

Physiological Monitoring in Diving Mammals

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LONG-TERM GOALS

The objective with this study is to develop and calibrate an invasive data logger to measure muscle O₂ saturation in large, freely diving whales. We intend to use this data logger to measure muscle O₂ saturation and determine how blood flow to muscle is altered during diving. These data will be important to determine if muscle blood flow is reduced during diving, and important to estimate how the dive response affects muscle N₂ levels and the risk of decompression sickness (DCS).

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OBJECTIVES

This project is separated into two aims:

Aim 1: Develop a new generation of tags/data logger for marine mammals that will contain a sensor to be implanted into the muscle. The logger will collect physiological data from muscle tissue in freely diving marine mammals. The sensor will be tested and calibrated in terrestrial mammals at Massachusetts General Hospital, Boston (see Appendix for a detailed description and current progress for the work by MGH).

Aim 2: The data logger will be tested in freely diving marine mammals in the field, and muscle O₂ saturation data will be collected.

Aim 3: Measure the concentration of aerobic/anaerobic enzymes, total myoglobin, and fiber type in muscle tissues of post-mortem stranded whales.

APPROACH

This project is separated into two aims: Aim 1a) Development of a new generation of tag/data logger for marine mammals that will contain a sensor to be implanted into the muscle. The logger will collect physiological data from muscle tissue in freely diving marine mammals. The sensor will be tested and calibrated in terrestrial mammals at Massachusetts General Hospital, Boston; Improve the ; Aim 2) The data logger will be tested in freely diving marine mammals in the field, and muscle O₂ saturation data will be collected; Aim 3) The aerobic and anaerobic enzyme and myoglobin levels in the muscle of selected species will be measured. The latter is important to convert O₂ saturation to O₂ content.

Aim 1: A near infrared spectrophotometer connected to a data logging device will be developed and used to measure myoglobin/hemoglobin O₂ saturation in freely deep diving whales (e.g. beaked whales, sperm whales, Fig. 1). The unit will be developed based upon the successful construction of an oximeter used in Weddel seals [1].

A delivery device will be fabricated to allow implantation of the optical probe into the muscle (Fig. 2). The flexible cable will allow the muscle to move freely, resulting in minimal discomfort. Initial experiments on terrestrial mammals and stranded or by-caught (post-mortem) marine mammals will assess the impact of the implantation to minimize the potential for inflammation and hematoma [2].

Aim 2: The data logger will be tested on a variety of diving marine mammals over 2 field seasons. We aim to perform trans-location experiments in Northern elephant seals (*Mirounga angustirostris*) with collaborators at University of California Santa Cruz (UCSC). This allows us to perform controlled field experiments and to determine if the data logger is able to collect the physiological data and to assure minimal impact to the animal.

Aim 3: Muscle tissues from deceased stranded whales (sperm and beaked whales) will be analyzed for aerobic/anaerobic enzymes, total muscle myoglobin concentration and fiber type.

WORK COMPLETED

Aim 1:

A modified version of the data logger (Fig. 1) was housed in a pressure vessel made as a dome out of aluminum. The dome was air filled, and remained at ambient pressure. This prevents electronic artifact caused by the pressure. In addition, not encasing the electronics in epoxy or in a non-conducting liquid allows for easy access to change batteries or modify the electronics. The housing was pressure tested to 3000 m without causing any leaks or affecting the electronics.



Figure 1. External data logger with muscle implanted ballistic containing the LED and photosensor



Figure 2. Data logger and peel-away catheter being used to implant sensor into the muscle



Figure 3. Sensor end housing LED and photo sensors before being implanted into the muscle

Aim 2:

We are planning trans-location experiments in Northern elephant seals (*Mirounga angustirostris*) with collaborators at University of California Santa Cruz (UCSC). The Northern elephant seal is a good research model as they commonly perform long and deep dives. Trans-location experiments have been performed by researchers at UCSC for over a decade (Andrews, Jones et al. 1997). There are several advantages to their experimental design. First, implanting the sensor in the muscles of seals will allow for assessment of possible tissue damage, important in assuring minimal physical impact on the large whales. Tissue damage will be assessed by visual observation. Second, as the pre-molting animals faithfully return to their haul out sites (Andrews, Jones et al. 1997), we will remove the device from the muscle and assess the impact of the implanted ballistic head. The knowledge from these experiments will guide us towards modifications for the tag and implantation process as well as minimize tissue damage to the larger whales.

Aim 3:

Muscle tissue will be collected from appropriate species and analyzed for concentration of aerobic/anaerobic enzymes, total concentration of muscle myoglobin and muscle fiber type by our colleagues at Baylor University (Dr. Trumble). The data obtained from these experiments will provide information of deep diving whales have an elevated capacity for exceeding their ADL, allow us to estimate the O₂ stores of the muscle and estimate the metabolic rate of the muscle during diving from the change in muscle O₂ saturation.

RESULTS

Aim 1:

Implantation device:

Figure 4 shows the delivery system with the peel away catheter holding the ballistic end. The red plastic discs that sit between the logger housing and the end allow us to adjust the implantation depth. The logger housing (Fig. 5) is pressed against the red discs. The entire delivery device is inserted into the rifle and fired towards the whale. The ballistic end penetrates the blubber and delivers the sensor into the muscle. A line attached to the delivery device allows us to remove the delivery device and catheter, leaving the sensor in the muscle and logger attached to the skin (Fig. 6 and 7). The logger is attached with superglue and pins that penetrate the skin.



