BCHEM 365
Lecture 14
December 11, 2018
Specimen preparation

Specimen for microscopy can be an entire organism or a dissected organ (whole mount), an aliquot collected during a biochemical protocol for a quick check of the preparation, or a small part of an organism (biopsy), smear of blood or spermatozoa.

In order to collect images from it, the specimen must be prepared into a thin and somewhat transparent piece of tissue mounted on a glass slide in a mounting medium (water, tissue culture medium or glycerol) with a thin square of glass (coverslip) mounted on top. The preparation involves, fixation, sectioning, and staining.

Fixation

Tissues are generally soft and fragile and begin a process of autolysis (decay) soon after excision. The purpose of fixation is to kill and preserve tissues permanently in as close to its natural state as possible, without any form of autolysis, and to harden the tissue to permit sectioning of ultrathin slices for visualizing detail. To do this, a **fixative** is used. Fixatives act by disabling intrinsic biomolecules—particularly proteolytic enzymes—which otherwise digest or damage the sample; protect a sample from extrinsic damage from putrefying bacteria, which may attack dead tissue; provide mechanical strength to the tissue. There are three types of fixation processes:

Fixation by heat: After a cell smear has dried at room temperature, the slide is gripped by tongs or passed through flame of a Bunsen burner several times to heat-kill and adhere the organism to the slide. This method is routinely used with bacteria and Archaea. Heat fixation generally preserves overall morphology but not internal structures. Heat denatures the proteolytic enzyme and prevent autolysis.

Perfusion: This is fixation via blood flow. The fixative is injected into the heart of the whole organism with the injection volume matching cardiac output. The fixative spreads through the

entire body, and the tissue doesn't die until it is fixed. This has the advantage of preserving perfect morphology, but kills the subject. Because large volumes are required for large animals, cost can be high.

Immersion in chemical fixative: The tissue is immersed in chemical fixative at 1:20 w/v and time allowed to have fixative percolate through the tissue. Large sized tissue require longer times than small ones. Chemical fixation is enhanced in slight vacuum.

Types of fixatives

Five major groups of chemical fixatives, aldehydes, mercurials, alcohols, oxidizing agents, and picrates are in common use. Their choice depends on the type of tissue and features to be examined.

Aldehydes

Formaldehyde (formalin) forms covalent bonds with the free ε-amino group of lysine in proteins, cross-linking them with methylene bridges (-CH_2-) so they are stabilized and locked into position. This cross-linkage does not harm the structure of proteins greatly, so that antigenicity (antibody binding) is not lost. Therefore, formaldehyde is good for immunoperoxidase techniques. Formalin penetrates tissue well, but is relatively slow. The standard solution is 10% neutral buffered formalin containing approximately, 3.7%-4.0% formaldehyde in phosphate buffered saline. The buffer prevents acidity that would promote autolysis and cause precipitation of formol-heme pigment in the tissues.

Glutaraldehyde, like formaldehyde, forms covalent bonds with the free ε-amino group of lysine in proteins and stabilizes the protein with methylene cross bridges. Glutaraldehyde causes deformation of alpha-helix structure in proteins and so is not good for immunoperoxidase staining. However, it fixes very quickly so it is good for electron microscopy. It penetrates very poorly, but gives best overall cytoplasmic and nuclear detail. The standard solution is a 2% buffered glutaraldehyde. Both formaldehyde and glutaraldehyde stabilize proteins, nucleic acids and mucosubstances of the tissue by making them insoluble.

Mercurials fix tissue by an unknown mechanism. They contain mercuric chloride and include fixatives as B-5 and Zenker's. These fixatives penetrate poorly and cause some tissue hardness and shrinkage, but give excellent nuclear detail. Their best application is for fixation of hematopoietic and reticuloendothelial tissues. Since they contain mercury, they must be disposed of carefully.

Alcohols, including methanol and ethanol, are protein denaturants and are not used routinely for tissues because they cause too much brittleness and hardness. However, they are very good for cytologic smears because they act quickly and give good nuclear detail.

Oxidizing agents including permanganate fixatives (potassium permanganate), chromic acid fixatives (potassium dichromate), and osmium tetroxide crosslink proteins via reaction with various side chains of proteins and other biomolecules, allowing formation of crosslinks that stabilize tissue structure.
Osmium tetroxide is often used as a secondary fixative when samples are prepared for electron microscopy. It is not used for light microscopy as it penetrates thick sections of tissue very poorly.
The following fixatives have specialized applications.

Salts of picric acid -the most important member of this group is Bouin solution. **Bouin solution** is a compound fixative used in histology. It is composed of picric acid, acetic acid and formaldehyde in an aqueous solution. It is especially good for gastrointestinal tract biopsies because this fixative allows crisper and better nuclear staining than 10% neutral-buffered formalin. It is not a good fixative when tissue ultrastructure must be preserved for electron microscopy, but preferable with soft and delicate tissue as it preserves texture. The acetic acid in this fixative lyses red blood cells and dissolves small iron and calcium deposits in tissue.
The effects of the three chemicals in Bouin solution balance each other.

Formalin causes cytoplasm to become basophilic but this effect is balanced by the effect of the picric acid. This results in excellent nuclear and cytoplasmic structure. The tissue hardening effect of formalin is balanced by the soft tissue fixation of picric acid. The tissue swelling effect of acetic acid is balanced by the tissue shrinking effect of picric acid. In its action, picric acid penetrates tissue well to react with histones and basic proteins to form crystalline picrates with amino acids and precipitate all proteins. It produces nuclear detail but does not cause as much hardness. Picric acid is an explosion hazard in dry form and stains everything it touches yellow, including skin –care must be taken with use. It is a good fixative for connective tissue, preserves glycogen well, and extracts lipids to give superior results to formaldehyde in immunostaining of biogenic and polypeptide hormones. However, it causes loss of basophilia unless the specimen is thoroughly washed following fixation.

