Optical Filters and Light Sources for FISH

Michael Sommerauer, Ingrid Feuerbacher, and Alexander Krause

Abstract

Brilliant fluorescence signals with almost no background and cross talk are the aim of FISH analysis in imaging systems. The precise selection of hardware components like optical filters and light sources plays a major role. Considering fluorescent dye characteristics is the base of configuring perfectly matched multicolor-FISH (mFISH) filters, which allow the simultaneous application of up to seven dyes. The spectral characteristics of filters are here explained with respect to microscope setups. Spectral cross talk, pixel-shift effects, and stable energy output will be the main issues in daily work. Specific hard-coated single-band filters with small bandwidth but maximum transmission avoid cross talk to a high degree; the use of multiband filters allows simultaneous imaging of up to four dyes; multiband systems with separate exciters and emitters in filter wheels can be controlled by software. The comparison of mercury and metal halide light sources to new light-emitting diode (LED)-based light sources is shown, which reveals the chance of stable and long-term light output of the new LED light sources. White-light LED sources are nowadays a perfect choice for replacing mercury-based lamps. The combination of hard-coated mFISH filters with stable LED light sources is a very helpful tool in daily work.

Keywords Light-emitting diode (LED) light sources, Comparative genomic hybridization (CGH) filter sets, Multicolor fluorescence in situ hybridization (mFISH) filter sets, Filter handling, Fluorophores, Mercury lamp, Exciters, Emitters, Beam splitter, Mounting of filters

1 Introduction

1.1 Fluorescence, Fluorescence Spectra, and Fluorescent Dyes

Fluorescence is a material intrinsic property which is common to all materials. It is the ability to absorb energy—here light ($E = h^{*}\nu$)—and to emit light of lower energy than the absorbed. The absorption is strongly depending on the wavelength. Absorption only occurs if a photon has enough energy to push an electron from the ground state S^0 into an excited state S^1. The extinction coefficient is a degree of the efficiency of the absorption [1].

Electrons in excited states are relaxing into the ground state in nanosecond scale. Hereby several pathways are possible. One way of releasing energy is the emission of light which can be detected as fluorescence; another way is relaxation without radiation. A degree of this emission is the quantum yield. In Fig. 1a the absorption and

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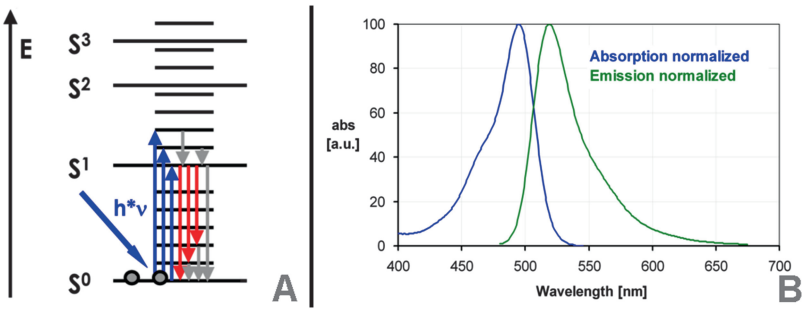

Fig. 1 (**a**) Jablonski term scheme. (**b**) Spectrum of FITC: The difference between the excitation maximum and the emission maximum is called Stokes shift

emission of a photon is illustrated. Further the term scheme shows that the electronic levels are divided into sublevels which belong to the vibronic states of substances. An electron can change these vibronic sublevels without any emission of light. This explains the energy difference between the light which is used for pushing the electron into an excited state and the light which is released by the electron during relaxation. The term scheme also shows an energy distribution for absorption and emission. The energy (E) can be transformed into a wavelength (λ) by the equitation $\lambda \sim 1/E$. Figure 1b shows the wavelength distribution or spectrum for the common fluorescence dye FITC [2]. The extinction coefficient and the quantum yield are characterizing each substance. The higher both parameters are, the better a substance (molecule or nanocrystal) can be used as fluorescence marker. In most cases the fluorescence markers are organic molecules based on aromatic ring systems. They are soluble in common solvents and can be chemically bound to proteins, DNA or RNA. This gives the user the possibility to label samples specifically [1].

1.2 Filter Characteristics

Fluorescence can be detected by using spectrometers or fluorescence microscopes. These instruments have light sources which are used for exciting the fluorescent dyes. In most cases such with a more or less continuous emission spectrum is applied, e.g., mercury, xenon, metal halide, or light-emitting diode (LED) light sources. Optical filters with a dye-specific transmission band are placed between these light sources and the labeled sample. For FITC (green) only a part of the blue emission of such a lamp is used. Every other wavelength is blocked by this so-called excitation filter or exciter. The detection of the fluorescence can be done with CCD cameras or with the eyes. In comparison with the light which is used for excitation, the emitted fluorescence is more than 10,000 times less intense. This means another filter which is called emission filter or emitter has to block the light which was used for excitation. Exciter and emitter have to be blocked against each other;

