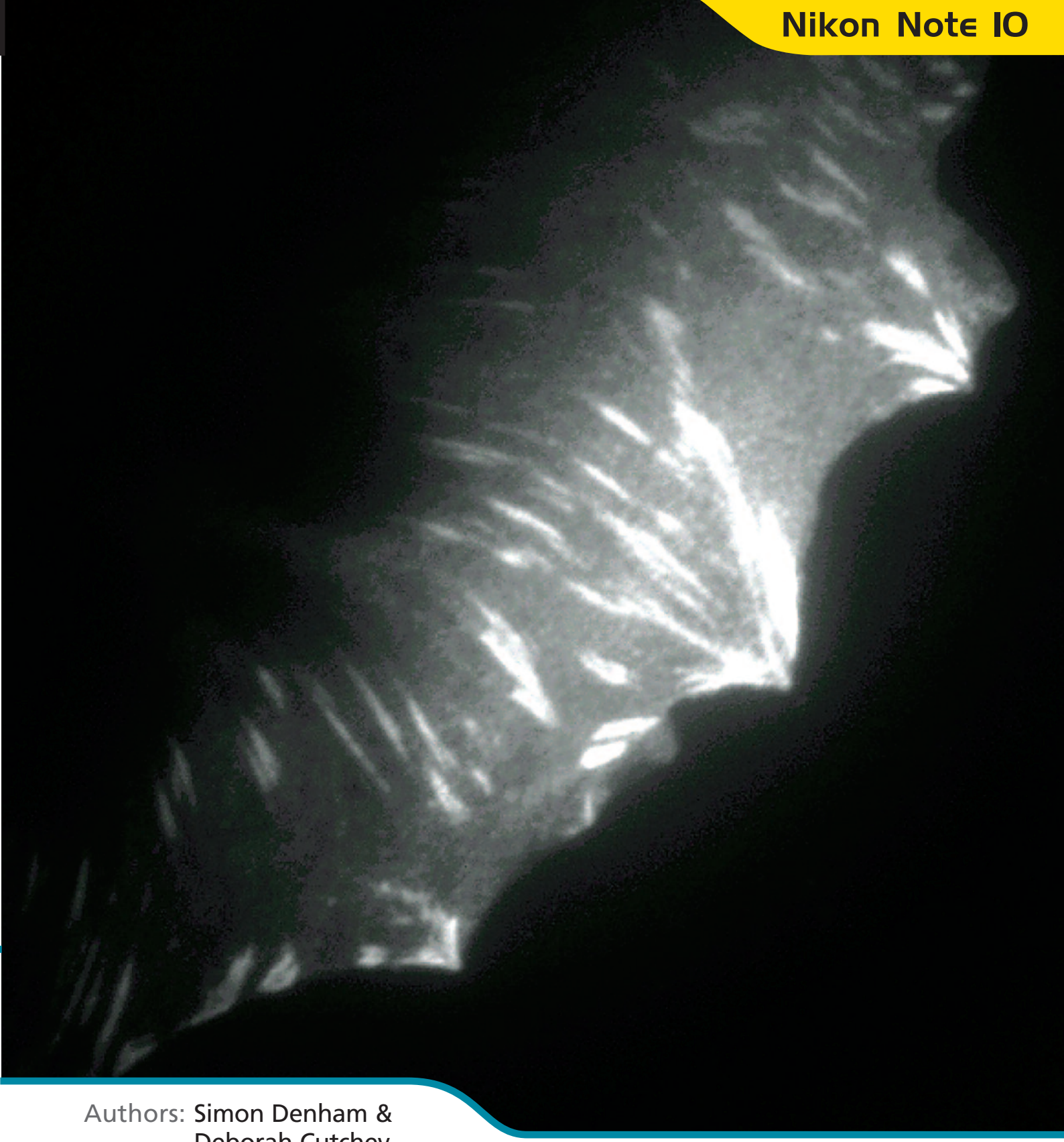

Authors: Simon Denham &
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TOTAL INTERNAL REFLECTION FLUORESCENCE (TIRF) MICROSCOPY:

High Contrast Imaging of Surface Events

Abstract:

TIRF microscopy is a technique for imaging the surface of cells that utilises a thin field of illumination to image fluorophores on or near to the coverslip. TIRF is a highly sensitive widefield camera-based technique providing an excellent signal-to-noise ratio compared with epi fluorescence or confocal imaging methods. TIRF can be used alone or in combination with other imaging techniques to give an "inside and out" view of a specimen. This technical note describes the theoretical basis of TIRF microscopy, the hardware required and examples of TIRF applications.

