Application Note

Imaging of Nanoparticle-Based Contrast Agents with the Vevo LAZR Photoacoustic Imaging System

VISUALSONICS

Introduction:

The goal of contrast agents as molecular biology tools is to identify biomolecular markers of disease using *in vivo* imaging technologies. The ability to distinguish between normal and diseased tissue *in vivo* in addition to obtaining molecular information about the tissue of interest has important applications in cancer research in particular. Nanoparticles are one such group of contrast agent which have been used for photoacoustic (PA) imaging, whereby light is used to generate acoustic waves from absorbers in the subject of interest. This technology allows for the visualization of vasculature structure as well as agents which can increase image contrast when targeted to specific receptors in the vasculature.

The Vevo® LAZR platform is a high-resolution combined photoacoustic and micro-ultrasound system which uses pulsed laser light at wavelengths from 680 to 970 nm to generate acoustic waves which are detected by a linear array transducer. The photoacoustic image can be co-registered on a B-Mode ultrasound image to provide anatomical information about the photoacoustic signal. Hemoglobin in red blood cells absorbs light at the wavelengths mentioned above (called the near-infra red or NIR range) and thus imaging of vasculature can be performed independent of blood flow. Nanoparticles (NPs) such as gold nanorods (GNRs) and single-walled Carbon nanotubes (SWNTs) have been designed to absorb light in the NIR and are biologically inactive allowing for long circulation times *in vivo*[1,2]. These NPs can act as contrast agents to not only enhance the endogenous vascular signal, but also they can be targeted to specific intravascular receptors such as alpha-5, beta-3 integrins which are overexpressed in tumor neovasculature[3].

In this study, we investigated untargeted GNRs (Nanopartz) and SWNT conjugated with arginine, glycine and asparagine (RGD) containing peptides, which bind to alpha-5, beta-3 integrins[1] (obtained from Sanjiv S. Gambhir at Stanford University) *in vivo* in a subcutaneous hindlimb tumor model. The increase in average photoacoustic signal was measured before and after administration of the nanoparticles.

Materials and Methods:

The Vevo LAZR photoacoustic imaging system (VisualSonics Inc, Toronto, Canada) was used to acquire all images. The array was retrofitted with a housing that held rectangular fiber optic bundles (25.4 x 1.25 mm) to either side, at an angle of 30° relative to the imaging plane. The rectangular bundles were bifurcated ends of a single bundle that was coupled to a tunable laser. The µUS system was synchronized with the laser and photoacoustic signals were acquired with a fluence < 20 mJ/cm^2, beamformed in software, and displayed at 5-20 Hz.

The LZ250D (center operating frequency of 21 MHz, axial resolution 75 µm) and LZ550D (center operating frequency of 40 MHz, axial resolution 40 µm) probes were used to acquire all images.

Images were acquired from athymic nude mice implanted with Lewis lung carcinoma (LLC) cells subdermally in the hindlimb 3 to 4 weeks before imaging. Animals were anaesthetized using isofluorane (1.5-2.0%); the animal was secured to a heated animal handling platform which allows for monitoring of the ECG, respiration, and temperature of the animal. Ultrasound gel was used to provide a coupling interface between the ultrasound probe and the animal.

The Vevo LAZR software allows for the acquisition of photoacoustic images to detect the presence of hemoglobin and other absorbers, and co-register it with B-Mode images. The wavelength of the pulsed laser light used to generate the photoacoustic effect can be changed anywhere from 680 nm to 970 nm. Images were acquired at 680, 750, 800, 850 and 970 nm.

Gold nanorods (Nanopartz, Loveland, CO) had a peak absorbance of 814 nm, an axial diameter of 25 nm and a length of 103 nm and a concentration of 3.146E13 NPs/ml (2.143 mg/ml). SWNT-RGDs (Stanford University, Palo Alto, CA) had a peak absorbance at 690nm, an axial diameter of 1-2 nm (without the RGD molecule) and a length of 50-300 nm, and a concentration of 1.2 µM. Both types of nanoparticles were coated with polyethylene glycol (PEG) to increase biocompatibility.

Photoacoustic Imaging Mode:

While pure optical imaging methods have limited depth and spatial resolution due to scattering of light, pure ultrasound is limited in its functional imaging capabilities since sound is not sensitive to chemical changes. The Vevo LAZR platform combines these optical imaging and ultrasound methods to offer increased imaging depth due to the low scattering and high resolution of ultrasound while offering functional imaging due to the different absorption spectra of oxygenated and deoxygenated hemoglobin. The ability to image at different wavelengths of pulsed light also allows for the characterization of different absorbers such as contrast agents which differ from endogenous absorbers.

