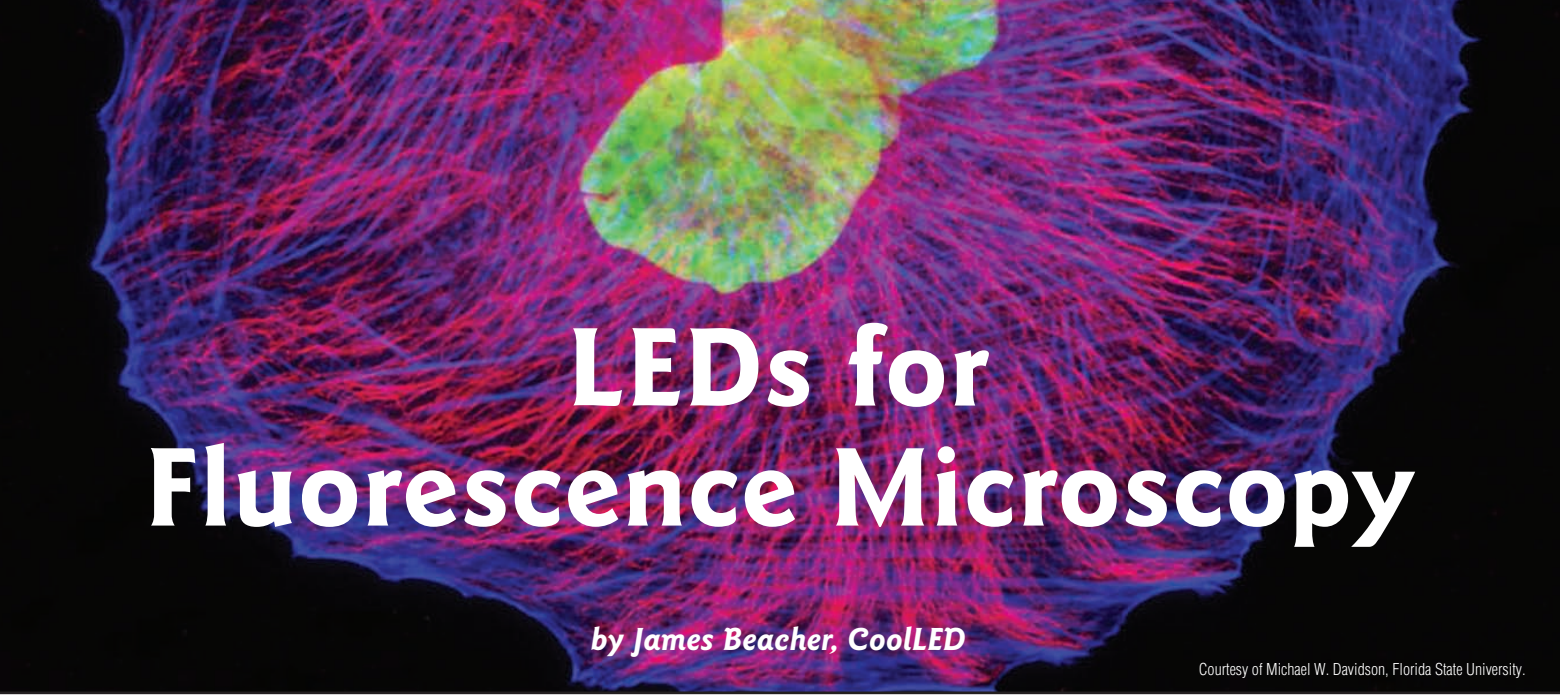

LEDs for Fluorescence Microscopy

by James Beacher, CoolLED

Courtesy of Michael W. Davidson, Florida State University.

LEDs offer reliability and repeatability for fluorescence applications.

