Enhancement of short coherence digital holographic microscopy by optical clearing

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Abstract: In this work, we used a short coherence digital holographic microscopy system to demonstrate cross-talk noise suppression and imaging performance enhancement by optical clearing. Performance of the system on both phantom and in vitro porcine skin tissues before and after the treatment of 70% v./v. glycerol-saline solution was investigated. Our results showed that optical clearing effectively inhibits the cross-talk noise and improves the image quality in the deep of the in vitro porcine skin tissues. The imaging depth was increased by about 30% after topical application of the glycerol-saline solution for 30 min.

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1. Introduction

Optical coherence tomography (OCT) [1] combines temporal coherence gating with confocal spatial filtering to discriminate light backscattered from different depths and reject multiply-scattered photons arising from scattering specimens. High lateral resolution of a conventional OCT system is usually limited to the focus region (twice the Rayleigh length), which is always much smaller than the depth resolved range of the system. Combined with conventional OCT, numerical reconstruction methods such as digital holography (DH) [2] and inverse scattering [3] have been demonstrated to enlarge the effective focus region [4,5]. However, because of using confocal beam scanning for volume data acquisition, these techniques suffer from reduced sensitivity [6], and additional efforts must be made to maintain phase stability to ensure proper reconstruction [5].

Compared with confocal scanning OCT, full-field OCT (FF-OCT) illuminates samples and detects backscattered light fields in a parallel manner. Both the time domain and Fourier domain OCT systems can work in this parallel configuration [7,8]. This configuration offers advantages of fast three-dimensional image acquisition and high phase stability in the en face image plane [9–11]. With the amplitude and phase of the scattered field detected by full-field systems, either inverse scattering or DH can retrieve the structure image outside the focus region. Tomographic imaging with depth-independent resolution and sensitivity can be possibly achieved. An algorithm was proposed and simulated to recover object structures both inside and outside the focus depth, by solving the inverse scattering problem for FF-OCT [12]. Short coherence digital holographic microscopy (SCDHM), which employed a similar system as used in FF-OCT but recorded the interference patterns of the defocused backscattered fields with a reference field, was able to perform optical tomography of specimens out of focus by digital focusing [13–15]. Holoscopy [6,16], combining full-field FD-OCT with DH, was proposed to obtain tomograms with diffraction-limited resolution and uniform sensitivity over several Rayleigh lengths.

For the aforementioned full-field coherence imaging techniques using numerical reconstruction methods, expected results were well demonstrated with tissue phantoms consisting of scatterers distributed within transparent substrates in a low density. However, experimental results of scattering biological tissues showed limited improvements of the effective imaging depth. That is because a sufficiently highly scattering or absorbing tissue is much more complex than tissue phantoms. Sample-induced aberrations produced by multiple scattering, inhomogeneities in the refractive index (RI), absorption, dispersion and diffraction [14], change the light behavior inside a biological sample, and result in degrading of image quality of deep layers. If only scattering is considered, multiple scattering can decrease the penetration depth, and cause artifacts which reduce the image resolution, contrast, and the maximum imaging depth. Especially for full-field approaches, multiple scattering often leads to significant optical crosstalk when scattering samples are studied [17,18]. The latter hinders shot-noise-limited detection and diffraction-limited imaging [6,12,17]. Hence, imaging performance of these numerical reconstruction methods is limited by the optical properties of scattering biological tissues, and their abilities of large depth imaging with uniform resolution are far away from fully demonstrated.

From the perspective of changing the optical property of a scattering tissue, the optical clearing (OC) technique enlarges the imaging depth by reducing the scattering of biological tissues and making them more transparent [19,20]. This technique diffuses optical clearing agents (OCAs) with high RI into tissues. The penetration of OCAs reduces the RI mismatch between the intracellular and the extracellular tissue components, resulting in a reduction of the scattering coefficient [20]. In the last two decades, OC have been combined with many optical imaging modalities to improve the imaging depth or contrast [21–27].

Towards taking full advantage of the large depth imaging ability of the numerical reconstruction methods conducted on full-field systems, as an example, this work combined the OC technique with a SCDHM system for in vitro biological scattering tissues imaging.
The bases of using OC for enhancing the imaging performance of SCDHM were discussed. An on-axis phase shifting SCDHM system was built. We considered the detected light fields from the sample arm as superposition of the desired wavefronts diffracting through scattering tissues and multiple-scattering-induced noise. Then two imaging modes were performed: transmission mode (TM) imaging was performed to show the improvement effects of OC on a measured wavefront propagating through an in vitro thin scattering tissue slice, including analyses of both the reconstructed intensity and phase images of a phantom; reflection mode (RM) imaging was performed to demonstrate crosstalk suppression and imaging performance enhancement by OC when tomographic imaging in vitro thick scattering biological tissues. The TM imaging experiments could be considered as a finite element analysis of effects of OC on wavefronts diffracting through in vitro thick scattering tissues. Experimental results demonstrated that OC improves the wavefront propagation, decreases the cross-talk noise effectively and enhances the imaging performance of a SCDHM system for observing deep layers within in vitro porcine skin tissues. The image resolution, contrast, and imaging depth can be improved.

2. Theory

For full-field coherence imaging techniques, a considerable amount of multiply-scattered light is collected by the detector due to the parallel illumination and detection manner. Crosstalk occurs when the light originating from the coherence gated imaging depth arrives at the detector and interferes with the multiply-scattered light having the same optical path length [17, 18, 28]. In parallel OCT, this cross-talk-generated noise can be suppressed efficiently by using spatially incoherent illumination or partially spatially coherent illumination [28, 29]. However, attenuating the degree of spatial coherence of light sources could result in incorrectly sampling the diffractive light fields [6] and reducing the axial imaging range [30] for numerical reconstruction techniques.

