Journal of Microscopy, Vol. 222, Pt 2 May 2006, pp. 135–140
Received 24 November 2005; accepted 23 January 2006

SHORT TECHNICAL NOTE

Filter cubes with built-in ultrabright light-emitting diodes as exchangeable excitation light sources in fluorescence microscopy

C. MOSER, T. MAYR & I. KLIMANT
Graz University of Technology, Institute of Analytical Chemistry, Technikerstrasse 4, 8010 Graz, Austria

Key words. Fluorescence microscopy, light-emitting diode, luminescence lifetime imaging, mercury arc lamp.

Summary

The use of ultrabright light-emitting diodes as a potential substitute for conventional excitation light sources in fluorescence microscopy is demonstrated. We integrated ultrabright light-emitting diodes in the filter block of a conventional fluorescence microscope together with a collimating Fresnel lens, a holographic diffuser and emission filters. This setup enabled convenient changes between different excitation light sources and resulted in high excitation efficiencies. Quantitative comparison of image intensities of test samples revealed that light-emitting diodes yielded intensities in the range of a mercury arc lamp depending on the wavelength. The use of ultrabright light-emitting diodes also enabled luminescence lifetime imaging without the need for image intensification.

Introduction

Solid-state light sources have gone through a breathtaking development during the past few decades and are now starting to replace conventional light sources in various applications (Schubert & Kim, 2005). Despite the continuous increase in luminous efficiency and overall light output of light-emitting diodes (LEDs), the maximum theoretical potential has not yet been reached.

Fluorescence microscopes are at present mainly operated with high-power lamps, such as mercury and xenon arc lamps, as excitation light sources. They have a long tradition as the excitation light source but suffer from disadvantages. Mercury arc (HBO) lamps emit a weak broad-spectrum irradiation that is overlapped by strong mercury emission lines. There are several useful emission lines in the ultraviolet (UV) and visible spectral range but none between 435 and 546 nm and none at wavelengths higher than 580 nm (Rost, 1992). When used as the excitation light source, only a small part of the emission spectrum illuminates the specimen and the rest is filtered by bandpass filters. As a result, a lot of excess light and heat are produced. Other drawbacks of high-power lamps are the short lifetime of the lamp together with the high cost of replacement, danger of explosion and generation of ozone (Rost, 1992). The emission of mercury arc lamps is not completely stable over time and they are therefore not well suited for quantitative measurement of fluorescence signals. Xenon arc lamps emit a broad band spectrum without prominent lines. In general, their emission is more stable but less intense, especially in the blue and UV region.

Lasers are another light source employed in fluorescence microscopy. They emit monochromatic light with high-power efficiency. Because of the narrow beam, lasers are commonly employed in scanning mode. Attempts to widen the narrow laser beam result in a non-uniform illumination pattern called speckles, due to the coherency of laser light. Efforts have been made to reduce speckles to some extent (Ambar & Aoki, 1985; Saloma *et al*., 1990).

Until now, LEDs have been mainly used in applications in fluorescence microscopy, where the lack of emission intensity is outweighed by other advantageous features. Their low cost inspired the construction of cheap fluorescence microscopes, e.g. for use in teaching. Haseloff (2003) described the construction of a low-cost fluorescence microsope for teaching by adapting a non-fluorescence microscope. A similar assembly is commercially available from Hund (Wetzlar, Germany). Another concept uses the glass microslide bearing the specimen as a waveguide (Silk, 2002). The low power consumption and mechanical robustness of LEDs are also of importance for specialized microscopes in space flight and field applications

Correspondence to: I. Klimant. Tel: +43 316 873 4328; fax: +43 316 873 4329; e-mail: klimant@tugraz.at

(Jones *et al.*, 2005). Furthermore, the possibility of modulating the LED emission at MHz frequency has made LEDs a valuable tool in fluorescence lifetime imaging, although this technique relies on the use of sophisticated and expensive image intensifier equipment (Herman *et al.*, 2001).

