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Difficulties in the detection of individual virions by flow cytometry

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**DIFFICULTIES IN THE DETECTION OF INDIVIDUAL VIRIONS
BY FLOW CYTOMETRY**

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Inna M. Wilson

August, 1998

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ABSTRACT

DIFFICULTIES IN THE DETECTION OF INDIVIDUAL VIRIONS BY FLOW CYTOMETRY

by Inna M. Wilson

This thesis addresses the detection of individual virions by flow cytometry. Limited research has been done on this subject due to the very small size of the viruses and limitations on the sensitivity of commercially available flow cytometers. The strategy of the research was staining virions with new cyanine-based nucleic acid dyes having over 1,000-fold higher fluorescence when bound to nucleic acids, and identification of the virions by their fluorescent signal. Flow cytometric studies were performed on different microorganisms and DNA molecules including *Escherichia coli*, an endogenous retrovirus produced by CHO cells, Baculovirus, Murine Leukemia Virus, and *Saccharomyces cerevisiae* chromosomes.

Results revealed that the sensitivity of the FACSort was probably sufficient to detect at least larger viruses based on the fluorescent signal of the stained genome. However, flow cytometric analysis of three different virus models did not show any discrete population identifiable as virions. Potential reasons for this are discussed.

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INTRODUCTION

The original purpose of the project was the direct detection of individual viral particles in a culture supernatant by flow cytometry. The strategy was to use the recently invented cyanine-based nucleic acid specific dyes that had over 1,000-fold higher fluorescence when bound to nucleic acids than when in free form. This property should help to decrease the background fluorescence of the samples. The virions would then be identified by measurement of the fluorescent signal of the stained viral genome. This strategy would not give precise information that the viral genome was detected since nucleic acid specific dyes bound to any nucleic acid present in the sample (besides viral genomes there could be free nucleic acids, ribosomes, mitochondria). Nonetheless, at least two factors could be used to indicate that the observed fluorescent signal was due to stained viral genome. First, the concentration of the assayed virus, determined by Transmission Electron Microscopy (TEM), was known. The number of the fluorescent events per volume of sample could be calculated during flow cytometric analysis (FCA) based on the rate of fluorescent events in a population of interest and the rate of sample injection. Comparison of the concentration obtained by FCA with the known virus titer would then indicate whether the observed signal was due to stained virions. Second, if non-viral nucleic acids were present in the sample, they would probably be of various sizes, whereas all virions added to the sample would have the same genome size. The number of bound fluorochromes was expected to correspond to the length of the nucleic acid. We, therefore, expected that in DNA histograms the stained viral genome would give a peak, whereas signal from the non-viral particles would be more widely distributed. We also investigated light scatter to detect viruses, or to discriminate densely packed virions from other types of

fluorescent particles. Finally, other virus models and DNA molecules were studied for better understanding of virus staining and instrument performance.

LITERATURE REVIEW

Flow Cytometric Studies of Microorganisms

Flow cytometry can answer many microbiological questions which require the precise measurement of individual cells or particles. Although various types of microorganisms have been studied by flow cytometry during the last twenty years, this technique has contributed much less to the analysis of microorganisms than to eukaryotic, and primarily, mammalian cells. The basic problem for using flow analysis arises because of the small size of many microorganisms. This chapter reviews the flow cytometric work done on yeast, bacteria, and viruses. Since the main strategy of our research was based on genome staining, the studies that used a similar approach are emphasized.

Yeast

Flow cytometry was used to study the cell cycle by measuring DNA in yeast such as *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, which are important models for studying the cell cycle regulation of higher eukaryotes (16, 35). Flow cytometry was also used to determine the total genome size of yeast strains such as *S. cerevisia* (26) and *Candida albicans* (14), which are found in nature at a variety of different ploidy levels.

Yeast cells have a larger size and DNA content than bacteria or viruses, which makes them technically simpler to study using flow cytometry. However, they are still smaller in size and have approximately 300 times less chromosomal DNA than

mammalian cells (44). Thus, when the DNA distribution of yeast is studied, the fluorescence intensity of the stained nuclear yeast DNA is much lower than that of mammalian cells. This creates significant difficulties for using flow cytometry to study yeasts. Another problem is a much larger ratio of mitochondrial to nuclear DNA in yeast. Mitochondrial DNA comprises 5-20% of the total yeast DNA (10), which may contribute significantly to fluorescence during staining of the yeast genome. This is exacerbated by the fact that the mitochondrial number can vary greatly depending on culture conditions.

Bacteria

The main problem with flow cytometric measurements of bacteria comes from their small size and small DNA content. For example, the DNA content of *Escherichia coli* is about 4.6×10^6 base pairs (2), which is about 10^3 times smaller than the genome of a diploid human cell and about 40 times smaller than the DNA content of an average human chromosome. The volume of *E. coli* is about 10^3 times smaller than that of human leukocytes (39). As a result, if the cell surface is characterized with antibodies, it will give about one hundred times lower signal than human cells if the density of the epitopes is the same on human and bacterial cells. This means that to measure the content of DNA or other parameters of bacteria by flow cytometry, the brightest dyes and highly sensitive instruments have to be used. Note, however, bacteria may have highly repeated molecules on their surfaces (e. g. lipopolysaccharide, teichoic acids) that may be used as epitopes for the detection of bacteria if specific antibodies are available. An additional problem can arise from the presence of the outer membrane of Gram-negative bacteria. This membrane may present a permeability barrier for some

dyes. Thus, fixation or treatment of the bacteria with permeabilizing agents might be necessary prior to staining.

Flow cytometry of bacteria has several obvious applications in both basic and applied bacteriology. Applications in basic research include studies of cell-cycle kinetics under various conditions as well as studies of regulatory mechanisms of bacterial cell growth and replication. Some cell cycle studies on *E. coli*, *Rhizobium meliloti*, and *Rhizobium japonicum* included measuring the nucleic acid content and size of the bacteria (32, 33). The cell cycle of bacteria is not completely understood. The reasons for this are: a) the cell cycle is more complicated than that of mammalian cells; b) the bacterial cell cycle depends a lot on the growth conditions. The currently accepted model of the cell cycle of bacteria was proposed by Cooper and Helmstetter in 1968 (6). This model suggests that initiation of chromosome replication may commence before the previous round of replication is completed. As a result, more than one chromosome can be present in a bacterial cell and, under particular conditions, the maximum number of chromosomes can be six. In such bacterial cultures, measuring of DNA by flow cytometry will result in DNA histograms that may exhibit several peaks.

One of the main points of interest in the use of flow cytometry in clinical bacteriology is the possibility it offers for rapid assays, in contrast to the conventional techniques, which involve a time delay. Current methods for detection and identification of bacteria require isolation and prolonged (up to several days) incubation of the samples in appropriate media. Testing of drug susceptibility also involves the incubation of microorganisms for at least 18-48 hours in the presence of biocide and observation of the growth inhibition zones. Some clinical conditions, such as bacteremia, require more rapid diagnosis and assessment of responses to antibiotics. Flow cytometry can detect a low number of organisms of a particular cell type in the

presence of large numbers of other cells, thus providing a rapid method for detection and possibly quantitation of the bacteria in biological fluids.

During the last two decades many studies have been directed toward the development of methods for detection and identification of bacteria. Mansour et al. (30) were able to detect 100 bacteria per ml blood from rabbits infected with *E. coli*, and 10 bacteria per ml of human blood infected *in vitro*. In this study the bacteria were stained with ethidium bromide and identified by light scattering and fluorescent signal. Identification of bacteria is a difficult task for flow cytometry since there are so many kinds of bacteria. Identification can be done by using monoclonal antibodies to some strain specific antigens. A few examples of species-specific antibodies detected by flow cytometry have appeared (27, 34, 37). Another approach for the identification of bacteria is based on the measurement of the guanine-plus-cytosine percentage (%G+C), which is constant for each particular species. Thus, Van Dilla et al. (42) demonstrated separation of *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa* by staining these bacteria with Hoechst 33342 and chromomycin A₃ (these dyes are sensitive to DNA base composition). In another study it was shown that %G+C can be used for the detection of bacteriophage T4 infection in *E. coli* (38).

Many studies include FCA for detection of antimicrobial activity. Durodie et al. (12) were able to demonstrate a clear distinction of the antibiotic-treated *E. coli* cells based on measurements of forward light scatter and protein content (staining of the cells was done with FITC). Another method measured single cell membrane potentials using potential-sensitivity dyes. For example, carbocyanine dyes (e.g. DiOC₅(3)) are positively charged, accumulate inside the cells, and fluoresce. When cells depolarize, the dye is redistributed and the fluorescence intensity decreases. This technique was used for detection of antibiotic susceptibility of *Staphylococcus aureus* (31). Comas et al. stained *E. coli* with membrane potential sensitive dyes (Rhodamine 123 and bis-

oxonol) and with nucleic acid specific dyes (propidium iodide, SYTO-13 and SYTO-17) to determine the biocidal effect of surfactants by flow cytometry (5).

Viruses

Detection of virus infected cells by flow cytometry has employed several different approaches. Herpesvirus infected cells were detected when the cell lines were stained with mithramycin (a nucleic acid specific dye) (11). Replication of the virus led to an alteration of the DNA content of infected cells, and, as a result, changed the normal cell profile. Two nucleic acid specific dyes with different base specificity (Hoechst 33258 and chromomycin A3) were used to measure a change of %G+C in *E. coli* cells after infection with bacteriophage T4 (38). Another way of detecting virus infected cells is to show that viral antigens are present on the surface or inside the cells. Lehman et al. (28) were able to detect Simian Virus 40 infected CV-1 cells by using antibodies specific to T and V viral antigens.

The more difficult task is the detection of individual virions by flow cytometry. It is obvious that the main problem is the small size of the virions. The smallest virions (*Parvoviridae*) are about 20 nm in diameter. The largest virions (*Poxviridae*) are measured about 300 nm long and 180 nm in diameter. Thus, the light scattering of the largest virus (pox virus) should be 10^5 times weaker than for *E. coli*, which is probably beyond the sensitivity limits of most commercially available cytometers (39). However, there are two studies that demonstrate the detection of T2 bacteriophage, reoviruses (23) and pox virus (39) by measuring light scattering using laboratory-built cytometers. Some modifications to commercially available flow cytometers can also be done to increase the light scatter sensitivity of the instrument (13, 45).

