Methods and Applications in Fluorescence

PERSPECTIVE

Kappaphobia is the elephant in the fret room

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Abstract

FRET is both a phenomenon and a spectroscopic technique, capable of measuring one geometric quantity: kappa-squared divided by the sixth power of the donor-acceptor distance. Kappa-squared is often replaced by a constant even though such a replacement may lead to serious errors. Kappaphobia, the fear of kappa or the reluctance to deal with kappa-squared adequately, is a looming presence in the FRET community. Unfortunately, this reluctance, or fear, is often tolerated, and sometimes encouraged. A decrease in kappaphobia will lead to an increase in the impact and success of FRET.

Introduction

The expression ‘the elephant in the room’ [1] is a metaphorical idiom in English for an important problem that is obvious, but no one wants to discuss because it makes people uncomfortable or is embarrassing. Kappa or kappa-squared is an orientation factor. The following ‘radio-analogy’ is useful for a conceptual understanding of this important quantity [2]. Sending and receiving a radio signal is completely analogous to emission and absorption of light. Radio is normally used in the ‘far-field,’ where the distance between sender and receiver is much larger than the wavelength of the radio wave. However, it is possible to use radio in the ‘near-field,’ where the wavelength is smaller than the sender-receiver distance. FRET [3] is completely analogous to radio in the near field. The radio efficiency depends on power, distance and resonance. Resonance is determined by the frequency overlap between sender and receiver (the receiver/acceptor, needs to be ‘tuned’ to the sender/donor), but also by the relative orientations of sender and receiver antennas. The receiver’s antenna needs to be aligned with the sender’s electromagnetic field. An antenna in radio has the same role as a dipole transition moment in FRET. Kappa or kappa-squared describes the antenna effect. Kappa is equal to \( \cos(\omega) \sqrt{1 + 3(\cos(\theta))^2} \), where \( \omega \), omega, is the angle between the accepotor antenna and the electric field generated by the donor at the location of the acceptor, and \( \theta \), theta, is the angle between the donor antenna and the line connecting the centers of the antennas. Diagrams of electric field lines and the role of these angles are shown in figure 1, reproduced from [4]. If omega equals 90 degrees, kappa is zero, but if both omega and theta are equal to zero, kappa is 2 and kappa-squared is 4. Kappa-squared is a number between 0 and 4 [4–6].

Kappaphobic mistake number 1: ‘kappa-squared is two-thirds’

Kappaphobes [7] will tell you that kappa-squared is equal to two-thirds. The experienced kappaphobes teach the next generation and tell their students: ‘and now you replace kappa-squared by two-thirds.’ But, you say, ‘it says here that kappa-squared is between 0 and 4’. ‘Yes, but the average is two-thirds, and there is a lot of motion going on. So, it is perfectly OK to take the average.’ ‘Are you sure?’ The Dale-Eisinger-Blumberg paper [5] says that you can use fluorescence depolarization to find a minimum and a maximum kappa-squared, and that you should use these extremes to find the range of distances. ‘Well, they are just trying to hold up the field, the Haas-Katchalski-Katzir-Steinberg paper says that kappa-squared is two-thirds [5].’ But that is not true! What Haas and his colleagues have actually shown is that the distance probability distribution due to uncertainties in kappa can be broad and the peak does not always correspond to the two-thirds value for kappa-squared.

Orientalional order is important for FRET, for two reasons, one: kappa is an orientational quantity, and two: kappa can be determined by measuring depolarization factors which are also orientational measures. The first FRET paper I read happened to be the Dale-Eisinger-Blumberg paper. I loved that paper! It
Figure 1. This is figure 4.5 on page 68 of [4], reproduced here with permission from the copyright holder Wiley-VCH Verlag GmbH & Co.KGaA. Examples are shown of donor and acceptor orientations with corresponding kappa-squared ($\kappa^2$) values. The donor antenna is the bar in the center of each circle. The bars on the circle are examples of acceptor antennas. The number next to each acceptor is the $\kappa^2$ value for the donor-acceptor pair. The red lines inside each circle are the electric field lines caused by the donor. The direction $\hat{d}$ is that of the acceptor antenna, $\hat{d}$ is that of the donor antenna and $\hat{E}_{D}$ is the direction of the electric field from the donor at the location of the acceptor. The circle on the left has examples where donor and acceptor antennas are parallel, including four cases where $\kappa^2 = 0$ ($\omega = 90^\circ$, $\theta = \text{ACOS}(1/3) = 54.7^\circ$). The next circle shows examples where the acceptor is along the donor electric field including four cases where $\kappa^2 = 2$ ($\omega = 0^\circ$, $\theta = \text{ACOS}(1/3) = 54.7^\circ$). Note that in these four cases $\kappa^2$ is not zero, but the donor antenna is perpendicular to that of the acceptor. The two circles on the right have $\kappa^2 = 0$ examples where the acceptor is perpendicular to the donor electric field: at the third circle donor and acceptor are in the plane of the page, at the fourth circle all acceptor antennas are perpendicular to the page.

