

This unit provides an overview of light microscopy, including objectives, light sources, filters, film, and color photography for fluorescence microscopy and fluorescence in situ hybridization (FISH). Computerized image analysis systems currently used in clinical cytogenetics are also discussed. Scanning and transmission electron microscopy as well as confocal microscopy are not covered in this unit despite their usefulness as invaluable tools for contemporary studies of biological systems (see van der Voort et al., 1987; Shotton, 1993 for further information).

## HISTORICAL FOUNDATIONS OF MICROSCOPY

Even in medieval times it was understood that curved mirrors and hollow glass spheres filled with water had a magnifying effect. In the early 17th century, men began experimenting with lenses to increase magnification. Galileo used the lensed telescope in 1609. Naturalists Jan Swammerdam (1637-1680) and Nehemiah Grew (1641-1712), anatomist Regnier Graaf (1641-1673), and physiologist Marcello Malpighi (1628-1694) made important discoveries using magnifying lenses. The Dutch merchant, Anton van Leeuwenhoek (1632-1723) meticulously produced his own lenses with which he was able to obtain a 200-fold clear magnification and build the first microscope. Later, the Royal Society encouraged Robert Hooke (1635-1703) and Nehemiah Grew to build their own microscopes. Hooke made his first public demonstration on November 15, 1677, at the Royal Society meeting where the "enchanted beasts" previously described by van Leeuwenhoek were observed by the awestruck crowd. Hooke's book, *Micrographia*, published in 1665, contains beautiful drawings based on his microscopic observations. It was not until the year 1773 that a Danish microbiologist, Otto Muller (1730-1784), used the microscope to describe the forms and shapes of various bacteria. Waldeyer coined the term "chromosomes" in 1888 to refer to those colored bodies he saw in dividing cells. In 1893, August Köhler, a German zoologist, described the principles of what we now refer to as Köhler illumination. This was a critical step in generating a uniform field of illumination and providing optimal image resolution. Köhler, along with Reichert, Lehman, and others, went on to de-

velop the fluorescence microscope. It was not until the 1950s, however, when Coons and Kaplan used a fluorescein-tagged antibody to localize antigens in tissue sections, that its value began to be realized. It was still difficult to unequivocally identify individual chromosomes except to classify them by grouping based on morphology (size and centromere location). This changed in 1968, when Caspersen developed his method of fluorescently banding chromosomes using quinacrine mustard. Although chromosome banding is now routinely done using the nonfluorescent dye Giemsa, fluorescent identification of Waldeyer's colored bodies using a multicolor fluorescence hybridization approach combines the advances made in both cytogenetics and microscopy.

## MICROSCOPY IN MODERN HUMAN GENETICS

Microscopy currently plays a crucial role in both research and diagnostic aspects of modern genetics. This typically involves the use of light microscopes for the analysis of microbiological, cytological, and pathological specimens, as well as the cytogenetic analysis of metaphase and interphase chromosomes. With recent advances in fluorescence technology, there has been growth, even in clinical laboratories, in the use of fluorescence microscopy. Analysis of cell types in blood and biopsy specimens, apoptosis assays in diseased tissue, cytogenetic analysis, and even surgical procedures using fluorescence have all been reported. Because this unit deals primarily with the use of microscopy and contemporary image analysis in mammalian cytogenetics, a brief explanation of some of the applications of fluorescence microscopy in this field is relevant.

The ability to label nucleic acids with fluorescent molecules and detect them in situ was developed in the early 1980s (Langer-Safer et al., 1982; Manuelidis et al., 1982). Fluorescence in situ hybridization (FISH; UNIT 4.3) technology has been applied to many different areas of cytogenetic investigation (Lichter and Ward, 1990). The first use involved determination of chromosome copy number in interphase nuclei using centromere-specific fluorescent probes (Manuelidis, 1985; Cremer et al., 1986; Devilee et al., 1988). This was later extended to the identification of aberrant or marker chro-

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mosomes (Taniwaki et al., 1993; Thangavelu et al., 1994; Blennow et al., 1995) or microdeletions (Ried et al., 1990) using chromosome painting probes or single-copy probes known to map to specific chromosomes. Giemsa banding was not compatible with fluorescence hybridization techniques, thereby making it difficult to obtain simultaneous identification of the chromosomes. Methods were soon developed, however, whereby banding patterns could be obtained through the hybridization of fluorescently labeled repetitive sequences in both humans (Baldini and Ward, 1991) and mice (Boyle et al., 1990a; Arnold et al., 1992). Concurrent with these advances came the start of the Human Genome Project and the application of FISH to gene mapping (Lichter et al., 1990; Ward et al., 1991; Lichter et al., 1992; Ried et al., 1992; Otsu et al., 1993; Trask et al., 1993). FISH mapping is a useful technique for the identification of other genes in a gene family, including functional and non-functional genes (Giordano et al., 1993), and for the mapping of genes across species barriers, a technique referred to as Zoo-FISH (Wienberg et al., 1990; Raudsepp et al., 1996; Fronicke and Scherthan, 1997; O'Brien et al., 1997; Chowdhary et al., 1998).

The fluorescent labeling and hybridization of entire genomes is useful for a number of different areas of investigation. Somatic cell hybrid lines (see Chapter 3) have proven very useful for isolating and mapping disease genes in both mice and humans (Harris, 1995). It is important to determine the genomic contribution of the species of interest in these lines not only during their derivation, but also periodically due to their unstable nature. This can be done by labeling the genomes of the parental species and hybridizing them to metaphases from the hybrid line to display the chromosomes contributed by each species (Durnam et al., 1985; Manuelidis, 1985; Schardin et al., 1985). The reciprocal experiment of labeling the somatic-line genome and hybridizing it to metaphases from each of the parental species identifies the specific chromosome(s) segregating in the hybrid (Boyle et al., 1990b; Doucette-Stamm et al., 1991). Comparative genome hybridization (CGH) is another technique involving the fluorescent labeling of entire genomes. In this instance, the genomes are from karyotypically normal reference and mutant (i.e., tumor) populations. The genomes, labeled with different fluorochromes, are pooled prior to hybridization. The hybridization ratio of the two fluorochromes along the length of each

chromosome is calculated to determine the gain or loss of chromosomal regions in the mutant cells (*UNIT 4.6*; Kallioniemi et al., 1992; Forozan et al., 1997; Ried et al., 1997). This technique is extremely useful in cases involving multiple chromosomal rearrangements for which specific bands cannot be identified by Giemsa staining, or for detecting small insertions or deletions (larger than 1 Mb).

Comparative cytogenetics is the study of changes in chromosome number and composition in different species as a function of their evolutionary divergence from one another. Chromosome painting has proved very useful in identifying homologous chromosome regions between species and has led to a better understanding of the evolutionary rearrangement of genomes (Wienberg et al., 1990).

