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Around-the-objective total internal reflection fluorescence microscopy

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Abstract

Total internal reflection fluorescence (TIRF) microscopy uses the evanescent field on the aqueous side of a glass/aqueous interface to selectively illuminate fluorophores within ~100 nm of the interface. Applications of the method include epi-illumination TIRF, where the exciting light is refracted by the microscope objective to impinge on the interface at incidence angles beyond critical angle, and prism-based TIRF, where exciting light propagates to the interface externally to the microscope optics. The former has higher background autofluorescence from the glass elements of the objective where the exciting beam is focused, and the latter does not collect near-field emission from the fluorescent sample. Around-the-objective TIRF, developed here, creates the evanescent field by conditioning the exciting laser beam to propagate through the submillimeter gap created by the oil immersion high numerical aperture objective and the glass coverslip. The approach eliminates background light due to the admission of the laser excitation to the microscopic optics while collecting near-field emission from the dipoles excited by the ~50 nm deep evanescent field.

1. Introduction

Fluorescence microscopy can detect low light levels and measure spatial distribution and dynamics (rotational and translational) of small ensembles, or single biomolecules, in biological samples. The method has uniquely contributed to cell biology studies requiring real-time monitoring of biomolecule dynamics. Much of the progress is related to development of high-quantum-efficiency fluorescent probes that can undergo many excitation/emission cycles before irreversible photobleaching [1]. Optical microscopy hardware has likewise evolved to promote light collection and transmission efficiency over a wider optical spectrum. Analysis techniques for high-precision localization of isolated single fluorescent molecules have advanced spatial resolution beyond the diffraction limit [2-4]. New microscope configurations have likewise improved spatial resolution to below the diffraction limit by using evanescent field excitation [5], surface plasmon coupled emission (SPCE) [6,7], supercritical angle fluorescence (SAF) [8], structured illumination (SI) [9], and the stimulated emission depletion (STED) of peripheral excited molecules in a diffraction limited spot [10]. Back focal plane (BFP) emission pattern imaging of isolated probes determined emitter dipole orientation by inspection [11] and lifted some of the degeneracy in orientation determination normally characteristic to dipole emitters [12].

Evanescent field excitation in total internal reflection fluorescence (TIRF) microscopy takes place when an excitation laser beam impinges on a glass/aqueous interface from the higher refractive index (glass) side at an angle greater than critical angle, θ_c , for total internal reflection (TIR). The beam totally reflects at the interface while producing an evanescent field in the lower refractive index (aqueous) side that decays exponentially with distance from the interface. TIRF is used extensively in cell biology applications, where it preferentially illuminates specimen thin sections within ~ 100 nm of the interface [13,14]. Two common TIRF microscopy configurations introduce the exciting laser beam to the interface either through the microscope objective [15] or via a prism external to the microscope in optical contact with the interface [16]. Through-the-objective TIRF (oTIRF) uses a coverglass and oil immersion objective with numerical aperture (NA) significantly larger than the water refractive index of 1.334. A laser beam propagating parallel to the optical axis but displaced laterally and focused at the BFP is refracted by the objective and impinges on the coverglass/aqueous interface at angles beyond θ_c . Propagating exciting light in the objective produces unavoidable background autofluorescence. Sample fluorescence emission necessarily occurs near the glass/aqueous interface, providing substantial SAF intensity collected in the objective. Through-the-prism TIRF (pTIRF) has the excitation laser beam totally reflecting at a coverglass/aqueous interface with the coverglass optically coupled to an external prism. Sample fluorescence is collected from the aqueous side or from the glass side with a water immersion objective to avoid frustrating the TIR. Although SAF is emitted, it cannot be collected in the objective. A new TIRF setup, around-the-objective TIRF (aoTIRF), combines through-the-prism excitation and SAF light collection. We demonstrate aoTIRF on upright and inverted microscopes using artificial and biological samples.

In aoTIRF, the oil immersion objective (NA 1.45) is focused on the coverslip/aqueous interface. A narrow exciting laser beam at near-skimming incidence to the interface is focused at the objective focal point by a low aperture lens. In the upright microscope, the focused excitation beam propagates into the immersion oil, separating the interface from the tip of the objective using a Littrow prism optically coupled to the oil. The incidence angle is 87° – 88° within the oil medium to assure that the focused beam clears the objective tip. After TIR at the intersection of the objective focal plane and optical axis, the exciting beam exits the immersion oil using a second Littrow prism. In the inverted microscope, propagating the exciting beam into the immersion oil is more complicated because the beam must approach the objective tip from below the microscope stage. We accomplished this with an additional reflection of the beam at a glass/air interface as the beam approaches or retreats from the objective.

We show that the upright and inverted microscope versions of aoTIRF produce an evanescent field with penetration depth of ~ 50 nm by observing fluorescent nanosphere diffusion through the excitation field. BFP imaging of fluorescent nanosphere emission detects the SAF emission from the object and demonstrates the lower background characteristics of aoTIRF excitation. Images from a green fluorescent protein (GFP)-tagged myosin light chain exchanged into cardiac papillary muscle fibers indicate the favorable contrast of aoTIRF compared to epi-illumination in the wide-field imaging microscope.

2. Methods

A. Chemicals

Carboxylate-modified fluorescent microspheres are from Molecular Probes (Eugene, Oregon). ATP, Na-Azide, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), trans-1,2-Diaminocyclohexane-N,N,N,N-tetraacetic acid (DCTA), ethylene glycol-bis (2-amino-ethylether)-N,N,N,N-tetra-acetic acid (EGTA), Triton X100, phosphocreatine, and creatine phosphokinase are from Sigma (St. Louis, Missouri). Recombinant human cardiac troponin C (TnC) is from Life Diagnostic (West Chester, Pennsylvania). Leupeptin is from Roche Applied

Sciences (Indianapolis, Indiana). All chemicals are reagent grade or ultrapure, if available. The GFP-tagged myosin regulatory light chain (RLC-GFP) was constructed as described [17].

