Filters for FISH Imaging

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Dan Osborn is the Product Marketing Manager at Omega Optical, Inc.; a thin film optical coating company specializing in the manufacture of filter solutions for applications in Basic Research and OEM components for Instrumentation. Dan spent 16 years as a researcher at Schleicher and Schuell Biosciences, later acquired by Whatman PLC. He has wide experience in nucleic acid and protein isolation, purification, and labeling schemes. He contributed to the launching of one of the earliest immobilized antibody arrays for quantitative Cytokine/Chemokine detection, as well as the development of a two-color fluorescent Hapten swap array. He came to Omega Optical in 2005 as Product Manager for the Fluorescent Microscopy Program. Since then, he has extended his role to Product Marketing Manager for Business Development and Marketing. He has refined Omega Optical's stock fluorescence filters and conducted development, testing and implementation of new products. He collaborates with field researchers, organizations, and manufacturers to ensure Omega Optical effectively supports current and future needs in fluorescence detection.

The application of in situ hybridization (ISH) has advanced from short lived, non-specific isotopic methods, to very specific, long lived, multiple color Fluorescent-ISH probe assays (FISH). Improvements in the optics, filter technology, microscopes, cameras, and data handling by software, have allowed for a cost effective FISH setup to be within reach of most researchers. The application of mFISH (multiplex-FISH), coupled to the advances in digital imaging microscopy, have vastly improved the capabilities for non-isotopic detection and analysis of multiple nucleic acid sequences in chromosomes and genes (1).

Filters and Fluorescent Imaging

In an upright microscope, the fluorescence illuminator follows an epifluorescent path (illumination from above) to the specimen. In the pathway is housed the filter blocks containing the dichroic mirror, excitation, and emission filters, which work to greatly improve the brightness and contrast of the imaged specimens, even when multiple fluorochromes are being used. Figure 1 illustrates the basic setup of the fluorescence illuminator on an upright microscope.

The principle components in the episcopic (reflected illumination) pathway consist of the light source (here depicted as a Mercury arc lamp), a series of lenses that serve to focus the light and correct for optical aberrations as the beam travels toward the filters, diaphragms which act to establish proper and even illumination of the specimen, and the filter turret, which houses the filter sets. In the diagram it can be seen schematically how the broad band excitation light from the light source is selectively filtered to transmit only the green component by the excitation filter in the turret, which is in turn reflected by the dichromatic mirror to the specimen. The red fluorescence emission is then transmitted back through the objective lens, through the mirror and is further filtered by the emission filter before visualization by eye or camera.

