RESEARCH ARTICLE

Accurately calibrated frequency domain diffuse optical spectroscopy compared against chemical analysis of porcine adipose tissue

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Abstract
Frequency domain diffuse optical spectroscopy (fdDOS) is a noninvasive technique to estimate tissue composition and hemodynamics. While fdDOS has been established as a valuable modality for clinical research, comparison of fdDOS with direct chemical analysis (CA) methods has yet to be reported. To compare the two approaches, we propose a procedure to confirm accurate calibration by use of liquid emulsion and solid silicone phantoms. Tissue fat (FAT) and water (H2O) content of two ex vivo porcine tissue samples were optically measured by fdDOS and compared to CA values. We show an average H2O error (fdDOS minus CA) and SD of 1.9 ± 0.2% and −0.9 ± 0.2% for the two samples. For FAT, we report a mean error of −9.3 ± 1.3% and 0.8 ± 1.3%. We also measured various body sites of a healthy human subject using fdDOS with results suggesting that accurate calibration may improve device sensitivity.

KEYWORDS
calibration, chemical analysis, diffuse optical spectroscopy, porcine adipose, tissue lipid, tissue water

1 | INTRODUCTION

Frequency domain diffuse optical spectroscopy (fdDOS) is a well-established clinical research technique capable of non-invasively analyzing tissue optical properties and chromophore parameters oxyhemoglobin (HBO₂), deoxyhemoglobin (HBR), water (H₂O), and lipid (FAT). fdDOS has been previously applied in a wide range of studies such as breast cancer research [1–5], sports medicine [6, 7], and critical care medicine [8–12]. One driving force behind fdDOS development has been meeting needs in modern clinical research as a non-invasive alternative or supplement to existing healthcare devices. However, to the best of our knowledge, comparison of fdDOS against direct chemical analysis (CA) has yet to be reported.
While the stability and precision of fdDOS instruments has been thoroughly studied [13–16], there are few resources discussing instrumental accuracy. Generally accepted practices validate new fdDOS devices by comparison to older, more established generations using a common set of phantoms [16]. Yet, the origins of optical properties for calibration phantoms often lack secondary validation or have sparse documentation. Much work in the optics community has been devoted to performing large multicenter trials for defining optical standards [15, 17–19]. However, this method can be labor and time intensive, potentially requiring the participation of multiple instruments, operators, and laboratories making it inconvenient for rapid and routine characterization of phantoms. Other studies have correlated fdDOS to clinical “gold-standards” such as magnetic resonance imaging (MRI) or dual-energy x-ray absorptiometry (DXA) [6, 20, 21]. Still, even ubiquitous gold-standards such MRI and DXA have undergone comparison to CA methods [22–24]. As a straightforward, gravimetric approach, CA is able to provide direct compositional verification for certain extractable tissue components such as H₂O and FAT [25, 26].

We first propose a series of steps to verify accurate calibration of an fdDOS instrument through usage of a liquid emulsion and solid silicone phantoms. Next, tissue H₂O and FAT quantification by fdDOS and CA of ex vivo porcine samples are compared. Lastly, we noninvasively examine the composition of various in vivo human body sites using fdDOS. While we exclusively utilized fdDOS, proposed procedures to verify accurate calibration and conduct comparisons to CA could potentially be applied to other types of quantitative diffuse optics devices.

## 2 METHODS

### 2.1 Optical instrumentation

fdDOS is a noninvasive optical technique operating in the near-infrared (NIR) spectral window (approximately 650–1000 nm) used to estimate optical properties and composition of turbid media. The technology behind fdDOS has been extensively detailed in previous reports [14, 27–30]. In review, fdDOS is fundamentally based on frequency-domain photon migration (FDPM) wherein optical laser sources are intensity modulated at frequencies in the MHz to GHz regime. Calibrated amplitude decay and phase delay of the detected signal at each discrete laser wavelength can be fit to the P₁ semi-infinite approximation for the radiative transport equation calculating optical absorption (μₐ) and reduced scattering (μₛ') [31–34]. A power law can then be utilized to extrapolate the scattering profile to all wavelengths in the NIR [14, 35]. By combination of broadband reflectance with FDPM techniques, μₐ across the NIR spectrum can be obtained allowing for the estimation of chromophore concentrations utilizing known extinction coefficients [14].

