Swayam Course - Analytical Techniques

Week: 6, Module16 - Flourescence Microscopy: Applications to live cell

Content Writer - Dr. Ruby Dhar, DBT-Biocare fellow, Department of Biochemistry,All India institute of Medical sciences,New Delhi

Introduction

Fluorescence microscopy, using a variety of fluorescent indicators (probes) that can be tailored in terms of their specificity for targets such as proteins, lipids, or ions has perhaps been the biggest step in allowing us to watch cell physiology. The underlying process of fluorescence involves the absorption of light energy (a photon) by an indicator followed by the emission of some of this light energy (as another photon) a few nanoseconds later.

Imaging of living cells and tissue is now in many fields of the life and physical sciences, and is instrumental in revealing a great deal about cellular dynamics and function. It is crucial when performing such experiments that cell viability is at the forefront of any measurement to ensure that the physiological and biological processes that are under investigation are not altered in any way. In recent years, technological advances, including sensor sensitivity, computing power, brighter and more-stable fluorescent proteins (FPs), and new fluorescent probes for cellular compartments, have given researchers the tools to study complex biological processes in great detail.

This module deals with basics of Fluorescence microscopy, it's applications and use in live cell imaging.

LAYOUT OF THE MODULE:

1.Introduction to Fluorescence microscopy

2.History of Fluorescence microscopy

3. Applications of Fluorescence Microscopy

4. Architecture of a Confocal Microscope

5. Types of Fluorescence microscope

6. Why Live cell imaging

7. How do we do live cell imaging

8. Fluorescent proteins vs Fluorophores

9. Important parameters to perform live cell imaging

10. Examples of application of cell Imaging

1. Introduction to Fluorescence microscopy

- Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the fluorescence lifetime.
- Fluorescence is a form of luminescence, in which susceptible molecules emit light from electronically excited states created by either a physical (for example, absorption of light), mechanical (friction), or chemical mechanism.
- Fluorescence microscopy is a powerful tool for modern cell and molecular biologists It provides a window into the physiology of living cells at subcellular levels of resolution.

Three very useful properties associated with Fluorescence are

- Sensitive
- Spatial resolution
- high specificity

Fluorescence process is governed by three important process:

- Excitation of a susceptible molecule by an incoming photon happens in femtoseconds (10E-15 seconds)
- Vibrational relaxation of excited state electrons to the lowest energy level is much slower and can be measured in picoseconds (10E-12 seconds).

- Emission of a longer wavelength photon and return of the molecule to the ground state, occurs in the relatively long time period of nanoseconds (10E-9 seconds).

This phenomenon is a stunning manifestation of the interaction between light and matter that forms the basis for the expansive fields of steady state and time-resolved fluorescence spectroscopy and microscopy.

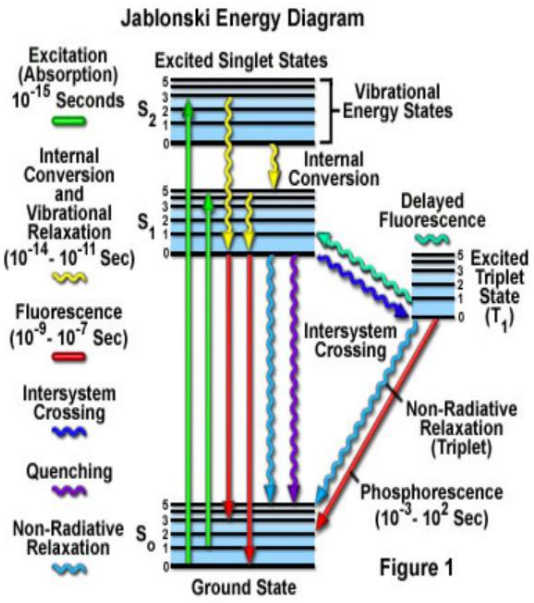


Figure 1

2. HISTORY OF FLUORESCENCE MICROSCOPY:

Several investigators reported luminescence phenomena during the seventeenth and eighteenth centuries, but it was British scientist **Sir George G. Stokes** who first described fluorescence in 1852 and was responsible for coining the term in honor of the blue-white fluorescent mineral fluorite (fluorspar).

Stokes also discovered the wavelength shift to longer values in emission spectra that bears his name.

The first fluorescence microscopes were developed between 1911 and 1913 by German physicists **Otto Heimstädt and Heinrich Lehmann** as a spin-off from the ultraviolet instrument. These microscopes were employed to observe autofluorescence in bacteria, animal, and plant tissues.