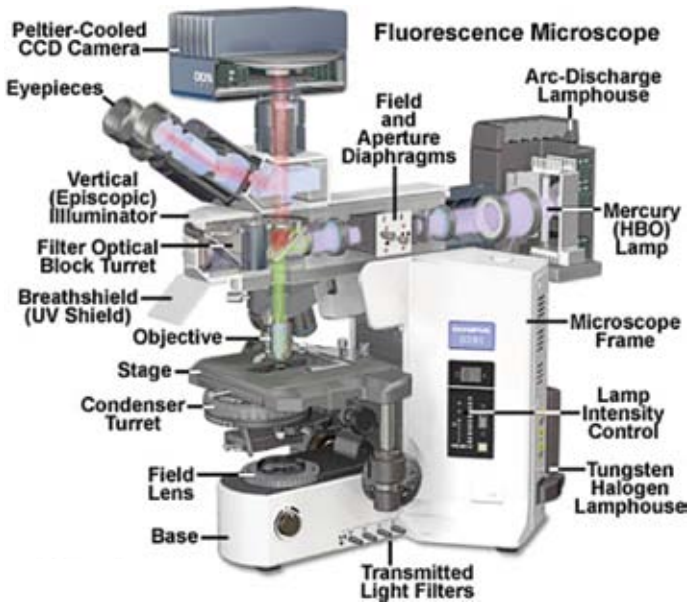


Figure 1.*

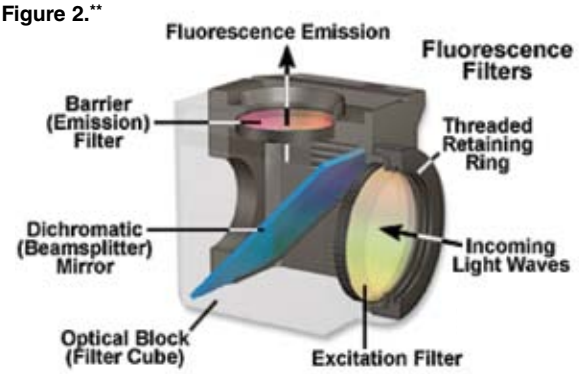


Figure 2.**

Omega XF207 Texas Red filter set:
XF1207 Exciter 580/20
XF3028 Emitter: 630/30
XF2020 Dichroic 600DRLP

An exploded view of the filter cube is shown in Figure 2. The excitation filter is shown in yellow and the emission filter in red to describe a typical band-pass Texas Red filter set.

Filter Descriptions

Band-pass filters can be described in several ways. Most common is the Center Wavelength (CWL) and Full Width Half Maximum (FWHM) nomenclature, or alternatively, by nominal Cut-on and Cut-off wavelengths. In the former, the exciter in Fig. 2 is described as a 580/20 or, a filter with nominal CWL of 580nm and a FWHM of 20nm. The half maximum value is taken at the transmission value where the filter has reached 50% of its maximum value (Fig. 3, Left). In the latter scheme, the filter would be described as having a Cut-on of 570nm and a Cut-off of 590 nm, no CWL is declared. The Cut-on describes the transition from attenuation to transmission of the filter along an axis of increasing wavelengths. The Cut-off describes the transition from transmission back to attenuation. Both values indicate the 50% point of full transmission.

Cut-on and Cut-off values are also used to describe two types of filters known as Long pass (or high pass) filters (Fig. 4) and short pass (or low pass) filters (Fig. 5).

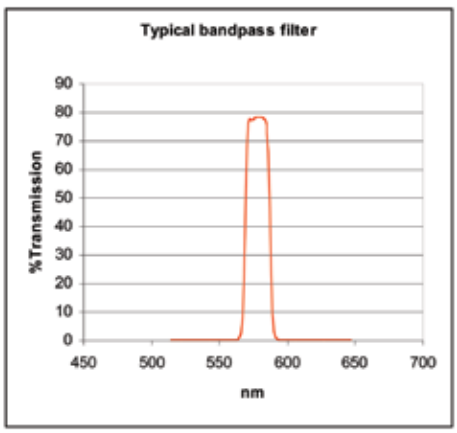


Figure 3.

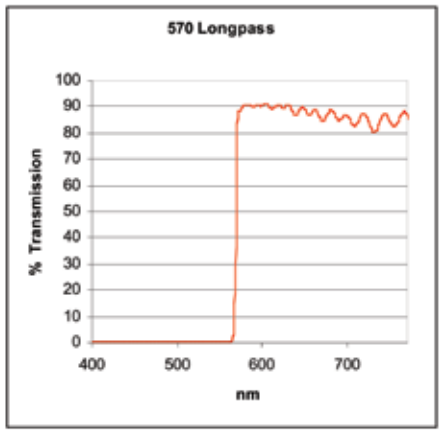


Figure 4.

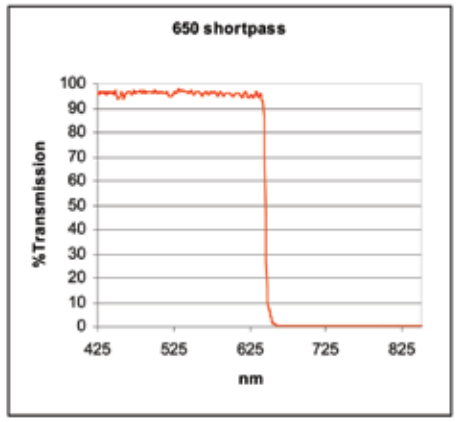


Figure 5.

