Chapter 14

Intracellular Signaling and Biochemical Assays

After reading this chapter, you should be able to:

- Discuss the rationale for studying proteins and signaling pathways in neuroscience research
- Explain why antibodies are useful for studying proteins and how they are produced
- Describe common biochemical assays used to investigate intracellular signaling

Techniques covered:

- **Antibody production**: making monoclonal and polyclonal antibodies
- **Techniques used to purify proteins**: affinity chromatography, immunoprecipitation assay
- **Techniques used to measure the amount and location of proteins within cells and tissue**: western blot (immunoblot), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunohistochemistry (IHC), immunoelectron microscopy (IEM), reporter proteins, pulse chase labeling
- **Techniques used to investigate protein-protein interactions**: co-immunoprecipitation, protein affinity chromatography, yeast two-hybrid assay, proximity labeling, fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation (BiFC)
- **Techniques used to investigate posttranslational modifications**: posttranslational modification (PTM) assays (kinase assay), detection of PTMs
- **Techniques used to investigate protein−DNA interactions**: electromobility shift assay (EMSA), chromatin immunoprecipitation assay (ChIP), luciferase assay

Proteins are molecular machines responsible for virtually all of the structural and functional properties of cells. Structural proteins maintain a cell's shape, size, and durability. Transmembrane proteins serve as receptors for neurotransmitters and hormones, allowing the cell to receive information from the

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extracellular environment. Other proteins act as downstream messengers from cell surface receptors and mediate the response to an extracellular cue. Some proteins serve as enzymes that facilitate the metabolic and physiological needs of the cell. Finally, some proteins regulate the transcription and expression of genes that code for other proteins to further affect a cell's physiology. Because of the important role that proteins play in neural structure and function, anyone interested in how the brain works should appreciate the techniques scientists use to investigate proteins.

Proteins interact with each other and other biomolecules much like neurons interact in functional circuits. Instead of acting in isolation, proteins communicate with upstream signaling molecules and subsequently affect downstream proteins and biochemical substrates. **Intracellular signaling** (or signal transduction) is a term used to describe the biochemical pathways that affect a cell's physiology and metabolism. Often, these pathways begin with the activation of a cell surface receptor, which leads to a series of biochemical events that culminate in a change in gene expression in the nucleus. Just as an organism senses a change in the environment and uses neural circuits to produce an appropriate response, an individual neuron senses a change in its environment and uses intracellular signaling pathways to change its transcriptional, physiological, and metabolic properties.

The purpose of this chapter is to survey the methods used to study the important roles that proteins play within neurons. We will begin with a general description of intracellular signaling events and why they are useful for understanding the properties of neurons. Next, we will describe two fundamental methods used to study proteins: producing and using antibodies, and purifying proteins of interest. The bulk of the chapter then surveys a variety of methods that answer useful questions about intracellular signaling events: (1) whether a protein is expressed in a particular population of cells and how much is present; (2) whether a protein interacts with another protein; (3) what types of posttranslational modifications can occur for a protein and which proteins cause these modifications; and (4) whether a protein is capable of interacting with DNA and regulating gene expression.

INTRODUCTION TO SIGNAL TRANSDUCTION AND INTRACELLULAR SIGNALING

The study of signal transduction pathways is fundamental to several subfields of neuroscience including clinical neuroscience, learning and memory, regulation of homeostasis, and neural development. For example, many researchers have investigated the signaling pathways responsible for axon guidance and synapse formation. Extracellular guidance molecules bind to membrane receptors, signaling which direction the neuron should grow. Intracellular signaling cascades then provide the metabolic and structural changes necessary for the axon to extend in the correct direction. When an axon reaches its postsynaptic target, other extracellular cues shut off this signaling cascade and new cascades develop to strengthen synaptic connections. Clearly, an understanding of the

biochemical events within a neuron is necessary to truly understand the nature of axon guidance, as well as other vital neuronal functions.

An example of a well-known cell signaling pathway is the JAK-STAT pathway (Fig. 14.1). Various extracellular proteins such as **cytokines** and **growth factors** bind to specific transmembrane receptors. Some of these receptors bind to proteins called Janus Kinases (JAKs). **Protein kinases** are proteins that, when activated, have the ability to add a phosphate group to other proteins. When a cytokine binds to its receptor, JAKs transfer a phosphate group to the receptor. This phosphate group causes a conformational change in the three-dimensional structure of the receptor that allows other proteins to bind called Signal Transducers and Activators of Transcription

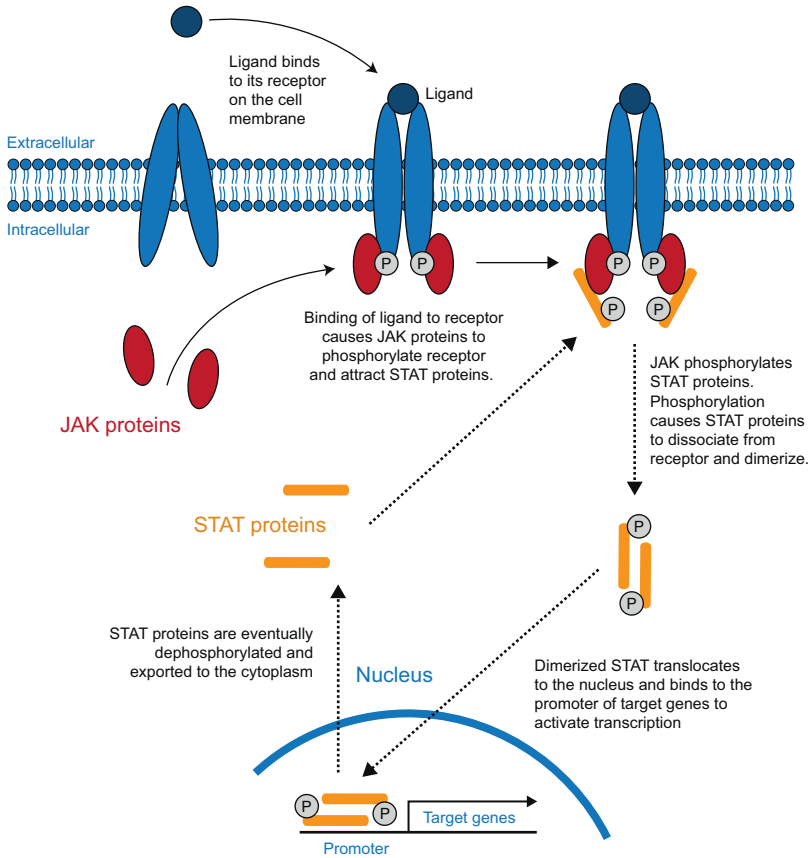

FIGURE 14.1 An example of an intracellular signaling pathway: the JAK-STAT pathway. Some receptors for ligands (e.g., cytokines and growth factors) interact with Janus Kinase (JAK) proteins. When a ligand binds to the receptor, JAKs add a phosphate group to the intracellular domain of the receptor. These phosphate groups attract STAT proteins to the receptor. JAKs add a phosphate group to STAT proteins, causing them to dissociate from the receptor and bind to each other. These STAT dimers then translocate to the nucleus, where they can affect gene transcription. Eventually the STAT proteins are dephosphorylated and are exported to the cytoplasm.

(STATs). When STAT proteins bind to the receptor, JAKs add an additional phosphate group to the STAT proteins, causing them to dissociate from the receptor and bind to each other. These new STAT dimers translocate to the nucleus and activate specific target genes.

The JAK-STAT pathway is one of several pathways that play a prominent role in the development and functional physiology of the nervous system. Several elements of this pathway are consistent with other signaling pathways: an extracellular cue binds to a membrane protein, causing an intracellular signaling cascade that eventually affects the expression of genes. These genes code for other proteins or genetic regulatory sequences that allow the cell to adapt to its environment.

FUNDAMENTAL TOOLS USED TO STUDY PROTEINS

This chapter describes many techniques that can be used to answer specific questions about intracellular signaling. Many of these techniques routinely make use of two fundamental biochemical tools that are essential for studying proteins: antibodies and protein purification techniques. Therefore, before discussing any specific assays, we first examine these fundamental methods.

Making and Using Antibodies

Antibodies are proteins used by an animal's immune system to detect foreign proteins, called **antigens** (Fig. 14.2A). The region of an antigen recognized by a specific antibody is called an **epitope**. Antibodies are extremely useful research tools because they bind specific proteins with high affinity. Therefore, they can be used to identify, collect, and purify specific proteins of interest. Most of the techniques presented in this chapter depend on antibodies (Table 14.1).

Antibodies used as research tools are classified as being either monoclonal or polyclonal. **Monoclonal antibodies** are collections of antibodies that recognize the same epitope (Fig. 14.2B). They are derived from single cell lines, usually cells obtained from mice. Once produced, these cell lines provide a renewable source of antibodies and all batches of antibody will be

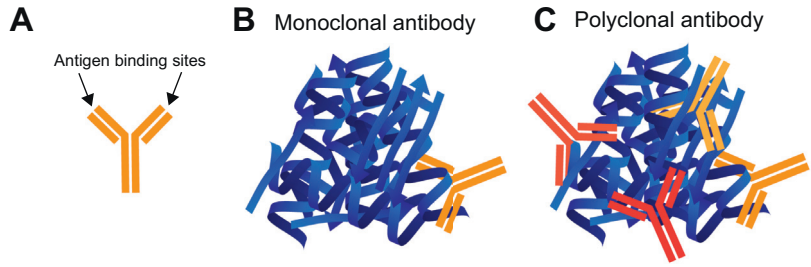

FIGURE 14.2 Monoclonal versus polyclonal antibodies. (A) An antibody has a characteristic "Y" shape. Two antigen binding sites are located at the proximal top ends of the Y. (B) A monoclonal antibody recognizes a single epitope of an antigen. (C) A polyclonal antibody is a mixture of several antibodies that recognize different epitopes of an antigen.

TABLE 14.1 Techniques That Utilize Antibodies.

Technique	Role of Antibody in Technique
Western blot	An antibody binds to a protein that has been run on a polyacrylamide gel to mark the presence, approximate size, and relative quantity of the protein in a sample.
Enzyme-linked immunosorbent assay (ELISA)	An antibody binds to a protein to measure the quantity of the protein in a sample.
Radioimmunoassay	An antibody binds to radioactive proteins of known concentration. A sample containing nonradioactive proteins is added to compete with the radioactive proteins, allowing a scientist to determine the protein concentration in a sample.
Immunohistochemistry	An antibody binds to a protein to show spatial expression in tissue or cells.
Immunoprecipitation	An antibody is bound to small beads. A sample is mixed with the beads, so the antibody binds and purifies a protein. The protein sample is then eluted off the beads.
Co-immunoprecipitation	An antibody is bound to small beads. A sample is mixed with the beads, so the antibody binds and purifies a protein. After the protein is eluted, the investigator uses a separate antibody in conjunction with a western blot or ELISA experiment to detect the presence of any secondary proteins that may interact with the immunoprecipitated protein.
Chromatin immunoprecipitation (ChIP)	An antibody is bound to small beads in a column. A sample is run through the column, so the antibody binds and purifies a protein. After the protein is eluted, the investigator uses PCR to determine the identity of any bound DNA sequences, demonstrating a protein–DNA interaction.
Antibody interference	An antibody binds to a protein, preventing the proper function of the protein.

identical. Because they are directed against the same epitope, these antibodies may not bind to homologous proteins from other species. In contrast, **polyclonal antibodies** are a collection of antibodies that recognize different epitopes of the same antigen (Fig. 14.2C). Therefore, these antibodies are more likely to bind to antigens and detect homologous proteins across species. However, the disadvantage of using polyclonal antibodies is that they are prone to batch-to-batch variability; therefore, the quality of successive batches of antibodies may not be consistent over time. Polyclonal antibodies are commonly produced in rats, rabbits, chickens, goats, sheep, and donkeys.