showed me that my PhD thesis work on orientational order in liquid crystals could be useful in biophysics. That paper made me a kappaphile [8], not realizing until much later that many FRET practitioners are kappaphobes. The initial value of the fluorescence anisotropy is 0.4, if there is neither any fast wobbling of the transition dipoles [5] nor any excitation of mixed transitions [6]. If there is some fast wobbling or excitation of coupled transitions, the initial value of the anisotropy is 0.4-times-the-square-of-a-depolarization-factor [4, 5]. So, depolarization factors can be measured using fluorescence anisotropy data. Thus, in the decade after the publication of the Dale-Eisinger-Blumberg paper several papers appeared with maximum and minimum values for kappa-squared using depolarization data. However, then came the big switch to fluorescence microscopy and it is not that easy to measure fluorescence anisotropy in the microscope because of aperture corrections [9, 10]. Eisinger has proposed the use of anisotropy standards in the microscope [11]. That idea may become extremely useful.

At first glance, the Dale-Eisinger-Blumberg paper [5] and the Haas-Katchalski-Katzir-Steinberg paper [6] seem to contradict each other as they have different conclusions: Dale, Blumberg and Eisinger had a somewhat pessimistic attitude and conclude that, where possible, fluorophores with rapid restricted motion at high wobbling amplitudes should be used to lower the kappa-induced uncertainty. On the other hand, Haas, Katchalski-Katzir and Steinberg are more optimistic and recommend using fluorophores that can be excited in overlapping transitions, so that kappa-induced uncertainty can be minimized. However, both indicate that fluorescence anisotropy or, equivalently of course, polarization is the key to solving the kappa-squared problem. Moreover, fluorescence anisotropy experiments cannot distinguish between rapid rotations and overlapping transitions unless responses can be resolved within picoseconds after excitation. In the appendix of [12], I have shown that there is no significant difference between the Dale-Eisinger-Blumberg approach and the Haas-Katchalski-Katzir-Steinberg approach [12]. We have reconciled these two approaches in [4] and [13] by introducing the concept of ‘the most probable kappa-squared’. This most probable value is defined as that kappa-squared value which corresponds to the most probable donor-acceptor-distance. Think of an ‘error-distribution’ of distances. Each kappa in the range of possible values corresponds with a possible distance. The resultant distance distribution has a peak somewhere in the middle. That is the most probable distance, and the corresponding kappa-squared value is, by definition, the most probable kappa-squared. Figure 2 allows you to find the most probable kappa-squared value if both the donor and acceptor depolarization factors are available.

There are at least two different types of errors associated with setting kappa-squared equal to two-thirds. There is the Peak-Location-Error meaning that if you assume that kappa-squared is two-thirds, you assume that the peak of the distance distribution is at the distance corresponding to two-thirds, which is incorrect in most cases as explained in figure 2. This error can be as large as 30% [4, 13], but it can be avoided by measuring the depolarization factors, which enables identification of the actual peak using figure 2. There is also the Broad-Distribution-Error meaning that there is an additional uncertainty caused by the width of the peak.
This error is in most cases between 0 and 5% but can approach 24% if both depolarization factors are close to 1 \cite{4, 13}. The words 'at least' in the statement above (that there are at least two errors) refer to the fact that it is necessary to know which averaging regime is applicable. Finding the most probable kappa-squared from depolarization factors is correct if your system is in the dynamic averaging regime, so that assuming incorrectly that the dynamic averaging regime applies, constitutes an additional error. Kappa-squared is also relevant outside the dynamic averaging regime, but in a different way, see below.

Time-resolved energy transfer measurements allow one to determine which averaging regime applies. Consider a drop in your sample containing a large number of donors and acceptors. Then visualize a flash of light, many photons traveling together, exciting donors in this drop. Immediately after excitation, antennas of the excited donors are starting, or actually continue, to wobble and their fields are reaching out to acceptors. We must now consider 3 possibilities: ONE: the rate of transfer is low compared to the rate of rotational jumps (or rate of coupling between transitions) during the time the donors are excited. So, in a short time a complete collection of possible transfer rates is created. All possibilities are there: high-kappa-rates and low-kappa-rates are represented. Each donor has enough time to jump through a wide range of orientations before transfer takes place. This situation corresponds with the **dynamic averaging regime**. In that averaging regime, the transfer takes place at a rate which is the overall average of all the transfer rates that are possible in the distribution of transfers existing in the drop. As a result, the TRE, the time-resolved energy transfer \cite{4}, is a rising single exponential reflecting the average transfer rate. TWO: the rate of transfer is high compared to the rate of rotational jumps. In this case, all possible transfer rates are present in the drop, but they apply independently of each other. For this **static averaging regime** each set of orientations is permanently represented throughout the decay and exhibits its own decay rate. As a result, the meaningful average is that of the efficiencies corresponding to these rates. In this regime the TRE is not mono-exponential. Note that it is also possible that the rotational rates are split into two bands, as discussed below. THREE: the system is in between the two regimes and then a more sophisticated analysis is necessary \cite{14}.

