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Buffalo milk fat globules and their biological membrane: *in situ* structural investigations

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Abbreviations: MFGM: milk fat globule membrane; Lo phase: liquid ordered phase; CLSM: confocal laser scanning microscopy; DIC: differential interference contrast; TAG: triacylglycerol; SM: sphingomyelin; PC: phosphatidylcholine

ABSTRACT

Milk fat globules and their surrounding biological membrane (the MFGM) are not well understood despite the importance of these milk components in human nutrition and the role of fat globules in determining the properties of dairy products. The objectives of this study were to investigate these unique colloidal assemblies and the microstructure of the MFGM in buffalo milk, which is the second largest global source of dairy products. *In-situ* structural investigations were performed at room temperature using confocal microscopy with multiple fluorescent probes (Nile Red, Rh-DOPE, the lectin WGA-488). Microscopic observations showed cytoplasmic crescents around fat globules and the heterogeneous distribution of glycosylated molecules and polar lipids with the occurrence of lipid domains. The lipid domains in the buffalo MFGM appear to form by the segregation of lipids with a high phase transition temperature (e.g. sphingomyelin and saturated phosphatidylcholine molecular species) and cholesterol resulting in a gel phase or a Lo phase forming circular domains. The structure of the buffalo MFGM results from a non-random mixing of components, consistent with observations for other species. Structural heterogeneities of the MFGM could affect the processability of buffalo fat globules and the bioavailability of milk lipids.

Keywords: milk fat globule membrane, phospholipid, sphingomyelin, high phase transition temperature lipid, lipid domain

1. Introduction

Milk is an exceptionally complex biological fluid used for the manufacture of a wide range of dairy products. Numerous studies have focused on cow's milk although milks from other animal species such buffalos, ewes, goats and camels are essential to the human diet in various parts of the world. Buffalo milk represents the second largest volume of milk produced globally after cow's milk with more than 97 million tonnes produced each year (FAOSTAT, 2012). Buffalo milk is also one of the richest milks from a compositional point of view (Ménard et al., 2010). Fat constitutes the main fraction of buffalo milk, with the almost twice the fat content of bovine milk (7.4-8.8% w/w vs. 3.6-4.7% w/w) (Solh, Staines, Honda, & Limley, 2007; Varricchio, Di Francia, Masucci, Romano, & Proto, 2007) and this fat is responsible for the high energetic and nutritive value of buffalo milk. Despite the nutritional value of milk fat and the influence of fat on dairy product properties, information about buffalo milk fat is scarce.

All milk fat is thought to be dispersed in colloidal assemblies called milk fat globules. The core of the milk fat globule is mainly composed of triacylglycerols (TAG, esters of fatty acids and glycerol; 98% of milk lipids). This core is surrounded by a biological membrane called the milk fat globule membrane (MFGM). This MFGM contains many bioactive compounds which are involved with several biological functions and health benefits, such as neonatal gut maturation, antibacterial infection, inhibition of colon development and lowering cholesterol absorption (Dewettinck et al., 2008; Lopez, 2011). Previous studies have demonstrated that buffalo milk fat globules are significantly larger in size (5.0 μm vs. 3.5 μm) and have a higher absolute zeta potential (|-11.0 mV| vs. |- 9.4 mV|) compared to bovine milk fat globules (Ménard et al., 2010). Few authors have characterized the buffalo MFGM (Abou-Dawood, Moussaa, El-Demerdash, & Ahmed, 1988; D'Ambrosio et al., 2008; Ménard et al., 2010).

This biological membrane is thought to be comprised of three layers, containing mainly of glycerophospholipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; phosphatidylserine, PS), sphingolipids (mainly sphingomyelin, SM), cholesterol and proteins (Keenan & Patton, 1995; Ménard et al., 2010). Polar lipids, which are mainly located in the MFGM, account for about 2.6 mg/g fat and about 190 mg/L of buffalo milk (Ménard et al., 2010), which is 28% higher than in bovine milk (Ménard et al., 2010). Also, buffalo milk contains less cholesterol than bovine milk (7.0-10.2 mg/100mL vs. 10.5-19.8 mg/100 mL) (Strzalkowska, Jozwik, Baghnicka, Krzyzewski, & Horbanczuk, 2009; Talpur, Memom, & Bhanger, 2007; Zotos & Bampidis, 2014). The average content of membrane protein was reported to be less in buffalo MFGM compared to bovine milk, either when calculated as a percentage of fat (4.2% vs. 4.9%) or as a proportion of the total MFGM material (29.5% vs. 33.9%) (Abou-Dawood et al., 1988). A recent proteomic study identified 50 proteins within buffalo MFGM, with the major proteins being as xanthine dehydrogenase/oxidase, butyrophilin, adipophilin, lactadherin and mucin, similar to bovine MFGM proteins (D'Ambrosio et al., 2008; Fong, Norris, & MacGibbon, 2007). Both the composition and the structure of the MFGM result from the mechanisms of secretion of fat globules from the epithelial cells of the mammary gland (Heid & Keenan, 2005). The compositional differences reported to date suggest there may be further underlying structural differences in the MFGM in buffalo milk and possible differences occurring during the *in vivo* secretion of these globules during milk production.

A number of studies performed using confocal microscopy have revealed heterogeneities in the organization of the MFGM (Evers et al., 2008), in the localization of membrane proteins (Lopez, Madec, & Jimenez-Flores, 2010; Lopez & Ménard, 2011) and the presence of lipid domains in the membranes of bovine (Gallier, Gragson, Jimenez-Flores, & Everett, 2010; Lopez et al., 2011; Lopez et al., 2010) and human milk (Lopez & Ménard, 2011; Zou et al.,

2012). This heterogeneous distribution has been attributed to the phase separation of polar lipids according to their temperature of phase transition (Tm). The exogenous phospholipid fluorescently head-labelled with rhodamine (i.e. Rh-DOPE; PE containing 2 oleic acids with very low phase transition temperature) preferentially partitions in the fluid Ld phase containing unsaturated polar lipids (i.e. containing one or two long-chain unsaturated fatty acids) (Lopez et al., 2010). Hence, areas of the MFGM not stained by Rh-DOPE may correspond to gel phase domains rich in polar lipids with a high Tm. Polar lipids not stained by Rh-DOPE could adopt a second possible phase consisting of liquid ordered (Lo) phase domains composed of high Tm lipids and cholesterol. These high Tm lipids include SM, which accounts for about 25% of polar lipids in the MFGM (Lopez, 2011) and contains long chain saturated fatty acids (Tm = 35 °C; Malmsten, Bergentahl, Nyberg, and Odham (1994)). Saturated PC are also high Tm lipids found in the MFGM (e.g. dipalmitoyl phosphatidylcholine, DPPC, Tm = 41.7 °C; Benesch and McElhaney (2014)). The surrounding membrane stained by Rh-DOPE may correspond to the fluid liquid disordered (Ld) phase of the unsaturated glycerophospholipids (PC, PE, PI, PS) which have a low Tm and are responsible for the fluidity of the biomembranes (Gallier et al., 2010; Lopez et al., 2010). A recent study performed by atomic force microscopy with a model membrane showed that milk SM segregates from the fluid Ld phase of unsaturated polar lipids to form domains in the gel phase (Guyomarc'h et al., 2014). This study also showed that cholesterol has a large effect on the domains formed by milk SM; the shapes and nanomechanical properties of the domains formed differ in the gel phase in the absence of cholesterol and in the Lo phase when cholesterol is present. The domains also protrude further from the fluid phase of the membrane in the absence of cholesterol (Guyomarc'h et al., 2014).

