Mapping cell–glass contacts of *Dictyostelium* amoebae by total internal reflection aqueous fluorescence overcomes a basic ambiguity of interference reflection microscopy

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Summary

The widespread ability of eukaryotic cells to produce thin cytoplasmic sheets or lamellae 100–200 nm thick can give rise to uncertainties in the interpretation of interference reflection microscopy (IRM) images when cell–substratum topography is the key interest. If allowed to spread upon a poly-L-lysine-coated surface, *Dictyostelium discoideum* amoebae typically form ultrathin lamellae of ≈100 nm thickness by cytoplasmic retraction. Whereas the cell body is grey, the lamellae appear very dark under IRM optics. These dark areas could be misinterpreted as stemming from a closer cell–substratum apposition beneath the lamellae than the cell body. This ambiguity can be avoided if the technique of total internal reflection aqueous fluorescence (TIRAF) is used in conjunction with a high refractive index glass (n = 1.83) as substratum. Contributions to the image generated by thin cytoplasm and also variable cytoplasmic refractive index are thereby minimized due to the extremely short range of the ‘illuminating’ evanescent wave. From our comparative IRM and TIRAF study of the ultrathin lamellae of *Dictyostelium* amoebae it is concluded that the cell–glass gap is relatively uniform beneath the entire cell. We briefly discuss the sensitivity of several cell types to TIRAF, the generation of ultrathin lamellae and the nature of the cell–glass gap.

Key words: IRM, TIRAF, TIRF, fluorescence, ultrathin lamellae, cell–substratum contact, *Dictyostelium*.

Introduction

It is well known that in certain circumstances cells can form very thin cytoplasmic sheets. The formation of lamellipodia, some 100–200 nm thick, at the advancing edge of locomoting fibroblasts in culture was first recognized by Abercrombie *et al.* (1971) using transmission electron microscopy (TEM), and further studied with interference reflection microscopy (IRM) by Izzard & Lochner (1980). Phagocytic engulfment can also involve enveloping sheets of cytoplasm 200 nm or less in thickness (Zenian *et al.*, 1979; McNeil, 1981). In these situations the lamellipodia apparently play a role in extending the cell periphery and probably in adhesion as well.

Alternatively, after spreading upon a poly-L-lysine-coated coverslip *Dictyostelium* amoebae initially generate extensive, peripheral, thin cytoplasmic sheets by a process of centripetal thinning (Gingell & Vince, 1982a). These *ultrathin lamellae*, which persist for some 10–20 min before active locomotion starts, appear black under IRM optics, in contrast to the mass of the cell body, which is mid-grey. Using quantitative IRM theory (Gingell & Todd, 1979; Gingell *et al.*, 1981), Gingell & Vince (1982a) argued that the black seen in IRM is generated when the cytoplasm thins down to around 100 nm, an interpretation supported by their scanning electron microscope (SEM) images. However, it is not widely appreciated that using IRM alone it is difficult, if not impossible, to differentiate between the contributions to the final image arising from the thickness of such lamellae and their separation from the substratum.

In the present paper we have used a fluorescence technique to show how this difficulty can be unambiguously resolved. Suppose cells are spread in an aqueous...
medium on a glass block. If a laser beam is directed into the block such that it is incident on the glass-water interface at an angle exceeding the critical angle no beam is transmitted into the water, but a localized disturbance called an evanescent wave is generated in the water adjacent to the glass. This wave decays exponentially within ∼30–100 nm of the glass, the exact value depending on the refractive index step and the wavelength of the laser beam. Any fluorochrome molecules located within this zone, but not beyond it, will therefore fluoresce, giving TIRF (total internal reflection fluorescence) images. Axelrod (1981) was the first to use this method in cell biology. He used a fluorochrome that adsorbed to cells and obtained bright TIRF images of cell–glass contact zones against a black background. Our ‘extracellular volume marker’ variant of this (Gingell et al. 1985) utilizes an aqueous fluorochrome, which does not adsorb significantly to either the cell or to the glass and does not penetrate into healthy cells. The cell contacts displace fluorochrome molecules and thus appear as dark patches on a brightly fluorescent background. We have recently shown how the cell–glass apposition distance can be calculated from fluorescence measurements when extracellular volume marker TIRF is used (Gingell et al. 1987). For brevity and clarity we now use the acronym TIRAF (total internal reflection aqueous fluorescence) for our technique.

