

Confocal Microscopy

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INTRODUCTION

A confocal microscope creates sharp images of a specimen that would otherwise appear blurred when viewed with a conventional microscope. This is achieved by excluding most of the light from the specimen that is not from the microscope's focal plane. The image has less haze and better contrast than that of a conventional microscope and represents a thin cross-section of the specimen. Thus, apart from allowing better observation of fine details it is possible to build three-dimensional (3D) reconstructions of a volume of the specimen by assembling a series of thin slices taken along the vertical axis.

BACKGROUND

Confocal microscopy was pioneered by Marvin Minsky in 1955 while he was a Junior Fellow at Harvard University.^[1] Minsky's invention would perform a point-by-point image construction by focusing a point of light sequentially across a specimen and then collecting some of the returning rays. By illuminating a single point at a time Minsky avoided most of the unwanted scattered light that obscures an image when the entire specimen is illuminated at the same time. Additionally, the light returning from the specimen would pass through a second pinhole aperture that would reject rays that were not directly from the focal point. The remaining "desirable" light rays would then be collected by a photomultiplier and the image gradually reconstructed using a long-persistence screen. To build the image, Minsky scanned the specimen by moving the stage rather than the light rays. This was to avoid the challenge of trying to maintain sensitive alignment of moving optics. Using a 60 Hz solenoid to move the platform vertically and a lower-frequency solenoid to move it horizontally, Minsky managed to obtain a frame rate of approximately one image every 10 sec.

MODERN CONFOCAL MICROSCOPY

Modern confocal microscopes have kept the key elements of Minsky's design: the pinhole apertures and

point-by-point illumination of the specimen. Advances in optics and electronics have been incorporated into current designs and provide improvements in speed, image quality, and storage of the generated images. Although there are a number of different confocal microscope designs, this entry will discuss one general type—the other designs are not markedly different.^[2]

The majority of confocal microscopes image either by reflecting light off the specimen or by stimulating fluorescence from dyes (fluorophores) applied to the specimen. The focus of this entry will be on fluorescence confocal microscopy as it is the mode that is most commonly used in biological applications. The difference between the two techniques is small. There are methods that involve transmission of light through the specimen, but these are much less common.^[3]

Fluorescence

If light is incident on a molecule, it may absorb the light and then emit light of a different color, a process known as fluorescence. At ordinary temperatures most molecules are in their lowest energy state, the ground state. However, they may absorb a photon of light (for example, blue light) that increases their energy causing an electron to jump to a discrete singlet excited state.^[4] In Fig. 1, this is represented by the top black line. Typically, the molecule quickly (within 10^{-8} sec) dissipates some of the absorbed energy through collisions with surrounding molecules causing the electron to drop to a lower energy level (the second black line). If the surrounding molecules are not able to accept the larger energy difference needed to further lower the molecule to its ground state, it may undergo spontaneous emission, thereby losing the remaining energy, by emitting light of a longer wavelength (for example, green light).^[5] Fluorescein is a common fluorophore that acts this way, emitting green light when stimulated with blue excitation light. The wavelengths of the excitation light and the color of the emitted light are material dependent.

Microscopy in the fluorescence mode has several advantages over the reflected or transmitted modes. It can be more sensitive. Often, it is possible to attach fluorescent molecules to specific parts of the specimen, making them the only visible ones in the microscope

