

Clearer view for TIRF and oblique illumination microscopy

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Abstract: In Total Internal Reflection Fluorescence (TIRF) microscopy, the sample is illuminated with an evanescent field that yields a thin optical section. However, its widefield detection has no rejection mechanism against out-of-focus blur from scattered light that can compromise TIRF images. Here I demonstrate that via structured illumination, out-of-focus blur can be effectively suppressed in TIRF microscopy, yielding strikingly clearer images. The same mechanism can also be applied to oblique illumination schemes that extend the reach of TIRF microscopy beyond the basal surface of the cell. The two imaging modes are used to image a biosensor, clathrin coated vesicles and the actin cytoskeleton in different cell types with improved contrast.

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OCIS codes: (180.2520) Fluorescence microscopy; (120.3180) Interferometry; (100.2960) Image analysis; (100.3010) Image reconstruction techniques; (000.1430) Biology and medicine;

References and links

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1. Introduction

Total Internal Reflection Fluorescence (TIRF) microscopy is an indispensable tool in cell biology [1]. By combining widefield detection, high numerical aperture lenses, and optical sectioning with evanescent fields, TIRF enables very sensitive imaging with high signal to background ratios. While TIRF is limited to studying the plasma membrane adjacent to the glass coverslip, an objective launched system is also compatible with highly-inclined and laminated optical sheet (HILO) microscopy, with which the entire cell volume can be interrogated [2, 3].

In practice, the optical sectioning capability in TIRF is reduced by far-field excitation, which can be caused by imperfections in the optical train of the instrument and light scattering of the evanescent field by the sample itself [4, 5]. This can lead to fluorescence excitation well outside the depth of focus of the detection objective. Since fluorescence is detected in a widefield format, which features a missing cone in its optical transfer function (OTF), out-of-focus blur cannot be rejected. In densely labeled cells, such out-of-focus blur can significantly reduce the contrast of the final TIRF image and hamper quantitative image analysis. Further, two-dimensional structured illumination microscopy (SIM) combined with TIRF is prone to reconstruction artifacts when out-of-focus blur is present, which has recently triggered a heated debate about SIM [6, 7].

In HILO microscopy, the illumination light is propagating at a shallow angle to the focal plane. Therefore, the beam will only coincide with the focal plane over a small field of view and out-of-focus excitation is inevitable. Further, if a conventional TIRF setup is used for HILO illumination, the waist of the illumination beam will exceed the depth of focus of the detection objective, leading to further out-of-focus fluorescence excitation.

So far, suppression of out-of-focus blur in TIRF microscopy has been achieved with either two photon excitation [8] or supercritical angle fluorescence (SAF) detection [9]. Two photon fluorescence requires high peak excitation intensities, restricts the choice of suitable fluorophores and increases the cost of a TIRF setup. SAF in turn reduces the detection efficiency of the instrument and modifies its point spread function, as a large portion of the backfocal plane of the objective has to be blocked [10]. Some of these issues have been

addressed with confocal [11] or virtual SAF [12, 13] in more recent work. To the best knowledge of the author, no method has been published to suppress out-of-focus blur in HILO microscopy.

Here, structured illumination [14, 15] is used to suppress out-of-focus blur in TIRF and HILO (referred to as sTIRF and sHILO, respectively) microscopy while keeping the advantages of widefield detection. By illuminating the sample with a coarse interference pattern, the missing cone of the OTF can be filled and thereby out-of-focus blur is rejected [14]. The two new imaging methods are demonstrated on biological imaging of PI 3-kinase biosensor localization, clathrin coated vesicles and the actin cytoskeleton in cancer cells.

2. Theory

Figure 1(a) illustrates light scattering in a conventional TIRF setup. The evanescent field travelling along the coverslip surface undergoes scattering by cellular features (e.g. dense organelles), resulting in photons that can propagate into the far field and can excite fluorescence outside of the evanescent field. Since TIRF is a widefield technique, the detection OTF features a missing cone, which provides no rejection for out-of-focus light. When illuminating the sample just slightly below the critical angle, which is referred here as oblique illumination or HILO, the sample is illuminated by a propagating, non-evanescent electromagnetic field which in a first approximation linearly increases in z -depth with propagation distance [Fig. 1(b)]. The increased illumination depth as well as light scattering can generate out-of-focus blur in HILO techniques.