Introduction:

Total Internal Reflection Fluorescence (TIRF) microscopy is a high signal-to-noise ratio technique that can be used to obtain a very thin optical section of a specimen whilst minimising background noise[1]. TIRF utilises the evanescent field created when a beam of light strikes an interface between two media to excite fluorescent dyes in the specimen. Although TIRF cannot image deep into a specimen, it allows imaging of the specimen near the coverslip with high contrast compared to other techniques.

TIRF microscopy requires two optical media with different refractive indices, such as glass (n=1.51) and water (n=1.333). When a beam of light hits an interface between the medium in which it is travelling and a medium of lower refractive index, part of the beam is refracted and part is reflected. The relative proportions of refracted and reflected light depend on the angle that the beam strikes the interface (termed the angle of incidence). As the angle of incidence increases, the amount of light reflected also increases. Once the angle of incidence exceeds an angle known as the critical angle all light is reflected. This is known as total internal reflection (equation 1).

Equation 1: Critical Angle for Total Internal Reflection

$$\theta_C = sin^{-1} (n_2 / n_1)$$

Where:

θ_C = Critical angle

n_1 = Refractive index of first medium (e.g. glass)

n_2 = Refractive index of second medium (e.g. water)

When light is totally internally reflected, some of the incident energy generates a very thin electromagnetic field that penetrates into second medium (figure 1). The intensity of this field decreases exponentially as it moves away from the interface and, as such, is called the evanescent field ('evanescent' meaning 'tending to vanish'). The evanescent field retains the frequency of the incident light and is capable of exciting fluorophores within approximately 100nm of the coverslip. The evanescent field excites fluorophores The exact depth of penetration is dependent on the wavelength of the incident light and the angle of incidence. The depth of penetration decreases as the angle of incidence increases and as the wavelength of the light decreases. As the evanescent field only excites fluorophores within an exceptionally small distance of the coverslip, it gives a very thin optical section that eliminates background fluorescence.

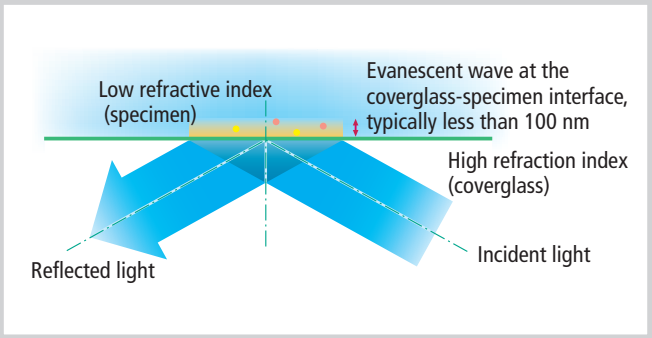

Figure 1: Creation of an evanescent wave at the coverglass-specimen interface

Technology

Early TIRF systems utilised a prism to direct the light to the interface. The development of high numerical aperture (NA) objectives has permitted 'prismless' total internal reflection using a through-the-lens approach. This technique is more convenient, gives better spatial resolution and allows greater specimen accessibility.

In the through-the-lens technique, the angle of illumination is varied by positioning the light beam off-axis at the back focal plane of the objective. The further off-axis the beam is positioned, the higher the angle of the incident beam and, therefore, the thinner the optical section. Illumination can be switched from TIRF to standard epi-fluorescence by repositioning the beam on-axis at the back focal plane (figure 2). These functions are fully motorised in the Nikon Laser TIRF attachment, and can be controlled via the microscope or PC software.

