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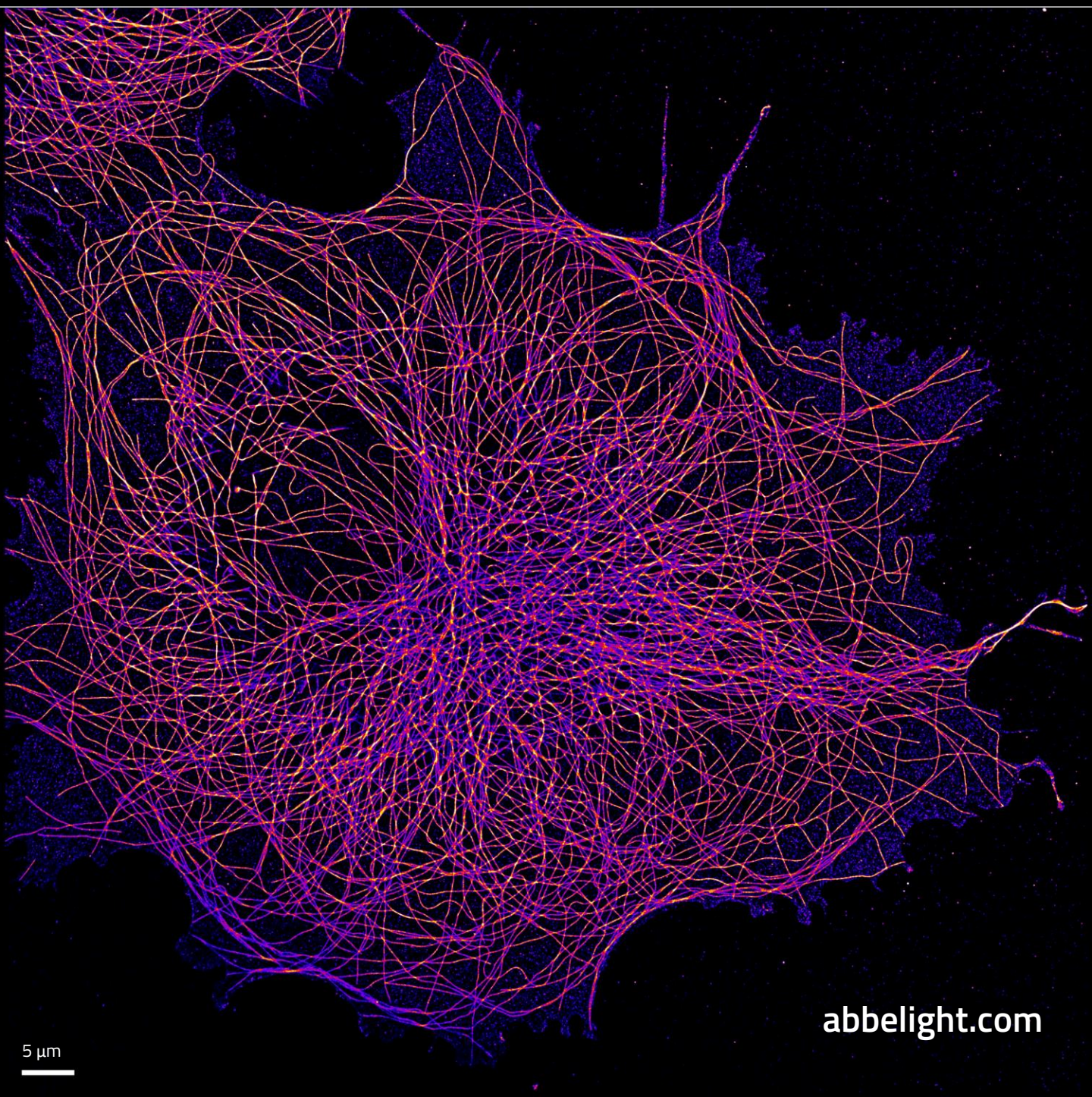
Pushing the Boundaries of TIRF Excitation for Various Sample Media



