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Fluorescence recovery after photobleaching of suspensions of vacuoles

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Abstract

In this work we derive theoretical expressions for the FRAP measured on a liquid suspension of vacuoles labelled by a fluorescent probe bound to the surface membrane of these vacuoles. The bleaching laser beam creates an inhomogeneity in the surface concentration of the probe molecules. We consider the case in which the randomization of these probe molecules on the vacuole surface occurs much faster than the fluorescent recovery due to the vacuole diffusion. For a given value of the bleaching parameter K, we found that the bleaching fraction of the fluorescent molecules and the fluorescence recovery rate are decreasing functions of the square ratio of the vacuole to the laser beam radius of the FRAP instrument.

Keywords: Theory of FRAP; Vacuoles transport; Diffusion

1. Introduction

The method of fluorescence recovery after photobleaching (FRAP), is employed for measuring the lateral rates of diffusion or of flow, of fluorescent macromolecules [1–7]. FRAP instruments use a laser beam for photobleaching and for fluorescence excitation. Different optical geometry of the beam has been proposed [8–12]. The most common instrument of FRAP is based on the so-called laser spot photobleaching in which a laser Gaussian beam is focused on the sample by a microscope objective [2,6,13].

The theory of FRAP has first been developed for planar samples. This theory may be applied to reconstituted membranes and to living cell surface membranes [14]. The theory is also valid for macromolecular solutions and living cell cytoplasm, provided that the convergence of the projected laser beam is negligible in the illuminated zone of the sample [15].

In the present work, we extend this theory to the case of the diffusion of vacuoles suspended in a liquid. Each vacuole is assumed to be labelled by fluorophores incorporated in its lipidic surface. Vacuoles bounded by a lipid membrane are found in samples of biological interest [16–18]. Different systems of vacuoles also exist in living cells, and some may be specifically labelled in vivo on their surface by fluorescent probes [19–22]. In these living cells, the vacuole motion is often saltatory. These movements, however, may be similar to a diffusion along the microtubules [23,24]. The present calculation contains steps which may be useful in a future theory of the FRAP of the living processes.

Photobleaching by the laser beam produces an inhomogeneity in the distribution of the fluorescent molecules on the vacuole surface. This process is usually followed by uniformization of the fluorophore concentration occurring when the molecules

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diffuse on the membrane or when the whole vacuole rotates with the Brownian motion. This rate of homogenization can be assumed to be rapid compared to the vacuole diffusion. But the rate of homogenization has also to be compared to the rate of fluorophore deactivation by the bleaching pulse.

We perform here the calculations for two extreme values of the rate of the probe concentration uniformization, namely a high value or a small one compared to the rate value of bleaching. It is found that the bleaching fraction and the fluorescence recovery rate decrease when the ratio of the vacuole radius to the laser beam radius increases, in the same way approximatively for both cases envisaged. Formulas are given which allow the coefficient of diffusion of the vacuoles to be determined from the shape of the fluorescence recovery curve.

2. Results

2.1. General considerations

Let consider a sample of spherical vacuoles in a liquid suspension. These vacuoles have an identical radius R. The number of these vacuoles per volume unit is designated by N. Each of these vacuoles contains fluorescent molecules uniformly distributed on their surface membrane. If $n(-)$ is the number of the probe molecules per vacuole, their membranous concentration is:

$$\gamma(-) = n(-)/4\pi R^2. \quad (1)$$

On the other hand the probe concentration in the suspension is obtained as follows:

$$C(-) = n(-)N. \quad (2)$$

During the photobleaching phase, the suspension is submitted to a laser beam of a Gaussian profile [2,6]. The radiative intensity of such a beam depends on the position of the illuminated point. Consequently, the number of bleached molecules varies at different points of the sample. In the present case the beam creates a double gradient in the sample: a concentration gradient of probes in a vacuole and a gradient in the number of fluorescent probe molecules per vacuole among the vacuoles suspended in the liquid. We assume here that the gradient of the probe concentration on the membrane surface dissipates very rapidly by diffusion on the membrane. On the other hand the gradient of the number of probe molecules per vacuole is dissipated by the diffusion of the vacuoles in the suspension which is much slower.

The validity of these assumptions will be examined in the discussion section of this work. The diffusion of the vacuoles provides a recovery of the fluorescence when the suspension is excited by the laser beam, which has been strongly attenuated in order to avoid photobleaching.

2.2. Photobleaching

We designate by $\gamma(\rho,t)$, the membranous concentration of the probe at the time t which varies from $-T$ to 0 during the photobleaching phase. The position of a point M of a vacuole is defined by the coordinates of the vector ρ reported in axes linked to the sample studied (for the representation of the main vectors defined in this work see Fig. 1).

2.2.1. Negligible diffusion of the probe on the membrane

We assume here that the probe diffusion on the membrane is negligible during the bleaching phase, and that the incident light bleaches the fluorescence according to a first order process [2]. That is to say:

$$\frac{d\gamma(\rho,t)}{dt} = -\alpha I(\rho)\gamma(\rho,t), \quad -T < t < 0 \quad (3)$$

where α is the rate constant of the bleaching reaction and $I(\rho)$ the laser beam intensity at the point defined by ρ. The membranous concentration of the probe at the beginning of the recovery phase is obtained by integrating Eq. (3). Taking account of the prebleaching concentration given by Eq. (1), one obtains:

$$\gamma(\rho,0) = \frac{n(-)}{4\pi R^2}\exp\left[\frac{-KI(\rho)}{I_0}\right] \quad (4)$$

where I_0 is the laser beam intensity on the optical axis of the beam.

K is the photobleaching parameter defined as [2]:

$$K = \alpha T I_0. \quad (5)$$

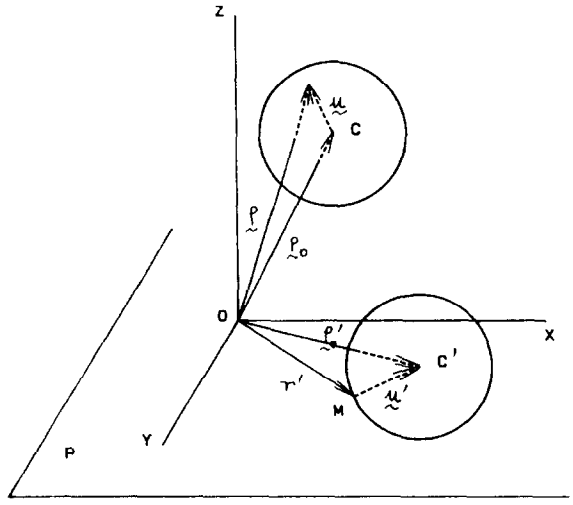

Fig. 1. Vectors used in the calculations of the FRAP of a vacuole suspension. The sample is a sheet of liquid comprised between two parallel planes and containing spherical vacuoles. P, the middle plane of the sample, is the $o\ x\ y$ plane. oz is the optical axis of the laser beam. $o\ x\ y\ z$ are the laboratory axes. A point M of P is defined by the two dimensional vector $\underset{\sim}{OM} = \underset{\sim}{r}'$. The center C' of a vacuole, the surface of which passes through M at the time t of the recovery phase, is given by the vector $\underset{\sim}{MC'} = \underset{\sim}{u}'$ and the position of C' in the laboratory axes are given by the coordinates of $OC' = \underset{\sim}{\rho}'_0 = \underset{\sim}{u}' + \underset{\sim}{r}'$. The center of the same vacuole was coincident with C at time 0, defined in the laboratory axes by the components of $\underset{\sim}{OC} = \underset{\sim}{\rho}_0$. A point S of the vacuole surface was at that time, given by $\underset{\sim}{CS} = \underset{\sim}{u}$ and in the laboratory axes by the components of $\underset{\sim}{OS} = \underset{\sim}{\rho} = \underset{\sim}{u} + \underset{\sim}{\rho}_0$.