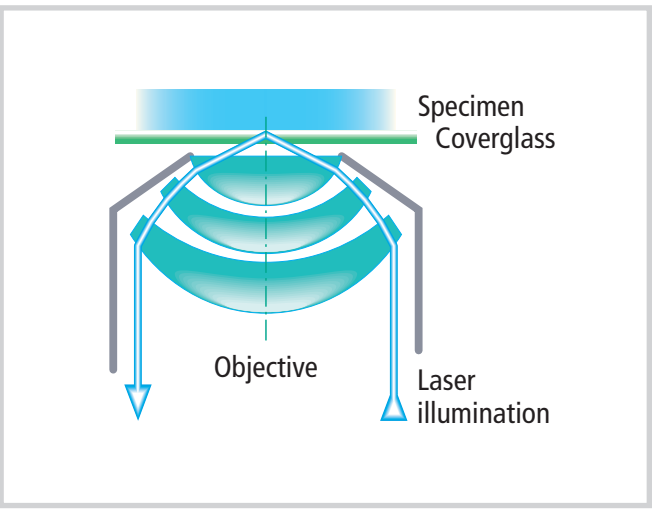

Figure 2: Through-the-lens laser TIRF.

The maximum angle of the emergent beam from the objective is determined by the NA of the lens, which must exceed the refractive index of the second medium (e.g. water, n=1.33) to achieve the critical angle. In practice, the NA of the lens should be as high as possible so super-critical angles can be achieved to create shallower evanescent fields and thinner optical sections. Objective lenses such as the Nikon CFI60 TIRF Apochromat range have an exceptionally high NA of 1.49, which is the highest practicable NA for use with standard immersion oil and glass coverslips (equation 2).

Equation 2: Maximum Angle of Marginal Ray

$$\theta_{max} = sin^{-1}(NA/n_{oil})$$

Where:

θ_{max} = Maximum angle of emerging light beam

NA = Numerical aperture of objective lens

n_{oil} = Refractive index of immersion oil

Increasing signal intensity

The overall signal in TIRF is low compared to epi-fluorescence imaging as only a very narrow depth of specimen and, therefore, a small quantity of fluorophore is illuminated. Because of this decreased signal, TIRF systems require high sensitivity cameras, such as an EMCCD, which multiply signal before read-out and, in some cases, are capable of detecting single photons.

Laser light in TIRF results in higher intensity imaging. Single wavelength excitation light also allows the depth of the evanescent field to be determined more accurately. White light TIRF offers a cost-effective alternative to laser TIRF with comparable optical sectioning performance. The Nikon White Light TIRF Multi-Fluorescence Imaging System uses a standard mercury light source to provide a variety of observation methods, including TIRF, epi-fluorescence, oblique angle illumination fluorescence, and surface reflective interference contrast (SRIC). SRIC is a form of Interference Reflection Microscopy that indicates the specimen's proximity and adherence to the coverslip. In the white-light system, excitation wavelengths are selected by use of filter cubes, making the system highly flexible and easy to update for new fluorophores.

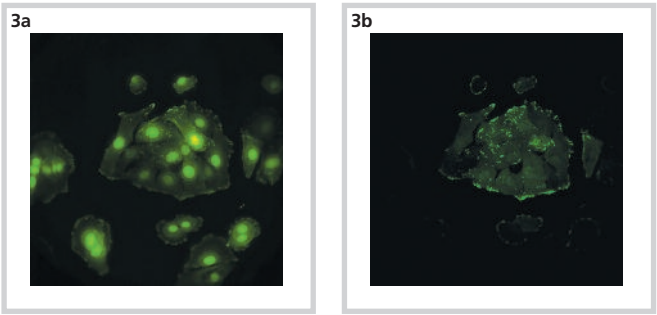

Figure 3: Epi (3a) vs TIRF(3b) . Epithelial cells expressing GFP-dSH2. Both were captured with Apochromat 60x 1.49 NA objective. The fluorescence (a) was illuminated with an Hg light source, the TIRF (b) was illuminated with a 488nm laser. Images taken by Simon Denham, Nikon Instruments. Samples provided by S.E. Ledevedec, Leiden University.

