REVIEW ARTICLE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Light-Emitting Diodes in Modern Microscopy—From David to Goliath?

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Received 22 August 2011; Revision Received 4 January 2012; Accepted 12 January 2012

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Published online 30 January 2012 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22023

Abstract

Proper illumination is essential for light microscopy. Whereas in early years incandescent light was the only illumination, today, more and more specialized light sources, such as lasers or arc lamps are used. Because of the high efficiency and brightness that light-emitting diodes (LED) have reached today, they have become a serious alternative for almost all kinds of illumination in light microscopy. LED have a high durability, do not need expensive electronics, and they can be switched in nanoseconds. Besides this, they are available throughout the UV/Vis/NIR-spectrum with a narrow bandwidth. This makes them ideal light sources for fluorescence microscopy. The white LED, with a color temperature ranging from 2,600 up to 5,000 K is an excellent choice for bright-field illumination with the additional advantage of simple brightness adjustments without changing the spectrum. This review discusses the different LED types, their use in the fluorescence microscope, and discusses LED as specialized illumination sources for Förster resonance energy transfer and fluorescent lifetime imaging microscopy. © 2012 International Society for Advancement of Cytometry

Key terms

light emitting diode; microscopy illumination; FRET; laser microscopy; FLIM; cytometry

SINCE H.J. Round first published "A note on carborundum" in 1907 (1) electroluminescence underwent a dramatic development—from curiosity to widespread application. The first commercial light-emitting diodes (LED) in the visible range were based on GaAs and were developed at General Electric (USA) by Nick Holonyak. The red emission from these devices could be seen in dim ambient light, but were essentially useless for illumination purposes. During the sixties and seventies of the last century, a wide spectrum of colors, from green up to the infrared range, was developed. However, their low efficiency only allowed applications in displays: With the exploration of new, highly transparent materials like GaN or GaNP, bright LED finally became available, making them interesting as backlights for cars or in traffic lights. The most important step in LED development was probably the introduction of the blue LED at the end of the eighties by Nichia (Tokushima, Japan) (2,3). Now, with the availability of bright devices ranging across the entire visible spectrum, large displays and the first applications in illumination emerged. Using a fluorescent chip in combination with bright blue emitting devices, a white LED became available. Today, due to their favorable power and efficiency characteristics, LED compete with fluorescent and halogen lamps in everyday illumination applications. Even applications requiring high light intensity such as fluorescence imaging are now compatible with modern LED technology.

In recent years, the spectrum was extended into the ultraviolet range, down to 245 nm. Especially, LED in the deep UV range between 255 and 300 nm and the line of 365 nm are interesting for life science applications as they can be used for uncaging experiments, tissue autofluorescence imaging, and time-gated luminescence microscopy due to the high energy at low wavelength (4,5).

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The light emission of the LED is localized at the pn-junction of the semiconductor crystal. When this junction is shaped as resonant waveguide, and the semiconductor material is properly doped, a laser diode (LD) is formed. Whereas LED have a bandwidth of tens of nanometers, LDs emit a single line with a typical bandwidth of about 1 nm.

With the development of bright LED with bandwidths throughout the visible spectrum, these devices gain increasing interest for microscopy applications. This ranges from replacing the halogen and arc lamps, to the opening of the field for new applications by virtue of their incredible features like cold light emission, simple control for pulsed applications, and a defined spectrum with only small variations throughout the working range (6,7). Their small form factor and a life span of several tens of thousands of hours that make replacement essentially unnecessary are especially interesting for custom-tailored designs of advanced optical systems.

LDs, although technically often the better alternative, exhibit considerable gaps in their availability within the spectrum, as only a few lines are available.

LED in Fluorescence Microscopy

The advantages of LED as light source for fluorescence imaging can best be appreciated when considering their substantial growth in biomedical optical applications, after their only very recent introduction. As with most components now in use in fluorescence microscopy, LED were not designed with this purpose in mind. Increased availability and lower cost are associated with market-driven large scale production. This development can be seen for lasers that became central components in consumer electronics devices like DVD and BluRay players. The same crossover effect is seen for LED as illumination source in low-light level biological fluorescence microscopy. Whereas the first red LED were barely bright enough to serve as indicator lights in dimly lit environments, today, the demand for energy savings in domestic, automotive, and traffic lighting has led to a tremendous improvement in their brightness (by four orders of magnitude over the last 30 years) and their availability in a wide variety of colors. Inevitably, they became useful for fluorescence imaging as well. LED first replaced established light sources for bright-field illumination (8), but since about 15 years also for fluorescence microscopy (9,10). A major cost factor in fluorescence microscopy is the limited lifetime of the mercury lamp, which is a few hundreds of operation hours, and considerably less when the lamps are frequently started. Replacement is time-consuming, expensive, and can be dangerous for some lamps. Furthermore, the lamp is toxic waste. It is therefore not surprising that the LED with its long lifetime of tens of thousands of hours, regardless of the number of starts, its low power consumption, the absence of warm-up or cool-down time, and its low cost have become a serious alternative for mercury and xenon lamps.