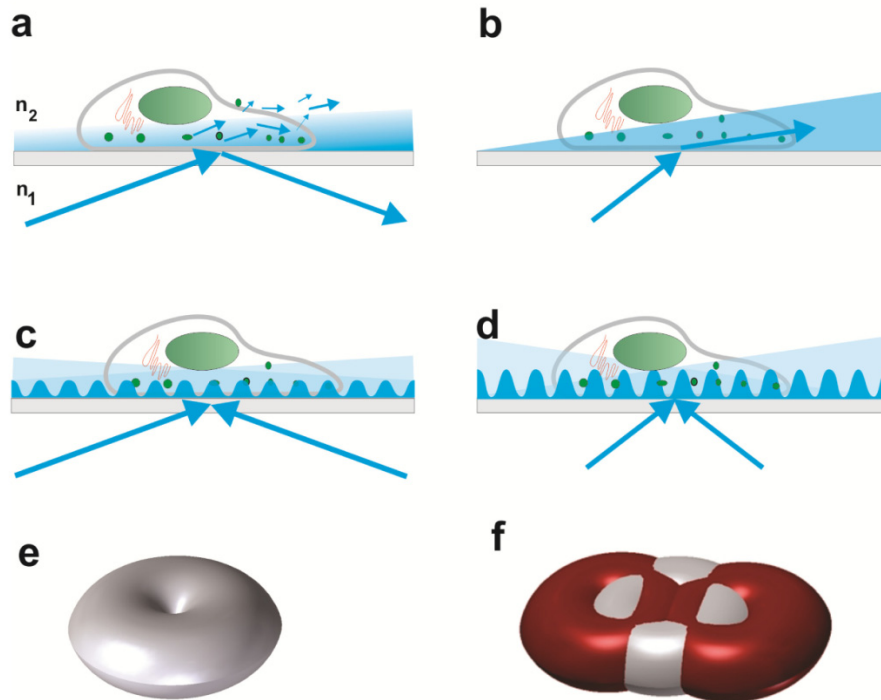


Fig. 1. Principle of sTIRF and sHILO. (a) Light scattering in TIRF microscopy. n_1 and n_2 : refractive indices of glass and water, respectively (b) oblique illumination below the critical angle. (c) Evanescent interference pattern in sTIRF. (d) Interference pattern in sHILO. (e) optical transfer function (OTF) for widefield detection. (f) Filling of the missing cone of the detection OTF with structured illumination.

By illuminating the sample with an evanescent [sTIRF, Fig. 1(c)] or farfield [sHILO, Fig. 1(d)] standing wave with a spatial frequency of half the diffraction limit, additional spatial

information of the sample is encoded that that can be reassigned in reciprocal space to fill the missing cone [Figs. 1(e)-1(f)]. Importantly, while Fig. 1(e) and 1(f) show three-dimensional OTFs, improved optical sectioning, realized by ‘filling-in’ the missing cone, applies to two-dimensional image slices as well [16].

While sTIRF may look similar to structured illumination microscopy (SIM) applied to TIRF microscopy [17–19], the illumination patterns serve a different purpose. In TIRF-SIM, the finest possible line spacing is applied to enlarge the OTF support and thereby boost lateral resolution. However, the resulting overall OTF has still a missing cone and hence no rejection of out-of-focus blur occurs, which in turn can lead to reconstruction artifacts [7]. Here, a coarse illumination pattern is employed to fill the missing cone, whereas lateral resolution is mainly unaffected [14].

3. Methods

3.1 Experimental setup

In previous setups for structured illumination, the line spacing of the illumination pattern was controlled by the incidence angle of the interfering laser beams [14, 15]. However, in TIRF microscopy, the incidence angle needs to exceed the critical angle and hence cannot be freely chosen. Instead, in sTIRF the opening angle between the propagation vectors of the two evanescent fields is varied to produce the desired line spacing of the interference patterns, as shown in Fig. 2(a). Similarly, the incidence angle in sHILO remains fixed and the line spacing of the interference pattern is also controlled by the opening angle.

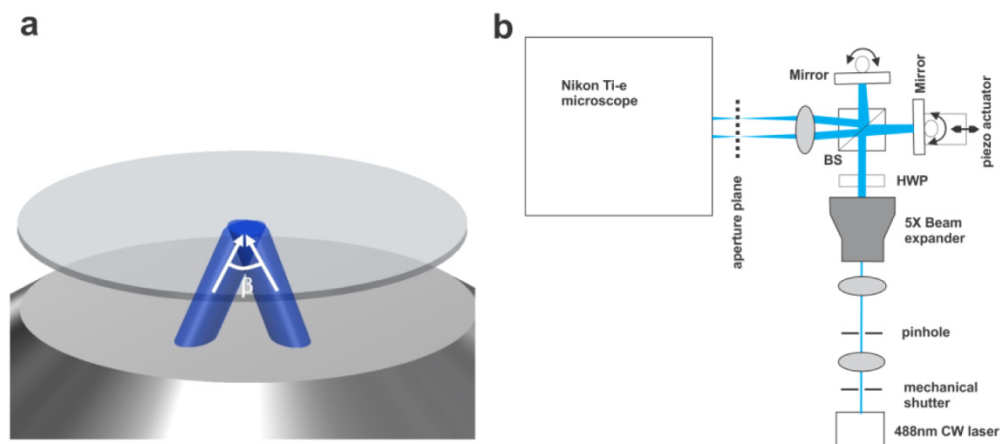


Fig. 2. Experimental setup. (a) Experimental realization of sTIRF and sHILO using a high NA objective. A rendering of the objective and the two laser beams that are incident on the coverslip is shown. β denotes the opening angle of the two beams projected into the lateral sample plane. (b) Schematic drawing of the microscope setup. HWP: half waveplate. BS: beam splitter.