Shortly thereafter, **Stanislav Von Provazek** launched a new era when he used fluorescence microscopy to study dye binding in fixed tissues and living cells.

In 1940s that Albert Coons developed a technique for labeling antibodies with fluorescent dyes, thus giving birth to the field of immunofluorescence.

3. APPLICATIONS OF FLUORESCENCE MICROSCOPY:

1.Localization of proteins and association

2.Studying nucleic acid by in situ hybridization

3.Measuring the concentration of various inorganic ions

- pH

- Calcium concentration

4. Live cell imaging

5. Live tracking in animal models

6. Colocalization and interaction studies

4. ARCHITECTURE OF CONFOCAL MICROSCOPE

Excitation light from laser is passed through the various collimating optics to either a variable dichroic mirror(Nikon, Zeiss or Olympus)

Or AOBS(Acoustic –Optical Beam Splitter; by Leica)

Now it is reflected through the objective and focus to a point on the sample.

Fluorescence emission light passes back through the objective, through the dichroic or AOBS to the light sensing PMT(photo-multiplier tube)

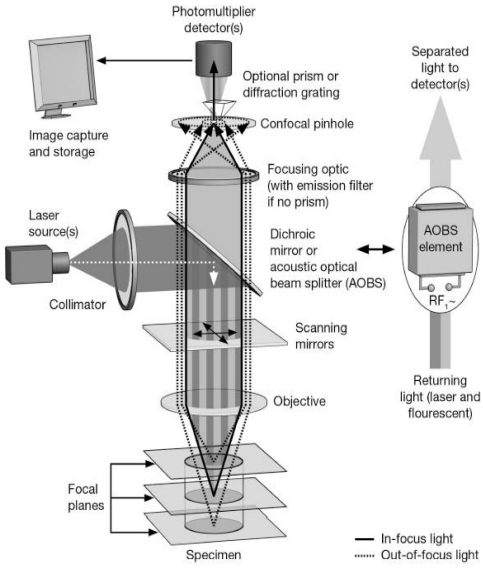


Figure Courtesy :Curr Protoc Neurosci. 2010 January ; 0 2

So, there never is a complete image of the sample -- at any given instant, only one point of the sample is observed. The detector is attached to a computer which builds up the image, one pixel at a time. In practice, this can be done perhaps 3 times a second, for a 512x512 pixel image. The limitation is in the scanning mirrors. Our confocal microscope (from Noran) uses a special Acoustic Optical Deflector in place of one of the mirrors, in order to speed up the scanning. This uses a high-frequency sound wave in a special crystal to create a diffraction grating, which deflects the laser light (actually, the first diffraction peak is used, with the zeroth-order peak being thrown away).

By having a confocal pinhole, the microscope is really efficient at rejecting out of focus fluorescent light. The practical effect of this is that your image comes from a thin section of

your sample (you have a small depth of field). By scanning many thin sections through your sample, you can build up a very clean three-dimensional image of the sample.

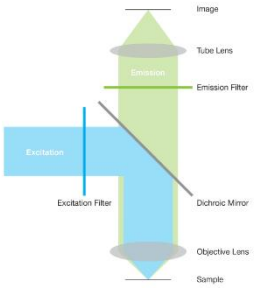
5. Types of Fluorescence Microscopy:

Wide-field Fluorescence Microscopy (WFFM) Techniques

Wide field Microscope:

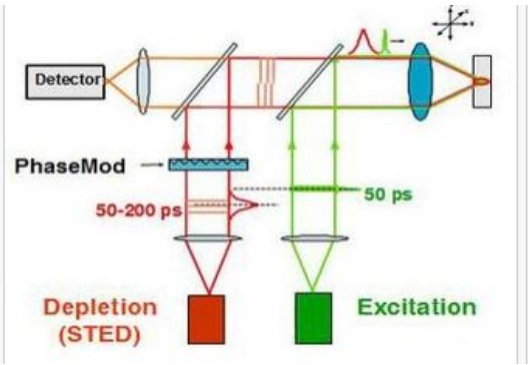
Widefield microscopes are excellent for producing 2D images of specimens as the entire field can be captured at once.

The resulting emission light, of longer wavelength, is observed through the microscope eyepieces or by a camera followed by computer digitization.



One of the most significant advancements has been the development of electron multiplied (EMCCD) and very low-noise, cooled CCD cameras.