Here we will define a function $\sigma(\underset{\sim}{u},R)$ of the space vector $\underset{\sim}{u}$ and of the number R, which allows to express conveniently that the application domain of any space function $f(\underset{\sim}{u})$ is restricted to a sphere of radius R.

By definition:

$$\sigma(\underset{\sim}{u},R) = 0 \text{ for } |\underset{\sim}{u}| \neq R \tag{6}$$

and

$$\int\sigma(\underset{\sim}{u},R)\,\mathrm{d}^3\underset{\sim}{u} = 4\pi R^2 \tag{7}$$

$$\int f(\underset{\sim}{u})\,\sigma(\underset{\sim}{u},R)\,\mathrm{d}^3\underset{\sim}{u} = R^2\int_0^{2\pi}\int_0^{\pi} f(R,\theta,\varphi)\sin\theta\,\mathrm{d}\theta\,\mathrm{d}\varphi. \tag{8}$$

Where $f(|\underset{\sim}{u}|,\theta,\varphi)$ is the expression of $f(\underset{\sim}{u})$ when $\underset{\sim}{u}$ is expressed in its polar coordinates $|\underset{\sim}{u}|,\theta,\varphi$. In the case of $f(\underset{\sim}{u}) = 1$, Eq. (8) becomes Eq. (7).

This function σ will help to define the number of probe molecules per vacuole, the center of which is defined by the vector $\underset{\sim}{\rho}_0$. That is to say:

$$n(\underset{\sim}{\rho}_0,t) = \int\sigma(\underset{\sim}{u},R)\,\gamma(\underset{\sim}{u}+\underset{\sim}{\rho}_0,t)\,\mathrm{d}^3\underset{\sim}{u}. \tag{9}$$

Let us apply Eq. (9) at the end of the photobleaching phase ($t = 0$). Taking into account Eq. (4), one finds:

$$n(\underset{\sim}{\rho}_0,0) = \frac{n(-)}{4\pi R^2}\int\sigma(\underset{\sim}{u},R)\exp\left[-K\frac{I(\underset{\sim}{\rho}_0+\underset{\sim}{u})}{I_0}\right]\mathrm{d}^3\underset{\sim}{u}. \tag{10}$$

2.2.2. Instantaneous diffusion of the probe on the vacuole

We assume now, that the probe diffusion is fast enough for the probe concentration of the vacuole surface to be uniform at every moment of the bleaching phase. One may then write:

$$\gamma(\underset{\sim}{\rho},t) = \bar{\gamma}(\underset{\sim}{\rho}_0,t) \tag{11}$$

where $\bar{\gamma}(\underset{\sim}{\rho}_0,t)$ is a concentration which depends on the position $\underset{\sim}{\rho}_0$ of the vacuole center in the suspension.

The photobleaching kinetics obeys then the following differential equation:

$$\frac{\mathrm{d}\bar{\gamma}(\underset{\sim}{\rho}_0,t)}{\mathrm{d}t} = -\alpha\bar{\gamma}(\underset{\sim}{\rho}_0,t)\bar{I}(\underset{\sim}{\rho}_0) \tag{12}$$

for $-T < t < 0$ where $\bar{I}(\underset{\sim}{\rho}_0)$ is the average intensity of the laser beam taken on different points of the vacuole surface.

One may write:

$$\bar{I}(\underset{\sim}{\rho}_0) = \frac{1}{4\pi R^2}\int\sigma(\underset{\sim}{u},R)\,I(\underset{\sim}{\rho}_0+\underset{\sim}{u})\,\mathrm{d}^3\underset{\sim}{u}. \tag{13}$$

Integration of (12) provides the membranous concentration, at time $t = 0$, from which one obtains the number of probes per vacuole as follows:

$$n(\underset{\sim}{\rho}_0,0) = n(-)\exp\left[-K\frac{\bar{I}(\underset{\sim}{\rho}_0)}{I_0}\right]. \tag{14}$$

In writing this equation we have taken into account the prebleaching concentration given by expression (1).

2.3. Probes number per vacuole during the recovery phase

The vacuoles bleached during the bleaching phase, leave the illuminated zone of the suspension and are replaced by non bleached vacuoles which diffuse into that same zone. The number of probe molecules per vacuole, the center of which takes the position $\underset{\sim}{\rho}_0$ at a time $t > 0$, is given by the following expression:

$$n(\underset{\sim}{\rho}'_0,t) = \int n(\underset{\sim}{\rho}_0,0)\, w(\underset{\sim}{\rho}_0,\underset{\sim}{\rho}'_0,t)\, \mathrm{d}^3\underset{\sim}{\rho}_0 \qquad (15)$$

where $w(\underset{\sim}{\rho}_0,\underset{\sim}{\rho}'_0,t)$ is the conditional probability that a vacuole having its center position defined by $\underset{\sim}{\rho}_0$ at time 0, is in the position $\underset{\sim}{\rho}'_0$ at time t [25].

For vacuole diffusing in the three dimensional space, one has:

$$w(\underset{\sim}{\rho}_0,\underset{\sim}{\rho}'_0,t) = (4\pi Dt)^{-3/2}\exp\left[-(\underset{\sim}{\rho}'_0 - \underset{\sim}{\rho}_0)^2/4Dt\right] \qquad (16)$$

where D is the diffusion coefficient of the vacuoles. It can easily be seen that Eqs. (15) and (16) imply the following diffusion equation:

$$\frac{\delta n(\underset{\sim}{\rho}_0,t)}{\delta t} = D\nabla^2 n(\underset{\sim}{\rho}_0,t). \qquad (17)$$

The solution of this equation both depends on the initial conditions which are determined by Eq. (10) or (14), and on the boundary conditions. To be more precise we have to specify the geometrical characteristics of the FRAP apparatus as follows: we choose three axes of coordinates (o, x, y, z) at right angles, which are linked to the liquid suspension sample. The optical axis of the laser beam is assumed to be directed along oz. The thickness of the sample is small along oz and of infinite dimensions along the axes ox and oy. Consequently, the transverse Gaussian radius of the beam may be considered as constant in the illuminated area of the sample [15]. The luminous intensity of the bleaching beam may be expressed as follows [2]:

$$I(\underset{\sim}{\rho}) = I(\underset{\sim}{r}) = I_0\exp\left[-\frac{2\underset{\sim}{r}^2}{w^2}\right] \qquad (18)$$

where $\underset{\sim}{r}$ is the projection of $\underset{\sim}{\rho}$ in the (x o y) plane and w is the Gaussian radius of the laser beam.

