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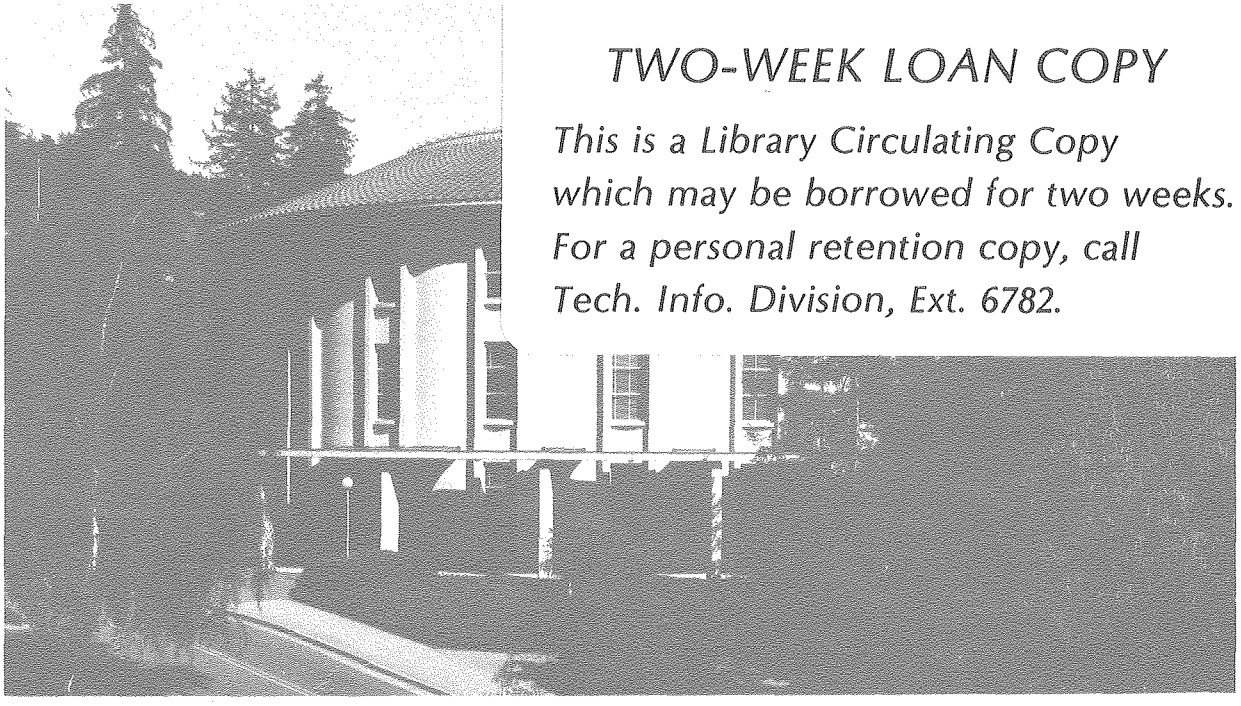

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THE USE OF FLUORESCENCE CORRELATION SPECTROSCOPY TO PROBE CHROMATIN IN THE CELL NUCLEUS

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SUMMARY

All systems in thermodynamic equilibrium are subject to spontaneous fluctuations from equilibrium. For very small systems, the fluctuations can be made apparent, and can be used to study the behavior of the system without introducing any external perturbations. The mean squared amplitude of these fluctuations contains information about the absolute size of the system. The characteristic time of the fluctuation autocorrelation function contains kinetic information.

In the experiments reported here, these concepts are applied to the binding equilibrium between ethidium bromide and DNA, a system where the fluorescence properties of the dye greatly enhance the effect of spontaneous fluctuations in the binding equilibrium.

Preliminary experiments employ well characterized DNA preparations, including calf thymus DNA, SV40 DNA, and calf thymus nucleohistone particles. Additional measurements are described which have been made in small regions of individual nuclei, isolated from green monkey kidney cells, observing as few as 5000 dye

molecules. The data indicate that the strength of dye binding increases in nuclei isolated from cells which have been stimulated to enter the cell growth cycle. The viscosity of nuclear material is inferred to be between one and two orders of magnitude greater than that of water, and decreases as the cells leave the resting state, and enter the cell growth cycle. Washing the nuclei also lowers the viscosity.

These experiments demonstrate that fluorescence correlation spectroscopy can provide information at the subnuclear level that is otherwise unavailable.

INTRODUCTION

Quantitative measurements in the cell nucleus are generally very difficult because of low signal levels and very small sample volumes. In contrast, fluorescence correlation spectroscopy requires small sample sizes for successful data collection, and relies on spontaneous fluctuations from equilibrium to generate the signal. Thus, the technique should be applicable to small delicate biological systems, such as the cell nucleus. The results presented here serve to establish that fluorescence correlation spectroscopy is a suitable technique for probing the cell nucleus.

The concept of fluctuation spectroscopy is extremely general. In principal, it can be applied to any system in thermodynamic equilibrium.[1-3] If such a system were subjected to an external perturbation, it might be displaced from equilibrium. After small perturbations, the system will relax in a manner characteristic of the kinetics of the original thermodynamic equilibrium. The

characteristic relaxation times can be measured and interpreted in terms of the kinetic parameters of the system near equilibrium.

Fluctuation spectroscopy takes advantage of spontaneous fluctuations undergone by any system in thermodynamic equilibrium. The state of the system near equilibrium should evolve according to the same kinetic parameters observed with the perturbation technique.

Fluctuation amplitudes contain information about the size of the system.[4,5] In the case of diffusion of molecules through an open volume, molecular concentrations can be inferred from fluctuation amplitudes.[6,7] Another application of fluctuation spectroscopy is the study of ion channels in nerve tissue, where fluctuations in electrical conductance are observed as channels in the nerve membranes open and close.[8-11] Scattered light can be used to monitor number fluctuations in the same way that fluorescence is used in fluorescence correlation spectroscopy.[3,12] The measurement of rotational diffusion by fluctuation spectroscopy has been considered theoretically.[13,14] Such measurements would have the important feature that the rotational correlation time should be much longer than the fluorescence lifetime.

The technique of fluorescence correlation spectroscopy has been reviewed recently.[1-3] There have been relatively few applications.[15-17] There has been somewhat more interest in a variation of the method, where the slow recovery after photobleaching is monitored.[18-21]

MATERIALS AND METHODS

Description of the Apparatus

The apparatus is similar to previously reported devices.[18,22] Features of the system are shown schematically in Fig. 1. Optical components are mounted on a vibration isolation table. The cooling fan for the laser and all fan cooled electrical equipment are placed away from the table. The microscope is mounted in a steel frame to reduce the effect of any possible vibrations. A Spectra-Physics model 160 argon ion laser provides illumination at 488 nm or 514.5 nm.

A feedback mechanism minimizes fluctuations in laser intensity, and monitors beam power. A stress plate modulator, SPM, is driven by a high voltage amplifier to stabilize the beam intensity. A spatial filter, SF, removes high frequency spatial variations, leaving a gaussian intensity profile. Lens L1 can be moved along the beam axis, so that the beam can be focussed by the microscope objective lens to a point above, below or within the sample plane. The sample and beam may be observed in a binocular eyepiece. Fluorescence is excited in the sample, collected by the microscope objective lens, and directed toward a photomultiplier, PM. A dichroic mirror, DM, reflects scattered laser light, but transmits fluorescent light from the sample. The aperture, A, restricts the field of view to the center of the sample, eliminating much of the background light intensity. Light transmitted by the aperture can be observed in a monocular eyepiece. Glass filters, GF, discriminate further against light outside the fluorescence emission band. The

photocurrent is converted to a voltage, and amplified, so that its average value is equal to the average beam monitor voltage. The monitor voltage is then subtracted from the photomultiplier signal, and the remaining fluctuations are processed by high pass, and variable low pass band filters. The processed signal can be observed on an oscilloscope. A Saicor model 43A Correlation and Probability Analyzer computes the autocorrelation function of the processed signal. The result may be observed on an oscilloscope as it is being generated. When computed, the autocorrelation function is recorded by an X-Y recorder and punched on paper tape.

Analysis of the Data

The fluorescence signal is denoted $i(t)$. Its average is $\langle i \rangle$, so that fluctuations from the average are $\delta i(t) = i(t) - \langle i \rangle$. Data are expressed in terms of the autocorrelation function, $G(\tau)$, of the fluctuations in fluorescence intensity.

$$G(\tau) = \lim_{T \to 0} \int_0^T \delta i(t) \delta i(t+\tau)\, dt \qquad (1)$$

Illumination is provided by a beam with a gaussian intensity profile, $I(r)$, having the form $I(r) = I_0 \exp(-2r^2/w^2)$, where w is the beam e^{-2} radius. When simple 2-dimensional diffusion in a thin sample is the only process contributing to the fluctuations, it has been demonstrated that the observed autocorrelation function, $G(\tau)$, will have the form[7,15,22]

$$G(\tau) = \frac{G(0)}{1 + \tau/\tau_D} \qquad (2)$$

Furthermore, $\langle i \rangle^2/G(0) = \pi w^2 LC$, where $G(0)$ is the mean squared fluctuation, L is the depth of the sample, and C is the average

number concentration of diffusing fluorescent molecules. The characteristic time, τ_D, can be written $\tau_D = w^2/4D$, where D is the is the diffusion coefficient of the diffusing fluorescent molecules.

