A beginner's guide to improving image acquisition in fluorescence microscopy

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This article presents an overview of optical microscopy and digs into the details of fluorescence microscopy, exploring the link between the signals in biological samples and the digital data from microscope cameras. Understanding this relationship can help you set the ideal image acquisition conditions to achieve the highest quality images and data.

Background and observation methods

An optical microscope is a tool used to image cells and small organisms and analyse the spatial and temporal distribution of specific features, including the co-localization of proteins and gene expression, using several observation methods. Compared to biochemical methods, optical microscopy has unique advantages, such as sensitivity for spatial distribution up to single-molecule resolution and the capability to handle diverse and non-purified samples, including *in vivo* and *in vitro* live samples.

The most common observation method is brightfield (BF). BF is often used for chemical or immunohistochemical stained samples to create a morphological map with colours that represent organic differences or labelled target proteins. Darkfield (DF) is a method used to enhance contrast. It is not 100% quantitative, but is an easy way to detect a target's existence, even if it is only several nanometres long. Phase contrast (PH) is the most common way to observe cultured cells. Because PH converts the optical phase gap caused by reflective index differences to contrast, the technique enables us to see colourless and transparent living cells without any stain. Differential interference contrast (DIC) is another method used to see colourless samples, but this method can visualize thickness difference and is more useful than PH for thicker samples. All these observation methods are easy and relatively affordable ways to visualize your sample based on its optical characteristics.

Two other important observation method types are fluorescence microscopy and laser-based microscopy. Although some highly advanced laser microscopes can visualize organisms and structures without any dye, their primary advantage is that they can precisely identify targeted genes or proteins, thanks to ever-improving fluorescence proteins, immunofluorescence stains and other dyes. These microscopes detect and measure fluorescence light emitted from excited fluorophores or proteins using an area detector, like a camera, or a point detector, like a photomultiplier tube (PMT). Laser-based microscopes have the highest resolution in depth direction as well as a background suppression feature called sectioning. With these features, laser microscopes can reconstruct the 3D structure of samples. On the other hand, when we simply say 'a fluorescence microscope', it usually means a non–laser-based fluorescence microscope, which is less expensive than laser-based microscopes and uses a camera to detect fluorescence light.

Now that we have covered the basics, let us explore the principles of microscopic fluorescence detection to better understand the link between acquired digital data and the biological sample.

The fundamentals of digital imaging for fluorescence microscopy

The microscope observes the fluorescence light emitted from dyes or proteins in a biological sample; then the camera detects this light and converts it into photoelectrons to be detected as a digital signal.

The detected signal value is a complex multiplier of the number of labelled targets (e.g. targeted proteins), excitation light intensity, and efficiencies of excitation and fluorescence emission/detection, including the camera's conversion efficiency from light to digital signal (Figure 1).

When you use the same system and image acquisition settings for different samples in one experiment, all components, except for the amount of the target, become

Summary: Fluorescence microscope imaging can be tricky since you need to obtain high-quality images without affecting cell viability. But with the right microscopy tools and techniques, you can run a successful experiment and get reliable image data. Discover how to strike a balance with microscope camera best practices.

1. Always consider your purpose of image acquisition based on your application and the priority of factors that contribute to image quality.
2. Understand the causes of background signals to minimize them.
3. Configure appropriate microscopy and image acquisition setting suitable for your purpose.
4. Leverage histogram and display adjustment to optimize the excitation intensity and exposure time, which are two major factors of image acquisition optimization.

constant values, making the detected signal value proportional to the amount of the target. This means, for example, that you could quantitatively compare a gene-edited sample with its wild type.

The anatomy of signal and background noise

This section explains the core factors that contribute to image quality and highlights the importance of carefully taking images during an experiment.

Actual signal vs background signal

The detected signal contains actual signal and background signal (background noise). To detect your target by identifying it from background signals, you need a high enough ratio of actual signal intensity vs background signal (Figure 2). This is called the signal-to-noise ratio (SNR). Aiming for a higher SNR leads to better image quality and quantitative analyses.