Under these conditions, the diffusion problem of FRAP is essentially two dimensional [15,26] and one may write:

$$n(\underset{\sim}{\rho}_0,0) = n(\underset{\sim}{r}_0,0) \qquad (19)$$

$$n(\underset{\sim}{\rho}'_0,t) = n(\underset{\sim}{r}'_0,t) \qquad (20)$$

where $\underset{\sim}{r}_0$ and $\underset{\sim}{r}'_0$ are the projections of $\underset{\sim}{\rho}_0$ and $\underset{\sim}{\rho}'_0$ on the plane (o x y).

For determining $n(\underset{\sim}{r}'_0,t)$ as a function of $n(\underset{\sim}{r}_0,0)$, we use the Fourier method according to which one may write [2]:

$$n(\underset{\sim}{r}'_0,t) = (2\pi)^{-2}\iint \mathrm{d}^2\underset{\sim}{\mu},\mathrm{d}^2\underset{\sim}{r}_0 \exp\left[-i\underset{\sim}{\mu}(\underset{\sim}{r}'_0 - \underset{\sim}{r}_0) - \underset{\sim}{\mu}^2 Dt\right] n(\underset{\sim}{r}_0,0) \qquad (21)$$

where μ is the two dimensional vector, the components of which are the Fourier transform variable μ_x and μ_y.

2.4. General expression of the fluorescence recovery of a probe bound to vacuoles in suspension

The fluorescence emitted by the sample during the recovery phase, is expressed as follows [2,5]:

$$F(t) = \frac{q}{A}\Delta l \int C(\underset{\sim}{r}',t)\, I(\underset{\sim}{r}')\, \mathrm{d}^2\underset{\sim}{r}' \qquad (22)$$

where q is a factor which takes into account the light absorption of the exciting laser, the fluorescence quantum yield of the probe and the photon loss in the optical detection system; A is the attenuation factor of the laser beam during the recovery phase; Δl is the sample thickness along o z and $C(\underset{\sim}{r}',t)$ is the probe concentration at the point $M(\underset{\sim}{r}')$ of the plane x o y.

$C(\underset{\sim}{r}',t)$ is obtained by summing the contribution of probes brought by all the fractions of membranes which pass through the volume element surrounding $M(\underset{\sim}{r}')$.

According to our general assumption, the distribution of the probe molecules is rapidly uniformized before the vacuole has perceptibly diffused. Then we may write:

$$C(\underset{\sim}{r}',t) = \frac{N}{4\pi R^2}\int n(\underset{\sim}{r}' + \underset{\sim}{u}',t)\, \sigma(\underset{\sim}{u}',R)\, \mathrm{d}^3\underset{\sim}{u}' \qquad (23)$$

where $\underset{\sim}{u}'$, a vector which links the point $M(\underset{\sim}{r}')$ to a vacuole center, is defined by the following relation:

$$\underset{\sim}{\rho}_0' = \underset{\sim}{u}' + \underset{\sim}{r}'. \tag{24}$$

Taking into account expressions (21) and (23), Eq. (22) may be written as follows:

$$F(t) = \frac{q}{A}\Delta l \frac{N}{16\pi^3 R^2} \iiiint d^3\underset{\sim}{u}', d^2\underset{\sim}{r}_0, d^2\underset{\sim}{r}' d^2\underset{\sim}{\mu}\sigma(\underset{\sim}{u}', R) \times I(\underset{\sim}{r}')\, n(\underset{\sim}{r}_0, 0) \exp\left[-i\underset{\sim}{\mu}(\underset{\sim}{r}' + \underset{\sim}{u}' - \underset{\sim}{r}_0) - \underset{\sim}{\mu}^2 Dt \right]. \tag{25}$$

2.5. Fluorescence recovery when the probe diffusion on the membrane is negligible during photobleaching

In order to obtain the fluorescence intensity, we have to replace the quantity $n(\underset{\sim}{r}_0, 0)$ in Eq. (25) by its value expressed in Eq. (10). In taking into account Eq. (2), one obtains:

$$F_K(t) = \frac{q\Delta l}{A} \frac{C(-)}{64\pi^4 R^4} \iiiint d^3\underset{\sim}{u} d^3\underset{\sim}{u}' d^2\underset{\sim}{r}' d^2\underset{\sim}{r}_0 d^2\underset{\sim}{\mu} \times \sigma(\underset{\sim}{u}, R)\sigma(\underset{\sim}{u}', R) \cdot I(\underset{\sim}{r}') \exp\left[-K \frac{I(\underset{\sim}{r}_0 + \underset{\sim}{u})}{I_0} \right] \times \exp\left[-i\underset{\sim}{\mu}(\underset{\sim}{r}' + \underset{\sim}{u}' - \underset{\sim}{r}_0) - \underset{\sim}{\mu}^2 Dt \right]. \tag{26}$$

We expand the first exponential of (26) in an infinite series and integrate the result with respect to $\underset{\sim}{r}', \underset{\sim}{r}_0$ *and* $\underset{\sim}{u}$ (see Appendix A).

One finds:

$$F_{Kn}(t) = F(-) + \sum_{n=1}^{\infty} F_{Kn}(t) \tag{27}$$

where

$$F(-) = \frac{q}{A}\Delta l C(-) I_0 \frac{\pi w^2}{2} \tag{28}$$

is the fluorescence intensity in the prebleaching phase,

$$F_{Kn}(t) = \frac{(-K)^n}{n!} \frac{F(-)}{\gamma} \frac{1}{8\pi} \int_0^{2\pi}\int_0^{\pi}\int_0^{\pi} \times \exp\left[-\frac{2R^2 n}{w^2} \frac{g(\theta, \theta', \chi)}{\gamma} \right] \sin\theta \sin\theta' d\theta d\theta' d\chi \tag{29}$$

$$g(\theta, \theta', \chi) = \sin^2\theta + \sin^2\theta' + 2\sin\theta\sin\theta'\cos\chi \tag{30}$$

$$\gamma = 1 + n(1 + 2t/\tau_D) \tag{31}$$

and

$$\tau_D = w^2/4D \tag{32}$$

is the time characteristic of the fluorescence recovery due to the vacuoles diffusion.

We may compute the triple integral of formula (29) by expanding the exponential in series. After integration, one obtains the following equation (Appendix A):

$$F_{Kn}(t) = \frac{(-K)^n}{n!} \frac{F(-)}{\gamma} \left\{ 1 + \sum_{p=1}^{\infty} \left(\frac{2R^2}{w^2} \frac{n}{\gamma} \right)^p L_p \right\} \tag{33}$$

where:

$$L_p = \frac{(-1)^p}{(p!)} \sum_{q=0}^{\mathrm{int}(p/2)} \sum_{u=0}^{q-2p} C_p^{2q} C_{2q}^{q} C_{p-2q}^{u} \times S(q+u) S(p-q-u) \tag{34}$$

int $(p/2)$ is the integral number of $p/2$, the C_h^k are the coefficients of a binome brought to the power h and $S(h)$ is given in the formula (A 13) of Appendix A. We note that for $R = 0$, the Eq. (33) is reduced to:

$$F_{Kn}(t) = \frac{(-K)^n}{n!} \frac{F(-)}{1 + n\left(1 + \dfrac{2t}{\tau_D}\right)} \tag{35}$$

which is the expression found for the two dimensional FRAP of a solution of fluorescent molecules diffusing freely [2].

