BOX 12–2 WORKING IN BIOCHEMISTRY

FRET: Biochemistry Visualized in a Living Cell

Fluorescent probes are commonly used to detect rapid biochemical changes in single living cells. They can be designed to give an essentially instantaneous report (within nanoseconds) on the changes in intracellular concentration of a second messenger or in the activity of a protein kinase. Furthermore, fluorescence microscopy has sufficient resolution to reveal where in the cell such changes are occurring. In one widely used procedure, the fluorescent probes are derived from a naturally occurring fluorescent protein, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Fig. 1).

When excited by absorption of a photon of light, GFP emits a photon (that is, it fluoresces) in the green region of the spectrum. GFP is an 11-stranded β barrel, and the light-absorbing/emitting center of the protein (its chromophore) comprises the tripeptide Ser^{65}–Tyr^{66}–Gly^{67}, located within the barrel (Fig. 2). Variants of this protein, with different fluorescence spectra, can be produced by genetic engineering of the GFP gene. For example, in the yellow fluorescent protein (YFP), Ala^{206} in GFP is replaced by a Lys residue, changing the wavelength of light absorption and fluorescence. Other variants of GFP fluoresce blue (BFP) or cyan (CFP) light, and a related protein (mRFP1) fluoresces red light (Fig. 3). GFP and its variants are compact structures that retain their ability to fold into their native β-barrel conformation even when fused with another protein. Investigators are using these fluorescent hybrid proteins as spectroscopic rulers to measure distances between interacting components within a cell.

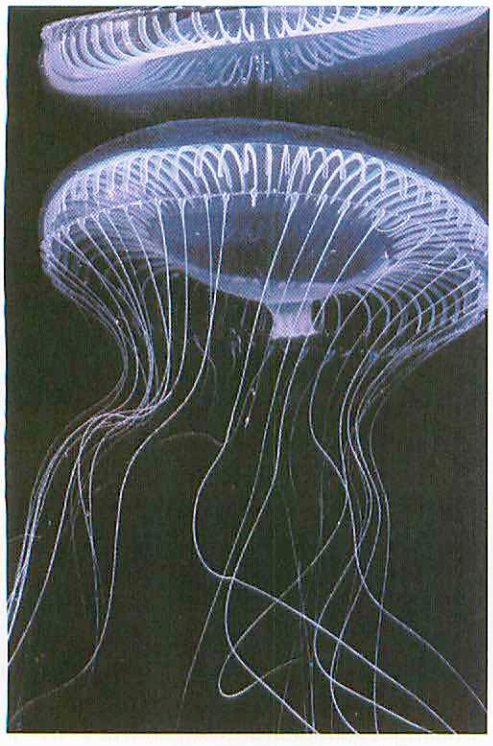

FIGURE 1 *Aequorea victoria*, a jellyfish abundant in Puget Sound, Washington State.

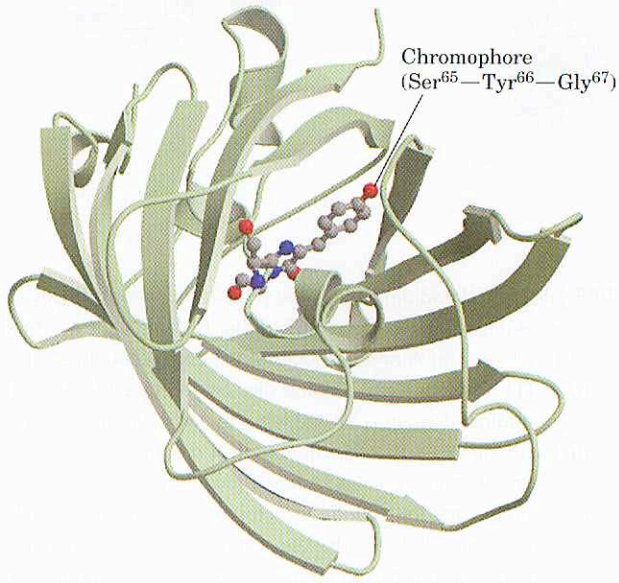

FIGURE 2 Green fluorescent protein (GFP), with the fluorescent chromophore shown in ball-and-stick form (derived from PDB ID 1GFL).