The lipid domains recently revealed in the MFGM could potentially be responsible for several biological processes (e.g. digestion of milk fat globules, interaction with pathogens and

119 viruses in the gut) and cellular functions. Also, the lipid domains present in outer bilayer of
120 the MFGM could be involved in functional properties of milk fat globules, important for dairy
121 applications.

122 The objective of this study was to perform a structural analysis of fat globules and their
123 biological membrane, *in situ* in buffalo milk. The microstructure was investigated using the
124 combination of optical microscopy with differential interferential contrast together with
125 confocal laser scanning microscopy (CLSM) with adapted fluorescent dyes able to label
126 TAG, total proteins, polar lipids and glycosylated molecules located in the MFGM.

127

128 2. Materials and methods

129

130 *2.1. Milk samples*

131 The buffalo milks used in this study were a mixture of the individual milks produced by 30
132 buffaloes of the Mediterranean breed *Bubalus bubalis* and collected from evening and
133 morning milking from Coopérative de Bufflonnes (Maurs, Cantal region, France). NaN_3
134 (0.02% w/v) was added to the milk to prevent the growth of bacteria. Milk samples were
135 stored at ambient temperature before fat globule size measurements and CLSM experiments.
136 The content of fat in the milk was determined using the Gerber method method (IDF, 2008).

137 *2.2 Fat globule size measurements*

138 ***2.3*** The fat globule size distributions were determined by laser light scattering, using a
139 Mastersizer 2000 (Malvern Instruments, Malvern, U.K.) equipped with a He/Ne laser (λ =
140 633 nm) and an electroluminescent diode (λ = 466 nm). The refractive indexes of milk fat

were set at 1.46 (at 466 nm) and 1.458 (at 633 nm) and the refractive index was set at 1.33 for water. The absorption coefficient used was 0.0001. The experiments were performed at room temperature. Aliquots of approximately 70 µL of whole milk were introduced into the measurement cell of the apparatus, which contained 100 ml of water, in order to reach 10% obscuration (optimal conditions for particle-size measurements with this apparatus). A solution of 35 mM EDTA/NaOH pH 7.0 buffer (>98% disodium salt dihydrat, Prolabo, Fortenay-sous-Bois, France) was added to the measurement cell to disrupt the casein micelles. All analyses were performed in triplicate with three replicate milk samples collected from different days, giving the total of nine measurements. The specific surface area and volume-weighted average diameter d_{43}, defined as $\Sigma n_i d_i^4/\Sigma n_i d_i^3$, where n_i is the number of fat globules of diameter d_i, were calculated by the software. ***Microstructural analysis***

Microstructural analysis was performed using an inverted microscope NIKON Eclipse-TE2000-C1si (NIKON, Champigny sur Marne, France). Confocal laser scanning microscopy (CLSM) was performed using an argon laser operating at an excitation wavelength of 488 nm with emission detected between 500 nm and 530 nm, a He-Ne laser operating at 543nm wavelength excitation with emission detected between 565 nm and 615 nm and a diode operating at 633 nm, detected with a long pass filter > 650 nm. The observations were performed using a ×100 (numerical aperture NA 1.4) oil immersion objective.

The staining protocols followed previously described methods (Lopez et al., 2010; Lopez & Ménard, 2011). Briefly, Nile Red (5H-Benzo α-phenoxazine-5-one, 9-diethylamino, supplied by Sigma-Aldrich, St. Louis, USA) was prepared at a concentration of 42 µg/mL in propanediol and used to stain the triacylglycerol core of the fat globules. Fast Green FCF (Sigma-Aldrich, St. Louis, USA) was prepared at a concentration of 10 mg/mL in water and

used to stain proteins. N-(Lissamine rhodamine B sulfonyl) dioleoylphosphatidyl ethanolamine (Rh-DOPE, Avanti polar lipids Inc., Birmingham, England) was provided at a concentration of 1 mg/mL in chloroform and used to label the phospholipids in the membrane surrounding buffalo milk fat globules. Wheat germ agglutinin Alexa fluor 488 (WGA488, Invitrogen, Cergy Pontoise, France) was prepared at a concentration of 1 mg/mL in phosphate saline buffer and used to label the glycosylated molecules in the membrane, i.e. carbohydrate moieties containing N-acetylglucosamine and N-acetyl neuraminic acid (sialic acid) residues.

The concentrations of the fluorescent dyes were adapted to the specific composition of buffalo milk. For observation of the fat globules, 100 μL of Nile Red and 10 μL of Fast Green FCF were added to 1 mL of buffalo milk samples. For observation of the MFGM polar lipids, 40 μL of the Rh-DOPE solution was placed in a glass vial and the chloroform was evaporated under nitrogen, to avoid the possible artefacts caused by this organic solvent. Then 1 mL of milk sample was introduced in the vial. For dual staining of the MFGM polar lipids and carbohydrate moieties, 10 μL of the WGA-488 solution was also added into the vial containing Rh-DOPE. Then, 1 mL of milk sample was added to the vial. The stained milk samples were kept at room temperature for at least 1 h prior to observation by CLSM. The microstructural analyses were performed at room temperature (19 ± 1 °C)

Optical microscopy using differential interference contrast (DIC, also called Nomarski, Cogswell and Sheppard (1992)) was also used to characterize buffalo milk fat globules. DIC images were superimposed on the fluorescent emission recorded in the CLSM images. The two dimensional images had a resolution of 512×512 pixels and the pixel scale values were converted into micrometers using a scaling factor. In the multiple labeled samples, different colors were used to locate the fluorescent probes (e.g. red color for phospholipids stained by

188 Rh-DOPE, green color for the carbohydrate moieties stained by WGA-488 and grey levels for
189 DIC images).

190 3. Results and discussion

191 *3.1. Size distribution of buffalo milk fat globules*

192 The buffalo milks used in this study contained 74.1 ± 5.8 g fat per kg milk, in agreement with
193 previous studies (Ahmad et al., 2008; Ménard et al., 2010; Varricchio et al., 2007). The size
194 distribution of the fat globules, determined using light scattering, ranged from 0.4 μm to 15.8
195 μm, with a mean volume-weighted diameter of 5.0 ± 0.4 μm (Figure 1A). The surface of fat
196 globules covered by the MFGM was 2.0 ± 0.2 m^2/g fat, which corresponded to about 148 m^2
197 per kg milk. The biological membrane surrounding fat globules represents a large interface
198 between the TAG core of fat globules and the aqueous environment, confirming the
199 importance of its characterization. These size parameters are consistent with previous reports
200 (Ahmad et al., 2008; Ménard et al., 2010).

