



# A rapid label-free method for quantitation of human immunodeficiency virus type-1 particles by nanospectroscopy

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## A B S T R A C T

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Infection of cells with human immunodeficiency virus type-1 (HIV-1) results in the production of both infectious and non-infectious virions. At present, several assays are available for the quantitation of virus particles based on the presence of either viral capsid protein or nucleic acid. However, the ability to detect the total number of virus particles, both infectious and non-infectious, has been an elusive goal that would advance the study of virus assembly and egress. A rapid optical detection scheme for real-time label-free quantitation of HIV-1 virus particles was developed. Virions produced in cell cultures transfected transiently were evaluated with a nanospectroscopic assay. Quantitation with the optical detection scheme correlated with routine conventional assays. Nanospectroscopy can be used for the detection of both infectious and non-infectious, wild type and mutant strains of HIV-1 in solution at concentrations as low as  $7 \times 10^{10}$  particles/ml, requiring volumes as small as  $2 \mu\text{l}$  per assay, and in significantly less time than standard techniques. This assay provides a rapid, reliable system for quantifying virus particles in solution and could be applied to the study of viral particle production in cell culture.

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## 1. Introduction

According to the UNAIDS Epidemic Update in 2009, the World Health Organization reports that human immunodeficiency virus type-1 (HIV-1), which is responsible for the development of Acquired Immunodeficiency Syndrome (AIDS), is a global epidemic infecting more than 30 million people. To date, there is no available vaccine against HIV-1; however the advent of Highly Active Antiretroviral Therapy (HAART) has reduced greatly the rates of disease transmission and progression (Broder, 2010; Richman et al., 2009). Quantitation of virus particles is a critical method used to monitor the effects of drug therapy in patients in clinical settings and to study viral fitness, replication, and inhibition in the research setting. The extent of the epidemic has necessitated the development of multiple types of assays for the detection of HIV-1. Some common methods used include an assay that measures the capsid protein by enzyme-linked immunoabsorbance (ELISA) (Higgins et al., 1986), a method that quantifies viral genomic RNA by

real-time PCR (Leutenegger et al., 2001; Taylor et al., 2009), methods which measure the number of infectious viruses in a sample (Derdeyn et al., 2000; Kimpton and Emerman, 1992) and a method that detects anti-HIV-1 antibodies in human serum (Sarngadharan et al., 1984).

Cells infected with HIV-1 produce approximately 1000–10,000 virus particles per cell (Dimitrov et al., 1993; Eckstein et al., 2001; O'Connell et al., 2011). However, a large proportion of HIV-1 particles released from an infected cell are defective (Bourinbaier, 1994). Most detection methods for virus can be used to infer the number of virus particles in a sample, but do not measure directly the total number of infectious and non-infectious virus particles. The need to know the total number of virus particles would aid in the study of virus particle assembly and egress. Several optical virus particle detection methods have been developed, but these often require labeling of the virus particles (Bhatta et al., 2011; Chen et al., 2009; Driskell et al., 2011; Lu et al., 2011; Niikura et al., 2009). A nanospectroscopy assay as a rapid, particle-based, label-free detection method able to discriminate among nanoparticles ranging from 40 to 200 nm in diameter was developed recently (Mitra et al., 2010). Samples are passed through a nanofluidic chamber and nanoparticles interact with a laser. The light scattered by this interaction is detected by optical interferometry. This method has been used recently to resolve viruses of mixed diameters within a single sample preparation (Mitra et al., 2010). This current work

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demonstrates that nanospectroscopy can be used to determine the concentration of virus in a sample and that this quantitation correlates with quantitative virus load by real-time PCR and p24 ELISA.

## 2. Materials and methods

### 2.1. Cells

The cell lines, 293 or 293T (ATCC, Manassas, VA), human kidney tumor epithelial cell lines, were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). Cells were propagated in a 5% CO<sub>2</sub> environment at 37 °C.

