Determination of Epithelial Tissue Scattering Coefficient Using Confocal Microscopy

Tom Collier, Dizem Arifler, Anais Malpica, Michele Follen, and Rebecca Richards-Kortum

***Abstract*—Most models of light propagation through tissue assume the scattering properties of the various tissue layers are the same. The authors present evidence that the scattering coefficient of normal cervical epithelium is significantly lower than values previously reported for bulk epithelial tissue. They estimated the scattering coefficient of normal and precancerous cervical epithelium using measurements of the reflectance as a function of depth from confocal images. Reflectance measurements were taken from *ex vivo* cervical biopsies and fit to an exponential function based upon Beer's law attenuation. The mean scattering coefficients derived were 22 cm^{-1} for normal tissue and 69 cm^{-1} for precancerous tissue. These values are significantly lower than previously reported for bulk epithelial tissues and suggest that scattering of bulk tissue is dominated by the stroma. They also suggest that computational models to describe light propagation in epithelial tissue must incorporate different scattering coefficients for the epithelium and stroma. Further, the lower scattering of the epithelium suggests greater probing depths for fiber optic probes used by optical diagnostic devices which measure reflectance and fluorescence in epithelial tissue. The difference in scattering between normal and precancerous tissue is attributed to increased nuclear size, optical density, and chromatin texture. The scattering coefficients measured here are consistent with predictions of numerical solutions to Maxwell's equations for epithelial cell scattering.**

***Index Terms*—Cervix, confocal microscopy, epithelial tissue, scattering.**

I. Introduction

A NUMBER of optical technologies have recently shown promise for detection of precancerous lesions *in vivo*, including reflectance spectroscopy [1]–[6], fluorescence spectroscopy [7]–[12], Raman spectroscopy [13], [14], confocal imaging [15]–[17], and optical coherence tomography (OCT) [18], [19]. Optical technologies are sensitive to both morphologic architectural and biochemical changes associated with the dysplasia to carcinoma sequence [20], [21]. As precancers develop, the morphology of epithelial cells is altered, with hyperchromasia, pleomorphism, an increased nuclear to cytoplasmic ratio, and increased metabolic activity. Stromal changes also accompany the development of intraepithelial neoplasia, with increased angiogenesis, decreased matrix density, and other alterations in the epithelial-stromal communication [22]. Recently, a number of studies suggest that optical spectroscopy and imaging can probe changes in both the epithelium and stroma [23]–[25]. As an example, fluorescence spectroscopy has been used to simultaneously probe changes in epithelial metabolism and stromal matrix density [23], [25].

Optical spectroscopy measurements can be made easily *in vivo* using fiber optic-based probes. Several analytic and numeric models have been proposed to mathematically separate the signal recorded from the epithelium and the stroma [23], [25], [26]. Monte Carlo models that simulate the random walk of photons in tissue have been used to suggest creative fiber optic probe designs that can physically separate signal from the epithelium and stroma [27]–[29]. However, in both cases, the accuracy of these models is limited by the relatively incomplete knowledge of the optical properties of each tissue layer. In particular, these models require accurate values for the absorption coefficient, the scattering coefficient, and the scattering anisotropy of both the epithelium and the stroma.

Several methods have been developed to compute the optical properties of tissue from measurements of collimated transmission, diffuse transmission, and diffuse reflectance [30]–[32] or from reflectance measurements made at varying source detector separations [33], [34]. In general, these methods assume that the optical properties of the sample are homogeneous. While optical properties have been derived using these approaches for a number of epithelial tissues, including the bladder, colon, skin, and esophagus [30], [35], [36], it is likely that the reported values are dominated by the optical properties of the stroma, which occupies the largest volume fraction of the tissue and which likely has the largest absorption and scattering coefficients.

In order to use multilayer models of tissue spectroscopy and to design sophisticated fiber optic probes to separate signals from different epithelial and stromal layers, it is critical to have accurate values for the optical properties of the epithelium. Qu and colleagues microdissected the epithelium of bronchial tissue and separately measured scattering of the epithelium and stroma. They measured scattering coefficients of 200 and 225 cm^{-1} at 700 nm for epithelial and stromal tissue, respectively [37]. An alternative to physical sectioning is optical sectioning. Using reflectance-based confocal microscopy, the depth dependence of reflected light from epithelial cells can easily be probed [15]–[17]. Several groups have measured the depth-dependent decay of reflected light in tissue phantoms using both OCT and confocal microscopy; intensity was observed to decay exponentially according to the scattering

