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Three-dimensional imaging of xenograft tumors using optical computed and emission tomography

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Abstract

The physical basis and preliminary applications of optical computed tomography (optical-CT) and optical emission computed tomography (optical-ECT) are introduced, as new techniques with potential to provide unique 3D information on a variety of aspects of tumor structure and function. A particular focus here is imaging tumor micro-vasculature, and the spatial distribution of viable tumor cells, although the techniques have the potential for much wider application. The principle attractiveness of optical-CT and optical-ECT are that high resolution ($<20\ \mu\text{m}$) and high contrast co-registered 3D images of structure and function can be acquired for relatively large intact samples. The unique combination of high contrast and resolution offers advantages over micro-CT and micro-MRI, and the lack of requirement for sectioning offers advantages over confocal microscopy, conventional microscopy, and histological sectioning techniques. Optical-CT/ECT are implemented using in-house custom apparatus and a commercial dissecting microscope capable of both transmission and fluorescence imaging. Basic studies to characterize imaging performance are presented. Negligible geometrical distortion and accurate reconstruction of relative attenuation coefficients was observed. Optical-CT and optical-ECT are investigated here by application to high resolution imaging of HCT116 xenograft tumors, about 1 cc in dimension, which were transfected with constitutive red fluorescent protein (RFP). Tumor microvasculature was stained *in vivo* by tail vein injection of either passive absorbing dyes or active fluorescent markers (FITC conjugated lectin). Prior to imaging, the tumors were removed (*ex vivo*) and optically cleared in a key process to make the samples amenable to light transmission. The cleared tumors were imaged in three modes (i) optical-CT to image the 3D distribution of microvasculature as indicated by absorbing dye, (ii) optical-ECT using the FITC excitation and emission filter set, to determine microvasculature as indicated by lectin-endothelial binding, and (iii) optical-ECT using the DSR2 filter set to determine the 3D distribution of viable tumor as indicated by RFP emission. A clear correlation was observed between the independent vasculature imaging modes (i) and (ii) and postimaging histo-logical sections, providing substantial validation of the optical-CT and optical-ECT techniques. Strong correlation was also observed between the RFP imaging of mode iii, and modes i and ii, supporting the intuitive conclusion that well-perfused regions contain significant viable tumor. In summary, optical-CT and optical-ECT, when combined with new optical clearing techniques, represent

powerful new imaging modalities with potential for providing unique information on the structure and function of tumors.

Keywords

optical computed tomography; emission tomography; fluorescent imaging; 3D imaging; OPT; projection tomography; tissue imaging; optical-CT; tissue clearing; microvasculature

I. INTRODUCTION

Optical-CT can be conceived as the optical analog of x-ray CT. Three-dimensional (3D) images of the distribution of optical attenuation throughout a sample were reconstructed from optical projection images of light transmitted through the sample. Optical-ECT is conceived as the optical analog of SPECT (single photon emission tomography). In optical-ECT, 3D images of the distribution of emitting light sources (e.g., fluorochromes) are reconstructed from emission images of light emitted from the sample. Some of the well-established benefits associated with combining image data from complimentary modalities, such as x-rayCT/SPECT or x-ray/PET, translate over to the optical-CT/optical-ECT combination. These include the facility for accurate registration of transmission and emission image data, and the potential for relating tissue structure and function as demonstrated here in relation to xenograft tumor imaging. A significant difference between the optical and x-ray modalities is that SPECT/x-rayCT are *in vivo* technologies. The poor optical transmission of biological tissue, however, necessitates *ex vivo* sample preparation for successful optical-CT/optical-ECT imaging. Despite this apparent limitation, accurate “*in vivo*” functional information may be entirely feasible because optical stains and fluorescent markers can be applied *in vivo*, to a live animal/subject, such that representative staining is achieved for subsequent imaging by optical-CT/optical-ECT.

