Chapter 1
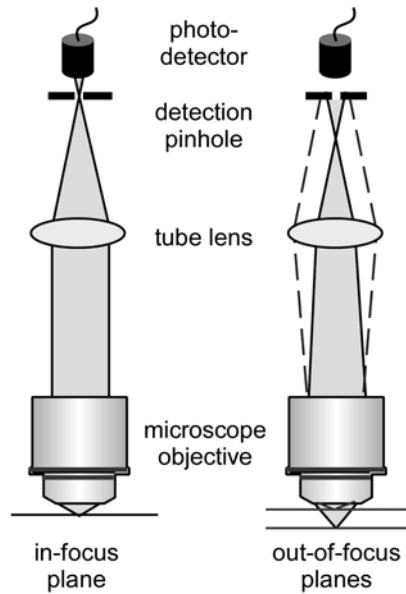
Confocal Fluorescence Microscopy

1.1 The principle

Confocal fluorescence microscopy is a microscopic technique that provides true three-dimensional (3D) optical resolution. In microscopy, 3D resolution is generally realized by designing the instrument so that it is primarily sensitive to a specimen's response coming from an in-focus plane, or by subsequently removing the contributions from out-of-focus planes. Several techniques have been developed to achieve this. For instance, 3D deconvolution [Agard and Sedat, 1983] uses both in- and out-of-focus information from a stack of images, taken at various focal planes, to reconstruct the 3D image. Another example is two- and three-photon absorption microscopy [Denk et al., 1990; Hell et al., 1996], where a nonlinear interaction with the specimen is used to confine the specimen's response to the focal plane only.

In confocal fluorescence microscopy, true 3D resolution is accomplished by actively suppressing any signal coming from out-of-focus planes. This is achieved by using a pinhole in front of the detector as schematically depicted in Fig. 1.1. Light originating from an in-focus plane is imaged by the microscope objective such that it freely passes the pinhole, whereas light coming from out-of-focus planes is largely blocked by the pinhole.

In a confocal fluorescence microscope (Fig. 1.2), the specimen is generally illuminated by a laser. The term "excitation" rather than "illumination" will be used in what follows, since it more explicitly refers to the contrast-generating process: the excitation of fluorophores, through absorption, causing detectable fluorescence. The light coming from the laser passes through an (excitation) pinhole, is reflected by a dichroic mirror, and focused by a microscope objective to a small spot in the specimen. The dichroic mirror reflects light of a shorter wavelength (e.g., 488 nm

Figure 1.1 The principle of confocal fluorescence microscopy. Light coming from out-of-focus planes is largely blocked by a pinhole in front of the detector.
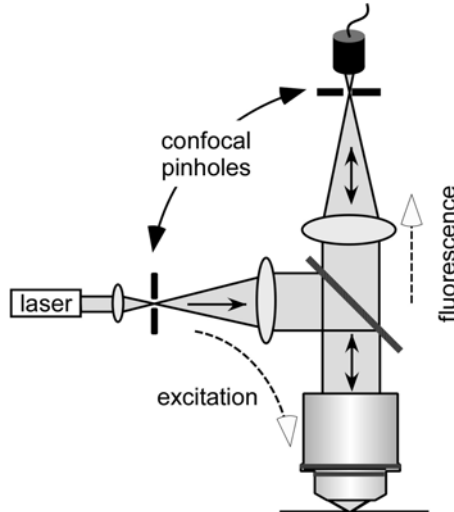
Figure 1.2 Excitation of the specimen in confocal fluorescence microscopy by a laser. A fraction of the fluorescence emitted by the fluorophores in the specimen is collected by the microscope objective and imaged onto the detection pinhole in front of a photo-detector.

from an Argon-ion laser) while transmitting that of a longer wavelength (e.g., the fluorescence >510 nm from fluorescein). Specific dichroic mirrors can be made for the relevant wavelength regions of excitation and fluorescence.

