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# Lateral mobility of plasma membrane lipids in bull spermatozoa: heterogeneity between surface domains and rigidification following cell death

**Shab Ladha[1], Peter S. James[2], David C. Clark[1], Elizabeth A. Howes[2] and Roy Jones[2,*]**

[1]Department of Food Biophysics, Institute of Food Research, Norwich Research Park, Colney Lane, Norwich, NR4 7UA, UK
[2]Laboratory of Sperm Function and Fertilization, Department of Signalling, The Babraham Institute, Babraham, Cambridge, CB2 4AT, UK

*Author for correspondence

## SUMMARY

**Compartmentalization of surface membrane antigens into discrete regions or domains is a characteristic feature of differentiated cells. In mammalian spermatozoa at least 5 surface domains are known, implying the presence of barriers or boundaries within the plasma membrane. Using the technique of fluorescence recovery after photobleaching (FRAP) to measure diffusibility of fluorescent lipid analogues 1,1′-dihexadecyl-3,3,3′3′-tetramethylindocarbocyanine ($DiIC_{16}$) and 5-(N-octa-decanoyl) aminofluorescein (ODAF), we have investigated lipid topology and dynamics in the plasma membrane of ejaculated bull spermatozoa. Contrary to reports in the literature, we have found that $DiIC_{16}$ stains only dead or damaged spermatozoa whereas ODAF intercalates into the plasma membrane of both live and dead cells, each type showing a distinctive staining pattern. FRAP analysis with ODAF revealed that diffusion coefficients on live spermatozoa are significantly faster on the acrosome and postacrosome ($29.3\times10^{-9}$ $cm^2$/second) than on the midpiece and principal piece ($11.8\times10^{-9}$ $cm^2$/second). Recovery (R) is >90% in all domains. ODAF diffusion also shows regionalized temperature-sensitivity with a 4-fold increase over the sperm head and a 1.8-fold increase on the tail between 20°C and 37°C. Remarkably, dead or permeabilized spermatozoa rapidly develop a large immobile phase (R<25%) over the whole plasma membrane. This rigidification is temperature insensitive and irreversible suggesting major changes in the physical state of membrane lipids. It is concluded that lipid diffusion in the plasma membrane of live bull spermatozoa is rapid and varies significantly between surface domains. Following permeabilization or cell death, however, a large immobile phase develops indicating substantial changes in membrane lipid disposition.**

Key words: Spermatazoon, FRAP, Surface domain, Lipid diffusion

## INTRODUCTION

The original Fluid Mosaic Model of membrane structure as proposed by Singer and Nicolson (1972) envisaged proteins and lipids as freely diffusing within a simple bilayer arrangement. Since then, a large amount of experimental work on a variety of cell types has established that asymmetry, both lateral and transverse, is the rule rather than the exception and current concepts of membrane structure centre on microheterogeneity and compartmentalization of components within distinct surface domains (reviews Edidin, 1993; Zachouski, 1993).

The problem of how cells generate asymmetry in their surface membrane and maintain it over relatively large distances (5-10 μm) against the randomizing effects of diffusion is fundamental to understanding many cellular processes, especially those that control differentiation and direct specialized function. The paradigm for many of these studies has been the polarized epithelial cell in which vectoral transport of membrane components ensures non-random insertion into either apical, lateral or basal compartments that is commensurate with their presumed function, e.g. lamin receptors are targeted to the basal membrane and ion channel transporters to the apical membrane (reviewed by Rodriguez-Boulan and Nelson, 1989). This heterogeneity is enforced by the presence of tight junctions that structurally separate apical from lateral plasma membrane domains and act as barriers to diffusion, at least in the exoplasmic face of the bilayer (Dragsten et al., 1981). However, in some terminally differentiated cells like spermatozoa that have a clearly perceived function, the problem of maintaining lateral segregation of antigens is more acute. Spermatozoa are transcriptionally inactive and hence are unable to turn over membrane components at the same rates as somatic cells. Nonetheless, they maintain a dynamic surface membrane that shows considerable heterogeneity (reviews Holt, 1984; Wolf, 1995). All mammalian spermatozoa contain 2 obvious surface macrodomains, head and tail, within which subdomains are present that on the head are known as acrosome, equatorial segment and postacrosome, and on the tail as midpiece and principal piece. The boundaries between these subdomains are established during late spermiogenesis and may be delineated by specialized structures within the plasma membrane, such as the posterior ring at the junction of the head and midpiece and

the annulus between the midpiece and principal piece, or, where such morphological correlates are not apparent, by differences in component lipids (Millette, 1979; Wolf et al., 1986a). The posterior ring and annulus appear as electron dense accretions within and below the bilayer and are regarded as analogous to tight junctions (Myles and Primakoff, 1984). Evidence for co-existent lipid domains is more indirect but fluorescence depolarisation and photobleaching (FRAP) analysis of spermatozoa stained with lipophilic reporter dyes have suggested significant differences in diffusion coefficients between the head and tail (Wolf and Voglmayr, 1984; Wolf et al., 1986b, 1990). Furthermore, fluorescent liposomes of phosphatidylserine (PS) have been found to fuse specifically with the equatorial segment of acrosome-reacted human spermatozoa (Arts et al., 1994). Maintenance of PS within this domain was $Ca^{2+}$-dependent since the addition of EDTA perturbed the boundary thereby allowing PS to diffuse laterally throughout the plasma membrane.

Whatever the nature of the boundaries between domains they are clearly not immutable as lipid and protein antigens can actively migrate across them depending on the developmental status of the spermatozoon. Thus, capacitation of boar spermatozoa in vitro induces redistribution of a sulfogalactolipid from the apical ridge of the acrosome to the equatorial segment (Gadella et al., 1995). Similarly, glycoprotein PH20 on guinea pig spermatozoa migrates from the postacrosomal domain to the inner acrosomal membrane following the acrosome reaction and on rat spermatozoa 2B1 antigen moves from the tail to the acrosomal domain concomitant with capacitation (Myles and Primakoff, 1984; Jones et al., 1990). Likewise, CE9 glycoprotein can breach the annulus and migrate from the principal piece to the midpiece during epididymal maturation (Nehme et al., 1993). Intriguingly, migration of these antigens is in a posterior to anterior direction implying that the barriers have a degree of unidirectionality about them.

