Simultaneous imaging of several focal planes in fluorescence microscopy for the study of cellular dynamics in 3D

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ABSTRACT

Fluorescence microscopy of live cells is an important tool to investigate cellular trafficking pathways. The existing microscope design is very well suited to image fast moving vesicles, tubules and organelles in one focal plane. More problematic is the imaging of cellular components that move between different focal planes. This is due to the fact that tracking of such cellular components requires that the focal plane of the microscope be changed. This has to be done with a focusing device, which is relatively slow. More importantly, only one focal plane can be imaged at a time. Therefore, while the cell is imaged at one focal plane, important events could be missed at other focal planes. To overcome these shortcomings, we present a modification of the classical microscope design with which two or more focal planes can be imaged simultaneously. In this design, the emission light collected by a single stationary objective lens is split into multiple channels. Light in each channel is focused on a CCD camera by a tube lens. By ensuring that the camera position with respect to the tube lens focal plane position is not the same in any two channels, distinct planes within the specimen can be simultaneously imaged. Here we discuss the implementation of a configuration with which four focal planes can be imaged simultaneously.

Keywords: Fluorescence microscopy, three-dimensional (3-D) tracking, multi-focal plane imaging.

1. INTRODUCTION

Fluorescence microscopy is an important tool to study cellular trafficking pathways in live cells. Significant progress has been made in the understanding of cellular events by conducting new types of experiments with fluorescent proteins. This has been possible due to advances in fluorescent labeling techniques along with the use of highly sensitive detectors.[1] The standard microscope design is very well suited to image cellular events in one focal plane. More problematic is the imaging of vesicles, tubules and organelles that leave the current focal plane. Their continued imaging requires that the focal plane be changed. This can be done using a focusing device such as a piezo-z focus. However, such focusing devices are relatively slow. Even though highly sensitive charge coupled devices (CCD) are available, that can be used to image at relatively high frame rates, the piezo devices typically take tens of milliseconds to move a fairly short distance. This makes it difficult to image fast moving vesicles, tubules and organelles that move between focal planes. Additionally, only one focal plane can be imaged at a time.

With the advent of total internal reflection fluorescence microscopy (TIRFM),[2] trafficking dynamics on the cellular membrane can be studied with high sensitivity and in great detail. However, these events on the cellular membrane cannot be correlated with dynamics inside the cell since in total internal reflection fluorescence microscopy only a small volume is illuminated near the cover glass.

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Due to these limitations of the current microscopes, it is very difficult to image relatively rapid intracellular trafficking processes that are not confined to one focal plane. Such trafficking events are of central interest to cell biologists. For example, the imaging of rapidly moving tubules or vesicles on the recycling pathway leading from sorting endosomes to exocytosis on the plasma membrane has not been possible with the existing microscopes.

We address these problems by a novel microscopy setup that enables imaging of two or more focal planes simultaneously. This has been achieved by a modification of the emission pathway of a standard fluorescence microscope and by using a multiple camera acquisition format to image more than one focal plane at the same time. Our proposed design is such that it can be implemented relatively easily by using attachments to an existing microscope. Additionally, a laser setup is being used that allows for the simultaneous excitation of the sample using total internal reflection and epifluorescence with different laser lines. We expect that this new imaging modality will allow the investigation of important cellular events that to date could not be studied with a conventional microscope.

2. MULTI-FOCAL PLANE IMAGING MICROSCOPE

The basic principle to the design of a microscope emission pathway with which different focal planes can be imaged simultaneously is illustrated in Figure 1. By moving the detector closer to the tube lens the focal plane shifts from the design position. Therefore placing detectors (in combination with beam splitters) at different positions with respect to the tube lens allows us to simultaneously image different focal planes.

