Optical spectroscopy for detection of neoplasia

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Fluorescence and reflectance spectroscopy provide the ability to assess tissue structure and metabolism *in vivo* in real time, providing improved diagnosis of pre-cancerous lesions. Reflectance spectroscopy can probe changes in epithelial nuclei that are important in pre-cancer detection, such as mean nuclear diameter, nuclear size distribution and nuclear refractive index. Fluorescence spectroscopy can probe changes in epithelial cell metabolism, by assessing mitochondrial fluorophores, and epithelial-stromal interactions, by assessing the decrease in collagen crosslink fluorescence that occurs with pre-cancer. Thus, fluorescence and reflectance spectroscopy provide complementary information useful for pre-cancer diagnosis. Tissue engineering provides three-dimensional cell cultures that can be used to further explore the relationship between tissue structure and biological events important in cancer development and progression. In the future, improving our understanding of the biological changes that can be assessed using spectroscopy will not only improve optical techniques but also provide new tools to better understand cancer biology.

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Abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists
EEM	excitation–emission matrix
FAD	flavin adenine dinucleotide
FDA	Food and Drug Administration
FDTD	finite-difference time-domain
LIFE	light-induced fluorescence endoscopy

Introduction

The screening for and detection of cancers as currently practiced are slow, ponderous and expensive. As such, these medical techniques are available to a limited part of the population of the world. Screening and detection could be vastly improved by optical technologies that are now within reach scientifically and which will provide improvement in speed and accuracy of diagnosis while decreasing the cost of finding and confirming lesions in the curable, pre-cancerous phase. Optical technologies provide the ability to visualize tissue structure and metabolism at the sub-cellular level, providing functional information to identify focal, pre-cancerous lesions.

The potential to use fluorescence imaging for tumor detection was noted as early as 1924 [1]. Early reports suggesting quantitative fluorescence spectroscopy could discriminate normal and malignant tissues appeared in 1965 [2]. In the 1980s, Alfano *et al.* [3] measured fluorescence spectra from cancerous and normal animal tissues *in vitro*. At this time, advances in fiber optics and sensitive detectors enabled development of clinical instrumentation for *in vivo* measurements. Progress has been summarized in recent review articles on fluorescence [4,5•] reflectance [6] and Raman spectroscopy [7] for cancer detection. The largest body of clinical data has been collected for fluorescence and reflectance spectroscopy; therefore, this article focuses on these techniques. Much effort has been directed towards development of fiber optic probes to measure tissue Raman spectra, and promising results have recently been reported [8].

Information available from optical spectroscopy of tissue

More than 85% of cancers arise in epithelial tissues. The vast majority of epithelial cancers are preceded by pre-cancerous changes that affect both the surface epithelium and deeper stroma. Biochemical and morphologic changes associated with pre-cancer perturb tissue absorption, scattering and fluorescence properties; thus, optical spectroscopy can probe pre-cancerous changes. When light impinges on tissue, it is typically multiply elastically scattered, following which absorption and perhaps fluorescence can occur. Further scattering and absorption can occur before light exits the tissue surface. Pre-cancers are accompanied by local metabolic and architectural changes at the cellular and subcellular level; for example, changes in the nuclear-to-cytoplasmic ratio of cells and changes in chromatin texture. These changes affect the elastic scattering properties of tissue [9–13,14•,15–18,19•,20]. Pre-cancers are characterized by increased metabolic activity, which affects mitochondrial fluorophores NAD(P)H and FAD, and changes the fluorescence properties of tissue [21•,22,23•,24,25].

