

RESEARCH ARTICLE

Ex vivo confocal Raman microspectroscopy of porcine *dura mater* supported by optical clearing

Ali Jaafar^{1,2,3*}  | Roman Holomb^{1,4} | Anton Y. Sdobnov^{5,6} | Zsombor Ocskay⁷ | Zoltán Jakus⁷ | Valery V. Tuchin^{5,8,9}  | Miklós Veres¹

¹Institute for Solid State Physics and Optics, Wigner Research Center for Physics, Budapest, Hungary

²Institute of Physics, University of Szeged, Szeged, Hungary

³Ministry of Higher Education and Scientific Research, Baghdad, Iraq

⁴Department of Information Control Systems and Technologies, Uzhhorod National University, Uzhhorod, Ukraine

⁵Science Medical Center, Saratov State University, Saratov, Russia

⁶Optoelectronics and Measurement Techniques Laboratory, University of Oulu, Oulu, Finland

⁷Department of Physiology, Semmelweis University School of Medicine, Budapest, Hungary

⁸Laboratory of Laser Diagnostics of Technical and Living Systems, Institute of Precision Mechanics and Control of the Russian Academy of Sciences, Saratov, Russia

⁹A.N. Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, Moscow, Russia

*Correspondence

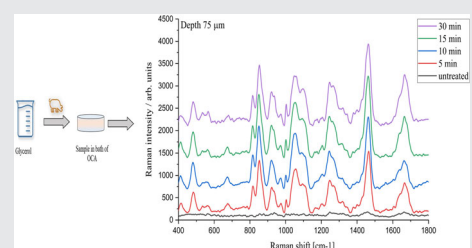
Ali Jaafar, Institute for Solid State Physics and Optics, Wigner Research Center for Physics, P.O. Box 49 H-1525, Budapest, Hungary.
Email: alijaafar@szzfki.hu

Abstract

The effect of tissue optical clearing (TOC) to increase the probing depth and observe in-depth structure of the ex vivo porcine *dura mater* was studied by confocal Raman microspectroscopy (CRM). Raman intensities were significantly increased at the depth of 250 μm for all collagen bands after treatment with glycerol. The influence of glycerol on collagen hydration was also investigated. The results indicate that the process of TOC can be divided into three main steps. The first one is a fast process of tissue dehydration accompanied by collagen shrinkage while the second relatively slow process is related to the glycerol penetration into the interfibrillar space of collagen combined with swelling of tissue. The third step is collagen dissociation caused by the high concentration of glycerol. To the best of our knowledge, this study is the first example to introduce the TOC technique in assisting CRM of ex vivo *dura mater* in-depth probing.

KEYWORDS

collagen type I, confocal Raman microspectroscopy, *dura mater*, glycerol, tissue optical clearing



Abbreviations: CRM, confocal Raman microspectroscopy; DM, *dura mater*; OCA, optical clearing agent; TOC, tissue optical clearing.

1 | INTRODUCTION

In the last few years, the progress in using biophotonics techniques for the real-time monitoring of the pathophysiological changes of the tissue and tissue diagnostics has been increased, because of their easy operation, low price, and minimal risks. One of the focuses of optical technology development is the design of clinical functional cerebral imaging systems to study deep inhomogeneities in tissues, aiming brain diagnostics, laser therapy and surgery. [1, 2] The interest in Confocal Raman microspectroscopy (CRM) for biomedical application is increased in the last decade. By recording the spectral distribution and intensity of the Raman scattered photons, this technique allowed to obtain information on the type and concentration of chemical bonds presents in a tissue, since the frequency of the vibrational band observed in the Raman spectrum is determined by the bonds and the geometry of the molecular structure. [3] In fact, the Raman spectrum of the tissue can be taken into consideration as its distinctive molecular fingerprint containing information about the chemical conformation.

Raman spectra from tissues samples are intrinsically weak due to the low amount of inelastically scattered photons. Therefore, number of technologies have been developed to improve the Raman intensity. Resonance Raman scattering, [4] surface-enhanced Raman scattering (SERS), [5] tip-enhanced Raman scattering, [6, 7] and stimulated Raman scattering (SRS) [8] are among the most effective approaches used nowadays.

In the case of the brain, the Raman spectrum corresponds to fingerprint signatures for the chemical composition of different tissue constituents like collagen, blood, proteins, lipids, nucleic acids and tumors. [9–11] Therefore, the evaluation of Raman bands gives information about the biochemical properties of the observed tissue. Even insignificant changes in tissue composition can lead to alterations in Raman band positions and intensities, indicating very good sensitivity and detection capabilities of this technique.

All optical imaging techniques including CRM, are facing critical issues limiting the probing depth and spatial resolution due to the high level of light scattering and low absorption properties of biological tissues. [12] Considering the brain imaging, sparing therapy and diagnostics of brain diseases, the significantly high scattering of *dura mater* (DM) in the visible and near-infrared spectra range limits the spatial resolution and depth of probing for using noninvasive optical imaging technologies. [13] One of the simplest and most effective methods to improve the scanning depth, image quality and to increase the spectroscopic data accuracy from blood

vessels network and the cerebral cortex structures is to temporarily change the optical properties of the DM. [14–16]

In the 1990s, tissue optical clearing (TOC) technique was developed and introduced with the main goal to improve the effectiveness of penetration of visible and infrared light into the deep layers of biological tissue and to enhance the probing depth for optical spectroscopic and imaging devices. The TOC allows changing the optical properties of the biological tissues, particularly, by highly reducing the light scattering properties of tissues. It was effectively used to improve the scanning depths and to enhance the contrast of optical imaging techniques, as well as to enhance the light focusing ability and the spatial resolution for many noninvasive optical diagnostic methods. [1, 17] Detailed description of interactions between the biological tissues and OCAs can be found in the literature. [1, 18–21]

Currently, numerous optical imaging methods such as Raman spectroscopy, [22] optical coherence tomography, [23, 24] 3D-confocal microscopy, [25] and polarized microscopies, [26] are used in combination with different OCAs like glucose, [27] dimethyl sulfoxide (DMSO), glycerol, [28] uDISCO, [29] *ScaleS*, [30] and *Scale*, [31] to increase sensitivity and improve light propagation in-depth tissues. Even though there are a vast number of research articles, very little attention has been given to investigate the effects of OCAs on different tissues and different optical imaging methods.

The cranial meninges have been demonstrated to play a critical role in traumatic brain injury. Meanwhile, the mechanical reaction of the brain has been intensively investigated, and the meninges have relatively been ignored. [32] Therefore, the importance of investigating the TOC of human DM collagen could be a useful technique to examine head injuries, aiding in the assessment of subdural bleeding associated with this tissue, particularly subdural hematomas. [15] Recent evidence suggests that DM endothelial cells have more angiogenic potential than brain endothelial cells that accelerated vascular regeneration in a head injury study. [33] Moreover, meningiomas are the most common brain tumors and thought to arise from DM. [34] Raman spectroscopy was successfully used to detect the differences between dura and meningioma, related to collagen and lipid content, [9] identification of glioma margins and grades, [35] determined water concentration with very high accuracy in brain tissue, [36] and surface-enhanced Raman scattering was also employed to detect and quantify three types of meningitis pathogens in the cerebral spinal fluid. [37]

In this study, we present the results of the experimental investigation of OCA influence on probing depth using porcine DM as a human's DM model with CRM

assessment. The influence of glycerol on Raman spectra and intensities of principal collagen peaks has been investigated using CRM. To the best of our knowledge there is no information about using CRM for investigation of DM combined with TOC technique in-depth probing.

2 | MATERIALS AND METHODS

2.1 | Properties of *dura mater*

Human DM is the supportive and protective barrier surrounding the brain. It consists of two layers: the outermost periosteal layer and the inner meningeal layer as illustrated in Figure 1. The first layer is attaching to the inner skull and contains branched net of blood vessels, cerebro-spinal fluid (CSF) drainage system, nerves, and large collagenous packed in lamellar bundles. The laminae are immersed in the interstitial fluid (an amorphous base substance). The second layer is a dense fibrous membrane connected with the DM of the spinal cord. [38] It should be noted that there are no abrupt boundaries between the DM layers. They are anatomically and functionally connected to each other, constituting a single whole.

