Cell-Substrate Contacts Illuminated by Total Internal Reflection Fluorescence

DANIEL AXELROD
Biophysics Research Division and Department of Physics, University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT A technique for exciting fluorescence exclusively from regions of contact between cultured cells and the substrate is presented. The technique utilizes the evanescent wave of a totally internally reflecting laser beam to excite only those fluorescent molecules within one light wavelength or less of the substrate surface. Demonstrations of this technique are given for two types of cell cultures: rat primary myotubes with acetylcholine receptors labeled by fluorescent α-bungarotoxin and human skin fibroblasts labeled by a fluorescent lipid probe. Total internal reflection fluorescence examination of cells appears to have promising applications, including visualization of the membrane and underlying cytoplasmic structures at cell-substrate contacts, dramatic reduction of autofluorescence from debris and thick cells, mapping of membrane topography, and visualization of reversibly bound fluorescent ligands at membrane receptors.

The regions of contact between a tissue culture cell and a solid substrate are of considerable interest in cell biology. These regions are obvious anchors for cell motility (1), loci for aggregation of specific membrane proteins (2–4), and convergence points for cytoskeletal filaments (2, 5, 6). Described here is a fluorescence microscope method for selectively visualizing specific molecules in cell-substrate contact regions while avoiding fluorescence excitation of the cell interior liquid medium and cellular debris. Other potential applications of this method include viewing fluorescence-marked receptors at very low cell surface concentrations, cytoplasmic filaments in thick cells, and fluorescent agonists that bind reversibly to the cell membrane.

The new method is an application of total internal reflection fluorescence (TIRF) to cellular microscopy and is an extension to fluorescence of the total internal reflection microscope illumination system introduced by Ambrose (7) to detect light scattered at cell-substrate contacts. TIRF microscopy utilizes a light beam in the substrate that is obliquely incident upon the substrate liquid interface at an angle greater than the critical angle of refraction. At this angle, the light beam is totally reflected by the interface. However, an electromagnetic field called the "evanescent wave" does penetrate into the liquid medium. The evanescent wave propagates parallel to the surface with an intensity I that decays exponentially with perpendicular distance z from the surface:

$$I = I_o \exp(-z/d) \tag{1}$$

The characteristic exponential decay depth d is:

$$d = \frac{\lambda}{4\pi n_2}\left(\frac{\sin^2\theta}{\sin^2\theta_c} - 1\right)^{-1/2}, \tag{2}$$

where n_1 = refractive index of the substrate; n_2 = refractive index of the liquid medium; θ_c = the critical angle of incidence $= \sin^{-1} n_2/n_1$; θ = the angle of incidence, $\theta > \theta_c$; and λ = the wavelength of incident light in vacuum. The decay depth d decreases with increasing θ. Except for θ close to θ_c (where $d \to \infty$), d is on the order of λ or smaller. I_o, the intensity of the evanescent wave at $z = 0$, is on the order of the incident light intensity except for angles of incidence very near the critical angle (8). Therefore, for most experimental configurations, a fluorescent molecule located in the evanescent wave at $z = 0$ will be excited with roughly the same efficiency as it would if it were located in the incident beam.

A fluorescent molecule located close to the surface in the evanescent wave can become excited and emit fluorescence; molecules much farther away will not be excited. The efficiency of excitation decays exponentially according to Eqs. 1 and 2. For typical experiments described here, identical fluorescent molecules located at 1, 10, 100, and 1,000 nm from the surface will emit relative fluorescence intensities of 0.99, 0.92, 0.43, and 0.0002, respectively. For cells adhering to the surface, only fluorescent molecules at or near the cell surface in the regions of closest contact with the substrate will be excited significantly.

TIRF has been employed previously to study surface interactions in a variety of molecular systems, including solutions of fluorescein (9) and serum albumin (10, 11) at glass surfaces, and antibodies at antigen coated surfaces (12). More recently, TIRF has been combined with fluorescence photobleaching recovery and fluorescence correlation spectroscopy to study the surface adsorption/desorption kinetics of fluorescent macromolecules (13, 14) and viruses (15).

