Flow Virometry: a Powerful Tool To Functionally Characterize Viruses

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ABSTRACT For several decades, flow cytometry has been a common approach to analyze cells and sort them to near-purity. It enables one to probe inner cellular molecules, surface receptors, or infected cells. However, the analysis of smaller entities such as viruses and exocytic vesicles has been more difficult but is becoming mainstream. This has in part been due to the development of new instrumentation with resolutions below that of conventional cytometers. It is also attributed to the several means employed to fluorescently label viruses, hence enabling them to stand out from similarly sized particles representing background noise. Thus far, more than a dozen different viruses ranging in size from 40 nm to giant viruses have been probed by this approach, which was recently dubbed “flow virometry.” These studies have collectively highlighted the breadth of the applications of this method, which, for example, has elucidated the maturation of dengue virus, served as quality control for vaccinia vaccines, and enabled the sorting of herpes simplex virus discrete viral particles. The present review focuses on the means employed to characterize and sort viruses by this powerful technology and on the emerging uses of flow virometry. It similarly addresses some of its current challenges and limitations.

KEYWORDS FACS, flow cytometry, flow virometry, HSV, herpes, review, sorting, viral particles, exosomes, herpes simplex virus, hsv, nanoparticles

Flow cytometry and its derivative fluorescence-activated cell sorting (FACS) have been methods of choice since the 1970s to analyze and purify individual cells. In many cases, their use has permitted the isolation of low-abundance cellular subpopulations with high purity in a single step. This has all been possible owing to the ability to detect and discriminate cells by both light scattering and fluorescence. In the latter case, this is often achieved with antibodies and fluorescent secondary reagents directed at the cell surface and/or internal constituents in permeabilized cells. Alternatively, the use of cells encoding genetically modified proteins that are fluorescently tagged has proven to be an equally valuable way to monitor cells. Flow cytometry has been particularly interesting in light of the many different markers that can be observed simultaneously, hence enabling the discovery and characterization of ever-expanding subpopulations of cells, most notably in the field of immunology. Moreover, one substantial appeal of flow cytometry and FACS analysis has been its ability to analyze and sort very large numbers of cells, thus providing data that have great statistical value.

THE SIZE OF MOST VIRUSES IS BELOW THE RESOLUTION THRESHOLD OF COMMON FLOW CYTOMETERS

Classical viruses range in size from the smallest nonenveloped 17-nm circovirus (1) to the much larger enveloped vaccinia viruses, which are brick shaped and up to 350 nm in size at their wider edge (2). Notable exceptions are the recently discovered giant viruses, which can reach over 1 μ m in size (3, 4). Most viruses fall below the resolution

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limit of standard flow cytometers, which has been pegged at 300 to 500 nm (5, 6). However, the issue is not so much that these instruments cannot detect such small entities but rather that the size of the viruses falls within the range corresponding to optical, electrical, and filtered sheath buffer background noise. For this reason, it has long been considered theoretically possible but challenging to characterize viruses by flow cytometry. The ability to fluorescently label viral particles, along with the availability of new generations of dyes and instrumentation, now makes such analyses achievable.

THE BIRTH OF FLOW VIOMETRY

The use of flow cytometry to characterize viruses was actually pioneered decades ago with the construction of a homemade flow cytometer to detect glutaraldehyde- or formaldehyde-fixed T2 phages, which are elongated viruses (approximately 70 nm wide and 200 nm long) (7). In contrast, the smaller, more spherical reovirus (diameter of 60 to 80 nm) could barely be detected in those studies. Flow cytometry was subsequently used to detect fixed T4 phages and the larger poxvirus (8). This approach has also been routinely used to enumerate marine viruses (9–12). This required careful preparation of the samples, fixing, labeling of the viral particles, and heating to promote the penetration of the dye (12, 13). Despite these exciting initial reports, the analysis of viruses by flow cytometry, which Grivel and colleagues termed “flow virometry” in 2013 (14), has become more mainstream only recently. Flow virometry has now been used to characterize an expanding array of additional viruses, including lambda phage (15), herpes simplex virus 1 (HSV-1) (16, 17), mouse hepatitis virus (MHV) (18), human immunodeficiency virus (HIV) (14, 19–21), Nipah virus (22), Junin virus (23), vaccinia virus (24), dengue virus (20, 25), human cytomegalovirus (HCMV) (26), and giant viruses (13). Flow virometry is becoming a powerful tool to characterize viruses.

