# Liposomes Under Real Cell Conditions Behave Like Real Cells with a Single Pulsed Electric Field

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## Abstract

Liposomes are widely assumed to present a straightforward physical model of cells. However, almost all previous liposome experiments with pulsed electric fields (PEFs) have been conducted in low-conductivity liquids, a condition that differs significantly

from that of cells in medium. Here, we prepared liposomes consisting of soy bean lecithin and cholesterol, at a molar ratio of 1:1, in higher-conductivity liquid that approximated the conditions of red blood cells in phosphate-buffered saline, with inner and outer liquid conductivities of 0.6 and 1.6 S/m, respectively. We found that a single 1.1 kV/cm, 400 μs PEF promoted cell-like spontaneous division of liposomes.

**Keywords**

Liposome; Vesicle; Pulsed electric field; Conductivity; Real cell condition; Lipid composition

## Introduction

Bioelectrics, which studies the relationship between electricity and biology, has been the subject of both basic and applied research of note [1–9]. Electropores induced on plasma membranes may trigger responses in cells and tissues to pulsed electric fields (PEFs) [10–17]. Numerous papers have attempted to validate this hypothesis, applying pulses to liposomes composed of artificial lipid membranes [1,18–20]. However, few studies have managed to completely mimic the phenomenon in cells. In the case of cells, plasmid DNA reportedly accumulates on the cell membrane facing the cathode side, and

subsequently, actin patches appear on the surface, after which plasmid DNA is taken into the cells [3,21]. In a study using liposomes, plasmid DNA traversed the lipid membrane without accumulating [22]. Rols et al. assumed actin as the source of the difference and suggested conducting a similar experiment using actin-containing liposomes.

Little dye was seen in liposomes without actin, but liposomes with actin proceeded taking dye more than 146 s, and the latter reaction was similar to those of cells under a PEF [23]. However, those liposomes were prepared in liquid with much lower conductivity than is seen in cytoplasm and extracellular liquid (phosphate-buffered saline [PBS]) [24–27]. Because liquid conductivity influences substance influx through electropores on cells [28–30] and low-conductivity conditions differ from those of cell experiments, experiments that approximate real cytoplasm and media will require adjustment of the conditions. Furthermore, although the liposomes exhibited accurate lipid molar fractions, it is unlikely that their composition was similar to that of real cell membranes. As membrane composition can influence reactions to PEFs, liposome compositions that are closer to those of a real plasma membrane are necessary. To that end, we adopted an emulsion method that made it possible to adjust the conductivities of the interior and exterior liquids of the liposomes. We applied PEFs to liposomes with

55 inner- and outer-liquid conductivities comparable to those of red blood cells (0.6 S/m)
56 and PBS (1.6 S/m) and whose membranes were composed of soybean lecithin and
57 cholesterol. We adjusted the conductivity of liposomes' inner liquid to that of red blood
58 cells because their value had already been reported, and most previous simulation
59 studies preferred this value [31]. Because soybean lecithin is not a purified lipid, but an
60 extract, we assumed that the composition of soybean lecithin was close to that of real
61 cell membranes. Although the actual components are uncertain, lecithin is composed
62 mainly of phosphatidylcholine (PC), which is a main component of cell membranes. We
63 also applied egg lecithin to the process but were unavailable to produce liposomes.
64

65 **Materials and methods**

66 **Materials**

67 For liposome preparation, we purchased soy bean lecithin, cholesterol, liquid paraffin,
68 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1- palmitoyl- 2- oleoyl- sn- glycero- 3-
69 phosphocholine (POPC), chloroform, glucose, and sucrose from Wako and Texas Red-
70 1,2- dihexadecanoyl- sn- glycero- 3- phosphoethanolamine (DHPE) from Takara. For the
71 liposome outer liquid, we used PBS(-) (from Wako), and for the liposome inner liquid, we
72 mixed PBS(-) with 308 mM glucose solution at a 15:26 mL volume ratio. The theoretical

73 osmotic pressure of PBS(-) was 306 mOsm/L. The osmotic pressure of the 308 mM
74 glucose solution was theoretically 308 mOsm/L. Conductivities of the inner and outer
75 liquids were 0.64 and 1.64 S/m, respectively. Cholesterol, DOPC, and POPC were
76 dissolved in chloroform at 180 mM and Tex Red-DHPE at 0.9 mM. All solutions were
77 stored at a temperature of −20˚C.
78
79 **Liposome preparation**
80 **Lecithin liposome**
81 Liposome production followed the water-in-oil emulsion method [32]. We estimated the
82 soy bean lecithin molar weight at 758.06 and then dissolved 0.53 mg of soy lecithin in
83 500 μL of liquid paraffin at 80˚C by vortex (1.4 mM). After storing the sample at a
84 temperature of 80˚C for 10 min with the cap of the microtube left open, we added 50 μL
85 of the inner liquid and vortexed it for 10 s and then immediately put the sample on ice
86 for 10 min to stabilize the emulsions. Next, 400 μL of the emulsion solution was put on
87 400 μL of the outer liquid, which had been on ice. The volume ratio of the emulsion
88 solution and the outer liquid was 400:400. After treating the sample under
89 centrifugation at 18,000 *g* and a temperature of 4 ˚C for 5 min, we disposed of as much of
90 the supernatant as possible and extracted the sediments, which was the aggregation of

liposomes.

**Lecithin-cholesterol liposomes and fluorescent liposomes**

To produce cholesterol-containing lecithin liposomes (at a molar ratio of lecithin/cholesterol = 1:1 ), we added 4 μL of cholesterol solution to a heated liquid paraffin-lecithin solution at a temperature of 80˚C and then mixed them by vortex to adjust the cholesterol concentration to 1.4 mM. For a lecithin/cholesterol molar ratio of 1:1.5 and 2:1 liposomes, the amounts of added cholesterol solution were 6 and 2 μL. For fluorescent labeling, 2 μL of Tex Red-DHPE was added simultaneously to make a final concentration of 3.6 μM (with a molar ratio of 0.13 mol%). The sample was stored at a temperature of 80˚C for 10 min with the cap of the microtube left open, and the rest of the protocol was the same as that of lecithin liposome.

