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Signal analysis for the estimation of mechanical parameters of viable cells using GHz-acoustic microscopy

Sebastian Brand[1,2], Nick Grube[1,2], Kay Raum[2,3]
[1]Fraunhofer Institute of Material Mechanics, Halle, Germany
[2]Dept. of Orthopedics, University of Halle, Germany,
[3]Julius Wolff Institute & Berlin Brandenburg School for Regenerative Therapies, Charité- Universitätsmedizin Berlin, Germany

Eric M. Strohm, Michael C. Kolios
Department of Physics
Ryerson University
Toronto, Ontario, Canada

***Abstract*—The current study aimed on the method development for the estimation of mechanical properties of individual biological cells. Samples investigated ultrasonically were kept under culture conditions and no chemical markers have been applied in order to not alter the cells chemical conditions. For the estimation of mechanical properties namely thickness and sound velocity ultrasonic waves in the GHz frequency range have been used. The major benefit of applying acoustic GHz-microscopy is the contact-free and therefore non-destructive mode of operation combined with a resolution of approx. 1 µm. Modulated but otherwise unprocessed radio frequency (rf) echo signals of HeLa cells have been acquired. Signals were processed using a numerical deconvolution technique which has proven to be reliable and robust in low-frequency ultrasonic applications. Echo positions of the cell membrane and substrate have been estimated. From the time of flight (TOF) of the cell membrane versus the TOF of the substrate outside the cell the local cell thickness was derived. Cell thickness and the TOF inside a cell lead to the estimation of the local sound velocity. For verification cell thickness has been measured by confocal laser scanning microscopy. Individual cells showed varying thickness values ranging from 5 µm to 15 µm depending on the cell topography. Sound velocities however varied between 1600 m/s and 1800 m/s. Deviations of thickness estimates between laser scanning microscopy and ultrasonic GHz-microscopy are in acceptable agreement. It was noticed that the cell-thickness estimation shows a higher accuracy in regions of the cell nucleus vs. the surrounding cytoplasm. That fact may be attributed to the much smaller cellular thickness in extra-nuclear regions of adhering cells. Thickness values obtained by laser scanning microscopy showed an artifact at the cells nuclear region, however, values obtained at both edges of the nuclei agree well with the ultrasonically obtained results. The current study contributes towards the estimation of intra-cellular changes of individual cells during chemo-therapy and the development of a low-frequency technique for an in-vivo monitoring of treatment responses in cancer therapy.**

Keywords: mechanical cell properties; acoustic microscopy; in-situ monitoring

I. INTRODUCTION

The estimation of mechanical properties at a microscopic scale is important for the understanding of the macroscopic behavior of materials. Acoustic microscopy enables the unique opportunity of a non-destructive assessment of material constants combined with a near-optical resolution. Since its invention in the 1970's acoustic microscopy underwent great improvements in both the understanding of the contrast corresponding to elastic properties and the technical equipment, i.e. allowing digital data acquisition of the unprocessed rf-data.

Acoustic microscopy can be performed in a broad range of frequencies. In situations when it is important to investigate internal structures ultrasonic waves of up to 250 MHz can be applied. The limitation of the applicable frequency is mainly dictated by the attenuation the acoustic wave encounters when propagating from and to the transducer. Wavelength and achievable resolution are inevitably connected and thus for increasing the resolution into the µm range the frequency has to be increased. Ultrasonic transducers with transmission frequencies of up to 2 GHz are available, allowing a resolution on a fluid-solid interface of approx. 0.5 µm [1] but the penetration depth is limited to approx. 1.5 times the wavelength below the surface [1]. However, due to the excellent focusing properties in fluids structural and elastic surface parameters of solids can be investigated on a µm scale using acoustic microscopy in the GHz-frequency range. If, however, the sample under investigation has properties close to a fluid acoustic microscopy allows internal imaging and quantitative evaluation of mechanical properties. In the case of biological cells this condition is fulfilled allowing the elastic inspection of single cells with a sub-cellular resolution. Ultrasonic scattering effects in biological soft tissue in the frequency range from 10 – 100 MHz have their origin in structural properties on a cellular and sub-cellular level [1]. Therefore, acoustic microscopy in the GHz-range offers the potential of contributing towards the understanding of these

scattering effects by investigating viable cells. A major benefit of acoustic microscopy is provided in the rich contrast in imaging which negates the need of specialized and potentially toxic dyes as used in optical microscopy. Additionally, cell culture media have acoustical properties similar to coupling fluids commonly used and therefore allow investigating viable cells under culture conditions and thus, the monitoring of property variations versus time and medication.