In previous work optical-CT has been developed in the context of high-resolution 3D dosimetry for verification of radiation therapy treatments.¹⁻⁷ In Ref. 3 a “macroscopic” scanner was built to perform optical-CT on samples with size ranging from 2 to 20 cm in diameter. This scanner was a first generation system, in that samples were scanned in a rastering manner by a single laser beam. The first generation systems had the principle advantage of accuracy, because scattered light can be effectively prevented from reaching the detector. The rastering process of data acquisition was slow, however, and the large laser spot-size limited spatial resolution to ~750 μm , too low to visualize tumor microvasculature. Techniques were developed^{4,5} to minimize potential artifacts in optical-CT arising from light reflection/refraction and these efforts continue.^{8,9} Second generation CCD “area-detector” optical-CT systems have been developed by several groups.^{7,10,11} Sharpe *et al.* implemented optical-CT for small optically cleared embryos, extended the technique to include emission tomography, and demonstrated applications in developmental embryology and embryonic gene expression.^{11,12} Here we develop this direction and present a novel implementation and application of optical-CT and optical-ECT to imaging tumor microvasculature and gene expression. Optical-CT/ECT are implemented on a standard dissecting microscope and independently on a bench-top system (the latter to be reported in a subsequent publication).³⁵ Optical clearing of tissue is a prerequisite for these techniques, unless projections data can be acquired of unscattered early arriving photons.¹³

The high sensitivity and resolution of CCD based optical detection technologies may enable optical-CT/ECT to address an “imaging gap” between the relatively low-medium resolution of clinical systems (x-ray-CT, SPECT, MRI, and PET) and the very high-resolution cellular imaging techniques of photo-microscopy, confocal microscopy, and optical-coherence-tomography. The former clinical imaging modalities have yielded revolutionary insight into

both anatomy and function of live tissue. Present challenges that are difficult to adequately address with these modalities include high-resolution 3D imaging of tumor microvasculature, viable tumor cell-distribution, and gene expression. Optical-CT/Optical-ECT has potential to address these challenges. Practically, the cost of producing and detecting visible light is vastly less than the costs of producing and detecting x rays, and light detection technologies are much more sensitive with far higher resolution than their x-ray counterparts. Given sufficient light penetration through the sample, and that the proportion of scattered light is small, very-high-resolution, low-cost optical tomography becomes a viable goal.

Successful optical-CT/ECT requires developments on two fronts: imaging/acquisition hardware and sample preparation. The sample preparation consists of carefully controlled optical-clearing procedures where even large ($\sim 2 \text{ cm}^3$), unsectioned tissue samples are rendered almost completely transparent by replacing intracellular water with high refractive index solutions that minimize scatter at the cell membrane interface. Optical-CT/ECT techniques are illustrated here by application to cancer imaging, specifically 3D microvasculature and distribution of viable (fluorescing) cells, in xenograft tumors. The ability to image 3D microvasculature in high resolution and in unsectioned samples is of significant present interest in cancer research. Anti-angiogenic agents are known to enhance the therapeutic effect of ionizing radiation. However, recent research suggests that complex relationships exist¹⁵ between radiation and the actions of the therapeutic agents,^{16,17} the fractionation of application,^{18,19} the restructuring of vascular networks,²⁰ and the distribution of hypoxic regions.^{21,22} The high-contrast, high-resolution 3D optical-CT/optical-ECT imaging demonstrated here indicates a powerful new technique for efficient investigation of these relationships. The principle advantage over conventional histological techniques is that tissue sectioning is not required, and hence undistorted 3D information can be obtained of the structure and function of the sample.

