Imaging protein–protein interactions in plants and single cells

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As in any society, the many different proteins found within cells must often work together to carry out their intricate business. The protein complexes that form can be stable, as seen with multisubunit enzymes or structures such as the proteasome, or transient, such as those seen during the assembly of transcription factors or the intermediates in a signaling cascade. Wherever they are found, the identification of the interacting protein partners can be a difficult and sometimes daunting task. Interestingly, when two proteins are in close proximity, light energy absorbed by one can be passed to the other by a process called resonance energy transfer (RET). During fluorescence resonance energy transfer (FRET), the input light energy is obtained by illuminating the sample, whereas in bioluminescence resonance energy transfer (BRET), the input light energy is derived from an *in situ* bioluminescence reaction (1). Light derived from bioluminescence is generally weak and does not photobleach the sample or cause autofluorescence, a problem particularly acute in plant cells because of chlorophyll. BRET is therefore an excellent choice for measuring protein interactions in plant cells but in the past has seen limited application precisely because the bioluminescence emission is so weak. The work of Xu *et al.* (2) in this issue of PNAS marries an exquisitely sensitive CCD camera with a beam splitter to solve the light intensity problem, and the ensemble allows BRET to be visualized not only in plant seedlings but also in single cells.

There are a number of techniques available that have been developed to address the thorny issue of protein–protein interactions inside cells. Perhaps the most frequently found in molecular biology literature is that of the yeast two-hybrid assay, where two protein fusions are constructed, one containing the DNA binding domain and the other containing the activation domain of a transcription factor; the transcription factor is reconstituted when the other moieties of each fusion protein interact. Two-hybrid assays can be used in high-throughput screening (3); however, because protein interactions must occur in the yeast nucleus, heterologous proteins are far from their normal environment. A second technique, also suitable for high-throughput analyses of protein interactions, is tandem affinity purification (TAP) (4), in which interactants with a target protein can be purified in quantities sufficient for mass spectroscopic sequencing by virtue of a sophisticated affinity label on the target. However, the proteins must be extracted for analysis, so that TAP does not necessarily reflect interactions in the context of the protein's natural environment inside the cell.

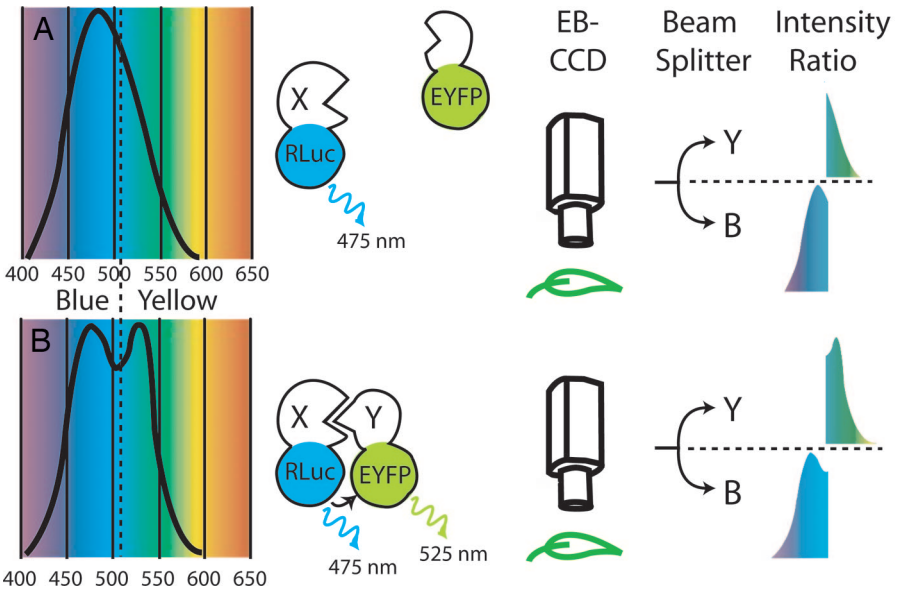

Fig. 1. Spectral changes associated with BRET measurements of protein interactions. (*A*) Energy released when RLUC oxidizes its bioluminescence substrate, coelenterazine, is given off as blue light with a spectral peak at 475 nm. Captured by a sensitive CCD camera, the emitted light can be passed through a beam splitter, and the light above (yellow-green; Y) and below (blue; B) 505 nm (dashed line) can be collected separately. The spectral emission of RLUC luminescence alone has more light in the blue region than it does in the green-to-yellow region, so the yellow-green/blue ratio is low. (*B*) When RLUC is held adjacent to a fluorescent protein, such as EYFP, energy transfer by resonance to EYFP changes the spectrum of light emission. More light is now emitted at a longer wavelength, and the ratio of the intensity of yellow-green light to blue light increases. When RLUC and EYFP are expressed as fusion proteins, an interaction between the fusion domains X and Y that brings the RLUC and the EYFP into proximity is observed as an increase in the yellow-green/blue ratio.