2.4.8 Fluorescent proteins[b]

In 1961, Shimomura et al. isolated a calcium-dependent bioluminescent protein from the jellyfish *Aequorea victoria*: *aequorin*. Together with this protein, they isolated a second protein that emitted green fluorescence upon UV excitation and named it the green fluorescent protein (GFP). Later, it was found that these proteins work together in the jellyfish to convert calcium-induced luminescent signals into green fluorescence. Upon stimulation, a chemical reaction provides the energy for excitation of *aequorin*. This energy is transferred through resonant energy transfer from *aequorin* to GFP, which in turn emits green fluorescence.

In 1992, the gene for GFP was first cloned [Prasher et al., 1992], and soon the significant potential of this molecule as a molecular fluorescent probe was demonstrated [Chalfie et al., 1994], using GFP expression to monitor gene expression and protein localization in living organisms. With the gene encoding for GFP available, it can be fused to any gene encoding for a particular protein. Expression of this protein then inherently results in a simultaneous expression of GFP and a fluorescent probe that is covalently linked to the expressed protein. An additional advantage is that expressed fusion proteins are generally not toxic to cells. The fluorescent protein technique avoids the problem of purifying, tagging, and introducing labeled proteins into cells as well as the task of producing specific antibodies for surface or internal antigens. However, each fusion protein should be carefully tested for its biological functionality. The GFP moiety is relatively large (~27 kDa) and may interfere with the function of the fused protein.

Genetic mutation techniques have been used to enhance GFP properties as well as produce a large number of variously colored mutants (denoted by BFP, CFP and YFP for the blue, cyan, and yellow fluorescent protein respectively). More recently, other fluorescent proteins, emitting in the orange and red spectral regions, have been isolated from the marine anemone *Discosoma striata* (known as Ds-red [Matz et al., 1999]).

The tripeptide fluorophore of GFP is enclosed in a barrel-like structure of eleven β-sheets and a central α-helix. This packing of amino acid residues inside the β-barrel is extremely stable, which results in a high-fluorescence quantum yield (up to 80%). This tight protein structure also confers resistance to fluorescence variations that could result from fluctuations in pH, temperature, and denaturants such as urea. Since the intrinsic fluorophore characteristics are closely related to the precise protein structure, minor genetic mutations can dramatically influence the spectral properties of the protein. For instance, the main drawback of the native GFP is its absorption maximum close to the UV (395 nm), which is generally not suited for live-cell microscopy. Introducing a single-point mutation, such as converting the serine at position 65 into a threonine residue (S65T), shifts the absorption maximum to a more convenient 488 nm. This mutation is present in the

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[b]For this section, extensive use has been made of the Nikon microscopy Internet site (http://www.microscopyu.com). For more detailed information on fluorescent proteins, their specific genetic mutations, and protocols for use in live-cell microscopy, the reader is referred to the literature (e.g., [Patterson, 1997 #82; Piston, 1999 #83; Tsien, 1998 #85]).

widely used enhanced GFP (EGFP), which is commercially available. In enhanced fluorescent proteins, the DNA sequence is optimized for expression in mammalian cells at 37°C.

Similar point mutations have been used to produce fluorescent proteins for which the excitation and emission spectra are shifted to shorter (EBFP and ECFP) or longer wavelengths (EYFP). Double-label studies using these different fluorescent proteins can be employed for fluorescent resonant energy transfer (FRET) studies. Table 2.3(b) summarizes the main spectral properties of the different fluorescent proteins. Figure 2.14 shows the excitation (i.e., the amount of fluorescence as a function of excitation wavelength) and fluorescence spectra of the most commonly used fluorescent proteins. The spectra have been normalized to permit a qualitative comparison. Minor changes in the spectra may occur for specific protein mutations, or they may depend on the specific environment.

