The Development of Fluorescence Microscopy

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Advanced article

Article Contents

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The fluorescence microscope (wide-field, scanning, confocal, one-photon excitation, multiphoton excitation) is an extremely useful and ubiquitous instrument in biological and medical laboratories. The fluorescence microscope provides enhanced contrast, single protein specificity and single molecule sensitivity. Progress in the technical development over more than 100 years of instrument design and fluorescent probe synthesis has contributed to the continuing widespread utility of the fluorescence microscope. Modern advances in instrumentation include the use of continuous wave and pulsed laser light sources, dichroic filters, photomultipliers, avalanche photodetectors and charge-coupled device detectors. The invention of the two-photon excitation microscope provides for microscopic imaging of live cells and whole organisms. The development of specific dyes, physiological probes and molecular probes (intrinsic and extrinsic) stimulated the widespread use of fluorescence microscopy in the life sciences. Recently, genetically expressed fluorescent proteins and quantum dots provide new research capabilities for the intravital fluorescence microscope.

Introduction

Following the advances made by Ernst Abbe (1840–1905) in his 1873 theoretical analysis of the role of diffraction in microscopic image formation, and the known capability to manufacture and provide biologists with microscope objectives that minimized the effects of spherical as well as chromatic aberrations (at least for three wavelengths) several practical problems were still unsolved (Abbe, 1873; Masters, 2007). In the past, as in the present, the problems of resolution, contrast, noise, optical aberrations, sensitivity and specificity are formidable for all types of fluorescence microscopes. This article will concentrate on the use of fluorescence techniques to provide the biologist with improved contrast and specificity with the optical microscope. There are two recent related articles on the history of the microscope; one on the history of the optical microscope in cell biology and medicine and the other on the history of the electron microscope in cell biology. **See also**: History of the Electron Microscope in Cell Biology; History of the Optical Microscope in Cell Biology and Medicine

The next problem to be solved was how to achieve improved specificity and contrast in optical microscopy and the solution was the application of fluorescence (incident light of short wavelength caused a fluorescent molecule to emit light of a longer wavelength) to microscopy. The development of the fluorescence microscope followed a series of technical advances in microscope design; additionally, there were new fluorescence techniques that were based on synthetic fluorescent molecules as well as intrinsic fluorescence molecules. In the former category are the new sources of ultraviolet light, the use of liquid filters to separate specific wavelengths or colours of illumination, the use of solid or liquid filters to separate the incident excitation light from the emitted light that occurred at longer wavelengths, the design of objectives and lenses that can transmit ultraviolet light, and solid-state detectors to detect the images. In the latter category are fluorescent molecules that are chemically bound to antibodies that can couple to a specific protein in a cell, and thereby provide high contrast in microscopy that has very high specificity (single protein). **See also**: Fluorescent Analogues in Biological Research

The phenomenon of fluorescence was extensively investigated in the 1800s and early 1900s. In 1833, Sir David Brewster (1871–1868) reported on several observations of what today is labelled fluorescence: the red radiation from green chlorophyll, the luminescence of solutions of quinine,

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and the luminescence of the mineral fluorspar that is composed of calcium fluoride. The term fluorescence was coined by Sir George Gabriel Stokes (1819–1903). In 1843, he described the luminescence in calcium fluoride as fluorescence. Stokes used the light from the sun as the source of excitation. Sunlight was first passed through a filter composed of a solution of cupric hydroxide in an aqueous solution containing ammonia, then the filtered light was focused onto the specimen, and the emitted light together with the filtered excitation sunlight was passed through an yellow filter composed of an aqueous solution of potassium dichromate. Stokes observed the fluorescence with his eyes. In what became known as Stokes law, the phenomenon of fluorescence is often characterized by the wavelength of the emitted light that is of a longer wavelength than the wavelength of the exciting light (Stokes, 1852).

The theory of fluorescence was extended by the physics professor Eugen Cornelius Joseph von Lommel in 1875; he posited that a substance must absorb light before it can emit the light as fluorescence. Others investigated the phenomenon of luminescence included Alexandre Edmond Becquerel who investigated the phenomena of fluorescence and phosphorescence. Becquerel invented a device to accurately measure the decay time of phosphorescence, that is the duration of the emission after the light source was turned off. Eilhard Wiedemann, a German physicist also investigated the fluorescence of sodium and potassium atoms in gases. These investigations supported the idea that the fluorescence spectra were characteristics of specific substances, and therefore that measurement of the fluorescence spectra could be used to characterize substances (McGucken, 1969). In 1935, Alexander Jablonski (1898–1980) used graphical diagrams to illustrate the process of the absorption of light and the subsequent fluorescent emission form an excited state; these were denoted singlet states (Jablonski, 1935). The field of spectral characterization of organic substances was popularized in the organic chemistry community by the numerous editions of Peter Pringsheim's (1928) book: *Fluoreszenz und Phosphoreszens im Lichte der neueren Atomtheorie*.

