Application Note

Quantification of Tumor Angiogenesis using High Frequency Ultrasound

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. There are numerous pathways involved in angiogenesis, one of the most commonly referred to pathway involves vascular endothelial growth factor (VEGF) and its receptors. Numerous potential therapeutics or experimental procedures are being tested for their ability to alter the angiogenic pathways crucial to the growth of tumors. There are various types of tumor models which are of interest, including transgenic, orthotopic, and subcutaneous, all of which are well suited for imaging using high frequency ultrasound.

Ultrasound imaging is a non-invasive, real-time imaging technique which allows for longitudinal studies on the same animal. Imaging sessions provide anatomical and functional data, and with the use of contrast agents molecular data as well. Imaging sessions are preformed rapidly, allowing for short anesthesia times and quick data acquisition.

The Vevo® 2100 High Resolution Ultrasound Imaging system, using linear array technology for the transducer design, provides axial resolution down to 30um; this type of resolution would allow for the detection of tumorigenesis well before the lesion is palpable. There are various Doppler imaging techniques available on the Vevo 2100 which allow for detection and quantification of vascularity in vessels larger than 30um. MicroMarker™ Contrast Agents can be used to assess vascularity in vessels down to the capillary level, while Target-Ready MicroMarker Contrast Agents can be conjugated to various antibodies to detect the expression of cell surface markers such as Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) which are involved in angiogenic pathways. Other vascular cell surface markers can be studied, such as integrins and CD31, which may be relevant to cancer research.

Ultrasound imaging is non-invasive therefore the same tumor can be studied over the course of an experiment, leading to much stronger data and requiring fewer animals to get significant results. The MicroMarker contrast agents are administered through the tail vein, or any other venous access point, and can be used throughout the course of a longitudinal study.

The goal of this poster is to show the utility of the Vevo 2100 in tumor angiogenesis imaging, images will be shown from a subcutaneous tumor model, however all techniques are applicable to orthotopic and transgenic tumor models. The various Doppler imaging modes will be explained, as will the use of MicroMarker Contrast agents.

Materials and Methods:

The Vevo 2100 High Resolution Ultrasound Imaging System (VisualSonics Inc, Toronto, Canada) was used to acquire all images. The MS-550D (center operating frequency of 40MHz, axial resolution 40um) probe was used to acquire all images.

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 animal.

Images were acquired from athymic nude mice implanted with 5x105 MeWo human melanoma cells (ATCC; Manassas, VA) subdermally in the hindlimb 3 to 4 weeks before imaging.

Doppler Imaging Modes:

Doppler imaging is used in ultrasound to detect the presence of blood flow and to evaluate direction and speed of flow. The most commonly used Doppler imaging modes in cancer research are Color Doppler and Power Doppler.

For additional information on the Doppler imaging modes please see the references listed at the end of this document.

Color Doppler

Color Doppler applies a color pixel where blood flow is detected; the specific color indicates direction of flow, blue is blood flowing away from the transducer while red is blood flowing towards

the transducer. Information on the mean velocity of blood flow is also provided on the image. Information is provided for blood vessels which are larger than the resolution of the ultrasound probe, in most cases that is larger than 30µm in size.

Color Doppler is typically used in cancer research to provide an overview of the vascular network within a tumor, and to give an indication of the direction of flow and the mean velocity.

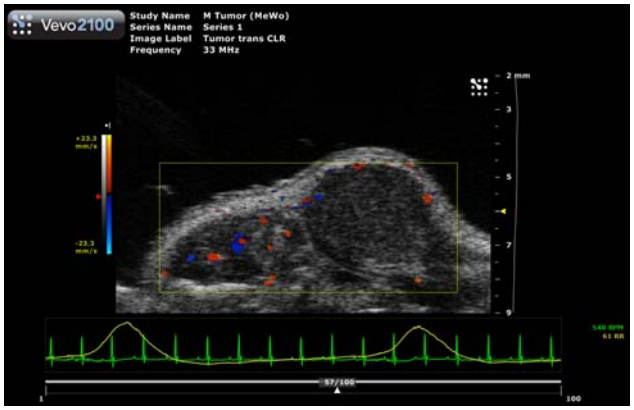

Figure 1 *– Color Doppler imaging of a MeWo tumor on the hind limb of a mouse. Blue indicates blood moving away from the ultrasound probe which is located at the top of the image, while red indicates blood moving towards the ultrasound probe. The region of interest is defined by the yellow color overlay box; while the mean velocity is located on the left hand side of the image, next to the color bars.*

