Topic Introduction

Arc Lamps and Monochromators for Fluorescence Microscopy

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Fluorescence microscopy requires high photon-flux densities in the specimen plane. These intensities are only achieved by lasers, arc lamps, and, most recently, light-emitting diodes (LEDs). Lasers and LEDs, however, are restricted to a limited number of wavelength regions, whereas with arc lamps it is possible to select arbitrary wavelengths and wavelength regions. Moreover, the lower cost of arc lamps, compared with lasers, makes them the light source of choice for the majority of fluorescence microscopy applications. Recently, so-called white light lasers have become commercially available, but their photon fluxes—although sufficient for laser scanning applications—are still not high enough for applications where extended areas need to be illuminated. This article discusses arc lamps and the design and performance of an arc lamp-based illumination system for fluorescence microscopy that allows the user to choose any wavelength from ultraviolet (UV) to infrared. The system permits rapid switching speed between colors, while maintaining quite stable and homogenous emissions.

ADVANTAGES OF ARC LAMPS AND MONOCHROMATORS FOR FLUORESCENCE MICROSCOPY

Arc lamps offer a sunlike spectrum reaching from the UV to the infrared, are relatively inexpensive, and, if used in the right configuration, can obtain short- and long-term stability values better than any other bright light source. With fiber coupling, they can be positioned at a distance from the microscope, thus minimizing detrimental effects caused by heat and vibrations. By using critical illumination, a degree of homogeneity can be achieved in the specimen plane that is not attained when using any other light source.

To exploit the wide spectral coverage of arc lamps, the desired wavelength range is selected from the white spectrum by placing interference filters into the beam path. Filter wheels, which are often used for this task, are relatively slow, requiring >50 msec to switch from one filter position to the next. A faster alternative is galvanometer technology, which directs the beam path through different filters (e.g., the Lambda DG-4 [Sutter Instrument] or the Oligochrome [TILL Photonics]). Although they require only 1–2 msec to move between filters, galvanometer-driven mirror systems limit the number of available colors to four or five and do not permit bandwidth adjustment. The most versatile filter system is a rapidly scanning monochromator, which selects the desired wavelength by tilting a grating in the illumination beam and can jump from one wavelength to the next in 1–2 msec.

CHARACTERISTICS OF FIBER-COUPLED ARC LAMPS

Spectrum

Three classes of arc lamps are used in fluorescence microscopy: mercury, xenon, and metal halide lamps. All three cover a wide spectral range (Fig. 1), but only xenon lamps show a modest line

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FIGURE 1. Spectral characteristics of three types of arc lamps.

structure, which is essential for use in a monochromator. The spectral composition delivered by an illumination device depends not only on the spectrum of the map but also on the band-pass characteristics of a given filter or the grating and the optical design used in the monochromator. Although filters show very steep spectral transitions from blocking to transmitting wavelengths, monochromators roll off less steeply outside their chosen wavelength band; thus, if a narrow spectral band in the spectrum of a lamp falls onto the slope of the monochromator transmission, the spectral maximum may not be where it is thought to be. The monochromator design described below thus uses a xenon arc lamp as its light source, because its spectrum does not show narrow spectral bands.

The output power of Xe-arc lamps does not vary by >50% when operating in the range from 320 to 700 nm. Photon fluxes of $>10^{23} \text{ m}^{-2} \text{ sec}^{-1}$ can be achieved in the specimen plane, implying a photon transfer efficiency close to the theoretical limits for the lamp. A typical fluorophore, like fluorescein isothiocyanate (FITC) or green fluorescent protein (GFP), emits $>10^3$ photons/sec under these conditions, which is enough for single-molecule detection.

Spectral Purity

Filter-based systems show better than 6 orders of magnitude blocking outside their transmission band. This level of spectral purity cannot be achieved with a monochromator, which typically shows stray-light levels in the range of 3.5–4 OD. The cure is simple: Equip the filter cube used for a given experiment with a suitable short-pass filter in the excitation beam, which transmits all light below the cut-on wavelength of the emission filter and blocks all light in the spectral region where the emission filter transmits. This preserves the full spectral flexibility of the monochromator design while at the same time allowing for the same >6 OD background rejection of a filter-based system.