Most fluorescent proteins in their natural state exist as dimers, tetramers, or higher-order oligomers. For *Aequorea victoria* GFP this phenomenon is only observed at very high protein concentrations. However, when fluorescent proteins are targeted to specific cellular compartments, such as the plasma membrane, the localized protein concentration can become high enough to permit dimerization. This is a particular concern when conducting FRET experiments, which are easily compromised by dimerization artifacts. Therefore, additional genetic modifications have been developed to produce strictly monomeric fluorescent proteins.

Since the initial demonstration of the utility of fluorescent proteins in 1994, the number of applications has increased sharply and continues to grow. Research continues to enhance the available color palette of fluorescent proteins as well as develop specific biosensors that use fluorescent protein technology.
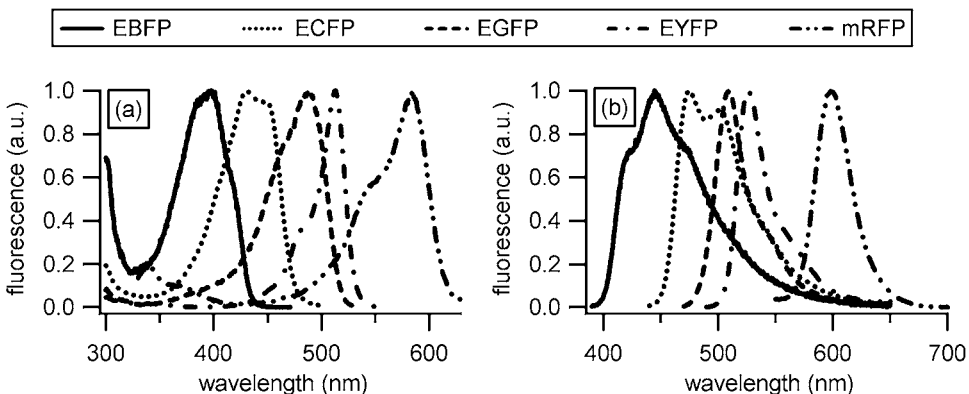
Figure 2.14 Excitation (a) and fluorescence emission (b) spectra of the most commonly used fluorescent proteins. The fluorescent protein mRFP is a variant of DsRed, which is a tetramer. mRFP contains 40 mutations with respect to DsRed and is a monomer. (Image courtesy of G.-J. Kremers, University of Amsterdam.)

Chapter 4

Digitization

A confocal fluorescence microscope is a serial rather than parallel image-acquisition device: the object is illuminated point by point and the generated fluorescence, imaged onto the detection pinhole, is measured sequentially for each illuminated point. In such an instrument, the image acquisition is discrete rather than continuous. The image is built up in discrete spatial steps, the fluorescence is sampled in discrete steps that correlate to a particular spatial position, and the image is displayed in some discrete manner as pixels of a certain intensity in the image. However, the object itself is continuous in all practical senses and will show structure at every level of detail. So the question arises: In how many steps does the object need to be sampled to form an image to faithfully represent reality? Naturally, extremely fine sampling in an almost infinite number of steps will provide a faithful image, but it will take infinitely long and provide an infinite amount of data. Too few steps, on the other hand, may result in a loss of information, or worse, in artifacts introduced into the image.

The issue of the best strategy for discrete sampling of a continuous signal is relevant to a great number of practical problems. For instance, digital sound recording, as for CD players, for example, requires digitization of continuous signals (the sound waves) without losing the information required for high-quality reproduction. But oversampling should be avoided to minimize the disk space required to digitize a particular song. Film and TV are other instruments that work with discrete steps—the frames in the film and the refresh rate of the screen—that appear continuous to the viewer when reproduced at sufficient speed. They also show the artifacts that arise from undersampling: stagecoach wheels that appear to rotate in the wrong direction as a consequence of a frame rate lower than the rotation rate of the wheels, causing undersampling of the motion. This phenomenon of artifacts that result from undersampling is known as aliasing.