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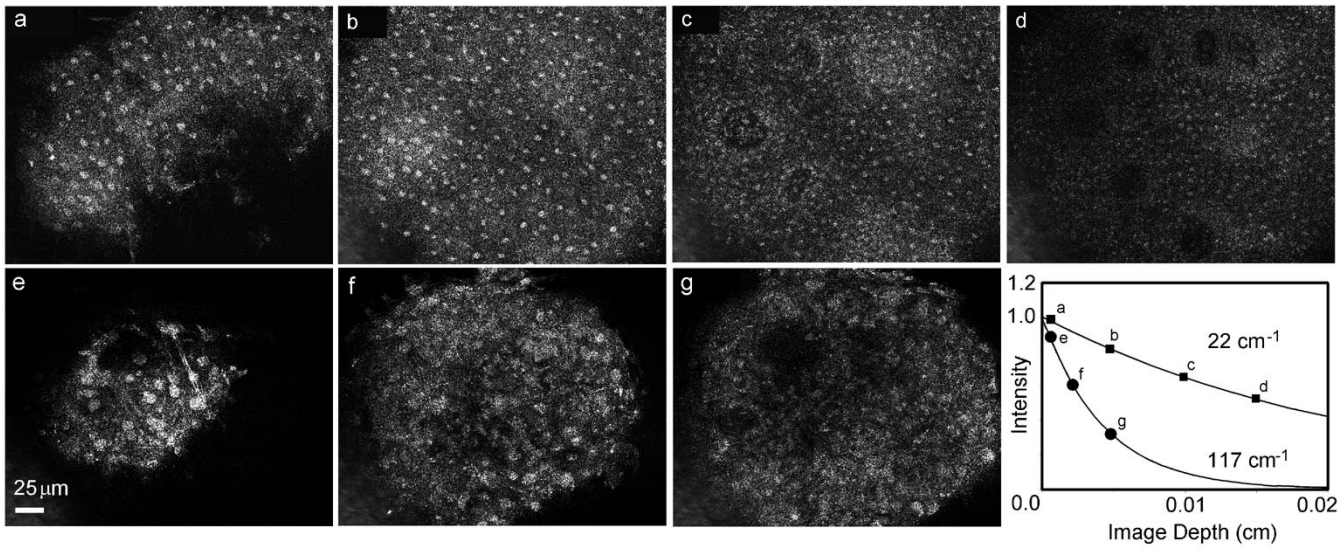

Fig. 1. Reflectance confocal images taken at various depths from a normal biopsy and a CIN 3 biopsy. Nuclei can be clearly visualized in images taken at varying depths within the epithelium of the normal biopsy at (a) 0 μm, (b) 50 μm, (c) 100 μm, and (d) 150 μm. Nuclei can only be visualized in the CIN 3 biopsy at image depths of: (e) 0 μm, (f) 25 μm, and (g) 50 μm. Attenuation of nuclear intensity is plotted for both biopsies with the estimated scattering coefficients illustrated.

coefficient [38]–[40]. With the reflected intensity measured using a confocal detection system from depth $z, I(z)$ can be expressed as

$$I(z) = I_0 e^{-\mu_t z} R(z) e^{-\mu_t z} = I_0 e^{-2\mu_t z} R(z) \qquad (1)$$

where I_o is the incident intensity, $R(z)$ is the depth dependent reflectivity, and μ_t is the attenuation coefficient, which is the sum of the absorption and scattering coefficients. Equation (1) assumes that excitation and reflected light travel parallel to the optical axis, which is equivalent to assuming a low numerical aperture. The equation can be used to analyze measurements of the intensity of reflected light measured as a function of depth to extract the attenuation coefficient of the epithelium, assuming that the reflectivity and the attenuation coefficient are not functions of depth within the epithelium. In the near infrared, where confocal measurements are frequently made, the absorption coefficient is significantly less than the scattering coefficient, so that this process can be used to estimate the epithelial tissue scattering coefficient [37].

The goal of the present study is to use (1) to analyze depth-dependent intensity measurements from confocal images of *ex vivo* cervical epithelium to estimate the scattering coefficient. In order to investigate the potential differences in scattering between normal and precancerous epithelium, measurements are made from both normal and precancerous tissue and compared with previous measurement of tissue scattering coefficients and numerical estimates of the scattering of normal and precancerous epithelial cells.

