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Advances in quantitative UV–visible spectroscopy for clinical and pre-clinical application in cancer

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Methods of optical spectroscopy that provide quantitative, physically or physiologically meaningful measures of tissue properties are an attractive tool for the study, diagnosis, prognosis, and treatment of various cancers. Recent development of methodologies to convert measured reflectance and fluorescence spectra from tissue to cancer-relevant parameters such as vascular volume, oxygenation, extracellular matrix extent, metabolic redox states, and cellular proliferation have significantly advanced the field of tissue optical spectroscopy. The number of publications reporting quantitative tissue spectroscopy results in the UV–visible wavelength range has increased sharply in the past three years, and includes new and emerging studies that correlate optically measured parameters with independent measures such as immunohistochemistry, which should aid in increased clinical acceptance of these technologies.

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Introduction

There is a great need to accurately quantify predictive biomarkers *in vivo* for the diagnosis, prognosis, and treatment of cancers. Since current approaches in cancer management are generic across patients and involve empirical routines [1], there is a growing emphasis toward developing individualized and personalized approaches that are based on detection of molecular, metabolic, and physiological biomarkers. Traditional biomarkers include features such as the tumor grade, size, and/or the number of local lymph nodes with metastasis. More recent reports, however, for example, sense molecular and/or genetic markers (such as point mutations in key genes such as RAS or p53, or the presence of molecular markers such as HER2/neu and/or EGFR), all of which in turn could influence the upregulation, expression, and stability of molecules related to hypoxia and angiogenesis, including important hallmarks of carcinogenesis such as HIF-1 and VEGF [1]. Optical spectroscopy is a technique that is exquisitely sensitive to several important biomarkers of cancer. There have been many reports of optical spectroscopy for the study of cancer, but only a few have employed spectral analysis algorithms that extract quantitative, physically meaningful parameters from the tissue spectra. Quantitative optical spectroscopy in the UV–visible wavelength regime has been used in the study of cancers, including the breast and cervix, by a variety of researchers over the past decade, and will be the focus of the subsequent review. While challenges in implementation remain, we believe that these methods may provide value in characterizing important biomarkers of cancer non-destructively and rapidly, in a practical clinical setting.

Biomarkers of cancer

Vascular and metabolic factors

Oxygenation and hypoxia

Oxygenation, particularly, the lack of it, is widely recognized as a crucial factor that influences the growth rate, metabolism, treatment resistance, and metastatic behavior of cancer cells [2,3]. Hypoxic microenvironments have routinely been identified in solid tumors of almost all tissues. Numerous studies have investigated the link between clinical outcomes and hypoxia using a variety of different methods to date [4]. All of these studies have demonstrated that hypoxia is clearly related to clinical outcome, which motivates the importance of measuring it *in vivo*. Currently, methods to measure tumor hypoxia can be divided into two classes, indirect and direct. The gold standard of direct tissue hypoxia measurement is through electrode polarography—an invasive process that yields point measurements of pO_2 (sampled from ~100–300 cells) at given spatial locations along the path of electrode insertion and retraction within the tissue. Oxygen concentration has also been imaged indirectly using perfluorinated compounds in combination with electron paramagnetic resonance (EPR) spectroscopy, MR spectroscopy, by using 2-nitroimidazoles with positron emission tomography (PET), or through immunohistochemical (IHC) analyses of biopsies [5].

Angiogenesis and blood volume

Irregular vasculature has previously been identified has a hallmark of cancer [6]. Given that tumor cells have a

constant need for new blood vessels to nourish their growth, solid tumors persistently sprout new segments of vessels to the existing vascular system leading to a highly irregular, leaky, and chaotic network of blood vessels [7]. The sprouting of new vessels is facilitated by overexpression of the vascular endothelial growth factor (VEGF), which is known to be upregulated under hypoxic conditions [6]. This increased tumor vascularization eventually paves the way for a small, localized tumor to become an enlarged mass and subsequently metastasize to other distant sites. *In vivo* measurements of tumor angiogenesis have been performed by both extrinsic contrast-enhanced MR imaging and spectroscopy and PET techniques [5,8]. Clinical gold standards involve immunohistochemical assays that measure tumor microvessel density and/or expression of angiogenic markers such as VEGF.

Reduction–oxidation state of the cell

Cellular respiration occurs via the electron transport chain in all aerobic cells and in the mitochondrial membranes of these cells, reactive oxygen species (ROS) are generated during oxidative phosphorylation. There are several complex cellular biochemical pathways that help cells protect themselves against low levels of ROS and free radicals by forming a network of redox buffers (which include the NAD(P)H/NAD(P)$^+$ species); nevertheless, these mechanisms might be rendered dysfunctional under abnormally high levels of ROS leading to oxidative stress in the tumor microenvironment [9]. Further, it is well known that the onset of hypoxia within solid tumors causes the cells to prefer anaerobic glycolytic pathways over aerobic oxidative phosphorylation to meet their energy needs, which in turn influences both the amount of ROS produced and the amount of NADH/NAD+ redox buffer available in these cells [10]. Thus, determining the redox status within the tumor could shed light on both the levels of hypoxia and concentration of ROS in the cells. Detection of the redox status of tumors, especially *in vivo*, still remains a challenging task [9]. There is currently no accepted clinical gold standard to estimate the redox status of tumors, though detection in tumors has been achieved via the use of paramagnetic nitroxide spin probes in conjunction with EPR/MR spectroscopic methods [11,12].

