Application Note

Evaluation of Tumor Microenvironment using the Vevo LAZR Photoacoustic Imaging System

VISUALSONICS

Introduction:

Tumor biology has been studied extensively, especially in the areas of angiogenesis, apoptosis, hypoxia/metabolism, MAP kinases and other types of molecular profiling. Pathways leading to cancer progression include oncogenes, DNA repair, angiogenesis, glycolysis metabolism and immune therapy. Oncogenes are paradoxical, in that they can activate both apoptosis and cell survival. Traditionally, they have been the most targeted genes via cancer therapeutics. While the number of genes involved in cancer onset has remained consistent, mutations in the form of translocations and fusions have risen steadily across 12 pathways, indicating the importance of developing molecular targets. Cancer metabolism can be differentiated from that of normal cells by the reliance on glycolysis for energy generation as opposed to mitochondria, indicating a lack of oxygen requirement. The abnormal metabolic pathway is brought about by a genetic alteration of PI3 kinase, an oncogene, which is triggered by hypoxia in the tumor microenvironment.

Angiogenesis is a large area of cancer research and refers to the process of new blood vessel formation. When such growth is irregular and asymmetrical, it is a defining hallmark of tumor development. Tumor angiogenesis is currently visualized using ultrasound, confocal, two-photon, Doppler and Color Doppler methods. Photoacoustic (PA) imaging offers new methods for quantification via oxygen saturation and new visualization possibilities through the endogenous heme signal.

The Vevo® LAZR platform is a unique photoacoustic imaging modality which combines the sensitivity of optical imaging with the low acoustic scattering and high-resolution of micro-ultrasound (μUS). Moreover, optical absorption is sensitive to biological processes such that photoacoustics may be used for functional imaging.

The differing optical absorption spectra of oxygenated (HbO_2) and deoxygenated hemoglobin (Hb) (Figure 1) make photoacoustics an ideal modality for *in vivo* imaging of vascular oxygen saturation (sO_2), early tumor detection and angiogenesis.

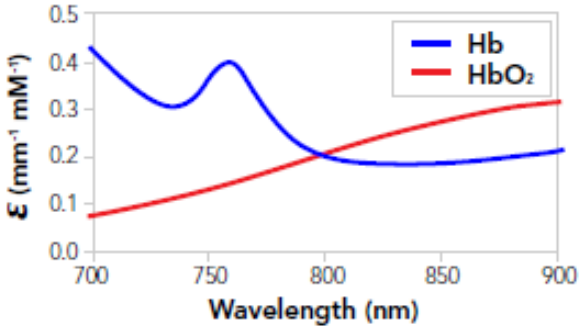

Figure 1 *– Absorption spectra for oxygenated (HbO_2) and deoxygenated (Hb) blood by S Jacques, based on Wray et al., 1988.*

Photoacoustics is a well suited and unique imaging modality for tumor preclinical research. It holds the ability to identify hypoxia via oxygen saturation measurements and employs the endogenous heme optical absorption spectra of neovasculature to achieve high contrast. With the Vevo LAZR technology sentinel lymph nodes can be accurately located using methylene blue dye and biopsied using fine needle aspiration techniques, bypassing the need to perform invasive surgical resections of nodes when aiming to detect the presence of mestasizing tumor cells. Furthermore, the ability to selectively detect nanoparticles, such as gold nanorods (GNRs) as well as targeted single-walled carbon nanotubes (SWNTs), *in vivo* allows for increasingly specific and enhanced investigation. This is facilitated by an integrated tunable laser (680 nm to 970 nm) which is part of the Vevo LAZR platform.

The goal of this application note is to describe the utility of photoacoustics imaging with the Vevo LAZR technology for

- Measuring oxygen saturation (sO_2)
- Sentinel lymph node detection
- Visualization of nanoparticles in tumors

Materials and Methods:

Animal Models

Photoacoustic imaging was performed on normal and tumor-bearing mice. Adult female CD1 mice were used for sO_2 measurements. Sentinel lymph node detection and subsequent dissection was performed on regular CD1 mice. Nanoparticles were visualized in athymic NUDE mice with hindlimb tumors.