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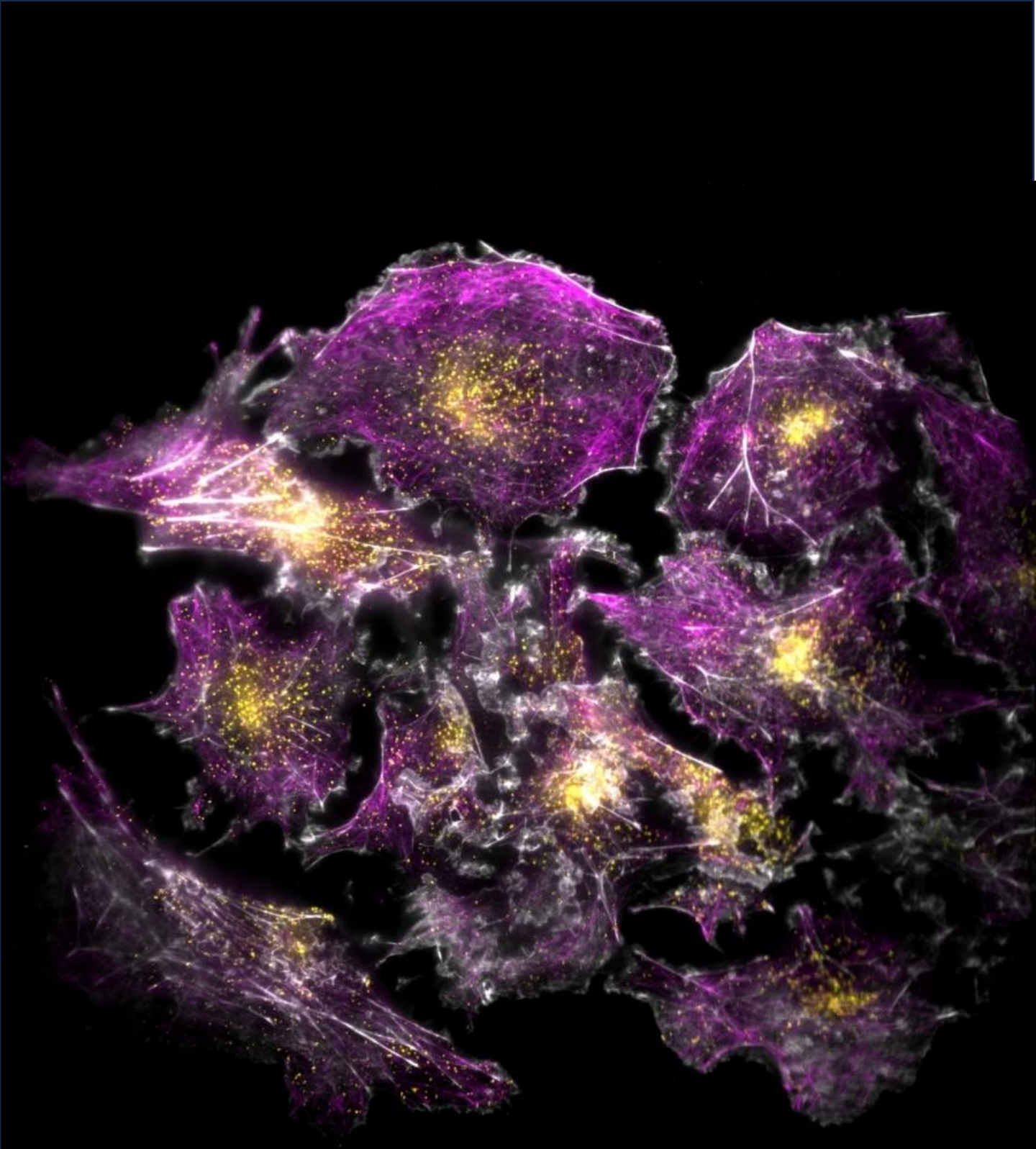