Morphological factors

There are significant changes in cellular morphology and structure that are associated with the onset and progression of cancer. Pathologists routinely use microscopic differences observed in cellular and nuclear features including shape, size, crowding, chromatin organization and DNA structure in biopsied tissues to diagnose, prognosticate, and stage disease [13]. Besides changes in cellular and/or nuclear structure, previous studies have observed changes in the amount of fatty acid or lipid content between normal and malignant tissues—particularly in breast tumors [14,15]. Further, there is clear evidence that there are important interactions between the invading tumor cells and stromal cells that allow tumor proliferation, invasion, metastasis, and malignant transformation [16]. These interactions are expected to cause significant changes in the organization and arrangement of the extracellular matrix surrounding the neoplasm. Both PET and MRI-based imaging have been applied to the detection of cell death. In MRI, the measured apparent diffusion coefficient (ADC) of water in tissue can provide information regarding tissue structure by imaging water content in intra-cellular and extracellular space, which in turn can be used to assess cell death occurring via necrosis or apoptosis [17,18]. The uses of PET imaging methods for sensing apoptosis and/or necrosis remain limited and involve the use of labeled apoptosis or necrosis markers (such as 18F-labeled annexin-V) [18].

Optical spectroscopy

Methods of optical science and engineering have been developed for cancer detection and diagnosis and more recently to assess response to therapy in a variety of tissue sites for applications in both pre-clinical and clinical studies [19,20,21,22,23,24•,25,26,27]. The interaction of light with complex media such as biological tissues is characterized by processes that depend on the physical nature of the light and the specific tissue morphology and composition [28,29]. The incident light can be scattered (elastically or inelastically) multiple times owing to microscopic differences in the index of refraction of cells and subcellular organelles within the tissues, and may be non-radiatively absorbed by chromophores present in the medium or by fluorophores, which release their excess energy by radiative decay, producing fluorescence. The remitted fluorescent light can, in turn, be scattered multiple times or absorbed. Although complex, these optical responses can be measured by a variety of spectroscopic techniques and processed through rigorous computational or theoretical models to obtain quantitative biochemical and morphological information about the tissues [30–32].

In optical spectroscopy, the wavelengths of illumination span the ultraviolet (UV) through the near-infrared (NIR) wavelengths. In steady-state reflectance spectroscopy, a broadband light source is used for illumination and a spectrum of the reflected light is collected [33], while in steady-state fluorescence spectroscopy a narrow spectral band of incident light (obtained via filtering a broadband source or from a narrowband laser) is used to excite fluorophores and the emerging fluorescence spectrum at each excitation wavelength is detected [34].

The diffuse reflectance spectrum is a function of the optical absorption and scattering coefficient spectra [28]. The absorption and scattering coefficients are

Table 1

Sources of optical contrast and features they correspond to in tissues.

Optical interactions	Contrast	Spectral range	Biomarkers
Absorption	HbO_2, Hb	UV-NIR	Blood saturation, angiogenesis
Absorption	Beta-carotene	vis	Adipose
Absorption	Lipid	NIR	Adipose
Absorption	Water	NIR	Water content
Fluorescence	NADH, FAD	UV–vis	Redox ratio, ROS, hypoxia
Fluorescence	Collagen, elastin	UV–vis	Metastasis, invasion
Scattering	μ_s'	UV-NIR	Apoptosis, necrosis, proliferation

wavelength-dependent and their value at each wavelength reflects the probability that a photon (of given wavelength) will be absorbed or scattered by the tissue when it traverses an infinitesimal step within the medium. The shape and magnitude of the absorption coefficient depends on the extinction coefficient and concentrations of dominant tissue chromophores including oxygenated hemoglobin (HbO_2) and deoxygenated hemoglobin (Hb), beta carotene, water, and lipids in the UV-NIR spectrum [35,36]. Since diffuse reflectance spectroscopy can measure both HbO_2 and Hb one can estimate both the total hemoglobin concentration (THb = HbO_2 + Hb) and the oxygenation saturation in tumors (SO_2 = HbO_2/THb). The optical scattering coefficient is known to be sensitive to the spatial architecture and organization of the tissue and therefore can be used as a means to quantify cellular morphology and structure [37–39]. The shape and magnitude of the intrinsic fluorescence spectrum depends on the concentrations of the tissue fluorophores, which include several important biochemical molecules such as nicotinamide adenine dinucletide (NADH) and flavin adenine dinucleotide (FAD) or structural proteins such as collagen, elastin, and keratin [40–42]. The ratio of NADH to FAD is called the optical reduction–oxidation (redox) ratio and can provide information about the reduction–oxidation state in the electron transport chain within the mitochondria. The optical redox ratio has been shown to increase with a decrease in cellular oxygenation [43,44]. Measurement of endogenous fluorescence could also provide a means to sense changes in the extracellular matrix composition. However, the measured fluorescence spectrum can be significantly distorted by tissue absorption and scattering and needs to be corrected to get the intrinsic, turbidity-free fluorescence spectrum that can then be used to quantify either absolute or relative tissue fluorophore contributions [34].

