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Imaging and characterisation of the surface of live cells

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Determining the organisation of key molecules on the surface of live cells in two dimensions and how this changes during biological processes, such as signaling, is a major challenge in cell biology and requires methods with nanoscale resolution. Recent advances in fluorescence imaging both at the diffraction limit tracking single molecules and exploiting super resolution imaging have now reached a stage where they can provide fundamentally new insights. Complementary developments in scanning ion conductance microscopy also allow the cell surface to be imaged with nanoscale resolution. The challenge now is to combine the information obtained using these different methods and on different cells to obtain a coherent view of the cell surface. In the future this needs to be driven by interdisciplinary research between physical scientists and biologists.

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Introduction

Modern biology has advanced to a stage where we now have detailed information about the structure of many of the molecules that make up the living cell, down to the angstrom level. One of the key challenges is to now understand how the molecular components of a cell interact with one another and are organised in two or three dimensions to form a fully functioning living cell. To address this problem and deal with the fundamental heterogeneity that is present in complex biological systems, new biophysical tools are needed that are capable of imaging the topography and function of living cells down to the level of individual proteins and molecular complexes. These methods need to be applied at the resting state and then have sufficient time resolution to follow the changes that take place during key biological processes, such as endocytosis and exocytosis, or signaling.

One main approach taken is fluorescence-based, which was for many years constrained by the diffraction limit of light until recent advances in obtaining subdiffraction limited resolution. The advantage of fluorescence-based approaches is that it is possible to image individual fluorophore-labeled molecules or macromolecular complexes, but the disadvantage is that the cell topography is not generally imaged at the same time and hence one cannot relate the organisation of the labeled molecules to the topography. This is especially important in the context of understanding function at the cell surface because it is becoming clear that its two dimensional organisation is functionally important and occurs on a hierarchy of length scales: protein clustering on the nanoscale, the underlying cytoskeleton on the micron scale and supra-molecular domains on the many micron scale. A highly complementary approach, therefore, is to directly image the cell surface using scanning probe microscopy and then probe and image the cellular function. The challenge here is the softness of living cells and obtaining sufficiently high resolution. Both approaches have the challenge of imaging dynamics on live cells which for many methods can lead to a trade-off between obtaining the image in a short enough time to follow important dynamics versus imaging sufficient points in order to have the required spatial resolution. This review focuses on recent advances in fluorescence and topographic imaging of the cell surface and its function on the nanoscale.

Nanoscale fluorescence imaging

Fluorescence imaging determines the location of fluorophore-labeled molecules and hence is limited by the number of distinctly detectable fluorophores that can be imaged at any one time. Recent advances have enabled nanoscale fluorescence imaging [1,2]. One microscopy is based on stochastic imaging of single photoswitchable fluorophores [3,4] where individual fluorophores are successively switched on, imaged and their position determined to build up the overall image; photoactivated localisation microscopy (PALM) [5•,6] or stochastic optical reconstruction microscopy (STORM) [1,7,8•,9]. The other microscopy is stimulated emission depletion (STED) microscopy [10,11] that effectively reduces the size of the imaging laser spot to obtain super resolution. Using STORM, PALM or STED, fluorescently labeled molecules have been resolved at 10 nm resolution or better. The single molecule approaches are largely limited to imaging the basal surface of the cell owing to

