Complete microscale profiling of tumor microangiogenesis
A microradiological methodology reveals fundamental aspects of tumor angiogenesis and yields an array of quantitative parameters for its characterization

Chia-Chi Chien a,b, Ivan M. Kempson a, Cheng Liang Wang a, H.H. Chen a, Yeukuang Hwu a,b,c, N.Y. Chen d, T.K. Lee a, Cyril Petiboise e, Kelvin K.-C. Tsai f,g, Ming-Sheng Liu f, Kwang-Yu Chang f, C.S. Yang g, G. Margaritondo h,**

a Institute of Physics, Academia Sinica, Nankang, Taipei 115, Taiwan
b Department of Engineering and System Science, National Tsing Hua University, Hsinchu 300, Taiwan
c Institute of Optoelectronics Sciences, National Taiwan Ocean University, Keelung 202, Taiwan
d National Center for High-performance Computing, Hsinchu 300, Taiwan

Université de Bordeaux, CNRS UMR 5248-CBMN, F33405 Talence-Cedex, France
f National Institute of Cancer Research and Translational Center for Glandular Malignancies, National Health Research Institutes, Tainan 704, Taiwan
g Center for Nanomedicine, National Health Research Institutes, Miaoli 350, Taiwan
h Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

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A B S T R A C T
Complete profiling would substantially facilitate the fundamental understanding of tumor angiogenesis and of possible anti-angiogenesis cancer treatments. We developed an integrated synchrotron-based methodology with excellent performances: detection of very small vessels by high spatial resolution (~1 μm) and nanoparticle contrast enhancement, in vivo dynamics investigations with high temporal resolution (~1 ms), and three-dimensional quantitative morphology parametrization by computer tracing. The smallest (~3–10 μm) microvessels were found to constitute >80% of the tumor vasculature and exhibit many structural anomalies. Practical applications are presented, including vessel microanalysis in xenografted tumors, monitoring the effects of anti-angiogenetic agents and in vivo detection of tumor vascular rheological properties.

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1. Introduction
Early research on tumor angiogenesis dates back to the 1940s (Kerbel, 2000) and the proposal of therapeutic anti-angiogenesis to the early 1970s (Folkman, 1971). The importance of angiogenesis is widely recognized (Hanahan and Folkman, 1996; Hanahan and Weinberg, 2000), but fundamental aspects are still unclear and the issues about therapy are not solved. Methodology is a relevant hindrance: the ideal objective of a complete three-dimensional (3D) picture of microangiogenesis in vivo remained so far elusive. We present here a solution: an integrated approach based on synchrotron microradiology for submicron, real-time analysis and parameterization of tumor microvasculature.

This methodology provides excellent performances compared to current techniques, in terms of spatial resolution (MRI, PET and CT) or sensitivity (MRI) (McDonald and Choyke, 2003; Weissleder, 2002). Its resolution is much better than conventional radiology. Fast imaging (~1 ms per frame) avoids image blurring by live specimen movements. The analysis can be applied to large volumes (several cm³), i.e., entire tumors and organs.

The methodology also includes tomographic reconstruction—starting from many projection images—that allows 3D profiling (Less et al., 1991) of the microvascular network, down to the smallest vessels (<3 μm) (Hwu et al., 1999). Computer tracing extracts microvascular characteristics—length, diameter, branching number, angulation and morphological heterogeneities—fundamental in the tumor physiology and development (Palmowski et al., 2008). This 3D examination is a substantial advantage over two-dimensional (2D) shallow (Winkler et al., 2009) analysis by immunohistochemistry.