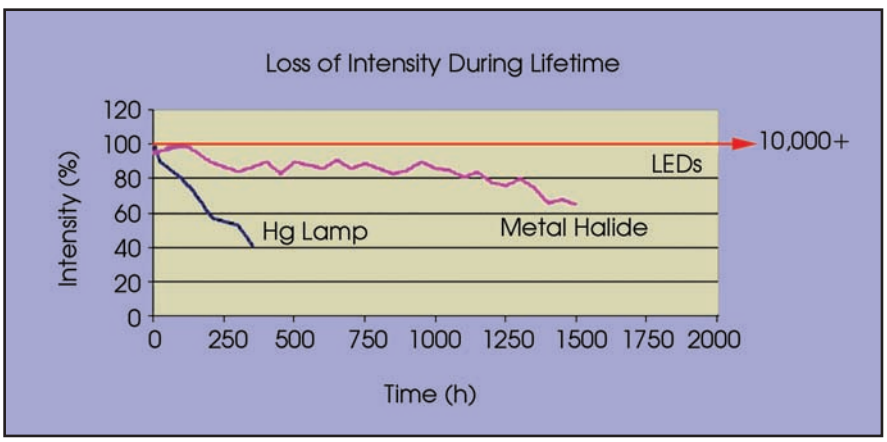

Figure 1. The operating intensities and lifetimes of various light sources are shown.

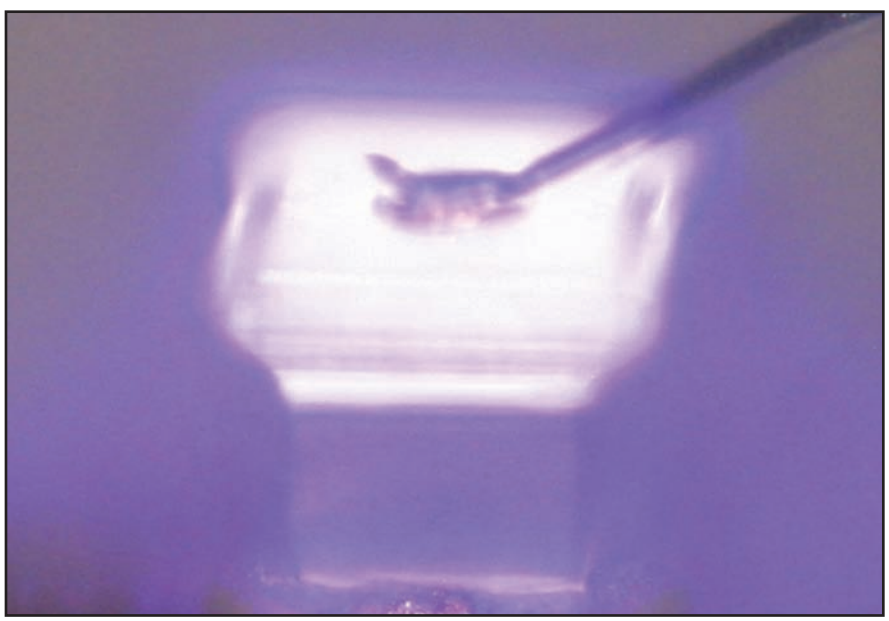

Figure 2. This bare LED die emits high intensity in all directions.

Until recently, fluorophore excitation has been achieved using broad-spectrum mercury gas discharge lamps with a combination of optical filters to remove many of the unwanted wavelengths. These lamps are widely used and accepted in microscopy applications. The devices' wide bands of excitation (from the UV across the visible spectrum) render them useful for most fluorescence applications. These arc lamps use a combination of high temperatures and pressure, meaning that, although the occurrence is rare, mercury bulbs can explode. Metal halide lamps combine mercury with other metals to produce their light, and they address some of the disadvantages of gas discharge lamps, but they are not the ultimate solution.

All gas discharge lamps take time to reach an operating equilibrium. Once this is achieved, the lamp requires a period of waiting after it is turned off before the bulb can be reactivated. As a result, lamps often are kept running all day to permit immediate use when required. This contributes heat to the microscope area, which can cause imaging problems. Lamp intensity fluctuates during use and decays throughout its lifetime, which can be as short as a few hundred hours (Figure 1). As a result, both qualitative and quantitative data can be unreliable. As pressure is increasing to produce repeatable results and to satisfy the FDA's good-practice guidelines (GxP), gas discharge lamps may introduce uncertainty into otherwise excellent results.

However, recent advances driven by

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mass market applications for LED technology, such as domestic and automotive lighting, are making it possible for LEDs to replace lamps as an excitation source for fluorescence microscopy. Thus, biologists can benefit from the advantages of these light sources.

