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Clean localization super-resolution microscopy for 3D biological imaging

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We propose clean localization microscopy (a variant of fPALM) using a molecule filtering technique. Localization imaging involves acquiring a large number of images containing single molecule signatures followed by one-to-one mapping to render a super-resolution image. In principle, this process can be repeated for other z-planes to construct a 3D image. But, single molecules observed from off-focal planes result in false representation of their presence in the focal plane, resulting in incorrect quantification and analysis. We overcome this with a single molecule filtering technique that imposes constraints on the diffraction limited spot size of single molecules in the image plane. Calibration with sub-diffraction size beads puts a natural cutoff on the actual diffraction-limited size of single molecules in the focal plane. This helps in distinguishing beads present in the focal plane from those in the off-focal planes thereby providing an estimate of the single molecules in the focal plane. We study the distribution of actin (labeled with a photoactivatable CAGE 552 dye) in NIH 3T3 mouse fibroblast cells.

The last decade has seen an enormous growth in fluorescence microscopy in general and super-resolution microscopy in particular. Some of the prominent super-resolution techniques include, fPALM,3 STORM,5 PALM,4 STED,6 SIM,7 GSDIM,8 and PAINT.9,10 All these techniques are primarily dependent on the photophysics of single molecules, and, specifically, their ON and OFF states. In addition, 3D super-resolution is made possible by Biplane (BP) fPALM that use diffraction limited spot size for imaging two axially separated object planes.12 These techniques have enabled visualization of nanoscale organization of proteins and lipids (which themselves range from < 1nm to tens-of-nanometers) in samples of a range of sizes, including in mammary cell spheroids.13 Temporal resolution is another key factor that is essential to study rapidly occurring biological processes.25 Of late, rapid versions of established super-resolution techniques (such as, RapidSTORM and QuickPALM) have been demonstrated.18–21 In STORM microscopy, fast photoswitchable probes have enabled live cell 3D super-resolution imaging.22 Since, these techniques can be employed over large scale of spatial dimensions and possess high temporal resolution, they qualify to become the next generation of state-of-art imaging techniques.

The common feature among almost all the super-resolution techniques involves the photophysics of fluorescent probes. STED microscopy utilizes photophysics to deplete (switch-off) the molecules at the periphery of the diffraction limited excitation spot resulting in fluorescence emission from a relatively small spot. Single molecule based localization microscopy techniques employ the bright (fluorescence) and dark states of fluorophore to render sub-diffraction resolution. True representation of the fluorophore map distribution is essential for both quantitative and qualitative understanding of the target biological process. This becomes even more critical for studies involving interactions at the single molecule level. So, it is imperative to devise optical and computational...
techniques to render 2D images that are free from false realization of single molecules in the focal planes.

Although, localization techniques are relatively simple, cost-effective and reduce photobleaching, they need special attention for reliable imaging. In the widefield mode where signals arrive from both the bottom and top of the sample (off-focal planes). This can result in images which are difficult to interpret. Our realization of localization microscopy is depicted in Fig. 1. Since this technique involves both activation and excitation, this calls for two independent light sources of different energy. The high energy light is used to activate the molecules by means of breaking chemical bonds or through conformational changes. Fig. 1 shows two light sources of different wavelengths, $\lambda_{405nm} (S_{actv})$ for activation and $\lambda_{532nm} (S_{exc})$ for excitation. The activation laser is purposefully routed through single mode fiber for obtaining a nearly Gaussian profile. This is followed by combining the beams using a beam-combiner (BC). The resultant beam is passed through the beam expander (BE) to expand the beam to an appropriate size before being focused by $L_3$ at the back-focal-plane of the objective lens $O$. For the present study, a beam expansion of $2 \times$ is found to be enough to ensure required field-of-view for visualizing the whole cell. The dichroic mirror (DM) ensures reflection of illumination light sources and allows the emitted fluorescence to pass through. The output fluorescent light is focused by the internal tube lens $L_4$ of the inverted microscope. To achieve higher magnification we have employed $L_5$ and $L_6$ with a focal length ratio of $1.6$. With a $100 \times$ magnification of the objective lens, the total magnification of the detection sub-system becomes $100 \times 1.6 = 160 \times$. Finally, the magnified image is made to pass through a set of filters $F$ to eliminate stray light and background before it falls on the sensitive EMCCD camera sensor.

Major steps involved in the existing super-resolution microscopy are : (1) Acquiring single molecule signatures (approximated by Gaussian spots) in the EMCCD camera, (2) Localization of single molecules, and (3) Combination of all the centroid of the corresponding Gaussian in a single frame. In the widefield mode, this results in molecules outside the focal plane being sometimes included within the rendered image. Although techniques for identifying z-plane information of single molecules have been achieved, this calls for precision instrumentation and additional complex optical arrangements. These methods introduce additional optical components, which increase the complexity of alignment and data analysis. In the proposed technique (Clean Localization), we utilize the existing information about the system PSF size to constrain the z-position of single molecules. The fact that the PSF far from focus widens rapidly provides a natural cutoff for identifying off-focal molecules. Here, we introduce an intermediate step to filter-out the single molecule signatures originating from other planes. This is primarily based on the size of Gaussian approximating single molecules. We define the single molecule model as,

$$G = A \exp \left[ \frac{(x-x_0)^2 + (y-y_0)^2}{2w_{xy}^2} \right]$$  

where, $w_{xy}$ represents the PSF width (measured in terms of FWHM of the Gaussian, G).
FIG. 2. (A) Schematic diagram of the optical setup for calibrating the in-focus and out-of-focus point emitters (sub-diffraction beads, size = 175nm). (B) Image of the bead at and out of the detector image plane at an inter-plane separation of 1 µm. At focus (z = 0), the bead size is about 289 nm, while this size increases with increasing axial distance from the focal plane in either direction (see, Fig. 2(C)). (C) Plot showing the size of the bead at the focal and off-focal planes. Scale bar = 3 µm.