the need to use total internal reflection geometry in most cases. There is the concern that cell topography or structure may be altered by contact with the glass surface, especially in cases where the cell spreads over the surface to form a good contact with the glass, yielding better signal to noise. By contrast, the STED approach is similar to confocal microscopy in terms of the samples that can be imaged, allowing images of the apical cell surface to be obtained. It is also possible to image at video rates [12•] in contrast to a frame rate of around 10 s per frame at best with PALM. One concern with STED, however, is that the high laser powers required may lead to photo-damage in experiments on live cells. Both methods have the general issue of how the molecule of interest is labeled and this often involves the transient expression of auto-fluorescent proteins, leading to increased expression of the protein of interest and potential changes in behaviour that are not relevant at physiological levels of expression. One method that has the potential to solve this problem is dStorm [13,14•], which uses normal dye fluorophores and a reductant in solution for photoswitching [15] and could in principle be used with fluorophore-labeled Fabs to study endogenous proteins on the cell surface. The other main issue for these methods is that the cell topography is not measured at the same time as fluorescence imaging. This makes it more difficult to relate the structure imaged to the overall organisation of the cell surface. This could be tackled by simultaneously imaging the cell membrane using a lipid dye or the actin cytoskeleton but at present, while two colour super-resolution imaging has been performed, this is not routine yet. One method that has been developed is based on measuring the position of two fluorophores in the z direction with about 10 nm precision, differential nanometry, and this has been applied to follow the relative displacement of key molecules involved in clathrin mediated endocytosis with one-second time resolution [16].

There have been two recent notable biological applications of these super-resolution methods. First, Hell and co-workers [17••] used STED to directly image so-called lipid rafts, showing that these are small ($\leq$20 nm) and shorter-lived ($\leq$20 ms) than widely expected. Second, Mark Davis and co-workers used PALM to image T-cell receptor (TCR) organisation on T-cells five minutes after contacting non-activating or activating lipid bilayers, which revealed that the receptors were present in small clusters of 5–20 complexes on the cell surface [18••]. Here it would have also been useful to know the location of the cell membrane in case the clusters detected just represented regions of close contact between the cell and the surface, where diffusion is hindered. In addition this result is in disagreement with measurements performed at the apical cell surface [23,26]. Most importantly, however, this experiment shows the potential of super-resolution methods to reveal the nanoscale organisation of proteins at the cell surface. Both examples

illustrate the potential of super-resolution imaging to now tackle key biological questions on the organisation of the cell membrane.

Probing protein organisation in the cell membrane

Perhaps the single most important piece of information is to know whether given proteins need to form stable complexes with other molecules in order to function, or whether they function autonomously. This issue has become especially controversial in the context of the cell surface, where, in some cases, it is unclear whether important groups of proteins are functional monomers or dimers or, in other cases, whether they are pre-clustered in protein islands. While the super-resolution imaging techniques are relatively new there has been much recent progress in exploiting diffraction limited two-colour single molecule fluorescence imaging and the complementary method of resonance energy transfer to address this issue.

Single molecule imaging is limited to protein densities below about 2 molecules/μm^2, although many proteins are present at the cell surface below this level and methods are available using antibodies to label endogenous proteins. Schutz and co-workers have recently developed a method allowing single-molecule analysis above this limit [19,20]. This involves bleaching all the molecules in a region and then analyzing the diffusion of labeled molecules back into this region at early times, when the density is lower. The methods used to analyse the labeled molecules at low densities employ similar principles and are based on single or dual-colour tracking of individual molecules using total internal fluorescence imaging. An example of the principle of one such approach, dynamic single-molecule colocalisation (DySCo; [23]) is shown in Figure 1. Associated molecules will either be brighter than monomers or give co-localised fluorescence from the two distinct fluorophores. The increase in brightness or colocalisation may be short lived if transient dimers are formed, allowing the lifetime to be determined. Recent work has focused on G-protein coupled receptors (GPCRs) [21•,22•], owing to their importance as drug targets, on the stoichiometry of the TCR in its resting state using DySCo [23], and on lipid raft-like structures [24•]. Two recent GPCR studies suggest the formation of transient dimers by labeled-ligand bound GPCRs, which last about 100 ms before dissociating [21•,22•]. Caveats with these analyses are the extent to which the observed behaviour is contingent on the cell responding to contact with the glass and confirming that the two GPCRs actually come into contact. Recent work by Schutz suggested that the presence of long-lived nanoscale platforms for a model GPI anchored protein, lasting about 2 s in contrast to data suggesting that raft-like structures are short-lived [24•]. On the apical surface confocal microscopy has been used to determine

