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

25 # ABSTRACT

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

41

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

44

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).

69 This biological membrane is thought to be comprised of three layers, containing mainly of
70 glycerophospholipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE;
71 phosphatidylinositol, PI; phosphatidylserine, PS), sphingolipids (mainly sphingomyelin, SM),
72 cholesterol and proteins (Keenan & Patton, 1995; Ménard et al., 2010). Polar lipids, which are
73 mainly located in the MFGM, account for about 2.6 mg/g fat and about 190 mg/L of buffalo
74 milk (Ménard et al., 2010), which is 28% higher than in bovine milk (Ménard et al., 2010).
75 Also, buffalo milk contains less cholesterol than bovine milk (7.0-10.2 mg/100mL vs. 10.5-
76 19.8 mg/100 mL) (Strzalkowska, Jozwik, Baghnicka, Krzyzewski, & Horbanczuk, 2009;
77 Talpur, Memom, & Bhanger, 2007; Zotos & Bampidis, 2014). The average content of
78 membrane protein was reported to be less in buffalo MFGM compared to bovine milk, either
79 when calculated as a percentage of fat (4.2% vs. 4.9%) or as a proportion of the total MFGM
80 material (29.5% vs. 33.9%) (Abou-Dawood et al., 1988). A recent proteomic study identified
81 50 proteins within buffalo MFGM, with the major proteins being as xanthine
82 dehydrogenase/oxidase, butyrophilin, adipophilin, lactadherin and mucin, similar to bovine
83 MFGM proteins (D'Ambrosio et al., 2008; Fong, Norris, & MacGibbon, 2007). Both the
84 composition and the structure of the MFGM result from the mechanisms of secretion of fat
85 globules from the epithelial cells of the mammary gland (Heid & Keenan, 2005). The
86 compositional differences reported to date suggest there may be further underlying structural
87 differences in the MFGM in buffalo milk and possible differences occurring during the *in vivo*
88 secretion of these globules during milk production.

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

94 2012). This heterogeneous distribution has been attributed to the phase separation of polar
95 lipids according to their temperature of phase transition (Tm). The exogenous phospholipid
96 fluorescently head-labelled with rhodamine (i.e. Rh-DOPE; PE containing 2 oleic acids with
97 very low phase transition temperature) preferentially partitions in the fluid Ld phase
98 containing unsaturated polar lipids (i.e. containing one or two long-chain unsaturated fatty
99 acids) (Lopez et al., 2010). Hence, areas of the MFGM not stained by Rh-DOPE may
100 correspond to gel phase domains rich in polar lipids with a high Tm. Polar lipids not stained
101 by Rh-DOPE could adopt a second possible phase consisting of liquid ordered (Lo) phase
102 domains composed of high Tm lipids and cholesterol. These high Tm lipids include SM,
103 which accounts for about 25% of polar lipids in the MFGM (Lopez, 2011) and contains long
104 chain saturated fatty acids (Tm = 35 °C; Malmsten, Bergentahl, Nyberg, and Odham (1994)).
105 Saturated PC are also high Tm lipids found in the MFGM (e.g. dipalmitoyl
106 phosphatidylcholine, DPPC, Tm = 41.7 °C; Benesch and McElhaney (2014)). The
107 surrounding membrane stained by Rh-DOPE may correspond to the fluid liquid disordered
108 (Ld) phase of the unsaturated glycerophospholipids (PC, PE, PI, PS) which have a low Tm
109 and are responsible for the fluidity of the biomembranes (Gallier et al., 2010; Lopez et al.,
110 2010). A recent study performed by atomic force microscopy with a model membrane showed
111 that milk SM segregates from the fluid Ld phase of unsaturated polar lipids to form domains
112 in the gel phase (Guyomarc'h et al., 2014). This study also showed that cholesterol has a large
113 effect on the domains formed by milk SM; the shapes and nanomechanical properties of the
114 domains formed differ in the gel phase in the absence of cholesterol and in the Lo phase when
115 cholesterol is present. The domains also protrude further from the fluid phase of the
116 membrane in the absence of cholesterol (Guyomarc'h et al., 2014).