The first reported applications of the use of LED as lamp replacement came from the hobbyist side. Jim Haseloff of the University of Cambridge (UK) provides detailed instruction on his website (11) for about a decade now on how to build the "Cheaposcope," a laboratory side project that is actually used in his research, which involves the fluorescent imaging of plants. Amateur developers have also helped establish the feasibility and inform microscope users of this approach. The retired microscopy enthusiast Ely Silk in 2002 published his adaptation outside the main stream scientific journals (12) and also maintains an excellent website with all relevant information and application examples (13). One of the proposed applications for a cheap fluorescent microscope offered by Silk was the screening of patient's sputum for tuberculosis in the developing countries, where the availability of an affordable battery-operated fluorescence microscope would be extremely helpful. In fact, the WHO earlier this year officially recommended LED-fluorescence microscopy for the detection of tuberculosis by Auramine-O staining (7,14). This obvious niche application (including other tropical diseases like malaria) has driven many companies to produce affordable LED-based microscopes (15,16, see Table 1). Amateur scientists prefer LED, because the disadvantages of the mercury lamp are clearly prohibitory to a nonprofessional setting.

Another reason for adopting LED illumination for fluorescence imaging is their small size, allowing very compact optical designs or specialized applications like microscopes designed for use in space missions (e.g., www.cytoscience.com). Here, a small payload and low energy consumption is essential. Also for lab-on-a-chip or remote telemedicine applications that involve fluorescence microscopy, LED are indispensable tools for the creation of cost-effective and miniaturized fluorescence imaging solutions (17,18).

The simplicity and small scale of these applications are exemplified by the recent integration of image cytometry and fluorescent microscopy on a cell phone using a LED-based optofluidic attachment (19). Although existing "high-end" microscopes offer extremely powerful solutions for all kinds of imaging approaches, these complex systems are generally not suitable for field-use in resource-limited settings. Tremendous efforts have been focused on developing microscopes and cytometers that satisfy the requirements of cost-effectiveness, portability and robustness, while still achieving a decent imaging performance (19,20). Another example is the holographic lensless microscope—based on 23 LED combined with 23 light fibers—which achieves a wide field-of-view of $\sim$24 mm^2 with a reasonable resolution below 1 μm that was recently reported by Bishara et al. (21).

Today, other fast developing branches are point-of-care equipment and applications where the LED is placed inside or onto organisms for live imaging, like the recent development of ingestible cameras for gastrointestinal inspections. One particularly interesting application, however, falling outside the scope of this review, is the use of implantable micro-LED arrays for the photonic stimulation of optogenetically modified neurons in freely behaving small experimental animals. This technology promises great translational potential as "optical pacemaker" for, for example, patients with spinal cord injuries, cardiac or neurological/neurodegenerative disease if safe targeted gene transfer techniques become available (22).

Table 1. LEDs in fluorescence microscopy, illuminators, adapters, and integrated solutions

COMPANY	PRODUCT	TYPE	LED POSITION	COLORS
Partec	CyScope	Integrated microscope	Replaces high-pressure mercury lamp (HBO)	455 nm (tuberculosis); 365 (malaria); two colors freely configurable for research configuration
Zeiss	Primo Star iLED	Integrated microscope	Integrated, above filter cube	455 nm/385 nm/470/N-White for excitation of red dyes
AMG	Evos	Integrated microscope	Integrated, above/with filter cube	Three light cubes, DAPI, GFP, RFP
Zeiss	Colibri	Microscope illuminator	Replaces HBO	Four, depending on modules and beam combiner settings, two of which can be switched with ms speed
Olympus	PrecisExcite	Microscope illuminator	Replaces HBO	up to three
Leica	SFL100/4000/7000		Replaces HBO	SFL4000 five colors, SFL7000 (one color?) has ms switching time, SFL100 470 nm
Thorlabs	Collimated LED source	Microscope illuminator	Replaces HBO	Single color
Prizmatix	Microscope-LED/dual-LED/multi-LED	Microscope illuminator	Replaces HBO	Modular design, up to three
ScopeLED	Fluorescence microscope illuminator FMI	Microscope illuminator	Replaces HBO	Four
CoolLED	pE-100 with PE combiner,/pE-1/pE-2	Microscope illuminator	Replaces HBO	pE-100 one color, combined up to two, pE-1 three colors, pE-2 four colors, fast switching
Mightex	TET-470	Microscope illuminator for brightfield microscope	Through the eyepiece	Single color, 470 or 590 nm
QBC Diagnostics	Paralens Advance	Microscope illuminator for brightfield microscope	Above the objective	Single color, 470 nm
LW Scientific	Lumin	Microscope illuminator for brightfield microscope	Above the objective	Single color, 470 nm
Fraen	AFTER (Amplified Fluorescence by Transmitted Excitation of Radiation)	Microscope illuminator for brightfield microscope	Above the condensor	Single, combinable to up to three, transmission mode!
Olympus	FluoLED	Microscope illuminator for brightfield microscope	Above the condensor	Single (480 nm EasyBlue), combinable up to three units transmission mode!
Volpi	Volpi IntraLED 2020 LED Light source	High power LED light source with 10 mm fiber optical output	Parallel light path via glass fiber, replaces HBO	White light
89 North	Heliophor	Pumped phosphor light engine	Replaces HBO but can also be used in different configurations	Up to six modules; The user can choose between: 405 nm (175 mW), 430 nm (200 mW), 480 nm (450 mW), 500 nm (425 mW), 530 nm (350 mW), 555 nm (365 mW), 580 nm (350 mW), 640 nm (240 mW), 670 nm (225 mW)

The manifold advantages of LED compared to tungsten and arc lamps are the main reason for their introduction in the life science, especially in microscopy.

