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Heterogeneity of milk fat globule membrane structure and composition as observed using fluorescence microscopy techniques

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ABSTRACT

Fluorescence microscopy studies of fat globules in milk and milk products using fat-soluble stains (Nile Blue or Nile Red) allow visualisation of the neutral fat (core) of the globules, but provide no information about the milk fat globule membrane (MFGM). We applied the lipophilic probes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid ($DiIC_{18}$(3)-DS) and *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64), as well as fluorescent conjugates of the lectin wheat germ agglutinin (WGA488, WGA594 and WGA647), to milk to stain specifically the MFGM in its native environment. Using fluorescence microscopy, we observed various distribution patterns of the probes. This demonstrates that the MFGM of fat globules in harvested milk is structurally and chemically heterogeneous both within and among globules from the same species and probably between species. We propose that the use of membrane-specific fluorescent probes has significant potential for providing real time structural and chemical information about the MFGM in matrices such as mammary gland tissue, harvested milk, and milk products.

1. Introduction

Fat globules in milk are surrounded by a thin layer of surface-active material, which is called the milk fat globule membrane (MFGM). Upon secretion of the fat droplet from the mammary secretory cell, the structure of the MFGM is trilaminar, consisting of a surface-active inner layer that surrounds the intracellular fat droplet, then a dense proteinaceous coat and finally a true bilayer membrane originating from the secretory cell apical plasma membrane (Keenan & Mather, 2002). Following secretion of the fat globule by the secretory cell, the MFGM is believed to change to some degree, e.g., by partial loss of the bilayer membrane, although the extent of the loss is unclear (Evers, 2004a).

Much of the current knowledge of the structure and molecular organisation of the MFGM has been obtained using morphological techniques such as (immuno)electron microscopy applied to fat globules that had been isolated from the original milk sample (Buchheim, 1982; Franke et al., 1981; Henson, Holdsworth, & Chandan, 1971; Horisberger, Rosset, & Vonlanthen, 1977; Mather, Jack, Madara, & Johnson, 2001; Monis, Rovasio, & Valentich, 1975; Pinto da Silva, Peixoto de Menezes, & Mather, 1980; Robenek et al., 2006; Sasaki & Keenan, 1979; Schmidt & Buchheim, 1992; Welsch, Buchheim, Schumacher, Schinko, & Patton, 1988; Wooding, 1971). We were particularly intrigued by recently published freeze-fracture electron microscopy results (Robenek et al., 2006), which revealed variability in the physical structure of the surface of the MFGM of individual globules. However, electron microscopy results apply to fat globules that may, or may not, have undergone substantial physical and/or chemical modification, and the environment of which has been drastically changed. Hence, the degree to which the results represent the structure of the membranes of fat globules in the original sample remains uncertain.

Fluorescence microscopy can be used to study fat globules without isolating them from the milk sample matrix. The commercially available fluorescent dyes Nile Blue and Nile Red have been used to study fat globules in milk and milk products, using either conventional fluorescence microscopy or confocal laser scanning microscopy (CLSM; note that this abbreviation is also used for the confocal laser scanning microscope, as defined by the context) (Everett & Olson, 2003; Evers, 2004b; Herbert, Bouchet, Riaublanc, Dufour, & Gallant, 1999; Lopez & Briard-Bion, 2007; Lopez, Briard-Bion, Beaucher, & Ollivon, 2008; Lopez, Camier,

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& Gassi, 2007; Michalski et al., 2004; Sutheerawattananonda, Fulcher, Martin, & Bastian, 1997; Trachoo, 2003; Ye, Singh, Taylor, & Anema, 2005). However, these neutral fat dyes stain the fat core of the globules and, therefore, provide no information about the MFGM. The fluorescent dye Acridine Orange has been used to visualise fat globules (King, 1958; Scolozzi, Martini, & Abramo, 2003) or cytoplasmic crescents associated with fat globules (Janssen & Walstra, 1982; Patton & Huston, 1988), but this dye is presumed to stain cytoplasmic matter rather than the MFGM itself (Scolozzi et al., 2003). Immunofluorescence techniques have revealed generally patchy distributions of butyrophilin and other membrane proteins (McManaman, Palmer, Wright, & Neville, 2002), but these techniques are known to introduce artefacts (Robenek et al., 2006).

It is in this context that we sought to develop new techniques that would specifically probe, in real time, the structural and compositional details of the MFGM in its native environment (i.e., milk) in a non-destructive manner. Thus, a range of commercially available fluorescent probes, individually and in combination, were screened by means of conventional fluorescence microscopy and CLSM. In this paper, we present staining patterns of the MFGM by two lipophilic probes, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid (DiIC$_{18}$(3)-DS) and *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64), and by the lectin wheat germ agglutinin (WGA).

2. Materials and methods

2.1. Chemicals

Reverse osmosis water was purified using a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Fluorescent probes (solids for reconstitution; molecular structures given in Fig. 1) were obtained from Molecular Probes (Eugene, OR, USA): DiIC$_{18}$(3)-DS, FM4-64 and wheat germ agglutinin (WGA*xyz*, where *xyz* indicates the particular Alexa Fluor® conjugate, e.g., WGA488). Single-probe stock concentrations were 1 mg mL^{-1} Milli-Q water, unless indicated otherwise. A dual-probe stock solution contained both FM4-64 and WGA488, each at 0.5 mg mL^{-1} phosphate buffered saline (pH 7.4). Ethanol (96%) was obtained from Merck (Darmstadt, Germany). *N*-acetylneuraminic acid (NANA) (minimum 95%) and *N*-acetylglucosamine (GlcNAc) (minimum 99.0%) were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of laboratory grade quality. Phosphate buffer (KH_2PO_4, 0.001 M; Na_2HPO_4, 0.01 M) was adjusted to pH 7.3 using 0.1 M HCl.