201 The fat globules, observed by the combination of differential interference contrast (DIC) light
202 microscopy and CLSM after staining with Nile Red, were spherical in shape and dispersed in
203 the aqueous phase of milk containing proteins (Figure 1B and D). A rough surface could be
204 observed on some fat globules (indicated by arrows in Figure 1C) and may arise from surface
205 heterogeneities or partial crystallisation of the TAG core. The overlay of DIC and fluorescent
206 CLSM images showed that the triacylglycerols are located in the core of fat globules (Figure
207 1D), similar to previous observations for bovine and human milk (Lopez et al., 2010; Lopez
208 & Ménard, 2011; Zou et al., 2012).

3.2 Cytoplasmic remnants attached to buffalo milk fat globules

Cytoplasmic remnants (CR) from lactating cells were observed to be connected to some buffalo fat globules of various sizes, using optical microscopy and fluorescence microscopy (Figure 2). The volume of CR varied from one globule to another and their thickness was 1 μm up to 5 μm, as determined in the fluorescence images (Figure 2). The CR can exceed the buffalo milk fat globule volume (Figure 2 D). The morphology of CR can be compared to a crescent attached to fat globules, consistent with another name for these remnants of cytoplasmic crescents. The majority of the buffalo fat globules did not contain CR and were enveloped compactly by the MFGM (Figure 2 D and E).

CR occur when the mechanisms involved in the closure of the apical plasma membrane behind the projecting lipid droplet lead to their secretion with a piece of cytoplasm remaining attached to fat globule in milk (Heid & Keenan, 2005). The occurrence of the CR has also been reported in previous CLSM studies of human milk (Evers et al., 2008; Lopez & Ménard, 2011) but CR have not been observed in bovine milk (Lopez et al., 2010) when the same CLSM methods and fluorescent probes have been applied. This is probably due to variations in CR concentration between the milk of different species, arising from differences in the membrane secretion process, which is poorly understood (Robenek et al., 2005). CR were observed for 7.2% of the fat globules within human milk but for less than 1% of the fat globules in bovine milk, quantified using microscopic observation with the assistance of a counting chamber slide (Huston & Patton, 1990). In goat milk, between 1% and 5% of milk fat globules had CR (Wooding, Peaker, & Linzell, 1970). The current study is the first to report the presence of CR in buffalo milk but the number of CR was not quantified, which may be worthy of further investigation.

232 Previous authors who observed CR in human milk employed Rh-DOPE or $DiIC_{18}(3)$-DS to
233 label the MFGM (Evers et al., 2008; Lopez & Ménard, 2011). DiIC18(3)-DS is a lipophilic
234 fluorescent probe that can penetrate the membrane by inserting its two acyl chains into the
235 interior of the phospholipid membrane (Evers et al., 2008). The labelling of the CR by Rh-
236 DOPE or DiIC18(3)-DS suggests that these remnants are rich in phospholipid, consistent with
237 their origin as fragments of the cytoplasm or apical plasma membrane (Heid & Keenan,
238 2005). The size of CR has been observed to range from a thin sliver to a size exceeding the fat
239 globule core (Heid & Keenan, 2005). CR contain all organelles and intracellular membrane
240 materials (except for nuclei) of the lactating cells (Heid & Keenan, 2005; Wooding et al.,
241 1970) and thus, these CR may be a good source of enzymes, micronutrients and growth
242 factors necessary for newborn development (Huston & Patton, 1990). RNA has also been
243 successfully isolated from the CR of human milk (Lemay et al., 2013; Maningat et al., 2009),
244 suggesting CR could impact on protein expression in host cells, similar to other RNA
245 containing membrane vesicles found in milk and other body fluids (Valadi et al., 2007; Zhou
246 et al., 2012). CR could similarly be examined as potential markers of milk quality.

247 ***3.3 Heterogeneous distribution of polar lipids in buffalo MFGM***

248 The fluorescent dye Rh-DOPE was used to assess the distribution of polar lipids in the
249 MFGM surrounding buffalo fat globules, *in situ* in buffalo milk at 19 ± 1 °C, as already
250 performed for bovine and human MFGM (Gallier et al., 2010; Lopez et al., 2010; Lopez &
251 Ménard, 2011; Zou et al., 2012).

252 Rh-DOPE integrated the buffalo MFGM and appeared fluorescent at the periphery of the fat
253 globules viewed as an equatorial section by CLSM (Figure 3, Figure 4 A and C). The interior
254 of buffalo milk fat globules, which is mainly composed of triacylglycerols, as shown in
255 Figure 1, was not labelled by Rh-DOPE (Figure 3 and Figure 4 C). In some cases, the entire

periphery of fat globules was fluorescent, showing the homogeneous distribution of the fluorescent dye, while in other cases, both fluorescent and non-fluorescent areas were visible, indicating the partial labelling of the MFGM by the exogenous phospholipid Rh-DOPE. Three dimensional observations (i.e. recording of thin optical sections at different z-depths of milk samples) allowed the characterisation of non-fluorescently-labelled domains at the surface of milk fat globules composed by the MFGM (noted Z-depth 1 in Figure 4A) and of fluorescent rings seen in an equatorial cross section of the fat globule (noted Z-depth 2 in Figure 4B). These non-fluorescent areas were mostly circular in shape with a size of approximately 0.5 to 2 μm and a number generally ranging from zero to nine in one side of fat globule (Figures 4). Differences in size and shape of the non-fluorescent areas, however, were also observed (Figure 4). For example, Figure 4 shows fat globules with circular non-fluorescent areas of size ranging from very small (Figure 4 B) to very large (Figure 4 E), while fat globules with irregular shaped non-fluorescent regions have also been characterized (Figure 4 F, G, H). The irregular shapes could result from the connection of several circular domains at the surface of fat globules or to different physical states of the lipids in the domains. Non-fluorescent domains not stained by the Rh-DOPE can diffuse in the plane of the membrane as a function of time, as observed at the surface of a fat globule (highlighted by arrows in Figure 5). This result shows that the MFGM is a highly dynamic system, as previously reported for the bovine MFGM (Lopez et al., 2010).

