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A Model-Based Approach for Microvasculature Structure Distortion Correction in Two-Photon Fluorescence Microscopy Images

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SUMMARY

This paper investigates a post-processing approach to correct spatial distortion in two-photon fluorescence microscopy images for vascular network reconstruction. It is aimed at *in vivo* imaging of large field-of-view, deep-tissue studies of vascular structures. Based on simple geometric modeling of the object-of-interest, a distortion function is directly estimated from the image volume by deconvolution analysis. Such distortion function is then applied to sub volumes of the image stack to adaptively adjust for spatially varying distortion and reduce the image blurring through blind deconvolution. The proposed technique was first evaluated in phantom imaging of fluorescent microspheres that are comparable in size to the underlying capillary vascular structures. The effectiveness of restoring three-dimensional spherical geometry of the microspheres using the estimated distortion function was compared with empirically measured point-spread function. Next, the proposed approach was applied to *in vivo* vascular imaging of mouse skeletal muscle to reduce the image distortion of the capillary structures. We show that the proposed method effectively improve the image quality and reduce spatially varying distortion that occurs in large field-of-view deep-tissue vascular dataset. The proposed method will help in qualitative interpretation and quantitative analysis of vascular structures from fluorescence microscopy images.

Keywords

Distortion Correction; Image Deconvolution and Restoration; Vascular Imaging

INTRODUCTION

Two-photon fluorescence microscopy has become a prominent tool for high-resolution *in vivo* imaging of biological tissues (Periasamy *et al.*, 1999, So *et al.*, 2000). Several studies

have demonstrated improved techniques for large field-of-view, three-dimensional imaging of *in vivo* specimens at a great depth (Kobat *et al.*, 2009, Schroeder *et al.*, 2011, Crosignani *et al.*, 2012, Zong *et al.*, 2014, Glancy *et al.*, 2014). However, achieving high spatial fidelity in obtained biological structures is still a challenge due to the distortion of the point-spread function (PSF) of the microscope, the inhomogeneity of tissue and immersion media (Boutet de Monvel *et al.*, 2001, de Monvel *et al.*, 2003, Von Tiedemann *et al.*, 2006), and most importantly, spatially varying distortion resulting from different specimen structures across the imaging field (Booth *et al.*, 2002, Dong *et al.*, 2003, Arigovindan *et al.*, 2010, Maalouf *et al.*, 2011, Temerinac-Ott *et al.*, 2012).

Image deconvolution is a standard approach that can reduce spatial distortion in microscopy images. It is based on the spatial invariance assumption of the PSF of the microscope. Recovering the objective image can thus be performed through an inverse operation, deconvolution, to reduce the blurring effect from the observed image. The PSF of a microscope can be calculated theoretically or measured empirically. In order to accurately determine the distortion in a microscope, it is often required to measure the PSF using sub-resolution fluorescent beads on the order of 100 nm in diameter and imaging them at half-diameter voxel size (Dong *et al.*, 2003). However, this approach has practical limitations for *in vivo* imaging applications since it cannot estimate the distortion from scattering and refraction of light from the tissue and its surrounding structures. Thus the PSF determined under ideal conditions is not applicable to many situations deep inside tissues. Without appropriate high resolution “guide-stars” throughout the tissue (Sibarita, 2005) to provide regional distortion correction as well as high spatial resolution scanning of the tissue, this classical PSF correction approach has limitations in deconvolution for deep tissue imaging.

We have shown previously that the voxel size is frequently limited to around one micron resolution for large field-of-view, *in vivo* imaging experiments in order to minimize or compensate for tissue motion, deformation and photo-dynamic damage during the image acquisition (Schroeder *et al.*, 2011, Bakalar *et al.*, 2012, Glancy *et al.*, 2014). Under these sampling conditions, aliasing can be significant and the descriptive information of PSF measured at the nanometer resolution becomes discordant for direct image deconvolution and restoration. These limitations render the standard approach using PSF measured at sub-resolution of the microscope for image deconvolution and distortion correction ineffective and impractical for *in vivo* fluorescence microscopic imaging at larger voxel sizes. Furthermore, PSF should ideally be measured under the same conditions as imaging the specimen itself. This implies that beads should be measured after invasively injecting into the specimen which is generally not feasible (Sibarita, 2005).

Several studies have shown promising results of image deconvolution using *in vivo* and *in situ* PSF estimation from confocal microscopy images (Boutet de Monvel *et al.*, 2001, de Monvel *et al.*, 2003, Von Tiedemann *et al.*, 2006). In these studies, small point-like structures were detected from the images and a theoretical PSF-model was fitted to the extracted point-like structure before image deconvolution. However, these approaches may still be hampered from the availability of the point-like biological structures in the specimen, the reliability of detecting relatively poor signal-to-noise ratio (SNR) sub-resolution beads or

point-like structures in the images, and the consistency of estimating spatially varying distortions at different geometrical locations in the 3D volume.