Use of antibodies specific to viral proteins does not seem to be a useful approach since the number of binding sites would be extremely low because of the small number of structural proteins in most virions. Also, finding a way to wash away unstained antibodies could be a challenge, whereas leaving the samples unwashed will probably result in high background fluorescence.

Staining viral genomes might be useful for detection of the individual virions. The content of nucleic acid ranges from 5,000 (*Parvoviridae*) to 280,000 (*Poxviridae*) nucleotides. This means that the fluorescent signal of viruses would be roughly from 17 to 1000 times weaker than the signal given by *E. coli* (genome size of *E. coli* is 4.6×10^6 base pairs). Recently a few very bright cyanine fluorochromes with DNA and RNA specificity have become commercially available (for example, TOTO-1, YOYO-1, SYTO dyes). These stains are characterized by very high fluorescence enhancement upon binding to the nucleic acid. They show very low fluorescence when they are free in solution (19). Rye et al. (36) have reported $F_{\text{bound}}/F_{\text{free}}$ values of 1,100 for TOTO-1 and 3,200 for YOYO-1. By using TOTO-1 and an ultrasensitive flow cytometer, restriction fragments of lambda phage DNA as small as 5,000 base pairs were detected (17).

Studying Viruses by Fluorescence Microscopy

Epifluorescence microscopy and flow cytometry have some elements in common. The phenomenon of fluorescence is the basis for both methods, and the same fluorescent dyes can be used. Therefore, studies done with viruses by using fluorescence microscopy may also provide useful background information.

The invention of brightly fluorescent cyanine-based dyes that have very high

binding coefficients for nucleic acids increased the sensitivity of the fluorescence microscopy technique. One study used fluorescently stained viruses as probes to label specific strains of bacteria. Virions attached to the bacterial cell surface were observed. Thus, Hennes et al. (22) demonstrated that cyanophages and bacteriophages stained with nucleic acid specific dyes (YOYO-1 and POPO-1) can be used to tag and identify specific types of bacteria within mixed microbial communities. Staining bacteriophages with another dye (YO-PRO-1) allowed a direct count of viruses in natural waters and laboratory cultures (21). It is interesting that the YO-PRO method in this study consistently yielded higher values for virus concentrations compared to TEM counts. The authors concluded that the YO-PRO staining gave a more accurate count of viruses, whereas the TEM method underestimated the true concentration of viruses due to some of the following reasons: a) viruses could be lost when the grids are rinsed, b) small viruses might not be recovered with 100% efficiency during ultracentrifugation prior to staining, and c) viruses and stains might not be uniformly distributed across the grid surface. An alternative explanation for the different estimates obtained with epifluorescence microscopy and TEM was that fluorescent particles other than virions were counted. Theoretically, these particles could include, mitochondria, free ribosomes, and some other particles that bind nucleic acids or stain in a similar manner to nucleic acids. If this was the case, the problem of non-specific staining could also appear when a similar staining procedure is used in flow cytometry experiments for the detection of virions.

Endogenous Retroviruses

Retroviruses are enveloped virions about 100 nm in size. The genome consists of two molecules of RNA which are (+) sense and vary in size from about 8 to 11 kb each (43). Historically, retroviruses were divided into four groups (type A through D) based on their morphology in negatively-stained electron microscopy pictures. The retrovirus used in our research (an endogenous retrovirus produced by a Chinese Hamster Ovary (CHO) cell line) was C-type. C-type viruses are extracellular particles with a condensed central core and barely visible spikes. Most mammalian and avian retroviruses are C-type retroviruses, for example MLV, HIV, etc. The presence of budding C-type endogenous retrovirus in CHO cells is well documented (1, 20, 25). Transmission of the endogenous retrovirus to other cells has never been detected (29). The noninfectious nature of the endogenous retrovirus was an advantage that was considered when a virus model was chosen for the research.

Baculoviruses

Autographa californica nucleopolyhedrovirus (AcNPV) was used as one of the virus models in the present study. This is an insect virus and belongs to a family *Baculoviridae*, genus Nucleopolyhedrovirus (43). Infectious AcNPV particles enter susceptible insect cells by facilitated endocytosis or fusion, and viral DNA is uncoated in the nucleus. DNA replication starts about 6 hours after infection. There are two phases of the Baculovirus infection cycle. During the early phase, the infected cells secrete extracellular virus particles, or budded viruses (BV). The envelopes of the budded viruses are loose-fitting. During the late phase occluded viruses (OV) are

produced inside the nucleus. OV are visible under the light microscope where they appear as dark bodies filling up the nucleus of infected cells. These occlusions may range in size from 0.15 to 15 μm and contain many virions embedded in a homogenous matrix made from polyhedrin protein (40). OV are released during lysis of the infected cells. Special insect cell lines (e. g. Sf9, Sf21) are used for production of AcNPV. The genome of wild-type AcNPV is a super-coiled, double-stranded, circular DNA molecule with a molecular weight of 130 kb (4, 7).

MATERIALS AND METHODS

Evaluation of Binding Properties of Nucleic Acid Specific Dyes

To estimate binding properties of the nucleic acid dyes, the human Raji cell line (ATCC # CCL-86) (American Type Culture Collection, Rockville, MD) was stained. Two types of dyes were assayed: permeant SYTO 11 to SYTO 15 and impermeant SYBR I, SYBR II, and YOYO 1 (Molecular Probes, Inc., Eugene, OR). The staining with impermeant dyes was performed with a detergent treatment prior to staining (0.25% Triton X-100 for 10 min). Serial dilutions of the dyes were prepared. Stained cells were analyzed on a FACSort TM (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA). The dyes were compared in terms of: a) fluorescence intensity upon binding to nucleic acid, b) background fluorescence, c) saturation concentration.

The samples contained dyes in concentrations which differed by one order of magnitude. This big difference in dye concentrations required running samples at different FL1 voltage settings in order to place the fluorescent signals on the FL1 scale. In order to standardize the fluorescent signals from all samples, it was necessary to run some of the samples twice at two different FL1 voltages. The difference in the FL1 voltage was taken into consideration when fluorescent signals were calculated in order to construct a titration curve. The ratio of the fluorescent signal of the same sample obtained at higher and lower FL1 voltages was calculated, and the fluorescence value of the samples run at a lower voltage was multiplied by this calculated coefficient. Thus, "Fluorescence Intensity" in Fig. 1A and Fig. 2 is displayed up to 210,000 and 600,000 arbitrary units respectively, although the maximum fluorescence with one voltage setting would be reach only 10,000.

***E. coli* System**

A stock culture of *E. coli* (strain PET226-GFp-CD4) was a gift from Dr. Mehrpouyan (BDIS). Bacterial cultures were grown for 5-6 h in Luria-Bertani broth (LB) medium containing 1% tryptone (Difco, Detroit, MI), 0.5% yeast extract (Sigma Chemical Co., St. Louis, MO), and 0.5% NaCl (Sigma Chemical Co.) at 37°C with continuous agitation. The cells were microcentrifuged for 10 min at 4°C at 5000 x g, resuspended in cold distilled water, and diluted 1: 400 in 0.05 M TRIS buffer (pH 8.0) prior to staining. The TRIS buffer was filtered through a 0.22 µm membrane filter (Millipore Corp., Bedford, MA) to remove microparticulates that would otherwise contribute to background noise during data acquisition. SYTO 11 was added to a final concentration of 2.5 µM and the cells were incubated for 5 min at room temperature. Ethanol fixation was performed for some samples as follows. The cells were microcentrifuged, and ethanol was added to the cells resuspended in cold distilled water to a final concentration of 70%. The samples were diluted (1: 200) in 0.05 M TRIS buffer (pH 8.0) prior to staining. DNA and RNA digestion was performed by adding RNase-free DNase I, or DNase-free RNase, or both enzymes (Boehringer Mannheim, Indianapolis, IN) to the ethanol-fixed and non-fixed stained samples (1 unit/µl for each enzyme).

Determination of Instrument Performance

Fluorescence

A fluorescence quantitation kit, Quantum 24 (Flow Cytometry Standards Corp., San Juan, PR), was used to determine fluorescence performance of the instrument. This

kit contains a mixture of beads (labeled with fluorescein) with a known fluorescence intensity and unstained reference beads. Since the calibration beads were designed to fluoresce green, the green fluorescence was measured. For each bead population, the Mean Fluorescence Intensity (MFI) was obtained using the CellQuest software (BDIS). The system was triggered on FSC and SSC, or on FL1. A Molecules of Equivalent Soluble Fluorochrome (MESF) standard curve was constructed by plotting the observed MFI versus the known MESF of the beads and fitting the best line. The expression of fluorescence was done in terms of MESF. This was necessary because the fluorescence of fluorescein decreases when it binds to a bead. So, the number of dye molecules on a bead is measured by comparison of the bead fluorescence with a fluorescein solution (number of molecules of fluorescein in solution). Fluorescence of CaliBRITE unlabeled beads (BDIS) and *E. coli* genome stained with SYTO 11 in terms of MESF were determined using the MESF standard curve.

Light scattering

One drop of fluoresbrite-carboxylate latex beads (Polysciences, Inc., Warrington, PA) of 0.16, 0.21, 0.57, 1.64, or 2.24 micron in diameter was added to a tube containing 0.5 ml of 0.05 M TRIS buffer (pH 8.0) previously filtered through a 0.22 micron filter. The samples were analyzed by flow cytometry. The system was triggered on FSC and SSC, and a log amplifier was used during data acquisition for all channels.

Retroviral Systems

Analyzing Background Fluorescence of Different Fluids

TRIS buffer (0.05 M, pH 8.0), 1X PBS buffer (BioWhittaker, Inc., Walkersville, MD), RPMI-1640 (BioWhittaker, Inc.) supplemented with 10% fetal bovine serum (FBS), and supernatant fluid from a CHO cell line were compared in terms of background fluorescence. All fluids were filtered through a 0.22 micron filter and stained with SYTO 11 (2.5 μ M). Stained and unstained samples were analyzed by flow cytometry. In the experiment the threshold instrument setting was adjusted in such a way that the number of events per second during data acquisition would be approximately the same (3,000 events/sec) for each sample. The threshold values (instrument setting) were used to compare the background fluorescence of the fluids. The range of the threshold instrument settings was from 0 to 1024, which closely corresponded to a channel scale with 1024 channels. Since log amplification during the experiment was used, the analysis was done using a fluorescence intensity scale from 0 to 10,000 arbitrary linear units. To convert the threshold instrument settings (channel number) into the corresponding values on the fluorescence intensity scale, the following equation was used: $X=10^{\text{Channel number} / 256}$, where X is the converted value of the threshold instrument setting.