> "In order to minimize the spectral bleedthrough of very closely spaced fluors in multicolor labeling schemes, specialized narrow band filter sets are needed."

A long pass filter is designed to reflect and/or absorb light in a specific spectral region, to go into transmission at the Cut-on value (here 570mn) and transmit light above this over a broad wavelength range. A short pass filter does the reverse, blocking the wavelengths of light longer than the Cut-off value for a specific distance, and transmitting the shorter wavelengths. It should be noted that these reflection and transmission zones do not continue indefinitely, but are limited by properties of the coating chemicals, coating design, and the physical properties of light.

Specialized Filters for FISH and mFISH

The imaging of multiple fluorescent probes requires special considerations towards the set up of the filter blocks in the microscope turret. One strategy is to use individual filter cubes for each probe in the specimen. This is an effective strategy for 6 color viewing (six being the standard number of filter positions in most upright research microscopes), as good spectral isolation of the different probe species can be obtained through careful filter design. This setup also reduces the potential bleaching of the probes by illuminating only one fluorescent species at a time. A potential drawback to this setup is image registration shifts caused by slight misalignments of the filters, producing a minor beam deviation that can be detected when switching between several different filter cubes. The dichroic mirror and the emission filter are the imaging elements of the filter cube and are the two components which can contribute to this effect.

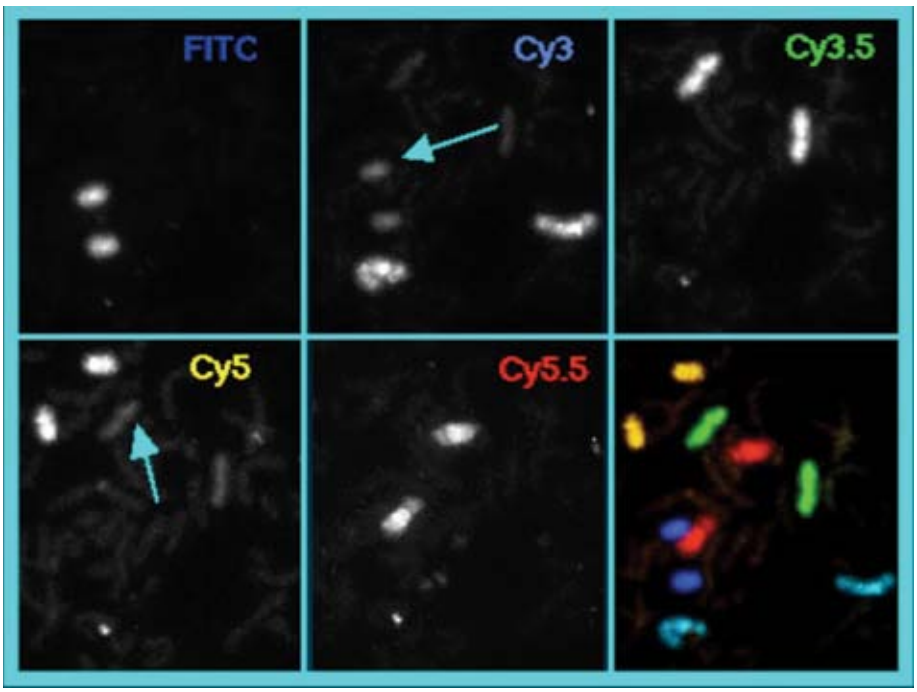


Figure 6. (Image courtesy of Octavian Henegariu, Yale University)

Another strategy is to utilize single multiband dichroic mirrors and emission filters and separate exciter filters either in an external slider or filter wheel. This will preserve the image registration and reduce mechanical vibrations, but the trade offs are a reduced brightness of the fluorescence, limitations on how many different probes can be separated, and reduced dynamic range and sensitivity due to the necessary color CCD camera.

