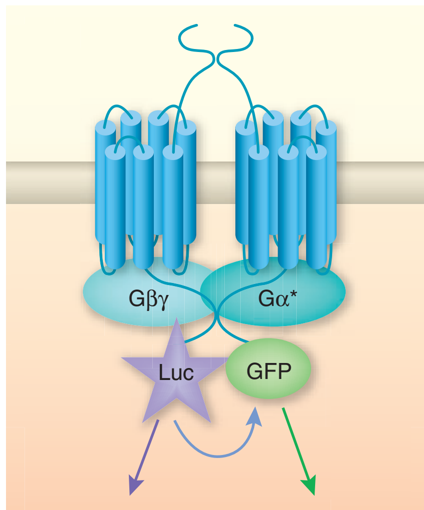

Figure 1 | Assessment of GPCR dimerization by BRET. GPCRs are tagged at their C termini with luciferase (Luc) or GFP, and each fusion protein is expressed in cells. If two GPCRs fused to different tags approach each other, resonance energy transfer from Luc to GFP results in a measurable change in the ratio of GFP fluorescence to Luc bioluminescence. GPCRs signal to G proteins, composed of a Gα subunit that binds GTP upon activation (asterisk) and a Gβγ complex. GPCR dimers have been proposed to couple to either one (as depicted) or two separate G proteins.

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tor was defective in ligand binding and the other in G-protein coupling—and yet the pair was fully functional, suggesting that the signal traveled from the ligand binding receptor to the signaling-competent second receptor[6] (**Fig. 1**). Even though these observations have not been uncontested[7] and arguments have been brought forward for the monomeric character of rhodopsin in particular[7], in general the concept of GPCR dimerization has gained wide acceptance[4].

Resonance energy transfer methods, using either bioluminescence (BRET) or fluorescence (FRET) for excitation, have become a major tool in the identification of GPCR dimers. In these experiments, either a fluorescent donor molecule is excited at its appropriate wavelength (FRET) or a donor is bioluminescent (BRET); a nearby fluorescent acceptor can then become excited by the donor via energy transfer. Because FRET and BRET are very sensitive to distances and work only over maximally ~10 nm, these methods are ideally suited to discover close proximity (**Fig. 1**). Many—but not all (for example, see ref. 8)— BRET and FRET studies provide evidence for close proximity of GPCRs, most likely in homo- or heterodimers[4]. A critical issue, however, is to distinguish true interactions from random events.

In this issue, James *et al.*[1] provide data suggesting that the extent of GPCR dimerization may have been overestimated. The authors use two types of analyses to distinguish BRET signals arising from specific dimers from those generated by random collisions in the often-crowded cell membrane (also called 'bystander BRET'). First, they vary the relative donor/acceptor ratio at a constant overall expression level, and second, they vary the expression level at a constant donor/acceptor ratio. Both methods give specific patterns for the dependence of the BRET ratio that distinguish true dimers from random interactions. This is verified with known monomeric membrane proteins such as CD86 and with known dimeric proteins such as CTLA-4. The authors then proceeded to study several GPCRs. The $GABA_B$-receptor, a well-known dimeric GPCR, gave signals expected for a dimeric protein, but two other GPCRS, the β_2-adrenergic and the cannabinoid receptors did not. This finding is certainly going to cause controversy, as several other authors have reported that β_2-adrenergic receptors form homo- and heterodimers. The advantage of the present work compared to earlier studies is the clear quantitative approach, and a direct comparison with clearly monomeric and clearly dimeric proteins, respectively, that serve as standards to calibrate the BRET results.

Future studies will now have to reassess reports on GPCR dimers. Obviously, BRET is not the only technique to investigate this issue. In particular, functional studies in cells and, more importantly, in intact tissues will be required to reveal the physiological significance of possible dimerization. The approach described by James *et al.*[1] will help as a calibration to determine the extent of GPCR dimerization. It remains to be seen which GPCRs function as dimers and which do not.