LED basics

An LED chip (die) is a semiconducting material doped with impurities to create a *p-n* light-emitting junction (Figure 2). Typically, the die are encapsulated to form a simple optical package. When an electrical current is applied to the LED, the wavelength of the photons released at the junction is determined (inter alia) by the semiconductor materials used. Thus, LEDs produce wavelengths of a defined bandwidth around 10 to 30 nm.

The light intensity of LEDs varies over the visible spectrum, with peaks in the violet, blue and red regions being particularly strong. Because there have been few mass market applications requiring high-power peaks in the green, yellow and orange regions, these spectral bands have experienced less development to date. However, as the number of applications for LEDs has grown, intensities in this region have increased also.

LEDs have been used in biological research for fluorescence excitation since the early 1990s. Early attempts were limited by their low efficiency and by the availability and suitability of commercially available products. Although LEDs have become more powerful, today's brightest LEDs are still only 25 to 35 percent efficient, and attention must be given to thermal management to ensure their stability, reliability and lifetime.

As a solid-state electronic device, an LED light source is capable of a precise level of control. No mechanical shutters or neutral density filters are needed to reduce the intensity of light falling on the sample when using LEDs because they can be instantly switched off and on and because light intensity is controllable in discrete (typically 1 percent) steps from 0 to 100 percent. This also means that vibration and response delay are reduced with the elimination of these moving parts.

The stability of LED intensity over short periods and throughout the device's lifetime is a key benefit in ensuring consistent

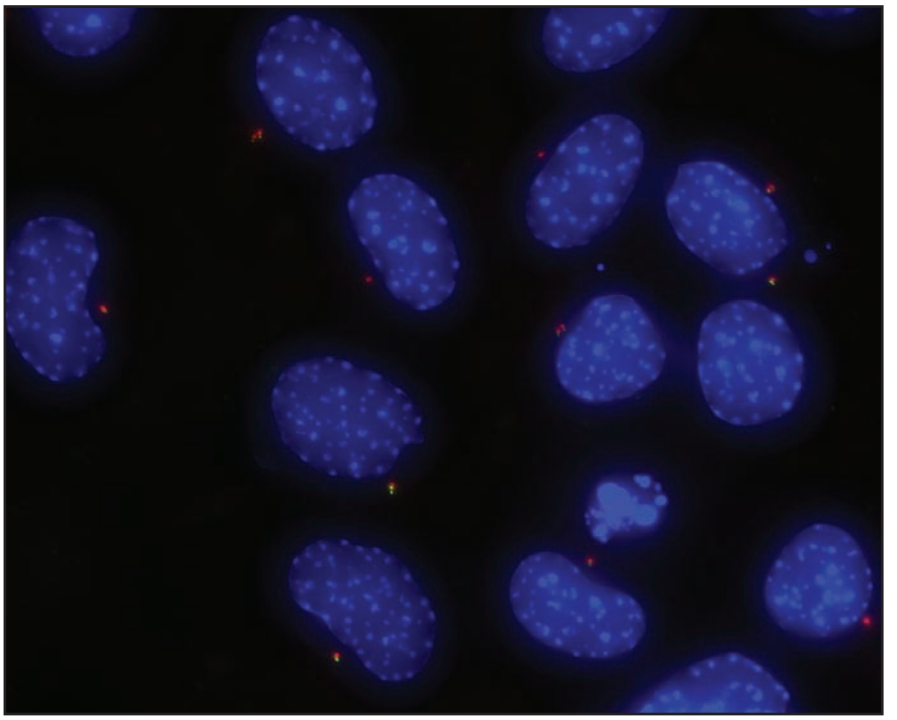

Figure 3. These mouse embryonic fibroblasts were stained with Al 568 antibody (red) and centrine GFP (green). The DNA was stained with DAPI (blue). The images were taken with the PrecisExcite using a 100-ms exposure. The 400-nm excitation was set at 10 percent, the 465-nm at 5 percent and the 525-nm at 80 percent. Courtesy of the Institute of Molecular Pathology in Vienna, Austria.

Figure 4. Each LED array module contains up to 96 individual LED chips.

and repeatable excitation. Although current LEDs are considered efficient, about 70 percent of their output is heat. To ensure optimal stability, cooling them is essential. Active cooling while the units are on will hold them at an optimal temperature for maximum excitation and stability, which makes repeatable results possible regardless of the age of the device. Wavelength switching can be achieved electronically, making submillisecond switching possible. Further optimization is likely in the future, but switching times of a few hundred microseconds already are available.

