Application driven assessment of probe designs for Raman spectroscopy

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Abstract: In vivo Raman spectroscopy has been utilized for the non-invasive, non-destructive assessment of tissue pathophysiology for a variety of applications largely through the use of fiber optic probes to interface with samples of interest. Fiber optic probes can be designed to optimize the collection of Raman-scattered photons from application-dependent depths, and this critical consideration should be addressed when planning a study. Herein we investigate four distinct probe geometries for sensitivity to superficial and deep signals through a Monte Carlo model that incorporates Raman scattering and fluorescence. Experimental validation using biological tissues was performed to accurately recapitulate in vivo scenarios. Testing in biological tissues agreed with modeled results and revealed that microlens designs had slightly enhanced performance at shallow depths (< 1 mm), whereas all of the beam-path-modified designs yielded more signal from deep within tissue. Simulation based on fluence maps generated using ray-tracing in the absence of optical scattering had drastically different results as a function of depth for each probe compared to the biological simulation. The contrast in simulation results between the non-scattering and biological tissue phantoms underscores the importance of considering the optical properties of a given application when designing a fiber optic probe. The model presented here can be easily extended for optimization of entirely novel probe designs prior to fabrication, reducing time and cost while improving data quality.

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1. Introduction

In vivo Raman spectroscopy is a non-invasive, non-destructive technique that can assess tissue pathology or physiological state and has been applied to a variety of applications [1]. The majority of these applications require the use of fiber optic probes to interface with samples of interest. Almost all fiber optic probe designs collect and integrate the signal obtained from a small volume of the sample beneath the probe tip. Due to the weak nature of Raman scattering, it is important to maximize the signal to noise ratio obtained from the tissue of interest at the particular depth of interest. Fiber optic probes can be designed to optimize collection of Raman-scattered photons from application-dependent depths. However, the target depth alone does not provide sufficient information to optimize probe design for a specific application; the tissue’s optical properties are critical and must be considered when planning a study. There is a need for a platform that allows investigators to 1) predict Raman scattered photon distribution in the sample of interest and 2) optimize fiber optic probe excitation and collection geometries to ensure maximum signal to noise at the desired depth. Such a tool must take into consideration Raman scattering, fluorescence, elastic scattering, and absorption in order to accurately predict how a probe design will perform for a given application. Although non-contact probes are being developed for a variety of applications, this work will focus on probes designed for contact with tissue.

The clinical application space driving innovative probe designs is vast, and has recently been reviewed [1]. Broadly, these applications can be grouped into three depth ranges: superficial (10-200 µm) [2–16], subsurface (200-2000 µm) [17–22], and deep (greater than 2000 µm)
[23–27]; examples of such applications are provided in Table 1. Raman probe designs have been developed within each of these depth categories to optimize data collection for specific applications as reviewed by Stevens et al. [28]. Distinct probe design strategies have been demonstrated to interrogate the extremes of superficial and multiple millimeter depths. To interrogate signals with increased performance at shallow depths, confocal detection designs using ball lenses and in some configurations beveled fibers, as well as excitation and collection geometries that use the same fiber, have achieved increased signal performance in the first 300 µm of tissue relative to unmodified fiber probe designs [3,5,6,13–16,29–32]. At the other extreme, the standard for detecting signals buried deep in scattering tissues is based on principles of random walk and multiple scattering. Spatially offset Raman spectroscopy (SORS) probes have been designed and constructed for specific applications such as detecting bone or calcifications. They rely on probabilistic paths indicative of detected photons that have traveled deeper into the sample when the source and detector elements of the probe have a greater separation [21,33,34]. Between these two extremes lies a spectrum of probe designs that incorporate various strategies to steer both the excitation and collection paths to measure relatively shallow or deep layers [28]. Each of these probe designs collects a volume-averaged signal from the sample beneath the probe tip. However, with proper care to consider the application, one can achieve dramatically improved signal detection from the targeted sample depth [10,30,35,36].

| Probe design          | Applications                                                |
|----------------------|-------------------------------------------------------------|
| Superficial (10 - 200 µm) | Epithelial cancers and precancers (bladder [10], cervix [2,16], colon [5,12], esophagus [3], lung [8], nasopharynx [15], oral cavity [9], skin [6,7], & stomach [4]) |
|                      | Barrett’s esophagus [13]                                    |
|                      | Dermatitis [11]                                             |
|                      | Inflammatory bowel disease [12]                             |
| Subsurface (200 - 2000 µm) | Cervical/stromal remodeling during pregnancy [17–19]       |
|                      | Tumor margin assessment (breast [21,27] & brain [22])       |
|                      | Vulnerable plaque assessment in arteries [20]               |
| Deep (greater than 2000 µm) | Detection of malignant breast calcifications [25]          |
|                      | Detection of urologic stones [26]                           |
|                      | Transcutaneous assessment of bone [23,24]                   |

Probe designers generally use ray tracing in air to inform light delivery and collection geometries for probe performance. Ray tracing software allows for determination of the excitation and collection cone angles and their overlap in non-scattering media, as well as photon distribution in scattering media. Accounting for the interactions between light and tissue is vital to accurately determining probe behavior. Robust prediction of light transport in tissue can be modeled using Monte Carlo (MC) simulations, in which the radiative transport equation can be solved for complex scenarios with an accuracy that depends upon the computational burden. Monte Carlo models were originally developed to track photon distribution and absorption in turbid media and have been extended for numerous and varied optical applications including fluorescence and Raman scattering [10,14,33,37–40], exceeding the current capabilities of ray tracing packages. Application-driven probe design must bridge these two approaches—ray tracing and light-tissue interaction models—to truly optimize fiber optic probes for biological targets.