TECHNOLOGICAL ADVANCEMENTS

The development of specialized flow cytometers has been a major contributor to the present success in studying nanoparticles such as most viruses and exosomes (7, 8, 27, 28). As such, many of the reported technological improvements described below were in fact focusing on exocytic vesicles, as recently reviewed (29, 30). There are indeed many parallels between the extracellular vesicle and flow virometry fields which will clearly benefit both. For example, one common issue is that very small particles disperse light differently than larger ones and divert the photons much more broadly (31). In standard flow cytometers, sample size is typically evaluated with detectors of forward light scatter (FSC). These routinely efficiently capture light emitted in an angle range of 0.5° to 15° to monitor cells but miss a significant portion of the light refracted by smaller objects. They do, however, efficiently record the background signal that is predominant at angles under 15°. New-generation cytometers dedicated to nanoparticles now offer “reduced wide-angle FSC” detection, which blocks light in the 0° to 15° range to reduce noise, and instead monitor light at angles between 15° and 70° (8, 27, 32). A second common issue is the power of the lasers and the sensitivity of the detectors, which perform very well for cells but less efficiently for nanoparticles. The issue is that elements with wavelengths smaller than the wavelength of the light illuminating the samples poorly refract light, with signals estimated to decrease with the sixth power of the particle size (33). To circumvent this limitation, some cytometers have lasers reaching wattage levels of up to 300 mW, as opposed to 10 to 20 mW in standard instruments (23). Interestingly, Hercher’s original pioneering work performed in 1979 to detect T2 phages employed a whopping 1-W laser (7). Newer cytometers additionally rely on high-performance photomultiplier tubes (PMT) or digital focusing systems (DFS) that are generally more sensitive than photodiode detectors (27). Note that this superiority is not absolute, as illustrated by avalanche photodiodes (APDs), which outperform PMTs at wavelengths above 650 nm (34). Another strategy has been to focus the samples in the midst of the flow stream of the cytometer and to reduce the internal chamber size to improve optimal illumination and single-particle detection.

FIG 1 Technological developments of flow cytometers. A schematic representation of a FACS apparatus, including the fluidic, optic, and electronic components, is drawn. Various viruses (differently colored) are also portrayed. Current improvements to the cytometers (in blue boxes) include filtering of the sheath buffer with a $0.1\text{-}\mu\text{m}$ -pore-size filter, optional filtering of the viral sample with a $0.45\text{-}\mu\text{m}$ -pore-size filter (for viruses smaller than that), better hydrodynamic focusing, more-powerful lasers, and forward light scatter (FSC) detectors with greater sensitivity and reduced wide-angle capabilities. Note that standard FSC detectors typically monitor light in the 0.5° to 15° range, where most of the background signal is found (red line) and where less light is emitted by nanoparticles (green lines). The reduced wide-angle FSC detector instead blocks any light below 15° and records the signal from 15° to 70° , greatly improving signal-to-noise ratios. SSC, side light scatter.

Finally, filtering the sheath buffer with a $0.1\text{-}\mu\text{m}$ -pore-size filter, instead of the standard $0.22\text{-}\mu\text{m}$ -pore-size unit, reduces background signals from impurities (23). Similarly, filtering samples themselves with a $0.45\text{-}\mu\text{m}$ cutoff, when possible, limits aggregates and artifacts (16, 17). Altogether, these improvements, schematized in Fig. 1, have reduced the resolution limit of newer flow cytometry and FACS instruments to around $100\ \text{nm}$ (23, 27), which is sufficient to probe many of the larger viruses by light scattering.