The procedures for producing monoclonal and polyclonal antibodies are described in Figs. 14.3 and 14.4, respectively. A great variety of antibodies are commercially available and range in cost from $50 to as much as $1000

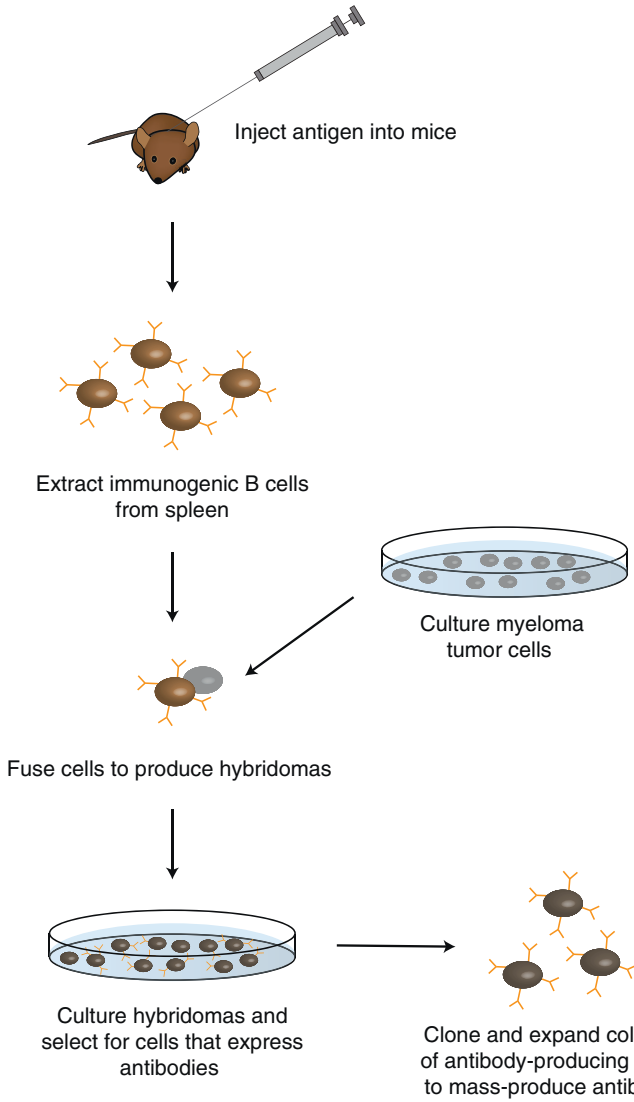

FIGURE 14.3 Producing a monoclonal antibody. A scientist injects an antigen into an animal, usually a mouse. The mouse's immune system naturally produces antibodies as a response. After several days, the scientist extracts immunogenic B cells that produce antibodies from the spleen. These cells are fused with tumor cells that divide indefinitely. After culturing these cells and selecting for hybrids that continuously produce an antibody of interest, a scientist can mass produce a monoclonal antibody.

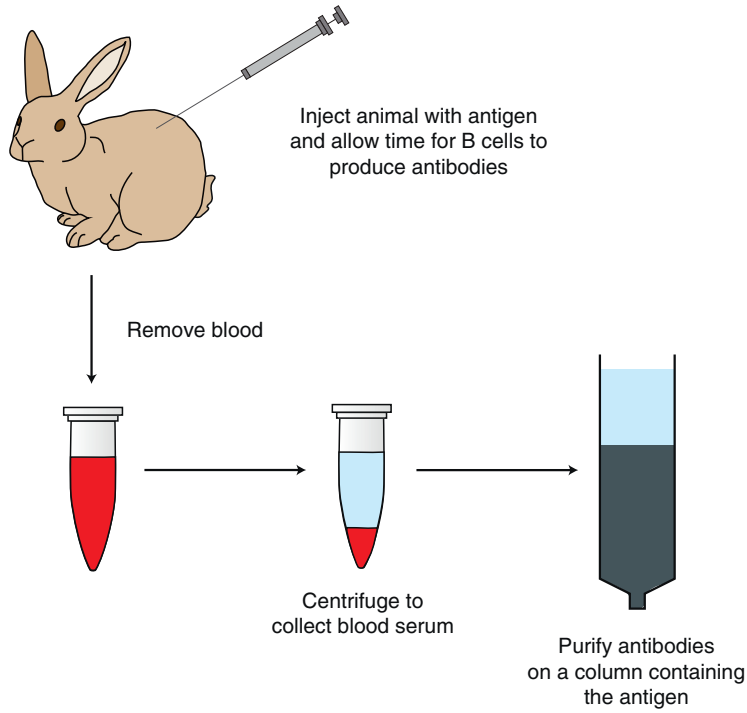

FIGURE 14.4 Producing a polyclonal antibody. A scientist injects an antigen into an animal, often multiple times over the course of weeks. The animal's immune system produces many antibodies against the antigen as a response. The scientist then collects blood, separates the serum from the red blood cells, and purifies the antibody from the serum using affinity chromatography.

($200–300 is common). If an antibody for a protein is not commercially available, individual labs may produce their own antibodies. Typically, though, this work is contracted to an antibody production service that produces custom antibodies on demand. If an antibody is difficult to produce, scientists can use recombinant DNA techniques (Chapter 10) to produce proteins tagged with a short amino acid sequence that can be easily detected by commercially available antibodies (Box 14.1).

Although several biochemical techniques depend on the use of antibodies (Table 14.1), a specific antibody may only work well for one, several, or (rarely) all of these techniques, depending on the particular binding affinity of the antibody–protein complex. For example, an antibody that works well for IHC may work very poorly for ChIP. Even within IHC, the antibody may work much better for labeling proteins in cell culture than labeling proteins in tissue sections. Often, commercial manufacturers indicate which biochemical techniques an antibody is best suited for based on internal testing or customer feedback. Even so, the only way to know if an antibody is useful for a

BOX 14.1 Creating and Using Tagged Proteins

Most of the techniques used to detect specific proteins rely on antibodies. However, antibodies do not exist for all native proteins. Furthermore, producing good antibodies can sometimes be difficult and expensive, and the antibodies are not guaranteed to be effective for all techniques. Therefore, many scientists use standard recombinant DNA techniques (Chapter 10) to add short amino acid tags to a protein of interest. The scientist can then use antibodies against the tag to examine or purify the tagged protein. Common tags include c-Myc, His, FLAG, GST, and HA. Because of the different binding efficiencies of common antibodies for these tags, FLAG and GST tags are often used for western blot and IP assays, while c-Myc and HA tags are often used in immunohistochemistry. These peptide tags have a variety of origins: the polyhistidine (His)-tag was developed commercially for protein purification, while HA is derived from human influenza hemagglutinin, a viral gene, which may explain the ability to easily produce antibodies to this peptide sequence.

particular technique is to try it. Ideally, the antibody should be tested on negative control samples (in which the protein of interest is not present) and positive control samples (in which the protein is known to be present) to ensure that the antibody is truly specific to the protein of interest.

While antibody-based techniques are overwhelmingly used in neuroscience research to identify and report the presence of proteins, antibodies cannot always be generated to recognize a target molecule of interest. Recently, scientists have developed an alternative to antibodies called **aptamers**. Aptamers are nucleic acid or peptide-based molecules that are engineered to bind to a specific target molecule (a chemical compound, protein, nucleic acid sequence, etc.). To create aptamers, a lab (or company) exposes the target molecule to a library of random DNA, RNA, and/or protein molecules to determine which molecules have the highest affinity. Aptamers have proven useful for identifying and labeling substances within the brain, such as amyloid plaques in murine and human brains.

Purifying Proteins

Purifying proteins allows an investigator to generate antibodies, determine a protein's sequence, and determine a protein's binding partners. Two common methods of purifying proteins include chromatography and immunoprecipitation (IP).

Chromatography

Chromatography allows a mixture of proteins (and other biomolecules) in a solution to be separated on a column. In this procedure, a scientist runs the

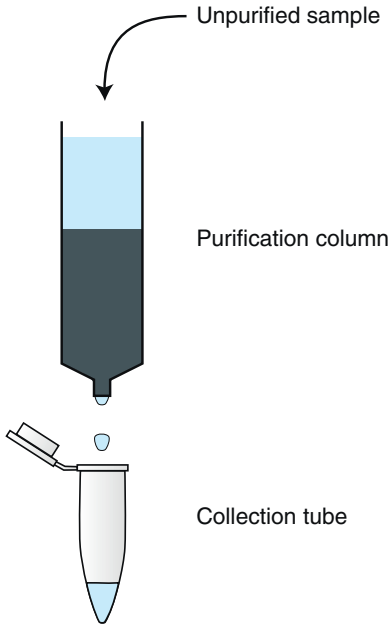

FIGURE 14.5 Chromatography. A biological sample is run through a column composed of a porous membrane. Different columns have different properties and separate proteins based on their size, charge, binding partners, etc.

solution through a column containing a porous matrix (Fig. 14.5). The proteins pass through the column differently depending on how they interact with the matrix, and therefore proteins can be differentially collected as they flow out the bottom of the column.

Within the matrix, proteins can be separated on the basis of many different properties. For example, in **gel-filtration chromatography**, proteins descend through a column based on their size. In **ion-exchange chromatography**, proteins are separated based on their charge. A more specific way to purify a protein, **affinity chromatography**, uses small molecules fixed to the porous matrix to bind to proteins that may have a high affinity, such as a substrate binding to an enzyme. After the solution is run through the column and the protein of interest is bound to the matrix, the proteins are eluted from the column by changing the salt concentration or pH and the purified sample is acquired in a small collection tube.

Immunoprecipitation

Immunoprecipitation uses antibodies to purify a protein of interest out of solution (Fig. 14.6). Specific antibodies are first bound to a mixture of beads. When a solution is mixed with these antibody-conjugated beads, the antibody will bind to its epitope, precipitating the protein out of solution. After the initial solution is washed away, a scientist can elute the protein off the beads

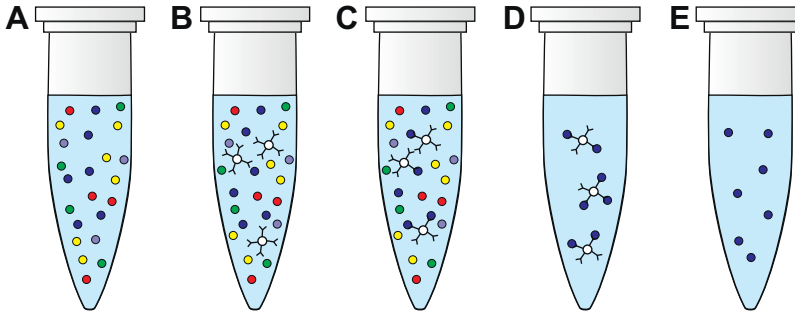

FIGURE 14.6 Immunoprecipitation. (A) A protein of interest exists in a heterogeneous sample with many other proteins. (B) A scientist adds small beads coated with antibodies that recognize the protein. (C) The antibodies bind to the proteins, causing them to precipitate out of solution and adhere to the beads. (D) The heterogeneous sample is washed away from the beads. (E) The proteins can be removed from the beads by changing the salinity or pH of the solution, and then the beads can be removed from the solution.

by removing the antibodies, usually by changing the salt concentration or pH of the solution. This elution product can be further purified, although this can also reduce the yield of the final product.

Taken together, the ability to purify proteins based on their binding partners, charge, size, and other properties, as well as the ability to use antibodies to purify proteins out of solutions, allows scientists to perform many cell signaling assays to investigate proteins in the nervous system.

INVESTIGATING PROTEIN EXPRESSION

A fundamental question in any cell signaling experiment is whether a particular protein is expressed in a population of cells and, if so, how much of the protein is present. There are a variety of methods that a scientist may use to answer these questions, some more quantitative and others with better spatial resolutions. Table 14.2 provides a brief comparison of the strengths and limitations of each technique.

Western Blot

The **western blot** (also known as an **immunoblot)** is the most commonly used method to measure the expression of a protein in a tissue or cell sample (Fig. 14.7). Before performing a western blot, scientists extract proteins from freshly dissected brain tissue or cultured cells using a simple protocol that lyses the cells and removes DNA contaminants. The sample is then processed using what is known as **sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)**, or simply **SDS-PAGE**. SDS is a negatively charged detergent that binds to hydrophobic regions of the protein, allowing it to

TABLE 14.2 Methods of Measuring Protein Expression.

Technique	Description
Western blot (immunoblot)	Allows for the detection of a protein in a sample, with measurements of relative concentration between samples.
Enzyme-linked immunosorbent assay (ELISA)	Allows for measurement of protein concentration in a sample. More sensitive than a western blot. Can quantify specific amount of protein, rather than determine the relative amount of protein (as in a western blot).
Radioimmunoassay	Precise measurement of protein concentration in samples in which the protein may be very dilute.
Immunohistochemistry	Measures the spatial distribution of the expression of a protein in cells and tissues. Not very quantitative.
Immunoelectron microscopy	Measures the spatial distribution of the expression of a protein within cells at very high spatial resolution.
Reporter proteins	Measures the spatial distribution of the expression of a protein without the need to use IHC. Can be performed in live cells or tissues.

become soluble. The detergent also causes the protein to take on a net negative charge, regardless of its amino acid composition. Therefore, the protein will migrate toward a positive electrode when voltage is applied during electrophoresis.

