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# 1 **Sonoporation at a low mechanical index**

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37 Keywords: Sonoporation, Low mechanical index, Microbubbles, Ultrasound contrast  
38 agent, HeLa cells, Cell penetration

## 39 **Abstract**

40 Purpose: The purpose of this study was to investigate the physical mechanisms of  
41 sonoporation, in order to understand and improve ultrasound-assisted drug and gene  
42 delivery. Sonoporation is the transient permeabilisation and resealing of a cell membrane  
43 with the help of ultrasound and/or an ultrasound contrast agent, allowing for the  
44 trans-membrane delivery and cellular uptake of macromolecules between 10 kDa and  
45 3 MDa.

46 Methods: We studied the behaviour of ultrasound contrast agent microbubbles near  
47 cancer cells at low acoustic amplitudes. After administering an ultrasound contrast  
48 agent, HeLa cells were subjected to 6.6-MHz ultrasound with a mechanical index of 0.2  
49 and observed with a high-speed camera.

50 Results: Microbubbles were seen to enter cells and rapidly dissolve. The quick  
51 dissolution after entering suggests that the microbubbles lose (part of) their shell whilst  
52 entering.

53 Conclusions: We have demonstrated that lipid-shelled microbubbles can be forced  
54 to enter cells at a low mechanical index. Hence, if a therapeutic agent is added to the  
55 shell of the bubble or inside the bubble, ultrasound-guided delivery could be facilitated  
56 at diagnostic settings. In addition, these results may have implications for the safety  
57 regulations on the use of ultrasound contrast agents for diagnostic imaging.

## 58 Introduction

59 Sonoporation is the transient permeabilisation and resealing of a cell membrane  
60 with the help of ultrasound and/or an ultrasound contrast agent, allowing for the  
61 trans-membrane delivery and cellular uptake of macromolecules between 10 kDa and  
62 3 MDa.<sup>1</sup> Many studies have demonstrated increased drug and gene uptake of sites  
63 under sonication.<sup>2-9</sup> These studies presumed, that a physical membrane disruption  
64 mechanism, *i.e.*, sonoporation, caused the increased uptake, as opposed to naturally  
65 occurring active uptake processes, such as endocytosis, that are controlled by the  
66 system biology.<sup>2-9</sup> Although mechanical disruption with the aid of ultrasound has been  
67 attributed to violent side effects of inertial cavitation and microbubble fragmentation,  
68 most notably, the increased uptake has also been observed at low acoustic amplitudes,  
69 *i.e.*, in acoustic regimes where inertial cavitation and microbubble fragmentation are not  
70 to be expected. An ultrasound contrast agent microbubble might act as a vehicle to carry  
71 a drug or gene load to a perfused region of interest. If the same ultrasound field that has  
72 been implicated in the sonoporation process can cause release of the therapeutic load,  
73 this load could be delivered into cells. Apart from plainly mixing ultrasound contrast  
74 agents with therapeutic agents, several schemes have been proposed to incorporate  
75 therapeutic loads to microbubbles. These include loads to the microbubble shell,<sup>10</sup>  
76 therapeutic gases inside the microbubble,<sup>11</sup> gas-filled lipospheres containing drugs,<sup>12</sup>  
77 and drug-filled antibubbles.<sup>13</sup> To understand and ameliorate ultrasound-assisted drug  
78 and gene delivery, the physics of controlled release and of sonoporation have been under  
79 investigation. That objective also forms the focus for this paper. Moreover, we studied  
80 the behaviour of ultrasound contrast agent microbubbles near cancer cells deliberately  
81 at low acoustic amplitudes in order to probe whether sonoporation in this regime  
82 was possible; and if so, to ascertain what the microscopic mechanism might entail; and  
83 finally, to assess and scrutinise the safety aspects of ultrasound exposure in this regime.

## 84 Mechanical index

85 The mechanical index (MI) gives an indication of mechanical damage of tissue due to  
86 inertial cavitation. It is defined by:

$$\text{MI} = \frac{p^-}{\sqrt{f_c}}, \quad (1)$$

87 where  $p^-$  is the maximum value of peak negative pressure anywhere in the ultrasound  
88 field, measured in water but reduced by an attenuation factor equal to that which would  
89 be produced by a medium having an attenuation coefficient of  $0.3 \text{ dB cm}^{-1} \text{ MHz}^{-1}$ ,  
90 normalised by 1 MPa, and  $f_c$  is the centre frequency of the ultrasound normalised by  
91 1 MHz. For  $\text{MI} < 0.3$ , the acoustic amplitude is considered low. For  $0.3 > \text{MI} > 0.7$ , there  
92 is a possibility of minor damage to neonatal lung or intestine.<sup>14</sup> These are considered  
93 moderate acoustic amplitudes. For  $\text{MI} > 0.7$ , there is a risk of cavitation if an ultrasound

94 contrast agent containing gas microspheres is being used, and there is a theoretical risk  
95 of cavitation without the presence of ultrasound contrast agents.<sup>15</sup> The risk increases  
96 with MI values above this threshold. These are considered high acoustic amplitudes.  
97 On commercial scanners, the MI has been limited to 1.9 for medical imaging.<sup>16</sup> At low  
98 MI, microbubbles pulsate linearly, whereas at high MI, their greater expansion phase  
99 is followed by a violent collapse. During the collapse phase, when the kinetic energy  
100 of the bubble surpasses its surface energy, a bubble may fragment into a number of  
101 smaller bubbles. Fragmentation has been exclusively observed with contrast agents  
102 with thin, elastic shells. Fragmentation is the dominant disruption mechanism for  
103 these bubbles.<sup>17</sup> Although the fragmentation of therapeutic load-bearing microbubbles  
104 must release their loads, the actual drug or gene delivery is in this case a passive  
105 process, dependent on diffusion rate and proximity to the target cells. Fragmenting  
106 microbubbles may not create pores in cells, since fragmentation costs energy. However,  
107 if a microbubble collapses near a free or a solid boundary, the retardation of the liquid  
108 near the boundary may cause an asymmetry. This asymmetry causes differences in  
109 acceleration on the bubble surface. During further collapse, a funnel-shaped jet may  
110 protrude through the microbubble, shooting liquid to the boundary.<sup>18</sup> The pore size  
111 created by a jet has been empirically related to the microbubble expansion.<sup>19</sup> If  
112 jets could be directed to cell layers, in case of a microbubble carrying a therapeutic  
113 load, the load could be delivered into cells. The jet formation is effected by the  
114 cavitation topology, synergistically interacting with local fluid dynamics arising through  
115 the bubble's expansion and contraction due to the ultrasound field. However, as the  
116 fluid forming the microjet is just the bulk fluid which carries no therapeutic agent, then  
117 there is no guarantee that, even with the formation of a sonopore due to jet impact with  
118 the cell membrane, therapeutic agent will enter the cell. It needs to be dislodged and  
119 mobilised from the bubble first. Furthermore, jetting has not been observed at low or  
120 moderate MI,<sup>20</sup> so that fragmentation is likely to occur before any delivery takes place.  
121 By pushing the loaded microbubbles towards the vessel wall using primary radiation  
122 forces,<sup>21</sup> release can take place closer to target vessels. In a recent study, Caskey *et*  
123 *al.* pushed bubbles into tissue-mimicking gels at MI=1.5.<sup>22</sup> We previously studied  
124 how microclusters consisting of lipid-encapsulated microbubbles can be formed using  
125 primary and secondary radiation forces, and how these clusters can be pushed towards  
126 vessel walls.<sup>23</sup> We found that, even at MI<0.15, microbubble clusters can be formed  
127 and pushed within seconds.

