Lighting up the cell surface with evanescent wave microscopy

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Evanescent wave microscopy, also termed total internal reflection fluorescence microscopy (TIR-FM), has shed new light on important cellular processes taking place near the plasma membrane. For example, this technique can enable the direct observation of membrane fusion of synaptic vesicles and the movement of single molecules during signal transduction. There has been a recent surge in the popularity of this technique with the advent of green-fluorescent protein (GFP) as a fluorescent marker and new technical developments. These technical developments and some of the latest applications of TIR-FM are the subject of this review.

The old adage 'beauty is not skin deep' can be misleading. On the contrary, fascinating cellular processes, such as membrane docking and fusion can be revealed if one literally takes a more 'superficial' view. The specialized technique of evanescent wave or total internal reflection fluorescence microscopy (TIR-FM) specifically illuminates fluorophores in a thin optical plane on the coverslip and provides vertical resolution and signal-to-noise (S/N) ratio that is unmatched by any other light microscopy technique; even single fluorescent molecules can be observed and their dynamics and kinetics studied.

Recent developments in TIR-FM and related technologies are providing exciting new opportunities for exploring the cell and its molecules in vivo and in vitro. This review gives a brief description of TIR-FM, with emphasis on practical considerations, recent exciting applications (especially in vesicle fusion and single molecule detection) and a prediction of what lies ahead. Interested readers are encouraged to read some of the excellent in-depth reviews on TIR-FM theory\(^1\), practical implementation\(^1,2\), single molecules\(^3,4\) and its other applications\(^5-7\).

What is total internal reflection?

The optical phenomenon of total internal reflection (TIR) can be observed in everyday life, from fibre optics to sparkling diamonds. The principle, based on Snell’s law, is straightforward (Box 1): if light travelling in a dense medium (high refractive index, \(n_1\)) strikes a less dense medium (of lower refractive index, \(n_2\)) beyond a certain ‘critical angle’, \(\theta_c\), the light will undergo TIR. This critical angle depends on the relative refractive indexes of the two media. If the \(n_1/n_2\) ratio is very small, such as occurs at an air–diamond interface (1.0/2.4), the critical angle is shallow (\(\theta_c = 24.6^\circ\)) and TIR is easily achieved. Jewellers capitalise on this trick by cutting diamonds so that TIR occurs multiple times, ‘trapping’ the light before it refracts out of the top face.

Fluorescing footprints in the dark

Biologists, too, capitalize on TIR. In practice, cells are grown on glass coverslips or transparent materials of high refractive index (e.g. \(\alpha\)-corundum – \(\text{Al}_2\text{O}_3\)), and a beam of light, usually from a laser, is optically coupled into the coverslip by a prism or the objective itself (Box 2). If light approaches the aqueous medium at \(\theta > \theta_c\), it totally reflects into the glass; however, if the light ‘rays’ (which are really electromagnetic wave fronts) simply bounced off the interface like a mirror, this would neither illuminate the cells, nor the biologists. In fact, at angles \(\theta > \theta_c\), some of the energy slightly penetrates the aqueous medium as an ‘evanescent wave’, propagating parallel to the interface due to ‘near field’ effects. This seemingly mysterious ‘tunnelling’ effect can be derived from Maxwell’s equations on the behaviour of electromagnetic fields at a dielectric interface. Axelrod et al. explain the theory in detail\(^8\) and interactive films are also available on the internet (see http://www-optics.unine.ch/research/microoptics/RigDiffraction/main.html and http://www.phy.ntnu.edu.tw/java/indexLayer.html#).

An important property of the evanescent wave is that the intensity falls off exponentially away from the coverslip (Box 1). The theoretical ‘penetration depth’ (the distance where the intensity has decreased to \(I/I_0\)) depends on the incidence angle, wavelength and polarization of light, as well as the refractive index of the coverslip and medium. Penetration depths of \(<100 \text{ nm}\) are easily achieved. The net effect is that only fluorophores near the coverslip, corresponding to the ‘footprint’ of the cell, are excited; for this reason, one of the first applications of the technique was to monitor cell–substrate contacts (for reviews, see Refs 5 and 6).

