



Application Brief: Tumor Microenvironment Imaging with Photoacoustic Technology

Executive Summary

Pharmaceutical development of therapeutic and imaging agents that improve tumor prognosis is an expensive R&D effort with many compounds in the preclinical testing pipeline (Olafsson et al., 2010). Introducing an efficient way to screen and reject unpromising compounds during the discovery research phase of development, such as *in vivo* preclinical imaging, could save some of the \$800 million in costs required to produce them, since a large portion of the cost is attributed to clinical trial expenditure (Agdeppa and Spilker, 2009).

High-frequency ultrasound enables the detection of anatomical information within mammalian systems, offering the ability to localize structures such as the tumor microenvironment. This minimally-invasive, longitudinal imaging process benefits cancer research by allowing identification and real-time monitoring of tumors within living tissues. This tool holds important diagnostic and therapeutic potential by permitting longitudinal studies of tumor progression and regression. This is achieved through visualization of neovasculature development through the endogenous heme signal.

Photoacoustics (PA) combines optical contrast with the high spatial resolution and deep tissue penetration offered by ultrasound. The linear transducer array technology enables the co-registration of images along a linear plane with both modalities, in order to study both anatomical structures and functional parameters within them. Photoacoustics obtains optical contrast from biological tissues by thermoelastic expansion upon illumination with a tunable laser. The expansion creates an ultrasonic wave, which is then detected by an ultrasound transducer. The tunable laser system enables characterization of tissue and contrast agents, such as nanoparticles. Such applications are especially beneficial for monitoring tumor development, measuring blood concentration changes within it, and quantifying networks of vasculature formation and carcinoma growth over time (Siphanto et al., 2005). Furthermore, the endogenous contrast provided by hemoglobin allows for label-free subcutaneous and cortical vascular imaging (Hu et al., 2010). To obtain even higher than natural contrast levels, photoacoustics is compatible with near IR-absorbing dyes and particles, and is capable of reaching contrast levels up to 10-fold greater than blood (Olafsson et al., 2010; Pan et al., 2010).

Combining photoacoustic technology with high-resolution ultrasound projections offered by the Vevo 2100 system provides tremendous benefits for cancer screening. Researchers can now benefit from the combined high-resolution ultrasound and optical contrast ability of the Vevo[®] LAZR photoacoustic imaging system to achieve clear, deep, images in 2D and 3D for optimal *in vivo* visualization and quantification of internal anatomy, tumor tissue, and hemodynamics.



Background on Tumor Imaging with Photoacoustics: Importance of Insight into Oxygen Saturation, Vasculature and the Role of Sentinel Lymph Nodes

Oxygen Saturation

Oxygen saturation (sO_2), the percentage of oxygen molecules carried by hemoglobin, is an important parameter in identifying tumor presence. The lower oxygen content of tumors, also known as 'hypoxia', is useful for prognosis, pathophysiology and treatment, since it shows strong correlation with tumor growth, malignant progression, and therapy resistance (Lungu et al., 2006). Until recently, the only sO_2 measurement methods were via staining for glucose transporter-1 (Glut-1) expression or pulse oximetry. Glut-1 is a protein upregulated in response to hypoxia, as tumors show increased glucose uptake in comparison to normal tissue. Pulse oximetry detects light passing through a tissue, via a photosensor or catheter, based on the differential absorption characteristics of hemoglobin and deoxyhemoglobin, and gives an output ratio of transmitted red to infrared light at a specific anatomical point, such as an earlobe. Staining for hypoxic cells with the bioreductive drug marker, pimonidazole, provides an indirect method of detecting sO_2 (Coeur et al., 2007). Pimonidazole binds covalently to thiol-containing proteins within hypoxic cells, forming immunohistochemically-detectable intracellular adducts, but must be administered 16 hours prior to tumor excision (Coeur et al., 2007). These quantification methods, although useful, must often be performed collectively to obtain anatomical, functional and physiological information. Matching up these various pieces of information, obtained from various animals, can introduce inaccuracies in measuring sO_2 levels, physiological and functional information and anatomical localization.