DM is a typical fibrous tissue that contains mostly collagen fibrils which constitute more than 90% of its thickness. The average diameter of collagen fibrils assessed by

electron microscopy is about 100 ± 5 nm. [39] Collagen fibrils are the main components of the DM layer which constitutes more than 90% of its thickness, measurement of its optical properties such as extremely high scattering coefficient and relatively low absorption coefficient in the visible and near-infrared wavelength regions, basically means measurement of the optical properties of the whole DM. [40] As in most fibrous structures, the indices of refraction of the main components of the DM, were found as 1.474 for collagen fibrils and 1.345 for the interstitial fluid (at the wavelength of 589 nm). [20]

2.2 | Reagents and sample preparation

Glycerol of 99.0% purity (purchased from Sigma-Aldrich Ltd.) with a refractive index of 1.47 was used in this study as an OCA. [41, 42] To study the TOC, fresh porcine DM was used. Porcine DM may serve as a good model for in vivo human DM studies considering factors such as: gross anatomical structure, housing, feasibility and ethical considerations. [43–45] Due to its structure, the DM is most similar to the sclera and skin dermis. The presence of blood vessels in the DM is the major difference between the structure of the DM and sclera. [38]

The DM samples were taken from 12 pigs provided by the local accredited abattoir (Albertirsa, Hungary) and kept cold on ice in phosphate-buffered saline. Before the measurement, DM was wiped with a paper towel. The

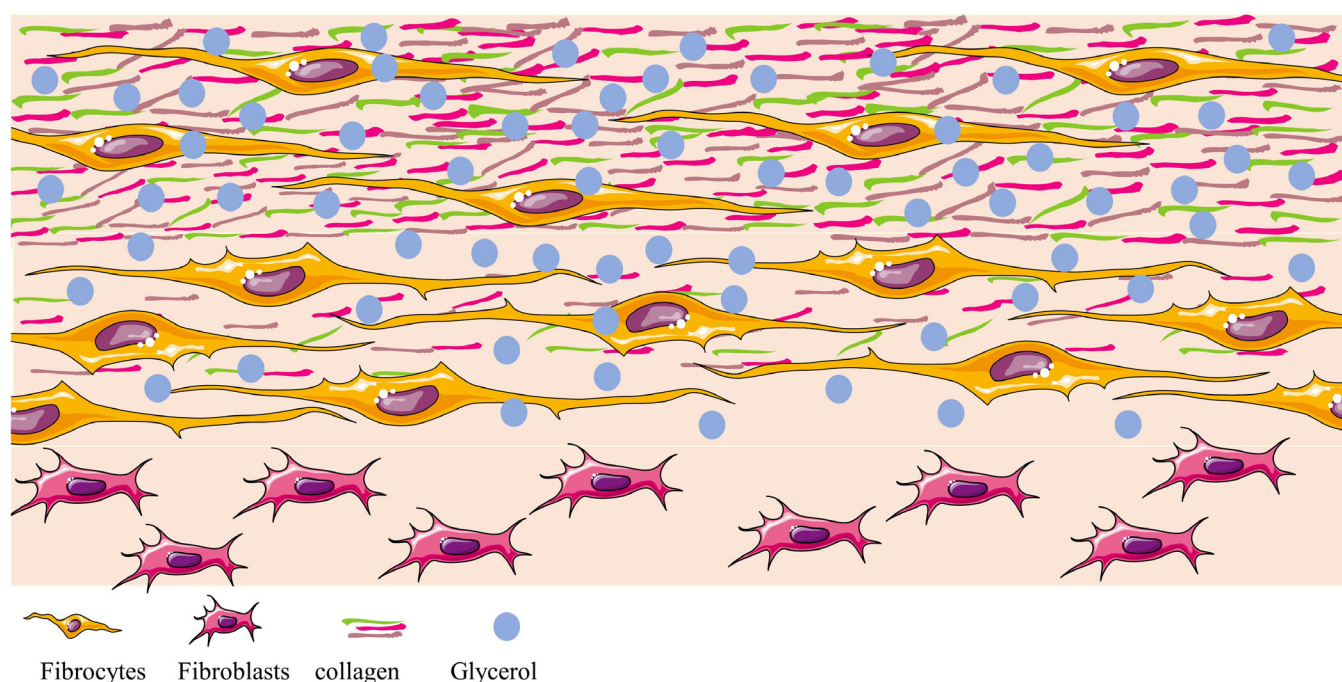


FIGURE 1 Schematically shows the distribution of glycerol molecules when a *dura mater* sample is immersed in glycerol, the average thickness approx. 0.5 mm. The shapes of the cells of different types were adapted from Servier Medical Art (<http://smart.servier.com/>)

tissue samples of 22 mm² size and approximately 0.5 mm thickness were prepared for investigation of TOC effect, a leather punch was used to cut the samples. Finally, the tissue samples were placed in Petri dishes filled with 5 mL of 99.0% glycerol for 5, 10, 15, and 30 min. DM without the OCA treatment (i.e., untreated tissue) was used as a control sample. The thickness of each sample was measured using a digital micrometer before and after TOC. All CRM measurements were performed by placing the tissue samples on silicon substrates for easier handling and examination through the outer endosteal layer.

2.3 | Confocal Raman microspectroscopy

Raman spectra were obtained in the backscattered geometry and confocal mode by using an inVia Raman microscope (Renishaw, UK). To obtain the depth profile (Z) of porcine DM, samples were placed on a computer-controlled three-axis motorized stage allowing the vertical movement of the sample with micrometer resolution (see Figure 2); A 633 nm laser source was used for the measurements with 1200 g/mm gratings. The measurements were performed with spatial resolution of 0.77 μm and a spot size of 1.5 μm . All spectra were recorded and analyzed in the molecular fingerprint spectral region from 400 to 1800 cm^{-1} . Before the Raman measurements, the system was spectrally calibrated using the 520 cm^{-1} Raman band of a silicon wafer.

The laser beam was focused on the sample by a $\times 50$ objective, and the Raman spectra were also collected with it. To prevent tissue damage, the delivered laser power on the DM surface was kept at 8.3 mW. The Raman spectra were collected with 5 s of laser exposure time at three different spots on each sample. Before measurements, remains of the OCA were removed from the sample surface using paper towel.

2.4 | Data analysis

All recorded spectra from each sample were averaged, then processed and analyzed using the Spectragryph software. The spectra processing includes the baseline correction using a polynomial function and curve smoothing using the Savitzky–Golay filter (3rd-order polynomial and nine points interval). [46] Principal component analysis (PCA) was applied to reduce the low variability components of the Raman spectra. The PCA analysis was performed with the built-in algorithm of the Renishaw WIRE software, by using the first four principal components. A more detailed description and the effect of PCA error reduction can be found in the literature. [47, 48]

3 | RESULTS AND DISCUSSION

Figure 3 illustrates an examples series of Raman spectra obtained from the untreated DM at different depths ranging (from 0 to 250 μm) with 25 μm step size. The four principal Raman peaks of the DM corresponding to collagen can be identified at 938 cm^{-1} (C–C stretching mode of collagen), 1246 cm^{-1} (amide III), 1268 cm^{-1} (amide III), and 1666 cm^{-1} (amide I). [9, 49, 50]

The DM tissue is highly scattering in the visible and near-infrared regions due to the mismatching between refractive indices of the collagen fibers and interstitial fluid. The tissue scattering significantly attenuates the power of probing light source and causes the focused beam to spread, which leads to reduction of the detected signal by CRM. As a result, the intensities of Raman bands get weaker with increasing depth. It is clearly seen from Figure 3 that the Raman intensity drastically diminished with the increased depth of investigation due to the limited focus light penetration into DM. The Raman bands cannot be efficiently

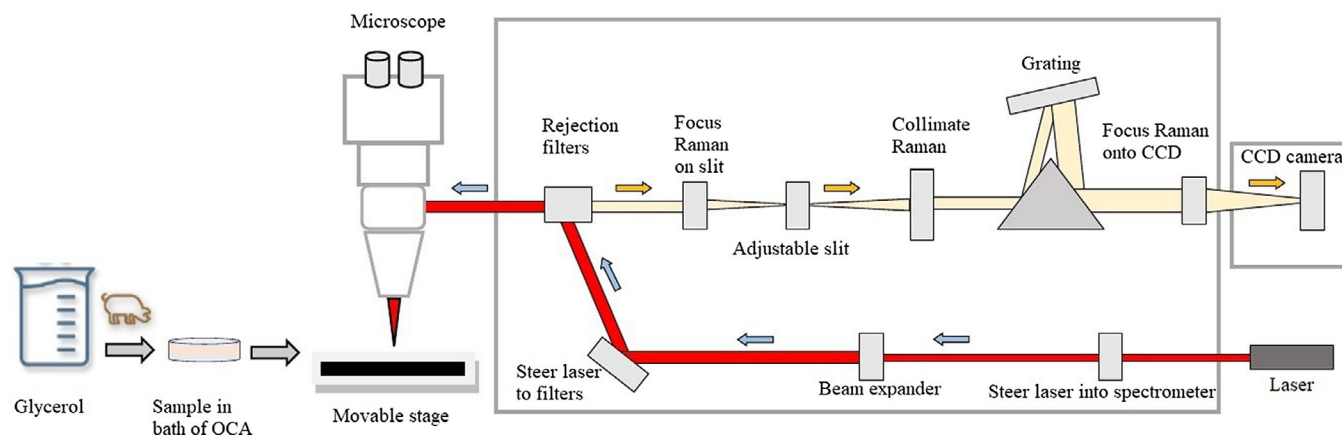


FIGURE 2 Schematic of the experimental setup of the inVia Raman microscope

resolved at the depth of 75–250 μm , which can be ascribed to fewer number of probing and detected photons caused by high scattering and absorption properties of the collagen tissue. Because of the cellular and

subcellular structures with varying refraction indices, fewer photons can travel to the deepest DM regions and at the same time, less Raman-backscattered photons can be detected from there, as well.