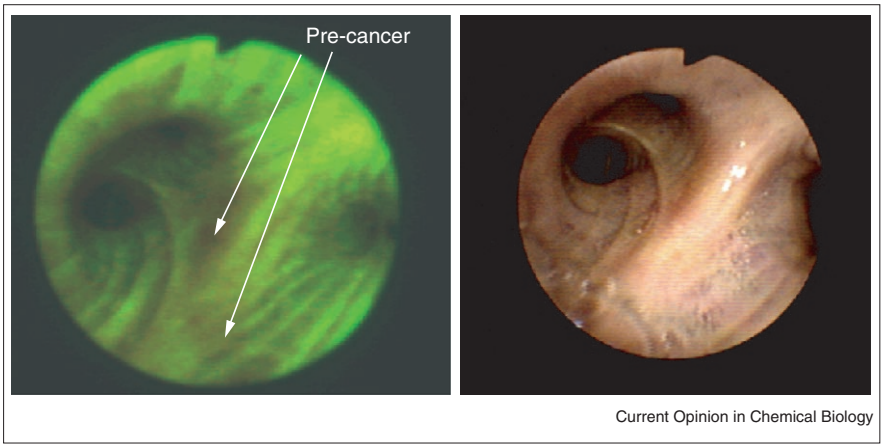
Developing clinical systems

Optical spectroscopy has the potential to provide immediate and accurate diagnostic information, and thus could significantly reduce costs associated with unnecessary biopsies, and treatment delays [26]. Clinical trials are required to confirm this potential. Phase I trials provide exploratory data about device safety and performance, which may allow sample size calculations for future studies [27]. Phase II trials evaluate the sensitivity and specificity of a device in a well-defined population. Phase III trials evaluate the performance and cost-benefit ratio of a new device compared with those of usual care in a multi-center setting.

Instrumentation and safe light exposure levels

Initially, research focused on measuring either reflectance spectra or fluorescence emission spectra at a single or a few

Figure 1



Comparison of LIFE (left image) device with standard white light bronchoscopy (WLB, right image) during lung examination. The image obtained using LIFE technology shows pre-cancer as redish brown color on background of green fluorescence of normal tissue (left). The area of abnormality is not visible in WLB (right). (Figure was kindly provided by Dr Calum MacAulay.)

excitation wavelengths. Recently, it has been shown that fluorescence and reflectance spectra contain complementary information useful for pre-cancer diagnosis [21•,22,28], and emphasis has shifted to approaches that utilize both [29–31]. A system to measure fluorescence excitation–emission matrices (EEMs) and reflectance spectra at different source-detector separations is being used in Phase II trials to provide a better understanding of the diagnostic ability of changes in absorption, scattering and fluorescence properties of tissue [32]. A system to measure fluorescence EEMs and reflectance spectra from the same tissue area, analyzing both the single and multiply elastically scattered light has been described by Georgakoudi [21•,22].

Some of these systems illuminate tissue with UV light; potential mutagenicity is a concern [33]. Standards for safe exposure of skin and eye to UV radiation have been developed [34,35]. For skin exposure, ACGIH (American Conference of Governmental Industrial Hygienists) guidelines provide a threshold limit value of 3 mJ/cm^2 for effective ultraviolet radiation relative to a monochromatic source at 270 nm. Tissue EEMs and reflectance spectra with good signal-to-noise ratio can be collected while remaining an order of magnitude below this limit [34]. The US Food and Drug Administration (FDA) suggests use of the ACGIH standard for development of optical devices for cervical pre-cancer detection [36].

Analysis of tissue optical spectra for neoplasia detection

Pattern recognition can be used to develop and evaluate strategies to classify tissue type based on a measured spectrum [37]. Emphasis in the field has shifted to model-based approaches. Monte Carlo techniques have been used to describe the fluorescence of inhomogeneous tissues and to examine the effects of the excitation beam profile and varying collection geometries [38]. Using this approach, Zonios and colleagues have used Monte Carlo techniques to predict the fluorescence spectra of normal and neoplastic human colon at 370 nm excitation [39]. Similar approaches have been used to model fluorescence spectra of normal and neoplastic skin [40], cervix [25] and brain [41]. Alternatively, analytic models have been developed wherein light propagation is modeled using the diffusion approximation to the radiative transfer equation [42,43,44•,45]. Zonios *et al.* [46] modeled reflectance spectra of colon tissue to extract hemoglobin concentration, hemoglobin oxygen saturation, effective scatterer density and size. They found that pre-cancerous areas showed increased hemoglobin concentration and increased effective scatterer size in a Phase I study. Tromberg and co-workers [47] modeled near infra-red reflectance spectra and found that cervical absorption and effective scattering are up to 15% lower in high-grade cervical pre-cancers compared to cervical tissue.

Recently, others have used the measured reflectance spectrum to deconvolve effects of scattering and reabsorption from tissue fluorescence spectra [48–51]. A limitation of this approach is that it does not take into account inhomogeneities in the distribution of fluorophores and absorbers. Hyde and colleagues [44•] recently presented an analytic model of reflectance and fluorescence that explicitly takes into account the layered nature of tissue.