Advantages of TIR-FM

It is the ability to make an optical slice with the dimensions of a thin electron microscopy (EM) section that lies at the heart of TIR-FM applications. How does this technique compare with much more familiar confocal systems? TIR-FM typically illuminates a vertical slice of \(<100\text{ nm}\) as opposed to a slice of \(~500–800\text{ nm}\) for 1- and 2-photon confocal systems, respectively. This thin optical sectioning means that...
the S/N ratio is much better than with confocal images, and cellular photodamage and photobleaching are minimal (but minimizing focal plane drift is also more crucial). TIR-FM images are captured frame-by-frame with charge-coupled device (CCD) cameras. These are sensitive and/or fast (but rarely both) and can now reach up to ~80% quantum efficiency and speeds of ~200 Hz (frames/s). Image intensifiers are required for single molecule sensitivity or can be used to minimize exposure times when imaging live cells. By contrast, most confocal systems scan the sample pixel-by-pixel, reject light with the pinhole and use photomultipliers. Generally, frame rates are slow (~0.1–5 Hz) and photon detection efficiency is relatively low. Nipkow disc-based confocal systems use video cameras for detection and can reach ~10-fold faster frame rates; however, the most important difference is that a confocal section is much thicker than an evanescent field.

But TIR-FM is not necessarily the panacea. Confocal microscopes can generate deep three-dimensional (3D) images of cells and photobleach areas of interest. Rather, TIR-FM is a complementary approach that can be combined with other microscopy techniques, such as brightfield, epifluorescence (EPI), confocal, fluorescence correlation spectroscopy (PCS), atomic force microscopy (AFM) and fluorescence lifetime imaging microscopy (FLIM), to mention a few.

The recent surge of reports using TIR-FM might create the impression that it is a brand new technique. In fact, the basic principles and approaches were described and applied in the early 1980s, championed largely by the efforts of Daniel Axelrod and other biophysicists. So why the lag in its popular application to cell biology? First, looking at fluorescent markers in live cells was difficult until the recent advent of green fluorescent protein (GFP) and its cyan, yellow and red derivatives (CFP/YFP/DsRFP). Second, choosing and implementing a TIR-FM system, although not difficult, is not trivial either. In this regard, the recent introduction of new objective lenses, condensers and other materials affords greater opportunities.

**TIR-FM practical considerations**

Most TIR-FM set-ups are custom built and numerous configurations are possible. The choices can be overwhelming. Does one need an inverted or upright microscope? Should one use mirrors or fibre optics to guide the light? Will the light be coupled through a prism or an objective (‘prismless’) type set-up? Unfortunately, there is no single ‘right’ solution; the optimal set-up depends on the needs of the user. Nevertheless, some rules and non-obvious issues should be borne in mind.

**Box 1. Some key TIR formulas**

**Snell’s law:**

\[
\frac{n_1 \sin \theta_1}{n_2} = \frac{n_2 \sin \theta_2}{n_1}
\]

\(\theta = \text{angle of incidence, } n = \text{refractive index}\)

**Critical angle:**

At the critical angle, \(\theta_c, \theta_c = 90^\circ; \sin 90^\circ = 1; \frac{n_1 \sin \theta_c}{n_2} = \frac{n_2}{n_1}\)

If \(n_1 = 1.515\) and \(n_2 = 1.36\), \(\theta_c = \sin^{-1}\left(\frac{1.36}{1.515}\right) = 63.85^\circ\)

**Evanescent field:**

\[
I_z = I_0 \exp\left(-\frac{z \lambda}{d_p}\right)
\]

\(d_p = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta_1 - n_2^2}}\)

\(I = \text{intensity, } z = \text{distance, } \lambda = \text{wavelength, } d_p = \text{penetration depth}\)

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**Box 2. Two common TIR-FM set-ups**

Cells are plated on coverslips (dashed black line; Fig. 1) and placed in a chamber filled with dye-free medium or aqueous buffer. In the prism-type set-up (a) excitation light from a laser (green) strikes a prism (shown as a half-cylinder here) that is optically coupled to the coverslip by immersion oil or other liquid of matching refractive index (yellow). The decaying evanescent field illuminates the cells. Light emitted from the fluorophore (red arrow) is collected in a high NA water immersion lens. Note that access to the sample is partially blocked by the objective. In the objective-type set-up (b), there is full access to the cell. However, unlike the prism-type set-up, excitation light and emitted light are both collected by the objective and must be blocked by appropriate dichroic and emission filters (not shown).

$$\alpha = n \sin \alpha,$$

where $n$ is the refractive index of the medium between the object and the lens and $\alpha$ is half the intake angle of the lens.