The methodology requires effective contrast agents. Hydrophobic, hydrophilic, micro-emulsified agents based on high-Z materials and nanoparticles were tested (Chien et al., 2010). Sub-μm BaSO4 particle solutions produced the best contrast without extravascular particle diffusion.
The orthotopic pancreatic tumorigenesis model (Fig. 2a–c) studies were performed by inoculating pancreatic carcinoma PANC1 cells with or without an identical number of RLT-PSCs (2 × 10^6 total cells in 100 μl 1:1 mixture of Matrigel and HBSS) into the parenchyma of the pancreatic body of immunodeficient NOD/SCID mice (NLAC). Three days prior to cell implantation, RLT-PSCs were irradiated with 8 Gy of radiation to functionally activate their matrix remodelling and tumor-promoting phenotypes (Jesnowski et al., 2005; Orimo et al., 2005). The mice were sacrificed at 3 days after cell implantation with surgical procedures similar to those for lung and liver cancer. For contrast agent infusion, the PE-10 catheter (BB31695/1, Scientific commodities, Inc., I.D.: 0.28 mm, O.D.: 0.64 mm) was inserted into the thoracic artery and secured by a 6-0 nylon ligature. BaSO₄ was then infused for 20 min with a flow rate of 10 μl/s. After perfusing the contrast agent, the cancerous tissues were removed and embedded in resin.

For the xenografted lung tumorigenesis model shown in Fig. 2d–f, A549 cells along with WI-38 pulmonary fibroblasts (1 × 10^6 cells in 100 μl 1:1 mixture of Matrigel and HBSS) were inoculated subcutaneously into the anterior thighs of NOD-SCID mice. The mice were given intraperitoneal injections of bevacizumab (10 mg/kg) or vehicle at the time of cell implantation and daily thereafter until sacrifice at day 3. The infusion of contrast agent followed the aforementioned procedures.

All the protocols for animal care and experiments related to this article were approved by the Institutional Animal Care and Use Committee (IACUC) of Academia Sinica and National Health Research Institutes, Taiwan.

### 2.4. Microradiology

The cancerous organs were removed and immersed in 0.9% normal saline solution for 8 h and dehydrated with a series of ethanol solutions (30, 50, 70, 90 and 100%); the immersion and dehydration procedures were repeated three times. Then, the specimen was exposed to a series of resin solutions mixed with ethanol (again 30, 50, 70, 90 and 100%) to replace ethanol with resin. The tissues were then kept in an oven at 70 °C for 8 h for EMBed 812 Resin polymerization.

Synchrotron microradiography (Hwu et al., 1999, 2004a, 2004b; Meuli et al., 2004) was implemented with unmonochromated (i.e., without filtering to select a narrow band of wavelengths) X-rays emitted by the O1-A beamline wavelength shifter of the National Synchrotron Radiation Research Center (Taiwan). The photon energy ranged from 4 keV to 30 keV and the average beam current in the synchrotron source accelerator was kept constant at 300 mA with the top-up operation mode. To obtain 4.59 × 3.43 mm images, the X-rays were first converted to visible light by a CdWQ₄ single crystal scintillator and then captured by an optical microscope with a CCD camera (model 211, Diagnostic instruments, 1600 × 1200 pixel). The radiation dose was reduced by attenuating the emitted X-ray beam with two pieces of 550 μm single crystalline silicon wafers placed before the animal.

The exposure time was ∼100 ms and the distance between the sample and the scintillator was ∼5 cm; a 2×, 5× or 10× lens in the optical microscope was used to obtain the desired field of view. The size of each pixel in the final image taken with the 2× lens was -2.87 × 2.86 μm^2. For microtomography reconstruction, specimens were fixed by paraformaldehyde and then embedded in resin as described above.

The conceptual details of synchrotron-based microradiology, including absorption and phase contrast, are discussed in Hwu et al. (1999, 2004a, 2004b; Meuli et al., 2004) and in the references therein.