In our previous study,[3] we described a microscope setup that enabled us to image two distinct focal planes simultaneously. In that setup a beamsplitter was inserted in the infinity corrected light path. In each of the two resulting light paths a tube lens was placed followed by a camera. For each of the cameras the focal plane which is imaged can be adjusted by changing the distance between the detector and the tube lens. A problem with this setup is that a standard microscope has to be changed in a significant way by replacing its standard emission optics with a custom setup.

Here we report a new approach to the design of a microscope that allows for the simultaneous imaging of several focal planes. Importantly, with this approach the standard microscope body does not need to be modified. Figure 2 shows the layout of a microscope for imaging up to four different focal planes simultaneously. The basic principle is to use standard dual camera adaptor. Our setup uses a Zeiss Axiovert 200 microscope and three dual camera adaptors (Zeiss, Thornwood, NY). One of the dual camera adaptor is inserted in a standard camera emission port of the microscope. Using a beamsplitter this attachment splits the emitted light into two light paths. In each of the two emission ports of this first adaptor another dual camera adaptor is placed. As a result four emission ports are available for imaging. The standard use of a dual video adaptor also requires the use of an extension tube to be placed between the emission port of the video adaptor and the camera. The purpose of this extension tube is to position the detector at the design focus position. If for each of the four emission ports the standard extension tube is used, by design all four cameras image the same focal plane. Replacing the standard extension tube with a shorter / longer extension tube a focal plane different from the default focal plane can be imaged.

TIRFM, is a widely used technique to image dynamic cellular events near the plasma membrane.[4–6] In this technique, only a thin layer near the cover glass is illuminated,[2] hence out of focus fluorescence does not interfere with the emitted signal. This has allowed events at the plasma membrane to be imaged with unprecedented sensitivity, including the tracking of single molecules in a live cell environment.[7–10] However, for example in the study of exocytic events, the advantage of TIRFM also has a negative aspect. Due to the shallow illumination layer exocytic events cannot be related to intracellular events. The intracellular origins of an exocytosing vesicle or tubule have great biological significance. However, with conventional TIRFM this origin can typically not be determined. To address this problem we have developed a multi-labeling strategy together with multi-focal plane imaging. To implement this strategy, for example, a receptor is labeled with two fluorescent markers (e.g. GFP and RFP). One of the labels is imaged at the cellular membrane with TIRFM, while the other label is imaged at a higher plane in epifluorescence. In this way the receptor can be studied with TIRFM sensitivity at the membrane and can be tracked inside the cell using standard epifluorescence imaging.

