Review

Quantitative real-time single particle analysis of virions

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ABSTRACT

Providing information about single virus particles has for a long time been mainly the domain of electron microscopy. More recently, technologies have been developed—or adapted from other fields, such as nanotechnology—to allow for the real-time quantification of physical virion particles, while supplying additional information such as particle diameter concomitantly. These technologies have progressed to the stage of commercialization increasing the speed of viral titer measurements from hours to minutes, thus providing a significant advantage for many aspects of virology research and biotechnology applications. Additional advantages lie in the broad spectrum of virus species that may be measured and the possibility to determine the ratio of infectious to total particles. A series of disadvantages remain associated with these technologies, such as a low specificity for viral particles. In this review we will discuss these technologies by comparing four systems for real-time single virus particle analysis and quantification.

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Measuring virus concentrations

From basic research on emerging viral diseases to clinical applications of viral gene therapy vectors—it is often vital to quantify viral amounts accurately. As a consequence a wide spectrum of methods is in use for the determination of virus concentrations. They may be grouped broadly into four categories: (a) determining levels of infectivity, (b) measuring the presence or function of viral proteins, (c) detecting the presence of viral or marker nucleic acid within the viral genome and (d) counting physical viral particles, whether labeled or unmarked (see Table 1).

Methods to determine infection levels include measurements of cytopathic effects such as plaque forming and 50% tissue culture infectious dose (TCID$_{50}$) assays but also flow cytometric measurements of cellular transduction after infection with viral particles carrying reporter genes such as green fluorescent protein (Metzner et al., 2008; Papanikolaou et al., 2013). While hemagglutination assays directly measure the propensity of viral proteins to crosslink susceptible cell types, serological methodologies also measure the presence of viral antigens, albeit indirectly, by determining antibody conversion. Generally, the use of antibody technology has had a great impact on virus quantification, since high specificity and sensitivity are achieved, i.e. in enzyme-linked

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Table 1
Methods of virus quantification. The table summarizes some of the most common methods used for the quantification of virus. TCID50 tissue culture infectious dose 50; ELISA enzyme-linked immunosorbent assay; PERT—product-enhanced reverse transcriptase assay; SRID—single radial immunodiffusion assay; (RT-)qPCR—(reverse transcriptase) quantitative polymerase chain reaction; NTA—nanoparticle tracking analysis; VC—VirusCounter; TRPS—tunable resistive pulse sensing; FFF-MALLS—field-flow fractionation multiple-angle laser light scattering; AFM—atomic force microscopy.

Category	Type	Time to result	Stringency	Comment
Viral quantification methods				
Infectivity	Plaque assay	Days–week(s)	High	Virus must replicate in culture
	Marker transduction	Days–week(s)	High	
	TCID50	Days–week(s)	High	
Protein	ELISA	Hours–day(s)	Low	Measured viral element not necessarily linked to viral particle
	Hemagglutination	Hours–day(s)	Low	
	PERT	Hours–day(s)	Low	
	Neuraminidase	Hours–day(s)	Low	
	Immunoblotting	Hours–day(s)	Low	
	SRID	Hours–day(s)	Low	
Nucleic acid	qPCR	Hours	Low	
	RT-qPCR	Hours	Low	
Particle	Electron microscopy	Hours	Medium	Inherent low specificity for virus
	Flow cytometry	Minutes–hour(s)	Medium	
	NTA	Minutes–hour(s)	Medium	
	Flow (VC)	Minutes–hour(s)	Medium	
	TRPS	Minutes–hour(s)	Medium	
	FFF-MALLS	Minutes–hour(s)	Medium	
	AFM	Minutes–hour(s)	Medium	

immunosorbent assay (ELISA) formats. However, stringency of ELISA approaches for virus quantification can be considered to be low, as the measured viral element is not necessarily linked to a virus particle (see Table 1). Even if considerably faster than cell-culture based methods, it will take hours to complete the assay. The advent of molecular techniques, especially polymerase chain reaction (PCR), has also left its mark on the quantification of viral particles. Both RNA and DNA levels can be measured using quantitative PCR approaches. Absolute quantification (i.e. copy numbers or numbers of particles) can be obtained from relative raw data by using standard dilutions of vector DNA or RNA. PCR may also be used to quantify protein levels. In product-enhanced reverse transcriptase (PERT) assays, production of DNA from viral RNA by the reverse transcriptase being present in the retroviral sample is quantified and used in turn to estimate virus particle concentration (Metzner et al., 2013). PCR and protein detection methods offer advantages in terms of the time needed to get results when compared to cell-culture based methods. Indeed, no culturing is necessary, which constitutes a significant advantage since culturing may not be possible in all cases. Nevertheless, stringency for this techniques can also be considered to be low, as the measured viral element—in this case the nucleic acid—is not necessarily part of avirion (infectious or otherwise), and calculations may significantly over-estimate the number of particles present (see Table 1 and Fig. 1)

