Super-resolution scanning laser microscopy through virtually structured detection

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Abstract: High resolution microscopy is essential for advanced study of biological structures and accurate diagnosis of medical diseases. The spatial resolution of conventional microscopes is light diffraction limited. Structured illumination has been extensively explored to break the diffraction limit in wide field light microscopy. However, deployable application of the structured illumination in scanning laser microscopy is challenging due to the complexity of the illumination system and possible phase errors in sequential illumination patterns required for super-resolution reconstruction. We report here a super-resolution scanning laser imaging system which employs virtually structured detection (VSD) to break the diffraction limit. Without the complexity of structured illumination, VSD provides an easy, low-cost and phase-artifact free strategy to achieve super-resolution in scanning laser microscopy.

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1. Introduction

High resolution imaging is essential for biomedical study and disease evaluation. However, the spatial resolution of conventional imaging systems is constrained by light diffraction, which precludes the observation of fine structures of biological specimens. Several approaches, including stimulated emission depletion (STED) microscopy, stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM),
have been investigated to achieve super-resolution imaging. In a STED system, the fluorophores are excited by an excitation laser and followed by a second depletion laser with a doughnut-shaped intensity profile. The STED laser can deactivate the fluorophores in the periphery of the excitation laser focus, allowing only the fluorescence from the sub-diffraction-limited center to contribute to super-resolution recording [1, 2]. However, the extremely intensive laser exposure limits its applications for live cell imaging of biological systems, such as delicate and fragile retina. Alternatively, STORM [3], PALM [4], or fluorescence PALM (FPALM) [5] can achieve super-resolution by mapping localizations of individual molecules with photo-switchable fluorescence probes. Although single molecule localization based imaging approach has been demonstrated for live cell imaging [6–8], the imaging speed is limited due to the requirement of acquiring multiple sub-images for super-resolution reconstruction. Therefore, its application for high temporal resolution monitoring of live systems is still challenging.

Moreover, aforementioned super-resolution imaging approaches require exogenous fluorescent dyes or proteins. Therefore, they are not practical for intrinsic signal (e.g., reflectance or transmission) imaging. Bertero and his colleagues proposed a computational strategy suitable for both fluorescence and intrinsic signal imaging by taking the super-resolution reconstruction as an inverse problem [9–11]. However, the inversion process is ill-posed and sensitive to potential noise, and the reconstruction is numerically complicated and time consuming. Alternatively, structured illumination microscopy (SIM) has been developed to surpass the diffraction limit by shifting high frequency signal of the sample into the passing band of imaging systems [12–14]. The SIM can be implemented in both fluorescence and intrinsic signal imaging. However, the wide-field spatially structured illumination patterns require sophisticated manipulation of the pattern generator, i.e., grating [12–14] or grid [15, 16], and is not suitable for extended application in confocal scanning laser microscopy (SLM). In theory, SIM can also be realized in a point scanning system through spatiotemporal modulation, either by modulating light source intensity in illumination arm or by placing a moving mask in light detection arm [17]. However, the spatiotemporal modulation of the illumination/detection arm is technically difficult. So far, experimental validation of the proposed spatiotemporal modulation is not yet demonstrated.

The purpose of this study is to demonstrate virtually structured detection (VSD) for super-resolution SLM, which requires neither dynamic modulation of the light source intensity in illumination arm nor the physical mask in light detection arm [17]. In the VSD based system, the spatiotemporal modulation is achieved by mathematical processing of digital images. Digital implementation of the spatiotemporal modulation in the detection arm has been proposed in a theoretical article [17]. In this paper, freshly isolated retinas were employed for experimental validation of the VSD based super-resolution system in intrinsic reflectance imaging of thick (> 100 μm) live tissues. Experimental results showed that individual photoreceptors, which were not differentiated in the diffraction limited SLM, could be clearly identified in the VSD based super-resolution imaging.

2. Method

2.1. Experimental setup

Figure 1 illustrates a schematic diagram of the VSD based super-resolution SLM. A superluminescent laser diode (SLD-35-HP, Superlum), with center wavelength $\lambda = 830$ nm and bandwidth $\Delta \lambda = 60$ nm, is used to produce near infrared (NIR) illumination of the specimen. A pair of galvo mirrors (GVS002, THORLABS) is used to steer the focused NIR light across the specimen to generate two-dimensional (2D) images. In order to control the vignetting effect, the Fourier plane of the objective is conjugated to the middle point between these two galvo mirrors. The reflected light from the sample is descanned by the 2D (X and Y) scanning system, and is relayed to the image plane (CCD). Instead of using a single
element detector for recording light intensity in conventional SLM, a CCD camera (AVT Pike F-032B) is employed to map light profile (i.e., intensity distribution) of individual sampling points. The stack of 2D light profiles is used to construct super-resolution image based on the VSD method. Using a 5X objective with numeric aperture (NA) 0.1, the diffraction limited imaging resolution is 5 μm; while theoretical resolution of the VSD based super-resolution imaging is 2.5 μm.