The Vevo LAZR platform simultaneously collects photoacoustic and micro-ultrasound data and displays the image data side-by-side or co-registered. The intensity of the photoacoustic signal corresponds to the degree to which a substance absorbs light at the particular wavelength being used. The wavelength range of the Vevo LAZR technology lies in the NIR range, also referred to as the 'therapeutic window' since few biological molecules absorb light in this range[4]. Endogenous absorbers include hemoglobin and melanosomes[5]. For this reason, vasculature can be imaged effectively with photoacoustics and necrotic and vascularized regions of a tumor may be distinguished.

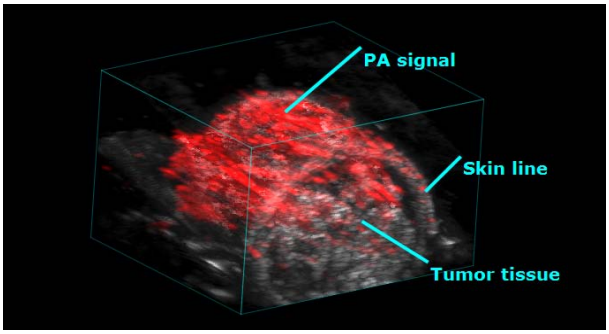

Figure 1 *– Photoacoustic imaging of a LLC tumor on the hindlimb of a mouse. The red signal is primarily derived from the absorption of light by hemoglobin in red blood cells.*

In addition, the software allows the user to select a region of interest and calculate the average intensity of the photoacoustic signal there. This is done by measuring the brightness of the signal at every pixel within the region of interest and taking an average. This photoacoustic measurement tool may also be used to detect changes over time when applied to images collected at different time points or on a cine loop.

Photoacoustic information can also be collected in 3D, where a motor is used to translate the probe over the complete area of the tumor. A photoacoustic measurement region applied to such a scan, where each frame of the cine loop represents an adjacent 'slice' of the tumor can be used to estimate an average photoacoustic intensity value for the entire tumor.

Gold nanorods:

2D and 3D scans of a subcutaneous hindlimb tumor were performed before and after tail-vein injection of a 200 µl bolus of undiluted untargeted gold nanorods (described previously) in a nude mouse. In addition, a 2D scan was performed during and for approximately 2 minutes after the bolus.

The average photoacoustic signal in the 2D scan performed during the bolus increased to a maximum of approximately 55% at 15 seconds. After approximately 2.5 minutes following bolus injection, the photoacoustic signal dropped back down to close to baseline.

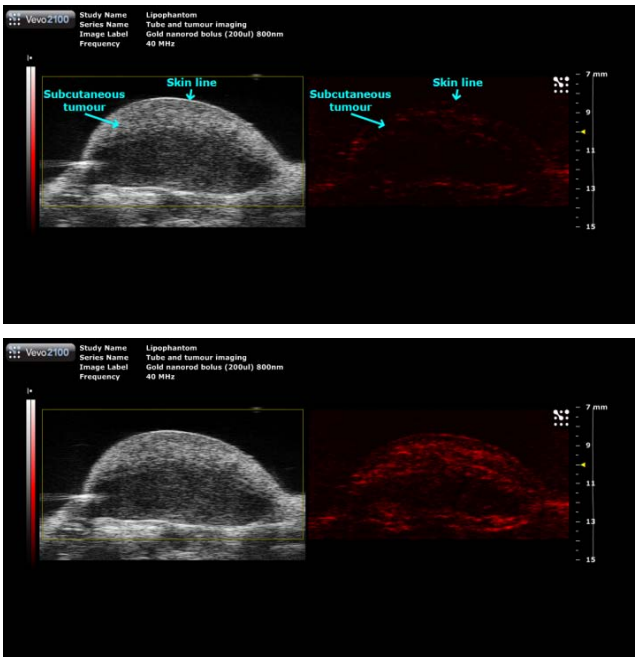

Figure 2 *– B-Mode and photoacoustic images of a subcutaneous tumor before and 2 minutes after a 200 µl bolus of GNR. Photoacoustic contrast in enhanced by GNRs in the vasculature.*