## 128 **Sonoporation**

129 There are five non-exclusive hypotheses for explaining the sonoporation phenomenon.  
130 These have been summarised in Figure 1: push, pull, jetting, shear, and translation.<sup>24</sup>  
131 It has been hypothesised that expanding microbubbles might push the cell membrane  
132 inward, and that collapsing bubbles might pull cell membranes outward.<sup>25</sup> These

133 mechanisms require microbubbles to be present in the close vicinity of cells. A separate  
134 release mechanism should then ensure localised delivery. Although jetting only occurs  
135 in a high-MI regime, it is very effective in puncturing cell membranes. Jetting has  
136 been observed through cells using ultrasound contrast agent microbubbles. However,  
137 the acoustic impedance of the solid cell substratum formed the boundary to which  
138 the jetting took place, not the cell itself.<sup>26</sup> Also, there has not been any proof yet  
139 of cell survival after jetting. In a separate study, we excluded the role of jetting as  
140 a dominant mechanism in sonoporation.<sup>27</sup> If a microbubble is fixed to a membrane,  
141 the fluid streaming around the oscillating bubbles creates enough shear to rupture the  
142 membrane.<sup>28</sup> Here again, separate release mechanism should then ensure localised  
143 delivery. Finally, it has been speculated that lipid-encapsulated microbubbles, in  
144 compressed phase, translate through cell membranes or channels in the cell membrane.  
145 In case of therapeutic loading, the load would be delivered directly into the target cell.  
146 The main advantage of the latter mechanism is that microbubble translation by means  
147 of ultrasonic radiation forces requires very low acoustic pressures. Hence, and potential  
148 damaging bioeffects due to inertial cavitation can be ruled out.

## 149 **Materials and methods**

### 150 **Sonoporation configuration**

151 In previous studies, increased gene uptake was demonstrated at  $MI < 0.3$ .<sup>29,30</sup> We  
152 used a similar sonoporation configuration for our experiments. An overview of the  
153 experimental setup is shown in Figure 2. A signal consisting of 50 cycles with a centre  
154 frequency of 6.6 MHz and a pulse repetition frequency of 10 kHz, *i.e.*, a duty cycle  
155 of 7.5%, was generated by an AFG 3102, dual channel arbitrary function generator  
156 (Tektronix, Inc., Beaverton, OR, USA), amplified by a 150A250 radio-frequency (RF)  
157 amplifier (Amplifier Research, Souderton, PA, USA) set to maximum gain, and fed  
158 to a custom-built 6.6-MHz ultrasound transducer with a hexagonal lithium niobate  
159  $y$ -36°-cut active element with a maximum width of 25 mm.<sup>31</sup> The peak-negative acoustic  
160 pressure was measured to be 0.5 MPa in a separate tank and in the sonication chamber  
161 itself. This corresponds to an MI of 0.2. The transducer was placed in a custom-built,  
162  $260 \times 160 \times 150$  (mm)<sup>3</sup> Perspex sonication chamber, in which an OptiCell<sup>®</sup> cell culture  
163 chamber (Nunc GmbH & Co. KG, Langenselbold, Germany) was placed. One side of the  
164 cell culture chamber contained a monolayer of  $1.6 \times 10^6$  HeLa cells that had been cultured  
165 in MEM with Earl's salts medium (PAA Laboratories GmbH, Pasching, Austria)  
166 supplemented with 10% v/v heat-inactivated fetal calf serum, GlutaMAX<sup>™</sup> (Life  
167 Technologies Gibco, Paisley, Renfrewshire, UK), 1% v/v of non-essential amino-acids  
168 (PAA), penicillin ( $100 \text{ units ml}^{-1}$ ) and streptomycin ( $100 \mu\text{g ml}^{-1}$ ) (PAA), at 37°C in  
169 a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were used when there was  
170 60–80% confluency. Ultrasound contrast agent was injected into the cell culturing

171 chamber before each experiment. Several lipid-shelled ultrasound contrast agents  
172 were tested in this study. In this paper, we present results of a 3.33% dilution of  
173 MicroMarker<sup>TM</sup> (VisualSonics B.V., Amsterdam, Netherlands), a lipid-shelled agent  
174 with a mean diameter of 2.5  $\mu\text{m}$ . A customised BXFM-F microscope unit with an  
175 LCach N 20 $\times$ /0.40 PhC (Olympus Deutschland GmbH, Hamburg, Germany) and a  
176 LUMPlanFL 60 $\times$ /0.90 water-immersion objective (Olympus) was placed on top of the  
177 sonication chamber. The colour charge coupled device (CCD) of a PHOTRON FastCam  
178 MC-2.1 high-speed camera (VKT Video Kommunikation GmbH, Pfullingen, Germany)  
179 was connected to the microscope. The sensor was rotated to make sure that in all  
180 recorded movies, the ultrasound is directed from the left to the right of the frame.

