Approach to multiparticle parallel tracking in thick samples with three-dimensional nanoresolution

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This Letter proposes a method referred to as distorted grating (DG) and double-helix point spread function (DH-PSF) combination microscopy (DDCM), which is capable of multiparticle parallel localization and tracking in a transparent sample thicker than 10 μm, the thickness of a cell. A special phase mask, combining the field depth extension capabilities of DG with the three-dimensional (3D) nanolocalization capabilities of the DH-PSF, is designed for multiparticle parallel localization. Time-lapse tracking of one particle moving along the z axis and parallel tracking of two particles are simulated. Results demonstrate that, with only a single snapshot, particles can be localized, tracking with 3D nanoresolution wherever they are. The theoretical localization precisions of DDCM, DH-PSF, and multifocus microscopy are compared. DDCM results in almost constant localization precisions in all three dimensions for a depth of field larger than 10 μm. DDCM is expected to become a tool in investigations of important dynamic events in living cells. © 2013 Optical Society of America

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Research on intracellular dynamic events is important to the understanding of cellular function and even life itself [1–3]. Single-particle tracking (SPT) provides information to the dynamics of interested molecules and vesicles in real time, without being obscured by the averaging inherent in bulk studies [4]. In SPT, thanks to the high localization accuracy, the particle can be localized with an accuracy as fine as 1.5 nm [5].

Almost all the biological functions inside cells involve molecular transport that is inherently three-dimensional (3D) in nature, so some research groups have reported their work on 3D SPT. These studies have used techniques such as astigmatism (cylindrical lens) [6], multiplane methods [7–9], fluorescence interference (iPALM) [10], double-helix point spread function (DH-PSF) [11,12], and designed corkscrew point spread function (PSF) [13]. Among all these 3D localization methods, DH-PSF has the largest effective imaging range on the z axis [i.e., the effective depths-of-field (DOFs)], even to more than 10 μm [12]. However, the localization accuracy in the z axis has to be sacrificed to extend the effective DOF, and we will discuss this later in this Letter. As to other approaches, their effective DOFs are too thin to image whole mammalian cells, which tend to be about 10 μm thick. In order to track molecules throughout a cell, combination of these 3D localization methods with z scanning is a possible method. But obviously, it is time consuming, and some information will be lost because molecules cannot be tracked until they are scanned again after a regular period. So, multifocus microscopy (MFM), including multifocal plane microscopy (MUM) [14–16] and distorted grating (DG)-based MFM [17], are more preferable. In MUM, although tracking over a 10 μm region has been achieved successfully [16], several cameras were used to image different focal planes, and this requires a large, expensive system. In one previous study, a focal stack of nine two-dimensional (2D) images was acquired with an aberration-corrected MFM [17]. The more images there are in a focal stack, the larger the axial range in which the molecules can be tracked, but the number of photons collected per molecule has to be divided into more parts per image, which decreases the accuracy.

In this Letter, a new approach to tracking multiple particles simultaneously is proposed. This technique involves 3D nanoresolution in a transparent sample thicker than 10 μm. In brief, the DG function and DH-PSF function are combined with a single one-phase mask, so the approach is named DG and DH-PSF combination microscopy (DDCM). With DDCM, multiple particles in a whole cell can be imaged and localized simultaneously with only one snapshot, no matter where the particles are located.

The phase mask for the DG is actually an off-axis Fresnel zone plate, which is capable of diffracting light as an ordinary optical grating. At the same time, the mask can focus beams with different strengths by introducing different phase contributions into different diffraction orders. In practice, the grating can be positioned anywhere within the detection light path [18].

DH-PSF is one example of the so-called self-imaging effect, which can be achieved by extracting only a few Laguerre–Gauss (LG) modes located along a straight line in the LG modal plane [19]. The LG modes selected for DH-PSF are (1, 1), (3, 5), (5, 9), (7, 13), and (9, 17) [11].

In order to localize single molecules throughout a cell with nanoresolution, the field depth-extending
Design of bifunctional phase mask. The image shows the superposition (c) of two phase patterns, corresponding to (a) DH-PSF, $\varphi_{DH}$ and (b) DG, $\varphi_{DG}$, respectively. Different shades of gray represent corresponding phase shifts, as shown in the gray bar.

capabilities of the DG are combined with the 3D imaging capabilities of DH-PSF. The DG part and DH-PSF part can be cascaded directly. However, such a configuration will increase the complexity of the system and consequently reduce the signal transfer efficiency, so the localization accuracy will be reduced correspondingly. In order to avoid such negative effects, a single-phase mask configuration is adopted in DDCM. In this configuration, one-phase mask $\varphi$ is designed to be the superposition of two phase patterns, $\varphi_{DG}$ and $\varphi_{DH}$, for the functions of DG and DH-PSF, respectively (Fig. 1): $\varphi = \varphi_{DG} + \varphi_{DH}$. For an incident plane wave of unit amplitude, the phase shift introduced by the phase mask is the product of the two phase factors $f_{DG} = \exp(i\varphi_{DG})$ and $f_{DH} = \exp(i\varphi_{DH})$, and $f = f_{DG} \cdot f_{DH}$. The corresponding inverse Fourier transform is $F = F_{DG} \ast F_{DH}$. $F_{DG}$ and $F_{DH}$ indicate the 2D Fourier transforms of $f_{DG}$ and $f_{DH}$, respectively. This means that when the designed phase plate is mounted in the Fourier plane of the detection light path, the 3D localization capability of DH-PSF will be replicated to three diffraction orders of DG.