Figure 4. The implantation device containing the tag, muscle oximeter sensor and implantation device.



Figure 5. Tag housing with subdermal pins. The housing is approximately 50 mm in diameter but can be varied depending on the size of the board. The housing has 3 components. Also shown is the muscle oximeter probe with 3 LEDs and photo sensor to estimate muscle O₂ saturation.

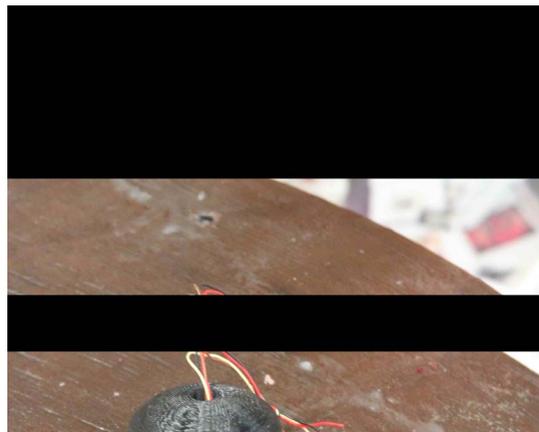


Figure 6. The oximeter sensor implanted into the muscle with the housing attached to the whale. The pins are shaped to hold the logger attached to the skin. The wires sticking out will be attached to the logger board and made long enough so they can slide in the interface between the muscle and blubber to avoid shearing.



Figure 7. Side view showing the depth of penetration of the pin. The number of pins can be varied and they can be attached with or without super glue, giving a wide range of attachment strength.

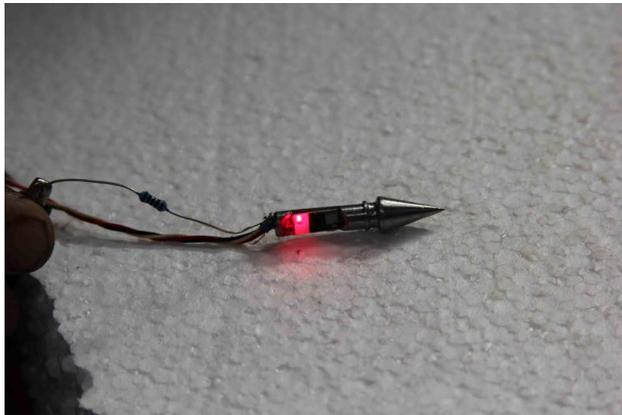


Figure 8. Photo showing that LED and photo sensors are not damaged following the ballistic implant.

All the components of the data logger (oximeter probe and data logger housing and electronics) and delivery system (custom-built rifle) for cetaceans have been tested either in cadavers or in freely diving elephant seals. We are currently modifying the delivery device and logger housing to allow us to deliver the tag and implant to a cetacean.

Aim 2:

Elephant seal Translocations:

In April 2013, 5 northern elephant seals were captured from the Año Nuevo (Table 1) and were instrumented with the oxygen sensor tag (Fig. 1-3). Ultrasound was used to determine blubber thickness and to ensure that the sensor end was placed percutaneously through the blubber and into major swimming muscle (*longissimus* or *lattissimus dorsi*). Seals were also fitted with a satellite and radio transmitter allowing us to track the animals at sea and land. Following surgery and until transport to the release site, seals placed in an enclosure that had a pool and haul-out area. While in the enclosure, the seals were observed every 1-2 hrs for signs of inflammation, infection, erratic behavior or changes in swimming gait. The initial three animals were allowed to recover and observed for ~48 hrs, but as no problems were noted the recovery duration was shortened to 24 hrs.

Following recovery, the seals were transported to the south end of Monterrey Bay and released. All animals returned to the Año Nuevo rookery in approximately 2-7 days (Table 1). The animals were again immobilized, and ultrasound confirmed that the sensor head was still embedded in the muscle following the transit back to Año Nuevo. In all 5 seals the instrumentation was easily and successfully removed, and no animal showed signs of infection, inflammation or trauma. In fact, 2 seals were found again a week after the probes and data loggers had been removed at Año Nuevo. There were still no signs of inflammation and infection at the implantation sites indicating that the implantation had no residual or harmful effects.

Data was recovered from each of the data loggers and is currently being analyzed. For the first two data loggers, it was discovered that the battery life was considerably shorter than expected; however, the first two seals provided data while recovering in the pool of ~18 hours. The data logger for the 3rd seal started prematurely while the seal was recovering from the implant procedure pool recovery data was again collected. For seal 4 and 5 we secured the magnetic switch and the loggers were activated once the animals were released. For these animals, we managed to collect free-diving data for between 8hr to 18 hrs before the battery power was drained. We are currently analyzing the data and hope to have this analysis complete in the spring 2014.

Table 1. Summary of seal morphometrics, recovery time, and time it took each seal to return after translocation.

Seal ID	Sex	Weight (kg)	Std length (cm)	recovery time (hrs)	return time (days)
6931	F	161	176	48	7
6939	M	198	193	48	2
G6841	M	169	186	48	4
G5944	F	173	180	8.5	6
G5882	F	162	166	4.5	6

Aim 3:

Aerobic and anaerobic enzyme activity levels and myoglobin concentration in marine mammals muscles.