Though specialized flow cytometers do handle viruses and exosomes more efficiently, standard flow cytometers can nonetheless be employed owing to parallel advancements in labeling viral particles by various means. This is important, as fluorescence detection remains to this day the best way to discriminate viruses from the typically nonfluorescent background noise (31, 35). An approach employed by several laboratories is that of labeling the surface of the viral particles with antibodies that are coupled either directly to a fluorescent moiety or indirectly via a secondary antibody. In some cases, these antibodies have also been prebound to 15-nm nanobeads to improve the detection of the virus by light scattering (see, e.g., reference 14). Another successful approach has been to label the genetic content of viruses, which can be

either DNA or RNA. In our hands and those of others, not all nucleic acid fluorescent dyes have performed well in this respect. For instance, Hoechst, DAPI (4',6-diamidino-2-phenylindole), and propidium iodine all perform poorly for detection of viruses by flow cytometry (8) (N. El Bilali and R. Lippé, unpublished observations). This is presumably because of the lower numbers of molecules that bind the viral genomes, which are much smaller than those of their cellular counterparts. Alternatively, it may be due to the inaccessibility of the dyes to the viral nucleic acid as it is buried inside a protein shell (capsid), which is itself covered by one or several distinct proteins (called the matrix or tegument layer depending on the virus) and, for enveloped viruses, a lipid bilayer. The discovery of brighter fluorescent dyes with better quantum yields and extinction coefficients has been very useful. Examples of nucleic acid dyes currently employed include SyBR green-I, YOYO-1, TOTO-1, and PicoGreen (10, 23, 36, 37). However, SyBR green-I does not always efficiently label viral particles unless they are first heated to up to 80 to 90°C (9, 12), which is incompatible with keeping viruses infectious. After a screen of many commercial dyes, we personally opted for membrane-permeable Syto 13 (green fluorescence) or Syto 62 (red fluorescence) to label herpes simplex virus 1 (HSV-1), owing to their good signals, very low background noise, and excellent sample penetrance (16, 17). These dyes work well for nonenveloped intermediates such as HSV-1 nuclear capsids as well as for fully enveloped extracellular mature virions. Most importantly, these Syto dyes are not detrimental to the infectivity of the virus at the concentration used (16, 17). Another means to label enveloped viruses is the use of lipid dyes. This approach was successfully employed for HIV, vaccinia virus, and dengue virus using DiD, DiO, and DiI, for example (14, 24, 25). DiR, a related fluorochrome in the far-red spectrum, may also be a good choice (38). It should be pointed out that exosomes and ectosomes, recently discovered microvesicles secreted by most cells into the extracellular environment (39), are routinely analyzed by flow cytometry (20, 32, 40–43). One dye commonly used to label them is PKH67, a green fluorescent molecule with an aliphatic tail that targets lipid bilayers (27, 40, 41). These properties make it a potential candidate for the study of enveloped viruses. Finally, it is possible to probe by flow cytometry or FACS analysis genetically modified viruses expressing fluorescently tagged virion components, including capsid, matrix/tegument, or envelope viral proteins (16, 17). With these tools at hand, it is clearly possible to analyze and sort viruses on both specialized and standard flow cytometers (Fig. 2).