Staining Supernatant Fluid from a CHO Cell Line Containing an Endogenous Retrovirus

Supernatant fluid from CHO cells containing endogenous retrovirus was provided by Dr. Marshall Dinowitz, Dr. Yuan Xu, and Ms. Alison Laney (Genentech, Inc., South San Francisco, CA). The virus titer was determined by TEM at Genentech and was 10^8 particles/ml. CHO supernatant fluid was diluted prior to staining to the final concentration 10^6 particles/ml, which should register about a 1,000 events/sec when the rate of sample injection is "High". Staining of non-purified and partially purified virus in the supernatant fluid was performed. The virus was partially purified by using the following techniques.

- a) Microcentrifugation for 2 min at 4°C at 5000 x g. The supernatant fluid was used.
- b) Filtration through 0.45, 0.2, or 0.22 micron membrane filters (Millipore Corp.).
- c) Concentration of the virus by using a Centricon 500 (Amicon, Inc., Beverly, MA) that retained particles with MW above 500 kD. The concentrated fluid was used.
- d) Different combinations of these techniques.

Non-purified or partially purified CHO supernatant fluid was diluted to 10^6 particles/ml and stained with SYTO 11 (2.5 μ M), YOYO 1 (4 μ g/ml), and SYBR II (10X). Staining with impermeant dyes (YOYO 1 and SYBR II) was done with and without a detergent treatment (0.25% Triton X-100 for 10 min). In the energy transfer experiment (see RESULTS, Energy Transfer Experiment) a red-excitable dye, Thiazole Blue (BDIS), was used at final concentration of 10 μ g/ml.

Staining Murine Leukemia Virus

A preparation of Murine Leukemia Virus (MLV) purified by ultracentrifugation was provided by Genentech. The titer of MLV was determined by TEM at Genentech and was 10^{10} - 10^{11} particles/ml. MLV samples were prepared in TRIS buffer or RPMI-1640 with 5% FBS. Prior to staining, MLV was inactivated by: a) low pH treatment (pH 3.8 for 25 min), or b) heat inactivation (56°C for 15 min). MLV was stained with SYTO 11 (2.5 μ M), YOYO 1 (4 μ g/ml), and SYBR II (10X). FCA was performed.

Baculovirus System

Producing and Amplifying AcNPV Baculovirus

AcNPV wild-type Baculovirus (catalog number 21103E) was purchased from PharMingen (San Diego, CA). The Sf9 insect cell line (PharMingen) was grown in TNM-FH medium (90% Grace's antheraea medium supplemented with 3.3 g/L lactalbumin hydrolysate and 3.3 g/L yeastolate, 10% FBS, 50 μ g/ml gentamicin) (PharMingen). The cells were maintained at 27°C in a non-humidified environment without CO₂. Sf9 cells were subcultured every 3-4 days. Infection with AcNPV wild-type Baculovirus was performed when Sf9 cells reached 90%-95% confluency. The cells were infected in 75 cm² tissue culture flasks (Falcon Labware, Lincoln Park, NJ). The initial infection was done at 3,000 plaque forming units (pfu)/flask (the virus titer in the stock solution was $1:10^8$ pfu/ml), and the supernatant fluid was harvested 6-7 days post infection. The signs of infection were monitored microscopically: infected cells were characterized by a larger size and the presence of black occlusion bodies when compared to uninfected control cells.

The harvested supernatant fluid was centrifuged at 5,000 x g for 10 min to remove cells and stored at 4°C. The supernatant fluid was used to infect Sf9 cells by transferring about 4 ml of the supernatant fluid into a flask containing about 36 ml of TNM-FH medium.

Isolating AcNPV Particles

About 5 liters of high titer Baculovirus stock solution were produced. The stock solution was spun at 10,000 x g for 5 min to remove cell debris (Sorvall RC-5B Superspeed Centrifuge; GSA rotor) (Du Pont Co., Newtown, CT). The supernatant fluid was centrifuged at 40,000 x g for 30 min (Sorvall RC-5B Superspeed Centrifuge; SS34 rotor) (Du Pont Co.) to pellet the virus. The pellets were resuspended in TRIS/EDTA (TE) buffer (10 mM TRIS (Research Organics, Inc., Cleveland, OH), 0.1 mM EDTA (Sigma Chemical Co.)), pH 7.5. A 5%/40% sucrose step gradient in 0.1X TE was prepared and placed under 3 ml of the resuspended virus in ultracentrifuge tubes (Ultra-Clear SW-41 ultracentrifuge tube) (Beckman Instruments, Inc., Palo Alto, CA). The tubes were centrifuged at 40,000 x g for 30 min (Beckman L8-80M Ultracentrifuge; SW-41 rotor) (Beckman Instruments, Inc.). After centrifugation the virus particles located between the two layers of sucrose were harvested and resuspended in TE buffer. The resuspended material was spun at 40,000 x g for 30 min to pellet the virus. The virus particles were resuspended in 0.5 ml of TE buffer and stored at 4°C.

Baculovirus Staining

The purified AcNPV was diluted 1:10,000 in 0.05 M TRIS buffer (pH 8.0), and stained with SYTO 11 (2.5 µM). Ethanol fixation was performed for some samples prior to staining. Ethanol was added to the AcNPV preparation to the final concentration of 70%

and incubated for 15 min at room temperature. Then the samples were centrifuged at 5,000 x g for 10 min, the supernatant fluid was discarded and the virus pellet was resuspended in TE buffer. The samples were stained with SYTO 11 only (2.5 μ M), or with SYTO 11 (2.5 μ M) and TB (10 μ g/ml). Some of the ethanol fixed stained samples were sonicated for 1 min in 8890 bath sonicator (Cole-Parmer Instrument Co., Vernon Hills, IL).

Yeast System

Preparation of Agarose Embedded Yeast Chromosomes

A single colony of *S. cerevisiae* (ATCC # 200148) was inoculated into 50 ml YPD (1% yeast extract, 2% bactopectone, 2% dextrose) and was grown at 30°C with agitation to an O.D.₆₀₀ of 1.0. A CHEF Yeast Genomic DNA Plug Kit (BioRad Laboratories, Hercules, CA) was used for isolation of yeast chromosomes as follows. Cells were centrifuged at 5,000 x g for 10 min at 4°C, and resuspended in cold 50 mM EDTA, pH 8.0. About 6 x 10⁸ cells were removed, centrifuged at 5,000 x g for 10 min at 4°C, resuspended in 0.63 ml of Cell Suspension Buffer, and equilibrated to 50°C. Lyticase stock (30 μ l) was added prior to mixing the cells with 2% CleanCut agarose solution. The cell/agarose mixture was transferred to plug molds and allowed to solidify at 4°C. The solidified agarose plugs were placed in Lyticase solution (170 μ l of Lyticase stock to 5 ml of Lyticase buffer), and incubated for 2 h at 37°C. The plugs were rinsed with sterile water and incubated in Proteinase solution (5 ml of Proteinase K Reaction Buffer and 200 μ l of Proteinase K stock) overnight at 50°C without agitation. The plugs were washed four times in 1X Wash Buffer, 1 h each at room temperature with gentle agitation. The plugs

were stored at 4°C. This preparation is referred to as Prepared yeast chromosomes (P-yeast chromosomes).

Staining *S. cerevisiae* Chromosomes

DNA MW markers (Sigma Chemical Co.) consisted of 15 *S. cerevisiae* chromosomes ranging from 225 to 2,200 kb. These are referred to as Commercial yeast chromosomes (C-yeast chromosomes) in this thesis. The markers were embedded in 1% low melting point agarose (International Biotechnologies, Inc., New Haven, CT). For FCA an agarose block (containing about 1 µg of DNA) was melted at 75°C for 45 min. Dilutions (1:1,000 and 1:10,000) in 0.05 M TRIS buffer (pH 8.0) that was filtered through 0.22 micron filter were prepared, stained with SYTO 11 (final concentration 2.5 µM), and analyzed in the flow cytometer. To perform nuclease treatment of the stained samples, the samples were incubated with RNase-free DNase I at 1 unit/µl for 60 min at room temperature.

Pulsed-Field Gel Electrophoresis

Pulsed-Field gel electrophoresis (PFGE) was carried out for C-yeast chromosomes and P-yeast chromosomes with a CHEF-DR II apparatus (BioRad Laboratories). About 100 ml of molten 1% Molecular Biology Certified Grade agarose (Low Melting Point) in 0.5X TBE buffer was poured into a casting stand (15x14 cm) and allowed to solidify at 4°C. The samples were loaded as solid plugs into wells 1 mm thick. The wells containing samples were sealed with the same agarose that was used for the gel. Electrophoresis was carried out for 2, 9, or 19 h in 0.5X TBE buffer at 200 V and 9°C with the 20 sec initial

and 178 sec final switching intervals. The flow rate of the buffer through the chamber was approximately 1 liter/min. After electrophoresis the gel was stained in ethidium bromide (0.5 µg/ml) for 30 min. The DNA was visualized by placing the gel on an UV transilluminator (300 nm). The photographs were obtained using black-and-white Polaroid film (Sigma Chemical Co.).

Flow Analysis of Yeast Chromosomes After PFGE

C-yeast chromosomes were run in PFGE for 2 or 19 h as described above. The gel was stained with SYBR I (10X in TBE) for 45 min. DNA bands were visualized on an UV transilluminator and cut out, trying to minimize the amount of agarose present in the blocks. As a negative control, an agarose block without a DNA band was used. The samples were melted at 65°C for 10 min, diluted in 0.05 M TRIS buffer (pH 8.0), stained with SYBR I (10X), and analyzed by flow cytometry. C-yeast chromosomes which had not been electrophoresed (a positive control) were diluted in TRIS buffer, stained with SYBR I, and analyzed by flow cytometry.

