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Optical properties of tissues quantified using morphological granulometry from phase-contrast images of thin tissue samples

Zhifang Li^a, Haiyu Chen^b, Hui Li^{a,*}, and Wei R. Chen^{a,c,*}

^aFujian Provincial Key Laboratory of Photonic Technology, Key Laboratory of Optoelectronic Science and Technology for Medicine, College of Photonic and Electronic Engineering, Fujian Normal University, Ministry of Education, Fuzhou, Fujian, China

^bDepartment of Cardiovascular Surgery, Fujian Provincial Hospital, Fuzhou, Fujian, China

^cBiophotonics Research Laboratory Department of Engineering and Physics, University of Central Oklahoma, Edmond, OK, USA

Abstract

A method combining morphological granulometry with Mie theory to determine optical scattering in biological tissues was proposed. Otsu's method was applied to binarize phase-contrast images. Binary morphological granulometry was used to estimate size density distribution of the tissue samples based on the binary phase-contrast images. Our results showed that the optical parameters associated with light scattering in tissue could be quantitatively determined by combining size density distribution with Mie theory. It was suggested that this unique method could be used to characterize biological tissues for disease diagnosis.

Keywords

Morphological granulometry; optical scattering properties; Mie theory

1. Introduction

Light scattering is an important property in tissue optics. Currently, there are different techniques to determine scattering parameters in bulk tissues, such as integrating spheres [1] and optical coherence tomography [2]. During the past several years, considerable attention has been devoted to the development of new methods to determine subcellular optical scattering properties, including quantitative phase imaging [3,4] and spatial light interference microscopy [5,6], based on phase-sensitive characteristics to directly extract the refractive index of cells. Their optical scattering properties are quantified by Fourier-transform light scattering of the phase images [7]. Recently, we proposed the grayscale morphological granulometry for phase-contrast image of tissue to extract the size

*Corresponding authors: Hui Li, Fujian Provincial Key Laboratory of Photonic Technology, Key Laboratory of Optoelectronic Science and Technology for Medicine, College of Photonic and Electronic Engineering, Fujian Normal University, Ministry of Education, Fuzhou 350007, Fujian, China. Tel.: +86 591 2286 8133; hli@fjnu.edu.cn. Wei R. Chen, Biophotonics Research Laboratory Department of Engineering and Physics, University of Central Oklahoma, Edmond, OK 73034, USA. Tel.: +1 405 974 5147; wchen@uco.edu.

distribution for scattering properties [8]. However, the size distribution can be easily influenced by noise based on grayscale morphological granulometry.

In this work, we used a phase-contrast microscope for acquiring the grayscale phase-contrast images of tissues. Then Otsu's method was used to binarize the grayscale phase-contrast images. Furthermore, morphological granulometry was employed to extract the particle size density distribution of the binary phase-contrast images. Finally, the particle size density distribution was combined with Mie theory to determine optical scattering coefficient of tissues, such as rat lung, liver and myocardium.

2. Methods and samples

2.1. Image preprocessing and morphological granulometry

Biological tissues are complex media and many of their textures show a multi-scale feature. Multiscale discrete particle model can provide an approximation of the complicated spatial refractive-index variations within bulk tissue. Two-dimensional fluctuations of refractive indices can be estimated from measurements of spatial variations in the optical phase, $\delta\phi$, of a beam transmitted through a thin slice of tissue. If the phase differences arise from statistically isotropic, inhomogeneous medium with different sizes but with the same variance of refractive indices δn^2 , the ensemble averaged variance is proportional to δn^2 , $\langle \delta\phi^2 \rangle \propto \delta n^2 df(d)$ [9], where $f(d)$ is the size density distribution of the inhomogeneous medium with a transverse dimension d .