Tubulin - AF647
Clathrin - CF568
Actin - Phalloidin-AF488

Abbelight, Simultaneous Multicolor TIRF microscopy imaging at large and homogenous field of view

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Pushing the Boundaries of TIRF Excitation for Various Sample Media

Author: Dr. Ipek Altinoglu

Total Internal Reflection Fluorescence (TIRF) microscopy is a powerful imaging technique widely used for studying molecular interactions and dynamics at the surface of cells. However, one of the main challenges in TIRF microscopy is dealing with samples that have varying refractive indices (RI), which can alter the TIRF imaging quality. Abbelight's TIRF excitation technique addresses this issue by using automated calibration to define the experimental and precise TIRF angle, ensuring high accuracy and reproducibility for samples with different RI, hence making the process more reliable and adaptable to diverse experimental conditions.

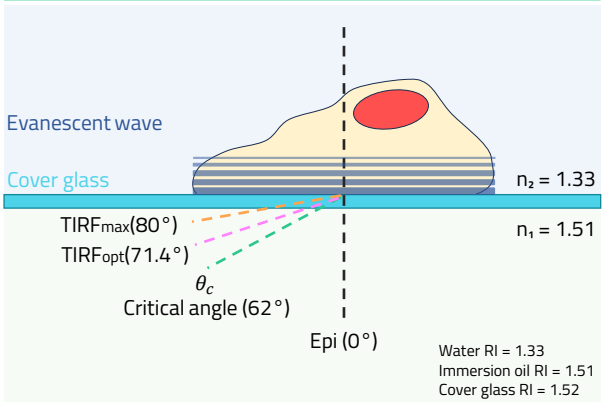
INTRODUCTION

Total Internal Reflection Fluorescence (TIRF) microscopy is a powerful technique based on the principle of total internal reflection, where light is directed at the interface between two media at an angle greater than the **critical angle, θ_c**. The critical angle is defined by Snell's law, $\theta_c = \sin^{-1}(n_2/n_1)$: It is determined by the refractive indices of the two media, the denser medium (n_1), such as immersion oil, and the less dense medium, such as water (n_2) [1]. When light is incident upon interface at or beyond this critical angle, it is totally reflected, creating an **evanescent wave** that decays exponentially with distance from the surface. This wave selectively excites fluorophores within a thin region, typically a few hundred nanometers from the surface, allowing for high optical sectioning of surface dynamics while minimizing background fluorescence from deeper areas (Figure 1).

Samples with different refractive indices such as Fluoromount-G (RI=1.40) and Vectashield (RI=1.45) can alter the light's reflection behaviour [2]. It increases the critical angle, thereby limiting the TIRF excitation range (which lies between the critical angle and the angle determined by the objective's NA). As a result, it requires an exceptionally focused beam and recalibration of the TIRF excitation. If the TIRF angle is not optimized, it can result in poor surface excitation, reduced image quality, and inaccurate data. Ensuring a perfect TIRF angle enables consistent, reliable and high-resolution imaging.

Abbelight's TIRF excitation strategy addresses these challenges with an adaptable, automated, and reproducible perfect TIRF angle configuration for different media, objectives, and stage accessories. The user-friendly software tool performs the automated calibrations on calibration samples that may have varying refractive indices, determining the positions for epifluorescence **(Epi)**, the critical angle (**Hilo**), the optimized TIRF angle (**TIRF$_{opt}$**), and the maximum TIRF angle (**TIRF$_{max}$**) (Figure 1, dotted lines). These angular values can be stored and readily reused, enabling consistent and reproducible illumination positioning, regardless of the objective, microscope, or stage accessories used.

TIRF principle



$$TIRF\ angles > \theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right)$$

Figure 1: Principle of TIRF imaging. The figure illustrates the illumination from the objective at different angles, transitioning from the immersion oil (Light green area) to the water (Light blue area) medium. When the angle exceeds the critical angle (Dotted green line), an evanescent wave (Blue straight lines) is generated at the surface of the coverglass. Critical angle is calculated using Snell's law. TIRFopt and TIRFmax are calibrated angles set by Abbelight's automated TIRF technology (see Figure 2).