Animal Handling

In order to validate the specificity of the oxygen saturation signal, the mouse jugular vein was exposed under anesthesia, a 25 gauge needle was inserted into the vein (pointing caudally), and an OXYLAB pO_2 E Series (Oxford Optronix, Oxfordshire, UK) probe was introduced through the needle and into the vein. Mice were exposed to varying concentrations of mixed oxygen (100% and 5%) and compressed air to create hypoxic and hyperoxygenated states in the animal for measuring oxygen saturation *in vivo*.

To detect sentinel lymph nodes, a CD1 mouse was anesthetized and a high-gauge butterfly needle attached to a syringe containing 1% methylene blue (Ricca Chemical Company, Arlington, TX) was inserted into the pad of the forepaw and secured with tape. During imaging, the animal received infusions of methylene blue (see 'Sentinel Lymph Node Detection' section below). After imaging, the animal was sacrificed and the lymph nodes were dissected to visually confirm staining.

For nanoparticle visualization, mice were subdermally implanted with hepatocarcinoma cells (Hep3B-Luc-C4) 4-5 weeks prior to imaging. They were anesthetized with 2% isoflourane and the tail vein was cannulated.

Vevo LAZR Photoacoustic Imaging System

The Vevo LAZR photoacoustic imaging system (VisualSonics, Inc, Toronto, Canada) was operated with a linear array transducer (MS250, center frequency = 21 MHz). 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 (680-970 nm). The system was synchronized with the laser and photoacoustic signals were acquired with a fluence < 20 mJ/cm2, beamformed in software and displayed at 5 Hz.

Oxygen Saturation Measurement

For sO_2 estimates, images were acquired using 'Oxyhemo' mode, which collects data at 750 and 850 nm and creates and displays a parametric map of estimated oxygen saturation and total haemoglobin at a rate of 1 Hz.

The software allows the user to create a region of interest (ROI) for the set photoacoustic images and displays the average value of sO_2 within the ROI. These values were compared to the reading from the Oxylab probe (Figure 3) to further correlate the sO_2 for each selected oxygen partial pressure (pO_2) value. These sO_2 values were then compared to predicted sO_2 values based on the measured pO_2. Predicted sO_2 values were obtained from an online calculation based on GR Kelman (JAP 21(4):1375-6, 1966).

Sentinel Lymph Node Detection

The axillary lymph node was located using B-Mode imaging and 2D and 3D photoacoustic imaging was performed at 680 nm and 760 nm. While imaging with 680 nm wavelength, 30 µl of methylene blue (Ricca) was infused into the forepaw. Subsequent 2D and 3D imaging was performed at 680nm and 760 nm every 5 minutes post-methylene blue injection for approximately 40 minutes. Pre- and post-bolus images were compared as well as images at the different wavelengths and any difference in signal was assessed for anatomical position to determine if it originated in the lymph node.

Nanoparticle Imaging

An image of the tumor was obtained with B-Mode imaging using Non-Targeted gold nanorods (GNRs) that enhance the endogenous vascular signal. A 150 µl amount of polyethylene-glycol coated GNRs (Nanopartz) was infused through the tail vein and photoacoustic imaging was performed at 800 nm before, during and after and the procedure. The wavelength chosen is the peak absorption wavelength for the gold nanorods (note that peak absorption of GNRs varies depending on the aspect ratio of the GNRs).

Pre- and post-infusion 2D and 3D images were compared to determine the amount of contrast enhancement.

Results

Oxygen Saturation Measurements

Photoacoustic derived sO_2 measurements were made and compared to expected sO_2 values from the literature at various oxygen partial pressures (pO_2). Changes in pO_2 values and sO_2 values correlated well with alterations in inhaled O_2 concentration (data not shown). pO_2 values measured with the OxyLab probe and sO_2 values measured with photoacoustic imaging correlated well (Figure 2).

