = 4$ for the truncation of the orders in the expansions of the light excitation in Eq.(S145) and the $f(x)$ function in Eq.(S115). $\alpha = 0.2$ (a); $\alpha = 0.4$ (b); $\alpha = 0.6$ (c); $\alpha = 0.8$ (d). $\sigma_{12} = 73 \text{ m}^2\cdot\text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2\cdot\text{mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$. The markers correspond to isodensity curves : 0.01 (dot small dashed), 0.03 (dot large dashed), 0.05 (dot) and 0.07 (dashed). In contrast to the sinusoidal modulation of large amplitude, we could not numerically compute the behavior at the largest modulation amplitude $\alpha = 1$. This behavior probably originates from the truncation of the expansion of light excitation in Eq.(21). As shown in Figure S5, one observes significant oscillations close to the discontinuity of the square wave function. They most probably generate singularities (e.g. negative values of the concentrations) at too large α values. This explains why we checked that the behavior of the $|\mathbf{1}_{\text{norm}}^{\mathbf{1},\text{out}}|$ did not significantly depend on the α value. We correspondingly adopted $\alpha = 0.8$ above.

Expression of the fluorescence intensity Fluorescence emission $I_F(t)$ is extracted from Eq.(S56) by using the temporal dependence (S145) of the exciting light source and the expression of $O(t)$ given in Eq.(S131). Adopting again the expression (S135), we derive

- Zeroth order:

$$\mathcal{J}_{\mathfrak{F}}^{\circ} = \left(\mathfrak{D}^{\circ} + \frac{2\alpha}{\pi} \sum_{p=0}^{\infty} \frac{\mathfrak{D}^{2p+1,\text{in}}}{2p+1} \right) I^0 \quad (\text{S159})$$

- First order:

$$\mathcal{J}_{\mathfrak{F}}^{\mathbf{1},\text{in}} = \left[\mathfrak{D}^{\mathbf{1},\text{in}} + \frac{4\alpha}{\pi} \mathfrak{D}^{\circ} - \frac{2\alpha}{\pi} \left(\sum_{p=0}^{\infty} \frac{\mathfrak{D}^{2(p+1),\text{out}}}{2p+1} \right) \right] I^0 \quad (\text{S160})$$

$$\mathcal{J}_{\mathfrak{F}}^{\mathbf{1},\text{out}} = \left[\mathfrak{D}^{\mathbf{1},\text{out}} + \frac{2\alpha}{\pi} \left(\sum_{p=0}^{\infty} \frac{\mathfrak{D}^{2(p+1),\text{in}}}{2p+1} \right) \right] I^0 \quad (\text{S161})$$

| Modulation | p | n | $10^4 \times I^{0,R}$ ($\text{ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) | $10^2 \times \omega^R$ ($\text{rad}\cdot\text{s}^{-1}$) | $10^2 \times 1_{\text{norm}}^{1,\text{out}} $ |
|-------------|-----|-----|---|--|--|
| Sinusoidal | – | – | 0.96 | 3.02 | 5.81 |
| Square wave | 1 | 4 | 1.20 | 2.82 | 8.16 |
| Square wave | 2 | 6 | 1.20 | 2.82 | 8.16 |
| Square wave | 3 | 8 | 1.20 | 2.82 | 8.16 |

Table S2: Coordinates and amplitude of the $|1_{\text{norm}}^{1,\text{out}}|$ extremum from a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to light square wave forcing ($\alpha = 0.8$) as a function of the truncation orders p and n in the expansions of the light excitation in Eq.(21) and the $f(\theta x)$ function in Eq.(30). The Table also provides the coordinates and amplitude of $|1_{\text{norm}}^{1,\text{out}}|$ extremum observed in a regime of sinusoidal modulation of small amplitude.

- Even order $n = 2k$ with $k \in \mathbb{N}^*$:

$$\mathcal{J}_{\mathfrak{F}}^{2\ell,\text{in}} = \left[\mathfrak{D}^{2\ell,\text{in}} + \frac{2\alpha}{\pi} \left(\sum_{p=0}^{k-1} \frac{\mathfrak{D}^{2\ell-(2p+1),\text{out}}}{2p+1} - \sum_{p=0}^{\infty} \frac{\mathfrak{D}^{2\ell+2p+1,\text{out}}}{2p+1} \right) \right] I^0 \quad (\text{S162})$$

$$\mathcal{J}_{\mathfrak{F}}^{2\ell,\text{out}} = \left[\mathfrak{D}^{2\ell,\text{out}} + \frac{2\alpha}{\pi} \left(\sum_{p=0}^{\infty} \frac{\mathfrak{D}^{2\ell+2p+1,\text{in}}}{2p+1} - \sum_{p=0}^{k-1} \frac{\mathfrak{D}^{2\ell-(2p+1),\text{in}}}{2p+1} \right) \right] I^0 \quad (\text{S163})$$

