

Probing the cellular size distribution in cell samples undergoing cell death

Emilie Franceschini, Laure Balasse, Sandrine Roffino, Benjamin Guillet

► 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-02200316v1

Submitted on 31 Jul 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Probing the cellular size distribution in cell samples undergoing cell death

Emilie Franceschini^{a,*}, Laure Balasse^b, Sandrine Roffino^{c,**}, Benjamin Guillet^b

^aAix-Marseille Université, CNRS, Centrale Marseille, LMA, Marseille, France ^bAix-Marseille Université, INSERM, INRA, C2VN, Marseille, France ^cAix-Marseille Université, CNRS, ISM, Marseille, France

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 $(r^2=0.79 \text{ and } r^2=0.72, \text{ respectively})$. The current study contributes to the

^{*}Corresponding Author: Dr. Franceschini Emilie, Aix-Marseille Université, CNRS, Centrale Marseille, LMA, Marseille, France ; Email, franceschini@lma.cnrs-mrs.fr; Phone, +33 4 84 52 42 86

^{**}Also at: Université Côte d'Azur, Nice, France

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

1 Introduction

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

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

In order to develop QUS techniques and interpret QUS parameters for 32 cancer therapy assessment, it is essential to understand which are the specific 33 changes (in cell morphology and/or in cellular mechanical properties) during 34 cell death that cause these changes in QUS parameters. The aforementioned 35 QUS studies are generally based on classical ultrasound scattering models 36 (*i.e.*, spherical Gaussian model or fluid sphere model) which assume ran-37 domly and independently distributed scatterers. Under this assumption, the 38 power of the backscattered signals increases linearly with the scatterer volume 30 fraction and this linear relationship has been used to monitor the ASD and 40 AAC. More recently, the concentrated ultrasound scattering model, namely 41 the polydisperse structure factor model (SFM), has been proposed to explain 42 the measured BSCs (or structure functions) from densely packed media (Han 43 et al. 2015, Franceschini et al. 2014, 2016). The polydisperse SFM describes 44 tissue as an ensemble of discrete scatterers and considers interference effects 45 caused by the correlations among scatterer positions (coherent scattering) 46 using a structure factor. Experiments on *in vitro* cell pellet biophantoms 47 and ex vivo mouse tumor models demonstrated that the SFM is the most 48 appropriate model to use for modeling densely packed cellular contents in 49 tumors (Han et al. 2015, Franceschini et al. 2014, 2016, Muleki-Seya et al. 50

⁵¹ 2016). The SFM has also been used in a two-dimensional (2D) numerical ⁵² study to explain the contribution of changes in cellular size variance to the ⁵³ increase in the BSC during a cell death process (Vlad et al. 2010). However, ⁵⁴ this 2D model-based approach cannot be applied to quantitatively comparing ⁵⁵ the 2D simulated BSCs and the experimentally measured BSCs.

The first objective of this work was to go further in the understanding of 56 the BSCs measured from cells undergoing apoptosis. Ultrasonic backscatter 57 measurements were performed at frequencies ranging from 10 to 42 MHz 58 on colon adenocarcinoma (HT29) cell samples treated with staurosporine, 59 an inducer of apoptotic cell death. The polydisperse SFM was examined in 60 order to explain the BSC measured from HT29 cells undergoing cell death. To 61 this end, a parameter estimation procedure was proposed in order to estimate 62 the volume fraction and the relative impedance contrast that could explain 63 the change in BSC pattern by considering the actual change in cellular size 64 distribution during cell death. 65

The second objective of this work was to blindly estimate the cellular size 66 distribution in cell samples undergoing cell death. For this purpose, a novel 67 approach was proposed, which consisted in fitting the polydisperse SFM to 68 two BSCs measured before and after therapy. This novel approach makes 69 it possible to estimate four QUS parameters: the mean scatterer radius, the 70 standard deviation of the scatterer size distribution, the total volume fraction 71 and the relative impedance difference. QUS parameters estimated by the 72 polydisperse SFM were compared with the ASD and AAC estimated by the 73 classical sparse scattering model, namely the fluid sphere model. Finally, the 74 relationship between the percentage of dead cells and QUS parameters was

⁷⁶ investigated.