A completely unrelated transmitted illumination technique,

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called interference reflection contrast, can reveal cell-substrate contacts as characteristically dark regions (16–18). Although not itself a fluorescence technique, interference reflection contrast has been used in conjunction with conventional epifluorescence to examine submembrane filament structure at contact regions (3, 5).

MATERIALS AND METHODS

Two different cell types were used to demonstrate the TIRF technique. Primary cultures of embryonic rat muscle were plated according to a previously published protocol (4) onto 32.5-mm diameter glass cover slips placed in 35-mm plastic tissue culture dishes. Myoblasts in these cultures fuse into multinucleated myotubes that display membrane acetylcholine receptors (AChR) in both diffuse and localized ("patch") distributions (19). Before being mounted in the TIRF microscope apparatus, these cells were treated with tetramethylrhodamine-labeled α-bungarotoxin (R-Bgt) (20) to visualize AChR by fluorescence as previously described (4). The other culture type was a normal human skin fibroblast line grown on the 32.5-mm cover slips in a medium of the same composition as used for the rat myotubes. The human fibroblasts were labeled by 3,3′-dioctadecyclindocarbocyanine (dil) by incubating the cells for 10 min at 37°C in 1 ml of Hanks' balanced salt solution to which was added 10 μl of 0.3 mg/ml dil in ethanol. Dil becomes incorporated into the plasma membrane bilayer with an orientation similar to that of the phospholipids (21) and becomes internalized into the cytoplasm rather slowly. Cells of both types were washed with Hanks' balanced salt solution many times before observation of fluorescence.

The TIRF apparatus, shown in Fig. 1, is based on the stage of an inverted fluorescence microscope (Leitz Diavert). The glass cover slip with the adherent cells is removed from its culture dish, wiped dry on its bottom surface, inverted, and placed in a glass-cover-slip-bottomed 35-mm dish mounted on the microscope stage. A thin Teflon spacer separates the bottom cover slip sealed in the dish from the inverted cell cover slip. (The Teflon spacer used to produce the photographs in this paper was 0.24 mm thick.) The region between the cover slips is filled with Hanks' balanced salt solution. A 1-cm^3 fused quartz cube is placed in optical contact with the upper surface of the cell cover slip via a thin intervening layer of glycerol.

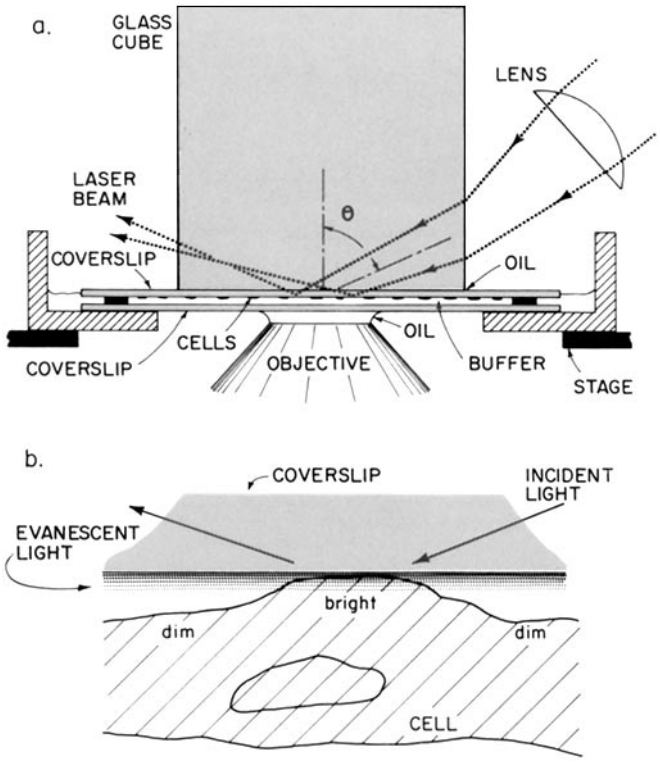


FIGURE 1 TIRF microscope apparatus for viewing cells in culture. (*a*) The optical system on the microscope stage, showing the direction of laser illumination and the position of the cover slip with adherent cells with respect to the glass (e.g., fused quartz) cube and the objective. (*b*) Magnified schematic of the evanescent wave at the cell cover slip/solution interface exciting fluorescence of those portions of a cell in close contact with the cover slip.

