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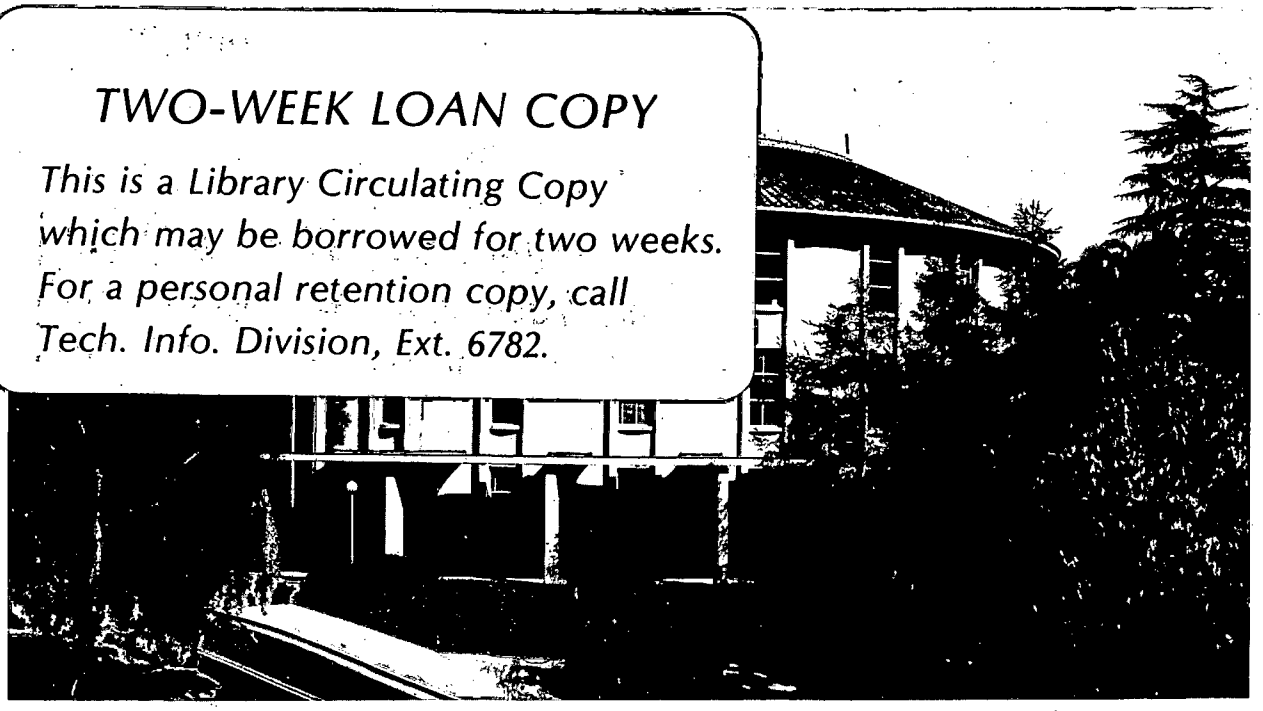

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DISCLAIMER

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ABSTRACT

Central to the application of fluorescence correlation spectroscopy, to measure the self-diffusion coefficients and average concentration of fluorescent molecules in a volume determined by a focused laser beam, is the determination of the focal spot size. As the focal spot size in the sample plane is varied by displacing either the focusing lens or sample position along the beam axis, the diffusion time and average number of molecules vary in a parabolic manner. Analysis of the parameters of the parabola leads to estimates of the beam radius at the waist. The results agree with theoretical predictions and provide an independent measurement of the beam profile.

INTRODUCTION

Fluorescence fluctuation or correlation spectroscopy provides an attractive method for determining self-diffusion coefficients as well as other kinetic parameters of molecules in solution at equilibrium. The attraction stems from the fact that the thermodynamically driven fluctuations about equilibrium provide the driving force thus obviating the requirement for application of extrinsic perturbations as are exemplified by temperature jump experiments.

In this method, introduced by Magde, Elson, and Webb[1,2,3], a focussed laser beam defines a volume, otherwise open, through which fluorescent molecules freely diffuse. Analysis of autocorrelation functions of the fluctuations of fluorescence light intensity leads to the kinetic parameters provided the focal spot size is known.

In this paper we present a simple method for the determination of the focal spot size which circumvents the difficulties encountered by previous practitioners of this method.

Generally, when it is desired to determine the size of a focal spot it is compared with an object whose dimensions have been determined previously by other methods. The difficulty increases as the beam size becomes smaller. Weissman, Schindler and Feher described a fluctuation correlation experiment in which a large sample volume is calibrated with polystyrene spheres[4]. Other approaches involve knife edges and thin fibers being translated across

the focal spot[5,6,7]. By measuring the amount of light scattered by the fiber or knife edge, the profile of the beam intensity can be inferred. This is also a calibration step. The sample is subsequently placed in the beam and the sample and beam are brought into focus simultaneoulsy, so that the sample is illuminated at the beam waist. Thus, the beam dimensions are measured in one experiment and the fluctuation measurements are made in a separate experiment or experiments.

I. PRINCIPLE OF MEASUREMENT

It is possible to perform both measurements simultaneously. Near the focal plane the beam shape varies in a known way. The fluctuation parameters will vary correspondingly so that the size of the beam waist and the other experimental parameters can be estimated from the same data.

