Volumetric optoacoustic imaging unveils high-resolution patterns of acute and cyclic hypoxia in a murine model of breast cancer

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Abbreviations list:

Oxygen partial pressure (pO_2)

Deoxyhemoglobin (HbR)

Oxyhemoglobin (HbO)

Total hemoglobin (HbT)

Oxygen saturation (sO_2)

Multi-spectral optoacoustic tomography (MSOT)

Volumetric multi-spectral optoacoustic tomography (vMSOT)

Near-infrared (NIR)

Field of view (FOV)

Graphics processing unit (GPU)

Volumetric hypoxic fraction (vHF)

Fast Fourier Transform (FFT)

Volumetric cyclic fraction (vCF)

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ABSTRACT

Mapping tumor heterogeneity and hypoxia within a living intact organism is essential for understanding the processes involved in cancer progression and assessing long-term responses to therapies. Efficient investigations into tumor hypoxia mechanisms have been hindered by the lack of intravital imaging tools capable of multi-parametric probing of entire solid tumors with high spatial and temporal resolution. Here we exploit volumetric multi-spectral optoacoustic tomography (vMSOT) for accurate, label-free delineation of tumor heterogeneity and dynamic oxygenation behavior. Mice bearing orthotopic MDA-MB-231 breast cancer xenografts were imaged non-invasively during rest and oxygen stress challenge, attaining time-lapse 3D oxygenation maps across entire tumors with 100µm spatial resolution. Volumetric quantification of the hypoxic fraction rendered values of 3.9-21.2% whereas the oxygen saturation (sO_2) rate declined at 1.7-2.3% per mm in all tumors when approaching their core. Three distinct functional areas (the rim, hypoxic, and normoxic cores) were clearly discernible based on spatial sO_2 profiles and responses to oxygen challenge. Notably, while sO_2 readings were responsive to the challenge, deoxyhemoglobin (HbR) trends exhibited little to no variations in all mice. Dynamic analysis further revealed the presence of cyclic hypoxia patterns with a 21% average discrepancy between cyclic fractions analyzed by sO_2 (42.2±17.3%) and HbR fluctuations (63±14.1%) observed in the hypoxic core. These findings corroborate the strong potential of multi-spectral optoacoustic tomography for advancing pre-clinical imaging of cancer and informing clinical decisions on therapeutic interventions.

Significance: vMSOT provides quantitative measures of volumetric hypoxic fraction and cyclic hypoxia in a label-free and non-invasive manner, providing new readouts to aid tumor staging and treatment decisions.

INTRODUCTION

Mapping tumor heterogeneity is a key approach for assessing the long term responses to therapy (1). The physiologic microenvironment of neoplastic lesions is dictated by abnormal metabolism and neovascularization, differing substantially from healthy tissues. A particularly important alteration is the presence of hypoxia, a condition of reduced level of oxygen partial pressure (pO_2) during which cells undertake key biological pathways associated to tumor growth. It appears that tumors exhibit highly heterogeneous and dynamic oxygenation patterns, ranging from normoxia to hypoxia. Disorders in oxygen distribution can further results in significant variations in perfusion to neighboring regions (2). This apparently chaotic behavior of cancerous tissues represents a major obstacle for understanding the disease (3). Tumor hypoxia is also closely associated with resistance to chemotherapy and radiation therapy (4).

To this end, the spatial distribution of pO_2 within the tumor has been profiled via invasive methods, such as CT-guided pO_2 sensors. This enabled assessing the pO_2 drop between the viable rim and the core in just a few isolated locations (5). Imaging with ^{19}F- magnetic resonance oximetry (6) and ^{18}F-fluoromisonidazole positron emission tomography (7) may provide higher spatial resolution but relies on exogenous oxygenation sensitive agents. Furthermore, those methods mainly reveal static oxygenation profiles, only comprising a partial picture of the complex tumor microenvironment, whilst studying the transient spatio-temporal characteristics is essential for advancing in knowledge (8). For this, dynamic oxygen challenge has been suggested as a method for studying the oxygen stress response in tumors (9), aiming at characterizing the low perfusion efficiency of neovasculature by assessing responses to rapid respiratory challenges in different tumor sub-regions. Those were prominently studied with MRI (10) by exploiting the sensitivity of R_1–based sequences to changes of dissolved O_2 in blood (11). The approach was however limited by a relatively low spatio-temporal resolution further representing an indirect oxygen tension measure. It has been now widely recognized that, in addition to chronic hypoxia, reduced perfusion in some regions of the tumor may lead to appearance of hypoxic–reoxygenation periods. These fluctuations between hypoxic and non-hypoxic states are referred as cyclic (dynamic) hypoxia (8). Cyclic hypoxia is associated with increased tumor aggressiveness, resistance to treatments and metastasis (12,13). Hence, the ability to map and characterize the dynamics of cyclic heterogeneities may eventually contribute to improving treatment outcomes (14). Almost exclusively, MR methods have been used for this purpose. Although pO_2 and deoxyhemoglobin (HbR) variations were detected in tumor bearing mice with electron paramagnetic (15) and with T2*- (16) and R_2*- (17) weighted magnetic resonance imaging, low spatio-temporal resolution remains the main limitation of MR-based methods preventing effective analysis of cyclic patterns in whole tumors. Moreover, conventional MRI instruments are restricted to HbR–correlated measurements lacking the clinical relevance of pO_2 (18). New methods to probe the tumor microenvironment over different time scales, from perfusion dynamics to neovasculature development, are then required for a better understanding of the processes involved in cancer progression.

