Chapter 1

Tools of the Cell Biologist

one

One of the first questions a cell biologist might ask in his or her search for a protein's function would be, "Where is it located in the cell?" Is it in the nucleus or the cytoplasm? Is it a surface membrane protein, or resident in one of the cytoplasmic organelles? Knowing the subcellular localization of a protein provides significant direction for further experiments designed to learn its function.

One of the primary tools a cell biologist would use to answer the question of subcellular location would be a microscope.

MICROSCOPY: ONE OF THE EARLIEST TOOLS OF THE CELL BIOLOGIST

Microscopy, in its various forms, has historically been the primary way in which investigators have examined the appearance and substructure of cells, and increasingly in recent decades, the location and movement of biological molecules within cells. We may speak broadly of two kinds of microscopy, **light microscopy (LM)** and **electron microscopy (EM),** although the field of microscopy recently has been broadened by the advent of **atomic force microscopy (AFM).**

The resolution of standard light microscopy is limited by the wavelength of visible light, which is comparable with the diameter of some subcellular organelles; but a variety of contemporary techniques now exist that permit light microscopic visualization of proteins and nucleic acid molecules. Chief among these new techniques are those using either organic fluorescent molecules or quantum nanocrystals ("quantum dots") to directly or indirectly "tag" individual macromolecules. Once the molecules of interest have been fluorescently tagged, their cellular location can be viewed via **fluorescence microscopy** (Figure 1-1).

Fluorescence Microscopy

In many situations, fluorescence microscopy is the first approach one might take to identify the subcellular location of particular proteins. One widely used technique to fluorescently tag a protein is based on the great precision and high affinity with which an antibody molecule can bind its cognate protein antigen. This antibody-based approach has been termed **immunolabeling**.

Because antibodies are relatively large molecules that do not cross the surface membrane of living cells, one must fix and permeabilize cells before an antibody can be used to view the location of a target protein. In recent years, it has become possible to view the location and movement of fluorescently tagged proteins inside **living cells**, using an approach that has been broadly termed **genetic tagging.** With this approach, one uses genetic engineering to create a plasmid expressing the protein of interest, which has been fused at its amino or carboxy terminus with either a directly fluorescent tag, such as **green fluorescent protein (GFP)**, or an indirect fluorescent tag, such as **tetra-cysteine**. Tetra-cysteine-tagged proteins when expressed in cells can bind subsequently added small, membrane-permeable fluorescent molecules such as the red or green biarsenicals FlAsH and ReAsH. The lines between immunolabeling and genetic tagging blur when one considers another type of genetic tagging, termed **epitope-tagging**, in which the recombinant protein is expressed with an antigenic amino acid sequence at one of its ends, to which commercial antibodies are readily available,

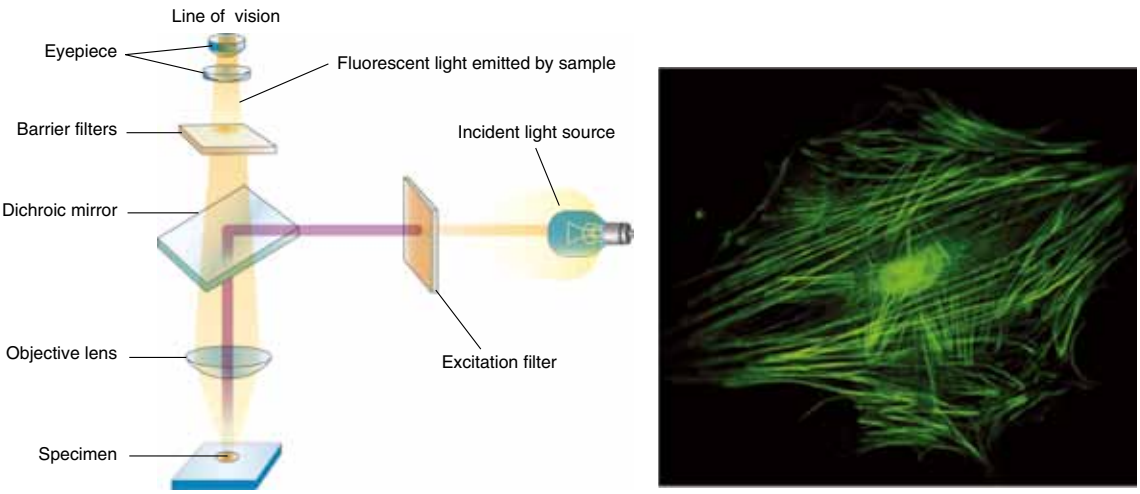

Figure 1-1 Fluorescence microscopy

A: Optical layout of a fluorescence microscope. Incident light tuned to excite the fluorescent molecule is reflected by a dichroic mirror, and then focused on the sample; fluorescent light (longer wavelength than excitation light) emitted by the sample passes through the dichroic mirror for viewing. **B:** Immunofluorescent micrograph of a human skin fibroblast, stained with fluorescent anti-actin antibody. Cells were fixed, permeabilized, and then incubated with fluorescein-coupled antibody. Unbound antibody was washed away before viewing. (*A: Modifi ed from Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J.* Molecular Cell Biology, *4th ed. New York, NY: W.H. Freeman, 2000; B: Courtesy E. Lazerides.*)

such as a "myc-tag."

Let us first consider immunolabeling in more detail, and then consider genetic labeling, using the example of GFP.

Immunolabeling

Specific antibodies directed against the protein of interest, used in combination with either light microscopy or EM, are useful tools for discovering the subcellular location of the protein.

A fluorescent tag (e.g., fluorescein) can be chemically coupled to the Fc domain of antibody for use in fluorescent light microscopy. For use in transmission EM, an electron-dense tag such as the iron-rich protein ferritin or nanogold particles can be coupled to the antibody. These two techniques are referred to as immunofluorescent microscopy and immunoelectron microscopy, respectively. Figure 1-1B shows an example of the use of immunofluorescence to visualize the actin "stress fibers" in a fibroblast; an example of immunoelectron microscopy is shown later in Figure 1-4.

So how would one go about obtaining antibodies to a particular protein?

Antipeptide Antibodies

One way to obtain antibodies here would be to chemically synthesize peptides corresponding to the predicted amino acid sequence of the protein product of the gene of interest. One would then chemically couple these peptides to a carrier protein, such as serum albumin or keyhole limpet hemocyanin (commonly used), and then immunize an animal such as a rabbit with the peptide-carrier complex.

This approach has one potential problem. If dealing with one of the newly discovered human genes whose protein product is completely uncharacterized, one would not have any information about the three-dimensional structure of this protein. Consequently, one would not know whether any particular amino acid sequence chosen for immunization purposes would be exposed on the surface of the native, folded protein as found in a cell. If the selected peptide corresponded to an amino acid sequence that is buried in the interior of the folded structure, antibodies directed against it would not be able to bind the native protein in the fixed cell preparations one would be using for microscopy. It turns out that amino- or carboxyl-terminal amino acid sequences are frequently exposed on the surface of many natively folded proteins; for this reason, peptides

corresponding to these terminal sequences are frequently chosen for immunization of rabbits. Also, hydrophilic sequences are generally found on the surface of folded proteins, and if one or more such sequences can be identified in the predicted amino acid sequence of the protein of interest, they too would be good candidates for immunization.

Because of the preceding considerations, antipeptide antibodies are not always successful in immunofluorescent localization experiments, where the target protein is in a native configuration. They are, however, often useful for the technique of **Western blotting.**

A convenient feature of antipeptide antibodies is that excess free peptide competes for the protein in the binding of the antibody and provides a useful control for the specificity of any antibody-protein interaction observed.

Antibodies against Full-Length Protein

The alternative to immunizing rabbits with synthetic peptides is to immunize them with either the entire protein, or a stable subdomain (e.g., the extracellular globular domain of a single-pass transmembrane protein). Immunization with the whole protein requires purification of relatively large amounts of the protein of interest (tens or hundreds of milligrams). Production of large amounts of protein (overexpression) from a cloned gene is greatly facilitated by the use of any of several plasmid or virus-based **protein-expression vectors**. Insertion of the coding sequence into an expression vector also allows creation of a "run-on" protein with a carboxyl-terminal "tag" sequence that permits subsequent rapid and efficient affinity purification. Commonly used tag sequences are "6× histidine" and "GST" tags. Such tags permit rapid and efficient affinity purification of the overexpressed protein.

