Rapid Enumeration of Respiratory Viruses

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Virus detection and enumeration has become increasingly important in fields ranging from medicine and biotechnology to environmental science. Although there are a wide variety of techniques that can be used to count viruses, there is demand for a rapid and more accurate means for virus enumeration. In this work, the performance of a flow cytometer that was designed and custom-built specifically for rapid detection of single viruses was evaluated. The instrument, designated a single nanometric particle enumerator (SNaPE), was characterized and calibrated using fluorescent polystyrene nanoparticles. The reliability of the instrument with respect to virus enumeration was demonstrated for three medically relevant viruses, adenovirus-5, respiratory syncytial virus, and influenza A, treated with a fluorescent nucleotide stain. In each case, the SNaPE yielded a virus particle concentration consistent with, but slightly lower than, transmission electron microscopy (TEM) results, as expected. In addition, on the basis of calibration of signal intensity, the average peak height for a given virus was correlated with genome size, as expected. In contrast to time-consuming analyses such as TEM and plaque titers, SNaPE analysis of pure virus samples (including sample handling, data collection, and data processing) can be completed within 1 h.

Viruses are capable of infecting plant, animal, and bacterial cells alike. The ability to routinely and rapidly quantify viruses has become increasingly important in fields as diverse as medicine and environmental science. In medicine, diseases such as polio, hepatitis, and AIDS are caused by viral infections. Diagnosis of infections such as these often requires not only identification of the virus but also determination of the viral load in body fluids and tissue samples. Viral enumeration techniques are needed not only for medical diagnosis, but for the development of advanced medical treatments, such as gene therapy and gene transfer. These promising new approaches utilize modified viruses to treat illness, and treatment efficacy cannot be determined without viral enumeration and monitoring.

Although the most well-known viruses are associated with epidemic diseases, the number of viruses able to infect humans constitutes only a small fraction of the viruses known to science. Viruses that infect microbiological organisms are equally important, since such organisms are capable of influencing and even regulating aspects of our environment. Recent studies have shed light on the role of viruses in the marine environment, and initial results indicate that their influence has been grossly underestimated. Limited understanding of virus regulation of microbiological organisms in natural water systems may be attributed, at least in part, to the lack of direct virus counting techniques prior to the late 1980s. It is now known that natural waters typically contain upward of 10^10 viruses/mL. These numbers make viruses the most abundant biological entities in the marine environment. There are current studies focused on toward evaluating the indirect role of viruses in climate, the marine food web, and marine nucleotide fluxes. Additionally, it is likely that viral loads in public water supplies will need to be evaluated regularly in the future in order to ensure public health and to better understand the spread of disease.

Within the field of virology, now just over 100 years old, the number of analytical techniques demonstrated for viral enumeration is quite varied. The small size of viruses (typically 10-300 nm in diameter) has historically made their enumeration and monitoring difficult. Cell culture techniques, similar to those


10.1021/ac011183q CCC: $22.00 © 2002 American Chemical Society Published on Web 03/08/2002
still commonly used today, were the first methods capable of virus enumeration.\textsuperscript{1,2}\textsuperscript{24} Electron microscopy techniques, such as transmission electron microscopy (TEM), allow for direct visualization of viruses and have been instrumental in advancing the field of virology;\textsuperscript{1,2}\textsuperscript{26} however, there remains a need for new techniques that are capable of rapid, cost-effective, and routine viral counting.\textsuperscript{3,4,7,9,12,23,24}\textsuperscript{28}

Plaque assay, a cell culture technique, was developed in 1952 by Renato Dulbecco and prevails as the most widely used method for virus enumeration.\textsuperscript{10} However, there are several disadvantages to plaque assay titers. Specifically, titers based on cell culture techniques are time-consuming. The time required for preparation, incubation, and evaluation is typically between 12 h and 2 weeks.\textsuperscript{4,26} Titers also have low sample throughput and are relatively inaccurate and imprecise, even when administered by skilled technicians.\textsuperscript{7,16,27} Another significant limitation of plaque assays is the fact that many viruses cannot be quantified by cell culture techniques. For example, the nonlytic Epstein-Barr virus does not form plaques and cannot, therefore, be titered by a plaque assay.\textsuperscript{29} Additionally, plaque assays require that the virus has been isolated and the host cell type is known. The Hanta virus is a good example of a case in which the virus remained uncharacterized for almost three decades.\textsuperscript{29} For environmental samples, these disadvantages impose prohibitive limitations, since in an overwhelming number of cases, the host is unknown or the virus has not been identified or isolated.