Power Doppler

Power Doppler is similar to Color Doppler in that a region of interest box is drawn over the area where the overlay is to be shown. However the data in Power Doppler represents intensity of flow rather than direction of flow. This type of imaging can be done in 2D or 3D and the percent vascularization (PV) can be quantified. The PV represents the percentage of pixels within the defined area which have a Power Doppler signal associated with them, indicating the presence of blood flow. In 3D imaging a motor is used to translate the ultrasound probe over the complete area of the tumor. Information is provided for blood vessels larger than 30µm in size.

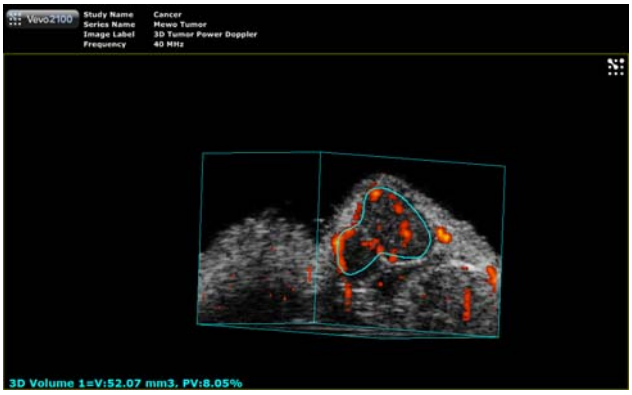

Figure 2 *– 3D Power Doppler imaging of a MeWo tumor on hind limb of mouse. The tumor volume and percent vascularity (PV) are quantified.*

MicroMarker Contrast Agents:

Non-Targeted MicroMarker Contrast Agents

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 through functional blood vessels.

The microbubbles are made up of a phospholipid shell containing a polyethylene glycol outer shell, along with a perfluorobutane/nitrogen gas core. The microbubbles are designed in such a way that the average diameter is 2-3µm in size, allowing for enhanced visualization of blood flow in vessels down to the capillary level.

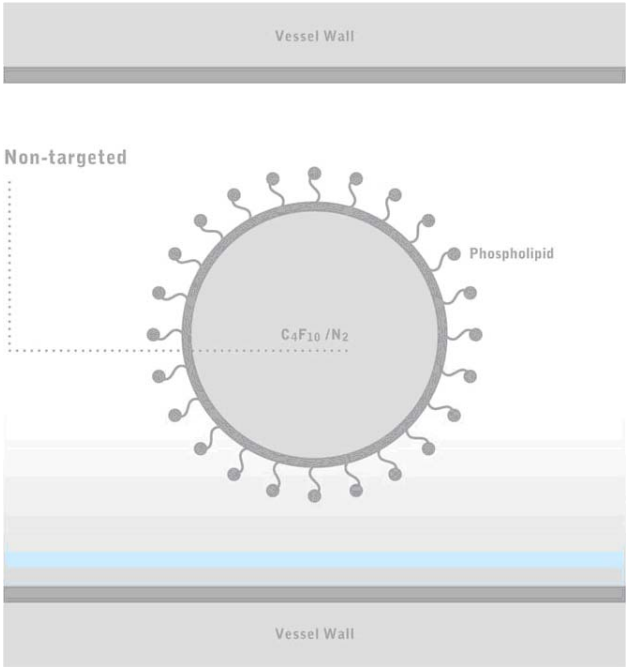

Figure 3 *– Non-Targeted MicroMarker Contrast Agent structure. Here the microbubble is shown within a blood vessel.*