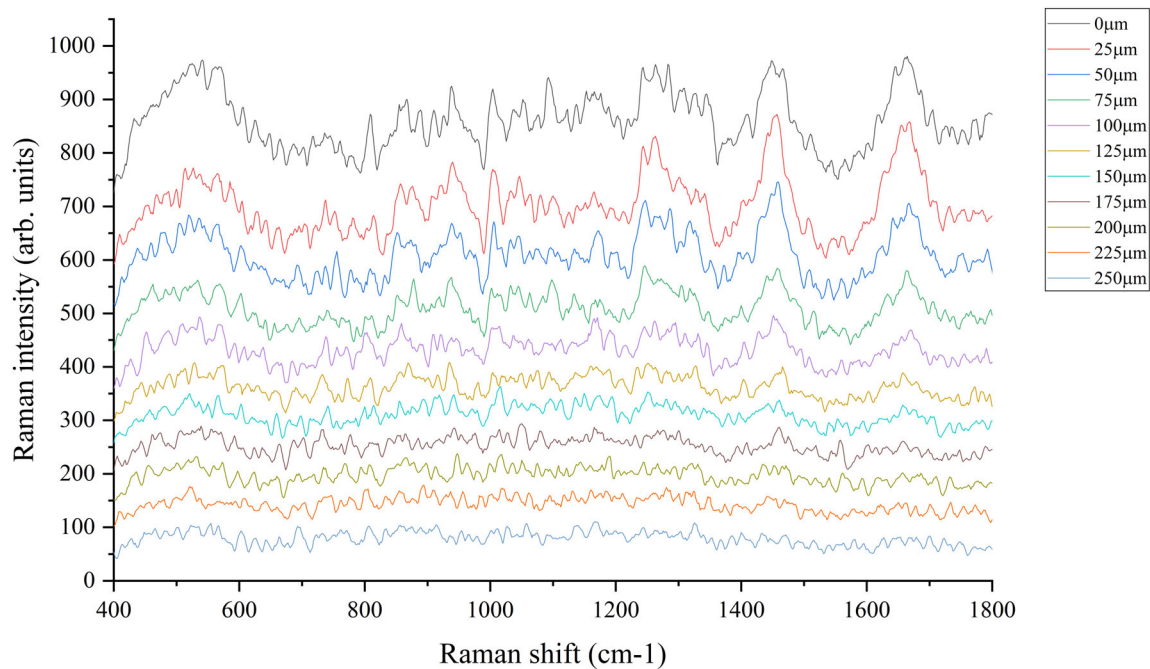


FIGURE 3 Evolution of the Raman spectrum of untreated porcine *dura mater* in the fingerprint region measured at different depths from 0 to 250 μm . The spectra were offset along the ordinate for clarity

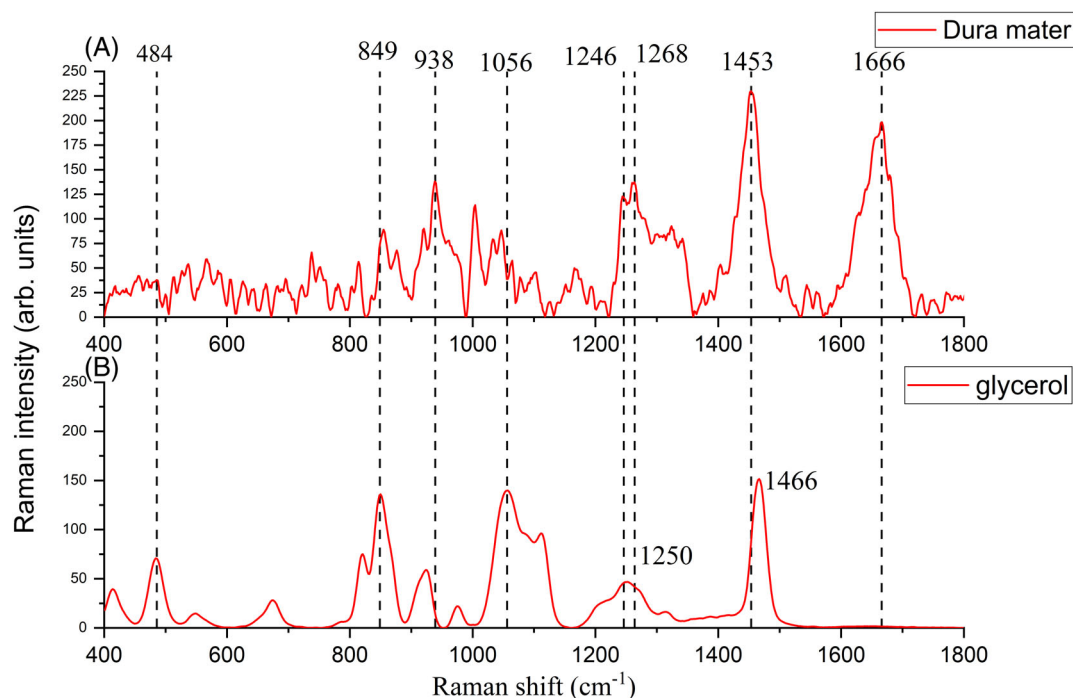


FIGURE 4 (A) Raman spectrum of *dura mater*. (B) 99.0% glycerol. The dashed lines indicate the spectral signatures for the porcine *dura mater* and glycerol

The treatment of DM with glycerol as OCA could improve the quality of the Raman bands in the spectrum of the tissue measured at deeper regions, thus allowing the deeper layer to be investigated and analyzed in detail. Figure 4 compares the Raman spectrum of DM and glycerol excited by a 633 nm laser. The main Raman peaks of glycerol are located at 484, 849, 1056, 1250, and 1466 cm^{-1} . However, Raman spectra of DM and glycerol are partially overlapped. It is possible to recognize the areas where the overlapped of the Raman bands are minimal, for example, the Raman bands at 484, 849, and 1056 cm^{-1} to check the presence of glycerol in the DM. In previous studies, it has been demonstrated that the application of glycerol on tissues could efficiently enhance the Raman to fluorescence ratio and signal contrast. [50, 51]

To study the optical clearing process of DM using 99.0% glycerol, the Raman spectra of untreated and treated porcine DM were obtained at different depths. The depth dependent Raman spectra of porcine DM after 5, 10, 15, and 30 min glycerol treatment are shown in Figure 5A–D, respectively. In order to allow direct comparison between TOC results at different depths, the DM

spectra were firstly baseline corrected and normalized on the amide I band (from 1590 to 1750 cm^{-1}). [52–54]

As it can be seen from Figure 5A–D, the vibrational bands observed in the Raman spectra of the treated DM are much higher in intensities in comparison with the untreated sample (see Figure 3). Such behavior is especially seen in the Raman spectra measured from the deeper layers of treated DM. Therefore, the TOC effect can directly be detected by CRM. As Figure 5 shows, the Raman bands of glycerol are initially visible only in the outermost superficial layers. With increasing treatment time, these bands are also visible in deeper layers of tissue, indicating that glycerol molecules diffuse into these deeper layers of tissue over time. To perform quantitative investigation of the time-dependence of the TOC effect at different depths, it is necessary to compare the spectra obtained at given depths after different treatment times. The results summarized in Figure 6 show the kinetic curves that reveal the effect of treatment time on the intensity of Raman bands of collagen located at 938, 1246, 1268, and 1666 cm^{-1} .

It can be seen in Figure 6 that the Raman band intensities are increasing after TOC treatment due to refractive