In fluorescence fluctuation experiments one measures the autocorrelation function $G(\tau)$, of the photomultiplier current, i. For the case of translational diffusion in an open volume,[2]

$$G(\tau) = G(0)/(1 + \tau/\tau_D) \tag{1}$$

which is completely specified by the two parameters, $G(0)$ and τ_D,

where

$$\tau_D = \frac{w^2}{4D} = \frac{w_o^2}{4D}\left(1 + \frac{\lambda^2 \Delta z^2}{\pi^2 w_o^4}\right) \tag{2}$$

$$G(0) = \frac{\langle i \rangle^2}{\pi w^2 LC} = \frac{\langle i \rangle^2}{\pi LC\, w_o^2\left(1+\frac{\lambda^2 \Delta z^2}{\pi^2 w_o^4}\right)}, \tag{3}$$

and w_o is the radius of the beam in the focal plane, D is the diffusion coefficient, C is the average concentration of fluorescent diffusing molecules in the illuminated volume, L is the depth of the illuminated volume, $\langle i \rangle$ is the average photocurrent, and Δz is the distance between the sample position and the position of beam minimum $z = z_o$.

Thus, both τ_D and $\langle i \rangle^2 / G(0)$ depend on Δz in essentially the same way. If measurements of the parameters are expressed in the form

$$\tau_D = a\,\Delta z^2 + b \quad \text{and} \quad \frac{\langle i \rangle^2}{G(0)} = c\,\Delta z^2 + d,$$

then $$w_o = \left(\frac{\lambda^2}{\pi^2}\frac{b}{a}\right)^{1/4} = \left(\frac{\lambda^2}{\pi^2}\frac{d}{c}\right)^{1/4}, \quad D = \frac{\lambda}{4\pi\sqrt{ab}} \quad \text{and} \quad CL = \frac{\sqrt{cd}}{\lambda}.$$

In this way measurements of the autocorrelation function at different values of Δz lead to essentially independent estimates of w_o while simultaneously giving values for the diffusion coefficient, D, and the two-dimensional concentration, CL.

Before demonstrating the technique we will first characterize the beam profile in front of the focusing lens. We can then predict the beam shape near the focal plane, after which the beam shape near the focal plane will be measured directly. Finally, we will infer the beam shape from parameters measured by fluorescence fluctuation spectroscopy and compare the values obtained for the beam waist radius, w_o.

II. RESULTS

A. Beam profile

The illuminating beam is that of an Argon ion laser operating at 488nm. After spatial filtering, to reject higher order modes, and recollimation, the beam is primarily gaussian in profile. To measure the intensity profile of the recollimated beam in the plane normal to the beam axis, a pinhole was translated across the beam. A photosensitive field-effect-transistor measured the light transmitted by the pinhole. The pinhole and detector were driven by a micrometer. Fig. 1 shows the beam intensity profile measured in this way. The points are measured values while the solid curve is the fit of a gaussian to the observed values. If $I(r) = I_0 \exp(-2r^2/d^2)$, then $d = 3.08$ mm.

B. Size of the focussed beam; prediction and direct measurement

Scalar diffraction theory predicts[8] that the laser beam intensity in a plane perpendicular to the propagation direction remains gaussian in profile near the focal plane,

$$I(r) = I_0 \exp(-2r^2/w^2) \tag{4}$$

$$w^2 = w_0^2 \left(1 + \frac{\lambda^2 \Delta z^2}{\pi^2 w_0^4}\right) \tag{5}$$

where w_0 is the beam radius as measured in the focal plane, λ is the wavelength of the laser light, and Δz is the displacement along the beam axis from the focal minimum, $z = z_0$.

The recollimated beam is focussed by a lens of focal length, $f = 25.4$ cm. The beam radius in the focal plane will be[8]

$$w_o = \frac{\lambda f}{\pi d} \tag{6}$$

where λ is the wavelength of the light, f is the focal length of the lens, and d is the e^{-2} radius of the collimated beam at the front surface of the lens. Thus we expect w_o = 12.8 μ. The uncertainty in this value is about 0.5 μ.

When the beam is focussed, the pinhole used to characterize it will no longer be small compared to the dimension of the beam. Translating the pinhole across the focussed beam would give a distorted measure of the intensity profile. Rather than translating the pinhole <u>across</u> the beam, the shape of the beam near the focal plane is confirmed by translating the pinhole <u>along</u> the beam axis. The pinhole is centered on the beam axis and the transmitted light intensity is measured as a function of displacement of the pinhole along the beam axis through the focal region. The arrangement is shown in Fig. 2.

If we let T be the fraction of light transmitted, then

$$T = \frac{\int_0^{r_o} \exp(-2r^2/w^2)\, r\, dr}{\int_0^{\infty} \exp(-2r^2/w^2)\, r\, dr} = 1-\exp(-2r_o^2/w^2) \tag{7}$$

where r_o is the radius of the pinhole.

Next, we define $W^2(T) = -2/\ln(1-T)$. From the expression for T, we see that W(T) is the beam radius expressed in units of r_o, the pinhole radius. That is, $W^2(T) = \frac{w_o^2}{r_o^2}\left(1 + \frac{\lambda^2 \Delta z^2}{\pi^2 w_o^4}\right)$

Microphotographs of the pinhole indicate that it is roughly circular with radius, $r_o = (23 \pm 1)\mu$. Airy diffraction rings of light transmitted by the pinhole give a value for r_o of $(23.5 \pm 0.5)\mu$. Fig.3 shows how $W^2(T)$ varies with the position of the pinhole along the beam axis. The solid line is a computer fit of the data assuming the parabolic dependence of $W^2(T)$ on Δz. From these data we find $w_o = 14\mu$ and $r_o = 22.6\mu$, which compare favorably with the expected values of 12.8μ for w_o and 23.4μ for r_o.

