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Multi-wavelength epi-illumination in fluorescence microscopy

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Abstract

Fluorescence is a process where a substance after having absorbed light (photons) emits a radiation the wavelength (colour) of which is longer than that of the absorbed light, and where this emission stops immediately after cessation of the excitation. This phenomenon is the basic element of fluorescence microscopy and its application.

Besides the "classical" excitation of fluorescence in a light microscope it is possible today to obtain the same emission effect via the modern technology of confocal laser scanning microscopy by an excitation with two or multiple photons having longer wavelengths than those of the emitted ones. Fluorescence occurs either as autofluorescence of biological and/or inorganic structures or as so called secondary fluorescence after a treatment of the specimen with special dyes (fluorochromes, fluorescent markers). To perform fluorescence in a microscope the following requirements have to be met: powerful energy sources (high-pressure mercury arc lamp, halogen lamp, etc.), adequate transmission filter systems (filterblocks) selecting the excitation light and the emitted radiation perfectly, and, last but not least, optical parts and outfits suitable for fluorescence, i.e. collector lenses, illuminators, beamsplitters, objectives, tube lenses and eyepieces.

Compared with the situation of today, fluorescence microscopy was for the first time applied with the use of transmitted light and darkfield microscopy. This was due to its limited ranges of application in those days. But with its increasing importance to histology, cytology, molecular biology and immuno-diagnosis the demand for a fundamental improvement of illumination and observation techniques came up more and more. This was the hour of birth of incident light fluorescence microscopy.

During its course of nearly 40 years of application and development this technique became one of the basic tools for routine and research work in biology, medicine, science and industry. The progress in incident light fluorescence microscopy was particularly determined by the research work of Ploem and his function as trend setter and also by the forward strategy of Leitz resp. Leica Wetzlar in developing the optical instruments required.

This course of development is described chronologically in the present contribution. A synoptical table of fluorescence microscopy techniques gives relevant information about microscopes, objectives, fluorochromes, light sources and filter systems concerning the fields of application and the methods referring to this.

Fluorescence

Fluorescence is a molecular phenomenon in which a substance absorbs light (excitation) and radiates light of longer wavelength (emission) for a very short period of time. When a fluorochrome absorbs light, energy in the form of photons is taken up, leading to excitation of electrons to higher energy states. The process of absorption of photons is extremely rapid and is immediately followed by a return to lower energy states, which is then accompanied by emission of photons, which can be observed if light is emitted in the visual range.

This short duration (nanoseconds) distinguishes it from other forms of luminescence such as phosphorescence. The difference in wavelengths between the excitation and emission peaks is referred to as the Stokes shift. Fluorochromes with a large Stokes shift are easy to excite and to observe their emission in a fluorescence microscope. With transmitted-light illumination, a small Stokes shift may make it impossible to illuminate a fluorochrome at its excitation peak and to observe the fluorescence colour at its emission peak.

Confocal laser scanning fluorescence microscopy enables two-photon excitation with photons of longer wave-length than the emitted light. The electron is then brought into its excited state with two photons of half the required energy, arriving simultaneously. In most cases the electron ends up in the same excited state as with normal single-photon excitation before it drops down to the ground state. Similarly, the fluorescence emitted is similar to that given off by normal single-photon excitation.

Fluorochromes

Many molecules, both non-organic and organic, show fluorescence emission, especially with excitation using high energy radiation. When plant or animal tissue is excited with UV-light very often a bluish fluorescence emission is observed which is called primary fluorescence, or autofluorescence [7, 8]. Naturally occurring substances often have very broad excitation and emission spectra. With the development of molecular biology, research has been focussed on special dyes with bright fluorescence to distinguish them from possible autofluorescence. The dyes used to selectively stain biologically important molecules are called fluorochromes. When conjugated to antibodies or nucleic acids, they are referred to as fluorescent markers or probes. Today fluorochromes are available with peak emissions in the violet, blue, green, orange, red and near-infrared regions of the spectrum. The possibility to excite fluorochromes with orange and red light and detect the red and infrared fluorescence with a photomultiplier or a CCD-camera has extended fluorescence microscopy beyond the visual range. The excitation and emission of a fluorochrome may shift with changes in cellular environment. Some dyes are especially chosen because their excitation or emission spectra are shifted, following changes in concentration of some ions in the intracellular medium such as calcium, sodium, and of the pH.

Early developments in epi-illumination fluorescence microscopy

For a review of the history of fluorescence microscopy the reader is referred to Kasten [11]. Epi-illumination (vertically incident exciting light) was used already by Policard and Paillet [32]. Several instruments were made by Leitz (Leica), Bausch & Lomb, Reichert and Zeiss, which were partially based on suggestions by Ellinger and Hirt [4, 5], Singer [37] and Mehler and Pick [16, 19]. An early Leitz (Leica) fluorescence epi-illumination system was described by Haitinger [6]. For more details on the evolution of epi-illumination fluorescence microscopy the reader is referred to Rost [34] and for early applications of incident-light fluorescence microscopy to Hauser [7].

A major contribution in epi-illumination fluorescence microscopy was the introduction of a dichromatic mirror for incident illumination with UV light by Brumberg and Krylova [2]. Epi-illumination has definite optical advantages because, unlike transmitted illumination where the condenser and the objective have independent optical axes which must be perfectly aligned. The objective functions both as a condenser and as a light-collecting objective, avoiding all alignment problems. The separation of fluorescence emission from excitation light, using a dichroic beamsplitter, is much easier than with transmitted light fluorescence microscopy, These possibilities did, however, not lead to a general acceptance by industrial microscope manufacturers of epi-illumination for routine fluorescence microscopy. The main reason for this could have been that transmitted-light darkfield UV excitation gave already excellent results in most applications of fluorescence microscopy [17]. Its replacement by UV epi-illumination would not have had significant advantages. The use of transmitted light using a dark-field condenser remained the industry standard until the late sixties.