One important issue of flow virometry is that of ensuring detection of single viral particles. Analysis of samples, particularly small ones, by flow cytometry or FACS requires great care to monitor and reduce the incidence of coincidental events, i.e., the inadvertent inclusion of two or more particles in a unique droplet while it is being exposed to the laser beam. Although light scattering can be used for cells to determine if single events pass through the cytometer, this is unfortunately not as easy with smaller objects as described above. Classical ways to evaluate this have therefore included serial dilutions of the nanoparticles, whereby the mean fluorescence intensity (MFI) of each particle should not change upon dilution if it truly represents a single event. Another method of choice is the use of electron microscopy examination to verify that the samples are indeed single entities (29, 31). Concurrently, an effective approach to monitor aggregates is to mix viruses prelabeled with different fluorochromes and to see how well one can resolve them by flow cytometry. It is worth noting that for large viruses such as HSV-1, one can also detect aggregates by light scattering since they would be above the 300- to 500-nm limit seen with most cytometers. We and others have successfully used these approaches (16, 17, 25, 35) (R. Lippé, unpublished data).

FUNCTIONAL ANALYZES OF VIRUSES BY FLOW VIROMETRY

Although early attempts to detect viruses by flow virometry served the purpose of enumerating them (9–12), flow virometry has since been instrumental in characterizing many aspects of viruses (Table 1). For instance, Williamson and colleagues used that approach in 2011 to purify unfixed T4 and lambda phages and to perform so-called

FIG 2 Efficient means to label viral particles for flow cytometry. The typical constitution of a virus is depicted (an enveloped virus is shown here). The viral genome is enclosed in a proteinaceous capsid, which can be surrounded by a protein layer called a matrix or tegument depending on the given virus. For enveloped virions, a cell-derived envelope that contains viral glycoproteins is additionally present. The figure indicates the current methods used to perform flow virometry, which includes genetic tagging of the diverse virion constituents, including the capsid, matrix/tegument, or viral glycoproteins (fFP; GFP or alternative fluorescent tags). It is also possible to detect the viruses with antibodies directed against surface molecules (glycoproteins for the enveloped viruses or capsid proteins for nonenveloped viruses). Some laboratories couple the antibodies to nanoparticles for greater detectability by light scattering. A third approach is to employ fluorescent lipophilic markers in the case of enveloped viruses (DiD, DiO, Dil) with the possible use of PKH67. The use of DiR, a member of the same family of dyes but with excitation/emission profiles in the infrared spectrum, should also be an option. Finally, one can label the RNA/DNA viral genome with several different dyes (Syto13, Syto62, SyBr green-I, YOYO-1, TOTO-1, and PicoGreen). Note that this list is not exhaustive, as many other dyes may also perform well.

“single virus genomics” (15). At issue are the amplification and sequencing of the genomes of uncultivated and uncharacterized viruses isolated from diverse environments (human, soil, water, etc.). As a proof of concept, the authors reported the successful separation by flow virometry of individual SYBR green-labeled lambda and T4 phages. They then individually trapped the sorted viruses on agarose beads and PCR amplified their genome *in situ* followed by sequencing of single viral particles. One interesting limitation that they encountered, though, was that imbedding of the viruses in agarose beads was required to preserve their integrity. Nonetheless, this innovative approach demonstrated that it is possible to study novel viruses without the need to first define ways to cultivate them, which can be a limiting factor (44).

Another example of the value of flow virometry was reported by our group back in 2012 (16). In that study, this approach was used to sort subpopulations of so-called nuclear A-, B-, and C-capsids produced during herpes simplex virus 1 (HSV-1) infections. Most interestingly in the case of C-capsids, this was achieved with purity greater than that seen with standard biochemical approaches, providing a new method to enrich these viral intermediates. This is an important improvement to understand the HSV-1 life cycle since only C-capsids incorporate the viral genome and evolve into mature virions (45–47). It is therefore now possible to characterize relatively pure preparations of these C-capsids and to compare them to their A-capsid and B-capsid counterparts.