Confocal configuration can be readily achieved using virtually synthesized pinholes [18] to reject out-of-focus light. The pinhole in conventional SLM and scanning laser ophthalmoscope (SLO) varies from 0.8X~10X Airy disc diameter [19–22] depending on specific applications. In comparison with a large pinhole, a small pinhole provides better resolution and enhanced sectioning ability [23]. However, a small pinhole can also reject photons that carry useful information which is required for the VSD based super-resolution reconstruction. In this paper, we set the virtual confocal pinhole as 2X Airy disc diameter as a trade-off.

2.2. Principle of VSD based super-resolution reconstruction

Point spread function (PSF) can be used to evaluate the resolution in spatial domain. For the system shown in Fig. 1, the PSF of the illumination path \( h_{il}(x, y) \) is identical to the PSF of the detection path \( h_{de}(x, y) \). Under incoherent illumination, the theoretical PSF is [24]:

\[
h_{il}(x, y) = h_{de}(x, y) = \frac{\Omega^2}{\pi} \left( \frac{J_1(\Omega \rho)}{\Omega \rho} \right)^2
\]

where \( J_1 \) is the first-order Bessel function, \( \Omega = 2\pi NA/\lambda \) and \( \rho = \sqrt{x^2 + y^2} \). The resolution of conventional SLM is defined as the radius of the Airy disc:

![Fig. 1. Schematic diagram of experimental setup. OB: objective; CO: collimator; L1-L3: lens; and BS: beam splitter. Focal lengths of lenses L1, L2 and L3 are 200 mm, 40 mm and 150 mm, respectively. The objective is 5X (NA = 0.1). The light source is provided by the SLD with center wavelength \( \lambda = 830 \text{ nm} \) and bandwidth \( \Delta \lambda = 60 \text{ nm} \). The theoretical resolution of this system is \( 0.61\lambda/\text{NA} = 5 \mu\text{m} \).](image)
\[ R = 0.61 \lambda / NA \] (2)

Given that \( \lambda = 830 \text{ nm} \) and \( NA = 0.1 \), the spatial resolution of the system is \( R = 5 \mu \text{m} \). In Fourier domain, the corresponding cutoff frequency of the PSF can be expressed as:

\[ f_c = 1 / R \] (3)

In other words, only frequency below the cutoff frequency is able to pass through conventional SLM system:

\[ -f_c \leq f \leq f_c \] (4)

For a single point light source, we assume here that the illumination intensity has a normalized number 1. Therefore, at the sampling position \((x_0, y_0)\), corresponding light intensity distribution on the sample is \( h_0(x-x_0, y-y_0) \). In a descanned system in Fig. 1, the light intensity distribution on the image plane (i.e., CCD) can be expressed as \( I_{\text{des}}(x, y, x_0, y_0) \). The non-descanned image can be obtained by shifting descanned images:

\[ I_{\text{non}}(x, y, x_0, y_0) = I_{\text{des}}(x-x_0, y-y_0, x_0, y_0) \] (5)

If we assume the reflectance ratio of the sample is \( s(x, y) \) and that the magnification of the system is ignored, then we will have [17]

\[ I_{\text{non}}(x, y, x_0, y_0) = \iint h_0(\mu-x_0, \nu-y_0)s(\mu, \nu)h_0(x-\mu, y-\nu)d\mu d\nu \] (6)

In order to achieve VSD, a digital mask is applied to multiply with the non-descanned image:

\[ I_{\text{mul}}(x, y, x_0, y_0) = I_{\text{non}}(x, y, x_0, y_0)m(x, y) \] (7)

We select a digital mask \( m(x, y) \) with sinusoidal function:

\[ m(x, y) = \cos[2\pi f_0(x \cos \theta + y \sin \theta) + \alpha] \] (8)

where \( \theta \) is the rotation angle of sinusoidal stripes and \( \alpha \) represents a constant phase. The carrier frequency \( f_0 \) is set here to the value of the cutoff frequency \( f_c \):

\[ f_0 = f_c \] (9)