Specifically for Raman scattering, a few groups have implemented these MC models. Matousek et al. developed an MC model for layered media for investigation of chemical powders [41]. Enejder et al. investigated system geometry effects on blood spectra [42] and Shih et al. used
an MC model to correct Raman spectra for tissue scattering and absorption artifacts [40].
Two separate MC models have been developed for evaluating superficial fiber optic probe
geometries for epithelial tissue detection applications [10,14]. Furthermore, Keller et al.
developed an MC model for investigating SORS approaches in turbid media [39]. Reble et al.
implemented a Raman MC model to systematically investigate the impact of tissue absorption
and scattering properties on the performance of probes with different collection fiber diameters
and spatial offsets from the excitation [35]. More recently, full Raman spectra models have been
developed [43–47], some of which have upgraded the platform to use graphics processing units
for increased speed [44–47]. Each of these models has proven valuable for investigating detection
of Raman signals in complex turbid media, although only one has incorporated fluorescence [47],
which has a direct effect on Raman signal quality. For example, Raman spectra collected from
liver tissue at 532 or 785 nm are overwhelmed by autofluorescence and consequently have poor
Raman SNR. Excitation at 1064 nm produces significantly lower autofluorescence, yielding high
SNR Raman spectra from liver tissue [48]. As a Raman fiber optic probe detects competing
fluorescent photons, consideration of fluorescence is vital for a model to successfully identify the
optimal probe design for a target application.

The goal of this paper is to investigate the impact of incorporating optical properties and
application-specific parameters when choosing or developing a Raman probe to measure depth
dependent signals using a MC model that tracks Raman and fluorescence photons paired with
experimental validation in biological phantoms. This approach allows researchers to more
accurately recapitulate and/or predict in vivo performance scenarios and optimize system designs.

2. Methods

2.1. Fiber optic probes

Four distinct fiber optic probe designs were experimentally tested and their performance was
modeled using the MC simulation explained below. All of the fiber optic probes investigated in
this study were designed for excitation at 785 nm, have seven collection fibers (300 µm diameter)
surrounding one excitation fiber, and a total outer diameter of 2.1 mm. All of the probes have
filters on the excitation fiber such that only 785 nm light passes to the sample and all of the
collection fibers have filters that block the diffusely reflected and elastically scattered 785 nm
laser light. Each probe that was evaluated has a distinct configuration, which are all drawn to
scale based upon results from ray tracing software (Radiant Zemax, USA) and shown in Fig. 1.
The four probes include 1) a conventional design that has no lenses or beveling with standard
0.22 NA fibers (±12.7° acceptance angles, 400 µm diameter excitation fiber, EmVision LLC,
USA; Fig. 1(A)); 2) a beveled collection fiber design that has both unmodified collection and
superficial overlap of the excitation and collection cone angles (±12.7 & 33.8-53° acceptance
angles, 400 µm diameter excitation fiber, Gaser probe by Visionex Inc, USA; Fig. 1(B)); 3) a
microlens design with a polished 3 mm diameter sapphire plano-convex lens coupled to a 1 mm
thick MgF₂ window that yields excitation and collection cone overlap deep within a sample
(2.7-26.1° acceptance angles, 200 µm diameter excitation fiber, EmVision LLC, USA; Fig. 1(C));
and 4) a microlens design with a polished 2 mm diameter sapphire plano-convex lens coupled to
a 1 mm thick MgF₂ window that results in excitation and collection cone overlap at the surface of
the sample (24.9-28.7° acceptance angles, 200 µm diameter excitation fiber, EmVision LLC,
USA; Fig. 1(D)). Probe-specific acceptance angles and collection fiber surface cross-sectional
areas are listed in Table 2

2.2. Monte Carlo model

A multimodal MC model, incorporating diffuse reflectance, fluorescence, and Raman scattering
was developed in MATLAB (The Mathworks, Inc., USA) by modifying a previously existing
Fig. 1. Probe collection geometries. Probe geometry cross section based on ray tracing: (A) unmodified, (B) beveled, (C) deep microlens, and (D) shallow microlens. Overlap between excitation and collection cones denote theoretical probe performance in air.

Table 2. Probe specifications for modeled collection fiber geometry

|                  | Unmodified | Beveled        | Microlens deep | Microlens shallow |
|------------------|------------|----------------|----------------|-------------------|
| Collection surface area | 0.086 mm²  | 0.049 mm²      | 0.658 mm²      | 0.407 mm²         |
| Acceptance angle  | ±12.7°     | ±12.7, 33.8-53°| 2.7-26.1°      | 24.9-28.7°        |

multilayer model for diffuse reflectance [37,49]. As described in the original publications, three coordinate systems were used: a Cartesian coordinate system was used to track photon movement, with origin centered at the midpoint of the excitation fiber on the surface of the media (XY plane); a cylindrical coordinate system, sharing the same origin and z-axis with the Cartesian system, was used to record internal photon absorption and scattering positions as a function of $r$ and $z$ based on the cylindrical symmetry of the system; and a moving spherical coordinate system that updates the z-axis to align with the photon propagation direction, used to sample the change in propagation angle of the photon by sampling deflection and azimuthal angles which are translated to directional cosines in the Cartesian grid system. Raman scattering and photon propagation were incorporated based on prior work [39,40,50]. Angular dependent photon injection and collection was included to investigate the interaction between optical properties and probe geometry at the sample interface. At the extremes of the modeled layers, diffusely scattered and, if the layers are sufficiently shallow, transmitted photons are recorded in the model using radial and angular grid coordinates to track photons exiting the media relative to the $z$-axis, which enables angular dependent collection definitions for each probe design investigated. The
general algorithm for the multimodal MC model can be seen in Fig. 2. Briefly, ≥ 5 × 10^6 seed photons are injected from the excitation fiber (Gaussian profile) into the top layer of the sample and propagate in the presence of absorption and elastic scattering. Each photon path is tracked as an XYZ coordinate relative to the model grid system as implemented by Wang et al [37]. After a photon migrates and deposits a discrete photon weight (based on Beer’s law). Raman scattering can occur (probability check: \( \xi < \log(1 + k_{RS}/10) \)), where \( \xi \) is a random number [0,1] and \( k_{RS} \) is the Raman scattering cross-section. If the probability check is passed and a Raman scattering event occurs, the photon is changed to a Raman scattered photon at \( \lambda_{RS} \) and the XYZ position and the remaining photon weight (\( W \)) of the Raman scattering event are stored. For any of these events (elastic or Raman scattering), a new photon trajectory is calculated and the photon propagates (based on appropriate wavelength- and layer-specific optical parameters) until either the photon weight is decremented and ended as per standard MC roulette routines or the photon escapes the model layers. If a photon is scattered through the surface of the topmost layer and remains within the detection region and acceptance angles defined for the collection fiber, the remaining Raman scattered photon weight is considered detected. At this time, the fluorescence for the collected Raman photon is also calculated based on the photon weight remaining at the time of the Raman scattering event. For this model, fluorescence generation is considered to be a ubiquitous process that occurs with each photon step along the random walk through the model.