The scientist loads the protein sample onto a polyacrylamide gel (Fig. 14.7A). In the presence of an electric field, the gel acts as a physical filter so that larger proteins travel relatively slowly and smaller proteins travel relatively quickly. This allows the investigator to separate a heterogeneous mixture of proteins into a spectrum of proteins with different molecular weights.

At this stage of the WB process, the investigator can visualize the protein bands on the polyacrylamide gel using a stain such as **Coomassie blue**. This dye will stain all of the proteins in each lane of the gel. The result can appear like a smear, with some prominent bands representing highly expressed proteins in the sample. A scientist might perform this step to demonstrate that the amount of protein in each lane is the same.

After the protein sample is run on a gel, the final step in a WB procedure is to identify the presence of a specific protein. Because the gel is relatively thick and extremely delicate, a scientist first transfers the proteins onto a thin, nitrocellulose membrane, referred to as the "blotting" process (Fig. 14.7B). This procedure uses current to transfer the proteins from the gel onto the membrane. Once

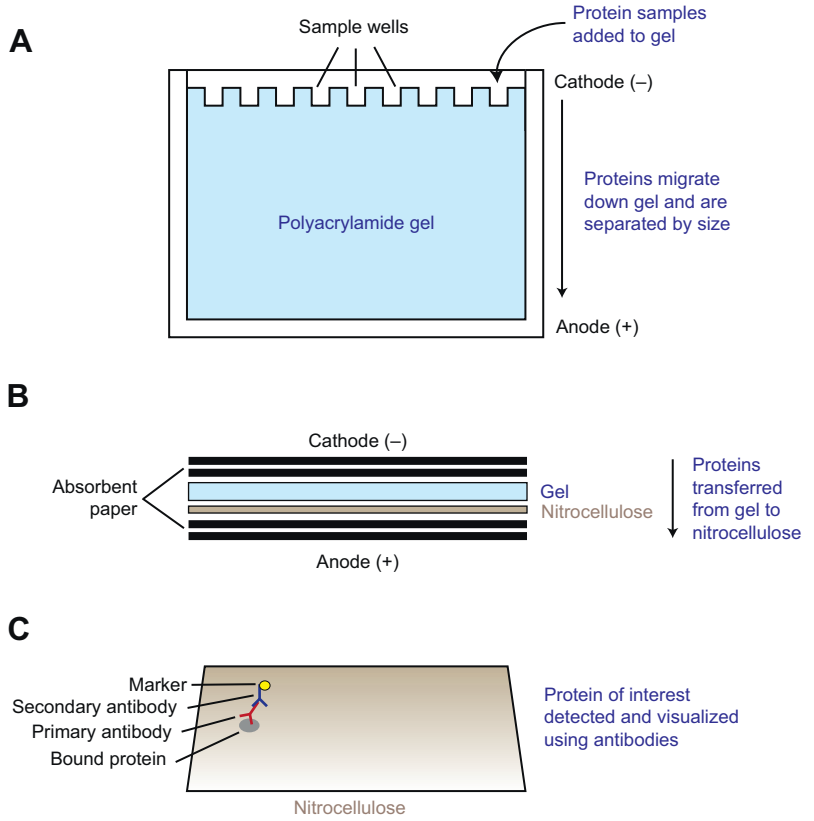

FIGURE 14.7 **The western blot.** (A) Protein samples are run on an SDS-PAGE gel, which separates proteins on the basis of size. Smaller molecular weight proteins migrate faster toward the anode. (B) The gel is placed on top of a thin nitrocellulose membrane and surrounded by absorbent paper. Applying an electric field causes the proteins within the gel to be transferred to the membrane. (C) To detect a specific protein of interest, a scientist incubates the nitrocellulose membrane in a solution with primary antibody, followed by a solution with a secondary antibody that recognizes the primary antibody. The secondary antibody contains a marker that can be used to visualize the protein on the membrane.

the proteins have transferred to the membrane, the investigator can detect the presence of a particular protein by incubating the membrane in a solution with an antibody specific to the protein (Fig. 14.7C). Then the membrane is incubated in a solution with a secondary antibody that recognizes and binds to primary antibody based on the species in which the primary antibody was produced. This secondary antibody is coupled to a radioactive isotope, a chromogenic enzyme, or a fluorescent dye. The labeled antibody marks the presence of the protein as a discrete band within a lane of the gel. The scientist can compare the location of the protein on the blot to a standardized protein ladder that highlights specific molecular weights in kilodaltons (kD).

Therefore, the final result of a western blot experiment is a depiction of the band of protein, along with its molecular weight and necessary controls (Fig. 14.8). The most important control in a WB is a "loading control," a measurement of a separate protein in all experimental samples to ensure that each lane contains the same quantity of total protein. For example, antibodies to β-actin or GAPDH, proteins expressed in all cells, are often used to ensure that each sample contains the same total amount of protein; therefore, if a WB shows that a protein of interest is relatively lower in one sample compared to others, it is not because the investigator simply added less starting material. When characterizing a new antibody, a scientist should perform a negative control with a sample in which the specific protein is absent, as well as a positive control with a sample in which the specific protein is present. These controls allow the scientist to verify that the antibody is specific and capable of reliably indicating the presence of the protein of interest.

An investigator may wish to know not only which cells express a protein of interest, but also where, within those cells, that protein is localized. In this case, the investigator can use a process called **cell fractionation** to divide a sample into its cellular components before a WB experiment begins (Fig. 14.9). Individual organelles within a cell vary in their weight and can be pulled out of solution through a series of centrifugation steps. A tissue or cell culture sample is first dissociated into a liquid homogenate and then centrifuged through a series of spins, each increasing in time and force. After each step, the pellet containing specific cellular components is collected. Finally,

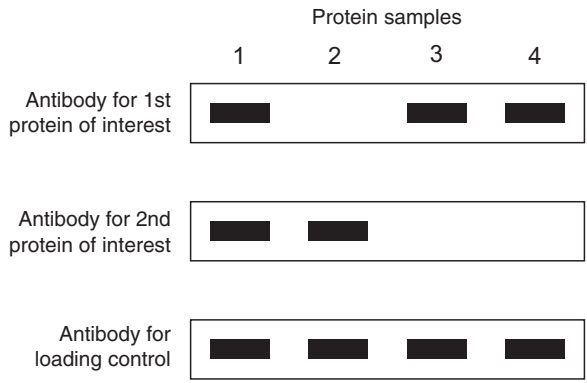

FIGURE 14.8 **Western blot data analysis.** Western blot data are often presented as blots on gels. These dark blots represent signal on the nitrocellulose membrane from the bound antibodies. The darker the band, the more protein present in the sample. Each row usually represents detection from a specific antibody. A loading control, an antibody that recognizes a protein that should be present in equal amounts in all samples, is included to show that each well contains the same amount of starting protein. Usually this loading control is an antibody against β-actin or GAPDH. A reasonable conclusion from this mock figure would be that the first protein of interest is detected in samples 1, 3, and 4, but not sample 2. A second protein of interest is detected in samples 1 and 2 but not 3 and 4.

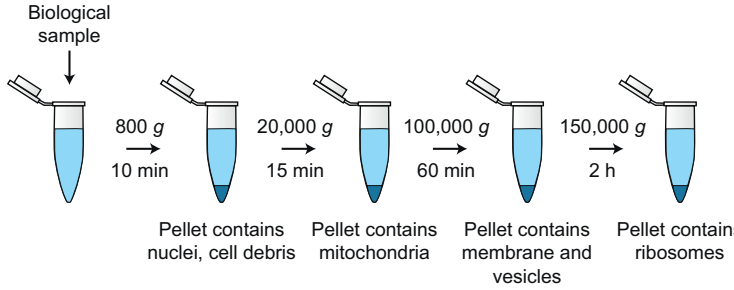

FIGURE 14.9 **Cell fractionation.** A biological sample can be centrifuged at successively higher speeds to collect specific organelles within cells.

the scientist can run each sample in a separate lane of a WB experiment to determine which fraction contains a particular protein.

Enzyme-Linked Immunosorbent Assay

An **enzyme-linked immunosorbent assay (ELISA)** is an alternative to a WB, used to measure the expression of a protein, especially a protein that may be expressed in extremely low quantities. A traditional western blot requires milligrams of protein, while an ELISA is able to quantify nanograms.

A scientist binds an antibody to the bottom surface of a plate (Fig. 14.10). A sample is added to the plate, and any protein present that can bind to the antibody becomes adhered to the surface. The sample is washed away, but the bound protein remains. A secondary detecting antibody that is linked to an enzyme is then applied to the plate, such that the protein of interest is sandwiched between both antibodies. A chemical substrate is added to the sample that is converted by the enzyme into a chromogenic or fluorescent signal. Finally, the scientist uses a specialized device to measure the signal and determine the quantity of protein present.

Radioimmunoassay

A **radioimmunoassay** is a highly sensitive method of measuring very low concentrations of proteins. Samples are usually derived from blood, extracellular fluid, or cerebrospinal fluid.

To perform an RIA reaction (Fig. 14.11A), a scientist first labels a known quantity of protein with a radioisotope, usually an isotope of iodine (^{125}I or ^{131}I), as iodine readily attaches to the amino acid tyrosine. The radiolabeled protein is then mixed with an antibody specific for that protein. This antibody–protein mixture is divided into several samples. To the first sample, the scientist adds a known amount of unlabeled, nonradioactive protein. This protein competes for antibody binding sites with the radioactive form of the protein. In the subsequent samples, the scientist continually increases the concentration of nonradioactive

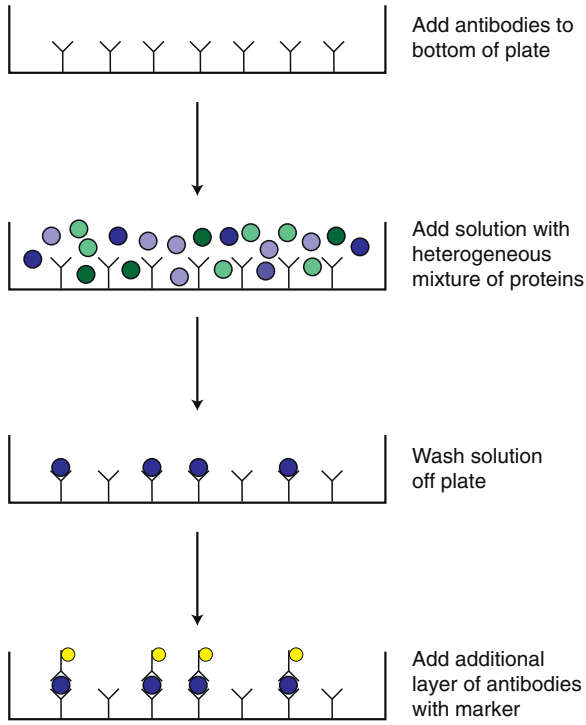

FIGURE 14.10 ELISA. A scientist coats the bottom of a plate with antibodies specific to a protein of interest and then adds a heterogeneous biological sample in solution. The proteins bind to the antibodies, and the rest of the sample is washed off the plate. Other antibodies conjugated to a visible marker that recognize the protein are added to the plate, allowing a scientist to quantify the amount of protein in the sample.

protein, increasing the amount of displaced radioactive protein. Finally, the scientist removes all proteins bound to antibodies from the samples using a secondary antibody so that only unbound proteins remain. The radioactivity of each sample is measured. From these data, a scientist can create a standard binding curve reflecting the ratio of bound to unbound protein versus the concentration of nonradioactive protein (Fig. 14.11B).

To measure the concentration of a protein from a biological sample, the scientist runs this unknown quantity of protein in parallel with the known preceding quantities. Once the scientist identifies the ratio of bound to unbound protein, it is possible to use the standard binding curve to deduce the concentration of the unknown sample (Fig. 14.11C).

An RIA is a very sensitive technique and can measure extremely small protein concentrations, such as the concentration of neuropeptides in the extracellular fluid of brain tissue. However, this technique requires specialized equipment and is relatively expensive. It also requires extra safety precautions due to the use of radioactivity.