In general, maximizing the actual signal (e.g. with a higher numerical aperture [NA] objective) and minimizing the background signals (e.g. with a darkroom, deeper cooling or a high quantum efficiency [QE] camera) are common ways to improve the SNR.

Please note that the gain setting, which defines the amplification factor in a camera for signals, does not improve the SNR because it impacts both actual signal and background signal.

Actual signal

As we mentioned before, using a high NA objective can help improve the SNR. Another important factor to obtain a stronger signal is a high QE. The QE indicates the conversion efficiency from incident light to photoelectron. Keep in mind that a camera cannot sense light if its QE is 0% at a certain wavelength. For example, we must choose a camera with a sensitivity over 720 nm to use near-infrared (NIR) dyes like Cy7 for an NIR window in biological tissues or for preventing cross-talk while multiplexing (Figure 3).

Background noise

Background signals can be categorized as: a) biological background signals, b) non-biological background

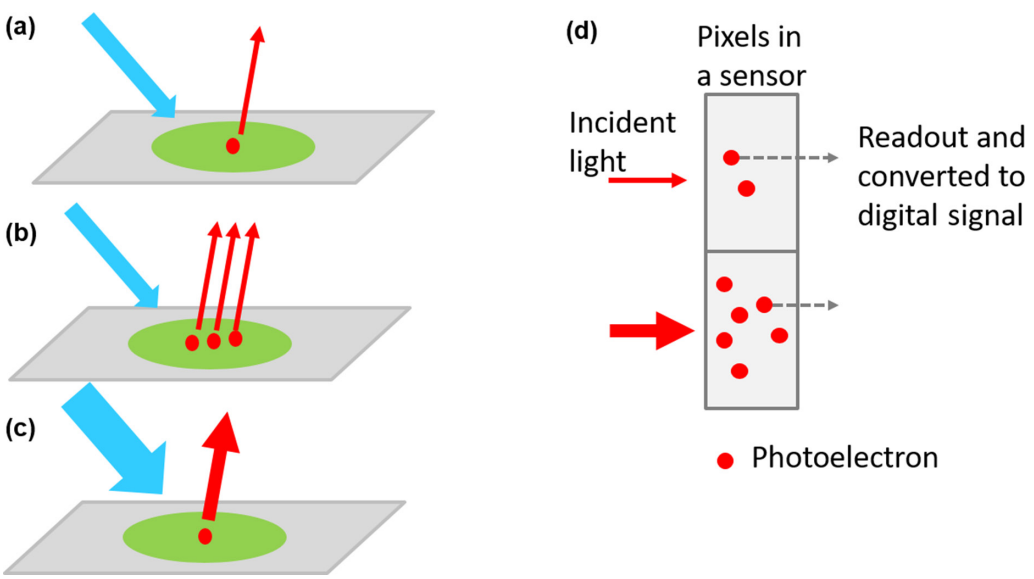

Figure 1. From sample to digital signal: **(a)** an excited labelled target emits fluorescence light. The light intensity increases when more targets are in the sample **(b)** or we apply stronger excitation light **(c)**. The signal intensity is proportional to the incident light intensity **(d)**.

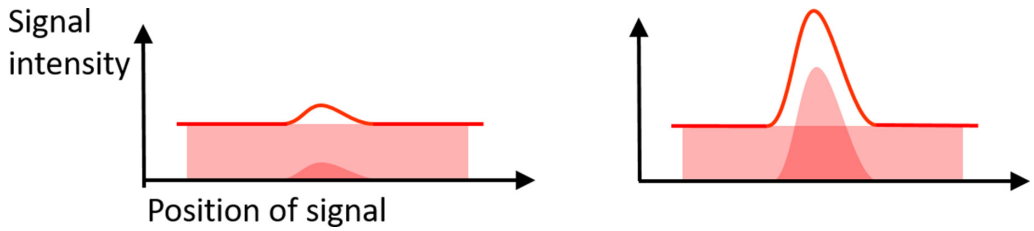

Figure 2. Left: low SNR: background noise makes it difficult to identify the actual signal. Right: high SNR: you can identify and measure the actual signal from the sample.

signals, c) statistical fluctuation of a photoelectron (shot noise) and d) noises in a camera.

