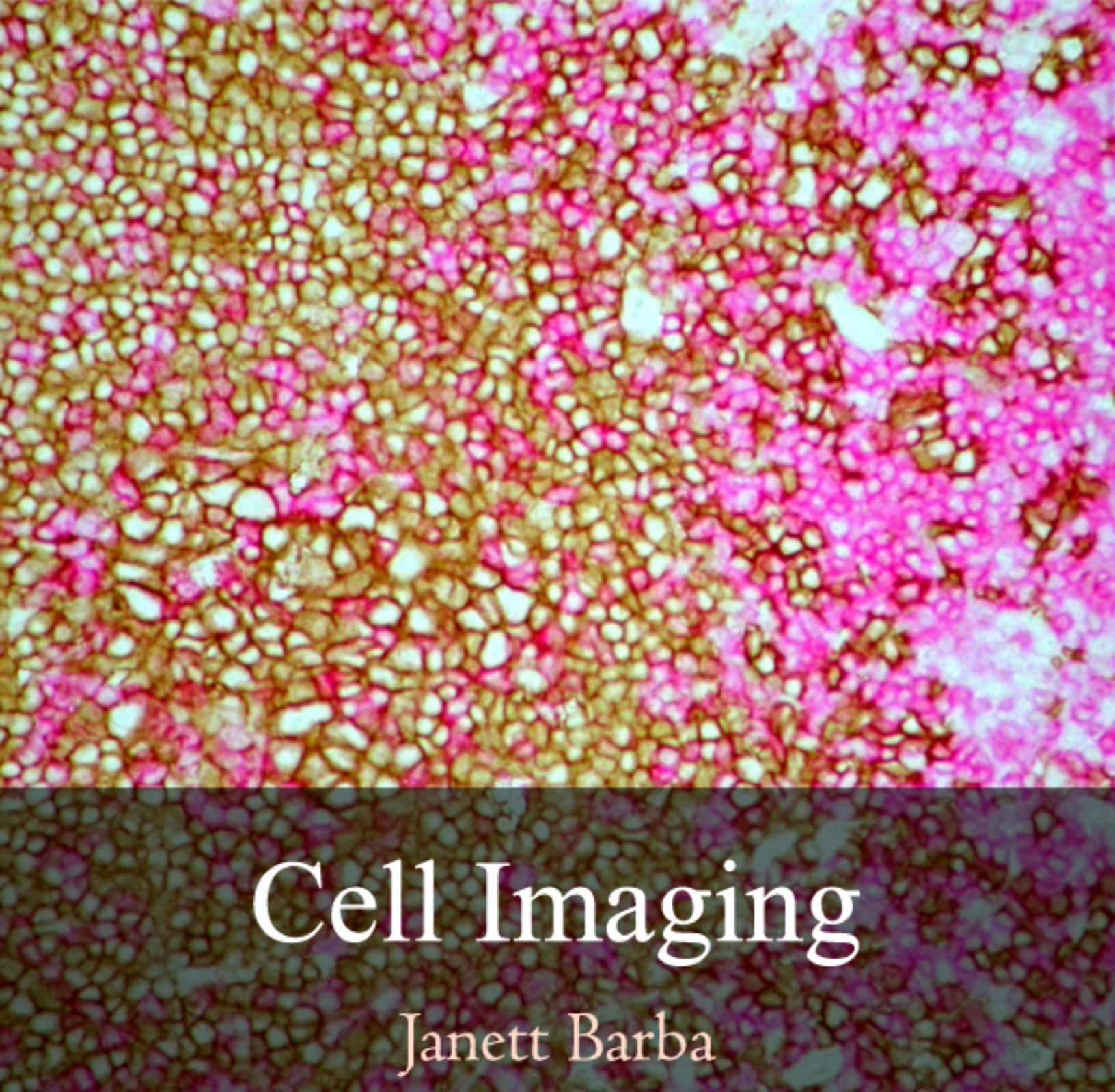

Cell Imaging
Janett Barba

First Edition, 2012

ISBN 978-81-323-4100-0

Published by:
White Word Publications
4735/22 Prakashdeep Bldg,
Ansari Road, Darya Ganj,
Delhi - 110002
Email: info@wtbooks.com

Table of Contents

Chapter 1

Staining

A stained histologic specimen, sandwiched between a glass microscope slide and coverslip, mounted on the stage of a light microscope.

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for

example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells.

In biochemistry it involves adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. Staining and fluorescent tagging can serve similar purposes. Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis.

Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers.

In vivo *vs* In vitro

In vivo staining is the process of dyeing living tissues—*in vivo* means "in life" (compare with *in vitro* staining). By causing certain cells or structures to take on contrasting colour(s), their form (morphology) or position within a cell or tissue can be readily seen and studied. The usual purpose is to reveal cytological details that might otherwise not be apparent; however, staining can also reveal where certain chemicals or specific chemical reactions are taking place within cells or tissues.

In vitro staining involves colouring cells or structures that are no longer living. Certain stains are often combined to reveal more details and features than a single stain alone. Combined with specific protocols for fixation and sample preparation, scientists and physicians can use these standard techniques as consistent, repeatable diagnostic tools. A counterstain is stain that makes cells or structures more visible, when not completely visible with the principal stain.

- For example, crystal violet stains only Gram-positive bacteria in Gram staining. A safranin counterstain is applied which stains all cells, allowing the identification of Gram-negative bacteria as well.

Often these stains are called vital stains. They are introduced to the organism while the cells are still living. However, these stains are eventually toxic to the organism, some more so than others. To achieve desired effects, the stains are used in very dilute solutions ranging from 1:5000 to 1:500000 (Howey, 2000). Note that many stains may be used in both living and fixed cells.

In vitro methods

Preparation

The preparatory steps involved depend on the type of analysis planned; some or all of the following procedures may be required.

Fixation–which may itself consist of several steps–aims to preserve the shape of the cells or tissue involved as much as possible. Sometimes heat fixation is used to kill, adhere, and alter the specimen so it will accept stains. Most chemical fixatives (chemicals causing fixation) generate chemical bonds between proteins and other substances within the sample, increasing their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid. Pieces of tissue may be embedded in paraffin wax to increase their mechanical strength and stability and to make them easier to cut into thin slices.

Permeabilization involves treatment of cells with (usually) a mild surfactant. This treatment will dissolve the cell membranes, and allow larger dye molecules access to the cell's interior.

Mounting usually involves attaching the samples to a glass microscope slide for observation and analysis. In some cases, cells may be grown directly on a slide. For samples of loose cells (as with a blood smear or a pap smear) the sample can be directly applied to a slide. For larger pieces of tissue, thin sections (slices) are made using a microtome; these slices can then be mounted and inspected.

Staining proper

At its simplest, the actual staining process may involve immersing the sample (before or after fixation and mounting) in dye solution, followed by rinsing and observation. Many dyes, however, require the use of a mordant: a chemical compound which reacts with the stain to form an insoluble, coloured precipitate. When excess dye solution is washed away, the mordanted stain remains.

Most of the dyes commonly used in microscopy are available as **certified stains**. This means that samples of the manufacturer's batch have been tested by an independent body, the Biological Stain Commission and found to be meet or exceed certain standards of purity, dye content and performance in staining techniques. These standards are published in detail in the journal Biotechnic & Histochemistry Many dyes are inconsistent in composition from one supplier to another. The use of certified stains eliminates a source of unexpected results.

Negative staining

A simple staining method for bacteria which is usually successful even when the "positive staining" methods detailed below fail, is to employ a negative stain. This can be achieved simply by smearing the sample on to the slide, followed by an application of nigrosin (a black synthetic dye) or Indian ink (an aqueous suspension of carbon particles). After drying, the microorganisms may be viewed in bright field microscopy as lighter inclusions well-contrasted against the dark environment surrounding them. Note: negative staining is a mild technique which may not destroy the microorganisms therefore it is unsuitable for studying pathogens.

Specific techniques

Gram staining

Gram staining is used to determine gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin counterstain to mark all bacteria. Gram status is important in medicine; the presence or absence of a cell wall will change the bacterium's susceptibility to some antibiotics.

Gram-positive bacteria stain dark blue or violet. Their cell wall is typically rich with peptidoglycan and lacks the secondary membrane and lipopolysaccharide layer found in Gram-negative bacteria.

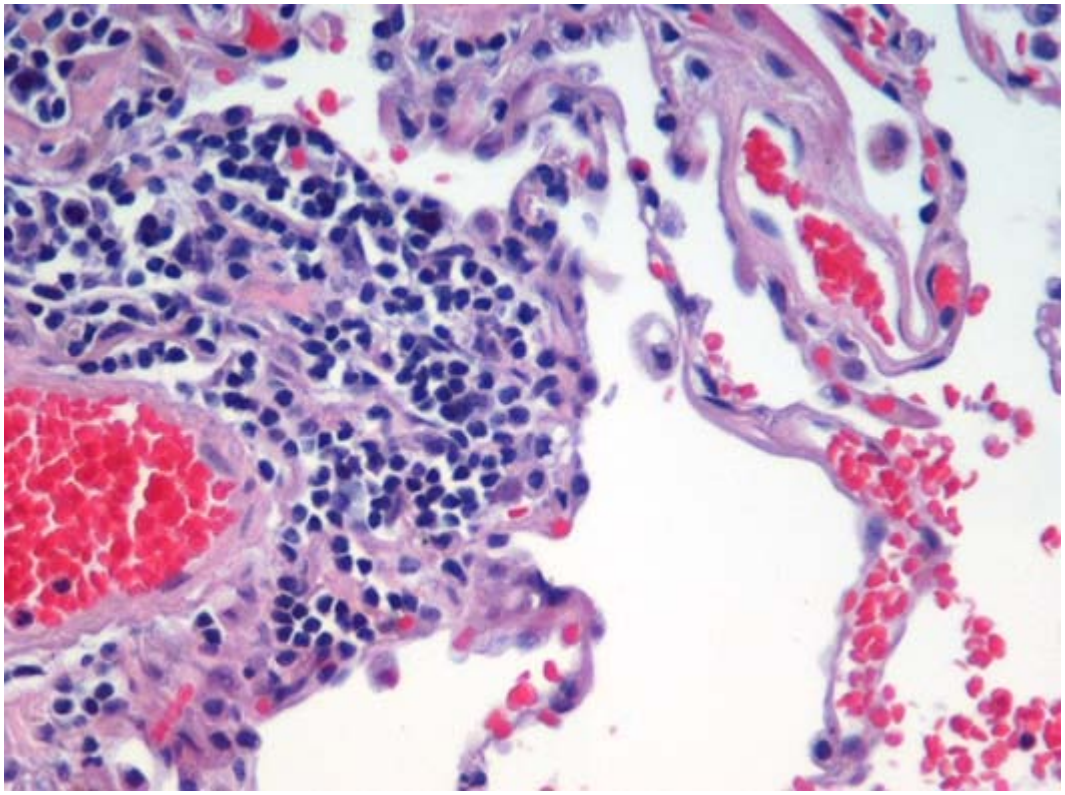
On most Gram-stained preparations, Gram-negative organisms will appear red or pink because they are counterstained. Due to presence of higher lipid content, after alcohol-treatment, the porosity of the cell wall increases, hence the CVI complex (Crystal violet - Iodine) can pass through. Thus, the primary stain is not retained. Also, in contrast to most Gram-positive bacteria, Gram-negative bacteria have only a few layers of peptidoglycan and a secondary cell membrane made primarily of lipopolysaccharide.

Ziehl-Neelsen stain

Ziehl-Neelsen staining is used to stain species of *Mycobacterium tuberculosis* that do not stain with the standard laboratory staining procedures like Gram staining.

The stains used are the red coloured Carbol fuchsin that stains the bacteria and a counter stain like Methylene blue or Malachite green.

Haematoxylin and eosin (H&E) staining



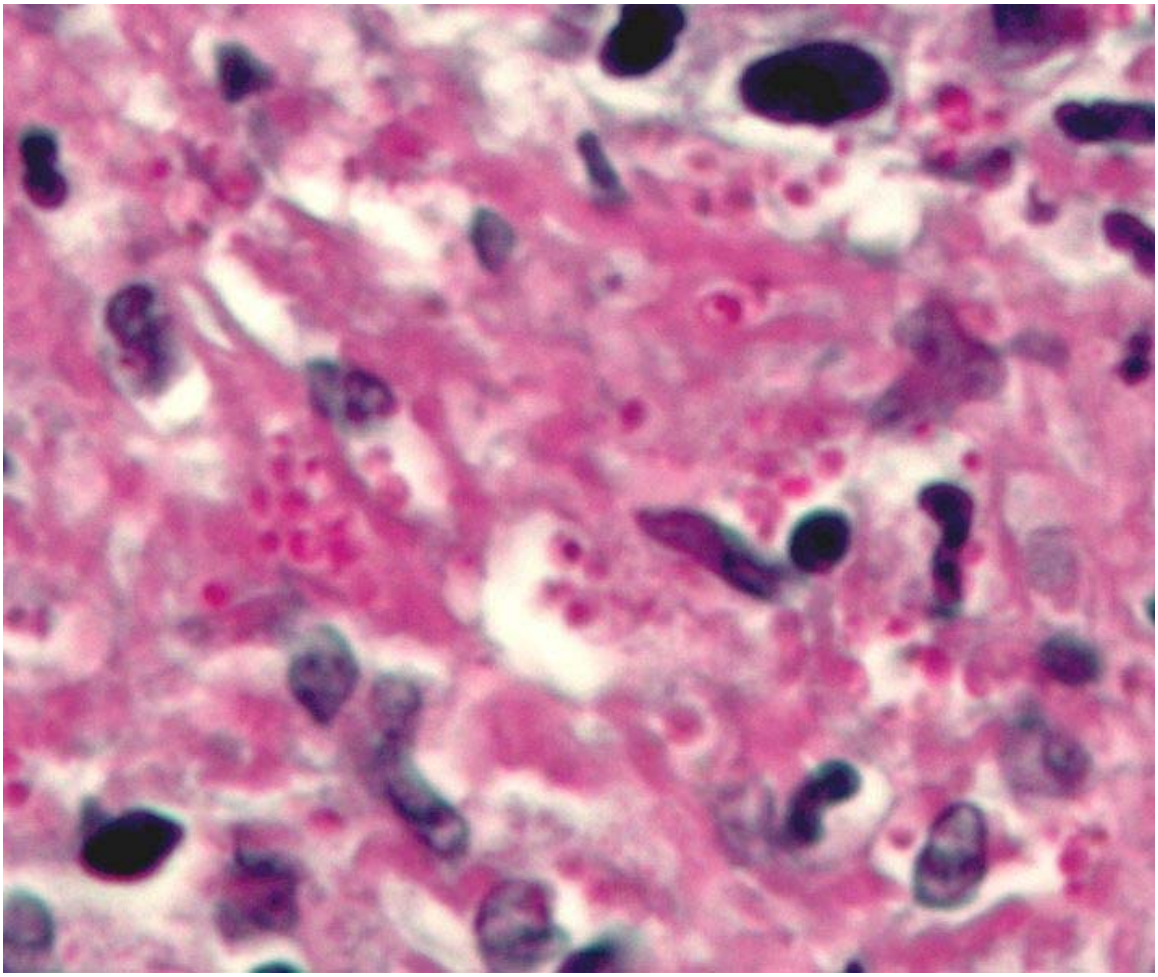
Microscopic view of a histologic specimen of human lung tissue stained with hematoxylin and eosin.

Haematoxylin and eosin staining protocol is used frequently in histology to examine thin sections of tissue. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, colouring them bright red. In a skilfully made H & E preparation the red blood cells are almost orange, and collagen and cytoplasm (especially muscle) acquire different shades of pink. When the staining is done by a machine, the subtle differences in eosinophilia are often lost.

Papanicolaou staining

Papanicolaou staining, or Pap staining, is a frequently used method for examining cell samples from various bodily secretions. It is frequently used to stain Pap smear specimens. It uses a combination of haematoxylin, Orange G, eosin Y, Light Green SF yellowish, and sometimes Bismarck Brown Y.

PAS staining



PAS diastase showing the fungus Histoplasma.

Periodic acid-Schiff staining is used to mark carbohydrates (glycogen, glycoprotein, proteoglycans). It is used to distinguish different types of glycogen storage diseases.

Masson's trichrome

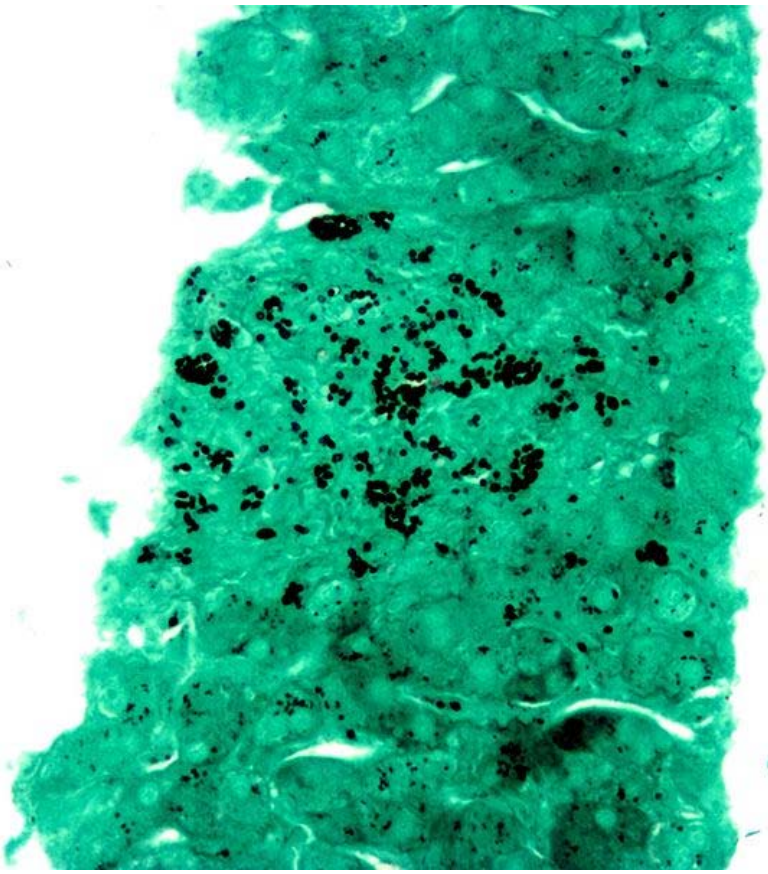
Masson's trichrome is (as the name implies) a three-colour staining protocol. The recipe has evolved from Masson's original technique for different specific applications, but all are well-suited to distinguish cells from surrounding connective tissue. Most recipes will produce red keratin and muscle fibers, blue or green staining of collagen and bone, light red or pink staining of cytoplasm, and black cell nuclei.

Romanowsky stains

The Romanowsky stains are all based on a combination of eosinate (chemically reduced eosin) and methylene blue (sometimes with its oxidation products azure A and azure B). Common variants include Wright's stain, Jenner's stain, Leishman stain and Giemsa stain.

All are used to examine blood or bone marrow samples. They are preferred over H&E for inspection of blood cells because different types of leukocytes (white blood cells) can be readily distinguished. All are also suited to examination of blood to detect blood-borne parasites like malaria.

Silver staining



Gömöri methenamine silver stain demonstrating histoplasma (black round balls).

Silver staining is the use of silver to stain histologic sections. This kind of staining is important especially to show proteins (for example type III collagen) and DNA. It is used to show both substances inside and outside cells. Silver staining is also used in temperature gradient gel electrophoresis.

Some cells are *argentaffin*. These reduce silver solution to metallic silver after formalin fixation. This method was discovered by Italian Camillo Golgi, by using a reaction

between silver nitrate and potassium dichromate, thus precipitating silver chromate in some cells. Other cells are *argyrophilic*. These reduce silver solution to metallic silver after being exposed to the stain that contains a reductant, for example hydroquinone or formalin.

Sudan staining

Sudan staining is the use of Sudan dyes to stain sudanophilic substances, usually lipids. Sudan III, Sudan IV, Oil Red O, and Sudan Black B are often used. Sudan staining is often used to determine the level of fecal fat to diagnose steatorrhea.

Conklin's staining

Special technique designed for staining true endospores with the use of malachite green dye, once stained, they do not decolourize.

Common biological stains

Different stains react or concentrate in different parts of a cell or tissue, and these properties are used to advantage to reveal specific parts or areas. Some of the most common biological stains are listed below. Unless otherwise marked, all of these dyes may be used with fixed cells and tissues; vital dyes (suitable for use with living organisms) are noted.

Acridine orange

Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, it is very similar spectrally to fluorescein. Like fluorescein, it is also useful as a non-specific stain for backlighting conventionally stained cells on the surface of a solid sample of tissue (fluorescence backlighted staining).

Bismarck brown

Bismarck brown (also Bismarck brown Y or Manchester brown) imparts a yellow colour to acid mucins. Bismarck brown may be used with live cells.

Carmine

Carmine is an intensely red dye which may be used to stain glycogen, while Carmine alum is a nuclear stain. Carmine stains require the use of a mordant, usually aluminum.

Coomassie blue

Coomassie blue (also brilliant blue) nonspecifically stains proteins a strong blue colour. It is often used in gel electrophoresis.

Crystal violet

Crystal violet, when combined with a suitable mordant, stains cell walls purple. Crystal violet is an important component in Gram staining.

DAPI

DAPI is a fluorescent nuclear stain, excited by ultraviolet light and showing strong blue fluorescence when bound to DNA. DAPI binds with A=T rich repeats of chromosomes. DAPI is also not visible with regular transmission microscopy. It may be used in living or fixed cells. DAPI-stained cells are especially appropriate for cell counting.

Eosin

Eosin is most often used as a counterstain to haematoxylin, imparting a pink or red colour to cytoplasmic material, cell membranes, and some extracellular structures. It also imparts a strong red colour to red blood cells. Eosin may also be used as a counterstain in some variants of Gram staining, and in many other protocols. There are actually two very closely related compounds commonly referred to as eosin. Most often used is eosin Y (also known as eosin Y ws or eosin yellowish); it has a very slightly yellowish cast. The other eosin compound is eosin B (eosin bluish or imperial red); it has a very faint bluish cast. The two dyes are interchangeable, and the use of one or the other is more a matter of preference and tradition.

Ethidium bromide

Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis - such cells have much more permeable membranes. Consequently, ethidium bromide is often used as a marker for apoptosis in cells populations and to locate bands of DNA in gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence.

Acid fuchsine

Acid fuchsine may be used to stain collagen, smooth muscle, or mitochondria. Acid fuchsine is used as the nuclear and cytoplasmic stain in Mallory's trichrome method. Acid fuchsine stains cytoplasm in some variants of Masson's trichrome. In Van Gieson's picro-

fuchsine, acid fuchsine imparts its red colour to collagen fibres. Acid fuchsine is also a traditional stain for mitochondria (Altmann's method).

Haematoxylin

Haematoxylin (hematoxylin in North America) is a nuclear stain. Used with a mordant, haematoxylin stains nuclei blue-violet or brown. It is most often used with eosin in H&E (haematoxylin and eosin) staining—one of the most common procedures in histology.

Hoechst stains

Hoechst is a *bis*-benzimidazole derivative compound which binds to the *minor groove* of DNA. Often used in fluorescence microscopy for DNA staining, Hoechst stains appear yellow when dissolved in aqueous solutions and emit blue light under UV excitation. There are two major types of Hoechst: *Hoechst 33258* and *Hoechst 33342*. The two compounds are functionally similar, but with a little difference in structure. Hoechst 33258 contains a terminal hydroxyl group and is thus more soluble in aqueous solution, however this characteristics reduces its ability to penetrate the plasma membrane. Hoechst 33342 contains a ethyl substitution on the terminal hydroxyl group (i.e. an ethylether group) making it more hydrophobic for easier plasma membrane passage

Iodine

Iodine is used in chemistry as an indicator for starch. When starch is mixed with iodine in solution, an intensely dark blue colour develops, representing a starch/iodine complex. Starch is a substance common to most plant cells and so a weak iodine solution will stain starch present in the cells. Iodine is one component in the staining technique known as Gram staining, used in microbiology. Lugol's solution or Lugol's iodine (IKI) is a brown solution that turns black in the presence of starches and can be used as a cell stain, making the cell nuclei more visible. Iodine is also used as a mordant in Gram's staining, it enhances dye to enter through the pore present in the cell wall/membrane.

Malachite green

Malachite green (also known as diamond green B or victoria green B) can be used as a blue-green counterstain to safranin in the Gimenez staining technique for bacteria. It also can be used to directly stain spores.

Methyl green

Methyl green is used commonly with bright-field microscopes to dye the chromatin of cells so that they are more easily viewed.

Methylene blue

Methylene blue is used to stain animal cells, such as human cheek cells, to make their nuclei more observable. Also used to staining the blood film and used in cytology.

Neutral red

Neutral red (or toluylene red) stains Nissl substance red. It is usually used as a counterstain in combination with other dyes.

Nile blue

Nile blue (or Nile blue A) stains nuclei blue. It may be used with living cells.

Nile red

Nile red (also known as Nile blue oxazone) is formed by boiling Nile blue with sulfuric acid. This produces a mix of Nile red and Nile blue. Nile red is a lipophilic stain; it will accumulate in lipid globules inside cells, staining them red. Nile red can be used with living cells. It fluoresces strongly when partitioned into lipids, but practically not at all in aqueous solution.

Osmium tetroxide (formal name: osmium tetraoxide)

Osmium tetraoxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance.

Rhodamine

Rhodamine is a protein specific fluorescent stain commonly used in fluorescence microscopy.

Safranin

Safranin (or Safranin O) is a nuclear stain. It produces red nuclei, and is used primarily as a counterstain. Safranin may also be used to give a yellow colour to collagen.

Stainability of tissues

Positive affinity for a specific stain may be designated by the suffix *-philic*. For example, tissues that stain with an azure dye may be referred to as azurophilic. This may also be used for more generalized staining properties, such as acidophilic for tissues that stain by acidic stains (most notably eosin), basophilic when staining in basic dyes and *amphophilic* when staining with either acid or basic dyes. In contrast, Chromophobic tissues do not take up coloured dye readily.

Electron microscopy

As in light microscopy, stains can be used to enhance contrast in transmission electron microscopy. Electron-dense compounds of heavy metals are typically used.

Phosphotungstic acid

Phosphotungstic acid is a common negative stain for viruses, nerves, polysaccharides, and other biological tissue materials.

Osmium tetroxide

Osmium tetroxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance. Because it is a heavy metal that absorbs electrons, it is perhaps the most common stain used for morphology in biological electron microscopy. It is also used for the staining of various polymers for the study of their morphology by TEM. OsO_4 is very volatile and extremely toxic. It is a strong oxidizing agent as the osmium has an oxidation number of +8. It aggressively oxidizes many materials, leaving behind a deposit of non-volatile osmium in a lower oxidation state.

Ruthenium tetroxide

Ruthenium tetroxide is equally volatile and even more aggressive than osmium tetraoxide and able to stain even materials that resist the osmium stain, e.g. polyethylene.

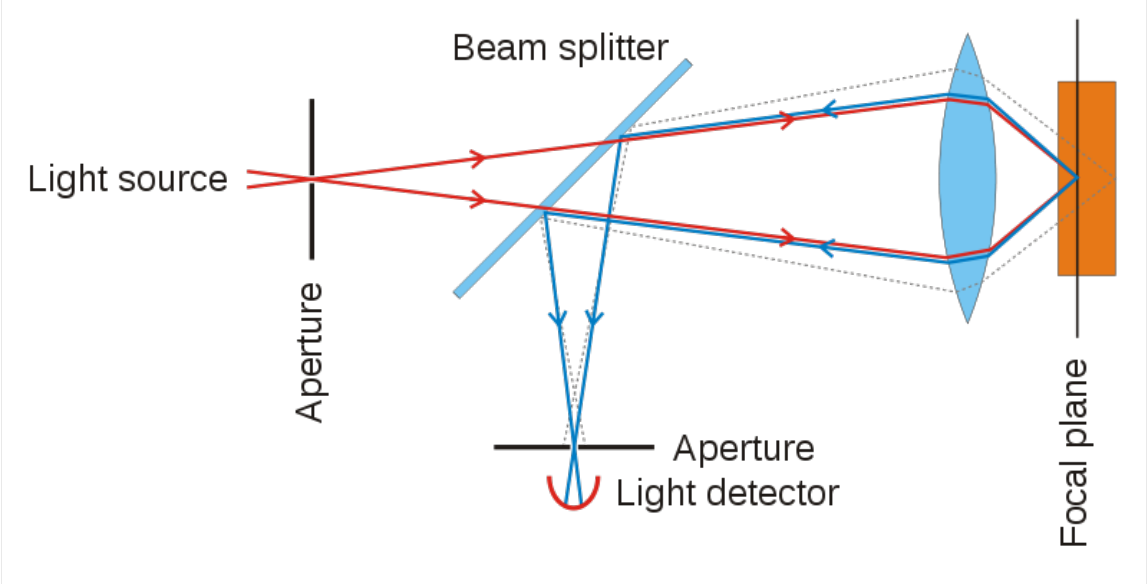
Other chemicals used in electron microscopy staining include: ammonium molybdate, cadmium iodide, carbohydrazide, ferric chloride, hexamine, indium trichloride, lanthanum nitrate, lead acetate, lead citrate, lead(II) nitrate, periodic acid, phosphomolybdic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate, sodium chloroaurate, thallium nitrate, thiosemicarbazide, uranyl acetate, uranyl nitrate, and vanadyl sulfate.