The Abbelight's TIRF technology is integrated with ASTER [3] technology to provide homogeneous and large-field TIRF microscopy and nanoscopy imaging, enabling users achieve imaging areas up to 150 x 150 µm² with 100x objectives and 230 x 230 µm² with 60x objectives.

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Together, Abbelight's TIRF microscopy solution includes the SAFe M45 for single and sequential multicolor imaging, and the SAFe M90 for simultaneous four-color imaging. These microscopy products are easily upgradable and compatible with nanoscopy solutions, supporting both 2D/3D single-color, sequential, and simultaneous multicolor SMLM imaging with the SAFe MN180 and SAFe MN360, respectively.

METHODS

Automated Calibration for Optimized TIRF angle for specific RI

The Abbelight's TIRF microscopy and nanoscopy software solution include a calibration section for automated angle determination. This process requires parallel data from the Image Plane (IP) (Figure 2A) to calculate the average intensity from the calibration sample, along with information from the Back Focal Plane (BFP) (Figure 2B) to define the position of the laser beam and identify the TIRF angle. To achieve this, we used Abbelight SAFe MN360 system, which allows simultaneous observation of both the BFP and IP, a capability that is also accessible with all Abbelight's products. The automated calibration tool acquires a theta (θ) stack of 200 angles, starting position from angle at 0°(Epi) up to end position angle at 83°where the light exits the BFP.

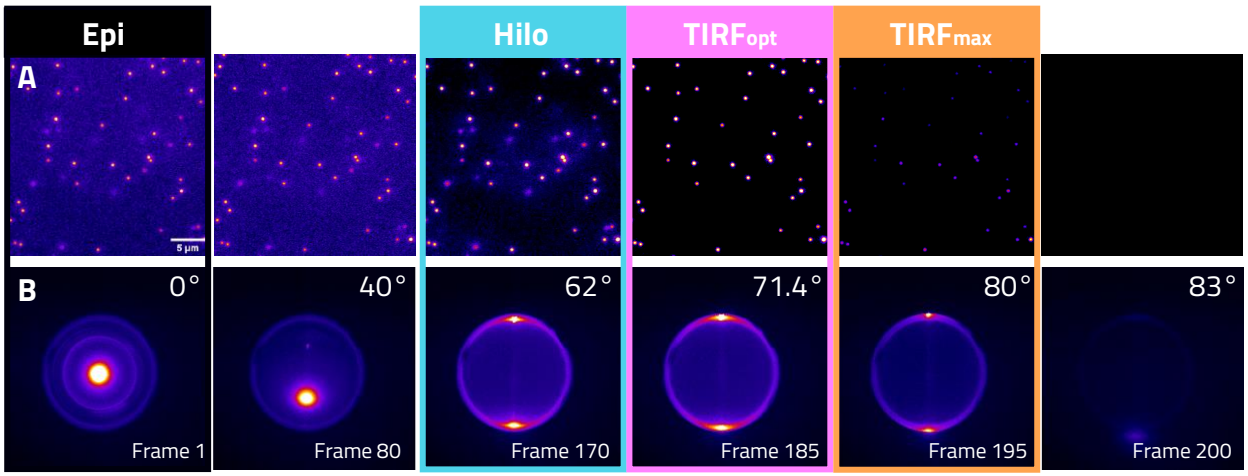

Figure 2: Abbelight's automated and experimental TIRF calibration for diverse sample.

For the IP, we used the Abbelight's calibration sample where beads dispersed from the coverslip into a gel with a refractive index of ~1.33. The stack of IP is used to detect each single beads and calculate the average intensity per frame for each angle (θ). The frame which includes highest SNR defined as Critical angle (Hilo) (Figure 2, Turkuaz selection) and corresponding angle from BFP assigned as a numerical value. TIRFmax is the maximum angle at which the laser beam is visible at the edge of the BFP, beyond which the beam exits, and its image is no longer detectable (Figure 2, Orange selection). Lastly, TIRFopt is defined as the angle positioned between the critical angle and the TIRFmax angle, ensuring optimal conditions for total internal reflection fluorescence (Figure 2, Pink selection).

