A liquid optical phantom with tissue-like heterogeneities for confocal microscopy

Danni Wang, Ye Chen, and Jonathan T. C. Liu*

Stony Brook University (SUNY), Department of Biomedical Engineering, Stony Brook, NY 11794, USA
*jonathan.liu@stonybrook.edu

Abstract: Phantoms play an important role in the development, standardization, and calibration of biomedical imaging devices in laboratory and clinical settings, serving as standards to assess the performance of such devices. Here we present the design of a liquid optical phantom to facilitate the assessment of optical-sectioning microscopes that are being developed to enable point-of-care pathology. This phantom, composed of silica microbeads in an Intralipid base, is specifically designed to characterize a reflectance-based dual-axis confocal (DAC) microscope for skin imaging. The phantom mimics the scattering properties of normal human epithelial tissue in terms of an effective scattering coefficient and a depth-dependent degradation in spatial resolution due to beam steering caused by tissue micro-architectural heterogeneities.

© 2012 Optical Society of America

OCIS codes: (170.1790) Confocal microscopy; (170.7050) Turbid media; (170.6900) Three-dimensional microscopy; (170.3880) Medical and biological imaging; (170.5810) Scanning microscopy

References and Links
1. J. T. C. Liu, N. O. Loewke, M. J. Mandella, R. M. Levenson, J. M. Crawford, and C. H. Contag, “Point-of-care pathology with miniature microscopes,” Anal Cell Pathol (Amst) 34(3), 81–98 (2011).
2. J. M. Jabbour, M. A. Saldua, J. N. Bixler, and K. C. Mairland, “Confocal endomicroscopy: instrumentation and medical applications,” Ann. Biomed. Eng. 40(2), 378–397 (2012).
3. R. Nordstrom, “Phantoms as Standards in Optical Measurements,” Proc. SPIE 7906, 79060H, 79060H-5 (2011).
4. J. Hwang, J. C. Ramella-Roman, and R. Nordstrom, "Introduction: feature issue on phantoms for the performance evaluation and validation of optical medical imaging devices," Biomed. Opt. Express 3(6), 1399–1403 (2012).
5. B. W. Pogue and M. S. Patterson, “Review of tissue simulating phantoms for optical spectroscopy, imaging and dosimetry,” J. Biomed. Opt. 11(4), 041102 (2006).
6. T. Moffitt, Y. C. Chen, and S. A. Prahl, “Preparation and characterization of polyurethane optical phantoms,” J. Biomed. Opt. 11(4), 041103 (2006).
7. S. L. Jacques, B. Wang, and R. Samatham, “Reflectance confocal microscopy of optical phantom,” Biomed. Opt. Express 3(6), 1162–1172 (2012).
8. L. Lui, P. A. Roman, S. A. Mathews, and J. C. Ramella-Roman, “Microfluidics based phantoms of superficial vascular network,” Biomed. Opt. Express 3(6), 1350–1364 (2012).
9. G. Lamouche, B. F. Kennedy, K. M. Kennedy, C. E. Bisaillon, A. Curatolo, G. Campbell, V. Pazos, and D. D. Sampson, “Review of tissue simulating phantoms with controllable optical, mechanical and structural properties for use in optical coherence tomography,” Biomed. Opt. Express 3(6), 1381–1398 (2012).
10. R. C. Chang, P. Johnson, C. M. Stafford, and J. Hwang, “Fabrication and characterization of a multilayered optical tissue model with embedded scattering microspheres in polymeric materials,” Biomed. Opt. Express 3(6), 1326–1339 (2012).
11. S. T. Flock, S. L. Jacques, B. C. Wilson, W. M. Star, and M. J. van Gemert, “Optical properties of Intralipid: a phantom medium for light propagation studies,” Lasers Surg. Med. 12(5), 510–519 (1992).
12. J. T. C. Liu, M. J. Mandella, J. M. Crawford, C. H. Contag, T. D. Wang, and G. S. Kino, “Efficient rejection of scattered light enables deep optical sectioning in turbid media with low-numerical-aperture optics in a dual-axis confocal architecture,” J. Biomed. Opt. 13(3), 034020 (2008).
13. P. D. Nimni, F. Martelli, and G. Zaccanti, “Intralipid: towards a diffusive reference standard for optical tissue phantoms,” Phys. Med. Biol. 56(2), N21–N28 (2011).
14. J. T. LaCroix and M. A. Haidekker, “Quantifying light scattering with single-mode fiber -optic confocal microscopy,” BMC Med. Imaging 9(1), 19 (2009).
1. Introduction