Fluorescent microscopes typically come equipped with standard filter cubes for the common DAPI stain, FITC, TRITC, and Texas Red fluors. Standard filter sets have mostly wideband excitation and emission filters (sometimes using long pass emission filters) in order to provide maximal brightness. When employing FISH, these standard sets can work well for 2, 3 and 4 color labeling, but spectral bleedthrough can rapidly become a problem. For instance, FITC is partially visualized through the Cy3 filter, and Cy3.5 can be seen through the Cy5 filter (2).

Figure 6 depicts five different labeled chromosome pairs, the crosstalk between channels is shown by the arrows in the top middle and bottom left images. Bottom right panel is an overlaid and pseudo-colored image of the series.

In order to minimize the spectral bleedthrough of very closely spaced fluors in multicolor labeling schemes, specialized narrow band filter sets are needed. Exciter filters of 10-20nm in bandwidth and emission filters of 20-40nm provide the specificity necessary to achieve

Figure 7.

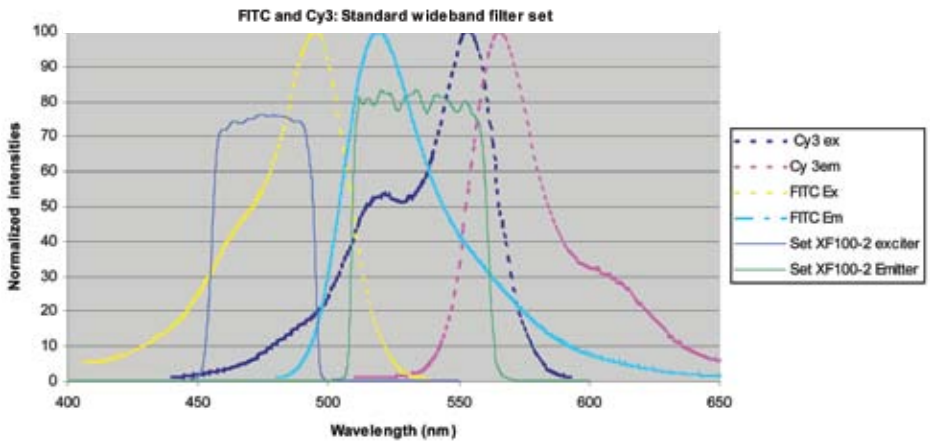


Figure 8.

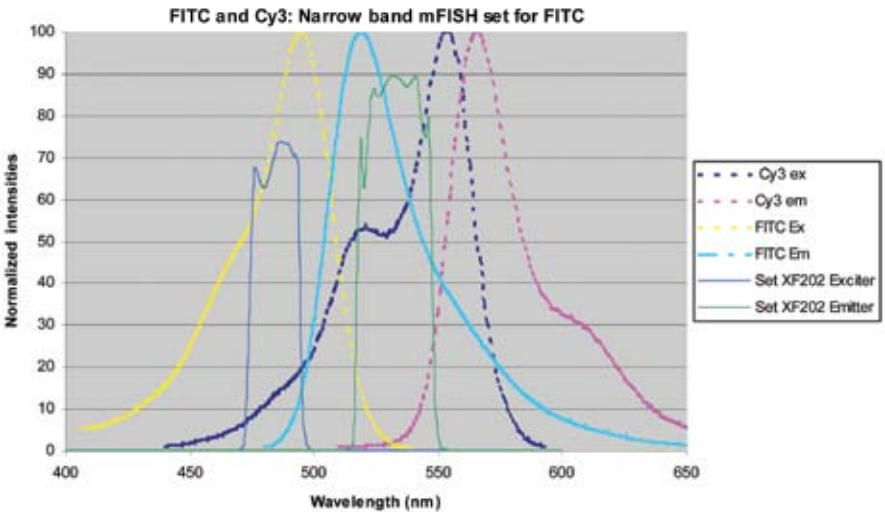
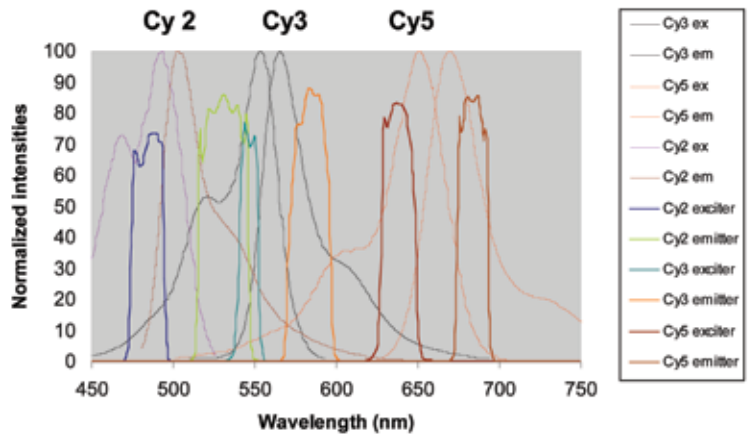