For our fdDOS device, laser sources centered at 659, 690, 791, and 829 nm were coupled using a custom optical fiber bundle which combined individual 400 μm optical input fibers to a common output. Modulation of the lasers (50–500 MHz) was driven by a vector network analyzer (TR1300/1, Copper Mountain Technologies, Indianapolis, Indiana) while an avalanche photodiode (APD S12023-10 with C5658, Hamamatsu Photonics K. K., Hamamatsu City, Japan) was utilized to detect the modulated light. A tungsten-halogen lamp (HL-2000-FHSA, Ocean Optics Inc., Largo, Florida) was used as a broadband source and coupled by a 2.5 mm diameter fiber bundle comprised of 50 μm optical fibers. Diffuse reflectance was collected using a 1 mm solid-core fiber (R Specialty Optical Fibers LLC, Williamsburg, Virginia) terminating to a commercial spectrometer (HS2048XL-U2, Avantes, Apeldoorn, Netherlands). Reflectance was corrected using a Spectralon standard (SRS-02-020, Labsphere Inc., North Sutton, New Hampshire). Data analysis was performed using MATLAB (MathWorks, Natick, Massachusetts).

### 2.2 Calibration of fdDOS

While the continuous-wave component of fdDOS utilize reflectance standards, determining the IRF of the FDPM component involves more complex procedures, detailed in previous reports [14, 27, 36]. In summary, for an FDPM measurement, the detected amplitude will contain effects both from photon interactions with the sample as well as instrumental factors, such as optical and coaxial cable coupling losses. Likewise, the measured phase delay will be inflated by instrumental components such as optical fibers and electrical traces:

\[
A_0(\mu_a, \mu_s', \rho, f) = A_{IRF}(f)A(\mu_a, \mu_s', \rho, f) \tag{1}
\]

\[
\phi_0(\mu_a, \mu_s', \rho, f) = \phi_{IRF}(f) + \phi(\mu_a, \mu_s', \rho, f) \tag{2}
\]

where A₀ and φ₀ are the measured, uncalibrated amplitude, and phase, respectively, A_{IRF} and φ_{IRF} are the amplitude and phase components of the IRF, A, and φ are the pure amplitude and phase contributions from the sample, and f is the frequency. In practice, A_{IRF}(f) and φ_{IRF}(f) are commonly characterized by performing a measurement on a known sample (KS), such as an optical calibration phantom [16, 17], A(μₐ, μₛ', ρ, f) and φ(μₐ, μₛ', ρ, f) due to absorption and scattering interactions in
the calibration phantom can be predicted using a model of photon propagation, such as the semi-infinite approximations for the radiative transport equation [32–34]. The IRF factors in Equations (1) and (2) can then be solved:

\[
A_{\text{IRF}}(f) = A_0(\mu_a, \mu'_a, \rho, f)A(\mu_a, \mu'_a, \rho, f)^{-1}
\]

(3)

\[
\phi_{\text{IRF}}(f) = \phi_0(\mu_a, \mu'_a, \rho, f) - \phi(\mu_a, \mu'_a, \rho, f)
\]

(4)

Assuming the fdDOS instrument is stable (e.g., components remain the same, optical and radiofrequency connectors not changed, minimal systemic drift, etc.), \(A_{\text{IRF}}(f)\) and \(\phi_{\text{IRF}}(f)\) can be applied to calibrate other measurements in the same session.

