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Remote focusing multifocal plane microscopy for the imaging of 3D single molecule dynamics with cellular context

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ABSTRACT

Three-dimensional (3D) single molecule fluorescence microscopy affords the ability to investigate subcellular trafficking at the level of individual molecules. An imaged single molecule trajectory, however, often reveals only limited information about the underlying biological process when insufficient information is available about the organelles and other cellular structures with which the molecule interacts. A new 3D fluorescence microscopy imaging modality is described here that enables the simultaneous imaging of the trajectories of fast-moving molecules and the associated cellular context. The new modality is called remote focusing multifocal plane microscopy (rMUM), as it extends multifocal plane microscopy (MUM) with a remote focusing module. MUM is a modality that uses multiple detectors to image distinct focal planes within the specimen at the same time, and it has been demonstrated to allow the determination of 3D single molecule trajectories with high accuracy. Remote focusing is a method that makes use of two additional objective lenses to enable the acquisition of a z-stack of the specimen without having to move the microscope's objective lens or sample stage, components which are required by MUM to be fixed in place. rMUM's remote focusing module thus allows the cellular context to be imaged in the form of z-stacks as the trajectories of molecules or other objects of interest are imaged by MUM. In addition to a description of the modality, a discussion of rMUM data analysis and an example of data acquired using an rMUM setup are provided in this paper.

Keywords: Cellular context, fluorescence microscopy, multifocal plane microscopy, remote focusing, single molecule localization, single molecule microscopy, single molecule tracking, three-dimensional microscopy

1. INTRODUCTION

Single molecule fluorescence microscopy is a powerful and versatile technique for the study of biological processes at the level of individual molecules. A particularly important application of the technique is the investigation of the subcellular trafficking of a macromolecule of interest such as a protein. In this context, various imaging modalities[1–3] have been demonstrated that not only enable the visualization of the three-dimensional (3D) trajectory of such a molecule, but also the highly accurate localization of the molecule in each frame of the recorded time sequence of images. Regardless of the specific imaging modality used, however, a single molecule trajectory alone is of limited biological significance as information about the cellular environment with which the molecule interacts is missing. In order to associate the captured trajectory with its cellular context, which may include the organelles and membranes with which the molecule interacts, cellular structures will also need to be imaged at the same time as the molecule. An imaging modality called remote focusing multifocal plane

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microscopy, or rMUM, is introduced here that solves the challenging problem of simultaneously imaging single molecule trajectories and the cellular context.

The content of this paper comprises an overview of previous work[4] and is organized as follows. In Section 2, the need for rMUM and the design of rMUM are described. In Section 3, different aspects of rMUM image data analysis are discussed. In Section 4, an example is given of data that was acquired using an rMUM setup.

2. REMOTE FOCUSING MULTIFOCAL PLANE MICROSCOPY

rMUM is a new fluorescence imaging modality that combines multifocal plane microscopy (MUM)[5] with a method of remote focusing.[6,7] MUM is a technique that uses multiple detectors positioned at different distances from the tube lens of the microscope to simultaneously image different focal planes within the sample. Within the axial range supported by the focal planes, simultaneous image acquisition by the detectors allows the continuous observation of objects that move rapidly in three dimensions, as the signals from these objects are captured by at least one of the detectors at any given time. MUM has been used to visualize the recycling pathways of a protein receptor[8] and to determine accurate 3D trajectories of single protein molecules[2,9] in live cells. In these studies, some limited cellular context was imaged by the MUM setup in addition to the moving objects of interest. In the two earlier studies,[2,8] a relatively small axial range of no more than approximately 1 μm was imaged that revealed sorting endosomes in proximity to the cell membrane. In the more recent work,[9] a large 10-μm axial range spanning the thickness of a cell was imaged that revealed the cell membrane. The limited cellular context in these studies provided sufficient information for the trafficking events investigated therein. In general, however, a MUM setup alone may not be able to sufficiently capture the cellular context that is needed in a given study. For example, in a subcellular trafficking study, widely spaced focal planes that span the thickness of a cellular sample[9] often need to be used with the MUM setup in order to observe the 3D movement of single molecules or other objects over a large axial range. Imaging of only the widely spaced focal planes, however, may lead to insufficient sampling of the 3D cellular context, which can include relatively slow-moving organelles distributed throughout the cellular volume.