Chapter 2

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (**CLSM** or **LSCM**) is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically complex objects. For opaque specimens, this is useful for surface profiling, while for non-opaque specimens, interior structures can be imaged. For interior imaging, the quality of the image is greatly enhanced over simple microscopy because image information from multiple depths in the specimen is not superimposed. A conventional microscope "sees" as far into the specimen as the light can penetrate, while a confocal microscope only images one depth level at a time. In effect, the CLSM achieves a controlled and highly limited depth of focus. The principle of confocal microscopy was originally patented by Marvin Minsky in 1957, but it took another thirty years and the development of lasers for CLSM to become a standard technique toward the end of the 1980s. In 1978, Thomas and Christoph Cremer designed a laser scanning process, which scans the three dimensional surface of an object point-by-point by means of a focused laser beam, and creates the over-all picture by electronic means similar to those used in scanning electron microscopes. This CLSM design combined the laser scanning method with the 3D detection of biological objects labeled with fluorescent markers for the first time. During the next decade, confocal fluorescence microscopy was developed into a fully mature technology, in particular by groups working at the University of Amsterdam and the European Molecular Biology Laboratory (EMBL) in Heidelberg and their industry partners.

Image formation



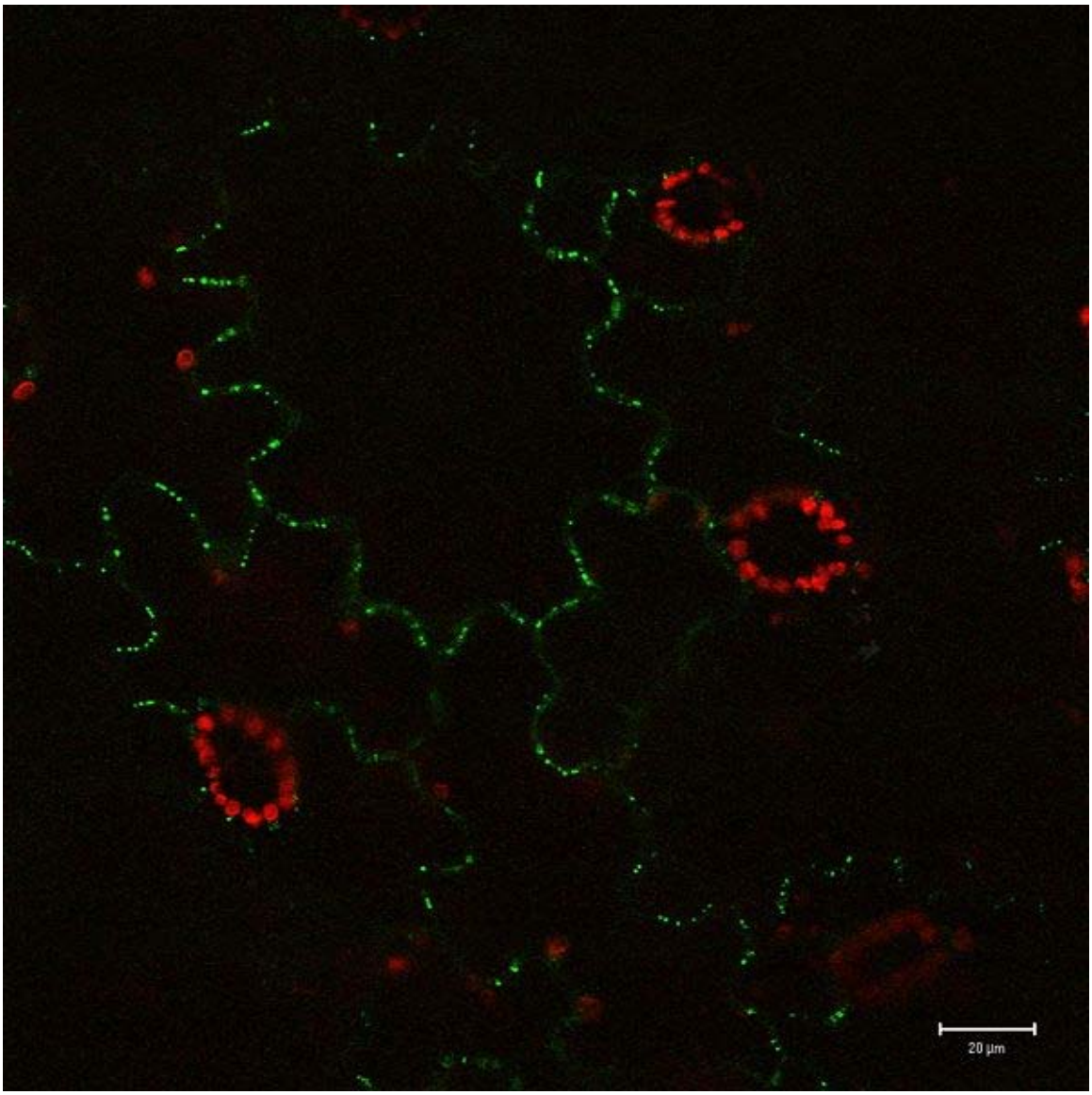
Principle of confocal microscopy.

In a confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small (ideally diffraction limited) focal volume within or on the surface of a specimen. In biological applications especially, the specimen may be fluorescent. Scattered and reflected laser light as well as any fluorescent light from the illuminated spot is then re-collected by the objective lens. A beam splitter separates off some portion of the light into the detection apparatus, which in fluorescence confocal microscopy will also have a filter that selectively passes the fluorescent wavelengths while blocking the original excitation wavelength. After passing a *pinhole*, the light intensity is detected by a photodetection device (usually a photomultiplier tube (PMT) or avalanche photodiode), transforming the light signal into an electrical one that is recorded by a computer.

The detector aperture obstructs the light that is not coming from the focal point, as shown by the dotted gray line in the image. The out-of-focus light is suppressed: most of the returning light is blocked by the pinhole, which results in sharper images than those from conventional fluorescence microscopy techniques and permits one to obtain images of planes at various depths within the sample (sets of such images are also known as *z stacks*).

The detected light originating from an illuminated volume element within the specimen represents one pixel in the resulting image. As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected light. The beam is scanned across the sample in the horizontal plane by using one or more (servo controlled) oscillating mirrors. This scanning method usually has a low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better

contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage or objective lens. The computer can generate a three-dimensional picture of a specimen by assembling a stack of these two-dimensional images from successive focal planes.



An example of a GFP fusion protein.

Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution. Biological samples are often treated with fluorescent dyes to make selected objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create

organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest).

Resolution enhancement

CLSM is a scanning imaging technique in which the resolution obtained is best explained by comparing it with another scanning technique like that of the scanning electron microscope (SEM). CLSM has the advantage of not requiring a probe to be suspended nanometers from the surface, as in an AFM or STM, for example, where the image is obtained by scanning with a fine tip over a surface. The distance from the objective lens to the surface (called the *working distance*) is typically comparable to that of a conventional optical microscope. It varies with the system optical design, but working distances from hundreds of micrometres to several millimeters are typical.

In CLSM a specimen is illuminated by a point laser source, and each volume element is associated with a discrete scattering or fluorescence intensity. Here, the size of the scanning volume is determined by the spot size (close to diffraction limit) of the optical system because the image of the scanning laser is not an infinitely small point but a three-dimensional diffraction pattern. The size of this diffraction pattern and the focal volume it defines is controlled by the numerical aperture of the system's objective lens and the wavelength of the laser used. This can be seen as the classical resolution limit of conventional optical microscopes using wide-field illumination. However, with confocal microscopy it is even possible to improve on the resolution limit of wide-field illumination techniques because the confocal aperture can be closed down to eliminate higher orders of the diffraction pattern. For example, if the pinhole diameter is set to 1 Airy unit then only the first order of the diffraction pattern makes it through the aperture to the detector while the higher orders are blocked, thus improving resolution at the cost of a slight decrease in brightness. In fluorescence observations, the resolution limit of confocal microscopy is often limited by the signal to noise ratio caused by the small number of photons typically available in fluorescence microscopy. One can compensate for this effect by using more sensitive photodetectors or by increasing the intensity of the illuminating laser point source. Increasing the intensity of illumination laser risks excessive bleaching or other damage to the specimen of interest, especially for experiments in which comparison of fluorescence brightness is required. When imaging tissues which are differentially refractive, such as the spongy mesophyll of plant leaves or other air-space containing tissues, spherical aberrations that impair confocal image quality are often pronounced. Such aberrations however, can be significantly reduced by mounting samples in optically transparent, non-toxic perfluorocarbons such as perfluorodecalin, which readily infiltrates tissues and has a refractive index almost identical to that of water.

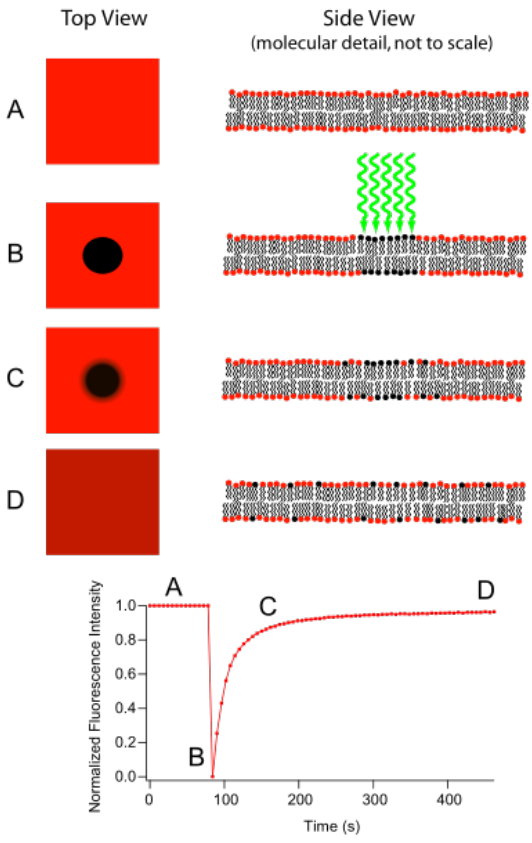
Uses

CLSM is widely-used in numerous biological science disciplines, from cell biology and genetics to microbiology and developmental biology.

Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea. It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for endoscopic procedures is also showing promise. In the pharmaceutical industry, it was recommended to follow the manufacturing process of thin film pharmaceutical forms, to control the quality and uniformity of the drug distribution. CLSM is also used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus.

Chapter 3

Fluorescence Recovery After Photobleaching



Principle of FRAP A) The bilayer is uniformly labeled with a fluorescent tag B) This label is selectively photobleached by a small (~30 micrometre) fast light pulse C) The intensity within this bleached area is monitored as the bleached dye diffuses out and new dye diffuses in D) Eventually uniform intensity is restored

Fluorescence recovery after photobleaching (FRAP) denotes an optical technique capable of quantifying the two dimensional lateral diffusion of a molecularly thin film containing fluorescently labeled probes, or to examine single cells. This technique is very useful in biological studies of cell membrane diffusion and protein binding. In addition, surface deposition of a fluorescing phospholipid bilayer (or monolayer) allows the characterization of hydrophilic (or hydrophobic) surfaces in terms of surface structure and free energy. Similar, though less well known, techniques have been developed to investigate the 3-dimensional diffusion and binding of molecules inside the cell; they are also referred to as FRAP.

Experimental Setup

The basic apparatus comprises an optical microscope, a light source and some fluorescent probe. Fluorescent emission is contingent upon absorption of a specific optical wavelength or color which restricts the choice of lamps. Most commonly, a broad spectrum mercury or xenon source is used in conjunction with a color filter. The technique begins by saving a background image of the sample before photobleaching. Next, the light source is focused onto a small patch of the viewable area either by switching to a higher magnification microscope objective or with laser light of the appropriate wavelength. The fluorophores in this region receive high intensity illumination which causes their fluorescence lifetime to quickly elapse (limited to roughly 10^5 photons before extinction). Now the image in the microscope is that of a uniformly fluorescent field with a noticeable dark spot. As Brownian motion proceeds, the still-fluorescing probes will diffuse throughout the sample and replace the non-fluorescent probes in the bleached region. This diffusion proceeds in an ordered fashion, analytically determinable from the diffusion equation. Assuming a Gaussian profile for the bleaching beam, the diffusion constant D can be simply calculated from:

$$D = \frac{w^2}{4t_{1/2}}$$

where w is the radius of the beam and $t_{1/2}$ is the time required for the bleach spot to recover half of its initial integrated intensity.

Applications

Supported Lipid Bilayers

Originally, the FRAP technique was intended for use as a mean to characterize the mobility of individual lipid molecules within a cell membrane. While providing great utility in this role, current research leans more toward investigation of artificial lipid membranes. Supported by hydrophilic or hydrophobic substrates (to produce lipid bilayers or monolayers respectively) and incorporating membrane proteins, these biomimetic structures are potentially useful as analytical devices for determining the

identity of unknown substances, understanding cellular transduction, and identifying ligand binding sites.

Protein Binding

This technique is commonly used in conjunction with green fluorescent protein (GFP) fusion proteins, where the studied protein is fused to a GFP. When excited by a specific wavelength of light, the protein will fluoresce. When the protein that is being studied is produced with the GFP, then the fluorescence can be tracked. Photodestroying the GFP, and then watching the repopulation into the bleached area can reveal information about protein interaction partners, organelle continuity and protein trafficking.

If after some time the fluorescence doesn't reach the initial level anymore, then some part of the fluorescence is caused by an immobile fraction (that cannot be replenished by diffusion). Similarly, if the fluorescent proteins bind to static cell receptors, the rate of recovery will be retarded by a factor related to the association and disassociation coefficients of binding. This observation has most recently been exploited to investigate protein binding.

Applications Outside the Membrane

FRAP can also be used to monitor proteins outside the membrane. After the protein of interest is made fluorescent, generally by expression as a GFP fusion protein, a confocal microscope is used to photobleach and monitor a region of the cytoplasm, mitotic spindle, nucleus, or another cellular structure. The mean fluorescence in the region can then be plotted versus time since the photobleaching, and the resulting curve can yield kinetic coefficients for the protein's binding reactions and/or the protein's diffusion coefficient in the medium where it is being monitored. The analysis is most simple when the curve is dominated by only the diffusional or only the binding components. For a circular bleach spot and diffusion-dominated recovery, the fluorescence is described by the Soumpasis equation and involves modified Bessel functions:

$$f(t) = e^{-h}\left(I_0(h) + I_1(h)\right)$$

where h=r^2/(2*D_f*t); r=radius of bleach spot; t=time; D_f=diffusion coefficient; $f(t)$ is the normalized fluorescence (goes to 1 as t goes to infinity).

For a binding-dominated reaction, in which the diffusion is much faster than the unbinding of the bleached protein and subsequent binding of unbleached protein, it is given by

$$f(t) = 1 - B_{eq}e^{-k_{off}t}$$

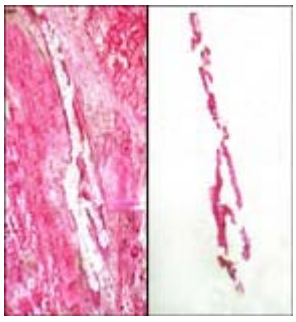
where B_{eq} is the fraction of the protein that is bound to other structures in the photobleached region at equilibrium, and k_{off} is the dissociation constant for the binding. Sometimes there are multiple binding states in which case there are just more exponential

terms of the same form. Many FRAP recoveries are not dominated overwhelmingly by just diffusion or just binding, so their curves are more complex; FRAP recoveries are analyzed in much more detail in Sprague and Pego et al.

Chapter 4

Laser Capture Microdissection and Pulse-Chase Analysis

Laser capture microdissection



Laser capture micro-dissection transfer of pure breast duct epithelial cells. Left panel shows tissue section with selected cells removed. Right panel shows isolated epithelial cells on transfer film.

Laser capture microdissection (LCM), also called Microdissection, Laser MicroDissection (LMD), or Laser-assisted microdissection (LMD or LAM) is a method for isolating specific cells of interest from microscopic regions of tissue/cells/organisms.

Extraction process

A laser is coupled into a microscope and focuses onto the tissue on the slide. By movement of the laser by optics or the stage the focus follows a trajectory which is predefined by the user. This trajectory, a so called *Element*, is then cut out and separated from the adjacent tissue. After the cutting process, an extraction process has to follow if an extraction process is desired.

Theoretically, there are several ways to extract tissue from a microscope slide with a histopathology sample on it:

- Press a sticky surface onto the sample and tear out. This will extract the desired region, but also bears the chance to carry particles or unwanted tissue on the surface, because an *allround sticky* surface is not selective.
- Melt a plastic membrane onto the sample and tear out. The heat is introduced by an, e.g., red or IR laser onto a membrane stained with an absorbing dye. As this adheres the desired sample onto the membrane, as with any membrane that is put close to the histopathology sample surface, there might be some debris extracted. Another danger is the introduced heat: Some molecules like DNA, RNA, or protein don't allow to be heated too much or at all for the goal of being isolated as purely as possible.
- Transport without contact. There are two different approaches:

1. Transport simply by gravity using an upright microscope or
2. Reliable and precise transport by *Laser Pressure Catapult*

Procedure

Under a microscope using a software interface, a tissue section (typically 5-50 micrometres thick) is viewed and individual cells or clusters of cells are identified either manually or in semi-automated or more fully automated ways allowing the imaging and then automatic selection of targets for isolation. Currently five primary isolation/collection technologies exist using a microscope and device for cell isolation. Four of these typically use an ultraviolet pulsed laser (355 nm) for the cutting of the tissues directly or the membranes/film, and sometimes in combination with an IR laser responsible for heating/melting a sticky polymer for cellular adhesion and isolation. IR laser provides a more gentle approach to microdissection.

The various technologies differ in the collection process, possible imaging modalities (Fluorescence microscopy/Bright field microscopy/Differential interference contrast microscopy/Phase contrast microscopy/ etc.) and the types of holders and tissue preparation needed before the imaging and isolation. Most are primarily dedicated Micro-dissection systems, and some can be used as research microscopes as well, only one technology (#2 here, Leica) uses an upright microscope, limiting some of the sample handling capabilities somewhat esp. for live cell work.

1.) The first technology may first cut around the sample then collect it by a patented "catapulting" technology. The sample can be catapulted from a slide or special culture dish by a defocused U.V laser pulse (generating a photonic force for propelling the material off the slide/dish. This is sometimes called Laser Micro-dissection Pressure Catapulting (LMPC) and the tissue is sent upward (up to several mm)to a microfuge tube cap (or other collector) containing buffer or a specialized tacky material in the tube cap that the tissue/cell/ nucleus/chromosome fragment will then adhere to. This active catapulting process avoids some of the static problems when using membrane-coated

slides. This system had historically been offered by PALM Microlaser Technologies and is now a Zeiss product called PALM MicroBeam.

2.) Another closely related LCM process cuts the sample from above and the sample drops via gravity into a capture device below the sample.

3.) When the cells (on a slide or special culture dish) of choice are in the center of the field of view, the operator selects the cells of interest using instrument software. The area to be isolated when a near-IR laser to activate transfer film on a cap placed on the tissue sample, melting the adhesive which then fuses the film with the underlying cells of choice; and/or by activating a UV laser to cut out the cell of interest. The cells are then lifted off the thin tissue section, leaving all unwanted cells behind. The cells of interest are then viewed and documented prior to extraction.

4.) The last UV based technology offers a slight difference to the 3rd technology here by essentially creating a sandwich of sorts with slide>sample>and membrane overlying the sample by the use of a frame slide whose membrane surface is cut by the laser and ultimately picked up from above by a special adhesive cap**.**

NON-LASER BASED MICRODISSECTION/ISOLATION

5.) This last technique cannot really be called Laser Capture Microdissection but is closely related. For lack of a better term is micro-chiseling; by using a piezoelectric driven micro-chisel that vibrates ultrasonic frequencies. This allows for fine etching or chiseling of cells and particles in small areas and for collection via a finely adjustable aspirator to a waiting tube collector. Another related technique for isolating via a micropositioned manipulator is available.

In addition to tissue sections, LCM can be performed on living cells/organisms, cell smears, chromosome preparations, and plant tissue.

Applications

The laser capture microdissection process does not alter or damage the morphology and chemistry of the sample collected, nor the surrounding cells. For this reason, LCM is a useful method of collecting selected cells for DNA, RNA and/or protein analyses. LCM can be performed on a variety of tissue samples including blood smears, cytologic preparations, cell cultures and aliquots of solid tissue. Frozen and paraffin embedded archival tissue may also be used . On formalin or alcohol fixed paraffin embedded tissues, DNA and RNA retrieval has been successful, but protein analysis is not possible (requires frozen section).

Pulse-chase analysis

In biochemistry and molecular biology, a **pulse-chase analysis** is a method for examining a cellular process occurring over time by successively exposing the cells to a labeled compound (pulse) and then to the same compound in an unlabeled form (chase). Radioactivity is a commonly used label.

Mechanism

A selected cell or a group of cells is first exposed to a labeled compound (the pulse) that is to be incorporated into a molecule or system that is studied. The compound then goes through the metabolic pathways and is used in the synthesis of the product studied. For example, a radioactively labeled form of leucine (^{3}H-leucine) can be supplied to a group of pancreatic B cells, which then uses this amino acid in insulin synthesis.

Shortly after introduction of the labeled compound (usually about 5 minutes, but the actual time needed is dependent on the object studied), excess of the same, but unlabeled, substance (the chase) is introduced into the environment. Following the previous example, the production of insulin would continue, but it would no longer contain the radioactive leucine introduced in the pulse phase and would not be visible using radioactive detection methods. However, the movement of the labeled insulin produced during the pulse period could still be tracked within the cell.

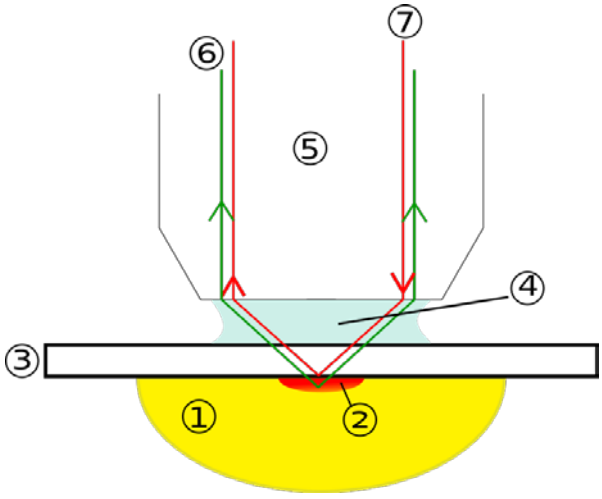
Uses

This method is useful for determining the activity of certain cells over a prolonged period of time. The method has been used to study protein kinase C, ubiquitin, and many other proteins. The method was also used to prove the existence and function of Okazaki fragments. George Palade used pulse-chase of radioactive amino acids to elicit the secretory pathway.

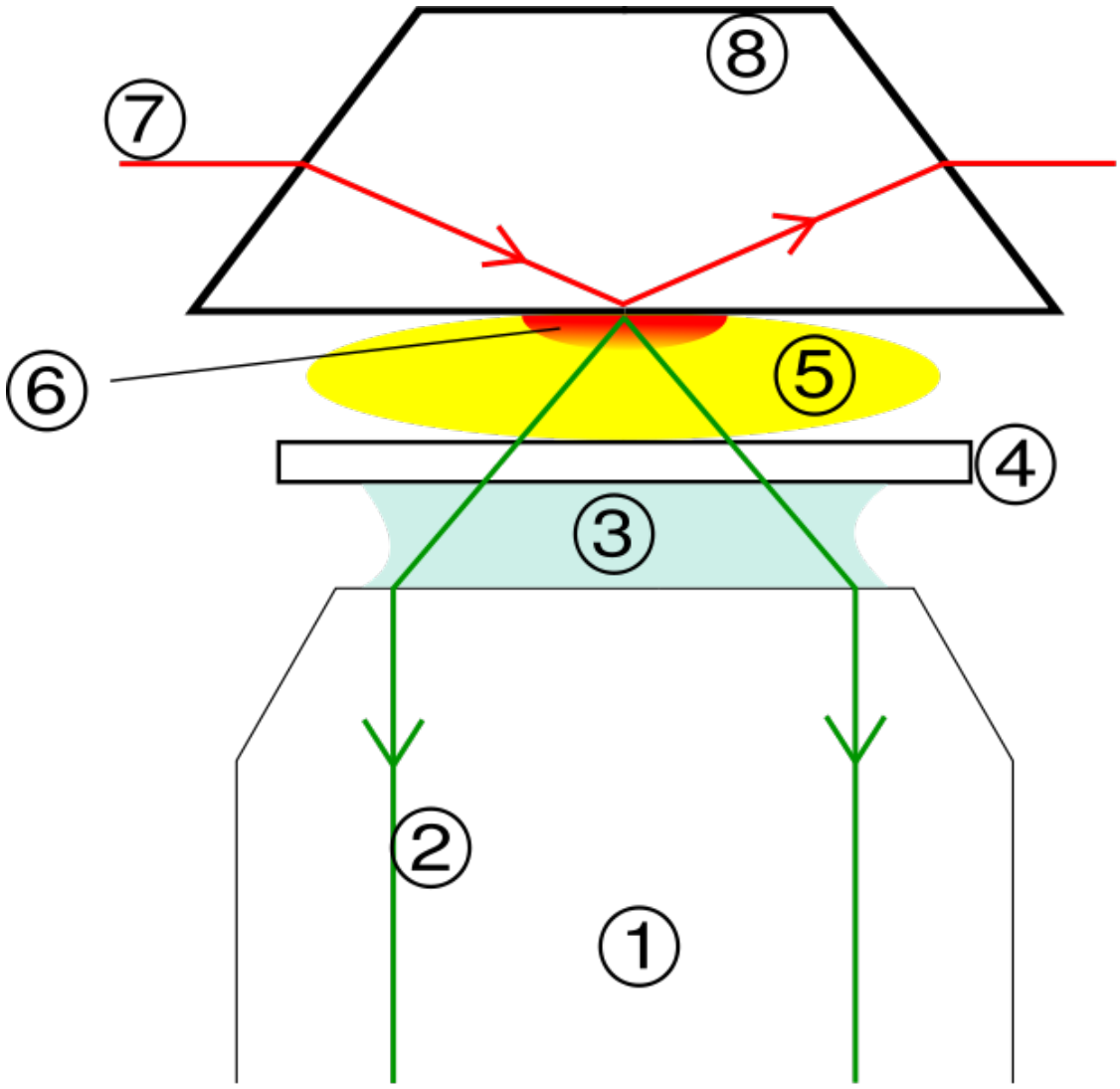
Chapter 5

Total Internal Reflection Fluorescence Microscope and Two-Photon Excitation Microscopy

Total internal reflection fluorescence microscope



total internal reflection fluorescence microscope (TIRFM) diagram
1. Specimen
2. Evanescent wave range
3. Cover slip
4. Immersion oil
5. Objective
6. Emission beam (signal)
7. Excitation beam



Total Internal Reflection Fluorescence Microscope trans- (TIRFM) diagram
1. Objective
2. Emission beam (signal)
3. Immersion oil
4. Cover slip
5. Specimen
6. Evanescent wave range
7. Excitation beam
8. Quartz prism

A **total internal reflection fluorescence microscope** (**TIRFM**) is a type of microscope with which a thin region of a specimen, usually less than 200 nm, can be observed.

Background

In cell and molecular biology, a large number of molecular events in cellular surfaces such as cell adhesion, binding of cells by hormones, secretion of neurotransmitters, and membrane dynamics have been studied with conventional fluorescence microscopes. However, fluorophores that are bound to the specimen surface and those in the surrounding medium exist in an equilibrium state. When these molecules are excited and detected with a conventional fluorescence microscope, the resulting fluorescence from those fluorophores bound to the surface is often overwhelmed by the background fluorescence due to the much larger population of non-bound molecules.