Numerical simulations are then used to demonstrate the capabilities of this bifunctional phase mask. In all simulations, the emission wavelengths of the emitters are set to 670 nm. The signal is collected by an oil immersion objective (NA 1.4, 100×). After a tube lens ($f_{tube} = 180$ mm), the light is modulated with a 4f relay system ($f = f_{tube}$), where the phase masks are mounted in its Fourier plane. The signal is finally detected with a detector whose pixel size is set to 16 μm. The phase pattern of DH-PSF in DDCM, $\varphi_{DH}$, is optimized on the same procedure introduced in [20]. All the phase masks are designed with 256 phase steps, and 336 × 336 pixels. And the pixel size is $7.5 \mu m \times 7.5 \mu m$.

In the first simulation, one emitter moving along the $z$ axis is imaged. Three situations are compared (Fig. 2). When only the DG function is employed, the images of the emitter are shown in Fig. 2(a). Only when the emitter is at several specific locations conjugated to the three focal planes of the three diffraction orders [up arrows in Fig. 2(a)] can it be imaged and localized effectively in 2D. If only DH-PSF plays the role [Fig. 2(b)], the emitter can be 3D localized only when it is located in a certain axial range, $h_{DH}$, which indicates the 3D localization capability of DH-PSF. In all simulations in this Letter, $h_{DH}$ is set to 4 μm, which means particles at $z = -2$ to 2 μm can be localized according to the angle of the line between the two lobes of the PSF, $\theta$.

This difficult choice is not necessary in DDCM [Fig. 2(c)]. In order to make full use of the localization ability of DH-PSF, the DG can be designed to be capable of imaging three layers’ spacing, $h_{DH}$, simultaneously. However, in DH-PSF, because $\theta$ will change from 0 to $\pi$ when the emitter moves from $-2$ to 2 μm along the $z$ axis, if the emitter is too close to the edge of this action range, a serious localization error may occur. As an extreme example, when the emitter is at $z = 2$ μm, it may be mistaken for the one at $z = -2$ μm, which is unacceptable for localization. So, the axial ranges for the three diffraction orders are designed to overlap partially; that is, the distance between the two focal planes for the first and zeroth diffraction orders, $h_{DG}$, is designed to be 3.4 μm, a bit smaller than $h_{DH}$. The total effective axial range of response for DDCM is 10.2 μm rather than 12 μm. In order to localize the emitter, the imaging plane must be divided into three parts [see Figs. 2(c) and 3(a)], which are divided with two dot-dashed lines, corresponding to the three available imaging areas for the three diffraction orders. The axial positions of the focus for these three areas are 3.4, 0, and $-3.4$ μm, respectively, as indicated with right arrows in Fig. 2(c). The same DH-PSF localization procedure [11] can be used for each

![Fig. 1](image1.png)

![Fig. 2](image2.png)
of the three imaging areas to determine the lateral position and the relative defocusing distance, $d_{\text{ve}}$, which is defined as the axial displacement of the emitter relative to the focus for the imaging area. The absolute axial position of the emitter can be calculated with Eq. (1):

$$d_{\text{abs}} = \begin{cases} d_{\text{ve}} + h_{\text{DG}}, & m = \pm 1 \\ d_{\text{ve}}, & m = 0 \end{cases},$$

where $m$ means the diffraction order. In DDCM, if the emitter moves out of the original DOF of the DH-PSF, it can also be captured in the imaging areas of the +1st or the -1st diffraction orders [Fig. 2(c)]

