Criteria for the rapid diffusion limit of fluorescence energy transfer

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Excitation energy transfer in a system where the spatial relationship between the donor and acceptor is not fixed generally leads to a nonexponential decay of donor fluorescence. A single-exponential decay, however, is expected as a limiting form when the diffusion of the donors and/or acceptors is sufficiently rapid. The exponential character at the rapid diffusion limit will greatly facilitate the analysis of experimental data. In this paper a theoretical framework is presented that allows the calculation of the criterion for the rapid diffusion limit. Explicit criteria are given for various donor-acceptor geometries, all for the case of energy transfer via the resonance interaction of the Förster type. The criteria, except for the cases of densely distributed acceptors under a wide surface, have a common form \( D \tau_D \alpha^2 / R_0^4 \lambda \), where \( \tau_D \) is the lifetime of donor fluorescence in the absence of acceptors, \( D \) is the sum of diffusion coefficients of the donor and acceptor, \( \alpha \) is the distance of closest approach between the donor and acceptor, \( R_0 \) is the critical distance for energy transfer, and \( \lambda \) is a geometrical constant with a value less than one. Exponential decays are not easily obtained when acceptors are densely distributed under a wide surface. The results are compared with the criterion given earlier by Thomas et al. [D. D. Thomas, W. F. Carlsen, and L. Stryer, Proc. Natl. Acad. Sci. U.S.A. 75, 5746 (1978)]. Experimental aspects, such as the effect of heterogeneity in a sample, are also discussed.

I. INTRODUCTION

Fluorescence energy transfer is a useful tool for studies of macromolecular and supramolecular architectures. Analysis of the rate of excitation energy transfer from an excited fluorophore (donor) to a nearby acceptor yields information about the distance between the donor and acceptor. The energy transfer rate can be estimated experimentally from the decrease in the lifetime, or in the quantum yield, of donor fluorescence in the presence of the acceptor. When the transfer occurs via the resonance interaction of the Förster type, the transfer rate is sensitive to the intermolecular distance of the order of 1–10 nm. Since the sizes of most biological macromolecules fall in this range, the distance determination by means of fluorescence energy transfer has found wide applications in structural biology. Typically the distance between two distinct sites in a macromolecule, or a macromolecular assembly, is estimated by labeling each site with a donor fluorophore and an acceptor dye. In addition to the distance, orientations of the donor and acceptor can be determined under special conditions.

The energy transfer technique can also be applied to systems of distributed acceptors (and/or donors) where the spatial relationship between the donor and acceptor is not fixed. An important parameter in such systems, which can be estimated from an energy transfer experiment, is the distance of closest approach between the donor and acceptor. If the donor is an internal chromophore of a biological macromolecule, e.g., the distance to an aqueous surface can be estimated by putting suitable acceptors in the external medium. Similar techniques have been successfully applied to the studies of chromophore disposition in soluble proteins or in biomembranes.

In the presence of distributed acceptors, the donor fluorescence does not, in general, decay exponentially, since the spatial distribution of acceptors around each donor is different from donor to donor. A single exponential decay, however, is expected when the diffusion of donors and/or acceptors is rapid enough to average out the distribution before significant energy transfer takes place (the rapid diffusion limit). Analysis is thus greatly facilitated by designing an experiment that meets a criterion for the rapid diffusion limit. This is particularly true for steady-excitation fluorometry where interpretation is very difficult, if not impossible, unless single exponential decay is ensured.

The criterion for the rapid diffusion limit has been discussed by Thomas et al. in general terms. Kouyama et al. have also given a criterion applicable to their experimental data. The criteria given by the two groups, however, are apparently quite different from each other. The purpose of the present paper, therefore, is to resolve the discrepancy and to derive criteria that are of general use. First we introduce in Sec. II a theoretical framework that allows the calculation of the rapid diffusion criteria. Then we present in Sec. III explicit criteria for various geometries of donor–acceptor boundary, all for the case of energy transfer of the Förster type. In Sec. IV we compare the criteria with that of Thomas et al., and discuss some experimental aspects.

II. THEORETICAL FRAMEWORK

A. Description of the problem

In a system containing \( M \) donors and \( N \) acceptors, the intensity \( F(t) \) of donor fluorescence after a pulsed excitation at time \( t = 0 \) is given by

\[
F(t) = \frac{1}{M} \sum_{j=1}^{M} \exp \left[ -\left( \frac{1}{\tau_D} + \sum_{j=1}^{N} k(R_j) t \right) \right],
\]

where \( \tau_D \) is the excited-state lifetime of the donor in the absence of acceptors, and \( k(R_j) \) is the rate of energy trans-
fer as a function of the separation vector $\mathbf{R}_y$ between donor $i$ and acceptor $j$. We define a normalized decay rate $\xi(t)$ by

$$\xi(t) = - [dF(t)/dt] / F(t).$$

(2)

Initially all the transfer rates make unbiased contributions to the decay rate:

$$\xi(0) = \frac{1}{\tau_D} + \frac{1}{M} \sum_{m=1}^M \sum_{j=1}^N k(\mathbf{R}_y).$$

(3)

Thereafter $\xi(t)$ generally decreases with time $t$, since those donors with a large $k(\mathbf{R}_y)$ are rapidly quenched and disappear from the sum in Eq. (3). The rapid diffusion limit is attained when $\mathbf{R}_y$ changes so rapidly as to keep the distribution of the values of $k(\mathbf{R}_y)$'s for the remaining donors practically constant during the whole decay process. In the limit $\xi(t)$ is independent of time and $F(t)$ decays exponentially.

Our goal is to find out an inequality for the diffusion coefficients of the donor and acceptor that ensures the rapid diffusion limit.

**B. Criterion for the rapid diffusion limit**

Below we develop a formal theory which leads to the inequality representing a criterion for the rapid diffusion limit. The framework follows essentially that of Steinberg and Katchalskii.10

We consider a system shown in Fig. 1(A), where each donor is embedded in a particle of arbitrary shape and acceptors are spherical. The particle may contain multiple donors as long as all donors occupy equivalent positions. Without losing generality, we neglect the radius of the spherical acceptors (and add a corresponding thickness to the donor particle). We assume that the system is dilute and neglect the effect of concentration on the diffusion coefficients of donor particles or of acceptors. Before proceeding further, we note that the following discussion is equally applicable to the system in Fig. 1(B) where the geometry of donor and acceptor is exchanged. The case of multiple acceptors in a particle may also be included since the acceptors can be replaced, mathematically, with a single, effective acceptor (see examples in Sec. III below).