Solution

To solve this problem, TIRFM was developed by Daniel Axelrod at the University of Michigan, Ann Arbor in the early 1980s. A TIRFM uses an evanescent wave to selectively illuminate and excite fluorophores in a restricted region of the specimen immediately adjacent to the glass-water interface. The evanescent wave is generated only when the incident light is totally internally reflected at the glass-water interface. The evanescent electromagnetic field decays exponentially from the interface, and thus penetrates to a depth of only approximately 100 nm into the sample medium. Thus the TIRFM enables a selective visualization of surface regions such as the basal plasma membrane (which are about 7.5 nm thick) of cells as shown in the figure above. Note, however, that the region visualised is at least a few hundred nanometers wide, so the cytoplasmic zone immediately beneath the plasma membrane is necessarily visualised in addition to the plasma membrane during TIRF microscopy. The selective visualisation of the plasma membrane renders the features and events on the plasma membrane in living cells with high axial resolution.

TIRF can also be used to observe the fluorescence of a single molecule, making it an important tool of biophysics and quantitative biology.

Two-photon excitation microscopy

Two-photon excitation microscopy is a fluorescence imaging technique that allows imaging of living tissue up to a very high depth, that is up to about one millimeter. Being a special variant of the multiphoton fluorescence microscope, it uses red-shifted excitation light which can also excite fluorescent dyes. However for each excitation two photons of the infrared light are absorbed. Using infrared light minimizes scattering in the tissue. Due to the multiphoton absorption the background signal is strongly suppressed. Both effects lead to an increased penetration depth for these microscopes. However, the resolution remains diffraction-limited. Two-photon excitation can be a superior alternative to confocal microscopy due to its deeper tissue penetration, efficient light detection and reduced phototoxicity.

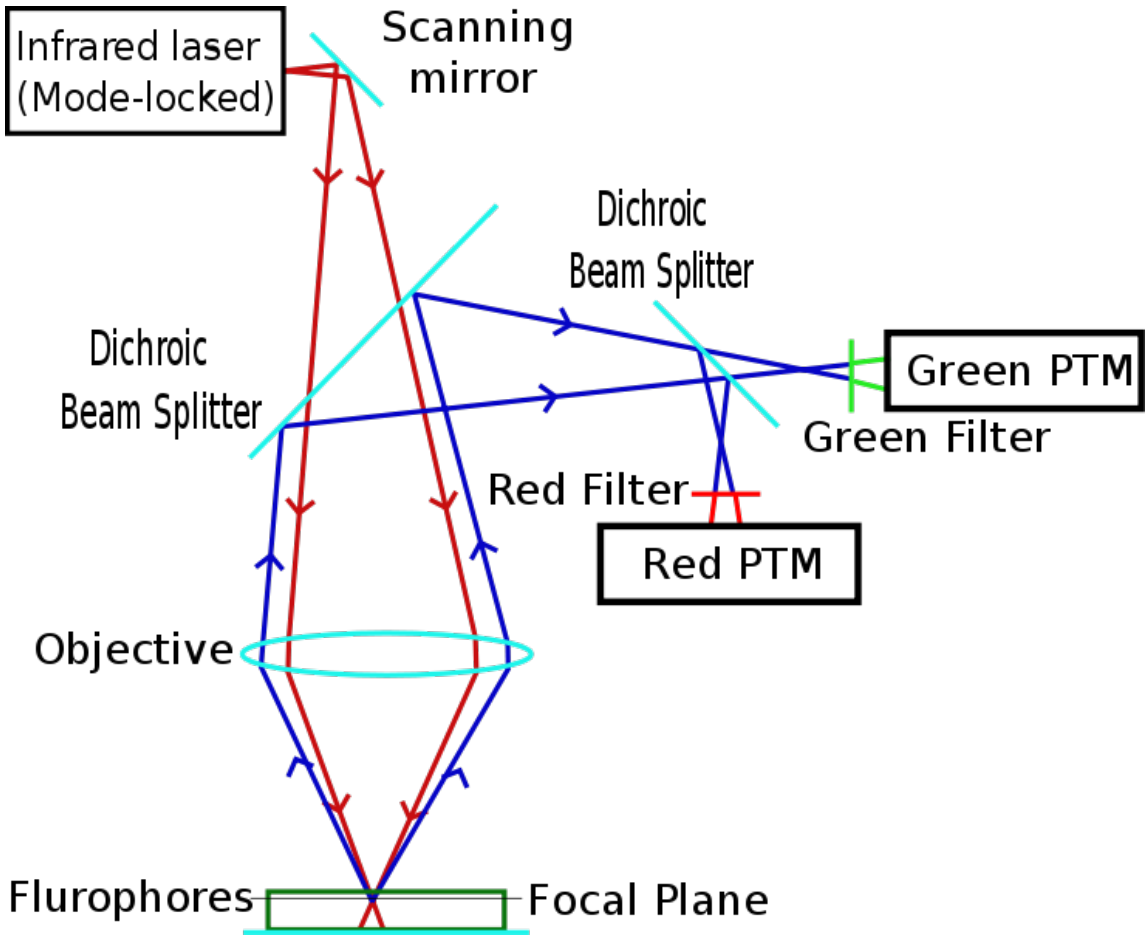
Concept

Two-photon excitation employs a concept first described by Maria Goeppert-Mayer (1906–1972) in her doctoral dissertation in 1931, and first observed in 1962 in cesium vapor using laser excitation by Isaac Abella.

The concept of two-photon excitation is based on the idea that two photons of comparably lower energy than needed for one photon excitation can also excite a fluorophore in one quantum event. Each photon carries approximately half the energy necessary to excite the molecule. An excitation results in the subsequent emission of a fluorescence photon, typically at a higher energy than either of the two excitatory photons. The probability of the near-simultaneous absorption of two photons is extremely low. Therefore a high flux of excitation photons is typically required, usually a femtosecond laser.

The most commonly used fluorophores have excitation spectra in the 400–500 nm range, whereas the laser used to excite the two-photon fluorescence lies in the ~700–1000 nm (infrared) range. If the fluorophore absorbs two infrared photons simultaneously, it will absorb enough energy to be raised into the excited state. The fluorophore will then emit a single photon with a wavelength that depends on the type of fluorophore used (typically in the visible spectrum). Because two photons are absorbed during the excitation of the fluorophore, the probability for fluorescent emission from the fluorophores increases quadratically with the excitation intensity. Therefore, much more two-photon fluorescence is generated where the laser beam is tightly focused than where it is more diffuse. Effectively, excitation is restricted to the tiny focal volume (~1 femtoliter), resulting in a high degree of rejection of out-of-focus objects. This *localization of excitation* is the key advantage compared to single-photon excitation microscopes, which need to employ additional elements such as pinholes to reject out-of-focus fluorescence. The fluorescence from the sample is then collected by a high-sensitivity detector, such as a photomultiplier tube. This observed light intensity becomes one pixel in the eventual image; the focal point is scanned throughout a desired region of the sample to form all the pixels of the image.

Development



A diagram of a two-photon microscope

Two-photon microscopy was pioneered by Winfried Denk in the lab of Watt W. Webb at Cornell University. He combined the idea of two-photon absorption with the use of a laser scanner. In two-photon excitation microscopy an infrared laser beam is focused through an objective lens. The Ti-sapphire laser normally used has a pulse width of approximately 100 femtoseconds and a repetition rate of about 80 MHz, allowing the high photon density and flux required for two photons absorption and is tunable across a wide range of wavelengths. Two-photon technology has been patented by Winfried Denk, James Strickler and Watt Webb at Cornell University. Microscope manufacturer Carl Zeiss AG currently holds this patent; Olympus Inc. has licensed it to sell two-photon microscopes.

The use of infrared light to excite fluorophores in light-scattering tissue has added benefits. Longer wavelengths are scattered to a lesser degree than shorter ones, which is a benefit to high-resolution imaging. In addition, these lower-energy photons are less likely to cause damage outside the focal volume. Compared to a confocal microscope, photon detection is much more effective since even scattered photons contribute to the usable signal. There are several caveats to using two-photon microscopy: The pulsed lasers

needed for two-photon excitation are much more expensive than the continuous wave (CW) lasers used in confocal microscopy. The two-photon absorption spectrum of a molecule may vary significantly from its one-photon counterpart. For very thin objects such as isolated cells, single-photon (confocal) microscopes can produce images with higher optical resolution due to their shorter excitation wavelengths. In scattering tissue, on the other hand, the superior optical sectioning and light detection capabilities of the two-photon microscope result in better performance.

Higher-order excitation

Simultaneous absorption of three or more photons is also possible, allowing for three-photon or multiphoton excitation microscopy.

Chapter 6

Digital Holographic Microscopy

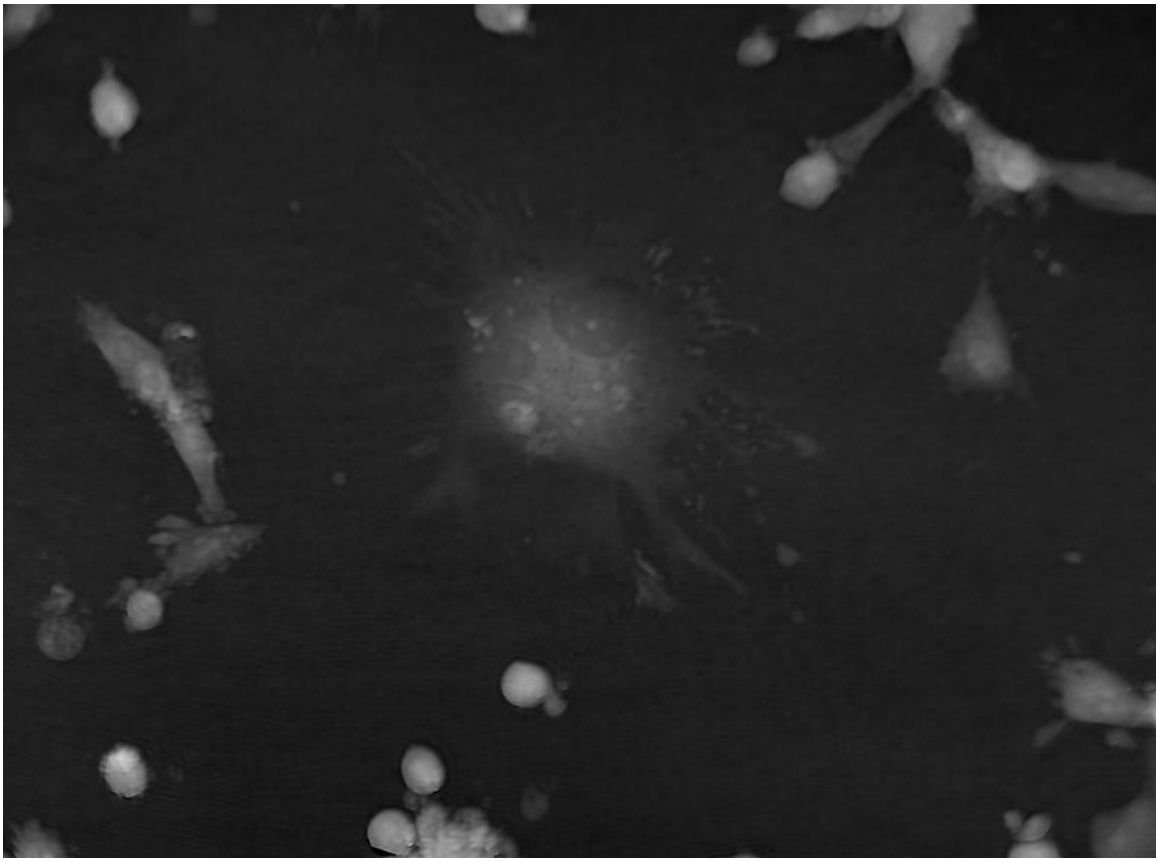


Figure 1. DHM phase shift image of cell details.

Digital holographic microscopy (DHM) is digital holography applied to microscopy. Digital holographic microscopy distinguishes itself from other microscopy methods by not recording the projected image of the object. Instead, the light wave front information originating from the object is digitally recorded as a hologram, from which a computer calculates the object image by using a numerical reconstruction algorithm. The image forming lens in traditional microscopy is thus replaced by a computer algorithm.

Other closely related microscopy methods to digital holographic microscopy are interferometric microscopy, optical coherence tomography and diffraction phase microscopy. Common to all methods is the use of a reference wave front to obtain amplitude (intensity) **and** phase information. The information is recorded on a digital image sensor or by a photo detector from which an image of the object is created (reconstructed) by a computer. In traditional microscopy, which do not use a reference wave front, only intensity information is recorded and essential information about the object is lost.

Digital holography has mostly been applied to light microscopy. However, digital holography has also been applied to electron microscopy. Holography was invented by Dennis Gabor to improve the electron microscope. For various reasons holography never made it into the electron microscope. Digital electron holography may finally bring home holography to its birth place and fulfill Gabor's vision.

Working principle

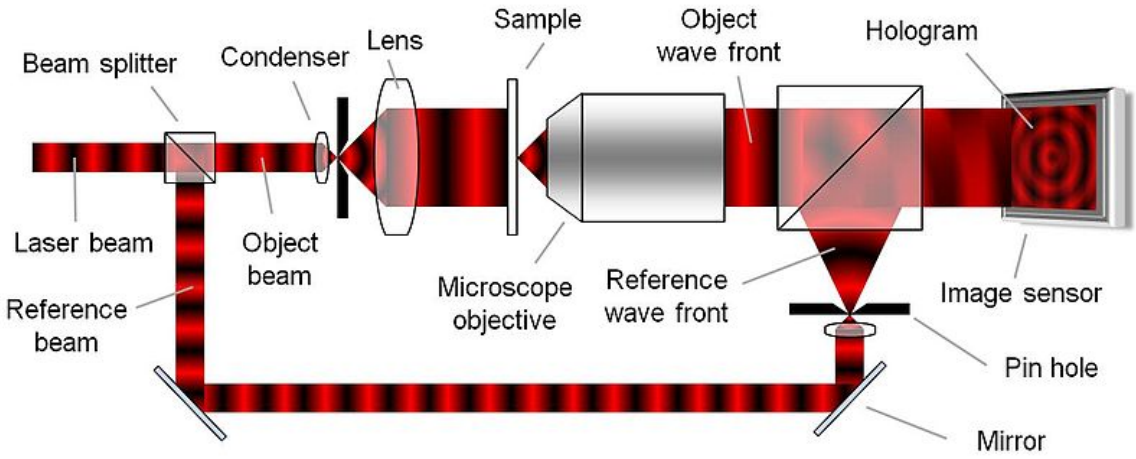


Figure 2. Typical optical setup of DHM.

To create the necessary interference pattern, i.e. the hologram, the illumination needs to be a coherent (monochromatic) light source, a laser for example. As can be seen in Figure 2, the laser light is split into an object beam and a reference beam. The expanded object beam illuminates the sample to create the object wave front. After the object wave front is collected by a microscope objective, the object and reference wave fronts are joined by a beam splitter to interfere and create the hologram. Using the digitally recorded hologram, a computer acts as a *digital lens* and calculates a viewable image of the object wave front by using a numerical reconstruction algorithm.

Commonly, a microscope objective is used to collect the object wave front. However, as the microscope objective is only used to collect light and not to form an image, it may be replaced by a simple lens. If a slightly lower optical resolution is acceptable, the microscope objective may be entirely removed.

Digital holography comes in different flavors, such as *off-axis Fresnel*, *Fourier*, *image plane*, *in-line*, *Gabor* and *phase-shifting* digital holography, depending on the optical setup. The basic principle, however, is the same; a hologram is recorded and an image is reconstructed by a computer.

The lateral optical resolution of digital holographic microscopy is equivalent to the resolution of traditional light microscopy. DHM is diffraction-limited by the numerical aperture, in the same way as traditional light microscopy. However, DHM offers a superb axial (depth) resolution. An axial accuracy of approximately 5 nm has been reported.

Advantages

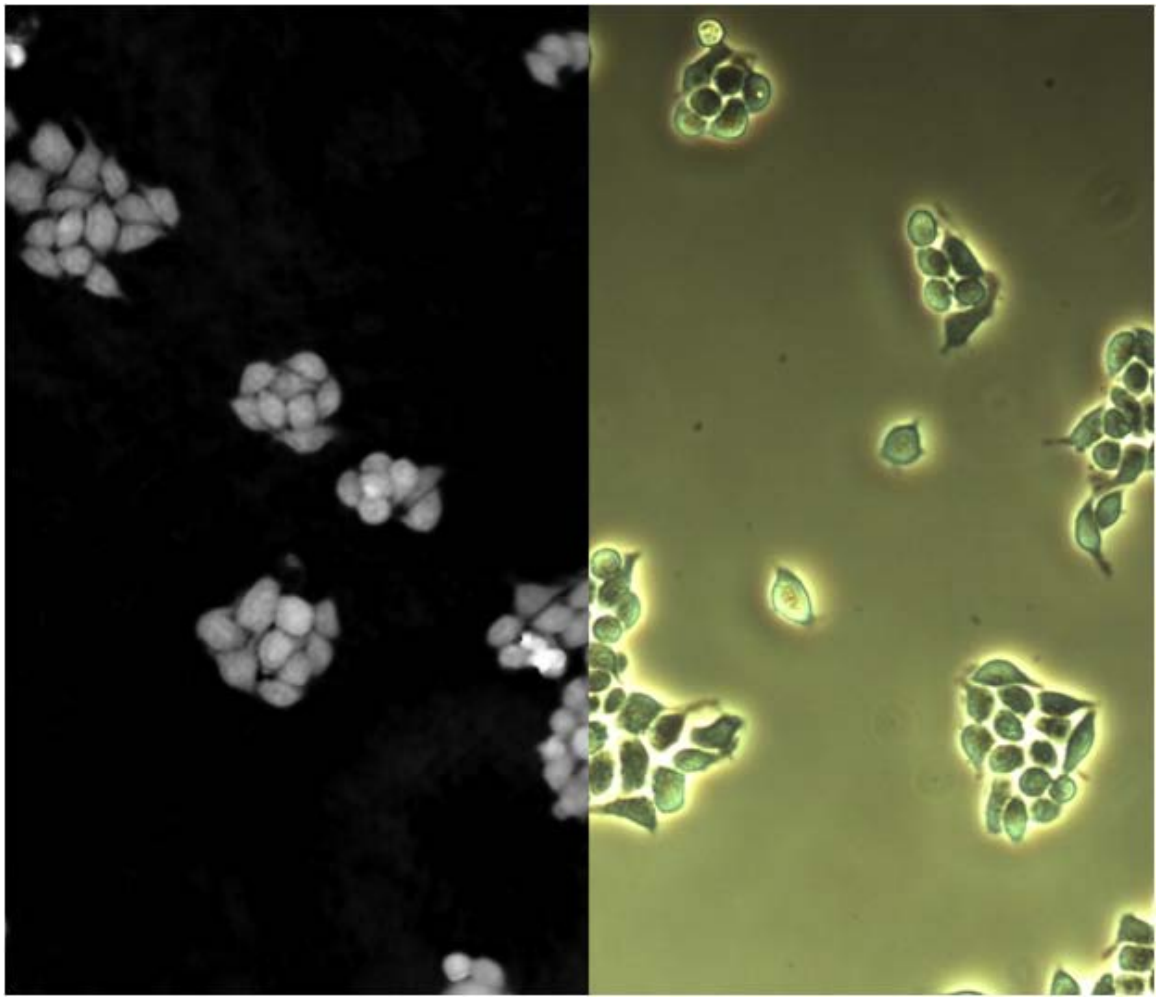


Figure 3. Comparison of a DHM phase shift image (left) and a phase contrast microscopy image (right).

Phase shift images
Besides the ordinary bright field image, a phase shift image is created as well. The phase shift image is unique for digital holographic microscopy and gives quantifiable

information about optical distance. In reflection DHM, the phase shift image forms a topography image of the object.

Transparent objects, like living biological cells, are traditionally viewed in a phase contrast microscope or in a differential interference contrast microscope. These methods visualize phase shifting transparent objects by distorting the bright field image with phase shift information. Instead of distorting the bright field image, transmission DHM creates a separate phase shift image showing the optical thickness of the object. Digital holographic microscopy thus makes it possible to visualize and quantify transparent objects and is therefore also referred to as *quantitative phase contrast* microscopy.

Traditional phase contrast or bright field images of living unstained biological cells, Figure 3 (right), have proved themselves to be very difficult to analyze with image analysis software. On the contrary, phase shift images, Figure 3 (left), are readily segmented and analyzed by image analysis software based on mathematical morphology, such as CellProfiler.

3-Dimensional information
An object image is calculated at a given focal distance. However, as the recorded hologram contains all the necessary object wave front information, it is possible to calculate the object at any focal plane by changing the focal distance parameter in the reconstruction algorithm. In fact, the hologram contains all the information needed to calculate a complete image stack. In a DHM system, where the object wave front is recorded from multiple angles, it is possible to fully characterize the optical characteristics of the object and create tomography images of the object.

Digital autofocus
Conventional autofocus is achieved by vertically changing the focal distance until a focused image plane is found. As the complete stack of image planes may be calculated from a single hologram, it is possible to use any passive autofocus method to digitally select the focal plane. The digital auto focusing capabilities of digital holography opens up the possibility to scan and image surfaces extremely rapidly, without any vertical mechanical movement. By recording a single hologram and afterwards stitch sub-images together that are calculated at different focal planes, a complete and focused image of the object may be created.

Optical aberration correction
As DHM systems do not have an image forming lens, traditional optical aberrations do not apply to DHM. Optical aberrations are "corrected" by design of the reconstruction algorithm. A reconstruction algorithm that truly models the optical setup will not suffer from optical aberrations.

Low cost
In optical microscopy systems, optical aberrations are traditionally corrected by combining lenses into a complex and costly image forming microscope objective. Furthermore, the narrow focal depth at high magnifications requires precision mechanics.

The needed components for a DHM system are inexpensive optics and semiconductor components, such as a laser diode and an image sensor. The low component cost in combination with the auto focusing capabilities of DHM, make it possible to manufacture DHM systems for a very low cost.

Applications

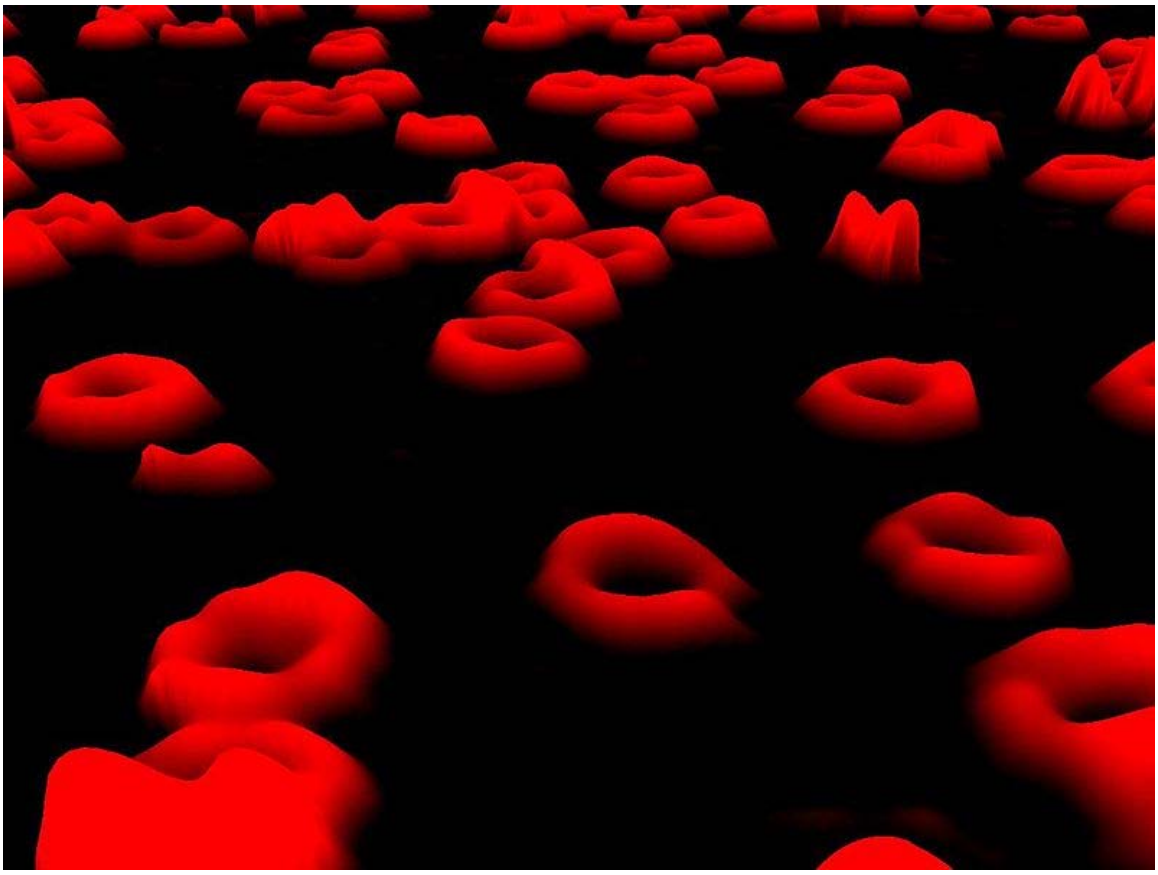


Figure 4. DHM phase shift image of human red blood cells.

Digital holographic microscopy has been successfully applied in a range of application areas. However, due to DHM's capability of non-invasively visualizing and quantifying biological tissue, bio-medical applications have received most attention. Examples of bio-medical applications are:

- **Label-free cell counting in adherent cell cultures.** Digital holographic microscopy makes it possible to perform cell counting and to measure cell viability directly in the cell culture chamber. Today, the most commonly used cell counting methods, hemocytometer or Coulter counter, only work with cells that are in suspension.

- **Label-free viability analysis of adherent cell cultures.** Digital holography has been used to study the apoptotic process in different cell types. The refractive

index changes taking place during the apoptotic process are easily measured with DHM.

- **Label-free cell cycle analysis.** The phase shift induced by cells has been shown to be correlated to the cell dry mass. The cell dry mass can be combined with other parameters obtainable by digital holography, such as cell volume and refractive index, to provide a better understanding of the cell cycle.

- **Label-free morphology analysis of cells.** Digital holography has been used in different contexts to study cell morphology using neither staining nor labeling. This can be used to follow processes such as the differentiation process where cell characteristics change. DHM has also been used for automated plant stem cell monitoring, and made it possible to distinguish between two types of stem cells by measuring morphological parameters.

- **Label free nerve cell studies.** Digital holographic microscopy makes it possible to study undisturbed processes in nerve cells as no labeling is required. The swelling and shape changing of nerve cells caused by cellular imbalance was easily studied.

- **Label-free high content analysis.** Fluorescent high content analysis/screening has several drawbacks. Label-free alternatives based on phase shift images have therefore been proposed. The capability of DHM to obtain phase shift images rapidly over large areas opens up new possibilities of very rapid quantitative characterization of the cell cycle and the effects of specific pharmacological agents.

- **Red blood cell analysis.** Phase shift images, created by diffraction phase microscopy, have been used to study red blood cell dynamics. Diffraction phase microscopy is very similar to digital holographic microscopy and creates phase shift images identical to the phase shift images created by digital holography.

- **Flow cytometry and particle tracking.** In-line digital holographic video microscopy has been used to analyze the radius and refractive index of particles in a microfluidic channel. These results can be applied on cells to enable real time digital holography flow cytometry.

- **Time-lapse microscopy of cell division and migration.** The autofocus and phase shift imaging capabilities of digital holographic microscopy makes it possible to effortlessly create label-free and quantifiable time-lapse. In Figure 5 a label-free time-lapse of dividing and migrating cells is shown.

- **Tomography studies.** Digital holographic microscopy allows for label-free and quantifiable analysis of subcellular motion deep in living tissue.

History

The first reports of replacing the photographic hologram of classical holography by digitally recording the hologram and numerically reconstructing the image in a computer was published in the late 1960s and in the early 1970s. Similar ideas were proposed for the electron microscope in the early 1980s. But, computers were too slow and recording capabilities were too poor for digital holography to be useful in practice. After the initial excitement, digital holography went into a similar hibernation as holography experienced about two decades earlier.

In the mid 1990s, digital image sensors and computers had become powerful enough to reconstruct images with some quality. In the 1960s, digital holography could either mean to compute an image from a hologram or to compute a hologram from a 3D model. The latter developed in parallel with classical holography during the hibernation of digital holography. During that time, digital holography was synonymous with what is now known as computer generated holography.