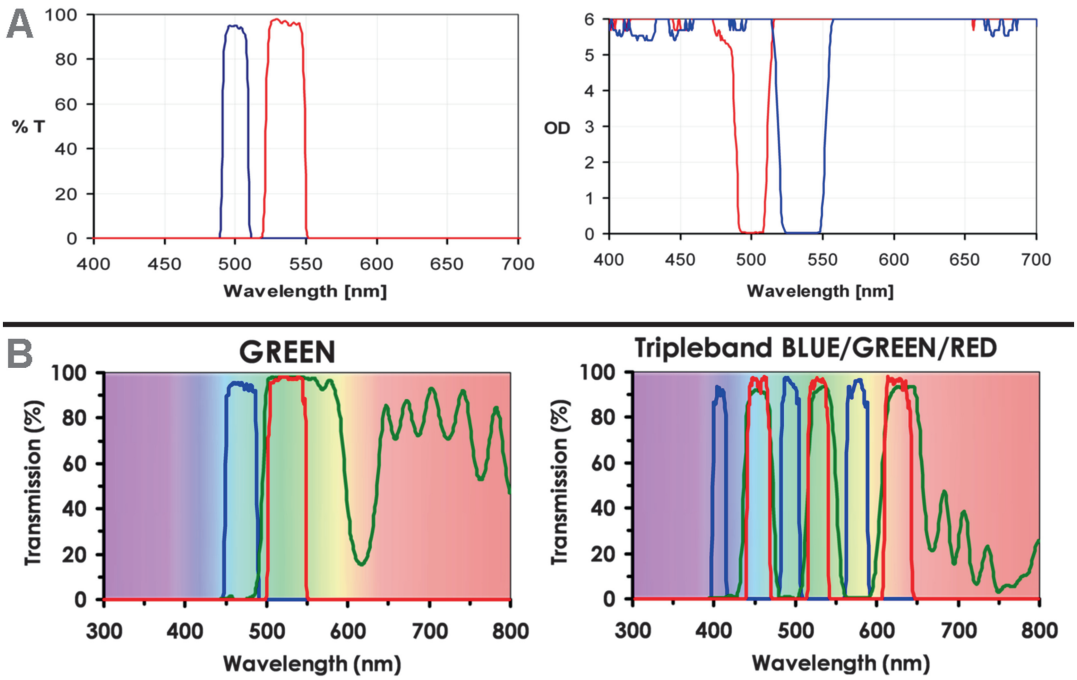

Fig. 2 (**a**) Spectral data of filters in linear and logarithmic scaling. (**b**) Single-band filter set for green (e.g., Sp. Green) and triple-band filter set for blue, green, and red dyes (e.g., DAPI, Sp. Green, Sp. Red). Exciter blue line, dichroic green line, and emitter red line

otherwise the fluorescence signals cannot be detected. Due to the big energy difference between excitation light and fluorescence, a filter must suppress undesired light very efficiently. The transmission (T) in these blocking areas should not exceed 0.0001 %. Instead of using the transmission, the blocking of a filter is determined by the optical density OD = −log (T). Figure 2a shows a pair of exciter and emitter.

Normally the fluorescence is measured perpendicular to the excitation. Therefore another optical component called "beam splitter" is used to reflect the light which is used for excitation and to transmit the fluorescence light of the sample. According to its function, the beam splitter has to be mounted under 45° into the light path. This beam splitter only supports the blocking of the excitation and emission filters against each other, but its own blocking properties are not sufficient [3]. For application of FISH in electron microscopy, see chapter by Hannes Schmidt and Thilo Eickhorst "Gold-FISH: In Situ Hybridization of Microbial Cells for Combined Fluorescence and Scanning Electron Microscopy".

1.3 Filters in a Microscope

In a fluorescence microscope, perfectly matched filter sets are used. A set consists of an exciter, an emitter, and a beam splitter as described (chapter by Ivan Iourov "Microscopy and Imaging

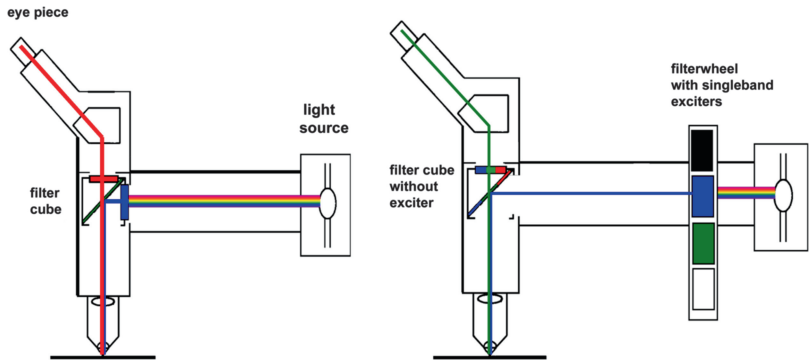

Fig. 3 Imaging with single-band and a multiband filter set

Systems"). In current microscopes each filter set is mounted in its own filter cube [4]. All three components are fixed in the right position and the correct angles. No further adjustments have to be made by the user. Such a cube can easily be put into the microscope and removed from it. For every dye a special filter set mounted in a cube has to be chosen (Fig. 2b). Nevertheless it is possible to make filter sets with two or three transmission bands, so that three different dyes can be detected simultaneously. These filter sets are called full dual- or triple-band filter sets as shown in Fig. 2b.

If the microscope has an additional filter wheel between the lamp housing and the microscope stand, further possibilities for placing filters are given. The dual-, triple-, and even quad-band filter sets—shortly described as multiband filter sets—can be divided into two sections. The beam splitter and emitter stay in the filter cube and the exciters are placed in the additional filter wheel. A filter set for a single color has one exciter. For each color channel of these multiband filter sets, one exciter (called single-band exciter, Fig. 3) can be placed in the described filter wheel. This allows a sequential imaging of the different colors, which can easily be automated. This avoids pixel shift (see below), because the same beam splitter and emitter stays in the light path.