The rapidly growing interest in molecular biology, however, led to the development of many (monoclonal) antibodies for the detection of important macromolecules in the cell. To study the detailed morphological location of several macromolecules in the cellular organelles, fluorescent markers with different colours were increasingly used. UV excitation – as used traditionally for fluorescence microscopy – was not optimally suited for detecting multiple fluorochromes simultaneously in a cell.

Around 1962 Ploem started work in collaboration with Schott on the development of dichroic beamsplitters for reflection of blue and green light for fluorescence microscopy using epi-illumination. At the time of his first communication [1965] and publication on epi-illumination with narrow band blue and green light [21], he was not aware of the development of a dichroic beamsplitter for UV excitation with incident light by Brumberg and Krylova [2]. Neither was the Leitz company, from which he obtained an "Opak" epi-illuminator with a neutral beamsplitter. This illuminator had to be modified to contain a slider in the incident light path containing four dichroic beamsplitters, for respectively UV, violet, blue and green excitation light. This device, developed at the University of Amsterdam, permitted the easy exchange of different dichroic beamsplitters in the incident light path (Fig. 1a). The wavelength of the excitation light could thus be easily and rapidly changed.

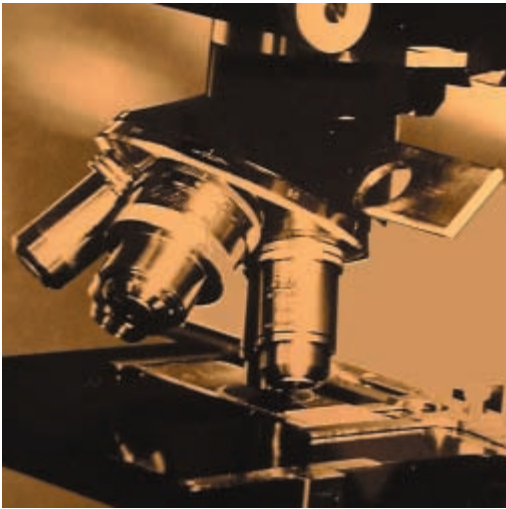


Fig. 1a:
Fluorescence multi-wavelengths epi-illuminator with four dichroic beam-splitters mounted in a slider, for incident illumination with UV, violet, blue and green excitation light. Constructed at the University of Amsterdam (Ploem, 1965).

Soon it became clear that excitation with narrow-band blue and green light opened optimal possibilities for the detection of the widely used immunofluorescence labels fluorescein-isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC). The use of blue and green excitation also minimized autofluorescence of tissue components, an undesired effect encountered with conventional transmitted illumination with UV light. FITC could now be excited with narrow band blue light (using a band interference filter with a half width of 16 nm), close to the excitation maximum at 490 nm (long wavelength blue), with clear observation of the green fluorescence peak emission at 520 nm. Autofluorescence of tissue components was minimized (Fig. 2a, b) resulting in a high image contrast. Excitation of FITC near its excitation maximum enabled such an efficient excitation that even a mercury high-pressure arc lamp, having no strong emission peak in the blue wavelength range, could be used. Furthermore epi-illumination with a green reflecting dichroic mirror enabled for the first time the excitation of Feulgen-pararosaniline with the strong mercury emission line at 546 nm (Fig. 3a, b).

In his second publication on the multi-wavelengths epi-illuminator, describing a Leitz prototype with four dichroic beam-splitters (Fig. 1b), Ploem [22] could acknowledge the contribution of Brumberg and Krylova [2]. The inaccessibility of Russian research in that time period, and the absence of any major industrial development of epi-fluorescence microscopy in Russia or East Germany was the reason that Leitz had not been aware earlier of such a development. The possibility to introduce epi-illumination with UV light, although useful for several applications, had not been a motive for a new technological development at Leitz, since they had already excellent transmitted dark field UV excitation available. The



Fig. 1b:
Leitz prototype (not commercialized) multi-wavelength epi-illuminator with four dichroic beam-splitters mounted in a slider (Ploem, 1967).



Fig. 1c:
Leitz epi-illuminator with four exchangeable filter cubes (blocks) [Kraft, 1972].

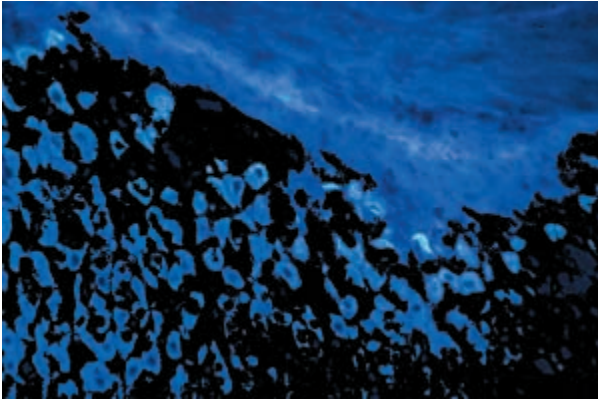


Fig. 2a: Tissue cells marked with an immunolabel (FITC) illuminated with wide-band UV excitation. Note the tissue structure with blue autofluorescence.