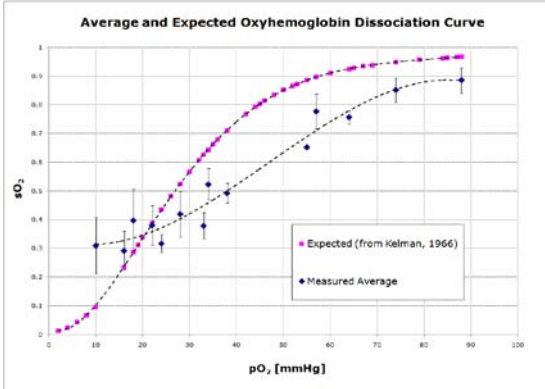

Figure 2 - *The oxyhemoglobin dissociation curve, showing expected sO_2 values (purple) and average sO_2 measurements taken at the corresponding pO_2 values (blue).*

While the slope of the oxyhemoglobin dissociation curve determined with measured pO_2 and sO_2 values was less than that of the expected oxyhemoglobin dissociation curve (Figure 2), a significant correlation between the measured and predicted values was observed (Figure 3) (R2= 0.8246, P<0.01). In addition, the 'S' shape of the expected oxyhemoglobin dissociation curve was approximated by the measured curve, demonstrating preliminary validation (Figure 2).

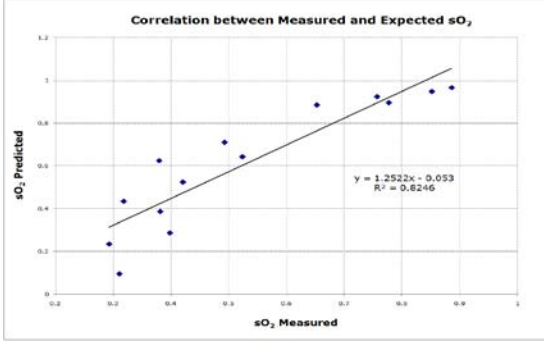

Figure 3 – *Correlation between photoacoustic derived sO2 values and sO_2 values derived from the literature (Kelman, 1966).*

Hypoxic status in the tumor microenvironment is an important application of the Vevo LAZR platform, and can be determined by oxygen saturation measurements. Current visualization includes co-registration of PA and B-Mode images and oxygenation mapping which allow for real-time monitoring of blood oxygenation (Figure 4).

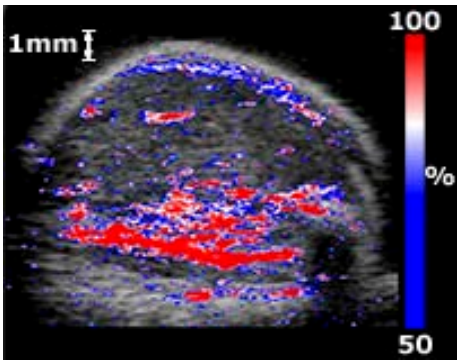

Figure 4 *– Photoacoustic oxygenation map co-registered with a high resolution image of a subcutaneous tumor.*

Sentinel Lymph Node Detection

The axillary lymph nodes of a mouse were detected using the Vevo LAZR platform with methylene blue as a contrast agent.
Post-injection images clearly display the position of the axial lymph node in a mouse model (Figure 5).

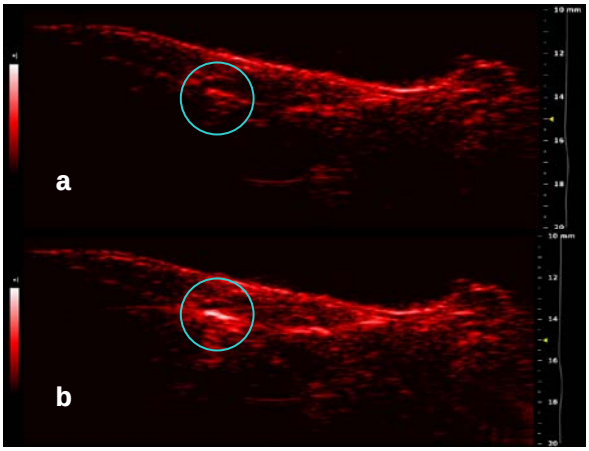

Figure 5 – *Post-methylene blue injection 2D photoacoustic image of mouse axial lymph node (identified in blue circle) at (a) 760 nm and (b) 680 nm*

Nanoparticle Detection and Quantification

The contrast of vasculature within a tumor was enhanced using GNRs and visualized with the Vevo LAZR technology. GNRs can be seen with photoacoustics when infused through the tail vein in both 2D (not shown) and 3D (Figure 6). These enhanced images of vascularity allow more detail and deeper structures to be seen within a tumor.

