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### ► To cite this version:

Emilie Franceschini, Laure Balasse, Sandrine Roffino, Benjamin Guillet. Probing the cellular size distribution in cell samples undergoing cell death. *Ultrasound in Medicine & Biology*, 2019, 45 (7), pp.1787-1798. 10.1016/j.ultrasmedbio.2019.01.006 . hal-02200316

**HAL Id: hal-02200316**

**<https://hal.science/hal-02200316>**

Submitted on 31 Jul 2019

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# Probing the cellular size distribution in cell samples undergoing cell death

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## Abstract

A polydisperse scattering model adapted for concentrated medium, namely the polydisperse structure factor model, was examined in order to explain the backscatter coefficients (BSCs) measured from packed cell samples undergoing cell death. Cell samples were scanned using high-frequency ultrasound in the 10-42 MHz bandwidth. A parameter estimation procedure was proposed in order to estimate the volume fraction and the relative impedance contrast that could explain the changes in BSC pattern by considering the actual change in cellular size distribution. Quantitative ultrasound parameters were estimated and related to the percentage of dead cells determined by flow cytometry. The standard deviation of scatterer size distribution extracted from the polydisperse structure factor model and the spectral intercept were found to be strongly correlated to the percentage of dead cells ( $r^2=0.79$  and  $r^2=0.72$ , respectively). The current study contributes to the

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understanding of ultrasonic scattering from cells undergoing cell death towards the monitoring of cancer therapy.

*Key words:*

Quantitative ultrasound, Cell death, Polydispersity, Structure factor model, Scatterer size distribution

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## 1 Introduction

2 Quantitative UltraSound (QUS) techniques for determining tissue mi-  
3 crostructure are promising tools to detect and quantify cell death, and thus  
4 monitor tumor response to therapy. The radiofrequency (RF) backscattered  
5 signals provide information about the tissue microstructure which is not re-  
6 solvable by conventional ultrasound B-mode images. QUS techniques exam-  
7 ine the frequency dependence of the signals backscattered from tissues and  
8 are used in a wide range of applications to differentiate normal versus dis-  
9 eased tissue and characterize tumors in the prostate (Feleppa et al. 1997),  
10 breast (Oelze et al. 2004), lymph node (Mamou et al. 2010) and thyroid  
11 (Lavarello et al. 2013). Spectral-based QUS parameters such as the spectral  
12 slope, the spectral 0-MHz intercept and the midband fit are derived from lin-  
13 ear regression analysis of RF power spectra. Model-based QUS parameters,  
14 such as the average scatterer diameter (ASD) and acoustic concentration  
15 (AAC), can be obtained by fitting an ultrasonic scattering model to the  
16 measured backscatter coefficient (BSC). QUS techniques for monitoring cell  
17 death were first conducted on cell pellet biophantoms exposed to chemother-  
18 apeutics (Czarnota et al. 1997, Kolios et al. 2002, Brand et al. 2008, Brand  
19 et al. 2009). Cell pellet biophantoms consist of centrifuged cells mimicking  
20 densely packed cells in tumors and serve as highly simplified *in vitro* versions  
21 of real tumors. These *in vitro* studies demonstrated that apoptosis causes  
22 an increase in backscatter intensity and a change in QUS parameters (*i.e.*,  
23 spectral slope, intercept, midband fit, ASD and/or AAC) (Kolios et al. 2002,  
24 Brand et al. 2008, Brand et al. 2009). High frequency QUS (>20 MHz) has  
25 also been applied to *in vivo* animal models exposed to cancer radiotherapy or

26 chemotherapy in order to differentiate between responding and non respond-  
27 ing regions within tumors (Vlad et al. 2008, Tadayyon et al. 2015). Recent  
28 clinical applications have also demonstrated that low frequency QUS (<10  
29 MHz) is a power tool to predict breast tumor response to therapy (Sannachi  
30 et al. 2015, Tadayyon et al. 2017), even though lower frequencies cannot be  
31 used to provide an actual description of the cellular structures.

32 In order to develop QUS techniques and interpret QUS parameters for  
33 cancer therapy assessment, it is essential to understand which are the specific  
34 changes (in cell morphology and/or in cellular mechanical properties) during  
35 cell death that cause these changes in QUS parameters. The aforementioned  
36 QUS studies are generally based on classical ultrasound scattering models  
37 (*i.e.*, spherical Gaussian model or fluid sphere model) which assume ran-  
38 domly and independently distributed scatterers. Under this assumption, the  
39 power of the backscattered signals increases linearly with the scatterer volume  
40 fraction and this linear relationship has been used to monitor the ASD and  
41 AAC. More recently, the concentrated ultrasound scattering model, namely  
42 the polydisperse structure factor model (SFM), has been proposed to explain  
43 the measured BSCs (or structure functions) from densely packed media (Han  
44 et al. 2015, Franceschini et al. 2014, 2016). The polydisperse SFM describes  
45 tissue as an ensemble of discrete scatterers and considers interference effects  
46 caused by the correlations among scatterer positions (coherent scattering)  
47 using a structure factor. Experiments on *in vitro* cell pellet biophantoms  
48 and *ex vivo* mouse tumor models demonstrated that the SFM is the most  
49 appropriate model to use for modeling densely packed cellular contents in  
50 tumors (Han et al. 2015, Franceschini et al. 2014, 2016, Muleki-Seya et al.

2016). The SFM has also been used in a two-dimensional (2D) numerical study to explain the contribution of changes in cellular size variance to the increase in the BSC during a cell death process (Vlad et al. 2010). However, this 2D model-based approach cannot be applied to quantitatively comparing the 2D simulated BSCs and the experimentally measured BSCs.

The first objective of this work was to go further in the understanding of the BSCs measured from cells undergoing apoptosis. Ultrasonic backscatter measurements were performed at frequencies ranging from 10 to 42 MHz on colon adenocarcinoma (HT29) cell samples treated with staurosporine, an inducer of apoptotic cell death. The polydisperse SFM was examined in order to explain the BSC measured from HT29 cells undergoing cell death. To this end, a parameter estimation procedure was proposed in order to estimate the volume fraction and the relative impedance contrast that could explain the change in BSC pattern by considering the actual change in cellular size distribution during cell death.

The second objective of this work was to blindly estimate the cellular size distribution in cell samples undergoing cell death. For this purpose, a novel approach was proposed, which consisted in fitting the polydisperse SFM to two BSCs measured before and after therapy. This novel approach makes it possible to estimate four QUS parameters: the mean scatterer radius, the standard deviation of the scatterer size distribution, the total volume fraction and the relative impedance difference. QUS parameters estimated by the polydisperse SFM were compared with the ASD and AAC estimated by the classical sparse scattering model, namely the fluid sphere model. Finally, the relationship between the percentage of dead cells and QUS parameters was

76 investigated.

## 77 **Background: ultrasonic scattering theory**

78 This section briefly describes three ultrasonic scattering models: the poly-  
 79 disperse SFM, the monodisperse SFM and the fluid-sphere model. These  
 80 theoretical models describe tissue as an ensemble of discrete scatterers and  
 81 are based on several approximations for soft tissue scattering ([Insana et al.](#)  
 82 [1990](#)): far-field regime, Born approximation, no multiple scattering and in-  
 83 cident plane wave propagation. Moreover, the scatterers are assumed to be  
 84 non-overlapping spheres with identical acoustic properties.