It is worth noting that negative values are allowed in the digital mask. Therefore, there is no direct current (DC) component in Eq. (8). The spatial integral of the image \( I_{\text{mul}}(x, y, x_0, y_0) \) is assigned to the position \((x_0, y_0)\):

\[ p(x_0, y_0) = \iint I_{\text{mul}}(x, y, x_0, y_0) dx dy \] (10)

Substituting Eqs. (6) and (7) into Eq. (10) yields [17]:

\[
\begin{align*}
p(x_0, y_0) &= \iint h_0(\mu-x_0, \nu-y_0)s(\mu, \nu)h_0(x-\mu, y-\nu)m(x, y)d\mu d\nu \times dx dy \\
&= \iint h_0(\mu-x_0, \nu-y_0)s(\mu, \nu) \left( \iint h_0(\mu-x, \nu-y)m(x, y)dx dy \right) d\mu d\nu \\
&= h_0(x_0, y_0) \otimes \left[ s(x_0, y_0) \left( h_0(x_0, y_0) \otimes m(x_0, y_0) \right) \right]
\end{align*}
\]
where the integration order is changed, the fact that PSFs are even functions is assumed, and $\otimes$ denotes convolution. Considering $h(x, y) = h(x, y)$, exchanging $h(x, y)$ and $h(x, y)$, and then rearranging Eq. (11) yield:

$$p(x_0, y_0) = [(m(x_0, y_0) \otimes h(x_0, y_0)]s(x_0, y_0) \otimes h(x_0, y_0)$$  \hspace{1cm} (12)

Equation (12) exactly represents the acquired image of conventional wide-field SIM in which the modulation function $m(x_0, y_0)$ is implemented spatially in the illumination arm [25]. Equivalency of Eq. (11) and Eq. (12) implies that modulations in the illumination arm and in the detection arm are equivalent to each other in theory. The Fourier transform of Eq. (11) is [17]:

$$\tilde{p}(f_x, f_y) = \mathcal{F}\{p(x_0, y_0)\} = \tilde{h}(f_x, f_y)\{\tilde{s}(f_x, f_y) \otimes \tilde{h}_m(f_x, f_y)\tilde{m}(f_x, f_y)\}$$ \hspace{1cm} (13)

where $f_x$ and $f_y$ are spatial frequencies, and $\mathcal{F}$ is the Fourier transform operator. $\tilde{m}(f_x, f_y)$ is the Fourier transform of Eq. (8):

$$\tilde{m}(f_x, f_y) = \frac{1}{2}[\sigma(f_x - f_0 \cos \theta, f_y - f_0 \sin \theta)e^{i\omega} + \sigma(f_x + f_0 \cos \theta, f_y + f_0 \sin \theta)e^{-i\omega}]$$ \hspace{1cm} (14)

where $\sigma$ is the Dirac delta function. Because of sifting property of the Dirac delta function, Eq. (13) can be rewritten as:

$$\tilde{p}(f_x, f_y) = \tilde{h}(f_x, f_y)\{\tilde{s}(f_x - f_0 \cos \theta, f_y - f_0 \sin \theta)e^{i\omega} + \tilde{s}(f_x + f_0 \cos \theta, f_y + f_0 \sin \theta)e^{-i\omega}]$$ \hspace{1cm} (15)

where constant coefficients are ignored. Thus, the higher frequency $\tilde{s}(f_x, f_y)$ is shifted toward the lower passing band of $\tilde{h}_m(f_x, f_y)$ and the retrievable frequency based on Eq. (15) is

$$-2f_c \leq f \leq 2f_c$$  \hspace{1cm} (16)

Therefore, the theoretical retrievable band width is doubled. In other words, the theoretical resolution is enhanced by a factor of two.

2.3. Sample preparation

A standard optical target (USA 1951 1X, Edmond) and freshly isolated frog (Rana Pipiens) retina was used for functional test of the system in Fig. 1. It is known that the frog retina consists of photoreceptors with variable diameters (rods: \(-5-8 \mu m;\) cones: \(-1-3 \mu m\)) [26, 27]. Therefore, the frog retina provides an excellent preparation to evaluate the SLM resolution before (5 \(\mu m\)) and after (2.5 \(\mu m\)) VSD data processing. Moreover, the frog retina has \(-200 \mu m\) thickness, and thus it readily allows testing the VSD based super-resolution imaging in deep (> 100 \(\mu m\)) tissue. Animal handling was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Preparation procedures of freshly isolated retinas have been documented in previous publications [28, 29]. Briefly, the frog was euthanized by rapid decapitation and double pithing. After enucleating the intact eye we hemisected the globe below the equator with fine scissors. The lens and anterior structures were removed before the retina was separated from the retinal pigment epithelium.
3. Results