A comprehensive study has been presented to discuss multiple-scattering effects in FF-OCT realized with a broadband spatially coherent light source [17]. The dependences of crosstalk on sample properties including the anisotropy (g) and the optical density (OD) were investigated. It was found that the worst cross-talk effects are obtained for scatterers with diameters approaching the wavelength, and scattering samples with larger OD [17]. This study indicates that for purely scattering samples, reducing the scattering coefficient (μs) could decrease the cross-talk noise, leading to higher contrast and resolution. Suppressing cross-talk noise by manipulating the optical properties of scattering samples, could be of significance to full-field coherence imaging techniques which use numerical reconstruction since no spatial coherence needs to be sacrificed.

OC was proposed as a method of reducing scattering by altering the optical properties of tissues [19, 20]. For soft tissues, collagen, elastic fibers, cells and cell compartments, may be considered as scattering particles with higher RI, which are surrounded by the interstitial fluid or cytoplasm with lower RI. For a first-order approximation, these scattering particles are assumed to be scattering spheres. According to the scattering spheres model, the scattering coefficient μs can be given by [31]

\[ \mu_s = \frac{3.28\pi r^2 \rho}{1-g} \left( \frac{2\pi r}{\lambda} \right)^{0.37} \left( \frac{n_s}{n_o} - 1 \right)^{2.09}, \]  

where g is the anisotropy factor, r is the radius of the scatterers, ρ is the volume density of the scatterers, λ is the wavelength of incident radiation, and ns and no are the refractive indices of the scattering particles and surrounding media, respectively. Equation (1) predicts the scattering coefficient μs with a high accuracy for spherical particles in the ranges of
According to Eq. (1), the more the RI of background media matches that of scattering particles, the smaller the scattering coefficient will be. Therefore, the major mechanism of OC to reduce scattering is that the diffusion of OCAs increases the RI of interstitial fluid and/or cytoplasm, and reduces the RI mismatch among of various tissue components. Moreover, topical application of hyperosmotic OCAs often leads to dehydration of tissue and increases packing density of scatterers [20], which may result in spatial correlations between scatterings and reduce scattering further [19,32].

Reduction of the scattering coefficient induced by OC can alter not only the scattering intensity but also the phase distribution of the scattered optical field. Suppose a thin slice of scattering medium with thickness $L$ is irradiated by a plane wave and imaged by quantitative phase imaging approaches. According to the scattering-phase theorem [33,34], the scattering coefficient $\mu_s$ and the anisotropy factor $g$ of the medium can be expressed as [34]

$$\mu_s L = \langle (\Delta \phi)^2 \rangle, \quad g = 1 - \frac{\langle |\nabla \phi|^2 \rangle}{2k^2 \langle (\Delta \phi)^2 \rangle},$$

(2)

where $\phi$ is the measured spatially resolved phase image, $\langle \rangle$ denotes spatial average, $\Delta \phi \equiv \phi - \langle \phi \rangle$, $|\nabla \phi|^2 = (\partial \phi / \partial x)^2 + (\partial \phi / \partial y)^2$, and $k = 2\pi n_b / \lambda$ with $n_b$ the background RI and $\lambda$ the wavelength of light in vacuum. Eq. (2) is valid under the condition of $\mu_s L \ll 1$, or $|\Delta \phi| \ll 1$. It can be seen that the scattering coefficient is proportional to the spatial variance of the phase while the anisotropy factor $g$ is related to the spatially averaged phase gradient intensity. If $\mu_s$ is reduced and $g$ remains unchanged or is increased, the phase distribution propagating through the medium will suffer less variance, and the variance along the image plane will be more moderate. Therefore, the reconstructed phase distribution will be closer to the original. If considering the bulk tissue as a stack of many thin tissue slices, the wavefront propagating through lower scattering slices will degrade at a slower rate against the depth. It means not only the incident light can penetrate through more tissue slices, but also both amplitude and phase of the light field backscattered from deep can be recorded more accurately. This is crucial for SCDHM, since the complex amplitude of the detected light field is needed to perform the numerical post-processing.

From the above discussion, combining OC with full-field coherence imaging techniques which use numerical reconstruction, is expected to reduce the multiple-scattering-induced cross-talk noise, improve the wavefront propagation, and image deeper in scattering tissues, without compromising spatial coherence of the illumination.

3. Materials and methods

3.1 On-axis phase shifting SCDHM system

The schematic of our on-axis phase shifting SCDHM system is shown in Fig. 1. The system was based on a Michelson interferometer with the Linnik geometry. The light source was a superluminescent diode (SLD, Inphenix) used in OCT, with a central wavelength of 835 nm and 3dB bandwidth of 38 nm, corresponding to a coherence length of ~8.07 $\mu$m in air. The SLD delivered an optical power of 4.2 mW to a single-mode fiber. After collimated, the light beam was focused by $L_1$, and the focal beam was separated by a 50:50 beam splitter. Then the foci were adjusted to be at the back focal planes of two identical objective lenses respectively. In the reference arm, the parallel light beam output from $O_1$ irradiated normally onto a reflector, which was mounted on a piezo stage (P-542.2SL, PI) for phase shifting or small range scanning. Besides, the whole piezo stage was fixed on an electric displacement platform (TSA30-C, Zolix, China) for large range scanning (not shown in Fig. 1). In the sample arm, the specimen was illuminated by a parallel beam, and light
backscattered from within the sample was collected by OL2 and delivered onto a CCD by L2. The whole system was implemented on-axis mode. Holograms were formed when the optical path lengths of the reference beam and the light field scattered from the sample were matched within the coherence length of the source. A 12-bit monochrome CCD camera (G-146B/C, Manta) with $1388 \times 1038$ pixels and a pixel size of $4.65 \, \mu m$ was used to digitize the holograms. The camera worked on a $2 \times 2$ binning mode and a subregion of $512 \times 512$ pixels of the binned image was used for post-processing.

