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Optical Filters for Laser-based Fluorescence Microscopes

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Introduction

Due to many desirable properties such as high brightness, stability, longevity, and narrow spectral bandwidth, lasers have been advantageously replacing conventional broadband light sources for fluorescence imaging applications. Not only have these features of lasers allowed for higher-sensitivity visualization and enhanced throughput in imaging applications, but also several unique properties of lasers—including narrow beam divergence, high degree of spatial and temporal coherence, and well-defined polarization properties—have spawned new fluorescence imaging techniques. However, the advent of lasers as fluorescence light sources imposes new constraints on imaging systems and their components. For example, optical filters used in laser-based imaging systems such as confocal and Total Internal Reflection Fluorescence (TIRF) microscopes have specific requirements that are unique compared to those filters used in broadband light source based instruments.

Optical Filters Optimized for Lasers

Various types of powerful, efficient, and cost-effective lasers have evolved during the last four decades. Lasers tend to be classified by the gain medium and pumping scheme. Until recently the most popular lasers for fluorescence imaging have been gas lasers, such as Ar-ion and Kr-ion lasers with popular lines at 488, 568, and 647 nm. In the last several years solid-state lasers have been replacing gas lasers due to substantially better (wall-plug) efficiency (leading to much lower heat generation and simplicity of laboratory setup), smaller size, and lower cost. Popular laser types include semiconductor diode lasers (especially at 405 and 635 nm), optically pumped semiconductor lasers (including a widely used version at 488 nm), and frequency-doubled diode-pumped solid-state (DPSS) lasers (such as the yellow 561 nm laser and the newer 515 and 594 nm lasers). Table 1 summarizes the most popular lasers used for fluorescence imaging applications.

Excitation Filters: Despite varying opinions, optical source clean-up filters (excitation filters) are important for laser sources to block the unwanted light at wavelengths away from the actual laser line, including spontaneous emission observed in solid-state lasers and the plasma lines of gas lasers. Not only should these filters provide deep blocking of typically more than optical density (OD) 6 of the unwanted light, but they should also provide exceptional transmission of at least 95% at the laser lines (see Figures 1 & 3). Additionally, these filters should be durable enough to withstand the high intensity of laser beams. Unlike the traditional soft-coated

fluorescence filters used for decades, newer hard-coated thin-film filters made with ion-beam sputtering have high laser damage threshold (LDT) ratings. Semrock's laser filters typically have LDT ratings of 1 Joule/cm² or better for use with intense pulsed lasers. For continuous wave (cw) lasers, this LDT rating corresponds to about 10 to 100 kW/cm². High optical durability, combined with the robust environmental reliability of hard-coated filters—which are virtually impervious to thermal and humidity induced degradation—eliminates the need to ever replace the filters for most fluorescence microscopy applications.

Table 1: Lasers for popular fluorophores. “DPSS” = diode-pumped solid-state laser. “OPS” = optically pumped semiconductor laser. “Doubled” = frequency doubled via a nonlinear optical crystal.

Laser Line	Laser Type	Popular Fluorophores
~ 405 nm	Diode	DAPI, Hoechst, Alexa Fluor 405™, BFP
~ 440 nm	Diode	CFP
473 nm	Doubled DPSS	GFP, FITC, Alexa Fluor 488™
488.0 nm	Ar-ion gas	
~ 488 nm	Doubled OPS	YFP, Rhodamine
514.5 nm	Ar-ion gas	
515.0 nm	Doubled DPSS	TRITC, Cy3™, RFP
561.4 nm	Doubled DPSS	
568.2 nm	Kr-ion gas	Texas Red, mCherry (mRFP)
593.5 nm	Doubled DPSS	
632.8 nm	HeNe gas	Cy5™, Alexa Fluor 647™
~ 635 nm	Diode	
647.1 nm	Kr-ion gas	

