Chapter 6

Visualizing Nervous System Structure

After reading this chapter, you should be able to:

- Explain the process of preparing neural tissue for histological procedures
- Describe methods for visualizing gross cellular morphology
- Describe methods for visualizing gene and protein expression
- Describe methods for visualizing neural circuitry and connections between different parts of the nervous system

Techniques covered:

- **Tissue preparation:** fixation, embedding, sectioning, tissue clearing
- **Visualizing cell morphology:** fundamental histological methods (basophilic stains, fiber stains), Golgi stain, intracellular/juxtacellular labeling
- **Visualizing gene and protein expression:** in situ hybridization, immunohistochemistry, array tomography, enzymatic histochemistry, reporter genes
- **Visualizing neural circuitry:** anterograde tracers, retrograde tracers, transsynaptic tracers

A person can learn a lot about a complicated system simply by examining its structure. For example, imagine a satellite image of a city taken from space. How might you learn about what it would be like to live there just by examining the city from that vantage point? You could start by separating the image into component parts—for example, distinguishing buildings from roads. Larger buildings clustered together could reveal popular hubs of activity, while smaller, scattered buildings might indicate residential communities. You would learn even more by identifying the different categories of buildings, classifying different structures as schools, grocery stores, gas stations, shopping centers, and so forth. Major highways and freeways would indicate routes for long-distance travel; narrower roads would show local areas of transit. By simply examining the structure of the city, one could form educated guesses about how the city functions.

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Similarly, one can learn a lot about the nervous system simply by examining its structure. Just like distinguishing between buildings and roads in a city, a neuroscientist can begin to examine the brain by discriminating between cells and fiber tracts. Cells, like buildings, can be classified into different structural and functional groups, with each cell type expressing a unique combination of genes and proteins that specify its role in the brain. Thick white matter tracts show long-distance sites of neural communication, while smaller fiber tracts show local communication networks within populations of neurons. Learning about the structure of distinct brain regions can lead to educated hypotheses about the function of different neurons and how they influence neural circuits and behavior. Indeed, in the early 20th century, Santiago Ramón y Cajal formed many hypotheses (many of which turned out to be correct) about the function of neural systems simply by examining their structures.

The goal of this chapter is to survey techniques used to investigate the structure and connectivity of the nervous system. Using these techniques, it is possible to classify cells based on location, morphology, gene/protein expression profiles, and connections with other cells. These methods can be used in combination with techniques described in Chapter 7 to visualize neural function.

TISSUE PREPARATION

Neural tissue is soft, delicate, and degradable. Therefore, in order to accurately study neural structure, an investigator's first goal is to keep the tissue as close to its living state as possible. Investigators process tissue by (1) fixing, (2) embedding, and (3) sectioning before proceeding with visualization methods. Fixing and embedding methods stabilize tissue to capture its current state, while sectioning makes the tissue thin enough for light to pass through such that internal structures can be examined under a microscope.

Fixation

Fixation is the process of using chemical methods to preserve, stabilize, and strengthen a biological specimen for subsequent histological procedures and microscopic analysis. This process preserves cells and tissues by strengthening molecular interactions, disabling endogenous proteolytic enzymes, and killing microorganisms that might otherwise degrade the specimen. Thus, fixation terminates any ongoing biochemical reactions by "fixing" proteins into place, a process that kills cells that are not already dead.

There are two different categories of chemical fixatives: cross-linking fixatives and dehydrating fixatives. Which fixative an investigator chooses to use depends on the type of subsequent histology to be performed. **Cross-linking fixatives** create covalent chemical bonds between proteins in tissue

and include organic compounds with aldehyde groups, such as formaldehyde, paraformaldehyde, and glutaraldehyde. These fixatives are good at preserving structure and are often used when processing cells and tissues for light microscopy. Another cross-linking fixative, osmium tetroxide, causes molecules to oxidize and is often used as a secondary fixative for electron microscopy. **Dehydrating fixatives** disrupt lipids and reduce the solubility of protein molecules, precipitating them out of the cytoplasmic and extracellular solutions. Common dehydrating fixatives include methanol and acetone.

A scientist can fix cells in culture simply by adding a chemical fixative to the culture chamber. Whole brains can be fixed either by immersion or perfusion. **Immersion** refers to placing small brains, or even entire animals, in fixative solutions. The amount of time necessary for the fixative to penetrate the entire tissue is variable, depending on the size of the brain: a fly brain may need to be fixed for only a few minutes, while a mouse brain may require days. **Perfusion** is the process of delivering a fixative through an animal's cardiovascular system. To perfuse an animal, a scientist anesthetizes the animal, carefully opens the rib cage to expose the heart, and then penetrates the left ventricle with a needle connected to a perfusion pump. The left ventricle pumps blood throughout the entire body and can therefore deliver a fixative to the nervous system. A scientist initially pumps a buffered saline solution to rinse the blood out of the veins and arteries, and then pumps a cross-linking reagent to fix all the tissues in the body. Perfusion fixation is thorough, quick (can be performed in 5−15 min, depending on the animal), and adequately fixes all cells in the brain if properly performed.

Some histological procedures do not require fixation or even require that a brain *not* be fixed. For example, the use of radioactively labeled ligands to localize neurotransmitter receptor-binding sites depends on those sites remaining intact, and fixation could disrupt their structure. Each histological method determines whether fixation is necessary, which fixatives to use, and when the fixation process should occur.

Embedding

Once a brain is fixed and extracted from an animal, it is sometimes encased in a protective substance before sectioning. **Embedding** is the process of surrounding a brain or tissue section with a substance that infiltrates and forms a protective shell around the tissue. This process stabilizes the tissue's structure and makes it easier to cut. Standard embedding materials include gelatin, paraffin wax, and plastic. Brains can also be embedded before sectioning on a freezing microtome or cryostat in a viscous solution called **optimal cutting temperature (OCT) compound**. The OCT compound is clear and viscous at room temperature, but becomes solid and opaque around −10°C, forming a hard shell around a neural specimen.

Sectioning

Sectioning is the process of cutting a brain into thin slices for subsequent histological procedures or microscopic examination. This process is almost always necessary to examine the structure of neurons within the brains of vertebrate model organisms. Sectioning the brain allows histological reagents to access cells within the slice, and also allows a scientist to examine brain structures with a microscope. There are three common machines used to section tissue (Fig. 6.1, Table 6.1):

- A **microtome** (*micro* = "small," *tome* = "cut") is an instrument used to section tissue using a retractable blade. A specialized microtome called a **freezing microtome** is commonly used to section frozen tissue. Before

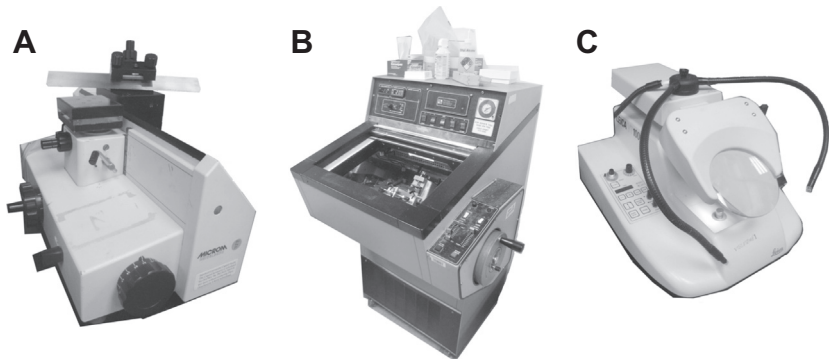

FIGURE 6.1 **Equipment used to section tissue.** (A) A microtome. (B) A cryostat. (C) A vibratome.