Comparing the phase variance theory of Schmitt and Kumar [9] with Otsu's method, we find that there is a similarity in both methods. In the phase-contrast image, the phase variance is equal to the gray variance. The algorithm of Otsu's method [10] assumes that the image to be thresholded may contain two classes of pixels or bi-modal histogram (e.g. object and background) and it calculates the optimal threshold by separating the two classes. Otsu's method searches for the threshold that maximizes the inter-class variance, defined as a weighted sum of variances of the two classes: $\sigma_w^2(t) = \omega_1(t)\sigma_1^2(t) + \omega_2(t)\sigma_2^2(t)$ weights ω_i are the probability of the two classes separated by a threshold t and variances σ_i^2 of these classes. [10]

The grayscale images are transformed into binary images by Otsu's thresholds. The binary image is in terms of the size distribution with the same index (Intensity value is 1 in binary image) relative to that of background (Intensity value is 0 in binary image). Thus, the binary image can be seen as an equivalent discrete particle randomly distributed in tissue slice.

The equivalent size distribution could be acquired using morphological granulometry on the binary phase-contrast image. Mathematical morphology is a branch of modern mathematics. It is most commonly applied to digital images, but it can be employed as well on graphs, surface meshes, solids, and many other spatial structures. One particular use of morphological granulometry is to varying sizes of grid to filter the different sizes of scatterers. Morphological granulometry is based on mathematical morphology. There are two basic morphological operations, erosion ($\ominus$) and dilation ($\oplus$) [11,12]:

$$I \ominus E = \min_{b \in E} \{I(x + b) - E(b)\}, \quad (1)$$

and

$$I \oplus E = \max_{b \in E} \{I(x + b) + E(b)\}, \quad (2)$$

where I represents image and E denotes structuring element. The opening operation is combining the erosion with dilation operations, and is defined as [12]:

$$I \circ E = I \oplus (I \ominus E). \quad (3)$$

Morphological granulometries are parameterized families of morphological openings that are used for granular filtering and for pattern and texture classification. Let $\Omega(d)$ be area of $I \circ dE$, and $\Omega(0)$ is area of I . $\Omega(d)$ is called size distribution. The normalized size distribution $\Phi(d)$ is a probability distribution function [12], can be expressed as:

$$\Phi(d) = 1 - \Omega(d)/\Omega(0) \quad (4)$$

And the derivative $f(d)$ is size density distribution, and given by:

$$f(d) = \Phi'(d) \quad (5)$$

2.2. Mie theory

The scattering parameters of tissue, including scattering coefficient, mean cosine of scattering angle, and reduced scattering coefficient, can be calculated by combining the measured size density distribution with Mie theory. The total scattering parameters can be approximated as the sum of the scattering parameters due to particles of a given diameter, d . Therefore, the scattering coefficient can be obtained as:

$$\mu_s = N_0 \sum \pi(d/2)^2 Q_{sca}(d) f(d_i), \quad (6)$$

where Q_{sca} represents the effective scattering factor, $N_0 = F_v/\Sigma 4/3 \pi (d_i/2)^3 f(d_i)$, where F_v is the volume fraction, and $f(d_i)$ is the distribution of particles with a diameter d_i . The total volume fraction occupied by the particle was estimated as $F_v = 1 - F_w - F_{pp}$, where F_w is the weight fraction of water in the tissue, and F_{pp} is the fraction of organic solids in the combined interstitial fluid and cytoplasmic spaces. The size density distribution $f(d_i)$ is extracted by morphological granulometry. The anisotropy factor can be written as

$$g = \sum \pi(d_i/2)^2 Q_{sca}(d_i) f(d_i) g(d_i) / \sum \pi(d_i/2)^2 Q_{sca}(d_i) f(d_i), \quad (7)$$

and the reduced scattering coefficient can be determined by

$$\mu'_s = \mu_s(1 - g). \quad (8)$$

However, it is shown that the correlated scattering has important effects on tissue optical parameters [13,14]. The packing fraction W_s can be applied for interparticle correlation effects when the diameters of the particles are much less than the wavelength, and given by [15]:

$$W_s = \frac{(1 - v)^2}{[1 + (m_1 - 1)v^2]} \times \left\{ (1 - v)^2 + (1 - v)v \frac{4m_1 m_2}{1 + 5m_2} + \frac{v^2 m_1^2 m_2}{1 + 4m_2} \right\} \quad (9)$$

where v is the volume fraction of subwavelength-diameter particle, m_1 presents the shape and correlation among scatters, and m_2 represents the variance in the particle size. For a suspension of identical rigid spheres, the parameters, m_1 and m_2 , are, 3 and 0, respectively. When phase contrast image was performed by Fourier transformation, and the shape of image intensity is usually an ellipse. The eccentricity e of the ellipse is between 0 and 1. $m_1 = 3 \times (1 - e)$. When the eccentricity is 0, the figure is a circle, and the scatter shape can be seen as the sphere. In our method, we replaced the size distribution $f(d)$ by the correlation-correction size distribution $W_s \cdot f(d)$ for the diameters of the particles are less than the wavelength.

The variation in the refractive index is determined using weighted averages of typical refractive-index values for the constituents of biological tissue. The mean refractive indices variation $\langle n \rangle$ is approximately 0.07 for biological tissue with the background refractive index of $\bar{n}_{bkg} = 1.35$ [13]. The particle refractive index is determined by calculating a quantity known at the mean indices variation, $\langle n \rangle$, and the particle refractive index is given by $\bar{n}_p = \bar{n}_{bkg} + \langle n \rangle = 1.42$ [14].

2.3. Samples

In our experiments, we used a phase-contrast microscope (Nikon TS100) to measure spatial variations in tissue refractive indices. Fresh lung, liver and myocardial tissues from a rat were obtained, then frozen and sectioned in slices with a thickness of 5 μm . The animal study was performed in accordance with the guidelines of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996), and was approved by the Animal Care and Use Committee of Fujian Medical University, Fuzhou, China. Ten samples for each organ from the same rat were imaged at magnifications of 100X were acquired with a CCD camera. The total magnification of $100 \times$ phase contrast image was simply calculated by multiplying a $10 \times$ eyepiece by a $10 \times$

objective, and the numerical aperture of the 100X phase contrast objective was 0.25. In addition, contributions from different tissue components, such as stroma and epithelium, are considered in terms of different shapes and distributions of scatterers in phase contrast images.

3. Results

Figures 1(a)–(c) showed typical gray phase-contrast images of rat liver, lung and myocardial tissue slices. Thus, we binarized the images by setting a threshold according to the value estimated by the Otsu method for the entire image field. This allowed us to interpret the refractive index in terms of the size distribution of tissue. Figures 1(d)–(f) showed the corresponding binary phase-contrast images of rat liver, lung and myocardial tissue slices. Figures 1(g)–(j) show that the phase contrast images were performed by Fourier transformation, made logarithmic transformation and binarized by setting a threshold value of 8. The shapes of images were ellipses. The eccentricities e of the ellipses were 0.63 ± 0.04 , 0.56 ± 0.03 and 0.73 ± 0.05 , respectively. The m_1 was determined by: $m_1 = 3 \times (1 - e)$.

The corresponding size density distributions for binary phase-contrast images of rat liver, lung and myocardial tissue slices were shown in Fig. 2. Particle diameters of tissue were usually chosen ranging from 50 nm to 20 μm [13]. When morphological granulometry was operated, the minimum diameter $d_{\min}$ of disk was set at 2 pixels, which is 44 nm. The minimum dimension of 44 nm was below the resolution limit of the optical phase microscope. We should note that the particle size distribution measured by morphological granulometry was an equivalent particle size distribution. Figure 2 demonstrated that different tissues had different maximum diameter scale $d_{\max}$ and different size density distribution, which controlled optical scattering properties of tissues. Thus it was suggested that the size density distributions based on the binary phase-contrast images could be a good parameter for differentiating the variations of tissue.