Besides the favorable trait of near-monochromicity, a major advantage is the direct brightness control by the driving current. Unlike tungsten lamps, the driving current does not noticeably influence the emission spectrum of the LED. Moreover, LED can be switched on and off very rapidly, allowing the formulation of complex automated sensing applications. High-frequency pulsing and modulated modes make them well suited for fluorescent lifetime imaging microscopy (FLIM) applications and for the detection of Förster resonance energy transfer (FRET). This will be discussed in a later section.

LED Optics—Different Types and Beam Characteristics

Today's LED come in a wide variety of colors, output powers, and form factors. Next to the 3, 5, and 10 mm "classic" LED that are embedded in clear epoxy lenses, surface-mounted device-LED (SMD-LED) are a rapidly growing class. These SMD-LED are mounted onto a ceramic carrier. An integrated parabolic mirror is usually incorporated to enhance the radiation profile. Ultra-high power SMD-LED are constructed by molding multiple SMD-LED within one hermetic ceramic package. Since heat removal becomes increasingly important with higher power consumption, cooling is often provided by a heat sink.

Unlike most filament or arc lamps, LED are often housed together with collimating lenses or reflectors. Typical ultra-narrow beam LED with integrated dome lenses can focus the beam down to $\pm$ 4°, narrow beam LED around $\pm$ 8–20°. Besides highly focused LED, wide-beam LED for homogeneous illumination are also available with nearly every beam divergence (i.e., 70–140°). By the use of polycarbonate collimating catadioptic lenses for high power LED with double reflection systems, it is possible to focus the beam to $\pm$ 1.5° for long distance or spot illumination. Short wavelength (UV) LED use glass lenses that are not degraded by UV-light.

LED Coupling

Although incandescent illumination is often incorporated in the microscope tripod itself, most light sources are housed externally, either directly at the microscope or coupled via fiber optics. Moreover, they usually need special electronics, which is often implemented in additional devices.

In contrast, both the LED itself and its driving electronics have a small enough form factor to be easily incorporated into the microscope. Although small LED for bright-field illumination can be introduced without taking care about heating, more powerful LED require proper heat management. These heat management solutions range from simple passive cooling solutions like a copper block, to more advanced solutions, such as are needed for the light output required for fluorescent illumination applications, which incorporate an active cooling element like a fan. Keeping the LED at the proper temperature increases its life span considerably.

The optical path of the microscope has to be matched to the beam characteristics of the LED. Because of the great variety in LED-housing, a beam divergence from nearly 120° down to 1.5° needs to be taken into account. Generally, a collimator composed of lenses or mirrors is used to generate a parallel beam, which can be directly coupled into the microscope. The smaller the active area of the LED, the simpler the design of the collimating optics becomes. While single chip solutions can be nearly regarded as a point source and require just a single lens, multichip devices demand more complicated optics to achieve homogeneous illumination.

Even if the same form factor of LED is used, for instance in multiwavelength applications, chromatic correction may be necessary as well, but this is usually omitted.

LED optical output can be controlled by simply setting the driving current. Here, a tremendous advantage is the very low drift of the excitation maximum with the driving current, which becomes almost negligible with proper heat regulation. The voltage dropping at a LED depends on the color and ranges from 1.2 V (red) up to more than 4 V (UV-LED).

Two basic regimes for dimming LED are established; current adjustment and pulse width modulation (PWM). Although current adjustment uses a linear regulation from zero to the maximum current, PWM switches the maximum current with a frequency typically between 1 and 200 kHz. The brightness is regulated by the ratio between the on-time and the off-time (i.e., the duty cycle).

Commercially available drivers allow either current adjustment or PWM. Some devices allow both, which is especially interesting for multiwavelength applications or specialized applications where fast switching is required, like for instance FLIM or FRET. In such arrangements, the current control is used for brightness setting while the PWM-input switches the LED on and off.

Most amateur implementations use the LED with oblique illumination. The compromise in illumination homogeneity makes this solution less suitable for scientific use, but it is still used for qualitative fluorescence imaging with a stereo microscope or for whole-animal imaging.

LED are preferentially incorporated at one of the four conjugate planes of the illumination. All of these positions have been exploited for this purpose (see Fig. 1). In a properly Koehler-illuminated microscope, the intrinsically structured lamp filament emission transforms in a bright and even illumination in the specimen plane. The most suited LED position is the filament plane of the mercury lamp. In this case, no further matching is required.

Most LED emit a "Lambertian" light cone, in which the intensity is proportional to the cosine of the angle from which it is viewed. As was discussed above, the width of the cone is variable and depends on the optical properties of the LED packaging. Most commercially available LED illuminator units and complete LED fluorescence microscope setups (see Table 1) use the established position of the arc lamp (Fig. 1A) in an extra housing. An image of the die (LED chip) is formed in the first conjugate plane at the aperture diaphragm. With the lamp housing removed, the LED can be moved inside the optical path to this point.