II. METHODS

A. Imaging hardware and acquisition

The principles of x-ray-CT and SPECT are well established.¹⁴ Projection/emission images in the optical analogs implemented here are acquired by a camera mounted on a standard dissecting microscope [Figs. 1(a) and 1(b)]. The sample is suspended in agarose gel in a scintillation vial mounted horizontally and connected to a stepping motor (MM-3M-R, from National Aperture Inc., Salem, NH) housed in an in-house “3D-enabling stage.” The 3D-enabling stage is compatible with any standard dissecting microscope and transforms the normally planar imaging device into a 3D optical-CT imaging system. The sides of the waterbath are made of 5 mm thick Lucite, and the base is constructed of antireflection coated glass. The motor rotates the vial inside a high refractive index matched fluid (a glycerol solution, refractive index ~ 1.47), which minimizes refraction at the glass interface, enabling accurate straight-line projection/emission images to be acquired over the central 2/3 of the vial. Towards the outer edges of the vial minor refraction effects are noticeable. In optical-CT (projection or transmission mode) light from the white-light source in the base of microscope traverses through the sample as illustrated in Fig. 2(A). Projections are acquired in a “step-and-image” manner at small angular increments (e.g., 1 deg) through a complete 360 deg revolution. Care is taken to align the central axis of rotation of the vial to be coincident with the central column of pixels in the camera image frame. This alignment ensures consistent projection data for the reconstruction algorithm. Precise synchronization of the step-and-image acquisition sequence is achieved using MATLAB code that instigated an incremental motor rotation whenever an image was written to disk.

The field of view necessary to image larger samples (up to 8 mm) necessitates use of a large $1\times$ objective lens. The aperture setting was closed as far as possible while still producing a

clear image of the sample. Lower aperture settings produce deeper depth-of-focus in the sample, and hence lead to ray paths that more closely approximate the simplified parallel ray imaging geometry described in Fig. 2(A). Projection images were acquired using an Axiocam HRM 12Bit 1040×1388 monochrome microscope camera (Carl Zeiss, NJ) controlled by commercial software. The exposure time of the image was set as high as possible subject to the constraint that no saturation occur on any of the projections.

The micro-optical-CT scanner presented in Fig. 1 represents a second generation and very fast scanning system when compared with the first generation optical-CT devices described elsewhere.^{1,3,6} The time for a typical acquisition of 360 projections was about 5 min. A second generation optical-CT scanner was also reported by Doran *et al.*,⁷ who used a CCD camera to image macroscopic 3D dosimeters. The speed advantage for the second generation scanners arises because an entire 2D projection image of the sample is acquired in a single acquisition. In the first generation scanners, the same projection was acquired by scanning the laser in raster fashion across the projection space. While a huge increase in speed is achieved, the second generation systems may be less accurate as projection images may contain significant contributions of scattered light.^{5,23}

In optical-ECT (emission mode) excitation light from the microscope mercury lamp causes longer wavelength fluorescent light to be emitted by the sample, which is then imaged as illustrated in Fig. 2(B). Acquisition proceeds in the same step-and-image procedure, with the same settings and constraints as described above. A key difference is that narrow bandwidth optical filters were used to select both the excitation and emitting wavelengths. High-quality filters greatly enhance effective image quality by reducing any contaminant autofluorescence. In addition, the time the sample was exposed to excitation light was minimized by using a camera driven shutter to only allow exposure during image acquisition. This minimized any fluorescence quenching and/or photo-bleaching during imaging. It is interesting to note that contrary to SPECT, no special collimation grids were required to achieve useful emission images. Collimating septa are required in SPECT to prevent x rays that are not emitted normal to the detector plane from contributing to the image. The optical equivalent of this effect is achieved in the optical-ECT system by the microscope lens and optics, which form the image from light passing through the sample as illustrated in the figure.

Once a complete set of projection or emission images has been acquired, 3D reconstructions of the sample were created using the commercial COBRA software (Exxim Computing Corp, Pleasantown, CA), which employs filtered backprojection for reconstruction. Input parameters were adjusted to reflect the geometries of the image acquisition as illustrated in Fig. 2. Unless stated otherwise, all optical-CT and optical-ECT scans presented here consisted of 240 projections acquired at 1.5 deg increments. In addition the projection images fed to the reconstruction algorithm were downsized to 512×688 using bilinear interpolation. Images were downsized in order to meet a reconstruction limit on the projection size in our COBRA/hardware implementation. Reconstructed output preserved the true aspect ratio of the object and cube dimensions were set to 512×512×512. It is recognized that downsizing from original projection data (1040×1388) to an output cube of (512×512) represents a significant loss of image information. The high quality of image data achieved despite this limitation is testament to the potential of the techniques.