In this investigation we have used 2 lipophilic dyes, $DiIC_{16}$ and ODAF, to report on lipid topology and dynamics in bull spermatozoa following FRAP analysis. Our aim has been to elucidate the mechanisms that maintain functional heterogeneity and segregation of membrane components into different surface domains. Contrary to previous reports (Wolf and Voglmayr, 1984) we have not been able to stain live spermatozoa with $DiIC_{16}$; only dead or permeabilized cells incorporate the dye and characteristically do so most strongly over the acrosome and midpiece domains. In contrast, all spermatozoa incorporate ODAF into their plasma membranes, there being a clear distinction between live and dead staining patterns. This difference is crucial for FRAP analysis as live spermatozoa show high recovery (R>90%) in all domains whereas dead spermatozoa develop a large immobile phase (R<25%) that is insensitive to temperature shifts. These results suggest a reappraisal of data on the dynamics of lipids in sperm plasma membranes and provide new insights into domain organisation and lipid boundaries in these highly specialized cells.

## MATERIALS AND METHODS

### Collection and treatment of spermatozoa

Bull semen was collected from 3 Holstein Friesian bulls using an artificial vagina and transported to the laboratory within 3 hours. Care was taken to prevent temperature shock. The bulls were part of an artificial insemination donor herd and were of proven fertility. Sperm motility was assessed by phase contrast microscopy at 23-25°C and their concentration in whole semen estimated using a hemocytometer. Where appropriate, bull spermatozoa were washed twice by centrifugation (500 ***g*** for 10 minutes) and resuspension in 5 vols of a bovine sperm washing medium (BSWM) containing 107.6 mM NaCl, 1.2 mM $MgSO_4$, 4.3 mM KCl, 1.2 mM $K_2HPO_4$, 24.1 mM sodium lactate, 0.5 mM sodium pyruvate, 5.5 mM glucose, 31.5 mM Hepes, 0.5 μg/ml streptomycin sulphate, and 0.6 μg/ml sodium penicillin G. In some experiments BSWM was supplemented with 0.4% BSA. The pH was 7.4 and osmolality 290-300 ml/kg. The integrity of sperm plasma membranes was assessed by staining whole cells with 5 μg/ml propidium iodide (PI) for 10 minutes at 23°C, washing once in BSWM and viewing with u.v. light. PI does not penetrate the plasma membrane of live spermatozoa (which remain unstained) whereas the nuclei of dead or membrane-damaged cells fluoresce red (Harrison and Vickers, 1990). For permeabilization studies, whole semen was diluted 10-fold in BSWM at 37°C and 1 ml dripped slowly into a prechilled glass test-tube on ice. After 5 minutes spermatozoa were reequilibrated to 37°C and the process repeated. Ram semen was collected from fertile rams maintained at the Babraham Institute and processed as described above except that Krebs Ringer-phosphate containing 5 mM glucose (KRPG; Mann, 1964) was substituted for BSWM.

### Labelling of spermatozoa with membrane reporter dyes $DiIC_{16}$ and ODAF

Two fluorescent membrane reporter dyes were used in these investigations: the cationic carbocyanine probe $DiIC_{16}$ (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and the anionic probe ODAF (5-(N-octa-decanoyl) aminofluorescein). Both dyes were obtained from Molecular Probes (Eugene, Oregon). These compounds partition into cell membranes with the fluorophore at the aqueous interface and the alkyl tail(s) embedded in the outer leaflet of the lipid bilayer.

The standard protocol for labelling bull spermatozoa consisted of diluting 10 μl whole semen with 40 μl BSWM and mixing with 50 μl of either 20 μM $DiIC_{16}$ in BSWM/4% (v/v) ethanol or 3.5 μM ODAF in BSWM/1% (v/v) ethanol. The concentration of ODAF was calculated from its extinction coefficient (87,000) in 100% methanol. Samples were incubated for 15 minutes at 23°C, spermatozoa washed twice by centrifugation (400 ***g*** for 5 minutes) with 0.5 ml BSWM and then resuspended to 50 μl in the same medium. Motility was checked by phase contrast microscopy and incorporation of reporter dye by epifluorescence illumination. Prior to FRAP analysis spermatozoa were immobilized by adding sodium azide to 0.2%. In other experiments, bull spermatozoa were washed twice in BSWM alone or BSWM + 0.4% (w/v) BSA before and after loading with reporter dyes. Ram spermatozoa were treated similarly except that whole semen was first diluted 1:10 in seminal plasma to reduce sperm concentration to approximately that found in bull semen (~$10^8$/ml) and KRPG was substituted for BSWM.

### FRAP analysis

Mobility of reporter dyes in the sperm plasma membrane was measured by FRAP analysis. The apparatus and arrangement of lasers has been described in detail in a previous paper (Ladha et al., 1994). It is essentially similar to that reported by Bloom and Webb (1983) with the important modification that the microscope (Nikon Optiphot) was equipped with 2 epi-illumination attachments to allow fluorescence illumination from 2 separate light sources. This enabled spermatozoa to be assessed under full-field fluorescence before subjecting them to point illumination for photobleaching experiments. ODAF was excited at 488 nm and $DiIC_{16}$ at 514 nm using a laser beam of Gaussian cross sectional intensity. The half-width at $1/e^2$ height of the laser beam at its point of focus was equal to 1.24 μm spot radius. The

beam was generated by a water-cooled argon ion laser and bleaching times were 5 milliseconds at 0.2 mW. Data points were collected every 5 milliseconds. On the sperm head the laser spot was sufficiently smaller than the total area available to allow recovery in 2 dimensions. Therefore, data from acrosome and postacrosome were analysed by non-linear least squares fitting to an equation defining the time dependence of fluorescence recovery seen with a beam of Gaussian cross-sectional intensity (Yguerabide et al., 1982; Ladha et al., 1994). However, on the midpiece and principal piece regions the diameter of the laser spot is equal to or greater than that of the flagellum. This only allows for recovery in 1 dimension as the situation represents photobleach recovery of a uniformly irradiated band on an infinite cylinder. Therefore, FRAP data from the midpiece and principal piece were analysed by non-linear least squares fitting to an expression defining the time dependence of fluorescence recovery $f(t)$ observed with a circular beam of uniform cross sectional intensity and had the form:

$$f(t) = \frac{F(t)-F(o)}{F(\infty)-F(o)} = \left(\frac{t}{\pi\tau_D}\right)^{\frac{1}{2}}(1-e^{-(\tau_D/t)}) + erfc\left(\left(\frac{\tau_D}{t}\right)^{\frac{1}{2}}\right);$$

where $F(t)$ is the observed fluorescence as a function of time, $F(o)$ is the intensity of the fluorescence immediately after the bleach pulse, $F(\infty)$ is the fluorescence at infinite time after the bleach pulse, $\tau_D$ is the characteristic diffusion time, $t$ is time and *erfc* is the complementary error function. This expression is a more accurate version of that described originally by Wolf (1991). The proportion of dye molecules free to diffuse within the membrane is expressed as the percentage recovery of the initial fluorescence level (R) and the diffusion coefficient (D) of the dye is calculated from the characteristic recovery time determined from the computed fit to the data curve (Ladha et al., 1994).

Samples of stained spermatozoa were drawn by capillary action into microslides (50 μm path length, rectangular cross-section; Camlab, Cambridge, UK), sealed with Critoseal (Hawksley, UK) and secured on a temperature-controlled microscope warm-stage. Spermatozoa orientated at 90° to the viewing axis were selected using full-field fluorescence and FRAP analysis performed on 4 surface domains; the acrosome, postacrosome, midpiece and principal piece of the tail. In preliminary experiments we investigated if the position of the photobleaching beam within a domain was important for consistency of response. For this purpose, three separate locations within the acrosome, postacrosome and midpiece were photobleached on 10 individual bull spermatozoa labelled with ODAF. Results showed that D values recorded within a domain were not significantly different from each other (data not shown). Thus, small errors in positioning of the laser beam should not affect results. However, to ensure as far as practicably possible that photobleached regions were comparable from one spermatozoon to another, a stereotyped procedure was adopted. The attenuated beam was first aligned on the centre of the anterior edge of the head and then moved posteriorly to the centre of the acrosome where a spot was photobleached. Next, the beam was focused on the neck region and moved anteriorly to the centre of the postacrosome or posteriorly to the centre of the midpiece. Lastly, the beam was moved down the tail beyond the midpiece into the principal piece where a fourth spot was photobleached. Single fluorescence recovery curves were collected for each region on at least 10 spermatozoa in each experiment and data curves summed and averaged prior to analysis. All experiments were carried out at 20°C unless stated otherwise and slides were equilibrated on the warm-stage for 5 minutes before recordings began. Data were collected for blocks of 10 spermatozoa in each experimental treatment over 40-50 minutes.

### Statistical analysis

Statistical treatment of data was carried out using analysis of variance and Student's *t*-test set to a matrix system. The level of significance was set at $<0.05$.

### Photomicroscopy

Fluorescence photomicrographs were taken on a Zeiss Axiophot photomicroscope with a 100 W mercury vapour lamp and a ×63 Plan phase objective lens with oil immersion. Colour photographs were taken on Kodak Ektar 1000 film.

## RESULTS

### Whole cell fluorescence on spermatozoa labelled with $DiIC_{16}$ and ODAF

When unwashed bull spermatozoa showing good progressive motility (>70% active) were incubated in BSWM containing 10% seminal plasma, 10 μM $DiIC_{16}$ and 2% ethanol, only 13.4% incorporated the dye into their membranes in the form of weak punctate fluorescence along the tail or on the acrosome (Fig. 1A, Table 1). Small particles of dye reagent were invariably in direct contact with these spermatozoa and appeared as intense pin-points of fluorescence so that on the sperm tail they frequently assumed a 'beads-on-a-string' appearance. Washing spermatozoa in BSWM increased the number of dye particles associated with spermatozoa but their distribution was highly irregular and <10% of cells were stained. Permeabilizing spermatozoa by cold shock, increasing the concentration of $DiIC_{16}$ to 20 μM or incubating at 34°C during labelling did not change the proportion of fluorescent cells significantly (Table 1). However, washing spermatozoa in BSWM containing 0.4% BSA had a noticeable effect on the staining pattern of those

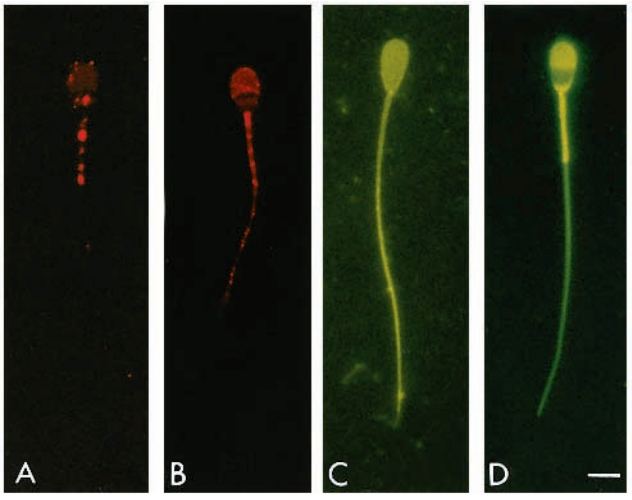


**Fig. 1.** Fluorescence staining of live and dead bull spermatozoa with $DiIC_{16}$ and ODAF. (A) Typical punctate incorporation of $DiIC_{16}$ over the head and midpiece domains of a dead spermatozoon after washing in BSWM. (B) Regionalized staining of a dead spermatozoon with $DiIC_{16}$ after washing in BSWM + 0.4% BSA. Note the strong fluorescence over the acrosome with a punctate pattern down the tail. This spermatozoon, and the one shown in A, are PI-positive. (C) Uniform incorporation of ODAF into a spermatozoon labelled in BSWM + 10% seminal plasma. Such spermatozoa are motile and PI-negative. This is the classic 'live-pattern' referred to in the text. (D) Regionalized incorporation of ODAF into a dead or permeabilized spermatozoon, the classic 'damaged, pattern'. Note the stronger fluorescence over the acrosome and midpiece domains than on the postacrosome and principal piece domains. Bar, 5 μm.

cells that incorporated the dye. The whole spermatozoon showed irregular fluorescence, typically with stronger staining on the acrosome and midpiece than the postacrosome or principal piece (Fig. 1B). Significantly, all of the spermatozoa that incorporated $DiIC_{16}$, irrespective of the labelling protocol and staining pattern, were immotile. Motile spermatozoa (motility varied from 30%-60% after washing depending on the ejaculate) did not stain with $DiIC_{16}$.