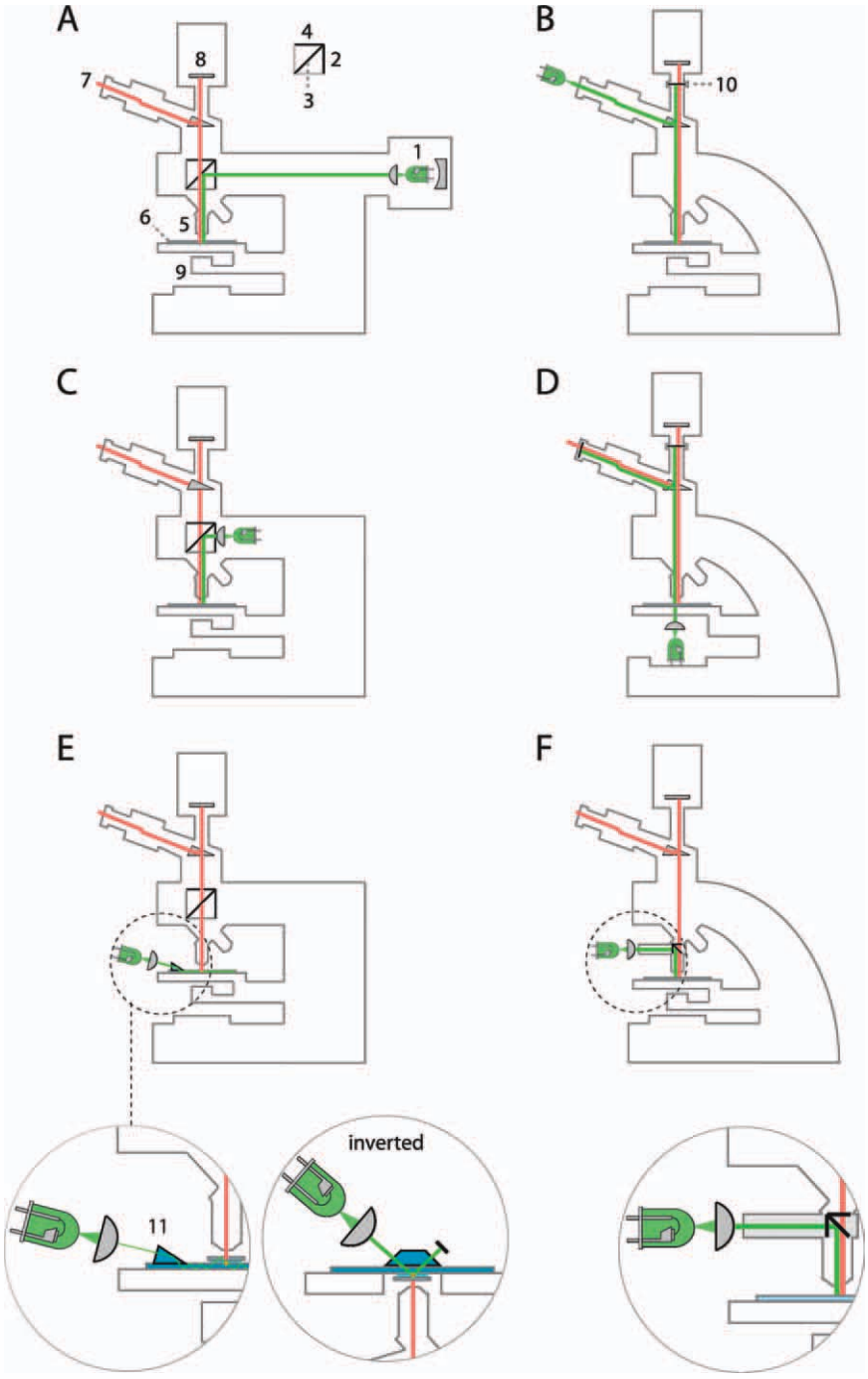

Figure 1. (**A**) Fluorescence illumination where a LED replaces the arc lamp at the same position. (1 LED, 2 excitation filter, 3 dichroic mirror, 4 emission filter, 5 objective, 6 specimen, 7 ocular, 8 camera, green path indicates excitation light, red path indicates fluorescence light). (**B**) LED directly mounted in the filter cube (10 emission filter). (**C**) LED mounted directly at the objective revolver with a dichroic mirror for reflecting the illumination light to the specimen but passing the fluorescence light to the ocular. (**D**) Ocular as illumination port. (**E**) Placement of the LED directly under the object. This arrangement not only allows simple bright field illumination but also, together with a highly blocking filter, transmission fluorescence imaging (11 coupling prism). (**F**) Illumination for fluorescence imaging without comprising the conventional light path of a bright field microscope. Either a prism is used to couple light into the side of the microscope slide or—as shown here for an inverted microscope—a prism is placed directly over the observation area onto the microscope slide and illuminated through the side windows. When the refractive index of the mounting medium is matched with the object and cover glass, a totally internally reflecting beam propagates through the object, generating fluorescence. The excitation beam does not enter the detection path.

As this requires no further adaptation of the microscope, we used this plane in our work with modulated LED for frequency-domain FLIM (23).

Another suitable position is the objective rear focal plane. Two solutions are on the market today. In a new design of fluorescence microscopes by Devos, the filter block turret is replaced by one that incorporates LED (Fig. 1C). This is made possible by the small size of the LED that allows a very compact design. After passing an excitation filter, the light is reflected by a dichroic mirror. The focus point lies in the rear focal plane of the objective. The fluorescence light from the object, as with the normal operation of a fluorescence microscope, passes through the dichroic mirror and through the emission filter to reach the image plane of the eye or a camera.

These adaptations refer to a standard fluorescence microscope with episcopic illumination for fluorescence excitation and a conventional filter block containing a dichroic mirror (excitation filter/dichroic mirror/emission filter).