After performing FDPM measurements at multiple-\(\rho\) on liquid phantoms [27], \(A_{\text{IRF}}(f)\) and \(\phi_{\text{IRF}}(f)\) can then be calculated. The primary advantage of multiple-\(\rho\), multiple-frequency FDPM over single-\(\rho\) calibration is estimation of the IRF without a priori knowledge of the liquid medium’s optical properties. However, multiple-\(\rho\) calibration has seen limited adoption due to factors of practicality. Liquid-based phantoms are typically not stable over time [37]. In addition, an apparatus to modify \(\rho\) accurately and precisely is needed, increasing the size and complexity of probes. Lastly, liquid phantoms, which may be several liters in volume [38, 39], are not convenient to transport, such as when traveling through airlines. In contrast, while calibrating fdDOS with a single-\(\rho\) is more widely applied, a key disadvantage is an assumption that the calibration standard has been accurately characterized. We propose calibration steps which attempt to leverage the advantages of the multiple-\(\rho\) approach to characterize silicone phantoms for future application at a single-\(\rho\).

### 2.3 Verifying accurate calibration

We outline steps to approximate accurate calibration of fdDOS by utilizing a liquid emulsion phantom and a set of solid silicone phantoms, shown in Figure 1. fdDOS was first used to measure a KS. We opted to formulate our own KS by precisely emulsifying water and soybean oil. In addition, the emulsion composition was verified by CA to ensure accurate characterization. \(\mu_a(\lambda)\) for KS was then calculated by:

\[
\mu_a(\lambda) = \sum_{i=1}^{n} C_i \epsilon_i(\lambda)
\]

(5)

involving \(n\) chromophores where \(C_i\) is the concentration, \(\epsilon_i\) is the extinction coefficient, and \(\lambda\) the center wavelength for each FDPM laser diode. The multiple-\(\rho\) FDPM fitting process was thus constrained to estimate \(\mu_a'(\lambda)\). \(H_2O\) and FAT content used for \(\mu_a(\lambda)\) calculations took into account the actual values obtained by CA rather than the nominal values measured during the mixing process. FAT extinction coefficients utilized were derived from porcine [40]. While porcine lard and soybean oil extinction coefficients are similar, small spectral differences observed below 700 nm and above 950 nm could contribute to the errors seen in results.

From here, measurements are performed at a single-\(\rho\). A set of solid, silicone phantoms \(P_n\), each with unique optical properties, was measured. Phantoms were fabricated following previously outlined procedures using nigrosin ink as the absorber and titanium dioxide for scattering [16]. The phantoms were in turn utilized to calibrate the fdDOS device and characterize another silicone phantom \(P_{UN}\) with unknown optical properties. Lastly, ex vivo porcine adipose tissue is measured. From

![Diagram](image-url)
here, we perform two validation steps to check the precision and accuracy of our approach:

1. Precision: Assuming our instrument is stable, then the IRF should be computed consistently, regardless of which $P_n$ phantom is selected. In this case, the reported optical properties for $P_{UN}$ would remain constant despite changing calibration phantoms. This tests the precision of our calibration approach.

2. Accuracy: If $P_n$ phantoms were accurately characterized, then composition analysis of porcine adipose tissue by fdDOS should yield similar results as CA.

### 2.4 Sample preparation procedures

To generate the KS, a liquid phantom was fabricated by blending FAT (soybean oil) and H$_2$O (distilled water) using soy lecithin as an emulsifying agent. Intravenous lipid emulsion (Intralipid) was considered as a possible candidate as suggested in other works [18, 38, 39, 41], but was not selected for two primary reasons. First, by concoction of our own emulsified solution, we can precisely control component ratios while also avoiding potential variations due to manufacturing tolerances. Second, Intralipid is slightly more complex in composition than our emulsion [42], which could add unnecessary steps to our CA process for separating substances. Lastly, raw materials of our phantom are more widely available whereas in certain regions, distribution of Intralipid is more regulated due to past safety concerns [43].

