Application Note

Application of the VevoCQ™ Software in Cancer Research:

An overview of quantification tools for perfusion kinetics and biomarker analysis

VISUALSONICS

Introduction

Angiogenesis, the process of new blood vessel growth from existing vessels, is a widely studied process and is aggressively studied in cancer research; additionally tumor perfusion is a target for potential therapeutics. In vivo assessment of both perfusion kinetics and expression of endothelial cell surface receptors, such as vascular endothelial growth factor receptor 2 (VEGFR2), would be ideal for most cancer research experimental protocols, especially if it could be accomplished non-invasively, rapidly and on a wide variety of animal models.

To study the development of microvasculature in vivo contrast agents are required when using imaging techniques such as high-frequency ultrasound. Recent development of nonlinear contrast imaging on the Vevo® 2100 micro-ultrasound imaging system provides a highly sensitive contrast imaging technique and with the release of the VevoCQ™ advanced contrast software analysis tool, perfusion kinetics as well as late phase targeted enhancement can now easily be quantified and color-coded parametric images allow for visualization of the spatial distribution of the derived parameters.

Ultrasound imaging is a non-invasive, real-time imaging technique which allows for longitudinal studies on the same animal. The Vevo 2100 high-resolution ultrasound imaging system provides axial resolution down to 30 µm; this type of resolution allows for the detection of tumorigenesis well before the lesion is palpable. MicroMarkerTM Contrast Agents can be used to assess vascularity and molecular expression of specific targets in vessels down to the capillary level.

The goal of this application note is to show the utility of the VevoCQ software analysis tool in cancer research applications, as a means of assessing tumor perfusion and the expression of various endothelial cell markers (VEGFR2, integrins…) in a subcutaneous tumor model.

Materials & Methods

The Vevo 2100 High-Resolution Ultrasound Imaging System (VisualSonics Inc, Toronto, Canada) was used to acquire all images. Micromarker Contrast Agents, Non-targeted and Target-Ready were used as in previously described VisualSonics protocols (PR_2100_Cb_NLC_for_ bolus_in_the_tumors_ver1.0, PR_2100_NA_NLC_ for_detection_of_biomarkers_ver1.0).

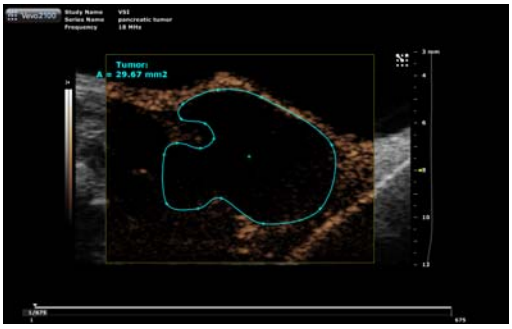
Images were acquired from mice implanted with tumor cells implanted subdermally in the hind limb 4 to 5 weeks before imaging; tumors used to study tumor perfusion were of a pancreatic carcinoma cell line, while tumors used to study VEGFR2 expression were from a hepatocarcinoma cell line.

Quantification of Tumor Perfusion

Non-Targeted MicroMarker Contrast Agents are used to enhance the visualization of blood flow down to the capillary level. They are injected i.v., typically through the tail vein and circulate freely through functional blood vessels.

The microbubbles are 2-3 µm in size and are made up of a phospholipid shell containing a polyethylene glycol outer shell, along with a perfluorobutane/nitrogen gas core.

A bolus injection of the Non-Targeted MicroMarker Contrast Agents allows for the visualization of the microvasculature within the tumor (Images courtesy of Dr. J Lazar, SUNY Downstate Medical Center, 2010); Figure 1 shows a time course of the bolus injection from the beginning where no contrast agent was present, through approximately 16 seconds after the bolus arrival at the tumor location.



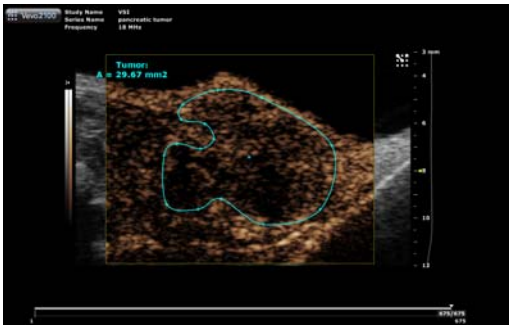


Figure 1 – Bolus injection of Non-Targeted MicroMarker Contrast Agents into subcutaneous tumor model; prior to injection (a) no signal was apparent within the tumor, however approximately 16 seconds after the arrival of the bolus in the tumor (b) there is significant contrast signal within the tumor

The VevoCQ software is used to quantify the contrast uptake kinetics as well as to visualize the spatial distribution of the various perfusion parameters as color-coded parametric images for qualitative analysis. Figure 2 displays the location of the four user defined regions of interest for the analysis preformed on this subcutaneous tumor, while Figure 3 shows the result of applying the curve fit algorithm to the generated echo-power data as a function of time for each region of interest.

