Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Elimination of autofluorescence in fluorescence correlation spectroscopy using the AzaDiOxaTriAngulenium (ADOTA) fluorophore in combination with time-correlated single-photon counting (TCSPC)

Ryan M. Rich, Mark Mummert, Zygmunt Gryczynski, Julian Borejdo, Thomas Just Sørensen, Bo W. Laursen, Zeno Foldes-Papp, Ignacy Gryczynski, Rafal Fudala

Materials and Methods

Fluorescence Correlation Spectroscopy

The physical data is extracted from the auto- or cross- correlation of fluorescence time traces, the general expression for which is as follows [1, 2]:

$$G_{ij}(\tau) = \frac{\langle \delta F_i(t)\, \delta F_j(t+\tau) \rangle}{\langle F_i(t) \rangle \langle F_j(t) \rangle} \qquad (1)$$

where δF_i and δF_j denote the variance of the fluorescence signal about the mean at time t and a later time, $t + \tau$, for two fluorescent time traces, i and j. Also $\langle F_i \rangle$ and $\langle F_j \rangle$ are the time averaged fluorescent signals. A model of diffusion, G_D, is fit in terms of the lag time, τ. The simplest of these models is the expression for pure diffusion of m fluorescent species [1].

$$G_D(\tau) = G(0)\ \sum_{m=1}^{n}\left(1+\frac{\tau}{\tau_m}\right)^{-1}\left(1+\frac{\tau}{\tau_m}\frac{\omega^2}{z^2}\right)^{-\frac{1}{2}} \qquad (2)$$

where ω and z are the width and height of the detection volume, respectively, τ_m is the average diffusion time of the m^{th} diffusing species, and G(0) is the correlation at $\tau = 0$. The average number of independent diffusion molecules that pass through the detection volume, N, is equal to the inverse of the correlation at $\tau = 0$.

$$G(0) = \frac{1}{\langle N \rangle} \qquad (3)$$

The diffusion constant, D, may then be calculated as

$$D = \frac{\omega^2}{4\tau_1} \qquad (4)$$

There are, of course, other phenomena that contribute to fluctuations in the fluorescence signal, including rotation and antibunching, but these are observed at time lags much shorter than those for which diffusion behavior is analyzed. For more detailed derivations of these models, we refer the reader to [1–3].

Time Gated Detection

The steady state intensity of a probe, Ip_{ss}, can be expressed in terms of its intrinsic lifetime, τ_p, and time span of the measurement after the pulsed excitation:

$$Ip_{ss} = \int_0^\infty Ip_0 e^{-t/\tau_p}\, dt \qquad (5)$$

where Ip_0 is the initial intensity of the probe, and the integration is performed over time, t, from t = 0 to infinity. We mark this t = 0 point as the instant after the excitation pulse. Now let us assume that the total steady state fluorescence intensity, I_{ss}, is the sum of that from the probe and that from the

autofluorescence background, and it is a function of the time at which the measurement is started in relation to the excitation pulse:

$$I_{ss}(t_x) = \alpha_p Ip_{ss}(t_x) + \alpha_b Ib_{ss}(t_x) \quad (6)$$

Here α_p and α_b are relative fractions of the probe and background intensity and $\alpha_p + \alpha_b = 1$. The start of the integration, t_x, is sometime after the excitation pulse such that $0 < t_x < \infty$. The expression for the total intensity is then as follows, with τ_b being the fluorescence lifetime of the background.

$$I_{ss}(t_x) = \alpha_p \int_{t_x}^{\infty} Ip_0 e^{-t/\tau_p}\, dt \quad + \alpha_b \int_{t_x}^{\infty} Ib_0 e^{-t/\tau_b}\, dt \quad (7)$$

If we assume a probe lifetime of 20 ns and a background lifetime of 4 ns with the start of the measurement delayed 30 ns ($t_x = 30$ ns), the signal from the probe will be substantially reduced (78%), but the background will be completely eliminated. In practice, photons are continuously collected throughout the entire measurement period, and afterwards a "time gate" is set so that photons arriving before the time gate ($0 < t < t_x$) are excluded from the calculation of the autocorrelation function. From these calculations, one may easily see the benefit of using ADOTA dye with its 19.4 ns lifetime. If the probe were to only have a lifetime of 10 ns, the 30 ns gating would reduce the signal by 95%. Of course, this is a hypothetical example. Most commonly employed organic probes have lifetimes much shorter (3 – 5 ns), making separation of the probe from autofluorescence impossible.

References

1. Lakowicz JR (1999) Principles of Fluorescence Spectroscopy. Kluwer Academic/Plenum

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3. Fore S, Yuen Y, Hesselink L, Huser T (2007) Pulsed-Interleaved Excitation FRET Measurements on Single Duplex DNA Molecules Inside C-Shaped Nanoapertures. Nano Lett 7:1749–1756