Journal of Fluorescence, Vol. 15, No. 2, March 2005 (© 2005)
DOI: 10.1007/s10895-005-2526-2

Propidium Iodide and PicoGreen as Dyes for the DNA Fluorescence Correlation Spectroscopy Measurements

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Received May 21, 2004; accepted July 24, 2004

Many experimental designs, in which nucleic acid conformational changes are of interest, require reliable fluorescence labeling. The appropriate fluorescence probe should have suitable optical properties and, more importantly, should not interfere with the investigated processes. In order to avoid chemical modifications the fluorescence label needs to be associated with nucleic acid via weak non-covalent interactions. There are a number of fluorescent probes that change their fluorescent properties (i.e. their quantum yield and/or spectral characteristics) upon association with nucleic acid. Such probes are frequently used to detect, visualize and follow processes involving nucleic acid and its conformational changes. In order to obtain reliable data regarding macromolecule or aggregate topology a detailed knowledge of probe–nucleic acid interactions on the molecular level is needed. In this paper we show that the association of propidium iodide with DNA alters its conformation and that it selectively labels plasmid fragments and/or its subpopulations in a concentration-dependent meaner. Another dye, PicoGreen, exhibits better properties. It labels nucleic acid uniformly and without any concentration-dependent artifacts.

KEY WORDS: Nucleic acid; fluorescence labeling; PicoGreen; propidium iodide.

INTRODUCTION

Fluorescent probes are commonly used for nucleic acid detection in gels and for in situ labeling during various microscopic and analytical protocols [1,2]. When used for imaging purposes without further needs of quantitative analysis the labeling conditions are not of a great relevance. However, when quantitative data regarding interactions on the molecular level are needed, then precise correlations between the amount of nucleic acid, the probe concentration and resulting fluorescence intensity are critical. A detail understanding of the molecular basis underlying interactions between nucleic acids and fluorescent labels must be prerequisite to the successful experiment when processes involving interactions between nucleic acids and other compounds are studied [3–5]. Labeling has to be quantitative and the probe should not interfere with the investigated processes. Fluorescent dyes whose intensity increases upon non-covalent binding to nucleic acids are frequently used in the detection and quantification of nucleic acid [1,6–9]. These dyes are very convenient, easy to use and can be employed during any stage in the process of interest. Nonetheless, there are reports that point to some difficulties associated with such dye applications. It has been shown, for example, that ethidium bromide has sequence specificity and that its association may affect

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1053-0509/05/0300-0179/0 © 2005 Springer Science+Business Media, Inc.

nucleic acid migration rate on gels [10,11]. In addition, there are reports that the amount of dye affects the quantitative experiment outcome [11,12]. The development of synthetic carriers for genetic material is a field where the fluorescence labeling of nucleic acids is a convenient way of determining resulting aggregate topology and all its intermediate structures. For example, the decrease in propidium iodide or ethidium bromide fluorescence intensity is considered to be a measure of nucleic acid condensation induced by polycations [13–17]. In order to follow processes where a large number of molecules forming the aggregate interact, the fluorescent label needs be a passive reporter of the process or at least the conditions under which it can be used need to be precisely determined. In this paper we address such problems by measuring how the amount and type of fluorescent dye affects DNA conformation. Fluorescence Correlation Spectroscopy (FCS) is used. This fluorescent technique allows for a statistical analysis of the events occurring on the single molecule level. Based on such data, conclusions regarding sample topology and homogeneity can be obtained. This approach has already been employed to detect plasmid condensation induced by cationic compounds [14–16].

MATERIALS AND METHODS

Materials

The pH βApr-1-Neo plasmid (10 kbp and contour length 3.4 μm) was a generous gift from the laboratory of Prof. Maciej Ugorski (Department of Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland). It was prepared as described elsewhere [18], with slight modifications during the final purification stage [15–16]. Propidium iodide and PicoGreen dies were purchased from Molecular Probes (Leiden, The Nederlands). All experiments were performed in a TE buffer (pH 7.95, 10 mM Tris, 1 mM EDTA).