### 2.2. HIV-1 viral vectors

Construction of wild-type (pAT2) and protease mutant, D30N (pAT2D30N) viral vectors has been described previously (Dykes et al., 2006). Both constructs are identical to strain NL4-3 except for the replacement of the viral *nef* gene with mouse Thy1.2. The Gagp6P10L construct was generated by site directed mutagenesis (QuikChange; Stratagene, La Jolla, CA, USA) of pRHAXX (Dykes et al., 2001) using the following primer pair: Gagp6P10L+ 5'-AGCAGACCAGAGCCAACAGCCCTAC and Gagp6P10L- 5'-CCTGGAGCTCTTCTGGTAGGGCTG- to create pRHAXXp6P10L. The *Apal* to *XmaI* fragment from pAT2WT was replaced with the same fragment from pRHAXXp6P10L to create pAT2gagp6P10L. The clone was sequenced to ensure no spurious mutations were introduced.

### 2.3. Generation of virus stocks

Wild type and mutant pAT2 constructs were used to transfect 293 and 293T cells transiently as described previously (Dykes et al., 2006). Briefly, 40 µg of DNA was incubated with 100 µl of Superfect (Qiagen, Germantown, MA, USA) and 1.2 ml of Opti-Mem (Invitrogen, Carlsbad, CA, USA) for 10 min. After the addition of DMEM, the transfection cocktail was added to the cell monolayers. Following a 4-h incubation period, the transfection medium was replaced with fresh culture media. Supernatants were harvested 72 h later and clarified by centrifugation at 400 × g. In some cases cell-free supernatants were (Ambion, Austin, TX, USA) treated with DNase for 1 h at 37 °C in the presence of 4 mM MgCl<sub>2</sub> (Piekna-Przybylska et al., 2011) in order to degrade any residual plasmid DNA from transfection. HIV-1 virus capsid protein (p24) quantitation was performed on serially diluted DNase treated stocks using a p24 ELISA (Perkin Elmer, Waltham, MA, USA). The number of copies of RNA/ml of virus was quantified by first extracting viral RNA with Trizol LS (Invitrogen, Carlsbad, CA, USA) from DNase treated samples followed by serial dilution and evaluation using the Roche COBAS Ampliprep/COBAS Taqman™ HIV-1 Test.

### 2.4. Nanospectroscopy sample preparation

In order to concentrate virus particles, 1 ml of transfected cell supernatant was spun at 50,000 × g for 1 h at 4 °C. The resulting supernatant was discarded and the pellet containing virus particles was resuspended in 10 µl of 4% paraformaldehyde to inactivate virus particles. In some cases, concentrated virus samples were mixed with a known concentration of 150 nm polymer microspheres (Thermo Scientific, Waltham, MA, USA). Concentrated virus samples were stored at –80 °C until evaluation using the nanospectroscopy method.

### 2.5. Quantitation of virus particles by nanospectroscopy

Quantitation of virus particles has been described previously (Mitra et al., 2010). Briefly, approximately 2 µl of sample is introduced into a 15 µm long nanofluidic channel with a cross-section of 400 nm × 500 nm. Vacuum pressure causes the sample to flow through the channel while a laser beam shines at a fixed focal point at the center of the channel. The light scattered by each individual particle flowing through the laser focus is overlapped with a reference laser beam on the surface of a photodetector and produces an interferometric signal whose amplitude gives a measure of the particle's size. Individual particle sizes are compiled to obtain a histogram of the size distribution of particles in each sample. Peaks in the size distribution histogram indicate the mean sizes of the different particle populations within any given sample analyzed. Fitting of these peaks with Gaussian curves, allows visualization of relative nanoparticle concentrations. The area under the fitted curve for the particles of interest (such as HIV virus particles) gives the total number of particle counts *N* during the experiment. The transit time of individual particles through the laser focus can be obtained from the real-time detector signal. Since the size of the focus and the cross-section of the channel are known, the particle's velocity (which is that same as the velocity of the buffer fluid) and the volume flow rate of the sample can be calculated. Hence, the total volume *V* of the sample that flows during the time frame of data collection can be computed. The quantity (*N/V*) gives the concentration of the particles in the sample.