Altogether, positions are automatically registered as Epi – Hilo – TIRF to the system for the objective used for calibration. This positions are modulable at imaging for coarse and fine adjustments. For each wavelength, theoretical penetration depth automatically calculated and displayed according to angle used.

By combining TIRF microscopy tools with the Multi-Dimensional Acquisition (MDA) module in Abbelight's NEO imaging software, it is possible to define different angles for different structures. For example, imaging the nucleus in Epi mode and transporter channels at the membrane in TIRF mode can be easily achievable, enabling advanced multi-color imaging microscopy applications.

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RESULTS

TIRF Microscopy imaging of various mounting mediums

The fundamental principle behind TIRF imaging relies on the refractive index mismatch between the immersion oil or coverslip and the sample or mounting medium. As the refractive index of the mounting medium approaches that of the coverslip or immersion oil, achieving the correct TIRF angle becomes more difficult, leading to reduced imaging quality and optical sectioning. Mounting media with a low refractive index, such as PBS or water, facilitate the precise TIRF angle, resulting in high-quality imaging. However, these media may compromise fluorescence preservation, leading to rapid photobleaching. Therefore, specialized mounting media like Fluoromount-G, ProLong Gold/Diamond, and Vectashield are preferred for fluorescence microscopy in fixed cells. These media contain antifade agents that help preserve fluorescence and minimize photobleaching, although achieving an ideal TIRF angle can be more challenging due to their higher refractive indices.

Here, we have used Abbelight's TIRF microscopy to show how the automated and perfect TIRF angle ensures high-resolution TIRF microscopy imaging in classical and challenging mediums.

Phosphate-Buffered Saline (PBS), RI=1.335

PBS is a clear, water-based buffer solution that is compatible with live-cell imaging. It is commonly used for short-term microscopy when samples need to be quickly observed. However, PBS lacks antifade agents, making it unsuitable for long-term storage of fluorescent samples or for imaging that involves prolonged light exposure. The refractive index of PBS is relatively low and is values as 1.335.

Here, we have used Abbelight's ready-to-use slide prepared in PBS and acquired a series of images at automatically calibrated angles EPI – Hilo – TIRF. The Actin filaments in Cos7 cells can be easily observed in fine details at the diffraction limit with the TIRF angle set to 71.4° without any adjustment.

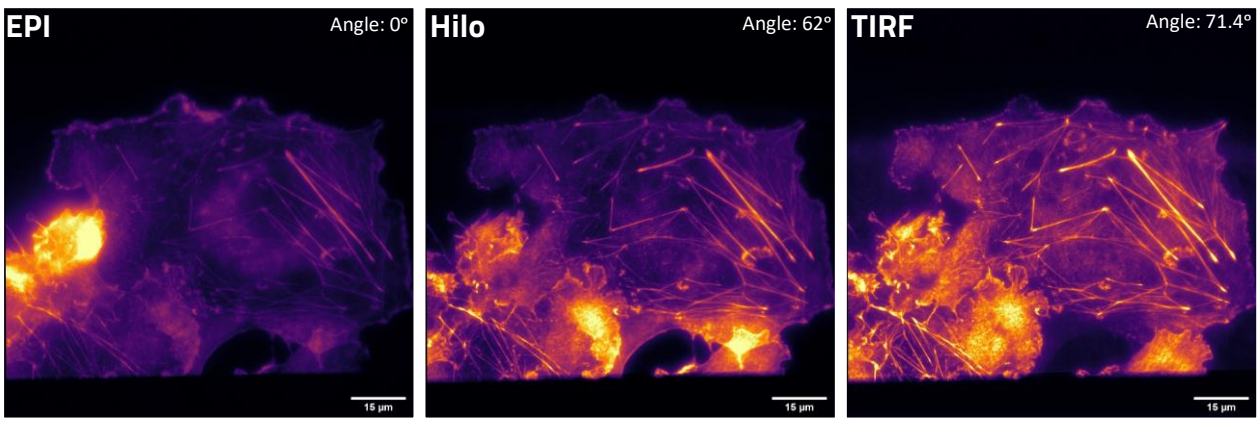

Figure 3: Epi – Hilo – TIRF microscopy imaging of Actin labelled with Phalloidin-AF488 in Cos7 cells mounted in PBS.