The sensing depth of light varies from several millimeters in the UV–visible spectrum to several centimeters in the NIR region [45]. In the UV–visible region, tissues are absorption dominant, which restricts the penetration depth. With increasing wavelength, the overall absorption coefficient decreases and the ratio of scattering to absorption coefficients increase. Thus, in the red and NIR wavelengths tissues are more transparent and photons can migrate through several centimeters of tissue that allows NIR spectroscopy to interrogate subsurface solid tumors such as those in the breast and neck nodes. UV–visible spectroscopy complementarily has a superficial sensing depth and can directly interrogate pre-cancerous growth and primary invasive carcinomas in the head and neck, anus, cervix, and recurrent chest wall disease in breast cancer. Optical spectroscopic probes can also be guided through endoscopes and biopsy needles to access tumors within body cavities as in breast cancers. This technology is also well suited for drug discovery in pre-clinical tumors in rodent models.

Table 1 shows the primary sources of optical contrast in the UV–visible-NIR range. As indicated in the table, not all optical sources of contrast are probed by all spectral regions of light and clearly the total number of intrinsic biomarkers that can be interrogated optically increases in the UV–visible spectral range.

The benefits of optical spectroscopy are that it is (1) quantitative, (2) fast, (3) can be used at the bedside, and (4) has exquisite sensitivity to intrinsic biomarkers already present in the tissue. The 'optical biomarkers' can be measured more frequently than conventional imaging methods such as contrast-enhanced PET, CT, and MRI. The synthesis of some of these contrast agents is expensive and requires specialized facilities (for example, cyclotron for PET). Further, multiple biomarkers can be measured simultaneously with light. With traditional imaging approaches, patients would need to be imaged by several different scanners to fully capture biomarkers of hypoxia and angiogenesis. Because of the frequency with which optical biomarkers can be measured, these technologies could conceivably be used to identify optimized temporal windows of opportunity for when more sophisticated functional imaging techniques could be used to get complete tumor coverage. Although the optical biomarker technology does not provide the tumor coverage that CT, PET, and MRI provide, it yields data from tissue sensing depths that are 'on par' with that evaluated via IHC, which is the current gold standard.

Instrumentation

Instrumentation overview

Optical spectroscopy involves illuminating the tissue with light and recording what comes back as a function of wavelength. This requires a few components that are common to all spectroscopic instruments: a light source to illuminate the sample, a means of filtering or selecting wavelengths, and a detector to quantify the collected light intensity. Light is commonly coupled through a fiber optic probe to the tissue site. There are several key considerations in designing such devices including spectral bandwidth, spectral resolution, wavelength range, measurement speed, and throughput; as well as practical concerns such as system cost, complexity, and size. These are interrelated with tradeoffs between them, for example, a smaller bandwidth will reduce throughput while increasing spectral resolution, so these factors must be tailored for the specific requirements of the application. Important aspects of these types of optical instrumentation are the geometry of illumination and collection light (which determine the sensing volume of the device), methods for accurate and routine calibration, and algorithms for converting measured optical spectra to quantitative, physical information.

Probe geometry

The probe geometry defines the sensing volume and depth of the measurement. There are two broad categories, contact and non-contact. This article will focus on contact geometries that are more commonly used for quantitative optical spectroscopy, as they allow for well-defined sensing depths and for quantitative measurements. Non-contact methods have the advantage of being more amenable to imaging, but make modeling and quantitation more challenging, although specialized approaches do exist.

The probing volume and depth are affected by the optical properties (absorption and scattering coefficients) of the medium, as well as the probe geometry, particularly the source–detector separation [46]. As the source–detector separation increases, the probing depth and volume increase, which increases the sensitivity of the probe to deeper tissue [46]. However, the extent to which the source and detector can be separated is limited by throughput of light through the tissue, which is influenced by the tissue absorption and scattering coefficients. Source–detector separations are significantly larger in the NIR (~centimeters) compared with the UV–visible range (~millimeters).

Epithelial pre-cancers are widely investigated as a potential application of optical spectroscopy. In these tissues, cancer typically originates in the epithelial tissue, and invades into the underlying stroma. As a result there has been much recent work investigating ways to separate the epithelial versus stromal components of the signal. One approach has used angled fibers that are more sensitive to shallower layers of the tissue [47,48]. These can be combined with flat-faced probes to enable a two-step determination of the shallow and deep tissue optical properties [49]. Another approach, differential path-length spectroscopy, utilizes two flat-faced fibers, one of which serves as both the source and collection fiber, along with a second separate collection fiber. Because the two fibers are placed close to one another, taking the difference in signal yields something that is dependent only on the shallowest interactions of light with the tissue. This allows one to characterize the optical properties of the shallow layers of epithelium [50].

Calibration

Calibration of optical measurements is essential to minimizing systematic errors and to ensure accurate retrieval of quantitative physiologic and morphological information. The primary goals are to (1) remove the wavelength dependence of system throughput, (2) eliminate variability in throughput over time, (3) eliminate of sources of noise, particularly any background signal, and (4) ensure accurate calibration of the measurement wavelengths. The first two calibrations require specialized methodology and equipment to achieve and will be discussed in detail below. Background correction is typically performed by simply measuring a spectrum with the light source turned off and subtracting this from the experimental measurements, and wavelength calibration can be performed by using a known narrow line source, such as a laser at a pre-selected wavelength.