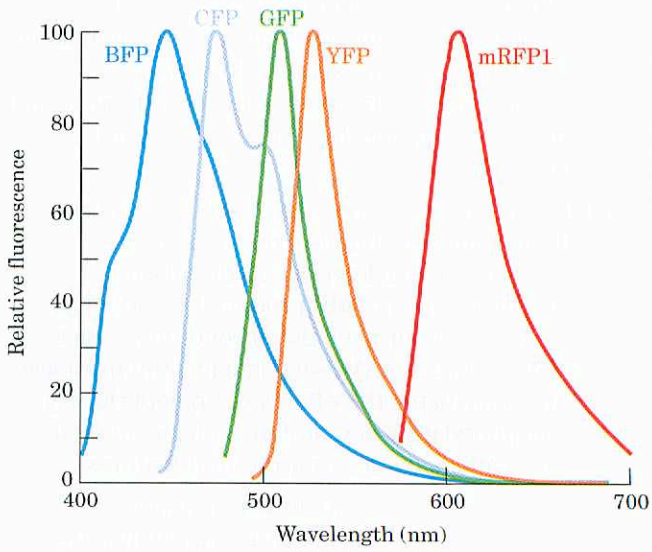

FIGURE 3 Emission spectra of GFP variants.

An excited fluorescent molecule such as GFP or YFP can dispose of the energy from the absorbed photon in either of two ways: (1) by fluorescence, emitting a photon of slightly longer wavelength (lower energy) than the exciting light, or (2) by nonradiative **fluorescence resonance energy transfer (FRET),** in which the energy of the excited molecule (the donor) passes directly to a nearby molecule (the acceptor) *without emission of a photon*, exciting the acceptor (Fig. 4). The acceptor can now decay to its ground state by fluorescence; the emitted photon has a longer wavelength (lower energy) than both the original exciting light and the fluorescence emission of the donor. This second mode of decay (FRET) is possible only when donor and acceptor are close to each other (within 1 to 50 Å); the efficiency of FRET is inversely proportional to the *sixth power* of the distance between donor and acceptor. Thus very small changes in the distance between donor and acceptor register as very large changes in FRET, measured as the fluorescence of the acceptor molecule when the donor is excited. With sufficiently sensitive light detectors, this fluorescence signal can be located to specific regions of a single, living cell.

FRET has been used to measure [cAMP] in living cells. The gene for GFP is fused with that for the regulatory subunit (R) of cAMP-dependent protein kinase, and the gene for BFP is fused with that for the catalytic subunit (C) (Fig. 5). When these two hybrid proteins are expressed in a cell, BFP (donor; excitation at 380 nm, emission at 460 nm) and GFP (acceptor; excitation at 475 nm, emission at 545 nm) in the inactive PKA (R_2C_2 tetramer) are close enough to undergo FRET. Wherever in the cell [cAMP] increases, the R_2C_2 complex dissociates into R_2 and 2C and the FRET signal is lost, because donor and acceptor are now too far apart for efficient FRET. Viewed in the fluorescence microscope, the region of higher [cAMP] has a minimal GFP signal and higher BFP signal. Measuring the ratio of emission at 460 nm and 545 nm gives a sensitive measure of the change in [cAMP]. By determining this ratio for all regions of the cell, the investigator can generate a false color image of the

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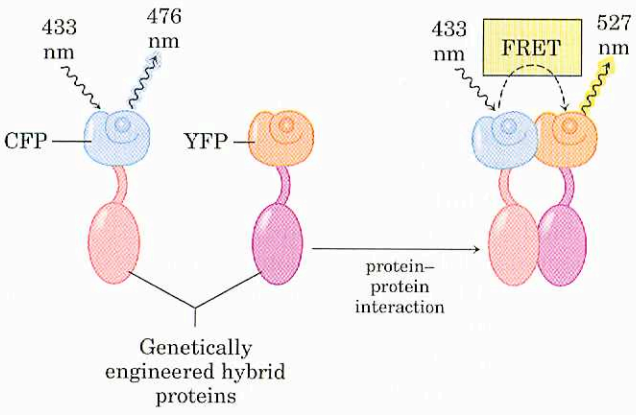

