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Interstitial diffuse optical probe with spectral fitting to measure dynamic tumor hypoxia

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Abstract

Understanding the dynamic nature of tumor hypoxia is vital for cancer therapy. The presence of oxygen within a tumor during radiation therapy increases the likelihood of local control. We used a novel interstitial diffuse optical probe to make real-time measurements of blood volume fraction and hemoglobin oxygen saturation within a tumor at a high temporal resolution. This device was initially characterized and benchmarked using a customized vessel designed to control hemoglobin oxygen saturation and blood volume in a solution of blood with different concentrations of an oxygen scavenger, tetrakis (hydroxymethyl) phosphonium chloride. The optical device was found to consistently monitor the changes in oxygen saturation and these changes correlated to the concentration of the oxygen scavenger added. In near-simultaneous measurements of blood volume and oxygen saturation in tumor-bearing mice, the changes in blood volume fraction and oxygen saturation measured with the interstitial diffuse optical probe were benchmarked against photoacoustic imaging system to track and compare temporal dynamics of oxygen saturation and blood volume in a patient-derived xenograft model of hypopharyngeal carcinoma. Positive correlations between our device and photoacoustic imaging in measuring blood volume and oxygen saturation were observed.

1. Introduction

Tumor hypoxia, a state of oxygen deficiency in a tumor: either chronic or acute [1] is the sum of the effects of diffusion, perfusion and metabolic consumption of oxygen.

Understanding tumor hypoxia and its dynamics is a critical factor in cancer therapy.

Deoxyribonucleic acid (DNA) damage is the primary way by which tumor cells are killed by radiotherapy. Oxygen enhances the effects of sparsely ionizing radiation by “fixing” damage

to DNA. That is, damage to DNA is made permanent such that the cell eventually dies when it attempts to replicate. Otherwise, the cell could repair the damage and continue to propagate. The pioneering work of Gray, *et al.* [2] was one of the first to investigate the importance of oxygen tensions in radiotherapy. They observed that a greater regression was achieved in a solid tumor in mice when a mouse was breathing pure oxygen than air. They also noted an exception to this observation when the tumor was very large at the time of irradiation. Based on their findings, they concluded that the concentration of dissolved oxygen in the tissue at the time of irradiation was a factor in the response to radiotherapy.

Multiple attempts have been made to increase oxygen in solid tumors before or during radiotherapy. The United Kingdom Medical Research Council conducted a clinical trial in integrating a hyperbaric oxygen chamber to increase tumor oxygenation and sensitize the tumor during radiotherapy [3–6]. They saw positive gains in the local control and improved survival rate for carcinoma cases of the head and neck and cervix. There was some evidence that suggested a benefit in the case of carcinoma of the bronchus, but not of the bladder. They also reported undesired increased toxicity to normal tissue. The Danish Head and Neck Cancer Study Group performed a randomized study to evaluate misonidazole, a hypoxic cell radiosensitizer, in the treatment of larynx and pharynx carcinoma [7]. This study yielded some positive results especially in the case of female patients who saw better loco-regional control but encountered some setback in the side-effects produced by the drug used in the study. Accelerated Radiotherapy with Carbogen and Nicotinamide (ARCON) represents another strategy used to improve the efficacy of radiotherapy by simultaneously targeting tumor cell repopulation and hypoxia. ARCON therapy consists of several radiotherapy fractions per day to offset cell repopulation, breathing of oxygen-rich gas (carbogen: 95% oxygen plus 5% carbon dioxide) to minimize diffusion-limited hypoxia, and taking nicotinamide to increase perfusion in the tumor thereby reducing perfusion-limited hypoxia [8]. Preliminary results from a two-year study showed that advanced laryngeal cancer in a high proportion of patients could be controlled [9]. The results of a phase III randomized trial of laryngeal cancer showed a lack of local tumor control in advanced laryngeal cancer, but a significant benefit to regional tumor control rate with the same levels of toxicity. A key setback observed in several of these studies which attempted to oxygenate cancer tissue is that they concentrated on the chronically hypoxic cells that are already “marked for death” since chronically hypoxic cells in tumors are not proliferative in general. There is a greater need to control cells that are at intermediate levels of oxygen saturation since these are the cells that drive most of the tumor oxygen dynamics.