Basic protocol for fixation

Paraformaldehyde fixation and paraffin wax embedding of tissues and embryos

MATERIALS

4% (w/v) paraformaldehyde (PFA) fixative, freshly prepared at 4ºC

Paraffin wax

50%, 70%, 95%, and 100% ethanol

Xylene

Silicone spray

20-ml snap-cap glass vials

60ºC oven and heating block with holes to hold 20-ml glass vials

Embedding molds and rings

Hot forceps or hot Pasteur pipette with end cut off

Hot Pasteur pipette with end drawn out and sealed

1. Place dissected organs in labeled 20-ml glass vials. Fill vials with 4% PFA fixative at 4ºC. Allow fixation to proceed at 4ºC for the desired time
2. Begin melting paraffin wax in 60ºC oven

3. After fixation is completed, pour off the fixative and replace with 50% ethanol. Immediately change the 50% ethanol to fresh 50% ethanol. Incubate 20 min and change the 50% ethanol again for a total of three changes of ethanol.
4. Continue dehydration by incubating for three changes in 70% ethanol, 20 min each time, room temperature.
5. Incubate in 95% ethanol for 20 min at room temp. Repeat two more times
6. Incubate in 100% ethanol for 20 min at room temperature. Repeat two more times
7. Replace 100% ethanol with xylene. Immediately change to fresh xylene and incubate 10 min. Follow with two more xylene changes and 10 min incubation at each change. (Note: xylene is toxic)
8. Pour off xylene and add 5 ml fresh xylene. Add an equal amount of molten wax using a hot glass pipette. Mix and leave samples overnight at room temperature
9. Transfer samples to 60^{o}C oven to melt the wax/xylene mixture
10. Pour off wax/xylene mixture into waste bottle. Immediately add fresh molten wax to the vial with a hot glass pipette. Put vial back into 60^{o}C heat block. Repeat procedure and return to 60^{o}C oven and incubate for 1 hr
11. Remove vials from oven and place again into 60^{o}C heating block Repeat steps 12 to 13 twice for a total of 3 hr of incubation
12. Prepare embedding molds according to manufacturer's instructions (e.g. coated with silicone spray) and fill one of the molds with molten paraffin was using a hot glass pipette. Immediately transfer sample to the wax-filled mold using hot forceps
13. Place an embedding ring on the mold and fill with paraffin wax. Label the embedding ring to facilitate future identification of samples. Orient samples within the mold using a hot drawn-out and sealed Pasteur pipette. Leave cast blocks at room temperature to harden completely
14. Remove cast blocks from embedding molds and store in a dry place at room temperature
15. Perform sectioning

Sectioning

Most tissue samples are too thick for their individual cells to be examined directly at high resolution and must be cut into very thin and transparent slices called **sections**. The thinner the section the crisper or finer the image. The process of sectioning loses information about the third dimension. If we have such a loss of the three dimensional features of the specimen, we need to use an alternative method for sectioning – **optical sectioning**.

Protocols in preparation for sectioning

Because tissues are generally soft and fragile, even after fixation, they need to be embedded in a supporting medium before sectioning. The usual embedding media are **waxes** or **resins**. In liquid form embedding media permeate and surround the fixed tissue; they can then be hardened by cooling to form a solid block, which is readily sectioned with a **microtome**. This is a machine with a sharp blade that operates like a meat slicer (Fig. 14.1) producing sections (typically 100 - 500 µm thick) which are then laid flat on the surface of a glass slide.

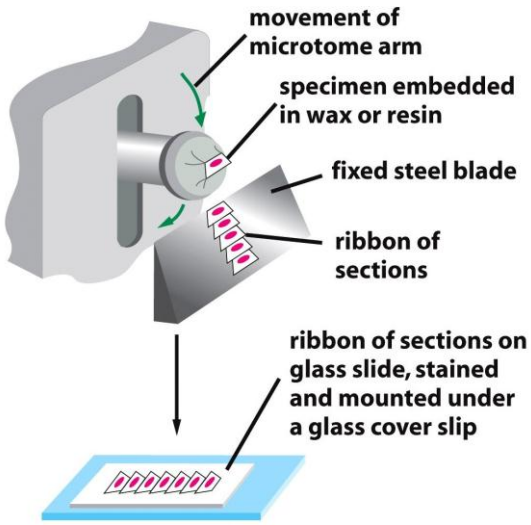


Fig. 14.1 Making tissue sections. This illustration shows how an embedded tissue is sectioned with a microtome in preparation for examination with light microscope.

Some samples are frozen and cut on a **cryostat**. This is a microtome that can keep a specimen in a frozen state, and produce frozen sections more suitable for immunolabelling.

Creating Contrast

The cell contains about 70% water by weight and so in their natural state there is very little material that can impede light to an appreciable extent. Even if fixed and sectioned, cells are almost colorless, transparent, lack contrast and so are invisible in ordinary light microscope. There are three main approaches to creating contrast in thin tissue sections that reveal the cells themselves and/or specific components, such as proteins and nucleic acids within them.

1. Staining with organic dyes that have specific affinity for particular subcellular components
2. Visualization of temporal and spatial expression patterns of differential gene expression observed as mRNA (***in situ* hybridization of tissue sections)** and protein **(immunohistochemistry)** in individual cells. Fixation and sectioning critically affect the success of both in situ hybridization and immunohistochemistry experiments
3. Use of fluorescent probes and markers

Staining

First, and traditionally, sections can be stained with organic dyes that have some specific affinity for particular subcellular components.

The morphology of specimen sections and the identity of specific areas are defined by lightly counterstaining sections. After staining, slides are dehydrated, mounted with coverslips, hardened, and cleaned for examination under the microscope.

1. Hematoxylin/eosin (H&E) stain.

   This dye has an affinity for negatively charged molecules and therefore reveals the distribution of DNA, RNA, and acidic proteins in a cell. **H&E stain** is a popular staining method in histology, a most widely used stain in medical diagnosis for revealing cancer. The staining method involves application of **hemalum**, which is a complex formed from aluminum ions and oxidized haematoxylin. This colors nuclei of cells blue. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colors eosinophilic structures in various shades of red, pink and orange. H&E stain differentiates both the nuclei and cytoplasm (Fig. 14.2).