Figure 9.



the degree of sensitivity and spectral resolution required in mFISH. Figure 7 shows a typical wide band FITC filter set overlaid on the excitation and emission peaks of FITC and CY 3. Although the filters are designed for covering a substantial area under the absorption and emission curves, there is a significant overlap with both the excitation and emission curves of Cy3, thus resulting in FITC channel contamination by Cy3. A solution is seen in Figure 8, where excitation and emission bands have been narrowed to improve the spectral resolution of FITC from Cy3, especially in the emission band. By limiting the red edge of the emission filter, a reduction in the area under the emission curve of the Cy3 dye of about 4-fold is achieved.

By incorporating the design strategy of narrow band, steep-edged filters, the spectral window for adding multiple fluorescent probes widens without the cost of adding emission bleed though between fluors.

This can be seen in Figure 9 where three fluors are effectively separated within a spectral window of less than 300nm.

A fourth dye, such as Cy 3.5, could easily be incorporated in this scheme as well in the 570-620nm region, but is left off to reduce congestion.

The demands on the filters used for mFISH are such that it is necessary to provide a specific category of products which are matched together to make optimal use of the available bandwidth for each mFISH fluor.

Table 1 shows the Omega Optical series of filter sets for the more prevalent fluors used in mFISH, along with excitation and emission filter bandwidths. Note all are single fluor sets except XF231 and 232 which used single exciters for each fluor and triple band dichroic and emission filters. This setup minimizes registration shift and stage movement by requiring only that an external filter slider or wheel be moved to excite the different dyes while the multiband dichroic

Table 1.

Set Name	Fluorophores	Filters		
XF06	DAPI, AMCA	Exciter: 365/50	Dichroic: 400DCLP	Emitter: 450/65
XF201	DEAC	Exciter: 436/8	Dichroic: 455DRLP	Emitter: 480/30
XF202	FITC, Cy2	Exciter: 485/20	Dichroic: 505DRLP	Emitter: 530/30
XF203	Alexa 532	Exciter: 520/18	Dichroic: 545DRLP	Emitter: 565/20
XF204	Cy3, TRITC, Alexa 546	Exciter: 546/10	Dichroic: 555DRLP	Emitter: 580/30
XF206	Cy3.5	Exciter: 572/15	Dichroic: 590DRLP	Emitter: 620/35
XF207	Texas Red, Alexa 594	Exciter: 580/20	Dichroic: 600DRLP	Emitter: 630/30
XF208	Cy5, Alexa 647	Exciter: 640/20	Dichroic: 650DRLP	Emitter: 682/22
XF210	Cy5.5	Exciter: 665/32	Dichroic: 692DRLP	Emitter: 710/40
XF231	DAPI/ FITC/ TRITC	Single exciters for each dye can be housed in external filter wheel, triple band dichroic and emitters housed in filter holder		
XF232	DAPI/FITC/ Texas Red	Single exciters for each dye can be housed in external filter wheel, triple band dichroic and emitters housed in filter holder		

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and emission filters are kept stationary in the microscope turret.