B. QA of imaging system

Three key characteristics of the imaging system need to be determined: geometrical accuracy of reconstruction, accuracy of reconstructed attenuation coefficients, and the modulation transfer function (MTF). Geometrical accuracy was evaluated using the wire phantom [Fig. 3 (A)] in a procedure similar to that outlined in Ref. 5. The wire phantom consists of a scintillation vial partially filled with agarose gel (0.75% by weight), containing a roughly spiral arrangement

of thin wires that were set into the gel in a vertical orientation. The phantom was scanned both by optical-CT and by x-ray-CT on a GE lightspeed scanner. The wires create strong contrast in both modalities. The 3D data sets were then manually aligned and registered using rigid body translation and rotation fusion tools in the DOSEQA software (Stonybrook University, www.doseqa.org). The wire positions in the x-ray-CT data were taken as the true gold-standard positions, and the accuracy of geometrical reconstruction in optical-CT was evaluated by comparing relative positions in the optical-CT data.

The accuracy of reconstructed attenuation coefficients was investigated by comparison with independent measurement using a spectrophotometer (Genesys Spectronic 20, Thermo Electron Corp, USA). 'Finger phantoms' [Figs. 3(B) and 3(C)] were constructed by filling a scintillation vial from 0.75% agarose gel (by weight), incorporating 3 or 4 agarose fingers of variable optical attenuation. The fingers were formed by setting and removing small pipette tips into the gel, thereby creating needlelike depressions. The depressions were then filled with the remainder agarose solution, at a temperature slightly above the setting temperature, which had been dyed with various concentrations of India ink. The true attenuation of the fingers was taken as that measured by pouring remainder finger gel into optical cuvettes and measuring transmission in the spectrophotometer. Spectrophotometer measurements were normalized to the control condition, a cuvette filled with plain gel containing no dye. For more intensely absorbing fingers, short path length cuvettes were used. Potential errors associated with variation in spectral absorption of the fingers was minimized by using a green filter (540 nm) in the optical-CT imaging, and selecting the transmission wavelength in the spectrometer at 510 nm.

The MTF of the system was determined from an optical-CT scan of a single human hair set vertically in a gelatin gel in a scintillation vial. The hair was set straight and vertical in the vial, and the diameter was measured as 0.07 mm by calipers. MTF was calculated by taking the fast Fourier transform (FFT) of a radon projection of a reconstructed axial slice. A correction was made for the thickness of the hair by dividing by the FFT of the hair.

C. Sample preparation

All samples were whole (i.e., unsectioned) at the time of imaging and optically cleared to enable visible light penetration through the sample. The opacity of biological tissue to visible light primarily arises from light scattering at the refractive index interface between the cell membrane (lipid) and the intracellular and extracellular tissue fluid (aqueous). The principle of optical clearing is to replace the water-based cellular fluid with a solution of high refractive index to match that of the cell membrane.²⁴ Achieving quality optical-clearing is a key step that enables the feasibility of both optical-CT and optical-ECT. Two general types of clearing techniques are described in the literature, reflecting potential clinical^{25,26} and *ex vivo* application.^{27,28} Topical applications of water-miscible solvents like glycerol or DMSO (dimethyl sulfoxide) are known to increase light penetration in skin,^{29,30} but these agents are insufficient for optical-CT of tissue samples of dimension 1 cm. More effective optical clearing is achieved by aromatic organic solvents such as benzyl-alcohol-benzyl-benzoate (BABB; refractive index 1.55)^{11,27} or methyl salicylate (refractive index 1.53).³¹ These solvents are not miscible in water and so a graded sequence of clearing steps is necessary to replace the water with clearing agent.