Note that, the PSF width was calibrated using sub-diffraction beads (Thermo Fisher Scientific, P7220, λill/λemi : 540/560nm, size = 175nm). The optical setup for z-calibration is shown in Fig. 2(A). These measurements served as the calibration curve for determining the z-position of individual molecules and subsequently the cutoff on the size (δS) of the single molecule (in the image plane) is determined. The variation of the size of beads at and off-focal planes are shown in Fig. 2(B) and plotted in Fig. 2(C). It may be noted that, the size of the Gaussian increases rapidly with axial distance. This is potentially beneficial for z-localization. For this purpose, we have fitted the width using a polynomial of order 3 (see, Fig. 2(C)). We have chosen a cutoff of 4 pixels in the image plane which is equivalent to a spatial dimension of approximately 386 nanometers. Alternately, measurements on several single molecules show that the corresponding FWHM of the detected Gaussian in the image plane is approximately, 3.3 pixels, which is equivalent to a Gaussian of 386nm in diameter. It may be noted that, other constraints also influence the size of PSF such as, the PSF size can be approximated by Gaussian within a distance of ≈ 1 µm from the focal plane. At large distances, the profile becomes more complex and its width cannot be determined by simple Gaussian fitting.

After calibration, a large number of 2D widefield images were acquired. Starting from the coverslip and continuing till the top of the specimen (here the NIH3T3 cell), images were acquired at an inter-plane separation of 1 µm. This separation is chosen based on the calibration using sub-diffraction fluorescent beads (see Fig. 2). Typically, 5000 frames acquired at 23.4 frames per second per z-position and analyzed employing the cut-off obtained from the calibrated data (see, Fig. 2). We study the spatial distribution of the actin cytoskeleton. The endogenous actin in NIH-3T3 mouse fibroblast cells was labeled with a monoclonal mouse antibody (clone AC-74; Sigma–Aldrich, St. Louis, MO) and anti-mouse secondary antibody conjugated to the photoactivatable dye CAGE 552 (Sigma–Aldrich, St. Louis, MO). Actin can form structures which span a range of length scales. The transmission image of the whole cell along with the imaged portion of the cell is shown in Fig. 3(A). The images at varying z depth of up to 9 microns from the coverslip with an inter-plane separation of 1 µm are shown in Fig. 3(B). Two sub-regions R1 and R2 are chosen for visual inspection. For comparison, the results of the proposed technique along with the existing localization technique are shown. Significant reduction in the number of single molecules in the focal plane are observed. The proposed technique selectively eliminates single molecules from the off-focal planes. While this will decrease the localization density, it helps to select only single molecules that fall within the focal plane and reject out-of-focus molecules. Note that, the depth of field achieved by restricting the PSF width to 386 nm is approximately 800nm (see, Fig. 2(C)). Figure 3 indicates that some structures are restricted in the axial plane (i.e. are only present in one or two z slices), while some persist throughout the entire width of the cell. Clean localization technique allows for super resolution images (each with nanometer localization precision) to be viewed in the context of the many micron thickness of the cell.
Localization precision plays an important role in determining the resolution of a super-resolved image. In general, localization precision is given by, \( \Delta l_p \approx \Delta_{psf} / \sqrt{N} \), where, \( \Delta_{psf} \) is the diffraction limited PSF and \( N \) are the number of photons detected from the single molecule. Fig. 4 show the localization precision of single molecules from both the traditional and the proposed localization technique along with the corresponding reconstructed images. It is evident that average localization precision of molecules in the focal plane (as analyzed by the proposed technique) is approximately 50nm. On the other hand, existing localization technique depicts localization precision of all the molecules along the path of illumination and not particularly restricted to the focal plane. While the reduction in the total number of localized molecules will reduce localization density, and in some circumstances overall resolution, the degree of molecular filtering is fully controllable by the user.

In conclusion, we proposed and demonstrated the importance of a molecule filtering technique and were able to reduce molecules from off-focal planes. It may however be noted that, the technique may not give an accurate map at large z-distance due to scattering and other aberration effects.
FIG. 4. Localization precision for traditional and Clean localization technique. The histogram in the inset is a zoomed view (in the range [0,200]) of the histogram for traditional localization technique. Moreover, the insets also show the corresponding reconstructed images for both traditional and Clean localization techniques.

Using the spread of the approximately Gaussian PSF for off-focal molecules provides a convenient way to quantify focal plane restricted single molecules in a widefield based localization microscopy. The proposed Clean localization technique employs a simple calibration using sub-diffraction beads without the need of precision z-piezo platform. Comparatively, proposed technique is able to constrain the z-position of single molecules from the widefield data and do not require additional optomechanical arrangements.

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