The binding of ethidium bromide and DNA can be described by the expression

$$A + B \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C \qquad (3)$$

where A represents open DNA binding sites, B represents free ethidium bromide, and C represents the complex. The forward and backward reaction rates constants, k_f and k_b, can be combined to give the binding constant, $K = k_f/k_b$.

When the fluctuations of interest arise from ethidium bromide-DNA binding, the expected autocorrelation function consists of three terms.[7,15]

$$G(\tau) = G_0(\tau) + G_+(\tau) + G_-(\tau) \qquad (4)$$

$G_0(\tau)$ describes the diffusion of DNA molecules. $G_+(\tau)$ can be thought of as arising from the diffusion of ethidium bromide molecules from one binding site to the next. By "next" binding site, is meant the next site to which the molecule binds, not the neighboring site. $G_-(\tau)$ can be thought of as reflecting fluctuations due to ethidium bromide molecules binding to and releasing from a binding site. In the experiments described in this report, both $G_0(\tau)$ and $G_+(\tau)$ have the same general appearance.

$$G_0(\tau) = \frac{G_0(0)}{1 + \tau/\tau_{DNA}} \qquad (5)$$

$$G_+(\tau) = \frac{G_+(0)}{1 + \tau/\tau_+} \qquad (6)$$

In these expressions, τ_+ and τ_{DNA} are the characteristic diffusion times of the processes from which $G_+(\tau)$ and $G_0(\tau)$ arise. In particular, $\tau_+ = \tau_D(1 + K[A])$, and $\tau_{DNA} = w^2/4D_{DNA}$, where D_{DNA} is the diffusion coefficient of DNA molecules, and τ_D is the diffusion time for free ethidium bromide. $G_0(0)$ and $G_+(0)$ can be used to estimate the number of observed DNA molecules and the number of observed bound dye molecules, respectively.

In all the experiments described here, the autocorrelation function will be dominated either by $G_0(\tau)$ or $G_+(\tau)$. This means that all the observed autocorrelation functions can be described in terms of the parameters in the general form $G(\tau) = G(0)(1 + \tau/T)^{-1}$, where T is the characteristic time of the system.

The third term, $G_-(\tau)$ is not observed in the measurements discussed here for at least three reasons. $G_-(\tau)$ has very rapid characteristic times.[15] In addition, the amplitude, $G_-(0)$ will be very small. Finally, signal to noise problems are necessarily more pressing in fast measurements, since fewer photons are detected per correlation time from each fluorescing molecule.[23]

The observed autocorrelation functions are analyzed by computer to obtain the amplitude of the autocorrelation function and the characteristic time. Before computing $\langle i \rangle^2/G(0)$ the average background light intensity must be subtracted from the observed photomultiplier signal to obtain the average fluorescence signal, $\langle i \rangle$. If data are being collected from a homogeneous sample, then a blank is prepared without dye to estimate background. When looking at cell nuclei, background can be measured by displacing the sample a short distance, so that the beam passes through the sample at a

point where no ethidium bromide will be bound to DNA. The fluorescence intensity from the displaced sample is taken as background.

Beam Waist Radius

The illumination beam is focussed in a plane near the sample. Illumination can be said to extend over an area πw^2, where w is the e^{-2} radius of the gaussian intensity profile. This area increases with the relative displacement of the sample and beam focal plane. For small relative displacements,[22]

$$w^2 = w_0^2(1 + \frac{\lambda^2 \Delta z^2}{\pi^2 w_0^4}) \qquad (7)$$

In this expression, Δz is the relative displacement, λ is the wavelength of the illumination, and w_0 is the radius at the beam waist, where $\Delta z = 0$.

Measurements have been made a number of times in which the focal plane is displaced by different amounts relative to the plane of the sample. Each experiment of this kind yields two estimates of the beam waist radius. The average of these determinations is (0.88 ± 0.12) μm. The standard error of the mean is less than 0.03 μm.

It has been shown that $\langle i \rangle^2/G(0)$ and the appropriate diffusion time, τ_D, τ_+, or τ_o should all vary quardratically with Δz.[22] In the process of verifying this dependence, one may simultaneously estimate the beam waist radius, the 2-dimensional concentration of diffusing molecules, and the diffusion coefficient. Furthermore, the quadratic dependence of the characteristic time on Δz is strong evidence that translational diffusion is involved in the observed fluctuations.

Fluorescence Intensity Per Diffusing Molecule

In these fluctuation experiments, total fluorescence intensity is measured and the number of diffusing molecules is determined. It is possible, then, to compute the average amount of light coming from one diffusing molecule. This is useful in distinguishing $G_+(\tau)$ from $G_0(\tau)$, when only one or the other contributes significantly to the observed autocorrelation function. The characteristic times may be indistinguishable in the two cases. However, the amount of fluorescence per diffusing molecule can be very large for DNA molecules, which may bind many chromophores. The fluorescence per diffusing molecule would be fixed at a lower value in the case of dye molecules diffusing from one binding site to another.

Relative Viscosities

If we presume that the observed diffusion coefficient is based on diffusion of ethidium bromide, slowed by binding, then we may write

$$D = \frac{D_{free}}{(1 + K[A])} \frac{\eta_n}{\eta_w} \tag{8}$$

where D is the observed diffusion coefficient, D_{free} is the diffusion coefficient of ethidium bromide in water, η_n and η_w are the viscosities of the nuclear material and water, respectively, and K[A] is the ratio of bound to free dye.

The concentration of bound dye can be estimated from $\langle i \rangle^2/G(0)$. Free dye concentrations should be close to the value in the medium, where dye is present in excess. The ratio of the concentrations gives K[A], and Eqn. 8 can then be used to compute the viscosity in the

nucleus relative to the viscosity of water.

One effect of bleaching should be to reduce the concentration of free dye in the beam, compared to the free dye concentration far from the beam. Thus, K[A] may be larger than the computed value. Bleaching reduces τ_+ and $\langle i \rangle^2/G(0)$ by comparable amounts.

Preparation of Samples

Calf Thymus DNA. Calf thymus DNA, obtained from Cal Biochem, was prepared in a solution of 10 mM Tris buffer and 1 mM EDTA at pH 7.9. The concentration of DNA base pairs was $2 \cdot 10^{-4}$ M, assuming an average molecular mass of 670 daltons/base pair. Ethidium bromide concentration was $1 \cdot 10^{-4}$ M. The sample taken for study had a volume of 20 µl. It was placed between a microscope slide and a cover slip having an area of 4.84 cm^2. Thus, the sample depth was approximately 40 µm. The edges of the sample were sealed with wax to retard evaporation of the solution. Excitation was at 488 nm.

It was found that thinner samples were unsuitable; data were not reproducible and were generally uninterpretable. It is believed that surface effects interfered with free diffusion in thin samples, by imposing local ordering in the solvent, or by creating a gel from the DNA. Similar effects were seen in very thin samples containing only Rhodamine 6G in water.

SV40 DNA. Purified Form II SV40 DNA in solution was prepared with ethidium bromide, making final concentrations of $3 \cdot 10^{-5}$ M ethidium bromide and 47 µg/ml DNA. The DNA mass concentration was determined from the absorption spectrum of the solution. A 10 µl sample was placed between a microscope slide and cover slip, and sealed with warm paraffin. It would have an average depth of about 20 µm.

Excitation was at 488 nm.

SV40 DNA in Agarose Gel. An agarose solution containing ethidium bromide was mixed with SV40 DNA solution at 45°C on a hot plate. A small volume of the mixture was sandwiched between a warmed microscope slide and cover slip, and sealed with paraffin. Final SV40 DNA concentration was about $1.5 \cdot 10^{-4}$ M DNA base pairs, or $3 \cdot 10^{-8}$ M DNA molecules. Final dye concentration was 10^{-6} M, and final agarose concentration was 1% by weight. Excitation was at 514.5 nm.

Calf Thymus Nucleohistone Particles. Calf thymus histones, not including H1, and calf thymus DNA, obtained from CalBiochem, were made to combine by slowly decreasing the salt concentration by dialysis. The concentration of DNA in the final solution was determined spectrophotometrically to be $7 \cdot 10^{-5}$ M base pairs. ethidium bromide was introduced into the solution, making a final dye concentration of 10^{-5} M. Samples were prepared from 5 µl of the final solution, and would have an average depth of about 10 µm.

Cell Nuclei. Cells were prepared from the green monkey kidney cell line designated TC-7.[24] Confluent populations were split into quarters, and transferred to culture dishes in fresh medium containing 10% fetal calf serum. Some of the cells were harvested 24 hours after transfer, in a state of active growth. Others were allowed to grow for at least a week, when they could be harvested at high cell density.

