Evanescent field excitation of fluorescence by epi-illumination microscopy

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By simple modification of the pattern of fluorescence excitation light in an epi-illumination inverted microscope, one can achieve conditions that produce total internal reflection fluorescence (TIRF) by evanescent wave excitation. Though traditionally requiring a collimated beam traversing through a special prism, TIRF also can be achieved by epi-illumination through the periphery of a 1.4 numerical aperture objective. An opaque disk of appropriate size is placed in the illumination path external to the microscope so as to cast a sharp, real-image shadow at the objective's back focal plane. This shadow allows a hollow cone of epi-illumination rays traveling at only super-critical angles to reach the glass/water interface at the sample plane. Three kinds of TIRF illumination patterns can be produced by variations of this scheme: (1) a small spot of illumination of 1.5 μm radius by use of a laser light source, (2) a large region of illumination by use of a laser-illuminated diffusing screen located upbeam from the opaque disk, and (3) a large region of illumination by use of a conventional mercury arc.

I. Introduction

Total internal reflection fluorescence (TIRF) is a useful technique for observing the equilibria and kinetics of chemical and biological processes at liquid–solid interfaces. In TIRF, the evanescent field of totally internally reflected light selectively excites fluorescent molecules within several hundred nanometers of the liquid–solid boundary without exciting background fluorescence from molecules farther away.[1]

To produce an evanescent field, the excitation illumination must be incident upon the plane of the interface at angles greater than the critical angle. As adapted to fluorescence microscopy, TIRF has employed a prism to introduce the incident light at a supercritical angle. Unfortunately, the presence of the prism somewhat restricts the motion of the sample stage, requires a laser source with its attendent interference fringes resulting from the coherence of the source, and may necessitate a nonconventional sample mounting system. In this paper, we show how TIRF can be performed inexpensively in any upright or inverted fluorescence microscope without any special prism. This new prismless TIRF configuration has several features not present in the older prism-based configurations: (1) the light source can be either a standard mercury arc or laser, (2) the sample may be mounted in a standard manner, (3) rapid interconversion to other forms of illumination can be performed, (4) either small spots (1.5 μm) or whole fields can be illuminated, and (5) whole fields can be made visually free of interference fringes. The new system also holds advantages for observing TIRF near thin metal films coated on glass—films which may find several applications to studies of cells and membranes in biophysics.

The new system is based on the use of a high numerical aperture (N.A. = 1.4) objective for epi-illumination. However, only those incident rays traversing a peripheral annulus are allowed to propagate freely through the objective; the more central rays are blocked. The peripheral rays are then incident upon the glass coverlip substrate of the sample at supercritical angles to the glass–water interface. This selective blockage of the subcritical rays is accomplished without alteration of either the objective or internal microscope optics.

We discuss three optical configurations: (1) TIRF laser illumination at a small (micron-sized) spot, potentially useful for measuring surface chemical kinetic rates and diffusion by fluorescence photobleaching or correlation spectroscopy[1], (2) whole field TIRF laser illumination free of apparent interference fringes, useful for imaging systems, and (3) TIRF with standard microscope mercury arc illumination. As a test of the effectiveness of prismless TIRF, fluorescence-labeled resealed red blood cell ghosts adhered to a glass surface are observed.

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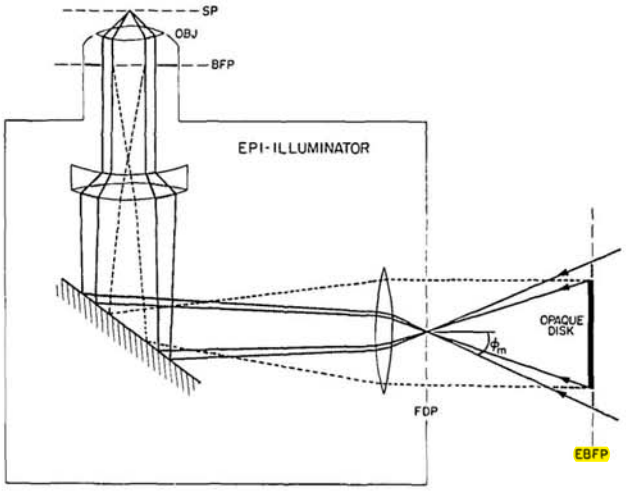

Fig. 1. Schematic diagram of supercritical rays in the epi-illuminator of a fluorescence microscope.