FIGURE 4 When the donor protein (CFP) is excited with monochromatic light of wavelength 433 nm, it emits fluorescent light at 476 nm (left). When the (red) protein fused with CFP interacts with the (purple) protein fused with YFP, that interaction brings CFP and YFP close enough to allow fluorescence resonance energy transfer (FRET) between them. Now, when CFP absorbs light of 433 nm, instead of fluorescing at 476 nm, it transfers energy directly to YFP, which then fluoresces at its characteristic emission wavelength, 527 nm. The ratio of light emission at 527 and 476 nm is therefore a measure of the interaction of the red and purple protein.

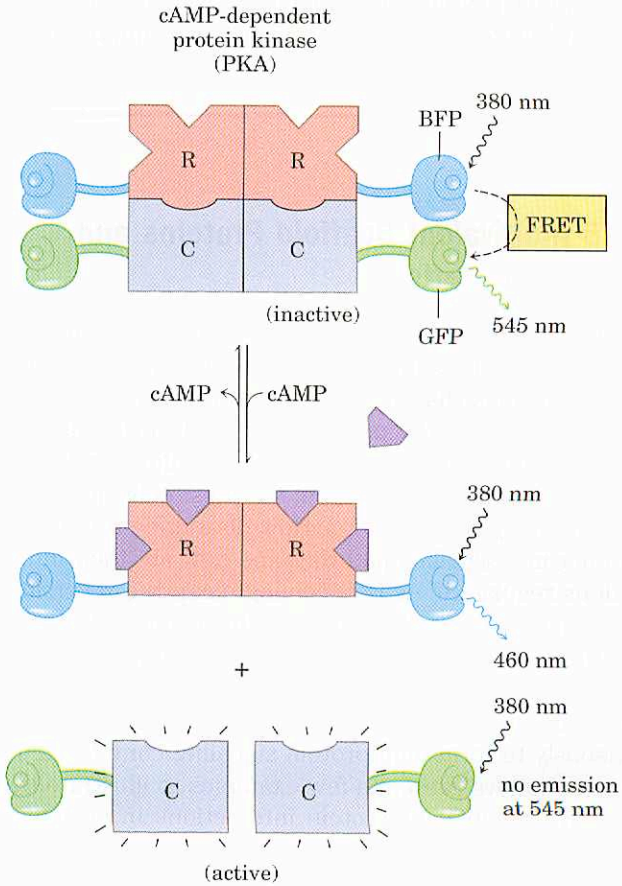

FIGURE 5 **Measuring [cAMP] with FRET.** Gene fusion creates hybrid proteins that exhibit FRET when the PKA regulatory and catalytic subunits are associated (low [cAMP]). When [cAMP] rises, the subunits dissociate, and FRET ceases. The ratio of emission at 460 nm (dissociated) and 545 nm (complexed) thus offers a sensitive measure of [cAMP].

BOX 12–2 WORKING IN BIOCHEMISTRY *(continued from previous page)*

cell in which the ratio, or relative [cAMP], is represented by the intensity of the color. Images recorded at timed intervals reveal changes in [cAMP] over time.

A variation of this technology has been used to measure the activity of PKA in a living cell (Fig. 6). Researchers create a phosphorylation target for PKA by producing a hybrid protein containing four elements: YFP (acceptor); a short peptide with a Ser residue surrounded by the consensus sequence for PKA; a Ⓟ–Ser-binding domain (called 14-3-3); and CFP (donor). When the Ser residue is not phosphorylated, 14-3-3 has no affinity for the Ser residue and the hybrid protein exists in an extended form, with the donor and acceptor too far apart to generate a FRET signal. Wherever PKA is active in the cell, it phosphorylates the Ser residue of the hybrid protein, and 14-3-3 binds to the Ⓟ–Ser. In doing so, it draws YFP and CFP together and a FRET signal is detected with the fluorescence microscope, revealing the presence of active PKA.