B. Solutions

The washing solution contains 4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 1.8 mM NaHPO₄, 5.5 mM glucose, 50 mM imidazole buffer, and 140 mM NaCl, pH 7.4. Skinning solution A contains 1% Triton X100, 50% glycerol, 10⁻⁸M Ca (pCa8), 1 mM Mg, 7 mM EGTA, 5 mM MgATP, 15 mM phosphocreatine, 15 U/mL creatine phosphokinase, 20 mM imidazole pH 7.0, 1 mM DTT, and ionic strength is adjusted to 150 mM with potassium propionate. Fiber Storage solution is Skinning A without Triton X100. Relaxing solution is Skinning A without Triton X100 and glycerol. Dissecting solution is Relaxing plus 15% glycerol. Extracting solution contains 5 mM CDTA, 50 mM KCl, 0.2 mM PMSF, 10 µg/mL leupeptin, 0.5 mM Na azide, 1 mM DTT, 40 mM tris pH 8.4, and 1% Triton X100. Skinning solution B is Relaxing plus 1% Triton X100. Reconstitution solution is Relaxing plus 40 µM RLC-GFP and 15 µM TnC. Contracting solution is Relaxing except that the Ca concentration is raised to 10⁻⁴ M (pCa4).

C. Sample Preparations

Clean #1 or #2 glass coverslips were sonicated for 10 min in ethanol then plasma cleaned (Harrick Plasma, Ithaca, New York) for 15–30 min. All experiments were conducted at room temperature.

Red-orange carboxylate-modified 40 nm diameter fluorescent spheres had excitation/emission maxima at 565/580 nm. The yellow-green 100 nm diameter fluorescent spheres had excitation/emission maxima at 505/515 nm. Sphere concentrations were computed using the formula from the manufacturer and diluted in distilled water. We used 10⁴–10⁶ fold dilutions from stock, giving a sphere concentration of $1.4 \times (10^{11} - 10^9)$ spheres/mL for the 40 nm diameter spheres or $3.6 \times (10^9 - 10^7)$ spheres/mL for the 100 nm spheres.

Nanospheres with 10⁶ fold dilution were flowed into the sample chamber and allowed to dry. After drying, some spheres are strongly attached to the substrate surface in a sparse random spatial distribution. The more concentrated sample (10⁴ fold dilution from stock) was then flowed into the sample chamber. All fluorescence nanosphere experiments were conducted with sparsely surface attached nanospheres in contact with aqueous solution containing a high concentration of nanospheres. These samples were investigated with epi-illumination or aTIRF excitation.

Cardiac muscle fibers were from the porcine left ventricular papillary muscle. The porcine heart left ventricle was opened and washed thoroughly with Washing solution, then bathed in Dissecting solution. Papillary muscle was dissected into bundles of ~200 single fibers each, then placed into Storage solution on ice for 1 h. The fiber bundles were then transferred to the Skinning A solution and kept at 4 °C for 24 h. The fibers were then transferred back to the Storage solution and kept at -20 °C until used. Fibers used for the RLC-GFP exchange were made from stored fiber bundles dissected down to smaller bundles containing ~10 fibers. The small fiber bundle was mounted on a microscope slide and glued down at the ends. A water resistant resin was extruded from a pipette tip to create a channel open at both ends containing the fibers. A coverslip was placed over the fiber. The fibers were washed with Relaxing (three times, 1 min each), then incubated in Skinning B solution for 60 min (three solution exchanges, 20 min each). The fibers were washed again with Relaxing solution (three times, 1 min each). The native regulatory light chain was extracted by incubating the fibers in Extracting solution for 60 min (three solution exchanges, 20 min each). The fibers were washed with Relaxing solution (three times, 1 min each), then reconstituted with Reconstitution solution for 1 h (four

solution exchanges, 15 min each). Finally, fibers were washed with Relaxing solution (three times, 1 min each) and transferred to the aoTIRF setup on the inverted microscope (Fig. 1).

D. aoTIRF Microscopy

Upright and inverted microscope configurations for aoTIRF are indicated schematically in Figs. 1(a) and 1(b). The exciting beam (dashed line) enters and exits the vicinity of the objective at incidence angles of 87° – 88° in both configurations. The 30° – 60° – 90° Littrow prisms (Edmund Scientific, Barrington, New Jersey) are needed for coupling the laser beam into and out of the coverslip. Total internal reflection at the glass/aqueous interface occurs at the focal point of the objective. The exciting beam in the inverted microscope configuration also totally internally reflects at the glass/air interface formed by the #2 coverslips. Prism size is larger in the inverted microscope configuration to accommodate the triple reflection. Exciting beam conditioning by the beam reducer and focusing lens is performed for both upright and inverted configurations.

Figure 2 shows an expanded view of the upright microscope configuration. The flat surface on the objective containing the lens has radius $R \approx 3.8$ mm. The working distance $W \approx 0.1$ mm, coverglass thickness $C \approx 0.15$ mm, gives $L \approx 0.25$ mm and arc length $A \approx L$. The maximum value for θ_2 (the complement of the incidence angle at the glass/aqueous interface) avoiding the objective edge (where segment A meets the objective) is ~ 3.8 deg, but we wanted to bring in the exciting beam nearer the midpoint of A , where $\theta_2 \approx 2.3$ deg. In this case, refraction at the air/glass interface of the Littrow prism requires the incidence angle for the laser exciting beam, θ_1 , to be ~ 3.5 deg.

