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Abstract. Recently, compression optical clearing (OC) was applied to detect dermal carotenoid using reflection spectroscopy. To enhance the precision and accuracy of reflection spectroscopy to better detect the spectral absorption of beta-carotene inside biological phantom, here, we simultaneously use compression and immersion OC using dimethyl sulfoxide. In addition, we analytically extract the absorption coefficient of beta-carotene using diffuse reflectance spectroscopy (as an analytical OC). Our results show that the presented analytical OC can be applied alone as a noninvasive method to measure cutaneous chromophores at deep tissues. Finally, we also improve the ability of the analytical clearing method mediated with experimental OC. Our result demonstrates that the combination of analytical and experimental clearing methods enhance the ability of diffuse reflection spectroscopy for extracting the absorption coefficient of beta-carotene as one of the chromospheres inside biological phantom. © 2018 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.23.9.095002]

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1 Introduction

Determining cutaneous carotenoid plays an important role in human health and nutrition studies.[1–4] High-pressure liquid chromatography (HPLC) is the main *in vitro* method for determining the amount of beta-carotene. In addition to the high cost, some amount of beta-carotene will be lost after biopsy of tissue, and it will not give a precise value.[3,5]

Due to the limitation of HPLC, optical methods, such as Raman spectroscopy and reflection spectroscopy, have been utilized to determine the amount of beta-carotene in human and animal skin. The accuracy of these optical methods requires evaluation. In 2010, Ermakov and Gellermann[6] investigated the correlation of *in vivo* skin tissue carotenoid obtained by resonance Raman spectroscopy with HPLC results. Darvin et al.[7] measured the content of beta-carotene in the inner palm by *in vivo* resonance Raman spectroscopy.

The absorption spectrum of beta-carotene is masked by the blood and melanin absorption spectra. Strong scattering of skin complicates additional beta-carotene registration due to broadening and decreasing of absorption peaks. Therefore, one can apply optical clearing (OC) to enhance determination of beta-carotene spectra. Ermakov and Gellermann[8] applied the compression mediated reflection spectroscopy to determine the amount of this choromophore. The mechanical compression results in a decrease in the value of the absorption coefficient of skin due to the decrease of total blood content at the location of compression.[9–11] Some additional decrease of light scattering is also expected due to a lower blood volume fraction (as red blood cells are strong scatterers) and an increase in packing

density of dermal collagen fibers.[9,11] Therefore, the absorption spectrum of beta-carotene can be detectable. Nakhaeva et al.[12] illustrated that the pressure exerted on the skin can weaken the effect of blood by reducing the depth of the dip in the green region of the spectrum that indicated the presence of blood in the tissue. Ansari et al.[13] also reported that the mechanical compression altered the value of the absorption coefficient. These changes in optical properties can affect the reconstructed concentration of desired chromophore. In addition, the amount of transported blood depends on the value of mechanical pressure and the stiffness of tissue that affects the accuracy of compression-mediated reflectance spectroscopy. Therefore, it is important to verify the accuracy of compression-mediated reflection spectroscopy to determine cutaneous beta-carotene using other OC methods, such as immersion OC.

Polyatomic alcohol (glycerol, polypropylene glycol) and organic solvents [dimethyl sulfoxide (DMSO) and benzyl ether, benzyl alcohol, tetrahydrofuran] have been applied for immersion OC.[14–19] Mao et al.[20] illustrated that some hydroxyl-terminated organic components' agents (1-butanol, 1,4-butanediol, 1,3-propanediol, PEG200, PEG400, and glycerol) induce significant OC effects of porcine skin. Their results show that the OC effect of skin caused by these alcohols is related to the number of hydroxyl groups.

As Tuchin et al. proposed, the mechanism of immersion OC can be explained by dehydration of tissue constituents and the replacement of interstitial water with an agent that has a higher refractive index (n).[14,21] Based on experimental results presented by Rylander et al.,[17] application of glycerol causes a 60% decrease in the reduced scattering coefficient in the context of *in vitro* rodent skin. Zhu et al.[22] illustrated that a combination of

PEG-400 and thiazone (as immersion agents) can reduce the scattering coefficient (at a wavelength of 630 nm) to 60% of the initial value after 40 min of topical treatment of this mixture on rat skin *in vitro*.