Advances in fluorescent dyes and instrumentation were crucial in the development of the fluorescence microscope. First, I will present some examples from the plethora of organic dyes and stains that were used in the fluorescence microscopy of biological specimens (Kasten, 1983, 1989). The development of the synthetic dye industry by William Perkin (1838–1907) in 1856 resulted in the synthesis and the chemical and spectral characterization of many new fluorescent dyes. Acridine orange was synthesized by Bender in 1989; acriflavine (a mixture of dyes) was synthesized by Erlich and Beneder in 1912; both dyes were used in histology. Auramine O was synthesized by Kern and Caro in 1883; it was used to stain bacteria. Caro synthesized Eosin B in 1875; it was used as a counterstain. The dye fluorescein was first synthesized in 1871 and it has a long history connected with cell staining. In 1881, the bacteriologist Paul Ehrlich (1854–1915) used fluorescein to observe the passage of aqueous humour in the eye. He also used various aniline fluorescent dyes to stain bacteria and thereby increase their contrast in the microscope. Rhodamine B and Rhodamine G was synthesized by Ceresole in 1887 and 1891, respectively, both dyes were used as vital fluorochromes to stain living tissues.

Before the 1911–1913 invention of the fluorescence microscope there were many stains and dyes for use in histology and cell biology (Kasten, 1989). Then with the invention and commercial availability of the fluorescence microscope many cells and tissues were labelled with dyes and their fluorescence was studied. Sigwald Bommer working in Giessen, Germany, stained histological sections with acriflavine and observed the fluorescence of the cell nuclei. Max Haitinger (1868–1946) introduced the term 'fluorochroming' or fluorescent staining of specimens, and he developed many of the staining techniques for the observation of specimens in the fluorescence microscope. Max Haitinger (1959) made advances in both staining and instrumentation as described in his 1938 monograph: *Fluorescenzmikroskopie Ihre Anwendung in der Histologie und Chemie*.

The Development of the Fluorescence Microscope

In 1903 Henry Friedrich Wilhem Siedentopf (1872–1940), who worked at the optical works of Carl Zeiss, collaborated with Richard Adolf Zsigmondy (1865–1929) and they invented the ultramicroscope to observe colloids. Although their microscope could not image colloids, it could detect each colloid as a bright spot of light. Siedentopf constructed a dark-field condenser that blocked the incident light from entering the microscope objective, thereby improving the specimen's contrast. Siedentopf reported that the use of ultraviolet light produced specimen fluorescence, and that the fluorescence was a problem since it reduced the specimen's contrast. Later in 1914, Siedentopf and Zsigmondy refined their original invention and produced a slit ultramicroscope and an immersion ultramicroscope. The Carl Zeiss firm manufactured and sold their ultramicroscope which was used for the study of colloids (**Figure 1**, **Figure 2** and **Figure 3**). It is of historical interest that modern microscopic techniques that are based on 'illumination with a light sheet' follow from the early works of Siedentopf and Zsigmondy on lateral illumination.

August Köhler (1866–1948) working in the Jena Zeiss factory developed in 1893 a new system of microscope illumination (later named Köhler illumination) for microscopic photographic purposes. In 1904, August Köhler invented the ultraviolet absorption microscope that preceded the fluorescence microscope. A camera was required to detect the very weak image. Köhler used the quartz monochromatic ultraviolet objective previously developed by Moritz von Rohr (1868–1940). The motivation to use the ultraviolet light was based on the fact that shorter