The emulsion fabrication process was similar to that reported by Ohmae et al. [37], with modifications to maintain the phantom in liquid form. A phantom containing 70% and 30% FAT and H$_2$O, respectively, was targeted. To achieve this, 455 ml of purified soybean oil, 195 ml of distilled water, and 6.261 g of soy lecithin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was measured separately. The soybean oil was then heated up to a constant temperature of 60°C in a sonicator bath (Branson CPX3800H-E, Emerson Electric, Danbury, CT). Soy lecithin was then quantitatively transferred into the soybean oil and then sonicated until the mixture appeared homogenized. The oil mixture was then transferred to a vacuum blender (i8800, Jiaxiang Electric Co., LTD, Guangdong, China). Distilled water was added, and the phantom was blended under vacuum conditions to minimize air bubble formation.

For the porcine tissue, the skin was first removed. We then considered the remaining top adipose layer as a homogenous layer. After the fdDOS measurement, smaller samples were excised from the center of the optical measurement sites for the CA step. We simulated various optical penetration depths to provide some guidance on the depth of excision [44, 45]. The tissue depth expected to contain >95% of photons was calculated for a range of adipose-like tissues. The thickness of excised samples was ensured to be greater than the maximum simulated photon penetration depth. Simulation parameters assumed a range of pure FAT (50%, 75%, and 100%), reduced scattering values ($\mu'_s = 1.00, 1.50$, and $2.00 \text{ mm}^{-1}$), $\rho$ equal to 16.5 mm, wavelength of 930 nm, anisotropy (g) as 0.8, and index of refraction (n) as 1.40 [7, 46, 47]. The maximum simulated photon penetration depth using our parameters was 10.06 mm.

### 2.5 fdDOS measurement procedures

All fdDOS measurements utilized a custom, 3D-printed probe containing the optical source fibers, detection fiber for the spectrometer, and a direct-contact APD. To protect sensitive APD electronics from potential moisture content of samples, a transparent plastic sheet (~0.025 inches in thickness) was taped to the face of the probe. All fdDOS data was collected in a single, continuous session within ~3 h. The fdDOS instrument was allowed to warm up after initialization for at least 30 min.

To accurately characterize KS, we performed a multiple-$\rho$, multiple-frequency FDPM measurement, as recommended by Campbell and O’Sullivan for liquid phantoms [48]. The probe measurement distances were set to $\rho = 16.5, 19.5, 22.5,$ and 25.5 mm. The probe was fixed in place by optical posts for semi-infinite measurements with the probe face resting (not submerged more than a few millimeters) on the liquid emulsion surface. For $P_n$, we utilized a set of five silicone phantoms with unique optical properties. Phantoms selected were empirically found to have a high signal-to-noise ratio across the four FDPM wavelengths (data not shown). The fdDOS probe was fixed in contact with the $P_n$ silicone phantoms with $\rho$ set at 16.5 mm. We included one more phantom to simulate effects when using an inaccurately characterized calibration phantom ($P_{IC}$). $P_{IC}$ was determined by intentionally swapping optical property values for $P_1$ and $P_5$.

For the porcine tissue, shown in Figure 2, a block of front leg tissue near the so-called “picnic shoulder” approximately 16 cm x 10 cm x 5 cm with a significant adipose layer (~2.8 cm in thickness) was acquired from a local butcher. The sample was not frozen and transported directly from the shop to the research area. Two regions of interest (A and B) were selected for fdDOS measurements and subsequent CA. Measurement sites were chosen to maximize tissue edge buffer, mitigating light leakage from the sides of the sample during optical measurements. Data was captured using the
FDPM and broadband components of the fDOS system. To estimate $H_2O$ and FAT levels, we performed chromophore fitting in the 900–1000 nm region. We considered this a reasonable dual-composition model for this tissue type. In addition, consumer-grade meat is exsanguinated, reducing the potential presence of hemoglobin factors within the adipose layer. The probe was manually held in place during measurements to ensure proper probe contact. Tissue was excised from the center of each optical measurement site for CA.

For in vivo human measurements, three anatomical locations were measured on a healthy male subject: abdomen (ABS), forearm (FOREARM), and thenar (THENAR). The measurement sites were selected as they were expected to be somewhat compositionally distinct. The fDOS probe was manually held during these measurements with $\rho$ set at 16.5 mm.