Water contents in the tissues can be different, and their weight fraction F_w of water were 0.790 ± 0.004 for rat lung, 0.705 ± 0.007 for rat liver, and 0.779 ± 0.002 for myocardium [16], respectively. And F_{pp} was the fraction of organic solids in the combined interstitial fluid and cytoplasmic spaces, chosen as 0.05 [14]. Consequently, the total volume fraction of scatterers of the tissues could be estimated as 0.29 for rat liver, 0.205 for rat lung, and 0.175 for myocardium.

The optical scattering parameters of liver tissues from a rat were calculated by our method by combining morphological granulometry and Mie theory at different wavelengths. The scattering coefficient μ_s , reduced scattering coefficient μ'_s , and anisotropy factor g of the rat liver tissue for ten samples for the same organ at the three different wavelengths (532 nm, 633 nm and 780 nm) were summarized in Table 1. Ding et al. [7] reported $\mu_s \sim 71.4 \text{ mm}^{-1}$ at the wavelength of 532 nm by using Fourier-transform light scattering. Beek et al. [1] reported that μ_s , μ'_s and g were $28.0 \pm 1.0 \text{ mm}^{-1}$, $1.3 \pm 0.1 \text{ mm}^{-1}$, and 0.952 ± 0.004 ,

respectively at the wavelength of 633 by using double integrating spheres. Beauvoit et al. [17] report the value of μ'_s is $1.6 \pm 0.2 \text{ mm}^{-1}$ at the wavelength of 780 nm.

The scattering coefficient μ_s , reduced scattering coefficient μ'_s , and anisotropy factor g of rat lung and myocardium tissue of 10 samples for the same organ at four different wavelengths were summarized in Tables 2 and 3, respectively. An equivalent particle size distribution was measured as a parameter to describe optical scattering properties of tissue. Beek et al. [1] reported that μ_s , μ'_s , and g of dog lung and myocardium by double-integrating-sphere were $23.0 \pm 0.5 \text{ mm}^{-1}$, $1.5 \pm 0.5 \text{ mm}^{-1}$, 0.935 ± 0.017 , and $19.1 \pm 1.6 \text{ mm}^{-1}$, $1.13 \pm 0.12 \text{ mm}^{-1}$, and 0.940 ± 0.004 , at the wavelength of 633 nm, respectively. Jacques [18] reported that the values of reduced scattering coefficient μ'_s of lung at three wavelengths (532 nm, 633 nm, 780 nm) were 2.4, 2.2, and 2.0 mm^{-1} , respectively, by analysis of literature data. An equivalent particle size distribution combined with Mie theory could deduce the scattering properties of tissues including the scattering coefficient μ_s , reduced scattering coefficient μ'_s , and anisotropy factor g .

4. Discussion

Our simulated values are different from that in the above references, likely due to the different sample preparations. Paraffin was embedded in tissue. When frozen sections were used in tissue pathology, the paraffin may decrease the scattering coefficient of tissue, because of the mismatch in refractive indices between the scatterers and their background.

The size model based on Fourier power spectrum, studied by Schmitt and Kumar [9], was a continuous size distribution of the scatterers. However, the simulation of optical properties of such a mixture, with an infinite number of different sphere sizes, was unrealistic. It was easier to use only a limited number of sphere sizes. Schmitt and Kumar [14] realized the problem, and chose ten sphere sizes to calculate the optical properties of sample. The size model based on morphological granulometry was a discrete size distribution, since scale-space analysis forced the classification of particles into several sizes with the integer times of 2. [19] Thus, the granulometry was more accurate than choosing ten sizes of power spectrum.

Based on diffraction phase microscopy, Fourier-transform light scattering was used to extract the scattering coefficient and anisotropy factor [7]. However, the anisotropy factor tended to be overestimated, due to the limited angular range measured [7]. We focused on the equivalent particle size distribution, because an equivalent particle size distribution could be used to obtain more details and accurate understanding about optical scattering property of tissue [20]. Both the scattering coefficient and anisotropy factor were inducible from particle size distribution using Mie theory, without the overestimated anisotropy.