All tissue samples imaged in the present work were first set in 0.75% agarose gel in scintillation vials. The tissue samples were positioned centrally and ~1 cm up from the bottom of the vial. Individual staining and fixing procedures varied between samples, and details are discussed in the corresponding sections below. The purpose of the agarose gel was to stabilize the sample inside the vial during rotation incurred in the optical-CT/ECT acquisition. Each sample (agarose and imbedded tissue) was then immersed in a succession of graded water/ethanol

solutions, until the tissue was completely dehydrated. Samples were then immersed in a succession of graded ethanol/methyl-salicylate solutions, until the ethanol was replaced by methyl-salicylate. This two-step process is necessary as methyl-salicylate is non-aqueous, but freely miscible in ethanol. The total clearing process for tissue samples of order one cubic cm typically takes about a week (2–3 days for each step) under normal diffusion processes at room temperature.

The full potential of optical-CT/optical-ECT is to image both structure and function in tissue samples. Imaging is performed on excised tissue samples however, such that accurate imaging of any functional parameter requires both natural optical labeling (e.g., *in vivo*) and preservation of the condition of that label through excision and sample preparation. In the examples that follow, we describe methods of optical stain/label application and tissue fixation designed to achieve this aim.

1. Tumor microvasculature—A suspension of three million HCT116RFP xenograft human colon tumor cells was injected subcutaneously on the hind legs of nude mice, following the procedures of an IACUC approved protocol. After three weeks, the tumors had grown to a typical size of 1 cm maximum length. Two methods of optically labeling/staining the tumor microvasculature were investigated; passive intravascular and active intravascular. In both methods the label/stain was administered by tail vein injection and subsequent natural circulation around the body. Five to ten minutes postinfusion, the mouse was sacrificed and the tissue of interest removed for sample preparation. The passive intravascular stain was a suspension of isotonic India ink, which had been spun down in a centrifuge to isolate the smaller ink particles. The carbon-based ink particles circulate in the blood stream and are trapped inside vessels, thereby marking patent microvasculature.³² Microvasculature appears as a high contrast network of dark vessels in optical projections of the cleared tumor samples [Fig. 6 (A)]. Transmission optical-CT projection images were acquired using the standard microscope white light source.

Active labeling was achieved by tail vein injection of a fluorescent probe (lectin conjugated with FITC). Tissue samples were fixed both by *in vivo* perfusion fixation techniques and by simple postexcision fixation using 4% paraformaldehyde. Lectin actively binds to endothelial cells of the micro-vasculature, fixing the fluorescent FITC probe to the target of interest. The lectin-FITC labeled microvasculature was thus amenable for optical-ECT imaging. A fluorescein (FITC) filter set was used for emission acquisition, to select the excitation and emission wavelengths as 494 and 518 nm, respectively. The exposure and gain settings for both optical-CT/ECT were set as described above in Sec. II A. The implementation of both passive and active labeling of microvasculature enables cross validation and comparison of both techniques.

2. Tumor RFP expression—The HCT116 tumor cell line described above had been transfected with a gene coding for constitutively expressed red fluorescent protein (RFP). Viable tumor cells therefore express red fluorescence when exposed to the excitation wavelength. Care was taken to perfusion-fix the tumors *in situ*, by aortic cannulation and gravitational drip feed of 4% PFA solution. Tumors were then immediately set in 0.75% agarose in a scintillation vial and re-perfused with graded solutions of water:ethanol, and then ethanol:methylsalicylate, to achieve optical clearing. A DSRed2 filter set was used during image acquisition, to select for the excitation and emission wavelengths corresponding to RFP (558 and 583 nm, respectively). Exposure of the sample to excitation light was minimized by utilization of a shutter that only permitted exposure during image acquisition. Consistent interprojection normalization was achieved by keeping the exposure time constant for each projection. This constant exposure time was set to maximize the signal (avoiding saturation) for the most emitting image.

