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Low to Very-High Frequency Ultrasound Biomicroscopy of Cell Death

G.J. Czarnota*

* Department of Radiation Oncology, and Imaging Research, Sunnybrook Health Sciences Centre
Department of Radiation Oncology, and Department of Medical Biophysics, University of Toronto
2075 Bayview Avenue, Toronto, ON, Canada M4N 3M5

Ultrasound has been used to detect tissue pathology since the 1960s. Although early studies were limited in terms of their appreciation of biology and the physics of ultrasound backscatter as it related to the biology of cell death, recent investigations combining rigorous and well controlled biological experimentation and quantitative ultrasound methods have provided valuable information. Our studies indicate that ultrasound may be used to detect and potentially quantify cell death *in vitro*, *in situ*, and *in vivo* at conventional ultrasound frequencies, higher-ultrasound frequencies and using ultrasound microscopy. These studies point to an important role of the cell's nucleus and its configuration in the formation of ultrasound backscatter in addition to cellular morphology.

Our research has established the use of high-frequency ultrasound to detect cell-death in tissues *ex vivo* and in live animals and provided a potential morphological link explaining the associated imaging changes with further experimentation *in vitro* [1]. Mouse brain tissue where apoptosis had been induced by photodynamic therapy was imaged *ex vivo* indicating spatially coincident increases in high-frequency ultrasound backscatter. Similarly, skin, where apoptosis had been induced by photodynamic therapy demonstrated comparable increases in ultrasound backscatter. Experiments *in vitro* which relied on drugs to induce nuclear condensation by arresting cells in metaphase of mitosis (colchicine) or inducing apoptosis (cisplatinum) have indicated a role for the condensation of the cell's nucleus in backscatter increases. Furthermore, subsequent enzymatic digestion of that condensed nuclear material using DNAse results in a normalization of backscatter further supporting a working hypothesis that nuclear configuration could alter backscatter from cells and tissues [2]. Nuclear structure has been subsequently further linked to backscatter properties in a high-frequency ultrasound examination of different cell types and their isolated nuclei in which speed of sound, attenuation coefficient and integrated backscatter coefficients were measured. Integrated backscatter coefficient values for cells and isolated nuclei showed much greater variation increasing from 1.71 x 10(-4) Sr(-1) mm(-1) for the smallest nuclei to 26.47 x 10(-4) Sr(-1) mm(-1) for cells with the largest nuclei. The findings have suggested that integrated backscatter coefficient values, but not attenuation or speed of sound, are correlated with the size of the nuclei [3].

Cell death has been detected also *in vivo* using high-frequency ultrasound and spectral analysis methods in animal model systems where xenograft tumours were treated using a number of different methods. The first preclinical tumour-based use of high-frequency ultrasound spectroscopy was to non-invasively monitor tumour treatment by following xenograft malignant melanoma tumour responses to photodynamic therapy (PDT) *in vivo*. Banihashemi *et al* observed a time-dependant increase in ultrasound backscatter variables after treatment. The observed increases in spectroscopic variables correlated with morphologic findings, indicating increases in apoptotic cell death, peaking at 24 hours after PDT. Analyses of changes in spectral slope strongly correlated with changes in mean nuclear size over time, associated with apoptosis, after therapy [4]. Vlad *et al* used high-frequency ultrasound in a similar manner to track the responses of xenograft tumours in vivo to radiotherapy. Data were collected with an ultrasound scanner using frequencies of 10 to 30 MHz.

Ultrasound estimates calculated from normalized power spectra and parametric images (spatial maps of local estimates of ultrasound parameters) were used as indicators of response. Two of the mouse models (FaDu and C666-1) exhibited large hyperechoeic regions at 24 hours after radiotherapy. The ultrasound integrated backscatter increased by 6.5 to 8.2 dB ($p<0.001$) and the spectral slopes increased from 0.77 to 0.90 dB/MHz for C666-1 tumours and from 0.54 to 0.78 dB/MHz for FaDu tumours ($P < 0.05$), in regions compared with preirradiated tumours. The hyperechoic regions in the ultrasound images corresponded in histology to areas of cell death. Parametric images were utilized in that study to further discern the tumour regions that responded to treatment [5].

Our most recent research focuses on Gigahertz-range very-high frequency ultrasound microscopy to probe cell composition at the single cell level. At 1 GHz, the wavelength of ultrasound is close to 1.5 um, and therefore a comparable spatial resolution can be achieved. Bulk measurements of ultrasound attenuation have shown an increase in the ultrasound attenuation for cells undergoing apoptosis [6,7] and large temporal changes in ultrasound backscatter as a function of time after response to chemotherapy exposure. We have used 100 MHz to 1 GHz ultrasound to study apoptosis and demonstrated at 400 MHz changes that subcellular changes in backscatter appear to be associated with regions of nuclear material aggregation that occurs during apoptosis. At high-frequencies (1 GHz) other factors seem to potentially predominate in the formation of backscatter images.

Looking towards medical applications, conventional (low) to mid-range ultrasound frequencies (1-20 MHz) are applicable clinically and have been used in medicine since the development of ultrasound technology. The detection of tissue changes related to cell death in the case of necrosis dates back nearly fifty years with decreases in backscatter being observed. It is only now, nevertheless, that methods in quantitative ultrasound are being applied at clinically relative frequencies for cell death detection. We have recently applied the use of 7 MHz ultrasound (3 to 10 MHz –6 dB bandwidth) to the detection of cell death using AML cells in vitro and tumours treated with radiation with or without anti-angiogenics in combination. Data from the experiments with cells has demonstrated an ability to detect as little as 10% apoptotic cells with data paralleling changes observed using high-frequency ultrasound. Moreover, ultrasound data detected from prostate cancer PC3 tumour xenografts where anti-angiogenic agents were used in combination with radiation to induce large macroscopic areas of cell death indicate that the detection of apoptosis may also be carried out in vivo. These emerging results suggest that the monitoring of treatment efficacy may be possible using low-frequency ultrasound and such evaluations are underway in patients receiving cancer therapy.

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