Virology 462-463 (2014) 199–206

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Review

Quantitative real-time single particle analysis of virions

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ARTICLE INFO

Article history:
Received 4 April 2014
Returned to author for revisions
5 May 2014
Accepted 4 June 2014

Keywords:
Nanoparticle tracking analysis
NanoSight
Tunable resitive pulse sensing
VirusCounter
Flow-field-fractionation
Multiple-angle laser light scanning
Virus titer
Single particle analysis

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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http://dx.doi.org/10.1016/j.virol.2014.06.005

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 overestimate 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

Please cite this article as: Heider, S., Metzner, C., Quantitative real-time single particle analysis of virions. Virology (2014), http://dx.doi.org/10.1016/j.virol.2014.06.005