There has been increasing interest in translating point-of-care optical microscopes into the clinic to observe subtle changes in pathological microenvironments for the early detection and diagnosis of diseases [1,2]. Technological modifications are constantly being investigated to improve the imaging performance and clinical utility of such devices. A reproducible and standardized method of assessing imaging performance would therefore be of importance in evaluating and comparing these new approaches. Furthermore, as technologies mature past the stage of prototyping and into preclinical and clinical testing, it is crucial to minimize the variations in device performance between various clinics and other test sites. The development of a reproducible phantom is an essential component in the verification and validation of these microscopes [3]. There have been many advances made in optical phantom design in recent years, in which appropriate phantoms are designed to model the optical properties that are most relevant for a specific imaging system and clinical application [4–10]. For example, phantoms mimicking optical, mechanical, as well as structural properties of tissue [6,10] have been developed for use with popular biophotonics techniques such as optical coherence tomography [8,9], spectroscopy [5] and conventional reflectance confocal microscopy [7]. Similarly, in this study, we have designed an optical-scattering phantom to verify the performance of a reflectance-based dual-axis confocal (DAC) microscope for imaging unprocessed fresh human skin. In particular, this phantom has been designed to replicate the extent of scattering and the refractive aberrations encountered during high-resolution optical imaging of the human epithelium.

High-resolution optical-sectioning microscopes are typically characterized by measuring their response to reflective targets. For example, an axial response is often measured by translating a flat mirror through the focus of a microscope in the vertical (depth) direction whereas the transverse response of a microscope is often characterized by translating a knife-edge reflector (a sharp transition between a non-reflective and highly-reflective surface) in the
lateral direction at the focus of a microscope. In order for the axial and transverse responses to be measured easily, a liquid-based or highly deformable phantom is ideal since it ensures consistent contact between all surfaces as they are translated spatially. In the past, various groups have used Intralipid™ as a simple liquid-based scattering phantom [11–15]. Intralipid is a homogeneous lipid emulsion, with a milk-like appearance, which exhibits a reproducible scattering coefficient and anisotropy factor [16], making it especially useful for characterizing low-resolution optical imaging devices such as photon-migration tomographs [17]. However, when interrogating human epithelial tissues with sub-cellular resolution, our group and others [18,19] have observed a degradation in spatial resolution that is not observed in experiments using Intralipid as a tissue phantom (see results). It has been established that although Intralipid models the dominant scattering properties of tissue, it lacks the micro-architectural heterogeneities to serve as a realistic scattering phantom for confocal microscopy. Structural heterogeneities such as cellular nuclei, glands, and vasculature, can cause significant beam steering and lensing (refractive effects) in diffraction-limited optical beams, thus leading to misalignments and degraded image resolution and contrast [18–20]. Therefore, we have formulated a tissue phantom that more closely mimics the confocal-imaging properties of human epithelium, our target of interest, both in terms of scattering magnitude and the heterogeneity-induced degradation in resolution.

An argument can be made that homogeneous tissue phantoms such as Intralipid are sufficient for ensuring the proper alignment and performance of optical-sectioning microscopes. However, since resolution and contrast are inextricably linked [21], an accurate assessment of microscope performance requires the use of phantoms that mimic both the contrast- and resolution-degrading properties of real tissues. Furthermore, a number of recent imaging techniques are being explored to improve the ability of light to propagate through tissues with diffraction-limited beam qualities [22–25]. The accurate evaluation of these techniques would benefit from a heterogeneous tissue phantom that reproducibly mimics the scattering and aberrating properties seen in real tissues.

2. Materials and methods

2.1. Dual-axis confocal (DAC) microscope

A custom dual-axis confocal (DAC) microscope was developed previously [12,26]. The DAC architecture allows for efficient rejection of out-of-focus and multiply scattered light, enabling relatively deep optical sectioning within tissues, as demonstrated through diffraction theory modeling [26], Monte Carlo scattering simulations, tissue-phantom measurements [12], and imaging studies [12,26]. In this study, the DAC microscope performance was characterized in reflectance mode. A unique feature of a DAC microscope is that the illumination and collection beams travel different paths through tissue (Fig. 1a), making the alignment particularly vulnerable to degradation via refractive beam-steering and lensing effects [18].