Fluoromount-G, RI = 1.40

Fluoromount-G is an aqueous-base mounting medium that is designed to protect fluorescent signals. It includes antifade agents which helps to reduce photobleaching and maintain the fluorescence intensity of samples for longer periods of time. The refractive index of Fluoromount-G is typically ~1.40.

Glucose Transporter 4 (Glut4), labelled with GFP in HeLa cells, was mounted in Fluoromount-G medium and imaged with Abbelight's TIRF microscopy. Under epifluorescence (Epi) illumination, the entire volume of the sample is uniformly exposed to light, which results in a lower resolution, particularly around the nucleus where

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overlapping fluorescence signals from the sample's volume are prominent. By contrast, adjusting the TIRF angle precisely to 74°(optimized for this sample) enabled selective illumination closer to the coverslip, where the cells are adhered. This adjustment significantly enhanced the signal-to-noise ratio (SNR) and overall image quality, providing clearer and more defined observations.

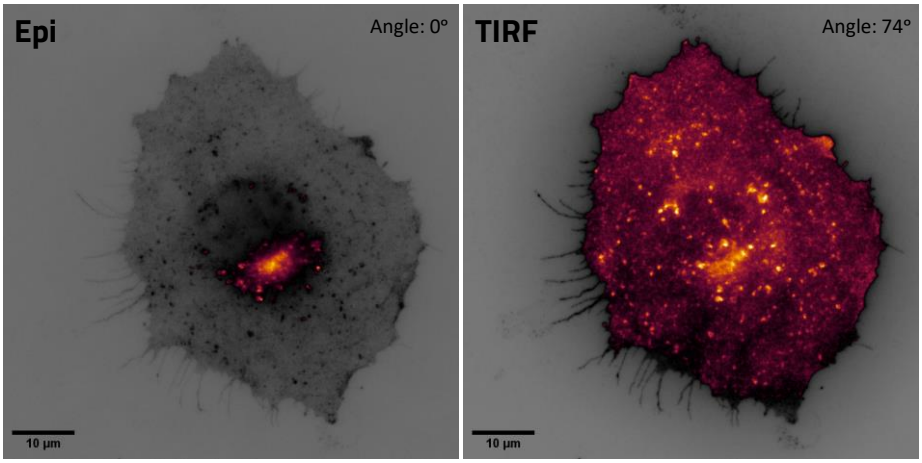

Figure 4: TIRF Microscopy imaging of GFP labelled Glut4 protein (Glucose Transporter 4) in HeLa cells mounted in Fluoromount-G, RI=1.40 . The Epi image is shown on the left, while the TIRF image is displayed on the right. Both images were processed using the same minimum and maximum contrast settings for consistency. The excitation angle used for each image is indicated in the top-right corner.

Vectashield, RI= 1.45

Vectashield is an anti-fade mounting medium that provides excellent protection against photobleaching. It contains anti-fade agents to preserve the fluorescence intensity of samples during imaging, allowing for longer observation times. The refractive index of Vectashield is ~1.45, which is well-suited for high-resolution fluorescence microscopy such as confocal and structured illumination microscopy. However, due to its refractive index being closer to that of coverslips and immersion oils, achieving an optimal TIRF angle for TIRF microscopy can be challenging.