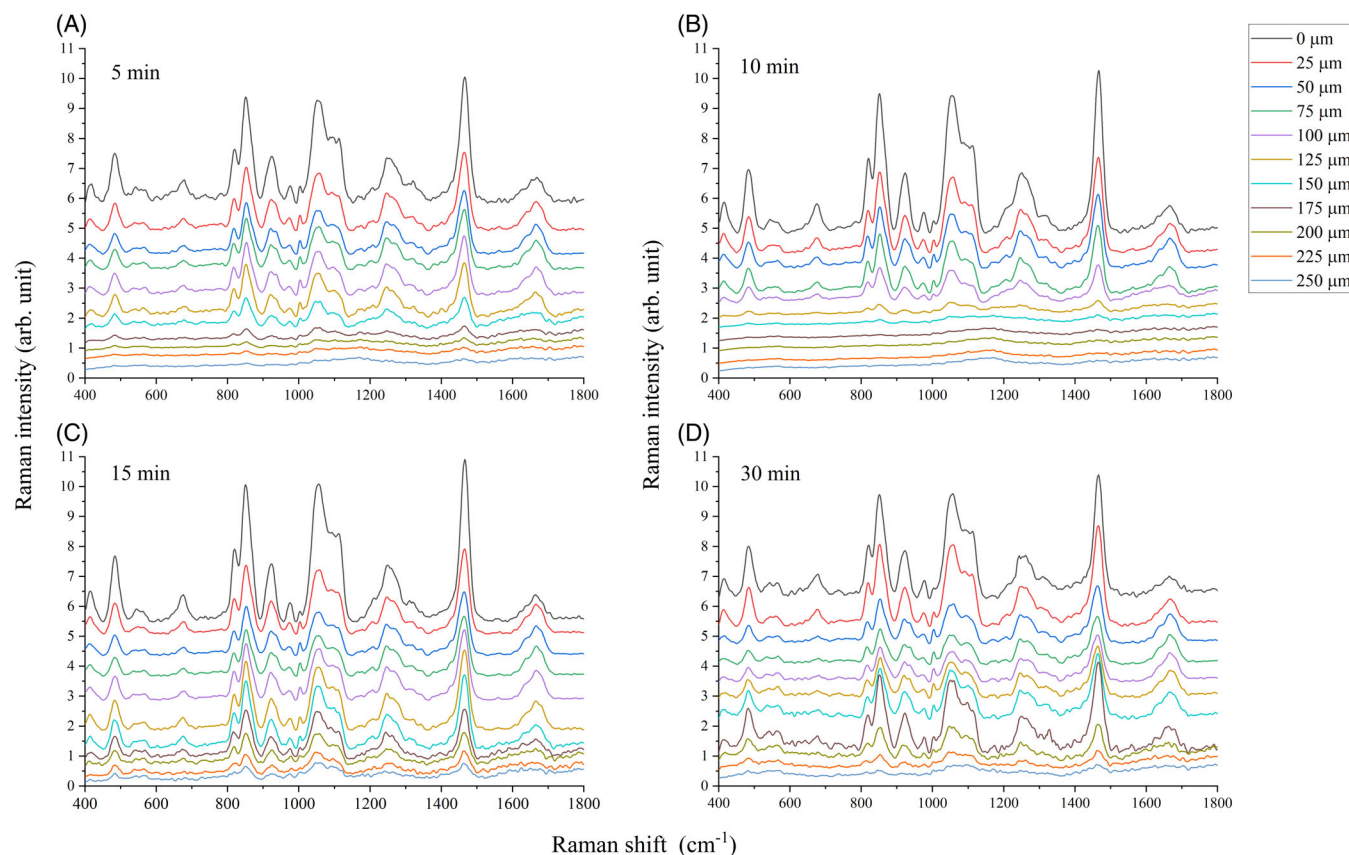


FIGURE 5 Evolution of averaged Raman spectra of porcine *dura mater* treated with 99.0% glycerol for (A) 5 min, (B) 10 min, (C) 15 min, and (D) 30 min, from depths of 0–250 μm in the fingerprint region (400–1800 cm^{-1}). All spectra were excited with a 633 nm laser and recorded using the same conditions

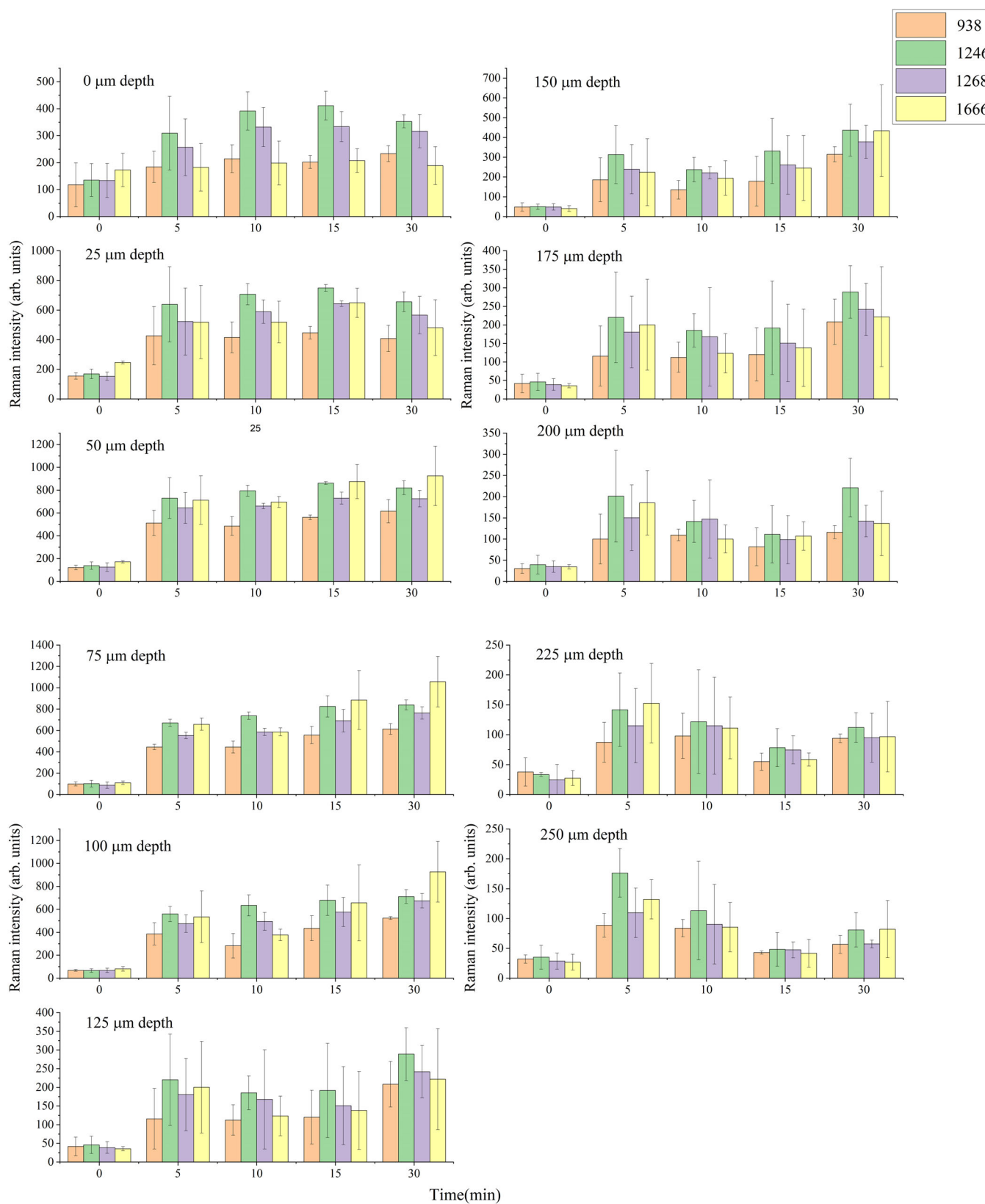


FIGURE 6 Evolution of the Raman bands of DM at 938, 1246, 1268, and 1666 cm^{-1} at different depths from 0 to 250 μm with time, after the application of 99.0% glycerol

index matching and dehydration process caused by glycerol, [55] thus, more compact organization of collagen fibers and less light scattering. Also, the application of OCA results in increased penetration depth of focused light through the DM tissue, [56, 57] leading to improvement of the Raman signal from in-depth tissues layers. The scattering of the laser light in the upper layers of turbid tissue is strongly anisotropic and balances elastic scattering and linear absorption. [58]

For the depths ranging from 0 to 50 μm , the intensities of Raman bands of DM are monotonically increasing after TOC followed by saturation at 15 min treatment after which the intensities start to decrease. In the depth range of 75–125 μm , the intensities of these Raman bands show a monotonic increase with respect to treatment time and achieve maximum at 30 min. This was most likely due to tissue dehydration caused by the osmotic properties of glycerol, [50, 53] as it can be seen in Figure 7.

For the depths ranging from 150 to 200 μm the intensities of Raman bands of DM are decreased after TOC. Also, for some bands, a similar decrease can be observed for 225 and 250 μm at 5–15 min treatment time. The possible reason for this effect can be rehydration due to the accumulation of water coming from deeper tissue layers (where there is no glycerol). Also, for these treatment times, fewer glycerol molecules reach the investigated depths. It is clearly seen that for 150–250 μm depths intensity of the Raman band starts to increase again after

30 min OCA treatment, meaning that glycerol penetrated deeper into tissue.

The 938 and 922 cm^{-1} Raman bands are characteristics for stretching vibrations of skeletal C–C bonds and the C–C bond found in collagen chains, respectively. The ratio of these band intensities (I_{938}/I_{922}) tends to increase with increasing collagen hydration, so it can be defined as a potential spectroscopic marker of collagen hydration. [59, 60] Figure 7 shows the collagen hydration rate for DM calculated at different depths ranging from 0 to 250 μm .