As a result of the substantial progress in the development of ultrabright LEDs, a palette of devices covering the whole visible spectrum together with the adjoining UV and infrared region has become available from various suppliers during the past few years. In parallel with this development, the meaning of 'ultrabright' has evolved, as LEDs with a power input of up to 5 W are now available. Although most commercial producers now offer LED illuminators for brightfield microscopy, adaptors for fluorescence are still not widely available.

In this study a versatile and simple way of applying ultrabright LEDs as light sources to a conventional fluorescence microscope is described. Due to the limited space in the filter cube, small and optimized components had to be selected. The optical setup comprises an ultrabright LED, a Fresnel lens as collimating component, optionally an optical filter and a diffuser to obtain uniform illumination of the specimen. Mountings were designed to integrate these parts into the filter block, in place of the excitation filter. The dichromatic mirror and barrier filter remain unaffected and are selected to fit the spectra of the LED and fluorophore. The main advantage of this design is the ability to switch between different excitation wavelengths (i.e. LEDs) and corresponding filter sets by moving the filter slider. Thus, it is also possible to change between LED and HBO illumination when placing an LED block and an arc-lamp filter set on the same slider. The setup was optimized in terms of emission intensity and uniformity. The performance was evaluated for several common fluorophores and compared with HBO lamp excitation. Additionally, luminescence lifetime imaging using a triggered CCD (charge-coupled device) camera was applied.

Materials and methods

Microscope

All experiments were carried out on a Zeiss (Gottingen, Germany) Axiovert 25 CFL inverted fluorescence microscope with epi-illumination and standard objectives (10× Achromat, 20× and 40× LD A-Plan, Zeiss). The original filter cube was designed to hold standard 25-mm circular excitation and barrier filters. A mercury arc lamp (HBO 50 W, 40 operating hours, Osram, Munich, Germany) was used as the excitation light source. Images were acquired by a SensiCAM camera (cooled CCD-Chip, 640 × 480 pix, black and white, 12-bit colour depth, PCO, Kehlheim, Germany). The equipment and image acquisition process for fluorescence lifetime imaging in the low microsecond range were adapted from Liebsch *et al.* (2000).

Specimen

Uniformity was optimized using adhesive fluorescent films (mactac, Soignies, Belgium). To compare the intensity of excitation light sources, fluorescent microspheres were chosen as the reference specimen. They are available with a number of standard fluorophores incorporated and provide reproducible fluorescence intensities. Melamine-formaldehyde resin microspheres stained with fluorescein 5(6)-isothiocyanate (diameter 6 μm, Ex_{max} = 490 nm, Em_{max} = 525 nm) and Nile blue (diameter 6 μm, Ex_{max} = 633 nm, Em_{max} = 672 nm) were purchased from Sigma-Aldrich (Vienna, Austria). Melamine-formaldehyde resin microspheres stained with Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride (diameter 8 mm, Ex_{max} = 440 nm, Em_{max} = 612 nm) were obtained from Microparticles (Berlin, Germany). Magnetic polystyrene microspheres of 6-μm diameter were purchased from Micromod (Rostock, Germany) and stained with Pt(II) meso-tetra(pentafluorophenyl)porphine showing excitation and emission maxima of 404 and 652 nm, respectively. To obtain specimens showing the selected fluorescence lifetime, magnetic polystyrene microspheres were stained with combinations of Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride and crystal violet as acceptor dye (Moser *et al.*, 2006). Optical filters from Zeiss, Omega Optical (Brattleboro, VT, USA) and Schott (Mainz, Germany) were used. The filter sets corresponding to each fluorophore are listed in Table 1. To achieve a fair comparison, the spectral bandwidth of the Zeiss excitation filter was adjusted to the emission of the LED by applying an additional long-pass filter when measuring Nile blue microspheres.

Table 1. Fluorophores and corresponding filter sets.

