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Total-internal-reflection fluorescence microscopy with W-shaped axicon mirrors

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A scheme based on a W-shaped axicon mirror device for total-internal-reflection fluorescence microscopy (TIRFM) is presented. This approach combines the advantages of higher efficiency compared with traditional TIRFM, adjustable illumination area, and simple switching between wide-field and TIRF imaging modes. TIRF images obtained with this approach are free of shadow artifacts and of interference fringes. Example micrographs of fluorescently labeled polystyrene beads, of *Convallaria majalis* tissue, and of Propidium-iodide-labeled Chinese hamster ovary cells are shown, and the capabilities of the scheme are discussed. © 2010 Optical Society of America

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Background reduction is an important issue in fluorescence microscopy. In many cases, (auto)fluorescence from out-of-focus excited and detected areas is the main source of background. For this reason, a variety of approaches ranging from confocal microscopy to total-internal-reflection fluorescence microscopy (TIRFM) [1] are employed to eliminate background and to improve the signal-to-noise ratio as well as the spatial resolution. The latter approach has found widespread application for investigations of processes at cell membranes [2] or for single-molecule sensitive imaging [3]. In TIRFM, light is reflected off an interface. This leads to the generation of an evanescent field at the interface. Since this field decays exponentially perpendicular to the interface, it can be used for fluorescence excitation in a thin volume element with a depth typically below 200 nm [4]. This excitation scheme thus suits itself for reducing the background in fluorescence microscopy.

TIRFM has been used mainly to image small fluorescent structures or single molecules located in a focal plane below a large number of fluorophores or for studies of dynamical cellular processes at cell membranes. Examples include protein orientation [5], endocytosis [6], and secretory granule tracking [7]. These experiments are done in two basic TIRFM configurations: the prism method and the objective-based method. A disadvantage of the prism technique is that the sample has to be imaged through the specimen itself, since the detection optics have to be located opposite to the prism [2]. This problem is avoided by the objective-based TIRFM method. A configuration involving a single laser beam that produces TIR from one incidence direction is the simplest geometry for this approach. Incidence angles greater than the critical angle are achieved using high-NA objectives (1.4 or higher). Yet a single illumination direction tends to produce shadows along the beam path because of evanescent field scattering by the cells [2].

The shadowing problem can be solved by using a multiple direction illumination TIRFM geometry, in which a cone of illumination over all the azimuthal angles around the optical axis and an intensity zero in the middle of the beam efficiently avoid this shadow artifact [2]. Commonly, an opaque disk is used in the multiple directions illumination TIRFM geometry. This allows only those rays converging at supercritical angles to strike the specimen. The TIRF signal is observed in the epidirection, but most of the incident light will be lost [2], which limits the applicability. To circumvent this restriction, Stout and Axelrod [4] employed an axicon prism in the arc lamp light path and converted the illumination profile into a dark-centered ring. Their method has the advantage of high transmission as well as absence of coherent light interference fringes; however, it has gained no practical importance due to the low light levels available from incoherent light sources.

In this Letter, we present a scheme of TIRFM by using W-shaped axicon mirrors. The W-axicon mirror device is a pair of concentric conical mirrors that have afocal properties and can be used to generate an annular shaped beam with a transmittance of nearly 100%. The W-shaped axicon mirrors were first introduced by Sincerbox *et al.* for doing dark-field microscopy [8]. Endo [9] used W-axicon optical resonators to generate arbitrary order Laguerre–Gaussian beams, whereas Shoham *et al.* [10] utilized basically the same principle to generate radially or azimuthally polarized beams.

Our approach is a multiple directions illumination TIRFM scheme. The W-axicon mirror substitutes for the "opaque disk" and ensures high optical throughput. Interference effects are eliminated by delivering the laser beam through a 50 m length multimode fiber acting as a mode scrambler. This ensures that the excitation light is distributed homogeneously over the sample. Like other multiple directions methods, our system is free of shadowing artifacts and offers high efficiency and an adjustable illumination area. At the same time, simple switching between wide-field and TIRF modes is possible.

A diagram of the W-axicon TIRF system is shown in Fig. 1. A Nd:VO_4 laser (Picotrain, HighQ Laser, Austria) with a wavelength of 532 nm and a diode laser with a wavelength of 660 nm (SD650-200, Sampling Laser Technology Inc., China) serve as excitation sources. The two beams are combined in a collinear geometry, coupled into a 50 m length multimode fiber (AFS105/125Y, NA 0.22,

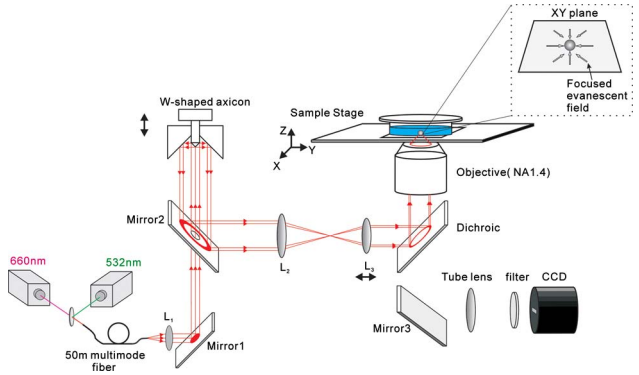

Fig. 1. (Color online) Experimental layout of the TIRF microscopy by using W-shaped axicon mirrors.