In this study, we determined aerobic and anaerobic enzymatic activity levels as well as myoglobin concentrations for Northern elephant seals (*Mirounga angustirostris*) primarily, but additionally harbor seals (*Phoca vitulina*) and California sea lions (*Zalophus californianus*) at various decomposition states. Additionally, we performed a degradation time series in six elephant seals for enzymes and myoglobin protein. Degradation was allowed to proceed over a 48 hour time frame at two temperatures (4° and 37°C) in order to tease out whether post mortem tissue is a viable source of information for metabolic studies.

Enzymes investigated here, and common to the marine mammal physiology literature, are those related to aerobic and anaerobic metabolism, for example citrate synthase (**CS**) and lactate dehydrogenase (**LDH**) respectively. The myoglobin (**Mb**) concentration was quantified as an indicator of aerobic potential, and to allow conversion of O₂ saturation to content.

Methods

Animals: Skeletal muscle biopsies (*Longissimus dorsi*) used during this study were collected to illustrate samples routinely collected from fresh marine mammals (Table 2). Muscle samples were

collected from live animals during physiological research. These fresh biopsies will be placed immediately into liquid nitrogen and subsequently stored at -80°C until analyzed.

A fresh dead elephant seal (ES3289; The Marine Mammal Center, TMMC, Sausalito CA) was sampled at the time of death to demonstrate sampling skeletal muscle at zero minutes post mortem (T_0). A harbor seal (HS2192; TMMC) was sampled at 24 hours post mortem (T_{24}) to demonstrate a beach cast animal. All skeletal muscle ($T_{0,24}$) removed from necropsied animals was immediately placed into a -80°C freezer.

Preliminary Results

The degradation of enzymes (citrate synthase (CS) and lactate dehydrogenase (LDH) and the myoglobin (Mb) protein for one elephant seal (ES3289) and one mouse over 2 hours at two temperatures (4°C and 37°C) was measured. Results are shown below in Figs. 9-11. In addition, enzyme and Mb values were measured for animals fresh dead from stranding and live biopsy for baseline “normal” values (Table 2). In this study, the freshest samples were taken biopsied from the LD muscle in live elephant seals (FJ-11 and FJ-13). When compared to each other, biopsied elephant seals showed very different values for CS, LDH and Mb (Table 1) indicating fluctuation between animals of the same species and age class (both juveniles). Day-old harbor seal (HS2192) LD muscle was compared to three previous studies (Reed *et al.*, 1994, Kanatous *et al.*, 1999, Polasek *et al.*, 2006). CS and Mb values for our day old harbor seal were lower than recorded values, however our LDH value was approximately 3 times higher. This further indicates the instability of these enzymes during assays.

Further sampling and decomposition of 6 adult Northern elephant seal biopsy samples (Oct 2013) will add additional data to bolster these results. It is expected that data will be analyzed by December 2014.

Table 2: Values from dead and alive pinnipeds in this study

Species	Muscle	Animal Weight (kg)	Age class	Decomp status	CS (umole/min/g)	LDH (umole/min/g)	Mb (mg/g)
Elephant seal (ES3289)	LD	38.4	Pup	Fresh Dead	38 +/- .96	791.2 +/- 10.0	30.8 +/- 4.8
Elephant seal (ES3289)	Pec	38.4	Pup	Fresh Dead	26.6 +/- 1.2	1119.3 +/- 21.8	24.7 +/- 2.5
Elephant seal (FJ-13)	LD	Not available	Juvenile	Alive-biopsy	11.3 +/- .32	1279.8 +/- 96.3	25 +/- 2.3
Elephant seal (FJ-11)	LD	Not available	Juvenile	Alive-biopsy	31.6 +/- 1.0	2409.9 +/- 185	34 +/- 0.0
Harbor seal (HS2192)	LD	69	Adult	Dead- 1 day	18.4 +/- .56	3232.6 +/- 83.5	24.7 +/- 2.5