3.1. Computer simulation of VSD based super-resolution imaging

Figure 2 shows computational simulation of the VSD based super-resolution method. The diffraction limited resolution of the simulated system was 5 \( \mu \text{m} \) while the period of the simulated sample was 2.5 \( \mu \text{m} \). In other words, the information of the sample (black curve in Fig. 2(B)) could not pass through the passing band of the PSF (red curve in Fig. 2(B)) except for the DC component. Therefore, conventional SLM could not differentiate the sinusoidal variations of the sample as shown in Fig. 2(C). Figure 2(D) confirmed that only DC component of the sample was detected by conventional SLM. In contrast, the VSD based super-resolution imaging detected the diffraction light profile of each sampling point (Fig. 2(E)). The diffraction light profile was modulated by digital sinusoidal masks, as shown in Figs. 2(F)-2(H). Then the spatial integration was applied to the modulated map [see Eq. (10)]. Figure 2(I) shows the reconstructed image through the VSD based super-resolution method. From Fig. 2(I) and its spectrum (Fig. 2(J)), we can see that the lost information outside of the critical frequency was partially retrieved.

Fig. 2. Computational simulation of the VSD based super-resolution imaging. \( \lambda = 830 \text{ nm}, \text{NA} = 0.1 \) and incoherent illumination was assumed. The resolution of this system was 0.61\( \lambda/\text{NA} = 5 \mu \text{m} \). (A) Sample of sinusoidal stripes. The period was 2.5 \( \mu \text{m} \). (B) Normalized spectra of the sample and the PSF of the system on x dimension. The PSF was defined by Eq. (1). (C) Conventional SLM image. (D) Normalized spectrum of the image (C) on x dimension. (E) Diffraction map of the sampling point at the center of the sample (A). (F)-(H) superimposed images [see Eq. (7)] between the diffraction map (E) and the sinusoidal maps with orientation angle \( \theta = 0, 60^\circ \) and \( 120^\circ \), respectively. (I) Reconstructed super-resolution image. The background of the image was removed. (J) Normalized spectrum of the image (I) on x dimension. Scale bars indicate 10 \( \mu \text{m} \).
3.2. VSD based super-resolution imaging of standard optical target

First of all, we used standard optical target (USAF 1951 1X, Edmond) to verify the VSD based super-resolution imaging. Figure 3(A) was the image acquired by conventional SLM. The period of the smallest grating (white and blue rectangles in Fig. 3(A)) of this test target is 4.4 μm. The theoretical resolution of conventional SLM, which employed a 5X objective with 0.1 NA, was 5 μm. Therefore, conventional SLM was not able to resolve the smallest gratings, as shown both in the white and blue rectangles in Fig. 3(A). In contrast, the smallest bars could be differentiated in both x (green rectangle in Fig. 3(B)) and y (red rectangle in Fig. 3(B)) directions. The reflectance profiles in Fig. 3(C) further confirmed the resolution enhancement of the VSD based super-resolution imaging in x direction. Likewise, Fig. 3(D) confirmed the resolution enhancement in y direction.

![Fig. 3. Implementation of the VSD based super-resolution imaging on the resolution test target. (A) Image of the test target acquired by conventional SLM. (B) Super-resolution image by VSD reconstruction. (C) Normalized intensity curves along x axis. The white curve was normalized intensity along x direction of the area specified by white rectangle in (A). The green curve was normalized intensity along x direction of the area specified by green rectangle in (B). (D) Normalized intensity curves along y axis. The blue curve was normalized intensity along y direction of the area specified by blue rectangle in (A). The red curve was normalized intensity along y direction of the area specified by red rectangle in (B).](image)

3.3. VSD based super-resolution imaging of isolated retina

The second step of this study was to verify the feasibility of the VSD based super-resolution imaging of photoreceptors in intact retina. We used the freshly isolated frog retina for this technical validation. The diameter of frog rods is ~5-8 μm, and cones ~1-3 μm [26, 27]. Therefore, conventional SLM which has lateral resolution 5 μm could only resolve partial amount of photoreceptors in Fig. 4(A). In contrast, the VSD based super-resolution microscopy, which has lateral resolution 2.5 μm, was able to detect more photoreceptors in...
Fig. 4(B). For example, the region specified by the white ellipse in Fig. 4(A) appeared to be a single blur structure, while in the same area in Fig. 4(B) seven individual photoreceptors could be resolved. The resolution enhancement of the VSD based super-resolution imaging could be also exemplified by comparing intensity profiles along lines drawn in Fig. 4(A) and Fig. 4(B). Three clear bumps (arrowheads in Fig. 4(C)) corresponding to three photoreceptors (green arrowheads in Fig. 4(B)) became much more prominent in the red curve compared to the white curve in Fig. 4(C).