Let's examine the stagecoach-wheel example in more detail. Film frames are taken at a frequency of 25 Hz. If the coach wheel has five spokes, the wheel appears

exactly the same at every 72-degree rotation. If the coach moves very slowly, the frame rate will be sufficient to capture the gradual movement of the spokes towards this 72-deg completion. This is shown in Fig. 4.1(a), in which the wheel turns with a 1 Hz frequency (corresponding to a coach speed of approximately 1 km/hour). In that case, the spokes frequency is 5 Hz, since all spokes are identical, and the wheel is clearly seen to rotate in a clockwise direction.

However, if the coach accelerates and the spoke frequency increases to 25 Hz, then between every frame of the film the wheel has turned exactly 72 deg. Whereas the coach clearly moves, the wheels appear to be stationary [Fig. 4.1(b)]. In other cases, the wheels may even appear to rotate in a counterclockwise direction, as shown in Fig. 4.1(c), in which the spoke frequency is approximately 20 Hz. This example shows that wheel movement can be reproduced faithfully only when the frame rate is sufficiently high compared to the frequency of the turning wheel. Artifacts are introduced at insufficient frame rates, or, in other words, sampling frequencies that are too low.

The above-described principle is true for every type of digitization, whether in the time domain (e.g., reproducing the coach-wheel movement in a limited number of film frames) or in the spatial domain (e.g., reproducing structural features in a limited number of image pixels). An example of the latter is shown in Fig. 4.2. Figure 4.2(a) shows an artificially generated image of a regular pattern in two dimensions. Figure 4.2(c) displays the same image magnified to enhance the pattern visibility. Figure 4.2(b) illustrates a re-sampling of Fig. 4.2(a) at a reduced frequency of 8% of the original pixel frequency with which 4.2(a) was created. Figure 4.2(d) shows the same image at higher magnification. This example demonstrates, again, that if the sampling frequency is too low, not only will image detail be lost, but artifacts will appear in the re-sampled image.
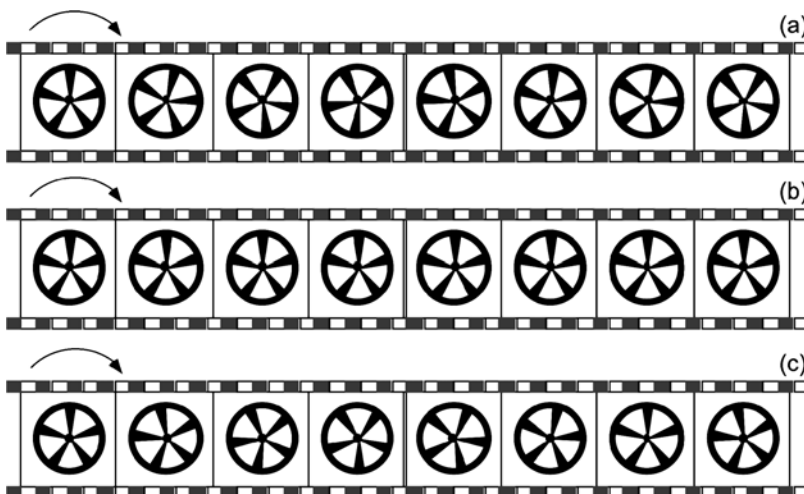
Figure 4.1 Whether coach wheels reproduced on film appear to rotate in a clockwise or counterclockwise direction, or even appear not to move at all despite coach movement, depends on the frequency of rotation of the wheel relative to the frame rate.
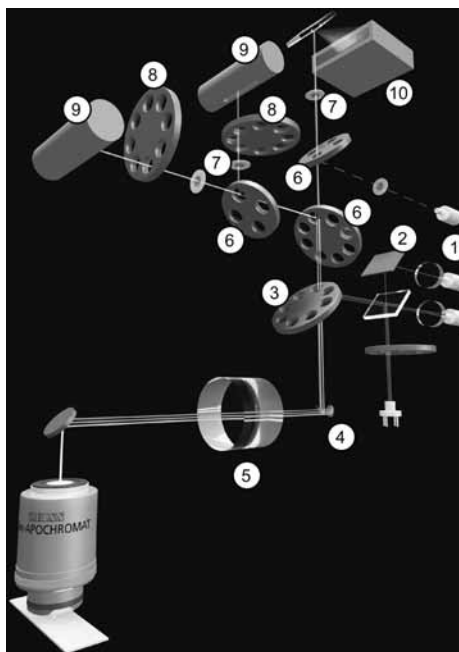
Figure 5.4 Optical layout of the Zeiss LSM 510 meta scanhead. (1) UV, visible (VIS) and infrared (IR) external lasers; (2) beam combiner; (3) dichroic beamsplitter; (4) scanning mirrors; (5) scanning lens; (6) dichroic beamsplitters; (7) confocal pinholes; (8) emission filters; (9) photomultipliers; (10) META detector. (Courtesy of Zeiss.)