77 Background: ultrasonic scattering theory

This section briefly describes three ultrasonic scattering models: the polydisperse SFM, the monodisperse SFM and the fluid-sphere model. These theoretical models describe tissue as an ensemble of discrete scatterers and are based on several approximations for soft tissue scattering (Insana et al. 1990): far-field regime, Born approximation, no multiple scattering and incident plane wave propagation. Moreover, the scatterers are assumed to be 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)

$$BSC_{\rm SFMp}(k) = n \int_0^\infty |\Phi(k, x)|^2 f(x) dx +$$

$$n \int_0^\infty \int_0^\infty \Phi(k, x_1) \Phi(k, x_2) H_{12}(k) f(x_1) f(x_2) dx_1 dx_2,$$
(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)

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

where γ_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)

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

where *a* is the mean radius and ζ 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 ζ corresponds to a narrow size distribution).

⁹⁰ 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)

$$BSC_{\rm SFMm}(k) = n |\Phi(k,a)|^2 S(k,a,\phi) = \frac{4}{9} n \gamma_z^2 k^4 a^6 FF(k,a) S(k,a,\phi),$$
(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).

⁹⁶ 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)

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

⁹⁷ where $n\gamma_z^2$ is the acoustic concentration (AAC) and d(=2a) is the scatterer ⁹⁸ diameter (ASD).

⁹⁹ Materials and Methods

100 Cell sample preparation

Experiments were conducted with HT29 cell samples treated with stau-101 rosporine, a drug which induces mainly cell apoptosis (Qiao et al. 1996). 102 Cells were grown at 37°C in T175 flasks filled with Dulbecco's modified Ea-103 gle's medium containing 4.5 g of glucose/liter and supplemented with 10%104 fetal calf serum. The cells were treated in culture with staurosporine in 105 T175 flasks at 60-70% confluence and then prepared as packed cell samples 106 (*i.e.*, cell pellets). The detailed protocol is described below. For forming 107 one packed cell sample, around 2×10^8 HT29 cells are needed (corresponding 108 approximately to three T175 flasks of non-treated cells or four T175 flasks 109 of treated cells). The supernatant (containing dead cells) was removed or 110 reserved from the T175 flasks, and cells were detached with accutase and 111 washed in phosphate-buffered saline (PBS). Cells from flasks were collected 112 and the supernatant, if reserved, was added to the collected cells. After ho-113 mogenization with a pipette tip, 100 μ L of this cell suspension was withdrawn 114 for flow cytometry analysis and 50 μ L for cell size analysis. The remaining 115 suspension was centrifuged for 5 minutes at 1200 g, then the medium was 116 aspirated and 500 μ L of PBS +/+ was added. After homogenization with a 117

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

A total of 24 cell pellet biophantoms were studied and divided into four series of experiments, each series including five treated cell samples and one non-treated cell sample. All the experiments in one series were conducted on the dame day, so that the non-treated cell sample could be used as control. 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 μ 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.
- Dose effect 2: Same as dose effect 1.
- Dose effect 3: Same as dose effect 1, except that the supernatant was
 not added during the preparation of the packed cell sample.

¹³⁶ - *Time effect:* HT29 cells were treated with 0.5 μ 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.

After the ultrasound measurement, the cell samples were fixed in 10% buffered formalin for three days, dehydrated in a graded ethanol series, cleared in methylcyclohexane and embedded in methyl methacrylate resin.
The resin-embedded samples were sectioned, and the sections stained with
toluidine blue. The histological sections allow us to observe structural changes
occurring in the dying cells and to verify that the cell spatial distribution was
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 μ M of staurosporine for 0, 12 and 24 hours. The cell size distribution was bimodal, with small cellular fragments (mean radius $a_s \approx 2.35 \ \mu$ m) and large cells (mean radius $a_l \approx 6 \ \mu$ 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:

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

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

¹⁵⁰ Flow cytometry using Annexin V/7-AAD was performed to quantify cell ¹⁵¹ death. For each condition, the 100 μ L withdrawn sample of cells was cen-¹⁵² trifuged at 500 g for 5 min and resuspended in 100 μ L of ice-cold Binding ¹⁵³ Buffer. The cell suspension was then incubated for 15 minutes on ice in the ¹⁵⁴ dark with 10 μ L of Annexin Vfluorescein isothiocyanate (FITC) prediluted ¹⁵⁵ 1:100 in Binding Buffer and 20 μ L of 7-AAD (Annexin V-FITC / 7-AAD kit, Beckman Coulter, Marseille, France). Then, 400μ L of ice-cold Binding Buffer was added before quantification with Gallios flow cytometer (Beckman Coulter, France). Approximately 2500 events were measured for each condition. The flow cytometry analysis makes it possible to estimate the percentage of viable cells and dead cells (in early apoptosis, late apoptosis and necrosis).

¹⁶² Ultrasound data acquisition and BSC measurements

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

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

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

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

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

¹⁹⁹ Optimization procedure for understanding the scattering from dead cells

The polydisperse structure factor model was first examined in order to explain the measured BSC_{meas} from packed cell samples undergoing cell death for all the 24 studied samples. Our hypothesis is that the changes in the measured BSC_{meas} during the cell death process is mainly due to the changes in cell size distribution. In order to confirm or contradict this hypothesis, it was assumed that:

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

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

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

$$(7)$$

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

223 Blind estimation of QUS parameters

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

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

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

$$C_1(ASD^*, AAC^*) = \sum_j \left\| \frac{BSC_{\text{meas}}(k_j) - BSC_{\text{FSM}}(k_j)}{BSC_{\text{meas}}(k_j)} \right\|^2.$$
(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 ζ , the total volume fraction ϕ and the relative acoustic impedance contrast γ_z . Because of the large number of unknown parameters, we propose an estimation procedure using two measured BSC_{meas} from non-treated sample (denoted BSC_{meas}^{nt}) and treated sample (denoted BSC_{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 γ_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 ζ of the treated cells, the mean scatterer radius a, the total volume fraction ϕ and the relative impedance difference γ_z , which are determined by minimizing the cost function C:

$$C_{2}(a^{*}, \zeta^{*}, \phi^{*}, \gamma_{z}^{*}) = \sum_{j} \left\| \frac{BSC_{\text{meas}}^{\text{nt}}(k_{j}) - BSC_{\text{SFMm}}(k_{j}, a, \phi, \gamma_{z})}{BSC_{\text{meas}}^{nt}(k_{j})} \right\|^{2} + \sum_{j} \left\| \frac{BSC_{\text{meas}}^{\text{t}}(k_{j}) - BSC_{\text{SFMp}}(k_{j}, a, \zeta, \phi, \gamma_{z})}{BSC_{\text{meas}}^{t}(k_{j})} \right\|^{2}.$$
(9)

The first term represents the fit of BSC_{meas}^{nt} with the monodisperse structure 235 factor model BSC_{SFMm} given by Eq. (4). The second term represents the fit 236 of BSC_{meas}^t with the polydisperse structure factor model BSC_{SFMp} given by 237 Eq. (1). The routine *fmincon* was employed to minimize the cost function 238 C_2 with the constraint conditions that $0 \le a \le 100 \ \mu m$, $1 \le \zeta \le 100$ and 239 $0 \le \gamma_z \le 0.30$. For the constraint condition on the parameter ϕ , two cases were 240 studied: no a priori information with $0 \le \phi \le 1$, or a constraint that assumes 241 concentrated medium with $0.68 \le \phi \le 1$. This point will be discussed later. The 242 constrained optimization routine *fmincon* in MATLAB (The Mathworks Inc., 243