Multi-spectral optoacoustic tomography (MSOT) is increasingly used in cancer research due to its unique capability for label-free noninvasive monitoring of hemodynamic parameters (19). A number of specific contrast agents have been further devised to sense key cancer bio-markers (20). MSOT was used to follow tumor growth and vascular development (21), to image tumor heterogeneity and perfusion (22-25) and to assess anti-cancer therapy efficacy (26). Previous works were yet limited to cross-sectional (2D) investigations unsuitable for an accurate characterization of differential real-time responses of

neighboring sub regions across entire solid tumors. Here we employed instead a volumetric multi-spectral optoacoustic tomography (vMSOT) approach based on simultaneous optoacoustic signal detection by spherical arrays (27), with the aim to non-invasively characterize dynamic high-resolution patterns of acute and cyclic hypoxia in murine breast cancer models.

Materials and Methods

Volumetric multi-spectral optoacoustic tomography (vMSOT)

The vMSOT system is based on a previously described real-time volumetric tomography technique (28). Briefly, ultrasound (pressure) signals are induced by short (<10 ns duration) pulses of light from an optical parametric oscillator laser tunable in the near-infrared (NIR) spectral range (680-950 nm). The optoacoustically-generated signals are simultaneously detected by a 512-element spherical array transducer having 10 MHz detection bandwidth (Fig. 1a). During the experiments, the light fluence at the mouse skin surface was maintained below 20 mJ/cm^2 for all wavelengths. The system renders an effective field of view (FOV) of 6 x 6 x 6 mm^3 with nearly isotropic 3D spatial resolution in the 80-150 µm range. For imaging, both the transducer and the mouse were immersed in a water tank to facilitate propagation of the optoacoustically-generated pressure waves. Reconstruction of individual volumetric image frames for each illumination wavelength was performed with a graphics processing unit (GPU) implementation of a back-projection formula (29).

In vivo animal handling

The mouse in vivo experiments were approved by the government of Upper Bavaria. All experiments were performed in full compliance with the institutional guidelines of the Helmholtz Center Munich. Orthotopic tumors were induced in 6 weeks old female immunodeficient SHrN® hairless NOD.SCID mice (Envigo, Rossdorf, Germany) by subcutaneous injection of 3 million MDA-MB-231 cells in a total volume of 60-80µL of PBS and Matrigel (1:1 v/v) into the inguinal gland of the mammary fat pad. *In vivo* imaging experiments were performed 30 days post cell inoculation, when the tumor reached an approximate size of 8 mm (Fig. 1b). The MDA-MB-231 cells were supplied by ATCC (ATCC, Manassas, USA) in May 2015, providing cell line authentication by STR analysis. The cell type was mycoplasma tested by a mycoplasma detection kit (Lonza, Basel, Switzerland) in March, 2019. Cells were used at 4 passages from thawing from frozen stocks. During acquisition, the mice were anesthetized with isoflurane (3% v/v for induction; 1.5%-2.0% v/v during imaging) in 100% O_2. A custom-made holder was used to maintain the mice in a stationary position with their fore and hind paws attached to the holder during the experiments. The mice were then immersed inside the water tank with the head kept outside water. The temperature of the water tank was maintained at 34°C with a feed-back controlled heating stick. A breathing mask with a mouth clamp was used to fix the head in an upright position and to supply anesthesia and oxygen.

Whole tumor imaging and analysis

While the effective FOV of the spherical array already covers a major portion of the tumor volume, high-resolution imaging of entire tumors and their surrounding areas was achieved by raster-scanning the array in the lateral (x-z) plane (Fig. 1a). Signal acquisition at multiple wavelengths (420,730,760,800 and 850 nm) was performed to allow for a high quality anatomical and functional tumor profiling. At each

position of the array, 100 volumetric frames per wavelength were averaged after applying a motion rejection algorithm that eliminated frames affected by breathing-related motion (30). The reconstructed volumes were then combined into a large-scale image of the entire region of interest (Fig. 1a). Images acquired at multiple wavelengths were then processed with a standard linear unmixing method to estimate, on a voxel-per-voxel basis, the distribution of oxyhemoglobin (HbO), HbR and total hemoglobin (HbT) as well as the oxygen saturation (sO_2) (31). Three dimensional visualization of the optoacoustic images was done with Amira (Visual Sciences Group).

Segmentation into functional sub-regions

Tumors were segmented into three sub-regions, namely, the rim, normoxic and hypoxic cores (n=6 mice). The segmentation between the tumor rim and its core was done manually by estimating the boundary between the linear and parabolic-shape portions of the oxygen saturation profiles at 100% O_2 level, rendering boundary sO_2 values in the 50-62% range (Supplementary Fig. 1). The segmentation between the normoxic and hypoxic cores was based upon critical O_2 tension range of 10-20 mmHg pO_2 for which binding of hypoxia markers occurs (32). Note that the upper pO_2 threshold of 20 mmHg corresponds to a sO_2 value of 18% according to the Kelman and Severinghaus model (33) of the oxygen dissociation curve (where pCO_2=40 mmHg, pH=7, T=37°C). Voxels with sO_2 values below 18% were thus regarded as hypoxic. The volumetric hypoxic fraction (vHF) measure was further calculated as the fraction of hypoxic voxels relative to the total voxel count in the tumor (voxels with insufficient signal-to-noise ratio were excluded).