Also the corresponding dual-band or triple-band exciters can be placed in these filter wheels for simultaneous imaging of the sample. Some FISH kits (e.g., UroVysion kit [5]) need this dual-band excitation (chapter by Thomas Liehr "Commercial FISH Probes"). A multiband filter set (Fig. 3) with a dual-band exciter or a full dual-band filter set itself can be used. The given example is using green and red dyes which have been imaged simultaneously. The green and red color can be easily detected in such a dual-band filter set. If both colors are co-localized, the addition of the two dyes can be detected as yellow (same principle as in every TV). A full triple-band filter set or the triple-band excitation of a multiband filter set will show the same results. Mostly the counterstaining (DAPI) is used as the third channel (blue channel). If the

concentration of DAPI is too high, the blue channel is overbalanced and complicates the detection of the other channels.

A few comments to the abovementioned pixel shift [3]. Pixel shift can occur by switching from one filter set to the other. The reason is the difference in the beam deviation which is caused by the wedge angle of every component in the light path. If both surfaces of an optical filter or beam splitter are exactly coplanar, the beam deviation is zero. A small deviation of the parallelism cannot be detected with cameras, but if the deviation at the camera chip is more than 6.7 µm, pixel shift is detected. The identical signal of the sample in different color channels is not registered at the same place.

To avoid this pixel shift, so-called zero pixel shift certified filter sets can be used. These sets have nearly coplanar substrate surfaces (wedge angle $\leq$ 10 arc sec). Small deviations can be adjusted with an autocollimator, when the filters (emitter and beam splitter) are mounted into the filter cube.

1.4 Choosing Filters

According to the possibilities of the microscope setup, filter sets can be chosen. Most essential is to know the spectral behavior of the dyes used. Each filter set has to be chosen according to the dye spectrum. That means the exciter should include the absorption maximum of the dye. Also the emission maximum should be included in the emission filter. Only in this case a good signal/noise ratio can be achieved. That means the smaller the Stokes shift is, the steeper the filters. Figure 4 shows a filter set for FITC which fulfills the abovementioned criteria.

By choosing dyes and filters, a closer look to the microscope-related equipment like the camera and the excitation source should be done. Dyes in the spectral region between DAPI (UV excitation and blue detection) and Texas Red (yellow excitation and red detection) can easily be determined by the eyes or any camera system (color or black & white). If Far Red dyes like Cy5 (red excitation and NIR detection) are used, it is essential to use a black and white camera system, because the eyes are insensitive in

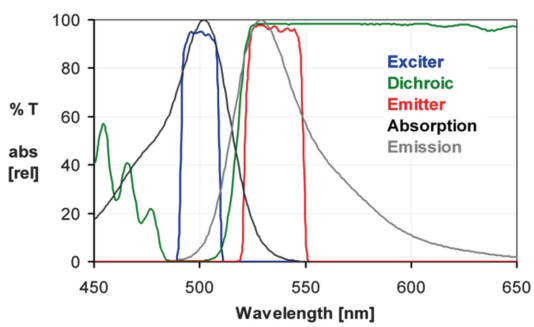

Fig. 4 Spectrum and filter set for FITC

these spectral regions. Color cameras will also cut a part of the emission spectra of these dyes. The excitation can be done with any kind of excitation sources like white-light LED, mercury, xenon, or metal halide lamps. If dyes like Cy5.5 (NIR) and Cy7 (IR) are used, black and white cameras and special LED or xenon excitation sources are essential. The output of mercury and metal halide lamps in the dark red and NIR are not powerful enough to excite these dyes properly. Every IR blocking filter or heat protection filter has to be removed from the microscope as well as from the camera.

1.5 Choosing the Right Light Sources

In the past mainly mercury or metal halide light sources, so-called arc lamps, were used. The principle of generating light is for all these sources the same. It is based on electrical discharge in a gas generated by high voltage. This leads to a broad emission of light which lasts from UV to the visible spectrum. In all those light sources, a major amount of mercury in the range of 20 mg is present. This can be seen at the characteristic peaks at 365, 405, 436, 546, 577, and 579 nm. Besides this emission, which is seen as white light, the lamps produce a lot of heat; only a few percent of their intake power is converted into light.

During the lifetime of these bulbs, the electrodes wear off. This strongly impacts the light output of these light sources (Table 1). LED light sources have generally a 10–100 times longer lifetime compared to arc lamps. They do not produce much heat, because the generation of light is different compared to arc lamps. Electrons and holes in a semiconductor material recombine and emit light of a defined wavelength range when current (DC) is switched on [6]. Depending on the semiconductor material, different emission bands from UV/NIR can be produced. Special high-power LEDs can be used for fluorescence excitation, but a single LED only covers a small range of the spectrum which is used for fluorescence excitation. A LED light source which can be used like a mercury lamp contains always several high-power LEDs of different wavelengths. In white-light LED light sources, up to six different LEDs

Table 1
Approximate lifetimes of different light sources of fluorescence microscopes

Light source	Lifetime (h)	Output at the end of lifetime (%)
Mercury 100 W	400	35 %
Mercury 50 W	200	40 %
Metal halide 120 W	1500–2000	Approx. 50 %
LED light source	20,000	>70 %

with bandwidths from 10 to 85 nm are combined. The result is a white-light spectrum between 350 and 680 nm. This allows exciting nearly all commonly used dyes even into the NIR range. In FISH samples DAPI is the dye with the lowest and Far Red or Cy5.5 is the one with the highest excitation wavelength. Every dye including the above mentioned ones can be properly excited.