### 181 Fluorescence configuration

182 An overview of the setup used for the fluorescence experiments is shown in Figure 3.  
183 It is almost identical to the setup described in the previous section. However, here,  
184 the signal consisting of 40 cycles with a centre frequency of 6.6 MHz and a pulse  
185 repetition frequency of 10 kHz, *i.e.*, a duty cycle of 6.1%, was amplified using a 2100L,  
186 +50-dB RF amplifier (Electronics & Innovation, Rochester, NY, USA) and fed to our  
187 custom-built 6.6-MHz ultrasound transducer.<sup>31</sup> Prior to injection in the OptiCell<sup>®</sup>, the  
188 MicroMarker<sup>®</sup> contrast agent was labelled using a DiD (DiI<sub>C18</sub>(5)) lipophilic fluorescent  
189 probe (Vybrant<sup>TM</sup> Molecular probes, Invitrogen, San Diego, CA, USA). A ratio of 1  $\mu\text{l}$  of  
190 DiD to 40  $\mu\text{l}$  MicroMarker<sup>®</sup> was homogenised by pipetting and incubating for 5 minutes  
191 at room temperature. Figure 4 shows how the DiD fluorescent probe bonded to the  
192 phospholipid.<sup>32</sup> Emitted  $\lambda=649\text{--}703\text{ nm}$  fluorescence was localised on the microbubble  
193 shell when exciting at  $\lambda=633\text{ nm}$ . A custom-made aluminium sonication chamber with  
194 internal dimensions of 130  $\times$  170  $\times$  35 (mm)<sup>3</sup> was locked into to the  $xy$ -stage of a 200M  
195 inverted confocal microscope (Carl Zeiss AG, Oberkochen, Germany) coupled with a  
196 LSM Axiovert 510 scanning device (Carl Zeiss), using an EC Plan-Neofluar 40 $\times$ /1.30  
197 Oil DIC M27 objective (Carl Zeiss AG), with automated  $z$ -stack functionality.

198 The peak-negative acoustic pressure was measured at the objective's field of view  
199 and corresponded to MI =0.2.

200 To ensure that the microbubbles were not naturally attracted to the cells,  
201 30  $\mu\text{l}$  MicroMarker<sup>®</sup> was diluted into 700  $\mu\text{l}$  of distilled water and tested for  
202 electrophoretic mobility ( $\zeta$ -potential) using a Zetasizer 3000 (Malvern Instruments,  
203 Malvern, Worcestershire, United Kingdom).

204 To measure the thickness of the cultured cells 10<sup>5</sup> HeLa cells were seeded into a  
205 OptiCell<sup>®</sup>. The cell plasma membrane was labelled with DiD lipophilic fluorescent  
206 probe (Vybrant<sup>TM</sup> Molecular probes) according to the manufacturers protocol. The  
207 membrane fluorescence was measured using a 200M confocal microscope. Cell thickness  
208 was calculated from the difference between the upper and lower slices where fluorescence  
209 was seen.

210 We recorded 23 movies under 6.6-MHz sonication at frame rates between 500 and  
211 2000 frames per second, representing 15 minutes of real-time exposure. Of these, 11  
212 movies were recorded using fluorescence. In addition we recorded 10 control movies,  
213 with a total duration of 22 minutes.

## 214 Results and discussion

215 Throughout this section, the optical  $z$ -axis is defined from distal-to-focus (negative) to  
216 proximal-to-focus (positive), with  $z = 0$  as the focal plane.

217 Figure 5 shows  $z$ -stacks of fluorescence emitted by the DiD dye attached to the  
218 membranes of four typical HeLa cells, representing the cell geometry. In total, the  
219 thicknesses of 42 cells were measured. The cultured cells were found to be  $13 \pm 2 \mu\text{m}$   
220 thick. Clearly, these cells had thicknesses much greater than ultrasound contrast agent  
221 microbubble oscillations amplitudes at  $MI=0.2$ .

222 We analysed our optical system and compared our results to bubbles and cells that  
223 were slightly out of focus, to rule out that the movement of the bubble takes place in a  
224 plane different from that of the cell. Figure 6 shows a  $z$ -stack of two ultrasound contrast  
225 agent microbubbles, similar to Figure 10 of Postema *et al.*<sup>33</sup> Proximal-to-focus Airy  
226 disks can be seen around the bubbles, whereas distal-to-focus the bubble boundaries are  
227 blurred. Note that the boundary contrast is maximal just proximal-to-focus.<sup>33</sup>

228 At a centre frequency of 6.6 MHz, we recorded 17 events of microbubbles entering  
229 HeLa cells. After entering, the microbubbles were observed to quickly dissolve. As an  
230 example, Figure 7 shows an event resampled at 3.4 Hz and 40 Hz, respectively, where  
231 two bubbles were pushed to a cell during 11 s of sonication. A microbubble “A” of  $4\text{-}\mu\text{m}$   
232 diameter entered the cell and dissolved, whereas a microbubble “B” of  $2\text{-}\mu\text{m}$  diameter  
233 stuck to the cell membrane.

234 Figures 8 and 9 show two similar events, where fluorescence-coated microbubbles  
235 were used. The left panels show a microbubble apparently penetrating through the cell  
236 membrane in optical focus. Approximately 70 ms after the ultrasound has been switched  
237 on a microbubble is seen to penetrate through the cell membrane in Figure 8. In Figure 9  
238 the microbubble is seen to penetrate through the cell membrane approximately 24 ms  
239 after the ultrasound has been switched on. The right panels show a  $z$ -stack through the  
240 entire cell, to record whether the apparent microbubble entry is actually into the cell.

241 For both events, Figure 10 shows average fluorescent intensities in two regions of  
242 interest, one inside the cell, and one control region. In both events, most fluorescence  
243 from apparent microbubble entry can be observed within  $5 \mu\text{m}$  proximal to optical focus,  
244 thus well within the cells themselves.

245 Figure 11 shows frames in optical focus from the events in Figures 8 and 9, before  
246 sonication and approximately 8 minutes after sonication. Clearly, fluorescence has  
247 transferred into the cells and remained inside the cells long after sonication.



248 At these low acoustic amplitudes, inertial cavitation, fragmentation, and jetting  
249 should not occur. Hence, as a mechanism in sonoporation at low MI, these phenomena  
250 might justifiably be neglected.

251 Our observations do not explain why some microbubbles enter a cell and others  
252 don't. The quick dissolution after entering suggests that the microbubble loses (part of)  
253 its shell whilst entering.

254 The  $\zeta$ -potential measurements showed that the microbubble shells had a charge of  
255  $-43.9 \pm 2.4$  mV. As cells have a natural negative charge,<sup>34,35</sup> the ultrasound contrast  
256 agent should be repelled by the cells, in our recordings we see that, once the ultrasound  
257 was turned on, the microbubbles would be attracted to the closest cell, independent of  
258 the direction of the sound field. This supports the recent finding that cell membranes  
259 can be acoustically active,<sup>36</sup> and therefore interact with microbubbles.

260 Other cell types than HeLa cells must be used in follow-up studies, to investigate  
261 differences in bubble–cell interaction.

## 262 Conclusions

263 We have demonstrated that lipid-shelled microbubbles can be forced to enter cells at a  
264 low MI. Hence, if a therapeutic load is added to the bubble, ultrasound-guided delivery  
265 could be facilitated at diagnostic settings.

266 In addition, these results may have implications for the safety regulations on the use  
267 of ultrasound contrast agents for diagnostic imaging.