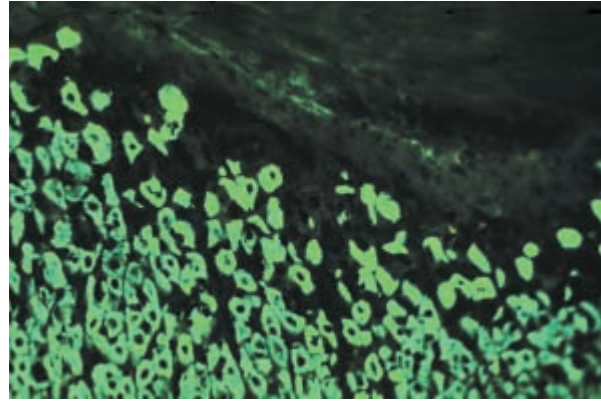


Fig. 2b: Same tissue and same immunostaining with FITC label illuminated with epi-illumination using narrow-band blue (490 nm) light. Note the increased image contrast (Ploem, 1967).

increasing world-wide use of routine immunofluorescence microscopy in medical diagnosis and molecular biology research could, however, profit from the new possibility of epi-illumination using narrow band excitation with blue and green light. Since standard high-pressure mercury arc lamps could be used, this seemed a practical proposition.

Subsequently Leitz developed a novel multi-wavelength fluorescence epi-illuminator (Leitz PLOEMOPAK) with four rotating dichroic beamsplitters for respectively UV, violet, blue and green light [13]. In successive generations of Leitz illuminators (containing four dichroic beamsplitters) barrier filters and a rotating turret for excitation filters were added. Finally an elegant epi-illuminator was constructed by Kraft [15] containing multiple sets of a combination of an excitation filter, a dichroic beamsplitter and a barrier or emission filter, mounted together in a filter cube, also called filter block (Fig. 4). Since this illuminator permitted the filter cubes to be rapidly turned into the optical light path, multi-wavelength illumination of the same section of tissue became a practical proposition. Moreover, the four filter cubes in the illuminator could be exchanged by the user (Fig. 1c). Different sets of four filter cubes could be assembled, chosen from many filter

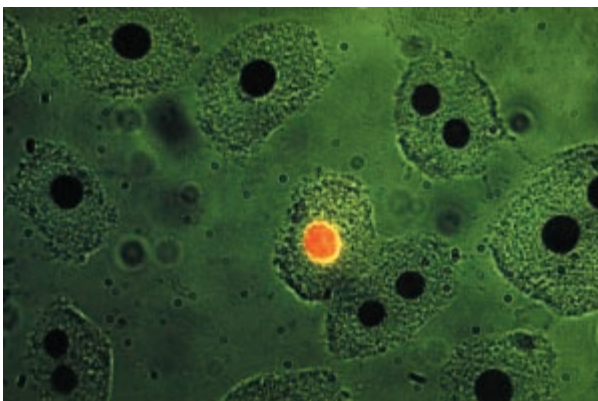


Fig. 3a: Liver tissue. Nuclei stained with Feulgen-pararosanilin for DNA, and visualized with transmitted green light. This stain was known as absorbing stain and not known to be fluorescent. One on the nuclei is illuminated with incident narrow-band green light (546 nm) resulting in a red fluorescence emission.



Fig. 3b: Liver tissue. Nuclei stained with Feulgen-pararosaniline for DNA. Epi-illumination with narrow band green light (546 nm) and a dichroic beam splitter for reflecting green light. Probably the first example of microscope excitation with green light (Ploem, 1965). Note large image contrast.



Fig. 4:
Complete Leitz (Leica) filter cube (block) for fluorescence microscopy containing: excitation filter, dichroic beam splitter and emission (barrier) filter.

cubes, containing combinations of excitation, barrier filters and dichroic beamsplitters, developed for different applications. Following suggestions by Ploem, Leitz also produced an inverted microscope with epi-illumination (Fig. 5a, b). For a review of the Leitz PLOEMOPAK illuminator for multi-wavelength fluorescence microscopy, the reader is referred to a review by Pluta [31].

The Leitz (Leica) filter cube system was so efficient that now, 39 years later, similar types of filter cubes are still used by most microscope manufacturers for multi-wavelength fluorescence microscopy. This development finally led within Leica to the development of automated multi-wavelength fluorescence epi-illuminators accommodating eight filter cubes for various wavelength ranges (Fig. 5c). When switching between filter cubes, pixel shift on the computer monitor is avoided or stays below the resolution power of a 35 mm film due to a 0-pixel shift technology. This illuminator is now used for fluorescence in situ hybridisation methods (FISH) in the study of chromosomes.

Ploem [24, 25, 26, 27, 28], van der Ploeg and Ploem [20] and Nairn and Ploem [18] further explored the filter combinations that had to be developed for many biomedical applications. This was done in collaboration with Schott and Leitz. Rygaard and Olson [35] developed a novel short-wave pass high transmission interference filter with a very high transmission for blue light and a sharp cut-off towards wavelengths longer than 490 nm.

Ploem [23] combined this SP filter with a 1 mm GG 455 filter from Schott, which blocked UV excitation, and suggested the development by Balzers of a similar filter (SP 560 = KP560) for excitation with green light and a filter for excitation with violet light (LP 425 = KP 425). The latter filter was applied in the investigation of neurotransmitters [25]. In Fig. 6a, b the resulting blue fluorescence can be observed.

From the optical industry side, early contributions and reviews on these developments were written by Kraft [14], Walter [39, 40], Trapp [38] and Herzog [8].