There were some different arrangements of the system between the TM imaging and the RM imaging experiments. In the TM imaging experiments, a neutral density filter with OD = 1 was placed between CL and L1 to adjust the optical power. Two identical $25 \times$ objective lenses (Daheng Optics, China) with NA = 0.4 were used, and provided an imaging field of about $224 \times 224 \, \mu m^2$ and a measured resolution of about $1.3 \, \mu m$. The reflector in the reference arm was a piece of silicon wafer which has a reflectivity of about $33\%$ at the wavelength of $835 \, nm$. The exposure time of the CCD camera was set to be $60 \, \mu s$. In the RM imaging experiments, the NDF was removed, and the two objectives were both replaced with two identical $10 \times$ objective lenses (Daheng Optics, China) with NA = 0.25. By changing the objectives, a larger imaging field of $484 \times 484 \, \mu m^2$ was obtained, with a measured resolution of about $2.1 \, \mu m$ and a depth of focus of about $30 \, \mu m$. The silicon wafer was replaced with an optical wedge ($4\degree$) made of MgF2, and the first surface was served as a reflector with a reflectivity of about $2.5\%$ at the used wavelength. The exposure time of the CCD camera was set to be $200 \, \mu s$. The light power of the output beam in the sample arm was measured to be about $1.92 \, mW$.

![Fig. 1. Schematic of the on-axis phase shifting SCDHM system. SLD, superluminescent diode; SMF, single-mode fiber; CL, collimator; NDF, neutral density filter; L1, L2, achromatic lenses ($f1 = 150 \, mm$ and $f2 = 200 \, mm$); BS, beam splitter; OL1, OL2, objective lenses; RF, reflector; PI, piezo stage; SS, sample stage.](image)

The system needed a piezo stage in the reference arm to conduct phase shifting and axial scanning. For one measurement of a certain depth, a three-step phase shifting technique [35] was employed to evaluate the complex amplitude of the object wave. After every phase shift of $\pi/2$, the phase-shifted interference pattern was recorded 4 times consecutively and the acquired holograms were accumulated as a new hologram for post process. The number of holograms for one accumulation was set as a compromise between the sensitivity and the
measurement speed. Then the complex amplitude of the object wave at the CCD plane was calculated with the three new holograms, and numerically focused to the object plane by the angular spectrum method [15]. The optimal digital refocus distance was determined by a widely used criteria which is based on amplitude analysis [36]. Phase shifting, axial scanning, and data acquisition were synchronized by one LabVIEW program, and the post process was done by another. It cost about 0.83 s for one measurement at a certain depth when running the former program on a computer with an Intel Core i5 CPU and 4GB RAM. An experimental sensitivity of about 48 dB and a phase resolution of about 0.035 rad were achieved for RM imaging a coverslip which was defocused by 200 μm.

3.2 Chemical agents and tissue preparations

Glycerol-water solution was one of the most effective OCAs [20]. In our work, glycerol solution with a concentration of 70% v/v was used by mixing glycerol with saline solution. The glycerol was purchased from Damao Chemical Reagent Factory (Tianjin, China), with a purity of 99.0%. With the method reported in [37], the RI of the 70% v/v. glycerol solution was measured to be 1.434 ± 0.002 (mean ± standard deviation) by an 835 nm OCT system described in [38]. To prepare thin tissue samples for the TM imaging experiments, colons freshly taken from 6 weeks old NIH mice (Guangdong Medical Laboratory Animal Center, China) were cleaned and cut into pieces with a size of about 10 × 10 mm². The thickness of each colon sample was measured by averaging results of five locations using the 835 nm OCT system. Then colon samples close in thickness were selected for the experiments and stored in saline solution at 4°C. These selected samples had a measured thickness within 90 ± 15 μm. The average RI of mouse colon was measured to be 1.38 ± 0.01 (mean ± standard deviation) at 835 nm in an additional experiment with the reported method [37] mentioned above. For the RM imaging experiments, thick tissue samples of fresh porcine skin obtained from an accredited abattoir were prepared in 20 × 20 mm² with a thickness of about 4 mm, cleaned and stored in saline solution at 4°C. According to [39], the in vitro porcine skin epidermis has a thickness of ~100 μm and an average RI of 1.402 at 850 nm, while the dermis has a thickness on the scale of millimeter and an average RI of 1.365 at 850 nm. All tissue samples equilibrated for 30 min. in a fresh volume of saline with room temperature before the experiments, and were measured within 12 hours of removal. Experimental procedures carried out on animals were in conformance with the laboratory animal protocol approved by Tsinghua University.

3.3 Transmission mode imaging

For TM imaging experiments, an optical resolution test board covered by a piece of mouse colon was used as an experimental phantom. The light double passing through the tissue sample and reflected by the chromium elements of the test board was detected for digital reconstruction. For one measurement of the covered test board, a precise depth scanning of the reflector in the reference arm was implemented to sample the whole interference region. Then the reconstruction image with the maximum intensity was regarded as the measurement result. The scanning interval of the piezo stage was set to be 0.25 μm for a scanning range of 15 μm. Such a dense sampling enabled the relative standard deviation of 20 measurements of the average intensity of a bare test board to be less than 3%.