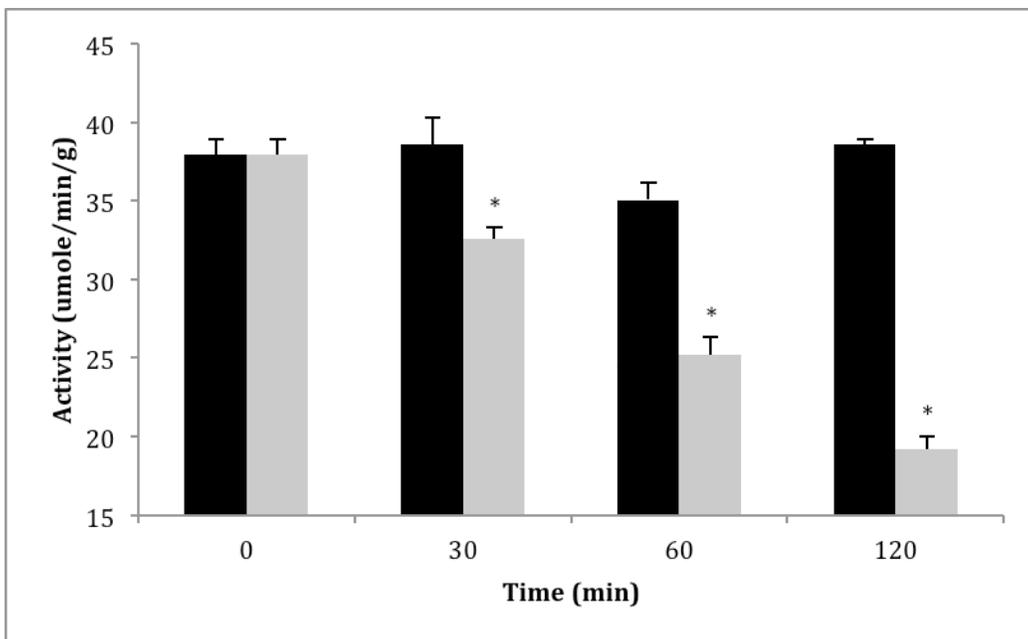


Figure 9. ES3289 CS (umole/min/g) degradation over 2 hours at two temperatures: 4°C (black) and 37°C (gray). Standard deviations represented by error bars.

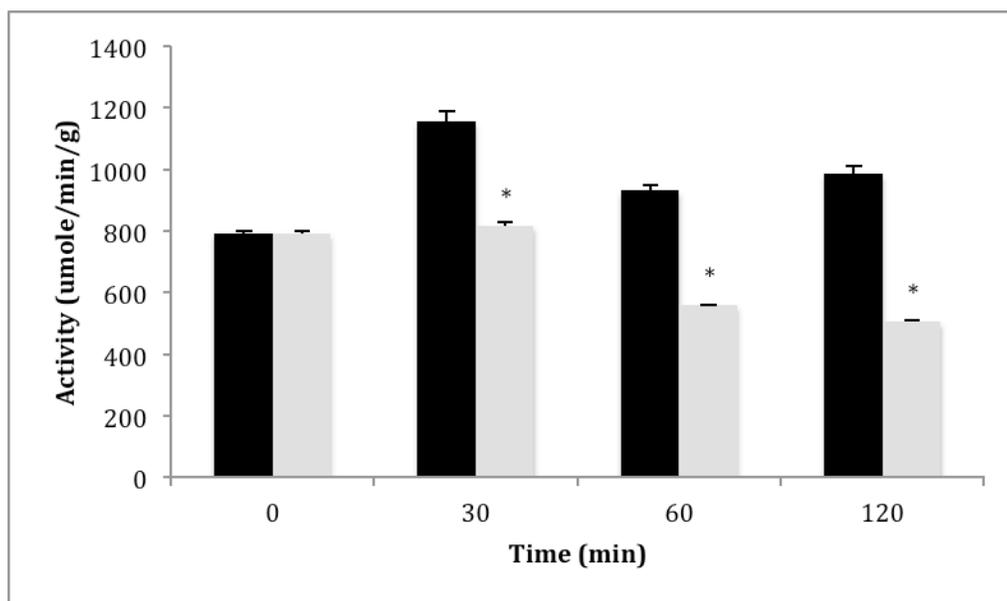
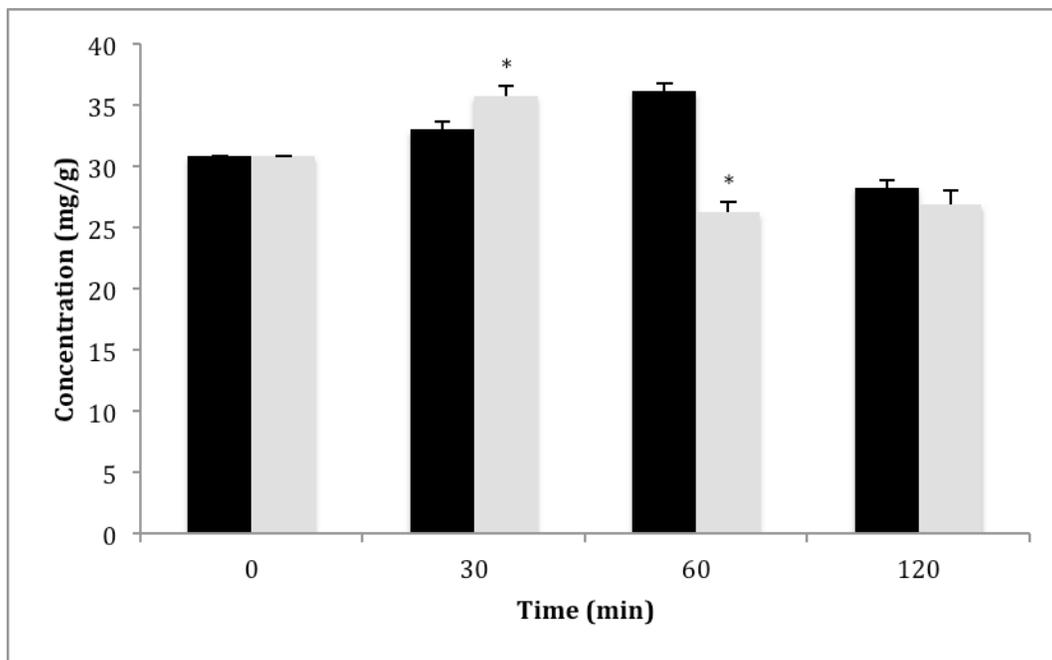


Figure 10. ES3289 LDH (umole/min/g) degradation over 2 hours at two temperatures: 4°C (black) and 37°C (gray). Standard deviations represented by error bars.



