LEDs in Microscopy: An Emerging Research Tool

LED light sources deliver convenience and green benefits to fluorescence microscopy. They are expected to remain effective research components, particularly for imaging and the preservation of sensitive samples.

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LED technology is playing an increasingly important role in lighting up our lives. Over the past 50 years, the technology has expanded from simple indicators in electronic gadgets to saving our planet terawatts of power by ousting incandescent bulbs in domestic lighting. But some not-so-well-known specialist lighting applications now exist for LEDs because of their high intensity, controllability and spectral output.

Fluorescence microscopy is one such application area where the move from bulbs to LEDs has brought much more than reduced running costs alone. The technique is employed widely in the life sciences to study biological specimens, from single cells to whole organisms. It involves using an optical microscope (Figure 1) to excite a sample with light at a specific wavelength. The light is reemitted at a longer wavelength through a process known as the Stokes shift — the difference in wavelength or frequency units between positions of the band maxima of the absorption and emission spectra of the same electronic transition, such as with fluorescence and Raman spectroscopy.

Using different fluorescent markers to tag the sample means that different regions can be made to fluoresce with different colors. This enables high-contrast multicolor images to be formed (Figure 2).

Meeting spectral needs

Sometimes living cells are imaged, while other times the cells are chemically fixed at a certain period in their lives. In all cases, the type and design of the light source used to illuminate the sample has a big impact on the required hardware needed with the microscope and, very importantly, the quality and validity of the images recorded.

Convenience for the end user and lab manager was a big factor in the early adoption of LED-based systems. The most common bulb — the 100-W high-pressure Hg bulb — is a short-lived source with a lifetime spanning around 300 hours. End users would typically keep a record of a bulb's "on" time on a small notepad, as running the bulb longer than the recommended life would increase the risk of an explosion. LED sources brought lifetimes into tens of thousands of hours.

Bulbs need a warm up and cool down period and so are left on all day. An LED source can be switched on and off electronically when needed, allowing it to be off when not in use; only when looking at a sample or taking a picture would the light source need to be switched on. Although the selling points for moving to LED sources were many, wide adoption stalled initially because of two major hurdles: higher intensity and spectral coverage.

LED sources are not broad spectrum but do emit a Gaussian-like spectral shape with an FWHM (full width half maximum) of around 10 to 40 nm. This forces the light source designer to include multiple LEDs to cover the researcher's spectral needs. This brought with it new optoelectronic and mechanical complexity in light-source design that was not necessary with traditional bulb sources. Capturing and collimating the Lambertian emission from the LED chip, and then combining multiple colors by the use of dichromatic mirrors, has become the standard approach. The Lambertian law of optics demonstrates that the luminous intensity observed from a diffusely reflecting surface is directly proportional

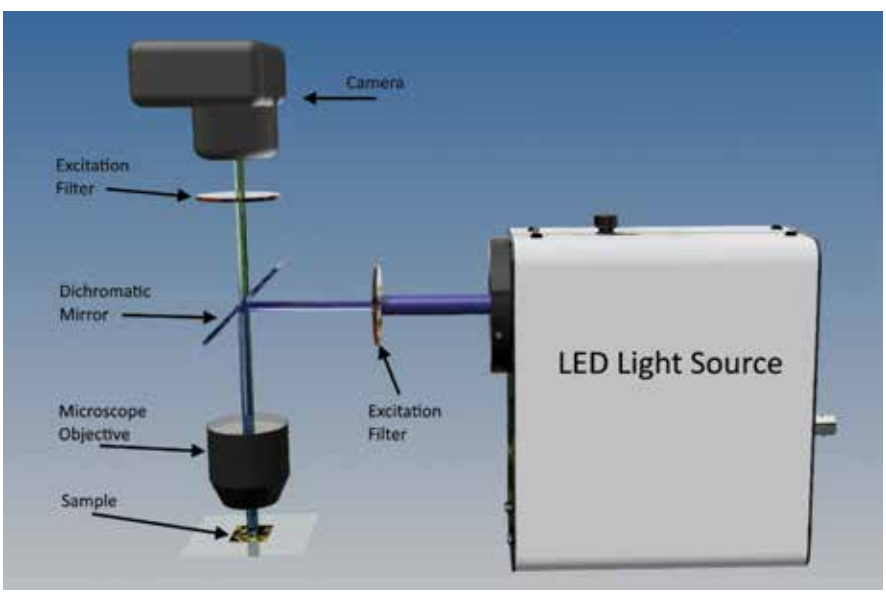

Figure 1. The basic components of a fluorescent microscope. Courtesy of Luther Hindley/CoolLED Ltd.

