

1 **Alternative culture systems for bovine oocyte *in vitro* maturation: liquid marbles and**
2 **differentially shaped 96-well plates**

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24 Abstract

25 *In vivo* matured oocytes exhibit higher developmental competence than those matured *in vitro*,
26 but mimicking the *in vivo* environment by *in vitro* conditions has been challenging. Till now,
27 conventional two-dimensional (2D) systems have been used for *in vitro* maturation of bovine
28 cumulus-oocytes-complexes (COCs). However, using such systems may cause cell flattening
29 and does not allow cumulus expansion in all dimensions, which is less physiological.
30 Therefore, implementing a low-cost and highly effective *in vivo*-like microenvironment
31 methodology may help to optimize oocyte *in vitro* maturation. Here, we used two different
32 systems to culture COCs and evaluate their potential influence on embryo development and
33 quality. In the first system, we used treated fumed silica particles to create a 3D
34 microenvironment (liquid marbles; LM) to mature COCs. In the second system, we cultured
35 COCs in 96-well plates with different dimensions (flat, ultra-low attachment round-bottom, and
36 V-shaped 96-well plates). In both systems, the nuclear maturation rate remained similar to the
37 control in 2D, showing that most oocytes reached metaphase II. However, the subsequent
38 blastocyst rate remained lower in the liquid marble system compared to 96-well plates and
39 control 2D systems. Interestingly, a lower total cell number was found in the resulting embryos
40 from both systems (LM and 96-well plates) compared to the control. In conclusion, oocytes
41 matured in liquid marbles or 96-well plates showed no remarkable change in terms of meiotic
42 resumption, embryo development, and quality in both systems. None of the surface geometries
43 influenced embryo development. These findings provide important inferences in many aspects
44 of oocyte and embryo development. Further investigation is needed to determine other aspects
45 like toxicity testing and ultrastructural changes in oocytes.

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49 **Introduction**

50 *In vivo* development of mammalian oocytes depends on continuous contact and
51 communication with cumulus and granulosa cells that are present in the follicle. This interaction
52 results in a dynamic composition of the follicular fluid which is required to fully support the
53 maturation of the oocyte [1,2]. *In vitro* maturation (IVM) systems aims to mimic the *in vivo*
54 environment by using a combination of a base medium supplemented with hormones (FSH,
55 LH), serum or serum replacement, and growth factors [3,4]. However, in the absence of a
56 genital tract, it is often a static system [5,6]. *In vitro* maturation of the oocyte is a crucial step
57 in the acquisition of its developmental competence [7]. Yet, this process is extremely sensitive
58 to environmental factors such as pH [8] or temperature variations [9]. Thus, further optimization
59 of this technique is required to support nuclear and cytoplasmic oocyte maturation and
60 therefore to improve the potential of the oocyte to develop into a blastocyst.

61 In conventional two-dimensional (2D) systems, cells are grown on flat, firm culture substrates,
62 which are economic and relatively easy to handle. However, in such culture conditions cells
63 can adhere and spread freely in the horizontal plane but they have limited possibility for
64 spreading in the vertical dimension [10]. Hence, the major drawback of 2D-culture systems is
65 that they do not fully imitate the *in vivo* microenvironment where cells are grown in a complex
66 three-dimensional (3D) matrix, which has an impact on cell-cell and cell-extracellular matrix
67 interactions, and consequently on cell responses (differentiation, proliferation, apoptosis, gene,
68 and protein expression) [11–16]. Furthermore, the cell morphology in 2D systems is different
69 from that in the natural structures of tissues, which might affect their functionality, secretion of
70 growth factors, organization of internal structures and cell signaling [17,18].

71 There is growing evidence suggesting that 3D cell culture models reflect more precisely the
72 actual microenvironment in which cells grow in native tissues [19]. These models allow cell
73 adhesion in all three dimensions whereas in the 2D system it is restricted to the x-y plan.
74 Likewise, 3D systems improve cell communication and soluble factors are more stable in those
75 systems compared to 2D systems [20,21]. In terms of *in vitro* embryo production, studies on

76 3D systems were designed to support oocyte IVM in several species using alginate microbeads
77 [22], alginate hydrogels [23], glass scaffolds [24], agarose matrix [25], or the hanging drop
78 method [26]. Recently, LM have also become interesting as 3D bioreactors. Liquid marbles
79 are culture medium droplets encapsulated with hydrophobic particles which prevent direct
80 contact between the liquid inside and the surrounding environment, thus, reducing the risk of
81 contamination, while the hydrophobic shell of the LM remains permeable for gases [27]. These
82 properties make LM a promising alternative as 3D microbioreactors for cell culture. This system
83 has been used for culturing microorganisms [28], embryoid bodies [29,30] or olfactory
84 ensheathing cell spheroids [31]. Liquid marbles have been also applied in ovine [32] and
85 porcine [23] oocyte IVM but it has not been validated yet in the bovine model.

86 Cellular responses are influenced by topographical surface features. For instance, human
87 epithelial cells presented differences in orientation, migration, and morphology when culturing
88 them on pillar or pit surfaces [33]. Similarly, concave and convex surfaces have been found to
89 influence stem cells' differentiation into osteoblasts [34], and V-shape surface has been related
90 to changes in cell shape and mRNA expression in fibroblasts [35,36] and osteoblast-like cells
91 [37]. However, all these studies used complex systems to recreate the different surface
92 geometries. Thus, a simpler alternative could be to use 96-well plates with different shapes,
93 which are available on the market and are easy to use and standardize. Three different 96-
94 well plates have been tested in several human cell lines (retinal epithelial, alveolar epithelial
95 and dermal fibroblastic) [38], nevertheless, to our knowledge, no studies have been performed
96 with these plates to evaluate the potential influence of different surface topographies during
97 oocyte IVM on embryo development.

98 In the present work, we evaluated the potential effects of using a 3D culture system during
99 oocyte IVM with two different methods: (1) a matrix system, using liquid marble
100 microbioreactors and (2) a non-matrix system, using differently shaped culture substrates (flat,
101 round, and v-shaped 96-well plates). We found both matrix and non-matrix 3D culture systems

102 had a similar effect as 2D culture systems in terms of oocyte nuclear maturation, while embryo
103 development was similar after oocyte maturation in the 96-well plates but lower in LM.