Other studies also proposed computational approaches to model the image degradation and estimate the structural information to improve image restoration. For example, a model-convolution approach was proposed to simulate the process of image blurring in fluorescence microscopy and extract microtubule information in spindle formation (Gardner *et al.*, 2010). Although this approach has potential to estimate the information content in fluorescence images accurately, it requires a priori modeling of the biological structure of interest and has not been applied to a large and extended 3D structure such as the capillary network. A continuous Gaussian radial basis function was also proposed to model the image formation and degradation, and was used to constrain the model parameters during the deconvolution process (Chen & Chen, 2014). Although this work showed encouraging results for 2D image restoration, its application in 3D fluorescence image and its use to restore spatially variant image distortion have not been addressed. In another study, an efficient alternate minimization algorithm for parametric blind image deconvolution was proposed to model the microscope image acquisition physical process and constrain the free parameters describing the PSF (Pankajakshan *et al.*, 2007). Although this work showed good results on simulated synthetic data, its limitation to restore spatially variant distortion in large field-of-view deep-tissue imaging has not been addressed. Furthermore, 3D field distortion in large field-of-view fluorescence imaging has been characterized (Pankajakshan *et al.*, 2012). It was shown that the PSF is spatially variant and that the severity of the variation increases rapidly with the distance from the center of the field-of-view.

The purpose of this study is to introduce a post-processing approach to correct the image distortion in large field-of-view, *in vivo*, two-photon fluorescence microscopy imaging of microvascular structures. Our approach estimates a 3D distortion function directly from microscopic images based on a simple geometric modeling of the underlying biological structures. The estimated distortion function is then used to restore the geometric shape and structure of the vessels through image deconvolution. The performance of the proposed method is validated by restoring the spherical geometry of large microsphere phantom images. We then apply the method to *in vivo* vascular imaging of mouse hind limb muscle to demonstrate that the proposed approach can effectively reduce the spatial distortion and improve the image quality of microvascular structures in large field-of-view, deep-tissue datasets.

MATERIALS AND METHODS

Sample Preparation

In vivo imaging was performed on 3–12 months old B6 mice according to an approved protocol reviewed by the NHLBI Animal Care and Use Committee. Mice were prepared for imaging as described recently (Glancy *et al.*, 2014). Briefly, animals were intubated and anaesthetized through a ventilator with 2% isoflurane and 30% oxygen. The mouse was restrained on a temperature-controlled platform and fitted onto the microscope stage. Fur on the lower hind-limb was removed with a depilatory agent and the Tibialis anterior muscle was exposed by resecting the skin and gently abrading the top two layers of the myofascial

collagen sheath. The exposed muscle was coupled to the microscope objective with an optical coupling gel (0.3% carbomer, 300 mM sorbitol, pH 7.4) with a refractive index (1.342) very similar to that of water (1.335) (Rothstein *et al.*, 2006). Di-8-ANEPPS (ANEPPS) was used as a vasculature dye and injected into the heparinized jugular vein until the fluorescent signal was sufficient to begin the microvasculature imaging.

To study the image distortion and compare different correction approaches, phantom imaging was performed using fluorescent microspheres with a diameter of 4.0 μm (FluoSpheres Yellow/Green, Molecular Probes, Eugene, OR). The size of the microspheres was chosen to be close to the capillary diameter for the *in vivo* study. The microspheres were diluted in water, placed on a microscope slide, and then coupled with water to the objective (25x/0.95 HCX IRAPO, Leica Microsystems, Germany). In a second set of experiment, fluorescent microspheres of 0.2 μm diameter were used to measure the PSF of the microscope for comparing different deconvolution results. They were diluted in water and a drop was dried on a microscope slide, and then coupled with water to the 25x/0.95 objective mentioned above.

Imaging Parameters

A Leica TCS SP5 II upright resonant scanning multi-photon microscope with a Leica 25X, 0.95NA water immersion objective was used throughout the entire experiment (Leica Microsystems, Germany). A Ti:sapphire laser (Spectra Physics, Irvine, California) was tuned to 720 nm for two-photon excitation in the UV range.

All *in vivo* vascular images were captured at 0.68 μm isotropic resolution with 8 line averages. For each image, 512×512 pixels were collected every 280 msec with non-descanned detectors and an 8 kHz resonant line scanning mirror. The vasculature dye ANEPPS channel consists of two dichroic mirrors with cutoff wavelengths at 510 nm and 560 nm (Semrock FF510-Di02 and FF560-Di01, respectively) and a 70 nm wide band-pass filter centered at 605 nm (Chroma Technology, ET605/70M). A short-pass filter with a cutoff wavelength at 680 nm is used to block the excitation light for all imaging experiments.

The 4.0 μm microsphere image stacks were captured at $0.4 \times 0.4 \times 0.4 \mu\text{m}$ and at $50 \times 50 \times 50 \text{ nm}$ isotropic resolution with 2 line averages using two dichroic mirrors with cutoff wavelengths at 450 nm and 650 nm (cover full emission range of microspheres). The PSF images from the 0.2 μm microsphere were scanned at $52 \times 52 \times 142 \text{ nm}$ resolution.

