Ram, S.; Prabhat, P.; Chao, J.; Abraham, A.V.; Ward, E.S.; Ober, R.J., "Localizing single molecules in three dimensions - A brief review," in *Signals, Systems and Computers, 2008 42nd Asilomar Conference on* , vol., no., pp.64-66, 26-29 Oct. 2008
doi: 10.1109/ACSSC.2008.5074362
keywords: {biological techniques;cellular biophysics;fluorescence;molecular biophysics;nanoparticles;optical microscopy;quantum dots;3D point source localization;Cramer-Rao lower bound calculation;MUM localization algorithm;fluorescence microscopy;imaging modality multifocal plane microscopy;live cell environment;nanoparticle tracking;quantum dots;single molecule tracking;Biomedical imaging;Cells (biology);Chaos;Fluorescence;Focusing;Immune system;Microscopy;Nanoparticles;Optical imaging;Quantum dots},
URL: http://ieeexplore.ieee.org/stamp/stamp.jsp?tp=&arnumber=5074362&isnumber=5074342

# Localizing single molecules in three dimensions - a brief review

Sripad Ram, Prashant Prabhat, Jerry Chao, Anish V. Abraham, E. Sally Ward and Raimund J. Ober

***Abstract*— Single molecule tracking in three dimensions (3D) in a live cell environment holds the promise of revealing important new biological insights. However, conventional microscopy based imaging techniques are not well suited for fast 3D tracking of single molecules in cells. Previously we developed an imaging modality multifocal plane microscopy (MUM) to image fast intracellular dynamics in 3D in live cells. Recently, we have reported an algorithm, the MUM localization algorithm (MUMLA), for the 3D localization of point sources that are imaged using MUM. Here, we present a review of our results on MUM and MUMLA. We have validated MUMLA through simulated and experimental data and have shown that the 3D-position of quantum dots (QDs) can be determined with high spatial accuracy over a wide spatial range. We have calculated the Cramer-Rao lower bound for the problem of determining the 3D location of point sources from MUM and from conventional microscopes. Our analyses shows that MUM overcomes the poor depth discrimination of the conventional microscope, and thereby paves the way for high accuracy tracking of nanoparticles in a live cell environment. We have also shown that the performance of MUMLA comes consistently close to the Cramer-Rao lower bound.**

## I. Introduction

Fluorescence microscopy represents a major tool for the study of intracellular trafficking processes in live cells. Recent technological advances have generated significant interest in studying the intracellular trafficking pathways at the single molecule level. However, the 3D tracking of single molecules within a cellular environment poses several challenges. Foremost being that with current microscopy techniques only one focal plane can be imaged at a particular time. However, cells are three dimensional (3D) objects and intracellular trafficking pathways are typically not constrained to one focal plane. As a result currently available technology is inadequate for detailed studies of fast 3D trafficking events. Thus the question arises whether or not images of the single molecule can be captured while it undergoes potentially highly complex 3D dynamics. Classical approaches based on changing the focal plane are often not effective in such situations since 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.

Previously we developed an imaging technology, multifocal plane microscopy (MUM), that facilitates the simultaneous imaging of multiple planes within a cell. Using MUM we have shown that different focal planes can be simultaneously imaged at various depths in a cell ([1]). We have carried out 3D live cell imaging in MUM to study the 3D intracellular dynamics of proteins on the recycling pathway undergoing exocytosis ([2]). Importantly, single molecule dynamics were also imaged at the same time as the cellular environment with which the single molecule interacts ([2]). While our prior results addressed the problem of providing qualitative results, the question of the tracking of the single molecules remained open, i.e. the estimation of the 3D coordinates of the single molecule at each point in time. A major obstacle to high accuracy 3D location estimation is the poor depth discrimination of a standard microscope. This means that the z-position, i.e. the position of the single molecule along the optical axis, is difficult to determine and this is particularly the case when it is close to being in focus. Aside from this, the question concerning the accuracy with which the 3D location of the single molecule can be determined is of fundamental importance. The latter is especially crucial in live cell imaging applications where the signal to noise ratio is typically very poor.

To address this concern, we have developed an estimation algorithm, the MUM localization algorithm (MUMLA), to determine the 3D coordinates of single fluorescent point sources imaged using MUM ([3]). We have exploited the specifics of MUM acquisition in that for each point in time more than one image of the point source is available, each at a different focal level. We have shown that by appropriately exploiting this data structure, estimates can be obtained that are significantly more accurate than could be obtained by classical approaches, especially when the point source is near the focus in one of the focal planes. We have calculated the Fisher information matrix for the problem of estimating the 3D location of a point source from MUM. By using the Cramer-Rao inequality ([4]), we have calculated a limit to the 3D localization accuracy of a point source can be determined from MUM. Through simulations and experimental data, we have shown that the performance of MUMLA comes

This work was supported in part by the National Institutes of Health (RO1 GM071048, R01 GM085575 and R01 AI039167) and in part by a postdoctoral fellowship from the National Multiple Sclerosis Society (FG 1798-A-1).