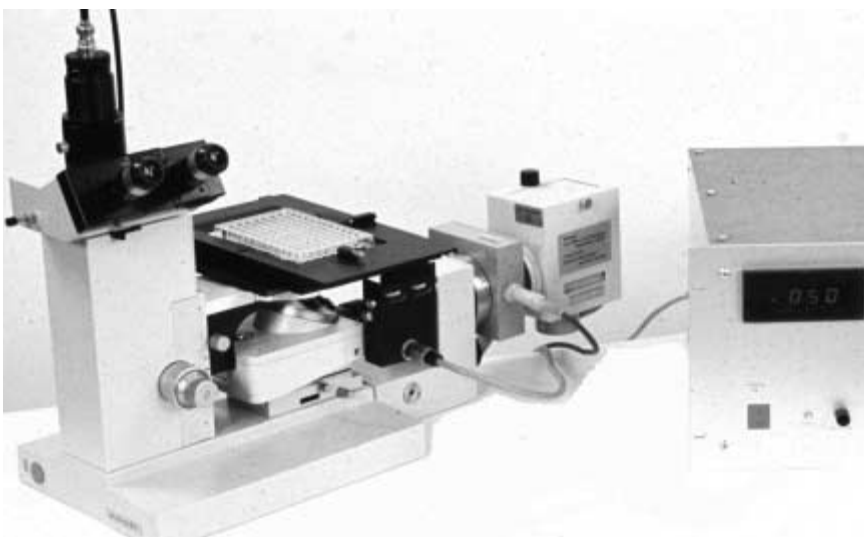


Fig. 5a: Leica inverted fluorescence microscope with multi-wavelength epi-illuminator.

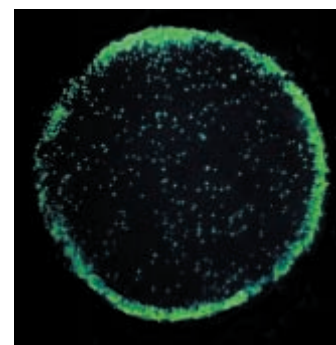
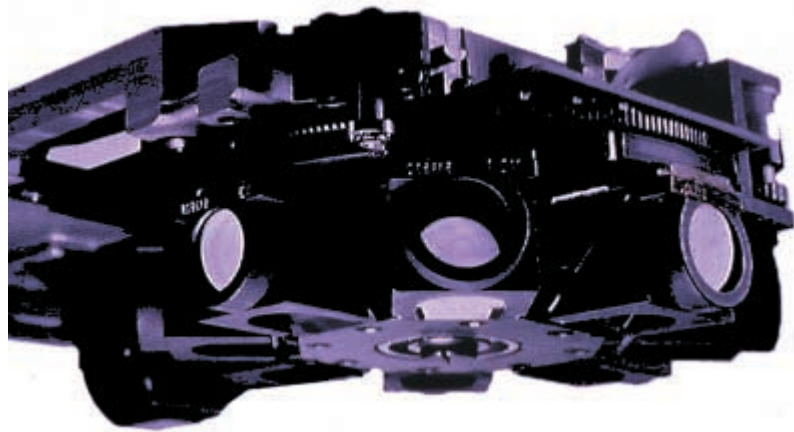


Fig. 5b:
Terasaki® plastic tray with human lymphocytes after a fluorescence cytotoxicity test for transplantation research. Epi-illumination with an inverted microscope.

Fig. 5c:
Leitz (Leica) motorized epi-illuminator
with eight filter cubes (blocks)



The main classes of filters used in epi-illumination fluorescence microscopy were defined in (1) the primary excitation filters LP (long pass) and SP (short pass) – in the German literature known as KP filter – and (2) the secondary filters such as barrier filters and emission filters [23]. The latter were also described as fluorescence selection filters; these are for instance used to limit the observation to the peak fluorescence at 520 nm of FITC. A recent extensive review on filters for fluorescence microscopy has been given by Reichman [33].

Cormane [3] was the first to demonstrate that narrow band blue light epi-illumination of the fluorescent label FITC gave an optimal contrast in immunofluorescence studies of human skin disease. Transmitted-light excitation with UV light used to cause such a strong autofluorescence of elastic fibres in the skin, so that visualization of the fluorescent antibody was severely hindered.

The pioneering work of Leitz in epi-illumination fluorescence microscopy coincided in the seventies with a worldwide increase in the application of immunofluorescence and other molecular biology methods like FISH in medical diagnosis and research. Hijmans et al [9, 10] were the first to demonstrate the usefulness of the new Leitz multi-wavelength excitation epi-illuminator for the selective detection of certain classes of immunoglobu-

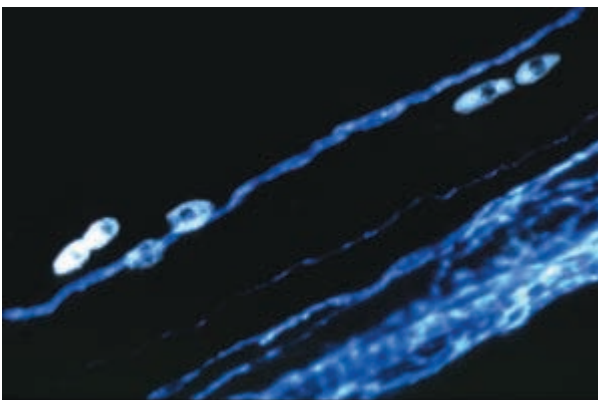


Fig. 6a: Mesenterium of the rat with small blood vessel surrounded by a blue fluorescent adrenergic (CA) nerve-plexus and yellow fluorescent mast (5-HT) cells. Formaldehyde-induced neurotransmitter fluorescence of CA and 5-HT fluorophores.