117 The lipid domains recently revealed in the MFGM could potentially be responsible for several
118 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

141 were set at 1.46 (at 466 nm) and 1.458 (at 633 nm) and the refractive index was set at 1.33
142 for water. The absorption coefficient used was 0.0001. The experiments were performed
143 at room temperature. Aliquots of approximately 70 µL of whole milk were introduced into
144 the measurement cell of the apparatus, which contained 100 ml of water, in order to reach
145 10% obscuration (optimal conditions for particle-size measurements with this apparatus).
146 A solution of 35 mM EDTA/NaOH pH 7.0 buffer (>98% disodium salt dihydrat, Prolabo,
147 Fortenay-sous-Bois, France) was added to the measurement cell to disrupt the casein
148 micelles. All analyses were performed in triplicate with three replicate milk samples
149 collected from different days, giving the total of nine measurements. The specific surface
150 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
151 number of fat globules of diameter d_i, were calculated by the software. ***Microstructural***
152 ***analysis***

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

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

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

172 The concentrations of the fluorescent dyes were adapted to the specific composition of
173 buffalo milk. For observation of the fat globules, 100 µL of Nile Red and 10 µL of Fast Green
174 FCF were added to 1 mL of buffalo milk samples. For observation of the MFGM polar lipids,
175 40 µL of the Rh-DOPE solution was placed in a glass vial and the chloroform was evaporated
176 under nitrogen, to avoid the possible artefacts caused by this organic solvent. Then 1 mL of
177 milk sample was introduced in the vial. For dual staining of the MFGM polar lipids and
178 carbohydrate moieties, 10 µL of the WGA-488 solution was also added into the vial
179 containing Rh-DOPE. Then, 1 mL of milk sample was added to the vial. The stained milk
180 samples were kept at room temperature for at least 1 h prior to observation by CLSM. The
181 microstructural analyses were performed at room temperature (19 ± 1 °C)

182 Optical microscopy using differential interference contrast (DIC, also called Nomarski,
183 Cogswell and Sheppard (1992)) was also used to characterize buffalo milk fat globules. DIC
184 images were superimposed on the fluorescent emission recorded in the CLSM images. The
185 two dimensional images had a resolution of 512×512 pixels and the pixel scale values were
186 converted into micrometers using a scaling factor. In the multiple labeled samples, different
187 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).

209 ***3.2 Cytoplasmic remnants attached to buffalo milk fat globules***

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

218 CR occur when the mechanisms involved in the closure of the apical plasma membrane
219 behind the projecting lipid droplet lead to their secretion with a piece of cytoplasm remaining
220 attached to fat globule in milk (Heid & Keenan, 2005). The occurrence of the CR has also
221 been reported in previous CLSM studies of human milk (Evers et al., 2008; Lopez & Ménard,
222 2011) but CR have not been observed in bovine milk (Lopez et al., 2010) when the same
223 CLSM methods and fluorescent probes have been applied. This is probably due to variations
224 in CR concentration between the milk of different species, arising from differences in the
225 membrane secretion process, which is poorly understood (Robenek et al., 2005). CR were
226 observed for 7.2% of the fat globules within human milk but for less than 1% of the fat
227 globules in bovine milk, quantified using microscopic observation with the assistance of a
228 counting chamber slide (Huston & Patton, 1990). In goat milk, between 1% and 5% of milk
229 fat globules had CR (Wooding, Peaker, & Linzell, 1970). The current study is the first to
230 report the presence of CR in buffalo milk but the number of CR was not quantified, which
231 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

256 periphery of fat globules was fluorescent, showing the homogeneous distribution of the
257 fluorescent dye, while in other cases, both fluorescent and non-fluorescent areas were visible,
258 indicating the partial labelling of the MFGM by the exogenous phospholipid Rh-DOPE. Three
259 dimensional observations (i.e. recording of thin optical sections at different z-depths of milk
260 samples) allowed the characterisation of non-fluorescently-labelled domains at the surface of
261 milk fat globules composed by the MFGM (noted Z-depth 1 in Figure 4A) and of fluorescent
262 rings seen in an equatorial cross section of the fat globule (noted Z-depth 2 in Figure 4B).
263 These non-fluorescent areas were mostly circular in shape with a size of approximately 0.5 to
264 2 μm and a number generally ranging from zero to nine in one side of fat globule (Figures 4).
265 Differences in size and shape of the non-fluorescent areas, however, were also observed
266 (Figure 4). For example, Figure 4 shows fat globules with circular non-fluorescent areas of
267 size ranging from very small (Figure 4 B) to very large (Figure 4 E), while fat globules with
268 irregular shaped non-fluorescent regions have also been characterized (Figure 4 F, G, H). The
269 irregular shapes could result from the connection of several circular domains at the surface of
270 fat globules or to different physical states of the lipids in the domains. Non-fluorescent
271 domains not stained by the Rh-DOPE can diffuse in the plane of the membrane as a function
272 of time, as observed at the surface of a fat globule (highlighted by arrows in Figure 5). This
273 result shows that the MFGM is a highly dynamic system, as previously reported for the
274 bovine MFGM (Lopez et al., 2010).

