Supporting materials to the article

Single-molecule spectroscopy of radical pairs, a theoretical treatment and experimental considerations

Noboru Ikeya,¹ Egor A. Nasibulov,*,²,³ Konstantin L. Ivanov,²,³ Kiminori Maeda,⁴ Jonathan R. Woodward¹

¹ University of Tokyo, Graduate School of Arts and Sciences, Tokyo, 153-8902, Japan
² International Tomography Center, Siberian Branch of the Russian Academy of Science, Novosibirsk, 630090, Russia
³ Novosibirsk State University, Novosibirsk, 630090, Russia
⁴ Saitama University, Graduate School of Science and Engineering, Saitama City, Saitama 338-8570, Japan

* Corresponding author, e-mail: nasibulov@tomo.nsc.ru

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I. Collection efficiency in our microscope

The total collection efficiency of the microscope, $\eta_{\text{system}}$, is written as follows:

$$\eta_{\text{system}} = \eta_{\text{obj}} \eta_{\text{dichroic}} \eta_{\text{mirror}} \eta_{\text{lens}} \eta_{\text{filter}} \eta_{\text{camera}}$$  \hspace{1cm} (1)

Here $\eta_{\text{obj}}$, $\eta_{\text{dichroic}}$, $\eta_{\text{mirror}}$, $\eta_{\text{lens}}$, $\eta_{\text{filter}}$ and $\eta_{\text{camera}}$ are the collection efficiencies of objective, dichroic mirror, reflection mirror, lens, filter and camera respectively. According to [1], the collection efficiency of objective $\eta_{\text{obj}}$ is determined by the following expression:

$$\eta_{\text{obj}} = \frac{1}{4\pi} \int_0^{2\pi} d\phi \int_0^{\sin^{-1} \frac{NA}{n_{\text{coverslip}}}} \sin \theta \, d\theta$$  \hspace{1cm} (2)

where $NA$ is the objective’s numerical aperture, and $n_{\text{coverslip}}$ is the refractive index of the coverslip.

Now, as we use a $NA = 1.49$ objective and $n_{\text{coverslip}} = 1.525$ coverslips, we substituted these values into the expression. Thus, we can obtain the collection efficiency of objective $\eta_{\text{obj}}$ as follows:

$$\eta_{\text{obj}} = 0.393$$  \hspace{1cm} (3)

Moreover, we use optical equipment with the following values

$$\eta_{\text{dichroic}} = 0.98, \eta_{\text{mirror}} = 0.9, \eta_{\text{lens}} = 0.998, \eta_{\text{filter}} = 0.98, \eta_{\text{camera}} = 0.81$$  \hspace{1cm} (4)

where the values come from the dichroic mirror, T470lpixr (Chroma), reflection mirror, CCM1-G01 (Thorlabs), lens lens, AC254-200-A (Thorlabs), filter, ET500lp (Chroma) and camera, ORCA-Flash4.0 V3 (Hamamatsu) used in our laboratory. Therefore, we obtain the following value for the total collection efficiency of the microscope, $\eta_{\text{system}}$:

$$\eta_{\text{system}} = 0.27$$  \hspace{1cm} (5)

This value is used for estimating the feasibility of SMS experiments with the FAD system using existing equipment.

II. Rate equation description for the FAD photochemistry scheme

First, the rate equations on the basis of the reaction scheme given by Figure 6 in the main article are expressed as follows:

$$\frac{d[S_0]}{dt} = -k_{ex}[S_0] + (k_F + k_{IC})[S_1] + k_d[T_P] + k_d[T_0] + k_{bet}[S^R]$$ \hspace{1cm} (6)

$$\frac{d[S_1]}{dt} = k_{ex}[S_0] - (k_F + k_{IC} + 3k_{ISC})[S_1]$$

$$\frac{d[T_P]}{dt} = 2k_{ISC}[S_1] - (k_d + k_R + k_1)[T_P] + 2k_R^T[T_0] + k_{-1}[H^+][T_P^R]$$
\[
\frac{d[T_0]}{dt} = k_{ISC}[S_1] + k_R^T[T_P] - (k_d + 2k_R^T + k_1)[T_0] + k_{-1}[H^+][T_0^R]
\]
\[
\frac{d[T_P]}{dt} = k_1[T_P] - (2k_R + k_{-1}[H^+])[T_0^R] + 2k_R[T_0^R] + 2k_R[S^R]
\]
\[
\frac{d[T_0^R]}{dt} = k_1[T_0] + k_R[T_P^R] - (2k_R + k_{-1}[H^+] + k_{hfc})[T_P^R] + k_{hfc}[S^R]
\]
\[
\frac{d[S^R]}{dt} = k_R[T_P^R] + k_{hfc}[T_0^R] - (k_{hfc} + 2k_R + k_{bet})[S^R]
\]

Here

\[
[T_P] = [T_+] + [T_-], [T_P^R] = [T_0^R] + [T_R^R]
\]

In the system of rate equations \([X]\) represents the population of the \(X\) state.