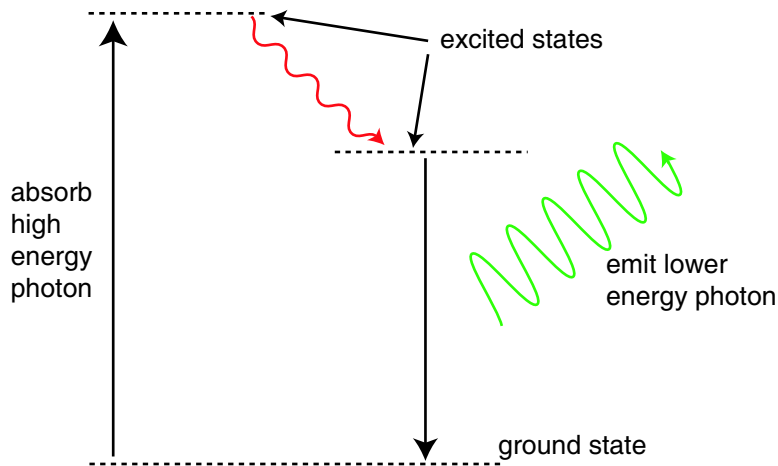


Fig. 1 Mechanism of fluorescence. The horizontal lines indicate quantum energy levels of the molecule. A fluorescent dye molecule is raised to an excited energy state by a high-energy photon. It loses a little energy to other molecules and drops to a lower excited state. It loses the rest of the energy by emitting light of a lower energy. (View this art in color at www.dekker.com.)

and it is also possible to use more than one type of fluorophore.^[6] Thus, by switching the excitation light different parts of the specimen can be distinguished.

FLUORESCENCE MICROSCOPY

In conventional fluorescence microscopy a dyed specimen is illuminated with light of an appropriate wavelength and an image is formed from the resulting fluorescent light. In Fig. 2 the excitation light is blue and the emitted light is green. The microscope uses a dichroic mirror (also called a “dichromatic mirror”) that reflects light shorter than a certain wavelength but transmits light of longer wavelength. Thus, the light from the main source is reflected and passes through the objective to the sample, while the longer-wavelength

light from the fluorescing specimen passes through both the objective and the dichroic mirror. This particular type of fluorescence microscopy, in which the objective used by the illuminating light is also used by the fluorescing light in conjunction with a dichroic mirror, is called epifluorescence. In the case of reflected light microscopy, a beamsplitter is used in place of the dichroic mirror.

CONFOCAL MICROSCOPY

To understand confocal microscopy it is instructive to imagine a pair of lenses that focuses light from the focal point of one lens to the focal point of the other. This is illustrated by the dark blue rays in Fig. 3. The light blue rays represent light from another point in

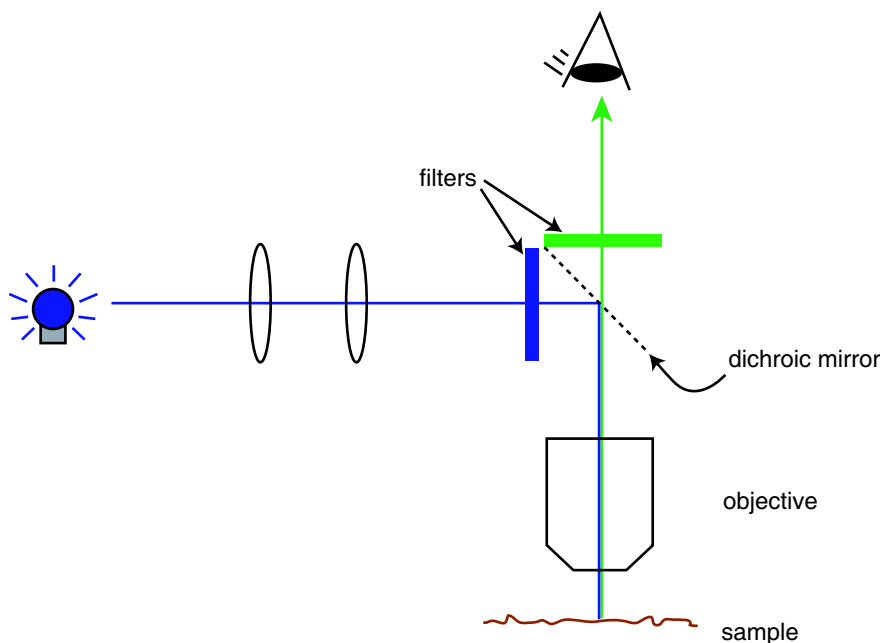


Fig. 2 Basic setup of a fluorescence microscope. Light from the source is reflected off the dichroic mirror toward the specimen. Returning fluorescence of a longer wavelength is allowed to pass through the dichroic mirror to the eyepiece. (View this art in color at www.dekker.com.)