Results from clinical trials of optical spectroscopy for neoplasia detection

Initial clinical trials compared the sensitivity and specificity of optical spectroscopy to biopsy gold standard [52]. Fluorescence-based algorithms provide sensitivity comparable with that of usual care, but significantly higher specificity. The increase in specificity could reduce the number of unnecessary biopsies during endoscopic procedures [26]. In a study of 75 patients suspected to have bladder cancer, a retrospective analysis of fluorescence spectra showed that use of fluorescence could have reduced the number of unnecessary biopsies by 75% while still correctly identifying 95% of all malignant lesions found by cystoscopy [53]. The use of light scattering spectroscopy has recently been examined for detection of dysplasia in Barrett's esophagus in Phase I trials with high sensitivity and specificity [10].

A small trial examining the combination of diffuse reflectance spectroscopy with fluorescence spectroscopy for detection of cervical pre-cancer suggested that the two techniques provide complementary diagnostic information [28]. Similarly, Georgakoudi [21•] conducted a Phase I study of cervical pre-cancers *in vivo* using a combination of fluorescence, diffuse reflectance and light scattering spectroscopies. The authors reported a significant improvement of sensitivity and specificity when a combination of the three techniques was used relative to any one of the approaches. Larger trials of fluorescence spectroscopy indicate that in some organ sites (e.g. cervical tissue) fluorescence is influenced by biographic covariates such as age and menopausal status [25]. In breast tissue, absorption is 2–3 times higher and scattering is 15–30% higher in pre-menopausal women than in post-menopausal women [54•]. Controlling for these factors may further improve diagnostic performance.

Multi-spectral fluorescence and reflectance imaging devices take advantage of the ability of optical systems to interrogate an entire organ site [55]. Recently, a device has been developed by SpectRx that incorporates the ability to measure both reflectance and fluorescence [56]. Encouraging sensitivities (97%) and specificities (70%) were reported, but may be optimistic due to overtraining.

FDA regulation and approval

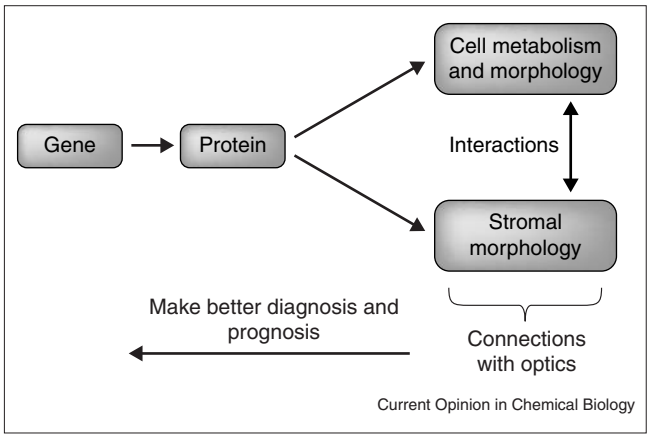
Several clinical systems for measuring tissue optical spectra have received FDA approval. In 1996, Xillix received approval for the light-induced fluorescence endoscopy (LIFE)-Lung Fluorescence Endoscopy System™. A multi-center clinical trial showed that the relative sensitivity of the combination of white light plus fluorescence endoscopy was 6.3-times higher than white light bronchosopy alone for pre-cancer detection (Figure 1) [57]. The SpectraScience™ Optical Biopsy™ System was approved in 2000 as an adjunct to lower gastrointestinal endoscopy. Prospective, multi-center, phase II clinical trials showed a statistically significant increase in sensitivity for the combination of visual endoscopy and spectroscopy to discriminate adenomatous and hyperplastic polyps [58]. In 1999, the FDA released a guidance document for industry for *in vivo* optical detection of cervical pre-cancer [36].

Biological explanation

Molecular targets for optical detection

Genetic alterations are responsible for cancer development and progression. Gene mutations lead to changes in the gene expression profile and cellular phenotype which are manifested in alterations in cell metabolism, morphology, protein secretion, properties of cytoskeleton, cellular membrane, etc. at the cellular level and in cell–cell and cell–stromal interactions in epithelial tissue. Correlation of genetic changes with cellular phenotype and biological activity is a key step in understanding the molecular biology of cancer. Work by Condeelis, Jain and Coffee provides important connections between specific genes and proteins and related changes in chromatin density [59], tumor metastases [60,61,62•], host-cell–tumor-cell interactions [63] and angiogenesis [64•,65]. It has been shown that these molecular events play a major role in cancer progression and can be valuable biomarkers for cancer diagnosis, grading and prognosis.

Figure 2



Optical spectroscopic and imaging techniques can detect changes in epithelial and stromal morphology and metabolism, which occur during carcinogenesis. Better understanding of the relationship between the observed optical signals and cancer molecular biology will give a new impetus to improve optical techniques for cancer screening and diagnosis.