Deconvolution Theory

The image formation of a microscope can be simplified as:

$$g=f \otimes h \quad (1)$$

where g is an observed image, f is an ideal image, h is the PSF, and $\otimes$ denotes the convolution operation. In order to correct the PSF distortion in the observed images, one needs to apply deconvolution procedures to obtain or approximate the ideal image. Deconvolution is a computational image processing technique to reduce image degradation

and blurring due to the PSF or system related distortions. Many deconvolution algorithms have been developed, including iterative, non-iterative, linear and nonlinear methods (Sun *et al.*, 2009). It is beyond the scope of this paper to compare different deconvolution algorithms. However, the nonlinear methods such as the commonly used Richardson-Lucy (RL) algorithms (Richards.Wh, 1972, Lucy, 1974) usually provide better results than linear ones (Biggs, 2010). In the RL algorithm, the deconvolution is performed through an iterative process which can be expressed as:

$$\hat{f}_{i+1} = \left(\frac{g}{\hat{f}_i \otimes h} \otimes \tilde{h} \right) \times \hat{f}_i \quad (2)$$

where $\hat{f}_i$ is reconstructed image at i^{th} iteration and $h(\tilde{x}) = h(-x)$. This process is executed iteratively until it reaches to a predefined maximum number of iterations or a convergence criterion, usually a pre-defined minimum root-mean-square error between $\hat{f}_i$ and $\hat{f}_{i+1}$.

In deconvolution microscopy, the PSF of the system is often measured empirically using nanometer size fluorescence beads that are close to the imaging resolution of the microscope. However, a single PSF estimate does not account for spatial variations of the imaging distortions due to refractive index inhomogeneities exist in scattering media such as biological tissues. As an extension to the standard deconvolution methods, blind deconvolution provides a more flexible approach for image restoration (Holmes, 1992, Biggs, 2010). Blind deconvolution is an adaptive technique to simultaneously estimating both the ideal image and the PSF as:

$$\begin{aligned} \hat{f}_{i+1} &= \left(\frac{g}{\hat{f}_i \otimes h_i} \otimes \tilde{h}_i \right) \times \hat{f}_i \\ \hat{h}_{i+1} &= \left(\frac{g}{\hat{f}_{i+1} \otimes h_i} \otimes \tilde{f}_{i+1} \right) \times \hat{h}_i \end{aligned} \quad (3)$$

In other words, it performs image restoration without direct knowledge about the PSF by adaptively modifying an empirical PSF during an iterative deconvolution process. This approach can be particularly useful to correct the PSF or a distortion function that varies spatially for different media across the imaging field.

Imaging Resolution and PSF

In order to measure the PSF of a microscope, the smallest beads should be used but they are weaker in signal intensity and bleach more rapidly. Therefore, microspheres with a diameter just under half of the microscope imaging resolution are typically used (Sibarita, 2005). However, these nanometers voxel sizes raise potential issues for large field-of-view, *in vivo* imaging due to tissue damage induced by two-photon absorption. In practice, voxel size is often limited to around a micron resolution in our *in vivo* imaging experiments (Glancy *et al.*, 2014) to prevent tissue deformation and photodamage. This requirement imposes limitations for applying a PSF measured at higher resolution to deconvolve images that are acquired at lower resolution settings. As shown in Figure-1, the effect of quantization or pixilation errors resulted from downgrading of the image resolutions can drastically degrade

the fidelity of the PSF measurements and limit the practicality of image deconvolution for distortion correction.

Model-Based Distortion Estimation

Mathematically, the distortion function or h in equation (1) can be derived from observed image g and source image f through the deconvolution process as mentioned previously. In this study we sought to derive the distortion function directly from the observed image with an ideal geometric model that represents the image object. We first demonstrate the feasibility of this approach by imaging large microspheres (4.0 μm in diameter) as a tissue phantom and use a spherical model to represent their ideal images. To better illustrate this method, equation (1) can be rewritten as:

$$g = m \otimes h, \quad (4)$$

where g is the observed microsphere image and m is a spherical model image describing the microspheres. Since microspheres are assumed ideally spherical, a solid model image m can be easily generated using the following inequality:

$$m = (x - x_0)^2 + (y - y_0)^2 + (z - z_0)^2 \leq r^2, \quad (5)$$

where (x_0, y_0, z_0) is the center of the image and r is the radius of the microsphere. Thus, the distortion function h can be estimated from the observed images g with above model image m through image deconvolution.

Phantom Image Analysis

To quantitatively evaluate the proposed approach, we processed the 4.0 μm microspheres image stack and analyzed the deconvolution results through following process. A model 3D image including a 4.0 μm diameter sphere was constructed using the same voxel size (0.4 μm isotropic) as the microsphere phantom imaging. We extracted a group of microsphere samples and estimated the distortion function for each microsphere image using the blind deconvolution algorithm. An average distortion function (DF) was then calculated by registering and averaging the DFs of all microspheres to improve the DF estimation. The effectiveness of the deconvolution was evaluated by using the averaged DF to restore all microsphere phantom images. The results were also compared with the conventional approach by using measured PSF for distortion correction.