A few assumptions have been made for the implementation of this code: 1) photon weight is deposited in the voxel at the end of the photon step, 2) secondary Raman scattering is neglected, 3) all Raman and fluorescence photons are modeled at a single wavelength for simplicity, 4) Raman scattering and fluorescence events are isotropic, and 5) the model is implemented with cylindrical symmetry extending from the center of the excitation fiber (z-axis) radially through the midpoint of a single collection fiber, which generates a continuous ring for photon collection instead of discrete fibers. The specific grid spacing utilized for modeling was maintained across all simulations for direct comparison, and was chosen to provide sufficient resolution for photon distribution: 100 radial bins that are 10 \( \mu \)m wide; 50 axial bins that are 50 \( \mu \)m deep; 30 angular grids of 3 degrees. The optical properties used as inputs in this model (Table 3) were based on both published values [51] (absorption and scattering coefficients, index of refraction, and anisotropy factors) as well as experimentally derived values (Raman cross section and fluorescence scaling coefficient). So that simulation tissue properties were not biased by differences in collection volume or filtering from the acquired Raman spectra of model components, the measurements of pure muscle and pure adipose tissue from all probe designs were averaged before model parameters were extracted. To account for signal detection of background fluorescence and scattering, a multiplicative scaling factor was incorporated in the fluorescence calculation as the only freely scaled parameter to match simulated predictions with experimental results [52]. As each photon propagation can be computationally addressed independently, this algorithm is well suited to parallelization. As such, the model was compiled into a standalone executable file and deployed using the Parallel Computing toolbox, taking advantage of all of the dedicated processing cores within a PC (primary machine processor: 6 core, i7-8086K @ 4GHz) for enhanced performance relative to serial calculations, similar to approaches previously used [53]. All reported modelling results are aggregated from at least 5 independent runs to ensure consistent performance. It is worth noting that there are several possible implementations for combining these optical phenomena within a single model: prior work has used this approach for either Raman scattering, fluorescence [37–39], or the two combined. The implementation in this work was chosen for model development because it specifically recapitulates the processes and detected signals obtained using fiber optic Raman probes.

To simulate the performance of the distinct probe designs in non-scattering media, as anticipated by a standard ray-tracing based approach, modeling was performed with scattering coefficients (\( \mu_s,Es \) and \( \mu_s,Em \)) set to zero, while all other parameters were held constant. In the
Fig. 2. Flowchart for tracking photons in multi-layered media with multimodal Monte Carlo simulation. Model parameters: $\lambda_{Ex}$ = wavelength, excitation photon; $s$ = photon step size; $d_b$ = distance to layer boundary; $\mu_T, Ex$ = total attenuation coefficient at $\lambda_{Ex}$; $\lambda_{RS}$ = wavelength, Raman scattered photon; $\xi$ = random number [0,1]; $k_{RS}$ = Raman scattering cross section; $\mu_s, Ex$ = scattering coefficient at $\lambda_{Ex}$; $\mu_a, Ex$ = absorption coefficient at $\lambda_{Ex}$; $\mu_s, RS$ = scattering coefficient at $\lambda_{RS}$; $\mu_a, RS$ = absorption coefficient at $\lambda_{RS}$; $W_{Ex}$ = photon weight at $\lambda_{Ex}$; $W_{RS}$ = photon weight at $\lambda_{RS}$. 
Table 3. Input parameters for Monte Carlo simulation

| Optical Properties | Muscle     | Adipose    |
|--------------------|------------|------------|
| Index of refraction (n) | 1.37\(a\) | 1.45\(a\) |
| \(\mu_a,Ex\) at 800 nm (1/cm) | 0.54\(a\) | 1.08\(a\) |
| \(\mu_s,Ex\) at 800 nm (1/cm) | 66.7\(a\) | 202\(a\) |
| \(\mu_s,RS\) at 900 nm (1/cm) | 0.32\(a\) | 1.25\(a\) |
| \(\mu_s,RS\) at 900 nm (1/cm) | 88.7\(a\) | 108\(a\) |
| Scattering anisotropy (g) | 0.93\(a\) | 0.9\(a\) |
| Raman cross-section (k_{RS}) | 0.92 | 7.43 |
| Fluorescence at 800 nm \((F_{Ex})\) | 0.214 | 0.446 |
| Fluorescence at 900 nm \((F_{RS})\) | 0.535 | 1.115 |

\(a\)Values reported in [50].