Escherichia coli is often used for the expression of cloned genes, but because of different codon usage between prokaryotes and eukaryotes (and corresponding differences in the levels of the various cognate tRNA), human genes are sometimes not satisfactorily expressed in *E. coli.* Furthermore, overexpressed proteins in *E. coli* often form insoluble aggregates called **inclusion bodies**, and posttranslational modifications such as glycosylation cannot occur in bacteria. For these reasons, a human gene might preferably be expressed in a eukaryotic expression system, using either a highly inducible expression vector in yeast or the insect baculovirus *Autographa californica* in insect Sf9 cells.

Once sufficient amounts of the protein have been purified, a **polyclonal antiserum** can be obtained by immunizing rabbits; alternatively, mice can be immunized for the production of **monoclonal antibodies.**

Genetic Tagging

Green Fluorescent Protein

GFP was first identified and purified from the jellyfish *Aequorea victoria,* where it acts in conjunction with the luminescent protein aequorin to produce a green fluorescence color when the organism is excited. In brief, excitation of *Aequorea* results in the opening of membrane Ca^{2+} channels; cytosolic Ca^{2+} activates the aequorin protein and aequorin, in turn, uses the energy of ATP hydrolysis to produce blue light. By quantum mechanical resonance, blue light energy from aequorin excites adjacent molecules of GFP; these excited GFP molecules then produce a bright green fluorescence. Thus, the organism can "glow green in the dark" when excited. The resonant energy transfer between excited aequorin and GFP is an example of a naturally occurring **fluorescence resonance energy transfer (FRET)** process (see later).

The gene for GFP has been cloned and engineered in various was to permit the optimal expression and fluorescence efficiency of GFP in a wide variety of organisms and cell types. Cloning has furthermore permitted the GFP coding sequence to be used in protein expression vectors such that a chimeric construct is expressed, consisting of GFP fused onto the amino- or carboxylterminal end of the protein of interest. Variant GFP proteins and related proteins from different organisms are now available that extend the range of fluorescence colors that are produced: blue (cyan) fluorescent protein (CFP), yellow fluorescent protein, and red fluorescent protein.

GFP is a β-barrel protein (its structure is shown in Figure 1-2). Within an hour or so after synthesis and folding, a self-catalyzed maturation process occurs in the protein, whereby adjacent serine, glycine, and tyrosine side chains in the

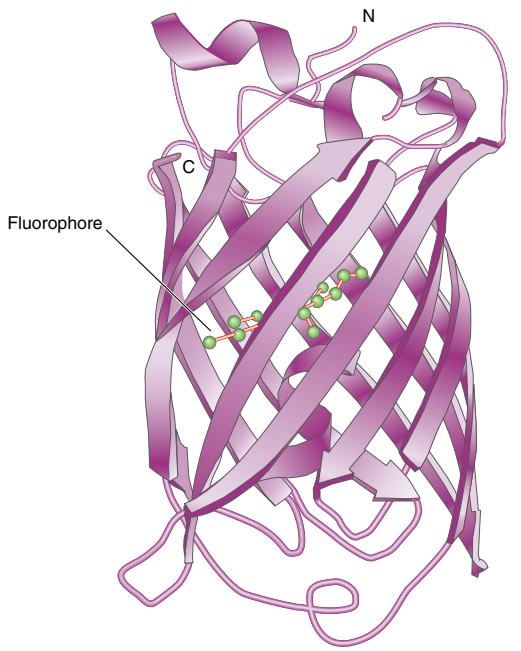

Figure 1-2 The structure of green fluorescent protein (GFP)

GFP is an 11-strand β-barrel, with an α-helical segment threaded up through the interior of the barrel. The amino- and carboxylterminal ends of the protein are free and do not participate in forming the stable β-barrel structure. Within an hour or so after synthesis and folding, a self-catalyzed maturation process occurs in the protein, whereby side chains in the interior of the barrel react with each other and with oxygen to form a fluorophore covalently attached to the through-barrel α-helical segment, near the center of the β-barrel cavity. (*Modified from Ormo M et al.*, Science,*273:1392-1395, 1995.*)

interior of the barrel react with each other and with oxygen to form a fluorophore covalently attached to a through-barrel α-helical segment, near the center of the β-barrel cavity. The GFP fluorophore thus produced is excited by the absorption of blue light from the fluorescence microscope, and then decays with the release of green fluorescence.

Because the amino- and carboxyl-terminal ends of GFP are free and do not contribute to the β-barrel structure, the coding sequence for GFP can be incorporated into expression vector constructs, such that chimeric fusion proteins can be expressed with a GFP domain located at either the amino- and carboxylterminal ends of the protein of interest. As mentioned earlier, the great advantage of genetic tagging of proteins with fluorescent molecules such as GFP is that this technique permits one to visualize the subcellular location of the protein of interest in a living cell. Consequently, one can observe not only the location of a protein but also the path it takes to arrive at that location. For example, using a GFP-tagged human immunodeficiency virus (HIV) protein, it was discovered that after entry into cells, the HIV reverse transcription complex travels via microtubules from the periphery of the cell to the nucleus.

The FRET technique can be used to monitor the interaction of one protein with another inside a living cell. As discussed earlier in this chapter, in *Aequorea*, blue light energy from aequorin is used to excite GFP by the quantum mechanical process of resonance energy transfer. Energy transfer like this can occur only when donor and acceptor molecules are close to each other (within 10 nm). Investigators are able to take advantage of this process to detect when or if two proteins in the cell bind each other under some circumstance. Both proteins of interest need merely be tagged with a pair of complementary (donor-acceptor) fluorescent proteins, such as CFP and GFP, and then coexpressed in the cell. CFP is excited by violet light, and then emits blue fluorescence. If the two proteins do not bind each other in the cell, only blue fluorescence will be emitted on violet light excitation; if, however, the two proteins do bind each other, resonant energy transfer from the donor CFP will be captured by the GFP-tagged partner, and green fluorescence will be detected (Figure 1-3).

Electron Microscopy

There are two broad categories of EM: **transmission EM** and **scanning EM.** First, we discuss the topic of transmission EM, including the special techniques of **cryoelectron microscopy.**

Transmission Electron Microscopy

Transmission electron microscopes use electrons in a way that is analogous to the way light microscopes use visible light. The various elements in a transmission electron microscope that produce, focus, and collect electrons after their passage through the specimen are all related in function to the corresponding elements in a light microscope (Figure 1-4). Rather than a light source, there is an electron source, and electrons are accelerated toward the anode by a

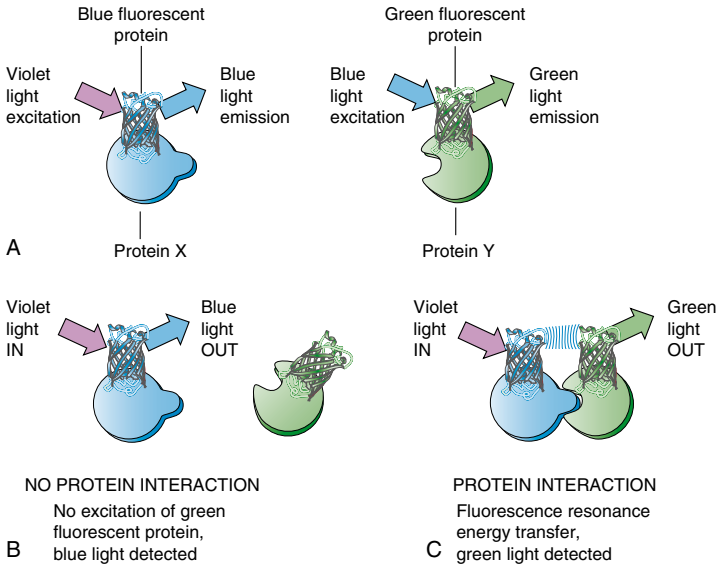

Figure 1-3 Fluorescence resonance energy transfer (FRET)