TEM is perhaps the second most common virus analysis technique. TEM can provide both qualitative and quantitative information on reasonably concentrated viral samples. Although viruses may be “visualized” using TEM, there are several experimental considerations that must be taken into account for quantitative evaluation. For example, the sample must be collected and homogeneously distributed on the TEM grid, which often requires centrifugation. In addition, the sample is imaged in a nonphysiological, high vacuum environment. In contrast to plaque assays, TEM analysis is not capable of distinguishing between infectious and noninfectious particles.\textsuperscript{3,30} For these reasons, as well as limited access to a relatively expensive and nonportable instrument, TEM is not an appealing technique for routine virus quantitative viral analysis.\textsuperscript{3,11,12,23,24,31,32}\textsuperscript{28}

Epifluorescence microscopy (EFM) has recently become a popular technique for enumeration of viruses. EFM is relatively inexpensive, relatively rapid, and both the instrument and sample handling methodology are adaptable for field measurements.\textsuperscript{22,23,31}\textsuperscript{39} However, there are drawbacks to EFM, including the need to filter, immobilize, and stain samples. In addition, with EFM, it is difficult to optically resolve and discriminate between viruses and other biological particles that are smaller than the diffraction limit (e.g., small bacteria, spores, etc.) in a complex sample.\textsuperscript{3,11,12,23,24,31}\textsuperscript{39}

A handful of relatively new techniques have emerged for enumeration and detection of viruses. Polymerase chain reaction (PCR)-based methods are perhaps the most promising of these new techniques, because they are sensitive, relatively rapid and usually do not require culturing.\textsuperscript{33–39} PCR-based methods require skilled technicians and are highly susceptible to biological, organic, and inorganic contaminants.\textsuperscript{33,36} Moreover, PCR analysis of viruses is limited by the fact that these methods require virus-specific sequence information, which in many cases is simply not available.\textsuperscript{3,35}\textsuperscript{1850} Analytical Chemistry, Vol. 74, No. 8, April 15, 2002

Recent work has demonstrated the ability of flow cytometry (FCM) to rapidly detect marine viruses stained with high-quantum-yield fluorescent dyes.\textsuperscript{24,37} However, the majority of commercially available FCM instruments are designed for analysis of samples containing particle sizes and fluorescence intensities much larger than that of a typical fluorescently stained virus.\textsuperscript{38,39} Because commercial flow cytometry instruments are expensive and not optimized for viral analysis, their use for virus enumeration is experimentally challenging and relatively rare.

A simple, rapid, and inexpensive flow cytometer that takes advantage of recent advances in optics developed for single molecule detection (SMD) has been developed and optimized in our lab for virus detection and enumeration. This instrument, designated the single nanometric particle enumerator (SNaPE), provides an alternative to standard virus enumeration methods with cost and simplicity advantages that facilitate routine analysis of virus samples for medical, environmental and other applications. Instrument performance was evaluated using three medically relevant viruses: adenovirus-5, respiratory syncytial virus, and influenza A virus.

