# Pixel shift in fluorescence microscopy

## 1. Introduction

Multicolor imaging in fluorescence microscopy is typically performed by sequentially acquiring images of different colors. An overlay of these images is used to study the relative spatial distribution of various types of cellular components. However, in order to ensure that such a composite image is a true representation of the biological phenomena under investigation, it is important to understand imaging artifacts such as “pixel shift” error in multicolor fluorescence imaging.

## 2. Meaning of “pixel shift”

Imagine a small cellular organelle or vesicle that has been labeled with three different fluorophores – DAPI, FITC and Texas Red. Assuming that the size of this vesicle is very small (i.e., a diffraction-limited imaging volume), it is fair to assume that the signal corresponding to all the three fluorophores comes from the same spot in the sample. Therefore, it is expected that the images corresponding to all the three fluorophores should overlap at the same location on the CCD camera. However, this is not always true. When the images of different colors do not overlap (to within the accuracy of a single pixel on the CCD) then there is a “pixel shift” between the different images (see Fig. 1).

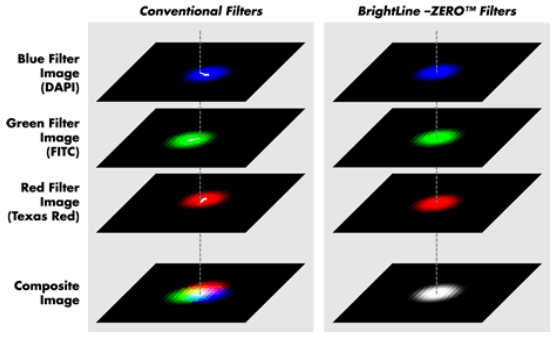


**Figure 1:** The phenomenon of pixel shift. When imaging a multicolored spot, for example a multiply labeled fluorescent microsphere with three separate filters, one corresponding to each color of fluorophore, pixel shift (left) causes the various colored spots to be out of alignment in the merged image at the bottom; whereas with a “zero pixel shift” filter set, spots are perfectly aligned (right). It is assumed here that all the other optical elements such as lenses in the imaging path are free of chromatic aberrations.

Unless the optical filters in the emission light path of a fluorescence microscope are specifically designed to eliminate pixel shift, imaging aberrations associated with pixel shift can lead to erroneous spatial interpretation of biological data.

## 3. Implications of pixel shift in biology

Colocalization analysis using fluorescence microscopy is a popular application that is sensitive to pixel shift. In this analysis it is desired to know whether two different proteins, for example, each labeled with a fluorophore of different color, interact with each other. Colocalization (appearing at the same spot) of the images of these two proteins implies their interaction and the lack of colocalization suggests that

the two proteins do not interact with one another. Such studies not only provide insight into the functioning of the cells but they are also essential tools in disease characterization and drug discovery and development.

If optical filters introduce pixel shift between images of different-colored fluorophores, colocalization analysis of such imaging data may not be reliable. Ideally, it is expected that optical filters should preserve the relative spatial information corresponding to different colors in the sample and this information should be reproduced in the sequentially acquired images (corresponding to different-colored fluorophores) on a camera. Such filters enable not only accurate colocalization studies, but they are crucial for most other multicolor imaging applications as well.

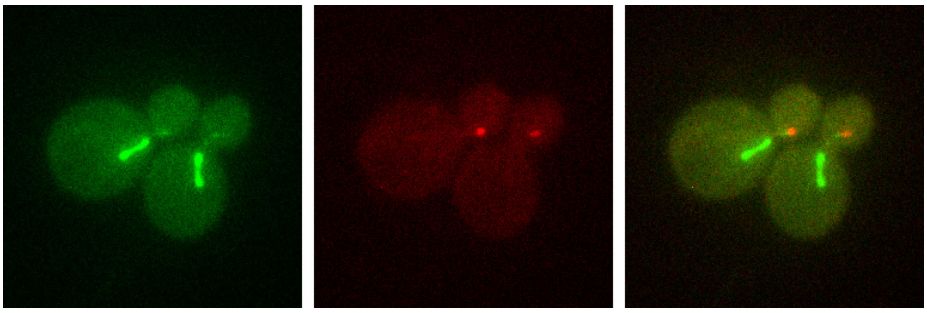

**Figure 2:** "Zero pixel shift" enables accurate interpretation of biological data. In this example tubulin was labeled with CFP and karyogamy protein was labeled with YFP in yeast cells, and the images of the two fluorophores were acquired using "zero pixel shift" filter sets mounted in a Carl Zeiss microscope equipped with a 100x, 1.45 NA objective. There is almost no pixel shift between CFP (green) and YFP (red) images, as evidenced by the fact that karyogamy protein clearly appears at the very tip of the microtubule in the merged image (c). Images courtesy of Mohan Gupta and David Pellman at Dana-Farber Cancer Institute and Harvard Medical School in Boston.