C. Beam shape inferred from fluctuation experiments

The apparatus used for fluorescence fluctuation measurements is shown in Fig. 4. It is essentially the same as the one described in reference 3, except that our system contains an external servo for additional stabilization of the beam intensity. The beam power is monitored by a photosensitive field-effect-transistor. The sample is a dilute solution of Rhodamine 6G. It is situated at the focus of a paraboloidal mirror which directs fluorescent light to a photomultiplier. The photocurrent is converted to a voltage and amplified, so that the photomultiplier and the beam monitor show equal average voltages. A difference amplifier subtracts the beam monitor signal from the photomultiplier signal in an effort to minimize the effect of fluctuations in beam intensity. The difference signal is correlated by a Saicor model SAI-43A Autocorrelator and Probability Analyzer. The output of the autocorrelator is punched on paper tape. A computer estimates the best least squares fit of the data to a

function of the form $G(\tau) = G(0)\ (1+\tau/\tau_D)^{-1} + B$ where B is a constant. Correlations imposed on the signal by electronic filters are not considered in the computations.

Measurements are made for various displacements of the sample along the beam axis. From each individual autocorrelation function, estimates of τ_D and $\langle i\rangle^2/G(0)$ are made. These values of τ_D and $\langle i\rangle^2/G(0)$ are then plotted against position, Δz, and are shown in Figs. 5 and 6, respectively.

Error bars shown in Figs. 5 and 6 are computed from the differences between observed values of $G(\tau)$ and fitted values. Consequently, they reflect only the precision of the fit. The observed values of $G(\tau)$ are, themselves, subject to uncertainties which would limit the accuracy of values derived from the computer estimates. Thus the error bars in the figures take into account only a fraction of the relevant uncertainties. The error bars were used to weight the data in the least squares estimates of parameters used to compute w_o, D and CL. The data were not weighted in computations of the variances of w_o, D and CL.

Assuming the stated dependence of τ_D and $\langle i\rangle^2/G(0)$ on Δz, these data lead to estimates for the diffusion coefficient, D, the two-dimensional concentration, CL, and to two estimates of w_o. The results are summarized in Table I. L, the depth of the sample cell, was 100 μ.

III. DISCUSSION

The uncertainties stated for w_o, r_o, D, and C are estimated from the differences between the observed and

fitted values. Consequently, any systematic errors are excluded from consideration in arriving at the stated uncertainties. For example, measurements using the pinhole near the focal plane are subject to uncertainty in the determination of the total light intensity in the beam. If the total light intensity were inaccurately measured, the fraction of light transmitted, T, would contain a small scale factor error.

Near the focal minimum the fraction of the light transmitted was essentially 1.0. Since the quantity plotted in Fig. 3 includes the factor $\ln(1-T)$, it would be sensitive to systematic errors in T. Thus, the stated uncertainty for the value of w_o measured in this way is probably an underestimate.

Nonetheless, the results presented are about as expected. The values given for the beam radius in the focal plane are in close agreement. It is significant that scale factor errors in τ_D and $\langle i \rangle^2/G(0)$ have no effect on the estimate of w_o. This is in contrast to the sensitivity to scale factor errors of the estimate of w_o obtained by transmission through the pinhole.

The value for the diffusion coefficient of Rhodamine 6G is consistent with the size of the molecule[9], although reports exist of somewhat lower values[3]. The value obtained for the radius of the pinhole agrees satisfactorily with dimensions obtained from the microphotograph and Airy rings. The measured two-dimensional concentration of the fluorescent

molecules is also about as expected. The dye solution was prepared to be 5×10^{-9} M by serial dilution from stock solutions. During the course of the experiments some of the molecules are destroyed by the exciting light and thus do not appear in $\langle i \rangle^2/G(0)$, leading to an underestimate of the two-dimensional concentration. We consider an underestimate of CL by a factor of five to be reasonable.

IV. CONCLUSIONS

The results presented here demonstrate that the focused beam behaves as predicted by scalar diffraction theory and that the beam shape can be characterized by measurements made by fluorescence correlation spectroscopy. It was mentioned above that other methods exist for determining beam size down to microscopic dimensions. In fact, one such measurement was presented here. The avantages of the correlation method are three-fold by comparison with direct physical measurements.

This method helps confirm that the autocorrelation functions arise from translational diffusion. In the case of rotational diffusion, for instance, the correlation time would not depend on beam size, while $\langle i \rangle^2/G(0)$ would[10,11].

This method also arranges for τ_D and $\langle i \rangle^2/G(0)$ to be estimated at the beam focal minimum even though no measurements need be performed at that precise location. A third advantage is that the beam waist is measured with the sample *in situ*. The last two points are especially important in practice. Typically, the fluorescent light is collected by an optical system different from the system that focuses the

exciting beam onto the sample. Both systems must be properly focused relative to each other and to the sample if reliance is to be placed on direct physical measurements of the beam shape.

