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Ultrasound, optical and photoacoustic imaging of Acoustic Cluster Therapy enhanced delivery to human tumors in mice

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***Abstract*— Acoustic cluster therapy (ACT) is designed to overcome some of the limitations of conventional microbubbles for ultrasound-assisted delivery of drugs to tumors. ACT consists of clusters of oil microdroplets and microbubbles. Diagnostic ultrasound imaging can be used to visualize the microbubble component and to activate (vaporize) the droplets to produce large (>20 μm) bubbles which lodge in the tumor's microvessels. A subsequent low-frequency ultrasound field then gently pulsates the activated bubbles which are in direct contact with endothelium, enhancing vascular permeability. We present *in-vivo* pre-clinical imaging experiments designed to better understand ACT behavior. Conventional microbubbles were observed to wash out of tumors within 6 min whereas activated ACT failed to wash out during the observation time of 15 min, confirming activation. Stationary echoes in tumors uniquely accumulated in fundamental B-mode images after ACT activation (but not with a microbubble agent, nor in nonlinear contrast mode) providing a signature for identifying ACT activation *in vivo*. ACT significantly enhanced CW800 dye uptake in tumor relative to dye alone. Photoacoustic CW800 signals appeared in the tumor periphery, suggesting potential for high-resolution, three-dimensional dynamic monitoring although with lower sensitivity than fluorescence imaging.**

Keywords — ACT, cancer treatment response, drug delivery, imaging biomarker, microbubble

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I. Introduction

The combination of ultrasound and intravenously injected microbubbles can improve drug delivery to tumors, also reducing systemic dose and toxicity [1]. Nevertheless, microbubbles have several limitations, which include limited capacity to carry drugs on their surface, small and brief local dose enhancement as the drug flows downstream and limited ability to interact with the vascular endothelium to increase permeation due to small surface area and relatively large distance from the endothelium. Acoustic cluster therapy (ACT) is designed to overcome some of these limitations. It consists of a dispersion of clusters of positively charged oil microdroplets that are electrostatically bound to negatively charged microbubbles (Fig. 1). The droplets could carry a dissolved drug or the whole product may be co-injected with a drug. Full details of ACT are provided in [2]. Diagnostic ultrasound is able to visualize the microbubbles and to activate (vaporize) the droplets to produce large (> 20 μm) bubbles which lodge in the tumor's microvessels and may briefly trap released or co-injected drug. A low-frequency ultrasound field is then employed to gently pulsate the activated bubbles which are in contact with a relatively large section of the vascular endothelium, increasing vascular permeability.

For fully effective future deployment of the ACT concept, imaging methods would ideally needed to monitor accumulation of non-activated agent for dosimetry, confirm and quantify activation and its spatial distribution, and monitor

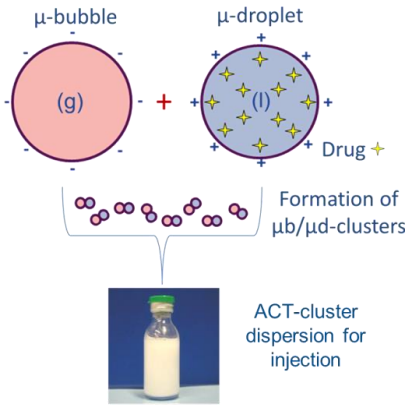

Figure 1. Illustration of the acoustic cluster therapy (ACT) concept. Although a drug-loaded microdroplet is shown, unloaded agent may be co-injected with a drug.

the delivery enhancement and its duration. Here we present *in-vivo* pre-clinical ultrasound, optical and photoacoustic imaging experiments designed to better understand ACT behavior. We extend previous work [3] by using different tumor models, apparatus for imaging and delivery enhancement, and signal processing.

