6

Non-Laser Light Sources for Three-Dimensional Microscopy

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INTRODUCTION

General Remarks on Choice of Excitation Light Sources

The first light source used for microscopy was the sun and the second was a candle flame. Both are hot plasmas that emit essentially black-body radiation (Fig. 6.1) with the addition of a few elemental lines. The introduction of light sources powered by electricity, both arcs and incandescent filaments, added a new level of convenience and flexibility but required improvements in the light-harvesting optics needed to illuminate the imaged area with light that was both intense and uniform.

Brightness

Because the recorded intensity of the image drops with the square of the magnification, the most important characteristic of a source for microscope illumination is its **brightness or radiance**. In this case, **brightness** refers not just to the ability of the source to produce many photons/second but also to its ability to produce these photons from a small volume so that a fair share of them can be conveyed to the small area of the specimen that is being imaged.

A large diffuse source, such as the 40 W fluorescent tubes used for lighting houses,[1] produces about the same number of photons/second as does the 50 W, short arc HBO-50 bulb used in fluorescence microscopy. However, in the former case, the photons emerge from a phosphor surface approximately 0.1 m^2 in size, while a cross-section through the brightest part of the HBO-50 has an area about one million times smaller.

The laws of optics specify what fraction of the light leaving a source can be focused into an image of this source. In Figure 6.2, light leaving h_1 that does not strike the optical system clearly, will not be focused onto the image plane at h_2. Although some of this lost light can be reclaimed by placing a spherical reflecting mirror so that the focal point f is centered on the source, there are still limits on how bright the image will be. If the optical system makes an enlarged image h_2 of the source h_1 at the condenser back-focal plane (BFP, not shown), then the fixed number of photons from the source will be spread over a larger area and the image will not be so bright. If the optical system demagnifies the source, the image will be smaller, BUT it will not be brighter, because, as shown in Figure 6.2, to demagnify the source, it must be farther from the optical system than the image. As a result, the NA (aperture) of the optical system collecting light from the source will be smaller by exactly the same ratio as the image has been demagnified. In other words, no matter how many lenses are used, the ability of an optical system to make a smaller image of the source is inexorably tied to using a collector lens with a lower NA (as seen from the source) and this means that a smaller fraction of the light emitted by each point on the source is actually collected. One just cannot squeeze all the light into a smaller image.[2] The best one can do is to design an optical system that makes an image the same size as the source (magnification = 1).

The moral of this story is that the only way to have bright illumination on the specimen is to start with a small bright source. It follows that the fraction of the light from the HBO-50 that can be conveyed by any optical system to an area of the specimen that is, for example, 100 µm on a side is about one million times greater than could be achieved using the phosphor surface of a 40 W fluorescent lighting tube. Source brightness is measured in terms of photons/second/square centimeter/steradian (radiance), where the steradian is a measure of the solid angle into which the light projects.

Whether this level of illumination is sufficient for the needs of microscopy or not depends on the contrast method in use (fluorescence needs about $10^6\times$ more than bright field), on the time available to accumulate the image, on the contrast of the image, and on the accuracy with which one needs to measure this contrast. That said, although it is usually quite easy to reduce the brightness of a light source by introducing neutral density (ND) filters, making it brighter requires major redesign.

Considering just the standard configuration in bright-field microscopy, about 5 W of optical power is emitted by a 100 W halogen lamp at its rated power consumption. As the filament is 4.2×2.3 mm in size, its cross-section is about 10 mm^2. The aspherical collector lens can cover an aperture of NA = 0.7 (45° half angle) or about 0.15 of the full solid angle. Using a back reflector this can be increased to approximately 0.30. Because of the optical

[1] Really, a very diffuse Hg arc, surrounded by a coating of powdered, inorganic phosphor.

[2] Another line of argument is to point out that, for the image to be brighter than the source, one would have to move energy from a cooler source to a hotter image. This is forbidden by thermodynamics.

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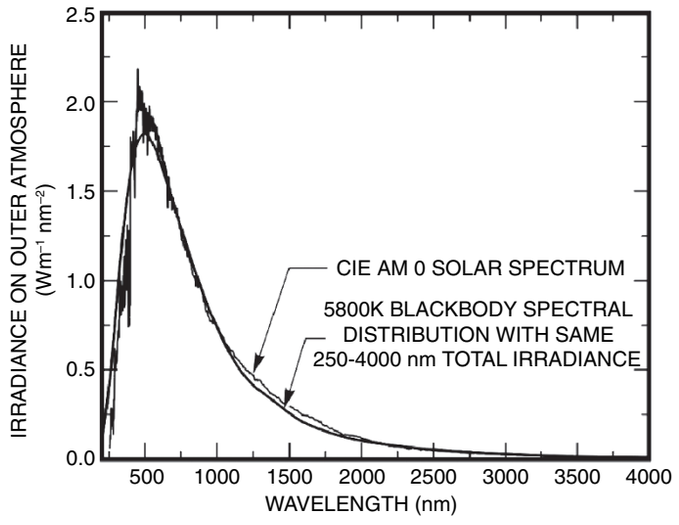

FIGURE 6.1. (A) CIE AM 0 Solar Spectrum**,** (B) 5800 K Blackbody spectral distribution with same 250 to 4000 nm total irradiance. (Spectra kindly provided by Oriel/Newport, Irvine, CA.)

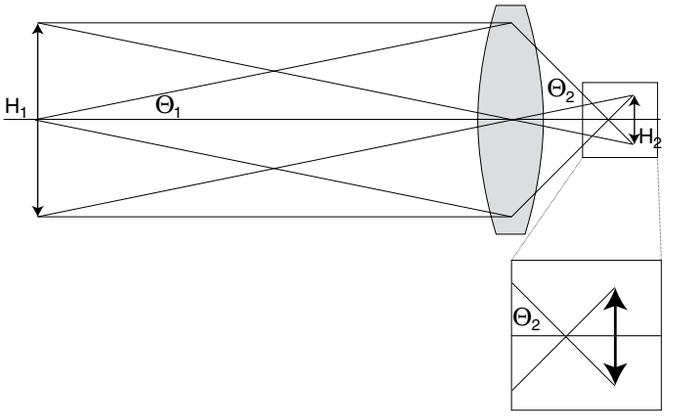

FIGURE 6.2. Relationship between demagnification and NA. When an optical system creates a demagnified image, h_2, the convergence angle, Θ_2, is larger than the divergence angle, Θ_1, leaving the source, h_1 and accepted by the optical system. Because the reduction in area caused by the demagnification is exactly compensated by the increase in NA^2, the image can never be brighter than the source.

rules mentioned above, even perfect optics can only transport about 0.001 of this optical power to illuminate a $100 \times 100\,\mu m$ area of the specimen.[3] This means that the power available to illuminate the field of a high magnification lens is less than $(5 \times 0.3 \times 0.001) = 0.0015\,W = 1.5\,mW$. Actual measurements of brightness at the focal plane are provided in Table 6.2 later in this chapter.

Assuming bright-field imaging for the moment, almost all of this light passes through the objective and intermediate optics and reaches the plane of a perfect charge-coupled device (CCD) camera, composed of a 1000×1000 array of $6\,\mu m$ pixels.[4] As such a sensor will be $6 \times 6\,mm$ in overall size; it will just match the size of the illuminated area on the specimen if a 60× lens is used. At this point, each pixel will intercept one millionth of the light at the specimen or 1.5 nW. As one watt of light corresponds to about 3×10^{18} photons/second and each $36\,\mu m^2$ CCD pixel can absorb about 20,000 photoelectrons before saturation, an exposure of 2×10^4 photons/$(1.5 \times 10^{-9}W) \times (3 \times 10^{18}$ photons/s/W$) = 3.7 \times 10^{-6}$ seconds. In other words, in the absence of other losses, even an exposure as short of $3\,\mu s$ would be enough to saturate the CCD (Inoué, 1997). Sadly, as the optical system just described focuses the filament into the focus plane, a blurred image of the filament will be present there to modulate whatever other contrast the specimen produces. Correcting this problem as described below and allowing for reflection and other losses will cut the signal by a factor of about 10. Adding an "interference green" filter and polarizer for optimal operation of monochrome differential-interference-contrast (DIC) imaging will cut the photon flux by another factor of 20. Should one decide instead to use this green light to excite fluorescence, the signal will likely be reduced by an additional factor of one million[5] and suddenly exposures of seconds or minutes are needed. The only solution is to use a brighter source.[6]

Uniformity

The second requirement of a light source for microscopy is **uniformity**; all parts of the imaged plane must receive the same illumination density. Initially this meant that the illumination level must be constant in any plane, a condition not too hard to arrange by focusing an image of a featureless source, such as the sun (lacking sunspots!) or a flame, directly onto the specimen. However, as the light-emitting area of arcs and most filaments cannot be called uniform, a new illumination system, **Köhler illumination**, was introduced, so that the non-uniformity was confined to aperture planes. While this optical solution does provide uniform intensity in the image planes, it means that there is still some non-uniformity in the angles at which light approaches each point of the focus plane.

The **Köhler illumination** system diagrammed in Figure 6.3 (Inoué, 1997) operates as follows: Light is collected from the near side of the source I using a high-aperture collector lens 1 while a spherical mirror returns light from the rear side of the source to form an image of the source to the side of the actual source (not shown in Fig. 6.3). This reflected light is also collected by the collector lens, increasing the apparent source size (Piller, 1977). The collector lens focuses the source at the back-focal plane of the condenser II (or of the objective in the case of epi-illumination III). Because of the reciprocal relationship of image and back-focal planes, anything that is in focus at one set of these planes is completely out of focus at the other. As a result, assuming that the condenser is correctly aligned and the source is planar, the focus plane in the specimen B is evenly illuminated. However, depending on the uniformity of the source and the aberrations of the optics, this light may not strike the specimen from all possible angles equally.[7]

The field aperture A lies in an intermediate image plane between the collector 1 and the condenser 2, from where it is

[3] As the most efficient optics operate at a magnification of 1 : 1, only the light emerging from a $100 \times 100\,\mu m$ area of the filament can be focused into an area of the same size on the specimen.

[4] The popular Sony ICX285AL chip has a pixel size of $6.45\,\mu m^2$.

[5] Of course, this factor depends strongly on the dye concentration, and distribution and the effective thickness of the sensed layer. Values in the range 10^{-3} to 10^{-9} would not be unusual.

[6] This discussion relates to low magnification operation where intensity is the main goal. At low magnification, it is necessary to illuminate a much larger area on the specimen. This involves exchanging optical elements to change the magnification between the plane of the field diaphragm and the plane of the specimen. This is possible because it is no longer important for the condenser to operate at high NA.

[7] Remember from diffraction theory that the highest resolution in transmission is only obtained when undiffracted light enters the objective from all angles up to that set by its NA.