An argon ion laser beam (wavelength 514.5 nm and power 0.2 W) is directed obliquely downward toward the quartz cube at a large enough angle of incidence so that the beam is totally internally reflected at the cell cover slip/Hanks' balanced salt solution interface and forms an evanescent wave in the solution near the surface. A converging lens in the beam path just before the cube serves to reduce the area of illumination and thereby increase the central intensity; the actual focal point of the lens need not be at the totally internally reflecting interface. An objective below the stage collects fluorescence excited by the evanescent wave. (Scattered incident light is filtered out by the conventional dichroic mirror and barrier filter combination of the epifluorescence illuminator upon which the objective is mounted.) With a sufficiently thin Teflon spacer between the cover slips (i.e., ≤0.10 mm), even the highest-aperture, shortest-working-distance liquid-immersion objectives can be employed successfully in this apparatus.

The dish and cell cover slip can be moved laterally by the stage translator, thereby enabling the field of view to be scanned. The cube is mounted on the microscope stage via separate translators and is normally kept fixed in position during cell scanning to preserve the alignment of the illumination from field to field.

In the system employed here, the glass cover slip, the quartz cube, and the intervening glycerol layer are not exactly matched in refractive index. For Eq. 1 to be valid, refractive index n_1 need only apply to the same material in which the angle of incidence θ is measured. The θ quoted in the figure legends is the angle between the beam in the quartz cube and the normal to the liquid/glass interface; d is calculated using $n_1 = 1.44$ for the quartz cube.

The prism that is in optical contact with the cover slip need not be cubical. Also, the prism, cover slip, and intervening oil may be composed of materials other than those employed here. In general, the higher the index of refraction of these materials, the shallower the depth d of the evanescent wave. In an extreme case, a titanium dioxide prism and slide (refractive index = 2.4) contacted by a suitably high index of refraction oil, with $\theta = 75°$ and $\lambda \cong 500$ nm, would produce an evanescent wave depth $d \cong 20$ nm in water.

The TIRF system described here is compatible with fluorescence excitation through the objective simply by redirecting the laser beam (or directing an independent light source) through the conventional epifluorescence illuminator. The system is also compatible with transmitted illumination systems such as phase contrast or schlieren optics (22) with a light source and condenser in their normal positions above the total internal reflection cube.

RESULTS

Fig. 2 shows a portion of a primary rat myotube labeled by R-Bgt in the vicinity of an endogenous AChR patch, illuminated respectively by TIRF, epifluorescence, and phase contrast. The TIRF photograph shows only patches; fluorescence from diffusely distributed AChR, cellular debris, and myotube interior is almost entirely absent. The prominence of the endogenous patches in TIRF demonstrates their close proximity to the substrate, as has been observed by other techniques (3, 4).

Fig. 3 is a low magnification view of human skin fibroblasts labeled by dil, as illuminated by TIRF, epifluorescence, and phase contrast. The TIRF photograph shows that contact points tend to occur in lines or bands that run roughly parallel to the straight or gently curved edges of the cells.

Fig. 4 is a higher magnification view of dil-labeled human fibroblasts under TIRF illumination at two different angles of incidence θ. The top photograph, utilizing a larger θ and hence a shallower evanescent wave, shows a rather selective fluorescence excitation in the bands of closest surface contact. The lower photograph, utilizing a smaller θ just slightly greater than the critical angle and a correspondingly deeper evanescent wave, shows a more extensive illumination of the cell's substrate-facing surface, although some areas are still too distant to become excited.