**DOPC liposomes and POPC liposomes**

To produce DOPC liposomes, DOPC and cholesterol liquids were mixed in 500 μL of liquid paraffin at the following ratios: 4:0 for DOPC-only liposomes, 4:4 for DOPC/cholesterol = 1:1 (molar ratio), 2:4 for DOPC/cholesterol = 1:2 (molar ratio), and 2:6 for DOPC/cholesterol = 1:3 (molar ratio). In the case of POPC, the POPC and

109 cholesterol liquids were mixed at the following ratios; 4:0 for POPC-only liposomes and
110 4:4 for POPC/cholesterol = 1:1 (molar ratio). The sample was stored at a temperature of
111 80˚C for 10 min with the cap of the microtube left open, and the rest of the protocol was
112 the same as that of lecithin liposome
113
114 **Calcium-ion influx detection**
115 *The same osmotic pressure between inner and outer liquid*
116 First, 50 μg of Fluo-8(R) sodium salt (Cosmo Bio) was dissolved in 62.5 μL of Milli-Q,
117 and 1 μL of the solution was diluted in 50 μL of the inner liquid for a final Fluo-8
118 concentration of 20 μM. For the calcium-ion flow experiment, liposomes were prepared
119 with the Fluo-8-containing inner liquid. After preparing the liposome sample, a solution
120 of D-PBS(+) preparation with a Ca and Mg reagent solution 100× (Nacalai Tesque) was
121 added at 1 % (v/v) to mix the calcium ions in the outer liquid.
122
123 *Different osmotic pressures between inner and outer liquid*
124 To strengthen calcium influx, we set the outer-liquid osmotic pressure at half of that of
125 the inner liquid. The inner-liquid composition was the same as that used to text Ca-ion
126 influx, but in the case of the outer liquid, the PBS (-) was diluted twice with Milli-Q. The

127 rest of the protocol was the same as that of no osmotic pressure difference.

128

129 **PEF application system and microscopy**

130 We used two slices of a platinum plate, which are 0.2-mm thick and 2.7-mm wide, as
131 electrodes. The electrodes were fixed with a 640-μm gap on a glass slide and connected
132 to the PEF generator. We set the glass slide–electrode device on a fluorescent
133 microscope (Leica, DMi8) combined with a digital camera (Canon, EOS 8000D). Movies
134 were recorded at 67 frames per second. The details of microscopy are provided in a
135 previous paper [33].

136

137 **Results**

138 We referred to HeLa cells, which were familiar to us, for the lipid molar ratio of cell
139 membranes, facilitating a comparison of liposome and cell results. As PC and
140 cholesterol constitute primarily of HeLa cell membranes, their molar ratio was 1:1, and
141 other lipids represented only minor parts [34,35], we mixed lecithin and cholesterol at a
142 molar proportion of 1:1.

143

144 **Liposomes divided spontaneously**

When we applied a single 1.1 kV/cm, 400 μs PEF to the liposomes, the liposomes divided spontaneously (Fig. 1A, B, and C). To determine the optimal PEF condition to induce division, we scanned the pulse duration and electric field intensity, showing that a 400 or 500 μs, 1.1 kV/cm PEF appeared to have the potential to induce division (Table. 1). A PEF with identical energy but a shorter pulse did not promote self-division (Table. 2). Because a single 1.1 kV/cm, 400 μs PEF can theoretically increase the PBS temperature by 2˚C, the thermal influence may be small. These results suggest that energy was not an important factor in the division.

Here Fig. 1.

Here Table. 1

Here Table. 2

**Do cholesterol and lecithin have division ability?**

As liposomes without cholesterol did not divide, it is possible that cholesterol has the potential to induce division. Varying the molar ratios of cholesterol, liposomes with more than 50% of cholesterol divided (Fig. 2A, B, C, and D).

163 Here Fig. 2.

164

165 PC is a main component of lecithin. Previous studies have used DOPC, POPC, and
166 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), all of which are classified as PC.
167 To evaluating which elements contributed to the liposome division, we prepared the
168 liposomes, with each lipid and cholesterol at a 1:1 molar proportion. DOPC produced
169 lecithin-like liposomes, but POPC did not (Fig. 3). Because POPC had higher phase
170 transition temperature than DOPC and temperature influences liposome production
171 [36], we anticipated that the surface aggregation on POPC liposomes may be aggregates
172 of gel-phase POPC. We did not prepare DPPC liposomes because DPPC has a much
173 higher phase transition temperature, making it unlikely for DPPC liposomes to produce
174 lecithin-like liposomes. No division occurred in DOPC liposomes, which most likely
175 resembled lecithin liposomes (Fig. 3A), even though the cholesterol fraction increased
176 by more than 50% (Fig. 3B and C). These results suggest that several lipids together
177 induced division, rather than only one element of phospholipids contributing to the
178 division.

179

180 Here Fig. 3.

181

182 **Conductivity of liquids related to the division**

183 When we lowered the conductivity of both inner and outer liquids to 105 μS/m, no
184 division occurred (lecithin/cholesterol = 1:1) (Table. 3). Because the emulsion method
185 required heavier inner liquids than outer liquids, we mixed glucose solution and PBS (-)
186 for the inner liquid to ensure that inner conductivity decreased to 0.6 S/m. For the same
187 reason, we did not execute the experiments with 1.6 S/m inner liquid. Instead, 0.6 S/m
188 for the inner liquid and 1.6 S/m for the outer liquid (the same as for the red blood cells in
189 PBS) were deemed appropriate.

190

191 Here Table. 3.

192

193 **Ca-ion influx had little relation to division**

194 To analyze ion influx under the PEF that induced the liposome division, we attempted
195 to observe Ca-ion influx into liposomes. However, we detected no Ca-ion flow into
196 liposomes after a single PEF (Fig. 4). Lecithin liposomes, the surfaces of which are not
197 smooth, collapsed immediately after PEF exposure (Supplementary Fig. 1A). When the
198 liposomes disintegrated, green fluorescence emerged from the outside of the liposomes,

199 indicating that Fluo-8 in the liposomes had seeped to the surroundings and connected to
200 Ca ions (Supplementary Fig. 1B). This proved that our method could detect Ca-ion
201 influx. Because a Ca ion is much smaller than dyes such as propidium iodide, Ca ions
202 are prone to flowing across lipid membranes faster than dyes [37]. Our failure to detect
203 a Ca influx indicated that the extent of liquid flow accompanied by the single 1.1 kV/cm,
204 400 μs PEF was small.
205
206 Here Fig. 4.
207
208 **Endocytosis-like phenomena are also induced**
209 Lecithin-cholesterol liposomes sometimes showed that lipid patches were detached from
210 the membrane surface as in endocytosis (Fig. 5A). Additionally, lipids aggregated on the
211 membrane just after PEF application (Fig. 5B). However, on DOPC-cholesterol
212 liposomes, which did not have division ability, lipid aggregation and patch uptake did
213 not occur (Fig. 5 C, D).
214
215 Here Fig. 5.
216

## Discussion

### Why did liposomes divide?

Why current conditions trigger liposome division and endocytosis-like behavior remains unknown. Previous studies have reported that electrofusion of liposomes promotes spontaneous liposome division, explaining the phenomenon in terms of thermodynamics; as the amount of lipid per liposome increased by electrofusion, the system became unstable, and consequently, liposomes divided to increase entropy and stabilize the system [38,39]. In our case, however, electrofusion did not precede division, so the previous theory did not explain our results. Because liposome division accompanied liposome deformation, it seemed possible to describe the phenomenon in terms of liposome volume change, but the influence of volume change is likely small because the osmotic pressures of the inner and outer liquids were almost identical. In fact, calcium-ion influx was too small to detect.

