

# Multi-angle Total Internal Reflection Fluorescent Microscopy (TIRFM) imaging of microtubules (MT) and fluorescent beads.

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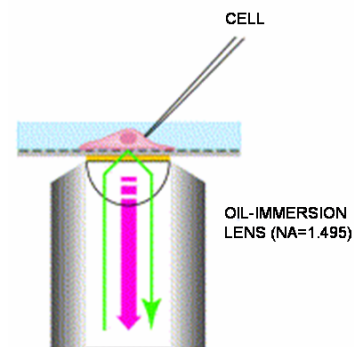
## Aim:

Microtubules (MT), a 25 nanometer wide cellular “skeleton” or cytoskeleton play numerous critical roles in providing mechanical support and structural tracks for the anchoring and transport of chromosomes, organelles, and vesicles. The assembly of microtubules coordinates cell division and migration and abnormal function of the assembly is involved in cell pathology such as neurodegenerative diseases and cancer. A key critical issue is the high resolution tracking of microtubules and microtubule tips. Previous studies showed that the MT target the cortex but a key open question is exactly where and how the MT moves in relationship to the cell surface (1). We showed that total internal reflection fluorescent microscopy (TIRFM), a technique that has very high signal to noise ratio, is ideal for tracking microtubules. To accurately track MT and other cellular objects we describe an approach to visualize the cell cortex in 3D using multiangle TIRFM. The main technical hurdle that we face is accurate calibration and quantification of TIRFM multiangle datasets, both of synthetic materials (e.g. beads) and microtubules and cells. We present new multiangle TIRFM quantitative visualization of MT and fluorescently labeled beads and describe an approach to determine the MT absolute position in 3D. We show that MT uniform thickness (~25 nm tube) makes it an ideal calibration standard.

## Methods:

Total internal reflection fluorescent microscopy (TIRFM) is a powerful tool to visualize fluorophores in a thin layer of a specimen, usually of the order of 100 nm (2) (3). The thickness of the layer illuminated by TIRFM is determined by the penetration depth of the evanescent field. Different penetration depths of evanescent waves can be generated by varying the angle of incidence of a laser beam. Imaging the sample by TIRFM at several penetration depths gives the information about the fluorophore distribution as a function of the axial z position.

In our experiments we either generate TIRFM using a classic objective-type setup using a beam of light that is focused to a point on the outer back focal plane of a high numerical aperture (NA=1.495) oil-immersion lens (60X) or using custom optics generating a ring of light on the back focal plane of the objective.



**Figure 1** Schematic view of the objective-type TIRFM.

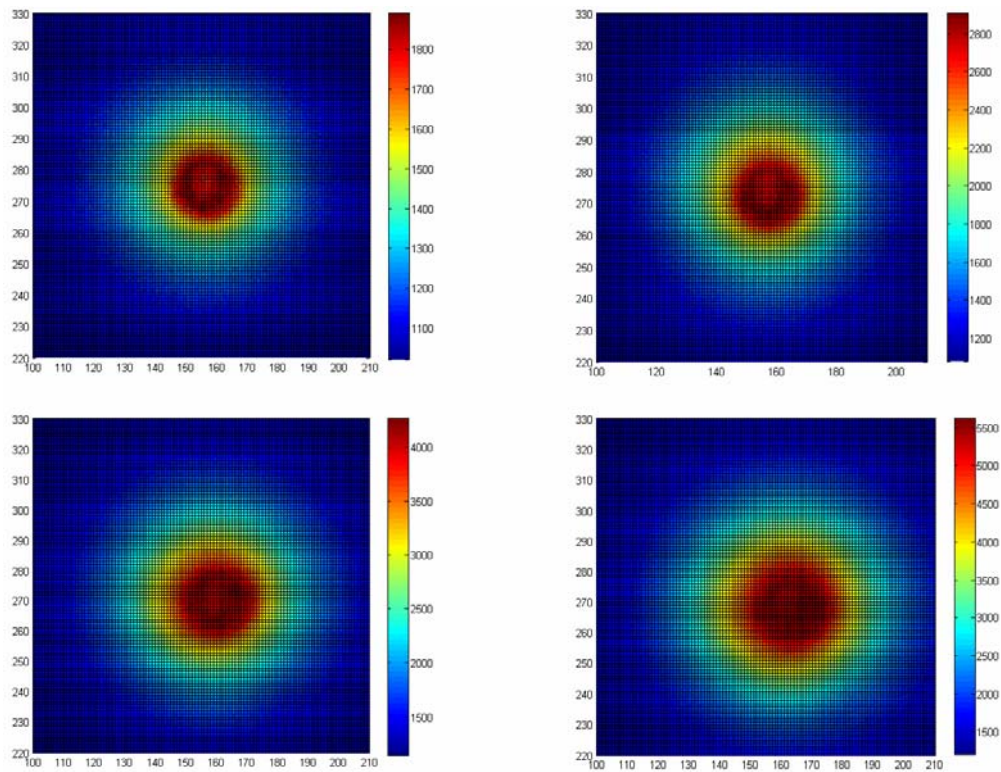
A ring of laser light passes through the microscope objective and undergoes total internal reflection at the interface between the glass and the aqueous solution. Totally internally reflected light penetrates the aqueous medium and forms evanescent waves which propagate parallel to the interface and exponentially decay in the direction normal to the interface. Fluorescent light excited by the evanescent waves is collected by the same objective. We change the penetration depth of the evanescent waves by changing the radius of the ring.