A schematic representation of the optical setup of sTIRF and sHILO is shown in Fig. 2(b). The sTIRF/sHILO microscope consists of a Nikon TI-E microscope and a homebuilt module that attaches to the backport of the microscope. Laser light from a solid state CW laser (Coherent Sapphire 488-300CW) is spatially filtered with a pinhole and expanded fifteen fold. The laser beam enters a Michelson type interferometer [20] and the returning two beams are focused into an aperture plane of the Nikon microscope. Phase stepping is achieved by moving one of the mirrors with a piezo stage (Physik Instrumente Hera P621-1CD). The relative phase steps were empirically adjusted to $2\pi/3$. The interference patterns are projected into the sample plane by a Nikon NA 1.45 100X objective. The incidence angle and azimuthal orientation of each beam can be manually controlled by tip-tilt mirrors in each arm

of the interferometer. Each of these mirrors is located in a conjugate image plane of the microscope. Thereby the angle of each beam can be modified while illuminating the same field of view and guaranteeing beam overlap in the sample plane. Fluorescence light is collected by the same objective, is separated from the excitation light by a dichroic mirror and a bandpass filter and is imaged via a side port onto a Hamamatsu Flash V4.0 scientific CMOS camera.

The line spacing of the interference pattern in sTIRF was adjusted with the opening angle β [see Fig. 2(a)] of the two evanescent fields and was set to 500-550nm between experiments, roughly twice the FWHM of an experimentally measured PSF (274 ± 6 nm) for green emission. Assuming 488 nm and an incidence angle of 66 degrees, this corresponds to an opening angle β of 44-55 degrees for 500 nm and 550 nm line spacing, respectively.

Polarization control was achieved with a half waveplate. Linear polarization was measured in the back focal plane (objective removed) with a linear polarizer to account for additional de-polarization by the dichroic mirror. The two beams were aligned such that both were close to s-polarized (both have a small p-polarization component as the polarization was not adjusted independently for each beam).

3.2 Reconstruction

sTIRF and sHILO images were reconstructed in Fourier space in a similar way as in two-dimensional structured illumination microscopy [21]. Structured illumination gives rise to the following spectral information: one object spectrum that is identical with standard widefield microscopy, which is here referred to as the DC band. Further, two spectral copies that are shifted by $+$ and $-$ the illumination pattern vector (whose magnitude equals to the pattern frequency) in respect to the origin of reciprocal space, which are here referred to as sidebands. The two sidebands contain low frequency Fourier components that were acquired with high optical sectioning strength and are here used to replace those Fourier components in the DC band that have been transferred with no sectioning strength due to the missing cone.

The illumination parameters (pattern frequency and relative phase steps) were measured directly from the raw data by analyzing the Fourier components of the illumination pattern [22]. This is possible since the coarse illumination pattern is transferred by the OTF with sufficient strength such that its Fourier components show up as distinct peaks in reciprocal space.

With the knowledge of the relative phase steps between the three images, the DC band and the two frequency shifted sidebands were separated pixel per pixel by solving a linear equation system [21], an operation that here will be referred to as “unmixing”. The two separated sidebands were then shifted numerically to their proper place in Fourier space. In a patch around the origin of reciprocal space, the absolute phase angle between the DC band and sidebands was determined and the angle difference was multiplied on the sidebands.

The object spectrum is reconstructed in 2D Fourier space as schematically sketched in Fig. 3(a)-3(e). Within a circle of $K_C/4$, with K_C being the cut-off frequency of the OTF, only the sum of the two sidebands is used, with the corresponding weighting mask shown in Fig. 3(c). In practice, $K_C/4$ was defined as half the frequency of the chosen interference pattern. Outside of this circle, only the Fourier components of the DC band were used. The corresponding weighting mask is shown in Fig. 3(d).

Theoretically, at a spectral radius of $K_C/4$, both the DC band and the sidebands should have the same optical sectioning strength and therefore the same Fourier components [see also Fig. 3(a)]. A thin annulus centered around $K_C/4$ was used to measure the relative strength of the DC band and the summed sidebands. The resulting scalar scaling factor m was then multiplied on the sideband components to equalize their strength to the DC band. Thereby the final object spectrum, schematically shown in Fig. 3(e), has a smooth transition at $K_C/4$ from the sideband components to the tail of the DC band.