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

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

252 **Results**

²⁵³ Flow cytometry, histology and cell size distribution

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

266

The histological study showed that in treated cells the staining of the 267 nuclear material is uniform (see Fig. 1a), whereas numerous cells treated 268 with staurosporine present characteristics of apoptosis (nuclear condensa-269 tion and fragmentation) and characteristics of necrosis (cell swelling) (see 270 Fig. 1b). Histological observations also demonstrated that whole cells are 271 densely packed in treated and non-treated cell samples. For each histological 272 slice, cellular surface fractions were calculated as the ratio between the white 273 color area (corresponding to the absence of cells) and the total area. The 274 cellular surface fractions were found to be comprised between 0.91 and 0.98 275 for all the studied (non-treated and treated) packed cell samples. This result 276 was expected since all the packed cell samples were prepared under the same 277 centrifugation force. The cellular volume fraction within the cell pellet bio-278 phantoms is related to the cellular area fraction that can be observed on those 279 histological images (Fig. 1). That is why we can reasonably assume that the 280 total volume fractions of cells are similar in all the 24 cell pellet biophantoms. 281 282

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

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. 295 Even when the counts of small cellular fragments (mean radius $a_s \approx 2.35$ 296 μ m) are greater than the counts of large cells (mean radius $a_l \approx 6 \mu$ m), 297 the percentage of volume fraction occupied by the large cells Φ_l is always 298 greater than 0.74 (see Table 1 and Figure 2(b)). Since the backscattering 299 cross-section is proportional to the square of the scatterer volume in the 300 low frequency, the contribution of cellular fragments $(a_s \approx 2.35 \ \mu m)$ to the 301 backscattering can be considered negligible. 302

³⁰³ Understanding of scattering: the change in cell size distribution mainly ex-³⁰⁴ plains the change in BSC pattern on dying HT29 cell samples

The volume fraction estimated by the optimization procedure using Eq. 305 (7) was found equal to $\phi_l^* = 0.73$ and agrees well with the expected maximum 306 packing for hard spheres (around 0.74). The relative impedance contrast 307 was found equal to $\gamma_z^*=0.25$. By taking the acoustic parameters of individ-308 ual viable cells estimated by (Falou et al. 2010) (c=1535 m/s and $\rho=1.09$) 309 and the acoustic properties of the surrounding medium close to those of wa-310 ter ($z_0=1.49$ MRayl), the relative impedance of viable cells is expected to 311 be approximately 0.13. Therefore, the estimated $\gamma_z^*=0.25$ seems to be in a 312 reasonable range of value, but maybe slightly overestimated. 313

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

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

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

To conclude, our approach hypothesizes that there is no change in impedance contrast during cell death and that both treated and non-treated cell samples have similar volume fractions of cells, so that only a change in scatterer size distribution has been considered. Despite the use of these simplifying assumptions, the good match obtained between theoretical and experimental BSCs in the majority of cases (Figs. 4 and 5) suggests that the change in whole cell size distribution is the predominant factor to explain the change in the BSC pattern in the HT29 cell samples during the dying process. (A more detailed discussion on these simplifying assumptions will follow later.)

Blind estimation of QUS parameters using the classical approaches: the spectral intercept correlates with the percentage of HT29 dying cells

For each cell pellet biophantom, five BSC_{meas} were measured (correspond-348 ing to the five acquired B-mode images), and for each measured BSC_{meas} , 340 the four classical QUS parameters (ASD^{*}, AAC^{*}, spectral slope, spectral 350 intercept) were estimated. When using the fluid sphere model, the ASD^{*} 351 increases and the AAC^{*} decreases as the percentage of dead cells increases. 352 Examples of ASD^* and AAC^* values are given in Fig. 6(a) for the series 353 of experiments dose effect 1. When using a straight line model, the spectral 354 slope decreases and the spectral intercept increases as the percentage of dead 355 cells increases (data not shown). 356