LED light sources typically are controlled by a remote pod that sits beside the microscope to allow the user to switch the wavelength and to adjust the intensity while looking at the samples. However, integration of the device within a microscope or imaging software package is possible, too, enabling automated routines and greater benefit from the fast-switching capability.

The lifetime of an LED is measured in tens of thousands of hours. Because it is available instantly, the instrument can be turned on for only the time needed to excite the fluorophore. We believe that, if an LED source is used in this way, its effective lifetime could be indefinite. This saves the cost of replacement bulbs and the time it takes for the difficult and time-consuming realignment of gas discharge lamps.

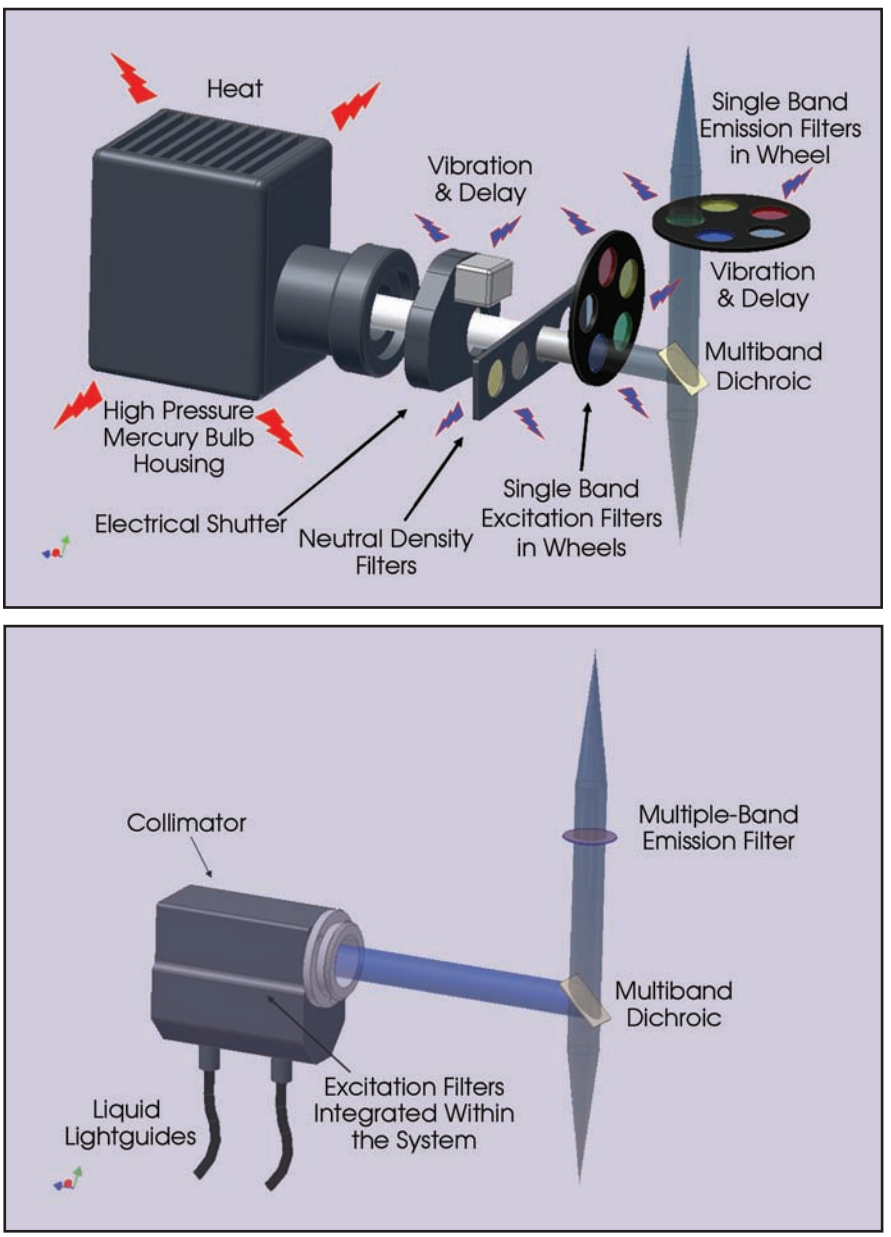

Figure 5. A traditional excitation setup (top) has more moving parts than an LED illumination configuration (bottom).