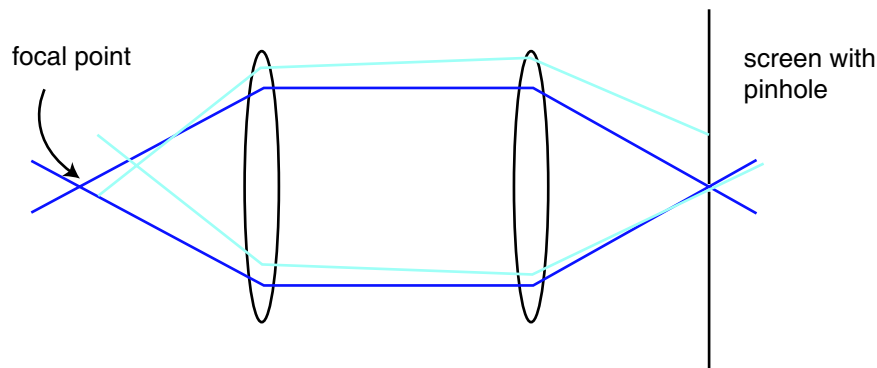


Fig. 3 Rejection of light not incident from the focal plane. All light from the focal point that reaches the screen is allowed through. Light away from the focal point is mostly rejected. (View this art in color at www.dekker.com.)

the specimen, which is not at the focal point of the left-hand-side lens. (Note that the colors of the rays are purely for purposes of distinguishing the two sets—they do not represent different wavelengths of light.) Clearly, the image of the light blue point is not at the same location as the image of the dark blue point. (Recall from introductory optics that points do not need to be at the focal point of the lens for the system of lenses to form an image.)

In confocal microscopy, the aim is to see only the image of the dark blue point.^[1] Accordingly, if a screen with a pinhole is placed at the other side of the lens system, then all of the light from the dark point will pass through the pinhole.^a Note that at the location of the screen the light blue point is out of focus. Moreover, most of the light will get blocked by the screen, resulting in an image of the light blue point that is significantly attenuated compared to the image of the dark blue point.

To further reduce the amount of light emanating from “light blue” points, the confocal microscope setup minimizes how much of the specimen is illuminated. Normally, in fluorescence microscopy the entire field of view of the specimen is completely illuminated, making the whole region fluoresce at the same time. Of course, the highest intensity of the excitation light is at the focal point of the lens, but the other parts of the specimen do get some of this light and they do fluoresce. Thus, light at a “dark blue” point may include light that has been scattered from other “light blue” points, thereby obscuring its fluorescence. To reduce this effect the confocal microscope focuses a point of light at the in-focus dark blue point by imaging a pinhole aperture placed in front of the light source.^[1] Thus, the only regions that are illuminated are a cone of light above and below the focal (dark blue) point (Fig. 9A).

Together the confocal microscope’s two pinholes significantly reduce the background haze that is typical of a conventional fluorescence image, as shown in Fig. 5.

^aActually not all the light from the focal point reaches the pinhole. Some of it is reflected and absorbed by the optics in between.

Because the focal point of the objective lens forms an image where the pinhole/screen is, those two points are known as “conjugate points” (or alternatively, the specimen plane and the pinhole/screen are conjugate planes). The pinhole is conjugate to the focal point of the lens, hence the name “confocal” pinhole.

HOW DOES A CONFOCAL MICROSCOPE WORK?

The confocal microscope incorporates the ideas of point-by-point illumination of the specimen and rejection of out-of-focus light.