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332 **List of Figures**

333 1 Possible mechanisms of sonoporation: a) push, b) pull, c) jetting, d)  
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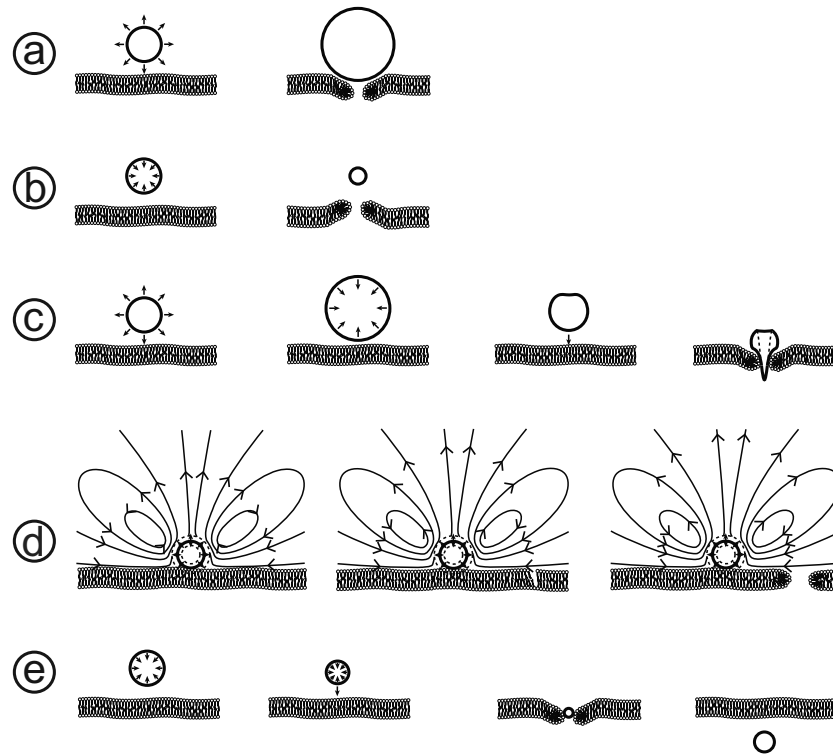


Figure 1: Possible mechanisms of sonoporation: a) push, b) pull, c) jetting, d) shear, e) translation. Based on Figure 9.2 in Postema *et al.*<sup>24</sup>

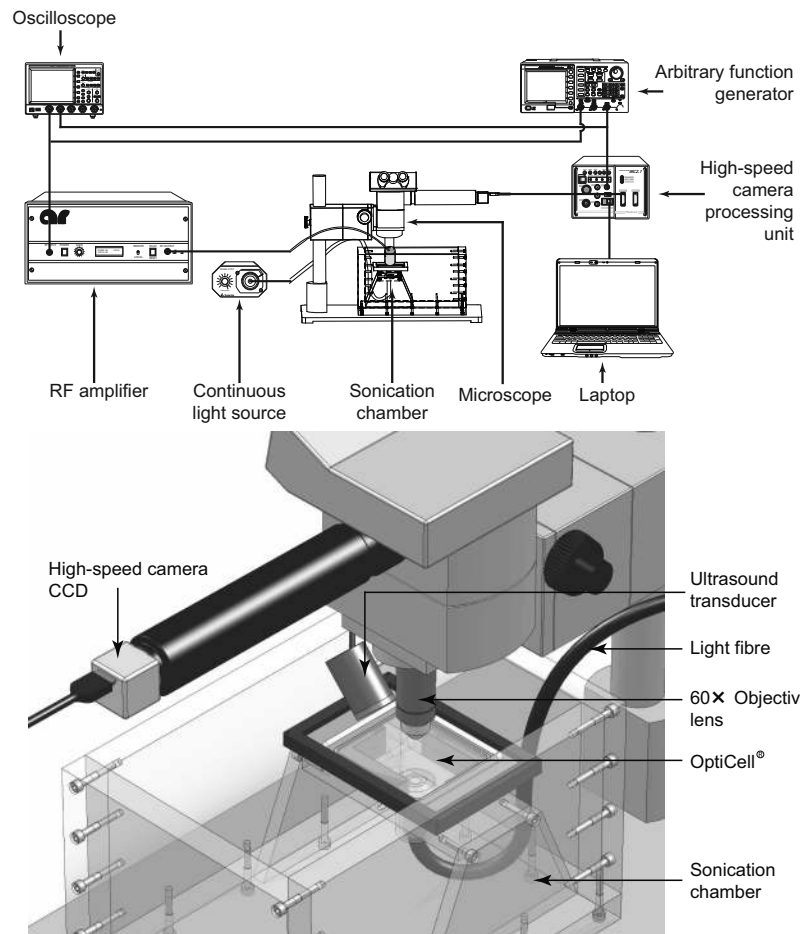


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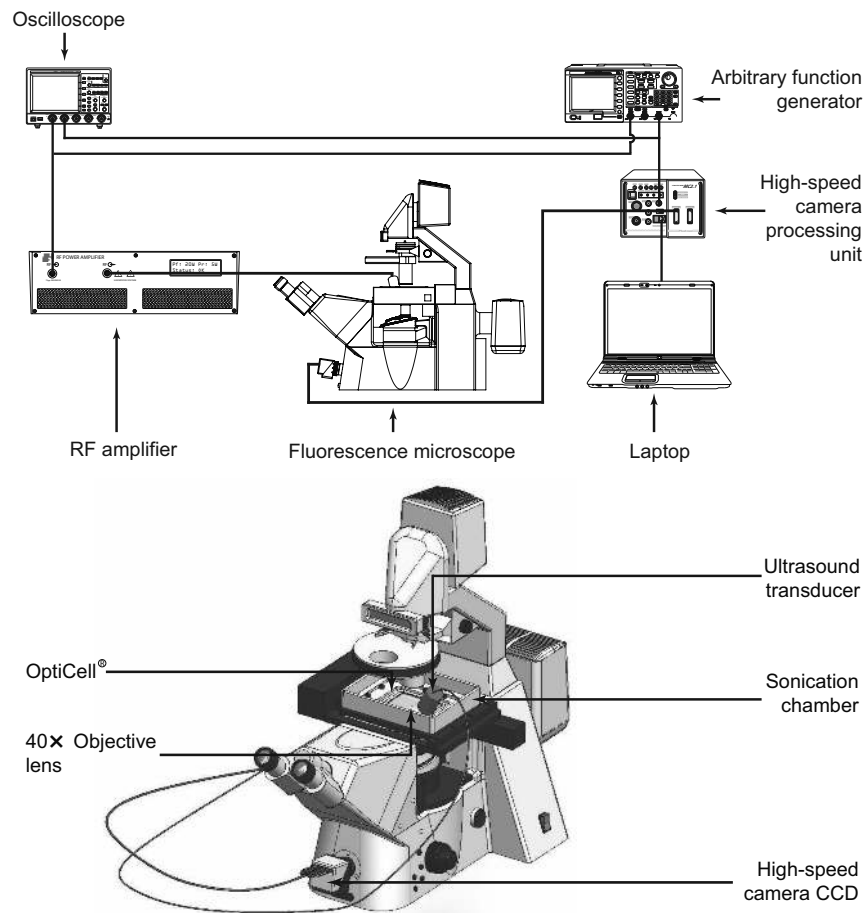


Figure 3: Experimental setup (*top*) and a close-up of the fluorescence configuration (*bottom*).