104 **Materials and methods**

105 **Experimental design**

106 **Experiment 1: Evaluation of liquid marble as 3-D model for**
107 ***in vitro* maturation**

108 Liquid marbles were tested as microbiorreactors for *in vitro* maturation of bovine oocytes. To
109 do so, a total of 941 cumulus-oocyte complexes (COCs) in seven replicates were used in three
110 different maturation systems: Liquid Marbles (LM) (n= 301 COCs) as 3D model, 2D droplets
111 (n = 309 COCs) as flat culture with similar oocyte/medium ratio to LM (5 COCs/30 μ L), and
112 Control group (n = 331 COCs) as our standard condition (60 COCs /500 μ L). After maturation,
113 COCs from all groups were randomly distributed for *in vitro* fertilization (IVF) and *in vitro* culture
114 (IVC; LM = 241, 2D droplets = 256, and Control = 262 COCs) or nuclear maturation
115 assessment (LM = 60, 2D droplets = 53, and Control = 69 COCs).

116 **Experiment 2: Evaluation of different surface geometries for**
117 ***in vitro* maturation**

118 A comparative study was carried out to evaluate the effect of three different bottom-shaped
119 multi-well plates during oocyte IVM on embryo development. Firstly, we conducted a pilot study
120 including 3 replicates (n = 1,414 COCs) to select the ideal work volume of maturation medium
121 per well and to analyze the effect of paraffin oil overlay in oocytes matured in multi-well plates.
122 Cumulus-oocyte complexes were matured in v-shaped 96-well plates under the following
123 conditions: five COCs in 30 μ L maturation medium with 30 μ L oil overlay (Oil V-shaped-30
124 (OV-30); n = 160 COCs) or without oil (V-shaped-30 (V-30); n = 120 COCs), ten COCs in 60
125 μ L maturation medium with 30 μ L oil overlay (OV-60; n = 163 COCs) or without oil (V-60; n =
126 155 COCs), and twenty COCs in 120 μ L maturation medium with 30 μ L oil overlay (OV-120; n

127 = 143 COCs) or without oil (V-120; n = 148 COCs). A control group (n = 252 COCs) was also
128 included. After maturation, IVF and IVC were performed routinely. Subsequently, based on the
129 results of the pilot study, three different surface geometries (flat (F), ultra-low attachment
130 round-bottom (R), and V-shaped (V) 96-well plates) were compared during maturation in six
131 replicates (n = 1,992 COCs) using the conditions described as for the OV-60, resulting in 4
132 groups: F-60 (n = 427 COCs), R-60 (n = 549 COCs), V-60 (n = 491 COCs) and control group
133 (n = 525 COCs). After maturation, COCs from all groups were randomly assigned to IVF and
134 IVC (F-60 = 374, R-60 = 504, V-60 = 440 and control group = 487 COCs) or nuclear maturation
135 assessment (F-60 = 53, R-60 = 45, V-60 = 51 and control group = 38 COCs). The experimental
136 design is depicted in Fig 1.

137 **Figure 1. Schematic representation of the experimental design.**

138 **Media and reagents**

139 Tissue culture media (TCM)-199 and antibiotics (gentamycin and kanamycin) were obtained
140 from Life Technologies Europe (Ghent, Belgium). Phosphate-Buffered Saline (PBS) was
141 purchased from Gibco™ 20012019, Thermo Fisher Scientific (Waltham, MA, USA). All other
142 products not indicated otherwise were provided by Sigma-Aldrich (Diegem, Belgium). Before
143 use, every media were filtered (0.22 µM; GE Healthcare-Whatman, Diegem, Belgium).

144 **Source of oocytes and *in vitro* maturation**

145 Bovine ovaries were obtained from a local slaughterhouse, transported to the laboratory, and
146 prepared for further processing within 2 h after collection. The ovaries were disinfected with
147 96% ethanol and cleaned for three times in physiological saline (37 °C) containing 50 mg/mL
148 of kanamycin. Cumulus-oocyte complexes were recovered with an 18-gauge needle and a 10
149 mL syringe from 4-8-mm-diameter follicles. Oocytes surrounded by three or more layers of
150 compact cumulus cells and a uniformly granulated cytoplasm were selected, washed in warm
151 HEPES - Tyrode's Albumin Lactate Pyruvate media (HEPES-TALP), and randomly assigned
152 to different IVM systems. Four IVM systems, as described below, were evaluated according to

153 the experimental group (see Experimental design). All treatment groups were cultured for 22
154 h in 5% CO₂ in the air at 38.5 °C.

155 Control. Sixty COCs were cultured in 500 µL maturation medium (TCM-199 Earle's salts
156 supplemented with 20 ng/mL epidermal growth factor and 50 µg/mL gentamicin) in flat-bottom
157 4-well dishes (Thermo Fisher®, Merelbeke, Belgium) without oil covering.

158 Encapsulation in liquid marbles. A single droplet of 30 µL maturation medium containing five
159 COCs was carefully placed on top of a layer of approximately 1 cm treated fumed silica powder
160 (Cabot Corp, Cab-O-Sil, TS-530), which was equally distributed in a 6 cm Petri dish (Fig 2A).
161 The Petri dish was mildly shaken in circular motions to ensure that the surface of the droplet
162 was completely and uniformly coated with the hydrophobic particles. To manipulate the LM,
163 the edge of a 1000 µL micropipette tip was cut to make its diameter to a small extent of the LM
164 diameter in order to ensure a proper grip, but big enough to avoid collapse. Before transferring
165 the marbles, the modified tip was coated with some powder to prevent its adhesion to the tip.
166 Then, the LM was picked up slowly (Fig 2B) and placed on a well of a 24-well plate (Thermo
167 Scientific) whose surface was previously covered with a small quantity of silica powder (Fig
168 2C). To avoid evaporation, the central space of the 24-well plate was filled with 5 mL sterile
169 HEPES-TALP medium. After IVM, the LM (Fig 2D-2E) was placed in maturation medium to
170 disrupt the silica powder's hydrophobicity, causing the marble's dissolution (Fig 2F). The
171 released COCs were washed three times in maturation medium to remove silica particles
172 before proceeding to the next step.