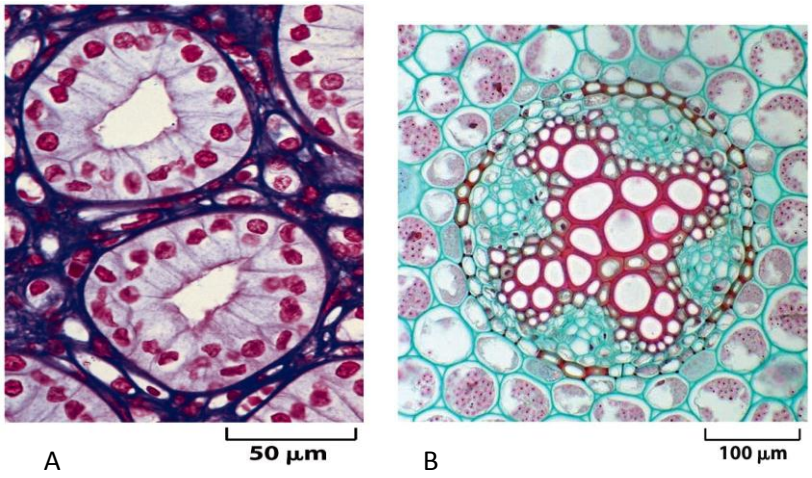


Fig. 14.2. **Staining of cellular components**. (A) This section of cells in the urine-collecting ducts of the kidney was stained with a combination of dyes, hematoxylin and eosin, commonly used in histology. Each duct is made of closely packed cells that form a ring, with nuclei staining red. The ring is surrounded by extracellular matrix, stained purple. (B) The section of a young plant root is stained with two dyes, safranin and fast green. Fast green stains cellulosic cell walls green, while safranin stains the lignified xylem cell walls bright red (Source: Wheater et al. 1987, Functional Histology, 2nd ed.)

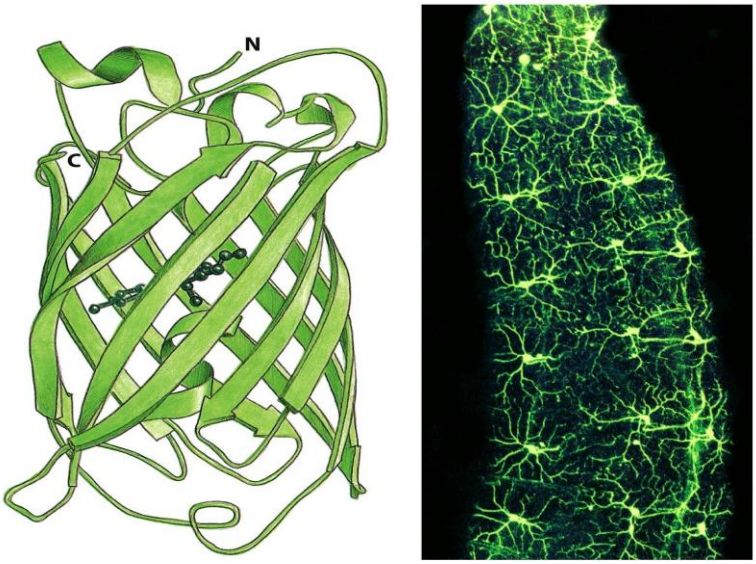
Fluorescent stains of living cells

Relatively few cells possess any inherent fluorescence (auto fluorescence) although some endogenous molecules are fluorescent and can be used for imaging, for example NAD(P)H. Relatively small fluorescent molecules are loaded into living cells using many different methods, including diffusion, microinjection, bead loading or electroporation. Relatively larger fluorescently labeled proteins are usually injected into cells, and after some time they are incorporated into the general protein pool of the cell for imaging.

Fluorescent proteins, such as green fluorescent protein (GFP) of the jellyfish (*Aequoria Victoria*) can be used to tag individual proteins in living cells and organisms by incorporation of the genes coding for this protein via genetic engineering enabling the creation of lines of cells or organisms that make their own visible fluorescent tags and labels, and produce glowing fluorescent color without the introduction of foreign fluorescent dyes.

This protein is encoded in the normal way by a single gene that can be cloned and introduced into cells of other species. The freshly translated protein is not fluorescent, but within an hour or so (less for some alleles of the gene, more for others) it undergoes a self-catalyzed post-

translational modification to generate an efficient and bright fluorescent center, shielded within the interior of a barrel-like protein (Fig. 14.3).



(A) (B)

Fig. 14.3. Green fluorescent protein (GFP). (A) The structure of GFP is made up of eleven β strands that form the shape of a barrel. Buried within the barrel is the active chromophore (dark green) that is formed post-translationally from the protruding side chain of three amino acid residues. (Source: Ormo et al. 1996 Science 273: 1392-1395). (B) The green fluorescent protein as a reporter. The image of a live fruit fly embryo with GFP gene joined to it using recombinant DNA technology. The image was captured by fluorescence microscopy and shows approximately 20 neurons with the long projections (axons and dendrites). (Source: Grueber et al. 2003. Curr. Biol. 13:618-625).

Extensive site-directed mutagenesis performed on the original gene sequence has resulted in useful fluorescence in organisms ranging from animals and plants to fungi and microbes. The fluorescence efficiency has also been improved, and variants have been generated with altered absorption and emission spectra in the blue-green-yellow range.

One of the simplest uses of GFP is as a reporter molecule, a fluorescent probe to monitor gene expression. A transgenic organism can be made with the GFP-coding sequence placed under the transcriptional control of the promoter belonging to a gene of interest, giving a directly visible readout of the gene's expression pattern in the living organism (Fig. 14.3).

In another application, **a peptide location signal can be added to the GFP to direct it to a particular cellular compartment,** such as the endoplasmic reticulum or a mitochondrion, lighting up these organelles so they can be observed in the living state.

The GFP DNA-coding sequence can also be inserted at the beginning or end of the gene for another protein, yielding a fusion product consisting of that protein with a GFP domain attached. In many cases, this GFP-fusion protein behaves in the same way as the original protein, directly revealing its location and activities by means of its genetically encoded contrast (Fig. 14.4).

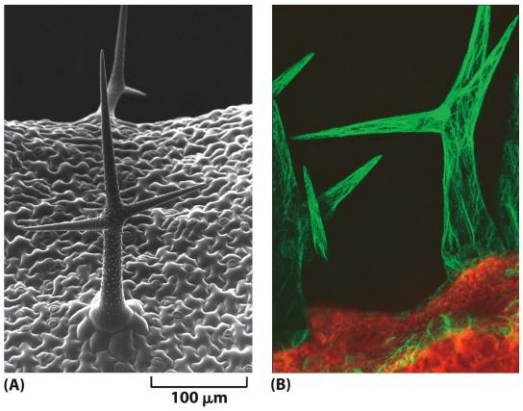


Fig. 14.4. GFP-tagged proteins. (A) The upper surface of the leaves of *Arabidopsis* plants covered with huge branched single hairs (trichomes) that rise up from the surface of the epidermis. This is imaged with scanning electron microscope. (B) Transformed *Arabidopsis* with a DNA sequence coding for talin (an actin-binding protein) fused to a DNA sequence coding for GFP. Confocal microscopy reveals the dynamics of the trichomes (green). The red fluorescence arises from chlorophyll in cells within the leaf.

