Get the Picture!

Easily incorporate microscopic imaging into small-scale applications

Stephan Briggs

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The power of today's high-resolution fluorescence imaging brings new capabilities to bear on everything from serum analysis to water quality inspection. Fluorescence filter cubes help scientists and engineers develop and create powerful new systems. Tight control over chromatic bands and optical wavefront, coupled with infinity-corrected optical train design, brings unprecedented interchangeability and performance to new form factors for analytical instrumentation.

Fig. 1 Incorporating fluorescence filter cubes that hold various filters into an infinity-corrected microscope design can minimize costs and development time.

The trend toward miniaturization is driving the need for custom fluorescence microscopes. Custom devices often are very expensive, but one can minimize cost and development time by incorporating a fluorescence filter cube into an infinity-corrected microscope design. Put those together with a capable detector, and one ends up with a video microscope unit that will economically and efficiently meet any application's needs.

Miniaturization drives optical needs

In fields as diverse as medicine and manufacturing, common practices are miniaturizing to smaller scales. Automobiles and telephones measure acceleration with MEMS devices. Laboratory instruments analyze microliters of biological fluids. Semiconductors squeeze more and more features into each square millimeter. The trend is likely to continue as future technologies look to create wearable sensors, implantable medical devices, and other miniature instruments.

Some of these instruments will incorporate sophisticated optics directly into their design, but even those that don't will require the use of optics to develop and validate their production methods. In production control or quality monitoring of small-scale fabrication one can't depend on visual examination, or even the few factors of magnification one can get with a traditional machine vision system. When critical features are on the microscopic scale, one's monitoring and inspection device needs to be a microscope.

On the face of it, that wouldn't appear to be a problem – there are plenty of commercially available microscopes to choose from – but off-the-shelf microscopes aren't designed specifically to solve your particular challenges. For example, a multi-wavelength confocal microscope offers enough flexibility to identify the locations of multiple fluorophores and register their positions accurately with respect to a brightfield image. But if you want a device to simply indicate the presence or absence of a specific fluorophore-labelled analyte in a nanoliter volume, an expensive confocal microscope will be far more than you need. A bare-bones off-the-shelf microscope, however, won't bring the capability to excite and detect small amounts of analyte.

The solution is to develop a semi-custom microscope tailored to your needs. The word "custom," however, triggers nightmares of long and expensive development cycles, followed by the additional expense of a production run. But one can eliminate excessive costs and minimize development time by incorporating fluorescence filter cubes in an infinity-corrected semi-custom video microscope unit (VMU).

Fluorescence filters

Every fluorescence microscope contains a set of three filters designed to separate excitation light from light emitted by fluorophores.

The first filter is the excitation light filter. This is a narrowband filter designed to let through only the wavelengths necessary to excite a specific fluorophore. For example, when labelling with indocarbocyanine (Cy3), one would ex-

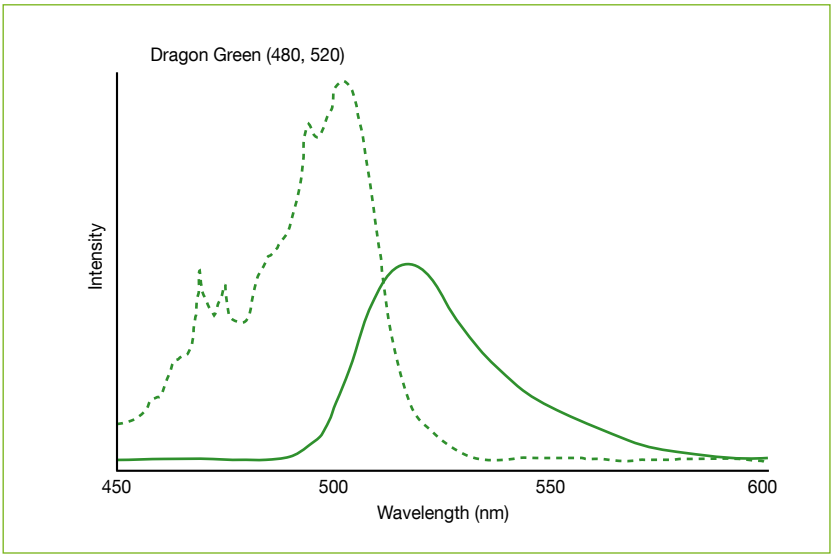

Fig. 2 High transmission, steep transition, uniform profile hard coated bandpass filters such as these are highly recommended for fluorescence applications to ensure the best signal to noise ratio for your system. (Graphic provided by Bangs Laboratories)

cite the sample with 550 nm light. Of course, white light – containing energy at 550 nm – would excite Cy3, but it also can excite other fluorophores, either those native to the sample or other artificially added fluorescent markers. Even more significant, this would also introduce a high level of background light at the emission wavelength of the dye. That is why one needs an excitation-side filter.

