Lesson: Fluorescence and Confocal Microscopy
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Introduction to fluorescence microscope

Although many of the remarkable discoveries in the field of life science have been made without the application of specialized technology, technology plays an important role in understanding new frontiers in life science. It provides as a means to test and to prove new ideas. It also provides new information to formulate new hypothesis. One of remarkable developments is microscopy and imaging techniques. These techniques occupy a center stage in biological as well in material science; an essential tool in assessing the properties of organic or inorganic substances. It provides the advantage of being able to observe and measure form and features to reveal the variability. With the development of more refine techniques couple with the discovery of an array of fluorophores, it has made possible to visualize and study the cellular and subcellular components and the diverse physiological processes like protein interactions, ion transports, nutrient mobility and metabolic processes taking place inside a living cell.

In light microscopy, the differential reflection, diffraction and absorption properties of different specimens are used to study the specimens. So improvements in microscopy are mainly emphasized on increasing the contrast between the specimen and the background. This contrast between the sample and the background is enhanced certainly by staining the sample with agents that absorb light in light microscopy. However this contrast has been greatly enhanced with the development of fluorescence microscopy. It provides better contrast than other optical microscopy techniques. This has been achieved by staining or tagging the target sample with fluorescence dye or with fluorescence molecules (fluorophores) amid the non-fluorescing background. On irradiation with high energy light, only the fluorescence molecules emit light enabling to visualize only the object of interest in the dark background. With the development of highly specific labeling probes, coupled with the ability to imaging of individual components and other macromolecular complexes, fluorescence microscopy brings a revolution in cell biology. (web link no.1)

History of fluorescence

Fluorescence has been described first by Irish Scientist Sir George G. Stokes in 1852 during the middle of nineteenth century. While working with a mineral named ‘fluorspar’ at the Cambridge University, he noticed that the mineral emitted red light when illuminated with blue light. Although this phenomenon has been encountered in microscopy in the early part of twentieth century by several scientists, including August Köhler and Carl Reichert, they

failed to recognised the fluorescence and instead reported it as a background noise in ultraviolet microscopy. The first fluorescence microscopes were developed by German physicists Otto Heimstädt and Heinrich Lehmann between 1911 and 1913 as variant from the ultraviolet microscopy. These microscopes have used to observe autofluorescence in bacteria, animal, and plant tissues. A new era of fluorescence microscopy aroused thereafter the development of a technique for labelling antibodies with fluorescent dyes by Albert Coons during the early 1940s. (web link no. 2)

Principles of Flourescence

Excitation and Emission

Every molecule can absorb light of certain wavelength. When these molecules are subjected to radiant energy, they absorb the energy and become excited to a higher energy state. The inherent property of every molecule to stay at the lowest energy state made much of the trapped energy to be released in the form of heat or light. However some atoms or molecules after absorbing light, it reradiates back the energy in the form of light within nanoseconds after absorption. This physical phenomenon is known as fluorescence and is first describe d by Sir George G. Stokes. He coined the term "fluorescence" named after the mineral 'fluorspar' he is working with. He also pointed out that, the light emitted by an excited molecule have a wavelength longer than the wavelength of light originally absorbed. Like, upon absorption of blue light, green light is emitted soon afterwards. Green is changed to yellow, yellow to reddish orange and invisible UV light to visible light. The time delay between the absorption and emission of the light in fluorescence is less than a microsecond. However, the phenomenon phosphorescence occurs when the emission persists even after the excitation light has been discontinued. (web link no.1)

A useful explanation of the various energy levels involved in excitation and emission process by a fluorophore is illustrated by the Jablonski energy diagram named after the polish physicist Alexander Jablonski.