Antibodies can be used to detect specific molecules

Antibodies are proteins produced by the vertebrate immune system as a defense against infection. They are unique among proteins because they are made in billions of different forms, each with a different binding site that recognizes a specific target molecule (or *antigen).* The precise antigen specificity of antibodies makes them powerful tools for the cell biologist. When

labeled with fluorescent dyes, antibodies are invaluable for locating specific molecules in cells by fluorescence microscopy. When they are labeled with electron-dense particles such as colloidal gold spheres, they are used for similar purposes in the electron microscope.

When we use antibodies as probes to detect and assay specific molecules in cells we frequently amplify the fluorescent signal they produce by chemical methods. For example, although a marker molecule such as a fluorescent dye can be linked directly to an antibody used for specific recognition, that is, the **primary antibody**, a stronger signal is achieved by using an unlabeled primary antibody and then detecting it with a group of labeled **secondary antibodies** that bind to it (Fig. 14.5). This process is called **indirect immunocytochemistry.**

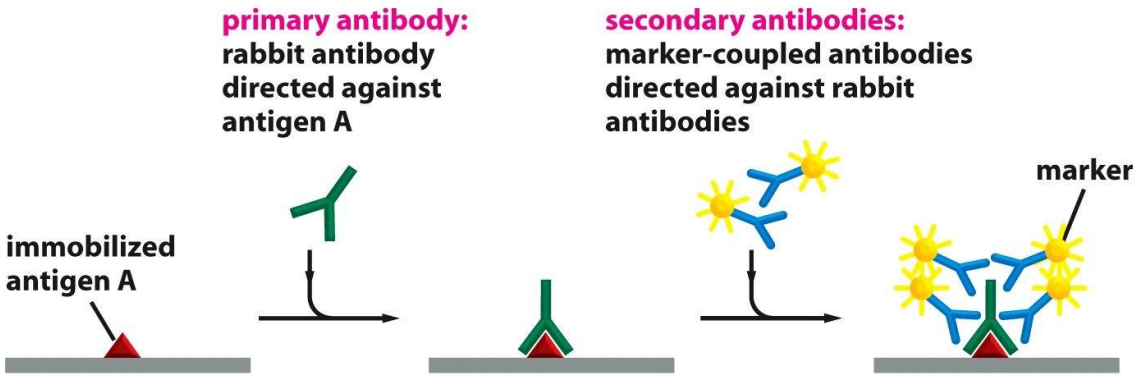


Fig. 14.5. Indirect immunocytochemistry. This detection method is very sensitive because many molecules of the secondary antibody are covalently coupled to a marker molecule that makes it readily detectable. Commonly used marker molecules include fluorescent dyes used in fluorescence microscopy, the enzyme horseradish peroxidase (for either conventional light microscopy or electron microscopy), colloidal gold spheres (for electron microscopy) and the enzymes alkaline phosphatase or peroxidase (for biochemical detection).

The most sensitive amplification methods use an enzyme as a marker molecule attached to the secondary antibody. The enzyme alkaline phosphatase, when in the presence of appropriate chemicals, produces inorganic phosphate that in turn leads to the local formation of a colored precipitate. This reveals the location of the secondary antibody and hence the location of the antibody-antigen complex.

Since each enzyme molecule acts catalytically to generate many thousands of molecules of product, even tiny amounts of antigen can be detected. An enzyme-linked immunosorbent assay (ELISA) based on this principle is frequently used in medicine as a sensitive test for pregnancy or for various types of infections. Although the enzyme amplification makes enzyme-linked methods very sensitive, diffusion of the colored precipitate away from the enzyme limits the spatial resolution of this method for microscopy, and fluorescent labels are usually used for the most precise optical localization.

Antibodies are made by injecting a sample of the antigen several times into an animal such as a rabbit or a goat and then collecting the antibody-rich serum. This *antiserum* contains a heterogeneous mixture of antibodies, each produced by a different antibody-secreting cell (a B lymphocyte). The different antibodies recognize various parts of the antigen molecule (called an antigenic determinant, or **epitope**), as well as impurities in the antigen preparation. Removing the unwanted antibody molecules that bind to other molecules sharpens the specificity of an antiserum for a particular antigen; an antiserum produced against protein X, for example, when passed through an affinity column of antigens X, will bind to these antigens, allowing other antibodies to pass through the column. Purified anti-X antibody can subsequently be eluted from the column. Even so, the heterogeneity of such antisera sometimes limits their usefulness. The use of monoclonal antibodies largely overcomes this problem. However, monoclonal antibodies can also have problems. Since they are single-antibody protein species, they show almost perfect specificity for a single site or epitope on the antigen, but the accessibility of the epitope, and thus the usefulness of the antibody, may depend on the specimen preparation. For example, some monoclonal antibodies will react only with unfixed antigens, others only after the use of particular fixatives, and still others only with proteins denatured on SDS polyacrylamide gels, and not with the proteins in their native conformation.

IMAGE DECTECTORS

In addition to the human eye and photographic film there are two types of electronic detectors employed in modern light microscopes. These are area detectors that form an image directly, for

example **video cameras** and **charge-coupled devices** (CCDs). Alternatively, **point detectors** can be used to measure intensities in the image; for example photomultiplier tubes (PMTs) and photodiodes. Point detectors are capable of producing images in scanning microscopy.

Optical sectioning

For ordinary light microscopy, a tissue has to be sliced into thin sections to be examined; the thinner the section the crisper/finer the image. The process of sectioning loses information about the third dimension. If we have such a loss of the three dimensional features of the specimen, we need to ask two questions:

1. How can we get a picture of the three-dimensional architecture of a cell or tissue?
2. How can we view the microscopic structure of a specimen that, for one reason or another, cannot first be sliced into sections?

The answer to these questions is to produce optical sections using the methods below?

1. By confocal microscopes
2. Multiple photon imaging
3. Deconvolution

Although an optical microscope is focused on a particular focal plane within complex three-dimensional specimens, all the other parts of the specimen, above and below the plane of focus, are also illuminated and the light originating from these regions contributes to the image as "out-of-focus" blur. Since the conventional epifluorescence microscope collects all of the information from the specimen, it is often referred to as a wide-field microscope. This can make it very hard to interpret the image in detail and can lead to fine image structure being obscured by the out-of-focus light. The 'out-of-focus blur' can be removed using a variety of **optical electronic techniques** to produce optical sections.