The expression between brackets, in Eq. (33), appears as a corrective term for the non negligible size of the vacuoles, and is a function of the square of the ratio of the vacuole radius to the laser beam radius.

The fraction of the fluorophore bleached is defined by the following expression [27]:

$$P = \frac{F(-) - F(0)}{F(-)}. \tag{36}$$

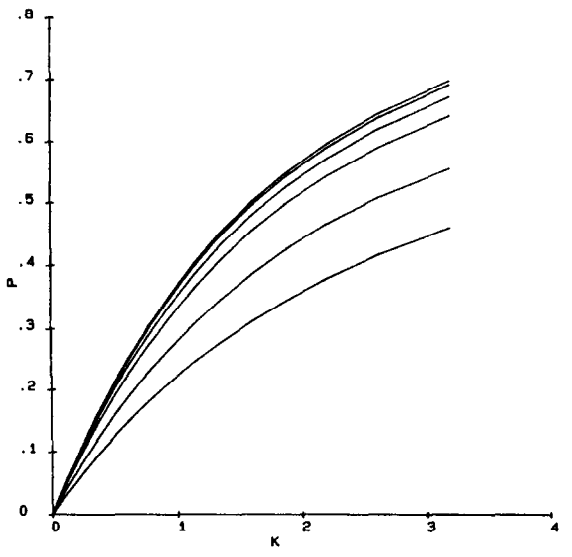

Fig. 2. Fraction of bleached molecules as a function of K when there is no marker diffusion on a vacuole membrane, during the photobleaching phase. From the upper curve to the lower one the values of R/w are successively: 0, 0.1, 0.2, 0.3, 0.5 and 0.7.

By using the Eq. (27) and (33) one obtains:

$$P = \sum_{n=1}^{\infty} \frac{(-K)^n}{(n+1)!} \left[1 + \sum_{p=1}^{\infty} \left(\frac{2R^2}{w^2} \frac{n}{n+1} \right)^p L_p \right]. \tag{37}$$

Here also the expression between the brackets of (37) appears as a corrective factor for the non negligible size of the vacuoles.

Finally one defines the fractional form of the fluorescence recovery as follows [2,5]:

$$X_K(t) = (F_K(t) - F_K(0))/(F_K(-) - F_K(0)). \tag{38}$$

Taking into account the definitions in Eqs. (27) and (36), one may write:

$$X_K(t) = 1 + \frac{1}{P} \sum_{n=1}^{\infty} F_{Kn}(t)/F(-) \tag{39}$$

which may be computed by expressions (33) and (34). For a given value of K, Fig. 2 shows that P decreases when R/w increases and one can see on Fig. 3 that the rate of the fractional recovery decreases as R/w increases.

The function defined by Yguerabide et al. (1982) is a simple way of analysing the fluorescence recovery curve of a sample containing freely diffusing molecules [27]. In its fractional form, the function of Yguerabide et al. (1982) may be written:

$$X_G(t) = \frac{t/t_{1/2}}{1 + t/t_{1/2}} \tag{40}$$

where

$$t_{1/2} = \beta \tau_D \tag{41}$$

and β is a numerical factor which is an increasing function of K. β tends to 1 when K tends to zero. β may be determined empirically by fitting $X_G(t)$ by the least square method to the rigorous theoretical expression of $X_K(t)$. The relative deviation of X_G from the rigorous expression remains smaller than 1% for $K \leq 3.2$.

In an analogous way, we determined β for the FRAP of the vacuole suspensions.

Fig. 4 represents the variation of β as a function of K for different R/w values. One can see that β increases linearly with K and that the slope of this line does not vary very much with R/w. When K

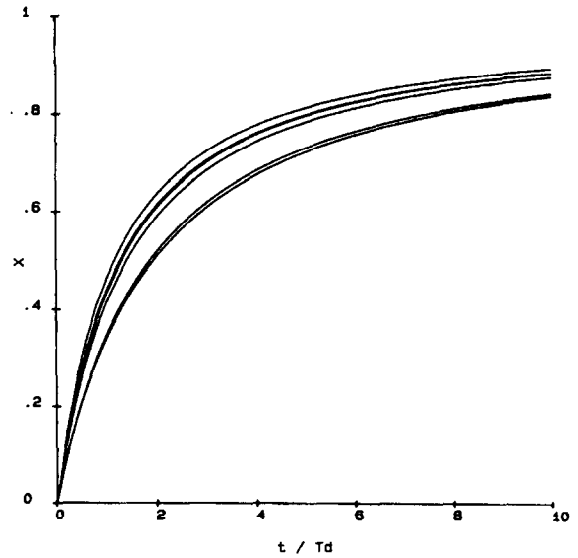

Fig. 3. Fractional fluorescence recovery as a function of t/τ_D when there is no marker diffusion during photobleaching. From the upper curve to the lower one, the values of K and R/w are respectively: 1.6, 0; 3.2, 0; 1.6, 0.3; 3.2, 0.3; 1.6, 0.7; 3.2, 0.7. The curves presenting the values of K and R/w: 3.2, 0 and 1.6, 0.3 are nearly identical.

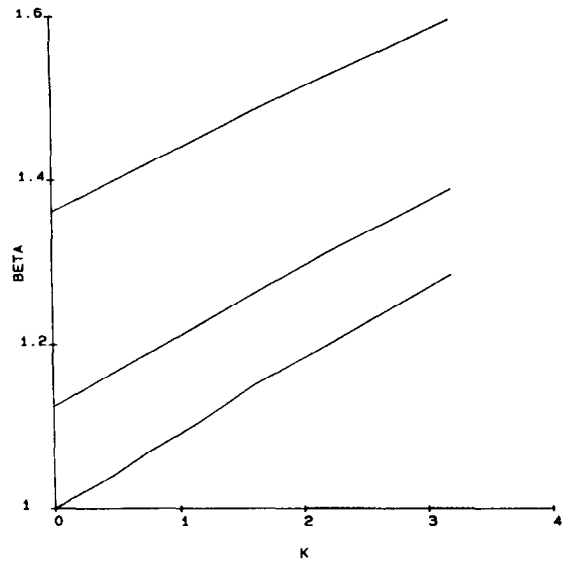

Fig. 4. Variation of the coefficient β with K when there is no marker diffusion during photobleaching. From the lower curve to the upper one the value of R/w are successively 0, 0.3, 0.5.

tends to 0, β no more tends to 1, but to a value $1 + a(R/w)$ where $a(R/w)$ is approximately proportional to $(R/w)^2$.

Finally when $R/w > 0.75$, the fluorescence recovery curve takes a S shape and the Yguerabide function does no longer correctly fit it.