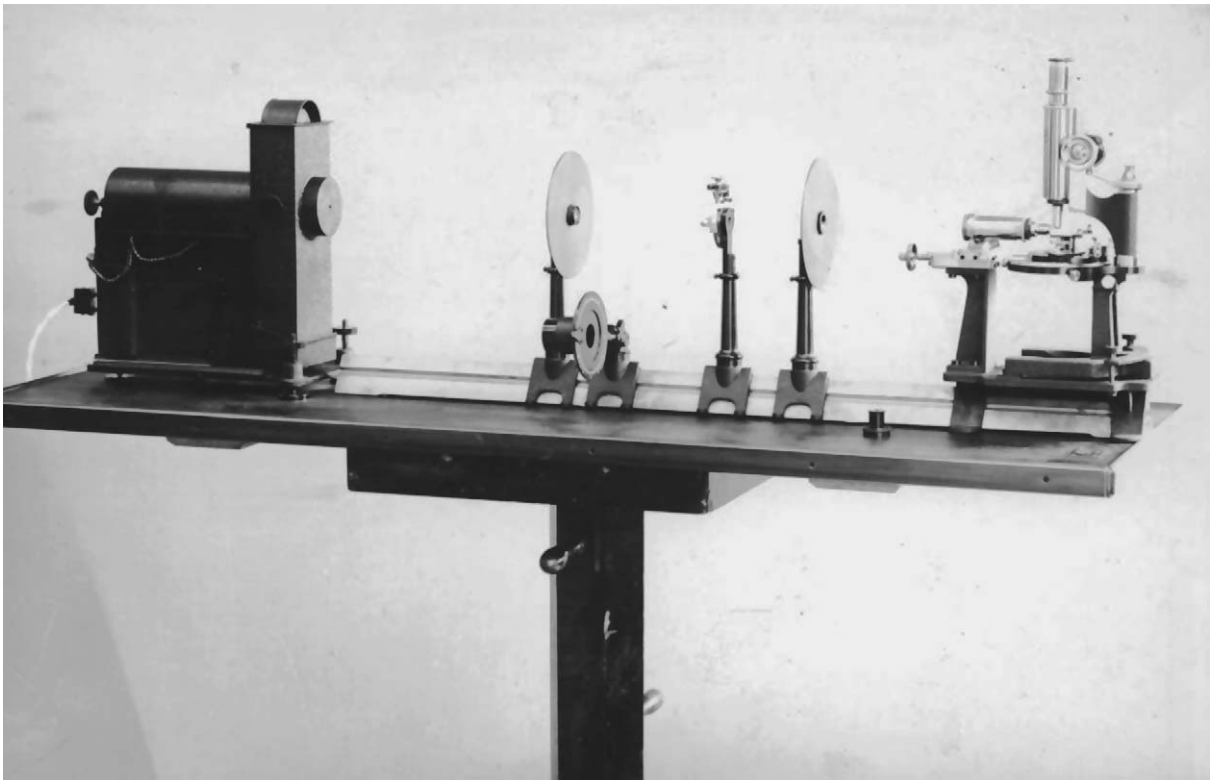

Figure 1 Slit ultramicroscope devised by Siedentopf and Zsigmondy (1902). Reproduced with permission from Carl Zeiss archives.

wavelengths of incident light results in increased resolution of optical microscopes (from the previous work of Abbe). Köhler noted that the ultraviolet light excitation on a crystal of barium platinum cyanide resulted in fluorescence in the visible spectrum. Together with Siedentopf, they made a fluorescence microscope with excitation at the wavelength of 275 nm from a spark source. They made microscopic observations of autofluorescence (self-fluorescence) of biological specimens excited with ultraviolet light. Siedentopf observed the ultraviolet image of unstained chromatin in the cell nucleus with incident light of wavelength of 275 nm. In spite of these successes, the spark gap was an unreliable source of ultraviolet light for microscopy.

The development of a steady source of ultraviolet light was a prerequisite to the first reliable fluorescence microscope. In 1903, Robert W Wood (1868–1956) working at Johns Hopkins University demonstrated how to isolate a band of ultraviolet light (300–400 nm) from an arc lamp with a dye solution of nitrosodiumethylaniline. Then, in 1910, H Lehmann of the Carl Zeiss factory in Jena used the modified Wood's light source (the addition of gelatin to Wood's solution plus another quartz chamber containing copper sulfate solution) to make a prototype bright-field fluorescence microscope (the ultraviolet exciting light entered the microscope objective to illuminate the specimen). This modified Wood's light source blocked all visible light. In 1914, Stanislaus von Prowazek (1875–1915) was the first to introduce vital cellular staining into fluorescence microscopy, that is his fluorescence staining of living protozoa.

At the same time the firm Carl Reichert of Vienna developed a competing fluorescence microscope with a new dark-field quartz condenser (the ultraviolet light did not enter the microscope objective). In 1911, Hans Stübel, a physiologist at Jena University, observed autofluorescence (naturally occurring fluorescence without the application of a fluorescence dye to the specimen) during his microscopic studies of cells and tissues. Subsequent observations by Herwig Hamperl (University of Vienna) who initiated the use of the fluorescence microscope in pathology confirmed the autofluorescence of the ocular lens of the eye, plant tissues, chlorophyll, amyloid, collagen, fibrin and elastic fibres. In 1913, the Zeiss firm introduced its commercial luminescence bright-field microscope. The fluorescence microscopes sold by the Zeiss firm and the Reichert firm used quartz lenses for the illumination with ultraviolet light, and glass lenses in the microscope. Both of these fluorescence microscopes worked in the transmission mode. They only differed in the type of condenser; the Zeiss microscope used a bright-field condenser, and the Reichert microscope used a dark-field condenser. In England, the Beck microscope company manufactured an ultraviolet microscope that had higher resolution than that obtainable with visible light. **See also**: Light Microscopy – Brightfield and Darkfield Illumination