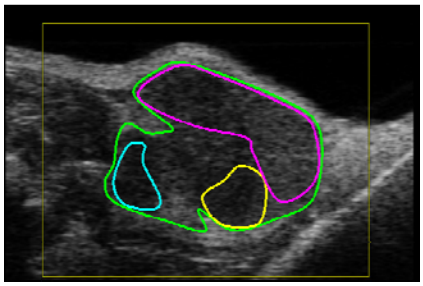


Figure 2 – Location of the four user defined regions of interest on the subcutaneous tumor

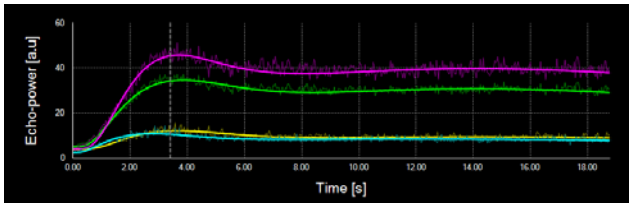


Figure 3 – Bolus perfusion model curve fit algorithm as applied to each region of interest on the subcutaneous tumor model

From the bolus perfusion model curve fit algorithm (Figure 4) various parameters are calculated for each region of interest, a selection of these parameters are shown in Table 1.

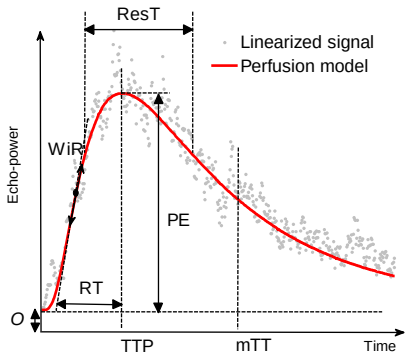


Figure 4 – Bolus perfusion model curve fit algorithm showing various calculated parameters; Rise Time (RT), Time to Peak (TTP), Wash-in Rate (WiR), Peak Enhancement (PE)

Table 1 – Selection of VevoCQ bolus perfusion model parametric outputs

	Whole tumor (green)	**Bottom right (yellow)**	**Upper area (pink)**	**Bottom left (turquoise)**
Peak Enhancement (a.u.)	29.61	8.21	41.73	8.32
Time to Peak (sec)	3.84	3.66	3.70	2.78
Wash-in Rate (a.u.)	15.50	4.51	22.56	6.09

Peak enhancement is a measure of relative blood volume, as long as one pays particular attention to contrast agent dose and system acquisition settings during image acquisition, while time to peak is an absolute time measurement representing filling kinetics and wash-in rate is the maximum slope of the curve fit function and therefore take into account both amplitude and time and is a measure of relative blood flow. Each one of these parameters is also displayed as a color-coded parametric image to allow for a visual representation of spatial distribution within the tissue of interest. Figure 5 provides these images for each one of the selected parameters.

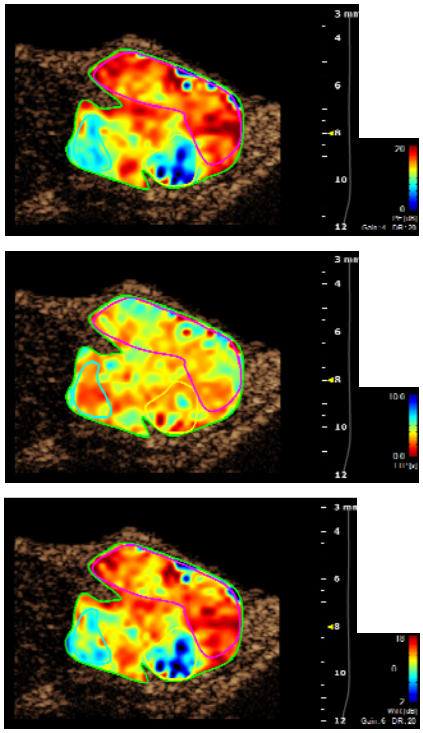


Figure 5 – Parametric images from bolus perfusion model (a) peak enhancement, (b) time to peak, (c) wash-in rate

Taken together the results from the VevoCQ software show that in this tumor there is heterogeneity in all perfusion parameters examined, especially peak enhancement and wash-in rate, measures of relative blood volume and flow respectively. The upper portion (outlined in pink throughout this analysis) of this tumor was found to have much higher perfusion compared to the bottom left (outlined in turquoise throughout this analysis) during this imaging session. This imaging was however completed non-invasively so that the same tumor could be studied over the course of a longitudinal study, as such it could act as it's own control for analysis purposes, lending to increased power in statistical analysis and strength of the overall study data set.