Using TIRAF we have now shown that our earlier interpretation of IRM images was indeed correct. Furthermore, we have been able to demonstrate that the depth of the aqueous gap separating the lipid bilayer from the glass is constant beneath the lamellae and is the same beneath the relatively thick cell body. This direct result, which has not been obtained on living cells by any other technique, has implications for the mechanics of cell adhesion.

### Materials and methods

Total internal reflection fluorescence, video imaging and recording equipment were used as described by Gingell et al. (1985). This is based on a Zeiss UEM microscope (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK), a Leica 85-1 argon ion laser operating at 486 nm (Lambda Photometrics Ltd, Harpenden, Herts, UK) and an 1SOcon low-light TV camera (English Electric Valve Ltd, Chelmsford, UK). All refractive indices quoted are for 486.1 nm or 488 nm.

*Dicyostelium discoideum* amoebae of the axenic strain Ax2 were cultured and harvested as described by Gingell & Vince (1982). Instead of washing by repeated centrifugation, cells were allowed to settle on glass slides and exposed to gently running 20 mM-NaCl solution. They were then washed off by more vigorous pipetting and collected in tubes to be maintained on ice.

Glass coverslips (Chance Propper, from E. Lamb & Co. Ltd, London, UK) were degreased and exposed to 4% HF in 40% HNO₃ for several seconds, before rinsing in copious distilled water. The latter was prepared in an all-glass still as described by Owens & Gingell (1987). Coverslips were left to equilibrate overnight in distilled water before use. Clean coverslips were treated with a 0.1% poly-L-lysine hydrobromide (M, = 30000–70000; Sigma Chemical Co. Ltd, Southampton, Hants, UK) solution for 1 min, then rinsed well in distilled water before use. Adequate polycation adsorption was shown by the strong adhesion of aldehyde-treated red blood cells (Todd & Gingell, 1980) in distilled water. No adhesion occurs on clean untreated glass.

The poly-L-lysine-treated coverslips were optically mated to the upper glass plate of the prism assembly (see fig. 1 of Gingell et al. 1985) with standard Zeiss immersion oil. The e-fold characteristic decay depth (ξ) of the evanescent wave for standard coverslip glass under the conditions used is 60 nm. For observations at low penetration (ξ = 35 nm) polished glass plates approximately 1 mm thick were flat cut (Ealing Optical Works, London, UK) from a high refractive index glass (n = 1.83) kindly supplied by Mr R. Dunning of Pilkinson Brothers, Ormskirk, Lancashire, UK. The plates displayed very low optical scatter when mated to the top surface of a prism, cut from the same glass, using Cargille immersion liquid M1 (n = 1.765; Cargille Laboratories Ltd, NJ, USA). The glass plates were cleaned in the same manner as the coverslips except that HF was omitted, since we found that it damaged the scatter-free finish. In this case HNO₃ was followed by a 30–45 min wash in 10% Decon 90 (Decon Laboratories Ltd, Hove, UK) with sonication. Poly-L-lysine coating was done as for the coverslips.

