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**TIRF:**  
**Evanescent waves for high  
resolution membrane  
fluorescence imaging**

## Abstract:

### Evanescent waves for high resolution membrane fluorescence imaging

Changes in the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) accompany many diverse biological phenomena from neuronal excitability and muscle contraction to activation of transcription and cell death. Thanks to the introduction of fluorescent intracellular  $\text{Ca}^{2+}$  dyes our understanding of  $\text{Ca}^{2+}$  signalling has greatly advanced; it is established that depending on the signalling molecule and cell type, the global increase in cytoplasmic  $\text{Ca}^{2+}$  is mediated by the mobilisation of  $\text{Ca}^{2+}$  from intracellular stores and by the opening channels on the plasma membrane, or by a combination of both mechanisms. In this application note, we use TIRF to monitor intracellular free calcium ion concentration  $[\text{Ca}^{2+}]_i$  on or very close to the plasma membrane of muscle cells including human myotubes

## Introduction

Total Internal Reflection Fluorescence (TIRF) microscopy is a method of monitoring membrane associated events including vesicular transport, protein surface membrane targeting, synaptic vesicle fusion and surface membrane receptor assembly. This technique offers several advantages over conventional line scanning confocal microscopy, including the possibility of acquiring data from an ultra thin optical section with a spatial resolution of 100 nm.

In TIRF microscopy a laser beam passes first through a high numerical aperture (N.A.) objective and then undergoes total internal reflection when it passes from a high-refractive medium (e.g., glass) into a low-refractive medium (e.g., cell, water). By using a high N.A. objective, the laser beam can leave the front optical plane of the objective with an angle greater than the critical angle and this results in total internal reflection, producing an evanescent wave which penetrates the low refractive index medium (cell membrane) adjacent to the coverglass. The energy of the evanescent wave drops off exponentially in the z direction starting at the interface between the low and high refractive index medium. The penetration depth of the evanescent wave into the low refractive index medium depends on the energy and wavelength of the incident light. In TIRF microscopy it is possible to collect fluorescent signals from a very thin optical section (up to 100 nm) adjacent to the cell membrane.

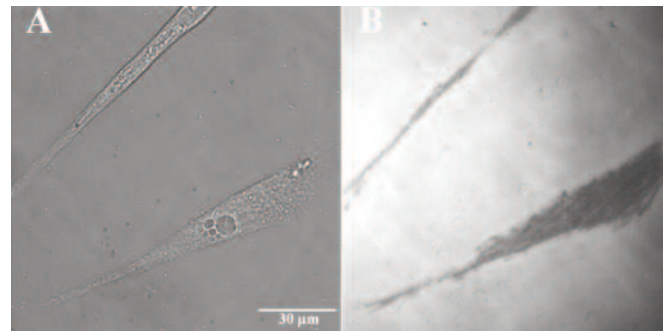
The most critical aspect of working with TIRF is the focus, which must be maintained at the glass-specimen contact site. A SRIC (Surface Reflective Interference Contrast) filter makes all parts of the specimen in contact with glass appear black, and should be initially used to adjust the focus at the glass-specimen interface. Nikon's Perfect Focus System (PFS) detects the boundary between the cover glass and the aqueous solution of the sample using a near infrared light beam, and controls the focus using the boundary as a reference. The PFS continuously corrects the focus to compensate for small focus changes (drift) that might occur during an experiment, especially timelapse experiments.

## Methods

Differentiated human muscle cells (myotubes) were grown on glass coverslips 0.12 mm thick as described previously<sup>1</sup> and were loaded with the fluorescent intracellular  $\text{Ca}^{2+}$  dye, fluo-4, according to standard procedures. Ryanodine (500  $\mu\text{M}$ ) was added to the incubation medium to block  $\text{Ca}^{2+}$  release, which would occur through the ryanodine receptor  $\text{Ca}^{2+}$  channel. After the loading protocol, the medium was replaced with fresh Krebs-Ringer solution (KR); the coverslip was mounted onto a thermostatically controlled perfusion chamber and individual cells were stimulated with 100 mM KCl (made iso-osmotic by replacing  $\text{Na}^+$  in the perfusion buffer, with  $\text{K}^+$ ) by means of a microperfusion system.