Two groups of experiments were conducted in transmission mode to demonstrate the effects of OC on both the intensity and phase distribution, respectively. For the intensity investigation, each piece of the colon sample was placed to cover the elements of group 6 and 7 of the test board. These elements were measured before, 1 min. and 5 min. after the topical application of 10 μl glycerol solution on the tissue. A coverslip was put on the colon slice to flatten the sample and prevent the natural dehydration during the measurements. For control experiments, colon samples were treated with 10 μl saline solution. Both the experimental and control groups collected measurement results of 5 samples. For the phase investigation, two
chromium elements of line 1 of group 4 of the test board were covered by the tissue slice and measured. By choosing such a pattern for imaging, the effects of OC on the phase distributions with three different intensity levels were studied. Other experimental procedures were the same as those of the former. Before every measurement, the test board was firstly focused to adjust the imaging area, and then defocused by 200 μm to simulate a structure layer out of focus.

To quantify the optical clearing efficacy on the intensity distribution, a defined parameter called relative average intensity $I_r$ was calculated as

$$I_r = \frac{I_{treated}}{I_{initial}}, \tag{3}$$

where $I_{initial}$ and $I_{treated}$ denoted the average intensities of the reconstructed intensity images of the test board before and after the treatment of OCA, respectively. The image resolution was determined by the reconstructed line pair with both the highest spatial frequency and a fringe contrast higher than 80%. Besides, two subregions both of 50 × 50 pixels corresponding to a chromium element and the substrate of the test board respectively were selected from the reconstructed intensity image, and the ratio of the average intensity of the former to that of the later was defined as the image contrast of the reconstructed intensity images of the test board. Then a relative image contrast $C_r$ was calculated as

$$C_r = \frac{C_{treated}}{C_{initial}}, \tag{4}$$

where $C_{initial}$ and $C_{treated}$ denoted the image contrasts of the reconstructed intensity images of the test board before and after the treatment of OCA, respectively. Furthermore, we defined a parameter called normalized correlation peak (NCP) as

$$NCP = \frac{(W_{covered} \otimes W_{orig})_{max}}{(W_{orig} \otimes W_{orig})_{max}}, \tag{5}$$

where $\otimes$ denoted the cross-correlation operation, $(..)_{max}$ meant taking the maximum, $W_{orig}$ and $W_{covered}$ were the reconstructed intensity images of the test board without and with covering of the colon sample, respectively. This parameter was used to evaluate correlations between the reconstructed intensity images under different treatment conditions and that of a bare test board.

To quantify the optical clearing efficacy on the phase distributions with different intensities, the spatial variance of the phase distribution $D = \langle (\Delta \phi)^2 \rangle$ and the spatial average of the phase gradient intensity $G = \langle |\nabla \phi| \rangle$ were computed in windows of 11 × 11 pixels across the entire reconstructed phase image. Then the $D$ map and $G$ map were respectively segmented into three zones corresponding to three different intensity levels. For each zone of each map, three subregions each with size of 50 × 50 pixels were selected and averaged, and the result was regarded as one measurement of $D$ or $G$ of that zone. All the subregions were carefully picked to avoid artifacts caused by the $2\pi$ phase jump. Then a relative spatial variance of phase $D_r$ and a relative spatial average of phase gradient intensity $G_r$ were defined as

$$D_r = \frac{D_{treated}}{D_{initial}}, G_r = \frac{G_{treated}}{G_{initial}}, \tag{6}$$
with $D_{\text{initial}}$ and $D_{\text{treated}}$ the spatial variances of the phases of one zone before and after the treatment of OCA, respectively; $G_{\text{initial}}$ and $G_{\text{treated}}$ the spatial averages of the phase gradient intensities of one zone before and after the treatment of OCA, respectively.

3.4 Reflection mode imaging

For RM imaging experiments, the underside of the porcine skin sample was submersed in saline solution to keep the hydration of the skin, with the epidermis exposed to the air for measurement. Prior to measurement, the focus plane of the objective was set on the skin surface. Then axial scannings of the coherence gating from about 20 μm above the skin surface to about 445 μm depth in tissue were implemented at the same site before, 15, 30, 45 and 60 min. after the topical application of 100 μl glycerol solution. The glycerol solution on the skin was gently removed with filter paper right before the axial scanning and added again right after the measurement. Considering the tradeoff between scanning range and total acquisition time, the scanning interval of the electric displacement platform was set to be 6.38 μm, and 100 depths were measured in about 83 s for one axial scanning. The scanning range in porcine skin samples was determined with the values of the thicknesses and average refractive indices of both the porcine skin epidermis and dermis mentioned in section 3.2. 5 pieces of porcine skin samples were measured for average.

To quantify the intensity attenuation against the depth, intensity images reconstructed from one axial scan were averaged respectively, and the results were regarded as one averaged pseudo A-scan. For image contrast, the OTSU algorithm [40], a widely used adaptive thresholding method, was applied to every intensity image, and then the whole image was segmented as object and background. We defined the ratio of the average of the object intensity and that of the background intensity as the image contrast. The imaging depth was defined as the depth beyond which the image contrast fell below 3.