Flow Cytometry

The stained preparations were analyzed using a FACSort (BDIS) equipped with a 15 mW air-cooled 488 nm argon-ion laser (for SYTO 11-SYTO 15, SYBR I and II, YOYO 1 excitation) and a 10 mW 635 nm red diode laser (for TB excitation). CellQuest software was used for data acquisition and analysis. The light scatter was collected with log amplification for yeast, bacteria, and viruses. For eukaryotic cells (Raji cell line) the light scatter was collected with linear amplification. Fluorescent signals were collected with log

amplification for all samples. The FL1, FL2 and FL3 detectors collected light from the particles excited by the 488 nm laser (blue laser). The spectrum of the collected light was 515 to 545 nm for the FL1 detector, and from 564 to 606 nm for the FL2 detector. The light with wavelength equal to or longer than 670 nm was collected by FL3 detector. The FL4 detector collected light with a wavelength from 653 to 669 nm from the particles excited by the 635 nm red diode laser.

RESULTS

Part I: Preliminary Study

Choice of Dyes

Brightly fluorescent cyanine-based dyes that have very high binding coefficients for nucleic acids were used. To estimate the binding properties of the nucleic acid dyes, we stained the Raji cell line. Permeant (SYTO 11 to SYTO 15) and impermeant (SYBR I, SYBR II, YOYO 1) dyes were assayed. These dyes fluoresce green (around 530 nm) when bound to DNA or RNA and excited with blue light. The dyes were compared for fluorescence enhancements upon binding to nucleic acids and background fluorescence. During the experiments a significant increase in background fluorescence occurred as the concentration of dyes increased. Dye concentrations were optimized to give high fluorescence at relatively low concentration.

The SYTO dyes are lower-affinity stains that are able to passively diffuse through cell membranes. These dyes stain both RNA and DNA. The comparison of SYTO dyes revealed two dyes which gave relatively high fluorescent signal (SYTO 11 and SYTO 13) (Fig. 1A). Even though SYTO 13 gave the highest signal, this dye showed lower affinity for nucleic acids than SYTO 11. High background fluorescence was also observed with increased concentrations of SYTO 13 (not shown). SYTO 11 was, therefore, the dye of choice for most further experiments. The saturation concentration for SYTO 11 was 2.5 μM (Fig. 1B).

Fig. 1. Fluorescence intensity of Raji cells stained with permeant SYTO dyes. The Raji cell line was stained with SYTO 11 to SYTO 15 dyes, and fluorescence intensity of the cells was analyzed by flow cytometry. The graphs were constructed by plotting dye concentrations (μM) versus mean fluorescence intensity (arbitrary units) of the cell population. A. SYTO 11 to SYTO 15 titration. B. SYTO 11 titration.

Fig. 2. Fluorescence intensity of Raji cells stained with impermeant dyes SYBR I, SYBR II, and YOYO 1. Fluorescence intensity of the cells was measured during flow cytometric analysis.

*Concentrations in μM are for YOYO 1 only. For SYBR I and SYBR II these numbers represent concentration factors with the stock considered 10,000 (as labeled by the manufacturer).

Impermeant dyes SYBR I, SYBR II, and YOYO 1 were compared (Fig. 2). All of these dyes are characterized by a very high affinity to nucleic acid. They showed fluorescent signals that were 2 to 4 times higher than the signal given by SYTO 11 even with a slightly lower voltage setting. However, the use of these dyes was limited because all three dyes produce very high background fluorescence (not shown), and SYBR II has some selectivity for RNA over DNA.

Staining *E. coli* Genome

Before attempting to analyze viral nucleic acid, the detection limits of the flow cytometer were determined. To do this *E. coli* nucleic acid was stained and analyzed by FCA. The size of the *E. coli* genome is 4.6 megabases, which is about 40 times smaller than the DNA content of an average human chromosome and about 200 times larger than the genome of endogenous retrovirus. The permeant nucleic acid dye, SYTO 11, has no known base specificity. Since for stains with no base specificity the fluorescence intensity is generally proportional to the total DNA content, it was assumed that analyzing the signal of the stained bacterial genome would give us an idea of the expected position of the viral signal (17 to 1000 times weaker than *E. coli*) in the FL1 channel. It was also assumed that the stained bacterial genome would indicate whether the flow cytometer would be able to detect a fluorescent signal as low as that expected from viral nucleic acid, and at which point instrumental noise and/or fluorescence of the background events begin to contribute so significantly that it would interfere with the virus detection.

Bacterial cultures were grown as described in Materials and Methods. Ethanol fixation of *E. coli* was performed prior to staining with SYTO 11. Non-fixed *E. coli* were

also stained. Comparison of the genome staining of fixed and non-fixed bacteria revealed the following (Fig. 3):

- a) Ethanol-fixed bacteria showed a sharper peak than non-fixed *E. coli* in a DNA histogram (Fig. 3C). Fixation made bacterial membranes more permeable for the dye, which probably was a reason for the more uniform staining of the genome. In non-fixed bacteria, the penetration of the dye depended on the biological state of each bacterial cell. Since the population was not synchronized there can be variations in the staining efficiency of the genome.
- b) Two peaks corresponding to the bacterial genome were consistently observed in the ethanol-fixed samples (Fig. 3C). According to the currently accepted model of the cell cycle of bacteria (6), initiation of chromosome replication may commence before the previous round of replication is completed. So, more than one chromosome may be present in bacterial cells. As a consequence, DNA histograms of such a culture will exhibit more than one peak. In our experiments we believe we observed peaks corresponding to 1 and 2 chromosomes, although more precise investigation of the issue would be required.
- c) Peaks observed in ethanol-fixed samples after staining with SYTO 11 were DNase sensitive (Fig. 4). According to the literature (39), RNA associated fluorescence tends to blur the DNA fluorescence, but attempts to eliminate RNA by RNase treatment prior to staining usually are not successful. In our experiments, RNase treatment alone did not effect the staining, whereas using both enzymes (DNase and RNase) led to a significant decrease in the fluorescent signal (Fig. 4).
- d) Fluorescence of the bacterial cells containing one chromosome (Fig. 3C, M1) was compared with fluorescence of background events in the ethanol-fixed *E. coli* samples (Fig. 3C; M2). The ratio of the mean fluorescence ($\bar{X}$ mean) of M1 to the mean fluorescence of M2 appeared to be about 140. Considering that the *E. coli* genome size is 4.6 megabases, the separation of bacterial signal from other particles in the sample was

Fig. 3. Staining the bacterial genome in ethanol-fixed and non-fixed *E. coli* cells with SYTO 11. Green fluorescence was measured by flow cytometry. Red events: stained bacterial cells; black events: background fluorescence. **A.** Ethanol-fixed cells. **B.** Non-fixed cells. **C.** DNA histogram of fixed and non-fixed *E. coli*. M1: fluorescence of bacterial cells containing one chromosome in ethanol-fixed samples. M2: background fluorescence of ethanol-fixed samples. Fluorescence is expressed in arbitrary units (X Mean).

Fig. 4. Nuclease treatment of *E. coli* after staining with SYTO 11. Stained bacterial cells were treated with RNase-free DNase I, or DNase-free RNase, or both enzymes. Orange events: stained *E. coli*; green events: background fluorescence and/or cell debris; purple events: *E. coli* with partially destroyed stained genomes.

sufficient for detecting about 32,000 nucleotides ($4.6 \text{ megabases}/140 = 32,857$ nucleotides). This also assumed that the staining efficiency of *E. coli* and virions would be similar. The peak of fluorescence of a 32,000 nucleotide genome would probably appear very close to or even overlap with the background events of the sample (Fig. 3C; M2). This result suggested that lower fluorescence of background events would be needed to be able to detect 20,000 nucleotide genome. In this respect, it is important to note that the fluorescence of the background events in the *E. coli* samples was higher than that seen in stained buffers (see below).

Detection Limits in Buffers

A fluorescence quantitation kit, Quantum 24, was used to determine fluorescence performance of the instrument (Fig. 5A). A MESF standard curve was constructed as described in Materials and Methods (Fig. 5C). It is important to note that the Quantum 24 kit suggests thresholding on forward light scatter. In this experiment FSC and SSC or FL1 thresholding were used. Data acquisition by either method revealed practically the same MFI values for the labeled bead populations. So, the MESF standard curve based on the MFI values obtained when FL1 thresholding was used was accurate (Fig. 5C). The difference was observed when the MFI of the reference blank was compared (not shown). The blank revealed lower MFI when FSC and SSC thresholding was used. When FL1 thresholding was used, it is possible that electronic noise contributed to the signal because the MFI value was higher for the blank population (Fig. 5A; M5). However, this was necessary information since the FL1 signal and an FL1 threshold were used for virus detection.

CaliBRITE unlabeled beads (BDIS) were potentially useful as an internal standard

Fig. 5. Determination of the FACSsort performance using Quantum 24 kit. **A.** A histogram of MFI of labeled beads (M1, M2, M3, M4) and noise (M5). **B.** A histogram of MFI of unlabelled CaliBRITE beads. M1: unlabelled CaliBRITE beads; M2: noise. **C.** MESF Standard Curve constructed using the Quantum 24 kit. Red line: the best fit line. MFI of CaliBRITE beads is indicated by the arrow. **D.** Fluorescence intensity of *E. coli* stained with SYTO 11 (dotted line) using extrapolated MESF standard curve.

in some of our experiments. It was necessary to analyze their fluorescence. CaliBRITE unlabeled beads gave a detectable signal that corresponded to approximately 4,000 MESF (Fig. 5B and 5C).

The fluorescent signal of *E. coli* stained with SYTO 11 was determined in terms of MESF. The minimum number of bound SYTO 11 molecules was expected to be about 800,000 if the dye binds at 1 molecule per 6 bases (usually nucleic acid dyes bind at 1 molecule per 2 to 6 nucleotides). The signal appeared to be slightly higher than 400,000 MESF (Fig. 5D), which was in the expected range. The Quantum 24 kit is designed for quantitation of fluorescence intensity in a low range between 2,000 to 50,000 MESF. The MESF standard curve had to be extrapolated in order to estimate the *E. coli* signal, which could cause an inaccurate determination of the fluorescence in MESF units. Quantum 24 beads were labeled with fluorescein, whereas *E. coli* was stained with SYTO 11. The optical properties of the dyes were compared. The quantum yield for unconjugated fluorescein is 0.9, and for SYTO 11 it is more than 0.4. The extinction coefficient for fluorescein is 82,000, and for SYTO 11 it is more than 50,000 $\text{cm}^{-1}\text{M}^{-1}$. Excitation and emission spectra of these dyes are similar (18, 19). These differences in the optical properties of the dyes might explain the MESF result when staining was performed with SYTO 11.