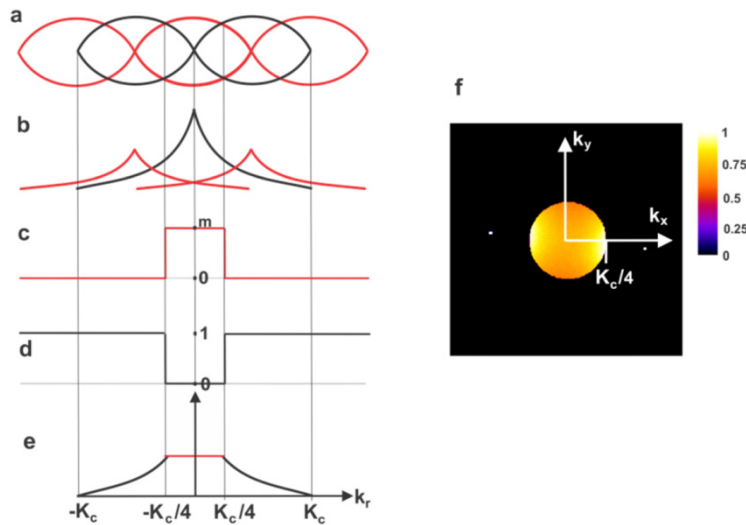


Fig. 3. Assembly of spectral information. (a) 3D OTFs of the DC band (black) and the two sidebands (red). (b) 2D projections of the OTFs. (c) Weighting mask for the two sidebands. m is a scalar scaling factor determined around $K_c/4$. (d) Weighting mask for the DC band (widefield spectrum). (e) reconstructed sample spectrum. Both weighting masks and final spectrum are rotationally symmetric around the optical axis. k_r is the radial spatial frequency coordinate and K_c the cut-off frequency of the optical transfer function. (f) Experimentally measured OTF for the summed sideband components within a circle limited by $K_c/4$. Color encodes the relative strength of the summed OTF. The two white dots indicate the Fourier components of the illumination pattern at $K_c/2$, which are the centers of the shifted sidebands and their corresponding OTFs.

A reconstructed image was obtained by an inverse fast Fourier transform of the assembled object spectrum. No apodization mask or deconvolution was applied to the reconstructed spectrum. It has to be noted that by adding the two sidebands together, a relatively uniform transfer function within the circle of a radius of $K_c/4$ results. This is shown in Fig. 3(f), where an experimentally measured OTF was used to estimate the combined transfer function: the raw OTF was centered on the corresponding peak of the illumination pattern, which is the center of the corresponding sideband. The two shifted OTFs were then summed within a circle with a radius of $K_c/4$.

The relative uniformity of the combined OTF within this circle is due to the fact that the individual transfer functions within that area are essentially wedge shaped, but mirrored to each other. One could use the experimentally measured and shifted OTFs for deconvolution of the sideband components, but no visual gain in image quality resulted. Instead, deconvolution was also avoided in the DC component of the reconstruction (from a spectral radius of $K_c/4$ to K_c) in order to allow a fair comparison to conventional TIRF images. Furthermore, a “deconvolution-free” reconstruction avoids experimental variations in measuring the OTF. In addition, no Wiener constant, apodization function or other deconvolution parameters need to be determined, which makes the reconstruction less biased and more reproducible.

Notably, the two sidebands contain information that could be used to reconstruct sample information beyond the diffraction limit (up to a frequency of $1.5 K_c$), with a reconstruction scheme as outlined in Wicker et al [16]. I choose not to use this information in this manuscript to allow a more fair comparison to the conventional TIRF images and to present a much simpler reconstruction algorithm. It has to be expected though that the weighted averaging of the different spectral components described by Wicker et al would be beneficial in the case of low SNR data.

Conventional TIRF and HILO images were produced from the raw structured illumination data to allow comparisons of the cellular data at the same time points. To this end, an FFT of the DC band obtained from the unmixing operation (black curve in Fig. 3b) was taken without using any of the sideband information. Alternatively, for perfect 120 degrees phase steps, a widefield image can be obtained by summing all three structured illumination images [15]. The advantage of using the unmixed DC band instead is that one can account for phase step errors.