A: The two proteins of interest are expressed in cells as fusion proteins with either blue fluorescent protein (BFP) (protein X) or GFP (protein Y). Excitation of BFP with violet light results in the emission of blue fluorescent light by BFP; excitation of GFP with blue light yields green fluorescence. **B:** If the two proteins do not bind each other inside the cell, excitation of the BFP molecule with violet light results simply in blue fluorescence. If, however, **C:** the two proteins do bind each other, they will be close enough to permit resonant energy transfer between the excited BFP molecule and the GFP protein, resulting in green fluorescence after violet excitation. (*Modifi ed from Alberts B, et al.* Molecular Biology of the Cell, *4th ed. New York, NY: Garland Science, 2002.*)

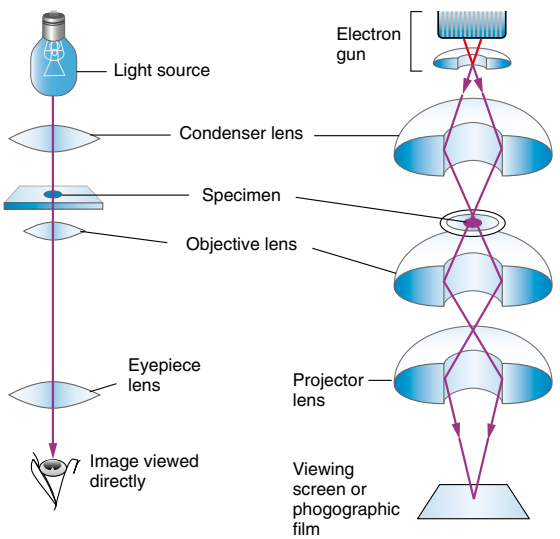

Figure 1-4 Comparison of the lens systems in a light microscope and a transmission electron microscope

In a light microscope (left), light is focused on the sample by the condenser lens. The sample image is then magnified up to 1000 times by the objective and ocular lenses. In a transmission electron microscope (right), magnets serve the functions of the condenser, objective and ocular (projection) lenses, focusing the electrons and magnifying the sample image up to 250,000 times. (*Modified from Alberts B, et al.* Molecular Biology of the Cell, *4th ed. New York, NY: Garland Science, 2002.*)

voltage differential. In an electron microscope, the electrons are focused not by optical lenses of glass, but instead by magnets.

Because electrons would be scattered by air molecules, both the electron trajectory and the sample chambers must be maintained in a **vacuum.**

We are perhaps more accustomed to thinking of an electron as a particle-like object rather than as an electromagnetic wave, but of course, quantum mechanically, electrons can behave as either particles or waves. As is the case for all waves, the frequency (and hence wavelength) of an electron is a function of its energy, which in turn is a function of the accelerating voltage that drives an electron from its source in an electron microscope. Typical electron microscopes are capable of producing accelerating voltages of approximately 100,000 V, producing electrons with energies that correspond to **wavelengths of subatomic dimensions.** This would, in theory, permit subatomic resolutions! A number of factors such as lens aberrations and sample thickness, however, limit the practical resolution to much less than this. Under usual conditions with biological samples, electron microscopic

resolution is approximately 2 nm, which is still more than 100 fold better than the resolution of a light microscope. This increased resolution, in turn, permits much larger useful magnifications, up to 250,000 fold with EM, compared with approximately 1000 fold in a light microscope with an oil-immersion lens.

Because of the high vacuum of the EM chamber, living cells cannot be viewed, and typical sample preparation involves fixation with covalent cross-linking agents such as glutaraldehyde and osmium tetroxide, followed by dehydration and embedding in plastic. Because electrons have poor penetrating power, an ultramicrotome is used to shave off extremely thin sections from the block of plastic in which the tissue is embedded. These ultrathin sections (50~100 nm in thickness) are laid on a small circular grid for viewing in the electron microscope.

Electrons would normally pass equally well through all parts of a cell, so membranes and various cellular macromolecules are given contrast by "staining" the tissue with heavy metal atoms. For example, the **osmium tetroxide** used as a fixing agent also binds to carbon-carbon double bonds in the unsaturated hydrocarbons of membrane phospholipids. Because osmium is a large, heavy atom, it deflects electrons, and osmium-stained membrane lipids appear dark in the electron microscope image. Similarly, **lead and uranium salts** differentially bind various intracellular macromolecules, thereby also staining the cell for EM.

The preceding discussion has been of how one would go about viewing the overall layout of the cell under an electron microscope, but most often we are interested in the subcellular location of a particular molecule, usually a protein. Here again a specific antibody against the protein can be brought into play, this time tagged with something electron dense; most often, this electron-scattering tag will be commercially available nanoparticles of colloidal **gold**, coated with a small antibody-binding protein, called **Protein-A** (Figure 1-5). Gold-tagged antibodies can also be used to stain various genetically tagged proteins containing tags such as GFP, a myc tag, or any other epitope.

In some circumstances, one may wish to obtain a more three-dimensional sense of a surface feature of the cell, or of a particular object such as a

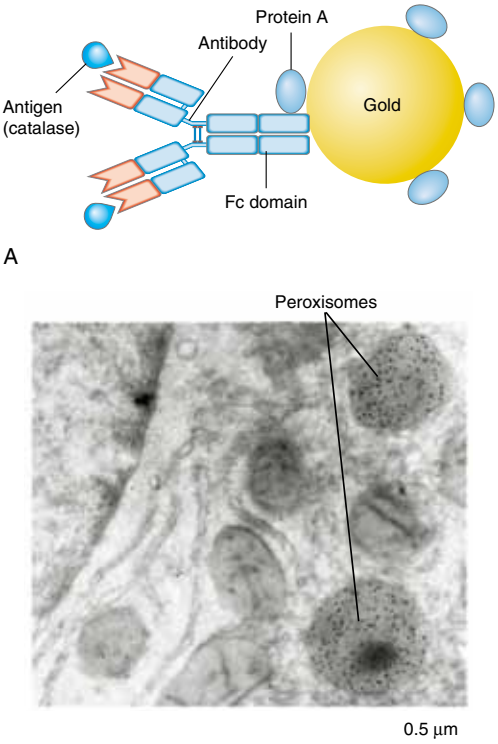

Figure 1-5 Protein A-coated gold particles can be used to localize antigen-antibody complexes by transmission electron microscopy (EM)

A: Protein A is a bacterial protein that specifically binds the Fc domain of antibody molecules, without affecting the ability of the antibody to bind antigen (the enzyme catalase, in the example shown here); it also strongly adsorbs to the surface of colloidal gold particles. **B:** Anticatalase antibodies have been incubated with a slice of fixed liver tissue, where they bind catalase molecules. After washing away unbound antibodies, the sample was incubated with colloidal gold complexed with protein A. The electron-dense gold particles are thereby positioned wherever the antibody has bound catalase, and they are visible as black dots in the electron micrograph. It is apparent that catalase is located exclusively in peroxisomes. (*A: Modified from Lodish H, Berk A, Matsudaira P, Kaiser CA, Krieger M, Scott MP, Zipursky SL, Darnell J.* Molecular Cell Biology, *5th ed. New York, NY: W.H. Freeman, 2004; B: From Geuze HF, et al.* J Cell Biol *1981;89:653, by permission of the Rockefeller University Press.*)

macromolecular complex. Two different techniques can be used to do this with a transmission electron microscope; One is called **negative staining**, and the other is called **metal shadowing.** In the case of negative staining, the objects to be viewed (e.g., virus particles) are suspended in a solution of an electron-dense material (e.g., a 5% aqueous solution of uranyl acetate), and a drop of this suspension is placed on a thin sheet of plastic, which, in turn,

is placed on the EM sample grid. Excess liquid is wicked off, and when the residual liquid dries, the electron-dense stain is left in the crevices of the sample, producing images such as that shown in Figure 1-6A.