However, LED make other fluorescence imaging arrangements, sometimes even using ordinary transmission microscopes, possible as well. The advantage is the high cost efficiency of the adaptation of existing microscopes that are for instance common in almost all physicians' practices. For these microscopes, a new illumination train has to be devised for the LED excitation light. A second suitable solution where the LED is placed at the objective rear focal plane therefore consists of a LED illumination holder that is screwed into the objective revolver head (Paralens and Lumin). This element accepts the objective on the other side. Inside the holder is a complete filter cube in which a dichroic mirror reflects the LED light onto the specimen (Fig. 1F).

Exchangeable dichroic, exciter and emitter filters are available for a number of diagnostic fluorescent assays.

Another unusual arrangement uses the microscope exit pupil conjugate plane as LED illumination entry position in the microscope (Fig. 1B). Even though this part belongs to the detection side, it still images the lamp filament, making it suitable as illumination port as well.

In a binocular microscope, one eyepiece can be used to excite the sample and the other to observe the fluorescence emission. The lack of a dichroic mirror means that the emission filter that is placed in the receiving eyepiece, or in the corresponding conjugate camera exit pupil plane, has to block all reflected and scattered excitation light. But it provides sufficient quality for most applications. Of course, the unit can be equally well placed in the exit pupil of the phototube, to observe fluorescence through both eyepieces.

One other commercial solution to retrofit bright-field microscopes with fluorescence capabilities deviates from the more conventional episcopic illumination and replaces the diascopic illumination train (for transmitted light detection) with a LED-based adapter (Fraen). As the condenser aperture diaphragm and the lamp filament are also conjugate illumination planes, placing the LED in any of these positions will create homogeneous illumination (Fig. 1D) of the specimen. Alternatively, using simple collimating optics, the LED is placed directly under the specimen, for example, on top of the condenser. Optical solutions can be derived that allow the LED illumination to operate without sacrificing the conventional transmitted light function.

However, since the excitation light reaches the observer's eyes (safety aspects!) or camera directly, high quality emission filters are required. Although (predominantly dark-field) diascopic illumination was the usual configuration before it was replaced by the now established episcopic arrangement, it is much less desirable for this reason.

Another elegant and simple solution is borrowed/inspired from illumination schemes known from prism-based total internal reflection microscopy (TIRFM) illumination arrangements, and was demonstrated for instance by Ely Silk.

Here, the glass object slide is made part of the optical elements that deliver the LED excitation light to the sample, perpendicular to the detection axis and without escaping into the objective (Fig. 1E). It should therefore work well in a microscope that is not equipped with (exciter-dichroic-emitter) fluorescence detection cubes, as a single emission filter will be sufficient to block the scattered excitation light. The solution consists of directly coupling the LED light into the glass object slide using a coupling prism (Fig. 1E, 11). The prism is optically coupled through immersion oil that matches the refractive indices of the prism and object slide glass. When collimated LED light is shone on the prism such that its reflection inside the prism directs the light in an angle above the critical angle for total internal reflection to occur, the light is effectively trapped in the glass slide acting as optical wave guide. When the specimen is observed using an air or water objective, and when the specimen is mounted in medium of sufficiently high refractive index, that is, similar to the glass of the glass slide and the cover slip, then the light will totally internally reflect at the interface of the glass slide and air, and the glass cover slip and air/water without escaping, illuminating the entire slide homogeneously. As the mounting medium is optically coupled, the excitation light will pass through it, exciting fluorophores that are contained within, causing these to fluoresce. The omnidirectional fluorescence does not undergo total internal reflection, and is therefore detected by the objective. This scheme can be varied with respect to the placement of the prism on the same or opposite side of the cover slip, depending on an upright or inverted orientation of the microscope. Furthermore, rather than using the glass slide as a wave-guide, the prism can also be placed directly over the cover slip on the opposite side. This configuration is closest to the original TIRFM application. The difference is that in TIRFM, the object is not optically coupled, but intentionally kept in low refractive index (watery) medium. Thus, the light cannot enter the object directly. However, now an exponentially decaying evanescent wave extends from the glass/water interface perpendicularly into the specimen and can excite fluorescence, producing very low background imaging in a thin optical slice of the object. This ingenious application for the coupling of alternative light sources bypasses the hardware of the microscope completely, and is therefore useful on any existing systems without special modification. It has been used in miniaturized, cost-effective solutions especially for the

advantage of not requiring a dichroic beam splitter arrangement (17–19,24).

LED as Specialized Illumination Sources for FRET and FLIM

One of the greatest advantages of LED is the simple control of the excitation energy without noticeable changes of their emission spectrum. Moreover, they allow fast switching, down to nanoseconds with no additional optical components.

FRET and FLIM benefit tremendously from this behavior. The transfer of excited state energy from a donor to an acceptor fluorophore by FRET carries consequences for the spectral emission yields (donor quenching and acceptor sensitized emission) and for the fluorescence decay characteristics of both fluorophores. The former intensity-based changes can be easily recorded by fluorescence microscopy. When measuring changes in FRET biosensors in which the donor and acceptor fluorophores, often a cyan and yellow fluorescent protein, are carried by the same sensor polypeptide, two acquisitions (donor emission upon donor excitation $F_D{}^D$, acceptor emission upon donor excitation $F_A{}^D$) suffice for a qualitative detection of FRET. Full quantification of the FRET efficiency, and the detection of interactions between individually labeled proteins, require an additional acquisition step (acceptor emission upon acceptor excitation $F_A{}^A$). Essentially, the $F_D{}^D$ and $F_A{}^D$ measurements are influenced by FRET as $F_D{}^D$ decreases (energy is removed from the donor excited state) while $F_A{}^D$ increases (as the energy is used to occupy the excited state of the acceptor, it will start to emit). Their ratio is therefore a highly sensitive indicator for FRET. Intrinsic uncertainties, however, prevent a straightforward interpretation of these signals in terms of efficiencies as the excitation and emission spectra of both fluorophores are rather broad that causes some overlap. Not all photons that contribute to $F_A{}^D$ are therefore emitted from the acceptor, but originate from the long-wavelength tail of the donor spectrum (bleed-through) rather than being generated by FRET. Moreover, some fluorescence originates from direct excitation within the short-wavelength tail of the acceptor excitation spectrum (direct excitation). As long as one is only interested in following the FRET process qualitatively and relatively (in time, or upon treatment), and the spectral contamination is constant (which is the case for a single FRET polypeptide construct), the dual emission, single excitation method yields very useable information. Any change in the ratio is attributable to FRET, although its absolute value cannot be determined. However, quantitative assays require rigorous signal calibration as variations in the spectral contamination due to uncertain environmental conditions or background fluorescence can become a real problem.

