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Fluorescence saturation in confocal microscopy

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Summary

The effects of fluorescence saturation on imaging in confocal microscopy have been studied. To include saturation it was necessary to deviate from the widely assumed linear relationship between the fluorescence and the illumination intensity. The lateral response for a point-like object, as well as the optical sectioning power, decreases depending on the degree of saturation. For very high illumination intensities the response for a saturated point object approached that of a conventional fluorescence microscope in which the fluorescence was not saturated. The decrease in the axial confocal response has been confirmed qualitatively by experiment.

Introduction

In the imaging theory of confocal fluorescence microscopy, the fluorescence is widely regarded as a linear process, i.e. the emitted fluorescence intensity is linearly dependent on the illumination intensity:

$$I_{\mathrm{flu}} \propto I_{\mathrm{i}} \tag{1}$$

and thus this model does not include any non-linear effects, such as saturation of the fluorophore or photobleaching. The earlier work of Wells *et al.* (1990) showed that saturation can occur very easily in laser scanning microscopes: typically 1 mW of radiant power in the focal area of a high NA objective can saturate the fluorophore. This inspired us to look, in more detail, at the effects of fluorescence saturation on imaging in confocal microscopy. We confined ourselves to the effects of fluorescence saturation and did not look at any effects due to photobleaching. The confocal set-up considered in this paper has an ideal point source and detector. To find an expression for the fluorescence intensity as a function of the illumination intensity we closely followed the approach of Van den Engh & Farmer (1992).

Saturation of fluorescence

When a molecule is illuminated not all the light, but only the fraction that hits its absorption cross-section σ, will be absorbed and lead to excitation. The rate of absorption $\aleph_{\mathrm{a}}$, in photons per second per molecule, is given by

$$\kappa_{\mathrm{a}} = \sigma I_{\mathrm{i}} \tag{2}$$

where I_{i} is the illumination intensity (photons $\mathrm{m^{-2}\,s^{-1}}$). After absorption of a photon the molecule very quickly relaxes to the lowest vibrational level of S_1 (Fig. 1). This internal conversion typically occurs in 10^{-12} s, and is complete prior to fluorescence emission. A return to the ground state can then take place either through radiative (fluorescence) or through non-radiative decay with decay rates of κ_{r} and κ_{nr}, respectively, so that the decay rate of the excited state is $\kappa_{\mathrm{e}} = (\kappa_{\mathrm{r}} + \kappa_{\mathrm{nr}})$. The average lifetime of the excited state S_1 now is defined as $\tau_{\mathrm{e}} = 1/\kappa_{\mathrm{e}}$ (Lakowicz, 1983). Saturation occurs when the rate of absorption is equal to the decay rate of the excited state κ_{e}. The illumination intensity at which saturation occurs, I_{s} (photons $\mathrm{m^{-2}\,s^{-1}}$), using Eq. (2) can be defined as

$$I_{\mathrm{s}} = \kappa_{\mathrm{e}}/\sigma. \tag{3}$$

Assuming some typical values for $\sigma = 10^{-20}\,\mathrm{m^2}$ and $\tau_{\mathrm{e}} = 10$ ns, we find that saturation occurs at the intensity $I_{\mathrm{s}} = 4 \times 10^{9}\,\mathrm{W\,m^{-2}}$, when converted to watts ($\lambda = 488$ nm). This kind of intensity in the object can already be achieved by focusing a laser beam of only 0·8 mW to a diffraction-limited spot with a diameter of approximately $\lambda = 488$ nm.

When the molecule is in the excited state it cannot absorb another photon, which means that in effect $\sigma = 0$ for an average time τ_{e}. Once the molecule has relaxed to its ground state it becomes receptive to excitation again, but has to wait for an average time τ_{p} (given by $\tau_{\mathrm{p}} = 1/\kappa_{\mathrm{a}}$) for the arrival of the next photon. Therefore, there is a probability, P_{a}, smaller than unity for a photon to be absorbed and to result in the excitation of the molecule, which can be written following Van den Engh & Farmer (1992) as

$$P_{\mathrm{a}} = \frac{\tau_{\mathrm{p}}}{\tau_{\mathrm{e}} + \tau_{\mathrm{p}}}, \tag{4}$$

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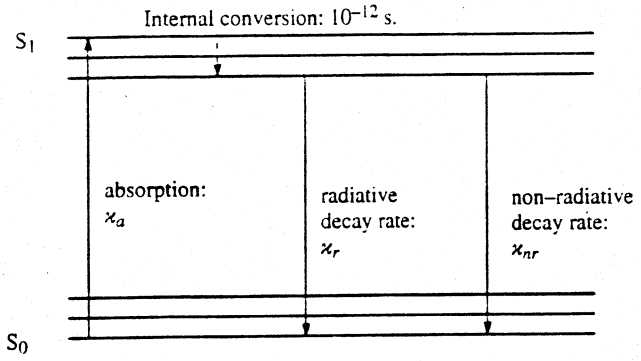

