Sripad Ram ; Prashant Prabhat ; Jerry Chao ; E. Sally Ward ; Raimund J. Ober; Three-dimensional single molecule tracking of quantum-dot labeled antibody molecules using multifocal plane microscopy. Proc. SPIE 7575, Colloidal Quantum Dots for Biomedical Applications V, 75750J (February 17, 2010); doi:10.1117/12.848940.

# 3D single molecule tracking of quantum-dot labeled antibody molecules using multifocal plane microscopy

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## ABSTRACT

Single molecule tracking in three dimensions (3D) in a live cell environment promises to reveal important new insights into cell biological mechanisms. However, classical microscopy techniques suffer from poor depth discrimination which severely limits single molecule tracking in 3D with high temporal and spatial resolution. We introduced a novel imaging modality, multifocal plane microscopy (MUM) for the study of subcellular dynamics in 3D. We have shown that MUM provides a powerful approach with which single molecules can be tracked in 3D in live cells. MUM allows for the simultaneous imaging at different focal planes, thereby ensuring that trajectories can be imaged continuously at high temporal resolution. A critical requirement for 3D single molecule tracking as well as localization based 3D super-resolution imaging is high 3D localization accuracy. MUM overcomes the depth discrimination problem of classical microscopy based approaches and supports high accuracy 3D localization of singe molecule/particles. In this way, MUM opens the way for high precision 3D single molecule tracking and 3D super-resolution imaging within a live cell environment. We have used MUM to reveal complex intracellular pathways that could not be imaged with classical approaches. In particular we have tracked quantum dot labeled antibody molecules in the exo/endocytic pathway from the cell interior to the plasma membrane at the single molecule level. Here, we present a brief review of these results.

**Keywords:** Multifocal plane microscopy, single molecule tracking, 3D super-resolution microscopy, localization accuracy, Fisher information matrix

## 1. INTRODUCTION

Fluorescence microscopy of live cells represents a major tool in the study of intracellular trafficking events. However, with current microscopy techniques only one focal plane can be imaged at a particular time. Membrane protein dynamics can be imaged in one focal plane and the significant advances over recent years in understanding these processes attest to the power of fluorescence microscopy.[1–3] However, cells are three dimensional objects and intracellular trafficking pathways are typically not constrained to one focal plane. If the dynamics are not constrained to one focal plane, classical microscopy techniques are inadequate for detailed studies of fast intracellular dynamics.[4–7] For example, significant advances have been made in the investigation of events that precede endocytosis at the plasma membrane.[8,9] However, the dynamic events post-endocytosis can typically not be imaged since they occur outside the focal plane that is set to image the plasma membrane. Classical approaches based on changing the focal plane are often not effective in such situations since the focusing devices are relatively slow in comparison to many of the intracellular dynamics. In addition, the focal plane may frequently be at the 'wrong place at the wrong time', thereby missing important aspects of the dynamic events.

To overcome these limitations, we developed an imaging modality, multifocal plane microscopy (MUM), that enables the visualization and tracking of sub-cellular events in 3D within a live-cell environment.[10–12] In MUM, the sample is simultaneously imaged at distinct focal planes. This is achieved by placing detectors at specific calibrated distances from the microscope tube lens. The detailed description of the principle and implementation of MUM are given elsewhere.[10,12,13] Here we present a brief review of MUM and its application to 3D single molecule localization/tracking and super-resolution imaging.

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## 2. CHALLENGES IN 3D SINGLE MOLECULE TRACKING IN CELLS

Modern microscopy techniques have generated significant interest in studying the intracellular trafficking pathways at the single molecule level. Single molecule experiments overcome averaging effects and therefore provide information that is not accessible using conventional bulk studies. However, the 3D tracking of single molecules poses several challenges. In addition to whether or not images of the single molecule can be captured while it undergoes potentially highly complex 3D dynamics,[14] the question arises whether or not the 3D location of the single molecule can be determined and how accurately this can be done. A major obstacle to high accuracy 3D location estimation is the poor depth discrimination of a standard microscope. Even with a high numerical aperture objective, the image of a point source does not change appreciably if the point source is moved several hundred nanometers from its focus position (Fig. 1a). This makes it extraordinarily difficult to determine the axial, i.e., z position, of the point source with a standard microscope, especially when the point source is close to the plane of focus.[13,15,16]