Figure 3 shows an expanded view of the inverted microscope configuration. Quantities R , W , L , C , θ_1 , and θ_2 are equal to their counterparts in Fig. 2. The bevel on the objective is θ_3 . Where the excitation beam enters the Littrow prism is critical to assure it will leave the prism to enter the #1 coverslip. With incidence at $K \approx 1$ mm, transition into the #1 cover-slip occurs at distance $P \approx 27$ mm well within the prism dimension of 37.9 mm. The #2 coverglass has thickness $B \approx 0.21$ mm, and is positioned to provide the glass/air interface for the first reflection of the exciting laser beam and to support the oil retainer. The first reflection position is at $S + R = (B + C)/\tan[\theta_2]$ from the optical axis, giving $S \approx 5.2$ mm. We made the oil retainer from a 2 mm thick strip of double sticky tape where $\varepsilon \approx 1.5$ mm. We find, then, that $H = 3.5$ mm and $H + F = 37.4$ mm.

The argon ion laser beam has a Gaussian intensity profile and a beam diameter of ~ 1.5 mm. It is conditioned for insertion into the aoTIRF setup using a beam diameter reducer and focusing lens (Fig. 1). The beam must fit into the ~ 250 μ m diameter approach-limiting aperture (Fig. 2 or Fig. 3) at distance R from the objective focal point. The exciting beam focus is at the objective focal point where TIR occurs. The beam diameter is reduced to 0.75 mm by a diameter reducer and then focused by a 12.7 mm $\times$ 150 mm (aperture diameter $\times$ focal length) lens.

We computed the intensity profile near the exciting beam focus for a Gaussian laser intensity profile with $\beta = (\text{aperture diameter})/(\text{beam diameter}) \approx 17$, as described previously [18-20]. It is represented as the rippled surface in Fig. 4(a) or Fig. 4(b), with intensity plotted on the y_p axis or the x_p axis in arbitrary units, respectively. The x_p and y_p axes define the focal plane of the exciting light with z_p in the direction of light propagation and y_p in the incidence plane at the glass/aqueous interface. Exciting light is linearly polarized along y_p in the p -polarization. The intensity profiles are practically identical in x_p and y_p and depend only on radial distance from the z_p axis. The planes in Figs. 4(a) and 4(b) shown slicing through the intensity profile at focus represent the objective field of view (FOV). The FOV is in the objective focal plane or, alternatively, the interface plane. The exciting beam reflects and refracts at the interface plane, but this is not depicted in the figure and does not affect the intersection of the intensity

profile with the FOV. The FOV is approximately the size of the field of view for the 60 $\times$, NA = 1.45 objective. The FOV plane subtends a short distance on y_p ($\pm 2 \mu\text{m}$) because the beam strikes the FOV plane at a glancing angle. The beam intensity profile in the x_p dimension has an $\sim 70 \mu\text{m}$ full width in the focal plane and fits entirely within the FOV.

The approach-limiting edge (Fig. 2 or Fig. 3) forms a barrier of $\sim 250 \mu\text{m}$ full width in the y_p dimension of the propagating beam coordinates at $z_p = 3.8 \text{ mm}$. The beam intensity z_p -dependence changes gradually, with 76% of its maximum peak height at the origin retained at $z_p = 3.8 \text{ mm}$, where the beam intensity profile in x_p or y_p has $\sim 80 \mu\text{m}$ full width. At $z_p = 10 \text{ mm}$, the beam intensity profile in x_p or y_p has $\sim 120 \mu\text{m}$ full width. Figure 5 shows the beam cross sections in y_p for various positions along z_p and indicates that the majority of the exciting light energy will transmit the approach-limiting edge.

Figure 6 shows close-up photographs of the upright [Fig. 6(a)] and inverted [Fig. 6(b)] configurations. The excitation laser pathway shows up in the Littrow prisms as a thin red line due to autofluorescence. Scattered light dimly illuminates the background, but the excitation beam pathway can be seen as it approaches and exits the sample area at the objective focal point. The sample region is clearly visible in the inverted configuration, where excitation light scatters as the excitation beam propagates between the objective and the glass/sample interface. The exciting light scattering is larger just at the edges of the objective lens, where there is a material discontinuity from the brass of the objective body to the glass of the lens.

Excited fluorescence collected by the objective is spectrally filtered and formed into an image by the microscope tube lens at the light sensitive area of a 12 bit CCD camera (Hamamatsu Orca ER) on the upright microscope (Zeiss Axioplan) or a 16 bit EMCCD camera (Hamamatsu C9100) on the inverted microscope (Olympus IX71). The emitted light pathway is altered for BFP imaging (see Subsection 2.E). We used the Olympus 60 $\times$ NA = 1.45 (TIRFM) objective for all measurements.

E. Back Focal Plane Microscopy

BFP imaging was done on the upright microscope. Excitation 488 nm light from an argon ion laser excites the sample under aoTIRF. The p -polarized incident light has electric field polarization in the incidence plane and produces an elliptically polarized evanescent electric field. Evanescent intensity is ~ 78 or 22% polarized normal or parallel to the interface [21].