Many fluorescent dyes have now been created, each with different excitation and emission characteristics. This has allowed for the simultaneous hybridization and discernment of multiple probes on a single slide (Johnson et al., 1991; Ried et al., 1992). A natural extension of this procedure involves the labeling of different probes with various combinations of fluorochromes, thereby enabling the hybridization of more probes than there are distinguishable dyes. With  $N$  fluorochromes, the number of possible labeling combinations is given by  $2^N - 1$ . This combinatorial labeling of individual chromosomes using five different fluorochromes is used for spectral karyotyping (SKY; Garini et al., 1996; Liyanage et al., 1996; Schröck et al., 1996) and M-FISH (Speicher et al., 1996) analysis of mouse and human metaphase chromosomes. This technique has proved very useful for the identification of chromosome aberrations in human tumors and mouse models of tumorigenesis (Barlow et al., 1996; Coleman et al., 1997; Veldman et al., 1997; Ghadimi et al., 1999). SKY analysis has also been applied to evolutionary studies and will improve the analysis of genomic relationships (Schröck et al., 1996). Another approach has been to label probes not only by using combinations of dyes, but also by varying the ratios in which they are used (Nederlof et al., 1992). If  $K$  different concentrations are used for each fluorophore, and if the highest concentration used is 1 and the other concentrations are defined as  $(1/2)^1, (1/2)^2, \dots, (1/2)^{K-2}$  and 0, the total number of possible valid combinations as described by (Garini et al., 1999) is given by:

$$K^N - (K - 1)^N$$

where "valid" refers to combinations that have different fluorochrome-concentration ratios. In other words, ratios of (1,1) and (0.5,0.5) for a two dye scheme have identical concentration ratios of 1.

Fluorescence technology is also making advances in the areas of cell biology, and more recently in studies to determine nuclear topography (Lawrence et al., 1989; Carter et al., 1993; Lawrence et al., 1993; Xing et al., 1993) and chromatin organization (Sachs et al., 1995; Yokota et al., 1995, 1997). Studies designed to analyze gene function are also incorporating advances in fluorescence microscopy, but the fluorescence in this case is not from a fluorochrome conjugated to a nucleic acid, but from a green fluorescent protein (GFP) isolated from jellyfish. By making constructs encoding the gene of interest fused to the GFP gene, researchers are able to determine the cellular sub-localization of their "glowing" gene product (Chalfie et al., 1994). GFP has also been used as a reporter gene in transgenic mice to determine the developmental stage and tissue-specific transcriptional activation of promoters (Fleischmann et al., 1998). The fusion of GFP to the CENPB gene, the product of which is known to localize to all human centromeres, has been used in conjunction with time-lapse fluorescence microscopy to follow the movement of centromeres throughout the cell cycle (Sullivan and Shelby, 1999).

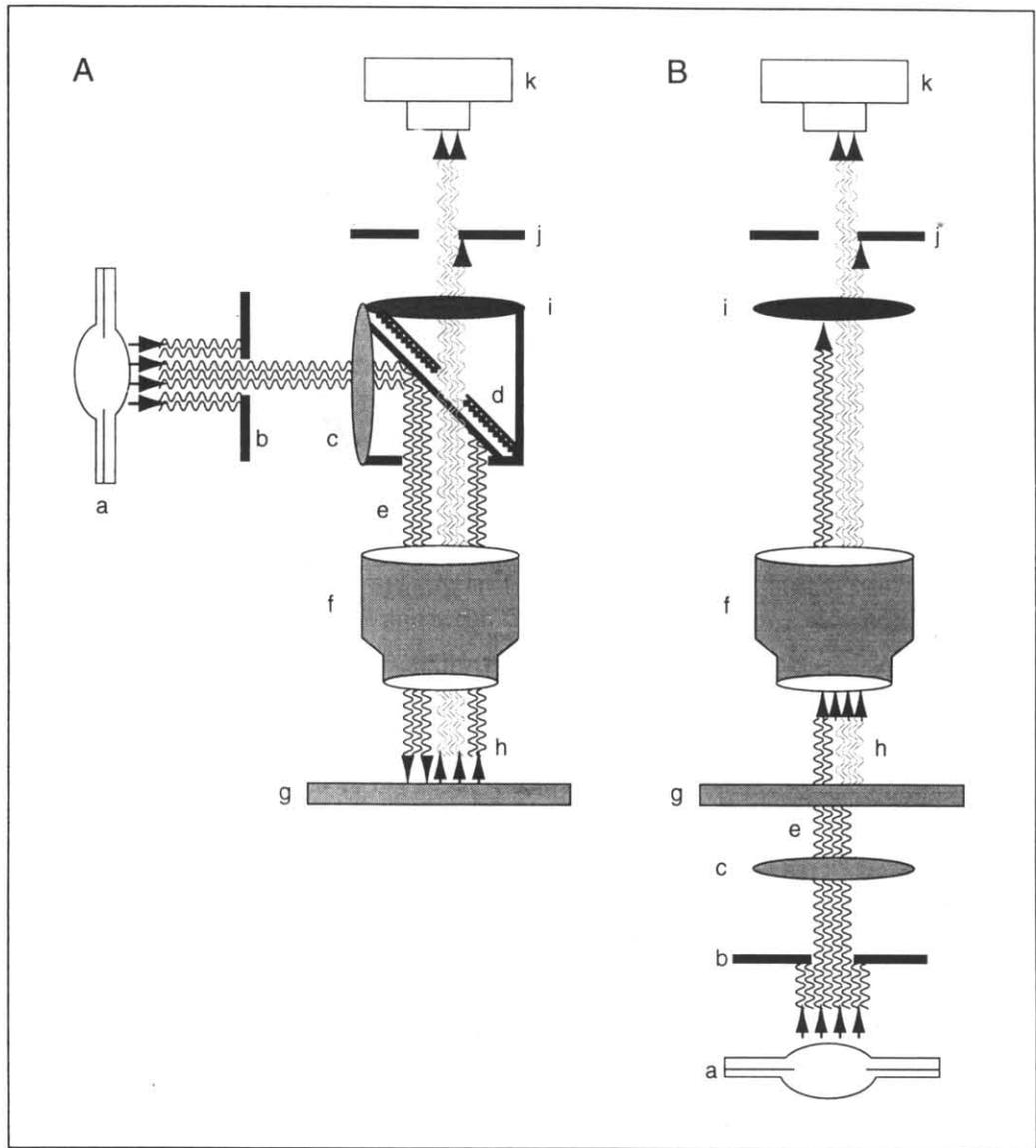
## TYPES OF MICROSCOPY

One source for an excellent and detailed discussion of microscopy and live cell analysis (in addition to other topics) is *Cells: A Laboratory Manual* (Spector et al., 1998). In general, the ability to generate a clear image is dependent on magnification, contrasts between internal and external milieu, and the ability to resolve discrete objects. There are many different microscope arrangements that can be used to enhance the contrast of specimens. These include phase contrast, polarized light, Nomarski or differential interference contrast (DIC), transmitted bright-field and dark-field illumination, and reflected-light illumination. Some of these configurations are used in conjunction with fluorescence microscopy. These are referred to as diasopic fluorescence in the case of transmitted light, and incident light fluorescence, epifluorescence, or episcopic fluorescence in the case of reflected-light illumination.

Köhler illumination results in an evenly lit field of view and is used for all light microscopy. There are many terms associated with

microscopy that indicate the direction of the incoming light with respect to the angle at which it intercepts the sample and the side of the sample through which it first passes (Fig. 4.4.1). Bright-field microscopy is the most commonly used light-microscopic technique. As the name indicates, the object of interest is darker than the surrounding background. Light from the illumination source is transmitted along a pathway parallel to the optical axis directly through the sample into the objective. This works well with samples that are either naturally (i.e., chloroplasts) or artificially (i.e., Giemsa) stained or have a high natural absorption. As light encounters the specimen, the intensity (or amplitude) is reduced compared to its surroundings, resulting in a darker appearance. The location of the illumination source defines the two types of bright-field microscopy. In transmitted-light bright-field microscopy, the illumination source is directly below the sample. As such, the light passes through the sample only once on its way from the source to the objective. This is the typical configuration of most microscopes used in the analysis of tissue culture samples and metaphase spreads, for example. In incident-light bright-field microscopy, the incoming light is first reflected down through the specimen and then back up through it into the objective (Fig. 4.4.1). Because many samples do not contain sufficient absorption properties to be discerned with normal bright field microscopy, one can generate contrast and reveal structures with low resolution by slightly rotating the condenser turret. This technique is referred to as oblique or anaxial illumination. Both transmitted illumination and incident illumination (epi-illumination) can be used for fluorescence. Older microscopes may have transmitted fluorescence illumination. Because transmitted light sources are more difficult to use and do not provide as clear an image, however, epi-fluorescence is more widely utilized.