II. Background

Consider excitation light rays directed through a glass coverslip onto a sample plane (at the interface between a glass coverslip and an aqueous medium) by an epi-illumination objective. The critical angle for total internal reflection (TIR) is given by

$$\theta_c = \sin^{-1}(n_1/n_2), \tag{1}$$

where n_1 and n_2 are the indices of refraction of liquid and solid, respectively. For a glass ($n_2 = n_g = 1.5$)/water ($n_1 = n_w = 1.33$) interface, $\theta_c = 61.4°$.

The maximum angle θ_m that light rays emerging from the objective can form with the normal to the sample plane depends upon the numerical aperture A of the objective:

$$A = n_g \sin\theta_m. \tag{2}$$

Since only those rays propagating at angles greater than the critical angle will produce an exponentially decaying evanescent field at the TIR interface, the condition imposed on the objective for TIR is:

$$\sin\theta_m > \sin\theta_c. \tag{3}$$

From Eqs. 1 and 2, we then derive the minimum A necessary for TIR:

$$A > n_w. \tag{4}$$

For an $A = 1.4$ objective, $\theta_m = 67.5°$. Therefore, exclusion of light at subcritical angles $\theta < \theta_c = 61.4°$ will still leave a hollow cone of supercritical angle light in the 61.4–67.5° angular range converging toward the sample, thereby creating an evanescent field that can selectively excite fluorophores in the vicinity of the interface.

The angle with which a ray emerges from the objective into the immersion oil and glass coverslip depends solely on its radial position from the optical axis as the ray passes the back focal plane (BFP) inside the objective; any two rays with the same radial position at the BFP, regardless of their angles at the BFP, will emerge parallel at some angle θ. Therefore, selective exclusion of subcritical angle ($\theta < \theta_c$) illumination light could be accomplished in principle by placing an opaque disk of appropriate radius at the BFP. In practice, the BFP of a microscope objective is inaccessible and even may be located within some lens element. However, if a real image shadow of an opaque disk were formed at the BFP, subcritical angle light would be excluded just as if there were a disk at that plane. The actual opaque disk is located in the excitation path outside the microscope, at a unique fixed plane equivalent to the BFP. This plane is equivalent in the sense that rays intersecting at the equivalent BFP (called the EBFP here) necessarily intersect at the BFP. This basic approach, common to all the arrangements described here, is depicted schematically in Fig. 1 as a ray diagram through a standard inverted microscope epi-illuminator.

III. Optical Arrangements

The first task is to locate the EBFP. This is done simply by coupling laser illumination light out of the $A = 1.4$ objective (in our case, an oil immersion Nikon 60× in an inverted Leitz Diavert microscope) with a plano-convex lens in concentric optical contact (via a layer of immersion oil) with a coverslip overlying the objective and allowing the emerging light to fall on a screen placed at any distance large compared to the diameter of the objective. On this screen, each radial distance from the optical axis of the objective corresponds to a unique emergent angle θ. An opaque object, such as a razor blade edge, placed part way across the laser beam at any location before it enters the epi-illuminator of the microscope will in general produce a shadow image on the screen. However, at only one location will the shadow image be sharp. This unique location is at the EBFP. For the Leitz Diavert or Ortholux epi-illuminator, the EBFP is ~10 cm upbeam from the field diaphragm plane of the epi-illuminator.

Light focused at the field diaphragm plane (FDP) will produce a focused spot at the sample plane, regardless of the range of angles of the light convergent upon the FDP. However, that range of angles does affect the range of radial positions of rays passing the BFP; in general, more convergent rays at the FDP will become more peripheral rays at the BFP. Therefore, the incident beam must be directed so that it converges toward the FDP with a angle of ϕ (see Fig. 1) sufficient to fill the objective aperture at the BFP. This angle ϕ can be increased until it reaches some maximal angle ϕ_m such that the shadow of the objective's limiting aperture is clearly visible on the screen. On the Leitz Diavert, $\phi_m \approx 10°$.