173 **Figure 2. Oocyte encapsulation in liquid marbles.** (A) One droplet of maturation medium
174 containing the oocytes was placed in treated fumed silica powder on a petri dish. The petri dish
175 was gently shaken to form the liquid marble. (B) A modified 1000 µL micropipette tip was used
176 to manipulate the liquid marbles. (C) Liquid marbles were placed individually in the wells of a
177 24-well plate containing a small amount of silica powder. The central space of the plate was
178 filled with 5 mL HEPES-TALP to prevent evaporation. (D) Resulting liquid marble drop. (E)

179 Five COCs encapsulated in a liquid marble drop before IVM, observed under a
180 stereomicroscope. (F) After maturation, liquid marbles were dissolved in maturation medium.

181 2D droplets. Droplets of 30 μ L maturation medium were prepared in a Petri dish (60 x 15 mm;
182 Thermo Fisher Scientific, Waltham, MA USA) and covered with 7.5 mL paraffin oil (SAGE,
183 CooperSurgical, Trumbull, CT, USA). Five COCs were matured in each droplet of maturation
184 medium.

185 Shaped culture in 96-well plates. Firstly, oocytes were matured in V-shaped 96-well plates
186 under the following conditions: five COCs in 30 μ L of maturation medium with or without
187 paraffin oil overlay of 30 μ L, ten COCs in 60 μ L of maturation medium with or without a paraffin
188 oil overlay of 30 μ L, and twenty COCs in 120 μ L of maturation medium with or without a paraffin
189 oil overlay of 30 μ L. Secondly, ten COCs were matured in 60 μ L of maturation medium with
190 paraffin oil overlay in flat, ultra-low attachment round-bottom, and V-shaped 96-well plates (all
191 from Corning®, Houten, Netherlands).

192 ***In vitro* fertilization and embryo culture**

193 Standard *in vitro* methods were used to generate bovine embryos, as previously described by
194 Wydooghe et al. [39]. Briefly, using a discontinuous 45/90% Percoll® gradient (GE Healthcare
195 Biosciences, Uppsala, Sweden), sperm capacitation of frozen-thawed straws from a known
196 fertile bull was performed. Consequently, the sperm pellet was washed in IVF-TALP medium
197 and a final concentration of 1×10^6 spermatozoa/mL was adjusted using IVF-TALP medium
198 enriched with BSA (Sigma A8806; 6 mg/mL) and heparin (25 mg/mL).

199 After 22 hours of IVM, oocytes from each treatment group in experiments 1 and 2 were pooled
200 to reach groups of 60 COCs. Then, oocytes were washed in IVF-TALP and subsequently co-
201 incubated in 500 μ L IVF-TALP with Percoll-purified spermatozoa for 21 h at 38.5 °C in 5% CO₂
202 in humidified air. After fertilization, zona attached sperm and cumulus cells were removed by
203 vortexing for 3 minutes in 2.5 mL Hepes-TALP. The presumed zygotes were randomly selected
204 and cultured in groups of 25 in 50 μ L droplets of synthetic oviductal fluid (SOF), 0.4% (w/v)

205 BSA (Sigma A9647), and ITS (5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium).
206 Each droplet was covered with 900 µL paraffin oil and incubated at 38.5 °C for 8 days in 5%
207 CO₂, 5% O₂, and 90% N₂.
208 Cleavage was evaluated 45 h post insemination and blastocyst yield was recorded on day 7
209 and day 8 post insemination. Both rates were calculated as a percentage over the presumed
210 zygotes.

211 **Evaluation of oocyte nuclear stage (maturation 212 assessment)**

213 After maturation, oocytes were denuded by vortexing for 8 min in 2.5 mL Hepes-TALP and
214 fixed with 4% paraformaldehyde (w/v). Then, oocytes were transferred to 0.1% (w/v)
215 polyvinylpyrrolidone (PVP) in PBS containing 10 µg/ml of Hoechst 33342 (Life Technologies,
216 Ghent, Belgium) for 10 min. Nuclear morphology was evaluated using a fluorescence
217 microscope (BRESSER Science ADL 601 F LED). The proportion of oocytes in each meiotic
218 stage – germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI),
219 metaphase II (MII), or degenerated – was recorded.

220 **Embryo quality assessment**

221 Embryo quality was determined by differential apoptotic staining for CDX2, a transcription
222 factor only expressed by trophectoderm cells, and caspase-3, a cysteine-aspartic acid
223 protease involved in the signaling pathways of cell apoptosis. The protocol was performed
224 accordingly to Wydooghe et al. [40]. Briefly, day 8 blastocysts were fixed in 4%
225 paraformaldehyde (w/v) at room temperature for at least 20 min and then stored in PBS
226 supplemented with 0.5% BSA at 4 °C until the staining was performed. Firstly, blastocysts were
227 incubated with ready-to-use anti-CDX2 primary antibodies (Biogenex, San Ramon, USA).
228 Embryos were next incubated with rabbit active caspase-3 primary antibody (0.768 ng/mL, Cell
229 Signaling Technology, Leiden, The Netherlands), followed by incubation in goat anti-mouse

230 Texas Red secondary antibody (20 µg/mL in blocking solution, Molecular Probes, Merelbeke,
231 Belgium) and then in goat antirabbit FITC secondary antibody (10 µg/ml in blocking solution,
232 Molecular Probes). Finally, the embryos were transferred to nuclear stain, Hoechst 33342 (50
233 µg/mL in PBS/BSA). A negative control was also included in which embryos were not
234 incubated with CDX2 and active caspase-3 antibodies. Samples were examined by a single
235 observer using fluorescence microscopy (Leica DM 5500 B) with a triple bandpass filter. With
236 this staining protocol, the number of trophectoderm (TE) cells, inner cell mass number (ICM),
237 total cell number (TCN = TE + ICM), ICM/TCN ratio, the total number of apoptotic cells (AC)
238 and the ratio of apoptotic cells (ACR; AC/TCN) were estimated.