Beside immersion OC, an analytical method also can be applied for measurement of chromophore inside tissues. For example, Zonios and Dimou[23] applied an analytical form of diffuse reflectance from semi-infinite phantom to determine the absorption coefficient of phantom containing polystyrene beads and hemoglobin from bovine blood. Välisuo et al.[24] applied a linear model based on the differential Beer–Lambert law to efficiently map the concentrations of blood and melanin in the skin. They calculated an optimal linear model around an operating point, taking into account the spectra of the selected wavelengths, the concentrations of these chromophores in the selected operating point, and the available wavelengths.

In the analytical OC method, a linear summation of different absorption spectra of key tissue constituents (blood, melanin, beta-carotene, and water) is considered to model the measured diffused reflectance with optimum values of chromophore concentration. It seems that immersion and analytical OC can be applied to enhance the capability of compression-mediated reflection spectroscopy to better reconstruct the concentration of chromophores inside tissues.

Motivation for this paper is threefold: first, we investigate the ability of both compression and immersion OC, as experimental OC, to improve the measurement of beta-carotene absorption. Second, we use an analytical OC method based on diffuse reflectance spectrum to detect the absorption spectrum of beta-carotene from experimental spectra. Finally, we show a new way to reconstruct effectively the absorption coefficient of beta-carotene obtained from diffuse reflection spectra based on both analytical and experimental clearing of samples. Hence, the originality of the current study is applying a combination of analytical and experimental OC methods to better measure the concentration of beta-carotene inside tissue phantom.

2 Materials and Methods

2.1 Experiment Set Up

As shown in Fig. 1, the experiment setup consists of a tungsten halogen lamp with 20-W power (HL2000; Ocean Optics Inc., Dunedin, Florida) and spectrometer (USB200, Ocean Optics)
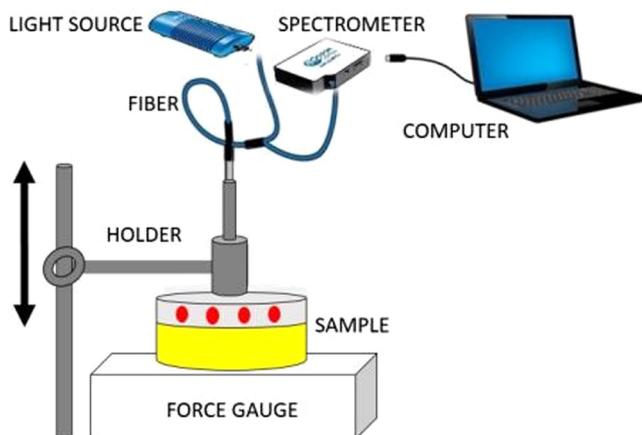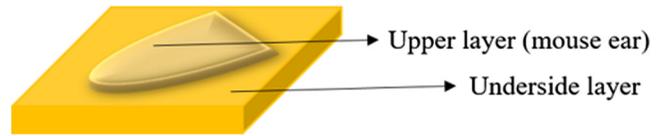

Fig. 1 The scheme of experiment setup.

Fig. 2 A schematic of phantom. Underside layer consists of Intralipid, agar- and beta-carotene, and ear of mouse applied as an upper layer.

with a spectral range of 350 to 1100 nm and an optical fiber probe (R200-REF, Ocean Optics, Dunedin). This probe consists of a bundle of seven optical fibers—six illumination fibers around one read fiber—each of which is 200 μm in diameter (NA = 0.22, length = 2 m) and a distance of source–detector separation of 200 μm. A $3'' \times 0.25''$ stainless steel ferrule houses the fiber bundle. We also use a force gauge to measure the pressure.

2.2 Phantom Preparation

We prepared a two-layer phantom (Fig. 2). The underside layer consisted of Intralipid® 20% (3.3 ml), distilled water (13 ml), agar (0.7 g), and beta carotene [≥97.0% (UV) Sigma-Aldrich] in different concentrations of 0.2, 0.3, 0.4, and 0.6 μM. The upper layer is a mouse ear (2-month old, 20 g) with an average thickness of 250 μm (one-half of its full thickness is composed of two layers: the epidermis and the dermis, and below the skin layers, cartilage forms the structural support for the mouse ear).[25] Eight ear specimens were obtained from Shahid Beheshti Medical Science University and stored at 4°C until experiments were performed (15 min). All animals participating in this experiment were cared for according to the ethic committee of Shahid Beheshti University, Iran.