Due to the spherical geometry of the microspheres, the object can be visualized with an ellipsoid. Therefore, we used the following quality measures or indices to evaluate the performance of our image restoration:

1. Diameters (D_{xy} and D_{xz}) of the object as measured on the XY and XZ planes.
2. Fractional Anisotropy (FA) of the object defined as:

$$FA = \sqrt{\frac{1}{2} \frac{\sqrt{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_3 - \lambda_1)^2}}{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}}, \quad (6)$$

where $\lambda_1, \lambda_2, \lambda_3$ are eigenvalues representing local invariant features of the object (Basser & Pierpaoli, 1996). FA has a scalar value between 0.0 and 1.0 that represents the degree of symmetricity or sphericity of an ellipsoid. For example, FA equals to 0.0 (i.e. $\lambda_1 = \lambda_2 = \lambda_3$) represents a perfect spherical or symmetrical object. FA closes to 1.0 (e.g., $\lambda_3 \gg \lambda_2 = \lambda_1$) represents an elongated ellipsoid such as a prolate spheroid.

To systematically measure these indices to evaluate the effect of image deconvolution, we developed the following procedures to automatically segment and calculate the indices automatically for each image stack: 1) apply a median filter with kernel $3 \times 3 \times 3$ to eliminate noises/outliers and stabilize signal intensity of image stacks, 2) calculate the maximum intensity projection of filtered images on XY and XZ planes, 3) apply a threshold at 30% of maximum intensity to filtered images to obtain a binary volumes, 4) perform a label geometry image filter (Padfield & Miller, 2008) to calculate the eigenvalues (λ), diameters (D), and Fractional Anisotropy (FA) for each binarized microsphere image volume. We applied these image analysis steps to the $4.0 \mu\text{m}$ microspheres imaging as described previously.

Microvascular Image Analysis

After the evaluation of our method in correcting the microsphere phantom data, we applied the proposed approach to *in vivo* microvascular images to reduce the spatial distortion of the vascular structures. Due to the discrepancies in imaging media and emission wavelengths between our phantom microspheres and the ANEPPS vascular dyes, the distortion function that was derived from the microspheres was not suitable to restore the spatial distortion in the microvascular images. Therefore, we extended our image processing approach to directly estimate the distortion function from the *in vivo* vascular structures and apply a top-to-down strategy for blind deconvolution in the 3D volume.

Since capillary vessels under regular blood pressure during *in vivo* imaging can be assumed to follow a simple geometry, a cylindrical model was used in our method to represent the microvascular structures of the capillary vessels. In order to establish such capillary model images, a small block of 3D capillary sub volume containing a single vessel structure was selected and extracted from the original 3D image stack. This sub volume was processed with median filtering ($3 \times 3 \times 3$ kernel size) and simple thresholding to segment the capillary structure. A 3D polynomial curve fitting was then applied to the segmented vessel in the binarized image to estimate its center line location.

Next, a 3D cylindrical model representing the extracted capillary structure was constructed by convolving a sphere with $4.76 \mu\text{m}$ diameter (7 voxels at $0.68 \mu\text{m}$ voxel size) with the estimated center line. This diameter is close to the capillary size of $5.0 \mu\text{m}$ as measured in previous experimental works (Krogh, 1921). From this reconstructed 3D model image and the original sub volume vessel image, a 3D distortion function (DF) was then estimated through the blind deconvolution algorithm, as shown in Figure-2.

To improve the estimation of the cylindrical vessel model in the selected sub volume, the fitting and reconstructing processes were executed iteratively to obtain the best polynomial

fitting between the reconstructed and original segmented vessels. To improve the quality of the estimated distortion function for the capillary structure, a default distortion function (DF_0) was computed by averaging multiple estimated DFs that were obtained from a group of capillary sub volume images at the same focal plane.

The entire 3D image stack was then divided into multiple equal size sub volume blocks with a small amount of overlap between neighboring blocks before estimating the image distortion at different spatial locations, as shown in Figure-3. To account for the spatial variation of the distortion function, the blind deconvolution algorithm was used to estimate individual DF for each image block. In our implementations, the block size of the image sub volumes was kept reasonably small (e.g. $64 \times 64 \times 64$) in order to effectively estimate the local distortion at each block locations. An overlap region (e.g. 25% – 50% of the block width) to the adjacent neighboring blocks was included in each image block to mitigate potential disrupted vessel signal or blocky artifacts occurred after the image restoration.