(1) Illuminate the sample with a pulsed light at time zero ($t = 0$) so that only one of the donors is excited. Consider a large volume $V$ around the donor: the volume contains $N$ acceptors numbered $1, \ldots, N$.

(2) Suppose that acceptor $j$ and the donor is separated by a vector $\mathbf{R}_j$ at $t = 0$. Define $p(\mathbf{R}_j^0, \mathbf{R}_j, t)$ as the joint probability that the separation vector becomes $\mathbf{R}_j$ at $t$ and that the excited donor has not given its energy to acceptor $j$ by time $t$. [We ignore, until step (3), the self-deactivation of donor excitation and the energy transfer to other acceptors.] The probability $p$ satisfies the following differential equation:

$$\frac{\partial p(\mathbf{R}_j^0; \mathbf{R}_j, t)}{\partial t} = - k(\mathbf{R}_j)p(\mathbf{R}_j^0, \mathbf{R}_j, t)$$

$$+ D \Delta_{\mathbf{R}_j} p(\mathbf{R}_j^0, \mathbf{R}_j, t),$$

(4)

where $k(\mathbf{R}_j)$ is the rate of energy transfer from the donor particle to acceptor $j$, $D$ is the sum of the diffusion coefficients of the donor and acceptor, and $\Delta_{\mathbf{R}_j}$ is the Laplacian operator with respect to $\mathbf{R}_j$. The boundary conditions for $p$ are

$$p(\mathbf{R}_j^0, \mathbf{R}_j, 0) = \delta(\mathbf{R}_j^0 - \mathbf{R}_j),$$

(5a)

$$\frac{\partial p(\mathbf{R}_j^0, \mathbf{R}_j, t)}{\partial n} = 0 \quad \text{at the surface of the particle},$$

(5b)

where $n$ is a vector normal to the surface of the particle containing the donor [or acceptor in Fig. 1(B)]. The last condition (5b), which states that the particle surface is impenetrable, differs from the boundary condition employed by Steinberg and Katchalskii,10 who imposed $p(\mathbf{R}_j^0, \mathbf{R}_j, t) = 0$ at the surface. Equation (5b) is appropriate when $k(\mathbf{R}_j)$ remains finite at the surface as in the case of energy transfer via the Förster mechanism.4 (The case $D = 0$ may be included as a limit.)

(3) Now the probability $m(\mathbf{R}_1^0, \ldots, \mathbf{R}_N^0; t)$ that the donor above remains excited by time $t$ without undergoing self-deactivation or giving its energy to any of the $N$ acceptors in the volume $V$ is given by the following product:

$$m(\mathbf{R}_1^0, \ldots, \mathbf{R}_N^0; t) = \exp(-t/\tau_D) \prod_{j=1}^N \int \nu p(\mathbf{R}_j^0, \mathbf{R}_j, t) d \mathbf{R}_j,$$

(6)

which also gives the decay kinetics of the donor fluorescence with the specified initial distribution of acceptors. In general, the decay is not exponential unless $D = 0$ or $D = \infty$.

(4) Repeat the pulsed excitation many times and accumulate the fluorescence decay data. [This procedure is equivalent to the averaging over different, but identical, donors in Eq. (1).] The final decay function $\overline{m}(t)$, corresponding to $F(t)$ in Eq. (1) for the case of $M \rightarrow \infty$, is obtained by averaging $m(\mathbf{R}_1^0, \ldots, \mathbf{R}_N^0; t)$ in Eq. (6) over the initial distribution of acceptors:

$$\overline{m}(t) = \frac{1}{V^N} \int \cdots \int_V m(\mathbf{R}_1^0, \ldots, \mathbf{R}_N^0; t) d \mathbf{R}_1^0 \cdots d \mathbf{R}_N^0$$

$$= \exp(-t/\tau_D) \left[ \frac{1}{V} \int \nu p(\mathbf{R}_1^0, \mathbf{R}_1, t) d \mathbf{R} d \mathbf{R}_1^0 \right]^N.$$

(7a)

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**Fig. 1. Geometry of donor–acceptor systems.** Open circles, donors; closed circles, acceptors. $\mathbf{R}$ represents the donor–acceptor separation vector and $\alpha$ the vector at the closest approach.
\[
\begin{align*}
&= \exp\left(-t/\tau_D\right) \left[ 1 - \frac{1}{V} \int_V \left(1 - \int p(R^0; R, t) \, d R^0\right) d R \right]^N. \\
&\quad \times \left(1 - \int p(R^0; R, t) \, d R^0\right) d R \right]^N.
\end{align*}
\]

By taking the limit of \( V \to \infty \) and \( N \to \infty \) while keeping \( N/V = C_0 \) (number of acceptors per unit volume), we obtain

\[
\overline{\eta}(t) = \exp\left(-t/\tau_D - \int \{C_0 - C(R, t)\} \, d R\right),
\]

where

\[
C(R, t) = C_0 \int p(R^0; R, t) \, d R^0
\]

satisfies Eqs. (4) and (5b) (\( p \) in these equations should read \( C \)) together with the initial condition

\[
C(R, 0) = C_0.
\]

The integrations are performed outside the particle.

(5) The normalized decay rate \( \xi(t) \) is calculated from Eq. (8) as

\[
\xi(t) = -\frac{\partial}{\partial t} \{\ln(\overline{\eta}(t))\} = \frac{1}{\tau_D} + \int \left\{C_0 - C(R, t)\right\} \, d R,
\]

\[
\xi(0) = \frac{1}{\tau_D} + C_0 \int k(R) \, d R.
\]

Thus, observed fluorescence initially decays as \( \exp[-\xi(0)t] \) whether diffusion takes place or not. Therefore, unless the diffusion is rapid enough, \( C(R, t) \) in Eq. (11) decreases with time particularly for those values of \( R \) where \( k(R) \) is large, leading to a slowing down of the fluorescence decay. This is a restatement of what we have said in Sec. II A above.

(6) Now the criterion for the rapid diffusion limit is that

\[
C(R, t) \approx C_0 \quad \text{for all} \quad R \quad \text{values outside the particle and for} \quad t \quad \text{up to several times} \quad \tau_D.
\]

It is clear that Eq. (4) is then valid when \( C(R, t) \) is fastest at \( R = a \) where the closest approach between the donor and acceptor is attained (Fig. 1). Since experiment requires that detectable transfer occurs by time \( t \approx \tau_D \), a practical criterion is

\[
C(a, \infty) \approx C_0.
\]

This condition ensures that \( \overline{\eta}(t) \approx \exp[-\xi(0)t] \) for all values of \( t \).