In addition, we have no reasonable explanation on why liposome divisions did not occur in previous studies. However, our study with less-conductive inner and outer liquids suggests that liquid conductivity is one of the determining factors. Indeed, one paper using buffered solution as an electroformation buffer showed vesicle budding without releasing daughter vesicle [40].

235

236 **Membrane conditions may be related to liposome division**

237 We increased Ca-ion influx by reducing the osmotic pressure of the outer liquid
238 compared with the inner liquid. Using 154 mM glucose solution with a conductivity of
239 0.7 S/m and a theoretical osmotic pressure of 154 mOsm/L as the outer liquid and
240 PBS(-)-glucose solution with a conductivity of 0.6 S/m and a theoretical osmotic
241 pressure of 308 mOsm/L as the inner liquid, Ca-ion influx was not detected in lecithin
242 liposomes (Supplementary Fig. 2A and B), whereas lecithin-cholesterol liposomes
243 disintegrated with Ca-ion influx (Supplementary Fig. 2C and D). These tendencies
244 coincided with those of previous studies that revealed that liposomes, including charged
245 lipids, such as cholesterol and POPG, were likely to collapse more easily due to PEF
246 exposure compared with those without charged lipids [41,42]. This prompted the
247 question of whether charged lipids can trigger liposome division. We therefore examined
248 the behavior of lecithin-POPG liposomes and lecithin-POPC liposomes. POPG has a
249 negative charge, whereas POPC does not, but both have similar hydrophobic backbones
250 with one saturated and one unsaturated fatty acid chain. Both POPG-containing
251 liposomes and POPC-containing liposomes divided (Supplementary Fig. 2E and F). This
252 suggested that it is not lipid charge but membrane condition that induces liposome

253 division. In fact, Riske et al. reported changes in lipid composition in a plasma
254 membrane during mitosis [43]. Determining which type of lipid can induce liposome
255 division with lecithin should be subjected to a screening wand, and we intend to follow
256 this line of inquiry in the future.
257

258 **Future tasks**

259 There are several important points to examine in the future: the relationship between
260 PEF conditions and the time lag from PEF application to division or endocytosis, the
261 influence of inner-liquid viscosity, the relationship between PEF conditions and the size
262 of mother liposomes and that of daughter liposomes, PEF direction and division or
263 endocytosis polarities, and how membrane composition or conditions affect the division.
264

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270 **Author contributions**

271 G.U, M.S, and T.O contributed to the experiments. S.K supervised and managed this
272 project.

273

274 **References**

275 [1] D.L. Perrier, L. Rems, P.E. Boukany, Lipid vesicles in pulsed electric fields:
276 Fundamental principles of the membrane response and its biomedical applications,
277 Adv. Colloid Interface Sci. 249 (2017) 248–271. doi:10.1016/j.cis.2017.04.016.

278 [2] Z.A. Levine, P.T. Vernier, Life cycle of an electropore: Field-dependent and
279 field-independent steps in pore creation and annihilation, J. Membr. Biol. 236 (2010)
280 27–36. doi:10.1007/s00232-010-9277-y.

281 [3] D. Miklavcic, M.-P. Rols, S. Haberl Meglic, C. Rosazza, A. Zumbusch, Gene
282 Electrotransfer: A Mechanistic Perspective, Curr. Gene Ther. 16 (2016) 98–129.
283 doi:10.2174/1566523216666160331130040.

284 [4] L. Rems, D. Miklavčič, Tutorial: Electroporation of cells in complex materials and
285 tissue, J. Appl. Phys. 119 (2016) 201101. doi:10.1063/1.4949264.

286 [5] K. Heileman, J. Daoud, M. Tabrizian, Dielectric spectroscopy as a viable biosensing
287 tool for cell and tissue characterization and analysis, Biosens. Bioelectron. 49 (2013)
288 348–359. doi:10.1016/j.bios.2013.04.017.

289 [6] S. Sundelacruz, M. Levin, D.L. Kaplan, Role of membrane potential in the regulation
290 of cell proliferation and differentiation, Stem Cell Rev. Reports. 5 (2009) 231–246.
291 doi:10.1007/s12015-009-9080-2.
292 [7] S.N. Iwasa, R. Babona-Pilipos, C.M. Morshead, Environmental Factors That
293 Influence Stem Cell Migration: An "Electric Field," (2017). doi:10.1155/2017/4276927.
294 [8] F. Chang, N. Minc, Electrochemical Control of Cell and Tissue Polarity, Annu. Rev.
295 Cell Dev. Biol. 30 (2014) 317–336. doi:10.1146/annurev-cellbio-100913-013357.
296 [9] K.R. Robinson, The responses of cells to electrical fields: A review, J. Cell Biol. 101
297 (1985) 2023–2027. doi:10.1083/jcb.101.6.2023.
298 [10] M.P. Stewart, R. Langer, K.F. Jensen, Intracellular delivery by membrane
299 disruption: Mechanisms, strategies, and concepts, Chem. Rev. 118 (2018) 7409–7531.
300 doi:10.1021/acs.chemrev.7b00678.
301 [11] J.L. Guerriero, D. Ditsworth, Y. Fan, F. Zhao, H.C. Crawford, W.X. Zong,
302 Chemotherapy induces tumor clearance independent of apoptosis, Cancer Res. 68
303 (2008) 9595–9600. doi:10.1158/0008-5472.CAN-08-2452.
304 [12] S.K. Frandsen, M.B. Krüger, U.M. Mangalanathan, T. Tramm, F. Mahmood, I.
305 Novak, J. Gehl, Normal and malignant cells exhibit differential responses to calcium
306 electroporation, Cancer Res. 77 (2017) 4389–4401.

307 doi:10.1158/0008-5472.CAN-16-1611.

308 [13] T.B. Napotnik, M. Reberšek, T. Kotnik, E. Lebrasseur, G. Cabodevila, D. Miklavčič,

309 Electropermeabilization of endocytotic vesicles in B16 F1 mouse melanoma cells, Med.

310 Biol. Eng. Comput. 48 (2010) 407–413. doi:10.1007/s11517-010-0599-9.

311 [14] M. Leguèbe, A. Silve, L.M. Mir, C. Poignard, Conducting and permeable states of cell

312 membrane submitted to high voltage pulses: Mathematical and numerical studies

313 validated by the experiments, J. Theor. Biol. (2014). doi:10.1016/j.jtbi.2014.06.027.

314 [15] E.E. Vaughan, D.A. Dean, Intracellular trafficking of plasmids during transfection is

315 mediated by microtubules, Mol. Ther. 13 (2006) 422–428.

316 doi:10.1016/j.ymthe.2005.10.004.

317 [16] E.B. Sözer, Z.A. Levine, P.T. Vernier, Quantitative limits on small molecule transport

318 via the electropermeome measuring and modeling single nanosecond perturbations,

319 Sci. Rep. (2017). doi:10.1038/s41598-017-00092-0.

320 [17] D. Voyer, A. Silve, L.M. Mir, R. Scorretti, C. Poignard, Dynamical modeling of tissue

321 electroporation, Bioelectrochemistry. 119 (2018) 98–110.

