

## Membrane Penetration Depth Analysis Using Fluorescence Quenching: A Critical Review

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### *Abstract*

Membrane penetration depth is an important parameter in the study of membrane structure and organization. Use of fluorescence quenching by membrane-bound quenchers to analyze the membrane penetration depths of fluorescent molecules or groups is reviewed in this article. Such quenching interactions are short ranged and thus convenient for structure analysis on the scale of molecular dimensions. The quenchers used are usually fatty acids or phospholipids, the latter being a better choice, that are covalently labeled with spin-label (nitroxide) groups or heavy atoms such as bromine. Analysis of such quenching data requires special treatment because of the anisotropic nature of the membrane and the motional constraints experienced by the fluorophore and quencher molecules in the membrane. One of the frequently used approaches for measurement of depth in membranes using fluorescence quenching has been by comparison of quenching efficiencies of various quenchers located at different depths in the membrane. The assumptions and limitations inherent in these studies are examined. A novel approach known as the 'parallax' method, avoids some of these problems. In this method, spin-labeled phospholipids are used as quenchers, and the quenching patterns are analyzed by a static quenching model applicable to a random distribution of fluorophore and quencher molecules in the plane of a membrane. The parallax method involves determination of the parallax in the apparent location of fluorophores, detected when quenching by phospholipids spin-labeled at two different depths is compared. By use of relatively simple algebraic expressions, the method allows calculation of depth in angstroms. It is concluded that membrane depth analysis by fluorescence quenching represents a powerful tool for investigation of membrane structure.

### *Introduction*

Biological membranes are complex assemblies of lipids and proteins that allow cellular compartmentalization and act as an interface through which cells communicate with each other and with the external medium. Although many important functions are associated with cell membranes, our understanding of these processes at a molecular level is limited, in part, by the lack of high resolution three dimensional structures of membrane-bound molecules. It is extremely difficult to crystallize membrane-bound molecules for diffraction studies. Only recently was the first complete x-ray crystallographic analysis of an integral membrane protein successfully carried out (1).

Due to the inherent difficulty in crystallizing membrane-bound molecules, most structural analyses of such molecules have utilized other biophysical techniques with an emphasis on spectroscopic methods. One such analysis involves determination of membrane penetration depth which usually refers to the location of a molecule or a specific site within a molecule in relation to the membrane surface. Knowledge of the precise depth of a membrane embedded group or molecule helps define the conformation and topology of membrane proteins and probes. In addition, properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds and extent of solvent penetration are known to vary in a depth dependent manner.

Such depth analysis in membranes using fluorescence quenching caused by membrane embedded quenchers constitutes the subject matter of this article. This review does not provide an exhaustive account of studies on depth analysis; rather, an attempt is made to critically analyze the existing methodologies. A brief overview of the physical basis of the quenching phenomenon and its relevance in membrane studies is provided below.

### *Fluorescence Quenching in Membranes*

Fluorescence quenching is the reduction in the measured fluorescence intensity when a fluorophore interacts with a quencher molecule. After absorption of a photon, but before emission of radiation, a fluorescent molecule remains in its excited state for a short period of time, usually referred to as the excited state lifetime. The excited state lifetime, which is the average period of time a fluorophore remains in the excited state, is typically in nsecs. Experimental determination of lifetime involves measuring the characteristic average decay time of an ensemble of fluorophores. If there is an interaction of a fluorophore in the excited state with a quencher, the fluorophore may be deactivated before emission of light can take place. The magnitude of quenching depends on the competition between the fluorescence process, the quenching process and other processes that lead to the deactivation of the excited state and is determined by their relative rates. The magnitude of quenching also depends on the concentration of the quencher, which determines the number of quencher molecules in close proximity to the fluorophore.

