Chapter 3

Practical Limits

The fluorescing capability of general fluorophores is limited, both in terms of the rate and number of emitted photons. These limitations, in combination with the limited detection efficiency of the microscope and the spatial size of the optical-probe volume, have direct implications for the rate at which images in confocal fluorescence microscopy can be acquired, as well as for the number of images that can be acquired from a certain specimen.

Consider a confocal fluorescence microscope working at typical high-resolution conditions as specified in Table 3.1. The fluorophore used in this example is fluorescein. The detection pinhole is, by assumption, set equal to the projected Airy disk. In this case, the lateral resolution, or width of the confocal probing voxel, is 90% of the wide-field case, whereas the axial resolution is 120% compared to the confocal case with an infinitely small detection pinhole (see Sec. 2.3).

The boundary conditions set by the fluorophore are shown in Table 3.2. They result from the dye concentration in the sample, the fluorescence lifetime, and the number of times the fluorophore can be excited before it irreversibly photobleaches (the Q-number; see Sec. 2.4) and is lost for fluorescence measurements. In practice, the average total of fluorescent photons emitted per fluorophore varies between 10^2–10^6. In some cases, removing oxygen or adding protective agents, can increase the Q-number and therefore the number of images that can be acquired from the specimen.

Table 3.1 Microscope characteristics.

λ_{exc}	494 nm
λ_{det}	518 nm
NA	1.3
oil immersion	$n = 1.52$
voxel width	210 nm
voxel length	765 nm
voxel volume	34×10^{-3} μm^3

The limitations set by the microscope optics and the fluorophore characteristics combine to provide the maximum rate at which fluorescence photons are emitted from a single voxel and the total number of photons this voxel emits (Table 3.3).

The efficiency with which fluorescence photons emitted by the sample are detected by the system further limits microscope performance. A dominant and unavoidable loss in any microscope is the objective lens's collection efficiency, which is

$$\frac{1}{2}\left(1-\sqrt{1-(\mathrm{NA}/n)^2}\right)=\frac{1}{2}(1-\cos\alpha), \tag{3.1}$$

where n is the refractive index of the immersion medium and α is the semi-aperture angle. For an oil-immersion microscope objective with NA = 1.3, just under 25% of the fluorescence is collected. Note that under particular circumstances, the solid angle of collection can be doubled by collecting light from both sides of the specimen using two high-NA objectives placed nose-to-nose [so-called 4π-microscopy; see Hell and Stelzer, 1992(a); Hell et al., 1994; Hell et al., 1995; and Sec. 5.4(a)].

The fraction of the collected light that emerges on the other side of the objective depends on its transmittance. Transmittance values vary depending on the type of objective and the wavelength between 50%–95%, with a typical value of around 90% (see Sec. 2.1). Next, the fluorescence photons must travel through the microscope, experiencing optical losses at the mirrors, filters, and relay lenses. In addition, optical losses are introduced by the detection pinhole, and these losses depend on the chosen size and the optical aberrations in the system. Large variations in transmission between various microscopic systems may exist, but as a typical value a 10% throughput seems reasonable.

Finally, the fluorescence photons must be converted into detectable events (i.e., electrons) by a photodetector. The most important detector characteristics are the (1) quantum efficiency (QE), or the fraction of the photons arriving at the detector that actually generate an output signal; and (2) noise *level*. This includes both additive noise, in the form of a dark current from the detector or electronic amplifier, and multiplicative noise, in the form of random variations in the actual detector-output pulses derived from identical input pulses.

Table 3.2 Fluorophore characteristics.

concentration	10^{-5} M
fluorescence lifetime	4.7 ns
Q-number	10^5

Table 3.3 Fluorescence from a single voxel.

# of fluorophores per voxel	200
emission rate (photons/s·voxel)	4×10^{10}
max. # of emitted photons/voxel	2×10^7

The QE depends on the type of detector and is generally a function of the wavelength of the photons to be detected. A photomultiplier tube (PMT) is the most commonly used detector in the confocal microscope. While the QE of a PMT may be as high as 30% in the green, this still means that 70% of the photons that finally reach the detector produce no signal. For most PMTs, the QE decreases for longer wavelengths, and they are practically unusable in the infrared.