Analysis of tumor dynamics

Dynamic tumor responses to breathing gas challenge were analyzed by recording time-lapse multi-spectral data with the spherical array remaining in a stationary position. The oxygen challenge experiments were performed by changing the percentage of oxygen in the breathing gas in the following order: 5 minutes of 20% O_2 (first baseline), 25 minutes of 100% O_2 and 15 minutes of 20% O_2 (second baseline). Prior to the challenge, the animals were given 15 minutes to stabilize at 20% O_2. To correct for respiratory motion artifacts, data was averaged over 2 seconds. Spatio-temporal volumetric sO_2 map was then calculated. Differential responses (ΔsO_2) to the oxygen stress were calculated on a per-voxel basis as signal difference between the averaged sO_2 during 100% O_2 breathing levels and the baselines, namely, each gas challenge yielded two differential responses for the first and second baselines. The ΔsO_2 voxels were grouped according to the three sub-regions, i.e. rim, normoxic core and hypoxic core.

Cyclic hypoxia analysis

The second segment of the dynamic oxygen challenge corresponding to 25 minutes of 100% oxygen level was used for assessing cyclic hypoxia in the tumors of n=6 mice. The analysis procedure is described elsewhere (34,35). Briefly, the following processing steps were applied to the time profiles of each voxel: 1) linear de-trending; 2) calculation of the autocovariance; 3) calculation of the power spectral density via Fast Fourier Transform (FFT); and 4) thresholding according to a confidence interval, assuming that the signal is affected by Gaussian noise. Following these steps, the peak of the power spectrum for each voxel was identified. Voxels having a peak within frequency band of 0-0.005 Hz were regarded as cyclic voxels (3 to 18 cycles per hour is considered the relevant physiological range (34,36)).

The volumetric cyclic fraction (vCF) was calculated as the ratio between the number of cyclic voxels and the total voxel count (excluding noisy voxels).

Statistical analysis

A total of n=6 animals were evaluated contributing 12 independent ΔsO_2 measurements. For each sub-region (rim, normoxic or hypoxic core) approximately 100,000 voxels were evaluated. To represent the entire distribution for each sub-region, pixels values were integrated using an average of all the pixels as well as the 25^{th} percentile, median and 75^{th} percentile values. The differences in ΔsO_2 values across the sub-regions were compared using Friedman's paired test followed by Dunn's post hoc test. The differences between vCF values of the tumors (n=6, calculated by either sO_2 or HbR analyses) were compared by an unpaired t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Analyses were carried out using Matlab and SPSS 25.0 (SPSS Inc., Chicago).

Histological validation

The mice were euthanized after being imaged with an overdose of ketamine (300 mg/kg) and directly stored at -80°C until performing whole body cryo-slicing. For this, the frozen specimen were embedded in Tissue-Tek® O.C.T.™ compound (Sakura Finetech Europe, Alphen aan den Rijn, The Netherlands). 10-µm-thick sections covering the entire tumor volume were extracted. Immunohistochemistry was performed on the frozen sections using antibodies against CD31 (Dianova, Hamburg, Germany) or CAIX (Novus Biologicals, Littleton, USA). Antibody staining was visualized with Alexa Fluor 488 or Alexa Fluor 594 conjugated secondary antibodies (Life Technologies, Thermo Fisher Scientific, Waltham, USA). All slices were further co-stained with DAPI (Molecular Probes, Thermo Fisher Scientific, Waltham, USA) to visualize cell bodies. Fluorescence compound slice images were recorded using an Imager M2 microscope (Carl Zeiss AG, Oberkochen, Germany). Image acquisition and analysis was done using the Zeiss Zen 2 microscope software.

RESULTS

Multispectral 3D tumor imaging

Position of the tumor in the inguinal mammary gland is clearly visible in the compounded volume image of the entire region surrounding the tumor, which was acquired by a large area scan of the mouse abdomen at 800nm (Fig. 1a). Due to the relatively low light attenuation at this wavelength, both tumor core and vast recruitment of neovascular networks from as deep as 7 mm are clearly visible (Fig. 1b). Note also the direction of arrows indicating some feeding vessels that branch from larger vessels and propagate toward the core. Image acquired at 420 nm reveals a different sort of anatomical information (Fig. 1c). For this wavelength, hemoglobin and melanin are highly absorbing, hence superior vascular contrast is achieved to the detriment of the maximal penetration depth of ~2 mm. Three distinct ring-type patterns can be observed across the tumor surface matching the photograph (Fig. 1c). An outer ring of epidermal and dermal layers is characterized by melanin absorption and fine vasculature patterns of the larger venules and arterioles (37). The middle ring with strong absorption signals is attributed to a

highly oxygenated rim layer located close to the surface. Finally, the inner ring belongs to the tumor core that breaches the dermal layer and is characterized by reduced signal intensity. The sO_2 map retrieved from the multi-spectral data at 730, 760, 800 and 850 nm (Fig.1d) suggests that the feeding vessels are well oxygenated while the core appears to be at an advanced hypoxic state with the highly perfused region at the edge, arguably belonging to the rim.