III. RESULTS AND DISCUSSION

A. Performance characteristics of the optical-CT imaging system

The optical-CT system was found to be geometrically accurate, within the central region of the reconstruction volume, to within experimental uncertainty (~ 0.25 mm). This is illustrated in Fig. 4(A), where needle positions determined by x-ray-CT (dark circles) and optical-CT (light points) are seen to coincide. The dark outer ring in Fig. 4 corresponds to refraction artifact at the external wall of the scintillation vial. The field of view of the optical-CT projection images was not wide enough to encompass the whole vial, and so the optical-CT reconstruction volume is seen to only encompass the inner seven needles in the figure. The sensitivity of this geometrical test was reduced by localized blurring artifacts in the optical-CT images observed to cause starlike appearance of the needles. These artifacts were more pronounced than those seen on needle phantoms scanned with first generation optical-CT systems.⁵ The cause is attributed to missing data streak artefacts arising from strong attenuation in the needles, and the relatively large diameter of the needle relative to pixel-size of the detector. Figure 4(B) shows the modulation-transfer-function for the imaging system. The figure indicates that spatial detail in the object at the $100\text{ }\mu\text{m}$ level is imaged with $>15\%$ magnitude. Substantial improvement on this characteristic ($<20\text{ }\mu\text{m}$) is likely under conditions of higher magnification, improved optics, and reduced motion point-spread-function.

The central axial plane through a finger phantom containing three fingers of known optical attenuation is illustrated in Fig. 5(A). This figure illustrates both the potential and some challenges of the optical-CT technique. The regions corresponding to the three fingers are clearly distinguished, however some streak and scatter artifacts are observed. The contrast in the fingers is caused by different concentrations of ink, which primarily absorbs rather than scatters light. Slight scattering (refraction) was noted, however, at the edges of the fingers corresponding to the interface between the dyed and nondyed gel. While the refractive indexes of dyed and nondyed gels are essentially the same, the imperfect melding at the interface causes some optical scatter. The plot of attenuation coefficients in the fingers determined by both optical-CT and independently by spectrophotometer is shown in Fig. 5(B). The attenuation values returned from the reconstruction software are relative and scaled to 16 bit depth. A linear correlation is observed, although the relatively high noise reflects the experimental challenges of creating small fingers of uniform known optical attenuation, and same refractive index as the host gel.

B. 3D optical-CT imaging of biological samples

1. Tumor micro-vasculature—A single optical transmission or projection image of the whole HCT116 tumor is shown in Fig. 6(A), and contrasted with micro-x-ray-CT and micro-MRI images acquired with state of the art small animal imaging systems^{33,34} [Figs. 6(B) and 6(C)] to highlight the improved contrast and resolution of optical-CT. The tumor had been optically cleared and passive micro-vascular staining achieved with the tail vein injection of India ink as described in Sec. II. Exquisite visualization of the micro-vasculature is observed, although the 3D information is lost in the projection. The 3D aspect of the vasculature can be visualized from the projection data by creating a movie of successive projection images. The tumor appears to rotate about a vertical axis, and the 3D structure is clearly apparent in exquisite detail (see EPAPS supplemental movie).³⁶ In this instance, and in other tumors we have imaged in this way, the vasculature is primarily seen on the periphery of the tumor, with just a few larger vessels penetrating to the tumor core. As these tumors were implanted subcutaneously, especially dense and intensive vasculature is seen along one side of the tumor where it was attached to the underlying fascia of the animal. While the movie-loop of projection images can give excellent qualitative visualization of the vasculature in 3D, more quantitative information is available from computed-tomographic reconstructions of the projections. Figure

7 shows such a reconstruction from 240 projections acquired at 1.5 deg increments. The pixel dimensions in the image are $\sim 30 \mu\text{m}^3$. The orthogonal views reveal the propensity for peripheral vasculature. Significant vascular penetration is observed to be limited to the right side of the tumor [Fig. 7(B)] indicating this region was relatively well perfused. These images represent the first time that vasculature structure has been imaged at such high spatial resolution and high contrast in a tumor of this size. While areas of high vascular density are clearly discernable, precise microvasculature linkages appear indistinct. This is largely attributed to artifacts from the limited depth-of-field of the microscope optics. A significant improvement in image quality is observed on the bench system with customized deep depth-of-field optics (subject of future publication).³⁵ These images are the first optical-CT images of microvasculature that we are aware of.

