Stratified squamous epithelial tissues such as cervix, oral cavity, and skin are essentially layered structures that consist of an epithelial layer, a basement membrane, and an underlying stroma. The depth distribution of endogenous fluorophores in these tissues varies with a number of factors including age, menopausal status, and disease progression (from normal to dysplasia to carcinoma). Hence fiber-optic probes are sensitive to the fluorescence that originates from different sublayers. Previously a multiseparation approach (varying the separation between illumination and collection fibers) was employed for depth-resolved fluorescence detection from turbid media. Monte Carlo modeling of this probe geometry for depth-sensitive fluorescence spectroscopy of a two-layered tissue phantom indicates that this probe is superior to that achieved with the multiseparation approach. We fabricated a first-generation angled fiber-optic probe in our lab. This angled probe consisted of a central collection fiber, which was fixed perpendicularly to the tissue surface, and two illumination fibers at a subset of illumination angles evaluated in the simulations. These illumination angles were 15° and 45° relative to the axis perpendicular to tissue surface, and the illumination fibers were placed on the opposite sides of the collection fiber. All fiber tips were polished to be flush with the tip of the probe. All fibers had a core diameter of 200 μm and a numerical aperture of 0.22. The center-to-center distance between the illumination and collection fibers at both illumination angles was approximately 450 μm. Illumination fibers for normal incidence and for an illumination angle of 30° were not included in the angled probe because sensitivity to the epithelial layer is similar for the 0° (normal incidence), 15°, and 30° angles of illumination at illumination-collection fiber separations greater than 400 μm.

The illumination fibers of the probe were coupled to an argon-ion laser (Innova 305 C, Coherent, Inc., Santa Clara, Calif.), which provided a maximum power of 0.19 W at 351 nm. The output power from the laser was attenuated to 1.90 mW by an adjustable aperture and a neutral-density filter and then coupled into the angled illumination fiber. The actual output powers of the illumination fibers were measured by a calibrated optical powermeter and were found to be approximately 650 and 300 μW for illumination angles of 15° and 45°, respectively. The collection fiber of the probe was coupled to the detection module of a fluorophotometer (Skinscan, J. Y. Horiba, Inc., Edison, N.J.), which comprises double emission scanning monochromators and a photomultiplier tube (PMT).

Two-layered agar phantoms were made to mimic human epithelial tissues based on previously published recipes. The thickness of the top layer was varied from 300 to 900 μm in 300-μm increments to cover the range of thicknesses of human epithelium, whereas that of the second layer and the lateral dimension were set at a large value (≥1 cm) to represent a semi-infinite medium. Plastic wraps (~10 μm in thickness) were placed between the two layers as well as between the probe tip and the top layer to prevent diffusion of phantom components. In the top layer, scattering was achieved with a suspension of polystyrene spheres (diameter of particles, 1.053 μm) at a concentration of 0.266% by volume (Polysciences, Inc., Warrington, PA).

Experimental proof of the feasibility of using an angled fiber-optic probe for depth-sensitive fluorescence spectroscopy of turbid media

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Received March 26, 2004

An angled fiber-optic probe that facilitates depth-sensitive fluorescence measurements was developed for enhancing detection of epithelial precancers. The probe was tested on solid, two-layered phantoms and proved to be effective in selectively detecting fluorescence from different layers. Specifically, a larger illumination angle provides greater sensitivity to fluorescence from the top layer as well as yielding an overall higher fluorescence signal. Monte Carlo simulations of a theoretical model of the phantoms demonstrate that increasing the illumination angle results in an increased excitation photon density and, thus, in increased fluorescence generated in the top layer. © 2004 Optical Society of America

OCIS codes: 060.2350, 300.2530, 300.6540, 170.3660, 170.3880.
The reason for using hemoglobin are similar to those reported in the literature for 580 nm (peak fluorescence emission of Rhodamine B) and 460 nm (peak fluorescence emission of NADH) and absorption coefficient of ink at the excitation wavelength to those at the peak emission wavelengths of 351–580 nm by use of the same geometry to account for the differences in the incident power at two angles of illumination. It is obvious that the fluorescence intensities detected at an illumination angle of 45° are at least one order of magnitude higher than those measured at an illumination angle of 15°, regardless of the top layer thickness. This trend is consistent with the results of previous Monte Carlo simulations. Another trend worth noting is that the ratio between the peak intensities at 467 nm (NADH fluorescence in the top layer) and 580 nm (Rhodamine B fluorescence in the bottom layer) is noticeably greater at an illumination angle of 45° than at 15° for top layer thicknesses of 300 and 600 μm. These results suggest that relatively more fluorescence is collected from the top layer when a 45° illumination angle is used.