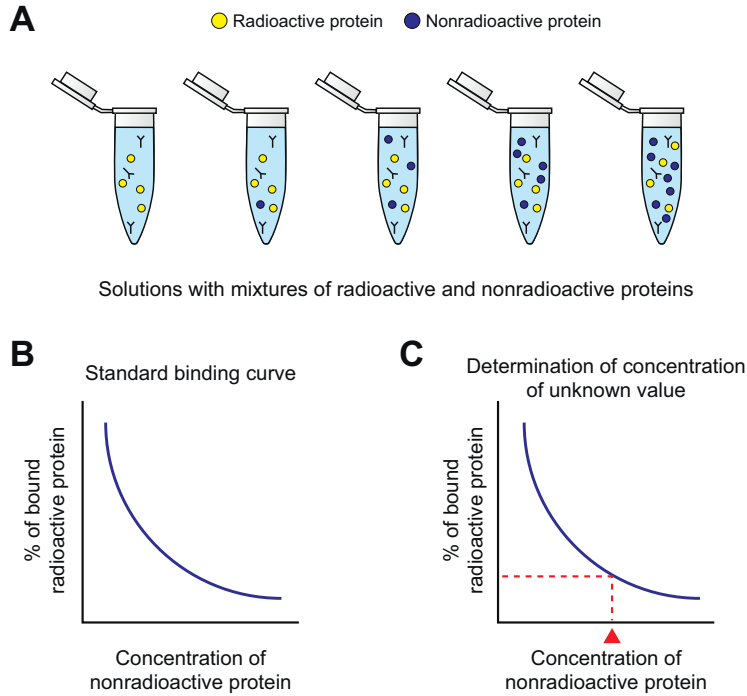

FIGURE 14.11 A radioimmunoassay. (A) A scientist mixes different concentrations of nonradioactive and radioactive versions of a protein in a series of reaction tubes with an antibody against the protein. The nonradioactive and radioactive proteins compete to bind with the antibody. The scientist then collects the antibodies and bound proteins using a secondary antibody so that only unbound proteins remain. (B) By measuring the radioactivity of each sample, the scientist can produce a standard binding curve, comparing the amount of radioactive protein bound to an antibody with the concentration of nonradioactive protein added to the sample. (C) This binding curve can be used to determine the unknown concentration of a protein from an experimental sample.

Immunohistochemistry

Immunohistochemistry (IHC) is described in more detail in Chapter 6 as a method to visualize the expression of proteins in cells or brain sections. The advantage of using IHC over WB, ELISA, and RIA methods is that it can visualize the spatial expression of a protein in specific populations of cells within a tissue sample. IHC can also sometimes show the location of a protein within a cell, such as the cytoplasm versus the nucleus (Fig. 14.12). **Immunoelectron microscopy (IEM)** is an extension of IHC used in combination with electron microscopy (Chapter 5) to visualize protein expression within subcellular structures at extremely high resolution. Similar to a cell fractionation procedure followed by a western blot, IEM can be used to determine if a protein is localized in a particular subcellular compartment.

Although IHC and IEC are great methods for showing the spatial presence and distribution of a protein, these techniques are not precise for quantifying or comparing the amount of protein present in different samples.

Visualizing Protein Dynamics Using Fused Reporter Proteins

To visualize proteins without the need for IHC, scientists can use recombinant DNA technology to attach a **reporter protein**, such as green fluorescent protein (GFP), to proteins of interest (Chapter 6). Tagging a protein of interest with a reporter protein is more time consuming than IHC, as various recombinant DNA constructs, gene delivery strategies, and/or transgenic strategies must be employed before an experiment can take place (Chapters 10–12). However, using reporter proteins overcomes some of the limitations of IHC, for example, allowing an investigator to determine the expression and location of a protein in living cells. Reporter proteins fused to proteins of interest also allow for time-lapse imaging to study real-time protein dynamics (Fig. 14.13).

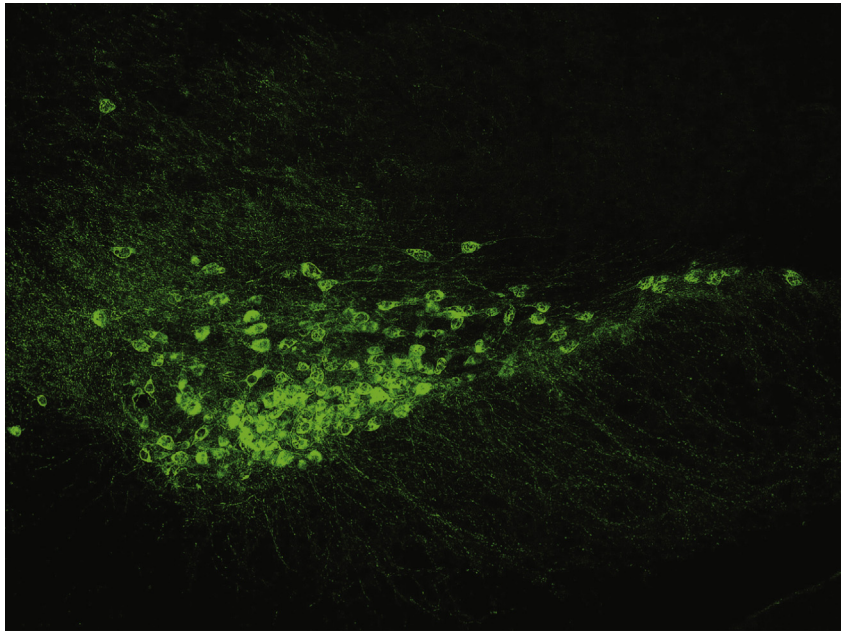

FIGURE 14.12 Immunohistochemistry reveals the presence and locations of proteins within specific cells. An example of the product of an immunohistochemistry experiment using an antibody for tyrosine hydroxylase in the substantia nigra in a mouse brain section. A secondary antibody tagged with a green fluorophore was directed against the primary antibody. Green fluorescence shows the specific cells that express tyrosine hydroxylase, as well as the location of tyrosine hydroxylase within the cytoplasm and axon fibers. The donut-shaped appearance of the cells demonstrates the absence of green signal in the nucleus, demonstrating that tyrosine hydroxylase is a cytoplasmic protein.

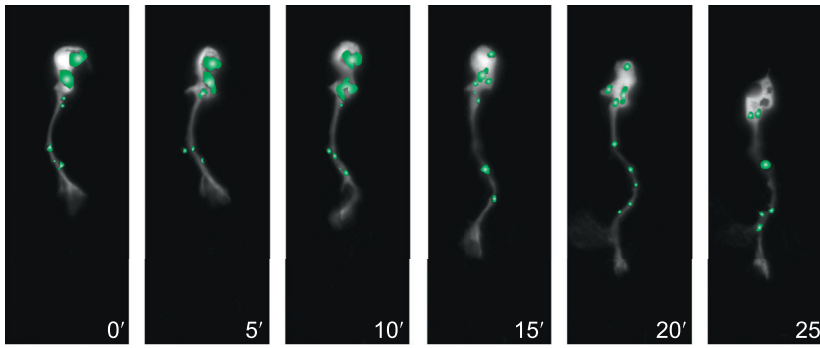

FIGURE 14.13 Example of time-lapse imaging with reporter proteins. In this example, a migrating neuron in culture expresses two different reporter proteins with different excitation and emission spectra: a cytoplasmic fluorescent protein allows visualization of the cell's morphology (white) and a GFP fusion protein that labels endosomes (green). Observing changes in the position of the green signal over time reveals intracellular trafficking dynamics, such as the timing of endosome translocation from the neural soma down the length of an axon.

For example, a transcription factor protein fused to GFP can reveal changes in localization in response to growth factors, demonstrating translocation from the cytoplasm to the nucleus.

One limitation to using fluorescent proteins is the phenomenon of photobleaching, in which illumination of the protein decreases fluorescence over time. However, investigators can exploit this phenomenon to examine protein turnover rates or trafficking. **Fluorescence recovery after photobleaching (FRAP)** uses strong laser illumination of a cell or tissue sample to bleach the fluorescent molecules in a particular region (Fig. 14.14). The investigator then measures the time course of fluorescence returning to the bleached region to reveal the kinetics of a fluorescently tagged protein's diffusion, binding, or dissociation, or active transport into the bleached region.

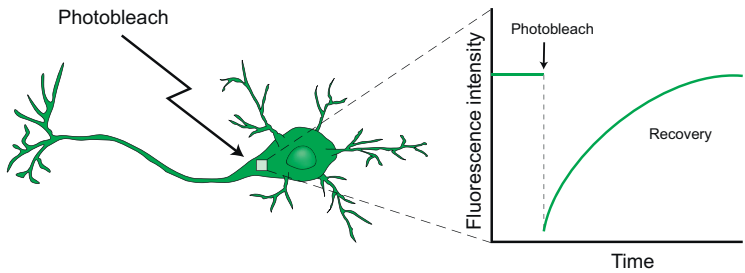

FIGURE 14.14 Fluorescence recovery after photobleaching (FRAP). Intense illumination is applied to a small region of a cell expressing a fluorescent reporter fused to a protein of interest. The recovery of fluorescent signal in the photobleached area is measured over time. The recovery time can be used to measure the kinetics of protein trafficking or turnover in various regions of the cell.

Some fluorescent proteins have been engineered so they are not fluorescent until hit with a specific wavelength of light (often UV) that **photoactivates** fluorescence. Photoactivatable fluorescent proteins can be fused to a protein and later activated, allowing selected subpopulations of the protein to be labeled and followed as they move around a cell. This is particularly useful for observing the behavior and dynamics of a highly abundant protein that may be difficult to track in a large population of fully fluorescent molecules. Some sophisticated photoactivatable proteins allow precise control over their fluorescence. For example, the photoactivatable protein Dronpa can be reversibly photoactivated; UV light increases fluorescence intensity, while blue light will quench the fluorescence. **Photoconversion** serves a similar purpose as photoactivation, but rather than activating a previously nonfluorescent molecule, a pulse of light causes a fluorophore to change its emission spectra from one color to another. For example, Kaede is a photoconvertible fluorescent protein that initially emits green light, but emits red light after UV illumination. This method allows for the total visualization of a protein of interest in green but the ability to study a smaller subset of the proteins in red.

Visualizing proteins of interest fused to reporter proteins allows for real-time measurement of protein expression, location, and dynamics within cells and tissues. However, these techniques require sophisticated fluorescent microscopes and equipment that may not be present or available in many traditional biochemistry laboratories. Chapter 7 discusses many of the requirements and methods of analysis for visualizing fluorescent activity in neural preparations.

Assaying Protein Trafficking with Pulse-Chase Labeling

Pulse-chase labeling is used to observe the movement of a substance through a biochemical or cellular pathway. A labeled probe is injected into animals or added to cultured cells for a brief period (the pulse) and then washed away and replaced by unlabeled molecules (the chase). By following changes in the localization of a marker in different subcellular compartments over time, a scientist can observe protein trafficking pathways. For example, a protein can be pulsed with a radioactively labeled amino acid in different specimens. By fixing specimens at different time points, a scientist can use autoradiography to determine the location of labeled probes at regular intervals to observe changes in the localization of the incorporated label. Pulse-chase experiments have been important in elucidating many biochemical pathways, including synthesis and release of neurotransmitters.

INVESTIGATING PROTEIN-PROTEIN INTERACTIONS

Almost all proteins function within the cell by interacting and/or forming a complex with other proteins. Therefore, a scientist might ask whether a

specific protein interacts with another protein of interest. Alternatively, a scientist might want to know the identity of *all* proteins that interact with a protein of interest. The following techniques address these questions.

Co-immunoprecipitation

One of the simplest methods to determine if a protein physically interacts with another protein is a **co-immunoprecipitation (Co-IP)** assay. This technique is an extension of the IP assay described previously (Fig. 14.6). A scientist lyses cells to extract proteins and then mixes the solution with beads linked to antibodies. An antibody directed toward one of the proteins in the complex immunoprecipitates the protein out of solution. Any proteins that are tightly associated with this protein will also precipitate out of solution and will be released in the elution process (Fig. 14.15). The eluted proteins can be separated and identified using an SDS-PAGE/western blot protocol with antibodies for the immunoprecipitated protein and potential binding partner (Fig. 14.16). If the identities of the potential binding partners are unknown, they may be determined using a technique called **mass spectrometry** (Box 14.2). Note that co-immunoprecipitation does not show if a protein *directly* interacts with another protein; the two proteins may exist in a complex, indirectly bound with intermediary proteins.