### 85 *The polydisperse structure factor model (SFM)*

When considering an ensemble of spheres differing only in size with radius  $x$  and scattering amplitudes  $\Phi(k, x)$ , the BSC is given by ([Griffith et al. 1987](#))

$$\begin{aligned} BSC_{\text{SFMp}}(k) = & n \int_0^\infty |\Phi(k, x)|^2 f(x) dx + \\ & n \int_0^\infty \int_0^\infty \Phi(k, x_1) \Phi(k, x_2) H_{12}(k) f(x_1) f(x_2) dx_1 dx_2, \end{aligned} \quad (1)$$

where  $k$  is the wavenumber,  $n$  is the scatterer number density,  $f$  is the probability density function (PDF) of the scatterer radii, and  $H_{12}$  is the partial structure function. In the present study, we use the analytical expression of Eq. (1), which exists when the scattering amplitude is derived from the fluid sphere form factor ([Han & O'Brien 2015](#))

$$\Phi(k, x) = \frac{\gamma_z}{4k} [\sin(2kx) - 2kx \cos(2kx)], \quad (2)$$

where  $\gamma_z$  is the relative acoustic impedance difference between the scatterer and the surrounding medium, and when the scatterer size distribution follows

a gamma PDF ([Griffith et al. 1987](#))

$$f_{(a,\zeta)}(x) = \frac{1}{\zeta!} \left( \frac{\zeta + 1}{a} \right)^{\zeta+1} x^\zeta e^{-(\zeta+1)x/a}, \quad (3)$$

where  $a$  is the mean radius and  $\zeta$  is the gamma width factor ( $\zeta > -1$ ). Note that the gamma width factor measures the width of the gamma distribution (a large value of the gamma width factor  $\zeta$  corresponds to a narrow size distribution).

#### *The monodisperse structure factor model (SFM)*

In the case of a monodisperse size distribution, *i.e.*, an ensemble of identical fluid spheres of radius  $a$ , the BSC is reduced to the following expression ([Franceschini & Guillermin 2012](#))

$$\begin{aligned} BSC_{\text{SFMm}}(k) &= n |\Phi(k, a)|^2 S(k, a, \phi) \\ &= \frac{4}{9} n \gamma_z^2 k^4 a^6 FF(k, a) S(k, a, \phi), \end{aligned} \quad (4)$$

where  $S$  is the monodisperse structure factor and  $FF$  is the fluid sphere form factor defined as:  $FF(k, a) = (3j_1(2ka)/(2ka))^2$ , where  $j_1$  is the spherical Bessel function of the first kind of order 1. Note that  $S$  can be analytically computed as described in Eqs. (A1)-(A4) in ([Franceschini & Guillermin 2012](#)).

#### *The fluid sphere model*

The fluid sphere model assumes a sparse distribution of spheres, such that the fluid spheres are considered to be randomly and independently distributed. In this peculiar case, the structure factor is equal to unity. Considering an ensemble of identical fluid spheres of radius  $a$ , the theoretical BSC



using the fluid sphere model is given by (Insana et al. 1990)

$$BSC_{\text{FSM}}(k) = \frac{4}{9}n\gamma_z^2k^4a^6FF(k, a), \quad (5)$$

where  $n\gamma_z^2$  is the acoustic concentration (AAC) and  $d(= 2a)$  is the scatterer diameter (ASD).

## Materials and Methods

### *Cell sample preparation*

Experiments were conducted with HT29 cell samples treated with staurosporine, a drug which induces mainly cell apoptosis (Qiao et al. 1996). Cells were grown at 37°C in T175 flasks filled with Dulbecco's modified Eagle's medium containing 4.5 g of glucose/liter and supplemented with 10% fetal calf serum. The cells were treated in culture with staurosporine in T175 flasks at 60-70% confluence and then prepared as packed cell samples (*i.e.*, cell pellets). The detailed protocol is described below. For forming one packed cell sample, around  $2 \times 10^8$  HT29 cells are needed (corresponding approximately to three T175 flasks of non-treated cells or four T175 flasks of treated cells). The supernatant (containing dead cells) was removed or reserved from the T175 flasks, and cells were detached with accutase and washed in phosphate-buffered saline (PBS). Cells from flasks were collected and the supernatant, if reserved, was added to the collected cells. After homogenization with a pipette tip, 100  $\mu\text{L}$  of this cell suspension was withdrawn for flow cytometry analysis and 50  $\mu\text{L}$  for cell size analysis. The remaining suspension was centrifuged for 5 minutes at 1200  $g$ , then the medium was aspirated and 500  $\mu\text{L}$  of PBS +/+ was added. After homogenization with a

118 pipette tip, the cells were transferred in an 8-well Nunc Lab-Tek II Chamber  
119 Slide System (Dominique Dutscher, Brumath, France) and was finally cen-  
120 trifuged for 5 minutes at 1700  $g$  to form densely-packed cell samples. Packed  
121 cell samples mimic the spatial distribution and packing of cells in tumors  
122 (Vlad et al. 2010). The chamber was then placed in a plastic dish and  
123 immersed in PBS to allow ultrasound measurement.

124 A total of 24 cell pellet biophantoms were studied and divided into four  
125 series of experiments, each series including five treated cell samples and one  
126 non-treated cell sample. All the experiments in one series were conducted on  
127 the same day, so that the non-treated cell sample could be used as control.  
128 The dose effect or time effect of staurosporine was investigated as follows:

- 129 - *Dose effect 1*: HT29 cells were treated for 24 hours with different drug  
130 doses of 0, 0.125, 0.25, 0.50, 0.75 and 1  $\mu\text{M}$  with addition of super-  
131 natant. Adding the supernatant during the preparation of the packed  
132 cell sample allows the percentage of dead cells to be increased.
- 133 - *Dose effect 2*: Same as dose effect 1.
- 134 - *Dose effect 3*: Same as dose effect 1, except that the supernatant was  
135 not added during the preparation of the packed cell sample.
- 136 - *Time effect*: HT29 cells were treated with 0.5  $\mu\text{M}$  of staurosporine for  
137 0, 6, 12, 24, 36 and 48 hours without addition of supernatant during  
138 the preparation of the packed cell sample.

139 After the ultrasound measurement, the cell samples were fixed in 10%  
140 buffered formalin for three days, dehydrated in a graded ethanol series,

141 cleared in methylcyclohexane and embedded in methyl methacrylate resin.  
 142 The resin-embedded samples were sectioned, and the sections stained with  
 143 toluidine blue. The histological sections allow us to observe structural changes  
 144 occurring in the dying cells and to verify that the cell spatial distribution was  
 145 homogeneous. An example of histological images is presented in Fig. 1.

#### 146 *Cell size and death analysis*

For each experimental condition, cell radii were measured with a Scepter  
 TM 2.0 cell counter (Millipore, Molsheim, France). Figure (2a) shows typical  
 examples of radius distribution for cells treated with 0.5  $\mu\text{M}$  of staurosporine  
 for 0, 12 and 24 hours. The cell size distribution was bimodal, with small  
 cellular fragments (mean radius  $a_s \approx 2.35 \mu\text{m}$ ) and large cells (mean radius  
 $a_l \approx 6 \mu\text{m}$ ). The probability density function (PDF) of the cell radius  $x$  was  
 fitted with a mixture of two gamma PDFs  $f$  with mixing parameter  $p$  and  
 was defined as:

$$F_{(a_s, \zeta_s, a_l, \zeta_l)}(x) = pf_{(a_s, \zeta_s)}(x) + (1 - p)f_{(a_l, \zeta_l)}(x) \quad (6)$$

147 where  $f_{(a_i, \zeta_i)}$  are the gamma PDF defined in Eq. (3) and the subscripts  $s$   
 148 and  $l$  are used for the smaller cellular fragments and for the larger cells,  
 149 respectively.