Agarose Gel Electrophoresis

Samples of plasmid were labeled beforehand with propidium iodide or PicoGreen than loaded onto 1.0% agarose gel and electrophoresed at 80 V in TAE buffer. Gels were scanned and analyzed with UV light.

FCS Measurements

Fluorescence measurements were performed on a ConfoCor® 1 (Carl Zeiss Jena, Germany), as described elsewhere [15,16,19]. In short, ConfoCor 1 is a PC-controlled fluorescence correlation-adapted AXIOVERT 135 TV microscope, equipped with an x–y–z adjustable pinhole, avalanche Photodiode SPCM-200-PQ, ALV-hardware correlator, and CCD camera. The Ar^+-laser beam (excitation wavelength, 514 nm) was focused by a water-immersion microscope objective at an open focal light cell. The size of the confocal volume element, calibrated with rhodamine-6G, was determined to be approximately 1 fL.

A digital hardware correlation interface processed fluorescence intensity fluctuations around a temporal average $I(t) = \langle I(t)\rangle + \delta I(t)$ and yielded a normalized autocorrelation function $G(t) = 1 + \langle \delta I(t)\delta I(t+\tau)\rangle / \langle I(t)\rangle^2$. The general solution of this three-dimensional autocorrelation function $G(t)$ for translational diffusion in an ellipsoidal confocal volume is:

$$G(t) = 1 + \frac{1}{N} \cdot \frac{1}{1 + (t/\tau_D)} \left\{ \frac{1}{1 + (t/\tau_D)\left(\omega_1^2/\omega_2^2\right)} \right\}^{1/2} \quad (1)$$

where ω_1 is the radius of the volume element in the xy-plane, ω_2 its half-length in the z-direction and N the particle number. When τ_1 and τ_D are known, the diffusion coefficient D can be determined from $\tau_D = \omega_1^2/4D$. All calculations were performed assuming that fluorescence decay (τ_f) and translational diffusion τ_d were well separated in time ($\tau_f \ll \tau_d$). When the theoretical expression did not fit satisfactorily to experimental data, a term describing a second diffusing species labeled with the same fluorophores was added. A detailed description of the theoretical analysis is given in review articles [20,21] and in our recent publication [19].

Samples were prepared for FCS measurements as follows. An appropriate amount of solution of DNA and TE buffer (ranging from 1 to 2 nM) was placed into the chamber with the glass bottom facing towards the ConfoCor water-immersion microscope objective. This was titrated with a propidium iodide or PicoGreen stock solution. Propidium iodide concentration was predetermined spectroscopically, whereas PicoGreen was used according to supplier guidelines. All experiments were carried out at room temperature. Experimental data were fit by FCS ACCESS evaluation software (1-component fitting, with triplet state consideration).

RESULTS AND DISCUSSION

The plasmid was labeled with various amounts of propidium iodide and its mobility in the aqueous phase was evaluated using Fluorescence Correlation

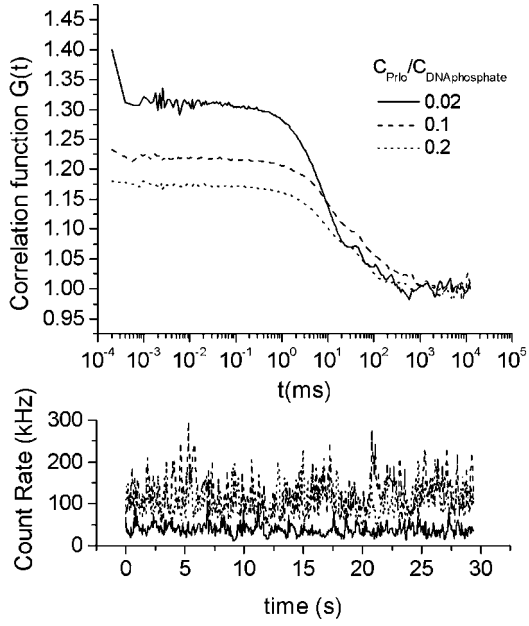

Fig. 1. Normalized fluorescence autocorrelation functions obtained for plasmid (βApr-1-Neo, 10 kbp and contour length 3.4 μm) labeled with propidium iodide and measured at three $C_{PrIo}/C_{DNA\,phosphate}$ ratios, namely 0.02 (solid line), 0.1 (dashed line) and 0.2 (dotted line). Corresponding fluorescence count rates are shown in the lower panel.