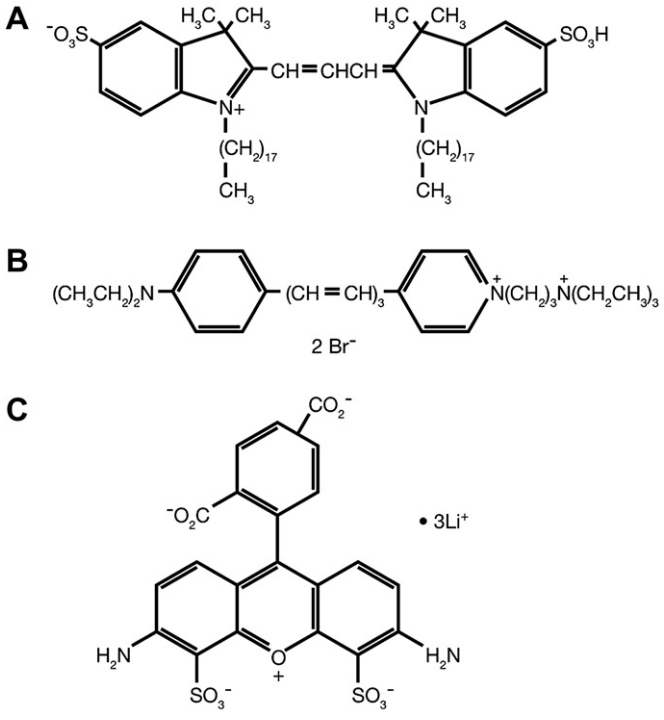

Fig. 1. Molecular structures of selected fluorescent probes used to stain the milk fat globule membrane: (A) DiIC$_{18}$(3)-DS; (B) FM4-64; (C) Alexa Fluor® 488 conjugate. Structures A and B reproduced with permission from Molecular Probes.

2.2. Milk samples

Bulk-tank bovine milk samples and ovine milk samples were obtained from a local dairy farm and a commercial sheep farm, respectively. Human milk was donated by a healthy volunteer.

2.3. Staining and imaging protocol

Unless stated otherwise, an aliquot of probe stock solution was added to the milk sample at a ratio of 1:100 (v/v) (single-probe stock) or 1:50 (v/v) (dual-probe stock), resulting in a final probe concentration of 10 μg mL^{-1} milk. The stained sample was mixed by gentle swirling and inversion of the vial containing it, and was incubated at room temperature for a specified period of time (see section 3). Stained milk samples were protected from light by wrapping vials in aluminum foil. A small aliquot (5–10 μL) of the stained milk was placed on a microscope slide and was carefully covered with a cover slip. Conventional fluorescence microscopy was performed using a Zeiss Axioskop 2 Plus microscope, fitted with an Axiocam MRC5 camera (Carl Zeiss, Göttingen, Germany). CLSM was performed using a Leica DMRBE TCS CLSM (Leica Microsystems GmbH, Wetzlar, Germany) with a 100 × oil immersion objective (1.4 NA). The CLSM filter parameters are listed in Table 1.

2.4. WGA488 controls

Fat globules were washed using deionised water to ensure that binding of WGA to fat globules could be studied in an environment free from competing binding sites for WGA (e.g., glycosylated plasma proteins). WGA488 stock was added to a solution containing NANA (0.2 M in phosphate buffer) at a ratio of 1:100 (v/v) and was incubated for 0.5 h at room temperature, before addition of washed fat globules (suspended in deionised water) to give a final concentration of fat similar to that of milk. The degree of fluorescence of the fat globules was assessed visually using conventional fluorescence microscopy after 60–90 min and again after about 5 h. Repeat experiments, in which the concentration of the WGA488 stock was increased up to a ratio of 6:100 (v/v), were conducted.

Table 1
CLSM filter settings suitable for the lipophilic probes DiIC$_{18}$(3)-DS and FM4-64, and for the lectin WGA Alexa Fluor®488 conjugate[a]

Probe	Channel 1		Channel 2	
	Beam splitter	Barrier filter	Beam splitter	Barrier filter
DiIC$_{18}$(3)-DS	RSP580[b]	LP515[c]		
FM4-64	RSP580	–	Mirror	LP590
WGA488	Mirror	LP515		
WGA488/FM4-64 combination	RSP580	BPFITC[d]	Mirror	LP590

[a] In all cases, a 488 nm excitation laser and a RSP510 beam splitter were used.
[b] RSP = Relative short pass.
[c] LP = Long pass.
[d] BPFITC: 515–545 nm.

A control sample consisted of washed fat globules added to a NANA-free phosphate buffer containing WGA488. Further control experiments were carried out as described above, but using free GlcNAc instead of free NANA.

3. Results

3.1. Lipophilic probes

An ethanolic DiIC$_{18}$(3)-DS stock solution immediately resulted in a small proportion (estimated <5%) of the fat globules being brightly stained. Subsequent incubation at room temperature for several hours resulted in the staining of further fat globules, but not as brightly as those stained initially. DiIC$_{18}$(3)-DS was located exclusively, but often heterogeneously, in the MFGM (Fig. 2). Because of the risk of ethanol perturbing the membrane structure by denaturing membrane proteins, aqueous stocks of DiIC$_{18}$(3)-DS were used in all subsequent work.

As DiIC$_{18}$(3)-DS does not fully dissolve in water at 1 mg mL^{-1}, the aqueous stock contained partly dissolved and partly dispersed probe. Comparisons between filtered (0.2 μm pore size) and unfiltered aqueous stocks of DiIC$_{18}$(3)-DS showed that staining was more effective when using the unfiltered stock. Presumably, the suspended probe in the unfiltered stock dissolved into the milk plasma after addition of this stock to the milk sample and thus increased the effective concentration of DiIC$_{18}$(3)-DS.