3.3 Sample Preparation

MV3 cancer cells were a kind gift from the Friedl lab at MD Anderson. A673 cancer cells were a kind gift from the Amatruda Lab at UT Southwestern, and were originally sourced from ATCC. hTERT-RPE1 cells were obtained from ATCC. Cells were cultured in DMEM supplemented with 10% FBS at 5% CO₂ and 37°C. Stable expression of the GFP-AktPH and GFP-tractin was achieved using lentivirus (Clontech). MV3 and A673 cells were seeded onto uncoated #1.5 glass coverslips approximately 18 hours before imaging. For the RPE cells, the same coverslips were coated with fibronectin. Rat IMCD (inner medullary collecting duct – a generous gift by Prof. Sanja Sever, Massachusetts General Hospital, Boston, MA) cells stably expressing EGFP-CLCa were generated through infection with retroviruses [23] coding for EGFP-CLCa in a pMIEG3 vector, followed by FACS sorting. Stable cells were grown under 5% CO₂ at 37°C in DMEM/Ham's F12 medium supplemented with 10 mg/ml streptomycin, 66 µg/ml penicillin and 10% (v/v) fetal calf serum (FCS, HyClone). For Total internal reflection fluorescence (TIRF) microscopy, cells were prepared as previously described [24]. U2OS cells were a kind gift from Dr. Dick McIntosh at University of Colorado, Boulder. Cells were maintained in DMEM supplemented with 10% FBS at 5% CO₂ and 37 °C. Cells were stably transfected with GFP-tractin using a lentiviral construct (Clontech). GFP-tractin positive U2OS cells were seeded onto 2 µg/ml bovine collagen coated #1.5 glass coverslips (Lab-Tek II) overnight before imaging.

4. Results

To test the optical sectioning capability of sTIRF, MV3 cancer cells labeled with AKT-PH-GFP, a biosensor for PI 3-kinase activity, were imaged. Figures 4(a)-4(c) show a comparison between conventional TIRF, linearly deconvolved (2D deconvolution using an experimentally measured OTF and Wiener filtering) TIRF and sTIRF, respectively. The sTIRF image shows a striking reduction in background level inside the cell and cell edges appear much sharper compared to both the conventional and linearly deconvolved TIRF images.

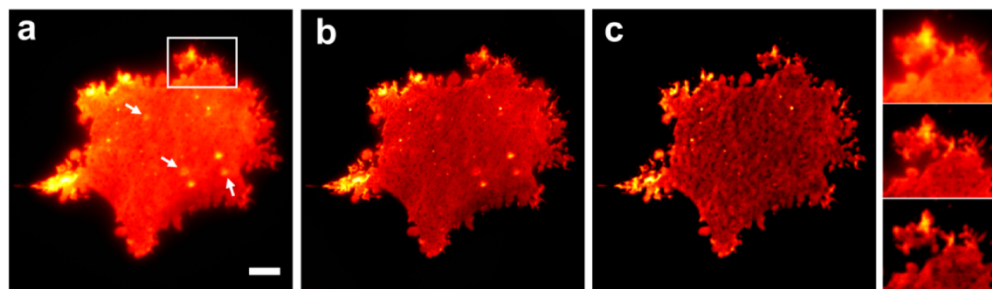


Fig. 4. Comparison of TIRF (a), linearly deconvolved TIRF (b) and sTIRF (c) by imaging an MV3 cancer cell labeled with AKT-PH-GFP, a biosensor for PI 3-kinase activity. Insets on the right show magnified views of the boxed region in (a). Arrows in (a) mark bright out-of-focus objects. Scale bar: 5 microns.

The linearly deconvolved TIRF image and the sTIRF image reveal fine intensity variations on the membrane, presumably representing the membrane topology, which are not

visible in the normal TIRF image. Several blurred spots inside the cell [three are indicated with arrows in Fig. 4(a)] are clearly visible in the conventional and deconvolved TIRF image, which may represent vesicles filled with the biosensor inside the cytosol. These bright out-of-focus particles are suppressed in the sTIRF image [Fig. 4(c)] owing to its increased optical sectioning capability. Thus although linear 2D deconvolution can increase the image contrast, it failed to remove out-of-focus blur. This is also evidenced in the residual blur around the cell edges, which can be seen in the insets in Fig. 4.

Such a biosensor is an extreme case, as the entire cytosol contains fluorophores, but it represents an important imaging application. However, sTIRF also improves the contrast on samples with less abundant labeling. Figures 5(a) and 5(b) show an RPE cell labeled with GFP-tractin, imaged with conventional TIRF and sTIRF, respectively. Bright spots may represent focal adhesions from which stress fibers protrude to the inside of the cell. The sTIRF image suppresses blur around dense focal adhesion clusters. Figures 5(c) and 5(d) show images of an A673 Ewing Sarcoma cell, labeled also with GFP-tractin.