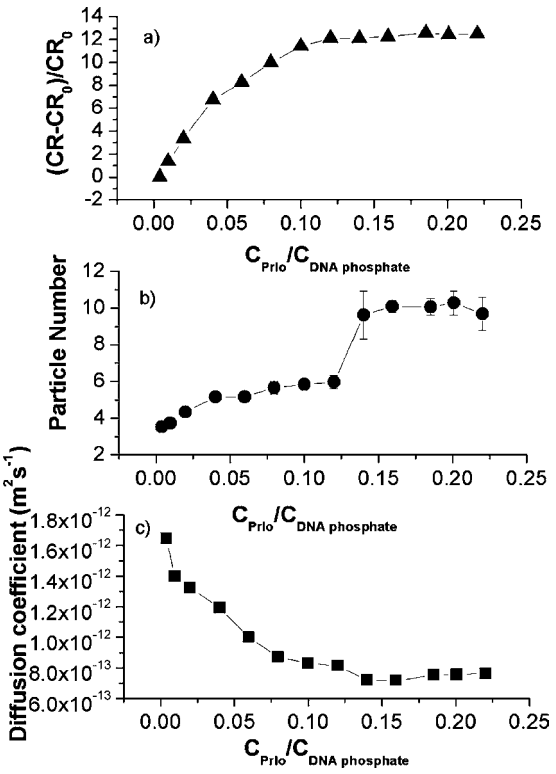

Fig. 2. Titration curve for the propdium iodide. The dependence of the βApr-1-Neo plasmid (10 kbp, contour length 3.4 μm) diffusion constant (D_t), Particle Number, and relative count rate $(CR–CR_0)/CR_0$ on the $C_{PrIo}/C_{DNA\,phosphate}$ ratio.

Spectroscopy. Figure 1 presents selected examples of the autocorrelation function for various ratios of probe and DNA phosphate groups. The autocorrelation function shows distinct differences between various labeling conditions. Calculated diffusion coefficients, particle numbers and relative fluorescence intensities for a specific amount of DNA as a function of dye concentration are summarized in Fig. 2.

The normalized fluorescence intensity increases monotonously with rising dye concentration and stabilizes at a level of 12 a.u., at which the dye/phosphate group ratio reaches 0.12 (Fig. 2C). The saturating character of fluorescence intensity change indicates that the dye successively fills all available binding seats on nucleic acid molecules. This rise in fluorescence intensity is accompanied by a systematic decrease in the diffusion coefficient. Its value falls from 1.6×10^{-12} to 8×10^{-13} m^2 s^{-1} with rising dye concentration (Fig. 2A). When the probe/phosphate group ratio reaches 0.15, the diffusion coefficient levels out at its minimum value of about 8×10^{-13} m^2 s^{-1}. The extent to which the diffusion coefficient changes cannot be explained by a simple increase in plasmid mass, which in turn is controlled by dye association (assuming that each propidium iodide molecule binds with four DNA bases, the mass should increase by less than 25%); larger mass differences are needed to account for the observed effects [20]. At first glance, the decreasing diffusion coefficient may be interpreted in terms of dye-induced conformational changes and/or plasmid aggregation. The second possibility can be excluded, because of the parallel rise in particle number (Fig. 2B). Aggregation should cause an opposite effect. Consequently, a decreasing diffusion constant should reflect an alteration in plasmid conformation upon the binding of propidium iodide (a similar effect was observed for the other dye—ethidium bromide [11]). A decreasing diffusion constant may indicate conformational changes for the plasmid: from small hydrodynamic size (i.e. the supercoil) to larger, more relaxed forms [22]. Additional information is available from the dependence of particle number on propidium iodide concentration. At the probe/phosphate group ratio of 0.15 there is a sharp rise in the number of particles detected. This result may indicate that there are two different plasmid populations that interact with the fluorescent dye in a different and concentration-dependent manner.