Depending on the degree of intermolecular motion during the lifetime of the excited state, there could be two major quenching mechanisms, static and dynamic (2-5). Static quenching occurs when the distance between the fluorophore and quencher does not change during the lifetime of the excited state. This is the case for quenching occurring in a solid, in a frozen or extremely viscous solution, or in a bound 'dark' ground state complex of fluorophore and quencher. In ordinary non-viscous solutions, on the other hand, quenching is largely dynamic because fluorophore-quencher distances change rapidly, *i.e.*, there is relative motion in nsec time scale. In such cases quenching interactions occur during periods of close approach of fluorophore and quencher. A special case of dynamic quenching occurs when the range of quenching interactions is sufficiently small so that only collisions between fluorophore and quencher result in quenching of fluorescence. This is called collisional quenching. The rate for such quenching processes is then limited by diffusion, and in cases where quenching is efficient, this rate is the diffusion-controlled collision rate.

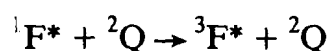
Fluorescence quenching by dipole-dipole energy transfer is the most extensively studied and characterized quenching process. Electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy acceptor over large distances (tens of angstroms). Förster proposed a theory for the dipole-dipole energy transfer process which postulated that the rate of transfer is inversely proportional to the sixth power of the distance between the donor and acceptor (6). This transfer of energy occurs without the appearance of a photon, and is primarily a result of dipole-dipole interactions between the donor and acceptor. The rate of energy transfer depends on the extent of overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the donor and acceptor transition dipoles, and the distance between these molecules.

It is well known dependence upon distance which has resulted in the widespread use of energy transfer to measure distances between the donor and acceptor (7). Such measurements require that the donor and acceptor be separated by a single distance which does not change during the excited state lifetime of the donor. However, for obtaining membrane depth, it is necessary to do energy transfer measurements for a random distribution of donor and acceptor molecules in two dimensions as in the case of membranes. Theoretical treatments for fluorescence quenching in two dimensions have been developed and used for such a case involving dipole-dipole energy transfer in biological membranes (8-11).

The analysis of membrane penetration depth by energy transfer, although widely used, has proved to be somewhat complex. In addition, the range of distances in which the method is best suited may not necessarily be the range of distances typical for membrane depth studies. This has given rise to disagreements in the reported depths for tryptophan residues of membrane-bound cytochrome  $b_5$  (12-15). Fluorescence quenching by dipole-dipole energy transfer usually involves long range interactions in which the donor and acceptor are typically separated by 30-50Å. On the other hand, there are the so called 'contact quenching' processes in which the fluorophores get quenched upon 'contact' with the quencher. Fluorescence quenching caused by the paramagnetic substances such as spin labels (nitroxides) and by molecules containing heavy atoms (such as bromine or iodine) fall into this category. Depth measurements utilizing such short range 'contact' quenching, rather than long range energy transfer, will be reviewed in this article.

Fluorescence quenching of a wide variety of fluorophores by molecules containing a nitroxide moiety in solution (16-19) and micellar environments (20,21) has been characterized. Likewise, it is known that molecules containing bromine atom can act as efficient quenchers of fluorescence in a number of cases (22,23). This is known as 'heavy atom quenching'. Quenching studies have been performed in model membranes (24,25) as well as in native membranes (26) using spin-labeled fatty acids or phospholipids. Lipid-protein interactions in model membranes have been studied in detail using spin-labeled phospholipids (27-29) and brominated phospholipids (30). From the temperature dependence of quenching in the liquid crystal phase and highly curved Stern-Volmer plots, it was concluded that quenching of membrane bound fluorophores by spin-labeled or brominated phospholipids is predominantly static in nature (27,30).

The mechanism of quenching in these cases is not very well understood. For quenching involving paramagnetic substances such as spin labels, an electron exchange mechanism involving an increase in the rate of transition from the usual excited (singlet) state of different spin quantum number (triplet) has been indicated (31). In this mechanism, the overall process of quenching of fluorescence of an excited fluorophore in its singlet state ( $^1F^*$ ) with a doublet quencher ( $^2Q$ ) is described as:



A weak complex is envisioned between  $^1F^*$  and  $^2Q$  that has no preferential orientation. The result of this electron exchange interaction between  $^1F^*$  and  $^2Q$  is an enhanced spin-orbit coupling in  $^1F^*$  which 'mixes' the singlet and triplet states of F to a greater degree than in the unperturbed system, making the originally forbidden singlet to triplet transition allowed. This leads to enhanced singlet-triplet intersystem crossing which results in loss of fluorescence. In cases involving heavy atoms such as bromine, it is believed that during close encounters of the fluorophore and quencher, the p orbital of the bromine atom overlaps the  $\pi$  orbital of the fluorophore and the perturbation produced by the bromine leads to the break down of spin selection rules (22,23). Under these conditions, the rate of singlet-triplet intersystem crossing increases, leading to a decrease in fluorescence quantum yield. In a recent report, the involvement of dipole-dipole interactions in quenching by brominated phospholipids in membranes has also been indicated (32).

Quenching of membrane-bound fluorophores by quenchers that are embedded in the membrane requires a separate treatment. The membrane is an anisotropic medium in which lipids are constrained to two dimensions, and lateral diffusion of lipids in membranes is sufficiently slow. Given a typical lateral diffusion coefficient for phospholipids in membranes of  $D = 10^{-8} \text{ cm}^2/\text{sec}$  (33), the distance between the fluorophore and the quencher does not change appreciably during the lifetime of the fluorophore (nsec). Thus, quenching phenomena observed in membranes are predominantly static in nature (4,5,27,30). However, dynamic components arising due to motions such as rotational diffusion and chain wobbling will make a contribution to quenching, if one considers the details of the quenching process (34).

#### *Depth Measurements using Short Range Quenching*

Jost and coworkers first suggested that quenching by spin labels (nitroxides) could be a useful way to measure distances in biological systems (35). Short range quenching caused by spin-label (36-49) or brominated (50-56) probes has been used in a number of cases to measure the location of fluorophores in membranes by comparison of the quenching efficiencies of these probes placed at various depths in the membrane. Some of these have been reviewed earlier (4,57). These studies exemplify the sensitivity of fluorescence quenching in relation to the membrane penetration depth of the fluorophore in question. The basic assumption in all these cases is that the fluorophore is closest to the depth of the quencher that gives the maximum quenching. While this is often true, there are cases where it is not valid. These are the cases in which