150 Flow cytometry using Annexin V/7-AAD was performed to quantify cell  
 151 death. For each condition, the 100  $\mu\text{L}$  withdrawn sample of cells was cen-  
 152 trifuged at 500  $g$  for 5 min and resuspended in 100  $\mu\text{L}$  of ice-cold Binding  
 153 Buffer. The cell suspension was then incubated for 15 minutes on ice in the  
 154 dark with 10  $\mu\text{L}$  of Annexin Vfluorescein isothiocyanate (FITC) prediluted  
 155 1:100 in Binding Buffer and 20  $\mu\text{L}$  of 7-AAD (Annexin V-FITC / 7-AAD

156 kit, Beckman Coulter, Marseille, France). Then, 400 $\mu$ L of ice-cold Binding  
157 Buffer was added before quantification with Gallios flow cytometer (Beck-  
158 man Coulter, France). Approximately 2500 events were measured for each  
159 condition. The flow cytometry analysis makes it possible to estimate the  
160 percentage of viable cells and dead cells (in early apoptosis, late apoptosis  
161 and necrosis).

#### 162 *Ultrasound data acquisition and BSC measurements*

163 Ultrasonic data were acquired using a high frequency ultrasound system  
164 (Vevo 770, Visualsonics Inc, Toronto, Canada). Two probes, RMV 710 and  
165 RMV 703, were used in B-mode. For the RMV 710 and the RMV 703  
166 probes, the oscillating single-element focused circular transducers had center  
167 frequencies of 20 and 30 MHz with -10 dB bandwidths of 10-32 and 18-42  
168 MHz, focuses of 15 and 10 mm and f-numbers of 2.1 and 2.5, respectively.  
169 Raw RF data were digitized at a sampling frequency of 250 MHz with 12-bit  
170 precision using a high-speed acquisition card (CS12501, Gage, Lockport, IL,  
171 USA).

172 During the experiments, the focus of each transducer was positioned 1 mm  
173 below the PBS/cell pellet biophantom interface. A translation stage (Physik  
174 Instrument, model M-403.4PD, Karlsruhe, Germany) controlled the probe  
175 motion. Five independent B-mode images were constructed from acquired  
176 RF echoes by translating the probe every 300  $\mu$ m. Examples of ultrasonic  
177 B-mode scans obtained with the 20-MHz center frequency probe are given in  
178 Fig. 3. For around 100 independent RF lines at the center of each B-mode  
179 image, echoes were selected in the focal zone with a rectangular window of  
180 0.75 mm. The power spectra of the gated RF signals were then averaged to

181 provide  $P_{\text{meas}}$ . This procedure was repeated for each probe and each packed  
182 cell sample.

183 The attenuation was calculated for each sample to allow for attenuation  
184 compensation during the BSC estimation. The attenuation measurement  
185 was performed with a focused transducer with center frequency of 22 MHz  
186 with -10 dB bandwidth 11-34 MHz, focus of 26 mm and f-number of 4. The  
187 transducer focus was positioned at the interface between the sample and  
188 the Plexiglass planar reflector. An insertion-loss broadband technique was  
189 used to calculate the attenuation (in neper per centimeter) by comparing the  
190 spectra of the echoes reflected by the well base surface with and without the  
191 sample being inserted in the echo paths (Chen et al.1997).

192 The measured  $BSC_{\text{meas}}$  were computed from  $P_{\text{meas}}$  using the reference  
193 phantom method (Yao et al. 1990). The experimental and processing meth-  
194 ods were described previously in detail in section III.C of (Franceschini et al.  
195 2014). This procedure yielded a  $BSC_{\text{meas}}$  compensated for the attenuation  
196 loss for each probe in the same region-of-interest. The two  $BSC_{\text{meas}}$  were  
197 then combined to yield a single  $BSC_{\text{meas}}$  over the combined bandwidths of  
198 the two transducers (*i.e.*, 10-42 MHz).

#### 199 *Optimization procedure for understanding the scattering from dead cells*

200 The polydisperse structure factor model was first examined in order to ex-  
201 plain the measured  $BSC_{\text{meas}}$  from packed cell samples undergoing cell death  
202 for all the 24 studied samples. Our hypothesis is that the changes in the mea-  
203 sured  $BSC_{\text{meas}}$  during the cell death process is mainly due to the changes  
204 in cell size distribution. In order to confirm or contradict this hypothesis, it  
205 was assumed that:

- 206 1. the main sources of scattering are the whole cells and the contribution  
207 of cellular fragments ( $a_s \approx 2.35 \mu\text{m}$ ) to the backscattering is negligible,
- 208 2. the large cells are gamma distributed, and the radius  $a_l$  and gamma  
209 width factor  $\zeta_l$  are known *a priori* and given by the Scepter TM 2.0 cell  
210 counter,
- 211 3. the total volume fraction  $\phi_l$  is similar for all the cell pellet biophantoms  
212 since all the samples were prepared under the same centrifugation force,
- 213 4. the impedance relative contrast  $\gamma_z$  does not change during the cell death  
214 process .

The unknown parameters are the total volume fraction  $\phi_l$  and the impedance relative contrast  $\gamma_z$ , which are determined by minimizing the cost function  $\mathcal{F}$  defined as (Franceschini et al. 2016):

$$\mathcal{F}(\phi_l^*, \gamma_z^*) = \sum_{i=1}^{24} \left\| \frac{BSC_{\text{meas}}^{Mi}(k_j) - BSC_{\text{SFMP}}^{Mi}(k_j, a_{l_i}, \zeta_{l_i}, \phi_l, \gamma_z)}{BSC_{\text{meas}}^{Mi}(k_j)} \right\|^2. \quad (7)$$

215 which synthesizes the 24 measurements  $M_{i=1\dots 24}$  from the 24 studied cell pel-  
216 let biophantoms. The measured  $BSC_{\text{meas}}^{Mi}$  corresponds to the  $BSC_{\text{meas}}$  aver-  
217 aged over the five measurements (corresponding to the five acquired B-mode  
218 images) for each cell pellet biophantom. A routine *fminsearch* in MATLAB  
219 (The Mathworks Inc., Natick, MA), *i.e.*, a Nelder-Mead simplex method, was  
220 employed to minimize the cost function  $\mathcal{F}$ . Afterwards, the theoretical BSCs  
221 computed with the estimated parameters were compared with the measured  
222  $BSC_{\text{meas}}$ .

## 223 *Blind estimation of QUS parameters*

224 Our second aim was to blindly estimate the QUS parameters. Two ap-  
 225 proaches were compared: 1) by fitting one measured BSC with a straight line  
 226 or with the classical fluid sphere model, or 2) by fitting two measured BSCs  
 227 before and after therapy with the polydisperse SFM.

228 *Estimation of QUS parameters by fitting one measured BSC.* Using a  
 229 straight line model, the spectral slope and the spectral intercept were calcu-  
 230 lated (Lizzi et al. 1986). The spectral slope is the linear slope of the BSC  
 231 as a function of frequency on a log-log scale. The spectral intercept is the  
 232 extrapolation of the BSC linear fit to zero frequency. The slope is related  
 233 to the effective scatterer size and the intercept is determined by the effective  
 234 scatterer size and acoustic concentration.