Images were acquired using a cooled, back-illuminated CCD camera with on-chip electron gain multiplication (C9100-13 Hamamatsu) connected to a PC. The current experiments were carried out using a Nikon 60x TIRF objective (N.A. = 1.49).

Cells of interest were identified using conventional brightfield microscopy using a Nikon Biomedical inverted research microscope with PFS (figure 1A). The PFS and epi-fluorescence illumination were switched on and the SRIC filter inserted. The focus was then adjusted so that the footprint of the cell on the glass coverslip was visible as a dark grey outline (figure 1B); this focal plane was selected and memorised.



**Figure 1:** (A) Brightfield photomicrograph of human myotubes. (B) The same cells observed with the SRIC filter. The dark grey outline represents the footprint of the cell on the glass coverslip. This focal plane was selected to perform time lapse imaging of  $\text{Ca}^{2+}$  influx.

The laser beam was first centred and made parallel to the optical axis of the objective by viewing the position of the laser spot projected perpendicularly to the microscope's objective onto the ceiling. After minimising the laser spot, total internal reflection was achieved by changing the angle of the laser beam by rotating the goniometer. The correct angle was determined by visualising the laser spot by means of a Bertrand lens. Once the angle parameters were set with the goniometer,  $\text{Ca}^{2+}$  influx could be assessed.

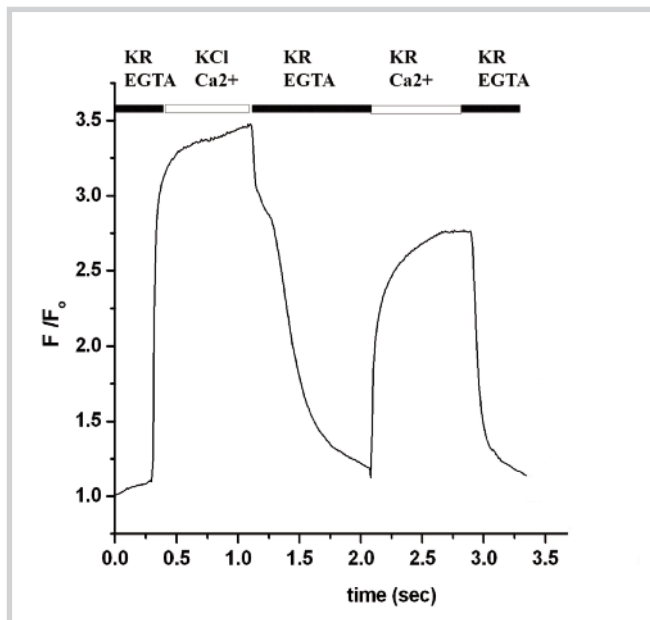
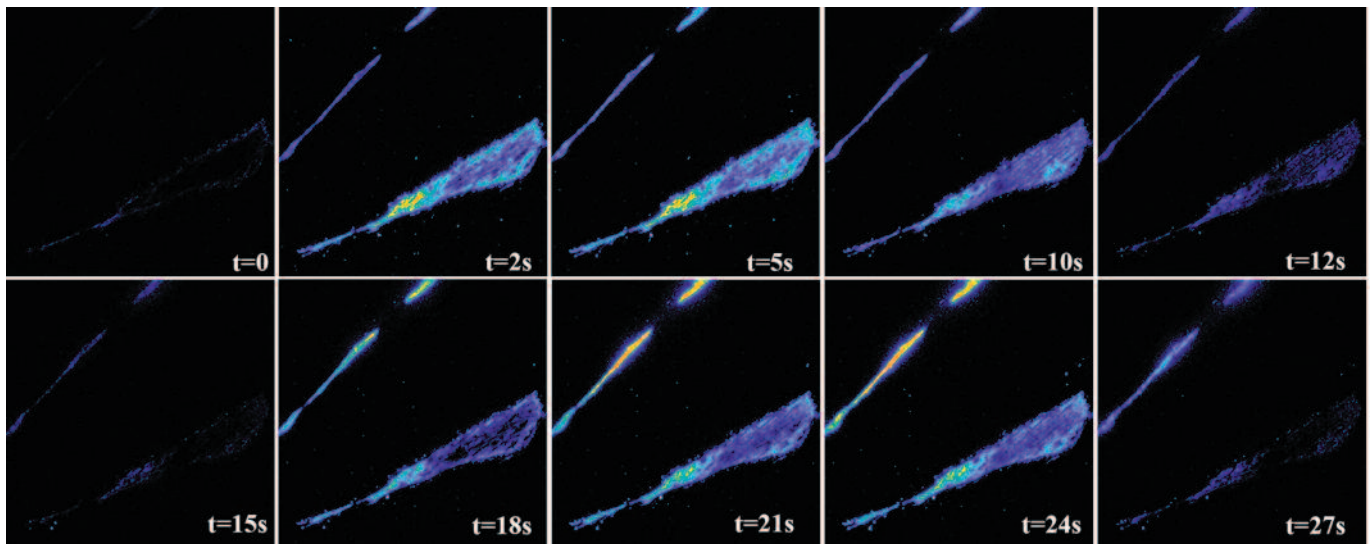