Protein Affinity Chromatography

A scientist can use protein affinity chromatography to isolate and identify proteins that physically interact with each other. To capture the interacting

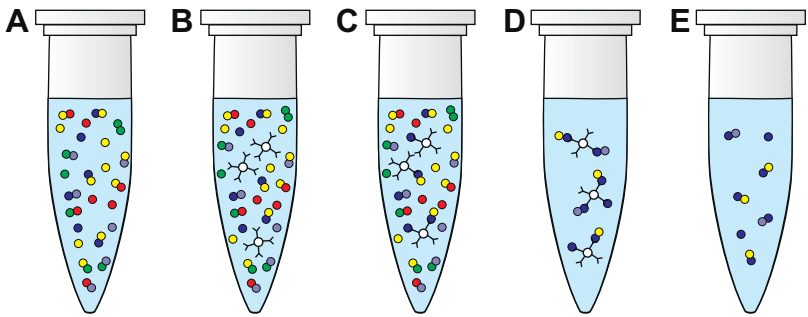

FIGURE 14.15 Co-immunoprecipitation. (A) A protein of interest exists in a heterogeneous sample with many other proteins. Some proteins are bound to each other. (B) A scientist adds small beads coated with antibodies that recognize the protein. (C) The antibodies bind to the proteins, causing them to precipitate out of the solution and adhere to the beads. Any proteins that interact with the protein will also adhere to the beads. (D) The heterogeneous sample is washed away from the beads and (E) The proteins can be removed from the beads by changing the salinity or pH of the solution, and then the beads can be removed from the solution. The scientist can now use other methods, such as a western blot or mass spectrometry, to identify the interacting proteins.

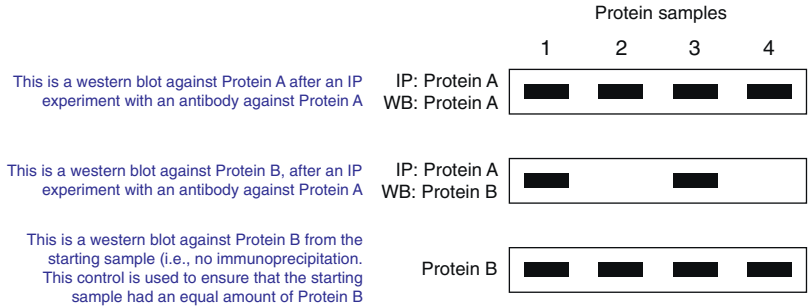

FIGURE 14.16 Co-immunoprecipitation data analysis. Co-immunoprecipitation experiments are often followed by western blots. As in all western blots, the dark blots represent signal detected from an antibody. The darker the band, the more protein present in the sample. A co-IP figure must report the antibody used to immunoprecipitate a protein as well as the antibody used to detect the protein in the western blot. A reasonable conclusion from this mock figure would be that Protein B interacts with Protein A in samples 1 and 3, but not in samples 2 and 4. This result is *not* because Protein B is absent from samples 2 and 4, as the bottom gel shows presence of this protein in all four samples. Perhaps Protein A and B did not interact in samples 2 and 4 because those samples were taken from conditions in which Protein A was in a different conformational state.

BOX 14.2 Mass Spectrometry

Mass spectrometry (MS) is a technique used to identify and sequence proteins that are present in a sample by breaking them down into fragments and then determining the mass to charge ratio of individual fragments. Automated programs compare the calculated mass of protein and peptide fragments from a sample with the masses computed from protein databases. Therefore, MS can identify unknown proteins in a sample. In "de novo" sequencing, MS can determine the amino acid sequence of an unknown peptide. MS can also be used to identify posttranslational modifications (PTMs), such as phosphorylation or methylation, as well as the amino acid residues on which they reside.

Determining the masses of proteins and peptides requires the sample to be in the gas phase, which can be accomplished in two ways: matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI, peptides are mixed with a UV-absorbing organic acid (which causes the proteins to become charged) and dried on a metal slide. The sample is then blasted with a UV laser. The protein matrix absorbs the laser light, causing it to go into the gas phase. In ESI, the sample is mixed with an acidic solution that charges the sample. The charged sample travels through a capillary into a heated area where solvent molecules are lost due to the heat. As the charge density becomes higher and higher, the droplets explode, becoming finer and finer until only single molecules remain.

Once the molecules enter a gas phase, their mass-to-charge ratio can be measured by a detector. In a "time of flight" (TOF) detector, ionized peptides are accelerated in an electrical field and fly toward a detector. The time it takes each peptide to reach the detector is determined by its mass and charge, with large peptides moving slowly and highly charged peptides moving relatively quickly. Other detectors use electrical or magnetic fields to selectively filter or detect different mass to charge ratios.

Continued

BOX 14.2 Mass Spectrometry—cont'd

Many molecules can have the same mass-to-charge ratio despite having very different chemical structures. For example, the peptides "PEPTIDE" and "TIDEPEP" would be indistinguishable by their mass alone. This situation commonly occurs with proteins, nucleic acids, and organic molecules. To distinguish between these molecules, they can be fragmented and the masses of their constituent components measured. Fragmentation of peptides can be caused by colliding a sample with gas molecules such as argon or nitrogen to cleave the peptide bonds. The result of this "collision induced dissociation" (CID) is overlapping fragments that reveal the original sequence of the peptide. For example, observing "EPEP" or "PEPT" in the preceding example would distinguish between the two peptides. Ideally, collisions create a virtual ladder of fragments, each differing by only one amino acid. In practice, a perfect ladder is rarely observed, but enough detail is present to match a peptide to known protein sequences.

MS is a core tool in the field of **proteomics**, the systematic characterization of the complete set of proteins in a system at a particular time, allowing for insight into signaling networks and protein interactions. Proteomics involves the analysis of many proteins at the same time; for example, identifying variations in protein expression in the brains of patients with Alzheimer's disease compared to healthy brains. MS (along with bioinformatics tools to manage the large amounts of data) allows for the profiling of these thousands of different proteins.

proteins, a target molecule is attached to polymer beads within a column. These target molecules have specific interactions with the protein of interest that will capture the protein as it passes through the column. They can be ligands for a receptor, substrates for an enzyme, or antibodies for an antigen. Cellular proteins are washed through the column, and the proteins that interact with the target attach to the matrix, while all other proteins pass through. The proteins that interact with the target are eluted off the beads, and their identity can be determined using a western blot. Just as in the co-IP assay, if the identities of the interacting proteins are unknown, they can be determined using mass spectrometry (Box 14.2). Also similar to a co-IP assay, this technique does not show if the two proteins directly interact or whether they exist in a larger protein complex.

Yeast Two-Hybrid Assay

A **yeast two-hybrid assay** investigates protein–protein interactions by exploiting a transcription system normally used by yeast cells. In a normal yeast cell, a transcription factor called Gal4 binds to a promoter region called an upstream activating sequence (UAS). Gal4 is composed of a binding domain (BD), which binds to the UAS, as well as an activation domain (AD), which initiates transcription of a target gene.

In a yeast two-hybrid assay, a scientist uses recombinant DNA technology to divide the Gal4 protein into these two separate domains (Fig. 14.17A). The Gal4 BD is fused to a protein of interest thought to interact with another protein. This protein is considered the "bait" in that its role in the experiment is to attract other binding partners. The scientist fuses the Gal4 AD to a potential binding partner. This binding partner is considered "prey" in that it could potentially bind with the bait. The two recombinant sequences are expressed in cultured cells. If the two proteins do not interact, the BD and AD fragments will be physically separated, and no transcription will occur. However, if the two proteins are able to bind, the Gal4 BD and AD fragments will become physically close enough to cause transcription of the downstream coding sequence (Fig. 14.17B). If the gene adjacent to the UAS is a reporter gene, such as *lacZ* or GFP, the scientist will be able to identify the interaction between the two proteins.

A yeast two-hybrid assay is useful to test hypotheses that two proteins interact, but this assay can also be used as a screen to identify potential binding partners for a protein of interest (Fig. 14.18). For example, a scientist can

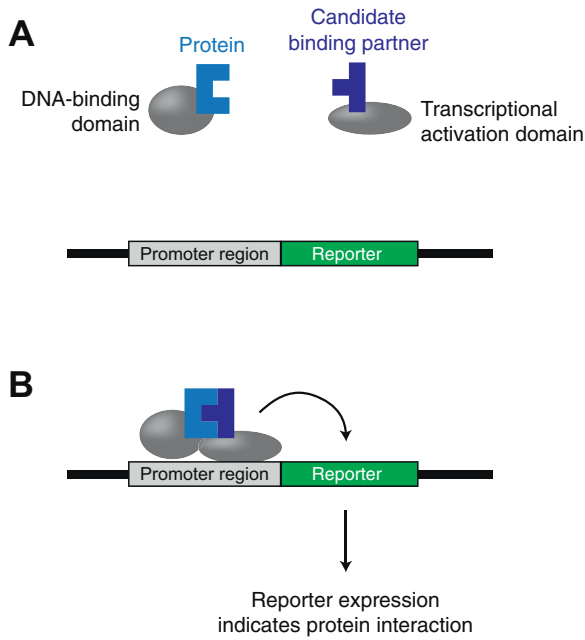

FIGURE 14.17 **Yeast two-hybrid assay.** (A) A scientist uses recombinant DNA technology to fuse the DNA binding domain of Gal4 to a protein of interest and the transcriptional activation domain of Gal4 to a potential binding partner. These constructs are introduced into host cells. If the two proteins do not interact, the DNA binding domain and transcriptional activation domain of Gal4 do not interact. However, (B) if the two proteins do interact, then the Gal4 complex is complete and can activate transcription of a reporter gene.

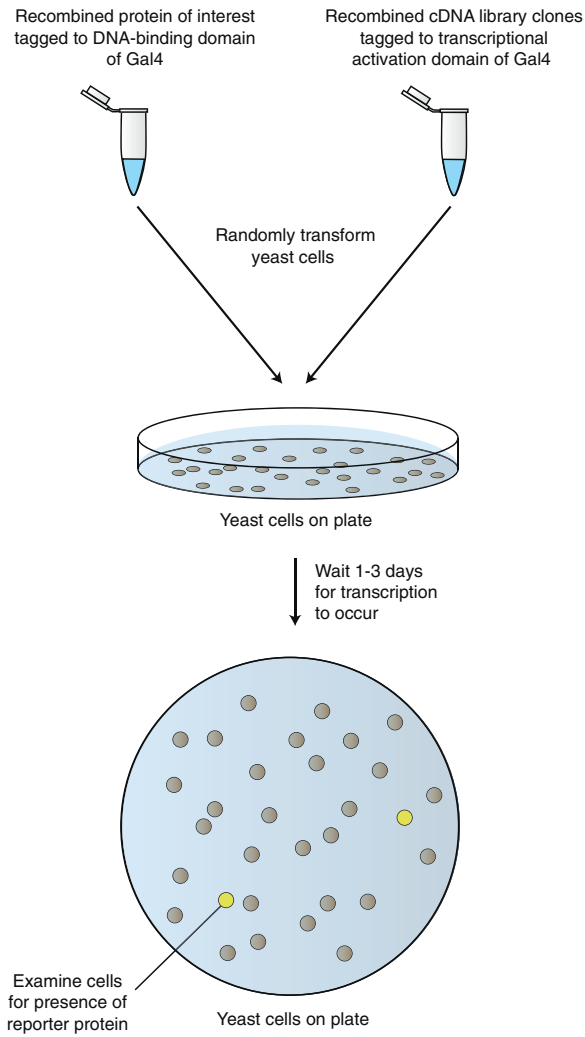

FIGURE 14.18 **Using a yeast two-hybrid assay as a screen.** Recombinant DNA technology is used to fuse thousands of genetic sequences from a cDNA library to the Gal4 transcriptional activator. These constructs, as well as a construct in which a protein of interest is fused to the Gal4 DNA binding domain, are randomly introduced into yeast cells. In some yeast cells, a target protein will interact with the protein of interest, and the Gal4 will activate expression of a reporter gene, changing the color of the cells. To determine the identity of the protein binding partner, the scientist can extract and sequence the DNA from these colonies.

identify unknown "prey" for a protein of interest by attaching the AD to a large mixture of DNA fragments from a cDNA library (see Chapter 10). Individual ligation products are then introduced into yeast cells containing the target protein. If one of the members of the library encodes a protein that interacts

with the target protein, the two Gal4 fragments will interact and activate transcription of the reporter. The scientist can then identify the yeast colony expressing the reporter, purify and sequence the DNA attached to the AD, and determine the identity of the binding protein. While this assay shows that the proteins *can* interact, it does not show that they interact in vivo or even that they can interact in mammalian cells.