The  $ASD^*$  and  $AAC^*$  were estimated by fitting one measured  $BSC_{\text{meas}}$   
 with the fluid sphere model (Insana et al. 1990). These parameters were  
 obtained by minimizing the mean square relative error between  $BSC_{\text{meas}}$   
 and  $BSC_{\text{FSM}}$  given in Eq. (5) (Franceschini et al. 2016)

$$\mathcal{C}_1(ASD^*, AAC^*) = \sum_j \left\| \frac{BSC_{\text{meas}}(k_j) - BSC_{\text{FSM}}(k_j)}{BSC_{\text{meas}}(k_j)} \right\|^2. \quad (8)$$

*Estimation of QUS parameters by fitting two measured BSCs before and  
 after therapy.* A novel approach was proposed to estimate QUS scattering  
 properties from the polydisperse SFM. This model parameterizes the BSC  
 with four parameters: the mean scatterer radius  $a$ , the gamma width factor  
 $\zeta$ , the total volume fraction  $\phi$  and the relative acoustic impedance contrast  
 $\gamma_z$ . Because of the large number of unknown parameters, we propose an  
 estimation procedure using two measured  $BSC_{\text{meas}}$  from non-treated sam-

ple (denoted  $BSC_{\text{meas}}^{nt}$ ) and treated sample (denoted  $BSC_{\text{meas}}^t$ ). To model backscattering from cells undergoing cell death, some simplifying assumptions were considered. First, the non-treated cells are modeled as an ensemble of identical fluid spheres, whereas the treated cells are modeled as an ensemble of fluid spheres following a gamma PDF. Secondly, it is assumed that the mean scatterer radius  $a$  and the relative acoustic impedance difference  $\gamma_z$  do not change during the cell death process, and that the total volume fractions of cells are approximately the same for non-treated and treated conditions. The unknown parameters are the gamma width factor  $\zeta$  of the treated cells, the mean scatterer radius  $a$ , the total volume fraction  $\phi$  and the relative impedance difference  $\gamma_z$ , which are determined by minimizing the cost function  $\mathcal{C}$ :

$$\begin{aligned} \mathcal{C}_2(a^*, \zeta^*, \phi^*, \gamma_z^*) = & \\ \sum_j \left\| \frac{BSC_{\text{meas}}^{nt}(k_j) - BSC_{\text{SFMM}}(k_j, a, \phi, \gamma_z)}{BSC_{\text{meas}}^{nt}(k_j)} \right\|^2 & \\ + \sum_j \left\| \frac{BSC_{\text{meas}}^t(k_j) - BSC_{\text{SFMP}}(k_j, a, \zeta, \phi, \gamma_z)}{BSC_{\text{meas}}^t(k_j)} \right\|^2. & \end{aligned} \quad (9)$$

235 The first term represents the fit of  $BSC_{\text{meas}}^{nt}$  with the monodisperse structure  
 236 factor model  $BSC_{\text{SFMM}}$  given by Eq. (4). The second term represents the fit  
 237 of  $BSC_{\text{meas}}^t$  with the polydisperse structure factor model  $BSC_{\text{SFMP}}$  given by  
 238 Eq. (1). The routine *fmincon* was employed to minimize the cost function  
 239  $\mathcal{C}_2$  with the constraint conditions that  $0 \leq a \leq 100 \mu\text{m}$ ,  $1 \leq \zeta \leq 100$  and  
 240  $0 \leq \gamma_z \leq 0.30$ . For the constraint condition on the parameter  $\phi$ , two cases were  
 241 studied: no *a priori* information with  $0 \leq \phi \leq 1$ , or a constraint that assumes  
 242 concentrated medium with  $0.68 \leq \phi \leq 1$ . This point will be discussed later. The  
 243 constrained optimization routine *fmincon* in MATLAB (The Mathworks Inc.,



244 Natick, MA), *i.e.*, the interior-point method, was used because unconstrained  
 245 optimization techniques sometimes gave unrealistic values for the estimated  
 246  $\gamma_z$  (up to values of 0.7).

247 In the sequel of the paper, the standard deviation of the scatterer diameter  
 248 distribution  $\sigma_D^* = 2a^*/\sqrt{\zeta^* + 1}$  will be reported instead of the gamma width  
 249 factor  $\zeta^*$ . For comparison with the fluid sphere model, the acoustic concen-  
 250 tration from the polydisperse SFM is computed as:  $AAC^* = \frac{\phi^* \gamma_z^{*2}}{\frac{(\zeta^*+3)(\zeta^*+2)}{(\zeta^*+1)^2} \frac{4\pi a^{*3}}{3}}$   
 251 [see Eq. (12) in [\(Franceschini et al. 2016\)](#)].

## 252 Results

### 253 *Flow cytometry, histology and cell size distribution*

254 The flow cytometry analysis revealed a mixture of apoptotic and necrotic  
 255 cells in treated cells. The percentage of necrotic cells ranged between 6.7%  
 256 and 29.2%, but no relationship with dose or time exposure was found. The  
 257 percentage of apoptotic cells increased when dose or time exposure to stau-  
 258 rosporine increased (from 5.8% at 0 h to 19.9% at 24 h, 25.7% at 36 h and  
 259 36.3 at 48 h for time effect experiment, or from 5.9% at 0  $\mu$ M to 33.0% at  
 260 0.25  $\mu$ M, to 48.5% at 0.50  $\mu$ M and 62.0% at 1  $\mu$ M for dose effect 1), except for  
 261 dose effect 2. For the latter, the percentage of apoptotic cells in treated sam-  
 262 ples was found equal to 60.2% at 0.25  $\mu$ M, to 42.6% at 0.50  $\mu$ M and 51.8% at  
 263 1  $\mu$ M, with no relationship with dose effect. In the following, the percentage  
 264 of dead cells corresponds to the sum of percentages of cells undergoing early  
 265 apoptosis, late apoptosis and necrosis.

266

267 The histological study showed that in treated cells the staining of the  
 268 nuclear material is uniform (see Fig. 1a), whereas numerous cells treated  
 269 with staurosporine present characteristics of apoptosis (nuclear condensa-  
 270 tion and fragmentation) and characteristics of necrosis (cell swelling) (see  
 271 Fig. 1b). Histological observations also demonstrated that whole cells are  
 272 densely packed in treated and non-treated cell samples. For each histological  
 273 slice, cellular surface fractions were calculated as the ratio between the white  
 274 color area (corresponding to the absence of cells) and the total area. The  
 275 cellular surface fractions were found to be comprised between 0.91 and 0.98  
 276 for all the studied (non-treated and treated) packed cell samples. This result  
 277 was expected since all the packed cell samples were prepared under the same  
 278 centrifugation force. The cellular volume fraction within the cell pellet bio-  
 279 phantoms is related to the cellular area fraction that can be observed on those  
 280 histological images (Fig. 1). That is why we can reasonably assume that the  
 281 total volume fractions of cells are similar in all the 24 cell pellet biophantoms.