## Results

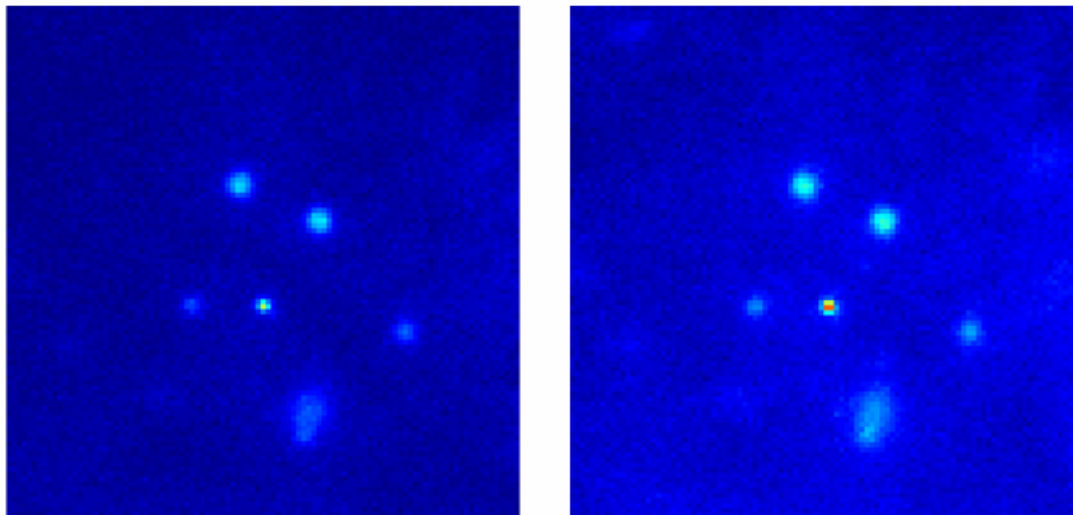
The samples used in the current experiments were fluorescent labeled large and small beads of sizes 20 microns and 20 nanometers respectively. Large bead represents object with well characterized curvature while a small bead represents point-like source.

Large beads were placed on the glass interface. The refractive indices of the aqueous solution and large beads were matched to prevent scattering of the evanescent waves. The small beads were directly placed on the surface of HELA cells. The placement was random. Figure 2 shows images of a sample large fluorescent bead acquired at different penetration depths. We can clearly see the response of the fluorescent signal with the change in height as well as with the change of penetration depth. Figure 3 shows two sample images of smaller beads which were placed on a cell.

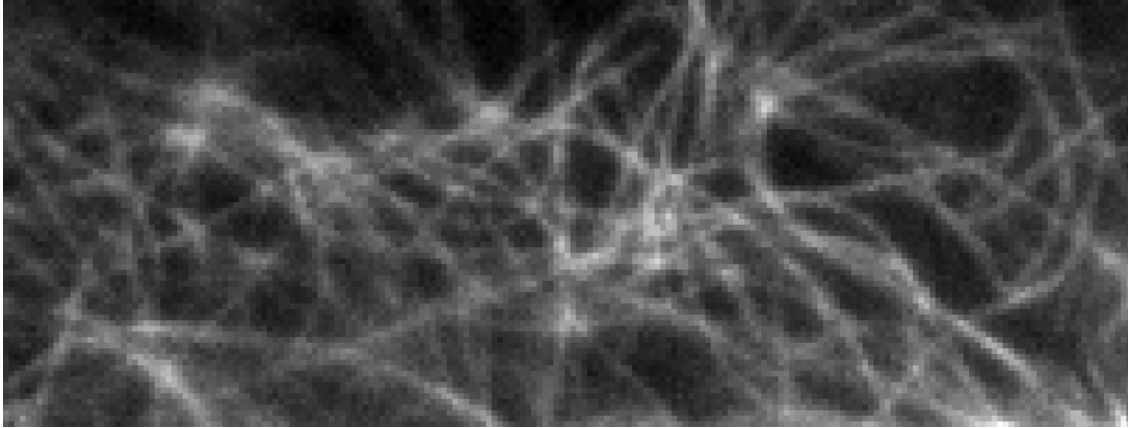
The change in pixel intensity of the beads represents their relative z position. The same images can be acquired as a function of time which is representative of cell membrane motion. Figure 4 is a sample grey scale image of the microtubules. It is a 2D projection of 3D structures. The change in the grey scale intensity reflects the curvature and motion of the microtubules.



**Figure 2** TIRFM images of the large fluorescently labeled sphere,  $d=20$  microns at different penetration depths which increases from top left to bottom right. Refractive index of the media is matched with the refractive index of the bead



**Figure 3** TIRFM images of the small fluorescent beads,  $d=20$  nm placed randomly on a surface of HELA cells. Penetration depth of the evanescent wave for the image on the right is larger than the penetration depth of the image in the left.



**Figure 4** Representative image of fixed microtubules stained with alpha-tubulin and seen by TIRFM. Image dimensions: 250x80 pixels. Pixel size is 200 nm. Intensity of the image reflects the 3D structure of the microtubules.

### **Conclusions and Discussions:**

We have performed experiments to capture the motion of cells and microtubules which are contained within the cell. The experiments and analysis are in a preliminary stage and in a future we hope to fully capture and model of these biologically important objects.

TIRFM images give 2D projections of the 3D objects illuminated by the evanescent waves at different penetration depths. 3D reconstruction can be performed on multiangle data set. Microtubules show promise as a standard object for calibration and doing quantitative TIRFM analysis in cells. Structural and mechanical parameters can be also included into the analysis of TIRFM imaging of MT motion in space and time.

### **References**

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