rMUM addresses the challenge of obtaining a finely sampled 3D cellular context by adding to a MUM setup a means by which z-stacks of the cellular context can be acquired at the same time as images of the fast-moving molecules or objects of interest. As a MUM setup requires that the position of the objective lens be fixed with respect to the sample, the traditional method of z-stack imaging by moving either the objective lens or the sample stage is not a feasible option. rMUM therefore enables z-stack imaging with the use of remote focusing,[6,7] a method that allows the acquisition of a z-stack without the need to move the objective lens or the sample stage. Remote focusing works by duplicating the specimen in the focal region of a second objective lens, which is then imaged by a third objective lens that is moved along the optical axis to acquire a z-stack of the specimen.

An rMUM setup thus comprises a standard fluorescence microscope, a *MUM-module* that images the 3D trajectories of single molecules or other objects of interest, and an *r-module* that images z-stacks of the cellular context. This is illustrated in Fig. 1, which shows the fluorescence collected by a standard inverted microscope to be separated into two light paths by a dichroic filter set. The fluorescence from the molecule or object of interest is directed towards the MUM-module, which in this case is capable of simultaneously imaging four different focal planes within the sample. Specifically, the MUM-module is implemented with beam splitters that further partition the fluorescence between four detectors, each of which is positioned at a distinct distance from the tube lens of the microscope. The fluorescence from the cellular context, on the other hand, is directed towards the r-module. In the r-module, a second objective lens creates a replica of the 3D cellular context in its focal region, and this replica is then imaged by a third objective lens which projects a magnified image onto a detector. The third objective lens is mounted on a piezo nanopositioner that moves it along the optical axis so that different z-slices within the replicated cellular context can be imaged.

To facilitate the association of a single molecule trajectory with its cellular context, the r-module should ideally produce an entire z-stack representation of the cellular context for every image that is acquired by a detector in the MUM-module. While this may not be practicable given the specific experimental conditions, it should generally be the case that a faster frame rate is used for the detector in the r-module. To simplify the subsequent data analysis, one can synchronize the detectors in the two modules such that the r-module detector acquires images at a rate that is greater than the rate of the MUM-module detectors by some integer factor.