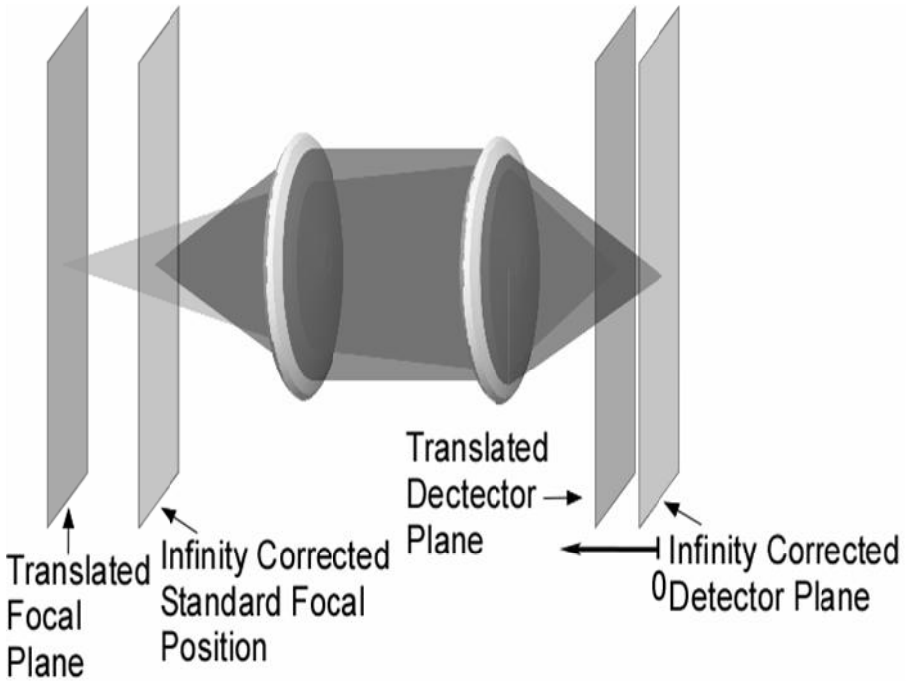

Figure 1. Emission light path of a microscope that shows how the imaged focal plane is shifted as a result of the detector being moved towards the tube lens.

3. CALIBRATION AND PARFOCALITY

In order to perform a quantitative analysis of the acquired data, it is important to know the spacing between focal planes within the sample that are being simultaneously imaged. For this, a calibration experiment is carried out to determine the relation between the focal plane spacings in the object space and the image space. In this experiment one of the cameras is set to image at the infinity corrected detector plane and the data captured from this camera is used as the reference. The other cameras are set to image distinct planes within the specimen. Analogous to our previous work,[3] images of fluorescent bead samples are simultaneously acquired in all the cameras while the z-focus of the objective lens is changed by a piezo focusing device (Polytec PI). The fluorescent intensity of the beads acquired in each camera is plotted as a function of the z-position of the focusing device. For a given camera, the location of the peak in the intensity plot represents the objective z-position at which the bead is in focus. The spacing of the focal planes in the specimen is then determined by taking the difference between this objective z-position and the objective z-position corresponding to the peak of the fluorescent intensity plot of the reference camera. By repeating this procedure for different camera spacings, a calibration graph can be obtained that provides a relation between the camera spacing in the image space and the corresponding plane spacing in the object space. Similar to our previous work,[3] the calibration graph obtained in the present study is a straight line.

If two cameras are supposed to image the same focal plane, e.g. with different colors, their parfocality is verified by imaging multicolor bead samples. For this purpose, two cameras are set to image the same plane in the object space but acquire in different colors. Similar to the calibration experiment, the images of the multicolor bead samples are simultaneously acquired in both the cameras while the z-focus of the objective lens is changed by a piezo focusing device. If the cameras are parfocal, then the z-position of the peak of the fluorescent intensity plot for both the cameras must coincide. The correction for parfocality is done by either increasing or decreasing the spacing of one of the cameras (usually the one that acquires longer wavelength) and then repeating the above experiment for verification.