Considering the genome of the endogenous retrovirus to be 20 kb (for both RNA strands) and the fact that nucleic acid dyes usually bind at 1 molecule per 2 to 6 bases, from 3,000 to 10,000 molecules of dye are likely to bind to each virion. The fluorescence of the stained *E. coli* genome expressed in MESF suggested that the number of SYTO 11 molecules bound per virion, which is about 200 times lower than that bound per bacterial cell, would be equivalent to 2,000 MESF. This is slightly lower than the CaliBRITE unlabeled bead signal but still within the detection limits of the FACSsort.

Using Light Scatter for Virus Detection

There have been studies that demonstrated the feasibility of the detection of bacteriophage T2 (about 110 nm), reovirus (about 70 nm) (23), and pox virus (39) based on light scattering with laboratory built cytometers. Using light scattering for virus detection in the present study was rejected for the following reasons:

- a) According to the literature (39) the light scattering of the largest virus (pox virus) would be roughly 10^5 times weaker than for *E. coli* (in the virus size range light scattering falls off with the square of the particle volume) if the measurement is done on a commercial cytometer with no specific modification. In our experiment with *E. coli*, the bacteria revealed very low light scattering (around 10^3 in FSC and 10^2 arbitrary units in SSC). This signal was barely adequate for thresholding (Fig. 6A). So, we would not expect FSC and SSC to be useful for virus detection.
- b) An experiment using latex beads of different size was performed (Fig. 6B). The smallest particles that we were able to detect in FSC were beads of 0.57 micron in diameter. The 0.21 and 0.16 micron beads were not detectable under these conditions.

Fig. 6. Determination of instrument performance in light scatter. A. Light scatter of *E. coli*. i: FL1 threshold; ii: SSC threshold. Note, side scatter of *E. coli* was barely adequate for separation of the bacterial population from instrumental noise. B. Light scatter of latex beads of 0.57 (blue, R2), 1.64 (red, R1), and 2.24 (green, R3) micron in diameter. The 0.57 micron beads were the smallest detectable beads. FL1: green fluorescence; FL2: orange fluorescence. The fluorescence of the latex beads is shown for easier gating of the different bead populations.

Conclusion of Preliminary Study

Based on the staining of the *E. coli* genome and determination of the instrument performance, the detection of at least larger viruses by measuring the fluorescent signal of the stained genomes seemed possible. The signal of stained viruses would be expected to be very close to the detection limits of the instrument. High background fluorescence would be a serious problem, and precautions should be taken to decrease the background.

Part II: Flow Cytometric Analysis of Viral Models

Analyzing Background Fluorescence of Different Fluids

All CHO cell lines shed retrovirus-like particles (classified as endogenous retroviruses). As a result, a negative control, such as the supernatant fluid of a CHO cell line without endogenous retroviruses, was not available. The presence of such a negative control would help to discriminate between the signal (autofluorescence or non-specific binding) coming from the medium contents (for example, serum) versus the signal coming from the stained viral genome. Another source of non-specific signal may be electronic noise from the flow cytometer. This source of noise is especially important in the current research since the measurements are done at a very high level of sensitivity.

It was known that the CHO supernatant fluid provided by Genentech contained RPMI-1640 supplemented with 10% FBS. The level of background fluorescence of different fluids was assayed. The background fluorescence of TRIS buffer, RPMI-1640 with 10% FBS, and PBS buffer was analyzed without staining and with SYTO 11 (Table 1). One of the instrument settings that can be controlled by a user is the threshold. The threshold setting selects for analysis by the cytometer only those events that have a signal higher than the level defined by the threshold. In the experiment the FL1 threshold was adjusted for each fluid in such a way that the number of events/sec during data acquisition would be approximately the same (3,000 events/sec). The threshold values were converted into arbitrary linear units as described in Materials and Methods and used to compare the background fluorescence. The term “background fluorescence” in this experiment means the fluorescence level of the dimmest particles. Changing the threshold settings reflected the fluorescence intensity of the dimmest particles. For example, if two samples have the

same number of events but the events have different fluorescence intensity, for the sample with the brighter events, the threshold would be brought up. For the sample with the dimmer events, it would be necessary to bring the threshold down. Although the same number of events was acquired for each sample, changing the threshold settings also reflected the total number of the events present in each sample. If there are two samples with a different number of events, but with the same fluorescence intensity, then for the sample with more events it would be necessary to bring the threshold up in order to acquire only 3,000 events/sec and remove the rest of the population. If the threshold were kept low, the data acquisition would be inefficient because the maximum rate of events during acquisition is about 8,000 events/sec. If a sample had a low number of events then the threshold should be brought down to acquire 3,000 events/sec.

Before staining, both the RPMI-1640 with 10% FBS and CHO supernatant fluid had much higher fluorescence compared to buffers (Table 1), probably because of the higher autofluorescence of material in these samples. Staining of the buffers resulted in slightly higher threshold values possibly because of the presence of free dye and/or insignificant amount of material that could be stained with SYTO 11. Staining with SYTO 11 brought threshold values much higher in both RPMI-1640 (2.6-fold increase) and CHO supernatant fluid (4.1-fold increase) likely because of staining of serum components present in both fluids. The fluorescence of the CHO supernatant fluid was higher than for RPMI-1640. This could be due to the presence of the stained retrovirus and/or some non-viral material (e. g. cell debris, free nucleic acids, mitochondria, ribosomes) that also was stained with SYTO 11. Two factors indicated that the observed signal was coming from non-viral material. First, the frequency of the events in the CHO supernatant fluid was much higher than expected from TEM. Second, the fluorescence intensity of the CHO supernatant fluid was higher than that expected from the retrovirus particles based on the *E. coli* fluorescence signal (*E. coli* stained with SYTO 11 was used as a control particle in this

experiment). If staining of retroviruses had occurred as anticipated, the fluorescent signal of the viral nucleic acid might be located in the same place or even lower on the FL1 scale as the signal from the non-viral material. In this case, it is likely that the non-viral fluorescence signal would camouflage the signal from viral nucleic acid.

Table 1

Comparison of background fluorescence of PBS buffer, TRIS buffer, RPMI-1640 with 10% FBS, and CHO supernatant fluid

Name of fluid	Threshold* (arbitrary linear units)		Fold increase (stained fluids vs. unstained)
	no dye	SYTO 11	
PBS buffer	2.7	4.0	1.5
RPMI-1640 + 10% FBS	13.3	35.2	2.6
CHO supernatant fluid	20.5	85.0	4.1
TRIS buffer	2.7	3.9	1.4

* The converted values of the threshold instrument settings were used to compare the background fluorescence of assayed fluids. The results from representative experiment are shown.

Staining Supernatant Fluid from a CHO Cell Line Containing an Endogenous Retroviruses

In order to identify viruses in flow cytometric analysis, two criteria must be met. First, the concentration of the endogenous retrovirus (determined by TEM) was known from an independent source. The number of the fluorescent events per volume of sample

can be calculated during flow cytometric analysis based on: a) the number of fluorescent events in the population of interest, and b) the rate of sample injection. The comparison of the event count obtained by flow analysis with the known virus titer was used as a criterion to determine whether or not the observed signal was due to stained virions. Second, if nucleic acids other than viral genomes are present in the sample, they probably are of various sizes, whereas all virions have the same genome size. The number of bound fluorochromes usually correspond to the DNA content. Thus, we would expect to observe a peak for the stained viral genome in DNA histograms, whereas any signal coming from non-specific staining should be more widely distributed.

Retrovirus-like particles from CHO supernatant are enveloped (see LITERATURE REVIEW, Endogenous Retroviruses). However, it is unclear if the envelope is loose-fitting or sealed. Assuming that both situations are possible, we used permeant (SYTO 11-15) and impermeant (YOYO 1 and SYBR II) nucleic acid dyes. Staining with these dyes did not reveal a peak in the DNA histogram (not shown); however, some fluorescent material was consistently observed in the samples. Results obtained after staining with SYTO 11 are shown on Fig. 7A. When serial dilutions were performed, the number of fluorescent events correlated with the dilution factors (not shown). This indicated that the observed signal was due to material in the sample, and not due to instrumental noise. The concentration of this fluorescence material in the sample was much higher than the concentration of virus as determined by TEM. Assuming that most of the fluorescence was the result of the staining of non-viral material, a partial purification of the supernatant was performed as described in Materials and Methods.

The only purification technique that decreased the amount of the fluorescent material was filtration of the supernatant through 0.2 micron filter (the size of retroviruses is approximately 100 nm). After filtration the events characterized by relatively high fluorescence and light scattering were eliminated (Fig. 7B). Neither concentration of the

supernatant fluid using a Centricon filtrator (eliminates small particles with MW up to 500 kD) nor microcentrifugation caused any significant decrease in the concentration of the fluorescent material. Combination of the concentration or microcentrifugation with filtration through 0.2 micron filter led to the same results as filtration only (not shown). However, even in the filtered samples it was not possible to isolate a population that could be identified as retroviruses based on the two criteria mentioned above.

Fig. 7. Flow cytometric analysis of the CHO supernatant fluid (containing the endogenous retrovirus) stained with SYTO 11. A. Unfiltered CHO supernatant. B. CHO supernatant fluid filtered through 0.2 micron membrane filter prior to staining. Events with relatively high fluorescence are indicated in red.

Energy Transfer Experiment

Another approach was used to separate viruses from other material in the supernatant. CHO supernatant fluid was stained with two permeant nucleic acid dyes: SYTO 11 and Thiazole blue (TB). SYTO 11 is blue-excitable and emits in FL1 (green fluorescence), whereas TB is a red-excitable dye and emits in FL4 (red fluorescence). An energy transfer was expected in this experiment since the emission spectrum of SYTO 11 overlaps the absorption spectrum of TB. So, when both dyes are present in a particle, excitation of SYTO 11 (with 488 nm laser) causes transfer of some energy to TB, which leads to emission in FL3 (Fig. 8). Both SYTO 11 and TB have high affinity for nucleic acids; so, nucleic acids will probably bind more fluorochrome molecules than other biopolymers present in the sample. An important condition for energy transfer is close distance (typically 10-100 Å) between donor and acceptor molecules. The distance between bound SYTO 11 and TB molecules presumably will be short, which facilitates energy transfer. Other biopolymers that may be present in the sample can bind the fluorochromes non-specifically but much less efficiently than nucleic acids (19). The distance between the dyes probably will be longer, which will result in a smaller degree of energy transfer. So, the virus population was expected to be stained more efficiently and reveal higher energy transfer than other material in the sample. It was anticipated that the virus population would shift up in FL3 due to energy transfer. The signal in FL1 was expected to shift down since some energy was transferred from SYTO 11 to TB. Another reason for the shift down in FL1 and possibly FL4 could be binding site competition between SYTO 11 and TB.