(7) To obtain an explicit expression of rapid diffusion criterion for a particular shape of the particle, we have to solve Eq. (4) for \( C(R, t) \) under the boundary conditions (5b) and (10). The condition (13), however, requires that the solution should be quasistationary at the rapid diffusion rate. Thus we seek for a stationary solution \( C^* (R) \) of Eq. (4) and impose \( C^* (a) = C_0 \). We expand \( C^* (R) \) in the form of a series

\[
C^* (R) = C_0 + C_1 (R)/D + C_2 (R)/D^2 + \cdots
\]

Comparing quantities of the order of \( 1/D \) we obtain

\[
\Delta_R \frac{\partial C_1 (R)}{\partial n} = 0 \quad \text{at particle surface, and} \quad C_1 (R \to \infty) = 0.
\]

The solution is expressed in terms of Green's function \( G \) that satisfies

\[
\Delta_R G(R^0; R) = -\delta(R^0 - R)
\]

and the boundary conditions (15):

\[
C_1 (R) = -C_0 \int k(R^0) G(R^0; R) \, d R^0.
\]

The rapid diffusion limit is attained when \( -C_1 (a)/D < C_0 \) or when

\[
\int k(R) G(R; a) \, d R \ll 1.
\]

This result is completely equivalent to the one introduced by Koyuma et al.,\(^9\) who sought for a time dependent solution of Eq. (4) and then let \( t \to \infty \). The result is also equivalent to the condition for the observation of exponential luminescence decay given earlier by Samson\(^1\) [our \( G(R; a) \) corresponds to \( \int \overline{\eta}(a, R, t) \, dt \) in the inequality (10) in his paper].

### C. The case of finite volume

Here we deal with the case where the diffusing acceptors (or donors) are contained in a closed volume \( V \) surrounded by a wall containing donors (or acceptors). An example is the reverse system studied by Thomas and Stryer\(^8\) which consisted of vesicular membranes with acceptors in the membrane phase and donors diffusing in the intravesicular aqueous phase. The case of finite volume requires slight modifications of the procedure in Sec. II B above.

Equations (4) through (7) remain valid if we understand that the "particle" above means in this case the wall of the closed volume. Note that all equations are unchanged upon reversal of the separation vector \( R \), or upon the exchange of the donor and acceptor. Mathematically, therefore, the case of diffusing acceptors inside a vesicle is equivalent to the reverse case of diffusing donors trapped inside a vesicle. The reverse case may call for additional comment: Formally, if multiple acceptors exist in the wall of the vesicle (or in any acceptor particle), they should be replaced with an equivalent pseudocounter (see Secs. III E and III F below). The vesicle system thus contains only one pseudocounter \( (N = 1) \) to which excitation energy is transferred at the rate \( k(R) \) which is the sum of transfer rates to the real acceptors. However, the final expression for the rapid diffusion criterion, the inequality (31) below, does not depend on \( N \). We therefore do not introduce the pseudocounter concept explicitly until we deal with the cases of densely distributed acceptors (Secs. III E and III F) where \( k(R) \) is significantly different from individual rates.

Instead of \( C(R, t) \) in Eq. (9) we define \( I(R, t) \) by

\[
I(R, t) = \exp(\widetilde{k}t) \int p(R^0; R, t) \, d R^0,
\]

\[C.\quad \text{Chem. Phys., Vol. 86, No. 6, 15 March 1987}\]
where
\[ \bar{k} = \frac{1}{V} \int_V k(R) dR. \]  
(20)

The function \( I(R, t) \) satisfies
\[ \frac{\partial}{\partial t} I(R, t) = [\bar{k} - k(R)] I(R, t) + D \Delta_n I(R, t), \]  
(21)

together with the boundary conditions
\[ I(R, 0) = 1, \]  
(22a)
\[ \frac{\partial I(R, t)}{\partial n} = 0 \text{ at the surface of the wall.} \]  
(22b)

Now steps (5) to (7) are modified as follows:

\[(5')\] The normalized decay rate \( \xi(t) \) is calculated from Eq. (7a), using Eqs. (4) and (19), as
\[ \xi(t) = -\frac{\partial}{\partial t} \left[ \ln [\bar{m}(t)] \right] = 1/\tau_D - \left[ \int_V \frac{\partial}{\partial t} p(R, t) dR dR^0 \right] \left[ \int_V p(R^0, t) dR^0 \right] \]  
(23)
\[ - \left[ \int_V I(R, t) dR \right]. \]

The initial decay rate \( \xi(0) \) is again given by Eq. (12).

\[(6')\] Comparison of Eqs. (12) and (23) suggests that the rapid diffusion limit is attained when
\[ N \int_V \int_V \left[ \int_V k(R) I(R, t) dR \right] dR \approx C_0 \left[ \int_V k(R) dR \right] \left[ \int_V I(R) dR \right] \]
or
\[ \int_V k(R) I(R, t) dR \approx \bar{k} \int_V I(R) dR \]  
(24)
holds for \( t \) up to several times \( \tau_D \). On the other hand, Eq. (21) shows that \( I(R, t) \) tends to decrease in the “sink” region where \( k(R) > \bar{k} \) and increase in the “source” region where \( k(R) < \bar{k} \). With time \( t \), therefore, the left-hand side of the relation (24) becomes smaller than the right-hand side unless the diffusion coefficient \( D \) is large enough to keep \( I(R, t) \) practically constant. If we define two vectors \( a \) and \( f \) such that \( k(R) \) is maximal at \( R = a \) (closest approach) and minimal at \( R = f \) (farthest separation), the rapid diffusion criterion is stated in the form \( I(a, \infty) \approx 1 \) and \( I(f, \infty) \approx 1 \), or
\[ I(a, \infty) - I(a, \infty) \approx 1. \]  
(25)

\[(7')\] As in step (7) above we seek for a stationary solution \( I^*(R) \) of Eq. (21) and impose \( I^*(f) - I^*(a) \approx 1 \). Expanding \( I^*(R) \) as \( I^*(R) = 1 + I_1(R)/D + I_2(R)/D^2 + \cdots \), we have for \( I_1(R) \):
\[ \Delta_n I_1(R) = k(R) - \bar{k}, \]  
(26)
\[ \frac{\partial I_1(R)}{\partial n} = 0 \text{ at the wall surface.} \]  
(27)