This tumor had also received vascular staining with FITC-lectin. The double staining was achieved simultaneously, by combining the FITC-lectin and ink stains in the same tailvein injection prior to sacrifice of the animal. The double staining approach enabled independent imaging of the vasculature by optical-ECT of the FITC-lectin distribution. Due to the potential for photo-bleaching under prolonged imaging, just 180 projection images were acquired at 2 deg increments. The corresponding reconstructions of the emitting FITC distribution within the tumor, acquired with the FITC filters, are shown in Figs. 7(D)–7(F). In general a clear correlation and agreement is observed between the optical-CT and optical-ECT images. Wellperfused regions appear bright in the optical-CT images (corresponding to light-absorbing dense micro-vasculature with high ink staining), and also as bright regions in the optical-ECT images (where the scale is inverted such that light pixel values correspond to high emission of light and hence high concentration of FITC).

2. Viable tumor RFP expression—The HCT116 tumor shown in Figs. 7 and 8 was also imaged in optical-ECT mode with DSRed2 filter set [Figs. 7(G)–7(I)]. In principle this image is highly significant as it represents the 3D distribution of RFP, which should correlate with the distribution of viable tumor cells. The correlations in Fig. 7 are striking and appear to clearly show that regions of high RFP expression light regions of Figs. 7(G)–7(I) correlate closely with the well perfused regions independently illustrated in Figs. 7 and 8 [the light regions stained with highly attenuating ink in Figs. 7(A)–7(C), and the light high-emission regions in Figs. 7(D)–7(F)]. This makes intuitive sense, as one would expect the more viable regions of the tumor to correlate with perfusion. A precise interpretation of Figs. 7 is complex due to the novelty of these techniques, and requires reference to more established imaging modalities. This is addressed below.

C. Tumor histology

Figure 8 shows conventional histological sections cut through the same HCT116 tumor imaged in Fig. 7. The sections were cut after embedding the tumor in a paraffin block, and care was taken to orient the sections such that they matched as closely as possible the middle panel views of the optical reconstructions in Figs. 7(B), 7(E), and 7(H). One slice [Fig. 8(A)] was stained with hematoxylin and eosin (H&E), a technique commonly used by pathologists to study tumor biopsies. Hematoxylin stains nuclear matter (e.g., DNA, RNA, chromatin) with a blue-purple hue, while eosin stains cytoplasmic constituents bright pink. Figure 8(A) provides strong supporting evidence for the conclusions derived above from the optical imaging modalities. The peripheral band of well-perfused viable cells, inferred from all three optical modalities, exhibits strong H&E staining and therefore viable cells in this region. The large central areas devoid of vasculature and viable cells, as determined from the optical modalities, are found to be devoid of H&E stain, indicating necrosis and cellular breakdown. Further correlation was observed between ink-stained tumor micro-vasculature in the optical and histological images. The ink stained vasculature was not readily visible in the H&E stain of Fig. 8(A) because the

ink stain was masked by the overwhelming deep-blue hematoxylin. The solution was to lightly stain a section with eosin as illustrated in Figs. 8(B) and 8(C). These sections are shown at 10× magnification to reveal the presence of dark ink-stained vasculature. The sections clearly show the presence of vasculature at the periphery of the tumor, and lack of vasculature in the necrotic regions.