Measuring oxygen in a tumor and adapting radiation treatment in real time can improve the efficacy of radiotherapy and make it more patient specific. Many researchers have demonstrated that the dynamics of hypoxia in tumors is on the order of treatment timescales (i.e., minutes). Observations from Dewhirst [10] indicate oxygen cycling of a few cycles per hour to many hours or days. In another publication, Dewhirst, *et al.* [11] observed cycle times ranging from 20 to 60 minutes using microelectrodes in window chambers. The observations of Cairns, *et al.* [12] showed kinetics on the scale of minutes. Also, Baudelet, *et al.* [13] showed periods of approximately 20 minutes using the BOLD-MRI technique. We have previously demonstrated the presence of cyclic hypoxia with a regular periodicity in a bladder cancer xenograft [14, 15].

It was the focus of this work to present a novel and inexpensive use of optical fiber spectroscopy (OFS) [16] to measure tumor oxygen dynamics directly in a tumor during oxygen inhalation. OFS uses diffusing broadband light and fiber-optics to provide excellent time resolution and avoid spatial specificity while simultaneously measuring blood volume fraction and hemoglobin oxygen saturation in vivo. Herein, we demonstrate proof of concept using controlled environment benchmarking and compare longitudinal, near-simultaneous measurement of blood fraction and oxygen saturation in several xenograft models with measurements obtained from photoacoustic (PA) imaging. **Material and Methods**

2. Materials and Methods

2.1 Material:

2.1.1 Hemoglobin oxygen saturation measuring chamber—A customized chamber was designed and built to hold a solution of blood at room temperature and pressure while preventing any undesired exchanges of matter with its surroundings. A commercially available 130 ml transparent plastic container had three holes bored on its lid using a 3/16" drill-bit. An adhesive was used to bond a 1.5" long, 3/8" outer diameter pipe to one of the holes to couple it with the inlet airflow pipe. Another inch pipe was bonded to the second hole that would allow for secure sealing and unsealing of the chamber for air flow to its surroundings. Cork was glued on the third hole, and two parallel holes (3 mm apart) were drilled through it to allow for a tight fit of optical fibers through them. Figure 1 is a photo of the chamber and a schematic of the chamber.

2.1.2 Mice—A solution containing the FaDu cell line [17] (1:1 tumor:matrigel) was inoculated onto the bilateral hind flanks of ten nude mice (Hsd: Athymic Nude-Foxn1^{nu}) at 0.1 ml per flank. Upon tumor establishment, xenografts were used to perform near-simultaneous measurements of oxygen saturation with OFS and PA imaging. All mice were kept in the Association for Assessment and Accreditation of Laboratory Animal Care-approved Wisconsin Institute for Medical Research (WIMR) Animal Care Facility. Food and water were provided ad libitum. Animals were housed in specific pathogen-free rooms, in autoclaved, aseptic, microisolator cages with a maximum of four animals per cage. All studies involving the mice were carried out under an approved animal use protocol. Mice were monitored twice weekly until tumors developed.

2.1.3 Production of Optical probes—The optical probe and needle holder were designed and manufactured in-house at the Biomedical Engineering Department at the University of Wisconsin – Madison, WI. The probe consisted of two replaceable, hollow 1.5" long stainless-steel needles with a short bevel point on one end. These needles were set 3 mm apart, parallel to each other and held in place by drilled holes in a plastic resin. Two optical fibers with 400 μ m core diameters (Thorlabs, USA) were each stripped and cleaved using an optical fiber cleaver (Furukawa Electric Co. Ltd., Japan). Fibers were inserted through one end of each needle until they were flush with the beveled end. The optical probe was then screwed to a plastic arm which was capable of multiple degrees of freedom of movement. Figure 2 is a *solidworks*® schematic of this probe. Figure 4 is a picture of the

interstitial optical fiber probe inserted in the bulk of the tumor in a mouse on the stage of the Vevo® LAZR Photoacoustic imaging system (FUJIFILM VisualSonics, Inc.).

2.2 Experimental Method

2.2.1 Hemoglobin oxygen saturation measurement in blood drawn from a pig

—Tetrakis (hydroxymethyl) phosphonium chloride (THPC), 80% solution in water was used to scavenge molecular oxygen in solution. Heparinized blood harvested from a pig was placed in a 37 ° C heated incubation chamber for at least one hour prior to experiments. Ten ml of blood and 10 ml of deionized (DI) water were measured and emptied in the measuring chamber. This was placed on a magnetic stirring plate and stirred for one minute. An initial light spectrum was measured with the OFS probe. Medical grade oxygen (100% pure) was blown into the chamber while stirring the solution. This lasted for at least 10 minutes to ensure that hemoglobin was 100% saturated with oxygen. From this point onward, THPC was added to the solution in 0.1 ml increments via a syringe. The solution was stirred, and the light spectra measured thrice for hemoglobin oxygen saturation at each concentration of THPC. THPC was added until the blood was completely deoxygenated. This experiment was repeated two times, and a control experiment was carried out with 20 ml of DI water and no blood. All experiments were carried out in a dark room to prevent external light from interfering with OFS measurements. Figure 3 is a depiction of the setup for these experiments.