By the mid 1990s, digital image sensors and computers had improved tremendously, but still lacked the required performance for digital holography to be anything more than a curiosity. At the time, the market driving digital image sensors was primarily low-resolution video, and so those sensors provided only PAL, NTSC, or SECAM resolution. This suddenly changed at the beginning of the 21st century with the introduction of digital still image cameras, which drove demand for inexpensive high-pixel-count sensors. As of 2010, affordable image sensors can have up to 60 megapixels. In addition, the CD and DVD-player market has driven development of affordable diode lasers and optics.

The first reports of using digital holography for light microscopy came in the mid 1990s. However, it was not until the early 2000s that image sensor technology had progressed far enough to allow images of a reasonable quality. At that time, the first commercial digital holography companies also started to appear. With increased computing power and use of affordable high-resolution sensors and lasers, digital holographic microscopy became feasible and is finding applications, primarily within the life science.

Chapter 7

Fluorescence Microscope

An upright fluorescence microscope (Olympus BX61) with the fluorescent filter cube turret above the objective lenses, coupled with a digital camera.

A **fluorescence microscope** is an optical microscope used to study properties of organic or inorganic substances using the phenomena of fluorescence and phosphorescence instead of, or in addition to, reflection and absorption. The term "fluorescence microscope" is colloquially synonymous with *epifluorescence microscope* but also refers to microscope designs such as the confocal microscope which also use fluorescence to generate the image.

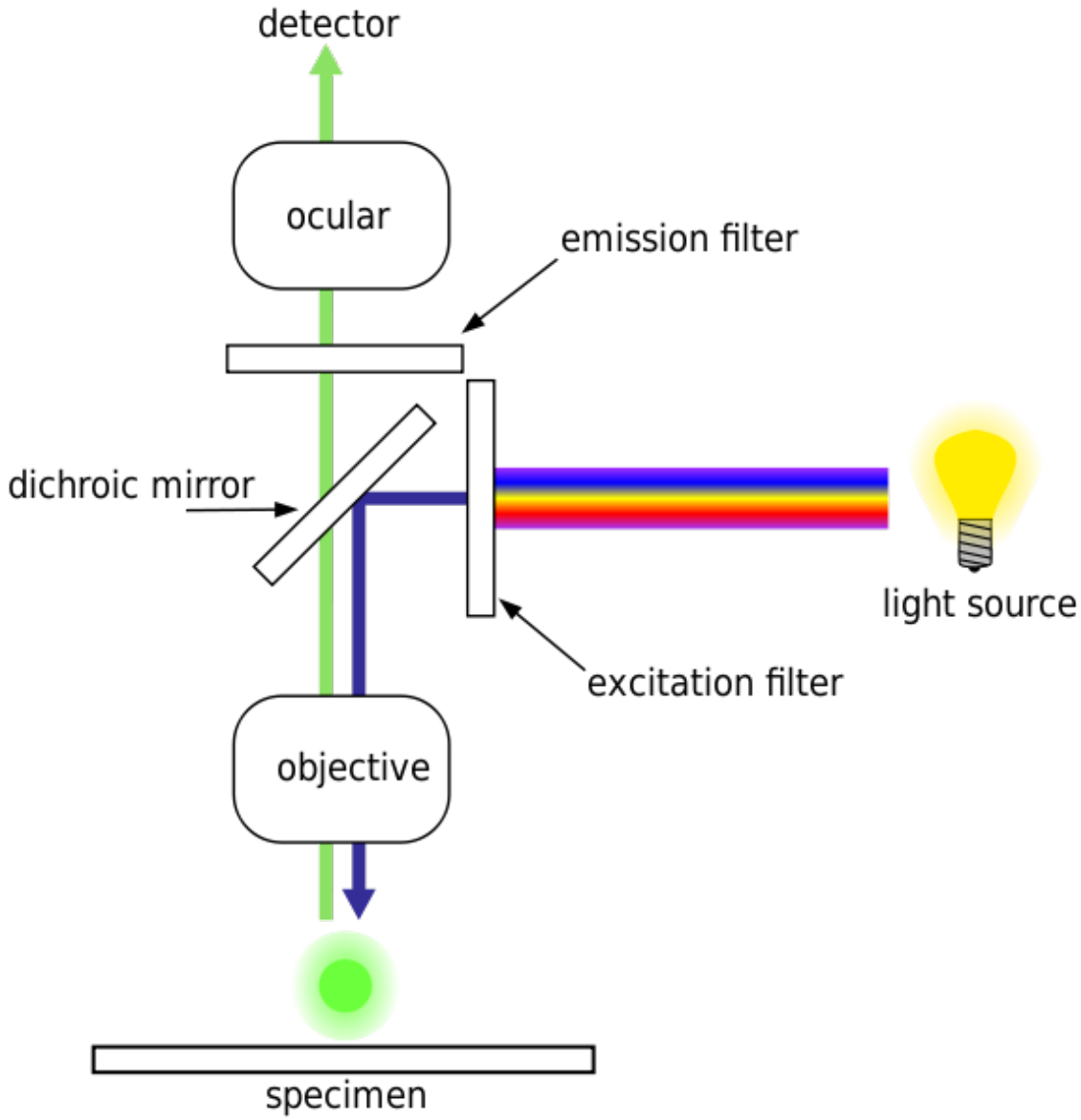
All fluorescence microscopy methods share the same principle. A sample is illuminated with light of a one wavelength which causes fluorescence in the sample. The light emitted by fluorescence, which is at a different, longer, wavelength than the illumination, is then detected through a microscope objective. Two filters are normally used in this technique; an illumination (or exitation) filter which ensures the illumination is near monochromatic and at the correct wavelength, and a second emission (or detection) filter which ensures none of the exitation light source reaches the detector. Fluorescence microscopy takes is a fundamentally different to generating a light microscope image compared to transmitted or reflected white light techniques such as phase contrast and differential interference. These two contrasting optical microscopy methods give very different but complementary data.

Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e. of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen. In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.

Most fluorescence microscopes in use are epifluorescence microscopes (i.e. excitation and observation of the fluorescence are from above (*epi–*) the specimen). These microscopes have become an important part in the field of biology, opening the doors for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).

Epifluorescence microscopy



Schematic of a fluorescence microscope.

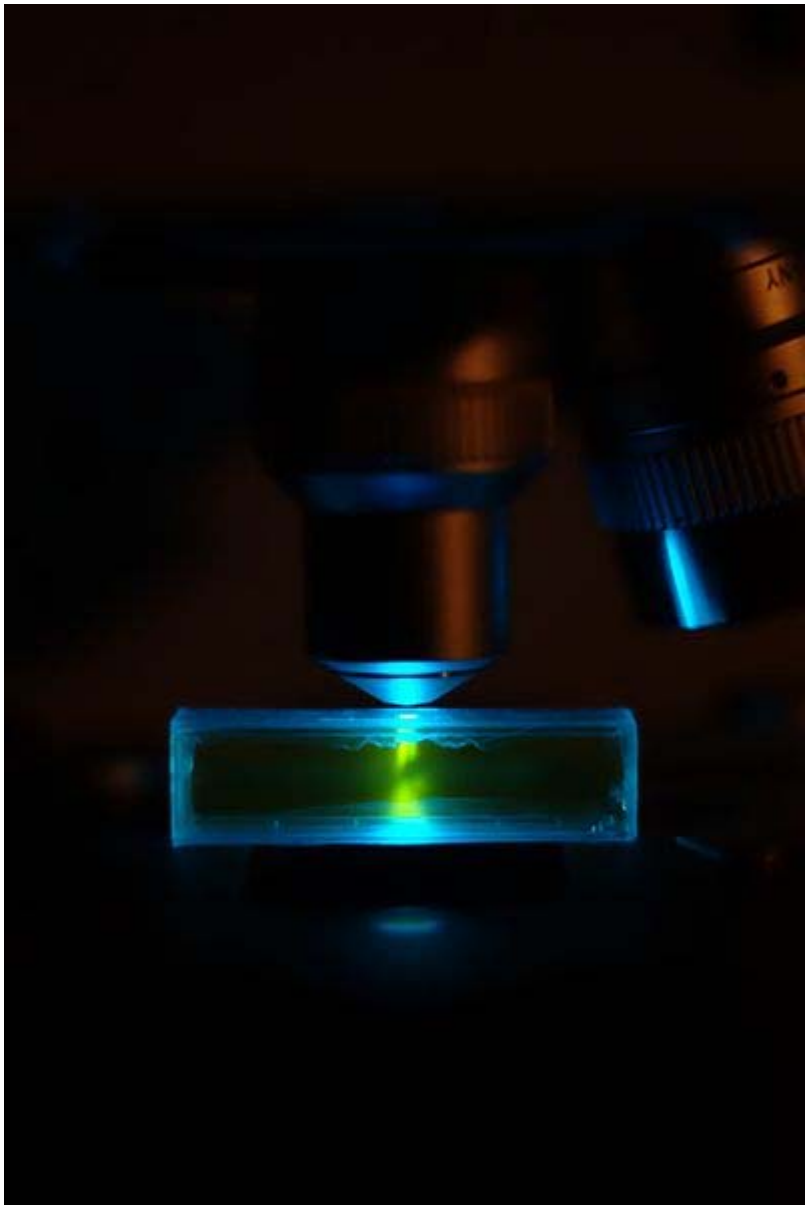
The majority of fluorescence microscopy, especially in the life sciences, is epifluorescence microscopy. The excitatory light is passed from above (or, for inverted microscopes, from below), through the objective lens and then onto the specimen instead of passing it first through the specimen. The fluorescence in the specimen gives rise to emitted light which is focused to the detector by the same objective that is used for the excitation. Since most of the excitatory light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and this method therefore gives an improved signal to noise ratio. An additional filter between the objective and the detector can filter out the remaining excitation light from fluorescent

light.

Light sources

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. There are two main types of light source used; xenon arc lamp or mercury-vapor lamps with an excitation filter and lasers. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and TIRF micrcroscopy while xenon and mercury lamps with an exitation filter are commonly used for widefield epifluorescence microscopes.

Sample preparation



A sample of herring sperm stained with SYBR green in a cuvette illuminated by blue light in an epifluorescence microscope. The SYBR green in the sample binds to the herring sperm DNA and, once bound, fluoresces giving off green light when illuminated by blue light.

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e. autofluorescence) can be used. In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimin in order to detect the

distribution of proteins or other molecules of interest. As a result there is a diverse range of techniques for fluorescent staining of biological samples.

Biolocal fluorescent stains

Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoescht which bind the minor groove of DNA, thus labelling the nuclei of cells. Others are drugs or toxins which bind specific cellular structures and have been derivitised with a fluorescent reporter. A major example of this class of fluorescent stain is fluorescently labelled-phalloidin which is used to stain actin fibres in mammalian cells.

There are many fluorescent reported molecules, called fluorophores such as fluorescein and DyLight 488, which can be chemically linked to a different molecule which binds the target of interest within the sample.

Immunofluorescence

Immuofluorescence is an antibody based technique which uses the highly specific binding of an antibody to its antigen in order to label specific proteins or other molecules within the cell. A sample is treated with a primary antibody specific for the molecule of interest A fluorophore can be directly conjugated to the primary antibody. Alternatively a secondary antibody, conjugated to a fluorophore, which binds speficically to the first antibody cam be used. For example a primary antibody raised in a mouse which recognises tubulin combined with a secondary anti-mouse antibody derivatised with a fluorophore could be used to label microtubules in a cell.

Fluorescent proteins

The modern understanding of genetics and the techniques available for modifying DNA allows scientists to genetically modify proteins to also carry a fluorescent protein reporter. In biological samples this allows a scientist to directly make a protein of interest fluorescent. The protein location can then be directly tracked, including in live cells.

Limitions

Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence. Photobleaching can severly limit the time over which a sample can be observerd by fluorescent microscopy. Several techniques exist to reduce photobleaching such as the use of more robust fluorophores, by minimizing illumination, or by using photoprotective a scavenger chemicals.

Fluorescence microscopy with fluorescent reporter proteins has enabled analysis of live cells by fluorescence microscopy, however cells are succeptible to phototoxicity,

particularly with short wavelength light. Furthermore fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.

Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been fluorescently labeled. For example observing a tissue sample prepared with a fluorescent DNA stain by fluorescent microscopy only reveals the organisation of the DNA within the cells and reveals nothing else about the cell morphologies.

Improvements and sub-diffraction techniques

The wave nature of light limits the size of the spot to which light can be focused due to the diffraction limit. This limitation was described in the 19th century by Ernst Abbe and limits an optical microscope's resolution to approximately half of the wavelength of the light used. Fluorescence microscopy is central to many techniques which aim to reach past this limit by specialised optical configurations.

Several improvements in microscopy techniques have been invented in the 20th century and have resulted in increased resolution and contrast to some extent. However they did not overcome the diffraction limit. In 1978 first theoretical ideas have been developed to break this barrier by using a 4Pi microscope as a confocal laser scanning fluorescence microscope where the light is focused ideally from all sides to a common focus which is used to scan the object by 'point-by-point' excitation combined with 'point-by-point' detection . However, the first experimental demonstration of the 4pi microscope took place in 1994 . 4Pi microscopy maximizes the amount of available focusing directions by using two opposing objective lenses or Multi-photon microscopy using redshifted light and multi-photon excitation.

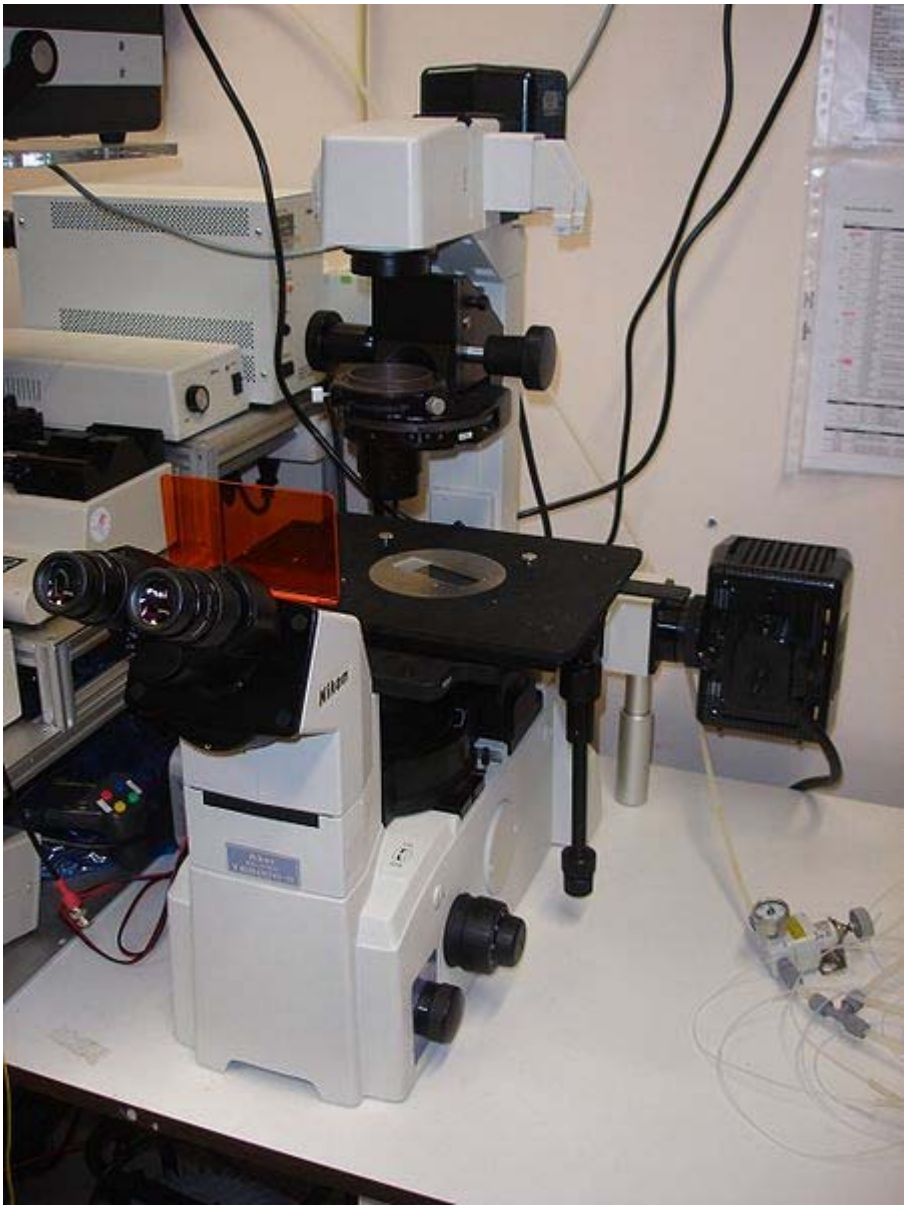
The first technique to really achieve a sub-diffraction resolution was STED microscopy, proposed in 1994. This method and all techniques following the RESOLFT concept rely on a strong non-linear interaction between light and fluorescing molecules. The molecules are driven strongly between distinguishable molecular states at each specific location, so that finally light can be emitted at only a small fraction of space, hence an increased resolution.

As well in the 1990s another super resolution microscopy method based on wide field microscopy has been developed. Substantially improved size resolution of cellular nanostructures stained with a fluorescent marker was achieved by development of SPDM localization microscopy and the structured laser illumination (spatially modulated illumination, SMI) . Combining the principle of SPDM with SMI resulted in the development of the Vertico SMI microscope . Single molecule detection of normal blinking fluorescent dyes like GFP can be achieved by using a further development of SPDM the so-called SPDMphymod technology which makes it possible to detect and count two different fluorescent molecule types at the molecular level (this technology is referred to as 2CLM, 2 Color Localization Microscopy) .

Alternatively, the advent of photoactivated localization microscopy could achieve similar results by relying on blinking or switching of single molecules, where the fraction of fluorescing molecules is very small at each time. This stochastic response of molecules on the applied light corresponds also to a highly nonlinear interaction, leading to subdiffraction resolution.

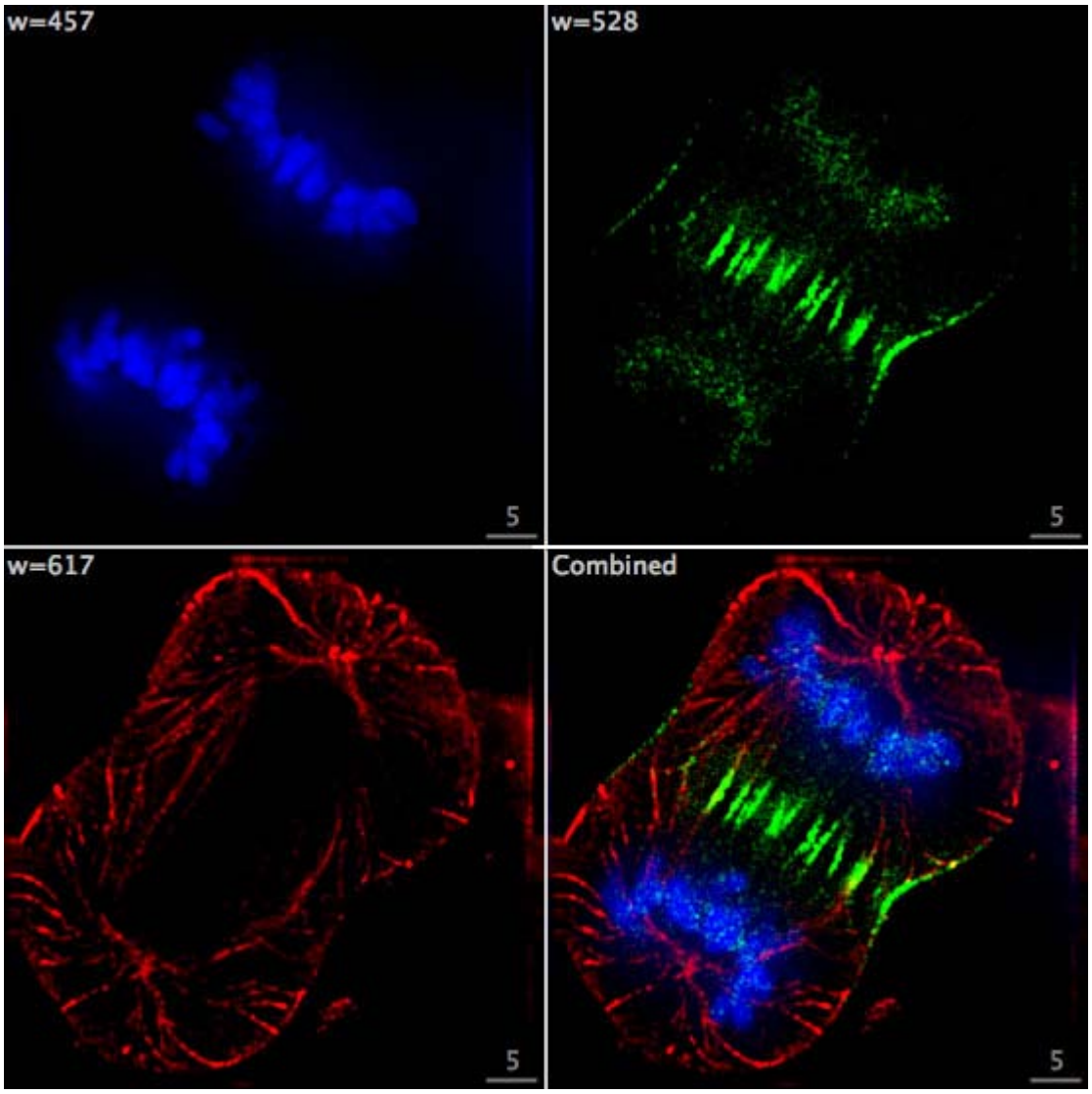
Fluorescence microscope gallery

An inverted fluorescence microscope (AMG EVOS fl) with an LCD display instead of occulars for fluorescence microscopy.

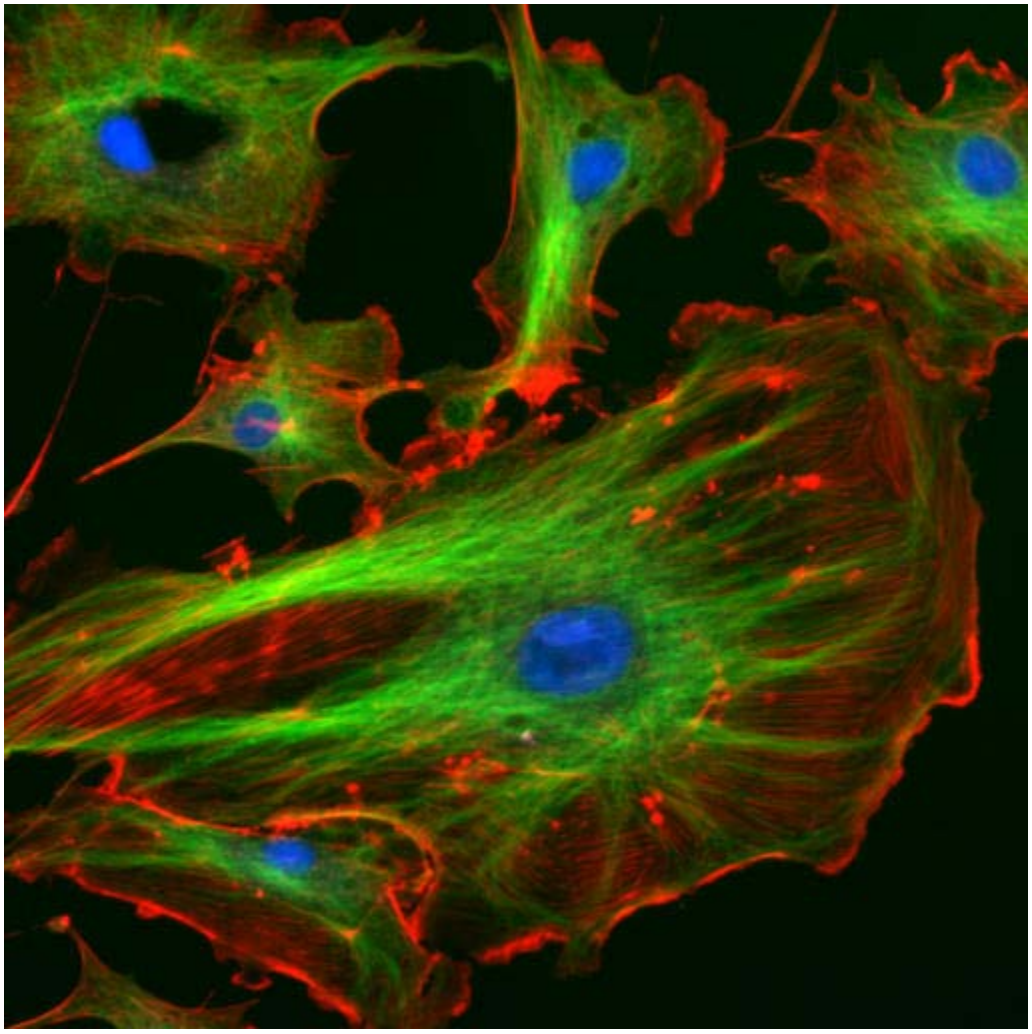


An inverted fluorescence microscope (Nikon TE2000) with the fluorescent filter cube turret below the stage. Note the orange plate that allows the user to look at a sample while protecting their eyes from the UV light.