- Odd order $n = 2k + 1$ with $k \in \mathbb{N}^*$:

$$\mathcal{J}_{\mathfrak{F}}^{2\ell+1,\text{in}} = \left[\mathfrak{D}^{2\ell+1,\text{in}} + \frac{4\alpha}{(2k+1)\pi} \mathfrak{D}^0 + \frac{2\alpha}{\pi} \left(\sum_{p=0}^{k-1} \frac{\mathfrak{D}^{2(\ell-p),\text{out}}}{2p+1} - \sum_{p=0}^{\infty} \frac{\mathfrak{D}^{2(\ell+p+1),\text{out}}}{2p+1} \right) \right] I^0 \quad (\text{S164})$$

$$\mathcal{J}_{\mathfrak{F}}^{2\ell+1,\text{out}} = \left[\mathfrak{D}^{2\ell+1,\text{out}} + \frac{2\alpha}{\pi} \left(\sum_{p=0}^{\infty} \frac{\mathfrak{D}^{2(\ell+p+1),\text{in}}}{2p+1} - \sum_{p=0}^{k-1} \frac{\mathfrak{D}^{2(\ell-p),\text{in}}}{2p+1} \right) \right] I^0 \quad (\text{S165})$$

As for the sinusoidal modulation of large amplitude, a square wave modulation of illumination is expected to cause modulation of the fluorescence intensity at an infinite number of radial frequencies, which are integer multiples of ω .

The zeroth order term $\mathcal{J}_{\mathfrak{F}}^0$ can be expressed using Eqs.(S84,S112,S116,S129,S133,S136). We obtained

$$\frac{\mathcal{J}_{\mathfrak{F}}^0 - I_F^0}{(Q_2 - Q_1) \alpha^2 I^0} = \frac{2}{\pi} \left[1 - \left(k_{12}^{h\nu,0} + k_{21}^{h\nu,0} \right) \tau_{12}^0 \right] z^{1,\text{in}} + \frac{2}{\pi} \sum_{p=1}^{\infty} \frac{z^{2p+1,\text{in}}}{2p+1}. \quad (\text{S166})$$

In contrast to the out-of-phase first-order response, Eq.(S166) shows that the zeroth order response of the fluorescence emission upon square wave light modulation of large amplitude significantly departs from I_F^0 , which is observed for a sinusoidal light modulation of small amplitude.

The first order terms can be expressed using Eqs.(S129, S130, S133, S134, S137) and (S129, S130, S133, S134, S138) to write

$$\frac{\mathcal{J}_{\mathfrak{F}}^{1,\text{in}} - \frac{4}{\pi} \alpha I_F^0}{\alpha (Q_2 - Q_1) I^0} = z^{1,\text{in}} \left[1 - \frac{8}{\pi^2} \alpha^2 \left(k_{12}^{h\nu,0} + k_{21}^{h\nu,0} \right) \tau_{12}^0 \right] - \frac{2}{\pi} \alpha \sum_{p=0}^{\infty} \frac{z^{2(p+1),\text{out}}}{2p+1} \quad (\text{S167})$$

$$\mathcal{J}_{\mathfrak{F}}^{1,\text{out}} = \alpha (Q_2 - Q_1) I^0 \left[z^{1,\text{out}} + \frac{2}{\pi} \alpha \sum_{p=0}^{\infty} \frac{z^{2(p+1),\text{in}}}{2p+1} \right]. \quad (\text{S168})$$

In reference to the $g(2^{n,\text{in}}, 2^{n,\text{out}})$ function introduced in the Main Text, one has thus

$$g(2^{n,\text{in}}, 2^{n,\text{out}}) = \frac{2}{\pi} \alpha \sum_{p=0}^{\infty} \frac{2^{2(p+1),\text{in}}}{2p+1}. \quad (\text{S169})$$