Here is another example in which minimization of pixel shift is critical in multicolor imaging. In this application, researchers study microtubule interactions with chromosomes and with the cell cortex to understand how cell signals regulate chromosome segregation and polarized morphogenesis (which relates to asymmetric cell shape and orientation). Karyogamy protein is required for correct positioning of the mitotic spindle and for orienting cytoplasmic microtubules, and it localizes at the tip of the microtubules in certain situations.

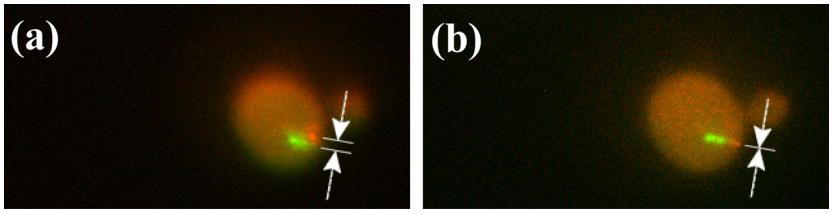


**Figure 3:** Pixel shift can lead to a wrong interpretation of the imaging data. Imaging conditions are the same as in Fig. 2. However, the image on the left is acquired with filter sets that are not corrected for pixel shift, whereas when the same sample is imaged with filter sets with "zero pixel shift" performance, karyogamy protein does appear at the tip of the microtubule. Images courtesy of Mohan Gupta and David Pellman at Dana-Farber Cancer Institute and Harvard Medical School in Boston.

As an example, high-resolution imaging (using a 100x, 1.45 NA objective) was performed on yeast cells in order to visualize the colocalization of karyogamy protein at the tip of an individual microtubule. Fig. 2 shows imaging results utilizing a filter set corrected for pixel shift error. In this experiment microtubules were labeled with CFP and karyogamy protein was labeled with YFP. By virtue of accurate pixel registration of both the colors karyogamy protein appears at the tip of the microtubule (Figs. 2 and 3b). Images acquired with filter sets that are not corrected for pixel shift (Fig. 3a) can produce erroneous interpretation of the biological function.

Correcting for pixel shift error in automated quantitative analysis of imaging data is even more critical because software algorithms may not be able to detect pixel shift error (as readily as a human eye can) unless the analysis algorithms are specifically designed to account for pixel shift error. Significant deviation can be observed from actual analysis if the regions of interest are fairly small.

## 4. Reasons for pixel shift

Typically optical filters are the only component of an imaging system that changes when creating a sequential, composite image (see Figs. 4 and 5). Assuming the microscope system is stable and well-isolated from vibrations (such as on a “floating table”), then the imperfections in the filters are the primary reason the image associated with one fluorophore shifts relative to that of another fluorophore. The major imperfection in optical filters which causes pixel shift is beam deviation created by a nonzero wedge angle (nonparallelism) of either the dichroic beamsplitter and/or the emission filter, since both of these filters are in the imaging path (see Fig. 4). A variation in the parallelism of filters in sets for different colors causes the emission beams of different colors to be deflected by different amounts, thereby producing a pixel shift between images of different colors.

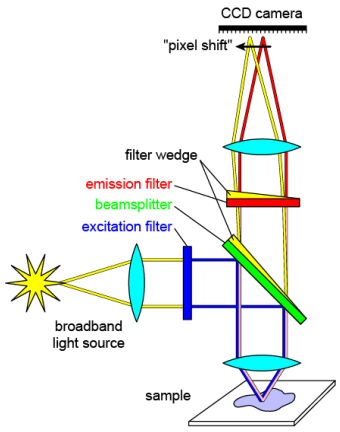


**Figure 4:** In an epifluorescence microscope, a wedge angle on the dichroic or emitter causes a beam deviation (gold path) that results in pixel shift. The wedge and beam deviation angles are exaggerated for illustration.

As alluded to above, pixel shift can also be caused by imperfections in the microscope itself. For example, vibrations associated with the movement of the filter turret can produce pixel shift either by

virtue of movement of the sample and/or the camera. Generally inverted microscopes are much more stable and therefore immune to such effects than upright microscopes. However, in either case a good vibration-isolated laboratory table is recommended when minimization of pixel shift is critical.