2.2.2 Near-simultaneous measurement of oxygen saturation using OFS probe and PA imaging in tumors—

Once a tumor on a mouse was palpable, (about 1 to 2 cm in diameter), the mouse was sedated with isoflurane and secured onto the stage of the photoacoustic system. A nose cone was placed on its nose for breathing controlled ratios of pure oxygen and air. The needles from the optical probe were inserted in the bulk of the tumor. B-mode ultrasound images were used to guide the needles into the tumor and to ensure that both needles were in the same plane for PA imaging. The settings on the Vevo® LAZR system were adjusted accordingly to obtain a PA image, a persistence value of 4 was set on the system for PA data acquisition. Figure 4 is the experimental setup showing the needles from interstitial probes inserted in the bulk of a tumor. The PA imaging laser operated at wavelengths of 750 nm and 850 nm, overlapping with the wavelengths used by the optical device. The OFS data was measured first followed by turning the optical instrument off and acquiring the PA data. The time delay between both techniques was about 8 seconds on average. Data was acquired for one hour at 1-minute intervals. During data acquisition, the ratio of oxygen to medical air was varied according to the graph in Figure 5. This was an attempt to exaggerate changes in hemoglobin oxygen saturation in the tumor for a good comparison in the two instruments. It should be noted that 21% of medical air is oxygen. So, at no point in time during experimentation was any mouse deprived of oxygen. The flow of isoflurane was also adjusted to ensure that the mouse's respiratory rate was held constant during the experiment.

At the end of the experiment, the anesthetized air was disconnected from the mouse. The mouse was put back in its cage for at least two days before the experiment was repeated on the tumor on its other flank.

2.3 Data Analysis

Mathematical fits for the spectra obtained from the optical device were modeled using a library of absorption coefficients [18, 19] at different wavelengths. A Matlab® 2018a script was written to minimize the diffusion equation model from Jacques and Pogue [16] onto the measured spectra to determine the biological composition of the medium [20, 21]. The work of Jacques [22] provides a detailed review of this spectral fitting routine. The red dots in Figure 6 are the experimentally obtained data from the spectrometer for a time point in a tumor, and the black curve shows the mathematical fit. See Figure S1 for similar spectra from the blood/water mixture at a concentration of THPC when the blood was fully oxygenated and when it was fully deoxygenated. This value was also adjusted to account for dilution as necessary. During the mathematical fitting process, random numbers were initially assigned to blood volume fraction (B), oxygen saturation (S), reduced-scatter coefficient (a'), and K. Using the aforementioned mathematical routine, the values of B, S, a' and K were iteratively changed until a set that resulted in the minimum value of the fitting routine were taken as the representative optical properties of the medium. The reduced-scatter coefficient here refers to the reduced-scatter coefficient at 500 nm wavelength such that $\mu'_s(\lambda) = a' \left(\frac{\lambda}{500nm} \right)^{-1}$, and K is the ratio of the collection efficiency of the measurement of the sample to that of the standard (Spectralon® Diffuse Reflectance Standard).

Raw data from the Vevo® LAZR system was converted into percent oxygen saturation and blood volume value data. An operator-selected region of interest (ROI) was generated to incorporate the area between the two ends of the optical fibers in the PA data. This region was to match the region of sensitivity as described in the tutorial by Kazanci and Jacques [25]. A dot product of a normalized Matlab® generated Gaussian window (that spanned the ROI) and the ROI was used to calculate the value of oxygen saturation and blood volume for this region in all the PA data.

The time series data from each technique in the near-simultaneous measurements of oxygen saturation and blood volume were compared by calculating the Pearson's correlation coefficient at the 95% confidence interval. The data from the PA imaging technique were interpolated to match the timestamps for OFS data. Pearson's correlation coefficients between the time series data of oxygen saturation from OFS and PA imaging were calculated. Similar calculations of the correlation coefficients were performed for the time series data for the blood volume using the OFS and PA imaging modalities.