This happens for instance if the concentrations of both fluorophores are not constant, as is the case when they are attached to different proteins that appear in unknown stochiometry with local variations throughout the cell. To overcome this problem, a third acquisition of $F_A{}^A$ is required to obtain information on the concentration of the sensor or acceptor component.

Furthermore, additional sample-independent control measurements are required on cells that exclusively express either the donor or acceptor in order to judge the absolute magnitude of the spectral contamination. This is discussed in detail elsewhere (25).

In any case, it is clear that the need for multiple acquisitions is a limiting factor when rapid FRET changes are expected in a cellular physiological response.

When using a fluorescence lamp, these acquisitions come with significant delays as the switching between colors are accompanied by the opening and closing of mechanical shutters, and the movement of filter cubes in the turret. Although fast switching solutions based on galvoscanners or Pockels-cells exist, they are expensive and require complicated construction. The development of electrically shifted optical bandpass filters using liquid crystals was discontinued because of the low selectivity of these devices.

Here, LED pave the way for fast and cost-effective solutions. In contrast to LDs, they are available throughout the visible spectrum. Moreover, on account of their high price, wavelength-adjustable lasers, like dye or supercontinuum lasers, do not present an acceptable alternative for most labs.

With an arrangement with two spectrally dedicated detectors, or an imaging beam splitter that projects donor and acceptor images side-by-side on the same detector chip, the only time-consuming step is the switching of the excitation light. With LED, this is done with effectively no delay as the acquisition times on the order of milliseconds are much longer than the switching time.

The opening of a mechanical shutter takes milliseconds and can cause image-degrading vibrations. The whole acquisition series would therefore take considerably longer in open-acquire-close cycles as compared to the very rapid direct switching of the illumination. Additional delays are introduced when the acquisition software waits for open/close signals before image acquisition.

The rapid switching capability of LED can be used to further increase acquisition speed by stroboscopic imaging. The LED flash and exposure time can be set significantly shorter than the camera read-out time, so that the sample is strobed rather than continuously illuminated. In an article on fast moving sperm cells, the effective time resolution could be enhanced by a factor of 20 using stroboscopic LED illumination (26). Furthermore, by minimizing the integrated illumination time, this approach reduces photobleaching and phototoxicity (27). Stroboscopic fluorescence excitation imaging is furthermore very useful in suppressing movement artifacts in living animals as it allows the synchronization with the cardiac cycle (28). All these functions can be incorporated in the direct control of the illumination by LED.

Assessing the fluorescence lifetime adds another independent characterization parameter, which is especially useful for the quantitative analysis of FRET.

In FRET, the fluorescence of the donor $F_D{}^D$ decays faster because the emission probability increases with dipole coupling to an acceptor.