Convergent angle ϕ is formed by a focusing lens FL selected and positioned so there is sufficient room for an opaque disk to be placed between it and the FDP. The radius of this opaque disk must be large enough to cleanly block all emergent light rays of angle $\theta \leq \theta_c$. The proper radius can be judged by comparing the

light pattern on the screen formed by the previously mentioned glass plano-convex lens in optical contact with the coverslip with the pattern formed through a simple bead of water on the coverslip. If no significant light is cast on the screen through the water bead (meaning $\theta \leq \theta_c$ is blocked) but a sharp-edged ring is seen with the plano-convex lens (meaning some $\theta_c < \theta < \theta_m$ is not blocked), then the opaque disk radius is chosen correctly. In our setup, the disk radius is 0.65 cm.

The choice of optical elements placed between FL and the source depends on the type of illumination desired: laser spot, laser wide area, or mercury arc wide area.

A. Laser Spot

In this simplest configuration, the laser beam is passed through a beam expander (in our case, to an ~6-mm radius) and then through the FL. The beam expander allows the FL to produce the maximal convergent angle of ϕ_m [see Fig. 2(a)]. A slightly more elaborate configuration, but one quickly interchangeable with the setup for the wide area illumination discussed below, passes a beam expanded to ~3 mm through a short focal length lens [in our setup, a 10× air immersion microscope objective lens, shown as OL in Fig. 2(b)]. Lens OL serves to converge the beam to a focus and then diverge it past that focus to a radius again sufficient to allow FL to produce a maximal convergent angle of ϕ_m. With OL and FL lenses corrected for spherical aberration (such as camera lenses), a region of illumination of average radius ~1.5 μm can be attained with this configuration; slight defocusing of OL or FL leads to a somewhat larger spot.

That the illuminated region is produced predominantly by a TIR evanescent field rather than by a freely propagating field can be qualitatively confirmed on a sample consisting of a coverslip coated with an insoluble orange-emitting carbocyanine dye in contact with a green-emitting fluorescein solution. Under TIR configured as above, the fluorescence appears as a small orange spot with surface defect details in the glass quite distinct; under epi-illumination (i.e., with the opaque disk withdrawn) the same sample appears as a featureless, diffuse, bright green field.

B. Laser Wide Area

The focal plane of OL in the above arrangement is equivalent to the FDP and to the sample plane; at each plane, the laser beam forms a diffraction-limited spot. If a larger area could be illuminated at the focal plane of OL, a larger area will be illuminated on the sample. A larger area can be produced by simply moving the OL slightly forward or back, but this soon leads to a circular dark area in the middle of the field of view, arising from the shadow of the opaque disk.

A better approach is to place a diffusing screen at the focal plane of OL. That diffusing screen plane is denoted as DSP in Fig. 2(c). The diffusing screen effectively converts the single point source of the laser into an extended source. The size of the extended source,

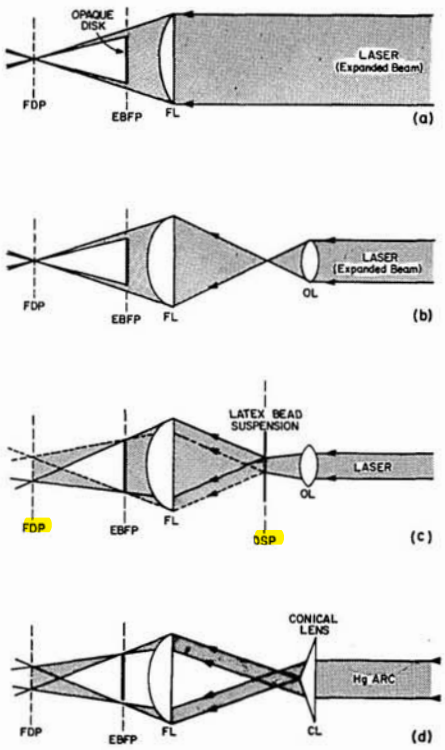

Fig. 2. Optical arrangements before the epi-illuminator for three configurations: (a) laser source focused with a single lens to a spot, (b) laser source focused to a spot with two lenses, (c) laser source converted to an extended source by a latex bead suspension for illuminating a large area (size of the illuminated region on the DSP is exaggerated for pictoral clarity), (d) mercury arc source, including the use of a conical lens to increase the power of supercritical light at the expense of subcritical light.