Nuclei were isolated from these cells in two ways. Washed nuclei were obtained by dissolving the cytoplasmic membranes with detergent, then washing the cytoplasm away by rinsing with buffer. Using milder conditions, the nuclei could be left clinging to a

microscope cover slip, with much of the cytoplasm and some of the cell membrane intact.

Washed nuclei were prepared by harvesting cells from one or two 100 mm. culture dishes with a rubber policeman or by incubating the cells for one minute at 37°C in trypsin. The cells were aspirated with isotonic saline. The cells were suspended, and agitated gently to break up clumps. A part of the suspension was taken for flow cytometry. The remainder was centrifuged at 1000 rpm for 60 seconds in a JA-21 rotor. The supernatant was discarded. The pellet was resuspended in 0.2 ml of 0.25% Nonidet P-40 detergent. The suspension was gently aspirated to prevent clumps from forming. Cell membranes dissolved immediately. The suspension was centrifuged at 1000 rpm for at least 60 seconds in a JA-21 rotor. The pellet was resuspended in 5 ml of saline solution containing $3 \cdot 10^{-8}$ M ethidium bromide. The suspension was centrifuged again, and all the saline solution was removed except approximately 0.2 ml. The nuclei were resuspended in the remaining saline solution. A 5 µl sample was placed on a microscope slide. A cover slip was placed over the drop, and sealed with warm paraffin, to retard evaporation.

In the second preparation method, six cover slips were placed in empty culture dishes. Transferred cells settled on the cover slips and attached themselves after several hours. When harvested, five cover slips were treated with trypsin, and the cells were removed into isotonic saline. They were used for flow cytometry. The sixth cover slip was placed on a small copper screen. The screen with the cover slip was placed in a dish containing 0.1% Triton X-100. Higher detergent concentrations were found to remove

the cell membranes immediately. At this strength of Triton X-100, the cell membranes would remain visible for a few minutes, and then would break up. Freshly transferred cells would break up in one or two minutes. Cells allowed to reach high density on the cover slip would require 2-3 minutes for the membranes to break away. At this point, the screen with the cover slip was removed from the detergent solution, and rinsed for 5 minutes in a dish containing saline and $3 \cdot 10^{-8}$ M ethidium bromide. The cover slip was then inverted, and placed on a microscope slide over a small depression containing the saline-dye solution. Excess solution was removed with a tissue until the cover slip rested on the slide. The edges were sealed with warm paraffin.

RESULTS

Results for each DNA preparation are described below. The beam waist radius measurements are given. The measurements based on the diffusion times are then described. Finally, the information leading to the two-dimensional concentrations is given.

Calf Thymus DNA and Ethidium Bromide

Measurements were made at several positions of the beam focal plane relative to the sample. Values of τ_0 and $\langle i \rangle^2/G(0)$ for various beam positions are shown in Fig. 2. From the data in the upper half of the figure, estimates are made of the beam waist radius, w_0, and the diffusion coefficient, D_{DNA}. From the data in the lower half of the figure, estimates are made of w_0 and the 2-dimensional concentration, CL. Some of these results, and similar values from other experiments are summarized in table I.

Beam waist radius. The beam waist radius derived from $\langle i \rangle^2/G(0)$ is (1.1 ± 0.2) μm. The value derived from diffusion times is (1.3 ± 0.2) μm. They are both higher than values from other experiments, but this sample was relatively thick. Blurring of the focal spot by thick samples would have an effect of about this amount.

Diffusion coefficient. From the behavior of τ_0 as a function of beam position, the diffusion coefficient is estimated to be $(2.5 \pm 0.2) \cdot 10^{-8}$ cm^2/sec. The molecular mass, diffusion coefficient, the mass density of the molecule, and the viscosity of the medium are related. Thus, the measured diffusion coefficient is consistent with a viscosity for the medium of 5-10 times that of water.

Two dimensional concentration. The behavior of $\langle i \rangle^2/G(0)$ as a function of beam position leads to a value of $(2.4 \pm 0.2) \cdot 10^{-7}$ Mμm for the 2-dimensional concentration, CL. If the sample were 40 μm deep, then the 3-dimensional concentration of diffusing molecules would be about $6 \cdot 10^{-9}$ M. The concentration of base pairs is known to be $2 \cdot 10^{-4}$ M, so there would be about $3 \cdot 10^4$ base pairs per diffusing molecule. Since each base pair weighs 670 daltons, on the average, the mass of the diffusing molecule would be $22 \cdot 10^6$ daltons.

The molecular mass inferred from $\langle i \rangle^2/G(0)$ is a modest overestimate. The molecular mass of calf thymus DNA molecules are known to be broadly distributed, but average about 10^7 daltons. Much of the difference between the inferred and expected molecular masses could lie with the sample depth, which may have been less than 40 μm if evaporation had reduced the sample volume. Another

possible reason for an overestimate is correlation between DNA molecules. The DNA concentration is relatively high in these preparations, and the medium is expected to be viscous. Thus, diffusion of neighboring molecules could be correlated, and the apparent molecular mass would be increased.

A third factor to consider when using non-homogeneous samples is that G(0) is weighted in favor of heavy molecules which would bind more dye. They would contribute disproportionately, making it appear that larger molecules were in the beam.[7] This effect is not expected to be appreciable, unless the distribution of molecular masss is skewed toward large molecules.

In view of these possibilities, and given the uncertainty in the expected molecular mass of calf thymus DNA, one can say that the observed figure differs only modestly from expectations.

Fluorescence Intensity Per Diffusing Molecule. The amount of light detected can be compared to the amount of light expected from the observed number of molecules. The optical collection efficiency was calibrated with Rhodamine 6G, and corrected for ethidium bromide, using the absorbance and fluorescence emmision bands of the two dyes, and the pass bands of the optical filters in the system.

The binding constant for the DNA-ethidium bromide system is such that essentially all the dye should be bound. Thus, the number of dye molecules per diffuser should have been $1.7 \cdot 10^4$, or fewer. The observed fluorescence intensity corresponds to $1.3 \cdot 10^3$ chromophores per diffusing molecule, where each chromophore is assumed to have the absorption and emission spectral characteristics of bound ethidium bromide, and a quantum efficiency for fluorescence

of 0.2.

Photobleaching can account for much of the difference. Some binding sites may be occupied by damaged dye molecules. Some binding sites may, themselves, be damaged. It is clear, however, that these data refer to the diffusion of molecules with many chromophores, represented by $G_0(\tau)$, rather than to the diffusion of chromophores from one binding site to another, corresponding to $G_+(\tau)$.

SV40 DNA

Diffusion time and 2-dimensional concentration. Diffusion times for SV40 DNA were similar to times measured for calf thymus DNA. The diffusion coefficient was inferred by measuring diffusion-times for different relative positions of the beam focal spot and sample. The diffusion coefficient was found to be $(1.5 \pm 0.7) \cdot 10^{-8}$ cm^2/sec. The uncertainty associated with the diffusion coefficient is considerable because the data were somewhat scattered. Corresponding to this diffusion coefficient is a hydrodynamic radius of about 0.15 μm assuming a viscosity close to that of water. Closed SV40 DNA has a strand length of about 1.5 μm, when laid out in a circle. In solution, of course, it is more compact. Thus the figure for the hydrodynamic radius of 0.15 μm is not unreasonable. Again, interpretation of such figures should take into account the fact that the viscosity of the medium has not been measured directly.

The measured value of $\langle i \rangle^2/G(0)$ can be combined with the measured mass concentration to make an estimate of the molecular mass. Making allowance for blurring of the focal spot, the computed molecular mass is $4.3 \cdot 10^6$ daltons. SV40 DNA molecules are

known to have a mass of $3.5 \cdot 10^6$ daltons. The discrepancy between the known and measured molecular mass is about 20%. The most significant sources of uncertainty in the measured value are sample depth, $\langle i \rangle^2/G(0)$, the measured mass density, and the beam waist radius. The estimated uncertainties of each of these is 10% or greater. Considering these factors, the agreement between computed and expected molecular masss is well within experimental error.

Fluorescence Intensity Per Diffusing Molecule As before, the fluorescence intensity can be used to compute the apparent number of chromophores per diffusing molecule, and then compare that figure to the average number of ethidium bromide molecules expected to bind to each SV40 DNA molecule.

Under the conditions of this experiment, almost 2100 chromophores are expected to bind to each SV40 DNA molecule. That is, all available sites are expected to be occuppied. The observed fluorescence intensity corresponds to 500 chromophores. Again, the effects of bleaching can account for much of the difference.

SV40 DNA in Agarose Gel.

Diffusion times and number concentrations. Diffusion times observed for ethidium bromide and SV40 DNA in agarose gels were comparable to times measured for calf thymus DNA and SV40 DNA in solution. They correspond in these measurements to diffusion coefficients of the order of 10^{-8} cm^2/sec. However, these diffusion times are interpreted in terms of individual diffusing ethidium bromide molecules slowed by binding to DNA. Based on binding properties, ethidium bromide molecules should have a diffusion coefficient of roughly $3 \cdot 10^{-8}$ cm^2/sec, in water, or lower in a more viscous

medium.