A 63× water-immersion objective (Carl Zeiss) was focused onto the interface under IRM optics. The amoebae, which rapidly settled and spread, were then examined under IRM and TIRAF in turn. Because of the extreme susceptibility of the lamellae to TIRAF conditions (see Discussion) we worked at very low light levels, necessitating high camera gain. The resulting noisy images were processed in an Arlunya TV integrating framestore (Agar Scientific Ltd, Stanstead, UK). Integrated images were photographed directly from the monitor screen, using a Nikon F3 camera and Ilford FP4 film. All microscopic observations were made in 20 mM-NaCl containing 10⁻²–10⁻⁴ M-fluorescein in the form of the sodium salt (Sigma) or fluoresceinated dextran (FD4, M, = 4100; Sigma) at 20°C. These extracellular markers give essentially identical images with chick fibroblasts (Gingell et al. 1985).

For electron microscopy, grids were coated with a film of Parlodion (Agar Scientific Ltd) prior to 0.1% poly-L-lysine solution. Cells were seeded directly onto the grids in a small chamber. A similar preparation on a coverslip was used to monitor the cell responses. After 17 min grids were transferred to 1/4 strength Karnovsky’s fixative and left for 1 h. They were then rinsed in 0.1M-cacodylate buffer, pH 7.4, stained in uranyl acetate, rinsed in buffer and air-dried (ethanol-treated grids lost their films). Specimens were examined in a Philips 300 instrument.
Results and discussion

Comparison of fluorescence and interference images

Destructive interference creating blackness in IRM images of cells arises between reflections from either: (1) the lower plasmalemma and the glass surface where the two are in close apposition; or (2) the upper and lower cell surfaces where thin sheets of cytoplasm have formed (Gingell, 1981; Gingell & Vince, 1982a). The existence of these two mechanisms can give rise to ambiguities in the interpretation of IRM images.

The fact that Dictyostelium amoebae form extensive thin sheets of cytoplasm on a poly-L-lysine substratum is shown in Fig. 1. The upper TEM image shows a cell that has developed a broad lamella; the lower shows another in the process of withdrawing cytoplasm from the surrounding lamella. In these whole mounts the low electron density of the peripheral areas clearly indicates that they are far thinner than the cell body. However, the thickness of the lamellae and their contact relationships with the substratum cannot be ascertained from such preparations. We shall show from our optical study that the distinctive IRM images of the cell peripheries do indeed stem entirely from ultrathin lamellar cytoplasm and that the cell–glass distance is the same beneath the cell body and the lamellae.

In Fig. 2A, B the lamella is being retracted, producing an arborescent periphery, similar to that seen in Fig. 1B. Under IRM (Figs 2A, 3C) the lamellae look much darker than the cell bodies due to destructive interference across the thin, almost parallel, sheet of cytoplasm. Under TIRAF, on low refractive index glass (Figs 2B, 3D) the lamellae appear brighter than the cell bodies because the evanescent wave partially penetrates the lamellae and stimulates a limited amount of fluorescence from the medium beyond it. A similar contrast reversal between IRM and TIRAF is seen in Fig. 3A, B, where two adjacent regions on the upper left of the cell (arrowed) have also thinned into lamellae. Since under TIRAF these areas appear substantially brighter than the cell body, which is certainly thicker than 1 μm, the paired images are consistent with the existence of very thin cytoplasmic lamellae.

Beneath the cell body in Fig. 2B a small brightly fluorescent area can be seen where the cell has lifted away from the glass (vertical arrow), while the corresponding IRM image (Fig. 2A) shows pale concentric fringes. Similar lifting is apparent in Fig. 3A, B (outlined arrows). Here the close proximity of tubules of the contractile vacuolar apparatus (white arrowheads in Fig. 3A), which are seen as moving black threads under IRM (Gingell & Vince, 1982a), suggests that the image may be associated with expulsion of water from a contractile vacuole. This has been seen under IRM to lift the cell transiently at the point of discharge.