3.4. Simultaneous localisation of polar lipids, glycoproteins and glycolipids in buffalo MFGM

The organisation of lipids and the localisation of the MFGM glycoproteins and glycolipids, *in situ* around buffalo milk fat globules, was investigated by CLSM with the simultaneous labelling of phospholipids with Rh-DOPE that stain the Ld phase and glycosylated molecules

that are specifically probed using the fluorescently labelled lectin wheat germ agglutinin (WGA-488). DIC optical microscopy in combination with CLSM permitted the observation of fat globules and the localisation of the components in the MFGM surrounding fat globules.

Figure 6 shows the heterogeneous lateral organisation of lipids and glycoproteins in the MFGM. Figure 6 A focuses on the top view of a fat globule (noted Z-depth 1) of about 10 µm diameter, while Figure 6 B-D shows an equatorial cross section of the same fat globule (at a higher Z-depth, noted Z-depth 2), with TAG in the core and the MFGM at the periphery. The lectin stain WGA-488 is reported to bind to N-acetylglucosamine and N-acetylneuraminic acid residues (Evers et al., 2008; Lopez et al., 2010), including the glycolipids such as gangliosides and the main glycoproteins of the MFGM such as butyrophilin, mucin and lactadherin (Evers et al., 2008; Heid & Keenan, 2005; Lopez et al., 2010). The distribution of these glycosylated molecules is non-homogeneous occurring in patches or networks in some areas of the MFGM of buffalo milk fat globules (Figure 6 and 7).

The co-localisation of polar lipids and glycosylated molecules reveals that most of the non-fluorescent areas observed when the MFGM was labelled with Rh-DOPE were also not fluorescent after labelling with WGA-488 (Figure 6). This is an additional argument in favour of the interpretation of the domains in which Rh-DOPE did not integrate, as being domains rich in SM and DPPC (i.e. high Tm lipids that are not glycosylated). Some exceptions were observed, however, where areas that were not stained by Rh-DOPE but stained by WGA-488 (areas indicated by arrows in Figure 7), indicating that some glycoproteins and/or glycolipids are located in the high Tm lipid rich domains. Also, some glycosylated molecules were observed at the periphery of the domains not labelled by Rh-DOPE (Figure 8). These results suggest that the glycosylated molecules are mostly present in the fluid phase of the MFGM but they can also participate in the high Tm lipid domains at times. Figure 7 E shows that

304 glycosylated molecules protrude in the aqueous phase surrounding buffalo milk fat globules,
305 and contribute in the formation of a glycocalyx.

306 The heterogeneous distribution of glycosylated molecules has also been reported in
307 previous studies for bovine, sheep and human milk (Evers et al., 2008; Gallier et al., 2010;
308 Lopez et al., 2011; Lopez et al., 2010). Non-fluorescent regions have been observed within
309 human and bovine MFGM when dual-staining with Rh-DOPE and WGA-488 has been
310 applied (Gallier et al., 2010; Lopez et al., 2011; Lopez et al., 2010; Lopez & Ménard, 2011)
311 and these non-fluorescent areas were attributed to SM-rich domains. Non-fluorescent areas
312 were also observed within the bovine, sheep and human MFGM when the milk was single
313 stained with WGA488 or when dual stained with WGA488 and the lipophilic probe FMN-64
314 (Evers et al., 2008), although these studies interpreted these non-fluorescent areas as the
315 regions where the MFGM is absent. The lateral organisation of polar lipids and proteins in the
316 MFGM could be involved in specific functions (including the mechanisms of lipid digestion
317 or protection of the neonates from infections), which currently remain unknown and require
318 further investigation.

319 ***3.5. The MFGM: a non-random mixing of components***

320 We observed that the MFGM is a non-random mixture of components characterised by the
321 lateral separation of compositionally distinct lipid phases. The occurrence of both fluorescent
322 and non-fluorescent areas observed with Rh-DOPE staining was interpreted as a
323 heterogeneous distribution of the polar lipids within the buffalo MFGM. Phase separation
324 would result in preferential partition of select lipids in the coexisting phases as a function of
325 their chemical and physical properties. Such heterogeneities in the partitioning of Rh-DOPE
326 stained lipids have been previously observed in the human and bovine MFGM (Lopez et al.,
327 2011; Lopez et al., 2010; Lopez & Ménard, 2011; Zou et al., 2012).

328 The non-fluorescent areas characterised at room temperature for the buffalo MFGM could
329 correspond to the preferential tight packing of polar lipids with a high gel to liquid crystalline
330 phase transition temperature (high Tm), leading to their lateral segregation from very low Tm
331 polar lipids in the liquid phase of the MFGM. These high Tm polar lipids consist of long-
332 chain saturated fatty acids (i.e. C16:0, P; C18:0, S; and fatty acids with a number of carbon >
333 20). According to the fatty acid composition of polar lipids in the MFGM (Sanchez-Juanes,
334 Alonso, Jancada, & Hueso, 2009) and the preferential concentration of PC and SM in the
335 outer layer of the MFGM (Deeth, 1997), these polar lipids could be SM (the main fatty acids
336 being C16:0, C20:0, C22:0, C23:0, C24:0; Lopez (2011)) and PC (i.e. DPPC, PSPC or SSPC).
337 Under the conditions examined (T=19 °C), these milk SM and saturated PC molecular species
338 are expected to be in the gel phase, as they have a Tm > 19 °C. Such high Tm polar lipids can
339 also segregate together with cholesterol in the plane of the biological membranes to form
340 ordered lipid domains in the Lo phase (Brown & London, 1998). The role played by
341 cholesterol in the MFGM is important, since a minimum molar percentage of cholesterol is
342 necessary to form the Lo phase with high Tm lipids, as reported in phase diagrams built with
343 controlled lipid compositions (Veatch & Keller, 2005). The coexistence of two ordered phases
344 (i.e. the Lo phase domains including high Tm lipids and cholesterol and the gel phase
345 domains composed only by high Tm lipids) with one fluid phase composed by unsaturated
346 polar lipids may occur, as already discussed for ternary model systems (Veatch & Keller,
347 2005). The ordered phases (gel and Lo phases) are immiscible with the MFGM fluid phase
348 and form micron-scale domains, permitting their observation by confocal microscopy. The
349 circular shape of the domains observed in the buffalo MFGM at room temperature is
350 consistent with the formation of Lo phase domains, since polar lipids organised in a gel phase
351 are semi-crystallised and form angles (Brown & London, 1998; Morales-Penningston et al.,
352 2011). Non-circular domains present in the outer bilayer of MFGM have been previously