and hence the size of the illuminated region on the sample, can be adjusted simply by moving OL forward or backward, here with no production of central dark areas on the sample.

A convenient diffusing screen is an aqueous suspension of microscopic latex beads sandwiched between two coverslips spaced apart by a thin (~50 μm thick) Teflon ring. An additional major advantage of a latex bead suspension is that the coherent light interference pattern produced on the sample from the superposition of scattered light waves from each bead flickers so rapidly due to bead diffusion that the TIR illumination appears to be uniform over much of the field of view.

Figure 3 shows fluorescent labeled erthrocyte ghosts adhered to a coverslip and photographed with this kind of illumination. With wide-area laser TIR [Fig. 3(a)] the image is sharp only when the objective is focused at the very bottom of the erythrocyte ghosts; there is very little out-of-focus fluorescence. On the other hand, with the opaque disk withdrawn, which yields predominantly non-TIR epifluorescence [Fig. 3(b)], the image in the regions of cell-surface contact is quite indistinct due to the overwhelming intensity of fluorescence from out-of-focus planes of the cells farther from the glass. If the microscope is refocused a

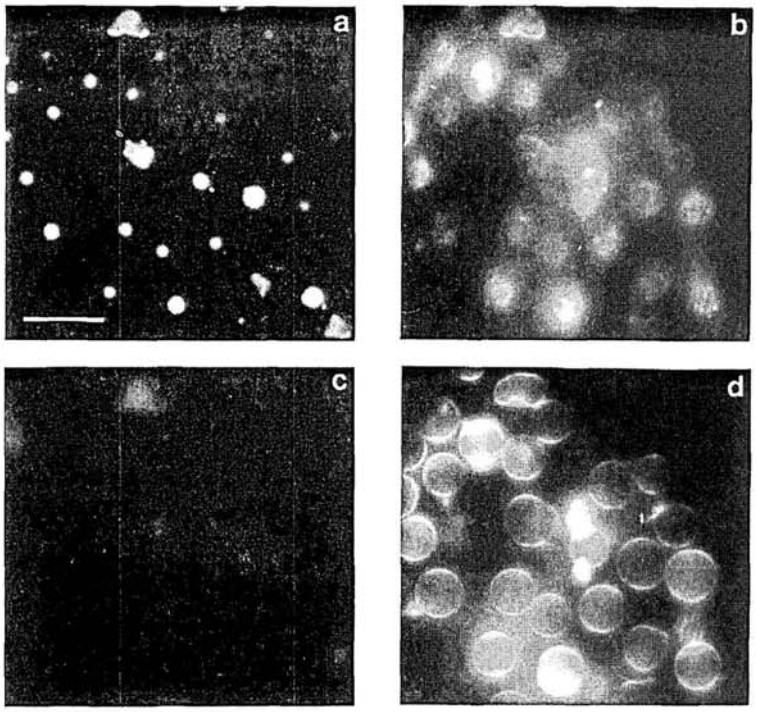

Fig. 3 TIRF on fluorescence-labeled erythrocyte ghosts, laser-illuminated by the configuration depicted in Fig. 2(c). (a) TIRF, focused at coverslip surface, (b) epi-illumination, focused at coverslip surface, (c) TIRF focused at midplane of spherical ghosts, (d) epi-illumination focused at midplane of spherical ghosts. Exposure time = 12 s for both TIRF and epi-illumination, on Kodak TMAX P3200 film. For TIRF the total laser power at $\lambda = 514.5$ nm was about 0.4 W before the OL; for epi-illumination this power was reduced by at least a factor of 10. Space bar = 10 μm.

short distance into the aqueous solution at the diameter plane of the spherical ghosts, TIR shows very little excitation of fluorophores [Fig. 3(c)] with the same photographic exposure as Fig. 3(a), but epi-illumination (i.e., the opaque disk withdrawn) shows a distinct tangential view of the circumference of the ghosts [Fig. 3(d)].