Within the lamella of the cell shown in Fig. 2 is a local thickening of the cytoplasm (horizontal arrow), which is much paler under IRM (Fig. 2A) than the surrounding thin lamella (Gingell, 1981). Under TIRAF (Fig. 2B) this thickening is as dark as the cell body, because it is too thick for the evanescent wave to penetrate. The lower cell of Fig. 2 has started locomotion and is trailing a lamella that has broken up into cellular debris; this too shows a localized region (horizontal arrow) where the lamella has thickened. If the dark zone (IRM) were due to destructive interference across a very small water gap beneath a part of the cell that is greater than several tenths of a micrometre thick, the paler zone would have to represent lift-off. In this case the TIRAF image would have to be brighter at this point, whereas it is darker. Thus cytoplasmic thickening is responsible, and from this it is virtually certain that ultrathin lamellae cause the dark IRM images.

These observations, taken alone, do not exclude minor optical perturbations from two sources. In previous work using immersion refractometry it was found that the refractive index of the lamella of Dictyostelium is slightly higher than the average for the whole cell (Gingell & Vince, 1982a). According to the calculations of Gingell et al. (1987) this could have a small influence on the TIRAF image, tending to brighten the lamella of a cell attached to coverslip glass (n = 1.528) by some 2%. Second, while there is no room for doubt from the arguments given above that a thin lamella is responsible for the images in Figs 2 and 3 it is conceivable that the lamella–glass separation differs slightly from the cell body–glass separation as well. This point has been settled by observing the TIRAF image on glass of high refractive index (n = 1.83), where the peripheral lamellae were found to be indistinguishable from the cell body (Fig. 4A, B). On high-index glass the wave decay characteristic can be made so low (Fig. 5) that the cytoplasmic thickness over all parts of the cell, including the lamella, considerably exceeds it (Gingell et al. 1987). We used $\varepsilon = 35$ nm, in which case the wave energy falls to 5% of the interfacial value at a distance of 105 nm. An additional advantage is that the increment in lamellar brightness due to a slightly higher cytoplasmic refractive index is reduced to 0.5%. Consequently, any differences in contrast seen under these conditions must be due to variations in the thickness of the zone between the plasmalemma and the glass. The fact that TIRAF on high-index glass shows uniform apposition (Fig. 4A, B) where IRM shows a cell with an extensive dark periphery, proves that the dark zone seen in IRM is due solely to destructive interference across the ultrathin lamella.
Fig. 1. TEM of *Dictyostelium* amoebae on Parlodion treated with poly-l-lysine. A. Cell showing a newly formed thin lamella of uniform electron density. B. Cell in which the lamella is being withdrawn, leaving behind remnants, again of uniform electron density. Bar, 2-5 μm.
**Mode of adhesion**

We have shown that IRM images of thin cytoplasmic lamellae can be a hidden trap for the unwary because they are difficult to distinguish from zones of very close contact. TIRAF observations on high-index glass avoid this difficulty and determine only the size of the cell–glass gap. However, TIRAF on ordinary coverslip glass may give ambiguous images of ultrathin lamellae because of the unavoidably longer range of the evanescent wave, even if a high angle of incidence is used. Providing the fluorescent marker molecules are able to diffuse beneath the cell (large molecules can be sterically excluded; Gingell et al., 1985) a dark image with TIRAF must imply a 'small' plasmalemma–glass gap. In *Dictyostelium* it appears to be uniform in thickness, without visible specialized adhesion sites. The dimensions of this zone are, however, responsive to changes in the ionic strength of the medium in a reversible way (Gingell & Vince, 1982b), thus implicating Debye electrostatic repulsion between fixed charges. The fact that amoebae attached to glass in 20 mM-NaCl cannot be removed by a shearing force of 6 N m$^{-2}$ (Owens, Gingell & Trommler, unpublished data) shows that...
they are not attached by virtue of a force balance in a colloid secondary minimum (Parsegian & Gingell, 1980), but that the molecules of the glycocalyx span the water gap and their ends are adsorbed directly to the glass. The increase in this gap seen in distilled water is evidently caused by electrostatic swelling of the polyanionic matrix of the glycocalyx. A similar conclusion has been reached regarding the adhesion of red blood cells to glass (Trommler et al. 1985). We are currently using TIRAF to investigate the kinetics of diffusion of fluorescently labelled markers through the glycocalyx, in a direction parallel to the lipid bilayer, and to measure the cell–glass separation.