To evaluate the possible interference originating from this second order term $g(2^{n,\text{in}}, 2^{n,\text{out}})$, we used Eq.(40) to numerically compute the dependence of $\mathcal{J}_{\mathcal{F}}^{1,\text{out}}$ on ω and I^0 . Figure S12 compares the absolute value of the normalized out-of-phase first order amplitude $|\mathcal{J}_{\mathcal{F},\text{norm}}^{1,\text{out}}| = |\mathcal{J}_{\mathcal{F}}^{1,\text{out}} / (Q_2 - Q_1) I^0 \alpha P_{\text{tot}}|$ obtained in the case of the square wave light modulation ($\alpha = 0.8$) with that obtained for a modulation of small amplitude. The position of the optimum is similar in both cases: we found $I^{0,R} = 1.04 \times 10^{-4} \text{ ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ and $\omega^R = 3.09 \times 10^{-2} \text{ rad}\cdot\text{s}^{-1}$ in the case of a square wave modulation, and $I^{0,R} = 0.96 \times 10^{-4} \text{ ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ and $\omega^R = 3.02 \times 10^{-2} \text{ rad}\cdot\text{s}^{-1}$ in the case of a modulation of small amplitude. The amplitudes of the optimum fairly compare as well: we computed $|\mathcal{J}_{\mathcal{F},\text{norm}}^{1,\text{out}}| = 7.43 \times 10^{-2}$ in the case of square wave modulation, and $\frac{4}{\pi} |\mathcal{J}_{\mathcal{F},\text{norm}}^{1,\text{out}}| = 7.40 \times 10^{-2}$ in the case of a sinusoidal modulation of small amplitude.

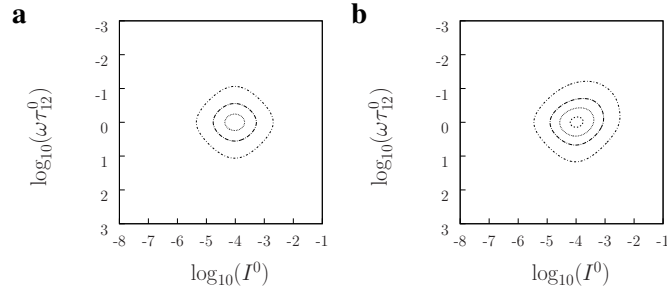


Figure S12: Theoretical dependence of the absolute value of the normalized amplitude of the out-of-phase oscillations of fluorescence intensity, $|\mathcal{J}_{\mathcal{F},\text{norm}}^{1,\text{out}}|$, from a solution of a photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$ submitted to square wave forcing (**b**; $\alpha = 0.8$) on the light flux I^0 (in $\text{ein}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) and the adimensional radial frequency $\omega\tau_{12}^0$. The numerical computation has been performed upon truncating at the 3rd and 4th truncation orders the expansions of the light excitation and the $f(x)$ function. The corresponding dependence in the regime of small amplitude sinusoidal modulation is displayed in **a**. $\sigma_{12} = 73 \text{ m}^2\cdot\text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2\cdot\text{mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$. The markers correspond to isodensity curves : 0.01 (dot small dashed), 0.03 (dot large dashed), 0.05 (dot) and 0.07 (dashed).

S3 Retrieval of concentrations from the observables

In the following section, we assume that, upon modulating light, the observable associated to the photoswitchable probe adopts the expression

$$S(t) = \mathfrak{S}^{\circ} + \sum_{n=1}^{\infty} \left[\mathfrak{S}^{n,\text{in}} \sin(n\omega t) + \mathfrak{S}^{n,\text{out}} \cos(n\omega t) \right], \quad (\text{S170})$$

which includes both contributions originating from the exchanging states **1** and **2**. In the absence of convolution with the exciting light intensity, the $\{\mathfrak{S}^{\circ}, \mathfrak{S}^{n,\text{in}}, \mathfrak{S}^{n,\text{out}}\}$ set identifies to the $\{\mathfrak{D}^{\circ}, \mathfrak{D}^{n,\text{in}}, \mathfrak{D}^{n,\text{out}}\}$ one in reference to Eq.(S131). In contrast, when one observes convolution of the concentrations with light intensity like for fluorescence emission, the $\{\mathfrak{S}^{\circ}, \mathfrak{S}^{n,\text{in}}, \mathfrak{S}^{n,\text{out}}\}$ terms correspond to the $\{\mathfrak{J}_{\mathfrak{F}}^{\circ}, \mathfrak{J}_{\mathfrak{F}}^{n,\text{in}}, \mathfrak{J}_{\mathfrak{F}}^{n,\text{out}}\}$ ones in Eq.(S135).