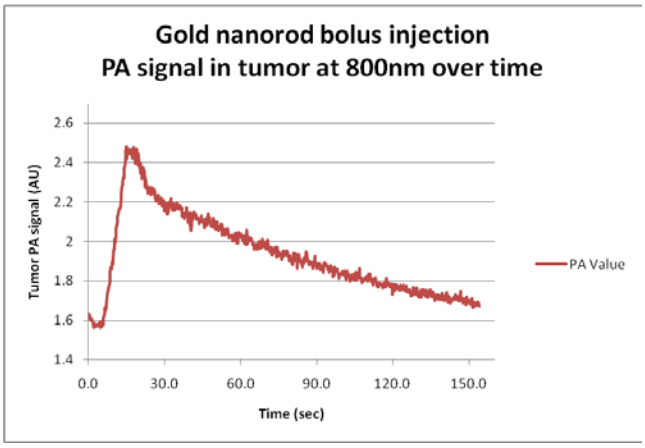

Figure 3 *–Photoacoustic signal (as measured by selecting a ROI that encompasses the tumor) in arbitrary units against time. The GNR injection was performed at approximately 5 seconds. The maximum signal increase is approximately 55%.*

Pre- and post-bolus 3D scans were compared by averaging the photoacoustic value for each frame. This provided an average photoacoustic value for the tumor both before and 3 minutes after gold nanorod injection. No significant difference was observed.

The gold nanorods are assumed to be taken out of the circulation by the reticuloendothelial system which includes the spleen and liver[6], so scanning of these organs was performed both *in vivo* and excised, one day after GNR injection. An *ex vivo* comparison was made between the spleen and liver of a treated animal comparted to that of a control animal which did not receive nanoparticle administration. A signal was present *in vivo* at 800 nm in both organs and was confirmed to be due to the sequestration of GNS by the clear difference in signal between control and treated organs by inspection of the images.

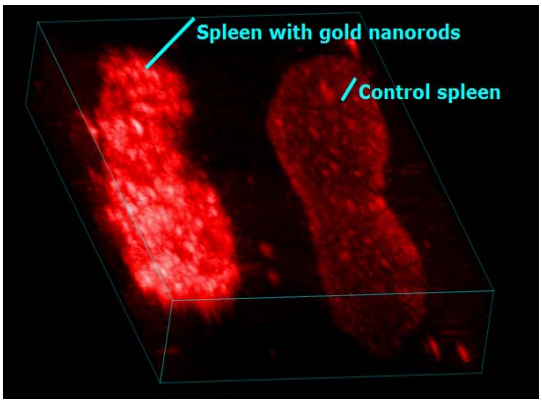

Figure 4 *– Ex vivo 3D photoacoustic images taken at 750 nm of the excised spleen of a mouse 1 day after a tail vein injection of 200 µl of GNRs and a control mouse spleen.*

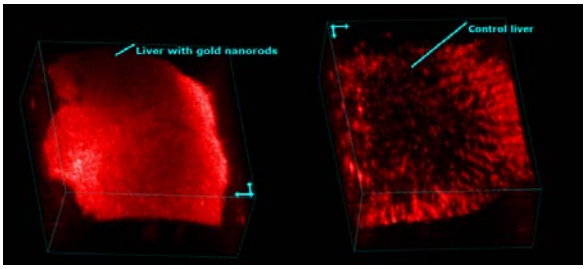

Figure 5 *– Ex vivo 3D photoacoustic images taken at 750 nm of the excised liver of a mouse 1 day after a tail vein injection of 200 µl of GNRs and a control mouse. Scale bars represent 1 mm.*

SWNT-RGD:

2D and 3D scans of a subcutaneous hindlimb tumor were performed before and after tail-vein injection of a 150 µl bolus of SWNT-RGD (described previously). In addition, a 2D scan was performed during and for approximately 3 minutes after the bolus.

The average photoacoustic signal in the 2D scan performed during the bolus increased steadily to 10% approximately 3 minutes after bolus injection.