**EXPERIMENTAL SECTION**

**SNaPE Instrument**. A schematic diagram of the custom-built flow cytometer is shown in Figure 1. The SNaPE instrument, described elsewhere in detail,\textsuperscript{40} has an air-cooled CW argon ion laser operating at 488-nm emission (18 mW) as the excitation source (Ion Laser Technology). A single microscope objective (100×, 1.2 NA, Edmund Scientifcs; Barrington, NJ) was used for focusing the excitation beam and collecting bursts of fluorescence produced by sample particles passing through the excitation volume. Fluorescence was directed toward a long-pass filter (o.d. 3 for λ < 460 nm) by a dichroic mirror (CVI Laser Corp.) that reflected ≥ 99.5% of 514 nm light. Sample fluorescence was incident on a PM T (Hamamatsu R928) biased at –950 V with a C3830 Hamamatsu power supply. The PM T was operated in current mode. A 488-nm holographic notch filter (Kaiser Optical Systems, Inc.) was used prior to the PM T to eliminate the majority of any scattered or reflected 488-nm light. A 500-μm pinhole was placed in the confocal image plane to spatially filter the sample image and produce an approximate probe volume of ~8 FL. The probe volume was calculated using a cylindrical approximation with a radius defined by the focused beam waist and a height...
Figure 1. Schematic diagram of the SNaPE instrument with an optical system that produced a 0.5 mW diffraction limited spot size of ~0.5 μm and a probe volume of ~8 fL. Samples were pumped through a fused-silica capillary (10-μm i.d., 140-μm o.d.) at 50 μL/hr using a microliter syringe pump. All optics were fixed, with focusing and alignment accomplished through positioning of the sample capillary using a three-dimensional translation stage.

determined by the capillary inner diameter. The calculated volume is consistent with volumes reported for single molecule detection studies utilizing similar confocal optical arrangements. Given the probe volume size and the standard flow rate of 50 μL/h, an average transit time (based on full width at half-maximum) is ~150 μs. The digitization rate is discussed in detail within the text, but the standard rate was 80 kHz.

Data Acquisition. The PMT signal was processed using a computer (Gateway Pentium 133) equipped with a 12-bit data acquisition card (PCI-DAS4426/12; Computer Boards) that was controlled using a program written with HPVee 5.01 (Hewlett-Packard) and the Computer Boards Universal Library software. Raw signal amplitude was inverted to yield positive peaks and corrected for a constant offset. Events were counted using the peak count feature in Grams/386 software (Galactic Industries). One of the criteria for peak identification was a threshold (i.e., a peak count feature in Grams/386 software (Galactic Industries). The controls were (1) TE buffer with and without OliGreen dye, (2) TE buffer with and without addition of OliGreen, and (3) adenovirus-5 without the addition of OliGreen.

Virus Samples. Active samples of adenovirus-5 (AD-5), respiratory syncytial virus (RSV), and human influenza A (FLU-A) virus were purchased in purified form (Advanced Biotechnologies, Inc., Columbia, MD). Biohazard precautions included hand and eye protection as well as sterilization of all surfaces and equipment before and after contact with the pathogens. Virus samples were supplied with reported virus concentrations, determined by TEM, of 5.10 × 10^10, 4.7 × 10^10, and 7.4 × 10^10 virus particles (vp)/mL for AD-5, RSV, and FLU-A, respectively. The manufacturer estimated the relative error of these TEM measurements to be ~25%. Virus purification methods, suspension buffers, and additional virus details are given in Table 1.

In addition to the reported TEM concentrations, the manufacturer also reported either tissue culture infectious dose (TCID_{50}) titer or protein assay results for the virus samples with an estimated relative errors of ~25% and 5% respectively. The number of plaque forming units (pfu) per mL can be estimated from TCID_{50} results by multiplying the TCID_{50} value by 0.7. This results in an estimated infectious particle concentration of 1.25 × 10^10 pfu/mL for the AD-5 sample, the only virus in this study to be enumerated by culture techniques. Protein concentrations, reported as 1.0 and 0.993 mg/mL for the RSV and FLU-A samples, respectively, cannot easily be converted to virus concentration but are indicative of virus content. Protein concentration was determined by the Pierce BCA method using a BSA standard.

All purified virus samples were diluted with sterile TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) to produce working samples with virus concentrations ranging from 5.00 × 10^7 to 1.28 × 10^10 vp/mL. This concentration range encompasses literature values reported for typical viral load in both environmental and medical samples. Oligonucleotide samples were stored at -70 °C for periods longer than 24 h or at 4 °C for periods less than 24 h, and all viruses were subjected to no more than 2 freeze/thaw cycles.

