Analysis of Hybridization and Measuring of Kinetic Parameters

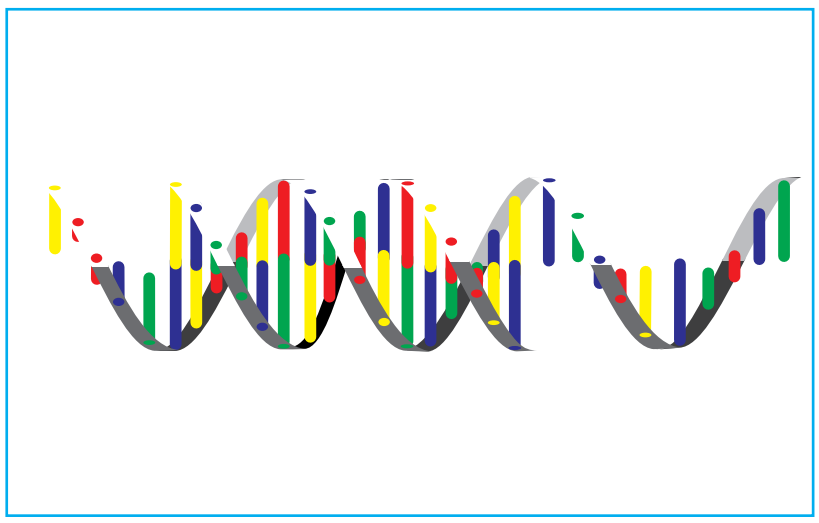


Fig.1: Hybridization of nucleic acids.

M. Kinjo (1,2), R. Rigler (1), K. Wyzgol (3) and S. Sterrer (3).
(1) Karolinska Institute, Stockholm, Sweden.
(2) Research Institute for Electronic Science, Hokkaido University, Sapporo, Japan.
(3) EVOTEC BioSystems GmbH, Hamburg, Germany.

INTRODUCTION

Hybridization, which is widely used for gene detection, furthers not only our understanding of the structure, organization and expression of genes, but also of higher order nucleic acid structures. Hybridization can be quantified in a variety of different ways by a range of different methods.

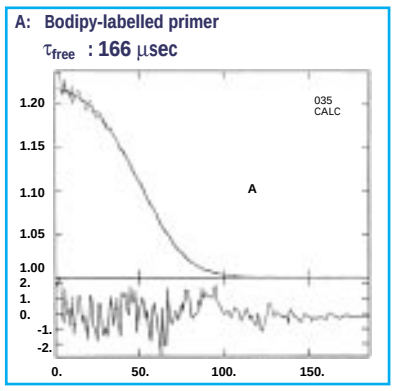
The use of fluorescence correlation spectroscopy (FCS) to monitor hybridization lends a new degree of sensitivity to the study of this process.

In FCS, the thermal fluctuations of molecules excited to fluorescence are observed and correlated. When excitation and observation are carried out in extremely small, confocally defined volume elements, fluctuations can be observed at the level of individual molecules.

The interaction between a fluorescent ligand and a larger target can be represented by the correlation of bound versus free ligands. This eliminates the need for separation, e.g. on a filter or by column chromatography.
We have evaluated the usefulness of FCS analysis for the study of the hybridization process using a test system based on the interaction between an 18-base oligonucleotide and M13 template DNA.

MATERIALS AND METHODS

Tetramethyl-rhodamine (TMR)-labeled sequencing primers (Fluoro Tide M13/pUC (-21) primer) were purchased from Molecular Probes and M13mp18(+)-strand DNA (7250 bases) from Pharmacia. FCS studies were performed with standard filter sets for rhodamine-6G using an argon laser (Coherent, 3W, 514 nm) and a C-APOCHROMAT 40x/1.2 W objective (Zeiss). For kinetic experiments, 50 nM TMR-labeled primer and 50 nM M13 DNA were incubated in 0.18 M NaCl at 40°C for varying lengths of time in a final volume of 20µl. All FCS measurements (each sample was measured four times for 30 s) were carried out at 20°C. For titration experiments, the concentration of template DNA was fixed at 50 nM and the concentration of the primer was varied. The incubation was carried out at 40°C for 130 min. Data was fitted using the FCS ACCESS software package.