As it can be seen from Figure 7, this ratio is dramatically decreased for all depths during the first 10 min of OCA application. The observed effect can be related to the strong dehydration process caused by glycerol (replacement of DM water molecules by glycerol). In addition, after 10 min of sample treatment the I_{938}/I_{922} ratio is slightly increase for 75–125 μm depths. It can be related to the rehydration of collagen in DM, due to the replacement of DM water in the upper layers with water accumulate from deeper tissue layers from depth 150–200 μm . [61] It can be seen that the ratio is decreasing after 5 min and increasing after 10 min of treatment then decreasing after 15 and 30 min of treatment. For longer treatment time, the glycerol diffusion into the sample results in its overall rehydration expressed as restoration of DM thickness (see Table 1 and Figure 9), providing two phases in TOC of collagenous tissue, fast shrinkage first and then swelling, because after penetration into

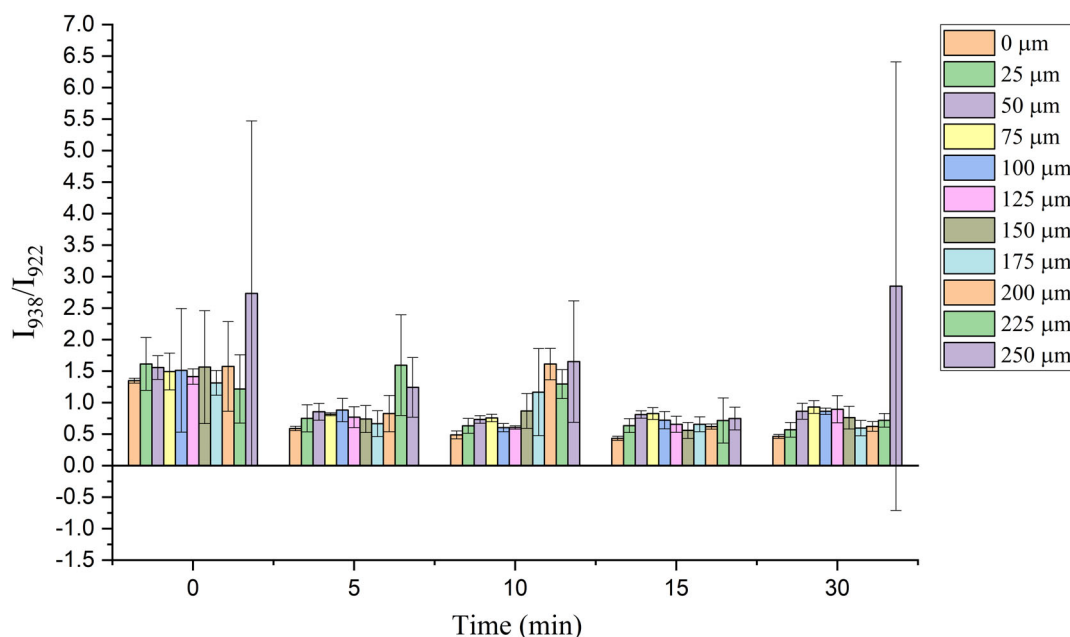


FIGURE 7 The intensity ratio of the 938 and 922 cm^{-1} Raman modes (I_{938}/I_{922}) as a function of treatment time for DM collagen from 0 to 250 μm depths range

TABLE 1 The thickness of DM samples (in mm) before and after 99.0% glycerol treatment for 5, 10, 15, 20, 25, and 30 min

Sample	S1	S2	Ave	STD
Before	0.540 ± 0.089	0.490 ± 0.088	0.515	0.035
After 5 min	0.460 ± 0.089	0.332 ± 0.090	0.396	0.091
Sample	S3	S4	Ave	STD
Before	0.376 ± 0.094	0.546 ± 0.099	0.461	0.120
After 10 min	0.378 ± 0.071	0.384 ± 0.044	0.381	0.004
Sample	S5	S6	Ave	STD
Before	0.664 ± 0.049	0.610 ± 0.057	0.637	0.038
After 15 min	0.500 ± 0.064	0.356 ± 0.034	0.428	0.101
Sample	S7	S8	Ave	STD
Before	0.540 ± 0.082	0.554 ± 0.042	0.547	0.009
After 20 min	0.382 ± 0.068	0.332 ± 0.034	0.357	0.035
Sample	S9	S10	Ave	STD
Before	0.360 ± 0.127	0.480 ± 0.065	0.420	0.084
After 25 min	0.426 ± 0.090	0.440 ± 0.089	0.433	0.009
Sample	S11	S12	Ave	STD
Before	0.442 ± 0.038	0.520 ± 0.058	0.481	0.055
After 30 min	0.510 ± 0.103	0.430 ± 0.059	0.470	0.056

Note: Two samples were measured for each treatment time and the thickness was measured in five different points on each of them. The measurements were performed on DM samples sandwiched between two glass plates by using a micrometer.

tissue, a glycerol molecule can accumulate approximately six molecules of water in tissue. [62, 63]

The Raman bands in the spectral region between 1600 and 1750 cm^{-1} typically consist of four major bands located at 1616, 1645, 1666, and 1681 cm^{-1} ($\nu[\text{C}=\text{C}]$ in phenylalanine and tyrosine; amide I related to both α -helix and β -sheet; amide I α -helical conformation; and amide I disordered structure, nonhydrogen bonds, and $\text{C}=\text{O}$ stretching, respectively). The Raman band at 1666 cm^{-1} was considered as a sensor mode of structural modifications of the amide group. [64] Therefore, spectral shift toward lower wavenumbers of the 1645 and 1666 cm^{-1} bands could be represented as an indication of the appearance of heavier structures in α -helix and β -sheet of collagen. [49] To estimate the spectral shift, Gaussian-Lorentzian deconvolution was applied for the 1590–1720 cm^{-1} range in order to obtain the Raman peak positions (see Figure 8). The full widths at half maximum (FWHM) of the 4 Gaussian-Lorentzian bands was permitted to change within a limit of 20 cm^{-1} .

To establish the principles for a quantitative use of CRM in assessing the collagen structural modification of porcine DM, a precise Raman spectra deconvolution was applied as a function of treatment time and depth (see Figure 9), which describes the physical importance and

the morphological alterations of the Raman bands. Raman band located at $\sim 1666 \text{ cm}^{-1}$ can be considered as a potential spectroscopic marker for structural alterations of the amide group. [64] The reason for the discussion was based on that the stretching mode ($\text{C}=\text{O}$) in the amide I is weakly bonded to the stretching mode of the $\text{C}-\text{N}$ bond and to the in-plane deformation mode of the $\text{N}-\text{H}$ amide bond.

As it is clearly seen in Figure 9, the band at 1666 cm^{-1} displays an upshift in wavenumber after different time treatments with glycerol. This indicate the alterations in molecular geometry of amide I, due to the degradation of collagen triple helix chains and their dissociation into simple string or double strings. [49] Collagen dissociation is one of the TOC mechanisms besides refractive index matching and dehydration. On the other hand, the band at 1666 cm^{-1} shows downshifts in wavenumbers after 10 min treatment at depths starting from 75 μm . This can be interpreted as the process of rehydration. [59, 60]

The Raman band at 1645 cm^{-1} , which encompasses of the amide I contributions from both α -helix and β -sheet, is more complicated to be explained. [65, 66] However, it shifts toward lower wavenumbers which could be considered as a marker for the existence of heavier structures in α -helix and β -sheet. [49]

One of the important points in assessing the TOC effect is the need to precisely measure the thickness of tissue samples during OC. Here, the measurement of thickness of DM was performed only before and after the application of the glycerol as an OCA. However, the measurements introduced in this article showed the limits of possible changes in the geometric parameters of the DM under the action of glycerol. This allows to utilize noninvasive optical imaging techniques such as OCT or confocal microscopy to monitor the thickness of DM, but the change in the index of refraction during the treatment of DM with OCA should also be taken into account. [1] The measured values, showing the limits of possible changes in the geometric parameters of DM after OCA treatment are summarized in Table 1. The results show that the differences in thickness shrinkage/swelling of DM can be correlated with variability from animal to animal. Despite the variability of data presented in Table 1, the averaged values calculated for all samples treated for 5–20 min show the shrinkage of DM tissue from 0.515 mm to about 0.396 mm in the first 5 min of treatment, caused by tissue dehydration. Then due to rehydration process, the thickness reverts to its original value after 30 min treatment. Swelling is expected for the longest time of treatment of DM tissue, as seen in Figure 10. The results of thickness measurement are in good agreement with the Raman analysis and support the three-step character of the TOC

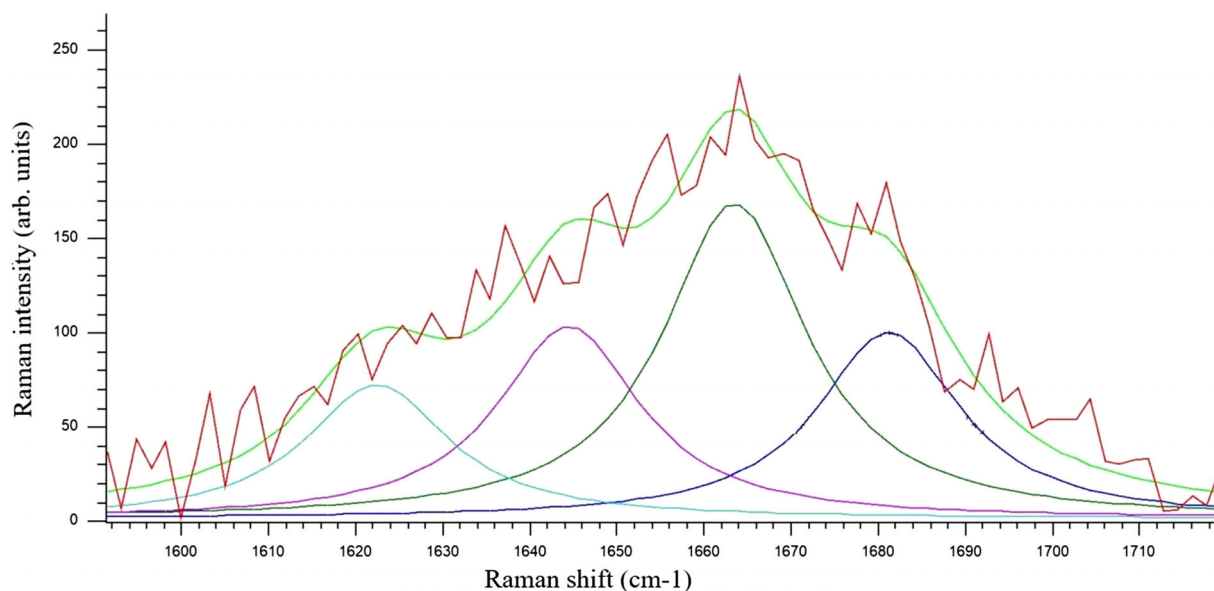


FIGURE 8 Four Gaussian-Lorentzian deconvolution of the amide I band of untreated *dura mater* (at the depth of 25 μm) with peaks centered at $1616 \pm 5 \text{ cm}^{-1}$, $1645 \pm 5 \text{ cm}^{-1}$, $1666 \pm 5 \text{ cm}^{-1}$, and $1681 \pm 5 \text{ cm}^{-1}$