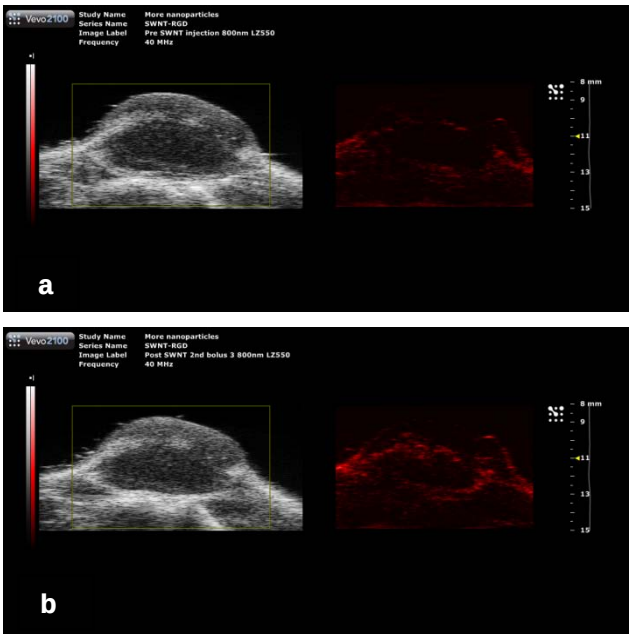

Figure 6 *– B-Mode and 800 nm photoacoustic images of a subcutaneous tumor before (a) and 5 hours after (b) a 150 µl bolus of SWNT-RGD. Photoacoustic contrast is enhanced by SWNT-GRDs targeted to tumor neovasculature.*

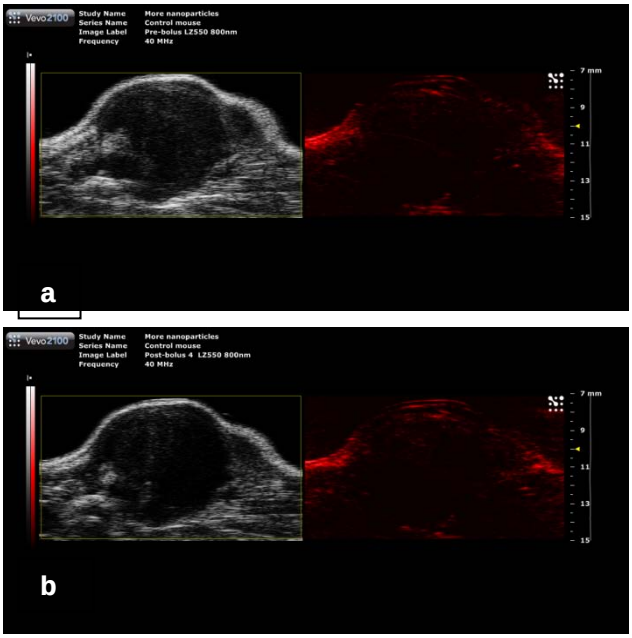

Figure 7 *– 2D (a) and 800 nm photoacoustic images (b) of a subcutaneous tumor before and 5 hours after a 150 µl bolus of saline as a control. There is little change in photoacoustic signal over time.*

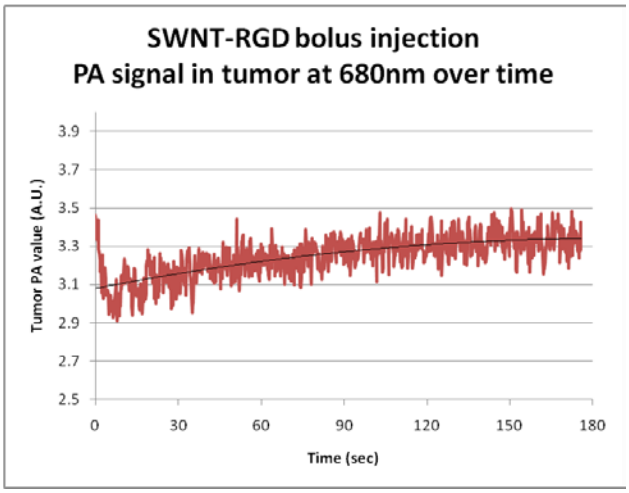

Figure 8 *– Photoacoustic signal (as measured by selecting a ROI that encompasses the tumor) in arbitrary units against time. The injection was performed at approximately 5 seconds. The maximum signal increase is approximately 10%.*

Pre- and post-bolus 3D scans were compared by choosing 50 consecutive frames that encompassed the middle of the tumor and averaging the photoacoustic value for each frame. This provided an average photoacoustic value for the tumor both before and at 0.5, 2, 3 and 5 hours after SWNT-RGD injection. A significant difference in signal was observed after 35 minutes at 750, 800, 850 and 970 nm and the difference increased after 2 hours at all wavelengths. It persisted above the baseline after 5 hours.