MC model implementation utilized for light-tissue simulations in this work, Raman scattering and fluorescence are only determined at the end of each step in the model. For non-scattering media, this step size increases significantly, such that many photons escape the modeled media thickness without Raman generation. However, as Raman scattering and fluorescence emission are inherently independent of elastic scattering properties and are considered isotropic in this model, a fluence-based calculation was instead utilized as a relative comparison of detection efficiency between designs. To implement this, the probe collection geometries, depicted in Fig. 1, were converted to binary masks. These masks were then applied to the tracked fluence distribution result from each model (accounting for layer-specific changes in index of refraction) to include voxels within the collection region for each distinct design after design-specific illumination. As the fluence is tracked for each voxel in the modeled media as a function of photon wavelength independent of elastic scattering, this computation facilitates an estimation of Raman collection efficiency based on probe geometry without requiring photons to complete movements along their trajectory. While this mask-based approach does not consider only the fraction of photons that will be Raman scattered into the solid angle of the collection fiber, the isotropic scattering permits this calculation as a valid relative comparison metric between designs.

2.3. Raman spectroscopy system

All Raman measurements were acquired using a cart-based clinical Raman spectroscopy system that used a 785 nm diode laser (Innovative Photonics Solutions, USA) coupled to an imaging spectrograph (Holospec f/1.8i, Kaiser Optical Systems, USA) and a thermoelectrically cooled charge-coupled device camera (Pixis 256BR, Princeton Instruments, USA), all operated by a laptop computer. The system was wavelength calibrated using a neon-argon lamp, and the Raman shifts were calculated using acetaminophen and naphthalene standards. Spectral response correction was determined using a National Institutes of Standards and Technology (NIST)-calibrated tungsten lamp. All measurements were background subtracted, noise smoothed, and fluorescence subtracted as described previously [54].

2.4. Biological tissue phantom experiments

Raman signals were acquired from semi-infinite porcine muscle and adipose tissues to simulate biological samples. The porcine tissues were commercially acquired and kept on ice throughout the experiment. Three hundred \(\mu\)m thick slices of muscle tissue were obtained via cryosection and were layered over the semi-infinite slab of adipose tissue until a total muscle tissue thickness of 1800 \(\mu\)m was achieved (Fig. 3(A)). Raman spectra (\(n\geq 45\), 3 accumulations, 500 ms acquisition time each) were acquired at all thicknesses with each probe. Ratios are presented for a peak specific
to muscle (1003 cm\(^{-1}\)) corresponding to symmetric aromatic ring breathing in phenylalanine [55]) and a peak specific to adipose tissue (1745 cm\(^{-1}\)) corresponding to C=O stretch [56]) (Fig. 3(B)). These peaks were chosen due to the spectral isolation in pure measurements such that stable peak ratios could be calculated. To minimize contribution from the other tissue phantom layers, peak-to-baseline intensity was calculated for each spectrum following preprocessing by subtracting the linear fit of background intensity (995-1017 cm\(^{-1}\), peak at 1003 cm\(^{-1}\) and 1720-1770 cm\(^{-1}\), peak at 1745 cm\(^{-1}\)) from the respective peak. The ability to detect the superficial (muscle) and deep (adipose) tissues was assessed using the signal to noise ratio (SNR) calculated as the ratio of Raman peak intensity to the shot noise calculated for the Raman and fluorescence background ($SNR = \frac{I_{Raman}}{\sqrt{I_{Raman} + I_{Fluorescence}}}$).

3. Results and discussion

3.1. Modeled theoretical probe performance

To evaluate the theoretical performance of distinct fiber optic probe designs indicated via ray tracing in air (Fig. 1), MC simulations were performed for the layered tissue components in the absence of elastic scattering ($\mu_{Els,5} \& \mu_{Em,5} = 0$). The modeled results of these simulations, each compiled from five independent runs per parameter set (with $1 \times 10^7$ seed photons, duration mean ± standard deviation: 114.6±3.7 seconds) are depicted in Fig. 4. This idealized demonstration, using purely absorptive media generating isotropic Raman scattering events, captures the expected performance of these probe designs with some notable trends. The signal acquired from the superficial layer is dependent upon both the probe design and the thickness of the layer. As expected from the overlap of the excitation and collection fibers for the beveled and the microlens designs, the superficial collection for these probes increases more rapidly in comparison with the unmodified design (Fig. 4(A)) and both designs detect significantly more signal overall (Fig. 4(D)). Indeed, the model predicts that the unmodified design does not collect appreciable signal from the superficial layer until there is over 1000 µm of the superficial layer present. Similarly, all the probes demonstrate higher signal collected from the deeper layer which generally

![Absorption and Scattering Diagram](image)

**Fig. 3.** Experiment setup: (A) Schematic of tissue phantom setup for depth response characterization with probe contacting the surface of the superficial layers (porcine muscle) above a semi-infinite base (porcine adipose, figure (A) not to scale). (B) Pure spectral components measured from porcine adipose (black) and muscle (light red) tissue samples of semi-infinite thickness (beveled probe design). Arrows in (B) indicate peak positions used in ratiometric analyses.
decreases as the superficial layer thickness is increased (Fig. 4(B)). Furthermore, as expected based on the overlap cone and the steep angles of the collection fibers, Fig. 4(C) indicates that the shallow microlens design is the most efficient at acquiring superficial signals while rejecting deeper sources of signal for the first few hundred micrometers of sample thickness, after which the deep microlens and beveled designs surpass the performance for the Raman ratio signal.

