Fluorescence and fluorescence microscopy

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The concept of specific cellular antigen staining by use of an immunofluorescent technique was introduced by AH COONS and co-workers about 60 years ago. Fluorescent probes are efficient tools and enable the detection of particular components in complex structures of organs including live cells. Under the condition of specific molecular interactions, fluorochrome labeled ligands allow the selective localization of cell compounds including antigens, nucleic acid structures or other molecules. Fluorescent techniques are especially useful to localize the intra- and extracellular position of biomarkers in the course of gene expression, during cytoplasmic synthesis and migration. The method can be equally applied to follow changes of their localization as response to a defined stimulus and to observe relative distributions of molecules with respect to their dependency from each other.

History of fluorescence

Light emission by bacteria or by decaying organic matter (luminescence) are natural phenomena and always been known to man, yet, scientific investigation of luminescence began when the Bolognian stone was discovered in 1602. The following table summarizes the historical steps of fluorescence according to data from T TOYOKUNI (Pharmacology Lectures, *Introduction to Biological Imaging: Fluorescent Probes*; Crump Institute for Molecular Imaging, UCLA/USA) and from the literature.

Table 1: Short history of fluorescence and fluorescent applications

1602:	The cobbler and alchemist V CASCIAROLO prepared by accident an artifical phosphor known as the *Bolognian stone* or *Bolognian phosphor* which glows after exposure to light. A complete description of V CASCIAROLO's stone and the first scientific study of luminescent phenomena were written in 1640 by F LICETUS (*Litheosphorus Sive De Lapide Bononiensi*).
1646:	The Jesuit priest A KIRCHER recorded an observation of the wood extract of *Lignum nephriticum*: an aqueous infusion of this wood exhibited blue color by reflected light and yellow color by transmitted light. The blue light is actually a type of light emission (fluorescence); therefore, A KIRCHER is often regarded as the discoverer of fluorescence. Perhaps he was also the first microscopist; in his "Ars magna lucis et umbrae" he used the term "smicroscopium", an instrument being named "Vitra muscaria" (flea glass).
1664:	R BOYLE was a pioneering chemical and physical experimenter. In 1664, he established (in *Experiments and Considerations Touching Colours*, London 1664) that many environmental factors exert influences on fluorescence properties of organic compounds such as dilution (the phenomenon of fluorophore-fluorophore interactions) and acids or alkalies (interactions with hydrogen and metal ions).
1838:	D BREWSTER used the term *internal dispersion* to describe fluorescence phenomena.

1852: G STOKES, professor of mathematics and physics, interpreted the light-emitting phenomenon and formulated the law that the fluorescent light is of longer wavelength than the exciting light (*Stokes law* or *Stokes shift*).

1853: G STOKES coined the term *fluorescence* from the term *internal dispersion*.

1871: The chemist A VON BAEYER synthesized a fluorescent dye, *fluoresceine*.

1880: G GRÜBLER & Company started to test and package the most desirable *dyes for research*.

1882: The bacteriologist and immunologist P EHRLICH employed the fluorescent dye uranin (sodium salt of fluoresceine) to track the pathway of secretion of aqueous humor in the eye which was the first case of the use of *in vivo fluorochroming* in physiology.

1887: K NOACK from the University of Giessen published a book listing some 660 *fluorescent compounds* arranged according to the color of their fluorescent light.

1897: The chemist R MEYER introduced the term *fluorophores* for chemical groups with which fluorescence was associated.

1904 A KÖHLER published his work about monochromatic lighting for the microscope, and he developed together with M VON ROHR a UV microscope at the CARL ZEISS company.

1911: M HAITINGER coined the term *fluorochrome*. In the following years, he became able to stain specimens with fluorochromes in order to study their *secondary fluorescence* (1933).

1913: The first *fluorescence microscopes* were developed by C REICHERT, O HEIMSTAEDT, H LEHMANN and S VON PROWAZEK as an outgrowth of the UV or luminescence microscope. The instrument was used to investigate the autofluorescence of bacteria, protozoa, plant and animal tissues, and bioorganic substances such as albumin, elastin and keratin.

1914: The protozoologist S VON PROWAZEK employed the fluorescence microscope to study dye binding to living cells. It was stated that *fluorochromes* introduced into the cell effectively *illuminate the partial functions of the cell* in the dark field of the fluorescence microscope; a great step forward in experimental cytology.

1929: P ELLINGER and A HIRT modified the fluorescence microscope so that it could be used to examine opaque specimens from most living organs; the instrument was called intravital microscope and is considered as the first *epi-fluorescence (or incident-light excitation) microscope*.