Figs. 3 and 4 are somewhat reminiscent of the patterns obtained by interference reflection contrast microscopy (16–18), except that interference reflection contrast requires no fluorescent labeling and shows cell-substrate contacts as dark regions against a bright background. The range of distances

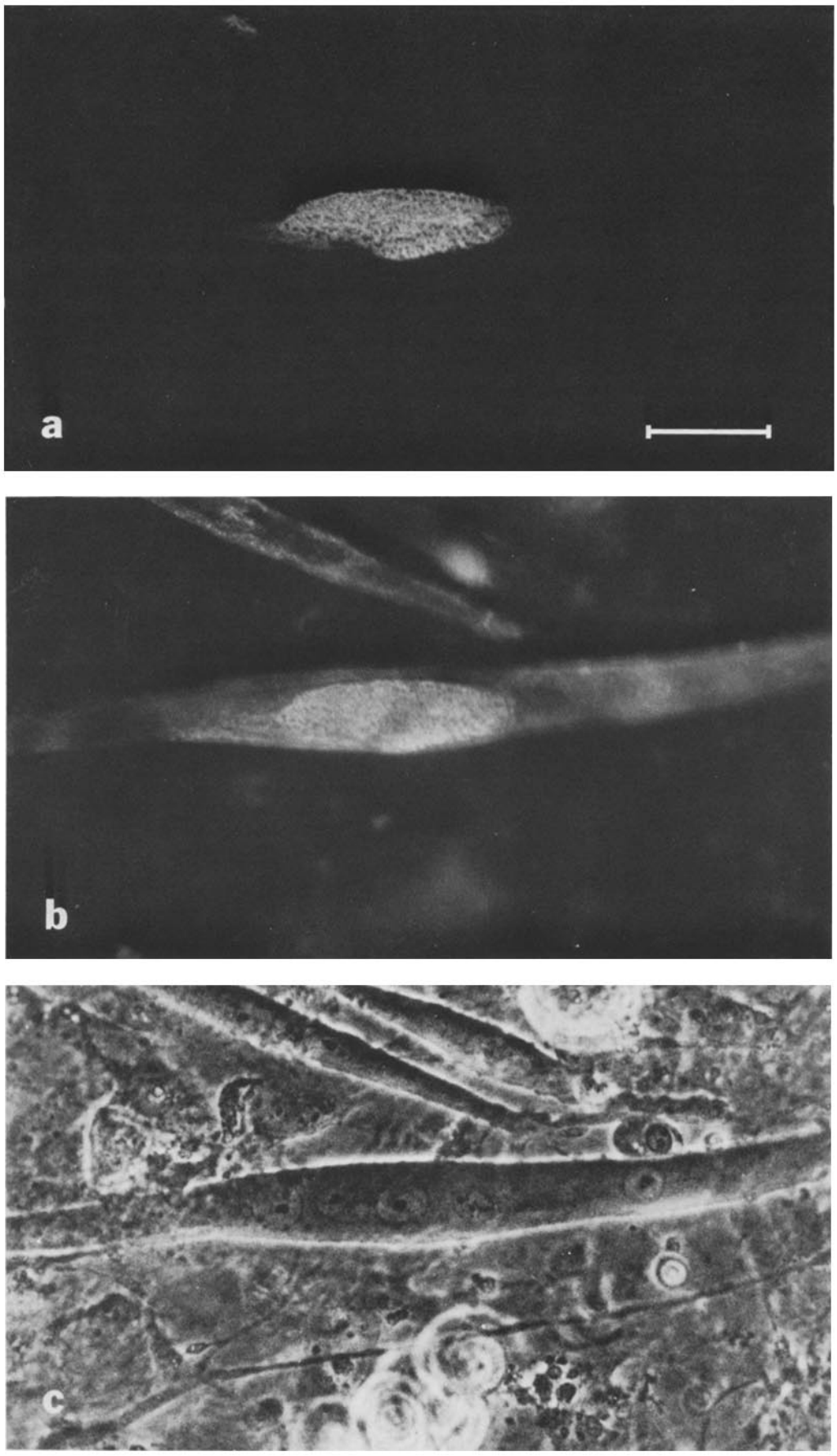
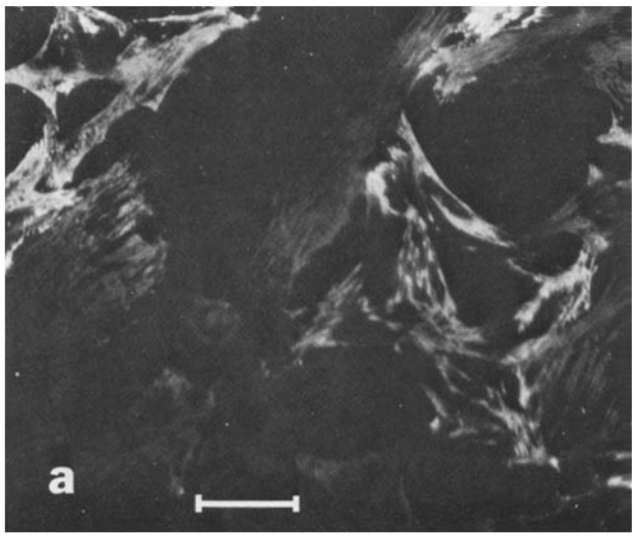
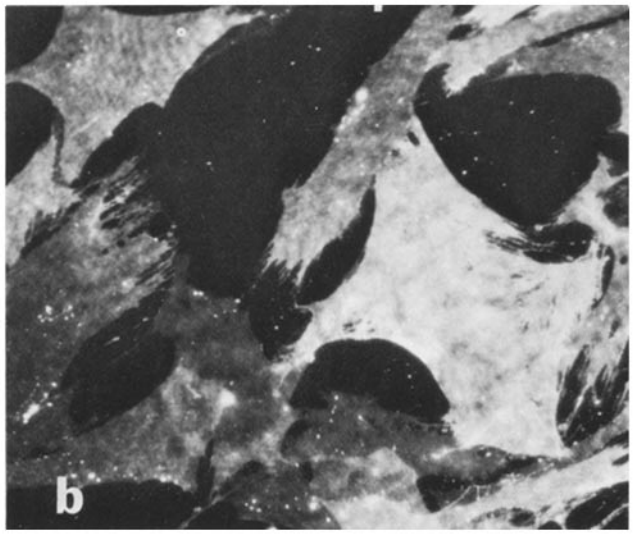