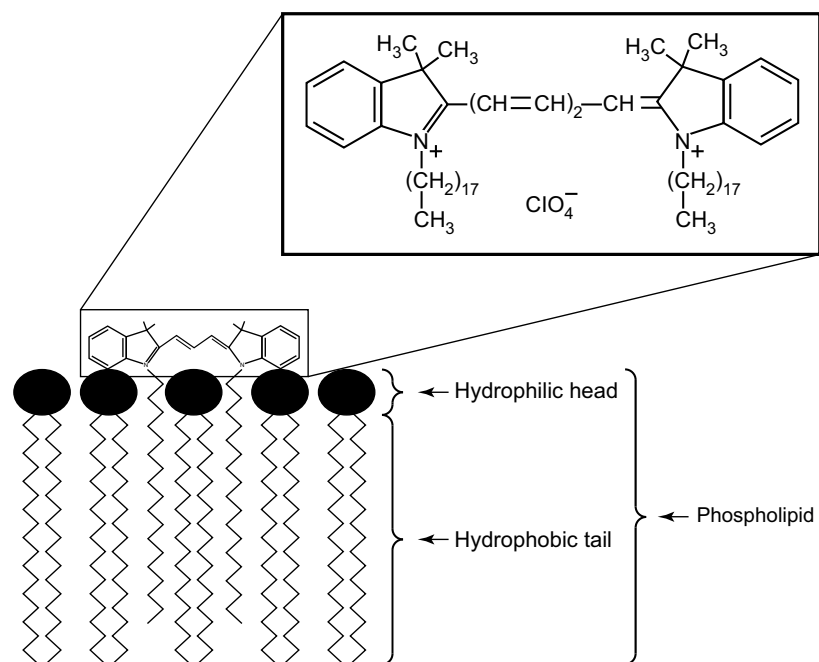


Figure 4: Schematic representation of DiD ( $\text{DiI}_{18}(5)$ ) lipophilic fluorescent probe bonding to phospholipid.<sup>32</sup>

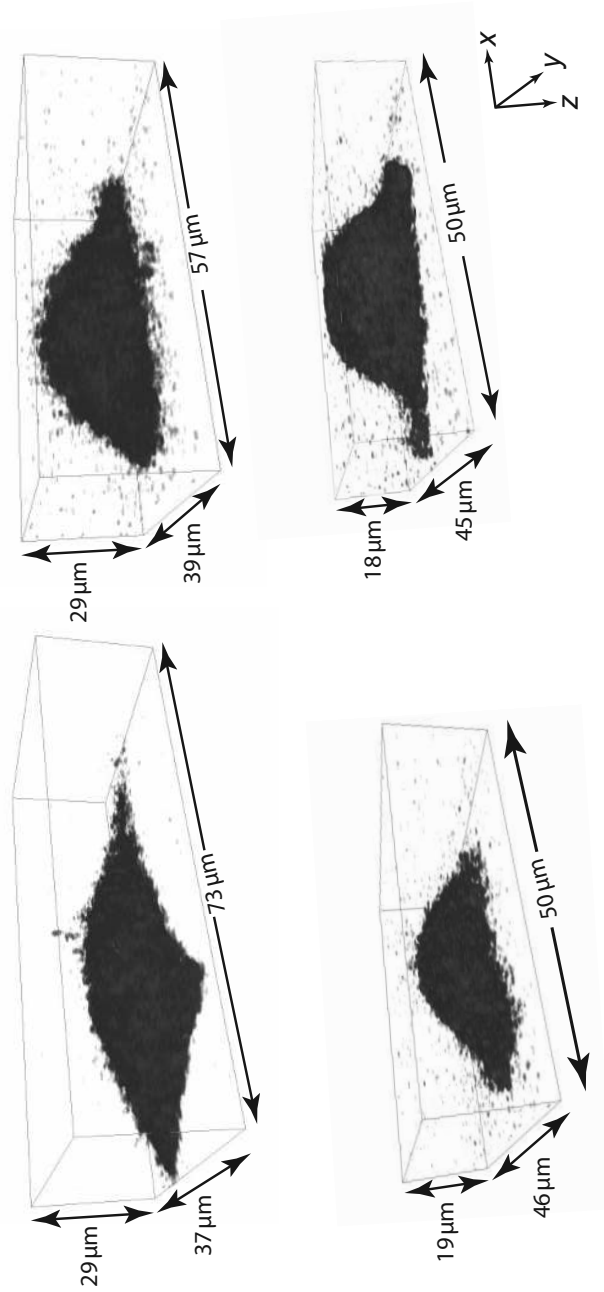


Figure 5:  $z$ -stacks of fluorescence emitted by the DiD dye attached to the membranes of four typical HeLa cells, representing the cell geometry.