In the past, several imaging techniques have been proposed for determining the z-position of point sources. Approaches that use out-of-focus rings[17–19] of the 3D point-spread function to infer the z position are not capable of tracking quantum dots[18] (QDs) and pose several challenges, especially for live-cell imaging applications, since the out-of-focus rings can be detected only when the particle is at certain depths. Moreover, a large number of photons needs to be collected so that the out-of-focus rings can be detected above the background, which severely compromises the temporal resolution. Similar problems are also encountered with the approach that infers the z position from out-of-focus images acquired in a conventional fluorescence microscope.[20] Moreover, this approach is applicable only at certain depths and is problematic, for example, when the point source is close to the plane of focus. The technique based on encoding the 3D position by using a cylindrical lens[21–23] is limited in its spatial range to 1 micron in the z direction.[22] Moreover, this technique uses epi-illumination and therefore poses the same problems as conventional epifluorescence microscopy in tracking events that fall outside one focal plane. The approach based on z-stack imaging to determine the 3D position of a point source has limitations in terms of the acquisition speed and the achievable accuracy of the location estimates, and therefore poses problems for imaging fast and highly complex 3D dynamics.[24,25] The approach based on optical Parallax,[26] which encodes the change in the z-position as a lateral shift, has intrinsic limitations on the depth range over which single molecules can be tracked. Confocal/two-photon particle tracking approaches[27–30] that scan the sample in three dimensions can only track one or very few particles within the cell and require high photon emission rates of the bead. It should be pointed out that the above mentioned techniques have not been able to image the cellular environment with which the point sources interact. This is especially important for gaining useful biological information such as identifying the final destination of the single molecules. Recently, a modified, confocal 3D tracking technique was reported[31] in which confocal detection of the fluorescence signal was simultaneously carried out above and below the particle being tracked in conjunction with widefield imaging of the sample. As noted in the report,[31] the confocal detection scheme is analogous to the detection scheme used in MUM. The widefield imaging capability offered by this technique allows for the simultaneous visualization of the cellular environment thereby overcoming one of the limitations of previously reported 3D confocal/two-photon particle tracking techniques. However, the modified technique could also only track one or few particles, and it was suggested that multiple particle tracking is feasible by multiplexing, provided the dynamics of the particles are sufficiently slow.[31] It should be pointed out that a dual focus, two-photon imaging technique has been reported which also uses a detection configuration similar to that of MUM.[32]

One of the key requirements for 3D tracking of single molecules is that the molecule of interest be continuously tracked for extended periods of time at high spatial and temporal precision. Conventional labels such as organic dyes and fluorescent proteins typically photobleach irreversibly after a few seconds. This severely limits the duration over which the tagged molecule can be tracked. On the other hand, the use of QDs, which are extremely bright and photostable fluorescent labels when compared to conventional fluorophores, enables long-term continuous tracking of single molecules for extended periods of time (several minutes to even hours). There have been several reports on single QD tracking within a cellular environment, for example on the plasma membrane[33–37] or inside the cells.[34,38–41] All of these reports have focused on QD tracking in two dimensions. However, the 3D tracking of QDs in cells has been problematic due to the above-mentioned challenges that relate to imaging fast 3D dynamics with a conventional microscope. Recently, a confocal particle tracking technique

was reported to track QDs in 3D.[30] As mentioned before, this technique tracks only one or few particles at a time and did not image the cellular environment with which the QD interacted with. The latter is especially important to gain useful biological insights.