These results were unexpected and are inconsistent with the work of Wolf and collaborators (Wolf and Voglmayr, 1984; Wolf et al., 1986b) who reported labelling of mouse and ram spermatozoa with $DiIC_{16}$ sufficient for FRAP analysis of their plasma membranes. Since the differences cannot be explained on the basis of the type of dye or supplier (Molecular Probes in each case), we then followed the staining protocol described by Wolf and Voglmayr (1984) for ram spermatozoa. For this purpose, bull semen was washed 3 times by dilution in 5 vols of KRPG and centrifuging at 500 ***g*** for 8 minutes. A sample containing ~$10^6$ spermatozoa was diluted into 200 μl of KRPG containing 0.4% BSA, 4.5 μM $DiIC_{16}$ and 0.5% ethanol and incubated at 34°C for 8 minutes. Cells were washed free of unbound dye by centrifuging (500 ***g*** for 10 minutes) through a layer of 10% BSA in KRPG and resuspending in KRPG plus 0.4% BSA. This procedure increased the proportion of fluorescent spermatozoa to between 15% and 35% but, again, they were immotile and typically stained more strongly on the acrosome and midpiece than on the postacrosome and principal piece of the tail (similar to Fig. 1B). Staining was also uneven within a domain with brighter patches against a diffuse background. Because labelling was clearly not homogenous it was not possible to perform FRAP analysis on these spermatozoa.

To investigate if bull spermatozoa are unusual in their inability to incorporate $DiIC_{16}$, ram spermatozoa were incubated with 10 μM $DiIC_{16}$ using the same protocols. In the presence of 10% seminal plasma, <7% of spermatozoa incorporated the dye (Table 1), usually in the form of punctate particles of dye irregularly distributed over the head. After washing in KRPG the proportion of fluorescent spermatozoa increased to 14% with variable labelling patterns ranging from punctate on the head or tail or whole cell. All of these spermatozoa were immotile. Cold shocking washed spermatozoa increased the percentage of labelled cells to 79%, all showing stronger fluorescence on the acrosome and midpiece than the postacrosome or tail. If spermatozoa were washed in KRPG containing 0.4% BSA then motility was better preserved and only 15% of cells incorporated the dye. These stained spermatozoa were consistently immotile and showed strong uniform fluorescence of greater intensity on the acrosome and midpiece than the postacrosome and tail. Live motile spermatozoa, however, remained unlabelled. Similar results were obtained if ram spermatozoa were labelled and washed through 10% BSA in KRPG as described by Wolf and Voglmayr (1984).

In contrast to the above, 100% of bull and ram spermatozoa incorporated ODAF into their plasma membranes, the majority (~90%) showing dull uniform fluorescence over their entire surface (Table 1; Fig. 1C). These uniformly-fluorescent spermatozoa were vigorously motile and did not label with PI. Those cells that were PI-positive were immotile and displayed characteristically strong fluorescence over the acrosome and midpiece domains with a weak signal on the postacrosome and principal piece of the tail (Fig. 1D). Washing or cold shocking spermatozoa produced a progressive increase in the percentage showing regionalized labelling with a concomitant rise in the proportion that were PI-positive (Table 1).

We interpret these findings to mean that only dead or membrane permeabilized bull and ram spermatozoa incorporate $DiIC_{16}$ and they do so in an uneven and regionalized manner. In the absence of 0.4% BSA, dye particles associate with the membrane to produce a punctate labelling pattern

**Table 1. Uptake of $DiIC_{16}$ and ODAF by bull and ram spermatozoa**

| Species | Treatment | Probe | Unlabelled (%) | Labelled | | PI positive* (%) |
|---|---|---|---|---|---|---|
| | | | | Uniform (%) | Regionalized (%) | |
| Bull | Unwashed | $DiIC_{16}$ | 86.6±3.0 | 0.0 | 13.4±3.0 | – |
| | Washed BSWM | ,, | 90.4±3.0 | 0.0 | 9.6±3.0 | – |
| | + cold shock | ,, | 86.5±3.8 | 0.0 | 14.0±3.8 | – |
| | + 0.4% BSA | ,, | 83.6±3.0 | 0.0 | 16.4±3.0 | – |
| | Unwashed | ODAF | 0.0 | 91.1±2.7 | 9.9±2.7 | 14.5±1.8 |
| | Washed BSWM | ,, | 0.0 | 89.8±1.2 | 11.2±1.2 | 19.6±2.2 |
| | + cold shock | ,, | 0.0 | 12.1±3.3† | 87.9±3.3† | 91.1±1.2† |
| | + 0.4% BSA | ,, | 0.0 | 92.5±2.5 | 7.5±2.5 | 18.5±1.8 |
| Ram | Unwashed | $DiIC_{16}$ | 93.4±1.6 | 0.0 | 6.6±1.6 | – |
| | Washed KRPG | ,, | 83.8±2.2‡ | 0.0 | 14.2±2.2‡ | – |
| | + cold shock | ,, | 20.2±4.1† | 0.0 | 79.8±4.1† | – |
| | + 0.4% BSA | ,, | 84.4±2.6‡ | 0.0 | 15.6±2.6‡ | – |
| | Unwashed | ODAF | 0.0 | 85.6±1.8 | 14.4±1.8 | 16.9±0.8 |
| | Washed KRPG | ,, | 0.0 | 90.4±0.9 | 9.6±0.9 | 18.3±1.7 |
| | + cold shock | ,, | 0.0 | 17.2±2.3† | 82.8±2.3† | 98.1±0.2† |
| | 0.4% BSA | ,, | 0.0 | 88.0±1.8 | 12.0±1.8 | 13.3±0.6‡ |