Sample Staining. Virus samples were stained with OliGreen dye (Molecular Probes; Eugene, OR), a high-quantum-yield fluorescence dye specific for nucleotides. Although OliGreen dye is reportedly semiselective for single-stranded (ss) DNA, it has been demonstrated that the dye binds to various nucleotide-based molecules, including double stranded (ds) DNA and RNA. OliGreen stock was diluted 200-fold, as recommended by the manufacturer, with TE buffer to produce working dye solution. Samples were stained with a volumetric sample-to-dye ratio of 1:1. Samples were allowed to incubate at ambient temperature for 20 min in the dark prior to analysis. Polypropylene containers and pipets were used in the preparation of all solutions containing OliGreen, since it is known to adsorb onto glass surfaces.

Control Samples. Several control samples were analyzed in order to establish the background count rate for the SNaPE instrument. The controls were (1) TE buffer with and without addition of OliGreen, (2) 18 MΩ water with and without addition of OliGreen, and (3) adenovirus-5 without the addition of OliGreen.

(46) Personal communication with ABI technical support personnel, 2001.
(48) Product specification sheets provided by ABI online (Columbia, MD) for item numbers 10-247-000 and 10-210-000, 2001.
(51) Data not presented here but available upon request.
**Table 1. Summary of Viral Samples Analyzed by the SNaPE Instrument**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Purification density gradient</th>
<th>Suspension buffer</th>
<th>Reported TEM concn (vp/mL)</th>
<th>Genome size (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus-5</td>
<td>Cesium chloride</td>
<td>10 mM Tris, 150 mM NaCl, 1 mM EDTA, 10% glycerol</td>
<td>5.10 × 10^10</td>
<td>30 000–38 000 ssDNA</td>
</tr>
<tr>
<td>Respiratory syncytial (A-2)</td>
<td>Sucrose</td>
<td>10 mM Tris, 150 mM NaCl, 1 mM EDTA</td>
<td>4.7 × 10^10</td>
<td>15 000–17 000 ssRNA</td>
</tr>
<tr>
<td>Influenza A/P/8/34 (H1N1)</td>
<td>Sucrose</td>
<td>PBS with 20 mM HEPES</td>
<td>7.4 × 10^10</td>
<td>12 000–15 000 ssRNA</td>
</tr>
</tbody>
</table>

*All suspension and dilution buffers were pH 7.5.*

**RESULTS AND DISCUSSION**

**Instrument Characterization.** In order to evaluate and characterize the capabilities of the SNaPE instrument prior to viral analysis, commercially available fluorescent polystyrene nanoparticles were utilized. Fluorescent polystyrene spheres are commonly used as calibration standards for flow cytometry, dynamic single molecule detection (SM D), and single particle tracking. Dilutions of supplied sphere stocks were made to produce samples with sphere concentrations ranging from 10^6 to 10^9 spheres/mL. The specified concentration range is typical of viral loads discussed in the literature for both environmentally and medically relevant samples analyzed by other enumeration techniques.

Initial settings for these parameters were chosen based on literature values for similar determinations using flow cytometry, and SM D. Using sphere standards before attempting virus enumeration. Initial settings for these parameters were chosen based on literature values for similar determinations using flow cytometry, and SM D.

**Acquisition Rate.** Digital acquisition rates ranging from 1 to 200 kHz were examined using a sample of 110-nm spheres at a concentration of 5.4 × 10^7 particles/mL and a flow rate of 50 μL/h. An acquisition rate of 80 kHz provided 12.5 μs time resolution and was, therefore, deemed sufficient for samples containing between 10^6 and 10^9 particles/mL. This acquisition rate also using sphere standards before attempting virus enumeration. Initial settings for these parameters were chosen based on literature values for similar determinations using flow cytometry, and SM D.
produced a manageable data file size (typically 1–5 MB) and was used for all further determinations unless otherwise noted.


\[ n_0 = \frac{N^2 \sigma^2}{d^2} \]  

where \( n_0 \) is the minimum sampling time in seconds, \( N \) is the number of seconds required to analyze 1 mL of the sample, \( z \) is the desired confidence interval (1.96 for 97% confidence), \( \sigma^2 \) is the population variance in particles/second, and \( d \) is the maximum allowed absolute error of the population estimate (typically 10% of the particles in 1 mL). Although the population variance is generally unknown, \( \sigma^2 \) may be substituted for the sample variance, \( \sigma^2 \). Equation 1 also assumes that the sampling time, \( n_0 \), is small in proportion to \( N \).