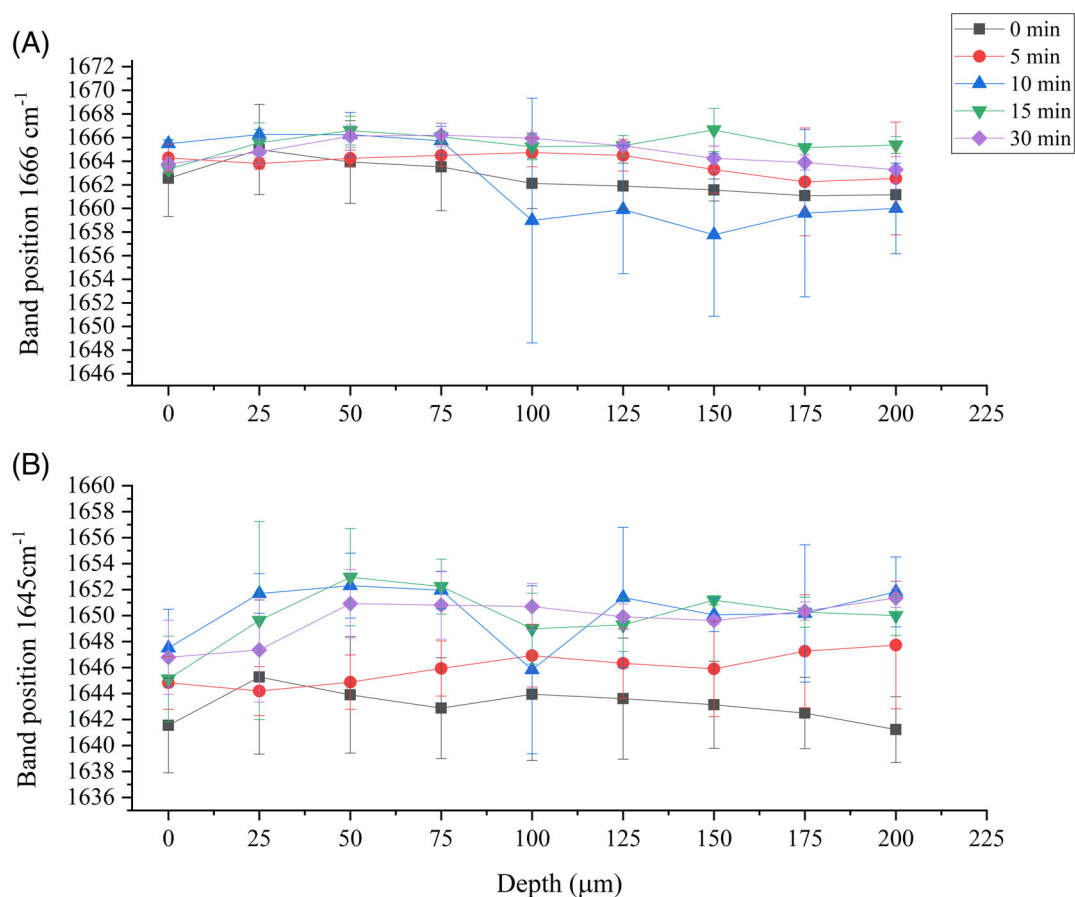


FIGURE 9 Evolution of the position of (A) 1666 cm^{-1} and (B) 1645 cm^{-1} Raman bands as function of depth at different treatment times

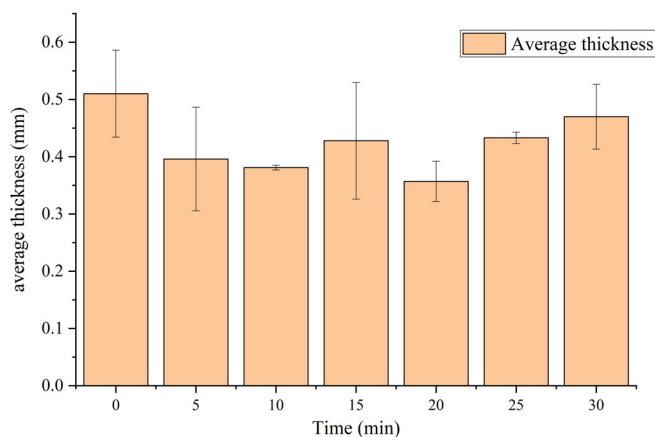


FIGURE 10 Average thickness of *dura mater* samples (in mm) as function of TOC treatment time

process. Such behavior is well fit to an expression of occludin proteins in tissues as a reaction to osmotic stress due to depolymerization of microfilaments or microtubules. [67, 68] Moreover, glycerol has been applied as an osmotic adjuvant to reduce intracranial hypertension. [69] It is important to note that in order to prevent the strong swelling of collagenous tissue under the action of OCA in ex vivo experiments, the treatment time must be <1 h. [70]

A previous study of glycerol effect on the thermal properties of collagen using optical displacement-enhanced heterodyne polarimeter shows improvement of thermal denaturation of collagen. [71] Studies of laser heating of costal cartilage, when using glycerol as an OCA in combination with magnetite nanoparticles, have shown to be effective. [72] Moreover, glycerol interaction with collagen is important in the context of the suitability of the collagenous tissue as a dural graft in the repair of spinal *dura mater* defects. [73, 74]

The inherently weak Raman scattering efficiency and the high scattering of the turbid tissue limits CRM use to superficial layers. To overcome this lack and to extend Raman scattering probing to deeper tissues by using optical clearing method with the assistance of metallic nanoparticles can be further used. Also, the optical clearing method in assisting SERS is a promising approach for the extension of the clinical applications to deeper tissues for instance using nanotags to detect metastatic sentinel lymph nodes. [75, 76]

4 | CONCLUSIONS

Ex vivo investigation of optical clearing of porcine DM with glycerol was performed by using confocal Raman microspectroscopy, including the detailed spectroscopic

depth profiling of the tissue. The results show that by using glycerol as an OCA for 30 min treatment time the information depth of porcine DM can be extended to 250 μm . This significant improvement is due to the reduction of light scattering in the tissue resulting in increased propagation depth of optical radiation into DM by keeping the focusing of the laser beam. As TOC is a dynamic technique, CRM was also successfully used to study the TOC processes related to collagen fibers. The results indicate that the process of tissue optical clearing can be divided into three main steps. The first one is a fast process of tissue dehydration accompanied by collagen shrinkage, while the second relatively slow process is related to the glycerol penetration into the interfibrillar space of collagen combined with swelling of tissue. The third step is related with collagen dissociation caused by high concentration of glycerol. The enhancement of laser light penetration into a DM tissue and, consequently, incensement of the probing depth by controlling the optical parameter is a useful technique and can be applied in laser therapeutic and optical diagnostic and imaging techniques of brain tissues. In addition, our results could be useful for forensic purposes and Burr holes application, where small holes in the skull are used to relieve pressure in the brain during fluid compression of brain tissue. Further work is necessary to define the optimal laser wavelength to study DM disease related to collagen with different OCAs and nanoparticles.

ACKNOWLEDGMENTS

This study was done under a scholarship of the Stipendium Hungaricum Scholarship Programme. This study was supported by the VEKOP-2.3.2-16-2016-00011 and VEKOP-2.3.2-16-2016-00002 grants, which are co-financed by the European Union and European Social Fund. VVT was supported by the grant of the Government of the Russian Federation 075-15-2019-1885. In addition, we would like to appreciate the support of Dr. Riana Gaifulina and Dr. Alaa Al-Halbosy for the technical support.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest relevant to this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ali Jaafar <https://orcid.org/0000-0002-7514-0951>