A few ‘kappa-engineering’ papers have appeared recently. One of these papers kills the kappa-squared-is-two-thirds claim very elegantly: a donor and acceptor are rigidly attached to a B-DNA backbone in such a way that the antennas are permanently collinear (both antennas are along the line connecting the centers of donor and acceptor). As a result, kappa-squared is equal to 4, or almost 4, permanently. That is a constant, all right, but it is not two-thirds \cite{15}.

**Kappaphobic mistake number 2: ‘kappa-squared is 0 if the donor transition moment is perpendicular to that of the acceptor’**

This mistake can be found in Lakowicz’s book \cite{16}. That book is excellent, but the mistake is there. And,
the mistake is repeated by other highly respected scientists. For example, Bastiaens mentions in one of his videos [17], that when the donor transition moment is perpendicular to that of the acceptor kappa is zero. Neither Lakowicz nor Bastiaens realized that this is very often true but not always. What is always true is that kappa is zero if the acceptor antenna is perpendicular to the electric field generated by the donor. The electric-field lines generated by the donor antenna are partly straight and partly curved. You can visualize the donor antenna as a sticklike cartoon figure with an extremely wide mouth right in the middle of the vertical stick (we assume here, arbitrarily, that the donor antenna is along the vertical). Above and below the stick in the same direction of the stick are the straight electric field lines generated by the electric oscillation in the stick. This cartoon figure has thousands of flat cigars in its mouth pointing in all horizontal directions, and with all these flat cigars in vertical planes. In any particular horizontal direction, the cigars overlap and vary in size between very short and very long. These cigars are the curved electric field lines, some of which are shown as red lines in figure 1. The acceptor can be located anywhere. For any acceptor sitting on the straight electric field line, kappa-squared is between 4, if the antennas are aligned, and 0, if the antennas are perpendicular. However, for any acceptor sitting at the top or bottom of the skinny side of the cigars, the situation is completely different. There, kappa is zero if the acceptor antenna is perpendicular to the electric field line. In that case, the donor acceptor is also vertical, so that the two antennas are parallel, but kappa equals zero. If, on the other hand, the acceptor antenna is along the ‘cigar’ at the skinny top, omega is zero, and kappa-squared reaches the local maximum, which turns out to be equal to 2 (the line through the centers of donor and acceptor makes an angle of ACOS (√(1/3)) with the donor antenna. This angle is about 54.7 degrees and is known as the magic angle in polarization spectroscopy). Note that the donor is vertical here, but the acceptor is horizontal, so that they are perpendicular to each other. So, it is possible that the donor is perpendicular to the acceptor at the same time that kappa is NOT equal to zero. This possibility is illustrated in figure 1. Note, that in many situations of perpendicular antennas, kappa is zero (4 examples in the third circle and 12 examples in fourth circle of figure 1), but not in all situations, so that it is incorrect to state that for all cases of perpendicular antennas kappa equals zero. Besides, as shown in figure 1, in some cases where the antennas are parallel, kappa can be zero too. Moreover, for acceptors located not on the vertical field line generated by the donor and not at the skinny top or bottom of the cigars, kappa is zero whenever the acceptor antenna is perpendicular to the cigar. In that case, the transition moments are neither parallel nor perpendicular (except at the tip of the cigar) and, yet, kappa is zero, and this reflects the fact that, of all the possible values of kappa-squared, an exact value of zero has the highest, actually infinite probability density [4, 5].

Kappaphobic mistake number 3: ‘in the dynamic averaging regime kappa-squared is two-thirds, and in the static averaging regime, kappa-squared is 0.476’