322 doi:10.1016/j.bioelechem.2017.08.007.

323 [18] T. Batista Napotnik, G. Bello, E.K. Sinner, D. Miklavčič, The Effect of Nanosecond,

324 High-Voltage Electric Pulses on the Shape and Permeability of Polymersome GUVs,

J. Membr. Biol. 250 (2017) 441–453. doi:10.1007/s00232-017-9968-8.

[19] E. Karatekin, O. Sandre, H. Guitouni, N. Borghi, P.H. Puech, F. Brochard-Wyart, Cascades of transient pores in giant vesicles: Line tension and transport, Biophys. J. 84 (2003) 1734–1749. doi:10.1016/S0006-3495(03)74981-9.

[20] R. Dimova, K.A. Riske, S. Aranda, N. Bezlyepkina, R.L. Knorr, R. Lipowsky, Giant vesicles in electric fields, Soft Matter. 3 (2007) 817–827. doi:10.1039/b703580b.

[21] C. Rosazza, H. Deschout, A. Buntz, K. Braeckmans, M.P. Rols, A. Zumbusch, Endocytosis and Endosomal Trafficking of DNA After Gene Electrotransfer In Vitro, Mol. Ther. - Nucleic Acids. (2016). doi:10.1038/mtna.2015.59.

[22] T. Portet, C. Favard, J. Teissié, D.S. Dean, M.P. Rols, Insights into the mechanisms of electromediated gene delivery and application to the loading of giant vesicles with negatively charged macromolecules, Soft Matter. 7 (2011) 3872–3881. doi:10.1039/c0sm01389g.

[23] D.L. Perrier, A. Vahid, V. Kathavi, L. Stam, L. Rems, Y. Mulla, A. Muralidharan, G.H. Koenderink, M.T. Kreutzer, P.E. Boukany, Response of an actin network in vesicles under electric pulses, Sci. Rep. 9 (2019) 8151. doi:10.1038/s41598-019-44613-5.

[24] M. Barthmes, J. Liao, Y. Jiang, A. Brüggemann, C. Wahl-Schott, Electrophysiological

343 characterization of the archaeal transporter NCX_Mj using solid supported
344 membrane technology, J. Gen. Physiol. 147 (2016) 485–496.
345 doi:10.1085/jgp.201611587.
346 [25] E.M. Schmid, D.L. Richmond, D.A. Fletcher, Reconstitution of proteins on
347 electroformed giant unilamellar vesicles, Elsevier Ltd, 2015.
348 doi:10.1016/bs.mcb.2015.02.004.
349 [26] W. Li, Q. Wang, Z. Yang, W. Wang, Y. Cao, N. Hu, H. Luo, Y. Liao, J. Yang, Impacts
350 of electrical parameters on the electroformation of giant vesicles on ITO glass chips,
351 Colloids Surfaces B Biointerfaces. 140 (2016) 560–566.
352 doi:10.1016/j.colsurfb.2015.11.020.
353 [27] D.J. Estes, M. Mayer, Giant liposomes in physiological buffer using electroformation
354 in a flow chamber, Biochim. Biophys. Acta - Biomembr. 1712 (2005) 152–160.
355 doi:10.1016/j.bbamem.2005.03.012.
356 [28] J. Dermol, O.N. Pakhomova, A.G. Pakhomov, D. Miklavčič, Cell electrosensitization
357 exists only in certain electroporation buffers, PLoS One. 11 (2016).
358 doi:10.1371/journal.pone.0159434.
359 [29] K.J. Müller, V.L. Sukhorukov, U. Zimmermann, Reversible electropermeabilization
360 of mammalian cells by high-intensity, ultra-short pulses of submicrosecond duration,

J. Membr. Biol. 184 (2001) 161–170. doi:10.1007/s00232-001-0084-3.

[30] J. Li, W. Tan, M. Yu, H. Lin, The effect of extracellular conductivity on electroporation-mediated molecular delivery, Biochim. Biophys. Acta - Biomembr. 1828 (2013) 461–470. doi:10.1016/j.bbamem.2012.08.014.

[31] A. Ivorra, Tissue Electroporation as a Bioelectric Phenomenon: Basic Concepts, in: 2010: pp. 23–61. doi:10.1007/978-3-642-05420-4_2.

[32] K. Nishimura, T. Hosoi, T. Sunami, T. Toyota, M. Fujinami, K. Oguma, T. Matsuura, H. Suzuki, T. Yomo, Population analysis of structural properties of giant liposomes by flow cytometry, Langmuir. 25 (2009) 10439–10443. doi:10.1021/la902237y.

[33] N. Ohnishi, Y. Fujiwara, T. Kamezaki, S. Katsuki, Variations of Intracellular Ca 2+ Mobilization Initiated by Nanosecond and Microsecond Electrical Pulses in HeLa Cells, IEEE Trans. Biomed. Eng. 66 (2019) 2259–2268. doi:10.1109/TBME.2018.2886602.

[34] D.B. Weinstein, J.B. Marsh, M.C. Glick, L. Warren, Membranes of animal cells. IV. Lipids of the L cell and its surface membrane., J. Biol. Chem. 244 (1969) 4103–11. http://www.jbc.org/content/244/15/4103.

[35] H.B. Bosmann, A. Hagopian, E.H. Eylar, Cellular membranes: The isolation and characterization of the plasma and smooth membranes of hela cells, Arch. Biochem.

379 Biophys. 128 (1968) 51–69. doi:10.1016/0003-9861(68)90008-8.

380 [36] Q. Li, X. Wang, S. Ma, Y. Zhang, X. Han, Electroformation of giant unilamellar
381 vesicles in saline solution, Colloids Surfaces B Biointerfaces. 147 (2016) 368–375.
382 doi:10.1016/j.colsurfb.2016.08.018.

383 [37] B.L. Ibey, J.C. Ullery, O.N. Pakhomova, C.C. Roth, I. Semenov, H.T. Beier, M.
384 Tarango, S. Xiao, K.H. Schoenbach, A.G. Pakhomov, Bipolar nanosecond electric
385 pulses are less efficient at electropermeabilization and killing cells than monopolar
386 pulses, Biochem. Biophys. Res. Commun. 443 (2014) 568–573.
387 doi:10.1016/j.bbrc.2013.12.004.

388 [38] H. Shiomi, S. Tsuda, H. Suzuki, T. Yomo, Liposome-based liquid handling platform
389 featuring addition, mixing, and aliquoting of femtoliter volumes, PLoS One. 9 (2014)
390 5–10. doi:10.1371/journal.pone.0101820.

391 [39] H. Suzuki, T. Yomo, T. Matsuura, H. Terasawa, K. Nishimura, Coupling of the fusion
392 and budding of giant phospholipid vesicles containing macromolecules, Proc. Natl.
393 Acad. Sci. 109 (2012) 5942–5947. doi:10.1073/pnas.1120327109.

394 [40] K.A. Riske, R.L. Knorr, R. Dimova, Bursting of charged multicomponent vesicles
395 subjected to electric pulses, Soft Matter. 5 (2009) 1983–1986. doi:10.1039/b900548j.