Thorlabs, USA), and collimated by lens L_1. Differently from the conventional multiple-direction illumination TIRFM geometry employing an opaque disk, the collimated beam passes through a plane mirror having a hole and hits the W axicon (LT Ultra-Precision Technology GmbH, Aftholderberg, Germany). There, the beam is reflected by the inner axicon mirror into a direction perpendicular to the original direction. The outer axicon mirror itself reflects the rays from the inner axicon mirror in a direction perpendicular to their previous direction. This generates a beam profile with an annular shape. The plane mirror with the hole is positioned at an angle of about 45° to the incident beam such that light from the incident beam passes through the aperture, while light reflected from the outer axicon mirror is reflected by 90° from this mirror [8]. The tip of the inner axicon is in line with the edge of the outer axicon, and the inner axicon is manufactured such that it can be slid forward or backward inside the aperture of the outer axicon to change the geometry of the W-axicon reflector. Sliding the inner part corresponds to adjusting the diameter of the annular beam and can be used to change from TIR to a wide-field excitation mode. The annular beam passes through the telescope formed by L_2 and L_3 and is reflected into the back aperture of the 63× oil immersion objective (HCX PL Apo, NA 1.4, Leica, Germany). Ideally, to obtain a large area of illumination with an evanescent field, L_3 should focus a very thin annulus onto the back focal plane of the objective. Instead, we directly use the annulus. Depending on the thickness of the annulus in the objective's backfocal plane, this leads to a range of incidence polar angles. In our experiment, the thickness of the annulus can be adjusted to an acceptable extent by the telescope formed by L_2 and L_3. A possibility to further reduce this narrow distribution of polar angles consists in using an open angle of the outer axicon mirror slightly larger than 45° in order to get reflected parallel rays diverging slightly from the optical axis. Then L_2 would focus the light into a very thin annulus, which can be cast on the back focal plane of the objective by L_3. The TIRF signal is detected in the epidirection. An electron-multiplying CCD camera (Andor IXonEM+, Andor Technology PLC, Northern Ireland) captures the two-dimensional TIRF image, with an objective (50 mm, f-number 1.8, Nikon, Japan) serving as a tube lens. A band-pass and two long-pass filters (605/70-2P, HQ680lp, and LP02-568RS, AHF Analysentechnik AG, Germany) block the excitation laser beams. The maximum laser powers used are 20 mW.

Fluorescence beads (TetraSpeck microspheres, 1 μm, fluorescent blue/green/orange/dark red, Molecular Probes, Inc., USA) immersed in a polymer film and *Convallaria majalis* slices (Leica, Germany) are used as test samples. The samples are imaged through 170-μm-thick glass coverslips.

Wild-type Chinese hamster ovary (CHO) clone cells (Toronto strain) were grown in Eagle's minimum essential medium (Gibco) supplemented with 8% heat inactivated foetal bovine serum (Gibco), 0.584 g/l L-glutamine (Gibco), 3.5 g/l D-glucose (Sigma), 2.95 g/l tryptose-phosphate (Sigma), 100 U/ml penicillin (Gibco), 100 mg/l streptomycin (Gibco), and basel medium Eagle vitamins (Sigma).

The penetration of Propidium iodide (PI) was achieved by electroporation. The electric-field generator (GHT Unipolar 2000 V, Betatech, France) delivers square-wave electric pulses. Two stainless-steel plate parallel electrodes (length = 10 mm, interelectrode distance = 10 mm) connected to the voltage generator provide a uniform electric field. Cells were seeded on a glass slide around 24 h before the observations. The culture medium was removed and replaced by a PI-containing pulsation buffer (i.e., 100 μM PI in 10 mM phosphate pH 7.4, 1 mM MgCl2, 250 mM sucrose). The electric field parameters were 10 pulses of 5 ms length, 0.35 kV/cm intensity, and 1 Hz frequency at room temperature. 5 min after the electric field application, the cells were incubated with 4% paraformaldehyde for 10 min to fix the system, washed with phosphate buffered saline, and mounted on a slide.