Two distinct but complementary approaches solve this problem: one is computational, the other is optical, both of which are three-dimensional microscopic imaging methods.

These methods make it possible to focus on a chosen plane in a thick specimen while rejecting the light that comes from out-of-focus regions above and below that plane. Thus one sees a crisp, thin *optical section.* The term 'optical section' refers to a microscope's ability to produce sharper images of specimens than those produced using a standard wide-field epifluorescence microscope by removing the contribution from out-of-focus light to the image and, in most cases, without resorting to physical sectioning of the tissue. From **a series of such optical sections taken at different depths** and stored in a computer, it is easy to reconstruct a three-dimensional image. The methods perform the same task for the microscopist as what the 'CT' scanner does for the radiologist investigating a human body: both machines give detailed sectional views of the interior of an intact structure. Such methods have revolutionized the ability to collect images from thick and fluorescently labeled specimens such as eggs, embryos and tissues.

The computational approach is often called **image deconvolution**.
Deconvolution is an image-processing method which removes the out-of-focus information from the digital image. Such images are computed from conventional wide-field microscope images. There are two basic types of deconvolution algorithm: **deblurring** and **restoration**. The approach relies upon knowledge of the point spread function of the imaging system.
To understand how it works, remember that the wavelike nature of light means that the microscope lens system produces a small blurred disc as the image of a point light source, with increased blurring if the point source lies above or below the focal plane. This blurred image of a point source is called the *point spread* function. An image of a complex object can then be thought of as being built up by replacing each point of the specimen by a corresponding blurred disc, resulting in an image that is blurred overall. Since it is assumed that the real image of the bead should be a point, it is possible to calculate the amount of distortion in the image of the bead imposed by the imaging system.

For deconvolution, we first obtain a series of (blurred) images, usually with a CCD camera, focusing the microscope in turn on a series of focal planes - in effect, a (blurred) three-dimensional image. The stack of digital images is then processed by computer to remove as much of the blur as possible and thus restore the actual image of the point by using a mathematical function, which can be applied to any subsequent images collected under identical settings of the microscope. The computation required is quite complex and used to be a serious limitation.

Deconvolution is now much faster using today's fast computers and improved software, and the method compares favorably with the confocal approach for producing optical sections. Deconvolution is practical for multiple label imaging of both fixed and living cells, and excels over the scanning methods for imaging relatively dim and thin specimens, for example yeast cells. Fig. 14.6 shows an example.

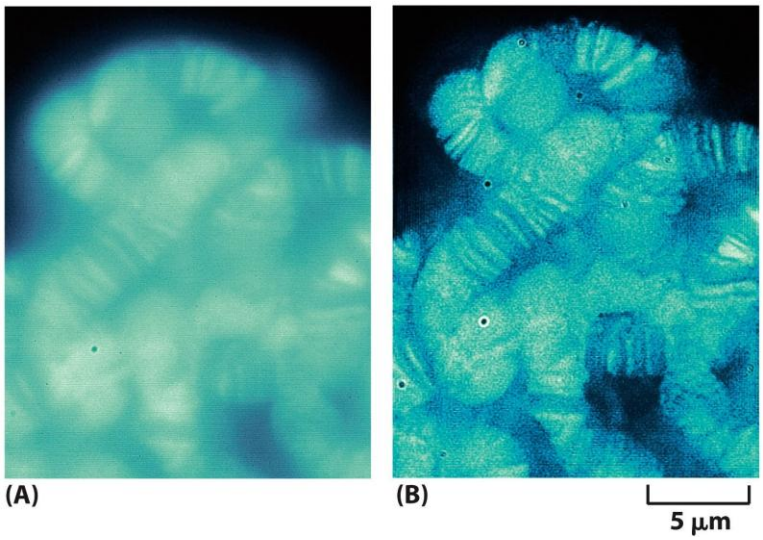


Fig. 14.6. Image deconvolution (A) a light micrograph of a polytene chromosomes from *Drosophila* stained with a fluorescent DNA-binding dye. (B) The same field of view after deconvolution clearly reveals the banding pattern on the chromosomes. Each band is about 0.25µm thick approaching the resolution limit of the light microscope. (Source: John Sedat Laboratory)

Laser scanning confocal microscopes

The confocal microscope produces optical sections by excluding out-of-focus light and achieves a result similar to that of deconvolution, but does so by manipulating the light before it is

measured; thus it is an analog technique rather than a digital one. The optical details of the confocal microscope are complex, but the basic idea is simple, as illustrated in Fig. 14.7.

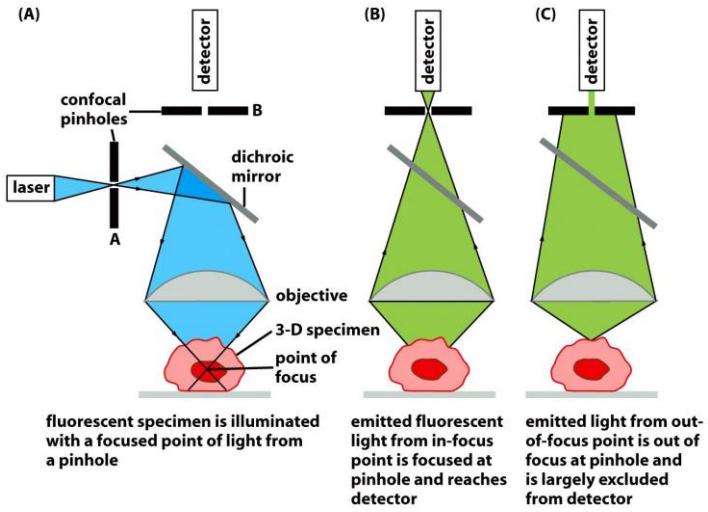


Fig. 14.7. The confocal fluorescence microscope. The simplified diagram shows that the basic arrangement of optical components is similar to that of the standard fluorescence microscope, except that a laser is used to illuminate a small pinhole whose image is focused at a single point on the specimen. (A) Emitted fluorescence from this focal point in the specimen is significantly focused at a second (confocal) pinhole. (B) Emitted light from elsewhere in the specimen is not focused at the pinhole and therefore does not contribute to the final image. (C) By scanning the beam of light across the specimen, a very sharp two-dimensional image of the exact plane of focus is built up that is not degraded by light from other regions of the specimen.