1935: The physicist A JABLONSKI descibed physically (energy diagrams) the observations made by G Stokes in 1852.

1941: AH COONS and co-workers described in the years 1941 and 1942 the concept of cellular antigen staining in tissue sections by an immunofluorescence technique (*fluoresceine -4-isocyanate*) for the microscope.

1948: T FÖRSTER described the physical effects of intermolecular energy migration and fluorescence which are now designated as *fluorescence resonance energy transfer* (FRET). Today, FRET detection methods are very efficient for probing cells in fluorescence microscopy which allow resolution at the nanometer scale.

1961: M MINSKY designed a confocal microscope (US Patent No. 3,013,467, filed 1957 and awarded 1961).

1971: The concept of *4Pi microscopy* was formulated by C CREMER and T CREMER in their patent specification P 21 16 521.9 (*Verfahren zur Darstellung bzw. Modifikation von Objektdetails, deren Abmessungen ausserhalb der sichtbaren Wellenlängen liegen*). The idea of the confocal laser scanning 4Pi microscope was published by C CREMER and T CREMER in 1978.

1982: Commercial development of the first *Laser scanning microscope* by CARL ZEISS company which laid the basis of modern microscopes for imaging in life science with fluorescent techniques such as FRET, FRAP, FLIP and FLAP. Until the 1990's, fluorescent markers were restricted to few fluorophores.

1992: SW HELL pioneered *STED (Stimulated Emission Depletion)-4Pi- fluorescence microscopy* (HELL SW and STELZER EHK, 1992; HELL SW et al., 1994; KLAR TA et al., 2000; DYBA M and HELL SW, 2002) enabling resolutions much below E ABBE's definition (1873) which is based on the diffraction limit of light. With the new techniques, resolutions of 30-50 nm (about λ/23) can be achieved; 4Pi microscopy has become an established nanoscopy method.

During the last decades, many new developments in fluorescence microscopy have revolutionized biological research with hitherto unpredicted perspectives (see below). Modern preparation techniques including non-destructive sample handling allow studies of living cells with very fast optical detection of molecular probes and very high specific contrast. The advancements include imaging in three dimensions by multi-photon excitation and techniques such as confocal, deconvolution, ratio-imaging and total internal reflection.

Then, new applications such as fluorescence in situ hybridization (FISH) or the use of green fluorescent proteins (GFP including variants thereof) are now available. They have refined the tools of molecular labelling (TRASK BJ et al., 1991; PRASHER DC et al., 1992; CHALFIE M et al., 1994; HEIM R et al., 1994; STEWART CN, 2006) and, also, initiated a renaissance in the microscopy field. Antibody molecules can be now labeled with any of a number of different fluorescent probes currently available from commercial sources. There exist a number of generalized protocols for the attachment of fluorophores to proteins including antibodies, nucleotides, lipids and oligosaccharides or other biological molecules; protocols are readily available in the newer literature, from commercial sources or from papers in the world wide web.

Principle of fluorescence

The light emitting phenomenon of fluorescence was interpreted for the first time by GG STOKES in 1852, its nature, however, was only understood much later, when A JABLONSKI (1935) could describe the underlying physics. In fluorescence microscopy, fluorescent molecules act like light sources that are located at specific cellular sites, indicating their location by light of a specific color. These light sources need energy to emit light (*emission* light), and this is given to the fluorochrome by the *excitation* light of the microscope light source; a specific range of wavelengths is necessary to excite a given fluorochrome. Chemically, fluorophores are aromatic hydrocarbons or heterocycles. The process of fluorescence is cyclical unless the molecule is destroyed by photobleaching.

Fluorescence is the result of a three-stage process that occurs in fluorophores. The processes involved are illustrated by the Jablonski diagram, (a) stage 1: absorbed light energy excites electrons to a higher level in the fluorochrome; (b) stage 2: these electrons lose a portion of their energy by vibration and rotation; (c) stage 3: the rest of the energy is then given off as fluorescent light as they return to their original state after about 10 ns; a photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower and therefore of longer wavelength.

In experimental work with a fluorophore, it is necessary to know the wavelength of photon absorption (absorption spectrum). The absorption maxima of fluorochromes used in biology are found in the ultraviolet (UV) and the blue light. The dependence of the fluorescence intensity is described by the emission spectrum; their maxima are shifted towards red (emission of a lower energy photon, see above). The difference in energy or wavelength is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. All fluorochromes show distinct spectral properties, i.e. every fluorochrome has its own excitation and emission spectra. This means that we are dealing with at least two different light beams which have to be separated, and the microscope must be adequately equipped to visualize the fluorescent light.