Proximity Labeling to Identify Protein–Protein Interactions

Proximity labeling investigates protein–protein interactions by identifying proteins that come in extremely close proximity to a protein of interest (Fig. 14.19). This technique takes advantage of one of the strongest noncovalent interactions identified in nature: the strong binding between the proteins biotin

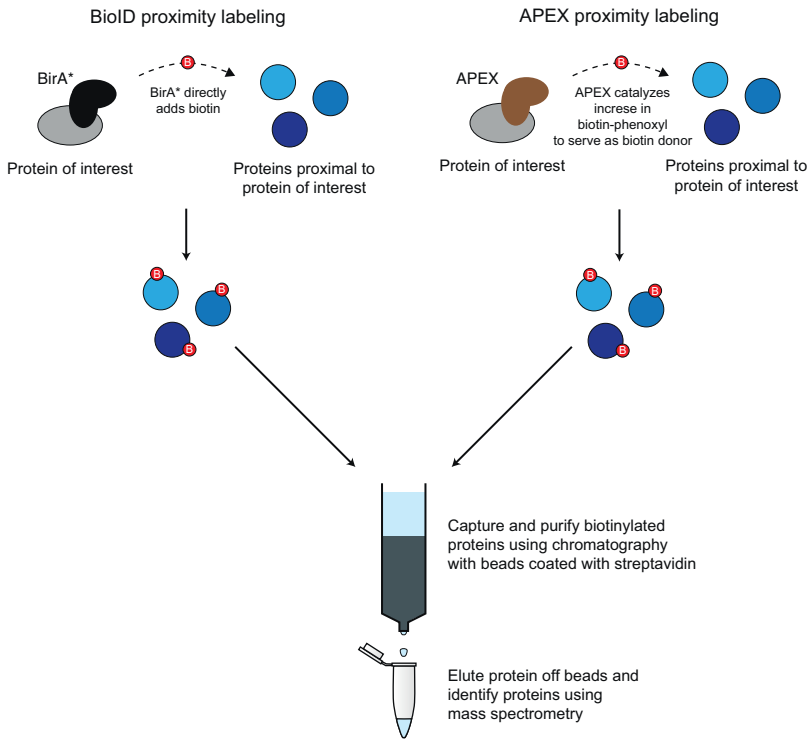

FIGURE 14.19 **Proximity labeling to determine protein-protein interactions.** To use proximity labeling, a protein of interest is fused with either a mutated biotin ligase (BirA*) or an APEX tag. BirA* directly adds biotin molecules to any proteins that come in close proximity with the protein. APEX causes the oxidation of biotin-phenol to a more reactive biotin-phenoxyl radical that also adds biotin molecules to nearby proteins. Biotin-labeled proteins from either strategy are purified using chromatography with beads bound to streptavidin. Once eluted from the beads, the proteins can be identified using mass spectrometry.

and streptavidin. For example, **BioID** is a proximity labeling technique that utilizes a mutated form of a protein called biotin ligase, BirA*, that causes biotin to bind to exposed lysine amino acid residues on nearby proteins. A scientist uses recombinant DNA techniques to fuse BirA* with a protein of interest. This fusion protein is expressed in cells (in vitro or in vivo) and can be localized to the endogenous location of the protein of interest (e.g., the synapse). During an experiment, cells are incubated with exogenous biotin. The fusion protein will therefore attach biotin molecules to any proteins that are in close proximity to the protein of interest. After multiple hours, the cells are lysed, and the contents are precipitated using chromatography using beads coated with streptavidin. Any proteins bound to the beads in high concentration were likely in close proximity with the BirA* fusion protein, and thus likely to interact with the protein of interest. These proteins can be identified using mass spectrometry.

Another system for efficient proximity labeling is an engineered ascorbate peroxidase (APEX). APEX causes catalysis of biotin-phenol to the radical biotin-phenoxyl, which reacts with electron-rich amino acids on neighboring proteins and causes their biotinylation. Biotinylated proteins can then be purified using chromatography and identified using mass spectrometry similar to the BioID system. An advantage of APEX over BioID is that it is able to label neighboring proteins in minutes rather than hours. However, the biotin-phenol reagent can be toxic to living cells and so APEX cannot be used for in vivo contexts.

Fluorescent Visualization of Protein–Protein Interactions

Fluorescence (or Förster) resonance energy transfer (FRET) is used to visualize interactions and proximity between two proteins of interest (Fig. 14.20A). Each protein is fused to a different fluorescent protein, such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The fluorescent proteins are chosen such that the emission wavelength of light of one protein (the "donor") can excite the other (the "acceptor"). For example, the emission wavelength of CFP is ~480 nm, the same wavelength of light that causes excitation of YFP. Therefore, if the two fusion proteins are expressed in a sample illuminated at the excitation wavelength of CFP, the CFP molecules will fluoresce such that the YFP molecules will also fluoresce, but only if the two proteins are in close proximity (closer than about 2 nm). FRET is measured as the ratio of fluorescence intensity of the donor to fluorescence intensity of the acceptor. FRET has traditionally been exploited to assess distances and orientations between separate molecules or different sites within a single macromolecule. This technique can also be used to report any substrate modification that leads to a conformational change resulting in the interaction of donor and acceptor fluorophores.

FRET measurements are sensitive to the concentration of the fluorescent molecules. **Fluorescence-lifetime imaging microscopy (FLIM)** is similar to FRET, but is insensitive to concentration, tissue thickness, or photobleaching.

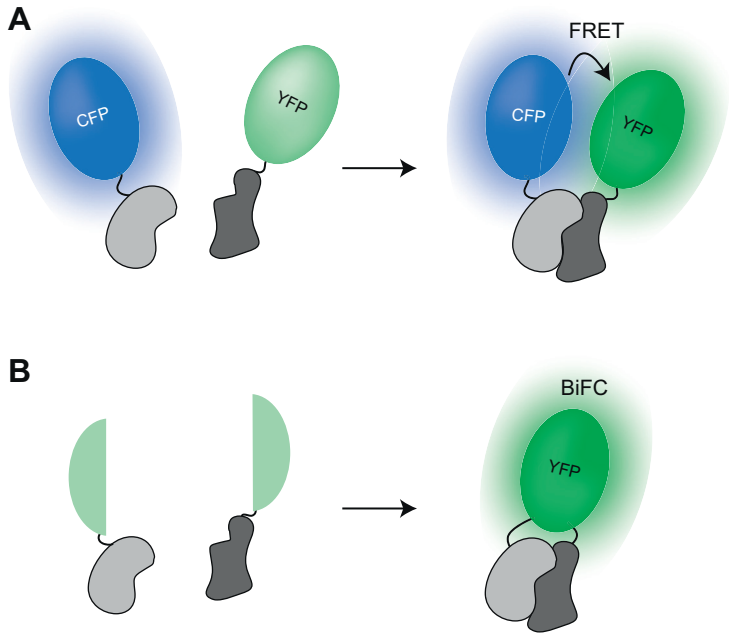

FIGURE 14.20 Fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC). (A) In FRET, one protein of interest is fused with cyan fluorescent protein (CFP) and another with yellow fluorescent protein (YFP). CFP is excited by light at ~436 nm, causing it to emit light at ~480 nm. YFP is excited by light at ~480 nm. Therefore, if the CFP fusion protein is in close proximity to the YFP fusion protein and the sample is illuminated at 436 nm, YFP will become excited and emit light at its emission wavelength of ~530 nm. If the two proteins are not in close proximity, YFP will not fluoresce. (B) In BiFC, a fluorescent molecule is split in two, and each half is fused with two proteins of interest. If the two proteins are in close proximity while exposed to the excitation wavelength of light, the two subunits can interact to emit light. If the two proteins are not in close proximity while exposed to the excitation wavelength of light, no light emission occurs.

Instead of measuring the raw intensity of the "acceptor" fluorescent protein (e.g., YFP) to determine molecular interactions, FLIM measures the time it takes the acceptor fluorescent molecule to decay from its excited state after a brief pulse of light that excites the donor fluorescent molecule. The decay time can serve as a reliable measure of proximity that is independent of the concentration of the fluorescent molecules.

A similar technique to FRET, **bimolecular fluorescence complementation (BiFC)**, also visualizes the interactions and proximity of two proteins of interest (Fig. 14.20B). Rather than fusing separate fluorescent reporter proteins to the two proteins of interest, two halves of a single reporter protein (e.g., GFP) are each fused to one of the proteins. The two fusion proteins are expressed in cells and illuminated at the excitation wavelength of light. If the

two proteins are not in close proximity, there is no fluorescence. However, if the two proteins are close in proximity, the two halves of the reporter protein can functionally interact and emit light.

INVESTIGATING POSTTRANSLATIONAL MODIFICATIONS

A **posttranslational modification (PTM)** is a biochemical modification that occurs to one or more amino acids on a protein after the protein has been translated by a ribosome. One of the most commonly studied PTMs is the addition or removal of a phosphate group, $-PO_4$ (Fig. 14.21). Other common PTMs are listed in Table 14.3. These modifications often change the ultrastructural and functional properties of a protein, causing substantial downstream signaling effects. For example, the phosphorylation state of a protein often indicates whether it is functional or inactive.

There are multiple questions a scientist may ask about a protein of interest regarding PTMs. For example, a scientist might want to know if a protein of interest receives a PTM. This question can be answered using mass spectrometry (Box 14.2) to analyze the molecular weights of protein fragments, identifying fragments with a PTM. Other questions include whether a specific enzyme is responsible for modifying a protein of interest, or how the PTM changes over time following a stimulus. These questions can be answered using the following techniques.

Detection of PTMs

In addition to detecting PTMs for a protein of interest using mass spectrometry, it is often also possible to produce an antibody to a protein of interest that is directed against a site on the protein that receives a posttranslational modification (Fig. 14.22). Such an antibody binds to its substrate protein only when the PTM is present. PTM-specific antibodies can be used in any of the previously described antibody-based techniques to assay the extent of modifications, or with spatially sensitive techniques such as IHC or IEM to determine the localization of modified proteins. Such analysis is especially informative when studying

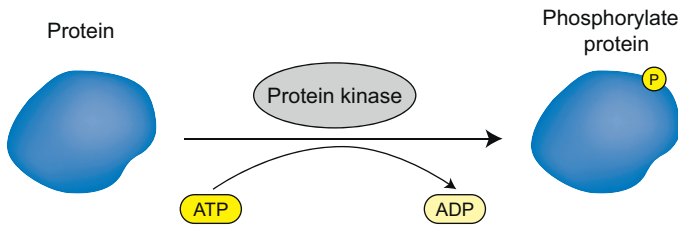

FIGURE 14.21 **Protein phosphorylation.** A protein kinase catalyzes a reaction in which a phosphate group from adenosine triphosphate (ATP) is covalently added to a substrate protein.

TABLE 14.3 Common Posttranslational Modifications.

Classification	Chemical Modification	Type of Enzyme Mediating the Modification
Phosphorylation	Addition of a phosphate group ($-PO_4$) to a residue of serine, tyrosine, threonine, or histidine	Protein kinase
Acetylation	Addition of an acetyl group ($-COCH_3$) at the N-terminus of a protein or a lysine residue	Acetyltransferase
Methylation	Addition of a methyl group ($-CH_3$) at a residue of lysine or arginine	Methyltransferase
Glycosylation	The addition of a glycosyl group to a residue of asparagine, hydroxylysine, serine, or threonine, resulting in a glycoprotein	(Multiple types of enzymes mediate this reaction)
Sulfation	Addition of a sulfate group ($-SO_4$) to a tyrosine residue	Tyrosylprotein sulfotransferase
Farnesylation	Addition of a farnesyl group ($-C_{15}H_{26}O$) to the C-terminus of a protein. Usually causes the protein to anchor to the cell membrane	Farnesyltransferase
Ubiquitination	Addition of a ubiquitin protein to a lysine residue. Usually targets proteins for proteasomal degradation.	Ubiquitin ligase

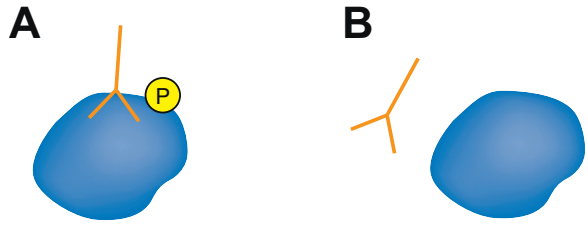

FIGURE 14.22 A PTM-specific antibody. (A) A PTM-specific antibody binds to an epitope on a protein that is phosphorylated at a specific amino acid residue, (B) but not when the phosphate group is not present.

dynamic changes in protein localization and activation. By exposing a given sample to various molecular signals, such as growth factors, a scientist can assay any changes in subcellular localization of a protein of interest that correlate with changes in posttranslational modification.

PTM-Specific Assays

PTM-specific assays are designed to test the hypothesis that a particular protein mediates the posttranslational modification of another protein. Each PTM has its own biochemical assay. Here, we describe a common type of assay: a kinase assay.