Fig. 6b: Same tissue and staining as is Fig. 6a: Epi-illumination with narrow-band violet excitation light (LP 3mm GG 400 and SP(KP)425 interference filter), a dichroic beam-splitter 495 nm, reflecting violet light and a barrier filter LP 460 nm. This filter-cube permitted for the first time the observation of blue fluorescent adrenergic nerve fibers, distinctly different from yellow fluorescent mast cells (Ploem, 1971).

lins in cells, using antibodies conjugated with green fluorescent FITC and red fluorescent TRITC. They applied the two-wavelengths excitation method using blue and green light and the selection of the peak fluorescence of FITC by an emission filter at 520 nm (Fig. 7). Brandtzaeg [1] and Klein et al. [12] made similar discoveries in identifying immunologically important cell types, using two-wavelength excitation with the Leitz epi-illuminator. In a staining of blood with "rosette" formation, the two-wavelengths excitation method using UV and green light can demonstrate erythrocytes around a mononuclear cell (Fig. 8).

Epi-illumination

In epi-illumination, a dichroic beamsplitter is used to deflect the incident light towards the specimen. The spectral characteristics of the dichroic mirrors have been designed in such a way that only the desired excitation wavelengths are deflected downwards through the objective onto the specimen, while the unwanted wavelengths are transmitted by the dichroic mirror and collected in a light trap [15] behind the dichroic mirror. The elimination of this unwanted excitation light results in a significant decrease of stray light and thus improves the image contrast. The dichromatic mirror deflects the desired (short wavelength) excitation light through the objective onto the specimen, but is transparent to the longer fluorescence wavelengths. The suppression filter (barrier filter) absorbs (or reflects) the excitation light reflected from the specimen and the lens surfaces of the objective, but is highly transparent to the fluorescence, which can thus reach the eyepieces. The efficiency of epi-illumination is related to the fourth power of the numerical aperture (NA) of an objective, serving in epi-illumination first as a condenser and then for observation as a light-collecting lens. At the time of marketing the first multi-wavelengths epi-illuminators only high-power objectives (x70, x100) were available with a high NA (0.95, 1.30). Following suggestions by Ploem [21, 22], Leitz was the first manufacturer to produce moderate power objectives like the oil-immersion x40 objective with a NA of 1.30 (Fig. 9). This new type of objective, especially designed for epi-illumination fluorescence microscopy, resulted in very bright images

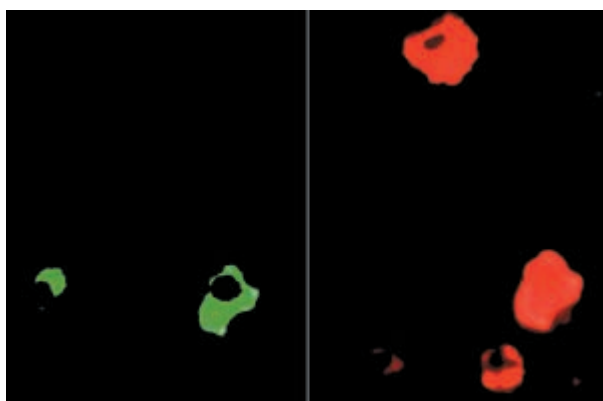


Fig. 7: Bone marrow cells stained with an anti-kappa TRITC conjugate and an anti-IgG FITC conjugate. Epi-illumination with narrow band green and blue light, resulting in red fluorescence of cells containing TRITC and green fluorescence of cells containing FITC. Some cells contain both FITC and TRITC.

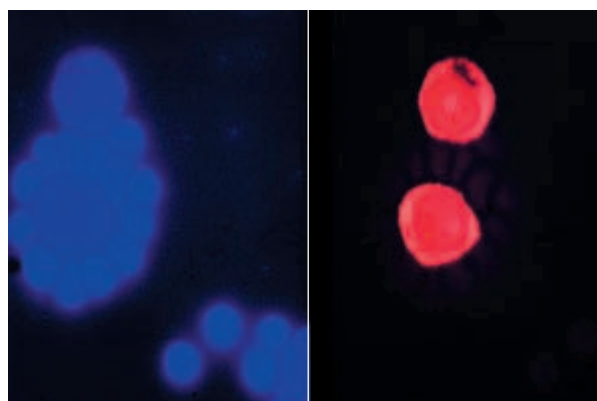


Fig. 8: Human blood cells, "rosette" formation. Erythrocytes stained with the blue fluorescent stain stilbene using epi-illumination with UV light. Lymphocytes stained with the orange-red stain eosine after excitation with green light (1965).



Fig. 9:
Leitz (Leica) early (1967) prototype ("Versuchs") oil immersion objective x40 with a NA 1.30 developed for trial experiments in fluorescence epi-illumination technology.

permitting short exposure times in routine fluorescence microphotography.

Optimal filling of the entrance pupil (aperture) of an objective

A problem in epi-illumination fluorescence microscopy has been the optimal filling of the entrance pupil of the objective with an image of the light source. A medium magnification of the light source of approximately 8 times was typical. This is related to the different arc sizes of the various high-pressure mercury and xenon lamps. In addition, entrance pupils of objectives vary considerably, e.g. 3.6 mm for an x100, NA 0.90 objective and 12 mm for an x10, NA 0.30 objective. Furthermore if only part of the arc can enter the entrance pupil, the fluorescence intensity obtained will be diminished. Recently Schöenborn [36] has reported on a completely innovated incident light path in the Leica DM R (HCS) microscopes. The path for epi-illumination has now been designed to allow individual adaptation of the illumination light path to the specific light sources used for different applications. Assuming a Koehler illumination in the incident light path, the combination of the illumination optics and the collector is to image the light source in the entrance pupil of the objective. For a given objective, the choice of the magnification of the light source depends directly on its geometric dimensions. Halogen lamps should be very well adjusted, since the coils of the lamp filament cause an extreme sensitivity to filament maladjustment. Relatively low magnifications are then advisable.