TABLE 6.1 Comparison of Different Brain Sectioning Methods.

Sectioning Tool	Essential Features	Standard Section Thickness
Microtome	• Brains are frozen • Preserves structures better than a cryostat	Medium: 25–100 μm
Cryostat	• Brains are frozen • Sections are cut in a cold chamber • Sections can be directly mounted onto glass slides	Thin: 10–50 μm
Vibratome	• Brains do not need to be frozen • A vibrating knife cuts brains • Can be used to cut tissue that will be kept alive	Thick: 100–400 μm

cutting, the tissue must be cryoprotected by soaking in a sucrose solution for 12–24 h to minimize artifacts caused by freezing. Using a freezing microtome, a scientist can collect 25–100 μm sections off the blade for subsequent histochemical processing. These sections are usually stored in a buffered saline solution until use. Other types of microtomes are used to section paraffin-embedded tissue or to create the ultrathin sections required for electron microscopy.

- A **cryostat** is essentially a microtome housed in a freezing chamber that allows sectioning at temperatures of −20 to −30°C. Maintaining the tissue and blade at low temperatures allows a scientist to section nonfixed, frozen tissue. Cryostats are able to cut thin, 10–50 μm sections. A scientist can mount sections onto slides within the cryostat or store sections in a buffered saline solution until use.
- A **vibratome** cuts tissue with a vibrating knife, somewhat similar to an electric vibrating toothbrush. The main function of a vibratome is to section tissues that are not frozen. The knife vibrates side-to-side so quickly that a soft brain can be cut into 100–400 μm sections. Thus, vibratomes are useful for avoiding artifacts, changes in morphology, or interruption of biochemical activity caused by freezing. A vibratome is also necessary for experiments in which a slice of brain tissue must be kept alive, as in slice culture or electrophysiology studies.

Once a brain is sectioned, a scientist can either mount the sections directly onto slides or can keep the sections floating in buffered saline for subsequent histological analysis.

Brains are typically cut in one of three standard orientations: coronal, horizontal, or sagittal (Fig. 6.2). The **coronal plane** divides the brain in the anterior-to-posterior direction, revealing a section of the brain that is complete from top-to-bottom (superior to inferior) and left-to-right (ear to ear). The **horizontal plane** divides the brain into superior (top) and inferior (bottom) sections, such that an area of the brain is complete from left-to-right and anterior to posterior. The **sagittal plane** shows the complete top-to-bottom of the brain, cutting it into left and right portions. A **midsagittal** cut is a section that perfectly divides the left and right hemispheres of the brain.

There are some preparations for which brain sectioning is not necessary. For example, scientists may wish to examine **whole-mount preparations** of intact brains or animals. Also, the brains of *Drosophila* and other invertebrates are usually small enough so that confocal or two-photon microscopy (Chapter 5) can provide good images of individual neurons within neural structures.

Tissue Clearing

Tissue clearing is the process of making intact tissue transparent, which allows for visualization of the nervous system without the need to section the

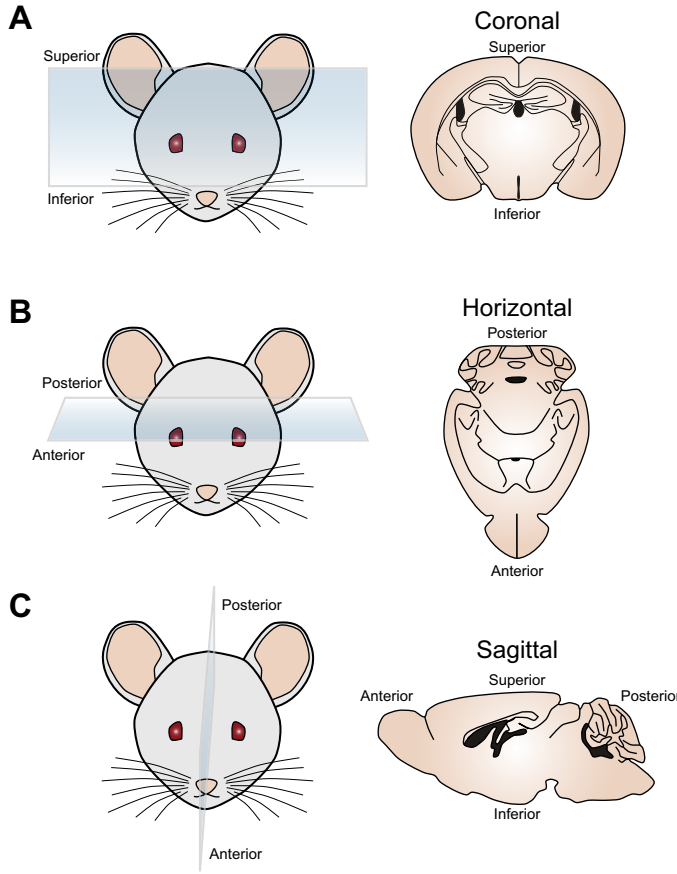

FIGURE 6.2 **Neuroanatomical planes.** Standard orientations for sectioning brain tissue. (A) Coronal plane. (B) Horizontal plane. (C) Sagittal plane.

tissue. For example, a scientist may want to visualize how a population of fluorescently labeled neurons projects throughout the mouse brain. To do this, the scientist can clear the entire brain, and then use light sheet microscopy (Chapter 5) to image the intact brain and trace the neurons. There are several techniques for tissue clearing, which vary based on the clearing reagents used. One popular technique, known as **CLARITY** (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/in situ hybridization-compatible Tissue hydrogel), fixes tissue using a hydrogel to preserve the physical structure of proteins and nucleic acids, but allows light-scattering lipids to be removed. This process makes intact tissue transparent and amenable to the structural visualization methods described in this chapter.

Now that we have discussed some common methods of preparing neural tissue for histological analysis, we describe fundamental methods of visualizing the gross morphology of the brain.

VISUALIZING MORPHOLOGY

Although lipids, proteins, carbohydrates, and other organic molecules provide the nervous system with its structure and durability, over three-quarters of the mass of the brain is water. This composition imparts significant consequences for any scientist interested in studying the structure of the brain, most notably that thin, unprocessed brain sections are almost completely transparent. Unless treated with histochemical stains, the structure of the nervous system will remain invisible and appear as a wafer-thin slice of gelatin mounted on a glass slide. Treating brain sections with various dyes enhances the contrast between different brain structures and allows an investigator to visualize cells, fibers, or other features of neural systems (Table 6.2). These methods each provide contrast to specific structures in the brain so that they stand out from other aqueous structures in a neural specimen.

Cell Body Stains

A **basophilic stain** is used to visualize cell bodies. The term *basophilic* is derived from *baso-,* meaning "base" and *-philic,* meaning "loving" and conveys the fact that these stains are basic (pH $>$ 8) and good for staining acidic molecules. As DNA and RNA molecules are acidic, basophilic dyes label structures enriched in nucleic acids, such as cell nuclei and ribosomes. A scientist can use these stains to examine the cytoarchitecture of individual neurons with high magnification or to visualize the macroscopic features of distinct brain regions, such as layers of cerebral cortex or subdivisions of hypothalamic nuclei (Fig. 6.3A).