Excitation λ	Dye	Emission λ	FACSort channel
488 nm $\longrightarrow$	SYTO 11 $\longrightarrow$	530 nm $\longrightarrow$	FL1
635 nm $\longrightarrow$	TB $\longrightarrow$	660 nm $\longrightarrow$	FL4
488 nm $\longrightarrow$	SYTO 11 $\searrow$	TB $\longrightarrow$	660 nm $\longrightarrow$ FL3

Fig. 8. The mechanism of energy transfer between SYTO 11 and TB. The emission spectrum of SYTO 11 overlaps the absorption spectrum of TB. Energy transfer occurs when both dyes are present in the same particle and the distance between dye molecules is between 10-100 Å. Energy transfer is displayed by fluorescence in FL3 channel when the dyes are excited by 488 nm light.

E. coli was stained with SYTO 11 and TB in order to demonstrate that energy transfer can take place with these two dyes (Fig. 9, Table 2). Bacteria stained with both dyes revealed about 15-fold increase in FL3 signal compared to bacteria staining with TB only (Fig. 9B and 9C). The FL3 signal was relatively high when bacteria were stained with SYTO 11 (Fig. 9A). However, *E. coli* stained with both dyes revealed a 2.5-fold decrease in FL1 fluorescence compared to bacteria stained only with SYTO 11 (Fig. 9A and 9C). This decrease likely resulted from both energy transfer and competition between dyes for the binding sites. If the entire loss was due to competition, the FL3 signal would also be expected to go down about 2.5 times. On the contrary, a 1.8-fold increase in the FL3 signal was observed when both dyes were used (Fig. 9A and 9C). A decrease in FL4 fluorescence of about 1.2-fold observed after staining with both dyes (Fig. 9B and 9C) was likely due to competition. This pattern of the fluorescence shift was expected, and demonstrated that energy transfer occurred between SYTO 11 and TB.

Fig. 9. Staining *E. coli* with SYTO 11 and TB. Red events: stained *E. coli*; black events: background fluorescence. The mean fluorescence values (arbitrary units) for stained *E. coli* in FL1, FL3, and FL4 are shown. Bacteria stained with both dyes (C) revealed about 15-fold increase in FL3 signal compared to bacteria stained with TB only (B). Bacteria stained with both dyes (C) showed a 2.5-fold decrease in FL1 fluorescence when compared to bacteria stained only with SYTO 11 (A). A decrease in FL4 of about 1.2-fold was observed after staining with both dyes (B and C). Statistics are also shown in Table 2.

Table 2

Energy transfer between SYTO 11 and TB during E. coli staining

Channel; dye name	Fluorescence (arbitrary units)	Increase (+) or decrease (-) in fluorescence (fold)*
FL1; SYTO 11	1344.48	-2.5
FL1; TB+SYTO 11	537.32	
FL4; TB	1030.43	-1.2
FL4; TB+SYTO 11	830.61	
FL3; SYTO 11	372.95	+1.8
FL3; TB+SYTO 11	659.17	
FL3; TB	44.22	+14.9
FL3; TB+SYTO 11	659.17	

* Fluorescence signals of the stained *E. coli* genome were compared before (staining with TB only or SYTO 11 only) and after (staining with TB and SYTO 11) energy transfer.

Since the energy transfer with these two dyes worked for *E. coli* cells, similar experiments were performed with the CHO supernatant fluid. The CHO supernatant fluid was stained with SYTO 11 and TB (Fig. 10, Table 3). Two populations were observed (Fig. 10). A 7.3-fold increase of fluorescence in FL3 for the population colored red, and about 2.4-fold increase for the population colored blue was seen. The shift down in FL1 was approximately 1.3 for both populations (red and blue). It is possible that the red events represented the virus population, and the blue events represented some non-viral material present in the CHO supernatant fluid. There were a few concerns about this conclusion. First, the virus population showed rather high FSC (the instrument settings were E01). One explanation could be aggregation of virions, although the possibility that those were aggregates of some other material present in the supernatant fluid could not be eliminated. Second, the number of events was about 100 times lower than expected. Retrovirus titer was estimated by TEM as 10^8 particles/ml. Based on the flow cytometry results, the concentration of virions was about 2.7×10^6 particles/ml. A possible explanation could be the fact that the system was triggered on FL1 and part of the population appeared to be below the threshold. Bringing the FL1 threshold down was not an option since it led to a significant increase in the event rate for background particles. If our suggestion about virus aggregates is correct, this would mean that thresholding on FL1 led to the selection of the large and bright aggregates. Since the FL1 signal went down due to energy transfer, part of the red population went below the threshold, which resulted in fewer events observed after staining with TB. The red population would now be only even bigger, brighter aggregates. The selection of the larger aggregates would explain a 3.3-fold increase in FSC (Table 3) for the “virus population” after staining with both dyes (Fig. 10B). Measuring mean fluorescence intensity of those aggregates would be also responsible for the higher FL3 signal after staining with both dyes. This would make it more difficult to interpret the results about energy transfer.

Fig. 10. Staining the CHO supernatant fluid containing an endogenous retrovirus with SYTO 11 and TB. Two fluorescent populations are present: one (red) with higher side scatter than the other (blue). A 7.3-fold increase of fluorescence for the red population and about 2.4-fold increase for the blue population was observed in FL3 channel. About 1.3-fold decrease in FL1 signal was seen for both (red and blue) populations. The statistics are shown in Table 3. The red population revealed the behavior consistent with that of nucleic acid containing particles (e. g. retroviruses) when energy transfer took place between SYTO 11 and TB.

Table 3

Energy transfer between SYTO 11 and TB during staining the CHO supernatant fluid containing the endogenous retrovirus

Channel, population (red or blue) ^a	Fluorescence (arbitrary units)		Increase (+) or decrease (-) in fluorescence (fold) ^b	
	Staining with SYTO 11 only	Staining with SYTO 11 and TB	Red population	Blue population
FL3, red	9.83	71.99	+7.3	-----
FL3, blue	5.33	12.75	-----	+2.4
FL1, red	12.83	10.41	-1.2	-----
FL1, blue	12.09	9.37	-----	-1.3
FSC ^c , red	4.42	14.50	+3.3	-----

^a Red population: aggregates of the endogenous retroviruses; blue population: non-viral material in CHO supernatant fluid.

^b Fluorescence signals for the red and blue populations were compared before (staining with SYTO 11 only) and after (staining with SYTO 11 and TB) energy transfer.

^c The numbers in this row represent measurements of FSC and an increase in FSC (not fluorescence) for the red population.

All experiments described above involved different staining methods of viruses in the CHO supernatant fluid. However, a population that could be unambiguously identified as retroviruses was not observed. One possible explanation was that the CHO supernatant contains non-viral material responsible for the high background fluorescence. Since the fluorescence signal of the stained viral genome was expected to be very low to begin with, it is likely that the background fluorescence camouflaged the signal from the retrovirus.

Murine Leukemia Virus

To avoid the problem of the background fluorescence, a purified sample of Murine Leukemia Virus (MLV) was obtained. This virus belongs to the C-type group of retroviruses and has structural properties very similar to the endogenous retrovirus secreted by CHO cells. The MLV was inactivated with low pH or heat as a precautionary biohazard measure. FCA of inactivated MLV did not reveal any discrete population (not shown). One reason for this could be that virions were physically destroyed during inactivation.

Baculovirus Model

The choice of *Autographa californica* nucleopolyhedrovirus (AcNPV) as a virus model was based on the relatively large size of the genome, about 130 kb, which is only 35 times smaller than the *E. coli* genome. AcNPV was produced and amplified in Sf9 insect cells. Virus particles were isolated from the harvested supernatant fluid as described in Materials and Methods. After sucrose step gradient ultracentrifugation, the virus particles appeared as a very well defined white band. Running an AcNPV sample in PFGE revealed the presence of a single band with a MW lower than 225 kb (not shown).

Fig. 11. AcNPV stained with SYTO 11. AcNPV Baculovirus was produced and purified by 5%/40% sucrose step gradient ultracentrifugation. A. Negative control (TRIS buffer and SYTO 11). B. Unfixed AcNPV diluted in TRIS buffer and stained with SYTO 11. C. Ethanol-fixed AcNPV stained with SYTO 11. Ethanol-fixed AcNPV was centrifuged at 5,000 x g, resuspended in TRIS buffer to the original volume, stained, and analyzed by flow cytometry.

Unfixed AcNPV stained with SYTO 11 revealed the presence of a population which was not observed in a negative control (TRIS buffer with SYTO 11) (Fig. 11A and 11B). In ethanol-fixed samples the staining seemed to be even more efficient, and the observed populations were characterized by even higher light scatter and fluorescent signal (Fig. 11B and 11C). However, it was difficult to isolate a particular population that could be recognized as AcNPV.

Ethanol-fixed samples of AcNPV were stained with SYTO 11 only and with SYTO 11 and TB (Fig. 12). Two populations showed a shift up in FL3 (1.1-fold increase for the green population (R2) and 1.5-fold increase for the orange population (R3), and shift down in FL1 (2.1-fold decrease for the green population and 1.2-fold decrease for the orange population). The behavior of these populations is consistent with energy transfer between SYTO 11 and TB when both dyes are present in the same particle. Thus, the green and orange populations might represent nucleic acid containing particles. The samples stained with SYTO 11 and TB were sonicated (Fig. 13). This resulted in a significant decrease in the number of events in the green (R2) and orange (R3) populations (4.2-fold decrease for the green and 3.4-fold decrease for the orange populations). The number of events in the blue population (R4) was about the same before and after sonication (Table 4). It is possible that the green and orange populations were aggregates of some material in the samples, presumably virus aggregates, which were broken up during the sonication. The nature of the blue population is unclear. Some of the samples stained with SYTO 11 and TB were treated with 0.2% Triton X-100, which might increase permeability of the viral envelopes. However, this did not result in any significant changes in the staining (not shown).