The image produced by the confocal microscope is far superior to those obtained by conventional light microscopy (Fig. 14.8).

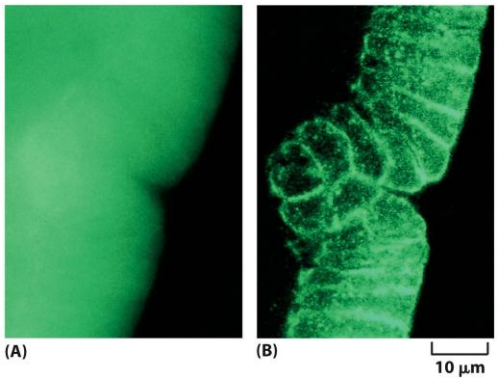


Fig. 14.8. Conventional and confocal fluorescence microscopy compared. The images are showing the gastrula-stage *Drosophila* embryo that has been stained with a fluorescent probe for actin filaments. (A) Conventional mode. (B) Confocal mode.

The microscope is generally used with fluorescence optics (check previous lecture) but instead of illuminating the whole specimen at once in the usual way, the optical system at any instant focuses a spot of light onto a single point at a specific depth in the specimen. It requires a very bright source of pinpoint illumination that is usually supplied by a laser whose light has been passed through a pinhole.

The fluorescence emitted from the illuminated material is collected and brought to an image at a suitable light detector. A pinhole aperture is placed in front of the detector, at a position that is *confocal* (having same focus) with the illuminating pinhole - that is, precisely where the rays emitted from the illuminated point in the specimen come to a focus. Thus, the light from this point in the specimen converges on this aperture and enters the detector.

By contrast, the light from regions out of the plane of focus of the spotlight is also out of focus at the pinhole aperture and is therefore largely excluded from the detector. To build up a two-dimensional image, data from each point in the plane of focus are collected sequentially by scanning across the field in a raster pattern (a pattern of horizontal scanning lines as on a television screen) and are displayed on a video screen. Scanning is usually done by deflecting the beam with an oscillating mirror placed between the dichroic (appearing to change color when viewed at different axis) mirror and the objective lens.

The confocal microscope has been used to resolve the structure of numerous complex three-dimensional objects, including the networks of cytoskeletal fibers in the cytoplasm and the arrangement of chromosomes and genes in the nucleus.

Comparison of deconvolution methods and confocal microscopy for three-dimensional optical microscopy

1. Confocal microscopes are generally easier to use than deconvolution systems and the final optical sections can be seen quickly.
2. In contrast, the CCD (charge-coupled device) cameras used for deconvolution systems are extremely efficient at collecting small amounts of light, and they can be used to make detailed

three-dimensional images from specimens that are too weakly stained or too easily damaged by the bright light used for confocal microscopy.

3. Neither method is good at coping with thick specimens.
4. Deconvolution methods quickly become ineffective any deeper than about 40 µm into a specimen, while confocal microscopes can only obtain images up to a depth of about 150 µm.

Molecules can be labeled with radioisotopes

In cell biology it is often important to determine the quantities of specific molecules and to know where they are in the cell and how their level or location changes in response to extracellular signals. Molecules of interest in the cell range from small inorganic ions, such as Ca^{2+} or H^{+} to large macromolecules, such as specific proteins, RNAs, or DNA sequences. Radioisotopes are used to trace the path of specific molecules through the cell.

In radioactive isotopes, the nucleus is unstable and undergoes random disintegration to produce a different atom. In the course of these disintegrations, either energetic subatomic particles, such as **electrons** (β particle), or radiations, such as gamma-rays (γ rays), are given off.

We can use chemical synthesis to incorporate one or more radioactive atoms into a small molecule of interest, such as a sugar or an amino acid, and the fate of that molecule (and of specific atoms in it) can be traced during any biological reaction.

Although naturally occurring radioisotopes are rare (because of their instability) they can be produced in large amounts in nuclear reactors, where stable atoms are bombarded with high-energy particles. As a result, radioisotopes of many biologically important elements are readily available (Table 14.1).

Table 14.1. Some radioisotopes in common use in biological research

Isotope	Half-life
^{32}P	14 days
^{131}I	8.1 days

^{35}S	87 days
^{14}C	5570 years
^{45}Ca	164 days
^{3}H	12.3 years

The isotopes are arranged in decreasing order of energy of the β radiation (energy) they emit. ^{131}I also emits γ radiation. The half-life is the time required for 50 % of the atoms of an isotope to disintegrate.

The radiation they emit is detected in various ways. Beta particles can be detected in a Geiger counter by the ionization they produce in a gas, or they can be measured in a scintillation counter by the small flashes of light they induce in a scintillation fluid. These methods make it possible to measure accurately the quantity of a particular radioisotope present in a biological specimen. Using either light or electron microscopy, it is also possible to determine the location of a radioisotope in a specimen by **autoradiography**, as we describe below. All of these methods of detection are extremely sensitive: in favorable circumstances, nearly every disintegration, and therefore every radioactive atom that decays can be detected.

Radioisotopes are used to trace molecules in cells and organisms

One of the earliest uses of radioactivity in biology was to trace the chemical pathway of carbon during photosynthesis. Unicellular green algae were maintained in an atmosphere containing radioactively labeled CO_2 ($^{14}CO_2$), and at various times after they had been exposed to sunlight, their soluble contents were separated by paper chromatography. Small molecules containing ^{l4}C atoms derived from CO_2 were detected by a sheet of photographic film placed over the dried paper chromatogram. In this way most of the principal components in the photosynthetic pathway from CO_2 to sugar were identified.

Radioactive molecules can be used to follow the course of almost any process in cells. In a typical experiment the cells are supplied with a precursor molecule in radioactive form. The

radioactive molecules mix with the preexisting unlabeled ones; both are treated identically by the cell as they differ only in the weight of their atomic nuclei. Changes in the location or chemical form of the radioactive molecule can be followed as a function of time. The resolution of such experiments is often sharpened by using a **pulse-chase labeling protocol**, in which the radioactive material (the pulse) is added for only a very brief period and then washed away and replaced by nonradioactive molecules (the chase).

Samples are taken at regular intervals, and the chemical form or location of the radioactivity is identified for each sample. Pulse-chase experiments, combined with autoradiography, have been important in elucidating the pathway taken by secreted proteins from the ER to the cell exterior.