Quantification of Endothelial Cell Markers such as VEGFR2

Target-Ready MicroMarker Contrast Agents can be conjugated to VEGFR2 antibodies to allow for the assessment of gene expression levels by late phase differential target enhancement.

Target-Ready MicroMarker Contrast Agents are similar in structure to the Non-Targeted MicroMarker Contrast Agents, however there is a streptavidin molecule attached to the polyethylene glycol (PEG) molecule which makes up the outer most layer of the shell. This molecule allows for the conjugation of any biotinylated molecule to the microbubble. The conjugated antibody, for example, can then bind to it's ligand on the surface of endothelial cells. A negative control, in the form of an isotype control antibody, is utilized to allow for the quantification of any non-specific binding of the contrast agent.

In this subcutaneous tumor model both VEGFR2 and isotype control antibody conjugated contrast agents were utilized. Figure 6 shows images of late phase targeted enhancement prior to destruction where the VEGFR2 conjugated contrast agent can be seen bound to this cell surface receptor in the tumor. Following a destruction pulse (necessary to clear the bound microbubbles in the imaging plane) a decrease in the contrast signal is apparent.

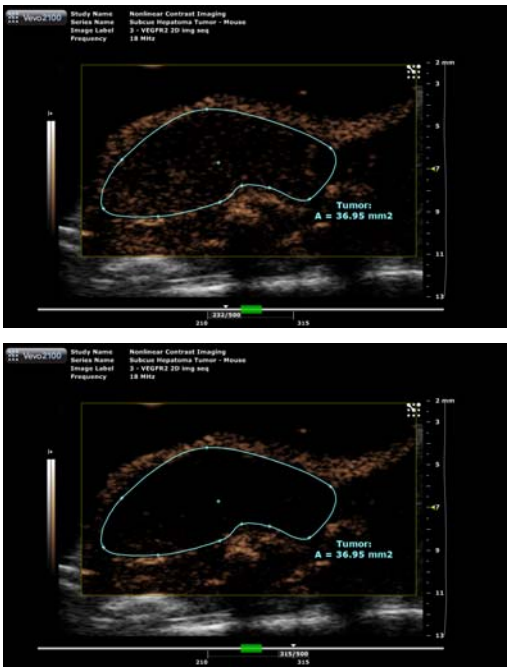


Figure 6 – Late phase targeted enhancement of VEGR2 conjugated Target-Ready MicroMarker Contrast Agents in a subcutaneous tumor model; prior to a destructive pulse being applied the bound contrast agent can be visualized (a) after which a decrease in the contrast signal is apparent (b)

Figure 7 shows the regions of interest used for analysis within the VevoCQ software, various areas were selected based on expression patterns of VEGFR2 in the entire tumor; while Figure 8 displays the destruction curve as applied to the echo power data as a function of time for the VEGFR2 data; the same analysis was completed using an isotype control antibody, to assess the level of non-specific binding in this model.

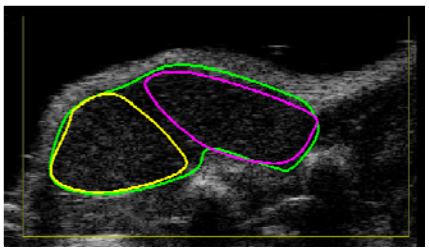


Figure 7 - Location of the three user defined regions of interest on the tumor

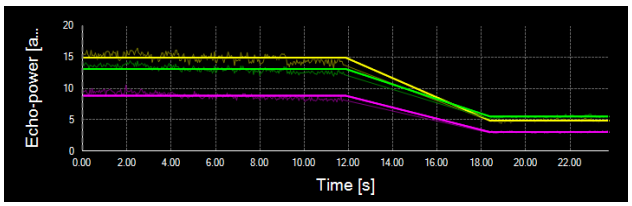


Figure 8 – Destruction curve as applied to each region of interest in the tumor

From the destruction curves the differential targeted enhancement was calculated (Figure 9) for each region of interest for both the VEGFR2 and isotype control antibodies; these are shown in Table 3.