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Table 1. Summary of Results

	w_o (μ)	r_o (μ)	D (cm^2/sec.)	C (M)
Expected value	12.8 ± 0.5	23.4 ± 0.5	2-5 x 10^{-6}	5 x 10^{-9}
Observed values				
Transmission through pinhole	14 ± 0.4	22.6 ± 0.5	--	--
τ_D versus Δz	13 ± 1	--	(5.5 ± 0.8) x 10^{-6}	--
$\langle i \rangle^2/G(0)$ versus Δz	12 ± 1	--		(1.1 ± 0.1) x 10^{-9}

Values are shown for beam waist radius, w_o; the radius of the pinhole, r_o; the diffusion constant for Rhodamine 6G in water, D; and the concentration of fluorescent molecules, C. Each type of microscopic measurement gives an estimate of w_o and one other physically significant parameter.

FIGURE CAPTIONS

Fig 1. The shape of the beam near the focal plane is determined by the beam profile at the front surface of the focusing lens. A pinhole is translated across the beam to map the beam profile before the lens. The pinhole has a diameter of 46μ. Data points indicate relative intensity transmitted by the pinhole. The solid line is the fit of a gaussian to the observed data. The beam profile is gaussian with e^{-2} radius of 3.08 mm.

Fig. 2. The shape of the beam near the focal plane is mapped by translating a pinhole along the axis of the beam. Transmitted intensity can be used to estimate the pinhole size and the beam waist size. Beam waist radius is w_0, displacement of the pinhole from the focal plane is Δz, and pinhole radius is r_0.

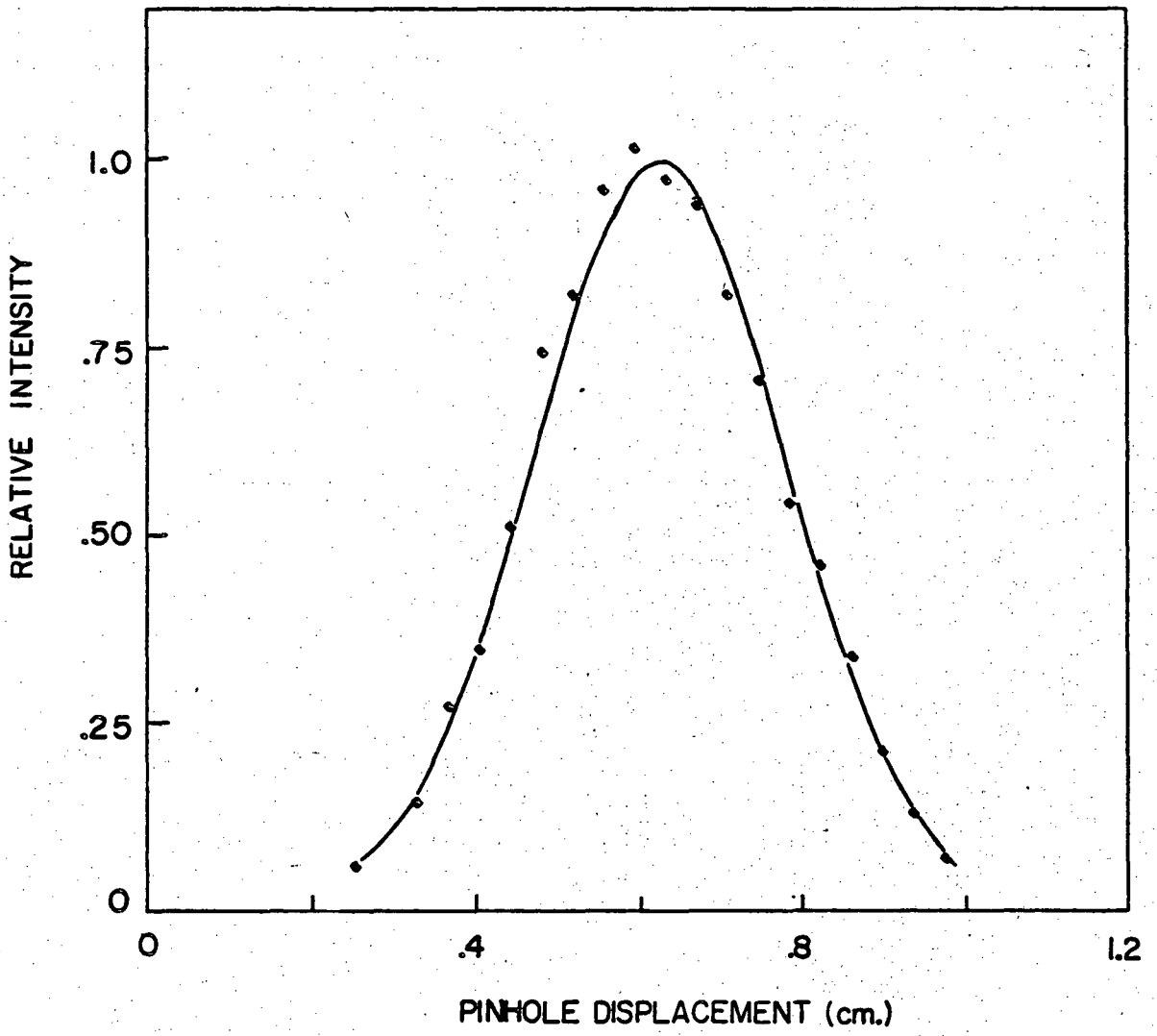
Fig. 3. W^2, the normalized beam radius is plotted against the displacement of the pinhole from the focal plane. From these data, the beam waist radius and the pinhole size are esitmated simultaneously. Values for w_0 and r_0 are $(14 \pm 1)\mu$ and $(22 \pm .5)\mu$, respectively.