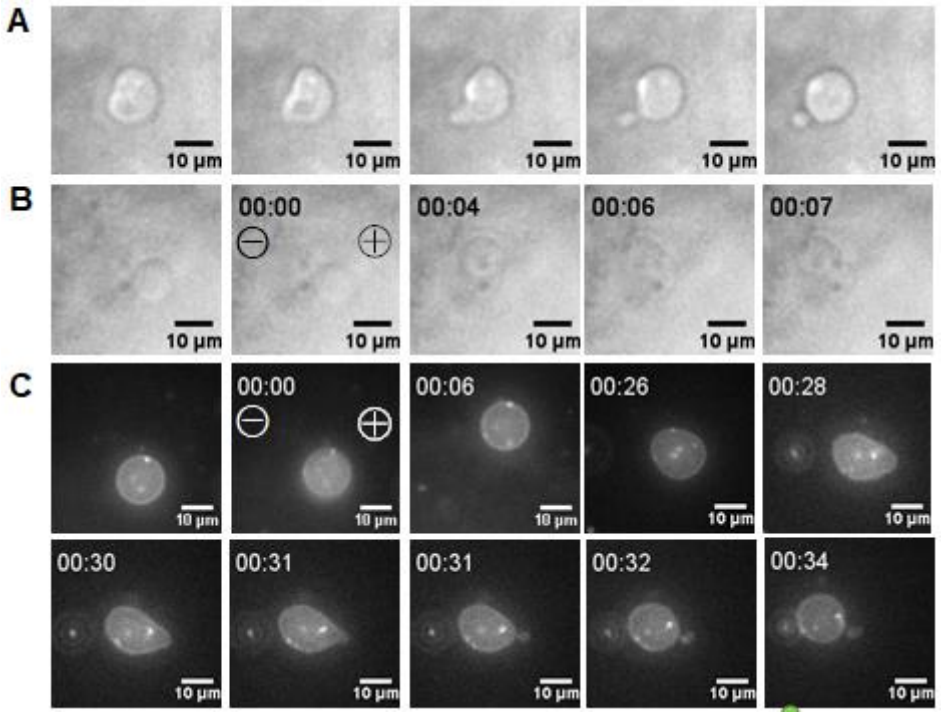
396 [41] T. Portet, R. Dimova, A new method for measuring edge tensions and stability of lipid

bilayers: Effect of membrane composition, Biophys. J. 99 (2010) 3264–3273. doi:10.1016/j.bpj.2010.09.032.

[42] R. Dimova, N. Bezlyepkina, M.D. Jordö, R.L. Knorr, K.A. Riske, M. Staykova, P.M. Vlahovska, T. Yamamoto, P. Yang, R. Lipowsky, Vesicles in electric fields: Some novel aspects of membrane behavior, Soft Matter. 5 (2009) 3201–3212. doi:10.1039/b901963d.

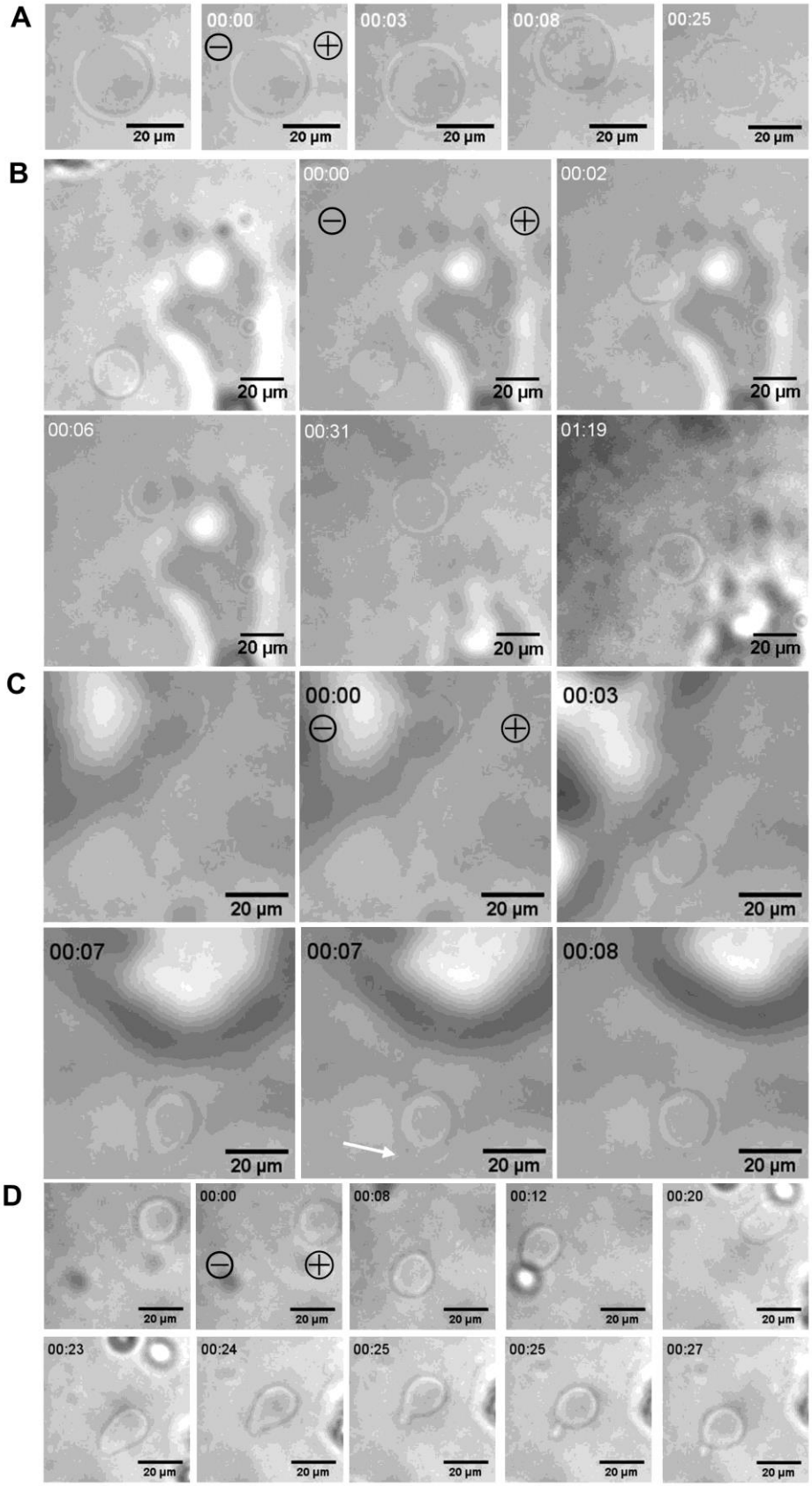
[43] S. Furse, G.C. Shearman, Do lipids shape the eukaryotic cell cycle?, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1863 (2018) 9–19. doi:10.1016/j.bbalip.2017.09.010.