Counting virion particles

The analysis of single virus particles has long been the remit of electron microscopy. Recently, technical progress in the field of microscopy as well as the adaptation of applications originally developed for use in nanotechnology crossed over to uses in virology and made the quantitative analysis of single viral particles as physical entities more feasible. Technologies include atomic force microscopy (AFM) (Ohnesorge et al., 1997), laser light scattering applications such as multiple-angle laser light scattering (MALLS) (Bousse et al., 2013; Wei et al., 2007) or nanoparticle tracking analysis (NTA) (Papanikolaou et al., 2013; Filipe et al., 2010; Kramberger et al., 2012; Anderson et al., 2011; Du et al., 2010), tunable resistive pulse sensing (TRPS, a method based on the Coulter principle) (Vogel et al., 2011; Farkas et al., 2013; Rybakova et al., 2013), and flow cytometry (FC) variants (Brussaard

et al., 2000; Ferris et al., 2011; Stepp et al., 2011, 2010; Kemp et al., 2012). Other methods that appear to fall into this category are not discussed in any greater detail, such as viral quantitative capillary electrophoresis (vqCE) (Mironov et al., 2011), since correlates of particle counts (such as nucleic acid amounts) are used for calculation of virus titers, similar to qPCR, rather than the presence of virion particles. However, vqCE is of special interest, since it is able to distinguish between the intact virus fraction and free DNA (Mironov et al., 2011)—an interesting aspect when trying to evaluate PCR based quantification data. This review will concentrate on technologies that show the most promise in the field and have as such progressed to the stage of commercial availability, namely field-flow fractionation (FFF)-MALLS, NTA, "flow virometry" using a VirusCounter (VC) device and TRPS (see Table 2).

FFF-MALLS

FFF-MALLS equipment offered by Wyatt Technology (http://www.wyatt.com) combines a separation step using variants of FFF with a detection step using MALLS. FFF is a liquid chromatography technique where sample separation occurs in a laminar flow channel with no column media to interact with the sample. Particles are eluted in order of increasing size, and separation of the sample is rapid and gentle. The eluted particles will be detected by MALLS, which provides simultaneous detection of light scattered from several angles, providing additional information compared to other laser light scattering approaches. By measuring the intensity and angular dependency of the scattered laser light, it is possible to deduce the radius of the particles i.e. determine size distributions (Chuan et al., 2008; Pease et al., 2009) and subsequently calculate the number of particles per volume (Bousse et al., 2013; Wei et al., 2007). FFF-MALLS data was compared to measurements of infectivity levels and qPCR for Influenza preparations. For samples from a range of sources, following vaccine production procedures, the highest values determined were observed for qPCR measurements followed by results from FFF-MALLS and measuring the correlate of infectivity (Bousse et al., 2013; Wei et al., 2007) (see also Table 3). As measuring infectivity is the most stringent method, these results could be expected (see also Tables 1 and 3). Similar to the comparison of different methods for measuring
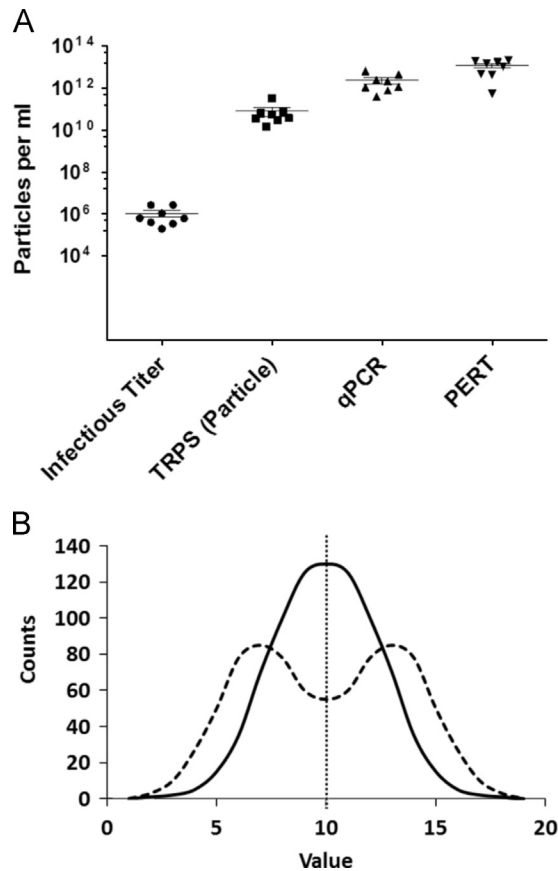
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Fig. 1. Comparison of strategies for measuring viral concentrations. (A) Levels of stringency in virus titer measurement. The type of parameter measured influences the stringency and thus the level of titers measured: measuring infectivity will always give lower titers than measuring particle numbers, protein or nucleic acid amounts based measurements. In this case, infectious titers (measured by flow cytometry after reporter gene transduction), total particle number (measured by TRPS), reverse transcriptase levels (measured by PERT assay) and viral RNA containing a GFP reporter (measured by RT qPCR) were determined in triplicate for 8 different preparations of lentiviral particles, derived from the stable producing cell line STAR-A-HV (ECACC no. 04072115). Viral preparations were concentrated by ultracentrifugation (2 h, 56,000g, 4 °C in a Beckmann XL70 ultracentrifuge using a SW32ti rotor). Concentrations for each preparation are shown together with the respective means and standard error of the mean (SEM). TRPS—tunable resistive pulse sensing; PERT—product-enhanced reverse transcriptase assay; qPCR—quantitative polymerase chain reaction. (B) Average vs. distribution. While the two distributions depicted are clearly distinct, they yield the same average (indicated by the vertical dotted line). When using ensemble methods, subpopulations may be hidden, indicating the importance of single particle analysis.