275 ***3.4. Simultaneous localisation of polar lipids, glycoproteins and glycolipids in buffalo***
276 ***MFGM***

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

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

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

293 The co-localisation of polar lipids and glycosylated molecules reveals that most of the non-
294 fluorescent areas observed when the MFGM was labelled with Rh-DOPE were also not
295 fluorescent after labelling with WGA-488 (Figure 6). This is an additional argument in favour
296 of the interpretation of the domains in which Rh-DOPE did not integrate, as being domains
297 rich in SM and DPPC (i.e. high Tm lipids that are not glycosylated). Some exceptions were
298 observed, however, where areas that were not stained by Rh-DOPE but stained by WGA-488
299 (areas indicated by arrows in Figure 7), indicating that some glycoproteins and/or glycolipids
300 are located in the high Tm lipid rich domains. Also, some glycosylated molecules were
301 observed at the periphery of the domains not labelled by Rh-DOPE (Figure 8). These results
302 suggest that the glycosylated molecules are mostly present in the fluid phase of the MFGM
303 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

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

381

382 4. Conclusion

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

394

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403

404 All authors have approved the final article.

405

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529 **FIGURE CAPTION**

530

531 **Figure 1:** Size distribution of buffalo milk fat globules determined using light scattering (A)
532 in combination with microscopy (B-D). (B) CLSM image with Nile Red labeled fat globule
533 cores appearing red and Fast Green FCF labeled protein appearing green, (C) Image taken
534 with differential interferential contrast and (D) overlay of images A and B. The scale bars are
535 10 µm in length. Arrows indicate fat globules with a rough surface.

536

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

544

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

547 globules. **(A)** CLSM image after labelling with Rh-DOPE (red colour), **(B)** overlay of DIC
548 optical microscopy image and CLSM image. The non-fluorescent areas (dark areas) observed
549 around the fat globules correspond to the lateral segregation of high phase transition
550 temperature polar lipids in domains (indicated by the white arrows). Images were captured at
551 room temperature (19 ± 1°C) using a 100x objective. The scale bars are indicated in the
552 figures.
553

554 **Figure 4**: Microscopy images showing the heterogeneous distribution of polar lipids within
555 the buffalo MFGM. (A, B, C) CLSM images after labelling with Rh-DOPE (red colour), (D-
556 H) overlay of DIC optical microscopy image and CLSM image. The non-fluorescent areas
557 (dark areas) observed around the fat globules correspond to the lateral segregation of high
558 phase transition temperature polar lipids in domains (indicated by the white arrows). Images
559 were captured at room temperature (19 ± 1°C) using a 100x objective. The scale bars are
560 indicated in the figures.
561

562 **Figure 5:** CLSM images taken at room temperature showing the diffusion of a non-
563 fluorescent lipid domain within the MFGM as a function of time (A) t = 0 sec, (B) t = 30 sec.
564 The polar lipids within the MFGM were stained using Rh-DOPE (red colour). The scale bars
565 are indicated in the figures.
566

567 **Figure 6:** Microscopy images showing the heterogeneous distribution of polar lipids and
568 glycosylated molecules (glycoproteins and glycolipids) in the buffalo milk fat globule
569 membrane characterised at different z-depths. In-situ observations performed by the
570 combination of DIC optical microscopy and CLSM, using Rh-DOPE to label the
571 phospholipids (red colour) and the lectin WGA-488 to stain the glycosylated molecules (green
572 colour). **(A)** overlay of DIC and fluorescence (Rh-DOPE and WGA) at z-depth 1
573 corresponding to the polar region of fat globules, (**B**) overlay of DIC and fluorescence (Rh-
574 DOPE and WGA-488) at z-depth 2 corresponding to the equatorial section of fat globules, (**C**)
575 overlay of DIC and CLSM image with WGA-488, (**D**) overlay of DIC and CLSM image with
576 Rh-DOPE. Images were captured using a 100x objective. The scale bars are indicated in the
577 figures. White arrows correspond to non-fluorescent domains, green arrows correspond to
578 glycosylated molecules located at the periphery or within a domain in which Rh-DOPE did
579 not integrate.
580