Widefield microscopy can produce high-resolution images. However, because the whole sample is illuminated, there are factors that can reduce the resolution. It can be difficult to tell how deep in the sample the fluorescence originated from, especially in thick samples such as live cells or tissues, as the emission light is incorporated throughout the sample.

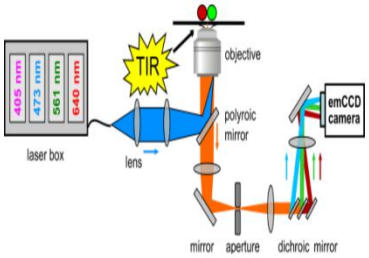


The emitted fluorescence can scatter, making the image appear blurry. This makes widefield microscopy sometimes unsuitable for producing 3D images.

Total Internal Reflection Fluorescence (TIRF) Microscopy

TIRF microscopy provides very good axial resolution (Z-direction, along the axis of illumination) to levels of approximately 200nm

Not only is the axial resolution better than most other techniques but this also can greatly reduce background light (thus increasing the signal to noise ratio)



The setup for TIRF microscopy is very simple and is similar to wide-field microscopy except that it employs an oblique angle for the excitation light impinging on the sample.

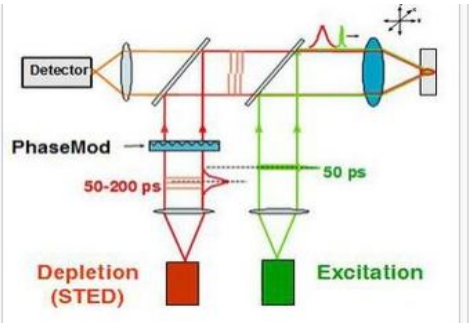
In contrast to confocal microscopy, which allows only the detection of one molecule at a time, we can detect hundreds of molecules in parallel with TIRF microscopy.

Stimulated Emission Depletion (STED)

Fluorescence Microscopy

STED functions by depleting fluorescence in specific regions of the

sample while leaving a center focal spot active to emit fluorescence.



STED microscopy, developed by Stefan Hell and colleagues, is a

relatively new super resolution technique that has been shown to improve fluorescence microscopy resolution by approximately an order of magnitude over traditional diffraction limited techniques such as LSCM (Laser scanning confocal microscopy)

STED can produce optical resolution to levels that were previously thought possible only with electron microscopy, and it has been used to examine key biological processes that no other technique could have examined

It creates super-resolution images by the selective deactivation of fluorophores, minimizing the area of illumination at the focal point, and thus enhancing the achievable resolution for a given system.

6. Why do we do live cell imaging?

Live-cell imaging is an important analytical tool in laboratories studying biomedical research disciplines, such as cell biology, neurobiology, pharmacology, and developmental biology. Imaging of fixed cells and tissues (for which photobleaching is the major issue) usually requires a high illumination intensity and long exposure time; however, these must be avoided when imaging living cells. Live-cell microscopy usually involves a compromise between obtaining image quality and maintaining healthy cells. Therefore, to avoid a high illumination intensity and long exposure time, spatial and temporal resolutions are often limited in an experiment. Imaging live cells involves a wide range of contrast-enhanced imaging methods for optical microscopy. Most investigations use one of the many types of fluorescence microscopy, and this is often combined with transmitted light techniques, which will be discussed below. Continual advances in imaging techniques and design of fluorescent probes improve the power of this approach, ensuring that live-cell imaging will continue to be an important tool in biology.

Live cell imaging techniques allow scientists to understand the following processes:

- Cell structure and processes in real time
- It can be visualized over time
- Observe dynamic changes which provides more insight into the operations of a cell than a snapshot provided by imaging fixed cells
- Spatial resolution of light microscopy allows observing subcellular structures, by using a range of fluorescent probes.
- Cellular integrity, endocytosis, exocytosis

- Imaging in live animal model system
- Less prone to experimental artifacts, it usually provides more reliable and relevant information than fixed cell microscopy and thus prone to less experimental artifacts, it usually provides more reliable and relevant information .

LIVE CELL IMAGING:

VARIOUS BIOLOGICAL APPLICATIONS AND MISROSCOPY APPROACHES USED

BIOLOGICAL APPLICATION TO BE USED	**MICROSCOPY APPROACH**
Cell Shape- Cell migration –organelle kinetics	Transmission Microscopy, bright field, phase contrast
Protein localization , structure of cell, organelles	Epifluorescence/Confocal/Spinning desk
3D imaging of live cells	Confocal/Spinning
3D imaging of tissues, model organisms microscopy	Multiphoton Microscopy/light sheet
Small animals	
Biosensing and protein-protein interactions	Fluorescence resonance energy transfer

Most modern wide-field epifluorescence, spinning disk confocal, or TIRF microscope setups rely on a similar set of optical and mechanical components, and all imaging modalities are often used for live cell imaging.