lentiviral counts, qPCR indicated higher concentrations than the particle based approach (compare Fig. 1). We believe that this is the result of different requirements for measurement; in qPCR only the nucleic acid needs to be present, while for FFF-MALS (or TRPS) more parts of the virus need to come together. The combination of separation and purification steps prior to detection is a promising concept for single particle quantification approaches, since sample purity is crucial for a precise measurement. Currently, the effort in terms of technical expertise and equipment is higher compared to the other techniques. However, this is mostly due to two different technologies having to be implemented separately.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) has been developed by NanoSight Ltd. (www.nanosight.com), which was recently acquired by Malvern Instruments Ltd. This real-time nanoparticle

visualization is based on a laser-illuminated microscope technique. A laser beam is passed through an optical prism, and the resulting refraction is used to compress the beam into an intense illumination region, where nanoparticles can be visualized. The Brownian motion of nanoparticles, i.e. virus particles, in liquid are detected by either a charge-coupled device (CCD) camera, or more recently a complementary metal–oxide–semiconductor (CMOS) sensor based system which transforms the optical information into electronic data. Modern CMOS sensors can process more frames per second and are thus well suited for the application: the video captured with this camera showing the movement of individual particles is subsequently analyzed using NTA software. It automatically calculates the distance each particle moves, and by knowing temperature and viscosity of the solvent, the particle diameter can be calculated using a variation of the Stokes–Einstein equation. Depending on the refractive index (R_i) of the nanoparticles, lower size limits for this detection method range between 10 nm for particles with high R_i such as colloidal gold, and 30 nm for lower R_i such as particles with biological origin. The upper size limit is determined by Brownian motion, as movement becomes too limited to track accurately, when the particles reach approximately 1 μm in diameter. As the NTA approach measures single particles simultaneously, a sufficient number of particles can be analyzed in 60 s according to manufacturer's claims; an important factor to make this possible is that samples contain between 10^7 and 10^9 particles per mL. As quantification is usually needed for samples with unknown concentration, the dilution steps needed to reach this (quite small) gap may result in significant longer time needed for sample preparation. Therefore, the time taken per measurement may vary between 5 min and 1 h (Filipe et al., 2010). Several groups tried to investigate the usefulness of the NanoSight device for viral particle quantification. Mironov et al. (2011) published that this technique provides a good indication of particles per volume when measuring Vaccinia virus samples, although it quickly reaches its technical limits when host cell debris or other background particles are present, indicating the need for efficient virus sample purification strategies. NanoSight's performance can also be extended by labelling virus particles with fluorophores to discriminate them from host cell debris or identify specific virus species (for more information see http://www.nanosight.com/technology/fluorescence-capability#6). Although particle-based methods are unable to distinguish between infectious and non-infectious particles, labelling (i.e. for viral proteins mediating cell entry) may be an option to increase the stringency of the method. Anderson et al. (2011) correlated NanoSight's results with plaque assay and quantitative PCR for quantifying bacteriophages and concluded that it is less precise, but provides results more quickly (within 5 min). The drawbacks they faced were that NTA only worked when phage samples were suspended in clear medium. They also commented on the high price of the equipment and the relatively small concentration range for which accurate results could be generated. Nevertheless, they stated that the NTA based method, once optimized, will likely be reproducible with accuracy comparable to plaque assays only significantly faster. When adenoviral preparations were analyzed using a cytopathic effect based cell culture assay and NTA, results varied by two to three logs. In all cases NanoSight measurement yielded the higher value (Kramberger et al., 2012) (see also Table 3). The same group used hemagglutinin (HA) titration and NTA for quantification of Influenza preparations (Kramberger et al., 2012). No particle numbers were, however, derived from HA values since appropriate standards for allowing an absolute quantification were missing. Thus, no direct comparison of infectious to total particles was possible in this case. When a lentiviral vector pseudotyped with vesicular stomatitis virus G (VSV-G) protein was analyzed using flow cytometry after reporter gene