S3.1 Extraction of \mathfrak{S}° , $\mathfrak{S}^{n,\text{in}}$, and $\mathfrak{S}^{n,\text{out}}$ from the overall signal $S(t)$

\mathfrak{S}° , $\mathfrak{S}^{1,\text{in}}$, and $\mathfrak{S}^{1,\text{out}}$ can be easily retrieved from the experimental trace of the observed signal $S(t)$ (either global or from each analyzed pixel).

As shown in Eq.(S170), \mathfrak{S}° can be obtained upon averaging $S(t)$ over an integer number m of periods of the modulated illumination

$$Int^0 = \frac{1}{mT} \int_0^{mT} S(t) dt = \mathfrak{S}^{\circ}. \quad (\text{S171})$$

In particular, upon observing fluorescence emission in a regime of sinusoidal light modulation of small amplitude, one has $Int^0 = I_F^0$.

The n -th order amplitudes $\mathfrak{S}^{n,\text{in}}$ and $\mathfrak{S}^{n,\text{out}}$ can be extracted from the fluorescence signal upon computing the integrals $Int^{n,\text{in}}$ and $Int^{n,\text{out}}$

$$Int^{n,\text{in}} = \frac{2}{mT} \int_0^{mT} S(t) \sin(n\omega t) dt = \mathfrak{S}^{n,\text{in}} \quad (\text{S172})$$

$$Int^{n,\text{out}} = \frac{2}{mT} \int_0^{mT} S(t) \cos(n\omega t) dt = \mathfrak{S}^{n,\text{out}}. \quad (\text{S173})$$

In particular

$$Int^{1,\text{in}} = \frac{2}{mT} \int_0^{mT} S(t) \sin(\omega t) dt = \mathfrak{S}^{1,\text{in}} \quad (\text{S174})$$

$$Int^{1,\text{out}} = \frac{2}{mT} \int_0^{mT} S(t) \cos(\omega t) dt = \mathfrak{S}^{1,\text{out}}. \quad (\text{S175})$$

Upon observing fluorescence emission in a regime of sinusoidal light modulation of small amplitude, one has $Int^{1,\text{in}} = I_F^{1,\text{in}}$ and $Int^{1,\text{out}} = I_F^{1,\text{out}}$, and $Int^{n,\text{in}} = I_F^{n,\text{in}} = 0$ and $Int^{n,\text{out}} = I_F^{n,\text{out}} = 0$ for $n > 1$.

S3.2 Quantifying a targeted component

In this subsection, we show how the observables \mathfrak{S}° and $\mathfrak{S}^{1,\text{out}}$ can be used to retrieve the concentration of a targeted component in two cases. In the first case, we consider that the photoswitchable probe is used for analyte labelling and that one aims at measuring P_{tot} , which quantifies the labelled analyte. This situation typically

refers to the dynamic schemes, which have been introduced in the subsections S1.1 (the label is a photoswitchable fluorophore) and S1.2 (the label is a fluorophore engaged in relaxation from both singlet and triplet excited states). In a second case, we consider that the photoswitchable probe is used for titrating an analyte **A** and that one aims at measuring the concentration of the titrated analyte A_{tot} . This situation has been addressed with the dynamic scheme introduced in subsection S1.3. As in subsection S1.3, we restrict our analysis to the specific case of (i) an analyte in excess ($A_{tot} \gg P_{tot}$) in a kinetic regime where (ii) photochemical reactions are rate-limiting with respect to reactions involving the analyte.

S3.2.1 Measuring the total concentration of the photoswitchable probe

The expressions of \mathfrak{S}° and $\mathfrak{S}^{1,\text{out}}$ identify to the ones of \mathfrak{D}° and $\mathfrak{D}^{n,\text{out}}$ or to $\mathfrak{J}_{\mathfrak{S}}^\circ$ and $\mathfrak{J}_{\mathfrak{S}}^{n,\text{out}}$ (when one observes convolution of the concentrations with light intensity like for fluorescence emission).