Analysis of the spatial sO_2 profiles

Spatial profiling of tumor oxygenation was performed by analyzing selected coronal slices at different depths (Fig. 2a). In this way, high-resolution sO_2 variations could be calculated as a function of distance from the center of the tumor (Fig. 2b). The corresponding sO_2 profiles (Fig. 2c and Supplementary Fig. 1) exhibit a substantial difference between the outer rim and the core with an average sO_2 drop of 1.7-2.3% per mm, as estimated by linear fitting. Segmentation of the rim and the core regions was based on thresholding the spatial sO_2 profiles, as shown for an exemplary slice in Fig. 2c. Comparison of the sO_2 map acquired at approximately 2 mm depth *in vivo* (Fig. 2d) with its corresponding *ex vivo* immunohistochemistry showed that high sO_2-values co-localize with high expressions of CAIX (Fig. 2e) and CD31 (Fig. 2f), which may indicate that both proteins facilitate enhanced oxygen supply to the rim. Note, however, that both CAIX and CD31 can also be found in the tumor core, although with less confinement and lower density as compared with the rim.

Oxygen challenge responses

Owing to their disparate perfusion patterns, it is generally anticipated for the distinct tumor sub-regions to exhibit different responses to dynamic oxygen challenge. As described in the previous section, the rim and core of each tumor were discerned by analyzing their corresponding spatial sO_2 profiles. Inside the core, a further segmentation into hypoxic and normoxic sub-regions was established via threshold-based approach by considering the critical O_2 tension for which binding of hypoxia markers occurs. Three distinct hypoxic foci are visible in a central sagittal slice showing the spatial architecture of neighboring hypoxic and normoxic sub-regions (Fig. 3a). A quantitative analysis of the relative volumes of the regions (Supplementary Table 1) allows for a more accurate assessment of hypoxia progression in the tumor. The relative volumetric fraction of the sub-regions was similar among all the imaged tumors, as well as the calculated vHFs (3.9-21.2%).

Additional insights on oxygen transport to the three sub-regions can be drawn by observing local sO_2 responses to the oxygen challenge (Fig. 3b and Supplementary Fig. 2) as well as the time-lapse video of the responses in single voxels (Supplementary Video 1). In all tumors, the rim and the normoxic core exhibit rapid response onset times of 1-2 min followed by a steady baselines. In contrast, hypoxic regions in the core exhibit moderate and unstable drops. These responses are not at all visible in the corresponding THb trends (Fig. 3c), which are characterized by a relative plateau in all tumors. The measured values of absolute sO_2 difference (ΔsO_2) between the baseline and 100% oxygen phase further show that each sub-region presents a distinct response to the oxygen stress challenge (Fig. 3d and Supplementary Fig. 3), which held true for all the imaged tumors. Paired comparisons between the sub-regions of each tumor indicated that segregation between rim, normoxic core and hypoxic core is statistically significant across all the measurements ($p<0.05$).

Cyclic hypoxia

We subsequently exploited the unique rapid volumetric multispectral imaging capabilities of the vMSOT system in order to assess cyclic hypoxia patterns by comparing periodic variations of HbR and sO_2. HbR is of particular interest since it directly corresponds to the commonly measured BOLD MRI signals. The frequency spectrum of the HbR signal from a typical voxel in a central sagittal slice through a tumor exhibits a dominant frequency at 0.001 Hz (Fig. 4a). This value corresponds to 3 to 4 cycles per hour and was in fact predominantly manifested in majority of the tumor mass in all measurements, both in the unmixed HbR and sO_2 signal channels (Fig. 4a). The fluctuations of sO_2 and HbR have a characteristic amplitude variation of ~30% (Fig. 4b). We estimated the vCF values in all the imaged tumors to be in the 17%-59% range (Supplementary Table 2), resembling the pO_2 ranges of 13% to 52% reported for ^{18}F-miso PET (38) and of 21±6% to 41±3% as measured by EPRI (15). An average discrepancy of 14% was observed between vCFs calculated from the HbR variations versus those based on the sO_2 (Fig. 4c). Notably, the hypoxic core sub-region yielded the most significant difference ($p<0.05$).

DISCUSSION

The importance of mapping the spatial distribution of pO_2 in solid tumors has been acknowledged ever since hypoxia has been linked to treatment outcomes (1). Herein, we provided unique estimates of the three-dimensional distribution of oxygenation patterns in murine breast cancer models with excellent spatial and temporal resolution owing to the new vMSOT approach. The imaging system provides sO_2 values by un-mixing of multi-spectral data, from which the equivalent pO_2 can be calculated. We exploited the unique capabilities of vMSOT to gather otherwise unattainable information on the static, dynamic and cyclic sO_2 behavior in distinct tumor sub-regions. Experiments in mice implanted with MDA-MB-231 tumor cells indicated that sO_2 linearly increases by 1.7% to 2.3% sO_2 per mm when moving away from the center of the core. This constitutes a useful metric for accurate assessment of the tumor perfusion and matureness (23). Volumetric quantification of hypoxic fraction rendered a similar range of 12.2±6.5%in all the studied tumors. This parameter was previously suggested for guiding clinical decisions pertaining optimization of tumor therapies (39). In this regard, the truly volumetric information provided by vMSOT is expected to significantly increase its accuracy with respect to 2D imaging approaches.