The shot noise is unique and can be compared to a coin toss. Just like a 50% probability of showing 'heads' might lead to 'tails' on two coin flips, any trial with the number N has a statistical fluctuation of $\pm\sqrt{(N)}$. The detected number of photoelectrons follows the same rule.

All the background noise examples are shown in Figure 4.

Resolution

A larger pixel size or binning image acquisition can capture more light and provide a higher SNR, but the larger pixel size lowers the resolution (Figure 5). Consider the best pixel size that aligns with the optical resolution.

Best practices for using microscope cameras

Although the ideal image acquisition settings vary depending on the application and sample, two common parameters are excitation light intensity and exposure time. Longer exposure time or stronger excitation light provides brighter fluorescence, leading to a higher SNR. However, this also worsens phototoxicity. This brings up an important question: how do you set the best image acquisition parameter to achieve longer live cell imaging experiments, while reducing damage to the cells caused by excitation light?

To determine the ideal exposure time, use an image histogram. The X axis of the histogram is signal intensity. The height of the histogram at each X value shows the number of pixels for the signal intensity (Figure 6).

Usually the black background pixels have a non-zero digital signal value even with no background light (Figure 7, left). This helps to avoid a negative value signal caused by the readout noise fluctuation shown in Figure 4d. The shape and distribution of the histogram tell us if the current exposure time is appropriate. If the histogram is too crowded in a low signal range, then the exposure time is too short (Figure 7, middle). If there is a sharp cliff at the maximum signal level, then the signal value is saturated (Figure 7, right). In this case, you can reduce the excitation intensity or shorten the exposure time.

Some image acquisition software have an automated display adjustment feature that provides better visibility while maintaining the original image data. In most cases, a monochrome camera's signal dynamic range (e.g. 16-bit = 65,536 levels) is wider than a display's dynamic range, which is usually 8 bit (=256 levels).

The display adjustment feature defines the link between signal intensity and display brightness. Usually, the intensity from the brightest signal in your sample is much lower than the maximum intensity the camera can

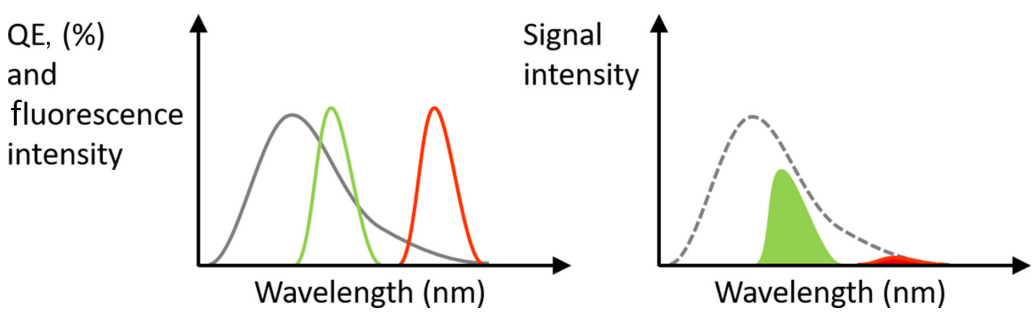

Figure 3. Left: the grey line is the QE of a camera. The green and red lines indicate the fluorescence emission spectra. Right: the detected signal value is equal to the area size, which is a multiplier of the QE and fluorescence spectra in the left figure. In this case, even if the fluorescence light has enough intensity, the detected signal could be weak for red fluorescence due to low QE.