2.6 | CA procedures

To verify the contents of KS, a rapid dehydration approach was utilized [25]. Approximately 2 g of the emulsion phantom was obtained and placed into a 50 ml beaker. About 0.12 g of table salt was added to the beaker, allowing the emulsion to rapidly break down and maximize moisture removal. The beaker was placed into a mechanical oven (Heratherm OMS60, Thermo Scientific, Waltham, Massachusetts) at 125°C for 2 h then placed into a desiccator (Nalgene 5312-0230, Thermo Scientific, Waltham, Massachusetts) to prevent the absorption of moisture from the surrounding air and to allow samples to cool. $H_2O$ was calculated by the formula:

$$H_2O = \left(\frac{\omega_f - \omega_f}{\omega_i}\right) \times 100 \quad (6)$$

where $\omega_i$ was the initial weight with added salt and $\omega_f$ was the final weight with added salt. FAT was calculated by $FAT = 1 - H_2O$ where contribution of soy lecithin was considered negligible compared to the rest of the emulsion.

Similarly, to obtain the $H_2O$ content of the porcine tissue, samples excised from locations A and B were blended separately until homogenized and then weighed out in duplicates of approximately 2 g on aluminum dishes. Each duplicate was then placed into the mechanical oven at 125°C for 4 h. After heating, the samples were passively cooled in a desiccator. Each sample was then weighed, and the $H_2O$ content was calculated using Equation (6). Note, that the addition of salt is not necessary to calculate the $H_2O$ content of porcine tissue.

To verify the FAT content of the porcine tissue, we utilized methods based on conventional Soxhlet continuous extraction [26, 49]. Approximately 2 g of sample A and 3.5 g of sample B were weighed out in separate cellulose thimbles (64840-U, Sigma-Aldrich, St. Louis, Missouri). Each thimble was then placed into the extraction tube of the Soxhlet apparatus with petroleum ether (184519-500ML, Sigma-Aldrich, St. Louis, MO) as the solvent in a connected round-bottom flask containing boiling chips. The apparatus was then heated until boiling. After 3 h, the thimble was removed, and the remaining petroleum ether in the flask was evaporated. The flask was then placed into the mechanical oven at 125°C for 30 min to further ensure complete absence of the solvent and cooled in the desiccator. Calculation of FAT used the following equation:

$$FAT = \left(\frac{\omega_{fi} - \omega_{fl}}{\omega_i}\right) \times 100 \quad (7)$$

where $\omega_{fi}$ is the initial total weight of the round-bottom flask after extraction with boiling chips, $\omega_{fl}$ is the initial weight of the flask with boiling chips, and $\omega_i$ is the initial sample weight prior to extraction.

FIGURE 2  An ex vivo porcine sample was obtained from a local butcher. The adipose layer was measured to be ~2.8 cm. The two measurement locations “A” and “B” were selected to minimize potential boundary layer effects at tissue edges.
RESULTS

3.1 Emulsion and silicone phantoms

After the emulsifying step, we verified our KS composition immediately after fdDOS data acquisition by CA with results shown in Table 1. Resulting H2O and FAT was 31.2 ± 0.8% and 68.8 ± 0.8%, respectively, which is within 2% compared to the expected values. The contribution of the emulsifier was assumed to be negligible.

| Component | Volume (ml) | Expected (%) | CA (%) |
|-----------|-------------|--------------|--------|
| FAT       | 455         | 70           | 68.8 ± 0.8 |
| H2O       | 195         | 30           | 31.2 ± 0.8 |

Note: Secondary verification of the emulsion phantom composition was performed by CA. Extracted values were within 2% of expected values. The contribution of the emulsifier was assumed to be negligible.

Optical properties for each Pn phantom are shown. Pn was characterized using KS as a calibration reference, as verified by CA. Pn phantoms were found to have unique sets of absorption and reduced scattering coefficients.