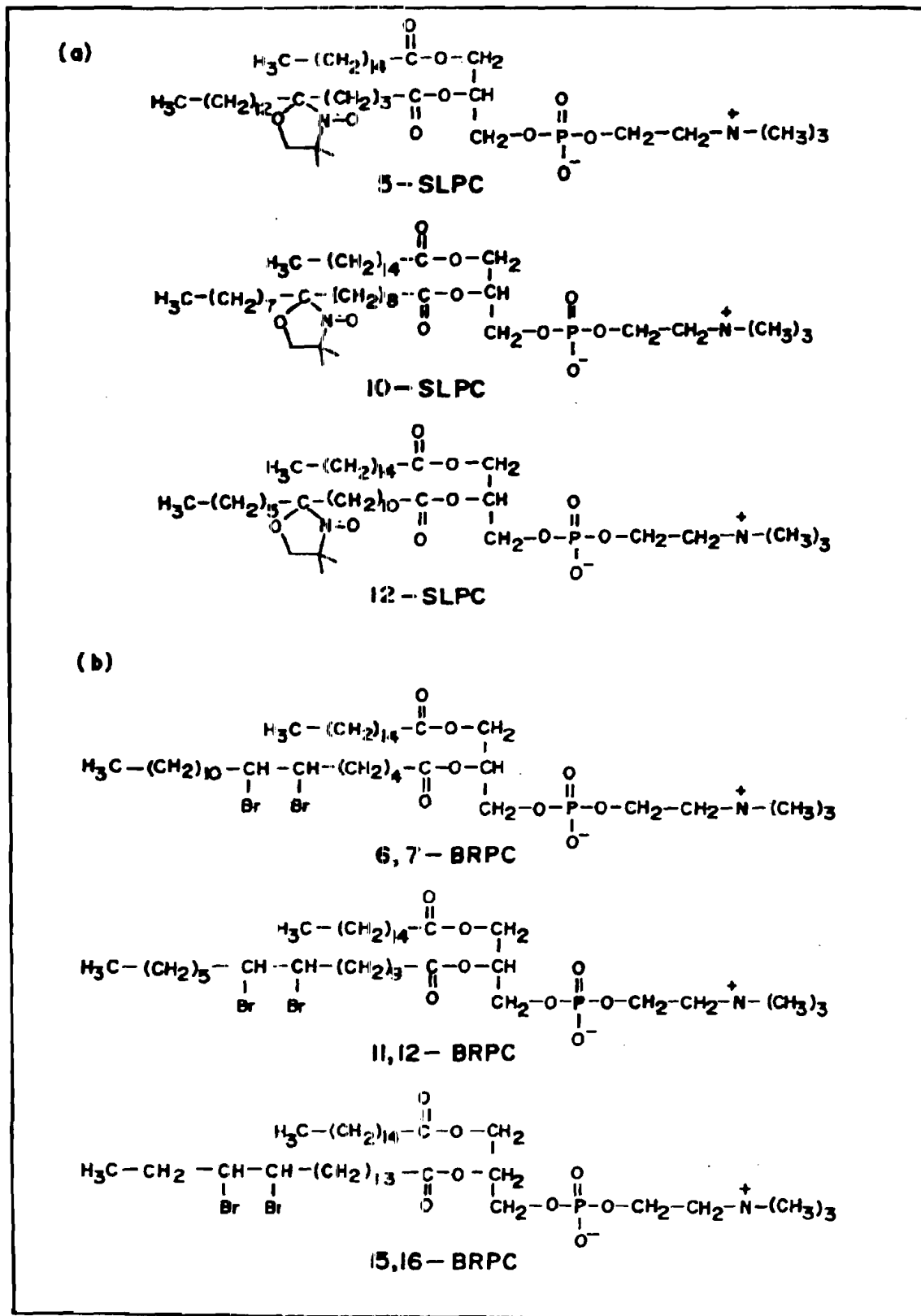


Figure 1: Chemical structures of some of the commonly used quencher phospholipids: (a) spin-labeled lipids and (b) brominated lipids.

deep quenchers (close to the center of the membrane) are used to study deeply buried fluorophores (58). Quenchers in one leaflet of the bilayer can then quench a fluorophore in the opposite leaflet, which is referred to as 'trans quenching'. Under certain conditions in which trans quenching occurs, the fluorophore will not be closest to the quencher that gives the maximum quenching.

In addition, these studies involve assumptions and have limitations, which must be carefully considered when interpreting the results. Most of these studies have used fatty acids (rather than phospholipids) with the spin label or bromine atom attached to different positions of the acyl chain. There are several limitations in using fatty acid probes as lipid analogues (5,59). These are: (i) spin-labeled fatty acids are not firmly held in relation to the bilayer; instead, they appear to exhibit marked vertical fluctuations as detected by electron-electron double resonance studies (60,61); (ii) free fatty acids are not normal membrane components, and if used in high enough concentrations (as is often required in quenching studies) may exert the lytic effects of detergents; (iii) the possibility of varying degrees of ionization of fatty acid probes must always be considered. The degree of ionization varies with pH, and consequently, the location of these probes in the bilayer may be pH dependent (62,63); (iv) fatty acids are much more water soluble than lipids, which results in partial partitioning depends on the attachment site of the spin label group on the acyl chain (42). Anomalous quenching of anthroyloxy probes has been attributed to this type of complication (40); and (v) fatty acids have been shown to perturb structure and function of membrane proteins (65-67). However, it is easier to incorporate fatty acids in membranes and this is useful in studies involving native membranes.