In addition to the use of specialized filter sets for mFISH protocols, other features of the filters which have improved image quality are broad band AR (anti-reflection) coatings and polished substrates.

The AR coating can provide transmission enhancements by reducing secondary surface reflections at glass interfaces. This improvement can be realized by up to a 7% increase in filter throughput (3). AR coatings on emission filters and dichroic mirrors also reduce the "ghost image" sometimes seen in optical pathways with multiple reflective surfaces.

Polished substrates are features also found predominantly on the emission filters and dichroic. By exposing the glass substrate on which the filter is manufactured upon to a two-sided polishing procedure, the substrate achieves a high degree of "parallelism", or even thickness. This process has the effect of reducing significant beam deviation of the transmitted image, thus allowing for minimal registration shifts when rapidly switching between filter sets.

Conclusion

The techniques of FISH and mFISH used in conjunction with the resolving power and automated digital imaging capabilities of the fluorescence microscope offer a powerful combination of advantages that stand to benefit many areas of biology, from basic research to prenatal disease detection, cancer research, pathology, and cytogenetics.

In the fluorescence microscope, careful consideration of the sample and system components is necessary to specify the correct filters for probe detection. Use of multiband dichroic and emission filters in a stationary turret with single exciters in an external slider or filter wheel can give near simultaneous probe detection with no registration shift, but there are likely compromises in overall brightness, color balance difficulty, and reduced resolution of the color CCD camera.

If sensitivity, spectral resolution, and minimal photobleaching are primary concerns, single narrow band filters sets with black and white CCD camera detection is the best option. Image registration shifts are minimized in today's filters by the use of polished substrates and virtually eliminated by using filter sets made to "zero shift" specifications.

The type and number of fluorescent probes also plays a role in the optimizing of the filters. For a small number of probes with adequate spectral separation it is possible to use traditional wide band-pass filter sets. In protocols where 5 or 6 probes are being used, it is necessary to use dye-specific narrow band filter sets to reduce spectral bleedthrough.

As methodologies in FISH and mFISH on the fluorescent microscope evolve, so must the software and hardware used to unravel the information contained in the specimen. A proper combination of filters, dyes, imaging hardware, and software is desirous for obtaining the resolution and contrast necessary for accurate image capture and analysis.

References

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2. Henegariu, O. 2001. Multi-color FISH. Retrieved October 8, 2008, from Tavi's Multicolor FISH Page:** http://info.med.yale.edu/genetics/ward/tavi/fi12.html
3. Johnson, B. 2006. *Anti-Reflection Coatings.* Omega Optical Application Note.

Troubleshooting

If there is no image:

-check that fluorescence light source is on and the light path is clear. Light can usually be seen illuminating the sample unless it is below 400nm (DAPI excitation).

-image is being sent to correct port, camera or eyepiece.

-correct filter block is in place for the desired fluor.

-if desired fluor emission is > than approx. 670nm (Cy5) it is not visible by most eyes. If not visible by camera, check that there is no IR blocking filter in camera.

If image has high bleedthrough from other fluors:

-make sure filter set is correct for single dye usage, not using long pass emission filter or wide band-pass filter set.

Glossary

- *Anti-reflection coating:* An optical thin film interference coating designed to minimize reflections that occur when light travels from one medium into another.
- *Dichroic Mirror:* Selectively transmitting and/or reflecting light according to its wavelength. A long pass dichroic transmits a broad spectral range while efficiently reflecting shorter wavelengths along a different optical channel.