Excitation filters for laser applications also have unique wavelength requirements. Some lasers, like gas lasers and DPSS lasers, have very precise and narrow laser lines. The ideal excitation filter for a particular laser line is a narrowband laser-line filter (typical bandwidth < 0.4% of the laser wavelength), keyed to the precise location of the laser line. Semrock's MaxLine® laser-line clean-up filters are ideal for this application. However, such a filter is not a good match for systems that might use multiple lasers with similar wavelengths (such as 473

nm and 488 nm for exciting GFP, or a 568 nm Kr-ion laser that might be upgraded to a 561 nm DPSS laser in the future), nor for systems that use semiconductor lasers. The spectral output from diode and optically pumped semiconductor lasers can vary appreciably from laser to laser, with temperature, and as the lasers age. Therefore for most laser microscopy systems broader excitation filters that appear similar to those used for broadband light source (e.g., arc lamp) microscopy systems are a good solution. For example, the excitation filter shown in Figure 1 is designed to be used with both 375 and 405 nm lasers, with the long-wavelength edge taking into account a ± 5 nm uncertainty in the wavelength of the 405 nm laser. These excitation filters are not identical to broadband light source filters, however. In addition to edge positions of laser filters being precisely keyed to the associated laser wavelengths, edge steepness as well as ripples in the passband are other important considerations. Whereas low ripple ensures high transmission at specific laser lines or over time as a semiconductor laser wavelength drifts, steep edges provide high optical noise discrimination.

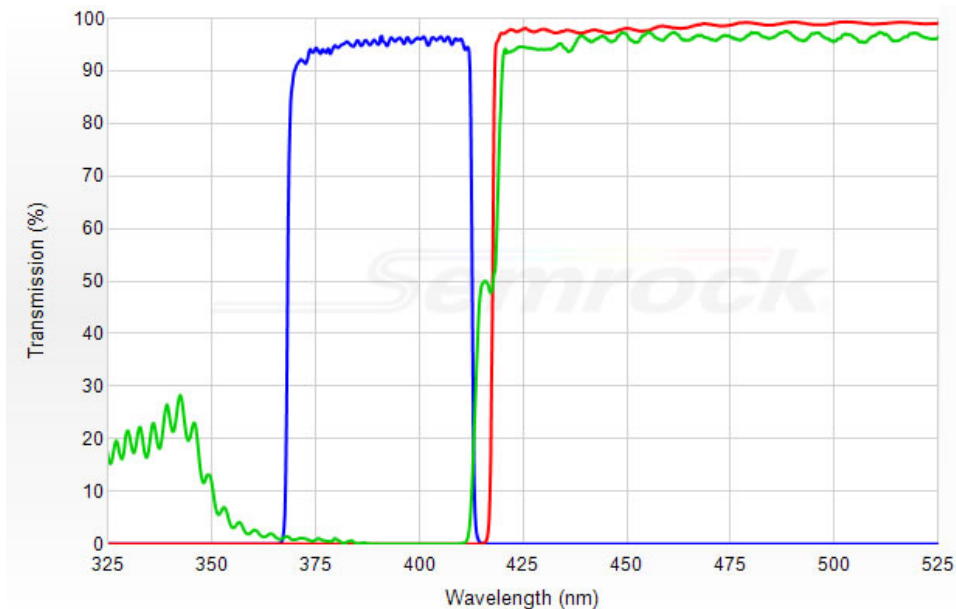


Figure 1: Measured spectral performance of a typical laser fluorescence filter set; blue line – **exciter**; green line – **dichroic**; red line – **emitter**.

Emission Filters: A typical Semrock emission filter provides high blocking ($> OD 6$) at all possible laser lines that might be used with the filter set, thus ensuring the darkest background signal level, while at the same time providing $> 97\%$ average transmission of the emission signal. It should be noted that not all emission filters for broadband light sources provide sufficient blocking at laser lines and therefore they can lead to an appreciable compromise in imaging contrast. As with an excitation filter, the edge wavelength of an emission filter should

be precisely keyed to the associated laser lines, and the edge steepness of the short-wavelength edge is particularly critical. Additional considerations for emission filters include the use of high optical quality glass for the substrate that exhibits low autofluorescence, excellent homogeneity, and low wedge angle for minimal beam deviation that can lead to pixel shift when exchanging filters.

Dichroic beamsplitters: Dichroics for laser applications should not only be made such that their reflection and transmission bands are compatible with the excitation and emission filters, but they also need to be coated with antireflection coatings in order to maximize transmission of the emission signal and eliminate coherent interference artifacts. Semrock laser dichroics are guaranteed to have > 98% transmission for s-polarization and > 94% transmission for average polarization at the laser wavelengths, as well as > 93% average transmission and very low ripple over extremely wide passbands—out to 900 and even 1200 nm. Dichroics should also have similar LDT ratings to those of the excitation filters and should have low ripple in the reflection bands to minimize variation of the excitation intensity.