4. Results

4.1 Transmission mode imaging: intensity image

Figure 2 shows the reconstruction results of the resolution test board through an in vitro mouse colon tissue slice at different stage of the OCA treatment. As a reference, Fig. 2(a) shows the reconstructed intensity image of the bare test board. Figure 2(b)-2(d) are the reconstructed images through a piece of mouse colon before, 1 min. and 5 min. after the topical application of glycerol solution, respectively. Figure 2(e)-2(g) are the reconstructed results of one controlled experiment using saline solution for treatment. It can be seen that the reconstructed pattern of the test board becomes visible and clear within 1 min. after the topical application of the glycerol solution to the tissue slice, especially for the elements of group 7 in the top-right corner. Both the intensity and resolution of the pattern are enhanced. However, the results of the control experiment show no obvious changes during the treatment of the saline solution. 20 horizontal lines adjacent to the white mark in every reconstructed image of both the experimental and control groups were averaged respectively and shown in Fig. 2(h) and 2(i). From Fig. 2(h), the maximums of the intensities after the addition of glycerol solution are about 2.7 times as large as that before OC, while the intensity distributions differ little between situations of 1 min. and 5 min. after the treatment. Moreover, the contrasts of the intensity curves also increase obviously after applying the glycerol solution. The results in Fig. 2(i) show no notable increases of either the intensity or the contrast after treatment of saline solution.
Fig. 2. Reconstructed intensity images of the phantom. (a) Reconstructed image of the test board covered by no tissue. (b)-(d) Reconstructed images of the test board through a piece of mouse colon without, 1 min. and 5 min. after the topical application of glycerol solution, respectively. (e)-(g) Reconstructed images of one controlled experiment using saline solution for treatment. The field of view of each reconstructed image was 224 × 224 μm². (h) Averaged results of horizontal lines adjacent to the white marks in (a)-(d) respectively, and (i) that in (a), (c)-(g) respectively. Note that the intensity scale is enlarged due to the accumulation of holograms.

To quantitatively evaluate the optical clearing efficacy on the reconstructed intensity distribution, the relative average intensities, the relative image contrasts, the image resolutions and the normalized correlation peaks of the reconstructed intensity images before, 1 min. and 5 min. after the treatment were calculated, and averaged for 5 samples of both the experimental group and the control group, respectively. Figure 3(a) shows that the relative average intensities of the reconstructed images after the treatment of glycerol solution are about 1.9 times of that before OC. The green and red boxes marked in the reconstructed images in Fig. 2 represent the two subregions selected for calculation of the image contrast. And it can be seen from Fig. 3(b) that the relative average contrasts both increase by about 4.3 times for the situations of 1 min. and 5 min. after the OC treatment. Moreover, the average image resolution is found to improve from worse than 7.81 μm before OC to 3.24 ± 0.61 μm (mean ± standard deviation) after the 1-min treatment. On the whole, as shown in Fig. 3(c), the reconstructed images enhanced by OC are more correlated to the original intensity distribution. For the control group, all the results after applying the saline solution keep the mean value almost as before the treatment. Error bars of these results depict the maximum and minimum of different samples, and could be resulted from the non-uniform thickness of samples, the inhomogeneity of tissue, the different effects of the agents on different tissues, and the measurement error.

In brief, the quality of the reconstructed intensity image of a wavefront propagating through an in vitro mouse colon tissue slice was improved by the OC treatment. The image intensity, contrast and resolution were all enhanced.
Fig. 3. Average results of the (a) relative average intensity, (b) relative contrast and (c) normalized correlation peak of the reconstructed intensity images before, 1 min. and 5 min. after the treatment, for both the experimental and control groups. Error bars show the maximum and minimum of different samples.

4.2 Transmission mode imaging: phase image

Figure 4 shows the reconstructed intensity and phase distributions of the resolution test board through an in vitro mouse colon tissue slice before and after the OCA treatment. As a reference, Fig. 4(a) and (e) show the reconstructed results of the test board without any tissue. Figure 4(b) and 4(f), 4(c) and 4(g), and 4(d) and 4(h) are the reconstructed intensity and phase images of the test board through a piece of mouse colon before, 1 min. and 5 min. after topically applying the glycerol solution, respectively. Figure 4(i) and 4(l), 4(j) and 4(m), and 4(k) and 4(n) are the reconstructed results of one controlled experiment correspondingly. It can be seen that besides the enhancements of the reconstructed intensity images, the chromium bars in the reconstructed phase images become more recognizable after the treatment of glycerol solution. It also can be found that the borders made of the $2\pi$ phase jumps become more regular, and the phase areas without $2\pi$ jumps become more continuous and less noisy, after the OC treatment. However, no such findings are observed in the results of the controlled experiment.
Fig. 4. Reconstructed images of the phantom. (a) and (e) are the reconstructed intensity and phase images of the bare test board. (b) and (f), (c) and (g), and (d) and (h) are the reconstructed results through a piece of mouse colon before, 1 min. and 5 min. after topically applying the glycerol solution, respectively. (i) and (l), (j) and (m), and (k) and (n) are the reconstructed results of one controlled experiment correspondingly. The field of view of each reconstructed image was 224 × 224 μm².