[Figure 11. ES3289 Mb (mg/g) degradation over 2 hours at two temperatures: 4°C (black) and 37°C (gray). Standard deviations represented by error bars.]

IMPACT/APPLICATIONS

This work is intended to enhance our understanding of how the dive response alters muscle blood flow and metabolism in large, freely diving whales. The results will provide information that will enable more realistic predictions of how the dive response varies during breath-hold diving at different activities. The study will also provide a new generation of data loggers that are able to collect physiological data in large whales with minimal impact.

Results from the completed study will help to improve our understanding about the physiology of marine mammals and improve modeling efforts that are aimed at estimating inert gas levels in breath-hold divers. The results can be used to determine how changes in dive behavior, from playback studies that measures avoidance patterns in deep diving whales, affect blood and tissue P_{N_2} levels. Thus, our results will enhance the fundamental understanding, interpretation and avoidance of the effect of anthropogenic sound, and enable knowledgeable decisions about sonar deployment, related training exercises and responses to NGO concerns. This should be of value to the US Navy Marine Mammal Program.

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APPENDIX

Oxygenation levels in muscle of free-diving mammals: Optical sensor development

1. Aims

We aim to monitor changes of oxygen levels in muscle of freely diving mammals, such as whales, by measuring changes in light absorption of oxygen-binding proteins in muscle tissue; namely, myoglobin and hemoglobin. Since the oxygenation level in tissue will be inferred from measurements of light absorption, discussions in this report and sensor development are about quantification of absorption coefficients. Our application in particular requires a compact, rugged, minimally invasive, calibration-free sensor able to quantify changes in the optical absorption of myoglobin and hemoglobin. In order to measure absolute changes in absorption without calibration, we are in the midst of implementing a near infrared (NIR), dual-wavelength, frequency-resolved method in reflectance mode to probe tissue oxygenation.

2. Background

Optical methods for absolute quantification of oxy-hemoglobin (HbO₂) and deoxy-hemoglobin (Hb) were developed by correlating NIR spectroscopy changes with an independent measurement of arterial hemoglobin saturation. Figure 1 illustrates the available spectroscopy methods for measuring optical properties of oxygenated tissues. Absolute determination of tissue optical properties, however, requires additional information over and above the detected intensity I at the tissue surface, which must then be combined with a model of light transport to derive absorption (μ_a) and scattering (μ_s) coefficients. The additional data can take many forms, e.g. the change in intensity with distance $I(x)$, the temporal dispersion of light $I(t)$ from an ultrashort input light pulse $I_0(t)$, or phase Φ , and modulation depth changes of intensity-modulated light I_o .

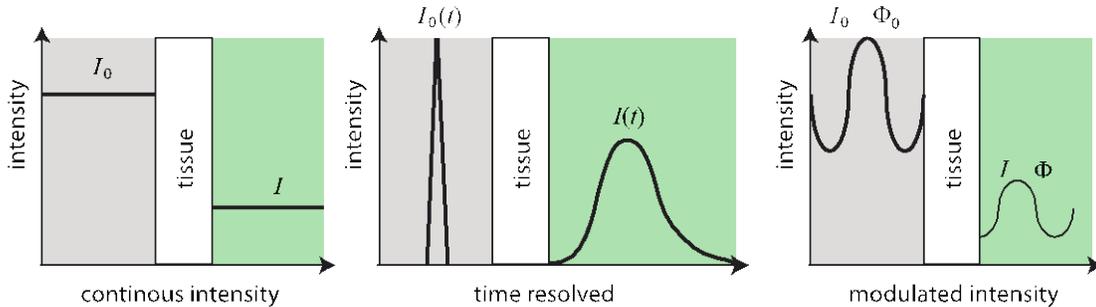


Figure 1: Basic principles of available spectroscopy methods for quantification of oxy-hemoglobin (HbO₂) and deoxy-hemoglobin (Hb). Continuous intensity method requires additional information to determine absolute optical coefficients. Time resolved and modulated intensity (or frequency resolved) methods allow absolute quantification of absorption and scattering coefficients.

We choose to implement a near infrared (NIR), dual-wavelength, frequency-resolved method in reflectance mode to probe tissue oxygenation. As compared to time resolved methods, frequency domain methods allow faster data acquisition times, which permit the study of dynamic processes, and relatively inexpensive equipment that is also smaller and demands less power.

At low frequencies f , the phase is approximately equal to

$$\Phi(r, f) \simeq \frac{r\pi f}{\nu} \sqrt{3 \frac{\mu'_s}{\mu_a}}, \quad (1)$$

where r is the separation between source and detector, ν is the speed of light in the medium, μ'_s is the reduced scattering coefficient. Measuring at two wavelengths λ_1 and λ_2 , the ratio of absorption coefficients is

$$\frac{\mu_1}{\mu_2} = \left(\frac{\Phi_2}{\Phi_1} \right)^2, \quad (2)$$

as long as the scattering coefficient remains the same. Besides the relationship stated in Eq. 2, there are many other mathematical relations that correlate phase (and modulation) to the optical properties of the medium. We chose the relationships above because of its simplicity. However, it will be experimental validation what determines which relation is going to be used.