II. Methods and Materials

A. Patients

Cervical biopsies were acquired from 25 patients at the colposcopy clinics of the University of Texas M.D. Anderson Cancer Center, Herman Hospital, and Lyndon Baines Johnson Hospital, Houston, TX. The patients were referred to these clinics for suspected dysplasia on the basis of an abnormal cervical cytology or for removal of cervical tissue using the loop electrical excision procedure (LEEP) due to a previous diagnosis of dysplasia. Informed consent was obtained from each patient, and the project was reviewed and approved by the Surveillance Committee at the University of Texas M.D. Anderson Cancer Center and the Institutional Review Board at the University of Texas at Austin. Samples were included in the analysis presented here if the diagnosis of the biopsy was normal squamous epithelium or cervical intraepithelial neoplasia 3 (CIN 3) which indicates that dysplastic cells have been discovered in the upper one-third of the epithelium.

B. Specimens

Two uterine cervix biopsies were acquired from each patient. One of the biopsies was taken from a colposcopically normal area and the other was taken from a colposcopically abnormal area of the cervix and immediately placed in growth media (DMEM, no phenol red). Colposcopic impression (normal/abnormal) was recorded for each biopsy. The biopsies were approximately 3 mm wide by 4 mm long by 2 mm thick. Reflectance confocal images were taken of the biopsies within 6 h of excision.

C. Confocal Imaging

Reflectance confocal images were obtained from each biopsy using an epi-illumination laser scanning confocal microscope described in [15]. The illumination source was a continuous wave diode laser operating at 810 nm. Images were acquired with a 25X 0.8 NA water immersion objective (Plan-Neofluar multi-immersion, Zeiss). The confocal system operates at a dimensionless pinhole radius of 2.5 to provide maximum optical sectioning for obtaining cellular detail. The measured lateral and axial resolution of the system are 0.8 and 2–3 μm, respectively.

Immediately prior to imaging, biopsies were removed from growth medium and rinsed with PBS. A 6% solution of acetic acid was then added to each sample and image frames were acquired at various epithelial depths up to the working distance of the microscope, 250 um. Images were acquired with the image plane parallel to the tissue surface. Images were digitized using a video frame grabber card and displayed at 7.5 frames per second on a computer monitor. Individual bitmap image files were saved from the frame grabber's video buffer. All specimens were then submitted for routine histologic examination using hemotoxylin and eosin stain. Sections were examined by an experienced board certified gynecologic pathologist (AM).

D. Image Processing

Images from each biopsy were processed to extract the reflected intensity from cell nuclei as a function of depth beneath the tissue surface. At each depth that an image was obtained, the mean gray scale intensity was measured from all visible nuclei. The mean reflected intensity was plotted as a function of depth from each biopsy. These data were fit to (1) by minimizing the mean square error between the data and the fit with the scattering coefficient as the only variable parameter.

III. Results

Images were available from 19 biopsies from ten patients diagnosed as normal or CIN 3; 15 of these were diagnosed as squamous normal tissue and four were diagnosed as CIN 3. Fig. 1 shows a series of images taken at varying image depths from a histologically normal biopsy and a biopsy with CIN 3. Nuclei can be visualized in every image. The rate of intensity decrease is noticeably greater in the biopsy with CIN 3; nuclei can clearly be visualized at an image depth of 150 μm in the normal biopsy, but only to an image depth of 50 μm in the CIN 3 biopsy. The mean reflected intensity as a function of depth for these samples is illustrated in Fig. 1. Fits to (1) are also shown; the extracted scattering coefficient was 22 cm^{-1} for the normal biopsy and 117 cm^{-1} for the CIN 3 biopsy assuming that the absorption coefficient is small.

Fig. 2 illustrates fits to (1) for the 15 normal biopsies. In Fig. 2, the mean reflected intensity at the surface has been normalized to unity. The fits for all image sets had a mean correlation coefficient of greater than 0.95. The mean scattering coefficient was 22 ± 5 cm^{-1} with a minimum and maximum value of 14 and 30 cm^{-1}, respectively. Fig. 3 illustrates the fits to (1) for the four biopsies with CIN 3. The correlation coefficient is above 0.95 for the two biopsies with the highest decay rate and above 0.75 for the remaining two. The average scattering coefficient is 69 ± 33 cm^{-1}; the minimum value is 44 cm^{-1}, and the maximum value is 117 cm^{-1}. Fig. 4 shows a scatter plot of the scattering coefficients for all specimens. Though the number of samples with CIN 3 is small, the average scattering coefficient is higher in the biopsies with CIN 3 than in the normal biopsies. The separation between normal and CIN 3 is clear. The results of a student's t-test indicate that the difference in mean scattering coefficients measured from the normal and CIN 3 biopsies is statistically significant ($P < 0.0001$).