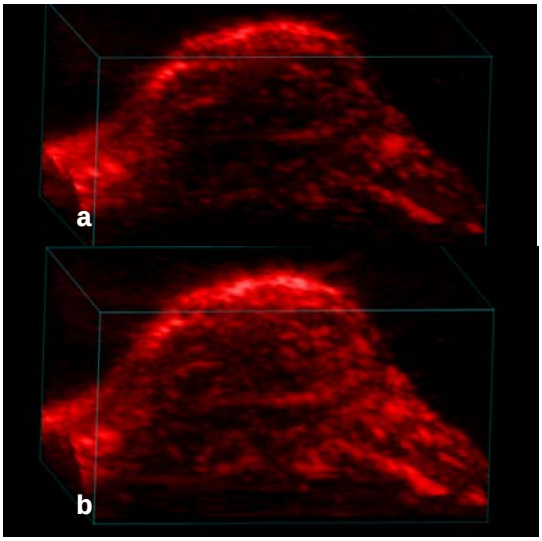

Figure 6 – *(a) Pre- and (b) post-gold nanorod 3D PA image of subcutaneous mouse tumor at 800 nm.*

Conclusions:

The images presented here clearly show the utility of the Vevo LAZR photoacoustic imaging system as a tool for *in vivo* imaging and quantification within the tumor microenvironment. The Vevo LAZR platform was demonstrated to measure oxygen saturation through the endogenous heme signal and therefore grade the hypoxic status within the tumor. Photoacoustic imaging of vascular sO_2, while not exactly matching the predicted values, can provide a good relative estimation. Detecting sentinel lymph nodes (SNLs)with the Vevo LAZR platform is another possibility, through the administration of methylene blue as a contrast agent. The ability to non-invasively identify SLNs allows for targeted fine-needle aspiration biopsies to be conducted, with diagnosis applications in areas such as breast cancer. Finally, we have shown visualization of tumor *in vivo* with nanoparticles using endogenously-enhancing GNRs. SWNTs can also be utilized for specific molecular targeting to intravascular receptors such as alpha-5, beta-3 integrins that are normally overexpressed in tumor neovasculature.

Anatomical, functional, physiological and molecular data can be visualized *in vivo* in real time on a single platform with inherent co-registration and the ability to record dynamic processes. Functional imaging is offered by the different absorption spectra of Hb and HbO_2, and the signal can be accurately positioned within internal anatomy offered by ultrasound. Early tumor detection and vascular dynamics studies in the microenvironment benefit from the superiour resolution and depth of the co-registration feature.

Furthermore, although not demonstrated here, the real-time signal acquisition not only allows for assessment of blood flow, but also permits Image-guided injection and resections with minimal invasiveness.

The ability to non-invasively monitor tumor progression offers the flexibility for long-term longitudinal studies to be carried out. Functional information such as oxygen saturation has been achieved by imaging the endogenous signal of optically-absorbing heme group found naturally in blood. Identification of tumor-bearing sites is easy through the endogenous signal, which highlights regions of blood flow and clearly demarks the highly convoluted and disorganized microvasculature charachteristic of tumors.

In this paper, we have shown the ability of the Vevo LAZR platform to measure sO_2, detect the correct positioning of SLNs and enhance tumor natural contrast via tumor-targeted gold nanoparticles. The versatility of the Vevo LAZR photoacoustic imaging system for imaging endogenous signals, contrast dyes and nanoparticles make it a multidisciplinary tool to assess all aspects of the tumor microvasculature.

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Recommended Papers:

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Recommended VisualSonics Protocols:

VisualSonics Vevo 2100 Imaging System, Operators Manual

Photoacoustic Imaging

Vevo LAZR Photoacoustic Protocols

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