In opposition to the widely accepted notion, the thickness of the dichroic beamsplitter should not introduce pixel shift in an ideal microscope using infinity-corrected objectives and a tube lens. This is because the beam is passing through the filters is collimated, and thus the beam emerging from the dichroic, although slightly offset laterally by an amount proportional to the thickness of the dichroic, is parallel to the incident beam. According to first-order optics (a good approximation in this case), lateral offset in the beam path does not create appreciable pixel shift. If the light transmitted through the angled dichroic is converging (or diverging), the dichroic will cause a significant shift of the image on the camera. A variation in the angle of the dichroic (i.e., not perfectly 45°) also does not cause pixel shift. However, it should be noted that in addition to nonparallelism, there are other imperfections in the dichroic and its mounting that can cause pixel shift, such as bending of the dichroic substrate. For a detailed discussion on the optical physics of pixel shift in a fluorescence microscope the reader is referred to our upcoming white paper on this topic [1].

## 5. Correcting for pixel shift

The term “pixel shift” only makes sense when defined relative to a reference image. In fluorescence microscopy, typically any one of a group of single-colored fluorescence images is considered as the reference image. In order to correct for pixel shift, two different filter sets (each for a different color) can be designed and manufactured such that they do not produce a pixel shift with respect to each other. This is a popular approach for eliminating pixel shift and is utilized in manufacturing “zero-pixel-shift” filter sets from Semrock (BrightLine ZERO™). When all images are taken with these “zero pixel shift” sets almost perfect overlap of images acquired in different colors is guaranteed.

Since pixel shift is primarily dictated by the wedge angles of the dichroic and the emission filter, it is the design and manufacturing of these two filters in a set (and not of the exciter) that accounts for pixel shift error. Due to the limitations of the older manufacturing techniques, such as electron-beam evaporation-based, soft-coated filter technology, historically it was difficult to make “zero pixel shift” filter sets. Because the emitter was based on multiple substrates laminated together, achieving a low wedge angle was nearly impossible without expensive post-processing of the finished filter. Then emitter and dichroic filter pairs with similar beam deviations had to be hand-selected, and subsequently the filters were carefully aligned (oriented) with respect to one another in a filter cube so that the beam deviations of the two filters canceled one another. However, once carefully aligned, the filters could not be removed from the cube or replaced except by the filter vendor. This approach for correcting the pixel shift error is primarily dictated by the limitations of the manufacturing process itself since it is difficult to control the wedge angle in multi-substrate thin-film optical filers.

With the advent of hard-coated filter technology such the Ion Beam Sputtering (IBS) pioneered by Semrock, it is possible to manufacture every filter with a single substrate and therefore a small wedge angle of the filters can be ensured simply by starting with high-quality substrates prior to deposition of the optical coatings. Hence there is no special alignment or assembly required to install the filters in a cube, and a microscope user can populate his or her own cubes, or exchange filters as often as desired.

Instead of using a “zero pixel shift” filter set, it is possible to use sophisticated software algorithms to correct for pixel shift in the images after they are captured. For example, at the beginning of an experiment, images of multicolored fiduciary markers (for example, 100 nm TetraSpeck™ beads from Invitrogen) are acquired using filter sets for different colors. An overlay of the images of beads in different colors is used to identify pixel shift of one color relative to another and this information is fed to the software algorithm during post-processing of the acquired images under investigation. However this approach is time-consuming, has limited accuracy, and often cannot be fully automated (requiring an operator to manually click on reference points on the images to enable the computer to implement the correction algorithms). In practical applications even the accuracy may be compromised if the pixel shift calibration done prior to the actual experiment is no longer valid during an experiment due a change in the environmental factors such as temperature or due to mechanical vibrations.

## 6. Pixel shift considerations in different multicolor imaging configurations

As noted above, in all multicolor imaging applications the combined wedge angle specifications of the emission filter and the dichroic beamsplitter dictate “zero pixel shift” performance. Also, since dichroics typically have a single-sided optical coating and have a thinner substrate compared to an emission filter they are relatively more prone to manufacturing-induced nonparallelism. Therefore in general dichroics tend to be the primary contributors to pixel shift error.