415 **Figures**




416

417 **Figure 1**. A) Spontaneous liposome divisions and PEF conditions. B)
418 Lecithin/cholesterol = 1:1 (molar ratio) liposomes divided spontaneously after a 1.1
419 kV/cm, 400 µs single PEF application. As we did not measure the time, there is no time
420 display in series A. All time displays are in seconds. "00:00" = timing of PEF application.
421 C. Liposomes with lecithin/cholesterol/texas red-DHPE = 1.4:1.4:0.0036 mM. ⊕ = the
422 anode side of the electrode; ⊖ = the cathode side of the electrode.




423

**Figure 2**. Reactions of several liposome types with 1.1 kV/cm, 400 μs single PEF. A) Lecithin-only liposomes did not divide. B) Lecithin/cholesterol = 2:1 (molar ratio) liposomes did not divide. C) Lecithin/cholesterol = 1:1 (molar ratio) liposomes budded daughter liposomes. The white arrow indicates the budding position. (D) Lecithin/cholesterol = 1:1.5 (molar ratio) budded daughter liposomes. All time displays are in seconds. "00:00" = timing of PEF application. ⊕ = the anode side of the electrode; ⊖ = the cathode side of the electrode.

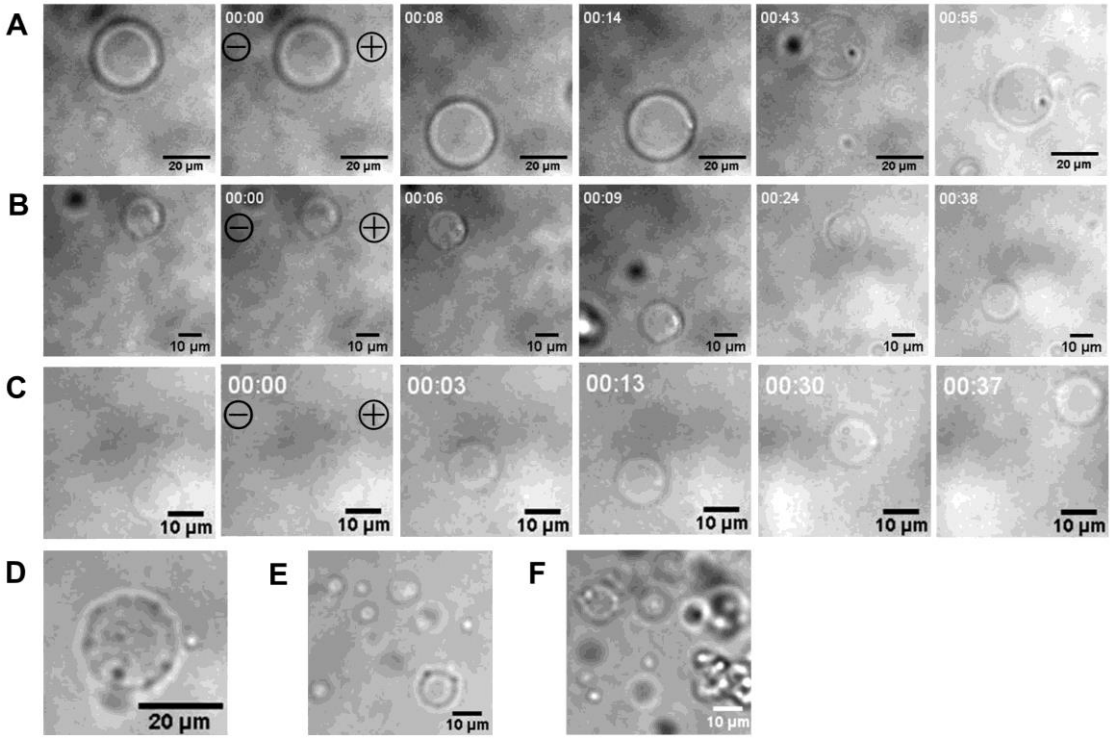



**Figure 3**. Liposomes without lecithin did not exhibit division. PEF was 1.1 kV/cm, 400 µs single pulse. A) DOPC/cholesterol = 1: 1 (molar ratio). B) DOPC/cholesterol =1:2 (molar ratio). C) DOPC/cholesterol = 1:3 (molar ratio). D) POPC-only liposome. E and F) POPC/cholesterol = 1:1 (molar ratio). All time displays are in seconds. "00:00" = timing of PEF application. ⊕ = the anode side of the electrode; ⊖ = the cathode side of the electrode.

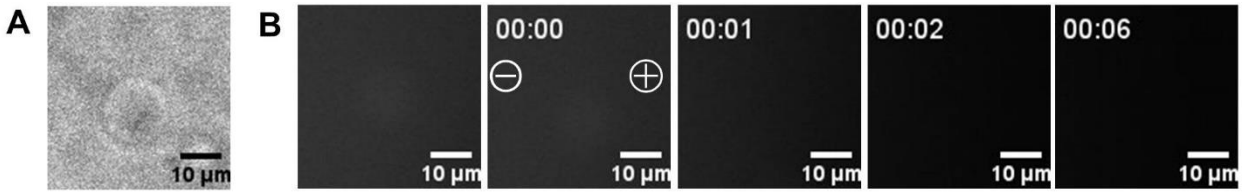



**Figure 4**. Calcium ion influx was not detected in lecithin/cholesterol = 1:1 (molar ratio) liposomes with 1.1 kV/cm, 400 µs single PEF. A) Bright-field photo of liposomes before PEF application. B) Fluorescent photos of liposomes with a GFP filter. There was no signal after pulse application. All time displays are in seconds. "00:00" = timing of PEF application. ⊕ =the anode side of the electrode; ⊖ = the cathode side of the electrode.