IV. CONCLUSIONS

Optical-CT and optical-ECT are relatively new imaging techniques, and this is the first application to imaging tumor structure and function. As with any new imaging modality, accurate interpretation of image content is gradually established by reference and correlation to alternative more established methodologies. The bulk of this effort is outside the scope of the present paper, which aims to introduce the physical basis and demonstrate preliminary application and potential for unique information on tumor structure and function. The application of optical-CT and optical-ECT presented here demonstrates substantial potential for imaging tumor microvasculature and viable tumor distribution, with unprecedented spatial resolution, contrast, and detail in 3D. The true potential of these techniques promises to be greater, as it should be feasible to image a wide range of other tumor and normal tissue structure and function, dependent on the development of smart bio-optical marker dyes and fluorochromes.

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36. <http://www.aip.org/pubservs/epaps.html><http://www.aip.org/pubservs/epaps.html> See EPAPS Document No. E-MPHYA6-33-018608 for a movie of sequential optical-CT transmission projections of a rotating optically cleared xenograft tumor with microvasculature staining that had been applied *in vivo*. This document can be reached via a direct link in the online article's HTML reference section or via the EPAPS homepage

Fig. 1. (a) Experimental setup for performing 3D optical-CT on a standard dissecting microscope. A=3D enabling stage, B=motor driver, C=microscope base containing white-light source, D=microscope camera, E=mercury lamp for fluorescent imaging. (b) Close up view of the sample contained in a scintillation vial (F) mounted horizontally and attached to a rotating clamp (G). The entire scintillation vial is immersed in optically-matched water-bath (H).

Fig. 2.
Simplified view of light paths through (A) the optical-CT (transmission) setup and (B) the optical emission CT setup.

Fig. 3. (A) Side view of a wire phantom used to verify geometrical accuracy of optical-CT. (B) and (C) side and bottom view of a finger phantom.

Fig. 4. (A) Overlay fusion image of a needle phantom scanned by both x-ray-CT (needles appear dark circles) and optical-CT (needles appear as light specs). (B) Modulation transfer function of the microscope system.

Fig. 5. (A) Reconstruction of central axial slice through a three-finger phantom. (B) Plot of attenuation coefficients of the fingers determined from optical-CT and spectrophotometer.

Fig. 6.

(A) A projection image of the HCT116 tumor after optical-clearing: (B) *in vivo* micro-CT of the same tumor on hind leg of mouse. The micro-CT image is reconstructed at 50 μm pixel resolution, and involved application of a new vascular contrast agent (fenestra). (C) *In vivo* T1 weighted contrast enhanced micro-MRI image of similar tumor of same size. MRI contrast was achieved with a continuous infusion of magnevist. The scanner is a 2T system, and acquisition parameters included TE=11 ms, TR=500 ms. The location of the tumor is indicated by the dashed line. The determination of subtle micro-vasculature in small tumors using micro-x-ray-CT and micro-MRI remains a challenge at the present time. The micro-CT and micro-MRI images were acquired at the Duke Center for In-Vivo Microscopy.

Fig. 7.

(A), (B) and (C) Orthogonal views of microvasculature of a HCT116 tumor after *in vivo* staining with light absorbing dye. The 3D microvascular data were acquired by optical-CT imaging incorporating a white light source. Light regions of high perfusion are clearly visible as containing relatively high amounts of light attenuating dye. (D), (E) and (F) Corresponding images of the 3D microvasculature of the same HCT116 tumor this time imaged by optical-ECT incorporating the FITC excitation and emission filter set. The light regions of these images correspond to increased presence of light emitting FITC, which was conjugated to lectin endothelial (micro-vasculature) marker protein. (G), (H) and (I) The distribution of viable tumor cells in the HCT116 tumor obtained by optical-ECT imaging of the same tumor, this time incorporating the DSRed2 filter set to select for RFP excitation and emission.

Fig. 8. Histological sections of the tumor depicted in Fig. 7. A tumor slice was stained with conventional H&E staining protocol to reveal morphology (A). Another slice was stained with eosin counterstain only and magnified images of select areas [represented by dotted circles in (A)] obtained to show ink-stained vasculature [(B) and (C)].