DEVELOPMENT OF AN OPTICAL FIBER-BASED REDOX MONITORING SYSTEM FOR TISSUE METABOLISM

Wen Qi Zhang1* | Alexandra Sorvina2 | Janna L. Morrison3
Jack R. T. Darby3 | Doug A. Brooks2 | Sally E. Plush2 | Shahraam Afshar Vahid1

1Laser Physics and Photonic Devices Laboratories, School of Engineering, University of South Australia, Adelaide, South Australia, Australia
2Clinical and Health Sciences, Sansom Institute for Health Research, University of South Australia, Adelaide, South Australia, Australia
3Early Origins of Adult Health Research Group, Health and Biomedical Innovation, UniSA: Clinical and Health Sciences, University of South Australia, Adelaide, South Australia, Australia

*Correspondence
Wen Qi Zhang, Laser Physics and Photonic Devices Laboratories, School of Engineering, University of South Australia, Adelaide, South Australia, Australia.
Email: wenqi.zhang@unisa.edu.au

Funding information
Australian Research Council, Grant/Award Number: FT170100431; National Health and Medical Research Council, Grant/Award Number: APP1066916

Abstract
An optical redox ratio can potentially be used to report on the dynamics of cell and tissue metabolism and define altered metabolic conditions for different pathologies. While there are methods to measure the optical redox ratio, they are not particularly suited to real-time in situ or in vivo analysis. Here, we have developed a fiber-optic system to measure redox ratios in cells and tissues and two mathematical models to enable real-time, in vivo redox measurements. The optical redox ratios in tissue explants are correlated directly with endogenous NADH/FAD fluorescence emissions. We apply the mathematical models to the two-photon microscopy data and show consistent results. We also used our fiber-optic system to measure redox in different tissues and show consistent results between the two models, hence demonstrating proof-of-principle. This innovative redox monitoring system will have practical applications for defining different metabolic disease states.

KEYWORDS
fluorescence, optical fiber, redox, two-photon microscopy

INTRODUCTION

In cellular metabolism, glucose is sequestered by cells to generate adenosine triphosphate (ATP), through different energy pathways including glycolysis, the citric acid cycle and oxidative phosphorylation [1]. These energy pathways require the coenzymes FAD/FADH2 and NAD+/NADH to complete the oxidation–reduction “redox” reactions, and as a consequence of this process FAD and NADH yield significant fluorescent emissions. The primary source of NADH fluorescence is protein-bound NADH in mitochondria [2], with free NADH and NAD(P)H having minimal

Financial disclosure: Janna L. Morrison was funded by a NHMRC Career Development Fellowship (APP1066916) and an Australian Research Council Future Fellowship (Level 3; FT170100431).

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2022 The Authors. Journal of Biophotonics published by Wiley-VCH GmbH.
fluorescent emissions [3]. An optical redox ratio is a measure of fluorescent emission for FAD relative to NADH (defined as FAD/(FAD+NADH) [4], hereinafter referred as redox ratio), and can be used to define cellular metabolism.

The measurement of redox ratios is gaining significant interest for different diseases with disrupted metabolism, including cancer [5–11], Alzheimer disease [12] and heart disease [13, 14]. Mass spectrometry has been used to characterize FAD/NADH [15], but this measurement requires sample destruction. Optical imaging provides a method to monitor and characterize redox ratios in a noninvasive manner, but optical penetration is a major limitation. Thus, while fluorescence microscopy [16, 17] and confocal fluorescence microscopy have been used [18, 19], two-photon excitation fluorescence microscopy [20, 21] can provided improved spatial resolution at increased tissue depth, and fluorescence lifetime imaging [7, 9, 22, 23] can provided additional temporal information. A minimally invasive technology is required to facilitate better in situ and in vivo tissue imaging and redox ratio determination to accurately report metabolic status at increased depth within a tissue.

Here, we report on a minimally invasive fiber-optic-based platform together with a new mathematical model to effectively measure redox ratio in different tissues. This fiber-optic-based system utilizes the endogenous fluorescent molecules (FAD, NADH), instead of relying on additional chemical coating of the optical fiber for long duration in situ or in vivo operations [24, 25]. The optical fibers carry the excitation laser light to the tissue, collect the autofluorescence from the tissue and then carry it back to the detector. The fiber-optic system also has the advantages over bulky and expensive multi-photon microscope systems, as it can provide real-time measurements at increased depth while utilizing a highly portable configuration. Optical fiber fluorescence sensing [9, 22, 26] is adaptable for miniaturization, and here we provide a pathway to in situ and in vivo metabolic assessment. This new fiber-optic-based system, uses hardware and an algorithm to measure the redox ratio values in fat, liver, skeletal muscle and heart tissues, collected from either fetal or adult sheep as proof-of-principle for imaging tissues with different metabolic states.