One big advantage of LED light sources is they can be switched on and off instantaneously without compromising the lifetime of the LEDs. They reach their maximum output level in less than 2 s. No warm-up or cooling-down time has to be considered. This means that a LED light source has only to be switched on when it is needed. This reduces their "on" time up to 95 % compared to mercury or metal halide light sources. Arc lamps normally burn the whole day because they have to warm up before and cool down after use. Considered a 40 h week, an arc lamp will be switched on 40 h, even if it is used only for 20 h a week. This means the mercury arc lamp has to be replaced after 10 weeks (lifetime 400 h). A LED light source will be only switched on for the time in use. In this example it is 20 h a week. After a bit more than 19 years, the 20,000 h lifetime is reached. During their lifetime LED light sources are maintenance-free. An easy integration into automated slide scanners is possible without having any mechanically moving items (e.g., filter wheels or shutters). One last benefit is that LED light sources are completely mercury-free, which helps to protect the environment.

1.6 LED Light Sources for FISH

The question is if arc lamps generally can be replaced by LED light sources when using the microscopes for comparative genomic hybridization (CGH; chapter by Thomas Liehr et al. "Comparative Genomic Hybridization (CGH) and Microdissection-Based CGH (micro-CGH)"), FISH (chapter by Thomas Liehr et al. "Two- to three-color FISH"), or multicolor FISH (mFISH; chapters by Thomas Liehr and Nadezda Kosyakova "Multiplex FISH and Spec tral Karyotyping"; Thomas Liehr et al. "FISH Banding Tec hniques"; Thomas Liehr et al. "cenM-FISH Approaches"; Thomas Liehr et al. "Heterochromatin Directed M-FISH (HCM-FISH)"; Anja Weise and Thomas Liehr "Subtelomeric and/or Subcentromeric Probe Sets"; Thomas Liehr et al. "Bar-Coding Is Back"). Here the white-light LED light sources are the best choice, but not every LED light source will show necessarily a good result.

Due to technical reasons, yellow and green LEDs are at present much less powerful compared to mercury or metal halide lamps in this specific region. This means that orange and red dyes might be not bright enough to guarantee evaluable results. New technologies however, like the "light pipe technology," allow to overcome this disadvantage of common LEDs [7] resulting in a comparable output power to a newly installed mercury/metal halide lamp (Fig. 5a). This means all LED light sources with a "boosted"