2.6. *Fluorescence recovery when the membranous uniformization of the probe concentration instantaneously occurs during photobleaching*

In this case, the expression of the initial number of probe molecules per vacuole, for a given position of the vacuole center is obtained by Eq. (14). The fluorescence of recovery is given in bringing the second member of expression (14) into Eq. (25), which leads to:

$$F_K(t) = \frac{q}{A}\frac{\Delta lC(-)}{16\pi^3 R^2}\iiiint \mathrm{d}^2\underset{\sim}{\mu}\,\mathrm{d}^3\underset{\sim}{u}'\,\mathrm{d}^2\underset{\sim}{r}'\,\mathrm{d}^2\underset{\sim}{r}_0 \times\sigma(\underset{\sim}{u}',R)\,I(\underset{\sim}{r}')\exp\left[-K\frac{\bar{I}(\underset{\sim}{r}_0)}{I_0}\right] \times\exp\left[-i\underset{\sim}{\mu}(\underset{\sim}{r}'+\underset{\sim}{u}'-\underset{\sim}{r}_0)-\underset{\sim}{\mu}^2 Dt\right]. \quad (42)$$

By expanding the first exponential of Eq. (42) in series, $F_K(t)$ becomes a sum expressed by formula (27) where:

$$F_{Kn}(t) = \frac{q}{A}\frac{\Delta lC(-)}{16\pi^3 R^2}\frac{(-K)^n}{n!}\iiiint \mathrm{d}^2\underset{\sim}{\mu}\,\mathrm{d}^3\underset{\sim}{u}'\,\mathrm{d}^2\underset{\sim}{r}'\,\mathrm{d}^2 r_0 \times\sigma(\underset{\sim}{u}',R)\,I(\underset{\sim}{r}')\left(\frac{\bar{I}(\underset{\sim}{r}_0)}{I_0}\right)^n \times\exp\left[-i\underset{\sim}{\mu}(\underset{\sim}{r}'+\underset{\sim}{u}'-\underset{\sim}{r}_0)-\underset{\sim}{\mu}^2 Dt\right]. \quad (43)$$

The calculation of these Integrals is outlined in Appendix B. One finds finally:

$$F_{Kn}(t) = \frac{(-K)^n}{n!}(F(-)/\gamma)\sum_{p=0}^{\infty}(2R^2/w^2)^p\sum_{q=0}^{p}a_n(p,q)/n^q \times\sum_{h=0}^{q}b(q,h)(4/\gamma)^h\cdot\sum_{k=0}^{h}4^k b(h,k)\sum_{j=0}^{\infty}(-1)^j/j! \times(2R^2/w^2)^{j+k}S(j+k)/\gamma^{j+k} \quad (44)$$

where the numerical coefficients $a_n(p,q)$ and $b(p,q)$ are given in Tables 1 and 2 for p and $q < 4$.

With these coefficients we have computed the series of formula (44) until the power of R^2/w^2 is equal to 4. The accuracy in $F_{Kn}(t)$ is then 1%, provided that $R/w < 1/3$ and $K < 3.2$.

From Eq. (44) one may find the fraction of bleached molecules and the fractional recovery of

Table 1
Coefficient $a_n(p,q)$ entering in the formula of $F_{kn}(t)$ in the case of an instaneous diffusion of the marker on the surface of the vacuoles during the photobleaching phase (formula 44 and B19)

$a_n(0,0)$	1
$a_n(1,0)$	$-2/3\ n$
$a_n(1,1)$	$2/3\ n$
$a_n(2,0)$	$4/15\ n + 4/9\ C_n^2$
$a_n(2,1)$	$-2\ a_n(2,0)$
$a_n(2,2)$	$2/15\ n + 4/9\ C_n^2$
$a_n(3,0)$	$-8/105\ n - 16/45\ C_n^2 - 8/27\ C_n^3$
$a_n(3,1)$	$-3\ a_n(3,0)$
$a_n(3,2)$	$-24/210\ n - 8/9\ C_n^2 - 8/9\ C_n^3$
$a_n(3,3)$	$4/315\ n + 8/45\ C_n^2 + 8/27\ C_n^3$
$a_n(4,0)$	$16/945\ n - 64/225\ C_n^2 + 16/45\ C_n^3 + 16/81\ C_n^4$
$a_n(4,1)$	$-64/945\ n + 172/225\ C_n^2 - 64/45\ C_n^3 - 64/81\ C_n^4$
$a_n(4,2)$	$16/315\ n - 16/30\ C_n^2 + 88/45\ C_n^3 + 32/27\ C_n^4$
$a_n(4,3)$	$-32/2835\ n + 16/450\ C_n^2 - 16/15\ C_n^3 - 64/81\ C_n^4$
$a_n(4,4)$	$4/945\ n - 4/225\ C_n^2 + 8/45\ C_n^3 + 16/81\ C_n^4$

Table 2
Coefficients $b(p,q)$ entering in the formula of $F_{kn}(t)$ in the case of an instaneous diffusion of the marker on the surface of the vacuoles during the photobleaching phase (formula 44 and B19)

$b(0,0)$	1
$b(1,0)$	1
$b(1,1)$	$-1/4$
$b(2,0)$	2
$b(2,1)$	-1
$b(2,2)$	$1/16$
$b(3,0)$	6
$b(3,1)$	-4.5
$b(3,2)$	$9/16$
$b(3,3)$	$-1/64$
$b(4,0)$	24
$b(4,1)$	-24
$b(4,2)$	4.5
$b(4,3)$	$-1/4$
$b(4,4)$	$1/256$

the fluorescence $X_K(t)$, by applying the definition given in Eqs. (36) and (39). We may then compute P and $X_K(t)$ by computer programs for different values of K and R/w.

Fig. 5 shows P as a function of K for different R/w values. Fig. 6 represents $X_K(t)$ for $K = 1.6$ at various values of R/w.

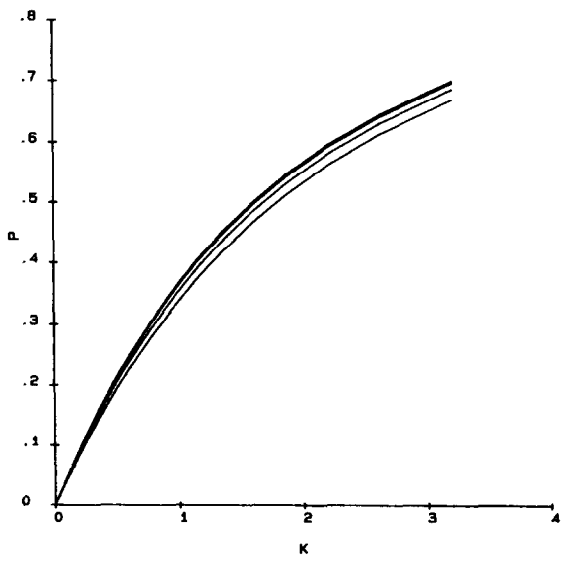

Fig. 5. Fraction of the bleached molecules as a function of K when the marker diffusion on a vacuole membrane is instantaneous during photobleaching. From the upper curve to the lower one the values of R/w are: 0, 0.1, 0.2, 0.3.