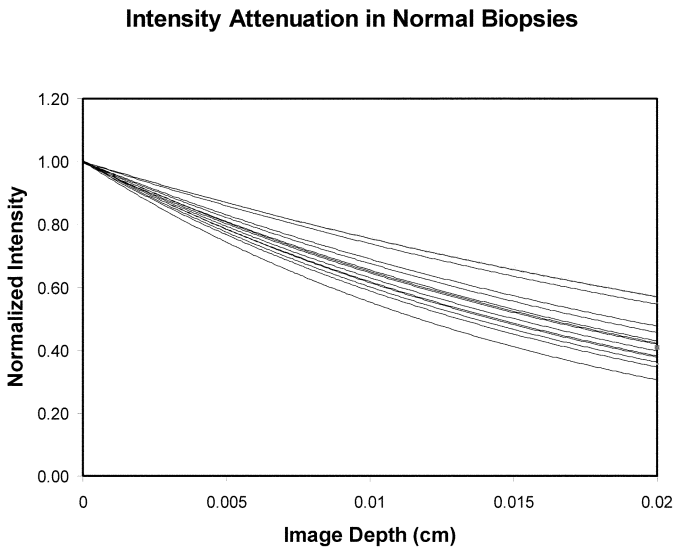

Fig. 2. Plot of the exponential fits from all normal biopsies; intensity at the tissue surface has been normalized to unity in each case. Mean scattering coefficient is $22 + / - 5$ cm^{-1}.

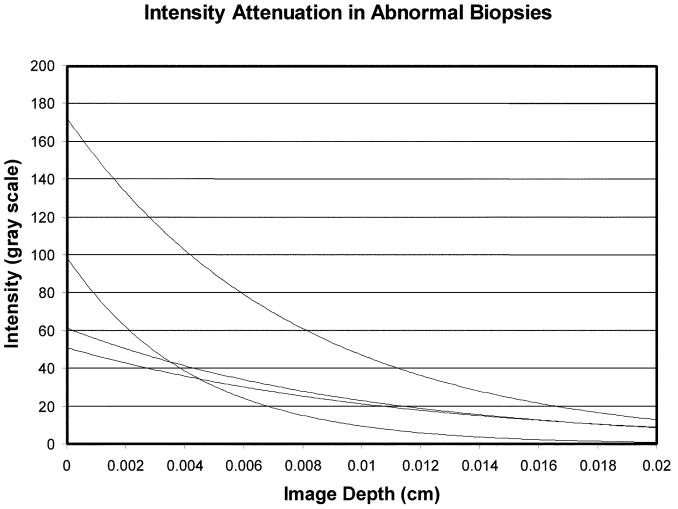

Fig. 3. Plot of the exponential fits from all CIN 3 biopsies. The mean scattering coefficient is $69 + / - 33$ cm^{-1}.

IV. Discussion

Table I presents scattering coefficients measured here for normal and precancerous cervix at 810 nm and compares these values to those reported in the literature for cervical, bladder, skin, and other epithelial tissues. Mourant studied the wavelength dependence of tissue scattering and noted that the scattering coefficient decreases with wavelength as $1/\lambda^{1.1}$ [45]. Table I calculates the scattering coefficient at 810 nm for other tissues assuming this relationship. The scattering coefficients measured here are significantly lower for normal cervical epithelium (22 cm^{-1}) than those previously reported for bulk scattering in cervical tissue (66–150 cm^{-1} at 800 nm) and in other epithelial tissues 53–370 (cm^{-1}). To our knowledge, they are also lower than values reported for any other tissue type. This suggests a significant disparity between the scattering properties of the cervical epithelium and the underlying connective tissue which appears to produce most of