II. Materials and Methods

A. Confirming acoustic cluster activation

An ovarian carcinoma (SK-OV-3, subline SLC-B) xenograft was grown as a subcutaneous tumor on the lower right flank of each of 6 athymic mice. Imaging and activation of ACT used a Toshiba Aplio-XG ultrasound scanner and a 1204BT linear array. Radiofrequency data and video image sequences were acquired for both fundamental and non-linear contrast modes, at 8 MHz, before, during and after intravenous ACT injection.

Initial studies confirmed that it was possible to detect the acoustic clusters arriving at the tumor without activating the agent, by setting the mechanical index ($MI = PNP/(f_c)^{½}$, where PNP = peak negative pressure and f_c = center frequency [4]) to a low enough value, typically less than 0.1. At such low MIs, acoustic clusters were visible as moving echoes in nonlinear contrast-specific imaging mode but were not visible in conventional fundamental B-mode images, which suggested that it was the microbubble component of the clusters that was producing the detectable nonlinear echo signal.

By increasing the MI, imaging evidence suggested that the agent was being activated. Above an MI of 0.1, stationary echoes were observed to appear in the fundamental B-mode images of a tumor which previously had shown little or no contrast enhancement on B-mode, consistent with the production of large trapped bubbles. To test that these stationary echoes were uniquely associated with the activation of ACT clusters, echo time-amplitude curve (TAC) properties were measured for mobile and stationary contrast agent components, in tumor regions of interest following Sonazoid™ (microbubble only) injections, compared with injections of ACT clusters. To be able to simultaneously generate separate time-amplitude curves for mobile versus stationary bubbles an image processing method was developed to segment stationary from moving echoes. This involved first characterizing the respiratory motion by using speckle tracking, then using this to apply a correction for such motion, after which images of echo temporal correlation were calculated and thresholded. After such processing, moving-bubble echoes exhibited low correlation. Stationary bubbles possessed high correlation and appeared in fundamental B-mode as new echoes since prior to injection.

B. Reporter dye delivery-enhancement studies

Unlike in our previous study [3], which employed partial immersion of the animal in a water-tank, reporter dye delivery-enhancement studies were conducted here using a clinically-translatable experimental apparatus (Fig. 2). Tail-vein injection of 100 µl, 2 nmol 800CW™ PEG infrared (IR) dye, with or without subsequent injection of ACT, was followed by delivery-enhancement insonification using 300 kHz ultrasound from a single element bowl transducer focused on the tumor.

ACT was administered in 3, 50 µl does, with 5-minute intervals between doses, and activated at the tumor site for 45 s immediately after injection using the Toshiba Aplio™ at 8 MHz operating in interleaved (10 frames/sec) non-linear contrast mode and fundamental B-mode, and at an MI of 0.4. Each administration of ACT and activation insonification was followed by insonification for delivery enhancement for 5 min using 300 KHz, 2-cycle pulses of ultrasound at a pulse repetition frequency of 2 kHz and MI of 0.12.

An IVIS™ SpectrumCT imaging system was then used to measure dye epifluorescence in the SK-OV-3 tumors compared to background.

As a preliminary study of photoacoustic monitoring of CW800 reporter-dye accumulation following ACT treatment, a subcutaneous pancreatic adenocarcinoma (MiaPaCa-2) tumor on the flank of a mouse was employed, scanned using a preclinical whole-body cross-sectional multispectral optoacoustic tomography (MSOT) system (iThera inVision256 MSOT™). MSOT images at various optical wavelengths in the range 680 – 900 nm were obtained and co-registered with ultrasound fundamental B-mode and non-linear contrast

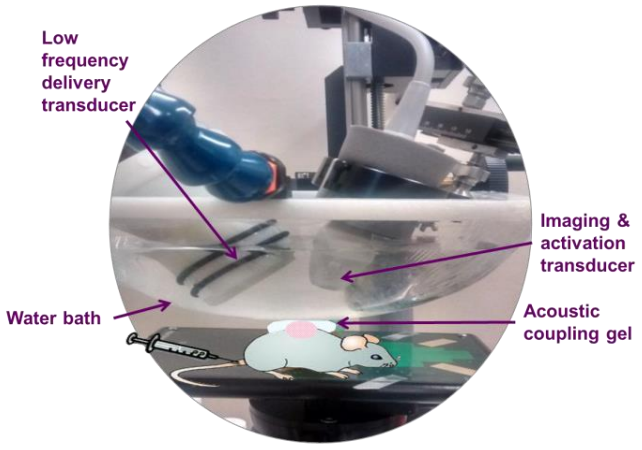

Figure 2. Apparatus for in-vivo ACT imaging, activation and delivery-enhancement.