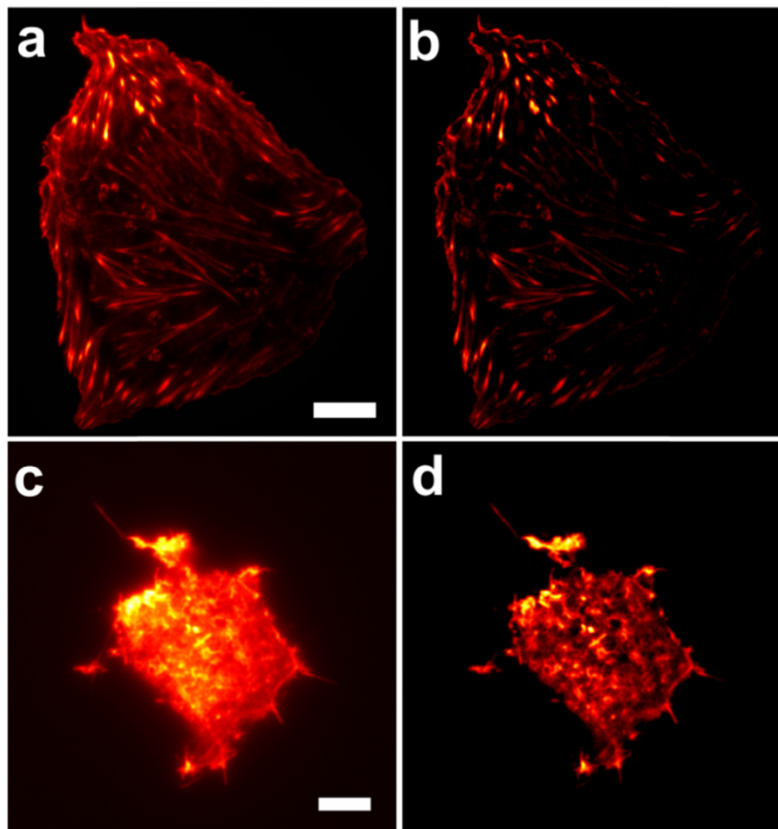


Fig. 5. Comparison of TIRF and sTIRF imaging of the actin cytoskeleton. A RPE cell labeled with GFP-tractin as imaged by TIRF (a) and sTIRF (b) microscopy. An A673 cancer cell labeled with GFP-tractin as imaged by TIRF (c) and sTIRF (d) microscopy. scale bars: (a) 10 microns, (b) 5 microns.

This particular cell appears to have adhered tightly to the glass coverslips and a dense actin network is visible, along with some image blur in the conventional TIRF image [Fig. 5(c)]. The sTIRF image [Fig. 5(d)] achieves higher image contrast and the cell boundary is much more clearly defined. As an example, the improved optical sectioning of sTIRF reveals that there is a gap between the main body and the large protrusion at the top, which is not resolved in the conventional image.

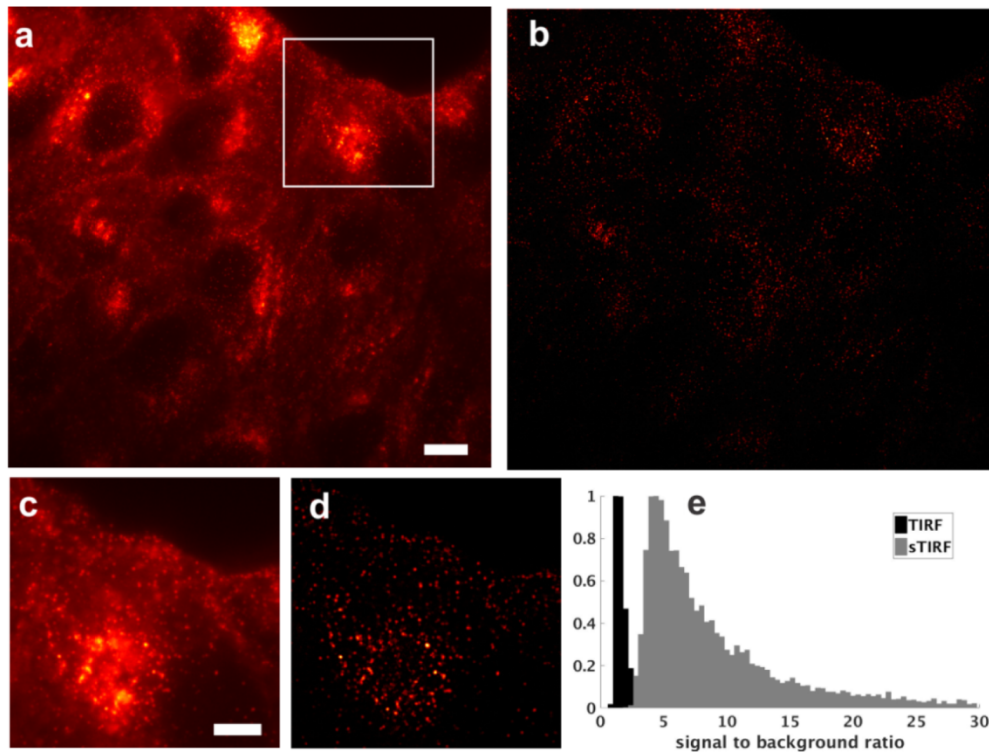


Fig. 6. Imaging clathrin coated vesicles in a monolayer of cells. IMCD cells labeled with EGFP-CLCa as imaged by TIRF [(a) and (c)] and sTIRF [(b) and (d)] microscopy. (c) and (d) show magnified versions of the boxed region in (a). (e) Normalized histogram of signal to background ratios of detected particles in (a) and (b). Image (b) has been gamma corrected with a gamma factor of 0.8 to better highlight weak features. Scale bars: (a) 10 microns, (c) 5 microns.