In the absence of light modulation or in a regime of sinusoidal light modulation of small amplitude, the expression of \mathfrak{S}° can be deduced from Eqs.(S49,S50,S55,S84)

$$\mathfrak{D}^\circ = \frac{\mathfrak{J}_{\mathfrak{S}}^\circ}{I^0} = Q_1 \frac{1}{1 + K_{12}^0} P_{tot} + Q_2 \frac{K_{12}^0}{1 + K_{12}^0} P_{tot} \quad (\text{S176})$$

where

$$K_{12}^0 = \frac{\sigma_{12} I^0}{\sigma_{21} I^0 + k_{21}^\Delta}. \quad (\text{S177})$$

In the presence of light modulation, we showed in the Main Text that $\mathfrak{S}^{1,\text{out}}$ can be reliably evaluated from its expression in the case of a sinusoidal light modulation of small amplitude. We correspondingly used Eqs.(S75,S78,S86) to yield

$$\mathfrak{D}^{1,\text{out}} = \frac{\mathfrak{J}_{\mathfrak{S}}^{1,\text{out}}}{I^0} = \varepsilon (Q_1 - Q_2) p_{21}^\Delta \frac{K_{12}^0}{(1 + K_{12}^0)^2} \frac{\omega \tau_{12}^0}{1 + (\omega \tau_{12}^0)^2} P_{tot} \quad (\text{S178})$$

where K_{12}^0 is given in Eq.(S177) and where

$$p_{21}^\Delta = \frac{k_{21}^\Delta}{\sigma_{21} I^0 + k_{21}^\Delta} \quad (\text{S179})$$

$$\tau_{12}^0 = \frac{1}{(\sigma_{12} + \sigma_{21}) I^0 + k_{21}^\Delta} \quad (\text{S180})$$

from Eqs.(S51,S67).

The theoretical expressions (S176,S178) make possible to directly retrieve the concentrations of the photoswitchable probe from the observed signal. However this approach requires the effort to acquire the values of all parameters involved in these expressions. Alternatively, quantification can proceed by calibration with the pure photoswitchable probe at a reference concentration. Eqs.(S176,S178) show that \mathfrak{S}° and $\mathfrak{S}^{1,\text{out}}$ are proportional to the overall concentration in photoswitchable probe. Thus quantification can be simply achieved by recording the observable from a calibrating solution of the photoswitchable probe at a known concentration P_{tot}^{cal} . The

concentrations of the photoswitchable probe, which are retrieved at zeroth- and first-order, are respectively

$$P_{tot}^0 = \frac{\mathfrak{S}^0}{\mathfrak{S}^{0,cal}} P_{tot}^{cal} \quad (\text{S181})$$

$$P_{tot}^{1,out} = \frac{\mathfrak{S}^{1,out}}{\mathfrak{S}^{1,out,cal}} P_{tot}^{cal}. \quad (\text{S182})$$

S3.2.2 Measuring the concentration of an analyte reacting with a photoswitchable probe

The expressions of \mathfrak{S}^0 and $\mathfrak{S}^{1,out}$ again identify to the ones of \mathfrak{D}^0 and $\mathfrak{D}^{n,out}$ or to $\mathfrak{J}_{\mathfrak{S}}^0$ and $\mathfrak{J}_{\mathfrak{S}}^{n,out}$ (when one observes convolution of the concentrations with light intensity like for fluorescence emission).

In the absence of light modulation or in a regime of sinusoidal light modulation of small amplitude, the expression of \mathfrak{S}^0 is again given in Eq.(S176) but with

$$Q_1 = \frac{Q_{1F} + Q_{1B} K_1^\Delta A_{tot}}{1 + K_1^\Delta A_{tot}} \quad (\text{S183})$$

$$Q_2 = \frac{Q_{2F} + Q_{2B} K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}} \quad (\text{S184})$$

$$K_{12}^0 = \frac{1 + K_2^\Delta A_{tot}}{1 + K_1^\Delta A_{tot}} \frac{\sigma_{12,F} I^0 + \sigma_{12,B} I^0 K_1^\Delta A_{tot}}{\left(\sigma_{21,F} I^0 + k_{21,F}^\Delta\right) + \left(\sigma_{21,B} I^0 + k_{21,B}^\Delta\right) K_2^\Delta A_{tot}}, \quad (\text{S185})$$

which originate from Eqs.(S34,S35,S40,S41,S49,S50,S52,S55,S84).