The second technique, metal shadowing, is

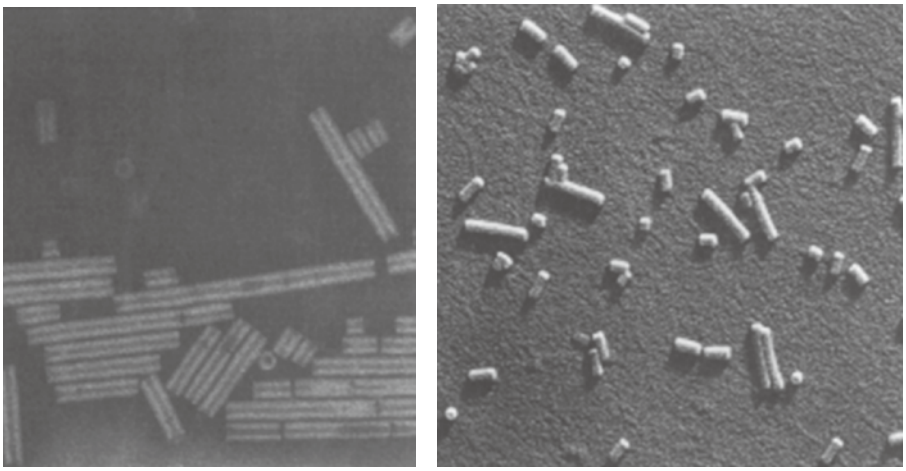

Figure 1-6 Electron microscopic images of negatively stained versus metal-shadowed specimens
A preparation of tobacco rattle virus was either **(A)** negatively stained with potassium phosphotungstate or **(B)** shadowed with chromium. (*Courtesy of M. K. Corbett.*)

illustrated in Figure 1-7. The chemically fixed, frozen, or dried specimen, on a clean mica sheet, is placed in an evacuated chamber, and then metal atoms, evaporated from a heated filament located at an overhead angle to the specimen, coat one side of the elevated features on the surface of the sample, creating a **metal replica.** When subsequently viewed in the electron microscope, electrons are unable to pass through metal-coated surfaces but are transmitted through areas in the sample that were in the shadow of the object and were therefore not metal-coated. The resulting image, usually printed as the negative, is remarkably three-dimensional in appearance (Figure 1-6B).

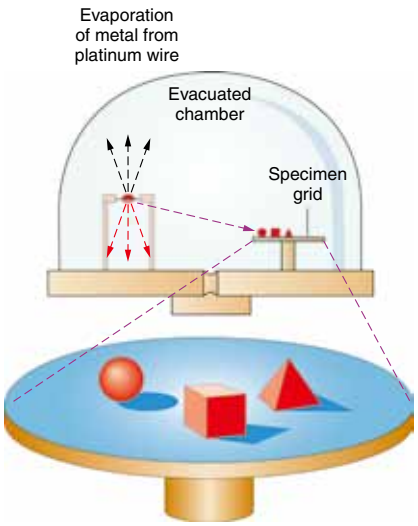

Figure 1-7 Procedure for metal shadowing
The specimen is placed in a special bell jar, which is evacuated. A metal electrode is heated, causing evaporation of metal atoms from the surface of the electrode. The evaporated metal atoms spray over the surface of the sample, thereby "shadowing" it. (*Modified from Karp G.* Cell and Molecular Biology, *3rd ed. New York: John Wiley & Sons, 2002.*)

In situations that involve a frozen sample (see the following section), after metal shadowing, the entire surface of the sample can then be coated with a film of carbon. After removal of the original cellular material, the metal-carbon replica is viewed in the electron microscope. When used in conjunction with a method of sample preparation called **freeze fracture**, metal shadowing has been useful in visualizing the arrangement of proteins in cellular membranes.

Cryoelectron Microscopy

The dehydration of samples that accompanies standard fixation and embedding procedures denatures proteins and can result in distortions if one wishes to view molecular structures at high

levels of magnification in the electron microscope. One solution to this difficulty is the technique of cryoelectron microscopy. Here the sample (often in suspension in a thin aqueous film on the sample stage) is rapidly frozen by plunging it into liquid propane (−42℃) or placing it against a metal block cooled by liquid helium (−273℃). Rapid freezing results in the formation of microcrystalline ice, preventing the formation of larger ice crystals that might otherwise destroy molecular structures. The frozen sample is then mounted on a special holder in the microscope, which is maintained at −160℃. In some cases, surface water is then lyophilized off ("freeze-etch") from the surface of the sample, which is then metal shadowed, producing images such as that in Figure 1-8.

In other cases, when there are many identical structures such as virus particles, computer-based averaging techniques, in combination with images from multiple planes of focus, can produce tomographic three-dimensional images with single nanometer resolution. This technique showed, for example, a previously unsuspected "tripod" structure for the HIV virus envelope spike (Figure 1-9).

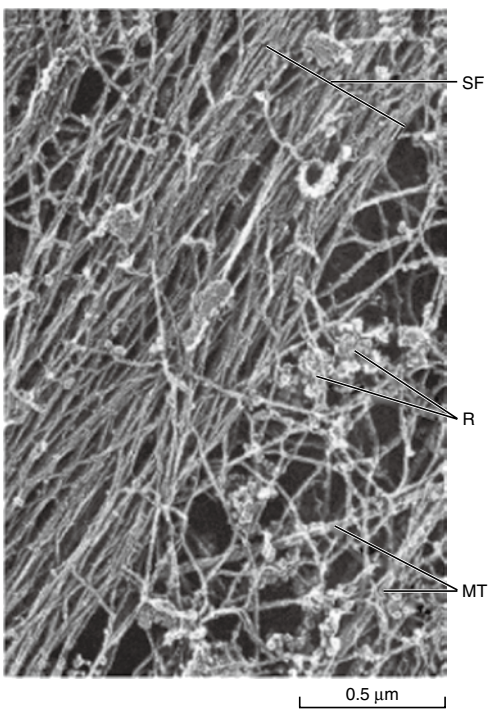

Figure 1-8 Cryoelectron microscopy of cytoskeletal filaments, obtained by deep etching

A fibroblast was gently extracted using the nonionic detergent Triton X-100 (Sigma, St. Louis), which dissolves the surface membrane and releases soluble cytoplasmic proteins, but has no effect on the structure of cytoskeletal filaments.The extracted cell was then rapidly frozen, deep etched, and shadowed with platinum, then viewed by conventional transmission electron microscopy. MT, microtubules; R, polyribosomes; SF, actin stress fibers. (*From Heuzer JE, Kirschner M.* J Cell Biol *1980;86:212, by permission of Rockefeller University Press.*)

Scanning Electron Microscopy

The surfaces of metal-coated specimens can also be viewed to good advantage with another type of electron microscope, the scanning electron microscope. Unlike the case with metal shadowing in transmission EM, in this case, the entire surface of the specimen is covered with metal. The source of electrons and focusing magnets in a scanning electron microscope are like those of a traditional transmission electron microscope, except that an additional magnet is inserted in the path of the electron beam. This latter magnet is designed to sweep (scan) the focused, narrow, pencil-like electron beam in parallel lines (a raster pattern) over the surface of the specimen. Back-scattered electrons, or secondary electrons ejected from the surface of the metal-coated specimen (usually coated with gold or gold-palladium), are collected and focused to generate the scanned image. The resolving power of a scanning electron microscope is a function of the diameter of the scanning beam of electrons. Newer machines can produce extremely narrow beams with a resolution on the order of 5 nm, permitting remarkably detailed micrographic images (Figure 1-10).

Atomic Force Microscopy

Atomic-force microscopy (AFM) was developed in the 1980s, and it has become an increasingly useful tool for cell biology. The principle of AFM is illustrated in Figure 1-11. A nanoscale cantilever/tip structure moves over the surface of the sample, and the up and down deflections of the cantilever tip are detected by a laser beam focused on its upper surface. Deflections on the order of a nanometer can be detected, producing resolutions comparable with or exceeding those of the best scanning electron microscopes.