To process all the sub volume blocks in the entire image stack, we first applied the default DF_0 to the top-most image blocks to reduce the image distortion in each block by the blind deconvolution algorithm. A top-to-down approach was then used to propagate the final DF from each upper block to the block below it and then reapply the blind deconvolution to restore all the subsequent blocks. This approach allows our algorithm to adjust the distortion function along the Z direction gradually and overcome the change of distortion function associated with different focal planes in the 3D space. For blocks that contain weak vessel signal, the initial distortion function DF_0 will be reused for the subsequent blocks below them to avoid the degradation of DF after the blind deconvolution. This implementation not only improves the estimation of spatially varying distortion function but also speeds up the image restoration for a large size of 3D image stacks.

To measure the computational time of the proposed image restoration method, the following software implementation and hardware configuration were used. The blind deconvolution algorithm was implemented in C/C++ programming language under Microsoft Windows 7 64bit operation system for both CPU and Graphics Processing Unit (GPU) architectures. The CPU implementation was using FFTW library (Matteo, 1999) and GPU version was using CUDA (Compute Unified Device Architecture) library (NVIDIA, 2013). The image stack management and measurement tools were written in Python using NumPy/SciPy library (Oliphant, 2007). All time measurements were performed on an Intel Core i7 X990 3.46 GHz CPU and a NVIDIA GeForce GTX 690 graphics card.

RESULTS

Microsphere Image Restoration

The effectiveness of image restoration using our model-based approach was first evaluated on the microsphere dataset and compared with the conventional approach using empirically measured PSF. To demonstrate the magnitude of spatial distortion in the microsphere phantom images, Figure-4 shows (a) the maximum intensity projection image of a $4.0 \mu\text{m}$ microsphere imaging at $0.4 \mu\text{m}$ resolution, (b) the PSF measured from a $0.2 \mu\text{m}$ bead and rescaled to the same $0.4 \mu\text{m}$ resolution, and (c) the distortion function estimated from the

proposed method. The distortion function derived from our approach provides considerably more detail about the spatial distortion and inhomogeneity of the signal intensity profile than the measured PSF under the same image resolution.

To compare the effectiveness of image restoration using the derived distortion function and conventional measured PSF, Figure-5 shows the microsphere phantom images processed with various iterations of the blind deconvolution algorithm. Averaged statistics of the geometric quality indices were computed from five 4.0 μm microspheres before and after the image deconvolution. Using the measured PSF for image deconvolution, the signal intensity profile of the microsphere remains highly distorted even with a large number of iterations (e.g. 1000). There was only subtle improvement in both geometric indices (diameters D_{xy} and D_{xz} , and fractional anisotropy FA) showing the ineffectiveness of using the measured PSF approach. In contrast, the results of applying our derived distortion function demonstrated a large improvement in recovering the signal intensity and geometric profile of the microsphere merely after ten iterations. A significant improvement in both geometric indices was measured after 100 iterations, especially in the Z direction, which shows our approach can effectively recover the symmetry and sphericity of the 4.0 μm microspheres.

Microvascular Image Restoration

After the microsphere phantom evaluation, we applied the proposed method to *in vivo* vascular images to assess the effectiveness of our approach restoring the microvascular network structure in the skeletal muscle.

Figure-6 shows an example 3D image stack of the mouse microvasculature scanned at 0.68 μm voxel size, and the results of image deconvolution performed with the measured PSF and the derived distortion function. Figure-6a shows the maximum intensity projection images of the z-stack before and after the image restoration. There was significant improvement in image clarity and edge sharpness across the entire field-of-view after the image deconvolution using the derived distortion function. In Figure-6b, 2D cross-sectional images also showed the signal intensity distortion of the microvasculature structures was substantially improved using our approach. Furthermore, 3D rendered images in Figure-6c displayed significantly improved image contrast and decreased edge blurring across the entire image volume using the derived distortion function for image deconvolution. Such improvement is further apparent toward the bottom of the image volume. Overall, our proposed approach demonstrated prominent image enhancement and reduced spatially varying distortion of microvasculature structure in the images.

To quantitatively compare the improvement of image quality using the proposed image restoration approach, Table-1 lists the signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR) measurements from a total of 16 3D vascular image stacks. The vessel and background signals were computed from a binary vessel mask obtained through a simple thresholding. Compared to the original image stack and the measured PSF, there was a large improvement in SNR and CNR using the derived distortion function for image deconvolution. To quantitatively compare the improvement of microvascular structure after the image deconvolution, Figure-7 shows the cross-sectional signal intensity profile and

diameter measured from 16 vessels extracted from the upper region of the image stacks. A 3D thinning step was performed using ImageJ to extract the centerline (i.e. skeleton) of each extracted vessel. The line intensity profiles of all vessels were then aligned to the centerline to compare the signal intensity dispersion of the lumen before and after the image deconvolution. A threshold at 35% of the maximum intensity was used to measure the vessel diameter. In good concordance with the qualitative improvement of the microvasculature structures as shown in Figure-6, these results also demonstrate improved vessel cross-sectional plane signal intensity profiles and diameter using our proposed method.