Optical coherence tomography is widely applied for estimation of optical properties of tissue *in vivo*, especially scattering coefficient. In comparison with optical coherence tomography, this method combined morphological granulometry with Mie theory determines the scattering properties including scattering coefficient, anisotropy factor and reduced

scattering coefficient, which more complete parameters for scattering properties of tissue. In selective photothermal interaction for cancer treatment with absorption enhancement of target tumors using either chemical dye [21–23] or nanoparticles [24], the absorption coefficient of dye or nanoparticles is much higher than the absorption coefficient of tumors. Using the absorption coefficient of dye or nanoparticles and accurate scattering properties including scattering coefficient and anisotropy factor of tumor which is extracted by this method in this study, simulations of photothermal effects [25,26] can yield more reliable predictions for laser irradiation of biological tissues.

5. Conclusions

In summary, a phase-contrast microscope was used to observe the micro index distribution of tissue. The gray phase-contrast images of tissues were binarized by setting a threshold according to the value estimated by the Otsu method. Morphological granulometry was used to estimate size density distribution of binary phase-contrast images of the tissue samples. The optical parameters associated with light scattering could be estimated by combining the uses of size density distribution and Mie theory. At the microscopic spatial scales, equivalent size distribution could be used in combination with the high-resolution (Objective: 20X, 40X, 60X) microscopes to describe the scattering properties of subcellular structures. Meanwhile, phase-contrast images of large area of tissues could be created by a montage of micrometer-resolution images, which were obtained by programmable scanning stage. Equivalent size distribution and the scattering properties of bulk tissue could be acquired. Thus, this work could be used to study tissue optics from microscopic to macroscopic spatial scales. This method provided quantitative optical properties of unstained slice and it could enable pathologists to make faster, more accurate diagnoses.

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Fig. 1.

(a)–(c) Gray phase-contrast images of rat liver, lung and myocardial tissue, respectively. (d)–(f) Binary phase-contrast images of rat liver, lung and myocardial tissue, respectively. (g)–(j) Fourier transforming binary phase-contrast images on logarithmic scale. Shape of image intensity is usually an ellipse. The eccentricities e of the ellipses are 0.63 ± 0.04 , 0.56 ± 0.03 and 0.73 ± 0.05 , respectively. Scale bar shows 11 μm .

Fig. 2.
Comparison of size density distributions of rat liver, lung and myocardial tissues.

Table 1

Optical scattering properties of rat liver at the four different wavelengths

Wavelength	$\mu_s \text{ mm}^{-1}$	g	$\mu'_s \text{ mm}^{-1}$
532 nm	90 ± 5	0.960 ± 0.005	3.6 ± 0.2
633 nm	60 ± 4	0.950 ± 0.005	3.0 ± 0.2
780 nm	31 ± 3	0.940 ± 0.007	1.8 ± 0.1

Table 2

Optical scattering properties of rat lung at the four different wavelengths

Wavelength	$\mu_s \text{ mm}^{-1}$	g	$\mu'_s \text{ mm}^{-1}$
532 nm	34 ± 5	0.860 ± 0.013	4.7 ± 0.4
633 nm	22 ± 4	0.83 ± 0.02	3.6 ± 0.3
780 nm	8.3 ± 1.4	0.80 ± 0.04	1.7 ± 0.2

Table 3

Optical scattering properties of rat myocardium at the four different wavelengths

Wavelength	$\mu_s \text{ mm}^{-1}$	g	$\mu'_s \text{ mm}^{-1}$
532 nm	35 ± 6	0.91 ± 0.02	3.1 ± 0.2
633 nm	23 ± 5	0.88 ± 0.02	2.8 ± 0.2
780 nm	11 ± 3	0.84 ± 0.04	1.7 ± 0.2