The efficacy of the probe stock in staining the MFGM (as assessed by the brightness of the stained fat globules as a function of incubation time and temperature) depended on the type of milk: human > bovine > ovine. As observed with the ethanolic DiIC$_{18}$(3)-DS stock, staining fat globules using aqueous DiIC$_{18}$(3)-DS stock often revealed heterogeneous fluorescence of the MFGM. Satisfactory staining of bovine milk fat globules was generally obtained within 2 h of incubation at 40–50 °C. We also observed that, in human milk, cytoplasmic crescents associated with fat globules were particularly brightly stained by DiIC$_{18}$(3)-DS (Fig. 3) irrespective of whether or not the sample had received an elevated heat treatment (75 °C, 5 min). Counterstaining the fat globules using Nile Blue confirmed that cytoplasmic crescents were attached to fat globules containing a neutral fat core (Fig. 3). Two-dimensional (2-D) CLSM imaging of heat-treated milk, dual stained with Nile Red and WGA488 (results not shown), provided further evidence that the crescents were not composed of neutral fat, thus eliminating the possibility that the observed structures (i.e., crescents) represented partially coalesced fat globules. Three-dimensional (3-D) CLSM images showed that the crescents were attached to the fat globules like blisters or sacs (Supplementary data).

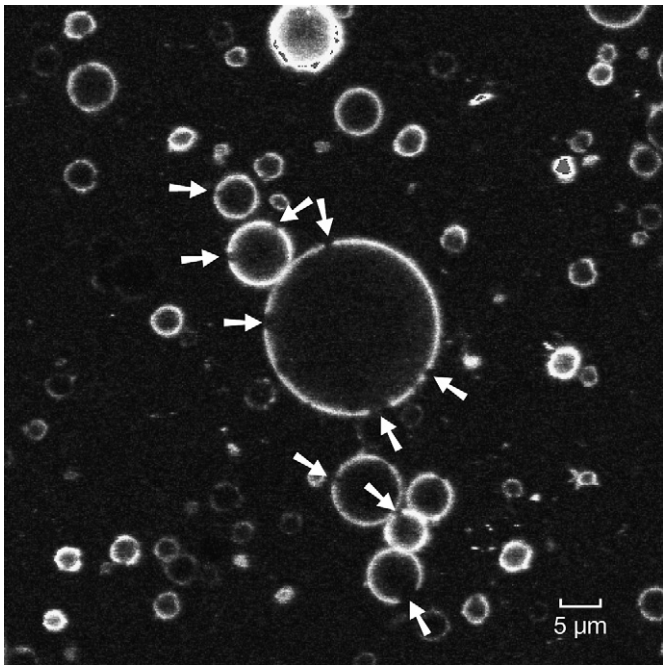

Fig. 2. CLSM image of human fat globules stained with DiIC$_{18}$(3)-DS (ethanolic stock). Fluorescence intensity varied over individual membranes. Arrows indicate non-fluorescent membrane areas.

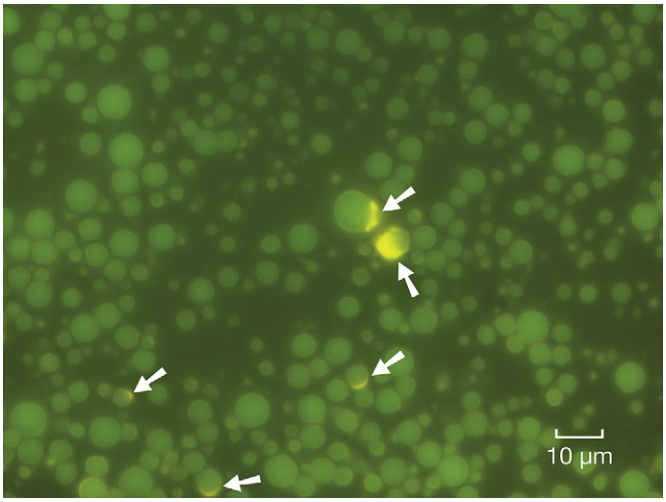

Fig. 3. Fluorescence microscopy image of heat-treated (75 °C, 5 min) human fat globules stained with DiIC$_{18}$(3)-DS (aqueous stock; added prior to heat treatment) and counterstained with Nile Blue (added after heat treatment). The image was acquired using a conventional fluorescence microscope with a band pass 450–490 nm excitation filter and a long pass 515 nm emission filter. Green colour: Nile-Blue-stained neutral fat. Yellow colour: DiIC$_{18}$(3)-DS-stained crescents. The size of the crescents varied from being a minor part of the fat globule to exceeding the volume of the fat globule.

The lipophilic styryl probe FM4-64, which, in contrast to DiIC$_{18}$(3)-DS, is fully water soluble at 1 mg mL^{-1}, appeared to be less effective in staining human MFGM and bovine MFGM, and to be less photostable than DiIC$_{18}$(3)-DS. As with DiIC$_{18}$(3)-DS-stained fat globules, a proportion of FM4-64-stained fat globules showed both fluorescent and non-fluorescent areas on the same MFGM (Fig. 4A). In some fat globules, the fluorescence of the MFGM was interrupted, indicating absence of the probe at these locations (Fig. 4A, locations 1, 3, 5 and 10). Variation in fluorescence intensity among different fat globules was less extreme than in the case of WGA488 (Fig. 4B; see below). The non-circular shape of some fat globules was the result of these fat globules moving during imaging.

3.2. WGA

In the presence of free NANA, WGA488 did not stain washed bovine fat globules (Fig. 5B) even when the WGA stock to milk ratio was up to six times greater than that typically used (1:100 v/v). However, in the presence of free GlcNAc, washed bovine fat globules were weakly fluorescent (Fig. 5C) and the average fluorescence intensity of the fat globules appeared to increase with the concentration of WGA and with the incubation time.