In the case of finite volume Green’s function \( G \) that would satisfy the boundary condition (27) does not exist. Instead we introduce a function \( H \) that satisfies
\[ \Delta_n H(R^0; R) = -\delta(R^0 - R), \]  
(28)
and the boundary condition
\[ \frac{\partial H(R^0; R)}{\partial n} = 1/S \text{ at the wall surface,} \]  
(29)
where \( S \) is the surface area of the wall. Then the solution of Eqs. (26) and (27) is expressed as
\[ I_1(R) = -\int_V [k(R^0) - \bar{k}] H(R^0; R) dR^0. \]  
(30)

Note that the above expression for \( I_1(R) \) satisfies the boundary condition (27). The rapid diffusion criterion for a finite volume is expressed as \( I_1(f) - I_1(a) \approx 1 \), or
\[ \frac{1}{D} \int_V [k(R) - \bar{k}] H(R; a) - H(R; f) dR \approx 1. \]  
(31)

For \( V \to \infty \) this result reduces to the criterion (18) since \( k \to 0, H \to G, \) and \( H(R; f) \to 0 \) for small \( R \) for which \( k(R) \) is significantly greater than zero.

III. RAPID DIFFUSION CRITERIA FOR ENERGY TRANSFER OF THE FÖRSTER TYPE

Now we calculate explicit criteria for several geometries of the particle or wall. We assume the Förster mechanism in the rest of this paper.

For energy transfer via the Förster mechanism, the rate \( k(R) \) is given by
\[ k(R) = \left( \frac{\kappa^2}{\tau_D} \right) \frac{R_0}{R^6}, \]  
(32)
where \( \kappa^2 \) is the orientation factor and \( R_0 \) is the critical distance for energy transfer. The magnitude of \( R_0 \) depends on spectroscopic properties of the donor and acceptor; for most donor–acceptor pairs \( R_0 \) ranges between 1 and 10 nm. Note that our \( R_0 \) is slightly different from the common definition in which the orientation factor \( \kappa^2 \) is included.

Since we are interested in the rapid diffusion limit, we can assume that at least the donors or acceptors rotate rapidly. Then \( \kappa^2 \) is given by
\[ \kappa^2 = \frac{1}{4} + \cos^2 \Theta, \]  
(33)
where \( \Theta \) is the angle between the separation vector \( R \) and the direction of the transition moment of the acceptor absorption (in the case of rapid rotation of the donor) or of the donor emission (rapidly rotating acceptor). Obviously \( \kappa^2 \) takes a value between 1/3 and 4/3; \( \kappa^2 = 2/3 \) for rapid rotation of both donors and acceptors. Since the rapid diffusion criteria are expressed in the form of an inequality, setting \( \kappa^2 = 2/3 \) does not introduce serious errors.

A. Spherical particle of radius \( a \) containing a donor or acceptor at the center

Take the origin at the center of the sphere and introduce polar coordinates \((r, \theta, \phi)\) as in Fig. 2, case A. For a separation vector \( R \) ending at \( r \), the transfer rate is given by
$$k(R) = (k^2/\tau_D) (R_0/r)^6.$$  (34)

With $a$, the separation vector at the closest approach, along z axis, Green's function is given by

$$4\pi G(R; a) = \frac{2}{(r^2 - 2ar + a^2)^{1/2}}$$
$$- \frac{1}{a} \ln \frac{a - r}{a} + (r^2 - 2ar + a^2)^{1/2}$$
$$r(1 - \rho)$$ (35a)

$$= 2 \sum_{n=0}^{\infty} \frac{a^n}{r^* + 1} P_n(\rho)$$
$$- \sum_{n=0}^{\infty} \frac{1}{n + 1} \frac{a^n}{r^* + 1} P_n(\rho),$$ (35b)

where $P_n(\rho)$ is the Legendre polynomial of order $n$ and $\rho = \cos \theta$. Introducing Eqs. (34) and (35b) into the inequality (18) we obtain the rapid diffusion criterion for $k^2 = 2/3$ as

$$D \tau_D^* a^4 / R_0^6 \gg 1/6 \approx 0.17.$$ (36)

If rotation of the particle is slow and $k^2$ in Eq. (33) is used ($\Theta = \theta$), the criterion is

$$D \tau_D^* a^4 / R_0^6 \gg 11/54 \approx 0.20.$$ (37)

In either cases the decay rate is given by

$$\xi(0) = 1/\tau_D + 8\pi C_0 R_0^6 / 9 \tau_D a^3.$$ (38)

B. A donor or acceptor under a flat surface at a depth of $a$

The geometry is shown in Fig. 2, case B. The case applies, e.g., to chromophores embedded in a membrane sheet or in a large membrane vesicle. For acceptors in a membrane, however, the surface density of the acceptors as well as the size of the membrane must be small (see Sec. III E below).

Take the coordinate system as in Fig. 2. Then

$$k(R) = \frac{k^2 R_0^6}{\tau_D} \frac{1}{(r^2 + 2ar + a^2)^{1/2}},$$ (39)

$$4\pi G(R; a) = 2/r.$$ (40)

The rapid diffusion criterion for $k^2 = 2/3$ is obtained from the inequality (18) as

$$D \tau_D^* a^4 / R_0^6 \gg (1/6)(\pi/4 - 1/3) \approx 0.075,$$ (41)

and the decay rate is given by

$$\xi(0) = 1/\tau_D + \pi C_0 R_0^6 / 9 \tau_D a^3.$$ (42)

If the donor (or acceptor) under the surface does not rotate and its transition moment makes an angle $\Theta$ with respect to z axis, the criterion calculated with a proper $k^2$ is

$$D \tau_D^* a^4 / R_0^6 \gg \frac{\pi}{64} + \frac{(5\pi/64 - 1/6) \cos^2 \Theta}{\approx 0.049 + 0.079 \cos^2 \Theta}.$$ (43)

Kouyama et al. have given a criterion for this particular case. Numerical values in their result are slightly different from those in Eq. (43) above, since the previous result was based on an approximate calculation. The decay rate for this case is

$$\xi(0) = 1/\tau_D + \pi C_0 R_0^6 (1 + \cos^2 \Theta) / 12 \tau_D a^3.$$ (44)

C. A sphere with a donor or acceptor at a depth of $a$

This is a generalization of the cases A and B above. The sphere may contain multiple, but equivalent, donors. Multiple acceptors may also be considered if their surface density is low and the radius of the sphere is small (see Sec. III E below). An example of application is a membrane vesicle with donors in the membrane phase and acceptors diffusing in the external medium. Another example is a large protein molecule with a buried chromophore.