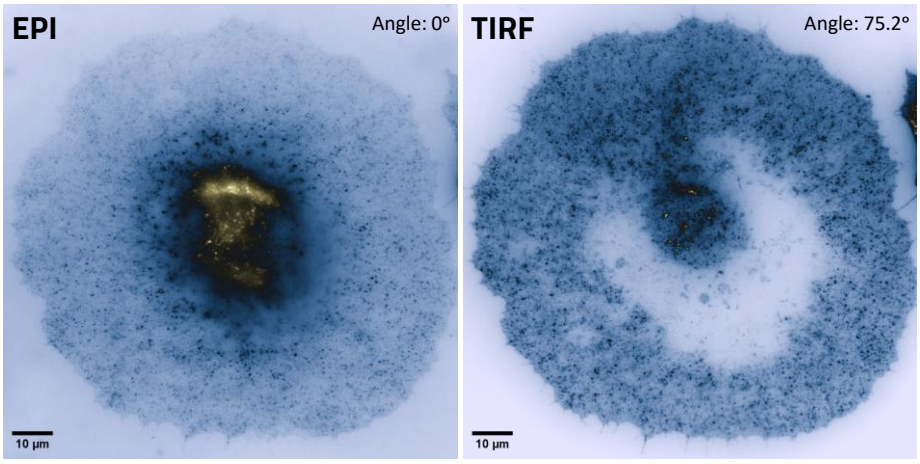

Figure 5: TIRF Microscopy imaging of GFP labelled Glut4 (Glucose Transporter 4) in HeLa cells mounted in Vectashield medium, RI=1.45. The EPI image is shown on the left, while the TIRF image is displayed on the right. Both images were processed using the same minimum and maximum contrast settings in ImageJ for consistency. The excitation angle used for each image is indicated in the top-right corner.

To address the challenge of achieving the optimal TIRF angle with Vectashield medium, we used the Abbelight's calibration software tool. The image captured with an optimized TIRF angle of 75.2° clearly demonstrates the Glut4-GFP protein localization on the membrane of HeLa cells, offering higher resolution compared to an EPI image. Additionally, the image was acquired in a large field of view (110 x 110 µm²), encompassing the entire cell and confirming the uniformity of ASTER illumination at TIRF.

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CONCLUSIONS

Sample preparation is a critical step for achieving high-quality images in all fluorescence microscopy and nanoscopy experiments. This is particularly true for TIRF microscopy which relies on specific angles to achieve resolution at the diffraction limit (~200 nm). The critical angle is directly influenced by the refractive indices of the immersion oil/cover glass (typically ~1.50) and the mounting medium (ranging between 1.33 and 1.51). To optimize TIRF imaging, three key considerations must be addressed:

1- **Choice of mounting medium:** Selecting a medium with a refractive index lower than that of the immersion oil/cover glass ensures access to the critical angle and facilitates the generation of an evanescent wave at the sample surface.
2- **Perfectly focused beam:** The TIRF excitation range is defined between the critical angle and the angle determined by the objective's NA. Achieving effective optical sectioning within this narrow range, especially when using a high RI mounting medium, requires a perfectly focused beam at the BFP.
3- **Precise calibration:** Accurate calibration is essential to define an optimized and reliable TIRF angle tailored to the specific refractive index of the medium being used.

Here we showed how Abbelight's TIRF microscopy solution can help achieve optimized, reproducible, perfectly focused and reliable experimental TIRF angle for samples prepared in both classical and challenging imaging mediums.

MATERIALS

SAMPLE PREPARATION

Abbelight's ready-to-use imaging slide featuring Actin labeled with Phalloidin-AF488 in Cos-7 cells, prepared in PBS medium was used for single-color TIRF microscopy imaging. For TIRF calibration, Abbelight's TIRF calibration samples were used.

HeLa cell samples labelled with Glut4-GFP, mounted in Fluoromount-G and Vectashield were provided by the Imaging Core Facility, Biozentrum, Basel University, for Abbelight's TIRF microscopy demonstration.

IMAGING

Abbelight's SAFe MN360 offering both TIRF Microscopy and Single Molecule Localization Microscopy (SMLM), and Neo Live imaging software v 2.17.0. was used to acquire images.

ImageJ software was then used to visualise and set the minimum and maximum contrast of the images. The NeuroCytoLab LUTs plug-in was used for image LUTs.

ACKNOWLEDGEMENTS

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