2.3 Optical Clearing Methods

Here, we used two different OC methods to detect absorption spectra of beta-carotene from reflectance spectra. First, the method of experimental OC (compression-immersion OC) is explained, and then the method of analytical method is stated.

#### 2.3.1 Experimental method: compression-immersion OC

Here, we utilized DMSO (≥99%, Sigma-Aldrich) as a chemical OC agent. A solution of DMSO 50% was prepared with ethanol as the solvent. Ear specimens were immersed in DMSO solution for 15 min. Then, the mechanical compression was applied by fiber probe on the specimen, and the reflectance spectra were measured, as depicted in Fig. 1.

2.3.2 Analytical method

As mentioned in Ref. 23, the analytical form of diffuse reflectance $R(\lambda)$ from semi-infinite phantom to determine optical properties can be stated as follows:

$$R(\lambda) = \frac{\mu_s'(\lambda)}{k_1 + k_2 \mu_a(\lambda)}, \qquad (1)$$

where k_1 and k_2 are the parameters depended on the probe geometry and refractive indices of the studied sample and were determined to be 0.025 mm^{-1} and 0.075, respectively. $\mu_s'(\lambda)$ is the wavelength-dependent reduced scattering coefficient, and $\mu_a(\lambda)$ is the wavelength-dependent absorption
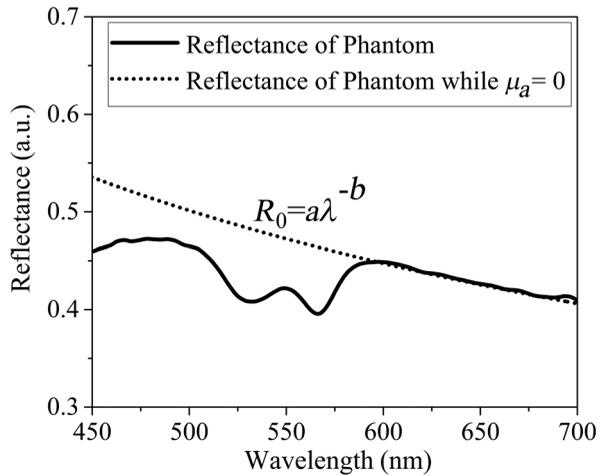
Fig. 3 Estimation of R_0 (dotted line) for measured results (solid line) using scattering power law. For the range $\lambda > 600$ nm, the spectrum graph (solid line) can be fitted by the scattering power law $a\lambda^{-b}$ (dotted line), where $a = 26.65$ and $b = 0.63$ (with 95% confidence bounds).
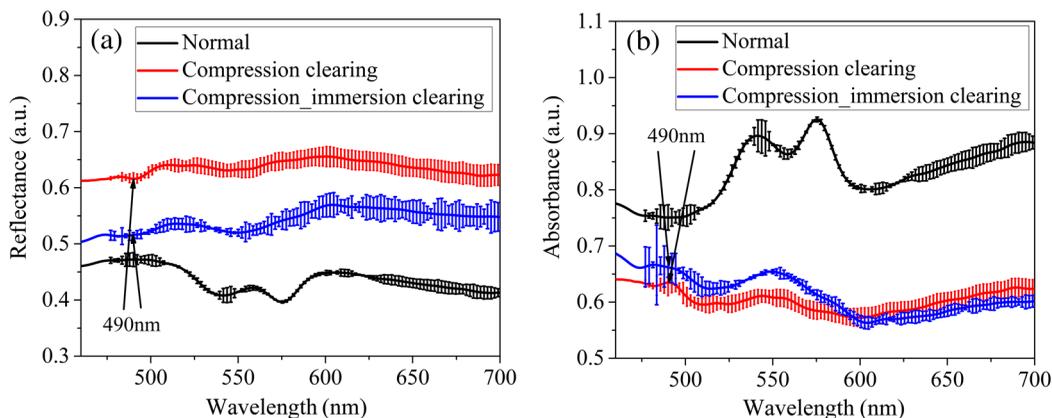
coefficient of tissue-like phantom. Assume R_0 indicates the diffuse reflectance for $\mu_a(\lambda) = 0$, so $\mu_s'(\lambda) = k_1 R_0$. This diffuse reflectance R_0 can be estimated from the wavelength region of 600 to 900 nm using the scattering power law, as mentioned in Ref. 26 (see Fig. 3). Finally, the absorption coefficient is obtained as follows:

$$\mu_a(\lambda) = \frac{k_1}{k_2} \cdot \left[\frac{R_0}{R(\lambda)} - 1 \right]. \tag{2}$$

In the visible spectrum, blood and melanin are the main chromophores that affect the absorption coefficient of our phantom, while variation of water and lipid does not have significant effects in this spectrum interval. Hence, the absorption coefficient of phantom can be expressed as follows:[27]

$$\mu_a(\lambda)_{\text{phantom}} = M\mu_a(\lambda)_{\text{mel}} + B\mu_a(\lambda)_{\text{Beta}} + Bl\mu_a(\lambda)_{\text{blood}}, \tag{3}$$

where

$$\mu_a(\lambda)_{\text{blood}} = \alpha Bl\mu_a(\lambda)_{\text{HBO2}} + (1 - \alpha)Bl\mu_a(\lambda)_{\text{HB}}, \tag{4}$$

where M, B, and Bl denote melanin, beta-carotene, and blood volume fractions, respectively, and α is the degree of oxygenation of hemoglobin. The absorption spectrum of the mentioned chromophores is selected from the results presented by the "natural phenomena simulation group in University of Waterloo" and data presented by Jacques in 2013.[28,29]
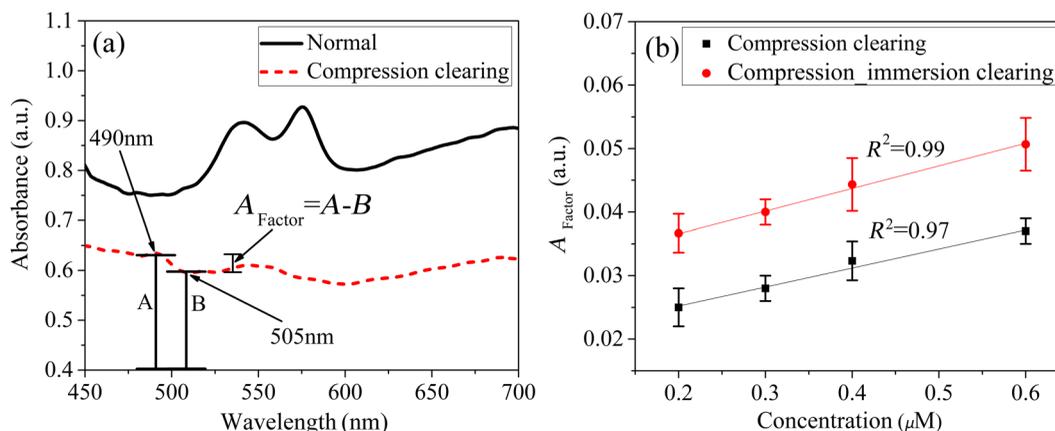
3 Results

3.1 Experimental Clearing

Figures 4(a) and 4(b) demonstrate the effects of experimental clearing methods on diffuse reflectance and absorbance [the absorbance $A(\lambda)$ is calculated by $A(\lambda) = -\log R(\lambda)$]. First, we apply compression OC and study the variation of diffuse reflectance (absorbance). The results including error bars show that the removal of blood dip between 500 and 570 nm depends on the amount of mechanical compression. This is because the transport of blood is a function of the amount of compression and Young's module of tissue. One can see in Fig. 4(a) that, after the application of pressure and when blood is removed, the reflection increases due to less blood absorption. Indeed, scattering is also changed a bit in this range (scattering by RBC), but, in this wavelength range, blood absorption prevails.