In the presence of light modulation, we again adopted the expression (S178) obtained in the case of a sinusoidal light modulation of small amplitude. The dependence on A_{tot} is now explicited in Eqs.(S183–S190)

$$p_{21}^\Delta = \frac{k_{21}^\Delta}{\frac{\sigma_{21,F} + \sigma_{21,B} K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}} I^0 + k_{21}^\Delta} \quad (\text{S186})$$

$$\sigma_{12} = \frac{\sigma_{12,F} + \sigma_{12,B} K_1^\Delta A_{tot}}{1 + K_1^\Delta A_{tot}} \quad (\text{S187})$$

$$\sigma_{21} = \frac{\left(\sigma_{21,F} I^0 + k_{21,F}^\Delta\right) + \left(\sigma_{21,B} I^0 + k_{21,B}^\Delta\right) K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}} \quad (\text{S188})$$

$$k_{21}^\Delta = \frac{k_{21,F}^\Delta + k_{21,B}^\Delta K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}} \quad (\text{S189})$$

$$\tau_{12}^0 = \frac{1}{\frac{\sigma_{12,F} I^0 + \sigma_{12,B} I^0 K_1^\Delta A_{tot}}{1 + K_1^\Delta A_{tot}} + \frac{\left(\sigma_{21,F} I^0 + k_{21,F}^\Delta\right) + \left(\sigma_{21,B} I^0 + k_{21,B}^\Delta\right) K_2^\Delta A_{tot}}{1 + K_2^\Delta A_{tot}}} \quad (\text{S190})$$

obtained from Eqs.(S36–S38,S51,S67).

Eqs.(S187–S189) first show that the values of I^0 and ω at resonance depend on A_{tot} . As classically practiced in any titration protocol, one should preliminarily have a guess of the concentration A_{tot} . Using this guess would permit to fix an initial set of resonance conditions (I^0, ω) and proceed as indicated below to extract a first evaluation of A_{tot} . This estimate would be subsequently used to fix a refined resonant (I^0, ω) set and extract a second evaluation of A_{tot} . This iteration should be performed until reaching convergence.

As shown in Eqs.(S183–S190), direct extraction of the concentration A_{tot} sought for requires a considerable amount of information about the dynamic system (**1F,1B,2F,2B**). In contrast, one can proceed by calibration

as proposed above. The information to be extracted from \mathfrak{S}° or $\mathfrak{S}^{1,out}$ is now contained in the amplitude of the \mathbf{P} response to the light modulation, normalized by its concentration. Hence to retrieve the \mathbf{A} concentration from a calibrating experiment requires to normalize \mathfrak{S}° or $\mathfrak{S}^{1,out}$ by the total concentration of \mathbf{P} . Then the most classical protocol is to proceed by a ratiometric analysis, where one collects the signal $S(t)$ upon adopting a same illumination but under two conditions of observation (for instance by recording the fluorescence emission at two different wavelengths), yielding to two sets of brightness, $\{Q_{1,1}, Q_{2,1}\}$ and $\{Q_{1,2}, Q_{2,2}\}$. The analyzed observables are now

$$\rho^0 = \frac{\frac{Q_{1F,1}+Q_{1B,1}K_1^\Delta A_{tot}}{1+K_1^\Delta A_{tot}} + \frac{Q_{2F,1}+Q_{2B,1}K_2^\Delta A_{tot}}{1+K_2^\Delta A_{tot}} K_{12}^0}{\frac{Q_{1F,2}+Q_{1B,2}K_1^\Delta A_{tot}}{1+K_1^\Delta A_{tot}} + \frac{Q_{2F,2}+Q_{2B,2}K_2^\Delta A_{tot}}{1+K_2^\Delta A_{tot}} K_{12}^0} \quad (\text{S191})$$

$$\rho^{1,out} = \frac{\frac{Q_{1F,1}+Q_{1B,1}K_1^\Delta A_{tot}}{1+K_1^\Delta A_{tot}} - \frac{Q_{2F,1}+Q_{2B,1}K_2^\Delta A_{tot}}{1+K_2^\Delta A_{tot}}}{\frac{Q_{1F,2}+Q_{1B,2}K_1^\Delta A_{tot}}{1+K_1^\Delta A_{tot}} - \frac{Q_{2F,2}+Q_{2B,2}K_2^\Delta A_{tot}}{1+K_2^\Delta A_{tot}}} \quad (\text{S192})$$