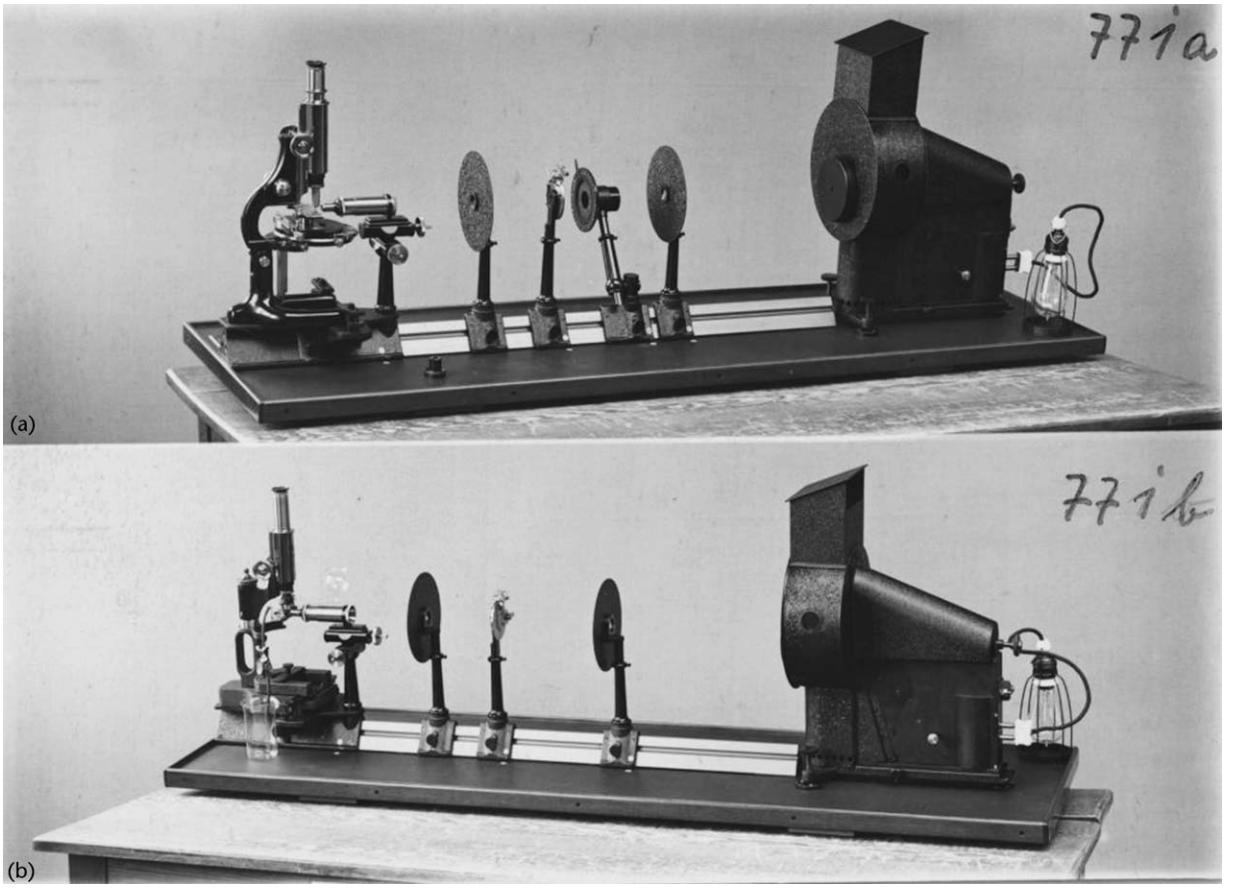

Figure 2 Slit ultramicroscope devised by Siedentopf and Zsigmondy (1923) (a) to observe ultramicroscopic particles in solid bodies and (b) to observe ultramicroscopic flowing particles. Reproduced with permission from Carl Zeiss archives.

Philipp Ellinger (1887–1952), a pharmacology professor who worked in Heidelberg in the 1920s, was instrumental in the development of the intravital fluorescence microscope. Between 1925 and 1932, Ellinger (the senior investigator) collaborated with August Hirt on the development of the intravital fluorescence microscope. This microscope was patented with the names of the inventors: Ellinger and Hirt. In 1929, the firm Carl Zeiss in Jena produced a commercial intravital microscope with vertical illumination, a water-immersion microscope objective and an ultraviolet light source for the investigations of the distribution of fluorescent dyes in the kidneys and the liver of frogs and mice. In 1933, Ellinger who was Jewish was forced to leave his position and subsequently Hirt, who was a member of the Nazi SS, falsely claimed that he was the sole inventor of the intravital fluorescence microscope (Kasten, 1991; **Figure 4**).