Dark-field microscopy can be used to obtain resolution of objects or features that are normally below the resolution of the light microscope. This is only possible with transmitted illumination because no direct light is allowed to enter the objective. Only incoming light diffracted, refracted, or reflected by the specimen enters the objective, resulting in a dark background. One note of importance is that the numerical aperture of the condenser must be higher than that of the objective. This technique is incompatible with the use of phase-contrast microscopy in association with fluorescence.



**Figure 4.4.1** The concepts of (A) incident and (B) transmitted light as applied to fluorescence microscopy. Many of the major components are similar to those used with either type of bright-field microscopy (with the elimination of the filters which are labeled c and i here). Indicated in the diagrams are the (a) light source, (b) stage condenser, (c) excitation filter, (d) dichroic mirror, (e) selected excitation wavelength, (f) objective, (g) microscope slide with specimen, (h) reflected (in A) or transmitted (in B) illumination (short wave; blue) and emitted fluorescence (long wave; red), (i) barrier or emission filter, (j) eyepiece condenser, and (k) eyepiece or camera.

Dark-field microscopy also requires scrupulously clean optics and slides, because any dirt will cause light to be scattered into the objective and mar the image quality.

Differential interference contrast (DIC) can be performed at high numerical apertures, gives better resolution than dark-field microscopy, and can be used in conjunction with fluorescence microscopy and live-cell imaging. This technique involves polarized light microscopy. The incoming light is first passed through a polarizing filter that only allows waves oriented in the same direction to pass through the filter.

Each plane-polarized light beam is then split into two separate beams containing perpendicularly oscillating components with a Wollaston prism (composed of two quartz prisms cemented together with their optical angles oriented at 90° to each other). Thickness and refractive index differences within the specimen generate opposing phase shifts in the two halves of the split beam. A second Wollaston prism placed after the objective recombines the halves of each split beam. Constructive or destructive interference occurs as a result of the phase shift between the two separate beams.

The light then passes through another polarizing filter (analyzer) and is visualized as differences in gray-scale levels across the specimen. The bas-relief of DIC is due to differences in refractive index, and not the three-dimensional topography of the specimen.

Phase-contrast microscopy is an alternative to DIC and also takes advantage of polarized light, but in a different manner (Zernike, 1955). A phase annulus (ring) in the condenser only allows a ring of light to reach the condenser. Focusing of this ring by the condenser lens generates a hollow cone of light that is projected onto the back focal plane of the objective. Some of the light waves are retarded as they pass through the sample. This is due to absorptive differences among cellular structures and differences in refractive index or thickness. As a result, their phase is shifted relative to those waves from the original light source, which have not encountered phase-dense objects. These phase shifts are usually not sufficient, however, to generate full constructive or destructive interference visible with normal bright field microscopy. A phase ring in the back focal plane of the objective absorbs 70% to 80% of the nondiffracted rays. This ring also shifts the phase by one quarter of the wavelength. This arrangement alters the amplitude and phase relationships of the diffracted versus nondiffracted light, thereby enhancing the contrast. Regions with a higher refractive index usually appear darker. Objects with too high a refractive index or thickness can result in a rather large phase shift and cause a contrast reversal (i.e., a positive phase shift of  $1.5\lambda$  would appear identical to a negative phase shift of  $0.5\lambda$ ).

There are many occasions when it is useful to follow the movement of cells or their organelles as a function of time. Studies of cell division, movement of chromosomes and centrosomes, and the polymerization of mitotic tubules are a few examples. Such analysis involves the successive microscopic imaging of live cells, rather than a single image of a fixed specimen. There are two different methods for accomplishing such an analysis. The first, time-lapse microscopy, involves the acquisition of individual images at distinct time points or intervals (e.g., every 10 min over an 8 hr period). These images can then be integrated into a single composite image and displayed simultaneously for an easy comparison of changes as a function of time. Video microscopy, however, involves near-continuous imaging over a prolonged time period, as one would do with a

standard video camera that has a rate of 30 frames/second. Due to the low levels of light generated in such a short time interval, however, special integration cameras must be used to increase the sensitivity. A thorough treatment of this technique, which is beyond the scope of this chapter, can be found in Spector et al. (1998).

## MICROSCOPE OBJECTIVES AND EYEPIECE LENSES

The compound light microscope must be equipped with the highest-quality optics (objectives and eyepieces), must be precisely aligned, and must have the proper filters installed to observe and record all relevant information from the objects under study. A detailed manual outlining the steps required to align the microscope is available from the manufacturer of the respective microscope and will not be considered in this unit, although the importance of proper alignment cannot be overstated. An excellent discussion of microscopy and photography is presented in *The ACT Cytogenetics Laboratory Manual* (Barch et al., 1997) and in *Human Cytogenetics: A Practical Approach* (Rooney and Czepulkowski, 1992). Both references are invaluable resources for the cytogenetics laboratory.

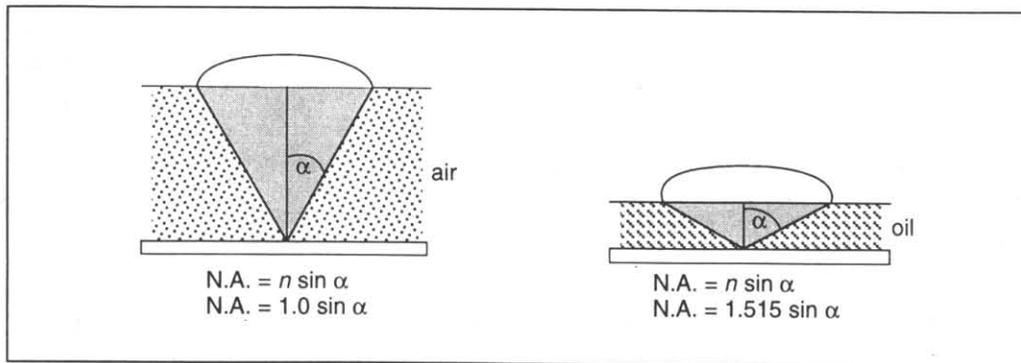
High-quality objective lenses are critical for obtaining maximum information in the study of biological specimens. Lenses condense light and magnify the image. The objective lens system must have high resolving power and correction for lens aberrations. The resolving power,  $R$ , of a lens is defined as the minimum distance by which two luminous points can be separated and still be discerned as distinct objects using that objective.  $R$  is described by the theory of optical diffraction as:

$$R = 1.22\lambda / (2 \times \text{N.A.})$$

where  $\lambda$  is equal to the wavelength of the incident light and N.A. is the numerical aperture, a measure of the light cone entering the objective at the fixed objective distance (James and Tanke, 1991; Rawlins, 1992). The value of N.A. is given by:

$$\text{N.A.} = n \sin \alpha$$

with  $n$  equal to the refractive index of the medium between the objective and the sample and  $\alpha$  equal to half the vertical angle of the light cone (Fig. 4.4.2). The N.A. is restricted for technical reasons to a maximum of 1.35 to 1.40