7. How do we do live cell imaging ?

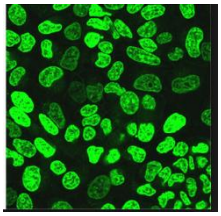
HOW DO WE LIVE TRACK CELLS, PROTEINS, ORGANELLES USING FLUORESCENCE BASED MICROSCOPY?

Using fluorescence microscopy, with help of fluorescent probes and fluorescent proteins cells can be tracked live to understand various structural changes, cell compartments, migrations and the concentrations of various ions. But certain parameters need to be kept in mind, while performing these assays. As part of their normal life cycle, most tissues and cells are never exposed to light, and it is known that ultraviolet (UV) light damages DNA, focused infrared (IR) light can cause localized heating, and fluorescence excitation causes phototoxicity to tissues and cells . The main cause of phototoxicity in living cells is the oxygen-dependent reaction of free-radical species, which are generated during the excitation of fluorescent proteins or dye molecules with surrounding cellular components. Thus, for live-cell imaging, it is best to reduce the amount of excitation light by optimizing the efficiency of the light path

through the microscope, and by using detectors that are optimized to detect most of the fluorescence emission. Low concentrations of fluorescent probes also need to be used to avoid causing nonspecific changes to the biological processes of interest.

live cell imaging of Hela cells by staining with nucspot live,

which stains the nucleus



There are two major ways which are used for live tracking:

- ❑ Fluorescent proteins
- ❑ Flourophores

FLOURESCENT PROTEINS:

Use of Fluorescent proteins, eliminates the use of external labeling, one can label cell compartments, or even whole cells, tissues or organisms, with the appropriate introduction of the FP gene in the genetic material of the cell or organism under study using Recombinant DNA technology

The discovery of the naturally occurring **green fluorescent protein (GFP),** which was first isolated from jellyfish, revolutionized live cell imaging.

- It emits green fluorescence light under blue light excitation. It was discovered by Osamu Shimomura, in 1962, who purified it from the Aequorea victoria jellyfish .
- It was only in 1992 that Douglas Prasher cloned the sequence of GFP and Martin Chalfie expressed this sequence in vivo .
- Later on, the group of Roger Tsien reported the first crystal structure of GFP, showing the way for the creation of GFP mutants, to finally obtain different color variants and improve the fluorescence signal and photostability .
- **Shimomura, Chalfie, and Tsien** were awarded in **2008 with the Nobel Prize in Chemistry** for their work on the genetically encoded fluorescent proteins.

© The Nobel Foundation. Photo: U. Montan
Osamu Shimomura

© The Nobel Foundation. Photo: U. Montan
Martin Chalfie

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Roger Y. Tsien

Mutation of the GFP gene led to the development of blue fluorescent protein (BFP) and a cyan fluorescent protein (CFP), which have altered absorption and emission properties from wild-type GFP . Yellow fluorescent protein (YFP) and DsRed fluorescent protein derivatives have varied emission properties and thus allow scientists to use several fluorescent protein probes within the same cell.

For example, a red fluorescent protein tdT tomato transgenically expressed in neural stem and progenitor cells was used to delineate the neurogenesis in adult mouse brains.

Live imaging of neurogenesis in the adult mouse hippocampus

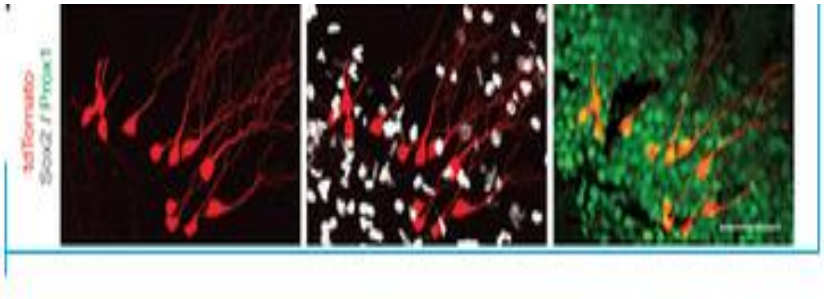


Fig Courtsey: *Science* 09 Feb 2018: Vol. 359, Issue 6376, pp. 658-662

8. Fluorophore (small molecule versus fluorescent protein)

Fluorescent dyes are small and exhibit favorable optical properties, such as brightness, photostability, and narrow bandwidth relative to fluorescent proteins.