Common basophilic stains used in brightfield microscopy include hematoxylin and thionine. A special category of basophilic stains, referred to as **Nissl stains**, specifically label RNA within cells, providing contrast to ribosomes and rough endoplasmic reticulum, which are enriched in neurons. Cresyl violet is one of the most used Nissl stains. Redistribution of Nissl-stained structures in injured or regenerating neurons allow Nissl stains to reveal the physiological state of some neurons.

In fluorescent microscopy, common markers of cell nuclei include DAPI, Hoechst (bis-benzamide), and propidium iodide (PI). These dyes intercalate within the helical spirals of DNA in the cell nucleus. Both DAPI and Hoechst emit blue light when excited by UV light, while PI emits red light when excited by green light. PI is useful in assays of cell death because it is membrane impermeant; therefore, it labels dead but not healthy cells.

Fiber Stains

Fiber stains label white matter tracts by staining **myelin**, the fatty substance that insulates and provides electrical resistance to axons. These stains are

TABLE 6.2 Histochemical Stains for Studying Neuroanatomy.

Stain	Use	Appearance	Comments
Cresyl violet	Cell nuclei, Nissl stain	Blue to purple	Useful for examining cytoarchitecture; stains each type of neuron slightly differently
Hematoxylin	Cell nuclei	Blue to blue-black	Often used in combination with eosin; known as H&E
Eosin Y	Cytoplasm	Pink to red	Counterstain with hematoxylin; acidophilic stain
DAPI	Cell nuclei	Fluorescent blue	Fluorescent DNA intercalating agent; excited by UV illumination
Hoechst (*bis*-benzamide)	Cell nuclei	Fluorescent blue	Fluorescent DNA intercalating agent; excited by UV illumination
Propidium iodide (PI)	Cell nuclei	Fluorescent red	Fluorescent DNA intercalating agent; excited by green light illumination
Weigert	Myelin	Normal myelin is deep blue; degenerated myelin is light yellow	Combines hematoxylin with other chemicals to selectively stain myelin
Weil	Myelin	Black	Combines hematoxylin with other chemicals to selectively stain myelin
Golgi stain	Fills neuron cell bodies and processes	Black	Stains individual neurons at random

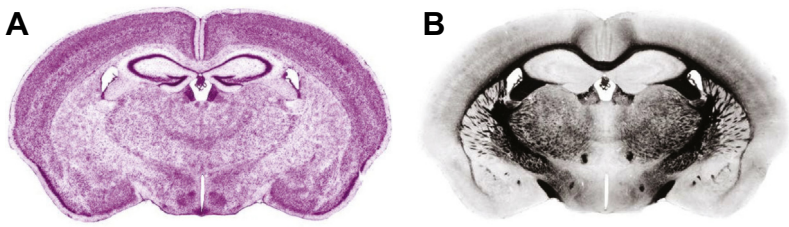

FIGURE 6.3 Basophilic and fiber stains. (A) Cresyl violet Nissl stain of a coronal mouse brain section. (B) An adjacent coronal section stained for myelin. *Courtesy of the High Resolution Mouse Brain Atlas at www.hms.harvard.edu/research/brain/atlas.html.*

useful for visualizing the major fiber tracts that project throughout the brain (Fig. 6.3B). However, the high density of fibers in most brain regions makes it impossible to trace individual axons from cell body to synapse. When fiber stains are used in combination with basophilic stains in adjacent tissue sections, a scientist can identify more anatomical structures than if either stain is used alone.

There are various protocols useful for labeling myelin, such as the Weigert or Weil methods that stain myelin dark blue or black. A scientist can also stain myelin using lipophilic fluorescent stains.

Golgi Stain

The **Golgi stain** is a classic technique used to completely label individual neurons and their processes (Box 6.1). There are two major advantages to using a Golgi stain: (1) neurons are labeled in their entirety, including cell bodies, processes, and even microscopic structures such as dendritic spines; (2) only 5%−10% of the total number of cells are labeled. Therefore, individual neurons stand out in a background of numerous unlabeled cells (Fig. 6.4). A scientist cannot control which neurons in a tissue slice become labeled, but modern techniques allow a scientist to duplicate the effects of a Golgi stain by expressing a visible marker in specific neural populations (Chapter 12). The Golgi stain is still used to define and trace the elaborate morphology of neurons, especially in neurodegeneration studies.

Intracellular and Juxtacellular Labeling

The preceding methods label cell bodies and/or axons after a brain has already been fixed and sectioned. It is also possible to label individual neurons during an experiment for subsequent histological analysis so that a neuron's activity

BOX 6.1 Historical Use of the Golgi Stain

The Golgi stain has an important place in the history of neuroscience. In the late 19th and early 20th centuries, Santiago Ramón y Cajal used this technique for his seminal studies of the cellular morphology of the nervous system. Among his many achievements was the discovery that neurons are distinct, independent cells that communicate via synapses. His work heavily contributed to the acceptance of the **neuron doctrine**: the hypothesis that neurons are the basic structural and functional units of the nervous system. Cajal also used the Golgi stain to reveal the morphological diversity of neurons, as well as to trace neural circuits in the retina, hippocampus, cerebellum, and other brain regions. In 1906, Cajal shared the Nobel Prize with Camillo Golgi, the scientist who developed the Golgi stain, for elucidating the structure of the nervous system.

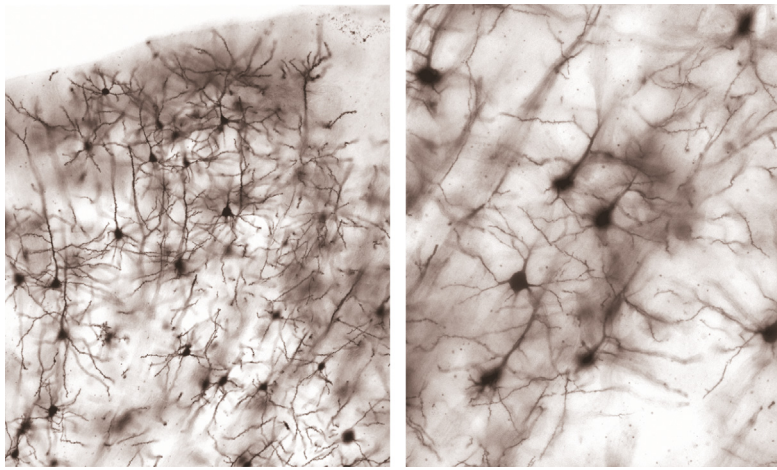

FIGURE 6.4 The Golgi stain reveals neuronal structure in fine detail. Two photographs of Golgi-stained mouse cortex at lower (left) and higher (right) magnification. *Courtesy of Dr. Jocelyn Krey.*

or function can be coupled to the neuron's structure and location in the brain. During electrophysiology experiments, a scientist can use a glass pipette to fill a neuron of interest with a chemical that can later be visualized using histological methods. This chemical is usually a variant of a molecule called **biotin**, such as biocytin or neurobiotin, which has properties that make it useful for subsequent histology (Box 6.2). A scientist can intracellularly label cells

BOX 6.2 Methods of Labeling Molecular Probes

A probe, such as an antibody in immunohistochemistry (IHC) or antisense strand in in situ hybridization (ISH), is not visible by itself. To be visualized, the probe must be conjugated to a label that is visible on its own or that reacts with other chemicals to create a visible product. The use of a particular label depends on a number of factors, including the degree of specificity required, whether other methods will be used in combination, and what types of microscopes are available. This box describes common types of labels (fluorescent, chromogenic, radioactive, and colloidal gold) and how they can be detected.