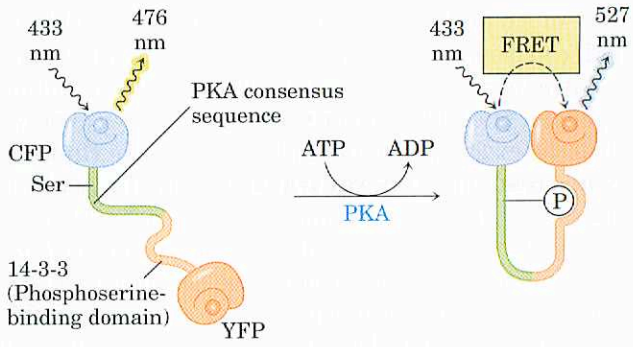

FIGURE 6 Measuring the activity of PKA with FRET. An engineered protein links YFP and CFP via a peptide that contains a Ser residue surrounded by the consensus sequence for phosphorylation by PKA, and the 14-3-3 phosphoserine binding domain. Active PKA phosphorylates the Ser residue, which docks with the 14-3-3 binding domain, bringing the fluorescence proteins close enough to allow FRET to occur, revealing the presence of active PKA.

12.5 Multivalent Scaffold Proteins and Membrane Rafts

About 10% of the 30,000 to 35,000 genes in the human genome encode signaling proteins—receptors, G proteins, enzymes that generate second messengers, protein kinases (>500), proteins involved in desensitization, and ion channels. Not every signaling protein is expressed in a given cell type, but most cells doubtless contain many such proteins. How does one protein find another in a signaling pathway, and how are their interactions regulated? As is becoming clear, the reversible phosphorylation of Tyr, Ser, and Thr residues in signaling proteins creates *docking sites* for other proteins, and many signaling proteins are *multivalent* in that they can interact with several different proteins simultaneously to form multiprotein signaling complexes. In this section we present a few examples to illustrate the general principles of protein interactions in signaling.

Protein Modules Bind Phosphorylated Tyr, Ser, or Thr Residues in Partner Proteins

We have seen that the protein Grb2 in the insulin signaling pathway (Fig. 12–6) binds through its SH2 domain to other proteins that contain exposed Ⓟ–Tyr residues. The human genome encodes at least 87 SH2-containing proteins, many already known to participate in signaling. The Ⓟ–Tyr residue is bound in a deep pocket in an SH2 domain, with each of its phosphate oxygens participating in hydrogen-bonding or electrostatic interactions; the positive charges on two Arg residues figure prominently in the binding. Subtle differences in the structure of SH2 domains in different proteins account for the specificities of their interactions with various Ⓟ–Tyr-containing proteins. The three to five residues on the carboxyl-terminal side of the Ⓟ–Tyr residue are critical in determining the specificity of interactions with SH2 domains (Fig. 12–22).

PTB domains (phosphotyrosine-binding domains) also bind Ⓟ–Tyr in partner proteins, but their critical sequences and three-dimensional structures distinguish them from SH2 domains. The human genome encodes 24 proteins that contain PTB domains, including IRS-1, which we have already met in its role as a scaffold protein in insulin-signal transduction (Fig. 12–6).

Many of the signaling protein kinases, including PKA, PKC, PKG, and members of the MAPK cascade, phosphorylate Ser or Thr residues in their target proteins, which in some cases acquire the ability to interact with partner proteins through the phosphorylated residue, triggering a downstream process. An alphabet soup of domains that bind Ⓟ–Ser or Ⓟ–Thr residues has been identified, and more are sure to be found. Each domain favors a certain sequence around the phosphorylated residue, so the domains represent families of highly specific recognition sites, able to bind to a specific subset of phosphorylated proteins.