Due to the thin optical sectioning of all TIRF techniques, time lapse observations can be prone to focus drift. The Nikon Perfect Focus System (PFS) is an ideal partner for TIRF microscopy, continually maintaining focus in the plane of interest. PFS constantly tracks the coverslip position in the Z direction and maintains focus in the selected plane by means of a unique offset lens. This allows time lapses to be captured over days with no need for focus adjustment. With the modular Nikon Eclipse Ti series, TIRF can also be combined with other imaging techniques such as laser-scanning confocal microscopy, or with trapping techniques such as laser tweezers to allow simultaneous manipulation and imaging of microscopic particles.

Applications

TIRF imaging provides new imaging possibilities that traditional widefield and confocal imaging cannot accomplish. TIRF has, for example, been used to selectively image bursts of actin activity at the plasma membrane during endocytosis[2]. Actin forms comet-like tail structures that appear to propel endosomes into the cytosol. Analysis of fast time-lapse TIRF images enables the speed of the actin tails to be calculated with a high degree of accuracy. Endosome propulsion has been confirmed by confocal imaging of endosomes filled with rhodamine-labelled dextran. The combination of the two techniques enabled the construction of an inside-and-out view of the cell.

The high signal-to-noice ratio of TIRF provides a powerful tool for mapping the flow of information in cell signalling networks and has been used to follow the transport of signalling proteins from the cytosol to the plasma membrane. The flow of proteins has been quantified by measuring the increase in fluorescence intensity at the plasma membrane[3]. As TIRF is a widefield technique, it also allows large numbers of cells to be imaged simultaneously.

The secretion of newly synthesised proteins is a process that has been studied extensively with both confocal and widefield epi-fluorescence microscopy. TIRF has added a new dimension to such studies[4]. With high temporal resolution and fine optical sectioning, TIRF enables observation of detailed kinetics of individual exocytotic events, which are usually masked by fluorescence from other sub-cellular structures.

TIRF is exceptionally well suited to live cell imaging, as only the plane that is visualised is illuminated. This minimises photobleaching and phototoxic effects to the specimen as a whole.

Conclusion:

TIRF is a highly sensitive technique that can be used to image a variety of cell surface events with an outstanding signal-to-noise ratio and nanoscale optical sectioning. TIRF is well suited to live cell time-lapse experiments, especially when partnered with the Nikon Perfect Focus System. High quality images of surface events can easily be obtained using a Nikon TIRF system equipped with optimised objectives, and a high-sensitivity EMCCD camera.

Simon Denham &
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Authors Background

Simon Denham is a Biological Imaging Systems Specialist for Nikon Instruments UK. Since 2002 Simon has lectured on the topic of digital imaging at exhibitions and Nikon's hugely successful Digital Imaging Seminars which have toured the UK for several years. He joined Nikon in 1997 after obtaining an HND in Applied Biology and an Honours degree in Environmental Science from the University of Plymouth.

Deborah Cutchey is a Bioscience Application and Product Specialist for Nikon Instruments UK. Deborah studied Biomedical Science at London Metropolitan University, graduating with First Class Honours. She is responsible for providing support to the Bioscience team, in particular the Advanced Research Team, in all things related to applications and product markets.

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- Harvard Medical School, USA
- University California San Francisco, USA
- Hokkaido University, Japan
- Singapore Bioimaging Consortium, Singapore
- Northwestern University Chicago, USA

- TIRF images events at or near the coverslip
- TIRF gives an excellent signal-to-noise ratio
- TIRF allows the imaging of single fluorescent molecules
- TIRF and confocal microscopy can be combined on the same imaging system to give an "inside and out" view

For more information on Nikon TIRF systems go to:

www.nikoninstruments.eu/Information-Center/TIRF

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