A practical alternative to the PMT is the solid state detector, of which the cooled CCD is the optimal example. The QE of this detector can be very high (70–80%) and extends well into the infrared. Also, each photon is recorded as an identical amount of current. Unfortunately, to keep the readout noise level acceptably low (±5 counts/measurement), this detector must be cooled to between −40° and −80°C, and read out at the relatively low rate of 25–250 kpixels/sec.

Table 3.4 summarizes the characteristics for the detection efficiency of a typical confocal microscope.

Finally, the required SNR of the image, which is directly related to the number of fluorescence photons that need to be detected, should be considered. For example, for a SNR = 10 with a detector dark count (N_d) of 10, a minimum of 100 photons must be detected for every pixel. This then provides 33 gray levels (N_G) in the image and 14 meaningful intensity levels (N_I; see Chapter 4). Based on these numbers, the maximum number of 3D images that can be acquired from a certain specimen, as well as the maximum rate of image acquisition, can be calculated.

The maximum number of detected fluorescence photons per voxel is limited by bleaching and detection efficiency to $2 \times 10^7 \times 5\% \approx 10^5$. Since at least 100 detected photons per image pixel are required, based on signal-to-noise considerations, and at least 4 pixels per imaged voxel (Nyquist's criterion, see Chapter 4), a maximum of ∼250 imaged optical sections exists. Taking into account that while imaging a certain optical section in the specimen, other optical sections both above and below experience bleaching at the same rate as the imaged section, it follows that approximately 6 full 3D images, with 40 optical sections per stack, can be acquired before the fluorescence has completely faded. (Note that Nyquist's criterion also requires that the axial spacing between these optical sections is less than half the axial resolution.)

Table 3.4 Detection efficiency.

	range	typical
collection	$\frac{1}{2}(1 - \cos\alpha)$	0.25
objective transmission	0.5–0.9	0.9
optical losses	0.01–0.2	0.1
detector QE		
PMT	0.05–0.3	0.2
CCD	0.3–0.9	
Total		5‰

Table 3.5 3D images and scan speed.

max. # of detected photons/voxel	10^5	(bleaching)
min. # of detected photons/pixel	100	(SNR)
max. collection rate (photons/voxel·s)	2×10^8	(fluorescence lifetime)
max. # of imaged optical sections	250	(4 pixels/voxel)
max. # of 3D images	6	(40 optical sections)
min. dwell time per pixel (μs)	2	(SNR & 4 pixels/voxel)
min. acquisition time per optical section (s)	0.5	(512×512 pixels)
min. acquisition time per 3D image (s)	20	(40 optical sections)

Similarly, with a maximum fluorescence emission rate of 4×10^{10} photons/s·voxel, and a detection efficiency of 5%, a maximum of 2×10^8 photons can be detected per second per voxel. With a minimum of 100 detected photons per image pixel and 4 pixels per imaged voxel, the minimum dwell time per pixel is 2 μs. For a total 3D image size of 512×512 pixels and 40 optical sections, this comes down to approximately 0.5 second per optical section, or 20 seconds for a full 3D image. Table 3.5 summarizes these numbers, which provide an approximate indication of the typical boundary conditions in terms of number of the 3D images and scan speed for high-resolution confocal fluorescence microscopy.

Clearly, the above numbers provide only a sample calculation and the actual numbers may vary considerably depending on the fluorophore, its concentration, the image resolution, and the detection efficiency of the particular confocal microscope. It shows, however, that for given fluorophore and microscope characteristics the number of 3D images that can be acquired is limited because of bleaching, whereas the acquisition time is limited because of the fluorescence lifetime of the fluorophore. The only way to increase the number of images or decrease the acquisition time for a given set of parameters is by reducing the resolution, i.e., increasing the voxel size. Consider, for instance, changing the microscope objective in the above example from a 1.3 oil-immersion to a 0.9 air-spaced objective, while keeping all other numbers constant. Such a reduction in NA increases the voxel size to 100×10^{-3} μm^3 and thus increases the total number of fluorophores that are emitted per voxel by a factor of ~3. At the same time, the collection efficiency remains at ~25%. Hence, the total number of 40-section 3D images that can be acquired before complete bleaching of the specimen increases to 18, whereas the acquisition time per section decreases to 0.15 s.