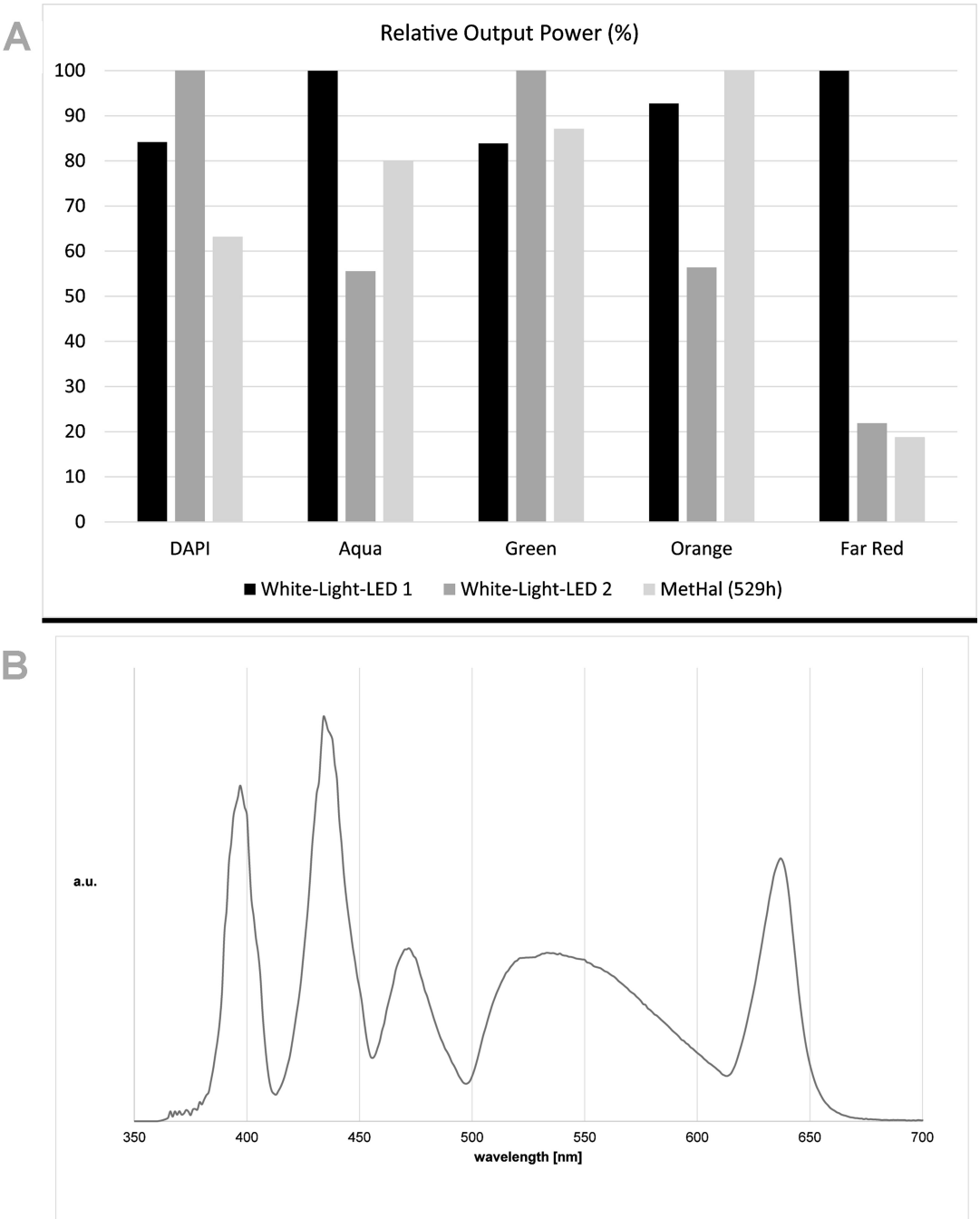

Fig. 5 (**a**) Comparison of the output power of metal halide and boosted LED light sources. (**b**) Spectrum of an LED light source with boosted yellow-green emission and red LED

yellow-green range can be used for FISH techniques. If mFISH is made, it is highly recommended that the LED light source has a red LED as shown in Fig. 5b.

If Cy7 is used as an IR dye, an additional NIR LED must be combined with the LED light source. This is technically no problem and can be easily integrated into any kind of fluorescence microscope.

2 Filter Sets for FISH

2.1 Filter Sets for FISH or CGH

The selection of the correct and suitable filter setup cannot be linked only with the type of dye which will be used in a typical FISH (chapter by Thomas Liehr et al. "Two- to Three-Color FISH") or CGH experiment (chapter by Thomas Liehr et al. "The Standard FISH Procedure"; chapter by Thomas Liehr et al. "Comparative Genomic Hybridization (CGH) and Microdissection-Based CGH (micro-CGH)"; chapter by Thomas Liehr "Commercial FISH Probes"). The use of only two or three dyes with wide spectral distance allows the use of completely different filters in comparison with multicolor applications with six to seven dyes and small spectral distance (see Sect. 1.4). Typically users start with blue, green, and orange signal combinations. This combination allows the use of filter sets with "standard specifications" that means filter sets which are typically used also in immunofluorescence applications. Older microscopes are often equipped with corresponding filter sets, e.g., blue or DAPI basic and green and red filter sets. A typical filter set is shown in Fig. 6a.

Fluorescence images taken with this type of filters will be always "bright," showing background signals from all components present in the sample which can be excited in the range from 450 to 490 nm. The emission window is "open." In some cases people complain that their signals will be not as specific as they want to have, e.g., red signals will be seen in the green filter set. To avoid this drawback, band-pass filters are used, there is only a specific "optic window," and background signals are blocked.

The signals will appear with more contrast and increased signal/noise ratio due to the black background. The most simple trick will be to exchange only the emitter by a suitable band-pass filter which fits to the exciter and dichroic in the setup.

If signals with very low intensity have to be detected, the excitation must be as efficient as possible in combination with very effective signal detection. This can be achieved by the new series of hard-coated band-pass filter sets which show maximum of transmission in the excitation and emitter band passes (Fig. 6b).

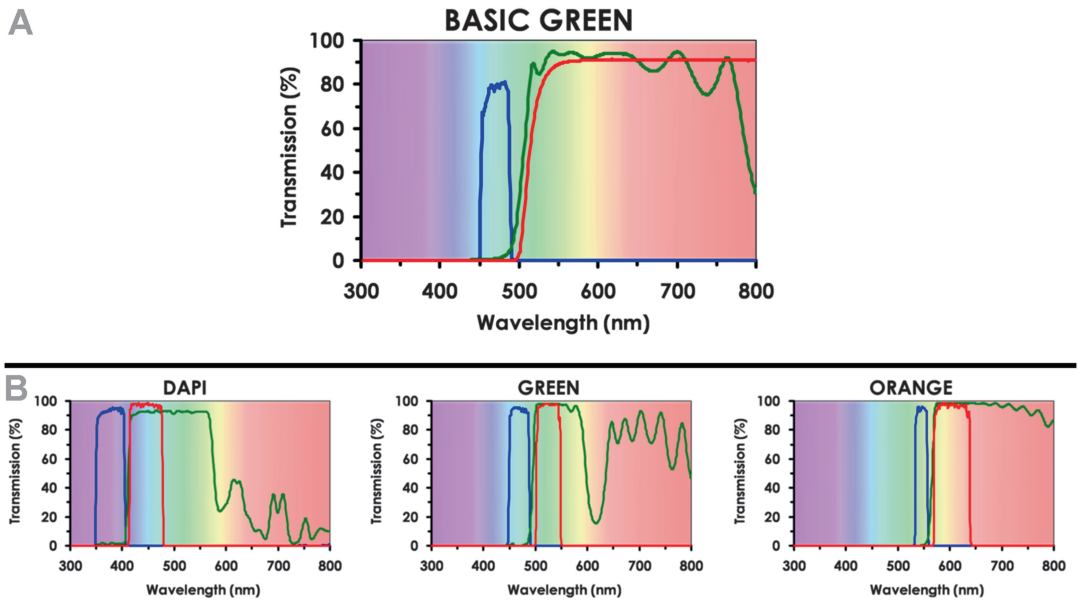

Fig. 6 (**a**) Basic green or FITC filter set with long-pass emitter. (**b**) Optimized spectra of three hard-coated filter sets used for DAPI, green, and orange dyes in FISH or CGH applications

The use of these high-efficient filter sets with >95 % transmission will reduce the exposure time in the range of 30–50 % in comparison with filter sets with around 70–80 % transmission. Additionally the pixel shift of these hard-coated filters will be very close to zero due to their perfect surface flatness (see Sect. 1.3).