Two different data acquisition methods are used in acoustic microscopy. Namely these are time-resolved and time integrated modes of operation. Whereas time resolved acoustic microscopy applies a short broad band pulse in time integrated mode a quasi-monochromatic tone-burst is used. Broad-band excitation provides an increased axial resolution and has been suggested to be the most promising method for quantitative analysis of viable cell. That beneficial shortness of the pulse however, also reduces the signal-to-noise ratio (SNR) of the received signal compared to tone-burst signals. In the current study the combination of tone-burst excitation with a numerical deconvolution technique is proposed. This off-line analysis is performed on the demodulated rf-echoes recorded from viable HeLa cells and local cell thicknesses and sound velocities were derived. For verification of the acoustically estimated cell thickness cell samples were fixated after the acoustic data acquisition and cell thickness has been determined by laser scanning microscopy, independently.

II. MATERIALS AND METHODS

A. Biological samples

Human cervix carcinoma cells (HeLa from ATTC) were cultured in minimum essential media (MEM) supplemented with 10% heat inactivated fetal bovine serum containing 0.1% gentamycin. Cell culturing was performed in a humidified atmosphere at 37°C containing 5% CO_2. Cells were thawed from frozen stock and cultured in Lab-Tek chamber slides containing 1.5 - 2 ml of the described medium. During ultrasonic data acquisition chamber slides containing adherent growing HeLa cells and the culture medium were placed in a custom made incubation chamber attached to the acoustic microscope. Throughout the whole investigation the temperature was kept constant at 37°C in a humidified atmosphere containing 5% CO_2. This procedure allowed conducting the experiment at cells under culture conditions. For conservation the complete cell culture was fixated in 10% (w/f) formalin in PBS and kept at 4°C until laser scanning microscopy was performed at the same cell.

B. Scanning Acoustic Microscopy

A KSI SAM 2000 (KSI, Herborn, Germany) acoustic microscope was used for the ultrasonic investigations on a single cell level. The transducer applied had a central frequency of 1000 MHz with a bandwidth of 10%. The transducers working distance was 80 µm. The acoustic lens had a semi aperture angle of 50° leading to a f-number of 0.65. For the experiments described here the scan range of the microscope in x- and y- direction was set to 200 x 200 µm. Cell samples were scanned with an increment of 390 nm in both directions. The acoustic microscope generated tone-burst pulses with a duration of 20 ns. The received echo signals are downshifted to a central frequency of 400 MHz, sequentially amplified, rectified and finally boxcar-integrated. This methodology allows a high SNR of the received data. However, during that processing all time of flight related information is erased from the signals. For estimating quantitative mechanical parameters the time-domain echo signals received from the cell samples were digitized after spectral downshifting and amplification. Modulation for spectral shifting is a linear transformation and therefore preserves all information contained in a signal. Modulated and amplified echo signals were digitized using a Gage Cobra Cs22G8 analogue to digital converter (ADC) (Gage Applied, Lockport, USA). The ADC sampled the signals at a rate of 2 GS/s with a resolution of 8 bit. At each scan position a 100 ns time gate of the echo signal was recorded resulting in 512*512 signals per scan. Digitized data were stored for off-line analysis. Scans were repeated at a series of distances between the transducer and the cell-sample.