Fig. 12. Staining ethanol-fixed AcNPV Baculovirus with SYTO 11 and TB. The events were classified into R1, R2, and R3 based on FSC/SSC properties since the properties were expected to be stable regardless of the dyes used. Green (R2) and orange (R3) events: Baculovirus aggregates; black events: background fluorescence. The nature of the blue events (R4) was unclear. The mean fluorescence values (arbitrary units) for R2, R3, and R4 in FL1 and FL3 channels are shown. About 1.1-fold increase for the green (R2) and 1.5-fold increase for the orange (R3) population was observed in FL3 after staining with SYTO 11 and TB. A decrease in FL1 channel was about 2.1-fold for the green (R2) and 1.2-fold for the orange (R3) population after staining with both dyes.

Fig. 13. Sonication of ethanol-fixed AcNPV Baculovirus stained with SYTO 11 and TB. The events were classified and colored as described in Fig. 12. The percentage of the events before and after sonication is shown for R2, R3 and R4. Note a significant depletion of the number of events in the green (R2) and orange (R3) populations after sonication. The number of events in the blue population (R4) was approximately the same before and after sonication.

Table 4

Sonication of the Baculovirus preparation stained with SYTO 11 and TB

Region*	% Total events		Increase (+) or decrease (-) in number of events after sonication (fold)
	Before sonication	After sonication	
R2	1.10	0.26	-4.2
R3	3.61	1.07	-3.4
R4	25.55	21.97	-1.1

* Regions R2, R3, and R4 were designated on Fig. 13.

Part III: Flow Cytometric Analysis of DNA Molecules

Staining *S. cerevisiae* Chromosomes

Staining the *E. coli* genome was successful; however, none of the three viral models revealed a population that could be identified as virions. DNA molecules that were smaller than the *E. coli* genome but larger than the viral genomes studied were analyzed to determine the smallest DNA molecule that can be detected by flow cytometry. C-yeast chromosomes consisting of 15 chromosomes of *S. cerevisiae* with sizes ranging from 225 to 2,200 kb were stained with SYTO 11. FCA of the C-yeast chromosomes revealed the presence of material characterized by high FL1 signal (Fig. 14B and 14C, green population), which was not observed in the negative control (Fig. 14A). The number of events in the green population differed approximately by 10 times when the 1:1,000 and 1:10,000 dilutions were compared. This correlation between the number of the fluorescent events and dilutions of the sample showed that the fluorescent signal was due to the material in the sample and not due to instrumental noise. To prove the nucleic acid nature of the observed material, DNase treatment of the samples was performed. To assure that DNase was functioning properly, stained *E. coli* was also treated with DNase (not shown). DNase treatment of the stained C-yeast chromosomes led to the almost complete disappearance of the green population (Fig. 14E and 14F). However, adding DNase to the samples contributed significantly to the background which made the analysis more difficult (Fig. 14D, 14E, 14F).

The problem in this experiment was that the fluorescent material covered about two orders of magnitude on the FL1 scale, whereas it was expected to cover only one order of magnitude since the range of the chromosome size was from 225 to 2,200 kb. This

Fig. 14. Staining *S. cerevisiae* chromosomes (C-yeast chromosomes) with SYTO 11. A. Negative control (TRIS buffer and SYTO 11). B. 1:1,000 dilution of chromosomes prior to staining. C. 1:10,000 dilution of chromosomes prior to staining. D. DNase treatment of negative control. E. DNase treatment of the chromosome sample diluted 1:1,000. F. DNase treatment of the chromosome sample diluted 1:10,000. Green events: yeast chromosomes; red events: background fluorescence. Note an increased amount of background fluorescence after nuclease treatment (D, E, F).

suggested that some material other than just yeast chromosomes was also present in the samples. Overall, the staining of the C-yeast chromosomes was successful. It seemed useful to use these chromosomes as size markers in FCA. It was necessary to find out which part of the signal was due to the stained yeast chromosomes, and which was due to staining of other material in the sample.

Pulsed-Field Gel Electrophoresis

To investigate the content of the yeast chromosome samples, the C-yeast chromosomes previously studied by flow cytometry (above) and P-yeast chromosomes were analyzed by PFGE. After running the C-yeast chromosomes in PFGE for 19 h, at least 13 of the 15 chromosomes were clearly visualized (Fig. 15A). A significant amount of nucleic acid material remained in the well. This material was likely to be complete yeast cells whose cell walls were not digested during isolation of the chromosomes. The P-yeast chromosomes in PFGE revealed a distribution of the bands similar to the C-type chromosomes. Also, a significant amount of material stayed in the wells (Fig. 15B). Material in the wells (might be yeast cells containing all 15 chromosomes) probably contributed to the highly fluorescent events observed during FCA of the C-yeast chromosomes (Fig. 14B and 14C).

Running the C-yeast chromosomes in PFGE for 9 h was performed to reveal low MW material present in the sample. This led to a poorer resolution of the yeast chromosomes, but showed the presence of low MW nucleic acid material that was not a yeast chromosome and was present in a large amount (relatively bright band) in the sample (Fig. 15C). This material may represent nonnuclear nucleic acid (could be mitochondrial

Fig. 15. Pulsed-Field Gel Electrophoresis of *S. cerevisiae* chromosomes. A. PFGE of C-yeast chromosomes (lane 1 and 3) for 19 hours. At least 13 out of 15 chromosome bands were visualized. B. PFGE of C-yeast chromosomes (lane 1) and P-yeast chromosomes (lane 3) for 19 hours. A significant amount of nucleic acid material remained in the wells. C. PFGE of C-yeast chromosomes for 9 hours. Note the presence of nonnuclear DNA (indicated by arrow) that was not observed during 19-hour run.

DNA) and probably contributed significantly to the events with lower fluorescence in the C-yeast chromosome sample (Fig. 14B and 14C).

Flow Analysis of Yeast Chromosomes after PFGE

The original idea was to separate the C-yeast chromosomes by PFGE, cut out each band containing one particular yeast chromosome from the gel, and then detect each chromosome by flow cytometry. This would allow us to use these chromosomes as size markers and find out which of them would give the lowest detectable signal in FCA. Preliminary results (not shown) indicated two problems. First, agarose present in the chromosome bands contributed significantly to the background fluorescence making analysis more difficult. Second, an insufficient amount of DNA was present in individual bands, but an increase in the amount of sample loaded on the gel led to poor resolution. In order to find out if the idea of observing separated yeast chromosomes in the flow cytometer after PFGE is feasible, the experiment was modified as described below.

The C-yeast chromosomes were run in PFGE for 2 h. This time was enough to allow the chromosomes to leave the wells but they were not separated yet, which led to an appearance of a band with a relatively high amount of DNA (C-yeast chromosomes-PFGE). The gel was stained with SYBR 1, and the visualized DNA band was cut out trying to minimize the amount of agarose. An agarose block without a DNA band (a negative control) and the C-yeast chromosomes without being electrophoresed (C-yeast chromosomes-no PFGE; a positive control) were also analyzed. Staining of the three samples (C-yeast chromosomes-PFGE, agarose block, and C-type chromosomes-no PFGE) was performed as described in Materials and Methods.

All three samples revealed the presence of the same population (Fig. 16; R2: a population colored green). However, the C-yeast chromosomes-PFGE and C-yeast chromosomes-no PFGE also revealed the presence of the second population (Fig. 16; R1: a population colored red), which was not observed in agarose block. It is likely that R1 represented yeast chromosomes. Fewer events in R1 were observed in the C-yeast chromosomes-PFGE sample compared to the C-yeast chromosomes-no PFGE sample. It is possible that some disruption and/or alteration of the structure of chromosomes occurred due to high temperature treatment during melting (especially after treatment with intercalating dye), and UV treatment (during photographing and cutting blocks out of gel).

After separation by PFGE, yeast chromosomes were observed in the flow cytometer although it was impossible to separate peaks for each chromosome on a DNA histogram (not shown). The total amount of DNA was not sufficient for adequate flow cytometric analysis. It is also possible that the structure of the chromosomes was altered because of the harsh treatment. In this case such DNA molecules could not serve as the size markers to detect the smallest molecule detectable in FCA.

Fig. 16. Flow cytometric analysis of *S. cerevisiae* chromosomes after PFGE. PFGE was carried out for C-yeast chromosomes for 2 hours. A visualized DNA band was cut out of the gel, melted at 65°C for 10 min, diluted in 0.05 M TRIS buffer (pH 8.0), stained with SYBR I (10X), and analyzed by flow cytometry. A. Negative control: an agarose block from the gel without a DNA band (the same staining procedure as for the C-yeast chromosomes). B. C-yeast chromosomes after running in PFGE for 2 hours. C. Positive control: C-yeast chromosomes without being electrophoresed (the same staining procedure as described above). Red events (R1): yeast chromosomes; green events (R2): background fluorescence. Note a significant amount of background fluorescence in all three samples.

DISCUSSION

Analysis of viruses by flow cytometry would have a variety of applications. Conventional techniques of identification and quantitation of viruses include counting of plaques formed in a monolayer of living cells. This method is time-consuming and limited to viruses for which a suitable cell system exists. FCA of virions would provide a fast method for precise counting of virions. Flow cytometry would also be a useful method for monitoring the presence of the viruses in, for example, cell culture supernatant fluid used for production of pharmaceuticals, and thus present a valuable approach for quality control. Flow cytometry might replace the more tedious and expensive TEM method widely used for this purpose currently. Since different types of viruses vary significantly in size, it might be possible to differentiate viruses based on their size, especially if measurement of light scattering becomes possible.

Fluorescence from dyes bound to nucleic acids was chosen in this study as a primary parameter for the detection of individual viruses. Using light scatter was rejected based on literature and experimental data. In our experiment with *E. coli*, forward and side scatter was barely adequate for thresholding, and would not be expected to be useful for viruses (Fig. 6A), the largest of which (pox virus) would have had about a 10^5 weaker signal than *E. coli* (39). With latex beads of different size, forward scatter sensitivity in the FACSort was barely sufficient to detect the 0.57 micron beads (Fig. 6B), while virions were expected to be smaller than 200 nm. This agrees with Dusenberry et al. (13), who reported that the forward scatter sensitivity of a FACScan was increased by replacing the photodiode with a photomultiplier tube, which has a background noise level about an order of magnitude lower. In the same study, the side scatter sensitivity was increased by replacing the existing focusing lens with a 3 x expansion telescope, which resulted in a

decrease in the cross-sectional area of the laser spot at the interrogation point. As a result, the intensity of the laser light was increased, which made the sensitivity of the instrument five-fold higher. Without engineering modifications to the flow cytometer, the detection of individual virions using light scattering was not possible.