Optical properties for Pn over the four FDPM wavelengths are shown. Reported optical properties for Pn were similar when using the Pn calibration phantoms (dashed line) as well as KS (solid circle), suggesting that both calibration options are equivalent. Shown Pn values are mean and SD. In contrast, optical properties reported by PIC demonstrates optical property errors when using an inaccurately characterized calibration standard (solid square markers).

Optical properties for each member of Pn were estimated using KS, as shown in Figure 3. Each member of Pn had a unique pair of optical properties ($\mu_a$ and $\mu_s'$) at each FDPM wavelength. Across all phantoms, $\mu_a$ values ranged from 0.0010 to 0.0074 mm$^{-1}$ while $\mu_s'$ values ranged 0.581 to 1.240 mm$^{-1}$.

Each member of Pn was in turn utilized as a calibration phantom to recover the optical properties of PUN, as shown in Figure 4. Using Pn, the $\mu_a$ differences compared to KS (mean Pn minus KS) for the four FDPM wavelengths were < 0.0001 and < 0.01 mm$^{-1}$ for $\mu_s'$. Highest SD for $\mu_a$ was 0.0002 mm$^{-1}$ occurring at the 791 nm wavelength while highest SD for $\mu_s'$ was 0.005 mm$^{-1}$ at the 659 nm wavelength. In contrast, comparing PIC values to KS (PIC minus Pn), $\mu_a$ differences were −0.0017, −0.0015, −0.0014, and −0.0015 mm$^{-1}$ for 659, 690, 791, and 829 nm wavelengths, respectively, while for $\mu_s'$, errors were −0.281, −0.272, −0.228, and −0.227 mm$^{-1}$.
3.2 Porcine adipose tissue (ex vivo)

H₂O and FAT were estimated for porcine tissue. Shown in Figure 5, H₂O and FAT errors were calculated considering CA as the gold-standard (fdDOS results minus CA results). Average and SD H₂O errors when using $P_n$ were $-1.9 \pm 0.2\%$ and $-0.9 \pm 0.2\%$ for Sample A and Sample B, respectively. In comparison, H₂O errors using $P_{IC}$ were $-5.7\%$ and $-4.7\%$ for Sample A and Sample B, respectively. FAT errors using $P_n$ were $-9.3 \pm 1.3\%$ and $0.8 \pm 1.3\%$ for Sample A and Sample B, while using $P_{IC}$ resulted in $-22.3\%$ and $-10.7\%$ errors, respectively. $P_n$ and KS calibration methods agreed within 1% of each other for both H₂O and FAT. CA values for the tissues were 7.58% and 9.05% H₂O for Samples A and B, respectively, and 85.96% and 82.38% for FAT. Excised sample thickness for Sample A was 17 mm while Sample B was 11 mm.

3.3 Human tissue (in vivo)

Tissue chromophore concentrations HBO₂, HBR, H₂O, and FAT on ABS, FOREARM, and THENAR measured on a healthy male subject are shown in Figure 6. Total hemoglobin ($THB = HBO₂ + HBR$) and tissue oxygenation...
TABLE 2  Average and SD chromophore values estimated by using $P_n$ and $P_{IC}$.