Since the dichroic beamsplitter is directly exposed to the powerful excitation beam, even weak autofluorescence from the filter will contaminate the emission signal. Therefore, a substrate with ultra-low autofluorescence, such as fused silica, should be used. Note that since the excitation light and the emission signal intensity levels differ by many orders of magnitude (typically a factor of 10^6) the requirement for the emission filter autofluorescence is not as stringent as for the dichroic beamsplitter. Nevertheless, light intensity levels on an emission filter in a TIRF microscope can be substantially higher than in a typical epifluorescence widefield fluorescence microscope, since the laser beam in a TIRF system is totally reflected off of the sample slide and redirected back down the emission path. Thus autofluorescence of emission filters should be considered more carefully in laser systems than in broadband systems.

The dichroic beamsplitter can have a significant impact on the image quality in certain applications, especially if the flatness (or curvature) of the dichroics is not suitable. Even though transmitted wavefront error (TWE) is not significantly affected by substrate curvature, the reflected wavefront error (RWE) can significantly compromise imaging quality. For example, sample illumination may deteriorate in a TIRF microscope when a non-flat dichroic is placed in the excitation light path [1]. Similarly, owing to the inherent bending stress introduced by hard coatings, aberrations may be introduced in an imaging beam that is reflected off a dichroic [2]. Therefore dichroics need to be made sufficiently flat for certain applications. For most laser microscope applications, the dichroic should be flat enough such that there is no noticeable shift in the focal spot of the illumination laser beam, where focal shift is typically defined by the

Rayleigh range [1, 2]. Similarly, a sufficient criterion for an imaging beam (i.e., focused onto a detector array such as a CCD) reflected off a dichroic, is that the diffraction-limited spot size should not change appreciably due to reflection off of the beamsplitter.

Optical filters working together as a set: Table 2 includes a summary of the critical characteristics of filters for laser systems. Overall, it is desirable that the optical filters should be able to achieve high blocking as well as high transmission of specific wavelengths of light without compromising the diffraction-limited image quality. This simple set of requirements not only influences the design of an individual filter but of the system of filters that are used in combination. Therefore, the designs of the excitation and emission filters as well as that of the dichroic beamsplitter should be complimentary to each other to obtain the highest fidelity fluorescence visualization.

Table 2: Summary of critical characteristics of optical filters specifically for laser imaging systems.

Critical Filter Characteristics			System Benefits
Exciter	Emitter	Dichroic	
Filter wavelengths keyed to one or more lasers and the associated bandwidth of the lasers			Maximize throughput, sensitivity, and contrast
Edge steepness			Maximize throughput and sensitivity for high speed and weak signal detection
Transmission in the filter passband			Maximize throughput and sensitivity for high speed and weak signal detection
		Flatness	Optimize sample illumination and minimize image aberrations
	Blocking at laser line(s)		Eliminate stray excitation light for darkest background signal level
	Substrate wedge		Minimize pixel shift when exchanging filters
		Anti-reflection coating	Maximize throughput and eliminate coherent interference artifacts
	Autofluorescence of the substrate glass		Eliminate contamination from undesired fluorescence for darkest background signal level
Ripple in the passband		Ripple in the reflection band	Minimize variation of excitation intensity
Laser Damage Threshold (LDT)		Laser Damage Threshold (LDT)	Eliminate need to replace filters Damaged by high laser intensity

For example, edge steepness of the excitation and emission filters is coupled. High edge steepness significantly affects utilization of the limited bandwidth of light. Therefore, the separation of the excitation and emission filters of laser sets should be exceptionally small. This separation is typically less than 1.5% of the longest laser wavelength in Semrock laser sets. At the same time, the cross-over edge location of the excitation and emission filters should have sufficient blocking (> OD 6) to make sure that the excitation light does not leak into the emission channel.

Image registration is also affected by the combined performance of the dichroic beamsplitter and the emission filter. It is important to use exceptionally flat glass substrates to minimize any wedge angles of the substrates (prior to applying thin-film coatings) as well as the curvature of the filters owing to coating stress. All-single-substrate bandpass filter construction, for fluorescence applications, makes it very straightforward to minimize the wedge (for example, several arc seconds for laser emitters and dichroics) when manufacturing the substrate. Semrock laser filter sets inherently provide excellent image registration performance—when interchanging these sets with one another, no appreciable pixel shift is observed. And images obtained with the laser filter sets exhibit excellent image registration not only with one another, but also with images obtained when no fluorescence filter cube is present (e.g., for differential interference contrast (DIC) or other brightfield modes).