Sample variance (\( \sigma^2 \)) ranged from 3.15 to ~9000 for concentrations ranging from \( 4.6 \times 10^5 \) to \( 1.35 \times 10^9 \) spheres/mL. The calculated minimum sampling time ranged from 0.18 to 0.41 s. Accordingly, for even the lowest concentrations, a sampling time of 0.41 s is expected to yield a concentration value within 10% of the true value with 97% confidence. However, a systematic study of measured particle concentration as a function of sampling time indicated that measurement precision improved with an increase in sampling time. Thus, in most cases, data were collected for >20 s.

**Simultaneous Events.** Given the relatively high concentration of particles in typical virus samples, it was important to establish the upper concentration limit for avoiding a discount based on two or more particles in the probe volume simultaneously. A simple Poisson distribution was used to estimate the probability, \( P(n) \), of \( n \) particles being detected concurrently.

\[ P(n) = \frac{(rt)^n e^{-(rt)}}{n!} \]  

where \( r \) is the average flow rate (in particles per second) through the excitation/detection region, and \( t \) is the particle residence time in the excitation/detection region. Under the conditions of the experiment, the probability of two particles occupying the excitation/detection volume simultaneously does not exceed 3% and for the majority of the samples analyzed, it is ~1% \( P(2) \) becomes significant at concentrations above \( 5 \times 10^7 \) particles/mL.

**Limit of Detection.** The optical system of the SNaPE instrument was optimized for the smallest and least fluorescent of the calibration particles to be examined (i.e., the 26- and 44-nm spheres). Therefore, analysis of samples containing the larger calibration standards required the use of neutral density filters for signal attenuation. Grams 386 software was used to extract peak height, width, area, and total count from calibration data sets. From the calibration study, \( \sigma \) fluorescence intensity histograms for the calibration spheres were readily obtained. Figure 2c shows a histogram of fluorescence intensities for both 26- and 110-nm spheres. These distributions were used to determine average fluorescence intensities for each sphere size. Average fluorescence intensity was then plotted against FITC equivalents (from manufacturer) in order to develop a calibration curve. A summary of data from all of the spheres is given in Table 2. A linear regression fit to the data for the 26-, 44-, and 110-nm spheres yields a slope of \( (2.14 \pm 0.01) \times 10^{-4} \) arb units/FITC equivalents, a y-intercept of 0.120 ± 0.005 arb units, and a correlation coefficient of 0.999.

The magnitude of the background noise (\( N_b \)) was determined from several blanks of 0.02-μm-filtered 18 MΩ water as well as nonevent time regions of calibration data sets. The measured \( N_b \) value (0.0129 arb units) represents one standard deviation from zero after baseline offset correction. The magnitude of \( N_b \) was used to set the peak-counting software threshold level. Although the event threshold could have been set as low as 3 \( N_b \) to obtain a minimum signal-to-noise ratio (\( S/N \) ) of 3, the threshold was set at 7.8 \( N_b \) for improved confidence and to avoid false positives. The y-intercept reported for the calibration curve corresponds reasonably well to the sum (0.11) of the measured noise, \( N_b \) (0.0129), and the software-set threshold magnitude of 0.1000. The calculated limit of detection is specified as 3 \( N_b \), which corresponds to ~180 FITC equiv. Thus, the 26-nm spheres are near the calculated limit of detection and, therefore, may be considered the measured limit of detection.