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

³⁶⁵ Blind estimation of QUS parameters using the novel approach

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

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

385 Discussion and Conclusion

386 Understanding scattering

It is generally assumed that the scattering within cell pellet biophantom 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 394 considering the nuclei as the main sources of scattering. More precisely, the 395 first two assumptions were re-formulated as follows: 1) the main sources of 396 scattering are the nuclei, and 2) the nuclei are gamma distributed and the 397 radius a_n and gamma width factor ζ_n can be calculated from the measure-398 ments with Scepter cell counter by considering a nucleus-to-cell ratio (a_n/a_l) 390 equal to 0.72. This novel optimization procedure makes it possible to esti-400 mate a volume fraction $\phi_n^* = 0.69$ and a relative impedance contrast $\gamma_{z_n}^* = 0.36$. 401 However, the estimated volume fraction did not match the expected volume 402 fraction of nuclei $\phi_n \approx \phi_c (a_n/a_l)^3 = 0.27$ (by considering a volume fraction of 403 densely packed whole cells $\phi_c=0.74$). Moreover, the value of the modified 404 cost function \mathcal{F}' was found to be larger when considering the nuclei as the 405 sources of scattering (when compared with the original cost function \mathcal{F} in Eq. 406 (7)). Therefore, the whole cell seems to play a major role in the BSC curves 407 for the HT29 cell samples treated with staurosporine, as observed previously 408 in biophantoms of viable K562 and CHO cells (Franceschini et al. 2014). 409 410

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

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

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

could not be fully explained by our approach (see Figs. 4(b) and 4(d)). 439 Investigating the sources that could explain this mismatch is difficult. It 440 can be due for example to a change in cellular size variance (as proposed in 441 this study) combined with a change in acoustic impedance. One might also 442 consider a more complex scattering process by considering both nuclei and 443 whole cells responsible for the scattering since histological observations reveal 444 that the size distribution changes in both the nucleus and the cell during the 445 cell death process. However, not all changes observed on histological images 446 will result in scattering changes. 447

448 Blinded estimation of QUS parameters

The spectral slope is linked to the effective scatterer size. When con-449 sidering a medium with scatterers differing only in size, the spectral slope 450 decreases with the increase in scatterer size variance. As an example, theo-451 retical BSCs were calculated in the 10-42 MHz bandwidth by using Eq. (1) 452 for a mean scatterer radius of 7μ m and various gamma width factors $\zeta = 90$, 453 50, 30 and 10. The corresponding spectral slopes were found to be equal to 454 4.10, 4.05, 4.00 and 3.8, respectively. In our experimental study, the decrease 455 in the spectral slope with the increase in the percentage of HT29 dying cells 456 is thus consistent with an increase in the cellular size variance. Similar obser-457 vations linked to the cellular size variance were previously observed in FaDu, 458 Hep-2 and C666-1 cell samples exposed to radiotherapy (Vlad et al. 2008, 459 Vlad et al. 2010). When using the fluid sphere model, the ASD^{*} was also 460 found to increase together with the percentage of dying HT29 cells, which is 461 consistent with the decrease in the spectral slope. 462

463

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

489 Acknowledgements

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

496 **References**

Brand S, Solanki B, Foster DB, Czarnota GJ, Kolios MC. Monitoring of
cell death in epithelial cells using high frequency ultrasound spectroscopy.
Ultrasound Med Biol 2009;35:482-493

- Chen X, Phillips D, Schwarz KQ, Mottley JG, and Parker KJ. The measurement of backscatter coefficient from a broadband pulse-echo system: a new formulation. IEEE Trans Ultras Ferroelectr Freq Control 1997; 44:515525
- Czarnota GJ, Kolios MC, Vaziri H, Benchimol S, Ottensmeyer FP, Sherar
 MD, Hunt JW. Ultrasonic biomicroscopy of viable, dead and apoptotic
 cells. Ultrasound Med Biol 1997; 23:961-965
- Falou O, Rui M, Kaffas AE, Kumaradas JC, and Kolios MC. The measurement of ultrasound scattering from individual micron-sized objects and its application in single cell scattering. J Acoust Soc Am 2010; 128:894-902
- Feleppa EJ, Liu T, Kalisz A, Shao MC, Fleshner N, Reuter V. Ultrasonic
 spectral-parameter imaging of the prostate. Int. J. Imag. Syst. Technol.
 1997; 8:11-25.
- Franceschini E, Guillermin R. Experimental assessment of four ultrasound scattering models for characterizing concentrated tissue-mimicking
 phantoms. J Acoust Soc Amer 2012; 132:3735-3747.
- Franceschini E, Guillermin R, Tourniaire F, Roffino S, Lamy E, Landrier
 JF. Structure Factor Model for understanding the measured backscatter
 coefficients from concentrated cell pellet biophantoms. J Acoust Soc Amer
 2014; 135:3620-3631.