**TIRAF can damage cells**

We are also investigating the sensitivity of cells to TIRAF. Under most circumstances, with chick limb bud or 3T3 fibroblasts, for example, illumination at incident power levels, which give acceptable images by eye, have no discernible effects upon the cells. However, after prolonged exposure to high-incident laser power (4–8 $\mu$J $\mu$m$^{-2}$ s$^{-1}$) a rapid loss of contrast is often observed, indicating permeabilization of the plasmalemma and entry of fluorescein into the cytoplasm. Since the use of lower laser power and/or pulsed observation obviates this effect we do not normally regard it as a serious limitation. In contrast,
Fig. 4. IRM (A) and TIRAF (B) images of the same amoeba generating an ultrathin lamella on a poly-L-lysine substrate adsorbed onto high refractive index glass (i = 35 nm). The black periphery of the IRM image indicates that the cytoplasm has thinned to some 100 nm in these areas whilst the interference fringes (arrowed) imply that this part of the cell is between 0-1 and 1 μm thick. Apart from two small brighter spots (circled), which correspond in both images and suggest areas of greater separation, TIRAF demonstrates the uniformity of the cell–glass gap beneath the entire cell. Laser illumination was kept sufficiently brief so that cytoplasmic flow into the lamella was not stimulated. Bar, 5 μm.

we have found Dictyostelium amoebae to be exceedingly sensitive to TIRAF. Cells can be observed under simultaneous IRM and TIRAF in such a way that IRM makes the major contribution to the combined image: within 30 s the dark peripheral lamellae increase to a relative irradiance characteristic of the cell body. With TIRAF alone, under conditions where the penetration depth of the evanescent wave is relatively high (i = 60 nm), the lamellae first appear pale, as in Fig. 2B, but then rapidly darken. These changes, which do not involve permeabilization of the cells to fluorescein, are caused by cytoplasmic flow from the cell bodies into the lamellae, so creating the localized thickenings referred to in the analysis of Figs 2, 3. When cells were exposed to TIR without extracellular fluorochrome, no thickening took place, but within half a minute of adding fluorescein the dark IRM image was lost. We believe this result implicates the products of photolysis of fluorescein (probably acting at the plasmalemma), but as neither 25 mM-thiourea (an OH free radical scavenger) nor superoxide dismutase (which scavenges superoxide) at 500 μg ml⁻¹ appear to reduce the sensitivity of the amoebae, we do not yet know their identity. As a final point, our experience with human monocytes and TIRAF has indicated that they too generate ultrathin lamellae by retraction but their sensitivity to TIRAF appears to be intermediate between that of Dictyostelium and fibroblasts.

Ultrathin lamellae

The lamellar response of amoebae closely resembles that of polymorphonuclear leucocytes (see Boyles & Bainton, 1979; Keller et al. 1979, 1983) and mononuclear leucocytes (Mellor, Gingell & Todd, unpublished data). It is therefore a common cellular response, not just an obscure and irrelevant feature of a primitive soil amoeba. The ultrastructural basis of lamella formation and withdrawal is little understood, but related studies on macrophages (Trotter, 1981; and for a review, 1985; Hartwig & Shevlin, 1986) and polymorphonuclear leucocytes (Malech et al. 1977; Boyles & Bainton, 1979) have shown that their lamellae have an extensive network of actin filaments in association with the adherent membrane. Dictyostelium amoebae form ultrathin lamellae on hydrophobic surfaces of methylated glass and polystyrene (Gingell & Vince, 1982) as well as on hydrophilic surfaces, including aminated glass, poly-L-lysine and clean untreated glass at high ionic strength (Owens, Bailey & Gingell, unpublished data). These diverse surfaces all appear to cause strong adhesion, but we are not yet in a position to test whether this is the common trigger.

References


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