Valery V. Tuchin <https://orcid.org/0000-0001-7479-2694>

REFERENCES

- [1] V. V. Tuchin, *Tissue Optics: Light Scattering Methods and Instruments for Medical Diagnostics*, 3rd ed., SPIE Press, Bellingham, WA **2015**, p. 988.
- [2] V. V. Tuchin, *Handbook of Optical Biomedical Diagnostics. Light-Tissue Interaction*, 2nd ed., Vol. 1, SPIE Press, Bellingham, WA, USA **2016**, p. 864.
- [3] P. J. Aarnoutse, J. A. Westerhuis, *Anal. Chem.* **2005**, *77*, 1228. <https://doi.org/10.1021/ac0401523>.
- [4] Y. Zhou, C.-H. Liu, Y. Sun, Y. Pu, S. Boydston-White, Y. Liu, R. R. Alfano, *J. Biomed. Opt.* **2012**, *17*, 116021. <https://doi.org/10.1117/1.jbo.17.11.116021>.
- [5] L. E. Jamieson, S. M. Asiala, K. Gracie, K. Faulds, D. Graham, *Annu. Rev. Anal. Chem.* **2017**, *10*, 415. <https://doi.org/10.1146/annurev-anchem-071015-041557>.
- [6] R. Zhang, X. Zhang, H. Wang, Y. Zhang, S. Jiang, C. Hu, Y. Zhang, Y. Luo, Z. Dong, *Angew Chem* **2017**, *129*, 5653. <https://doi.org/10.1002/ange.201702263>.
- [7] C. Gullekson, L. Lucas, K. Hewitt, L. Kreplak, *Biophys. J.* **2011**, *100*, 1837. <https://doi.org/10.1016/j.bpj.2011.02.026>.
- [8] K. S. Shin, A. T. Francis, A. H. Hill, M. Laohajarsang, P. J. Cimino, C. S. Latimer, L. F. Gonzalez-Cuyar, L. N. Sekhar, G. Juric-Sekhar, D. Fu, *Sci. Rep.* **2019**, *9*, 1. <https://doi.org/10.1038/s41598-019-56932-8>.
- [9] S. Koljenović, T. B. Schut, A. Vincent, J. M. Kros, G. J. Puppels, *Anal. Chem.* **2005**, *77*, 7958. <https://doi.org/10.1021/ac0512599>.
- [10] A. Mizuno, T. Hayashi, K. Tashibu, S. Maraishi, K. Kawauchi, Y. Ozaki, *Neurosci. Lett.* **1992**, *141*, 47. [https://doi.org/10.1016/0304-3940\(92\)90331-Z](https://doi.org/10.1016/0304-3940(92)90331-Z).
- [11] A. Mizuno, H. Kitajima, K. Kawauchi, S. Muraishi, Y. Ozaki, *J. Raman Spectrosc.* **1994**, *25*, 25. <https://doi.org/10.1002/jrs.1250250105>.
- [12] Y. Zhang, H. Liu, J. Tang, Z. Li, X. Zhou, R. Zhang, L. Chen, Y. Mao, C. Li, *ACS Appl. Mater. Interfaces* **2017**, *9*, 17769. <https://doi.org/10.1021/acsami.7b04205>.
- [13] A. N. Bashkatov, E. A. Genina, V. I. Kochubey, Y. P. Sinichkin, A. A. Korobov, N. A. Lakodina, V. V. Tuchin, *Control. Tissue Opt. Prop. Appl. Clin. Study.* **2000**, *4162*, 182. <https://doi.org/10.1117/12.405939>.
- [14] E. A. Genina, A. N. Bashkatov, V. I. Kochubey, V. V. Tuchin, *Opt. Spectrosc.* **2005**, *98*, 470. <https://doi.org/10.1134/1.1890530>.
- [15] E. C. Cheshire, R. D. G. Malcomson, S. Joseph, M. J. B. Biggs, D. Adlam, G. N. Rutt, *Forensic Sci. Med. Pathol.* **2015**, *11*, 395. <https://doi.org/10.1007/s12024-015-9691-7>.
- [16] E. Genina, A. Bashkatov, V. Tuchin, *J. Biomed. Photonics Eng.* **2017**, *3*, 010309. <https://doi.org/10.18287/jbpe17.03.010309>.
- [17] V. V. Tuchin, I. L. Maksimova, D. A. Zimnyakov, I. L. Kon, A. K. Mavlutov, A. A. Mishin, *J. Biomed. Opt.* **1997**, *2*, 401. <https://doi.org/10.1117/12.281502>.
- [18] A. Y. Sdobnov, M. E. Darvin, E. A. Genina, A. N. Bashkatov, J. Lademann, V. V. Tuchin, *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2018**, *197*, 216. <https://doi.org/10.1016/j.saa.2018.01.085>.
- [19] L. M. C. Oliveira, V. V. Tuchin, *The Optical Clearing Method: A New Tool for Clinical Practice and Biomedical Engineering*, Springer Nat. Switz. AG, Basel, Switzerland **2019**, p. 177.
- [20] V. V. Tuchin, *Optical Clearing of Tissues and Blood*, PM 154, SPIE Press, Bellingham, WA **2005**, p. 1. <https://doi.org/10.1117/3.637760>.
- [21] V. V. Tuchin, I. L. Maksimova, D. A. Zimnyakov, I. L. Kon, A. K. Mavlutov, A. A. Mishin, *Proc. SPIE - Int. Soc. Opt. Eng.* **1996**, 2925, 118. <https://doi.org/10.1117/12.281502>.
- [22] M. V. Schulmerich, J. H. Cole, K. A. Dooley, M. D. Morris, J. M. Kreider, S. A. Goldstein, *J. Biomed. Opt.* **2008**, *13*, 021108. <https://doi.org/10.1117/1.2892687>.
- [23] O. Zhernovaya, V. V. Tuchin, M. J. Leahy, *Photonics Lasers Med.* **2016**, *5*, 151. <https://doi.org/10.1515/plm-2016-0004>.
- [24] Y. Liang, W. Yuan, J. Mavadia-Shukla, X. Li, *J. Biomed. Opt.* **2016**, *21*, 081211. <https://doi.org/10.1117/1.jbo.21.8.081211>.
- [25] Y. Y. Fu, S. C. Tang, *Microvasc. Res.* **2010**, *80*, 512. <https://doi.org/10.1016/j.mvr.2010.06.003>.
- [26] O. Nadiarnykh, P. J. Campagnola, *Opt. Express* **2009**, *17*, 5794. <https://doi.org/10.1364/oe.17.005794>.
- [27] K. V. Larin, V. V. Tuchin, *Quantum Electron.* **2008**, *38*, 551. <https://doi.org/10.1070/qe2008v038n06abeh013850>.
- [28] X. Zhu, L. Huang, Y. Zheng, Y. Song, Q. Xu, J. Wang, K. Si, S. Duan, W. Gong, *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *166*, 11480. <https://doi.org/10.1073/pnas.1819583116>.
- [29] C. Pan, R. Cai, F. P. Quacquarelli, A. Ghasemigharagoz, A. Loubopoulos, P. Matryba, N. Plesnila, M. Dichgans, F. Hellal, A. Ertürk, *Nat. Methods* **2016**, *13*, 859. <https://doi.org/10.1038/nmeth.3964>.
- [30] H. Hama, H. Hioki, K. Namiki, T. Hoshida, H. Kurokawa, F. Ishidate, T. Kaneko, T. Akagi, T. Saito, T. Saido, A. Miyawaki, *Nat. Neurosci.* **2015**, *18*, 1518. <https://doi.org/10.1038/nn.4107>.
- [31] H. Hama, H. Kurokawa, H. Kawano, R. Ando, T. Shimogori, H. Noda, K. Fukami, A. Sakaue-Sawano, A. Miyawaki, *Nat. Neurosci.* **2011**, *14*, 1481. <https://doi.org/10.1038/nn.2928>.
- [32] D. R. Walsh, A. M. Ross, D. T. Newport, Z. Zhou, J. Kearns, C. Fearon, J. Lorigan, J. J. E. Mulvihill, *Acta Biomater.* **2021**, *134*, 388. <https://doi.org/10.1016/j.actbio.2021.07.043>.
- [33] B. I. Koh, H. J. Lee, P. A. Kwak, M. J. Yang, J. H. Kim, H. S. Kim, G. Y. Koh, I. Kim, *Nat. Commun.* **2020**, *11*, 1. <https://doi.org/10.1038/s41467-020-17545-2>.
- [34] M. J. Riemenschneider, A. Perry, G. Reifengerger, *Lancet Neurol.* **2006**, *5*, 1045. [https://doi.org/10.1016/S1474-4422\(06\)70625-1](https://doi.org/10.1016/S1474-4422(06)70625-1).
- [35] Y. Zhou, C.-H. Liu, B. Wu, X. Yu, G. Cheng, K. Zhu, K. Wang, C. Zhang, M. Zhao, R. Zong, L. Zhang, L. Shi, R. R. Alfano, *J. Biomed. Opt.* **2019**, *24*, 1. <https://doi.org/10.1117/1.jbo.24.9.095001>.
- [36] R. Wolthuis, M. van Aken, K. Fountas, J. S. Robinson, H. A. Bruining, G. J. Puppels, *Anal. Chem.* **2001**, *73*, 3915. <https://doi.org/10.1021/ac0101306>.
- [37] K. Gracie, E. Correa, S. Mabbott, J. A. Dougan, D. Graham, R. Goodacre, K. Faulds, *Chem. Sci.* **2014**, *5*, 1030. <https://doi.org/10.1039/c3sc52875h>.
- [38] V. I. Ziablov, I. N. Shapovalov, K. D. Toskin, V. V. Tkach, V. V. Zhebrovskii, *Arkh. Anat. Gistol. Embriol.* **1982**, *82*, 29.
- [39] A. N. Bashkatov, E. A. Genina, Y. P. Sinichkin, V. I. Kochubey, N. A. Lakodina, V. V. Tuchin, *Biophys. J.* **2003**, *85*, 3310. [https://doi.org/10.1016/S0006-3495\(03\)74750-X](https://doi.org/10.1016/S0006-3495(03)74750-X).
- [40] A. N. Bashkatov, E. A. Genina, V. V. Tuchin, Tissue optical properties. in *Handb. Biomed. Opt.*, CRC Press, Boca Raton, FL, **2016**, p. 67. <https://doi.org/10.1201/b10951-9>.