581 **Figure 7**: Microscopy images showing the heterogeneous distribution of polar lipids and
582 glycosylated molecules (glycoproteins and glycolipids) in the buffalo milk fat globule
583 membrane. In-situ observations performed by the combination of DIC optical microscopy
584 and CLSM, using Rh-DOPE to label the phospholipids (red colour) and the lectin WGA-488
585 to stain the glycosylated molecules (green colour). **(A)** DIC image, **(B)** overlay of DIC and
586 fluorescence with Rh-DOPE, **(C)** overlay of DIC image and fluorescence with WGA-488, **(D)**
587 overlay of DIC image with Rh-DOPE and WGA-488, **(E)** equatorial cross section of the same
588 fat globule showing the overlay of DIC image with Rh-DOPE and WGA-488. Images were
589 captured using a 100x objective. The scale bars are indicated in the figures. White arrows
590 correspond to non-fluorescent domains, green arrows correspond to glycosylated molecules
591 located at the periphery or within a domain in which Rh-DOPE did not integrate.
592
593
594 **Figure 8:** Microscopy images showing the heterogeneous distribution of polar lipids and
595 glycosylated molecules (glycoproteins and glycolipids) in the buffalo milk fat globule
596 membrane. In-situ observations performed by the combination of DIC optical microscopy
597 and CLSM, using Rh-DOPE to label the phospholipids (red colour) and the lectin WGA-488
598 to stain the glycosylated molecules (green colour). (A) overlay of DIC image with Rh-DOPE
599 and WGA-488, (B) overlay of DIC image with fluorescence with WGA-488, (C) overlay of
600 DIC image and fluorescence with Rh-DOPE, (D) DIC image. Images were captured using a
601 100x objective. The scale bars are indicated in the figures. White arrows correspond to
602 glycosylated molecules located at the periphery or within a domain in which Rh-DOPE did
603 not integrate.
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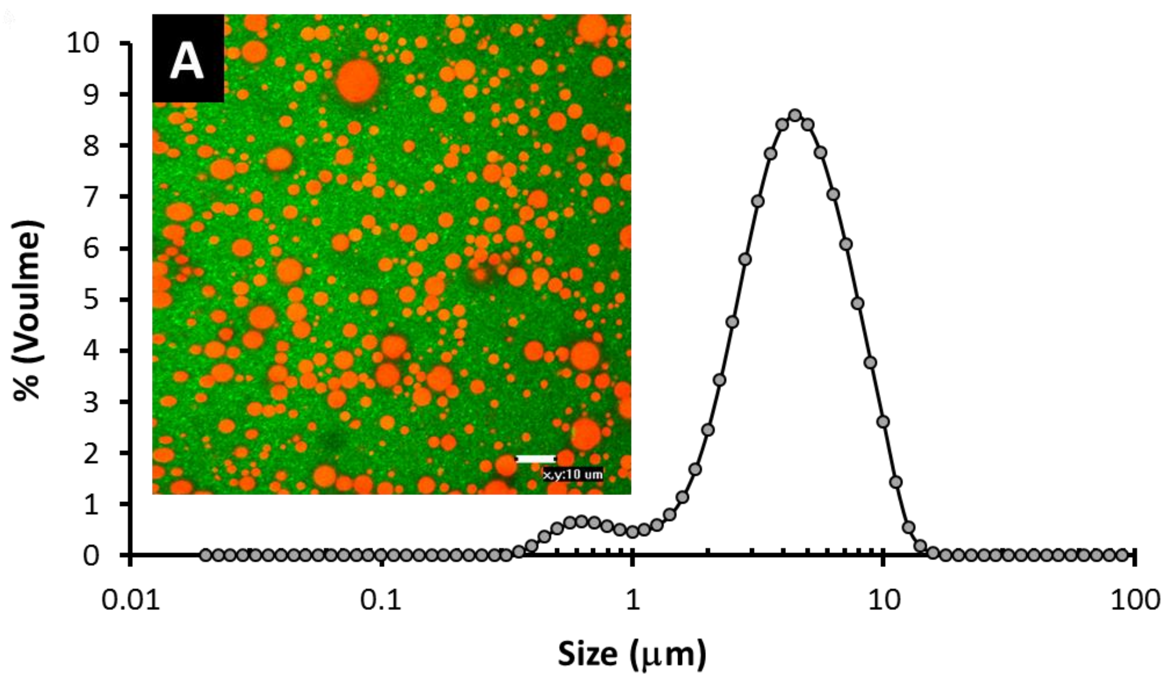
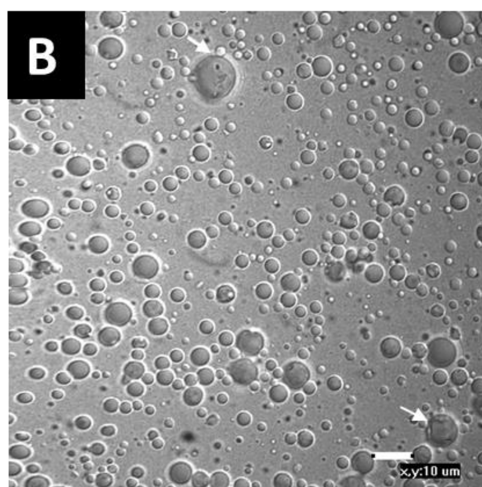
608 # FIGURES