The wavelength response of a detection system can be calibrated by using a calibrated light source. Such sources are commercially available and are traceable to NIST standards for radiance [51]. This procedure fundamentally involves coupling a calibrated light source (via an integrating sphere or other means) into the optical detection system. The instrument will then record the intensity as a function of wavelength, which can be compared to the known radiance of the light source as a function of wavelength. The ratio of these gives the instrument response, which can then be used to correct any measured spectrum (see [52] for a detailed description of theory and procedures).

Standards for the purpose of quantitative spectroscopy must possess known absorption, scattering, and fluorescence properties. This means tissue phantoms must be constructed with specified or determinable optical characteristics. There are a variety of potential components for which this is possible, see Pogue *et al.* [53] for an in depth review of reflectance phantoms, and [54–59] for a discussion of fluorescence standard development. Measurement of such standards must be frequent enough to account for system drift.

Table 2

A comparison of the merits of a variety of different approaches for extraction of tissue optical properties and intrinsic fluorescence.

Model	Computational requirements	Optical property limitations	Source detector separations
Diffusion [69–74]	Low	Absorption $\ll$ Scattering	Several mm
P3 [75,81]	Low	Absorption $\cong$ Scattering	$<$1 mm
Monte Carlo [77–80]	High	Any	Any

Algorithms

Quantitative determination of the underlying absorption, scattering, and fluorescence properties allows insight into tissue physiology and morphology. However, extraction of the parameters from the measured diffuse reflectance and fluorescence spectra is a challenging problem. Thus, it is imperative that modeling algorithms be developed and employed by which these relationships can be elucidated. There are a wide variety of algorithms and approaches, although they can be roughly subdivided into analytical and numerical approaches. Analytical approaches (generally simplified approximations of the Boltzmann radiative transport equation) have the advantage of low computational requirements, but are not widely applicable to a large variety of wavelength ranges or experimental conditions. On the contrary, numerical approaches (such as stochastic Monte Carlo modeling) have a high computational complexity but are more generally applicable and accurate. In spite of this, Monte Carlo-based approaches have been employed to extract quantitative physiologic and morphologic information for both fluorescence [60•] and diffuse reflectance spectra [61–63] by employing scaling and similarity relationships to enable the optical spectra to be estimated for a wide range of optical properties in near real-time using only a few baseline simulations. Application of these models generally involves a least squares fitting approach, whereby the modeled optical properties of the medium are varied such that they produce minimal error between the measured and modeled spectra. Table 2 lists the most common algorithms employed for quantitative extraction of tissue optical properties in the UV–visible and their relative advantages and disadvantages.

Summary of pre-clinical and clinical applications

In the past decade truly quantitative approaches have been used to perform quantitative biology of cancers and pre-cancers *in vivo* using steady state optical spectroscopy, with most of the reports emerging in the past three years. A majority of these studies have been carried out in the UV–visible range. The studies in the NIR primarily focus on frequency-domain and time-domain approaches and are beyond the scope of this review

Table 3

Summary of clinical implementations of quantitative UV–vis optical spectroscopy, organized by organ site of interest published in the past three years. Abbreviations: [Hb] = deoxyhemoglobin concentration, [HbO_2] = oxyhemoglobin concentration, SO_2 = hemoglobin oxygen saturation, $\mu_s'(\lambda)$ = wavelength-dependent reduced scattering coefficient.

Organ	Reference	Implementation	Spectroscopic technique	Sources of contrast quantified
Breast	Palmer *et al*. 2006 [70]; Zhu *et al*. 2006 [74]	*ex vivo*	Diffuse reflectance	[Hb], [HbO_2], SO_2, [β-carotene], $\mu_s'(\lambda)$
	Sun *et al*. 2006 [71]	*ex vivo*	Diffuse reflectance	[Hb], [HbO_2], SO_2, $\mu_s'(\lambda)$
	Zhu *et al*. 2008 [75]	*ex vivo*	Fluorescence	NADH, collagen, retinol/vitamin A
	Volynskaya *et al*. 2008 [73]	*ex vivo*	Diffuse reflectance Fluorescence	[HbO_2], [β-carotene], $\mu_s'(\lambda)$ NADH, collagen-like
	van Veen *et al*. 2005 [72]	*in vivo*	Differential pathlength	[Hb], [HbO_2], SO_2, [β-carotene], vessel diameter, scatter slope
	Brown, *et al*. 2009 [83]	*in vivo*	Diffuse reflectance	[Hb], [HbO_2], SO_2, [β-carotene], $\mu_s'(\lambda)$
Cervix	Chang *et al*. 2006 [67•]	*in vivo*	Two-layer fluorescence	[Hb], [HbO_2], SO_2, epithelial scattering, NADH, FAD, keratin, collagen
	Mourant *et al*. 2007 [69]	*in vivo*	Diffuse reflectance	[Hb], [HbO_2], SO_2
	Chang *et al*. 2009 [92]	*in vivo*	Diffuse reflectance	[Hb], [HbO_2], SO_2, $\mu_s'(\lambda)$
Lung	Bard *et al*. 2005 [66]	*in vivo*	Differential pathlength	[Hb], [HbO_2], SO_2, [β-carotene], vessel diameter, scatter slope
	Aerts *et al*. 2007 [64••]	*in vivo*	Differential pathlength	[Hb], [HbO_2], SO_2
	Fawzy, M *et al*. 2006 [68]	*in vivo*	Diffuse reflectance	[Hb], [HbO_2], SO_2, scatterer density and size
Oral mucosa	Amelink *et al*. 2008 [65]	*in vivo*	Differential pathlength	[Hb], [HbO_2], SO_2, [β-carotene], vessel diameter, scatter slope
Skin	Zonios etal., 2006 [76]; Zonios *et al*. 2008 [77]	*in vivo*	Diffuse reflectance	[Hb], [HbO_2], SO_2, melanin, scatterer density and size