One drawback with imaging a point onto the specimen is that there are fewer emitted photons to collect at any given instant. Thus, to avoid building a noisy image each point must be illuminated for a long time to collect enough light to make an accurate measurement.^[1] In turn, this increases the length of time needed to create a point-by-point image. The solution is to use a light source of very high intensity, which Minsky did with a zirconium arc lamp. The modern choice is a laser light source, which has the additional benefit of being available in a wide range of wavelengths.

In Fig. 4A the laser provides the intense blue excitation light. The light reflects off a dichroic mirror, which directs it to an assembly of vertically and horizontally scanning mirrors. These motor-driven mirrors scan the laser across the specimen. Recall that Minsky’s invention kept the optics stationary and instead scanned the specimen by moving the stage back and forth in the vertical and horizontal directions. As awkward (and slow) as that method seems it does have among others the following two major advantages:^[7]

- The specimen is everywhere illuminated axially, rather than at different angles as in the case of the scanning mirror configuration, thereby avoiding optical aberrations. Thus, the entire field of view is illuminated uniformly.
- The field of view can be made larger than that of the static objective by controlling the amplitude of the stage movements.

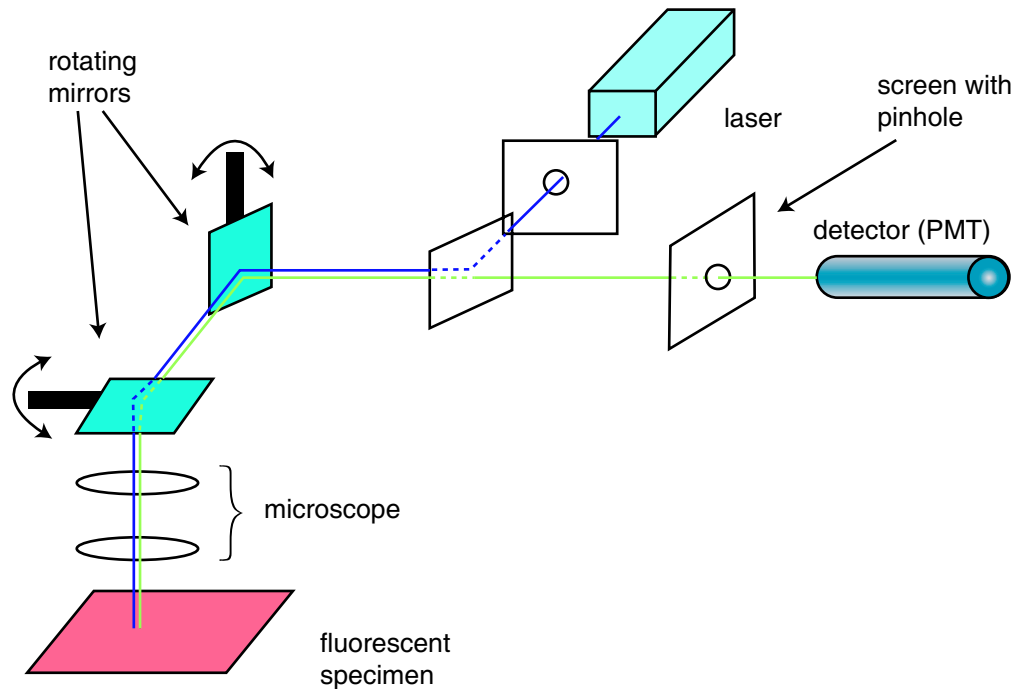


Fig. 4 Basic setup of a confocal microscope. Light from the laser is scanned across the specimen by the scanning mirrors. Optical sectioning occurs as the light passes through a pinhole on its way to the detector. (*View this art in color at www.dekker.com.*)

In Fig. 4 the dye in the specimen is excited by the laser light and fluoresces. The fluorescent (green) light is descanned by the same mirrors that are used to scan the excitation light (blue) from the laser and then passes through the dichroic mirror. Thereafter, it is focused onto the pinhole. The light that makes it through the pinhole is measured by a detector such as a photomultiplier tube.