Sample depths were inferred from strong reflections at the glass-water interfaces. Using the sample depths and values of $\langle i \rangle^2/G(0)$, the number concentration was computed to be about $4 \cdot 10^{-7}$ M. The SV40 DNA concentration is expected to be about $3 \cdot 10^{-8}$ M. The bound ethidium bromide concentration was expected to be 10^{-6} M. It would seem, then, that $G_+(\tau)$ was observed. This conclusion is supported by measurements of total fluorescence intensity.

Fluorescence Intensity Per Diffusing Molecule. From the binding properties, it is expected that about 75 ethidium bromide molecules would bind to each DNA molecule in the sample, on the average. The values computed from the fluorescence intensity for the apparent number of bound chromophores, fall in the range of 2-4. Such values suggest that $G_+(\tau)$ was observed, rather than $G_0(\tau)$. It is possible, of course, that both $G_+(\tau)$ and $G_0(\tau)$ contributed significantly to the overall autocorrelation function.

Nucleohistone Particles

Beam waist radius. Observations were made at several positions of the beam relative to the sample. Consequently, the beam waist radius was measured twice. It was found to be $(0.90 \pm 0.08)\mu m$.

Diffusion times and concentrations. Results for nucleohistone particles are included in Table I. The diffusion coefficient was about $3.5 \cdot 10^{-8}$ cm^2/sec which is comparable to, but slightly greater than the measured diffusion coefficients for calf thymus DNA and SV40 DNA.

The concentration of DNA measured spectrophotometrically

corresponded to about half the DNA used as starting material. The concentration derived from $\langle i \rangle^2/G(0)$ corresponds to half the spectrophotometric figure.

The observed concentration is not very far from expectations, and is consistent with the results for calf thymus DNA.

Fluorescence Per Diffusing Molecule. If the DNA were present without histones, one would expect essentially all of the ethidium bromide to be bound, given the excess of DNA. Then there would be roughly 2000 bound chromophores per observed diffusing molecule. In fact, the apparent number of chromophores, inferred from fluorescence intensity is roughly 130. In measurements involving DNA in the absence of histones, the fluorescence intensity underestimated the number of bound chromophores by a factor of about 5. Chromatin is known to bind less dye than free DNA, and the binding of dye to chromatin can be complex.[25-27] Unfortunately, the uncertainties in measurements presented here are too large to justify careful quantitative comparisons. Still, the number of chromophores per diffusing molecule obtained from these observations is consistent with the differences in binding expected between free DNA and chromatin.

Cell Nuclei

Isolated TC-7 nuclei appear either disc shaped or prolate. The long dimension is 10-15 μm and the shorter dimension is 5-10 μm. They are never seen to move. Nucleoli, a few microns across, are often present in the nucleus. They tend to fluoresce more strongly than surrounding areas. The enhanced fluorescence tends to fade after brief illumination, At dye concentrations of

$3 \cdot 10^{-8}$ M, fluorescence is limited to the region being illuminated directly. The beam itself occupies a small portion of the nucleus.

In the process of isolating washed nuclei, there may be a substantial loss of material, making it difficult to locate nuclei. It is possible, therefore, that a biased selection of washed nuclei may have taken place. In some preparations, the optical contrast between the washed nuclei and the medium is very low. Finding nuclei in such preparations may be very tedious, if not impossible. Broken nuclei were never observed.

Unwashed nuclei on cover slips are seen embedded in a small mass of cytoplasm. Within the remaining cytoplasm are small bright spots that are probably points of attachment of the cell to the cover slip. The cytoplasm fluoresces strongly. This is believed to be from RNA bound ethidium bromide. It is not known to what extent the cytoplasm covers the side of the nucleus facing the medium. Unwashed nuclei from cells at low density have open spaces between them where background fluorescence is comparable to blank fluorescence. Unwashed nuclei from cells at high density are completely surrounded by cytoplasm, leaving few or no open spaces between them. Nuclei are never seen to move. Cytoplasm is never seen to become dislodged, and the preparation can remain suspended on the cover slip for days. Comparison of microphotographs of the cells before lysing with Triton washing with the isolated nuclei indicates that no more than a few percent of the nuclei may be lost in preparation.

DNA histograms are shown in Fig. 3 for cell populations from which nuclei were isolated for use in fluorescence correlation experiments. The data obtained in the fluctuation experiments are

shown in Figs. 4, 5, and 6. Data in these figures and other information is summarized in Table II. Four classes of nuclei are indicated. Some nuclei were prepared by lysing cells, and washing in suspension. These are referred to as washed nuclei. Others were prepared by dissolving the outer membranes of cells attached to cover slips. These are referred to as unwashed nuclei. The cells themselves were harvested at high cell density after growing for at least a week, or were harvested 24 hours after transfer, at low cell density. Nuclei from confluent cells are designated resting. Nuclei from cells harvested 24 hours after transfer are referred to as being stimulated to grow. Thus, each nucleus is described by the state of the cell population from which is was isolated, and by the procedure used to isolate it.

In the process of obtaining nuclei for fluctuation experiments, a part of each population of cells was examined in the flow cytometer. In Fig. 3, DNA histograms are shown which are representative of cells corresponding to each class of nuclei examined in fluctuation experiments. DNA histogram a shows a distribution of DNA content per cell that is typical of cells in G_0 or G_1. The small peak corresponding to G_2 and M cells contains some counts from two cells, stuck together. The suspension of cells was filtered through a nylon screen to minimize this problem. In this histogram, a very small peak corresponding to triplets can also be seen. The peaks are relatively sharp. TC-7 cells are especially suitable for flow cytometry in this respect. Much of the existing peak width is due to variability in the detection of fluorescence intensity.

In DNA histogram b, many cells in S and G_2 + M are detected. Synchrony is not ideal, and the population is relatively heterogeneous. The distribution in DNA histogram c is essentially identical to a. They both correspond to populations of resting cells. The distribution in d is different from that in b. Most of the cells represented in d were apparently delayed in G_1, although some are well into S. The broadening of the G_1 peak is due to cells leaving G_1 and entering S. These cells had attached themselves to glass cover slips instead of the specially coated plastic culture dishes used for the cells in a and b. That probably accounts for the delay.

In fluctuation measurements, each nucleus was observed in two locations, on the average. Each site in a nucleus was treated as being independent in the histograms shown in Figs. 4, 5, and 6. Thus, each nucleus contributes two data points, on the average, to each of three histograms.

Figure 4 shows the distributions of diffusion times in the four classes of nuclei. Washed isolated nuclei from growing and resting cells have indistinguishable distributions. The diffusion times correspond to diffusion coefficients of 6.9 and $6.8 \cdot 10^{-9}$ cm^2/sec. Assuming ethidium bromide has a diffusion coefficient of $6 \cdot 10^{-6}$ cm^2/sec in water, we may write

$$(1 + K[A])\eta_n/\eta_w = 875 \pm 75$$

for washed isolated nuclei,

where K[A] is the ratio of bound to free ethidium bromide, and η_n and η_w are the viscosities of the nucleus and water, respectively.

Similarly, the distributions are indistinguishable for unwashed nuclei on cover slips derived from stimulated and resting cells. However, they are both very different from the distributions seen for washed nuclei isolated from stimulated and resting cells. The mean diffusion coefficients for nuclei on cover slips are 2.4 and $2.5 \cdot 10^{-9}$ cm^2/sec, corresponding to

$$(1 + K[A])n_n/n_w = 2500 \pm 240$$

for unwashed nuclei on cover slips.

This is greater by a factor of almost three than the value seen for washed nuclei.

Similar information can be inferred from $\langle i \rangle^2/G(0)$. Histograms of measurements of $\langle i \rangle^2/G(0)$ are given in Fig. 5. Washed isolated nuclei from resting and stimulated cells have very similar distributions. Assuming that the concentration of free dye in the nucleus is $3 \cdot 10^{-8}$ M, and that the nuclei are 8 μm deep, the means correspond to

$K[A] = 75 \pm 6$ for washed nuclei from resting cells,

and $K[A] = 110 \pm 9$ for washed nuclei from stimulated cells

prepared by washing the isolated nuclei in suspension.

In the case of unwashed nuclei on cover slips, the results are somewhat different. The distribution of $\langle i \rangle^2/G(0)$ for nuclei on cover slips from resting cells is shifted to lower values, relative to washed nuclei from resting cells. The values of $\langle i \rangle^2/G(0)$ for nuclei from stimulated cells are shifted considerably higher. The means of these distributions correspond to

$K[A] = 48 \pm 4$ for unwashed nuclei from resting cells

$K[A] = 160 \pm 9$ for unwashed nuclei from stimulated cells.

Diffusion times and $\langle i \rangle^2/G(0)$ should both increase as binding increases. Comparing unwashed nuclei from cells stimulated to grow, to washed nuclei from cells stimulated to grow, it is found that both diffusion times and $\langle i \rangle^2/G(0)$ decrease by roughly a factor of 2, after washing. In the case of nuclei from resting cells, washing causes $\langle i \rangle^2/G(0)$ to increase while diffusion times decrease, when going from washed to unwashed nuclei.