Staining of milk samples with WGA488, and examination by conventional fluorescence microscopy, showed that WGA promoted the aggregation of fat globules on the microscope slide over time. However, fat globule aggregation was limited during the first 5–10 min after mixing of the probe stock and the milk sample, and most fat globules were stained by WGA488 within this time

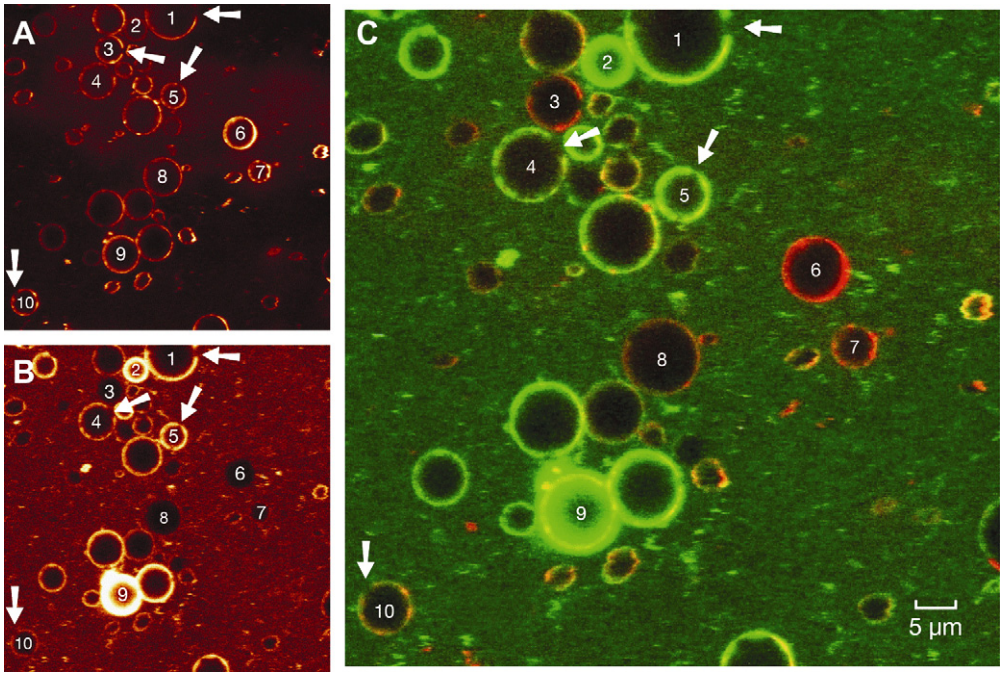

Fig. 4. Dual staining of human fat globules. (A) CLSM image, channel 2 (see Table 1), showing fat globules stained with the lipophilic styryl probe FM4-64. (B) CLSM image, channel 1 (see Table 1), showing fat globules stained with WGA488. (C) Overlay of images of Fig. 5A and B. FM4-64 and WGA488 were given false colours (red and green respectively). A yellow/orange colour means co-location of the two probes, with fluorescence at about the same pixel intensity. Although some fat globules were stained by both probes, the much greater fluorescence intensity of one of the probes may mean that only one colour is seen in the combined image. Hence, for proper analysis, the individual channel images need to be compared. Arrows indicate non-fluorescent areas on dual-stained fat globules. Other features are discussed in the body of the text.

interval. Hence, assessment of the stained sample was typically started within 10 min of the addition of the WGA stock to the milk sample. Although human and bovine milk fat globules were adequately stained with WGA488 at 10 μg mL^{-1} of milk, ovine milk required higher concentrations of WGA488 (30–50 μg mL^{-1} of milk) to achieve a similar intensity of staining. Background fluorescence was caused by WGA488 binding to glycosylated compounds in the milk plasma.

Significant variation in fluorescence intensity was observed among different fat globules, these ranging from weakly or non-fluorescent (e.g., Fig. 4B, locations 3, 6–8) to highly fluorescent (e.g., Fig. 4B, locations 2 and 9). The fluorescence intensity varied also within the membranes of individual fat globules; in some fat globules, the fluorescence of the MFGM was interrupted, indicating absence of the probe at these locations (e.g., Fig. 4B, locations 1, 4, 5 and 10).

Dual staining, in which the stock solution contained WGA488 in combination with the lipophilic probe FM4-64, showed that some fat globules that appeared to be very weakly stained or not stained with WGA488 were stained with FM4-64 (Fig. 4C, fat globules 3, 6–8). In several dual-stained fat globules, non-WGA-fluorescent membrane areas matched the non-FM4-64-fluorescent membrane areas on the same fat globules (arrows, Fig. 4C).

Optical sectioning of the stained fat globules using CLSM enabled assessment of the surface distribution of the probe (Figs. 6 and 7). Non-fluorescent areas on ovine MFGM were frequently circular and varied in size. Curiously, on one occasion, we observed some larger non-fluorescent areas either touching one another or being connected by narrow non-fluorescent "channels" (arrows, Fig. 6). So far, we have not observed such "channels" on any of the human or bovine milk fat globules stained with WGA488.

In contrast to the case with $DiIC_{18}(3)$-DS, heat treatment experiments showed that WGA488 progressively lost its fluorescent properties with increasing temperature (particularly above 60 °C; results not shown). Hence, WGA-Alexa Fluor® conjugates must be added to the milk sample after heat treatment at elevated temperatures.

Fig. 5. Testing the degree of non-specific binding of WGA488 to bovine MFGM: (A) control sample (sugar-free phosphate buffer with WGA-stained fat globules); (B) washed fat globules in the presence of NANA and WGA488; (C) washed fat globules in the presence of GlcNAc and WGA488. Bar = 20 μm.