239 **Statistical Analyses**

240 The statistical analyses were performed using R-core (version 4.2.1; R Core Team, Vienna,
241 Austria). The oocyte/zygote/embryo was considered as the unit of interest. Generalized mixed-
242 effects models were used to test the effect of IVM conditions on oocyte nuclear maturation,
243 cleavage, and embryo development rates. The effect of IVM conditions on blastocyst
244 differential staining parameters was fitted in mixed linear regression models. For all the models,
245 the replicate was set as random. Results are expressed as least square means and standard
246 errors. The differences between treatment groups were assessed using Tukey's post hoc test.
247 The significance and tendency levels were set at $p < 0.05$ and $p < 0.1$, respectively.

248 **Results**

249 **Experiment 1: Evaluation of liquid marbles as a 3D 250 model for *in vitro* maturation**

251 **Effect of liquid marbles on oocyte nuclear maturation**

252 Nuclear maturation assessment by Hoechst staining demonstrated that oocytes in the three
253 groups resumed meiosis (i.e., no germinal vesicles were found). Most oocytes in all treatment
254 groups reached the metaphase II stage with no significant differences among groups ($p > 0.05$;

255 Table 1). Likewise, the proportion of oocytes that reached germinal vesicle breakdown,
256 metaphase I, or degenerated was similar among groups ($p > 0.05$).

257 **Table 1. Nuclear maturation assessment of oocytes matured in: (A) control group, (B)**
258 **2D droplets, and (C) liquid marbles (LM).**

Treatment	No. oocytes	GV	GVBD	MI	MII	Degenerated
Control Group	69	0 ± 0	0 ± 0	13 ± 6.9	87 ± 4.1	0 ± 0
2D Droplets	53	0 ± 0	0 ± 0	13.2 ± 6.4	84.9 ± 4.9	1.8 ± 0.2
LM	60	0 ± 0	5 ± 2.8	3.3 ± 0.8	88.3 ± 4.1	3.3 ± 0.2

259 Germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II
260 (MII) and degenerated ratio of oocytes analyzed with Hoechst staining. Results are expressed
261 as least square means ± standard error (LSM ± SE). No statistical differences were found
262 between groups.

263 **Effect of liquid marbles on embryo development and embryo
264 quality**

265 Firstly, we determine the effect of oocyte maturation conditions on cleavage and blastocyst
266 rates. Although there were no significant differences in the cleavage rates between groups
267 (LM: $79.9 \pm 3.1\%$; 2D droplets: $85.7 \pm 2.9\%$; control group: $86.0 \pm 3.1\%$; $p > 0.05$; Fig 3A),
268 oocytes matured in LM showed lower day 7 ($17.6 \pm 3.4\%$) and day 8 ($26.1 \pm 3.7\%$) blastocyst
269 rates compared to 2D droplets ($26.4 \pm 4.2\%$, $p = 0.048$, and $38.8 \pm 4.2\%$, $p = 0.008$,
270 respectively) and control ($29.8 \pm 4.9\%$, $p = 0.01$, and $40.1 \pm 4.6\%$, $p = 0.007$, respectively).

271 **Figure 3. Cleavage, day 7, and day 8 blastocyst rates are expressed as a percentage of
272 presumed zygotes.** (A) Experiment 1. Oocytes were *in vitro* matured in liquid marbles (LM),
273 2D droplets, and a control group. (B) Experiment 2. Oocytes were *in vitro* matured in flat, v-
274 shaped, and ultra-low attachment round-bottom 96-well plates, and a control group. Different

275 superscripts (a and b) represent statistical differences ($p < 0.05$) among groups. Results are
276 expressed as least square means \pm standard error (LSM \pm SE).

277 Further on, we determined the effect of maturation conditions on embryo quality by differential
278 apoptotic staining of blastocysts. Differences among IVM culture systems in blastocyst quality
279 parameters are shown in Table 2. Maturation in LM produced blastocysts with lower TCN and
280 TE than in control ($p < 0.01$). Maturation in 2D droplets reduced the TCN, ICM, and TE ($p <$
281 0.01) and increased the AC/TCN ratio compared to control ($p = 0.03$).

282 **Table 2. Effect of the oocyte *in vitro* maturation in LM on embryo quality.**

Treatment	No.	Cell numbers			AC	ICM/TCN ratio	AC/TCN ratio
		blastocyst	TCN	ICM			
Control Group	52	117.8 \pm 8.6 ^a	36.1 \pm 2.2 ^a	81.6 \pm 4.0 ^a	1.9 \pm 3.7	30.9 \pm 1.3	2.0 \pm 0.5 ^a
2D Droplets	51	82.3 \pm 5.1 ^b	26.2 \pm 2.2 ^b	56.3 \pm 4.0 ^b	2.8 \pm 3.7	32.6 \pm 1.4	3.9 \pm 0.5 ^b
LM	53	91.3 \pm 5.1 ^b	31.6 \pm 2.2 ^{ab}	59.8 \pm 4.0 ^b	3.0 \pm 3.6	33.7 \pm 1.3	3.5 \pm 0.5 ^{ab}

283 Total cell number (TCN), trophectoderm cells (TE), inner cell mass (ICM), apoptotic cells (AC),
284 ICM/TCN ratio, and AC/TCN ratio of day 8 blastocyst differentially stained. Different
285 superscripts per column (a and b) represent statistical differences ($p < 0.05$) among groups.
286 Results are expressed as least square means \pm standard error (LSM \pm SE).

287 **Experiment 2: Evaluation of different surface
288 geometries for *in vitro* maturation**

289 Initially, a pilot study was performed to establish the optimum volume of maturation medium
290 and the effect of paraffin oil overlay during oocyte IVM in 96-well plates on embryo
291 development. The V-30 group was excluded from the treatment groups due to excessive
292 maturation medium evaporation after IVM. Although there were no significant differences in

293 the cleavage, blastocyst day 7 and blastocyst day 8 rates for all the treatment groups compared
294 to the control ($p > 0.05$), OV-60 had a numerically higher blastocyst rate at day 8. Therefore,
295 we selected IVM culture conditions as described for this group for follow-up experiments. For
296 detailed information on the pilot study results, see supporting information (S1 Table).