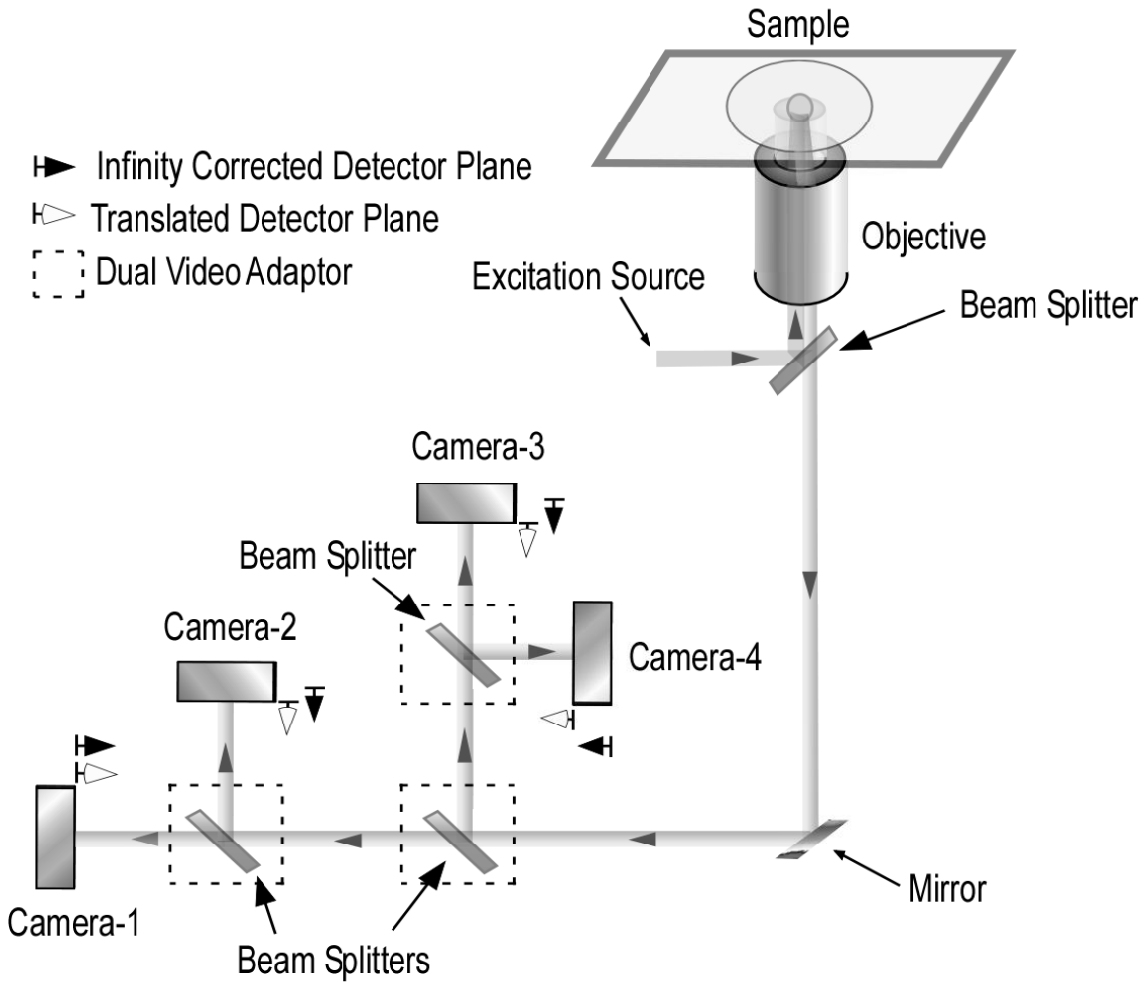

Figure 2. Layout of multi-focal plane microscope which can image up to four distinct focal planes simultaneously.

4. IMAGE ANALYSIS AND SOFTWARE

The multi-focal plane imaging modality gives the flexibility to conduct new types of experiments to study cellular dynamics in three dimensions. For experimental reasons the different cameras might not acquire images at the same frame rate. This requires that the resulting images from the different cameras need to be temporally synchronized. Additionally, the images from the various cameras also need to be spatially aligned. Data from just one acquisition can be very significant in size (as large as 10GB).

Processing of such complex and large data sets for visualization and analysis presents significant challenges. A robust framework is required for the development of tools for the visualization and analysis of such data. In our laboratory we have developed the Microscopy Image Analysis Tool (MIATool) software package for the display and analysis of complex microscopy data[11] (www4.utsouthwestern.edu/wardlab/miatool). This package is written in Matlab (Mathworks, Inc., Natick, MA), and uses an object oriented design approach.[12] Due to the capabilities of MIATool this provides an ideal environment for developing methods to analyze data resulting from multi-focal plane experiments. One of the aspects of MIATool is that it uses as much as possible image pointers rather than the images themselves to carry out operations such as temporal synchronizations. This provides significant savings in execution speed, RAM and disk space.

However, for the efficient processing of data acquired on a multi-focal plane imaging station significant further

software development needs to be carried out. Suitable software tools are needed for the tracking of vesicles, tubules and organelles in three dimensions, the analysis of calibration data, the temporal and spatial alignment of images (combined with the necessary magnification adjustments). Due to the complexity of the data none of these are straightforward analysis steps. With the currently available software, routine laboratory use of the multi-focal imaging workstation is highly problematic because of the extensive amount of time that is required to analyze even small data sets. It should be pointed out that the present tracking problem is significantly different from a standard tracking problem in three dimensional context, which typically uses a 3D image stack. This is due to the fact that a full three dimensional reconstruction of the cell is not possible, since with our present imaging modality only a limited number of planes are imaged.