In confocal microscopy, there is never a complete image of the specimen because at any instant only one point is observed. Thus, for visualization the detector is attached to a computer, which builds up the image one pixel at a time. For a 512×512 -pixel image this is typically done at a frame rate of 0.1–30 Hz. The large range in frame rates depends on a

number of factors, the most important of which will be discussed below.

The image created by the confocal microscope is of a thin planar region of the specimen—an effect referred to as optical sectioning. Out-of-plane unfocused light has been rejected, resulting in a sharper, better-resolved image. Fig. 5 shows an image created with and without optical sectioning.

THREE-DIMENSIONAL VISUALIZATION

The ability of a confocal microscope to create sharp optical sections makes it possible to build 3D renditions of the specimen. Data gathered from a series of

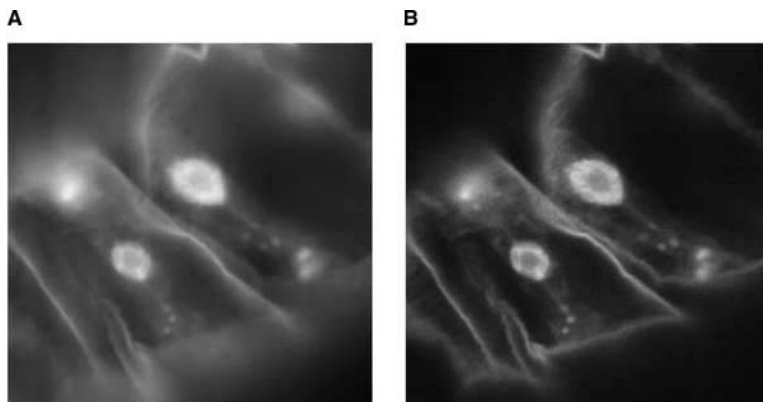


Fig. 5 Images of cells of *spirogyra* generated with and without optical sectioning. The image in (B) was created using a slit rather than a pinhole for out-of-focus light rejection. Most of the haze associated with the cell walls of the filamentous algae is absent, allowing clearer distinction of the different parts.

optical sections imaged at short and regular intervals along the optical axis are used to create the 3D reconstruction. Software can combine the 2D images to create a 3D rendition. Representing 3D information in a meaningful way out of 2D data is nontrivial, and a number of different schemes have been developed. Fig. 6 shows a 3D reconstruction, from slices of a suspension of 2 μm diameter colloidal particles using “alpha blending”—a technique that combines images by first making each of their individual pixels less or more transparent according to a computed weight called the “alpha” value.^[8] The result is a 3D-like structure.

OTHER CONSIDERATIONS

A confocal microscope, as with every instrument, has some limitations and often compromises must be made to optimize performance. The following is an outline of some of the most important of them.

Resolution

As with conventional microscopy, confocal microscopy has inherent resolution limitations due to diffraction. In the discussion above it is assumed that the point source used produces a point of light on the specimen. In fact it appears in the focal plane as an Airy disk, whose size depends on the wavelength of the light source and the numerical aperture of the objective lens.^[9] (The numerical aperture of a lens is a measure of how well it gathers light.) The graph of Fig. 7B shows a plot of the intensity of light as a function of radius of an Airy disk—the image is circularly symmetric, as shown in Fig. 7A.