The second filter is a dichroic mirror. The dichroic filter serves to direct the excitation light to the sample and the returned emitted light to the detector. Typically the dichroic will be a long-pass filter, reflecting the shorter excitation light and allowing the fluorophores' longer wavelength emitted light to pass through. The excitation and emission wavelengths in many fluorophores are very close, or even overlap. To minimize background and maximize signal these filters often need a very sharp wavelength cutoff.

The third and final filter is the emission-side filter. This filter is a bandpass filter designed to maximize transmission of the desired fluorophore emission and minimize all other wavelengths. Although there are some standard filter designs partnered to specific fluorophores, the bandpass may need to be adjusted to eliminate background light from native fluorophores or other artificial fluorescent markers present in a particular application.

Filter cubes and infinity-corrected designs

Conceptually, then, the process is straightforward. Excitation light is directed through a notch filter and reflects off a dichroic surface onto a sample. Scattered and emitted light from the sample strikes the dichroic, where the shorter wavelengths reflect and the longer wavelengths pass through. The passed wavelengths strike the emission filter, which rejects all wavelengths outside the region of interest – the desired fluorophore emission signal.

Every element in an optical train must be aligned, so three separate filters require three separate alignment processes. A fluorescence filter cube contains all three necessary filters pre-aligned in one convenient single assembly. One advantage to the filter cube is the ability to rapidly prototype new configurations.

Consider, for example, the situation where one is selecting a fluorescent marker for a microfluidic water quality monitor. If one selects a GFP label then the excitation filter could be a bandpass for wavelengths between about 460 and 490 nm, the dichroic could be a 495 nm long-pass filter, and the emission-side filter could be a bandpass from about 500 to 540 nm. To evaluate the effectiveness of, for example, a 605-nm-emission fluorescent nanocrystal – a quantum dot label – one could choose a 415 to 455 nm excitation filter, a 510 nm long-pass dichroic, and an emission bandpass filter from about 600 to 615 nm. Using filter cubes, three separate tedious alignments are replaced by a simple drop-in replacement.

There is one complication, however. When optical elements are introduced into a converging or diverging beam, they modify the propagation, introducing tilt, defocus, or other aberrations.

Fig. 3 Infinity-corrected semi-custom video microscope units hold various incorporated fluorescence filter cubes.

Company

Edmund Optics

Barrington, New Jersey, USA

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Replacing one filter cube with another changes those beam propagation characteristics, meaning that an optical system that performs perfectly with one filter cube may be degraded with an alternate filter cube in place.

That problem can be avoided if the filter cube is inserted in the optical train where the beam is collimated. That is the situation in a microscope, for example, where the object plane is at the focal length of the objective. In such a design, a tube lens focusses the parallel beam created by the objective, creating an intermediate image that is magnified by the eyepiece. This is called an infinity-corrected design. The beam in the space between the objective and the tube lens is parallel to the optical axis. When one filter cube in this collimated space is replaced by another, deviations in the beam wavefront are minimized. When replacing one filter cube with another, one must be careful to use cubes with comparable wavefront error specifications. Optical elements with dissimilar wavefront errors can disrupt the imaging quality and create false negative results.

Advancing technology leads to new applications

The advantage of the infinity-corrected design isn't limited to replacing one filter cube with another. It's also possible to insert additional optical elements – as long as one is careful with the wavefront quality specifications for the additional elements. This means chemists, biologists, and industrial engineers don't need to be optical designers to be able to develop VMU designs tailored to their own applications.

Modern optical coatings can combine aggressive filter specifications with excellent surface quality and resistance to harsh environments. Filters can maintain these characteristics even when they are very small, which means it is now possible to create portable instruments with the advanced capabilities of large benchtop instruments. Even better, these advanced capabilities are available in off-the-shelf components, which reduces lead time, cost, and risk.

These capabilities also mean that many applications that could previously only be done with a complex, expensive laboratory microscope can now be performed with a semi-custom VMU. Essentially, for specific applications small VMUs can now duplicate the performance of large microscopes, with tight spectral requirements and a much smaller footprint. Image quality is also retained, as the filter cubes can be produced with tenth- or even twentieth-wave surface error.

Advancing Technology; New Capabilities

The concept of a prealigned fluorescence filter cube has an almost incontestable logic. Anyone who has spent time in a lab knows that even simple alignments must be implemented with great care. In a microscope, small misalignments can cause unacceptably large image shifts. Because fluorescence filters come in sets of three, any time one configures a system for use with a different fluorophore, three separate alignments are necessary. Filter cubes ensure the three components are aligned with respect to one another, and the mechanical reference surfaces establish fixed alignment when replacing one cube with another. Alignment is only the beginning of the story, however; the individual components must also be of consistent high quality. The dichroic filter, for example, is used both to reflect light and transmit light. That means both the reflected wavefront and transmitted wavefront must be optimized. If different dichroic filters have different wedge angles or degraded surface quality, then changing out one filter cube with another will introduce image shifts. In the past, there was no way to combine steep spectral edges with high surface quality, because the coating process itself introduced stresses that deformed the filter shape. Advances in filter fabrication technology now allow high-quality coatings with very low residual stresses. Technology has advanced so quickly that one can now consider even incorporating multi-band filter cubes that are optimized for simultaneously exciting and imaging more than one fluorophore.