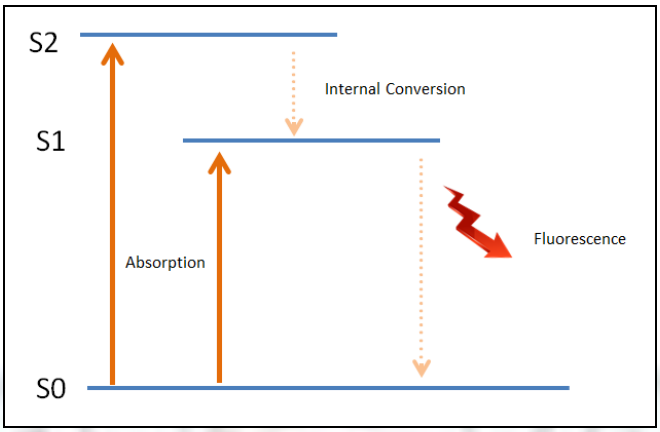

Figure: Jablonski diagram displaying the energy states of a molecule

Source: Author

The diagram illustrates three states of a molecule - the ground state (S0), the first (S1) and Second (S2) excited energy states as a stack of horizontal Lines. S0 represents the ground state (unexcited energy state of a molecule). S1 and S2 represent the excited states (outer electrons are transferred into a higher orbit). S2>S1>S0 represents the gradation in terms of energy states.

When a molecule in their ground state absorbs light energy, all the energy of the photon is transferred to the molecule. The amount of energy absorb by the molecule is inversely proportional to the wavelength of the photon

E=*h* X c/ λ, Where *h* is the plank's constant c and λ are the speed and wavelength of light in vacuum.

After absorbing the energy the molecule is transferred to S1 State. However if the amount of energy absorb is greater than that requires to transferred to the S1 state, than the molecule to transferred to the higher S2 state.

The excited molecule stayed at the excited state for the period on order of nanoseconds and return to the ground state by losing the absorb energy. Electrons excited at a higher orbital state (S2) first return to the S1 state. This transition between electron orbitals is known as 'internal conversion'. The extra energy from this transition is lost through vibrational relaxation. Electrons at the S1 state than return to the ground state. If the drop back from higher energy state S1 to lower state is accompanied by the release of photon of light whose energy is equal to the energy gap between the ground S0 and first single state S1. This emitted light is the fluorescence.

Excitation and emission spectra and stokes Shift

A Fluorophore on illumination absorbs light energy. If the energy absorbed is greater than that is necessary to excite it orbital electrons to S1 state, then electrons are transferred to the S1 state. This suggested that a range of wavelength with energies more than the minimum required transferring the orbital electrons to the higher energy state S1. So the excitation spectrum of a fluorophore is the range of wavelengths which can excite the fluorophore. Once excited the fluorophore return to the ground state by release of photons of energy. The emitted light can be at different wavelengths. This range of wavelengths the emitted photon have is the emission spectra of the fluorophore.

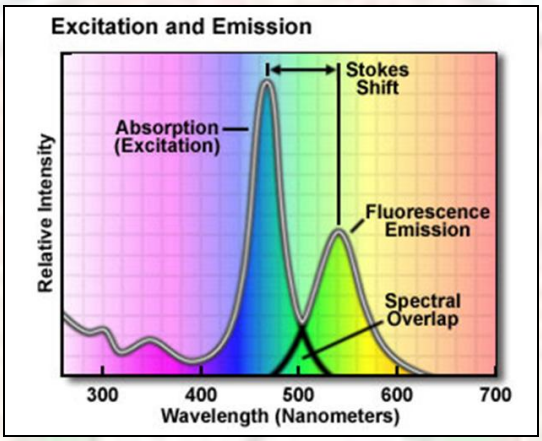

Figure: The figure represents an excitation and absorption spectra of a fluorophore.

Source: Author

The emitted spectra is at a longer wavelength than the excitation spectra. This difference between the exciting and emitted wavelengths is termed as Stoke's shift named after the Sir George G. Stokes. This shift in wavelengths may be attributed to the energy loss due to vibrational and internal conversions that takes place by the excited electrons. Although there is a shift between the excitation and emission wavelengths, the excitation and emission spectra are often the mirror image of the other. The excitation and emission spectra of fluorophore can be determined by illuminating the fluorophore with different wavelengths and measuring the wavelengths of light emitted. (web link no. 1.3)

Fluorescence microscope

This difference in wavelength between the absorption and emission wavelength or Stoke's shift is the critical property that is being utilized in fluorescence microscopy; illuminating the

sample with one wavelength and filtering the emitted light to allow only the longer wavelength to visualize thus making to see only the objects that are fluorescent against the dark background. The figure below represents the light pathway in an epifluorescence microscope.