Here we were able to segment the tumor into three sub-regions, namely the rim, hypoxic and normoxic cores. While the rim area was subjectively differentiated by relying on its distinct features in the spatial sO_2 profiles, the core was segmented into hypoxic and normoxic sub-regions based on a theoretical threshold of critical pO_2 for which binding of hypoxia markers occurs. Note that translation between pO_2 and sO_2 according to the oxygen dissociation curves might be prone to errors due to the temperature, pCO_2 or pH variability. The effectiveness of our segmentation methodology was further substantiated by demonstrating that oxygen saturation of the tumor sub-regions is significantly different. It is long known that chronic hypoxia in tumors is closely associated with limited perfusion (2) . Thereby, we hypothesized that a dynamic oxygen challenge would correspondingly reveal different local responses among the sub-regions. This was tested by exploiting the unique real-time 3D imaging capabilities of the vMSOT system. Notably, the ΔsO_2 analysis yields a good distinction ($p<0.05$) between the sub-regions in all mice. It was shown that the rim and the normoxic sub-regions of the core exhibited significant variations in response to the oxygen stress, while the sO_2 in the hypoxic core decreased only mildly. Note that declining sO_2 trends during oxygen stress have been previously reported in prostate tumor

cores (23). It is worth noticing that while sO_2 was responsive to the challenge, THb was barely altered in all mice. This indicates a clear advantage of the method with respect to alternative imaging modalities unable to differentiate between the oxygenated and deoxygenated states of hemoglobin. The dynamic ΔsO_2 analysis was hence exploited as a new robust method for direct segmentation of tumor sub-regions, which can be used to complement measurements done by other modalities that do not measure pO_2 or sO_2 directly but can only observe changes in trends (e.g. BOLD-MRI). Yet, it should be noted that the accuracy of sO_2 quantification by vMSOT might be affected by wavelength-dependent light attenuation in heterogeneous tissues (40,41). For this reason, ΔsO_2 represents a more robust metric able of eliminating common biases. The lack of variations in the THb profiles may be attributed to the limited vasoconstriction effects and the vascular tone within tumors (42,43), which may further diminish due to the generally low levels of vessels functionality in MDA-MB-231 tumors (44).

Hemoglobin is an intrinsic contrast molecule for BOLD-MRI, which has been previously used for non-invasive characterization of cyclic hypoxia. The latter is known to have physiological effects different from those of acute hypoxia (8). Our study is the first to detect the presence of cyclic hypoxia optoacoustically. We were able to analyze the cyclic oscillations of sO_2 and HbR simultaneously by solely relying on endogenous contrast. vMSOT provided a volumetric analysis of the entire tumor volume at spatial and temporal resolution, which may contribute to an enhanced reliability of the cyclic fraction estimations. We observed discrepancy between the vCF values calculated via sO_2 versus HbR. On average, 14% of the tumor volume manifested cyclic hypoxia behavior in the HbR channel but not in the sO_2. Notably, the hypoxic core exhibited the highest vCF values. This is consistent with a previously reported observation that cyclic hypoxia is more likely to appear in tumor regions where oxygen saturation of the blood entering that region is relatively low (45). Moreover, the hypoxic core was also the region manifesting the most significant discrepancy of 21% between the sO_2 and HbR fluctuations. Due to the close relationship between sO_2 and pO_2, these results suggest an over-estimation of cyclic fraction by the BOLD signal and other imaging methods solely using HbR-sensitive readouts for the cyclic analysis. Such a bias may have a clinical significance in treatment decisions which are based on the cyclic fraction values. Multi-spectral optoacoustic tomography has recently offered great prospects for clinical translation in the field of metastatic lymph node detection (46) and breast cancer diagnostics (47-49). Therefore, preclinical studies with vMSOT may inform clinical decisions by e.g. providing error estimates of the vCF measurements for different tumor types. The new imaging capabilities demonstrated in this work further foster optoacoustic imaging as a valuable tool for the screening, diagnosis, treatment planning and monitoring of cancer.

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Figures

Figure 1. **A** Simplified schematic of the imaging set-up. The volumetric multi-spectral optoacoustic data was recorded with a spherical matrix array transducer probe that was also translated to acquire images from larger field of view. A typical 3D image of the thorax and abdominal region acquired at 800nm from a tumor-bearing mouse is shown to the right. TV=Thoratic vessels. TFV=Tumor feeding vessels. In=Intestine. FV=Femoral vessels. IV=Ischiatic vessels. **B** A zoomed-in depth encoded image of the tumor area showing vessel recruitment toward the core (white arrows). **C** Photo of the tumor and a volumetric optoacoustic image (maximum intensity projection along y axis) acquired at 420nm. Three separate layers of dermis, rim and core are visible. **D** Spectrally-unmixed sO_2 image of the same tumor showing the presence of hypoxic core and its highly oxygenated feeding vessels. Scale bar - 1mm.

Figure 2. **A** A 3D stack of coronal slices extracted from the volumetric sO_2 map of the tumor. **B** A representative slice showing the presence of three distinct tumor sub-regions. **C** Radial sO_2 profile as a function of the distance from the tumor core (R^2=0.78). The calculated border between the rim and core is labeled by a dashed line. **D** *In vivo* sO_2 map of a different slice with its corresponding CAIX-DAPI histopathology **E** and CD31-DAPI staining **F** qualitatively matching the regions of enhanced oxygen supply and reduced oxygen supply. Scale bar - 1mm.

