Editor's Introduction: Optical Methods for Biomedical Diagnosis

Valery V. Tuchin
Saratov National Research State University
National Research Tomsk State University
Institute of Precision Mechanics and Control, Russian Academy of Sciences

I.1 Historical Aspects and Brief Overview

The history of light application for monitoring tissues and cells for the purpose of disease diagnosis is presented in Refs. 1–14. In 1831, Bright had reported that sunlight or light from a candle was able to shine through the head of a patient with hydrocephalus.[10] The ability of light to transilluminate tissues was later noted by Curling in 1843, and by Cutler in 1929 for monitoring breast lesions.[11,14,15] In 1911, Hasselbalch undertook studies of ultraviolet transmission through the skin, and by the early 1930s textbooks providing good scientific data on optical transmission, absorption, and fluorescence of tissues were available. The properties of skin in the near-infrared (NIR) range were reported by Pearson and Norris in 1933 and by Hardy and Muschenheim in 1935.[4] Due to strong light scattering and autofluorescence, such early studies allowed for understanding only some of the general optical properties of tissues.

Millikan was the first to suggest the dual-wavelength optical spectroscopy method for correction of light scattering, and he was successful in metabolite analysis in humans.[1,12,16] In the 1930s, 1940s, and early 1950s, many studies of the spectroscopy of hemoglobin in tissues were undertaken.[1] *In vivo* measurements of NIR transmittance spectra of the human earlobe and cheek done by Il'ina revealed many new important details about tissue spectra, such as the presence of a water band at 980 nm[17]

The use of NIR light for deep transillumination of mammalian tissues, including the adult human head, and the diagnostic value of NIR light for the assessment of hemoglobin oxygen saturation and the cytochrome *aa3* oxidation–reduction (redox) state in thick tissues were demonstrated in 1977 by Frans Jobsis.[18,19]

For many years, Britton Chance was a pioneer in the development of tissue optics and biomedical spectroscopy.[1,2,12,20–22] He applied spectroscopy for physiological studies of bioenergetics, for trend measurements of hemoglobin oxygenation, and for investigation of cytochrome oxidation. For more precise quantification of the absorbing species in tissues and therefore of the potential utility of this approach for clinical sensing, Chance and co-workers[22] and Delpy and co-workers[23] suggested time-resolved spectroscopy using pulse transillumination and detecting—the so-called time-domain (TD) technique. Later, this approach was further developed by Patterson and co-workers[24] and Jacques[25] to be applied for reflectance measurements, and was used by many investigators for tissue studies and designing of optical diagnostic instruments.[1,2,5–14,26–28]

In 1990, Lakowicz and Berndt[29] extended the time-resolved spectroscopy of tissues by using a frequency-domain (FD) approach, which is mathematically equivalent to the time-domain approach, but allows for a more robust and sensitive measuring technique to be designed.[30] The subsequent discovery of a new type of waves—photon-density waves[8]—and their interference[31] raised the possibility that the FD approach might be able to improve significantly the spatial resolution of tissue spectroscopic analysis.[32]

Many studies on *in vitro* and *in vivo* tissue spectrophotometry using continuous-wave (CW), TD, or FD techniques are overviewed in Refs. 1–14, 26–28, and 32. The development of the cooled charge-coupled device (CCD), time- and spatial-resolved techniques, and other instruments has proceeded at an increasing pace to a wider area of NIR spectroscopy investigations and biomedical applications. At present, more than 500 NIR spectroscopy clinical instruments are available commercially for monitoring and imaging of a tissue's degree of oxygenation, concentration of oxidized cytochrome, and tissue hemodynamics.[10]

The relative simplicity of measuring the human skin reflectance and fluorescence spectra meant that these values were first obtained many years ago. Nevertheless, only in the last three decades have quantitative spectral techniques for *in vivo* monitoring and diagnosis of certain cutaneous and systematic diseases been introduced.[3,13] Historical review of these developments can be found in Refs. 3 and 33.

Various fluorescence techniques, such as those based on autofluorescence and on microscopy using fluorescent markers, time-resolved (phase and time-gated), laser scan, and multiphoton technologies, have been used to study human tissues and cells *in situ* noninvasively.[9,13,14,34–38] Fluorescence

techniques are applicable to medical diagnoses of various pathologies affecting many tissues, including those involving the eye. Many robust and powerful combined optical diagnostic techniques, such as fluorescence/light scattering and fluorescence/Raman scattering, have also been designed.[35–37,39]

Raman spectroscopy, which is a great tool for studying the structure and dynamic function of biologically important molecules,[40] also has been used extensively for monitoring and diagnosis of disease *in vitro* and *in vivo* during the past three decades. Examples of its applications include cataracts, atherosclerotic lesions in coronary arteries, precancerous and cancerous lesions in human soft tissues, and bone and teeth pathologies.[13,39,41–43] The successful use of Raman spectroscopy is due to improvements in instrumentation in the NIR spectrum, where fluorescence is significantly reduced.

Among prospective noninvasive blood glucose sensing methods, optical techniques such as NIR and middle-infrared (MIR) (2.5–50 μm) spectrophotometry, fluorescence, and Raman spectroscopy are of great interest to investigators.[34,44] MIR spectroscopy—and particularly attenuated total reflectance Fourier transform infrared spectroscopy—is also important for *in vivo* monitoring of the human skin components.[13,45] MIR and Raman spectroscopy are both examples of so-called vibration spectroscopy, which is characterized by highly specific bands that are dependent on species concentration.[41–45]

Light scattering spectroscopy (LSS) is a novel technique capable of identifying and characterizing pathological changes in human tissues at the cellular and subcellular levels. It can be used to diagnose and detect disease, including through noninvasive monitoring of early cancerous changes in human epithelium.[13,46]

Quasi-elastic light scattering spectroscopy (QELSS), as applied to monitoring of dynamic systems, is based mainly on the correlation or spectral analysis of the temporal fluctuations of the scattered light intensity.[47] QELSS, which is also known as light-beating spectroscopy or correlation spectroscopy, is widely used for various biomedical applications, but especially for blood or lymph flow measurement and cataract diagnostics.[6,13,48–51] For studying optically thick tissues when multiple scattering prevails and photon migration (diffusion) within tissue is important for the character of intensity fluctuations, diffusion wave spectroscopy (DWS) is available.[8,13]

Optothermal or photothermal spectroscopy (OTS/PTS), which is based on detection of the time-dependent heat generation induced in a tissue by pulsed or intensity-modulated optical radiation, is widely used in biomedicine.[13,52–54] Among the various OTS methods, the optoacoustic (OA) and photoacoustic (PA) techniques are of great importance. They allow one to estimate the optical, thermal, and acoustic properties that depend on peculiarities of a tissue's structure.

I.2 Optical Diffuse Techniques

I.2.1 CW spectrophotometry

The specificity of optical diffuse techniques that use a CW light source and detection, applied to *in vivo* spectroscopy of thick tissues (e.g., the female breast or the newborn head) could be described by the following semi-empirical exponential equation for the collimated transmittance $T_c(\lambda)$:[13,55]

$$T_c(\lambda) = x_1 \exp[-\mu_a(\lambda) L(\lambda) x_2], \tag{I.1}$$

where $L(\lambda)$ is the total mean path length of the photons. This equation reflects the wavelength (λ) dependency on absorption, $\mu_a(\lambda)$ and reduced (transport) scattering coefficients $\mu_s'(\lambda)$; x_1 takes into account multiply scattered but nonabsorbed photons, which do not arrive at the detector, and the measurement geometry; x_2 compensates for measurement error of the slab thickness d and inaccuracies in the reduced scattering coefficient $\mu_s' = \mu(1-g)$, and μ_s and g are the tissue scattering coefficient and anisotropy factor of scattering, respectively. For a slab of thickness d, the diffusion equation can be used to calculate a total mean path length L of the photons.[24]

Equation (I.1) was successfully used for fitting of the *in vivo* measurement spectra of the female breast and estimations of the concentrations of the following absorbers: water (H_2O), fat (f), deoxyhemoglobin (Hb), and oxyhemoglobin (HbO):[55]

$$\mu_a = C_{H_2O}\,\sigma_{H_2O} + c_f \sigma_f + c_{Hb}\,\sigma_{Hb} + c_{HbO}\sigma_{HbO}, \tag{I.2}$$

where σ_i is the cross section of the absorption of the ith component. By varying the concentrations of the four tissue components, the measurement spectra could be fitted well using Eq. (I.2); the correlation coefficients were better than 0.99 in all cases.[55]

For many tissues, *in vivo* measurements are possible only in the geometry of the backscattering.[13,14] The corresponding relation for light reflectance R can be based on diffusion approximation. For backscattering optical spectroscopy, we have to know, in addition to the measured coefficient of reflection, the depth from which the optical signal is coming. For a spatially separated light source and detector (for example, two fibers normal to the tissue surface), that depth is defined by the photon-path distribution function for the photons migrating from a source to a detector. This spatial distribution function for a homogeneous scattering medium has a "banana" shape. The curve of the most probable direction of a photon migration of the "banana" region reaches a maximum depth, z^{max}, which depends on the source–detector separation r_{sd}:[13,56]

$$z^{\max} \approx (1/2\sqrt{2})r_{sd} \tag{I.3}$$

Instead of Eq. (I.1), which is used for *in vivo* studies in transillumination experiments, a modified form of the Beer–Lambert law is used to describe the optical attenuation in backscattering geometry:[13,56]

$$I/I_0 = \exp(-\varepsilon_{ab} \cdot c_{ab} \cdot r_{sd} \cdot \mathrm{DPF} - G_s), \tag{I.4}$$

where I is the intensity of detected light, I_0 is the intensity of the incident light, ϵ_{ab} is the absorption coefficient measured in $\mu\mathrm{mol}^{-1}$ cm^{-1}, c_{ab} is the concentration of absorber in μmol, DPF is the differential path length factor accounting for the increase of the photons' migration paths due to scattering, and G_s is the attenuation factor accounting scattering and geometry of the tissue.