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microinjection and other techniques. In prism-type set-ups, the water-immersion objective can hamper such manipulation, and in inverted microscopes, closed chambers must be used. Other advantages of objective-type set-ups are that more emitted light is detected and its intensity drops off monotonically with distance. For prism-type set-ups, the incidence angle of the laser beam can be varied precisely over a wide range and the penetration depth is therefore well controlled. By contrast, with objective-type set-ups it is more difficult to control the angle of incidence precisely. In fact, with standard high numerical aperture (NA) oil-immersion objectives, TIR is difficult to achieve at all. First, for TIR to occur, there must be a difference in refractive indexes. For objective-type set-ups, this means that the NA of the lens must be greater than the refractive index of the cell cytosol (1.36–1.38). With a 1.4 NA lens, only ~2.8% of the aperture (on the periphery) can be used for TIR. In practice, the critical angle is just surpassed (~1.9° beyond); therefore, correctly coupling the laser into the objective is tricky. Better results are achieved with a new 1.65 NA lens from Olympus. However, special high refractive index coverslips (which are expensive) and immersion oils (which are toxic) must be used. Lenses of 1.45 NA that are compatible with standard coverslips and immersion oils should be available soon from several manufacturers including Olympus and Zeiss. Importantly, these 1.45 and 1.65 NA objectives allow ~10% and ~25% of the aperture, respectively, to be used for TIR over a larger range of angles, which is a considerable improvement.

**Observing vesicle fusion**

There is an element of truth to the statement ‘you have to see it to believe it’. This is perhaps best exemplified in the field of membrane trafficking and fusion. Hundreds of biochemical, genetic and EM experiments have indicated that regulated and constitutive vesicles traffic to, and fuse with, the plasma membrane. However, these approaches are, to varying degrees, indirect and cannot investigate the complex spatial–temporal dynamics of the process.

Patch-clamping was a technological breakthrough and opened the door for detecting changes in surface area (capacitance) or release of oxidative material with high temporal resolution. From studies of regulated exocytosis in neuroendocrine cells and kinetic modelling, various pools of vesicles, including ‘locked’ and ‘ready-releasable’ pools were inferred. Although very powerful, the approach has its shortcomings; only the fusion event is monitored and prior vesicle trafficking, tethering and docking are not detected. Specificity is difficult as all fusion events are detected and most studies are therefore limited to regulated inducible exocytosis. There is also little spatial information on where exocytosis occurs. By contrast, EM has fantastic spatial resolution, but only gives ‘snapshots’ of the process.

TIR-FM offers an attractive compromise in that good spatial–temporal resolution can be achieved. Chromaffin cells are a good model system as they have the advantage of being generally well characterized and packed full of relatively large (~250 nm diameter) acidic granules that can be labelled with fluorescent acidotropic dyes. Almers and colleagues observed that, after a stimulus, granules fused with the plasma membrane and secreted dye into the medium as a bright ‘puff’ that could be detected by TIR-FM. Simultaneous capacitance measurements and complementary EM studies confirmed that indeed granule fusion was specifically detected by TIR-FM. Moreover, vesicles became exponentially brighter as they approached the plasma membrane and nanometre-sized vertical changes, caused by membrane docking, gave a large increase in fluorescence; an optical property unique to TIR-FM (but see caveats with prism-type set-ups). Since Almers’ TIR-FM study of exocytosis, a rapidly increasing number of TIR-FM studies with refined techniques have exploited this approach for monitoring regulated and constitutive exocytosis.

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Exocytosis in action: kissing, hotspots and fusion
To give an overview of the potential of this technique, some major and unexpected findings from the aforementioned exocytosis studies are summarized. The first characteristic is that vesicles, whether granules, constitutive vesicles or synaptic vesicles, can be followed from where they first dimly appear in the evanescent field (~100–400 nm) until they fuse with the plasma membrane as bright flashes. Another feature of TIR-FM is that, in calibrated systems, fluorescence changes can be extrapolated to changes in axial position. This means that, although the cells are only imaged in two dimensions, 3D information on the position of vesicles can be obtained. One technical improvement has been the use of acousto-optical deflectors (AODs) to rapidly switch the incidence angle of the laser beam in prism-type set-ups. Another variation is to use dual shutters to switch rapidly between standard widefield EPI and TIR. EPI provides a good overview of events near the plasma membrane and deeper in the cell, whereas TIR fluorescence selectively excites approximately the bottom 100 nm (see Fig. 1). When images of EPI and TIR were false coloured and merged, the entire sequence of late constitutive exocytosis—from cargo exit out of the Golgi, trafficking and fusion—could be followed (Fig. 1d). There are other useful applications of AODs in TIR-FM; they can be used as ultra-fast shutters and can quickly change the excitation wavelength in systems equipped with multi-line lasers.

The tracking of vesicles has led to some surprising observations. Granules and synaptic vesicles were observed to approach the plasma membrane at an angle, presumably guided there by the cytoskeleton. The lateral diffusion of granules and synaptic vesicles decreased approximately five fold when the granules ‘docked’ near the plasma membrane, suggesting restrictive diffusion, perhaps due to the cortical actin meshwork. Interestingly, the ‘priming time’, or time taken to go from a morphologically docked state, varied considerably (−0.25 s for synaptic vesicles, ~1 s for chromaffin granules and ~40 s for constitutive vesicles).