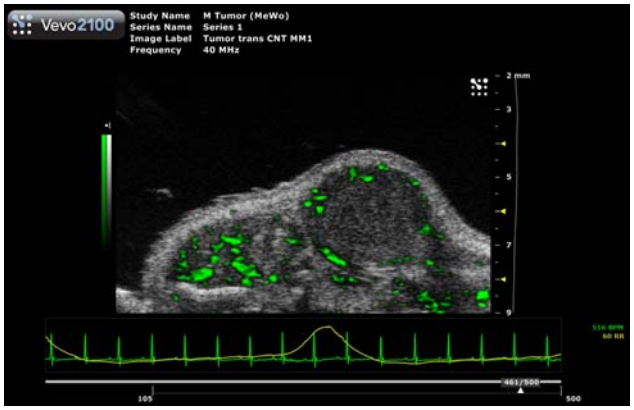

Figure 4 *– MicroMarker Contrast Agent imaging in a MeWo tumor on the hind limb of a mouse. The green contrast overlay is shown wherever the MicroMarker Contrast Agents are present enhancing the visualization of blood flow.*

The slope with which the contrast agents enter a specific region, and the plateau signal intensity they reach allow for quantification of the relative blood velocity (slope) and relative blood volume (plateau level) in a 2D area. If a region of interest is drawn around the tumor a wash-in curve is generated, the software allows for curve fit functionality, where the equation for the curve fit is[1]:

$$Y = A\ (1 - e^{-Bt})$$

Where A is the plateau value, representing the relative blood volume; and the B is the slope, representing the relative blood velocity; while Y is the contrast signal.

Figure 5 *– MicroMarker Contrast Agent bolus wash-in graph for the entire tumor volume. The curve fit functionality provides two values, A is the plateau value which is a measure of relative blood volume, and B is the slope of the curve which is a measure of relative blood velocity.*

The MicroMarker Contrast Agent imaging can also be done in 3D, where a motor is used to translate the ultrasound probe over the complete area of the tumor. This type of imaging provides a measure of relative blood volume for the entire tumor, detecting blood flow down to the capillary level. The relative blood volume is calculated as the percent agent (PA), which represents the percentage of pixels within the defined volume which have a contrast agent signal associated with them, indicating the presence of blood flow.

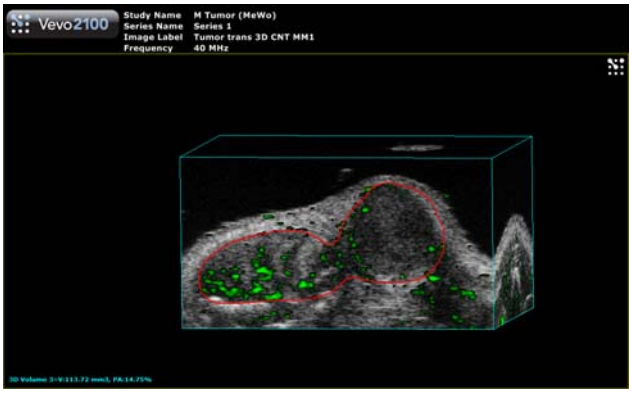

Figure 6 *– 3D MicroMarker Contrast Agent imaging in a MeWo tumor on the hind limb of a mouse. The percent agent (PA) is calculated and is a measure of relative blood volume.*

Target-Ready MicroMarker Contrast Agents

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 anything biotinylated to be conjugated to the microbubble, such as a primary antibody for vascular endothelial growth factor receptor 2 (VEGFR2). The conjugated antibody, for example, can then bind to it's ligand on the surface of the endothelial cells. This microbubble has an average diameter of 2-3um. Although VEGFR2 is used as an example here any antibody or ligand which would bind to a vascular cell surface marker can be bound to the Target-Ready MicroMarker Contrast Agent, i.e. integrins or CD31.

