Fluorescence Microscopy – Instrumentation

This section examines fluorescence microscopy instruments, focusing on diascopic and episcopic microscopes.

The Fluorescence Microscope and Its Basic Design

Incident-light fluorescence utilizes a vertical illuminator (episcopic illumination), while transmitted-light fluorescence employs a diascopic illuminator with either a brightfield or darkfield condenser. The following discussion is also relevant to inverted fluorescence microscope stands.

The transmitted-light fluorescence setup is simple and includes a bright light source, IR blocking filter, excitation filter below the condenser, darkfield-type condenser, specimen, suitable objective, suppression filter below the eyepiece, and eyepiece (see **Fig. 1**). It's useful for fluorescein isothiocyanate (FITC) fluorescence and broad-band excitation experiments with a 3 mm BG12 excitation filter but not suitable for less transparent or thick specimens. With the numerical aperture NA and the magnification of the objective *M*, the brightness *B* of a transmitted-light fluorescence microscope is given by

$$B \sim (NA_{Obj} \times NA_{Cond})^2/M^2.$$

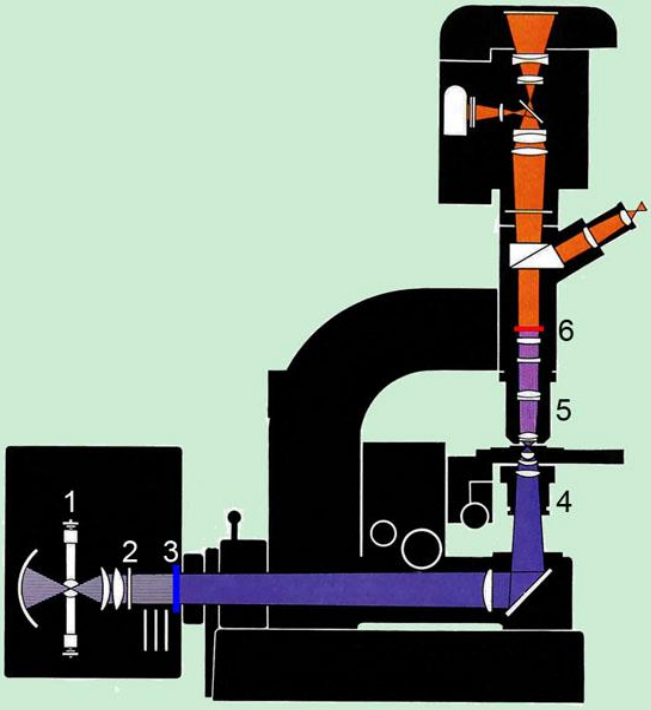


***Fig. 1**: Transmitted light fluorescence microscope. Light source (**1**), heat filter (**2**), exciter filter (**3**), condenser (**4**), objective (**5**), suppression filter (**6**).*

In 1938, Max Haitinger demonstrated a Leitz fluorescence microscope using episcopic illumination. This incident-light fluorescence setup operates differently from the transmitted-light setup. It consists of a bright light source, an IR blocking filter (heat-filter), an excitation filter, a dichroic beam splitter, a suitable objective (used as a condenser), the specimen, the same objective again, a suppression filter below the eyepiece, and the eyepiece (see **Fig. 2**). The performance of this type of fluorescence microscope is consistent regardless of sample thickness. The brightness *B* of this setup is given by

$$B \sim NA_{Obj}^4/M^2.$$

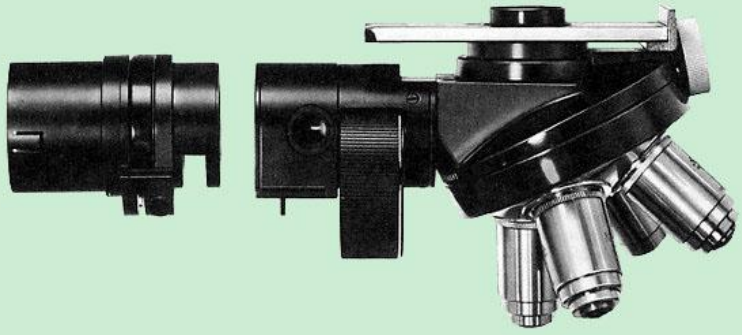


***Fig. 2**: Leitz Ploemopak 1 for Leitz Orthoplan (1972).*

In 1967, **Johan Sebastiaan Ploem (b1927)** introduced a new episcopic design using a dichroic (dual-band or dual-wavelength) beam splitter. Following this invention, Leitz developed the Ploemopak turret with four dichroic mirrors and matching barrier filters at 45° to the light path. Later, they released the Ploemopak 2, featuring a revolving disc holding up to four filter cubes, each containing a dichroic mirror, barrier filter, and exciter filter. This design rapidly popularized incident-light fluorescence microscopy in various fields (see **Fig. 3**, **Fig. 4**, and **Fig. 5**). The filter cube design is also used in inverted fluorescence microscopes.