Other surprising discoveries were made. Many constitutive vesicles were ‘docked’, often for several minutes. A significant fraction of docked vesicles did not fuse and detached from the membrane. A dual-colour study indicates that granules can ‘kiss’ the membrane and secrete soluble cargo yet retain membrane proteins associated with the granule, implying a ‘kiss and glide’ model. Partial fusion from the tips of membrane tubules was also observed. Also, when fusion events were positionally mapped,
Table 1. Spectral properties of selected fluorophores

<table>
<thead>
<tr>
<th>Spectral property</th>
<th>Fluorophores</th>
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<tbody>
<tr>
<td></td>
<td>Cy3</td>
</tr>
<tr>
<td>Max absorption $\lambda_{\text{m}}$ (nm)</td>
<td>549</td>
</tr>
<tr>
<td>Max emission $\lambda_{\text{m}}$ (nm)</td>
<td>573</td>
</tr>
<tr>
<td>Extinction coefficient, $\varepsilon$ ($\text{M}^{-1} \text{cm}^{-1}$)</td>
<td>150 000</td>
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<tr>
<td>Quantum yield $\Phi$</td>
<td>0.33</td>
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<tr>
<td>Rate of photobleaching at $10 \text{ MWm}^{-2}$ (s$^{-1}$)</td>
<td>0.007</td>
</tr>
<tr>
<td>Total number of emitted photons/fluorophore $\times 10^6$</td>
<td>38</td>
</tr>
</tbody>
</table>

*Values in brackets refer to the rate of photobleaching and total number of emitted photons/fluorophore $\times 10^6$ at 1 MWm$^{-2}$ (s$^{-1}$). We thank Michael Anson (NIMR, London, UK), who kindly provided the information for this table.

exocytic ‘hot spots’ were discovered in neurons$^{11}$, chromaffin cells$^{17}$ and epithelial-like PtK$_2$ cells$^{22}$ (although these were random in Cos-1 fibroblasts$^{10}$).

These observations with TIR-FM allow new questions to be addressed. How are vesicles directed to these ‘hot spots’? How are the kinetics of docking, priming and fusion modulated? Why do some vesicles escape fusion? Are there molecular kinetic ‘timers’ involved? The answers are not yet clear, but remember that these questions only surfa...
Clearly, in addition to showing where molecules are localized, the visualization of single molecules can offer detailed mechanistic information. In summary, ideal substrate analogues for single molecule studies need not only to be bright and stable but homogeneous and well characterized on the macroscopic level.

Future prospects
What does the future of TIR-FM hold for cell biologists? In our opinion, it will be pushed largely on two fronts: continuing technical advances and the application of TIR-FM to new areas of cell biology. One advance will be the continued merging of TIR-FM with other microscopy techniques such as FCS, AFM, and FLIM. Another will be the ongoing development of dual- and multi-coloured TIR-FM so that multiple dyes can be observed in live cells. Two recent reports have used dual colour emission detection for dyes that can be excited at a single wavelength; in principle, implementing TIR-FM with multiple excitation is also feasible. Improvements in the detection efficiency and speed of cameras as well as the properties of dyes will aid in single molecule detection. The application of fluorophores such as flavin nucleotide analogues and quantum dots for single molecule work offers exciting possibilities because of their unique optical properties and compatibility with biological systems.

A number of interesting applications are possible on the cellular front. Although most membrane trafficking studies have focused on exocytosis, studies of endocytosis with TIR-FM should be possible, and equally exciting. Although for brevity we have not focused on the cytoskeleton here, TIR-FM is clearly an elegant approach for specifically studying the cytoskeleton near the substrate (e.g. see the local illumination of actin and microtubules in Fig. 1). For instance, TIR-FM revealed that ‘comet-like’ actin tails, originating at the plasma membrane, propelled pinosomes into mast cells. Molecular and genetic manipulation of cells, coupled with optical detection and analysis, will further extend the range of the approach.

The fact that many signalling events are initiated by recruitment and/or clustering of lipid rafts underscores the complex lipid and protein dynamics at the membrane. Clearly, there is an enormous potential for using TIR-FM to study signal transduction. For example, single Cy-labelled epidermal growth factor (EGF) molecules were detected by TIR-FM. Binding to the EGF receptor and subsequent dimerization of EGF caused spots to double in intensity and FRET to occur. These two reports are an exciting prelude of what we predict to be a growing field. There is no doubt that live imaging of cellular dynamics near the plasma membrane has a bright future – with evanescent wave technology it appears even brighter.

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References