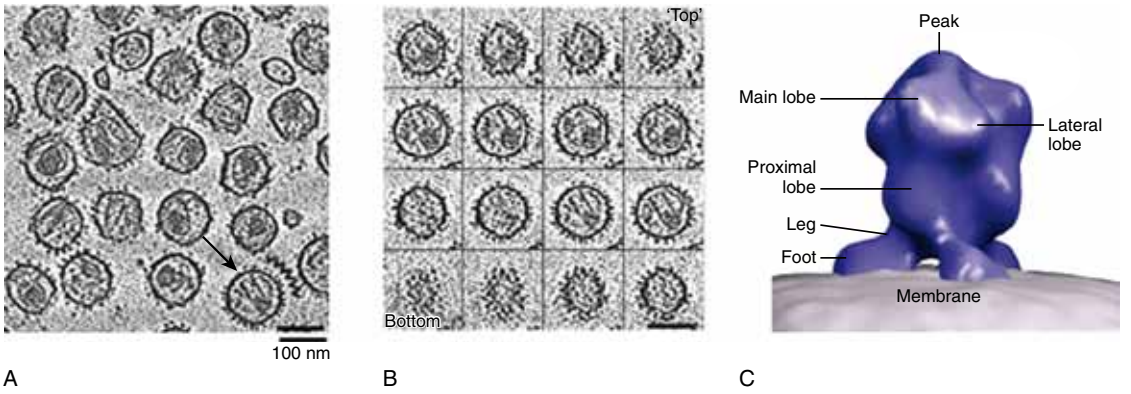

Figure 1-9 Cryoelectron microscopy and tomography of human immunodeficiency virus (HIV)

Concentrated virus (HIV or SIV) in aqueous suspension was placed on a grid and rapidly frozen by plunging the grid into liquid ethane at −196℃. The frozen sample was then placed in a cryoelectron microscopy grid holder for viewing at a magnification of ×43,200. The sample holder was tilted at a succession of angles for consecutive images, from which tomograms were computed. **A:** Sample virus field; the virus shown in this field is simian immunodeficiency virus (SIV), which has a higher density of surface spike proteins than HIV. The virus particle indicated by the *arrow* was chosen for tomographic analysis. **B:** Computationally derived transverse sections through the selected virus particle (from top to bottom). **C:** Tomographic structure of the virus envelope spike complex, which is a trimeric structure of viral gp120 (globular portion of the spike) and gp41 (transmembrane "foot") proteins, in the form of a twisted tripod. (*From Zhu P, et al.* Nature *2006;441:847, by permission.*)

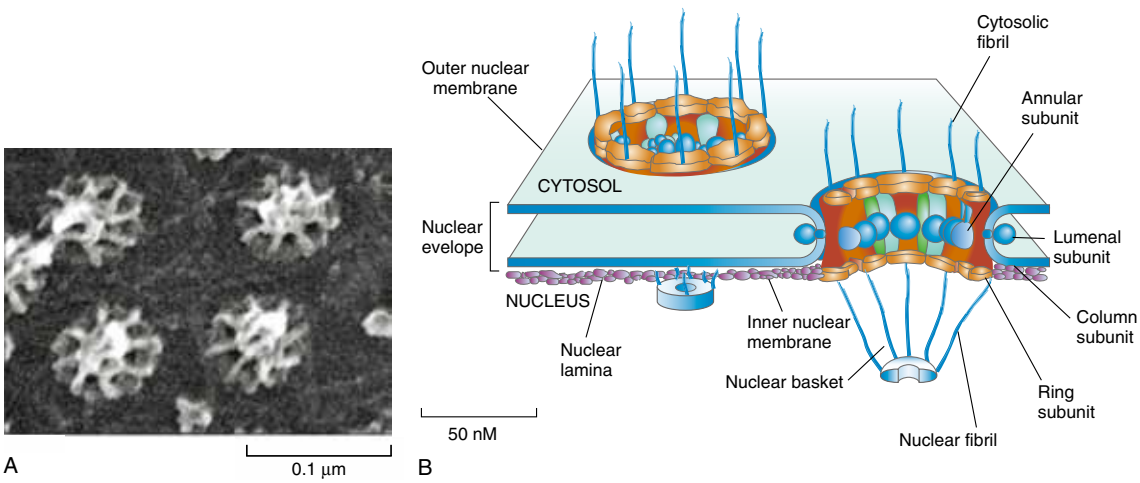

Figure 1-10 High-resolution scanning electron micrograph of nuclear pore complexes

A: Purified nuclear envelopes were prepared for scanning electron microscopy. The electron micrograph shows the image of nuclear pores as viewed from the nuclear side of the pore. **B:** Current model for the structure of a nuclear pore. (*A: From Goldberg MW, Allen TD.* J Cell Biol *1992;119:1429, by permission of Rockefeller University Press; B: Modified from Alberts B, et al.* Molecular Biology of the Cell, *4th ed. New York, NY: Garland Science, 2002.*)

Samples to be scanned by AFM need not be metal plated and put in a vacuum as is required for scanning EM; and a particular advantage of AFM over scanning EM is that samples immersed in aqueous buffers, or even living cells in culture medium, can be scanned by an AFM device. In this way, for example, the real-time opening and closing of nuclear pores in response to the presence or removal of Ca^{2+} (in the presence of ATP) has been demonstrated by AFM of isolated nuclear envelopes, and a novel cell-surface structure called the *fusion pore* was identified on the apical surface of living pancreatic acinar cells (Figure 1-12).

Not all applications of AFM technology are topologic. For example, by increasing the downward force of the probe tip on the sample, nanodissections can be performed, such as taking

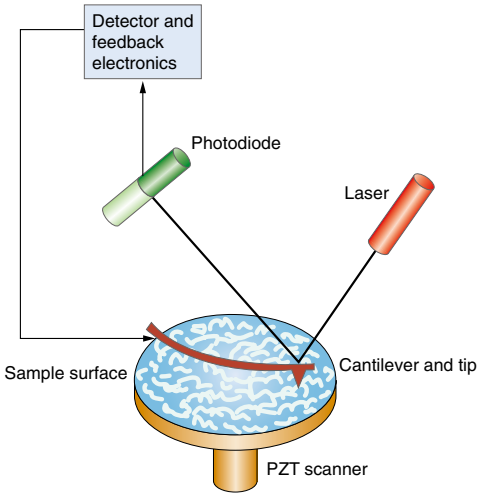

Figure 1-11 Atomic-force microscopy (AFM)

In AFM, the sample is scanned by a microscale probe, consisting of a sharp tip attached to a flexible cantilever. The deflection of the probe as it moves over the sample is measured by the movement of a laser beam reflected from the top of the cantilever onto an array of photodiodes. (*Modified from the Wikipedia article "Atomic Force Microscope," http://en.wikipedia.org/wiki/Atomic_force_microscopy.*)

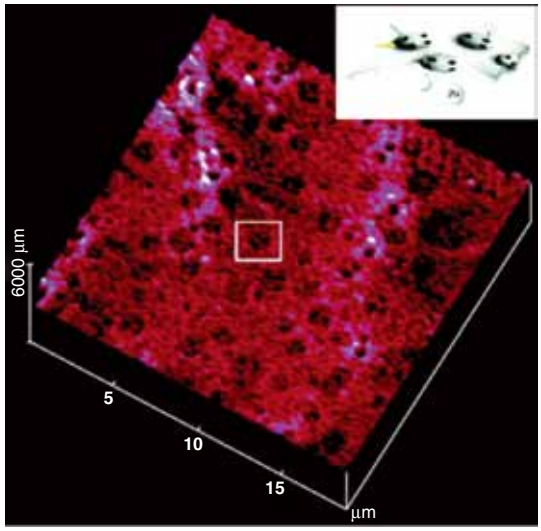

Figure 1-12 Atomic-force microscopy (AFM) image of fusion pores in the membrane of a living cell

The apical plasma membrane of living pancreatic acinar cells was scanned by AFM, producing this image of multiple pore structures. Pores are located in permanent pit structures (one of which is framed by the *white box*) on the apical surface membrane. **Inset:** Schematic depiction of secretory vesicle docking and fusion at a fusion pore. Fusion pores (*blue arrows*), 100 to 180 nm wide, are present in "pits" (*yellow arrows*). ZG, zymogen granule. (*From Horber J, Miles M.* Science *2003;302:1002, reprinted with permission from AAAS.*)

a "biopsy" sample from a specific region of a single chromosome (Figure 1-13)!