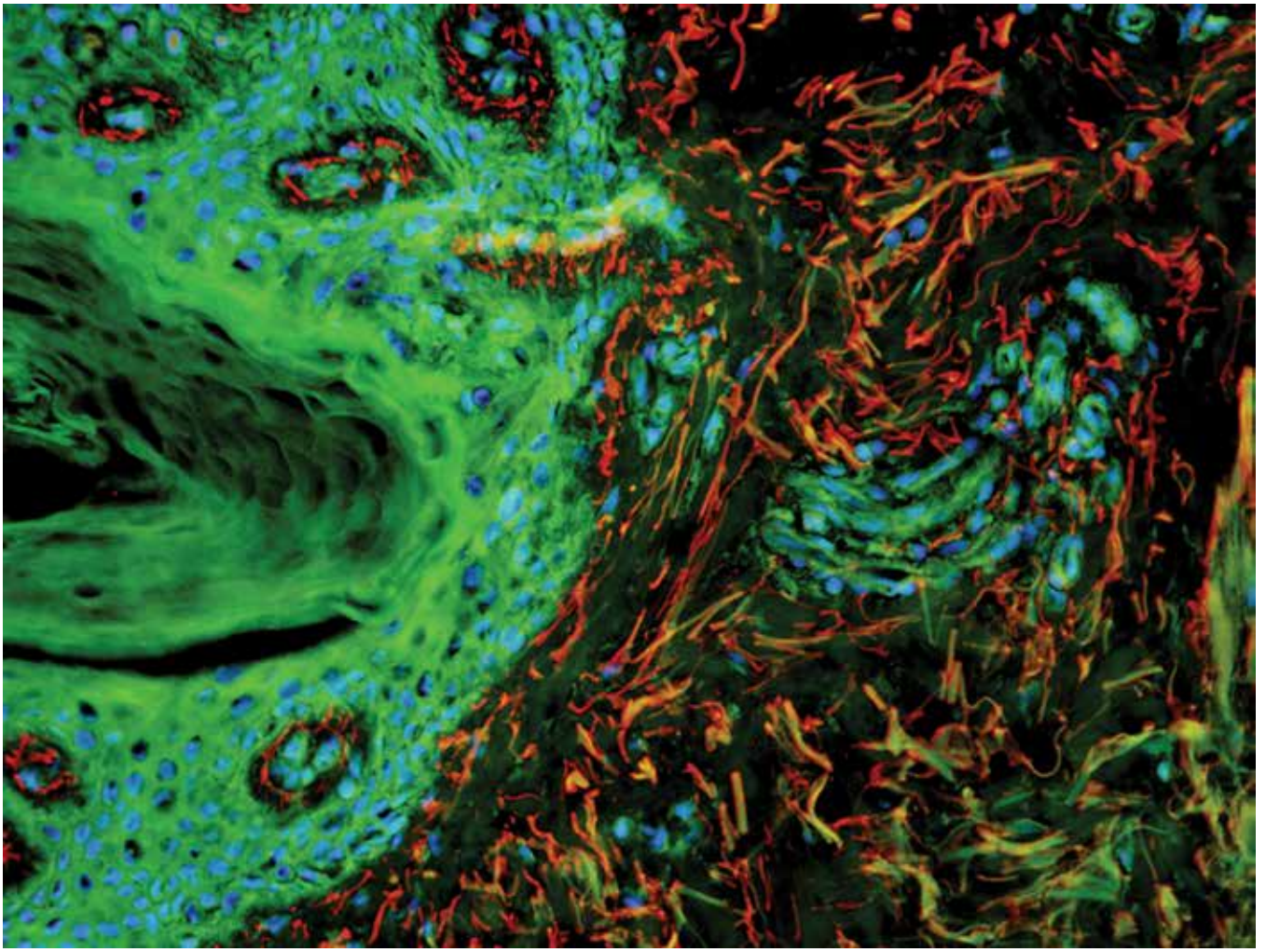

Figure 2. Fluorescent skin image taken using a CoolLED pE-300white. Courtesy of Peter Call/CoolLED Ltd.

to the cosine of the angle θ between the direction of the incident light and the surface normal.