2.2 Filter Sets for Multicolor FISH (mFISH)

The use of six to seven dyes in an mFISH experiment (chapters by Thomas Liehr and Nadezda Kosyakova "Multiplex FISH and Spec tral Karyotyping"; Thomas Liehr et al. "FISH Banding Tec hniques"; Thomas Liehr et al. "cenM-FISH Approaches"; Thomas Liehr et al. "Heterochromatin directed M-FISH (HCM-FISH)"; Anja Weise and Thomas Liehr "Subtelomeric and/or Subcentromeric Probe Sets"; Thomas Liehr et al. "Bar-Coding Is Back") requires very well-matched filter combinations. Even best-blocked filters cannot completely avoid spectral interferences due to the spectral overlapping of the dyes. As a rule of thumb, the spectral distance of neighbored dyes should be about 50–60 nm, e.g., the difference between Sp. Gold and Sp. Orange is about 30 nm, and between Sp. Gold and Sp. Red, the distance is about 55 nm. Sp. Gold and Sp. Orange can't be selectively separated by using filters. Sp. Gold and Sp. Red in the same sample can be detected selectively. The bleed through between these two dyes is only a few percent, if specific band-pass filters are used (Table 2). At present series of mFISH filters are available from different companies. As example we will present a complete series of hard-coated filter sets, which show maximum transmission for each filter set matched to the mFISH dyes (Fig. 7). Due to their small spectral

Table 2
Calculated spectral overlapping of the fluorophores DAPI, Sp. Aqua, Sp. Green, Sp. Gold, Sp. Orange, Sp. Red, Cy5/Far Red, Cy5.5, and Cy7 into the specific mFISH filter sets

Filter set	DAPI	Sp. Aqua	Sp. Green	Sp. Gold	Sp. Orange	Sp. Red	Cy 5/Far Red	Cy 5.5	Cy 7
DAPI	100 %	30 %	0 %						
Sp. Aqua	0 %	100 %	1 %						
Sp. Green		0 %	100 %	3 %	0 %				
Sp. Gold			2 %	100 %	49 %	1 %			
Sp. Orange			0 %	36 %	100 %	11 %			
Sp. Red				0 %	15 %	100 %	1 %		
Cy 5						12 %	100 %	53 %	1 %
Cy 5.5						0 %	53 %	100 %	6 %
Cy 7							0 %	12 %	100 %

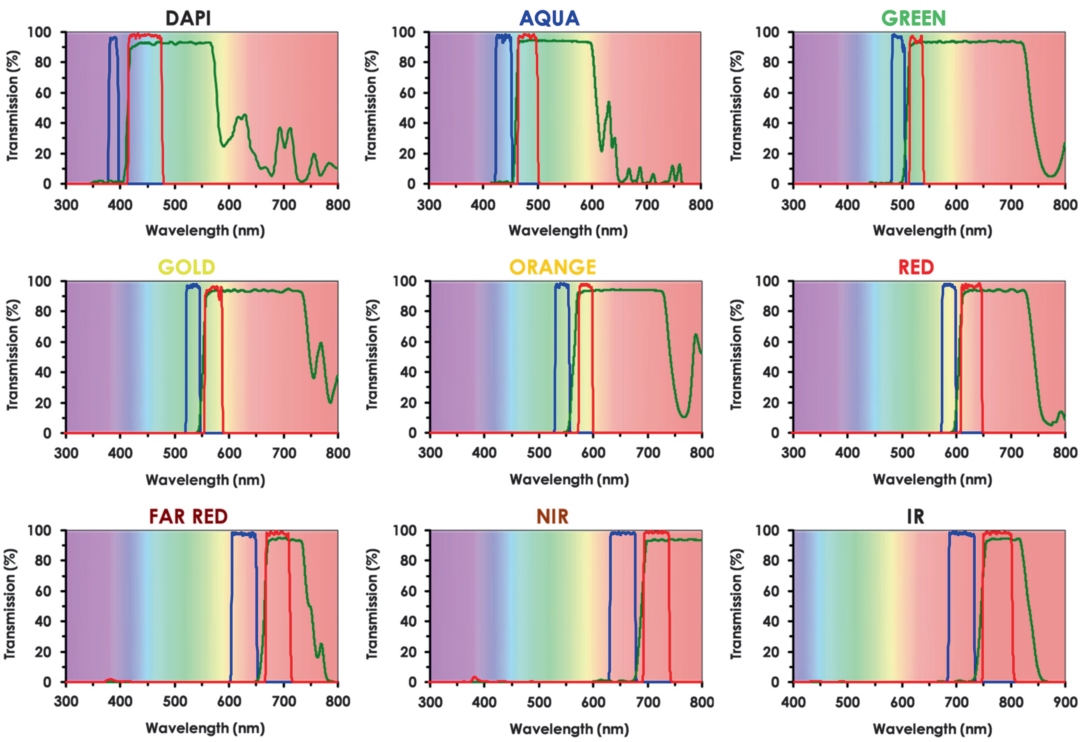

Fig. 7 Filter spectra of the series of specific narrowband hard-coated mFISH filter sets designed for mFISH dyes (list of dyes see Table 2)