The picture that emerges from the presented experimental data is rather complicated. Two subpopulations of plasmid need to be assumed. These subpopulations are likely to have different affinities towards the fluorescent probe and different hydrodynamic sizes. In addition, both

parameters are dependent on fluorescent probe concentration. Taking into account all available data, the following model can be proposed. At very low dye concentrations, there are two plasmid subpopulations. One does not bind fluorophore at all, while the other changes its conformation upon associating with propidium iodide with rising dye concentration, which results in a decreasing diffusion coefficient. However, only the latter subpopulation is detectable. When dye concentration is higher the second plasmid subpopulation becomes visible, causing a sudden rise in particle number (Fig. 2B).

In order to test the two subpopulation hypothesis, gel electrophoreses in carefully selected conditions have been performed. As shown in Fig. 3, there is only one fluorescent bend at lower probe concentrations. Two bends are clearly visible when the propidium iodide/phosphate group ratio reaches 0.2 (Fig. 3 panel a), in accordance with the FCS experiment (Fig. 2C). When a saturating amount of fluorescent probe was added (Fig. 3b), the two bands of DNA are clearly visible and their separation depends on propidum iodide concentration in the initial staining. The same effect was observed when ethidium bromide was used as a detecting agent (results not shown).

The experimental data show that propidium iodide is not a good indicator when the conformational alteration of DNA is concerned. Obtained results strongly depend on dye concentration, and data interpretation is difficult and requires a number of assumptions. All this makes the application of propidium iodide very questionable when DNA conformation and/or its alteration needs to be monitored.

Similar experiments have been performed using a second fluorescent probe—PicoGreen. The results of these experiments are presented in Fig. 4. They show that the dependencies of all measured parameters on the amount of fluorescent probe are qualitatively different from that for propidium iodide. The increase of dye-DNA aggregate mass is less than 30% for both propidium iodide and PicoGreen, which is not sufficient to cause the variations detected experimentally [21]. Therefore, the differences between propidium iodide and PicoGreen, in particular the dependence of their diffusion constant on fluorescent dye concentration, cannot be explained by various extents of dye binding alone. Despite steadily increasing fluorescence intensity, the diffusion coefficient remains constant throughout the entire PicoGreen concentration range and is equal 10^{-12} m^2 s^{-1}. This shows that PicoGreen does not alter plasmid conformation and uniformly labels all molecules in the sample within the concentration range used. This is supported by the dependence of particle number on dye concentration. The final number of detected particles was 10–12, in agreement with the propidium iodide experiment. This implies that there are no unlabelled particles in the preparation.

Surprisingly, when the gel electrophoreses was performed using PicoGreen as a label the same bends distribution was observed as in the case of propidium iodide (Fig. 3). There are two DNA similar subpopulations detected with the two dyes. This result makes the picture emerging from FCS measurements even more difficult to explain. A number of additional assumptions need to be made in order to make the proposed plasmid