Fig. 4. Apparatus for Fluorescence Correlation Spectroscopy. The laser is an Argon ion laser operating at 488 nm. The servo helps to stablize light intensity from the laser. A microscope objective lens, L1, focusses the beam into a spatial filter, SF, which removes unwanted spatial intensity variations from the beam. The beam is recollimated by lens L2, and focussed on the sample, S, by a movable lens, L3. The sample lies at the focus of paraboloidal mirror, PM. Light from the sample is directed to an EMI model QB9558 photomultiplier tube, PMT. Glass filters, GF, discriminate against scattered exciting light. The photomultiplier signal is amplified to be equal in average value to the beam monitor signal. The difference of the two signals is autocorrelated by a Saicor, model SAI-43A Autocorrelator and Probability Analyzer. Results are punched on paper tape and analyzed by computer.

Fig. 5. Diffusion time, τ_D, as a function of displacement of the sample from the focal plane. The solid line is a weighted least squares fit of the values of τ_D to a parabola. Diffusion times are estimated from autocorrelation functions of the fluorescence intensity fluctuations. From these data, beam

waist radius, w_o and diffusion constant, D, are estimated simultaneously. Values for w_o and D are $(13\pm1)\mu$ and $(5.5\pm0.8)\times10^{-6}$ cm^2/sec, respectively. Error bars represent only the precision of the computer fit to the observed autocorrelation function.

Fig. 6. $\langle i\rangle^2/G(0)$ as a function of displacement of the sample from the focal plane. The solid line is a weighted least squares fit of values of $\langle i\rangle^2/G(0)$ to a parabola. Values for $\langle i\rangle^2/G(0)$ are taken from autocorrelation functions of fluorescence intensity fluctuations. From these data, the beam waist radius w_o, and the 2-dimensional concentration, CL, are estimated simultaneously. Values for w_o and CL are $(12\pm1)\mu$ and $(1.1\pm0.1)\times10^{-7}$ M respectively. The cell depth, L, is 100μ . Error bars represent only the precision of the computer fit to the observed autocorrelation function.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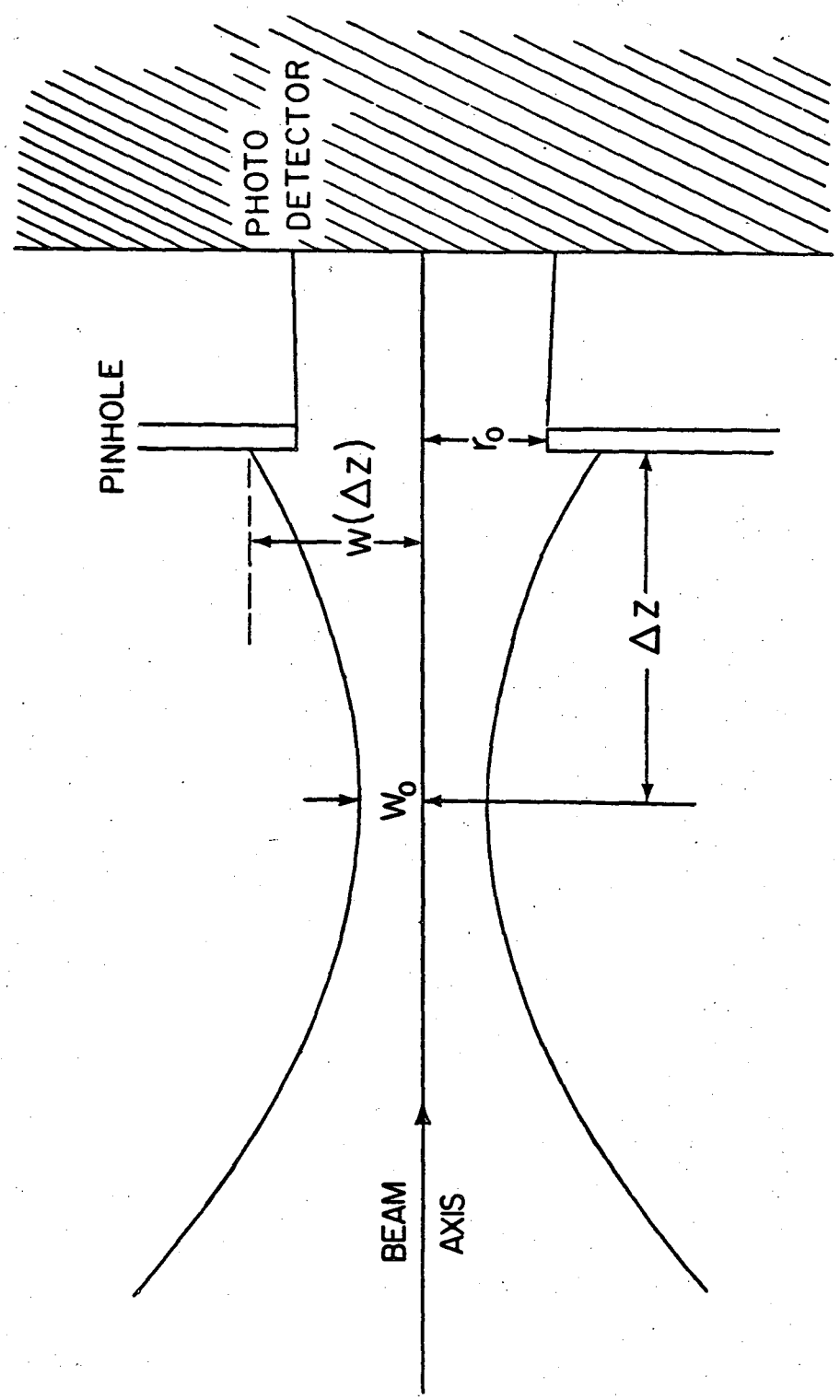