5. THREE DIMENSIONAL TRACKING OF VESICLES, TUBULES AND ORGANELLES IN LIVE CELLS

To enable the study of protein dynamics across multiple focal planes, we developed a novel double labeling protocol. In this protocol, two distinct fluorescent markers are chosen and the cell is prepared such that it expresses the protein of interest with either of the two fluorophores attached to it. By exciting one of the fluorophores in TIRF mode and the other fluorophore in epifluorescence mode, the dynamics of the protein of interest can be simultaneously monitored across multiple planes that span from the cell surface to the cell interior. In the present study, we used the fluorescent protein markers enhanced Green Fluorescent Protein (eGFP)[13] and monomeric Red Fluorescent Protein (mRFP)[14] and our protein of interest is the neonatal Fc receptor, FcRn.[15] Human endothelial (HMEC) cells were transfected to express two fusion proteins, namely FcRn-mRFP and FcRn-eGFP.

In fluorescence microscopy, epifluorescence illumination is typically used to image the protein dynamics inside the cell. Since epifluorescence illumination extends across the entire depth of the cell, protein dynamics close to the cell surface cannot be clearly imaged in this mode of illumination due to the fluorescence haze that arises from the interior of the cell. TIRF illumination, on the other hand, extends over a small region close to the cell membrane selectively exciting the fluorophores near the cell surface. Hence TIRF illumination is typically used to study the protein dynamics on the cell surface such as vesicle docking, exocytosis and endocytosis. In our earlier study,[3] the FcRn trafficking pathway from the cell interior to the cell surface was studied in HMEC cells by simultaneously tracking the movement of FcRn containing tubules in two focal planes. One focal plane was inside the cell and was illuminated in epifluorescence mode to image FcRn-RFP. The other focal plane was on the cell membrane proximal to the cover slip and was illuminated in TIRF mode to image FcRn-GFP. The use of the double labeling protocol in conjunction with the combined TIRF and epifluorescence illumination enabled us to visualize and track the movement of FcRn containing tubules across the cell. The analysis of the data acquired from both focal planes displayed a variety of dynamics. Numerous FcRn containing vesicles and tubules were seen to be moving in the top plane (i.e., the focal plane inside the cell) and in the cell-membrane plane. In the top plane, FcRn was also seen in sorting endosomes.[16] For example, a tubule was observed leaving a sorting endosome. This tubule was seen in the top plane for a number of frames, then leaves this plane and appeared on the focal plane of the cell membrane. The tubule then partially fused with the cell membrane in subsequent frames. Similarly the movement of FcRn positive vesicles could also be tracked across the cell.

In our present study, we have used a similar approach to simultaneously image up to four distinct focal planes. Figure 3 shows a schematic of a cell with the positions of the four focal planes. Here, the bottom plane i.e. the cellular membrane is illuminated in TIRF mode to image FcRn-eGFP and the higher planes (i.e., the intermediate planes 1 and 2, and the top plane) are illuminated in epifluorescence mode to image FcRn-mRFP. By imaging four focal planes, instead of two, additional information is available that can be used to study the trafficking pathways of proteins that span a larger depth inside the cell. Imaging data acquired from HMEC cells that express FcRn-mRFP and FcRn-eGFP indicate that the movement of FcRn-positive vesicles and tubules across four distinct focal planes can be observed and tracked simultaneously, yielding the three dimensional dynamics of FcRn. It should be pointed out that the present technique can also be used to track the dynamics of two (or more) distinct proteins in three dimensions within a cellular environment.