353 reported at room temperature for human milk (Lopez & Ménard, 2011) but have not been
354 observed for the bovine MFGM (Lopez et al., 2010). This difference in the morphology of the
355 lipid domains observed in the MFGM from various species could possibly be due to the
356 differences in the composition of the polar lipids such as the concentration and individual
357 lipid species with high Tm (for example SM and DPPC) and the relative proportion of low
358 Tm lipids, high Tm lipids and cholesterol (Veatch & Keller, 2005). The relative proportion of
359 SM, which is assumed to be the major component of the Lo phase domains (Gallier et al.,
360 2010; Lopez et al., 2010), is lower in the buffalo MFGM compared to the bovine MFGM
361 (24.8% vs. 26.9% of polar lipid) (Ménard et al., 2010). It has been reported that the
362 morphology of the SM-rich domains and the composition of the bovine MFGM could be
363 linked to fat globule size (Gallier et al., 2010; Lopez et al., 2011). Although some studies
364 have reported that small bovine fat globules appear to have fewer domains compared to large
365 fat globules (Gallier et al., 2010), others did not find any significant differences arising from
366 the characterisation of domains in small fat globules (diameter < 2 µm) (Lopez et al., 2011).
367 The lipid composition of the outer bilayer of the MFGM could be considered as a ternary
368 system consisting of high Tm polar lipids (e.g. SM and saturated PC), cholesterol and low Tm
369 polar lipids (e.g. unsaturated glycerophospholipids) with differences in membrane structure
370 and dynamics arising from compositional differences between species.

371

372 The organisation of lipids in the MFGM can govern the functional properties of this
373 biomembrane, affect the localisation of membrane-associated proteins and the chemical
374 reactions that occur in the MFGM or that are mediated by this membrane. Lipid phase
375 separation could also influence the binding of molecules to the MFGM, such as bacteria and
376 viruses (Lopez, 2011).The MFGM plays a key role in the physical stability of fat globules in
377 milk including protection against coalescence and aggregation. Buffalo milk fat globules are

important in the preparation of dairy products such as yoghurt and cheese. Hence, the lipid domains formed in the MFGM, could be involved in a number of functions, warranting further investigation of these microstructural features.

4. Conclusion

Buffalo fat globules and their biological membrane are poorly characterised despite the importance of buffalo milk to human nutrition and dairy processing. This study investigated the microstructure of the buffalo MFGM improving our knowledge of this dynamic and complex biological membrane. We showed that the MFGM is heterogeneous with respect to lipid, cholesterol and protein assemblies. Confocal experiments revealed that polar lipids segregated in the outer bilayer of the MFGM into two or more liquid phases (e.g. gel, Lo and Ld phases) that can coexist and have different physical properties, with gel and Ld phases being most probable. Glycoproteins and glycolipids were mainly present in the Ld fluid phase but also existed in the lipid domains (gel or Lo phases) at times. Whether or not the lipid domains in buffalo MFGM are lipid rafts remains unknown and would be worthy of further study.

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All authors have approved the final article.

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FIGURE CAPTION

Figure 1: Size distribution of buffalo milk fat globules determined using light scattering (A) in combination with microscopy (B-D). (B) CLSM image with Nile Red labeled fat globule cores appearing red and Fast Green FCF labeled protein appearing green, (C) Image taken with differential interferential contrast and (D) overlay of images A and B. The scale bars are 10 μm in length. Arrows indicate fat globules with a rough surface.

Figure 2: Microscopy images showing the presence of cytoplasmic remnants (CR) attached to buffalo milk fat globules. (A) DIC image, (B) CLSM image of fat globules stained with Rh-DOPE, (C) overlay of images A and B, (D and E) CLSM images with Rh-DOPE. Images were captured using a 100x objective. The scale bars are indicated in the images. Thick arrows indicate cytoplasmic remnant, thin arrows show the non-fluorescent domain in the biological membrane surrounding fat globules. TAG core = triacylglycerol core of milk fat globules.

Figure 3: Microscopy images showing an equatorial cross-section of fat globules and the heterogeneous distribution of polar lipids within the buffalo MFGM surrounding the milk fat

globules. **(A)** CLSM image after labelling with Rh-DOPE (red colour), **(B)** overlay of DIC optical microscopy image and CLSM image. The non-fluorescent areas (dark areas) observed around the fat globules correspond to the lateral segregation of high phase transition temperature polar lipids in domains (indicated by the white arrows). Images were captured at room temperature (19 ± 1°C) using a 100x objective. The scale bars are indicated in the figures.

Figure 4: Microscopy images showing the heterogeneous distribution of polar lipids within the buffalo MFGM. (A, B, C) CLSM images after labelling with Rh-DOPE (red colour), (D-H) overlay of DIC optical microscopy image and CLSM image. The non-fluorescent areas (dark areas) observed around the fat globules correspond to the lateral segregation of high phase transition temperature polar lipids in domains (indicated by the white arrows). Images were captured at room temperature (19 ± 1°C) using a 100x objective. The scale bars are indicated in the figures.

Figure 5: CLSM images taken at room temperature showing the diffusion of a non-fluorescent lipid domain within the MFGM as a function of time (A) t = 0 sec, (B) t = 30 sec. The polar lipids within the MFGM were stained using Rh-DOPE (red colour). The scale bars are indicated in the figures.

Figure 6: Microscopy images showing the heterogeneous distribution of polar lipids and glycosylated molecules (glycoproteins and glycolipids) in the buffalo milk fat globule membrane characterised at different z-depths. In-situ observations performed by the combination of DIC optical microscopy and CLSM, using Rh-DOPE to label the phospholipids (red colour) and the lectin WGA-488 to stain the glycosylated molecules (green colour). **(A)** overlay of DIC and fluorescence (Rh-DOPE and WGA) at z-depth 1 corresponding to the polar region of fat globules, (**B**) overlay of DIC and fluorescence (Rh-DOPE and WGA-488) at z-depth 2 corresponding to the equatorial section of fat globules, (**C**) overlay of DIC and CLSM image with WGA-488, (**D**) overlay of DIC and CLSM image with Rh-DOPE. Images were captured using a 100x objective. The scale bars are indicated in the figures. White arrows correspond to non-fluorescent domains, green arrows correspond to glycosylated molecules located at the periphery or within a domain in which Rh-DOPE did not integrate.

Figure 7: Microscopy images showing the heterogeneous distribution of polar lipids and glycosylated molecules (glycoproteins and glycolipids) in the buffalo milk fat globule membrane. In-situ observations performed by the combination of DIC optical microscopy and CLSM, using Rh-DOPE to label the phospholipids (red colour) and the lectin WGA-488 to stain the glycosylated molecules (green colour). **(A)** DIC image, **(B)** overlay of DIC and fluorescence with Rh-DOPE, **(C)** overlay of DIC image and fluorescence with WGA-488, **(D)** overlay of DIC image with Rh-DOPE and WGA-488, **(E)** equatorial cross section of the same fat globule showing the overlay of DIC image with Rh-DOPE and WGA-488. Images were captured using a 100x objective. The scale bars are indicated in the figures. White arrows correspond to non-fluorescent domains, green arrows correspond to glycosylated molecules located at the periphery or within a domain in which Rh-DOPE did not integrate.