**Figure 4.4.2** Diagrammatic explanation of numerical aperture. As  $\alpha$  approaches  $90^\circ$ ,  $\sin \alpha$  approaches 1.0. The refractive index of the medium between the sample and the objective is designated as  $n$ .

and is usually indicated on the side of the objective. High N.A. results in the smallest lateral resolution, smallest axial resolution and maximum capture of light photons. This is particularly important in fluorescence microscopy, where the amount of emitted light is often very small. The brightness of the captured light is affected by many different parameters, including the concentration of the fluorophore, the transmission of light through the optics, the total magnification, and the numerical aperture of the objective (and the condenser, in the case of transmitted fluorescence). The relative image brightness in epifluorescence microscopy is given by the equation:

$$B = (\text{N.A.}_{\text{obj}})^4 \text{Mag}^2$$

When the so-called “object space” between the objective and coverslip contains air (as with a “dry” objective), the numerical aperture cannot exceed 0.95. However when immersion oil with a refractive index of 1.515 is used between the two surfaces, an N.A. of 1.35 to 1.40 can be obtained. This is because the refractive index of the oil is identical to that of the glass slide, coverslip and objective. This prevents the light from being refracted as it passes from the specimen through these other materials. Immersion media include various natural and synthetic oils (with varying  $n$  values), water ( $n = 1.333$ ), and glycerol ( $n = 1.455$ ). Immersion objectives are usually produced for use with a specific type of immersion medium and are so indicated on the side of the objective; “dry” objectives will not function as immersion objectives. Immersion oil with low self-fluorescence is required for fluorescence microscopy, and the objective manufacturer can help obtain the proper type.

In addition to resolving power, which is a function of both magnification and numerical aperture, modern light microscope objectives

must correct for problems of spherical and chromatic aberration. Spherical aberration is produced by failure of the curved surface(s) of a lens to direct all light rays passing through the lens to the same focal point. A coverglass of the incorrect thickness or a refractive-index mismatch (i.e., wrong immersion oil) can also cause spherical aberration and an inability to focus. Early microscopes (single or compound lenses) suffered from loss of fine detail due to chromatic aberration that resulted in rings of color around small objects. White light passing through the lens is broken up into its constituent colors. Different wavelengths are diffracted to different extents, and hence have different focal points. After 1820, achromatic lenses were developed, allowing great advances in biology and medicine (Kapitza, 1996; Inoué and Spring, 1997). Achromatic objectives are rather simple in that spherical aberration is corrected for the middle range of the light spectrum, thereby directing all broken-up wavelengths to the same focal point. Plan-achromatic objectives are more complex and have the advantage of less curvature-of-field aberration than ordinary achromatic objectives. Curvature of field is caused when light passing through the periphery of the objective is focused closer to the back focal plain of the lens than light passing through the center. The result is a discrepancy in focal plane between the center and periphery of the field of view. Plan-apochromatic objectives are costly, complex, flat-field objectives that offer the greatest correction for chromatic and spherical aberration. The type of correction supplied by the objective is also indicated on its side.

## FLUORESCENCE MICROSCOPY

Many dyes absorb light energy at one wavelength and subsequently emit part of this energy as light (fluorescence) at a different wave-

length. Compounds with these properties are termed fluorochromes or fluorophores. In order to grasp the principles of fluorescence, it is necessary to further understand the laws describing light waves. Energy behaves in accordance with Planck's law, which states:

$$E = hv = hc/\lambda$$

where  $E$  is energy,  $h$  equals Planck's constant,  $\nu$  equals light frequency,  $c$  equals light velocity, and  $\lambda$  equals light wavelength. Thus, energy is linearly proportional to the light frequency and inversely proportional to its wavelength. The quantum of energy ( $E$ ) is greater for radiations of shorter wavelengths, such as UV, than for radiations of longer wavelength, such as infrared. Wavelengths in the UV spectrum (300 to 400 nm) and visible light spectrum (400 to 700 nm) are used in fluorescence microscopy (James and Tanke, 1991).

The spectral characteristics of individual fluorochromes and fluorescent proteins are dependent upon the regions of the light spectrum where absorption (excitation) and emission of light energy occur. Stokes' law states that the average wavelength of emitted fluorescence is longer than the average excitation wavelength for any given fluorochrome. DAPI (4,6-diamino-2-phenylindole) is a fluorescent dye with affinity for A-T residues in DNA. This is often used to give a chromosome banding pattern (R-banding) complementary to that obtained with Giemsa staining (UNIT 4.2). A simple digital image-processing step, "inverse contrast," is thus able to transform a DAPI-stained metaphase spread into a Giemsa-like staining pattern. An additional sharpening step then improves the banding to near G-band quality. It is also useful as a DNA counterstain for interphase nuclear studies. The energy absorbed at 350 nm raises an electron to a higher excitation state. Some of the energy is then lost to vibration. When the electron falls back to its starting, or ground, state the energy is released as fluorescent light. Because the amount of energy is less than the input, the emitted light has a longer wavelength (470 nm) and is said to be red-shifted. Some fluorochromes, however, may require an excitation energy that overlaps with the emission energy of another fluorochrome. This fact must be considered when setting up an experiment involving the simultaneous use of more than one fluorophore. It is crucial to note that spectral properties of some fluorochromes are subject to significant environmental effects and vary depending on measur-

ing conditions—e.g., medium, pH, and substrate bound (Haugland, 1992; Mason, 1993). Understanding the spectral properties of various fluorochromes is important when choosing components of the microscope used in fluorescence microscopy. This includes the light source (Xe or Hg) as well as the filter(s).

To generate and observe fluorescence requires a fluorochrome bound to a molecule. This can be a fluorochrome bound either directly to a nucleic acid probe or indirectly, through the use of haptens (such as biotin and digoxigenin) and fluorescently-conjugated moieties with which they interact (avidin and anti-digoxigenin antibodies, respectively). Another source of fluorescence tagging is fusion of the green fluorescent protein (GFP) or any of its many derivatives directly to a protein of interest. A light source and optical filters are required to produce the correct wavelength(s) of light energy required for excitation of the fluorescent moiety. The light passing through or being emitted by the sample must then pass through another set of optical filters, such that emitted light energy of only the desired wavelength reaches the detection system (i.e., eye or other detector; Fig. 4.4.1). Details of these requirements are elaborated in the following sections.

### Light Sources

The standard light source for bright-field microscopy is a tungsten-filament bulb. The intensity of the illumination can be controlled by changing the amount of current flowing to the lamp via a rheostat dial. The light sources used in fluorescence microscopy are either mercury or xenon arc lamps. The choice of lamp is determined by the wavelengths of excitation energy needed to excite the fluorescent molecule being used as a probe. Mercury lamps have three main peaks of excitation light around 440, 550, and 580 nm, whereas xenon lamps are more uniform in their intensity across this range. An additional mercury peak around 365 nm in the UV range is important for the imaging of DAPI-stained objects (i.e., DNA). Both types of bulbs are available in a number of different wattages. Brighter lamps result in more intense fluorescence and therefore a shorter exposure time is required. A 100-W bulb also has a longer operating life than a 50-W bulb (200 versus 100 hr). Another important parameter is the gap between the anode and cathode in the bulb itself. This is known as the arc gap. Small gaps provide a small arc that can emulate a point source for epi-Köhler illumina-