2.2. Axial response

For axial-response measurements, a chrome mirror was translated at a velocity of 0.66 µm/s through the microscope focus in the axial (z) direction using a motorized micrometer (Newport Inc., PN: 12CCCL). The axial response is a plot of the system output intensity as a function of axial mirror displacement (relative to the focus at z = 0). The axial resolution is defined as the full width at half-maximum (FWHM) of the Gaussian profile of the axial response [26]. An axial response measured in a non-scattering water sample is acquired as a reference measurement in all experiments.

2.3. Transverse response

A knife-edge target consisting of a reflective chrome mirror (40% reflectivity) that transitions sharply (~20 nm transition width) into uncoated glass (0.4% reflectivity) is positioned at the
microscope focal plane. The knife edge is oriented along the x direction and is translated at a velocity of 0.66 µm/s in the y direction as shown in Fig. 1a. The transverse response is plotted as the normalized system output as a function of the knife-edge position. The transverse resolution is defined as the distance between the 90% to 10% intensity points of the transverse response. A transverse response measured in a non-scattering water sample is acquired as a reference measurement in all experiments.

2.4. Effective scattering coefficient

We have defined an empirical “effective scattering coefficient (µs-eff)” to characterize each phantom, which quantifies the signal degradation as a function of penetration depth due to both true scattering losses (µs) as well as losses from tissue-heterogeneity-induced aberrations and refractive beam steering and/or lensing effects. For these measurements, the peak output intensity of the microscope is obtained when the mirror is placed at the focus of the microscope. This peak signal is recorded as the focus of the microscope, and the mirror (always positioned at the focus), are adjusted over a range of shallow imaging depths in each sample. Since the in-focus signal at shallow depths is predominantly due to ballistic (unscattered) photons [12], the µs-eff is calculated according to the Beer-Lambert relation, Eq. (1):

\[ \mu_{s-eff} = \frac{1}{L_i - L_2} \ln \left( \frac{I_2}{I_1} \right), \]

where \( L_i \) is the path-length of the ballistic (unscattered) photons through tissue at location \( i \), and \( I_i \) is the corresponding detected peak signal. For a DAC microscope, \( L_i = 2 \frac{d_i}{\cos \theta} \), where \( d_i \) is the physical depth of the imaging plane at location \( i \). \( L_i \) is the total round-trip pathlength traversed by ballistic photons through the tissue (and reflected off the mirror) when the illumination and collection axes of a DAC microscope have a crossing half-angle of \( \theta \) (see Fig. 1a). Here we emphasize again that \( \mu_{s-eff} \) is not a true scattering coefficient, but is an empirical constant to describe signal loss as a function of depth due to both true scattering (\( \mu_s \)) AND refractive misalignments and optical aberrations.

2.5. Phantom design and testing

An aqueous suspension of polydisperse silica beads was prepared at 0.15 mg/mL for initial attempts to develop an optical phantom for our DAC system. The sample (MIN-U-SIL®40, U.S. Silica, Berkeley Springs, WV) contains ground silica beads with a median diameter of 10.5 μm and a maximum bead diameter of 40 μm. This bead mixture mimics the size of refractive objects in tissue ranging from sub-cellular organelles (<10 μm) to cell bodies (10–50 μm) and microvasculature (>10 μm).

The suspension concentrations were adjusted by water dilution as needed. In certain samples, 20% Intralipid (Sigma Aldrich) was added as a homogeneous scattering component. These phantoms were evaluated by performing axial and transverse response measurements on our tabletop DAC microscope. Since the observed degradation in resolution is smaller in magnitude in the transverse direction compared to the axial direction, the initial screening of phantom formulations was performed by gauging only the axial responses of the DAC microscope at a wavelength of 660 nm (index of refraction of silica at 660 nm is ~1.45).

2.6. Full-thickness human epithelium

Normal human skin tissues were obtained from consenting patients at the dermatology clinic of Memorial Sloan-Kettering Cancer Center (MSKCC). The skin-biopsy locations varied. All procedures were approved by the Institutional Review Board of MSKCC (IRB# 08-006). The full-thickness epithelium (approx. 75- to 125-μm thick) was peeled from excised skin samples soaked in warm isotonic saline (50–60°C). No preservatives were added and no additional processing was done to the fresh tissue samples.
3. Results and discussion

The optical properties of skin tissues are highly variable between patients and at different locations within an individual. Our goal has been to develop a phantom with reproducible optical properties that lie within the range of values observed by us and others in human samples [18,19]. As mentioned previously, measurements in Intralipid (Figs. 1d, 1e) reveal that this homogeneous phantom does not recapitulate the heterogeneities in real tissues that cause beam steering and aberrations (Figs. 1b, 1c). All signals are reported in dB (10log(I/I_max)).