In addition, a set of nontopologic uses of AFM technology exists that might be regarded as biophysical but which have cell biological ramifications. In these cases, the cantilever tip is used to measure interactive or deforming forces. For example, ligands or reactive molecules can be attached to the tip of the cantilever. After binding of the tip to the sample, one can measure the force required to either lift the tip or move the object

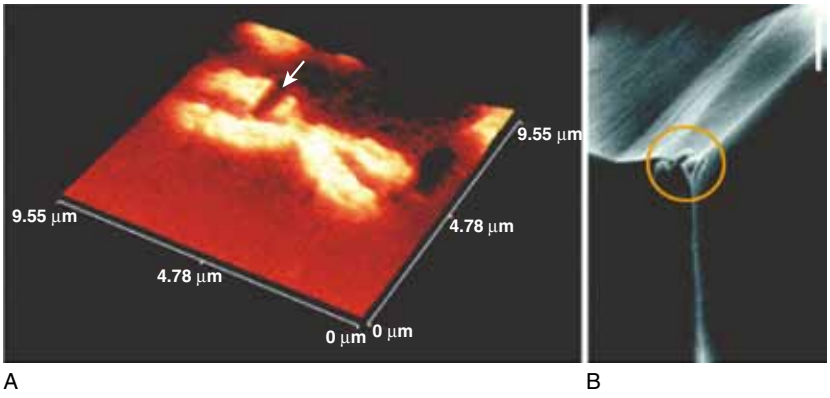

Figure 1-13 Atomic-force microscopy (AFM) "biopsy" of a human chromosome

Metaphase chromosome spreads were prepared and fixed on glass microscope slides by standard techniques. Airdried, dehydrated chromosomes were first scanned by AFM in noncontact mode; for dissection **(A)**, the probe was dragged through a previously identified location on a selected chromosome with a constant applied downward force of 17 micronewtons. **(B)** Scanning electron microscopic image of the tip of the probe used for the dissection shown in **A;** the material removed from the chromosome on the tip of the probe is *circled.* DNA in the sample could subsequently be amplified by polymerase chain reaction. (*From Fotiadis D, et al.* Micron *2002;33:385 by permission of Elsevier Science, Ltd.*)

to which the tip is bound. Experiments such as these yield insights into such processes as the force required to unfold modular protein domains, the strength of lectin-glycoprotein interactions, and so forth.

MORE TOOLS OF CELL BIOLOGY

In a search for the functions of novel genes demonstrated by the Genome Project, there are, of course, many other techniques in addition to microscopy that might be brought into play. The techniques of **animal cell culture, flow cytometry**, and **subcellular fractionation** are considered in the following sections.

Cell Culture

Many bacteria (auxotrophs) can be successfully grown in a medium containing merely a carbon source (e.g.,sugar) and some salts. Animals (heterotrophs) have lost the ability to synthesize all their amino acids, vitamins, and lipids from scratch and require many such nutrients to be provided preformed in their diet. Mammals, for example, require 10 amino acids in their diet. Mammalian cells grown in culture require the same 10 amino acids, plus 3 others (cysteine, glutamine, tyrosine) that are normally synthesized from precursors by either gut flora or by the liver of the intact animal. By the 1960s, all the micronutrient growth requirements for mammalian cells had been worked out (amino acids, vitamins, salts, trace elements), and yet it was found that it was still necessary to supplement the growth medium with serum (typically 5%~10%) to achieve cell survival and growth. Eventually, it was shown that *serum provides certain essential proteins and growth factors:* (1) **extracellular matrix proteins** such as cold-insoluble globulin (a soluble form of fibronectin), which coat the surface of the petri dish and provide a physiologic substrate for cell attachment; (2) **transferrin** (to provide iron in a physiologic form); and (3) three **polypeptide growth factors:** platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor. It is now possible to provide all the required components of serum in purified form to produce a completely defined growth medium. This can be useful in certain circumstances, but for routine growth of cells, serum is still used.

Embryonic tissue is the best source of cells for growth in culture; such tissue contains a variety of cell types of both mesenchymal and epithelial origin; but one cell type quickly predominates: cells of mesenchymal origin, resembling connective tissue fibroblasts. These fibroblastic cells proliferate more rapidly than the more specialized organ epithelial cells, and hence soon outgrow their neighbors. Special procedures must be used if one wishes to study other differentiated cell types from either embryonic or adult tissue, such as liver epithelial cells, breast epithelial duct cells, and so forth, and it is often not easy to maintain the differentiated phenotype of these cells after prolonged growth in culture. Cultured fibroblasts, however, have proved useful for explorations of the fundamental details of mammalian molecular and cell biology.

To obtain cells for growth in culture, a tissue source is gently treated with a diluted solution of certain proteolytic enzymes, such as trypsin and collagenase, often in the presence of the chelating agent ethylenediamine tetra-acetic acid (EDTA). This procedure loosens the adhesions between cells and breaks up the extracellular matrix, thereby producing a suspension of individual cells. The cells are suspended in growth medium and transferred to clean glass or (more commonly) specially treated plastic petri dishes. After transfer, the cells settle to the bottom of the dish, where they attach, flatten out, and begin both moving around on the surface and proliferating. Eventually, the cells (fibroblasts) cover the bottom surface of the dish, forming a monolayer; at this point, the growth and movement of the cells greatly slow or cease. This is known as **contact inhibition of growth.** At this point, typically 3 to 5 days after seeding, the cells can again be treated with a trypsin solution to remove them from the dish; an appropriate aliquot of the cells is then resuspended in fresh growth medium and reseeded into a new set of petri dishes. This process of cell transfer is called *trypsinizing* the cell cultures.

Cell Strains versus Established Cell Lines

Cells freshly taken from the animal initially grow well in culture, but eventually their rate of proliferation slows and stops. Depending on the animal of origin and its age, this typically occurs after anywhere from 20 to 50 cell doublings. This phenomenon is termed **cellular senescence**, and the slowing of proliferation that precedes it is termed *crisis* (Figure 1-14). In some cases, especially with rodent cells, rare variants arise in the culture that have escaped the senescent restriction on cellular proliferation, and now grow indefinitely. Such cells are termed **established cell lines.** In the case of mouse embryo cells, for example, this frequently occurs, and a well-known cell line derived from mouse embryo cells in this way is called the *3T3* cell line. Cell lines are sometimes referred to as being "immortal" because of their ability to proliferate indefinitely in culture. Spontaneously arising cell lines such as mouse 3T3 cells usually have abnormalities in chromosome content and can have precancerous properties.

In the case of primary human cell cultures,

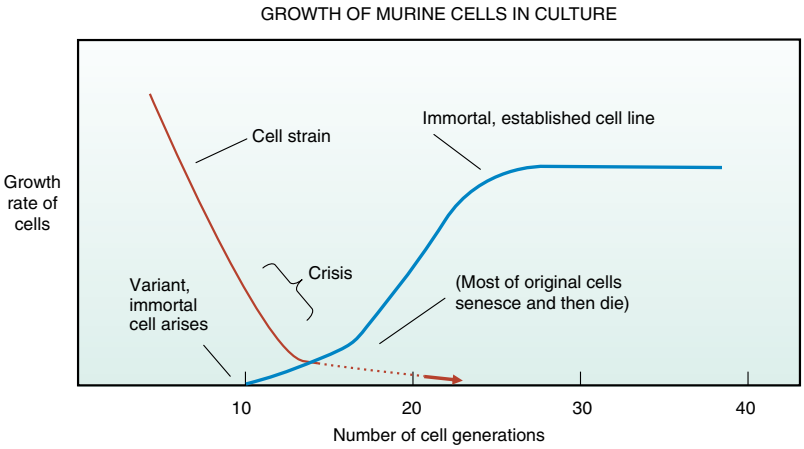

Figure 1-14 Cell strain versus established cell lines

Murine cells (e.g., mouse embryo cells) initially grow well in culture, and during this period of growth, such cells are termed a "cell strain." But the growth rate falls after several generations, and the cells enter "crisis," following which almost all cells senesce and die. Often, however, a rare variant cell will arise in the culture, capable of indefinite growth (i.e., "immortal"). The descendants of this variant cell become an "established cell line." These immortalized cells are typically aneuploid. (*Modified from Todaro GJ, Green H.* J Cell Biol *1963;17:299-313.*)