Franceschini E, de Monchy R, Mamou J. Quantitative characterization of tissue microstructure in concentrated cell pellet biophantoms based on the structure factor model. IEEE Trans Ultras Ferroelectr Freq Control 2016; 63:1321-1334.

Griffith WL, Triolo R, Compere AL. Analytical scattering function of a
polydisperse Percus-Yevick fluid with Schulz distributed diameters. Phys
Rev A 1987; 35:2200-2206.

Han A, Abuhabsah R, Blue JP, Sarwate S, OBrien WD. The measurement
of ultrasound backscattering from cell pellet biophantoms and tumors ex
vivo. J Acoust Soc Am 2013; 134:686693.

Han A, O'Brien WD. Structure function for high-concentration biophantoms of polydiperse scatterer sizes. IEEE Trans Ultras Ferroelectr Freq
Control 2015; 62:303-318.

Hunt J.W., Worthington A.E., Xuan A., Kolios M.C., Czarnota G.J. and Sherar M.D., A model based upon pseudo regular spacing of cells combined with the randomisation of the nuclei can explain the significant changes in high-frequency ultrasound signals during apoptosis. Ultrasound Med Biol 28, 2002, 217226.

Insana MF, Wagner RF, Brown DG, Hall TJ. Describing small-scale
structure in random media using pulse-echo ultrasound. J Acoust Soc Am
1990; 87:179-192

Kolios MC, Czarnota GJ, Lee M, Hunt JW, Sherar MD. Ultrasonic
spectral parameter characterization of apoptosis. Ultrasound Med Biol
2002; 28:589-597

545 Lavarello RJ, Ridgway WR, Sarwate SS, Oelze ML. Characterization

of thyroid cancer in mouse models using high-frequency quantitative
ultrasound techniques. Ultrasound Med Biol 2013; 39:2333-2341.

Lizzi FL, Ostromogilsky M, Feleppa EJ, Rorke MC, Yaremko MM.

549 Relationship of ultrasonic spectral parameters to features of tissue mi-

crostructure. IEEE Trans Ultrason Ferroelect Freq Contr. 1986; 33:319-329

⁵⁵¹ Mamou J, Coron A, Hata M, Machi J, Yanagihara E, Laugier P, Feleppa

E. Three-dimensional high-frequency characterization of cancerous lymph
 nodes. Ultrasound Med Biol 2010; 36:361-375.

 $_{553}$ nodes. Ultrasound Med Biol 2010; 36:361-375.

Muleki-Seya P, Guillermin R, Guglielmi J, Chen J, Pourcher T, Konofagou
E, Franceschini E. High frequency quantitative ultrasound spectroscopy of
excised canine livers and mouse tumors using the structure factor model.
IEEE Trans Ultras Ferroelectr Freq Control 2016; 63:1335-1350.

Oelze ML, O'Brien WD, Blue JP, Zachary JF. Differentiation and characterization of rat mammary fibroadenomas and 4T1 mouse carcinomas using quantitative ultrasound imaging. IEEE Trans. Med. Imaging 2004; 23:764-771.

⁵⁶² Oelze ML, O'Brien WD. Application of three scattering models to char-⁵⁶³ acterization of solid tumors in mice. Ultrasonic Imaging 2006; 28:83-96.

Pasternak MM, Sadeghi-Naini A, Ranieri SM, Giles A, Oelze ML, Kolios
MC, Czarnota GJ, High-frequency ultrasound detection of cell death:
spectral differentiation of different forms of cell death in vitro. Oncoscience
2016; 3:275-287.

Qiao L, Koutsos M, Tsai LL, Kozoni V, Guzman J, Shiff SJ, Rigas B. Staurosporine inhibits the proliferation, alters the cell cycle distribution and induces apoptosis in HT-29 human colon adenocarcinoma cells. ⁵⁷¹ Cancer Letters 1996; 107:83-89.

Sannachi L, Tadayyon H, Sadeghi-Naini A, Tran W, Sonal G, Wright F,
Oelze M, Czarnota GJ. Non-invasive evaluation of breast cancer response
to chemotherapy using quantitative backscatter parameters. Medical
images analysis 2015; 20:224-236.