Registering photoacoustic imaging with high-resolution anatomical imaging provides the ability to visualize, quantify and monitor vascular dynamics in the tumor microenvironment in real-time at a high frame rate in under a minute. Photoacoustics has been demonstrated to non-invasively image tumor oxygenation and determine the hypoxia status using the differential spectroscopic absorption between oxyhemoglobin and deoxyhemoglobin. This imaging modality can easily differentiate healthy, symmetrical vasculature from the irregular, distorted vasculature characteristic of tumor angiogenesis via the lowered sO_2 content residing in diseased tissue. While pimonidazole staining, Glut-1 staining and pulse oximetry give sO_2 readings at specific time points, photoacoustic imaging offered by the Vevo LAZR platform provides *in vivo*, real-time longitudinal data on tumor sO_2 levels.

Hypoxia occurs as a result of the chaotic and disorganized vascular network that develops in tumors, which impacts blood oxygen distribution. The role of neovascularization in atypical tissue development is another important prognostic indicator of tumor angiogenesis. Tumors are typically characterized by asymmetrical morphology and abnormal vascular network branching in the microcirculation, where gases, wastes, nutrients and thermal energy are exchanged (Hu et al., 2010). Modalities that have traditionally been used in vascular imaging include magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and ultrasound imaging. Optical microscopy, including intravital, confocal, two-photon, Doppler optical coherence tomography and orthogonal polarization spectral imaging are able to overcome the lower resolution and contrast inherent in MRI, CT, PET and ultrasound. However, they can also be invasive, lack depth, require contrast agents and lack the ability to perform longitudinal studies. To study vascular dynamics *in vivo* within the tumor microenvironment, there is a need for additional visualization tools to image both structure and function over time without injury to the animal. By utilizing the safe, endogenous heme signal as a contrast agent and therefore a measure of oxygenation, photoacoustics provides insight in to the hypoxia status of tumors in a real-time, non-invasive manner.



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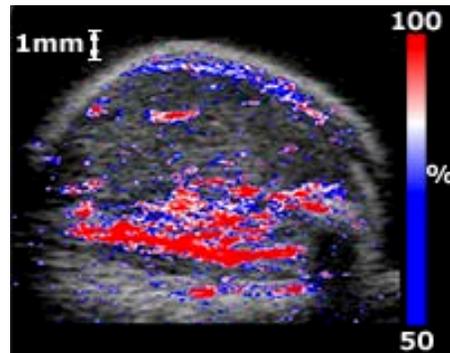


Figure 2 – Photoacoustic oxygenation saturation map superimposed on a B-Mode image of a subcutaneous tumor.

Sentinel Lymph Nodes

Sentinel lymph nodes (SLNs) are the first lymph nodes to receive metastatic cancer cell drainage from a tumor, and therefore play important roles in cancer staging and prognosis (McCormack et al., 2009). If evidence of circulating tumor cells can be found in SLNs, systemic therapies can be employed and axillary lymph node dissection can be avoided (Erpelding et al., 2010). An invasive biopsy, consisting of SLN identification and confirmation with radioactive colloids and blue dyes, resection, and histological assessment of excised SLNs, is the current staging method used to assess if tumor cells have migrated to the lymph bed (Erpelding et al., 2010; McCormack et al., 2009). However, this surgical procedure has revealed false positive results in 74.2% of breast cancer patients who underwent SLN resection, therefore creating a demand for a less invasive prognostic screening procedure (Krag et al., 2007).

Methylene blue, an optically-absorbing dye, has demonstrated its utility in accurately and non-invasively visualizing and mapping sentinel lymph nodes both in humans and animals *in vivo*, via co-registered photoacoustic and ultrasound imaging (Erpelding et al., 2010). By providing the ability to localize lymph nodes within surrounding anatomy, this imaging capability holds the potential to guide fine-needle aspiration biopsies of SLNs for axillary lymph node staging, as opposed to dissections, holding important clinical implications for breast cancer patients.