A word of caution is called for. These data are averaged over all observed nuclei in the population. They should not be interpreted literally as applying to "cells stimulated to grow." They apply to the population of cells stimulated to grow, some of which may be found in any of the states of the cell cycle. No attempt was made to correlate features in histograms to subgroups of the populations of growing cells.

The viscosity of the nucleus might account for some of the observed changes by effecting both $\langle i \rangle^2/G(0)$ and the diffusion times. The binding equilibrium might shift to smaller amounts of bound material in a diffusion controlled reaction, when the viscosity increases. Of course, diffusion times would increase with viscosity.

Pursuing this line of thought, viscosities can be estimated from data for diffusion times, and two dimensional concentrations. Such computations were made for observations at different sites in observed nuclei. It was assumed that the average nuclear depth was 8 μm, the concentration of free dye was $3 \cdot 10^{-8}$ M, and that

the diffusion coefficient of ethidium bromide in water is $6 \cdot 10^{-6}$ cm^2/sec. The distributions of viscosities inferred in this way are shown in Fig. 6 for the four classes of nuclei. The means of the distributions of relative viscosities and ratios of bound to free dye for each population are stated in columns E and F of Table II.

Generalizations apply to the data. The inferred viscosity of nuclei from cells stimulated to grow is less than the inferred viscosity of corresponding nuclei from resting cells. The inferred viscosity in unwashed nuclei is greater than the inferred viscosity in washed nuclei.

Fluorescence Intensity Per Diffusing Molecule. The fluorescence intensity per diffusing molecule was computed for each observation in cell nuclei. The average value in each of the four classes of nuclei corresponded to that expected for one ethidium bromide molecule per diffusing molecule. This is strong evidence that $G_+(\tau)$ was observed, rather than $G_0(\tau)$. Of course, it is unlikely that $G_0(\tau)$ would be observed, since chromatin is not expected to be mobile on the time scale of these measurements.

DISCUSSION

There is support in the literature for some of these results. Reports describe increased ethidium bromide binding by chromatin isolated from growing cells.[28-30] The magnitudes of the increases vary, but fall in the range of 30-200%. The increase in binding inferred from experiments described here is about 40% for washed nuclei. For unwashed nuclei, the increase is about 3-4

times. Reports also claim that chromatin becomes more condensed in early S phase in intact HeLa cells.[31] This would have the effect of increasing K[A] in nuclei from cells stimulated to grow over values for nuclei from resting cells.

It is also true, of course, that stimulated cells are synthesizing chromatin. Consequently, the concentration of DNA binding sites might be expected to increase. This would increase K[A] in stimulated cells compared to resting cells.

Morphological changes might also effect values listed in column E of Table II. Column E is presented, assuming an average nuclear depth, L, of 8 μm. If nuclear depth changes were to account for changes listed in column E, then L would have to be smaller in stimulated cells than in resting cells. There is no reason to expect such a thing to happen.

The results in Table II can be interpreted in biochemical terms. As the cells are stimulated to grow, transcription and replication mechanisms become activated. These and related activities might be associated with decreases in viscosity. Also, unwashed nuclei are subjected to extremely mild conditions compared to washed nuclei. Thus, the lower viscosities of washed nuclei could be attributed to the loss of nuclear material, or the breakdown of nuclear structures in the washing process.

It may be mentioned that in vivo measurements of the type reported here have been undertaken with limited success. Very few live cells take up ethidium bromide. In rare cases where data were obtained in vivo, they corresponded closely to the in situ measurements reported here.

A matter of interest is the extent to which available binding sites are filled. If [B] is the concentration of free dye, then K[B] is the ratio of filled to empty binding sites. Since [B] is less than or equal to $3 \cdot 10^{-8}$ M, we may conclude that K[B] is much less than one. Thus, well over 90% of the binding sites are unoccupied.

Another point of interest is the question of dye binding in the nucleolus. It is possible to translate the nucleus across the beam axis, so that the beam passes through the nucleolus. Initially, fluorescence intensity from the nucleolus is very strong. Within minutes, the signal decays to levels found elsewhere in the nucleus. Rapid recovery is not observed. Values of τ_+, and $\langle i \rangle^2/G(0)$ measured in the nucleolus are not significantly different from values measured elsewhere in the nucleus. These observations suggest that ethidium bromide is bound preferentially in the nucleolus, but that the binding sites become damaged, or they become blocked by damaged dye molecules. In any event, the binding that is particular to the nucleolus is not readily observed in the fluctuation measurements.

It may be asked whether measurements from different sites in the nucleus are statistically different from each other. This question was not considered in detail. However, certain general observations were made. When repeated measurements were made at the same point in the nucleus, the variability among measurements was about 10%. Similarly, average variations among measurements from different sites in the same nucleus were approximately 20%. Measurements from different nuclei, including nuclei prepared on

different days could vary by 50% or more. No attempt was made to correlate variations to morphological features.

CONCLUSIONS

One goal of the research reported here is to establish fluorescence correlation spectroscopy as a suitable technique for probing the cell nucleus. To that end, measurements have been made consistently and reproducibly on well defined and easily prepared cell nuclei. Many questions of biological interest can now be considered in this manner. In principal, vital stains can be used, instead of ethidium bromide, that could extend fluorescence correlation spectroscopy to individual living cells as they pass through the cell cycle.

To be sure, there are difficulties, and many situations can be handled more naturally with other experimental procedures. But in matters involving cell nuclei and relatively few molecules, it is now reasonable to consider fluorescence correlation spectroscopy as one of only a few workable probes.

Hydrodynamic properties and information about binding are emphasized in the experiments described above. However, by choosing appropriate conditions, $G_0(\tau)$ can be made to dominate, giving primarily hydrodynamic data. The observed fluorescence intensity per diffusing molecule can help distinguish among various possible sources of the correlated fluctuations.

Hopefully, extensions of this work will answer many important questions about the biology of the cell nucleus.

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FIGURE CAPTIONS

Figure 1. The apparatus used for Fluorescence Correlation Spectroscopy is shown schematically. Illumination is provided by an argon ion laser. An electro-optical feedback device minimizes intenstiy fluctuations in the laser beam. A spatial filter rejects high spatial frequency noise in the beam intensity profile. The sample and beam can be observed simultaneously in the microscope. The sample and focal plane of the beam can be displaced relative to one another by moving either the sample stage of the microscope or lens L1. A photomultiplier detects fluorescence from the sample and the signal is processed electronically, as described in the text, to compute the autocorrelation function of the fluctuations in the fluorescence intensity arising from the diffusion of fluorescent molecules through the illuminated region.

Figure 2. The focal plane of the illuminating beam may be displaced relative to the plane of the sample. The area of the illuminated region depends quadratically on the relative displacement. Both the diffusion time, τ_0, and the number of diffusing fluorescent molecules in the beam, $\langle i \rangle^2/G(0)$, are proportional to the area of the illuminated region. The beam waist radius can be estimated from both types of measurement. In addition, one may estimate the diffusion coefficient from the correlation times, and the two-dimensional concentration from values of $\langle i \rangle^2/G(0)$.

Figure 3. Histograms of DNA content per cell are generated by flow cytometry, after staining with propidium iodide. Each histogram

refers to a different preparation of TC-7 cells. a) Cells were grown in culture dishes for at least one week. They were harvested after forming a confluent population. b) Cells were stimulated to grow by splitting confluent populations into quarters, and transferring the cells to culture dishes in fresh medium with 10% fetal calf serum. Cells were harvested 24 hours after transfer. c) Cells were grown on microscope cover slips in culture dishes for at least one week. They were harvested after forming a confluent population. d) Cells were stimulated to grow on cover slips by splitting confluent populations into quarters and transferring cells in fresh medium with 10% fetal calf serum into culture dishes containing clean cover slips.

Figure 4. Diffusion times were determined by fitting the observed fluorescence fluctuation autocorrelation functions to functions of the form given by Eqn. 8. The data refer to the diffusion of ethidium bromide molecules in small regions of the nucleus, slowed by the binding to and release from chromatin binding sites. Distributions of diffusion times are shown for nuclei isolated from the four classes of cells indicated in Fig. 3. a) Washed nuclei from resting cells were isolated from a population of confluent cells by lysing cells in suspension, and washing the nuclei with saline. b) Washed nuclei from cells stimulated to grow were isolated from cells harvested 24 hours after being transferred in fresh medium containing 10% fetal calf serum. The cells were lysed in suspension, and the nuclei were washed in saline. c) Unwashed nuclei from resting cells wer obtained

from a population of confluent cells, attached to microscope cover slips. The cells were treated with detergent to disrupt the plasma membranes, leaving part of the membrane, the nucleus and some of the cytoplasm attached to the cover slip. d) Unwashed nuclei from cells stimulated to grow were obtained from cells stimulated to grow on cover slips by transferring in fresh medium containing 10% fetal calf serum. Cells were treated with detergent 24 hours after transfer to disrupt the plasma membranes, leaving the nucleus part of the plasma membrane and some of the cytoplasm attached to the cover slip.