Calibration requires to preliminarily investigate the dependence of ρ^0 and $\rho^{1,out}$ on A_{tot} . Then quantification can be simply achieved by recording the observable from a calibrating solution where the photoswitchable probe is used to sense a known concentration A_{tot}^{cal} . The concentrations of the analyte, which are retrieved at zeroth- and first-order, are respectively

$$A_{tot}^0 = \frac{\rho^0}{\rho^{0,cal}} A_{tot}^{cal} \quad (\text{S193})$$

$$A_{tot}^{1,out} = \frac{\rho^{1,out}}{\rho^{1,out,cal}} A_{tot}^{cal}. \quad (\text{S194})$$

S4 Improvement of the spatial resolution

The light intensity-dependence of the first-order response of the concentration in photoswitchable fluorophore to the illumination modulation allows us for improving the spatial resolution of the out-of-phase fluorescence imaging protocol with respect to usual fluorescence imaging at zeroth-order. To evaluate the corresponding improvement, we computed the spatial profile of the fluorescence emission resulting from focusing a sinusoidally modulated light beam of wavelength λ (regime of small amplitude modulation) in a solution of photoswitchable fluorophore upon using both types of imaging protocols.

We assumed the light beam to be Gaussian and correspondingly adopted

$$I^0(r, z) = I^{0,R}(0, 0) \left[\frac{w(0)}{w(z)} \right]^2 \exp \left[\frac{-2r^2}{w^2(z)} \right] \quad (\text{S195})$$

with

$$w(z) = w(0) \sqrt{1 + \left(\frac{z}{z_R} \right)^2} \quad (\text{S196})$$

for the spatial dependence of light intensity. In Eq.(S195), r and z respectively correspond to the radial distance from the center axis of the beam and the axial distance from the beam's narrowest point (the "waist"), $w(z)$ is the radius at which light intensity drop to $1/e^2$ of its axial value, and $I^{0,R}(0, 0)$ is the intensity at the center of the beam at its waist, which is supposed to satisfy the resonance condition (14). In Eq.(S196), $z_R = \frac{\pi w^2(0)}{\lambda}$ designates the Rayleigh range.

S4.1 Fluorescence imaging at zeroth order

The fluorescence emission at zeroth order of the light modulation, I_F^0 , is given in Eq.(S84), where I^0 , $I^{0,2}$, and I^0 depend on the coordinates (r, z) . Using Eqs.(S49,S50,S52) and introducing the average light intensity at resonance, $I^{0,R} = \frac{k_{21}^{\Delta}}{(\sigma_{12} + \sigma_{21})}$, one obtains

$$I_F^0 = Q_1 \left[1 + \left(\frac{Q_2}{Q_1} - 1 \right) \frac{\sigma_{12}}{(\sigma_{12} + \sigma_{21})} \frac{\frac{I^0}{I^{0,R}}}{\frac{I^0}{I^{0,R}} + 1} \right] I^0 P_{tot}. \quad (\text{S197})$$

Upon introducing the fluorescence emission at zeroth order of the light modulation at resonance, $I_F^{0,R}$, one eventually derives

$$\frac{I_F^0}{I_F^{0,R}} = \frac{1 + \left(\frac{Q_2}{Q_1} - 1 \right) \frac{\sigma_{12}}{(\sigma_{12} + \sigma_{21})} \frac{\frac{I^0}{I^{0,R}}}{\frac{I^0}{I^{0,R}} + 1}}{1 + \frac{1}{2} \left(\frac{Q_2}{Q_1} - 1 \right) \frac{\sigma_{12}}{(\sigma_{12} + \sigma_{21})}} \frac{I^0}{I^{0,R}}. \quad (\text{S198})$$

In a homogeneous solution, one has thus

$$\frac{I_F^0(r, z)}{I_F^{0,R}(0, 0)} = \frac{1 + \left(\frac{Q_2}{Q_1} - 1 \right) \frac{\sigma_{12}}{(\sigma_{12} + \sigma_{21})} \frac{\frac{I^0(r, z)}{I^{0,R}(0, 0)}}{\frac{I^0(r, z)}{I^{0,R}(0, 0)} + 1}}{1 + \frac{1}{2} \left(\frac{Q_2}{Q_1} - 1 \right) \frac{\sigma_{12}}{(\sigma_{12} + \sigma_{21})}} \frac{I^0(r, z)}{I^{0,R}(0, 0)}. \quad (\text{S199})$$