In some applications, the fluorescence intensity could be increased by factor 1.8 and for industrial applications in the UV range even by factor 3.5 in the DM R (HCS) microscopes. The increase in the UV range has been supported by a new chromatically corrected 6-lens collector of special high transmission glass types. For the Leica DM R (HCS) microscope system two modules are now available: Illumination optics with the HC F module have a magnification factor of 11.5 and for the HC RF module the magnification factor is 4.8. In addition the Leica DM R (HCS) epi-illumination stand has an extra illuminating lens in combination with the HC F module that produces very high fluorescence intensity and good homogeneity in a field of view of 25 mm. Users who need particularly good homogeneity even have the possibility to disengage an illumination lens of the HC F module, which only slightly decreases the fluorescence intensity. The image scale of the light source in the objective pupil is reduced from 11.5 to a value of about 9.5, resulting in an increase of about 20% of the useful field of view. To further increase the homogeneity, optical diffusion disks can be inserted in the module. Interestingly, it has been observed that the most pleasant image impression for viewing fluorescence is obtained when image intensity is not completely constant but slightly falls to the edge of the image. This effect is the result of the physiology of the eye (so-called "lateral retardation"). Conversely, video techniques with image analysis demand extremely constant illumination. This can be achieved by switching the optical diffusion disk in the HC RF module to the video mode, resulting in more homogeneity in a central field of about 16 mm (corresponding to the area covered by a 1/2" camera with a TV adaptor x0.5).

Leica has also further improved objectives for fluorescence microscopy. The selection of optical glasses with low autofluorescence properties resulted in an enhancement of the image contrast.

A problem in fluorescence microscopy was that no sharp images could be obtained from relatively thick specimens. This is due to the unwanted contribution of pre-focal and post-focal planes – using objectives with a high numerical aperture – to the final microscope image. This results in less well-defined images. The Leica spectral confocal microscope can however obtain sharp images taken at high resolution from several focal planes within the specimen at high lateral and vertical resolution.

The colour separation of multiple fluorochromes in a specimen has been further improved by spectral analysis. Computer assisted spectral confocal scanning microscopy has thus become the ultimate tool for fluorescence microscopy in biomedical and materials research.

Filter cubes for various applications in molecular biology

The explosion in molecular-biology applications in the last decade has led to a development of many filter combinations for the various applications. These sets of filters are now mounted in a large variation of filter cubes (blocks). Fluorescence epi-illuminators enable the exchange of 2–8 filter cubes by hand-operation or motorized exchange, as for example needed in fluorescence hybridisation studies. This paper is not intended to give a comprehensive and detailed discussion of the multitude of applications enabled by the various filter cubes. Instead only a schematic overview of filter cubes and keywords is given, indicating possible applications (Table 1).

Reflection contrast microscopy (RCM)

The development in epi-illumination microscopy by Leica has led to a further development: reflection contrast microscopy [29, 30]. Reflection contrast microscopy provides strong signals from specimens stained with routine absorbing immunomarkers such as peroxidase-DAB, immunogold-silver and immuno-phosphatase (Fig. 10, 11). Because of the strong signals that can be obtained – the high image contrast and no deterioration of the image by pre- and post focal images –, RCM of thin sections fulfils all the theoretical optical criteria for optimal light microscope resolution. The optical results can be defined as high definition images.

The strong reflection of most immunostains provides such a high contrast that this staining can be combined with many classical (absorbing) histochemical stains for important macromolecules that show a moderately strong reflection. The latter stains can often be used to provide fine morphological orientation and in many cases a more precise location of the immunomarker is achieved than with fluorescent markers alone. In addition, such light microscope observations can be confirmed by electron microscopy through examination of a next ultra-thin section (having the same immunostaining) with EM.

In confocal laserscanning microscopy, RCM can be performed without a

Table 1: Examples of fluorescence microscopy applications.

Only the optical parts and fluorochromes are listed here when they were mentioned in the cited papers. The language and terminology in this table are restricted to the terminology used in the text of the cited papers. Filter name terminology in German is listed in Italics. The half-width of band interference filters (BP) may be referred to by authors as either e.g. +00 or /00 nm, indicating the center wavelength and the half power bandwidth (e.g. BP 525/20) or the short and long-wave half power points (e.g. BP 450-490). Short and long pass filters are identified by the letters SP and LP and the edge wavelength in nm (e.g. SP 490, LP 520 or >520 nm). For references of the authors to manufacturers of optical parts the reader is referred to the cited literature.

*Emission or Laser line. ^Short Pass SP (KP) excitation filter. &Narrow band-pass (BP) excitation filter.
 ~Multi band-pass filter (MBP). %Dichroic beam splitter (DBS) or dichroic mirror (DM) and
 %-Polychroic beam spitter (PBS). #Barrier filter (LP). \$Emission band-pass (BP) interference filter,
 and emission multiple band-pass filter (MBP). @Filter cube, block (FC).