Figure 1
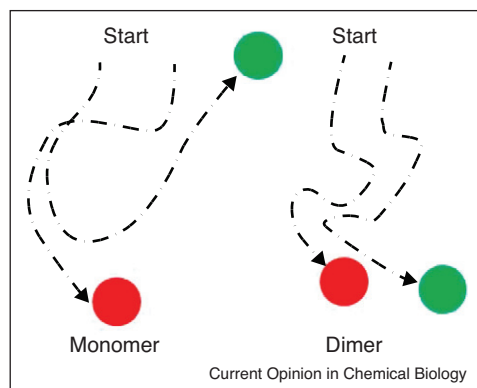
Principle of the DySCo method. Unassociated molecules show little correlated motion (*left*) whereas associated molecules track within a short distance of one another for multiple frames (*right*). Unassociated molecules may track together by chance over a short distance (*left*, middle of tracks) but the probability of this occurring for multiple frames is small.

the oligomerisation state of proteins [26•]. Here coincident bursts of fluorescence are detected when associated molecules pass through overlapped focused laser beams allowing them to be detected and analysed [25]. This has been used to show that on the apical surface of the T cell the mobile form of the TCR is monovalent rather than consisting of 'dimers of dimers' [26•]. The advantage of taking measurements at the apical surface is that it might prove to more faithfully reflect the 'resting state' of the cell surface.

Resonance energy transfer techniques, since they rely on non-radiative energy transfer between donor and acceptor fluorophores over distances less than ~100 Å, are complementary methods to detect the association of proteins, which are ideally suited to identifying very short range protein interactions, such as those present in protein oligomers [27]. Bioluminescence resonance energy transfer (BRET utilises luciferase-coupled donors and GFP-coupled acceptors. Compared to other types of RET experiment, such as fluorescence RET (FRET), BRET has the advantages of a very high signal/noise ratio gained from luminescence detection and the fact that it is unaffected by photobleaching, autofluoresence or other optical effects [27]. A key issue for all RET experiments, however, is that background signals may arise from random interactions if the levels of donor and acceptor levels are high. This is particularly problematic in situations where the transfer efficiency for oligomeric interactions is comparable to those arising from random interactions, because the subunits are large or interact weakly, making discrimination between random and oligomeric interactions difficult. These problems are well illustrated by studies of G protein-coupled receptors, whose widely accepted capacity to form oligomers on the basis of BRET experiments [28,29] has recently been questioned. *Bona fide* oligomeric interactions can, however, be readily identified by using existing theoretical principles [30–32] in two types of BRET experiments [33•]: in the first type, the combined number of donors and acceptors is held constant, while in the second type the expression level is varied and the acceptor/donor ratio kept constant. The principle underlying the first type of experiment, i.e.

Figure 2
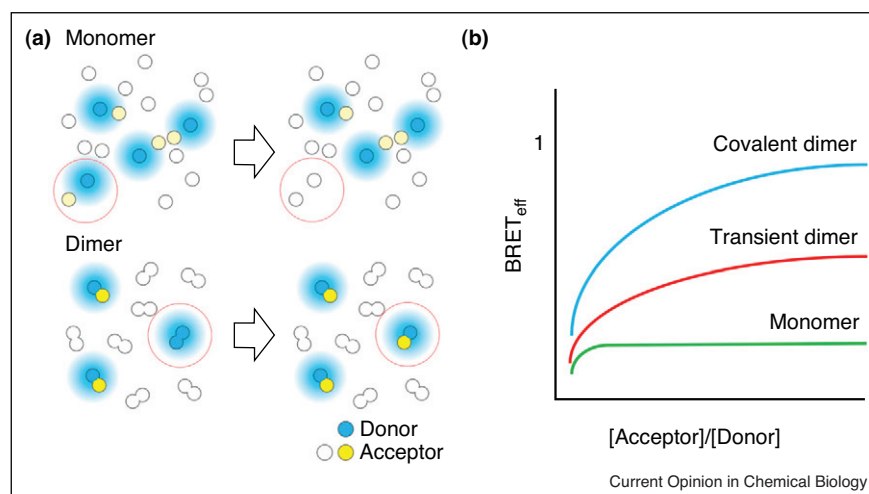
Contrasting dependence of bioluminescence resonance energy transfer efficiency ($BRET_{eff}$) on acceptor:donor ratio for dimers and monomeric proteins in a 'type 2' BRET experiment [31]. **(a)** In the experiment shown, the acceptor/donor ratio is increased by replacing one of four donors with an acceptor, *keeping overall expression level constant.* For the monomers (upper panel), at relatively high acceptor/donor ratios the $BRET_{eff}$ (i.e. amount of energy transfer per donor) remains constant because the 'acceptor environment' of the remaining three donors is unchanged. Conversely, for the dimers, $BRET_{eff}$ doubles as an unproductive donor/donor dimer becomes a productive donor/acceptor dimer. **(b)** Graphical representation of the relationship between $BRET_{eff}$ and the acceptor/donor ratio when the latter is varied systematically.