**Table 4.4.1** Useful Microcopy-Related Web Sites

Resource	WWW site
<b>Microscopy</b>	
Ried Lab	<a href="http://riedlab.nci.nih.gov">http://riedlab.nci.nih.gov</a>
National High Magnetic Field Laboratory (NHMFL), Florida State University	<a href="http://micro.magnet.fsu.edu/primer/resources/general.html">http://micro.magnet.fsu.edu/primer/resources/general.html</a>
Microscopy Online	<a href="http://www.microscopy-online.com/">http://www.microscopy-online.com/</a>
MicroWorld: Internet Guide To Microscopy	<a href="http://www.mwrn.com/guide/light_microscopy/material.htm">http://www.mwrn.com/guide/light_microscopy/material.htm</a>
Center for Light Microscope Imaging and Biotechnology, Carnegie Mellon University	<a href="http://www.stc.cmu.edu/">http://www.stc.cmu.edu/</a>
History of the Light Microscope, University of Tennessee, Memphis	<a href="http://www.utmem.edu/personal/thjones/hist/hist_mic.htm">http://www.utmem.edu/personal/thjones/hist/hist_mic.htm</a>
Universal Imaging Corporation (many links to useful and informative sites)	<a href="http://www.image1.com/info/links/index.html">http://www.image1.com/info/links/index.html</a>
<b>Microscope companies</b>	
Leica Microsystems Imaging Solutions Ltd.	<a href="http://www.leica.co.uk/ia/homepage.htm">http://www.leica.co.uk/ia/homepage.htm</a>
Nikon	<a href="http://www.nikonusa.com/">http://www.nikonusa.com/</a>
Olympus America	<a href="http://www.olympus.com/oai.html">http://www.olympus.com/oai.html</a>
Carl Zeiss	<a href="http://www.zeiss.co.uk/">http://www.zeiss.co.uk/</a>
<b>Karyotype systems</b>	
Applied Imaging	<a href="http://www.aicorp.com/">http://www.aicorp.com/</a>
Applied Spectral Imaging	<a href="http://www.spectral-imaging.com/">http://www.spectral-imaging.com/</a>
Leica Microsystems Imaging Solutions	<a href="http://www.leica.co.uk/ia/cyto/Products/550cw.htm">http://www.leica.co.uk/ia/cyto/Products/550cw.htm</a>
MetaSystems	<a href="http://www.metasystems.de/">http://www.metasystems.de/</a>
Optical Insights, LLC	<a href="http://www.optical-insights.com/">http://www.optical-insights.com/</a>
Perceptive Scientific Instruments	<a href="http://www.persci.com/">http://www.persci.com/</a>
Vysis	<a href="http://www.vysis.com/620048.html">http://www.vysis.com/620048.html</a>
<b>Microarrays</b>	
Large-Scale Gene Expression and Microarray Links and Resources	<a href="http://industry.ebi.ac.uk/~alan/MicroArray/">http://industry.ebi.ac.uk/~alan/MicroArray/</a>
Brown Lab MicroArray Homepage, Stanford University	<a href="http://cmgm.stanford.edu/pbrown/array.html">http://cmgm.stanford.edu/pbrown/array.html</a>
<b>Fluorescence reagents and antibody distributors</b>	
Amersham Pharmacia Biotech	<a href="http://www.apbiotech.com/product/product_index.html">http://www.apbiotech.com/product/product_index.html</a>
Antibody Resource Page	<a href="http://www.antibodyresource.com/">http://www.antibodyresource.com/</a>
Chemdex Corporation (Web site listing various chemical companies)	<a href="http://www.chemdex.com/suppliers/">http://www.chemdex.com/suppliers/</a>
Clontech Laboratories	<a href="http://www.clontech.com/">http://www.clontech.com/</a>

*continued*

nation. It is important to monitor the bulb use because older bulbs result in weaker fluorescence signals. This will affect exposure settings, which is more important when using photographic film, because it is easier to retake the image with a digital-imaging device. Also, mercury bulbs should not be used >200 hr because there is a risk of explosion that can damage the microscope. Changing and aligning the bulb requires patience and skill and is

often best performed by the microscope service representative.

### Filters

Excitation filters (Fig. 4.4.1, component c) allow the passage of selected wavelengths of light from the illumination source that corresponds to the excitation spectrum of the fluorochrome. Other wavelengths are either absorbed or reflected by this filter. A second filter, the

**Table 4.4.1** Useful Microcopy-Related Web Sites, continued

Resource	WWW Site
Diatron	<a href="http://www.diatronscience.com/">http://www.diatronscience.com/</a>
Kirkegaard & Perry Laboratories	<a href="http://www.kpl.com/">http://www.kpl.com/</a>
Molecular Probes	<a href="http://www.molecularprobes.com/">http://www.molecularprobes.com/</a>
NEN Life Science Products	<a href="http://www.nenlifesci.com/prodsrv.c.htm">http://www.nenlifesci.com/prodsrv.c.htm</a>
Intergen Company (formerly Oncor)	<a href="http://www.oncor.com/intergentoc.htm">http://www.oncor.com/intergentoc.htm</a>
Packard Instrument Company	<a href="http://www.packardinst.com/">http://www.packardinst.com/</a>
PharMingen	<a href="http://www.pharmingen.com/">http://www.pharmingen.com/</a>
Quantum Biotechnologies	<a href="http://www.qbi.com/">http://www.qbi.com/</a>
Roche Molecular Biochemicals (formerly Boehringer Mannheim Biochemicals)	<a href="http://biochem.boehringer-Mannheim.com/">http://biochem.boehringer-Mannheim.com/</a>
Rockland	<a href="http://www.rockland-inc.com/cgi-bin/gen.cgi?page=cy.html">http://www.rockland-inc.com/cgi-bin/gen.cgi? page=cy.html</a>
Universal Imaging Corporation	<a href="http://www.image1.com/">http://www.image1.com/</a>
Vector Laboratories	<a href="http://www.vectorlabs.com/">http://www.vectorlabs.com/</a>
Ventana Medical Systems	<a href="http://www.ventanamed.com/">http://www.ventanamed.com/</a>
<b>Digital cameras, filters, and accessories</b>	
Bioptechs: Live-Cell Micro-Observation Products	<a href="http://www.bioptechs.com/">http://www.bioptechs.com/</a>
Chroma Technology	<a href="http://www.chroma.com/">http://www.chroma.com/</a>
Cambridge Research & Instrumentation	<a href="http://www.cri-inc.com/">http://www.cri-inc.com/</a>
Data Translation	<a href="http://www.datx.com/">http://www.datx.com/</a>
Diagnostic Instruments	<a href="http://www.diaginc.com/">http://www.diaginc.com/</a>
Hamamatsu	<a href="http://usa.hamamatsu.com/">http://usa.hamamatsu.com/</a>
Instrutech	<a href="http://www.instrutech.com/">http://www.instrutech.com/</a>
Eastman Kodak Company	<a href="http://www.kodak.com/">http://www.kodak.com/</a>
Ludl Electronic Products	<a href="http://www.ludl.com/">http://www.ludl.com/</a>
Roper Scientific Photometrics	<a href="http://www.photomet.com/">http://www.photomet.com/</a>
Roper Scientific Princeton Instruments	<a href="http://www.prinst.com/">http://www.prinst.com/</a>
Photon Technology International	<a href="http://www.pti-nj.com/overview.html">http://www.pti-nj.com/overview.html</a>
Sutter Instrument	<a href="http://www.sutter.com/">http://www.sutter.com/</a>

emission or barrier filter, is necessary on the imaging side of the sample for blocking transmission of unabsorbed wavelengths of excitation light and allowing transmission of the emitted fluorescence light to the detector (Fig. 4.4.1, component i). This is important because some of the excitation light is reflected off various microscope surfaces and would be brighter than the fluorescence if it was not reflected out of the emission path by the dichroic mirror and also suppressed by the barrier filter. Fluorescence microscopes with incident or epi-illumination also utilize a dichroic mirror (chromatic beam splitter) to aid in separation of fluorescence emission light from unabsorbed reflected excitation light. Dichroic mirrors have a surface coating that reflects light excitation wavelengths toward the sample and

passes emission wavelengths to the detector or eyepiece (Fig. 4.4.1, component d).