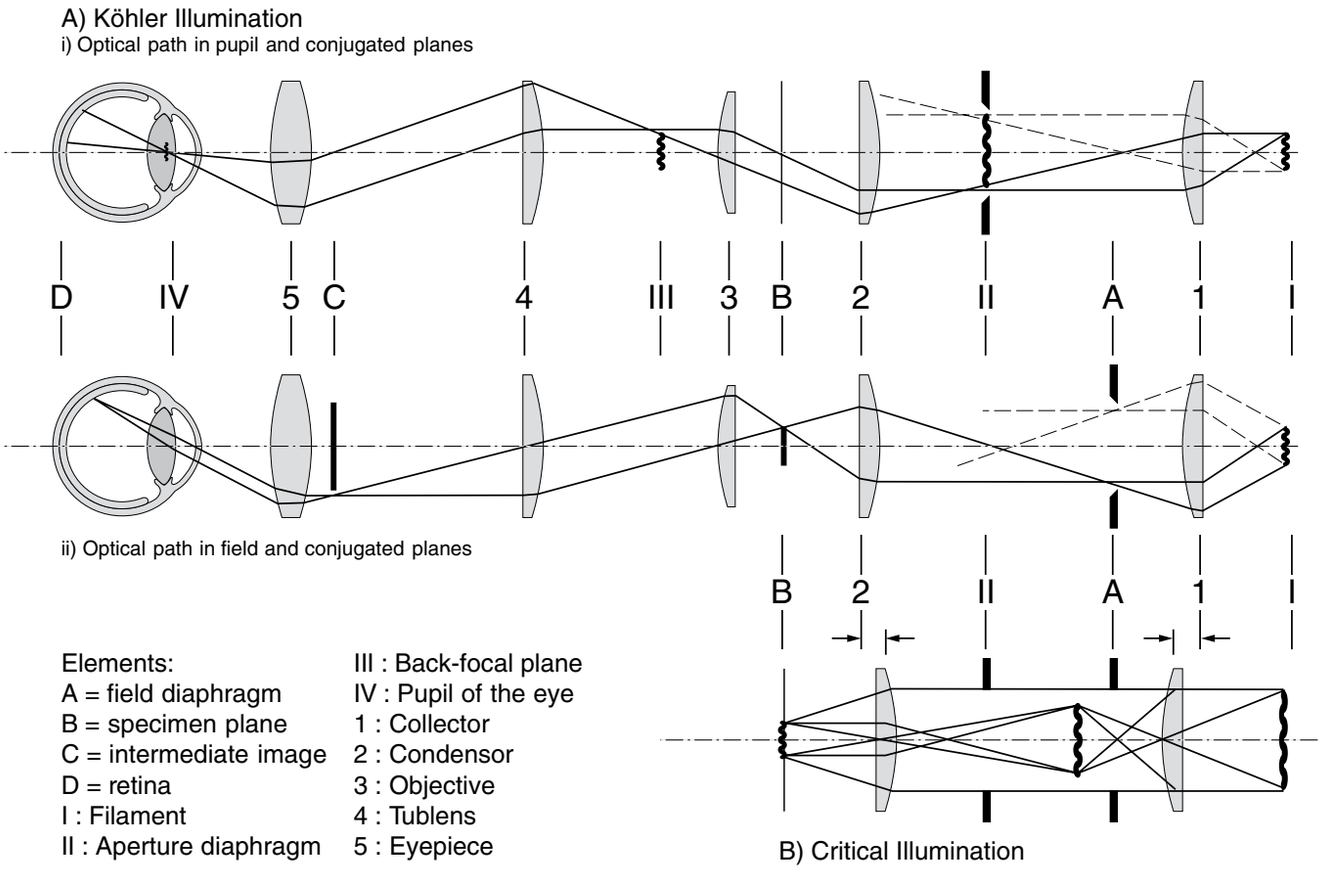

FIGURE 6.3. Köhler illumination with the optical elements: (1) collector, (2) condenser, (3) objective, (4) tube lens, and (5) eyepiece. (A.i) Ray paths focused on pupil and focal planes: (I) source, (II) aperture diaphragm, (III) back-focal plane of objective, (IV) eyepoint above ocular/pupil of the eye. (A.ii) Ray paths focusing on image planes: (A) field diaphragm, (B) focus plane in specimen, (C) intermediate image, (D) retina/CCD. (B) Critical illumination in which the source is focused directly on the focus plane in the specimen. This leads to more light striking the focus plane but precludes the use of the field diaphragm to limit excitation to the area of the specimen actually being imaged.

imaged at plane B in the specimen by the condenser. Its size can be adjusted to restrict the area illuminated to that being viewed without affecting the angle of the illuminating light.

Because it makes maximum use of neither the full surface of the source nor the full angular distribution of the emitted light, Köhler illumination is not very photon efficient. In addition, because the condenser lens should have the same NA as the objective, it must be focused with equal care if the field diaphragm is to remain properly focused into the image plane. Because, on most upright microscopes, the condenser and stage move together, moving the stage to change focus is a process that necessarily moves the condenser out of Köhler alignment. This, and the fact that microscope slides and coverslips are likely to vary in thickness by tens of micrometers, means that, ideally, one should readjust the condenser position whenever the specimen is changed or the focus plane substantially shifted.[8]

Of course, the Köhler illumination diagram has some hidden assumptions that are really not justified in practice. The chief of these is that the source is planar and that, therefore, light emerging from it can be focused by "perfect" lenses into other planes according to the geometric optics of the diagram. In fact, both arcs and most filament sources are approximately spherical in shape. Consequently, even if the optical system focuses light from a central plane of this sphere into the back-focal plane (BFP) of the condenser lens, many photons emerging from planes within the luminous sphere that are either in front of or behind the central plane will be proportionally out of focus at the condenser BFP, and at every other conjugate BFP in the diagram. The corollary of it being out-of-focus at the BFPs is that it will be partially in focus at the image plane and therefore, that in spite of our best efforts, the illumination will still have some structure at the focus plane.

Two more relevant variables of the illumination optics are the total magnification between the source and the BFP of the condenser and what might be termed its "length." All collector lenses have large NA to capture as many photons as possible, and a focal length short enough to keep the light source small without getting the glass so close to the source that the heat cracks it. However, as the need to dissipate the heat dictates that the source and collector be mounted at some distance from the condenser lens, a variety of optical arrangements are used to convey the light across the intervening space.

[8] The same argument also applies to inverted microscopes. Ideally, the objective and condenser would remain fixed to each other and only the stage would move.

At the other end of the illumination optics, the condenser lens should have an NA as large as that of the largest objective with which it will be used but its focal length is not specified.[9] A longer focal length implies a larger BFP and, if this is to be "filled" with an image of a filament, then the optics up to that point may have to form an enlarged image of the filament. Often epi-illumination Köhler optics are designed to make a relatively small image of the arc at the BFP of the objective so that this image is not truncated (and the light lost) by the relatively small BFP of, for example, a 100× NA 1.3 objective. However, this means that the larger BFP of a 40× NA 1.3 objective will be severely under-filled, a factor that reduces the rate at which excitation light diminishes above and below the focus plane and thereby affects the WF PSF (Hiraoka *et al.*, 1990).

The length is important because, although so-called telan lenses can be introduced to convey all the light from the focal point of the collector to the BFP of the condenser, light originating from planes in front of or behind this plane will "hit the wall" of the optical system and be lost or create reflections. The longer the optical conveyer system, the more it is true that the light reaching the condenser BFP will have originated near to the focal plane of the collector. This makes such light both more coherent and more structured because it conveys the image of the inhomogeneity of the filament at the focal plane of the collector.

To restore some semblance of order to the system, it is common to insert a flat, ground-glass surface just outside the collector lens, and let this planar surface become the effective source. This solves the out-of-focus light problem but substantially reduces (by >90%, depending on its roughness) the brightness of the source. For this reason, the ground glass is normally used only when imaging with coherent, transmitted light (phase contrast, DIC, etc.) and not when the main aim is just to get as many photons onto the specimen as possible (fluorescence, dark-field).

The alternative to **Köhler illumination** is **critical illumination**, a system that requires a highly uniform emitting surface I because this surface is focused directly into the imaged plane within the specimen B [Fig. 6.3(B)]. Because it images the entire source, a larger solid angle can be used than with Köhler illumination and therefore it can deliver more photons/second/square centimeter at the image plane. The downside is that any non-uniformity in the emitter will appear as a brightness change in the final image. The coherence of the illumination in this system is that of the source. If a large source is imaged, coherence is quite low. Flickering variations in brightness caused by convection within the plasma of the arc can make it tiring to view the specimen when the plasma is imaged directly onto the image plane, but a time-average of such an arc image is quite uniform. A solution to this brightness–coherence–uniformity conundrum was proposed by Gordon Ellis, who suggested that the image of the arc be focused on the entrance of a 1mm diameter multimode optical fiber that is then bent around two perpendicular axes. Mixing in the fiber scrambles the light so that the exit surface appears to be a uniformly illuminated circle. Illuminators of this type are discussed below in the section on light scrambling.

If the collection mirror used is elliptical or parabolic rather than spherical, and the arc tube is mounted axially (Fig. 6.4), it can collect up to 85% of the total emitted light. Unfortunately, the projected ray bundle has a dark central spot where rays are obscured by the end of the plasma tube. In addition, because arcs are more stable and uniform when the axis of the envelope is vertical, light sources using this type of collector usually project their output downwards.

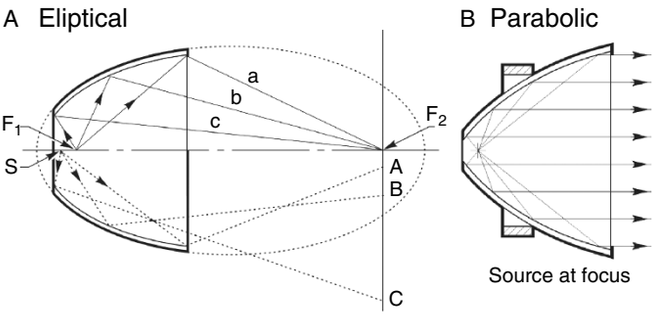

FIGURE 6.4. (A) Elliptical collector with both the focal points F_1 and F_2 and eccentricity $e < 1$, every ray (a–c) emitted from focal point F_1 will be imaged at focal point F_2. (B) Parabolic collector (eccentricity $e = 1$), with a source in the focal point. The source will be imaged into infinity (a parallel wave front). (Diagrams kindly provided by Oriel/Newport, Irvine, CA.)

The coatings on all collection mirrors should be dichroic to let heat wavelengths pass through. Reflectors designed for various wavelength bands are sold by Oriel Corp. (Stratford, CT) and Optical Radiation Company (Electroforming Division, Azusa, CA) among others, and sold as "cold mirrors" or "hot mirrors," depending on the wavelengths transmitted or reflected. If some scrambling is done and the source image is expanded, elliptical mirror sources can provide a Köhler illumination scheme suitable for disk-scanning instruments (see below), though a somewhat more uniform output can be produced if the elliptical surface is slightly deformed. These same companies and ICL Technologies (Sunnyvale, CA) make modules and lamp housings that utilize such aspheric reflectors. Many models are available for different arc lamps.

Wavelength

The third important variable is **wavelength**. Although the plasma and filament sources discussed so far have been "white light" sources with fairly uniform brightness across the visible wavelengths, the same is not true of arc sources. Photons are produced whenever an excited electron loses energy. They will be visible photons (i.e., photons having a wavelength of between 380 and 780 nm) if the amount of energy lost by the electron is in the range of 3.3 to 1.59 electron volts (eV). Excited electrons can be produced in many ways, but the traditional methods that produce them efficiently and from small volumes include heating the electrons in a tungsten filament to 3200 K or 3400 K, or heating a Hg or Xe plasma to a much higher temperature. These plasma sources also have specific emission bands that are defined by the specific energy levels of the atoms involved [Fig. 6.5(A,B)].[10]

From Figure 6.5(B), one can see that an Hg arc is almost 10× brighter at 365 nm than at 400 nm. Not surprisingly the 300 W Xe arc makes about 4× more total light than the 100 W Xe arc. However, because the brightest part of the image of the 300 W

[9] Except for the long working distance (LWD) condensers used on inverted microscopes to allow use with thick cells chambers or access to the specimen with micro-electrodes and the like.

[10] All irradiance spectra kindly provided by Newport Co. (Irvine, CA).

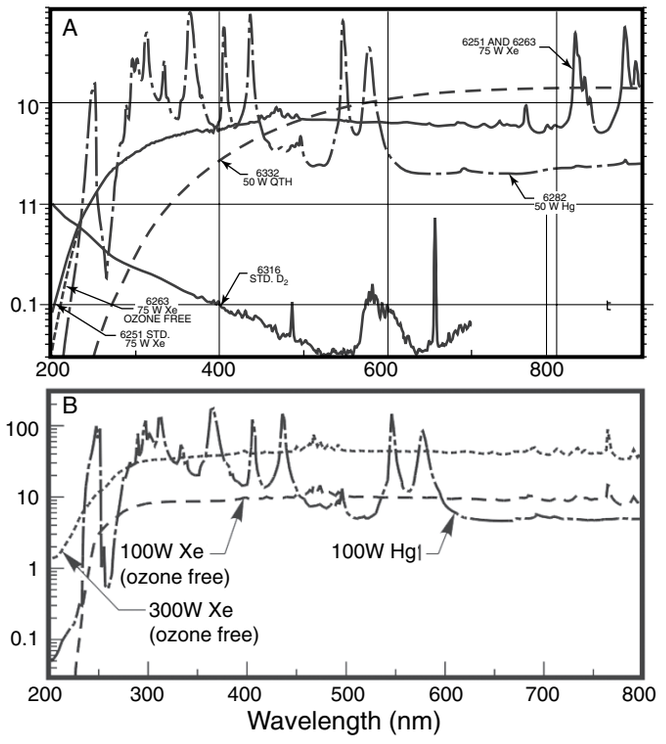

FIGURE 6.5. Wavelengths resolved irradiance of various light sources. (A) *Lower line*: 30 W deuterium arc lamp; *upper line*: 75 W xenon arc lamp; *dashed line*: 50 W halogen filament; *dashed/dotted line*: 50 W mercury arc lamp. (B) *Dashed line*: 100 W xenon arc lamp; *dashed/dotted drawn line*: 100 W mercury arc lamp; *dotted drawn line*: 300 W xenon arc lamp. (Spectra kindly provided by Oriel/Newport, Irvine, CA.)

source is almost 8× larger than the comparable area of the 100 W source, its brightness is only half as high.

Coherence

A fourth important source parameter is its **coherence**. Although coherence is closely related to brightness, in that bright sources are likely to also be highly coherent, the term *brightness* is more used to describe the ability of a source to focus a large number of photons into a small area, whereas *coherence* is more of a measure of the ability of wave functions that describe these photons to interfere with each other, either at the focus plane (good) or between the reflections from every dust particle and imperfection in the optical system (bad). In coherent light, a large fraction of the wave functions passing a given point are in-phase with each other.

Although light from a laser is extremely coherent, even here there are limitations. Laser light emerges not at a single wavelength but over a very narrow wavelength band. Consequently, even if all the photons started off in phase, after some distance (the coherence length) those with slightly longer wavelength will become out of phase with those of slightly shorter wavelength. What this means practically is that light scattered by a dust particle on one side of a piece of glass can only interfere with light scattered on the other side if the thickness of the glass is less than the coherence length.[11] Interference of this type is the source of "laser speckle," the pattern of random interferences that converts what should be a uniform beam into a pattern of black and white blobs. Because in microscopy it is preferable that the contrast seen in the image represent only the interference that occurred within the specimen, light sources of low coherence are preferred.