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the less intuitive of the two approaches, is shown in Figure 2. These applications of the theory were disputed [34,35], but control experiments with very well characterised known monomers and dimers had already borne out their implementation [33$^\bullet$], as had similarly constructed FRET experiments [36]. Furthermore, the most recent work on GPCRs utilizing single molecule fluorescence-based methods, as discussed above, has also ruled out constitutive dimerisation [21$^\bullet$,22$^\bullet$]. Overall, we suggest that conclusions regarding their organisation are so significant in terms of understanding receptor function that they ought not to be based on only one analytical approach and hence there is a requirement to use multiple methods to confirm a given finding.

Imaging the cell topography

Scanning ion conductance microscopy (SICM) and atomic force microscopy (AFM) are both forms of scanning probe microscopy (SPM) that can be used to image the topography of live cells. SICM, originally developed by Paul Hansma et al. [37], is based on a nanopipette in a conducting solution, normally physiological buffer (Figure 3). The pipette is made by melting capillary glass normally under computer control using a commercial pipette puller. The pipette inner radius, which determines the resolution is typically between 10 and 50 nm and depends on the melting temperature of the glass used. The application of a voltage, typically a few hundred mV, between an electrode in the pipette and in

Figure 3
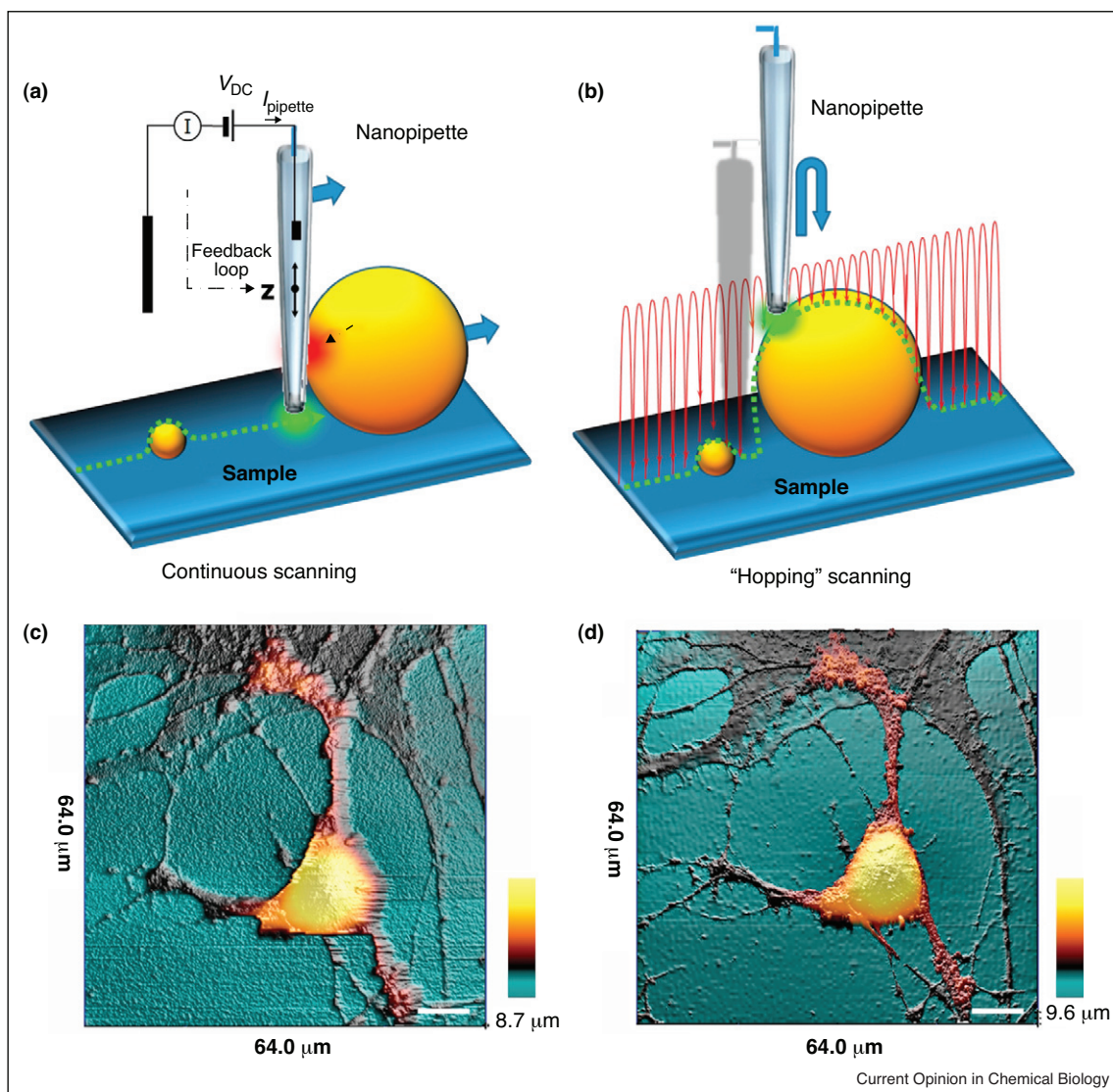
Principle of hopping mode ion conductance microscopy. (**a**) Using continuous distance feedback control the pipette collides with a spherical object possessing a steep vertical slope. (**b**) In hopping mode the pipette is withdrawn to a position well above the sample before approaching the surface allowing a spherical object to be scanned. (**c,d**) Topographical images of the same fixed hippocampal neuron obtained first with hopping mode (**d**) and then with continuous scanning (**c**), using the same nanopipette.