To quantify the optical clearing efficacy on the reconstructed phase distributions, relative spatial variance of phase $r_D$ and relative spatial average of phase gradient intensity $r_G$ of three zones of the reconstructed phase distributions before, 1 min. and 5 min. after the treatment were calculated, and averaged for 5 samples of both the control and experimental groups, respectively. The green boxes marked in Fig. 4(a) represent the three zones with an average intensity ratio of about 1:15:12, which are produced by illumination of a Gaussian beam on the chromium bars and substrate of the test board. The bars in Fig. 5 represent the average results of the experimental group, and the crosses depict that of the control group. As illustrated in Fig. 5(a) and 5(b) respectively, applying the glycerol solution results in obvious decreases of $D_i$ and $G_i$, for the three zones, while for the control group no significant changes of $D_i$ and $G_i$ are observed after the application of the saline solution. In Fig. 5(a), the value of the averaged $D_i$ of zone 1 decreases by about 38% after the clearing treatment, which is less than about 57% for zone 2 and 60% for zone 3. In Fig. 5(b), the value of the averaged $G_i$, decreases by about 29% after the clearing treatment for zone 1, which is less than about 50% for zone 2 and 53% for zone 3. It seems that both $r_D$ and $r_G$ of zones with higher intensities decrease by a larger proportion after OC. The differences of the reduction proportions between zone 1 and zone 2 or 3 should be mainly due to the dependence of the phase noise on the fringe contrast within the holograms. For a temporal phase shifting digital holography system, it was shown that the phase noise in the reconstructed phase distributions is inversely proportional to the fringe contrast [35]. After the OC treatments, the reflective
light fields from zone 2 and 3 had larger intensities which were comparable to that of the reference beam, leading to interference fringes with higher contrasts and lower phase noises in zone 2 and 3. For zone 1 with a much lower intensity, larger phase noises were produced and resulted in overestimation of both $D_r$ and $G_r$.

![Graph showing average results of phase variance and phase gradient intensity](image)

**Fig. 5.** Average results of the (a) relative spatial variance of phase $D_r$, and the (b) relative spatial average of phase gradient intensity $G_r$ of three zones of the reconstructed phase distributions before, 1 min. and 5 min. after the treatment, for both the experimental and control groups. Bars: average results of the experimental group; crosses: average results of the control group. Error bars show the maximum and minimum of different samples.

In summary, the quality of the reconstructed phase image of a wavefront propagating through an *in vitro* mouse colon tissue slice was improved by the OC treatment. Both the spatial variance of phase and the phase gradient intensity were reduced. The reconstructed phase distribution was closer to the original. Optimizing the reflectivity of the reference mirror could enhance the improvement effects.

### 4.3 Reflection mode imaging: volume image

Figure 6 shows the intensity images reconstructed at several depths of axial scans of a piece of porcine skin, and these axial scans were performed at the same site with OC treating intervals of 0, 15, 30, 45 and 60 min., respectively. It can be seen clearly that there is a considerable amount of noise in the reconstructed images before the OC treatment. The skin structures submerged in noise becomes indistinguishable at the depth larger than 232.3 μm. This degradation of image quality should be due to cross-talk artifacts induced by multiple scattering. After the application of the glycerol solution, effective rejection of these artifacts is demonstrated and clear improvements in both resolution and imaging depth are observed. In the depth range from 91.5 μm to 185.3 μm, dermal papillae are well resolved with a higher resolution and contrast. And structures at depth from 232.3 μm to 326.4 μm which could be collagenous fibers of the reticular dermis are also discriminable. Especially, the structures imaged at the depth of 326.4 μm show a noticeable enhancement. Moreover, it can be found that the image brightness decreases at every depth after applying the glycerol solution. And the treatment duration longer than 45 min. results in evidently reduced intensity at the shallow layers. Therefore, we could conclude that topical application of the used glycerol solution for 30 to 45 min. is the most effective condition for this experiment.
Fig. 6. Intensity images of a piece of porcine skin reconstructed at different depths, with OC treating intervals of 0, 15, 30, 45 and 60 min., respectively. The field of view of each reconstructed image was $484 \times 484 \mu m^2$. Note that the value limits of the color bar at each depth is adjusted to provide a better presentation.

To further investigate the changes of contributions of singly-scattered signal and multiply-scattered cross-talk noise to the reconstructed images during the OC treatment, the averaged pseudo A-scans of the porcine skin corresponding to OC treating intervals of 0, 15, 30, 45 and 60 min. were calculated and shown by a logarithmic scale in Fig. 7(a). As a reference, the baseline was obtained by an averaged pseudo A-scan of the air. The left peaks in the figure represent the skin surface. It can be seen that the changes of the attenuation slopes of the averaged pseudo A-scans have experienced two periods. The first period is the first 15 min. after applying the glycerol solution, during which the slope becomes larger while the whole A-scan becomes lower. And the second period is from 15 min. to 60 min. after the OC
treatment, during which both the slope and the whole A-scan decrease. These changes in the two periods can be explained as follows. The averaged pseudo A-scans contain both the singly-scattered signal and multiply-scattered cross-talk noise. Before the OC treating, the cross-talk noise dominates the deep part of the A-scan. The value of such an A-scan is significantly larger than that predicted by the single-backscattering model [41] used in confocal scanning OCT. Then for the first 15 min., the overall cross-talk noise decreases rapidly with the process of the clearing, especially at large depths. The singly-scattered signal contributes more and more to the averaged pseudo A-scan. As a result, the slope increases though the intensity of the A-scan drops. For the second period, the trend of continuous decrease of the slope is similar as that observed by confocal scanning OCT [42]. This indicates that the singly-scattered signal is mainly responsible for the averaged pseudo A-scan, and the attenuation slope decreases as the scattering is reduced by OC.