Values are means ± s.e.m. of 5 separate ejaculates.
*The data apply to $DiIC_{16}$ as well as ODAF stained samples.
Significance is relative to unwashed control samples: †$P<0.001$, ‡$P<0.05$.

whereas, in the presence of 0.4% BSA, labelling is more uniform and consistently stronger over the acrosome and midpiece than the postacrosome or principal piece of the tail. Live motile spermatozoa do not incorporate $DiIC_{16}$ into their plasma membrane using any of the protocols described. ODAF on the other hand, intercalates into the plasma membrane of all spermatozoa, live and dead, irrespective of the labelling protocol. Live motile cells display a uniformly distributed fluorescent signal of equal intensity over all surface domains (henceforth referred to as the 'live-pattern'; Fig. 1C) whereas damaged or dead spermatozoa show intense staining on the acrosome and midpiece relative to the postacrosome and principal piece (henceforth referred to as the 'damaged-pattern'; Fig. 1D). In subsequent experiments, therefore, ODAF was used as a reporter dye for FRAP analysis of lipid diffusibility in different surface domains of ejaculated bull spermatozoa. The second epi-illumination attachment on the microscope enabled spermatozoa to be classified as live-pattern or damaged-pattern before photobleaching.

### FRAP analysis of ODAF diffusion in bull sperm plasma membranes

Spermatozoa with a live-pattern of staining with ODAF showed very high recovery of fluorescence after photobleaching over the acrosome, postacrosome, midpiece and principal piece (R=89%-94%; Table 2 and Fig. 2). However, D values for the same cells varied significantly between domains, being highest over the acrosome ($29.3\pm1.5\times10^{-9}$ $cm^2$/second) and lowest on the principal piece ($11.8\pm2.6\times10^{-9}$ $cm^2$/second; Table 2). In contrast, spermatozoa exhibiting a damaged-pattern of labelling gave very low and variable recovery values after photobleaching to the extent that calculation of D values became unreliable since the SEM was very large. Fig. 2 illustrates representative recovery curves for a typical live-pattern spermatozoon relative to a damaged-pattern spermatozoon. The latter spermatozoa represent the 10-12% of dead or dying cells normally found in fresh bull semen. To reproduce this effect under more controlled conditions, spermatozoa were deliberately permeabilized by cold shock to induce a high incidence of damaged-pattern labelling (Table 1). FRAP analysis of these cells again revealed a very high immobile fraction on the acrosomal and postacrosomal domains to the extent that, for reasons given earlier, accurate D values could not be calculated (Table 2). On the midpiece and principal piece domains, however, intermediate recoveries of 40% and 68%, respectively, were obtained giving calculated D values of $9.4\times10^{-9}$ $cm^2$/second and $6.9\times10^{-9}$ $cm^2$/second, respectively. This was a further indication that the plasma membrane over the sperm tail behaves differently to that on the head.

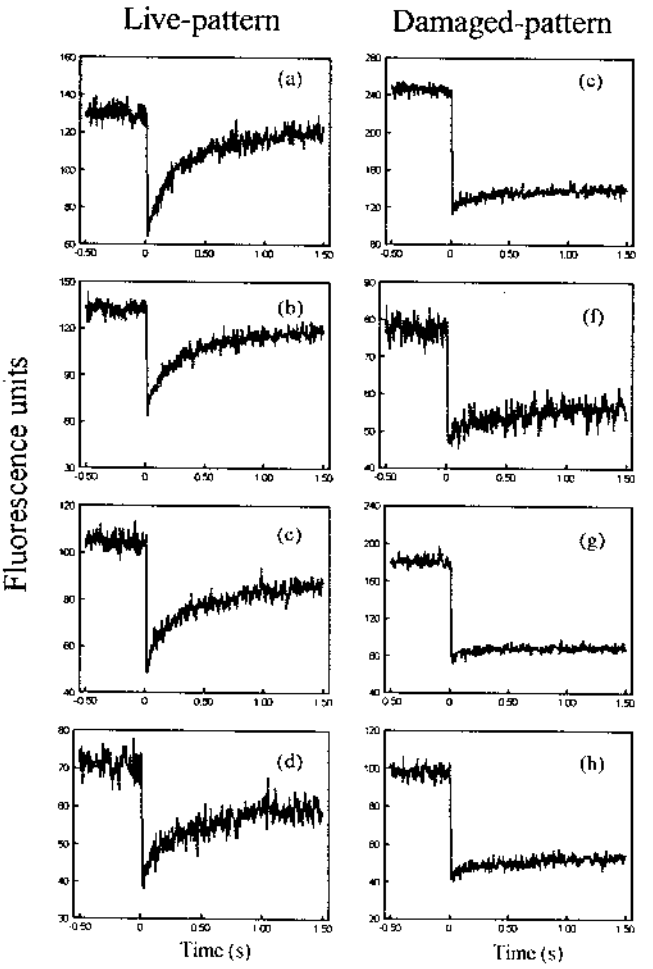


**Fig. 2.** Typical FRAP curves from live-pattern and damaged-pattern bull spermatozoa stained with ODAF. (a and e) Acrosome. (b and f) Postacrosome. (c and g) Midpiece. (d and h) Principal piece. Time '0' in each graph represents the bleach point.

Since ODAF is a single conjugated fatty acid and may behave differently to phospholipids in the plasma membrane, preliminary measurements were also made on the diffusion of fluorescein-conjugated phosphatidylethanolamine (F-PE) in live and dead spermatozoa. Using the same labelling protocols as described for ODAF, highly purified F-PE (Wall et al., 1995) stained live motile spermatozoa uniformly all over the plasma

**Table 2. ODAF diffusion in different surface domains of live- and damaged-pattern bull spermatozoa**

| | Live-pattern | | Damaged-pattern | | Cold shocked | |
|---|---|---|---|---|---|---|
| Surface domain | D ($\times10^{-9}$ $cm^2$/second) | % R | D ($\times10^{-9}$ $cm^2$/second) | % R | D ($\times10^{-9}$ $cm^2$/second) | % R |
| Acrosome | 29.3±1.5 | 94.4±0.7 (171) | NM | <10.0 (10) | NM | <10 (10) |
| Postacrosome | 20.3±1.7 | 90.5±2.1 (150) | NM | <10.0 (10) | NM | <10 (10) |
| Midpiece | 13.3±1.2 | 90.0±1.7 (124) | NM | <10.0 (10) | 9.4±1.3 | 40.6±0.4 (10) |
| Principal piece | 11.8±2.6 | 89.6±2.6 (83) | NM | <10.0 (10) | 6.9±0.8 | 68.4±0.9 (10) |

Values are means ± s.e.m.