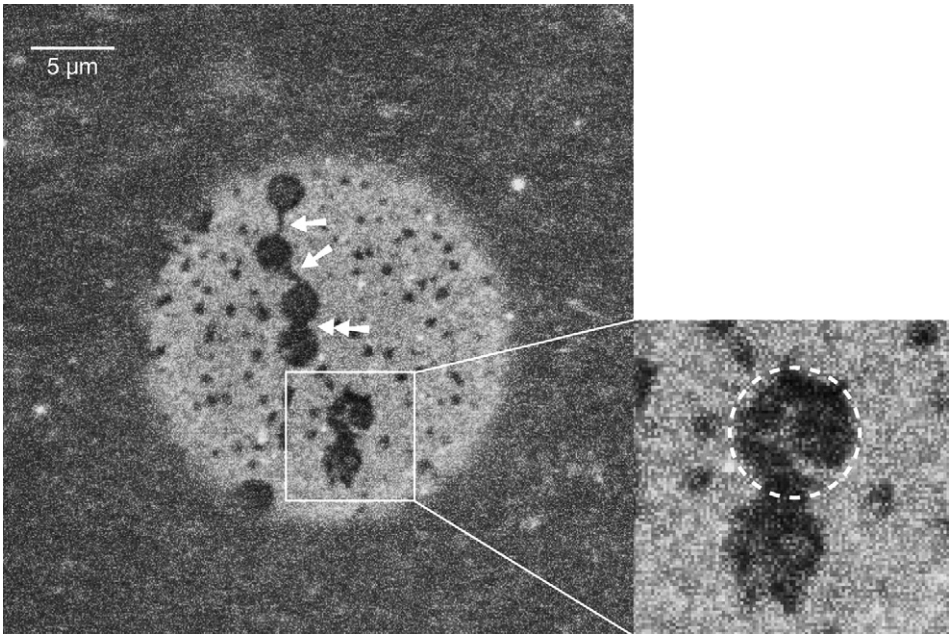

Fig. 6. CLSM image showing part of the WGA488-stained membrane of an ovine fat globule. Non-fluorescent areas of different sizes were predominantly circular. Some large non-fluorescent areas near the top were connected to each other by "channels" (arrows). Inset: Smaller non-fluorescent areas appear to fuse to form larger non-fluorescent areas. The dashed circle denotes the expected final circular non-fluorescent area.

Using bovine milk, alternative WGA probes, such as WGA594 and WGA647 (Fig. 8), were also found to be suitable, although, in the case of WGA647, an elevated probe concentration in the sample was required (about six times that for WGA488), as the fluorescence of WGA647-stained fat globules was significantly weaker than that of WGA488-stained fat globules.

4. Discussion

4.1. WGA controls

Conflicting results for WGA control studies using GlcNAc have been reported in the literature. In washed bovine milk fat globules and human milk fat globules, GlcNAc appeared to completely inhibit WGA binding (Farrar, Harrison, & Mohanna, 1980). Similarly, non-specific binding of WGA in the presence of *N*-acetylchitopentaose was reported to be very low (Horisberger et al., 1977). However, in equine milk, GlcNAc had no inhibiting effect (Welsch et al., 1988).

To our knowledge, no studies of WGA binding to fat globules have been performed that have involved control studies using both NANA and GlcNAc; this is interesting, as WGA recognises both residues (Sharon & Lis, 2003). Our results showed that, in the presence of free NANA, non-specific binding of WGA to bovine fat globules was virtually absent, whereas, in the presence of free GlcNAc, WGA binding to bovine fat globules was WGA-concentration dependent, but was lower in extent than the binding of WGA to fat globules in the absence of free GlcNAc. The time-dependent and concentration-dependent binding of WGA to MFGM in the presence of free GlcNAc may have been due to MFGM-bound GlcNAc oligomers and/or terminal NANA successfully competing for WGA rather than to non-specific binding of WGA with MFGM.

4.2. Location and orientation of probes in the membrane

A schematic of the locations and orientations of the tested probes in the MFGM is shown in Fig. 9. Dialkylcarbocyanines are believed to locate in membranes by injecting the two alkyl chains into the lipid interior of the membrane and by orientating the long axis of the fluorophore parallel to the membrane surface (Molecular Probes Handbook; www.probes.invitrogen.com). The absence of fluorescence in some locations of the membranes of certain $DiIC_{18}(3)$-DS-stained fat globules could denote either the absence of a bilayer membrane at these locations or the presence of co-existing fluid and gel phases in the membrane, with the fluorescence denoting the gel phase; in co-existing fluid and gel phases, long chain dialkylcarbocyanine probes, such as $DiIC_{18}(3)$-DS, prefer

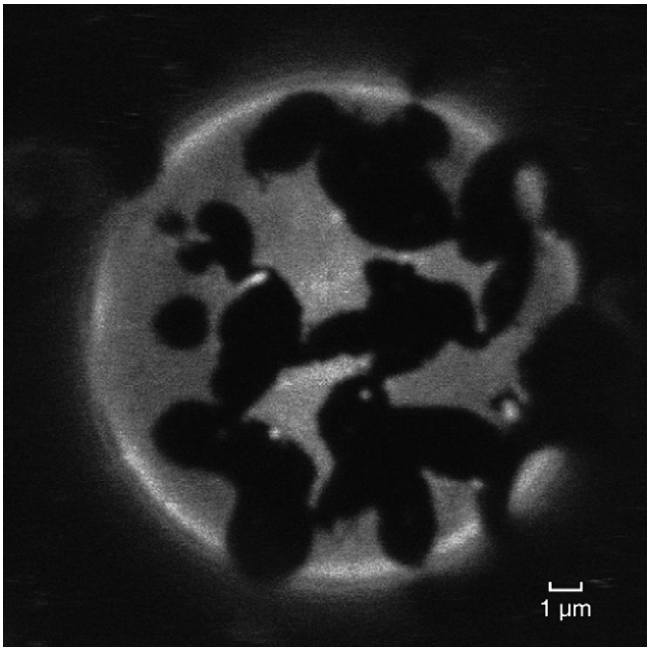

Fig. 7. CLSM image of part of the membrane surface of a bovine fat globule stained with WGA488. The milk sample had been heated (75 °C, 5 min) before addition of the WGA. A significant proportion of the membrane area was not fluorescent.