FIGURE 2 Rat myotube in primary culture, labeled with R-Bgt. (*a*) TIRF. $\theta = 72.5°$; $d = 120$ nm. Note that only the large AChR patch in the middle of the field and a small one near the top become visible. (*b*) Epifluorescence of the same field. (*c*) Phase contrast of the same field. A × 40 phase-contrast water-immersion objective with a numerical aperture of 0.75 and a free working distance of 1.4 mm was used. Bar, 30 μm.





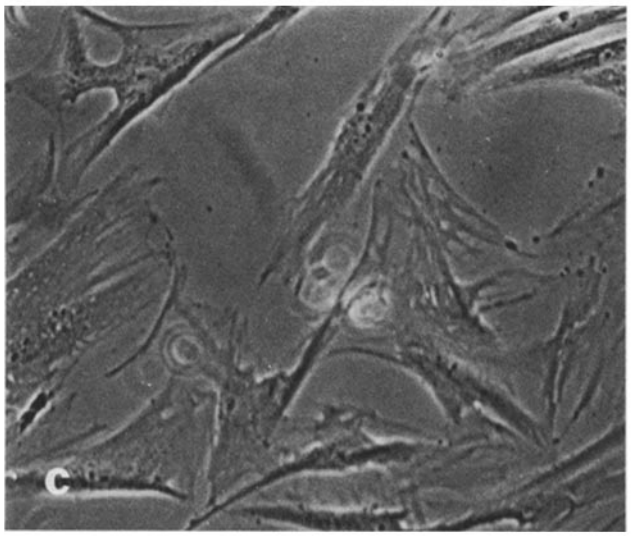


FIGURE 3 Human skin fibroblasts labeled with dil and viewed at low magnification. (a) TIRF. $\theta = 72.5°$; $d = 120$ nm. (b) Epifluorescence of the same field. The variable fluorescence from cell to cell is characteristic of dil labeling. The somewhat mottled intensity and interference fringe pattern is a result of the coherence of the laser illumination. (c) Phase contrast of the same field. A × 10 phase-contrast air-immersion objective with a numerical aperture of 0.25 and a free working distance of 7.1 mm was used. Bar, 100 μm.

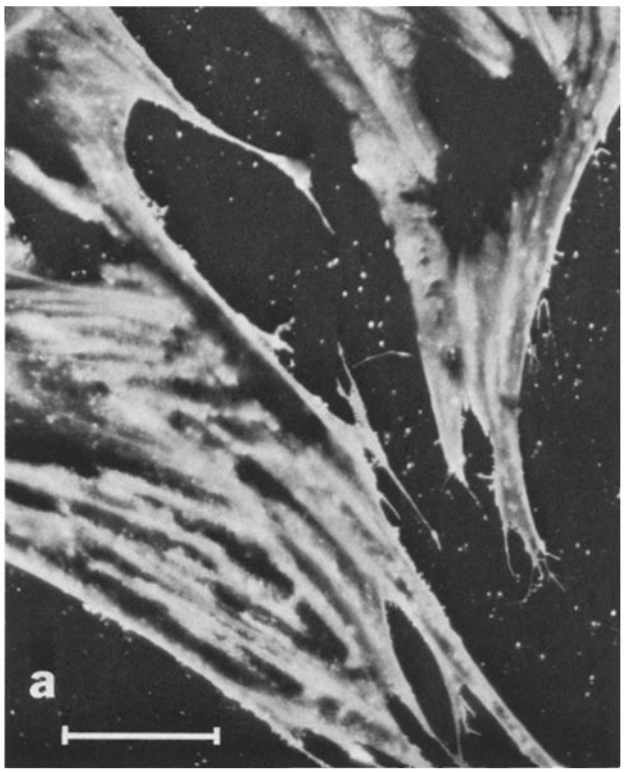


FIGURE 4 Human skin fibroblasts labeled with dil and illuminated by TIRF at two different angles of incidence. (a) $\theta = 74.3°$; $d = 105$ nm. (b) $\theta = 67.9°$; 406 nm. The critical angle for refraction in our system is $\theta_c = 67.5°$. A × 50 water-immersion objective with a numerical aperture of 1.00 and a free working distance of 0.68 mm was used. Bar, 30 μm.

between cell and substrate to which the two techniques are sensitive is comparable (16). TIRF is best suited for examination of specific label-receptive molecules or probes in the contact region.

DISCUSSION

This paper presents a novel fluorescence illumination system for cell cultures that selectively excites fluorescent molecules in regions of close contact with the substrate. The features of TIRF lead to potential applications as follows: (*a*) TIRF greatly reduces fluorescence from cytoplasmically internalized label, cellular debris, and autofluorescence in thick cells, relative to fluorescence from membrane regions close to the substrate. This feature may allow detection of lower concentrations of fluorescence-marked membrane receptors than would otherwise be possible. (*b*) Study of the submembrane structure of the cell-substrate contact in thick cells can be facilitated. A fluorescently labeled cytoskeletal structure in the contact region can be visualized without interference from an out-of-focus background from fluorescent cytoskeletal structure farther from the substrate. (*c*) By varying the incidence angle, the topography of the membrane facing the substrate can be mapped. (*d*) Reversibly bound fluorescent ligands on membrane receptors might be visualized without exciting background fluorescence from unbound ligand in the bulk solution. In this manner, certain cell surface receptors might be studied without the necessity of blocking them by irreversible antagonists.

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