images obtained at 8 MHz using the above-mentioned Aplio™ scanner. A novel system, reported elsewhere [5], was used to register the ultrasound images to the MSOT images. Single-wavelength and spectrally-unmixed MSOT images were compared with IVIS™ fluorescence images.

III. Results and Discussion

A. Acoustic cluster activation effectiveness

Stationary echoes in tumors uniquely accumulated in fundamental mode after ACT injection and ultrasound activation, but they were not seen when Sonazoid™ alone was injection, nor in nonlinear contrast mode whether ACT or Sonozoid™ was injected, nor when ACT was injected and an insufficient (non-activating) MI was employed. This behavior was apparent by subjective observation of image sequences (Fig. 3) and after image processing to automatically identify activated clusters, providing a signature with which to identify and count the level of ACT activation *in vivo* (Fig. 4). The peak number of stationary activated ACT bubbles tended to increase as a function of MI, from MI = 0.1 to 0.4 (Fig. 5).

Clear differences were observed between the temporal dynamics of Sonazoid™, ACT, and moving and stationary components of ACT, as a function of MI. From the TAC properties (Fig. 6), the peak for Sonazoid™ mobile contrast accumulation in tumors was 5 – 10 s post-injection, compared

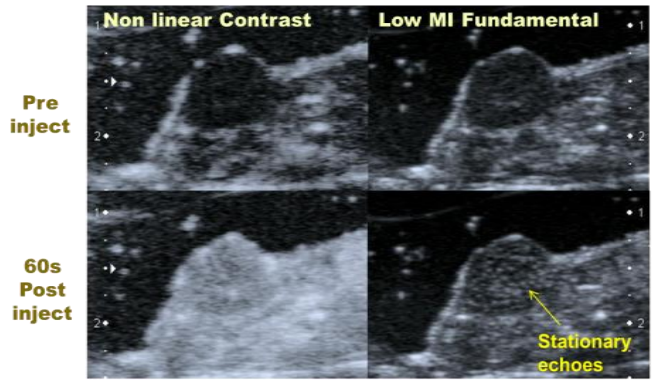

Figure 3. Ultrasound images of an SK-OV-3 tumor pre- and 60 s post-ACT injection (MI = 0.4).

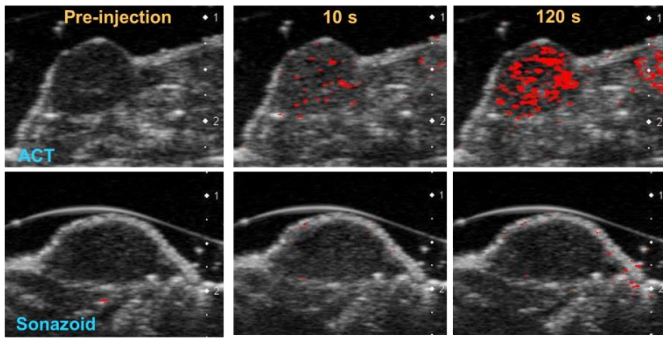

Figure 4. Example frames from image sequences at MI = 0.4, overlaid with contrast echoes segmented as stationary (red). Left: pre-injection. Middle: 10 s after injection. Right: 120 s after injection. Top: ACT was injected. Bottom: Sonazoid™ was injected.