Fluorescent Labels

Fluorescent molecules are ubiquitous in biological research. Fluorescent proteins, such as green fluorescent protein (GFP), can be genetically encoded and serve as reporter proteins. Other nonprotein **fluorophores** can be attached to a variety of molecular probes. Because these fluorescent probes have different excitation and emission spectra (Chapter 5), scientists can use multiple fluorescent labels to visualize distinct genes, proteins, and structures in the same neural specimen. Fluorescence is a sensitive method of detection, as bound fluorophores will emit light against a dark background. Attaching multiple fluorophores to a probe can amplify the signal to enhance detection.

BOX 6.2 Methods of Labeling Molecular Probes—cont'd

There are a variety of fluorophores available for labeling probes. Commonly used fluorescent labels include the organic cyanine and Alexa fluor dyes, which are commercially available and often used to label antibodies. **Quantum dots** are semiconductor nanocrystals that are extremely bright and resistant to photobleaching, making them particularly useful for live-cell imaging over long time periods.

Chromogenic/Colorimetric Labels

Chromogenic or **colorimetric** labels are enzymes that react with a substrate to produce a colored product that is visible under a light microscope. The most commonly used combinations are alkaline phosphatase (AP) with the substrates NBT/BCIP to produce a blue to purple stain; horseradish peroxidase with the substrate DAB to produce a brown to black stain; and β-galactosidase (genetically encoded by *lacZ*) with X-gal or IPTG substrate to produce a blue to purple stain. Commercially available substrates that produce fluorescent rather than colored products are also available, though colorimetric substrates are more frequently used.

Though not a chromogenic label itself, **biotin** is commonly conjugated to antibodies or incorporated into ISH oligonucleotide probes. The biotinylated probe can then be detected using the biotin-specific binding partner avidin/streptavidin conjugated to a chromogenic label like AP or HRP. Avidin and biotin can form large complexes and greatly enhance the signal compared to using a probe alone. **Digoxigenin** (abbreviated *dig*, sounds like "dij") is another common molecule that is incorporated into ISH probes as an alternative to radioactive labels. Anti-digoxigenin antibodies conjugated to chromogenic enzymes are used to detect the presence of the dig-labeled probe.

Radioactive Labels

Radioactive isotopes, or radioisotopes, have an unstable atomic nucleus that randomly disintegrates to produce a different atom. In the course of disintegration, these isotopes emit either energetic subatomic particles, such as electrons, or radiation, such as gamma rays. By using chemical synthesis to incorporate one or more radioactive atoms into a small molecule of interest such as a sugar, amino acid, or neurotransmitter, the fate of that molecule can be traced during any biological reaction.

Radioisotopes are visualized using **autoradiography**. Specimens are coated with a photographic emulsion and left in the dark while the radioisotope decays. Each decay event causes a silver grain to precipitate in the emulsion, so the position of the radioactive probe is indicated by the position of the developed silver grains. Because it is a direct chemical reaction, each silver grain represents one decay event. Therefore, radioactive labels allow quantitative studies of biological probes.

Primarily used to label ISH oligonucleotide probes, ^{35}S and ^{33}P are radioisotopes incorporated into the probe sequence, allowing sensitive detection, even for genes expressed at low levels. However, it can be tricky to calibrate the amount of time needed for exposing the emulsion to get the right signal-to-noise ratio, and the exposure may require weeks to months to observe a signal.

^{3}H and ^{125}I are radioisotopes that are used to label proteins and nucleic acids to investigate a number of processes, such as receptor localization, proliferation, and protein trafficking. Radioactively labeled ligands added to a specimen can bind to active receptors, allowing autoradiographic detection for the presence of those receptors.

Continued

BOX 6.2 Methods of Labeling Molecular Probes—cont'd

Gold Labels

Colloidal gold labels are conjugated to antibodies for probe detection in electron microscopes (Chapter 5). Gold provides an electron-dense material that can be visualized as a dark spot in EM. Avidin/streptavidin can also be conjugated to gold for detection of biotinylated probes in EM. Gold particles of different sizes can be used to detect multiple targets in the same sample.

Label Type	Comments	Visualization Method
Fluorescent dyes	Green dyes: FITC, Alexa 488, Cy2	Fluorescence microscopy
	Red dyes: Rhodamine, Texas red, Alexa 594, Cy3	
	Infrared dyes: Cy5	
Quantum dots	Spectral selectivity based on size	Fluorescence microscopy
Alkaline phosphatase (AP)	NBT/BCIP substrate produces blue/purple product	Usually light microscopy, though fluorescent substrates are available
Horseradish peroxidase (HRP)	DAB substrate produces brown/black product	Light or electron microscopy of colored DAB product
β-galactosidase (encoded by *lacZ* gene)	X-gal or IPTG substrate produces blue/purple product	Usually light microscopy, though fluorescent substrates are available
Biotin	Biotinylated antibodies or proteins are detected using avidin conjugated to another label described (fluorescent, chromogenic, or gold)	Light, fluorescent, or electron microscopy depending on type of label conjugated to avidin
Digoxigenin	Used to label ISH probes, detected with antidigoxigenin probe conjugated to another label, frequently AP	Light or fluorescence microscopy, depending on substrate for label
Radioactive isotope (^{35}S,^{33}P, ^{3}H,^{125}I)	ISH probes: ^{35}S,^{33}P, ^{3}HProteins: ^{3}H,^{125}I	Light microscopy
Gold	Electron-dense label used for detecting immunostaining in EM	Electron microscopy

in vivo or in slices. During an extracellular recording session, the electrode tip can be apposed to the neuron's membrane for juxtacellular labeling. This labeling can completely fill a neuron's dendrites or axonal arborizations.

VISUALIZING GENE AND PROTEIN EXPRESSION

In addition to classifying cells based on their morphology and location in the brain, a scientist can also categorize cells based on their gene and protein expression profiles. The genes expressed by a neuron determine whether it is excitatory or inhibitory, what kinds of neurotransmitters it releases and responds to, its physiological characteristics, and many other functional properties. Therefore, determining the genes and proteins expressed by a cell provides vital clues to its role in the brain. Conversely, a scientist may identify a gene or protein of interest, and then want to know where in the brain it is expressed. The following methods provide tools an investigator can use to visualize gene and protein expression in the nervous system.

In Situ Hybridization

In situ hybridization (ISH) is used to visualize the expression of nucleic acids, usually mRNA, allowing a scientist to determine when and where a specific gene is expressed in the nervous system. This technique is good for identifying which neurons express a gene, although it does not reveal where the functional protein product is expressed within the cell.

To perform an in situ hybridization experiment, a scientist must identify the genetic sequence of an mRNA to be studied. Next, the scientist creates a single-stranded nucleic acid probe that has a complementary base-pair sequence. The probe is tagged with radioactive nucleotides or other molecules that allow detection (Box 6.2). A tissue sample is exposed to the tagged probes such that the complementary strands can form a probe-mRNA duplex, marking the location of neurons that produce the mRNA sequence (Fig. 6.5A). This tissue sample is almost always a brain section, although it is possible to perform in situ hybridization on cultured cells or even unsectioned, whole-mount brains and animals.

In situ hybridization techniques require specific control experiments. Because an investigator uses a complementary ("antisense") strand of mRNA as a probe, a common negative control is a probe with the exact same ("sense") sequence of the mRNA of interest. This control probe should not produce any signal, as no hybridization should occur. Another good control to ensure that the antisense strand of mRNA is specific to the gene of interest is to perform additional experiments with other antisense strands that hybridize to a different region of the same mRNA.