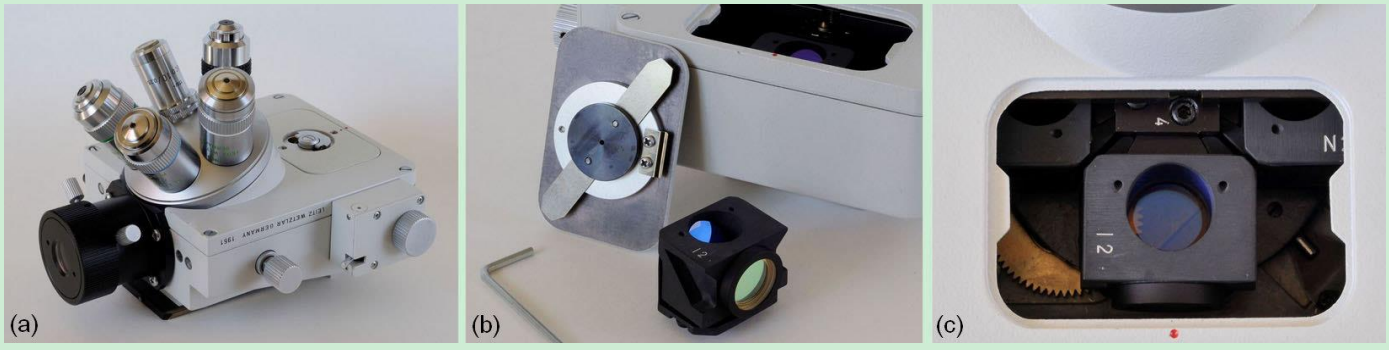


***Fig. 3**: (**a**) Leitz Ploemopak 2.2 for Leitz Ortholux II. (**b**) Leitz filter cube I2 removed from Ploemopak 2.2. (**c**) Leitz filter cube I2 inside Ploemopak 2.2.*

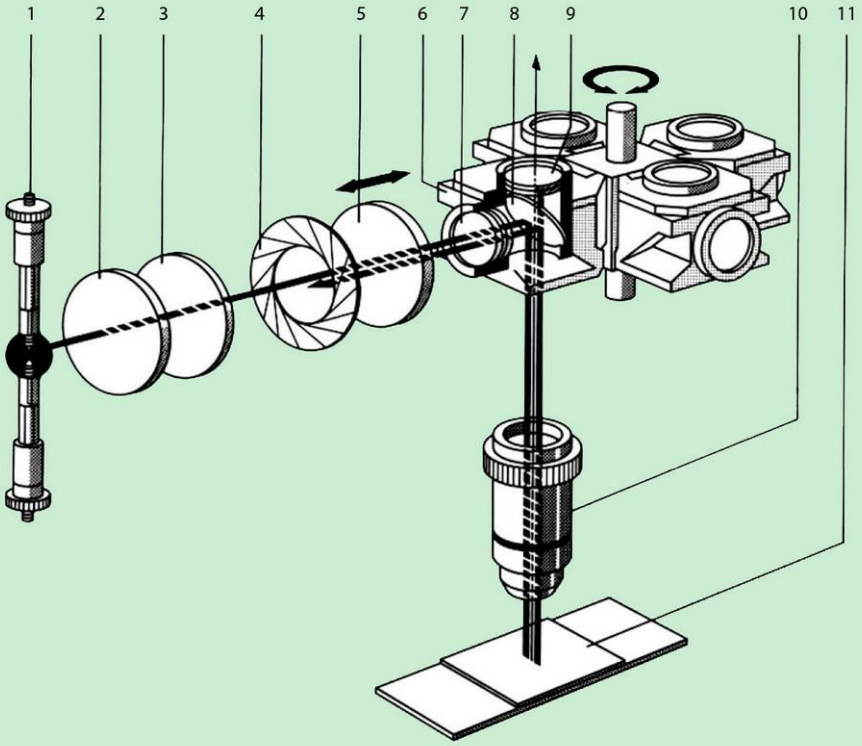


Fig. 4*: Schematic of an incident-light fluorescence microscope with Leitz Ploemopak 2. Light source (1), heat absorbing filter (2 mm KG1) (2), red-suppression filter (4 mm BG38) (3), field diaphragm (4), adjustable lens for focusing field diaphragm (5), exchangeable filter cube (6), exciter filter (7), dichroic mirror (8), suppression filter (9), objective (10), specimen (11). The BG38 is part of the Ploemopak 2, while the KG1 is added inside the filter slot of the lamp housing.*