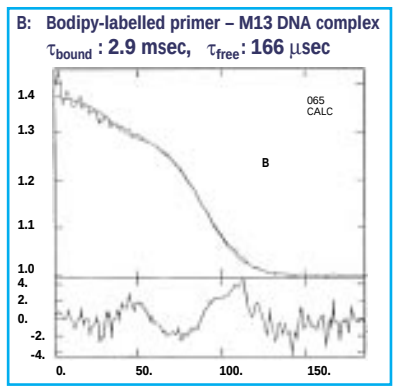


Fig.2: Autocorrelation function of an M13 primer labeled with TMR (Molecular Probes), free and complexed with M13 DNA; (A) free (50nM, τ_D = 166 µs), (B) 100 % complexed (τ_D = 2.9 ms).

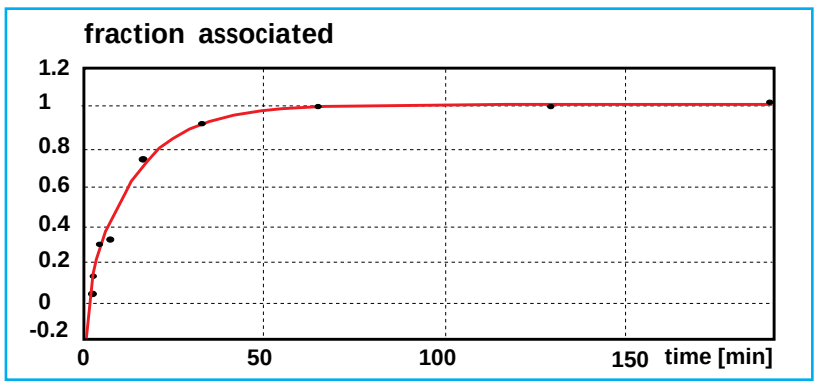


Fig.3: Time course of the interaction of primer with single-strand M13 template (50 nM primer, 50 nM template, 0.18 M NaCl, 40°C, k_{ass}=1.1x10^{5}M^{-1}s^{-1}).

RESULTS

The size of the confocal volume element determined by calibration with the free dye was about 0.2 fl. The formation of a complex between primer and template was easily identified by the change in the autocorrelation function (Fig. 2). Due to the difference between the translational diffusion time of the free primer (166 µs) and the bound primer (primer-template complex, 2.9 msec) we were able to quantify the fraction of primer hybridized without having to separate non-hybridized primer. The fraction of primer which hybridized with the template DNA was plotted versus time (Fig. 3). From these data the rate constant k for the association was determined by using the formula $y(t) = 1-[1/(1+kC_o t)]$ where C_o = initial concentration of single-strand primer and single-strand template. From titration experiments, the number of binding sites for this primer in M13 DNA could be obtained.

The hybridization was saturated at a primer to template ratio of 2:1. This indicates that two primers may bind to one M13 template DNA and suggests the presence of an additional site with similar affinity under the conditions applied.

LITERATURE

M. Eigen and R. Rigler: *Sorting single molecules: Applications to diagnostics and evolutionary biotechnology. Proc. Natl. Acad. Sci. USA 91, 5740-5747, 1994.*

M. Kinjo and R. Rigler: *Ultrasensitive hybridization analysis using fluorescence correlation spectroscopy, Nuc. Acids. Res. 10, 1795-1799, 1995.*

R. Rigler: *Fluorescence correlations, single molecule detection and large number screening. Applications in biotechnology. J. of Biotechnology 41, 177-186, 1995.*

SUMMARY

FCS has proved to be a powerful tool capable of rapidly characteriz-ing the hybridization of nucleic acids in solution without the need for separation of the non-bound fraction. At nanomolar concentrations it was possible to quantify the stoichiometry, kinetics and thermodynamics of the interaction.

Apart from the application of FCS for the diagnosis of pathogens, the possibility of analyzing hybridization under physiologic conditions and measuring of kinetic parameters makes FCS an important tool for antisense studies.

Carl Zeiss Jena GmbH
Zeiss Gruppe • D-07740 Jena
Telefon: + 49-3641-643385
Telefax: + 49-3641-643311

EVOTEC BioSystems GmbH
D-22529 Hamburg • Grandweg 64
Telefon: + 49-40-56081-0
Telefax: + 49-40-56081-222