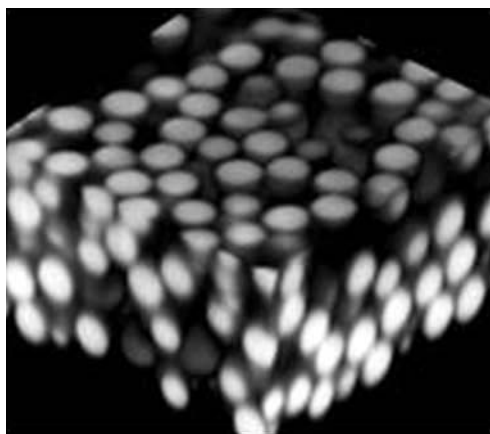


Fig. 6 Three-dimensional reconstruction of a series of 2D images of PMMA spheres suspended in a cyclohexylbromide and decalin solution. The image was created using “alpha blending.”

The Airy disk limits the maximum resolution that can be attained with the confocal microscope—the best resolution is typically about 200 nm. Ideally, the image of a point would just be a single intense point right at radius = 0. However, the finite size of the Airy disk sets the scale for which details can be resolved. According to the Rayleigh criterion, the minimum separation between two Airy disks for which they are distinguishable is equal to their radius. This corresponds to the maximum of one Airy disk superimposed on the minimum of the other.

Resolution along the optical axis is also limited by diffraction effects. As in the lateral direction there is a periodic, but elliptical distribution of intensity in the shape of an Airy disk.^[10]

Pinhole Size

The optical sectioning capability of a confocal microscope derives from having a pinhole to reject out-of-focus light rays. In turn, the strength of the optical sectioning (the rate at which the detected intensity drops off in the axial direction) depends strongly on the size of the pinhole.^[11]

It is tempting to assume that making the pinhole as small as possible is the best choice. However, as the pinhole size is reduced, so too are the number of photons that arrive at the detector from the specimen. This may lead to a reduced signal-to-noise ratio. To offset the weaker signal more fluorescence is needed from the specimen. This usually can be done, to a limit, by raising the intensity of the excitation light. But high intensities can damage the specimen, and in the case of fluorescence, also degrade the fluorophore. Moreover, it has been shown that optical sectioning does not improve considerably with the pinhole size below a limit that approximates the radius of the first zero of the Airy disk.^[2,11] (Note that the study considered imaging in coherent mode; however, the result still qualitatively applies to inherently incoherent confocal fluorescence microscopy.) Thus, a good approximation is to make the pinhole about the size of the Airy disk.

Intensity of Incident Light

An important component of a confocal microscope is the photodetector that captures light from the specimen. In confocal fluorescence imaging the pinhole along with the optics preceding it significantly reduce the intensity of the emission that reaches the detector. Thus, the detector’s sensitivity and noise behavior are vitally important.^[3] The sensitivity is characterized by the quantum efficiency, which, as in any measurement involving quantum interactions, is limited by Poisson statistics.^[12] That is, the accuracy of the measurement is improved by increasing the number of photons