297 **Effect of surface geometry on oocyte nuclear maturation,
298 embryo development, and embryo quality**

299 Hoechst staining was used to analyze the meiotic progression of the oocytes after IVM in flat,
300 v-shaped, and ultra-low attachment round-bottom 96-well plates. No differences were found in
301 the proportion of mature, immature, or degenerated oocytes in the three tested geometries and
302 the control group ($p > 0.05$; Table 3).

303 **Table 3. Nuclear maturation assessment of oocytes matured in: (A) control group, (B)
304 F-60, (C) V-60, and (D) R-60.**

Treatment	No. oocytes	GV	GVBD	MI	MII	Degenerated
Control Group	38	2.6 ± 0	7.9 ± 4.4	2.6 ± 0.1	86.8 ± 5.5	0 ± 0
F60	53	3.8 ± 0	11.3 ± 4.4	0 ± 0	84.9 ± 4.9	0 ± 0
V60	51	0 ± 0	5.9 ± 3.3	3.9 ± 0.1	90.2 ± 4.2	0 ± 0
R60	45	4.4 ± 0	4.4 ± 3.1	0 ± 0	91.1 ± 4.2	0 ± 0

305 Germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II
306 (MII) and degenerated ratio of oocytes analyzed with Hoechst staining. Results are expressed
307 as least square means ± standard error (LSM ± SE).

308 Cleavage, day 7 and day 8 blastocysts rates were similar in the three 96-well plates and the
309 control group ($p > 0.05$; Fig 3B). Differences among treatments in blastocyst quality parameters
310 determined by differential apoptotic staining are shown in Table 4. *In vitro* maturation in flat,
311 ultra-low attachment round-bottom and v-shaped 96-well plates resulted in blastocysts with

312 lower TCN and TE than control ($p < 0.05$). Blastocysts in the R-60 group also presented lower
313 ICM compared to control ($p = 0.01$). However, there were no differences in the ICM/TCN or
314 AC/TCN ratios among treatments and control ($p > 0.05$).

315 **Table 4. Effect of the oocyte *in vitro* maturation in three different surface geometries on**
316 **embryo quality.**

Treatment	No.	Cell numbers				ICM/TCN	AC/TCN
		blastocyst	TCN	ICM	TE	AC	ratio
Control Group	80	134.6 ± 5.2 ^a	51.0 ± 2.5 ^a	83.6 ± 3.6 ^a	1.8 ± 2.6	38.6 ± 3.9	1.7 ± 0.3
F-60	83	112.4 ± 5.1 ^b	44.8 ± 2.4 ^{ab}	67.5 ± 3.5 ^b	2.0 ± 2.5	34.5 ± 3.9	2.2 ± 0.3
V-60	84	112.2 ± 5.1 ^b	44.1 ± 2.4 ^{ab}	68.0 ± 3.5 ^b	1.7 ± 2.5	39.4 ± 3.8	1.7 ± 0.3
R-60	70	97.6 ± 5.6 ^b	39.8 ± 2.7 ^b	57.8 ± 3.8 ^b	1.61 ± 2.5	41.9 ± 4.2	1.7 ± 0.3

317 Total cell number (TCN), trophectoderm cells (TE), inner cell mass (ICM), apoptotic cells (AC),
318 ICM/TCN ratio, and AC/TCN ratio of day 8 blastocyst differentially stained. Different
319 superscripts per column (a and b) represent statistical differences ($P < 0.05$) among groups.
320 Results are expressed as least square means ± standard error.

321 **Discussion**

322 Currently, most standard oocyte IVM is performed in two-dimensional culture systems, which
323 are both economical and practical. However, these systems are only a poor representation of
324 the physiological environment within the follicle. Therefore, novel alternatives to mature
325 oocytes in a three-dimensional milieu might enhance the interaction between the oocyte, the
326 cumulus cells, and different factors within the culture medium, and consequently improve the
327 developmental competence of the oocyte. In this study, we used liquid marbles as
328 microreactors to perform serum-free IVM for the first time in the bovine model. Moreover, we

329 also tested a simple and practical non-matrix system using differently shaped 96-well plates.
330 We found that both matrix and non-matrix 3D culture systems had a similar effect as 2D culture
331 systems in terms of oocyte nuclear maturation, while embryo development was similar after
332 oocyte maturation in the 96-well plates but lower in LM.

333 We proved that meiotic resumption is not affected in bovine oocytes by the use of LM, since
334 the proportion of oocytes that reached metaphase II was similar in both 2D- and 3D-systems,
335 which concord with previous studies in the cat [41] and sheep [32,41]. In our study, although
336 oocytes matured in LM were able to reach the blastocyst stage, the blastocyst yield was
337 reduced compared to oocytes matured in 2D. However, embryos derived from oocytes
338 matured in LM exhibited a lower total cell number count than those natured in a 2D system. It
339 has been demonstrated that material toxicity can decrease the total cell number and affect
340 embryo rates [42]. However, the toxicity of treated fumed silica particles has not been tested
341 in oocytes. Therefore, we hypothesize that treated fumed silica particles could exhibit some
342 toxicity in bovine oocytes since our findings differ from the results obtained by Bebbere et al.,
343 who matured ovine oocytes in LM formed with the same particles, showing better blastocyst
344 rate compared to those matured in 2D conditions [41]. Additionally, in a previous study of the
345 same group, better blastocyst yield was reached after the maturation of ovine oocytes in
346 polytetrafluoroethylene marbles compared to the group in 2D [32]. Therefore, apart from the
347 possible toxicity, the presence of serum in their maturation medium, the oocyte/medium ratio,
348 or species-specific differences might explain discrepancy of the results. Moreover, additional
349 manipulation during the LM preparation compared to the standard system may also affect the
350 developmental capacity of the gametes.