With the coordinate system shown in Fig. 3, the transfer rate is given by

$$k(R) = \frac{k^2 R_0^6}{\tau_D} \frac{1}{(r^2 - 2ar + c^2)^{1/2}},$$ (45)

where $c = b - a$. We take $k^2$ as 2/3. Green's function is given by Eq. (35) in which $a$ is replaced with $b$. To avoid complicated integration we neglect the second term on the right-hand side of Eq. (35a). Inspection of Eq. (35b) suggests that this approximation leads to an overestimation of the integral in the inequality (18) by a factor of at most 2, which may be disregarded safely. After a lengthy procedure of integration we obtain finally the rapid diffusion criterion:

$$D \tau_D^* a^4 / R_0^6 \gg \frac{1}{6} \left[ \frac{\pi^{1/2}}{\gamma^{1/2}} \arctan (\pi^{1/2}) \right. $$
$$+ \left. 1 - 2\gamma - r^2 - (2/3)\gamma^2 \right] / (1 + \gamma)^3,$$ (46)

where $\gamma \equiv c/b = (b - a)/b < 1$. The right-hand side takes a value between 0.075 and 0.333. The decay rate (for $k^2 = 2/3$) is given by

FIG. 2. Coordinate systems used in Secs. III A and III B. $r = (r, \theta, \phi)$ is a vector representing the position of the acceptor in the coordinate system. $\rho = \cos \theta$.

FIG. 3. Coordinate systems used in Secs. III C and III D.

\[ \dot{\xi}(0) = \frac{1}{\tau_D} + 8\pi C_0 R_0^6 / 9 \tau_D a^3 (1 + \gamma)^3. \]  
(47)

In the limit of \( b \to \infty \) and \( \gamma \to 1 \), relations (46) and (47) reduce, respectively, to relations (41) and (42). For \( \gamma \to 0 \), Eq. (38) is recovered from Eq. (47) but the right-hand side of the inequality (46) becomes 1/3 instead of 1/6 in the inequality (36). The discrepancy is due to the omission of the second term of Eq. (35a).

**D. A hollow sphere of radius \( b \) with a donor or acceptor in the wall at a depth of \( a \)**

The geometry is shown in Fig. 3. Aside from an obvious application to membrane vesicles, this case may apply to a large particle with a deep surface depression below which a chromophore is situated. The wall may contain multiple, but equivalent, donors. Multiple acceptors are allowed when their surface density is low and the radius \( b \) is small (see Sec. III F below).

The transfer rate is again given by Eq. (45) where \( c \) in this case is defined by \( c \equiv b + a \). We take \( \kappa^2 \) as 2/3. The average transfer rate \( \bar{\kappa} \) in Eq. (20) is then calculated to be

\[ \bar{\kappa} = 2R_0^6 / 3\tau_D a^3 (1 + \gamma)^3, \]  
(48)

where \( \gamma \) in this case is defined by \( \gamma \equiv c / b = (b + a) / b > 1 \). The function \( H(R;b) \) in the inequality (31) is given by

\[
4\pi H(R;b) = \frac{2}{(r^2 - 2br + b^2)^{1/2}} + \frac{1}{b} \times \ln \frac{2b}{b - pr + (r^2 - 2br + b^2)^{1/2}} - \frac{1}{b}
\]

\[
= 2 \sum_{n=0}^{\infty} \frac{r^n}{b^{n+1}} P_n(\rho) + \sum_{n=1}^{\infty} \frac{1}{b} \frac{r^n}{b^{n+1}} P_n(\rho) - \frac{1}{b}.
\]  
(49a)

The farthest separation between the donor and acceptor is achieved on the axis at \( z = -b \). Thus \( H(R;r) \) is given by Eq. (49) in which \( \rho \) is replaced with \( -\rho \). For both \( H(R;r) \) and \( H(R;\rho) \) we neglect the second term on the right-hand side of Eq. (49a). The error introduced should be within a factor of 3/2 and is negligible when \( a \) is much smaller than \( b \). Under this approximation the rapid diffusion criterion is calculated to be

\[ Dr_D a^4 / R_0^6 \gg \frac{1}{6} \left[ \frac{1}{\gamma^{1/2}} \arctan \left( \frac{1}{\gamma^{1/2}} \right) - \frac{6\gamma^3 + 10\gamma}{3(\gamma + 1)^4} + \frac{(\gamma - 1)^4}{2(\gamma + 1)^2 \gamma^{1/2}} \ln \frac{\gamma^{1/2} + 1}{\gamma^{1/2} - 1} \right]. \]  
(50)

The right-hand side takes a value between 0 and 0.075. The decay rate is given by Eq. (47) with \( \gamma \) defined above. The acceptor concentration \( C_0 \) in Eq. (47) is the concentration inside the sphere. When acceptors are buried in the wall, \( C_0 \) is the number of acceptors divided by the volume in which the donors diffuse:

\[ \dot{\xi}(0) = \frac{1}{\tau_D} + 8\pi R_0^6 / 3\tau_D a^3 A (1 + \gamma)^3, \]  
(51)

where \( A \) is the average surface area (at radius \( b \)) per acceptor. Relations (41) and (42) are recovered from relations (50) and (47) by taking the limit of \( b \to \infty \) and \( \gamma \to 1 \). As \( \gamma \to \infty \) (\( b \to 0 \)), the right-hand side of the inequality (50) approaches zero, ensuring an exponential decay of fluorescence. This is an expected but trivial result.

Although we have assumed that the hollow sphere is closed, existence of an opening toward an external space does not seriously affect the results provided the transfer rate \( k(R) \) is negligibly small in the external space. This is because the major term on the right-hand side of the inequality (50), the first term containing \( \arctan \), comes from the term \( k(R) H(R;\rho) \) in the integrand in the inequality (31). Connection to the external space further reduces the contribution from other terms since \( k \) and \( H(R;\rho) \) tend to zero, while \( H(R;\rho) \) is not significantly altered at \( R \approx a \) where \( k(R) \) is large. In applications to membrane vesicles with donors in the membrane phase, e.g., leakage of acceptors through the membrane does not affect the fluorescence kinetics provided the donor position is closer to the internal surface of the membrane (so that the energy transfer to external acceptors is negligible). The case of a donor situated below an (approximately spherical) depression on a large particle is also dealt with by relations (47) and (50). The reverse cases where acceptors are in the wall, however, are not straightforward. In open systems, the acceptor concentration \( C_0 \) in Eq. (47) is the number of acceptors in the sample divided by the sample volume including the external space; Eq. (51) does not apply. Significant deviation of \( \dot{\xi}(0) \), the decay rate, from 1/\( \tau_D \) thus requires a large number of hollow spheres. At the same time, however, the volume fraction of the hollow particles, including their internal spaces, should be much less than one, since otherwise the excluded volume effect leads to the violation of the multiplication law on the right-hand side of Eq. (6). Also the path(s) between the internal and external spaces should allow effectively free diffusion of donors. These conditions, together with the requirement that the acceptor density in the wall be low (Sec. III F), severely restrict the realization of the rapid diffusion limit in systems of open hollow spheres with acceptors in the wall.