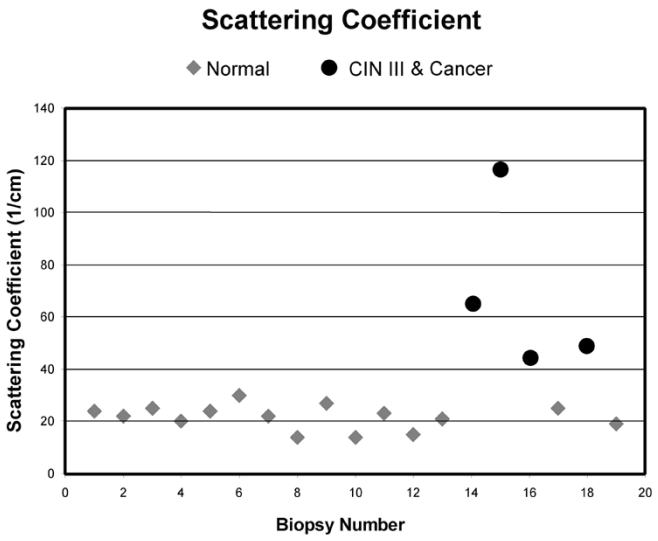

Fig. 4. Scatter plot of the scattering coefficients for all biopsies. Difference in the mean scattering coefficients of normal biopsies and CIN 3 biopsies are statistically significant ($P < 0.0001$).

TABLE I
REPORTED SCATTERING COEFFICIENT VALUES FOR EPITHELIAL TISSUES

Reported Scattering Coefficients				
Organ Site	**Reference**	**Wavelength Measured (nm)**	**μ_s (cm^{-1})**	**μ_s (cm^{-1}) @ 810 nm**
Cervix (normal)	This Paper	810	22	22
Cervix (precancerous)	This Paper	810	69	69
Cervix	36	800	66-100	65-99
Uterus	30	635	394	301
Breast	41	810	150	150
Breast	42	1100	100-500	140-700
Bronchial tissue epithelium	37	700	200	170
Esophagus	35	630	70	53
Skin	30	633	187	143
Skin	43	810	370	370
Skin	44	1000	160	127

the scattering. Further evidence for this conclusion can be made by considering the scattering properties of connective tissue alone. The scattering coefficient has recently been extracted from RAFT cultures composed of rat tail collagen and human fibroblasts [46] using second harmonic generation imaging. A value of 137 cm^{-1} was measured. The agreement between the values for bulk cervical tissue and the RAFT culture suggests that a majority of the scattering in bulk tissue is caused by the underlying connective tissue.

The scattering coefficients reported here for precancerous tissue (69 cm^{-1}) are significantly higher than the measurements for normal epithelium (22 cm^{-1}). We suspect that the increased scattering in the dysplastic tissue is due to changes in nuclear morphology. Quantitative measurements of the nuclear size and nuclear density from the same confocal image sets indicate that both are increased in CIN 3 [15]. Changes in chromatin optical density and texture within the nucleus are also suspected to cause increased scattering in the dysplastic tissue. We compared the scattering coefficient values measured here with values calculated using our finite-difference time-domain (FDTD) algorithm described in [47]. Estimating the volume fraction of nuclei and average nuclear size from the confocal images and

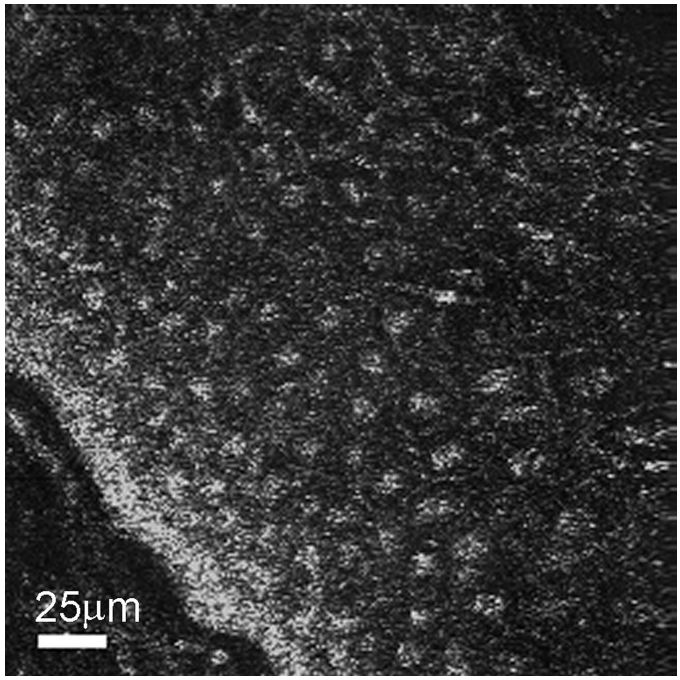

Fig. 5. Confocal image of *ex vivo* transverse sectioned biopsy. Depth-dependent reflectance is apparent with brighter nuclei near the basement membrane at the lower left corner of the image compared to those near the biopsy surface at the upper right corner of the image.

using the scattering cross section obtained using our FDTD algorithm, we predict the scattering coefficient for the normal and dysplastic tissue to be 13 and 142 cm^{-1}, respectively, at 810 nm. These values compare reasonably well with the values extracted from the confocal images (22 and 69 cm^{-1}) and give further evidence of increased scattering in the abnormal tissue.