Figure 8: Microscopy images showing the heterogeneous distribution of polar lipids and glycosylated molecules (glycoproteins and glycolipids) in the buffalo milk fat globule membrane. In-situ observations performed by the combination of DIC optical microscopy and CLSM, using Rh-DOPE to label the phospholipids (red colour) and the lectin WGA-488 to stain the glycosylated molecules (green colour). (A) overlay of DIC image with Rh-DOPE and WGA-488, (B) overlay of DIC image with fluorescence with WGA-488, (C) overlay of DIC image and fluorescence with Rh-DOPE, (D) DIC image. Images were captured using a 100x objective. The scale bars are indicated in the figures. White arrows correspond to glycosylated molecules located at the periphery or within a domain in which Rh-DOPE did not integrate.

FIGURES

Figure 1

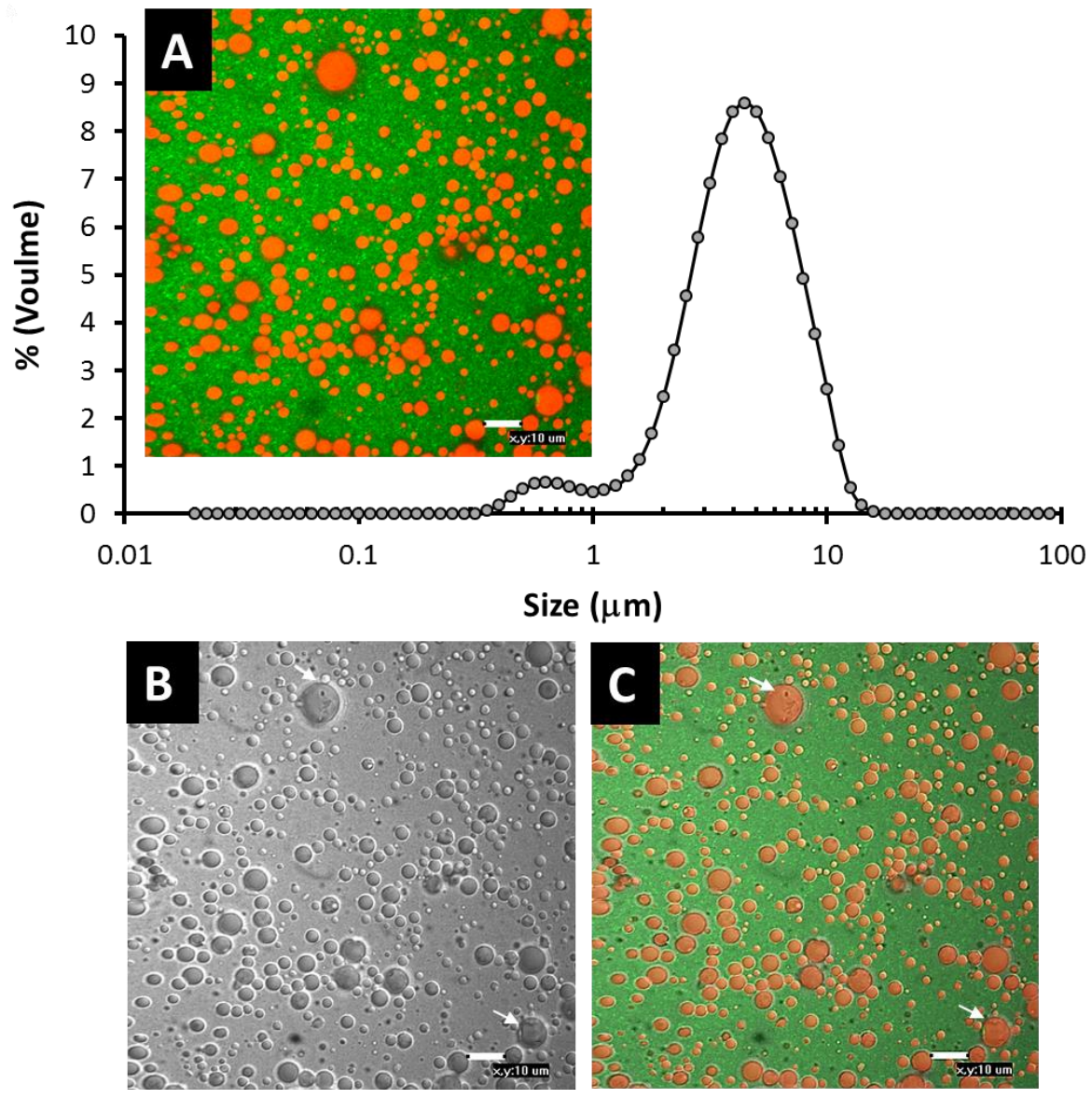


Figure 2

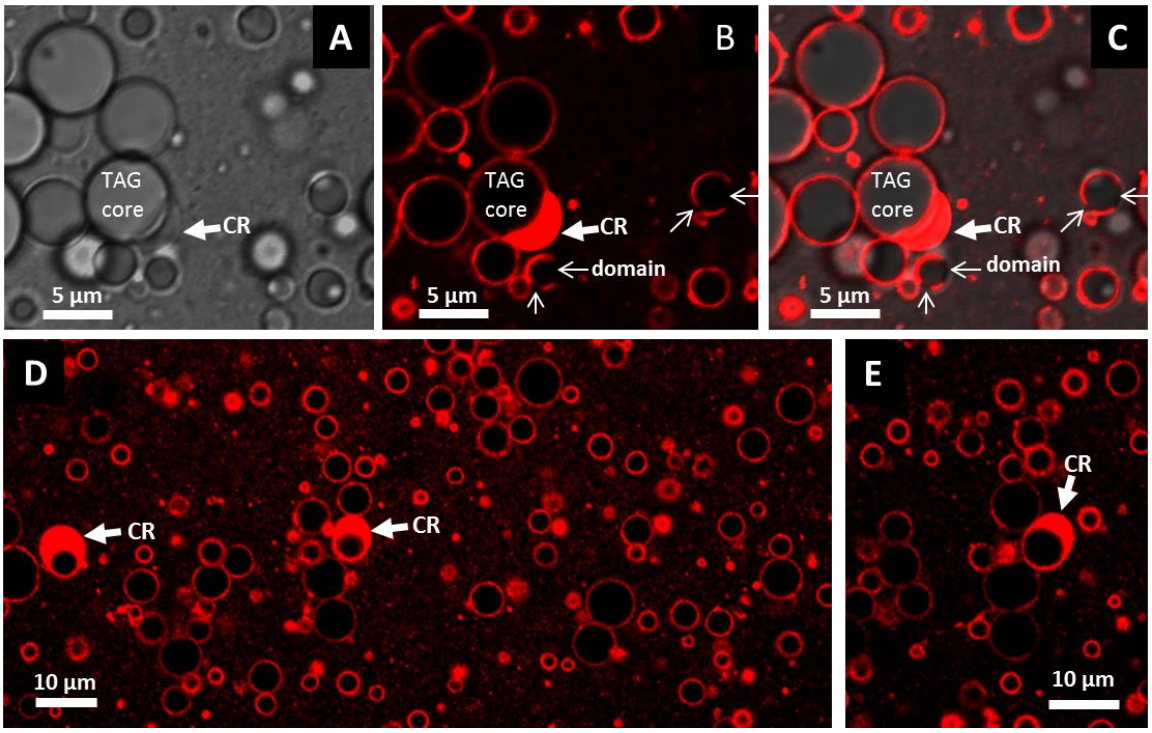

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Figure 3