Figures in parentheses represent the number of spermatozoa measured.

Significance tests were assessed between different surface domains of live-pattern spermatozoa only. For D values: acrosome vs postacrosome, $P<0.001$; postacrosome vs midpiece, $P<0.001$; midpiece vs principal piece, not significant. For % R: all interactions not significant. NM, non mobile.

membrane (i.e. similar to live-pattern ODAF stained cells) and gave D values between 3.0 and $10.0\times10^{-9}$ $cm^2$/second with >60% recovery. Permeabilized and dead spermatozoa on the other hand, stained more intensely on the acrosome and midpiece domains and showed no recovery in any region after photobleaching (C. Golding, P. James and S. Ladha, unpublished observations). Thus, ODAF and F-PE behave similarly in sperm plasma membranes and the observed differences between live and dead cells are not dependent on the nature of the probe.

### Effects of temperature on lateral diffusion of ODAF in bull spermatozoa

To eliminate the possibility that ODAF was associating non-specifically with the sperm surface (e.g. electrostatically) rather than reporting the dynamics of the lipid environment within the membrane, we next investigated the effects of temperature on D values and %R in different surface domains. Temperature is well known to influence the phase disposition of lipids in bilayer structures with profound consequences for membrane fluidity that should be reflected in FRAP data. Conversely, if the probe is bound by ionic interactions to the bilayer surface rather than penetrating into it, its behaviour would be less temperature sensitive. As shown in Fig. 3, lowering the temperature to 5°C significantly reduced D values on live-pattern spermatozoa in the acrosomal, postacrosomal and midpiece domains relative to those at 20°C. At 37°C the acrosomal and postacrosomal membranes showed an ~4-fold increase in D relative to 20°C whereas the midpiece domain was substantially less responsive (1.8-fold increase). Fluorescence recovery was high (>90%) in all regions over the entire temperature range with the exception of the midpiece where it fell to 67% at 5°C. These temperature effects on D are similar to those found in human erythrocytes labelled with ODAF. Values of $7.0\times10^{-9}$ $cm^2$/second and $31.0\times10^{-9}$ $cm^2$/second were obtained at 5°C and 37°C, respectively, representing a 4.4-fold increase (S. Ladha, unpublished results). Although this is somewhat less than that reported by Bloom and Webb (1983) for mouse erythrocytes (~20-fold increase between 5°C and 37°C) it is clear that diffusion of ODAF in the plasma membrane of live spermatozoa behaves in a manner consistent with the known response of lipids to temperature shifts and that the results obtained earlier cannot be explained on the basis of an ionic interaction between the dye and the ectodomain of the plasma membrane.

Damaged-pattern spermatozoa were also subjected to photobleaching at 5°C and 37°C. However, neither temperature altered the kinetics of recovery of the large immobile phase which again remained too low and variable to calculate accurate D values (data not shown).

### Can ODAF redistribute spontaneously in the plasma membrane during transition from live-pattern to damaged-pattern?

The ability to distinguish live- and damaged-pattern spermatozoa labelled with ODAF allowed us to ask if the former could give rise to the latter by spontaneous redistribution of the probe. The strong fluorescence from the acrosomal and midpiece domains of permeabilized cells labelled in suspension could be caused by uptake of ODAF into the outer acrosomal and mitochondrial membranes as well as the overlying plasma membrane. Alternatively, ODAF may relocate laterally within the plane of the membrane to produce a regionalized pattern as the cell dies. To investigate this phenomenon, bull spermatozoa were labelled with ODAF in 10% seminal plasma, washed into BSWM + 0.4% BSA and incubated at room temperature in the dark. At different time intervals aliquants were withdrawn and examined by full-field u.v. illumination. Results showed that at time 0 minutes the incidence of live-pattern spermatozoa was 88%, damaged-pattern 7% and indeterminate pattern 5%. After 3 hours classic live-patterns had decreased to <2% and a different form appeared showing uniform fluorescence on the head and principal piece but with strong labelling on the midpiece (referred to as 'bright-midpiece' pattern). Bright-midpiece pattern spermatozoa were motile and comprised 86% after 3 hours with 12% showing classic damaged-patterns. These proportions did not change significantly over the next 24 hours. Unlabelled control samples were incubated in parallel to the above and loaded with ODAF at times 0 and 24 hours in the usual way. These samples contained ~85% live-pattern and ~10%-15% damaged-pattern spermatozoa after 24 hours incubation but bright-midpiece patterns never rose above ~10%. Double labelling with PI revealed that bright-midpiece pattern spermatozoa had intact plasma membranes whereas those showing damaged-patterns were permeabilized (results not shown). It would seem, therefore, that in spermatozoa pre-labelled with ODAF a redistribution of the dye onto the acrosomal domain does not take place as cells degenerate and that the bright-midpiece pattern may be artifactual, reflecting a localized effect of mitochondrial metabolism on fluorescence intensity in the overlying plasma membrane rather than a real increase in the concentration of ODAF molecules in the midpiece domain.

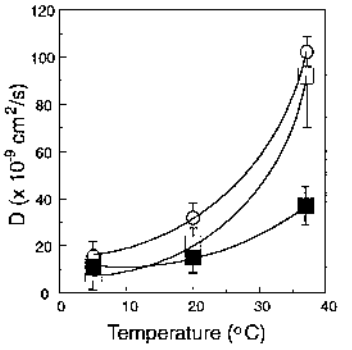


**Fig. 3.** Effects of temperature on lateral diffusion coefficients of ODAF in live-pattern bull spermatozoa. ODAF diffusion is more temperature-responsive on the acrosome (○) and postacrosome (□) than on the midpiece (■). Values are means ± s.e.m. of 3 separate experiments.