Then, we simultaneously studied the influence of both compression and a chemical clearing agent on tissue phantoms. Blue lines including error bars in Figs. 4(a) and 4(b) reveal a noticeable change in absorbance due to utilizing DMSO, as the chemical OC agent, in OC of a mouse ear. Immersion of ears in DMSO leads to a decrease in optical scattering of them, so one can see an increase in optical depth (diffusion of DMSO in phantom causes a reduction in the scattering coefficient by refractive index matching).[30] Results depict that the combined OC (before 600 nm) can enhance the absorption spectra, whereas these two methods have similar effects in an optical window (a wavelength longer than 600 nm).

As shown in Fig. 4 and during application of OC, beta-carotene absorption spectrum appears in its specified wavelength.

Fig. 4 The influence of compression and immersion OC on the spectrum of reflectance and absorbance. The black line shows the (a) reflectance and (b) absorbance before applying experimental clearing. The red line depicts the variations of reflectance (absorption) due to compression OC (300 N/m²). The blue line presents the effects of the combination of immersion and compression OC on (a) reflectance and (b) absorbance. Eight ears are used while doing the experiment for investigating the repeatability of this method. Arrows point out absorption peak of beta-carotene.

Fig. 5 (a) Illustration of A_{Factor} obtained from absorbance spectra of optically cleared phantoms, where A is the absorbance at the wavelength 490 nm and B is the absorbance at the wavelength 505 nm. The black solid line depicts the spectrum before applying compression OC, and the red dotted line illustrates the absorbance spectra of optically cleared phantom after applying compression. (b) Relation between A_{Factor} and concentration of beta-carotene for different phantoms. The black line shows the influence of compression OC, and the red line presents the effects of the combination of compression and immersion OC on A_{Factor}.
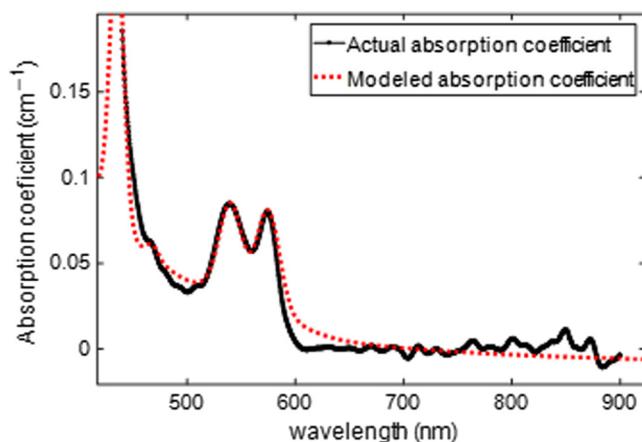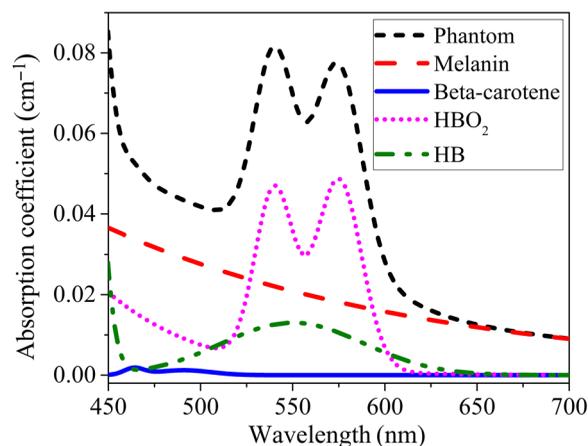

Fig. 6 Modeling measured absorption coefficient (black solid line) using diffuse reflectance presented by the red dotted line.

Fig. 7 Extracted absorption coefficient based on the optimum volume fraction of main chromophores.

We define an indicator $A_{Factor} = A - B$, as shown in Fig. 5(a), to clarify the effect of OC on absorbance spectrum (A is the absorbance at $\lambda = 490$ nm and B is the absorbance at $\lambda = 505$ nm). Figure 5(b) depicts the amount of A_{Factor} for different values of beta-carotene concentration. The results show a linear relation between the concentration of beta-carotene and A_{Factor}.

3.2 Analytical Clearing

Figure 6 compares the modeled absorption coefficient spectra and actual absorption coefficient obtained from diffuse reflectance spectra, as explained in Sec. 2.3.2. As shown in Fig. 6, these two curves are correlated with a high correlation coefficient of $R^2 = 0.94$, so the fitting parameters indicating the volume fraction of chromophores can be obtained from modeled spectra.