| TISSUE | HBO₂ (µM) | HBR (µM) | THB (µM) | STO₂ (%) | H₂O (%) | FAT (%) |
|-------|-----------|----------|----------|-----------|--------|---------|
| $P_n$ | ABS       | 17.2 ± 0.6 | 11.2 ± 0.1 | 28.4 ± 0.6 | 60.6 ± 0.8 | 27.8 ± 0.4 | 41.9 ± 0.5 |
|       | FOREARM   | 101.8 ± 2.0 | 47.2 ± 0.8 | 149.0 ± 1.8 | 68.3 ± 0.7 | 63.0 ± 1.2 | 4.8 ± 1.5 |
|       | THENAR    | 93.3 ± 2.0 | 48.2 ± 0.7 | 141.5 ± 1.8 | 65.9 ± 0.7 | 78.1 ± 1.2 | 0.0 ± 0.0 |
| $P_{IC}$ | ABS     | 8.6      | 11.7      | 20.3      | 42.4      | 29.1      | 48.0       |
|       | FOREARM  | 118.6    | 67.9      | 186.5     | 63.6      | 76.6      | 0.1        |
|       | THENAR   | 104.9    | 68.3      | 173.2     | 60.6      | 94.3      | 0.0        |
| $Δ$   | ABS      | -8.6     | 0.5       | -8.1      | -18.2     | 1.3       | 6.1        |
|       | FOREARM  | 16.8     | 20.7      | 37.5      | -4.7      | 13.6      | -4.7       |
|       | THENAR   | 11.6     | 20.1      | 31.7      | -5.4      | 16.3      | 0.0        |

Note: The difference ($Δ$) between $P_{IC}$ and mean $P_n$ values are also shown.

(STO₂ = HBO₂/THB × 100) were also calculated. Particularly muscular tissue also contains myoglobin, but was not distinguished from hemoglobin in this study.

Mean and SD chromophore values using $P_n$ and $P_{IC}$ for the three body sites are shown in Table 2. The difference ($Δ$) in results when using the two calibration methods are also shown.

4 | DISCUSSION

In order to compare fdDOS with CA, we proposed a calibration scheme to check the precision and accuracy of our system. Secondary verification of KS composition was achieved by CA, which showcases an important advantage of liquid emulsion phantoms over silicone phantoms for our study. With silicone phantoms, while one may attempt to carefully homogenize absorbing dyes and scattering particles, secondary verification with physical evidence (i.e., separation of the dye and scatterer from the silicone matrix) cannot be easily performed. In contrast, a small volume of our FAT and H₂O emulsion phantom can be sampled and decomposed by CA.

Using fdDOS, $P_n$ phantoms were calibrated using a compositionally verified KS. For the calculation of $μ_a$, we opted to use H₂O and FAT values calculated by CA, which we considered our gold-standard technique. The precision of the fdDOS instrument was demonstrated by then using each $P_n$ phantom to calibrate the fdDOS instrument and recover optical properties of $P_{UN}$. As a reminder, each $P_n$ phantom was shown to have a unique set of optical properties. Thus, given a precise fdDOS device, calculation of the IRF is expected to be consistent in this step resulting in agreement of $P_{UN}$ optical properties across $P_n$ phantoms. However, to simulate an improperly characterized $P_n$ phantom, we switched the optical properties of $P_1$ and $P_5$ labeling this case as “$P_{IC}$.”

By switching optical properties, $P_{IC}$ carried $μ_a$ and $μ'_s$ errors of roughly 0.0015 and 0.310 mm⁻¹ across the four FDPM wavelengths. We show that these calibration errors are not properly removed during IRF calculations and are instead translated into a similar magnitude of error when reporting optical properties of $P_{UN}$. Overall, optical property variations when using $P_n$ phantoms were exceptional, well within previously estimated margins of error for fdDOS instruments [15, 16].

On ex vivo porcine tissues, we attempted to match the fdDOS sampling volume and CA volume with the aid of photon propagation simulations, shown in Table 3 [45]. The 930 nm wavelength was selected as the peak sensitivity to FAT. When excising samples from the porcine slab, the depth target was set to be greater than the maximum simulated interrogation depth.

However, while a minimum sample thickness was set, maximum thickness was not defined. In our simulations, we predicted a photon sampling depth of approximately 10 mm. However, after the tissue excision process for CA, sample A was actually measured to be 17 mm in thickness. While this may not play a factor given a homogeneous volume, we suspect some non-negligible degree of heterogeneity in the biological tissue. Subcutaneous adipose tissue in porcine may develop in layers over the maturity of the animal [50]. Thus, it is possible porcine adipose tissue, may actually vary in composition depending on the depth.