Fluorophore	Excitation filter	Dichromatic mirror	Barrier filter
FITC	Omega 475AF20	Omega 500DRLP	Omega 510AF23
Nile blue	Zeiss BP575-625 + Schott RG610	Omega 630DRLP	Schott RG665
$Ru(dpp)_3Cl_2$	Omega 475AF20	Omega 500DRLP	Schott OG550
Pt-Por	Omega 405DF10	Omega 420DCLP	Schott RG570

FITC, fluorescein 5(6)-isothiocyanate; $Ru(dpp)_3Cl_2$, Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride; Pt-Por, Pt(II) meso-tetra(pentafluorophenyl)porphine.

Light-emitting diodes

Blue ultrabright LEDs from Luxeon (San Jose, CA, USA; Luxeon V, 470 nm, 5 W, lambertian emission) and Roithner Lasertechnik (Vienna, Austria; Roithner Lasertechnik, 470 nm, 350 mA, 120°) were tested with fluorescein 5(6)-isothiocyanate and Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride microspheres.

Pt(II) meso-tetra(pentafluorophenyl)porphine excitation was achieved by a 395-nm LED (Roithner Lasertechnik; 350 mA, 120° emission). LEDs from Luxeon (Luxeon III, 617 nm, 1400 mA, lambertian emission) and Roithner Lasertechnik (625 nm, 350 mA, 120° emission) were characterized by measuring Nile blue microspheres.

In general, LEDs emit light at a certain colour equivalent to a narrow spectral range. However, the LEDs tested here have a spectral bandwidth of at least 50 nm at 10% of the maximum emission. Therefore, the use of an excitation filter is necessary to reduce background in the fluorescence image if spectra of the LED and barrier filter overlap. Blue LEDs were used with a DT-blue filter (Linos Photonics, Gottingen, Germany) and red LEDs with a 645-nm short-pass filter (OIB, Jena, Germany). The UV LED was used without an optical filter.

Mountings

Detailed dimensions of the mountings are given in Fig. 1. Figure 2 shows a filter block bearing the LED setup mounted on a filter slider together with a filter block bearing a conventional filter set. The jacket acts as a carrier for the Fresnel lenses and additional optical components. The LEDs were mounted to the centre of the capsules by means of heat-conductive paste and an adhesive. Both mountings were turned from aluminium to support heat conduction. They were designed to achieve a maximum distance between the LED and dichromatic mirror in the narrow space that is limited by the filter block and the surrounding body of the microscope. All components were selected with regard to small size. Collimating Fresnel lenses made of acrylic plastic (positive focal length 11 mm, cut to a diameter of 21 mm) were purchased from Fresneltech (Forth Worth, TX, USA). A 10° circular holographic diffuser was purchased from Edmund Optics (Blackwood, NJ, USA) and cut to fit the capsule.

Fig. 2. Photograph of the filter block with capsule and jacket mounted and light-emitting diode switched on.

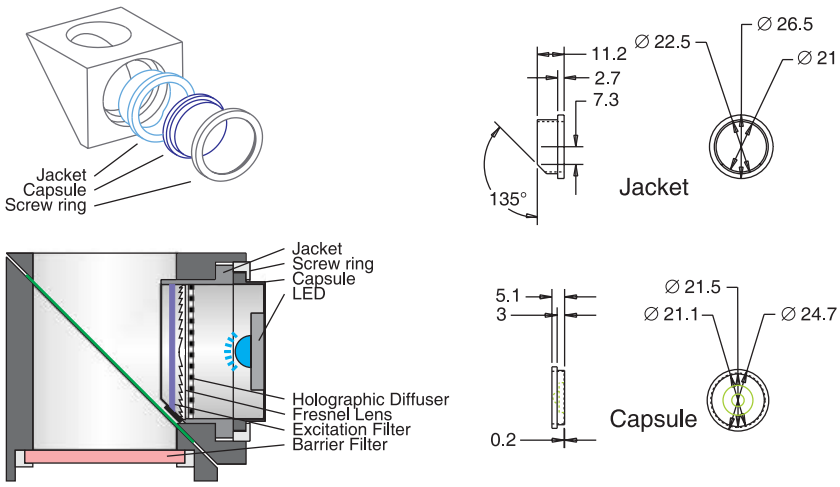

Fig. 1. Schematic drawing of the mountings and filter block. All dimensions in mm. LED, light-emitting diode.