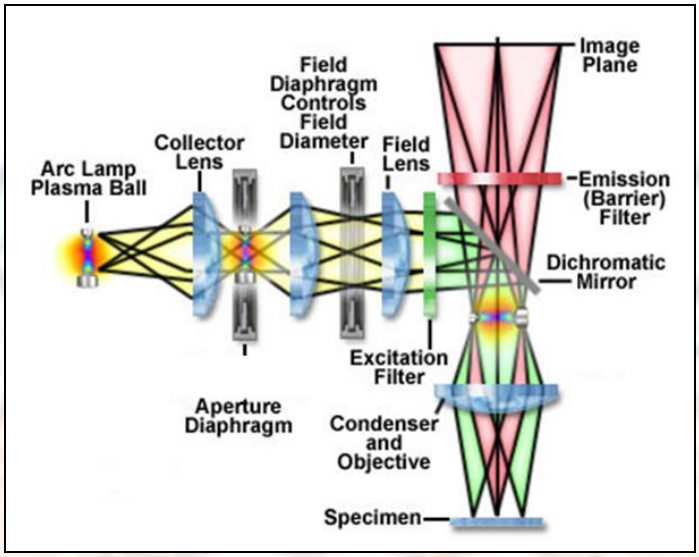

Figure: A graphical representation of light pathway of a fluorescence microscope.

Source: Author

This is the preferred type of fluorescence microscopy in the modern microscopy techniques. An epifluorescence microscope is basically a reflected light microscope in which the reflected light is with a longer wavelength than that of excitation wavelength. The illuminator (the horizontal tube where the light passes) is interposed between the detector (eye piece or imaging camera) and the nosepiece housing the objectives. In this orientation the objective lens performed two functions - magnification and imaging of the specimen and as a condenser of the light which illuminates the specimen.

In this type of microscope, the light of different wavelength often in the ultraviolet region is produced from an arc-discharge lamp or other source positioned at one end of the vertical illuminator called lamphouse. The light from the source travels along the horizontal illuminator, passes through an adjustable aperture diaphragm then through a variable, centerable field diaphragm and then impinged over the excitation filter. After passing through a wavelength selective filter called 'excitation filter', the selected wavelengths falls on the specimen through objective lens after getting reflected from a dichromatic mirror or beam splitter position at 45 degree to the light path. The objective lens collects the

fluorescence light emitted from the object. This then passes through the dichromic mirror and barrier filter (allows passage of only longer wavelength light to the detector). All these three types of filters referred to as special interference filters, have an advantage of selecting lights of very specific wavelengths.

In most of the fluorescence microscopes of reflected type, the excitation filter, dichromic mirror and the barrier filter are held together by means of a small block or cube shaped filter holders.

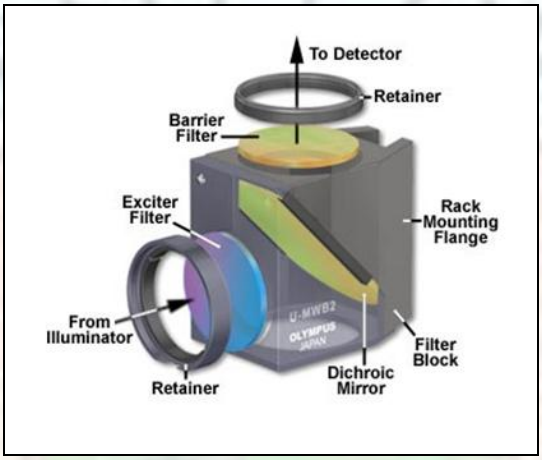

Figure: A typical filter cube with the excitation Dichromatic and emission filters

Source: Author

Most of the fluorescence microscopes accommodate three or more such cubes into a revolving circular turret, enabling to switch imaging between different fluorophores with different excitation and emission wavelengths easily and rapidly.