S. Ram is in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

P. Prabhat and J. Chao are in the Department of Electrical Engineering, UT Dallas, Richardson, TX and in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

A. V. Abraham is in the Department of Computer Science, UT Dallas, Richardson, TX and in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

E. S. Ward is in the Department of Immunology, UT Southwestern Medical Center, Dallas, TX.

R. J. Ober is in the Department of Electrical Engineering, UT Dallas, Richardson, TX and in the Department of Immunology at UT Southwestern Medical Center, Dallas, TX; Corresponding Author, Email: ober@utdallas.edu.



Authorized licensed use limited to: Univ of TX SW Medical Ctr at Dallas. Downloaded on June 17, 2009 at 11:03 from IEEE Xplore. Restrictions apply.

consistently close to the limit of the localization accuracy for a wide spatial range ($\sim$ 2.5 microns depth). We have also shown that MUM overcomes the poor depth discrimination of the conventional microscope, and thereby paves the way for high accuracy tracking of nanoparticles in a live cell environment.

In this article we present a brief review of our results ([3], [5], [6]) on MUMLA for the 3D localization of point sources from MUM images.

## II. Results

### A. Multifocal plane microscopy

We have developed an imaging modality, multifocal plane microscopy, that can simultaneously image two distinct focal planes within the specimen. This is achieved by placing detectors at specific, calibrated distance from the tube lens. A detailed discussion of the design and construction of MUM can be found in [1], [2], [3], [7].

### B. The MUM localization algorithm MUMLA

MUM was developed for 3D tracking of subcellular objects in live cells ([1], [2]). To use MUM for 3D single molecule/particle tracking applications, it is necessary to be able to determine the 3D position of the particle at each point in time. For this, we have developed the MUM localization algorithm (MUMLA) ([3]). For a two plane MUM setup, MUMLA is based on the following approach: for each pair of point source images acquired in the two MUM planes, a pair of 3D point spread function profiles are simultaneously fitted to obtain the point source position that best matches the acquired data (see [3] details). The fact that the algorithm can rely on information not only from one defocus level but from two provides significant additional constraints to the estimation problem that result in an improved performance.

We have tested MUMLA through Monte-Carlo simulations as well as experimental data for a wide spatial range. Our results from simulated data show that MUMLA correctly predicts the z-position ([3]). We generated experimental data by imaging stationary quantum dot samples on a two plane MUM setup. Specifically, the quantum dot sample was imaged at different defocus levels by moving the objective at 50 nm or 200 nm steps and at each level several MUM images of the sample was acquired. Our results showed that MUM was able to follow the stepwise movement and was able to recover the correct step size of the objective over a wide spatial range of 2.5 microns ([3]).

### C. Depth discrimination and MUM

The depth discrimination property of an optical microscope is an important factor in determining its capability for 3D imaging and tracking applications. In a conventional microscope, even for 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. This makes it extraordinarily difficult to determine the axial, i.e. z-position, of the point source with a conventional microscope. On the other hand, in MUM images of the point source are simultaneously acquired at different focus levels. These images give additional information that can be used to constrain the z-position of the point source. This constraining information largely overcomes the depth discrimination problem near the focus.

To quantify the influence of depth discrimination on the z-localization accuracy of a point source, we calculate the Fisher information matrix ([4]) for the problem of estimating the z-position of a point source from a conventional microscope image as well as a two plane MUM image (see [3] for details). According to the Cramer-Rao inequality ([4]), the covariance of any unbiased estimator $\hat{\theta}$ on an unknown vector parameter $\theta$ is always bounded from below by the inverse Fisher information matrix, i.e., $\mathrm{Cov}(\hat{\theta}) \geq \mathbf{I}^{-1}(\theta)$. Because the performance of estimators are typically given in terms of the standard deviation, we define the 3D localization measure of $z_0$ as the square root of the corresponding leading diagonal entry of $\mathbf{I}^{-1}(\theta)$.

