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Microscopy using randomized speckle illumination

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ABSTRACT

It is well known for structured illumination microscopy (SIM) that the lateral resolution by a factor of two beyond the classical diffraction limit is achieved using spatially structured illumination in wide-field fluorescence microscope. In the state of art SIM systems, grating patterns are generally generated by physical gratings or by spatial light modulators such as digital micro mirrors (DMD), liquid crystal displays (LCD). In this study, using a combination of LCD and ground glasses, size controlled randomized speckle patterns are generated as an illumination source for the microscope. Proof of concept of using speckle illumination in SIM configuration is tested by imaging fixed BPAE cells.

Keywords: Speckle, Structure illumination, Fluorescence microscopy, Optical imaging

1. INTRODUCTION

Biomedical engineering and optics play a very vital role in the areas such as imaging[1-3] and translational medicine[2, 4-10]. In recent years, there has been a lot of advancement specifically in biomedical optics, lasers and imaging[11-14]. It is important to note that in all these areas and specifically in optical imaging, illumination plays a very significant role.

Structured Illumination Microscopy (SIM) is the most widely accepted wide field microscopy technique to attain high resolution in both lateral and axial directions[15-17]. This method consists of modifying the illumination with well-defined fixed periodic illumination pattern (like grids). A set of images are captured, each corresponding to different phases of the projected structured illumination pattern. In SIM method, objective information with a high spatial frequency is down-modulated depending on the frequency of the illumination grating in the Fourier domain[18]. Another notable illumination technique is the laser speckle, which has found tremendous applications in field such as optical metrology, microscopy [19-21]. The speckle imaging has already been employed in many super resolution imaging techniques as the random pattern has many interesting statistical characteristics and together combining nonlinear analysis to extract high spatial frequency components of samples.

In this manuscript, we demonstrate here the experimental implementation of the concept of using microscopy with randomized speckle within well-defined periodic patterns as illumination. A comparison of spatial features resolvable through the new proposed illumination method is compared with SIM method. The obtained results confirm that apart from offering large working distance, the resolution achievable with this illumination technique is superior compared to conventional wide field microscope configuration.

2. RESULTS

2.1 Generation of randomized speckle within well-defined patterns

The schematic diagram of the microscope with illumination modified as having randomized speckle within well-defined fixed patterns is shown in Figure 1. A multiline laser source combiner with diode lasers of wavelengths 561 nm, 514 nm and 488 nm serve as excitation sources (Coherent Inc, OBIS laser, USA). The microscopic objective lens used in the study was 50X Mitutoyo Plan Apo infinity corrected (50X, NA0.55, 13 mm working distance), long working distance microscope objective. The light from the laser was first passed through a ground glass to generate speckle illumination. HOLOEYE, LC 2012 liquid crystal spatial light modulator was used to further modulate the speckle light. The light modulated by the HOLOEYE, LC 2012

liquid crystal is then passed through a polarizer to tune and project the desired pattern of illumination. The illumination pattern was then reflected by a dichroic beam splitter onto the back aperture of the microscope objective. The fluorescence emission from the microscope objective lens was then collected through a tube lens (ITL 200, Thorlabs). LB-1844-A convex lens (Thorlabs, $F=50$ mm) was placed at the focal plane of the tube lens. The reflected laser beam separated from the beam passing through the tube lens by a filter and finally captured by the EMCCD camera (iXON 887 Andor, Canada). During measurements in SIM mode, no ground glass was used, light from the laser was directed directly towards SLM (no speckle generation).

Figure 1. Experimental setup of fluorescence microscope with illumination modified by randomized speckle within periodic fixed patterns. Lens 1 is 50X Mitutoyo Plan Apo infinity corrected (50X, NA0.55, 13 mm working distance) and Lens 2 is planoconvex lens is LB-1844-A convex lens (Thorlabs, $F=50$ mm). SLM is HOLOEYE, LC 1012 liquid crystal spatial light modulator.

2.2 Lateral resolution

Figure 2. Lateral resolution measurement using 200 nm fluorescent beads. (a) Illumination using fixed pattern (like SIM configuration). (b) Illumination by randomized speckle within fixed pattern. Note, 200 nm beads were selected because the beads are below the sub-diffraction limit of the microscope objective lens we used. The blue box highlight the proof of improved resolution.