283 The cellular radius distribution measured by cell counter was fitted with  
 284 a mixture of two gamma PDFs using Eq. (6). Table 1 summarizes the mean  
 285 radii and gamma width factors for small cellular fragments ( $a_s, \zeta_s$ ) and large  
 286 cells ( $a_l, \zeta_l$ ), as well as the percentage of volume fraction occupied by large  
 287 cells  $\Phi_l$ . For all series of experiments, the non-treated cells exhibit a quasi-  
 288 unimodal size distribution ( $\Phi_l \geq 0.99$ ), a mean cell radius around  $6.5 \mu\text{m}$  and  
 289 a narrow size distribution ( $\zeta \geq 42$ ). After cell death, the percentage of volume  
 290 fraction occupied by large cells  $\Phi_l$  decreases, and the gamma width factor  $\zeta$   
 291 decreases, especially for the dose effect 1 and the time effect. Considering

all the 20 treated cell samples, the averaged radius of large cells is equal to  $a_l = 6.6 \pm 0.9 \mu\text{m}$ , which is close to the mean radius of non-treated cell samples.

Figure 2(b) gives examples of histograms of the cell volume distribution. Even when the counts of small cellular fragments (mean radius  $a_s \approx 2.35 \mu\text{m}$ ) are greater than the counts of large cells (mean radius  $a_l \approx 6 \mu\text{m}$ ), the percentage of volume fraction occupied by the large cells  $\Phi_l$  is always greater than 0.74 (see Table 1 and Figure 2(b)). Since the backscattering cross-section is proportional to the square of the scatterer volume in the low frequency, the contribution of cellular fragments ( $a_s \approx 2.35 \mu\text{m}$ ) to the backscattering can be considered negligible.

*Understanding of scattering: the change in cell size distribution mainly explains the change in BSC pattern on dying HT29 cell samples*

The volume fraction estimated by the optimization procedure using Eq. (7) was found equal to  $\phi_l^* = 0.73$  and agrees well with the expected maximum packing for hard spheres (around 0.74). The relative impedance contrast was found equal to  $\gamma_z^* = 0.25$ . By taking the acoustic parameters of individual viable cells estimated by (Falou et al. 2010) ( $c = 1535 \text{ m/s}$  and  $\rho = 1.09$ ) and the acoustic properties of the surrounding medium close to those of water ( $z_0 = 1.49 \text{ MRayl}$ ), the relative impedance of viable cells is expected to be approximately 0.13. Therefore, the estimated  $\gamma_z^* = 0.25$  seems to be in a reasonable range of value, but maybe slightly overestimated.

Figure 4 shows some examples of  $BSC_{\text{meas}}$  versus frequency curves averaged over the five measurements (corresponding to the five acquired B-mode images) and the corresponding theoretical  $BSC_{\text{theo}}$  computed with the poly-

317 disperse SFM. Also given in Fig. 4 are the goodness-of-fit statistics,  $R^2$ , as  
 318 defined in Eq. (2) in Ref. (Oelze & O'Brien 2006). The theoretical  $BSC_{\text{theo}}$   
 319 was computed with the polydisperse SFM using the radius distribution of  
 320 large cells measured by the cell counter (see  $a_l$  and  $\zeta_l$  given in Table 1) and  
 321 the fitting parameters  $\phi_l^*=0.73$  and  $\gamma_z^*=0.25$  obtained from the optimiza-  
 322 tion procedure. For 19 BSC measurements out of 24 (*i.e.*, 79% of the BSC  
 323 curves), the theoretical and experimental BSCs share a similar pattern with  
 324 goodness-of-fit  $R^2$  values higher than 0.8, as it can also be observed in Fig. 4.  
 325 However, large differences between theory and experiments can also occur.  
 326 For example, the experiment with  $0.75\mu\text{M}$  of the dose effect 1 shows a nega-  
 327 tive value of the goodness of fit ( $R^2=-0.19$ ) meaning that a mere horizontal  
 328 line passing through the average value of the  $BSC_{\text{meas}}$  would fit data better  
 329 than does the polydisperse SFM (see Fig. 4(b)). Note that only one BSC  
 330 measurement out of 24 shows a negative value of the goodness of fit. Oth-  
 331 erwise, 4 BSC measurements over 24 show goodness-of-fit value comprised  
 332 between 0.5 and 0.8.

333 The  $BSC_{\text{meas}}$  and  $BSC_{\text{theo}}$  averaged over the 10-32 MHz bandwidth (cor-  
 334 responding to the frequency bandwidth of the RMV710 probe) are compared  
 335 in Fig. 5 for all the 24 cell pellet biophantoms. A good correlation ( $r^2=0.78$ )  
 336 is found between the averaged  $BSC_{\text{meas}}$  and  $BSC_{\text{theo}}$  (Fig. 5).

337 To conclude, our approach hypothesizes that there is no change in impedance  
 338 contrast during cell death and that both treated and non-treated cell sam-  
 339 ples have similar volume fractions of cells, so that only a change in scatterer  
 340 size distribution has been considered. Despite the use of these simplifying  
 341 assumptions, the good match obtained between theoretical and experimental

342 BSCs in the majority of cases (Figs. 4 and 5) suggests that the change in  
 343 whole cell size distribution is the predominant factor to explain the change  
 344 in the BSC pattern in the HT29 cell samples during the dying process. (A  
 345 more detailed discussion on these simplifying assumptions will follow later.)

346 *Blind estimation of QUS parameters using the classical approaches: the spec-*  
 347 *tral intercept correlates with the percentage of HT29 dying cells*

348 For each cell pellet biophantom, five  $BSC_{\text{meas}}$  were measured (correspond-  
 349 ing to the five acquired B-mode images), and for each measured  $BSC_{\text{meas}}$ ,  
 350 the four classical QUS parameters (ASD\*, AAC\*, spectral slope, spectral  
 351 intercept) were estimated. When using the fluid sphere model, the ASD\*  
 352 increases and the AAC\* decreases as the percentage of dead cells increases.  
 353 Examples of ASD\* and AAC\* values are given in Fig. 6(a) for the series  
 354 of experiments *dose effect 1*. When using a straight line model, the spectral  
 355 slope decreases and the spectral intercept increases as the percentage of dead  
 356 cells increases (data not shown).

357 The percentage of dead cells was plotted against the classical QUS pa-  
 358 rameters for the 24 studied cell pellet biophantoms, as shown in Fig. 6(b)  
 359 with the spectral intercept parameter. The classical QUS parameters yield  
 360 good correlation with the percentage of HT29 dying cells:  $r^2=0.71$  for ASD\*,  
 361  $r^2=0.61$  for AAC\*,  $r^2=0.67$  for the spectral slope and  $r^2=0.73$  for the spec-  
 362 tral intercept. The highest correlation coefficient of  $r^2=0.73$  suggests that  
 363 the spectral intercept may be a pertinent parameter to assess the cell death  
 364 index in an HT29 tumor exposed to chemotherapy.

### 365 *Blind estimation of QUS parameters using the novel approach*

366 When considering the QUS parameters ( $ASD^*$ ,  $AAC^*$  and  $\sigma_D^*$ ) estimated  
367 by the novel approach based on the polydisperse SFM with the constraint  
368  $0 \leq \phi \leq 1$ , the standard deviation of the scatterer diameter distribution  $\sigma_D^*$   
369 was found to increase with the percentage of dead cells, providing a good  
370 correlation coefficient  $r^2=0.79$  (see Fig. 6(c)). No correlation was found  
371 between the percentage of dead cells and the other QUS parameters estimated  
372 by the novel approach ( $ASD^*$  or  $AAC^*$ ).

