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High-resolution 3D reconstruction of microtubule structures by quantitative multi-angle total internal reflection fluorescence microscopy

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ABSTRACT

Total internal reflection fluorescence microscopy (TIRFM) has been widely used in biomedical research to visualize cellular processes near the cell surface. In this study, a novel multi-angle ring-illuminated TIRFM system, equipped with two galvo mirrors that are on conjugate plan of a 4f optical system was developed. Multi-angle TIRFM generates images with different penetration depths through the controlled variation of the incident angle of illuminating laser. We presented a method to perform three-dimensional (3-D) reconstruction of microtubules from multi-angle TIRFM images. The performance of our method was validated in simulated microtubules with variable signal-to-noise ratios (SNR) and the axial resolution and accuracy of reconstruction were evaluated in selecting different numbers of illumination angles or in different SNR conditions. In U373 cells, we reconstructed the 3-D localization of microtubules near the cell surface with high resolution using over a hundred different angles. Theoretically, the presented TIRFM setup and 3-D reconstruction method can achieve 40 nm axial resolution in experimental conditions where SNR is as low as 2, with ~35 different illumination angles. Moreover, our system and reconstruction method have the potential to be used in live cells to track membrane dynamics in 3-D.

1. Introduction

Total internal reflection fluorescence microscopy (TIRFM) has been widely used in biomedical research to observe a thin layer with the penetrating depth up to 600 nm from the interface of glass coverslip and aqueous solution by creating an evanescent field that decays exponentially along *z*-axis [1]. The axial resolution of TIRFM can achieve ~100 nm [2,3], better than confocal microscopy (300–500 nm) [4,5]. Due to the high axial resolution, fluorescence intensities out of the focus plane are eliminated, and the SNR of images are improved greatly [6,7]. Therefore, TIRFM is particularly useful for recording the biological activities near the cell surface, where fundamental cellular processes take place, such as exocytosis and endocytosis of secretory vesicles, dynamic remodeling of cytoskeleton elements and signaling activation at the cell surface [8,9]. In order to eliminate uneven illumination and interference fringes introduced in traditional TIRFM illumination, spinning the azimuthal angle of the illumination beam and accurately control of the incidence beam orientation during the exposure time has been proposed [10–12]. Each TIRFM image is actually the projection of a three-dimensional (3-D) volume and cannot alone produce an accurate localization of structures in the *z*-dimension. Through changing of the incidence angle of illuminating laser beam, a set of multi-angle TIRFM images with different penetration depths can be practically used to reconstruct the 3-D fine structures within the cells.

In this study, a novel multi-angle ring-illuminated TIRFM system was developed. The two one-dimensional galvo mirrors that are on conjugate plan of a 4f optical system were modulated to provide the ring pattern light illumination and multi-angle

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alternation. The voltages on the galvo mirrors controlled the alternation of the incidence angle of the TIRF illuminating laser beam. A practical experimental approach was employed to measure the correlation between the incidence angle of the illuminating laser beam, the voltage of galvo mirrors and the corresponding penetration depth of generated evanescent field. The 3-D volume of the sample can be potentially reconstructed from the multi-angle TIRFM images with calibrated incidence angles of the illuminating laser beam and their calculated corresponding penetration depths. We presented a method to perform 3-D reconstruction of cell microtubule structures from multi-angle TIRFM images. The performance of our method was validated in computer simulated microtubule structures with variable signal-to-noise ratio (SNR), while the axial resolution and accuracy of reconstruction were evaluated in selecting different numbers of illumination angles or in different SNR conditions. Using over a hundred different illuminating angles, we reconstructed the 3-D localization of microtubule structures near the plasma membrane of a U373 human astrocyte with high axial resolution. In theoretical estimation, the established TIRFM setup and 3-D reconstruction method can achieve 40 nm axial resolution in experimental conditions where SNR is as low as 2, with only ~35 different illumination angles. Furthermore, multi-angle TIRFM imaging and image reconstruction method will have vast applications in biology to quantitatively analyze the 3-D localization and distribution of objects in the TIRF field.