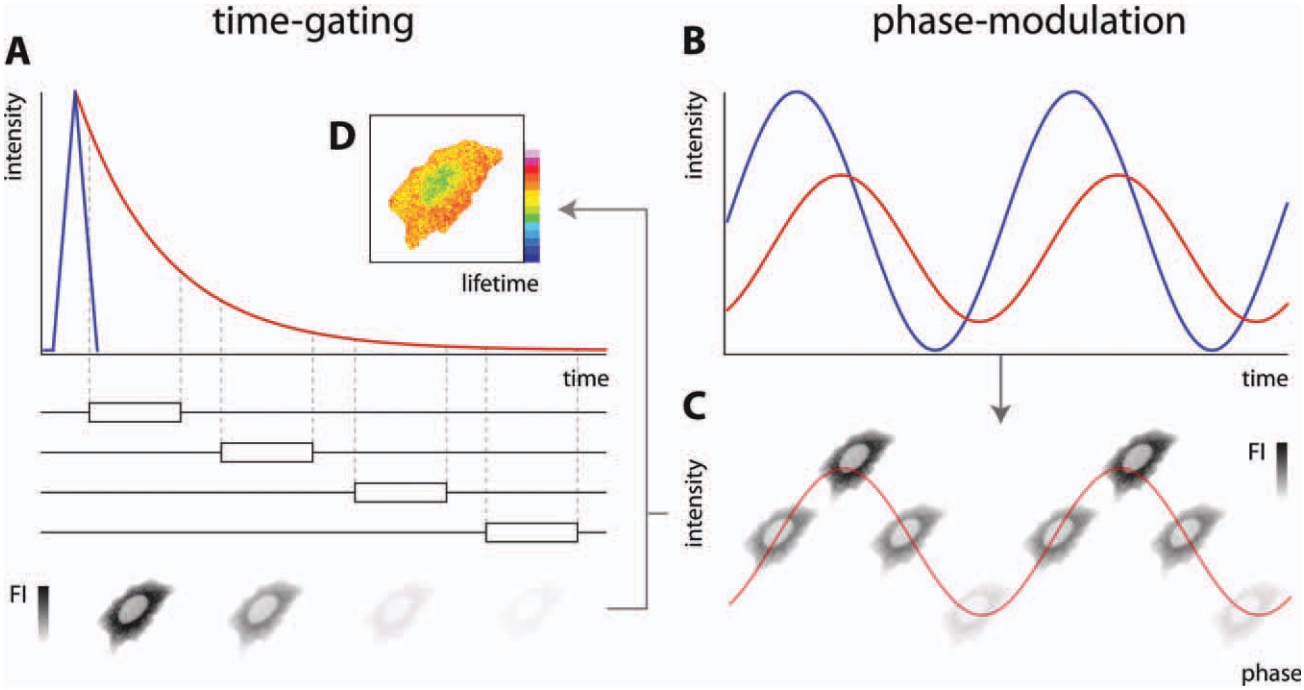

Figure 2. (**A**) In time-gating fluorometry, a picosecond-resolution gated (image intensifier-based) camera is triggered to record images at defined time intervals (boxes) after each light pulse (blue trace). The exponential decay of the intensity (FI, fluorescence intensity) in these images (red trace) is described by the fluorescence lifetime and can be obtained by fitting. TCSPC can be seen as an extreme form of time-gating with high numbers of very short time bins. Rather than taking images, a sensitive point detector records the arrival times of individual photons. The scanning variant of time-gating collects many photons in time bins, but not in the form of images directly. It provides a coarser ''bar graph'' representation of the decay curve than TCSPC. (**B**) In phase-modulation fluorometry, the information on the lifetimes is contained in the shift in phase and modulation depth of the fluorescent signal (red trace) following high-frequency periodic excitation (blue trace). (**C**) This temporal information becomes available by the addition of discrete phase steps in the gain of the image intensifier. In effect, the emission sine wave can now be reconstructed from the resulting intensity changes in the phase images, which can be taken at the limiting temporal resolution of the imaging system (lower right panel). The excitation signal can be obtained by the measurement of a reference object; reflecting foil, scattering medium, or the solution of a fluorophore with a known lifetime. (**D**) The resulting map of decay times from these methods is the lifetime image and can be displayed in an appropriate false-color lookup table.

In the ideal case, fluorescence decay shows first order kinetics, that is, it decays exponentially where the decay constant, called the fluorescence lifetime, is determined with spatial resolution in FLIM.

The relative decrease in fluorescence lifetime is directly proportional to the efficiency of FRET, and is independent of fluorophore concentration, excitation intensity, or light path; a feature that is very useful for quantitative fluorescence FRET measurements in living cells. Details on the use of FLIM for FRET are given elsewhere (25–27).

The major difficulty with FLIM is the short fluorescence lifetime on the order of nanoseconds for most fluorophores that are used in cell biology. This implies an accuracy of a few tens of picoseconds for a resolution of a few percent of FRET change.

Two principles for obtaining the fluorescence lifetime are established, time domain techniques like time-correlated single photon counting (TCSPC) and time-gating fluorometry, and phase-modulation fluorometry (see Fig. 2). In TCSPC a laser flash (fs- up to ps- second duration) excites the probe and starts a counter. The arrival of a fluorescence signal stops the counter. The assumption of counting single photons only holds if the excitation probability is sufficiently low. For this reason, excitation power does not have to be very high, making this method suitable for use with ultra-short pulsed LED. Repeated measurements per pixel provide the arrival time statistics from which the fluorescence lifetime can be determined. Scanning over the surface of the probe yields the 2D image. Time-gating fluorometry collects photons in a number of defined nanosecond-length time bins after each pulse. In scanning microscopes, electronic counters are activated one after the other at each image location to form the time bins. Gating can also be performed in a wide-field microscope. Here, fluorescence images are directly acquired in the time bins. The emission decay response is reconstructed in 2D from the intensity profile of the successive images. Camera gating is achieved with the use of image intensifier-coupled cameras.

In phase fluorometry, a modulated fluorescence signal (typically 50–100 MHz) is used and the phase shift (and demodulation) between excitation and emission signal carries information about the fluorescence lifetime. As this type of FLIM is also typically performed using image intensifier-coupled cameras, the 2D image is available without the need for scanning. The latter methods, implemented in wide-field microscope systems, are typically faster than their TCSPC counterpart.

Table 2. High-frequency modulated and pulsed LEDs for fluorescence lifetime imaging microscopy (FLIM)

COMPANY	PRODUCT	PULSE-WIDTH/MODULATION FREQUENCY	COLORS
Lambert Instruments	Multi-LED	80–120 MHz harmonic modulation, depending on wavelength	Four, covering the range of 360–640 nm
Picoquant	PLS series subnanosecond pulsed LEDs	Down to 500 ps FWHM, up to 40 MHz repetition rate	11, covering the range from 255 to 600 nm
Jobin Yvon Horiba, IBH	NanoLED	1.1–1.4 ns, 1 MHz repetition rate	Eight, covering the range from 455 to 625 nm
Thorlabs	Modulated LED source for FLIM microscopy	10–100 MHz harmonic modulation	Four: 365, 405, 470, 630 nm, other wavelengths up to 940 nm on request

Conventional light sources like mercury lamps or lasers are not suited for this application or are very expensive. Although it is possible to modulate the mercury lamps at high frequency, their spectral noise will diminish the useful signal. Fast switching with opto-electronic shutters is rather expensive and therefore rarely available. Continuous wave lasers can be modulated using optoelectronic equipment like Pockels-cells or acousto-optical modulators as it is done for intrinsic switching in mode-locked and multiphoton lasers.