The Allen Brain Institute in Seattle has provided researchers with a searchable, high-throughput collection of in situ hybridization images for

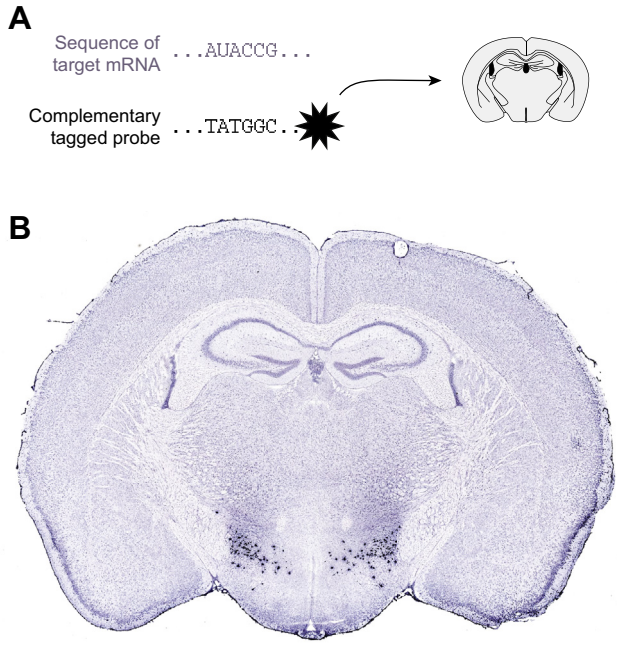

FIGURE 6.5 In situ hybridization shows where a gene is expressed. (A) Labeled oligonucleotide probes are synthesized with a complementary sequence to an mRNA transcript of interest. These probes are incubated with a neural specimen such that the probes hybridize with the mRNA. (B) A representative in situ hybridization image from the Allen Mouse Brain Atlas showing a coronal brain section stained with a probe directed against the mRNA transcript for the hypocretin neuropeptide. Cells expressing hypocretin are visible in black toward the bottom of the brain section. *From the Allen Mouse Brain Atlas at https://mouse.brain-map.org.*

every gene expressed in the mouse brain. Using this online resource (www.brain-map.org), a scientist can view a series of brain sections with an in situ hybridization reaction for a gene of interest (Fig. 6.5B). Alternatively, a scientist can type in the name of a brain region and receive a list of the most highly expressed genes in that area of the brain. This resource allows scientists to quickly obtain information about gene expression throughout the mouse brain without having to personally perform in situ reactions. The only limitation to this resource is that it does not examine gene expression for conditions other than baseline conditions, so gene expression could be different depending on environmental stimuli.

It is also possible to use fluorescent molecules to label probes for in situ hybridization. **Fluorescent in situ hybridization (FISH)** also uses antisense probes, but in FISH, fluorescent dyes are used to label the probe-mRNA duplex. One major benefit to using FISH is that multiple probes can be used in the same tissue section to determine whether certain cells coexpress genes

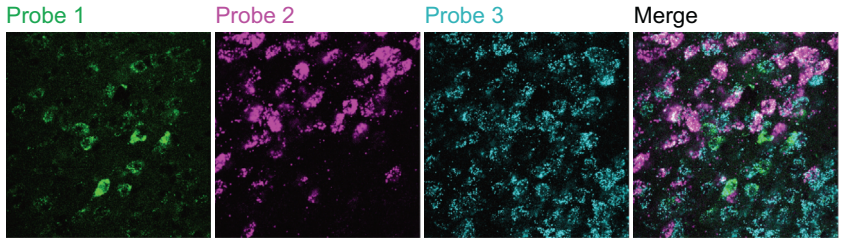

FIGURE 6.6 **Fluorescent in situ hybridization (FISH).** Cells labeled using RNAscope to visualize the expression of three different mRNA transcripts in the same specimen. The three probes are labeled in different colors, and a merged image shows the expression of all three transcripts. In this example, Probes 1 and 2 label distinct cell types and Probe 3 labels all cells in this specimen.

of interest. Modern commercial approaches to FISH, such as **RNAscope**, allow scientists to order previously designed probes for virtually any gene in the genome and use enhanced procedures to fluorescently label multiple different RNA transcripts in the same section (Fig. 6.6).

Immunohistochemistry

Immunohistochemistry (IHC; *histo* = "tissue") is used to visualize the expression of proteins. IHC applied to cells is often referred to as **immunocytochemistry** (ICC; *cyto* = "cells"), and IHC using fluorescent reagents is referred to as **immunofluorescence**. The root *immuno-* refers to the fact that these techniques depend on antibodies to recognize and bind to specific proteins (antigens). By exploiting the immune system of other animals, such as mice, sheep, rabbits, and goats, scientists and commercial suppliers have generated a wide number of antibodies for many proteins. Table 6.3 lists a few of the many antibodies that are commonly used in neuroscience to stain specific categories of cells. See Chapter 14 for details about antibody production.

To perform an IHC experiment, a scientist incubates a sample with an antibody that recognizes a specific antigen. This **primary antibody**, the antibody that binds to the protein, can be conjugated directly with a fluorescent molecule or chromogenic enzyme. This procedure is known as **direct IHC** (Fig. 6.7A), but usually results in very weak signal that is difficult to visualize. Alternatively, in **indirect IHC**, a scientist adds a **secondary antibody** that recognizes the primary antibody (Fig. 6.7B). Because multiple secondary antibodies can bind to the primary antibody, the signal is amplified. The secondary antibody will only react with the primary antibody if it is directed against the correct immunoglobulin molecule of the animal species in which the primary antibody was raised. For example, if the primary antibody was produced in a rabbit, then the secondary antibody must be anti-rabbit and

TABLE 6.3 Antibodies that Label Specific Neural Cell Types.

Cell Type	Antibodies
Neurons	Tuj1 (neuron-specific β-tubulin); NeuN
Neuronal subtypes	GAD (GABAergic neurons); vGLUT (glutamatergic); TH (dopaminergic); 5-HT (serotonergic); ACh (cholinergic)
Young neurons	Doublecortin (DCX); NeuroD
Progenitors/radial glia	Nestin; Pax6; RC2; vimentin; NF (neurofilament)
Axons	Tau-1, L1, Tag-1
Synapses	PSD95, synapsin, synaptophysin
Dendrites	MAP2
Astrocytes	GFAP
Oligodendrocyte progenitor cells (OPCs)	PDGFRα, NG2, A2B5, O4 (late progenitor)
Mature oligodendrocytes	MBP (myelin basic protein); PLP (proteolipid protein); CC1
Microglia	TMEM119; Iba1

produced in a different animal. The secondary antibody is conjugated with a fluorescent molecule or chromogenic enzyme that allows the scientist to visualize the expression of the protein (Fig. 6.7C).

It is critical that an investigator determines whether the result of an IHC experiment is specific for the protein of interest. Using both a positive control, a specimen known to contain the antigen of interest, and a negative control, a specimen known *not* to contain the antigen of interest, is useful for determining whether the antibody works properly. Another good control experiment for primary antibody specificity is to preincubate the primary antibody with a known solution of its antigen: this process should form antibody/antigen complexes so there is no free antibody to bind to a sample. In indirect IHC, incubating the specimen with the secondary antibody but no primary antibody is important to test for nonspecific binding of the labeled secondary antibody.