### Table 1. Values of \( \mu_a \) [cm\(^{-1}\)], \( \mu_s \) [cm\(^{-1}\)], and \( g \) (left to right) for the Top and Bottom Layers of the Two-Layered Phantom at the Excitation Wavelength and at the Peak Emission Wavelengths of NADH and Rhodamine B

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Layer</th>
<th>( \mu_a ) [cm(^{-1})]</th>
<th>( \mu_s ) [cm(^{-1})]</th>
<th>( g )</th>
</tr>
</thead>
<tbody>
<tr>
<td>351</td>
<td>Top</td>
<td>4.2, 118.5, 0.91</td>
<td>3.0, 132.1, 0.91</td>
<td>2.2, 113.9, 0.91</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>22.4, 331.8, 0.91</td>
<td>7.4, 369.9, 0.91</td>
<td>3.8, 318.9, 0.91</td>
</tr>
<tr>
<td>460 (NADH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>580 (Rhodamine B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To facilitate discrimination of the fluorescence from the top layer more efficiently at the 45° illumination angle than at the 15° illumination angle. When the thickness of the top layer is 900 μm, the
Fig. 2. Ratio between peak intensities at 467 nm (NADH fluorescence in the top layer) and 580 nm (Rhodamine B fluorescence in the bottom layer) for top layer thicknesses of 300, 600, and 900 μm. Results are shown for two phantoms made according to the same recipe.

peak intensity ratios at the two illumination angles are similar, suggesting that the maximum depth from which the detected fluorescence originates at both illumination angles is within 900 μm.

To elucidate the underlying basis of the above findings we determined the excitation fluence distribution within the two-layered phantom from Monte Carlo simulations for the 15° and 45° angled illumination geometries. The fluence [cm⁻²] refers to the radiant energy propagating in all directions through a given location and was calculated by division of the photon energy deposited within a local volume by the local absorption coefficient, which essentially reflects the distribution of excitation light inside this tissue. Figure 3 shows the simulated excitation fluence as a function of depth for top layer thicknesses of 300, 600, and 900 μm in a theoretical model of the two-layered phantom, which provides insight into the basis for depth-sensitive fluorescence detection with the angled illumination probe geometry. Because fluorescence starts with absorption of excitation light, the distribution of excitation light in the tissue determines where the fluorescence originates. The shallower the distribution of excitation light, the shallower the volume from which the detected fluorescence originates.

According to Fig. 3, the distribution of excitation light in the tissue phantom is confined to a more superficial tissue volume as the angle of illumination is increased. Thus it can be inferred that the detected fluorescence also originates from a more superficial volume as the angle of illumination is increased, which leads to two important conclusions: (1) The sensitivity to the fluorescence from the top layer increases with increasing angle of illumination and (2) the detected fluorescence signal increases with increasing angle of illumination because of decreased photon attenuation. These conclusions are expected to be true even when there is a difference between the excitation and the emission wavelength’s optical properties because the distribution of excitation light in tissue is determined only by the illumination geometry and optical properties of the phantom at the excitation wavelength.

In this Letter we have experimentally demonstrated depth-sensitive detection of fluorescence from a two-layered turbid medium by using an angled illumination probe. A more-sophisticated angled probe that includes additional angles of illumination (at a smaller separation of 250 μm) is being developed by our group. This next-generation probe will be tested systematically on solid phantoms and on epithelial tissues in vivo.

The authors acknowledge support from the Prize Fellowship, University of Wisconsin—Madison, and from National Institutes of Health grant PO1 CA082710 05. The e-mail address of Q. Liu is qliu@cae.wisc.edu.

References