this escape from crisis to form an established cell line never occurs. Human and mouse cells before crisis are referred to as **cell strains**, or sometimes, more colloquially, "primary cells." The latter term, however, is more appropriately used for cells freshly taken from the animal, before trypsinization, to produce a secondary culture. At the heart of cellular senescence are repetitive, noncoding sequences called **telomeres**, which are found at both ends of the linear chromosomal DNA molecules of eukaryotic cells. Because of the biochemistry of DNA replication, terminal sequence information is lost each time a linear DNA molecule is replicated. The telomeric sequences of eukaryotic chromosomes protect coding DNA, because it is the "junk" telomeric DNA at the ends that shortens when chromosomal DNA is replicated. In very early embryo cells, as well as in adult germ-line cells and certain stem cells, an enzyme called **telomerase** is expressed, which maintains the length of the telomeres during cell proliferation. In most somatic cells, however, telomerase is not expressed; as a result, each time the cell replicates its DNA and divides, the telomeric DNA sequences shorten. After a certain number of cell doublings, the shortened telomeric DNA reaches a critical size limit that is recognized by the cellular machinery responsible for activating the senescence program (i.e., the cessation of further cell proliferation).

One of the critical steps in the conversion of a normal cell into a cancer cell is reactivation of telomerase expression. Because cancer cells

are therefore able to maintain telomere length, they escape senescence and are "immortal". Consequently, cancer cells, if adapted to growth in culture, grow as established cell lines. For many years there were no established lines derived from normal human cells; the one established human cell line that was available was the **HeLa** cell line. These widely used cells were derived in the 1950s from the cervical cancer tissue of a woman named Henrietta Lacks. Normal animal cells must attach and spread out to grow (the "**anchorage requirement**" **for growth**); but HeLa cells, like some other established lines derived from cancer cells, have lost the anchorage requirement for growth, and can be grown in suspension like bacteria or yeast cells.

Flow Cytometry

Flow cytometry is a method to count and sort individual cells based on cell size, granularity, and the intensity of one or another cell-associated fluorescent marker. The device that is most commonly used to perform the analysis is called a **fluorescence-activated cell sorter (FACS),** and the layout of a typical FACS instrument is shown in Figure 1-15. In the device, cells pass single file into sheath liquid, which in turn passes through a special vibrating nozzle that creates roughly cell-sized droplets. Most droplets contain no cell, but some droplets contain a single cell (droplets that contain no cell or aggregates of two or more cells are detected and discarded). Just before the cells enter the nozzle, each cell is illuminated by a laser beam that causes any cell-associated dye to fluoresce. Forward- and side-scattered light is also measured. Based on these measurements, individual droplets are given either no charge (i.e., empty droplets or droplets with clumps of cells) or a positive or negative charge, and are then deflected (or not, if uncharged) by a strong electric field, which sends them to a particular sample collector.

The FACS device can be used simply to measure the characteristics of a population of cells **(cytometry),** or to sort and isolate subpopulations of cells **(cell sorting).** Earlier devices had a single laser source, and four light detectors, one each for forward scatter (a measure of cell size), side scatter (cellular granularity),

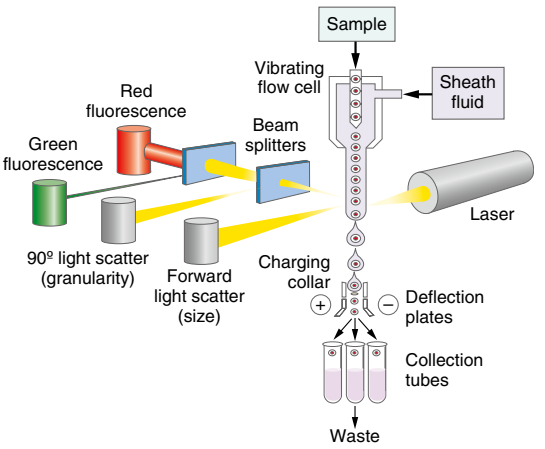

Figure 1-15 Fluorescence-activated cell sorting (FACS) Antibodies tagged with red or green fluorescent molecules and specific each for one of two different cell surface proteins (e.g., CD4 and CD8) are used to label a population of cells (e.g., a population containing the CD4 and or the CD8 protein on their surface). The labeled cells pass into a vibrating flow cell, from which they emerge within individual fluid droplets. The droplets are excited by a laser beam. Forward-scattered laser light, side-scattered laser light, and red and green fluorescent light from the droplet are measured. Based on these measurements, individual droplets will be given a positive (+) or negative (−) charge, and then diverted to collection tubes via charged deflection plates. (*Modified from Roitt IM, Brostoff J, Male D.* Immunology, *5th ed. St. Louis: Mosby Year-Book, 1998.*)

and red or green fluorescence. Coupled with the use of red and green fluorescently tagged monoclonal antibodies directed against particular surface proteins, devices such as these played a large role in working out the role of various populations of precursor cells in the process of lymphocyte differentiation.

Second- and third-generation instruments now use as many as 3 lasers, and can detect and sort cells based on as many as 12 different fluorescent colors. FACS analysis is quite useful in studies of, for example, cytokine production by individual T-cell populations, expression of activation markers, and apoptosis induction in cell population subsets. FACS not only uses monoclonal antibodies for staining of surface markers, but it can also be used to sort and clone hybridoma cells present at low frequency in a postfusion population. This can permit the rescue of rare hybridoma clones expressing useful monoclonal antibodies that might otherwise be lost to overgrowth by nonproducing hybrids. When coupled with reporter gene constructs, such as those expressing proteins tagged

with GFP, rare cells expressing the reporter can be identified and captured for further growth and analysis. Additional applications of flow cytometry are discussed in Chapter 2.

Subcellular Fractionation

Subcellular fractionation is a set of techniques that involve cell lysis and centrifugation. These techniques were intimately involved in the discovery, over the course of the last half of the preceding century, of all the various compartments, membrane structures, and organelles that are now known to make up the internal structure of a cell. They are also part of the working repertoire of any contemporary cell biologist.

Cell Lysis

Cells can be lysed in any of a variety of ways; the optimum method depends on the cell or tissue type, and the intent of the investigator. One common way to gently break open tissue culture cells, for example, is a device called a *Dounce homogenizer*. A **Dounce homogenizer** consists of a glass pestle with a precision-milled ball at the end; the dimensions of the ball are such that it slides tightly into a special tube in which the cell suspension is contained. Several up and down strokes of the pestle suffice to break open the majority of the cells while leaving nuclei and most organelles intact.