A plot of blood volume fraction and hemoglobin oxygen saturation against time are shown in Figure 8. The analysis is capable of following hemoglobin saturation, blood volume, fat, water and melanin content over time. However, in this case, only blood volume and hemoglobin saturation are potentially dynamic. Since the probe is interstitial, melanin is not absorbing as it would be through the skin.

3. Results

Analysis of the spectra measured from the blood solutions yielded values of hemoglobin oxygen saturation, blood volume fraction, scatter coefficient and the value of K. The

changes in oxygen saturation strongly correlated with increases in the concentration of the oxygen scavenger (THPC) in the solution. Initial measurements of the solution showed that oxygen saturation was high in the solution but not at a 100% saturation. After stirring the solution in 100% medical grade oxygen gas, the oxygen saturation went up to 100%. The oxygen saturation generally stayed at 100% until THPC exceeded 2% by volume in solution. The oxygen saturation linearly decreased with the concentration of THPC thereafter. After the addition of approximately 5% of THPC, hemoglobin in solution was wholly desaturated. The blood volume measurements were between 5 and 10% of the volume of the solution throughout the addition of THPC. Figure 7 are graphs showing how oxygen saturation, blood volume fraction, scatter coefficient, and K changed with the addition of THPC and associated standard deviations from the three spectra that were measured at each concentration of THPC.”

The data acquired during the near-simultaneous measurement of hemoglobin oxygen saturation and blood volume using the interstitial optical probes and the Vevo® LAZR system for PA imaging were collected at one-minute intervals for one hour. A convolution of the time series with a rectangular window with a width of 3 was performed to smooth the data. Figure 8 shows the results from measurements performed on five tumors. Results from other tumors can be found on the supplementary material, Figure S3. Although tumors were inoculated on both rear flanks of the mice, some mice only produced tumors on one flank. Additionally, one mouse died during experimentation, and the data from that experiment was omitted in our analysis. The needles from the interstitial probes inserted in the tumors produced artifacts in PA images for some of the tumors that made it difficult to analyze and report results with confidence. Data from such images were also omitted.

The leftmost column of Figure 8 shows PA images of the tumor in the plane of the interstitial needles. The tumor is highlighted in red to distinguish it from the rest of the mouse. The darkened semi-circular regions within the bulk of the tumor illustrate the operator selected ROI from which the time series of oxygen saturation and blood volume for PA imaging analysis were obtained. See Figure S2. The regions coincided with the volume just distal to the tips of the interstitial needles. The acquired time series are plotted on graphs for each tumor in the middle and rightmost columns of Figure 8. The middle column of Figure 8 shows the smoothened blood volume plots of the time series obtained via PA in broken red lines and the time series obtained via OFS in solid blue lines. The rightmost column shows the smoothened oxygen saturation plots of the time series obtained via PA in broken red lines and the time series obtained via OFS in solid blue lines.

A correlative analysis was performed from the raw data of both the OFS and PA imaging techniques. The time series of both blood volume and oxygen saturation from the PA data were interpolated to match the timestamps for which the OFS data was acquired. Table 3.1 lists the 95% confidence interval of the Pearson’s correlation coefficients of the results from this analysis of the tumors. The closer the correlation coefficient interval is to 1, the stronger the positive correlation between both time series. On the other hand, if the interval is closer to -1, it indicated that the time series from both techniques trended in opposite directions.

4. Discussion

The results of the optical measurement of blood drawn from a swine using OFS demonstrated that the hemoglobin oxygen saturation, as measured by our optical device, strongly correlated with the addition of THPC in solution pass 2% concentration by volume. Prior to the addition of 2% of THPC, the measurement indicated that hemoglobin was at 100% saturation. This was because THPC first scavenged all molecular oxygen in solution before interacting with oxygen from hemoglobin since pure oxygen gas was initially blown through the solutions.

Blood volume measurements stayed mostly between 5 and 10%. Some of the large error bars in the early part of the measurement were because scavenging of oxygen molecules by THPC is not an instantaneous process. The fraction of blood volume measurement in DI water was zero throughout the addition of THPC as seen on the top right corner of Figure 7. The value of blood volume is the sum of oxygenated and deoxygenated hemoglobin. This implies that the oxygen saturation measured in this experiment, where only DI water was used due to noise. The ramification of this simultaneous measurement of blood volume and oxygen saturation in solution indicates that the oxygen saturation values can only be reliably interpreted when there is sufficient blood in solution. This can be illustrated with this example. Having one molecule of oxygenated and deoxygenated hemoglobin each will produce the same value of 50% oxygen saturation as would 10000 molecules of oxygenated and deoxygenated hemoglobin each.