3. Activities

The development milestones achieved during the past 12 months are the following:

- Integration of second light source
- Integration, testing and characterization of phase detection stage
- Reduction in size of hardware footprint
- Experimental validation of spectroscopic and analytical methods to quantify changes in optical absorption

Integration of second light source

A second light source, 660 nm (120 mW) laser diode (LD), was integrated into the sensor system as shown schematically in Fig. 1a. In addition, a radiofrequency (RF) switch and laser diode (LD) driver were also integrated to control the illumination of the volume interrogated at 808 nm (200 mW) and 660 nm. Figure 1b illustrates the interrogation process of a single wavelength in terms of electric signals. The intensity of the LD is modulated at 70 MHz producing a sinusoidal signal with specific amplitude and phase, Fig 1b blue curve. As the signal travels through the interrogated volume, the amplitude decreases and the phase shifts, Fig 1b red curve. The extent of the reduction in amplitude and the shift in phase are related to the optical properties of the volume, i.e., the absorption and scattering coefficients. Interrogation with a second wavelength gives additional amplitude reduction and phase shift information.

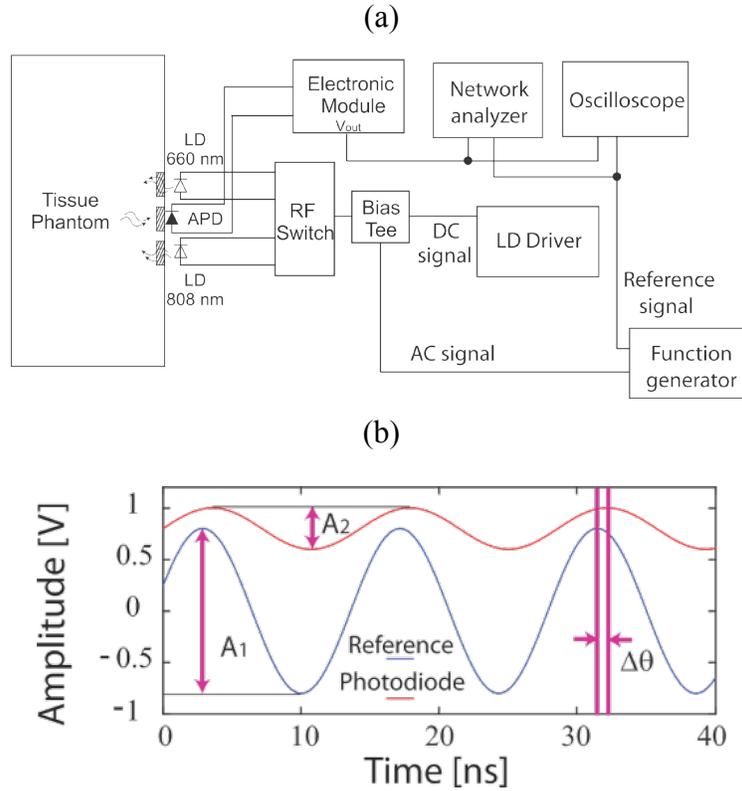


Figure 2. (a) Experimental set up: avalanche photodetector (APD); APD's electronic module; 660 nm and 808 nm laser diodes (LDs); LD driver; function generator to modulate LDs emission intensity; network analyzer for measuring phase shifts; oscilloscope. (b) Graphic representation of the measured reference (blue) and detected (red) signals: amplitude A_i and phase shift $\Phi = \Delta\theta$, where θ is the phase and Δ represents the difference between reference and detected signals.

Integration, testing and characterization of phase detection stage

The laboratory experimental set up is shown in Fig. 2. As tissue model or phantom we used water, India Ink and Intralipid™ to mimic the optical absorption and scattering properties of tissue. Absorption and scattering were varied by changing the concentration of India Ink and Intralipid™, respectively. The dimensions of the phantom container are large enough to make sure that light within the phantom propagates by diffusion only, Fig. 2a. Hence, our light sensor detects modulated diffused light only, whose signal is then compared to the reference signal by a network analyzer, Fig. 2b. A phase detector eventually substituted the network analyzer.

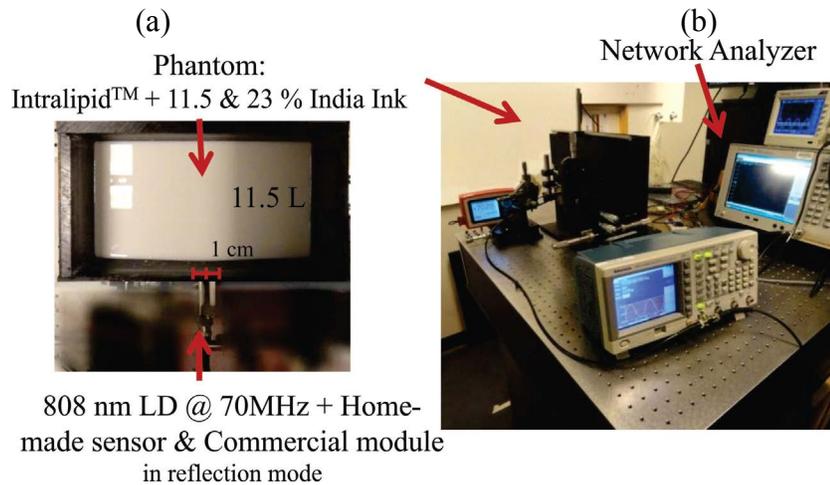


Figure 3. Laboratory experimental setup for testing, characterization and validation of optical sensor: (a) tissue phantom to emulate the absorption and scattering properties of biological tissue; (b) network analyzer to measure phase shifts in response to changes in tissue phantom optical absorption.