In order to describe the statistics of fluorescence, we evaluate the \(\tau_{ON}\) and \(\tau_{OFF}\) times.

For calculating the on-time constant, we write the following equations that can be solved using the approximation of quasi-stationary concentrations:

\[
\frac{d[S_1]}{dt} = 0 \Rightarrow [S_0] = \frac{k_F + k_{IC} + 3k_{ISC}}{k_{ex}} [S_1]
\]

\[
P_{ON} = [S_0] + [S_1] = \frac{k_{ex} + k_F + k_{IC} + 3k_{ISC}}{k_{ex}} [S_1]
\]

\[
\frac{dP_{ON}}{dt} = -\frac{3k_{ISC}k_{ex}}{k_{ex} + k_F + k_{IC} + 3k_{ISC}} P_{ON} + k_d[T_P] + k_d[T_0] + k_{bet}[S^R]
\]

Hence, we obtain

\[
\bar{\tau}_{ON} = \frac{1}{k_{OFF}} = \frac{k_{ex} + k_F + k_{IC} + 3k_{ISC}}{3k_{ISC}k_{ex}}
\]

Here \(\bar{\tau}_{ON}\) represents the average on-time.

Next, for evaluating the off-time constant, using the following steady state condition,

\[
\frac{d[T_P]}{dt} = \frac{d[T_0]}{dt} = \frac{d[T_P^R]}{dt} = \frac{d[T_0^R]}{dt} = \frac{d[S^R]}{dt} = 0
\]

we can obtain the following rate equations:

\[
0 = 2k_{ISC}[S_1] - (k_d + k_1^T + k_1)[T_P] + 2k_R^T[T_0] + k_{-1}[H^+][T_0^R]
\]
\[
0 = k_{ISC}[S_1] + k_R[T_P] - (k_d + 2k_R^T + k_1)[T_0] + k_{-1}[H^+][T_0^R]
\]
\[
0 = k_1[T_P] - (2k_R + k_{-1}[H^+])[T_0^R] + 2k_R[T_0^R] + 2k_R[S^R]
\]
\[
0 = k_1[T_0] + k_R[T_P^R] - (2k_R + k_{-1}[H^+] + k_{hfc})[T_P^R] + k_{hfc}[S^R]
\]
\[
0 = k_R[T_P^R] + k_{hfc}[T_0^R] - (k_{hfc} + 2k_R + k_{bet})[S^R]
\]

Eliminating \([S_1]\) using the two expressions containing \([S_1]\) terms, we obtain
\[ 0 = -(k_d + 3k_R^T + k_1)[T_p] + 2(k_d + 3k_R^T + k_1)[T_0] + k_{-1}[H^+][T_p^R] - 2k_{-1}[H^+][T_0^R] \]  

(11)

Using equations (10), (11) and \( P_{OFF} = [T_p] + [T_0] + [T_p^R] + [T_0^R] + [S^R] \), we can express the average off-time under steady state, \( \overline{\tau_{OFF}} \), using the reaction rates of the proposed FAD scheme.

\[
\overline{\tau_{OFF}} = \frac{1}{k_{ON}} = \frac{P_{OFF}}{k_d[T_p] + k_d[T_0] + k_{bet}[S^R]}
\]

(12)

Calculations were performed using the free software Maxima. Using home-written Maxima code to describe the expressions above and the following reaction rate coefficients for FAD photochemistry determined by Murakami et al. [2], we obtained numerical solutions of the steady state off-time. These results allow us to estimate the off-time in the absence and in the presence of a magnetic field.

\[
k_d = 3.0 \times 10^5 \text{ s}^{-1}, \quad k_R^T = 1.0 \times 10^8 \text{ s}^{-1}, \quad k_1 = 7.0 \times 10^6 \text{ s}^{-1},
\]