TABLE 1 Flow virometry applications

Virus	Size (nm) ^a	Particular application	Reference(s)
Bacteriophages			
T2	70 × 200	Discriminating viruses	7
T4	70 × 200	Single-virus genomics	15
Lambda	50–60	Single-virus genomics	15
Mammalian viruses			
Vaccinia virus	350 × 270 × 250	Vaccine quality control	24
HCMV	200–300	Developing neutralizing antibodies	26
HSV-1	125 (capsids)	Nuclear capsid sorting	16
	250 (virions)	Tegument variability; viral fitness	17
Nipah virus	150–500	Surface protein analysis	22
HIV	120–150	HLA DR/LFA1 heterogeneity	14
		Envelope conformation	19
Junin virus	100–300	Viral subgroups; impact of host	23
MHV	80–120	Size marker for liposomes	18
Reovirus	60–80	Discriminating viruses	7
Dengue virus	40–60	Virion maturation	20, 25
Marine viruses	Various	Enumeration	9–12
Giant viruses			
Cedratvirus	1,200	Isolation	13
Mimivirus	500–650	Isolation	13

^aViruses are not necessarily homogenous in size and can vary, so the data represent average values.

Moreover, flow virometry sorting can be extended to analyze intermediates of other viruses.

Viral stocks typically contain a mixture of defective and infectious particles, the former usually vastly outnumbering the latter. Classical approaches to characterize viruses such as Western blotting and PCR usually analyze bulk samples that include these defective particles and whose protein content can be very different from that of their infectious equivalent. In addition, there is substantial evidence that even infectious virions vary significantly from one to another. Though it is possible to visualize and analyze discrete viruses with imaging techniques (e.g., atomic, electron, and fluorescence microscopy), it has been a challenge to individually monitor very large numbers of virions and to correlate their content with viral fitness. Flow virometry has changed this since one can now sort viruses based on their intrinsic properties. As a proof of concept, we recently evaluated the heterogeneity of the tegument proteins among individual HSV-1 virions. This tegument is a complex layer that is found between the viral capsid and envelope and which contains 23 to 24 distinct viral proteins and possibly even more host proteins (17, 48, 49). For the evaluation, we used a battery of green fluorescent protein (GFP)-labeled viruses and analyzed up to 100,000 viral particles per condition. The data confirmed some significant discrepancies among the virions but most importantly revealed upon sorting of viable virions that this can impact viral fitness (17). This hinted that flow virometry can indeed sort subpopulations of viable viruses for the purpose of assessing their specific infectivity in cells. Most interestingly, it may now be possible to inject those purified subpopulations into animals to test if this translates to changes in virulence.

The first study where flow virometry was employed to sort infectious viral particles was performed by Gaudin and Barteneva, who characterized Junin viruses, which contain a RNA genome (23). By sorting viruses stained with fluorescent antibodies directed against the envelope viral glycoprotein precursor (GPC), they demonstrated that viral infectivity correlates with virion size and GPC content. Moreover, they showed that the proportions of competent virions (GPC positive [GPC⁺] and RNA⁺) differ among viral stocks. This infectious-particle-to-noninfectious-particle ratio was also strongly influenced by the type of cells producing the virions. They went further and showed that viral infectivity additionally depends on the presence of tetraspanin and the lipid raft marker cholera toxin B. This was an important milestone that indicated

that flow virometry is a very useful tool to study the impact of virion content on infectivity.

Flow virometry has very interestingly benefitted vaccine quality control in the case of vaccinia virus, as described in reference 24. In that study, the authors examined the properties of diverse vaccinia virus preparations generated for their oncolytic activity and cancer vaccine potential. Using fluorescently labeled virions analyzed and sorted by flow virometry, Tang and colleagues (24) revealed a high level of heterogeneity in viral particle size and ratios of infectious to noninfectious particles. They also reported that the virus tended to aggregate over time in those preparations depending on the storage conditions, thus influencing how these vaccines should be preserved. This was the first instance where flow virometry proved an interesting avenue to evaluate the quality of vaccine preparations.