**E. A sphere or radius \( b \) with acceptors uniformly distributed at a depth of \( a \)**

The geometry is shown in Fig. 4. A vesicular membrane containing multiple acceptors is classified in this category or in case C above depending on the surface density of the acceptors and the size of the vesicle (see below).

![FIG. 4. Coordinate systems used in Secs. III E and III F. The large closed circle represents the (arbitrarily chosen) position of the pseudocell.](image)
Since the distributed acceptors act as a unit, we can replace them with a single pseudoacceptor. The pseudoacceptor receives excitation energy from an external donor at an effective rate given by the sum of transfer rates to real acceptors. For consistency with the preceding sections we locate the pseudoacceptor, arbitrarily, on the real acceptor on positive z axis (large closed circle in Fig. 4). For a donor at R from the pseudoacceptor the effective rate \( k(R) \) is given, for \( \kappa^2 = 2/3 \), by

\[
k(R) = \frac{2R_0^6}{3\tau_D} \frac{2\pi b^2}{A} \int_1^a \frac{dp}{(r^2 - 2\epsilon r + \epsilon^2)^{3/2}}
\]

where we have smoothed out the acceptor distribution; \( A \) is the average surface area (at radius \( b \)) per acceptor and \( c = b - a \). Introducing Eqs. (52) and (35b) into the inequality (18) we obtain the rapid diffusion criterion as

\[
D_{\tau_D} \cdot \frac{a^4}{R_0^6} \geq \frac{2\pi ab}{9} \frac{3 + \gamma^2}{A(1 + \gamma)^3},
\]

where \( \gamma = c/b = (b - a)/b < 1 \). The decay rate is given by

\[
\xi(0) = 1/\tau_D + 32\pi^2 C, R_0^6 b^3/9\tau_D a^3(1 + \gamma)^3,
\]

where \( C \) is the concentration of the acceptor spheres (number of spheres per unit volume). Equation (54) reduces to Eq. (47) upon introduction of the relation \( C_0 = C_0(4\pi b^2/A) \) where \( C_0 \) is the number of acceptor molecules per unit volume. For \( A = 4\pi b^2 \) (one acceptor in the sphere), the inequality (53) at the limit of \( \gamma \to 0 \) \( (b-a) \) agrees with the inequality (46) at \( \gamma \to 0 \).

The use of smoothed distribution leads to an underestimate of \( k(R) \) at donor positions immediately above the real acceptors. Since the integral in the inequality (18) is sensitive to the value of \( k(R) \) at \( R = a \), the right-hand side of the criterion (53) is also an underestimate when \( A \) is large. A safe criterion is obtained by adding the right-hand side of the inequality (46) to the right-hand side of the inequality (53), since addition of Eq. (45) to Eq. (52) lifts the underestimation.

The above consideration also leads to a distinction between cases C and E. Comparison of the relations (46) and (53) shows that case C applies when \( ab/A < 1 \) (small sphere with low acceptor density) and case E applies when \( ab/A \gg 1 \). In the intermediate range the right-hand sides of the two inequalities should be added. In any case the decay rate \( \xi(0) \) is given by Eq. (47) when \( C_0 \) is defined properly.

For given \( a \) and \( A \), the right-hand side of the inequality (53) tends to infinity as \( b \to \infty \) \( (\gamma \to 1) \). With large spheres, therefore, the rapid diffusion limit is not easily reached. In particular the limit is never attained when acceptors are distributed in an infinite plane. In real systems such as membrane sheets, however, the size of the plane is finite. The inequality (53) may then be used as the rapid diffusion criterion with \( b \) being the radius of the planar sheet.

**F. A hollow sphere of radius \( b \) with acceptors uniformly distributed in the wall at a depth of \( a \)**

The geometry is shown in Fig. 4. An application to be considered is a vesicular membrane system.

As in Sec. III E above we smooth out the acceptor distribution and replace the real acceptors with a pseudoacceptor on positive z axis. The effective transfer rate \( k(R) \) is given by Eq. (52) where \( c \) in this case is defined by \( c = b + a \). The average transfer rate \( \bar{k} \) for the pseudoacceptor is given, for \( \kappa^2 = 2/3 \), by

\[
\bar{k} = \frac{8\pi R_0^6}{3\tau_D} a^3 b \lambda (\gamma + 1)^3,
\]

where \( \lambda = c/b = (b + a)/b > 1 \). The function \( H(R; a) \) in the inequality (31) is given by Eq. (49b). The transfer rate \( k(R) \) in Eq. (52) is smallest at the center of the sphere. Therefore, \( H(R; f) \) is given by

\[
4\pi H(R; f) = 1/r.
\]

The rapid diffusion criterion is thus calculated as

\[
D_{\tau_D} \cdot \frac{a^4}{R_0^6} \geq \frac{2\pi ab}{9} \frac{3^2 - 1}{A(1 + \gamma)^3}.
\]

The decay rate is given by Eq. (51).

As to the consequence of the smoothing of the acceptor distribution and to the distinction between cases D and F, the comments in Sec. III E above apply: For safety, the right-hand sides of the inequalities (50) and (57) should be added. Case D is for \( ab/A \ll 1 \) and case F for \( ab/A \gg 1 \).

As in case E above the rapid diffusion limit is not easily reached when the sphere is large. For example, Thomas et al.\(^4\) have studied a system in which donors were trapped in the inner aqueous phase of membrane vesicles with acceptors distributed in the membrane phase. The system was characterized by \( D = 4 \times 10^{-10} \text{cm}^2/\text{s}, \tau_D = 2 \text{ms}, R_0 = 4.88 \text{nm}, b = 15 \text{nm}, \) and \( n \) was found to be \( 1 \text{nm} \) for \( A \) between 200 and 1000 \text{nm}^2. \) These numbers do satisfy the inequalities (50) and (57) with a safety factor of \( 10^3 \). For vesicles with a size of cells \( (b \gtrsim 1 \mu \text{m}), \) however, the acceptor density \( (1/A) \) needs to be increased in proportion to \( b \) in order to obtain the same transfer efficiency [see Eq. (51)]. Then the inequality (57) will be violated.