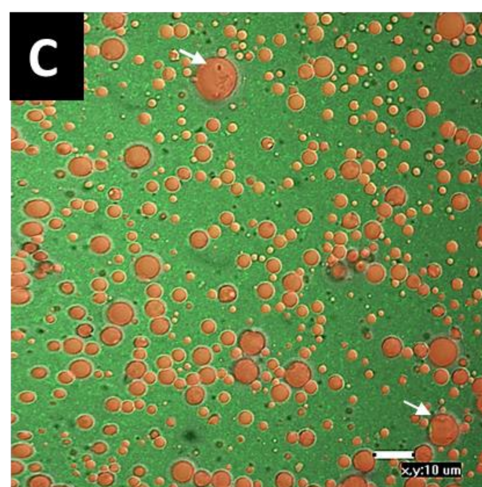
609

610 ## Figure 1

611



612 



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614 **Figure 2**

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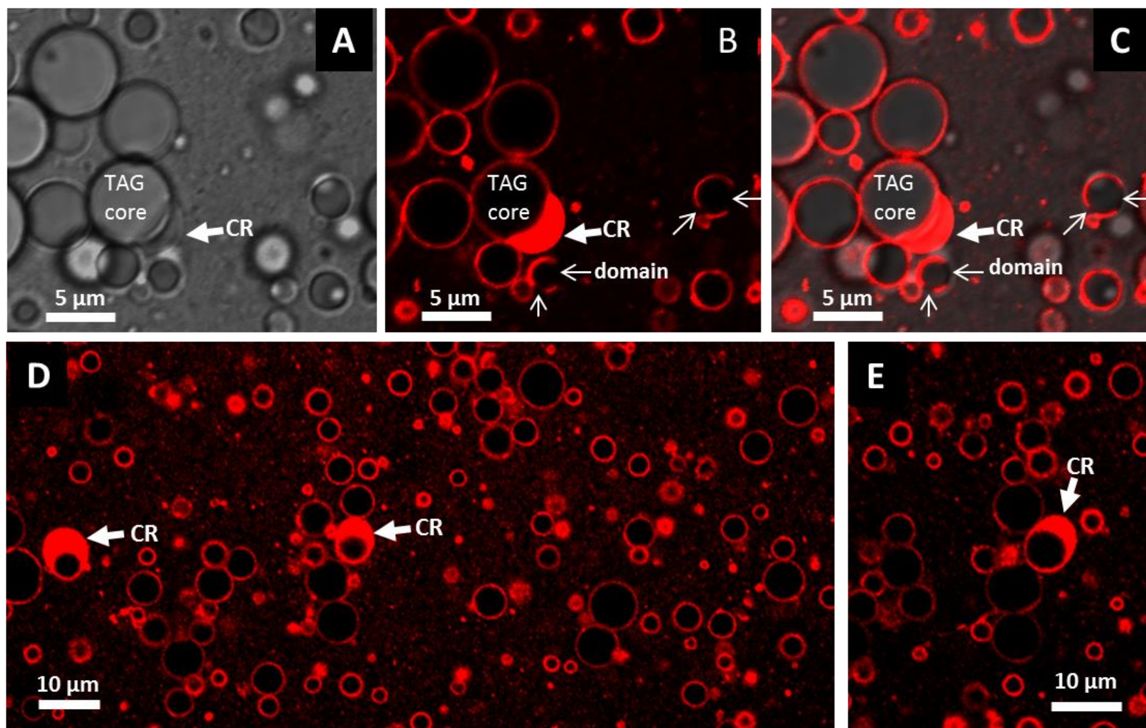