### 2.5. 3D vessel tracing algorithm

The tracing algorithm was based on the following procedure: first, the image voxels were segmented into several connected clusters. A

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cluster is defined as “connected” if every voxel in this cluster is connected through at least one path of connections (two voxels are connected when they are in the 6 nearest neighboring site, i.e. $x \pm 1$, $y \pm 1$, or $z \pm 1$). Second, image voxels in a connected cluster were encoded based on the idea of region-growth method. Every voxels in this connected cluster was encoded by a number representing the sequential distance of the shortest path from the origin. A codelet $i$ was defined by voxels with coding number $i - 1$, $i$, $i + 1$ and its center of mass (CM) was calculated based on all these voxels. The tracing for one connected cluster was performed by simply following the codelets sequentially with increasing indices through all the encoded voxels. The skeleton of the image was obtained by connecting all the CMs of each codelet for a segment or branch. When a codelet had disconnected voxels, then it met the branches, i.e. there was a branch point (BP) there. When no further voxels could be traced, then the codelet met the end point (EP). When the codelet reached voxels that had been traced before, then it met a loop, i.e. there was a loop point (LP) there. Finally, after all were traced, the skeleton, BPs, EPs, LPs, cross section area, branch angles and other relevant quantitative parameters for the image could be calculated.

2.6. SOM text

The SOM presents additional images and movies illustrating details of the results of the methodology, essential to appreciate its effectiveness for the different tasks of complete profiling of tumor angiogenesis. The use of video files is particularly important due to the nature of the approach.

3. Results and discussion

We applied the methodology to four types of tumors: subcutaneous, lung, pancreas, and liver. Fig. 1(a–c) shows representative images of subcutaneous cancer taken at different times after injecting the BaSO$_4$ particle solution (Supporting Material S1). Note the fine details of the microvasculature network at an early tumor development stage. The contrast agent amount within the squares is ~86.3 μg for Fig. 1a and 182.3 and 13,498 μg for Fig. 1b and c (all three specimens contained the same total amount).

Fig. 1(d–f) are examples of 3D tomographic imaging: side views of a liver tumor with microangiogenesis confined to its volume (Supporting Material S2). One clearly sees the differences between the cancer microvasculature and the healthy tissue, in particular the microvessel size and density (Supporting Material S3) and the vascular structure.

Fig. 1g illustrates the extraction of essential 3D microvasculature parameters by ad hoc software. The result here is the vessel diameter distribution for the projection image of Fig. 1c.

Fig. 1h is a tomographic cross sectional view of pancreas cancer, with bright spots corresponding to strong contrast agent absorption. After zooming (right), vessels with ~3 μm diameter are clearly detected (these are not artifacts since they are tubular and connected to larger vessels). Thus, our resolution, ~1 μm and 3D analysis can reveal the smallest tumor vessels and measure their geometry for a complete profiling.

The quantitative analysis in 3D was performed with a computer algorithm using the complete data matrix to trace trees or dendritic structures based on the region-growth encoding and codelet with triple codes (Dellian et al., 1996). After tracing the vascular structure, this software automatically yields parameters such as the distributions of vessel sizes, vessel density and branching points (Fig. 1g and Supporting Material S4).

The methodology can detect tiny differences between very similar xenografted tumors, as shown by the analysis of stroma-mediated tumors (Kuperwasser et al., 2004; Orimo et al., 2005) in Fig. 2 (and Supporting Material S5). Pancreatic carcinoma PANC1 cells and pancreatic stromal stellate cells (RLT-PSCs)—activated by ionizing radiation (Jesnowski et al., 2005) (RAS specimen) or not irradiated (control (Ctrl) specimen)—were orthotopically co-implanted into the pancreatic parenchyma. Compared to the normal tissue of Fig. 2a, images taken 3 days after implantation, Fig. 2b and c, show the exuberant microvascular network surrounding the inoculation site.

Computer analysis (Supporting Material S6) unveils very small differences between the RAS and Ctrl specimens. Fig. 2d shows that ~61% and ~38% of the vessel segments (between branch points) for the Ctrl specimen have diameters <10 μm and <5 μm; the RAS specimen values are ~71% and ~46%. Fig. 2e shows that 72% (Ctrl) and 67% (RAS) of the vessels are shorter than 75 μm. Thus, the RAS specimen has shorter and narrower branches.

The vessel “tortuosity” is quantitatively described by the ratio of the vessel length between branch points to their distance. Fig. 2f shows that for the Ctrl and RAS specimens 25% and 23% of the
vessels has a ratio <1.5. The portion is 35% for normal tissue, thus the cancer microvasculature is substantially more tortuous than normal pancreas. Other extracted parameters are the total number of branch points, starting points, end points and loop points. The corresponding results are larger for RAS than for Ctrl by ~9.6%, ~17.8%, ~30.1% and ~9.9%.