Figure 3. **A** sO_2 map of a sagittal slice of the tumor superimposed on an anatomical (single-wavelength) optoacoustic image. Segmentation into three sub-regions (rim, normoxic core and hypoxic core) is shown in the zoom-in. Scale bar - 1mm. **B** Average (with 1 standard deviation) sO_2 response of each sub-region to the dynamic oxygen challenge of a single animal (mouse #1). **C** The corresponding total hemoglobin signal exhibiting no significant variations in response to the oxygen challenge. **D** The corresponding voxel distribution plot presenting the percentile differences between the oxygen saturation for 100% and 20% O_2 (center line represents the median, whereas the upper and lower thresholds represent the 25^{th} and 75^{th} quartiles).

Figure 4. **A** Frequency map (single slice) of the hypoxic cycles in the tumor, as analyzed by HbR fluctuations and by sO_2 fluctuations. The HbR-based maps exhibit higher density of cyclic voxels in comparison to the sO_2-based maps. Scale bar - 1mm. **B** Power spectrum and the corresponding amplitude fluctuations of two representative voxels. One voxel (orange arrow in A) exhibits cyclic behavior in both HbR and sO_2 channels whereas the voxel labeled by a purple arrow only manifest cyclic behavior in the HbR channel. **C** distribution of vCF in all tumors reveals significant differences between the sO_2- and HbR-based analyses (n=6).