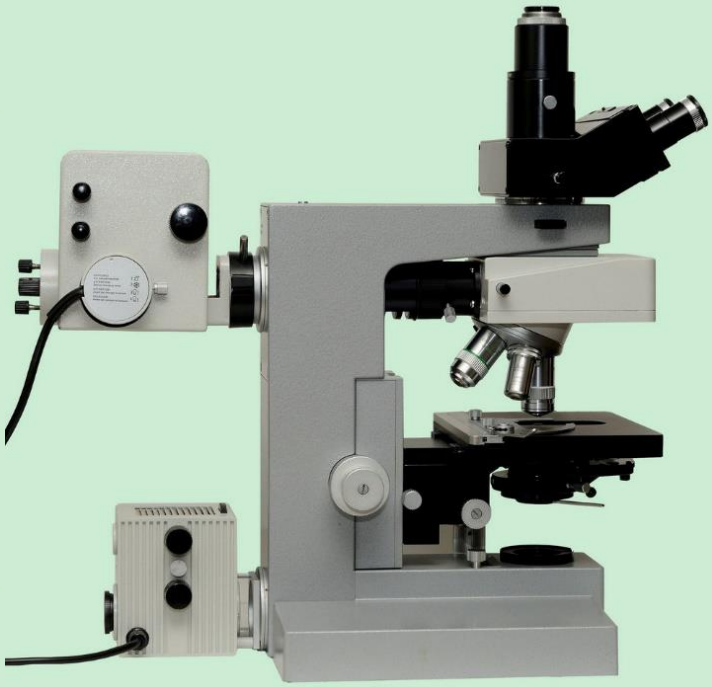


Fig. 5*: Leitz Ortholux II with Ploemopak 2.2 (1975).*

From the early 21st century, top microscope makers have provided illuminators with high-performance diodes at key wavelengths. By using a computer-controlled array of filters, prisms, and mirrors, any main excitation light can be produced and directed towards the sample. An appropriate suppression filter is still required between the specimen and eyepiece. One example is the Zeiss Colibri illuminator.

Since the brightness B for incident-light fluorescence microscopy is given by $B \sim NA_{Obj}^4/M^2$, it is important to use objectives with higher numerical aperture. Additionally, objectives with high transmission of shorter wavelengths must be used when the specimen is to be excited with UV or blue light. Two excellent objectives for fluorescence microscopy are shown in **Fig. 6**.

***Fig. 6**: Leitz objectives used for fluorescence microscopy (PL FLUOTAR 40/0.70 and NPL FLUOTAR 16/0.45).*

Light Sources Suitable for Fluorescence Microscopy

In 1933, Haitinger employed a low-pressure mercury arc lamp for fluorescence microscopy. In 1937, the high-pressure mercury arc lamp was introduced.

There are four primary types of light sources suitable for fluorescence microscopy:

- Arc-discharge, high-pressure Mercury HBO lamp (e.g., OSRAM HBO50 Mercury Short Arc lamp)
- Arc-discharge, high-pressure Xenon XBO lamp
- Incandescent, halogen lamp (e.g., 12 V / 100 W iodine-quartz lamp)
- Array of high-intensity diodes of different wavelengths (e.g., Zeiss Colibri)

A 100W halogen lamp is used for many applications, often in combination with other contrast techniques, such as phase contrast or DIC. Occasionally, a 50W Mercury HBO lamp is also utilized. Unlike the incandescent lamp, the mercury lamp does not provide uniform intensity across the spectrum from ultraviolet to infrared. Much of the intensity of this type of lamp is in the near ultraviolet with major peaks of intensity occurring at 313 nm, 334 nm, 365 nm, 406 nm, 435 nm, 546 nm, and 578 nm [**3**, page 223]. The 50W Mercury lamp's intensity at major peaks is much higher than that of the 100W halogen lamp

(~40 times at 546 nm). However, its apparent brightness (2000 lm) is about 30% less than the halogen lamp (2800 lm). The 100W halogen lamp seems brighter, but the intense UV from the Mercury HBO lamp can harm the observer's eyes. Always wear UV protective goggles when adjusting the HBO lamp and never look directly into it. Refer to the manufacturer's documentation for safe handling. Use a heat-absorbing filter (2 mm KG1) with both types of illuminators to block IR radiation.

Several companies made mirror housings for switching between lamps. These housings allow microscopists to choose UV light from an arc-discharge lamp or light from an incandescent lamp. Mixed light from at least two sources can also illuminate a specimen (see **Fig. 7** and **Fig. 8**).