## 2 | EXPERIMENT

### 2.1 | Animals and tissue collection

All experimental protocols were reviewed and approved by the Animal Ethics Committee of the South Australian Health and Medical Research Institute (SAHMRI) and abided by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes developed by the National Health and Medical Research Council. The ewes were housed in an indoor facility with a constant ambient temperature of 20–22°C and a 12-hour light/dark cycle. Each ewe was housed in an individual pen with ad libitum access to food and water and in view of other sheep. All investigators understood and followed the ethical principles outlined in Reference [27].

Four ewes and their fetuses were humanely killed via overdose with sodium pentobarbitone (8 g; Vibrac Australia, Peakhurst, Australia) at 119–121 days gestation (term 150 days). The uterus was removed by hysterotomy, and the fetus was removed and weighed. The maternal and fetal tissues were dissected, and samples of the heart, skeletal muscle, fat and liver were stored on ice-cold phosphate-buffered saline (PBS) and protected from light. To minimize variations from sample to sample, fetal and adult tissue samples were prepared at the same time. The spectral data was acquired from fetal and adult tissue samples in random order. The samples used for one-photon laser measurements were about 2 cm³ in volume, while the samples used in two-photon microscope were diced into thin slices.

### 2.2 | Two-photon determination of redox ratio

Two-photon imaging was performed on a Zeiss LSM 710 META NLO inverted microscope (Carl Zeiss, Jena, Germany) supplemented with a two-photon Mai-Tai®, tunable Ti:Sapphire femtosecond pulse laser (710.920 nm; Spectra Physics, Mountain View, CA). Endogenous fluorescence signals from heart, skeletal muscle, fat and liver tissues were recorded using a polychromatic multichannel detector (META spectral detector), MBS-InVis: MBS 690+, FW1: Rear, excitation wavelengths 750 and 810 nm and emission of FAD was detected at 548 nm (533–562 nm interval) and the emission of NAD(P)H was determined at 489 nm (474–504 nm interval) by exposure to two-photon illumination [31–33]. To avoid crosstalk
between these two fluorophores, different excitation wavelengths were used; 750 nm for NAD(P)H and 810 nm for FAD [34].

Quantitative measurements of fluorescence intensity were made on digital images. The intensity value of a pixel is related to the number of photons present in a field of view, making microscopy data quantitative [35]. The mean of NAD(P)H- and FAD-fluorescence intensity was defined using Zen 2011 software (Carl Zeiss, Jena, Germany). The total number of fields of view per section for each sample was two, and results were calculated from the intensity values of NAD(P)H and FAD. The redox ratio was calculated for the NADH and FAD signals using Equation (4).

### 2.3 One-photon determination of redox ratio

The main body of the fiber-optic setup is the fiber probe (Figure 1), which consists of three fibers in a bundle that have been glued together in a hypodermic needle. Two fibers are used for excitation; one connected to a 375 nm laser and the other to a 405 nm laser. The third fiber is used for the collection of the fluorescence emission and is therefore connected to a spectrometer. The sharp tip of the needle and the ends of the fibers are polished flat together.

The light coming out of one of the fibers forms a cone pointing away from the needle tip. Only the part of the tissue sample that is located within this cone volume will be illuminated by the light. This is the same for the collection of the fluorescent emission, that is, only the portion of the emission that scatters within the volume of the cone will be collected by the fiber. We measured each of the samples by hovering the hypodermic needle 2–3 mm above the tissue sample. Although the excitation and collection fibers were separated, the diameter of the fiber was only 220 μm and there was overlap between the cones intercepted by the tissue samples 2.3 mm away (Figure 1). The fibers used have a numerical aperture of 0.39, which corresponds to a half angle of the cone of ~23°. The overlapping area between excitation and collection fibers was calculated (Figure 2); with more than 80% of the excitation area covered by the collection cone at distances longer than 2 mm.

The benefit of separating excitation and detection fibers is to suppress autofluorescence generated along the optical path including free space optics and the optical fibers. The autofluorescent light exiting the fiber is directional. Only a small fraction of the light will be recaptured by the collection fiber. This is in contrast to what occurs when a single shared piece of fiber is used for excitation and collection. Furthermore, the emission from the tissue radiates in all directions, and it does not depend on the angle of incidence of the excitation beam.