The HIV envelope proteins (Envs) play an important role in viral propagation as they modulate the attachment of the virus to the cell and entry by fusion (50). Functional Envs are composed of trimers of gp120 and gp41 proteins produced by proteolytic cleavage of a unique precursor. They also undergo conformational changes upon binding to their cognate cellular receptors (50). Moreover, uncleaved monomeric or dimeric Envs are also produced during the infection (51). This results in multiple conformations of the proteins, whose plasticity impacts their biological activity as well as viral fitness (52). One open issue was whether active and inactive Envs coexist on a given viral particle and whether they could influence viral infectivity both negatively and positively. To resolve this issue, Margolis and colleagues (19) resorted to flow virometry to discriminate the various conformations of the HIV envelope proteins on single virions using a battery of gp120-specific antibodies. Their study revealed that, by and large, HIV virions exclusively carry at their surface active Env proteins or inactive Env proteins but not both. The authors consequently concluded that viral particles harboring both active and inactive Envs are unlikely to be significant contributors to the infection. These were important findings as they affect potential strategies to control the virus based on neutralizing antibodies. Most importantly in the context of this review, the use of flow virometry was instrumental in that study. This was again the case in yet another study focusing on HIV where Arakelyan et al. (14) monitored the heterogeneity, among discrete HIV particles, of HLA-DR and LFA-1, two host components incorporated into the viral envelope. In their study, they captured the viruses with 15-nm-diameter magnetic nanobeads coated with antibodies against gp120 and probed the host proteins described above by flow virometry using fluorescently labeled monoclonal antibodies. That analysis revealed that the incorporation of cellular proteins into the viral envelope differs extensively from one particle to the next. Most importantly, this variability escaped detection by conventional methods, highlighting the power of flow virometry.

Nipah virus is a highly pathogenic level 4 virus that is consequently difficult to study (53). To circumvent these difficulties, Landowski et al. (22) relied on virus-like particles to study viral entry kinetics and to determine the abundance of two key viral proteins on these particles, namely, the G attachment and F fusion proteins. They did this by labeling the surface of the particles with various virus-specific antibodies and appropriate fluorescent secondary reagents. Once again, flow virometry proved invaluable for these studies and they could additionally monitor conformational changes in the G protein upon its binding to its cellular receptor. This indicated that flow virometry could be useful not only to study naturally occurring viral entities but also to probe man-made virus-like particles.

Another example where flow virometry was an ideal tool is the study of dengue virus. This virus has an RNA genome that encodes a single polypeptide that ultimately produces several proteins by proteolytic cleavage (54). One of those proteins is the structural prM membrane protein. PrM is incorporated on the surface of newly assembled immature virions, which assemble in the endoplasmic reticulum (ER) in a manner similar to that seen with other members of the flavivirus family (55). Those virions reach the *trans*-Golgi apparatus, where viral maturation occurs. This involves, in part, the

proteolytic processing of prM into the M protein via a furin-like cellular protease (56). The M protein can then interact with and modulate the viral envelope E protein, which has fusogenic activity (57). However, one issue was whether extracellular virions released from the cells exhibit a mix of mature and immature M proteins on their surface. As one cannot address this issue by bulk analysis of virions, Zicari et al. probed the maturation of the dengue virus by flow virometry (25). To this end, Dil-labeled virions were captured on magnetic nanobeads coupled to antibodies reacting against the viral envelope E protein and the captured virions were incubated with prM-specific antibodies. These were then eluted and analyzed by flow virometry, gating on the Dil label, and quantifying prM in a different fluorescent channel. Their data indicated that half of the virions produced contained only the mature M protein, while other particles proved heterogeneous (25). This revealed that it is possible to monitor virion maturation by flow virometry.

Finally, flow virometry was also pivotal in the pursuit of a vaccine against cytomegalovirus, a major congenital virus that affects newborns (58). At issue are recently discovered potent neutralizing antibodies that target the gH/gL/UL128/UL130/UL131 pentameric complex on the surface of naturally occurring human cytomegalovirus (HCMV) but that are absent from laboratory and vaccine strains (59–61). To study viral heterogeneity and to characterize the pentameric complex, flow virometry was again harnessed to characterize individual virions with the goal of generating better neutralizing antibodies in clinical trials (26).