Radioisotope labeling is a uniquely valuable way of distinguishing between molecules that are chemically identical but have different histories, such as those that differ in their time of synthesis. In this way, for example, it was shown that almost all of the molecules in a living cell are continually being degraded and replaced, even when the cell is not growing and is apparently in a steady state. This "turnover," which sometimes takes place very slowly, would be almost impossible to detect without radioisotopes.

Today, nearly all common small molecules are available in radioactive form from commercial sources, and virtually any biological molecule, no matter how complicated, can be radioactively labeled. Compounds can be made with radioactive atoms incorporated at particular positions in their structure, enabling the separate fates of different parts of the same molecule to be followed during biological reactions.

As mentioned previously, one of the important uses of radioactivity in cell biology is to localize a radioactive compound in sections of whole cells or tissues by autoradiography. In this procedure, living cells are briefly exposed to a pulse of a specific radioactive compound and then incubated for a variable period to allow them time to incorporate the compound before being fixed and processed for light or electron microscopy. Each preparation is then overlaid with a thin film of photographic emulsion and left in the dark for several days, during which the

radioisotope decays. The emulsion is then developed, and the position of the radioactivity in each cell is indicated by the position of the developed silver grains. If cells are exposed to ^{3}H-thymidine, a radioactive precursor of DNA, for example, it can be shown that DNA is made in the nucleus and remains there (Fig. 14.9).

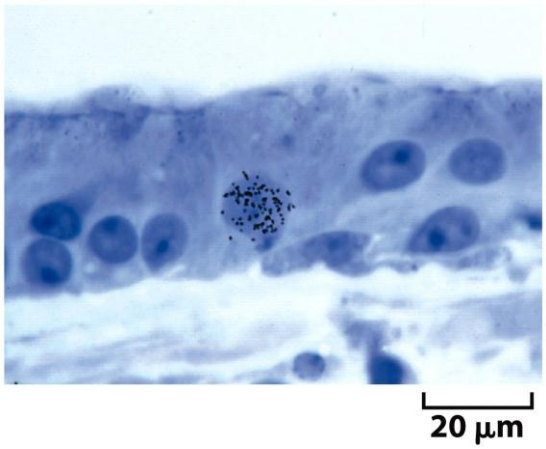


Fig. 14.9. Autoradiography. This tissue has been exposed for a short time to ^{3}H-thymidine. Cells that are replicating their DNA incorporate this labeled DNA precursor into their nuclei. The silver grains on the photographic film are seen as black dots.

By contrast, if cells are exposed to ^{3}H-uridine, a radioactive precursor of RNA, it is found that RNA is initially made in the nucleus and then moves rapidly into the cytoplasm. Radiolabeled molecules can also be detected by autoradiography after they are separated from other molecules by gel electrophoresis: the positions of both proteins and nucleic acids are commonly detected on gels in this way.

Summary

Many light-microscope techniques are available for observing cells. Cells that have been fixed and stained can be studied in a conventional light microscope, whereas antibodies coupled to fluorescent dyes can be used to locate specific molecules in cells in a fluorescence microscope. Living cells can be seen with phase-contrast, differential-interference-contrast, dark-field, or bright-field microscopes. All forms of light microscopy are facilitated by digital image-processing techniques, which enhance sensitivity and refine the image. Confocal microscopy and image deconvolution both provide thin optical sections and can be used to reconstruct three dimensional images.

Techniques are now available for detecting, measuring, and following almost any desired molecule in a living cell. Fluorescent indicator dyes can be introduced to measure the concentrations of specific ions in individual cells or in different parts of a cell. Fluorescent proteins are especially versatile probes that can be attached to other proteins by genetic manipulation. Virtually any protein of interest can be genetically engineered as a fluorescent-fusion protein, and then imaged in living cells by fluorescence microscopy. The dynamic behavior and interactions of many molecules can now be followed in living cells by variations on the use of fluorescent protein tags, in some cases at the level of single molecules. Radioactive isotopes of various elements can also be used to follow the fate of specific molecules both biochemically and microscopically.

THE STEREOMICROSCOPE

A second type of light microscope, the stereomicroscope is used for the observation of the surfaces of large specimens (Fig. 14.10). The microscope is used when 3D information is required, for example for the observation of whole organisms or tissue culture samples (Fig. 14.11). Stereomicroscopes are useful for micromanipulation and dissection where the wide field of view and the ability to zoom in and out in magnification is invaluable. A wide range of objectives and eyepieces are available for different applications. The light sources can be from above, from below the specimen, encircling the specimen using a ring light or from the side, giving a dark-field effect. These different light angles serve to add contrast or shadow relief to the images. Fluorescent stereomicroscopes are also available and are used for screening transgenic animals labeled with GFP and its variants.

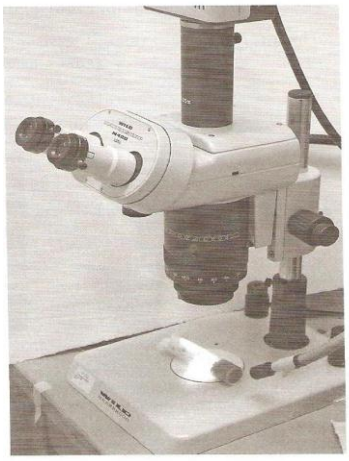


Fig. 14.10. A research grade stereomicroscope

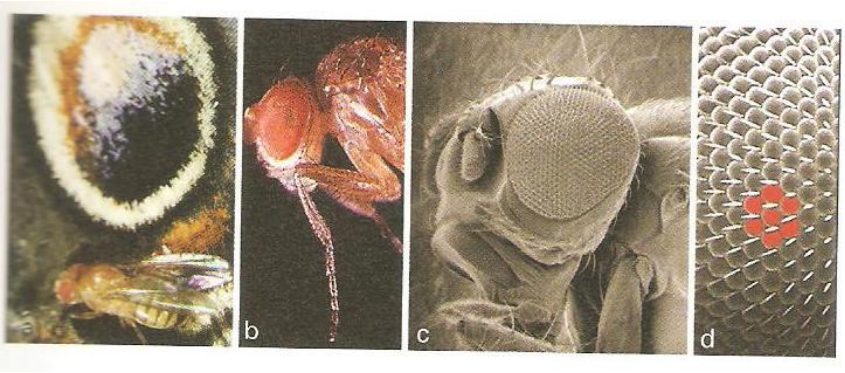


Fig. 14.11. Image surfaces using the stereomicroscope and the scanning electron microscope.