Fluorescence spectra and quantum yields are in general more dependent on environmental factors than the absorption spectra and extinction coefficients. This spectral sensitivity makes fluorescence versatile for probing the behaviour of cells. Many environmental factors are known to have influences on fluorescence properties.

- Environmental polarity: fluorescence spectral shifts to longer wavelenghts occur in polar solvent because energy of the excited state is lost to the solvent when polar solvent molecules reorient around the more dipolar structure of excited fluorophores shortly after excitation. For example, aminonaphthalene fluorophores are effective probes of environmental polarity in a protein's interior.
- Fluorophore-fluorophore interactions: quenching of one fluorophore by another occurs in the case of high loading concentrations or in high labeling densities. This is for example the basis of protease detection in which protease catalyzed hydrolysis of heavily labeled (and almost quenched) protein substrates relieves the intramolecular self-quenching and, thus, yielding brightly fluorescent peptide fragments. Fluorophore-
  fluorophore interactions can be employd to monitor molecular assembly or fragmentation processes. Then, fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electrical excited states of two fluorophores in which excitation is transferred from a donor molecule to an acceptor molecule without the emission of photon.
- Interactions with hydrogen or metal ions: the binding of hydrogen or metal ions changes the electronic structure of a number of fluorophores, thus affecting the absorption and the fluorescence properties of the probes. For example, various fluoresceine labeled proteins have quantum yields in the range of 0.2 to 0.7 at pH 8.0 which drop rapidly with decreasing pH. This characteristic can be used to develop probes for pH, calcium (and other metals) and inorganic ions.
- Interactions with biomolecules: due to their structural nature, fluorescent probes can bind noncovalently to biomolecules in cells in a specific or non-specific manner. The binding can highly affect the fluorescence quantum yield and wavelength which is the basis of DNA and RNA probes, protein probes and others; examples are ethidium bromide, DAPI and acridine dyes. Then, fluorogenic enzyme substrates are probes which are converted by specific enzymes into products that have either increased fluorescence or shifted spectra. These are useful for monitoring enzyme activities.

Fluorescence microscopy

Basically, a fluorescence microscope is a conventional light microscope equipped by an excitation light source and an array of filters. The whole construction has to ensure that the

excitation light path through the objective lense is the opposite light path that fluorescence light takes on the way to the eyepiece or the camera, respectively. The performance of a fluorescence microscope is especially sensitive to the choice of the filter sets.

The precise location of cellular components labeled with specific fluorochromes can be monitored because a fluorescence microscope is able of imagining the distribution of a single molecular species based on the properties of fluorescence emission. The fluorescence microscope needs four essential elements, (a) a fluorophore labeled specimen; (b) a light source for excitation; (c) wavelength filters to isolate emission from excitation photons; (d) and last not least, the optical device for the observation equipped with an image recording unit.

An intense light source is needed to provide the wavelengths to excite the fluorescence of a fluorochrome. Either mercury or xenon lamps with high intensity illumination are needed. Because all fluorochromes are subject to the process of photobleaching, excitation brightness and time length must be restricted to the rigth amount needed. This can be done with neutral density filters or a motorised attenuator.

Following the light path in the microscope, the next step is the selection of filters which only permit the range of excitation wavelengths to pass through. This is performed by an excitation filter with bandpass filter characteristics. After restriction of the light to the color that is needed to excite the fluorochrome, this one is directed to the specimen via a dichromatic mirror. This mirror has to reflect light below a given wavelength and to let longer wavelengths pass through. Then, the excitation light travels through the objective (which acts like a condensor) to the specimen and fluorescence takes place. Here, the excited fluorochromes emit the fluorescence of longer wavelengths being captured by the objective lens and moving on to the dichromatic mirror. The design of the mirror will now let the longer wavelengths pass through. Finally, the last filtering is done by the emission filter (barrier filter) which restricts the light color to fit best with the fluorochrome emission so that no unwanted wavelengths are observed. The emission filter is usually a bandpass filter precisely restricted to one spectrum; a longpass filter can also be used but with less optimised restrictability.

To limit the amount of irradiation of the fluorochrome, one can focus and scan the specimen initially using phase-contrast optics with transmission from the UV source blocked. Because fluorochromes are prone to photobleaching, one can add bleaching retardants such as DABCO (1,4-diazobicyclo-[2,2,2]-octane) to the mounting media. Another approach is to use a second fluorescent probe that emits light at another wavelength (e.g. DAPI); nuclei can be stained with these dyes by including a few microliters of dye in the second antibody incubation or in subsequent wash buffer. The absorption and emission curves of these dyes do not overlap the narrow band filters for FITC or rhodamine.