Figure 5. The number of diffusing ethidium bromide molecules in the illuminated region is given by $\langle i \rangle^2/G(0)$. Data in Figs. 4 and 5 were obtained from the same autocorrelation functions. Both the diffusion time, τ_+, and $\langle i \rangle^2/G(0)$ will increase when the ratio of bound to free dye concentrations increases.

Figure 6. By combining the data for τ_+ with the corresponding $\langle i \rangle^2/G(0)$ values, the viscosity of the nucleus relative to the viscosity of water can be inferred. Histograms of the inferred relative viscosity are shown for the nuclei referred to in Figs. 4 and 5. These values for the inferred viscosity assume that the diffusion coefficient of ethidium bromide in water is $6 \cdot 10^{-6}$ cm^2/sec, that the concentration of free ethidium bromide in the beam is $3 \cdot 10^{-8}$ M, and that the light path through the nucleus is 8 μm.

Table I. Results for Well Characterized DNA-Ethidium Bromide Preparations.

The autocorrelation function of fluctuations in the fluoresecnce intensity was measured for different preparations of DNA in the presence of ethidium bromide. Each autocorrelation function provides an estimate of the absolute number of diffusing fluorescent molecules, and the diffusion coefficient. The molecular mass may be inferred from the number concentration, if the mass concentration is also known.

DNA PREPARATION	KNOWN DNA BASE PAIR CONCENTRATION (mM)	ETHIDIUM BROMIDE CONC. (mM)	EXPECTED NUMBER CONC. (nM)	OBSERVED NUMBER CONC. (nM)	EXPECTED DNA MOL. MASS (megadalton)	INFERRED DNA MOL. MASS (megadalton)	10^8 X DIFFUSION COEF. (cm^2/sec)
CALF THYMUS DNA	0.2	0.1	13	6	10	22	2.5
SV40 DNA	0.07	0.03	13.5	11	3.5	4.3	1.5
SV40 DNA IN AGAROSE GEL	0.15	0.001	1000	410	____	____	1.4
CALF THYMUS NUCLEOHISTONE PARTICLES	0.063	0.01	4.3	2.1	10	21	3.5

Caption for Table II.

Table II. Results for Cell Nuclei.

Nuclei were isolated from resting and growing populations of cells, either by gently disrupting plasma membranes or by lysing the cells and washing the nuclei. For each of the four preparations of nuclei, measurements were made of the diffusion coefficients and the number concentrations of ethidium bromide in small areas of individual nuclei. Both quantities are related to the ratio of the bound to free dye concentrations. From the diffusion coefficient and the number concentration, the ratio of bound to free dye and the viscosity of the nuclear material are inferred.

Table II.

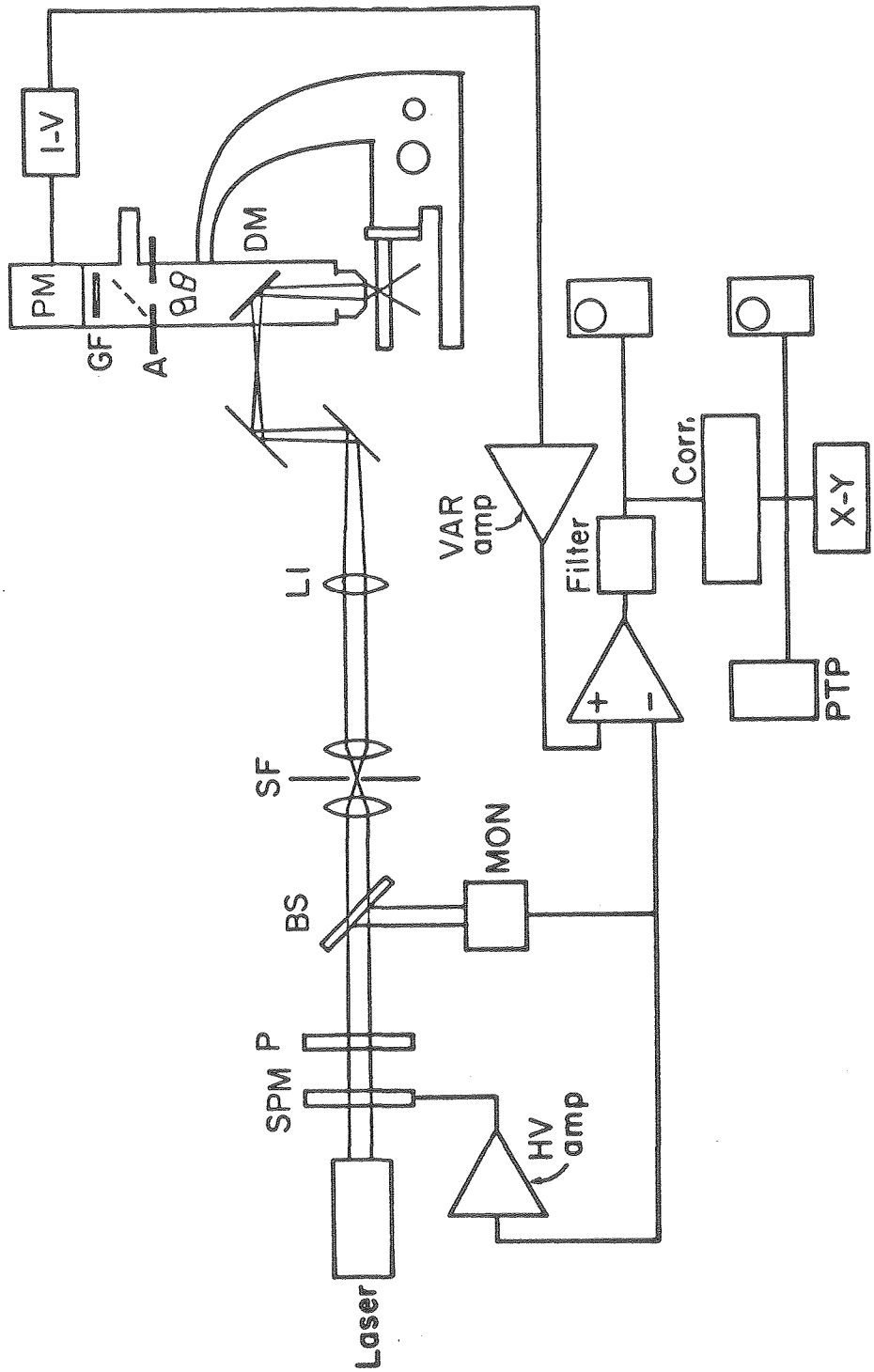
PREPARATION OF NUCLEI	A[1] AVERAGE τ_+ (msec)	B[2] 10^9 X DIFFUSION COEFFICIENT (cm^2/sec)	C[3] $(1 + K[A])\frac{n_n}{n_w}$	D[1] $\langle i \rangle^2/G(0)$	E[4] K[A]	F[1] $\frac{n_n}{n_w}$
WASHED NUCLEI FROM RESTING CELLS	285 ± 25	6.8 ± 0.6	870 ± 75	27000 ± 2000	77 ± 6	15.1 ± 2.0
WASHED NUCLEI FROM STIMULATED CELLS	280 ± 20	6.9 ± 0.5	880 ± 65	38000 ± 3000	108 ± 9	10.0 ± 0.8
UNWASHED NUCLEI FROM RESTING CELLS	800 ± 75	2.4 ± 0.2	2500 ± 230	17000 ± 1300	48 ± 4	56.5 ± 6.8
UNWASHED NUCLEI FROM STIMULATED CELLS	700 ± 75	2.5 ± 0.2	2400 ± 230	56000 ± 3000	160 ± 9	18.2 ± 3.0

(1) Values are means ± standard error of the mean.

(2) Diffusion coefficient, assuming a beam radius of 0.88μm.

(3) Values are given by the observed diffusion coefficient divided by $6 \cdot 10^{-6}$ cm^2/sec.

(4) $\langle i \rangle^2/G(0)$ measures the number of bound dye molecules in the volume $\pi w^2 L$. L is taken to be 8μm, w is taken as 0.88μ, and the free dye concentration is taken to be $3 \cdot 10^{-8}$ M. Values in column E are the ratios of free to bound dye concentrations.