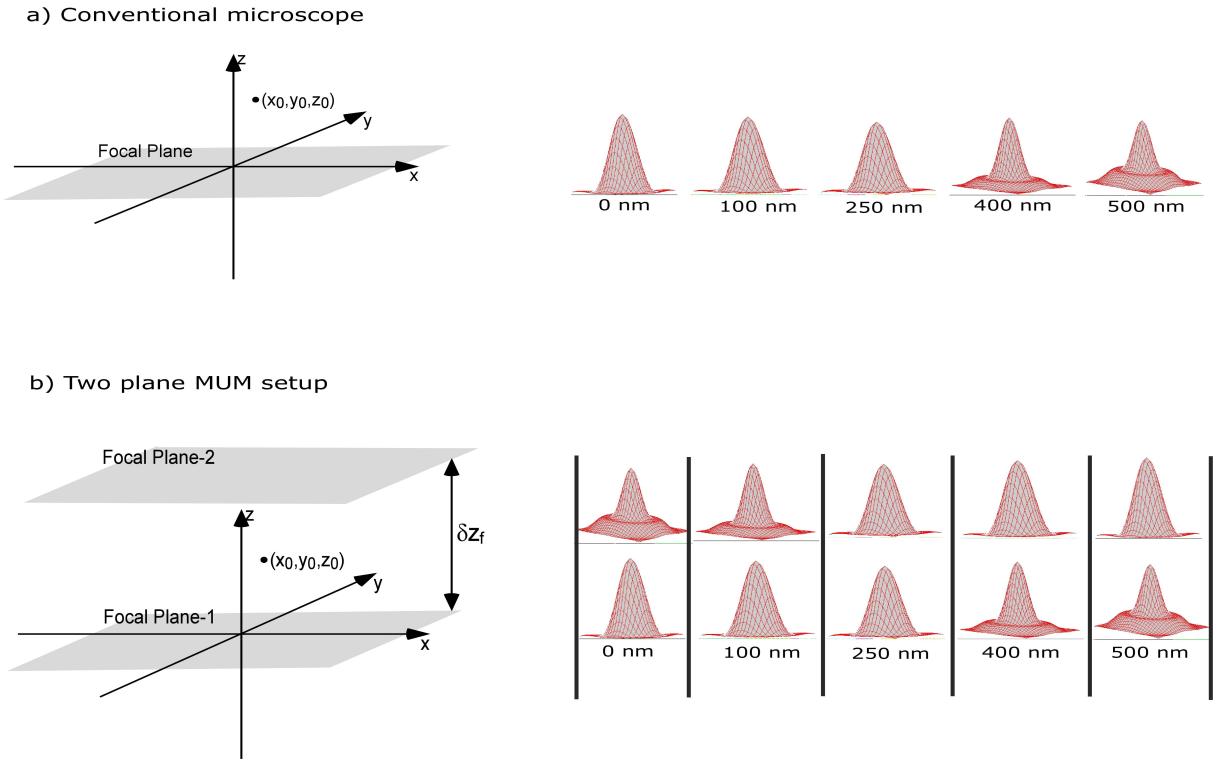


Figure 1. Comparison of conventional microscope and MUM. Panel a shows the schematic of the object space for a conventional microscope where a point source is located at $(x_0, y_0, z_0)$ with respect to the focal plane of the objective lens. The panel also shows the simulated intensity profiles of the image of a point source at different defocus levels when acquired through a conventional microscope. Panel b shows the schematic of the object space for a two plane MUM setup. The panel also shows the simulated intensity profiles of the image of a point source at different defocus levels when acquired through to a two plane MUM setup.

## 3. MUM AND 3D SINGLE MOLECULE TRACKING

We have developed a MUM localization algorithm, MUMLA, which determines the 3D location of point sources that are imaged using MUM in live cells.[13] In particular, MUMLA exploits the specifics of MUM in that at each time point more than one image of the point source is available from different focus levels. These images give additional information that can be used to constrain the z position of the point source (Fig. 1b). By making use of this data structure, we were able to achieve significantly better accuracy in determining the z-location of the point source than that could be obtained with classical approaches.[13] Further, the data structure also largely overcomes that depth discrimination problem of conventional microscope especially near the plane of focus.[13,16] We have demonstrated through simulations and experimental data that MUMLA is applicable over a wide spatial range ($\tilde{2}.5$ microns depth).

It should be pointed out that MUM supports multicolor imaging. This has enabled us to image QDs in three dimensions and also to image, at the same time, the cellular environment with which the QD-labeled molecules interact. The latter was realized by labeling the cellular structures with spectrally distinct fluorescent fusion proteins. Using this we have imaged the entire 3D trafficking itinerary of QD labeled antibody (QD-IgG) molecules in live cells at the single molecule level. In particular we have tracked single QD-IgG molecules on the exocytic pathway from a sorting endosome deep inside the cell to exocytosis on the plasma membrane.[12] We have also imaged individual QD-IgG molecules on the endocytic pathway starting from the plasma membrane

to the sorting endosome.[13] We observed that QD-IgG molecules exhibit complex itineraries before and after exo/endocytosis. Our results suggest that there exist direct and indirect pathways by which molecules traffic between sorting endosomes and the plasma membrane.