When r_{sd}, DPF, and G_s are kept constant, the changes of absorbing medium concentration can be calculated using measurements of the changes of the optical density (OD), $\Delta(\mathrm{OD}) = \Delta(\log(I_0/I))$:[56]

$$\Delta c_{ab} = \Delta(\mathrm{OD})/\varepsilon_{ab} r_{sd} \mathrm{DPF}. \tag{I.5}$$

Using optical spectroscopy or imaging, the changes in the optical density are measured as follows:

$$\Delta(\mathrm{OD}) = \log(I_0/I_{\mathrm{test}}) - \log(I_0/I_{\mathrm{rest}}) = \log(I_{\mathrm{rest}}) - \log(I_{\mathrm{test}}), \tag{I.6}$$

where I_{rest} and I_{test} represent, respectively, the light scattering intensity of the object (e.g., brain tissue, skeletal muscle) detected during rest and during testing that involves induced brain activity, cold or visual testing, training, or some other experimental condition. For example, based on the OD changes at the wavelengths 760 and 850 nm, one can get either the absorption images for the two measuring wavelengths or functional images (oxygenation or blood volume) within the detection region of study:

$$\Delta(\mathrm{OD})_{oxy} = \Delta(\mathrm{OD})_{850} - \Delta(\mathrm{OD})_{760};\ \Delta(\mathrm{OD})_{total} = \Delta(\mathrm{OD})_{850} + k_{bvo}\Delta(\mathrm{OD})_{760}, \tag{I.7}$$

where $(\mathrm{OD})_{850}$ and $(\mathrm{OD})_{760}$ are the optical densities measured at the wavelengths 850 and 760 nm, and k_{bvo} is the modification factor for reducing the cross-talk between changes of blood volume and oxygenation.

The typical *in vivo* backscattering spectrum (400–700 nm) for a tissue contains the absorption bands of hemoglobin (the Soret and Q-bands).[13,57,58] It also encompasses some absorption from compounds such as flavins, beta-carotene, bilirubin, and cytochrome, among others. On the basis of measurement of the spectral differences of normal and pathological tissues, the corresponding spectral signature "identifiers" can be created. For *in vivo* medical diagnosis, the spectral "identifiers" typically use the ratios of the

integrated reflection coefficients within selected spectral bands or the measurement of the spectrum slope for the selected spectral bands. As an internal standard for evaluating the absolute concentrations of the blood components in a tissue, the water band at 980 nm can be used.[57]

I.2.2 Eye tissues

Even such transparent tissues as the human cornea scatter light because the total and axial (collimated) transmissions are not identical.[13,59] Due to the low scattering, water absorption peaks are evident at 300, 980, 1180, 1450, 1900, and 2940 nm. They provide for poor transmission of light through the cornea in the ultraviolet (UV) and infrared (IR) spectral regions.

Average spectral transmittance derived from cornea transmittance measurements in the spectral range 320–700 nm on 10 subjects (14–75 years) was modeled by the following functions for the total transmittance $T_t(\lambda)$ (acceptance angle close to 180 deg) and axial transmittance $T_c(\lambda)$ (acceptance angle of about 1 deg):[60]

$$\mathrm{Log}\, T_t(\lambda) = -0.016 - 21 \cdot 10^8 \lambda_0^{-4}, \quad \mathrm{Log}\, T_c(\lambda) = -0.016 - 85 \cdot 10^8 \lambda_0^{-4}, \tag{I.8}$$

where λ_0 is the wavelength in nanometers.

The normal human eye lens is less transparent than the cornea for the visible light, because, in addition to scattering, absorption by different chromophores including 3-hydroxy-L-kynurenine-*O*-β-glucoside and age-related protein (responsible for lens yellowing in older adult subjects) is important.[13,35,61]

The sclera shows poor transparency because of strong light scattering by its structure elements (a system of polydispersive, irregularly arranged collagen cylinders immersed in the ground substance with a lower refractive index).[13] Such a fibrous structure allows for easy control of the human sclera transmittance at a refractive index matching that of collagen fibers and ground material through its impregnation by the immersion liquid.[13]

I.2.3 Time-domain method

Time-dependent radiation transfer theory (RTT) makes it possible to analyze the time response of scattering tissues.[1,2,5–14,22–26,55,62] When probing the plane-parallel layer of a scattering medium with a short laser pulse, the transmitted pulse consists of a ballistic (coherent) component, a group of photons having zigzag trajectories, and a highly intensive diffuse component. Both unscattered photons and photons undergoing forward-directed single-step scattering contribute to the intensity of the component consisting of photons traveling straight along the laser beam. This component is subject to

exponential attenuation with increasing sample thickness—a factor that accounts for the limited utility of such photons for practical diagnostic purposes in medicine.

The group of snake photons with zigzag trajectories includes photons, which experience only a few collisions each. They propagate along trajectories that deviate only slightly from the direction of the incident beam and form the first-arriving part of the diffuse component. These photons carry information about the optical properties of the random medium.

The diffuse component is very broad and intense since it contains the bulk of incident photons after they have participated in many scattering acts and, in turn, migrate in different directions and have different path lengths. The diffuse component carries information about the optical properties of the scattering medium, and its deformation may reflect the presence of local inhomogeneities in the medium. However, the resolution obtained by this method at a high light-gathering power is much lower than that obtained by measuring straight-passing photons. Two principal probing schemes are conceivable—one recording transmitted photons and the other taking advantage of their backscattering.

The time-dependent reflectance is defined as[24,25]

$$R(r_{sd},t) = \frac{z_0}{(4\pi cD)^{3/2}}\, t^{-5/2} \exp\left(-\frac{r_{sd}^2 + z_0^2}{2cDt}\right) \exp(-\mu_a\, ct), \tag{I.9}$$

where t is time, $z_0 = (\mu_s')^{-1}$, and $D = 1/3(\mu_s' + \mu_a)$ is the photon diffusion coefficient in centimeters. To convert the last value to cm^2/s, it should be multiplied by c, the velocity of light in the medium (with units of cm/s).

In practice, μ_a and μ_s' are estimated by fitting Eq. (I.9) with the shape of a pulse measured by the time-resolved photon counting technique. An important advantage of the pulse method is its applicability to *in vivo* studies, in that μ_a and μ_s' can be evaluated separately using a single measurement for a definite source–detector distance r_{sd}.

I.2.4 Frequency-domain method

The frequency-domain (FD) method measures the modulation depth of scattered light intensity m_U $AC_{detector}/DC_{detector}$ and the corresponding phase shift relative to the incident light modulation phase $\Delta\Phi$ (phase lag).[1,2,5–14,29–32,62–64] Compared with the TD measurements, this method is simpler and more reliable in terms of data interpretation and noise, because it involves amplitude modulation at low peak powers, slow rise time, and hence smaller bandwidths than the TD method. Higher signal-to-noise ratios are attainable as well. Medical device FD equipment is more economic and portable.[32] However, the FD technique suffers from the simultaneous transmission and reception of signals, and it requires special efforts to avoid

unwanted cross-talk between the transmitted and detected signals. The current measuring schemes are based on heterodyning of optical and transformed signals.[13,32]

The development of the theory underlying this method resulted in the discovery of a new type of waves: photon-density waves, which are progressively decaying waves of intensity. Microscopically, individual photons make random migrations in a scattering medium, but collectively they form a photon-density wave at a modulation frequency ω that moves away from a radiation source. Photon-density waves possess typical wave properties; for example, they undergo refraction, diffraction, interference, dispersion, and attenuation.[1,2,5–14,29–32,62]

In strongly scattering media with weak absorption far from the walls and from a source or a receiver of radiation, the light distribution may be regarded as a decaying diffusion process described by the time-dependent diffusion equation for photon density. For a point light source with harmonic intensity modulation at frequency $\omega = 2\pi\nu$ placed at the point $\vec{r} = 0$, an alternating component (AC) of intensity is a going-away spherical wave that has its center at the point $\vec{r} = 0$ and that oscillates at a modulation frequency with modulation depth

$$\mathrm{m}_U(\vec{r},\omega) = m_I \ \exp\left(\vec{r}\sqrt{\mu_a/D}\right)\exp\left(-\vec{r}\sqrt{\omega/2cD}\right), \tag{I.10}$$

and undergoes a phase shift relative to the phase value at point $\vec{r} = 0$ equal to

$$\Delta\Phi(\vec{r},\omega) = \vec{r}\sqrt{\omega/2cD}\,, \tag{I.11}$$

where m_{I} is the intensity modulation depth of the incident light.