Microscope, Objective, Macromolecules Literature References	Fluoro- chrome	Light Source Excitation Filter	Dichroic Beam Splitter Filter cube	Emission Filter
Laser scan microscopy (LSC) x20 Surface markers DNA staining Köneman et al Cytometry 41:172–177,2000	FITC PI	Argon laser blue 488 nm*		530+30 nm ^{\$} BP green 625+28 nm ^{\$} BP red
Inverted fluorescence microscope x40 Fluorescence lifetime imaging Murata et al Cytometry 41:178–185,2000	Ho 7-AAD	DCM laser UV 335 nm*	FT 395% (DM, DBS)	450+33 nm ^{\$} BP violet
Digital fluorescence microscopy x100/0.60-1,32, SP(KP) 630 in front of CCD camera to block far red and infrared light PNA FISH De Pauw et al. Cytometry 32:163–167,1998	CY3 DAPI	green515-560 ^{&} UV 340-380 ^{&} nm BP		LP 580 red [#] LP 430 blue [#]
Confocal fluorescence microscopy x60/1.4 Photobleaching studies Van Oostveldt et al. Cytometry 32:137–146,1998	FITC	Ar ⁺ -ion line blue 488 nm* BHS ^{&}		LP OG 530 [#] yellow
Digital fluorescence microscopy, x63/1.4 Excitation filter wheel Chromosome studies Poon et al. Cytometry 36:267–278,1999	DAPI Cy3	hybrid/xenon mercury lamp 405 nm violet 546 nm green DAPI/Cy3 filter set		
Fluorescence microscopy Intratumor heterogeneity of chromosomes Di Vinci et al. Cytometry 37:369–375,1999	TRITC FITC DAPI	triple-bandpass filter~ (MBP)	PM [%]	(emission)
Fluorescence microscope x100/1.35 Daunorubicin sequestration Bour-Dill et al. Cytometry 39:16-25,2000	LB Daunorubicin NBD DiOC	mercury 100W BP 330-385 ^{&} BP 460-449 ^{&} BP 460-490 ^{&}	DM 410 [%] DM 570 [%] DM 510 [%]	(emission) BP 420-460 ^{\$} LP 590 [#] red BP 510-550 ^{\$}
Fluorescence microscope Automated comet assay Böcker et al. Cytometry 35:134–144,1999	PI	mercury 100W SP 580 [^]	E2 [@] (FC)	(emission) LP 590 [#]

Microscope, Objective, Macromolecules Literature References	Fluoro-chrome	Light Source Excitation Filter	Dichroic Beam Splitter Filter cube	Emission Filter
Motorized epi-illuminator x100/1.3 Chromosome studies with multicolor FISH Kuzobek et al. Cytometry 36:279–293,1999	DAPI FITC Texas-red	mercury 100W UV blue green	four filter cubes [@] (FC)	(emission) BP 425 ^S blue BP525 ^S green BP 615 ^S red
Inverted fluorescence microscope, CCD Three-color imaging of chromosomes Coco-Martin et al. Cytometry 32:327–336,1998	DAPI FITC TRITC	UV BP 365/12 ^{&} BP 450-490 ^{&} BP 546/12 ^{&}		emission > 379 [#] nm > 520 [#] nm > 593 [#] nm
Digital fluorescence microscope and (CCD) x40/0.75 Image analysis of granulocytes Sz cs et al. Cytometry: 33:19–31,1998	R 123 EB	Multi-band pass filter (MBP) 490~ nm blue 570~nm green	Triple-band (polychroic) Beamsplitter (PBS) ^{%~}	Triple-band emission filter 520~ nm 580~ nm
Fluorescence microscope with motorized epi-illuminator, delayed luminescence imaging and CCD, x63/1.32 FISH Karyotyping Tanke et al. Cytometry 33:453-459, 1998	cascade bl F LR Cy5 Cy7 Platinum coproporphyrin	mercury 100W BP ^{&} 500-560 nm	A [@] HQ-FITC [@] HQ-TRITC HQ-Cy5 [@] HQ-Cy7 [@] DBS 580 [%] nm	BP ^S 600-700 nm
Fluorescence microscope with motorized filter changer and CCD camera Two colour ratio method in FISH Morrison and Legator. Cytometry 27:314–326, 1997	DNA probes: 1 green 2 orange (1) WCP (2) CEP	mercury 100W BP ^{&} 510/20 nm BP ^{&} 572/18 nm	Triple-band (polychroic) beamsplitter (PBS) ^{%~}	Triple band-pass emission filter: MBP [~] 535 nm MBP [~] 606 nm
Fluorescence microscope with CCD camera Comparitive genomic hybridization Bornfleth et al. Cytometry 24:1–13, 1996	DAPI FITC Texas red	mercury 50W Triple band-pass excitation filter		Triple band-pass emission filter
Fluorescence microscope and CCD camera, x20/0.75 Segmentation of nuclear images Price et al. Cytometry 25:303–316,1996	DAPI	mercury 100W BP ^{&} 365/10	DM [%] 400 nm	none
Microscope with CCD camera x30/0.5 Multiparameter fluorescence imaging of lymphocytes using labelled monoclonal antibodies Galbraith et al. In Proc. of the SPIE , Vol 1063, New Technologies in Cytometry,1989	FITC PE Cy5 Cy3	manual, six-position slider for fluorescence filters		
Special optical system for epi-illumination microscopy with wavelength switching using a polychroic beam-splitter Heiden and Tribukait Cytometry, 20:95–101, 1995.	DAPI TRITC	mercury 100W BP ^{&} 365/33 nm BP ^{&} 546/23 nm	Polychroic PBS [%] 365 reflection 546 reflection	BP ^S 435-500 nm BP ^S 590-750 nm

special objective or other stray light reducing optical measures, due to the elimination of stray light by illumination through a pinhole. Most absorbing immunostains give a very strong reflection of laser light and show very limited fading. They often permit the use of a small pinhole size (e.g. 10 micron), which is about five to ten times smaller than can be used with most fluorochromes in confocal fluorescence laserscanning microscopy. The use of reflecting immunomarkers can therefore result in a very significant increase in optical resolution. Specimens double-stained with both a