In the fiber setup, the laser power and collected spectra are not calibrated to the excitation power, a known parameter when using the commercial two-photon microscope. Therefore, it is necessary to introduce a new approach to compare the measured results to those of the commercial two-photon system. The autofluorescence from the tissue in the visible wavelength range includes fluorescence signal from NADH and FAD molecules, where the 375 nm laser excites both molecules while the 405 nm laser excites mainly FAD molecules. Therefore, by employing the following normalization on the obtained data, it is possible to eliminate the dependency on the excitation power. Note that when using the fiber-optic setup with tissue samples, the area of detection cannot be carefully selected, which means that if the tissues are not homogeneous, the emission spectra will contain other fluorescent components. For

![Figure 1](image1.png) **Figure 1** Schematics of the fiber-based redox measurement system

![Figure 2](image2.png) **Figure 2** The overlap ratio is defined as the overlap area divided by the whole spot area
instance, we observed additional peaks in the emission spectra around 650–700 nm (red dotted curves in Figure 3 “1P Liver”) that were emitted by molecules such as chlorins and porphyrins that are not of interest in this current application [9]. To overcome this issue with the optical fiber setup, we excluded these wavelengths from normalization. We used a 450 nm long-pass filter in the fiber setup to remove the excitation light from the collected emission. To avoid these wavelength regions, we normalized all collected spectra to the spectral power within 460–580 nm, per Equation (1),

\[
\frac{\tilde{I}^{\text{em}X}(\lambda)}{I^{580\text{em}X}(\lambda) d\lambda} = \frac{\tilde{I}^{\text{em}X}(\lambda)}{\int_{460}^{580} \tilde{I}^{\text{em}X}(\lambda) d\lambda},
\]

where \(X\) can be 375, 405 (for fiber-based system), 750, 810 (for two-photon microscope system) denoting the excitation wavelength in nm and “em” indicates emission profile. Applying this normalization to all spectra, we obtained the following figures for both one-photon and two-photon measurements. It is worth mentioning that during one-photon measurements, five spots on each sample were measured while two fields of view on each sample were measured during two-photon measurements. The single-photon measurements were obtained immediately after the tissue samples were collected and the two-photon measurements were taken up to 3 hours later (after transporting the samples on ice to a different location).

All of the spectra in Figure 3 show distinct features and there is good correlation between both the single-photon and two-photon techniques. For example, when excited with 375/750 nm lasers, the fetal fat tissues emit a stronger blue color (460–490 nm; blue solid curves) than the adult fat tissues (red solid curves). Furthermore, when excited with 405/810 nm lasers, the peaks of the emission spectra of both fetal and adult fat tissues shift towards longer wavelengths. Similar observations were also made for the other tissues studied (Figure 3). To quantitatively compare the results of the optical fiber setup and those of the two-photon microscopy, it was necessary to derive a new mathematical model to connect the normalized spectra to the optical redox ratio.

3 | COMPARISON

In the previous section, we obtained the fluorescent spectra in Figure 3 for various tissue samples using both the fiber-optic single-photon excitation approach and the conventional two-photon microscope approach. To quantitatively compare the distinctive features in the spectra, we first defined ratios within two regions of interest as Equations (2) and (3),

\[
R_b = \frac{\int_{460}^{489} \tilde{I}^{\text{em}405}(\lambda) d\lambda}{\int_{460}^{489} \tilde{I}^{\text{em}375}(\lambda) d\lambda} \quad \text{or} \quad R_b = \frac{\int_{460}^{489} \tilde{I}^{\text{em}810}(\lambda) d\lambda}{\int_{460}^{489} \tilde{I}^{\text{em}750}(\lambda) d\lambda},
\]

**FIGURE 3** Spectral comparison of one-photon (top) and two-photon (bottom) excitation methods. The blue curves are the emission spectra obtained from fetal tissues, the red curves are the emission spectra obtained from adult tissues. The solid curves are the emissions excited by the 375/750 nm lasers while the dotted curves are the emissions excited by the 405/810 nm lasers.
Two 29 nm wide bands were taken from the spectrum where we saw the maximum difference between the emission spectra excited with 405/810 nm and the 375/750 nm laser. The 29 nm bandwidth was chosen based on the spectral resolution (separation between two points in wavelength) of the two-photon microscope system. The ratios were then calculated for all tissue samples (Figure 4).

The ratio plots show clear differences between tissues; with for example liver and fat tissues, showing similar ratios for single-photon and two-photon measurements. However, the fetal tissues had larger separations between $R_b$ (blue) and $R_g$ (green) than the adult tissues. While no differences between the adult heart vs fetal heart tissues or the adult skeletal vs fetal skeletal muscle tissues were observed, a comparison between adult heart tissues vs adult skeletal muscle tissues and fetal heart tissues vs fetal skeletal muscle tissue were observed, which could be related to the different metabolic activity of these tissues. To quantify the two methods, we plotted the $R_b$ and $R_g$ of the one-photon results vs the two-photon results and calculate the Pearson correlation coefficient between the results (Figure 5). A highly positive correlation of .92 was obtained showing that the measurements are comparable.