Fig. 1. Jablonski diagram. Absorption/excitation can take place in 10^{-15} s. The rates at which the molecule relaxes to the ground state through fluorescence and non-radiative decay are κ_r and κ_{nr}, respectively.

assuming that the photons arrive at random over the time interval $\tau_e + \tau_p$. Combining Eqs. (2), (3) and (4) yields

$$P_a = \frac{I_s}{I_i + I_s}. \tag{5}$$

The amount of light absorbed by the molecule will be proportional to the product of the illumination intensity I_i and the probability for absorption. Hence,

$$I_{ex} = I_i \frac{I_s}{I_i + I_s}. \tag{6}$$

The fluorescence now is proportional to I_{ex}:

$$I_{flu} = Q \cdot I_{ex} = \frac{\tau_e}{\tau_{flu}} \cdot I_{ex} \tag{7}$$

where Q is the efficiency of the fluorescence, defined as the quotient of the lifetime of the excited state and the fluorescence lifetime, which in the case of fluorescent decay is given by $\tau_{flu} = 1/\kappa_r$. In an elegantly devised flow cytometry experiment Van den Engh & Farmer (1992) found a good agreement with Eq. (6). Now that we have derived an expression for the fluorescence intensity, we will discuss its consequences for imaging in the confocal microscope.

Confocal imaging under saturation conditions

In the case of a point object, the confocal response can be written as the product of the detection sensitivity distribution, equal to the illumination distribution $I_i(u, v)$ in confocal imaging, and the fluorescence intensity distribution (Brakenhoff *et al.*, 1979); hence,

$$I(u, v) = I_i(u, v) \cdot I_{flu}(u, v) \propto I_i(u, v) \frac{I_i(u, v) \cdot I_s}{I_i(u, v) + I_s}. \tag{8}$$

We have introduced the dimensionless optical coordinates u and v defined as

$$u = kz \sin^2 \Omega \quad v = k(x^2 + y^2)^{1/2} \sin \Omega \tag{9}$$

where Ω denotes the angular semi-aperture and $k = 2\pi/\lambda$. We calculated the illumination distribution, $I_i(u, v)$ using the vectorial diffraction theory presented by Richards & Wolf (1959). The illumination intensity is defined as $I_i(u, v) = \boldsymbol{E}(u, v) \cdot \boldsymbol{E}^*(u, v)$, where $\boldsymbol{E}$ is the electric field vector for circular polarization and $\boldsymbol{E}^*$ its complex conjugate. The optical coordinates are in agreement with those used by Richards & Wolf (1959).

Usually the imaging properties of the confocal fluorescence microscope are calculated assuming that the emission intensity is a linear function of the illumination intensity. This is when $I_i \ll I_s$, a situation we shall refer to as 'linear fluorescence' later. When this approximation is applied to Eq. (8), it is found that

$$I(u, v) \propto I_i^2(u, v). \tag{10}$$

When, however, $I_i \gg I_s$, a condition we shall refer to as 'complete saturation', the emission intensity asymptotically approaches I_s (as we saw before), so that the confocal response of the point-like object becomes

$$I(u, v) \propto I_s I_i(u, v) \tag{11}$$

which is equivalent to that of the point response in the conventional linear fluorescence microscope. This means that the image of a completely saturated object in the confocal microscope would be equivalent to its linear fluorescent image in the conventional microscope. We therefore expect that the response for 'partly' saturated point objects will be somewhere in between the linear confocal and the linear conventional point response. Results for the in-focus lateral point responses for various ratios of I_i and I_s are plotted in Fig. 2(a). In the case where $I_i(0, 0) = I_s$, so that $\tau_e = \tau_p$ for the peak intensity, the full with at half maximum (FWHM) of the response has increased by approximately 18% compared with the linear confocal fluorescence response. Furthermore, when $I_i(0, 0) = 100\, I_s$ the response approaches that of the linear conventional fluorescence microscope.