By definition, the 3D localization measure provides a quantitative measure of how accurately the location of the point source can be determined. A small numerical value of the 3D localization measure implies very high accuracy in determining the location, while a large numerical value of the 3D localization measure implies very poor accuracy in determining the location. In a conventional microscope when the point source is close to the plane of focus, e.g. $z_0 \leq 250$ nm, the 3D localization measure predicts very poor accuracy in estimating the z-position. For example, for practical experimental conditions when $z_0 = 250$ nm the 3D localization measure predicts an accuracy of 31.79 nm and when $z_0$ = 5 nm the 3D localization measure predicts an accuracy of >150 nm. Thus in a conventional microscope it is problematic to carry out 3D tracking when the point source is close to the plane of focus (see [3] for details).

On the other hand, for a two plane MUM setup the 3D localization measure of $z_0$ predicts consistently better accuracy in determining the z-position of the point source when compared to a conventional microscope. For example, for $z_0$ values in the range of 0 - 250 nm, the 3D localization measure of $z_0$ predicts an accuracy of 20 - 25 nm in determining the z-position.

It should be pointed out that for the MUM setup, the predicted z-position accuracy is relatively constant for a range of $z_0$ values (e.g. $z_0 = 0 - 1000$ nm), which is in contrast to a conventional microscope where the predicted z-position accuracy varies over a wide range of values. This implies that the z-location of a point source can be determined with relatively the same level of accuracy for a range of $z_0$ values which is favorable for 3D tracking applications. In particular, the finite value of the 3D localization measure for $z_0$ values close to zero implies that the z-position of the point source can be accurately determined in a MUM setup when the point source is close to the plane of focus.

Our 3D localization measure calculations explicitly take into account the shot noise characteristics of the signal from the point source. Specifically, the detected photon counts from the point source in the acquired data are modeled as



Authorized licensed use limited to: Univ of TX SW Medical Ctr at Dallas. Downloaded on June 17, 2009 at 11:03 from IEEE Xplore. Restrictions apply.

independent Poisson random variables. Additionally, we take into account the presence of additive noise sources and the effects of pixelation in the data. We consider two additive noise sources, i.e., additive Poisson and additive Gaussian noise sources. The Poisson noise component is used to model the effects of background photons that arise, for example, due to autofluorescence of the cell-sample/imaging-buffer and scattered photons. The Gaussian noise component is used to model the measurement noise that arises, for example, during the readout process in the imaging detector.

We have also calculated the 3D localization measure for the experimental data and here we observed that the standard deviation of the z-position estimates was consistently close to the 3D localization measure of $z_0$ for a wide spatial range. This shows that MUMLA attains the theoretically best possible accuracy (see [3] for additional details).

## REFERENCES


[1] P. Prabhat, S. Ram, E. S. Ward, and R. J. Ober, "Simultaneous imaging of different focal planes in fluorescence microscopy for the study of cellular dynamics in three dimensions," *IEEE Transactions on Nanobioscience*, vol. 3, no. 4, pp. 237–242, 2004.

[2] P. Prabhat, Z. Gan, J. Chao, S. Ram, C. Vaccaro, S. Gibbons, R. J. Ober, and E. S. Ward, "Elucidation of intracellular pathways leading to exocytosis of the Fc receptor, FcRm, using multifocal plane microscopy," *Proceedings of the National Academy of Sciences USA*, vol. 104, pp. 5889–5894, 2007.

[3] S Ram, P. Prabhat, J. Chao, E. S. ward, and R. J. Ober, "High accuracy 3d quantum dot tracking with multifocal plane microscopy for the study of fast intracellular dynamics in live cells," *Biophysical Journal,* In Press.

[4] C. R. Rao, *Linear statistical inference and its applications.*, Wiley, New York, USA., 1965.

[5] S. Ram, E. S. Ward, and R. J. Ober, "How accurately can a single molecule be localized in three dimensions using a fluorescence microscope?," *Proceedings of the SPIE*, vol. 5699, pp. 426–435, 2005.

[6] S Ram, J. Chao, P. Prabhat, E. S. Ward, and R. J. Ober, "A novel approach to determining the three-dimensional location of microscopic objects with applications to 3d particle tracking," *Proceedings of the SPIE*, vol. 6443, pp. D1–D7, 2007.

[7] P. Prabhat, S. Ram, E. S. Ward, and R. J. Ober, "Simultaneous imaging of several focal planes in fluorescence microscopy for the study of cellular dynamics in 3D," *Proceedings of the SPIE*, vol. 6090, pp. 115–121, 2006.




Authorized licensed use limited to: Univ of TX SW Medical Ctr at Dallas. Downloaded on June 17, 2009 at 11:03 from IEEE Xplore. Restrictions apply.