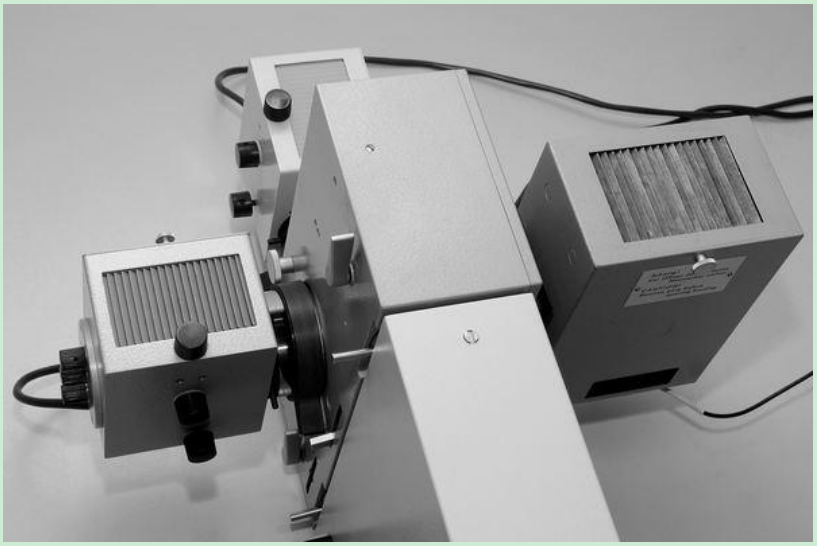


***Fig. 7**: Leitz mirror housing 500 with three lamps.*

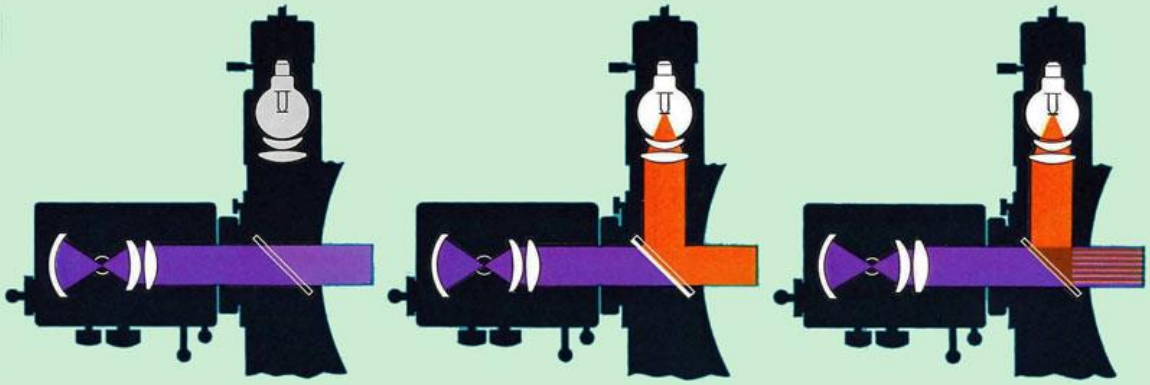


***Fig. 8**: Top view of Leitz mirror housing 250 with two different illuminators showing three different positions (UV light, incandescent, mixed).*

Filters for Fluorescence Microscopy

Selecting the correct filters is imperative for successful fluorescence microscopy. In the following, we are focusing primarily on filters available inside the filter cubes of the Leitz Ploemopak and similar cubes offered by other companies (such as Zeiss, Nikon and Olympus). Heat absorbing filters (e.g. KG1) that are inserted close to the collector lens of the illuminator (see **Fig. 9**) are not further discussed. - Of course, appropriately selected individual filters and mirrors can certainly replace any cube design.

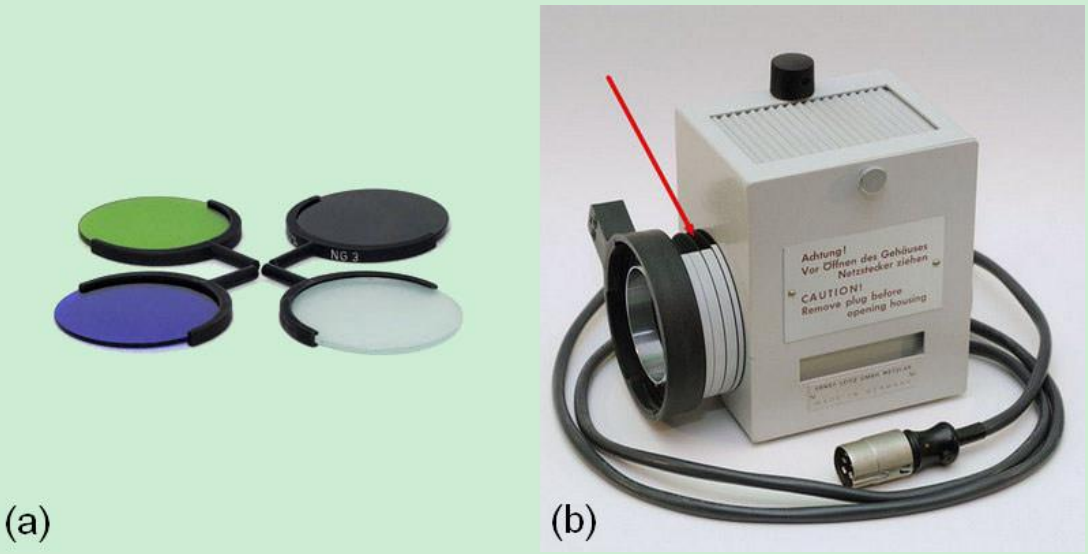
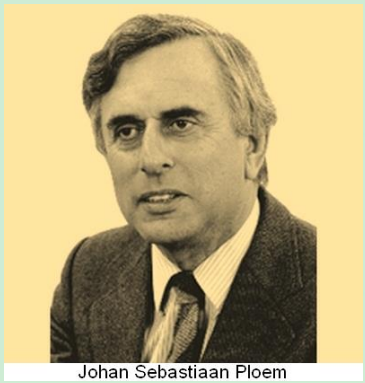


***Fig. 9**: (**a**) Leitz filters for Leitz illuminator (green, blue, gray, and diffuse). (**b**) Leitz Lamp Housing. Red arrow indicates filter slot for various Leitz filters*

Fluorescence microscopy uses various filters, including short pass, long pass, dichromatic beam splitters (dichroic mirrors), wide pass, and narrow band pass filters. Combinations of these filters are common. Refer to **Table 1** for details.