While IHC is straightforward in theory, there are a number of steps that must be optimized for IHC to work in practice. For clean, interpretable results, a scientist may need to adjust the method of fixation, antibody concentration, length of time the antibody is incubated with tissue, and accessibility to the antigen. Each batch of antibody is different, even antibodies directed against the same antigen. Therefore, an investigator should optimize experimental conditions for each new antibody before performing IHC experiments on multiple samples.

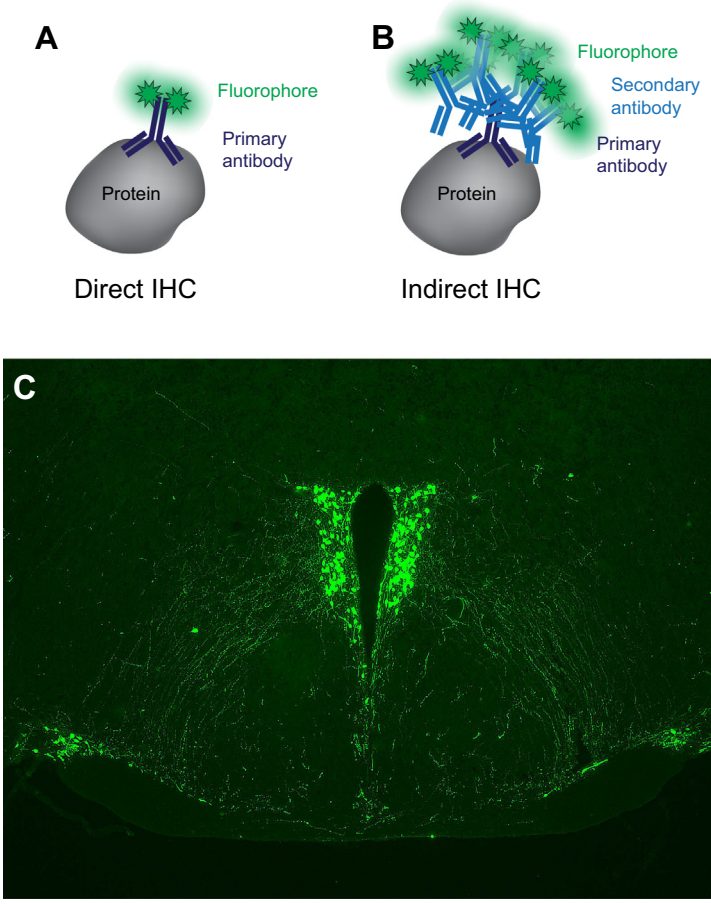

FIGURE 6.7 **Immunohistochemistry.** (A) In Direct IHC, primary antibodies are conjugated directly to a label that can be visualized. (B) In Indirect IHC, primary antibodies attract labeled secondary antibodies, further amplifying the signal. (C) An example of a fluorescent IHC experiment using a primary antibody against oxytocin and a secondary antibody conjugated with a fluorescent green fluorophore. Labeled cells are located toward the middle of this image (in the paraventricular hypothalamic nucleus), as well as on the bottom left and right sides of this image (in the supraoptic hypothalamic nucleus). Fluorescent label is also visible within fiber tracts emanating from the paraventricular hypothalamic nucleus.

A technique called **array tomography** uses immunohistochemistry on ultrathin serial sections to improve spatial resolution and eliminate out-of-focus fluorescence during imaging. This method allows investigators to create highly detailed three-dimensional images of protein expression in the nervous system. An ultramicrotome is used to cut and collect ribbons of ultrathin (50−200 nm) serial sections on glass microscope slides. A scientist can perform immunohistochemistry to detect the presence of an antigen, but then

the antibody can be removed and another IHC reaction performed. As many as 10 IHC reactions can be performed on the same section. Because the sections are so thin, antibodies have greater access to antigens in the section. The serial sections can then be reconstructed to provide high-resolution, three-dimensional information about the spatial relationships of these proteins. In addition, the sections can be used for electron microscopy, allowing an unprecedented investigation of protein expression combined with powerful magnification and resolution.

Enzymatic Histochemistry

An enzyme is a biomolecule capable of catalyzing a biological reaction. These proteins can be detected using immunohistochemistry. However, another method to detect enzymes is to use the enzyme's endogenous activity to create a visible reaction product—a process known as **enzymatic histochemistry**. The scientist incubates a brain section in a solution containing a chromogenic chemical capable of serving as a substrate for the enzyme. A colored by-product indicates the presence of the enzyme. For example, endogenous acetylcholinesterase (the enzyme that breaks down acetylcholine) is often used as a marker for acetylcholine in brain sections. Another commonly detected enzyme is cytochrome oxidase, which aids in visualizing metabolically active neurons, such as whisker barrels in the rodent sensory cortex (Fig. 6.8).

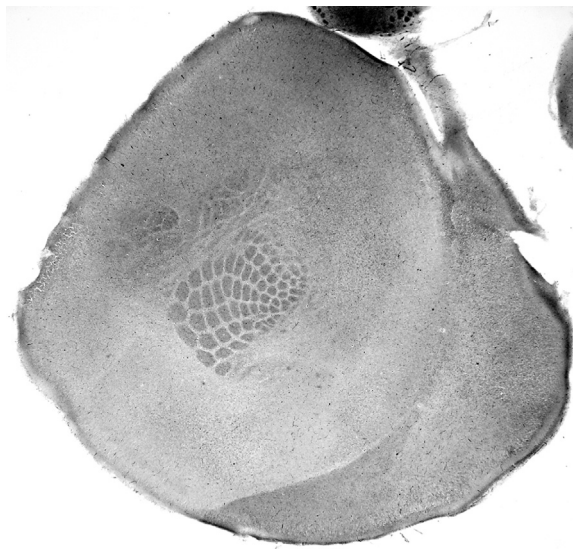

FIGURE 6.8 Enzymatic histochemistry. Visualization of rodent whisker barrel cells in somatosensory cortex in mouse. These cells express the enzyme cytochrome *c* oxidase; incubating sections with the substrate (cytochrome *c*) and a colorimetric indicator (e.g., DAB) allows the whisker barrels to be visualized. *Courtesy of Dr. Pushkar Joshi.*

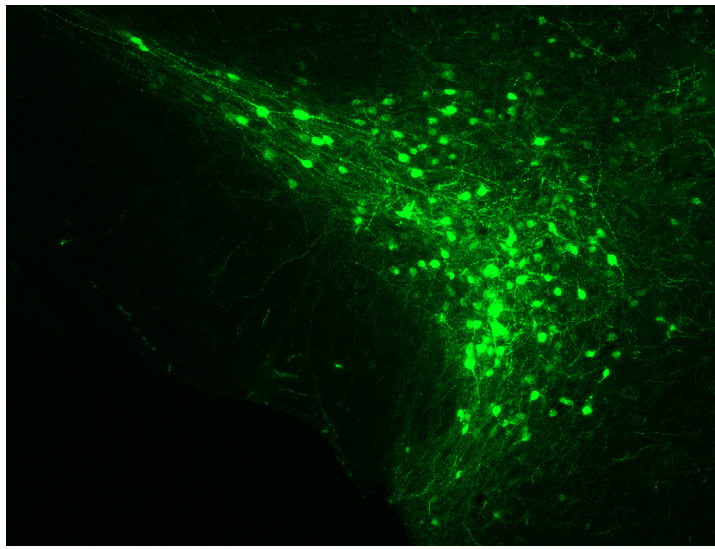

FIGURE 6.9 Visualization of cell types using reporter proteins. Representative image of tachykinin-1-expressing cells in the parasubthalamic nucleus expressing the green fluorescent protein (GFP) reporter. Cell bodies and axonal fibers are visible.