Sigwald Bommer used a fluorescence microscope in 1929 in his discovery of the autofluorescence of both lipofuscin granules and also elastic fibres in tissues. This microscope was subsequently used for intravital observation of living skin, liver and kidney, and to study the microcirculation of organs and glands in the living animal. By 1932 various firms manufactured ultraviolet microscopes and they were used to study the microvasculature, living skin, liver and kidney.

Several later developments improved the fluorescence microscope and resulted in its widespread use by cell biologists (Ploem and Tanke, 1987). In 1947, Siegfried Strugger (University of Münster), who previously developed the vital dye neutral red to stain plant cells, used acridine orange (a cellular stain) to differentiate live cells from dead cells. Strugger wrote a book on these studies: *Fluorescence Microscopy and Microbiology*. A major advance in the incident-light fluorescence microscope was the 1948 invention by Evgenii Brumberg; he called his technique as 'illumination from above'. It consisted of an interference mirror that directed the illumination light to the microscope objective, and separated the exciting light and the fluorescence light that was collected by the microscope objective (Brumberg, 1959). Brumberg's invention was subsequently developed by Johan Sebastiaan Ploem (1967). It consisted of the dichromatic beam-splitting plate (dichroic mirror) that is used to separate the excitation light from the longer wavelength fluorescence. **See also**: Fluorescence Microscopy; Fluorescence Microscopy

The sensitivity and specificity of the binding of antibodies and antigens was applied to fluorescent labels and that resulted in the ability to image single proteins in cells

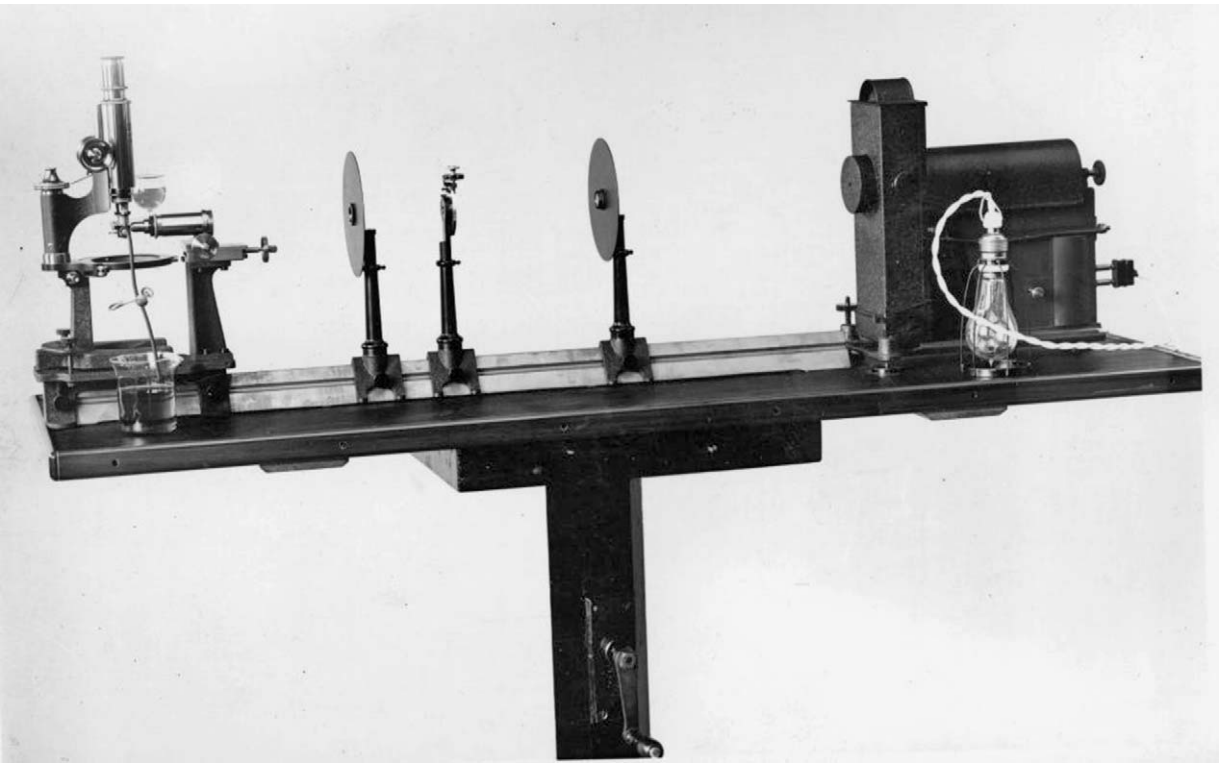

Figure 3 Slit ultramicroscope devised by Siedentopf and Zsigmondy (1930) with a cuvette to investigate ultramicroscopic flowing particles. Reproduced with permission from Carl Zeiss archives.