In 1971 Steinberg stated that in the static averaging regime the average of kappa-squared is probably 0.476 [16, 18]. Then, in 1983 Steinberg and his coworkers were more specific [19]: they derived the Steinberg-Haas-Katchalski-Katzir-curve. This curve is a graph of the average kappa-squared versus the donor-acceptor distance in the static averaging regime, showing kappa-squared increasing with distance. At low distance kappa goes to zero and the curve is almost flat, but at distances much larger than the average Förster distance kappa-squared reaches 2/3 asymptotically as the curve flattens again. In the middle it increases linearly and passes the 0.476 value near the average Förster distance. This trend is in agreement with Dale’s 1978 approximate linear relation, $\kappa^2 = \frac{3}{4}(1 - E)$, which says that, in the static averaging regime, the average orientation factor is 0 if the average transfer efficiency is equal to 1 (very small donor-acceptor distances), and reaches two-thirds at very low efficiency values (very large donor-acceptor distances) [20]. The fact that the average kappa-squared goes to zero when the efficiency reaches 1, seems to be counterintuitive, but not if you imagine this case as the limit of all possible Förster-distance-cases: This distance is proportional to kappa-squared. So, if you plot the efficiency versus distance for all possible values of the Förster-distance (corresponding to all possible kappa-squared values), you will see a series of similar curves with the kappa-squared-is-4-case on the right, and all other kappa-cases being similar but shifted to the left. Therefore, the kappa-is-zero-case is the limiting curve which is essentially a ‘curve’ that is collapsed to a vertical line segment with its low-distance-high-efficiency maximum exactly at distance-is-zero. In the literature: depolarization factors and excitation in overlapping transitions are mentioned in the dynamic averaging regime, but not in the static regime. This inconsistency needs to be corrected: the static regime does not forbid that immediately after excitation antennas wobble a little bit around an average direction nor does it exclude that transitions are coupled or overlap each other. How can the difference between the static and the dynamic averaging regime in general be understood, allowing for the appearance of depolarization factors in both regimes? The key issue is that the wobbling of antennas and/or the coupling of antennas can happen in two steps: a fast step and a slow step. In both the dynamic and static regimes, the fast
step occurs immediately after excitation. Then the depolarization factors are established, and the distribution of kappa-squared values is created. The difference is that, in the dynamic regime, the second step proceeds transfer, whereas in the static regime the transfer comes before the second step:

**Timeline in the dynamic averaging regime**

Excitation…Fast wobbling (coupling)…Slow wobbling & rotations…Transfer

**Timeline in the static averaging regime**

Excitation…Fast wobbling (coupling)…Transfer…Slow wobbling & rotations

Therefore, in the dynamic regime the distribution of kappa-squared values is complete before transfer takes place and all transfer rates in the distribution are available to each donor-acceptor pair at the moment of transfer. As a result, the overall transfer takes place at the average rate. However, in the static averaging regime the complete distribution is available but not to each donor-acceptor pair at the same moment! The transfer rates compete with each other. In this case the average efficiency must be calculated and the relevant average kappa-squared can be obtained from the average efficiency. In the dynamic averaging regime, the average kappa-squared is not universally equal to two-thirds as explained above. In the static regime, the results of [19] and [20] show that kappa-squared is also not universally equal to 0.476. In the static averaging regime kappa-squared varies between zero (or, more generally, the minimum kappa-squared [21]) and two-thirds, assuming that distance and orientation are independent. It is also possible though, that distance correlates with orientation.

**Kappaphobic mistake number 4: ‘2 peaks in the efficiency distribution means that the distance distribution also exhibits 2 peaks’**

For the special case where both depolarization factors are equal to one and the donor-acceptor distance is unique, the efficiency distribution is given in [22]. At low average efficiency there is one peak, but at high average efficiency the distribution has two peaks, one at low efficiency and the other at high efficiency. Yet, **only one distance is involved.** This mistake is highly relevant for single-molecule FRET, also known as smFRET, a new subfield that is poised to dominate FRET [23–28]. Schuler et al do mention kappa-squared in the context of smFRET and seem to favor working with the kappa-squared-is-two-thirds assumption but fail to realize that kappa-squared can have an effect on the efficiency distribution [22, 23].

The applicability of the kappa-squared-is-two-thirds assumption can be tested by polarization sensitive detection [24], but Schuler and coworkers do not do so. Kyrychenko and coworkers [25] address the variability of the orientation factor using molecular dynamics simulations in conjunction with an experimental study. They propose to improve the accuracy of distance measurements by comparing experimental and simulated FRET efficiencies for a model donor-acceptor pair of enhanced cyan and enhanced yellow FPs connected by a flexible linker. This should have been a great paper, but it has disturbing flaws: The authors show the same schematic kappa-squared special cases as Lakowicz does in his book on page 308, except that the diagrams are much better, but still highly misleading and incorrect for the kappa-squared-is-4-case as noted in the comment at the citation of reference [16]. In their figure 5A they show three special cases of kappa-squared, $\kappa^2 = 4$, $\kappa^2 = 1$ and $\kappa^2 = 0$. These special cases are depicted right below the equation for kappa-squared, where also the definition of the angles is shown. It should have been obvious that the diagram for kappa-squared $=4$ is directly in violation of the equation for kappa-squared. I cannot trust a molecular dynamics simulation performed by scientists who apparently have a flawed understanding of kappa squared. If this misunderstanding was the only problem, I could still be notably impressed by their work. However, in figure 5C they show their probability distribution for kappa squared obtained with molecular dynamics with a minimum at kappa-squared $=0$, showing counts going all the way to zero, and a maximum at 3.5. This range is expected, of course, but the surprising issue here is that the probability distribution reaches a minimum at zero, whereas theoretically it should be infinite [5, 22]. To me it is really disturbing that Kyrychenko and coworkers have failed to comment on this extreme incompatibility: the probability density at the kappa-squared minimum should be infinite, but they find it to be zero, and seem not to have noticed that there is a potential problem. Note that the simulations by Corry et al show kappa-squared distributions that do have a strong maximum at kappa-squared is zero, as expected [29]. Hakansson and his colleagues provide a detailed analysis of the problem of energy migration within pairs of donors [26]. They do this using the well-known extended Förster theory. They demonstrate that it is possible to overcome the kappa-squared problem by combining FRET with fluorescence depolarization data [26]. Sindert and coworkers discuss an interesting paradox: if you want to use kappa-squared is two-thirds you should use long flexible linkers, but that gives rise to uncertainties in the donor-acceptor distance. On the other hand, avoiding distance uncertainties by using shorter linkers triggers kappa-squared problems [27]. Hellenkamp and 43 co-authors have presented a very impressive multi-lab FRET study on B-DNA constructs, designed to exhibit kappa-squared values close to two-thirds by using flexible linkers and avoiding donors or acceptors attached to the end points [28].
Kappaphobic mistake number 5, a prediction of a future headline: ‘FRET has shown that DNA is NOT a double helix after all. No, it is a spiral!’