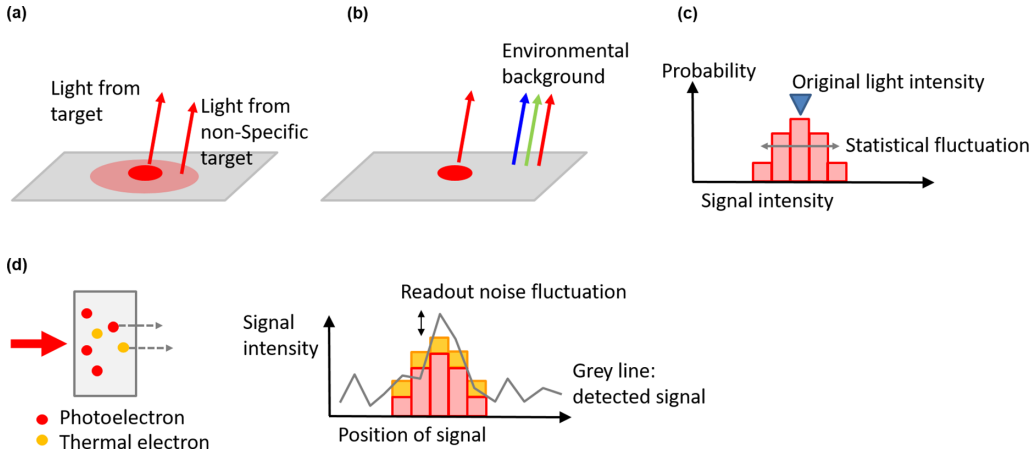

Figure 4. Examples of background noises: **(a)** biological background from a non-specific stain or autofluorescence, **(b)** environment light from the room reflected on a slide, **(c)** shot noise and **(d)** noises in a camera that contain thermal electrons generated in a sensor (left) and readout noise (right). Thermal electrons can be reduced by cooling the sensor.

handle. In this case, matching the display dynamic range to the data dynamic range (the range from background level to the brightest signal) enables you to get better visibility while maintaining the original image data (Figure 8). A histogram helps to illustrate this adjustment.

Six steps to set up a microscope camera's acquisition parameters

To summarize, here are six general steps to properly set up a microscope camera for an experiment. Please note that the best procedure depends on your specific application and samples.

1. Determine the observation magnification.
2. Adjust the focus on your sample and find the observation target. Consider using a higher gain or binning mode on the camera to shorten the process and minimize phototoxicity. We also recommend that you use an automated or manual display adjustment to observe the signal under its best condition and close the excitation light shutter whenever you are not observing the image.
3. Set the gain and binning mode back for image acquisition.
4. Try the gentlest excitation light intensity, and check if you can observe the signal in a realistic exposure