4 | A NEW MODEL FOR OPTICAL REDOX RATIO

4.1 | Two-laser system

The first definition of optical redox ratio was proposed by Britton Chance as $I_F/I_N$ [36], where $I_F$ and $I_N$ are
fluorescent emission intensities of FAD and NADH, respectively. This was extended by Shiino to the commonly used form of (hereafter referred to as the conventional model) [4]:

\[
R_e = \frac{I_F}{I_N + I_F},
\]

such that the optical redox ratio is bound between 0 and 1. However, in practice, the choice of spectral components for the equation is rather arbitrary. In general, the method employed by most researchers is one that uses two different excitation wavelengths with a short one to excite both FAD and NADH molecules and a long one to excite only FAD. Then spectral intensity at the peaks of excitation spectra were used for \( I_N \) and \( I_F \), respectively. When using two-photon microscopy, the power of excitation lasers is kept the same. However, when an optical fiber system is used in vivo, many parameters such as the movement of the fiber probe and alignment of the tissue against the fiber tips can change the collected spectral information. A better redox model is needed to eliminate the uncertainties in the measured results. To this end, we have derived a new optical redox ratio model from Beer–Lambert law to address these problems.

According to Beer–Lambert law, for \( N_Y \) mols per unit volume of fluorescent molecule (\( Y \) can be NADH or FAD), the number of photons \( N_Y \) that are emitted per unit length with M incident photons can be represented as

\[
N_Y = \eta_Y \left( 1 - 10^{-\alpha_X^Y} \right) M \approx \log(10) \eta_Y \alpha_X^YN_YM,
\]

where \( \eta_Y \) is the quantum yield, \( \alpha_X^Y \) is molar absorbance of \( Y \) molecule at excitation wavelength X. A first order approximation is applied since the molar density of the fluorescent molecule in tissues is small.

For each emitted photon, the probability of being in the range of \( \omega \) and \( \omega + d\omega \) is described by the probability density function (PDF) in the spectral domain, \( p_Y(\omega) \).

The intensity of the emitted photons from \( Y \) excitation with wavelength X can then be written as

\[
I_{em}^X(\omega) = N_Y h\omega p_Y(\omega)
\]

\[
= \log(10) \eta_Y \alpha_X^YN_YM h\omega p_Y(\omega)
\]

\[
= \log(10) \eta_Y \alpha_X^YN_Y I_{ex}^X \frac{\omega}{\omega^X} p_Y(\omega),
\]

where \( I_{ex}^X \) is the intensity of the excitation light at wavelength X, \( \omega^X \) is the angular frequency corresponding to wavelength X. For NADH or FAD, we define normalized spectra \( j_Y(\omega) \) as:

\[
j_Y(\omega) = \frac{I_{em}^X(\omega)}{\max(I_{em}^X(\omega))}.
\]

Using Equation (9), we can write the PDF as:

\[
p_Y(\omega) = \frac{j_Y(\omega)}{h\omega} \times \left( \int \frac{j_Y(\omega)}{h\omega} d\omega \right)^{-1}.
\]

The PDFs of NADH and FAD molecules can be obtained by measuring the emission spectra of NADH and FAD molecules individually and then applying Equations (9) and (10). Since the PDFs of the two molecules are used in the calculations, we plot them instead of the emission spectra in Figure 6.

Here, we use an approach that is similar to the conventional one, that is, we consider two excitation lasers at 375 and 405 nm. We also assume that the absorption of NADH at 405 nm and beyond is zero as it can be seen from Figure 7, which shows the absorption spectra of FAD and NADH molecules.

Using Equation (8), we can obtain the following emission spectra

\[
I_{F}^{em405}(\lambda) = 405I_{ex}^{405} \log(10) \eta_F \alpha_{F}^{405} N_F \frac{p_F(\lambda)}{\lambda},
\]

\[
I_{F}^{em375}(\lambda) = 375I_{ex}^{375} \log(10) \eta_F \alpha_{F}^{375} N_F \frac{p_F(\lambda)}{\lambda},
\]

FIGURE 6 Probability density function of FAD (green) and NADH (blue) emission as a function of wavelength
The total emission spectrum obtained following excitation with a 375 nm laser is the sum of $I_{em}^{375}(\lambda)$ and $I_{N}^{375}(\lambda)$:

$$I_{em}^{375}(\lambda) \approx I_{F}^{em375}(\lambda) + I_{N}^{em375}(\lambda), \quad (14)$$

while the total emission spectrum excited with 405 nm laser is

$$I_{em}^{405}(\lambda) \approx I_{F}^{em405}(\lambda). \quad (15)$$

Note that the emission spectrum of the NADH molecules overlaps with absorption cross section of the FAD molecules shown in Figure 7. The overall absorption of the FAD molecules in cells is rather small, and hence it does not cause significant depletion of the excitation light, which enables us to simplify the equations. We did not consider the re-absorption of NADH emission in this part of the work.