5.3.2 Objectives

A large range of microscope objectives is available for confocal fluorescence and two-photon absorption microscopy. Because of the required beam scanning, the imaging properties should be constant over a large field of view (denoted by the word "Plan"). Careful selection of the immersion medium (air, water, glycerol, or oil) to match the refractive index of the specimen-mounting medium is equally important, in view of possible refractive index mismatch aberrations (Sec. 1.4). Finally, the transmittance and chromatic aberration correction at the required wavelengths of excitation and detection should be considered.

5.3.3 Dichroics

Dichroic mirrors are generally used to direct the excitation light to the specimen and separate the emitted fluorescence from scattered laser light. Additional dichroic mirrors split the emitted fluorescence over the different detection channels. Because of fabrication constraints, dichroic mirrors are generally long-wave pass: they reflect a certain wavelength and transmit at longer wavelengths. When using multiple excitation wavelengths, complicated dichroic mirrors provide a number of reflection bands and transmittance in between the reflected wavelengths and above the longest excitation wavelength.

The AOBS from Leica provides a tunable alternative for the excitation dichroic beamsplitter. Also in detection, the Leica system provides a tunable setup through combining a prism and movable reflective razor blades (Fig. 5.3).

5.3.4 Detection pinhole

Except for the Zeiss system, all commercially available confocal microscopes use a single detection pinhole for all detection channels. Hence, in multicolor studies, a compromise is required in terms of the detection-pinhole size. Alternatively, the Zeiss system provides independent detection pinholes for all separate detection channels.

5.3.5 Detection

Multiple detection channels are standard in all systems. Generally, dichroic mirrors are used to split the fluorescence over the different channels and photomultiplier tubes accomplish detection. Increasingly, spectral detection (i.e., resolving the fluorescence intensity as a function of wavelength) is being implemented in various ways in the different systems. Spectral information can be used to isolate the contribution of each separate fluorophore to the total signal, potentially without crosstalk between different channels.

5.3.6 Speed

The scanning Nipkow disk (*Perkin-Elmer*) provides high-speed, real-time confocal operation. In addition, this scanning strategy may in some cases significantly reduce bleaching and phototoxicity. Another approach to fast confocal microscopy is the effective line-scanning and slit-detection system of Zeiss (LSM 5 live).

5.3.7 Software

Extensive software packages are available from most vendors to control the operation of the microscope, to set up photobleaching experiments, fluorescence resonance energy transfer studies, time-lapse experiments, etc. They also often include 3D image-analysis tools and deconvolution algorithms.

5.4 Related 3D fluorescence techniques

Ever since the invention of confocal fluorescence microscopy, scientists have conducted a continuous search for alternative techniques that could, using fluorescence, provide even better resolution in three dimensions. Various concepts have been developed. Two of these have actually reached the commercial market: two-photon absorption microscopy and 4π microscopy. In this section, the main concept behind these techniques is briefly introduced. The reader is referred to the literature for a more detailed treatment of these imaging modes.