Fig. 1. (a) Schematic of experimental setup with the DAC microscope, in which the illumination beam is colored blue and the collection beam path is colored green. (b) The axial response of the DAC microscope when imaging through full-thickness human epithelium (approx. 75- to 125-μm thick) is highly variable and shows a degradation in resolution (~3 dB) compared to a reference water sample. (c) Transverse response of the DAC microscope when imaging through full-thickness human epithelium shows resolution degradation (highly variable) compared to a reference (water) sample. (d) The axial and (e) transverse responses
when imaging through ~100 μm of Intralipid do not exhibit a noticeable degradation in resolution.

The first step in all experiments was to confirm the alignment and diffraction-limited resolution of our DAC microscope by measuring the axial and transverse responses in water. These experiments led to consistent measurements of axial resolution = 2.1 ± 5% μm, and lateral resolution = 1.2 ± 5% μm.

During initial experiments, the axial responses with 0.15-mg/mL samples of MIN-U-SIL®40 beads, at a depth of ~100 μm, revealed a significant degradation in resolution (Fig. 2). At a concentration of 0.15 mg/mL, the measured μs-eff of the 40-μm bead phantom was 77.3 mm⁻¹, which is much higher than the typical reported values for the true scattering coefficient, μs, of skin [27,28]. As mentioned previously, note that our empirical definition of μs-eff quantifies signal loss as a function of depth due to both true scattering, μs, as well as tissue-heterogeneity-induced refractive effects (beam steering, lensing, and aberrations). The reproducibility of measurements performed with this phantom was low due to temporal stability issues (Table 1). We found that lower-concentration bead suspensions were more stable. Therefore, subsequent investigations utilized lower concentrations of bead suspensions that exhibited reproducible optical properties for at least 10 minutes.

The axial responses with MIN-U-SIL®40 bead suspensions at concentrations of 0.15 mg/mL, 0.075 mg/mL, and 0.0075 mg/mL are shown in Fig. 2. The measured axial resolutions are 3.1 μm, 3.7 μm, and 2.2 μm, respectively, and the μs-eff are calculated to be 77.3 mm⁻¹, 11.8 mm⁻¹, and 1.9 mm⁻¹, respectively. Comparing its axial response with the control (water) sample, the lowest-concentration (0.0075 mg/mL) bead suspension does not reveal a significant degradation in axial resolution. In addition, compared to the published values of μs for human skin [27,28] the μs-eff of the highest-concentration (0.15 mg/mL) bead suspension is too high, and the μs-eff of 0.0075 mg/mL bead suspension is too low. The 0.075 mg/mL suspension exhibited a μs-eff that is comparable to skin, but the variability between measurements was relatively high. Therefore, it was determined that the optimal phantom formulation of a MIN-U-SIL®40 bead suspension (in water), with an appropriate μs-eff and high reproducibility, should lie within a concentration range of 0.0075 mg/mL to 0.075 mg/mL.

Fig. 2. The axial response of the DAC microscope when investigating different concentrations of MIN-U-SIL®40 bead suspensions.

In aqueous suspensions with beads alone, there is a direct coupling between μs-eff and the level of resolution degradation. In addition, high bead concentrations exhibit poor temporal stability (<10 minutes). Therefore, we decreased the bead concentration to the lowest level where a notable level of degradation in the axial resolution was still observed. Then, to compensate for the loss of scattering events due to decreased bead concentration, the base of
the solution was changed to a low concentration of Intralipid, thereby allowing us to tune the $\mu_{\text{c-eff}}$ independently of the resolution degradation.

Intralipid was added into bead suspensions at a concentration of 5% or 3% by volume. The axial and transverse responses with MIN-U-SIL®40 bead suspensions (0.050 mg/mL) in 5% and 3% Intralipid are shown in Fig. 3a (axial response) and Fig. 3b (transverse response). The axial resolution in both cases is ~3.4 µm (a 62% degradation vs. baseline resolution), and the transverse resolution in both cases is ~2.0 µm (a 67% degradation vs. baseline). The $\mu_{\text{c-eff}}$ is calculated to be 11.0 mm$^{-1}$ for the beads in 5% Intralipid, and 8.1 mm$^{-1}$ for the beads in 3% Intralipid.