Less sample damage

Any light source can photobleach biological samples that are exposed to it long enough, but broadband excitation, including UV, can damage a sample in a very short period. Even with wideband blocking filters, damage is not uncommon.

Using the lower out-of-band transmission — particularly low UV — available with LEDs reduces phototoxicity, permitting users to image more often without damaging cells, which makes it easier to keep track of cell movement. It is expected that, by using LEDs, one can achieve the same quality of imaging with less than 25 percent of the exposure time of shuttered lamps. Reduced autofluorescence from less exposure also produces crisper images.

Light sources mounted directly to the microscope can introduce heat, vibration and electromagnetic interference, all of which can damage the samples and compromise the results. In an LED system, electromagnetic interference can be removed from the sample and microscope with lightguides. This is attractive for electrophysiology and for work carried out in a Faraday cage.

The inherent stability of thermally controlled LEDs is important for experiments that may run for many days or weeks. Rather than requiring lamp stability and lifetime to be factored in, LEDs make it possible for every image to be taken under identical excitation conditions from the beginning of the experiment to the end. Such conditions are particularly important in systems using automation and robotics, and LEDs offer the additional benefit of not needing to be replaced or realigned, as do bulbs.

Dual staining procedures using fluorophores such as GFP with red fluorescent proteins such as mCherry are becoming common. Here, controlling the intensity of individual wavelengths independently allows excitation to be optimized in a way that does not saturate GFP and that still excites the red fluorescent protein well (Figure 3). Mechanical neutral density filters, shutters and lamps cannot provide this level of control. In addition, Förster resonance energy transfer (FRET) can be carried out effectively using LEDs for cyan or yellow fluorescent

protein excitation. Fast switching and stability are important for FRET.

Applications requiring very short periods of excitation lend themselves to LED light sources; for example, in aligning a sample before imaging with a laser-scanning confocal microscope.

It is important for the user of an LED light source to match the optical filters in the microscope to the selected peak wavelength of the LED excitation wavelength. Although LEDs offer narrowband excitation, reducing the level of unwanted light, there are still "tails" of emission that must be removed to reduce unwanted background light in the fluorophore's emission region.

The PrecisExcite LED excitation system from CoolLED is designed for fluorescence microscopy applications. The company's experience with semiconductor LED die enabled it to use optimized LED parts rather than the standard ones available to produce a light flux at the LED in excess of 70 W/cm^2. The user can choose from a range of wavelengths by selecting an LED array module containing up to 96 individual LED chips (Figure 4). Currently, the interchangeable modules are available at 400, 445, 465, 505, 525, 595 and 635 nm, and additional wavelengths are under development.

Patented technology developed by the company enables maximum light transfer from the LED to the liquid lightguide and, thus, to the sample under excitation. Light is recombined at the microscope using a collimator that fits into the light port. This arrangement provides two benefits: (1) heat, electromagnetic interference and vibration are kept remote from the microscope and samples, and (2) the lightguides act as excellent diffusers, achieving an even homogeneous field of excitation at the microscope.

Future of LEDs

Most high-end applications need the unique benefits of stability, repeatability, control and reduced sample damage provided by LEDs. Specific applications, such as live-cell imaging, require the fast switching and reduced photobleaching of an LED light source.

Gas discharge lamps will continue to play a role because their broadband capability remains attractive for general and standard applications. However, many cell biologists already recognize that most of their work is performed with a small number of specific fluorophores. A flexible LED system optimized for their particular application provides all of the benefits without the limitation of a gas discharge lamp's mechanical system.

As LEDs become more powerful, and as more wavelengths become available, LED sources will present the same benefits as a gas discharge lamp while offering greater control and lower running costs. A significant change to illumination on a microscope will be the redundancy of moving parts such as filter wheels (Figure 5). This will reduce costs and should decrease image capture times dramatically. At that time, an all-around safer, highly controllable LED product will exist that will enhance the quality of fluorescence microscopy. □

Meet the author

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