**IV. DISCUSSION**

**A. Use of the rapid diffusion inequalities**

The rapid diffusion criteria are given in the form of inequalities. In applications one has to decide how strictly the criterion should be fulfilled. The derivation in Sec. II shows that the extent of departure from an exponential decay, \( [\xi(0) - \xi(\infty)]/\xi(0) \), is given approximately by the value of the left-hand side of the inequality (18) or (31). For the explicit criteria in Sec. III, \( [\xi(0) - \xi(\infty)]/\xi(0) \) is approximated by the ratio of the value of the right-hand side to the value of the left-hand side in the rapid-diffusion inequalities. If the ratio is 0.1, e.g., the normalized decay rate \( \xi(t) \) remains within 10% of the initial rate \( \xi(0) \).

Assuming an exponential decay in the analysis of experimental data generally leads to an underestimate of the initial decay rate. The maximal error, however, does not exceed the above ratio. The error in \( \xi(0) \) is transmitted to the error in the distance of closest approach, \( a \), through the relation (47) or a similar relation. Thus 10% error in \( \xi(0) \) is permissible in most applications, since the relative error in \( a \) will then be a few percent unless \( \xi(0) \) is close to 1/\( \tau_D \) and since the error in \( R_0^6 \) is usually greater than 10%.
words the inequality sign \( \gg \) reads, practically, "greater at least by a factor of 10", except where the efficiency of energy transfer is very poor \( [\xi(0) \approx 1/\tau_D] \).

**B. Nature of the rapid diffusion criteria**

The results in Sec. III show that, except for the cases of many acceptors in the particle (cases E and F), the rapid diffusion criteria for the energy transfer of the Förster type have a common form:

\[
D\tau_D a^4/R_0^6 \gg \lambda, \tag{58}
\]

where \( \lambda \) is a geometrical constant that depends only on the ratio between the distance of closest approach \( a \) and the radius \( b \) of the particle. The value of \( \lambda \) is less than one. Thus the inequality (58) with \( \lambda = 1 \) may be used as a universal criterion, irrespective of the particle geometry. (Note that this universal criterion is well on the safe side for single-acceptor particle systems but is not sufficient for multi-acceptor particle systems for which \( \lambda \) may exceed one depending on the acceptor density. For practical applications, consult an appropriate inequality in Sec. III and see Sec. IV A above.)

The inequality (58) is interpreted as follows: At the closest approach between the donor and acceptor, the rate of energy transfer is \( R_0^6/\tau_D a^6 \) (the orientation factor of the order of one being neglected). The distance between the donor and acceptor remains on the order of \( a \) for the time of the order of \( a^2/D \). Once the distance becomes larger, the transfer rate is greatly diminished owing to the steep dependence on the distance. Thus the product \( (R_0^6/\tau_D a^6) \cdot (a^2/D) \) is the probability for the occurrence of energy transfer during a single encounter. The inequality (58) states that this probability should be much less than \( 1/\lambda \), or much less than one: Significant energy transfer must be the result of many encounters. Donors are equivalent under this condition. A convex surface is less advantageous than a concave one, since diffusion along the convex surface is less effective in bringing the donor and acceptor apart.

In cases E and F where acceptors are densely distributed under a surface, the transfer rate at the closest approach is given, approximately, by \( R_0^6/\tau_D a^4A \) [see Eq. (52)]. In other words, of the acceptor area \( A \) a fraction \( a^2/A \) gives the maximal transfer rate \( R_0^6/\tau_D a^4 \). A donor at the closest approach remains in the neighborhood of the acceptor sphere of radius \( b \) for the time of the order of \( b^2/D \). Of this time a fraction \( a/b \) is passed in the transfer region, i.e., within a distance \( a \) from one of the distributed acceptors. Thus the probability of energy transfer during a single encounter between a donor and acceptor sphere is given by a product \( (R_0^6/\tau_D a^4A) \cdot (b^2/D) \cdot (a/b) \). The inequalities (53) or (57) state that the product should be much less than one: Single encounters must be ineffective. In particular the rapid diffusion limit is not attained when the surface is infinitely wide, since those donors that diffuse along the surface fail to escape from acceptors.

The discussion above does not apply to the cases (D and F) of finite volume of which the radius \( b \) is comparable to or smaller than \( a \). Here donors cannot escape from acceptors. Obviously, however, the decay of donor fluorescence should be exponential when the diffusion within the small volume is faster than the maximal rate of energy transfer. Moreover, as the volume becomes smaller \( (b/a \to 0) \) the need for the diffusion is lessened since the variation of the transfer rate in the volume becomes negligible. Thus the right-hand sides of the inequalities (50) and (57) tend to zero as \( \gamma \to \infty \) \( (b/a \to 0) \).

**C. Criterion by Thomas et al.**

Thomas et al.\(^4\) have suggested that the criterion for the rapid diffusion limit is

\[
D\tau_D/s^2 \gg 1, \tag{59}
\]

where \( s \) is the "mean distance between the donors and acceptors." This criterion is apparently quite different from ours in the inequality (58). For example, it is intuitively clear that the decay of donor fluorescence cannot be exponential when \( a/R_0 \ll 1 \). This situation is excluded by the inequality (58), not by the inequality (59). In terms of the discussion in the preceding subsection, the inequality (59) is the condition that ensures an excited donor many encounters with acceptors; the important condition that the transfer probability during a single encounter be negligible is left out.

The criterion (58) ensures an exponential decay of fluorescence. It does not, however, specify whether, or how much, the decay is accelerated by energy transfer. For example, the inequality (58) alone does not exclude the trivial case where energy transfer is totally ineffective \( (a \to \infty \) \( \text{or} \) \( R_0 \to 0) \). Equation (47) shows that the inequality

\[
C_0 R_0^6/a^3 \gg 0.1 \tag{60}
\]

must be satisfied for energy transfer to be effective \( (\text{to accelerate fluorescence decay by more than 10\%}) \).

Multiplication of the inequalities (58) and (60) yields

\[
D\tau_D/s^2 \gg 0.1\lambda s/a, \tag{61}
\]

where we have used the relation \( a_0 \approx 1/s^3 \) (for the case of one acceptor per particle). The right-hand side takes a value not much less than one, since \( a \) that can be investigated is of the order of \( R_0 \) which is at most 10 nm and \( s \) cannot be much smaller than 1 nm \( (C_0 \approx 1 \text{ M}) \). Thus the inequality (59) is very approximately a necessary condition, although it is not sufficient.