The length of a photon-density wave, Λ_Φ, and its phase velocity, V_Φ, are defined by

$$\Lambda_\Phi^2 = 8\pi^2 cD/\omega \text{ and } V_\Phi^2 = 2cD\omega \tag{I.12}$$

.

Measuring $m_U(\vec{r},\omega)$, $\Delta\Phi(\vec{r},\omega)$ allows one to separately determine the transport scattering coefficient μ_s' and the absorption coefficient μ_a and then to evaluate the spatial distribution of these parameters.

For typical female breast tissue at 800 nm ($\mu_s' = 15\ \mathrm{cm}^{-1}$, $\mu_a = 0.035\ \mathrm{cm}^{-1}$) for $\omega/2\pi = 500$ MHz, and $c = (3 \times 10^{10}/1.33)$ cm/s, the wavelength is $\Lambda_\Phi \cong 5.0$ cm and the phase velocity is $V_\Phi \cong 1.77 \times 10^9$cm/s.

A number of FD systems demonstrating achievements in the field of optical *in vivo* diagnostics applied for clinical study have been described.[13,32] For example, to obtain quantitative measurements of the absolute optical parameters of various types of tissue, a portable, high-bandwidth

(0.3–1000 MHz), multi-wavelength (674, 811, 849, and 956 nm) frequency-domain photon migration instrument was designed.[63–66]

I.2.5 Photon-density wave interference method

The photon-density wave interference method was described first in Ref. 31 and is also known as the phase and amplitude cancellation method and as the phased-array method. It appears very promising as a means to improve the spatial resolution of the modulation technique.[13,32] This idea is based on the use of either duplicate sources and a single detector or duplicate detectors and a single source, so that the amplitude and phase characteristics can be compensated and the system becomes a differential. If equal amplitudes at 0-deg and 180-deg phases are used as sources, an appropriate positioning of the detector can lead to a null in the amplitude signal and a crossover between a 0- and 180-deg phase shift—that is, 90 deg.

In a heterogeneous medium, the apparent amplitude's null and the phase's crossover may be displaced from the geometric midline. This method is extremely sensitive to perturbation by an absorber or scatterer. A spatial resolution of approximately 1 mm for the inspection of an absorbing inhomogeneity has been achieved, and the same resolution is expected for the scattering inhomogeneity. Another good feature of the technique is that at the null condition, the measuring system is relatively insensitive to amplitude fluctuations common to both light sources. Inhomogeneities that affect a large tissue volume common to the two optical paths cannot be detected, however. The amplitude signal is less useful in imaging since the indication of position is ambiguous. Although this can be accounted by further encoding, the phase signal is more robust, and a phase noise less than 0.1 deg (signal-to-noise ratio more than 400) for a 1-Hz bandwidth can be achieved.[32]

I.2.6 Spatially modulated spectroscopy and imaging

In diffuse techniques, spatially resolved measurements have been generally limited to "multi-distance" measurements, tracking the spatial dependence of a reflected or transmitted light field generated from a point-like illumination and detection with a number of source–detector separations r_{sd}.[13] The Fourier transform equivalent to the real spatial domain is the spatial-frequency domain (SFD).[65,66] In diffractive optics, spatially structured illumination techniques are used for manipulating of optical images. Spatially modulated laser beams also have been used effectively in studies of scattering objects, including samples of tissues and blood.[13,67] This technique has mostly been applied to investigate low-scattering objects or thin tissue slices and blood layers. However, it was approved successfully for investigation of whole cataractous human eye lenses based on averaging of interferential fringes to eliminate speckle modulation.[13]

The interactions of spatially modulated light beams with diffuse media are described in Refs. 65, 66, and 68–73. Instead of laser beams, low-cost incoherent conventional white light sources are widely used with this approach. Spatially modulated imaging (SMI) provides a wide-field mapping of scattering tissues in the SFD. The spatial modulation transfer function (*s*-MTF) of a turbid tissue encodes both depth and optical property information, enabling both quantitation and tomographic imaging of the spatially varying tissue's optical properties.[71] Similarly to time-resolved methods, the SMI method can be described analytically using diffusion-based theory, or numerically using Monte Carlo simulations in the framework of an RTT-based approach. The optical properties of tissues can be recovered by analysis with the analytic diffusion model using an inversion method, such as a least-squares multifrequency fitting algorithm or a more rapid two-frequency lookup table approach.[65,66,68–73]

The spatially modulated photon density can be considered as "standing" photon-density waves. The basic principles underlying generation of spatially modulated photon-density plane waves were formulated in Ref. 71. Their properties were also described by using spatial-frequency spectral representation. As a first approximation, a diffusion theory was used to get analytical expressions valid for a relatively large transport albedo $\Lambda' = \frac{\mu_s'}{\mu_a+\mu_s'}$ and small spatial frequencies. However, based on Monte Carlo modeling of the transport equation, the results can be extended to low albedo and high spatial frequency modes.

For the time-independent form of the diffusion equation for a homogeneous medium with a semi-infinite geometry and a normally incident periodically modulated plane wave, the source function S_d can be presented in the form[71]

$$\mathrm{S}_d = S_{do}(z)\cos(k_{\mathrm{x}}x + \alpha)\cos(k_{\mathrm{y}}y + \beta) \tag{I.13}$$

with spatial frequencies $f_x = (k_x/2\pi)$ and $f_y = (k_y/2\pi)$, and spatial phases α and β, extending infinitely in the tangential spatial dimensions x and y, with some arbitrary dependence on depth z.

If the medium's response is proportional to the input intensity, this sinusoidal modulation will give rise to a diffuse fluence rate U with the same frequency and phase:

$$U = U_0(z)\cos(k_{\mathrm{x}}x + \alpha)\cos(k_{\mathrm{y}}y + \beta). \tag{I.14}$$

A plane wave with both x and y modulation gives rise to a photon-density wave propagating with a scalar attenuation coefficient:

$$\mu'_{\text{eff}} = \sqrt{\mu_{\text{eff}}^2 + k_{\text{x}}^2 + k_{\text{y}}^2} = \frac{1}{\delta'_{\text{eff}}}, \tag{I.15}$$

where

$$\mu_{\text{eff}} = \sqrt{3\mu_a(\mu'_s + \mu_a)} \tag{I.16}$$

and δ'_{eff} is the effective light penetration depth into a scattering medium.

Although spatial anisotropy may exist in real tissues, to understand scalar photon-density wave attenuation in multiply scattering media, 1D projection can be used for simplicity; that is, $k = k_x$, with constant illumination along y ($k_y = 0$). At zero spatial frequency ($k = 0$), the effective light penetration depth into a scattering medium, δ'_{eff}, is equivalent to that of a planar (none-modulated) illumination, $\delta_{\text{eff}} = (1/\mu_{\text{eff}})$. In general, however, μ'_{eff}(and δ'_{eff}) are functions of both optical properties and the spatial frequency of illumination. Thus, at known parameters of illumination, it is possible to evaluate the optical properties of tissues.

The amplitude of the periodic wave, $U_0(z)$, is independent of the tangential spatial dimensions x and y. As a consequence, existing planar geometry solutions of the diffusion equation can be used to describe spatially modulated photon density by simply substituting μ_{eff} with the new μ'_{eff} term.