A black film was fixed to the dichromatic mirror where it touched the chamfered part of the mounting jacket to block the LED's stray light from reaching the barrier filter directly.

Measurement and image evaluation

Microspheres were diluted in microplate wells. After sedimentation to the transparent bottom of the microplate, the layer of microspheres was examined through the microscope with the HBO lamp and subsequently with the LED configurations. Dichromatic mirrors and barrier filters remained unchanged for each individual fluorophore. All images of one type of fluorophore were taken with the same CCD integration time.

The fluorescence intensity of the microspheres in the images was analysed by a MATLAB-script (The Mathworks, Natick, MA). First, a background image, taken from an empty well filled with water, was subtracted from the analysed images. Subsequently, microspheres in the image were detected by LoG (Laplacian-of-Gaussian operator) edge detection and circular Hough transform (Sonka *et al.*, 1999). Grey values of the central region of all beads were averaged to give a measure of the fluorescence intensity.

Results and Discussion

Despite significantly better ratios of fluorescence excitation : power input compared with arc lamps, ultrabright LEDs driven at maximum power generate a substantial amount of heat. Nevertheless, the LEDs can be run in continuous mode because the heat is guided by the aluminium capsule and dissipated in the massive metal filter block as heat sink. Depending on the LED, a decrease in emission intensity of up to 10% is observed during the first minute at continuous maximum power operation before reaching a constant level. This observation is attributed to the reduced efficiencies of LEDs at higher chip temperatures. The effect is reversible by shutting down the LED for cooling. The plastic Fresnel lens and holographic diffuser are not affected by the moderate temperature increase inside the mounting. Compared with operation with the arc lamp, the excitation filter is less loaded with excess light that would be converted to heat. However, shutters protecting sensitive samples from light between observations become obsolete, as LEDs can be dimmed and switched on and off electrically.

When applying the LED filter cube without Fresnel lens and holographic diffuser, the sample is excited only in the centre of the field of view and the emission intensity is low (Fig. 3a). Collimating lenses of conventional design with a short focal length are too bulky and would prevent the installation of filters and diffusers. Consequently, a collimating Fresnel lens is inserted between the LED and optical filter. As a result, the beam of light closely resembles the beam generated by the arc lamp. The emission filter is passed by collimated light, which is necessary for proper filtering.

The dimensions and emission patterns of LEDs vary by supplier and type. Therefore, the position of the Fresnel lens had to be adjusted individually with respect to the intensity and uniformity of the emission light for each type of LED (Table 2). In general, the best results were obtained when positioning the Fresnel lens such that the chip of the LED was located approximately the in focal plane of the lens. The uniformity of the illumination over the whole imaging area depends on the optics of the LED. The 5-W Luxeon LEDs come with lambertian emission optics that, when collimated, result in a non-uniform illumination of the specimen (Fig. 3b). The pattern of illumination corresponds to the geometry of the LED chip. To avoid this obstacle, the holographic diffuser was inserted into the light path. Thus, uniform illumination was achieved at the cost of some excitation intensity (Fig. 3c). However, compared with conventional diffusing glasses, the losses from side scattering are substantially lower when applying a holographic diffuser with defined diffusion angle. The 1-W