the bath leads to a flow of Na^+ and Cl^- ions to the electrodes. The ion flow is firstly limited by the small aperture at the tip of the pipette and is further reduced as the pipette approaches the cell and ion flow is shut down. This reduction in ion flow as the pipette approaches an ion-impermeable surface is used for distance feedback. Typically the pipette approaches the surface so that the ion current has been reduced by 0.1–1% from the limiting current when far from the surface [38]. The major advantage of this method is that the nanopipette can sense the presence of the surface when still an inner radius away, typically 10–50 nm, so there is no direct contact with the soft cell surface and the forces exerted on the cell when scanning are negligible. A common misconception, owing to its name, is that the method maps surface conductivity but in the high-salt conditions used for imaging the Debye length is less than 1 nm so the method is insensitive to differences in surface charge or the opening of ion channels, and the distance feedback only responds to changes in surface topography.

Like all SPM methods, because the SICM probe senses locally at the tip it is not possible to scan highly con-voluted surfaces since the side of the probe may touch the sample before the tip has sensed the presence of a surface. To address this issue SICM has recently been modified so that the distance feedback control is not continuous and the pipette always approaches the surface from above (Figure 3). This has enabled complex neuronal networks to be scanned [39•]. The advantages of this approach are clearly shown in Figure 3 where topographic features are much more clearly resolvable. Since the pipette is no longer raster scanned over the surface it also becomes possible to use adaptive resolution where the pipette makes fewer or more measurements of the surface topography depending on the surface roughness. This has two advantages: firstly it becomes possible to perform a lower-resolution scan to identify the region of interest and secondly the imaging time is reduced significantly.