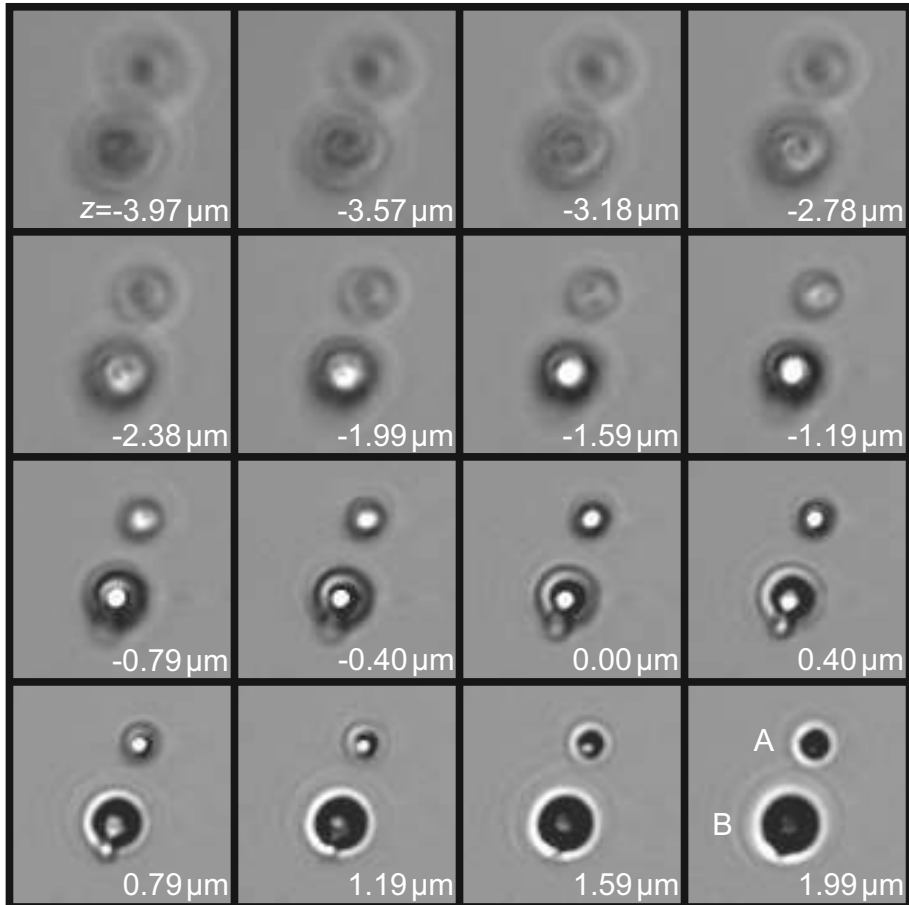


Figure 6:  $z$ -stack of two ultrasound contrast agent microbubbles. Proximal-to-focus Airy disks can be seen around the bubbles, whereas distal-to-focus the bubble boundaries are blurred. Microbubble A has a diameter of  $2\ \mu\text{m}$ , whereas microbubble B has a diameter of  $3\ \mu\text{m}$ . Each frame corresponds to a  $11 \times 11\ (\mu\text{m})^2$  area.

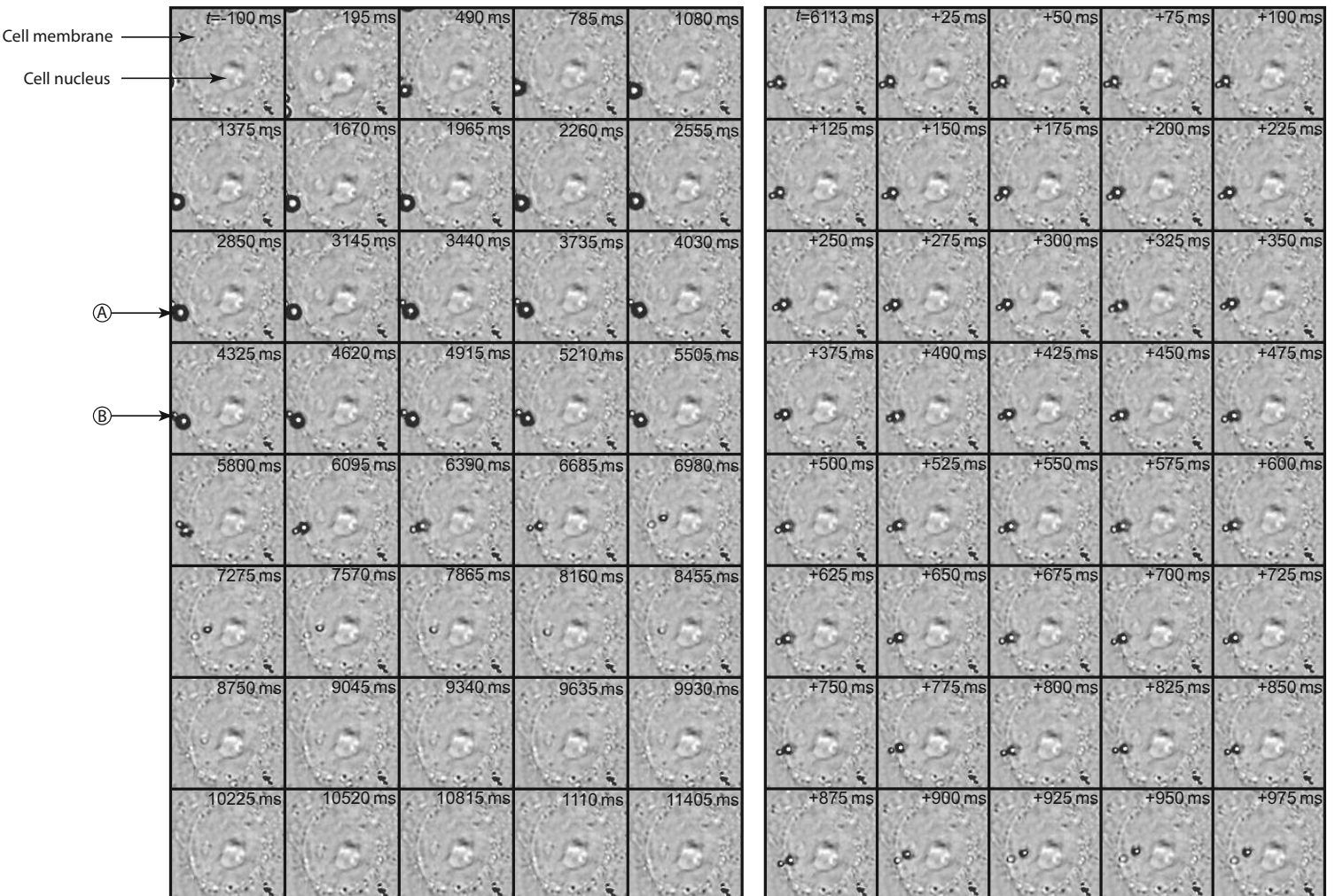


Figure 7: A sonoporation event including microbubble dissolution during 11 s of sonication (*left*) and selected frames of the microbubble entering a cell (*right*). Microbubble “A” entered the cell and dissolved, whereas microbubble “B” struck to the cell membrane. Each frame corresponds to a  $23 \times 23 (\mu\text{m})^2$  area.