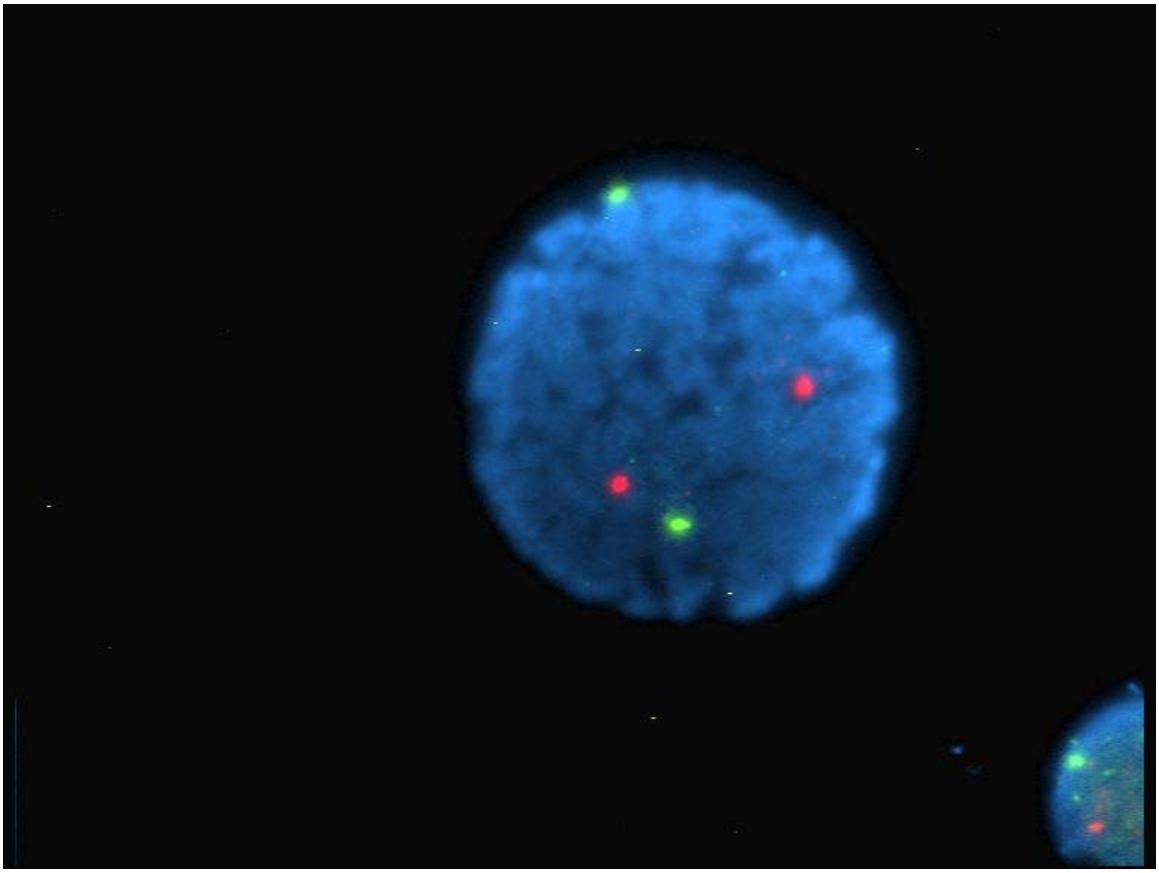
Fluorescence micrograph gallery



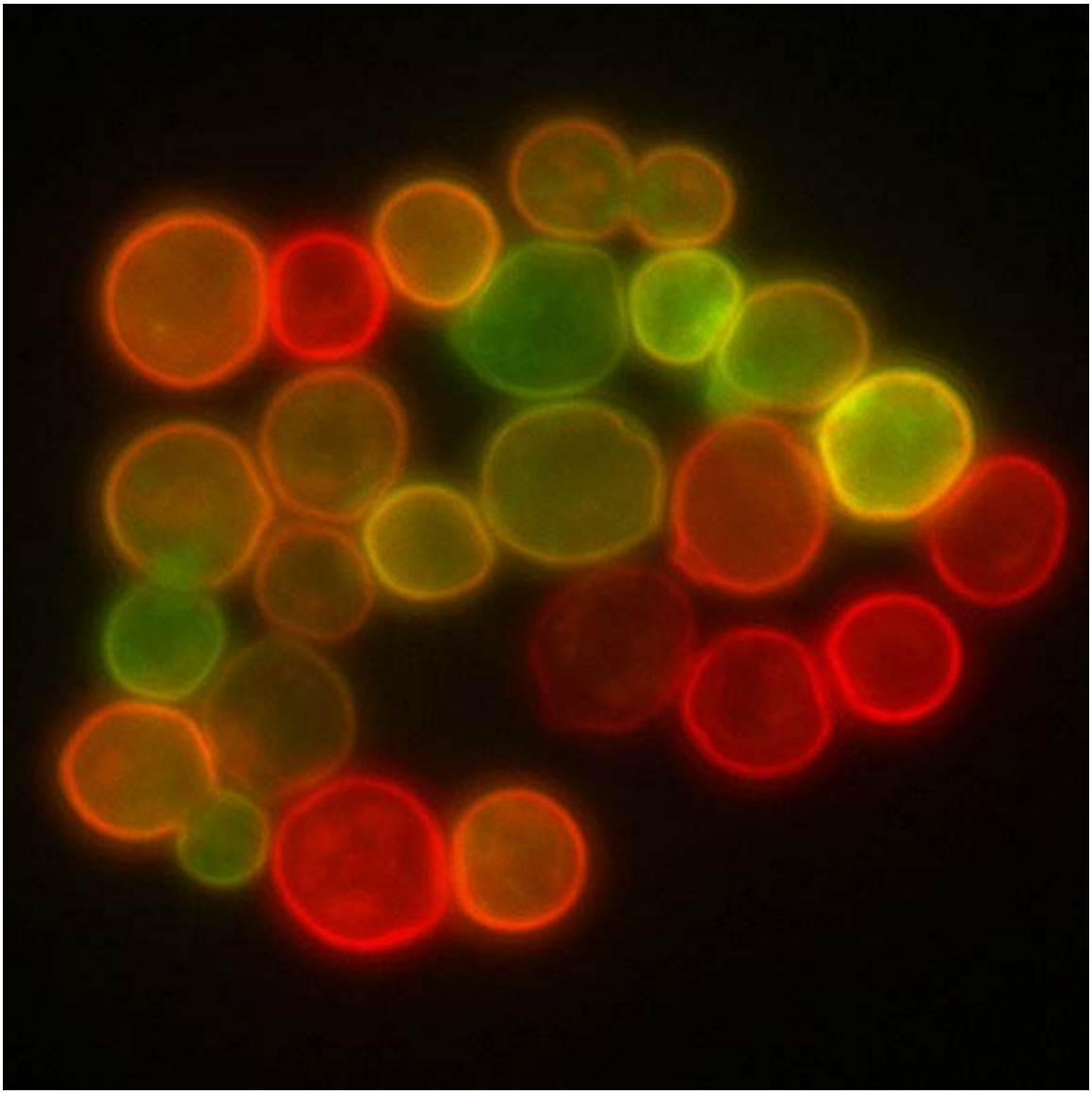
Epifluorescent imaging of the three components in a dividing human cancer cell. DNA is stained blue, a protein called INCENP is green, and the microtubules are red. Each fluorophore is imaged separately using a different combination of excitation and emission filters, and the images are captured sequentially using a digital CCD camera, then overlaid to give a complete image.



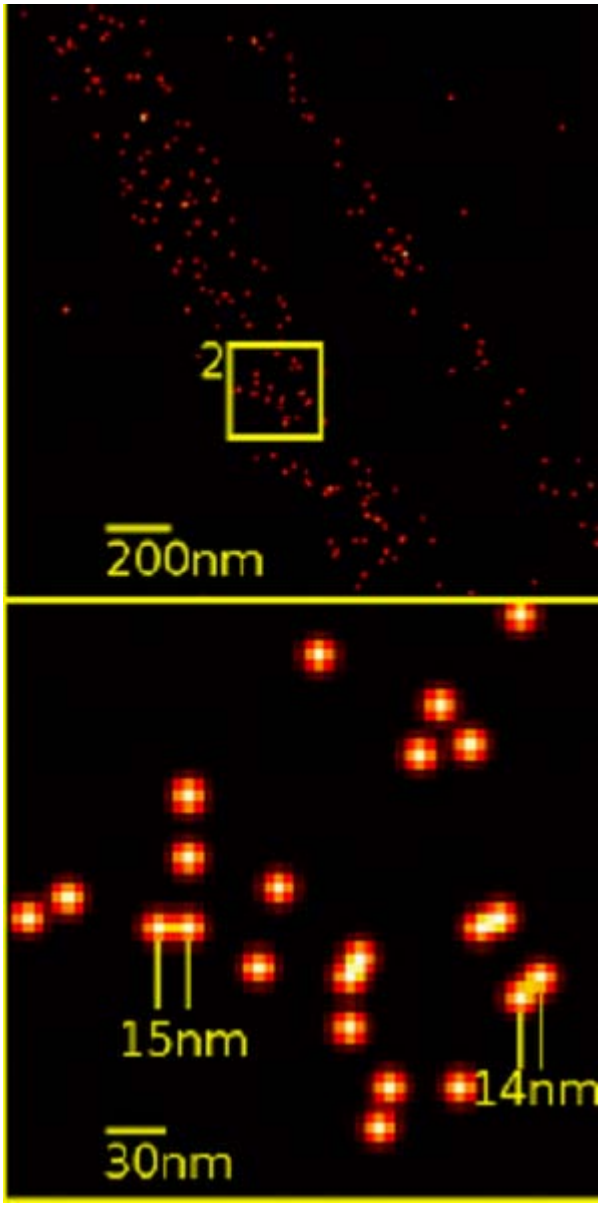
Endothelial cells under the microscope. Nuclei are stained blue with DAPI, microtubules are marked green by an antibody bound to FITC and actin filaments are labeled red with phalloidin bound to TRITC. Bovine pulmonary artery endothelial (BPAE) cells



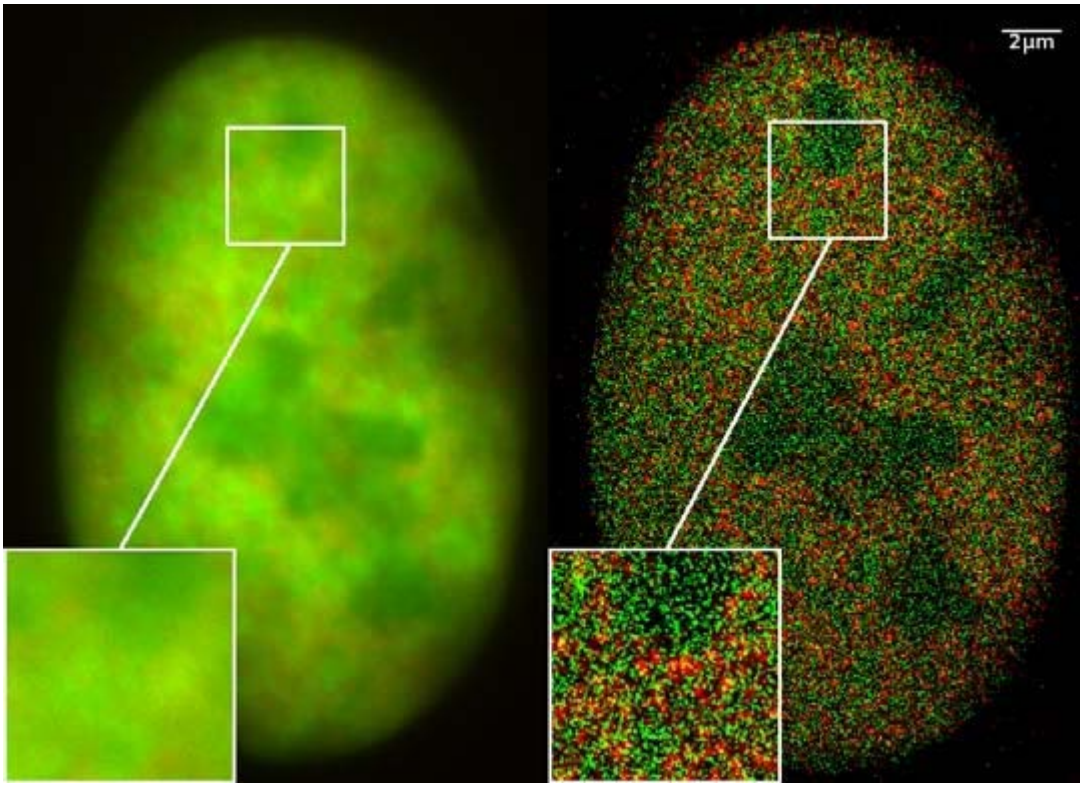
Human lymphocyte nucleus stained with DAPI with chromosome 13 (green) and 21 (red) centromere probes hybridized (Fluorescent in situ hybridization (FISH))



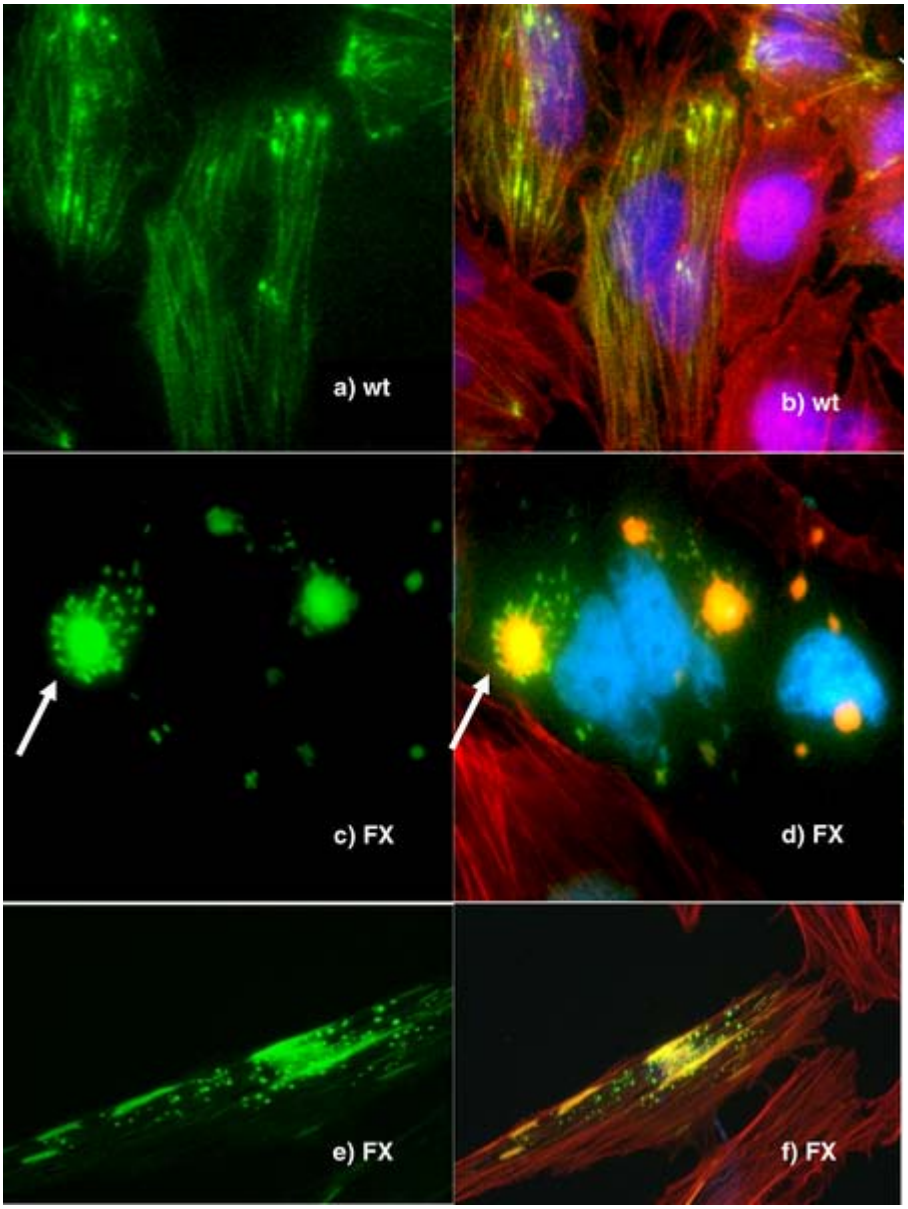
Yeast cell membrane visualized by some membrane proteins fused with RFP and GFP fluorescent markers. Imposition of light from both of markers results in yellow color.



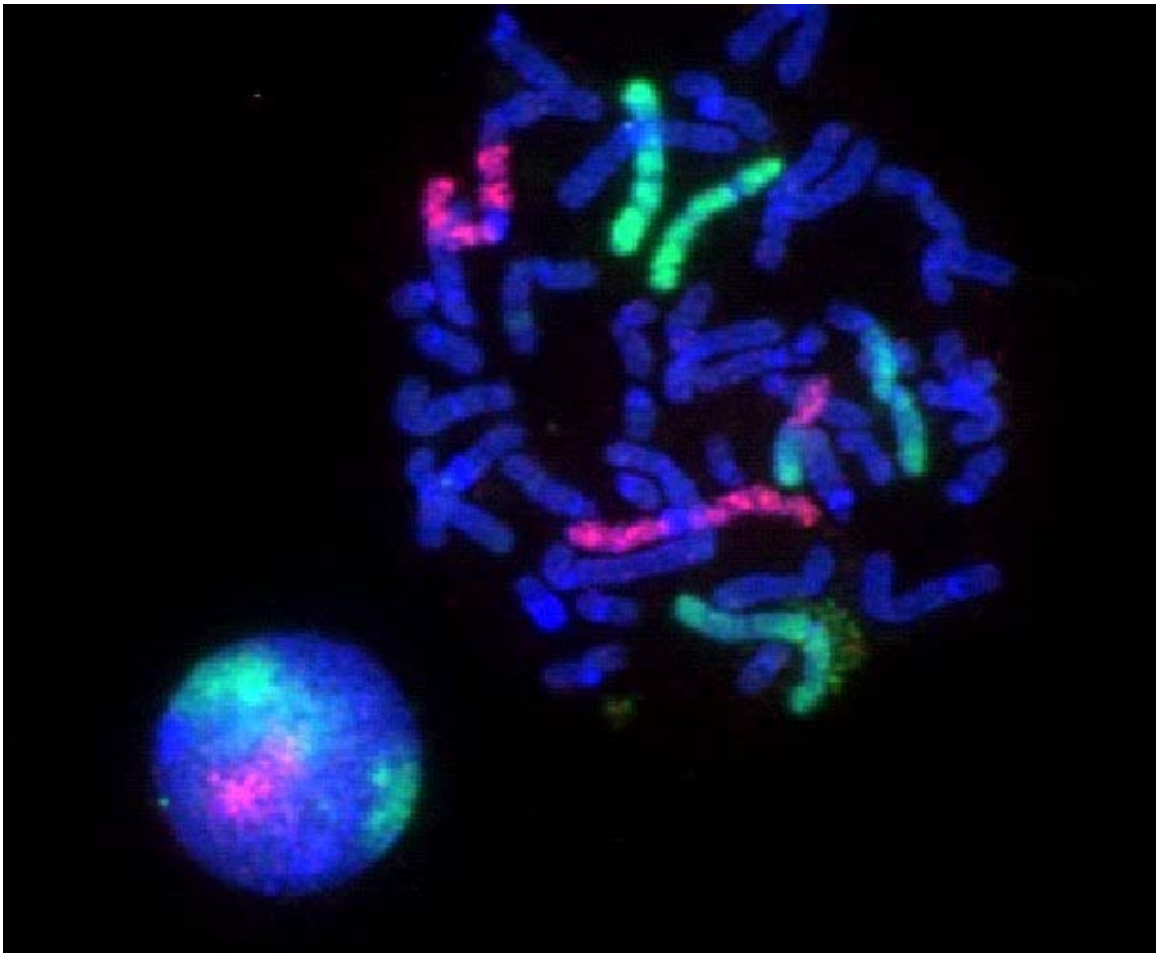
Super Resolution Microscopy: Single YFP molecule detection in a human cancer cell. Typical distance measurements in the 15 nm range (5 nm standard deviation) measured with a Vertico-SMI/SPDMphymod microscope



Super Resolution Microscopy: Co-localzation microscopy (2CLM) with GFP and RFP fusion proteins (nucleus of a bone cancer cell) 120.000 localized molecules in a wide-field area (470 μm^2) measured with a Vertico-SMI/SPDMphymod micrsocpe



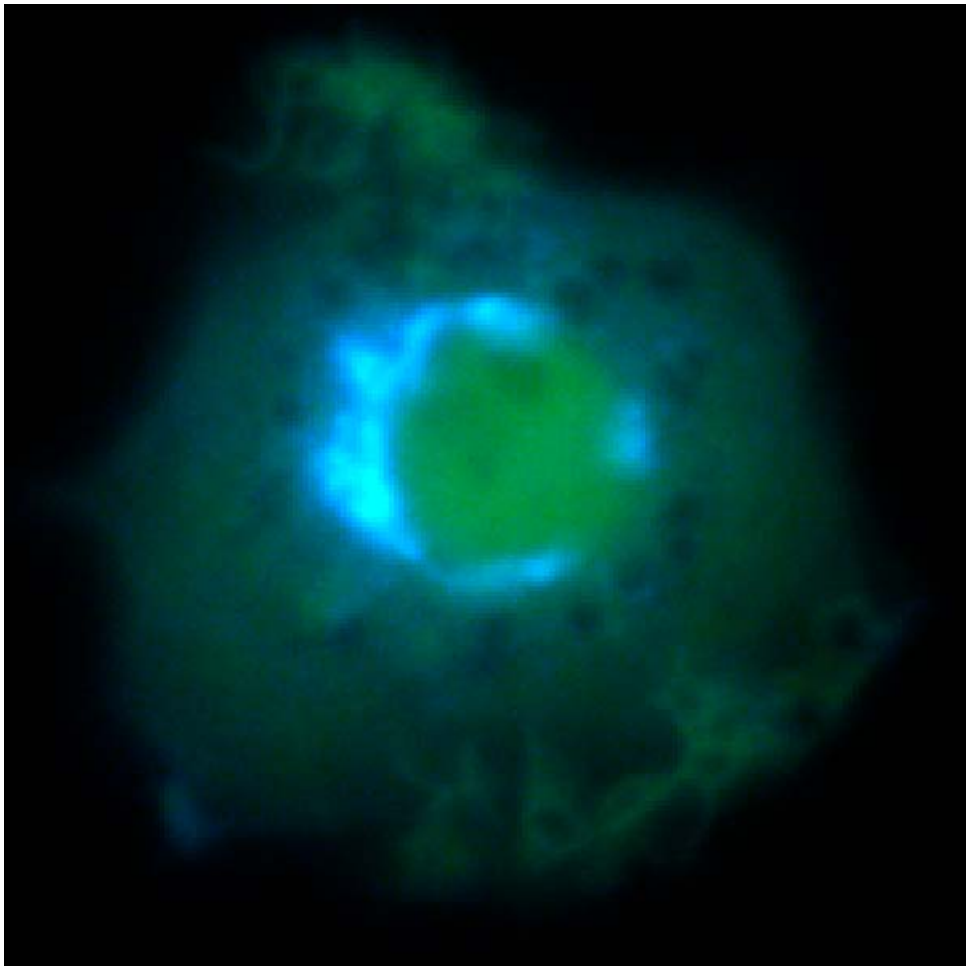
Fluorescence microscopy of DNA Expression in the Human Wild-Type and P239S Mutant Palladin.



Fluorescence microscopy images of sun flares pathology in a blood cell showing the affected areas in red.

Chapter 8

Förster Resonance Energy Transfer



Fluorescently-labeled guanosine 5'-triphosphate hydrolase ARF reveals the protein's localization in the Golgi apparatus of a living macrophage. FRET studies revealed ARF activation in the Golgi and in the formation of phagosomes.

Förster resonance energy transfer (abbreviated **FRET**), also known as **fluorescence resonance energy transfer**, **resonance energy transfer** (**RET**) or **electronic energy transfer** (**EET**), is a mechanism describing energy transfer between two chromophores.

A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore (in proximity, typically less than 10 nm) through nonradiative dipole–dipole coupling. This mechanism is termed "Förster resonance energy transfer" and is named after the German scientist Theodor Förster. When both chromophores are fluorescent, the term "fluorescence resonance energy transfer" is often used instead, although the energy is not actually transferred by fluorescence.[2] In order to avoid an erroneous interpretation of the phenomenon that (even when occurring between two fluorescent chromophores) is always a nonradiative transfer of energy, the name "Förster resonance energy transfer" is preferred to "fluorescence resonance energy transfer" – the latter enjoys common usage in scientific literature. FRET is analogous to near field communication, in that the radius of interaction is much smaller than the wavelength of light emitted. In the near field region, the excited chromophore emits a virtual photon that is instantly absorbed by a receiving chromophore. These virtual photons are undetectable, since their existence violates the conservation of energy and momentum, and hence FRET is known as a *radiationless* mechanism. From quantum electrodynamical calculations, it is determined that radiationless (FRET) and radiative energy transfer are the short- and long-range asymptotes of a single unified mechanism.[3]

Theoretical basis

The FRET efficiency (*E*) is the quantum yield of the energy transfer transition, *i.e.* the fraction of energy transfer event occurring per donor excitation event:

$$E = \frac{k_{ET}}{k_f + k_{ET} + \sum k_i}$$

where k_{ET} is the rate of energy transfer, k_f the radiative decay rate and the k_i are the rate constants of any other de-excitation pathway.

The FRET efficiency depends on many parameters that can be grouped as follows:

- The distance between the donor and the acceptor
- The spectral overlap of the donor emission spectrum and the acceptor absorption spectrum.
- The relative orientation of the donor emission dipole moment and the acceptor absorption dipole moment.

E depends on the donor-to-acceptor separation distance *r* with an inverse 6th power law due to the dipole-dipole coupling mechanism:

$$E = \frac{1}{1 + (r/R_0)^6}$$

with R_0 being the Förster distance of this pair of donor and acceptor, i.e. the distance at which the energy transfer efficiency is 50%. The Förster distance depends on the overlap

integral of the donor emission spectrum with the acceptor absorption spectrum and their mutual molecular orientation as expressed by the following equation:

$$R_0{}^6 = \frac{9000\, Q_0\, (\ln 10) \kappa^2\, J}{128\, \pi^5\, n^4\, N_A}$$

where Q_0 is the fluorescence quantum yield of the donor in the absence of the acceptor, κ^2 is the dipole orientation factor, n is the refractive index of the medium, N_A is Avogadro's number, and J is the spectral overlap integral calculated as

$$J = \int f_{\mathrm{D}}(\lambda)\, \epsilon_{\mathrm{A}}(\lambda)\, \lambda^4\, d\lambda$$

where f_{D} is the normalized donor emission spectrum, and ε_{A} is the acceptor molar extinction coefficient. κ^2 =2/3 is often assumed. This value is obtained when both dyes are freely rotating and can be considered to be isotropically oriented during the excited state lifetime. If either dye is fixed or not free to rotate, then κ^2 =2/3 will not be a valid assumption. In most cases, however, even modest reorientation of the dyes results in enough orientational averaging that κ^2 = 2/3 does not result in a large error in the estimated energy transfer distance due to the sixth power dependence of R_0 on κ^2. Even when κ^2 is quite different from 2/3 the error can be associated with a shift in R_0 and thus determinations of changes in relative distance for a particular system are still valid. Fluorescent proteins do not reorient on a timescale that is faster than their fluorescence lifetime. In this case $0 \leq \kappa^2 \leq 4$.

The FRET efficiency relates to the quantum yield and the fluorescence lifetime of the donor molecule as follows:

$$E = 1 - \tau'_{\mathrm{D}}/\tau_{\mathrm{D}}$$

where τ'_{D} and τ_{D} are the donor fluorescence lifetimes in the presence and absence of an acceptor, respectively, or as

$$E = 1 - F'_{\mathrm{D}}/F_{\mathrm{D}}$$

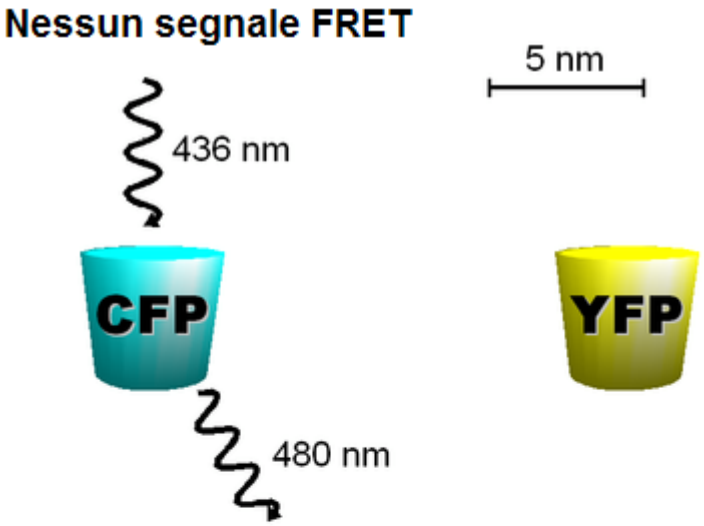
where F'_{D} and F_{D} are the donor fluorescence intensities with and without an acceptor, respectively.

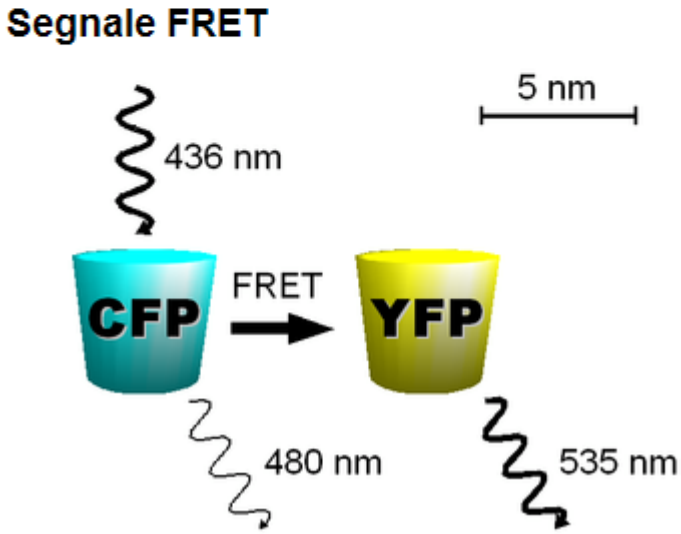
Experimental Confirmation of the Förster resonance energy transfer theory

The inverse sixth power distance dependence of Förster resonance energy transfer was experimentally confirmed by Stryer and Haugland using a donor and an acceptor separated on an oligoproline helix. Haugland, Yguerabide and Stryer also experimentally

demonstrated the theoretical dependence of Förster resonance energy transfer on the overlap integral by using a fused indolosteroid as a donor and a ketone as an acceptor.

Methods





Example of FRET between CFP and YFP (Wavelength vs. Absorption): a fusion protein containing CFP and YFP excited at 440nm wavelength. The fluorescent emission peak of CFP overlaps the excitation peak of YFP. Because the two proteins are adjacent to each other, the energy transfer is significant–a large proportion of the energy from CFP is transferred to YFP and creates a much larger YFP emission peak.

In fluorescence microscopy, fluorescence confocal laser scanning microscopy, as well as in molecular biology, FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein-protein interactions, protein-DNA interactions, and protein conformational changes. For monitoring the complex formation between two

molecules, one of them is labeled with a donor and the other with an acceptor, and these fluorophore-labeled molecules are mixed. When they are dissociated, the donor emission is detected upon the donor excitation. On the other hand, when the donor and acceptor are in proximity (1-10 nm) due to the interaction of the two molecules, the acceptor emission is predominantly observed because of the intermolecular FRET from the donor to the acceptor. For monitoring protein conformational changes, the target protein is labeled with a donor and an acceptor at two loci. When a twist or bend of the protein brings the change in the distance or relative orientation of the donor and acceptor, FRET change is observed. If a molecular interaction or a protein conformational change is dependent on ligand binding, this FRET technique is applicable to fluorescent indicators for the ligand detection.