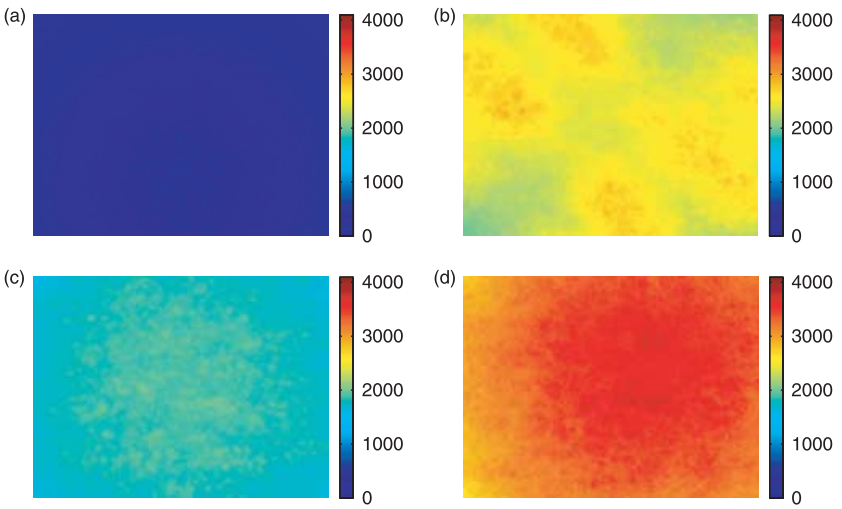

Fig. 3. False colour intensity images of a grainy green fluorescent film demonstrating the uniformity of illumination with a blue Luxeon 5-W light-emitting diode (LED) (a–c) and Roithner Lasertechnik 1-W LED (d). (a) Without Fresnel lens and without holographic diffuser; (b) with Fresnel lens but without holographic diffuser and (c) with Fresnel lens and holographic diffuser in the excitation light path. (d) Taken with 1-W blue Roithner Lasertechnik LED with Fresnel lens but without holographic diffuser. All images were taken at the maximum LED forward current with 1-ms CCD integration time using the 40× objective. Field of view is 330 × 250 µm.

Table 2. Operating conditions and results of image evaluation for the fluorophores tested.

Fluorophore	Excitation light source	LED current (A)	Distance x (mm)	Fluorescence intensity		
				Mean intensity (a.u.)	CV (%)	% of HBO
FITC	HBO	–	–	7048	15	100.0
	Roithner Lasertechnik 470 nm	0.37	12.5	5314	16	75.4
	Luxeon 470 nm + diffuser	0.80	12.5	2775	16	39.4
Nile blue	HBO	–	–	4434	12	100.0
	Roithner Lasertechnik 625 nm	0.37	14.0	2290	13	51.7
	Luxeon 617 nm	0.80	13.5	3861	18	87.1
	Luxeon 617 nm + diffuser	0.80	13.5	2114	11	47.7
	Luxeon 617 nm + diffuser	1.40	13.5	3665	10	82.7
$Ru(dpp)_3Cl_2$	HBO	–	–	2492	16	100.0
	Roithner Lasertechnik 470 nm	0.37	12.5	1512	16	60.7
	Luxeon 470 nm + diffuser	0.80	12.5	716	21	28.7
Pt-porphin	HBO	–	–	11292	11	100.0
	Roithner Lasertechnik 395 nm	0.37	12.5	2585	8	22.9

Mean intensities and coefficient of variation (CV) are normalized to 100 ms CCD integration time. x is the distance between the bottom of the capsule and the Fresnel lens. FITC, fluorescein 5(6)-isothiocyanate; $Ru(dpp)_3Cl_2$, Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride; LED, light-emitting diode; a.u., arbitrary unit.