A **kinase assay** is used to determine whether one protein is capable of phosphorylating another protein. As mentioned previously, protein kinases are enzymes that catalyze the covalent transfer of phosphate from ATP to a substrate protein. A scientist can incorporate a radioactive label, such as [^{32}P] orthophosphate, into molecules of ATP. This radioactive ATP acts as the phosphate donor for the protein kinase substrate. To perform this assay, the scientist combines the hypothetical protein kinase, the substrate protein, and radioactive ATP into a reaction tube (Fig. 14.23A). Next, the scientist isolates the substrate protein using immunoprecipitation and visualizes the presence of ^{32}P using autoradiography (Fig. 14.23B). If the target protein incorporates ^{32}P, then this protein is capable of being phosphorylated by the protein kinase.

Kinase assays are easily the most common PTM assay represented in the literature, as at least 2% of the genes in the mammalian genome code for protein kinases. However, there are many different PTM-specific assays, for example, methyltransferase or acetyltransferase assays, which are used to determine whether a protein is capable of adding a methyl or acetyl group, respectively, to a target protein.

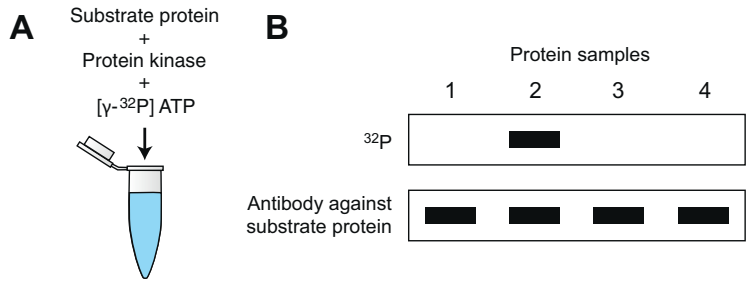

FIGURE 14.23 A kinase assay. (A) To determine if a protein kinase is able to phosphorylate a protein, a scientist mixes the kinase, the protein, and radioactive ATP into a reaction tube. 15 min later, the scientist immunoprecipitates the protein. (B) The immunoprecipitated proteins are run on a gel. ^{32}P will only be present if the protein kinase is able to phosphorylate the substrate protein. To ensure that the amount of substrate protein was equal in each sample tested, a scientist typically performs a western blot using an antibody against the substrate protein. A reasonable conclusion from this mock figure would be that the protein kinase under investigation was able to phosphorylate a protein in sample 2 but not samples 1, 3, or 4.

INVESTIGATING PROTEIN–DNA INTERACTIONS

The final step in many intracellular signaling pathways is the activation of a protein that directly affects gene transcription. Scientists therefore often ask whether a specific protein of interest is able to interact with DNA and affect the expression of a specific target gene. Three techniques are commonly used to examine interactions between proteins and DNA: the electrophoretic mobility shift assay, the ChIP assay, and the luciferase assay. Because these techniques answer slightly different questions about protein–DNA interactions, scientists often use them collectively in the same studies.

Electrophoretic Mobility Shift Assay

An **electrophoretic mobility shift assay (EMSA)**, also known as a **gel shift assay**, is used to determine if a protein is able to directly interact with a short, specific sequence of DNA. Before the experiment begins, the investigator hybridizes two complementary DNA strands (about 30–40 bp in length) and labels the strands with a radioactive probe. The investigator also purifies the protein that is hypothesized to interact with the strand of DNA.

During the experiment, the radiolabeled DNA is run in several lanes of a polyacrylamide gel (Fig. 14.24A). The speed at which the DNA molecules move from one side of the gel to the other during electrophoresis is due to their size, with lower molecular weight molecules migrating faster. In one lane, the DNA is run by itself. In another lane, the DNA is run with the purified protein. If the protein interacts with the DNA strand, the size of the DNA–protein complex will be greater than the DNA strand alone, and therefore, the band of DNA will be shifted upwards on the gel (Fig. 14.24B).

Several controls are necessary for a proper EMSA experiment: (1) in addition to the DNA sequence of interest, the investigator could run a slightly scrambled DNA sequence to ensure that the DNA–protein interaction is specific to the exact DNA sequence of interest. (2) The investigator could perform a "competitive" binding assay in which nonradiolabeled DNA is added to the reactions with the radiolabeled DNA. If there is sufficient nonradiolabeled DNA, the amount of protein bound to the radiolabeled DNA will decrease, and the size of the radiolabeled DNA band will be shifted back to normal. (3) An antibody to the purified protein could be added to a lane with the protein and labeled DNA. This will further increase the size of the DNA–protein complex and create a "supershifted" band, demonstrating that it is indeed the protein of interest that is causing the shift in the first place. (4) The investigator could run a nonspecific protein known not to interact with DNA, such as a membrane-bound protein, to ensure that there is nothing "sticky" about the DNA strand in the EMSA reaction.

One of the strengths of an EMSA assay is that it demonstrates that a protein can directly bind to a sequence of DNA. One of the limitations is that all reactions take place in a test tube. Therefore, the only conclusions that can be reached using this assay is that a protein is capable of binding DNA, but not that it actually does so in a living cell.

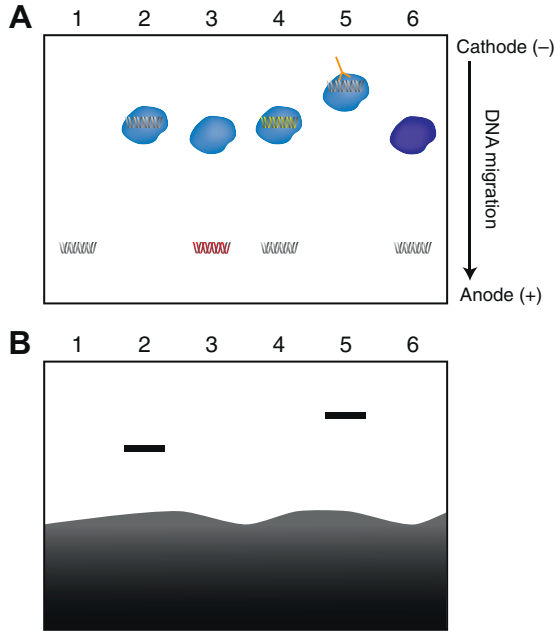

FIGURE 14.24 Electrophoretic mobility shift assay (EMSA). (A) To determine if a protein is capable of interacting with a short sequence of double-stranded DNA, a scientist radioactively labels short DNA sequences, mixes these sequences with purified proteins, and runs the mixture on a gel. Any DNA sequences that interact with a protein will migrate much slower on the gel. In this example, lane 1 represents DNA alone. Lane 2 represents DNA with a protein that interacts with the DNA. Lane 3 represents a "scrambled" control, in which the nucleotide sequence is jumbled and there is no protein interaction. Lane 4 represents a reaction in which a sufficient amount of nonradiolabeled DNA is added to the mixture, taking a high percentage of the interactions with the binding protein, and causing most of the radiolabeled DNA to be unbound. Lane 5 represents a reaction that includes an antibody to the protein. The antibody–protein interaction increases the macromolecular complex, "supershifting" the DNA so it moves more slowly down the gel. Lane 6 represents a reaction in which a protein is included that is known not to bind to DNA, such as a membrane-bound protein. (B) Data analysis for the EMSA reaction described above. The dark gradient at the bottom represents the unbound, radioactive DNA strands that have migrated to the bottom of the gel.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is used to determine if a protein interacts with a specific region of DNA. The advantage to the ChIP method over an EMSA assay is that the starting material comes from living cells, most often cultured cells, though fresh tissue is sometimes used. However, unlike an EMSA, it is impossible to determine if a protein directly interacts with a sequence of DNA. It is possible that the protein could interact with DNA in a larger macromolecular complex with other proteins but not come into contact with DNA directly.

A scientist begins a ChIP experiment by quickly fixing cells in a diluted formaldehyde solution so that DNA–protein complexes are **cross-linked** and remain intact (Fig. 14.25). Next, the DNA is sheared into approximately 600–1000 bp fragments using a device called a sonicator. If the cross-linking is adequate, the proteins will continue to be bound to the fragmented DNA. At this stage, the scientist immunoprecipitates the DNA–protein complexes using an antibody against the protein of interest.

After the immunoprecipitation step, the purified proteins are "reverse cross-linked" such that the proteins become unbound to the DNA and the DNA fragments can be purified. If the scientist hypothesizes that the protein of interest interacted with a specific DNA sequence, specific primers can be used to sequence the DNA or amplify the DNA fragments using PCR. The final result of a ChIP assay is often a photograph of an agarose gel that reveals the end product of PCR reactions (Fig. 14.26). Alternatively, quantitative real-time PCR can be performed to better quantify the amount of PCR product in each

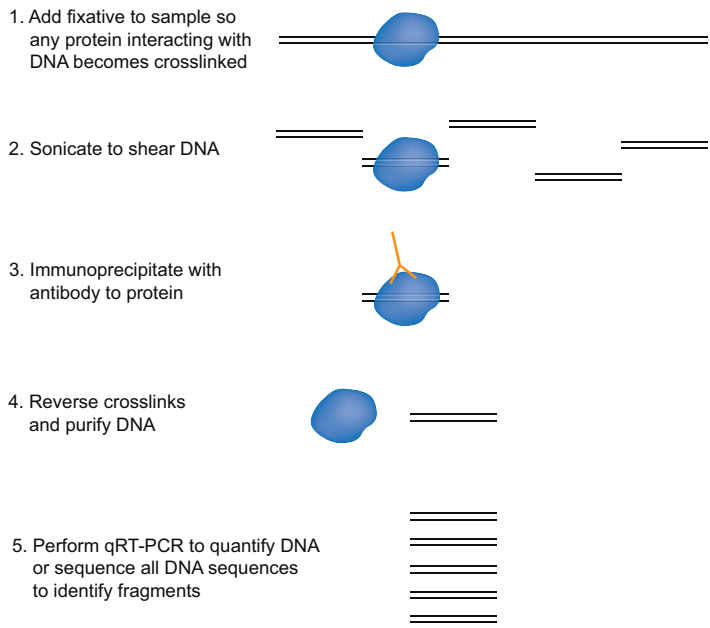

FIGURE 14.25 Chromatin immunoprecipitation (ChIP). ChIP can be used to identify DNA–protein interactions. A scientist collects a DNA sample from cells or tissues. (1) A fixative is added to cross-link any protein–DNA interactions in the sample. (2) DNA is sonicated into short fragments. The cross-links allow proteins to remain bound to DNA. (3) An antibody against a protein of interest is used to immunoprecipitate the protein, along with any bound DNA fragments. (4) Cross-links are reversed so that protein becomes unbound to DNA. Only the short DNA sequences that interacted with the protein of interest remain. (5) The DNA fragments can be identified using primers to amplify the sequences using PCR and run on a gel, or analyzed using qRT-PCR. Alternatively, all remaining DNA fragments can be sequenced.

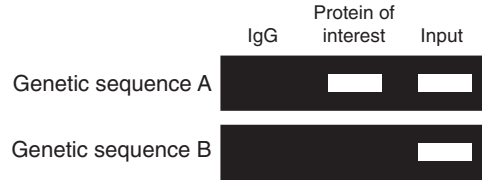

FIGURE 14.26 ChIP assay data analysis. To analyze ChIP using gel electrophoresis, a scientist amplifies specific genetic sequences from the ChIP reaction product using PCR. The PCR products are run on an agarose gel to visualize the bands at specific sizes. The labels at top are the immunoprecipitating antibodies. IgG is a random immunoglobulin that should not bind DNA and therefore should not show any signal. The input lane represents the solution *before* running the immunoprecipitation, so all DNA should be present. A reasonable conclusion from this mock figure would be that the protein of interest interacts with DNA sequence A but not sequence B.

lane. If a scientist does not have a hypothesis as to which DNA sequences are bound to a protein of interest, it is possible to determine the identity of all the DNA sequences bound to the immunoprecipitated protein using high-throughput sequencing techniques (Chapter 9) in a process called **ChIP-seq**.

Several positive and negative controls are necessary for a proper ChIP experiment. An important positive control is an input lane containing PCR products from DNA obtained after sonication but prior to immunoprecipitation. This reaction should result in a strong signal and ensure that the DNA of interest was properly obtained in the first place. Another important positive control is an immunoprecipitation reaction with an antibody for a protein known to bind to any region of DNA, such as a histone protein. ChIP results using a histone antibody should therefore produce a strong DNA signal. Negative controls include performing an immunoprecipitation with nonspecific antibodies and using primers that amplify nonspecific DNA regions.

Luciferase Assay

A **luciferase assay** is used to determine if a protein can activate or repress the expression of a target gene. Unlike the ChIP or EMSA assays, which only assess the ability of a protein to interact with a region of DNA, a luciferase assay is able to establish a *functional* connection between the presence of the protein and the amount of gene product that is produced. This assay is unable to determine whether the protein directly interacts with DNA itself, as the protein could indirectly affect transcription by activating or repressing a separate protein or protein complex that affects transcription.