Another kappa-engineering paper on the variation of kappa also concerns a donor and acceptor bound to DNA. In this case, to the terminals of a DNA strand [30]. The key result of this important and beautiful study is shown in figure 3. This graph shows that with increasing distance between the donor on one end of the DNA fragment and the acceptor on the other end, the efficiency is NOT a decreasing function of distance, but a curve approaching unit efficiency at small distances equal to twice the pitch of the helix and also near 3 times this pitch. The curve has minima at distances equal to 1.5 and 2.5 times the pitch. This is clearly a kappa-squared effect: at distances equal to integer times the pitch, the antennas are close to parallel ($\kappa^2$ is high), but at distances equal to an integer-plus-a-half times the pitch the antennas are close to perpendicular to each other ($\kappa^2$ is low). However, in the future, if kappaphobia persists, it is entirely possible, that some scientist will re-examine these data: falsely assuming that kappa is just a constant, conclude that the only way these minima and maxima can be explained is that DNA is actually a spiral and not a double helix. At that point, FRET would indeed become the veritable laughingstock of spectroscopy!

It is interesting to compare the study by Hellenkamp et al [28] to that of Iqbal et al [30]. Both are FRET, mostly smFRET, studies. Both are dealing with FRET between probes attached to DNA. However, Hellenkamp et al designed their study to have kappa-squared close to two-thirds everywhere by choosing long flexible linkers and by avoiding attaching the probes to the end points, while Iqbal et al made sure that kappa squared strongly depends on the helical configuration of the system by attaching the fluorophores to the ends of the DNA. Unfortunately, both missed an opportunity to measure kappa squared, as explained below (see point 3 of the next section).

WHAT NEEDS TO BE DONE?

1. Get over kappaphobia by learning about kappa. Kappaphobia hurts progress in FRET. Attention to kappa improves the measurement of distances, distance distributions, efficiency distributions, and will lead to further discoveries and applications. My advice to future FRET scientists is this:
Do NOT believe what I and others tell you about kappa but check it out for yourself. Read the early papers in the 1978—2002 era, when kappa-phobia really did not exist. The best educational papers are in my opinion [5, 6, 18, 19, 20 and 31]. References [6 and 7] are interesting because of their content, but also because of the debate that started then: is kappa a big or small problem? References [18, 19 and 20] are about many aspects, but in particular about the differences between the static and the dynamic regimes (very important). Reference [31] discusses the kappa dependence on FRET and donor-acceptor distance for the case where several chromophores are present, not just one donor-acceptor pair. There are, of course, papers on kappa-squared, published after 2002 that are excellent, for example the papers by Corry et al [29, 32]. Interestingly, [29] confirms our finding that when only one distance is active, but a multitude of kappa values exist, the efficiency distribution shows two peaks [21].

2. Apply the Vogel Logic Tree [33] ‘Interpreting a FRET efficiency in terms of distance involves caveats! First, can you assume a homogenous or defined distribution of distances in the population? If not, anything goes! Next, can you assume an isotropic distribution of orientations? If not, again anything goes! If you can assume isotropic, is it dynamic averaging, static, or in between?’

3. Eighteen years ago, Dale showed theoretically that kappa-squared can be measured if the donor-acceptor-connection line is along the long axis of a macromolecule, using both donor-only and acceptor-only fluorescence anisotropy decay in combination with FRET including measuring the transfer anisotropy [4, 34]. Interestingly, both Iqbal et al [30] and Hellenkamp et al [28] could have done that experimentally in their modified DNA structures. Both groups did excellent work, but unfortunately overlooked the possibility of measuring kappa squared. Of course, it is possible that other methods of measuring kappa-squared will be discovered, possibly by doing FRET in oriented systems [29, 32].