Leitz Filter Cube	Exciter Filter	Dichroic Mirror	Barrier Filter
A (UV → blue)	2x 2 mm **UG1**	**TK400**	**K430**
I2 (blue → green)	2x **KP490**, 1 mm **GG455**	**TK510**	**K515**
N2 (green → red)	2 mm **BG36**, **KP560**, **K530**	**TK580**	**K580**
H2 (violet, blue → green)	2x **KP490** (= **KP500**)	**TK510**	**K515**
G (UV, violet, blue → green, red)	3mm **BG12**	**TK510**	

***Table 1**: Selected Leitz filter cubes for the Ploemopak 2.2 and 2.1.*


Johan Sebastiaan Ploem

Short pass filters let the shorter wavelengths pass and discriminate longer wavelengths (see **Fig. 10**). The Leitz designation is **KP** for “kurz pass”. This type of filter cuts off wavelengths above a certain value. For instance, Leitz filter cube **I2** (see **Fig. 16**) contains two **KP490** that let wavelengths up to 490 nm pass. For most fluorescence microscopes using transmitted light, we distinguish between filters that can be placed very close to the lamp’s collector lens (just after the IR blocking filter) and those that must be placed below the condenser. For a setup using episcopic illumination, the filter cube containing the short pass filter is placed after the IR block filter and after a 4 mm red suppression filter. BTW, the red suppression filter is built into the Leitz Ploemopak 2 and should not be removed.

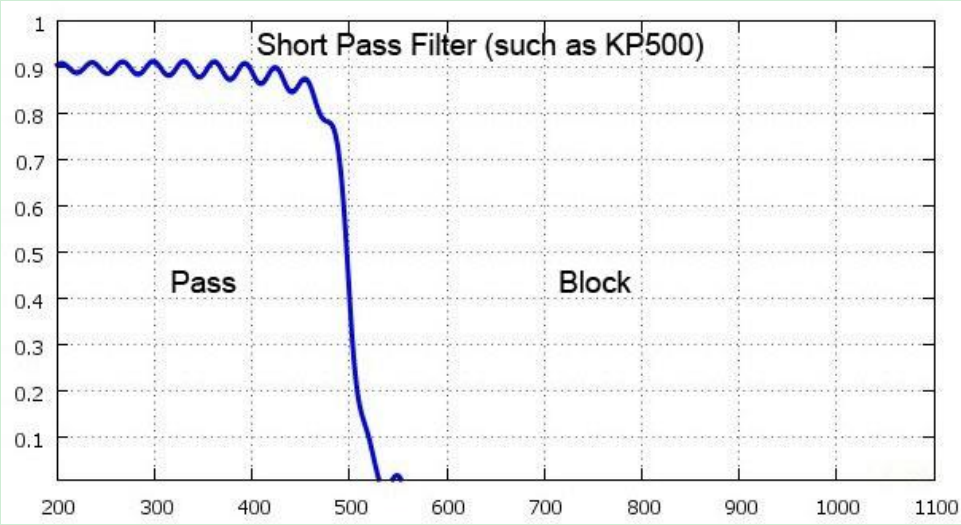


***Fig. 10**: Sketch of short pass filter. Normalized transmission is shown as a function of wavelength in nanometers.*

Long pass filters let the longer wavelengths pass and discriminate shorter ones (see **Fig. 11**). The Leitz designation is either **LP** for “lang pass” or **K** for “Kante” (meaning “edge”), which is an acronym for long-wave pass interference filter. This type of filter, which is often used as the suppression filter in fluorescence microscopy, lets pass wavelengths above a certain frequency. For instance, Leitz filter cube **A** contains a **K430** that lets wavelengths above 430 nm pass.