### 2.6. Electron microscopy

The virus plus bead samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. They were rinsed in buffer, post-fixed in 1.0% osmium tetroxide for 60 min, rinsed in distilled water, treated with 0.1% tannic acid for 30 min, rinsed in distilled water and then treated with 2.0% filtered aqueous uranyl acetate overnight at 4 °C. The specimens were pelleted and trapped in 3.0% agarose, dehydrated in a graded series of ethanol to 100%, transitioned into propylene oxide, then into EPON/Araldite epoxy resin overnight. The next day the specimens were embedded into silicone molds in fresh epoxy resin and polymerized at 60 °C overnight. One micron sections were cut and placed on glass slides then stained with Toluidine Blue. A diamond knife was used to cut thin sections at 70 nm thickness which then were placed onto 200 mesh nickel grids. The grids were stained with aqueous uranyl acetate and lead citrate. The grids were examined and photographed using a Hitachi 7650 transmission electron microscope with an attached 11 megapixel Gatan Erlangshen digital camera. Measurements were done using Gatan Digital Micrograph software.

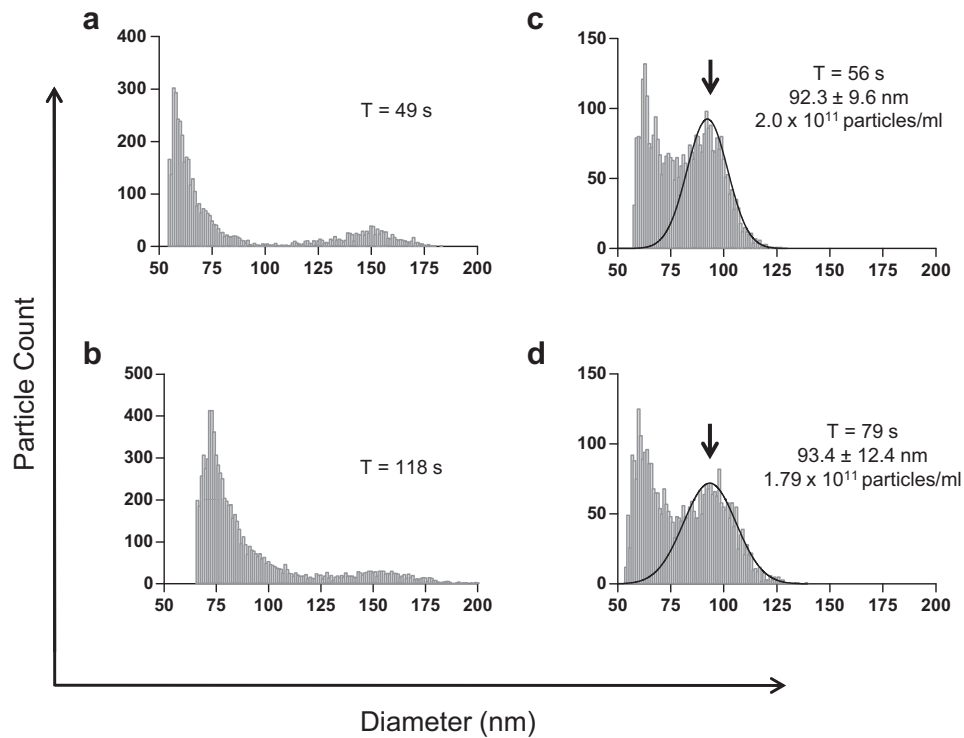
### 2.7. Statistics

The particle counts as measured by the nanospectroscopy method were compared to particles counts based on p24 capsid or viral RNA quantitation using a two-sided Pearson's correlation coefficient with 95% confidence levels. Statistical values were generated using SAS 9.2 (SAS Institute).

## 3. Results

### 3.1. Quantitation of HIV-1 virus particles

In order to validate the ability of this assay to detect cell-free HIV-1 virus particles in suspension, four different samples were evaluated initially. The first samples consisted of supernatants from mock-transfected cells. These samples allowed us to determine the size of nanoparticles present in the culture media as well as