Single 100 nm diameter fluorescent spheres immobilized on the TIR interface are in diffusive contact with identical spheres, as described in Subsection 2.C. Excited fluorescence was collected by the objective and spectrally filtered using a fluorescein filter set. Figure 7 shows the emission pathway optical setup, where OP is the object plane, OBJ is the objective, TL is the tube lens with focal length f_{TL} , RL is a removable lens, and CCD is the image plane for the camera. Light emitted by the sample (black arrow at OP) emerges from the back of the objective in parallel rays. An object at the BFP (light double arrow) is imaged onto the CCD camera with insertion of RL. The BFP is telecentric, implying that an object displaced laterally in the focal plane does not change the position of its intensity pattern. RL is positioned to form a real image of the BFP at the CCD plane by placing a translucent ruler at the BFP and adjusting the RL position until its real image is formed at the CCD plane. RL is corrected for chromatic and spherical aberrations and is removable to facilitate conversion between BFP and normal imaging.

F. Emission Near an Interface

When a dipolar is near a dielectric planar interface, the radiated fields are significantly altered [22]. An emitting fluorophore has a dipolar emission field that is the superposition of

propagating transverse waves forming the far field and nonpropagating longitudinal (evanescent) waves forming the near field. The near field is not detected unless perturbed by a nearby interface creating detectable propagating transverse waves. Converted near-field propagating waves appear in the glass medium at angles beyond θ_c and are referred to as SAF [8]. Oil immersion objectives with $NA \gtrsim 1.3$ capture SAF. Subcritical angle fluorescence (UAF) is from far-field emission.

The interface also affects total radiated power. Hellen and Axelrod pointed out that, for a fluorophore under steady illumination, the dissipated power must equal the absorbed power, implying that the fixed power, rather than the fixed amplitude, dipole radiator is the appropriate model for probe emission near an interface [22].

The high-NA collection of light in a microscope objective mixes light contributions from emitter dipole Cartesian components due to the collection of light from a large solid angle surrounding the dipole. The Cartesian dipole moments produce differently polarized light that is combined by the objective such that their individual contributions cannot be separated by using an analyzing polarizer. Separating the contributions into the Cartesian components of the dipole moment is the basis for fluorescence polarization measurement of dipole orientation. Axelrod described the solution to the problem in a homogeneous medium [23]. We addressed the probe emission perturbation by the interface and the complications for polarized light collection by a high-NA objective in the dipolar emission intensity from single probes. Our computations gave the two-dimensional (2D) intensity pattern in the objective BFP for comparison with experiment and showed the effect of probe orientation on the pattern as described previously [12]. BFP intensity pattern computation and display was performed with Mathematica 7 (Wolfram Research, Champaign, Illinois).

G. Random Walk Simulation

Fluorescent spheres diffuse through the aoTIRF illumination volume, giving a time-dependent fluorescence intensity depending on sphere diffusion constant D , detection volume (V_d) size and shape, and on constraints imposed on sphere position by the presence of the planar glass/aqueous interface where TIR occurs. We model sphere diffusion as a three-dimensional (3D) random walk subject to constraints, compute the time-dependent fluorescence intensity, and compare computed with observed signals to deduce the size and shape of V_d .

The random walk simulation of diffusion with step size δ , characteristic time between steps of τ , and with constraints imposed by planar glass/aqueous interface was carried out as described previously [18]. The Einstein-Smoluchowski relation gives the diffusion constant, $D = kT/(6\pi S\eta)$, where kT is thermal energy, S is the sphere radius, and η is the fluid viscosity. The diffusion constants for the 40 and 100 nm diameter spheres in water at room temperature, where $\eta = 0.01$ g cm/s are 1.1×10^{-7} and 4.4×10^{-8} cm²/s, respectively. We measured 2D sphere displacement in Brownian motion where the 3D movement was projected onto 2D using the CCD camera on the upright microscope. Measurements were conducted on bulk diffusing spheres in the proximity (<1 μ m) of the glass/aqueous interface. Results agreed with the Einstein-Smoluchowski relation for the 100 nm spheres, but gave a diffusion constant $\sim 1/2$ that expected for the 40 nm spheres. The measurements suggest the 40 nm spheres were aggregates or their movement was inhibited by transient adsorption to the glass interface.

We chose a $\delta = 2$ nm step size that is more than 20-fold smaller than any linear dimension in our system. Characteristic times, $\tau = \delta^2/2D$, are ≤ 1 μ s and $\sim 10^4$ fold shorter than any time-dependent measurement we can make. The real sphere executes multiple random steps for each simulated step; however, the dimensions of the system and the time frame of our measurements prohibit any detectable effect from this computational short cut. Simulations provided time-dependent trajectories of the sphere center coordinate ($x_0(t)$, $y_0(t)$, $z_0(t)$).

The evanescent field intensity, I , on the aqueous side of the glass/water interface is given by

$$I = I_0 \exp\left[-\frac{z}{d}\right] \quad \text{where } d = \frac{\lambda}{4\pi \sqrt{(n_g \sin \theta)^2 - n_w^2}}, \quad (1)$$

and for I_0 the evanescent field intensity at $z = 0$, exciting beam incidence angle θ , exciting light wavelength λ , and the refractive indices for glass and water n_g and n_w . Values appropriate for our application are $\theta = 87.7^\circ$, $\lambda = 488$ nm, $n_g = 1.516$, and $n_w = 1.334$, giving $d \approx 54$ nm. Fluorescence intensity, F , emitted from a finite-size sphere is proportional to excitation intensity [I in Eq. (1)] times the fluorophore spatial density integrated over the sphere volume. Excitation intensity from the evanescent field varies over the spatial dimensions of the sphere. When fluorophores are uniformly distributed on the sphere surface

$$F = F_0 \exp\left[-\frac{z_0}{d}\right], \quad (2)$$

where F_0 is a geometric factor varying with sphere size and normalized arbitrarily to be 1 for a point source. Finite-size spheres have $F_0 = 1.02$ for $S = 20$ nm and 1.15 for $S = 50$ nm. Equation (2) defines the z dimension of V_d as penetration depth d . Other dimensions of V_d are defined by the resolution limit or the CCD camera pixel size, whichever is larger. We performed quantitative sphere diffusion measurements with the upright microscope, where the resolution limit for the objective matches 2×2 binned pixels for the Orca ER corresponding to ~ 220 nm. V_d is a thin slab with height given by d and base dimensions identically 220 nm. The simulations show that the characteristic time for sphere diffusion through V_d is highly sensitive to d .