The quantitative 3D analysis, like the imaging and the tomographic reconstruction, can be extended to whole tumors and entire organs. We find that the anomalous vasculature typically occupies ~3–4% of total tissue volume (Supporting Material S7).

The methodology is suitable to detect the impact of anti-angiogenesis agents like the (VEGF)-blocking agent bevacizumab (Zerbini et al., 2008). On a nude mice model, subcutaneous tumors induced by A549 lung cancer cells were analyzed with or without intraperitoneal administrations of bevacizumab. Retroviral labelling of the A549 cells with firefly luciferase visualized the tumor by bioluminescence imaging (BLI). Fig. 2f shows large angiogenic tumors near and within the BLI signal 3 days after cell inoculation.

Bevacizumab does significantly affect the observed angiogenesis (Fig. 2g) (Helmlinger et al., 1997; Jain, 1999). A computer analysis of tomography images, Fig. 2h and i, reveals indeed differences in the vasculature morphology (Supporting Material S8): the vessels are larger, longer and less tortuous after bevacizumab treatment.

The methodology can image in vivo the microvasculature flow dynamics in growing tumors (Elleaume et al., 2000; Hwu et al., 2004b; Kim et al., 2007; Myojin et al., 2007). Fig. 3 is a sequence revealing the contrast agent flow adjacent to a subcutaneously implanted tumor (the same as in Fig. 1c). We monitored the contrast agent accumulation with time in different tumor regions and the surrounding stroma through the pixel intensity of the tomographic images, corresponding to the X-ray absorption and proportional to the contrast agent amount. After injecting the agent, this intensity first increases (Fig. 3b and e and Supporting Material S9) and then saturates (Fig. 3f and g).

The contrast agent initially accumulates in a small stroma region (Fig. 3d, arrow). Note that some microvasculature areas are more...
Appendix A. Supplementary data

References


Fig. 3. Micrographs from an image sequence (50 s intervals) reveal the contrast agent flow in the microvasculature of a subcutaneously implanted tumor. The arrow in (c) marks a vessel filled with contrast agent later than the others, already visible in (b). The arrow in (g) marks a blood-pool-like area with high contrast agent accumulation (scale bar: 1 mm). (h) Reconstructed slice (0.924 mm×0.924 mm) of healthy mouse liver showing vessels with the incomplete contrast agents filling.

easily perfused by the blood stream than others—a valuable information for understanding the tumor mass viability (Sahai, 2005). One sees cell invasivity where vasculature is dense and functional and hypoxic areas where it is underdeveloped and/or less functional.

High magnification shows that the local contrast agent accumulation is not due to clustered vessels but to extravasation (Roberts and Palade, 1997), likely related to the altered permeability of tumor microvessels—and to the well known capillary fenestration after strong angiogenic stress in tumor areas (Hobbs et al., 1998). We do not observe accumulation in normal tissues. The methodology thus identifies tumor areas where fenestration occurs, which is considered a major facilitator of tumor development (Roberts and Palade, 1997) and potentially of targeted drug delivery (Deeken and Loscher, 2007).

Finally, the methodology can also detect unfilled microvessels. In this case (Fig. 3h), phase contrast (Hwu et al., 2004a, 2004b; Meuli et al., 2004) modulates the intensity at the vessel boundary delineating the walls—prevailing over absorption contrast. This facilitates, for example, the detection of circulation dynamics inhomogeneities.

In essence, complete profiling of tumor microangiogenesis requires 2D and 3D imaging down to micron-size vessels and over entire tumors, evaluation of quantitative parameters characterizing the microvessel network and time-resolved analysis of the anomalous blood dynamics. The methodology here described fully meets all of these requirements. It can thus be used to clarify the microvascular aspects of tumor growth at early and advanced stages, and its possible exploitation for therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.biotechadv.2011.12.001.