The process of light amplification by the stimulated emission of radiation that produces laser light (see Chapter 5, *this volume*) necessarily produces very coherent light. Although it is often assumed that coherent light can be produced in no other way, this is untrue. If one thinks of light as an oscillating electro-magnetic field that propagates in one direction, and chooses to measure this field at a particular instant and in a location of space that is smaller in dimensions than its wavelength, the wavefields of all the photons passing through this volume at any instant are added to produce a single electromagnetic wave vector; that is, the light emerging from this tiny volume is completely coherent. Were one to focus light from a filament onto a 0.2 μm diameter aperture, the light emerging from the hole would be coherent and all of it could be focused into a diffraction-limited spot.[12] The problem is that it would also be a very dim spot (about 6 nW of white light by extension of the calculations noted above). As the aperture gets bigger, the coherence is reduced.

Incoherent light, such as sunlight from a cloudy sky, and coherent light from a laser, are each limiting theoretical constructs. Even though, as presented in Chapter 1, using these limiting conditions simplifies the process of writing equations that describe the image formation process in the microscope, neither coherence condition can be realized in practical microscopy. In conventional microscopy, little attention is paid to the degree of coherence of the illuminating light except when considering diffraction and interference effects. Roughly speaking, light is considered to be incoherent when it does not produce speckle effects and coherent when it does.

Speckle is bright if the interference of light scattered by the feature is constructive with that from the background and it is dark if destructive interference occurs [Fig. 6.6(A); Briers, 1993]. The apparent size of the scattering feature and that of the individual speckles are related to the resolution limit (or NA) of the optics. In the case of incoherent illumination [Fig. 6.6(B)], overlap between speckle patterns having different wavelengths partially cancels them out to produce a lower contrast pattern. Because speckle is an interference phenomenon, any movement of the optical system or the specimen will result in a complex change of the speckle pattern in time.

Even in imaging situations that are well described by incoherent phenomena, coherent effects can often be detected if the results are examined with sufficient optical resolution (Reynolds *et al.*, 1989). In general microscopy, light with low coherence is desired for bright-field and reflection modes, while light with higher coherence is required for phase and interference modes. The process of fluorescent emission involves so many intermediate steps between excitation and emission that any coherence in the illuminating light is usually lost, and the light emitted from the specimen is basically incoherent.

If the coherence of the illuminating light is too high, microscopy in the reflected or backscattered light (BSL) mode[13] yields images with fringes caused by interference of the coherent

[11] The time required for light to move one coherence length is the coherence time.

[12] Conversely, the extent to which this is NOT possible is a good measure of incoherence.

[13] BSL is the more general term for reflected light, the term "reflected" should be reserved for the coherent scattering that occurs from smooth surfaces.

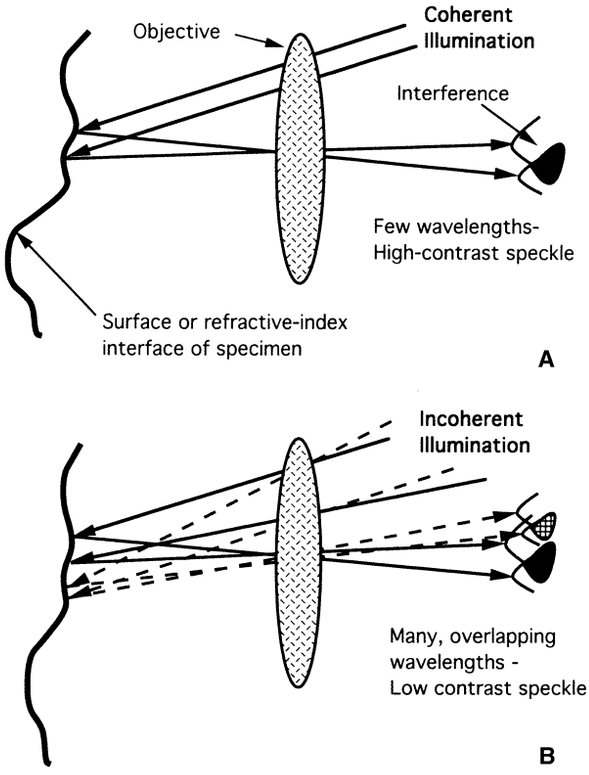

FIGURE 6.6. Speckle patterns. High-contrast patterns result from interference between two reflection maxima in a highly coherent system. (A) Low-contrast speckle results from the summation of interference intensity patterns in a system with incoherent illumination. (B) Speckle spot size is a function of system resolution.

light reflected from any of the optical surfaces: lenses, mirrors, dust windows, and, in particular, the coverslip surface. This complex interference can appear as defined rings, but more commonly, it appears as a high-contrast granular speckle superimposed upon the image, making real image details hard to interpret. Furthermore, when the specimen is transparent and has multi-layered microstructure, the speckle spots become even more complex figures.

The coherence of non-laser light sources can be modified by changing the magnification of the Köhler illumination system to reduce the effective source size. Doing so makes the light less intense but more suitable for interference microscopy.

While the sun is considered an incoherent source, under the imaging conditions of high-resolution microscopy, sunlight has enough coherence to impart speckle to the image. Tungsten ribbon lamps as well as light-emitting diode (LED)-based lamps have relatively low spatial coherence because of the large size of the emitter. Arc lamps have higher coherence unless a large area of the plasma is used as the source. As the short-arc Xe/I source mentioned below uses a plasma spot that, after demagnification, is one third the area of the Airy disk, as a source for a confocal microscope, the illumination is almost fully spatially coherent (Hell *et al.*, 1991). However, Hell and colleagues argue that because temporal coherence, associated with a bandwidth of 360 to 570 nm, corresponds to a frequency bandwidth of 3.07×10^8 MHz, to the extent that the intensity spectrum of the arc approximates a continuum, it is appropriate to use

$$\text{coherence length} \leq c/\text{frequency bandwidth}$$

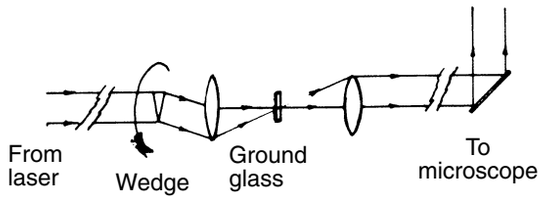

FIGURE 6.7. Phase randomization scheme for laser use. (After Hard, *et al.*, 1977.)

from Born and Wolf (1980). This yields an upper limit of coherence length of approximately 1 µm. Points in the specimen whose optical path difference are greater than 1 µm cannot interfere to give rise to noticeable interference artefacts in transparent microstructures.

A major function of Köhler illumination systems is to make the illumination homogeneous at the image planes and to control its coherence somewhat. However, it is essentially a coherent system and does not "scramble" light to any extent. Additional scrambling is sometimes needed to decrease spatial inhomogeneity, spatial coherence, or temporal coherence. Although most scramblers have been designed to work with highly coherent laser light, the same methods can also be used to reduce the coherence of light from other sources.

Scrambling and Filtering the Light

Three methods have been proposed to reduce coherence. Hard and colleagues (1977) proposed a method to phase-randomize laser light for illuminating a conventional microscope by inserting a rotating optical-wedge-and-ground-glass combination into the light path (Fig. 6.7). Because the wedge and the ground glass rotate, any remaining temporal coherence becomes cyclical. This method requires very stable placement and accurate alignment of the rotating device and the laser relative to the microscope (Reynolds *et al.*, 1989).

The second method to reduce coherence is to focus the laser light into a flexible length of single multi-mode optical fiber (Fig. 6.8). The internal reflections in the bent fiber are constantly changing because the fiber is vibrated at up to 100 kHz (Ellis, 1979) and this makes the exit beam appear uniform in intensity over time (rather than having the Gaussian profile characteristic of lasers). The phase is scrambled by the varying path lengths of the light passing through the fiber on different trajectories, but the high radiance and monochromaticity are preserved. Technical Video Ltd. (Woods Hole, MA) markets a non-vibrating version with a fixed, single quartz fiber segment bent to a specific radius in two perpendicular planes. Applied Precision Instruments (API, Issaquah,

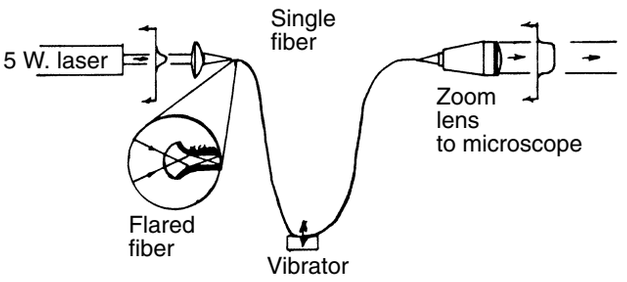

FIGURE 6.8. Phase randomization scheme for laser (after Ellis, 1979).

WA) also offers such a system as part of their Deltavision three-dimensional (3D) imaging system.

All these methods minimize speckle by making it change with time. Speckle is not apparent as long as the recording system records for a time period that is longer than the period of the scrambler. As the motorized system is quite slow, it is only suitable for photographic recordings. Although the fiber scrambler can be oscillated more rapidly, even this is too slow to cause a significant reduction in coherence over the 1.6 µs pixel time that characterizes many beam-scanning confocal instruments.

As light scramblers can be damaged if subjected to a high total light flux, provision must be made to remove heat and other unwanted wavelengths before they enter the scrambler system. Ideally, only wavelengths critical to image formation should leave the source. Dichroics that reflect only specific wavelengths should be used to separate the useful light and allow the unwanted heat to escape. In single-sided disk scanners, this would not only reduce heating but also decrease flare and scattering from the top surface of the disk. Heat-absorbing glass is the most common heat filter, but a filter consisting of a chamber filled with a salt solution chosen to screen out infrared light has much higher heat capacity. Aside from heat removal, liquid filters can also be made to function as bandpass or cut-off filters by the careful choice of the salt used. An extensive description of useful solutions is described by Loveland (1970).

TYPES OF SOURCES AND THEIR FEATURES

The following section discusses the important parameters of various common non-laser light sources with respect to the goals of microscopy. In this sense "parameter" means a degree of freedom a user can change to optimize illumination for the application. Unfortunately these parameters are not independent of each other, and all components of an illumination system have to be treated together as a unit. Overall performance depends on the geometry of the source, the focal length, magnification and NA of the collecting optics, and these in turn depend on the shape and position of the mirrors and lenses. Much can be learned by removing the lamp housing and adjusting the controls used to position the source, the reflector, and the collector to project an image on the source on a distant wall. (When using arc sources, take care not to aim it in such a way that the direct light gives anyone a sunburn or strikes anyone in the eye.)

The important parameters of non-laser sources are:

1. Structure (spatial distribution, source geometry, coherence, alignment, collecting optics, etc.).
2. Available wavelengths.
3. Stability in time and wavelength.
4. Source radiance (brightness).
5. How source parameters such as intensity, color, and spatial distribution are controlled.

Structure

The Actual Source of the Light

Microscope sources are optimized to produce the maximum light intensity or brightness (photons/s/cm^2/steradian) from a tungsten ribbon or the arc of an electrical discharge. The filaments of halogen lamps are often bent to resemble disks or wide, flat bands to match the input aperture of the light-collection optics. Arc lamps generally generate light in a ball discharge at the tip of a pointed electrode (Fig. 6.9). The two electrodes in xenon arcs have different shapes. The anode has a bigger diameter and a flatter tip. As a result, the light will be most intense where electric flux lines are closest together near the point of the cathode [Fig. 6.9(B)]. As the pointed electrode erodes, the field at the tip decreases and the plasma ball becomes larger and therefore less intense.

These sources are geometrically similar but are different in size. The brightest part of the arc in a common HBO-100 arc lamp is about 0.3×0.5 mm in cross-section, the tungsten filament of a 100 W halogen lamp is about 4×2 mm wide. Both source dimensions are set by the manufacturer, and there is no option to vary them.

Electrons passing through the depletion region of any forward-biased semiconductor diode lose energy equal to eV_g, where V_g is the bandgap of the semiconductor. This energy is converted into a photon having an energy equal to the bandgap energy. Silicon diodes emit in the near-infrared (IR) region, but diodes made of other semiconductors emit in the visible and even the near ultraviolet (UV). When such a diode is configured primarily to produce light, it is called a light-emitting diode or LED (Schubert, 2003). Recently, technological developments have increased available power levels and now LEDs are used in applications where their long life and high efficiency are important, such as traffic lights. They are of interest to microscopists because they are compact and efficient light sources that can emit a high flux of quasi-monochromatic photons from a small area.