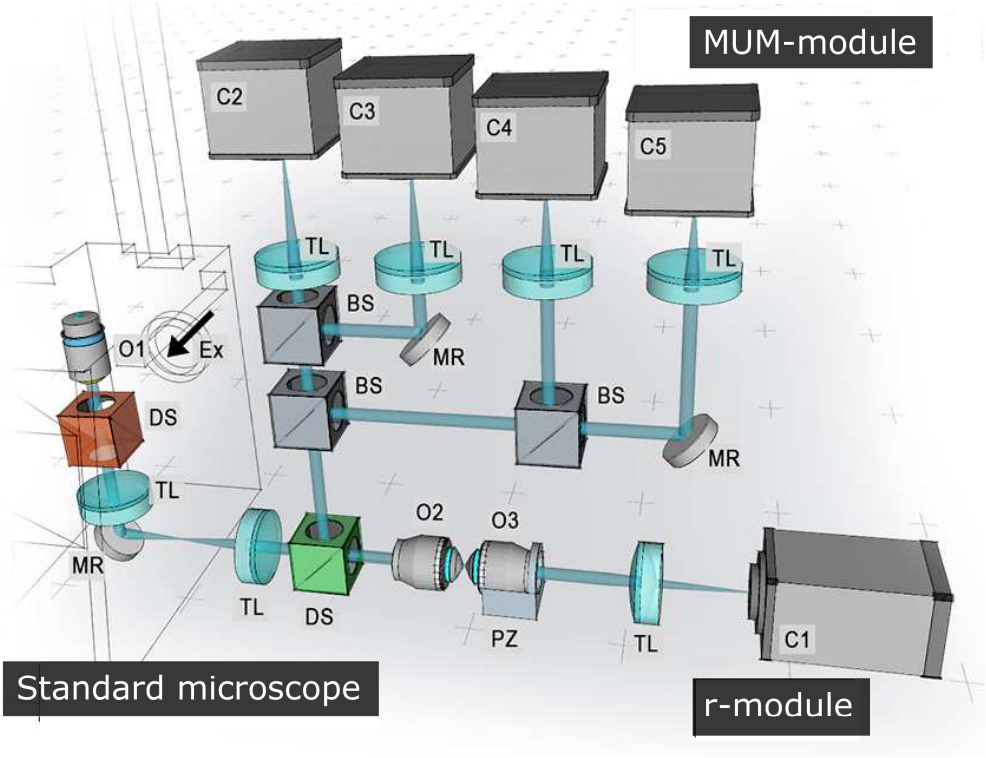

Figure 1. Remote focusing multifocal plane microscopy (rMUM). An rMUM setup consists of a standard inverted microscope, a MUM-module, and an r-module. A high-level representation of the microscope comprises an objective lens (O1), a dichroic filter set (DS), a tube lens (TL), and a mirror (MR). The black arrow (Ex) depicts excitation light that is used to illuminate the fluorophore-labeled specimen. The fluorescence collected from the specimen by objective lens O1 is split into two light paths by wavelength. The light path that enters the MUM-module is partitioned between multiple detectors using beam splitters (BS). In this example, this light path is split between four detectors (C2, C3, C4, C5) that are positioned at distinct distances from the tube lens of the microscope. Four different focal planes within the sample can therefore be imaged at the same time with this MUM-module. The light path that enters the r-module passes through a second objective lens (O2) that creates an optical replica of the specimen in its focal region. The replica is then imaged by a third objective lens (O3) which projects a magnified image onto a detector (C1). The objective lens O3 is mounted on a piezo nanopositioner (PZ) so that z-stacks of the specimen can be obtained by changing its position using the nanopositioner.

To help maximize the speed at which images are acquired by the r-module, the acquisition of z-stacks can be performed in a bidirectional manner. This means that after a z-stack is acquired by moving the piezo nanopositioner from one end of the specified axial range to the other, the acquisition of the next z-stack follows immediately as its frames are acquired in reverse z order as the piezo nanopositioner is returned step-by-step towards the end of the axial range from which it came. This is in contrast to a unidirectional mode of acquisition in which every z-stack is acquired in the same direction. Unidirectional acquisition is slower because of the time it takes to return the piezo nanopositioner to the starting end of the axial range before the next z-stack can be acquired. The two modes of z-stack acquisition are illustrated in Figs. 2(a) and 2(b).

3. DATA ANALYSIS

To obtain an accurate representation of a single molecule trajectory imaged with the MUM-module, a localization algorithm needs to be applied to the MUM image data that can accurately determine the 3D position of the molecule at each point in time as captured by the set of simultaneously acquired images of distinct focal planes within the sample. For example, the MUM-module of Fig. 1 would produce images of the molecule from four different focal perspectives at each acquisition time point, and a suitable localization algorithm should be able