FUTURE DEVELOPMENTS AND CHALLENGES

Flow virometry is a recent and exciting development of flow cytometry. The examples described above underscore the breadth of the avenues by which flow virometry can contribute to various fields and applications (summarized in Table 1). Unlike other methods, flow virometry can track discrete viral particles in numbers well beyond what is possible with other techniques, providing the additional benefit of greater statistical value. Moreover, flow virometry can in principle be performed with the standard flow cytometers that are present at most facilities, which means that most laboratories have access to this tool. Nonetheless, it will be most stimulating to see how new generations of dyes and flow cytometers are adapted to study nanoparticles such as viruses and their intermediates as they mature into fully functional virions. An open issue is whether these new cytometers will discriminate by light scatter analysis the smallest of viruses, which seems achievable based on the recent detection of silica and gold nanoparticles (62, 63). It should also be noted that 50- to 100-nm-diameter metal particles can also be analyzed by side scattering (63).

A key development is the recent possibility of combining flow cytometry and mass spectrometry in a single instrument, a method referred to as mass cytometry or cytometry by time of flight (CyTOF) (64). This innovative approach relies on labeling of samples with antibodies coupled to rare metal isotopes rather than fluorescent moieties, where abundance is measured by mass spectrometry (65). Two advantages of mass cytometry over flow cytometry are the lack of spectral overlaps in analyzing several fluorescent antibodies at once and the irrelevance of background autofluorescence (65, 66). Consequently, mass cytometry can routinely handle 40 different parameters in parallel, instead of the 6 to 12 parameters monitored by modern flow cytometers (67). However, mass cytometry also has limitations as it destroys samples during the ionization stage, so its use is not yet amenable to sorting live cells or infectious viruses (66). It is also slower than flow cytometry and can handle only hundreds of cells every second (65), so greater speeds will be highly beneficial. Despite these limitations, mass cytometry is likely to become a game changer.

A major challenge is that of distinguishing viral particles from similarly sized extracellular vesicles such as exosomes, given that the two types of particles are coproduced by infected cells. In principle, this could be achieved by immunisolating or gating onto one or the other with selective markers. However, the challenges of identifying unique molecules may not be trivial given that some viral components are

packaged in exosomes (68, 69), virions can incorporate host proteins (70), and viruses modulate the content of exosomes (71).

A persistent issue with flow cytometry is absolute quantification of molecules, which is problematic at the moment for lack of proper standards. The accuracy of size measurement for small particles is also in need of improvement as commercial beads are not suitable, given their different refractive indices and since estimates determined by forward scatter analysis are not linear for small entities (24, 31, 72–75). It would be interesting to see if well-characterized, fixed, and uniform viruses, particularly the more rigid nonenveloped ones, could eventually be employed as biological FACS standards. Interestingly, mouse hepatitis virus, though an envelope virion, was already used as a size marker for liposomes (18).

Other foreseeable developments are new applications of flow virometry and the analysis of the biology of an expanding list of viruses. For instance, it should now be possible to sort desired viral subpopulations to high levels of purity for subsequent mass spectrometry analysis, an avenue that we are actively pursuing to characterize HSV-1 viral intermediates (El Bilali and Lippé, unpublished). Other potential uses include the diagnostics of virus infections in a clinical setting whereby flow virometry could focus on genome-containing infectious particles rather than on genome-free defective particles (e.g., by labeling them with nucleic acid dyes). It could additionally serve as a purification tool to characterize existing or newly discovered pathogens. Similarly, one can envision the further development of therapeutics focusing on the most virulent viral particles containing, for example, more of a given protein (17). It may also be possible to prepare highly enriched and homogeneous populations of virions for animal studies. Many additional and unforeseen developments will likely also arise from this powerful approach.

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