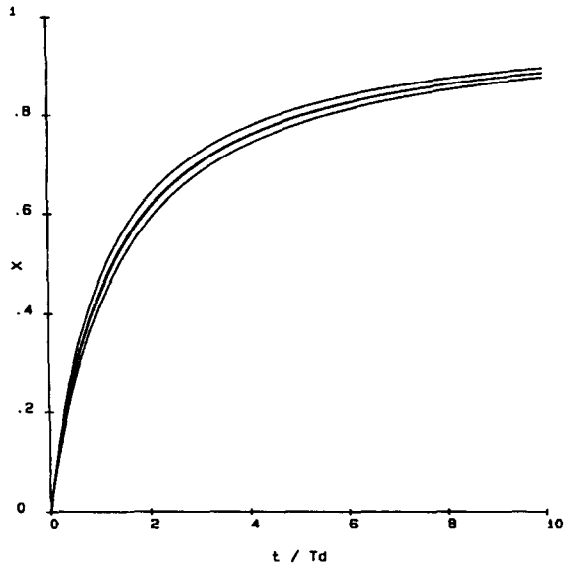

Fig. 6. Fractional fluorescence recovery as a function of t/τ_D when the marker diffusion on a vacuole membrane is instantaneous during photobleaching. From the upper curve to the lower one the values of K and R/w are respectively: 1.6, 0; 3.2, 0; 1.6, 0.3; 3.2, 0.3. The curves representing the values of K and R/w: 3.2, 0 and 1.6, 0.3 are nearly identical.

Finally the coefficient β of Yguerabide et al. [27] has been determined. As can be seen in Table 3, these different parameter values are very similar to those obtained when the probe diffusion is negligible during the bleaching phase. The comparison has been limited to $R/w < 0.3$ and $K < 3.2$.

Table 3
Parameters characterizing the FRAP of a vacuole suspension

	M		N		I	
K	P	β	P	β	P	β
0	0	1	0	1.124	0	1.125
0.225	0.105	1.02	0.093	1.143	0.094	1.145
0.475	0.204	1.04	0.183	1.165	0.184	1.168
0.765	0.301	1.07	0.270	1.19	0.275	1.194
1.14	0.403	1.104	0.364	1.222	0.372	1.228
1.6	0.501	1.15	0.454	1.261	0.467	1.271
2.2	0.596	1.2	0.543	1.311	0.562	1.323
3.2	0.700	1.285	0.644	1.389	0.670	1.406

P = fraction of bleached molecules.
$\beta = t_{1/2}/\tau_D$.
Vacuoles: the membrane diffusion during photobleaching is negligible (column: N) or instaneous (column: I). R/w is equal to 0.3.
Molecules: diffusing freely in a two dimensional sample (column: M, [27]).

3. Discussion

The present work allows to determine the translationai diffusion coefficient of a vacuole suspension by the FRAP method. We considered that the vacuoles were labelled on their surface by fluorophores. This situation may be found in the case of lipidic vacuoles labelled with fluorescent lipids dissolved in their peripheric membranes or when the membrane contains a membranous protein labelled with a fluorescent marker.

As we have already seen, the bleaching by the laser is inhomogeneous and introduces an heterogeneity in the distribution of the probe concentration in the membrane. We assumed that this inhomogeneity disappeared at the beginning of the recovery phase in a short time compared to the time characteristic of the fluorescence recovery due to the vacuole diffusion. This uniformization of the membrane concentration may be performed by the diffusion of the labelled molecules on the surface membrane. But one may also envisage to consider an average randomization due to the Brownian rotation of the vacuole itself. Let us quantitatively evaluate these different processes.

The coefficient of the translational diffusion of a spherical particle in a three dimensional fluid medium is given by the following well known Einstein's formula:

$$D = kT/6\pi\eta R \tag{45}$$

where k is the Boltzmann constant, T the absolute temperature, η the solvent viscosity and R the particle radius. For vesicles of diameter 1 μm suspended in an aqueous medium at 25°C, we found, by applying formula (45) $D = 4.4\ 10^{-9}$ cm^2 s^{-1}.

With the microscope of the FRAP apparatus equipped with an objective of magnification 40, one easily obtains a laser beam radius $w = 1.8$ μm. The ratio of the vesicle Radius R to w is 0.28. By Eq. (32), one may compute:

$$\tau_D = 1.8\ s. \tag{46}$$

The diffusion coefficients of fluorescent lipids and of labelled membranous proteins have been measured by FRAP on planar bilayers in the liquid crystalline phase. These diffusion coefficients are in the range 10^{-9} cm^2 s^{-1} $< D_m <$ 10^{-7} cm^2 s^{-1} [14].

On the other hand, the time which characterizes the homogenization by diffusion of molecules on a spherical surface, is given by the following Eq. [8]:

$$\tau_s = R^2/6D_m. \tag{47}$$

Applying relation (47) to the lipid vacuole of 1 μm diameter leads to the following range of values of τ_s: 4.2 ms $< \tau_s <$ 420 ms which are smaller than the τ_D computed above.

Finally, we computed the rotational Brownian relaxation time of a sphere using the following formula [25]:

$$\tau_R = \frac{4}{3}\frac{\pi R^3\eta}{kT}. \tag{48}$$

For a sphere of 1 μm diameter, suspended in an aqueous liquid at 25°C, the application of Eq. (48) gives $\tau_R = 120$ ms. Therefore $\tau_s << \tau_D$. For a decreasing value of R, τ_D decreases more slowly than τ_R and τ_s, as seen by formulas (32), (45), (47) and (48). On the other hand τ_D increases in the same way as w^2 while τ_R and τ_s remain independent of w.

In conclusion, the assumption that the distribution of a marker is homogeneous at the vacuole surface, in the beginning of the fluorescence recovery phase, appears valid in a number of practical cases.

Let us remark that one can label specific types of vacuole by fluorescent markers in living cells [20,22,23]. These vacuoles are generally moving by saltatory motions along microtubules. In some cases, these motions may be similar to a diffusion in one dimension [23,24].

We expect that a number of aspects of the present theoretical work will help the interpretation of FRAP applied to the vacuole motion in living cells.

Appendix A. Calculation of $F(t)$ when the diffusion of the probe is negligible during photobleaching

We write formula (26) in a way which indicates the order of integration as follows:

$$F_k(t) = q\Delta lC(-)/(A64\pi^4R^4)\int\int \mathrm{d}^3\underset{\sim}{u}\,\mathrm{d}^3\underset{\sim}{u}'\sigma(\underset{\sim}{u},R)$$

$$\times\sigma(\underset{\sim}{u}',R)\int d^2\underset{\sim}{\mu}\exp\left[-i\underset{\sim}{\mu}\cdot\underset{\sim}{u}'-\underset{\sim}{\mu}^2Dt\right]$$
$$\cdot\int d^2\underset{\sim}{r}'I(\underset{\sim}{r}')\exp\left[-i\underset{\sim}{\mu}\cdot\underset{\sim}{r}'\right]\int d^2\underset{\sim}{r}_0$$
$$\times\exp\left[-KI(\underset{\sim}{r}_0+\underset{\sim}{u})/I_0\right]\exp\left[i\underset{\sim}{\mu}\cdot\underset{\sim}{r}_0\right]. \quad (A1)$$