article. Table 3 contains a summary of the relevant articles published in the past three years, organized by organ site of interest, describing the methodology and implementation used and sources of intrinsic contrast studied [64••,65–77]. Since the breast and cervix have been studied by the largest number of groups, we will focus our attention on these two organ sites. Moreover, since quantitative approaches are desirable because of the insight they provide into the biology/physiology of the disease beyond diagnosis, we will devote special attention to those studies that exploit this concept. Important references pertaining to these organ sites, which were published before 2005, are referenced and discussed in their respective subsections.

Breast

Although anatomical imaging for the screening, diagnosis, and management of breast cancer has made a significant impact on current breast cancer care, gaps remain in which the superficial sensing of UV–visible optical spectroscopy may be able to augment traditional technologies in a useful way. These potential applications range from use as an adjunct to core-needle diagnostic biopsy, as an indicator of pre-therapy tumor oxygenation, as an intra-operative tool for assessment of surgical tumor margins, as a directed-sampling tool for traditional histopathology, and as a tool to monitor response to chemotherapy or radiotherapy in humans or pre-clinical rodent models. In the following sections, we will review the reports in which quantitative UV–visible spectroscopy has been applied in the breast.

Pre-clinical applications

UV–visible spectroscopy is particularly well suited to studying biological models of carcinogenesis and therapeutic response in animal models owing to a good match between the penetration depth at this wavelength and the size of animal tumors. The primary advantage of the use of quantitative spectroscopy in these models would be to non-invasively provide longitudinal snapshots of the tumor environment that would traditionally require excision of tissue and serial histochemical or immunohistochemical processing for analysis.

As an example, the Ramanujam group applied diffuse reflectance spectroscopy coupled with an inverse Monte Carlo-based method to quantify biomarkers in a chemotherapy model of 4T1 mammary carcinomas grown in the flanks of nude mice [78]. In this study, optical measurements were compared to traditional immunohistochemical and histological markers for hypoxia and tumor necrosis, to evaluate their potential as surrogate measures of response to chemotherapy that could be applied non-invasively and dynamically throughout the course of therapy. 4T1 tumors were grown to 4–6 mm diameter in 50 nude mice, at which point half were given the chemotherapy agent Doxorubicin at the maximum tolerated dose (MTD) while the remaining animals served as controls. The tumors were monitored non-invasively over two weeks post-treatment. Deoxyhemoglobin (Hb) concentration was found to correlate with hypoxia as measured by pimonidazole IHC staining, whereas the mean reduced scattering coefficient (μ_s') was found to correlate with tumor necrosis as assessed by hematoxylin and eosin staining. The authors showed that both IHC and quantitative spectroscopy indicated a statistical increase in tumor oxygenation in the Doxorubicin treated animals (the treated group showed an average increase of over 30% in tumor oxygenation on day 10 relative to baseline, while the control group showed a decrease of nearly 10% on day 10 relative to baseline; $P<0.05$). The ability to serially and non-invasively interrogate tumor response to therapy in pre-clinical models could have potentially important implications in the development of new cancer treatments.

Clinical studies

Table 4 contains a comparison of the trends in optically measured parameters across the clinical studies reviewed

Table 4

Comparison of optically measured physiological parameters between studies indicating how the parameters changed with breast cancer. Arrows indicate whether the parameter was significantly higher (↑), lower (↓), or no different (=) in malignant tissues (C) compared to non-malignant tissues (N). Blank areas indicate that the parameter was not measured or reported.

Reference	Samples (C, N)	Absorption			Fluorescence		Scattering		
		Blood content	Blood saturation	β-carotene	Collagen	NADH	$<\mu_s'>$	*a*	*b*
Ex vivo									
Ghosh *et al*. 2001 [82]	10, 10						↑		
Palmer *et al*. 2006 [70]	17, 24	=	↓	↓			↑		
Zhu *et al*. 2006 [74]	35, 50	=	↓	↓			↑		
Zhu *et al*. 2008 [75]	54, 70	=	↓	↓	↑	↑	↑		
Volynskaya *et al*. 2008 [73]	9, 31	=		↓	↑	=		=	
In vivo									
van Veen *et al*. 2005 [72]	10, 40	↑	↓	=					=
Brown *et al*. 2009 [83]	20, 56	=	↓	=			=		

a = Mie scatter density; *b* = Mie scatter size; $<\mu_s'>$ = mean reduced scattering coefficient.

below. As seen in Table 4, the studies within each implementation (*ex vivo* and *in vivo*) are remarkably consistent, and what variations exist could be attributed to sample size. In addition to being consistent with each other, the findings of these studies are also consistent with independent measures of these parameters reported in the literature. For instance, studies using the Eppendorf pO_2 electrode (reviewed in Vaupel *et al.* [79]), and immunohistochemical markers like HIF-1α [80] and CA IX [81] have shown that breast tumors are generally characterized by a decrease in oxygenation.