To quantify the improvements in image quality that result from the improved blur reduction in sTIRF, the signal to background ratio (SBR) of clathrin coated vesicles was measured in IMCD cells expressing eGFP-CLCa. The IMCD cells form densely packed, epithelial monolayers, as can be seen in Fig. 6(a). This made TIRF imaging challenging as the cytosolic background did not disappear owing to increased light scattering of the evanescent field. Here, sTIRF [Fig. 6(b)] drastically improved the contrast compared to the conventional TIRF image [see also Figs. 6(c)-6(d)]. Single particles were identified in the TIRF and sTIRF image using a locally adaptive detection algorithm described by Aguet et al [25]. For each detected particle the SBR was determined by dividing the peak intensity by the local background level. Non-fluorescent background, i.e. camera digitizer offset, was subtracted from the data before calculating this ratio. Figure 6(e) shows normalized histograms of the SBR of particles detected in TIRF (10111 detected particles) and sTIRF (8308 detected particles). The mode of the distribution (i.e. its peak) increased from an SBR of 1.5 in TIRF to 4.4 in sTIRF. The median SBR increased from 1.7 to 7.7 in TIRF and sTIRF, respectively.

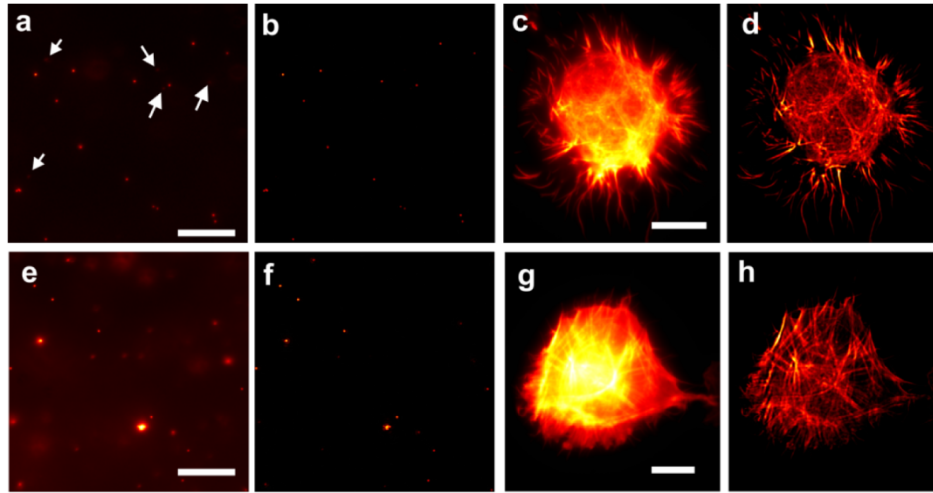


Fig. 7. Comparison of oblique illumination with and without structured illumination by imaging fluorescent beads in Agarose and U2OS cells labeled with tractin-GFP. 200nm fluorescent beads in 2% Agarose as imaged with oblique illumination (a) and sHILO (b) at an incident angle of 62.2 ± 0.1 degrees. Arrows mark beads that are slightly out of focus. An U2OS cell labeled with tractin-GFP, as imaged with oblique illumination (c) and sHILO (d) at an incident angle of 62.4 ± 0.3 degrees. 200nm fluorescent beads in 2% Agarose as imaged with oblique illumination (e) and sHILO (f) at an incident angle of 59.3 ± 0.7 degrees. An U2OS cell labeled with tractin-GFP as imaged with oblique illumination (g) and sHILO (h) under an incident angle of 58.5 ± 0.3 degrees. The images in (a), (b), (e) and (f) have been gamma corrected with a gamma factor of 0.8 to better highlight weak features. Scale bars: 5 microns.

To image beyond the plasma membrane, the incidence angle of the two laser beams can be adjusted slightly below the critical angle, resulting in the oblique illumination scheme sHILO. 200nm fluorescent beads (Polysciences Inc, PA) mixed with 2% Agarose (resulting in a concentration of $\sim 5 \times 10^{10}$ particles/ml) were used to evaluate the sectioning performance of HILO and sHILO at shallow angles. Incident angles were calibrated using a large (12mm thickness) optical flat made of N-BK7. The incidence angle was determined by measuring the distances from where the laser beams undergo total internal reflection at the glass-air interface to the position of the optical axis of the objective. The optical axis was marked with an auxiliary laser beam that was precisely adjusted to travel along the optical axis of the objective. Potential sample stage tilt was checked by going to the critical angle at opposing sides of the pupil and measuring the incident angles with the optical flat. Standard deviations of the incident angles were determined by repeating the measurements, thus they reflect the measurement error in determining the beam positions, but not other potential error sources (e.g. curvature/tilt of sample coverslip).