**D. Heterogeneity**

So far we have assumed that the parameters such as \( R_0, a, \text{and} \ b \) are constant throughout the system. Actual systems, however, are often heterogeneous. For example, it is very difficult to prepare a suspension of membrane vesicles with a uniform radius. Below we discuss the consequence of such heterogeneity.

The derivation in Sec. II shows that heterogeneities do not introduce serious problems in open systems (cases A, B, C, and E in Sec. III) where a donor can interact with any of the acceptors. The decay of donor fluorescence will be exponential if all types of donor-acceptor pairs in the system fulfill the rapid diffusion criterion appropriate to the geometry. Whether the criterion is satisfied or not, the initial decay rate \( \xi(0) \) is given by a modification of an appropriate equation in Sec. III: The second term on the right-hand side of the equation, \( \xi(0) - 1/\tau_D \), should be summed over different
types of acceptors and the whole expression should be averaged over different types of donors.

In a system composed of closed subsystems such as the hollow spheres treated in cases D or F in Sec. III, exponential decays are not easily realized. Except for the case of "leaky" subsystems discussed in Sec. III D, the subsystems are independent from each other. Even if each subsystem satisfies the rapid diffusion criterion and contributes an exponential decay, the sum is not exponential unless the decay rate $\xi(0)$ is common to all subsystems. Heterogeneity, among the subsystems, in any of the parameters in $\xi(0)$ leads to a nonexponential decay. When acceptors are contained in vesicular membranes, for example, $\xi(0)$ is sensitive to the radius $b$ of the vesicles as shown in Eq. (51). Unless the radius is controlled very carefully, the decay will deviate from exponential. In the reverse system where donors are in the membrane phase, $\xi(0)$ is not sensitive to $b$ unless $b > a$ as is seen in Eq. (47). Instead one may have to worry about the statistical variation of $C_D$. An acceptor concentration, $C_A$, of 1 mM in a hollow sphere of radius 10 nm means only 2.5 acceptors, on the average, per sphere.

E. Experimental aspects

The major purpose of a rapid diffusion experiment is to estimate the distance of closest approach, $a$, between the donor and acceptor. With time-resolved techniques, attaining the rapid diffusion limit is not necessarily an absolute requirement since the measurement of the initial decay rate with desired precision, if possible, suffices for the determination of $a$. Often, however, the precision is guaranteed only for (nearly) exponential decays, which call for the rapid diffusion limit.

Attaining the limit is essential in steady-state experiments in which one illuminates the sample with continuous light and measures the quantum yield $Q$ of donor fluorescence. In general the dependence of $Q$ on $a$ can be worked out only by complicated numerical calculations. At the rapid diffusion limit, however, the yield $Q$ is related to the initial decay rate $\xi(0)$ simply by

$$Q_D / Q = \tau_D \xi(0),$$

(62)
where $Q_D$ is the quantum yield in the absence of acceptors. Thus, using an appropriate expression for $\xi(0)$ given in Sec. III, the distance $a$ is determined in a straightforward manner. The value of $a$ obtained may then be used to check if the rapid diffusion criterion is really satisfied. This is theoretically a safe procedure, since the value of $a$ calculated from Eq. (62) is the lower limit [$\tau_D \xi(0)$ is the upper limit of $Q_D / Q$, i.e., $Q_D / Q < \tau_D \xi(0)$ for nonexponential decays]. Of course direct confirmation by a time-resolved measurement is preferable.

In designing a rapid diffusion experiment, both inequalities (58) and (60), or preferably corresponding relations in Sec. III appropriate to the system, should be consulted. The inequality (58) is the condition for observing an exponential decay and serves as a criterion for the choice of a suitable donor–acceptor pair: For a given range of $a$ to be explored, the inequality (58) sets an upper limit for $R_a$. The inequality (60) then sets a lower limit of the acceptor concentration. If the required concentration cannot be achieved, due to solubility or other problems, a donor with a longer $\tau_D$ should be sought for.

Thus, as has been discussed by Thomas et al., a donor with a long excited-state lifetime is desirable in rapid diffusion experiments. The terbium chelates used by these authors have $\tau_D$ of a few milliseconds. Since the diffusion coefficient of a relatively small molecule in water is of the order of $10^{-6}$ cm$^2$/s, a down to 1 nm satisfies the inequality (58) even for $R_a$ as large as 5 nm. With $R_a = 5$ nm and $a = 1$ nm, the acceptor concentration can be reduced down to $\approx 10 \mu$M.

Ordinary fluorophores with $\tau_D$ in the nanosecond range, however, may also be used by choosing a small $R_a$ and by working at high acceptor concentrations. In the experimental system of Koyama et al., e.g., the donor was a reduced chromophore of bacteriorhodopsin with $\tau_D$ of 20 ns. The acceptor chosen was cobalt methenylidenediacetate, giving $R_a$ of 1.26 nm and the diffusion coefficient in water of $5 \times 10^{-6}$ cm$^2$/s. Thus the rapid diffusion limit is expected when $a > 1$ nm. At $C_D = 250$ mM they observed an acceleration of 10% in the rate of fluorescence decay, which was exponential as expected. Analysis gave $a = 1.2$ nm.

The discussion in Secs. IV B and IV D above show that, when dealing with supramolecular systems such as membranes, preferred tactics is to put acceptors in the solution phase and a donor(s) in the large object. The reverse system of acceptors in the large object is likely to suffer from complications such as the difficulty in attaining the rapid diffusion limit, the heterogeneity problem, and the excluded volume effect. From biological point of view, on the other hand, an intrinsic chromophore is the preferred target since it often constitutes an active center and since perturbations resulting from labeling can be avoided. Natural chromophores, however, do not always have a long fluorescence lifetime, although they can almost always serve as acceptors. Chemical treatment may confer desired fluorescence characteristics to the chromophore, as in the example of bacteriorhodopsin above, but this is not always possible.

In any case the best strategy is to investigate the same object with various combinations of donor–acceptor pairs and seek for consistency. Such strategy has successfully been employed in the study of the transmembrane location of the retinal chromophore of bacteriorhodopsin in the purple membrane of Halobacterium halobium. Not all measurements were made at the rapid diffusion limit, but the initial decay rates in all systems were consistent with a unique transmembrane location of the chromophore.

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