For the microscope objective (50X with NA 0.55, 13 mm working distance) used in our study, 510 nm is the Abbe's resolution limit for 561 nm wavelength. Experimentally, to measure the spatial resolution of the microscope, we imaged TetraspeckTM fluorescent microspheres ($0.2\mu\text{m}$, $\lambda_{\text{ex}} = 560$ nm and $\lambda_{\text{em}} = 580$ nm) on $170\mu\text{m}$ thick coverslips as test samples (Figure 2). Figure 2a shows the fluorescent bead images obtained from SIM microscope configuration. Figure 2b shows the fluorescent bead images obtained from our microscope configuration, in which the illumination is randomized speckle within well-defined fixed pattern. The image of bead features obtained using randomized speckle within well-defined patterns show improved lateral resolution

over conventional SIM configuration. This result clearly shows the improved lateral resolution for the conventional SIM configuration (with fixed patterns) when combined with randomized speckle illumination.

2.3 Proof of concept

Figure 3. Images of BPAE cells obtained by (a) conventional wide field microscopy and (b) our system. (c) The dotted line scans over the cell images at the same location in a, b are plotted as intensity Vs position in c.

Many illumination techniques meant to improve certain imaging capabilities of the microscope. However, not suitable for wide-field modes and hence have restricted use in microscopy applications such as cell biology. As a proof of concept of using this novel illumination method for cell biology, we have compared this illumination method as compared to conventional widefield microscope by imaging bovine pulmonary artery endothelial cells (BPAE cells) slice. Mitochondria of the BPAE cells have been labelled with MitoTracker® Red CMXRos and F-actin with Alexa Fluor® 488 phalloidin. It can be observed that the respective fluorescence images of mitochondria and F-actin were clearly visible with 561 nm and 488 nm excitations. Figure 3 shows image obtained by using randomized speckle within well-defined fixed patterns has much improved lateral resolution over conventional wide-field microscopy.

3 DISCUSSION

In widefield microscopy configuration, modulation of illumination light has shown tremendous impact on the resolution of the imaging system. Here we have combined periodic pattern together with random speckle pattern for illumination. The improvement in lateral resolution of the imaging system using this illumination concept is experimentally validated by imaging individual 200 nm beads and adjacent beads which are known to be below diffraction limit of the system (Figure 2). As a proof of concept for high resolution fluorescence microscopy for cell imaging, we have performed imaging of mitochondria and F-actin of BPAE cells with better clarity as compared to conventional microscope (Figure 3).

Image processing for the captured images: For our configuration, we have used a combination of ground glass and transmissive SLM (HOLOEYE, LC 2012) to generate illumination pattern. The SLM is based on a Liquid Crystal (LC) micro-display with 1024 X 768 pixel resolution. This LC-SLM can change grayscale level of any pixel between 0 and 255 with switching rate of 60Hz. Three images were captured using an EMCCD camera with the phase of the illumination pattern shifted 120° between them. This method was repeated for two more

orientations of the illumination pattern rotated by 120° and 240°. For random speckle illumination, there are no standard algorithms to extract data. However, since we have used a combination of speckle and fixed pattern as in SIM, Fourier-based image reconstruction was performed to the captured images according to the method proposed by Gustafsson[15]. Finally, to subdue the noise in the images, deconvolution was performed using Iterative Parallel Deconvolution using ImageJ software. Precise knowledge of illumination pattern is vital for image reconstruction. Since the speckle projection in the fixed periodic pattern can change with either the wavelength or speckle projection, the image reconstruction will not be as same as performed for fixed periodic pattern (without speckle). We believe a better algorithm would enhance the data treatment and this would be one area we will be focusing for our future work.

The speed of data acquisition of this method has similar challenges as in standard SIM techniques. In our system, speed and sensitivity of the CCD camera and SLM switching time is the critical factors which limits the system performance. The LC-SLM switching frequency is 60 Hz, thus has a negligible impact on the speed of data acquisition. However, use of DMD (switching frequency 10,000 Hz) instead of LC-SLM is an alternate option to improve the data acquisition speed. In the current version, we used a 512 x 512 pixels commercial EMCCD camera (iXON 887 Andor) with a maximum full-frame rate of 25 fps.

4 CONCLUSION

In this report, we have demonstrated using experimental evaluation, the potential of using randomized speckle within fixed periodic pattern as illumination to improve widefield microscopes resolution. As compared to fixed illumination pattern as used in SIM, the randomized speckle within fixed pattern further enhances the lateral resolution. As a proof of concept, we have demonstrated by using the novel illumination concept in a fluorescence microscope configuration to image BPAE cells. It is envisaged that this simple illumination concept can offer a wide range of potential applications in a variety of fields such as microscopy, metrology, diagnostic imaging to name a few.

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