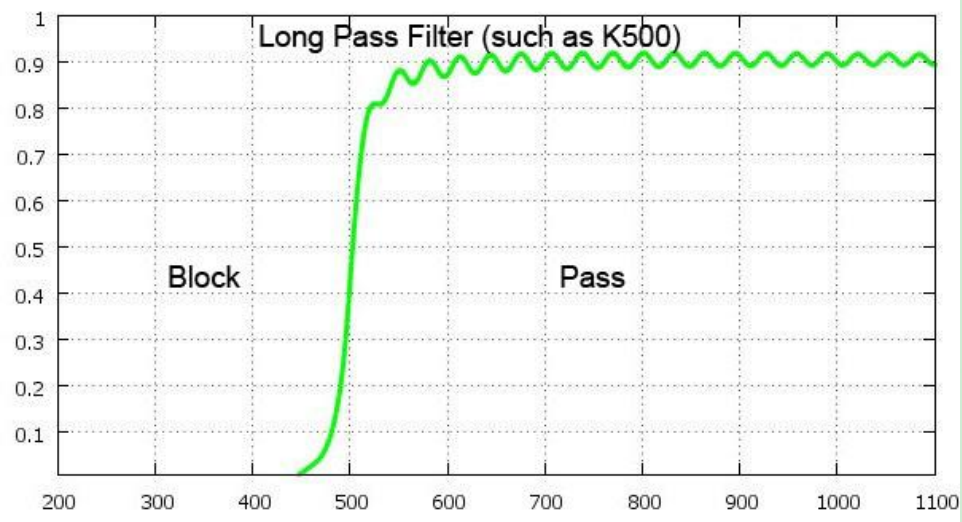


***Fig. 11**: Sketch of long pass filter. Normalized transmission is shown as a function of wavelength in nanometers.*

Dichromatic beam splitters (also known as dichroic mirrors) are like long pass filters in that they let pass wavelengths above a certain frequency (see **Fig. 12**). The filter (or mirror) is usually put at a 45° angle to reflect the incoming light towards the specimen. Dichromatic beam splitters are only useful for episcopic illumination. They are not used for transmitted light fluorescence microscopy. The Leitz designation is **TK** for "Teilerkante". All Leitz filter cubes contain such a filter (e.g. **TK510** in cube **I2** or **TK400** in cube **A**). The position of the edge of the dichroic mirror above which wavelengths pass is chosen to be slightly lower than the one for the long pass filter (e.g. in cube **N2**, we have a **TK510** and a **K515**). As shown in **Fig. 12**, the dichroic mirror will also allow a narrow band of UV radiation to pass. This fact emphasizes the importance of using a barrier filter in form of a long pass filter to suppress any unwanted UV radiation.

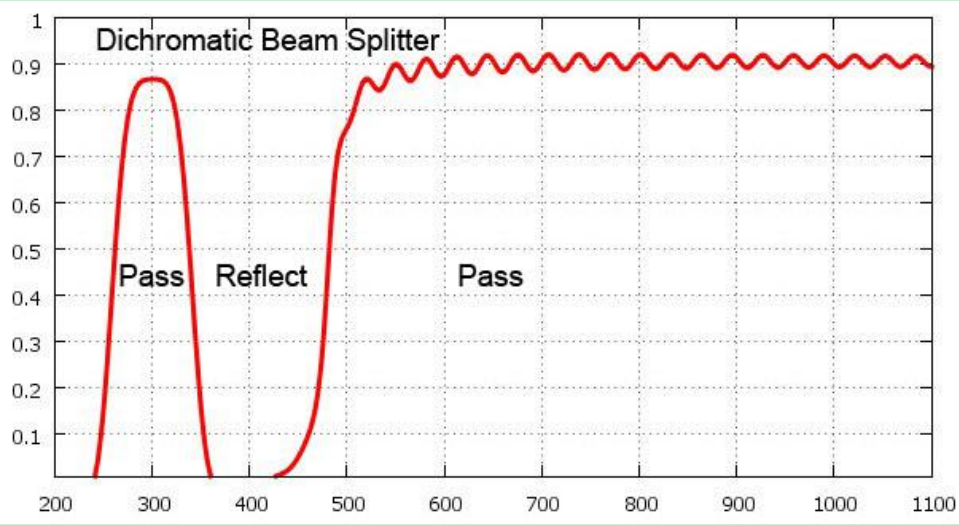


***Fig. 12**: Sketch of dichromatic beam splitter (dichroic mirror). These filters let UV light pass. Normalized transmission is shown as a function of wavelength in nanometers.*

Wide pass filters (colored glass filters) are the simplest type of filters (see **Fig. 13**). Leitz commonly uses the following designations: **GG** = gelbes Glas (yellow glass), **UG** = ultraviolettes Glas (ultraviolet glass) and **BG** = blaues Glas (blue glass). These filters offer a broader range of wavelengths that are permitted to pass and hence have a broad half-value width. Wavelengths below and above this broader range are suppressed with varying efficiency. For instance, a 3mm **BG12** blue exciter filter allows wavelengths between ~320 to ~500 nm to pass with a transmission maximum at around 400 nm. BTW, wavelengths above ~900 nm (beginning of IR band) pass through a 3mm **BG12** filter although we do not want IR to pass. It is good to know that the red suppression filter inside a Ploemopak 2 takes care of unwanted IR excitation.