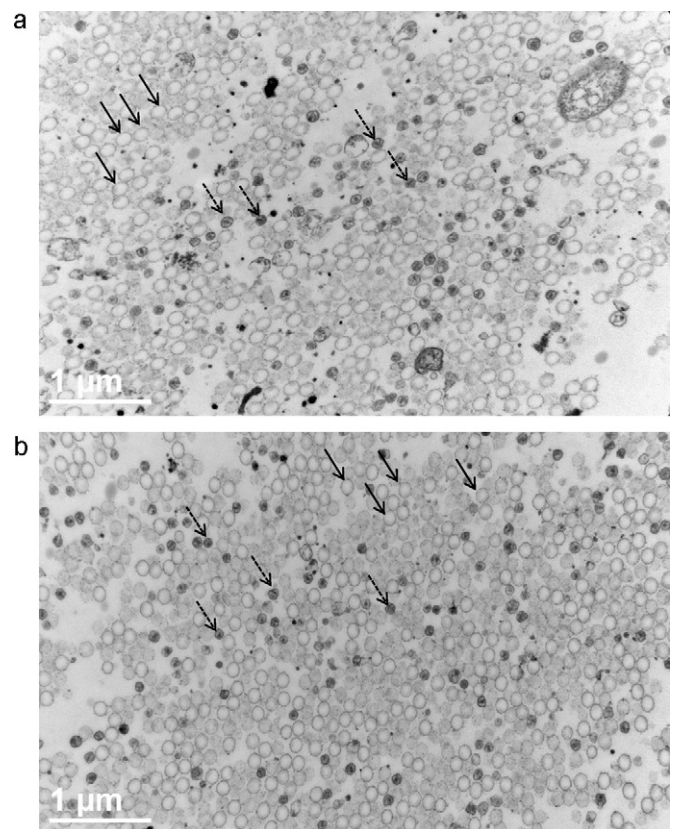


**Fig. 1.** Detection of wild-type and mutant HIV-1 viral particles using nanospectroscopy. Histogram and Gaussian fits of the size distribution of particles in samples evaluated from (a) mock-transfected cell supernatants or supernatants of cells transfected with (b) wild-type, (c) gagp6P10L, or (d) D30N HIV-1 viral constructs. Samples (a) and (c) were mixed with 150 nm polymer microspheres as a size and concentration reference prior to evaluation. When available, the amount of time for data acquisition (T), mean size (of HIV-1 particles), and calculated particle concentrations are indicated to the right of each graph. Arrows indicate virus particle peaks.

any nanoparticles produced from the cells during culture. Fig. 1a shows that only small nanoparticles approximately 60 nm in diameter were detected as well as the 150 nm polystyrene beads added to size the particles. Next, cells were transfected with an HIV-1 construct deficient in Gag-p6. Gag-p6 is a protein responsible for sequestering the cellular protein Tsg101, which tethers virus particles and prevents their release (Freed, 2003; Gottlinger et al., 1991; Huang et al., 1995). A virus deficient in Gag-p6 would have reduced amounts of virus released. Fig. 1b shows the particle distribution curve for the Gag-p6 mutant. Only the presence of particles smaller in size than HIV-1 and the 150 nm polystyrene beads can be seen. To verify the detection of wild-type virus particles, cell supernatants from cells transfected with the wild type pAT2 construct were evaluated. Fig. 1c shows a single Gaussian distribution of  $2.0 \times 10^{11}$  virions/ml centered at 92.3 nm in diameter, representative of the predicted size of HIV-1 virions (Bennett et al., 2009; Takasaki et al., 1997). The virus particle distribution and concentration of a protease inhibitor resistant mutant, D30N (Mahalingam et al., 1999), was tested next. D30N should not have reduced numbers of virus particles released, but would have reduced numbers of mature infectious particles. Evaluation of cell supernatants from cells transfected with D30N showed a similar viral peak compared to wild type, representative of  $1.79 \times 10^{11}$  virions/ml centered at 93.4 nm in diameter (Fig. 1d). In order to confirm that virus particles were present, electron microscopy (EM) was used to verify the presence of virus particles in the virus pellets produced from culture supernatants. Fig. 2 shows EM of wild type and D30N samples.

### 3.2. Reproducibility of the optical nanospectroscopy assay

To determine the precision of the optical detection scheme, three replicate samples of HIV-1 wild type were prepared in parallel. Samples mixed with  $150 \pm 4$  nm diameter polymer



**Fig. 2.** Electron microscopy of wild type and D30N. Wild type (a) and D30N (b) virus samples were visualized using transmission electron microscopy. The dashed arrows indicate examples of virions and the solid arrows indicate examples of polystyrene beads.