I.3 Fluorescence Spectroscopy

I.3.1 Fundamentals and methods

Fluorescence arises upon light absorption and is related to an electron's transition from the excited state to the ground state within a molecule. In the case of thin samples (e.g., biopsies that are a few micrometers in thickness), the fluorescence intensity I_{F} is proportional to the concentration c and the fluorescence quantum yield η of the absorbing molecules.[34,74–76] In a scattering medium, the path lengths of scattered and unscattered photons within the sample are different, and should be accounted for.[34]

At excitation of biological objects by ultraviolet light ($\lambda \leq 300$ nm), fluorescence of their components, such as proteins and nucleic acids, should be observed. Fluorescence quantum yields of all nucleic acid constituents, however, are approximately 10^{-4} to 10^{-5}, corresponding to lifetimes of the excited states in the picosecond time range. Autofluorescence (AF) of proteins is related to the amino acids tryptophan, tyrosine, and phenylalanine, which have absorption maxima at 280 nm, 275 nm, and 257 nm, respectively, and emission maxima between 280 nm (phenylalanine) and 350 nm (tryptophan).[34,74–76] Fluorescence from collagen or elastin is excited between 300 and 400 nm and shows broad emission bands between 400 and 600 nm, with maxima around 400 nm, 430 nm, and 460 nm. In particular, fluorescence of

collagen and elastin can be used to distinguish various types of tissues (e.g., epithelial versus connective tissues) and their pathology.[9,13,28,58,61,74–82]

The reduced form of coenzyme nicotinamide adenine dinucleotide (NADH) is excited selectively in a wavelength range between 330 nm and 370 nm. NADH is most concentrated within mitochondria, where it is oxidized within the respiratory chain located within the inner mitochondrial membrane. Its fluorescence is an appropriate parameter for detection of ischemic or neoplastic tissues.[75] Fluorescence of free and protein-bound NADH has been shown to be sensitive to oxygen concentration. Flavin mononucleotide (FMN) and dinucleotide (FAD), which have excitation maxima around 380 nm and 450 nm, respectively, have also been reported to contribute to intrinsic cellular fluorescence.[75]

Porphyrin molecules—for example, protoporphyrin, coproporphyrin, uroporphyrin, and hematoporphyrin—occur within the pathway of biosynthesis of hemoglobin, myoglobin, and cytochromes.[75] Abnormalities in heme synthesis, such as are observed in porphyrias and some hemolytic diseases, may considerably enhance the porphyrin level within tissues. Several bacteria (e.g., *Propionibacterium acnes* and bacteria within dental caries lesions) accumulate considerable amounts of protoporphyrin. Therefore, measurements of intrinsic fluorescence appear to be a promising method for detecting acne or caries.

At present, various exogenous fluorescing dyes can be applied to probe cell anatomy and cell physiology.[75] In humans, such dyes as fluorescein and indocyanine green are already used for fluorescence angiography or blood volume determination. *In vivo* fluorescence probes rely on a family of fluorescent proteins (FP). Based their emission maxima, blue, green, yellow, and red fluorescent proteins may be distinguished. The most popular is the green fluorescent protein (GFP). After cloning of the FP gene, various FP variants with different excitation and emission properties have been produced. When genes coding for a specific cellular protein are fused with FP or its variants, functional and site-specific tracking in living cells or even whole organisms becomes possible.

Fluorescence spectra often give detailed information on fluorescent molecules, including their conformation, binding sites, and interactions within cells and tissues. Fluorescence intensity can be measured as a function of either the emission wavelength or the excitation wavelength. The fluorescence emission spectrum $I_F(\lambda)$, which is specific for any fluorophore, is commonly used in fluorescence diagnostics. Fluorescence spectrometers for *in vivo* diagnostics are commonly based on fiber-optic systems and use of an optical multichannel analyzer (OMA; a diode array or a CCD camera) as a detector of emission radiation.[58,74–78]

Various comprehensive and powerful fluorescence spectroscopies, such as microspectrofluorimetry, polarization anisotropy, time-resolved with pulse excitation and frequency-domain, time-gated, total internal reflection

fluorescence spectroscopy and microscopy, fluorescence resonant energy transfer method, confocal laser scanning microscopy, and their combinations are available now.[34,74–82] These methods support the following applications (see Chapter 5 in Volume 2):[75]

1. 3D topography of specimens measured in the reflection mode for morphological studies of biological samples
2. High-resolution microscopy measured in the transmission mode
3. 3D fluorescence detection of cellular structures and fluorescence bleaching kinetics
4. Time-resolved fluorescence kinetics
5. Studies of the motions of cellular structures
6. Time-gated imaging so as to select specific fluorescent molecules or molecular interactions
7. Fluorescence lifetime imaging
8. Spectrally resolved imaging

Fluorescence is also beneficial in the practical work carried out by medical staff members. Concepts and applications of fluorescence imaging for surgeons are discussed in recent book[83] and in reviews and original papers.[84–86] Indocyanine green (ICG) is a NIR fluorescent dye that has been used in medical diagnostics for almost six decades, yet has great potential in the development of new imaging systems for several surgical specialties due to its unique molecule binding and spectral properties.[84,85] The introduction of new clinical applications has occurred especially rapidly during the last few years. ICG fluorescence imaging in the areas of plastic and reconstructive surgery, neurosurgery, and cardiac, vascular, oncological, and hepatic surgery is reviewed in Ref. 85.

The inability to identify microscopic tumors and assess surgical margins in real time during oncologic surgery, which may lead to incomplete tumor removal, prompted authors of a recent paper to develop a wearable-goggle augmented imaging and navigation system (GAINS); this system can provide accurate intraoperative imaging of tumors and sentinel lymph nodes (SLNs) in real time without disrupting normal surgical workflow.[86] The optical system projects both NIR fluorescence from the tumors and the natural-color images of tissue onto a head-mounted display without latency. Human pilot studies in breast cancer and melanoma patients using a NIR dye show that GAINS detected SLNs with 100% sensitivity. Clinical use of GAINS to guide tumor resection and SLN mapping promises to improve surgical outcomes, reduce rates of repeat surgery, and improve the accuracy of cancer staging.

I.3.2 *In vivo* human skin fluorescence

Currently, reflectance and fluorescence spectroscopies are probably the most developed among the available optical methods for investigating skin *in vivo*.

Reflectance and fluorescence from skin carry information about the structure of the epidermis and dermis, the quantity and density of blood vessels, the concentration and spatial distribution of chromophores and fluorophores in the skin, and the nature of skin metabolic processes. Typical applications include the *in vivo* quantitative analysis of skin erythema and pigmentation, determination of cutaneous color variation, monitoring of dermatological treatment effects, determination of skin photo-aging, diagnosis of skin tumors, and study of skin biophysics.[3,76,77,79–81,87,88]

The potential advantages and possible applications resulting from combined use of reflectance and fluorescence spectroscopy of the skin for the evaluation of erythema and pigmentation indices, the determination of hemoglobin oxygenation and concentration, and the investigation of the efficacy of topical sunscreens are discussed in Chapter 3 in Volume 2.[76]

Most of the biological components that are either related to the skin tissue structure or are involved in metabolic and functional processes generate fluorescence emission in the UV-visible spectral region. As a result, different morpho-functional conditions of the skin related to histological, biochemical, and physiochemical alterations can be characterized, in principle, on the basis of information available in fluorescence excitation–emission maps (EEMs).[6,76,78,80]

Among the various endogenous skin fluorophores, different forms of NAD and keratin located in the epidermis and in collagen located in the dermis can be found. The reduced (NADH) and oxidized (NAD^+) forms of NAD have roles in cellular metabolism, and the intensity of their specific fluorescence (fluorescence maxima near 460 nm and 435 nm, respectively) is used in differential diagnostics for metabolism dysfunction.[76]

Collagen and elastin are found predominantly within the papillary and reticular layers of the dermis. For these substances, both excitation light and emission light are attenuated because absorption by melanin and fluorescence intensity in the 400–480 nm range is subject to attenuation by other skin chromophores—hemoglobin, porphyrins, carotenoids, and so on.[76,81]

Recent studies of endogenous and exogenous fluorescence skin cancer diagnostics for clinical applications are overviewed in Ref. 88.

I.3.3 Advantages of multi-photon fluorescence

A new direction in laser spectroscopy of tissues and cells has emerged with the introduction of multi-photon (two-photon, three-photon) fluorescence scanning microscopy. This technique makes it possible to image functional states of an object or, in combination with autocorrelation analysis of the fluorescence signal, to determine the intercellular motility in small volumes.[13,38,89–92] Multi-photon fluorescence employs sharply focused ballistic photons at a long wavelength that provide fluorescence excitation by the second or third harmonic of the incident radiation. Fluorescence comes to a

wide-aperture photodetector exactly from the focal area of the excitation beam.

A unique advantage of multi-photon microscopy is the possibility of investigating three-dimensional distributions of chromophores excited with ultraviolet radiation in relatively thick samples. Such an investigation becomes possible because chromophores can be excited (e.g., at a wavelength of 350 nm) with laser radiation whose wavelength for two-photon-excitation fluorescence (TPEF) (700 nm) falls within the range where a tissue has high transparency. Such radiation can reach deep layers and produces less damage in tissues. Fluorescent emission in this case lies in the visible range (wavelengths greater than 400 nm). It emerges comparatively easily from a tissue and reaches a photodetector, which registers only the legitimate signal from the focal volume without any extraneous background noise.

TPEF of target molecules in a tissue is a nonlinear process induced by the simultaneous absorption of two NIR photons, whose total energy is sufficient to excite the electronic state of the molecular transition. In general, photons with different wavelengths λ_1 and λ_2 can be used

$$\frac{1}{\lambda_{1f}} \cong \frac{1}{\lambda_1} + \frac{1}{\lambda_2}, \tag{I.16}$$

where λ_{1f} is the wavelength necessary to excite the fluorescence at single-photon absorption. However, the excitation by the same light source (i.e., $\lambda_1 = \lambda_2$ and $\lambda_2 \cong 2\lambda_{1f}$) is more practical.