TABLE 3  Simulation results for >95% photon penetration depth in millimeters for a range of FAT and $μ_a'$ values

| FAT | 50% $μ'_a$ | 75% $μ'_a$ | 100% $μ'_a$ |
|-----|------------|------------|-------------|
| 50% | 10.06      | 9.35       | 8.84        |
| 75% | 9.65       | 8.90       | 8.30        |
| 100%| 9.31       | 8.53       | 7.97        |

Note: Photon interrogation depth was estimated to be approximately 8–10 mm.
measured. This is further supported by our sample B results, in which the thickness of the extracted tissue was considerably thinner (11 mm) and closer to our expected photon interrogation depth. Errors comparing fdDOS and CA for sample B were significantly smaller despite originating from the same animal extracted from a location directly adjacent to sample A. Therefore, we speculate this small amount of heterogeneity may have been the main contributing factor to the higher FAT error calculated for sample A. Nevertheless, we find that fdDOS and CA methods on ex vivo porcine tissue samples agreed reasonably with H₂O error ranging 1–2% and FAT error within 1–9% when using an accurately calibrated system. In contrast, when \( P_{IC} \) was utilized as calibration, errors roughly doubled with H₂O and FAT error ranging 5–6% and 11–22%, respectively.

Various locations on a human subject were measured to showcase different tissue compositions. As a clinical research device, we wished to illustrate how measurements on human tissue may be affected using an inaccurate calibration reference. We highlight that when using \( P_{IC} \) as calibration, no distinction in FAT content was reported between FOREARM and THENAR. Furthermore, ABS tissue was reported to be hypoxic (STO₂ = 42.4%) if \( P_{IC} \) was used. In contrast, when using \( P_n \), STO₂ for all tissue types fell within 60–70%. These findings suggest that the accuracy of fdDOS calibration may not only affect reported chromophore values as an offset factor, but may also have a small impact on chromophore trends.

However, we noted that larger trends were still detectable even when using \( P_{IC} \). For example, ABS was expected to contain the most FAT. This held true whether using \( P_{IC} \) or \( P_n \) for calibration. Still, our results suggest that accurate calibration may improve the sensitivity of fdDOS in some scenarios, such as determining more exact tissue oxygenation levels, or when measuring small tissue compositional and hemoglobin changes.

Lastly, the relative complexity of our approach, particularly the usage of the Soxhlet apparatus, may be challenging for other groups looking to apply our full methodology. However, steps for accurate calibration can still be performed with conventional tools. First, a liquid emulsion phantom is fabricated, and a small amount of its volume is dehydrated to confirm the water and oil content. Household tools can be used for these steps, such as a blender for mixing and a convection oven for the dehydration step. Next, a multi-distance measurement is utilized to optically characterize the emulsion. Finally, silicone phantoms can be calibrated using the emulsion phantom as a reference. For our purposes, steps involving the Soxhlet apparatus was done to further validate our calibration efficacy by physically extracting measured FAT and H₂O within adipose tissue.

Limitations of this study include the assumption of a homogenous model during analysis of porcine adipose tissue. For future studies, we will more closely match the thickness of cut and simulated photon sampling depth. Another limitation of this study is our exclusive use an fdDOS device. For future investigations we wish to expand our approach to other spectroscopic methods such as time-domain and spatial-frequency domain devices.

5 | CONCLUSION

In this report, we proposed and demonstrated a method to verify accurate fdDOS calibration for comparison against CA. We find agreement in ex vivo porcine adipose tissue. In vivo human tissue was also examined, discussing the potential benefits of an accurate calibration procedure, and showcasing potential errors if using an inaccurately characterized calibration phantom. We hope usage of these calibration and validation steps can aid labs to accurately characterize tissues and tissue-simulating phantoms in the field of diffuse optics.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jesse H. Lam, Kelsey J. Tu, and Sehwan Kim conceived the study. Jesse H. Lam acquired optical data while Kelsey J. Tu performed the chemical analysis. Jesse H. Lam, Kelsey J. Tu, and Sehwan Kim analyzed the data and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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