Ex vivo *studies*

A number of studies have investigated the application of quantitative optical spectroscopy to excised breast tissues. In an early report, Ghosh *et al.* employed a diffusion approximation-based approach to quantitatively determine *ex vivo* breast optical properties in the UV–visible spectral range [82]. The authors performed spatially resolved reflectance measurements at multiple source–detector separations on previously frozen intact human breast tissues, and extracted the absorption and reduced scattering coefficients from fits of the diffusion approximation to the spatially resolved reflectance. They showed that malignant breast tissues were more highly absorbing and scattering than normal tissues.

More recently, Palmer *et al.* developed an inverse Monte Carlo model-based approach to the analysis of diffuse reflectance and multiexcitation-wavelength intrinsic fluorescence from tissues [63,83], which has been applied to the study of normal and diseased human breast tissues *ex vivo* by Palmer *et al.* and Zhu *et al.* [70]. Application of this analysis method to breast tissues measured *ex vivo* [70] resulted in quantification of the absorber concentrations (oxyhemoglobin and deoxyhemoglobin, beta carotene) as well as ancillary quantities such as total hemoglobin content and hemoglobin concentration, computed from the extracted absorber concentrations; scattering properties (reduced scattering coefficient); and relative contributions of intrinsic fluorophores present in the tissue. Across these three independent studies, the hemoglobin saturation and β-carotene concentration of malignant tissues was significantly lower than that of non-malignant tissues, and the mean reduced scattering coefficient (μ_s') of malignant tissues was consistently significantly higher than that of non-malignant tissues. In addition, there was a clear relationship between the adipose tissue content and the measured β-carotene concentration consistent with the understanding that lipid-soluble β-carotene is stored in fatty tissues. In addition, Zhu *et al.* found that the relative contributions of collagen (related to fibrous stroma), NADH (related to cellular metabolism), and retinol/vitamin A fluorescence (related to β-carotene/adipose content) in the samples were reflective of the structural and biochemical make-up of the tissues [75], further supporting the notion that quantitative spectroscopy may be used to assay tissue composition. Malignant and fibrous/benign tissues were marked by higher relative collagen and NADH fluorescence, and lower retinol fluorescence, than adipose tissues.

In a similar study, Volynskaya *et al.* employed a modified diffusion approach for quantitative reflectance and intrinsic fluorescence spectroscopy of the breast *ex vivo* [73]. In this work, the concentrations of oxyhemoglobin and β-carotene, and scattering-related parameters were extracted from diffuse reflectance measurements, while the contributions of NADH and collagen were extracted from single-excitation-wavelength intrinsic fluorescence measurements. Deoxyhemoglobin was not assumed to be present as an absorber, and therefore no computation of hemoglobin saturation was possible; however, owing to the shallow penetration depth reported by the authors for this instrument (~100 μm), this is probably a valid assumption owing to diffusion of atmospheric oxygen into the very superficial regions of the tissue. Consistent with other reports, the concentration of β-carotene was found to be higher in normal (more fatty) tissues than in tissues with benign changes or invasive carcinoma, which are expected to be less fatty owing to the invasion of fibrous stroma and invading cancer cells. Also, the contribution of collagen fluorescence was sensitive to differences in fibrous stroma extent between the tissue types studied. The relative contribution of collagen fluorescence was higher in malignant and fibrous/benign tissues than normal tissues, whereas the contribution of NADH fluorescence was useful in discriminating fibrocystic and malignant tissues from benign fibroadenomas.

In vivo *studies*

An exciting prospect is the application of quantitative optical spectroscopy to the breast *in vivo*, which could be useful for breast cancer care in a variety of ways. For instance, it could be used to guide selection of sites to sample in diagnostic biopsy, could provide complementary information about the tumor environment or could be useful in prognosis, prediction, or monitoring of response to therapy. A multitude of non-invasive diffuse optical spectroscopy (DOS) and diffuse optical tomography (DOT) applications employing NIR wavelengths have been reported in the literature for the study of breast cancer, which are outside the scope of this review. However, to date only a few groups have employed UV–visible optical spectroscopy for the direct measurement of breast tissue properties *in vivo*, of which only a couple have employed model-based approaches.

In the report by van Veen *et al.*, differential path-length spectroscopy (a variant of reflectance spectroscopy) was used to measure the optical properties of normal and

diseased breast via an optical biopsy technique in 12 patients [72]. A thin fiber-optic probe was modified to fit into a commercially available biopsy needle such that optical measurements and biopsy samples could be co-registered for pathologic assessment, and was inserted into areas of interest in the breast using palpation of the tumor for guidance. A model based on Mie scattering theory and exponential attenuation (Beer-Lambert Law) was fit to the acquired differential reflectance spectra, and scattering properties and absorption properties (including the concentrations of oxyhemoglobin and deoxyhemoglobin, β-carotene, and blood vessel diameter). The authors found that malignant tissues ($n = 10$) were characterized by significantly lower hemoglobin saturation and higher blood volume than normal breast tissues ($n = 40$). This is not surprising, since malignant tissues are known to exhibit regions of hypoxia (low oxygen tension) as well as increased vascularity due to angiogenesis. Interestingly, the β-carotene concentration was higher, and the scattering slope lower, in malignant tissues versus normal tissues, although these differences were not significant.