1. James, J.R., Oliveira, M.J., Carmo, A.M., Iaboni, A. & Davis, S.J. *Nat. Methods* **3**, 1001–1006 (2006).
2. Cone, R.A. *Nature New Biol.* **236**, 39–43 (1972).
3. Poo, M. & Cone, R.A. *Nature* **247**, 438–441 (1974).
4. Bulenger, S., Marullo, S. & Bouvier, M. *Trends Pharmacol. Sci.* **26**, 131–137 (2005).
5. Liang, Y. *et al. J. Biol. Chem.* **278**, 21655–21662 (2003).
6. Hlavackova, V. *et al. EMBO J.* **24**, 499–509 (2005).
7. Chabre, M. & le Maire, M. *Biochemistry* **44**, 9395–9403 (2005).
8. Vilardaga, J.P., Bünemann, M., Krasel, C., Castro, M. & Lohse, M.J. *Nat. Biotechnol.* **21**, 807–812 (2003).

Atomic force microscopy of membrane proteins separating two aqueous compartments

Yves F Dufrêne

A novel atomic force microscopy (AFM) setup allows researchers to image and manipulate unsupported membrane proteins separating two aqueous compartments. This promises to permit new detailed measurements of protein conformational changes and interactions under native-like conditions.

During the past 15 years, rapid advances have occurred in applying AFM to biological samples, thereby contributing to improve our molecular understanding of their structure-function relationships. In this relatively new form of microscopy, a sharp tip is scanned over the surface of the sample, sensing the interaction force between tip and sample. Because the instrument does not rely on an incident beam as in light or electron microscopy, the specimen can be visualized at nanometer—and even subnanometer—resolution, directly in buffer solution. Another key advantage of AFM

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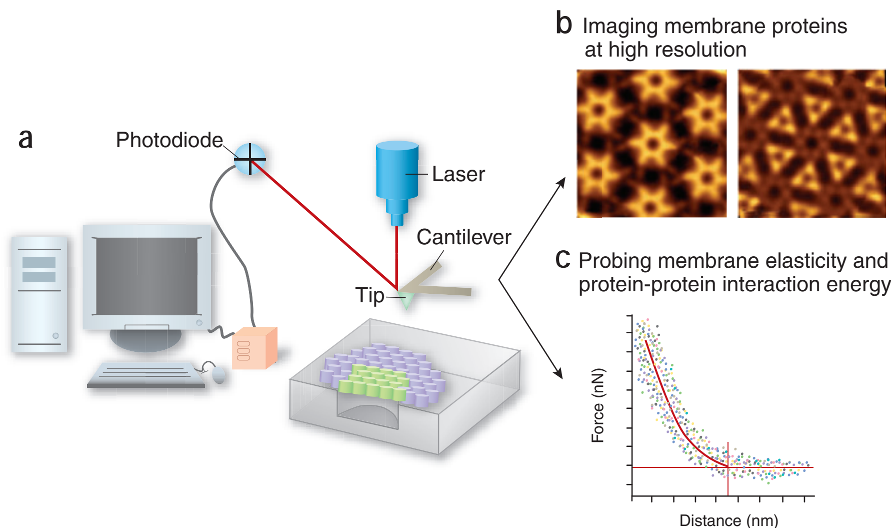

Figure 1 | Principle and applications of the two-chamber AFM set-up. (**a**) Nonsupported membrane patches are probed by AFM in buffer, by adsorbing membranes on holey silicon surfaces. (**b**) This setup allows researchers to image membrane proteins separating two aqueous compartments (over which gradients can be established) at sufficient resolution to delineate 15-Å-wide protein pores. (**c**) Used in the force-spectroscopy mode, the device allows measurement of the membrane elastic properties and the lateral interaction energy between proteins.

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is its ability, when used in the force spectroscopy mode, to measure the minute forces within or between single biomolecules, providing new insights into the molecular bases of events such as protein folding and molecular recognition.

Two-dimensional crystals of membrane proteins, and more recently native membranes, have proven to be particularly well suited for high-resolution AFM imaging and manipulation[1–4]. Owing to continuous progress in instrumentation, sample preparation methods and recording conditions, structural information can now be routinely obtained on membrane proteins to a resolution of 0.5–1 nm and under physiological conditions, which makes AFM a complementary tool to X-ray and electron crystallography. Notably, as recently reviewed by experts in the field, there is a strong indication that AFM is evolving from an imaging technique to a 'lab on a tip' multifunctional nanotool, permitting the study of the unfolding pathways, the assembly, oligomeric states and molecular interactions of membrane proteins at the single-molecule level[4].

