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***En face* speckle reduction in optical coherence microscopy by frequency compounding**

Caroline Magnain^{1,*}, Hui Wang¹, Sava Sakadžić¹, Bruce Fischl^{1,2}, and David A. Boas¹

¹Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital/Harvard Medical School, Charlestown, Massachusetts 02129, USA

²Computer Science and AI Laboratory, MIT, Cambridge, Massachusetts 02139, USA

Abstract

We report the use of frequency compounding to significantly reduce speckle noise in optical coherence microscopy, more specifically on the *en face* images. This method relies on the fact that the speckle patterns recorded from different wavelengths simultaneously are independent; hence their summation yields significant reduction in noise, with only a single acquisition. The results of our experiments with microbeads show that the narrow confocal parameter, due to a high numerical aperture objective, restricts the axial resolution loss that would otherwise theoretically broaden linearly with the number of optical frequency bands used. This speckle reduction scheme preserves the lateral resolution since it is performed on individual A-scans. Finally, we apply this technique to images of fixed human brain tissue, showing significant improvements in contrast-to-noise ratio with only moderate loss of axial resolution, in an effort to improve automatic three-dimensional detection of cells and fibers in the cortex.

Optical coherence tomography (OCT) is an optical technique providing three-dimensional (3D), high resolution volumes up to several hundreds of micrometers in depth, originally applied to ophthalmology studies [1–3], and more recently is used to study the structural properties of brain tissue [4,5]. The cortex of the human brain is a complex medium composed of various cells, such as neurons and glia, as well as fibers. Cytoarchitecture, that is, the type of neurons found in specific areas (e.g., pyramidal, stellate, spindle cells) and their organization (e.g., laminar structure), and myeloarchitecture (e.g., fiber caliber, orientation, and myelin content) are critical to understanding the structure and function of the brain. Diseases or injuries involving the brain will disrupt these features and lead to neurological deficiencies (e.g., neurofibrillary tangles in Alzheimer's disease, damaged fibers in traumatic brain injury). Optical coherence microscopy (OCM), using a high numerical aperture (NA) objective, yields a lateral resolution high enough to image single neurons and fibers as well as tumorous cells [6–12]. One of the main drawbacks of OCT/OCM and the quantitative analysis of the acquired data is the speckle noise inherent to any coherence technique, reducing the image contrast and blurring boundaries between

*Corresponding author: cmagnain@nmr.mgh.harvard.edu.

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structures. Speckle reduction techniques are mainly based on spatial compounding [13,14] and digital filters [15,16]. However, those methods require multiple acquisitions and 2D filters, reducing the lateral resolution. Frequency compounding for reducing speckle noise has been introduced by Schmitt *et al.* [13]. In this approach, the light source of an OCT setup is split into multiple nonoverlapping frequency bands. The speckle pattern arising from those different bands is incoherent, and thus after summation of the processed data, the speckle noise can be significantly reduced. However, the axial resolution increases linearly with the number of bands used. Frequency compounding has been used *in vivo* for intra-vascular frequency domain imaging [17], for example, where spatial compounding is impractical due to the rotation of a catheter and the heart motion. Another scheme for frequency compounding is to use two or more independent, nonoverlapping light sources and two or more separate detectors [18]. The sources can be chosen to yield the same axial resolution. Unfortunately, the complexity of the system increases. All the papers published to date related to frequency compounding look at the speckle reduction in the cross-sectional plane of the OCT image, generally acquired with low spatial resolution. Here, we investigate the speckle reduction based on frequency compounding in the *en face* images obtained by OCM. By using a high NA, we can prevent the degradation of the axial resolution, thanks to the confocal gating provided by the objective. We demonstrate our method with microbeads in agarose. Finally we assess the speckle reduction obtained by frequency compounding in the human brain by imaging neurons and fibers in the cortex and quantifying the improvement in the contrast-to-noise ratio.