A significant amount of excitation power is lost by scattering at the diffusing screen with this wide-area TIR illumination system (apart from the loss of all subcritical rays), but a standard 3W argon laser is still bright enough to produce satisfactory images. The predominance of evanescent field excitation in this system has been confirmed qualitatively by viewing a carbocyanine-labeled surface in contact with a fluorescein solution, as described in Sec. III.A.

The erythrocyte ghosts were prepared as follows. One drop of freshly drawn human blood was placed in 1 ml of Dulbecco's phosphate-buffered saline solution (DPBS). 20 μl of a solution of 0.5 mg/ml di-I (3,3′-dioctadecylindocarbocyanine, a gift from Dr. Alan Waggoner) in ethanol solution was added to the cells, and the dye was allowed to label the membranes for 5 minutes. The cells were then centrifuged for 10 minutes, after which the supernatent, containing unincorporated di-I, was drawn off. To the cells remaining, 0.5 ml DPBS was added to resuspend them. The cells were then gradually hemolyzed by adding 1.5 ml distilled water in 0.5 ml increments. This method of hemolysis leaves the erythrocyte ghosts in a more or less spherical shape. 0.7 ml of this suspension was placed between two glass coverslips, and the erythrocyte membrane ghosts where allowed to settle to the bottom before they were observed.

C. Mercury Arc, Wide Area

100-W mercury arc lamps are much more commonly used than lasers for microscope fluorescence illumination than lasers. Unfortunately, with standard prism-based TIRF, the power of a mercury arc light that can be directed through a TIRF prism at supercritical angles is insufficient to produce visible images. The prismless method, as used in Sec. III.A. with an arc instead of a laser, can direct more supercritical light into a smaller sample area. Nevertheless, the light available is still marginal because of the blockage of the subcritical light. Much of this light can be utilized, however, by employing a conical lens immediately in front of the mercury arc housing [see Fig. 2(d)]. The conical lens (custom manufactured in our case by Vogelin Optics, Minneapolis, from standard optical glass with a 78° apex half angle and a 25-mm diam serves to direct much of the arc light into a somewhat fuzzy ring. The FL (the same lens as used in the laser illumination systems in approximately the same position) then converges this ring past the opaque disk situated at the usual EBFP position (at which the radius of the ring is just slightly larger than the radius of the disk) toward a