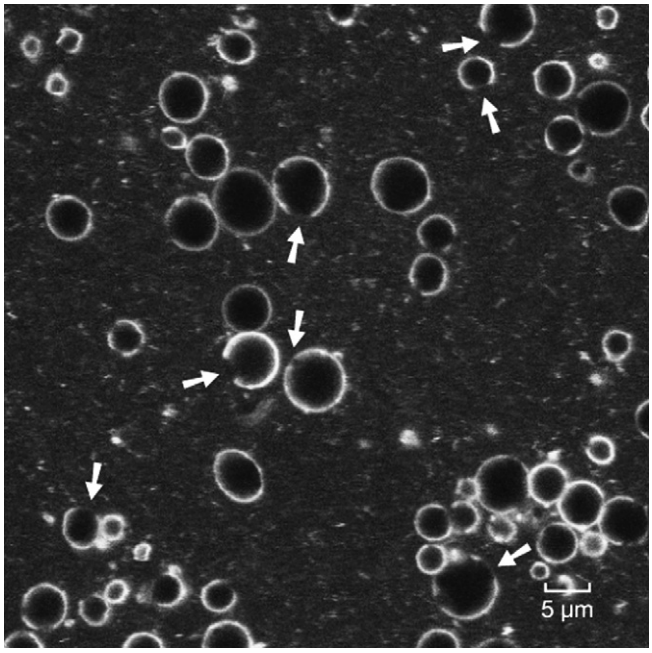

Fig. 8. CLSM image of bovine fat globules stained with WGA647. Fluorescence intensity varied among fat globules. Membrane fluorescence of single fat globules was sometimes not homogeneous (arrows denote non-fluorescent areas). Some fat globules appear to be non-spherical because they moved during imaging.

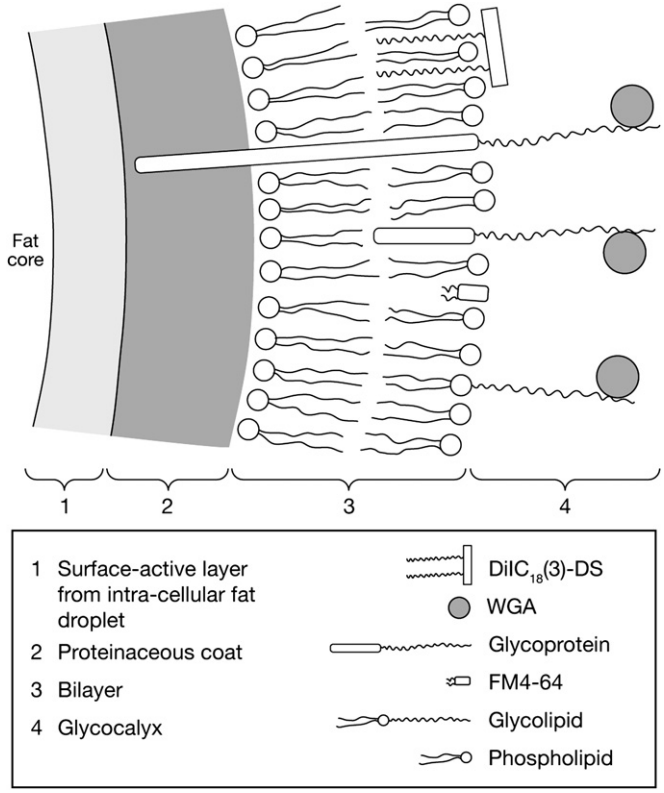

Fig. 9. Locations and orientations of $DiIC_{18}(3)$-DS, FM4-64 (adapted from Molecular Probes) and WGA*xyz* in the MFGM. Highly schematic. Not to scale. Currently, we have no evidence for or against location of the lipophilic probes in the inner leaflet of the bilayer.

the gel phase (Molecular Probes Handbook; www.probes.invitrogen.com). However, the similar fluorescence patterns observed when using the styryl probe FM4-64 and the lectin WGA (Fig. 4), and the observed increased uptake of the probe into the MFGM with increasing temperature (Evers, 2008) (i.e., with increasing fluidity of the MFGM), support the former (i.e., the absence of bilayer), and not the latter, explanation.

FM4-64 is believed to insert into the outer phospholipid leaflet of membranes and is thus useful for identifying membrane boundaries (Molecular Probes Handbook; www.probes.invitrogen.com). Small differences in polarity of styryl probes can have a large effect on the rate of uptake and retention properties (Molecular Probes Handbook; www.probes.invitrogen.com) and we speculate that styryl probes other than FM4-64 may also be useful MFGM markers.

WGA binds specifically to GlcNAc and NANA residues (Sharon & Lis, 2003) and thus is expected to locate in the glycocalyx by binding to relevant glycoproteins and glycolipids. WGA has previously been reported to bind to bovine MFGM (Farrar et al., 1980; Horisberger et al., 1977), human MFGM (Buchheim, Welsch, & Patton, 1988; Farrar et al., 1980; Horisberger et al., 1977; Welsch et al., 1990), ovine MFGM (Welsch et al., 1990) and equine MFGM (Welsch et al., 1988, 1990), but no investigation involved studying the fat globules in their native environment, or made use of CLSM. The current study showed that fluorescent conjugates of WGA stain the fat globules of at least three species (human, cow and sheep) and that the distribution of WGA in the MFGM can be assessed using CLSM (Figs. 4, 6–8), thus providing chemical information (degree and distribution of glycosylation) about the MFGM. Because the nature of the association of WGA with the MFGM (binding to NANA and GlcNAc residues) is fundamentally different from that of the lipophilic probes (which embed in the phospholipid bilayer), and because a large range of fluorescent WGA conjugates covering the wavelength range 400–700 nm are commercially available, WGA is an ideal probe for use in multiple-staining techniques.

4.3. Scope of the developed technique

WGA is a multivalent protein and its binding to monovalent sugar residues could, in principle, lead to glycoprotein and glycolipid aggregation, resulting in WGA-stained patches, but this was not found to occur in the apical membrane of Madin–Darby canine kidney cells (Kovbasnjuk & Spring, 2000). Furthermore, rather than observing WGA-stained patches amongst a background of unstained membrane, we observed unstained patches in a membrane largely stained by WGA. In addition, the dual-staining results (Fig. 4) suggest that, in some fat globules, the non-fluorescent areas of the membrane as revealed by the lipophilic probe FM4-64 and those as revealed by the lectin WGA are the same. We interpret these non-fluorescent areas to represent areas where a bilayer membrane is absent. It seems unlikely that the presence of the lipophilic probe causes the loss of significant sections of the MFGM, particularly considering that we observed similar heterogeneous staining using two very different lipophilic probes (i.e., a styryl probe and an indocarbocyanine probe). Taken together, these results provide indirect evidence that the staining patterns observed with WGA reflect the pre-existing, and not an artefactual, membrane state.