Our OCT/OCM system uses a superluminescent diode from Thorlabs (LS2000B SLD, Thorlabs Inc., Newton, New Jersey) with a 200 nm bandwidth ($\Delta\lambda$) centered at $\lambda_0 = 1274$ nm [7]. From a single acquisition, we can split the recorded spectrum into N nonoverlapping optical frequency bands (OFBs) of equal bandwidth $\Delta\lambda/N$ and different central wavelengths. The phases of the fully developed speckles from separated spectra are uncorrelated. As a result, the N reconstructed volume images can be summed to obtain a volume image with reduced speckle noise. One problem with this method is that the axial resolution worsens with the number of OFBs used for frequency compounding since the bandwidth is reduced. The axial resolution can be estimated based on the OFB with the highest central wavelength:

$$l_{c,m} = \frac{2\ln(2)}{\pi} * \frac{(\lambda_0 + \Delta\lambda - \Delta\lambda/2N)^2}{2n\Delta\lambda/N}, \quad (1)$$

with n being the refractive index and N the number of OFBs. In high resolution OCM, the confocal gate maintains the high axial resolution although the coherence gate increases with frequency compounding.

We inspected the axial resolution for the $10\times$ (Zeiss N-Achroplan $10\times$ W, NA 0.3) and $40\times$ (Olympus LUMPLANFL/IR $40\times$ W, NA 0.8) water immersion objectives with respect to frequency compounding. We acquired images from a mirror at the focus at 14 different depths, while we increased the reference arm length. We then processed the data using frequency compounding for $N=1$ (no speckle reduction) to 6. Figure 1 displays the boxplot of the axial resolution, measured as the full width at half maximum of each profile, with

respect to the amount of frequency compounding, for (a) the $10\times$ objective and (c) the $40\times$ objective. The solid line corresponds to the theoretical values calculated from Eq. (1). As expected, the axial resolution increases linearly with N and follows the theory, except when N equals 1 and 2. The theoretical axial resolutions for OFBs 1 and 2 are 2.7 and 5.8 μm , respectively. However, the pixel size in z , fixed by the light source and the number of pixels of the camera, is 3.3 μm , larger than half the resolution, which explains the discrepancy between theory and experiment for lower N . Finally, we experimentally quantified the confocal parameter for each objective by translating a mirror in the sample arm. The intensity on the mirror with respect to the depth is reported in Fig. 1 [$10\times$: (b), $40\times$: (d)] for the different OFBs (dots). Each curve has been corrected for the sensitivity decay introduced by the finite spectrometer resolution [19]. As expected, frequency compounding has no effect on the confocal parameter. We fit the curve for each objective by a Gaussian beam, defined as

$$I(z) = I_0 \left(\frac{\omega_0}{\omega(z)} \right)^2 = \frac{I_0}{1 + \left(\frac{z - z_0}{z_r} \right)^2}, \quad (2)$$

where z_0 is the focus depth and z_r is the Rayleigh range, which is half the confocal parameter b and is shown by the red line. We found the confocal parameter to be about 100 μm for the $10\times$ objective and 6 μm for the $40\times$ objective. The theoretical values for those objectives are about 5 and 38 μm , respectively; however, we under fill the back aperture of the objective in our system and thus achieve a longer Rayleigh range. The confocal parameter for OCM, using the $40\times$ objective, is smaller than the axial resolution obtained with frequency compounding using three or more OFBs. We therefore investigated the possibility that the narrow confocal parameter would maintain an axial resolution better than 6 μm when we use frequency compounding to significantly reduce speckle noise in the *en face* images while keeping the high lateral resolution of OCM, by acquiring data with a z -step inferior to the confocal parameter, for instance, a step of 1 μm .

We embedded 1 μm microbeads (Polybeads Microspheres μm , Polysciences, Inc.) in an agarose gel. We imaged the microbeads using both the $10\times$ and the $40\times$ objectives at every 1 μm in depth over a total of 50 μm by moving the z -stage. For each volume image acquired, we processed the data for different numbers of OFBs ($N = 1$ to 6). For each volume image, we kept the *en face* image corresponding to the focus plane and stacked them together to reconstruct the whole volume. We then found the center of microbeads and recovered their profiles in depth. Each profile was then registered to obtain the average profile.

Figures 2(a) and 2(b) show the normalized depth profile of the microbeads obtained with the $10\times$ and the $40\times$ objectives, respectively, with respect to the number of OFBs. The diameter of the microbeads is measured as the full width at half maximum and reported in Fig. 2(c). While the diameter obtained with the $10\times$ objective follows the analytical expression given by from Eq. (1) (oblique line) and increases linearly with respect to N , the results from the $40\times$ objective show that the axial resolution remains relatively constant and smaller than the

confocal parameter (horizontal line), confirming that the confocal gating maintains an axial resolution despite increasing the number of OFBs.