4. Accept that the static averaging regime can exhibit depolarization or mixed transitions. Use depolarization factors to find the minimum-kappa-squared (or get it from the TRE, see point 7, below). The generalized Förster equation should depend on the minimum-kappa-squared as follows [21]:

\[
distance - ratio = \left( \frac{1 - \left(1 - \frac{3}{2} \kappa_{\text{minimum}}^2 \right) E}{E} \right)^{1/6}
\]

5. Extend the analysis of relative distance distributions to include transfer anisotropy. We have found the most probable kappa-squared value with its analysis of peak-location error and broad-distribution error [4, 13] for the case where the transfer anisotropy is NOT known. The importance of transfer anisotropy is discussed in [5 and 35]. Regions of different maxima and minima have been found numerically (though with some regions of error near the extremes) [5], and analytically [35]. However, the corresponding relative distance distributions with estimates of uncertainties have not yet been explored. Most likely, the Broad-Distribution-Error will be much smaller if transfer anisotropy is taken into account.

6. Measure your TRE, the time-resolved-efficiency, the donor decay alone minus the donor decay in the presence of acceptor, all that divided by the donor decay alone [4]. This TRE allows you to follow the transfer in time. In the simplest case the TRE is a rising exponential: \(1 - \exp(-k_T t)\), where \(k_T\) is the transfer rate and \(t\) is the time elapsed after excitation with a flash of light. This single exponential probably means that the dynamic averaging regime applies. In the static averaging regime, the TRE combined with the depolarization factors gives you the chance of employing the kappa-squared complexity to measure the width of the distance distribution. Why? Because the TRE is the Laplace transform of the distribution of transfer rates. As pointed out to me by my colleague Paul Blank, the inverse Laplace transform of the TRE yields the distribution of rates. And, if there is one unique distance, knowledge of the depolarization factors predicts exactly what the distribution of rates should be. Mathematica has the capability to perform a symbolic inverse Laplace transform, and Matlab can do a numerical inversion of Laplace transforms. If your transformed TRE deviates from the distribution of rates dictated by kappa-squared and the depolarization factors, it must be because the distance is not unique but distributed. That deviation, therefore, has critical information about the distance distribution. It may be possible to measure the width of the distance distribution independently using X-ray diffraction. The initial slope of the TRE reflects the average transfer rate. The failure to reach unity at larger times means that the contribution of the minimum-kappa-squared is significant [4], or, that the donor-acceptor link is so floppy that the contribution of large distances is significant. It is also useful to
study the sensitized emission by the Acceptor which will respond to the kappa-squared distribution in a way that is succinctly different from the behavior of the Donor. The Donor timeline is this: initially, say within the first 0.1-inverse-average-transfer-rate, all kappa-squared values are equally active so that then the average value of 2/3 dominates the transfer. Next, the contribution of the maximum kappa-squared will decay and for a very short time control the transfer, and then the next highest will decay and so on. Eventually, only the minimum kappa-squared is still active, and, if that value is zero (as a result of the absence of fast wobbling or coupling of transitions) no transfer will occur until a rotation causes kappa to become nonzero. As a result, the sensitized emission by the Acceptor is initially dominated by the average kappa squared. Next the highest values of kappa become dominant and the Acceptor’s emission reaches a maximum followed by a decay that becomes slower and slower as the lower kappa values take over. Note that we are referring here to the static averaging regime where the TRE is definitely NOT a single rising exponential and the sensitized emission is highly complex, but predictable. In the dynamic averaging regime, the TRE is a single rising exponential and the emission of the Acceptor is a combination of two exponentials, that of the TRE and that of the natural emission of the Acceptor.

7. Do Stryer-Haugland experiments to include kappa-variations. The original goal of this study was to check the distance-to-the-negative-6-power-dependence, and, also check the kappa-squared dependence, but this last goal was dropped [36], because, as Dick Haugland told me in 2002, they did not know how to do that back in 1967. Now, it is possible to examine the kappa-squared dependence explicitly as shown in [30], a rare example of a study focusing on kappa-squared. More such studies are needed, for example, comparing kappa-squared-dependence in flexible polypeptides compared to less flexible systems. Red-edge spectroscopy is standard in homotransfer, but in heterotransfer varying the excitation (or emission) wavelength will also help to control kappa-squared [6].

8. Simulations of kappa-squared have allowed Lillo and coworkers [37] to eliminate orientations incompatible with the structure of their system and to reduce the possible range of values. Vanbeek and coauthors showed a marked correlation between distance and kappa [38]. Hoefling et al demonstrate that by combining single molecule FRET experiments with the mutual dye orientation statistics obtained from Molecular Dynamics simulations improved estimates of distances and distributions are obtained [39].