The two-photon absorption cross section for biological molecules σ_2 is typically very small (approximately 1 GM $= 10^{-58}$ $m^4 s^{-1}$), so intense photon fluxes on the order of 10^{30} photons per second per square meter ($s^{-1}m^{-2}$) are required. Pulsed excitation (approximately 10^{-13} s), which allows for reduction of the heat load on the tissue and selective excitation of individual electronic transitions of biological molecules, is preferable.

For a pulse laser with repetition rate $f_p = 1/T$ and a duration of the rectangular pulses τ_p, the time-averaged intensity of the TPEF is expressed as[13,89]

$$\left\langle \hat{I}_{2f} \right\rangle_p = \kappa \pi \sigma_2 \eta \frac{P_{ave}^2}{\tau_p f_p} \left[\frac{(NA)}{hc\lambda_{exc}} \right]^2, \tag{I.17}$$

where κ is the coefficient taking into account the collection efficiency of the fluorescent photons, $\eta = \eta(\lambda_{em})$ is the fluorescence quantum yield, $P_{ave} = (\tau_p{:}f_p)P_{peak}$ is the average power, P_{peak} is the peak power, and NA is the numerical aperture of the microscope objective.

It follows from Eq. (I.17) that the excitation of fluorescence emission by a pulse laser with a wavelength $\lambda = 1000$ nm, an average power of 1 mW at a repetition rate of 80 MHz, and a pulse duration of 100 fs, focused by the

objective with NA = 1.4 onto the tissue with a typical two-photon cross section $\sigma_2 = 10$ GM $= 10^{-57}$ M^4c^{-1} provides a rate of fluorescence photon counting equal to 10^5 Hz.

Three-photon fluorescence microscopy of a tissue possesses the same advantages as two-photon microscopy but ensures a somewhat higher spatial resolution and provides an opportunity to excite chromophores with shorter wavelengths.

I.4 Second-Harmonic Generation (SHG)

Second-harmonic generation (SHG) is a new high-resolution nonlinear optical imaging modality for study of intact tissues and cellular structures.[13,92–94] SHG is a second-order nonlinear optical process that can arise only from media lacking a center of symmetry—for example, an anisotropic crystal or at an interface such as a membrane. This technique can be used to image highly ordered structural proteins without any exogenous labeling. Collagen is a main component of connective tissues due to its helix secondary structure, which is noncentrosymmetric; it has a dominant uniaxial second-order nonlinear susceptibility component aligned along the fiber axis and, therefore, satisfies the conditions for SHG. In the skin, SHG light is generated mostly within the dermis, rather than in cellular layers such as the epidermis or subcutaneous fat.

SHG techniques offer a number of advantages connected with the incident wavelength's division and its selectivity to tissue structure, which allow one to reject surface reflection and multiple scattering of the incident light in the upper epithelial layer without any gating technique. As in the case of multi-photon excited fluorescence, SHG arises from a very small tissue volume within a focal volume of the sharply focused NIR laser beam. As a result, it provides a high spatial resolution, in-depth probing, and separation of excitation and detection signals. In spite of the high power density in the focal spot, a very short pulse (50–200 fs) allows for generation of harmonics in the living tissue with no damage to it due to the low overall energy.[13,92–94]

In general, the nonlinear polarization for a material can be expressed as[93]

$$\mathbf{P} = \chi^{(1)}\mathbf{E} + \chi^{(2)}\mathbf{EE} + \chi^{(3)}\mathbf{EEE} + \ldots, \tag{I.18}$$

where $\mathbf{P}$ is the induced polarization, $\chi^{(n)}$ is the nth order nonlinear susceptibility, and $\mathbf{E}$ is the electric field vector of the incident light. The first term describes normal absorption and reflection of light; the second describes SHG, sum, and difference frequency generation; and the third describes both two- and three-photon absorption, as well as third-harmonic generation and coherent anti-Stokes Raman scattering (CARS).

SHG, unlike two-photon fluorescence, does not arise from an absorptive process. Instead, an intense laser field induces a nonlinear, second-order

polarization in the assembly of molecules, resulting in the production of a coherent wave at exactly twice the incident frequency (or half the wavelength).

The SHG pulse is temporally synchronous with the excitation pulse. A simplified expression for the SHG signal intensity has the form[93]

$$I(2\omega) \propto \left[\chi^{(2)} \frac{E(\omega)}{\tau_p}\right] \tau_p, \tag{I.19}$$

where $E(\omega)$ is the laser pulse energy. As in TPEF [see Eq. (I.17)], the signal is quadratic with peak power, but since SHG is an instantaneous process, a signal is generated only for the duration of the laser pulse.

I.5 Vibrational Spectroscopy

Middle-infrared (MIR) and Raman spectroscopies use light-excited vibrational energy states in molecules to obtain information about the molecular composition, structures, and interactions in a sample.[40–45,95,96] In MIR spectroscopy, infrared light from a broadband source (usually 2.5–25 μm or 4000–400 cm^{-1}) is directly absorbed to excite the molecules to higher vibrational states. In Raman scattering, event light is inelastically scattered by a molecule when a small amount of energy is transferred from the photon to the molecule (or vice versa). The energy difference between incident and scattered photons is expressed in a wavenumber shift (cm^{-1}).

The MIR and Raman spectroscopy techniques have been successfully applied to various areas addressed by clinical studies, such as cancerous tissues examination, the mineralization process of bone and teeth, tissues monitoring, glucose sensing in blood, noninvasive diagnosis of skin lesions based on benign or malignant cells, and monitoring of treatments and topically applied substances (e.g., drugs, cosmetics, moisturizers) to the skin.[41–45,95–102]

Raman spectroscopy is widely used in biological studies, ranging from studies of purified biological compounds to investigations at the level of single cells.[40,96] At present, combinations of spectroscopic techniques such as MIR and Raman spectroscopy with microscopic imaging techniques are being explored to map molecular distributions at specific vibrational frequencies on samples so as to locally characterize tissues or cells.[95–99] Chemical imaging is expected to become increasingly more important in clinical diagnosis in the future.

Because the penetration depth of MIR light in tissue extends to only a few micrometers, the attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) method is well suited to study changes of the outermost cell layers of the tissue.[95]

The Raman technique exhibits certain characteristics that make it particularly suitable for studying the skin, both *in vitro* and *in vivo*.[45,95,100]

Confocal detection is particularly useful to studying the outer skin layers, such as the stratum corneum and viable epidermis. Since the dermis is much thicker than the epidermis (1–4 mm thick), it can easily be studied using a non-confocal detection scheme, given a detection volume that is large compared to the thickness of the epidermis.[95]

I.6 Coherent Anti-Stokes Raman Scattering (CARS)

Coherent anti-Stokes Raman scattering (CARS) is a third-order nonlinear optical process in which three excitation fields interact to produce a fourth field, which is detected [see Eq. (I.18)].[13,98,99] In general, two laser beams with frequencies ν_{pump} and ν_{S} are tuned to get their difference ($\nu_{\text{pump}} - \nu_{\text{S}}$) to be equal to the frequency ν_{vib} of a vibrational transition of the sampling molecules. Then the probing laser beam with frequency ν_{probe} generates resonantly a fourth enhanced field with frequency $\nu_{\text{AS}} = (\nu_{\text{pump}} + \nu_{\text{vib}})$. Typically only two laser beams are used to generate CARS signal, because a so-called frequency-degenerate optical scheme with $\nu_{\text{pump}} = \nu_{\text{probe}}$ can be applied.

The intensity of CARS signal depends quadratically on the modulus of the induced third-order polarization $\mathbf{P}^{(3)}$ in the sample [see Eq. (I.18)]

$$I_{AS} \propto |\mathbf{P}^{(3)}|^2, \tag{I.20}$$

where $\mathbf{P}^{(3)}$ depends on the third-order optical susceptibility that can be presented as a sum of the nonresonant and resonant contributions

$$\mathbf{P}^{(3)} = \left[\chi^{(3)}_{\text{nonres}} + \chi^{(3)}_{\text{res}}\right]\mathbf{E}_{\text{pump}}\mathbf{E}_{\text{prob}}\mathbf{E}_{s} \tag{I.21}$$

The main advantages of CARS compared to conventional Raman spectroscopy, besides the opportunity to amplify the signal by more than four orders of magnitude, are the direct signal generation, narrow band, and complete absence of the influence of autofluorescence as the signal is generated at wavelengths shorter than the wavelength of excitation. Since three nonlinear methods—CARS, TPEF, and SHG—are technically implemented using the similar experimental equipment, they are often used together as part of a multimodal approach to obtain more information about the fundamental processes in tissues and cells.[13,98,99,103,104]