S4.2 Out-of-Phase Fluorescence imaging at first order

The out-of-phase fluorescence emission at first order of the light modulation, $I_F^{1,out}$, is given in Eq.(S86), where $Q_1^{1,out}$, and I^0 depend on the coordinates (r, z) . Assuming light modulation to occur at resonance ($\omega^R = 2k_{z1}^{\Delta}$), we use Eqs.(S51,S52,S67,S75) to extract

$$I_F^{1,out} = -\varepsilon Q_1 \left(\frac{Q_2}{Q_1} - 1 \right) \frac{2\sigma_{12} \frac{I^0}{I^{0,R}}}{(\sigma_{12} + \sigma_{21}) \left(1 + \frac{I^0}{I^{0,R}} \right) \left[\left(1 + \frac{I^0}{I^{0,R}} \right)^2 + 4 \right]} I^0 P_{tot}. \quad (\text{S200})$$

Upon introducing the out-of-phase fluorescence emission at first order of the light modulation at resonance, $I_F^{1,out,R}$, one eventually derives

$$\frac{I_F^{1,out}}{I_F^{1,out,R}} = \frac{16 \frac{I^0}{I^{0,R}}}{\left(1 + \frac{I^0}{I^{0,R}} \right) \left[\left(1 + \frac{I^0}{I^{0,R}} \right)^2 + 4 \right]} \frac{I^0}{I^{0,R}}. \quad (\text{S201})$$

In a homogeneous solution, one has thus

$$\frac{I_F^{1,out}(r, z)}{I_F^{1,out,R}(0, 0)} = \frac{16 \frac{I^0(r, z)}{I^{0,R}(0, 0)}}{\left(1 + \frac{I^0(r, z)}{I^{0,R}(0, 0)} \right) \left[\left(1 + \frac{I^0(r, z)}{I^{0,R}(0, 0)} \right)^2 + 4 \right]} \frac{I^0(r, z)}{I^{0,R}(0, 0)}. \quad (\text{S202})$$

S4.3 Comparison of the spatial resolutions from the fluorescence imaging protocols at zeroth- and first-order

Figure S13 displays the results from Eqs.(S199,S202). One can notice that the width of the fluorescence profile is larger with the zeroth-order protocol than with the out-of-phase first-order one. More specifically, the gain in spatial resolution is more pronounced along the optical axis (Figure S13a) than in the focal plane (Figure S13b).

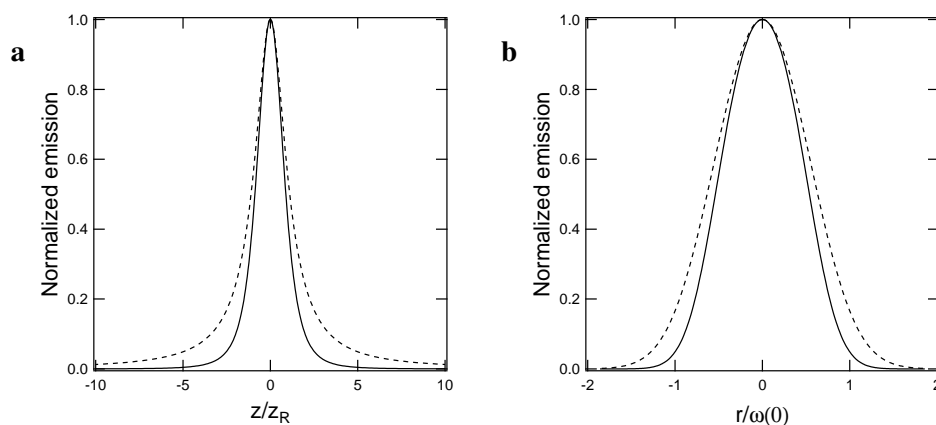


Figure S13: Normalized fluorescence intensity profiles resulting from applying a sinusoidally modulated Gaussian light beam (regime of small amplitude modulation) in a solution of photoswitchable fluorophore $\mathbf{1} \rightleftharpoons \mathbf{2}$. The longitudinal (along the optical axis: $r = 0$; **a**) and lateral (in the focal plane: $z = 0$; **b**) profiles have been calculated with Eqs.(S199) and (S202) using adimensional units involving the Rayleigh range z_R and the beam waist at $z = 0$, $w(0)$. Dashed line: zeroth-order fluorescence imaging; Solid line: out-of-phase first-order fluorescence imaging. The numerical computation has been performed with $\sigma_{12} = 73 \text{ m}^2 \cdot \text{mol}^{-1}$, $\sigma_{21} = 84 \text{ m}^2 \cdot \text{mol}^{-1}$, $Q_2/Q_1 = 0$.

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