We observed fluorescent nanospheres within V_d using aoTIRF. We recorded and characterized the brightest transient fluorescent events for a given sphere size and light collection interval as they diffuse through V_d . The brightest events characterize the sphere position optimal for light collection that occurs when the sphere is adjacent to the interface. The simulated trajectories were selected to include at least one instance where sphere position is adjacent to the interface. We estimated the expected intensity using Eq. (2), with $z_0(t)$, for $x_0(t)$ and $y_0(t)$ within a single pixel, and F summed over a time segment equal to the light collection interval.

3. Results

A. Sphere Diffusion Through V_d

Figure 8(a) shows three sequential images of 100 nm diameter fluorescent spheres under aoTIRF illumination in the upright microscope. The images are 20 ms exposures of the CCD camera to light but are separated in time by 59 ms due to overhead in the imaging software (Slidebook, Intelligent Imaging Inc., Denver, Colorado). The images show the transient appearance of a diffusing sphere in the evanescent field. The fluorescent sphere in the lower part of each panel is fixed to the interface and indicates the fluorescence intensity for a sphere constantly in the most intense region of the evanescent field. The brightest pixel for the stationary sphere has 641 counts for the 20 ms collection time. The brightest pixel for the transient sphere in the upper right corner of the middle panel has 214 counts for the same collection time. The transient events observed characterize the brightest events for the diffusing sphere. The average background is ~ 16 counts. Typically ~ 20 single-sphere events like that depicted in Fig. 8(a) were observed over 250 exposures in the time-lapse movie of spheres diffusing through V_d . Table 1 indicates the statistics for the events and their comparison to

simulation. Similar experiments were carried out for 40 nm diameter fluorescent spheres, with results also summarized in Table 1. Observed and simulated characteristics agree favorably.

Figure 8(b) shows three sequential images of 100 nm diameter fluorescent spheres under aoTIRF illumination in the inverted microscope configuration. Exposure time was 100 ms on the EMCCD camera and frames are separated in time by 135 ms. Spheres in the upper left and lower portion of the frames are stationary. The transient event is in the middle of the frame. The intensity of the transient sphere somewhat exceeds that of the stationary spheres, probably because the stationary spheres are photobleached due to exposure to light or because the transient is from an aggregate of spheres. Other stationary spheres in the FOV (not shown) match the transient sphere intensity. The inverted microscope configuration would benefit from the routine use of quartz glass prisms and coverslips, as is the practice with conventional pTIRF [24].

Figure 8(c) is epi-illumination of the bulk solution of 40 nm diameter spheres observed in the upright microscope configuration. The images are 10 ms exposures of the CCD camera to light, but are separated in time by 80 ms. This sphere remained within the ~600 nm objective depth of focus for >400 ms. The epi-illumination time-lapse images contrast sharply with the aoTIRF images, where spheres were not observed in sequential frames.

B. Back Focal Plane Imaging of an Interface Immobilized Sphere

Single 100 nm diameter fluorescent spheres immobilized on the TIR interface and in diffusive contact with identical spheres are illuminated under aoTIRF with emission spatial distribution observed at the BFP using the setup depicted in Fig. 7. As pointed out by Mattheyses and Axelrod, the microscope objective maps a ray propagation angle from an in-focus source into off-axis radial positions at the BFP [25]. The BFP intensity pattern from a high-aperture objective separately maps far- and near-field emission. Patterns from emitting sources close to the interface show an intense circular band where critical angle light intersects the BFP. Light falling outside the band is SAF, and light falling within the band is UAF [14]. We interpreted the BFP pattern, emitted from a dipole excited by oTIRF illumination, in terms of fluorescent dipole orientation and distance from the interface [12].

Figure 9 shows the BFP image from a 100 nm diameter sphere emitting fluorescence excited by elliptically polarized aoTIRF illumination (top) and the pattern computed from a single emitting dipole lying 50 nm above the interface and pointing horizontally along the x axis direction (bottom). The 100 nm sphere lies on the planar glass/aqueous interface with its center at 50 nm above the interface. The computed BFP patterns have reflection symmetry through the x axis at the junction of the two images; hence, we can compare patterns with the half-plane representation. The aoTIRF excitation is p -polarized in the incidence plane with the evanescent electric field intensity polarized ~78 or 22% normal or parallel to the interface. Excitation light propagates along the interface parallel to the x axis with polarization either normal or along the x axis. The emission pattern most closely resembles the pattern expected for dipoles aligned to the parallel electric field. Dipoles aligned normal to the interface would produce a BFP pattern with circular symmetry.