Table 2

Commercially available techniques for single-particle quantitiative analysis of virus particles. FFF—field-flow fractionation; NTA—nanoparticle tracking analysis; VC—VirusCounter; TRPS—tunable resistive pulse sensing.

	FFF-MALLS	NTA	Flow (VC)	TRPS
Single particle quantification techniques				
Hardware	Eclipse/dawn	NanoSight	VirusCounter	qViroX, qNano
Developer/ producer	Wyatt	Malvern	Virocyt	Izon
	www.wyatt.com	www.nanosight.com	www.virocyt.com	www.izon.com
Detection principle	Light scattering	Light scattering	Fluorescent in-flow detection	Coulter principle
	Label-free	Label-free	Dual-label	Label-free
Time/sample	Depending on separation	5 min to 1 h	30 min (staining) < 5 min detection	< 10 min
Size range (in nm)[a]	> 50	30–1000	> 25 (> 9000 nt)	60–2000
Concentration range[a]	> 10E+06[b]	10E+07–10E+09	10E+05–10E+09	10E+05–10E+12
Pros	Separation step included, size measurement	Additional fluorescence measurement enhancing specificity[c]	Easy handling, quick results	Small, inexpensive hardware, charge and size measurements
Cons	Elaborate set-up, little testing on virus	Narrow concentration range	No additional parameters measured	Little peer-reviewed literature for use on virus
Costs	For information on pricing please contact manufacturer			
References	Bousse et al., 2013; Wei et al., 2007; Chuan et al., 2008; Pease et al., 2009	Papanikolaou et al., 2013; Kramberger et al., 2012; Anderson et al., 2011; Du et al., 2010; Gutierrez-Granados et al., 2013; Cervera et al., 2013; Cayatte et al., 2013	Ferris et al., 2011; Stepp et al., 2011, 2010; Kemp et al., 2012	Vogel et al., 2011; Farkas et al., 2013; Rybakova et al., 2013; Brussaard et al., 2000

[a] According to manufacturer.
[b] According to Bousse et al. (2013).
[c] According to http://www.nanosight.com/technology/fluorescence-capability#6.

Table 3

Measuring the ratio of total to infectious particles. T:I ratio of total to infectious particles; FFF—field-flow fractionation; NTA—nanoparticle tracking analysis; VC—VirusCounter; TRPS—tunable resistive pulse sensing.