Fig. 4. VSD based super-resolution imaging of freshly isolated frog retina. (A) Image of the retina acquired by conventional SLM. (B) Super-resolution image of the retina by VSD reconstruction. (C) Reflectance profiles of the white and red line areas in A and B. The white curve and the red curve were normalized intensity profiles along the white line in (A) and the red line in (B), respectively.

4. Discussion

In summary, the VSD based super-resolution imaging has been experimentally validated to break the diffraction limit. The theoretical simulation result indicated that the VSD based super-resolution method was capable of enhancing the lateral resolution by a factor of two (Fig. 2). Experimental imaging of optical target (Fig. 3) and isolated retina (Fig. 4) verified that the VSD based super-resolution imaging revealed detailed structures that were not detectable in conventional SLM. Although the VSD based method can be implemented in an imaging system with a high magnification objective, we selected a relatively low magnification objective (5X) in this study for technical validation of super-resolution imaging of retinal photoreceptors.

The VSD based super-resolution imaging has two technical merits over conventional SIM. First, the VSD based super-resolution imaging is modulation artifact free. In conventional SIM systems, dynamic phase (e.g., 0°, 120° and 240°) modulation of structured illumination (e.g., sinusoidal gratings) is necessary for super-resolution reconstruction. Therefore, precise phase manipulation of illumination patterns is necessary, which makes conventional SIM vulnerable to modulation artifacts [30, 31]. In contrast, the VSD based super-resolution imaging applies virtual modulation digitally, and thus it is modulation artifact free. Second, the VSD based super-resolution imaging provides improved sectioning ability. In conventional wide-field SIM, only modulation with positive magnitude on the illumination is allowed [12]. Therefore, there is a DC component in Eq. (8):

\[ m(x, y) = 1 + \cos\left[2\pi f_0 (x \cos \theta + y \sin \theta) + \alpha\right] \]  

Equation (15) consequently becomes:

\[ \tilde{p}(f_x, f_y) = \tilde{h}_d(f_x, f_y) \tilde{s}(f_x, f_y) + 0.5h_d(f_x, f_y) \tilde{s}(f_x - f_0 \cos \theta, f_y - f_0 \sin \theta)e^{i\alpha} + 0.5h_d(f_x, f_y) \tilde{s}(f_x + f_0 \cos \theta, f_y + f_0 \sin \theta)e^{-i\alpha} \]  

Equation (17) consequently becomes:

\[ m(x, y) = 1 + \cos\left[2\pi f_0 (x \cos \theta + y \sin \theta) + \alpha\right] \]
The first item in Eq. (18) contains the zero spatial frequency that does not attenuate with defocus in a wide-field illumination system [32]. Although the structured illumination can theoretically remove the first item in Eq. (18), the first item may become dominant and overwhelm the second and third items that contain super-resolution information in a complex structure, especially in thick tissues. Thus, it has been challenging to conduct wide-field SIM in deep (> 100 μm) tissues. In contrast, a confocal configuration, such as the VSD based super-resolution imaging, can provide improved sectioning capability, due to attenuated zero spatial frequency signals correlated with out-of-focus volumes [33]. In order to further improve the sectioning ability, we are currently pursuing a VSD based super-resolution optical coherence tomography (OCT). We anticipate the OCT technique can provide improved penetration capability and enhanced imaging resolution in deep tissues. Moreover, we anticipate that VSD can have extended application in super-resolution assessment of retinal structures in vivo. High resolution is important for advanced retinal study and diagnosis. Adaptive optics (AO) [34] has been used to compensate for optical aberration of ocular optics to achieve diffraction limited resolution [35, 36]. Further integration of VSD into SLO promises a possible strategy to break the diffraction limit in in vivo imaging of the retina.

5. Conclusion

The VSD based super-resolution imaging has been demonstrated as a new strategy to break the diffraction limit. The VSD method is modulation artifact free, with improved sectioning ability, compared to wide-field SIM. In addition to confocal SLM demonstrated in this paper, the VSD method can be integrated into other imaging modalities, such as line-scan confocal imager [37, 38], multifocal scanning system [39], OCT, SLO, etc..

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