## DISCUSSION

In this study we have used FRAP analysis to investigate lipid topology and dynamics in the plasma membrane of bull spermatozoa. Results show that lipid diffusion differs significantly between the head and tail domains of live motile spermatozoa but following permeabilisation or cell death the plasma

membrane undergoes extensive rigidification to the extent that a very large (~70%) immobile phase develops in all regions. This suggests major changes in the physical state of component lipids coincident with the loss of membrane integrity.

### Validation of ODAF and $DiIC_{16}$ as membrane reporter probes for FRAP analysis

$DiIC_{16}$ has been widely used to measure lipid diffusibility in cell membranes, and hence its failure to label live bull spermatozoa was unexpected. However, it would appear that this is not, in fact, unusual. Neuroectoderm cells from *Pleorodeles waltl* embryos (Dupou et al., 1987), eggs from *Xenopus laevis* (Dictus et al., 1984), spermatozoa from the spiny starfish (Sase et al., 1995) and cultured bovine endothelial cells (Tournier et al., 1989) have either not incorporated $DiIC_{16}$ at all or been labelled to a very limited and variable extent. Our results, therefore, are consistent with these findings but contrast with those of Wolf and Voglmayr (1984) and Wolf et al. (1986b) who used $DiIC_{16}$ to stain ram and mouse spermatozoa prior to FRAP analysis. These workers reported regionalized incorporation of the dye into the acrosomal and midpiece domains. We have observed similar labelling patterns on dead and permeabilized bull and ram spermatozoa but not on live motile cells. Furthermore, uptake of $DiIC_{16}$ into dead spermatozoa is enhanced by BSA. One explanation for this effect is that BSA binds the fatty acid moiety of $DiIC_{16}$ and acts as a carrier for lipid exchange with the membrane. Alternatively, $DiIC_{16}$ may remain bound to BSA which itself is known to associate non-specifically and intimately with sperm plasma membranes (Dow and Bavister, 1989). Whatever the mechanism, FRAP measurements on dead or permeabilized spermatozoa stained with $DiIC_{16}$ cannot be regarded as reflecting real-time lipid dynamics in fully intact and functional cells.

For this reason we have focused on ODAF as a reporter dye for FRAP analysis, its advantages being: (a) it is incorporated homogenously into the plasma membrane overlying all domains of live motile spermatozoa and is independent of the presence of BSA in the medium; (b) it labels live and dead spermatozoa differentially thereby enabling them to be distinguished from each other before FRAP analysis; (c) D measurements on live spermatozoa respond in predictable directions to shifts in temperature; (d) ODAF and F-PE behave in a similar fashion on live and dead spermatozoa. It is reasonable to presume, therefore, that the kinetics of diffusion of ODAF in cell membranes are an accurate reflection of lipid organisation and dynamics.

The simplest interpretation of the dead-pattern staining obtained with ODAF is that it represents incorporation of dye into the outer acrosomal and mitochondrial membranes accessed through holes in the overlying plasma membranes. Regionalized staining cannot be explained by lateral redistribution of ODAF within the plane of the membrane as the cell loses its viability. The bright midpiece-pattern that appears on spermatozoa after they have been pre-labelled with ODAF followed by cold shocking or ageing in vitro may be related to the pH dependence of the dye's fluorescence emission. Proton displacement from the surface membrane overlying the midpiece (e.g. by $Ca^{2+}$ released from internal stores of calmodulin) would cause a localized rise in pH to which the fluorescent head groups on ODAF would respond with a stronger signal.

A more difficult question to answer is why $DiIC_{16}$ stains only dead or permeabilized spermatozoa and not live ones. $DiIC_{16}$ is cationic and might be expected to interact electrostatically with the net negative change on live spermatozoa. That it does not do so, even in the presence of BSA, and yet incorporates into the membrane of permeabilized spermatozoa indicates that far-reaching changes in surface potential and lipid disposition must accompany cell death (see later).

### Lipid domains in sperm plasma membranes

It is well known that lipids in membranes are less constrained than component glycoproteins and hence have faster diffusion coefficients (D for lipids = $10^{-7}$ to $10^{-8}$ $cm^2$/second versus D for glycoproteins = $10^{-9}$ to $10^{-11}$ $cm^2$/second). However, lipids still show complex interactions with each other and with neighbouring glycoproteins and consequently they have slower diffusion rates than in single- or multicomponent liposomes. As a result of a large number of investigations using electron spin resonance, fluorescence energy transfer and FRAP analysis there is now good evidence for lipid heterogeneity and domain formation in cell membranes (Zhang et al., 1991; Welti and Glaser, 1994; Mouritsen and Jørgensen, 1995). These domains may extend over a range of time and length scales, varying from a few nanoseconds to hours and from tens of molecules to the whole cell surface. In *Xenopus* eggs (diameter ~1 mm) labelled with HEDAF for example, D in the animal pole was $1.5\times10^{-8}$ $cm^2$/second whereas in the vegetal pole it was $7.6\times10^{-8}$ $cm^2$/second (Dictus et al., 1984). After fertilization, this discrepancy increased 100-fold. At the micrometre scale, significant differences have been found between the apical and basal plasma membranes of vascular endothelial cells (Tournier et al., 1989) and in focal versus non-focal contact points of cultured chicken fibroblasts (Geiger et al., 1982). Microheterogeneity is also present at the nanometre level, e.g. for 'collar' lipids surrounding transmembrane glycoproteins (Mouritsen and Jørgensen, 1992). The significant decrease in D from the acrosome to the principal piece of live bull spermatozoa stained with ODAF, therefore, is entirely consistent with the concept of large regionalized lipid domains. Bull spermatozoa are not unique in this respect as we have obtained similar results with ram, boar and mouse spermatozoa (results not shown). Our data, however, contrast with those reported by Wolf and co-workers (Wolf, 1995) who found faster diffusion over the midpiece than the acrosome of ram and mouse spermatozoa stained with $DiIC_{16}$. This discrepancy may reflect different properties of the probes ($DiIC_{16}$ has two fatty acyl chains to ODAF's one) or, more likely, is due to a failure to distinguish between live and dead spermatozoa as suggested earlier. In this respect, it is noteworthy that the midpiece and principal piece domains are less perturbed by cold shock than the acrosomal domain, a finding consistent with long-standing observations that the acrosome is more sensitive to stress than other regions of the spermatozoon and that fertilizing capacity usually declines before motility (Mann, 1964). Whatever the reason, a major barrier to lipid diffusion must exist between the head and tail macrodomains.