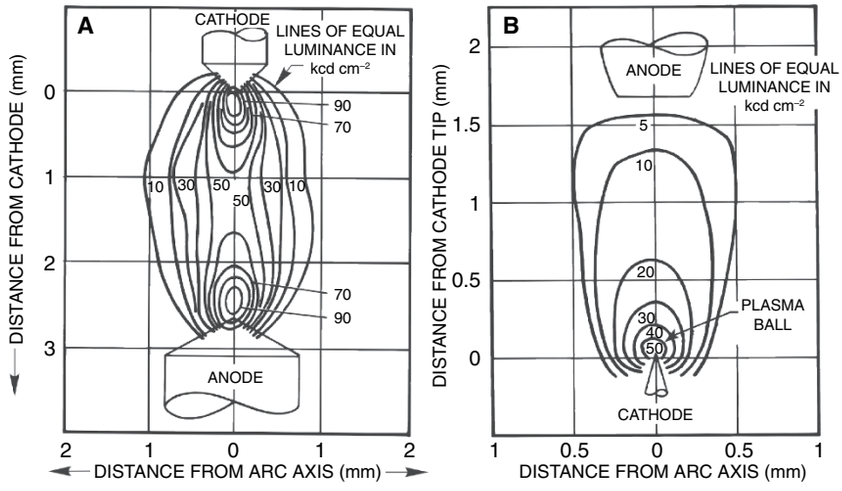

FIGURE 6.9. Iso-intensity plots from the discharge of a mercury arc lamp (A) and xenon arc lamp (B). Values in kcd/cm^{-2}. Note the different size scales and the fact that the Hg arc has two intense plasma balls while the xenon has only one, and this is smaller and less intense than the Hg. (Plots kindly provided by Oriel/Newport, Irvine, CA.)

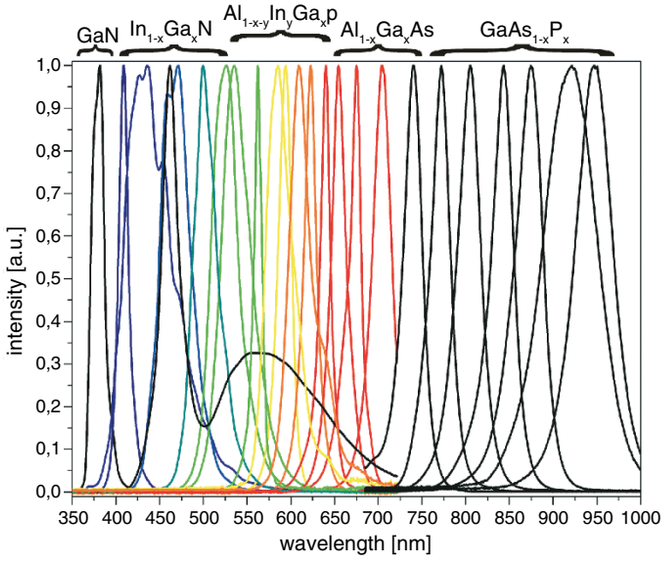

FIGURE 6.10. Spectral distributions of monochrome LEDs. The compounds used for the LED dies are shown above. The black line with a sharp peak at 460 nm and a smaller, wider peak at about 575 nm, is the emission curve of a so-called "white" LED — a blue emitter surrounded with a phosphor that absorbs at 575 nm and emits at longer wavelengths.

An LED source consists of an area of semiconductor crystal approximately 0.3 mm × 0.3 mm in size called a **die**. The most common crystals used are $Ga_{1-x}Al_xAs_{1-y}P_y$, GaN, and ZnSe and each emits in a different waveband (Fig. 6.10). Normally one or more dies are embedded in a larger LED structure for protection, light collection, and electrical and thermal handling. The advantage of LED technology is that one can combine these small units to build up a light source of the shape best suited to the needs of the application. Possible source geometries are limited only by heat dissipation and the permitted package density of the surface mount device (SMD) technology used to integrate a number of dies onto the printed circuit board. Very dense, bright, custom-designed sources can be fabricated in this way. Figure 6.11 shows the general structure of an LED and how they have changed over recent years (Steigerwald, 2002). Die dimensions up to 1.5 × 2 mm are now available.

How to Cope with the Heat?

The most important aspect of any microscopy light source is an efficient heat sink. Incandescent and the arc-based lamps produce a lot of heat because of their low optical conversion efficiency (5%–10%). The holders and housings of these lamps are made of a material resistant to high temperature and designed to dissipate ~100 W of heat. As a result, they cannot be mounted inside a microscope system. Although present LEDs have similar efficiency, all the photons produced are emitted over a narrow range of wavelengths (see below) and they operate at a **much** lower temperature. This means that less electrical power is needed for the same optical output and they can be more compact; for instance, they can be bonded to a small metal heat sink, cooled by a small, computer-controlled fan. This technology makes it possible to mount LED sources inside the microscope system, closer to the specimen, and avoid loss of light intensity in transit. Despite this flexibility, it is important to remember that LED-based sources do need an efficient heat sink because operating much above room temperature causes lower lifetime and a loss of optical output efficiency (Perduijn *et al.*, 2004; Fig. 9.12).

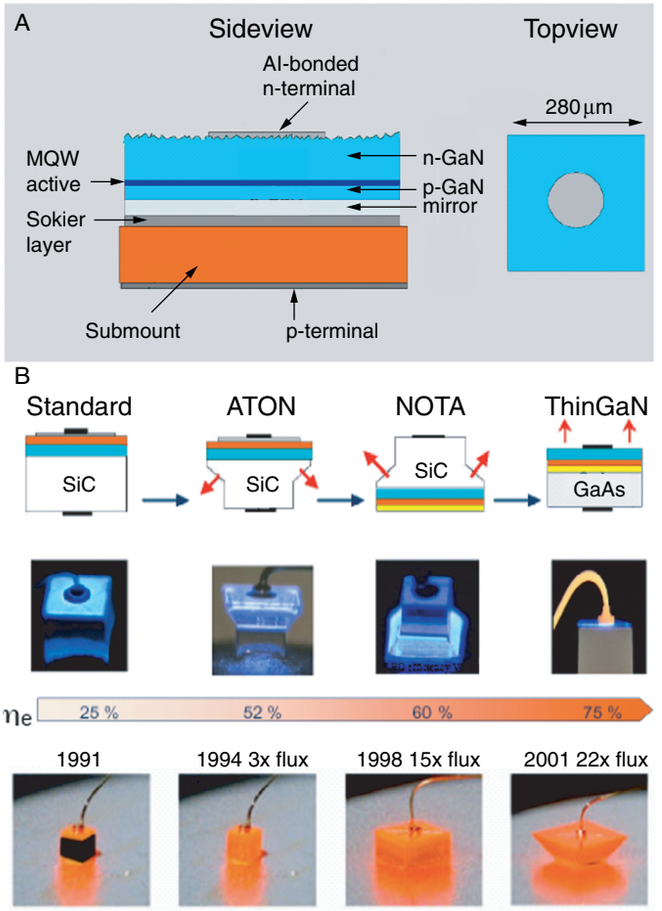

FIGURE 6.11. (A) General structure of an LED with the different layers marked. The mirror is used to reflect the back-emitted light to the right direction. The metal bond pad on the top is for the electrical contact. (B) Changes in the geometry of LED dies in recent years have led to larger dies and have increased the efficiency with which light is emitted. (With kind permission from OSRAM Optical Semiconductors, Regensburg, Germany.)

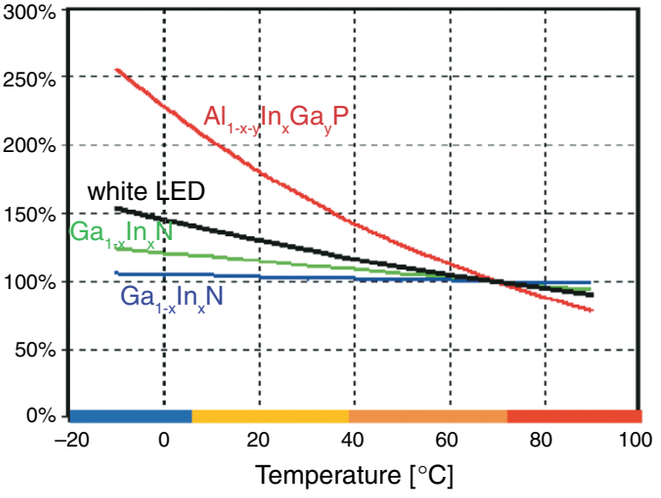

FIGURE 6.12. The dependence of light flux on LED temperature, standardized to 70°C for dies made using different types of LED crystals. Because dies emitting at longer wavelengths have a smaller bandgap energy, they are more affected by changes in temperature. Formulas for the compounds, note above.

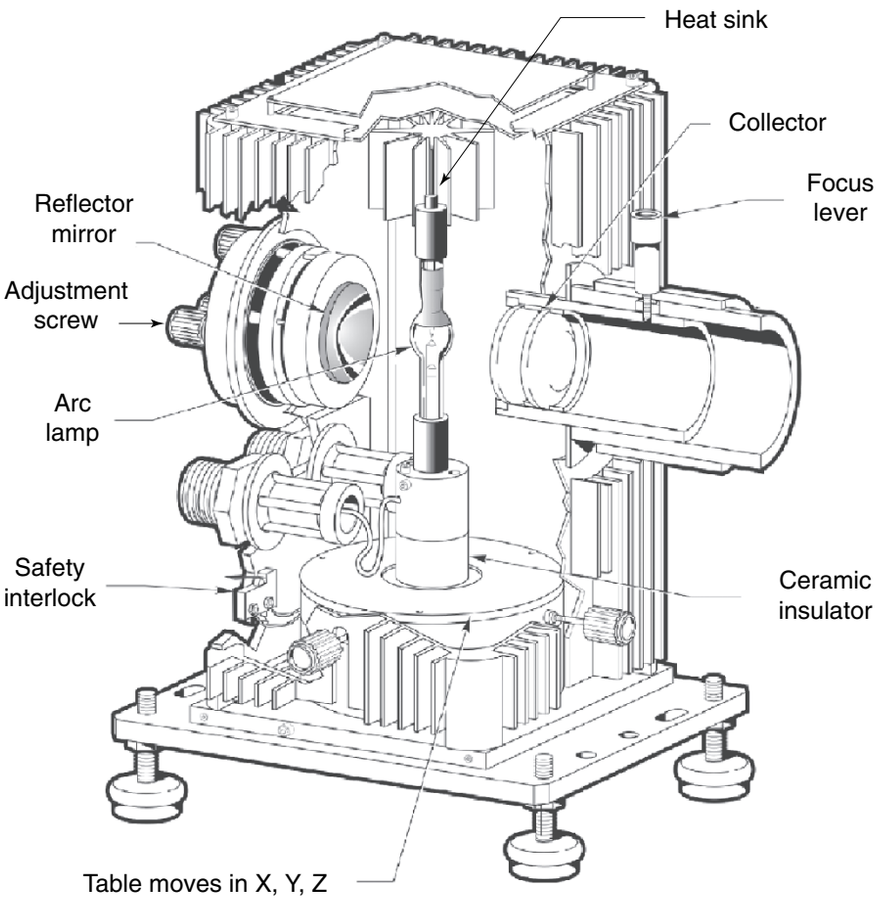

FIGURE 6.13. Diagram of lamp housing. The discharge of the arc is located at the focal points of the rear reflector and the first condenser lens, both of which can be moved in three directions to permit alignment with the optical system.

Reflecting and Collecting the Light: Source Optics

As incandescent and arc lamps are almost isotropic emitters that radiate equally in all directions, a spherical reflector can be placed behind the source to create the image of the source beside the actual arc. A collector lens is mounted so its focal point coincides with the center of the source; it catches a large fraction of the light and focuses it at the BFP of the condenser (Fig. 6.13). Due to the small source size and the desire for a short compact structure, collector lenses are usually aspheric and have high chromatic aberration.

LED-based sources use three different principles to reflect and collect the light generated inside the die. The first approach was to use the clear, molded plastic LED package itself to collect and focus the light. Although suitable for low-level bright-field applications, this method is rarely used in microscopy because of the limited optical power available from a single LED (e.g., the white light LED NEPW500, manufactured by Nichia with a $0.3\,mm^2$ die). The second approach is to work without a collector and print the LED dies directly onto the printed circuit board. The density of the packaging achievable in this way is only limited by the need to bond the single dies with the connecting wires and the ability to dissipate heat. The disadvantage is one loses any light that the LED die emits to the side (e.g., the monochrome arrays available from Laser2000 with 88 single $0.3\,mm^2$ dies packaged on a ceramic plate). The third possibility is to place the LED die into a mirrored well that acts as a reflector (e.g., the "Golden Dragon" manufactured by ORAM) and then arrange these units onto a printed circuit board. Because the reflectors are bigger than the dies, this results in a lower packing density.

As every die is a separate source, when an LED array is built up of several dies, one needs a different method to combine the light from them than one would use for a conventional lamp. The most efficient way to collect the emitted light is to use a microlens array placed the proper distance in front of the LED array. This array can be made of molded plastic or of glass and must be designed so that every LED die has its own collecting lens (Fig. 6.14). The main goal of this configuration is to catch as much light as possible and deliver it into the acceptance angle of the microscope illumination optics so that it fills the condenser aperture diaphragm with axial, parallel light as homogeneously as possible.