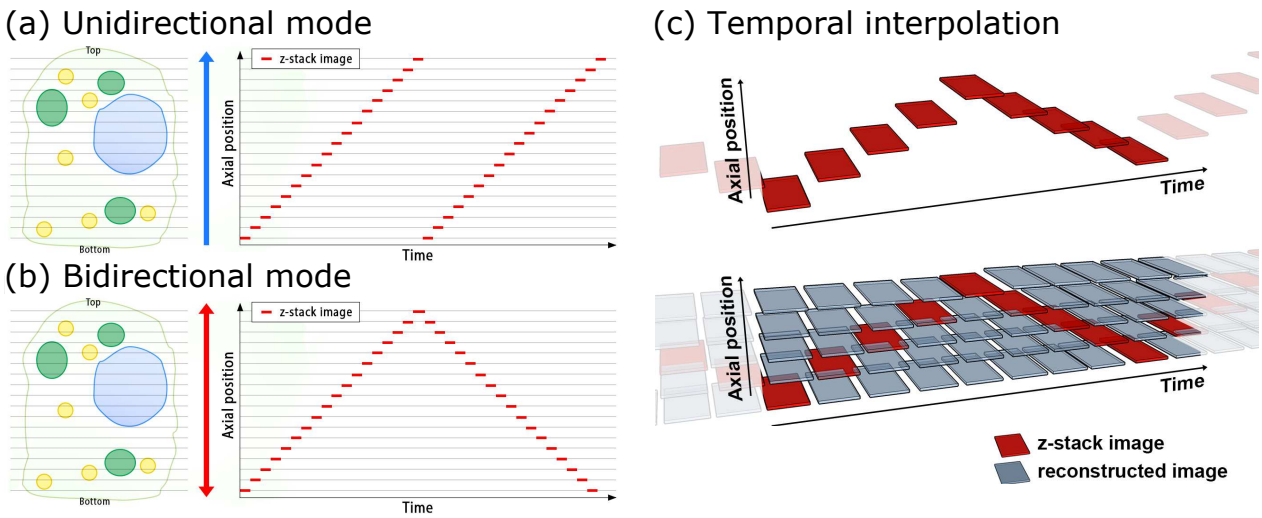

Figure 2. Acquisition and temporal interpolation of z-stack images of the cellular context. (a) In the unidirectional acquisition mode, all z-stacks are acquired in the same direction along the optical axis. After a z-stack is acquired, the piezo nanopositioner is returned to the same starting z position before the next z-stack is acquired. (b) In the bidirectional acquisition mode, successive z-stacks are acquired in opposite directions along the optical axis. No extra time is taken between the acquisition of one z-stack and the next because the piezo nanopositioner does not need to be returned to the same starting z position. (c) Assuming that a z-stack comprises images captured from five z focus levels, only one image (red) corresponding to one of the five z focus levels is captured at any given acquisition time point (top panel). Each of the four unavailable images at each time point can be reconstructed (gray), however, by interpolating the pixel values of images captured from the same z focus level at other time points (bottom panel). The illustration assumes that z-stacks are acquired in bidirectional mode.

to accurately estimate the molecule's 3D positional coordinates using the four images. One such algorithm is *MUM localization algorithm* (MUMLA),[2,9] which performs the localization by globally fitting an appropriate point spread function to the set of images corresponding to different focal planes. The performance of MUMLA has been demonstrated to come close to a Fisher information-based theoretical bound.[2,10]

The ultimate goal in the analysis of rMUM image data is to properly associate the 3D single molecule trajectories imaged by the MUM-module with the cellular context imaged by the r-module. This association requires that the coordinate system used for the MUM-module data analysis is aligned with the coordinate system of the r-module data. This can be achieved by imaging a 3D fluorescent bead sample with the rMUM setup, independently estimating the 3D locations of the same beads from images acquired by the MUM-module and the r-module using a localization algorithm such as MUMLA, and using a registration method to determine a transformation between the two coordinate systems based on the two sets of location estimates. A possible registration method is one that determines an affine transformation using a generalized least squares algorithm.[11] Assuming that the obtained transformation maps the MUM-module coordinate system to the r-module coordinate system, applying it to the estimated coordinates of a single molecule trajectory will then allow the proper association of the trajectory with its cellular context in one common coordinate system.

For visualization, one may want to create a representation, such as a movie, in which a single molecule of interest is shown together with the cellular context at every time point along its trajectory. Since the frames of a z-stack are acquired over time, however, at any given time point only one image of the cellular context captured from a particular z focus level is available. To display a more complete rendering of the cellular context at each time point of a trajectory, the unavailable images corresponding to other z focus levels can be reconstructed using images acquired at other time points. Specifically, the unavailable image corresponding to z focus level ζ can be reconstructed by interpolating the pixel values of images acquired at other time points from focus level ζ. A high-level schematic that illustrates this approach is shown in Fig. 2(c).