Demanding applications such as imaging of single molecules, for which lasers are ideally suited as the source, may impose unprecedented constraints on the blocking of laser beams in the emission channel while maximizing the collection of every possible photon from the fluorophores. In such situations, conventional bandpass emission filters may be replaced by long-wave-pass filters (Fig. 1). Long-wave-pass emission filters also allow capture of maximum signal from fluorophores that have widely separated absorption and emission spectra. Instead of the emission filter, some researchers choose to use only a notch filter keyed to the specific laser line, as these filters provide maximum transmission on both sides of the laser wavelength. In our observation, some demanding applications (especially TIRF systems) even benefit from using a second emission filter or a notch filter in conjunction with all the filters of a laser set. The main purpose of the second filter, which should be physically separated from the first emission filter, is to ensure that higher-angle scattered excitation light does not make it through the entire imaging path to the detector [1].

Use of Multiple Lasers Simultaneously

Many newer imaging methods are based on the implementation of multiple-laser imaging systems: two-, three-, and even four-color systems are very common. Samples are

labeled with multiple fluorophores that need to be visualized distinctly under specific conditions. Thus it is necessary to combine (multiplex, or “MUX” for short) or demultiplex multiple laser beams. Dichroic beam combiners/splitters for this application have unique requirements relative to those used for imaging only. LaserMUX™ beam combiners from Semrock (Fig. 2) provide high transmission (>95%) combined with excellent reflection (> 98%) of the important laser lines, thereby minimizing loss.

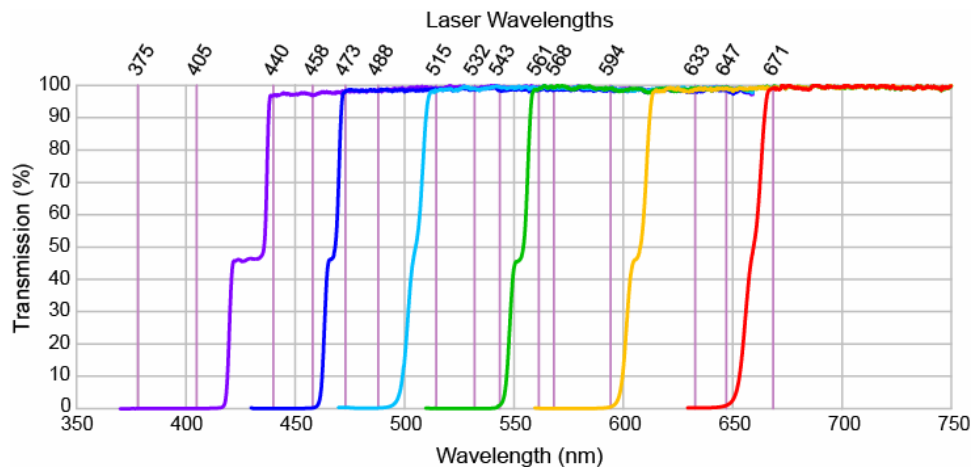
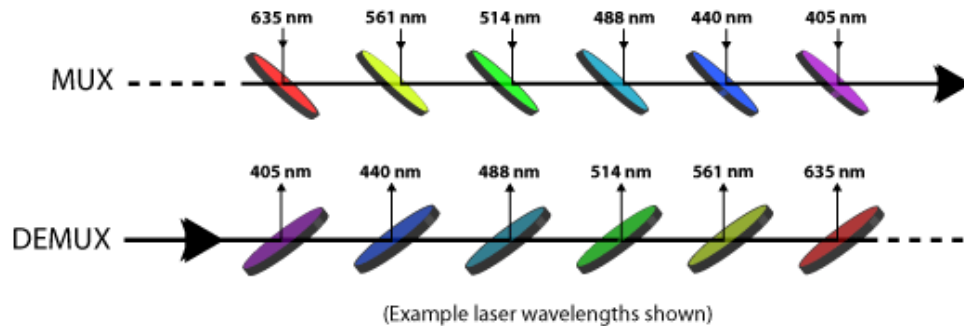


Figure 2: Combining laser beams for multicolor imaging. TOP: diagram that illustrates how LaserMUX™ dichroic beamsplitters are used for combining (multiplexing) or separating (demultiplexing) laser beams. BOTTOM: typical measured spectra of LaserMUX filters.