A similar analysis was performed on a control animal which received a 150 µl bolus of saline and was imaged at similar time points. The average photoacoustic values for the tumor changed little over the same time course as the SWNT animal. When the percent change in photoacoustic signal of the SWNT-RGD and control tumors was compared, an increase in signal was observed for the SWNT-RGD injected animal above control at all time points with the exception of the first time point at 680 nm.

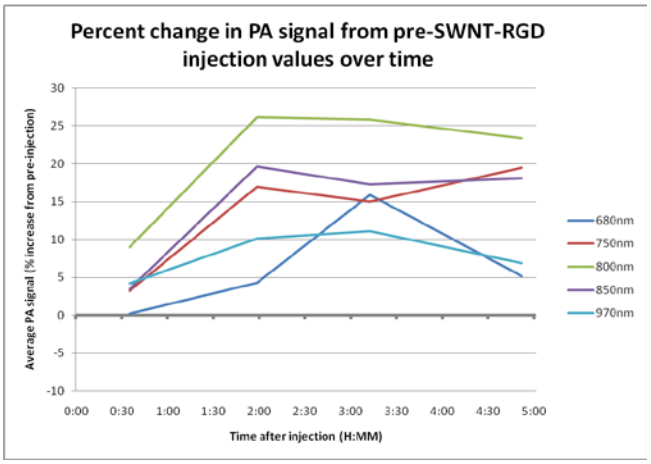

Figure 9 *– Percent change in photoacoustic signal from pre-injection values (as measured by selecting an ROI that encompasses 50 frames of a 3D scan of the tumor) against time. The photoacoustic signal increases and remains above baseline at all wavelengths for at least 5 hours.*

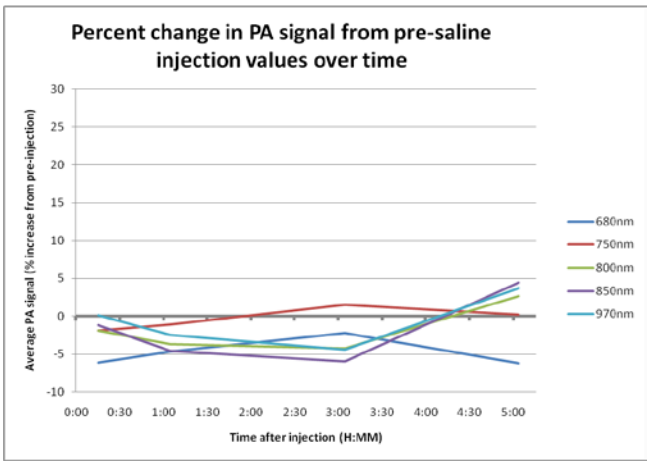

Figure 10 *– Percent change in photoacoustic signal from pre-injection values (as measured by selecting an ROI that encompasses 50 frames of a 3D scan of the tumor) against time. The photoacoustic signal for all wavelengths never increases above 5% of baseline.*

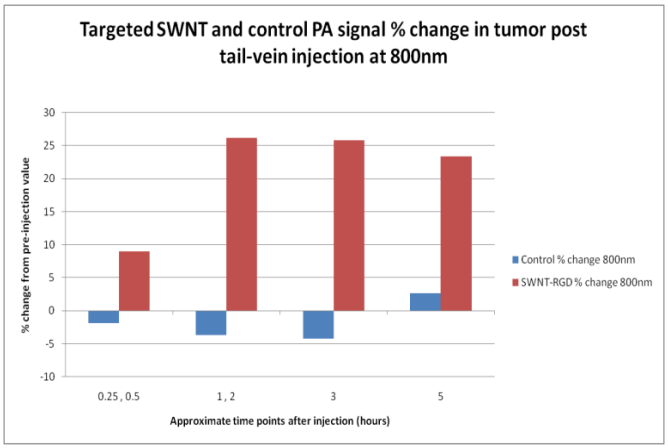

Figure 11 *– A comparison of percent change in photoacoustic signal at 800 nm from pre-injection values at various time points for SWNT-RGD and control animals. The SWNT-RGD photoacoustic signal is higher than control at all time points.*

The SWNT are removed from the circulation by the reticuloendothelial system which includes the spleen and liver, so imaging of these excised organs was performed two days after SWNT-RGD injection. An *ex vivo* comparison was made between the spleen and liver of a treated animal compared to that of a control animal. A clear difference in signal between control and treated organs was observed by inspection of the images at all wavelengths.