As shown in Fig. 7, analytical OC could extract and quantify the absorption coefficient of each chromophore within tissue-like optical phantom without experimental clearing methods for detection of beta-carotene.

4 Discussion

Here, two methods for clearing of tissue-like phantom to extract the absorption of beta-carotene are studied.

4.1 Experimental Clearing (Compression-Immersion OC)

First, we used mechanical compression to weaken the effect of high blood absorption and obtain the amount of beta-carotene in our phantoms. Ermakov and Gellermann[8] investigated the correlation between results obtained by compression mediated reflection spectroscopy and Raman spectroscopy. They illustrated the feasibility of this OC to extract the absorbance spectrum of beta-carotene. Because the blood distribution inside biological tissue depends on the amount of mechanical pressure and the stiffness of tissue, the accuracy of compression mediated reflection spectroscopy can be a key limitation for detecting chromophore at deep depths.
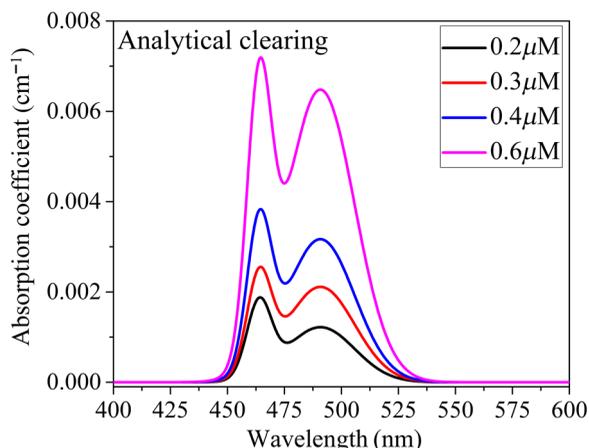
Hence, we used DMSO as a chemical clearing agent during compression-immersion clearing to obtain beta-carotene

absorption because DMSO is a well-known skin penetration enhancer and its permeability rate (176 ± 42 g/m^2 h) is ~10 times greater than that of water.[17,31] In addition, DMSO induces a reversible conformational change of protein when it substitutes for water.[17] By contrast, for example, glycerol does not have a high enough penetration rate since it can hardly penetrate into dermis to achieve OC by topical application because of the barrier of stratum corneum.[19] As one can see in Fig. 4(b), applying DMSO as the OC agent improves the detection of the absorption spectrum of beta-carotene.
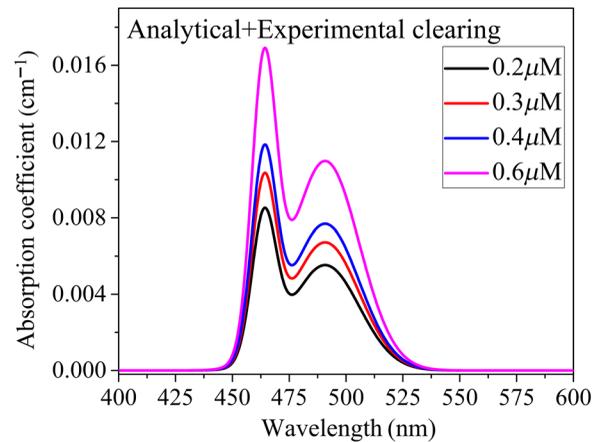
In this experiment, we use a phantom consisting of a mouse ear and beta-carotene at a depth of ~0.2 mm from the surface of phantom. (In fact, we apply a bilirubin-free phantom having a low amount of melanin that influences the results.) Using DMSO and compression helps to decrease the influence of blood and mismatched refractive index to better detect beta-carotene absorption spectra. The efficacy of our method to measure the beta-carotene content in human skin depends on the scattering coefficient of skin. The light scattering in skin depends on different elements, such as melanin and other tissue structures. The presence of melanin ($n = 1.7$) on the background of low refractive index of cell cytoplasm and interstitial fluid ($n = 1.34$) causes high scattering in the epidermis layer of the skin.[32] However, very white skin having a low melanin content has a high scattering coefficient.[33–35] Hence, in the range of 400 to 700 nm, high absorption and scattering limits the ability of experimental and analytical OC to efficiently detect the desired chromophores.