The suitability of thresholding was also assessed during the preliminary study. Two approaches were used. The first included staining the *E. coli* genome of 4.6 megabases, which is about 200 times larger than the genome of the endogenous retrovirus of interest. The bacteria were identified by the fluorescence signal. The separation of the bacterial signal from other particles in the sample suggested that it should be possible to detect a 32,000 nucleotide genome. This assumed that the staining efficiency of *E. coli* and virions would be similar. While this detection limit is higher than needed for the detection of a 20,000 nucleotide genome, the fluorescence of the background events in the *E. coli* samples was higher than that seen in stained buffers. If virus samples are purer than the *E. coli* samples (e. g. purified virus in a buffer), the threshold could be reduced. As a result, the detection of genomes even smaller than 32,000 nucleotides might be possible. The second approach was fluorescence quantitation (using Quantum 24 kit) in terms of a minimum number of fluorescein equivalents that would give a signal detectable when thresholding on green fluorescence. When the same method of fluorescence quantitation was used for *E. coli* (Fig. 5D), the MESF result was slightly more than 400,000. The Quantum 24 kit is designed for quantitation low level signals (up to 50,000 fluorochromes); so, it is possible that measuring a high fluorescent signal of *E. coli* based on the MESF standard curve obtained with Quantum 24 kit was not very precise. Quantum 24 beads were labeled with fluorescein, whereas *E. coli* was stained with SYTO 11. Fluorescein has a higher quantum yield and extinction coefficient than SYTO 11. These differences in the optical properties of the dyes explain the MESF results obtained during *E. coli* staining with SYTO 11. Based on genome size, retrovirus would give a signal

about 200-fold lower than bacteria, equivalent to approximately 2,000 MESF. This is slightly lower than CaliBRITE bead signal (about 4,000 MESF) but above the noise level seen in buffers.

As a virus model, CHO supernatant fluid containing endogenous retroviruses was originally used. Staining the supernatant fluid with different nucleic acid dyes did not reveal any discrete population that could be identified as the retrovirus. Filtration of the supernatant fluid through 0.2 micron filter was the only purification technique that decreased background fluorescence, but it did not lead to any significant improvement in the staining. The frequency of the events in the sample was much higher than expected from TEM. This suggested that the CHO supernatant fluid contained a significant amount of non-viral material that was binding the dyes. Comparison of the fluorescence of the events in the CHO supernatant fluid and other fluids (buffers and RPMI-1640 with 10% FBS) supported this suggestion (Table 1). CHO supernatant fluid stained with SYTO 11 revealed events with higher fluorescence than in any of the assayed fluids. It was also significantly higher than the background observed during staining of *E. coli* or AcNPV (not shown). So, it is likely that some material present in CHO supernatant fluid camouflaged the retrovirus fluorescence, especially because the fluorescence signal of the stained viral genome was expected to be very weak to begin with.

A preparation of MLV purified by ultracentrifugation was used in order to avoid the problem of the background fluorescence. However, the virions were probably physically destroyed during inactivation. No discrete population was observed during FCA.

Since the results of studying retroviral system (an endogenous retrovirus from CHO supernatant fluid and MLV) were unsatisfactory, it was useful to analyze a different type of viruses. The Baculovirus model seemed to be the most suitable for the study because of the relatively large genome (about 130 kb), which is only 35 times smaller than the *E. coli* genome. High titer stock solution of wild-type AcNPV was produced and

purified. Based on the fluorescence obtained from the *E. coli* staining, a detectable signal from the Baculovirus was expected. However, staining the Baculovirus with SYTO 11 did not reveal any discrete viral population. Staining with SYTO 11 and TB revealed two populations (green and orange; Fig. 12) with behavior consistent with energy transfer expected from nucleic acid containing particles. The number of events in these population was significantly depleted after sonication, which suggested that these populations represented aggregates of the Baculovirus. Staining the CHO supernatant fluid with SYTO 11 and TB (Fig. 10) allowed separation of two populations, one of which had behavior consistent with that of nucleic acid containing particles. However, the unexpectedly high FSC and the low number of events made conclusions about the retrovirus nature of the population difficult.

Staining the *E. coli* genome was successful, whereas the analysis of three different viral models did not reveal any discrete populations. An attempt was made to analyze DNA molecules that were smaller than the *E. coli* genome but larger than the viral genomes. Staining the isolated chromosomes would help to determine if viral structural characteristics (e.g. presence of an envelope) may be responsible for the low staining efficiency. Staining *S. cerevisiae* chromosomes (ranging from 225 to 2,200 kb) with SYTO 11 revealed the presence of DNase sensitive material, which likely was yeast chromosomes (Fig. 14). Unlysed yeast cells with a complete set of 15 chromosomes and nonnuclear nucleic acid probably contributed significantly to the events with high and low fluorescence respectively. Impurity of the sample made it difficult to interpret the results, and may explain poor peak resolution of the chromosomes. Purification of the sample was performed by PFGE. However, the presence of agarose residue in the samples and insufficient amount of DNA after PFGE did not allow adequate FCA. It is also possible that the structure of the chromosomes was altered during handling and analysis. This is one more reason why such DNA molecules could not be used as a model for the study.

Despite the fact that the fluorescence performance of the cytometer seemed to be sufficient for the detection of at least larger viruses (e. g. Baculovirus), none of the three viral models analyzed by flow cytometry showed any discrete population that could be identified as virions. It is possible that the problem arises from poor staining of the viruses. An envelope in some types of viruses may serve as a barrier for some dyes, and result in decreased staining efficiency. However, the presence of the envelope does not explain why staining with a permeant dye, such as SYTO 11, would be ineffective. The accessibility of the viral nucleic acid for permeant dyes may also depend on how densely the nucleic acid is packed. In this respect it would be useful to mention some studies done on eukaryotic cells.

Routinely the human chromosomes can be efficiently stained in their native form (24). However, some authors suggest that a large portion of nuclear DNA in native chromatin is unstainable, and for certain dyes the amount of unstainable DNA varies depending upon chromatin structure (3, 9). There is evidence of variations in the accessibility of mammalian DNA to intercalating dyes, generally associated with changes in chromatin structure. For example, a decrease in DNA accessibility to acridine orange was demonstrated during spermiogenesis (15) and erythropoiesis (41). During differentiation of Friend leukemia cells, it was shown that the binding of intercalating dyes (acridine orange, ethidium bromide, DAPI, etc.) was remarkably diminished in differentiated cells (9). Changes in chromatin during mitosis altered binding of intercalators (8). Studies of *Euglena gracilis* also demonstrated differences in DNA fluorescence between exponentially growing versus stationary cells stained with intercalating dyes (3). The mechanism of this restriction of dye binding to DNA in chromatin is poorly understood. Some authors emphasize the role of the histones and other nuclear proteins in chromatin organization, and suggest that compact conformation of chromatin decreases the accessibility of DNA to intercalating molecules (3, 9). The more condensed the nucleic acid the fewer binding sites

available for the dyes. This is supported by the studies showing that removing the nuclear proteins by acid treatment results in a significant increase of binding of intercalating dyes in mammalian cells (9) and *Euglena gracilis* (3). All these results support the idea that high condensation of nucleic acid may significantly diminish the staining efficiency of a genome. Similarly, if the viral nucleic acid is very densely packed, it may exhibit a low efficiency of genome staining. The interaction between protein core and viral genome, which is not completely understood for many viruses, may therefore play a role in limiting the accessibility of the nucleic acid for the dyes.

It is interesting that changes in chromatin structure do not effect the staining efficiency of the eukaryotic cells when nonintercalating dyes (e. g. Hoechst 33258, mithramycin, chromomycin A₃) are used (3, 9). Probably the mode of binding is an important factor determining the staining efficiency of nucleic acids. The mode of binding of cyanine-based dyes has been studied recently. YOYO 1 was found to exhibit two binding modes: intercalation and external binding. At low dye to base pair ratios, intercalation takes place, whereas at high dye to base pair ratios, external binding begins to contribute (19). The mode of binding of SYTO dyes is still unknown. Optimization of the dye to base pair ratio may be important for the improvement of the staining procedure of the virions.

Further work will be needed to clarify the reasons for low staining efficiency of the virions. The optimal conditions for viral nucleic acid staining must be defined in FCA. Investigating the mode of dye binding and optimization of the dye to base pair ratios may be useful. More information about viral structure, the genome organization, degree of genome condensation and dimensional relationship between core proteins and nucleic acids will help to determine the optimal staining conditions. Modifications of the flow cytometer may be necessary to increase the sensitivity of the instrument, allowing analysis of both fluorescence signals and light scatter for the detection of the virions.

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APPENDIX

Abbreviations

AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
BSA	Bovine serum albumin
BV	Budded virus
CHO	Chinese Hamster Ovary (cell line)
$F_{\text{bound}}/F_{\text{free}}$	Fluorescence of bound dye/ Fluorescence of free dye
FBS	Fetal bovine serum
FCA	Flow Cytometric Analysis
FITC	Fluorescein isothiocyanate
FL1	FL1 detector (green fluorescence)
FL2	FL2 detector (orange fluorescence)
FL3	FL3 detector (red fluorescence)
FL4	FL4 detector (red fluorescence)
FSC	Forward scatter
g/L	Grams per liter
%G+C	Guanine-plus-cytosine percentage
h	Hour(s)
kb	Kilobases
kD	Kilodalton
LB	Luria-Bertani (broth)
MESF	Molecules of Equivalent Soluble Fluorochrome

MFI	Mean Fluorescence Intensity
min	Minute(s)
MLV	Murine Leukemia Virus
MW	Molecular Weight
OV	Occluded Virus Particles
PBS	Phosphate buffered saline
PFGE	Pulsed-Field Gel Electrophoresis
pfu	Plaque forming unit(s)=virus
sec	Second(s)
SSC	Side scatter
TB	Thiazole Blue
TBE	Tris borate/EDTA
TE	Tris/EDTA
TEM	Transmission Electron Microscopy
TNM-FH	<i>Trichoplusia ni</i> Medium-Formulation Hink
UV	Ultraviolet (light)

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