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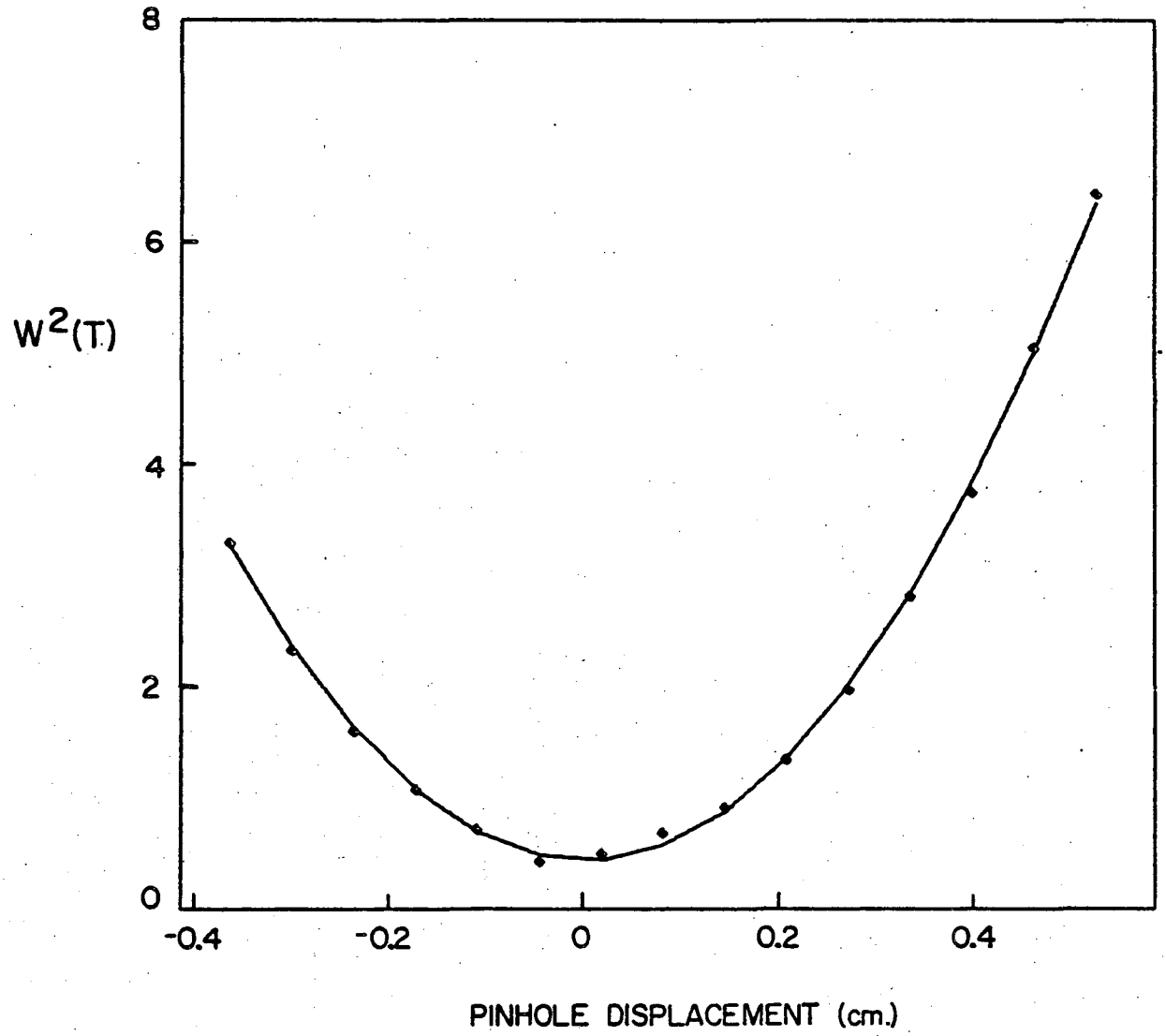