To adjust for spatially varying distortion in the 3D image stack, we divided the entire image volume into small sub image blocks and then applied the blind deconvolution algorithm with a top-to-down approach as described in the methods section. Figure-8 shows an example for the distribution of spatially varying distortion functions that were estimated from different spatial locations within a mouse vascular image stack. There were apparent changes in signal intensity profile among the estimated distortion functions in 3D space demonstrating our approach can effectively estimate and adjust for spatially varying distortion across different image locations. To compare the spatial distortion estimated from image blocks at different depths, Figure-9 shows 2D cross-sectional displays of the distortion functions in three orthogonal planes. It is evident that in each block there was an increased amount of signal intensity distortion along the Z direction than the XY plane. Furthermore, such distortion was more prominent with the presence of larger vessels which appear more blurring in the image block.

DISCUSSION AND CONCLUSION

An image post-processing approach for correcting image distortion of microvascular structures in two-photon fluorescence microscopic imaging was assessed in this paper. The proposed method estimates the distortion function directly from image based on a geometrical model representing the structure of interest. This estimated distortion function was applied subsequently to recover the source image through blind deconvolution to adjust for spatial variation in the volumetric dataset. The performance of the proposed method was compared to the conventional approach using measured PSF. Our method showed significant improvement over the measured PSF in restoring the spherical geometry of the microsphere phantom dataset. We also demonstrated that using the estimated distortion function from *in vivo* vascular structure data and combining with a block-by-block blind deconvolution approach effectively improved the overall image quality and restored the tubular microvasculature of the skeletal muscles. The proposed approach can improve the objectivity and accuracy for image interpretation and quantification of *in vivo*, large field-of-view, two-photon microscopy imaging studies.

For the microsphere phantom dataset, we showed there was a large discrepancy in the signal intensity profile between the measured PSF and our derived distortion function as shown in Figure-4. The image deconvolution using the PSF measured from submicron beads leads to ineffective image restoration of the shape of the microspheres compared to the derived distortion function. We speculate this difference was resulted from different voxel size and

immersion media between the PSF and microsphere phantom imaging, and spatially varying light refraction of the large microsphere.

As in Figure-5, our results show that when applying the distortion function derived directly from the microsphere phantom data, the geometrical profile of the 4.0 μm microspheres was quickly recovered and converged to a symmetrical shape after a hundred iterations of blind deconvolution. The geometric indices were greatly improved and the blurring around the microsphere edges was also significantly reduced. On the other hand, the shapes of the 4.0 μm microspheres remain highly distorted and blurred after a thousand iterations of blind deconvolution using the measured PSF for image restoration. Furthermore, similar results, as shown in supplemental figures, were obtained using publicly available image deconvolution plugins in ImageJ (Schneider *et al.*, 2012); such as Deconvolution-Lab (Biomedical Imaging Group, 2009) and Parallel Iterative Deconvolution (Piotr, 2013).

For the vasculature image dataset, we showed our 3D cylindrical model combined with the proposed top-to-down image deconvolution approach can reduce the image blurring and restore the tubular structure of the capillary vessels. As shown in Figure-6, the maximum intensity projection image shows a more uniform range of signal intensity in all vessels after the image restoration. The vascular structure appears sharper and has a more symmetrical shape from the 2D cross-sectional view. In the 3D rendered display, the appearance of large vessel structures near the bottom of the image volume was also improved significantly. In addition to reduced background noise in the image, the edges of the capillary structure were more refined and the depth perception of the whole image stack was considerably increased after the image restoration.

Besides the improvement in image quality, another advantage of using the derived distortion function for image restoration is a significant increase in computation speed compared to the measured PSF. For all our vascular datasets that were acquired with the ROVAR system (Schroeder *et al.*, 2011), sequential image stacks were acquired with the size of each image stack around $512 \times 512 \times 768$ pixels at 0.68 μm voxel size. The computation time for the proposed image restoration procedures using the derived distortion function averaged about 10 minutes to process an image stack based on our CPU implementation. Comparatively, the computation time using the measured PSF averaged 10 to 20 times longer for the deconvolution processing with the same hardware configuration. It is worth to mention that the computation time is directly related to the number of iterations required for the deconvolution algorithm to converge. In our method, the number of iteration is varied among different sub volume blocks depending on the amount of information within the blocks. The deconvolution can converge quickly (10 to 12 iterations) for sub volume blocks containing more background signal such as the bottom 2 blocks in Figure-9. The deconvolution requires 20 to 25 iterations to converge when the blocks have more vessel signals such as the block 3, 4, and 5 in the Figure-9.

To qualitatively compare the distortion function derived from the vascular image with the distortion function of the microsphere phantom, it was observed that there was a dim rippled signal intensity pattern in the XZ plane of the derived vascular distortion function (Figure-2e) compared to both the measured PSF and the derived distortion function in the

microsphere phantom (Figure-3b and 3c). We speculate this more apparent signal intensity pattern in the estimated vascular distortion function was a result of light scattering and refraction from the surrounding tissue structures which was absent in the microsphere distortion function or the measured PSF. Thus, using the conventionally measured PSF or the microsphere derived distortion function for image deconvolution can lead to ineffective restoration of the tubular structure as shown in our microvascular images.