Non-scattering samples such as gasses and liquids represent a simplified, predictable subset of materials that can be interrogated with optical probes. Without the presence of scattering components, the predictable performance of these probe designs is dominated by the overlap of the collection solid angles and the excitation cones, the depth of the sources of generated signal, and the source-detector offsets. Hence, the need for complicated models to predict probe performance in such samples is unnecessary, as Beer’s law and angular dependence should define the performance and efficiency of the designs. In fact, as models such as this one often require a photon to complete an entire step before a potential Raman scattering event can occur, non-scattering media would significantly increase the depth for initial Raman scattering events in the tissue that would alter performance for these distinct probe geometries. To circumvent...
this limitation, the fluence-based approach was used to calculate the relative Raman scattering based on excitation and collection cone overlap without the need for completion of model steps. When model layers become more complex from the introduction of scattering properties into the media of interest, with values that can vary dramatically across biological tissues, the use of Beer’s law which assumes purely absorptive media is no longer valid. As such, design of Raman fiber optic probes necessitates the consideration of the influence of the scattering properties on the performance. A common limitation in the design process of fiber optic probes is to merely consider the theoretical performance in non-scattering media, but this will fail to recapitulate the practical probe performance for turbid samples thus delineating the value of computational models that enable the prediction of experimental performance for these designs while investigating the parameters of interest.

3.2. Experimental and modeled biological tissue phantom characterization

To test performance of the four Raman probes in measuring biological tissue, experiments and Monte Carlo simulations using porcine adipose tissue with increasing thickness of overlying porcine muscle tissue were performed. Experimental and simulation results from muscle tissue specific peak SNR analysis indicate an intuitive overall increase in the muscle tissue signal as muscle tissue layer thickness increases (Fig. 5(A)) in all probe designs. The corresponding modelling results for the biological tissue phantom are presented for direct comparison in Fig. 5(B) ($5 \times 10^6$ seed photons, duration mean ± standard deviation: $38.9 \pm 6.9$ minutes). The Monte Carlo simulation time is highly correlated with the number of interactions tracked, and thus an increase in scattering coefficient or tissue thickness consequently increases simulation time (300 µm thick muscle layer: $28.30 \pm 0.34$ minutes; 1800 µm thick muscle layer: $48.22 \pm 0.34$ minutes). The simulation had high correlation with the experiment (Pearson's r ranged from 0.91-0.98 in the four designs (Fig. 5(B))). As expected, drastic differences are observed upon comparison with depth-dependent probe performance in non-scattering media (Fig. 4). In the non-scattering results, the unmodified design (Fig. 1(A)) is not sensitive to superficial tissue until over 1 mm of muscle tissue is placed above the underlying adipose due to the absence of overlap in the excitation-collection cones. Although the unmodified design has the lowest SNR in the experimental and modeled scattering phantom results (Fig. 5(A,B)), measurable signal is detected from the 300 µm thick muscle layer, and gradually increases with correspondingly thicker muscle tissue due to scattering at even the most superficial depths. The three modified designs similarly detected muscle signal at smaller thicknesses than predicted from the non-scattering simulations due to the introduction of tissue scattering. The scattering coefficient(s) of the tissue(s) of interest modulate the depth sensitivity for all probes in both the experiment and model.

Experimental SNR analysis indicated that both of the microlens designs outperformed the beveled design for superficial muscle signal collection, (Fig. 5(A)) while, as expected, the microlens shallow design was least sensitive to the underlying adipose signals (Fig. 5(C)). This is likely explained by the small collection area and relatively narrow acceptance angles for the microlens shallow design compared to the other designs, which improves rejection of photons generated deep within the tissue (Table 2). Conversely, the overlap of excitation-collection cones in the beveled and microlens deep designs should accept light from both superficial and deep positions, accounting for the differences in depth sensitivity. This contrasts with the non-scattering results in Fig. 4(A), where these designs exhibit a continued increase in superficial Raman SNR as that layer increases in thickness, yet the microlens shallow design reaches a plateau in SNR. The deep microlens design (Fig. 1(C)) has a wide acceptance cone and initial overlap of the excitation and collection cones making it less susceptible to scattering properties.

Experimental results from underlying adipose tissue SNR analysis predictably decrease as overlying muscle tissue thickness increased in all probe designs (Fig. 5(C)). Similarly, the simulated detection of adipose tissue Raman photons decreased monotonically for all probe
Fig. 5. Combined experimental and modeled Raman scattering results for distinct probe designs applied to the biological tissue phantom. Experimental (left: A,C,E,G) and modeled (right: B,D,F,H) signals indicate close agreement (Pearson’s r linear correlation coefficient values per design) for probe performance as a function of superficial layer thickness for superficial layer SNR (A, B) and deep layer SNR (C, D), defined relative to the detected shot noise. Signal ratios for superficial vs. deep signals (E, F) and total integrated Raman signals (G, H) further indicate agreement between model and experimental results, with modified probe designs outperforming the unmodified design, and the enhanced performance of the shallow microlens probe for obtaining signal from superficial Raman scatterers. All data are displayed as mean ± standard deviation of the acquired spectra and modeled signals.
designs to a point where there was no difference in performance between designs (Fig. 5(D)). Collection of deep Raman signal in the unmodified design and beveled probe had stark differences between scattering and non-scattering scenarios. In the unmodified design, low signal is observed until about 600 µm thickness of muscle tissue is overlaid in the non-scattering results, whereas deep signal is highest with thin layers of muscle overlaid in the scattering context. These differences are likely due to no excitation-collection overlap at superficial depths (Fig. 1(A)) where the fluence is highest, which is overcome by tissue scattering in the biological experiments and tissue phantom simulation. The beveled probe also lacks excitation-collection cone overlap at shallow depths, however, the overlap occurs at a more shallow point than the unmodified design (Fig. 1(A,B)), and thus appreciable signal is still observed with only 100 µm of muscle tissue and increases until 600 µm of muscle tissue, after which the signal decreases due to a sparsity of deep layer photons reaching the detector. In the scattering results, the beveled probe’s sensitivity to deep signals monotonically decreases with increasing muscle layer thickness as scattering enables collection of deep tissue Raman photons outside of the excitation-collection cone overlap that limits performance in the non-scattering regime. The deep and shallow microlens probes have similar trends in deep Raman signals with and without scattering, likely due to the immediate overlap of excitation-collection cones (Fig. 1(C,D)). The correlation between experimental and simulated deep Raman SNR results in biological phantoms was high, ranging from a Pearson’s r of 0.966-0.995 in the four designs (Fig. 5(D)). However, the shallow microlens design displayed noteworthy differences in performance between the experimental and model results for deep SNR (Fig. 5(C,D)). The experiment indicates a consistently lower SNR from deep tissue and the simulation shows a higher SNR compared to the other probe designs at all superficial layer thicknesses. Potential explanations include possible inaccuracies in excitation and collection cone angles which were derived from ray-tracing, which may have introduced error into the model. In addition, there may be inconsistencies between the theoretical probe design on which the ray-tracing is modeled and the actual fabricated probe and wear and tear on the probe which could slightly alter the excitation or collection efficiency. Furthermore, although the authors conducted the experiment as carefully and quickly as possible, experimental errors such as imperfect contact with the tissue surface or biological phantom deterioration as the experiment progressed could also contribute to the discrepancies observed between the experiment and simulation.