In the second simulation, two moving emitters ($E_1$ and $E_2$) are traced. $E_1$ and $E_2$ are located at (-2, -2.5, 5.1) and (2, -2, 5.1) μm originally; they then move bottom-up and top-down along two spiral trajectories described as $(\mp 2 \pm 0.3 \cos(2\pi t/10.2), \pm 2 \pm 0.3 \cos(2\pi t/10.2), \mp 5.1 \pm 0.1t)$ μm. For each emitter, the number of photons per exposure is set to 2000. Three typical images at three points in time ($t = 0, 3.4, \text{and } 5.1$ s) are shown in Fig. 3(a). In order to make the images closer to real situations, images shown here are pixelated and corrupted by Poisson noise and Gaussian noise. The variance of the Poisson noise here is set to 2. The expected value and variance of the Gaussian noise here are set to 0 and 2, respectively. It is obvious that even with the same noise level, the signal-to-noise ratios (SNRs) for the images are different. It is reasonable that the image recorded at $t = 5.1$ s has the highest SNR while the emitters are both at $z = 0$, the focal plane related to the zeroth diffraction order. Images of $E_1$ and $E_2$ are highlighted with red and blue circles, respectively. When the two emitters are 10.2 μm apart ($t = 0.0$), they can be imaged simultaneously, but in different imaging areas. When the emitters are in the overlapped axial ranges mentioned above, they might be shown in two adjacent imaging areas corresponding to two diffraction orders ($t = 3.4$ s). Either imaging area can be used for localization. Besides, in practice, this is also a natural and easy way to calibrate the three coordinate systems for the three imaging areas. When the emitters are both in the same axial range of one diffraction order, they can be captured in the imaging area of this diffraction order ($t = 5.1$ s). 103 successive images are recorded at the predetermined time interval, 10 ms. Each image has to be divided into three parts, as mentioned above. For each imaging area, the positions of the emitters are estimated with the same method [11], and then the estimated positions coming from the three areas are moved to the same coordinate system by coordinate system translation. The estimated trajectories of the two emitters are shown in Fig. 3(b).

Next, the theoretical localization precision for an unbiased estimator of DDCM is analyzed and compared with the DH-PSF system and MUM [16] by using Fisher information analysis. The imaging system for DH-PSF is similar to that for the DDCM introduced above, except for the phase mask. The effective DOF of DH-PSF for the comparison is set to 10.2 μm. In MUM, the axial position of the midplane between the two focal planes corresponding to the second and the third detectors is set to $z = 0$. In all cases, the number of total photons detected on the pixelated detector is set to 1000, and the background level is set to 2 photons/pixel. The attainable localization precision can be described by the square root of the Cramér–Rao bound (CRB), which is actually the inverse of the Fisher information matrix. The same methodology [21] is adopted for all cases, except that the image function is replaced by DDCM PSF, MUM PSF, and DH-PSF. All computations used for each PSF simulation are based on scalar diffraction theory and a wave-optics analysis of the respective imaging system. Their lateral ($x$ and $y$) and axial ($z$) localization precisions as a function of the axial position $z$ with respect to focus are shown in Fig. 4. The lateral localization precisions of DDCM and MUM are generally comparable for almost all the range [Figs. 4(a) and 4(b)], while the axial localization precisions of DDCM are more constant [Fig. 4(c)]. DH-PSF is superior to the other two but only for a certain axial range around the plane $z = 0$, but its localization precision deteriorates dramatically for the axial positions beyond this range. For DH-PSF, the $z$ position is dependent on the angle of the line between the two lobes, with the same effective value range (from 0 to $\pi$), which means that the larger the DOF is, the lower the localization accuracy will be. DH-PSF with such a large DOF is not ideal for the accuracy of the localization.

One assumption for DDCM is that the emitters are distributed sparsely enough to be resolved by the optical system; hence, DDCM is also useful in time saving when combined with single-molecule-localization microscopies [22–24]. The DG discussed in this Letter is only one-dimensional. Actually, the DOF extension ability can be improved further by replacing the phase pattern of the DG for the bifunctional phase mask with a 2D DG [15]. In DDCM, the bifunctional phase mask can be implemented with spatial light modulators, which have already been used in many similar occasions, for example, the DH-PSF system itself [11]. However, since the phase pattern for the phase mask used in DDCM does not change during the experiments, fixed phase masks are more preferable. The bifunctional phase mask can be fabricated by gray-level lithography. Since the phase masks for DH-PSF and DG have already been both fabricated successfully [25, 26], fabrication of the designed dual-function phase mask for DDCM should not be a big challenge. In DDCM, photons for each diffraction order are about one-third of the total photons. One tough problem that might become a bottleneck in the development of

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**Fig. 3.** Two-particle tracking with DDCM. Two emitters were simulated to move bottom-up and top-down spirally. (a) Three typical images of the emitters at $t = 0.0, 3.4, \text{and } 5.1$ s are shown. (b) Trajectories of $E_1$ (blue) and $E_2$ (red) are drawn with 103 time-lapse images.
this method is the decreased localization accuracy caused by this shortage of photons. Thanks to the development of advanced labeling materials, this deficiency can be counteracted with brighter materials, such as polymer dots \cite{27} and ultrabright fluorophores created by reductive caging \cite{28}.

In conclusion, a new MPT method, called DDCM, is put forward and numerically demonstrated. The critical element is a specially designed bifunctional phase mask which combined the DOF-extending capabilities of the DG with the 3D nanolocalization capabilities of DH-PSF. The simulations demonstrate that with this phase mask, multiple particles in a sample more than 10 µm thick can be tracked simultaneously. DDCM may have important applications in the fields of high-resolution biomedical dynamic imaging and in the study of vital processes in living cells.

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