9. Do fluorescence measurements in oriented systems so that orientational dynamics of transition dipoles can be studied [29, 32]. The location of the transition dipole moments in the structure of the fluorophores can be determined from excited state calculations [29]. Transition moments determining kappa-squared are NOT permanent dipoles, but oscillating dipoles and should be visualized either as bars, as in figure 1 above, or as arrows pointing in both directions as in figure 1 of [32], and figure 1 of [39]. Accordingly, the sign of kappa does not have a meaning [38].

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Conflict of interest statement

The author has declared that no conflicting interests exist.

Funding declaration

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References and footnotes

[1] https://en.wikipedia.org/wiki/Elephant_in_the_room

[2] Theodor Förster was in contact with radio engineers. It is not known for what purpose, but it is certainly possible that he wished to simulate kappa-squared effects. In the photosynthesis literature the radio analogy is implicitly applied as well 2019 http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

[3] ‘Fluorescence Resonance Energy Transfer,’ but this interpretation incorrectly suggests that fluorescence is either transferred or undergoes resonance. Brent Krueger and his students have proposed ‘Fluorescence-detected Resonance Energy Transfer’ (Vanbeek, D.B., M.C. Zwer, J.M. Shorb and B.P. Krueger 2007 Biophys. J. 92 4168–4178). This reading is correct, but I prefer ‘Fluorescence with Resonance Energy Transfer,’ which also correctly lists all relevant quantities involved and describes the FRET process (I introduced this reading of the acronym in 2002 Reviews in Molecular Biotechnology 82 181–196). Another reading of the acronym is Förster Resonance Energy Transfer, introduced by R.M. Clegg, (2006, Reviews in Fluorescence. Volume 3, editors C.D. Geddes and J.R. Lakowicz), this book.

[4] VanDerMeer B W, Vandermeer D M and Vogel S S 2014 Estimating the distance separating membrane studied by microscopic polarization Biophys. J. 126 537–74

[5] Fisz J 2007 Fluorescence polarization spectroscopy at combined high-aperture excitation and detection: application to one-photon excitation fluorescence microscopy J. Phys. Chem. A 111 8606–21

[6] Eisinger. J 1987 Personal communication. He suggested that polarization standards could be designed using a fluorometer with high-quality polarizers. Droplets from a series of such standards could then be observed in a microscope where the measured polarization would be different because of aperture effects, allowing for aperture corrections

[7] VanDerMeer B W Kappa-squared from nuisance to new sense Rev. Mol. Biotechnol. 82 181–96

[8] Raman spectroscopy, Einstein coefficients and Förster radius

[9] Principles of Fluorescence Spectroscopy chapter 10, p.309. There are several kappaophobic mistakes in this book: On page 308 the standard equation for kappa-squared is given (equation 10.13 in this book) with a reference to figure 10.1 on page 305, showing a diagram with the angles appearing in the equation and below this, 3 examples of kappa-squared is 4, 1, and 0, respectively. An improved version of this figure appears in reference 24 below, figure 5A. The 3 examples are explained on page 309 in Lakowicz’s book, where it says: ‘For aligned and parallel transition dipole κ = 4, and for oppositely directed and parallel dipole κ = 1’. This statement is wrong for several reasons: 1) if you reverse the direction of one or both dipole the value of kappa-squared does not change, and 2), immediately following from reason 1, both diagrams, the one labeled σ = 4 and the one labeled σ = 1, correspond to kappa-squared = 1. Correct examples of the kappa-squared is 4-case are shown in figure 3 above: 2 examples at the circle on the left and 2 examples at the next circle

[10] Steinberg I Z 1971 Long range nonradiative transfer of electronic excitation energy in proteins and poly peptides Annual Rev. Biochem. 40 83–114

[11] Steinberg I Z, Haas E and Katchalski-Katzir E 1983 Long-range nonradiative transfer of electronic excitation energy in proteins and polypeptides Time-Resolved Fluorescence Spectroscopy in Biochemistry and Biology ed R B Cundall and R E Dale (New York: Plenum Press) pp 411–50

[12] Dale R E 1978 Fluorescence depolarization and orientation factors for excitation energy transfer between isolates donor and acceptor fluorophore pairs at fixed intermolecular separations Acta Phys. Pol. A54 743–56

[13] The special case for κ_{max}=0 has been published by Vogel S S, VanDerMeer B W and Blank P S 2014 Estimating the distance separating fluorescent protein FRET pairs Methods 66 131–8 The equation in question is 4 is new and based on the generalization of Dale’s result in reference 20 that in the special static regime (where depolarization and/or mixed transitions are not present) the average kappa-squared correlates with the average efficiency as going linearly from 2/3 at low efficiency to authors for correction of errors in the sign of some of the vector components in their expressions for kappa-squared
0 at high efficiency. The generalization of this trend to the generalized static regime (where depolarization and/or mixed transitions do exist) is \( \kappa^2 \approx \frac{1}{3} - \left( \frac{2}{3} - \kappa^2_{\text{spin}} \right) F \)