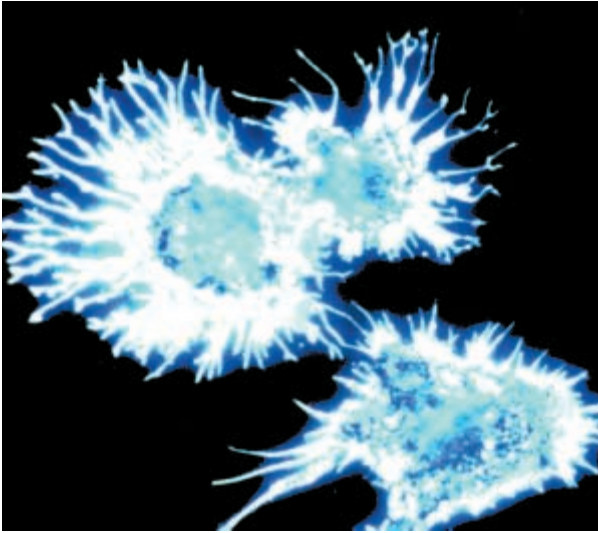


Fig. 10: Reflection contrast microscopy (RCM). Mouse peritoneal macrophages adhering to a coverslip and stained with a monoclonal antibody to surface antigens. Immunoperoxidase staining. Thin extensions of filopodia, which are not visible with brightfield microscopy, are visible because of the strong reflection of the DABox product.

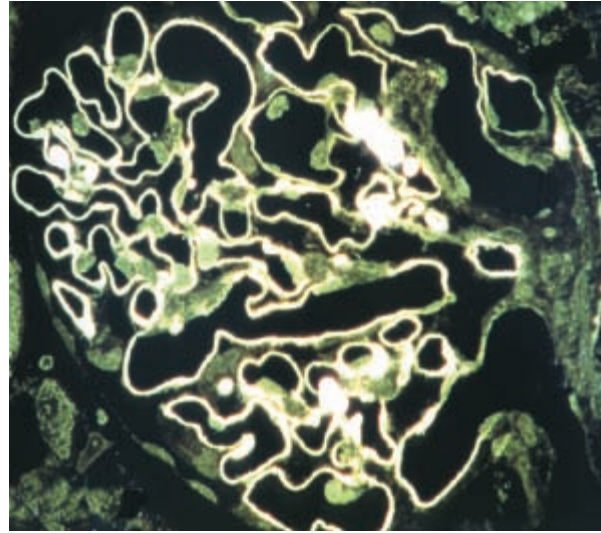


Fig. 11: Ultrathin section (ca. 35 nm) of kidney tissue: peroxidase DAB staining. Linear staining patterns of the glomerulus observed with reflection contrast microscopy present often sharper images than with fluorescence microscopy.

fluorochrome and the Leica Spectral Confocal Laserscanning Microscope providing new possibilities for multiple markers can easily examine a reflecting immunomarker.

RCM uses a fluorescence microscope stand, epi-illuminator and high pressure lamps. In the fluorescence epi-illuminator an extra polarizer filter cube, containing a polarizer, reflector, and an analyzer must be inserted (Fig. 12). Also a reflection contrast (RC) diaphragm module (containing the central stop system) must be brought in the incident light path. This RC diaphragm module adapts sliding sets with central stops and/or aperture diaphragms. Furthermore a special objective developed for reflection contrast microscopy, the Leica RC M-plan oil-immersion x100/1.25 RCM objective equipped with a $\lambda/4$ plate at the front lens, should be added to the set of objectives (for fluorescence microscopy) on the turret of the objective revolver.



Fig. 12: A filter cube (block) for reflection contrast microscopy, containing a polarizer, a neutral (50 %) beam-splitter and an analyzer. This filter cube (block) can be inserted into one of the positions of a Leica epi-illuminator for fluorescence microscopy.

In reflected-light microscopy, contrast is based on differences in reflection intensities (= reflectance). In this type of microscopy it should be reminded that reflection of light occurs at every optical boundary, that is when the refractive index and/or the absorption index change. For biological specimens showing reflectance of less than 1% and mostly less than 0.2%, it is almost impossible to obtain a reflected light image with a conventional microscope, due to the unwanted reflections inside the microscope tube. In reflection contrast microscopy, a combination of two methods is used to reduce glare due to scattered light. The incident light is made into a ring-shaped cone of light by the insertion of a central stop (an aperture diaphragm provided with a central stop, creating an annular aperture) in the incident light path at a plane conjugated with the back focal (aperture) plane of the objective. As a second method, unwanted scattered reflected light is reduced by the use of the "antiflex" method. Light reflected inside the microscope is suppressed by using crossed polarizers, with the result

that only the light reflected from the specimen is passed on to the eyes. To that purpose an objective is provided with a quarter-wave plate in the front lens of the objective. The passing of the light through the $\lambda/4$ plate (downwards to the specimen and upwards after reflection from the specimen) change the polarization direction of the light with $2 \times 45^\circ = 90^\circ$.

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