The experimental results for the ratio of superficial to deep signals show a rise with increasing muscle layer thickness (Fig. 5(E)). The results indicate that the microlens shallow design has a higher proportion of signal arising from the more weakly scattering superficial muscle than any of the other designs. Specifically tailored to preferentially collect superficial signals, this design may provide a midpoint between other volume-averaging designs and confocal Raman probes that have been based on tightly focusing ball lens approaches. [29] The simulated Raman ratio results show an intuitive increase in muscle to adipose tissue signal ratio as the muscle thickness increases in all probe designs, where the modified probe designs have slightly higher muscle to adipose ratios (Fig. 5(F)). The unmodified design’s Raman ratio is deep signal dominated in experiment and simulation arising from the low overlap of excitation-collection cones at superficial depths compared to the other designs. The beveled and deep microlens designs have strong differences between the biological tissue phantom and the non-scattering simulation, where the non-scattering Raman ratio rises exponentially for both designs with increasing superficial layer thickness, whereas the biological tissue ratios rise in a linear fashion. This is likely caused by the increasing area of excitation-collection cone overlap with increasing superficial layer thickness in these designs without accounting for the attenuation of light with depth caused by absorption in the non-scattering simulation. The shallow microlens design’s ability to favor superficial photons is not as obvious in the non-scattering results, as the superficial advantage of the design is hampered at increasing thicknesses of muscle tissue due to poor overlap of excitation and collection cones after about 1 mm distance from the probe surface. The superiority of the
shallow microlens design in detection of superficial photons and in rejection of deep is much more obvious in the scattering context, which improves with increasing muscle tissue thickness. Both the experimental and modeling results indicate that the probes detect a majority of their Raman scattered photons from the deeper adipose when superficial muscle is $\leq 1500 \, \mu m$ in thickness in the biological phantom. There is a discrepancy between the behavior of the microlens shallow design in the experiment and simulation, with a higher ratio observed in the experiment that is essentially double the values of the other probes. In contrast, the ratio of the microlens shallow probe is indistinguishable from the other designs. As described above, this could be due to a number of factors, including inaccuracy in the ray-tracing of excitation-collection overlap, imperfections in probe fabrication, wear and tear on the probe, and experimental error.

The total integrated Raman signal detected in the biological phantom experiments and the corresponding MC model from each probe as a function of muscle tissue thickness are shown in Fig. 5(G,H). Experimentally, the modified probe designs all detect similar signal amounts, which are elevated compared to the unmodified design for all superficial layer thicknesses. According to the modeling, the shallow focused microlens probe is predicted to detect the most photons at all muscle tissue thicknesses and slightly outperform the two other modified designs (Fig. 5(H)). Despite having a smaller cross-sectional area than the microlens deep design (Table 2), the microlens shallow probe has the largest excitation and collection cone overlap (Fig. 1) at the sample interface, as well as the most tightly focused excitation cone due to the 3 mm microlens. The deep focused microlens and the beveled design collect about 75% of the signal compared to the shallow focused microlens. The unmodified design collects by far the fewest photons of all of the designs at all depths simulated. This is likely a result of having the lowest overlap of excitation and collection cones (Fig. 1(A)) due to source-detector offset and narrow acceptance angles (Table 2). While the non-scattering integrated Raman signal trends (Fig. 4(D)) did correlate with biological experiments and simulations (Fig. 5(G,H)), strong differences were observed in depth-specific contexts and underscores the importance of considering tissue properties when measurement of a specific depth is desired (Fig. 4(D)).

3.3. Experimental and modeled fluorescence from biological tissue

Total fluorescence photons detected by each probe design were recorded for both experimental and modeled performance as a function of superficial layer thickness (Fig. 6(A,B)). Experimentally, this data is captured as the autofluorescence and scattering background that is typically subtracted (and ignored) during Raman spectral analysis. As the shape and contribution of these signals generally lacks strong and distinct features between tissue layers compared to the narrow Raman peaks depicted in Fig. 3(C), only the integrated fluorescence signal is compared. The trends observed in the MC simulations predict that the detected fluorescence signal (Fig. 6(B)) resembles a combination of the detected Raman signals originating from both the superficial and deep layers (Fig. 5(A,C)). Generally, there is a modest decrease in total detected fluorescence background as the thickness of superficial muscle is increased, both in the model and experimental results. This trend agrees with the expected results based on the higher likelihood of fluorescence being generated within the adipose tissue relative to the superficial muscle layer (Table 3). As the more fluorescent adipose tissue is buried beneath more muscle tissue, the signal is less efficiently collected. Despite fluorescence generation from both excitation and Raman scattered photons, the influence of tissue scattering and absorption properties have a significant impact on the collection for all probe geometries. Experimentally, this background signal does not decrease greatly as the superficial layer dominates the theoretical collection volume. The model predicts a much stronger falloff in the fluorescence detected as a function of depth compared with the experiment. One potential explanation for this trend is that modeling tissue autofluorescence at only 2 distinct wavelengths does not accurately capture the biological tissue process which is detected as a function of wavelength at the same time as the Raman scattering signals. It may
also be the case that the high scattering from the deep adipose tissue leads to a higher likelihood of detecting the fluorescence photons that have been multiply scattered through the superficial muscle where, even though fewer fluorescence events are likely to occur, the photon path lengths should increase. These longer mean free paths in the superficial muscle may account for the limited differences in overall fluorescence collection efficiency between probe designs since the fluorescence generation is significantly more frequent than Raman scattering, and as such, there should be much greater volume averaging of these emitted photons into all collection angles. According to these results, the modified probe designs that more efficiently collect photons from directly beneath the excitation fiber are more efficient at detecting the broad spectrum autofluorescence from tissue.