Figure 1 shows the chemical structures of some commonly used spin-labeled and brominated phospholipids in depth studies. Depending on the attachment site of the quenching probe on the fatty acyl chain, the depth of the quencher changes. There are relative merits and demerits of spin-labeled and brominated lipids as quenching probes. Both types of lipids have been shown to form membrane vesicles which have physical properties similar to liquid crystalline bilayer membrane vesicles made with other phospholipids (14,27,30,68). Bromine has a small molecular volume, about the same as a methyl group, and so perturbation of the membrane is minimum with brominated lipids. In addition, the high electron density of the bromine atom allows the determination of the location of bromine atoms in the membrane by x-ray diffraction. The membrane penetration depths of bromine atoms in membranes made with a series of brominated lipids have been determined (69). Spin labels, on the other hand, have the advantage that they can quench virtually all types of fluorophores including tryptophans (4). This is not true for brominated probes. Spin labels are also stronger quenchers than brominated probes and thus function at a lesser concentration. Another advantage is that because of the paramagnetic nature of the spin label, the same sample can be used for electron spin resonance (ESR) studies. Perturbation caused by spin labels has been shown not to be a major problem for depth studies (58). Several lines of evidence indicate that the spin label groups in spin-labeled phospholipids lie close to the corresponding position expected for an unlabeled phospholipid in membrane bilayers (58). This question has been recently addressed by studying the positions of the spin label groups in vesicles by  $^{13}\text{C}$

nuclear spin lattice relaxation (70). These authors concluded that even if some amount of deviation is present in the positions of the spin labels, it is not enough to create a significant problem for depth measurements. The accuracy of such measurements has very recently been checked by comparing the depths obtained by spin label quenching to that obtained from quenching by lipids labeled with bromine atoms at different positions of the fatty acyl chain (71). Since the positions of the bromines in membranes are known (69), the depth of the spin labels could be calibrated. This analysis shows that the depths obtained are accurate to around 2Å, thus justifying the assumed positions of the spin labels in membranes.

These depths studies utilizing short range quenching by spin label or brominated probes (36-56) have tended to be more qualitative. This is because interpretation of quenching data is limited by lack of knowledge about the dependence of quenching upon the distance between the quencher and the fluorophore. To overcome this problem, relatively simple and general mathematical expressions that are applicable to fluorescence quenching in membranes have recently been derived (58,72,73). This theoretical framework developed for analysis of quenching by membrane-bound fluorophores is based on Perrin's static quenching model (74) as applied to a random distribution of fluorophore and quencher molecules in two dimensions. The equations obtained allow straightforward and direct determination of membrane depth in angstroms by comparing the quenching obtained with quenchers at two different depths, *i.e.*, by the apparent degree of parallax in fluorophore position as viewed by quenchers at two different depths. This method, known as the 'parallax' method, also has the additional advantage that only phospholipids spin labeled at definite positions are used for depth analysis, and consequently, artifacts due to fatty acids are eliminated. Analysis of depth in this way is quantitative, yet less complicated than methods utilizing fluorescence energy transfer. In addition, the theoretical framework of the method is general, so that the method can be extended to quenching by probes other than spin labels (brominated probes or energy transfer probes). A limitation of the parallax method is that for cases in which multiple fluorophores are present in the membrane (*e.g.*, for proteins having multiple tryptophans in the membrane embedded portions), only an average depth is obtained. Nevertheless, it is still useful since it represents the minimum depth of penetration *i.e.*, at least one of the fluorophores is located deeper than the average depth obtained. This method is widely applicable to reconstituted systems and has been applied to determine penetration depths of the fluorescent groups in a series of nitrobenzoxadiazol (NBD)-labeled lipids (58,72) and anthroxyloxy-labeled fatty acids (5). The depths of the NBD groups obtained in this way have been further confirmed from independent spectroscopic and ionization properties of NBD-labeled lipids (75). In addition, the parallax method has been utilized to probe the locations of the membrane embedded tryptophan residues in the reconstituted nicotinic acetylcholine receptor from *Torpedo californica* (59,76), in synthetic channel peptides (77) in membrane-bound diptheria toxin (82) and in membrane-bound annexins (78), a class of  $Ca^{2+}$ -dependent membrane binding proteins.