Source Alignment

The rule of thumb says the smaller the source, the more important it is to align it properly. For arc lamps and incandescent lamps, one must align the reflector and collector in such a way the source and its image from the reflector lie side by side and are centered in the aperture diaphragm of the illumination path. Only in this way will one be able to fill the aperture diaphragm approximately homogeneously (Fig. 6.15). Modern systems usually either incorporate special viewers so that one can check alignment or use self-aligning sources such as the self-aligning HBO-100 source available from Carl Zeiss (Fig. 6.16). The heart of the self-aligning source is a photodiode with four quadrants. Some of the light emitted by the arc in the direction towards the reflector passes through a small hole onto the optical axis where it is captured by

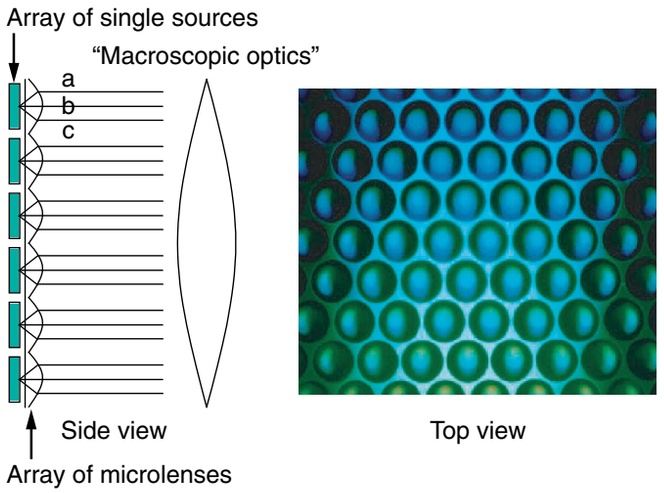

FIGURE 6.14. Principle of a micro-lens array. (A) Each mircro-lens efficiently couples the light from a single LED source to the macroscopic optics of the microscope. As a result, the macroscopic collector optics can have a lower aperture, and therefore, a larger focal length at a reasonable diameter. In addition, such optics have lower spherical and chromatic aberrations. (B) Hexagonal micro-lens array made of quartz glass. This example is normally used in telecommunication to couple two bundles of optical fibers together.

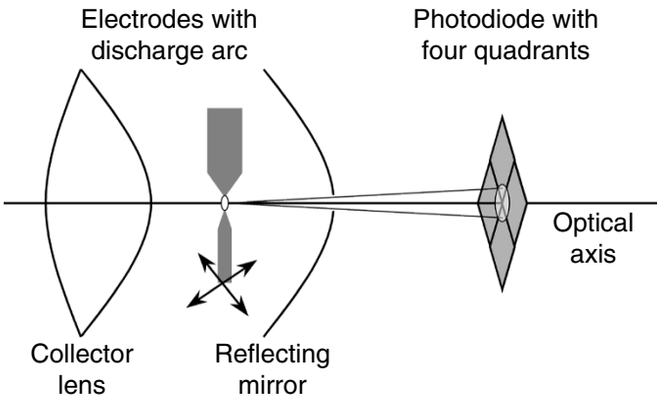

FIGURE 6.16. Components for a self-aligning source. Light passing through small hole in the reflector strikes a quadrant detector. Signals from this detector are used to move the arc in y and z until the output of all four diodes are equal.

the quadrants of the photodiode. A microcontroller measures the intensity of all four quadrants and moves the burner on an xy-stage until there is no difference between them. As the right reflector position depends on the particular optics of the microscope, the z-position of the reflector still has to be aligned manually.

For LED-based sources, alignment is simpler. In bright-field microscopy the main goal is to fill the aperture diaphragm as homogeneously as possible. This can be accomplished by mounting a large (5–10 mm) LED array just behind the aperture diaphragm. The problem in this case shifts to the alignment between the microlens array and the LED sources. As the focal length of the microlenses has to be relatively short and the LED sources are very small, the tolerance to a misalignment is also small.

Wavelength

Historically, many fluorophores were selected and used because they are excited by the intense lines of Hg arc lamps. This was not only because of the increased brightness but also because the narrow bandwidth made it more easy to design effective dichroic emission filters and beam-splitters. In addition, microscope objectives are often designed to give optimal correction at these wavelengths (Herman, 1998). As the laser wavelengths available seldom match these wavelengths, the microscopist using a laser confocal microscope must sometimes exchange familiar fluorochromes for new, less familiar ones that can be excited at laser wavelengths. Because histochemistry is complex enough without having to try entirely new stains and chemistry, it is important to have a light source flexible enough to excite the most suitable dye for each application.

The sun has very high brightness and the continuous spectrum of a black-body radiator with a surface temperature of 5800 K (Fig. 6.1). A clock-driven heliostat was used by Petran and colleagues (1985) to track it as a light source for confocal microscopy. The sun's broad continuous spectrum allows easy selection of wavelengths for difficult specimens.

Aside from the sun, only a synchrotron can provide as bright and continuous a spectrum (van der Oord, 1992; Gerritsen *et al.*, 1992, 1994). Although using the sun or a synchrotron as a light source might be ideal in many ways, the nuisance of having to depend on geography, season, cloudiness, and time of day led to the development of portable sources.

Incandescent lamps also emit essentially black-body radiation, the spectral shape of which depends on the temperature (Fig. 6.17). However, because the filament is only 3200 K or 3400 K, the light is less intense and has more red light than sunlight. Although it is possible to increase the blue component by raising the tempera-

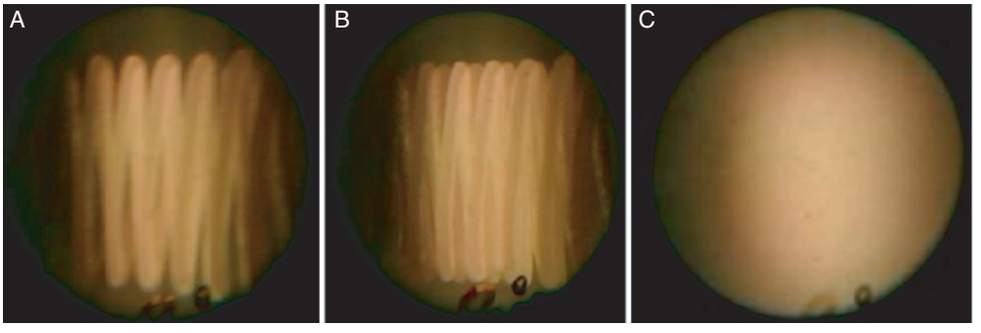

FIGURE 6.15. Image of the filament of a 100 W halogen source. (A) Image of filament and its reflection superimposed; (B) filament and its image side-by-side, right (C) after insertion of ground glass to randomize image of filament. The illumination optics do not magnify the image of the filament quite enough to fill the BFP completely. Even with the ground glass in place one can see a visible drop of intensity at the outer edges of the BFP.

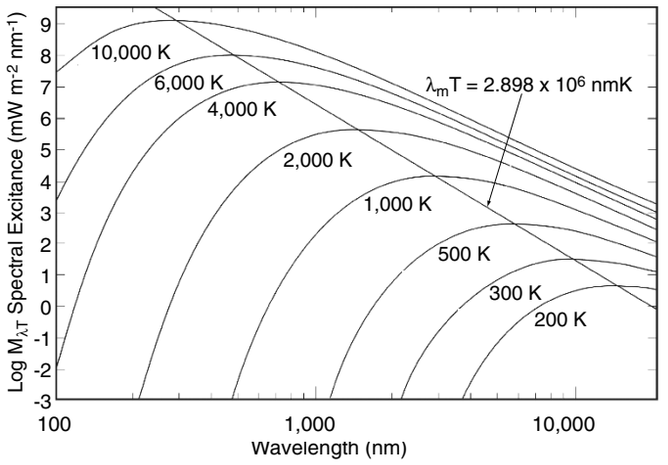

FIGURE 6.17. Spectral distribution of black-body radiation at various temperatures. Note that the vertical scale is logarithmic. The total luminosity of a black body varies with the fourth power of the temperature.

ture, doing so increases the rate at which the tungsten sublimes. The tungsten vapor condenses on the inside surface of the quartz envelope where it absorbs light from the source and heats the envelope. In addition, the filament becomes thinner until it fails.

The halogen gas in a quartz–halogen source interrupts this process by first reacting with the evaporating tungsten to form tungsten–halide compounds. These then decompose when they strike the hot filament, effectively returning the tungsten to the hottest (and thinnest) part of the filament. This permits the filament to be operated at a higher temperature with little darkening and a longer time before failure. Higher temperatures may also deform the filament structure, causing it to move from its correct location in the optical system.

The optimal wavelengths for exciting classic fluorescent dyes (436 nm, 546 nm, 579 nm, 365 nm, 405 nm) coincide with Hg arc emissions lines [Fig. 6.5(A)]. The carbon arc (not shown) has a single very intense line at 400 nm. Mixed-gas Hg-Xe arcs have many intense, useful spectral lines in the UV and visible. Xenon and zirconium arcs have spectral lines in the near IR.

The emission spectra of arc sources can be classified in three ways: (1) continuous, (2) line spectra, and (3) mix of lines and continuous spectra. Figure 6.5(A) shows the spectra of arcs using mercury and xenon, as well as a tungsten halogen incandescent source, plotted with the same horizontal scale.

The super-pressure xenon arc provides intense broadband illumination without prominent spectral lines in either the UV or the visible because the high pressure broadens the xenon spectral lines. Because the intensity of the continuum of a 75 W high-pressure xenon lamp is 2× higher than the continuum of a 100 W Hg arc lamp, the xenon lamp is better suited for low efficiency fluorophores not excited well by the prominent Hg lines. The availability of broadened spectral lines sometimes allows the simultaneous activation of several fluorochromes with differing emission wavelengths, including those in the UV.

The only disadvantage of the xenon lamp is the high pressure in the bulb (approximately 30 bar at room temperature and approximately 10× that at operating temperature). This makes it necessary to use protective gloves, goggles, and a shield to shelter the chest when changing a bulb in case it should explode.

The LED technology makes it possible to supply the right excitation wavelength for each fluorophore. Most wavelengths are now available from UV (365 nm) up to IR (>800 nm; Fig. 6.10) with intensities sufficient for widefield fluorescence. The FWHM of a quasi-monochrome LED varies from 20 to 40 nm, which is similar to the width of the excitation band of many fluorophores. Compared to laser light, the wider bandwidth of the LEDs make it easier to excite a variety of dyes, and compared to the continuous spectrum of an arc lamp they are cooler, smaller, and provide an easier way to choose the wavelength one wants and to do so rapidly. That said, one still needs to use excitation filters to remove the tails of their emission wavelength distribution.

Using conventional fluorescence microscopy as many as five different fluorescent labels have been imaged simultaneously in the same living cell (De Basio *et al.*, 1987).[14] This certainly could have been done as well using a disk-scanning confocal microscope having an efficiently configured arc source.

When imaging living cells, success requires using minimum light intensity at the specimen plane and choosing illumination wavelengths that interfere as little as possible with the life process under study. Using a continuous spectrum source, one can often make small wavelength changes that reduce specimen mortality. Because fluorophore excitation maxima are altered by their molecular environment, and because this is especially true for a fluorophore attached to a functioning macromolecule in a living cell, fine tuning the excitation wavelength can result in increased emission. Using strip- or wheel-type, continuously graded interference filters, such as those manufactured by Ocean Optics (Dunedin, FL), the microscopist can often precisely adjust the illuminating wavelength to minimize interference with the process under study and to maximize the excitation of a fluorophore in a particular cellular environment.

Stability in Time and Wavelength

Ramp-Up and Short-Time Stability

Everyone who has used an arc lamp knows the buzzing noise that occurs when the lamp is switched on and the plasma arc discharge is building up between the two electrodes. All sources based on plasma-discharge or incandescence need a considerable time to reach thermal stability.

Figure 6.18 shows typical intensity ramp-up curves for the various sources.[15] All lamps that produce significant heat show a dependence of the emission on the source temperature. This even applies to LED-based sources. It can take up to 1 h until the source is sufficiently stable to make reproducible measurements or to make a good time-lapse movie. Once operating temperature has been reached, the halogen lamp is the most stable source over time periods of a few milliseconds because of the high thermal inertia of the tungsten filament. LED-based sources react very fast (in a few microseconds) and, therefore, they are affected by any high-frequency instability in the power supply. In general, the most unstable source is the arc lamp. Not only is the arc itself a chaotic, flickering discharge but its light output can also be affected by ambient electromagnetic fields or an unstable power supply.

Stability can be increased by using the signal from a light sensor as a feedback signal to control the excitation power. The Oriel 68950 power supply controller (Newport, Inc., Irvine, CA)

[14] De Basio and colleagues measured five cell parameters: (fluorescent dye) nuclei (Hoechst 33342), mitochondria (diIC-[5]), endosomes (lissamine rhodamine), B-dextran, actin (fluorescein), cell volume (Cy7-dextran).

[15] These curves were measured with an upright microscope, using a source mounted in a common epi-illumination lamp housing. A 50/50 beam-splitter was used and a radiospectrometer placed at the BFP of the condenser.