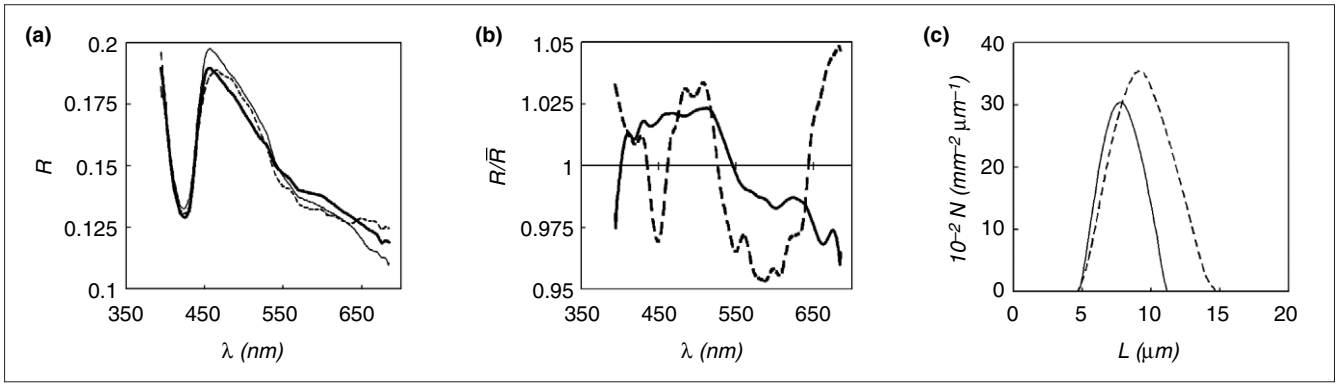
In this section, we discuss the studies that correlate the observed optical changes with cancer-related biomarkers and cancer molecular biology (Figure 2). We focus on two key areas: first, using elastic light scattering spectroscopy to quantitatively characterize nuclear morphology and chromatin structure; and second, using fluorescence spectroscopy to probe changes in epithelial–stromal interactions at the very earliest stages of carcinogenesis.

Light-scattering spectroscopy and nuclear morphology and chromatin structure

In 1998, Perelman *et al.* [9] developed a simple analytical model based on the approximate solution of the transport equation for the diffuse reflectance signal to extract the single scattered component of light diffusely scattered by tissue. Single scattering of light by cells was approximated using Mie theory, to extract the distribution of nuclear sizes (Figure 3). This technique showed promising results in the diagnosis of pre-cancer in four organ sites *in situ* [9,10,66•].

In a modified approach, polarized illumination/detection was used to extract single-scattering events in the presence of strong blood absorption and diffuse stromal scattering [11,12,67]. Anderson [68] and Jacques *et al.* [69] used this technique to image superficial and deep features in skin. Backman *et al.* [11] and Sokolov *et al.* [12] analyzed the wavelength dependence of the single-scattered light to extract nuclear sizes of epithelial cells. Myakov *et al.* [67] developed a fiber-optic probe for polarized reflectance

Figure 3

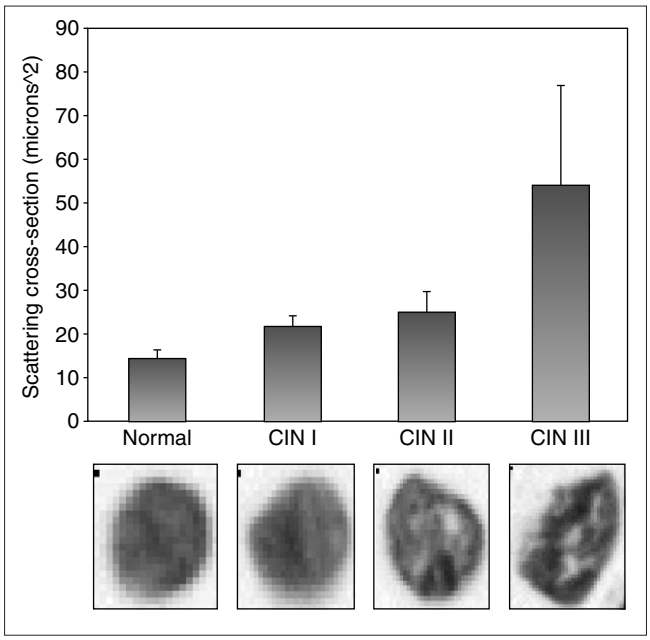


Reflectance spectroscopy of Barrett's esophagus. **(a)** Diffuse reflectance spectra obtained from normal tissue (solid line), a pre-cancerous site (dashed line), and a model fit (thick solid line); **(b)** the corresponding single scattering of epithelial cells; **(c)** nuclear size distributions derived from the single-scattered component using Mie theory. Figure reproduced from [9] with permission. Copyright 2002 by the American Physical Society.