As shown in Table 1, the phantom formulation of 0.050 mg/mL MIN-U-SIL®40 beads in 5% Intralipid produces measurements with the lowest standard deviation. Since reproducibility is a critical feature for phantoms, this phantom formulation represents the optimal choice.

Table 1. Summary of phantom testing

| Sample | $\mu_{\text{c-eff}}$ (mm$^{-1}$) ± std. dev. | Axial resolution (µm) ± std. dev. | Transverse resolution (µm) ± std. dev. |
|--------|-------------------------------------|---------------------------------|------------------------------------|
| Water  | NA                                  | 2.1 ± 5%                        | 1.2 ± 5%                           |
| 20% Intralipid | 43.9 ± 14% (n = 6) | 2.1 ± 10%                        | 1.2 ± 10%                           |
| 10% Intralipid | 23.1 ± 14% (n = 6) | 2.1 ± 10%                        | 1.2 ± 10%                           |
| 5% Intralipid | 12.0 ± 6% (n = 6) | 2.1 ± 10%                        | 1.2 ± 10%                           |
| 0.15 mg/mL MIN-U-SIL®40 | 77.3 (n = 1) note: not stable | 3.1 ± 26%                        | NA                                 |
| 0.075 mg/mL MIN-U-SIL®40 | 11.8 ± 18% (n = 6) | 3.7 ± 26%                        | NA                                 |
| 0.0075 mg/mL MIN-U-SIL®40 | 1.9 ± 16% (n = 6) | 2.2 ± 10%                        | NA                                 |
| 0.05 mg/mL MIN-U-SIL®40, 3% Intralipid | 8.1 ± 16% (n = 6) | 3.4 ± 16%                        | 2.0 ± 12%                           |
| 0.05 mg/mL MIN-U-SIL®40, 5% Intralipid | 11.0 ± 8% (n = 12) | 3.4 ± 12%                        | 2.0 ± 8%                           |

Finally, we characterized the depth dependence of the degradation in axial resolution for our DAC microscope imaging through the most ideal phantom formulation (0.050 mg/mL MIN-U-SIL®40 beads in 5% Intralipid). Note that until this point, all resolution measurements were made at a phantom depth of approximately 100 µm in order to match the typical thickness of human full-thickness skin epithelium samples. Fig. 4 shows how the axial resolution varies as a function of penetration depth through the phantom. The results show that the degradation in resolution reaches a relatively constant value at depths above 100 µm. This may be due to the fact that the tissue heterogeneities only create significant aberrations.
and beam steering near the beam waist at the focus of the microscope. Further simulations and experiments are needed to explore this hypothesis, both in heterogeneous phantoms and in real tissues.

![Graph](image)

**Fig. 4.** The axial resolution (FWHM) of the DAC microscope as a function of imaging depth through a heterogeneous phantom. Error bars correspond to 1 standard deviation from the mean.

### 4. Summary

In this study, we present a formulation for a liquid optical phantom with tissue-like heterogeneities for confocal microscopy and have validated its performance with a DAC microscope. This phantom is highly reproducible, stable, simple to prepare, and mimics the scattering properties of the normal human epithelium. This phantom is a necessary component for assessing the alignment, sensitivity, resolution and contrast of various prototypes of optical sectioning microscopes under development and in various stages of preclinical and clinical testing. In particular, since our ultimate goal is to translate miniature microscopes into the clinic for *in vivo* image-guided tumor resection and diagnostics [29], it is critical to develop a standardizable testing method and phantom to ensure the consistent performance of devices at multiple testing sites. In addition, our phantom will be useful for the development of novel imaging approaches that further mitigate the effects of tissue scattering and aberrations.

### Acknowledgments

This research was supported by the National Institute of Biomedical Imaging and Bioengineering (NIBIB)—R00 EB008557 (Liu); the National Institute of Dental and Craniofacial Research (NIDCR)—R01 DE023497 (Liu); the Undergraduate Research and Creative Arts (URECA) program at Stony Brook (support for Y. Chen), and the Office of the Vice President for Research (OVPR) at Stony Brook University, a State University of New York (SUNY). The authors would like to acknowledge Dr. Milind Rajadhyaksha and Dr. Bjorg Larson from the Memorial Sloan Kettering Cancer Center for fresh human skin samples.