Usable Output Power

In arc lamps, radiation originates from spatially confined plasma, whose dimensions increase with increasing lamp power. This has consequences for the maximum photon flux that can be concentrated onto a given sample area. According to the Helmholtz–Lagrange invariability principle, every attempt to concentrate light originating from a spot with diameter d_1 onto a smaller spot of diameter d_2 (i.e., $d_2 < d_1$), invariably increases the NA at the target spot over the NA of the source spot. (The invariability principle states that $n \times \text{NA} \times d$ [called the *étendu*] remains constant throughout an optical system, where n is the index of refraction of the medium, NA is the numerical aperture, and d is the diameter of a source of radiation or a receiving element.) In a microscope, the NA of the objective that

is in use limits the NA of the “target side.” Hence, increasing the wattage of a lamp (and thus its diameter) beyond a certain value only increases the total photon flux but not the flux density at the sample. This is illustrated by the following example: Imagine using a 40× oil-immersion objective with NA 1.3. The goal is to concentrate as much light as possible onto the field that is covered by a 0.65-in camera chip positioned under the microscope (225 μm). If we assume that the lamp condenser collects light with high NA (e.g., 0.65), then the usable spot of the arc lamp has a diameter of $1.3/0.65 \times 225 \mu\text{m} = 450 \mu\text{m}$. Thus, every arc lamp with a hot spot $>450 \mu\text{m}$ will create light that cannot be used. Figure 2 shows why the hot spot size of a 150-W xenon arc lamp is a good match for the experiment described.

Stability

Until very recently, mercury arc lamps have been the most commonly used light source for fluorescence microscopy. They show the smallest arc dimensions and hence can provide the highest photon-flux densities of all arc sources, particularly within the lines of the typical mercury spectrum. However, they show poor long-term stability; output power drops 30% within 200 h. This not only means a tedious lamp change but also a high cost of ownership. Thus, metal halide lamps (which were first used in consumer projection devices) have recently replaced mercury arc lamps as the most popular light source for fluorescence microscopy. The bulbs are easily exchanged and they show lifetimes of >1000 h. Whereas long-term stability is good, short-term stability is poor owing to arc flicker, which occurs irregularly every few seconds and causes short-term fluctuations of several percent. Although this does not matter for longer time-lapse studies that allow for longer integration times, or for nonquantitative experiments in which temporal stability is not essential, more demanding quantitative experiments are best served by xenon arc lamps, which provide the best short-term and long-term stability of all arc lamps. They operate for 2000–3000 h and, provided their constant current power supply does not degrade performance, RMS (root mean square) intensity fluctuations remain in the range of 3×10^{-3} . To achieve this degree of stability, xenon lamps need to be convection cooled. Forced-air cooling aggravates arc wander, which is caused by convection inside the quartz envelope, and is the major source of lamp instability in xenon lamps.

Fiber Delivery

Most commercially available microscopes have been designed with an epifluorescence condenser optimized for direct coupling of an attached mercury arc lamp. These lamps usually have a power consumption of 50–100 W; hence they can still be convection cooled. The higher wattage and optical

FIGURE 2. Intensity distribution (false colors) of the arc of a 150-W xenon arc lamp.

packaging of metal halide lamps renders forced-air cooling a necessity, and to avoid unwanted heat and vibration transfer from the lamp to the microscope, metal halide lamps are usually coupled to the microscope by means of a liquid light guide. However, a metal halide lamp with its built-in reflector has not been designed for concentrating light onto the smallest possible spot. Therefore, to collect a large fraction of its radiation, liquid light guides of 5 mm diameter must be used. Their high NA of >0.5 brings the Helmholtz–Lagrange invariability principle into play again, and thus only a small fraction of the light carried by the light guide is usable. As an example, consider a $60\times$ oil-immersion objective, whose maximum NA for illumination is 1.33 (every greater angle suffers total internal reflection and thus does not reach the sample). The maximum demagnification achievable without loss is $1.33/0.5 = 2.66$; thus, the image size of the light guide cannot be reduced below $5/2.66 = 1.88$ mm. With a $60\times$ objective, this would correspond to a field size of 112 mm, in contrast to the standard microscope field size of 22 mm. As a consequence, only 3.8% of the radiation transported by the light guide is usable. The advantage of the large diameter of the liquid light guide is that no sophisticated optics are required to couple its light into a microscope. By overilluminating both the field and pupil of the objective, a homogeneous illumination is almost always guaranteed.

A very different situation arises if quartz fibers are used. They have much smaller diameters and a smaller NA (usually 0.22), and maximum homogeneity is only achieved by means of critical illumination; that is, by imaging the exit window of the fiber into the specimen plane. This requires sophisticated condenser designs, one for each type of microscope, and careful alignment. However, if set up correctly, such a system provides the best transfer efficiency and maintains it over an indefinite period of time, because quartz fibers (unlike liquid light guides, which show significant aging) do not degrade with age.