In contrast to typical fluorescence microscopy procedures, we chose not to wash out unbound probe from the specimen as we wanted to preserve the native environment of the fat globules. Consequently, background (i.e., milk plasma) staining was observed with all probes. Although the contrast between the fluorescent membranes and the background was generally sufficient to allow imaging, the technique would benefit from the elimination, or reduction, of background fluorescence. As the milk plasma contains both membranous matter and glycosylated proteins, it would

appear that background fluorescence can probably be reduced significantly if, after staining, the fat globules are isolated from the milk plasma. Future work should investigate whether washing stained fat globules alters the distribution of the probes in the MFGM. Alternatively, background suppression tools, such as spectral fingerprinting available on modern CLSMs, might successfully eliminate the fluorescence arising from the milk plasma.

4.4. Membrane heterogeneity in and among fat globules within species

Morphological features of the MFGM, as shown by electron microscopy, suggest that the MFGM is heterogeneous and contains domains of different shapes (Buchheim, Welsch, & Patton, 1988; Robenek et al., 2006). Similarly, for each of the tested fluorescent probes, we observed a variety of distribution patterns in the MFGM. Some fat globules were completely stained with a given probe, whereas the membranes of other fat globules contained both fluorescent and non-fluorescent areas. Although circular non-fluorescent areas were frequently observed (e.g., Fig. 6), non-fluorescent areas with other shapes were also regularly observed. Some of these patterns resembled those observed by electron microscopy (Buchheim, Welsch, & Patton, 1988; Robenek et al., 2006). Furthermore, the suggestion that significant sections of the bilayer appeared to be absent – the MFGM not being stained by either a lipophilic dye or the lectin WGA (Fig. 4) – sheds new light upon an existing controversy in the literature. On the basis of electron microscopy, it had been suggested that most of the bilayer is lost from milk fat globules in bovine milk post-secretion by the secretory cell (Bauer, 1972; Henson et al., 1971; Wooding, 1971, 1974). In contrast, biochemical data have suggested that the structure of the MFGM is largely preserved in secreted milk (Mather & Keenan, 1998). The current dual-staining results indicate that the truth may be somewhere in between, i.e., that most fat globules still possess a substantially intact bilayer, but that, in a certain proportion of fat globules, part of the bilayer has been lost.

Differences in fluorescence intensity between different WGA-stained fat globules suggest differences in the degree of glycosylation between different fat globules, which is in agreement with electron microscopy results (Horisberger et al., 1977). In addition, using dual staining, we observed on some occasions that some areas of the fat globule surface stained with WGA488, but not with FM4-64 (results not shown). These results suggest that, in areas lacking a lipid bilayer as indicated by the absence of staining with FM4-64, the surface-active inner layer and/or the proteinaceous coat between the bilayer and the fat core contain glycosylated compounds. This finding corroborates an earlier observation of this phenomenon by electron microscopy (Horisberger et al., 1977). It is probable that this glycosylated matter at least includes the glycoprotein butyrophilin, which is anchored into the proteinaceous coat (Keenan & Mather, 2006) and is present in relatively high concentrations in the surface-active inner layer (Robenek et al., 2006). In contrast to globules that contained areas that were stained with WGA and not FM4-64, some fat globules that presumably contained a bilayer (i.e., stained by FM4-64) were virtually unstained by WGA488 (Fig. 4). Again, this corroborates electron microscopy data of Horisberger et al. (1977), who observed fat globules that for the most part were not stained by WGA. We conclude that the distribution of glycoproteins and/or glycolipids in the MFGM is variable, the variation being caused in part by variation in the proportion of the fat globule surface that is covered by a bilayer membrane. This variation may, in part, arise from alveoli that are in different physiological states during lactation as indicated by the micro-heterogeneity of the lactating mammary gland (Molenaar, Davis, & Wilkins, 1992).

4.5. Species differences

Distinct differences in the uptake of the fluorescent probes by fat globules in the milk samples from three species were observed, which suggest structural and/or compositional differences in the MFGM between species. Notable differences between particularly bovine MFGM and human MFGM exist. Electron microscopy has shown the existence of high molecular weight (>400,000) glycoproteins in human MFGM (Buchheim, 1986; Buchheim, Welsch, Huston, & Patton, 1988), present as numerous thin filaments, whereas these have not been detected in bovine milk or ovine milk (Buchheim, Welsch, Huston, et al., 1988; Buchheim, Welsch, & Patton, 1988). These high molecular weight glycoproteins are lost on heating at elevated temperature (80 °C, 10 min) and contain NANA (Buchheim, Welsch, & Patton, 1988). Heat treatment experiments on bovine milk and human milk showed that heat-treated human fat globules (75 °C, 5 min) were not fluorescent upon staining with WGA488, whereas, for heat-treated bovine milk, not only did fat globules retain their WGA488-binding ability, but also a small proportion of the globules became highly fluorescent (Evers, 2008). This suggests that, in human milk fat globules, WGA binding occurs predominantly via these high molecular weight proteins, whereas, in bovine milk fat globules, WGA binding occurs via different glycoproteins. However, as both the ovine milk samples and the bovine milk samples were from pooled sources, in contrast to the human milk samples which were from a single source, and as the composition of milk varies with the stage of lactation, diet etc., factors inherent in the milk other than the structure of the MFGM may have affected the uptake of the probes by the fat globules. Further work should elucidate which factors affect the uptake of the tested fluorescent probes by the fat globules from milks of different species. Additionally, further screening of fluorescent probes should be conducted as it is conceivable that analogues of the fluorescent probes tested in this study possess superior staining properties for fat globules from certain species of milk.