We integrate Equations (15) and (14) and obtain

$$\int I_{F}^{em405}(\lambda)d\lambda = 405 I_{F}^{em405} \log(10) \eta_{F} \alpha_{F}^{405} N_{F} P_{F}, \quad (16)$$

$$\int I_{em}^{375}(\lambda)d\lambda = 375 I_{em}^{375} \log(10) \eta_{F} \alpha_{N}^{375} N_{F}^{P} + 375 I_{em}^{375} \log(10) \eta_{N} \alpha_{N}^{375} N_{N}^{P}, \quad (17)$$

where

$$\mathcal{P}_{F} = \int \frac{P_{F}(\lambda)}{\lambda} d\lambda. \quad (18)$$

Redefining the normalized spectra as

$$\tilde{I}^{em405}(\lambda) = \frac{I^{em405}(\lambda)}{\int I^{em405}(\lambda)d\lambda} = p_{F}(\lambda) \frac{\lambda}{\lambda_{F}^{em}}, \quad (19)$$

and

$$\tilde{I}^{em375}(\lambda) = \frac{I^{em375}(\lambda)}{\int I^{em375}(\lambda)d\lambda} = \eta_{F} \alpha_{F}^{375} N_{F} P_{F} + \eta_{N} \alpha_{N}^{375} N_{N} P_{F},$$

the ratios of the two normalized spectra can be written as:

$$\frac{\tilde{I}^{em405}}{\tilde{I}^{em375}}(\lambda) = \eta_{F} \alpha_{F}^{375} N_{F} P_{F} + \eta_{N} \alpha_{N}^{375} N_{N} P_{F} = \eta_{F} \alpha_{F}^{375} N_{F} P_{F} + \eta_{N} \alpha_{N}^{375} N_{N} P_{F}.$$  

The quantum yield of NADH and FAD are 0.019 and 0.033, respectively [37, 38]. Therefore $\eta_{F}/\eta_{N} \approx 1.7$. The molar absorbance around 375 nm for NADH is 2.1 mM$^{-1}$ cm$^{-1}$ and FAD is 9.2 mM$^{-1}$ cm$^{-1}$, therefore $\alpha_{F}^{375}/\alpha_{N}^{375} \approx 4.4$. Replacing $\eta$s and $\alpha$s in Equation (23), we have

$$\frac{\tilde{I}^{em405}}{\tilde{I}^{em375}}(\lambda) = \frac{N_{F}}{N_{F} + \eta_{F} \alpha_{F}^{375} N_{F} P_{F} + \eta_{N} \alpha_{N}^{375} N_{N} P_{F}}, \quad (22)$$

where

$$p(\lambda) = \frac{P_{N}(\lambda)}{P_{F}(\lambda)}, \quad \mathcal{F} = \frac{\mathcal{P}_{N}}{\mathcal{P}_{F}}.$$  

Defining the Redox ratio as

$$R_{F} = \frac{N_{F}}{N_{F} + N_{N}}, \quad (25)$$

and rearranging Equation (24), we find

$$R_{F} = \frac{\tilde{I}^{em375}}{\tilde{I}^{em405}} \left( \mathcal{F} - 7.5 \right) - \frac{\tilde{I}^{em375}}{\tilde{I}^{em405}} \left( p(\lambda) - 7.5 \right). \quad (26)$$

Equation (27) shows a direct relationship between redox ratio and normalized emission spectra of FAD and NADH molecules. Using this equation, we can calculate...
the optical redox ratio without the need to calibrate to the excitation laser powers \( I_{em405} \) and \( I_{em375} \). This equation also gives us a gateway to directly compare the result from our single-photon fiber setup to the two-photon microscope system.

According to Equation (27), the optical redox ratio should be a constant across the whole wavelength span. However, due to the noise and errors in the measured spectra, the actual calculated redox varies with wavelength. Furthermore, Equation (27) has a singularity when the denominator goes to zero. It is important to choose a wavelength range far away from this singular point to obtain any meaningful values. For this work, we choose a range around 540 nm where the calculated optical redox value is relatively constant over that wavelength range for each sample.

To compare our model Equation (27) with the standard two-photon approach, we calculated the optical redox ratio using the spectral data from the two-photon microscope. In this way, we isolate the effects of the model from other influential factors such as differences in excitation wavelengths or responses of different spectrometers. The difference being observed here between the optical redox ratios will be due to the model itself.

We show that apart from the fetal liver and adult fat tissue samples, the data followed a near linear fit \( R^2 = 0.94 \) (Figure 8). The discrepancy between the optical redox values of fetal liver (solid circle) and adult fat (hollow diamond) tissue may be attributed to the endogenous background fluorescence signal from other autofluorescent molecules in the liver. With liver and fat tissue, the spectra (Figure 3) excited with 405 nm laser (dotted curves in the top row) showed additional peaks from 650 nm for fetal (blue) and adult (red) tissue, respectively. These peaks can also be seen in the two-photon spectra (in the bottom row of Figure 3), but are not as obvious as in the one-photon spectra due to low spectral resolution. These peaks influenced the normalization process, and this error exists in both the conventional method and our model. A potential solution for this problem is to insert a low-pass filter around 600 nm in both optical fiber and two-photon microscope setups to remove these peaks.