Preparation of specimens for electrom microscope

Unlike the light microscope, new procedures are needed for embedding, cutting, and staining tissues.

Since the specimen is exposed to a very high vacuum in the electron microscope, living tissue is usually killed and preserved by fixation, first with **glutaraldehyde**, which covalently cross-links protein molecules to their neighbors, and then with **osmium tetroxide**, which binds to and stabilizes lipid bilayers as well as proteins (Fig. 14.12).

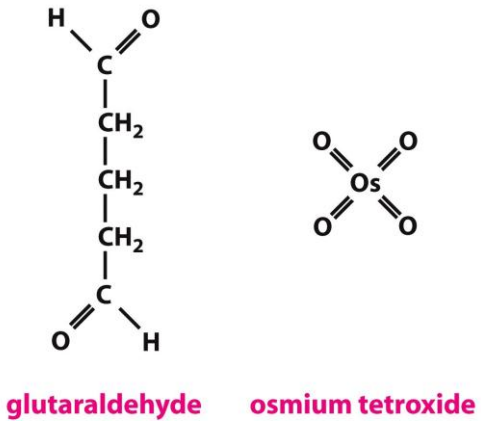


Fig. 14.12. Two common chemical fixatives used for EM.

Because electrons have very limited penetrating power, the fixed tissues normally have to be cut into extremely thin sections 50-100 nm thick, about 1/200 times the thickness of a single cell, before they are viewed. This is achieved by dehydrating the specimen and permeating it with a monomeric resin that polymerizes to form a solid block of plastic; the block is then cut with a fine glass or diamond knife on a special microtome. These thin sections, free of water and other volatile solvents, are placed on a small circular metal grid for viewing in the microscope (Fig. 14.13).

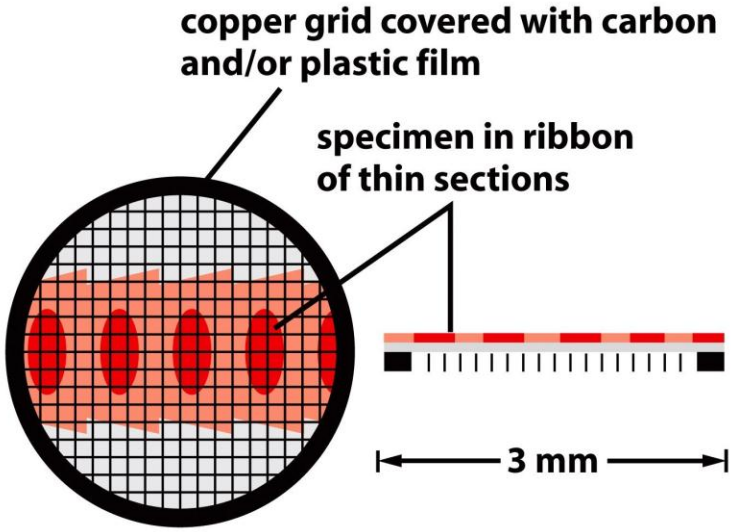


Fig. 14.13. The copper grid that supports the thin sections of a specimen for EM.

Contrast in the electron microscope depends on the atomic number of the atoms in the specimen: the higher the atomic number, the more electrons are scattered and the greater the contrast. Biological tissues are composed of atoms of very low atomic number (mainly carbon, oxygen, nitrogen, and hydrogen). To make them visible, they are usually impregnated (before or after sectioning) with the salts of heavy metals such as uranium, osmium, and lead. The degree of impregnation, or "staining," with these salts reveals different cellular constituents with various degrees of contrast. Lipids, for example, tend to stain darkly after osmium fixation, revealing the location of cell membranes.

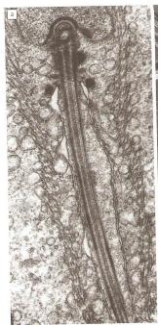


Fig. 14.14. Transmission electron microscopy. A thin section of developing sperm cells stained with uranyl acetate and lead citrate.
Source: Wilson and Walker, 2006

All of the water has to be removed from any biological specimen before it can be imaged in the EM. This is because the electron beam can be produced and focused only in vacuum. The major drawback of EM observation of biological specimens therefore is the non-physiological conditions necessary for their observation. Nevertheless, the improved resolution afforded by the EM has provided much information about biological structures and biochemical events within cells that could otherwise not have been collected using any other microscopical technique.

Extensive specimen preparation is required for EM analysis, and for this reason there can be issues of interpreting the images because of artefacts from specimen preparation. For example, specimens have been traditionally prepared for the TEM by fixation in glutaraldehyde to cross-link proteins, followed by osmium tetroxide to fix and stain lipid membranes. This is followed by dehydration in a series of alcohols to remove the water, and then embedding in a plastic such as Epon for thin sectioning.

Small pieces of the embedded tissue are mounted and sectioned on an ultramicrotome using either glass or a diamond knife. Ultrathin sections are cut to a thickness of approximately 60 nm. The ribbons of sections are floated onto the surface of water and their interference colors are used to assess their thickness. The desired 60 nm section thickness has a silver/gold interference colour on the water surface. The sections are then mounted onto copper or gold EM grids, and are subsequently stained with heavy metals, for example uranyl acetate and lead citrate.

For the SEM, samples are fixed in glutaraldehyde, dehydrated through a series of solvents and dried completely either in air or by critical point drying. This method removes all of the water from the specimen instantly and avoids surface tension in the drying process, thereby avoiding artefacts of drying. The specimens are then mounted onto a special metal holder or stub and coated with a thin layer of gold before being viewed in the SEM. Surfaces can also be viewed in the TEM using either negative stains or carbon replicas of air-dried specimens.

Recent developments in EM methods

New methods of fixation which prevent development of artefacts of specimen preparation and to observe the specimen more closely to its living state are now available. These include rapid (milliseconds) high pressure freezing which preserves the biochemical state of the cell . Many of these frozen samples can be observed directly in the EM or they can be chemically fixed using freeze substitution methods. Here, fixatives are infused into the preparation at low temperature, after which the specimen is slowly warmed to room temperature.

Using cryo-electron tomography (Cryo-ET) the 3D structure of cells and macromolecules can be visualized at 5-8 nm resolution. Cells are frozen and mounted in an apparatus that moves the specimen through a range of tilt angles. A 2D digital EM image is collected at each one of these tilt angles, and, using computer software, a 3D representation of the specimen can be constructed.