measurements and demonstrated its application in oral cavity *in vivo*. Subsequently, polarized light-scattering spectroscopy was used by Gurjar *et al.* [70] to image *ex vivo* colonic polyps. Mean nuclear diameter, nuclear size distribution, and refractive index of nuclei were determined at each pixel. The authors suggest that the refractive index can be quantitatively related to chromatin content of nuclei.

All these studies assumed nuclei were homogeneous spherical objects, such that scattering could be described using Mie theory. However, nuclei have heterogeneous internal structure that influences their scattering. The heterogeneity is due to chromatin structure, which is one of important and earlier biomarkers of cancer progression.

Figure 4



Changes in scattering cross-sections of nuclei of cervical epithelial cells throughout cancer progression. The pictures below the graph show the corresponding images of Feulgen-thionin stained cell nuclei. Figure adapted from [19•] with permission. Copyright SPIE 2002.

Mourant and co-workers have intensively studied the wavelength and angular dependence of scattering of tumorigenic and nontumorigenic fibroblasts [13,14•,15,16]. The authors concluded that the backscattering properties of cells are dominated by scatterers with 0.5–1.0 μm radii; up to 40% of cellular scattering arises from nuclei; and that backscattering at angles greater than 110° can be correlated with the DNA content of the cells. Although these studies provide important insight into cell scattering, they do not provide a direct link between morphology and scattering properties. Models of cell scattering can address this important question.

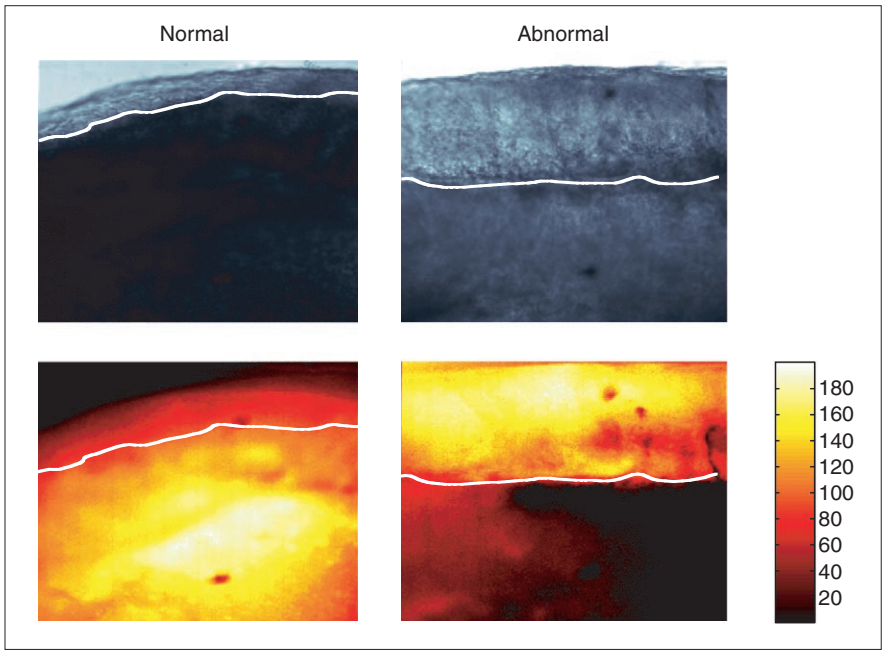
Recent electromagnetic models incorporate internal complexity to calculate the angular distribution of scattered light (phase function) from individual cells of arbitrary shape and dielectric structure. These models are based on numerical solution of Maxwell's equations using a finite-difference time-domain (FDTD) technique [17,18]. As the grade of dysplasia increases, the simulations predict that the scattering cross section significantly increases, because of increases in nuclear size, optical density and texture (Figure 4) [19•]. Furthermore, the wavelength dependence of this scattering can be calculated using FDTD simulations [20]. These simulations are in good agreement with the experimental measurements of cellular scattering reported by Mourant *et al.* [13].