Choice of filters for fluorescence microscopy is determined by the fluorochrome and counterstain used in sample preparation. Any list of available filters and/or filter sets would be incomplete and therefore misleading to anyone getting started with fluorescence microscopy. A list of vendors and their Web sites has therefore been included at the end of this unit (Table 4.4.1) and a continually updated version will be maintained on the Ried Lab web site (see Table 4.4.1). A discussion with each manufacturer stating the nature of the experiment, illumination source available, and fluorochromes to be used (particularly for samples labeled with multiple dyes) is highly recommended prior to the purchase of any filter or filter set. Fluorochromes commonly used in

fluorescence microscopy and for which filters are typically needed are DAPI, Hoechst, quinacrine or propidium iodide as DNA counterstains, as well as fluorescein isothiocyanate (FITC), Texas red, rhodamine, and other fluors emitting in the far-red portion of the spectrum as probe-specific labels. Filters are usually supplied in sets and consist of an excitation filter, a dichroic mirror, and an emission or barrier filter. These are often contained within a device known as a filter cube, which slides easily into the filter holder or turret. Excitation and emission filters may be either colored glass or the more expensive interference filters (a glass substrate carrying vacuum-deposited thin layers of metallic salt compounds). Interference filters are more efficient than their colored glass counterparts in allowing only the desired wavelength through to the specimen.

Filter sets also differ in the amount of light they permit to pass. Short-pass filters allow all light shorter than a particular wavelength to pass through to the specimen, while long-pass filters only allow the passage of longer wavelengths. These types of filters are therefore not very restrictive in their transmission range. Band-pass filters are more selective in that they transmit one (or more) particular region(s) or band(s) of the light spectrum. This means that wavelengths both shorter and longer than the excitation wavelength are blocked. Narrow-band-pass filters have a much more restricted range of transmitted wavelengths compared to wide-pass (or broad-pass) filters. Broad-pass filters, because they allow more light through, result in a brighter image but include a broader spectrum of wavelengths. Band-pass filters now have high light transmission values (>90%) and very narrow band characteristics that allow selective excitation of one or more fluorochromes.

Dual- and triple-band-pass filters, which permit concurrent visualization of two or three fluorochrome combinations, are available. These are useful for the simultaneous excitation and detection of multiple fluors hybridized to the same sample and abrogate the need to change filters between imaging each fluorochrome. The simplest application is the imaging of two gene-specific probes for mapping. To take advantage of these filter sets, one needs a means of imaging that distinguishes the different colors. This can be as simple as a camera and color film or as technologically advanced as a color CCD or video camera. An example of a triple-pass filter cube is the one used for spectral karyotyping (SKY). This contains fil-

ters that allow alternating regions of excitation and emission wavelengths. This is necessary due to the overlapping excitation and emission spectra of the five fluorochromes used in the hybridization. Quadruple-pass filters do have some compromises, however, in that the brightness may be affected (P. Millman, Chroma Technology, pers. comm.). Filters and filter sets can be purchased from microscope companies or directly (and usually at a reduced cost) from filter manufacturers (e.g., Chroma Technology).

Other filters are important for altering the intensity of light entering the system. Neutral-density filters decrease the overall amount of transmitted light without altering the intensity ratios of different wavelengths. This may be desired if the signal intensity is strong and the fluorochrome is particularly sensitive to photobleaching. Heat filters can be extremely important in removing excessive heat radiating from the bulbs. KG-1, BG-38, and Hot-Mirrors are some examples of heat filters. Each has a different wavelength at which it reduces the transmitted heat. Choosing the correct heat filter depends on the wavelengths one needs for excitation of the sample. Requiring shorter excitation wavelengths allows one to choose a heat filter that prevents a larger portion of the spectrum from reaching the sample. Heat filters protect not only the specimen from damage, but are also useful for protecting sensitive elements such as polarizers and other filters.

## IMAGE ACQUISITION

After identifying an object that is worthy of documenting, an image must be acquired, particular portions of the object resolved and defined, and a careful analysis performed to generate useful information. In the past, images were photographed or studied directly at the microscope. High-technology image analysis systems are now available that allow computerized image capture, image enhancement, manipulation of captured images, mass storage, retrieval, and computer analysis. Imaging systems are now commercially available for storage and analysis of DNA gels and autoradiograms, sperm morphometry and motion analysis, and interphase and metaphase cytogenetic studies. These systems have found their places in clinical diagnostic laboratories as well as in basic research laboratories.

Preparation of the mammalian karyotype has always been a time-consuming and labor-intensive process. Significant strides have been made in automating cytogenetic analysis and

now several commercial imaging systems are available that offer either semiautomatic or interactive karyotyping capabilities. These systems save time because they eliminate the dark-room work required to make photographic prints and the physical cutting and pasting of chromosomes to produce a karyotype. Each system is composed of the following basic components: a microscope with a charge-coupled device (CCD) camera, a video monitor to view the image, a computer with the appropriate image capture and storage capabilities, and a high-resolution printer for generating a copy of the image. Because the metaphases and karyotypes are digital images, long-term storage and fading of photographs is avoided, and quick transmission of high-quality data is possible via computer networks.

Photography is sometimes useful for imaging metaphase cells, cell morphology, expression of proteins (e.g.,  $\beta$ -galactosidase) in transfected tissue culture cells, and stained histological specimens. The following section will therefore cover some of the essentials.

### Film and Photography

Several types of film are suitable for black-and-white photography of metaphase chromosomes banded by a variety of methods. These include Agfapan 100, Ilford FP4, and Kodak TP 2415. Sometimes certain filters are required to increase contrast and improve object definition. Black-and-white photography of certain objects (e.g., G-banded chromosomes) is enhanced by use of a green filter in combination with panchromatic film. Suitable green filters include Wratten 58 (Eastman Kodak) or a 550-nm interference filter (Thomson and Bradbury, 1987). Kodak TP 2415 film is widely used by cytogenetics laboratories. This film has a fine grain and variable contrast influenced by the choice of photographic developer. Kodak HC110 developer allows for a wider range of contrast that is determined by the developer dilution. Kodak D19 and D76 can also be used to develop TP2415 film, but they do not provide the flexibility of HC110.

Once a film and developer are chosen, a test roll of film should be shot, varying the ASA/DIN to determine which settings provide optimum contrast. An ASA of 50 for bright-field microscopy and 200 for fluorescence microscopy are commonly used. Kodak TP-2415 is also suitable for black-and-white photography of fluorescent images to avoid expensive page charges for publication of color images.

In FISH studies of interphase or metaphase cells, nucleotides modified with biotin (or digoxigenin) are incorporated into the probe. The cells are incubated with the probe, and a conjugate of avidin (or anti-digoxigenin) and a fluorochrome (e.g., fluorescein, rhodamine, or Texas red) then binds to the probe, emitting fluorescence when exposed to light of appropriate wavelength and intensity. It is also possible to directly label the probe with fluorophores, thereby eliminating the need for detection with antibodies or avidin. Propidium iodide may be used as a counterstain to aid in visualization of nonfluorescent objects (i.e., chromosomes). A derivative of the fluorescent dye DAPI is used for chromosome banding to permit unequivocal chromosome identification. Dual- or triple-band-pass filters allow simultaneous visualization of different fluorochromes and DAPI-stained (banded) chromosomes. If only single-band-pass filters are used, the fluorescent image is photographed, the filters are switched, and then the DAPI-banded chromosomes are photographed.