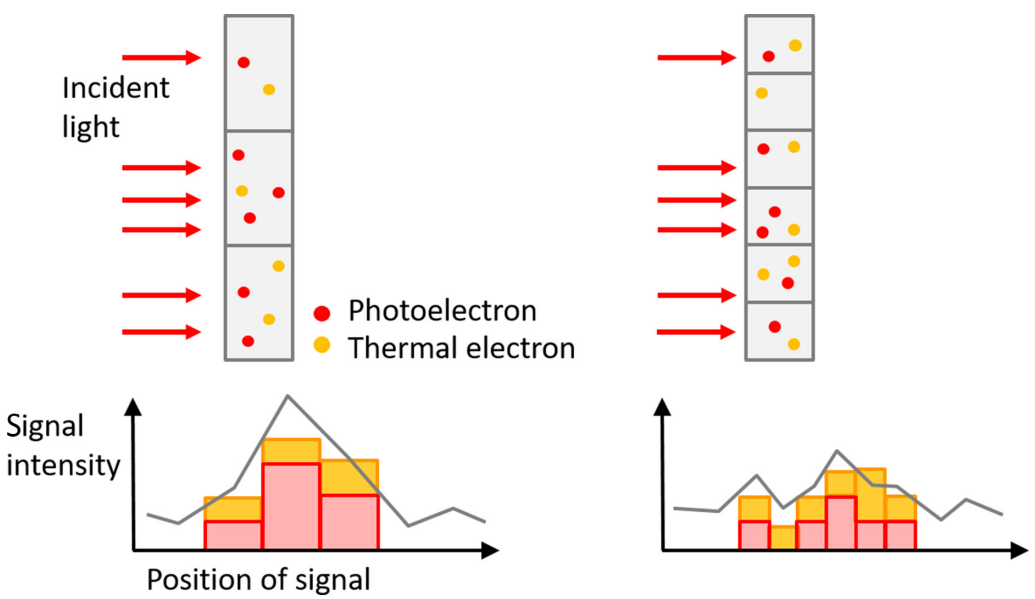

Figure 5. Left: a larger pixel size provides a higher sensitivity, but lower resolution. Right: a smaller pixel size provides a higher resolution, but lower sensitivity.

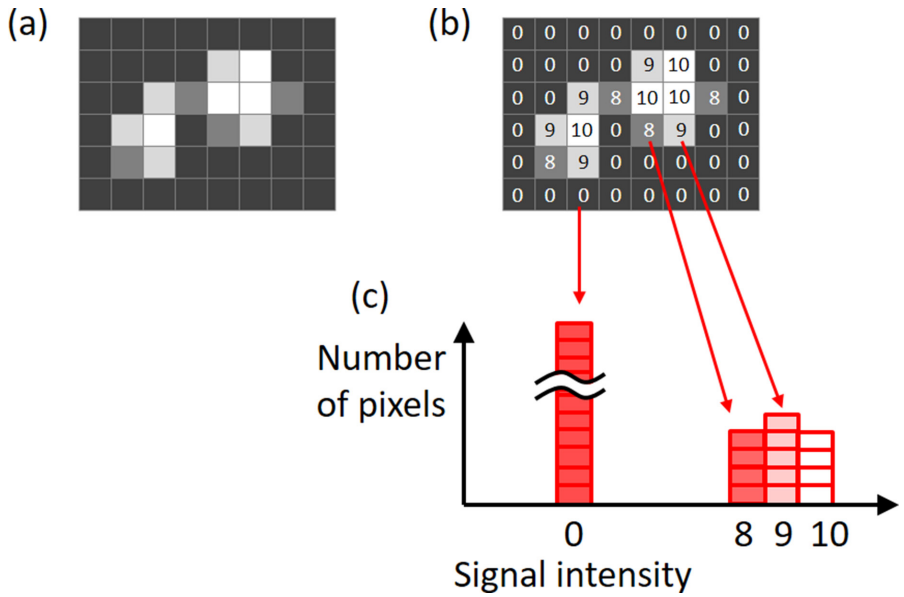

Figure 6. A histogram of an image. **(a)** Original image, **(b)** signal intensity of each pixel shown in the original image, **(c)** a histogram created based on the original image.

time. If you cannot identify the signal, or if the SNR is too low, try a longer exposure time.

5. If the exposure time is unrealistically long or longer than the maximum exposure time allowed for the image acquisition speed, try a slightly higher excitation intensity step by step.
6. Check the histogram to confirm there is no saturation.

How the technique is being used in an area of cutting edge or emerging research

Since these are fundamental physics of any microscopic image acquisition using a camera, this knowledge can be applied to any of the latest microscopy techniques like spinning disc confocal or super resolution microscopy with 2D detector. It is worth noting that an image is the major source of information we get from fluorescence microscopy, and therefore, the image quality determines any quality of downstream analysis. Even with the latest AI-based image analysis, higher SNR images provide better reliable, reproducible, accurate result than low SNR images. We can leverage this basic knowledge and tips for any of the fluorescence microscopy techniques. Thanks to emerging gene editing and multiplexing technique, we are expanding our capability to visualize complex pathway of biological phenomena tracing over tens of molecules and expression of multiple genes. This combination of multiplexing, latest immunofluorescence and gene editing gives more visibility on the biology, and fluorescence microscopy is a key for the temporal and special distribution analysis of the phenomena. To fully leverage these latest techniques, please keep these fluorescence digital imaging basics in your mind.