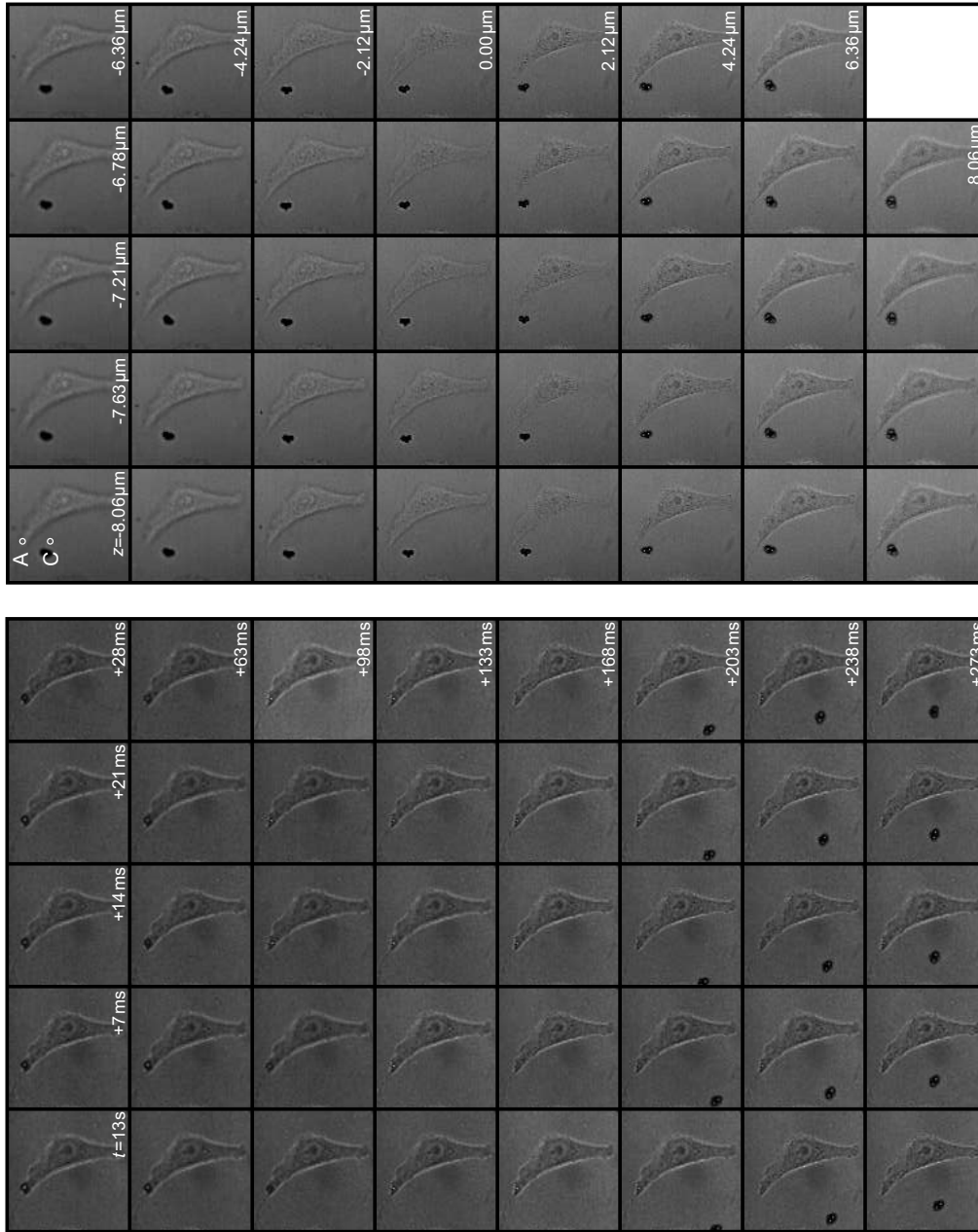


Figure 8: Microbubble of  $5\text{-}\mu\text{m}$  diameter apparently penetrating through the cell membrane in optical focus (*left*);  $z$ -stack through the entire cell, to record whether the apparent microbubble entry is actually into the cell (*right*). Areas (A) and (C) are regions of interest inside and outside the cell, respectively. Each frame corresponds to a  $76 \times 76 (\mu\text{m})^2$  area.