Yet an important constraint has limited the widespread use of the technique in membrane research, that is, the need to firmly attach the specimens onto a solid support for analysis, meaning the very central concept of a native membrane separating two aqueous compartments is not preserved. The two major problems associated with supported membranes are (i) that membrane properties such as elasticity, fluidity and diffusion properties may be dramatically altered, therefore limiting the biological relevance of the measurements, and (ii) that a number of exciting experiments, such as the exploration of ion channels subjected to specific ion gradients, are not accessible. In this issue of *Nature Methods*, Simon Scheuring's group circumvented these limitations by developing an elegant two-chamber AFM setup that allows investigators to probe the structure, elasticity and energy of interaction of membrane proteins separating two aqueous compartments, and over which membrane gradients can be established[5]. As illustrated in **Figure 1a**, the basic idea is to analyze, using *in situ* AFM, membrane patches that are adsorbed on holey Si surfaces, with different hole diameters (90–250 nm) and periodicities (200–500 nm). Taking advantage of this approach, Scheuring and colleagues report three major findings. First, both the inner and the outer surfaces of nonsupported *Corynebacterium glutamicum* membranes can be imaged at high resolution, allowing unambiguous visualization of structural details such as ~15-Å-diameter pores (**Fig. 1b**). Second, using the AFM force spectroscopy mode, the tip can be pushed onto nonsupported membranes to determine their elastic properties and yield-force, and in turn to assess the lateral interaction energy between proteins (**Fig. 1c**). Third, the authors demonstrate the setup's possibilities for functional studies by monitoring pH changes in attoliter chambers induced by purple membrane proton pumping.

The new AFM setup reported here should have an important impact on many life science disciplines. In membrane research, investigators will have the opportunity to couple structural and functional AFM analyses of single proteins in nonsupported membranes, including the observation of conformational changes of channels, pumps and receptors owing to ion, pH or solute gradients, and the probing of the unfolding and assembly forces, the oligomeric states and molecular interactions of fully native membrane proteins. These single-molecule analyses offer exciting opportunities in biomedicine, particularly for studying the structure-function relationships and misfolding events of membrane proteins that are major targets for drug discovery, such as G protein–coupled receptors[4,6]. Exciting prospects are also expected in cell biology and microbiology, in which AFM-based techniques will allow investigating the structure, properties and functions of isolated cytoplasmic membranes and of microbial cell walls.

Yet, there are still a number of technological challenges remaining to be addressed for exploiting the full potential of AFM in life sciences, and more particularly in membrane protein research. Time resolution is a first issue in biological AFM imaging. Today, recording a high-resolution image of a biological sample usually takes at least 30 seconds, which is clearly much greater than the time scale at which dynamic processes generally occur in biology. But fast-speed instruments are being developed[7–9], giving access to unprecedented timescales (<1-s resolution) and opening up fascinating new perspectives to explore molecular and cellular dynamics such as conformational changes. Another crucial challenge is to develop the AFM toward an ultrasensitive probe for localizing specific receptors and ligands on membranes and cell surfaces, which is currently achieved using adhesion and dynamic recognition force mapping with biofunctionalized AFM tips[10]. Progress in developing these approaches will provide insight into the supramolecular organization of membranes and cell surfaces, as well into the molecular basis of protein folding, molecular recognition and cellular interactions.

1. Engel, A. & Müller, D.J. *Nat. Struct. Biol.* **7**, 715–718 (2000).
2. Oesterhelt, F., *et al. Science* **288**, 143–146 (2000).
3. Scheuring, S. & Sturgis, J.N. *Science* **309**, 484–487 (2005).
4. Müller, D.J. *et al. Curr. Opin. Struct. Biol.* **16**, 489–495 (2006).
5. Gonçalves, R.P. *et al. Nat. Methods* **3**, 1007–1012 (2006).
6. Fotiadis, D. *et al. Nature* **421**, 127–128 (2003).
7. Viani, M.B. *et al. Nat. Struct. Biol.* **7**, 644–647 (2000).
8. Ando, T. *et al. Proc. Natl. Acad. Sci. USA* **98**, 12468–12472 (2001).
9. Humphris, A.D.L., Hobbs, J.K. & Miles, M.J. *Appl. Phys. Lett.* **83**, 6–8 (2003).
10. Hinterdorfer, P. & Dufrêne, Y.F. *Nat. Methods* **3**, 347–355 (2006).