Strohm EM, Czarnota GJ, Kolios MC. Quantitative measurements of
apoptotic cell properties using acoustic microscopy. IEEE Trans Ultras
Ferroelectr Freq Control 2010; 57:2293-2304.

Tadayyon H, Sannachi L, Sadeghi-Naini A, Al-Mahrouki A, Tran WT,
Kolios MC, Czarnota GJ. Quantification of ultrasonic properties of in
vivo tumor cell death in mouse models of breast cancer. Translational
Oncology 2015; 8:463-473.

Tadayyon H, Sannachi L, Gangeh MJ, Kim C, Ghandi S, Trudeau M,
Pritchard K, Tran WT, Slodkowska E, Sadeghi-Naini A, Czarnota GJ.
Chemotherapy response and survival in breast cancer patients using
quantitative ultrasound, Scientific reports 2017; 7:45733.

Taggart LR, Baddour RE, Giles A, Czarnota GJ, Kolios MC. Ultrasonic
characterization of whole cells and isolated nuclei. Ultrasound Med Biol
2007; 33:389-401.

⁵⁹⁰ Vlad RM, Alajez NM, Giles A, Kolios MC, Czarnota GJ. Quantitative
⁵⁹¹ ultrasound characterization of cancer radiotherapy effects in vitro. Int J
⁵⁹² Radiat Oncol Biol Phys 2008;72:12361243.

⁵⁹³ Vlad RM, Saha RK, Alajez NM, Ranieari S, Czarnota GJ, Kolios ⁵⁹⁴ MC. An increase in cellular size variance contributes to the increase in ⁵⁹⁵ ultrasound backscatter during cell death. Ultrasound Med Biology 2010;

⁵⁹⁶ 36:1546-1558.

597	Weiss EC, Anastasiadis P, Pilarczyk G, Lemor RM, Zinin PV. Mechanical
598	properties of single cells by high-frequency time-resolved acoustic mi-
599	croscopy. IEEE Trans Ultras Ferroelectr Freq Control 2007; 54:2257-2271.
600	Yao LX, Zagzebski JA, Madsen EL. Backscatter coefficient measurements
601	using a reference phantom to extract depth-dependent instrumentation
602	factors. Ultrasonic Imaging 1990; 12:58-70.

$_{604}$ Tables

Table 1: Mean radius a_s (in μ m) and gamma width factor ζ_s for small cellular fragments. Mean radius a_l and gamma width factors ζ_l for large cells, and corresponding percentage of volume fraction occupied by large cells Φ_l . Results are presented for the four series of experiments (i.e. ST dose and time effect) and for the different conditions (dose effect $C_{i=1...6}=0, 0.125, 0.25, 0.50, 0.75$ and 1 μ M and time effect $C_{i=1...6}=0, 6, 12, 24, 36$ and 48 hours).

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

Table 2: Examples of QUS parameters (ASD^{*} and σ_D^* in μ m and AAC^{*} in dB.cm⁻³) estimated by the polydisperse SFM for the *time effect* experiment. The novel approach was tested by considering two different contraint conditions: $0 \le \phi \le 1$ or $0.68 \le \phi \le 1$.

617

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

618

Figure Captions

- **Figure 1:** Histological images of HT29 cell pellet biophantoms (a) non treated and (b) treated with staurosporine 1 μ 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 μ M for 36 hours.
- Figure 4: Comparison between measured BSC_{meas} (solid lines) and theoretical BSC_{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_{theo} in the 10-32 MHz bandwidth predicted by the polydisperse structure factor model with the mean BSC_{meas} in the 10-32 MHz bandwidth.

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



Figure 1: Histological images of HT29 cell pellet biophantoms (a) non treated and (b) treated with staurosporine 1 μ 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 μ M for 36 hours.



Figure 4: Comparison between measured BSC_{meas} (solid lines) and theoretical BSC_{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_{theo} in the 10-32 MHz bandwidth predicted by the polydisperse structure factor model with the mean BSC_{meas} in the 10-32 MHz bandwidth.



Figure 6: (a) Examples of ASD^{*} and 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 σ_D^* estimated by the polydisperse SFM as a function of the percentage of dead cells for the four experimental series.