FRET studies are scalable: the extent of energy transfer is often quantified from the milliliter scale of cuvette-based experiments to the femtoliter scale of microscopy-based experiments. This quantification can be based directly (sensitized emission method) on detecting two emission channels under two different excitation conditions (primarily donor and primarily acceptor). However, for robustness reasons, FRET quantification is most often based on measuring changes in fluorescence intensity or fluorescence lifetime upon changing the experimental conditions (e.g. a microscope image of donor emission is taken with the acceptor being present. The acceptor is then bleached, such that it is incapable of accepting energy transfer and another donor emission image is acquired. A pixel-based quantification using the second equation in the theory section above is then possible.) An alternative way of temporarily deactivating the acceptor is based on its fluorescence saturation. Exploiting polarisation characteristics of light, a FRET quantification is also possible with only a single camera exposure.

CFP-YFP pairs

The most popular FRET pair for biological use is a cyan fluorescent protein (**CFP**) - yellow fluorescent protein (**YFP**) pair. Both are color variants of green fluorescent protein (**GFP**). While labeling with organic fluorescent dyes requires troublesome processes of purification, chemical modification, and intracellular injection of a host protein, GFP variants can be easily attached to a host protein by genetic engineering. By virtue of GFP variants, the use of FRET techniques for biological research is becoming more and more popular.

BRET

A limitation of FRET is the requirement for external illumination to initiate the fluorescence transfer, which can lead to background noise in the results from direct excitation of the acceptor or to photobleaching. To avoid this drawback, Bioluminescence Resonance Energy Transfer (or **BRET**) has been developed. This technique uses a bioluminescent luciferase (typically the luciferase from *Renilla reniformis*) rather than CFP to produce an initial photon emission compatible with YFP.

FRET and BRET are also the common tools in the study of biochemical reaction kinetics and molecular motors.

Photobleaching FRET

FRET efficiencies can also be inferred from the photobleaching rates of the donor in the presence and absence of an acceptor. This method can be performed on most fluorescence microscopes; one simply shines the excitation light (of a frequency that will excite the donor but not the acceptor significantly) on specimens with and without the acceptor fluorophore and monitors the donor fluorescence (typically separated from acceptor fluorescence using a bandpass filter) over time. The timescale is that of photobleaching, which is seconds to minutes, with fluorescence in each curve being given by

$$(\text{background}) + (\text{constant}) * e^{-(\text{time})/\tau_{\text{pb}}}$$

where τ_{pb} is the photobleaching decay time constant and depends on whether the acceptor is present or not. Since photobleaching consists in the permanent inactivation of excited fluorophores, resonance energy transfer from an excited donor to an acceptor fluorophore prevents the photobleaching of that donor fluorophore, and thus high FRET efficiency leads to a longer photobleaching decay time constant:

$$E = 1 - \tau_{\text{pb}}/\tau'_{\text{pb}}$$

where τ'_{pb} and τ_{pb} are the photobleaching decay time constants of the donor in the presence and in the absence of the acceptor, respectively. (Notice that the fraction is the reciprocal of that used for lifetime measurements).

This technique was introduced by Jovin in 1989. Its use of an entire curve of points to extract the time constants can give it accuracy advantages over the other methods. Also, the fact that time measurements are over seconds rather than nanoseconds makes it easier than fluorescence lifetime measurements, and because photobleaching decay rates do not generally depend on donor concentration (unless acceptor saturation is an issue), the careful control of concentrations needed for intensity measurements is not needed. It is, however, important to keep the illumination the same for the with- and without-acceptor measurements, as photobleaching increases markedly with more intense incident light.

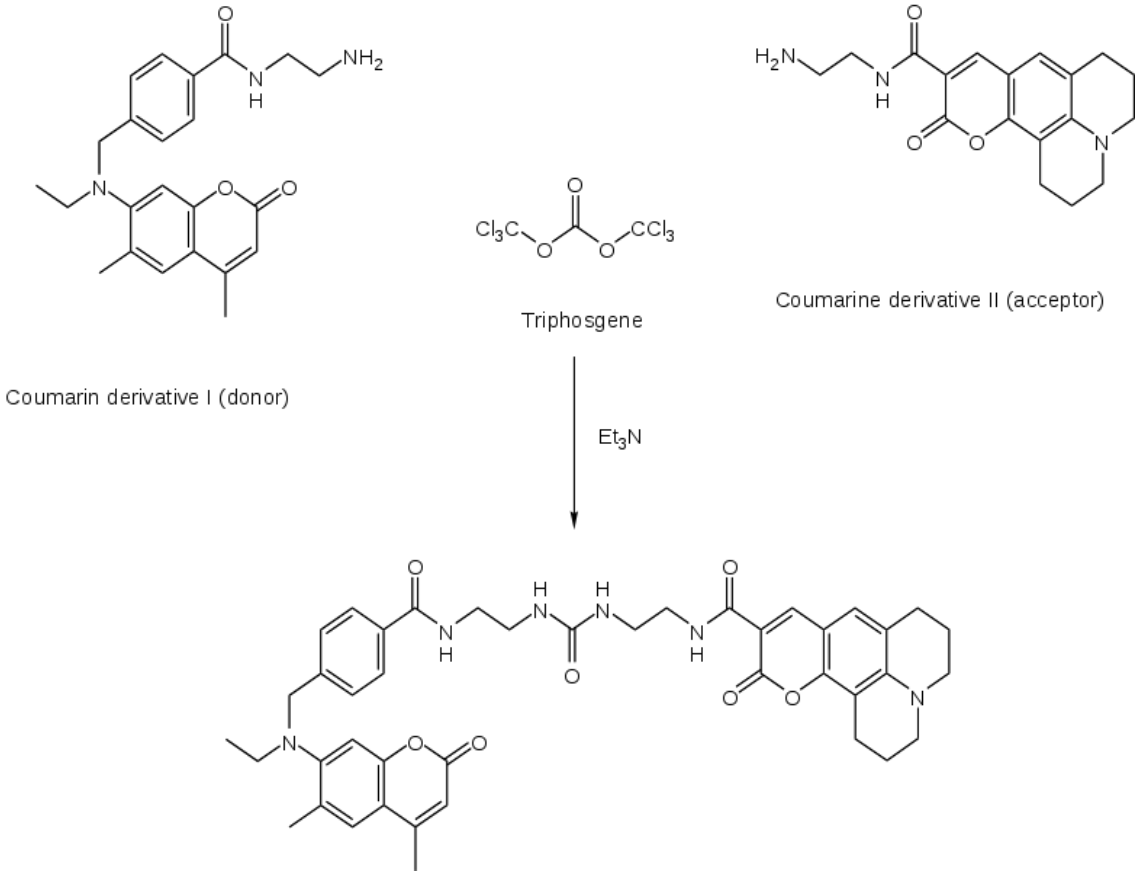
Other methods

A different, but related, mechanism is Dexter Electron Transfer.

An alternative method to detecting protein-protein proximity is the bimolecular fluorescence complementation (BiFC) where two halves of a YFP are fused to a protein. When these two halves meet they form a fluorophore after about 60 s - 1 hr.

Application

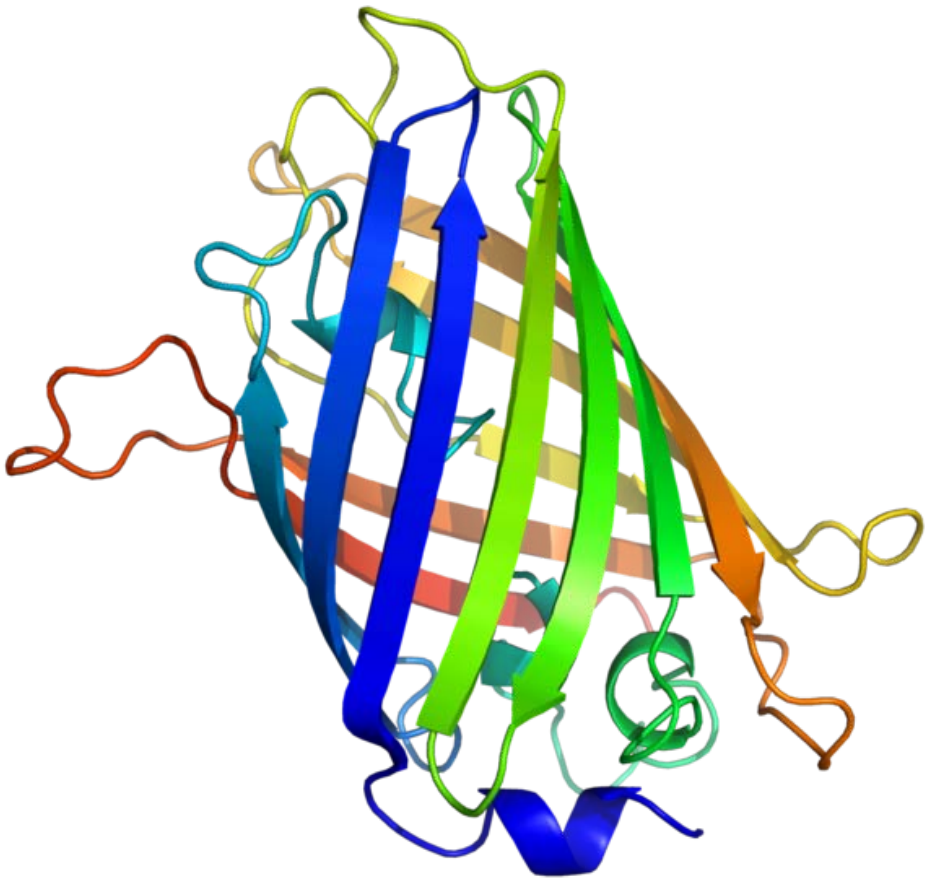
FRET has been applied in an experimental method for the detection of phosgene. In it, phosgene or rather triphosgene as a safe substitute serves as a linker between an acceptor and a donor coumarine (forming urea groups). The presence of phosgene is detected at $5x10^{-5}$M with a typical FRET emission at 464 nm.



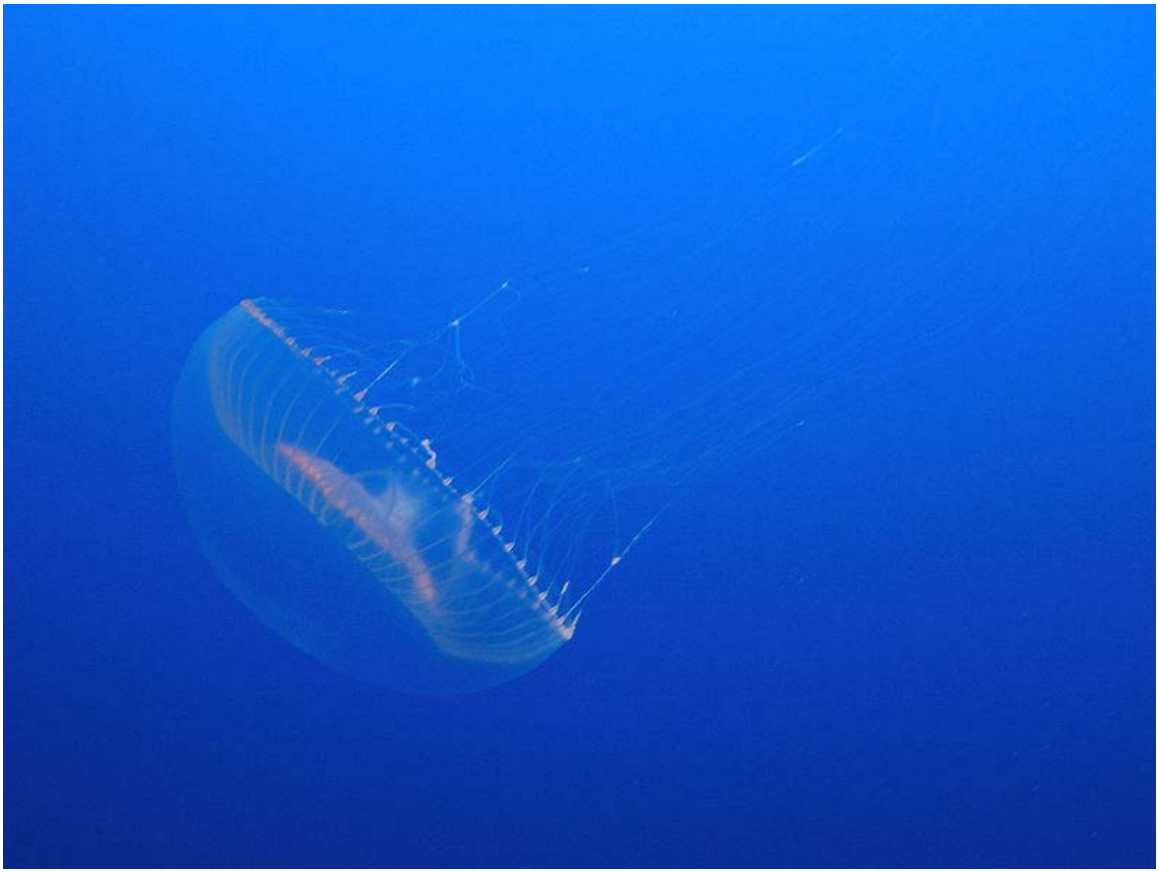
FRET is also used to study lipid rafts in cell membranes.

Chapter 9

Green Fluorescent Protein



GFP ribbon diagram. From PDB 1EMA.



Aequorea victoria

The **green fluorescent protein** (**GFP**) is a protein composed of 238 amino acid residues (26.9kDa) that exhibits bright green fluorescence when exposed to blue light. Although many other marine organisms have similar green fluorescent proteins, GFP traditionally refers to the protein first isolated from the jellyfish *Aequorea victoria*. The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm, which is in the lower green portion of the visible spectrum. The GFP from the sea pansy (*Renilla reniformis*) has a single major excitation peak at 498 nm. In cell and molecular biology, the GFP gene is frequently used as a reporter of expression. In modified forms it has been used to make biosensors, and many animals have been created that express GFP as a proof-of-concept that a gene can be expressed throughout a given organism. The GFP gene can be introduced into organisms and maintained in their genome through breeding, injection with a viral vector, or cell transformation. To date, the GFP gene has been introduced and expressed in many bacteria, yeast and other fungi, fish (such as zebrafish), plant, fly, and mammalian cells, including human. Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien were awarded the 2008 Nobel Prize in Chemistry on 10 October 2008 for their discovery and development of the green fluorescent protein.

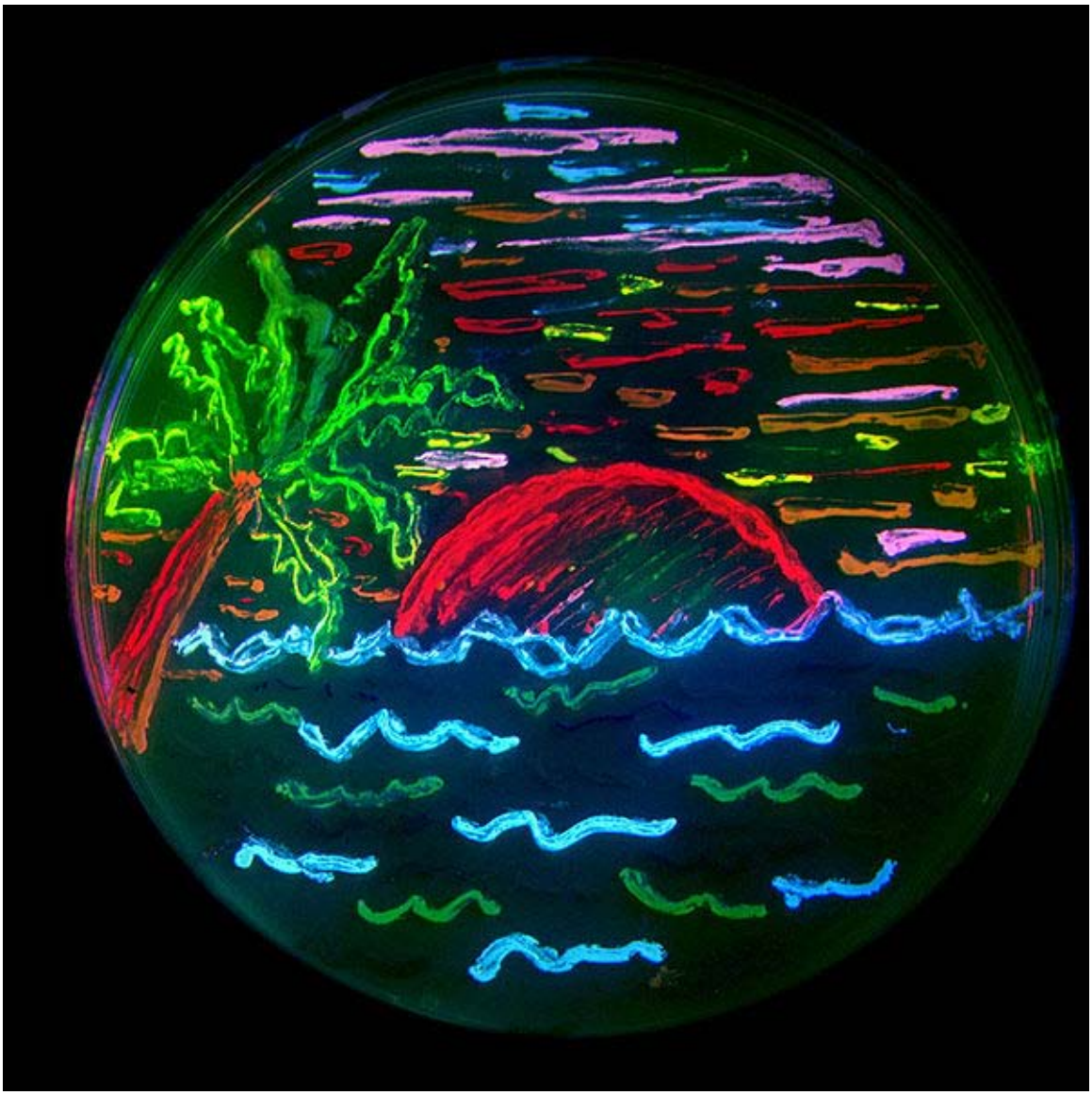
History

Wild-type GFP (wtGFP)

In the 1960s and 1970s, GFP, along with the separate luminescent protein aequorin, was first purified from *Aequorea victoria* and its properties studied by Osamu Shimomura. In *A. victoria*, GFP fluorescence occurs when aequorin interacts with Ca^{2+} ions, inducing a blue glow. Some of this luminescent energy is transferred to the GFP, shifting the overall color towards green. However, its utility as a tool for molecular biologists did not begin to be realized until 1992 when Douglas Prasher reported the cloning and nucleotide sequence of wtGFP in *Gene*. The funding for this project had run out, so Prasher sent cDNA samples to several labs. The lab of Martin Chalfie expressed the coding sequence of wtGFP, with the first few amino acids deleted, in heterologous cells of *E. coli* and *C. elegans*, publishing the results in *Science* in 1994. Frederick Tsuji's lab independently reported the expression of the recombinant protein one month later. Remarkably, the GFP molecule folded and was fluorescent at room temperature, without the need for exogenous cofactors specific to the jellyfish. Although this near-wtGFP was fluorescent, it had several drawbacks, including dual peaked excitation spectra, pH sensitivity, chloride sensitivity, poor fluorescence quantum yield, poor photostability and poor folding at 37°C.

The first reported crystal structure of a GFP was that of the S65T mutant by the Remington group in *Science* in 1996. One month later, the Phillips group independently reported the wild-type GFP structure in *Nature Biotech*. These crystal structures provided vital background on chromophore formation and neighboring residue interactions. Researchers have modified these residues by directed and random mutagenesis to produce the wide variety of GFP derivatives in use today. Martin Chalfie, Osamu Shimomura and Roger Y. Tsien share the 2008 Nobel Prize in Chemistry for their discovery and development of the green fluorescent protein.

GFP derivatives



The diversity of genetic mutations is illustrated by this San Diego beach scene drawn with living bacteria expressing 8 different colors of fluorescent proteins.

Due to the potential for widespread usage and the evolving needs of researchers, many different mutants of GFP have been engineered. The first major improvement was a single point mutation (S65T) reported in 1995 in *Nature* by Roger Tsien. This mutation dramatically improved the spectral characteristics of GFP, resulting in increased fluorescence, photostability, and a shift of the major excitation peak to 488 nm, with the peak emission kept at 509 nm. This matched the spectral characteristics of commonly available FITC filter sets, increasing the practicality of use by the general researcher. A 37 °C folding efficiency (F64L) point mutant to this scaffold yielding enhanced GFP (EGFP) was discovered in 1995 by the lab of Ole Thastrup. EGFP allowed the practical use of GFPs in mammalian cells. EGFP has an extinction coefficient (denoted ε) of

55,000 $M^{-1}cm^{-1}$. The fluorescence quantum yield (QY) of EGFP is 0.60. The relative brightness, expressed as ε•QY, is 33,000 $M^{-1}cm^{-1}$. Superfolder GFP, a series of mutations that allow GFP to rapidly fold and mature even when fused to poorly folding peptides, was reported in 2006.

Many other mutations have been made, including color mutants; in particular, blue fluorescent protein (EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein (ECFP, Cerulean, CyPet), and yellow fluorescent protein derivatives (YFP, Citrine, Venus, YPet). BFP derivatives (except mKalama1) contain the Y66H substitution. The critical mutation in cyan derivatives is the Y66W substitution, which causes the chromophore to form with an indole rather than phenol component. Several additional compensatory mutations in the surrounding barrel are required to restore brightness to this modified chromophore due to the increased bulk of the indole group. The red-shifted wavelength of the YFP derivatives is accomplished by the T203Y mutation and is due to π-electron stacking interactions between the substituted tyrosine residue and the chromophore. These two classes of spectral variants are often employed for fluorescence resonance energy transfer (FRET) experiments. Genetically-encoded FRET reporters sensitive to cell signaling molecules, such as calcium or glutamate, protein phosphorylation state, protein complementation, receptor dimerization, and other processes provide highly specific optical readouts of cell activity in real time.

Semirational mutagenesis of a number of residues led to pH-sensitive mutants known as pHluorins, and later super-ecliptic pHluorins. By exploiting the rapid change in pH upon synaptic vesicle fusion, pHluorins tagged to synaptobrevin have been used to visualize synaptic activity in neurons.

Redox sensitive versions of GFP (roGFP) were engineered by introduction of cysteines into the beta barrel structure. The redox state of the cysteines determines the fluorescent properties of roGFP.

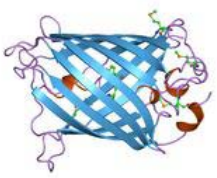
The nomenclature of modified GFPs is often confusing due to overlapping mapping of several GFP versions onto a single name. For example, **mGFP** often refers to a GFP with an N-terminal palmitoylation that causes the GFP to bind to cell membranes. However, the same term is also used to refer to monomeric GFP, which is often achieved by the dimer interface breaking A206K mutation. Wild-type GFP has a weak dimerization tendency at concentrations above 5 mg/mL. mGFP also stands for "modified GFP," which has been optimized through amino acid exchange for stable expression in plant cells.

Structure

GFP has a typical beta barrel structure, consisting of one β-sheet with alpha helix(s) containing the chromophore running through the center. Inward-facing sidechains of the barrel induce specific cyclization reactions in the tripeptide Ser65–Tyr66–Gly67 that lead to chromophore formation. This process of post-translational modification is referred to as *maturation*. The hydrogen-bonding network and electron-stacking interactions with

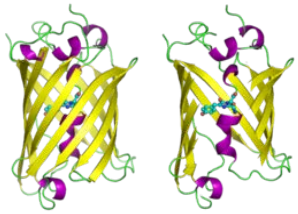
these sidechains influence the color of wtGFP and its numerous derivatives. The tightly packed nature of the barrel excludes solvent molecules, protecting the chromophore fluorescence from quenching by water.

Green fluorescent protein



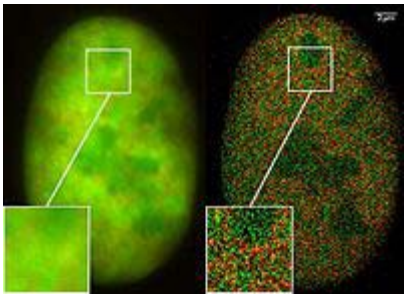
Structure of the Aequorea victoria green fluorescent protein.

Identifiers	
Symbol	GFP
Pfam	PF01353
Pfam clan	CL0069
InterPro	IPR011584
SCOP	1ema
Available protein structures:	



GFP molecules drawn in cartoon style, one fully and one with the side of the beta barrel cut away to reveal the chromophore (highlighted as ball-and-stick). From PDB 1GFL.

Use



Superresolution with two fusion proteins (GFP-Snf2H and RFP-H2A), Co-localisation studies (2CLM) in the nucleus of a bone cancer cell. 120.000 localized molecules in a widefield area(470 µm2)

The availability of GFP and its derivatives has thoroughly redefined fluorescence microscopy and the way it is used in cell biology and other biological disciplines. While most small fluorescent molecules such as FITC (fluorescein isothiocyanate) are strongly

phototoxic when used in live cells, fluorescent proteins such as GFP are usually much less harmful when illuminated in living cells. This has triggered the development of highly automated live-cell fluorescence microscopy systems, which can be used to observe cells over time expressing one or more proteins tagged with fluorescent proteins. For example, GFP had been widely used in labelling the spermatozoa of various organisms for identification purposes as in *Drosophila melanogaster*, where expression of GFP can be used as a marker for a particular characteristic. GFP can also be expressed in different structures enabling morphological distinction. In such cases, the gene for the production of GFP is spliced into the genome of the organism in the region of the DNA that codes for the target proteins and that is controlled by the same regulatory sequence; that is, the gene's regulatory sequence now controls the production of GFP, in addition to the tagged protein(s). In cells where the gene is expressed, and the tagged proteins are produced, GFP is produced at the same time. Thus, only those cells in which the tagged gene is expressed, or the target proteins are produced, will fluoresce when observed under fluorescence microscopy. Analysis of such time lapse movies has redefined the understanding of many biological processes including protein folding, protein transport, and RNA dynamics, which in the past had been studied using fixed (i.e., dead) material.

The Vertico SMI microscope using the SPDM Phymod technology uses the so-called "reversible photobleaching" effect of fluorescent dyes like GFP and its derivatives to localize them as single molecules in an optical resolution of 10 nm. This can also be performed as a co-localization of two GFP derivatives (2CLM).

Another powerful use of GFP is to express the protein in small sets of specific cells. This allows researchers to optically detect specific types of cells *in vitro* (in a dish), or even *in vivo* (in the living organism). Genetically combining several spectral variants of GFP is a useful trick for the analysis of brain circuitry (Brainbow). Other interesting uses of fluorescent proteins in the literature include using FPs as sensors of neuron membrane potential, tracking of AMPA receptors on cell membranes, viral entry and the infection of individual influenza viruses and lentiviral viruses, etc.