351 Besides the LM system, in the current study, we evaluated the effect of different geometry
352 surfaces on oocyte developmental competence. Although we tested a range of medium
353 volumes and evidenced the importance of paraffin oil overlay when low volumes are used, we
354 did not find differences in oocyte nuclear maturation nor embryo development, or quality
355 between the three surface geometries tested. Although blastocyst derived from oocytes

356 matured in 96-well plates presented a lower total cell number count than oocytes matured in
357 standard conditions, culture conditions did not adversely affect the developmental capacity of
358 the oocytes. These results are in agreement with Shafaie et al., the only previous study that
359 compared flat, round-bottom, and V-shaped 96-well plates in cell culture. In that study, different
360 human cell lines, namely A549 (alveolar epithelial), ARPE-19 (retinal epithelial) and Malme-
361 3M (dermal fibroblast), cells attached and spread differently in each plate, but the phenotype
362 and functionality of the cells were not affected by the surface topography [38].

363 As cell culture is tending to move towards more physiological systems such as 3D-methods, it
364 is important to study the impact of those systems on fundamental cellular processes. In *in vitro*
365 embryo production, oocyte maturation and embryo development and quality are the main
366 outcome parameters to evaluate culture systems. In our study, we showed that oocyte meiotic
367 resumption was not affected by any of the culture systems. However, only nuclear maturation
368 was assessed and further studies to evaluate differences in cytoplasmic maturation may be
369 performed. Interestingly, our experiments with different surface topographies exhibited a
370 similar blastocyst rate in comparison with traditional culture but LM reduced it. Yet, alternative
371 culture systems entail higher costs and complexity than conventional IVM (Table 5). Liquid
372 marbles demonstrated the highest level of complexity and risk of loss of oocytes during the
373 elaboration of the marble, with the longest handling time, which might be an additional
374 explanation for the lower blastocyst yield. On the other hand, 96-well dishes made the handling
375 of COCs during preparation and recovery more difficult due to the smaller diameter of the wells.

376 **Table 5. Characteristics of *in vitro* maturation systems.**

Maturation technique	Description	Hands-on-time (min)		Ease-of-use	Cost (€)
		Preparation	Recovery		

Four well dish without oil (control)	Sixty COCs in 500 μ L of maturation medium in flat-bottom 4-well dishes without oil covering	1	0.5	+	1.3
Droplets on petri dish under oil (2-D Droplets)	Five COCs in droplets of 30 μ L of maturation medium in a Petri dish (60 x 15 mm) and covered with 7.5 mL paraffin oil	4	3	++	3.2
Liquid marbles	Five COCs in 30 μ L of maturation medium are placed on top of a layer of treated fumed silica powder to form a LM, which is transferred to a well of a 24-well plate with a cut 1000 μ L pipette tip	15	7	++++	3.3
96-well plates with different dimensions	Five COCs in 60 μ L of maturation medium covered by 30 μ L of paraffin oil overlay in flat, ultra-low attachment round-bottom, and V-shaped 96-well plates	4	5	+++	Flat: 3.8 V-shaped: 3.9 Round-bottom: 22.3

377 Comparison of *in vitro* maturation systems in terms of working principle, hands-on-time (i.e.
378 based on the manipulation time in minutes required to handle 60 COCs from the dish in which
379 they are selected and washed in HEPES-TALP to the final *in vitro* maturation (IVM) system
380 (preparation) and from the IVM system to the fertilization dish (recovery)), ease-of-use and
381 approximate cost for one maturation process (i.e. based on cost of the dish, oil if necessary
382 and silica powder for liquid marbles preparation). + = low; ++ = moderate; +++ = high; ++++ =
383 very high

384 Conclusion

385 For the first time, two different 3D culture systems were tested in bovine IVM. We showed that
386 there are limited differences in development and quality in embryos resulted from oocytes
387 cultured in the 2D control system compared with LM and 96-well plate systems. Importantly,
388 no adverse results were observed in terms of embryo development when cultured in 96-well

389 plates and, although there was a slight decrease in embryo yield in LM, these results contribute
390 towards the development of a more accurate, simple high throughput, *in vitro* maturation
391 systems that represent the *in vivo* dynamic environment. Maybe further investigation is needed
392 like in toxicity testing and ultrastructural changes of the oocyte.

393 Acknowledgments

394 The authors thank Petra Van Damme for her technical assistance. This project has received
395 funding from the European Union's Horizon 2020 research and innovation programme under
396 the Marie Skłodowska-Curie grant agreement No 860960.