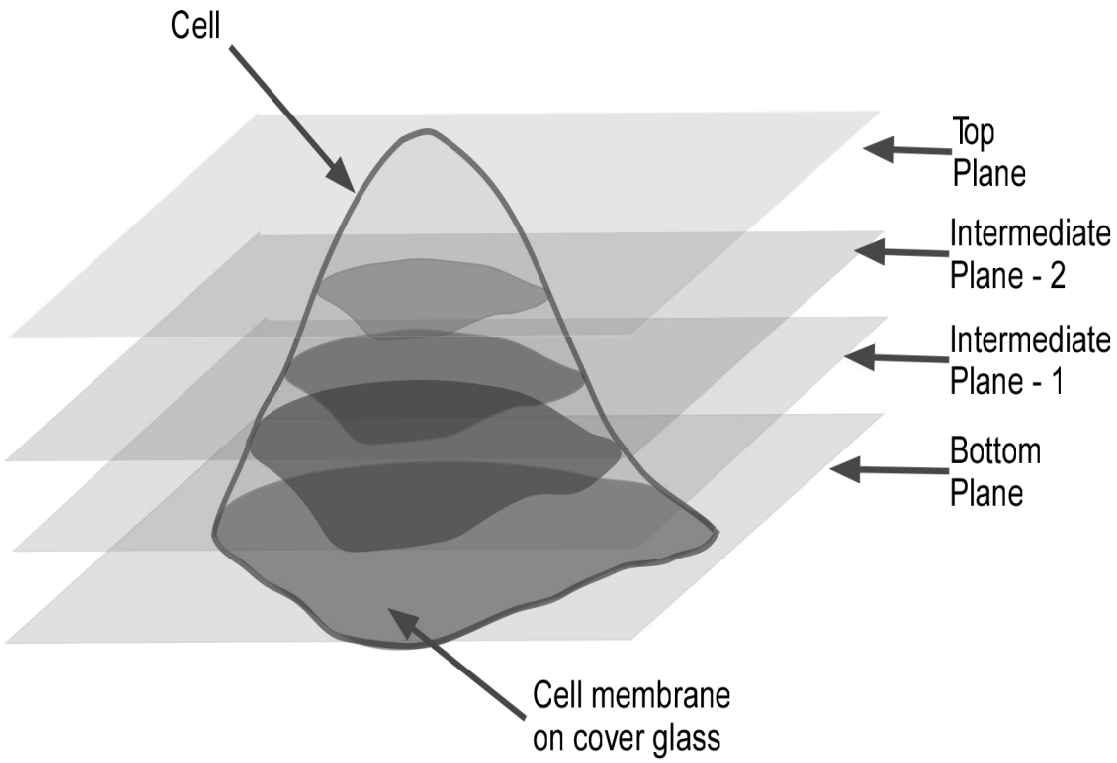

Figure 3. Sketch shows a cell and indicates the positions of four focal planes that are imaged.

6. DISCUSSION

Methodologies suitable for the tracking of a labeled object, such as a bead, using a confocal microscope have been proposed in some studies.[17–20] To track a bead, its current position is measured and the z-focus of the microscope is adjusted in a feedback manner. In this approach, the objects that are to be tracked can typically only be identified in a post-acquisition examination of the acquired imaging data. Hence, for image based sensors and the trafficking of vesicles, tubules and organelles, this approach appears less well suited than the approach presented in our study. Since our design does not require any moving part, it eliminates vibrational disturbances associated with a standard focusing device. In addition, it also allows simultaneous imaging of focal planes that can be significantly apart. However, if needed, our current approach can be combined with changes of the focal planes using a focusing drive. Even though our study was carried out for fluorescence microscopy, its underlying principle may also be applied to other imaging approaches such as transmitted illumination. Our work is not the first time that a multi-focal imaging approach has been proposed. After our first paper[3] appeared we became aware of another report[21] in which multi-focal plane imaging was proposed to determine the focal plane of a microscope in a high throughput microscopy application.

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