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619 **Figure 3**

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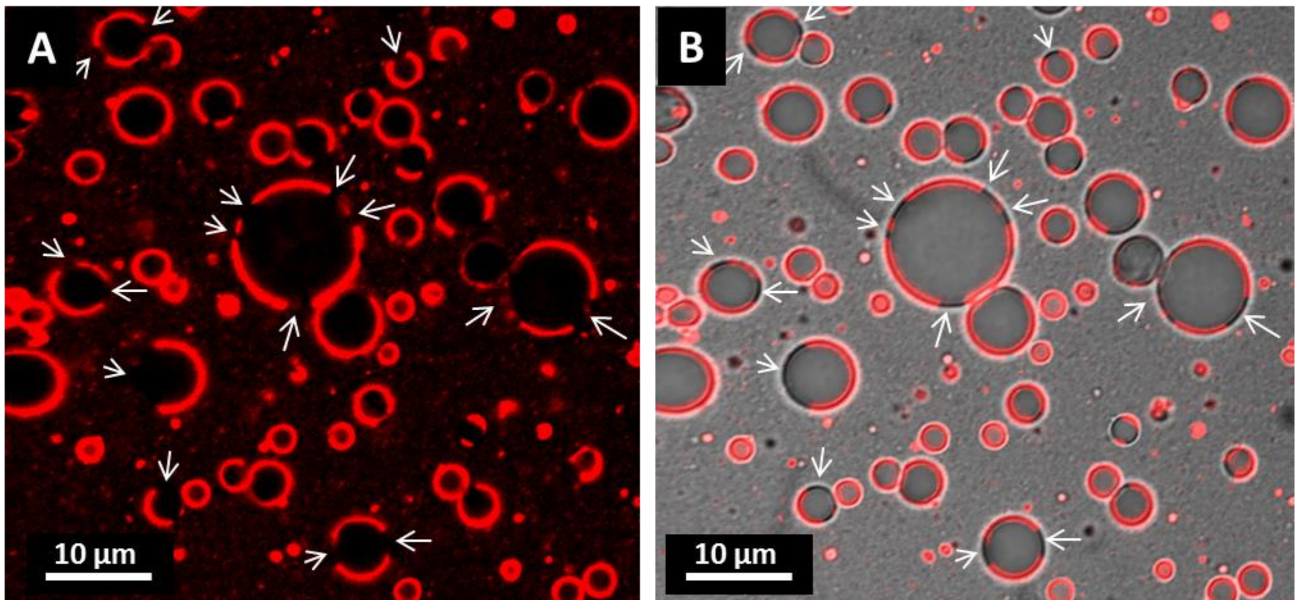