In our image restoration algorithm, dividing a large image stack into small 3D blocks for blind deconvolution is an important step to overcome spatially varying distortion exists in the whole volume. In order to improve the connectedness and smoothness of vessel signal intensity in the neighboring image blocks, we incorporated an overlapped region among all neighboring blocks. In our experiments, a reasonable performance was achieved using a 50% width of the block dimension as the size of the overlapped region. It is noted that reducing this size may result in disruptive signal intensity of the vessel or potential blocky background artifacts.

In our study, we found that the estimated distortion function was elongated more notably in the Z direction than the XY plane. As shown in Figure-9, there is an increase of signal intensity distortion that visually correlates with the size and signal strength of the vessel. To ensure a proper convergence in blind deconvolution adjusting for the spatial varying distortion estimates, we implemented a top-to-down approach to propagate the final distortion function from the upper block to the current block and use it as the initial distortion function for the blind deconvolution. This approach allows our algorithm to adjust the distortion function along the Z direction more efficiently and overcomes the change of distortion function associated with different focal planes as well as vessel contents in the 3D image stack.

In the case when there was very weak vessel signal or only background pixels existed in a given block, the initial distortion function from the top-most block was reused for the blind deconvolution to avoid degradation of the distortion function estimation. A threshold value was pre-calculated from an empty image block using the mean value of the estimated distortion function. This threshold was used to reset the initial distortion function of next block to the default one in the case there was very low vessel signal in the current block. This reset-to-default approach was a quick step in our current implementation to resolve low vessel signal cases.

A better approach which requires more computation would be to calculate an average distortion function from other neighboring blocks that have stronger vessel signal, and then apply such average distortion function to the current processing block. Such implementations may also lead to a multi-pass deconvolution strategy by refining the distortion functions after each pass among neighboring blocks. The resultant distortion estimate can be recalibrated by averaging each distortion function with its neighbors after completing a whole volume deconvolution. The calibrated distortion functions can then be reused as the initial distortion function for corresponding blocks during the next pass of deconvolution process. Of course, the trade-off would be a longer computation time.

A limitation of the current approach is that the prior information used for calculating the initial distortion function was based on a simple cylindrical geometry considered only a fixed capillary diameter. Though we find this useful in creating an initial condition for blind deconvolution to adjust for spatially variant distortion at different image locations, this approach may still be unlikely to remove all distortions in capillary or vascular structures that are significantly different from the initial condition. For studies requiring precise quantitative measures of the capillary or vascular dimensions, this approach may thus have its intrinsic limitations. However, as demonstrated in the paper, this approach is very effective in improving the image quality of vascular network of the tissue which is of primary interest to many investigators. If precise quantitative information is required, one may consider the use of injected or other intrinsic “guide stars” inside the tissue to precisely measure the image distortion. We have not evaluated systematically the effect of using different capillary diameters or geometric models for initial distortion function estimation. It is possible that iterative approaches could be developed to consider more than a single average capillary dimension. This is, however, outside of the initial goals of this work for improving the image quality of our *in vivo* vascular network dataset.

Another potential limitation in the proposed approach is the use of the blind deconvolution algorithms. In general, these algorithms do not converge to a unique solution unless certain specific constraints are applied to the image and/or the PSF. As a result, the distortion function reconstructed by deconvolving the images with a geometric model of the vessel structures will likely contain biases introduced by the a priori model. Notably, the deconvolution is unlikely to retrieve much information about the distortion function in directions where image contrast is flat. As in our examples shown in Figure-2 and Figure-9, the reconstructed distortion function displays strong astigmatism between X and Y, and with a size more than twice along Y than along X. This is unlikely to be genuine, but more likely reflects the fact that the vessels to be reconstructed were oriented nearly along the Y axis. This astigmatism may not affect the deconvolution results too much because the example considered is in effect two-dimensional. However, this limitation may still affect the effectiveness of the proposed approach in reconstructing the distortion function in situ from real specimen images.

In conclusion, we propose a model-based image restoration approach allows for correcting the geometrical distortion of microvascular structure in two-photon, large field-of-view, deep-tissue imaging. The results in our microsphere phantom study showed the proposed method restored the spherical geometry indices and reduced the computation time for image deconvolution compared to the conventional measured PSF method. Our approach also showed a large improvement in image quality and restoration of vessel structures from our *in vivo* microvascular image stacks. The proposed method should also be applicable to other types of fluorescent imaging applications such as confocal or wide-field microscopy to reduce the image distortion and assist in qualitative and quantitative image analysis of vascular networks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LAY DESCRIPTION

Image distortion in fluorescence microscopy affects qualitative interpretation and particularly quantitative comparison of biological structures. Conventional image deconvolution approaches using empirically measured point-spread function (PSF) from submicron beads have limitations in correcting for large structural distortion in *in vivo* deep-tissue scans that require spatially varying models of the PSF. In this paper, we present a model-based image restoration approach to reduce spatial distortion and image blurring of microvascular structures in two-photon fluorescence microscopy images. Our results showed significant improvement in quantitative geometry measurements of the microsphere phantom compared to conventional measured PSF approach. We also showed that the method improve the image quality of vessel structures in mouse skeletal muscle. This image restoration approach can assist in qualitative and quantitative analysis of large field-of-view deep-tissue two photon fluorescence microscopy images.