- [41] V. Genin, D. Tuchina, A. J. Sadeq, E. Genina, V. Tuchin, A. Bashkatov, *J. Biomed. Photonics Eng.* **2016**, 7, 825. <https://doi.org/10.18287/jbpe16.02.010303>.
- [42] M. H. Khan, B. Choi, S. Chess, K. M. Kelly, J. McCullough, J. S. Nelson, *Lasers Surg. Med.* **2004**, 34, 83. <https://doi.org/10.1002/lsm.20014>.
- [43] F. H. Michael Frink, H. Andruszkow, *J. Biomed. Biotechnol.* **2011**, 2011, 1. <https://doi.org/10.1155/2011/797383>.
- [44] R. B. Emilia Mazgajczyk, K. Ścigała, M. Czyż, *Acta Bioeng. Biomech.* **2012**, 14, 51.
- [45] A. Kinaci, W. Bergmann, R. L. A. W. Bleys, A. van der Zwan, T. P. C. van Doormaal, *Comp. Med.* **2020**, 70, 170. <https://doi.org/10.30802/AALAS-CM-19-000022>.
- [46] Spectragryph: optical spectroscopy software: Description, (n.d.). https://www.ffmpeg2.de/spectragryph/about_descr.html.
- [47] S. Mujica Ascencio, C. S. Choe, M. C. Meinke, R. H. Müller, G. V. Maksimov, W. Wigger-Alberti, J. Lademann, M. E. Darvin, *Eur. J. Pharm. Biopharm.* **2016**, 104, 51. <https://doi.org/10.1016/j.ejpb.2016.04.018>.
- [48] C. Choe, J. Lademann, M. E. Darvin, *Analyst* **2016**, 141, 1981. <https://doi.org/10.1039/c5an02373d>.
- [49] G. Pezzotti, M. Boffelli, D. Miyamori, T. Uemura, Y. Marunaka, W. Zhu, H. Ikegaya, *J. Biomed. Opt.* **2015**, 20, 065008. <https://doi.org/10.1117/1.jbo.20.6.065008>.
- [50] A. Y. Sdobnov, V. V. Tuchin, J. Lademann, M. E. Darvin, *J. Phys. D Appl. Phys.* **2017**, 50, 285401. <https://doi.org/10.1088/1361-6463/aa77c9>.
- [51] D. Huang, W. Zhang, H. Zhong, H. Xiong, X. Guo, Z. Guo, *J. Biomed. Opt.* **2012**, 17, 015004. <https://doi.org/10.1117/1.jbo.17.1.015004>.
- [52] P. J. Caspers, G. W. Lucassen, E. A. Carter, H. A. Bruining, G. J. Puppels, *J. Invest. Dermatol.* **2001**, 116, 434. <https://doi.org/10.1046/j.1523-1747.2001.01258.x>.
- [53] A. Y. Sdobnov, M. E. Darvin, J. Schleusener, J. Lademann, V. V. Tuchin, *J. Biophotonics* **2019**, 12, 1. <https://doi.org/10.1002/jbio.201800283>.
- [54] N. Nakagawa, M. Matsumoto, S. Sakai, *Skin Res. Technol.* **2010**, 16, 137. <https://doi.org/10.1111/j.1600-0846.2009.00410.x>.
- [55] J. M. Hirshburg, K. M. Ravikumar, W. Hwang, A. T. Yeh, *J. Biomed. Opt.* **2010**, 15, 055002. <https://doi.org/10.1117/1.3484748>.
- [56] P. Matousek, *Appl. Spectrosc.* **2007**, 61, 845. <https://doi.org/10.1366/000370207781540178>.
- [57] Y. A. Menyaev, D. A. Nedosekin, M. Sarimollaoglu, M. A. Juratli, E. I. Galanzha, V. V. Tuchin, V. P. Zharov, *Biomed. Opt. Express* **2013**, 4, 3030. <https://doi.org/10.1364/boe.4.003030>.
- [58] B. H. Hokr, V. V. Yakovlev, *Opt. Express* **2013**, 21, 11757. <https://doi.org/10.1364/oe.21.011757>.
- [59] Q. Zhang, K. L. Andrew Chan, G. Zhang, T. Gillece, L. Senak, D. J. Moore, R. Mendelsohn, C. R. Flach, *Biopolymers* **2011**, 95, 607. <https://doi.org/10.1002/bip.21618>.
- [60] T. T. Nguyen, T. Happillon, J. Feru, S. Brassart-Passco, J. F. Angiboust, M. Manfait, O. Piot, *J. Raman Spectrosc.* **2013**, 44, 1230. <https://doi.org/10.1002/jrs.4355>.
- [61] S. R. Utz, V. V. Tuchin, E. M. Galkina, *Vestn. Dermatol. Venerol.* **2015**, 4, 60.
- [62] V. Hovhannisyan, P.-S. Hu, S.-J. Chen, C.-S. Kim, C.-Y. Dong, *J. Biomed. Opt.* **2013**, 18, 046004. <https://doi.org/10.1117/1.jbo.18.4.046004>.
- [63] J. J. Towey, A. K. Soper, L. Dougan, *J. Phys. Chem. B* **2012**, 116, 13898. <https://doi.org/10.1021/jp3093034>.
- [64] M. G. Tosato, R. S. Alves, E. A. P. Dos Santos, L. Raniero, P. F. C. Menezes, K. M. S. Belletti, C. E. O. Praes, A. A. Martin, *Photomed. Laser Surg.* **2012**, 30, 85. <https://doi.org/10.1089/pho.2011.3059>.
- [65] R. J. Lakshmi, V. B. Kartha, C. M. Krishna, J. G. R. Solomon, G. Ullas, P. U. Devi, *Radiat. Res.* **2002**, 157, 175. [https://doi.org/10.1667/0033-7587\(2002\)157\[0175:TRSFTS\]2.0.CO;2](https://doi.org/10.1667/0033-7587(2002)157[0175:TRSFTS]2.0.CO;2).
- [66] R. Calheiros, N. F. L. Machado, S. M. Fiuza, A. Gaspar, J. Garrido, N. Milhazes, F. Borges, M. P. M. Marques, *J. Raman Spectrosc.* **2008**, 39, 95. <https://doi.org/10.1002/jrs.1822>.
- [67] C. Li, L. L. Chen, Y. Y. Wang, T. T. Wang, D. Di, H. Zhang, H. H. Zhao, X. Shen, J. Guo, *Int. J. Nanomedicine* **2021**, 16, 1663. <https://doi.org/10.2147/IJN.S291286>.
- [68] S. Cells, J. P. Wiebe, A. Kowalik, R. L. Gallardi, O. Egeler, B. H. Clubb, *J Androl* **2000**, 21, 625.
- [69] S. Gwer, H. Gatakaa, L. Mwai, R. Idro, C. R. Newton, *BMC Pediatr.* **2010**, 10, 1. <https://doi.org/10.1186/1471-2431-10-23>.
- [70] R. LaComb, O. Nadiarnykh, S. Carey, P. J. Campagnola, *J. Biomed. Opt.* **2008**, 13, 021109. <https://doi.org/10.1117/1.2907207>.
- [71] C. M. Wu, H. H. Chen, K. H. Tseng, H. W. Chen, *Appl. Sci.* **2015**, 5, 1184. <https://doi.org/10.3390/app5041184>.
- [72] Y. Alexandrovskaya, K. Sadovnikov, A. Sharov, A. Sherstneva, E. Evtushenko, A. Omelchenko, M. Obrezkova, V. Tuchin, V. Lunin, E. Sobol, *J. Biophotonics* **2018**, 11, e201700105. <https://doi.org/10.1002/jbio.201700105>.
- [73] C. Calikoglu, M. Cakir, Y. Tuzun, *Eurasian J. Med.* **2019**, 51, 133. <https://doi.org/10.5152/eurasianjmed.2018.17422>.
- [74] W. Liu, X. Wang, J. Su, Q. Jiang, J. Wang, Y. Xu, Y. Zheng, Z. Zhong, H. Lin, *Front. Bioeng. Biotechnol.* **2021**, 9, 1. <https://doi.org/10.3389/fbioe.2021.628129>.
- [75] B. Khlebtsov, D. Bratashov, A. Burov, N. Khlebtsov, *Photonics* **2021**, 8, 1. <https://doi.org/10.3390/photonics8050144>.
- [76] Z. Bao, B. Deng, Y. Zhang, X. Li, Z. Tan, Z. Gu, B. Gu, Z. Shao, W. Di, J. Ye, *Biomaterials* **2021**, 276, 121070. <https://doi.org/10.1016/j.biomaterials.2021.121070>.

How to cite this article: A. Jaafar, R. Holomb, A. Y. Sdobnov, Z. Ocskay, Z. Jakus, V. V. Tuchin, M. Veres, *J. Biophotonics* **2022**, 15(4), e202100332. <https://doi.org/10.1002/jbio.202100332>