As with bright-field photography, camera settings will vary depending on the type of microscope, and optimal settings must be determined empirically. To reduce fading of the fluorescent signal, use the shortest exposure times that are adequate to record the image. When film is exposed for long periods of time, as is often necessary when photographing fluorescent images (e.g., mammalian chromosomes using FISH techniques), the film's sensitivity gradually becomes lower than its labeled value, necessitating a longer exposure time than indicated. This phenomenon is known as reciprocity failure and it varies with film type. Most camera systems have a correction setting for reciprocity failure, and this is one variable that must be assessed for optimal photography.

Because of the high cost of producing color prints, many laboratories use color slide film for photographing FISH images. There are numerous color slide films available; many investigators find that Kodak Ektachrome color slide film (HC400) works well. This high-speed film (ASA 400) results in better photographic capture of weak probe signals. Although the "fast" film produces a grainier print than does ASA 100 film, the additional graininess does not interfere with enlargements  $\leq 8 \times 10$ -in. It is possible to expose ASA 100 film with an ASA setting of 400 and have the development "pushed" by the color photographic laboratory. Pushing increases film sensitivity, but it also

results in very red chromosomes when propidium iodide is used as the counterstain, and this makes it more difficult to print accurate images.

Although film has high spatial resolution, it does suffer from low quantum efficiency. Quantum efficiency (Q.E.) is defined as the number of photons detected divided by the number that reaches the detector. All detectors have Q.E. values that vary with wavelength, but for historical reasons and illustrative purposes we will consider 546 nm light (546 nm is the mercury arc line for which achromatic objective lenses are optically corrected). The Q.E. of good black-and-white film is ~1% (one in one hundred photons reaching the film actually contributes to the signal). The Q.E. of color film depends on the color emulsion referred to and the wavelength(s) of light. The Q.E. of a typical video CCD camera (see discussion of Digital Image Acquisition) is ~3%, that of an intensifier for low-light video-rate microscopy is 30%, that of a scientific grade 12-bit digital CCD camera is 35% to 50%, and the Q.E. of a very expensive "slow scan back-illuminated" 16-bit digital CCD is 90%. For comparison, the original commercial laser scanning confocal microscopes (LSCM) used a photomultiplier tube with a Q.E. of <10%, while current confocal microscopes use detectors with Q.E. values of 10% to 25%. In other words, digital CCD cameras detect 30 to 90 times more photons than film, and convert the intensity information of the scene into a computer-ready format for quantitation and display (Inoué and Spring, 1997).

### Digital Image Acquisition

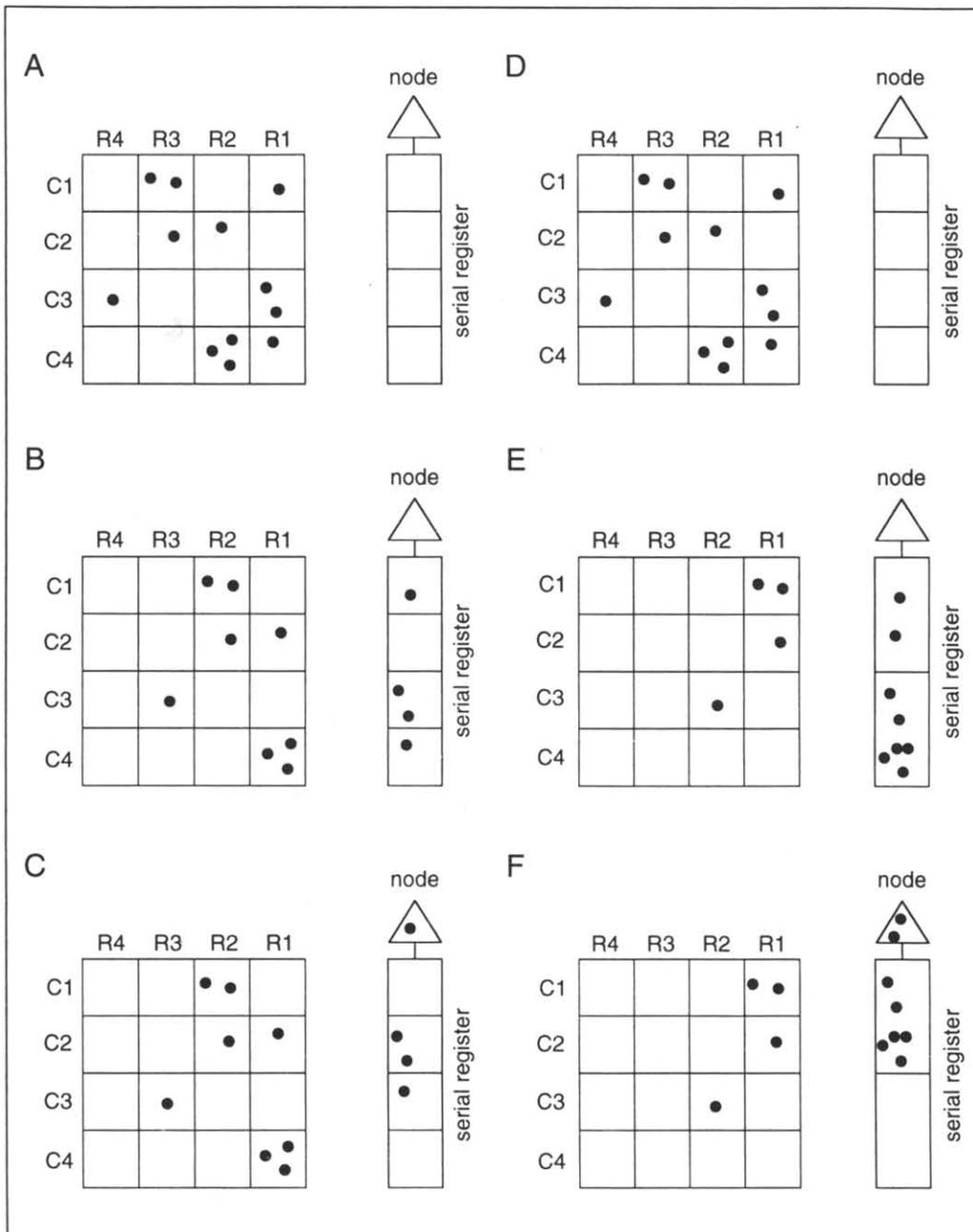
Instead of traditional photographic cameras, charge-coupled device (CCD) cameras, developed in the 1970s and 1980s, are used in digital-imaging systems. Modern high-performance CCD cameras were originally used in quantitative astronomy, where CCDs revolutionized the field. They are currently used in the security and surveillance industry, military installations, airports, banks, radar tracking, and in quality-control applications. CCDs are much smaller and more robust than the old tube cameras and have become ubiquitous at home in both hand-held video camcorders and digital "photography." One-dimensional CCD arrays are also the enabling technology for the flat-bed scanners used to digitize photographs and printed pages. These CCD cameras are used increasingly in image processing and image analysis. Several companies now market CCD

cameras for diverse applications in video microscopy.

CCD cameras have been developed using various technologies for specific functions. Most CCD cameras sample an image 30 times per second, but integrating CCD cameras have the ability to delay the readout for several seconds rather than milliseconds, thus offering good performance in low-light applications such as fluorescence microscopy. One-chip color CCD cameras are available for fluorescence microscopy. A red, green, or blue filter (striped filters) is placed in front of each pixel. These are broad-band filters and are not perfectly spectrally matched to the fluorochromes. Three-chip color CCD cameras, with one color per chip, are frequently used in pathology applications. These offer a reasonably high resolution, but require ample light. They are nonintegrating and relatively insensitive.