(13)

\[
k_{-1} = 2.7 \times 10^9 \text{ s}^{-1} \text{M}^{-1}, \quad [H^+] = 10^{-2.3} \text{ M}, \quad k_R = 1.7 \times 10^6 \text{ s}^{-1},
\]

\[
k_{hfc} = 8.0 \times 10^7 \text{ s}^{-1}, \quad k_{bet} = 1.3 \times 10^7 \text{ s}^{-1}
\]

Estimation of the steady-state on time is also important. Although it is not magnetic field sensitive, it strongly influences the fraction of time that a magnetic field can influence the fluorescence intensity. It is estimated using the following equation:

\[
k_{ON} = \frac{3k_{ex}k_{ISC}}{k_{ex} + k_F + k_{IC} + 3k_{ISC}}
\]

(14)

Although no literature data exists for the rate coefficient for intersystem crossing for FAD, instead we use the intersystem crossing quantum yield published for riboflavin (0.4) [3] and fluorescence rate coefficient for FAD at pH 2.3[4] to give the following parameters:

\[
k_f = 3.55 \times 10^8 \text{ s}^{-1}, \quad k_{ISC} = 3.64 \times 10^9 \text{ s}^{-1}, \quad k_{IC} = 1.28 \times 10^9 \text{ s}^{-1}
\]

(15)

These values yield the following dependence of the steady state on time with laser power:
III. The average waiting time calculation from the FAD rate equation

The mean of the waiting time <τ> is the first emission time of an excited molecule. The calculation method of the average waiting time τ is described in ref. [5,6]. Here, we present a method to calculate the waiting time of FAD. First, to calculate the mean of the waiting time <τ>, we modify the FAD reaction scheme as shown in supporting Figure 1. The modification is to express the first emission event.

Supporting Figure 1. Modified reaction scheme of the proposed FAD photochemistry

Then, we can define the probability distribution of waiting times, f(τ) = k_f P_{S_1}(τ), where P_{S_1}(τ) is the probability of being in the singlet excited state, S_1. In ensemble-averaged spectroscopy, the probability of being in state X can be expressed as the population of the state X as follows:

P_X(t) = \frac{[X](t)}{\sum_Y [Y](t)}

(17)

where the denominator means the total population of the all states.

Using this expression, the modified rate equation is written as follows:

\[ \frac{dP_{S_0}}{dt} = -k_{ex}P_{S_0} + k_{IC}P_{S_1} + k_dP_{T_p} + k_dP_{T_0} + k_{bet}P_{S_R} \]
\[ \frac{dP_{S_1}}{dt} = k_{ex}P_{S_0} - (k_F + k_{IC} + 3k_{ISC})P_{S_1} \]
\[ \frac{dP_{T_p}}{dt} = 2k_{ISC}P_{S_1} - (k_d + k_{T_R} + k_1)P_{T_p} + 2k_{R}P_{T_0} + k_{-1[H^+]P_{T_p}} \]
\[ \frac{dP_{T_0}}{dt} = k_{ISC}P_{S_1} + k_{T_R}P_{T_p} - (k_d + 2k_{R} + k_1)P_{T_0} + k_{-1[H^+]P_{T_0}} \]

(18)
\[
\begin{align*}
\frac{dP_T^R}{dt} &= k_1 P_T - (2k_R + k_{-1}[H^+])P_T^R + 2k_R P_T^0 + 2k_R P_S^R \\
\frac{dP_T^0}{dt} &= k_1 P_T + k_R P_T^R - (2k_R + k_{-1}[H^+] + k_{\text{hfc}})P_T^0 + k_{\text{hfc}} P_S^R \\
\frac{dP_S^R}{dt} &= k_R P_T^R + k_{\text{hfc}} P_T^0 - (k_{\text{hfc}} + 2k_R + k_{\text{bet}})P_S^R
\end{align*}
\]

By Laplace transformation of the above rate equation, we can solve for the probability distribution of the waiting time, \(f(\tau)\). The Laplace transform of the rate equations is as follows:

\[
k_p(u \phi_{S_0}(u) - 1) = -k_{ex} k_F \phi_{S_0}(u) + k_{ic} \dot{f}(u) + k_d k_F \phi_{T_0}(u) + k_d k_F \phi_{T_0} + k_{bet} k_F \phi_{S_0}(u) \tag{19}
\]

\[
u \dot{\phi}(u) = k_{ex} k_F \phi_{S_0}(u) - (k_F + k_{ic} + 3k_{ISC}) \dot{\phi}(u)
\]