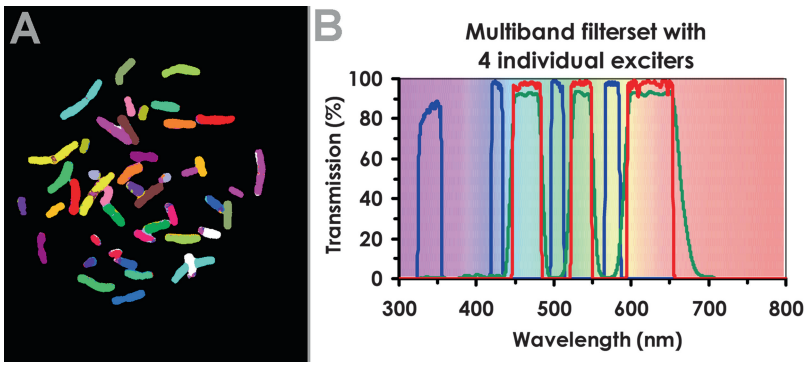

Fig. 8 (**a**) Metaphase spread labeled with DAPI, DEAC, FITC, Sp. Orange, Texas Red, and Cy5 taken with mFISH filter series, pseudo colors (MetaSystems, Altlussheim, Germany). (**b**) Spectrum of a hard-coated multiband filter set with four single-band exciters (blue lines), polychroic (green line), and multiband emitter (red line). Exciters will be mounted in separate filter wheel

bandwidth, the analysis will be mainly done with CCD cameras in combination with software programs. The theoretical overlap between the different channels is given in Table 2 [8]. Figure 8a shows the result of an mFISH experiment taken with this corresponding mFISH filter series.

A very elegant filter technique in mFISH analysis will be the use of multiband filter sets as described in Sect. 1.3 instead of single-band-pass filters. The multiband set for blue/aqua/green/orange (Fig. 8b) consists of four separate single-band exciters which must be mounted in an exciter filter wheel. The polychroic beam splitter and polychroic emitter will be mounted in the filter cube. Additionally available, precisely matched dual- or triple-band exciters allow the detection of two to four dyes simultaneously, a very helpful tool for quick visual detection of overlapping signals. Changing of exciters can be controlled by software. The background signal will be not as dark as in single-band mFISH filter sets, as long as the emitter has three band passes (like three "windows") instead of one specific. Microscope setups with a filter wheel in the emission path allow the use of specific band-pass emitters, which can be controlled as well by software. This configuration will be most flexible but affords precise controlling.

3 Filter Handling

3.1 Cleaning

Coated substrates should only be touched at the edges. Handling of exposed coatings with bare fingers has to be avoided.

3.1.1 Exciters and Emitters

Gently cleaning should be done only if necessary. Loose particles should be removed with a bulb puffer or filtered pressurized air cleaner. If necessary, the surfaces should be gently wiped using alcohol (ethanol, isopropanol, or methanol) and a lint-free towel. A new surface of the towel should be used with each wipe. Touching or wiping of A/R (antireflective) coated surfaces should be avoided. Fingerprints on the surface of the excitation filter will burn and might shorten the lifetime of the exciter.

3.1.2 Beam Splitters

Loose particles should be removed with a bulb puffer or filtered pressurized air cleaner. Touching or wiping of A/R coated surfaces should be avoided. If filters or beam splitters need a special cleaning, they should be sent back to the manufacturer. Exciters are exposed to the light source. Exciters in additional sliders or filter wheels close to a light source must be protected with appropriate heat protection filters. A heat protection filter has to be mounted in a filter wheel or in the lamp housing of the light source. The filters and beam splitters in the microscope should be checked from time to time.

3.2 Mounting

Most of the filter cubes allow mounting and demounting all filters and beaming splitters. The fixation of the filters is done with screws or special filter rings; if necessary special tools are delivered with the microscope or the filter cube. Some microscope manufacturers glue the filters into the filter cubes. In this case the filters have to be changed by specialists. To obtain the most optimal performance of a filter set, the filters and the beam splitter should be orientated in a filter cube.

Exciters and emitters are mostly labeled with arrows on the side of the filter ring. Often the arrow(s) point into the direction of propagation of the light, but this is not a general rule. Follow the instructions of the manufacturer carefully. Beam splitters (dichroics, polychroics, mirrors) have to be mounted with the coated side toward the light source (Fig. 9a). A dot, arrow, small scratch, or bevel edge on the beam splitters indicates the coated side.

If the beam splitter is not labeled, it can be illuminated with any light source. When viewing the beam splitter with the reflecting side up, only a predominantly single reflection of the light source can be seen. The thickness of the beam splitter at the far edge is not visible. When viewing the beam splitter with the reflecting side down, a double reflection of the light source occurs. The thickness of the beam splitter at the far edge is visible (Fig. 9b).