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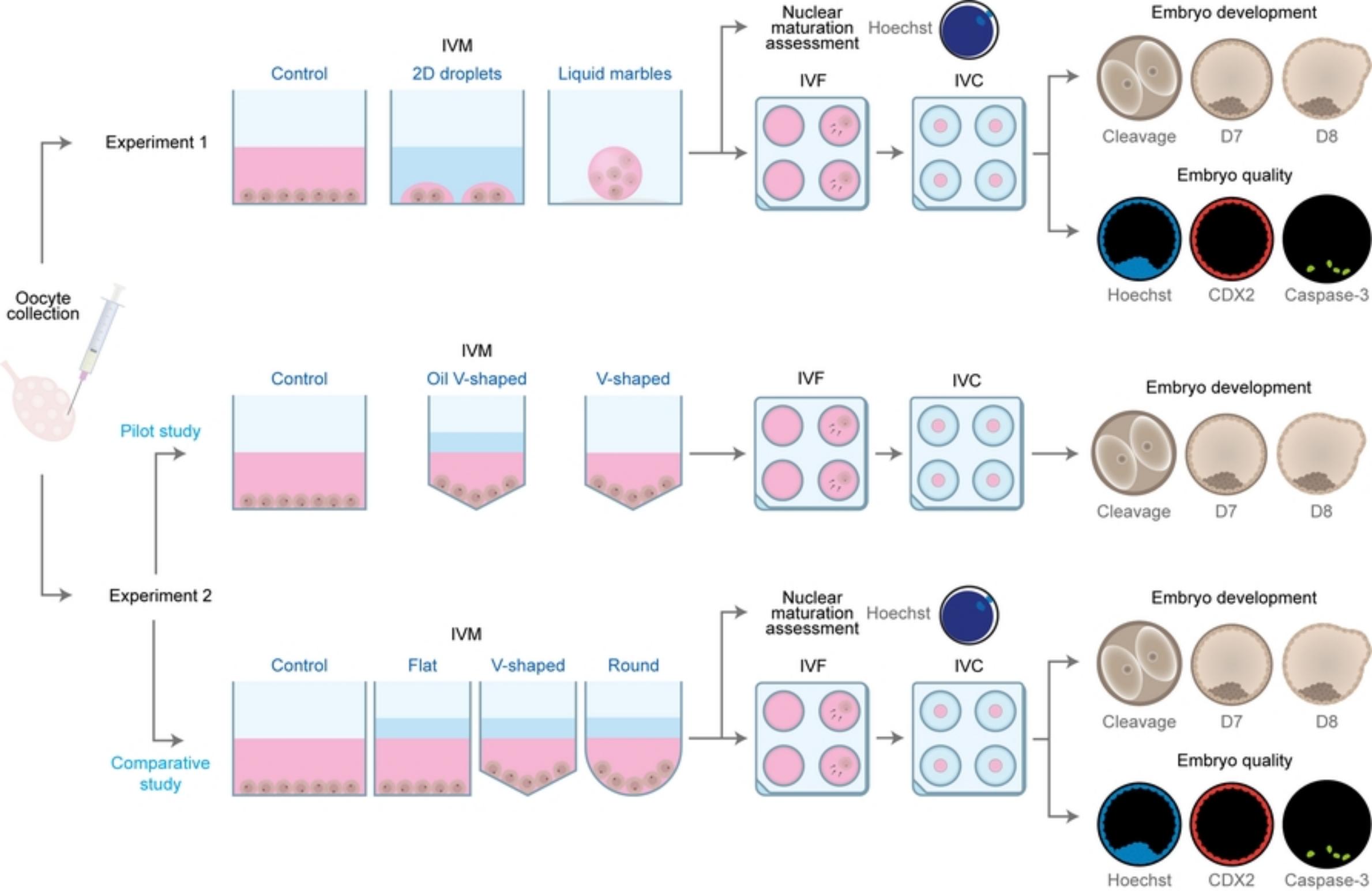