A property of far more importance in practice is the optical sectioning power, $I(u)$, defined as the response for homogeneously fluorescent, infinitely thin, plane objects orientated perpendicular to the optical axis. $I(u)$ can generally be obtained from the 3-D Fourier transform of the point response, $\hat{I}(f_u, f_v)$, through

$$I(u) = \mathfrak{F}^{-1}[\hat{I}(f_u, f_v)|_{f_v=0}] \tag{12}$$

where f_u and f_v are the spatial frequencies corresponding to the u and v coordinates and $\mathfrak{F}^{-1}$ is the 3-D inverse Fourier transformation. We calculated the optical sectioning power for the ratios of I_i and I_s we used for the lateral point responses. The results, $I(u)$, are plotted in Fig. 2(b). The sectioning power, as expressed by the FWHM, has increased by approximately 16% when $I_i(0, 0) = I_s$. A further decrease in the optical sectioning power is seen when the

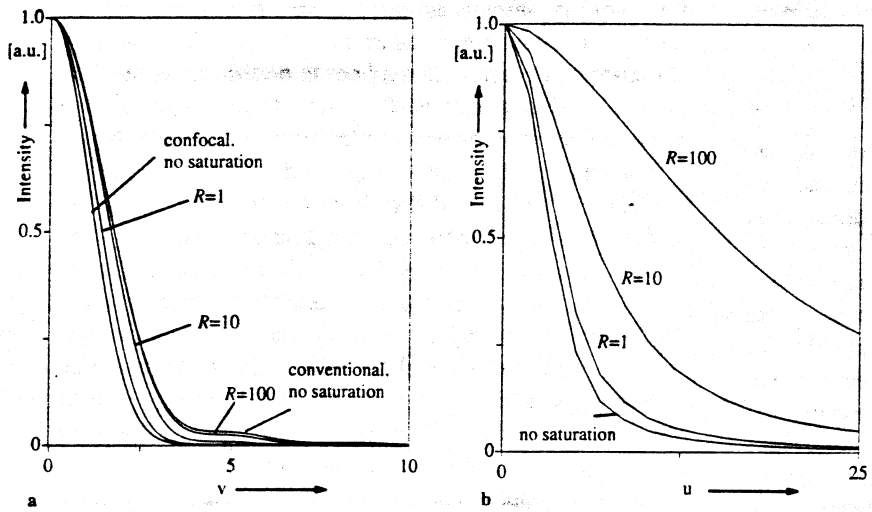

Fig. 2. (a) The in-focus confocal point responses, $I(0, v)$ and (b) the optical sectioning power $I(u)$ of a point source – point detector confocal microscope for several degrees of saturation $R = I(0,0)/I_s$.

illumination intensity increases. As we have seen before, the confocal response of a completely saturated point object is equivalent to the linear conventional point response. Since a conventional fluorescence microscope has no optical sectioning power, no optical sectioning power for completely saturated plane objects in a confocal microscope can be expected.

Experiment

We have measured, in a qualitative way, the responses of a homogenously fluorescent half space using our stage scanning confocal microscope, with a diffraction-limited illumination spot and a square detection pinhole with sides of approximately 200 nm in object space (Brakenhoff *et al.*, 1985; Van der Voort *et al.*, 1985). The object was a bath of a 10^{-5} M rhodamine 6G solution in water (a concentration below the quenching limit; Pringsheim, 1949). To exclude photobleaching as one of the mechanisms for the deterioration in the resolution we acquired the three images in a low, high, low excitation intensity sequence P_1, P_2, P_3, with $P_1 = P_3 \approx 0{\cdot}3$ mW and $P_2 \approx 11$ mW.

The theoretical responses of fluorescent half spaces can be found by the integration of $I(u)$ with respect to u, so that with increasing illumination intensity we expect a decrease in the slope of the depth response. For rhodamine 6G the cross-section for absorption is typically $a = 3 \times 10^{-20}$ m^2, while τ_e, which is dependent on the environment (like pH), in this case is estimated to be 4 ns. We further have assumed that rhodamine is a high efficiency dye so that $\tau_e \approx \tau_{flu}$. From these data we estimated the laser power for which saturation occurs at approximately 0·7 mW (NA = 1·3, λ = 530·9 nm). For real quantitative work this may be a hazardous assumption, but it will do in this qualitative experiment, which is intended to show the effect of saturation on the axial confocal imaging properties. We measured the responses for an estimated power in the focus of approximately 0·3 mW and 11 mW, so that we were below and well above saturation. Images were obtained on a 64 × 64 × 63 3-D raster. The normalized data plotted in Fig. 3 show the average pixel value in a 64 × 64 image plane as a function of the z coordinate of the plane. From Fig. 3 a decrease of the slope of the response can be observed at high illumination intensity. At low illumination intensities the distance between the 85% and 15% points is approximately 0·75 μm, while at high illumination intensity this distance has become approximately 2·5 μm.