Critical angle emission occurs at the bright narrow band identified in the computed pattern in Fig. 9 at the arrow. The band is apparent but less distinctive in the observed BFP pattern, probably because most of the emitting dipoles on the sphere are closer than 50 nm due to the narrow penetration depth of the excitation evanescent field. Critical angle emission separates UAF and SAF emission. We see that the fluorescent sphere emits in both regions due to its proximity to the interface. Artifacts are produced in the sphere emission pattern at 3 and 9 o'clock by scattered light. Figure 6 shows that laser light scattering is larger just at the edges of the objective lens, where there is a material discontinuity from the brass of the objective

body to the glass of the lens. This light enters the objective by propagating roughly parallel to the optical axis and is focused to two spots near the BFP edges but within the UAF emission region. Background subtraction to form the image at the top of Fig. 9 removes most of the autofluorescence excited in the objective by the focused scattered light, but the artifact is visible. The autofluorescence is smaller than that produced by oTIRF because emission is excited by a tiny fraction of the exciting light entering the objective in aoTIRF compared to the total intensity of the exciting beam focused at the BFP in oTIRF. The BFP is within a glass element of the Olympus objective, producing a very bright spot under oTIRF excitation in the SAF region, prohibiting sample fluorescence detection.

C. aoTIRF Imaging of a Cardiac Papillary Muscle Fiber

The striated pattern characteristic to transmitted light microscopic images of skeletal and cardiac papillary muscle fibers comes from the interdigitated myosin and actin filaments, where thick filament bands are due to myosin scattering of transmitted light. The muscle sarcomere is the elementary contractile structure consisting of thick filaments and opposed actin filaments. The thick filament is bipolar, with myosins segregated to align similarly along the $+z$ axis and in the reverse polarity along the $-z$ axis when the thick filament center is at $z = 0$. Actin filaments are unipolar but oppositely directed within the sarcomere, where they are connected to the Z-disks defining the sarcomere boundary. Actin and myosin polarity is complementary within the sarcomere, permitting the actomyosin interaction to shorten the sarcomere during contraction. Myosin contains the enzymatic ATPase activity in contracting fibers that isolates to the myosin head domain, usually called the cross-bridge in the fiber. The cross-bridge also contains the actin binding site.

Epi-illumination fluorescence from myosin cross bridges labeled with a chromophore also appears striated. Contrast between light and dark regions depends on the depth of focus. Epi-illumination bright-field and confocal images of the striated muscle fiber have lower and higher contrast levels between light and dark regions. The shorter depth of focus in the confocal image removes out-of-focus light, giving the greater contrast. oTIRF images have a subdiffraction limit depth of focus and surpass the contrast of the confocal images. We showed that inserting a thin metal film at the glass/aqueous interface in oTIRF further narrows the detection volume depth from which fluorescence is detected [6].

Figure 10 shows images from cardiac papillary muscle fiber tissue preparations that have been chemically skinned to permeabilize the membrane. Fluorescence is detected from RLC-GFP specifically replacing native RLC on myosin cross bridges in the muscle fibers. The muscle fibers are in the relaxed state where cross bridges spend the predominant amount of their time detached from the actin filament. Figures 10(a) and 10(b) are the same GFP-tagged cardiac papillary muscle fiber under epi-illumination and aoTIRF illumination. Figure 10(c) is from another cardiac papillary fiber under oTIRF. All images were taken with the inverted microscope.

Figures 10(a) and 10(b) differ due to the depth of focus. In epi-illumination, wide-field excitation light propagates from the objective and transmits the sample. Emitted fluorescence is collected from excited dipoles within the focal depth of the high NA objective and detected by the CCD camera. The ~ 600 nm focal depth gives an image formed from emitted light from a relatively thick section of tissue. Relative myosin filament lateral displacements in shallow versus deep sections of the fiber tend to lower contrast between the thick and thin filament regions. The ~ 50 nm deep aoTIRF evanescent excitation images myosin filaments in a thinner section, where they are in better register. The image has larger contrast between the thick and thin filament regions; however, the shallow illumination produces less total fluorescence because fewer dipoles are excited. The signal-to-noise ratio is not as favorable as the oTIRF image [Fig. 10(c)], where the evanescent excitation intensity is ~ 100 nm deep, but contrast

between light and dark bands is larger in the aoTIRF versus oTIRF images. The *M*-line is a myosin cross-bridge depleted part of the thick filament, where myosin tails reverse direction to enable myosin cross bridges to pull actin filaments toward the thick filament center. It is a narrow dark line in the center of the thick filament image and subtly discernable in both the oTIRF and aoTIRF images.

The cardiac papillary muscle fiber specimen contains more connective tissue than the similarly prepared skeletal muscle fiber. Nonfluorescent connective tissue displaces the cardiac fiber from the glass/aqueous interface, making tight contact between fiber and glass more spatially intermittent. The brighter stripe in the center of the middle panel is from a small section of myosin containing specimen in closer contact with the interface. Skeletal muscle fibers have less connective tissue and make a tighter contact with the glass/aqueous interface over the entire field of view than can be seen in previously published work [6].

D. aoTIRF with Other Infinity-Corrected Objectives

Diffusing spheres were observed under aoTIRF excitation using a Zeiss 100× NA = 1.45 infinity-corrected objective on the upright microscope. The objective external dimensions are consistent with application of aoTIRF and the approach-limiting edge is more accommodating because distance *R* (Fig. 2) is smaller at 3 mm and the objective working distance *W* is slightly larger at 0.11 mm. Under aoTIRF, we observed diffusing spheres as intense light pulses surrounding steady intensity from stationary spheres attached to the glass in the glass/water interface of the illuminated region. The phenomena matched observations from the Olympus objective.