**Detection Efficiency.** Although the confocal excitation/collection geometry utilized in the SNaPE instrument allows for detection of nanometric particles by minimizing background fluorescence, it also restricts detection efficiency. Multiple (\( \geq 3 \)) data sets were collected for each sphere size at known concentrations to determine the fraction of the manufacturer’s reported concentration detected by the SNaPE system. Percentages were found to be independent of particle concentration within the range examined, and thus, percentages for a given sphere size were combined, regardless of concentration. Table 2 summarizes the detection efficiencies measured for each sphere size (assuming the manufacturer’s reported concentration is accurate), which vary from 4 to 10%. The overall average detection efficiency for the SNaPE instrument is 8 ± 3%. This value is consistent with
calculated geometric approximation of ~5% detection efficiency (based on the capillary inner diameter and the diffraction-limited spot size of the excitation beam). It is important to note that the overall consistency of the efficiencies presented in Table 2 indicate the instrument’s ability to accurately quantify nanometric particles within the size, concentration, and fluorescence intensity range examined.

VIRUS ANALYSIS

There are a vast number of potential medical applications for a technique capable of rapid virus enumeration. Adenoviruses, for example, are the most common viruses used in gene therapy techniques. AD-5 is a spherical virus with an approximate diameter of 90 nm. The nonenveloped AD-5 has a ssDNA genome that contains 30 000 to 38 000 nucleotides. Gene transfer therapies utilizing adenovirus vectors have the potential to treat and (or) prevent illnesses, including cardiovascular disease, cancer, cystic fibrosis, Parkinson’s disease, and multiple sclerosis. Advancement of adenovirus mediated gene therapy techniques will require routine adenovirus enumeration. Currently, adenovirus concentrations are most commonly evaluated by plaque assay techniques that require up to two weeks to complete. In this case, it is important to distinguish infectious and noninfectious particles. For the AD-5 viruses examined in this work, the host for the titer was 293/human embryonic kidney cells.

Figure 3a shows the AD-5 concentrations determined by SNaPE analysis plotted against the manufacturer’s reported TEM concentrations. A linear regression to the data yields a correlation coefficient of 0.999 with a slope and yintercept of 0.45 ± 0.04 SNaPE particles/TEM vp and 2.26 × 107 SNaPE particles/ML, respectively. The quality and linearity of the plot demonstrates the reproducibility and reliability of the SNaPE instrument. The manufacturer’s reported concentration for the AD-5 stock solution, as determined by TEM, is 5.1 × 1010 vp/ML. The SNaPE instrument gives a stock concentration of 2.3 × 1010 particles/ML once the 8% detection efficiency is taken into account. The manufacturer also reported a TCID50 of 1.8 × 108 (viruses)/ML. It is expected that cell culture techniques result in the lowest relative concentrations, since they are sensitive only to those viruses capable of infecting the host cell. In contrast, TEM cannot distinguish between infectious and noninfectious viruses and generally results in higher particle counts.

Table 2. Instrumental Response for Fluorescent Polystyrene Calibration Spheres and OliGreen Stained Viruses

<table>
<thead>
<tr>
<th>nominal sphere diam/ virus</th>
<th>SNaPE %</th>
<th>av intensity</th>
<th>( \frac{1}{\sqrt{N}} )</th>
<th>FITC equiv</th>
</tr>
</thead>
<tbody>
<tr>
<td>26 nm</td>
<td>6.8</td>
<td>0.3</td>
<td>0.154</td>
<td>0.003</td>
</tr>
<tr>
<td>44 nm</td>
<td>4</td>
<td>2</td>
<td>0.199</td>
<td>0.003</td>
</tr>
<tr>
<td>110 nm</td>
<td>7</td>
<td>4</td>
<td>1.71</td>
<td>0.01</td>
</tr>
<tr>
<td>460 nm</td>
<td>10</td>
<td>2</td>
<td>89.8</td>
<td>0.7</td>
</tr>
<tr>
<td>2.6 μm</td>
<td>9</td>
<td>1</td>
<td>10300</td>
<td>200</td>
</tr>
<tr>
<td>advenvirus-5</td>
<td>1.71</td>
<td>0.02</td>
<td>7571†</td>
<td></td>
</tr>
<tr>
<td>influenza A</td>
<td>0.517</td>
<td>0.006</td>
<td>2890†</td>
<td></td>
</tr>
<tr>
<td>respiratory syncytial</td>
<td>0.637</td>
<td>0.009</td>
<td>2462†</td>
<td></td>
</tr>
</tbody>
</table>

* Percent of manufacturer’s reported concentration. † One standard deviation for a large population (N > 10 000). ‡ Specified in arbitrary units and corrected for attenuation. § Standard error of the mean is reported for average intensity. ‡ Manufacturer’s reported FITC equivalents. † Calculated FITC equivalents based on calibration curve (see ref 40).