For live cell imaging, the fluorescent probes should also penetrate in the cell without causing damage.

Probe entry mechanisms include esterification of dyes to promote their cellular uptake, the use of synthetic vesicles containing probe, and mechanical techniques such as microinjection and electroporation.

Fluorophores can be divided into three general groups:

- Organic dyes
- Biological fluorophores
- Quantum dots

ORGANIC DYES:

Synthetic organic dyes, such as fluorescein, were the first fluorescent compounds used in biological research. Derivatives of these original compounds have been produced to improve their photostability and solubility. These dyes are also derivatized for use in bioconjugation, especially **fluorescein isothiocyanate (FITC), rhodamine (tetramethyl rhodamine isothiocyanate, TRITC**

BIOLOGICAL FLOUROPHORES:

Green fluorescent protein (GFP) is a wellknown Biological Fluorophores routinely used for Flourescence microscopy and as a gene expression reporter. Since that time, derivatives of

the original GFP, phycobiliproteins (allophycocyanin), phycocyanin, phycoerythrin, and phycoerythrocyanin) and many other proteins have been designed for use in biological expression systems, and their use is now commonplace in biological research.

Advantage of GFP based fluorophores is that expression plasmids can be introduced into either bacteria, cells, organs or whole organisms, to drive expression of that fluorophore either alone or fused to a protein of interest in the context of the biological processes studied.

QUANTAM DOTS:

Quantum dots are nanocrystals with unique chemical properties that provide tight control over the spectral characteristics of the fluor.

Quantum dots were developed in the 1980s and since the 1990s have been increasingly used in fluorescence applications in biological research.

Quantum dots are nanoscale-sized (2-50 nm) semiconductors that, when excited, emit fluorescence at a wavelength based on the size of the particle; smaller quantum dots emit higher energy than large quantum dots, and therefore the emitted light shifts from blue to red as the size of the nanocrystal increases. And because quantum dot size can be tightly controlled, there is greater specificity for distinct excitation and emission wavelengths than other fluors.

Quantum dots have the following advantages quantum dots remained fluorescent for 4 months in an *in vivo* imaging study Additionally, quantum dots can be coated for use in different biological applications such as protein labeling. While the use of quantum dots in biological applications is increasing, there are reports of cell toxicity in response to the breakdown of the particles (4) and their use can be cost-prohibitive.

But, the disadvantage is that , the size of the fluorescent protein can change the normal biological function of the cellular protein to which the fluorophore is fused, and biological fluorophores do not typically provide the level of photostability and sensitivity offered by synthetic fluorescent dyes.

Fluorescent dyes can be chemically modified through organic synthesis. Thus, they can be

tailored to the cellular environment or illumination method. There are now hundreds of commercially

available organic dyes that span the visible wavelength range

NEAR INFRA RED DYES: Fluorescent *dyes* based on small organic molecules that function in the near *infra red* (NIR) region are of great current interest in chemical biology.

They allow for imaging with minimal autofluorescence from biological samples, reduced light scattering and high tissue penetration.

A noteworthy recent addition to the suite of small-molecule fluorophores is the near-infrared

(IR) silicon-rhodamine dyes and their extension to DNA stains and cytoskeletal stains

with improved spectral properties for long-term and in vivo imaging.

Another novel class of fluorogenic molecules consists of the carbofluoresceins and carborhodamines, which can be caged or masked to modulate their fluorescent properties. This unique attribute makes these

molecules valuable as sensors or reporters, as well as for superresolution microscopy applications.

9. Important parameters to perform live cell imaging:

To minimize the photobleaching effect of the fluorescent samples and obtain high resolution pictures, we need to keep a check both in terms of the sample handling and also the specific requirements in the instrument .

Important hardware factors that should be considered in the design of a live cell imaging microscope

1. **Excitation and emission light path**: The wavelengths of excitation and emission filters should be optimized to match the fluorophore used to limit unnecessary light exposure and optimize detection of fluorophore emission. It is also important to reduce background light by turning off the room lights and minimize scattered light in wide-field imaging by

closing the field diaphragm as much as possible.

2. **Shutters:** Fast, motorized shutters should be used to turn off the excitation light when not needed to take an image. It is particularly important to note that software-controlled shutters often have a significant.