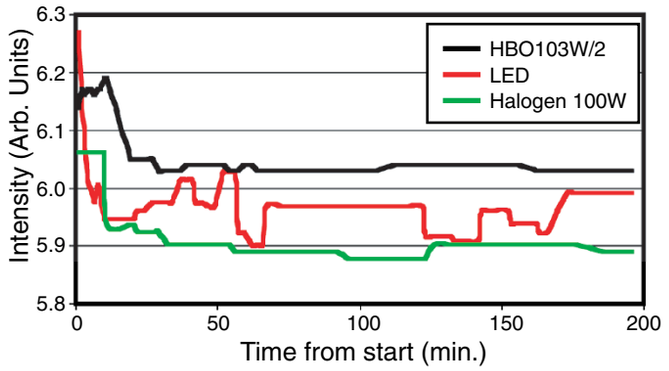

FIGURE 6.18. Ramp-up of brightness and long-term stability for various microscope light sources.

improves the stability of arc and halogen sources from 0.4% to 0.01% (Fig. 6.19). However, these figures are for total light output, and they cannot prevent local flickering in the particular region of the plasma that happens to be focused onto your specimen. Some suppliers of deconvolution systems, such as Applied Precision Instruments (Issaquah, WA), constantly monitor arc output during the CCD exposure time. They then use this information to normalize the CCD output for each plane of the *z*-stack.

Long-Time Stability, Degradation

When warmed up and powered by a regulated power supply, tungsten halogen sources are suitable for making photometric measurements.

Generally speaking, arcs are less stable than filament lamps because the points of the electrodes slowly erode. The larger radius of curvature that results reduces the concentration of current flow (and brightness) near the tip and also increases the power level needed to sustain the arc. Eventually the arc will not ignite. The intensity of the xenon arc can be deeply and rapidly modulated in time to keep the electrodes cool, as is the case for the electronic flash used for photography. Alternatively, the position of the arc plasma can be stabilized either by a periodic magnetic field imposed by a rotating permanent magnet or by the superimposition of a small, high frequency AC current on the main DC excitation current (Woodlee *et al.*, 1989).

As the photoelectric effect is fully reversible, it is not surprising that the LED has the lowest operating temperature and, as a result, is the most stable source. In addition, as long as it is operated at the proper voltage/current, the LED has a much longer lifetime than all other sources. One usually has to change an arc about every 200 h and incandescent lamps about every 500 h, but the LED sources have lifetimes in the range of a few thousands hours without significant loss of intensity. Some manufacturers promise a lifetime of 100,000 h before the source intensity drops to 70%.

Stability in Wavelength

In the discharge of an arc lamp, the gas pressure affects the composition of the spectrum. As the pressure rises, the prominent lines broaden and the continuum rises [e.g., compare the continuum brightness of a mercury and a high-pressure xenon lamp in Fig 6.5(A)]. On the other hand, the peak wavelengths of prominent lines shift just a few nanometers (<5).

The continuous spectrum of an incandescent lamp depends only on the temperature of the filament (Planck's law) and on the gas present in the bulb (Fig. 6.17). At a fixed current, a change in color temperature can only occur if evaporated tungsten condenses on the glass bulb. A change in the current to an LED causes a shift of the emission peak similar in magnitude to that seen in lines of the arc lamps. This may be due to the LED die not being perfectly homogeneous. The size of the shift depends on the type and quality of the crystal material used.

As the shifts are small compared to the width of the lines, one can neglect the effect for arc lamps. For LEDs, one either has to calibrate the dependence of the wavelength on the operating current or operate the die in a different way (see sections on Radiance and Control).

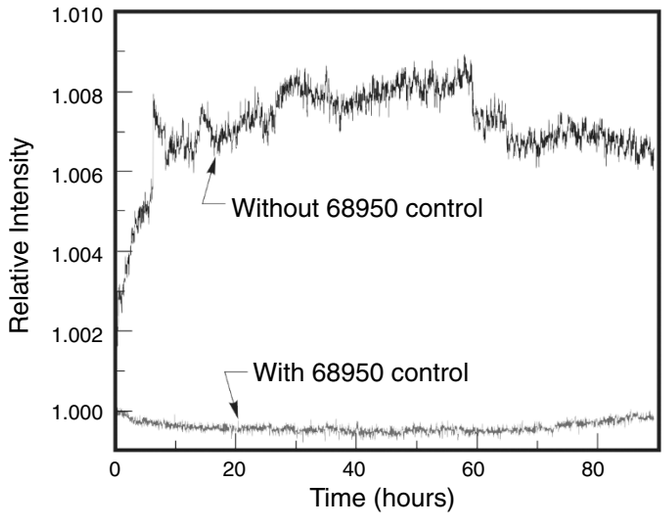

FIGURE 6.19. Stability plot of halogen source, with and without optical-feedback power stabilizing. Although quartz halogen sources are very stable, temperature rise increases output, and air-conditioning and other factors can produce transients. These can be compensated for by using the output from a monitor photodiode as the input to a negative feedback system controlling the power supplied to the lamp. (The plot was kindly provided by Oriel/Newport, Irvine, CA and reflects the performance of their 68950 power-supply controller.)

Radiance

Arc lamps are several orders of magnitude more radiant than tungsten filament lamps [Fig. 6.5(A)]. The HBO-100 (100 W high-pressure mercury arc lamp) is the most radiant of the commonly used lamps, whatever the wattage, because it has a very small source size (compared to the HBO-200, for example). Because of the optics rule mentioned at the beginning of this chapter, the larger arcs are only useful to illuminate larger areas of the specimen rather than to illuminate a single spot with maximum intensity. The main limitation on arc radiance is that the electrode tips erode or even melt as the power level increases (Fig. 6.5).

New arc sources of very high radiance have been described by Steen and Sorensen (1993). In these sources, commercial Hg, Xe, or Hg/Xe arc lamps have been modified to permit direct water cooling of the electrodes and the superposition of large-amplitude short-duration (20 µs) current pulses on the DC operating current. Increases in output of up to 10-fold were observed during each pulse. Hell and colleagues (1991) describe a new generation of short arc lamps with extremely short electrode distances (0.5 mm) using a Xe/I fill and tungsten carbide electrodes in a quartz bulb. The tungsten iodide TlJ dopant gives rise to a radiating plasma spot only 150 µm in diameter. Over the 450 to 550 nm band, the radiance exceeded that of the conventional Xe short arc by a factor of 3 to 5 and that of an HBO-100 mercury lamp by 12. The new arc lamp

has useful radiance from 360 to 570 nm. These developments push the arc source radiance closer to the realm once thought available only from lasers. Tables of the radiance of various arcs and lasers can be found in the catalogues of Melles Griot (Rochester, NY) and Oriel Corp. (Stratford, CT, now a division of Newport Inc.).

When driven by a 120 Hz square wave, the modulated (Hg-I) arc lamp introduced by LTM Corp. (Sun Valley, CA) produces a very useful spectrum with an efficiency of 110 lumens/W, compared to 30 lumens/W for a normal xenon arc. The deep square-wave modulation of the Hg-I arc reduces average heat production, allowing the lamp housing to be compact enough for placement near the microscope.

As the bulk depletion region inside the LED die is an isotropic emitter (lambert), one might assume that the light leaving the front surface of the die would also be isotropic in all directions. However, as the light generated in the volume of the crystal must pass through the crystal/air interface, any rays that strike this surface at less than the critical angle will be reflected and reabsorbed by the crystal. Approximately 50% of the light generated internally is lost in this way and less light is emitted at bigger angles.

The radiance of the high-brightness LEDs available today is still far less than that of the prominent lines of the arc lamps (Fig. 6.20). In the continuous operation mode, the brightest 2×2 mm LED die (Luxeon, 5 W Emitter, Fa. Lumileds, San Jose, CA) today delivers around 50% of the continuum radiance of a 75 W XBO at the same wavelength, and is bright enough to get an acceptable fluorescent signal from a well-stained specimen (Braun and Merrin, 2003). Unfortunately these high-power dies are not yet very stable and because of the high thermal load they degenerate very fast. More to the point, they are still not available in all wavelengths. At present, better results are obtained using smaller emitters (1 W emitter, Luxeon) to build up a light source. With a proper heat sink these are now very stable.

In pulsed-mode operation (see subsection under Control), the available radiance can be a factor of 20 higher than for the same unit used in continuous mode. When grabbing fluorescence pictures quickly, this mode is the most suitable one, as one can trigger the camera with the light pulse to ensure efficient usage of the emitted light. This mode of operation is also now being used in the machine vision area of industry where LEDs have become a long-life substitute for xenon flash lamps for illuminating moving objects (see, e.g., available light sources at http://www.laser2000.de). For other manufacturers, see the links in Table 6.1.

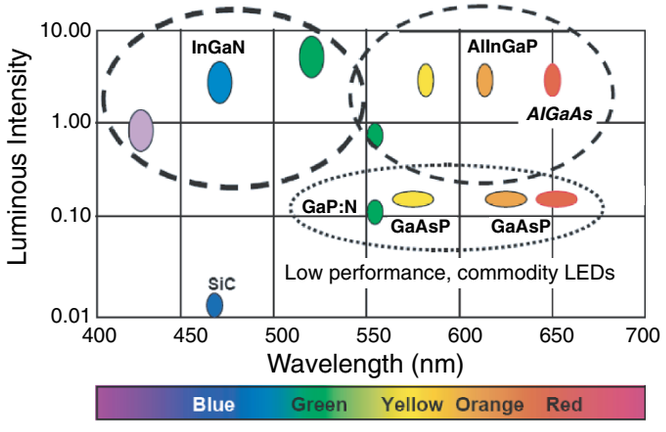

FIGURE 6.20. LED luminous intensity for various colors and dye compounds. Efficiency is measured in lumens/watt and refers to the efficiency with which electric power is converted into optical output.

TABLE 6.1. Useful Links

Microscopes, optics, and light sources
http://www.zeiss.com/
http://www.lot-oriel.com/
http://www.mellesgriot.com
http://www.chroma.com/
http://www.edmundoptics.com
http://www.optics.org/
http://www.wahl-optoparts.de/
http://www.oceanoptics.com
LEDs
http://www.luxeon.com/products/family.cfm?familyId=1
http://www.osram-os.com/
http://www.nichia.com/
http://we.home.agilent.com/USeng/nav/-11143.0/home.html
http://www.led.com/
http://www.optotech.com/
http://www.toyoda-gosei.com/led/index.html
http://www.stockeryale.com/
http://ledmuseum.home.att.net/ledleft.htm
http://www.laminaceramics.com
Basics and history
http://micro.magnet.fsu.edu/primer/
http://inventors.about.com/library/inventors/bllight.htm

Control

The quartz–halogen lamp is simply driven by a stabilized DC power supply converting the plug voltage into an adjustable voltage of 2 to 12 volts. Varying the voltage controls the temperature of the filament and thereby the spectral properties and intensity of the light.

Arc lamps are usually also driven by a current-stabilized power supply. The current can be decreased to 70% to lower the optical output and conserve the electrodes if one does not need the full optical power. Below 70% the plasma becomes unstable. Because of the decreasing temperature of the discharge, the vapor pressure drops and the discharge stops. Modern arc lamps have a heating filament wrapped around the bulb. This filament heats the bulb, restoring the vapor pressure, and allowing the current to be decreased down to 30% without stopping the discharge.

Neither arcs nor halogen lamps can be switched on rapidly. To change the emitting wavelength or intensity quickly one has to use mechanical shutters and filter wheels and switching times are usually longer than 100 ms.

The stabilizing circuitry of the arc power supply can stabilize the voltage, the current, or the total power (voltage × current). If the voltage is stabilized, the current (and the brightness) will slowly decrease as the electrodes become worn round. If the current is stabilized, the brightness will stay fairly constant[16] until the electrodes become too rounded for the arc to "strike." However, because an ever-higher voltage is required to maintain the fixed current, as the electrodes wear the power sent to the arc slowly increases. As a result, it can overheat and sometimes explode. Although power supplies that stabilize the total power level will avoid overheating, the light output will slowly drop with the current as the voltages needed to maintain the arc increases. All this suggests that it is best not to run an arc too long and that it might be a good idea to monitor the voltage across the arc to detect warning signs.

[16] The total light will stay about the same but it will be less concentrated at the tip of the electrode.

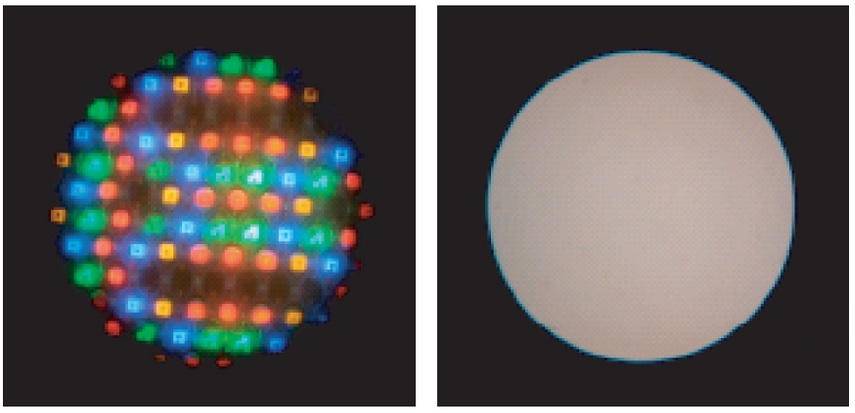

FIGURE 6.21. What an actual, functioning LED microscope source looks like. A close-packed array with four monochrome (470, 525, 590, 620 nm) colors on one matrix.