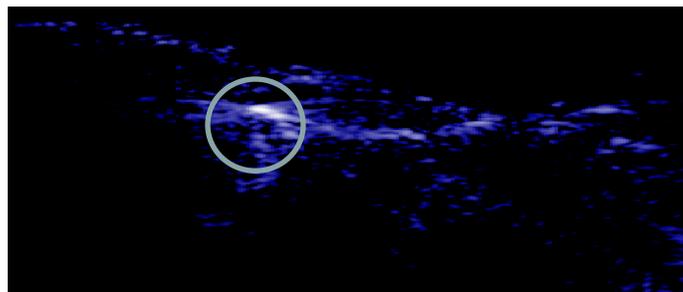


Figure 3 – Post-methylene blue injection 2D photoacoustic image of mouse axillary lymph node (identified in blue circle) at 680 nm.



Nanoparticle Detection and Quantification

Although intrinsic markers, such as hemoglobin in blood, can be used for contrast via photoacoustic imaging, many diseases do not exhibit endogenous contrast, creating a need for exogenous, signal-enhancing agents (De La Zerda et al., 2008). Typical examples of such contrast agents are dyes and nanoparticles. Nanoparticles are extremely versatile and have high biocompatibility, with easily tunable optical absorption (Chen et al., 2010). Although a plethora of nanoparticle types exist for various applications, those made from metals and other inorganic materials are the ones most suited to *in vivo* imaging. The properties of metals, such as gold nanoshells, are altered dramatically at the nano-level and render enhanced contrast through ultrasound and MRI imaging. Nanoparticles can be coupled to ligands or binding factors for proteins and antibodies expressed during tumor neovasculature (i.e. VEGFR2 protein) or various disease states, therefore allowing them to bind to specific tissues. Nanoparticles have been shown to enhance contrast up to 10-fold of that found naturally in blood (Pan et al., 2010) and the imaging agent further allows researchers to follow its progress for longitudinal studies of tumor formation.

There are various kinds of nanotechnology available for cancer-specific applications:

- **Gold nanorods (GNRs)** are non-targeted nanoparticles that accumulate at tumor sites and enhance the endogenous vascular signal at the peak absorption wavelength of 800 nm. GNRs absorb energy in the near-infrared range (NIR) and can emit it as heat to specifically kill tumors through a process called 'photodynamic therapy'.
- **Single-walled carbon nanotubes (SWNTs)** can be conjugated with specific targeting agents, such as alpha-5, beta-3 integrins, which are over-expressed in tumor vasculature, in order to detect the location and estimate the concentration of cancers *in vivo*.
- **Nanoshells** have a physical selectivity for cancer lesion sites based on their size, called 'enhanced permeation retention'. They can be conjugated with specific molecular agents for antigens expressed on cancer cell surface or the tumor microenvironment. Nanoshells can further absorb externally-applied energy, such as optical energy provided by a laser, selectively killing tumor cells by intense heat. Nanoshells have demonstrated safe injection into animal models, making it an important potential cancer therapeutic.
- **Nanoparticle-targeted molecular imaging** allows for visualization of cells and molecules that are otherwise undetectable through conventional imaging techniques. This feature gives researchers the ability to monitor therapeutic intervention *in vivo* inside the cell, as well as differentiate between apoptotic and activated cancer cells. Such applications are critical to successful treatment and diagnosis.



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Photoacoustic imaging achieved through the Vevo LAZR platform allows detection and quantification of nanoparticles *in vivo* and in 2D or 3D (see figures 4 and 5). No other imaging modality capable of investigating nanoparticles *in vivo* with a tunable laser for optimizing detection settings is currently available commercially. The Vevo LAZR photoacoustic imaging system represents a novel and advanced imaging modality for studying tumor microenvironment and therapeutic applications for cancer.