We quantified the *en face* speckle reduction by calculating the speckle contrast C , defined as the standard deviation of the speckle noise $\sigma(S)$ over its mean intensity $\langle I_S \rangle$. All the acquisitions were OCM, using the $40\times$ objective. Figure 3(a) shows C as a function of the number of OFBs ($N=1$ to 6, 16, and 25) measured on plain agarose (blue symbols). The different symbols represent the values obtained at three different depths separated by $25\ \mu\text{m}$. The contrast of the original images ($N=1$) is close to the theoretical value of a fully developed, mixed-polarization speckle of $C=0.52$ [20]. As expected, the speckle contrast is reduced by a factor close to $\sqrt{N}$ [13], represented on Fig. 3(a) by the solid line, independent of the depth of the imaging plane. The slightly lower decrease in speckle contrast than expected results from the speckle patterns not being completely independent. The correlation coefficient between the speckle patterns obtained for $N=2$ (OFB central wavelengths separated by $100\ \text{nm}$) is around 0.05. In the case of $N=25$, the correlation ranges from 0.17 for neighboring OFBs (OFB central wavelengths separated by $8\ \text{nm}$) to 0.05 for distant OFBs. Figure 3(b) shows the *en face* images of a $10\ \mu\text{m}$ microbead embedded in agarose after frequency compounding using N (1 to 6, 16, and 25) OFBs. The reduction of speckle noise is clearly visible, which in return increases the signal-to-background ratio, shown by the ratio of the microbead intensity over the background intensity. Moreover, the frequency compounding method is performed on individual A-scans and hence does not reduce the lateral resolution of the *en face* images.

To summarize, using frequency compounding on a single OCM acquisition will significantly reduce the speckle noise of the *en face* images by a factor of $\sqrt{N}$, where N is the number of OFBs. Even though the axial resolution after frequency compounding should worsen, the narrow confocal parameter inherent to the high NA objective used in OCM will filter out the out-of-focus contributions, resulting in an apparent axial resolution close to the confocal parameter itself.

Figure 3(c) shows the application of the frequency compounding method to fixed human brain images obtained by OCM, using the $40\times$ objective. We used fixed human brain (immersed in 10% formalin over a period of at least two months), obtained from the Massachusetts General Hospital Autopsy Suite (Boston, Massachusetts), to show the application of frequency compounding to improve histological studies by OCM. A subregion of a several square millimeter *en face* area (approximately $3\ \text{mm} \times 5\ \text{mm}$, containing the gray matter from the pial surface to the boundary with the white matter) within the entorhinal cortex (age 45 years old, F, post mortem interval $<24\ \text{h}$, neurologically normal) was blocked. We focused our imaging on layer II, containing islands of larger pyramidal and stellate neurons, fibers, and glia. We imaged the first $50\ \mu\text{m}$ of the several-millimeter-thick tissue as we did for the $1\ \mu\text{m}$ microbeads, using the $40\times$ objective and moving the sample every $1\ \mu\text{m}$ in the z direction. The top left image of Fig. 3(c) ($N=1$) shows an *en face* OCM image of the human brain at the focal plane. As can be seen, the signal-to-noise ratio is poor due to the speckle noise, which makes it difficult to distinguish and segment neurons, fibers, and potential glia. We applied frequency compounding using N (2 to 6, 16, and 25) to improve the contrast and reveal the important features of the cortex.

We defined a region where neither neurons nor fibers were visible to calculate the speckle contrast C . The results obtained at three different depths spanning 50 μm are reported in Fig. 3(a) in green. The value of the original images ($N=1$) is slightly over the theoretical value of 0.52, probably due to the presence of fine features (fibers) in the defined region. The results follow the theoretical noise reduction indicated by the solid line. Figure 3(c) shows the reduction of the speckle noise by frequency compounding ($N=1$ to 6, 16, and 25) on the brain *en face* images, and the overall contrast-to-noise ratio (CNR) is clearly improving with N . The neurons (larger bright cells) are more differentiated from the background tissue while retaining their shape and size, which will help improve automatic segmentation algorithms. The fibers were barely visible on the original image but can be more clearly distinguished after frequency compounding.