Figure 3. SNaPE-generated virus calibration curves (a) and an analysis of observed viral fluorescence event intensities (b). Part a contains calibration curves for (●) AD-5, (○) RSV, and (▲) FLU-A. Error bars represent ± 1σ from the mean with solid lines as linear regression fits to the data. Regression fits are given in the text. Part b shows the correlation between observed average viral fluorescence intensity in FITC equivalents and reported virus genome size for (○) AD-5, (▲) RSV and (▲) FLU-A. Error bars for viral genome size represent literature value ranges; FITC error bars represent the standard error of the mean value. The solid line represents a linear regression fit to the data. The inverse of the measured slope indicates a nucleotide-to-dye binding ratio of 4 ± 2 nucleotides/FITC.
90,000 hospitalizations and 4500 deaths annually.\(^\text{87}\) RSV is an enveloped negative-sense ssRNA virus (15,000–17,000 nucleotides) that is variable in shape and has a diameter of 120–300 nm.\(^\text{1,77,78,88}\) The development of an RSV vaccine, which is a high research priority according to the CDC, will require routine RSV enumeration.\(^\text{88}\) Clinical laboratories typically diagnose RSV infection via antigen detection assays utilizing antibody staining techniques.\(^\text{88}\) Research labs may have capabilities to enumerate RSV using enzyme-linked immunoabsorbent assays (ELISA), which require roughly 6 h.\(^\text{87}\)

The calibration of SNaPE counts versus manufacturer's reported TEM concentration for RSV is shown in Figure 3a. The data are highly correlated with the TEM results (R of 0.993) and the linear regression analysis yields a slope of 0.53 ± 0.05 SNaPE particles/TEM vp and a y-intercept of 3.59 × 10^6 SNaPE particles/mL. The reported stock concentration (by TEM) is 4.7 × 10^{10} vp/mL. The SNaPE analysis yields a value of 2.5 × 10^{10} particles/mL. RSV was not titered.

Influenza A can be either spherical or filamentous in shape (~50–200 nm in diameter) and contains 12,000–15,000 nucleotides of ssRNA.\(^\text{1,77,78,88}\) The FLU-A virus is common, and medical diagnosis is often based on symptoms rather than virus detection. However, detection and enumeration of the FLU-A virus are usually used for tracking outbreak sources and identifying emerging vaccine resistant strains.\(^\text{88}\) Although culture techniques such as plaque assays and TCID\(_{50}\) liters require 5–10 days, they are still the most common techniques for influenza virus enumeration.\(^\text{88}\) Although less common, ELISA assays may also be employed to provide influenza detection and enumeration in a matter of hours.\(^\text{88}\)

SNaPE calibration against TEM for FLU-A is given in Figure 3a. The linear regression analysis resulted in a slope of 0.59 ± 0.01 SNaPE particles/TEM vp and a y-intercept of 3.90 × 10^7 SNaPE particles/mL with an R of 0.999. The reported stock concentration (by TEM) is 7.4 × 10^{10} vp/mL. The SNaPE analysis yields a value of 4.4 × 10^{10} particles/mL after detection efficiency correction, FLU-A was not titered.

**Measurement Precision.** For all three viruses, the calibration curve slopes (against TEM) are consistent and are within 15\% of the regression slope when all of the data are combined (m\(_{\text{reg}} = (0.52 ± 0.04) \text{ particles/vp}). The combined SNaPE data set contains 55,700 measurements. The average relative error in virus particle concentration as determined by SNaPE is 18 ± 9\% Relative error for TEM is ~25\%, and relative error for the AD-5 TCID\(_{50}\) is ~25\%.