Technique	Virus	Description[a]	Infectivity by	Infectious titer	Total particles	Ratio T:I	Reference
Determining the infectivity index							
FFF-MALS	Influenza	V/e/80–120/13.5	Plaque assay	2.00E+07	2.00E+08	10	Bousse et al. (2013)
	Influenza	V/e/80–120/13.5	TCID50	2.51E+08	7.94E+09	31.63	Wei et al. (2007)
NTA	Adenovirus	I/n/90/35–36	TCID50	2.00E+09	1.79E+11	89.50	Kramberger et al. (2012)
	Adenovirus	I/n/90/35–36	Plaque assay	4.00E+10	4.19E+11	10.48	Du et al. (2010)
	Bacteriophage	I/n/50–110(head)/33–244	Plaque assay	n.a.	n.a.	1.5–5[b]	Anderson et al. (2011)
	Lentivirus (VSV-G)	VI/e/80–100/9.75	Reporter gene transduction	7.25E+08	7.40E+11	1,02E+03	Papanikolaou et al. (2013)
Flow (VC)	Adenovirus	I/n/90/35–36	TCID50	4.70E+06	1.10E+09	234.04	Stepp et al. (2010)
	Baculovirus	I/e/20 × 260/80–180	Plaque assay	5.80E+06	1.40E+08	24.14	Ferris et al. (2011)
	Coronavirus	IV/e/120/27–32	TCID50	1.60E+06	2.50E+08	156.25	Stepp et al. (2010)
	Cytomegalovirus	I/e/150–200/200	TCID50	2.10E+08	3.30E+10	157.14	Stepp et al. (2010)
	Denguevirus	IV/e/50/10–11	TCID50	8.90E+07	1.90E+09	21.35	Stepp et al. (2010)
	Herpes Simplex Virus	I/e/150–200/152	TCID50	2.10E+08	3.70E+10	176.19	Stepp et al. (2010)
	Influenza	V/e/80–120/13.5	TCID50	3.60E+06	1.60E+10	4.44E+03	Stepp et al. (2011)
	Parainfluenza	V/e/150/15	TCID50	1.00E+08	2.90E+08	2.90	Stepp et al. (2010)
	Respiratory Syncytial virus	V/e/150/15	TCID50	3.20E+07	4.60E+10	1.44E+03	Stepp et al. (2010)
	Rubella	IV/e/65–70/8.7–11.8	TCID50	1.00E+07	5.90E+10	5.90E+03	Stepp et al. (2010)
TRPS	Lentivirus (amphoMLV)	VI/e/80–100/9.75	Reporter gene transduction	1.08E+06	7.99E+10	7.42E+04	unpublished data

[a] Description data is given in the following format: baltimore class/naked (n) or enveloped (e)/diameter (in nm)/genome size (in kb), Source: http://viralzone.expasy.org/.
[b] Estimated from correlation data.

transduction and NanoSight, only 0.1% of nascent virions was found to be infective (Papanikolaou et al., 2013) (see also Table 3), providing important information on the quality of the vector preparation. In addition, NTA total particle titers of fluorescently tagged non-infectious HIV-1 virus-like particles were shown to be in good agreement with measurements based on electron microscopy and p24 ELISA (1.24×10^{11}; 1.14×10^{11} and 1.30×10^{11}, respectively) (Gutierrez-Granados et al., 2013; Cervera et al., 2013). Recently, NTA has been used to compare size and amounts of human cytomegalovirus (HCMV) virions and dense bodies (DB), a type of non-infectious particles produced by HCMV (Cayatte et al., 2013).

Virus Counter

Virocyt (http://www.virocyt.com) has developed a flow cytometer-like device (VirusCounter; VC) suitable for virus quantification in liquid samples. Recently, a modified version of the device has been presented, better suited for industrial applications (for more info see http://virocyt.com/news/new-rapid-virus-counter-system-introduced-at-world-vaccine-congress/). VC uses two separate fluorescent dyes to stain virus samples: one specific for proteins, the other for nucleic acids. Both, a certain size of virus (> 25 nm) and a certain length of viral genome (> 9000 nt/bp) are necessary to guarantee a sufficient level of staining for

detection. Only events positive in both channels are counted as viral particles. The non-specific staining process eliminates the need for target-specific reagents such as fluorescence-labelled antibodies required for conventional flow-cytometry methods and is therefore more cost-effective (Ferris et al., 2011; Schulze-Horsel et al., 2008). Staining for protein/nucleic acid also reduces signal from inorganic contamination, i.e. nano-sized salt precipitates in buffer solutions. VC is the only method mentioned in this review which relies on labelling for measurement. This may introduce non-specifically stained signals. Generally, labelling procedures may change sample properties (such as diameter or zeta potential) and will increase the time to measurement. However, only fluorescence is measured and staining the virus particles requires 30 min incubation only and subsequent analysis can be accomplished in around 10 min per sample according to the manufacturer's documentation. In trials run by the authors measurement times were generally shorter (5 min). The viral particle concentration is calculated from the number of particles generating positive events in both channels and the sample flow rate; the range for reliable measurements is $5*10^5$ to $1*10^9$ virus particles per mL. As for FFF-MALS, NTA or TRPS, there are currently only a few research groups testing and/or evaluating the VC. Stepp et al. (2010) determined $TCID_{50}$, concentration by transmission electron microscopy (TEM) and compared the results to measurements from the VC for a broad range of virus species from Adeno—via Herpes Simplex to parainfluenzavirus (see also Table 3). They could show good agreement between the different measurement methods. Ferris et al. (2011) generated recombinant baculovirus samples which were tested in house by plaque assay and subsequently sent to collaborators which performed plaque assays or measurements with VC. After de-blinding, they were able to show that VC provides statistically significant results—determined by linear regression analysis of log-transformed average results and Pearson correlation analysis—to plaque assays, with a considerable advantage in terms of time needed to get results. VC was also used to control viral titers in a baculovirus-based expression system (Kemp et al., 2012). When Influenza A/H1N1 samples were investigated for virus concentration using $TCID_{50}$, EM and VC, the titers were 3.6×10^6, 1.6×10^{10} and 1.6×10^{10} particles per mL respectively (Stepp et al., 2011). Also in this case a relatively big gap between infectious and total particle numbers is observed. Generally, two reasons contribute to the difference between infectious and total virus counts: (i) cells produce particles resembling virus which are not infectious, either as a consequence of viral infection (i.e. Hepatitis B) or as a consequence of cellular physiological processes (i.e. exosomes); (ii) artefacts of preparation contribute to the gap: formation of aggregates will increase total particle count, while loss of infectivity may occur during preparation procedures. For example, the lentiviral particles we have used in our TRPS study are particularly prone to the latter (see Fig. 1). Ultracentrifugation negatively affects the molecules responsible for viral entry.