Fig. 6. Experimental and modeled fluorescence signals for biological tissue phantom measurements with distinct probe designs. The general agreement in performance between experimental and modeling results for the distinct probe designs indicates that the designs more sensitive to the superficial layer of the biological phantom have an increased detection of fluorescence and background signals as well. The experimental variability in fluorescence signals from the biological tissue phantom indicates little detectable difference in the average fluorescence background between probe designs as a function of superficial layer thickness. All data are displayed as mean ± standard deviation of the acquired spectra and modeled signals.

3.4. General discussion

The tissue phantom simulation results all agree with theoretical understanding of bulk material properties for photon distribution in turbid media. As there is a layer-dependent probability for Raman scattering of the excitation photons, the intuitive trends observed in Fig. 5(A) & (C) are due to the increasing thickness of a layer with lower Raman cross-section above a stronger Raman scattering layer. It is likely that the decreased detection of Raman photons observed in Fig. 5(C) is due to the complex interaction of superficial layer thickness, scattering coefficients, and the probability for Raman scattering in that top layer. Based on the scattering coefficients utilized for the tissue simulations, the reduced mean free paths for the photons are equal to or greater than the source-detector separation for the excitation and collection fibers modeled and tested. This indicates that in the media modeled here, which are dominated by scattering, the expected path length in the diffusive regime is at least as large as the separation of the excitation and collection fibers for any probe design. As a result, it is expected that significant penetration and radial spread of the the photons into the superficial muscle layer and further to the adipose layer will account for numerous interaction steps and the potential for Raman scattering and fluorescence generation along their random walk. This also impacts the likelihood of signal collection for other probe geometries with significantly lower (confocal ball lens) or greater (SORS multi-ring) offsets that
will be differently impacted by the specific tissue layer scattering and absorption properties. In this work, while the superficial layer remains thin, appreciable excitation photons are Raman scattered (and fluorescently emitted) in the deep layer and propagate radially through the layers towards the detectors. At greater muscle layer thicknesses, the probability of deep-generated forward scattering (based on tissue anisotropy) Raman and fluorescence photons returning back to the collection fibers decreases, but there is an increased volume of muscle layer in which Raman scattering can occur, despite its reduced Raman cross-section. Furthermore, since the deep adipose layer is more likely to generate Raman photons, as the superficial muscle thickness increases, there is an overall decrease in the total detected signal (Fig. 5(D)). The apparent decrease in fluorescence and background scattering detected suggests that tissue scattering and fluorescence quantum yield are important parameters to consider in combination with probe collection geometry, as there is a relationship between signal generation and detected scattering that is not solely defined by effective quantum yield. By direct comparison with non-scattering modeling results, it is apparent that designing fiber optic probes for biological tissues without consideration of application-specific properties does not recapitulate \textit{in vivo} performance. The results presented here agree with photon distribution theory for turbid media and indicate the importance of accounting not only for relative changes in scattering and absorption within a tissue of interest, but also the relative strength of competing optical processes for a target application at a particular wavelength.

Overall, the experimental trends with increasing superficial layer thickness correlate well with the MC model, although some deviation in probe-specific performance was observed. The most prominent difference is the apparent overestimation of the SNR for collection of deeper layer Raman signal by the microlens shallow design (Fig. 5(D)) and the higher estimated total integrated signal (Fig. 5(H)) for this probe as well. A potential explanation for the discrepancy in shallow microlens probe performance between the experimental and modeling results may be that the collection active surface area and acceptance angle range, as determined from ray-tracing in air, are not accurately estimated for this design and lead to the overestimation of the deep collection performance in the model. This could be related to the narrow excitation cone as well as the acceptance angles of the collection fiber, as modeled in air, which, if altered, would both significantly impact the predicted performance of an individual probe design as a function of depth. Another possible explanation is the difference in depth sensitivity for experimental data that arises from single peak analysis which is frequently not realistic in tissue based Raman spectroscopy studies, and is compared to modeling for Raman cross sections determined from an entire spectrum. Extending the model to a full spectrum implementation may provide a better recapitulation of some of the experimental results obtained with distinct probe designs [43,44]. Indeed, the spread of results between the probes evaluated demonstrates how small changes in probe design can significantly alter their performance. This validation is an important first step in developing a probabilistic tool for optimizing the choice or design of a fiber optic probe. Given the potential changes in efficiency and performance that appear in the model by altering such parameters, this computational approach to comparing application-specific probe designs may help better target ideal specifications prior to fabrication when beginning a study. In addition, the modified Raman probe designs with increased excitation-collection cone overlap at the tissue surface demonstrate significant improvement in collection efficiency through experimental testing and MC models. Regardless of application, modifications such as the inclusion of an optical window at the tip of an unmodified probe would likely increase the excitation-collection cone overlap and collection fiber surface area so that maximum Raman signal can be detected.