I.7 Light-Scattering Spectroscopy

Based on classical measurements of light scattering, innovative techniques capable of identifying and characterizing pathological changes in human tissues at the cellular and subcellular levels have been proposed.[13,46,58,105–109] Light-scattering spectroscopy (LSS) provides structural and functional

information about a tissue. This information can be used, in turn, to diagnose and monitor disease. One important application of biomedical spectroscopy is the noninvasive detection of early cancerous human epithelium.[46,106,109] The enlarging, crowding, and hyperchromaticity of the epithelium cell nuclei are common features to all types of precancerous and early cancerous conditions. LSS can be used to detect such early cancerous changes and other diseases in a variety of organs, such as the esophagus, colon, uterine cervix, oral cavity, lungs, and urinary bladder.[109]

Cells and tissues have complex structures with a very broad range of scatterer size—from a few nanometers, the size of a macromolecule; to 7–10 μm, the size of a nucleus; and to more than 20–50 μm, the size of a cell itself.[13,106] A great variety of cell organelle structures are small compared to the wavelength used in LSS. Light scattering by such particles, which is known as Rayleigh scattering, is characterized by a broad angular distribution; the scattering cross-section dependence on the particle's linear dimension a is characterized as a^6 and that on the light wavelength λ is characterized as λ^{-4}. When the particle is not small enough, coupled dipole theory or another approach such as Rayleigh–Gans approximation (RGA) can be used. RGA is particularly applicable to particles with a size comparable to the wavelength and may be useful for studying light scattering by small organelles such as mitochondria and lysosomes. With use of RGA, scattering in the forward direction prevails, and the total scattering intensity increases with the increase in the particle relative refractive index m as $(m-1)^2$ and with its size as a^6.

The scattering by a particle with dimensions much larger than the wavelength, such as a cell nucleus, can be described within the framework of the van de Hulst approximation, which enables scattering amplitudes in the near-forward direction to be obtained.[106] For large particles, the scattering intensity is highly forward directed, and the width of the first scattering lobe is approximately λ/a; the larger the particle, the stronger and narrower the first lobe. The forward scattering intensity exhibits oscillations with the wavelength. The origin of these oscillations is interference between the light ray passing through the center of the particle and a light ray not interacting with it. The frequency of these oscillations is proportional to $a(m-1)$, so it increases with the particle size and refractive index. The intensity of the scattered light also peaks in the near-backward direction, but this peak is significantly smaller than the forward-scattering peak.

These results agree well with the rigorous scattering theory developed for spherical particles (Mie theory).[110] To discriminate among the cell structure peculiarities originating from a pathology, the difference in light scattering can be used. The structures with large dimensions and high refractive index produce the scattered field that peaks in the forward and near-backward

directions, whereas smaller and more optically "soft" structures scatter light more uniformly.

The photons returned after a single scattering in the backward or near-backward directions produce the so-called single-scattering component. The photons returned after multiple scattering events produce the diffuse reflectance. Although the spectra of both single-scattering and diffusive signals contain valuable information about tissue properties, the type of information they provide is different. The single-scattering component is sensitive to morphology of the upper tissue layer, which in case of any mucosal tissue almost always includes or is limited by the epithelium. Its spectroscopic features are related to the microarchitecture of the epithelial cells—that is, the sizes, shapes, and refractive indices of their organelles, inclusions, and sub-organellar components. Thus, analysis of this component might be useful in diagnosing diseases limited to the epithelium, such as preinvasive stages of epithelial cancers, dysplasias, and carcinomas *in situ* (CIS).[46,106,109]

The single-scattering component is more important in diagnosing the initial stages of epithelial precancerous lesions, whereas the diffusive component provides valuable information about more advanced stages of the disease. However, single scattering events cannot be directly observed in tissues *in vivo*, because only a small portion of the light incident on the tissue is directly backscattered.

Several methods to distinguish single scattering have been proposed. Field-based light-scattering spectroscopy[111] and optical coherence tomography (OCT)[13,112] were developed for performing cross-sectional tomographic and spectroscopic imaging. In these extensions of conventional OCT,[13] information on the spectral content of backscattered light is obtained by detection and processing of the interferometric OCT signal. These methods allow the spectrum of backscattered light to be measured either for several discrete wavelengths[111] or simultaneously over the entire available optical bandwidth from 650 to 1000 nm[112] in a single measurement.

A much simpler polarization-sensitive technique is based on the fact that initially polarized light loses its polarization when traversing a turbid tissue.[113,114] A conventional spatially resolved backscattering technique with a small source–detector separation can be used as well.[46] In that case, however, the single scattering component (2–5%) should be subtracted from the total reflectance spectra.

The promise of LSS for diagnosing dysplasia and CIS was tested in human studies in three different types of *in vivo* epithelium: columnar epithelia of the colon and Barrett's esophagus, transitional epithelium of the urinary bladder, and stratified squamous epithelium of the oral cavity.[109] The spectrum of the reflected light was analyzed to determine the nuclear size distribution. In all studied organs, a clear distinction was apparent between

dysplastic and nondysplastic epithelium. Both dysplasia and CIS have a higher percentage of enlarged nuclei and, on average, a higher population density—characteristics that can be used as the basis for spectroscopic tissue diagnosis.[109]

I.8 Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT) was first demonstrated in 1991.[115] Imaging was performed *in vitro* in the human retina and in atherosclerotic plaque as examples of imaging in transparent, weakly scattering media as well as highly scattering media. This is an urgent field of research, with applications attracting more and more end-users. State-of-the-art monographs, tutorials, and special issues of journals describing principles and biomedical applications of OCT are widely available.[13,51,116–131]

OCT is analogous to ultrasonic imaging that measures the intensity of reflected NIR light, rather than reflected sound waves from the sample. Time gating is employed so that the time required for the light to be reflected back, known as the echo delay time, can be used to assess the intensity of backreflection as a function of depth. Unlike in ultrasound, the echo time delay, which is on an the order of femtoseconds in optics, is measured by using an optical interferometer illuminated by a low coherent light source.

This technique is conventionally implemented with the use of a dual-beam Michelson interferometer. If the path length of light in the reference arm is changed with a constant linear speed v, then the signal arising from the interference between the light scattered in a backward direction (reflected) from a sample and the light in the reference arm is modulated at the Doppler frequency

$$f_D = \frac{2v}{\lambda}. \tag{I.22}$$

Owing to the small coherence length of a light source, the Doppler signal is produced by backscattered light only within a very small region (on the order of the coherence length l_c) corresponding to the current optical path length in the reference arm. For the light source with a Gaussian line profile

$$l_c = \frac{2\ln(2)}{\pi} \cdot \frac{\lambda^2}{\Delta\lambda}, \tag{I.23}$$

where $\Delta\lambda$ is the Gaussian line bandwidth.

If a superluminescent diode (SLD) with a bandwidth of 15–60 nm ($\lambda \approx$ 800–860 nm) is employed, the longitudinal resolution falls within the range of 5–20 μm. For a titanium–sapphire laser with a wavelength of 820 nm, the bandwidth may reach 140 nm. Correspondingly, the resolution is 2.1 μm.[116]

Transverse resolution of OCT is defined by a light beam spot, which is typically from 5 to 20 μm.

In the literature, one can find descriptions of several different OCT systems, ranging from conventional amplitude or time-domain OCT to advanced systems combining, for example, spectral-domain OCT (SD-OCT) with multi-photon tomography (MPT) for 3D multimodal *in vivo* imaging.[13,51,112,115–131] Time-domain OCT is a single-point detection technique. It can be used to generate two-dimensional OCT images up to the video rate, although such systems have a limited sensitivity or a limited space–bandwidth product (resolved pixels per dimension). For some applications, two-wavelength fiber OCT is effective. Ultrahigh-resolution fiber OCT systems are also available. Frequency- and Fourier-domain OCT techniques are based on backscattering spectral interferometry and, therefore, are also called spectral-domain OCT (SD-OCT). Such systems are widely used in biomedical studies and in clinics. Doppler OCT (DOCT) combines the Doppler principle with OCT to obtain high-resolution tomographic images of static and moving constituents in highly scattering tissues. Optical microangiography (OMAG) is an OCT technique that utilizes a constant modulation frequency to separate the signal associated with the movement in the RBS vascular bed from the backscatter signal. Correlation-map OCT (cmOCT) applies two-dimensional OCT images to reconstruct blood vessel distribution within the skin.

The specificity of conventional OCT can be improved by providing measurements of polarization properties of probing radiation when it propagates through a tissue. This approach was implemented in the polarization-sensitive OCT technique (PS-OCT). In its turn, phase-sensitive OCT (PhS-OCT) provides quantitative dispersion data that are important in predicting the propagation of light through tissues, in photorefractive surgery, and in tissue and blood refractive index measurements. PhS-OCT systems are often used in tissue elastography. Indeed, the prospective technique called optical coherence elastography (OCE) takes advantage of high-resolution OCT to provide quantitative evaluation of a tissue's mechanical properties.