4. Discussion

TIRF imaging of biological samples is widely used to take advantage of the subdiffraction limit focal depth (*z*-dimension) resolution provided by the evanescent field. This feature provides a substantial improvement over confocal fluorescence imaging *z*-dimension spatial resolution. oTIRF is popular in microscopy applications because modern high-NA objectives make oTIRF simpler to implement than the older pTIRF setup. The oTIRF and pTIRF setups are suitable for both upright and inverted microscopy, but are distinguishable by complementary advantages and disadvantages. Practical application of oTIRF requires the highest NA objectives available ($NA \geq 1.4$) that are SAF collection capable ($NA > 1.3$). However, the NA restricts the maximum incidence angle of the excitation light to 73° – 79° for NA of 1.45–1.49 objectives. In practice, incidence of the exciting laser beam is less than the maximum to allow sufficient energy to reach the sample. Autofluorescence in oTIRF is due in part to emission from glass in the objective transmitting the high-intensity focused excitation light and is directly observable in BFP imaging from oTIRF excited samples. The pTIRF setup is not limiting for exciting light incidence angle or by autofluorescence that cannot be reduced with use of quartz prisms and coverslips [26]. Nonetheless, pTIRF as normally implemented does not detect SAF because emission is collected via a water immersion objective. aoTIRF circumvents the loss of SAF in a prism-based TIRF setup by delivering exciting light and collecting emitted light from the glass side of the interface in optical contact with the microscope objective. Exciting light propagates to the interface for TIR externally to the objective, thus avoiding the largest source of un-preventable autofluorescence. Implementation of aoTIRF provides the minimum evanescent field depth (~50 nm), but does not permit a practical ability to alter field depth by changing exciting light incidence angle.

The aoTIRF setups were demonstrated for the upright and inverted microscope configurations (Figs. 1-3). The upright microscope configuration is simpler to implement because it is more tolerant of variation in the geometric arrangement of the apparatus. The Littrow prisms can be the smaller or larger variety (12.5–38 mm base) and just one glass coverslip thickness (#1) is

needed. The inverted microscope configuration is complicated by the triple reflection, requiring two glass coverslip thicknesses (#1 and #2). Care is needed to maintain the tolerances in the geometric arrangement of the apparatus, as shown in Fig. 3. The inverted microscope setup is carefully constructed, but everything can be done reproducibly by hand. For either configuration, we found that 60–75 mm long #1 coverslips (available from Electron Microscopy Sciences, Hatfield, Pennsylvania) were most practical because they enable the two prism configuration, where the exiting laser beam is coupled out of the coverslip in the same manner as the entering laser beam was coupled into the coverslip. The exiting light produces the clean intensity profile of a totally internally reflecting beam on a white card placed a few feet from the microscope. Achieving a clean exit intensity profile provides a good guide for correctly conditioning the entering beam with the beam diameter reducer and focusing lens (Fig. 1).

On first implementing aoTIRF, it is desirable to practice on the fluorescent spheres sample to demonstrate that the exciting beam is truly undergoing TIR. Stray scattering can contaminate the FOV with an improperly conditioned exciting beam. This practical difficulty is easily identified and eliminated by imaging the stationary and diffusing fluorescent spheres (40 or 100 nm diameter). The stationary spheres are firmly adsorbed to the interface by allowing a very dilute bulk sample to evaporate within the sample chamber. The stationary spheres provide the target for focusing the microscope objective, and they contrast with the diffusing sphere transients observed when the more concentrated bulk sample of spheres is placed in the sample chamber. Under aoTIRF, diffusing spheres are rapidly blinking lights surrounding the stationary sphere images. Quantitative measurements (like we did) can be done; however, the diffusing sphere fluorescence transients under aoTIRF are obviously different from those produced by stray light excitation.

Light scattering and the autofluorescence it excites are a part of the background that we seek to minimize. Autofluorescence in pTIRF is mitigated by use of quartz substrates and low-fluorescence immersion fluids. In oTIRF, intense propagating excitation light excites background autofluorescence from the glass elements in the objective. The aoTIRF setup forgoes propagating excitation light through the objective. Autofluorescence from excitation light scattering collected by the objective was detected while imaging the BFP under aoTIRF illumination; nonetheless, there was a significant reduction in autofluorescence when comparing BFP images in aoTIRF versus oTIRF. In oTIRF, the focused excitation laser light at the BFP excited autofluorescence that was too bright compared to the emitting sample to permit imaging. Figure 9 is the BFP image produced under aoTIRF.

We tested aoTIRF on two high-aperture oil immersion objectives. They provided equivalent results despite their different external dimensions. We found that, for common oTIRF objectives, the chief difficulty in applying aoTIRF is accommodating the clearance to the objective focal point for the exciting laser beam defined by the approach-limiting edge. The clearance affects two factors that are limited by practical considerations. The distance R from the objective optical axis to the approach-limiting edge limits the allowable incidence angle of the exciting beam. The objective working distance W limits both incidence angle and the allowable width of the focused exciting beam. Permissive geometries have smaller R and larger W . The Olympus 60 $\times$ NA = 1.45 objective sets R = 3.8 mm and W = 0.1 mm as desirable minimums for these parameters.

A GFP-tagged cardiac papillary muscle fiber was imaged with aoTIRF. Results compare favorably with oTIRF images of similarly prepared tissues. The aoTIRF illumination has a somewhat higher contrast between light and dark bands, but has a lower signal-to-noise ratio for the image. Both effects are likely due to the lower evanescent field penetration depth under aoTIRF. The cardiac papillary fiber is a challenging tissue to image under TIRF because the

connective tissue associated with the preparations often tends to displace to fiber too far from the glass/aqueous interface to make contact with the evanescent field. Selected regions did make close contacts and were excited by the evanescent field.