**Evaluation of Reliable Concentration Range.** Four separate controls were used to evaluate the "blank" count rate (TE buffer with OliGreen; and water, TE buffer, and AD-5 without OliGreen). The TE buffer with added OliGreen resulted in the highest measured background, (4 ± 2) × 10^6 events/mL. The combined average of the blanks without OliGreen was (3 ± 4) × 10^6 events/mL. Background counts obtained for the controls without OliGreen are likely due to fluorescent impurities in the samples as well as electronic noise. The confocal design for the SNaPE instrument limits the probe volume to 8 ± 3\% of the capillary volume. Therefore, to calculate the true particle concentration, it is necessary to multiply the measured concentration by a correction factor (0.08 ± 0.12 ± 3 with error). Because this same correction factor is applied to all events, such as electronic noise, the control samples (i.e., blanks) result in an artificially high background. The high background imposes a lower concentration limit for reliable analysis of samples to > 10^6 particles/mL. The lower limit could be improved by reducing the digital discrimination threshold. The necessity of avoiding multiple particles within the probe volume imposes an upper limit on sample concentration, 5 × 10^6 particles/mL. Thus, the analytical range for reliable enumeration under the specified conditions is roughly 5 × 10^5–5 × 10^6 particles/mL.

**Additional Information Content.** Evaluation of fluorescence intensity from each event measured using the SNaPE can provide information about either the nucleotide-to-dye ratio or the viral genome size. The average observed SNaPE peak height for each virus was converted to an FITC equivalent using the instrument calibration discussed in the Instrument Characterization Section. The resulting values, summarized in Table 2, were plotted as a function of reported number of nucleotides for each viral genome, Figure 3b. For the limited data set available, there is a high degree of correlation between observed fluorescence intensity and virus genome size regardless of genome type (i.e., DNA vs RNA). The linear regression fit to the data produced a slope of 0.279 ± 0.006 FITC equivalents/viral nucleotide, a y-intercept of (1.9 ± 0.1) × 10^{-3} FITC equivalents, and a correlation coefficient of 0.999. This data indicates a nucleotide-to-dye ratio of 4 ± 2, which is within the range of values reported for other nucleotide systems using similar dyes.\(^\text{85,73,90,91}\)

**Time and Sample Requirements.** The single most important advantage of the SNaPE instrument for viral analysis is the speed with which accurate enumeration can be achieved for pure virus samples. Analysis (including dilution, staining, data collection and processing) is easily accomplished within 1 h, with a large portion of the time (~35 min) being dedicated to data processing. Processing time could be substantially reduced with application-specific software. An additional advantage includes minimal sample volume requirements (e.g., ≤100 \text{\mu L}). TEM and EFM measurements require sample immobilization that can require several hours and much larger sample volumes.

**Instrument Issues.** The entire SNaPE instrument was fairly inexpensive to construct (~$20,000) and is easily expandable to accommodate additional analysis channels. Since the data acquisition board can simultaneously monitor 4 channels, the only additional hardware required for multichannel analysis would be dichroic optics and additional detectors. Unlike single molecule detection, single particle detection does not require sophisticated detectors (e.g., avalanche photodiodes) and electronics (e.g., photon counting). Depending on the number of analysis channels and sorting capabilities, typical commercially available FCM instruments are more expensive.\(^\text{92}\)

**Ease of Use.** Since the SNaPE system has a single excitation source and detection channel, the alignment and operation of the

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system is straightforward. The capillary system used in the SNaPE, while limiting detection efficiency, is easy to use and is relatively robust, with each capillary being able to analyze an average of ~50 samples before replacement (which is a simple 10-min procedure). Use of a capillary also provides the opportunity to completely stop sample flow for in situ reactions or longer integrations for possible spectral analysis of single particles. With a host of literature addressing capillary-based reactions, such as the polymerase chain reaction, applications of this type of instrumentation are numerous.

CONCLUSIONS

The demonstrated capabilities of the SNaPE instrument have potential applications in diverse fields, including medical and environmental research. The specific viruses analyzed in this study are medically relevant and are prime candidates for routine viral analysis.

ACKNOWLEDGMENT

The authors gratefully acknowledge funding from the National Science Foundation.

Received for review November 15, 2001. Accepted January 20, 2002.