In fluorescence microscopy the concentration of the light emitting fluorophores is very low. To produce a detectable level of emission, the fluorophores needs to be excited with strong intensity light sources. The most common lamps used in fluorescence microscopes are mercury and xenon arc lamps. Being potentially dangerous they are encased to special lamp houses. Both the lamps produced lights of wavelengths throughout the UV, Visible and near infrared region. The Xenon lamp produced relatively even in wavelength coverage while mercury produced more in the UV region. Depending on the requirements both the lamps has their own importance. Both the lamps begin to give variations in intensity overtime due to the blunting of cathode and anode and the movements in arc positions that cause flickering. These bulbs need to be replaced once they reached the lifetime of 200hrs for

mercury and 400hrs for the xenon lamp. Thus the power supply of these lamps comes with a timer to track the number of hours being used. (web link no. 4)

Fluorophores

Molecules with the ability to emit fluorescence upon excitation with light are known as fluorophores or fluorochromes. Many organic molecules or compounds like chlorophyll, vitamins, resins, butter, etc. have intrinsic fluorescence when irradiated with light. This natural phenomenon is known as autofluorescence. Of the most notable biological fluorophore is Green fluorescence protein (GFP) from the jellyfish ***Aequorea Victoria***. Use of GFP and its variants as fusion protein provides invaluable service in live-cell imaging experiments. However, many of the biological fluorophores cannot be used to label biological specimens. Even the emission spectrum of most of these fluorophores is broad and thus limits their uses. So over the past several decades, many chemically synthesized fluorophores have been used in labeling a wide range of biological systems. Such chemical compounds often have ring structures with conjugated double bonds. These compounds usually have very narrow gap between the ground and excited energy states and even a low energy photons can be used to excite the fluorophore. Availability of a wide range of fluorophores with sufficient wavelength range allows the flexibility of simultaneous imaging of different cellular, subcellular and molecular components. The "*The Molecular Probes Handbook- A Guide to Fluorescent Probes and Labeling Technologies"* which is available online (web link 4) describes over 3,000 probes for a wide range of bio molecular labeling and detection experiments.

Fluorophores currently used offer sufficient range of wavelengths. The absorption and excitation wavelengths of a fluorophore and its ability to undergo repeated excitation/emission cycles are the intrinsic properties of the fluorophore and it is depended upon the energy state of the electrons at the outermost orbital. The absorption and emission efficiencies of fluorophores are usually quantified in terms of the molar extinction coefficient (EC) and quantum yield (QY) respectively. Extinction coefficient is the likehood of absorption of a photon of the excitation light by the fluorochrome. Larger the extinction coefficient more is likehood of maximum the absorption at the wavelength. The quantum yield is the measure of ratio of fluorescence emission to those absorbed. Higher is the quantum yield more is the output florescence. The utility of a fluorophore thus depends on the extinction coefficient, quantum yield and lifetime of fluorescence of the fluorophore. (web link no. 5)

Fading

Although a fluorophore in principle can cycle between the ground and excited states infinitely and emit fluorescence, there are conditions that effect the fluorescence emission reducing the fluorescence intensity. This reduction in intensity may be due to photo bleaching or due to the phenomenon called quenching. Photo bleaching is the irreversible destruction of the fluorophore in the excited state under high intensity illumination condition. Photo bleaching can occur due multiple photochemical events. However in most cases it is thought to be due to the interaction between the excited state fluorophore and oxygen. The excited state fluorophore on interaction with oxygen transfer its energy and excited the oxygen to a higher energy state. The excited oxygen being reactive undergoes chemical reaction with other organic molecules thus altering the fluorophore rendering the fluorophore to fluoresce. Quenching on the other hand is a reversible loss of fluorescence due to noncovalent interactions between the fluorophore and other molecules. It may also be due to the transfer of energy by the fluorophore to nearby acceptor molecule. This particular phenomenon is the known as fluorescence resonance energy transfer (FRET) and this phenomenon is used in studying molecular interactions.