XBL7812-13067

Fig. 1

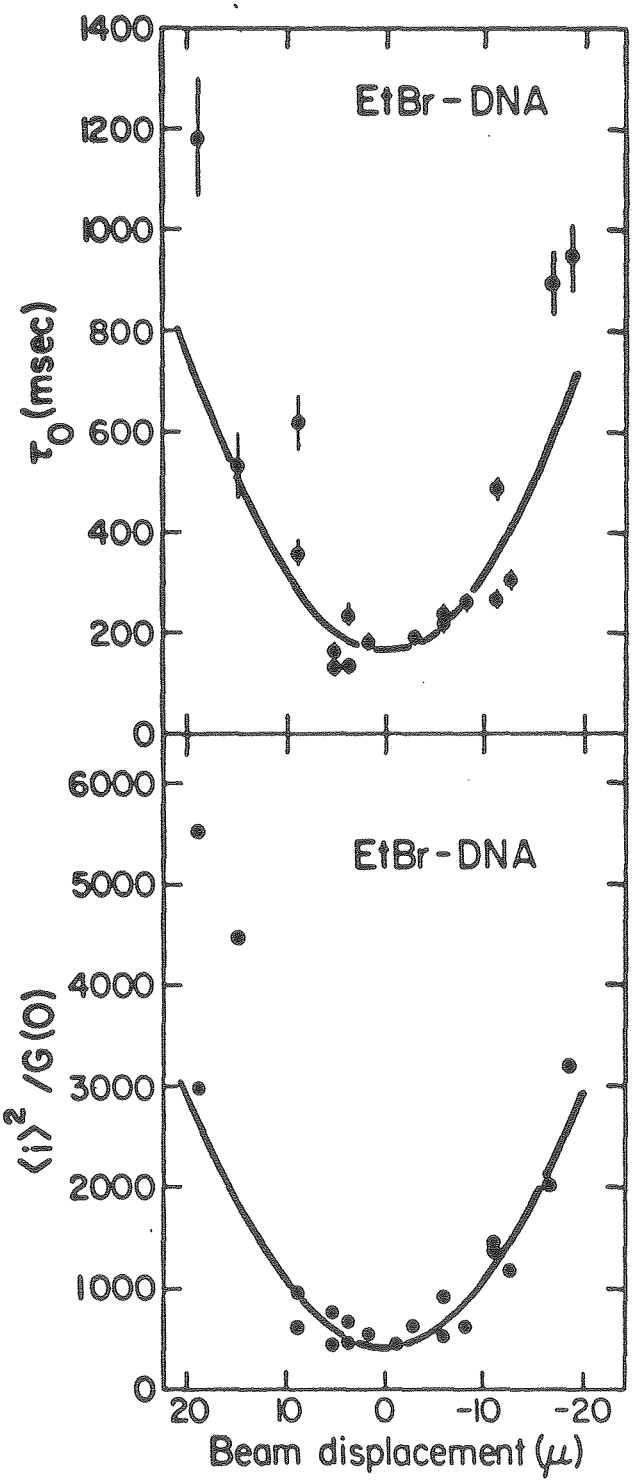


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Fig. 2

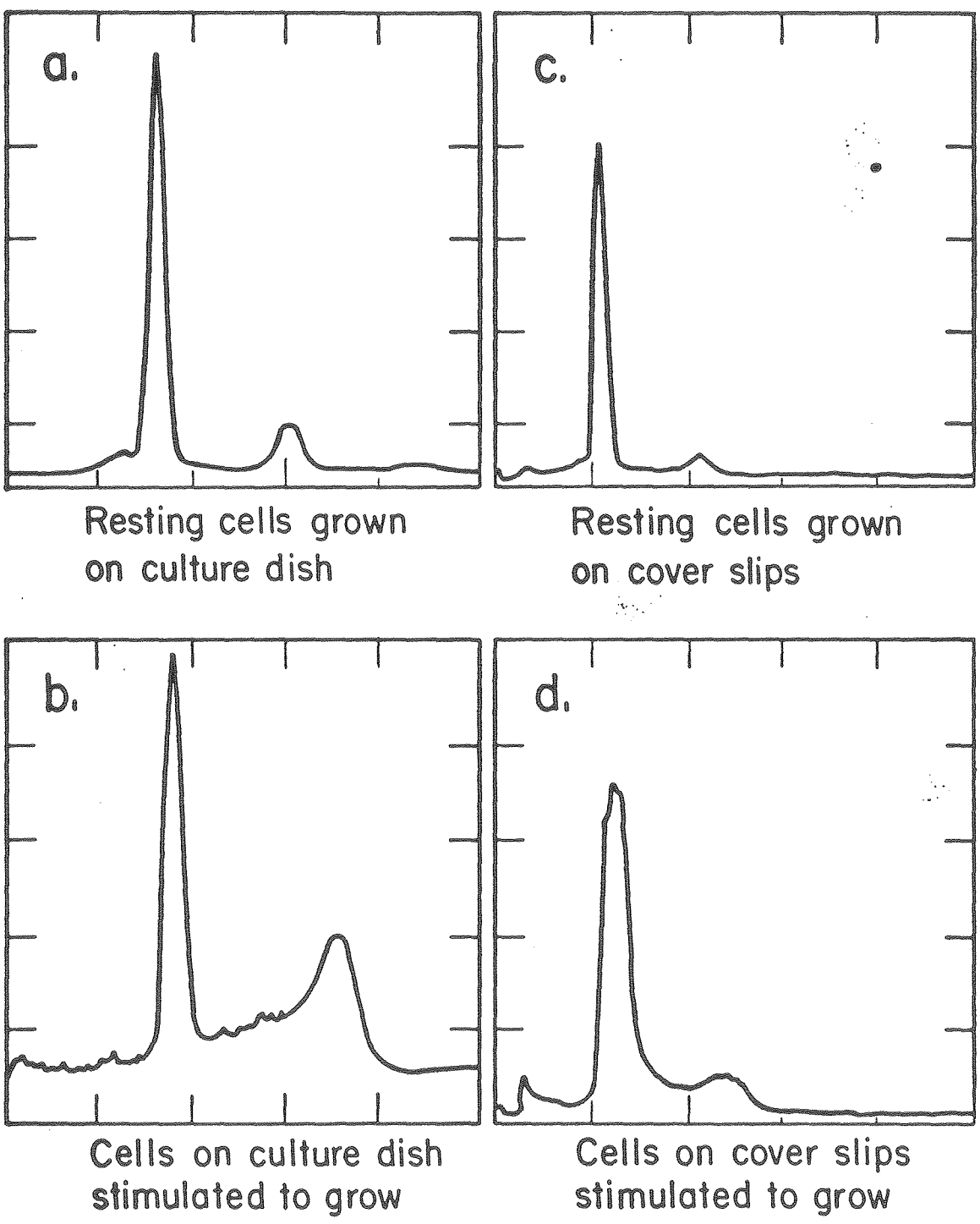
DNA Content
a.
c.
Resting cells grown on culture dish
Resting cells grown on cover slips
b.
d.
Cells on culture dish stimulated to grow
Cells on cover slips stimulated to grow
XBL 7910-5066