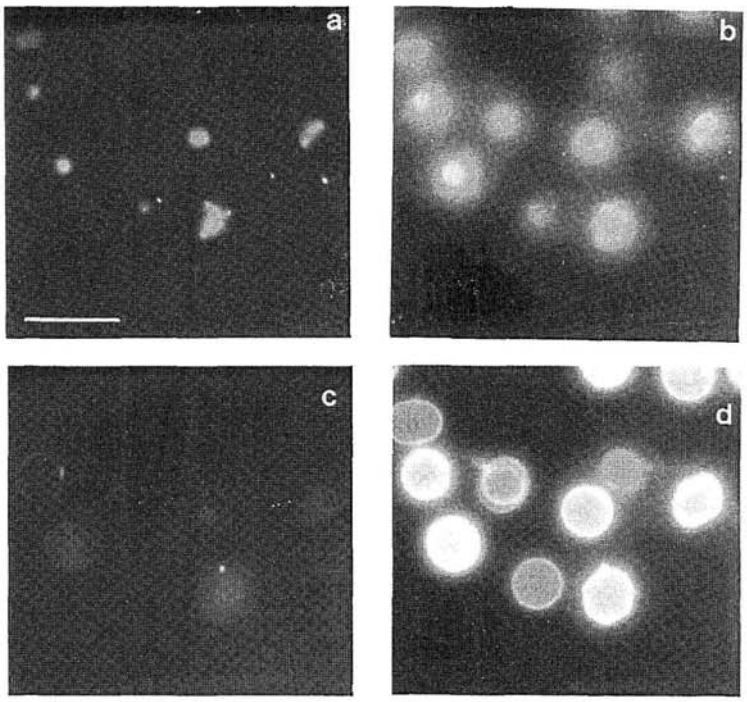

Fig. 4. Same as Fig. 3, except illuminated by the mercury arc source with the configuration depicted in Fig. 2(d).

focus at the field diaphragm plane. The result is a wide-area TIR illumination (again confirmed by the carbocyanine/fluorescein sample) entirely free of laser interference fringes. Figure 4 shows fluorescent erthyrocyte ghosts photographed under this kind of illumination in a sequence analogous with the wide-area laser illumination of Figs. 3.

IV. Discussion

We have outlined new arrangements for setting up TIRF microscopy without any special prisms using several kinds of illumination not previously attainable: (1) focused-spot laser, (2) wide-area laser, free of apparent interference fringes, and (3) wide-area mercury arc. We discuss here some of the advantages and drawbacks of these configurations.

First, TIRF should be compared with another method of optical sectioning that is becoming increasingly popular: confocal microscopy. TIRF selectively excites fluorophores only within a few hundred nanometers of a water–glass interface. If this is the region of interest in a particular experiment, TIR may be preferred over confocal microscopy, which has a characteristic depth at least several times larger and a much lower light-gathering efficiency. TIR is much less expensive to set up, particularly in the configurations presented here which are modifications to the optics external to a standard epifluorescence microscope equipped with a 1.4 aperture objective. Of course, if one wishes optically to section the entire thickness of a sample, rather than just the region near an interface, confocal microscopy is the required method.

The new configurations have some significant advantages compared to previous TIRF setups. In the past, TIR has been achieved by placing a prism directly in optical contact with the surface of the slide or coverslip opposite to the surface to which the sample adheres. That arrangement can be physically awkward and may reduce the range of travel of the sample. In addition, the exact location of TIR illumination relative to the microscope's optical axis can drift as the sample is translated. An additional constraint in that system is that the objective, located on the opposite side of the sample chamber from the prism, must have a working distance long enough to focus on the sample across a glass–liquid–glass sandwich. For high aperture oil immersion objectives whose aberration corrections require a sample immediately on the far side of a glass coverslip, viewing across an additional aqueous space can reduce clarity. In general, for 1.4 aperture objectives, viewing through the coverslip to which the fluorophores are in close proximity theoretically increases the total amount of fluorescence gathered relative to viewing through the water with the same objective.[2] Some samples are best mounted on metal-coated coverslips to quench fluorescence emanating from within the first 10 nm of the surface.[3] Viewing through the water sandwich in that case, as in previous TIRF configurations, leads to confusing and out-of-focus reflections of fluorescence at the metal surface, an effect avoided by viewing through the glass coverslip.

The possibility of attaining smaller focused TIRF spots in the present configuration than in prism-based

TIRF allows for more spatially selective studies of molecular dynamics (by, for example, photobleaching methods[1]) than previously attainable. The minimum spot size is determined by diffraction and objective lens aberrations. If the loss of more illumination power can be tolerated, a slight defocusing at the field diaphragm plane combined with a small diaphragm or pinhole placed at that plane could be used to control the size and profile of the illuminated region.

The demonstrated capability for surmounting apparent interference fringes in wide-area TIRF laser illumination allows for more faithful imaging. The option of using a mercury arc rather than laser makes TIRF accessible with any standard epifluorescence system.

One disadvantage of the opaque disk TIR system is that a large portion of the original excitation light is lost as it is blocked by the disk. This is not generally a problem with an argon laser, but it is a problem with a mercury arc. The use of the conical lens as described does allow utilization of some light that would otherwise be lost, but the mercury arc system still requires a brighter sample to produce a visible image.

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