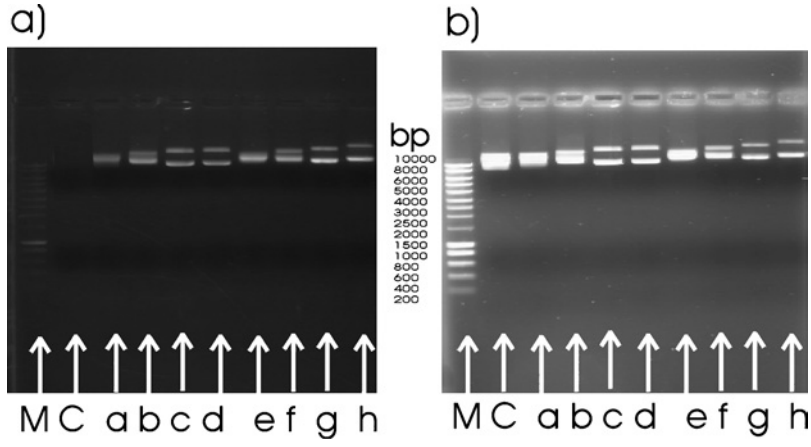

Fig. 3. Gel electrophoresis. Panel (a) gel electrophoresis at different $C_{PrIo}/C_{DNA\ phosphate,}$ ratios (lines a, 0.02; b, 0.1; c, 0.2; d, 0.4) and different $C_{PicoGreen}/C_{DNA\ phosphate}$ ratios (lines e, 0.05; f, 0.09; g, 0.13; h, 0.15). Lines: M – marker, C – pure DNA plasmid βApr-1-Neo (10 kbp, contour length 3.4 μm), not stained. Panel (b) UV visualization of the gel shown on the panel (a) after 0.5 hr incubation in the ethidium bromide bath (concentration of the ethidium bromide 0.5 μg/ml).

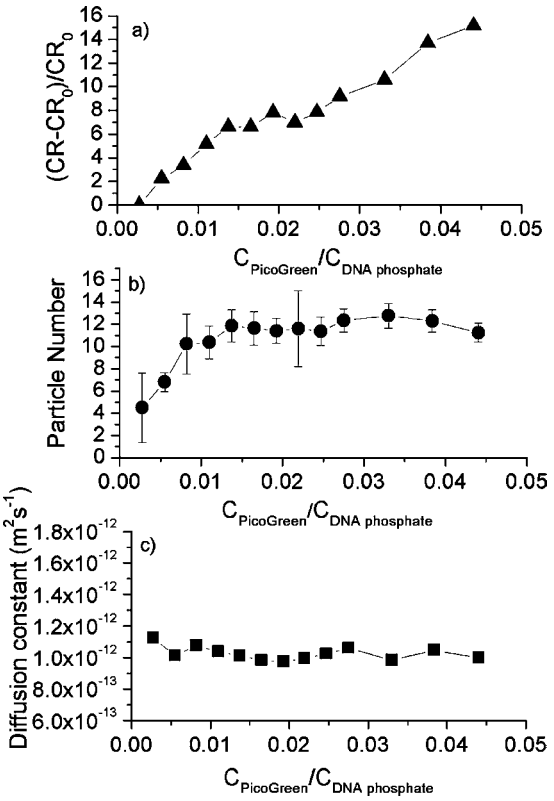

Fig. 4. Titration curve for the PicoGreen. The dependence of βApr-1-Neo plasmid (10 kbp, contour length 3.4 μm) diffusion constant, D_t, Particle Number, relative count rate $(CR–CR_0)/CR_0$ and correlation on the $C_{PicoGreen}/C_{DNA\ phosphate}$ ratio.

subpopulation coexistence self-consistent. There is, however another possibility that would explain the differences between the two dyes. As it has been shown previously, the DNA molecule may change its conformation locally, upon interaction with other compounds, meaning that the dye may intercalate within the certain regions of nucleic acid whereas remaining part of the molecule conformation is not altered. Such possibility will result with a number of distinct fluorescent regions. Such regions will be detected with FCS as separate events causing the increasing number of particles detected with simultaneous decrease of the diffusion constant. This interpretation implies that both dyes alter the nucleic acid conformation but to a different extend and/or in a different manner what may result from differences in labeling molecules locations. This hypothesis requires further experimental and theoretical analysis.

CONCLUSIONS

Data presented in this paper show that FCS is a very sensitive technique for determining the quality of DNA labelling with fluorescent probes. It was possible to show that when propidium iodide was used to stain plasmids, two different subpopulations of labelled molecules or different conformations were detected. Plasmid conformation depends on the type of the dye used, as manifested by the dependence of the diffusion coefficient and particle number on propidium iodide and PicoGreen concentrations. Presented data shows that when processes where information concerning the topology of nucleic acid is of interest the selection of the appropriate fluorescent label should be of a major concern.

ACKNOWLEDGMENTS

Financial support by the Czech Academy of Sciences (Martin Hof via 1ET400400413 informacni spolecnost) and Grant Agency of the Czech Republic (Teresa Kral via 203/05/2308) is gratefully acknowledged.

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