Figure 1

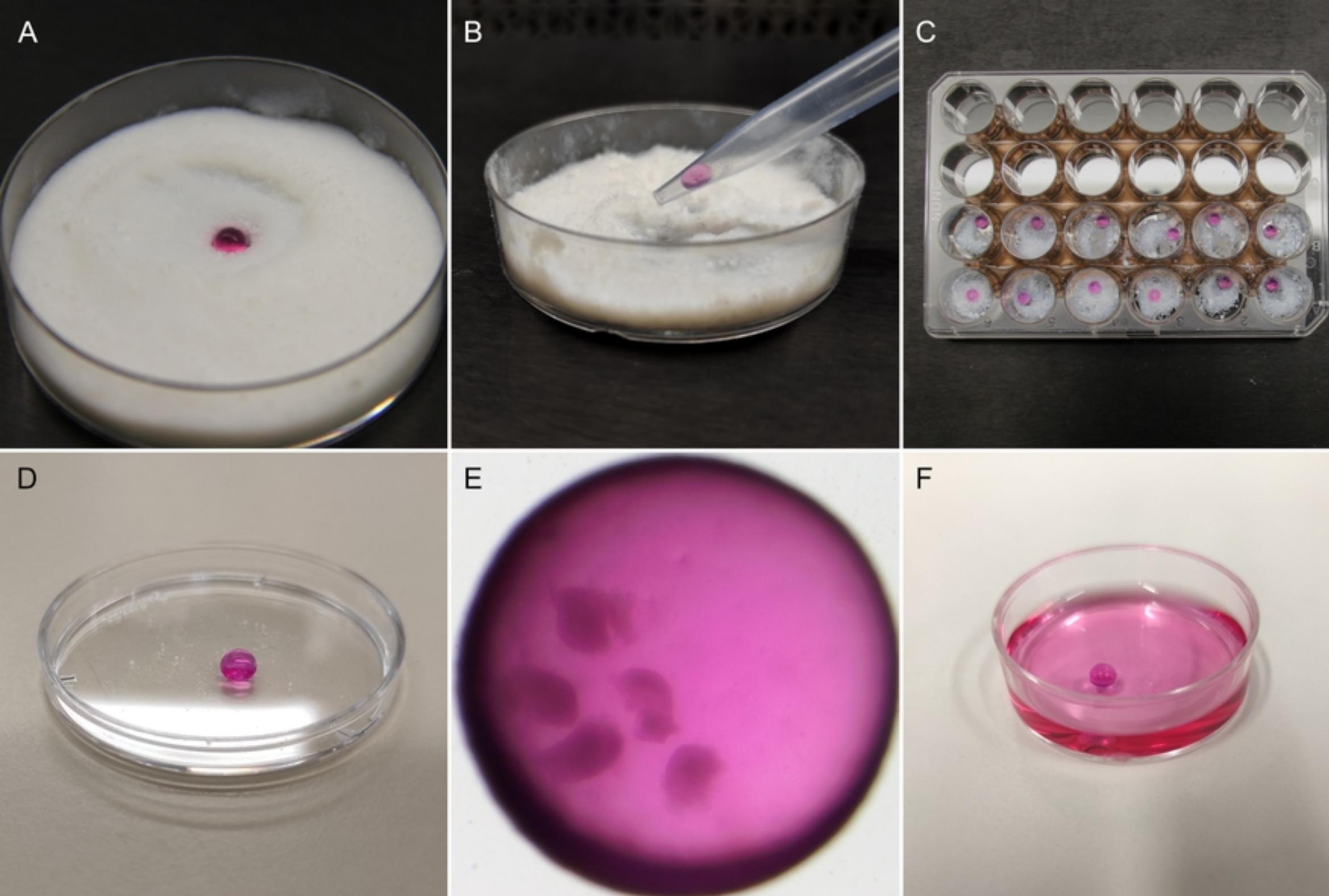


Figure 2

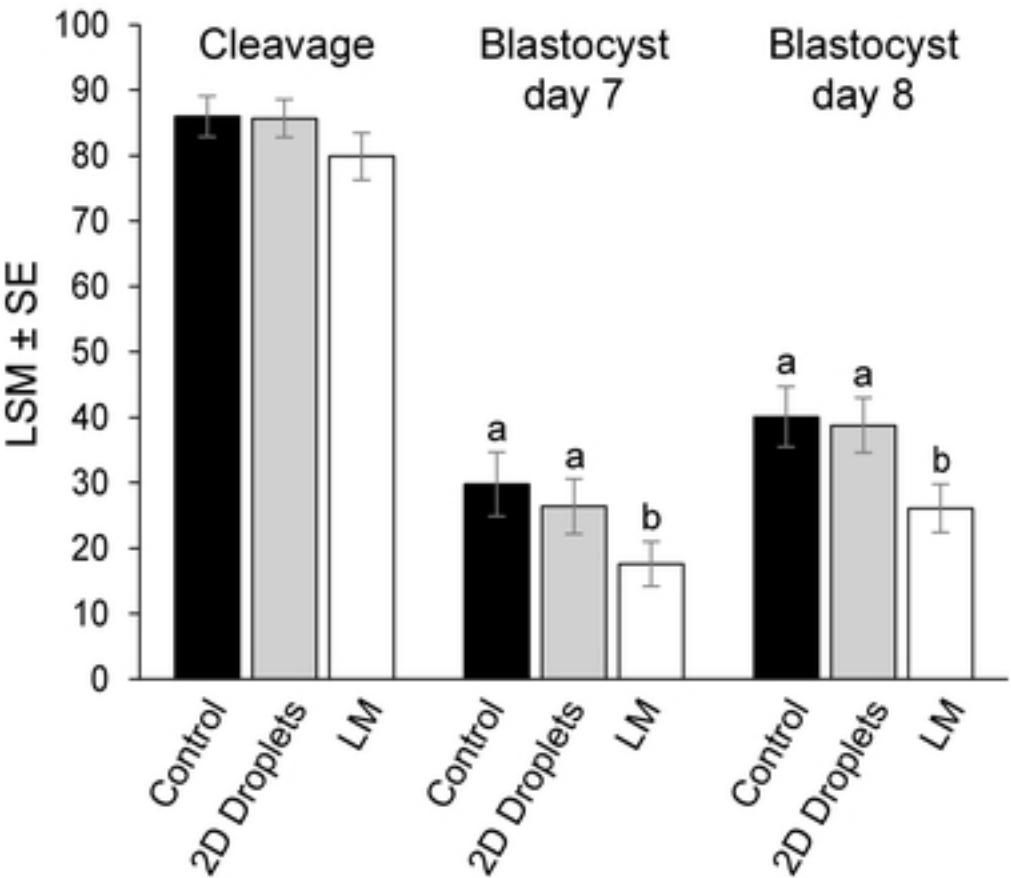
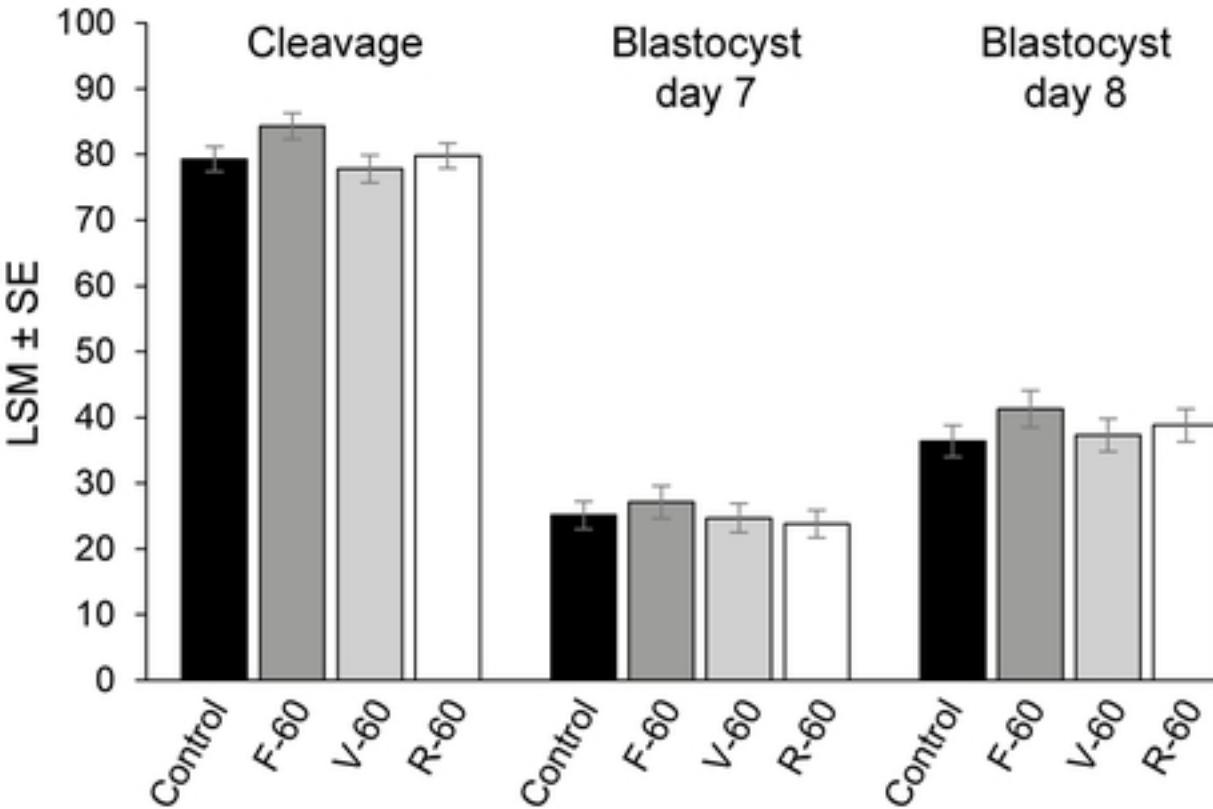
A**B**

Figure 3