We expand the exponential function in series. $F_K(t)$ takes the form of (27) and:

$$F_{Kn}(t) = (-K)^n/(n!)\cdot q\Delta lC(-)/(A64\pi^4R^4)\cdot J_{\underset{\sim}{u},\underset{\sim}{u}'} \quad (A2)$$

$$J_{\underset{\sim}{u},\underset{\sim}{u}'} = \int\int d^3\underset{\sim}{u}d^3\underset{\sim}{u}'\sigma(\underset{\sim}{u},R)\,\sigma(\underset{\sim}{u}',R)\,J_{\underset{\sim}{\mu}}(\underset{\sim}{u},\underset{\sim}{u}') \quad (A3)$$

$$J_{\underset{\sim}{\mu}}(\underset{\sim}{u},\underset{\sim}{u}') = \int d^2\underset{\sim}{\mu}\exp\left[-i\underset{\sim}{\mu}\cdot\underset{\sim}{u}'-\underset{\sim}{\mu}^2Dt\right]J_{\underset{\sim}{r}'}(\underset{\sim}{\mu})\,J_{\underset{\sim}{r}0}(\underset{\sim}{\mu},\underset{\sim}{u}) \quad (A4)$$

$$J_{\underset{\sim}{r}'}(\underset{\sim}{\mu}) = \int d^2\underset{\sim}{r}'I(\underset{\sim}{r}')\exp\left[-i\underset{\sim}{\mu}\cdot\underset{\sim}{r}'\right] \quad (A5)$$

$$J_{\underset{\sim}{r}0}(\underset{\sim}{\mu},\underset{\sim}{u}) = \int d^2\underset{\sim}{r}_0\left[I(\underset{\sim}{r}_0+\underset{\sim}{u})/I_o\right]^n\exp\left[i\underset{\sim}{\mu}\cdot\underset{\sim}{r}_0\right]. \quad (A6)$$

In Eqs. (A5) and (A6), we explicitly introduce the intensity given by expression (18). Then by integration of (A5) and of (A6) one finds:

$$J_{\underset{\sim}{r}'} = \left(I_0\pi w^2/2\right)\exp\left[-\underset{\sim}{\mu}^2w^2/8\right] \quad (A7)$$

where $\underset{\sim}{\mu}^2 = \mu_x^2+\mu_y^2$ and where μ_x and μ_y are the components of $\underset{\sim}{\mu}$.

$$J_{\underset{\sim}{r}0}(\underset{\sim}{\mu},\underset{\sim}{u}) = \left(\pi w^2/2n\right)\exp\left[-\underset{\sim}{\mu}^2w^2/(8n) - i\mu_xX-\mu_yY\right] \quad (A8)$$

where X and Y are the components of $\underset{\sim}{u}$ on the axes ox and oy.

The second member of the Eqs. (A7) and (A8) are then brought into (A4) and the integration with respect to μ_x and μ_y is performed. One finds:

$$J_{\underset{\sim}{\mu}}(\underset{\sim}{u},\underset{\sim}{u}') = \left(2I_0\pi^3w^2/\gamma\right)\exp\left[-2n/\left(\gamma w^2\right)\times\left((X+X')^2+(Y+Y')^2\right)\right] \quad (A9)$$

where X' and Y' are the components of $\underset{\sim}{u}'$ on the axes ox and oy respectively and γ is given by (31).

We express then X, Y, X', Y' in polar coordinates as follows:

$$X = |\underset{\sim}{u}|\sin\theta\cos\varphi$$
$$Y = |\underset{\sim}{u}|\sin\theta\sin\varphi$$
$$X' = |\underset{\sim}{u}'|\sin\theta'\cos\varphi'$$
$$Y' = |\underset{\sim}{u}'|\sin\theta\sin\varphi'. \quad (A10)$$

In order to calculate the integral of (A3), we apply the Eq. (8) and introduce the final expression found after integration, into (A2). We may then write the Eq. (29).

The function L_p of formula (33) is defined as:

$$L_p = (-1)^p/(p!8\pi)\times\int_0^{2\pi}\int_0^{\pi}\int_0^{\pi}g(\theta,\theta',\chi)\sin\theta\sin\theta'd\chi. \quad (A11)$$

When calculating the integrals of expression (A11) one takes into account the following results [28]:

$$\int_0^{2\pi}\cos^{2q+1}\chi d\chi = 0$$
$$\int_0^{2\pi}\cos^{2q}\chi d\chi = 2\pi C_{2q}^q/2^q \quad (A12)$$
$$S(p) = (1/2)\int_0^{\pi}\sin^{2p+1}\theta d\theta = (2p(2p-2)\ldots4.2)/((2p+1)(2p-1)\ldots5.3) \quad (A13)$$
$$\int_0^{\pi}\sin^{2p}\theta d\theta = 0.$$

(A11) leads to the formulas (34).

Appendix B. The calculation of $F_{Kn}(t)$ when the homogenization of the membranous concentration of the probe during the photobleaching occurs instantaneously

Eq. (43) may be written as follows:

$$F_{Kn}(t) = (-K)^n/n!)q\Delta 1C(-)/(A16\pi^3R^2)J_{\underset{\sim}{u}'} \quad (B1)$$

where

$$J_{\underset{\sim}{u}'} = \int \sigma(\underset{\sim}{u}', R) \cdot J_{\underset{\sim}{\mu}}(\underset{\sim}{u}')\mathrm{d}^3\underset{\sim}{u}' \tag{B2}$$

$$J_{\underset{\sim}{\mu}}(\underset{\sim}{u}') = \int \mathrm{d}^2\underset{\sim}{\mu} \exp\left[-\underset{\sim}{\mu}^2 Dt - i\underset{\sim}{\mu}\cdot\underset{\sim}{u}'\right] J_{\underset{\sim}{r}'}(\underset{\sim}{\mu}) J_{\underset{\sim}{r}0}(\underset{\sim}{\mu}) \tag{B3}$$

$$J_{\underset{\sim}{r}'}(\underset{\sim}{\mu}) = \int \mathrm{d}^2\underset{\sim}{r}' I(\underset{\sim}{r}') \exp\left[-i\underset{\sim}{\mu}\cdot\underset{\sim}{r}'\right] \tag{B4}$$

$$J_{\underset{\sim}{r}0}(\underset{\sim}{\mu}) = \int \mathrm{d}^2\underset{\sim}{r}_0 \left(\bar{I}(\underset{\sim}{r}_0)/I_0\right)^n \exp\left[i\underset{\sim}{\mu}\underset{\sim}{r}_0\right]. \tag{B5}$$

In order to calculate $\bar{I}(\underset{\sim}{r}_0)$, we introduce expression (18) and the polar coordinates of $\underset{\sim}{u}$ (Eq. (A10) into formula (13). Applying relation (8), we find:

$$\bar{I}(\underset{\sim}{r}_0)/I_0 = (4\pi)^{-1} \exp\left[-2\underset{\sim}{r}_0^2/w^2\right] \int_0^{2\pi}\int_0^{\pi} \times \exp\left[-2(a^2+b)/w^2\right] \cdot \sin\theta \mathrm{d}\theta\mathrm{d}\varphi \tag{B6}$$

where

$$a^2 = R^2\sin^2_\theta$$

$$b = 2R\sin\theta(x_0\cos\varphi + y_0\sin\varphi) \tag{B7}$$

x_0 and y_0 are the components of $\underset{\sim}{r}_0$.One may write:

$$\exp\left[-2(a^2+b)/w^2\right] = \sum_{s=0}^{\infty} \left(-2/w^2\right)^s (a^2+b)^s/s!.$$