Molecular origin of fluorescence changes during dysplasia

Many clinical studies have shown that tissue fluorescence decreases with cancer progression [3,4]. However, only recently has the molecular basis for these changes been identified using a new model system that closely resembles

Figure 5

Representative images from paired normal and pre-cancerous fresh tissue sections. The bottom row shows fluorescence images of normal and abnormal cervical biopsies obtained with 380 nm excitation under 10× objective. The fluorescence images are artificially colored: yellow represents brighter fluorescence signal and red is dimmer fluorescence. The top row shows the corresponding bright-field images. The white line indicates location of a basement membrane. Figure adapted from [23•] with permission. Copyright American Society for Photobiology 2001.



living human epithelial tissue [23•,71]. Drezek *et al.* [23•] demonstrated that there is a statistically significant increase in epithelial fluorescence at 380 nm excitation in pre-cancerous epithelium relative to normal epithelium. The increase in epithelial fluorescence is accompanied by a statistically significant decrease in fluorescence of stromal collagen at 380 nm excitation (Figure 5). The increase in epithelial fluorescence was attributed to an increase in the concentration of NADH in epithelial cells that correlates with higher metabolic rate of pre-cancerous epithelial cells. The decrease in stromal fluorescence indicates a possible decrease in concentration of collagen crosslinks with pre-cancer. It is known that cancerous cells express a class of metalloproteases — collagenases that are involved in degradation of extracellular matrix [72,73]. However, the striking discovery of these studies is the fact that the observed decrease in collagen fluorescence occurs at the earliest stages of pre-cancer before stromal invasion, indicating that changes in epithelial cell–stromal interactions can be probed by fluorescence spectroscopy and imaging. In subsequent studies by Georgakoudi and co-workers, the same trends in fluorescence intensities of epithelium and stroma were observed *in vivo* in Phase I clinical studies of cervix [28] and esophagus [24].

Drezek *et al.* [25] developed a forward Monte Carlo model to understand how NADH and collagen fluorescence contribute to the fluorescence spectra of epithelial tissue *in vivo*. Cervical tissue spectra were modeled for normal and pre-cancerous tissue using input data from the tissue culture experiments [23•]. The model accurately predicted both the shape and the relative intensity of fluorescence of normal and dysplastic cervix. In normal cervix at 380 nm excitation, approximately 20% of remitted tissue fluorescence is due to epithelial NAD(P)H, while the remaining 80% is due to stromal collagen. In dysplastic cervix, approximately 30–40% of remitted fluorescence is due to epithelial NAD(P)H, while 60–70% is due to stromal collagen.

The model of tissue fluorescence can explain the observed changes in fluorescence spectra in connection with tissue morphology and architecture. However, it does not provide information about molecular mechanisms that lead to the observed changes. To bridge this gap, new tissue phantoms based on the methods of tissue engineering have been recently introduced to biomedical optics.

Engineered tissue constructs for biomedical optics

Tissue engineering provides three-dimensional cell cultures that can be used to explore the relationship between tissue structure and biological events important in the development and progression of cancer, such as cellular motility, cell–substrate interactions, and cell stimulation by growth factors [74]. For example, Agarwal *et al.* [75] used two-photon laser scanning microscopy to image an engineered tissue model of the human respiratory mucosa which consisted of a three-dimensional co-culture of human lung fibroblasts, type I collagen, and human alveolar or bronchial epithelial cells. They demonstrated that epithelial cells modulate collagen density and contraction.

Recently, Sokolov *et al.* [76•] used methods of tissue engineering to create biologically relevant tissue constructs to systematically study optical properties of human epithelium and its components. The authors described a step-by-step reconstruction of epithelial tissue, where each

consecutive step increases the similarity between human tissue and the construct. Highly reproducible constructs with fully controlled composition can be prepared at different levels of complexity using different cell types (normal, pre-cancerous, or cancerous) and can be analyzed without restrictions involved in obtaining actual surgical specimens from patients. The optical spectroscopic and imaging analyses can be combined with immunochemical staining and other standard biochemical techniques to identify molecular origins of optical signals.

Conclusions

In vivo optical spectroscopy provides a real-time tool to assess the metabolic and morphologic changes in the epithelium and stroma associated with the development of pre-cancer. The increase in diagnostic specificity has the potential to reduce the number of unnecessary biopsies and to reduce delays in treatment. Multi-spectral imaging approaches have increased diagnostic sensitivity, identifying pre-cancers not seen with traditional white light endoscopy. In the future, improving our understanding of the biological changes that can be assessed using spectroscopy will not only give a new impetus to improve optical techniques but also provide new tools to better understand cancer biology (Figure 2).

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- •• of outstanding interest

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