**Table 1**  
Nanospectroscopy acquisition data, particle sizes, and concentrations for all samples.

Sample	Time of data acquisition (s)	Rate of flow (particles/ms)	Mean particle diameter (nm) <sup>a</sup>	Concentrated virus (particles/ml) <sup>a</sup>	Culture concentration (particles/ml) <sup>b</sup>
Mock	49	0.4	–	–	–
HIV-1 wt	56	0.5	92.3 ± 9.68	2.0 × 10 <sup>11</sup>	2.0 × 10 <sup>9</sup>
Gagp6P10L	118	0.5	–	–	–
D30N	79	0.55	93.4 ± 12.4	1.79 × 10 <sup>11</sup>	1.79 × 10 <sup>9</sup>
WT-1	60	0.6	89.5 ± 10.27	6.08 × 10 <sup>11</sup>	6.08 × 10 <sup>9</sup>
WT-2	27	0.7	89.7 ± 9.42	6.65 × 10 <sup>11</sup>	6.65 × 10 <sup>9</sup>
WT-3	17	0.4	91.3 ± 10.66	8.31 × 10 <sup>11</sup>	8.31 × 10 <sup>9</sup>
1×	22	1.8	91.0 ± 10.01	3.64 × 10 <sup>11</sup>	3.64 × 10 <sup>9</sup>
2×	28	0.5	91.8 ± 9.82	1.88 × 10 <sup>11</sup>	1.88 × 10 <sup>9</sup>
4×	58	0.5	89.2 ± 9.97	7.81 × 10 <sup>10</sup>	7.81 × 10 <sup>8</sup>

<sup>a</sup> Dashed lines indicate incidence instances where data was not acquired or was unavailable.

<sup>b</sup> Values represent the particle concentration of the original transfected cell supernatants.

microspheres, were evaluated independently using the nanospectroscopic method. Particle size distribution values and calculated concentrations of each replicate are shown in Fig. 3 and Table 1. Minimal variability was observed in particle size distributions ( $90.2 \pm 1.0$  nm) with calculated concentrations ranging from 6.08 to  $8.31 \times 10^{11}$  particles/ml (SDEV =  $1.16 \times 10^{11}$ ), representing a precision of ~16%.

### 3.3. Correlation of nanospectroscopy with p24 and viral load assays

The relationship between particle concentrations generated using optical nanospectroscopy was compared to routinely used

HIV-1 detection methods. A single sample of HIV-1 wild type virus was DNase treated, serially diluted, and prepared for evaluation using three separate detection methods: nanospectroscopy, p24 assay, and viral load assay. Scatterplots of the resulting detection values are shown in Fig. 4 and Table 1. The Pearson correlation between the p24 and nanospectroscopy assays was 0.9954,  $p = 0.0612$  (Fig. 4). The Pearson correlation between the viral load and nanospectroscopy assays was 0.9654,  $p = 0.1680$  (Fig. 4). In both instances, values obtained using the nanospectroscopy assay correlated positively with values obtained using the conventional p24 or viral load assays.