More recently, the Ramanujam group has applied an inverse-Monte Carlo-based method to the breast *in vivo* in 35 patients to date (JQ Brown *et al*., abstract 5009, 30th Annual San Antonio Breast Cancer Symposium, December 2007; JQ Brown *et al*., 2009). In contrast to van Veen *et al*., the authors used ultrasound guidance to interface a fiber-optic probe to normal and diseased tissues through a biopsy cannula, in patients undergoing breast cancer surgery. Application of the Monte Carlo-based inversion model to measured reflectance spectra resulted in quantification of scattering parameters (wavelength-dependent reduced scattering coefficient) and absorption properties (concentrations of oxyhemoglobin and deoxyhemoglobin and β-carotene). Like van Veen *et al*., the authors found that considered together, malignant tissues ($n = 20$) exhibited lower oxygenation than non-malignant tissues ($n = 56$) as measured by hemoglobin saturation. However, it was shown that not all malignant tissues assayed were hypoxic, which is consistent with the body of literature on breast tumor oxygenation. The presence of a significant number of tumors with normal oxygenation levels was correlated with expression of the HER2/neu receptor in those tumors (as determined by immunuhistological analysis). This was attributed to an increase in angiogenesis promoted by downstream gene targets of HER2/neu—in fact, the authors found a clear relationship between total hemoglobin concentration and oxygenation in the tumors, indicating that better perfused tumors have higher oxygenation levels owing to increased O_2 availability.

Uterine cervix

Optical spectroscopy has been studied extensively as a diagnostic aid for cervical cancer. The primary application to the cervix is as a diagnostic tool for detecting early dysplastic changes that might progress into cancer, particularly in the developing world where there is a lack of infrastructure for well-organized screening and diagnostic programs. In contrast to the breast, most researchers have performed these studies in the human *in vivo*, owing to the accessibility of the organ, and no requirement to make an incision creating minimal risk to the patient. However, most reports of UV–visible spectroscopy in the cervix have involved the development of empirical relationships between spectral features and disease diagnosis. While these methods have demonstrated an excellent ability to diagnose disease, there is a fundamental lack of understanding about the sources of contrast that would be helpful in understanding the progression of the disease from a physiological standpoint. Therefore, in the following section we review those studies that have employed quantitative spectroscopy for the study of the uterine cervix *in vivo*.

Table 5 contains a summary of optically measured parameters, stratified by origin of contrast (stroma, epithelium, or both), which allows ready comparison of the findings of the reviewed studies. As seen in the table, the trends in optically measured parameters with increased dysplasia are largely preserved across the studies. In addition to being internally consistent, these findings are also consistent with independent measures of these parameters reported in the literature. For instance, using immunohistochemical stains for vascular endothelial cells, Abulafia *et al*. and Dellas *et al*. [84,85] have shown that stromal microvessel density, which is proportional to vascular volume (and hence blood content), increases with cervical pre-cancer grades. Decreases in collagen fluorescence, and light scattering due to collagen, arising from the stroma, are due to a breakdown of the collagen network with increasing dysplasia in the stroma [86]. Conversely, scattering in the epithelium is expected to increase owing to increased nuclear size and density, as shown in the elegant microscopy study by Collier *et al*. [87].

Homogeneous models

In some of the first reports of quantitative UV–visible spectroscopy to the uterine cervix, Georgakoudi *et al*. used diffuse reflectance measurements and laser-induced fluorescence to extract absorption, scattering, and intrinsic fluorescence spectra from the cervix *in vivo* [88,89]. Using a linear combination of collagen and NAD(P)H fluorescence excitation-emission matrices (EEMs) to fit to the measured intrinsic fluorescence EEMs, the authors found that benign changes and precancerous lesions were marked by a decrease in collagen fluorescence as compared with normal tissues due to a breakdown of the extracellular matrix [90] or increased epithelial thickness. Furthermore, NAD(P)H contribution to total fluorescence was found to increase for pre-cancerous lesions, which was attributed to the increased metabolic activity of the cells within these lesions [91]. The effective

Table 5

Comparison of optically measured physiological parameters between studies indicating how the parameters changed with cervical pre-cancer. The origin of optical contrast is indicated in the table for each study. Arrows indicate whether the parameter was significantly higher (↑), lower (↓), or no different (=) in high-grade pre-cancer (HG) compared to normal and non high-grade pre-cancerous tissues (NHG). Blank areas indicate that the parameter was not measured or reported.

Reference	Samples (HG, NHG)	Origin of contrast							
		Stroma			Epithelium				Stroma + epithelium
		Blood content	Blood saturation	Collagen	NADH	Nuclear size	Nuclear density	$<\mu_s'>$	$<\mu_s'>$
Georgakoudi *et al*. 2002 [88]	34, 50			↓	↑	↑	↑		
Mourant *et al*. 2007 [69]	11, 77	=	=						
Chang *et al*. 2009 [92]	15, 64	↑	=						↓
Chang *et al*. 2006 [67•]	139, 354	↑		↓				↑	

scattering slope was decreased in precancerous lesions (attributed to breakdown of collagen in the stroma), but analysis of light scattered from the superficial epithelium suggested an increase in both the size and density of scattering centers (which was attributed to increased nuclear size and density in the epithelium).

Mourant *et al*. applied unpolarized and polarized reflectance spectroscopy to 36 patients undergoing standard colposcopy [69]. From fits to the spectra in the visible, the concentration and oxygenation of hemoglobin was quantified. The hemoglobin oxygenation was higher in high-grade pre-cancers versus non-high-grade pre-cancers, although the difference was not significant.