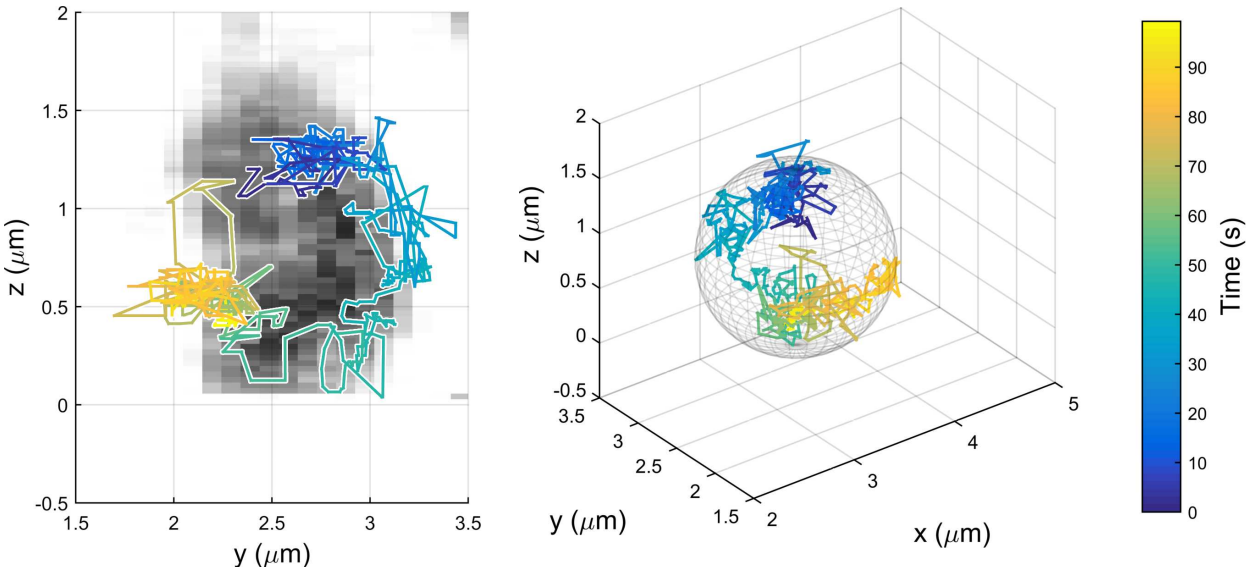

Figure 3. Trajectory of an anti-prostate-specific membrane antigen antibody molecule overlaid with a reconstructed image (left plot) and a 1.6-μm diameter spherical representation (right plot) of an endosome with which the antibody interacts. The antibody trajectory was captured by the four-plane MUM-module, and images of the endosome were captured by the r-module, of an rMUM setup. The antibody was labeled with Qdot 655, and the endosome was labeled by way of GFP-Rab5A. Images were acquired from a live GFP-Rab5A-transfected LNCaP cell. See Section 4 for further details.

4. AN EXAMPLE

Quantum dot (Qdot 655)-labeled anti-prostate-specific membrane antigen antibody and endosomes labeled by way of the protein Rab5A tagged with green fluorescent protein (GFP) were imaged in live GFP-Rab5A-transfected human prostate cancer (LNCaP) cells using an rMUM setup. To track the rapidly moving antibody molecules, Qdot 655 fluorescence was diverted to and imaged by the MUM-module, which consisted of four detectors that simultaneously captured images from four different focal planes within the imaged cell. The focal plane spacings used were 0.749 μm, 0.771 μm, and 0.798 μm. Endosomes with which the antibody interacts form the desired cellular context, and accordingly the GFP signal was directed to and imaged by the r-module. The r-module acquired z-stacks spanning an axial range of 7.35 μm in steps of 0.35 μm, in bidirectional mode. Images were acquired at a rate of 10 frames per second by both the MUM-module detectors and the r-module detector.

The trajectory of an antibody molecule, determined using MUMLA, is displayed with its cellular context in Fig. 3. In the left panel of the figure, a yz-projection of the trajectory is shown overlaid on top of a reconstructed image of the endosome with which the antibody molecule interacts. The rows of pixels in the reconstructed endosome image correspond to distinct z focus levels, and their intensity values were obtained using the interpolation approach depicted in Fig. 2(c). In the right panel of Fig. 3, a 3D view of the same antibody trajectory is shown. In this representation, the endosome is approximated as a sphere with a 1.6-μm diameter. Both representations suggest that the trajectory of the antibody molecule is spatially associated with the endosome.

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