The blood drawn from this pig was arterial blood collected via the carotid. This was well-oxygenated blood. Initial measurements of oxygen saturation were high but not 100%. Blowing medical grade oxygen gas through it and bringing to 100% oxygen saturation indicated that the hemoglobin molecules of the red blood cells still had an affinity for oxygen and were functional for this purpose. In a similar study, 70% oxygen saturation was achieved by blowing medical grade nitrogen gas through the fully saturated blood solution, and then bringing it back to full saturation by passing oxygen gas through the solution again.

The plot of the reduced scatter coefficient showed slightly lower values at lower concentrations of THPC than at higher values. This is consistent with the fact that the composition of the blood solution is initially comprised of oxygenated hemoglobin and later, deoxygenated hemoglobin. The plot of K value has a prominent rise and fall of values between 1.5 and 5% concentration of THPC. This coincides with the beginning of the desaturation of hemoglobin and when hemoglobin is fully desaturated.

The correlative analysis of both the blood volume and oxygen saturation time series of the OFS and PA technique showed significant positive correlations in measurements. The 95% confidence interval of Pearson's correlation coefficients are listed in Table 3.1. These intervals are mostly positive and significant, except for the case of M3R that were negative and not significant. The coefficient of correlation of oxygen saturation was negative and not significant for the case of M4L. These intervals were generally more positive for blood measurement than oxygen saturation. The reason for this is due to spatial inaccuracies in determining the sensitive volumes for comparisons. The signal for the optical device is

averaged over a volume, whereas the signal for the PA is averaged over a two-dimensional slice. Also, blood volume in an area has two degrees of freedom: blood entering an area and blood leaving that area. On the other hand, oxygen saturation has three degrees of freedom: saturated hemoglobin coming in with blood flow, saturated hemoglobin flowing out and oxygen consumption in the area.

During this set of experiments, the concentration of oxygen in the anesthesia infused gas that the mice were breathing was increasing over the first fifty minutes and then dropped to just medical air in the last ten minutes. One would expect the oxygen saturation measured to increase and then drop to normal following the trend in Figure 5. However, this was not the case. There was a general decrease in oxygen saturation measurement in both the OFS and PAI technique over the duration of experimentation. Although counterintuitive, this was consistent with observations of previous researchers [26, 27], where anesthesia impairs the animal's ability to efficiently exchange gas in the lungs due to paralysis from the anesthesia, and modified perfusion. The insertion of the optical needles in the interstitium might also have some effects on this trend.

The photoacoustic system uses the 750 nm and 850 nm wavelengths from a laser that cause tissue to thermally expand when it absorbs this light. This leads to the generation of ultrasound signals captured by the transducer. The signal is affected by the concentration of oxygenated and deoxygenated hemoglobin. Deoxygenated hemoglobin is more absorbing at 750 nm than oxygenated hemoglobin, and the opposite is true at 850 nm. A pair of linear simultaneous equations with known absorption coefficients of the two forms of hemoglobin is developed for the two wavelengths using the Beer law, and the percentage of oxygen saturation of hemoglobin can be calculated. This is the approach used by the Vevo® LAZR system to determine the value of percent oxygen saturation [28]. This PA system can reliably determine the relative value of oxygen saturation from time point to time point. However, it is not a good instrument to determine the absolute value of oxygen saturation. On the other hand, once one of the parameters of the OFS technique is known, the others can be fitted to produce reliable values for their concentrations. In the case of the optical device, all parameters were fitted relative to the value assigned to water as 65% of the tissue volume. The results in this experiment show that the technique of spectral fitting, applied to tumors, can concomitantly measure blood volume fraction and hemoglobin oxygen saturation. This was achieved by averaging over 3 mm of tissue that includes the microvascular spacing of a few hundred microns. Because of this spatial averaging, one is less subject to the risk of having a measurement confined to a necrotic zone in the microenvironment. Because of the very high time resolutions possible and the possibility for a long duration of measurements, this device allows one to investigate acute hypoxia, and without an expensive scanner.