Excitation and emission filters and dichroic beamsplitters for simultaneous multiple-laser imaging are particularly challenging. The bandpass filters must have multiple transmission and blocking bands (two, three, and even four), while maintaining all of the characteristics of laser filters described above, such as high transmission and blocking, high edge steepness, and wavelengths precisely keyed to the right laser lines. Dichroics with multiple edges are required to selectively segregate the excitation light for different fluorophores from their emission signals. The ability to reliably manufacture multiedge filters, each edge designed with high steepness,

allows for excellent signal-to-noise ratio for multiple fluorophores, while minimizing crosstalk (Fig. 3).

An example of a multiple-laser system capable of several different types of imaging applications is shown in Figure 4. Different types of optical filters are highlighted. In this example three lasers are combined, and the system demonstrates the possibility of confocal scanning with both descanned and non-descanned detection paths, each of which has simultaneous multiple-color detection. Alternatively, without the scanning function, it may be used with a TIRF-compatible objective to perform TIRF imaging.

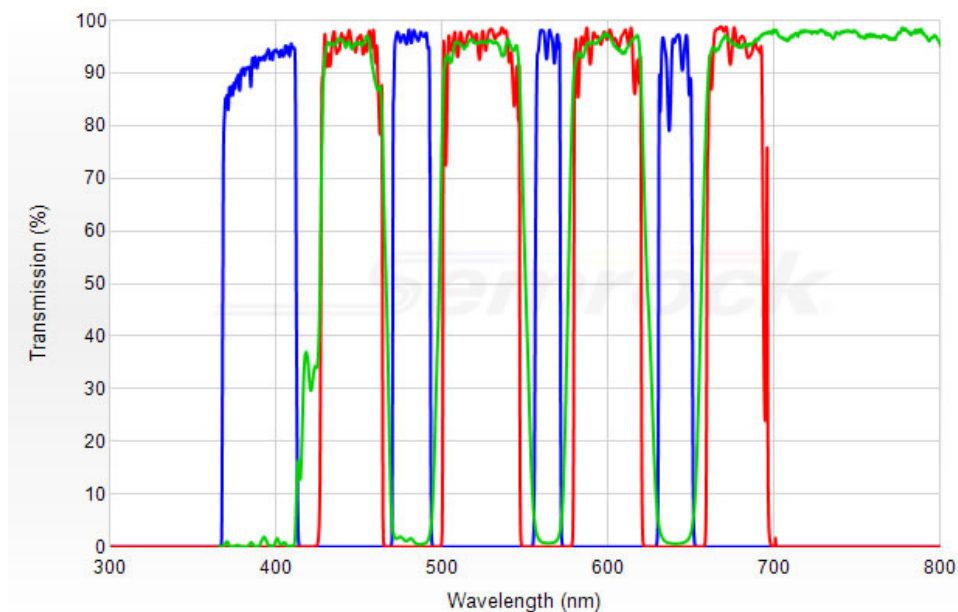


Figure 3: Semrock laser quad-band set that is compatible with 375 nm, 405 nm, 473 nm, 488 nm, 491 nm, 559 nm, 561 nm, 568 nm, 633 nm, 635 nm and 647 nm lasers; blue line – **exciter**; green line – **dichroic**; red line – **emitter**.

Concluding remarks

Optical filters play a vital role in obtaining maximum performance from complex, expensive, laser-based microscopes and it only makes sense to invest in optical filters that match the performance of the imaging system. What about the future of laser-based imaging systems? In order to gain a better insight into the mechanisms of cellular and sub-cellular interactions, complex imaging protocols are emerging. Super-resolution imaging techniques that are at the forefront of technological advances typically utilize lasers for illumination [3]. These techniques have enhanced the resolution of imaging well below the diffraction limit of a conventional microscope (limited to hundreds of nanometers resolution). Several fluorescence imaging techniques, such as STED, PALM, and STORM, to name a few, have demonstrated

different approaches for the visualization of fluorescently labeled samples with tens of nanometers of resolution. Such imaging techniques are based upon the premise of being able to accurately “localize” individual fluorophore molecules. Since the accuracy of localization of a given fluorophore increases dramatically with the number of photons acquired from a given fluorophore molecule [4], highly efficient optical filters play an increasingly important role in such cutting edge applications.