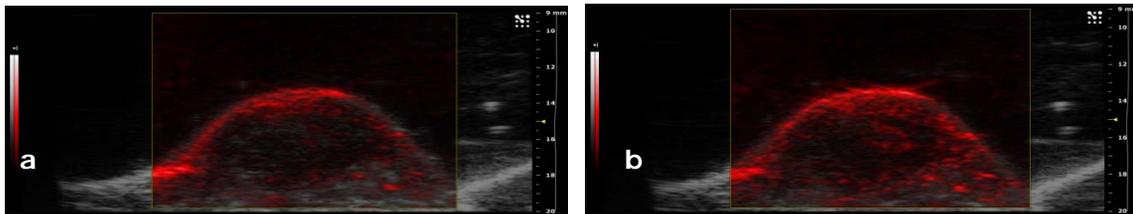


Figure 4 – (a) Pre- and (b) post-gold nanorod (Nanopartz) 2D photoacoustic image of subcutaneous mouse tumor at 800 nm.

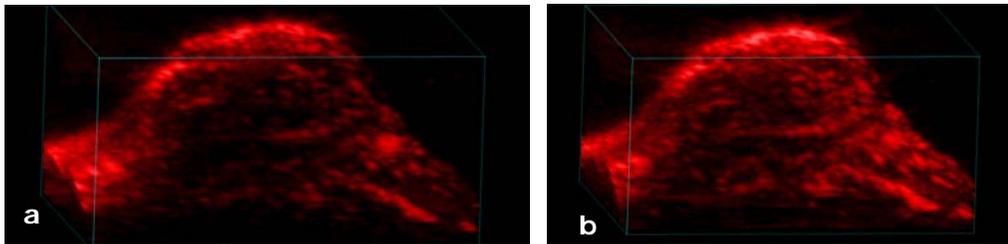


Figure 5 – (a) Pre- and (b) post-gold nanorod (Nanopartz) 3D photoacoustic image of subcutaneous mouse tumor at 800 nm.



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VisualSonics' Value Proposition

Tumor visualization and detection has in the past been limited to histological, immunocytochemical assays and molecular analysis. Although informative, these techniques have proven to be either very invasive, as in the case of SLN biopsies, or subject to human error, as attributed to generally non-reproducible histological assays. Furthermore, they cannot monitor disease progression in a quick real-time fashion.

Photoacoustics has the potential for a unique contribution to breast cancer therapy by reducing the morbidity associated with the 74.2% false positive outcomes from SLN biopsies, instead providing accurate, non-invasive detection and staging. Furthermore, it holds the capability of detecting circulating tumor cells via intrinsic contrast agents, such as melanin, present in cancer cells. Nanoparticles and exogenous contrast agents can help with the detection of other cellular attributes, such as the CD44 marker present on dissociated tumor cells.

Below is a summary of the unique value proposition VisualSonics delivers to researchers with the Vevo LAZR photoacoustic imaging system:

1. Inherent co-registration of photoacoustic and ultrasound signal for placing sensitive and specific signal into an anatomical and molecular context, and discerning function within morphology. The function is useful in early orthotopic tumor detection and detailed vascular dynamics studies within the tumor microenvironment.
2. High-resolution imaging allowing for visualization at the micro-scale – a critical parameter in small animal research to understand tumor microenvironment.
3. High specificity via the use of endogenous and exogenous contrast agents, cell-specific dyes, biomarker specific agents and nanoparticles.
4. Real-time visualization permits Image-guided procedures, such as fine-needle aspiration biopsy in SLNs, or implantation and subsequent monitoring of tumor cells.
5. Endogenous photoacoustic signal tools HemoMeaZure and OxyZated measure hemoglobin content and oxygen saturation, respectively, without the use of contrast agents.
6. Live 2D and 3D imaging for tumor morphology, vasculature mapping and oxygen saturation measurements.
7. Multispectral photoacoustic ('multiplexing') can be achieved with the NanoStepper tool, which allows laser wavelength control by the user and export of co-registered photoacoustic and anatomical information in digital RF format. This imaging capability increases the signal to noise ratio and is crucial for researchers studying multiple contrast agents simultaneously. It allows blood vessel differentiation from lymphatic tissue and identification of specific cell populations in the tumor microenvironment with targeted molecular agents.
8. Multiplexing of multiple sources in one image, such as contrast agents, gives the ability to image, for example both SLNs (via methylene blue) and metastatic cells inside the node.
9. LAZRTight™ small animal enclosure ensures optimal safety for the operator, and consists of physiological monitoring, integrated anesthesia, transducer mounting system, 3D image controller, and microinjection system.



References

- Agdeppa ED, and Spilker ME, "A review of imaging agent development," *AAPS J.* **11**(2), 286–299 (2009).
- Chen LC, Wei CW, Souris JS, Cheng SH, Chen CT, Yang CS, Li PC, Lo LW. Enhanced photoacoustic stability of gold nanorods by silica matrix confinement. *J Biomed Opt.* 2010 Jan-Feb;15(1):016010.
- Coer A, Legan M, Stiblar-Martincic D, Cemazar M and Sersa G. Comparison of two hypoxic markers: pimonidazole and glucose transporter 1 (Glut-1). **IFMBE Proceedings**, 2007, Volume 16, Part 13, 465-468.
- De la Zerda A, Zavaleta C, Keren S, Vaithilingam S, Bodapati S, Liu Z, Levi J, Smith BR, Ma TJ, Oralkan O, Cheng Z, Chen X, Dai H, Khuri-Yakub BT, Gambhir SS. Carbon nanotubes as photoacoustic molecular imaging agents in living mice. **Nat Nanotechnol.** 2008 Sep;3(9):557-62. Epub 2008 Aug 17.
- Erpelding TN, Kim C, Pramanik M, Jankovic L, Maslov K, Guo Z, Margenthaler JA, Pashley MD, Wang LV. Sentinel lymph nodes in the rat: noninvasive photoacoustic and US imaging with a clinical US system. **Radiology.** 2010 Jul;256(1):102-10.
- Hu S, Rao B, Maslov K, Wang LV. Label-free photoacoustic ophthalmic angiography. **Opt Lett.** 2010 Jan 1;35(1):1-3.
- Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Ashikaga T, Weaver DL, Miller BJ, Jalovec LM, Frazier TG, Noyes RD, Robidoux A, Scarth HM, Mammolito DM, McCready DR, Mamounas EP, Costantino JP, Wolmark N; National Surgical Adjuvant Breast and Bowel Project. Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial. **Lancet Oncol.** 2007 Oct;8(10):881-8.
- Lungu GF, Li ML, Xie X, Wang LV, Stoica G. In vivo imaging and characterization of hypoxia-induced neovascularization and tumor invasion. **Int J Oncol.** 2007;30:45–54.
- McCormack D, Al-Shaer M, Goldschmidt BS, Dale PS, Henry C, Papageorgio C, Bhattacharyya K, Viator JA. Photoacoustic detection of melanoma micrometastasis in sentinel lymph nodes. **J Biomech Eng.** 2009 Jul;131(7):074519.
- Olafsson R, Bauer DR, Montilla LG, Witte RS. Real-time, contrast enhanced photoacoustic imaging of cancer in a mouse window chamber. *Opt Express.* 2010 Aug 30;18(18):18625-32.
- Pan D., Pramanik M., Senpan A., Allen J., Zhang H., Wickline, SA., Wang LV., and Lanza GM. "Molecular photoacoustic imaging of angiogenesis with integrin-targeted gold nanobeacons." **FASEB J**, Nov 2010; 10.1096