The complexity and cost has led most LED light sources to contain up to six discrete wavelengths.

One novel and patented method employs a wavelength-grouping concept that involves spectrally neighboring LED wavelengths that are grouped into one channel from which the user can select. Four groups of spectrally separate LEDs can then be combined as needed for high-speed applications. The trick is to recognize that certain closely grouped wavelengths would rarely, if ever, be used on the same sample. LED-based light sources with up to 16 wavelengths are now available using this method for improved intensity, spectral coverage and lower cost.

The power available from current LED chips does not come close to the radiance produced by the plasma arc in the 100-W Hg bulb. Bulbs emit energy over a very wide spectrum, but at given wavelength regions of around 20 nm, a well-placed LED will compete very well; it even outshines the Hg bulb in most regions from 360 to 800 nm.

Overdriving LEDs is common in fluorescence applications, placing higher importance on thermal management. Cooling techniques range from Peltier cooling to putting the bare unpackaged LED chip onto a large copper heatsink.

For a long time, the green region of the spectrum suffered as the weakest area compared to bulbs. This area, known as the "green gap" in solid-state lighting, is still a very weak region for LEDs in general. Users can overcome this challenge by employing a phosphor material in a number of forms. One method patented by Philips uses a fluorescent rod that gets excited by a row of bright blue LEDs. This adds some expense and inefficiency when used on a typical fluorescent microscope compared to single chip LEDs. Recent developments in the power of blue LED chips have enabled a simpler solution with a phosphor being placed directly onto the LED. The phosphor in this case is chosen to give maximum green region intensity only, unlike white LEDs. Through the Stokes shift, a red color is created by bright green LED excitation light (Figure 3).

Enhanced imaging

Acquiring good quality images can be viewed as the end goal in microscopy. However, because of phototoxic effects, the act of observing a sample can have significant impact on the experiment. Improving the image signal-to-noise and the negative effects of imaging can both be improved by the inherent nature of the LED.

The need for bulb alignment to improve evenness of illumination was eliminated when liquid-filled light guides became