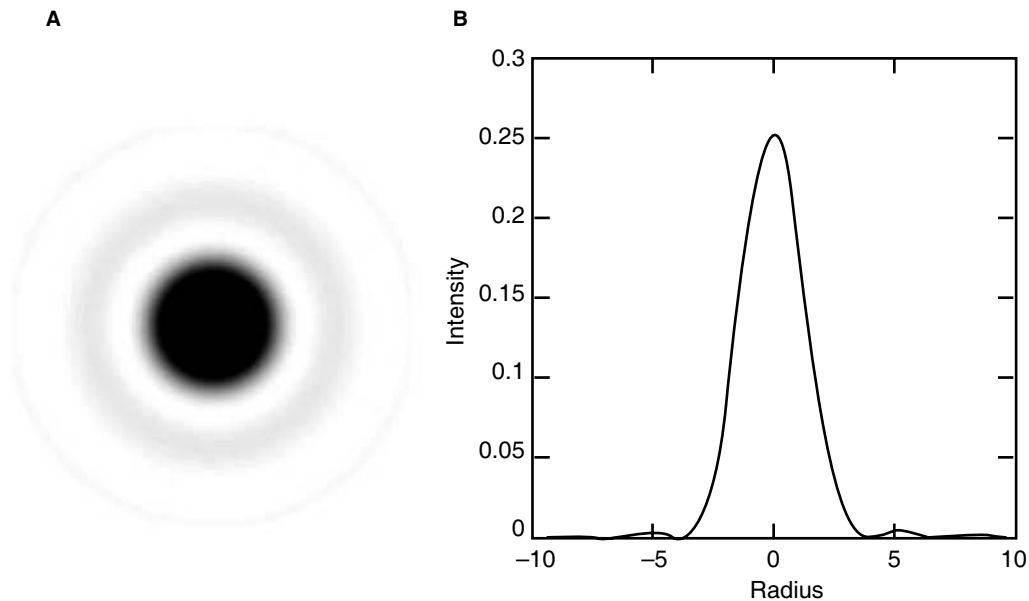


Fig. 7 Airy disk similar to that of an image of a very small particle. (A) The image is “overexposed” and in reverse color to allow distinction of the faint secondary peak. (B) A graph of the intensity change with radius.

arriving at the detector. In practical terms this can be done by averaging data from many frames—which has the drawback of slowing down the effective frame rate of the microscope. Or, it can be done by increasing the intensity of the fluorescence signal.

Fluorescence can be increased by dyeing the specimen with a larger concentration of fluorophore molecules or by raising the intensity of the excitation light. However, each of these methods increases excitation up to some limit. For high fluorophore concentrations, the individual molecules can quench each other.^[5] They may also reduce the amount of fluorescence deep inside the specimen—fluorophores nearest the light source can absorb enough light to significantly reduce the portion available to the rest of the specimen.^[3] Increasing fluorescence by increasing the excitation light intensity leads eventually to saturation of the fluorophore. Higher intensities drive a larger fraction of fluorophore molecules into excited states, which in turn leads to a smaller fraction of ground state molecules. Ultimately, the rate at which fluorophores are excited matches their decay rate, causing ground state depopulation. Fluorescence then ceases to increase with excitation intensity.^[13]

Fluorophores

Among the most important aspects of fluorescence confocal microscopy is the choice of fluorophore. It is typically influenced by several factors. The fluorophore should tag the correct part of the specimen. It must be sensitive enough for the given excitation wavelength.

For living specimens it should not significantly alter the dynamics of the organism; and an extra consideration is the effect of the specimen on the fluorophore—its chemical environment can affect the position of the peaks of the excitation and emission spectra.^[14]

Photobleaching

A major problem with fluorophores is that they fade (irreversibly) when exposed to excitation light (Fig. 8). Although this process is not completely understood, it is believed in some instances to occur when fluorophore molecules react with oxygen and/or oxygen radicals and become nonfluorescent.^[13,15] The reaction can take place after a fluorophore molecule transitions from the singlet excited state to the triplet excited state. Although the fraction of fluorophores that transitions to the triplet state is small, its lifetime is typically much longer than that of the singlet state. This can lead to a significant triplet state fluorophore population and thus to significant photobleaching.^[5]

Several strategies have been developed to reduce the rate of photobleaching.^[5,13] One method is to simply reduce the amount of oxygen that would react with the triplet excited states. This can be done by displacing it using a different gas.^[5] Another method is by the use of free-radical scavengers to reduce the oxygen radicals. Shortening the long lifetime of the triplet excited state has also been shown to be effective.^[16] Other ways include using a high numerical aperture lens to collect more fluorescence light and thus use less excitation light.^[17] Also, keeping the magnification as