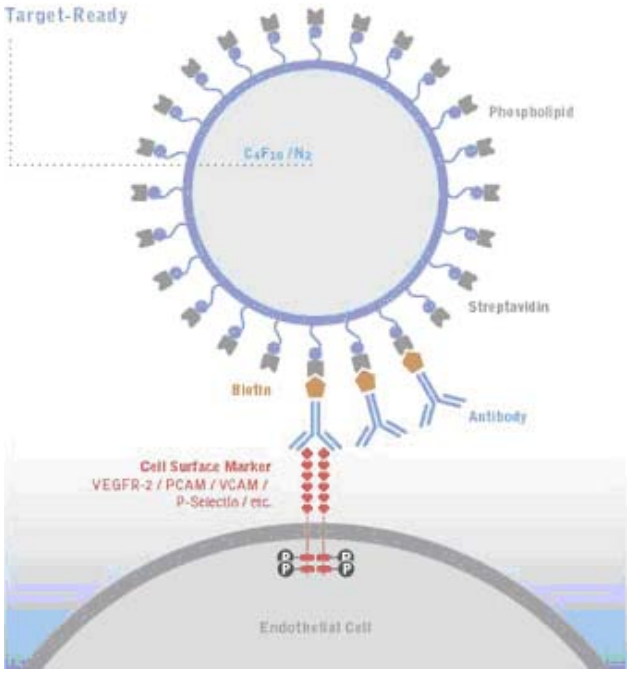

Figure 7 *– Target-Ready MicroMarker Contrast Agent structure.*

A negative control should be included in each experiment, such as an isotype control antibody conjugated microbubble, so that any non-specific binding can be measured. The quantification involves destruction of the bound contrast agent, and the amount of contrast agent bound in a given area of interest is represented by the change in contrast intensity. The relative expression of a particular marker, VEGFR2 for example, can be quantified using this technique; it is calculated by subtracting the non-specific signal (isotype control) from the specific signal (VEGFR2), the results are expressed as a change in contrast signal.

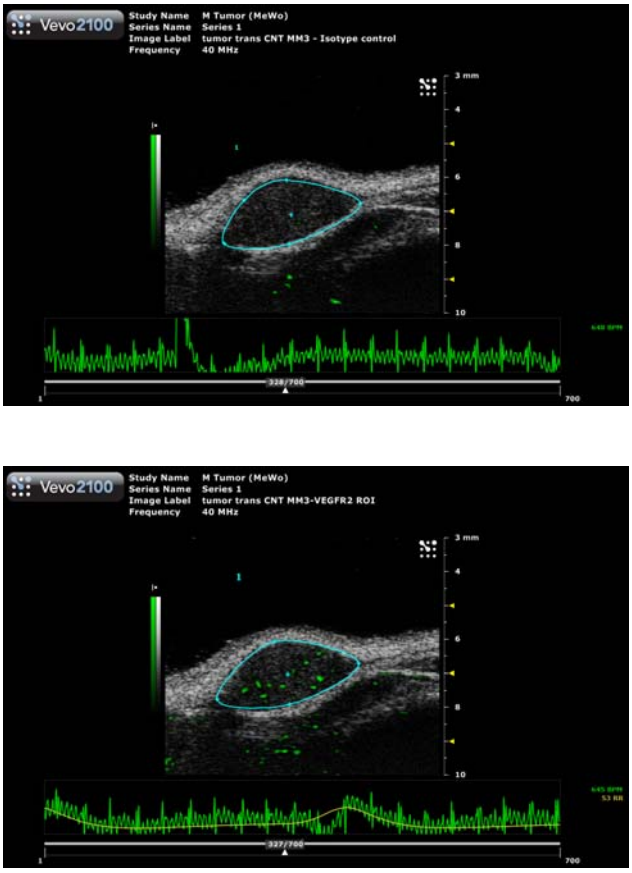

Figure 8 *– Target-Ready MicroMarker Contrast Agent imaging of a MeWo tumor on the hind limb of a mouse. Both Isotype Control (A) and VEGFR2 (B) conjugated microbubbles were imaged, the green contrast overlay shows the location of the bound microbubbles. A region of interest was drawn around the tumor and the amount of bound contrast agent was quantified as the change in contrast signal for each antibody(C).*