Luciferase is an enzyme used for bioluminescence by various organisms in nature, most famously the firefly. The scientist produces a construct in which the regulatory region of a target gene is fused with the DNA coding sequence for luciferase (Fig. 14.27). A separate DNA construct encodes the protein hypothesized to affect transcription. The scientist transfects a cell culture system, such as HEK 293T cells, with both constructs. If the protein is able to upregulate transcription of the target gene, the cells will express luciferase. If the protein downregulates transcription, the cells will express less luciferase than normal. The scientist examines the expression of luciferase about 2–3 days after the initial cell transfection. The cells are lysed, and the cell contents are placed in a reaction tube. If the proper substrate is added, luciferase will catalyze a reaction that produces light. This light is detected with a luminometer, a device that precisely quantifies how much light is produced in each reaction tube. The amount of light produced provides a quantitative measure of the effect of the protein on expression of the target gene.

Due to variability in conditions within each well in a cell culture plate, a proper luciferase assay experiment should repeat each condition at least in triplicate. The scientist should compare luciferase expression between conditions in which (1) no protein is present, (2) protein is present, and (3) the promoter sequence regulating luciferase expression is mutated so that the protein is no longer able to bind. If a scientist knows enough about the structure of a protein such that it is possible to mutate the protein's DNA-binding domain or enhance the ability of the protein to affect transcription, such conditions would also strengthen the conclusions of a luciferase assay experiment.

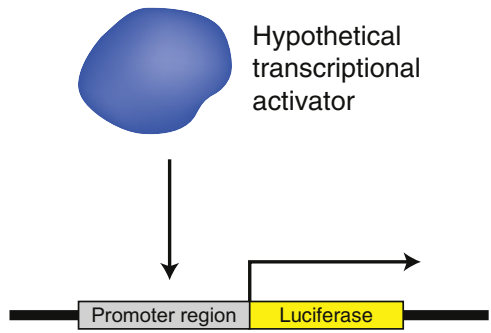

FIGURE 14.27 A luciferase assay. To determine if a protein is able to activate (or suppress) transcription of a gene of interest, a scientist uses recombinant DNA technology to produce a construct in which the gene's promoter is placed adjacent to a luciferase reporter gene. This construct, as well as a construct coding for the protein, is introduced into cultured cells. If the protein is able to activate transcription, the cell will produce the luciferase reporter. The amount of luciferase produced can be quantified using a luminometer.

CONCLUSION

The purpose of this chapter was to survey common methods used to investigate protein expression and signaling. Although these methods are numerous, they essentially fall into four categories: (1) determining whether a protein is expressed in a particular population of cells and how much; (2) determining whether a protein interacts with other proteins; (3) determining the types of posttranslational modifications a protein can receive and which proteins cause these modifications; and (4) determining whether a protein is capable of interacting with DNA and/or regulating gene expression. Anyone outside the fields of biochemistry and cell signaling should not be intimidated by the huge variety of techniques. Focusing on the experimental questions rather than the details of the methods allows intracellular signaling studies to be understandable to any neuroscientist (Box 14.3).

BOX 14.3 Walkthrough of an Intracellular Signaling Experiment

Let us say that you work in a laboratory that studies a specific aspect of neural development: how a neuron's axon correctly extends to its postsynaptic target. The tip of an axon is referred to as the axonal growth cone, and it reaches its destination by responding to extracellular guidance cues produced by other cells. There are three general steps that occur to guide the growth cone: (1) an extracellular guidance cue is secreted to guide the axon's position; (2) a receptor on the axon's surface recognizes that cue and transduces it into an intracellular signaling cascade that affects other proteins within the developing neuron; and (3) various downstream organelles receive and respond to the signal, including the cytoskeleton that must be reorganized to move the growth cone toward the site where the initial cue was detected. If you know the identity of the receptor and the extracellular cue, you might be interested in determining how the receptor translates the cue into a response. What is the intracellular signaling pathway that leads to the cytoskeletal changes necessary for the growth cone to turn toward the cue? Let us focus on identifying the next step of signaling that occurs after the receptor is activated: the activation of a second protein that binds to the intracellular domain of the receptor.

There are multiple approaches for identifying proteins that interact with the receptor. For example, you could immunoprecipitate the receptor and then perform mass spectrometry to identify any other proteins that coprecipitated. However, this approach would not tell you if the two proteins interacted directly or were simply part of a larger complex of proteins. Another approach would be to perform a yeast two-hybrid assay. This technique is used to identify protein-binding partners that directly interact with the receptor. To perform this assay, you would begin by genetically engineering a construct that attaches the receptor's intracellular domain to the DNA-binding domain of a transcriptional activator, such as Gal4, to act as the bait. Then you would create or use a cDNA library to attach different cDNA molecules to the activation domain of Gal4 to act as the prey. Finally, you would introduce the bait and prey constructs into cells that express a reporter gene, often *lacZ* or GFP, under the control of a promoter that is recognized by the transcriptional activator (*UAS* for the Gal4 activator). Thus, the bait

BOX 14.3 Walkthrough of an Intracellular Signaling Experiment—cont'd

will bind to the promoter but cannot initiate transcription of the reporter until a specific prey with the activation domain interacts with the bait to create the full activator. Then, the reporter gene will be transcribed and can be detected.

To detect cells that express *lacZ,* you could grow thousands of cells on an agar plate that also contains X-gal, the substrate that causes a visible blue by-product to form in those cells. The final step would be to pick blue yeast colonies off the plate, extract and sequence the DNA of the "prey," and identify the protein that interacts with the receptor based on the cDNA sequence. This screen allows you to conclude that the prey you found *can* interact with the intracellular domain of the receptor. Most likely, you will have discovered a number of positive candidates to sequence and should confirm the interaction's specificity to determine which would be a good candidate for future experiments.

This yeast two-hybrid experiment is a good start, but you could learn even more about the receptor–protein interaction by attaching different functional regions of the receptor's intracellular end to use as bait. This experiment would tell you with finer resolution exactly what part of the receptor is interacting with the prey or allow you to capture only prey that will interact with a specific region—say, the kinase domain—or a particular region you know to be functionally important. To convince yourself and other scientists of the interaction between the receptor and intracellular protein, you could perform additional, complementary experiments. For example, co-immunoprecipitation, as just described, is often used to verify yeast two-hybrid results. You could also use protein affinity chromatography. In cell signaling studies, there is no such thing as too much evidence; figures often contain several different methods to validate the same result.

Now that you have a candidate (or several candidates, based on the results of the yeast two-hybrid experiments), you could follow up with other experiments to make sure that your candidate protein makes sense as a protein that mediates the intracellular response to a guidance cue. One question you might ask right away is whether the candidate protein is expressed in the right place (the axon growth cone) and at the right time (the embryonic or postnatal period during which the axon grows toward its target). If the interacting protein is not present in axonal growth cones or at a time when axons are growing out, the interaction may not be relevant to the growth cone guidance behavior you are interested in, or the interaction may not actually occur. To examine protein expression, you could use immunohistochemistry to stain for the expression of the protein in histological preparations of the growth cone at different developmental timepoints. You could also collect tissue samples containing the growth cones from different timepoints and perform western blots to examine the expression of the protein at each time point.

If you determine that a candidate protein binds to a receptor, is expressed in the growth cone, and is expressed during the developmental period in which the growth cone migrates toward its target, you have laid the foundation for many future experiments. For example, you could ask whether the interaction between the receptor and the protein causes the protein to be phosphorylated. You could also use techniques described in other chapters, such as CRISPR-Cas9 or RNA interference (Chapter 12), to investigate the consequence of disrupting the expression of the intracellular protein.

SUGGESTED READING AND REFERENCES

Books

Berg, J.M., Tymoczko, J.L., Gatto Jr., G.J., Stryer, L., 2019. Biochemistry, ninth ed. W. H. Freeman.

Fields, R.D., 2008. Beyond the Synapse: Cell-Cell Signaling in Synaptic Plasticity. Cambridge University Press.

Moss, T., Leblanc, B., 2009. Protein Interactions: Principles and Protocols, third ed. Humana Press, NY.

Wilson, K., Walker, J.M., 2018. Principles and Techniques of Biochemistry and Molecular Biology, eighth ed. Cambridge University Press.

Review Articles

Araujo, S.J., Tear, G., 2003. Axon guidance mechanisms and molecules: lessons from invertebrates. Nat. Rev. Neurosci. 4, 910–922.

Grant, S.G.N., Blackstock, W.P., 2001. Proteomics in neuroscience: from protein to network. J. Neurosci. 21 (21), 8315–8318.

Greer, P.L., Greenberg, M.E., 2008. From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron 59, 846–860.

Guan, K.L., Rao, Y., 2003. Signaling mechanisms mediating neuronal responses to guidance cues. Nat. Rev. Neurosci. 4, 941–956.

Tronson, N.C., Taylor, J.R., 2007. Molecular mechanisms of memory reconsolidation. Nat. Rev. Neurosci. 8, 262–275.

Primary Research Articles—Interesting Examples from the Literature

Branon, T.C., Bosch, J.A., Sanchez, A.D., Udeshi, N.D., Svinkina, T., Carr, S.A., Feldman, J.L., Perrimon, N., Ting, A.Y., 2018. Efficient proximity labeling in living cells and organisms with TurboID. Nat. Biotechnol. 36, 880–887.

Cowan, C.W., Shao, Y.R., Sahin, M., Shamah, S.M., Lin, M.Z., Greer, P.L., Gao, S., Griffith, E.C., Brugge, J.S., Greenberg, M.E., 2005. Vav family GEFs link activated Ephs to endocytosis and axon guidance. Neuron 46, 205–217.

Flavell, S.W., Cowan, C.W., Kim, T.K., Greer, P.L., Lin, Y., Paradis, S., Griffith, E.C., Hu, L.S., Chen, C., Greenberg, M.E., 2006. Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. Science 311, 1008–1012.

Kim, M.S., Pak, Y.K., Jang, P.G., Namkoong, C., Choi, Y.S., Won, J.C., Kim, K.S., Kim, S.W., Kim, H.S., Park, J.Y., Kim, Y.B., Lee, K.U., 2006. Role of hypothalamic FOXO1 in the regulation of food intake and energy homeostasis. Nat. Neurosci. 9, 901–906.

Kondo, M., Takei, Y., Hirokawa, N., 2012. Motor protein KIF1A is essential for hippocampal synaptogenesis and learning enhancement in an enriched environment. Neuron 73, 743–757.

Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., et al., 2014. Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature 510, 162–166.

Patterson, G.H., Lippincott-Schwartz, J., 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. Science 297, 1873–1877.

Rhee, H.-W., Zou, P., Udesh, N.D., Martell, J.D., Mootha, V.K., Carr, S.A., Ting, A.Y., 2013. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. Science 339, 1328–1331.

Roux, K.J., Kim, D.I., Raida, M., Burke, B., 2012. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. J. Cell Biol. 196, 801–810.

Soden, M.E., Jones, G.L., Sanford, C.A., Chung, A.S., Guler, A.D., Chavkin, C., Lujan, R., Zweifel, L.S., 2013. Disruption of dopamine neuron activity pattern regulation through selective expression of a human KCNN3 mutation. Neuron 80, 997−1009.

Spence, E.F., Dube, S., Uezu, A., et al., 2019. In vivo proximity proteomics of nascent synapses reveals a novel regulator of cytoskeleton-mediated synaptic maturation. Nat. Commun. 10, 386.

Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., Afzal, V., Ren, B., Rubin, E.M., Pennacchio, L.A., 2009. ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature 457, 854−858.

Protocols

Aparicio, O., Geisberg, J.V., Sekinger, E., Yang, A., Moqtaderi, Z., Struhl, K., 2005. Chromatin immunoprecipitation for determining the association of proteins with specific genomic sequences in vivo. Curr. Protoc. Mol. Biol. https://doi.org/10.1002/0471143030.cb1707s23 (Chapter 21), Unit 21.3.

Golemis, E.A., Serebriiskii, I., Gyuris, J., Brent, R., 2001. Interaction trap/two-hybrid system to identify interacting proteins. Curr. Protoc. Neurosci. https://doi.org/10.1002/0471142727.mb2001s82 (Chapter 4), Unit 4.4.

Harlow, E., Lane, D., 1999. Using Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Pandey, A., Andersen, J.S., Mann, M., 2000. Use of mass spectrometry to study signaling pathways. Sci. STKE 2000, PL1.

Walker, J.M., 2002. The Protein Protocols Handbook, second ed. Humana Press, Totowa, NJ.

Websites

Resource for information on protein methods − expression, purification, antibody production: http://www.piercenet.com/method/protein-methods-library.