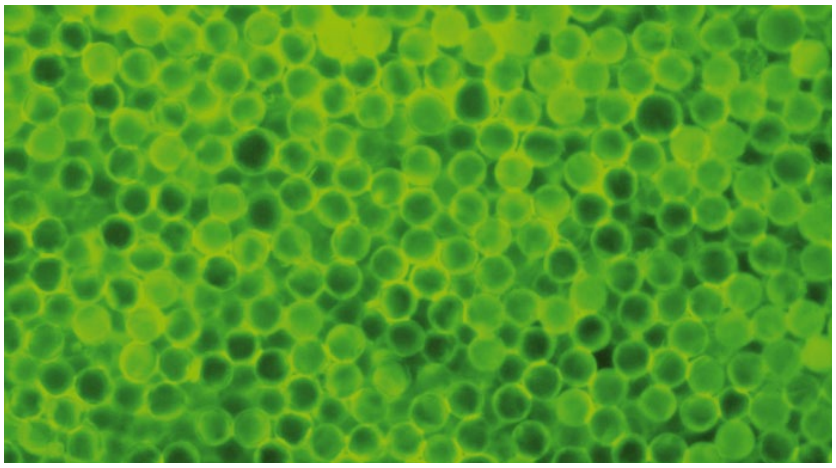

Fig. 4 Green Fluorescent Protein stained polystyrene microspheres with mean diameters of 10 microns showing a peak excitation wavelength of 480 nm, with a peak emission wavelength of 520 nm.

Design implications of filter cube integration

Consider the hypothetical case of developing a rapid assay for environmental exposure to certain biotoxins. You might develop a fluorescent bead-based assay, where beads adhere to a surface in the presence of the toxin. The sensitivity of your assay would be related to the minimum detectable fluorescence, so you'd use a fluorescence microscope in the lab to validate the chemistry of your assay. In addition, you could evaluate the performance of distinct types of fluorescent beads in your sample environment. You would select different filter sets in your lab microscope to match the different beads and select the fluorescent label that provides the clearest distinction between the desired signal and the undesirable background. Once you had refined the chemistry and selected the desired fluorescence characteristics, you would then construct an instrument to perform your assay.

You could build your custom instrument around the capabilities of your commercial fluorescence microscope, but, in addition to being expensive, that also raises issues of intellectual property ownership. You could produce a custom design but, as described earlier, the costs and risks of custom optical system development are significant. This is where you turn to the capabilities of fluorescence filter cubes in infinity-corrected microscopes.

Your goal is to duplicate the essential functions of the lab microscope in a fit and form that matches your application. You could begin with an infinity-corrected microscope objective matched to a camera and imaging lens to provide the proper magnification. Then you could incorporate a light source and an off-the-shelf fluorescence filter cube matching the set within your lab microscope. This modular approach reduces development costs and risks, yet still provides the desired form, fit, and function.

You can further optimize your design by customizing elements within the design. For example, fluorescence filter cube interchangeability gives you the opportunity to replace the standard filter set with custom filters. You can also select smaller lenses and a smaller filter cube to reduce the overall footprint of your design, yet still keep the ability to customize the optical performance. You could use an identical optical design to produce analytical instruments designed for different targets, simply by having two different versions of the filter cube. Design and testing cycles are significantly shorter, and the use of off-the-shelf components reduces lead time.

The fluorescence filter cube design approach is well suited for use in field-portable units as well. Because the infinity-corrected design allows filter sets to be interchanged, a modular design can allow the same instrument to be modified in the field for different uses.

The value of filter cubes in infinity-corrected systems

Incorporating fluorescence filter cubes in infinity-corrected video microscope unit designs combines the power of custom optical design size with the ease of purchasing off-the-shelf. Optical filters themselves offer high wavefront quality, excellent resistance to environmental exposure, and outstanding spectral characteristics. Those traits have opened up a new range of capabilities in VMUs. The flexibility of filter cubes and the ease of incorporating them in infinity-corrected systems are enabling new compact instruments for cell sorting, automated inspection, high contrast imaging, and other applications yet to come.

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Author

Stephan Briggs is a Biomedical Engineer at Edmund Optics' Barrington, NJ, USA office. He is responsible for researching innovative optical techniques in microscopy and medical imaging systems to noninvasively diagnose and treat.

Edmund Optics GmbH, Zur Giesserei 8, 76227 Karlsruhe, Germany, Tel: +49 721 6273730, E-mail: info@edmundoptics.de