As each electron passing the depletion region emits one photon, LEDs can be controlled by any current-stabilized electrical source.[17] Depending on what is needed, the LED configuration as well as the control circuit can be easily changed. If only a single LED device is used (e.g., a "white" LED, usually one that couples a blue primary emission with red and green light from blue-excited phosphors), only a single-channel current source is needed and the intensity is controlled by changing the current flowing through the LED. It is more common to use more complex LED structures combining LED dies with different emission wavelengths to obtain either narrow-band light for multi fluorescence or "white light" in bright-field microscopy (Fig. 6.21). Such devices are controlled by a multi-channel current source. By rapidly switching these currents on and off, it is possible to change intensity or emission wavelength on a microsecond or even nanosecond scale. This is a very important feature for short-time scale methods such as fluorescence lifetime imaging measurements (FLIM; Hermann *et al.*, 2001). The switching on this timescale is called pulse mode.

Because the peak emission of a given type of a LED can be shifted by changing the current level, it is often more suitable to operate multi-LEDs in the pulse mode. One sets the peak current to produce the desired output wavelength and then changes the average source brightness by varying the pulse width at a fixed peak current. Although, compared to continuous mode, more total light is available in this way, using a higher current for more than a short pulse will lead to thermal damage. The "damage threshold" current pulse width must be evaluated for each LED.

The spectral output of the LED can be controlled very precisely in this way. The optical output follows the current pulse without significant delay. Pulse-modulation frequencies up to megahertz are possible.

MEASURING WHAT COMES THROUGH THE ILLUMINATION SYSTEM

The procedures for measuring the light throughput of any microscope with a photometer are thoroughly described in a step-by-step manner in the book *Photomicrography* (Loveland, 1970). He even describes making a photometer for such a purpose, but a useful substitute can be made by attaching almost any small photodiode (or even a small "solar cell") to an inexpensive digital voltmeter set on a sensitive current range. Using this, one can measure light in the most obscure locations within the microscope. "Photon bookkeeping" based on such measurements is the only way to pinpoint those parts of the light path where preventable loss is occurring.

Microscopists not interested in building a photometer can obtain one of the commercial units. World Precision Instruments (Sarasota, FL) markets a fiber-optic monochromator and photomultiplier subsystem that can be used to examine light at the intensities present at any location in a confocal optical system. A 50 µm fiber is standard with this system and other vendors are listed in Table 6.3.

Young (1989) described the use of a feedback-controlled LED to generate known amounts of light from small (5–50 µm) sources. Using this system in either the source plane or the image plane, he was able to calibrate the input–output characteristics of a microscope system over four orders of magnitude.

For a measurement of radiance resolved by angle and wavelength simultaneously one must use a professional radiospectrometer such as the CS-1000 made by Minolta. Such a device can provide very detailed information on the quality of an illumination source and the illumination path.

Selective light loss can occur anywhere along the optical path, heat filters, tilted interference filters, and dust windows, as well as obvious lens elements [consider that every lens surface causes the loss of at least 1% of the incident light despite anti-reflection (AR) coatings]. In the past, the transmission characteristics of objectives were seldom displayed in manuals, and even the general characteristics of UV versus non-UV lenses are still often hard to obtain (some figures are listed in Chapters 7, 27, and 29, *this volume*). Popular photography magazines often feature the color bias of various camera lenses, and these show that the color effect of a given AR coating is different for the large NA rays than for those near the optical axis, due to the quality and the optical properties of the lens coating. The AR coating on dust windows may block the transmission of UV or IR illumination. Epi-illumination requires broad transmission in both the illumination and the viewing direction.

The Bare Minimum

Even if one isn't inclined to be a full-time photon sleuth, it is wise at least to monitor the performance of the illumination system under a few commonly used standard conditions. For example, one should monitor how much light emerges from a favorite high-NA objective when it is set up for Köhler illumination with a particular filter cube and with the field diaphragm set to just illuminate the full field of this objective. This can be measured with a 1 cm^2 photometer paddle held in front of the objective. Assuming that one does not want to oil-couple the sensor to the objective, make

[17] On the other hand, use of a voltage-stabilized source will almost certainly damage these devices. At a fixed voltage the current increases with temperature, causing thermal runaway.

sure that the front of it is free from oil so that the fraction of the light escaping into the air is set only by total internal reflection from the flat front element (not an ideal situation but at least one that is repeatable). Then do the same with the most commonly used low magnification lens and maybe a couple of different filter cubes. Changes in these numbers will warn of misalignment, dust, aging arc bulbs, damaged filters, or help one determine the final resting place of that bit of paper that fell down inside.

An alternative to the photometer paddle is an Ulbricht sphere (also called an integrating sphere). Using an Ulbricht sphere, there is no problem with rays being reflected by the surface of the detector because the entrance of the detector is just a hole. Using this device, it is easy to measure what fraction of light reaches the specimen. On an upright microscope, remove the condenser and the *xy*-stage and mount the Ulbricht sphere below the objective with the entrance hole at the specimen plane. It may help to use the halogen lamp with the bright-field contrast in reflection to align the parts to each other. Mount the lamp of interest at the reflection port for illumination. All the light passing the objective is captured by the sphere. Table 6.2 shows the optical power delivered to the specimen plane measured in this way for various light sources and for two objectives with different fields of view.

TABLE 6.2. Optical Power of Different Light Sources in the Specimen Plane

Filterset No. Used	Excitation Wavelength (pass width) in nm	Examples for Fluorescence Dyes	HBO103W2	XBO75	LEDs	HAL100 (at 12 V)
			Optical Power [mW]			
Objective PlanNeoFluar 40×/0.75, field of view 0.625 mm diameter						
#2	365 (50)	DAPI, Hoechst33342	30.8	0.1	4.4 Nichia 3 W 365 nm	0
#47	436 (20)	CFP, ChromomycinA	10.5	3.5	1.4 Luxeon 3 W 450 nm	0.2
#9	450–490	GFP, Fluorescein	6.4	12.7	1.9 Luxeon 1 W 470 nm 4.8 Luxeon 3 W 470 nm 10.0 Luxeon 3 W 450 nm	3.2
#46	500 (20)	Calcium Green, YFP	1.6	4.4	0.4 Luxeon 1 W 505 nm 1.5 Luxeon 3 W 505 nm	1.5
#14	510–560	RhodamineB	20.2	12.7	1.3 Luxeon 1 W 530 nm 2.4 Luxeon 3 W 530 nm 0.1 Luxeon 1 W 505 nm	7.1
#20	546 (12)	Cy3, Rhodamine	11.1	2.8	☹	1.4
#26	575–625	Cy5	125.0	9.7	0.5 Luxeon 1 W 590 nm	8.3
Objective fluar 10×/0.5, field of view 2.5 mm diameter						
#2	365 (50)		61.4	3.4	8.5 Nichia 3 W 365 nm	0
#47	436 (20)		43.9	10.5	3.4 Luxeon 3 W 450 nm	1.8
#9	450–490		27.2	33.8	4.8 Luxeon 1 W 470 nm 11.1 Luxeon 3 W 470 nm 23.0 Luxeon 3 W 450 nm	11.1
#46	500 (20)		8.5	11.7	1.5 Luxeon 1 W 505 nm 4.3 Luxeon 3 W 505 nm	4.5
#14	510–560		79.0	36.7	2.8 Luxeon 1 W 530 nm 4.0 Luxeon 3 W 530 nm 0.9 Luxeon 1 W 505 nm	22.6
#20	546 (12)		44.2	8.3	☹	4.2
#26	575–625		243.1	30.1	2.7 Luxeon 1 W 590 nm	30.1

The different filter sets were chosen to show a representative profile of the optical power of the light sources at different wavelengths. The bandpasses of filter sets #9 and #46 lie between two prominent lines of the HBO103W2. The XBO75 is much more radiant. The optical power of the HAL100 is between one third and one half of the continuum of the HBO103W2. This is sufficient to excite the brightest dyes. We have made preliminary measurements of two examples for LED-based sources. In each case, a single 1 W Luxeon emitter was used with a single collector lens with no further alignment. This means that the values for the LEDs in Table 6.2 represent a minimum for the optical power at the specimen plane using only a single, 1 W die. With a more radiant emitter, or more individual dies and proper collecting optics, the optical power at the specimen plane can easily be increased by a factor of 4 to 8. This would put the LED radiance between the HAL100 and the arc sources.

TYPES OF CONFOCAL MICROSCOPES THAT CAN USE NON-LASER LIGHT SOURCES

The notion that confocal microscopes must use laser illumination is widespread because most commercial confocal microscopes are single-beam instruments (Leica, Nikon Real Time, Olympus, Zeiss), and these currently use only laser illumination. In fact, none of these microscopes even make a provision for the user to connect a non-laser light source for use in the confocal mode.

Nevertheless, it is not true that single-beam confocal microscopes **require** laser light. Minsky (the inventor of the first confocal microscope) used a zirconium arc illuminator in the functional prototype stage-scanning microscope he built in the 1950s (Minsky, 1988). Many current commercial disk-scanning confocal microscopes come only with non-laser sources because only such sources can provide the broad beam needed to simultaneously illuminate the many confocal apertures in the field of view.

Using a tandem-scanning confocal microscope with a high-NA water-immersion objective, transparent ciliate protozoa such as paramecium and vorticella can easily be viewed by eye swimming in water in the confocal BSL mode. BSL images are formed using the light that is scattered by the index of refraction difference between organelles and water. By carefully adjusting the rotation speed of the aperture disk, one can view the rapidly beating cilia with stroboscopic illumination. A field of beating cilia viewed *en face* appears as dots (cross-sections of the cilia) slowly moving in circles. Though easily viewed by eye, this motion is difficult to capture electronically or photographically because the brain is able to extract meaning out of successive images with the slight trailing-edge blur that renders electronically captured single images meaningless.

Tandem Scanning: Basic Description

The tandem-scanning mechanism consists of a symmetrical, spinning Nipkow aperture disk at the intermediate image plane of the objective. Thousands of apertures arranged in spirals both send beams to be focused on the object and sample the light returning to form the intermediate image. The double-sided optical system developed by Petran uniformly illuminates the excitation area of the disk that is to be imaged onto the object. Through a series of mirrors and beam-splitters, the image returning from the spots in the specimen is focused onto the lower surface of the opposite side of the disk where the in-focus light passes through a mirror image conjugate set of holes (Fig. 10.4, *this volume*). This series of mirrors and beam-splitters permits the illuminating and sensing apertures to be distinct, thereby preventing light reflected by the solid part of the disk on the illumination side from reaching the imaging side of the system.

The light-source optics must fulfill two functions. They must illuminate the area of the disk that will be utilized to form the final image (usually 1 to 2.5 cm diameter). In addition, this light must leave the disk with the correct angle of divergence to fill the BFP of the objective lens. On disks with very small holes, diffraction at the holes will usually ensure that the second condition is satisfied, so the problem becomes how to get the maximum amount of light incident on the active area of the disk. This is important because, as only 1% to 2% of the disk is open, the system is very wasteful of light. Careful matching of a large-NA collector lens and an optimized condenser is needed to ensure that sufficient light reaches the specimen to form an image in a reasonable time.

Single-Sided Disk Scanning: Basic Description

In a single-sided, disk-scanning optical system, the spinning Nipkow aperture disk is again located at the intermediate image plane of the objective but, because the beam-splitter is above the disk, the same apertures now serve as both sources and pinholes (Fig. 10.4, *this volume*). The aperture disk is tilted and covered with "black chrome" to reduce reflections of the source from reaching the eyepiece. Furthermore, a polarizer placed in the illumination path, a quarter waveplate above the objective, and an analyzer at the eyepiece form an "anti-flex" system to further reduce the effect of disk reflections.[18] Because it lacks any mirrors between the disk and the objective, the single-sided system is self-aligning.

Boyde and Petran (1990) directly tested the light budget in the two types of disk confocal systems and found little difference in light efficiency, though the tandem system appeared to have better contrast.

EXPOSURE TIME AND SOURCE BRIGHTNESS

In conventional microscopy, data for every point in the image is collected in parallel. This leads to a short capture time compared to any scanning process. The capture time is only limited by the time needed to fill the pixels in the CCD camera. Depending on the magnification, the fluorescent dye concentration, and the quality of the CCD itself, recording times vary from a few milliseconds to several seconds. Living cell observations with moving specimens are no problem. Figure 6.22 shows comparison pictures of cells taken with an HBO arc lamp and an LED-based source. Although the lower brightness of present LEDs required a recording time about four times longer than that needed with the HBO source, the quality of the images is comparable.

In scanning microscopy, the image is formed by scanning a point or a group of points over the surface to be imaged, and this scanning process takes a finite amount of time. If a raster scan is used, the image is completed when the raster is finished, and so the confocal microscope is a sampling system in both time and space. To view specimens that move or change accurately, the scan time must be short compared to the expected rate of change. This requires not just a fast scanning system but also a light source

[18] While at the same time, reducing light throughput by about two thirds.