For a quantitative evaluation, portions of the A-scans from 102.4 μm to 325 μm in depth relative to the skin surface were fitted by linear lines, and the slopes of which were compared. The fitting results are shown in Fig. 7(b). The slope of the red dot line is increased by about 90% when compared with that of the black solid line. And the slope of the purple dash line is reduced by about 27% when compared with that of the red dot line. To quantitatively compare the contributions of both singly-scattered signal and cross-talk noise to the reconstructed images as a function of depth, the fitted lines in Fig. 7(b) were extended and subtracted from the corresponding A-scans in Fig. 7(a), respectively. For the situation of 0 min., the value of the red dot line in Fig. 7(b) was subtracted from the black solid line in Fig. 7(a). Assuming the fitted lines in Fig. 7(b) represent only the singly-scattered signals (except the black solid line), the residual values shown in Fig. 7(c) can be expressed as

$$\text{Residual} = \ln(1 + \frac{\text{cross-talk noise}}{\text{singly-scattered signal}})$$

(7)

It can be seen that the residual is a logarithmic expression of the ratio of cross-talk noise to singly-scattered signal. The black dot line in Fig. 7(c) represents the situation that the contribution of singly-scattered signal equals to that of cross-talk noise. It is clear that the
cross-talk noise overwhelms the singly-scattered signal beyond the depth of about 250 μm before the clearing treatment, while this situation is improved with the process of OC. For treatment with glycerol solution for 30 min., this cross-over depth reaches about 380 μm. It is noteworthy that the calculation of the residual may underestimate the contribution of the cross-talk noise, since which may still make a small contribution to the fitted lines in Fig. 7(b) for situations of after the clearing treatment. As a validation, we also fitted the attenuation slopes within the same range of averaged A-scans measured at the same site of the same porcine skin by the confocal scanning OCT mentioned above, before and 60 min. after the OC treatment respectively. The attenuation slope measured before OC by OCT was calculated to be about 9.85 mm\(^{-1}\), which is larger than 4.66 mm\(^{-1}\) of before and 8.84 mm\(^{-1}\) of 15 min. after the OC treatment measured by SCDHM as shown in Fig. 7(b). And after a 60 min. OC treatment, the attenuation slope measured by OCT was about 6.57 mm\(^{-1}\), which is larger than 6.43 mm\(^{-1}\) by SCDHM. These results demonstrate that a lot of cross-talk noise exists in the reconstructed images before the clearing treatment, and can be reduced by a large portion by OC, if cannot be totally rejected.

To quantify the optical clearing efficacy on the imaging depth, the contrasts of the intensity images reconstructed at the same depths as given in Fig. 6 with OC treating intervals of 0, 15, 30, 45 and 60 min. were calculated, and averaged for 5 samples, respectively. The results are shown in Fig. 8(a). It can be seen that the averaged image contrasts decrease with depth for all treating intervals but at different rates. The averaged contrasts after OC decays slower with depth than before, and the average values become larger at every depth. T-test was performed to determine significant difference between the image contrasts before OC and that with different OC treating intervals at the eight given depths. The values were considered significantly different if $p<0.05$. The image contrasts (mean ± standard deviation) before OC were 4.70 ± 0.26, 4.11 ± 0.16, 4.10 ± 0.24, 3.63 ± 0.18, 3.31 ± 0.11, 2.97 ± 0.11, 2.91 ± 0.02, and 2.797 ± 0.001, corresponding to the eight depths from 45.8 μm to 373.4 μm. The statistical analysis demonstrated that with the OC treating interval of 30 min., the image contrasts at 91.5 μm (4.66 ± 0.28), 185.3 μm (4.30 ± 0.11), 232.3 μm (4.20 ± 0.64), and 326.4 μm (3.98 ± 0.68) were significantly different from the corresponding values of the image contrasts before OC. With the treating interval of 45 min., the image contrasts at 138.3 μm (4.77 ± 0.51) and 279.4 μm (3.91 ± 0.85) were significantly different from the corresponding values before OC. With the treating interval of 60 min., the image contrast at 373.4 μm (3.13 ± 0.22) was significantly different from the corresponding value before OC. For the depth of 45.8 μm, there were no significant differences between the image contrast before OC and that with any of the four OC treating intervals. This could be explained as follows. At the depth of 45.8 μm, the cross-talk noise was low and the effect of the cross-talk noise on the image contrast was limited. Therefore, after the OC treatment, no significant enhancement of the image contrast was shown. This could be verified from Fig. 8(a) that the average image contrasts varied little during the whole 60 min. OC treatment at 45.8 μm. The statistical analysis indicated that for this group of experiments, OC treating intervals of 30 to 60 min. led to significant enhancement of the image contrast, especially in the deep of the in vitro porcine skin samples. Increase of contrast in the deep enlarges the imaging depth. In the situation of clearing for 30 min., the imaging depth is increased by about 30% when compared with that before OC. Figure 8(b) shows the average ratios of the contrasts of reconstructed images after clearing treatment to that before OC, for three depths. It can be seen that the maximum of the averaged relative contrast appears later for deep than for shallow, and its value becomes larger with depth. This indicates the process of OC with depth, and the greater enhancement of image contrast in deep. The largest averaged relative contrast is about 1.3 with a clearing duration of 45 min. at the depth of 279.4 μm.
On the whole, the application of OC reduced the cross-talk noise effectively when using a SCDHM system for tomographic imaging an in vitro porcine skin tissue. Image contrasts in deep tissue could be enhanced significantly, leading to a larger imaging depth.