### 4.2 One-laser system

In the previous sections, we used two lasers and assumed that the 405/810 nm laser mainly excites the FAD molecule. However, it is not necessary to use two lasers to measure the redox ratio. In this section, we show how our model allows redox ratio measurement with only one excitation laser and one point on the emission spectrum.

To compare our model Equation (27) with the conventional method vs our model Equation (27) based on the two-photon spectra. The suffixes “F” and “M” in the legend denote fetal and adult tissue samples, respectively.

This enables the development of a portable system with photo-diodes instead of a spectrometer.

Rewriting Equation (22) for an arbitrary excitation wavelength \( X \), we have:

\[
I_{emX}^{emX}(\lambda) = \frac{I_{emX}(\lambda)}{\int I_{emX}(\lambda) d\lambda}
\]

\[
= \frac{\eta_{\lambda} \alpha_{X} N_{F} p_{F}(\lambda) + \eta_{\lambda} \alpha_{X} N_{F} p_{F}(\lambda)}{\lambda \eta_{\lambda} \alpha_{X} N_{F} p_{F} + \lambda \eta_{\lambda} \alpha_{X} N_{F} p_{F}}.
\]

This can be rearranged, to find \( N_{N} \) as:

\[
N_{N} = -\frac{\eta_{\lambda} \alpha_{F}^{X}\left(p_{F}(\lambda) - \lambda p_{F}^{emX}(\lambda)\right)}{\eta_{\lambda} \alpha_{N}^{X}\left(p_{N}(\lambda) - \lambda p_{N}^{emX}(\lambda)\right)}\cdot N_{F},
\]

which can then be substituted in Equation (26) to have

\[
R_{e} = \frac{1}{\frac{\eta_{\lambda} \alpha_{F}^{X}\left(p_{F}(\lambda) - \lambda p_{F}^{emX}(\lambda)\right)}{\eta_{\lambda} \alpha_{N}^{X}\left(p_{N}(\lambda) - \lambda p_{N}^{emX}(\lambda)\right)}} \cdot (30)
\]

We compare the values of redox ratios based on Equation (30) and the conventional model Equation (4) using the same spectral data from the two-photon microscope with the excitation wavelength of 750 nm.
Figure 9 shows a good linear fit between the results of the two models, and reduces the discrepancy for the fetal liver and adult fat tissue samples since in Equation (30) we only used spectra excited using 750 nm laser. The influence of the fluorescent emission of for example chlorin and porphyrin molecules was greatly reduced.

To further validate our model, we prepared a batch of model solutions with known concentrations of NADH and FAD. Single laser excitation approach was used to measure the redox ratios with excitation wavelength \( X = 375 \) nm and emission wavelength \( \lambda = 450 \) nm. We did not use a long-pass filter in this test since the 375 nm laser is far enough from the emission spectra. A linear relation can be found between the designed and the measured redox ratios (Figure 10), which confirms the validity of our model.

5 SINGLE-PHOTON OPTICAL FIBER APPROACH

Using the two-laser model Equation (27) and one-laser model Equation (30), we can calculate the redox ratios for all tissue samples using the single-photon (375/405 nm with fiber) and two-photon (750/810 nm with microscope) excitation spectra and compare them with each other. Both Equations (27) and (30) have undefined points when the denominators go to zero. Therefore, during calculation, we avoid these points by choosing the wavelength of 550 nm.

The relation between optical redox ratios calculated based on the single-photon and two-photon excitation spectra are plotted in Figure 11, in which (a) shows the results using the two-laser model while (b) shows the results using one-laser model. The error bars in the figure indicate the standard deviation of optical redox ratios due to a wavelength change over 50 nm around the center 550 nm. Both models show linear response between the optical redox ratios calculated using one-photon and two-photon excitation data. The two-laser model have a higher \( R^2 \) value of 0.89 in comparison to the one-laser model of 0.82. This outcome is expected as less information was used to compute the redox value in the one-laser model and therefore it is more sensitive to fluctuations in the spectra. And it is important to mention that the tissue samples used for single-photon and two-photon measurement were from the same animal but made in different areas of the tissue and measured hours apart due to the need of transporting the samples to two-photon microscopy lab. Therefore, variations in the redox ratio were expected. The general linear response between the single-photon and two-photon measurement indicates that the optical fiber single-photon approach can be used for redox measurement.
Herein, we propose an optical fiber-based single-photon excitation redox measurement approach accompanied by two optical redox ratio models to enable the design of a portable redox detection system. The first model involves the use of two lasers as the conventional approach to measure redox but without the need for laser power calibration. The second model allows the measurement of redox with only one laser that excites both FAD and NADH molecules and does not require power calibration as well.