Cooled, slow-scan CCDs seem to be the best suited for fixed-cell studies because of their high quantum yield, excellent linearity, resolution, dynamic range, and spatial fidelity. Other CCDs include intensified high-gain CCDs that can detect very dim images, and video-rate CCDs with high sensitivity, for use in rapid kinetic studies. CCD camera technology is progressing rapidly and new products may offer advantages over existing cameras. For most users, a 12-bit digital CCD camera with the appropriate color filters will be the most appropriate image-capture tool. For special applications, confocal microscopy or digital deconvolution is used to obtain optical sectioning and Z-series capture, and Sagnac interferometer-based spectral imaging is used for 5-color fluorescence applications such as the 24-color spectral karyotyping (SKY; Schröck et al., 1996).

The CCD is an array of individual light sensors, each of which is a linear photometer (Aikens, 1990; Photometrics, 1995). Silicon CCDs utilize silicon crystals, whose covalent bonds are broken by incoming photons, thereby liberating electrons and generating electron-hole pairs. The CCD itself contains a rectangular array (or matrix) of wells where the liberated electrons are collected and stored until their quantity (i.e., charge) is measured. The capacity of each well may vary in accordance with the manufacturer, with capacities that can range up to  $1 \times 10^6$  electrons per well. Each well represents a pixel of digital information, the size of which (in  $\mu\text{m}^2$ ) is related to the magnification of the objective and the size of the array. Other energy sources, such as heat, can generate charge not related to the electromagnetic en-



**Figure 4.4.3** The 4 × 4-pixel CCD array illustrated above (A) has accumulated energy packets in several of the wells. (B) The energy packets are transferred one row at a time to the serial register. (C) They are then shifted into the output node one pixel at a time and on to the processor where they are quantitated and recorded. The concept of 2 × 2-pixel binning is demonstrated in panels D to F. Modified with permission from Photometrics (1995).

ergy released by the fluorochrome. This “dark current” can be reduced through cooling of the silicon array.

The signal output of a CCD array is a voltage that is linearly proportional to the charge present in each pixel. But how is the charge in each well, hereafter referred to as a charge packet, measured? Think of the array as being divided into rows and columns (Fig. 4.4.3). Once the CCD is exposed to the emitted light, charge

accumulates in each of the wells. The information in each well is shifted by one row, with the first row being transferred to an output node (i.e., an extra row outside the array used for the transfer of information to the signal-processing device). The packets in the node are then shifted column by column to the processor and quantitated. Once all the wells in the node have been measured, the next row is transferred and the process repeated until the entire array has been

read. The matrix is then capable of being exposed again to the light. The charge packets can be transferred thousands of times without significant loss of charge. This charge transfer efficiency (CTE) measurement is an important factor in choosing a camera, especially where the charge packets are small and any loss may result in significant image degradation. Because this is a complex process, methods have been established for reducing the readout time. One such technique, called binning, combines the charge from adjacent pixels; readout time is reduced as this effectively reduces the number of pixels that must be read. This comes at the price of reduced spatial resolution, however. One can control the amount and direction of binning. For example,  $2 \times 2$  binning combines the energy packets of four pixels; two pixels in the horizontal direction and two pixels in the vertical direction (Fig. 4.4.3, panels D to F). CCD performance is affected by a number of factors including linearity, charge-transfer efficiency, resolution, noise, dynamic range, quantum efficiency, and signal-to-noise ratio (Aikens, 1990; Photometrics, 1995).

#### **Image Analysis and Storage**

After the photons in a scene are collected on the CCD sensor, the number of electrons present at each picture element (pixel) is then quantified and read out as a signal whose intensity is proportional to the number of electrons, and hence to the number of photons. For video cameras, the data is reformatted to a broadcast standard so that it can be displayed on a television monitor. This video signal can also be redigitized to an 8-bit (256-intensity level) image by using a video frame grabber in the computer. Some video cameras and frame grabbers can handle color video capture and transfer ("24-bit color"). For digital cameras, the camera electronics convert the number of electrons into a digital value that is sent directly to a custom board in the computer, where the value is deciphered by a custom driver. Most digital cameras read out 12-bit (4096-intensity level) image data, because this is a good combination of cost and dynamic range. Three sequential red, green, and blue digital camera images can be merged to give a better color image than possible with color video cameras. Often the same software can be used for acquiring video or digital images (assuming appropriate frame grabber or digital camera drivers), as well as image processing, analysis, color merging, and storage. Typical configurations include a microprocessor that allows multiple image dis-

plays on a single monitor, networking capabilities for multiple workstations, choice of printers (including color), full-image contrasting, and, for FISH, hybridization spot enhancement and image sharpening.

Computerized imaging systems for cytogenetic applications vary in the extent of automation. Some possess slide-scanning and metaphase-finding capabilities, some identify specific chromosomes, while others require the user to point to each chromosome with a mouse or other pointing device and assign it a number. The software then places each chromosome onto a standard karyotype template. The chromosome images can be rotated, trimmed, inverted, labeled, and, in some systems, straightened. There are also variations in the degree of image enhancement or manipulation available. Software is available for the quantification of FISH signals, measurement analysis for gene mapping and spot-counting analysis for aneuploidy detection. Color image ratio measurement is a feature of software developed for comparative genomic hybridization technology (UNIT 4.6). Similarly, sophisticated software is available for spectral karyotyping (SKY). This technique employs a labeling scheme in which different chromosome painting probes are labeled with various combinations of five different fluorochromes. The software is able to identify each chromosome based on the different fluorescent-label-dependent patterns of the chromosomes, and arrange them in a karyotype. It is also capable of quantitative analysis for hybridization intensity and simultaneous display of fluorescent, DAPI-banded, and classification chromosomes, along with an idiogram. Image annotation and zooming are features on many software programs, and case and patient databases are available for importing image files and compiling data for statistical and epidemiologic studies. Image analysis systems, both for routine karyotyping and for FISH applications, are undergoing constant improvement. Because price and features continue to change, manufacturers should be contacted directly for current information.

With the increase in information per image and the decreasing cost of computer memory and storage space, image files have become larger—some on the order of 25 Mb per file. The easiest way to store files is on an external hard drive or server. These become full with time, and long-term storage becomes an issue. Popular disk storage include such media as Jaz (1000 or 2000 Mb per disk at \$0.09/Mb), Zip (100 or 250 Mb at \$0.10/Mb), CD ReWritable

(\$0.10/Mb), or CD-Recordable (\$0.003/Mb). The new DVD format should eventually allow even higher capacity and cheaper storage. Many universities and corporations have information service departments that can provide long-term archiving of data (for a price). Make sure that these units are compatible with the system (PC or Mac) as well as the software being used for image acquisition, enhancement, manipulation, and presentation. Typical software for these latter functions includes, but is not limited to, NIH Image, Adobe Photoshop and MacDraw Pro. Image acquisition software is more specific to the microscope/camera system being used and the type of analysis to be performed (e.g., FISH, immunocytochemistry, CGH, SKY, or microarray analysis). Careful consideration is therefore recommended when purchasing software from a company other than that from which the hardware was obtained. Special interface boards and cumbersome transfer of files through different programs may be required to make the hardware and software systems compatible. At a minimum, be sure that the imaging software can save the images in the industry standard TIFF (tagged image file format), so that one can share the data with colleagues. The JPEG and GIF file formats use data compression and are thus good formats for displaying image data on Web pages.

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