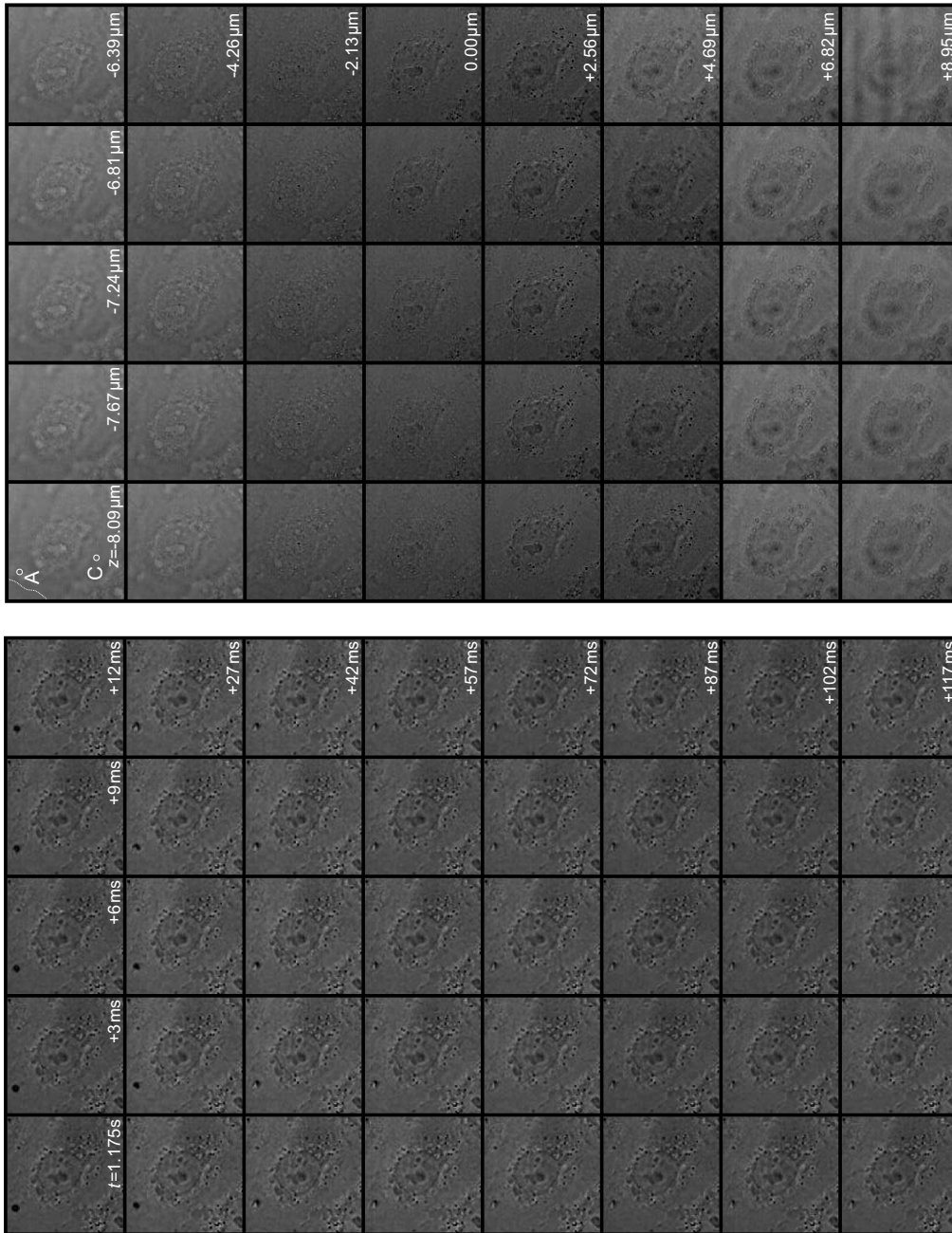


Figure 9: Microbubble of  $4\text{-}\mu\text{m}$  diameter apparently penetrating through the cell membrane in optical focus (*left*);  $z$ -stack through the entire cell, to record whether the apparent microbubble entry is actually into the cell (*right*). Areas (A) and (C) are regions of interest of high fluorescence and low fluorescence, respectively, inside the cell. The white dotted lines in the upper left frame of the right panel indicates the cell membrane. Each frame corresponds to a  $52 \times 52 (\mu\text{m})^2$  area.

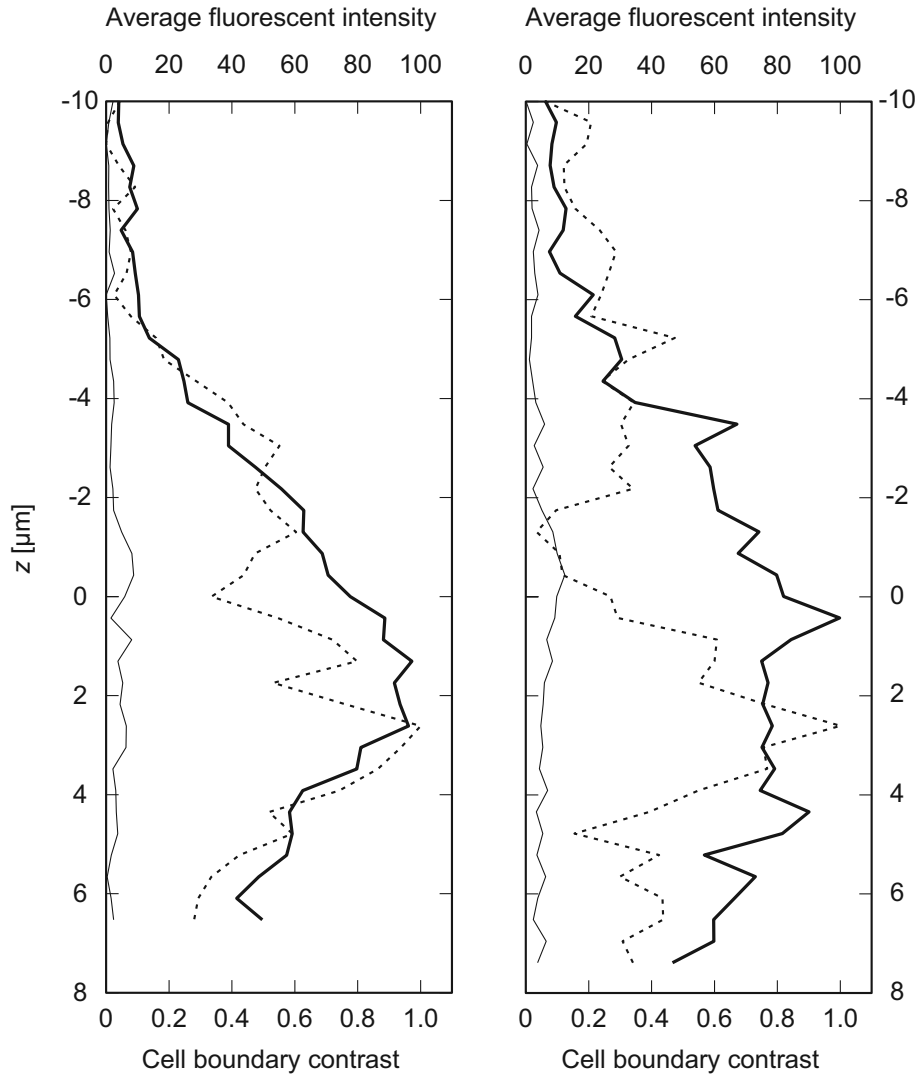


Figure 10: Average fluorescent intensities in the regions of interest (ROI) of Figures 8 (*left*) and 9 (*right*). Bold lines represent ROI (A) inside the cells, whereas hairlines represent ROI (C) the control regions. The dotted line represents the cell boundary contrast. Note that the cell boundary contrast is maximal just proximal-to-focus.

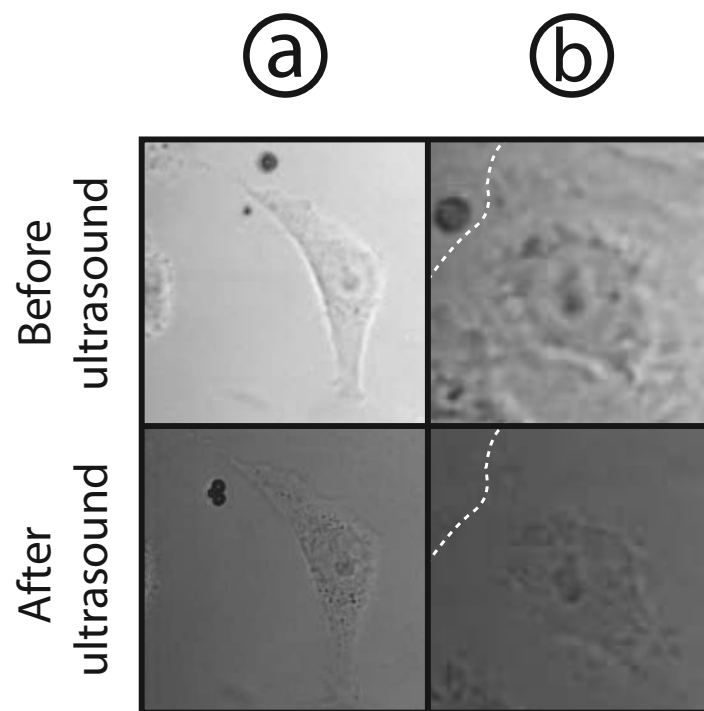


Figure 11: Columns (a) and (b) represent frames in optical focus from the events in Figures 8 and 9, respectively, before sonication and approximately 8 minutes after sonication. The white dotted lines in the right frames indicate the cell membrane boundary. The left frames correspond to  $76 \times 76 (\mu\text{m})^2$  areas, whereas the right frames correspond to  $45 \times 45 (\mu\text{m})^2$  areas.