Tunable resistive pulse sensing

TRPS, originally termed scanning ion occlusion sensing (SIOS) is the technology behind the qNano and qViroX devices marketed by Izon Ltd. (www.izon.com), which utilizes a stretchable membrane to achieve size-adjustable nanopores for counting and measuring the size of particles passing through the pore (Vogel et al., 2011). In addition, measurement of zeta-potential is possible (Kozak et al., 2012). This parameter describes the electrokinetic potential in a colloidal system. It provides information similar to charge data, and can help to predict attachment behavior or stability of a viral preparation (Zhang et al., 2008; Arjmandi et al., 2012). The

adjustable pore is the central part of this technique which offers the possibility to measure particles in a range from 60 nm to 2 μm, thus excluding some of the smaller virus families. The pore is located in the elastic polyurethane center of a cruciform support which is placed within the fluid cell of the qViroX instrument. By stretching the membrane mechanically, the pore is size-adjusted according to individual needs. The fluid cell is characterized by Ag/AgCl electrodes localized in the upper and lower compartments of the cell, which are used to apply a potential difference across the pore. By building an electrolytic fluid bridge, the current across the pore can be measured by qViroX's software. Samples are applied to the upper compartment of the fluid cell and as particles pass through the pore, so-called blockade events in the ionic current are created leading to resistive pulses, which have a linear relationship to the particle volume. Fundamentally, this is an application of the Coulter principle. Particles can pass through the pore due to their charge. For weakly charged or neutral particles there is the possibility to apply pressure in order to increase the flow of particles through the pore, which is also a helpful tool to measure samples of low concentration. qViroX is calibrated with carboxylated polystyrene particles in defined sizes and known concentrations. Samples are analyzed under the same conditions. Size distributions are calculated using a comparison between the blockade magnitude distributions of the calibration particles and unknown samples (Vogel et al., 2011). TRPS is reported to be able to measure particle size and size distribution of nanoparticles with good accuracy, precision and sensitivity in several different nanoparticles including silica nanoparticles, DNA-coated nanoparticles, adenovirus and liposomes (Yang et al., 2012). One group tested qViroX for size measurements (but not for concentration measurements) of Adenovirus samples and drew the conclusion that size measurements by TRPS were in good agreement to electron microscopy results (Vogel et al., 2011). Vogel et al. (2011) described the nanopores and standard particles they used in detail, analyzed them by EM and described reproducible size measurements for standard particles over a range of membrane stretches. In our group TRPS was used to quantify lentiviral particles derived from a stable lentiviral producer cells line called STAR-A-HV (Ikeda et al., 2003). Our results showed that measurements of particle concentration (7.99×10^{10} particles per mL) were considerably higher than infectious titers (1.08×10^6 particles per mL) but lower that results from PERT (1.16×10^{13} particles per mL) or RT-qPCR protocols (2.33×10^{12} particles per mL) (see also Fig. 1 and Table 3), indicating that only 0.001% of nascent virions are infectious. This represents a 100fold decrease compared to results achieved with VSV-G pseudotyped lentiviruses and NTA measurement (Papanikolaou et al., 2013), most likely due to the less stable amphotropic Murine Leukemia virus (MLV) Env present in STAR-A-HV-derived viral particles. Indeed, when we used sucrose cushions for concentrating the preparation, infectious titers improved on average ten-fold (unpublished data). However, a comparison is difficult since different methods for particle quantification were used. In addition to the work done in our group, TRPS has been used to quantify Rotavirus particles. The results from this study also suggest a gross overestimation of Rotavirus particles due to empty capsids and cellular debris (Farkas et al., 2013). This indicates that as with the other methods, great care has to be given to sample preparation, as the nanopore will get clogged when too crude samples are used. In our hands, TRPS membranes tended to block more easily than VC fluidics—an issue that may be rectified by changing the properties of the membranes surface, i.e. by coating with low adsorption materials. Bacteriophage tail-like particles have also been quantified by TRPS (Rybakova et al., 2013), indicating that the form of particles does not affect measurement. TRPS can also be used to analyze the interaction between two elements or particles in bio-sensing