[22] Vogel S S, Nguyen T A, VanDerMeer B W and Blank P S 2012 The impact of heterogeneity and dark acceptor states on FRET: implications for using fluorescent protein donors and acceptors PLoS ONE 7 e49593

[23] Schuler B, Soranno A, Hofmann H and Nettels D 2016 Single-molecule FRET spectroscopy and the polymer physics of unfolded and intrinsically disordered proteins Annual Rev. Biophys. 45 207–31

[24] Sisamakis E, Valeri A, Kalinin S, Rothwell P J and Seidel C A M 2010 Accurate single-molecule FRET studies using multiparameter fluorescence detection Methods Enzymol. 475 455–514

[25] Kryszekeno A, Rodnin M V, Ghatak C and Ladokhin A S 2017 Joint refinement of FRET measurements using spectroscopic and computational tools Anal. Biochem. 522 1–9

[26] Hakansson P, Iaksson M, Westlund P O and Johansson L B A 2004 Extended-Forster theory for determining intraprotein distances. 1. The kappa(2)-dynamics and fluorophore reorientation J. Phys. Chem. B108 17243–50

[27] Sindbert S, Kalinin S, Nguyen H, Kienzler A, Clima L, Bannwarth W, Appel B, Müller S and Seidel C A M 2011 Accurate distance determination of nucleic acids via Förster resonance energy transfer: implications of dye linker length and rigidity J. Am. Chem. Soc. 133 2465–80

[28] Hellenkamp B et al 2018 Precision and accuracy of single-molecule FRET measurements a multi-laboratory benchmark study Nat. Methods 15 699–76

[29] Corry B and Jayatilaka D 2008 Simulation of structure, orientation, and energy transfer between alexafluor molecules attached to MscL Biophys. J. 95 4168–78

[30] Iqbal A, Arsic S, Okumus B, Wilson T J, Giraud G, Schuler B, Soranno A, Hofmann H and Nettels D 2016 Single-molecule FRET spectroscopy and the polymer physics of unfolded and intrinsically disordered proteins Annual Rev. Biophys. 45 207–31

This is what he emailed me back. He added: ‘Frankly, if most FRET practitioners just followed this simple logic tree, 90% of my issues with erroneous FRET would disappear. I always try to report our FRET data as a FRET efficiency, not as a distance.’ And: ‘A carefully measured FRET efficiency should be accurate. For many studies this should be sufficient!’

[34] Dale R E 2002 Presentation at the American Biophysical Society Meeting discussed in Reference 3

[35] VanDerMeer B W 1999 Orientalational aspects in pair energy transfer Resonance Energy Transfer ed D L Andrews and A A Demidov (New York: John Wiley & Sons, New York) chapter 4 pp 151–72

[36] Stryer L and Haugland 1967 Energy transfer: a spectroscopic ruler PNAS 58 716–726, and, personal communication with Dick Haugland in 2002: Dick Haugland told me in 2002 that the original plan was to study all relevant factors in the transfer systematically. They did: the distance dependence was studied systematically, and, the effect of the refractive index was examined. However, they did not know how to vary kappa-squared efficiently. Remember, this was before the Dale-Eisinger-Blumberg paper [6] and the Haas-Katchalski-Katzir-Steinberg paper [7]. Now, as shown in reference 22, it is possible. It is interesting to note that the spectroscopically calculated \( R_0 \) assuming kappa-squared is 0.67 did deviate significantly from the one suggested by the data. The discrepancy is just within the kappa-squared range, that is, it can be reconciled with assuming that kappa-squared is close to 4. Already in 1967 there was this early sign that kappa-squared is not simply 2/3. However, this discrepancy can also be explained as resulting from averaging of the separation distance, since these molecules are quite flexible and the solution was of low viscosity [page 93 in VanDerMeer, B. Wieh, George Coker III and Simon Y-S. Chen 1994 Resonance Energy Transfer Theory and Data (New York, Weinheim and Cambridge, VCH Publishing Inc.])

[37] Lillo M P, Beechem J M, Szpikowska B K, Sherman M A and Mas M T 1997 Design and characterization of a multisite fluorescence energy-transfer system for protein folding studies: a steady-state and time-resolved study of yeast phosphoglycerate kinase Biochemistry 36 11261–72

[38] Vanbeek D B, Zwoer M C, Sbornik J M and Krueger B P 2007 Fretting about FRET: correlation between \( \kappa \) and R 2007 Biophys. J. 92 4168–78

[39] Hoefling M, Lima N, Haenni D, Seidel C A M, Schuler B and Grubmüller H 2011 Structural heterogeneity and quantitative FRET efficacy distributions of polyprolines through a hybrid atomistic simulation and Monte Carlo approach PLoS One 6 e19791