The other important recent development in the use of SICM is that it is now being used to image cellular function as well as topography as shown in Figure 4. The pipette probe has previously been used for local delivery [40] and single channel recording [41] but its combination with an electrode to measure local chemical

Figure 4
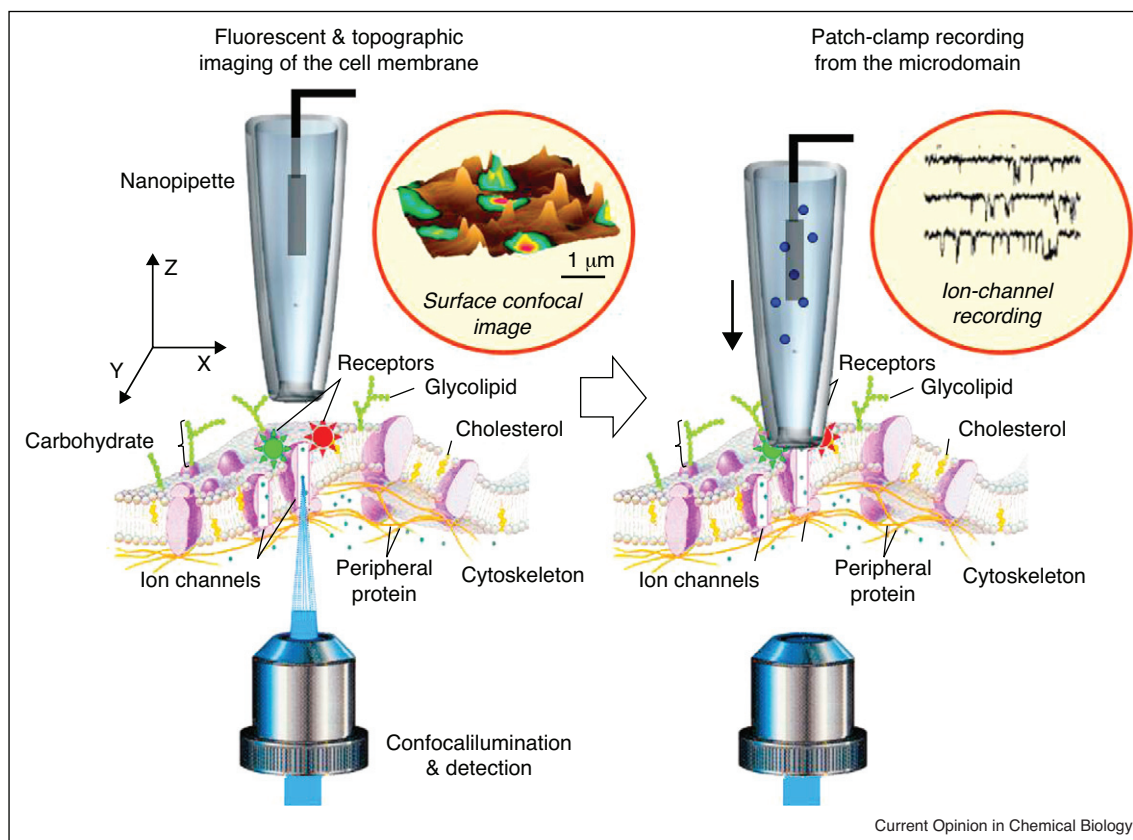
Scanning ion conductance microscopy can be combined with confocal fluorescence imaging to obtain simultaneous topographic and fluorescence images of the cell surface. The same nanopipette can be used to image function by detecting ion channels in the cell membrane.

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fluxes, scanning electrochemical microscopy, opens up a range of new possibilities in the nanoscale mapping of chemical species and fluxes at the cell surface [42]. The local delivery of reagents has been used to map β2 adrenergic receptors (β2AR) on live heart cells by using fluorescence to detect where local application of ligand from the pipette led to production of cAMP inside the cell [43••]. Since the topography of the cells was measured at the same time this allowed the receptors to be localised to the t-tubules in normal heart cells. In diseased cells this organisation was lost.