The latest developments in fluorescence microscopes have led to microscopes with improved resolution. Special filtering may be used to separate different frequencies and wavelengths in the biological specimens. High quality objectives are important in fluorescence microscopy; high-numerical aperture and the lowest useable magnification will give optimal signal strength. For good transmission and low background, fluorite or apochromate objectives should be selected. Finally, the imaging device has an influence on the structures resolved and at what level the specimen fluorescence may be detected.

Cameras and imaging device

The fluorescent image can be viewed directly or it is collected by a camera. The imaging device is of considerable importance for fluorescence microscopy and includes both high resolution and sensitivity as well as variable exposure times. The use of a CCD (Charged Coupled Device) camera is preferred due to its digital, linear and quantitative properties. Cameras should at least offer high signal sensitivity, low noise and the ability to quantify the intensity of intensity distribution. Monochrome cameras may be preferred. With fluorescence filters correctly chosen, they image the fluorescence intensity of each fluorochrome separately and can handle the respective images later on within a specific color space by use of appropriate software. The resulting images can be analysed or further processed. Postprocessing of images are useful to uncover details. Deconvolution algorythms, for example, will provide informations about the distortion of a small light spot (refraction limited) by the operating microscope and can be used to equalize images.

Advanced fluorescent techniques and high resolution

Much of our current understanding of cell structure, cell function and biomolecular complexes comes from studies using light and electron microscopy. Yet, detailed understanding of many biological processes such as function and regulation of biomolecules at the single molecule level needs a set of tools that enables one to image single molecules or even atoms and with which interaction forces between these basic structures become possible to be measured. It can be expected that innovations in nanotechnology and nanobiology and a strategy that combines different tools for biological and physical studies will open up new applications for the analysis of molecular processes by enhanced cell imaging and by high resolution imaging of single molecules and nanostructures.

New techniques in fluorescence microscopy were introduced with the development of the confocal microscope. The concept of confocal microscopy was originally invented by M MINSKY (patented in 1961) when studying neural networks in preparations of brain tissue to image biological events as they occur in living systems. Due to lack of intense light sources and computer capacity to handle large amounts of data, the invention of MINSKY remained for a long time unnoticed. Then, in the late 1970s and 1980s, advances in laser and computer technology led to the development of practical laser scanning confocal microscopes.

Confocal microscopy offers several advantages over conventional widefield optical epi-fluorescence microscopes, for example the ability to exclude secondary fluorescence in areas removed from the focal plane (the elimination or reduction of background information away from the focal plane), and the capability to collect serial optical sections from thick specimens. This is achieved with an optical microscope as central part and integrated electronic detectors, several laser systems combined with wavelength selection devices, a beam scanning assembly and a computer for image display, processing and storage. The main source of this approach is the use of laser beams and spatial filtering techniques to eliminate out-of-focus light in specimens whose thickness exceeds the current plane of focus.

Each type of laser produces light of only one or a few well defined wavelengths and minimizes the need for excitation filters. The light is both spatially and temporarily coherent producing a beam of light that is very bright and tightly focused in order to obtain a bright spot of light onto the specimen. A confocal image is collected from only one plane of the specimen.

Light microscopical methods are usually limited by diffraction (ABBE E, 1873; ABBE E, 1878; ABBE E, 1904), i.e. the limit of resolution that can be obtained in optical imaging techniques is related to the wavelength of the light. This diffraction limit originates from the fact that it is impossible to focus to a spot smaller than half of its wavelength. In optical microscopy, the maximal resolution is in the order of 250-300 nm. Because a large body of biomolecular studies such as experiments on cell-signaling events or the interaction of individual proteins on the molecular scale need imaging techniques that have a higher resolution, several approaches have been developed.

For the study of receptor-ligand interactions which occur in very close proximity and which are beyond the spatial resolution of a light microscope, the physical limits can be overcome by the technique of *Fluorescence Rresonance Energy Transfer* (FRET) or also called Förster energy transfer according to T FÖRSTER (1946, 1948) who was the first to describe this physical phenomenon. FRET is a nonradiative energy transfer between two different fluorophores. The first (donor) fluorophore is excited by light and transfers its energy without radiation to the second (acceptor) fluorophore; the important consequence of this energy transfer is that there is no emission of light by the donor. As a result of this transfer, the donor is quenched and does not show fluorescence while the acceptor becomes excited and shows fluorescence (sensitized emission). FRET is a distance-dependent interaction where the radiationless energy transfer occurs over a very limited distance of 1-10 nm. A positive FRET signal gives information about the range of distance of the FRET partners and can be quantified as FRET efficiency. The fact that FRET occurs typically in the 1-10 nm range means that these separation distances are very useful for the study of ligand-receptor interactions in life research inasmuch as these distances reflect the dimensions of biological macromolecules (see VAN DER MEER BW et al., 1994).