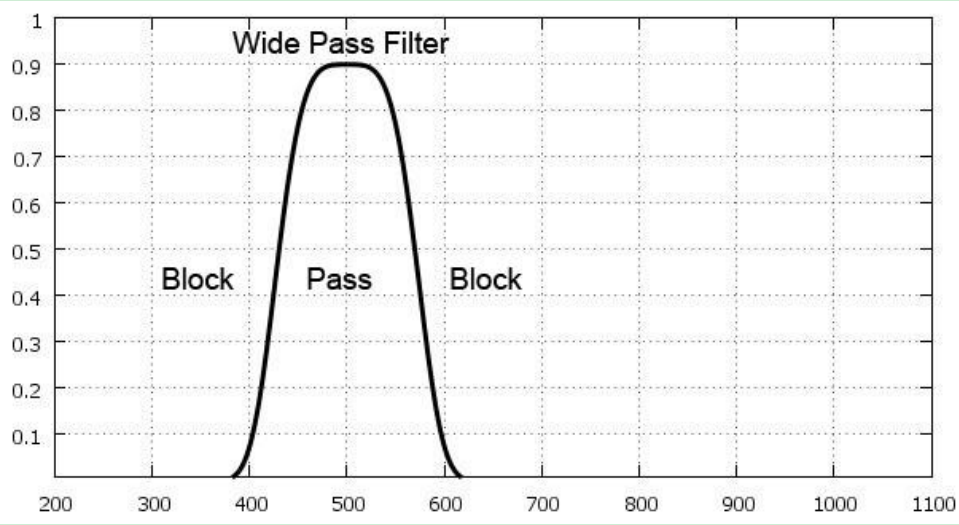


***Fig. 13**: Sketch of wide pass filter (such as BG12). Normalized transmission is shown as a function of wavelength in nanometers.*

Narrow band pass filters are used to allow only wavelengths over a narrow range to pass (see **Fig. 14**). The Leitz designation is **S** for “schmal” (narrow). Very often narrow band pass filters are the combination of one short pass filter and one long pass filter. For instance, the Leitz filter cube **N2** contains a **KP560** and a **K530** to form a narrow band pass filter for wavelengths between ~530 to ~560 nm. **Fig. 15** illustrates how a narrow band pass filter is constructed by combining a short pass filter with a long pass filter.

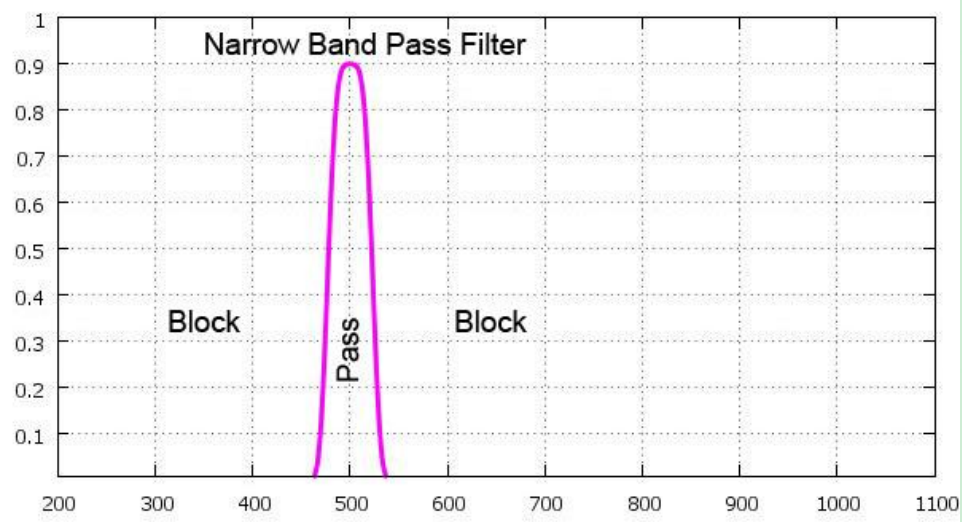


***Fig. 14**: Sketch for narrow band pass filter. Normalized transmission is shown as a function of wavelength in nanometers.*

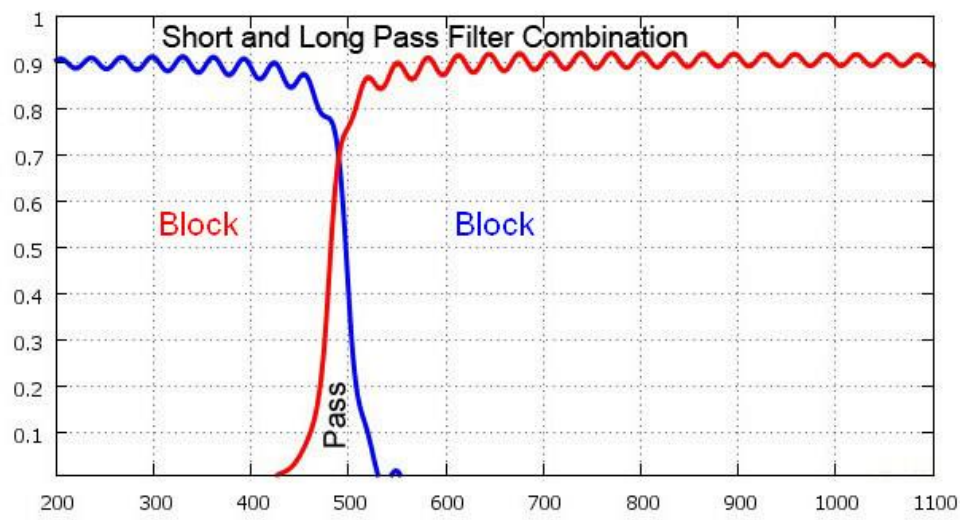


***Fig. 15**: Sketch for narrow band pass filter constructed by combining a short pass filter with a long pass filter. Normalized transmission is shown as a function of wavelength in nanometers.*

After having introduced the most important filter types, we discuss the excitation types used for fluorescence microscopy. We distinguish between three basic types of excitations, narrow-band excitation, broad-band excitation and selective excitation.