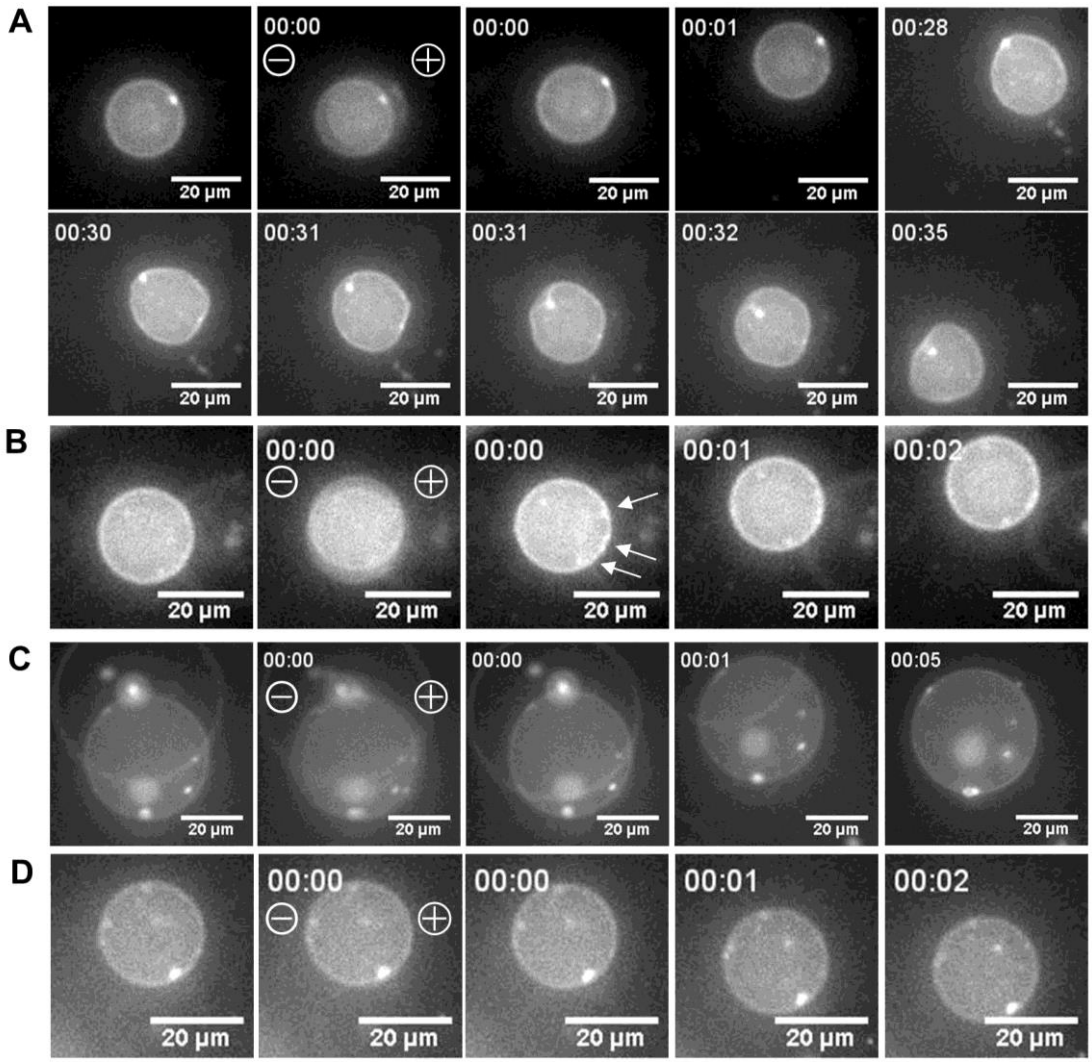



**Figure 5**. (A) Lecithin/cholesterol = 1:1 (molar ratio) liposomes sometimes exhibited endocytosis-like behavior after a single 1.1 kV/cm, 400 μs PEF application. B) Lecithin/cholesterol = 1:1:1 (molar ratio) liposomes showed patch-like aggregation of lipids on the membrane just after PEF application. White arrows indicate aggregated points. C and D) DOPC/cholesterol = 1:1 (molar ratio) liposomes did not show patch-like aggregation of lipids on the membrane. All time displays are in seconds. "00:00" = timing of PEF application. ⊕ = the anode side of the electrode; ⊖ = the cathode side of the electrode.

519 **Tables**

520 **Table 1.** Various PEF duration vs. whether the liposome division happened. Electric
521 field was 1.1 kV/cm.

| Pulse duration / us | Division |
|---|---|
| 100 | × |
| 200 | × |
| 300 | △ |
| 400 | ○ |
| 500 | ○ |

522 ○ = liposome division occurred almost every time; △ = division occurred some of the
523 time; × = division did not occur.

524

525

526 **Table 2.** Two different PEF conditions with the same energy of 1.1 kV/cm PEF (400 μs).
527 Both conditions did not trigger liposome division.

| Electric field (kV/cm) | Pulse duration (μs) | Division |
|---|---|---|
| 6.9 | 10 | × |
| 21.9 | 1 | Discharged |

528 × indicates no liposome division

529

530

531 **Table 3.** Several conductivity patterns of inner and outer liquids. PEF was 1.1 kV/cm
532 (400 μs). $\sigma_{ex}$ is the conductivity of the outer liquid, whereas $\sigma_{in}$ is the conductivity of the
533 inner liquid.

| Condition | Division |
|---|---|
| $\sigma_{ex}$ = 1.6 S/m, $\sigma_{in}$ = 0.6 S/m | ○ |
| $\sigma_{ex}$ = 105 μS/m, $\sigma_{in}$ = 0.6 S/m | × |
| $\sigma_{ex}$ = 1.6 S/m, $\sigma_{in}$ = 105 μS/m | × |
| $\sigma_{ex}$ = 105 μS/m, $\sigma_{in}$ = 105 μS/m | × |

534 ○ indicates liposome division and × indicates no liposome division. For $\sigma_{in}$ = 105 μS/m,
535 we used sucrose solution and for $\sigma_{ex}$ = 105 μS/m, we used glucose solution.