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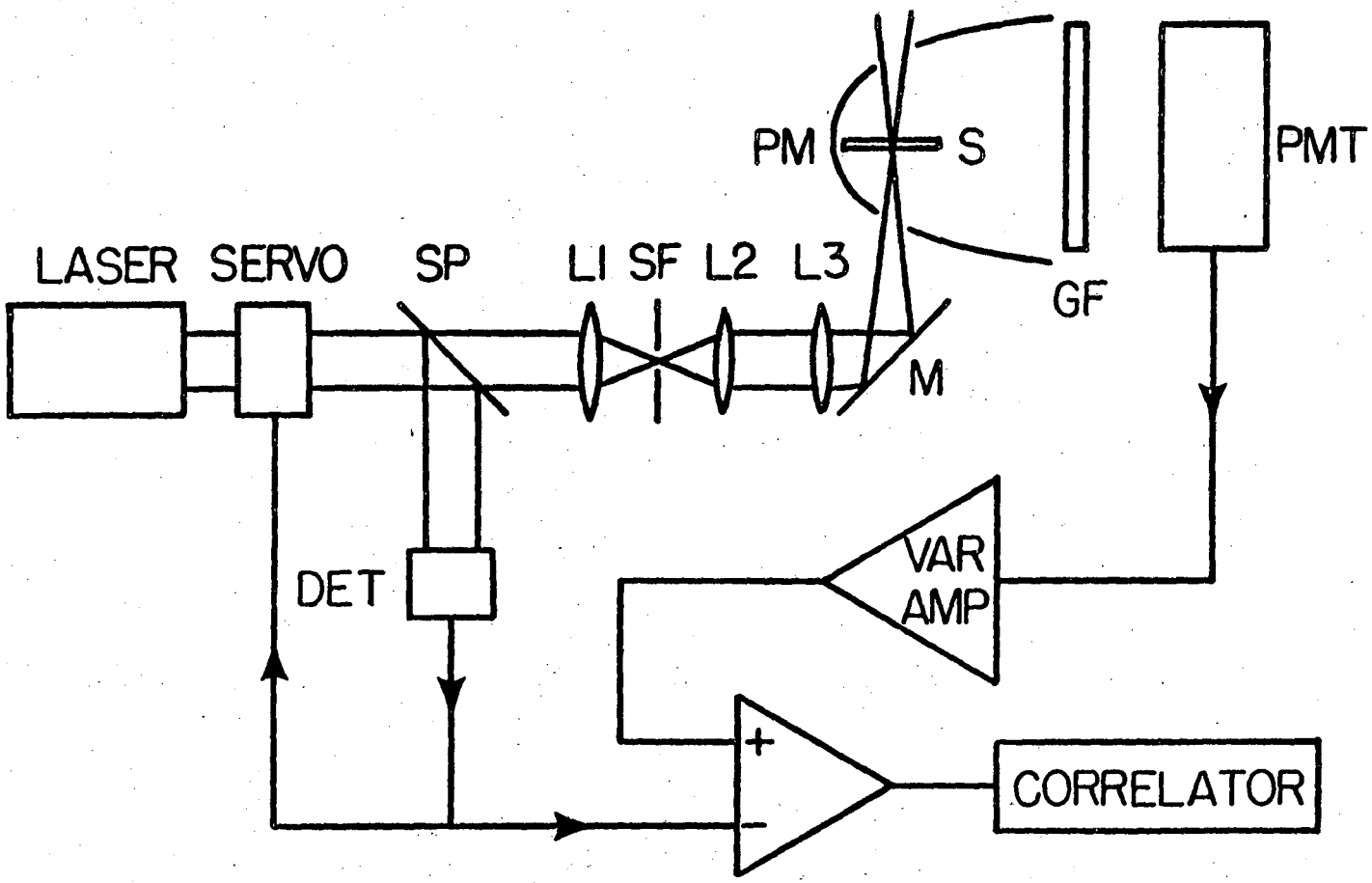

