in both chemistry and molecular biology. Secondly, the amount of protein generated is very low, thus exceptionally sensitive detection systems, such as electrophysiology or fluorescence, are required in these studies. In order to overcome some of these limitations, a modified method was developed. In this approach, a custom-made pair of orthogonal tRNA and aaRS is genetically introduced into a cell. The aaRS is engineered such that it only recognizes the UAA and efficiently acylates the corresponding tRNA. Subsequently, the UAA, which has to be nontoxic and cell permeable, is added to the cell culture medium, taken up by the host organism, and incorporated into the protein by the specific tRNA/aaRS pair. This approach was first used for incorporation of UAAs into bacteria, but has since been optimized for use in yeast, mammalian cells, primary neuronal cultures, and even in animals such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. These studies showed that increased incorporation efficiencies were obtained, leading to substantially higher yields of modified proteins. The primary challenge of this technology is that the specific aaRS has to be generated for each UAA which is done by extensive mutational studies and rounds of positive and negative selections. However, the approach is developing rapidly and recent advancements include identification of a broader number of orthogonal tRNA/aaRS pair as well as genetic engineering of the ribosome and the termination release factor to allow more efficient UAA incorporation.

The methodology has been applied to a variety of proteins including ion channels, G protein-coupled receptors (GPCRs) and histones to examine protein–protein interactions, conformational changes, signaling processes, and the role of posttranslational modifications.

A specific and widespread application is the incorporation of photo-cross-linking UAAs such as benzoyl-phenylalanine or azido-phenylalanine which upon UV radiation cross-link with nearby reactive species. This was used to map the interaction of peptide and small-molecule ligands to GPCRs (Figure 6.7) and also to map the intracellular interaction to a G-protein. Another application is the introduction of photo-caged UAAs that enables the researcher to control particular protein functions. This approach has, for example, been used to generate a light-activatable potassium channel functioning as an optical switch for suppressing neuronal firing (Figure 6.7). Very recently, incorporation of a fluorescent UAA has enabled in situ imaging of protein dynamics of the Shaker Kv channel. The methodology is also used to incorporate reactive UAAs that can subsequently be specifically chemically modified which is also being exploited commercially by a biopharmaceutical company to develop improved protein-based therapeutics.

A general constraint of these technologies is that the genetic code only contains three stop codons which limits the number of different UAAs that can be incorporated into a single protein. To potentially overcome this limitation, extended codons (quadruplets) have been employed. In this approach, an mRNA containing the quadruplet codon is being read by a modified UAA-tRNA containing the corresponding extended anti-codon. The quadruplet codon approach has successfully been used to incorporate UAAs into proteins, and also to incorporate two different UAAs into two different sites of a single protein, showing that the quadruplet codons are not only orthogonal to their host organism but also to each other. In order to use the quadruplet codon strategy to incorporate multiple UAAs by the orthogonal tRNA/aa-RS methodology, orthogonal tRNA/aa-RS pairs for each UAAs have to be developed. This has recently been achieved by evolving orthogonal tRNA/aa-RS from two different widely used tRNA/aa-RS pairs which has enabled simultaneous incorporation of two different UAAs in response to a stop codon and a quadruplet codon. More recent advances in this field include the de novo design of orthogonal tRNA/aa-RS pairs and engineering of an orthogonal ribosome that reads quadruplet codons more efficiently.

6.3.2 Peptide/Protein Ligation

A conceptually different strategy for modification of proteins is to employ methods based on solid-phase peptide synthesis (SPPS) for generation of proteins. This would allow incorporation of principally any amino acid. SPPS has in a few cases been applied for the synthesis of proteins, although yields are generally rather low. The first example was the synthesis of ribonuclease A (124-residues)