Full-field or parallel OCT (FF-OCT) uses linear or two-dimensional detector arrays of, respectively, N and N^2 single detectors. The advantage of parallel OCT is that when using linear or 2-D detector arrays, the SNR can be roughly $\sqrt{N}$ and N times larger, respectively, compared to the single detector signal.

Optical coherence microscopy (OCM) is a biomedical modality for cross-sectional subsurface imaging of tissue that combines the ultimate sectioning abilities of OCT and confocal microscopy (CM). In OCM, spatial sectioning due to tight focusing of the probing beam and pinhole rejection provided by CM is enhanced by additional longitudinal sectioning provided by OCT coherence gating.

Application of the fiber-optic light-delivering and light-collecting cables allows one to build a flexible OCT system that facilitates endoscopic analysis of human tissues and organs—in particular, high-speed *in vivo* intra-arterial imaging. The feasibility of OCT ultrathin needle probes for imaging of breast cancer, dystrophic skeletal muscles, tendons, connective tissues, and air-filled lungs has already been demonstrated.

The speckle OCT method has been shown to be a viable alternative to the Doppler OCT in 2D imaging of blood flow. Flow information can be extracted using speckle fluctuations in conventional time-domain OCT. One optical coherence elastography (OCE) technique that takes advantage of the high resolution of OCT and the high sensitivity of speckles is based on speckle tracking; it has been widely studied to evaluate the skin's mechanical properties both qualitatively and quantitatively.

One important advance related to OCT systems is the combination of this technology with other optical diagnostic modalities so as to achieve a synergetic effect in diagnostic ability. Technically this combination could be realized by using miniature fiber optical probes, shared light sources, optical pathways and scanning systems, and so on. In fact, dual OCT/confocal microscopy (CM) systems have already been described. A dual imaging en face OCT/CM system was used in ophthalmology for imaging of the anterior chamber of the eye and in dentistry. The combination of dual en face OCT/CM with fluorescence imaging gives a universally applicable instrument in microscopy. All-fiber-optic-based endoscopy for simultaneous OCT and fluorescence tissue imaging provides clear visualization of structural morphologies (OCT) and fluorophore distribution (the fluorescence module). Multi-photon tomography (MPT) and SD-OCT can be used for 3D multimodal *in vivo* imaging of normal skin, nevi, scars, and pathologic skin lesions. Photothermal OCT (PT-OCT) using gold nanorods (GNRs) as contrast agents has been shown to be a potentially powerful tool for molecular imaging. Adaptive optics-assisted OCT is currently under development and holds promise for subcellular imaging in biology and medicine.

I.9 Dynamic Light-Scattering Spectroscopy and Tomography

I.9.1 Photon-correlation spectroscopy

Quasi-elastic light-scattering spectroscopy (QELSS), photon-correlation spectroscopy, spectroscopy of intensity fluctuations, and Doppler spectroscopy are synonymous terms for technologies based on the dynamic scattering of light—a capability that underlies a noninvasive method for studying the dynamics of particles on a comparatively large time scale.[13,14,47–51] The implementation of the single-scattering mode and the use of coherent light sources are of fundamental importance in this case. The spatial scale of testing

for an ensemble of biological particles is determined by the inverse of the wave vector $|\bar{s}|^{-1}$:

$$|\bar{s}| = (4\pi n/\lambda_0)\sin(\theta/2), \tag{I.24}$$

where n is the refractive index and θ is the angle of scattering. With allowance for self-beating due to the photomixing of the electric components of the scattered field on a photodetector, the intensity autocorrelation function can be expressed as $g_2(\tau) = \langle I(t)I(t+\tau)\rangle$ For Gaussian statistics, this autocorrelation function is related to the first-order autocorrelation function by the Siegert formula:

$$g_2(\tau) = A\left[1 + \beta_{sb}|g_1(\tau)|^2\right], \tag{I.25}$$

where τ is the delay time; $A = \langle i\rangle^2$ is the square of the mean value of the photocurrent, or the baseline of the autocorrelation function; β_{sb} is the parameter of self-beating efficiency, $\beta_{sb} \approx 1$; and $g_1(\tau) = \exp(-\Gamma_T\tau)$ is the normalized autocorrelation function of the optical field for a monodisperse system of Brownian particles. $\Gamma_T = |\bar{s}|^2 D_T$ is the relaxation parameter and $D_T = k_B T/6\pi\eta r_h$ is the coefficient of translation diffusion, k_B is the Boltzmann constant, T is the absolute temperature, η is the absolute viscosity of the medium, and r_h is the hydrodynamic radius of a particle. Many biological systems are characterized by a bimodal distribution of diffusion coefficients, when fast diffusion (D_{Tf}) can be separated from slow diffusion (D_{Ts}) related to the aggregation of particles. The goal of QELSS is to reconstruct the distribution of scattering particles by sizes, which is necessary for the diagnosis or monitoring of a disease.

The homodyne and heterodyne photon-correlation spectrometers, the laser Doppler anemometers (LDAs), differential LDA schemes, and laser Doppler microscopes (LDMs), and laser scanning and speckle CMOS-based full-field imagers have a wide area of medical applications. In particular, they have been used to investigate eye tissues (cataract diagnosis), hemodynamics in individual vessels (vessels of eye fundus), and blood microcirculation in tissues.[13,47–51,123–126,132–139]

I.9.2 Diffusion wave spectroscopy/diffuse correlation spectroscopy

Diffusion wave spectroscopy (DWS) and diffuse correlation spectroscopy (DCS) are dynamic light scattering techniques related to the investigation of the dynamics of particles within very short time intervals.[8,13,48,49,51,140–145] A fundamental difference between these techniques and QELSS is that DWS and DCS are applicable in the case of dense media with multiple scattering, which is critical for tissues. In contrast to the case of single scattering, the autocorrelation function of the optical field $g_1(\tau)$ is sensitive to the motion of a particle on the length scale on the order of $\lambda[L/l_{tr}]^{-1/2}$, which is generally much less than λ because $L >> l_{tr}$ (L is the total mean photon path length and l_{tr} is

the transport length of a photon, $l_{\text{tr}} = 1/(\mu_a + \mu_s\prime) \approx 1/\mu_s'$). Thus, DWS/DCS autocorrelation functions decay much faster than the autocorrelation functions employed in QELSS.

Experimental implementation of DWS/DCS is very simple. A measuring system should provide irradiation of an object under study by a CW laser beam and measurement of intensity fluctuations of the scattered radiation within a single speckle with the use of a single-mode receiving fiber, photomultiplier, photon-counting system, and a fast digital correlator working in the nanosecond range.[8,13,140–145] The use of the DWS/DCS technique in medical applications has been demonstrated for blood microcirculation monitoring in the human forearm, skeletal muscle, and brain.[140–145] The autocorrelation function slope is the indicative parameter for determination of the blood flow velocity. The normalized autocorrelation function of field fluctuations can be represented in terms of two components related to the Brownian and directed motion of scatterers (erythrocytes or lymphocytes):

$$g_1(\tau) \approx \exp\{-2[\tau/\tau_{\text{B}} + (\tau/\tau_{\text{s}})^2]L/l_{tr}\}, \tag{I.26}$$

where $\tau_{\text{B}}^{-1} = |\bar{s}|^2 D_{\text{B}}$ characterizes Brownian motion as $\tau_s^{-1} \cong 0.18 G_{\text{V}}|\bar{s}|l_{\text{tr}}$ (the directed flow), and G_{V} is the gradient of the flow rate. In Eq. (I.26), directed flow dependent on τ^2 is compared to the τ dependence for Brownian motion because particles in flows travel ballistically; also τ_{B} and τ_{S} appear separately because the different dynamical processes are uncorrelated.

I.10 Optothermal Spectroscopy and Tomography

I.10.1 Optothermal interactions

The optothermal (OT) or photothermal (PT) method detects the time-dependent heat generated in a tissue via interaction with pulsed or intensity-modulated optical radiation.[13,52–54,146–155] The thermal waves generated by the release of heat result in several effects that have given rise to various imaging techniques: optoacoustics (OA) and photoacoustics (PA); optothermal radiometry (OTR) and photothermal radiometry (PTR); and photorefractive techniques.[13,52–54,146–155] In the past, the term "optoacoustics" was used to refer primarily to the time-resolved technique utilizing pulsed lasers and measuring profiles of pressure in tissue, and the term "photoacoustics" primarily described spectroscopic experiments with CW-modulated light and a photoacoustic cell. Nowadays, the term "photoacoustics" is much more frequently used for time-resolved techniques. The informative features of the PA method allow one to estimate tissue thermal, optical, and acoustical properties, which depend on tissue structure peculiarities.