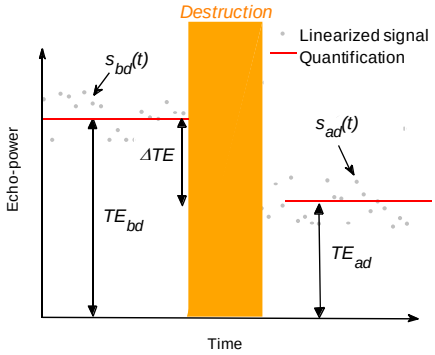


Figure 9 – Differential targeted enhancement model showing various parameters; TE_{bd} and TE_{ad} refer the targeted enhancement before and after destruction respectively and s is the linearized signal, while ΔTE is the differential targeted enhancement

Table 3 – Differential Targeted Enhancement (a.u.) in the subcutaneous tumor model

	Whole tumor (green)	**Left area (yellow)**	**Right area (pink)**
VEGFR2	7.56	10.00	5.79
Isotype Control	1.47	2.08	1.08

A color-coded parametric image allows for a visual representation of spatial distribution of the bound contrast agent bubble in this example. Figure 10 provides these images for both the VEGFR2 and isotype control conjugated microbubbles.

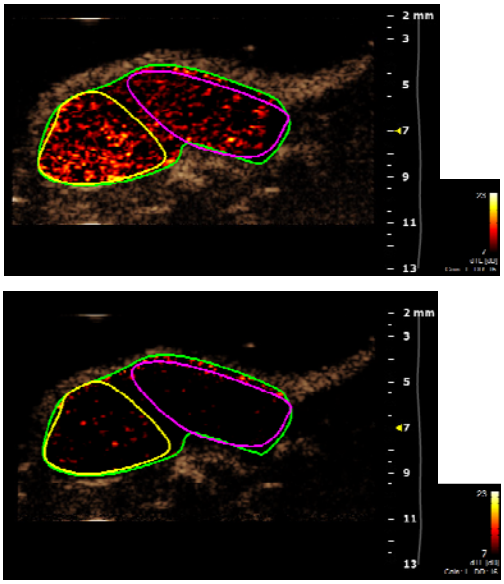


Figure 10 – Parametric images of the differential targeted enhancement for both the VEGF2 (a) conjugated Target-Ready MicroMarker Contrast Agent and the isotype control (b) conjugated agent

Here the differential targeted enhancement values as well as the parametric images clearly show the expression levels of VEGFR2 in this tumor model are enhanced on the left area of the tumor compared to those levels observed on the right.

In this example again all of the imaging was preformed non-invasively such that the same animal could be used over the course of a longitudinal study. One possible scenario could be testing the effect of a therapeutic drug on the expression of VEGFR2; again using the same animal as it's own control in a study data set lends power to the final outcome while reducing the number of animals necessary to complete the experimental design.

Conclusions

The images and techniques described here clearly show the utility of the VevoCQ software as well as the Vevo 2100 High-Resolution Ultrasound Imaging System as a tool for in vivo imaging and quantification of tumor perfusion and VEGFR2 expression in cancer research. The non-invasive nature of ultrasound imaging allows the same tumor to be studied over the course of an experiment, leading to much stronger data and requiring fewer animals to get significant results.

Recommended Papers

O'Connor, JPB, RAD Carano, AR Clamp, J Ross, et al. Quantifying Antivascular Effects of Monoclonal Antibodies to Vascular Endothelial Growth Factor: Insights from Imaging. Clin Cancer Res 15(21):6674-82, 2009.

Foster, FS, J Mehi, M Lukacs, D Hirson, et al. A New 15-50 MHz Array-Based Micro-Ultrasound Scanner for Preclinical Imaging. Ultrasound Med Biol 35(10):1700-8, 2009.

Olive, KP, MA Jacobetz, CJ Davidson, A Gopinathan, et al. Inhibition of Hedgehog Signaling Enhances Delivery of Chemotherapy in a Mouse Model of Pancreatic Cancer. Science 324(5933):1457-61, 2009.

Bagi, CM, J Christensen, DP Cohen, WG Roberts, et al. Sunitinib and PF-562,271 (FAK/Pyk2 inhibitor) Effectively Block Growth and Recovery of Human Hepatocellular Carcinoma in a Rat Xenograft Model. Cancer Biol Ther 8(9):856-65, 2009.

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