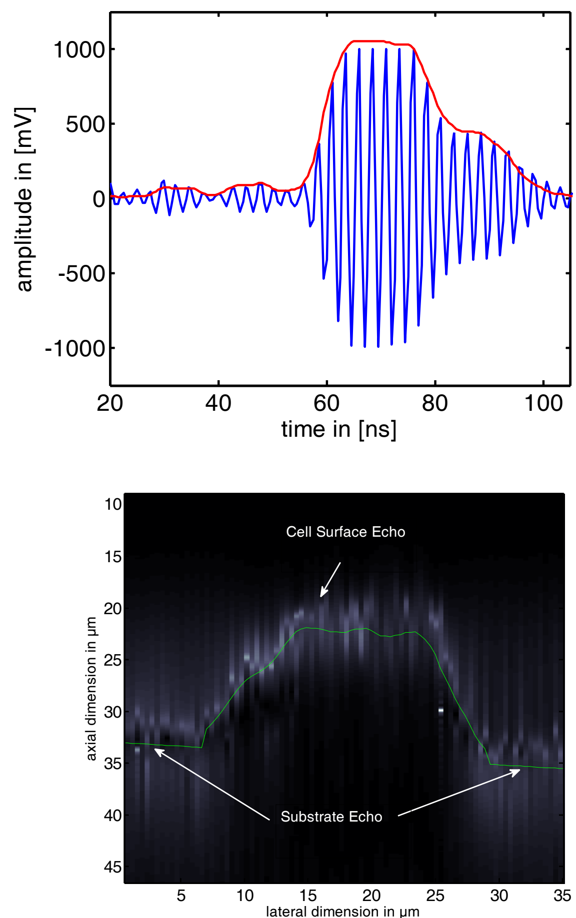


Figure 1: RF-data acquired from the acoustic microscope. Top: received echo signal after spectral downshifting to 400 MHz, digitized at 2GS/s. Bottom: Reconstructed and deconvoluted signals of a HeLa cell. It represents a cross section through the cell.

C. *Ultrasonic Signal Analysis*

For the estimation of local cell thicknesses and sound velocity additional signal analysis procedures were employed for reconstructing the original signal and the detection of echoes corresponding to the interfaces the acoustic wave encountered during propagation. Prior to echo localization all signals were demodulated for transformation into the original frequency band. Following signals were deconvolved using a numerical deconvolution algorithm which has been proven to be reliable and robust in lower frequency ultrasonic applications [5]. The deconvolution algorithm used is based on the method proposed by Honarvar [5]. It applies a Wiener filter using a reference signal which incorporates the systems transfer properties and a "noise-desensitizating factor" Q. Q^2 is commonly set to $10^{-2}\ |H(\omega)|^2_{max}$, where $H(\omega)$ is the spectral transfer function of the equipment. This transfer function was assessed by recording a pulse reflected from the glass bottom of the chamber slide, shown in fig.1. The deconvolved spectrum obtained by Wiener filtering was further improved by applying autoregressive spectral extrapolation. The spectral extrapolation algorithm models the autoregressive process from a spectral range of the input signal showing a high SNR. For performing a reliable and robust autoregressive spectral extrapolation the maximum-entropy algorithm of Burg was used [4]. Spectral extrapolation was repeated for 10 different frequency ranges within the -10 dB range of the reference spectrum for each signal recorded from the cell-sample. The order of the autoregressive model was set to 15. Extrapolation results were averaged prior to performing inverse Fourier transformation for converting signals into time domain. Results obtained from the inverse Fourier transform contained peaks which correspond to the echo positions. Time of flight (TOF) values were estimated from the upper cell membrane as shown in fig.1, the substrate behind the cell and the substrate outside the cell. The sound velocity of the cell culture medium was estimated temperature dependent in a separate experiment and was 1535 m/s at 37°C. Cell thickness was then computed by:

$$d = \frac{(t_{cell_top} - t_{outside_cell})}{2} v_{medium}$$

Where d is the local cell thickness, t_{cell_top} the TOF of the cell membrane, $t_{outside_cell}$ the TOF of the glass substrate outside the cell v_{medium} the sound velocity in the coupling fluid. The local sound velocity v_{cell} was estimated from the thickness estimates und the time delay between the top and bottom (t_{cell_top} and t_{cell_bottom}) echo of the cell by:

$$v_{cell} = \frac{d}{t_{cell_bottom} - t_{cell_top}}$$

D. *Laser Scanning Microscopy*

For verification of the acoustically obtained thickness estimates confocal laser scanning microscopy (CLSM) was performed using a Leica TCS SL CLSM (Leica, Wetzlar, Germany). The lens used was a HCX APO L63x 0.9W with a lateral resolution of 217 nm. For this purpose cell samples were fixated as described and laser scanning was performed within one week after fixation of the samples. Prior to the experiment the fixation solution was replaced by PBS. For imaging cells were stained using propidium iodide at a concentration of 20 µg/ml. This dye stains both the nuclear DNA and the RNA in the cytoplasm. Due to the narrow axial focus of 626 nm the CLSM is able to estimate cell thicknesses by recording images at successively decreasing distances between the lens and the specimen. At each lateral position the increase in signal is analyzed vs. the axial distance from the lens. The height is then estimated by the relative distance between the glass substrate and the detection of the fluorescent dye. This approach for the estimation of local cell thickness has been described by Townsend et al. [2] and showed good agreement with scanning electron micrographs of corresponding cell cross sections.

III. Results and Discussion

Figure 2 shows an acoustic micrograph of viable HeLa cells under culture conditions. The contrast in the image arises from the relative differences in attenuation between the individual cell compartments. Quantitative analyses for the estimation of local cell thickness and sound velocity were performed on cells indicated as "cell-1" and "cell-2". For verification the cross sections marked "X-1" and "X-2", have been compared with results obtained by laser scanning microscopy. Lateral thickness profiles of cell cross sections along the red markers indicated in Fig.2 are provided in fig.3. Thicknesses were estimated by laser scanning microscopy (top graphs) and by analyzing the echo signals obtained by acoustic microscopy. The decreasing thickness estimates in the central part of the cross section are assumed to be caused by an imaging artifact during laser scanning microscopy. Good agreement between CLSM and SAM estimates can be seen at the edges of the central part of the cell, surrounding the nucleus. Differences in the absolute values of thickness estimates are explained by the difference in physiological condition. Cells investigated by CLSM were fixated, while cells investigated ultrasonically have been viable.

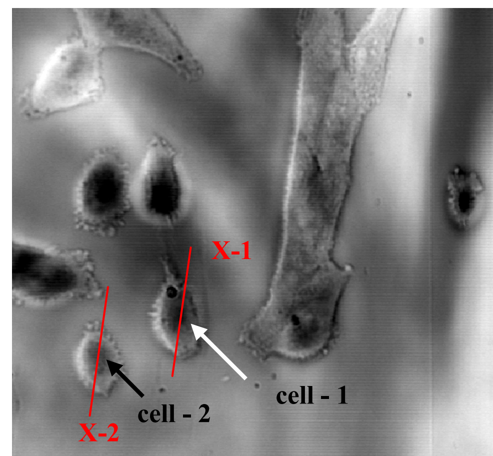


Figure 2: Acoustic micrograph of viable HeLa cells. Local cell thickness and sound velocity was estimated from acoustic data of cell-1 and cell-2. Red marks indicate the position of thickness comparison between laser scanning microscopy and acoustic microscopy.

Cell shrinkage during fixation was observed before. Townsend et al. reported an approx. 10-20 % decrease in local cell thickness after fixation [2]. In table 1 the averaged thickness estimates and standard deviations estimated by SAM and CSLM are provided. Estimates were obtained from the cross sections shown in Fig. 3 and show good agreement between the two methods. From the acoustically obtained data echo positions were computed of the interfaces of the cell surface, between cell and substrate and the substrate outside the cell. Local values of sound velocity inside the cell were then derived. The two dimensional distributions of the local cell thickness and sound velocity are plotted in Fig. 4. The spatial distributions shows higher values of both thickness and sound velocity in the nuclear region compared to estimates in the cytoplasm of these cells. Mean values and standard deviations of the sound velocity of these cells (shown in Fig.4) are provided in table 2. The estimates show acceptable agreement with published values of sound velocity on a cellular level [3]. The current study shows that accurate values for cell thickness and sound velocity can be achieved by acoustic GHz-microscopy using the advantages of tone-burst excitation combined with appropriate post processing of the echo signals.