Phase shifts as a function of changes in the optical absorption of the tissue phantom are shown in Fig. 4a. Optical absorption was varied systematically by changing the concentration of Ink. The figure shows a 4% discrepancy between the phase detector and network analyzer performance. Figures 4b and c illustrate the difference in size between phase detector and network analyzer.

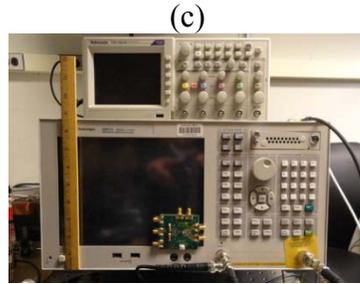
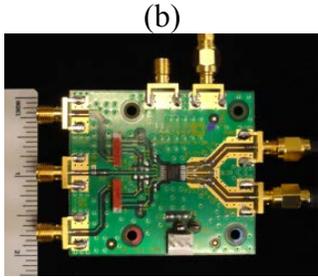
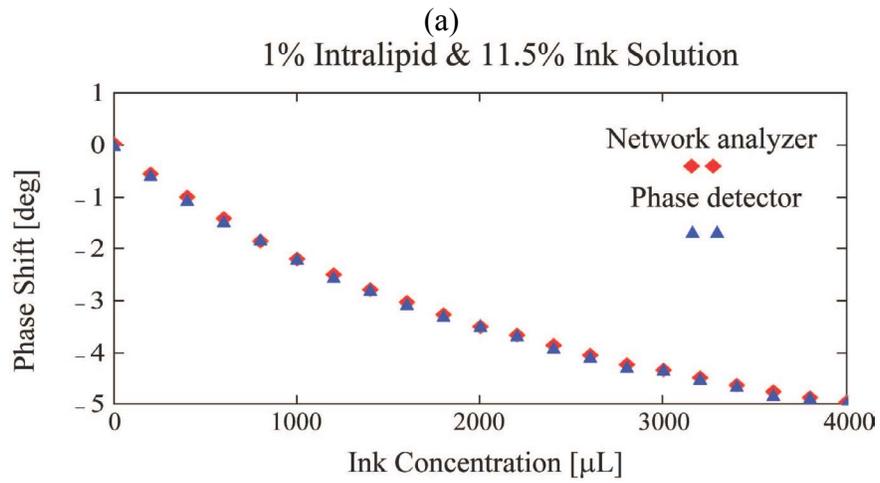


Figure 4. (a) Phase shifts in response to changes in tissue phantom optical absorption measured by (b) phase detector circuit and (c) network analyzer (Agilent Technologies E5071C). Phase detector measurements are within 4% of the network analyzer measurements.

Reduction in size of hardware footprint

Figure 5 shows the hardware components of the oxygen sensor as building blocks, which can be rearranged as needed.

