We believe that a general validation of Schroeder's method requires absolute proof that only outer monolayer *trans*-parinaric acid is quenched and quenched completely by energy transfer to TNBS. We believe that Schroeder can validate his conclusions about tumour cell bilayers by measuring r_∞, the limiting value of the anisotropy at long times following excitation. Because r_∞ is independent of the fluorescence lifetime and because the residual fluorescence in the presence of TNBS labelling probably arises predominantly from the inner monolayer, an elevated r_∞ in the presence of TNBS would provide good evidence for the difference in the mobility of inner and outer monolayer *trans*-parinaric acid probes.

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SCHROEDER AND KINDEN REPLY— We thank Sklar and Doody for their comments and appreciate their concern. It is regrettable that these authors treat a complex biological membrane system, the LM cell plasma membrane, as if it were a model membrane. No experimentally determined values for LM cell membranes were presented. In contrast, we have continued our work in this area and substantiated the use of trinitrophenyl (TNP) groups as fluorescence quenchers and asymmetry determination agents in several systems[1–9]. The erroneous assumptions made in the above critique are as follows.

First, comments (1)–(4) of Sklar and Doody assume the width of the LM cell membrane to be less than 50 Å. In contrast, from micrographs obtained with an RCA EMU-36 electron microscope, we have determined the width of the LM cell plasma membrane to be ~80 Å. Thus, half the width of the membrane would be 40 Å, not 23 Å as proposed by Sklar and Doody. The additional width of the LM cell membrane seems to be due to the presence of proteins. This is especially important because at least 10 times more TNP groups were attached to protein amino substituents than to phospholipids. The fluorophore of *trans*-parinaric acid is about 15 Å from the carboxyl end, similar to the distance of the fluorescent anthroyl group in anthroyl-stearate from its carboxyl end[10,11]. Therefore, quenching across the bilayer would be expected to be less than 0.004 times as efficient as in the outer monolayer. Studies of model systems indicate that there is little energy transfer from one leaflet to the acceptor in the opposite one if R_0 is much less than 40 Å (ref. 12).

Table 1 Fluorescence decay of *trans*-parinarate in LM cell plasma membranes

Cell or membrane treatment	$t_{1/2}$ (ns)	
Untreated membranes	5.9	8.9
Membranes + 100 μM TNP–glycine	5.8	9.1
Pretreatment of whole cells with 4 mM TNBS to trinitrophenylate outer monolayer amino groups[1]	6.0	9.1

$t_{1/2}$ Represents the halftime for decay for each decay component. Plasma membrane vesicles were resuspended in phosphate-buffered saline (100 μg ml^{-1}) and *trans*-parinarate was incorporated as described previously[1]. Trinitrophenylated membranes were prepared also as stated previously[1].

Second, our reported increases in polarization were not due to altered fluorescence lifetime. Evidence obtained with an Ortec 9200 nanosecond spectrometer (courtesy of Dr Lynwood Yarbrough, University of Kansas School of Medicine) showed that the fluorescence lifetime of *trans*-parinarate was not altered. Two decay components in unquenched membranes were observed as shown in Table 1. Addition of 100 μM TNP-glycine to plasma membrane vesicles (100 μg protein per ml phosphate-buffered saline, 24 °C, *p*H 7.4) maximally quenched fluorescence but did not alter the halftimes of decay. TNP-glycine has been used as a non-penetrating quencher in very low-density lipoprotein to quench completely the fluorescence of *trans*-parinarate[3–5]. As shown in Table 1, trinitrophenylated membranes also did not show altered halftimes for *trans*-parinarate fluorescence decay. In all cases of maximal quenching by TNP-glycine or TNP groups attached to the membrane, the fluorescence decay curves were superimposable. In addition, similar data were obtained using a second fluorescence probe molecule, 1,6-diphenyl-1,3,5-hexatriene[2]. Lastly, *trans*-parinarate was incorporated into lipid vesicles made from plasma membrane lipid extracts. The quenching agent TNP-glycine showed no differences in polarization between the inner and outer monolayer. This would be expected because lipid vesicles would have a randomized symmetric distribution of lipid molecules across the bilayer.

Thus, our data in no way support the general photophysical artefact alluded to by Sklar and Doody. Indeed, if the quenching data are artefactual, all three probe molecules (*trans*-parinarate, 1,6-diphenyl-1,3,5-hexatriene and *N*-phenyl-1-naphthylamine) and membranes from cells supplemented with choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine and ethanolamine should have given essentially the same data—increased fluorescence polarization in the presence of quenching agent. This was not the case[2]. *N*-phenyl-1-napthylamine did not show differences in polarization in the presence of quenching agents. *trans*-Parinarate incorporated into plasma membranes from *N,N'*-dimethylethanolamine and *N*-monomethylethanolamine-supplemented cells also did not show differences in polarization in the presence of quenching agents.

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