Centrifugation

After cell lysis, centrifugation can then be used to separate the various components of the cellular homogenate, based on their particular size, mass, and/or density. In one common approach, used for the rough fractionation of a homogenate, the lysate is centrifuged in a stepwise fashion at progressively greater speeds and longer times, collecting pelleted material after each step. A low-speed spin will pellet unbroken cells and nuclei; centrifugation of the supernatant from the low-speed spin at a higher, intermediate speed and for a longer duration will bring down organelles such as the mitochondria; centrifugation of the supernatant from the intermediate speed pellet at yet greater speeds and even longer durations will pellet microsomes (ER) and other small vesicles (Figure 1-16). This type of procedure

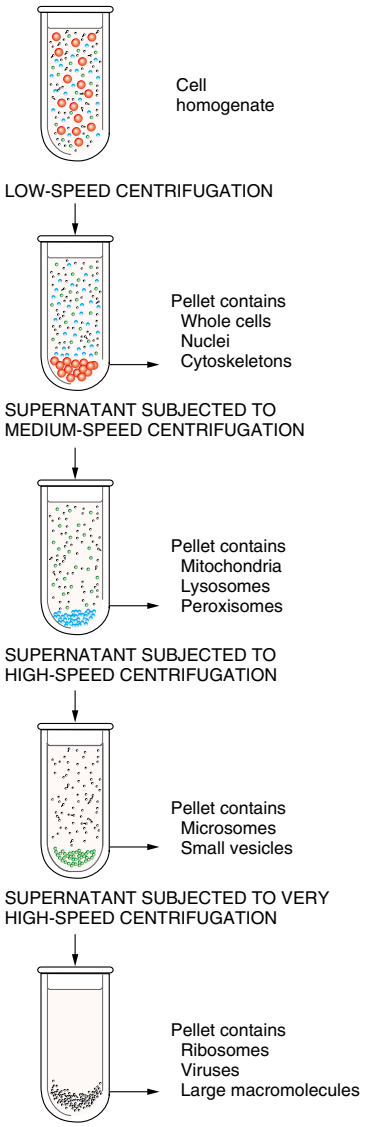

Figure 1-16 Differential centrifugation

A cell lysate is placed in a centrifuge tube, which, in turn, is mounted in the rotor of a preparative ultracentrifuge. Centrifugation at relatively low speed for a short time (800 *g*/10 minutes) will suffice to pellet unbroken cells and nuclei. The supernatant of the low-speed spin is transferred to a new tube, and centrifuged at a greater speed and longer time (12,000 *g*/20 minutes) will pellet organelles (mitochondria, lysosomes, peroxisomes); centrifugation of that supernatant at high speed (50,000 *g*/2 hours) will pellet microsomes (small fragments of endoplasmic reticulum and Golgi membranes); centrifugation at very high speeds (300,000 *g*/3 hours) will pellet free ribosomes or viruses or other large macromolecular complexes. (*Modified from Alberts B, et al.* Molecular Biology of the Cell, *4th ed. New York, NY: Garland Science, 2002.*)

is termed **differential centrifugation.** Differential centrifugation can be usefully applied to separate

subcellular components that differ greatly in size or mass. But the pelleted materials thus obtained are usually contaminated with many different components of the cell; in the case of Dounce homogenates, for example, the low-speed nuclear fraction contains not only unbroken cells but also large sheets of plasma membrane wrapped around the nuclei; mitochondrial pellets contain lysosomes and peroxisomes.

Further purification or more detailed analyses can be obtained by two other techniques of centrifugation: **rate-zonal centrifugation** (also known as **velocity sedimentation**) and **equilibrium density gradient centrifugation** (sometimes called **isopycnic density gradient centrifugation**). In both of these techniques, an aliquot of cellular material (whole-cell lysate or a resuspended pellet from differential centrifugation) is added as a thin layer on top of a gradient of some dense solute such as sucrose.

In the case of **rate-zonal centrifugation** (Figure 1-17A), the sample is layered on a relatively shallow sucrose gradient (e.g., 5%~20% sucrose), and then spun at an appropriate speed (based on the size and mass of the material in the sample); in this case, cellular material is not pelleted; instead, the centrifugal field is used to separate materials based on their size, shape, and density; the shallow sucrose gradient serves simply to stabilize the sedimenting material against convective mixing. After the sample components have been resolved based on their sedimentation velocity (but typically before any of the material has actually formed a pellet on the bottom of the centrifuge tube), the centrifuge is stopped, the bottom of the tube is pierced, and sequential fractions of the resolved material are collected for assay. In this way, for example, ribosomes and polyribosomes were first isolated and characterized. The velocity at which a particle moves during centrifugation can be characterized by a number called its "**sedimentation coefficient,**" often expressed in **Svedbergs (S).** The value of S is a function of the mass, buoyant density, and shape of an object. Large and small mammalian ribosomal subunits, for example, have sedimentation coefficients of 60S and 40S, respectively, whereas the whole ribosome has a sedimentation coefficient of 80S.

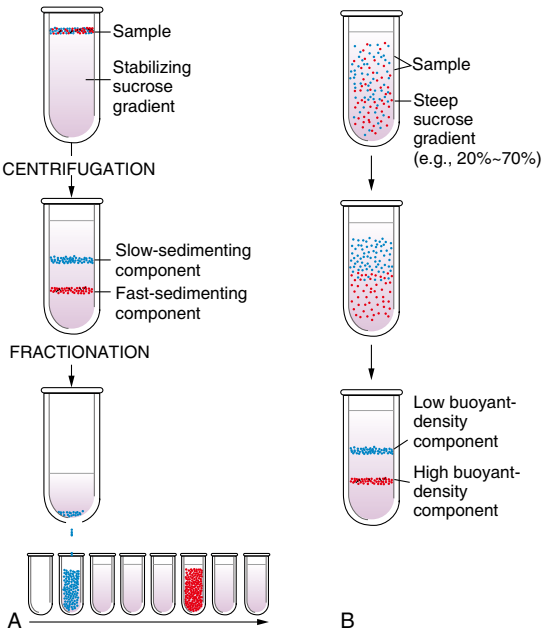

Figure 1-17 Rate-zonal centrifugation versus equilibrium density gradient centrifugation

A: In rate-zonal centrifugation, the sample is layered on top of a shallow sucrose gradient. During centrifugation, the various components in the sample then move toward the bottom of the tube based on their sedimentation coefficients. After resolution of the components, the bottom of the plastic tube is pierced and fractions are collected. **B:** Equilibrium density centrifugation resolves components in the sample based on their molecular density. The sample is either layered onto or incorporated into a steep sucrose gradient; during centrifugation, individual components move in the centrifugal field until they reach a density in the gradient that is identical to the buoyant density of the sample component. At this point, each component stops moving and forms a band in the gradient. (*Modified from Alberts B, et al.* Molecular Biology of the Cell, *4th ed. New York, NY: Garland Science, 2002.*)

In the previously described applications of centrifugation, objects are separated based largely on their relative mass and size. Alternatively, cellular materials can be resolved based on their **buoyant density.** Various proteins, for example, can differ widely in molecular mass, but all proteins have approximately the same buoyant density (approximately 1.3 g/cm^3); carbohydrates have densities of approximately 1.6 g/cm^3; RNA has a density of about 2.0 g/cm^3; membrane phospholipids have densities on the order of 1.05 g/cm^3; and cellular membranes, composed of both lipid and protein, have densities of approximately 1.2 g/cm^3. These differences in intrinsic molecular densities

permit the resolution of a variety of cellular substituents by the technique of **equilibrium density gradient centrifugation** (Figure 1-17B). Here again, the sample would be layered on top of a gradient of dense solute. For resolving cellular membranes and organelles, the solute would be sucrose, and a 20% to 70% sucrose gradient typically would be used, generating densities ranging from 1.1 to 1.35 g/cm^3. For resolving proteins and nucleic acids, higher density gradients made with cesium chloride would be used. During centrifugation over the course of several hours, cellular components migrate in the tube until they reach a point in the density gradient equal to their own buoyant density, at which point they cease moving and form a disk or "band" at their equilibrium position in the gradient. Rough ER membranes, smooth ER membranes, lysosomes, mitochondria, and peroxisomes all have unique buoyant densities, for example, and are readily separated from each other by this method.

SUMMARY

Cell biologists have many powerful and sophisticated tools to deploy in their investigations of the function of uncharacterized cellular proteins. Microscopy techniques, in the forms of fluorescence microscopy, EM, and AFM, are among the most useful of these tools, as are the allied techniques of immunology. Tissue culture techniques provide a source of defined, uniform cell types for protein expression and analysis, and flow cytometry technology permits rapid and extremely sensitive analysis of cell populations. Epitope tagging of the proteins encoded by cloned complementary DNA molecules permits their efficient affinity purification, especially in conjunction with the standard techniques of subcellular fractionation and liquid chromatography. Two-dimensional gel electrophoresis and Western blotting are powerful analytic methods for resolving and characterizing complex mixtures of proteins.

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