Figure 1

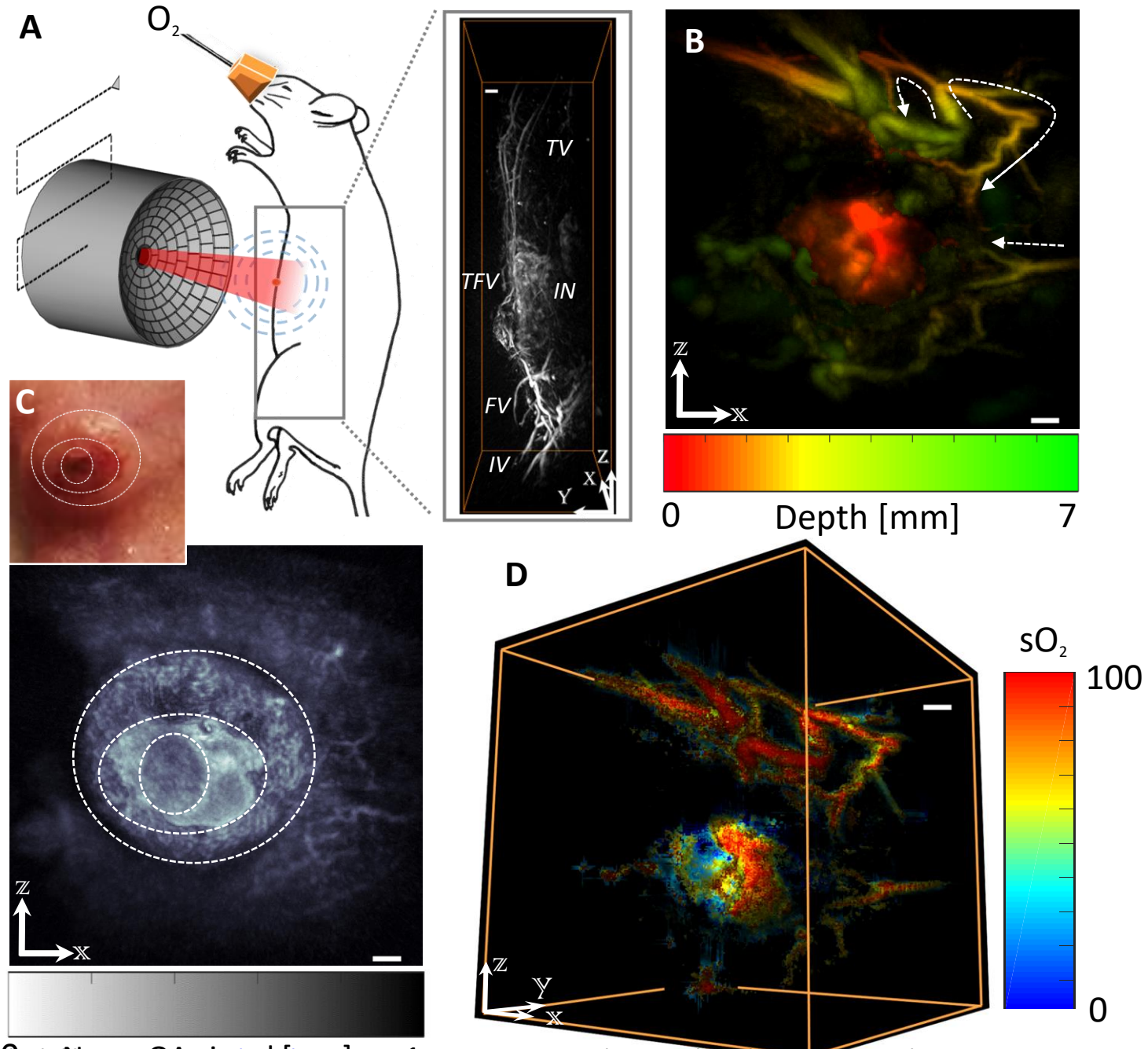


Figure 2

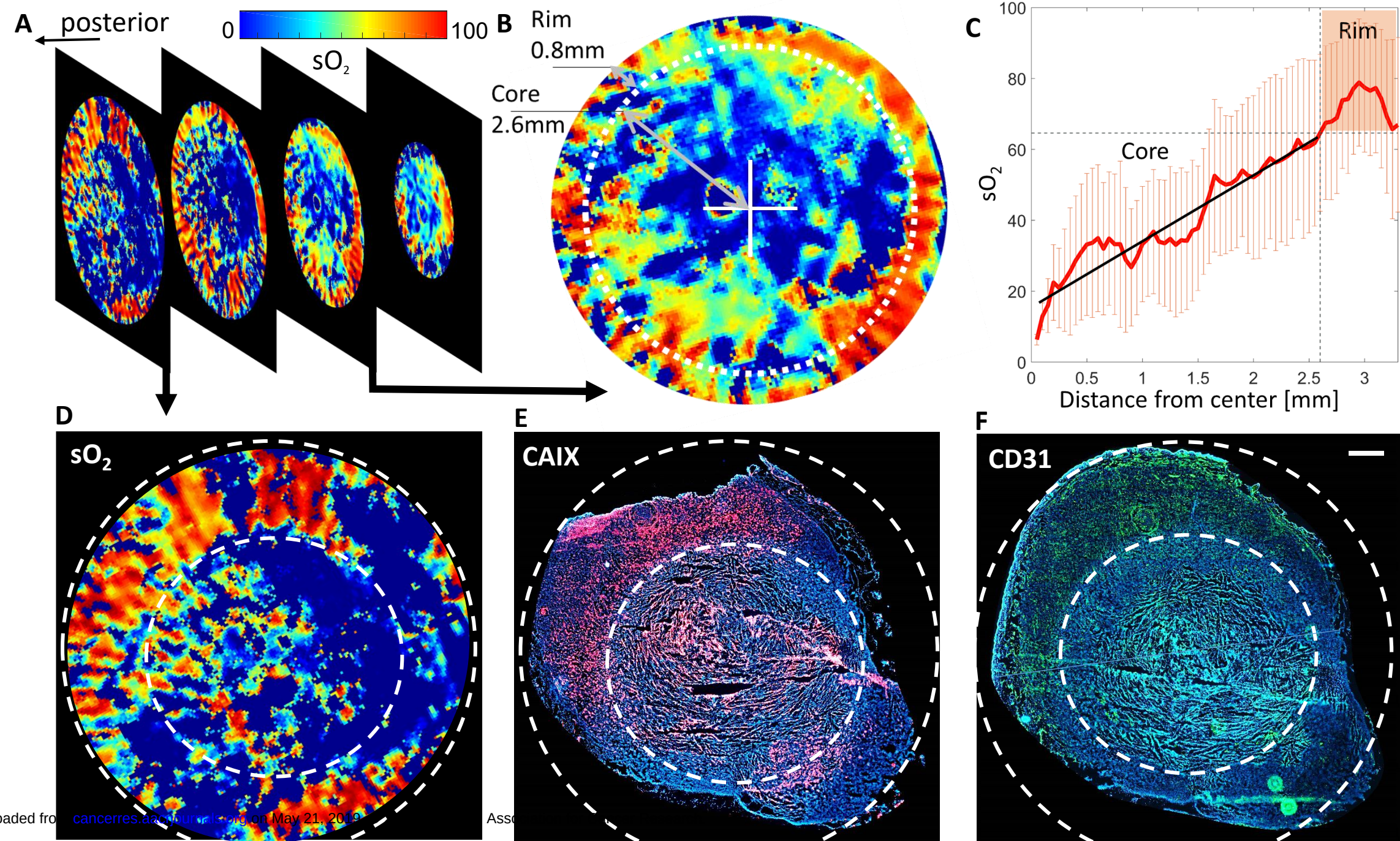


Figure 3