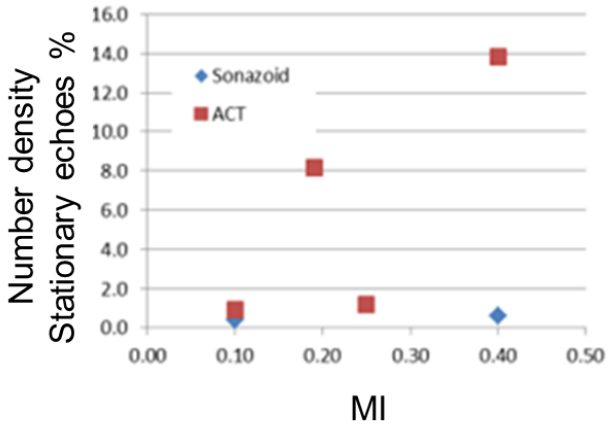

Figure 5. Maximum stationary echo number density as a function of incident MI value for Sonazoid™ and ACT.

with 30 – 120 s for activated ACT clusters. Sonazoid™ microbubbles washed out of the tumor within 6 min whereas activated ACT failed to wash out within the observation time (15 min), confirming activation.

B. Reporter dye delivery-enhancement

ACT significantly enhanced CW800 uptake in SK-OV-3 tumors, by a factor of 1.4 fluorescence intensity relative to dye alone (Fig. 7), as measured by fluorescence in the tumor relative to background. This is of particular significance since this dye is designed for use as an agent for enhanced permeation and retention (EPR). The uptake enhancement level achieved using ACT was comparable to that observed previously in mice [3] with the same infrared dye but using a different (human prostate) tumor model and insonation arrangement, which lends confidence to the reproducibility of results using ACT for enhancing delivery of surrogate therapeutics to tumors.

In the MiaPaCa-2 tumor, six days post-dye injection and ACT treatment, dye accumulation observed in the epifluorescence image was consistent with the behavior expected for an EPR agent with fluorescence remaining high in the tumor (Fig. 8a). Prior to that, tumor-to-background ratio increased from 2.37 to 6.77 between 150 min and 6 day post-injection and ACT treatment. MSOT spectrally-unmixed images using all wavelengths demonstrated ability to isolate hemoglobin, oxyhemoglobin and CW800 chromophores (Fig. 8d). Using these and images obtained at the dye's absorption peak of 770 nm (Fig. 8c), photoacoustic CW800 signals were observed in the tumor periphery (Figs. 8c and 8d), suggesting potential for using MSOT to measure the spatio-temporal dynamics of reporter dye uptake at high resolution in three dimensions if more dye were to be injected.

IV. Conclusions

Stationary new echoes in fundamental B-mode ultrasound images identify activated ACT clusters *in vivo*, and ACT significantly enhances uptake of an infrared dye acting as a surrogate therapeutic. These findings demonstrate reproducibility of previous observations in a different tumor model and using different insonification apparatus. The