Although the reduction of fluorescence can occur due to many reasons but photo bleaching is the most common problem experienced in fluorescence microscopy. It can be reduced by using only the necessary intensity of illuminating light. The intensity and aperture diaphragm can control the amount and the area of illumination to minimize general bleaching of the fluorophore. Another way to reduce the bleaching is to mount the specimen in a mounting media bath with efficient bleaching retardant. However the use of antifading fluorophores will be the best solution to the problems of photo bleaching and quenching.

Introduction to Confocal Microscopy

Confocal laser scanning microscope (CLSM) has helped in creating sharp images of a specimen that would otherwise appear blurred (when viewed with a conventional microscope) and thus has amounted to widespread applications in recent years. Exclusion of most of the light from the specimen that is not from the microscope's focal plane has helped in achieving images with better contrast and less haze. In addition to a better visualization of fine details through thin cross-sections of the specimen, it has also become possible to build three-dimensional renditions of a volume of the specimen through assemblage of a sequence of thin slices taken along the vertical axis.

History of CLSM

The principle of confocal microscopy employed in all modern day confocal microscopes was discovered in 1955 by Marvin Minsky. Minsky's goal was to image neural networks in unstained preparations of brain cells. The desire towards the development of confocal approach was to image biological events as they occur in living tissues. The earlier version of confocal microscope as discovered by Minsky used a zirconium arc as the point source of light. A point of light is focused across a specimen sequentially and then through collection of some of the returning rays, a point-by-point image is constructed. To maintain sensitive alignment of moving optics, the specimen was scanned by moving the stage rather than the light rays. A frame rate of approximately one image every ten seconds was thus devised by Minsky. Illumination of the entire sample at the same time could avoid most of the unwanted light that obscures an image. A second pinhole aperture allows the light being returned from the specimen to pass through it rejecting the light rays that were not directly from the focal point. The image is gradually reconstructed through collection of the remaining desirable light rays by a photomultipier.

Modern Confocal Microscopy

The key principles of Minsky's conventional design of confocal microscope (pinhole apertures and point-by-point illumination of the specimen) have been maintained in the modern confocal microscopes. Improvisation in speed, image quality and data storage have been made through advances in optics and electronics which has been incorporated into the development of numerous currently available designs. A laser is used as the light source instead of a tungsten or mercury lamp. Additionally, a sensitive PMT detector and computer-controlled movement of scanning mirrors and other scanning devices facilitates the collection and display of images. Following image acquisition, numerous image processing software packages are used to analyze the images stored on digital media.

Imaging fluorescent specimens using a conventional optical microscope often results in interference of secondary fluorescence (emitted by the specimen that appears away from the region of interest) with the resolution of those in focus. This is mostly observed for specimens having a thickness greater than two micrometers. Confocal laser scanning microscope (CLSM) has bridged the gap between the traditional conventional wide field microscope and transmission electron microscope in terms of resolution. CLSM provides a

marginal improvement in both lateral and axial resolution. CLSM excludes from the image the "out of focus" light even in thick fluorescently labeled specimens. The method of image formation in a confocal microscope is fundamentally different from a conventional microscope. Optical sections are produced by scanning the specimen usually through laser. This refers to the noninvasive method whereby the instrument uses focused light rather than physical means to section the specimen at discrete steps. A single projection (Z projection) of the image or a 3D representation of the image (3D reconstruction) is formed through merging the Z series of optical sections collected through confocal microscope.

Generation of entire information of the specimen from the focal plane of a CLSM essentially comprises of three steps:

1. Two galvanometric scanners scan the object line-by-line through a focused laser beam deflected in X and Y directions (Raster scanning). With the scanning field resolved into 512X512 pixels, an image acquisition rate of maximally 2-3 frames/sec can be achieved.
2. Photomultiplier (PMT) mediated pixel-by-pixel detection of the fluorescence emitted by the scanned specimen details.
3. Electrical signals provided by PMT are then digitally recorded to provide information about the specimen.