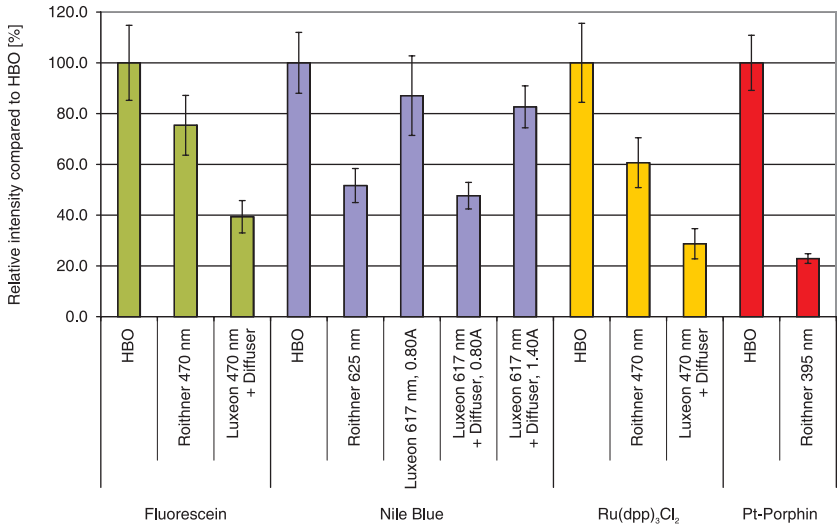

Fig. 4. Relative fluorescence intensities obtained for different types of fluorescent microspheres, used as reference specimen. Detailed values and operating conditions are shown in Table 2.

Roithner Lasertechnik LEDs with a 120° emission angle showed good uniformity without a holographic diffuser and therefore achieve a higher overall intensity (Fig. 3d).

Beads stained with common fluorescent dyes were chosen as reference specimens for quantitative comparison of setups and light sources because of their better measurement reproducibility compared with biological samples. However, we expect the proportions of intensity found in this work to also be valid for other fluorescent dyes and applications in the spectral regions under investigation.

The results of the direct comparison of LEDs and the HBO lamp are summarized in Table 2 and Fig. 4. Results were obtained using a 40× objective. A minimum of four images were evaluated, each showing 60–500 randomly distributed beads. Similar intensity ratios were also obtained for 10× and 20× objectives. Objectives with higher magnification are not available for our instrument. Nevertheless, we expected analogue results because the setup is based on mimicking the light path of conventional arc lamps.

The comparison reveals that the optics attached to every LED play an important role in optimizing intensity and uniformity. Although Luxeon LEDs were driven at higher power and provided brighter emission, their performance in this setup was impaired by the need to apply holographic

diffusers to obtain uniform illumination. Therefore, Roithner Lasertechnik LEDs achieved similar or better results. The increased level of coefficient of variation for the 617-nm Luxeon LED without holographic diffuser may be attributed to the non-uniformity of illumination, whereas the main source of SD is the variability of the beads themselves.

Compared with HBO excitation, the optimized LED setups show around 80% excitation efficiency for fluorescein 5(6)-isothiocyanate and Nile blue. UV excitation of Pt-porphin is also possible but is not as efficient as the UV emission of the HBO lamp. The application of luminescence lifetime imaging in the time domain down to 1-µs decay times is demonstrated by means of luminescently encoded beads. Encoded beads are of increasing importance in multiplexed bioanalysis (Yingyongnarongkul *et al.*, 2003). We applied luminescence lifetime imaging to decode randomly ordered bead arrays. The luminescence decay of the encoded beads was adjusted by staining beads with a combination of Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride and crystal violet as the fluorescence resonance energy acceptor (Moser *et al.*, 2006). Thus, beads can be clearly distinguished and attributed to a bead class (Fig. 5).

The whole concept was tested on a Zeiss Axiovert 25 CFL microscope but can also be applied to other instruments.

Conclusion

The integration of all necessary components, including the LED and collimating lens, into the filter block results in a compact unit for each excitation wavelength and in efficient excitation of the specimen. A holographic optical diffuser proved to be suitable to obtain uniformity of illumination when the patterns of LED chips disturb the images. Additionally, the use of ultrabright LEDs in combination with a modulated camera and trigger devices allows luminescence lifetime imaging at low levels of fluorescence without the need for an image intensifier. Providing similar fluorescence intensities in the spectral range of commonly used fluorophores, the new generation of ultrabright LEDs can be utilized as useful excitation sources in fluorescence microscopy and has the potential to replace arc lamps especially when the advantageous features, such as low cost and long life, are desired. As the luminous output of each type of LED varies from one device to another, a further increase in intensity can be achieved by selecting the most efficient LEDs from a large number. Although mercury arc lamps are still the superior excitation source in the UV, in the near future they will be outperformed by LEDs in the visible range of light due to ongoing development in the field of solid-state light sources.