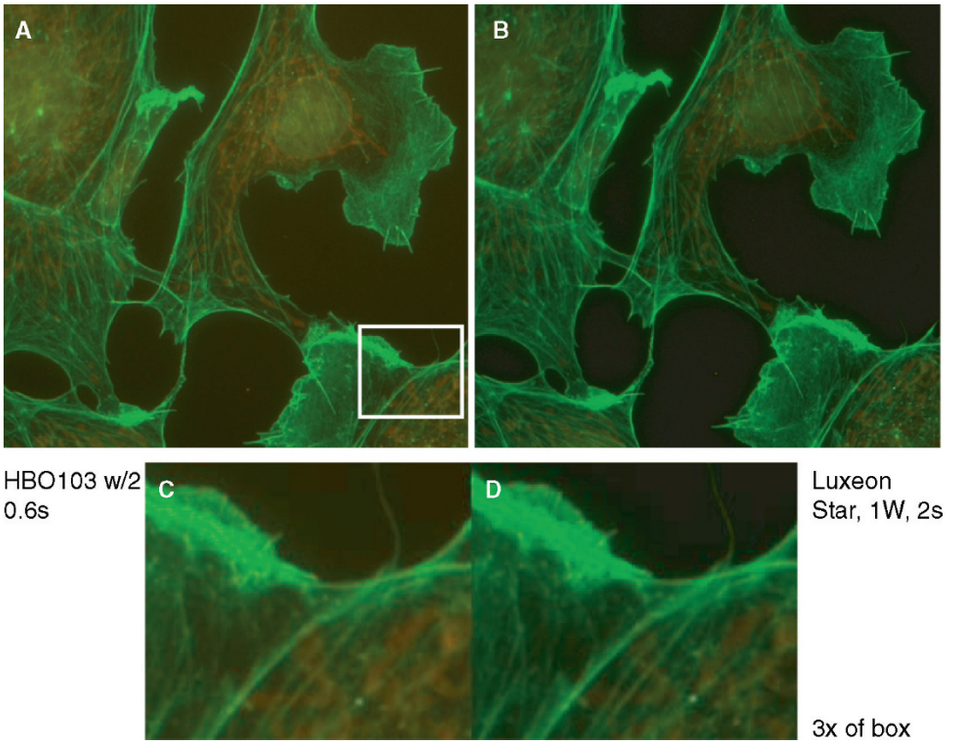

FIGURE 6.22. Fluorescent actin filaments imaged using HBO and LED sources. (A) HBO103W/2, exposure 600 ms, (B) Luxeon Star LED, 1 W, exposure 2 s. In the enlarged insets, one can see that some of the broadband light from the HBO gets past the excitation filter to excite the red MitoTracker Red dye as well as the Bodipy FL phallacidin, green dye, while the narrowband light from the LED does not do so. (Specimens are fixed BPAE cells stained on a Molecular Probes FluoCells prepared #1 slide. Imaged with an Axioplan 2, 63× /1.4 oil objective, Axiocam HrC, Filterset #9.)

bright enough to elicit from the specimen sufficient signal to make a usable image during the available scan time. In other words, shorter scan times need brighter sources. In practice, it has been the inability of arcs and LED-based sources to match the brightness of the laser that has prevented disk-scanning instruments from seriously challenging laser instruments for viewing low-intensity fluorescent specimens.

Because the rate at which signal can be derived from a fluorescent specimen depends fundamentally on the rate at which excitation photons impinge on the imaged area, some idea of the relative merits of the two approaches can be gained by measuring this quantity. When comparing disk- and laser-scanning data rates, it is important to normalize for the size of the area illuminated on the specimen from which data are being recorded because the former collects data in parallel while the latter collects only from one point at a time. Given comparable pinhole sizes and optical efficiencies, the crucial factor for a disk-scanning microscope is the rate at which the narrow-band, excitatory radiation strikes the area of the specimen that can be imaged by a high-quality imaging detector, such as a cooled CCD. A 512×512 CCD operating at the total magnification needed for proper Nyquist sampling of 0.25 µm resolution data (i.e., 0.1 µm pixels at the specimen; Chapter 4, *this volume*), images an area about $50 \times 50\,\mu m^2$ on the specimen.

In 1990, the illumination systems of the commercial, double-sided disk-scanning confocal microscopes could concentrate only 2 to 3 µW of narrow-band light into a 50×50 µm area, while the single-sided instruments could produce 6 µW (personal communication, V. Cejna, Technical Instruments, San Jose, CA). By contrast, the laser sources on the confocal laser-scanning microscope (CLSM) can easily deliver 100× more power (without producing significant singlet-state fluorescence saturation!) and can consequently produce data from (and bleaching of!) the specimen at a proportionally higher rate. This improvement is only partially offset by the fact that the CCD detector is about 3 to 6 times more quantum efficient than the photomultiplier tube used in the laser systems.

On the other hand, because disk-scanning instruments use many simultaneous apertures, the absolute limit on data acquisition presented by singlet-state fluorescence saturation (Chapters 2, 16, and 21, *this volume*) is far less of a limitation on these instruments. As a result, if higher radiance non-laser sources are developed and low-read-noise, electron-multiplier CCDs (EM-CCDs) replace the conventional CCDs now commonly used, the disk-scanning approach could eventually produce even higher useful frame rates than single-beam laser instruments and do so with the same number of photons from the specimen.

Another way to speed up the data rate is to use larger CCD sensors (1000^2). These would permit parallel detection of data from a larger area of the specimen while still maintaining the same pixel size. Although this strategy would increase the effective data acquisition rate by an amount proportional to the number of sensors in the detector, it would do so only at the price of viewing ever larger fields of the specimen. In other words, while it can be useful to survey larger fields in the same amount of time, it is not the same thing as increasing the source brightness or optical efficiency to permit imaging a particular cell more rapidly.

As current disk-scanning systems utilizing better illumination sources and improved optics claim power levels at the specimen that are 5 to 10 times higher than those measured in 1990, the balance may soon shift to the disk scanners.

FUTURE TRENDS

Viewed in one way, the arc lamps produce enough light right now to saturate or bleach common fluorescent dyes. The weak point of these sources is the difficulty of rapidly controlling operating parameters such as emitted intensity or spectral distribution. As these sources have been optimized over a period of many years, a quantum leap in performance is unlikely. On the other hand, arcs using different gas mixtures and electrode materials are constantly being developed and small improvements are likely to continue. The same is true for tungsten–halogen sources.

Viewed in another way, 100 years after the first use of Hg arcs in microscopy, and 40 years after the advent of the LED, microscopy has a new light source with many exciting possibilities. LEDs have all the features that arc lamps lack and they will soon be efficient enough to be run on a small battery. Although their weak point is still their marginal intensity, if one looks at the LED optical efficiency line over the last few years (Fig. 6.23), one notices a very interesting trend. LED brightness is projected to increase by about a factor of 3 in the next 5 years. Efforts are under way to use different growth mechanisms to produce LED die crystals with a geometry that decreases the loss of light through internal reflection. If this effort is successful, LEDs should be able to succeed in all fluorescence applications.

Improvements in the coupling between the LEDs and the microlens array and diffractive optical elements will increase the efficiency of the collection optics. Molded plastic technology is becoming ever more flexible in being formed to satisfy the requirements of this application. The brightness of a multi-color source mounted as a single flat array, such as that shown in Figure 6.21, could be increased by 3 or 4× by coupling together light from three to four planar sources, each emitting at a different wavelength, using an arrangement of dichroics and prisms similar to that used to separate R, G, and B light in a three-chip color CCD sensor. This would allow each LED color to be emitted at every location in the BFP. Three times as many dies would increase total light output by 300%.

Because LEDs have **no** emission at either 1/2 or 2× the design wavelength, it should be possible to develop more inexpensive and efficient light-source/filter sets for particular fluorescent dyes.

Somewhat farther over the horizon is the promise of organic LEDs (OLEDs). First developed in the early 1990s, OLEDs are built of organic molecules and light is generated by exciting the molecular orbitals of chromophore groups. Organic molecules sitting in a plastic lattice can be formed into almost any shape one can imagine. The brightness of these sources is now similar to that of conventional LEDs 20 years ago (Zhou, 2001). Although the operating lifetime of these sources is still very short, this technology is important as a potential area-display for consumer goods, such as video cellular phones, so substantial improvements may occur.

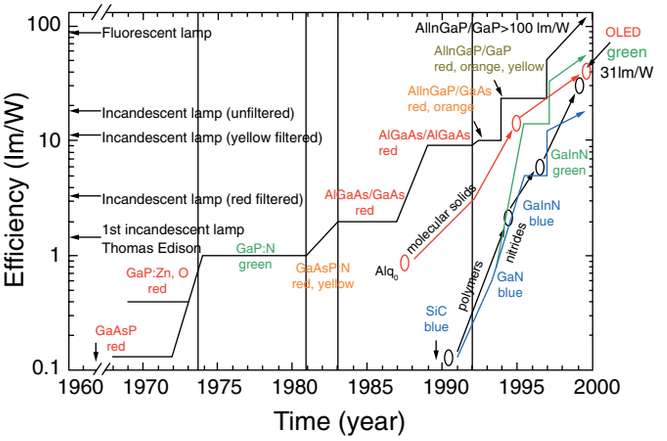

FIGURE 6.23. Development of the optical efficiency of LEDs in the last 40 years.

Significant improvements in arc sources (~10×) could be realized by using a modified elliptical collector (Luthjens *et al.*, 1990) with one of the newer Xe/I sources and by optimizing the magnification of the illumination optics to concentrate more light from the brightest part of this source into the 50 µm field covered by a 512 × 512 CCD.

Another development that should be mentioned is the emergence of a number of companies providing stand-alone light sources for use in microscopy (Table 6.3). Although initially these tended to be fiber-optic illuminators suitable for use with dissecting microscopes, more recently sources suitable for use in high-performance epifluorescence microscopy have been offered. Besides utilizing optimized arcs in elliptical collector mirrors and high-speed filters wheels for rapidly shifting the output wavelength, they also provide fiber-optic light scrambling.

TABLE 6.3. Companies Making Stand-Alone Microscopy Light Sources

Company	Model	Type of Source	Rapid λ Shifting?	Wavelength range, nm	Interfaces to . . .	URL
API	Uniform light source	HBO100/2	Yes	320–700	Sold only w/system	http://www.api.com/
Dolan-Jenner	Cold light sources	Arcs, e.g., metal halide	No	UV–VIS	Optical fiber	http://www.dolanjenner.com
EXFO	X-Cite 120PC	120 W metal halide	No	UV–VIS	Custom coupling optics	http://www.exfolifesciences.com
Illumination Technologies	Cold light sources	Halogen	No	VIS	Optical fiber	http://www.illuminationtech.com
Schott	Cold light sources	Arcs, e.g., metal halide	No	UV–VIS	Optical fiber	http://www.schott.com
StockerYale	Cold light sources	Halogen	No	VIS	Optical fiber	http://www.stockeryale.com
Sutter Inst	Lambda DG4	175 W XBO based	Yes, 1.2 ms	300–700	Optical fiber → flexible	http://www.sutter.com/
Till Photonics	Polycrome V	150 W XBO based	Yes, 400 nm/ms	320–680	Optical fiber → flexible	http://www.till-photonics.de
Technical Video Ltd	Fiber optic light scrambler	HBO100W/2	No	320–800	Optical fiber → All	http://www.technicalvideo.com

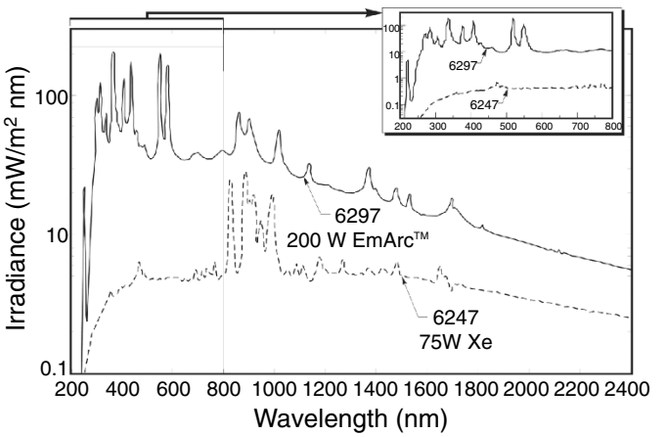

FIGURE 6.24. Spectral distribution of a 200 W EmArc, mercury–halide arc source and a 75 W xenon arc lamp (*dashed line*) 200 to 2400 nm. (Spectra kindly provided by Oriel/Newport, Irvine, CA.)

In a way, the really good news is that there is a great demand for high brightness light sources in areas of the economy that have considerably more market power than microscopy. Perusal of the Internet will show a wide variety of ingenious sources responding to both the general need to get more light for fewer watts of electric power and the more specific need to keep the source size small. The digital projectors used at scientific meetings are just the early scouts of a digital revolution about to overtake the movie industry. These projectors will need bright sources. Figure 6.24 shows the performance of one of the early candidates, a 200 W enhanced metal arc source that uses a mixture of gasses to produce an order of magnitude increase in light output in the visible, compared to a short arc 75 W Xe arc (USHIO, Inc. Cypress, CA). Although, at over US$2000 each, it is unlikely that many microscopists will use such sources immediately, there is every reason to think that eventually prices will come down.

ACKNOWLEDGMENTS

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