Discussion

We have seen that the resolution of the confocal microscope decreases when the fluorescence process is saturated due to excessive illumination intensities. For optimal resolution the illumination peak intensity $I_{ill}(0,0)$ should be well below the saturation intensity I_τ, as determined by the fluorophore that resides in the excited state for the longest time. The use of relatively low illumination intensities will, in practice, lead to weak fluorescence intensities. Consequently, in order

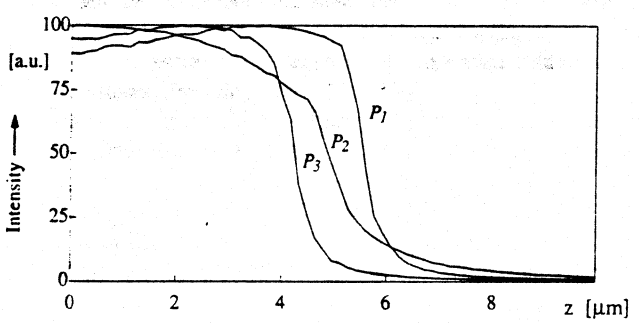

Fig. 3. Depth response of a rhodamine 6G 10^{-5} M solution in water. λ_{ex} = 530·9 nm, λ_{em} > 590 nm, $P_1 = P_3 \approx 0{\cdot}3$ mW, $P_2 \approx 11$ mW. (Confocal microscope: square detection pinhole with sides of 200 nm in object space, oil-immersion objective: 100 × /1·3.)

to obtain a reasonable signal-to-noise ratio in the image, long integration times per voxel and therefore long image-acquisition times result. For efficient detection at such low light levels, photon-counting techniques or the use of cooled CCDs (with their inherent temporal integration capability) may be considered. In practice, for high-resolution imaging, fast image acquisition at too high illumination intensities is not desirable and it may well be that a relatively slow, object scanning confocal microscope with stationary optics (which in principle has superior imaging properties over beam scanning microscopes) will be the best choice. For real-time applications it may be desirable to work with I_i larger than I_s, so that more emission photons can be acquired per unit of time. Speed can be obtained with loss of resolution, which can be kept moderate as long as the illumination intensity does not become excessive. Alternatively, to attain higher imaging rates while still avoiding saturation we can use scanning schemes with slit illumination and detection (Brakenhoff & Visscher, 1992); such systems are commercially available today. However, in slit scanning systems speed is obtained at the cost of resolution.

When imaging specimens that contain more than one type of dye, we have to be careful to work well under the saturation intensity as defined by the fluorophore with the longest excited state lifetime. Otherwise, it is possible to have completely different (linear and non-linear) imaging properties throughout the specimen depending on which dye is present at a certain object point. In principle, a space-invariant point spread function of the microscope can no longer be defined.

In what way does the occurrence of saturation affect quantitative measurements of, for instance, DNA contents or of the concentration of specific dyes? In principle, the fluorescent signal stays linear with the fluorophore concentration, even when the linearity with the illumination intensity is lost. Although we have to be careful when interpreting the acquired data, an intensity drop or rise at a specific point in the object might result not only from a decreased or increased dye concentration but also from a change of the fluorescence lifetime, or lifetime of the excited state due to environmental parameters (pH). This interpretation question is not specific to the case of fluorescence saturation but also occurs when there is still a linear relationship between illumination and fluorescence intensity (see Eq. 7). The occurrence of saturation makes the interpretation even more difficult.

The difference in saturation behaviour throughout the specimen may also be used as a contrast parameter for imaging. By taking the ratio of two images obtained at low and high illumination intensities, these differences should become clear. This kind of imaging may be useful for the identification of certain fluorescent components in the object, in a qualitative way. In principle, we should be able to determine the lifetime of the excited state in a carefully performed quantitative experiment by measuring the confocal response at different illumination intensities. However, quantitative interpretation of the data is expected to be difficult due to interfering photo destructive processes, especially at high intensities.

Conclusion

We have discussed the effects of fluorescence saturation on confocal imaging. We have shown that saturation of the fluorescent probe results in a decrease in the lateral resolution and in the optical sectioning power. Nevertheless, the imaging properties in the confocal microscope, in the worst case, become equal to those in a linear conventional microscope. Because saturation is related to the lifetime of the excited state, which is an object or object–environment-dependent parameter, the imaging properties can vary on a voxel by voxel basis. This means that the characterization of the microscope with a space-invariant point spread function is no longer possible. For quantitative fluorescence imaging, it will be necessary, in practice, to tune the illumination intensity well below the saturation intensity, as determined by the longest lifetime in the specimen.

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