As with Non-Targeted MicroMarker Contrast Agent imaging 3D imaging can be preformed using the Target-Ready MicroMarker Contrast Agents to quantify the relative expression of a specific marker for an entire tumor volume. Similarly a percent agent (PA) value would be calculated, representing the amount of bound contrast agent. Again the non-specific binding (isotype control) value is subtracted from the specific (VEGFR2, for example) value, to give the relative expression of that marker in the entire tumor.

Conclusions:

The images presented here clearly show the utility of the Vevo 2100 High Resolution Ultrasound Imaging System as a tool for *in vivo* imaging and quantification of tumor angiogenesis. With the resolution of the Vevo 2100 pre-palpable tumors can be imaged and followed longitudinally throughout the course of a study. 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.

Color and Power Doppler provide the ability to study the changing vascularity of a tumor over time without the use of contrast agents. In addition, by using Non-Targeted MicroMarker Contrast Agents the imaging session provides information on the vascularity of the tumor down to the capillary level. Molecular information about the expression of certain markers can be obtained by using the Target-Ready MicroMarker Contrast Agents.

Additionally, all imaging is done *in vivo* and the vasculature is studied in real time allowing for quantification of actively perfused blood vessels. The MicroMarker Contrast Agents will only enhance the visualization of blood flow, or detect the expression of specific endothelial cell surface markers, in blood vessels which are actively conducting blood allowing the contrast agent to flow through the vessel. This becomes increasingly important when studying tumors as the vascular network of a tumor is disorganized. Vessels may be forming, and therefore counted using traditional techniques such as histology; however they are not actively perfused and are therefore not contributing to the growth or survival of the tumor.

The techniques discussed above allow for acquisition of real time data on actively perfused vessels, and therefore would easily allow for the study of acute or chronic changes in perfusion or expression of specific markers in response to a potential therapeutic or experimental procedure.

References:

[1] Wei, K, AR Jayaweera, S Firoozan, A Linka, et al. Quantification of Myocardial Blood Flow with Ultrasound-Induced Destruction of Microbubbles Administered as a Contrast Venous Infusion. *Circulation* 97(5):473-83, 1998.

Recommended Papers:

Molecular Imaging of Vascular Endothelial Growth Factor Receptor 2 Expression Using Targeted Contrast-Enhanced High-Frequency Ultrasonography.
Lyshchik, A, AC Fleischer, J Huamani, DE Hallahan, et al. J Ultrasound Med 26:1575-86, 2007.

US Imaging of Tumor Angiogenesis with Microbubbles Targeted to Vascular Endothelial Growth Factor Receptor Type 2 in Mice.
Willmann, JK, R Paulmurugan, K Chen, O Gheysens, et al. Radiology 246(2):508-18, 2008.

Functional Neoangiogenesis Imaging of Genetically Engineered Mouse Prostate Cancer Using Three-Dimensional Power Doppler Ultrasound.
Xuan, JW, M Bygraves, H Jiang, F Valiyeva, et al. Cancer Res 67(6):2830-9, 2007.

Recommended VisualSonics Protocols:

VisualSonics Vevo 2100 Imaging System, Operators Manual

Doppler Imaging

Vevo 2100 App Protocol – Imaging Using Color and Power Doppler (PN:12072)

Principles of Doppler: Pulsed-Wave Doppler, Color Doppler, Power Doppler

MicroMarker Contrast Agent Imaging

Vevo 2100 Application Protocol – 3D Imaging Using the Untargeted MicroMarker (PN:12073)

VisualSonics Application Protocol – Image Enhancement by Bolus Injection using Untargeted MicroMarker (PN:12083)

VisualSonics Application Protocol – Detection of Biomarkers using MicroMarker Target-Ready (PN:12076)

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