Finally, we compared frequency compounding to axial averaging as a method to reduce speckle noise while keeping the lateral resolution intact. We used the same data acquired on the human brain tissue and presented in Fig. 3. By averaging the signal in depth on the original data, we keep the confocal parameter as a way to maintain the axial resolution. We averaged the signal around the focus plane over the depths corresponding to the axial resolution theoretically obtained by frequency compounding [Fig. 1(c), solid line]. This simple method, even though decreasing the speckle contrast, does not achieve as large an improvement in speckle contrast as the frequency compounding since the averaging is performed using a single acquisition where the adjacent pixels in depth are correlated.

We then compared the CNR calculated on images resulting from these two methods to show how the tissue features (*i.e.*, neurons and fibers) are enhanced with regard to the background tissue. CNR is defined as [16,21,22]

$$\text{CNR} = \frac{\mu_R - \mu_B}{\sqrt{\sigma_R^2 + \sigma_B^2}}, \quad (3)$$

where μ_R and μ_B are the mean intensities of the region of interest and the background, respectively, and σ_R and σ_B their standard deviations. In our images, we consider the neurons our region of interest and the remainder is our background. The CNR calculated might be underestimated since both fibers and other small cells are included in our background. Figure 4(a) reports the CNR for both speckle reduction methods with respect to the number N of OFBs. As shown in the graph, the CNR yielded by frequency compounding is better than that by axial averaging. Moreover, if we take a closer look at the resulting images for $N=6$ in Figs. 4(b) and 4(c), we observe that some fibers that are visible after frequency compounding (b) disappear after axial averaging (arrows).

In conclusion, we have demonstrated that the application of frequency compounding to reduce speckle noise in *en face* images obtained by OCM is very effective and only results in a slight loss of axial resolution, limited by the confocal parameter that is defined by the objective used. We also showed that frequency compounding increases CNR better than axial averaging, while maintaining the smaller features, such as individual fibers and other

cells. This technique can be applied to histology studies performed by OCM. This speckle reduction method only required one acquisition, so the imaging time is not reduced either. The reduction in speckle noise will be instrumental in the automatic 3D segmentation of neurons and fibers in the brain. This could help improve cell classification by OCM in various regions of the brain. Coupled with a vibratome and automatic XYZ stages [23], our OCM can be extended to characterize various brain regions by the cytoarchitecture and myeloarchitecture over several cubic centimeters.

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Fig. 1.

Measured axial resolution on a mirror with respect to the number N of OFBs for the (a) $10\times$ and (c) $40\times$ objectives. The central mark is the median across 14 different imaging depths, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, and outliers are plotted individually. The solid line represents the theoretical axial resolution provided by Eq. (1). Confocal parameters achieved for the two objectives, (b) $10\times$ and (d) $40\times$. Experimental data are shown by dots, and the fits from Eq. (2) are the red lines.

Fig. 2.

Average bead profile image for (a) the $10\times$ objective and (b) the $40\times$ objective with regard to the number of OFBs for frequency compounding. (c) shows the axial resolution obtained on the microbeads with the $10\times$ (blue circle) and the $40\times$ (green circle) objectives after the reconstruction with respect to the number N of OFBs. We compare these results to the theory (oblique solid line). The horizontal line represents the confocal parameter of the $40\times$ objective.

Fig. 3.

(a) Speckle contrast C in agarose (blue) and human brain tissue (green) at three different depths, the first depth measured at z_1 (+), $z_1 + 25 \mu\text{m}$ ($\times$), and $z_1 + 50 \mu\text{m}$ (o) with respect to the number of OFBs ($N = 1$ to 6, 16, and 25). The black line represents the theoretical reduction of speckle contrast by a factor of $\sqrt{N}$. (b) 10 μm microbead (field of view: $25 \mu\text{m} \times 25 \mu\text{m}$) and (c) *en face* images of layer II of entorhinal cortex of the human brain (field of view: $200 \mu\text{m} \times 200 \mu\text{m}$) with respect to the number of OFBs ($N = 1$ to 6, 16, and 25). The intensity has been rescaled from 0 to 1 for each image.

Fig. 4.

(a) CNR of the human brain tissue images for the two different speckle reduction methods: frequency compounding (green) for $N=1$ to 6 and axial averaging (blue) for the corresponding theoretical axial resolution. The images show the human brain tissue (b) after frequency compounding for $N=6$ and (c) after axial averaging over $18 \mu\text{m}$.