applications (http://www.izon.com/applications/bio-diagnostics/gold-nanoparticles-for-biosensing/), by following the size distribution. This may represent a possibility for specific detection, similar to the use of immuno-gold staining in electron microscopy. Antibody-functionalized nanoparticles could be associated with viral samples. Subsequently, the increase of particle size would demonstrate the specific interaction between nanoparticle and virus particle (http://www.izon.com/capabilities/particle-interaction-monitoring/). The qViroX has the smallest bench footprint of all the devices mentioned and is easily transportable.

Discussion: the pros and cons of physical virion quantification

Importantly, the quantitative single particle assays rely on parameters devoid of biological connotations (with the exception of protein/nucleic acid labelling for the VC) for the quantification of virus particles. In contrast, the established virus quantification methods are based on infectivity, protein or nucleic acid levels. However, the same information is ultimately presented: the number of particles per volume. The process of inferring this information gives rise to inherent differences when comparing results with other types of information (see Fig. 1A). For example, infectious titers, whether measured by plaque assays or by level of cellular transduction with a marker protein (as is the case in Fig. 1A) will always give lower concentration than methods based on the presence of nucleic acid, protein or particles (see also Table 3). This is explained by the level of stringency associated with the measurement parameters; while for protein or nucleic acid based methods the presence of said factors alone is enough, in measurement of infectivity all (or most) elements of the virus need to be in place correctly to allow infectivity. Measuring the presence of particles somehow lies in between: factors making up the virus need to be in the correct shape or form of aggregation. However, functionality is not required. This effect is even exaggerated in Fig. 1A, since a very crude ultracentrifugation protocol was used, largely destroying the infection capability of lentiviral particles pseudotyped with MLV amphotropic envelope protein. In addition, PERT assay, RT-qPCR and TRPS were used to quantify eight different preparations in triplicates (shown in Fig. 1A). In this case the stringency of TRPS seems to be ten-fold higher than that of PERT or qPCR. Similar results were obtained when Influenza samples were investigated using TCID$_{50}$, EM, and VC for virus quantification (Stepp et al., 2011). Also for Influenza, comparing FFF-MALS data to infectious titer correlates and qPCR confirms this, as does analysis of adenovirus samples with the NTA method (Kramberger et al., 2012) (see Table 3 for an overview). However, these differences in the measurement principles make it possible to investigate important questions in virology. While cells may produce a vast quantity of physical viral particles, only a portion of these will be fully infectious (Ibiricu et al., 2013; Meckes and Raab-Traub 2011). Non-infectious particles produced upon viral infection can contribute to viral spread in numerous ways, i.e. by providing an immune decoy. Comparing such different types of vesicles, i.e. according to size using the described technologies is possible and has been described for the dense bodies produced by HCMV infected cells (Cayatte et al., 2013). In gene therapy, the percentage of infectious particles in a given viral vector stock will significantly influence the efficacy of gene transfer and thus is a main parameter for vector stock quality control. All methods presented have the capability to measure total virus particles, thus allowing the determination of total to infectious particle ratios (ratio T:I; see Table 3). Another open question concerns the relationship between viral particle production (especially in the case of enveloped viruses) and the production of other lipid vesicles of a similar size range, such as exosomes (Meckes and

Raab-Traub 2011; Raposo and Stoorvogel 2013), which may contribute to particle counts but not to infectivity levels as they are common contaminants of viral preparations (Cantin et al., 2008). Exosomes have recently garnered a greater degree of interest, not only for their functions in cell-to-cell communication, but also for their potential use as a source of biomarkers (Properzi et al., 2013) or as gene delivery/therapy vectors (El-Andaloussi et al., 2012). Generally, the interplay of virus particles and exosomes is an interesting topic (Meckes and Raab-Traub 2011; Wurdinger et al., 2012). Single-particle analysis techniques including NTA (El-Andaloussi et al., 2012; Twu et al., 2013) and TRPS (de Vrij et al., 2013; Ng et al., 2013) have also been used for the characterization of exosomes. Most single-particle analysis techniques will also allow additional parameters to be measured, such as virus diameter or charge. In this respect, an inherent advantage of single particle analysis is that distributions of distinct values, rather than averages, are generated. Averaged data may hide subpopulations (see Fig. 1B) important for answering research questions, for example the discrimination of exosomes and viral particles.