Conclusion

While many complex factors contribute to image and data quality in the microscopy process, knowing these

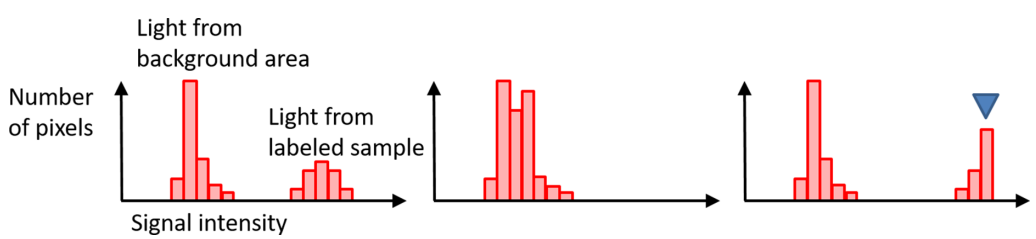

Figure 7. A histogram at normal exposure (left), underexposure (middle) and overexposure with saturation at the blue marker (right)

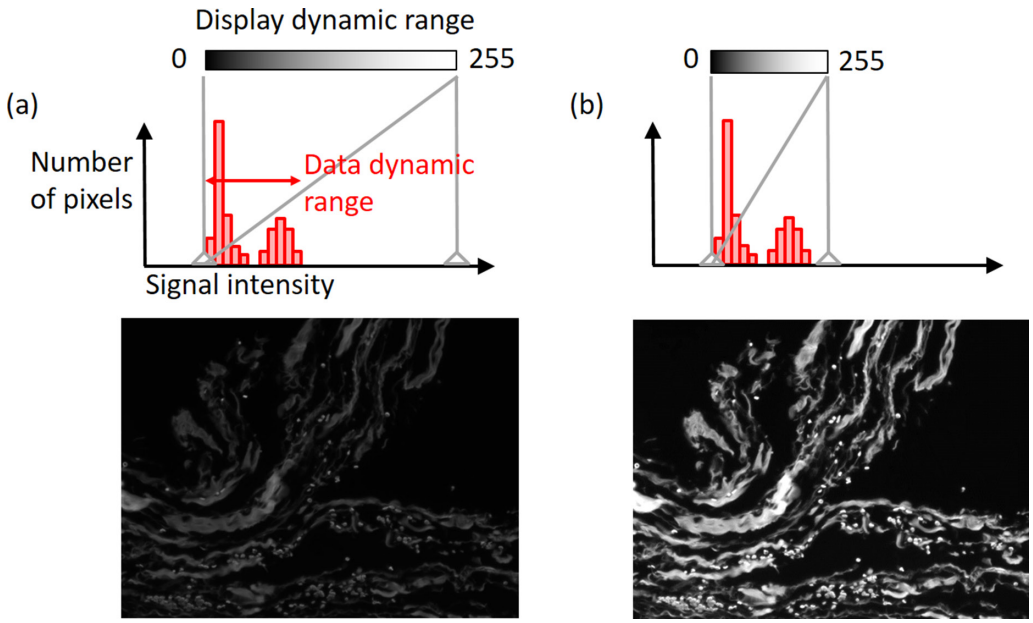

Figure 8. Display adjustment: (top) a histogram with the display setting indicator in a grey solid vertical line, (bottom) the example image. Left example image (**a**): original display setting. Right example image (**b**): display condition adjusted while maintaining the original image data.

digital imaging basics and tips can help you determine the best acquisition setting for each experiment. Maximizing the signal, minimizing the background and optimizing the sample condition are the essential elements to improve data quality for any application and experiment. ■

Further Reading

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