In PA techniques, microphone or piezoelectric transducers, which are in acoustic contact with the sample, are used as detectors to measure the

amplitude or phase of the resultant acoustic wave. In the PTR technique, distant IR detectors and array cameras are employed for estimation and imaging of the sample surface temperature. The intensity of the signals obtained with any of the PT or PA techniques depends on the amount of energy absorbed and transformed into heat as well as on the thermo-elastic properties of the sample and its surrounding. When nonradiative relaxation is the main process in a light beam decay and extinction is not very high, $\mu_a d << 1$ (d is the length of a cylinder within the sample occupied by a pulse laser beam), the absorbed pulse energy induces the local temperature rise, which is defined by

$$\Delta T \cong E\mu_a d/c_p V\rho, \tag{I.27}$$

where c_P is the specific heat capacity for a constant pressure, $V = \pi w^2 d$ is the illuminated volume, w is the laser beam radius, and ρ is the medium density. Supposing there is an adiabatic expansion of the illuminated volume upon being heated at a constant pressure, one can calculate the change of the volume ΔV. This expansion induces a wave propagating in the radial direction at the sound speed, v_a. The corresponding change of pressure Δp is proportional to the amplitude of mechanical oscillations

$$\Delta p \approx (f_a/w)(\beta v_a/c_P)E\mu_a, \tag{I.28}$$

where β is the coefficient of volumetric expansion and f_a is the frequency of the acoustic wave.

Equations (I.27) and (I.28) present principles of various PT and PA techniques. The information about the absorption coefficient μ_a at the selected wavelength can be received from direct measurements of the temperature change ΔT (optical calorimetry), volume change ΔV (optogeometric technique), or pressure change Δp (PA technique).

I.10.2 PA technique

For a highly scattering tissue, measurement of the stress-wave profile and amplitude should be combined with measurement of the total diffuse reflectance so as to extract separately both the absorption and scattering coefficients of the sample. The absorption coefficient in a turbid medium can be estimated from the acoustic transient profile only if the subsurface irradiance is known. For the turbid media irradiated with a wide laser beam (more than 0.1 mm), backscattering causes a higher subsurface fluence rate compared with the incident laser fluence.[13] Therefore, the z-axial light distribution in tissue and the corresponding stress distribution have a complex profile with a maximum at a subsurface layer. In contrast, when the heating process is much faster than the medium expansion, then the stress amplitude adjacent to the irradiated surface $\delta p(0)$ and the stress exponential tail into the depth of tissue sample $\delta p(z)$ can be found.[146,147,151] The stress is confined

temporarily during laser heat deposition when the laser pulse duration is much shorter than the time of stress propagation across the light penetration depth in the tissue sample. Such conditions of temporal pressure confinement in the volume of irradiated tissue allow for the most efficient pressure generation.

I.10.3 PTR technique

The pulse laser heating of a tissue causes perturbations of its temperature and corresponding modulation of the thermal (infrared) radiation. This pair of reactions is the basis for pulse photothermal radiometry (PTR).[13,52,54,153–155] The maximum intensity of living objects' thermal radiation approximates a wavelength of 10 μm. A detailed analysis of PTR signal formation requires knowledge of the internal temperature distribution within the tissue sample, tissue thermal diffusivity, and the absorption coefficients at the excitation μ_a and emission μ_a' (10 μm) wavelengths. And, working backward, knowledge of some of the previously mentioned parameters allows one to use a measured PTR signal to reconstruct, for example, the depth distribution of μ_a.

The pulse PTR method holds much promise for the study of the optical and thermal properties of tissues *in vitro* and *in vivo*.[13,52–54,153–155] For example, sequences (pairs) of infrared emission images recorded following pulsed laser irradiation have been used to determine the thermal diffusivity of biomaterials with high precision.[155]

I.11 Conclusion

Since publication of the first edition of the *Handbook* in 2002, optical methods for biomedical diagnostics have been further developed in many well-established, now-traditional directions, which were first summarized in the first edition. In addition, new trends have appeared. In recent years, a number of handbooks, textbooks, and special issues of journals have been published that are good companions to the coverage of topics provided in the second edition of the *Handbook*.[13,83,90,92,93,105,114,117,120,124,126,147,148,156–209]

The comprehensive *Biomedical Photonics Handbook* by Vo-Dinh covers many topics related to optical biomedical diagnostics, based on a variety of light–tissue, light–cell, and light–molecular interaction phenomena; it also includes descriptions of biosensing approaches.[159] The second edition of *Tissue Optics* by Tuchin mostly concentrates on the optics of soft and hard tissues, characterization and control of their optical properties, and light-scattering and coherent-domain methods for biomedical spectroscopy and imaging.[13]

As an introduction to the field of biomedical optics and biophotonics, three very enjoyable textbooks by Prasad,[161] Wang and Wu,[147] and Splinter and Hooper,[165] containing a number of actual examples, problems, and questions for students, might be recommended.

Optical coherence tomography is a new trend in biomedical diagnostics that is continually delivering novel modalities with better facilities in image resolution and real-time imaging.[13,117,119,120,126] The first book on OCT by Bouma and Tearney,[117] a comprehensive review paper by Fercher et al.,[119] the second volume of Tuchin's two-volume monograph,[126] the most recent exciting OCT book by Drexler and Fujimoto,[120] and the special issue of *Journal of Biomedical Optics* (*JBO*) edited by Larin et al.[131] summarize and analyze the cutting-edge OCT technologies and their biomedical applications. A brief overview of OCT fundamentals, techniques, and applications is provided by Tuchin.[13]

Another important trend in optical biomedical diagnostics is application of polarized light for tissue characterization and imaging.[114,164,178–180] The importance of the problem was underlined by the publication of a special issue of *JBO* devoted to this topic and edited by Wang et al.,[114] a monograph by Tuchin et al.,[164] and a tutorial paper by Ghosh and Vitkin.[179] Discussions of polarized light's interaction with tissues and applications for diagnostic purposes, in particular for glucose sensing, are presented in monographs by Tuchin.[13,101,177,178]

Further developments of multi-photon excitation microscopy and other methods of nonlinear microscopy are discussed in the handbook by Masters and So,[90] a book edited by Pavone,[92] and a book on SHG imaging by Pavone and Campagnola.[93]

Trends in nanobiophotonics as a novel synergetic science underlying diagnosis, prevention, and treatment of diseases including cancer, systematic conditions, and inflammatory diseases are overviewed in the special section of *JBO* edited by Tuchin et al.[171] and in a collective monograph edited by Tuchin.[177]

In vivo flow cytometry[181–185] and noninvasive blood flow imaging[123–125] in tissues are important directions for research in biomedical diagnostics. A few overview papers, book chapters, and books are available on blood flow imaging, published by Leahy and his group.[123–125]

In the previous edition of the *Handbook*, a lot of attention was paid to the characterization of optical properties of biological tissues using innovative approaches for inverse problem solution. Recent work on this front includes the collection of new experimental data and their critical analysis for different tissues in a wide spectral range.[186–191]

A comprehensive presentation of fundamentals, basic research, and medical application of biophotonics is provided in a three-volume monograph edited by Popp et al.[192–194] Some practical aspects of optical biomedical diagnostics and treatments, especially those targeting skin disease and cosmetology, are discussed in books by Wilhelm et al.,[166] Ahluwalia,[168] Baron,[172] and Querleux.[203] Photonics for solution of specific dental problems is covered in Kishen and Asundi's book.[163] All aspects of glucose noninvasive optical sensing and its impact on tissues are analyzed in the book by Tuchin.[101]

Tissue optical clearing has emerged as a hot topic in the field of optical biomedical diagnostics owing to the considerably enhanced ability of different optical methods to suppress light scattering.[13,210–240] The book on tissue and blood optical clearing by Tuchin,[212] many recent review papers,[210,211,213,216,218,219,223,240] several book chapters,[215,217,220,221,224] and a special section of *JBO*[214] are devoted to this growing area of research and the applications of temporal (reversible) control of tissue optical properties using immersion clearing. Mechanical compression and stretching are also prospective tools to improve optical imaging and diagnostics as well as the therapeutic abilities of light.[13,212,240–266]

Very recently, a brilliant textbook on *Quantitative Biomedical Optics* (which, I believe, students will accept with enthusiasm) was published by Bigio and Fantini.[267] Three more special sections of journals on urgent problems of biomedical optics and biophotonics, with a large number of papers related to optical biomedical diagnostics and specifically to polarization and optical clearing methods, were issued.[268–270]

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Valery V. Tuchin is a professor and chairman of Optics and Biophotonics at National Research Saratov State University. He is also the head of laboratory at the Institute of Precision Mechanics and Control, RAS, and the supervisor of the Interdisciplinary Laboratory of Biophotonics at National Research Tomsk State University. His research interests include biophotonics, tissue optics, laser medicine, tissue optical clearing, and nanobiophotonics. He is a member of SPIE, OSA, and IEEE, guest professor of HUST (Wuhan) and Tianjin Universities of China, and adjunct professor of the Limerick University (Ireland) and National University of Ireland (Galway). He is a fellow of SPIE and OSA, and has been awarded the Honored Science Worker of the Russia, SPIE Educator Award, FiDiPro (Finland), Chime Bell Prize of Hubei Province (China), and Joseph W. Goodman Book Writing Award (OSA/SPIE).