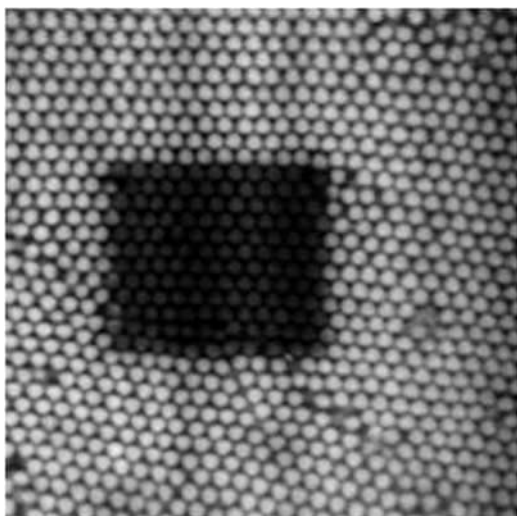


Fig. 8 Dyed suspension of densely packed polymethylmethacrylate beads with significant photobleaching. The rectangular region near the center faded after about 30 sec of exposure to excitation light.

low as is permissible spreads the excitation light over a larger area, thereby reducing the local intensity.

While photobleaching makes fluorescence microscopy more difficult, it is not always undesirable. One technique that takes advantage of it is fluorescence photobleaching recovery (FPR) or fluorescence recovery after photobleaching (FRAP). It involves exposing a small region of the specimen to a short and intense laser beam, which destroys the local fluorescence, and then observing as the fluorescence is recovered by transport of other fluorophore molecules from the surrounding region. Quantities such as the diffusion coefficient of the dyed structures can then be determined.^[18]

LIVING CELLS

Confocal microscopy has been used effectively for the 3D study of dynamics in living cells. However, the imaging of living specimens adds the challenge of maintaining the life and normal function of the organism.^[19] There are of course difficulties involved in preparing the sample for viewing as is the case in conventional microscopy. But on top of that the effect of photo-damage on the specimen caused by the focused high-intensity excitation light must be taken into account. This is compounded by the repeated exposure required for tracking the cellular dynamics—a problem that is worsened for 3D data collection. Fluorescence also introduces the problem of the fluorophore influencing the cell behavior as well as the risk that oxygen molecules reacting with fluorophores in triplet excited states may generate free radicals that damage the cell.^[19]

Despite the challenges, a wide variety of sophisticated fluorophores have been developed to study different aspects of cell biology. They are designed to mark specific parts of the cell interior and often can simply be introduced to the cell wall.^[19] The fluorophore molecules make their way into the cell and attach to the intracellular structures of interest such as the mitochondria and the Golgi apparatus. This is not always the case, however, as some fluorophores must be injected directly into the cell.^[19] Labeling is even applied to the study of “nonphysical” structures of the cell—some fluorophores have been developed for the measurement of dynamic processes such as membrane potentials and ion concentrations.^[5]

Multicolor Fluorescence

To distinguish between small features such as proteins within a cell it is useful to tag them with different fluorophores and image them as separate colors. There are two ways to do this: in one method fluorophores are selected to correspond with the wavelengths of a multiline laser and in the other their response to the same excitation wavelength causes emission at different wavelengths.^[20] In both cases the resulting emission is separated with appropriate filters and directed to different detectors. However, there can be cross talk between channels of the emitted light.^[14] For most of the commonly used fluorophores there is usually some overlap between their emission spectra, making perfect channel separation impossible by filtering alone. To first order this can be corrected by determining the level of overlap of emission of each individual fluorophore into the channels of the other fluorophores and subtracting it out mathematically.

FAST CONFOCAL MICROSCOPY

Most confocal microscopes generate a single image in 0.1–1 sec.^[21] For many dynamic processes this rate may be too slow, particularly if 3D stacks of images are required. Even for a single 2D image, slow frame rates translate into long exposure times of the specimen to intense laser light, which may damage it or cause photobleaching. Two commonly used designs that can capture images at high speed are the Nipkow disk confocal microscope and a confocal microscope that uses an acousto-optic deflector (AOD) for steering the excitation light.

Acousto-Optic Deflector

Speeding up the image acquisition rate can be achieved by making the excitation light beam scan more quickly