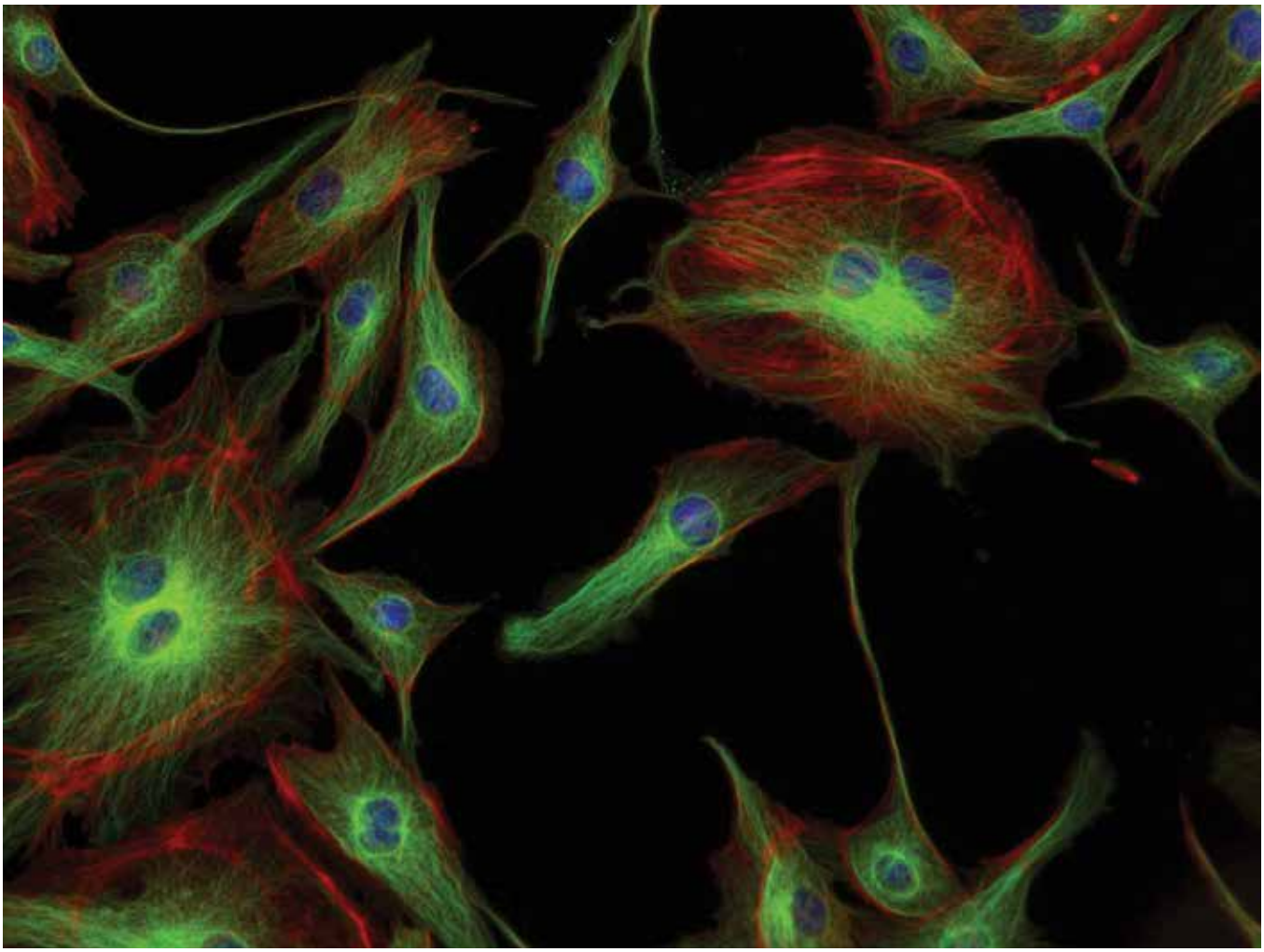

Figure 3. Fluorescent bovine pulmonary artery endothelial cells. The cell nucleus is shown blue, the green is tubulin and the red shows actin. Courtesy of Jordi Recasens/Izasa Scientific.

common with the introduction of metal halide systems. This improved homogeneity as the guide acts as a good scrambler of light. The solid-state nature of LED devices has allowed direct microscope attachment to be used without the need to realign, employing Köhler illumination — a technique for sample illumination in modern scientific light microscopy. With this method, optics in the light source can image the LED chip onto the back aperture of the microscope objective lens. This objective lens, working in reverse, scrambles and spreads the light evenly across the field of view at the sample. Light guides are still used with some LED systems, however, as they remove weight and vibration from the microscope.

Improvements in the image signal-to-noise ratio are achieved with high-quality filters that feature good blocking and transmission regions. In a typical excitation and emission filter for imaging the common fluorophore DAPI (Figure 4), the excitation filter needs to block out very high levels of energy from the blue region of the Hg spectrum.

By comparison, an LED used to excite DAPI emits a very low level of energy outside the excitation band of interest, including in the blue region where imaging of the comparatively weak sample occurs. The result is better system signal-to-noise in images that use LED sources, as they reduce background levels at the sample. Work by Sandrine Prost and colleagues from the University of Edinburgh has shown that with independent wavelength-controllable LED sources, signal-to-noise is dramatically improved over bulb systems and even over some available white wide-spectrum LED sources[1].

Results are affected by observing the sample, as cells are not suited for exposure to high-intensity light. Negative effects include photobleaching and phototoxicity, causing a reduction in signal over time and the death of living cells or abnormal behavior because of the illumination. Minimizing illumination time on the sample is paramount to reducing these effects. Traditional bulb sources require a mechanical shutter to control exposure. This can sometimes cause very long latency delays leading to the sample being needlessly illuminated on either side of the camera exposure time.

