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OPTIMIZATION OF LASER BEAMS IN FRAP EXPERIMENTS OF MICROSCOPICAL OBJECTS

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In fluorescence recovery after photobleaching (FRAP) experiments the sample is irradiated on a small spot, the diameter of which must be related to the sample size and the diffusion constant to be measured. This paper considers the conventional FRAP set-up where a laser beam is directed through a microscope vertical illuminator to the sample. The requirements of an intermediate optical system producing a Gaussian beam with a waist of given radius in the microscope object plane are considered, and the optical parameters determined.

1. Introduction

Fluorescence recovery after photobleaching (FRAP) provides a means to measure the diffusion of molecules included in lipid bilayers, cell plasma membranes and cell organelle envelopes [1–4]. Diffusion and interaction of macromolecules in solution can also be studied by this method as well as molecular motions in the cytoplasm and nucleoplasm. In a conventional FRAP apparatus a Gaussian laser beam enters a microscope vertical illuminator and is focussed on the sample placed on the microscope stage where the beam defines an illuminated area [2–4]. Some authors put a diaphragm on the beam [5,6] or produce an illuminated area with a striped pattern [7–9]. However, more often the whole beam is used, in order to provide the maximum bleaching power and the minimum possible illuminated area. The illuminated area radius is an important parameter for the following reasons:

(1) For a given diffusion coefficient the characteristic time of the fluorescence recovery depends on the square of the illuminated area radius.

(2) The illuminated area radius must be much smaller than the object under study.

(3) The illuminated area radius must not be too small if the sample is thick, otherwise the illuminated volume will not be cylindrical and this will complicate calculation of the diffusion coefficient.

Furthermore, it is desirable that the waist of the Gaussian beam be localized in the sample mean plane. In this way the illuminated volume has a minimum radius variation along the optical axis.

These considerations lead to the following statement: The Gaussian beam emerging from a laser has to be transformed in such a way that the beam waist should be localized in the microscope sample plane and have a given radius. This aim can be achieved with an intermediate optical system which consists of at least a single thin convergent lens [11].

In this work we describe a method which permits one to determine the intermediate optics. To our knowledge no general solution to this useful and practical problem has been published before.

2. Gaussian beam characteristics

Laser beams of propagation mode TMoo are characterized by a maximum of the light amplitude on the propagation axis and a Gaussian distribution of the amplitude around the axis [10,11]. Let W be the distance from the axis at which the amplitude is equal to $1/e$ of its value. W varies along the propagation axis and has its miminum value w in the beam waist. The value of w and the point O where the optical axis intersects the waist plane completely define the Gaussian beam. The confocal parameter is defined by the following equation [12]:

$$\rho = \pi w^2/\lambda \tag{1}$$

where λ is the light wavelength.

The radius W at a distance Z from O is given by the following relation:

$$W = w\left(1 + \left(\frac{Z}{\rho}\right)^2\right)^{1/2} \tag{2}$$

A Gaussian beam is transformed in another Gaussian beam by centered lens systems. The characteristics of the emerging beam are related to those of the incident beam by the following equations:

$$\rho' = f^2\rho/(x^2 + \rho^2) \tag{3}$$

$$x' = f^2 x/(x^2 + \rho^2) \tag{4}$$

where f is the focal length of the system, and x and x' the distance between the object and the image focal planes from the waists of the incident and emerging beams, respectively. The sign convention adopted here is described in refs. 10 and 11.

3. Emerging beam from the laser

The characteristics of the beam emerging from a laser can be computed from the resonator configuration. The formula corresponding to various configurations have been given in the literature [10].

In the case of the ionized gas lasers used in FRAP experiments, the conventional resonator configuration includes a plane back mirror and a front spherical mirror made from the inner face of the output window. The waist of the emerging beam is located behind the rear mirror. Its distance t_1 from the output window, and the beam confocal parameter ρ_1, are given by the following equations:

$$t_1 = \frac{nRl}{R + l(n^2 - 1)} \tag{5}$$

$$\rho_1 = \frac{R(l(R-1))^{1/2}}{R + l(n^2 - 1)} \tag{6}$$

where l, R and n are the cavity length, the curvature radius of the spherical mirror and the refractive index of the output window, respectively.