Atomic force microscopy is also widely used to image cells although often the images thus obtained show the underlying cytoskeleton suggesting deformation of the cell membrane. This is a strength and a weakness since it is often useful to know the location of the cell cytoskeleton but on the contrary the deformation can result in mechanical stimulation of the cell. A recent study by Shaffer and co-workers has directly compared AFM and SICM imaging of the same fixed mammalian cells and this shows that SICM deforms the cells far less than AFM [44•]. Since the Young's modulus of a live cell is an order of magnitude less than fixed cells this study shows for the first time that SICM is the best SPM method for minimal deformation of the cell surface when imaging cell topography. However, for mechanically harder cells such as yeast or bacteria AFM has the advantage of higher resolution and with the use of functionalised tips allows the distribution of single polysaccharides and proteins to be mapped [45–47].

Future outlook

Recent advances in scanning probe microscopy and fluorescence microscopy mean that nanoscale imaging of the surface of live cells is now possible with a spatial and temporal resolution that allows biological problems to be tackled. The challenge now is to exploit these methods to provide new biological insights. Here it is not only the performance of the methods that is important but also their reliability and robustness in order to be able to perform experiments routinely, allowing conditions and approaches to be optimised and to deal with the inevitable variability of biological experiments. It is therefore encouraging that AFM and SICM are commercially available as are also STORM and STED microscopes so researchers no longer need to build these instruments themselves. Fluorescence microscopy needs to be able to routinely image two or more colours with nanoscale resolution so that relative positions of molecules can be imaged and the formation of complexes directly observed. SICM has been combined with fluorescence imaging but this needs to be extended below 300 nm so that the fluorescence and topographic resolutions are more comparable, and the issue of the registry between the fluorescence and topographic images needs to be addressed so that the two images can be overlaid. There is also a need to continue to improve the imaging rate in SICM in order to follow faster events. Currently the fastest rates are imaging 1 μm × 1 μm in 10 s. Given the fundamental limits of the piezos and resonance frequencies of the SICM this may require some redesign of the instruments as was done for AFM to obtain video rate imaging [48]. The larger topographic features often encountered on cells are also a limiting factor. Methods to image cellular function as well as the position of molecules or of the cell membrane are also needed. This can be done using fluorescent reporters inside the cell as was done to detect triggering of β2AR receptors, or in a label-free mode by detecting, for example, ion-channel activity or chemical fluxes. Here maybe lies one key challenge for the future since it is likely that with nanoscale imaging a wide variety of structures or molecular associations may be detected and one needs a method to determine which are functional.

The other key challenge is to obtain a coherent picture of the cell membrane structure from the analysis of different cell lines, with only two or a small number of proteins labeled, performed on either the apical or basal cell surface. This will probably require the use of a number of complementary imaging methods. Differentiated cells with clear and reproducible cell structures such as sperm cells, cardiomyocytes, neurons or epithelial cells allow the merging of data taken on different cells. In addition, the underlying cell cytoskeleton provides another possible structure to which cell organisation can be related. Studies of endogenous proteins are also required to ensure minimal perturbation of the membrane organisation and for any studies of the basal cell surface it is important to ensure that there has been no artefactual reorganisation on contacting the surface. In the future, nanoscale images need to be recorded on cells with reproducible structures under minimally perturbed conditions so that information from different studies can be combined. Only in this way can our understanding of the organisation and function of the cell surface take full account of its complexity.

Concluding remarks

In conclusion methods are now available that allow imaging of the cell surface with resolution comparable to scanning electron microscopy and can be performed on live cells under physiological conditions. This is an exciting advance and the challenge is now for the physical scientists who developed these methods to work with biologists interested in the biology of the cell surface. The active involvement of physical scientists will not only yield new and better imaging tools but will also bring rigorous physical insights to the interpretation of what is observed. Interdisciplinary science will therefore be essential to converting this opportunity into reality over the next few years.

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