IMPROVEMENTS OF TIRF MICROSCOPY FOR STUDIES OF LOCAL Ca^{2+}-SIGNALS IN CARDIOMYOCYTES FROM *RATTUS NORVEGICUS* AND *SQUALUS ACANTHIAS*

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Total internal reflection fluorescence (TIRF) microscopy (Truskey, G. A., *et al.*, *J. Cell Science*, 103: 491-499, 1992) has the ability to image structures and events in an extremely thin focal plane (≅ 200 nm) immediately adjacent to a glass/fluid interface. In our laboratory this technique has been used to image "Ca^{2+} sparks" in rat ventricular cells and demonstrate that sparks are absent in elasmobranch cardiomyocytes which lack functional internal Ca^{2+} stores (Cleemann, L., *et al.*, in "*Analytical and quantitative cardiology*", eds. S. Sideman and R. Beyer, pp 57-65, Plenum, New York, 1997). The present report describes developments aimed at facilitating TIRF microscopy of cells subjected to rapid perfusion and patch clamp procedures.

In previous experiments an upright microscope was used to obtain fluorescence images (Fig. 1A) of cells adhering to the upper surface of a prism at the site where the excitation beam undergoes total internal reflection (TIR). This approach has the short comings that a) each batch of cells must occupy the experimental chamber 60-90 min before cell-adhesions are strong enough to produce detailed TIRF images, and b) it is difficult to gain access with patch clamp electrode and rapid perfusion manifold even with a "long distance" (2-3 mm) water immersion objective.

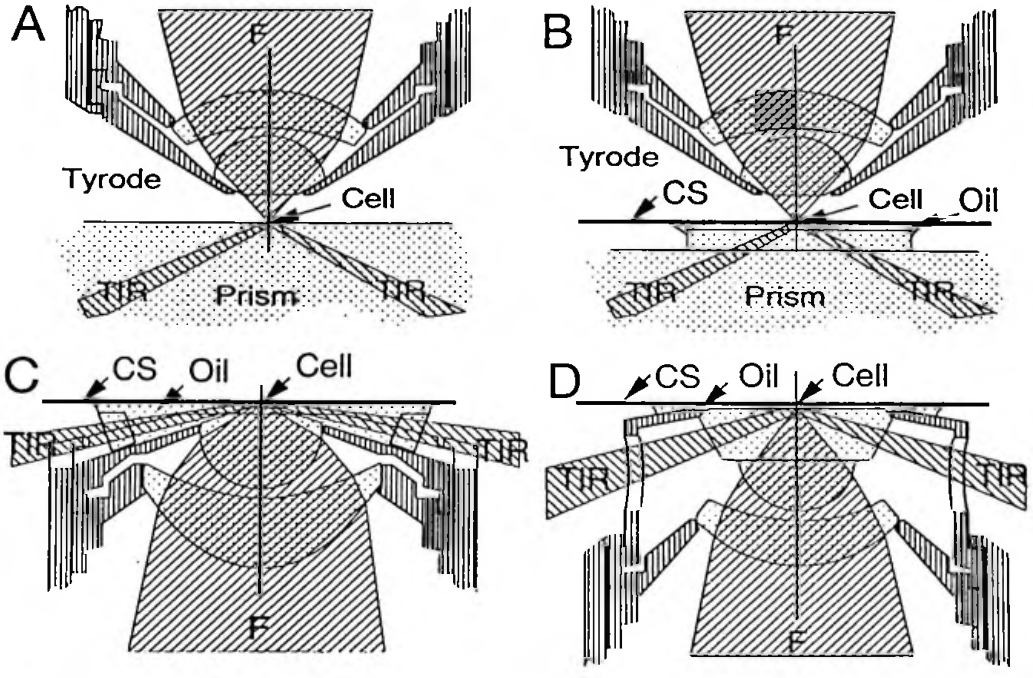

Figure 1. Use of upright (A & B) and inverted (C & D) microscopes for TIRF imaging. The panels show different configurations for the beams (hatched) with total internal reflection (TIR) used for excitation and fluorescent light (F) for imaging. The shown refractive elements (dotted) include the first two lenses of the objective, prisms, windows (C) and immersion oil. CS is the cover slip.

The first problem was solved by plating the freshly dissociated cardiomyocytes onto thin cover slips (25 mm ∅), staining (fluo-3 AM) and equilibrating the cells for 1-2 hours, and then using the cover slip, one after the other, as the exchangeable bottom in specially designed perfusion chamber. The cover slips were coupled to the underlying prism with immersion oil and a small glass prism cemented to the original larger prism (Fig. 1B). This configuration produced a light pass of high quality for the TIR-beam, and made it easier to scan for suitable cells as the prisms and thereby the field of illumination (50 μm x 150 μm) remained completely stationary. The requirement for a clean optical pass was highlighted by a dramatic increase in stray light when an occasional impurity or air bubble in the oil drifted into TIR-beam.

The quality of TIRF imaging was studied using shark (*Squalus acanthias*) and rat (*Rattus norvegicus*) ventricular myocytes plated onto cover slip either without treatment or treated with collagen or polylysine (0.01% solution, dried at 40 ^{0}C) and exposed to rapid flow from the perfusion system. We found that a) strong well defined fluorescence patterns were stable in a rapid and varying stream of solution, b) cells without visible indication of adhesions were generally swept away by the flow, and c) treatment with collagen or polylysine provided only a modest improvement of cell adhesion, and often reduced the survival of cells. Rapid increase of the extracellular Ca^{2+} concentration (0.2 mM $\rightarrow$ 2 mM) produced Ca^{2+} waves and sparks in rat ventricular cells, while, as previously reported, sparks were never observed in shark cells. These experiments where the perfusion manifold was mounted directly on the objective showed, however, that TIRF microscopy with an upright microscope (Fig 1A,B) is incompatible with rapid perfusion, since the stream of solution, between the objective and the cells produces an unacceptable amount of "schlieren" and astigmatism.

It was decided therefore to use an upright microscope thereby removing the flowing solution from the imaging path and gaining free access from above. This has been implemented by restricting the excitation beam to the peripheral rays of a paraffin objective with a numerical aperture (1.4) larger than the refractive index of water (Stout, A.L., *et al. Applied Optics.* 28:5237-5242, 1989). We pursued an alternative approach by allowing the excitation beam to enter over the objective (Fig. 1C) through glass windows on either side of a pool of immersion oil. Though some material (brass) was removed from the top of the objective, the space was still very tight making alignment extremely demanding. It would seem advisable, therefore, to design a special TIRF objective with wider separation between excitation and emission beams thereby providing a cleaner optical path and more room for adjustment of the angle of incidence (Fig. 1D). Never-the-less, the present arrangement (Fig. 1C), demonstrated the feasibility of this imaging approach and the advantages of improved access.

The present studies confirmed the high resolution of TIRF microscopy, the requirement for strong cell adhesion and the qualitative differences between shark and rat EC-coupling and tested practical solutions to the problems arising from the need for long equilibration times and separate light paths for excitation and emission beams.

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