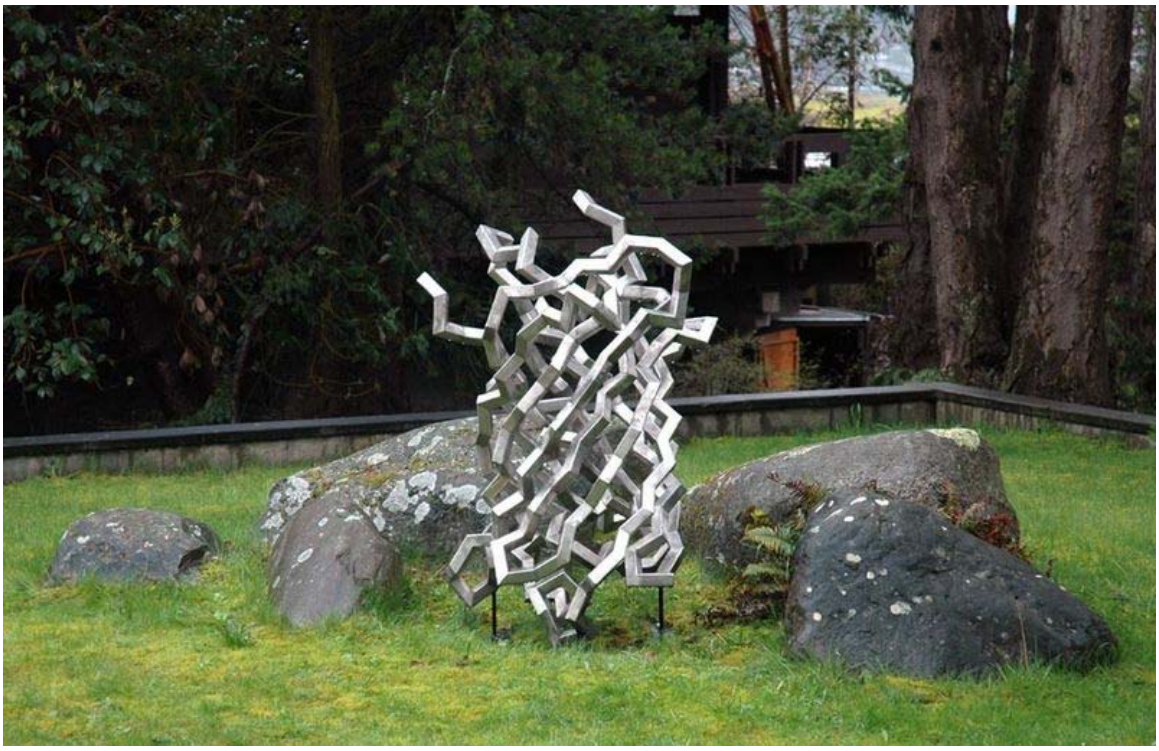
It has also been found that new lines of transgenic GFP rats can be relevant for gene therapy as well as regenerative medicine. By using "high-expresser" GFP, transgenic rats display high expression in most tissues, and many cells that have not been characterized or have been only poorly characterized in previous GFP-transgenic rats. Through its ability to form internal chromophore without requiring accessory cofactors, enzymes or substrates other than molecular oxygen, GFP makes for an excellent tool in all forms of biology.

GFP in nature

The purpose of both bioluminescence and GFP fluorescence in jellyfish is unknown. GFP is co-expressed with aequorin in small granules around the rim of the jellyfish bell. The secondary excitation peak (480 nm) of GFP does absorb some of the blue emission of aequorin, giving the bioluminescence a more green hue. The serine 65 residue of the GFP chromophore is responsible for the dual-peaked excitation spectra of wild-type GFP. It is

conserved in all three GFP isoforms originally cloned by Prasher. Nearly all mutations of this residue consolidate the excitation spectra to a single peak at either 395 nm or 480 nm. The precise mechanism of this sensitivity is complex, but, it seems, involves donation of a hydrogen from serine 65 to glutamate 222, which influences chromophore ionization. Since a single mutation can dramatically enhance the 480 nm excitation peak, making GFP a much more efficient partner of aequorin, *A. victoria* appears to evolutionarily prefer the less-efficient, dual-peaked excitation spectrum. Roger Tsien has speculated that varying hydrostatic pressure with depth may effect serine 65's ability to donate a hydrogen to the chromophore and shift the ratio of the two excitation peaks. Thus, the jellyfish may change the color of its bioluminescence with depth. However, a collapse in the population of jellyfish in Friday Harbor, where GFP was originally discovered, has hampered further study of the role of GFP in the jellyfish's natural environment.

GFP in fine art



Julian Voss-Andreae's GFP-based sculpture *Steel Jellyfish* (2006). The image shows the stainless-steel sculpture on display at Friday Harbor Laboratories on San Juan Island (Wash., USA), the place of GFP's discovery.

Julian Voss-Andreae, a German-born artist specializing in "protein sculptures," created sculptures based on the structure of GFP, including the 5'6" (1.70 m) tall "Green Fluorescent Protein" (2004) and the 4'7" (1.40 m) tall "Steel Jellyfish" (2006). The latter sculpture is currently located at the place of GFP's discovery by Shimomura in 1962, the University of Washington's Friday Harbor Laboratories.

Eduardo Kac has also done some work with GFP, the most notable one being the "GFP Bunny," Alba. Kac commissioned a French laboratory to create a green-fluorescent rabbit, which is the subject of a series of his art pieces.

Transgenic pets

Alba, a fluorescent rabbit, was commissioned by Eduardo Kac using GFP for purposes of art and social commentary. The US company Yorktown Technologies markets to aquarium shops green fluorescent zebrafish (GloFish) that were initially developed to detect pollution in waterways. NeonPets, a US based company markets green fluorescent mice to the pet industry as NeonMice. Green fluorescent pigs, known as Noels were bred by a group of researchers led by Wu Shinn-Chih at the Department of Animal Science and Technology at National Taiwan University.

Chapter 10

Fluorescence Lifetime Imaging Microscopy

Fluorescence Lifetime Imaging Microscopy or **FLIM** is an imaging technique for producing an image based on the differences in the exponential decay rate of the fluorescence from a fluorescent sample. It can be used as an imaging technique in confocal microscopy, Two-photon excitation microscopy, and multiphoton tomography.

The lifetime of the fluorophore signal, rather than its intensity, is used to create the image in FLIM. This has the advantage of minimizing the effect of photon scattering in thick layers of sample.

Fluorescence lifetimes

A fluorophore which is excited by a photon will drop to the ground state with a certain probability based on the decay rates through a number of different (radiative and/or nonradiative) decay pathways. To observe fluorescence, one of these pathways must be by spontaneous emission of a photon. In the ensemble description, the fluorescence emitted will decay with time according to

$$F(t) = F_0 e^{-t/\tau}$$

where

$$\frac{1}{\tau} = \sum_f w f i k_i .$$

In the above, t is time, τ is the fluorescence lifetime, F_0 is the initial fluorescence at $t = 0$, and k_i are the rates for each decay pathway, at least one of which must be the fluorescence decay rate k_f. More importantly, the lifetime, τ, is independent of the initial intensity of the emitted light. This can be utilized for making non-intensity based measurements in chemical sensing.

Measurement and processing

Fluorescence lifetime imaging yields images with the intensity of each pixel determined by τ, which allows one to view contrast between materials with different fluorescence decay rates (even if those materials fluoresce at exactly the same wavelength), and also produces images which show changes in other decay pathways, such as in FRET imaging.

Pulsed illumination

Fluorescence lifetimes can be determined in the time domain by using a pulsed source. When a population of fluorophores is excited by an ultrashort or delta pulse of light, the time-resolved fluorescence will decay exponentially as described above. However, if the excitation pulse or detection response is wide, the measured fluorescence, M(t), will not be purely exponential. The instrumental response function, IRF(t) will be convolved or blended with the decay function, F(t).

$$M(t) = IRF(t) \otimes F(t)$$

The decay function (and corresponding lifetimes) cannot be recovered by direct deconvolution using Fourier transforms because division by zero will produce errors and noise will be amplified. However, the instrumental response of the source, detector, and electronics can be measured, usually from scattered excitation light. The IRF can then be convolved with a trial decay function to produce a calculated fluorescence, which can be compared to the measured fluorescence. The parameters for the trial decay function can be varied until the calculated and measured fluorescence curves fit well. This process is known as reconvolution or reiterative convolution, and can be performed quickly by several software packages.

TCSPC

Time-correlated single photon counting (TCSPC) is usually employed because variations in source intensity and photoelectron amplitudes are ignored, the time resolution can be upwards of 4 ps, and the data obeys Poisson statistics (useful in determining goodness of fit during reconvolution). More specifically, TCSPC records times at which individual photons are detected by something like a photo-multiplier tube (PMT) or an avalanche photo diode (APD) after a single pulse. The recordings are repeated for additional pulses and after enough recorded events, one is able to build a histogram of the number of events across all of these recorded time points. This histogram can then be fit to an exponential function that contains the exponential lifetime decay function of interest, and the lifetime parameter can accordingly be extracted.

Gating Method

Pulse excitation is still used in this method. Before the pulse reaches the sample though, some of the light is reflected by a dichroic mirror and gets detected by a photodiode that

activates a delay generator controlling a gated optical intensifier (GOI) that sits in front of your CCD detector. The GOI only allows for detection for the fraction of time when it is open after the delay. Thus, with an adjustable delay generator, one is able to collect fluorescence emission after multiple delay times encompassing the time range of the fluorescence decay of the sample.

Phase modulation

Alternatively, fluorescence lifetimes can be determined in the frequency domain by a phase-modulated method. The intensity of a continuous wave source is modulated at high frequency, by an acousto-optic modulator for example, which will modulate the fluorescence. Since the excited state has a lifetime, the fluorescence will be delayed with respect to the excitation signal, and the lifetime can be determined from the phase shift. Also, y-components to the excitation and fluorescence sine waves will be modulated, and lifetime can be determined from the modulation ratio of these y-components. Hence, 2 values for the lifetime can be determined from the phase-modulation method. Consequently, if the lifetimes that are extracted from the y-component and the phase do not match, it means that you have more than one lifetime species in your sample.

Applications

FLIM has primarily been used in biology as a method to detect photosensitizers in cells and tumors as well as FRET in instances where ratiometric imaging is difficult. The technique was developed in the late 1980s and early 1990s (Bugiel et al. 1989. König 1989 , before being more widely applied in the late 1990s. In cell culture, it has been used to study EGF receptor signaling and ErbB1 receptor trafficking. FLIM imaging is particularly useful in neurons, where light scattering by brain tissue is problematic for ratiometric imaging. In neurons, FLIM imaging using pulsed illumination has been used to study Ras, CaMKII, Rac, and Ran family proteins. FLIM has been used in clinical multiphoton tomography to detect intradermal cancer cells as well as pharmaceutical and cosmetical compounds.

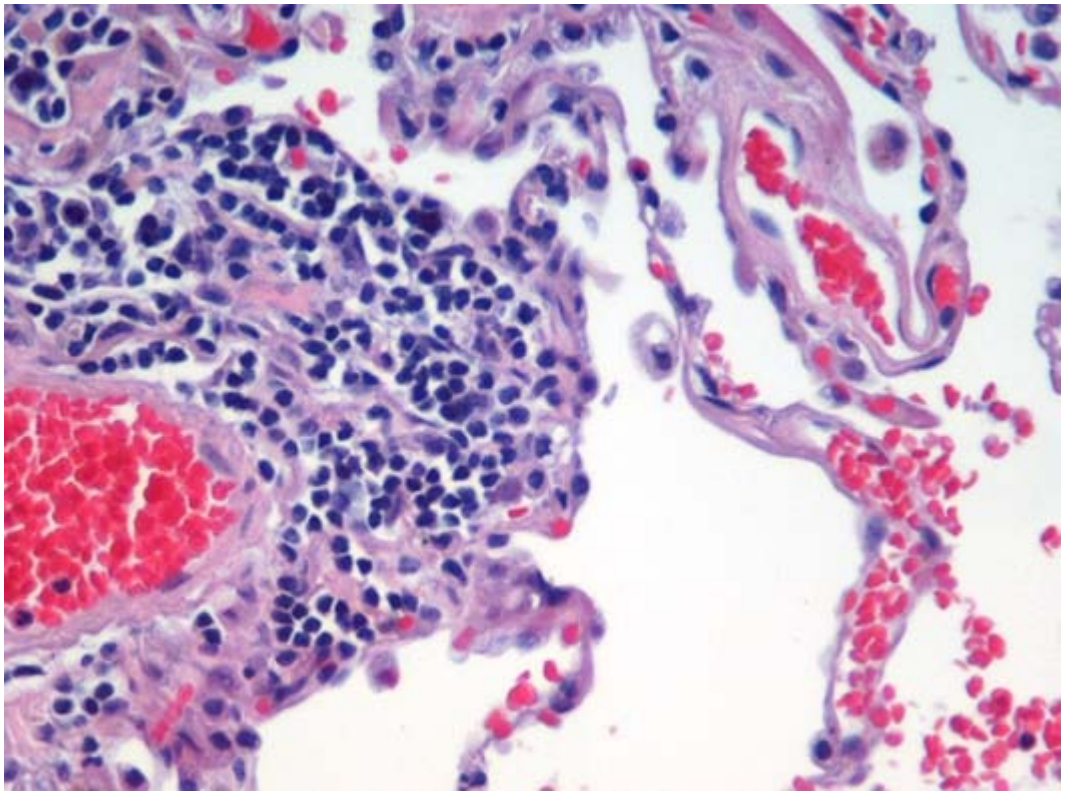
FRET Imaging

Since the fluorescence lifetime of a fluorophore depends on both radiative (i.e. fluorescence) and non-radiative (i.e. quenching, FRET) processes, energy transfer from the donor molecule to the acceptor molecule will decrease the lifetime of the donor. Thus, FRET measurements using FLIM can provide a method to discriminate between the states/environments of the fluorophore.. In contrast to intensity-based FRET measurements, the FLIM-based FRET measurements are also insensitive to the concentration of fluorophores and can thus filter out artifacts introduced by variations in the concentration and emission intensity across the sample.

Chapter 11

Histology

A stained histologic specimen, sandwiched between a glass microscope slide and coverslip, mounted on the stage of a light microscope.



Microscopic view of a histologic specimen of human lung tissue stained with hematoxylin and eosin.

Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. It is performed by examining a thin slice (section) of tissue under a light microscope or electron microscope. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains. Histology is an essential tool of biology and medicine.

Histopathology, the microscopic study of diseased tissue, is an important tool in anatomical pathology, since accurate diagnosis of cancer and other diseases usually requires histopathological examination of samples. Trained medical doctors, frequently board-certified as pathologists, are the personnel who perform histopathological examination and provide diagnostic information based on their observations.

The trained scientists who perform the preparation of histological sections are *histotechnicians,* histology technicians (HT), histology technologists (HTL), medical scientists, medical laboratory technicians, or biomedical scientists. Their field of study is called *histotechnology*.

Histology

Fixing

Chemical fixation with formaldehyde or other chemicals

Chemical fixatives are used to preserve tissue from degradation, and to maintain the structure of the cell and of sub-cellular components such as cell organelles (e.g., nucleus, endoplasmic reticulum, mitochondria). The most common fixative for light microscopy is 10% neutral buffered formalin (4% formaldehyde in phosphate buffered saline). For electron microscopy, the most commonly used fixative is glutaraldehyde, usually as a 2.5% solution in phosphate buffered saline. These fixatives preserve tissues or cells mainly by irreversibly cross-linking proteins. The main action of these aldehyde fixatives is to cross-link amino groups in proteins through the formation of CH_2 (methylene) linkage, in the case of formaldehyde, or by a C5H10 cross-links in the case of glutaraldehyde. This process, while preserving the structural integrity of the cells and tissue can damage the biological functionality of proteins, particularly enzymes, and can also denature them to a certain extent. This can be detrimental to certain histological techniques. Further fixatives are often used for electron microscopy such as osmium tetroxide or uranyl acetate

Formalin fixation leads to degradation of mRNA, miRNA and DNA in tissues. However, extraction, amplification and analysis of these nucleic acids from formalin-fixed, paraffin-embedded tissues is possible using appropriate protocols.

Frozen section fixation

Frozen section is a rapid way to fix and mount histology sections. It is used in surgical removal of tumors, and allow rapid determination of margin (that the tumor has been completely removed). It is done using a refrigeration device called a cryostat. The frozen tissue is sliced using a microtome, and the frozen slices are mounted on a glass slide and stained the same way as other methods. It is a necessary way to fix tissue for certain stain such as antibody linked immunofluorescence staining. It can also be used to determine if a tumour is malignant when it is found incidentally during surgery on a patient.

Processing - dehydration, clearing, and infiltration

The aim of Tissue Processing is to remove water from tissues and replace with a medium that solidifies to allow thin sections to be cut. Biological tissue must be supported in a hard matrix to allow sufficiently thin sections to be cut, typically 5 μm (micrometres; 1000 micrometres = 1 mm) thick for light microscopy and 80-100 nm (nanometre; 1,000,000 nanometres = 1 mm) thick for electron microscopy. For light microscopy, paraffin wax is most frequently used. Since it is immiscible with water, the main constituent of biological tissue, water must first be removed in the process of dehydration. Samples are transferred through baths of progressively more concentrated ethanol to remove the water. This is followed by a hydrophobic clearing agent (such as

xylene) to remove the alcohol, and finally molten paraffin wax, the infiltration agent, which replaces the xylene. Paraffin wax does not provide a sufficiently hard matrix for cutting very thin sections for electron microscopy. Instead, resins are used. Epoxy resins are the most commonly employed embedding media, but acrylic resins are also used, particularly where immunohistochemistry is required. Thicker sections (0.35µm to 5µm) of resin-embedded tissue can also be cut for light microscopy. Again, the immiscibility of most epoxy and acrylic resins with water necessitates the use of dehydration, usually with ethanol.

Embedding

After the tissues have been dehydrated, cleared, and infiltrated with the embedding material, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material (such as agar, gelatine, or wax) which is then hardened. This is achieved by cooling in the case of paraffin wax and heating (curing) in the case of the epoxy resins. The acrylic resins are polymerised by heat, ultraviolet light, or chemical catalysts. The hardened blocks containing the tissue samples are then ready to be sectioned.

Because Formalin-fixed, paraffin-embedded (FFPE) tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA and RNA) may be recovered from them decades after fixation, FFPE tissues are an important resource for historical studies in medicine.

Embedding can also be accomplished using frozen, non-fixed tissue in a water-based medium. Pre-frozen tissues are placed into molds with the liquid embedding material, usually a water-based glycol, OCT, TBS, Cryogel, or resin, which is then frozen to form hardened blocks.

Sectioning

Sectioning can be done in limited ways. Vertical sectioning perpendicular to the surface of the tissue is the usual method. Horizontal sectioning is often done in the evaluation of the hair follicles and pilosebaceous units. Tangential to horizontal sectioning is done in Mohs surgery and in methods of CCPDMA.

For light microscopy, a steel knife mounted in a microtome is used to cut 10-micrometer-thick tissue sections which are mounted on a glass microscope slide. For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections which are mounted on a 3-millimeter-diameter copper grid. Then the mounted sections are treated with the appropriate stain.

Frozen tissue embedded in a freezing medium is cut on a microtome in a cooled machine called a cryostat.

Staining

Biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used. Hematoxylin and eosin (H&E stain) is the most commonly used light microscopical stain in histology and histopathology. Hematoxylin, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink. Uranyl acetate and lead citrate are commonly used to impart contrast to tissue in the electron microscope.

Special staining: There are hundreds of various other techniques that have been used to selectively stain cells and cellular components. Other compounds used to color tissue sections include safranin, oil red o, Congo red, fast green FCF, silver salts, and numerous natural and artificial dyes that were usually originated from the development dyes for the textile industry.

Histochemistry refers to the science of using chemical reactions between laboratory chemicals and components within tissue. A commonly performed histochemical technique is the Perls Prussian blue reaction, used to demonstrate iron deposits in diseases like hemochromatosis.

Histology samples have often been examined by radioactive techniques. In historadiography, a slide (sometimes stained histochemically) is X-rayed. More commonly, autoradiography is used to visualize the locations to which a radioactive substance has been transported within the body, such as cells in S phase (undergoing DNA replication) which incorporate tritiated thymidine, or sites to which radiolabeled nucleic acid probes bind in in situ hybridization. For autoradiography on a microscopic level, the slide is typically dipped into liquid nuclear tract emulsion, which dries to form the exposure film. Individual silver grains in the film are visualized with dark field microscopy.

Recently, antibodies have been used to specifically visualize proteins, carbohydrates, and lipids. This process is called immunohistochemistry, or when the stain is a fluorescent molecule, immunofluorescence. This technique has greatly increased the ability to identify categories of cells under a microscope. Other advanced techniques, such as nonradioactive *in situ* hybridization, can be combined with immunochemistry to identify specific DNA or RNA molecules with fluorescent probes or tags that can be used for immunofluorescence and enzyme-linked fluorescence amplification (especially alkaline phosphatase and tyramide signal amplification). Fluorescence microscopy and confocal microscopy are used to detect fluorescent signals with good intracellular detail. Digital cameras are increasingly used to capture histological and histopathological image

Common laboratory stains

Stain	Common use	Nucleus	Cytoplasm	Red blood cell (RBC)	Collagen fibers	Specifically stains
Haematoxylin	General staining when paired with eosin (i.e. H&E)	Blue	N/A	N/A	N/A	Nucleic acids—blue ER (endoplasmic reticulum)—blue
Eosin	General staining when paired with haematoxylin (i.e. H&E)	N/A	Pink	Orange/red	Pink	Elastic fibers—pink Collagen fibers—pink Reticular fibers—pink
Toluidine blue	General staining	Blue	Blue	Blue	Blue	Mast cells granules—purple
Masson's trichrome stain	Connective tissue	Black	Red/pink	Red	Blue/green	Cartilage—blue/green Muscle fibers—red Keratin—orange
Mallory's trichrome stain	Connective tissue	Red	Pale red	Orange	Deep blue	Cartilage—blue Bone matrix—deep blue Muscle fibers—red
Weigert's elastic stain	Elastic fibers	Blue/black	N/A	N/A	N/A	Elastic fibers—blue/black
Heidenhain's AZAN trichrome stain	Distinguishing cells from extracellular components	Red/purple	Pink	Red	Blue	Muscle fibers—red Cartilage—blue Bone matrix—blue
Silver stain	Reticular fibers, nerve fibers, fungi	N/A	N/A	N/A	N/A	Reticular fibers—brown/black Nerve fibers—brown/black Neutrophil granules—purple/pink
Wright's stain	Blood cells	Bluish/purple	Bluish/gray	Red/pink	N/A	Eosinophil granules—bright red/orange Basophil granules—deep purple/violet Platelet granules—red/purple
Orcein stain	Elastic fibres	Deep blue [or crazy red]	N/A	Bright red	Pink	Elastic fibres—dark brown Mast cells granules—purple

						Smooth muscle—light blue
Periodic acid-Schiff stain (PAS)	Basement membrane, localizing carbohydrates	Blue	N/A	N/A	Pink	Glycogen and other carbohydrates—magenta

Table sourced from Michael H. Ross, Wojciech Pawlina, (2006). *Histology: A Text and Atlas*. Hagerstown, MD: Lippincott Williams & Wilkins. ISBN 0-7817-5056-3.

The Nissl method and Golgi's method are useful in identifying neurons.

Alternative techniques

Alternative techniques include cryosection. The tissue is frozen using a cryostat, and cut. Tissue staining methods are similar to those of wax sections. Plastic embedding is commonly used in the preparation of material for electron microscopy. Tissues are embedded in epoxy resin. Very thin sections (less than 0.1 micrometer) are cut using diamond or glass knives. The sections are stained with electron dense stains (uranium and lead) so that they can possibly be seen with the electron microscope.

History

In the 19th century, histology was an academic discipline in its own right. The 1906 Nobel Prize in Physiology or Medicine was awarded to histologists Camillo Golgi and Santiago Ramon y Cajal. They had dueling interpretations of the neural structure of the brain based in differing interpretations of the same images. Cajal won the prize for his correct theory and Golgi for the staining technique he invented to make it possible.

Histological classification of animal tissues

There are four basic types of tissues: muscle tissue, nervous tissue, connective tissue, and epithelial tissue. All tissue types are subtypes of these four basic tissue types (for example, blood cells are classified as connective tissue, since they generally originate inside bone marrow).

- Epithelium: the lining of glands, bowel, skin, and some organs like the liver, lung, and kidney
- Endothelium: the lining of blood and lymphatic vessels
- Mesothelium: the lining of pleural and pericardial spaces
- Mesenchyme: the cells filling the spaces between the organs, including fat, muscle, bone, cartilage, and tendon cells
- Blood cells: the red and white blood cells, including those found in lymph nodes and spleen
- Neurons: any of the conducting cells of the nervous system
- Germ cells: reproductive cells (spermatozoa in men, oocytes in women)

- Placenta: an organ characteristic of true mammals during pregnancy, joining mother and offspring, providing endocrine secretion and selective exchange of soluble, but not particulate, blood-borne substances through an apposition of uterine and trophoblastic vascularised parts
- Stem cells: cells able to turn into one or several of the above types

Note that tissues from plants, fungi, and microorganisms can also be examined histologically. Their structure is very different from animal tissues.

Related sciences

- Cell biology is the study of living cells, their DNA and RNA and the proteins they express.
- Anatomy is the study of organs visible by the naked eye.
- Morphology studies entire organisms.

Artifacts

Artifacts are structures or features in tissue that interfere with normal histological examination. These are not always present in normal tissue and can come from outside sources. Artifacts interfere with histology by changing the tissues appearance and hiding structures. These can be divided into two categories:

Pre-histology

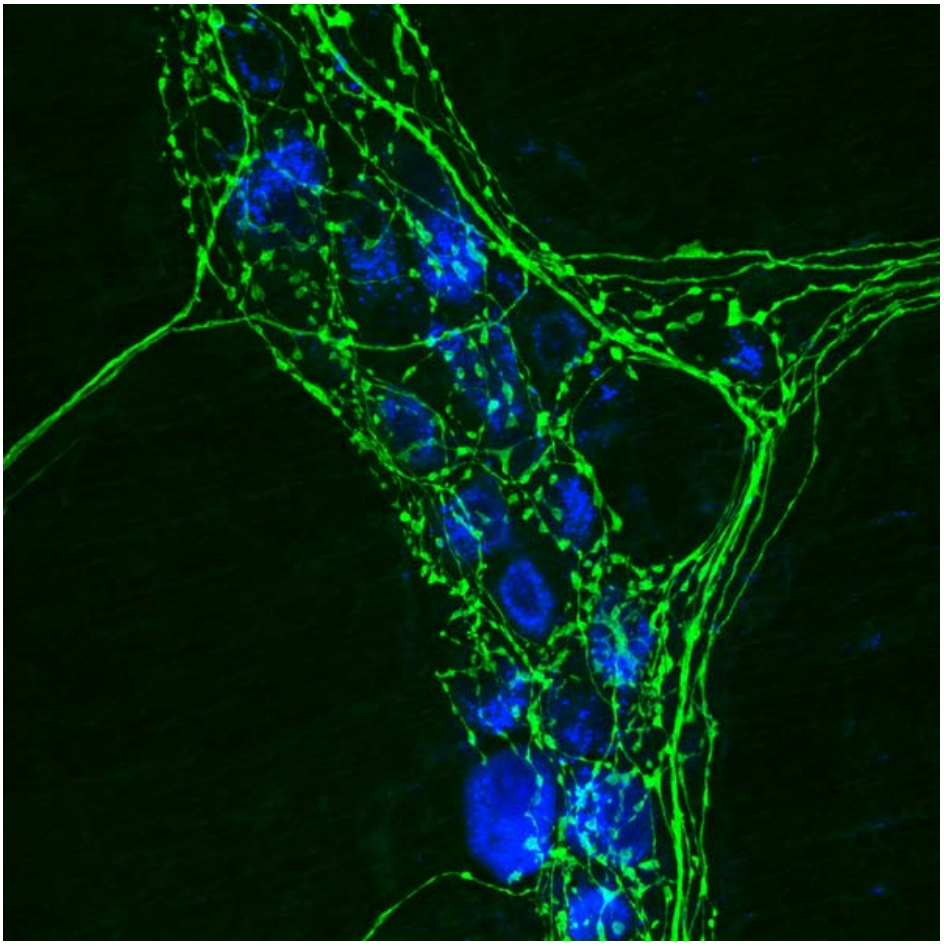
These are features and structures that have being introduced prior to the collection of the tissues. A common example of these include: ink from tattoos and freckles (melanin) in skin samples.

Post-histology

Artifacts can result from tissue processing. Processing commonly leads to changes like shrinkage, washing out of particular cellular components, color changes in different tissues types and alterations of the structures in the tissue. Because these are caused in a laboratory the majority of post histology artifacts can be avoided or removed after being discovered. A common example is mercury pigment left behind after using Zenker's fixative to fix a section.

Chapter 12

Immunohistochemistry



Immunohistochemistry labels individual proteins, such as TH (green) in the axons of sympathetic autonomic neurons.

Immunohistochemistry or **IHC** refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to immunocytochemistry). Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine.

Sample preparation

While using the right antibodies to target the correct antigens and amplify the signal is important for visualization, complete preparation of the sample is critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes. This requires proper tissue collection, fixation and sectioning. Depending on the purpose and the thickness of the experimental sample, either thin (about 4-40 μm) sections are sliced from the tissue of interest, or if the tissue is not very thick and is penetrable it is used whole. The slicing is usually accomplished through the use of a microtome, and slices are mounted on slides. "Free-floating IHC" uses slices that are not mounted, these slices are normally produced using a vibrating microtome.