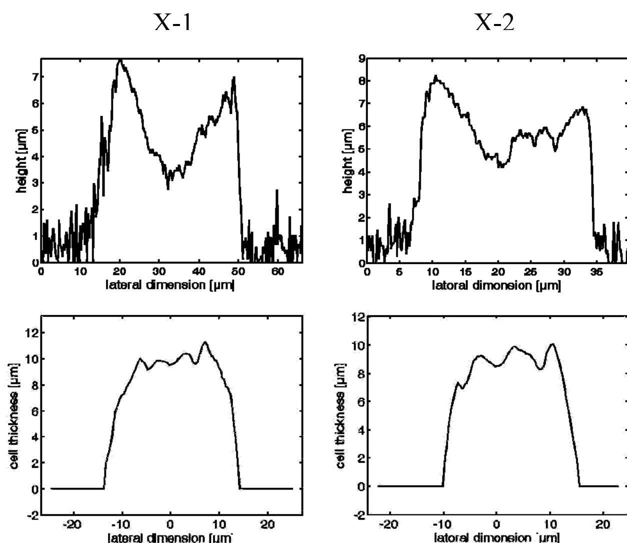


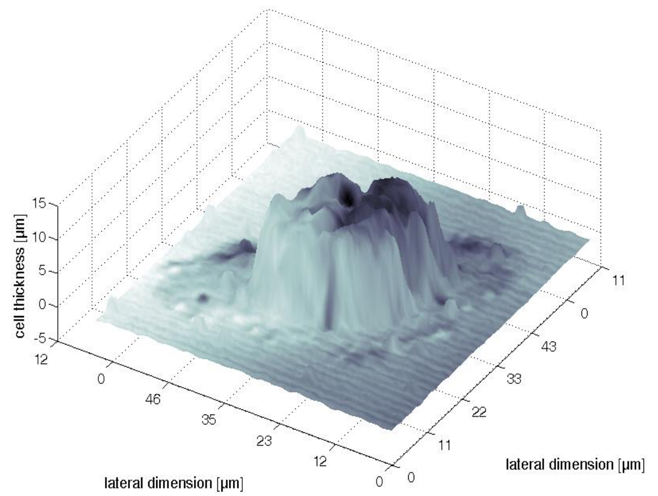
Figure 3: Lateral thickness profile of HeLa cells according to the cross sections marked in fig. 2. Top: Thickness profiles estimated by laser scanning microscopy. Bottom: Thickness estimates as obtained by acoustic microscopy.

TABLE I. CELL THICKNESS ESTIMATES OBTAINED BY SAM AND CLSM

Cell #	D_{SAM} *[µm]*	sd(d_{SAM}) *[µm]*	D_{CLSM} *[µm]*	sd(d_{CLSM}) *[µm]*
X-1	8.63	2.23	5.04	1.28
X-2	7.87	2.44	5.95	1.03

TABLE II. INTRACELLULAR SOUND VELOCITY

Cell #	v_{mean}	sd(v)
X-1	1688 m/s	116.14 m/s
X-2	1614 m/s	78.89 m/s



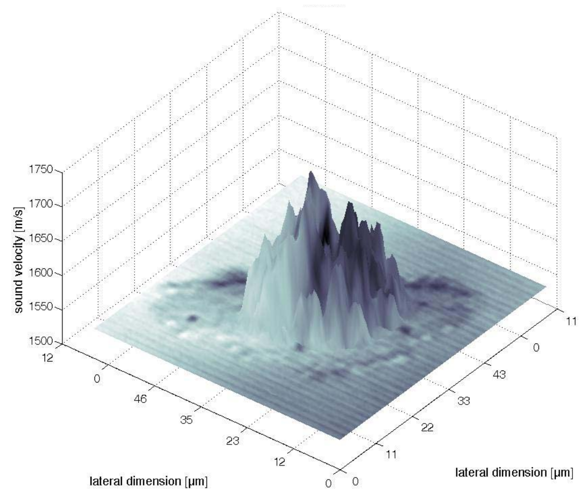


Figure 4: Distribution of cell thickness and sound velocity in a viablel HeLa cell..Top: Thickness estimates derived from SAM measurements. Bottom: Sound velocity computed from surface and substrate echo, recorded by SAM.

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