373 Table 2 gives examples of QUS parameters ( $ASD^*$ ,  $AAC^*$  and  $\sigma_D^*$ ) es-  
374 timated by the novel approach for the *time effect* experiment. Also given  
375 in Table 2 are the expected parameters: the expected ASD and standard  
376 deviation  $\sigma_D$  given by the cell counter, and the expected AAC calculated by  
377 considering  $\phi_l^*=0.73$  and  $\gamma_z^*=0.25$ . The strong constraint  $0.68 \leq \phi \leq 1$  allows us  
378 to obtain QUS parameters close to the expected parameters, whereas the con-  
379 straint  $0 \leq \phi \leq 1$  leads to large differences between the expected and estimated  
380 ASD and AAC (Table 2). As a consequence, the actual standard deviation  
381 of actual cell size distribution and those estimated by the polydisperse SFM  
382 are strongly correlated ( $r^2=0.69$ ) when using the constraint  $0.68 \leq \phi \leq 1$ , and  
383 are moderately correlated ( $r^2=0.46$ ) when using the constraint  $0 \leq \phi \leq 1$  (data  
384 not shown).

## 385 **Discussion and Conclusion**

### 386 *Understanding scattering*

387 It is generally assumed that the scattering within cell pellet biophantom  
388 or tumor mainly arises from the nuclei or from the whole cells (Oelze et

al. 2006; Taggart et al. 2007; Franceschini et al. 2014; Muleki-Seya et al. 2016). The nucleus and whole cell are tightly interconnected and their size and properties change simultaneously during cell death (Vlad et al. 2009). That is why we conducted a study to assess if only a change in nuclear size could explain the change in BSC magnitude, as explained below.

The optimization procedure proposed in Eq. (7) was slightly modified by considering the nuclei as the main sources of scattering. More precisely, the first two assumptions were re-formulated as follows: 1) the main sources of scattering are the nuclei, and 2) the nuclei are gamma distributed and the radius  $a_n$  and gamma width factor  $\zeta_n$  can be calculated from the measurements with Scepter cell counter by considering a nucleus-to-cell ratio ( $a_n/a_l$ ) equal to 0.72. This novel optimization procedure makes it possible to estimate a volume fraction  $\phi_n^*=0.69$  and a relative impedance contrast  $\gamma_{z_n}^*=0.36$ . However, the estimated volume fraction did not match the expected volume fraction of nuclei  $\phi_n \approx \phi_c (a_n/a_l)^3 = 0.27$  (by considering a volume fraction of densely packed whole cells  $\phi_c=0.74$ ). Moreover, the value of the modified cost function  $\mathcal{F}'$  was found to be larger when considering the nuclei as the sources of scattering (when compared with the original cost function  $\mathcal{F}$  in Eq. (7)). Therefore, the whole cell seems to play a major role in the BSC curves for the HT29 cell samples treated with staurosporine, as observed previously in biophantoms of viable K562 and CHO cells (Franceschini et al. 2014).

At the sub-cellular scale, the change in acoustic properties during cell death is still not well understood. Previous acoustic microscopy experiments (at 375 MHz or at 0.9-1 GHz) were performed to measure acoustic properties

414 on single viable and apoptotic cells (Taggart et al. 2007, Strohm et al. 2010).  
 415 It was found that the apparent attenuation increased by 61% after apoptosis,  
 416 whereas the other acoustic properties (sound speed, acoustic impedance, den-  
 417 sity, bulk modulus) were similar (Strohm et al. 2010). However, this apparent  
 418 attenuation increase is difficult to interpret because it can be attributed to  
 419 an increase in the cell absorption or backscatter, or a change in density of  
 420 cell interior (Taggart et al. 2007), or due to diffraction effects on the cellular  
 421 radius of curvature in the transducer focal region (Weiss et al. 2007). Pre-  
 422 vious QUS studies (<40 MHz) were also conducted on packed cell samples  
 423 exposed to cancer therapy and suggest that the increase in backscatter inten-  
 424 sity can be due to the increase in acoustic impedance during the increasing  
 425 compaction of nuclear material (Kolios et al. 2002, Pasternak et al. 2016)  
 426 The nuclear condensation and fragmentation as well as the change in cellular  
 427 size variance were also suggested to provide changes in scatterer size, spacing  
 428 and distribution that might cause increasing backscatter intensity (Hunt et  
 429 al. 2002, Brand et al. 2008, Vlad et al. 2010).

430 Because of the difficulty of differentiating between the influences of the  
 431 change in acoustic impedance and the change in scatterer size variance, it  
 432 was assumed, in a first approximation, that there is no change in acoustic  
 433 impedance during cell death. Despite this simple approximation, it is very  
 434 interesting to observe a good match between theoretical and experimental  
 435 BSCs in Figs. 4 and 5 for the majority of cases (*i.e.*, 19 BSC measurements  
 436 out of 24). This suggests that the change in size distribution of large cells  
 437 mainly contributes to the change in BSC pattern in dying HT29 cell samples.  
 438 For 6 BSC measurements out of 24, the BSC experimental measurements



could not be fully explained by our approach (see Figs. 4(b) and 4(d)). Investigating the sources that could explain this mismatch is difficult. It can be due for example to a change in cellular size variance (as proposed in this study) combined with a change in acoustic impedance. One might also consider a more complex scattering process by considering both nuclei and whole cells responsible for the scattering since histological observations reveal that the size distribution changes in both the nucleus and the cell during the cell death process. However, not all changes observed on histological images will result in scattering changes.

#### *Blinded estimation of QUS parameters*

The spectral slope is linked to the effective scatterer size. When considering a medium with scatterers differing only in size, the spectral slope decreases with the increase in scatterer size variance. As an example, theoretical BSCs were calculated in the 10-42 MHz bandwidth by using Eq. (1) for a mean scatterer radius of  $7\mu\text{m}$  and various gamma width factors  $\zeta = 90, 50, 30$  and  $10$ . The corresponding spectral slopes were found to be equal to  $4.10, 4.05, 4.00$  and  $3.8$ , respectively. In our experimental study, the decrease in the spectral slope with the increase in the percentage of HT29 dying cells is thus consistent with an increase in the cellular size variance. Similar observations linked to the cellular size variance were previously observed in FaDu, Hep-2 and C666-1 cell samples exposed to radiotherapy (Vlad et al. 2008, Vlad et al. 2010). When using the fluid sphere model, the ASD\* was also found to increase together with the percentage of dying HT29 cells, which is consistent with the decrease in the spectral slope.

464 In the present study, a novel approach was proposed to estimate QUS  
 465 parameters from the analysis of HT29 cell samples before and after treat-  
 466 ment. Plotting the percentage of dead cells against the standard deviation of  
 467 scatterer diameter distribution  $\sigma_D^*$  estimated by the polydisperse SFM shows  
 468 a good correlation coefficient  $r^2=0.79$  (Fig. 6(c)). The blind estimation of  
 469 the change in cellular size variance may thus be a complementary parameter  
 470 to assess the cell death index since the changes in cellular size variance re-  
 471 flect actual structural changes occurring during cell death. However, further  
 472 study should be conducted on *in vivo* tumors to confirm the added value  
 473 of this novel QUS parameter. Indeed, the assumptions of small changes in  
 474 mean scatterer radius and in cellular volume fraction that are valid for the  
 475 HT29 cell pellet biophantoms may not be adequate for actual tumors. For  
 476 example, the cellular volume fraction within actual tumors could vary dur-  
 477 ing the cell dying process, due to cell swelling during necrosis and/or due  
 478 to cell shrinkage and formation of apoptotic bodies during apoptosis. More-  
 479 over, tumors have more complex structures than cell pellet biophantoms.  
 480 The blood microvessels, extracellular matrix and tumor heterogeneity (re-  
 481 sponding and non-responding regions, different forms of cell death) may play  
 482 a role in tumor scattering, as shown by (Han et al. 2013). Future studies  
 483 should focus on 1) comparing the changes in backscattering between tumors  
 484 and cell samples of the same cell line under the same chemotherapy to go  
 485 further in the understanding of scattering from tumor, and 2) comparing the  
 486 standard QUS parameters with those estimated by the polydisperse SFM on  
 487 preclinical tumors under therapy to help in interpreting the standard QUS  
 488 parameters.

