COMMENTARY

Protein conformation: through a lens, darkly

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This commentary describes the study by Calleja and co-workers, who have used fluorescence resonance energy transfer between two fluorescent proteins fused to one protein kinase (protein kinase B/Akt) to reveal conformational changes in a functional protein. The findings of their study offer an intriguing insight into the behaviour of an

enzyme that is essential for multiple aspects of mammalian signalling.

Key words: AGC kinases, enhanced acceptor fluorescence, conformational change, dark yellow fluorescent protein (YFP^{dark}), fluorescence resonance energy transfer (FRET), protein kinase B (PKB)/Akt.

INTRODUCTION

Microscopy has come a long way in the last few years. Microscopists have traditionally seen cells as immobile, considering particular structures as static, architectural features built out of a Gordian knot of proteins. The arrival of better microscopes and sophisticated immunolabels added resolution, so proteins could be localized in their own right rather than as parts of structures. More recently, the increasing use of green fluorescent protein (GFP) and its variants has allowed the imaging of individual components of living cells. The ability to study living cells, without fixation or permeabilization, has brought motion into the picture, showing that some proteins turn over or redistribute unexpectedly rapidly. However, this still leaves proteins viewed as rigid, immutable building blocks. A new study by Calleja et al. [1] in this issue addresses this matter, by using fluorescence resonance energy transfer (FRET) between two GFP markers fused to one protein kinase to reveal conformational changes in a functional protein. Of particular note, Calleja et al. [1] have developed a 'dark' form of one of the fluorescent proteins, providing an elegant control for such studies. This represents another step forward for microscopy, allowing the dynamic behaviour of proteins, as well as their localization, to be dissected within living cells.

WATCHING PROTEIN CONFORMATION BY FRET

In many respects, FRET is the ideal technique for studying protein conformational changes. During FRET, energy is transferred directly between two fluorescent molecules, without the emission of photons. The efficiency of energy transfer is sensitive to both the precise alignment of the two molecules and to the sixth power of the distance between them. Thus a very small difference in the interaction between the two fluorophores can make a very large difference in the rate of transfer. The approach used by Calleja et al. [1] involved transfecting cells with a construct encoding the protein of interest, protein kinase B (PKB)/Akt, sandwiched between two fluorescent proteins, GFP and yellow fluorescent protein (YFP). Under normal conditions, the PKB/Akt holds the fluorophores together, resulting in a high efficiency of FRET. When PKB/Akt is activated, the resulting conformational change alters

this intramolecular interaction enough to cause a considerable loss of the FRET signal. GFP fluorescence is very sensitive to environmental conditions, which could potentially cause artefacts if the enzyme changes location. The development of a dark YFP provides a key control, demonstrating that the changes observed are indeed caused by changes in FRET rather than the local environment of the PKB/Akt. Consequently, Calleja et al. [1] are able to view with confidence the localized conformational activation of a protein within living cells, which would have been more or less unthinkable for most microscopists 10 years ago.

The idea of using linked fluorescent proteins as a biosensor is not new. Cameleons, in which a calmodulin is sandwiched between cyan fluorescent protein (CFP) and YFP, have been around since 1997 [2,3]. In cameleons, the rate of FRET is increased as the local Ca²⁺ concentration rises as the calmodulin changes conformation and pulls the fluorophores together. This has provided versatile probes for Ca²⁺ signalling in diverse living cells and even whole organisms [4,5]. Other similar probes for protein-protein interactions have also been developed. The advance by Calleja et al. [1] is to apply this technique to a working enzyme. Instead of using one construct as a probe for something else, the GFP-Akt-YFP construct is both enzyme and indicator.

DON'T TRY THIS AT HOME . . .

If FRET is such a potent tool, how come it has not revolutionized more of biology? The predominant problem lies in the difficulty of the techniques for measuring FRET, but there are considerations of the constructs also. How far does sandwiching a protein between two other fluorescent peptides affect its function? Apparently not too much in the case of PKB/Akt, although the main evidence from Calleja et al. [1] is that the kinase behaves in the expected way. This could rapidly lead to a circular argument if used incautiously. The cleanest approach is to use a gene replacement. In *Dictyostelium*, CFP and YFP fusions of G-protein α - and β -subunits could be shown to restore normal biological function when expressed in the appropriate knockout mutants [6]. This approach would be far too work-intensive to be widely used in mammalian cells.

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The imaging is also technically challenging. The simplest approach is to excite one fluor and measure the emission from the other. This is technically simple, but terribly prone to artefacts and limited by noise. The relatively low efficiency of FRET makes the signal small, whereas the broad spectral range of both excitation and emission of GFPs ensures that competing signals cannot be blocked out. Approaches in which cells are measured in a fluorimeter allow FRET to be easily distinguished from the background [6], but often largely miss the point; it is not possible to visualize where in the cell the interaction is occurring. Instead, it is possible to perform corrections for the effects of each fluor, but these are rendered inaccurate if the local concentrations alter.

A more rigorous technique, as used by Calleja et al. [1], is to measure the fluorescence lifetime of the donor. Because fluorescence lifetime imaging microscopy (FLIM) relies on the properties of the fluor, not the amount of signal, it is essentially concentration-independent. However, the equipment used to measure fluorescence lifetimes is sophisticated, difficult to use and currently extremely expensive. There are additional problems with each of the main methods of lifetime detection, so Calleja et al. [1] combine results from both. Frequency domain measurements, in which the fluorescence lifetime is measured by modulating the beam which excites the fluors, cannot distinguish light which is in and out of focus, and thus have poor resolution in the vertical axis. In time-domain measurements, cells are excited by a very short light pulse, and the times of arrival of the emitted photons are registered, collated and used to calculate the fluorescence lifetime. This sort of processing inevitably requires very long exposures:

each of the time-domain images taken by Calleja et al. [1] used 120 s, which is far too long for many of the most interesting processes in living cells.

In summary, the work of Calleja et al. [1] offers an intriguing insight into the behaviour of an enzyme that is essential for multiple aspects of mammalian signalling, with the prospect of other similar revelations to come, but FLIM is too tricky to offer the revolution microscopists would love, for the time being at least.

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