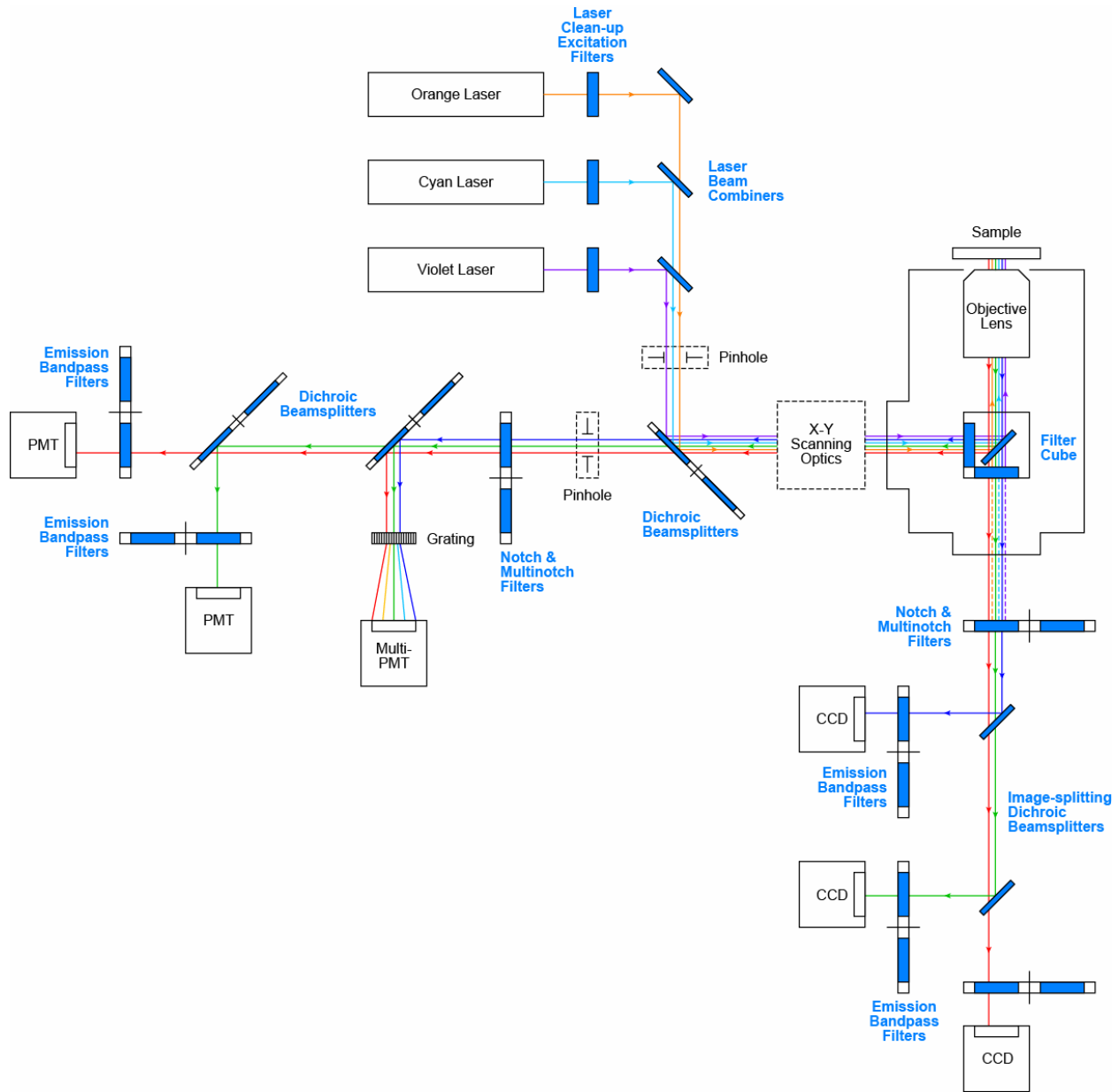


Figure 4: An example of a complex multiple-laser imaging system. Laser specific optical filters are highlighted in blue.

References

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