To demonstrate that the signal generated by this W-axicon-based scheme is TIRF, we imaged polymer-immersed fluorescence microspheres (1 μm diameter) deposited on a 170-μm-thick cover slide (Fig. 2). The TIRF nature of the detected signal is demonstrated by changing the diameter of the illuminated annular beam. As has been pointed out above, sliding the inner axicon inside the aperture of the outer axicon changes the diameter of the annular beam. When the incident angle on the sample is adjusted to a size greater than the critical angle, the illumination light will undergo total reflection, thereby providing TIRF excitation. By contrast, when the incident angle on the sample is less than the critical angle, the illumination light can enter the bulk of sample and several out-of-focus planes can be illuminated, and wide-field fluorescence microscopy is realized, which will lead to excitation of beads above the focal plane.

Similar results are obtained with a sample consisting of *Convallaria majalis* slices. Under the excitation of

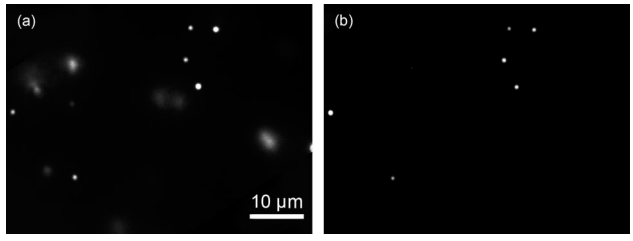

Fig. 2. Comparison of wide-field and TIRF images of 1 μm fluorescence microspheres: (a) wide-field fluorescence image and (b) TIRF image.

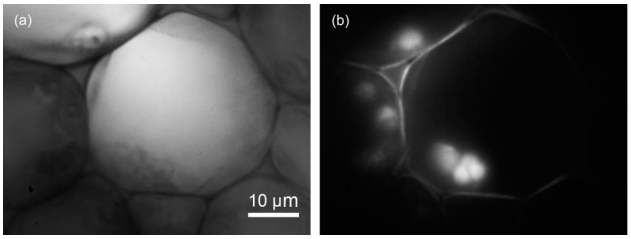

Fig. 3. Comparison of wide-field and TIRF images of *Convallaria majalis*: (a) wide-field fluorescence image and (b) TIRF image.

532 nm wavelength, the sample will exhibit strong intracellular autofluorescence [Fig. 3(a)]. In the corresponding TIR mode image, pronounced contrast from the cell walls is obtained, whereas no diffuse autofluorescence is visible [Fig. 3(b)].

The setup also suits itself to record TIRF images of living samples. We used wide-field microscopy to image PI-labeled CHO cells, shown in Fig. 4. Under the effect of electroporation, PI penetrates the membrane and slowly accumulates inside the nucleus. At short times after incubation, mainly the membrane is labeled. With the TIRFM illumination scheme, the membrane structure located in the evanescent field near the interface is clearly visible. Since the refractive index of the cytoplasm is approximately 1.38 or higher, the 1.4 NA objective is barely adequate for work on cells. Commercially available oil immersion objectives with NAs of 1.45 or higher would be a better choice for many biological samples.

The images from Fig. 2 and 3 were recorded by changing the position of the inner axicon mirror. They demonstrate that the proposed excitation scheme is suited to TIRFM with the mentioned advantages. It is noteworthy that illumination in the wide-field mode using the W-axicon setup does not exactly correspond to normal wide-field fluorescence microscopy, where the whole sample depth is illuminated. By contrast, using a W axicon with excitation angles below the critical angle results in illumination with a hollow cone of excitation light of approximately 10 μm thickness. This has been termed shallow angle fluorescence microscopy [11]. Fluorescence background in the images shown in Fig. 2(a) and 3(a) is, therefore, expected to be somewhat reduced compared to normal wide-field excitation.

In conclusion, we present an approach for implementing a TIRFM experiment based on a W-shape axicon mirror device. We demonstrated that our system has advantages over conventional TIRFM. It is free of shadow artifacts, has higher efficiency, offers adjustable illumination areas free of interference fringes, and can easily be switched between wide-field and TIRF imaging modalities. Examples of TIRF micrographs of fluorescence beads, *Convallaria majalis* tissue, and of PI-labeled CHO cells are given.

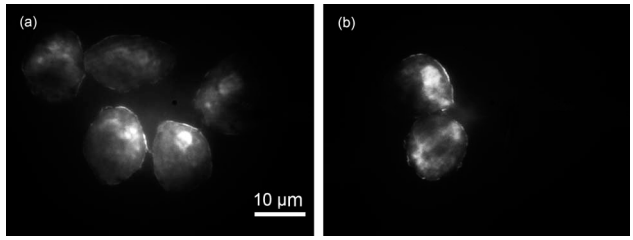

Fig. 4. TIRF images of PI-labeled CHO cells.

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