How does a confocal microscope work?

The ideas of point-by-point illumination of the specimen and rejection of out-of-focus light have been incorporated into confocal microscope. In contrast to the conventional microscope (object-to-image transformation takes place simultaneously and parallel to all objects), the specimen is irradiated in a point-wise fashion (serially) and the specimen detail irradiated is measured point by point as well. Confocal systems are known as point-probing scanners where the information is obtained about the entire specimen by guiding the laser beam across the specimen. The distinguished feature of a CLSM is the confocal aperture (called pinhole) being arranged in a plane conjugate to the intermediate image plane and hence to the object plane of microscope. PMT thus detects the light that has passed through the pinhole.

Pinhole Size

Pinhole helps in rejecting out-of-focus light rays i.e. excluding the light coming from the object points outside the focal plane and eventually from detection. However, optical sectioning potential depends strongly on the size of pinhole. Ideally pinhole diameter is infinitely so small that the detector looks at a point (point detection). The number of photons arriving at the detector from the specimen are reduced as the pinhole is reduced leading to a reduced signal-to-noise ratio. Invariably, pinhole is made as small as possible. Also, variation in the diameter of pinhole can lead to an adaptation in the practical requirements. The image is said to be non-confocal when the pinhole aperture is fully open. Additionally the stray light is suppressed with the usage of pinhole (helps in better image contrast).

Intensity of incident light

Yet another important component of a CLSM is the photodetector that helps in capturing the light from the specimen. The measurement accuracy is enhanced by increasing the number of photons arriving at the detector which is achieved either by increasing the intensity of fluorescence signal or averaging data from many frames. Raising the intensity of exciation light or dyeing the specimen with a larger concentration of fluorophore helps in increasing fluorescence. Pinhole (along with the optics preceding it) reduces the intensity of emission reaching the detector. One major disadvantage with imaging a point onto the specimen is that there are fewer emitted photons collected at any given instant. For a precise measurement, each point must be illuminated for a long time to collect sufficient light and to avoid a noisy image. This could further enhance the time required to create a point-by-point image. Using a light source of high light intensity like a laser point source is a solution which provides an added benefit of being present in a wide range of wavelengths. Mercury and Xenon light sources are too weak for confocal systems. Strong bundled light is generated by Lasers. There are different types of Lasers: Argon, Argon-Krypton, HeliumNeon, etc. Laser sources generate monochromatic light of a discrete wavelength- "LASER line". Different lasers are needed for the spectral range. Depending on the hardware of the microscope, some of the following lines might be available (in nm): 352, 364, 405, 430, 458, 476, 488, 496, 514, 543, 561, 596, 633. Imaging of only one point at a time in CLSM never allows a complete image of the specimen. The image is thus build one pixel at a time through a detector, which is attached to the computer. Image created of a thin planar region of the specimen is much sharper and better resolved (rejection of out-of-plane

unfocused light result). Accumulation of data from a series of optical sections imaged at short and regular intervals along the optical aixs helps in building 3D renditions of the specimen.

Fluorophores

Application of a particular fluorophore is the most crucial aspect of CLSM. This is governed by several factors like its sensitivity for the provided excitation wavelength. The correct part of the specimen should be tagged. The dynamics of the organism should not be considerably altered. Also, the chemical environment of the specimen can affect the position of the peaks of excitation and emission spectra, therefore, an additional consideration on the effect of the specimen on fluorophore should also be considered.

Dynamics of living cells

Imaging live specimens amounts to a challenge in maintaining the life and normal function of an organism, although CLSM has been used efficiently for the study of dynamics in living cells. As in conventional microscopy, CLSM also involves difficulties in preparing the sample for viewing. Additionally, the effect of photodamage on the object should also be considered. Repeated exposure of high intensity excitation light, which is required for tracking the cellular dynamics also compounds to this effect. The generation of free radicals through reaction of oxygen with fluorochromes in triplet excited states additionally introduces the problem of a fluorochrome influencing a cell's behavior.