As evidenced in Table 1, important biological investigations span many depth regimes. A confocal ball lens probe designed and evaluated by Wang \textit{et al.} remains the optimal design reported for detecting superficial (<100 µm) signals of biological tissues with limited contribution from deeper layers [29]. At the other extreme, spatially offset designs demonstrated by numerous
investigators have proven most effective at interrogating deep layers of highly scattering media [21,27,41]. Between these extremes of the depths of optical detection, volume integrating fiber probes can be tailored for preferential depth detection and increased sensitivity to a specific application of interest. Carefully tuning the performance of a fiber optic probe for a tissue specific application thus requires quantitative information regarding the impact of probe geometry and the relevant tissue properties. Implementing a simulation based approach, where such properties can be evaluated in silico enables a quantitative comparison of how a shallow or deep target signal may manifest. This would be of great importance when targeting small structures, or those with subtle spectral differences relative to the surrounding tissues, both scenarios for which optimization of probe design can greatly impact in vivo performance. This work demonstrates improved signal collection efficiency obtained when integrating microlens components into a probe tip design that can expand the overlap in excitation and collection cone angles as well as increase the surface area for light detection compared with conventional fiber probe designs. Beveled design probes have been commonly used in the tissue optics field, however, are not widely available. As such, custom designs that can be tailored to depth specific applications, such as those incorporating microlens components may fill this need, if proper care is taken to consider the application specific performance. Beyond high collection efficiencies, microlens designs can be optimized for superficial and deep targets based on the choice of lens as demonstrated in the experimental and simulation results. As expected, beveled and shallow microlens designs have superior performance in superficial samples whereas the deep focused microlens design has the highest collection efficiency in deep tissue layers. The conventional design has the lowest overall collection efficiencies at all tissue layer thicknesses, providing a volume averaged signal with limited depth sensitivity. All of these evaluations have utilized muscle and adipose tissues as biological phantoms paired with MC simulation results, and thus direct comparison with other studies is straightforward [21,29]. The volume-integrating probes evaluated here demonstrate variable performance with depth as a function of sample optical properties (biological phantoms vs. ray-tracing based fluence simulations with non-scattering optical properties), most notably the reduced scattering coefficient and Raman cross section. At extreme scattering values, either low or high, the improvements of a particular configuration may diminish. These results further support the need for probe development within the context of tissue properties for a targeted application, which may perform differently from theoretical models commonly employed for optical design.

Utilizing a numerical simulation may be simpler than precisely controlling tissue phantoms or biological samples, however, models rely on assumptions with associated limitations. Like all probabilistic or stochastic models, this simulation requires many photons to develop an accurate result. To exacerbate this requirement, a competitive model implementation for the distinct optical phenomena integrated in this simulation would require the use of extremely large numbers of photons if scaling factors are not utilized in order to obtain consistent and reliable results. To circumvent this requirement, here, fluorescence generation is not based on a probabilistic calculation but rather is assumed to occur uniformly at every absorption event with a layer dependent quantum yield. Thus, for every scattering step of an excitation photon, a discretely weighted computational fluorescence absorption and emission event occurs at the location of the deposited photon weight based on layer parameters. Like many MC models for Raman scattering and fluorescence, this model neglects secondary Raman events and assumes perfect coupling between adjacent model layers. Inclusion of these events and sources of scatter would likely be incremental, but could more accurately represent optical interactions, especially due to the relatively high number of fluorescence events that have the potential to cause secondary fluorescence and Raman scattering. These events could also be included as model noise for a more direct SNR comparison with experimental values.
Despite these limitations, this model provides valuable insight into the complex interaction of multiple optical processes detected by fiber optic probes. These simulation results afford a probabilistic basis for comparison with experimental data as a means to evaluate the performance of these distinct probe designs, including collection angle and surface area for photon collection. Greater sample complexity can be incorporated within the model to include finite Raman scattering objects instead of uniform layers, a system that would require careful experimental control for testing. By computationally evaluating Raman probe designs in scenarios that recapitulate tissue studies rather than ray-tracings in air, accurate selection of optimized configurations can be enabled for a specific application such as those listed in Table 1.

4. Conclusions

A platform for optimizing selection or design of a Raman fiber optic probe that incorporates application specific optical properties for prediction of probe performance has been developed and evaluated. The expanded Monte Carlo model includes Raman scattering, fluorescence, elastic scattering, and absorption, and agrees with experimental results from biological tissue including prediction of collection efficiency as a function of Raman scattering depth. Fluorescence is a competing optical phenomenon with a much higher likelihood than Raman scattering in most biological tissues. The apparent interaction of sample fluorescence and scattering properties is an important relationship that cannot be overlooked when optimizing fiber optic probes for Raman spectroscopic applications. Fiber optic probe-based Raman spectroscopy has been applied to many biological targets. Modeling and experimental testing demonstrate variations in performance based not only on probe design but also target sample properties. As such, the depth and relative Raman scattering of the desired signal source and the optical parameters of the sample are necessary considerations prior to the start of a fiber probe based study. Simulations based on fluence estimation from ray-tracings in air indicate theoretical performance, with limited superficial detection governed by acceptance angle and excitation and collection fiber cone overlap. By comparison, these same probe designs displayed greater depth dependent performance for superficial sensitivity and depth rejection in biological muscle and adipose tissues, underscoring the importance of incorporating application-specific optical properties during probe design and optimization.

Although this investigation focused on four particular probe geometries, this robust Monte Carlo model allows extension to other probe designs for any application with known optical properties. The model presented here can be easily extended for other existing probe geometries, and can be used to optimize entirely novel probe designs prior to fabrication, reducing time and cost while improving data quality for fiber optic Raman spectroscopy applications.

5. Supplementary materials

The scripts for the presented Monte Carlo model outlined in Fig. 2 are available from the Vanderbilt Biophotonics Center for use or modification, upon request. Please contact the corresponding author for further details.

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