Figure 2: Changes in fluo-4 fluorescence in human myotubes perfused with 100 mM KCl or after the re-addition of 2 mM  $\text{CaCl}_2$ .

## Results

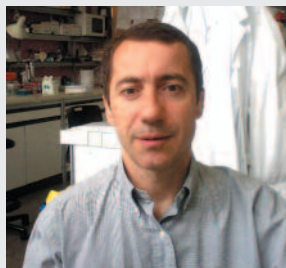
The trace and the pseudo-coloured images show the changes in  $[\text{Ca}^{2+}]_i$  occurring in the myotube shown (figure 2). Changes in fluorescence were obtained with the PFS 'on' and by illuminating fluo-4 loaded cells with a sapphire laser (488 nm), thus only the fluorescence emitted at the fixed focal plane was visible. The addition of 100 mM KCl (which mimics depolarisation) caused a rapid influx of  $\text{Ca}^{2+}$  independent of  $\text{Ca}^{2+}$  release mediated by the ryanodine receptor in intracellular  $\text{Ca}^{2+}$  stores activation, since the latter was blocked by incubation with 500  $\mu\text{M}$  ryanodine. In muscle cells, the depolarisation-induced  $\text{Ca}^{2+}$  influx is termed 'excitation coupled  $\text{Ca}^{2+}$  entry' or ECCE and is thought to be mediated by the dihydropyridine receptor L-type  $\text{Ca}^{2+}$  channel<sup>2,3</sup>. Removal of KCl and addition of Krebs Ringer with 0.5 mM EGTA blocked  $\text{Ca}^{2+}$  influx. The subsequent addition of 2 mM  $\text{Ca}^{2+}$  to the extracellular medium also resulted in  $\text{Ca}^{2+}$  influx, but the latter  $\text{Ca}^{2+}$  influx was not mediated by ECCE.

## Conclusion:

TIRF technology is a powerful tool for studying membrane-associated events with high spatial resolution. In this study we have shown that TIRF can be used to monitor changes in  $[\text{Ca}^{2+}]_i$  occurring at or very close to the plasma membrane of human myotubes by using TIRF in combination with the fast  $\text{Ca}^{2+}$  indicator, fluo-4. Similar studies can be performed on other cell types<sup>4</sup> to gather information on the role of  $\text{Ca}^{2+}$  influx in normal and pathophysiological events.



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## Authors' Background

**Susan Treves** PhD is Research Group Leader at the Departments of Anaesthesia and Biomedicine, Basel University Hospital, Basel, Switzerland and Assistant Professor, University of Ferrara School of Medicine, Ferrara, Italy. Main research interests include calcium homeostasis in excitable and non excitable cells and skeletal muscle excitation-contraction coupling. E-mail: [susan.treves@unibas.ch](mailto:susan.treves@unibas.ch)

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

■ TIRF is a powerful tool for studying membrane-associated events with high spatial resolution

■ A critical aspect of TIRF is to maintain focus at the glass-specimen contact site

■ Nikon's Perfect Focus System ensures focus during timelapse experiments

■ TIRF can be used to monitor changes in intracellular calcium concentration at, or very close, to the plasma membrane of human myotubes

For more information on Nikon TIRF systems go to:

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