## 489 **Acknowledgements**

490     We gratefully thank Samantha Fernandez from the European Center for  
491     Research in Medical Imaging (CERIMED) for technical support in the prepa-  
492     ration of cell samples and flow cytometry analysis. This work was carried  
493     out thanks to the support of the French Agence Nationale de la Recherche  
494     (ANR) under the A\*MIDEX project (ANR-11-IDEX-0001-02) funded by the  
495     Investissements d’Avenir French Government program.

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603



604 **Tables**

605 **Table 1:** Mean radius  $a_s$  (in  $\mu\text{m}$ ) and gamma width factor  $\zeta_s$  for small  
606 cellular fragments. Mean radius  $a_l$  and gamma width factors  $\zeta_l$  for  
607 large cells, and corresponding percentage of volume fraction occupied  
608 by large cells  $\Phi_l$ . Results are presented for the four series of experi-  
609 ments (i.e. ST dose and time effect) and for the different conditions  
610 (dose effect  $C_{i=1\dots 6}=0, 0.125, 0.25, 0.50, 0.75$  and  $1 \mu\text{M}$  and time effect  
611  $C_{i=1\dots 6}=0, 6, 12, 24, 36$  and  $48$  hours ).

		<i>Dose 1</i>	<i>Dose 2</i>	<i>Dose 3</i>	<i>Time</i>
$C_1$	$a_s, \zeta_s$	-, -	2.5, 15.2	2.4, 9.3	2.5, 15.1
	$a_l, \zeta_l$	6.3, 53.6	6.4, 42	6.8, 55.6	6.4, 95
	$\Phi_l$	1	1	0.99	0.99
$C_2$	$a_s, \zeta_s$	2.7, 5.1	2.6, 11.6	2.3, 2.3	2.3, 8.8
	$a_l, \zeta_l$	7.3, 23.9	5.5, 13.7	7.3, 73.1	6.7, 50.2
	$\Phi_l$	0.91	0.96	0.93	0.97
$C_3$	$a_s, \zeta_s$	2.7, 5.8	2.6, 13.5	2.7, 5.4	2.6, 6.5
	$a_l, \zeta_l$	7.2, 16.4	5.6, 11.7	7.5, 57.9	6.9, 50.4
	$\Phi_l$	0.92	0.97	0.93	0.93
$C_4$	$a_s, \zeta_s$	2.6, 7.7	3.7, 4.1	2.3, 2.3	2.3, 3.9
	$a_l, \zeta_l$	5.3, 10.1	7.1, 36.2	7.2, 58.3	7.1, 55.3
	$\Phi_l$	0.86	0.81	0.85	0.74
$C_5$	$a_s, \zeta_s$	2.6, 10.1	2.4, 13.8	2.4, 3.9	2.4, 5.0
	$a_l, \zeta_l$	5.5, 19.4	4.8, 18.3	6.9, 55.2	7.6, 48.8
	$\Phi_l$	0.84	0.92	0.80	0.75
$C_6$	$a_s, \zeta_s$	2.4, 12.4	2.5, 10.3	2.4, 5.5	2.0, 4.0
	$a_l, \zeta_l$	5.7, 10.6	5.2, 22.7	6.5, 39.4	8.5, 29
	$\Phi_l$	0.93	0.88	0.74	0.80

613 **Table 2:** Examples of QUS parameters ( $\text{ASD}^*$  and  $\sigma_D^*$  in  $\mu\text{m}$  and  $\text{AAC}^*$  in  
614  $\text{dB}\cdot\text{cm}^{-3}$ ) estimated by the polydisperse SFM for the *time effect* ex-  
615 periment. The novel approach was tested by considering two different  
616 constraint conditions:  $0 \leq \phi \leq 1$  or  $0.68 \leq \phi \leq 1$ .

617

		Expected parameters	QUS with constraint $0 \leq \phi \leq 1$	QUS with constraint $0.68 \leq \phi \leq 1$
6 h	$\text{ASD}^*, \sigma_D^*$ $\text{AAC}^*$	13.4, 1.86 64.3	4.7, 0.57 70.9	11.7, 1.17 63.3
12 h	$\text{ASD}^*, \sigma_D^*$ $\text{AAC}^*$	13.8, 1.91 63.9	9.0, 2.36 55.8	12.2, 1.33 64.9
24 h	$\text{ASD}^*, \sigma_D^*$ $\text{AAC}^*$	14.2, 1.89 63.1	7.9, 2.04 58.5	11.3, 1.31 66.1
36 h	$\text{ASD}^*, \sigma_D^*$ $\text{AAC}^*$	15.2, 2.15 62.7	4.6, 1.26 73.6	11.6, 1.53 67.0
48 h	$\text{ASD}^*, \sigma_D^*$ $\text{AAC}^*$	17.0, 3.10 63.3	4.5, 1.63 83.7	15.3, 2.30 66.9

618

619 **Figure Captions**

620 **Figure 1:** Histological images of HT29 cell pellet biophantoms (a) non treated  
621 and (b) treated with staurosporine 1  $\mu\text{M}$ .

622 **Figure 2:** a) Typical examples of cell radius distribution. The solid lines  
623 are the direct measurements using the Sceptor cell counter and the  
624 dashed lines correspond to the fitting curves with the cell radius prob-  
625 ability density function  $F(x)$  given by Eq. (6). (b) Histograms of the  
626 cell volume distribution  $V(x) = F(x)(4/3)\pi x^3$ . The histograms are  
627 normalized to one by dividing each count from the cell volume distri-  
628 bution to the maximum count.

629 **Figure 3:** a) Ultrasonic probe and cell pellet biophantom in a well im-  
630 mersed in PBS. b) and c) Examples of B-mode images of non-treated  
631 and treated cell pellet biophantoms obtained with the 20-MHz center  
632 frequency probe. The treated cell sample corresponds to the sample  
633 treated with staurosporine (ST) at 0.5  $\mu\text{M}$  for 36 hours.

634 **Figure 4:** Comparison between measured  $BSC_{\text{meas}}$  (solid lines) and theo-  
635 retical  $BSC_{\text{theo}}$  predicted by the polydisperse structure factor model  
636 (dashed lines) for the dose effect 1 (a) and b) and for the time effect  
637 (c) and (d).

638 **Figure 5:** Comparison of mean  $BSC_{\text{theo}}$  in the 10-32 MHz bandwidth pre-  
639 dicted by the polydisperse structure factor model with the mean  $BSC_{\text{meas}}$   
640 in the 10-32 MHz bandwidth.