Fig. 3

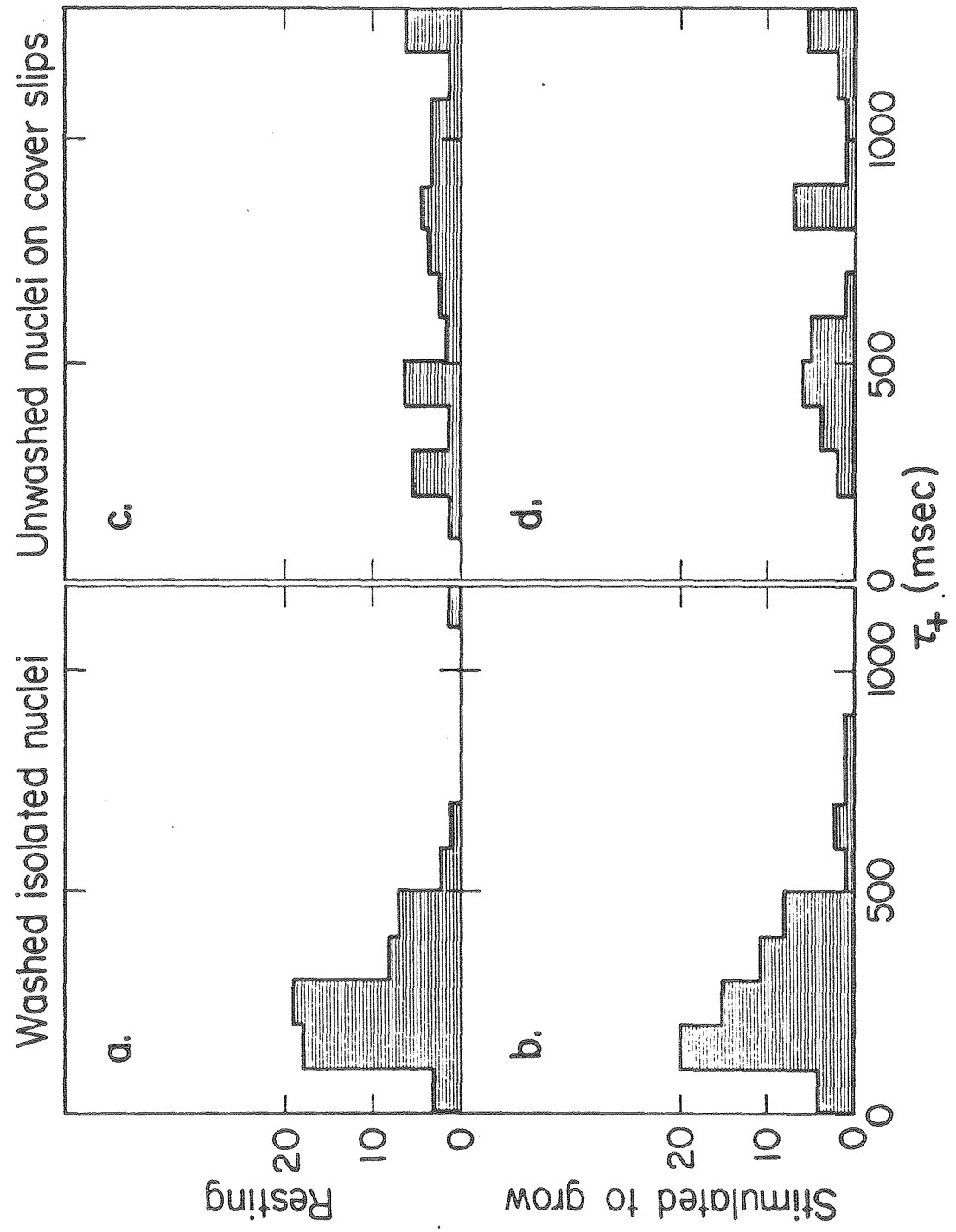

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Fig. 4

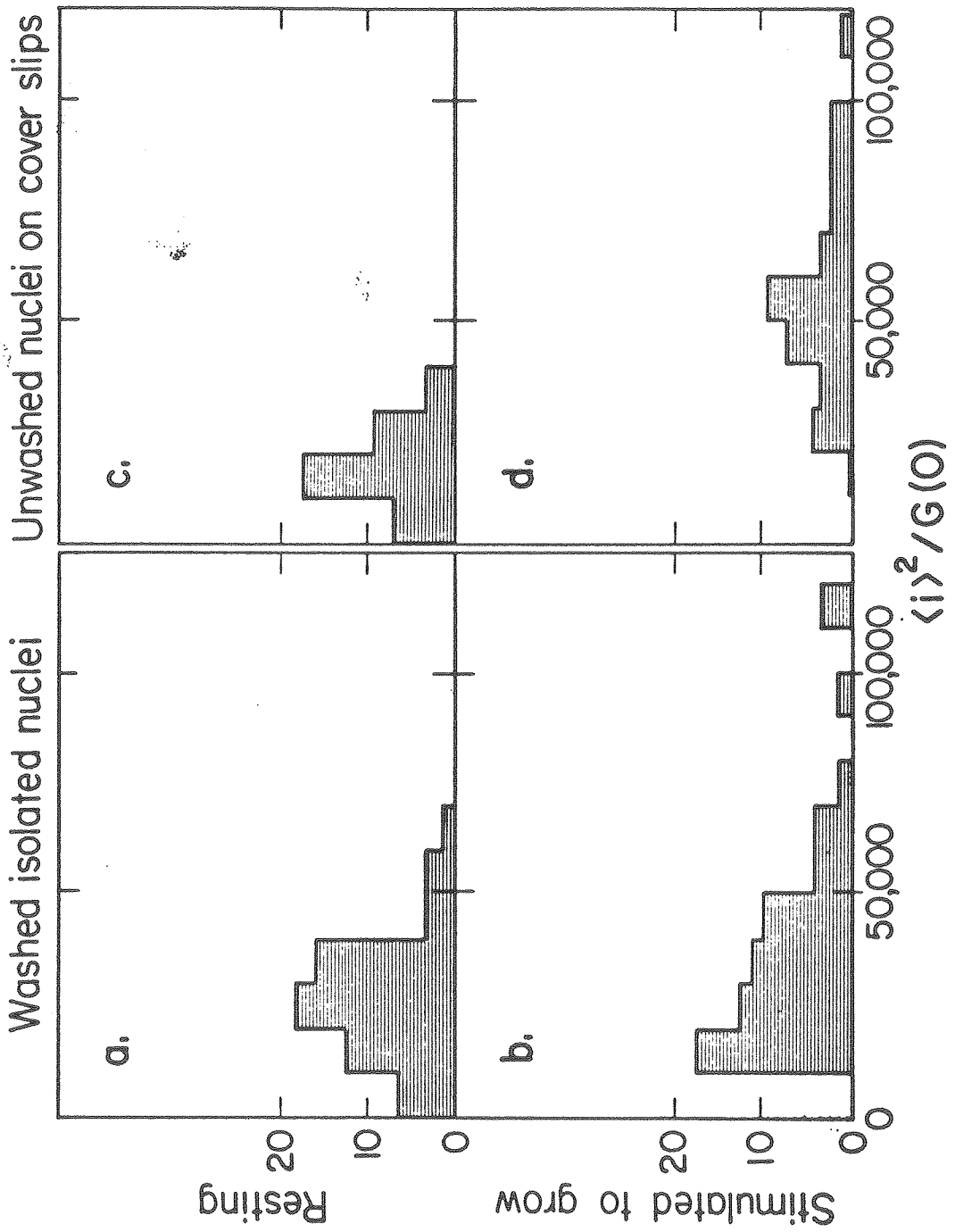

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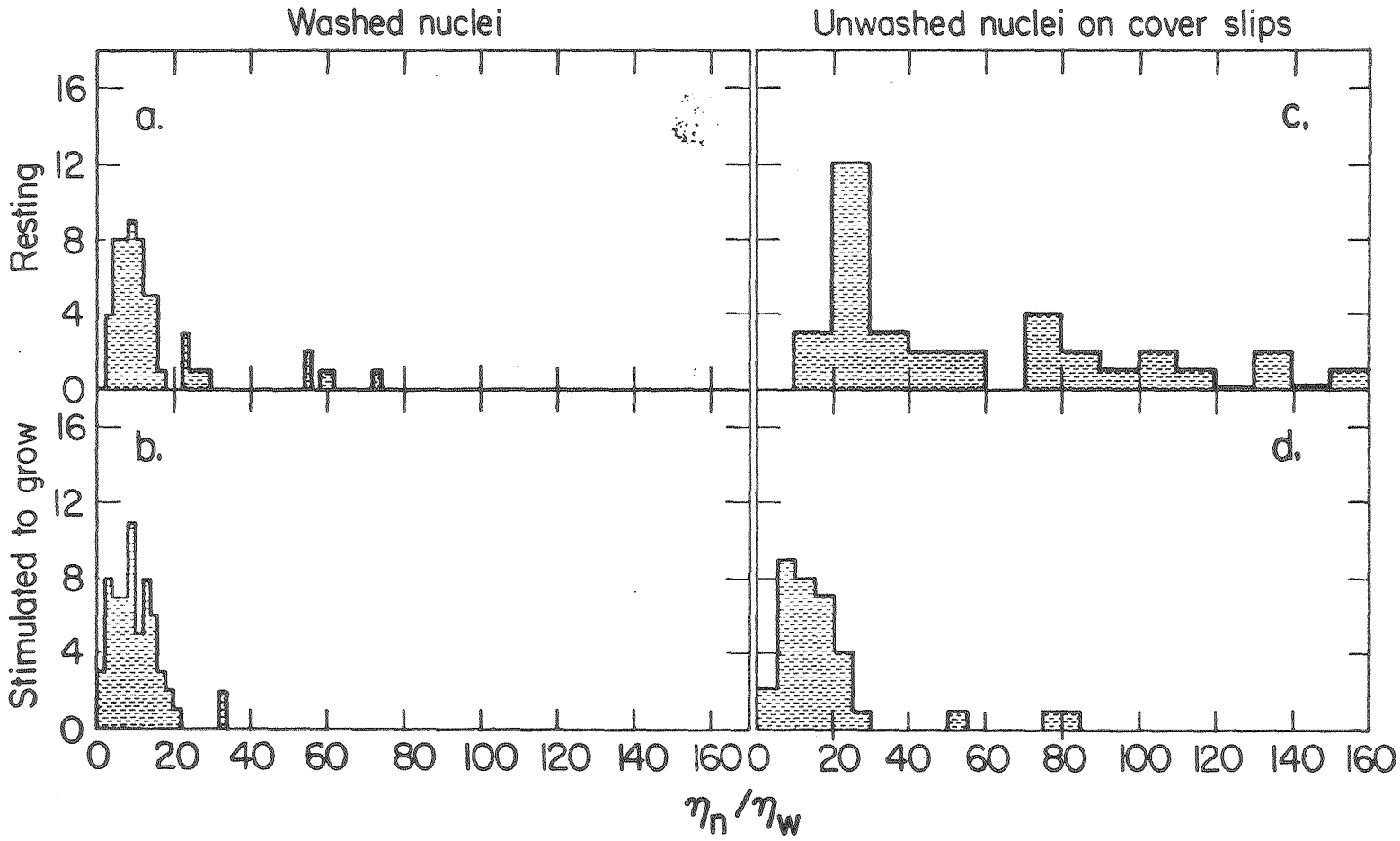
Fig. 5



XBL 7910-5062 A

Fig. 6