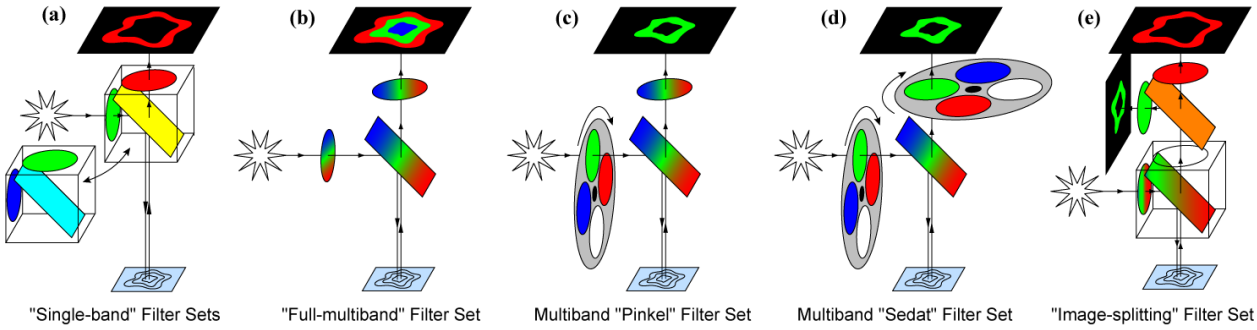


**Figure 5:** Examples of multicolor imaging configurations. Note that “zero pixel shift” considerations are different depending upon the imaging configuration. See text for details.

In order to design two single-band filter sets with “zero pixel shift” performance (Fig. 5a), the dichroic beamsplitter and the emission filters of both of these sets are manufactured with very low wedge angle specifications. The wedge angle specifications of Semrock’s BrightLine ZERO™ sets guarantee that the

worst-case image shift when interchanging sets is less than one pixel, as measured relative to the mean image position for a large sample of filter sets. Here one pixel of shift is based on a microscope with a 200 mm focal length tube lens and a camera with a pixel spacing of 6.7 µm.

In a “full-multiband” imaging configuration (Fig. 5b), a multiband beamsplitter and a multiband emission filter are used together with a multiband exciter to visualize different colors simultaneously. Assuming that all the optical elements in the microscope are color corrected, no pixel shift should be observed in the images acquired using a full-multiband filter set because neither the beamsplitter nor the emission filter needs to be changed in order to visualize different colors. Even in the “Pinkel” filter set configuration (Fig. 5c), pixel shift can be eliminated altogether since the same multiband beamsplitter and multiband emission filter are used to image different colors. In this configuration only the excitation filters (positioned in a filter wheel) are switched to achieve independent, sequential imaging of the different-colored fluorophores on a sensitive monochrome camera. Although the approach works for some applications – especially those that require very high-speed filter changes – image fidelity suffers as a result of the fluorophore crosstalk that occurs because all emission bands are present in every measurement. For applications that cannot tolerate the increased crosstalk, one can utilize single-band emitters in a filter wheel that is synchronized to the exciter wheel – the “Sedat” filter set configuration (Fig. 5d). This approach is more expensive, and the pixel shift caused by imperfections in the emitters must still be corrected or eliminated. It is worth noting that the variation in the wedge angles of the emitters is usually much smaller compared to those of the dichroics and therefore Semrock’s Sedat filter sets tend to exhibit pixel shift performance almost as good as certified BrightLine ZERO™ sets. In multicolor imaging applications that utilize an “image splitting dichroic” in the emission path of a microscope (Fig. 5e) for simultaneous multicolor imaging, it is best to use software tools with a calibration routine to correct for pixel shift.

## 7. Limitations and concluding remarks

It should be noted that even the “zero pixel shift” optical filters can provide image registration accuracy of up to at best only a single pixel. Therefore, if sub-pixel image registration is needed, advanced software algorithms together with calibration protocols (see Section 5) might be required for correction. Also, “zero pixel shift” sets are designed to provide excellent image registration among images acquired from multiple fluorophores; however, they do not necessarily provide perfect image registration between a bright field image (acquired with no fluorescence filters in the emission path) and a fluorescence image. This is because the specifications of the optical components in the emission path of standard bright field microscope are different from the specifications of the optical filters used in fluorescence imaging [1]. By matching the beam deviations in the bright field imaging path with the fluorescence imaging light path, it is possible to obtain good image registration between these two images (see, for example Semrock’s bright field filter set, BRFLD-A-000-ZERO). Furthermore, “zero pixel shift” filter sets from different manufacturers are typically not compatible with each other, and therefore

software-based pixel-shift correction might be required when filter sets from different manufacturers have to be used together.

Given the low cost and the long lifetime of the modern (hard-coated) fluorescence filters, when buying new filter sets it might be prudent for microscope users to add the “zero-pixel shift” option in order to enable “zero pixel shift” performance compatibility for future multicolor imaging applications.

## 8. References

[1] P. Prabhat and T. Erdogan, “Physics of pixel shift in fluorescence microscopy”. Semrock White Paper Series, www.semrock.com, 2011.

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