One candidate for such a barrier is the posterior ring which represents a circumferential line of fusion between the nuclear envelope and the overlying plasma membrane adjacent to the basal plate. Functionally, the posterior ring could behave like a tight junction to prevent free diffusion between compartments

as found in epithelial cells (Rodriguez-Boulan and Nelson, 1989). Other mechanisms for generating lipid domains (and hence diffusion boundaries) include selective coupling to the cytoskeleton via integral membrane proteins, thermodynamic phase separations brought about by intrinsic properties of the lipids and out of plane fluctuations caused by compositional heterogeneity (reviewed by Mouritsen and Jørgensen, 1992). The latter point is of major relevance to spermatozoa since their lipids are highly unsaturated (~60% of phospholipid-bound fatty acids consist of 22:5 and 22:6), complex (~45% are ether-linked plasmalogens) and generally have low cholesterol (range 26 mol% to 45 mol%; Poulos et al., 1973; Parks et al., 1987). It is not known if there is unequal distribution of one or several classes of phospholipids between the sperm head and tail but the regionalized effects of temperature on D (Fig. 3) and the differential recovery observed after cold shock (Table 2) suggest that this may be the case. If so, then it would exacerbate thermodynamically-driven phase separations of the kind that have been observed in uni- and multilamellar liposomes (Jain, 1983; Pedersen et al., 1996). Perhaps the most direct evidence for the coexistence of dynamic fluid and gel phase lipids in sperm membranes (ram and boar) is provided by differential scanning calorimetry which has detected 2 endothermic transition temperatures at 26°C and 60°C (Wolf et al., 1990; Canvin and Buhr, 1989). Human spermatozoa, however, appear to be different in this respect as Laurdan fluorescence spectroscopy failed to detect any thermotropic transitions between 10°C and 42°C indicating that in this species the plasma membrane is in a single liquid-crystalline phase throughout (Palleschi and Silvestroni, 1996). This may be related to their higher content of cholesterol (>50 mol%; Drobnis et al., 1993) the modulating effects of which are complex. In large amounts (>30 mol%) cholesterol 'seals' membranes by suppressing density fluctuations whereas in low amounts (~10 mol%) it has the opposite effect and enhances heterogeneity (Cruzeiro-Hansson et al., 1989). Cholesterol-rich and cholesterol-poor microdomains are present in plasma membranes (Schroeder et al., 1991) so the potential for localized variations in fluidity and interface formation are considerable. Overall, therefore, ample evidence exists for the presence of micro and macro lipid domains in cell membranes that would contribute significantly to generating and maintaining lateral heterogeneity. Such concepts would explain many of our present observations on the differential diffusion of ODAF between the head and tail of live bull spermatozoa.

### Origin of immobile lipids in bull sperm plasma membranes

One of the most striking features of our results is the sudden appearance of a large (>70%) immobile lipid fraction in all surface domains concomitant with loss of membrane integrity. This rigidification is rapid, irreversible and remarkably stable between 5°C and 37°C. It is not unusual for membrane glycoproteins to show a substantial immobile fraction that can be explained on the basis of cross-linking to cytoskeletal structures, percolative diffusion and directed transport (Zhang et al., 1993). Immobile lipids, however, are less common and have been found mainly in specialized intracellular organelles and in cells undergoing differentiation (Dictus et al., 1984; Metcalf et al., 1986). One potential complication with spermatozoa is that in permeabilized or dead cells the ODAF probe has access to intracellular membranes (e.g. acrosomal membranes) and FRAP data, therefore, will represent a composite of different membrane types. Evidence, however, indicates that intracellular membranes in spermatozoa are highly fluid. Cowan et al. (1987) obtained 85% recovery for $DiIC_{14}$ and a D of $5.0\times10^{-9}$ $cm^2$/second on the inner acrosomal membrane of guinea pig spermatozoa and we have obtained similar values with ODAF (data not shown). In addition, immobile lipid appears over the whole surface of spermatozoa following permeabilization irrespective of whether juxtaposed intracellular membranes are present or not, e.g. on the principal piece. Thus, lack of measurable D values in dead sperm membranes cannot be explained solely on the basis that ODAF is labelling different membrane types.

Other possible explanations for the rigidification phenomenon are: (a) cross-linking of negatively charged phospholipids caused by an influx of $Ca^{2+}$ ions. $Ca^{2+}$ has been shown to form salt bridges between polar head groups of phosphatidylserine and induce microaggregation (Ohnishi and Ito, 1974). These cross-linked lipid 'blocks', however, can be dispersed by increasing temperature whereas no such response could be detected in the sperm membrane; (b) fatty acyl chain shortening, cross-linking and decomposition induced by oxygen free radicals (Johansson and Magnusson, 1990; Block, 1991). Given the unusual susceptibility of sperm phospholipids to peroxidative damage (Jones and Mann, 1976; Aitken and Clarksen, 1987; Alvarez and Storey, 1989), extensive cross-linking of unsaturated acyl chains could conceivably cause rigidification to the extent observed here. Oxygen free radicals, however, have paradoxical effects on membrane fluidity (Watanabe et al., 1990) and further work is required to assess their contribution to this phenomenon; (c) formation of non-lamellar hexagonal-II phase lipid structures (Quinn, 1985). Little is known about the behaviour of plasmalogens in cell membranes or liposomes although some phospholipids (e.g. cardiolipin) can form non-bilayer structures depending on the pH, salt concentration and acyl chain length (Sneddon et al., 1983). These hexagonal-II phase arrangements can be remarkably stable but their presence in the plasma membrane of spermatozoa, alive or dead, must await the application of appropriate detection systems.

In conclusion, therefore, whilst acknowledging the unusual nature of lipids in the sperm plasma membrane, elucidating their sudden transition from a highly fluid phase to a rigid immobile phase following permeabilization has wide-ranging implications for understanding lipid dynamics in many other cell systems.

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