and tissues. Albert Coons (1912–1978) and Melvin Kaplan (1921–) are the inventors of immunofluorescence (1950) in which a fluorescent dye (fluorescein isocyanate) is chemically attached to an antibody; this technique resulted in an enormous increase in specificity (due to the antigen–antibody specificity) and resulted in many advances in cell biology. For example, in 1982, Mary Osborne and Klaus Weber used the fluorescence microscope together with fluorescent monoclonal antibodies to visualize the cytoskeleton protein tubulin in cells. **See also**: Immunofluorescence; Immunohistochemical Detection of Tissue and Cellular Antigens; Tubulin and Microtubules

Recent Advances in Fluorescence Microscope Instrumentation

The invention of the laser (1960) and its further development in the past five decades have provided numerous light sources for the fluorescence microscope. Although the early ruby and gas lasers provided a very limited number of wavelengths, the modern dye lasers and compact solid-state femtosecond lasers provide a variety of wavelengths, as well as pulse durations, and peak powers; recent developments in tunable laser light sources may open up further excitation potentials. In 1982, the firm Carl Zeiss introduced their first laser scanning microscope. An excellent tutorial on modern laser sources describes the various techniques to generate laser pulses, techniques to characterize these lasers and their use in two-photon and other nonlinear microscopies (Girkin, 2008).

The modern fluorescence microscope can be configured with a variety of photon detectors: photomultiplier tubes (PMT), avalanche photodiodes (ADP) and charge-coupled device (CCD) imaging systems (Janesick, 2001). Each device has its advantages and limitations; considerations of cost, signal-to-noise ratio, sensitivity, image acquisition speed is important in selecting a detector of fluorescence microscopy (Yazdanfar and So, 2008).

The 1931 publication of Maria Göppert-Mayer's doctoral dissertation (University of Göttingen) on the theory of two-photon quantum transitions (two-photon absorption and emission) in atoms was the theoretical foundation of multiphoton microscopy (Göppert-Mayer, 1931; English translation Masters, 2008a, b; Masters, 2000). The invention of two-photon microscopy is usually attributed to the group of Denk, Strickler and Webb who published their experimental findings in *Science* (Denk *et al.*, 1990). The use of fluorescence in a nonlinear optical microscope was previously suggested by Sheppard (Sheppard and Kompfner, 1978). The main advantages of two-photon excitation microscopy include the following: the use of near infrared light to excite molecules with absorption bands in the ultraviolet is less toxic to cells and organisms than the use of ultraviolet light, the near infrared light permits