In contrast, RET between fusion proteins can be used to study specific interactions both *in vitro* and *in vivo* (1). RET involves transfer of energy between one molecule in an excited state (a donor) to an adjacent molecule (an acceptor) in a manner analogous to making one violin string vibrate by playing an adjacent string at the same pitch. This nonradiative energy transfer depends strongly on the distance between the donor and the acceptor, so that the amount of energy transferred between the two serves as a measure of the distance separating them (5). The range over which this molecular ruler can be used is small, typically between 2 and 8 nm, which by fortunate coincidence approximately corresponds to the size of many proteins. FRET, the most widely used version of the technique, came into its own with modified fluorescent proteins that were mutated to fluoresce at different wavelengths (6). When two fluorescent proteins with overlapping excitation and emission spectra are genetically fused to a different protein,

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FRET between the fluorochromes occurs when they have been brought close to one another by interactions between fusion proteins. This technique can produce excellent spatial resolution of interacting proteins, even at the subcellular level (7). However, despite its many advantages, the requirement for external illumination has several inherent problems, such as autofluorescence, photobleaching, and direct excitation of the acceptor fluorochrome.

BRET, first described by the Johnson laboratory in 1999 (8), overcomes many of the problems associated with FRET by replacing external illumination by the low-intensity bioluminescence generated by *Renilla* luciferase (RLUC). Furthermore, because the fluorescence of the enhanced yellow fluorescent protein (EYFP) acceptor can be measured by exciting the fluorochrome directly, the levels of both fusion proteins can be monitored independently to facilitate comparisons between different experiments. The technical advances in Xu *et al.* (2) represent important technical improvements that bring BRET closer to FRET's level of sensitivity. Previously, increasing the BRET signal involved longer exposure times, unlike FRET, for which an increase in excitation intensity can increase the signal. The modifications include a very sensitive CCD camera and a beam splitter to collect blue and green-yellow light separately (Fig. 1). BRET measurements are termed ratiometric because they take the form of a ratio between the intensity of green-yellow light compared with that of blue light, which means that such measurements have a built-in control for the amount of light given off by the luciferase.

The biological questions addressed by this recent technical tour de force eloquently demonstrate how far BRET has come. In the first case, dimerization of the constitutive photomorphogenesis 1 (COP1) protein, previously documented in a luminometer without cellular imaging (9), has now been visualized directly in tobacco seedlings. COP1 is a nuclear ubiquitin E3 ligase involved in mediating photomorphogenesis by targeting specific transcription factors for degradation by the proteasome (10). The surprise here is that COP1 is expressed and dimerizes not only in the shoots but also in the roots, which do not normally receive much light. Exposure times are still a bit long for whole-seedling imaging (on the order of minutes) but are clearly short enough to monitor changes in COP1 dimerization as a result of light treatments, for example. COP1 is actually a good illustration of another BRET advantage: The illumination required for FRET might by itself induce the changes one wishes to observe. In the second case, CCAAT/enhancer binding protein α (C/EBPα) dimerization was imaged in the nucleus of single mammalian cells. C/EBPα is a basic-leucine zipper (bZIP) transcription factor involved in regulating cell growth, differentiation, and metabolism in a variety of cell types and regulated by a variety of mechanisms (11). BRET is therefore a suitable tool to dissect control over dimerization and nuclear entry in living cells.

So what does the new technique promise? Imagine being able to follow the transient association of two proteins when a signaling pathway is activated or to watch as transcription factors assemble together as different stimuli are presented to the cell. Observing proteins as they interact in their native habitat and react to different environmental conditions, as well as following when and where they interact and the conditions under which they do, represent important new avenues of research for which specific questions can now be answered. Seeing is believing, as the saying goes. This new imaging technique is bound to make many new believers.

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