An important advantage of LEDs is that they can be switched on and off very rapidly and may thus not require additional mechanical shutters.

3. **Objective lens:** As outlined earlier, specimen irradiance increases drastically with magnification. Thus, to limit photodamage to the specimen, the lowest

magnification should be used as determined by the experimental question.

4. **Camera:** To detect dim fluorescent signals, it is essential to use cooled scientific grade cameras with the lowest readout noise available. Lower noise allows detection of dimmer signal. While interline CCD cameras have historically shown the best performance for live cell imaging, the camera field has developed rapidly in the recent years

5. **Motorized stage:** Multipoint acquisition allows parallel data collection from many fields of view, which is especially important for longer time-lapse experiments or drug treatments that can only be done once per specimen. For high-magnification experiments, the accuracy with which a motorized stage returns to a previous position is key.

The two main experimental challenges in collecting meaningful live cell microscopy data are :

- minimize photodamage while retaining a useful signal-to-noise ratio
- provide a suitable environment for cells or tissues to replicate physiological cell dynamics.
- MICROSCOPE ENVIRONMENTAL CONTROL
- Cultured cells and tissues will only behave normally in a physiological environment and control of factors such as temperature and tissue culture medium composition is thus critically important to obtaining meaningful data in live cell imaging experiments.
- The conditions required to successfully maintain cell health on the microscope stage obviously depend on the organism, and in this section, we provide a general overview of options available to maintain environmental control with a focus
- on live cell imaging of mammalian cell types.
- **Temperature :** The most basic level of environmental control is maintaining correct temperature to ensure that observed cell dynamics are an accurate representation of in vivo cell behavior.
- For cells from warm-blooded animals, the specimen thus needs to be warmed. Although the most commonly used temperature is 37 C for mammalian cell lines.
- It should be noted that physiological body temperatures can vary considerably (i.e., 42 C in chickens), and depending on the experiment, one may want to take this into consideration. In contrast, Drosophila cells will not behave normally or even survive at 37 C.

- **Media and Ph Composition**
- Most tissue culture media are buffered to physiological pH by sodium bicarbonate and 5% CO2. Thus, pH in an open tissue culture dish on a microscope stage will rapidly increase and leave the physiological range within minutes as CO2 outgases into the atmosphere. One common approach to control pH is the use of CO2-independent media or addition of 10–25 mM HEPES to increase buffering capacity.
- **IMAGING CHAMBERS:**

For live cell microscopy, cells are typically grown on coverslips and viewed with an inverted microscope from below. Most microscope objectives are designed for no.1.5 coverslips (0.17 mm thick), and use of coverslips of a different thickness will result in spherical aberration..

- This Incubation Chamber allows the experimentalist to carry out experiments under CO^2 and H_2O control.

- Advantages of these Chambers

- Unique, diffusion grid, combined with air input and return vents provide an air flow pattern for consistent, even heating, with no hot or cold spots in the chamber.
-
- External heater that can be placed far enough from the system to eliminate electrical and vibrational interference from the heater
- High degree of temperature precision and stability
- Minimal focal drift after equilibrium is achieved– Accuracy ±0.1ºC at the sample itself, and 0.2ºC across the microscope stage (allowing for uniform heating of multiwell dishes)\
- Airflow pattern and temperature uniformity eliminate dramatic changes in environmental temperature when the incubator door opens

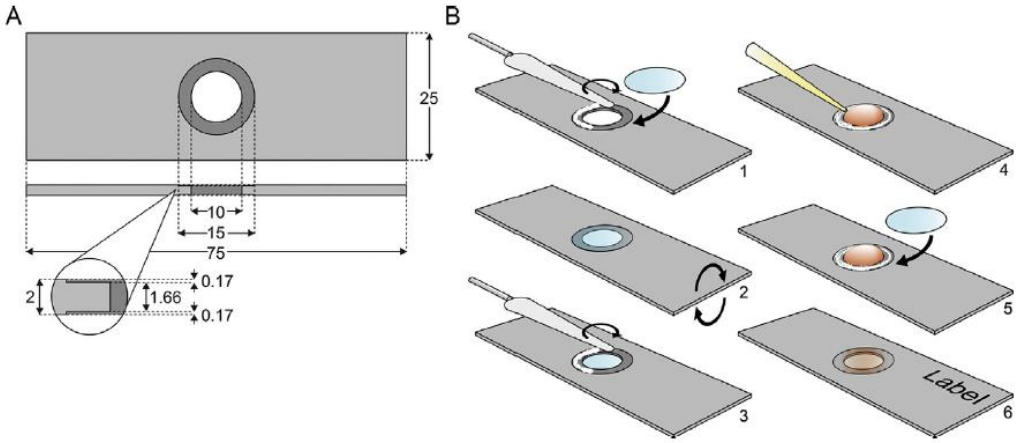


Diagram of reusable aluminium slides

The slide fits a 15mm round coverslip. A bead of silicon grease is distributed on one side of the slide and a clean cover slip is attached.