Reporter Proteins

A **reporter protein** is a fluorescent protein or enzyme that can visibly mark cells of interest (Fig. 6.9). Because reporter proteins are genetically encoded, they can be targeted to specific cells using unique promoter sequences (Chapter 12). Reporter proteins can also be fused with other proteins to indicate the spatial and temporal expression of that protein. Common reporters include **green fluorescent protein (GFP)** and its multicolored derivatives including mCherry, tdTomato, cyan fluorescent protein (CFP), and many others. Classical reporter genes, now rarely used, include *lacZ* (which encodes the enzyme β-galactosidase) and luciferase (the firefly protein).

VISUALIZING CIRCUITRY

So far, this chapter has reviewed methods of investigating cells based on their morphology, location, and gene/protein expression profiles. Of course, a neuron never functions in isolation; its role in the brain is governed by its unique combination of inputs and outputs and the neural circuits to which it belongs. Therefore, scientists often want to know the specific cells in the brain that provide synaptic input to a neuron of interest, as well as the specific cells to which a neuron projects.

Anterograde and Retrograde Tracers

A neuronal **tracer** is a chemical probe that labels axon paths to illuminate connectivity in the nervous system. Tracers are described by the direction they travel in a neuron. **Anterograde** refers to transport from the cell body through the axon to the presynaptic terminal; **retrograde** refers to tracing in the opposite direction—transport from the synaptic terminal back to the cell body (Fig. 6.10). Some tracers are strictly capable of only anterograde or retrograde transport. Others work in both directions. Classical tracers include horseradish peroxidase (HRP), biotinylated dextran amine, and Fluoro-Gold. These tracers are fluorescent or produce a colorimetric product that can be visualized using light microscopy (Box 6.2). Modern scientists often use viruses as an anterograde tracer to deliver a genetically encoded fluorescent protein, such as GFP, to a brain region of interest. The fluorescent protein fills the soma, but also diffuses down the length of an axon to show anterograde targets. For modern retrograde tracing experiments, scientists can inject commercially available fluorescent **retrobeads**, lipophilic beads conjugated to a fluorescent molecule that are taken up by synapses and retrogradely transported back to cell bodies.

To use an anterograde or retrograde tracing technique, a scientist almost always injects the tracer into the brain of a live animal. After a waiting period (1−14 days), the brain can be removed, processed, and examined for the presence of the tracer. An anterograde tracer will be present in the cell bodies and presynaptic terminals of the injected neurons, highlighting areas to which those neurons project. A retrograde tracer will be present in the cell bodies of neurons that project to the injection site.

Transsynaptic Labeling

Traditional anterograde or retrograde tracing experiments label only direct connections in a neural circuit. However, functional brain circuits are not simply

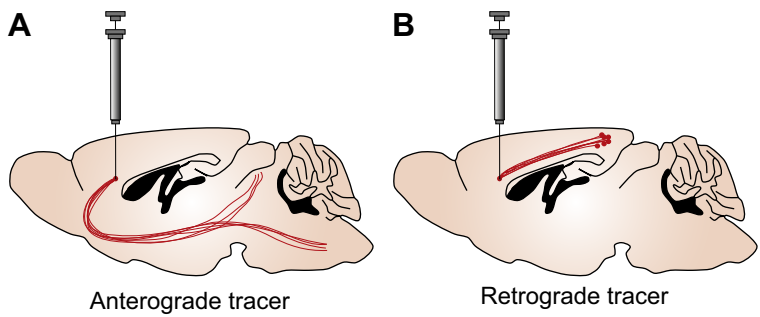

FIGURE 6.10 **Anterograde and retrograde tracers.** (A) Anterograde tracers show efferent projections from a neural population of interest. Axon terminals are visible in downstream neural populations as dense puncta. (B) Retrograde tracers identify neurons that project to a neural population of interest. Upstream populations are visible as labeled cell bodies.

composed of direct one-to-one connections, but also contain complicated pathways involving second- or third-order neurons. Scientists wishing to study these multisynaptic circuits can use a variety of **transsynaptic tracers**—tracers that can cross synapses and label multiple neurons in a circuit.

Viruses

The use of viruses as transsynaptic tracers exploits the ability of viruses (especially viruses that naturally infect neural tissue) to invade neurons and then produce infectious progeny. Some strains of virus release progeny capable of crossing synaptic junctions and subsequently infecting downstream neurons in a circuit. The virus acts as a self-amplifying tracer that progressively labels neurons in a circuit as they become infected. The most common viruses used to label transneuronal circuits are pseudorabies virus and herpes simplex virus.

It is also possible to use a **modified rabies virus** as a monosynaptic retrograde tracer such that it labels only one synapse back from the originally infected cells (Fig. 6.11). This method utilizes several genetic targeting strategies, discussed in more detail in Chapter 12. In a modified rabies virus, the rabies G glycoprotein, which allows the virus to leave host cells, is deleted (often referred to as RVΔG). In addition, the virus is pseudotyped to express an envelope protein (such as EnvA) that cannot naturally bind to and infect cells in the host organism. Thus, modified rabies virus can only infect and propagate from cells that express both G protein and receptors for EnvA. These components are delivered to genetically defined cell types using techniques described in Chapters 11 and 12, allowing for monosynaptic retrograde tracing from a specific cell type of interest.

The major limitation to using viruses as transsynaptic tracers is that these viruses can actually kill neurons and lead to generalized infection in the animals. The longer the animal is infected, the more likely that higher-order neurons in a circuit will be labeled, but the greater the likelihood that the first- and second-order neurons will die as the infection proceeds.

Plant Lectins

Lectins are proteins that exhibit extremely high binding affinities for specific sugars. Some plant lectins have affinities for specific glycoproteins on neuronal plasma membranes. After binding, they can be internalized and transported within the neuron. Some examples of plant lectins that can also be transported across synapses include wheat germ agglutinin and tetanus toxin fragment C. These lectins can be detected using antibodies against the lectin itself or by conjugation with a chromogenic enzyme (Box 6.2). In addition to the standard method of injecting the tracer into an area of interest, researchers can create transgenic animals (Chapter 12) that express transneuronal lectin tracers under the control of neuron-specific promoters to examine cell-type specific circuits.