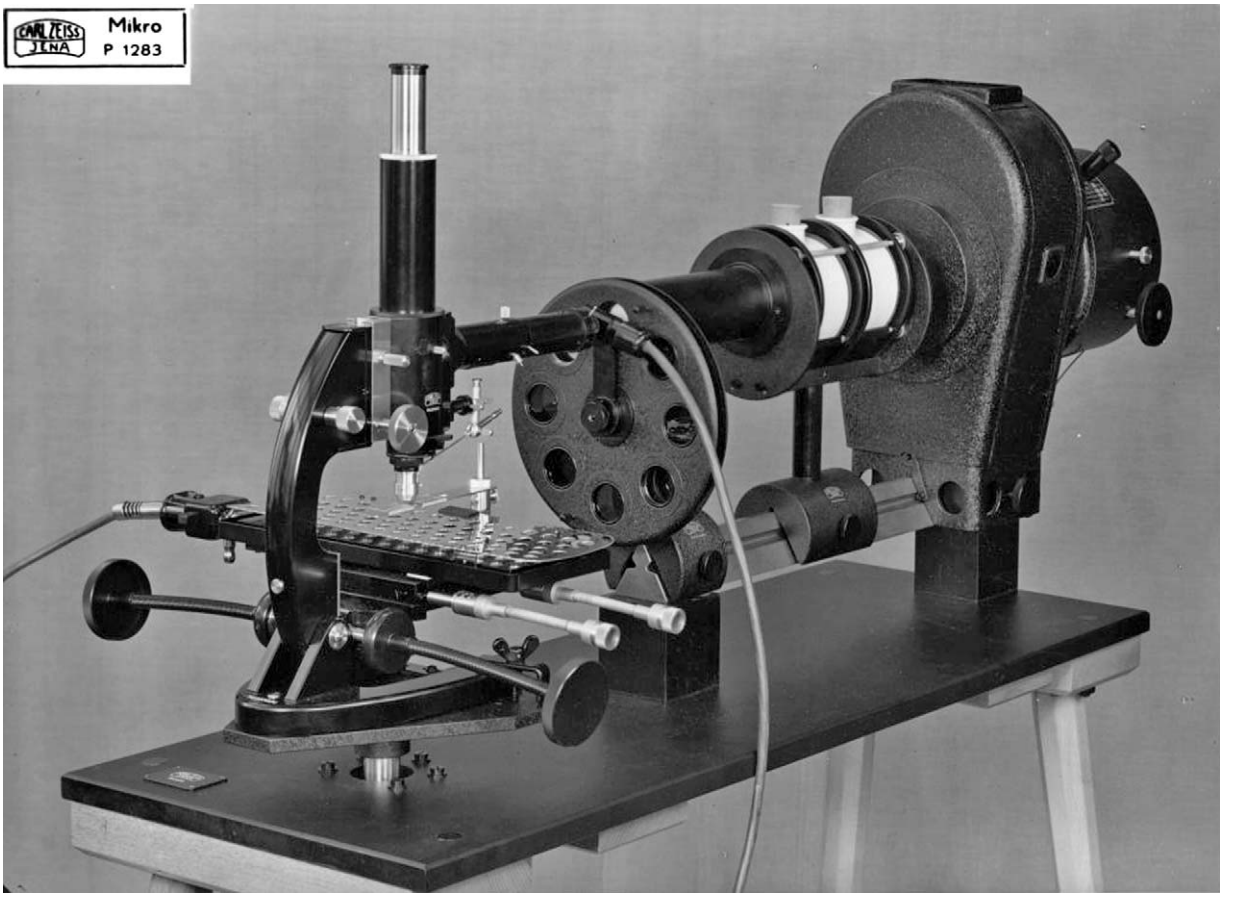

Figure 4 Luminescence microscope developed by Zeiss/Jena after design of Ellinger–Hirt. It used incident illumination to observe biological specimens in intravital fluorochroming. Reproduced with permission from Carl Zeiss archives.

deeper penetration (up to about 1mm), and there is optical sectioning based on the physics of the excitation (Masters, 1996, 2003, 2006; Masters and So, 2008). Two-photon microscopy is especially advantageous for deep tissue imaging. **See also**: Two-photon Fluorescence Light Microscopy

An important technical development is the use of fluorescence lifetime imaging microscopy (FLIM) and the plethora of biomedical applications (Elson *et al.*, 2002). The FLIM technique involves the determination of the average fluorescence decay time for each pixel in the microscope field of view and then forming a map of the decay data. The contrast of such a FLIM is based on the different average decay times for the components of the specimen and that is the physical basis for the image contrast in the FLIM technique.

Recent Advances in Fluorescent Probe Development

Green fluorescent protein (GFP) is a moderately large protein that is composed of 238 amino acids (26.9 kDa) and was discovered by Osamu Shimomura and his coworkers as a protein associated with aequorin, the chemiluminescent protein from the *Aequorea victoria* jellyfish. It can be expressed (actually overexpressed) in a variety of organisms (bacteria, yeast, other fungi, the zebrafish), plants, as well as mammalian cells including human cells, with the use of modern techniques of molecular biology. The important impact of this work in cell biology led to the awarding of the Nobel Prize in Chemistry in 2008 to Martin Chalfie, Osamu Shimomura and Roger Y Tsien for the development of GFP in biology (Tsien, 1998). The emission spectrum of this group of GFP mutants of genetically expressed proteins can cover a range of colours: green, blue, cyan, yellow and a mutant that fluoresces in the infrared wavelength range, and thus provide a range of wavelength separable fluorescent proteins that provide cells and tissues with specific protein fluorescence. **See also**: Green Fluorescent Protein (GFP)

A seminal recent advance in neurobiology is the technique of combining several spectral mutants of the fluorescent protein and then using fluorescence microscopy to image neurons in almost 90 different colours of genetically expressed proteins in neurons of the brain. Jeff Lichtman and his colleagues working at Harvard University found a

technique for transgenic mice to express various mixtures of four colours of fluorescent proteins in their neurons: yellow, red, cyan and either orange or green. This technique is called 'Brainbow' and shows great promise in tracing all the neurons in a cubic millimeter of mouse brain (Livet *et al*., 2007).