One can also obtain w_1 and t_1 by eqs. 7 and 8 which follow from eqs. 1 and 2:

$$w_1 = 2\lambda/\theta \tag{7}$$

$$t_1 = \frac{\pi w_1^2}{\lambda}\left(\left(\frac{W_L}{w_1}\right)^2 - 1\right)^{1/2} \tag{8}$$

Where θ is the beam divergence angle and W_L the beam radius on the output window. These two parameters are usually provided by the laer manufacturer.

4. Incident beam on the microscope

The incident beam enters the microscope tube laterally through a vertical illuminator, is reflected by a dichroic mirror, and finally focussed on the sample by passing through the objective in the direction opposite to that of the transmitted and fluorescence beams [4]. Lenses other than the objectives are often placed in the path of the conventional exciting source of the microscope vertical illuminator. We assume here that arrangements have been made in such manner that the laser beam does not pass through these lenses, otherwise one would have to take into account these lenses.

Microscopes can be equipped with objectives of various magnifications. Their design is such that the sample plane and the intermediate image plane

are in fixed positions relative to the microscope frame, positions which are the same for all the objectives [13].

Let O_3 and I be the points where the microscope optical axis intersects the sample plane and the intermediate image plane, respectively, and let F_3 and F_3' be the object and image foci of the objective. The Newton relation of geometrical optics laws can be written as follows [14]:

$$O_3F_3 = x_3 = f_3/g \tag{9}$$

$$IF_3' = x_N = f_3 g \tag{10}$$

where f_3 and g are the focal length and magnification of the objective, respectively. These parameters can be provided by the microscope manufacturer [13].

We need a Gaussian beam having its waist located in O_3 with an imposed radius w_3. The waist position O_3 and the confocal parameter ρ_2 of the Gaussian beam incident on the objective may be easily obtained by applying eqs. 3 and 4, assuming that the light path through the objective is opposite to that of the real beam light path:

$$O_2F_3' = x_2 = x_3 f_3^2/\left(x_3^2 + \rho_3^2\right) \tag{11}$$

$$\rho_2 = \rho_3 f_3^2/\left(x_3^2 + \rho_3^2\right) \tag{12}$$

We shall more conveniently define the abscissa of O_2 by its distance t_2 from the fixed point I, given by eq. 13:

$$t_2 = x_N - x_2 \tag{13}$$

Taking into account eqs. 1, 9, 10 and 12 we can write:

$$t_2 = \frac{\left(\pi w_3^2/\lambda\right)^2 f_3 g}{f_3^2/g^2 + \left(\pi w_3^2/\lambda\right)^2} \tag{14}$$

$$w_2 = \frac{w_3 f_3}{f_3^2/g^2 + \left(\pi w_3^2/\lambda\right)^2} \tag{15}$$

It should be noted that x_2 and t_2 must be positive with the sign convention chosen. It can be easily shown that t_2 and ρ_2 are linked together by the following relation:

$$\rho_2 = \sqrt{t_2\left(x_N - t_2\right)}$$

This relation entails the following properties:

(1) O_2 is located between I and F_3'

(2) For a given value of w_2 there are two possible positions of O_2 symmetrically located about the middle of IF_3'.

(3) The following relations hold:

$$\rho_2 \leqslant x_N/2 \quad \text{or} \quad w_2 \leqslant \left(\frac{x_N}{2\pi}\right)^{1/2}$$

The equality is obtained when $t_2 = x_N/2 = \rho_2$

5. Matching the laser beam to the microscope

We are then led to determine an optical system which transforms the beam emerging from the laser with a waist located in O_1 and a radius w_1, into a new beam entering the microscope with a waist located in O_2 and a radius w_2.

The distance between O_1 and O_2 is given by the following relation:

$$D = t_1 + D' + t_2 \tag{16}$$

where D' is the distance between the laser output window and the point I; t_1 and t_2 are given by eqs. 5 and 14. D' is a fixed distance if one ensures that the laser and the microscope are held firmly fixed to the experiment table. Let us determine the focal length f_2 and the location of a thin lens L_2 which achieves the beam matching. Let H be the point where the optical center of the lens is located and let us define $d_1 = HO_1$, $d_2 = HO_2$.

One can easily see that L must be convergent and situated between O_1 and O_2, which entails that f_2, d_1, d_2 must be positive.