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QUICKstep and GS-TAP: new moves for protein-interaction analysis

Pascal Braun, Michael E Cusick & Marc Vidal

Affinity purification combined with mass spectrometry (AP-MS) is an increasingly important tool for both high-throughput and low-throughput analysis of stable protein complexes in cells. Two groups further expand the capabilities of this experimental approach.

A powerful and widely used method to characterize cellular protein complexes uses affinity purification to isolate such complexes from cellular extracts, and then mass spectrometry to identify the protein components of the purified complexes (known as affinity purification-mass spectrometry, or AP-MS). Detecting stable protein complexes by AP-MS nicely complements binary interaction-detection assays such as the yeast two-hybrid or fluorescence resonance energy transfer, as well as other assays described in this issue. Because AP-MS detects multimeric protein complexes, it occupies a special niche among protein-interaction assays. Recent efforts to perform AP-MS screens in high throughput have been remarkably successful. Building on previous efforts[1,2], two groups recently identified hundreds of protein complexes from the yeast *Saccharomyces cerevisiae*[3,4]. AP-MS has also been successfully used in human cells to explore protein interactions in signal-transduction pathways[5,6].

Despite these substantial successes, several serious technological challenges have limited the general applicability of AP-MS. Proteins that copurify nonspecifically with the isolated protein complex present a significant background, making it often hard to identify the real interactors in a sea of false positive interactors. Overexpression of tagged proteins, which is necessary for many applications of AP-MS, especially in human cells, can induce potentially deleterious overexpression phenotypes and alter the composition of protein complexes. Lastly, despite considerable advances in mass spectrometry technologies that have allowed accurate analysis of ever smaller sample sizes, the amount of starting material required for such screens is still high, often unattainably so.

These problems can be mitigated by adaptations to standard AP-MS strategies, as described in two publications in this special issue of *Nature Methods*. In one report by Mann and Selbach, a new strategy, abbreviated QUICK for quantitative immunoprecipitation combined with knockdown, can be implemented to easily distinguish specific protein interactions from nonspecific false positives while avoiding the need for troublesome overexpression[7]. In another report, Superti-Furga, Bauch and colleagues describe the development of a new affinity purification tag that leads to higher yields of isolated protein complexes from mammalian cells, establishing a big step toward the large-scale exploration of human protein complexes by mass spectrometry[8].

With the QUICK strategy, Mann and Selbach aimed to develop a method for coimmunoprecipitation of endogenous proteins to distinguish specifically interacting proteins from those that represent the nonspecific background.[7] They use SILAC, or stable isotope labeling of cell cultures, which marks proteins from two cell populations by the metabolic incorporation of distinct heavy and light amino acids[9]. In QUICK, the SILAC methodology is adapted for the immunoprecipitation of endogenous proteins. In one cell population, the target protein is specifically knocked down by small interfering RNA. The knockdown causes the expression of the target protein to decrease greatly; this cell population represents the control. For the subsequent coimmunoprecipitation, lysates of the two cell populations are mixed in equal parts, and thus both the test sample and the control sample contribute equally to background proteins that bind nonspecifically to the beads or the antibody. In contrast, the target protein and its specific interaction partners are isolated in much greater yield from the experimental sample than from the control population (**Fig. 1a**). All isolated proteins are then identified by mass spectrometry. Because all proteins in both populations have been distinctly metabolically labeled, proteins that show up in the mass spectrum with similar intensities in both heavy and light incarnations thus represent nonspecific interacting proteins, or false positives. In contrast, proteins that have an intensity ratio >1 are likely specific interaction partners.

In a pilot test, this QUICK method identified and confirmed several interactors of the signaling proteins β-catenin and Cbl. The authors identified four specific interactors of β-catenin among 140 total identified proteins, and all four are known interactors of β-catenin. Included among the nonspecific interactors was γ-catenin, most likely because it cross-reacted with the β-catenin antibody used for the immunoprecipitation. Without QUICK, γ-catenin might have passed as a potential interactor because 'it makes sense'. For Cbl, the authors identified Sortin Nexin 18 as a new interactor in addition to three known interacting proteins.

The utility of QUICK is in the combination of SILAC with the isolation of endogenous proteins from an unaltered *in vivo* setting. This combination can identify interacting proteins without potentially deleterious overexpression. This strength of QUICK, however, is at the same time a great disadvantage, as it requires antibodies that can efficiently immunoprecipitate the endogenous native protein. Presently, such antibodies are rare and are unavailable for most proteins apart from well-studied ones.

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