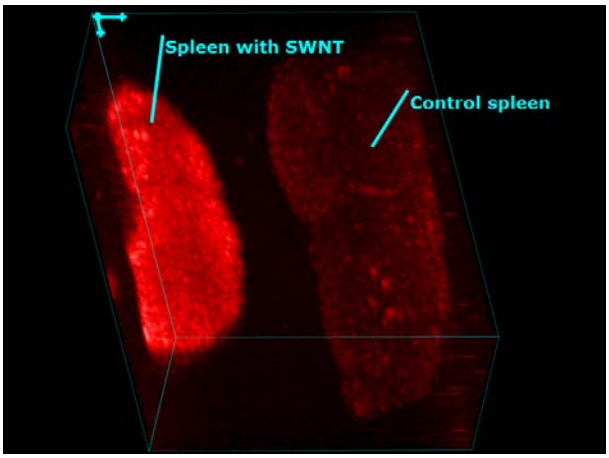

Figure 12 *– Ex vivo 3D photoacoustic images taken at 750 nm of the excised spleen of a mouse 1 day after a tail vein injection of 150 µl of SWNT-RGDs and a control mouse spleen. Scale bars represent 1 mm.*

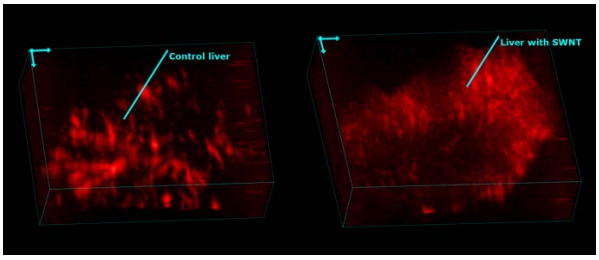

Figure 13 *– Ex vivo 3D photoacoustic images taken at 970 nm of the excised liver of a mouse 1 day after a tail vein injection of 200 µl of GNRs and a control mouse liver. Scale bars represent 1mm.*

Conclusions:

The Vevo LAZR platform can detect and quantify changes induced by intravenous administration of the nanoparticle-based contrast agents GRNs and SWNTs.

A bolus injection of untargeted GNRs increased the vasculature signal in a 2D photoacoustic scan temporarily by approximately 55%. They were quickly removed from the circulation, presumably sequestered by the spleen and liver, where they remained at least 24 hours after injection.

The percent increase in signal observed using the SWNT-RGD was less than with the GNRs. However, the signal increased over many minutes and hours, suggesting that the SWNT-RGDs remain in circulation and collect in the tumor tissue, (presumably on alpha-5, beta-3 integrins in the neovasculature). This was confirmed by comparing this increase in signal to a control animal which received an injection of saline.

A similar signal increase in the spleen and liver of the injected animal as with the GNRs indicates their sequestration there.

We have demonstrated that the Vevo LAZR platform is capable of detecting signal from two different nanoparticle-based contrast agents in the vasculature of subcutaneous tumors and in the liver and spleen of nude mice. The possibility of visualizing nanoparticles *in vivo* in real-time to understand cellular and molecular processes highlights the potential of the system to be an *in vivo* molecular modality.

The labeling of tissue from different disease models with contrast agents non-invasively and in longitudinal studies with our system increases the research potential of those studying cellular and molecular imaging.

References:

[1] De La Zerda, A. *et al.* Carbon nanotubes as photoacoustic molecular imaging agents in living mice. *Nature nanotechnology*. **3**, 557-562, 2008.

[2] Pan, D. *et al.* Molecular photoacoustic imaging of anigogenesis with integrin-targeted gold nanobeacons. *Federation of American Societies for Experimental Biology*. Nov 19, E. pub., 2010.

[3] Janssen, M.L. *et al.* Tumour targeting with rediolabelled alpha(v) beta(3) integrein binding peptides in a nude mouse model. *Cancer Res.* **62**, 6146-6151, 2002.

[4] Emelianov, S.Y. *et al.* Photoacoustics for molecular imaging and therapy. *Physics Today.* **62** (8), 34-39, 2009.

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[6] Sadauskas, E. *et al.* Kupffer cells are central in the removal of nanoparticles from the organism. *Particle and Fibre Toxicology.* **4** (10), 2007.

Recommended VisualSonics Protocols:

VisualSonics Vevo LAZR Imaging System, Operators Manual

PA Imaging

Vevo LAZR Photoacoustic Imaging Protocols

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