According to refs. 10 and 11 one can write:

$$d_1 = f_2 + \left(\frac{\rho_1}{\rho_2}\right)^{1/2}\left(f_2^2 - \rho_1\rho_2\right)^{1/2} \tag{17}$$

$$d_2 = f_2 + \left(\frac{\rho_2}{\rho_1}\right)^{1/2}\left(f_2^2 - \rho_1\rho_2\right)^{1/2} \tag{18}$$

Furthermore d_1 and d_2 are obviously linked by the following relation:

$$d_1 + d_2 = D \tag{19}$$

which, taking into account eqs. 17 and 18, can be

written:

$$D - 2f_2 = \left[\left(\frac{\rho_1}{\rho_2}\right)^{1/2} + \left(\frac{\rho_2}{\rho_1}\right)^{1/2}\right]\left(f_2^2 - \rho_1\rho_2\right)^{1/2} \tag{20}$$

By taking the square of the two sides of eq. 20 and rearranging the equality, one obtains the following equation of degree two in f_2

$$f_2^2 \frac{(\rho_1 - \rho_2)^2}{\rho_1\rho_2} + 4Df_2 - D^2 - (\rho_1 + \rho_2)^2 = 0 \tag{21}$$

This equation has a single positive solution from which one can calculate d_1 and d_2 by eqs. 17 and 18.

As an example we have drawn in fig. 2 the variation of f_2 and d_1 with w_3 for the experimental set-up of fig. 1 in which the intermediate system was reduced to a single lens.

The light source was an argon laser emitting light at 488 nm, the emerging waist w_1 was equal to 0.46 mm, and the distance between the laser waist and the intermediate image plane of the microscope in the vertical illuminator was 3.49 m. The objective was a Zeiss Neofluar of magnification 40.5 and focal length 4.5 mm [13].

The intermediate optics could also be formed by two convergent lenses (fig. 1). By varying the distance between the lenses, the focal length of the doublet continuously changed and permitted a continuous range of w_3 values to be obtained. Furthermore, a pinhole was placed between the two lenses realizing a spatial filter which improved the Gaussian profile of the beam.

The focal lengths of the lenses being given, one could determine the location of these lenses as a function of w_3 in a manner similar to that described above for the single lens. As a first step, one established the equation which is obeyed by