The Ramanujam group has applied an inverse-Monte Carlo method for UV–visible quantitative spectroscopy in the cervix *in vivo* in 38 patients [92]. Model-based analysis of reflectance spectra allowed quantification of hemoglobin parameters (species content and oxygenation) as well as scattering (wavelength-dependent μ_s'). Total hemoglobin content was statistically elevated in high-grade pre-cancers ($n = 15$) versus normal tissues or low-grade pre-cancers ($n = 64$), whereas scattering was significantly reduced in high-grade pre-cancers versus normal tissues or low-grade pre-cancers. The increased hemoglobin content with increased dysplasia was attributed to angiogenic processes. In contrast to the report by the Richards-Kortum group [67•], decreased scattering was observed with increasing dysplasia. However, simulation of the sensing depth of the optical probe geometry used in the study suggested that these decreases in scattering arose primarily in the cervical stroma, not the superficial epithelium. Thus, the decrease in scattering could be attributed to breakdown or remodeling of the cervical stroma [86], which is consistent with the collagen fluorescence results from Georgakoudi *et al*. ([989]) and Chang *et al*. ([67•]).

Two-layer models

Chang *et al*. developed an analytical model for estimation of tissue optical properties and intrinsic fluorescence in a two-layered medium [93], which they applied to the analysis of fluorescence spectra measured from the cervix *in vivo* in 292 patients [67•]. The authors stratified their results by menopausal status and age; however, within each particular demographic, the general trend was for increased epithelial scattering and increased hemoglobin content with increasing dysplasia, consistent with increased nuclear atypia and angiogenic processes associated with dysplasia. Furthermore, keratin fluorescence and fluorescence from enzymatically activated collagen crosslinks were observed to decrease with dysplasia. An interesting aspect of this study was the observation that significant inter-patient variability existed between groups of patients separated by menopausal status and age. The value of quantitative analysis is well-demonstrated here, in that the authors were able to identify which particular biomarkers were sensitive to menopausal status and age, which would have important implications for the development of an effective predictive model that could be applied to all patients.

Challenges and conclusions

There are several challenges in implementing optical spectroscopy for *in vivo* applications. For example, with respect to implementation of algorithms, most notably in modeling the diffuse reflectance spectra, there is a requirement to constrain the problem to a defined set of absorbers and scatterers when using a single source–detector separation. Exclusion of a source of absorption or scattering can lead to systematic biasing of results [94]. This can be problematic in tissue, where all such sources may not be well characterized, or whose properties may vary depending on their microenvironment. Secondly, most models in use generally make the assumption that the interrogation volume can be represented by a homogeneous model. This may not be appropriate in practice, with tissue showing highly complex morphology particularly in many disease states. This could be particularly problematic for probes with multiple source detector separations, each of which is essentially interrogating a slightly different non-homogeneous tissue volume. This can be accounted for using a non-homogeneous model of

light-tissue interaction, but this dramatically increases the complexity of the problem [95]. In addition absorbers are not uniformly distributed, but are localized to specific tissue compartments, for example hemoglobin is localized within red blood cells, which are themselves contained within blood vessels. This local concentration of hemoglobin leads to a shielding effect that distorts the perceived absorption spectrum of hemoglobin [96]. It is possible to correct for this effect by modulating the apparent absorption coefficient according to the packing diameter and absorption coefficient, which effectively reduces the apparent absorption coefficient for high μ_a [72,96].

Probe pressure is another challenge, with the potential problem of pressure influencing the local hemodynamics. Nath *et al.* conducted a detailed study investigating this effect on fluorescence spectra acquired from the human cervix [97]. Utilizing three levels up to approximately 0.9 N, they did not find significant differences in the fluorescence spectra acquired from these patients. However, this study looked only at measured fluorescence intensities and did not extract quantitative physiologic information, or consider diffuse reflectance spectra. On the contrary, Reif *et al.* had conflicting findings that suggested that there was a pressure dependence on the absorption and scattering properties of the mouse thigh muscle [98]. Notably, the vessel size decreased, scattering increased, and hemoglobin saturation decreased. Ti *et al.* reported that such changes may be highly tissue-dependent [99], so whether this is a significant concern would probably depend on the application.

Yet another challenge lays in the fact that source power, and other factors in system throughput, such as fiber bending, often fluctuate during the course of an experiment. This is an issue because calibration measurements are typically performed before or after an experiment, so real-time fluctuations cannot be accounted for. Yu *et al.* [100] developed a self-calibrating probe that addresses these concerns by incorporating a reference fiber into the fiber optic probe that simply records light reflected directly back through a coupler incorporated within the probe. This gives a real-time calibration of the system throughput while measurements are being made.

While these challenges in implementation exist, quantitative UV–visible optical spectroscopy may prove to be a viable alternative to more invasive, or less practical, methods for evaluating biomarkers of cancer for a variety of applications. Recent reports in which quantitative optical cancer biomarkers are clinically validated with currently accepted methods are a welcome addition to the field, as they will set the stage for optical technologies to gain widespread clinical acceptance. It is our hope that the number of groups employing quantitative approaches to tissue optical spectroscopy will only increase, and that continued research will reduce or eliminate any barriers to widespread clinical application of these technologies.

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