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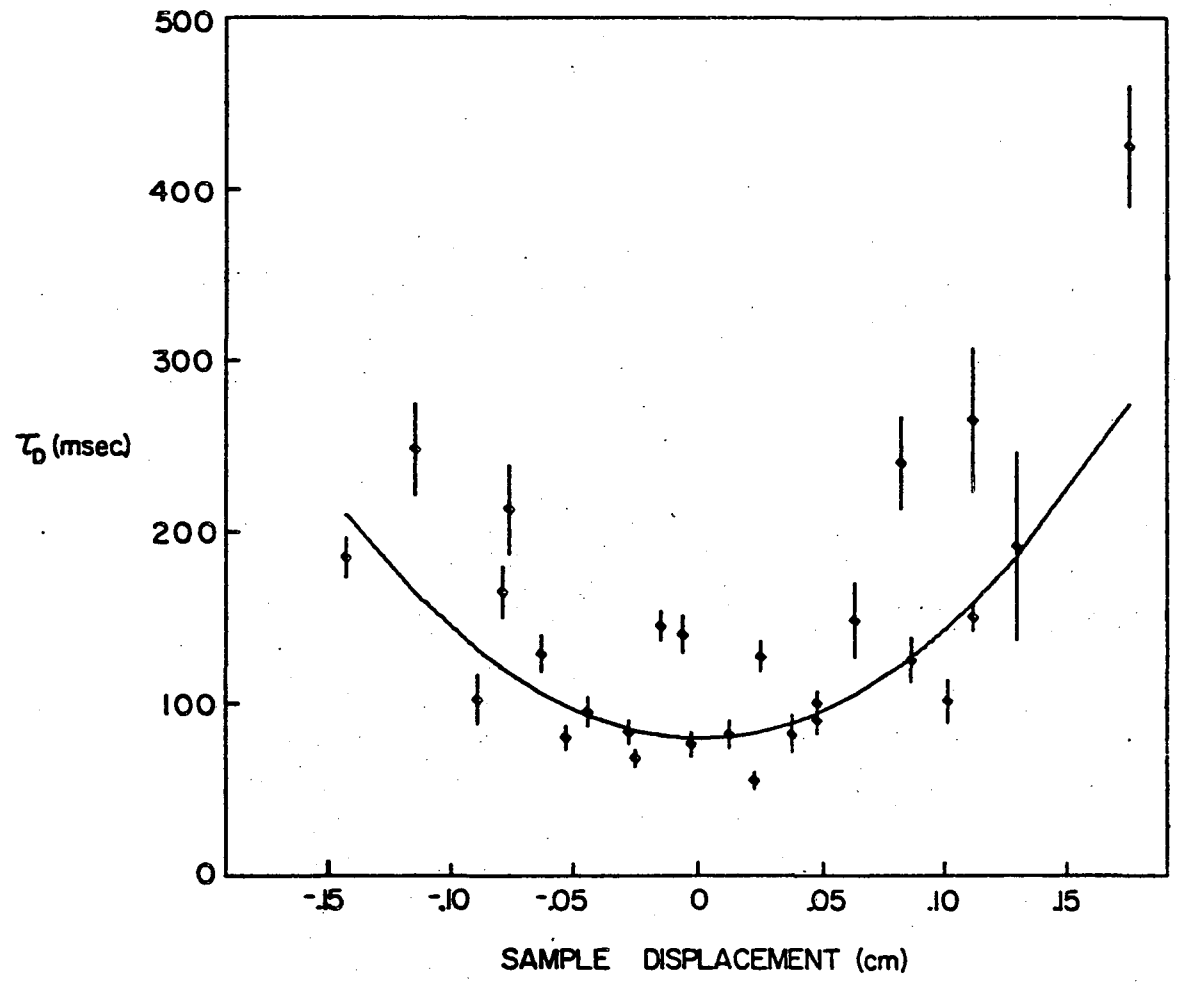
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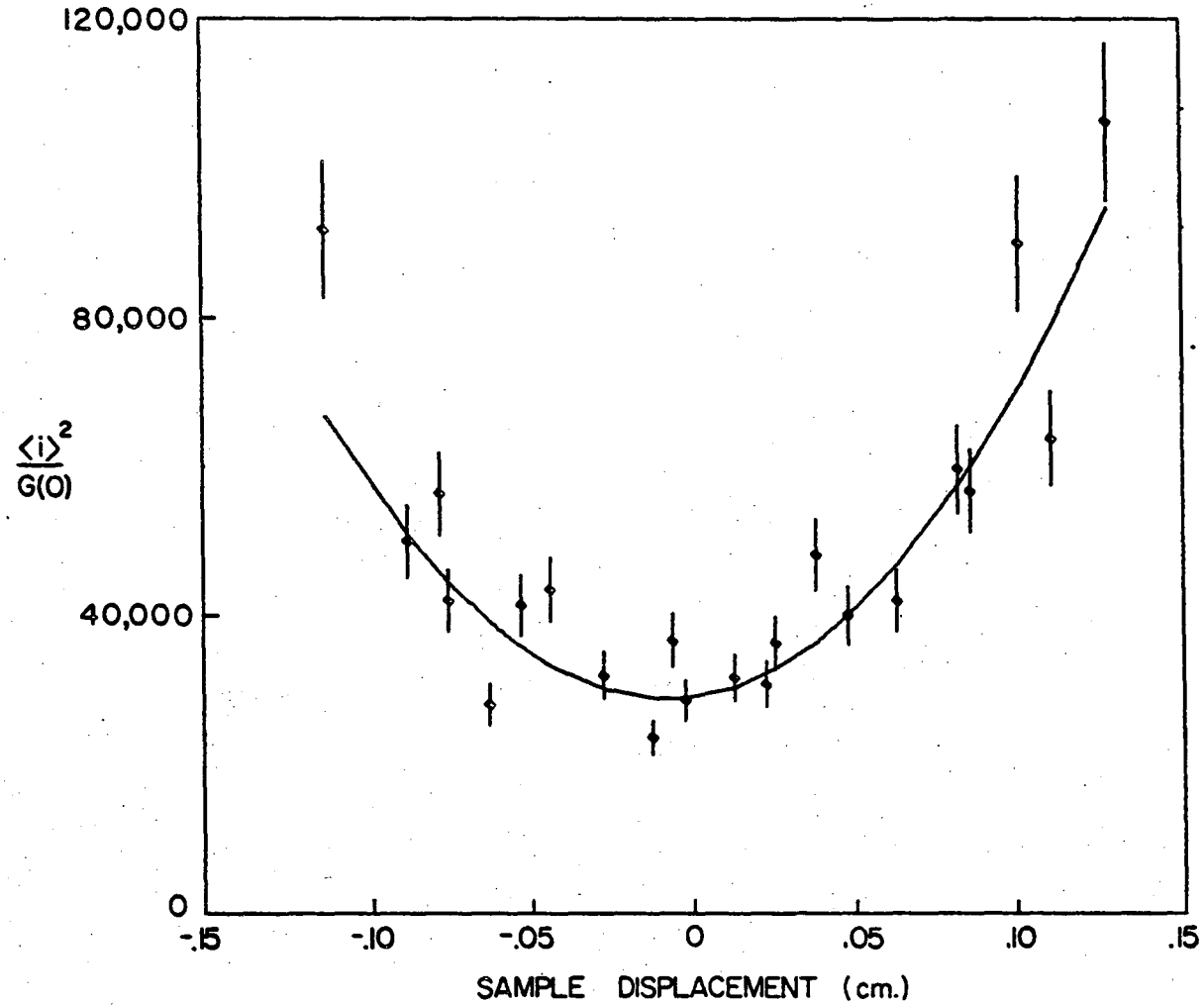
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