A number of optical techniques such as photoactivation, photoconversion including photobleaching techniques such as *Fluorescence Recovery After Photobleaching* (FRAP), *Fluorescence Loss In Photobleaching* (FLIP) or *Fluorescence Localization After Photobleaching* (FLAP) can be utilized to observe single molecule fluorescence and to study dynamic processes such as active transport or passive diffusion in a living cell. *Total Internal Reflection Fluorescence Microscopy* (TIRFM) is a special technique for the investigation of molecular interactions with surfaces.

The *4Pi-confocal fluorescence microscope* and the *STED* method (Stimulated Emmission Depletion) invented by C CREMER and T CREMER (1978) and by SW HELL (HELL SW and STELZER EHK, 1992; HELL SW et al., 1994; KLAR TA et al., 2000; DYBA M and HELL SW, 2002) represent now the most significant advances in fluorescence microscopy. STED microscopy allows optical measurements with sub-wavelength resolution for the study of biological specimens at the scale of nanometers. Thus, STED was used to study distributions of fluorescent antibody labeled proteins in a number of different tissue preparations (KITTEL RJ et al., 2006; SIEBER JJ et al., 2006; WILLIG KI et al., 2006; NÄGERL UV et al., 2008).

The STED-microscope relies on pairs of synchronized laser pulses. In this setup, excitation is performed by a subpicosecond laser pulse that is tuned to the absorption spectrum of the dye. The excitation pulse is focused into the sample producing an diffraction limited spot of excited molecules. The excitation pulse is immediately followed by a depletion pulse (dubbed “STED pulse”). The STED pulse is red-shifted in frequency to the emission sspectrum of the dye, so that its lower energy photons act only on the excited dye molecules quenching them to the ground state by stimulated emission.

The net effect of of the STED pulse is that the affected excited molecules cannot fluoresce because their energy is dumped and lost in the STED pulse. By arranging the STED pulse in a

doughnut mode, ideally only the molecules at the periphery of the spot become quenched. In the center of the doughnut, where the STED pulse is vanishing, fluorescence remains unaffected. By increasing the intensity of the doughnut-shaped STED pulse, the fluorescent spot can be progressively narrowed down (in theory to the size of a molecule).

In this way, STED-microscopy overcomes the diffraction limit in a fundamental way with great potential in biomolecular cell research because spatial resolutions of 30-50 nm (about $\lambda/23$) are now achievable. This extraordinary gain is really striking because limitations in resolution by diffraction effects of the light (about half of the wavelength of the light used) usually applies to all farfield methods (cf. *The microscope as a scientific tool*).

Comparable approaches are possible with near-field scanning optical microscopy (SNOM) (LEWIS A et al., 1984; POHL DW et al., 1984; DÜRIG U et al., 1986). In SNOM (as in the case of atomic force microscopy), a sharp probe is used to map the topographic features on the sample surface. Besides topography, NSOM also generates optical images. The SNOM probe is a tapered optical fiber. Laser light is coupled into the fiber and used to excite fluorophores while the probe scans the sample surface. The optical fiber with a final diameter of 20-120 nm is metal-coated and serves to confine the light to the tip region. The tip is finally processed by an etching step to give a flat and circular endpoint and aperture which functions as the light source. Its diameter determines the optical resolution of the microscope. In fluorescence mode, the probe funnels the incident light wave to dimensions that are below the diffraction limit. Typicall, the optical near-field generated at the aperture has significant intensity only in a layer of <100 nm from the aperture because the intensity of the evanescent light decays exponentially. Thus, the probe can excite fluorophores only within a layer of <100 nm from the probe (i.e. the near-field region). The sample fluorescence is subsequently collected by optics and transformed into an optical image.

SNOM combines the high resolution of scanning probe microscopy with the contrast of optical microscopy. It can achieve single-molecule detection sensitivity on the cell surface. The usefulness of this technique is demonstrated by a number of studies (BETZIG E et al., 1991; BETZIG E and TRAUTMAN JK, 1992; BETZIG E and CHICHESTER RJ, 1993; BETZIG E et al., 1993; HA T et al., 1996; ENDERLE T et al., 1997; KIRSCH AK et al., 1999; DE LANGE F et al., 2001; FREY HG et al., 2004; BAYLIS RM et al., 2007).

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Full citation of publications is given in chapter *References*
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