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624 **Figure 4**

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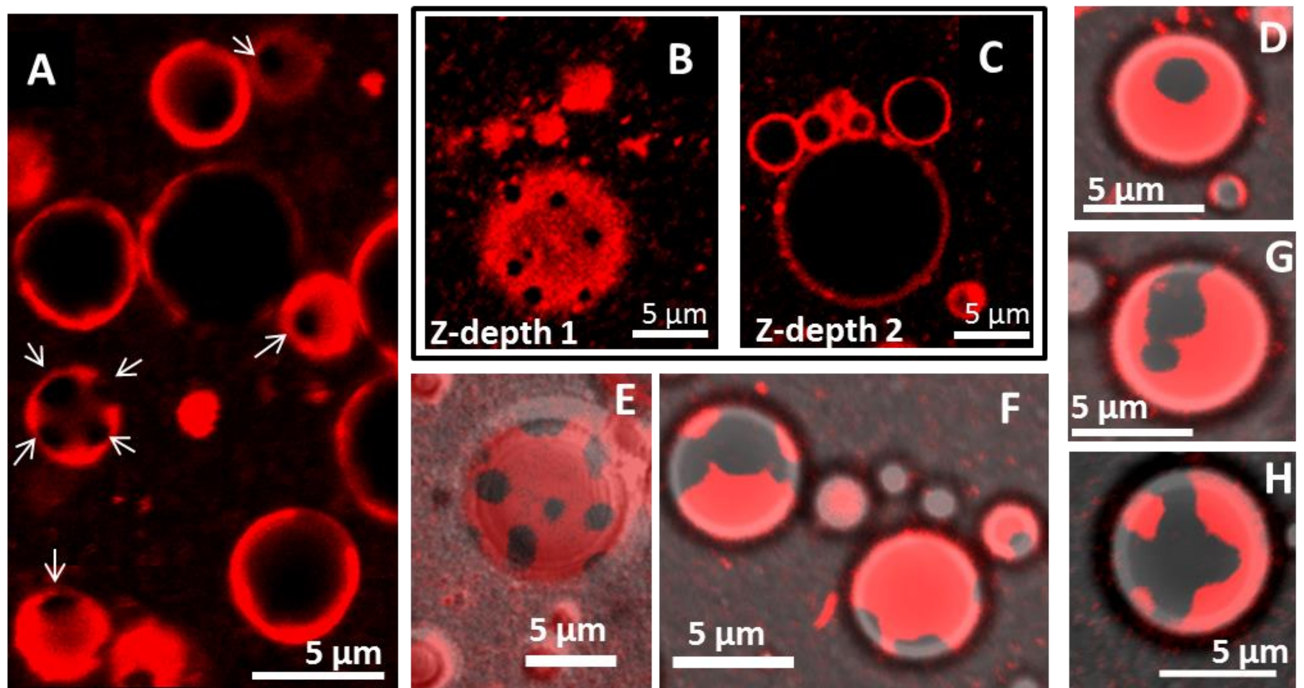

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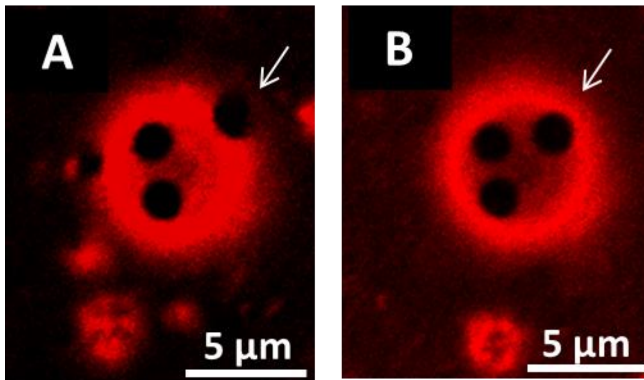
629 **Figure 5**



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633 **Figure 6**

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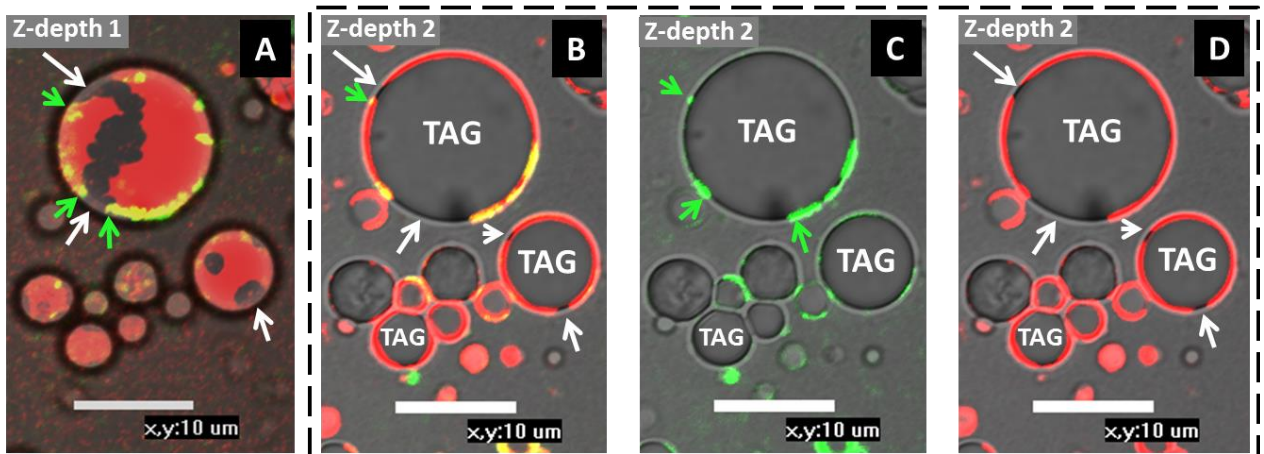