Another modern development for fluorescence microscopy is a new class of bright fluorescent probes: the semiconductor nanocrystals (quantum dots) (Alivisatos *et al*., 2005). These probes have been used for fluorescent microscopy at the cellular level and the applications include: immunolabelling, cell tracking, *in situ* hydridization, Förster resonance energy transfer (FRET) and *in vivo* imaging. **See also**: Fluorescence Resonance Energy Transfer

In contrast to organic fluorescent probes that are susceptible to rapid photobleaching and have narrow, overlapping emission bands, these inorganic quantum dots show unique optical properties. Quantum dots of different diameters can be excited by a single wavelength with minimal signal overlap. Quantum dots are less susceptible to photobleaching and are therefore more stable than organic dye molecules. In addition, the two-photon absorption cross-section is significantly larger for quantum dots than for organic dyes. Finally, their fluorescent lifetimes are in the range 10–40 ns which is significantly longer than for organic dyes. Quantum dots possess the problem of randomly alternating between two states: an emitting state and a nonemitting state, and that phenomenon is called blinking. The biocompatibility, biomolecule conjugation, toxicity and specificity as well as life science applications in cell lineage, cell motility assays, *in situ* hybridization and FRET for this new class of fluorescent probes has been discussed previously (Alivisatos *et al*., 2005).

Limitations of Fluorescence Microscopes

Two factors limit the utility of the fluorescence microscope in addition to the general limitations of resolution, noise and optical aberrations that affects all types of microscopes. First, the process of fluorescence is dependent on the deposition of energy into the biological specimen. This is correct for both one-photon fluorescence microscopy and two-photon excitation microscopy in which there is usually concomitant single-photon absorption within the biological specimen. Second, the use of exogenous fluorescent probes can affect both cellular structure and function. It should be clearly emphasized that the use of genetically expressed fluorescent proteins (GFP) in cells results in the overexpression of the GFP (Tsien, 1998). It is critical to perform careful control experiments to validate that the cellular function, processes and structures that are being investigated are not adversely modified by the overexpression of the GFP.

Fortunately, there are alternative techniques to avoid or mitigate these limitations. In general, cell, tissue and organism viability studies demonstrate that the use of pulsed far infrared radiation (from a femtosecond pulsed laser) is far less toxic and destructive to cellular function compared to the use of ultraviolet radiation to excite fluorophores with absorption bands in the ultraviolet (Denk *et al*., 1990; Sheppard, 1978). Another alternative to the use of extrinsic fluorophores or the use of GFP is to use the intrinsic cellular fluorophores such as reduced adenosine nucleotides (NAD(P)H) and flavour proteins as probes in fluorescence microscopy (Masters, 2008a, b).

The use of molecular probes is not without their problems and careful work requires validation of the interpretation of the images with a number of independent techniques, that is, fluorescence microscopy and electron microscopy (Perinetti *et al*., 2009). In particular, there is always the question of whether the fluorescent probe perturbs the cellular morphology or the function that is under investigation.

One alternative solution is to develop label-free biomedical imaging microscopes. Coherent anti-Stokes resonant spectroscopy (CARS) microscopy, developed in the Xie group at Harvard University, affords a microscopic technique that is label-free; the chemical specificity is obtained by tuning the laser excitation to specific vibronic transitions of known lipids and other chemical compounds that are naturally occurring within cells and tissues (Duncan *et al*., 1982; Zumbusch *et al*., 1999). Another example of the use of label-free biomedical imaging is the development in stimulated Raman scattering microscopy (Freudiger *et al*., 2008). Stimulated Raman scattering microscopy in contrast to coherent anti-Stokes Raman scattering microscopy (CARS) has the advantage that it provides background-free images with a readily interpretable chemical specificity and contrast.

The apparent resolution of cellular structures that are below the optical resolution of the light microscope will be imaged at the resolution of the light microscope; that is, cytoskeleton components will appear to be of diameters much greater than their actual diameters. Recent advances in superresolution optical microscopy represent a slow convergence of the optical resolution of light microscopes and electron microscopes (Hell and Wichmann, 1994; Huang *et al*., 2009; Nägerl *et al*., 2008; Shtengel *et al*., 2009). **See also**: Far-field Light Microscopy

Summary

The modern fluorescence microscope has emerged as an important tool in the life sciences. The concomitant developments in instrumentation, specifically light sources and detectors, as well as probe development have resulted in fluorescence with unprecedented sensitivity and specificity. Modern fluorescence microscopes have the capability of single-molecule imaging, subdiffraction limited resolution and the specificity of imaging single proteins in living cells and organisms. The use of two-photon excitation fluorescence microscopes permits long-term live-cell

imaging and the imaging of deep tissues such as the *in vivo* brain. All of these advances also contribute to the further development of intravital microscopy of living tissues. There is a strong and active synergy between the life sciences and new developments in fluorescence microscopy.

See also: Fluorescence *In Situ* Hybridization (FISH) Techniques; Single-molecule Light Microscopy

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