536

537

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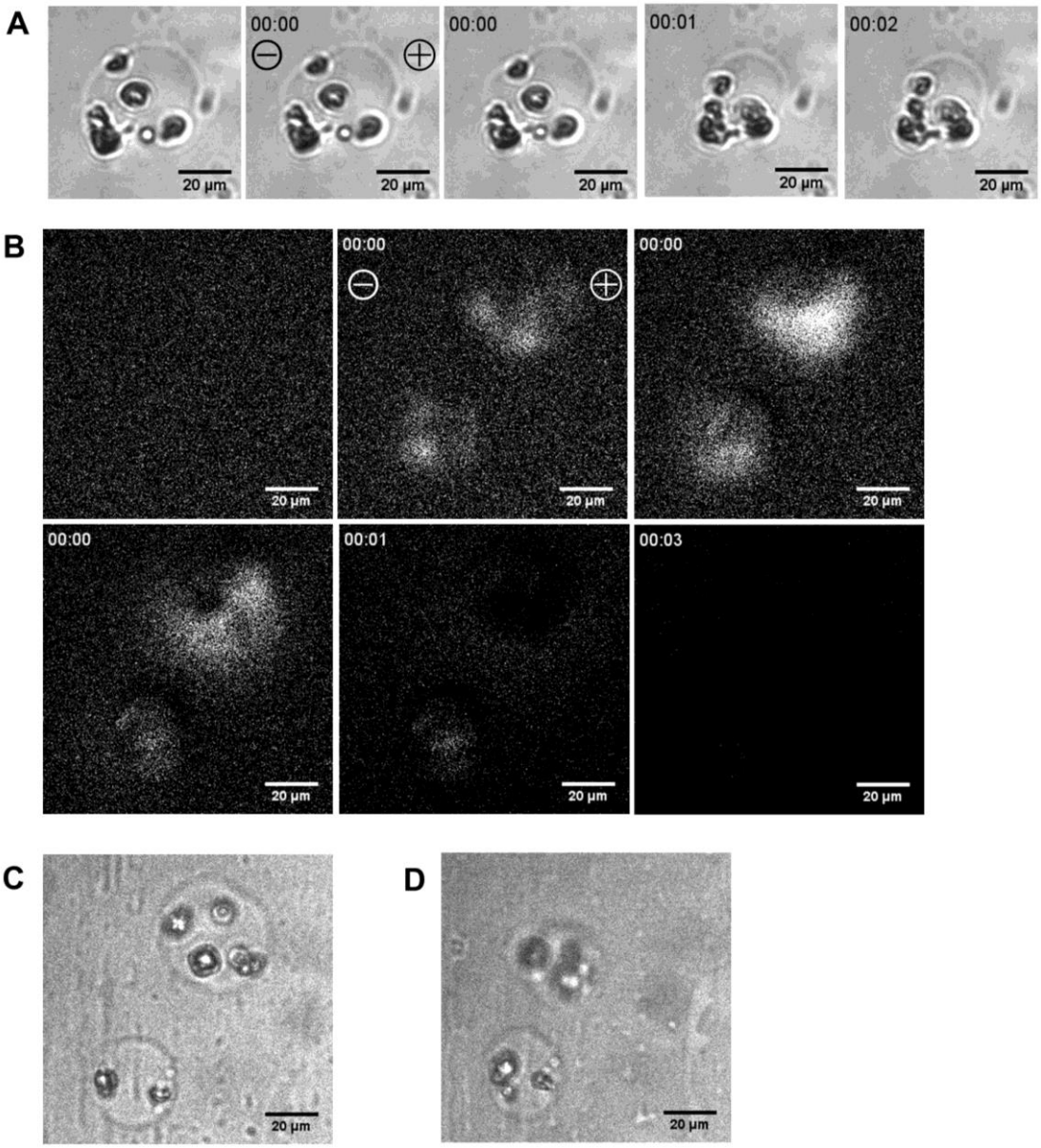
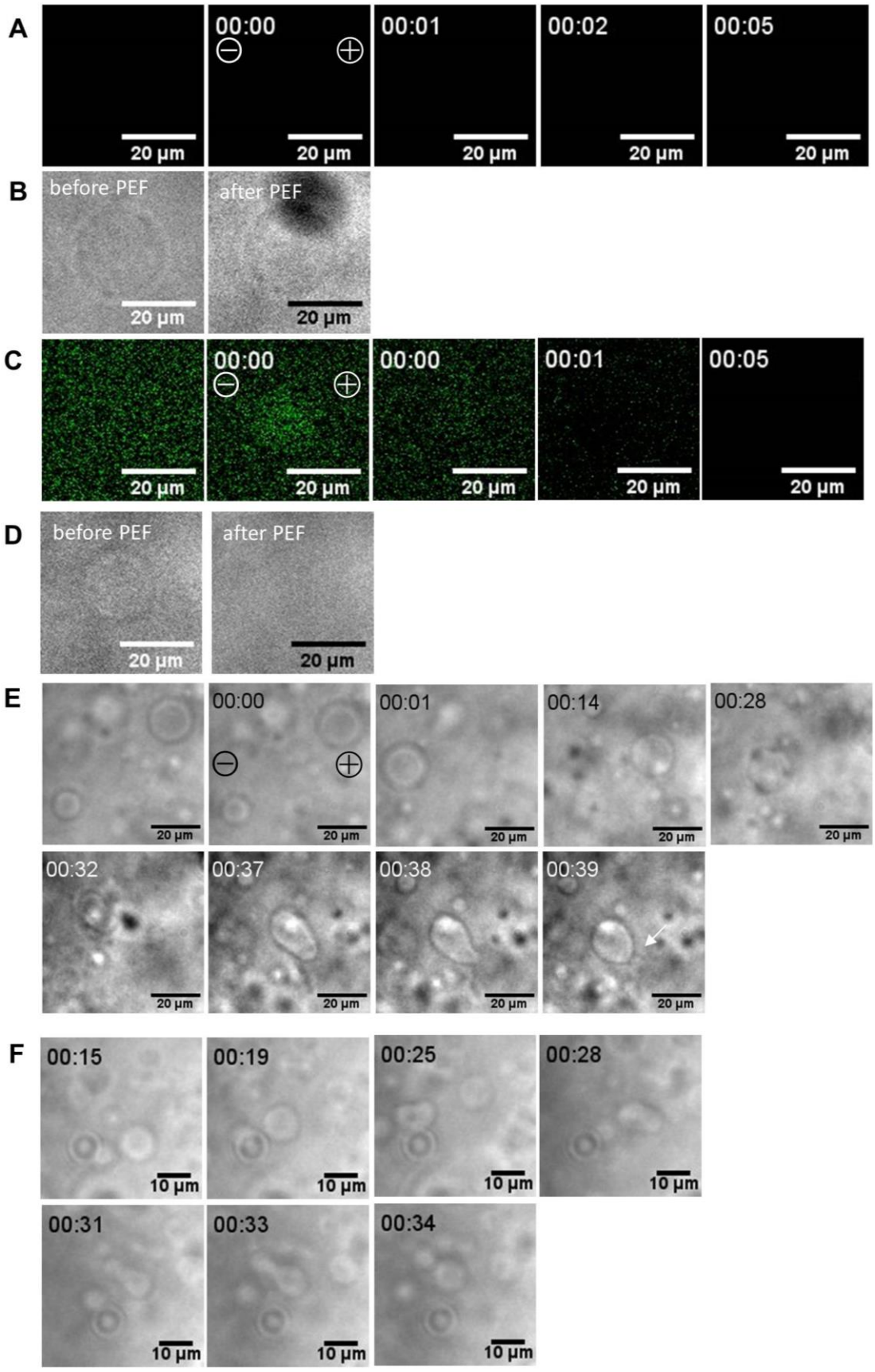
540

## Supplementary Figures




**Supplementary Figure 1.** A) Lecithin/cholesterol = 1:1 (molar ratio) liposomes with a rough membrane surface collapsed just after application of a single 1.1 kV/cm, 400 µs PEF. B) Just after integration of lecithin/cholesterol = 1:1 (molar ratio) liposomes, a fluorescent signal emerged within 1 s. C) Bright-field photo of the liposomes before PEF application. D) Bright-field photo of the liposomes after PEF application. All time displays are in seconds. "00:00" = timing of PEF application. ⊕ = the anode side of the electrode; ⊖ = the cathode side of the electrode.



551 **Supplementary Figure 2.** A) Lecithin-only liposomes did not exhibit calcium influx
552 under low-osmotic-pressure outer liquid. B) Lecithin-only liposomes remained after
553 PEF exposure. C) Lecithin/cholesterol = 1:1 (molar ratio) liposomes showed Ca-ion
554 influx just after PEF application under low-osmotic-pressure outer liquid. D)
555 Lecithin/cholesterol = 1:1 (molar ratio) liposomes disappeared after PEF exposure. E)
556 Lecithin/POPG = 2:1 (molar ratio) liposomes divided. White arrow indicates the
557 daughter liposome. F) Lecithin/POPC = 2:1 (molar ratio) liposomes divided. All time
558 displays are in seconds. "00:00" = timing of PEF application.
559