### **Conclusions**

The potential of fluorescence quenching methods in depth analysis of membrane-

bound fluorophores is the focus of this review. One of the most exciting applications of such methods would be to determine depths of specific sites in membrane proteins. In the absence of precise diffraction data, quenching studies could prove to be a powerful tool to elucidate the conformation and topology of membrane probes and proteins. For topological studies, it may be necessary to generate vesicles having an asymmetric transbilayer distribution of quencher lipids, *i.e.*, there will be quencher lipids only in one half of the bilayer. This approach has been utilized to study the topology of the integral membrane protein cytochrome  $b_5$  (79). The asymmetric transbilayer distribution of quenchers in this case was attained by using phospholipid exchange protein (PLEP). For vesicles containing spin-labeled lipids, selective reduction of the spin labels in the outer leaflet by ascorbate, a hydrophilic reducing agent, offers yet another way to generate asymmetric vesicles (68,80,81).

The quenching methods are limited for proteins which have tryptophans or tyrosines. Nevertheless, it could be still possible to extract information about location of specific sites for proteins which lack these residues. One possible way is to covalently label the site of interest with a fluorescent probe and then analyze the depth of the labeled site. However, non-specific labeling of the protein could become a major problem in such a case. An alternative approach will rely on the technique of site specific (directed) mutagenesis, in which the specific residue will be changed to a tryptophan, whose depth could then be determined. An implicit assumption in all these is that the overall conformation of the protein is not affected by labeling or single amino acid exchange.

Thus, in spite of possible complications caused by the probe itself and the complex nature of quenching in membranes, depth analysis utilizing fluorescence quenching represents a powerful tool for investigation of membrane structure. The studies reported here point out the unique advantages of the method and its future potential. These should be reflected in an increasing number of applications of this approach in investigations of membrane structure.

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1. Q: Amarnath Maitra (Delhi Univ., Delhi): In one of your slides you have shown a representative fluorescence curve which appears to me to be a convolution of more than one peak. Since you require an absolute value of fluorescent intensity to determine the distance, do you not think that you are to deconvolute the intensity peak so as to get as accurate intensity as possible?  
A: We do not need an absolute value of fluorescence intensity for depth measurements. What we need is the quenching ratio obtained with two quenchers. Fluorescence spectra of solvated molecules are always broad.
2. Q: David Grainger (Oregon Graduate Inst., Oregon): We have substantial evidence currently in press that these membrane probes occupy molecular areas in monolayers many times that found for phospholipids (150 Å). At levels 30 mol% probe that you report, vesicle lipid phases and protein aggregation states in the vesicle could be much different than that you might imagine. Can you comment on that?  
A: We do not require more than 1 mol% of these fluorophores (such as NDB-labelled lipids) for depth measurements. The perturbation is thus minimal. There is also the problem of extrapolating monolayer results to bilayer systems. Aggregation and/or phase separation of the protein is a real concern. However, although such artifacts will affect absolute values of quenching, they will not alter the quenching ratios (necessary for depth calculation) in any major way.
3. Q: Mary Roberts (Boston College, MA): Do you quench the remaining 70% Trp fluorescence in AcChR if you add an aqueous quencher? What is the protein asymmetry in reconstituted vesicles?  
A: We have been able to apparently quench a lot of the residual fluorescence by using aqueous quenchers like nitrate. However, high concentrations (up to 0.6 M) of quenchers are required for this. At such a high concentration of quencher, inner filter effect is significant due to absorbance of the quencher. When corrections are made for inner filter effect, the quenching is drastically reduced. AchR being an asymmetric shaped protein, 70-90% of the protein is oriented in a right side out manner in the reconstituted system.

4.Q: P. Yager (Univ. of Washington, Seattle, WA): In a case in which the probe is at high concentrations and may perturb the structure of the protein, would fluorescence lifetime measurements be the best?

A: Fluorescence lifetime measurements would definitely help in getting some more information about the fluorophore environments. However, these measurements are necessarily more difficult than steady-state intensity measurements and, in addition, they will not directly yield depth. Lifetimes are known to be unaffected in any major way in case of static quenching.

5.Q: Olaf Andersen (Cornell Univ. Med. College, New York, NY): What is the significance of the value of the depth at which you find the tryptophan?

A: This is a question we also worried about. Does the depth obtained reflect distances measured from the transition dipole moments? In any event, it is difficult to precisely ascertain the distances involved at this level. However, the uncertainty caused by this is comparable to the experimental error.