641 **Figure 6:** (a) Examples of  $ASD^*$  and  $AAC^*$  estimated by the fluid sphere  
642 model for the series of experiments *dose effect 1*. For each studied  
643 dose, five symbols are represented and correspond to the five acquired  
644 B-mode images. (b) Spectral intercept as a function of percentage of  
645 dead cells for the four experimental series. (c) Standard deviation of the  
646 scatterer diameter distribution  $\sigma_D^*$  estimated by the polydisperse SFM  
647 as a function of the percentage of dead cells for the four experimental  
648 series.

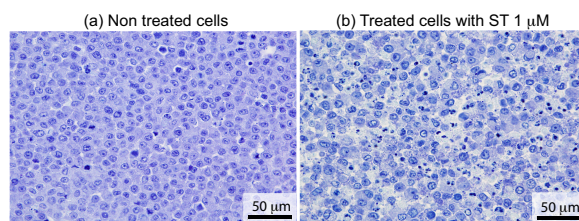


Figure 1: Histological images of HT29 cell pellet biophantoms (a) non treated and (b) treated with staurosporine 1  $\mu$ M.

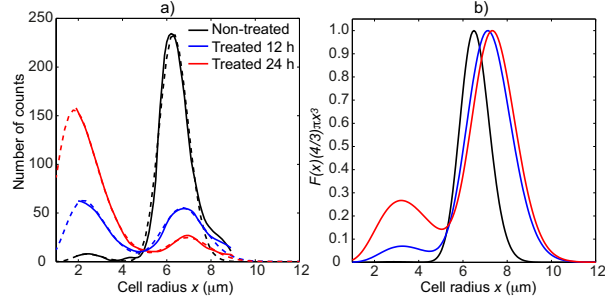


Figure 2: (a) Typical examples of cell radius distribution. The solid lines are the direct measurements using the Scepter cell counter and the dashed lines correspond to the fitting curves with the cell radius probability density function  $F(x)$  given by Eq. (6). (b) Histograms of the cell volume distribution  $V(x) = F(x)(4/3)\pi x^3$ . The histograms are normalized to one by dividing each count from the cell volume distribution to the maximum count.

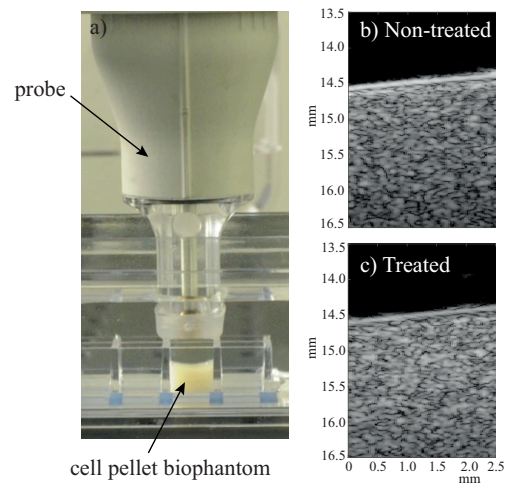


Figure 3: a) Ultrasonic probe and cell pellet biophantom in a well immersed in PBS. b) and c) Examples of B-mode images of non-treated and treated cell pellet biophantoms obtained with the 20-MHz center frequency probe. The treated cell sample corresponds to the sample treated with staurosporine (ST) at  $0.5 \mu\text{M}$  for 36 hours.



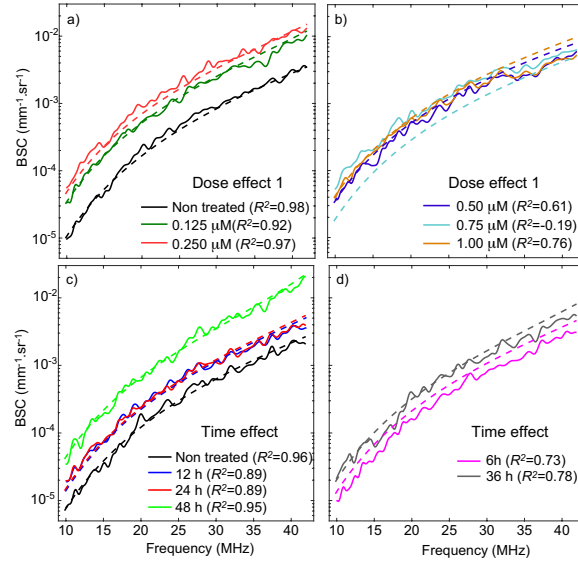


Figure 4: Comparison between measured  $BSC_{\text{meas}}$  (solid lines) and theoretical  $BSC_{\text{theo}}$  predicted by the polydisperse structure factor model (dashed lines) for the dose effect 1 (a) and b) and for the time effect (c) and (d).

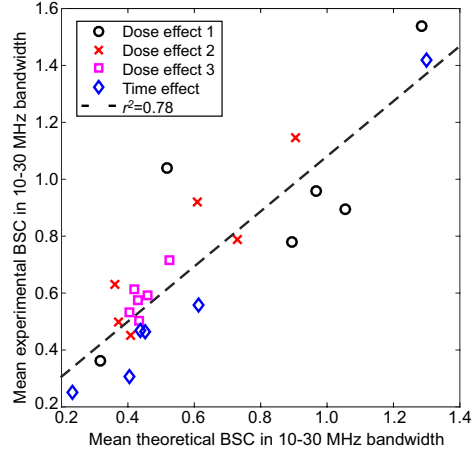


Figure 5: Comparison of mean  $BSC_{\text{theo}}$  in the 10-32 MHz bandwidth predicted by the polydisperse structure factor model with the mean  $BSC_{\text{meas}}$  in the 10-32 MHz bandwidth.

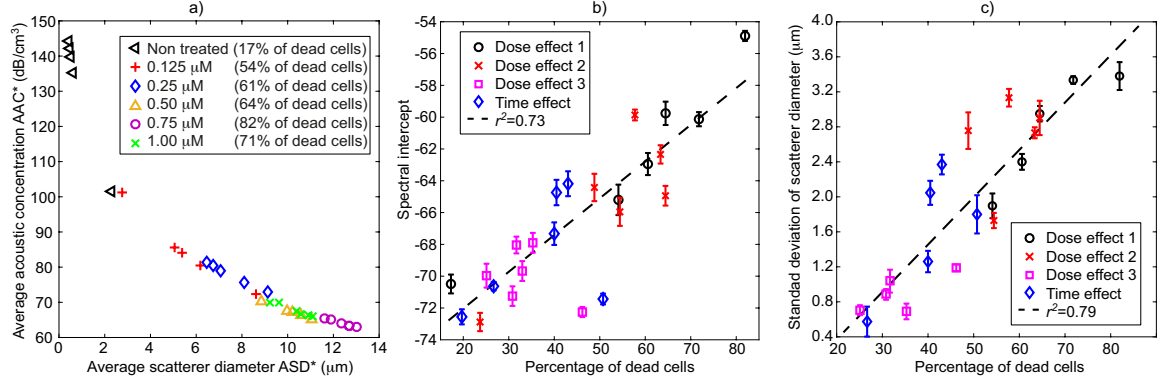


Figure 6: (a) Examples of  $\text{ASD}^*$  and  $\text{AAC}^*$  estimated by the fluid sphere model for the series of experiments *dose effect 1*. For each studied dose, five symbols are represented and correspond to the five acquired B-mode images. (b) Spectral intercept as a function of percentage of dead cells for the four experimental series. (c) Standard deviation of the scatterer diameter distribution  $\sigma_D^*$  estimated by the polydisperse SFM as a function of the percentage of dead cells for the four experimental series.