A drop of cell culture Medium is added and again mounted with a coverslip

Fig: Book on Fluorescence microscopy by Andreas Ettinger, Torsten Wittmann

REDUCING PHOTOBLEACHING :

Photobleaching—light-induced degradation of fluorophores—is a significant problem in live cell imaging.

- Use appropriate current fluorophores and fluorescent proteins that match the excitation and emission specifications of the microscope being used for imaging.
- Use as minimal an exposure and light power level as possible.
- Avoid lengthy exposure to "wasted" light, such as extended periods of time viewing the sample through the oculars while not collecting data.
- When designing the experiment, consider all parameters carefully to minimize the time in capturing the images

Many commercial vendors have marketed several products which can provide protection from photobleaching for live cell imaging.

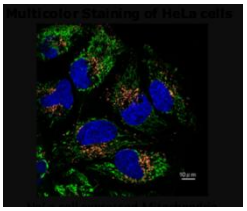
- Trolox Antifade Reagent has antiphotobleaching and antiblinking properties .
- Prolong™ Live Antifade Reagent, which is based on the Oxyrase antioxidant technology.

These antifade Reagent has been validated to provide protection for a wide range of organic dyes as well as fluorescent proteins. It has also been rigorously tested and shows little to no measurable effect on cell vitality, proliferation, or incidence of apoptosis for at least 48 hours.

10. Examples of application of cell Imaging :

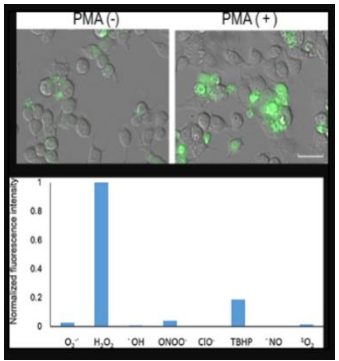
This section elaborates the various applications of live cell imaging using fluorescence microscopy.

1. AcidiFluor ORANGE: pH probe for acidic organelles



With the Acidifluor™ ORANGE probe, fluorescence increases greatly when under acidic condition. This probe can stain acidic organelles, such as lysosomes, late endosomes and secretory granules.

2. **Reactive Oxygen Species Research**



Reactive oxygen species (ROS) play key roles in many intracellular processes and are known to be involved in various diseases such as carcinogenesis, inflammation, and

many others. Many species of ROS exist and each one is likely to have a specific role in living cells.

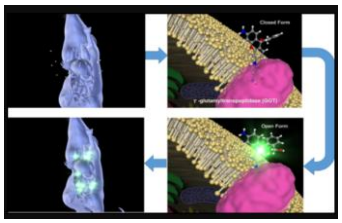
HYDROP: for specific detection of hydrogen peroxide

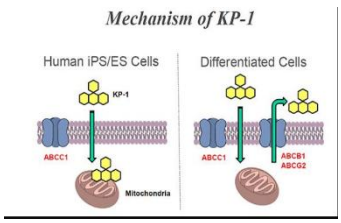
High specificity to hydrogen peroxide (H_2O_2)

Suitable for time-lapse imaging because it photo-bleaches slowly

λex: 492nm – λem: 518nm

3. **ProteoGREEN-gGlu (gGlu-HMRG)** is a fluorescent probe to detect γ-glutamyl transpeptidase (GGT) activity. The probe irreversibly reacts with GGT which is highly expressed on the surface of cancer cells or cancer tissues, and then turns into green fluorescent materials. Since the green materials are rapidly taken into the cells, ProteoGREEN-gGlu can detect the cancer tissues which highly express GGT.