We have carried out a rigorous theoretical analysis to quantify the depth discrimination capability of MUM and a conventional microscope. By using the theory concerning the Fisher information matrix,[42] we have calculated the 3D localization measure, which provides a limit to the best possible accuracy with which the coordinates of a point source can be determined.[13,16] We have also verified through simulations and experimental data that MUMLA in fact attains this limit.[13]

## 4. MUM AND SUPER-RESOLUTION IMAGING

The recent past has witnessed rapid progress in the development of localization based super-resolution imaging techniques.[43–50] These techniques typically use photoactivatable/photoswitchable fluorescent labels and exploit the fact that the location of a point source can be determined with a very high (nanometer) level of accuracy.[51] This in conjunction with the working assumption that, during photoactivation/photoswitching, sparsely distributed (i.e., spatially well separated) labels get turned on, enabling the retrieval of nanoscale positional and distance information of the point sources well below Rayleigh's resolution limit. Originally demonstrated in two-dimensional (2D) fixed cell samples, these techniques have also been extended to tracking single molecules in two dimensions in live cells.[49,52–54] However, live-cell imaging was carried out using classical microscopy-based imaging approaches, which pose problems for 3D tracking in terms of imaging events that fall outside the plane of focus. Thus, these techniques do not support continuous (time-lapse) 3D imaging of fluorophores, which limits their applicability to 3D imaging applications.

More recently, there have been several reports of widefield, 3D super-resolution imaging techniques.[23,55,56] The approach based on using a cylindrical lens[23] is limited in its spatial range to $1\mu m$ in the z-direction.[22] The technique based on double-helix point spread function[55,57–59](DH-PSF), makes use of the fact that the DH-PSF rotates as the z-position of the point source changes. Because the image profile of the DH-PSF has two peaks as opposed to one peak as is the case for a conventional point spread function, the rotation of the DH-PSF can be determined with relatively high accuracy.[55,58] Using this concept, 3D super-resolution imaging of single molecules was demonstrated in a $2\mu m$ thick sample.[55] This technique is conceptually analogous to the technique that uses a cylindrical lens to achieve 3D super-resolution imaging,[23] in which the z-position is encoded in the width of the image profile. A potential shortcoming of the DH-PSF technique is the depth range over which single molecules can be tracked due to the ambiguity in estimating angles when the rotation exceeds $2\pi$ radians. The technique[56] based on two-photon temporal-focusing reported 3D super-resolution imaging up to a depth of $10\mu m$ in fixed-cell samples and in live cells. In this approach, the use of temporal focusing in conjunction with two photon excitation enables widefield illumination/photo-activation of a thin region in the sample. By sequentially scanning the sample at different focal planes, 3D super-resolution imaging was carried in fixed and live cells. However, the requirement of sequential scanning may impose limitations on the speed with which samples can be imaged.

It is important to note that both 3D single molecule tracking and localization based 3D super-resolution imaging impose the same requirement that the single molecule be localized in 3D with very high accuracy. Because MUM supports high accuracy 3D localization of point sources as well as simultaneous imaging of spectrally different fluorophores, it is possible to carry out multicolor, 3D super-resolution imaging of fixed and live-cell samples with MUM.

### 4.1 Quantifying the resolving power of MUM

The resolution of an optical system is a measure of the system's ability to distinguish two closely spaced point sources. Classical resolution criteria such as the Rayleigh's criterion, although extensively used, are well known to be based on heuristic that are not well suited for modern imaging approaches. To address these concerns, we have introduced information-theoretic resolution criteria for optical microscopes for 2D and 3D imaging applications.[60,61] Using an analogous approach, we have quantified the 3D resolution limits of MUM and compared it to that of a conventional optical microscope.[62,63] Our results show that in many practical conditions, MUM

provides significantly improved 3D resolvability of closed spaced point sources when compared to a conventional optical microscope.[62,63]

**Acknowledgments**
This research was supported in part by grants from the National Institutes of Health (R01 GM085575 and R01 AI039167) and in part by a postdoctoral fellowship from the National Multiple Sclerosis Society (FG 1798-A-1).

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