Light path in a CLSM

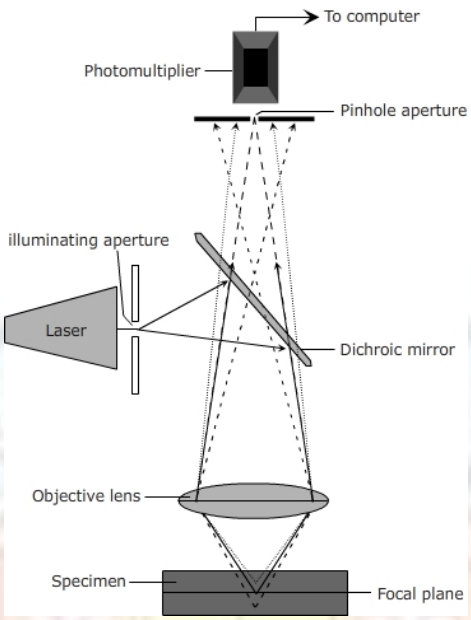

Figure: A schematic diagram of the optical components and light paths within a modern version of a confocal laser scanning microscope.

Source: Author

The microscope illuminates specimens using a laser beam focused by an objective lens down to a diffraction-limited spot. The position of the spot is controlled by scanning mirrors, which allow the beam to be swept over the specimen in a precise pattern. As the beam is scanned over the specimen, an image of the specimen is formed in the following manner. Light of short wavelength is emitted by a laser light source, passes through a tiny aperture, and is reflected by a dichroic mirror into an objective lens and focused onto a spot in the plane of the specimen. Fluorochromes in the specimen absorb the incident light and emit light of longer wavelength, which is able to pass through the dichroic mirror and come to focus in a plane that contains a pinhole aperture. Thus, the aperture and illuminated plane in the specimen are confocal. The light then passes into a photomultiplier tube that amplifies the signal and is transmitted to a computer which forms a processed, digitized image. Any light rays that are emitted from above or below the optical plane in the specimen are prevented from passing through the aperture and thus do not contribute to the formation of the image. As a result, out-of-focus points in the specimen become invisible. The diagram here illustrates the illumination of a single spot in the specimen. Different sites within this specimen plane are illuminated by means of a laser scanning process.

Advantages over conventional wide field microscope

Optical sectioning

Most images collected from relatively thick specimens produced using epifluorescence microscopy are not very clear. This happens because the image is made up of the optical plane of interest together with the contributions from above and below the focal plane of interest. Optical sectioning refers to the microscope's ability to produce sharper images of specimens than those produced using a standard wide-field epifluorescence microscope by removing the contribution of out-of-focus light to the image, and in most cases, without resorting to physically sectioning the tissue. Such methods have revolutionized the ability to collect images from thick and fluorescently labeled specimens such as eggs, embryos and tissues.

Information about the spatial structure of the object

Movement of the specimen along the optical axis (Z) by controlled increments helps in optical sectioning, thus allowing a greater number of slices to be recorded at different planes. The result is a 3D data set whose quality and accuracy depends on the thickness of the slice and spacing between successive slices. This can further be used to produce various aspects of 3D data set through computation.

Visualization of the dynamic changes

A possibility of visualizing and quantifying the changes is provided through the acquisition of confocal image series with a high time resolution.

Additionally glare from out-of-focus structures in the specimen is reduced and resolution is increased both laterally in the X and Y directions and axially in the Z direction.

Alternative methods to CLSM

Spinning disk confocal microscopes

This employs a different scanning system from the CLSM. Rather than scanning the specimen with a single beam, multiple beams scan the specimen simultaneously, and optical sections are viewed in real time. Addition of laser light sources and high-quality CCD detectors to the instrument has significantly improved the modern spinning disk microscopes. These are generally used in experiments where high-resolution images are collected at a fast rate (high spatial and temporal resolution), and are used to follow the dynamics of fluorescently labeled proteins in living cells. (web link no. 6)

Multiple photon microscopes

It has evolved from the CLSM. However, the difference lies in the fact that light source is a high-energy pulsed laser with tunable wavelengths. Usage of red light in such microscopes allows collection of optical sections from deeper within the specimen as compared to CLSM. This is generally chosen for imaging fluorescently labeled living cells since red light is less damaging to living cells than the shorter wavelengths (usually employed by confocal microscopes). Fluorophores are excited by multiple photons rather than single photons. There is less chance of over exciting (photobleaching) the fluorescent probe and causing photodamage to the specimen since the fluorochrome excitation is restricted to the point of focus in the specimen. Multiple photon excitation of a fluorophore is possible only where energy levels are high enough statistically confined to the point of focus of the objective lens.