5. Conclusion

We demonstrated a near-field excitation/emission TIRF microscopy technique that combines advantages of oTIRF with pTIRF. The technique has evanescent illumination of dipoles at a glass/aqueous interface with excitation light external to the microscope objective and collection of supercritical angle fluorescence from the excited dipoles. The approach eliminates background light due to the admission of the high-energy laser beam to the microscopic optics, as in oTIRF, while collecting near-field emission from the excited dipoles, as is impossible with water immersion objectives used in pTIRF. We call the approach around-the-objective TIRF (aoTIRF) because the evanescent field is created by a conditioned laser beam that propagates through the submillimeter gap created by the oil immersion high-NA objective collecting emitted light and the glass coverslip. Setups were developed for both upright and inverted microscope configurations. The aoTIRF technique was tested with 40 and 100 nm diameter fluorescent spheres in water that were diffusing into and out of the detection volume, with BFP imaging of single 100 nm fluorescent stationary spheres adsorbed to the glass substrate of the interface, and with imaging of cardiac papillary muscle fibers labeled with exchanged myosin RLC-GFP. Results confirm that the evanescent field has penetration depth of ~50 nm, that the background autofluorescence from the objective glass is significantly reduced, and that the technique is useful for imaging biological samples.

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Fig. 1. Schematic aotIRF diagrams for (a) upright and (b) inverted microscope configurations. Dashed lines show the beam path through the prisms to the glass/aqueous interface where the exciting beam totally internally reflects. The inverted configuration has two other total internal reflections at glass/air interfaces. Coverslips and Littrow prisms are optically coupled by immersion oil. The upright microscope configuration also shows the beam conditioning elements consisting of a beam waist reducer from 10 $\times$ and 20 $\times$ microscope objectives and a low-aperture focusing lens. Beam conditioning is needed in both upright and inverted microscope configurations but is shown only for the upright.

Fig. 2.

Detailed schematic aotIRF upright microscope configuration. Symbols defining distances or angles are used in the text. Distance C is ~ 0.15 mm for a #1 coverslip. The approach-limiting edge requires $\theta_2 \leq 3$ deg and is the strictest constraint on laser beam shape. The upright configuration works with both small and large base Littrow prisms. Part of the objective is shown with the incident laser beam path. The exiting laser beam pathway is a mirror image of the incident path with reflection through the objective symmetry axis.

Fig. 3.

Detailed schematic aotIRF inverted microscope configuration. Symbols defining distances or angles used in Fig. 2 are equivalently defined here. Numeric distances are given in millimeters. Distance B is ~ 0.21 mm for a #2 coverslip. Oil is confined by the objective, the lower edge of the #1 coverslip, the short edges of the #2 coverslips, and the oil retainers. The inverted configuration requires the large base Littrow prisms. The exiting laser beam pathway is a mirror image of the incident path with reflection through the objective symmetry axis.

Fig. 4.

Two views of the exciting laser beam intensity profile (rippled surface) and (FOV plane for the microscope objective near the focal plane for the exciting light. The x_p and y_p axes lie in the focal plane of the exciting light with z_p in the direction of light propagation and y_p in the incidence plane at the glass/aqueous interface. The vertical axis is intensity in arbitrary units for the intensity profile and micrometers for the (a) y_p or (b) x_p spatial dimensions.

Fig. 5.

The exciting laser beam cross section for various z_p values, where the z_p axis is parallel to the direction of light propagation, and the $1/e^2$ intensity level. The beam full width at $z_p = 3.8$ mm must be $<250 \mu\text{m}$ for the exciting light to propagate past the approach-limiting edge without producing excessive light scattering.

Fig. 6. Close up photographs of the (a) upright and (b) inverted aoTIRF configurations. The slightly red pencil of light inside the prisms is the 488 nm laser beam exciting autofluorescence.

Fig. 7.

The BFP microscopy emission pathway optical setup where OP is the object plane, OBJ is the objective, BFP is the back focal plane, TL is the tube lens with focal length f_{TL} , RL is the chromatic and spherical aberrations corrected removable lens, and CCD is the image plane for the camera.

Fig. 8.

(a) Sequential images of 100 nm diameter fluorescent spheres under aoTIRF illumination in the upright microscope configuration. The images are 20 ms exposures of the CCD camera to light but are separated in time by 59 ms. (b) Sequential images of 100 nm diameter fluorescent spheres under aoTIRF illumination in the inverted microscope configuration. Exposure time was 100 ms on the EMCCD camera and frames are separated in time by 135 ms. (c) Sequential images of 40 nm diameter spheres diffusing in bulk solution under epi-illumination and observed in the upright microscope configuration. The images are 10 ms exposures of the CCD camera to light but are separated in time by 80 ms. Scale bars are shown for each panel.

Fig. 9.

The BFP image from a 100 nm diameter sphere emitting fluorescence excited by aoTIRF illumination (top half) and the pattern computed from a single emitting dipole lying 50 nm above the interface and pointing horizontally along the x -axis direction (bottom half). The arrow at the bottom of the figure designates the bright ring due to critical angle emission.

Fig. 10.

Fluorescence images of cardiac papillary muscle fibers. Fluorescence is detected from GFP tagged myosin regulatory light chain specifically replacing the native regulatory light chain on myosin cross bridges. Images in (a) and (b) compare the same sample but under epi-illumination and aoTIRF illumination. (c) is from another cardiac papillary fiber under oTIRF. All images were taken with the inverted microscope and the scale bar applies to all panels.

Table 1

Event Statistics Observed for Nanospheres Diffusing in Water at Room Temperature

Sphere Radius <i>S</i> (nm)	Mean Relative Intensity $\pm$ SD		Light Collection Interval (ms)	Event Count
	Observed	Simulated ^a		
20	0.42 $\pm$ 0.11	0.46 $\pm$ 0.02	10	54
50	0.26 $\pm$ 0.04	0.25 $\pm$ 0.02	20	30

^a Simulated using the random walk algorithm described in Section 2.