2. System setup

The multi-angle ring-illumination TIRFM system was established based on a classical objective-type TIRFM, but we employed two-dimension galvometer (2D-GM) system to control the tilting and radial angle of the illumination laser beam (see Fig. 1). The 2D-GM used in our system is for 10 mm beam diameter, the focus distance of scanning lens is 50 mm and the objective lens is 60X (1.45 NA). The full-scale sine wave response frequency of 10 mm diameter 2D-GM used in current setup is 100 Hz and the acquisition speed of EMCCD is ~10 fps, so in one exposure process the illumination angle can spin at least 10 times, which enable uniform illumination. The total internal reflection angle is affected by the polarization, here we introduced a quarter wave plate to modulate the laser to circularly polarized light, so that penetration depth will not change when the illumination ringing at fixed tilting angle. The angle repeatability of 2D-GM is 15 μrad, and we can subdivide the supercritical angle more than 100 times, which enable superb resolution of the 3-D reconstruction image along the z-axis. In order to diminish optical artifacts, 2D-GM is installed with the reflectors of two one-dimension GMs that are on conjugate plan of a 4f optical system.

The incident angle of the illuminating laser beam was controlled by the galvo mirrors, which moving the spinning ring-pattern light at the back focal plan of the objective in different voltage conditions.

Selective frames from a multi-angle TIRFM image sequence taken at different incidence angles (corresponding to different voltages on the galvo mirrors) were shown in Fig. 2.

3. Methods

3.1. TIRFM imaging theory

Theoretically, each TIRFM image I with the incidence angle $\{\theta_i\}_{i=1}^N$ of the actual fluorescence labeled object C is the projection of a 3-D volume, can be expressed by an integral along the axial direction depending on the penetration depth $\{dp(\theta_i)\}_{i=1}^N$,

$$I_z(\theta_i)=\varnothing I_0(\theta_i)\int_0^\infty Q(z)\cdot \mathrm{PSF}(z)\cdot C(z)\cdot e\left[-z/dp(\theta_i)\right]\mathrm{d}z, (i=1,\cdots,N) \quad (1)$$

where $\varnothing$ stands for the quantum efficiency of the CCD camera and fluorophores, $Q(z)$ and $\mathrm{PSF}(z)$ are the photon collection efficiency and point spread function [13,14]. $\{I_0(\theta_i)\}_{i=1}^N$ is the intensity at the dielectric surface ($z=0$), which can be derived according to the Fresnel formula [15].

$$I_0(\theta_i)=\frac{4\cos^2\theta_i\left(2\sin^2\theta_i-(n_t/n_i)^2\right)}{(n_t/n_i)^2\cos^2\theta_i+\sin^2\theta_i-(n_t/n_i)^2}, (i=1,\cdots,N) \quad (2)$$

where n_i, n_t denote refractive indices of the glass coverslip and the specimen respectively. The analytical solution of penetration depth for evanescent wave is

$$dp(\theta_i)=\frac{\lambda}{4\pi\sqrt{n_i^2\sin^2\theta_i-n_t^2}}, (i=1,\cdots,N) \quad (3)$$

After calculating the penetration depth, the actual axial profile of evanescent field above the dielectric surface can be simply described as [16]

$$I_z(\theta_i)=I_0(\theta_i)\bullet e[-z/dp(\theta_i)], (i=1,\cdots,N) \quad (4)$$

3.2. Cell culture and immunofluorescence staining

U373 human astrocytes were cultured in Dulbecco's Modified

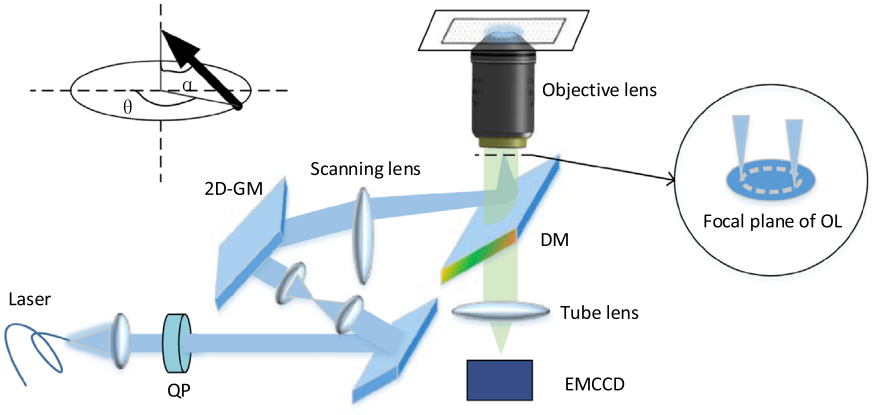

Fig. 1. The setup of the multi-angle ring-illumination TIRFM system. QP: quarter wave plate; 2D-GM: two-dimension galvometer; DM: dichroic mirror; α: tilting angle; θ: radial angle.

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