Because of the method of fixation and tissue preservation, the sample may require additional steps to make the epitopes available for antibody binding, including deparaffinization and antigen retrieval; these steps often makes the difference between staining and no staining. Additionally, depending on the tissue type and the method of antigen detection, endogenous biotin or enzymes may need to be blocked or quenched, respectively, prior to antibody staining. Unlike immunocytochemistry, the tissue does not need to be permeabilized because this has already been accomplished by the microtome blade during sample preparation. Detergents like Triton X-100 are generally used in immunohistochemistry to reduce surface tension, allowing less reagent to be used to achieve better and more even coverage of the sample. Although antibodies show preferential avidity for specific epitopes, they may partially or weakly bind to sites on nonspecific proteins (also called reactive sites) that are similar to the cognate binding sites on the target antigen. In the context of antibody-mediated antigen detection, nonspecific binding causes high background staining that can mask the detection of the target antigen. To reduce background staining in IHC, ICC and any other immunostaining application, the samples are incubated with a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include normal serum, non-fat dry milk, BSA or gelatin, and commercial blocking buffers with proprietary formulations are available for greater efficiency.

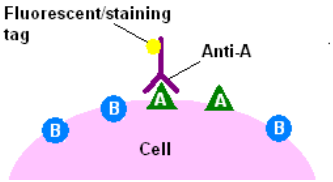
Sample Labeling

Antibody types

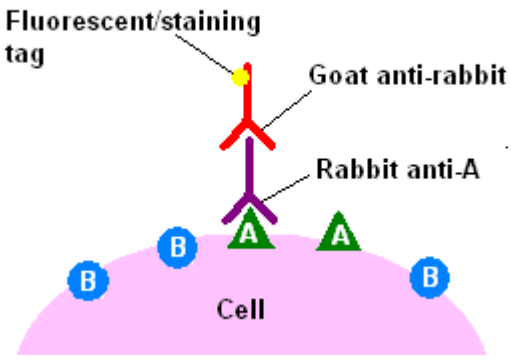
The antibodies used for specific detection can be polyclonal or monoclonal. Polyclonal antibodies are made by injecting animals with peptide Ag and, after a secondary immune response is stimulated, isolating antibodies from whole serum. Thus, polyclonal antibodies are a heterogeneous mix of antibodies that recognize several epitopes. Monoclonal antibodies show specificity for a single epitope and are therefore considered more specific to the target antigen than polyclonal antibodies. For IHC detection strategies, antibodies are classified as primary or secondary reagents. Primary antibodies are raised against an antigen of interest and are typically unconjugated (unlabelled), while secondary antibodies are raised against immunoglobulins of the primary antibody species. The secondary antibody is usually conjugated to a linker molecule, such as biotin, that then recruits reporter molecules, or the secondary antibody is directly bound to the reporter molecule itself.

IHC reporters

Reporter molecules vary based on the nature of the detection method, and the most popular methods of detection are with enzyme- and fluorophore-mediated chromogenic and fluorescentdetection, respectively. With chromogenic reporters, an enzyme label is reacted with a substrate to yield an intensely colored product that can be analyzed with an ordinary light microscope. While the list of enzyme substrates is extensive, Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. An array of chromogenic, fluorogenic and chemiluminescent substrates is available for use with either enzyme, including DAB or BCIP/NBT, which produce a brown or purple staining, respectively, wherever the enzymes are bound. Reaction with DAB can be enhanced using nickel, producing a deep purple/black staining. Fluorescent reporters are small, organic molecules used for IHC detection and traditionally include FITC, TRITC and AMCA, while commercial derivatives, including the Alexa Fluors and Dylight Fluors, show similar enhanced performance but vary in price. For chromogenic and fluorescent detection methods, densitometric analysis of the signal can provide semi- and fully-quantitative data, respectively, to correlate the level of reporter signal to the level of protein expression or localization.



The direct method of immunohistochemical staining uses one labelled antibody, which binds directly to the antigen being stained for.



The *indirect method* of immunohistochemical staining uses one antibody against the antigen being probed for, and a second, labelled, antibody against the first.

Target antigen detection methods

The *direct method* is a one-step staining method and involves a labeled antibody (e.g. FITC-conjugated antiserum) reacting directly with the antigen in tissue sections. While this technique utilizes only one antibody and therefore is simple and rapid, the sensitivity is lower due to little signal amplification, such as with indirect methods, and is less commonly used than indirect methods.

The *indirect method* involves an unlabeled primary antibody (first layer) that binds to the target antigen antigen in the tissue and a labeled secondary antibody (second layer) that reacts with the primary antibody. As mentioned above, the secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. This method is more sensitive than direct detection strategies because of signal amplification due to the binding of several secondary antibodies to each primary antibody if the secondary antibody is conjugated to the fluorescent or enzyme reporter. Further amplification can be achieved if the secondary antibody is conjugated to several biotin molecules, which can recruit complexes of avidin-, streptavidin or NeutrAvidin proteinbound-enzyme. The difference between these three biotin-binding proteins is their individual binding affinity to endogenous tissue targets leading to nonspecific binding and high background; the ranking of these proteins based on their nonspecific binding affinities, from highest to lowest, is: 1) avidin, 2) streptavidin and 3) Neutravidin protein.

The indirect method, aside from its greater sensitivity, also has the advantage that only a relatively small number of standard conjugated (labeled) secondary antibodies needs to be generated. For example, a labeled secondary antibody raised against rabbit IgG, which can be purchased "off the shelf," is useful with any primary antibody raised in rabbit. With the direct method, it would be necessary to label each primary antibody for every antigen of interest.

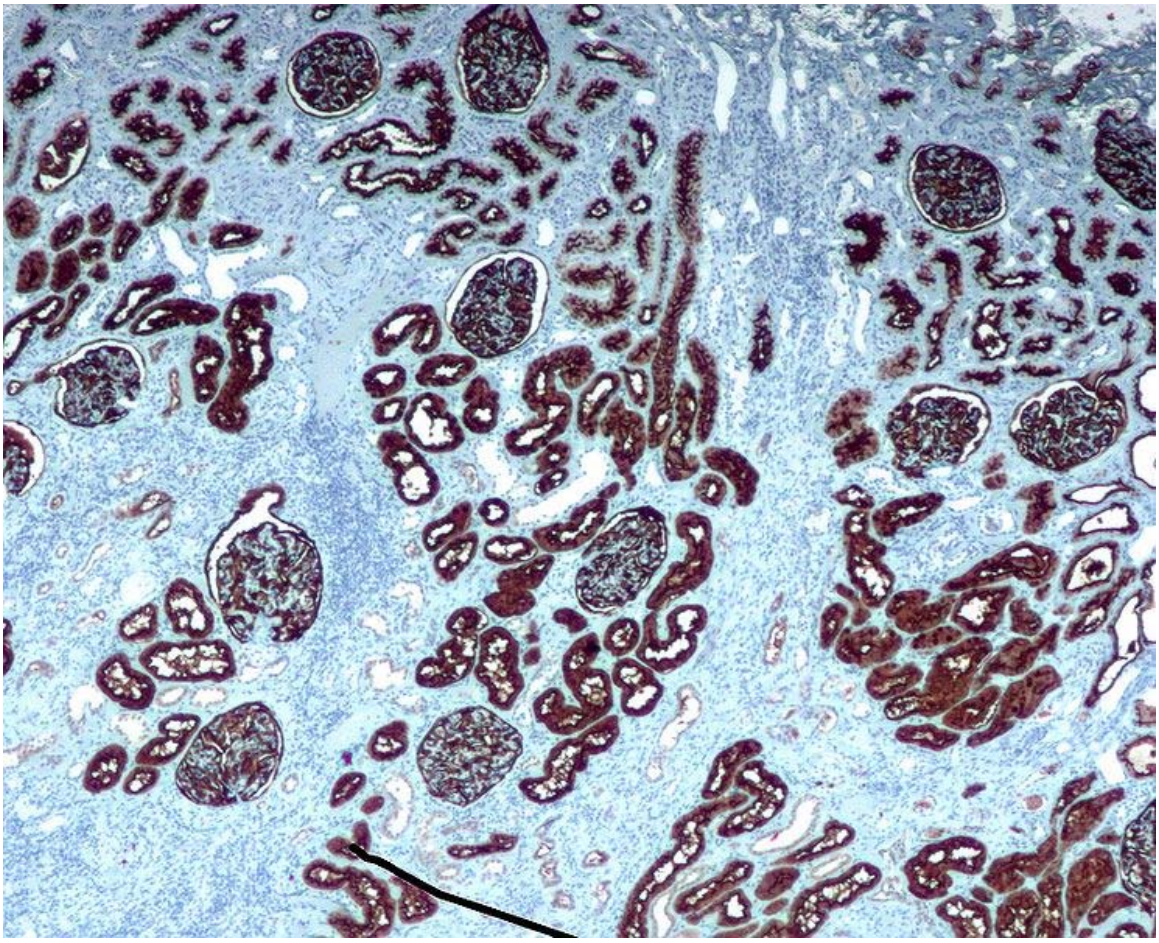
Counterstains

After immunohistochemical staining of the target antigen, a second stain is often applied to provide contrast that helps the primary stain stand out. Many of these stains show specificity for discrete cellular compartments or antigens, while others will stain the whole cell. Both chromogenic and fluorescent dyes are available for IHC to provide a vast array of reagents to fit every experimental design, and include: hematoxylin, Hoechst stain and DAPI are commonly used.

IHC Troubleshooting

In immunohistochemical techniques, there are several steps prior to the final staining of the tissue antigen, and many potential problems affect the outcome of the procedure. The major problem areas in IHC staining include strong background staining, weak target antigen staining and autofluorescence. Endogenous biotin or reporter enzymes or primary/secondary antibody cross-reactivity are common causes of strong background staining, while weak staining may be caused by poor enzyme activity or primary antibody potency. Furthermore, autofluorescence may be due to the nature of the tissue or the fixation method. These aspects of IHC tissue prep and antibody staining must be systematically addressed to identify and overcome staining issues.

Diagnostic IHC markers



Immunohistochemical staining of normal kidney with CD10.

IHC is an excellent detection technique and has the tremendous advantage of being able to show exactly where a given protein is located within the tissue examined. It is also an effective way to examine the tissues .This has made it a widely-used technique in the neurosciences, enabling researchers to examine protein expression within specific brain structures. Its major disadvantage is that, unlike immunoblotting techniques where staining is checked against a molecular weight ladder, it is impossible to show in IHC that the staining corresponds with the protein of interest. For this reason, primary antibodies must be well-validated in a Western Blot or similar procedure. The technique is even more widely used in diagnostic surgical pathology for typing tumors (e.g. immunostaining for e-cadherin to differentiate between DCIS (ductal carcinoma in situ: stains positive) and LCIS (lobular carcinoma in situ: does not stain positive)).

- Carcinoembryonic antigen (CEA): used for identification of adenocarcinomas. Not specific for site.
- Cytokeratins: used for identification of carcinomas but may also be expressed in some sarcomas.
- CD15 and CD30 : used for Hodgkin's disease

- Alpha fetoprotein: for yolk sac tumors and hepatocellular carcinoma
- CD117 (KIT): for gastrointestinal stromal tumors (GIST)
- CD10 (CALLA): for renal cell carcinoma and acute lymphoblastic leukemia
- Prostate specific antigen (PSA): for prostate cancer
- estrogens and progesterone staining for tumour identification
- Identification of B-cell lymphomas using CD20
- Identification of T-cell lymphomas using CD3

Directing therapy

A variety of molecular pathways are altered in cancer and some of the alterations can be targeted in cancer therapy. Immunohistochemistry can be used to assess which tumors are likely to respond to therapy, by detecting the presence or elevated levels of the molecular target.

Chemical inhibitors

Tumor biology allows for a number of potential intracellular targets. Many tumors are hormone dependent. The presence of hormone receptors can be used to determine if a tumor is potentially responsive to antihormonal therapy. One of the first therapies was the antiestrogen, tamoxifen, used to treat breast cancer. Such hormone receptors can be detected by immunohistochemistry. Imatinib, an intracellualar tyrosine kinase inhibitor, was developed to treat chronic myelogenous leukemia, a disease characterized by the formation of a specific abnormal tyrosine kinase. Imitanib has proven effective in tumors, that express other tyrosine kinases, most notably KIT. Most gastrointestinal stromal tumors express KIT, which can be detected by immunohistochemistry.

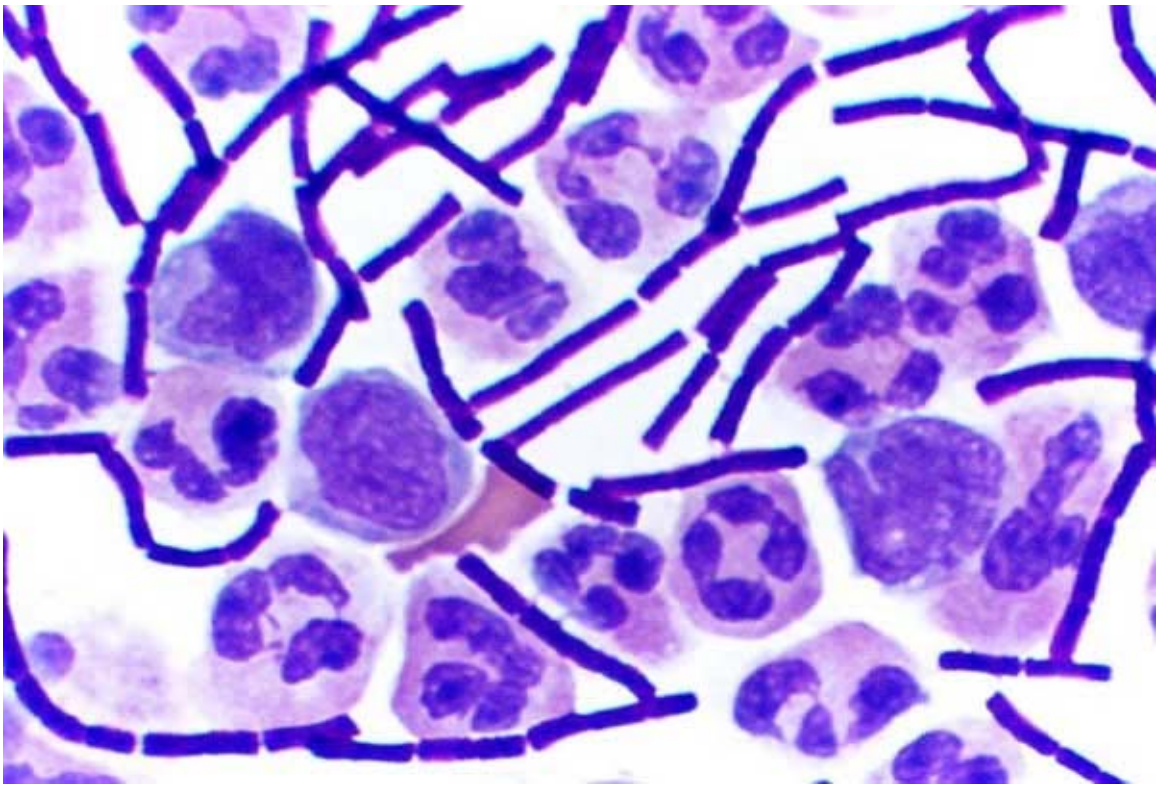
Monoclonal antibodies

Many proteins shown to be highly upregulated in pathological states by immunohistochemistry are potential targets for therapies utilising monoclonal antibodies. Monoclonal antibodies, due to their size, are utilized against cell surface targets. Among the overexpressed targets, the members of the epidermal growth factor receptor (EGFR) family, transmembrane proteins with an extracellular receptor domain regulating an intracellular tyrosine kinase, Of these, HER2/neu (also known as Erb-B2) was the first to be developed. The molecule is highly expressed in a variety of cancer cell types, most notably breast cancer. As such, antibodies against HER2/neu have been FDA approved for clinical treatment of cancer under the drug name *Herceptin*. There are commercially available immunohistochemical tests, Dako HercepTest and Ventana Pathway. Similarly, EGFR (HER-1) is overexpressed in a variety of cancers including head and neck and colon. Immunohistochemistry is used to determine patients who may benefit from therapeutic antibodies such as Erbitux (cetuximab). Commercial systems to detect EGFR by immunohistochemistry include the Dako pharmDx.

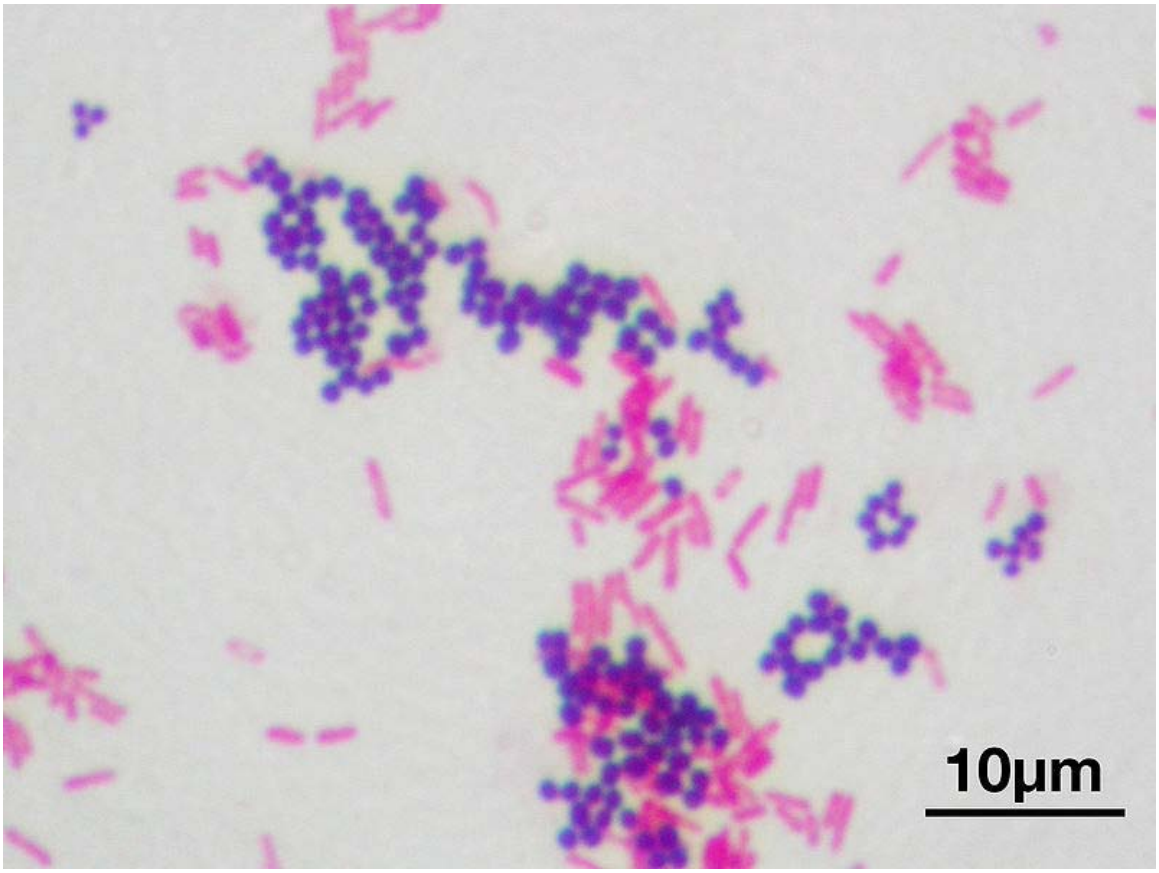
Chapter 13

Gram Staining and Romanowsky Stain

Gram staining



Gram-positive anthrax bacteria (purple rods) in cerebrospinal fluid sample. If present, a Gram-negative bacterial species would appear pink. (The other cells are white blood cells).



A Gram stain of mixed *Staphylococcus aureus* (Gram positive cocci) and *Escherichia coli* (Gram negative bacilli), the most common Gram stain reference bacteria

Gram staining (or **Gram's method**) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical, primarily the presence of high levels of peptidoglycan, and physical properties of their cell walls. The Gram stain is almost always the first step in the identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique, thus forming *Gram-variable* and *Gram-indeterminate* groups as well.

The word Gram is always spelled with a capital, referring to Hans Christian Gram, the inventor of Gram staining.

History

The method is named after its inventor, the Danish scientist Hans Christian Gram (1853–1938), who developed the technique while working with Carl Friedländer in the morgue of the city hospital in Berlin. Gram devised his technique not for the purpose of distinguishing one group of bacteria from another but to enable bacteria to be seen more readily in stained sections of lung tissue. He published his method in 1884, and included in his short report the observation that the Typhus bacillus did not retain the stain.

Uses

Gram staining is a bacteriological laboratory technique used to differentiate bacterial species into two large groups (Gram-positive and Gram-negative) based on the physical properties of their cell walls. Gram staining is not used to classify archaea, formally archaeabacteria, since these microorganisms yield widely varying responses that do not follow their phylogenetic groups.

The Gram stain is not an infallible tool for diagnosis, identification, or phylogeny, however. It is of extremely limited use in environmental microbiology, and has been largely superseded by molecular techniques even in the medical microbiology lab. Some organisms are Gram-variable (that means, they may stain either negative or positive); some organisms are not susceptible to either stain used by the Gram technique. In a modern environmental or molecular microbiology lab, most identification is done using genetic sequences and other molecular techniques, which are far more specific and information-rich than differential staining.

Medical

Gram stains are performed on body fluid or biopsy when infection is suspected. It yields results much more quickly than culture, and is especially important when infection would make an important difference in the patient's treatment and prognosis; examples are cerebrospinal fluid for meningitis and synovial fluid for septic arthritis. .

Staining mechanism

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stains purple, whereas Gram-negative bacteria have a thinner layer (10% of cell wall), which stains pink. Gram-negative bacteria also have an additional outer membrane that contains lipids, and is separated from the cell wall by the periplasmic space. There are four basic steps of the Gram stain, which include applying a primary stain (crystal violet) to a heat-fixed (death by heat) smear of a bacterial culture, followed by the addition of a trapping agent (Gram's iodine), rapid decolorization with alcohol or acetone, and *counterstaining* with safranin. Basic fuchsin is sometimes substituted for safranin since it will more intensely stain anaerobic bacteria but it is much less commonly employed as a counterstain.

Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride (Cl^-) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple.

Iodine (I^- or I_3^-) interacts with CV^+ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. Iodine is often referred to as a mordant, but is a trapping agent that prevents the removal of the CV-I complex and, therefore, color the cell.

When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer lipopolysaccharide membrane, and the inner peptidoglycan layer is left exposed. The CV–I complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CV–I complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain will be removed from both Gram-positive and negative cells if the decolorizing agent is left on too long (a matter of seconds).

After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Counterstain, which is usually positively charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color.

Some bacteria, after staining with the Gram stain, yield a *Gram-variable* pattern: a mix of pink and purple cells are seen. The genera *Actinomyces*, *Arthobacter*, *Corynebacterium*, *Mycobacterium*, and *Propionibacterium* have cell walls particularly sensitive to breakage during cell division, resulting in Gram-negative staining of these Gram-positive cells. In cultures of *Bacillus*, *Butyrivibrio*, and *Clostridium*, a decrease in peptidoglycan thickness during growth coincides with an increase in the number of cells that stain Gram-negative. In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain.

Examples

Gram-negative bacteria

The proteobacteria are a major group of Gram-negative bacteria. Other notable groups of Gram-negative bacteria include the cyanobacteria, spirochaetes, green sulfur, and green non-sulfur bacteria.

These also include many medically relevant Gram-negative cocci, bacilli, and many bacteria associated with nosocomial infections.

Gram-positive bacteria

In the original bacterial phyla, the Gram-positive forms made up the phylum Firmicutes, a name now used for the largest group. It includes many well-known genera such as *Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*. It has also been expanded to include the Mollicutes, bacteria like *Mycoplasma* that lack cell walls and so cannot be stained by Gram, but are derived from such forms.

Gram-indeterminate bacteria

Gram-indeterminate bacteria do not respond to Gram staining and, therefore, cannot be determined as either Gram-positive or Gram-negative

Romanowsky stain

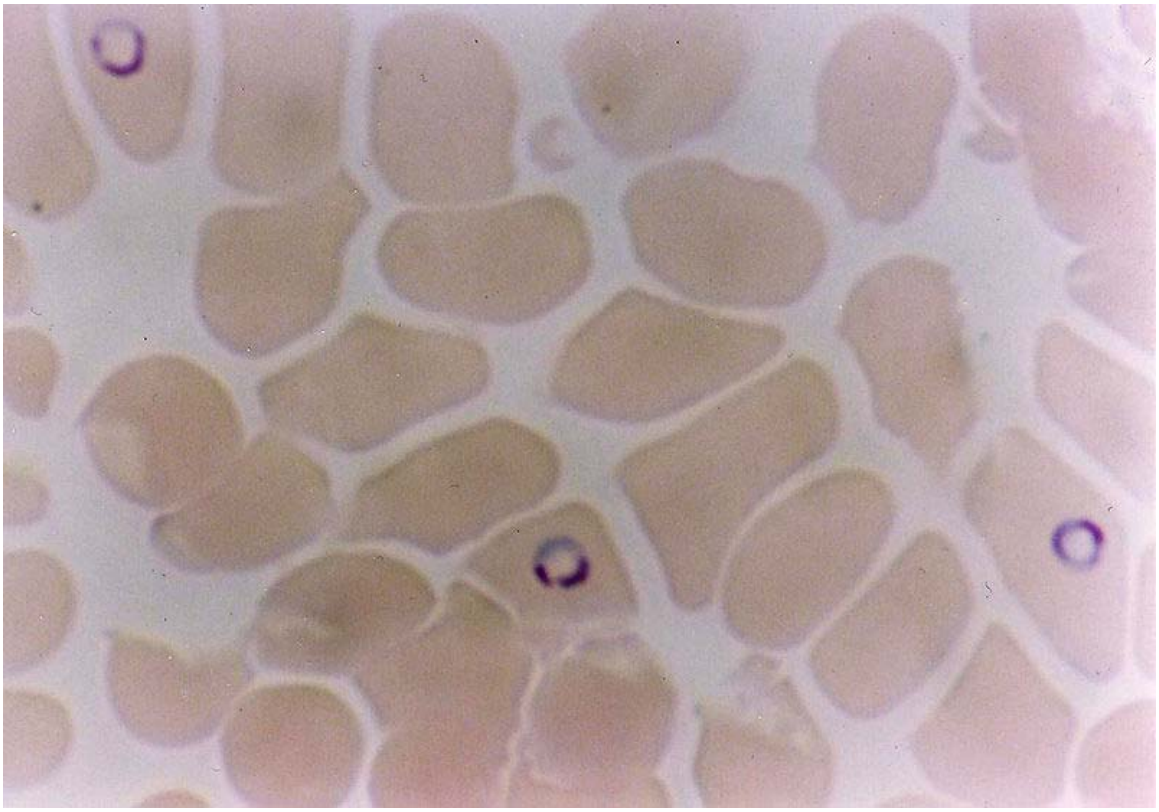
Romanowsky staining is a prototypical staining technique that was the forerunner of several distinct but similar methods, including Giemsa, Jenner, Wright, Field, and Leishman stains, which are used to differentiate cells in pathologic specimens.

Microscopic examination of stained blood films

Ernst Malachowski

Paul Ehrlich had used mixtures of acidic and basic dyes for this purpose in 1879: eg Fuchsine (acid) and methylene blue (base). In 1891 Ernst Malachowski and Dmitri Leonidovich Romanowsky independently developed techniques using a mixture of Eosin

Y and modified Methylene blue that produced a surprising hue unattributable to either staining component: a beautiful, distinctive shade of purple. Requirement for the occurrence of the Malachowski-Romanowsky-Giemsa effect are: I A cationic dye: The best dye is azure B and, though azure A gives the nuclear purple colour, the cytoplasmic blue is inferior. No other cationic dye such as methylene blue is suitable. 2 An anionic dye: Most commonly eosin Y is used. Because the aqueous dye solutions were unstable, methanol was introduced as a solvent, and William Boog Leishman and James Homer Wright advocated use of methanol as a fixative prior to staining. Gustav Giemsa improved this technique by standardizing the dye solutions and adding glycerol to increase stability.



Giemsa-stained thin blood film showing *Plasmodium falciparum* infections

The demethylation of Methylene Blue in aqueous solution using heat and alkali produces a mixture of Azure A, Azure B, Methylene Violet and Methylene Blue. Eosin Y is then added to produce a "neutral" dye. The precipitate is then dissolved in a mixture of methanol and glycerol to form a stock solution; this is diluted with water or an aqueous buffer to form a 'working' solution that is used in the staining of pathology specimens. The 'working' solution is stable for 3 hours.

Immunochromatographic capture procedures (Rapid Diagnostic Tests) are nonmicroscopic diagnostic options for the laboratory that may not have appropriate microscopy expertise available.