\[
k_F u \phi_{T_0}(u) = 2k_{ISC} \dot{\phi}(u) - (k_d + k_R + k_{-1}) \phi_{T_0}(u) + 2k_R \phi_{T_0} + k_{-1}[H^+] \phi_{T_0} \tag{20}
\]

\[
u \phi_{T_0}(u) = k_{ISC} \dot{\phi}(u) + k_d \phi_{T_0}(u) - (k_d + 2k_R + k_{-1}) \phi_{T_0} + k_{-1}[H^+] \phi_{T_0} \tag{21}
\]

\[
\phi_{T_0}(u) = k_{ISC} \dot{\phi}(u) + k_d \phi_{T_0}(u) - (k_d + 2k_R + k_{-1}) \phi_{T_0} + k_{-1}[H^+] \phi_{T_0} \tag{22}
\]

\[
u \phi_{T_0}(u) = k_F \phi_{T_0}(u) + k_{ISC} \phi_{T_0}(u) - (k_{ISC} + 2k_R + k_{bet}) \phi_{T_0}(u)
\]

Where,

\[
\phi_x(u) \equiv \mathcal{L}[P_x(\tau)] = \int_0^\infty e^{-ut} P_x(\tau) d\tau
\]

Next, to solve the mean of the waiting time \(\langle \tau \rangle\) with the probability distribution of waiting times \(f(\tau)\), we Laplace transform as follows:

\[
\hat{f}(u) \equiv \mathcal{L}[f(\tau)] = \int_0^\infty e^{-ut} f(\tau) d\tau \tag{23}
\]

Then, we obtain the following expression using the Taylor expansion of \(e^{-ut}\)

\[
\hat{f}(u) = \int_0^\infty e^{-ut} f(\tau) d\tau = \int_0^\infty \sum_{n=0}^{\infty} \frac{(-ut)^n}{n!} f(\tau) d\tau = \sum_{n=0}^{\infty} \frac{(-u)^n}{n!} \int_0^\infty \tau^n f(\tau) d\tau = \sum_{n=0}^{\infty} \frac{(-u)^n}{n!} \langle \tau^n \rangle \tag{24}
\]

By substituting 0 for the differential coefficient of the nth order, the mean value of the nth order of the waiting time \(\langle \tau^n \rangle\) can be obtained.

\[
\hat{f}^{(n)}(0) = (-1)^n \langle \tau^n \rangle
\]

Using the above calculations and measured reaction rate coefficients for FAD (13) and (15), we obtain the following numerical values associated with the waiting time for FAD.
\[
\langle \tau \rangle = \begin{cases} 
2.42774 \times 10^{-6} + \frac{7.68169}{k_{ex}} s (B = 0 \, T) \\
3.003751 \times 10^{-6} + \frac{7.68169}{k_{ex}} s (B = 0.2 \, T)
\end{cases}
\]

\[
\langle \tau^2 \rangle = \begin{cases} 
1.54172 \times 10^{-11} + \frac{1.18017 \times 10^2}{k_{ex}^2} + \frac{7.45909 \times 10^{-5}}{k_{ex}} s^2 (B = 0 \, T) \\
2.41693 \times 10^{-11} + \frac{1.18017 \times 10^2}{k_{ex}^2} + \frac{9.33273 \times 10^{-5}}{k_{ex}} s^2 (B = 0.2 \, T)
\end{cases}
\]

IV. Calculation error of the number of photons within the waiting time for finite integration times

Here, to consider whether the selected finite integration time can be regarded as large relative to the waiting time, we compare the Mandel Q parameter calculated with number of photons for a finite integration time to the same parameter for infinite integration time calculated with fixed waiting times.

\[
Q(\tau_{int}) = \frac{\langle N^2(\tau_{int}) \rangle - \langle N(\tau_{int}) \rangle^2}{\langle N(\tau_{int}) \rangle} - 1
\]

\[
Q(\infty) = \frac{\langle \tau^2 \rangle - \langle \tau \rangle^2}{\langle \tau \rangle^2} - 1
\]

Supporting Figure 2. Difference of calculation results for the Mandel Q parameter under selected finite integration times versus infinite integration time with no applied magnetic field.
Supporting Figure 3. Relative calculation error of the Mandel Q parameter under selected integration times versus infinite integration time in the presence and absence of an applied magnetic field.
Supporting Figure 4. Differences in the calculation results for the MFE on the Mandel Q parameter under selected finite integration times versus infinite integration time.

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