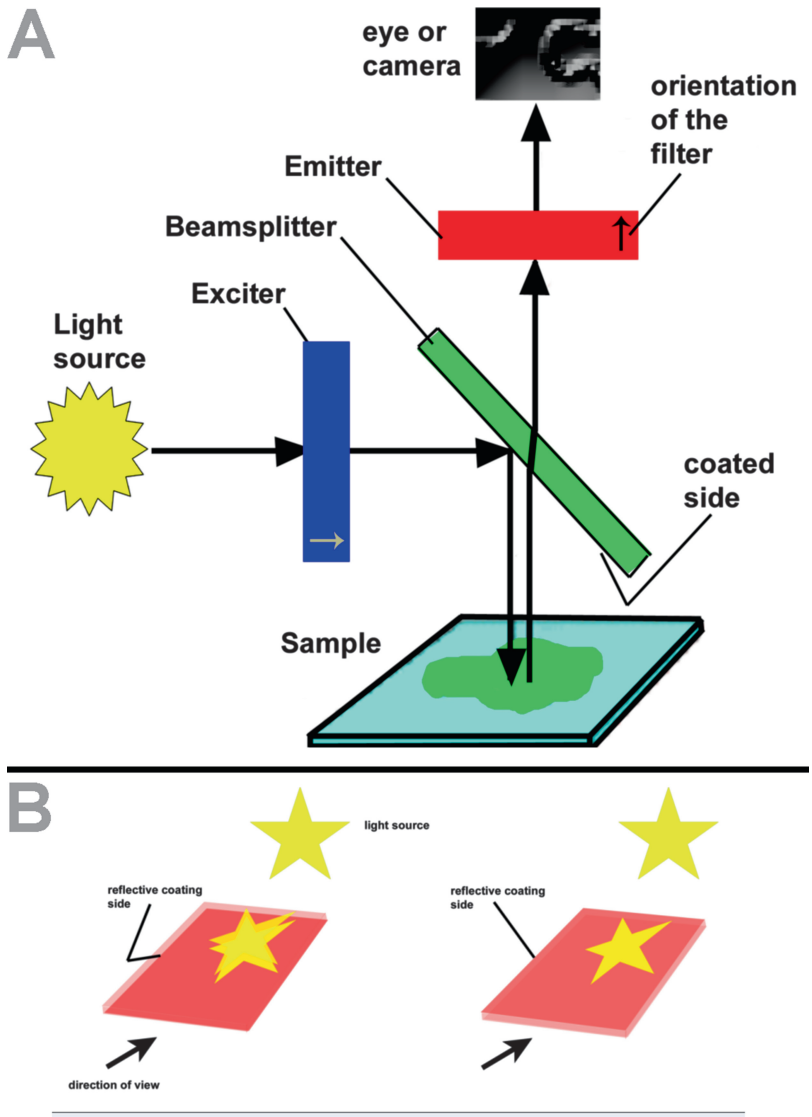

Fig. 9 (**a**) Orientation of filter sets in a filter cube. (**b**) Determination of the reflecting surface of a beam splitter

4 Troubleshooting

4.1 Uneven Illumination of the Sample

If every filter set shows the same uneven illumination, align the excitation source. If the light source is connected by a light guide, make sure that the light guide is mounted properly on both ends. If only one of the filter sets shows uneven illumination, check if the exciter is burned. You will see brown or black spots, which can't be removed by cleaning the filter. Exchange the excitation filter with a hard-coated filter. Hard-coated filters won't burn and age anymore.

4.2 A Lot of Background and Low Signal Intensity

Make sure that you are using the right filter set for the dye(s) in the sample. Maybe you are using a long-pass filter set. Use a specific band-pass filter set. If you are using a specific band-pass filter set and the described problems occur, try a new filter set. The used filter set might be aged (older than 10 years or daily used over years).

4.3 I See Nothing in My Blue, Green, Yellow, Orange, or Red Filter Set(s)

1. Turn on the lamp, open the shutter, or switch your filter wheel into the right position.
2. Make sure that the light is guided to the camera or the eyepiece of the microscope.

If only one filter set shows the problem, please check if the filters are mounted in the right way into the cube.

4.4 I Only See Very Dim Signals in All Color Channels

1. Make sure that the arc lamp didn't reach the end of life.
2. Check the intensity control of the light source, if the light source has an internal attenuation.
3. If your metal halide light source is coupled via liquid light guide to the microscope stand, check if the light guide is aged due to heat and UV of the light source. Change the liquid light guide after two to three lamp changes. This won't be an issue with LED light sources.

4.5 I See Nothing in My Dark Red, NIR, or IR Filter Set (e.g., Far Red, Cy5, Alexa 647, Cy5.5, Cy7)

1. Turn on the lamp, open the shutter, or switch your filter wheel into the right position.
2. Make sure that you have an IR sensitive camera. None of the abovementioned dyes can be seen with the eyes.
3. Remove every IR blocking glass from the light path. Check if older cameras have an IR blocking filter as protection glass in front of the CCD chip. Your camera supplier will help you.
4. If you are working with dyes like Cy5.5 or Cy7, use a LED, xenon, or metal halide light source. Mercury lamps are unsuitable for these dyes.

4.6 I See Only a Very Bright Even Illumination (White Light or a Specific Color)

1. Check if the exciter and emitter fit to each other. The labels of the filter rings often show the transmission band (e.g., 450–490, 470 ± 20, or 470/40). Compare the labeling with the provided spectral data. For further help, call your filter supplier.
2. If you are using a multiband filter set, make sure that your filter wheel is in the right position. If no exciter is in the light path, you will see scattered light of your excitation source. Be careful; bright unfiltered light can harm your eyes.

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