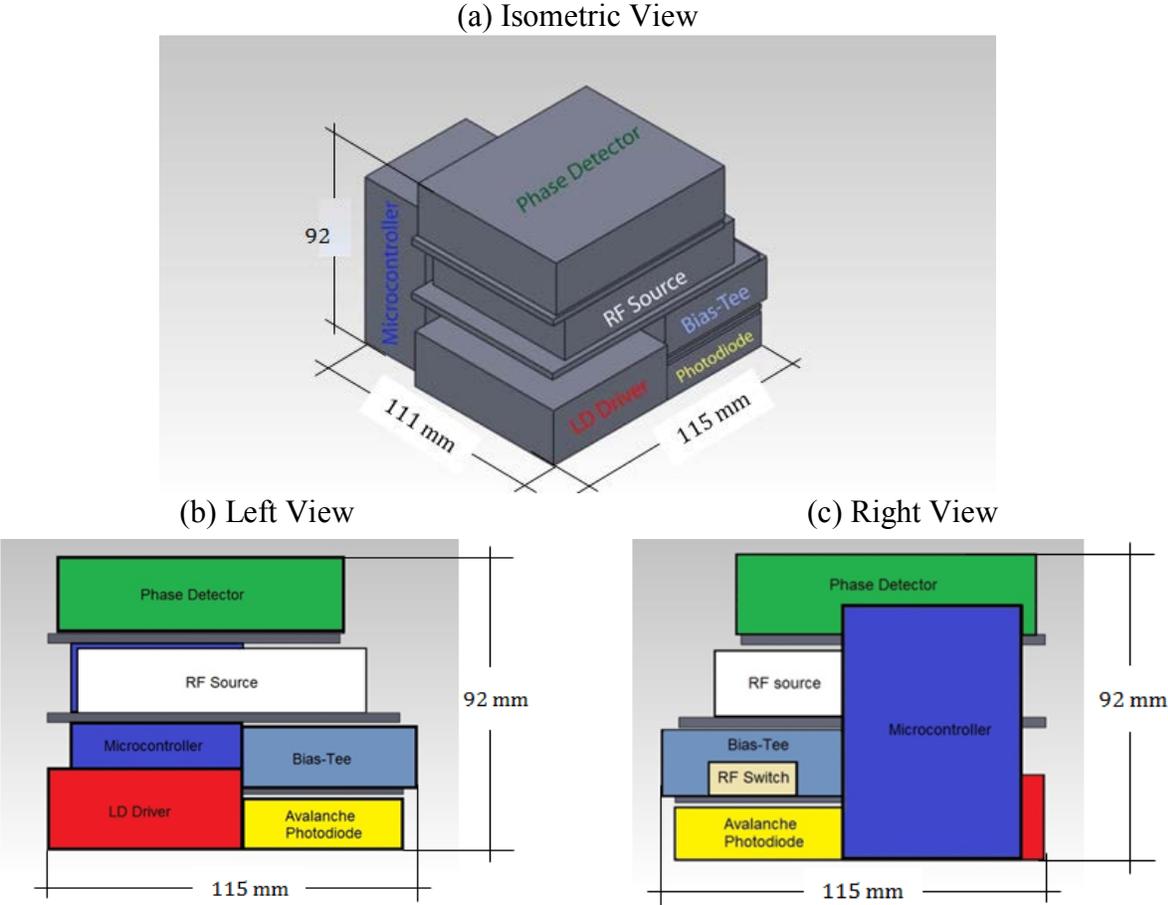


Figure 5. (a) Isometric, (b) left and (c) right views of system components illustrated as boxes and arranged vertically. As arranged, the components occupy a 115 mm by 111 mm by 92 mm volume.

Experimental validation of spectroscopic and analytical methods to quantify changes in optical absorption

As mentioned in the Background section, in light diffusion processes within tissue, the ratio of tissue absorption coefficients at two different wavelengths is proportional to the ratio of phases at the same two wavelengths squared, Eq. 2. Figure 5 shows measured phase ratios (symbols) as a function of changes in concentration of India Ink, and reference phase ratios (red line) as a function of changes in concentration of India Ink for different IntralipidTM and India Ink concentrations in solution. In Figure 6 the reference phase ratios are analytical values obtained from the squared root of the known absorption ratios. Measured phase ratios (experimental) are within 10% of the reference phase ratios (analytical). These measurements demonstrate that ratios of optical absorption coefficients can be inferred from phase measurements as proposed by Eq. 2.

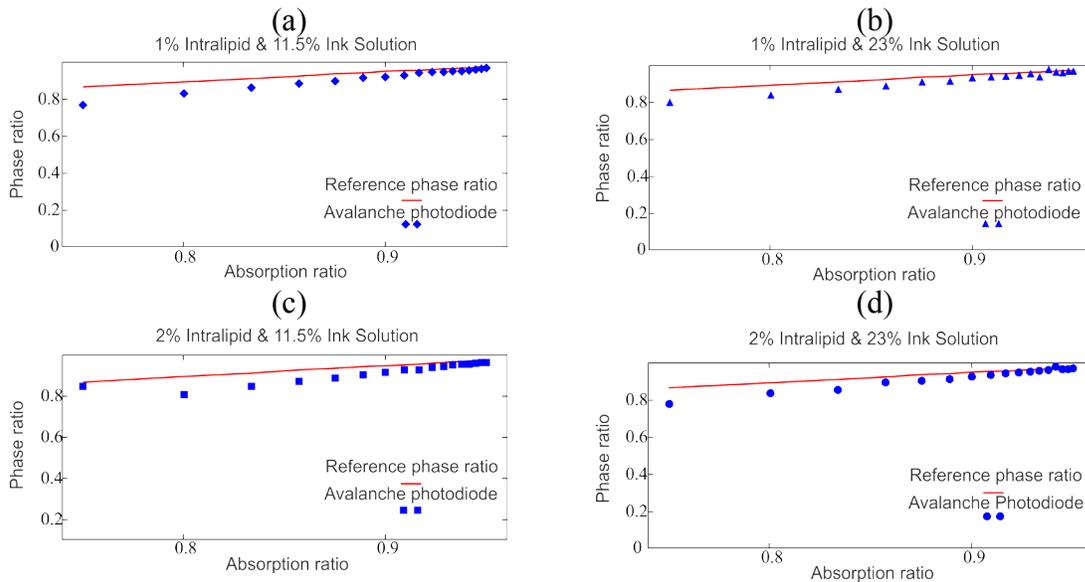


Figure 6. Experimental (symbols) and analytical (line) phase ratios as a function of absorption ratios, i.e. changes in concentration of Ink, for 1% and 2% IntralipidTM solution and 11.5% and 23% concentrations of diluted India Ink. Experimental values are within 10% of analytical values.

3. Summary

In summary, we

- completed the hardware implementation of the dual-wavelength frequency-resolved spectroscopy method, as summarized in the Background section,
- demonstrated in tissue phantoms that absolute quantitation of the ratio of absorption coefficients can be inferred from measurements of shifts in phase of modulated light illuminating the tissue phantom.

4. Ongoing Development

In the Development Stage corresponding to this report, the illumination of the samples was done with the LDs and light detector in contact with the tissue phantoms. This approach would require inserting the LDs into the muscle of the free-diving mammal. Since we are modulating the LDs at 70 MHz,

electronics must be properly shielded to avoid electromagnetic interferences. If the LDs and light detector were in the muscle, RF shielded cables would have to be used to communicate with the sensor system on the skin surface. The diameter of an RF shielded cable is about 7 mm. Inserting a bundle of 7 mm diameter cables is not a feasible, minimally invasive approach. To overcome this issue the light will be inserted to and collected from the muscle using optical fibers. As today, we are coupling the light of the developed and validated system into fibers.