A limitation of the current study is that confocal images were acquired from only three to five different depths. Increasing the number of images in each image stack would provide further evidence to indicate whether the signal decay is truly exponential. In the exponential fitting performed here, it has been assumed that the reflectance of the nuclei is constant as a function of depth within the epithelium. Confocal reflectance images taken of a transverse section of fresh cervical tissue (Fig. 5) indicate that this may not be the case. In Fig. 5, it is evident that the nuclei deep within the tissue, near the basement membrane, reflect more light than those near the top of the epithelium. This is consistent with FDTD models of the scattering cross section of superficial and basal epithelial cells where it has been hypothesized that increased nuclear optical density and increased refractive index heterogeneities within the nuclei cause increased backscattering from the basal cells [47]. The confocal image of the transverse section provides a direct measure of R(z), the reflectivity as a function of depth. In future work, this can be used to improve estimation of the depth dependent scattering coefficient. In addition, the scattering coefficients estimated here are not corrected for the finite NA of the detection system. The maximum incident angle is 37° with this system, so this may overestimate the scattering coefficient by as much as $1/\cos(37°) = 125\%$. Finally, we assume that the absorption coefficient is negligible compared

to the scattering coefficient at 810 nm. This is likely reasonable as the absorption coefficient of amelanotic epithelial tissues, including the cervix, has been reported with measurements below 1 cm^{-1} [30], [37].

The results presented here have important implications for computational tissue modeling of epithelial tissue fluorescence and reflectance and for optical probe design for *in vivo* data and image acquisition. The disparity of scattering between the epithelium and stroma suggests that single-layer tissue models that assume tissue is homogeneous are not well suited to describe propagation of light through epithelial tissue. The low scattering coefficient of the epithelium implies deeper light penetration within tissue, likely below the basement membrane and into the stroma.

Pfefer [27], Pogue [28], and Ramanujam [29] have shown that parameters such as the fiber diameter, source-to-collection fiber separation, and probe-to-tissue surface distance all affect the mean path length and collection efficiency of optical probes and imaging systems. Pogue demonstrated a fiber optic probe where the signal intensity is not significantly affected by the medium's absorption and scattering coefficients, but depends only on the properties of the fluorophores. This design was based on Monte Carlo simulations of a homogeneous medium with a scattering coefficient of 100 cm^{-1}, well above what we find for the epithelial layer of cervical tissue. Ramanujam presented a concept to locate the depth of a target layer of precancerous tissue between a normal epithelial layer and a stromal layer. This method was based on exciting and recording the fluorescence through apertures of variable diameter. Assuming the same scattering coefficient for all three layers (100 cm^{-1}), she showed that the variable aperture imaging method could be used to determine the depth dependence of the fluorescence contrast provided by the precancerous epithelium. Again, these calculations assume a much larger epithelial scattering coefficient than measured here. Pfefer presented an extensive Monte Carlo modeling study to investigate design parameters of the fiber optic probes. Assuming that the optical properties are homogeneous, with scattering coefficients ranging from 70–140 cm^{-1}, he studied the impact of tissue optical properties on fluorescence spectra. For example, Pfefer found an increase in axial selectivity with decreasing fiber radius. The conclusions of all these studies may not be applicable to cervical tissue, since the cervix has a layered structure and a lower scattering coefficient for the epithelial layer than used in these calculations. The design of fiber optic probes and imaging systems is critical to optimizing diagnostic information, and further work using the appropriate scattering coefficients is needed.

The lower scattering values also suggest improved imaging depths for many imaging modalities. The fundamental limit for reflectance based imaging within tissue is high scattering. A change in the scattering coefficient from 100 (the often assumed value) to 22 cm^{-1} results in a twofold increase in imaging depth for epi-illumination imaging. This is particularly significant for imaging modalities such as confocal and two-photon microscopy that use longer wavelengths where scattering of light within tissue is the most significant source of signal loss.

Finally, the increase in epithelial scattering as tissue progresses from normal to precancerous itself may be a useful diagnostic tool. It is relatively easy to process the signals from imaging probes as well as probes which collect diffuse reflectance in order to detect an increase in scattering within the tissue. This measure could provide an additional feature in classification algorithms designed to distinguish normal from dysplastic tissue.

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