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644 **Figure 7**

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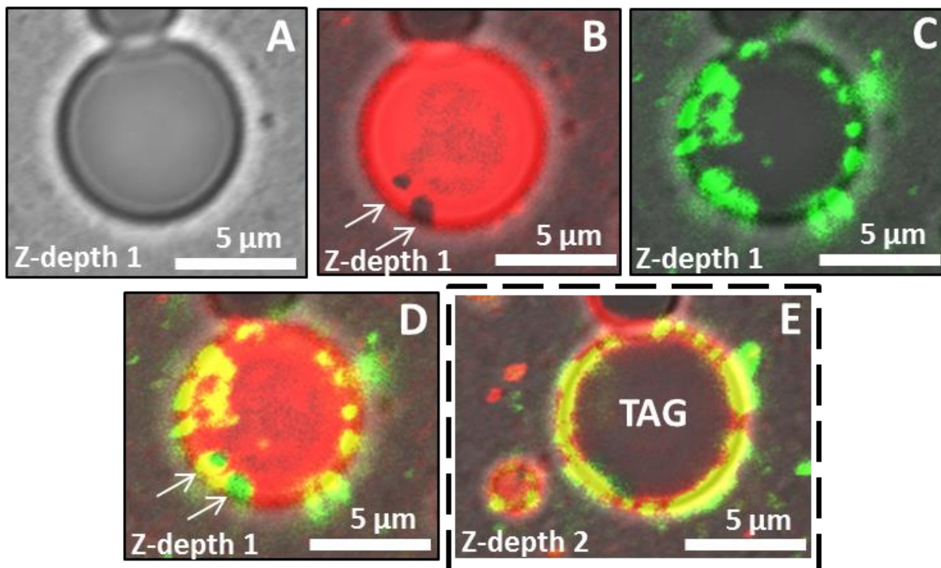

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649 **Figure 8**

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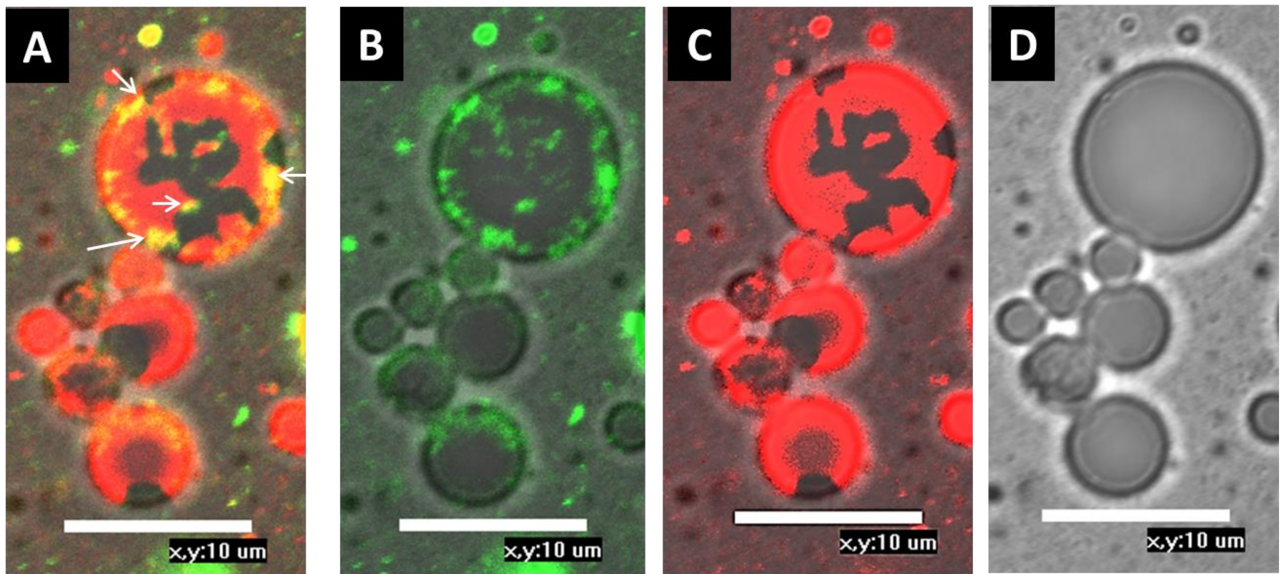

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