LDs and diode-pumped solid-state lasers can be switched and modulated, but they are available for a limited number of lines (colors).

LED, however, can be both pulsed in the nanosecond regime (28,29) and can be modulated at tens of MHz (23,30,31). Harmonically modulated near-infrared LED have also been used to image fluorescence lifetimes in small experimental animals (32).

Some of these solutions are also offered commercially (Table 2).

Together with recent developments in directly demodulating solid-state cameras for FLIM, modulated/pulsed LED hold the promise for affordable and flexible, multispectral FLIM/FRET applications for the larger community of biomedical scientists and clinicians.

Assets and Drawbacks

With recent developments in brightness and efficiency, LED possess considerable advantages over established illumination

Table 3. High-power LEDs for flexible fluorescence and light microscopy application

COMPANY	PRODUCT	DESCRIPTION	COLORS
Luxeon	High power Luxeon Star, Hexagonal package, 1 W	High power LED, diverse wavelengths, power chip technology, actually world's brightest LEDs lambertian, high dome diameter: 20 mm	455 nm, 150 mW at 350 mA 470 nm, 10 lm at 350 mA 505 nm, 30 lm at 350 mA 530 nm, 25 lm at 350 mA 590 nm, 36 lm at 350 mA 617 nm, 55 lm at 350 mA 625 nm, 44 lm at 350 mA
Roithner, Austria	High power LED HP803 series	High power LED emitter, 3 W electrical power, highest flux, very long operating life ($\gg$50,000 h) higher energy efficiency than incandescent and most halogen lamps low voltage DC operation instant light on/off (less than 100 ns) superior ESD protection, base diameter 7.4 mm, height 6.87 mm	White, type 6,000 K, 130–160 lm at 700 mA Warm white, type 3,300 K, 140–170 lm at 700 mA 470 nm, 20–30 lm at 700 mA 530 nm, 80–110 lm at 700 mA 590 nm, 36 lm at 700 mA 625 nm, 50–70 lm at 700 mA
Roithner, Austria	High power LED, C20A1 series	High power LED, aluminium disk package, diameter 20 mm, 1×1 mm^2 chip size. 1 W electrical power	395–405 nm, 15–20 mW at 350 mA 470 nm, 12–14 cd, 17–20 mW at 350 mA 525 nm, 25 cd, 13–17 mW at 350 mA and further wavelength at 590, 625, 650, 670, 680, 690, 720, 740, 760, 770, 820, and above.

sources like arc lamps or lasers. The excitation energy within the desired bandwidth already almost matches that of arc lamps (0.3 W/nm), but their high efficiency (>100 Lux/W) makes them the preferred solution for battery-powered devices, which is especially useful in remote locations or for portable devices. Other than laser devices, LED are available in a wide range from ultraviolet up to infrared with only small gaps (see Table 3).

Because of the small dimension of the active pn-region, they can be regarded as point sources, greatly facilitating their application, especially for homogeneous illumination. However, different integrated optics such as lenses or mirrors influence the radiation-profile, which needs to be taken into account when designing illumination devices.

LED exhibit a narrow band spectrum between 20 and 40 nm of half bandwidth. This is compatible with most fluorescence applications, such that excitation filters can be omitted. Additional filters are only required for dyes where the shift between absorption and emission is rather small, or when imaging two spectrally closely positioned dyes where small shifts are important, like for FRET and other ratiometric approaches. Another advantage of the narrow excitation band is the noise reduction due to the removal of unintended excitation of fluorescence in the short wavelengths that are not completely blocked by common excitation filters. For instance, the DAPI-filter set will not completely block the UV components of the mercury lamp, which can excite flavines, NADP or other fluorescent proteins whose emission then contribute to the background fluorescence. The absence of UV light also contributes to the lower phototoxicity that is associated with LED illumination. The use of 365-nm diodes can easily prevent this problem. Nevertheless, safety prevention regulations (e.g., by adding a respective blocking filter in the eye path) apply when using LED with emission wavelengths lower than 400 nm, because this light is harmful to the eyes.

Established fluorescence microscopy arrangements use a filter block, which guides the illumination light to the objects and reflects the fluorescent light to the detector (eye or camera). Given their small size, LED illumination can be incorporated directly into the filter block (Fig. 1C, e.g., in the Devos microscope), thus eliminating the need for the external lamp. For multiple wavelength applications, several LED are required with optical merging. This is also the basic arrangement for fast switching between different wavelengths.

LED have an extremely high durability and a long life span. This however, depends on the condition during operation as well. High temperature of the pn-junction, caused by high driving currents, decreases the life span from 50,000 down to 20,000 h, which is still considerably more than the life span of an arc lamp (400 h). In low-light application like in bright-field applications, LED can run on a lower current where the LED life span probably exceeds this of the entire apparatus (>100,000 h). Given the much lower cost for the LED and the driving electronics, LED-applications are by far more economic than arc lamps or lasers. Given these specific advantages of LED illumination, we expect these devices to firmly occupy standard and advanced microscopy applications in the near future.

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