Figure 1. The effect of voxel size versus the PSF measurement. (a) Maximum intensity projection images showing the signal intensity profile of a 0.2 μm bead imaging at 52 nm resolution which represents the PSF of the microscope. Simulation shows a deterioration of the PSF information due to pixilation caused by an increased voxel size at (b) 0.4 μm and (c) 0.68 μm .

Figure 2. Image processing workflow for estimating the spatial distortion from a vascular image stack. Example 3D rendered images of a selected vessel segment showing the process: (a) original image, (b) thresholded binary image mask, (c) 3D polynomial fitting and center line estimation, (d) the reconstructed vessel model. Blind deconvolution was applied to the original image (a) and the model image (d) to estimate the 3D distortion function as shown in (e). 2D cross-sectional images of the distortion function in three orthogonal planes showing a larger distortion along the Z direction.

Figure 3.

Image processing workflow for applying the derived distortion function (DF_0) in Figure-2e to the entire microvascular image stacks. The 3D image volume was divided into multiple equal size image blocks with small amount of overlap between neighboring blocks. A top-to-down approach was implemented to propagate the distortion function (DF_0) from the upper block to lower ones. Blind deconvolution was used to estimate the spatially varying distortion function in each image block for 3D image restoration.

Figure 4. Example of image distortion in a 4.0 μm microsphere scanned at 0.4 μm isotropic voxel size in water. (a) Maximum intensity projection images of the microsphere in XY and XZ orthogonal planes. (b) The PSF measured from a 0.2 μm bead and rescaled to the same 0.4 μm resolution, (c) the derived distortion function using the proposed model-based methods. Compared to the derived distortion function, the PSF measurement shows significantly truncated information to describe the spatial distortion of the microsphere image. The white bar within each image indicates 1.0 μm scale.

Figure 5. Comparison of image deconvolution for the 4.0 μm microsphere phantom using the measured PSF and the derived distortion function (DF) as shown in Figure-4b and 4c respectively. For the measured PSF, signal intensity profile and geometric indices of the microsphere remain almost unchanged even after a large number of iterations. For the derived DF, signal intensity profile of the microsphere shows a large improvement by the blind deconvolution algorithm after 10 iterations. Both geometric indices, diameters (D) and fractional anisotropy (FA), show significant improvement after 100 iterations. A threshold at 30% of the maximum intensity was used for all measurements. The white bar indicates 4.0 μm scale.

Figure 6.

(a) Maximum intensity projection, (b) 2D cross-sectional, and (c) 3D rendered images of a mouse microvasculature image stack comparing the effect of image restoration. Original image stack is displayed in the left column. Distortion corrected images using the measured PSF and the derived distortion function are displayed in the middle and right columns respectively. Detailed comparison of the image restoration for the microvascular structure can also be viewed in the supplementary video files. The white bar indicates 10.0 μm scale.

Figure 7. Comparison of cross-sectional vessel signal intensity profile and diameter measured from the original versus restored image stacks using the measured PSF and the derived distortion function (DF). A threshold at 35% of the maximum intensity was used for the diameter measurements. Tick marks on the x-axis indicate the voxel position to the centerline.

Figure 8.

(a) 3D rendered image showing the distribution of spatial varying distortion functions that were estimated from consecutive sub volume blocks of a mouse vascular image stack. (b) Merged view of the estimated distortion functions and microvascular structure after the image deconvolution. (c) Two example vertical column blocks as shown in the maximum intensity projection display were extracted to demonstrate the results of the top-to-down 3D image deconvolution approach. (d) The left, middle, and right columns of each block show the original images, estimated distortion functions, and the restored images respectively. The white bar indicates 10.0 μm scale.

Figure 9. 3D close up display of the left column blocks in Figure-8c at different image depths and their corresponding estimated distortion functions in 2D cross-sectional views. There was an increased amount of signal intensity distortion along the YZ and XZ directions compared to the XY plane. The signal intensity in the estimated distortion was more prominent with the presence of larger vessels and appeared more blurry in the image block. The white 3D sphere at top left corner indicates 5.0 μm diameter scale.

Table 1

Comparison of SNR and CNR for the original versus restored image volumes. Statistics were obtained using 16 3D vascular image stacks. SD: standard deviation, DF: distortion function.

(mean $\pm$ SD)	Original	Measured PSF	Derived DF
SNR	9.15 $\pm$ 0.98	4.65 $\pm$ 0.60	16.64 $\pm$ 1.30
CNR	7.44 $\pm$ 0.97	4.06 $\pm$ 0.57	15.97 $\pm$ 1.33