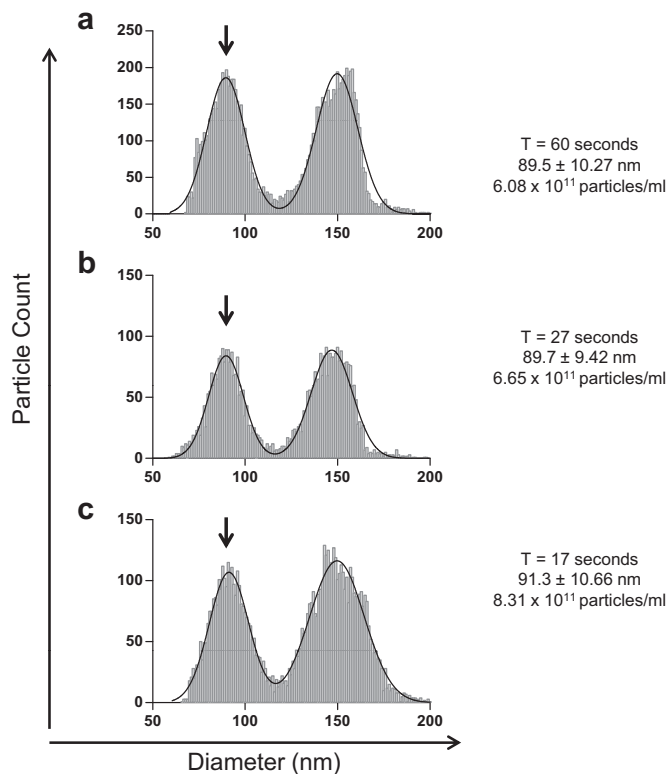
## 4. Discussion

Current viral quantitation methods are both highly sensitive (able to detect as few as 40 copies of viral RNA/ml) and laborious requiring anywhere from a few hours of sample preparation (quantitative PCR and p24 capsid ELISA) to days (Tissue Culture Infectious Doses<sub>50</sub>) before results are available. In this study, a nanospectroscopic detection scheme was used to demonstrate the rapid quantitation of unlabeled cell-free HIV-1 virions without requirement for the preparation of a standard curve, purification of virus particles, expensive reagents, or extensive sample manipulation. Nanospectroscopy is a viable method for detecting virus particles and has the added advantage of detecting infectious (wild type) and non-infectious (D30N) virus.

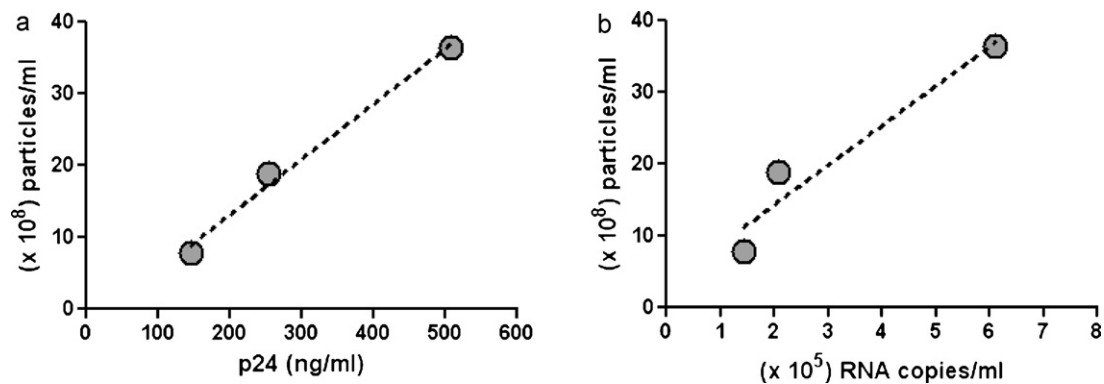
Determining the size of nanoparticles using this method is dependent on two factors; the size of the particle and the refractive index. In this study, 150 nm polystyrene beads were used as an internal control for sizing particles. According to the manufacturer, the refractive index of the beads was 1.59, resulting in virus particle sizes ranging from 89.5 to 92.3. Assuming that the refractive index of HIV-1 is similar to other viruses with a value of 1.5 (Oster, 1950), then correcting for the difference in the refractive index, results in an actual HIV-1 particle size of 102–105 nm, similar to previous studies (Bennett et al., 2009; Takasaki et al., 1997).

Knowledge of the mean particle diameter of virions present in a clinical sample may be useful in diagnostic applications where specific viral infections are suspected. Patient samples collected from blood, cerebral spinal fluid, vaginal lavage, saliva, or urine could potentially be collected, concentrated, and evaluated to aid in the rapid diagnosis of a biological agent.