We used the fiber-optic system to measure the auto-fluorescent signal in tissue samples from sheep and compared them to the signal collected using a two-photon microscope. Using the two redox models, we calculated the redox ratios from both single-photon and two-photon datasets and compared them to each other. Strong correlation was found between our approach and the conventional two-photon microscope approach indicating that the fiber-optic-based system together with the new model is a good candidate for in vivo redox measurement. In practice, the current fiber-optic setup can be adapted for vivo measurements. The optical fiber bundle can be delivered to the region of interest through a hypodermic needle, then the needle can be retracted with fiber tip left inside. The outside portion of the optical fiber can be fixed to the skin using adhesive tape to prevent retraction caused by movement.

With maturation of laser diode and photosensitive diode technologies, the fiber-optic system, together with the redox models, allow the development of small, cheap, portable and wearable redox monitoring system that can be deployed in large scale to gather real-time in vivo data. However, certain improvements in the engineering aspect of the fiber-optic system are required for future work. For example, optical coatings can be applied directly to the tip of the fiber to remove background fluorescence and filter out unnecessary spectral components to improve the performance of the system. Appropriate packaging designs of the optical fiber for specific applications can also improve the mechanical strength and durability of the system when in practical use.

**ACKNOWLEDGEMENTS**

Open access publishing facilitated by University of South Australia, as part of the Wiley - University of South Australia agreement via the Council of Australian University Librarians.

**CONFLICT OF INTEREST**

The authors declare no potential conflict of interests.

**AUTHOR CONTRIBUTIONS**

Wen Qi Zhang, Janna L. Morrison, Doug A. Brooks, Sally E. Plush and Shahraam Afshar Vahid were involved in conceptualization for design and application. Wen Qi Zhang was involved in writing—original draft, Janna L. Morrison, Shahraam Afshar Vahid, Doug A. Brooks and Sally E. Plush were involved in project management. Wen Qi Zhang, Alexandra Sorvina, Janna L. Morrison and Jack R. T. Darby were involved in investigation. All were involved in review and editing.
DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Wen Qi Zhang https://orcid.org/0000-0002-6524-6138
Alexandra Sorvina https://orcid.org/0000-0002-5918-3038
Janna L. Morrison https://orcid.org/0000-0002-8602-8519
Jack R. T. Darby https://orcid.org/0000-0001-7114-3920
Sally E. Plush https://orcid.org/0000-0002-9999-9154
Shahraam Afshar Vahid https://orcid.org/0000-0003-2322-9775