Narrow-band Excitation: When the excitation and emission maxima of fluorescence are closely spaced, narrow-band excitation is used. However, the use of a short pass filter (Leitz designation **KP**) and a long pass filter (Leitz designation **K**) are sufficient. For instance, Leitz filter cubes **A** and **N2** are used when the distance between excitation and emission maxima is small (~20 to ~40 nm).

Broad-band Excitation: Dyed glass filters (e.g. **UG** filters or **BG** filters) with a broad half-value width are used to excite the specimen over a broader range of different wavelengths. These exciter filters are recommended wherever the excitation and emission maxima of the fluorochrome are widely separated. A suitable filter cube for broad-band fluorescence is the Leitz filter cube **G**, which consists of 3mm **BG12** for excitation, a dichroic mirror **TK510** and a barrier filter **K515**. We are using this filter cube for studying the fluorescence of chlorophyll.

Selective Excitation: This type of excitation is very similar to narrow-band excitation except that asymmetrical interference filters with a particularly steep cut-off are used. For instance, the Leitz filter cube **I2** (see **Fig. 16**), which contains 2 **KP490**, 1mm **GG455**, **TK510**, and **K515**, is used for selective blue excitation with green emission. The 1mm **GG455** color glass filter prevents the low transmission of the **KP** filter to pass through, limiting the excitation to ~455 to ~490 nm.

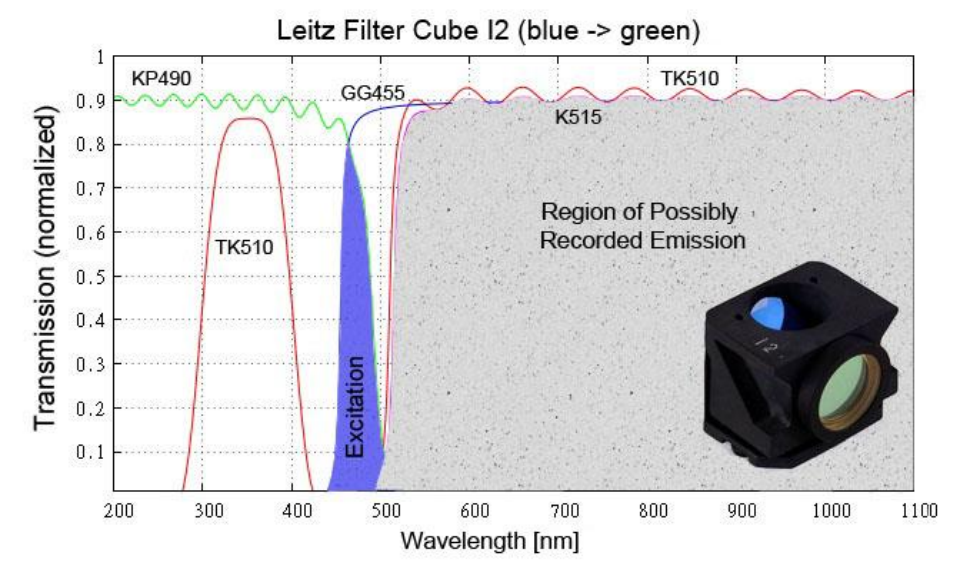


***Fig. 16**: Sketch of transmission properties of Leitz Cube I2 (blue -> green). This cube contains 2x KP490 and 1mm GG455 for excitation, a TK510 dichroic mirror and a K515 barrier filter. The complex spectral profile of the dichroic mirror TK510 is shown (red line). The blue region labeled "Excitation" is produced by the overlap of the short pass filter KP490 (green line) and the wide pass filter GG455 (blue line). The region of all recordable emissions is shaded in grey. The inset in the lower right corner shows a picture of the I2 cube. This sketch does not accurately depict the situation for wavelengths larger then ~750 nm.*

Additional Information

In addition to Rost's two-volume reference on fluorescence microscopy [**1** and **2**], we recommend Koch's Leitz publication [**3**] and Herman's book [**4**]. A short introduction to Fluorescence Microscopy is available from [**5**].

References

*[**1**] F.W.D. Rost, Fluorescence microscopy, Volume 1, Cambridge University Press (1992).*

*[**2**] F.W.D. Rost, Fluorescence microscopy, Volume 2, Cambridge University Press (1995).*

*[**3**] K.-F. Koch, Fluorescence Microscopy – Instruments, Methods, Applications, 512-123/Engl., Ernst Leitz GmbH, Wetzlar (1972).*

*[**4**] B. Herman, Fluorescence Microscopy, 2nd edition, Taylor & Francis Group, New York (1998).*

*[**5**] Gregor Overney, Fluorescence Microscopy – A Short Introduction, Micscape Magazine, UK (2025).*