5. Discussion

The enhancement of the image contrast in deep tissue by OC could be explained based on previous experiments and Monte Carlo studies [17,43]. The main scattering interaction of the incident beam in the scattering tissue can be classified into three types [43]: single backscatter \(a\), small-angle forward scatter \(b\), and wide angle scatter \(c\). Confocal scanning OCT detects photons from the first two types of scatters. As proposed by [43], these detected photons can be categorized into least scattered photons (LSP) and multiple scattered photons (MSP). The LSP experiencing few scattering events and very small angle scattering, including the interaction type \(a\) and part of type \(b\), provide the useful localized information. The MSP undergoing wider angle scattering and coming from the interaction type \(b\), degrade the detected signal. Compared with confocal scanning OCT, SCDHM can receive photons from all the three types of scatters by area detectors. MSP from the interaction type \(c\) are mainly responsible for the cross-talk noise. After the OC treatment, the scattering coefficient is reduced, which leads to an increase of the LSP in the deep [43], a decrease of the \(b\) type MSP for the entire depth [43], and a significant reduction of the \(c\) type MSP [17]. This leads to the enhancement of the image contrast in deep tissue which was observed in our experiments by a full field short coherence system, and in the work of [44] by a confocal scanning OCT system. For the case of increasing the anisotropic factor by OC, the LSP signal is increased in the deep and the \(b\) type MSP signal is decreased [43]. If the size of the scatterers is changed and becomes much larger or smaller than the wavelength, the \(c\) type MSP will be reduced in different degrees [17]. For biotissues of which the cells can be aggregated into clusters by the treatment of OCAs, blood, for example, increasing the anisotropic factor could lead to the reduction of the \(c\) type MSP, and therefore enhance the image contrast. For the tissues used in our experiments, the reduction of the scattering coefficient is believed to be the main mechanism of optical clearing and the primary reason of the enhancement of the image contrast.

The axial resolution of short coherence tomographic imaging techniques is usually degraded rapidly against the depth in highly scattering medium. In the RM imaging experiments, the effect of OC on the axial resolution was not investigated because of the
undersampling of the axial scanning. According to the Monte Carlo studies mentioned above, the reduction of the scattering coefficient can diminish the tail which usually results from the $c$ type MSP [17], and also enhance the axial resolution in deep probing depth which is provided by the LSP and the $b$ type MSP [43]. Therefore, we can expect that the axial resolution of SCDHM could be greatly enhanced by OC.

There seems to be a contradiction between results of those two imaging modes. In the TM imaging experiments, the intensity of the reconstructed image was increased after the OC treatment, while in the RM imaging experiments the intensity of the reconstructed image was decreased at every depth after the OC treatment. This could be explained as follows. In the experiments of TM imaging, the thin and weak-scattering mouse colon slices would induce little crosstalk noise when imaged by a full field short coherence imaging system. Single backscattered photons made a large contribution to the reconstructed images even before OC. After the OC treatment, the covered colon slice became more transparent, therefore the intensity of the reconstructed image was increased. However, in the experiments of RM imaging, in vitro thick tissues of porcine skins were imaged. Large amount of crosstalk noise was generated due to the high scattering characteristic of the porcine skins, and made a dominant contribution to the reconstructed images before OC. After the OC treatment, the crosstalk noise was decreased, leading to reduction of the intensities of the reconstructed images. This reduction of intensity at every depth, during the whole OC treatment, which could be seen from Fig. 6 and Fig. 7(a), indicated that even if the single backscattered photons from some depths could be increased with a certain OC treating interval, the contribution of the increase of the single backscattered photons to the reconstructed images was smaller than that of the reduction of the crosstalk noise. This could further indicate that the intensity of crosstalk noise decreases with the reduction of the scattering coefficient faster than exponentially for in vitro porcine skins. Overall, the difference between results of those two imaging modes could be due to the different scattering characteristics of in vitro mouse colon samples and in vitro porcine skins tissues. And it could be also due to the fast decrease rate of crosstalk noise with the reduction of the scattering coefficient for in vitro porcine skins.

In the RM imaging experiments, the imaging depth enhanced by OC was limited by the low sensitivity of the system. The averaged pseudo A-scans acquired at OC treating intervals of 45 and 60 min. in Fig. 7(a), almost approach the baseline at the depth of about 400 μm. This could be because optical clearing reduces the scattering intensities to beyond the sensitivity of the system. In Fig. 6, enhancement of the structures at the depth of 326.4 μm indicates that the image quality in deep could be improved significantly by OC if the scattering intensities after OC are still high enough to be recorded. Therefore a system with a higher sensitivity could image much deeper in the tissue with the aid of OC. For our system, the sensitivity can be increased by means such as using CCD with larger full well charge capacity, using water-immersion objectives, anti-reflection coating all the optical elements, optimizing the reflectivity of the reference mirror to maximize the fringe contrast, and raising the accumulation number of holograms.

Since the cross-talk noise almost exists in all full-field coherence imaging techniques, the OC technique may provide a solution to decrease the cross-talk noise when using these techniques for tomographic imaging scattering tissues, without compromising spatial coherence of the illumination.

6. Conclusions

Full-field coherence imaging techniques which using numerical reconstruction methods for tomographic imaging, have the advantages of depth-independent resolution and sensitivity. However, these techniques are vulnerable to multiple scattering and cross-talk noise, which severely deteriorate the image quality and limit the imaging depth. A short coherence digital holographic microscopy system was used to demonstrate cross-talk noise suppression and
imaging performance enhancement by OC. Experimental results demonstrated that OC improved the wavefront propagation, decreased the cross-talk noise effectively and enhanced the imaging performance of a SCDHM system for tomographic imaging in vitro porcine skin tissues. The image resolution, contrast, and imaging depth were all improved.

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