One limitation of this method is the requirement for high concentrations ( $>10^{10}$  particles/ml) of virus present for detection and quantitation. Samples at concentrations down to  $10^7$  could be detected (data not shown), however, collection times for samples at this concentration, in which reliable numbers of particles could be measured, were  $>30$  min. Pressure driven flow through the nanofluidic channels could not be maintained longer than 30 min without affecting the flow rate, a critical parameter for the determination of



**Fig. 3.** Reproducibility of the nanospectroscopy assay. Histograms of the size distributions of three replicate wild-type HIV-1 (WT) virus samples prepared in parallel and evaluated individually using the nanospectroscopy assay. Samples were mixed with 150 nm polymer microspheres as a size and concentration reference prior to evaluation. Images (a), (b), and (c) represent replicates WT-1, WT-2, and WT-3 respectively. The amount of time for data acquisition (T), mean size (of HIV-1 particles), and calculated particle concentrations are indicated to the right of each graph. Arrows indicate virus particle peaks.



**Fig. 4.** Correlation of nanospectroscopy with viral load and p24 capsid quantitation. Scatter plots of (a) p24 assay vs. nanospectroscopy assay and (b) viral load assay vs. nanospectroscopy assay are shown. Three 2-fold serial dilutions of HIV-1 wild type DNase-treated virus samples were evaluated and compared using three different detection methods. Pearson correlations were (a)  $r = 0.9954$ ,  $p = 0.612$ ; (b)  $r = 0.9684$ ,  $p = 0.1680$ . Viral particle concentrations seen in Table 1 were adjusted for 100-fold concentration prior to sample evaluation using the nanospectroscopic method.

sample concentrations. The flow rate was reduced due to the accumulation of particulate matter in the nanochannels that reduced the particle speed. If a particle takes  $>1$  ms to traverse the focus of the laser, Brownian motion influences the path of the particle and the size cannot be determined. Despite these limitations, quantitation of the samples containing virus was obtainable in the range of  $7.81 \times 10^{10}$ – $8.31 \times 10^{11}$  particles/ml. Modification of the detection apparatus to include a sample concentrator would significantly improve the use of the assay for both clinical and research applications.

Another limitation of this method may be the presence of impurities in a sample during analysis; nanoparticles with similar size distributions to the desired analyte may be irresolvable from the particles of interest. Nanospectroscopy has been used previously to resolve mixed populations of HIV (100 nm diameter) and Sindbis (70 nm diameter) virus particles (Mitra et al., 2010). This study demonstrates the effective resolution of HIV-1 virus particles from small nanoparticles present in serum routinely used in tissue culture. This feature may be desirable as a rapid method to detect impurities of particle preparations in either industrial or pharmaceutical applications such as the manufacture of virus-like particles for vaccines or gene therapy.

Another limitation of this assay is that knowledge of the size and refractive index of at least one of the component particles in a sample is required for calibration of the particle size distribution. This obstacle can be resolved easily through the use of 150 nm polystyrene beads in sample preparations for instrument calibrations. Essentially, any particles of known size and material can be mixed with the virus for this calibration.

Intra-assay precision was determined by quantitation of three aliquots of a single concentrated virus preparation and was found to be 16%. To compare quantitation of virus particles with commercially available diagnostic techniques, aliquots of a single virus stock preparation in parallel were evaluated using viral load and p24 antigen ELISA kits. These methods provide different surrogate measures of virus concentration. Although the viral load assay is extremely sensitive, storage and processing of samples prior to analysis may result in viral RNA degradation and consequentially a significant underestimation of virus content. Conversely, the p24 antigen ELISA may in some cases provide an overestimation of viral concentration because some viral capsid protein is not incorporated into virus particles. In both instances, a linear correlation between results was obtained between this optical detection scheme and p24 ELISA and viral load assay. Nanospectroscopy could not be correlated with EM, because EM methods make it difficult to back calculate the virion concentration in the original sample.

Since nanospectroscopy provides the ability to quantify the total number of viral particles in cell culture media, these results, when evaluated in conjunction with the total number of infected cells could aid in the exact determination of viral burst size in both transfected and chronically infected cells, contributing to the study of viral replication fitness and egression from host cells.

In summary, this nanospectroscopic detection method for the quantitation of viral nanoparticles in solution is rapid and useful. This method, unlike other commercially available assays, is able to determine directly the total amount of HIV-1 virus particles present in a solution without the requirement for labeling or sample purification prior to analysis.

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