4.6. Cytoplasmic crescents

The secretion of fat globules usually appears to involve tight envelopment of the intracellular fat globule by the secretory cell plasma membrane. However, cytoplasm can sometimes be entrained between the membrane and the fat globule core, resulting in cytoplasmic crescents of various sizes. The proportion of fat globules that bear cytoplasmic crescents varies with species and with individuals within species (Huston & Patton, 1990; Janssen & Walstra, 1982; Patton & Huston, 1988). Crescent-bearing fat globules can be shown morphologically using electron microscopy (e.g., Huston & Patton, 1990; Keenan, Dylewski, Woodford, & Ford, 1983; Keenan, Mather, & Dylewski, 1988; Patton & Huston, 1988; Wooding, 1975, 1977) or fluorescence microscopy (Janssen & Walstra, 1982; Patton & Huston, 1988). Fluorescence microscopy studies in the past invariably involved the use of the dye Acridine Orange. To date, only 2-D images of crescent-bearing fat globules have been shown in the literature. With conventional fluorescence microscopy and CLSM, we visualised crescents using membrane-specific fluorescent probes in both 2-D and 3-D format. Dual labelling of heat-treated milk, using $DiIC_{18}(3)$-DS and Nile Blue, enabled the sizes of the crescents to be easily compared with the sizes of the associated fat globules (Fig. 3). This showed considerable variation in the relative sizes of the crescents, which supports claims that crescents can vary from as small as thin slivers to as large as exceeding the fat globule core volume (Heid & Keenan, 2005). 3-D CLSM imaging of crescent-bearing fat globules stained with $DiIC_{18}(3)$-DS showed that the crescents were always shaped like rounded sacs (Supplementary data). In contrast, electron

microscopy images of crescents frequently show crescents of non-rounded shapes, including pointy sacs or pointy protrusions (e.g., Keenan et al., 1988; Keenan & Mather, 2006; Patton & Huston, 1988; Wooding, 1975, 1977). Further assessment of these images showed that sometimes pieces of membrane were missing from the crescent (image in Keenan et al., 1988), or that the shape of the crescent or the fat globule was jagged (e.g., images in Patton & Huston, 1988; Wooding, 1975, 1977), indicating that these shapes did not accurately represent crescents and fat globules in their native state. This strongly suggests that these pointy shapes were artefacts caused by the electron microscopy sample preparation technique.

Fat globule crescents stained with $DiIC_{18}(3)$-DS were consistently much brighter than the fat globule membrane itself. CLSM optical depth scan images (results not shown) showed that the greater fluorescence intensity of the crescent originated for the most part from stained matter inside the crescent. The fluorescence within the crescent was not homogeneously distributed. This pointed to the presence of both stainable and non-stainable matter being present within the crescent, which agrees with previous studies (Patton & Huston, 1988; Wooding, 1977). Electron microscopy has shown that the crescent can be filled with intracellular membranous matter (Keenan & Mather, 2006). Apparently, this membranous matter is stained by the lipophilic fluorescent probe $DiIC_{18}(3)$-DS, which indicates that this probe can actually penetrate the bilayer membrane when the inner leaflet is in contact with an aqueous environment.

It is believed that gaining an understanding of the molecular mechanism of crescent formation would advance the understanding of milk fat globule secretion in general (Heid & Keenan, 2005; Keenan & Mather, 2006). Electron microscopy is unlikely to deliver crucial kinetic data, considering that the process of fixation may disturb secretory events leading to promiscuous associations and interactions that are unrelated to normal secretory processes (Keenan & Mather, 2006; Mather & Keenan, 1998; Wooding, 1975). In contrast, the use of membrane-specific probes, such as those presented in the current study, in combination with CLSM has significant potential for providing insightful data, as this novel technique should allow secretory processes to be studied in real time and under physiological conditions.

4.7. Potential of the CLSM fluorescent probe technique and models of the MFGM

The CLSM technique developed here appears to have significant potential for providing, in a non-destructive manner for native fat globules, physico-chemical evidence for MFGM structural features to corroborate, or otherwise, features observed by other means such as electron microscopy and immunofluorescence microscopy. The very significant advantages of this technique over immunofluorescence and various electron microscopy techniques include: (a) the staining of fat globules in the absence of fixatives, detergents or solvents; (b) a very simple staining procedure (simply mixing probe stock with the milk sample followed by incubation); (c) the ability to conduct time-series experiments (e.g., to study time-dependent changes in the MFGM); and (d) the ability to study selectively particular characteristics of the MFGM (e.g., by using pH-sensitive probes, different lectins). We expect that future work will focus on perfecting staining protocols, expanding the range of suitable membrane probes and extending the technique to imaging fat globules in mammary gland tissue of lactating animals to understand better the origins of the observed heterogeneity. Consequently, we believe that the use of MFGM-specific probes in combination with CLSM will become an important research and analytical tool for studying fat globules in mammary tissue, in harvested milk and in milk products — in the last case especially in relation to interactions of the MFGM with milk product components.

Finally, most models of the MFGM represent the membrane as a trilaminar structure and focus on showing the location of membrane components (Danthine, Blecker, Paquot, Innocente, & Deroanne, 2000; Keenan & Mather, 2006). Future models of the membrane of fat globules in expressed milk should recognise the diversity of membrane structures within species as well as between species; clearly, further work is needed to explore this diversity. In particular, it is important to recognise that, in harvested milk, some fat globules appear to be completely covered by a trilaminar membrane, whereas the bilayer originating from the secretory cell apical plasma membrane is not fully intact in other fat globules. The non-bilayer patches may be circular or non-circular in shape. Further work is needed to verify whether parts of the bilayer are indeed lost through so-called membrane blebbing, as has previously been suggested on the basis of electron microscopy (Wooding, 1974).

5. Conclusions

Fluorescence microscopy using MFGM-specific probes has revealed chemical and structural heterogeneity at the micron and submicron scale in the MFGM, not only of individual globules but also among globules. In providing chemical as well as physical information on MFGMs in their native environment, our results confirm, complement and substantially extend previously reported morphological results for the MFGM of fat globules isolated from milk. The techniques developed show significant potential for advancing knowledge of the MFGM in its native environment.

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Appendix. Supplementary data

3-D images of heat-treated human fat globules stained with $DiIC_{18}(3)$-DS. The crescents were of a rounded shape and were attached to the fat globules like blisters or sacs. The 3-D effect is obtained by viewing the image using stereo glasses (green for left eye and red for right eye). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.idairyj.2008.06.001.

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