EXPERIMENTAL SETUP

The Illumination System

As noted above, transferring light from a source to a target without sacrificing brightness (photon-flux density) requires an optical system with good imaging quality. Moreover, for a monochromator system covering an extended wavelength range, this level of optical performance has to be maintained over the spectral range in question. The best results are obtained by combining an inherently achromatic reflective design (toroidal mirror) with a chromatically corrected pair of aplanatic lenses (Fig. 3). Light from a xenon arc lamp (XA) is collected with a high NA of 0.56. A positive quartz aplanatic lens (PAL) reduces this numerical aperture by $1/1.46$ (where 1.46 is the refractive index of quartz) while at the same time creating a virtual $1.46\times$ magnified image of the arc, which is reimaged by a toroidal mirror (TM). The toroidal surface is the approximation of an elliptical surface, which provides 1:1 imaging for source and target spots separated from each other. A second (negative) aplanatic lens (NAL) causes a further reduction in the numerical aperture, while at the same time increasing image size accordingly. The material of the NAL (LF-5, refractive index 1.6) is chosen so that it forms an achromatic pair with the PAL. The combination of the three optical elements

FIGURE 3. Schematic diagram of the optical setup of a monochromator-based illumination system. See text for details and explanation of abbreviations.

PAL, TM, and NAL, reduces the numerical aperture to $0.56/(1.46 \times 1.6) = 0.24$ while at the same time increasing image size 2.33 times. This magnified image fills the entrance slit (S1) of the monochromator.

The Monochromator

White light passing entrance slit S1 is collimated by an off-axis parabolic mirror (PM) and directed onto a diffraction grating (DG), having 1302 lines/mm. The diffracted light is collected by an UV-transmitting achromatic lens (AL), which projects the resulting spectrum onto an exit slit (S2). By adjusting the tilt of DG, the spectrum moves over the exit slit and different spectral regions are allowed to pass. The light that passes through S2 is coupled into a quartz–quartz fiber (QF) with a 1.25-mm diameter and NA of 0.22.

Standard systems are equipped with an entrance slit width of 1.5 mm and an exit slit width of 2.4 mm. Given a focal length of the parabolic mirror of 50 mm and a focal length of the achromatic lens of 76 mm, the bandwidth (half-width) is 14 nm. By changing both the entrance and exit slit widths, the spectral bandwidth can be changed. By changing only one of them, the intensity can be controlled while maintaining half-width.

Dynamic Performance

When mounted on a galvanometric scanner, the angle of the monochromator grating can be adjusted, which alters the wavelength that passes through the exit slit. An adjustment occurs in 1–1.5 msec, depending on the galvo-excursion required for a given wavelength change. Scanner accuracy is maintained to better than 0.01 nm over the lifetime of the system.

By choosing a resting wavelength not transmitted by the microscope optics (e.g., 260 nm), the monochromator can be used as a shutter, too. An alternative is to choose an experiment-dependent resting wavelength, in which the blocked wavelength is determined by the blocking region of the shortpass filter within the filter cube, to the desired wavelength and back.

With a fast scanner, the exposure time can be set to millisecond precision. Unlike with rotating filter wheels, the exposure time can be set independently for each wavelength, and complex wavelength protocols can be executed under computer control. As with most modern single-lens reflex (SLR) cameras, image brightness can be determined by the exposure time. Because photodamage depends much more on the integral photon dosage and not on the intensity, neutral density filters are unnecessary.

One drawback is the nonzero transition time of the scanner during which illumination with undesired wavelengths occurs. The relative error increases as exposure time decreases. This can become an issue with calcium ratio experiments, in which for an exposure time of 3 msec, the error is 7% and can be exacerbated if the dye has more absorption in the transition wavelengths than at the final wavelength (Gryniewicz et al. 1985). Correct calcium concentrations may still be derived from ratio experiments, provided the calibration values $R_{\min}$ and $R_{\max}$ are determined using the same exposure times that are used in the experiment (Messler et al. 1996). Another cure is to “blindfold” the detector during wavelength transitions. This works with photomultipliers, gatable image intensifiers, or charge-coupled device cameras with fast electronic shutter capabilities (interline transfer sensors). Frame transfer cameras are slower, requiring up to a millisecond for this shift, so that pixels are likely to be illuminated by transition wavelengths.

CONCLUSION

Xenon arc lamps remain the light source of choice for wide-field fluorescence microscopy when wavelength flexibility, broad spectral coverage, and high-intensity stability are required. Monochromators based on galvanometers that carry a diffraction grating provide the most flexible means of

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achieving rapid shuttering and wavelength selection. The arc lamp and monochromator should be used together when performing quantitative time-lapse fluorescence imaging.

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