In addition, measuring size distributions will help to determine the aggregation state of particles in solution. This is an important parameter in several applications, i.e. when assessing the quality of viral vectors for gene therapy, or vaccine preparations. Aggregation data has been derived from AFF-MALS data for Influenza (Wei et al., 2007) and NTA measurements for lentiviral preparation (Papanikolaou et al., 2013). TRPS experiments have been used to investigate aggregation in an adenoviral sample (Vogel et al., 2011). Depiction of size distribution in histograms allows for an easy interpretation (Papanikolaou et al., 2013; Vogel et al., 2011). In addition to size distribution, information from zeta potential measurements can help, since particles with higher zeta potential (i.e. > 25 mV) will repulse each other more readily, thus increasing monodispersity of preparations. Generally, the "average vs. distribution" topic resembles the difference between results from immunoblot analysis and flow cytometry; while the first will provide information about the average presence of a protein of interest in cells, the latter will provide information about the distribution over the cells (or viruses) comprising the sample.

An advantage shared by all the methods, is the time it takes for sample analysis, which is measured in minutes rather than in days or hours (see Tables 1 and 2). Particle numbers are counted directly and not inferred from infectivity, protein or nucleic acid levels. There is no need for culturing of viral particles and potentially a broad range of viruses can be measured with the same technology. All methods contribute information to determine the ratio of total to infectious particles with similar levels of stringency. However, problems are also shared between the different techniques. A peer-reviewed literature is still scarce. Sample preparation—mostly filtration to remove larger particulate material—is vital, both to ensure that the optimal measurement conditions determined by the device (i.e. avoiding blocking of fluidics) are met and to keep contamination levels low. Measurements to determine background noise levels in buffer are also critical. Measurements using single particle detection are of moderate stringency and specificity. FFF-MALS, TRPS and NTA measure the presence of particles in a specific size range: they may be virus particles, exosomes, cellular debris of appropriate size or inorganic contamination from buffers (indicating once more the strong influence of sample preparation on results). Subsequently, measurements in complex sample matrices, such as serum, will be difficult. The VC partially addresses this problem by staining viral particles for both nucleic acid and protein. While this may distinguish viral particles from cellular debris and inorganic contamination, it may not sufficiently discriminate virus from exosomes, which carry both protein and RNA. What is required is a possibility to label virus specifically, i.e. using

fluorescent tagged antibodies and then detect this in parallel along with the usual parameter. This has already been implemented for NTA and may be most easily implemented for the VC, since fluorescence detection is already in place. Currently, the potential for high-throughput measurement is limited, meaning options to measure several samples in parallel are lacking. However, the most recent version of the VC includes a 96-well compatible autosampler. Also, a combination of fractionation and detection, as suggested by the FFF-MALS system, may both make handling easier and allow a better estimation of contamination levels. Generally, handling requires experience from the operator. In this aspect, the VC system may prove to be the most robust. Multiple additional parameters such as size or charge (zeta potential) of particles can be measured by TRPS (Kozak et al., 2012), FFF-MALS (Wei et al., 2007) and NTA (Kramberger et al., 2012), but not the VC. This is interesting when trying to find subpopulations in viral preparation, i.e. for the quality control of viral vector or vaccine production, to identify contamination or aggregation events.

Comparing costs for the different systems is somehow difficult. On the one hand, prices for the devices themselves may vary considerably, depending on conditions of purchase agreements and specific device set-up (e.g. there are three different set-ups available for NTA, two devices available for VC). On the other hand, costs of consumables have to be taken into consideration: mainly the membranes containing the nanopores for TRPS and the fluorescent labelling cocktail for VC measurements. Contacting the manufacturers on costs for devices and consumables is, therefore, indispensable.

However, the final question still remains: what method to choose in order to quantify virus particles? Unfortunately, there is no clear single answer available—since sufficient comparative data on the use of the different systems is lacking—except that the combination with quantitative real-time single particle analysis methods will likely provide important supportive information, and ultimately, access to equipment may determine the choice of technology.

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