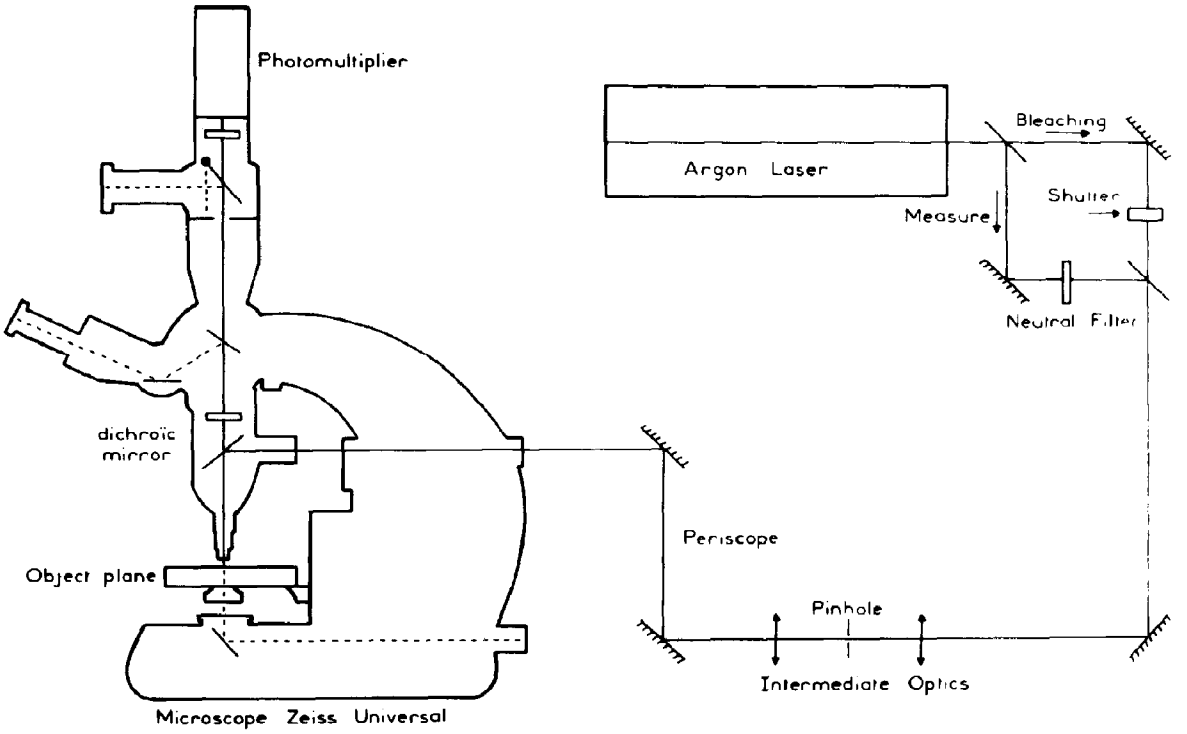

Fig. 1. A FRAP experimental apparatus.

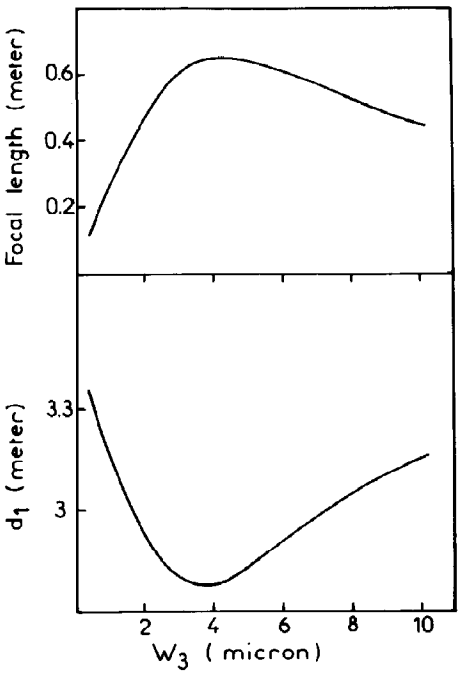

Fig. 2. The calculated focal length of a thin lens and the distance of this lens from the emerging laser beam waist as functions of the waist radius w_3 in the microscope object plane. The objective is a Zeiss 40 X Neofluar and the laser is a 164 Spectrophysics argon laser, emitting at 488 nm. Other parameters are given in the text.

the distance between the lenses. This equation was easily resolved by an iterative method (see appendix). We then used a system of lenses of focal length 50 mm. Each lens was mounted on a micrometric table which could be moved along a microbench.

Finally, it should be noted that the microscope manufacturers provide values of the objective magnification and focal length valid for a wavelength situated in the middle of the visible spectrum.

Since the objective show chromatic aberrations, their characteristics depend on the light wavelength. For this reason, one can consider that the calculation given above only provides an approximate value of the beam radius w_3 in the sample plane. Its exact value, necessary to determine the diffusion coefficient, must be measured by direct methods such as those described in ref. 15.

Appendix

Let L_a and L_b be the lenses of the doublet which forms the intermediate optics, f_a and f_b their focal lengths, and e the distance between them. The system focal length is

$$f_2 = f_a f_b / \Delta \tag{22}$$

with

$$\Delta = f_a + f_b - e \tag{23}$$

Eqs. 17 and 18 still hold, if d_1 and d_2 are the distances of w_1 and w_2 to the object and image principal planes, respectively [11]. The condition, eq. 19, is replaced by

$$s_1 + s_2 + e = D \tag{24}$$

where s_1 and s_2, the distances of w_1 and w_2 to L_a and L_b, respectively, are given by the following relations [14].

$$s_1 = d_1 + f_a - f_a^2/\Delta - f_2$$
$$s_2 = d_2 + f_b - f_b^2/\Delta - f_2 \tag{25}$$

Taking into account eqs. 17, 18 and 22–25 one can write an equation of order 4 for Δ where the only parameters are D, f_a, f_b, ρ_1 and ρ_2 which are assumed to be known.

Resolving this equation by a numerical method allows one to obtain Δ from which the positions of L_a and L_b can be calculated by determining e and s_1 using eqs. 23, 22, 17, 18 and 25.

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