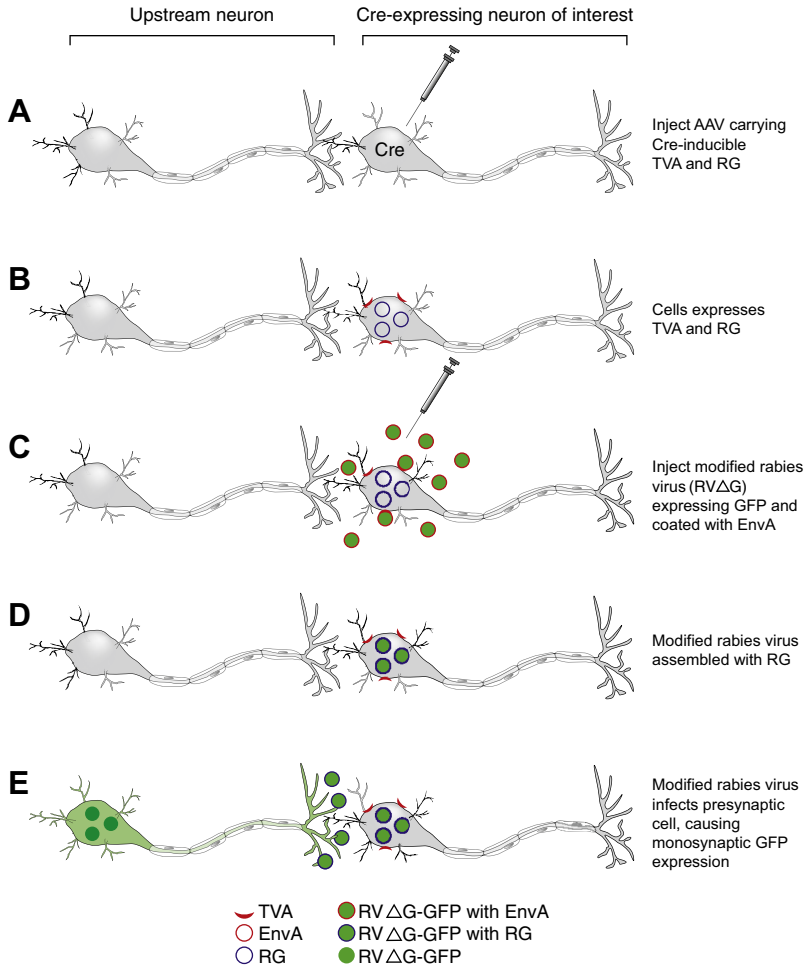

FIGURE 6.11 Monosynaptic retrograde tracing using modified rabies virus. (A) A neural population of interest expresses the gene for Cre recombinase (Chapter 12). Adeno-associated virus (AAV) carrying Cre-inducible constructs of TVA and RG protein is injected into the region of interest. (B) Several days later, the population of interest expresses TVA and RG. (C) Modified rabies virus (RVΔG) expressing green fluorescent protein is injected into the same site. The virus expresses a mutated RG glycoprotein (ΔG) such that it cannot infect cells. It is also coated with an envelope protein, EnvA. The TVA protein serves as the receptor for EnvA, restricting infection to the neural population of interest. (D) The infected cells produce new rabies virus and also the RG glycoprotein necessary to make the infectious viral coat. (E) The newly packaged modified rabies virus exits the cells and infects presynaptic cells upstream. The virus retrogradely travels to the cell body, causing the cell to fluoresce green. This virus cannot infect second-order neurons because there is no RG protein present in these cells.

CONCLUSION

The organization and structure of the nervous system provide the first clues about function and how particular neurons and circuits can give rise to behaviors. This chapter has surveyed fundamental methods of investigating the morphology, expression patterns, and connectivity of cells. In the next chapter, we will survey methods that visualize the electrical and biochemical activity within neurons.

SUGGESTED READING AND REFERENCES

Books

Blurry, R.W., 2010. Immunocytochemistry: a Practical Guide for Biomedical Research. Springer, New York, NY.

Kalyuzhny, A.E., 2016. Immunohistochemistry: Essential Elements and Beyond. Springer.

Kiernan, J.A., 2015. Histological and Histochemical Methods: Theory and Practice, fifth ed. Scion Publishing.

Lin, F., Prichard, J.W., Liu, H., Wilkerson, M.L. (Eds.), 2022. Handbook of Practical Immunohistochemistry: Frequently Asked Questions. Springer.

Polak, J.M., Van Noorden, S., 2003. Introduction to Immunocytochemistry, third ed. BIOS Scientific Publishers, Oxford.

Review Articles

Callaway, E.M., Luo, L., 2015. Monosynaptic circuit tracing with glycoprotein-deleted rabies viruses. J. Neurosci. 35, 8979−8985.

Jones, E.G., 2007. Neuroanatomy: Cajal and after Cajal. Brain Res. Rev. 55, 248−255.

Pollack, J.D., Wu, D.Y., Satterlee, J.S., 2014. Molecular neuroanatomy: a generation of progress. Trends Neurosci. 37, 106−123.

Ueda, H.R., Ertürk, A., Chung, K., Gradinaru, V., Chédotal, A., Tomancak, P., Keller, P.J., 2020. Tissue clearing and its applications in neuroscience. Nat. Rev. Neurosci. 21, 61−79.

Primary Research Articles—Interesting Examples From the Literature

Betley, J.N., Cao, Z.F.H., Ritola, K.D., Sternson, S.M., 2013. Parallel, redundant circuit organization for homeostatic control of feeding behavior. Cell 155, 1337−1350.

Braz, J.M., Basbaum, A.I., 2008. Genetically expressed transneuronal tracer reveals direct and indirect serotonergic descending control circuits. J. Comp. Neurol. 507, 1990−2003.

Chen, B., Schaevitz, L.R., McConnell, S.K., 2005. Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex. Proc. Natl. Acad. Sci. USA 102, 17184−17189.

Chung, K., Wallace, J., Kim, S.-Y., Kalyanasundaram, S., Andalman, A.S., Davison, T.J., Mirzabekov, J.J., Zalocusky, K.A., Mattis, J., Denisin, A.K., Pak, S., Bernstein, H., Ramakrishnan, C., Grosenick, L., Gradinaru, V., Deisseroth, K., 2013. Structural and molecular interrogation of intact biological systems. Nature 497, 332−337.

Fakhry, A., Ji, S., 2014. High-resolution prediction of mouse brain connectivity using gene expression patterns. Methods 73, 71−78.

Faucherre, A., Lopez-Schier, H., 2014. Dynamic neuroanatomy at subcellular resolution in the zebrafish. Methods Mol. Biol. 1082, 187−195.

Iyer, M., Essner, R.A., Klingenberg, B., Carter, M.E., 2018. Identification of discrete, intermingled hypocretin neuronal populations. J. Comp. Neurol. 526, 2937−2954.

Micheva, K.D., Smith, S.J., 2007. Array tomography: a new tool for imaging the molecular architecture and ultrastructure of neural circuits. Neuron 55, 25−36.

Wang, F., et al., 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed paraffin-embedded tissues. Mol. Diagnost. 14, 22−29.

Protocols

Helmchen, F., Konnerth, A., Yuste, R. (Eds.), 2011. Imaging in Neuroscience: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kay, K.R., Smith, C., Wright, A.K., Serrano-Pozo, A., Pooler, A.M., Koffie, R., Bastin, M.E., Bak, T.H., Abrahams, S., Kopeikina, K.J., McGuone, D., Frosch, M.P., Gillingwater, T.H., Hyman, B.T., Spires-Jones, T.L., 2003. Studying synapses in human brain with array tomography and electron microscopy. Nat. Protoc. 8, 1366−1380.

Liehr, T. (Ed.), 2018. Fluorescence in situ Hybridization (FISH), second ed. Springer.

Paletzki, R.F., Gerfen, C.R., 2019. Basic neuroanatomical methods. Curr. Protoc. Neurosci. 90, e84.

Tomer, R., Ye, L., Hsueh, B., Deisseroth, K., 2014. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat. Protoc. 9, 1682−1697.

Volpicelli-Daley, L.A., Levey, A., 2004. Immunohistochemical localization of proteins in the nervous system. Curr. Protoc. Neurosci. (Chapter 1, Unit 1.2).

Wilson, C.J., Sachdev, R.N., 2004. Intracellular and juxtacellular staining with biocytin. Curr. Protoc. Neurosci. (Chapter 1, Unit 1.12).

Websites

Allen Brain Atlas. http://www.brain-map.org.

IHC World. http://www.ihcworld.com.

Mouse Brain Architecture. http://brainarchitecture.org.

The Antibody Resource. http://www.antibodyresource.com.