The binome to the power of s may be expended and we find for the integral of (B6), the following expression:

$$J_{\theta,\varphi}(\underset{\sim}{r}_0) = \sum_{s=0}^{\infty} \left(-2/w^2\right)^s/s! \sum_{p=0}^{s} C_s{}^p \times \int_0^{2\pi}\int_0^{\pi} a^{2p} b^{s-p} \sin\theta \mathrm{d}\theta\mathrm{d}\varphi. \tag{B8}$$

Let us define:

$$J_\varphi(p,q) = \int_0^{2\pi} \sin^{2p}\varphi\cos^{2q}\varphi \mathrm{d}\varphi.$$

If one of the number p or q is odd and the other even, we have [28]:

$$J_\varphi(p,q) = 0.$$

If either both p and q are odd or even, one has [28]:

$$J_\varphi(p,q) = 2\pi(2p-1)(2p-3)\ldots5.3(2q-1)(2q-3) \times \ldots5.3/(2(p+q)(2(p+q)-2)\ldots4.2. \tag{B9}$$

In order to perform the integrals of (B8) we take the Eqs. (A12), (A13) and (B9) into account. By ordering the series in the order of the increasing powers of R^2, (B6) becomes:

$$\bar{I}(\underset{\sim}{r}_0)/I_0 = (4\pi)^{-1} \exp\left(-2\underset{\sim}{r}_0^2/w^2\right) \sum_{h=0}^{\infty} \left(2R^2/w^2\right)^h S(h) \times \sum_{k=0}^{h} \left(-2\underset{\sim}{r}_0^2/w^2\right)^k T(k,h) \tag{B10}$$

where:

$$T(k,h) = (h!)^{-2}(h-k)!)^{-1}.$$

We then take the power n of $\bar{I}(\underset{\sim}{r}_0)/I_0$ and we find from (B10):

$$\left(I(\underset{\sim}{r}_0)/I_0\right)^n = \exp\left[-2n\underset{\sim}{r}_0^2/w^2\right] \sum_{p=0}^{\infty} \left(2R^2/w^2\right)^p \times \sum_{q=0}^{p} a_n(p,q)\left(2\underset{\sim}{r}_0^2/w^2\right)^q. \tag{B11}$$

The values of the numerical coefficients $a_n(p,q)$ are given in Table 1.

We take (B11) into account, and we write (B5) as follows:

$$J_{\underset{\sim}{r}_0}(\underset{\sim}{\mu}) = \sum_{p=0}^{\infty} \left(2R^2/w^2\right)^p \sum_{q=0}^{p} a_n(p,q) H_q(\underset{\sim}{\mu}) \tag{B12}$$

where

$$H_q(\underset{\sim}{\mu}) = \left(2/w^2\right)^q \int \mathrm{d}^2\underset{\sim}{r}_0 \exp\left[-2n\underset{\sim}{r}_0^2/w^2 + i\underset{\sim}{\mu}\cdot\underset{\sim}{r}_0\right]\underset{\sim}{r}_0^{2q}. \tag{B13}$$

The integration of (B13) is obtained by adopting the following integration vector:

$$\underset{\sim}{V} = \underset{\sim}{r}_0 - i\underset{\sim}{\mu}w^2/4n \tag{B14}$$

and taking into account the following relations [28]:

$$\int du \exp\left[-2nu^2/w^2\right]u^{2p}$$
$$= \left(w^2/n4^p\right)\left(\pi w^2/2n\right)^{1/2}\cdot(2p-1)(2p-3)$$
$$\ldots\ldots 5.3\int d^2\underset{\sim}{V}\exp\left[-2n\underset{\sim}{V}^2/w^2\right]\underset{\sim}{V}^{2P}$$
$$= \pi p!\left(w^2/2n\right)^{p+1}.$$

After integration, we may write (B13) as follows:

$$H_q(\underset{\sim}{\mu}) = \pi w^2/(2n^{q+1})\exp\left[-\underset{\sim}{\mu}^2w^2/8n\right] \times \sum_{h=0}^{q} b(q,h)\left(w^2\underset{\sim}{\mu}^2/2n\right)^h \tag{B15}$$

where the $b(q,h)$ are numerical coefficients which are given in Table 2. Bringing (B15) into (B12) gives the following equation:

$$J_{\underset{\sim}{r}_0}(\underset{\sim}{\mu}) = \pi w^2/(2n)\exp\left[-\underset{\sim}{\mu}^2w^2/(8n)\right] \times \sum_{q=0}^{\infty}\left(2R^2/w^2\right)^p\sum_{q=0}^{p}a_n(p,q)/n^q \times \sum_{h=0}^{q} b(q,h)\left(w^2\underset{\sim}{\mu}^2/2n\right)^h. \tag{B16}$$

We notice that (B4) is identical to (A5). Its value is given by (A7). Bringing (B16) and (A7) into (B3) gives an expression of $J_{\underset{\sim}{\mu}}(\underset{\sim}{u}')$ formally identical to the expression of $J_{\underset{\sim}{r}_0}(\underset{\sim}{\mu})$ but where $\underset{\sim}{\mu}^{2h}$ is replaced by:

$$K_h(\underset{\sim}{u}') = \int d^2\underset{\sim}{\mu}'\exp\left[-w^2\gamma\underset{\sim}{\mu}^2/(8n) - i\underset{\sim}{\mu}\cdot\underset{\sim}{u}'\right]\underset{\sim}{\mu}^{2h} \tag{B17}$$

and where γ is given by (31).

The integral of (B17) has a form which is similar to that of (B13). After integration one obtains:

$$K_h(u') = \pi\left(8n/w^2\gamma\right)^{h+1}\exp\left[-\left(2n/w^2\gamma\right)\left(X'^2+Y'^2\right)\right] \times \sum_{k=0}^{h} b(h,k)\left(8n/\left(w^2\gamma\right)\left(X'^2+Y'^2\right)\right) \tag{B18}$$

where X' and Y' are the projections of $\underset{\sim}{u}'$ on the axes ox and oy.

Expressing these projections in polar coordinates (Eq. (A10)), applying formula (8) and taking into account (B18), we obtain for (B2):

$$J_{\underset{\sim}{u}'} = 8\pi R^2/(w^2\gamma)(\pi w^2/2)^n \times \sum_{p=0}^{\infty}\left(2R^2/w^2\right)^p\sum_{q=0}^{p}a_n(p,q)/n^q \times \sum_{h=0}^{q} b(q,h)(4/\gamma)^h\sum_{k=0}^{h}b(h,k) \times \int_0^{2\pi}\int_0^{\pi}\exp\left[-(2nR^2/w^2)\sin^2\theta'\right] \times (8nR^2\sin^2\theta'/w^2\gamma)^k\sin\theta'\,d\theta'\,d\varphi. \tag{B19}$$

We expand the exponential function of (B19) and apply (A13). Bringing the result in (B1), one finds Eq. (44).

We give the coefficients $a_n(p,q)$ and $b(p,q)$ for p and $q \leqslant 4$ in Tables 1 and 2.

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