When the incident angle was set to 62.2 ± 0.1 degrees (for the Agarose sample, ~ 62 degrees was estimated to be the critical angle), oblique illumination reduced the fluorescence excitation outside of the depth of focus, as evidenced by only a few slightly out-of-focus beads being visible in Fig. 7(a), which are removed by sHILO [Fig. 7(b)]. When imaging U2OS cells labeled with tractin-GFP using oblique illumination at an incidence angle of 62.4 ± 0.3 degrees, significant background blur can be observed [Fig. 7(c)]. Using sHILO at the same angle, this background is removed, revealing fine actin structures. The optical sectioning capability of oblique illumination drastically deteriorates when going below the critical angle. This is illustrated when imaging the same Agarose-bead sample (albeit at a different lateral position) at an incidence angle of 59.3 ± 0.7 degrees. Beads at various levels of defocus create a strong background [Fig. 7(e)], which is effectively removed with sHILO at the same incidence angle [Fig. 7(f)]. When imaging another U2OS cells, oblique

illumination at 58.5 ± 0.3 degrees resulted in an image with large out-of-focus blur that overshadows most in focus information [Fig. 7(g)]. The use of sHILO under the same incidence angle provides again excellent contrast [Fig. 7(h)] and fine cytoskeletal detail becomes visible.

5. Discussion

Structured illumination was employed in TIRF and oblique illumination microscopy to reject out-of-focus light and improve image contrast. Previous methods that aimed to reduce such background in TIRF required either the use of pulsed laser sources, which drive up the cost of the system and potentially increase photo-toxicity, or either drastically reduce the detection sensitivity (SAF), dispense with widefield imaging (confocal SAF) or have to acquire multiple images (virtual SAF). The method presented here maintains the sensitivity of widefield microscopy with the versatility of one photon excitation, but requires the acquisition of three images for every timepoint. Further, the use of structured illumination is not expected to noticeably improve the subdiffraction sectioning strength of the evanescent field. Its main use lies in suppressing blur arising from regions outside of the depth of focus, which is significantly larger than the penetration depths of evanescent fields employed in TIRF microscopy.

sTIRF excels on densely labeled samples and can also help with samples that strongly scatter the evanescent field [9]. For sparsely labeled samples and when good TIRF conditions can be achieved, the advantages of sTIRF are expected to be limited, as shown in the example of imaging focal adhesions. However, the increased optical sectioning provided by sHILO will be beneficial to a wide range of samples, including sparsely labeled ones: the reach of TIRF microscopy can be extended while suppressing the increased amounts of out-of-focus blur. In contrast to conventional three-dimensional SIM where the entire sample volume is illuminated, HILO and sHILO minimize sample irradiance similar to light sheet fluorescence microscopy and thereby reduce photo-bleaching and damage to the sample. Furthermore, it is expected that sHILO will lead to more robust reconstructions of very densely labeled specimens compared to conventional SIM, as SIM reconstructions tend to fail in the presence of overwhelming out-of-focus fluorescence (and the associated shot noise) [26].

Azimuthal rotation of the laser beam, also known as ring-TIRF, has been reported to reduce spurious interference patterns in TIRF microscopy and thereby increasing the illumination uniformity [27, 28]. However, ring-TIRF cannot suppress out-of-focus fluorescence caused by stray light from the instrument and light scattering by the sample itself. In contrast, while sTIRF can suppress out-of-focus blur, it will still be prone to spurious interference artifacts, as the two laser beams are mutually coherent. There is currently no easy way to combine the advantages of both structured illumination and the temporally incoherent illumination from many directions.

Structured illumination microscopy has been questioned for its potential for introducing imaging artifacts. In the case of TIRF-SIM, filling the missing cone and improving the overlap of the sidebands by using the coarse interference patterns of sTIRF is expected to drastically reduce some common reconstruction artifacts and thereby simplify image analysis.

In summary, owing to its robust out-of-focus rejection, improved image contrast and its simple implementation, sTIRF and sHILO should be straightforward extensions to existing TIRF setups and greatly expand their capabilities.

Funding

Cancer Prevention and Research Institute of Texas CPRIT grant RR160057 to Reto Fiolka

Acknowledgment

I would like to thank Dr. Sandy Schmid for lending the microscope and TIRF objective. Further I am grateful to Dr. Marcel Mettlen for help with the operation of the microscope,

initial help in hardware control and sample preparation. Further I would like to thank Dr. Erik Welf, Dr. Tadamoto Isogai and Dr. Kevin Dean for the preparation of the MV3, RPE and A673 cells and useful discussion of the manuscript. Further, I am grateful to Dr. Kevin Dean and Dr. Philippe Roudot for help with the particle detection software.