Deconvolution

Optical sections can be produced using an image processing method called deconvolution. Early versions of deconvolution (to remove out-of-focus information from the digital image) method were relatively slow. Computing a single section through some algorithms could take hours to compute a single optical section. It is much faster now-a-days using today's fast computers and improved software, and the method compares favourably with the confocal approach for producing optical sections. There are two basic types of deconvolution algorithm: deblurring and restoration. The approach relies upon knowledge of the point spread function of the imaging system. Multiple-label imaging of both fixed and living cells can be performed and this offers an advantage over other scanning methods for imaging

relatively thin specimens. Additional background from the images collected with CLSM, spinning disk or multiple photon microscope can also be removed. One advantage of deconvolution is that the microscope is not restricted to the specific wavelengths of light used in the lasers commonly found in confocal microscope. (web link no. 7)

Summary

- A considerable imaging improvement has been provided by confocal microscopes over conventional widefield microscopes.
- Data collection in three dimensions and sharper, detailed images are allowed through CLSM.
- CLSM is especially useful for measuring dynamic processes in biological application.
- Confocal microscopy enabled short-timescale dynamics has been made possible through development of numerous newer designs of CLSM.

Exercises

1. Illustrate the function of a pinhole in a confocal microscope.
2. How does raster scanning mechanism help in obtaining a better image in a CLSM?
3. Highlight the advantages of a confocal microscope over normal fluorescence microscope.

Glossary

Autofluorescence: the phenomenon of emission of light by naturally occurring organic molecules when irradiate with light. They are useful to give contrast to the fluorescently labeled probes.

Dichroic mirror: A dichromatic or dichroic mirror is an accurate filters which allows to pass light of certain specific wavelength while reflecting the others. Dichroic mirrors are made up of alternating layers of optical coatings with different refractive indexes on a glass substrate.

Epifluorescence microscope: The type of fluorescence microscope where a single objective is used for focusing both the excitation wavelength light on the specimen and emitted fluorescence from the specimen to the detector.

Excited state: the unstable state of a molecule where the orbital electrons after absorbing the energy get transferred to a higher energy level. This state of a molecule is short lived and return to the minimum energy state after losing the absorbed energy.

Fluorophore: organic or inorganic molecules with the ability to emit fluorescence upon irradiation with light are known as fluorophores.

Ground state: Non excited normal state of a molecule where it is at the lowest minimum energy state.

Photomultiplier: An electrical device designed for the detection of weak electromagnetic radiations, usually light. It consists of a photocathode and an electron multiplier (amplifies and produces a detectable pulse of current).

Point spread function: The three-dimensional diffraction pattern of light emitted from an infinitely small point source in the specimen which is then transmitted (through a high numerical aperture objective lens) to the image plane of a microscope.

Solenoid: An electromechanical device used in relaying energy from one device to another. It is made up of a coil which produces a magnetic field when electric current is passed through it.

Ultraviolet microscopy: In ultraviolet microscopy the shorter wavelength of ultraviolet light improves the image resolution beyond that of optical light microscopes due to limited diffraction it experienced. This technique is used for inspection of devices with very small features such as modern semiconductors where they are non destructive to the UV light.

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Suggested Readings

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Web Links

1. http://www.olympusmicro.com/primer/techniques/fluorescence/fluorointrohome.html
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4. http://www.springer.com/life+sciences/biochemistry+%26+biophysics/book/978-0-387-25921-5
5. http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html
6. http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/index.html
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