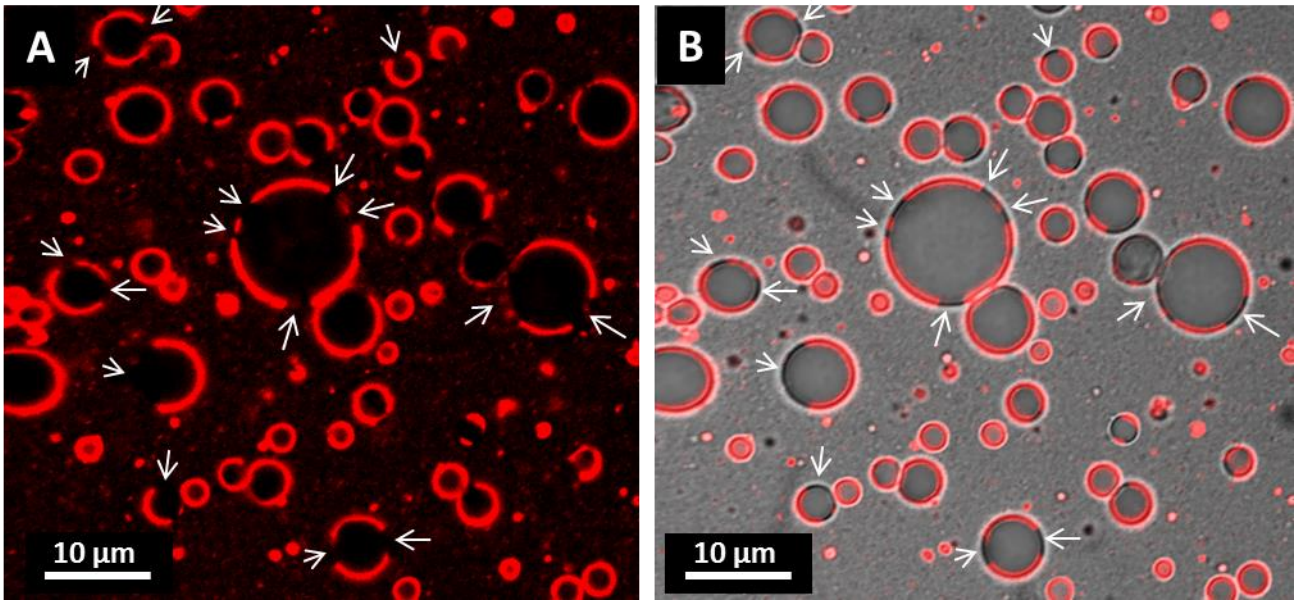


Figure 4

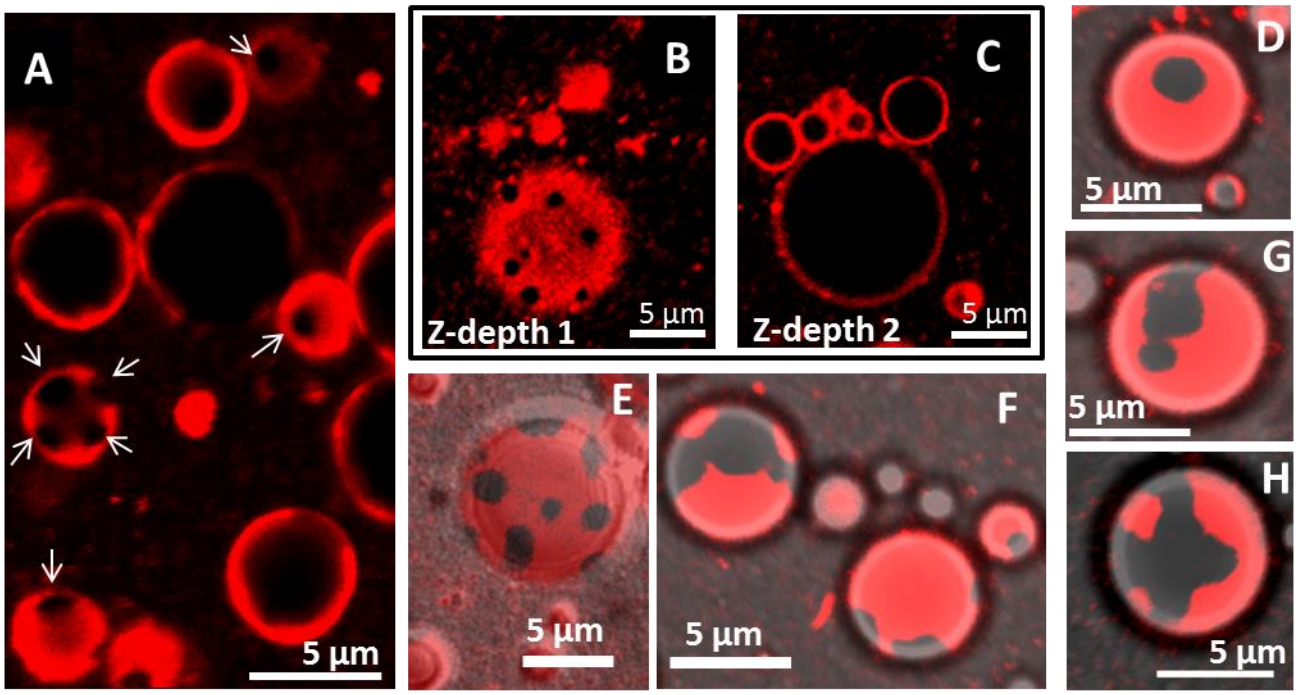


Figure 5

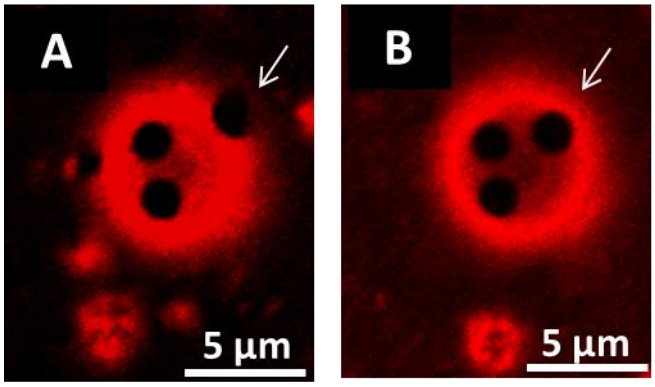
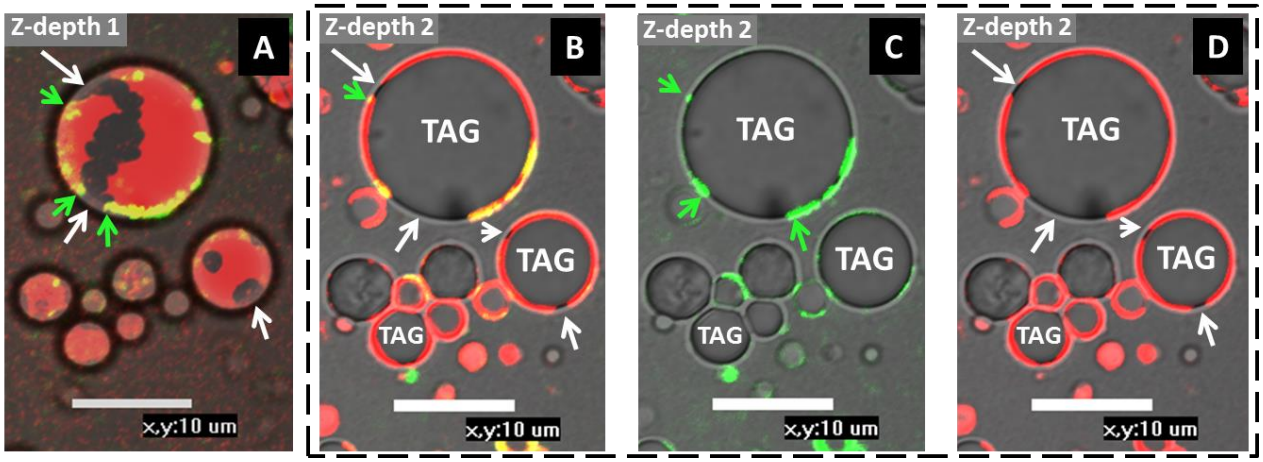
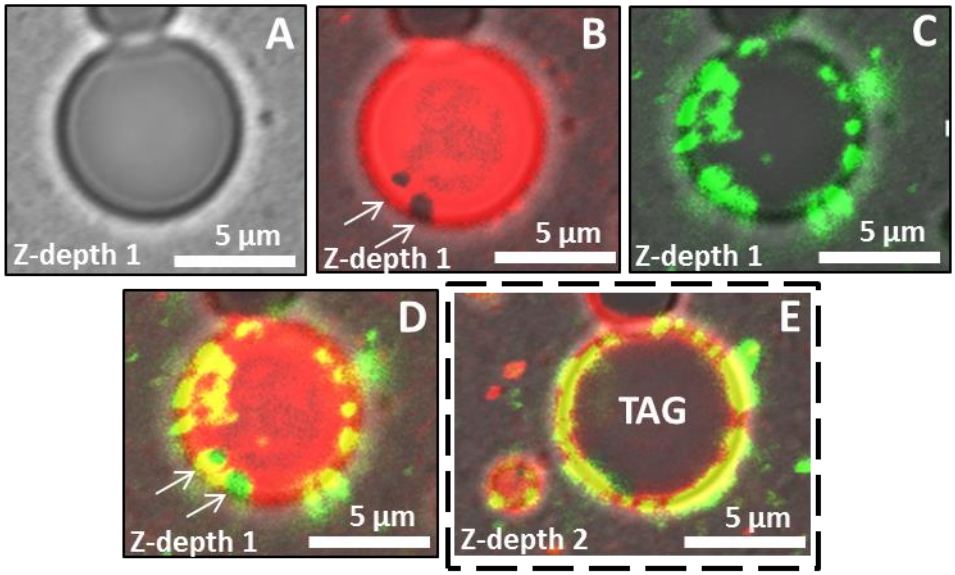


Figure 6



644 **Figure 7**

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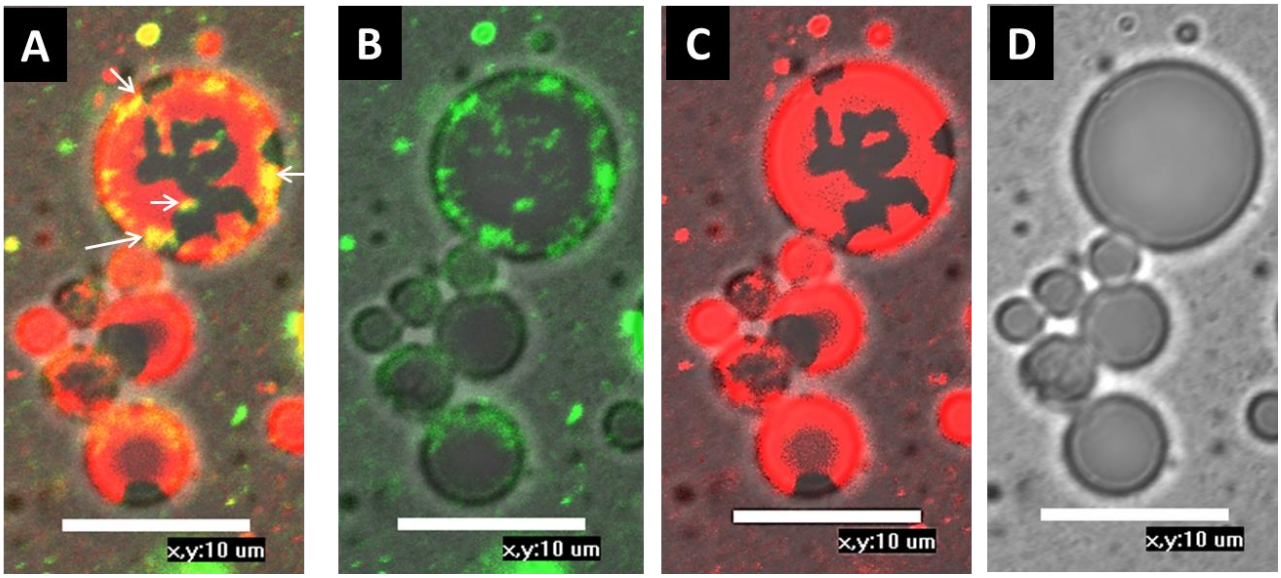
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649 **Figure 8**

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