The problem has been dramatically reduced with automatically controlled LED sources. Direct TTL control of the LED improves this over USB communication methods by providing on/off times in microseconds. Many high-end cameras provide a TTL output signal while the camera is exposing; this signal can be fed directly to the LED light source to switch the required light on, then off, to match the camera exposure very precisely. As sequential imaging of two or three fluorophores is common, the latest LED sources allow a step sequence of wavelengths to be programmed into the light source,

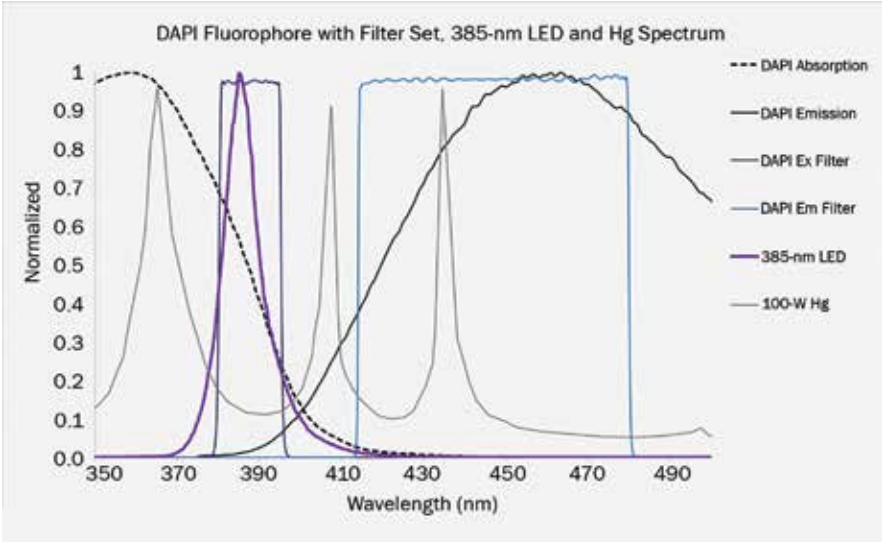

Figure 4. This graph shows a 385-nm LED and Hg spectrum overlaid on DAPI absorption and emission, with an example excitation and emission filter. Courtesy of Gerard Whoriskey/CoolLED Ltd.

which will then be stepped through sequentially with each camera exposure. This hardwired method reduces unneeded sample exposure because of mechanically controlled shutters and PC-based control methods that do not have real-time operating systems.

Recent work by Claire Brown and colleagues from McGill University has shown that by controlling light exposure in the sample, photobleaching and phototoxic effects can be reduced even further[2]. When a fluorescent molecule undergoes excitation by absorption of a photon, it moves into the excited singlet state, and ideally reemits most of this energy as a new photon with longer wavelength. However, it is possible that the fluorophore will transition into the triplet state — there, it can become toxic. If more photons are absorbed by a molecule in the triplet state, the extra energy can lead to the breaking of covalent bonds. This could also contribute to phototoxic and photobleaching effects.

In studies that involve laser excitation with a rapid line scanning method (available in confocal systems), photobleaching and phototoxicity reduced dramatically. This is attributed to short time-limited pulses of light exposure followed by a resting period, allowing the triplet state molecules to return to the safe ground state. Work continues in this area, with interest in LED-based sources expected to deliver similar outcomes because of their control and fast-switching capability.

Very recently, 340-nm LEDs at good power have entered the market, allowing calcium imaging with the fluorescent calcium indicator, Fura-2. This application captures activity in neuronal networks to allow research of Alzheimer's and other similar conditions. Work by Peter Tinning and colleagues from the University of Strathclyde has shown that with a strong 340/380-nm LED system, the Fura-2 concentrations loaded into cells can be reduced to as much as 25 percent over the standard cell preparation protocol[3].

The benefits of research are not just in saving money by getting more work out of a given volume of dye; it more importantly reduces the toxic effects on cells that a fluorophore can cause. The outcome is that observed reactions on the sample are more typical of natural behavior.

References

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