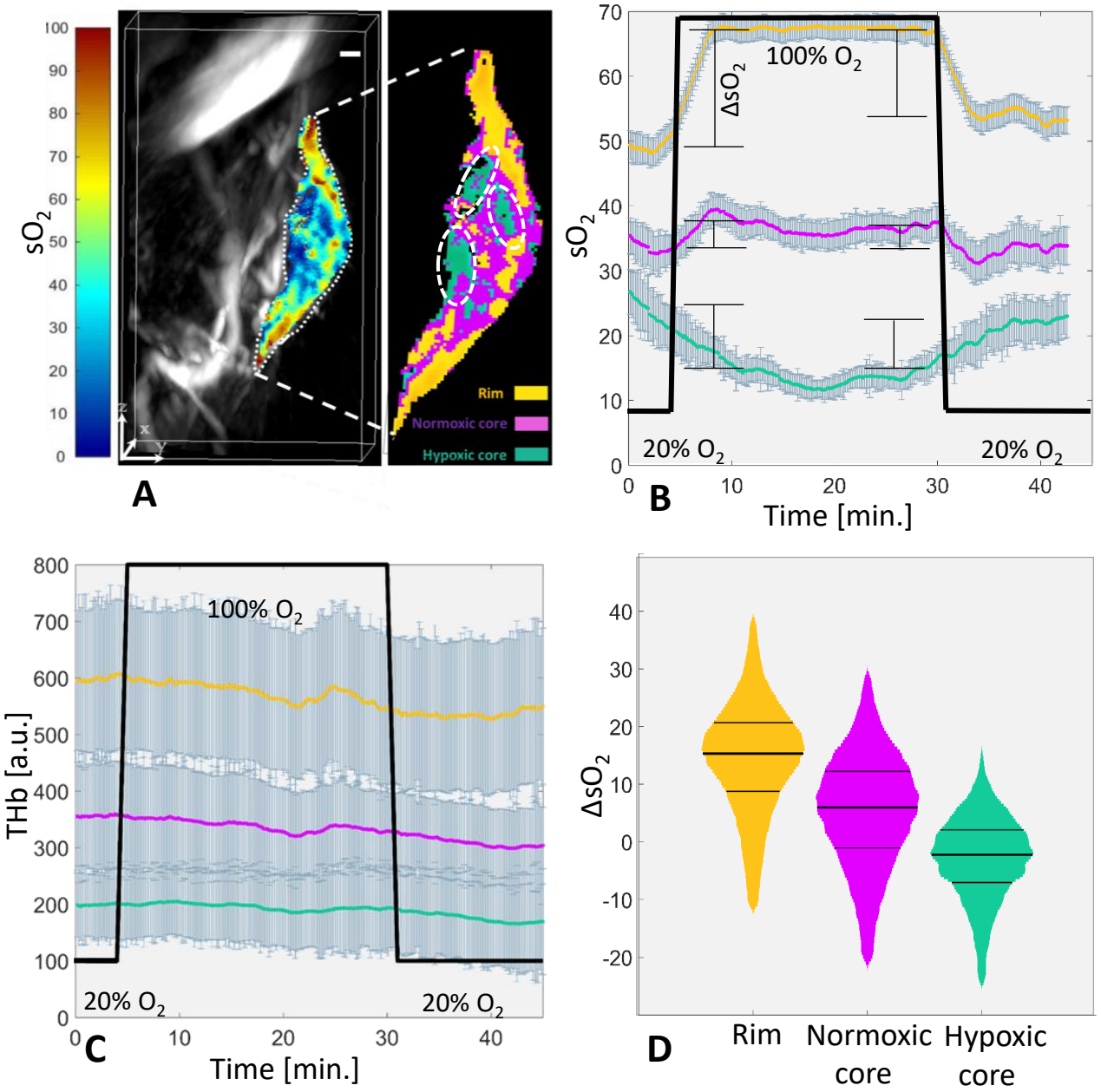
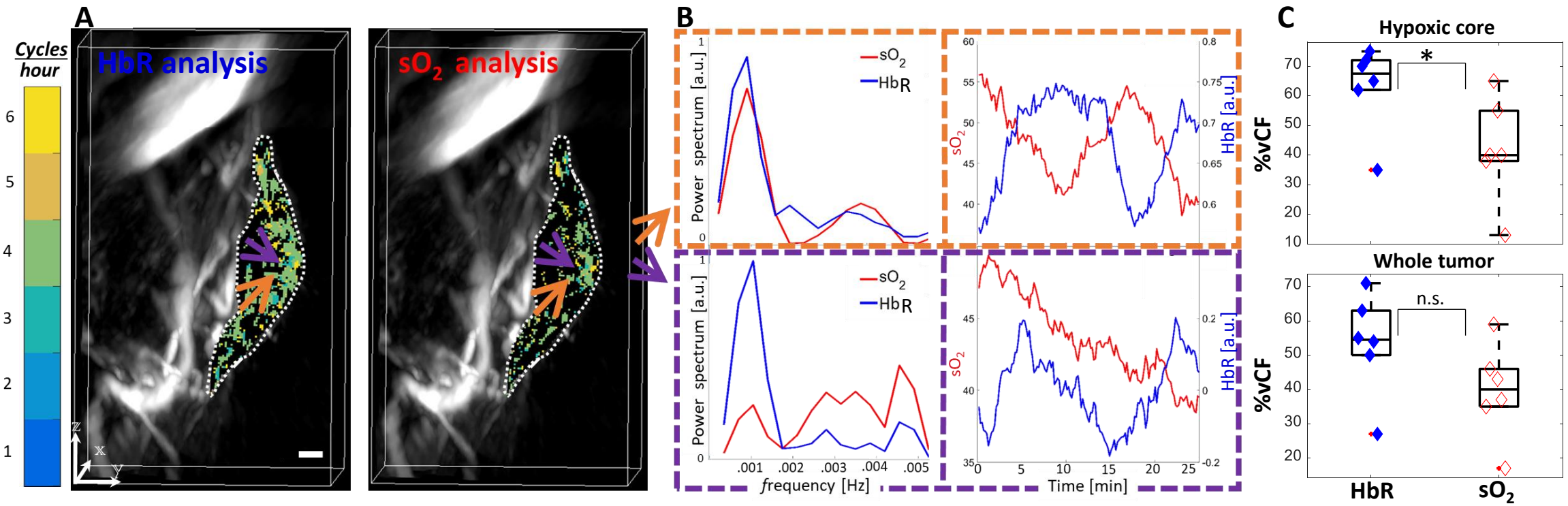


Figure 4



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