This optical interstitial probe works on the same principle of OFS as the side firing optical probe of Yu, *et al.* [29], except that the side firing probe can be limited by the depth of the tissue under interrogation. This interstitial optical probe allows for interrogation of deep-seated tissue. Other competing interstitial probes such as the Oxylite [30] optical probe and Polarographic (Eppendorf) probes [31] may measure oxygen at too small a spatial scale (sensing oxygen within 10 microns near the tip). Also, the Eppendorf probe consumes oxygen, making a long duration measurement in the same location impossible. The

microenvironment of any tumor is quite heterogeneous [32]. So, poor results may be obtained if the electrodes are placed in a necrotic region of the tumor. For example, curve B of Figure 2 in Bussink, *et al.* [33] would be the same had the probe been in a necrotic region. Our spectral device averages hypoxia over a spatial scale of 3 mm, much larger than other devices and larger than the microvascular spacing. It is suggested that this spatial averaging would make for a more robust temporal measurement.

5. Conclusion

A diffuse optical probe was developed and has demonstrated that it is capable of simultaneously measuring hemoglobin saturation and blood volume in a blood solution and patient-derived mouse xenografts with a high temporal resolution. It has advantages over other interstitial probes by averaging over the microvascular spatial scales and enabling long duration and high time resolution measurements. The value to cancer research is the potential to acquire high-quality data for acute hypoxia studies and other time related and therapy response behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1: Mixing chamber (~ 130-ml volume) holding 10 ml pig blood and 10 ml water. Photo. (B) Schematic, showing optical fibers above the blood mixture.

Figure 2:

This a *solidworks*® schematic of the assembled interstitial optical probe. The stainless-steel needles and optical fibers are sandwiched between two pieces of plastic resin. There are the two rectangular pieces of silicon on both ends that are helping to keep the needles and fibers from slipping.

Figure 3:

Placed on a magnetic stirrer, is the chamber that holds a solution to be measured. The solution in this setup is blood drawn from a pig. The blue coated optical fibers emanating from the cork material on the lid of the chamber transmits light into the solution and carries light that has interacted with the solution to a spectrometer for digitization and calculation. The solution has a magnetic bar for stirring the solution.

Figure 4:

An anesthetized mouse is placed on the stage of a Vevo® LAZR Photoacoustic Imaging System and restrained with adhesive tape while breathing air containing the anesthesia isoflurane and different ratios of oxygen to medical air. The optical fiber inserted in the bulk of the tumor is lit by broadband of light from a light source.

Figure 5:

A graph showing the temporal variation in the ratio of pure oxygen to medical air that a mouse was breathing during each experiment. The broken red line indicates temporal variation in pure oxygen, while the solid black line shows the variation in medical air. Ratios were changed at 10 minutes interval during the one hour.

Figure 6:

A representative spectrum that was taken at a time point of probing a tumor (dots in red) to get the mathematical curve (line in black). The values obtained for B, S, 'a' and K correspond to blood volume fraction, hemoglobin oxygen saturation, the reduced scatter coefficient at a 500 nm wavelength respectively, and the constant K is the ratio of the collection efficiency of a sample measurement to the standard.

Figure 7:

These are four graphs showing changes in the optical properties of blood as the concentration of tetrakis hydroxymethyl phosphonium chloride (THPC) was changing. The plots on the top left, top right, bottom left and bottom right, show how hemoglobin oxygen saturation, blood volume, reduced scatter coefficient and the value of K changed with the concentration of THPC respectively.

Figure 8:

Results from near-simultaneous measurements of temporal variations in blood volume and oxygen saturation with interstitial optical fibers and PA imaging. The pictures on the leftmost column are highlighted images of the tumor in red from PA imaging. The dark semi-circular region in the bulk of the tumor, are the selected ROI from which the time series of blood volume and oxygen saturation of the PA technique were obtained. The middle column is made of graphs of the time series of blood volume obtained via PA (in broken red lines) and OFS (in solid blue lines). Similarly, the rightmost column is made of

graphs of the time series of oxygen saturation obtained via PA (in broken red lines) and OFS (in solid blue lines)

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Table 3.1:

These are 95% confidence interval of the Pearson's correlation coefficients between the time series of blood volume (second row) and oxygen saturation (third row) comparisons between the OFS and PA imaging techniques.

	M1R	M2L	M3L	M3R	M4L	M4R	M5L	M5R	M6R
Blood vol.	(0.466, 0.772)	(0.939, 0.978)	(0.522, 0.802)	(−0.594, −0.163)	(0.702, 0.882)	(0.748, 0.903)	(0.720, 0.890)	(0.554, 0.816)	(0.884, 0.958)
Oxy-Sat	(0.399, 0.736)	(0.761, 0.908)	(0.773, 0.914)	(−0.144, 0.358)	(−0.327, 0.178)	(0.488, 0.783)	(−0.187, 0.315)	(0.499, 0.788)	(0.296, 0.675)