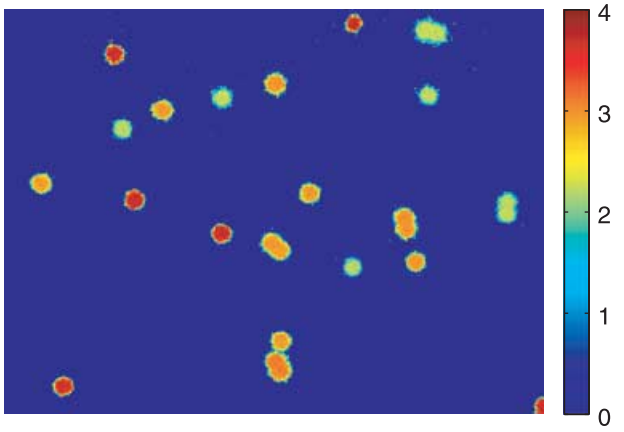

Fig. 5. Fluorescence lifetime image of beads with lifetimes of 3.6, 3.0 and 2.2 µs. Field of view is 330 × 250 µm.

Acknowledgements

The authors are very grateful to Eberhard Schutting who provided the schematic drawings of the design and to Günter Fellner for manufacturing the aluminium capsules and jackets.

References

Ambar, H. & Aoki, Y. (1985) Mechanism of speckle reduction in laser-microscope images using a rotating optical fiber. *Appl. Phys. B*, **38**, 71–78.

Haseloff, J. (2003) http://www.plantsci.cam.ac.uk/Haseloff/imaging/cheaposcope/cheaposcope.htm. Last checked in January 2006. Department of Plant Sciences. University of Cambridge, Cambridge, UK.

Herman, P., Maliwal, B.P., Lin, H.J. & Lakowicz, J.R. (2001) Frequency-domain fluorescence microscopy with the LED as a light source. *J. Microsc.* **203**, 176–181.

Jones, D., Broeckman, E., Derschum, H., Mandy, F. & Ries, H. (2005) LED fluorescence microscopy made for space for use on earth. *Royal Microscop. Soc. Proc.* **40** (2), 91–96.

Liebsch, G., Klimant, I., Frank, B., Holst, G. & Wolfbeis, O.S. (2000) Luminescence lifetime imaging of oxygen, pH, and carbon dioxide distribution using optical sensors. *Appl. Spectrosc.* **54**, 548–559.

Moser, C., Mayr, T. & Klimant, I. (2006) Microsphere sedimentation arrays for multiplexed bioanalytics. *Anal. Chim. Acta*, **558**, 102–109.

Rost, F.W.D. (1992) *Fluorescence Microscopy*, Vol. I. Cambridge University Press, New York.

Saloma, C., Kawata, S. & Minami, S. (1990) Laser-diode microscope that generates weakly speckled images. *Opt. Lett.* **15**, 203–205.

Schubert, E.F. & Kim, J.K. (2005) Solid-state light sources getting smart. *Science*, **308**, 1274–1278.

Silk, E. (2002) LED fluorescence microscopy in theory and practice. *Microscope*, **50**, 101–118.

Sonka, M., Hlavac, V. & Boyle, R. (1999) *Image Processing, Analysis, and Machine Vision*. Brooks/Cole Publishing, Pacific Grove.

Yingyongnarongkul, B., How, S., Diaz-Mochon, J.J., Muzerelle, M. & Bradley, M. (2003) Parallel and multiplexed bead-based assays and encoding strategies. *Comb. Chem. High Throughput Screen*, **6**, 577–587.