4. **Kyoto Probe 1 (KP-1): Specifically label human iPS and ES cells**

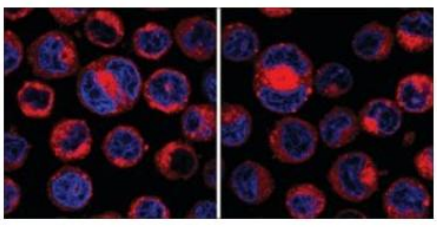
Able to distinguish human iPS cells and/or human ES cells from differentiated cells

Usable for flow cytometry or live cell imaging

Staining while culturing

λex: 515nm – λem: 529nm

CellVue® Claret Far Red Fluorescent Cell Linker Midi Kit for General Membrane Labeling

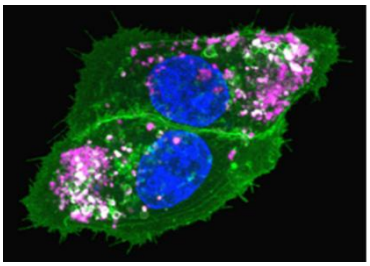


Fluorescent Antibody Labeling

Due to their large size (~150 kDa), antibodies have historically been limited to applications in fixed, permeabilized cells. However, the specificity of antibodies is useful for monitoring endogenous proteins in cells, so several recent strategies have been developed for antibody-based live-cell imaging.

These approaches typically utilize only a portion of a full-length antibody, either the antigen binding fragment (Fab, ~55 kDa), the single-chain variable fragment (scFV, ~28 kDa), or the smallest recognition unit called a nanobody or VHH domain (~12 kDa), derived from single-chain camelid antibodies

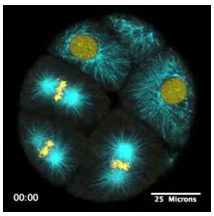
Live Cell Tracking:



U2-OS cells, were transduced with CellLight Plasmabrane(GFP)

,Qtracker 655 Cell Labeling (Magenta) and NuclearMask (Blue)

Live cell imaging of a 16-Cell Sea Urchin Embryo



This figure shows how 16-cell Sea Urchin embryo divides and undergo various phases of cell Cycle.

Time-lapse microscopy video of untreated 16-cell purple urchin embryo with EMTB-3G (microtubules) in shown cyan and mC-H2B (chromatin) in yellow.

SUMMARY

1.Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the fluorescence lifetime.

2.Fluorescence microscopy is a powerful tool for modern cell and molecular biologists It provides a window into the physiology of living cells at subcellular levels of resolution.

3. Applications of Fluorescence microscopy ranges from live imaging, protein localization, ion concentration

4. Live cell imaging helps in better understanding of the dynamics and cell structure in real time. Observe dynamic changes which provides more insight into the operations of a cell than a snapshot provided by imaging fixed cells

5. Live cell imaging can be achieved by either using Fluorescent proteins or Fluorophores.

6. Fluorescent proteins can be used to label cell compartments, or even whole cells, tissues or organisms, with the appropriate introduction of a fluorescent tag in the genetic material of the cell or organism under study using Recombinant DNA technology

7. The discovery of the naturally occurring **green fluorescent protein (GFP),** which was first isolated from jellyfish, revolutionized live cell imaging. Later on many mutants of GFP has been generated to produce Fluorescent tags with different colors.

8. **Shimomura, Chalfie, and Tsien** were awarded in **2008 with the Nobel Prize in Chemistry** for their work on the

genetically encoded fluorescent proteins.

9. Fluorescent dyes are small and exhibit favorable optical properties, such as brightness, photostability, and narrow

bandwidth relative to fluorescent proteins.

10. **Fluorophores can be divided into three general groups:**

- Organic dyes
- Biological fluorophores
- Quantum dots

11. The two main experimental challenges in collecting meaningful live cell microscopy data are :

- minimize photodamage while retaining a useful signal-to-noise ratio
- provide a suitable environment for cells or tissues to replicate physiological cell dynamics.

12.For live cell imaging ,cells need to be grown under proper conditions so that they can grow properly. Depending on

the cell type, appropriate temperature, CO_2 and Humidity needs to ne maintained.

13 Photobleaching—light-induced degradation of fluorophores—is a significant problem in live cell imaging.

Hence appropriate current fluorophores and fluorescent proteins that match the excitation and emission

specifications of the microscope being used for imaging. Also we need to use as minimal an exposure and light power

level as possible.

14. There are several commercially available dyes, which can bind to specific organelles inside the live cells, even without fixation and thus helps in live tracking of these organelles.

15. In this module we also learned how specific fluorophores like HYDROP, helps in monitoring the release of H2O2 in live cells, in experimental conditions where there is increase oxidative stress.