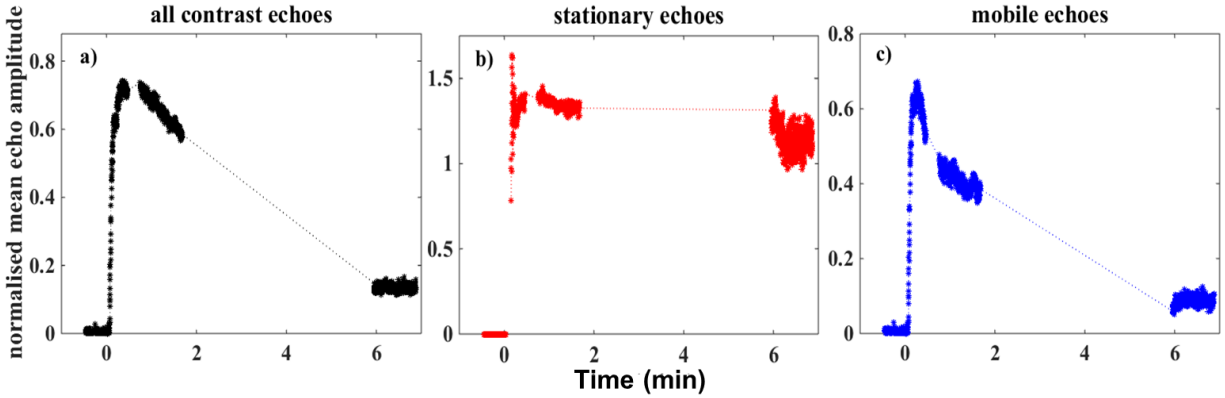

Figure 6. Example TAC curves showing rate of tumor wash-in and wash-out, MI=0.4 a) all echoes, b) segmented new stationary echoes, c) segmented moving echoes.

imaging/insonification apparatus employed here has potential for translation to the clinic, especially if both imaging and delivery-enhancement ultrasound beams can be generated by the same transducer.

MSOT photoacoustic imaging shows potential for high-resolution three-dimensional dynamic monitoring, albeit with lower sensitivity than epifluorescence imaging.

The methods developed to automatically segment stationary bubble (activated acoustic clusters) echoes from moving bubble (microbubble) echoes may also be of value in fields such as the study of molecularly targeted bubbles, to determine which bubbles have bound to their target. For future applications of ACT, in delivering actual therapeutics as well as dye surrogates, the method developed for counting integrated amplitude of stationary new echoes as a measure of activation efficiency would be worth evaluation as a predictor of enhancement of uptake and hence response to treatment.

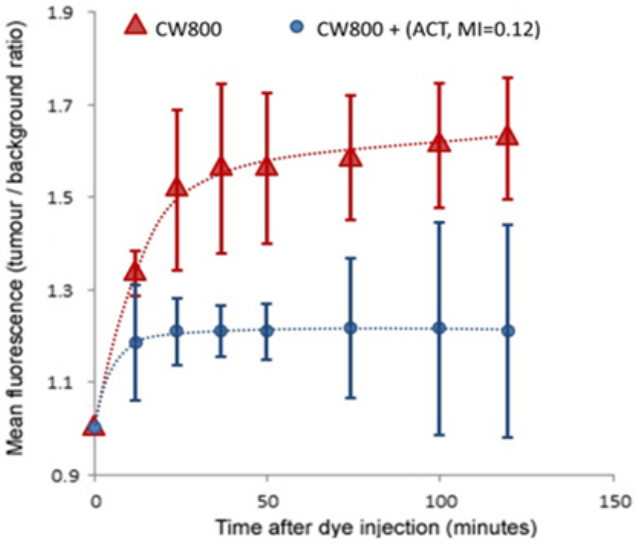

Figure 7. CW800 infrared dye uptake enhancement using ACT. The tumor/background epifluorescence for CW800 dye alone is compared with that for CW800 dye followed by ACT activation and delivery enhancement sonification with pulsed 300 kHz and MI = 0.12 (means, ±1 standard deviation, N = 3).

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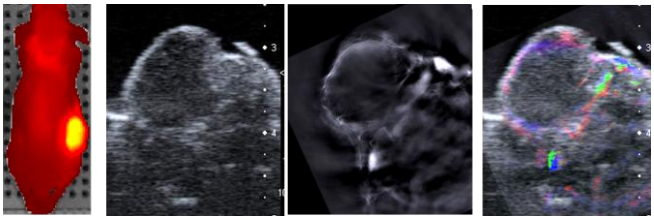

Figure 8. Images of a subcutaneous pancreatic adenocarcinoma xenograft (MiaPaCa-2) 6 days after injection of 2 nmol CW800 dye followed by ACT activation and delivery enhancement sonification. (a) Epifluorescence image of the whole mouse showing the tumor as strongly fluorescing. (b) Ultrasound B-mode image at 8 MHz of the tumor in cross-section. (c) co-registered MSOT tumor cross-sectional image at 770 nm. (d) Image from (b) overlaid with a spectrally unmixed MSOT image showing oxyhemoglobin (red), hemoglobin (blue) and CW800 (green).