REFERENCES
[1] J. M. Berg, J. L. Tymoczko, L. Stryer, J. M. Berg, J. L. Tymoczko, L. Stryer, Biochemistry, 5th ed., W H Freeman, New York 2002.
[2] K. Blinova, R. L. Levine, E. S. Boja, G. L. Griffiths, Z.-D. Shi, B. Ruddy, R. S. Balaban, Biochemistry 2008, 47(36), 9636.
[3] P. D. Reiss, P. F. Zurendonk, R. L. Veech, Anal. Biochem. 1984, 140(1), 162.
[4] A. Shiino, M. Haida, B. Beauvoit, B. Chance, Neuroscience 1999, 91(4), 1581.
[5] Z. Zhang, D. Blessington, H. Li, T. M. Busch, J. Glickson, Q. Luo, B. Chance, G. Zheng, J. Biomed. Opt. 2004, 9(4), 772.
[6] S. Palmer, K. Litvinova, E. U. Rafailov, G. Nabi, Biomed. Opt. Express 2015, 6(3), 977.
[7] F. Poulon, M. Mehidine, M. Juchaux, P. Varlet, B. Devaux, J. Pallud, D. Abi Haidar, Sci. Rep. 2017, 7(1), 13995.
[8] J. H. Ostroder, C. M. McMahon, S. Lem, S. R. Millon, J. Q. Brown, V. L. Seewaldt, N. Ramanujam, Cancer Res. 2010, 70(11), 4759.
[9] D. Abi Haidar, B. Leh, M. Zanello, R. Siebert, Biomed. Opt. Express 2015, 6(4), 1219.
[10] K. Alhallak, L. G. Rebelo, T. J. Muldoon, K. P. Quinn, N. Rajaram, Biomed. Opt. Express 2016, 7(11), 4364.
[11] R. Tain, H. N. Xu, X. J. Zhou, L. Z. Li, K. Cai, Adv. Exp. Med. Biol. 2016, 832, 923, 401.
[12] D. G. Smith, R. Cappai, K. J. Barnham, Biochim. Biophys. Acta – Biomembr 2007, 1768(8), 1976.
[13] J. L. Morrison, A. Sorvina, J. R. T. Darby, C. A. Bader, M. C. Lock, M. Seed, T. Kuchel, S. E. Plush, D. A. Brooks, J. Biophotonics 2017, 11, e201700242.
[14] A. Sorvina, C. A. Bader, M. C. Lock, D. A. Brooks, J. L. Morrison, S. E. Plush, J. Biophotonics 2017.
[15] P. Arpino, Mass Spectrom. Rev. 1989, 8(1), 35.
[16] J. M. Levitt, A. Baldwin, A. Papadakis, S. Puri, J. Xylas, K. Münger, I. Georgakoudi, J. Biomed. Opt. 2006, 11(6), 064012.
[17] C. Mujat, C. Greiner, A. Baldwin, J. M. Levitt, F. Tian, L. A. Stucenski, M. Hunter, Y. L. Kim, V. Backman, M. Feld, K. Münger, I. Georgakoudi, Int. J. Cancer 2008, 122(2), 363.
[18] B. R. Masters, A. Kriete, J. Kukulies, Appl. Optics 1993, 32(4), 592.
[19] J. Pawley Ed., Handbook of Biological Confocal Microscopy, 3rd ed., Springer, New York NY 2006.
[20] S. Huang, A. A. Heikal, W. W. Webb, Biophys. J. 2002, 82(5), 2811.
[21] W. L. Rice, D. L. Kaplan, I. Georgakoudi, PLOS One 2010, 5(4), e100075.
[22] D. S. Kittle, F. Vasefi, C. G. Patil, A. Mamela, K. L. Black, P. V. Butte, Sci. Rep. 2016, 6, 1.
[23] W. Q. Zhang, J. L. Morrison, J. R. T. Darby, S. E. Plush, A. Sorvina, D. A. Brooks, T. M. Monro, S. A. Vahid, Nanophotonics Australasia 2017, International Society for Optics and Photonics, Melbourne, Australia 2018 p. 104564R.
[24] W. Chudyk, C. Sotolongo, E. Mueller, Environ. Monit. Assess. 2014, 186(1), 415.
[25] A. Mayevsky, B. Chance, Science 1982, 217(4559), 537.
[26] D. Grundy, J. Physiol. 2015, 593(12), 2547.
[27] J. R. T. Darby, A. Sorvina, C. A. Bader, M. C. Lock, J. Y. Soo, S. L. Holman, M. Seed, T. Kuchel, D. A. Brooks, S. E. Plush, J. L. Morrison, J. Biophotonics 2020, 13, 3.
[28] J. L. Morrison, A. Sorvina, J. R. T. Darby, C. A. Bader, M. C. Lock, M. Seed, T. Kuchel, S. E. Plush, D. A. Brooks, J. Biophotonics 2018, 11, 3.
[29] J. L. Morrison, A. Sorvina, J. R. T. Darby, S. L. Holman, A. Sorvina, S. E. Plush, M. Massi, D. A. Brooks, J. L. Morrison, J. Biophotonics 2021, 14(4), e20200322.
[30] J. Hou, H. J. Wright, N. Chan, R. Tran, O. V. Razorenova, E. O. Potma, B. J. Tromberg, J. Biomed. Opt. 2016, 21(6), 60503.
[31] K. Blinova, C. Combs, P. Kellman, R. S. Balaban, J. Microsc. 2004, 213(Pt 1), 70.
[32] A. Diaspro, G. Chirico, M. Collini, Q. Rev. Biophys. 2005, 38(2), 97.
[33] M. E. Dickinson, C. W. Waters, G. H. Bearman, R. Wolleschensky, S. Tille, S. E. Fraser, Multiphoton Microscopy in the Biomedical Sciences II, International Society for Optics and Photonics, San Jose, CA 2002, p. 123.
[34] J. L. Morrison, J. Cell Biol. 2009, 185(7), 1135.
[35] B. Chance, B. Schoener, R. Oshino, F. Itshak, Y. Nakase, J. Biol. Chem. 1979, 254(11), 4764.
[36] A. Baici, R. Joppich-Kuhn, P. L. Luisi, A. Olomucki, M. O. Monneuse-Doublet, F. Thomé-Beau, Eur. J. Biochem. 1978, 83(2), 601.
[37] S. D. M. Islam, T. Sudsorf, A. Penzkofer, P. Hegemann, Chem. Phys. 2003, 295(2), 137.

How to cite this article: W. Q. Zhang, A. Sorvina, J. L. Morrison, J. R. T. Darby, D. A. Brooks, S. E. Plush, S. Afshar Vahid, J. Biophotonics 2022, 15(4), e202100304. https://doi.org/10.1002/jbio.202100304