4.2 Analytical Clearing (Extracting the Absorption Coefficient of Beta-Carotene)

Johansson[36] measured the reflectance spectra of a thin cerebral phantom to estimate its absorption coefficient. Using linear regression, the absorption spectra were fitted to extract the main chromophore spectrum inside cerebral phantom. We applied this idea to perform analytical OC. That means, the blood and melanin spectra were digitally removed from the measured absorption spectra, then the absorption spectra of the desired chromophore should be apparent. The spectral elimination of dominant chromophores enhanced the capability of this method to detect the low value of concentration of chromophore

Fig. 9 Extracted spectral absorption coefficient of different values of concentration of beta-carotene using combination of analytical and experimental OC.

at deep tissues without applying any experimental clearing. For example, Fig. 8 shows the spectral absorption coefficient for different values of concentration of beta-carotene inside the phantom. In general, a combination of all three considered modalities should be helpful for improving capabilities of the OC approach because of the reduction of tissue scattering and blood absorption impact on analytical reconstruction of chromophore concentration with the masked spectrum. To prove this claim, we also combine analytical clearing with experimental clearing methods. As shown in Fig. 9, the value of the extracted absorption coefficient of beta carotene is improved. Figure 10 also compares the value of the extracted absorption coefficient at a wavelength of 490 nm for these two methods. It is clearly specified that the estimated value of the absorption coefficient using this hybrid method (the combination of analytical and experimental clearing) is larger than experimental or analytical clearing alone. However, compression and immersion OC should be applied when analytical OC is not enough to get reliable data.

Fig. 8 Extracted spectral absorption coefficient of different values of concentration of beta-carotene using analytical OC.
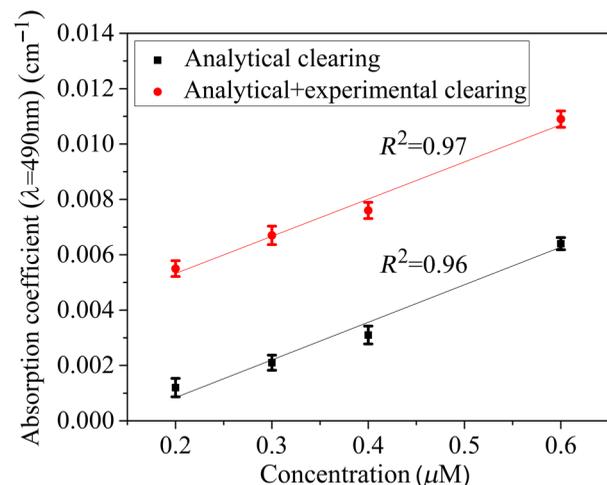
Fig. 10 Relation between extracted absorption coefficient of beta-carotene at a wavelength of 490 nm with a concentration of beta-carotene inside phantoms for analytical clearing method and combination of analytical and experimental clearing method.

5 Conclusion

This study aimed to improve compression OC using the analytical OC to measure beta-carotene inside the phantom. First, we utilize compression-immersion OC to decrease both the scattering coefficient of specimen and the effect of blood spectra. Our results show that this method enhances the ability of reflectance spectroscopy to predict the amount of beta-carotene. While the accuracy of this method depends on the value of pressure and the stiffness of the biological tissue, we also apply analytical OC to detect beta-carotene. Results depict that the analytical OC can measure the absorption coefficient of beta-carotene. Indeed, the analytical OC does not require any chemical agent or compression that might change or destroy the structure of tissues (as a noninvasive method). We finally apply the experimental clearing method besides analytical clearing to improve analytical capabilities of the OC approach for the extraction absorption coefficient of beta-carotene. The results show that analytical OC can be applied independently and in combination with compression or immersion, or both, when it is needed. The presented OC techniques can be applied for determining chromophores, such as melanin and lipid, in deep tissues that have good abilities for *in vivo* oncology and nutrition research, especially for spectral imaging of tumors.

Disclosures

The authors have no relevant financial interests in this article and no potential conflicts of interest to disclose.

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