

# Detecting RNA viruses in living mammalian cells by fluorescence microscopy

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**Traditional methods that rely on viral isolation and culture techniques continue to be the gold standards used for detection of infectious viral particles. However, new techniques that rely on visualization of live cells can shed light on understanding virus–host interaction for early stage detection and potential drug discovery. Live-cell imaging techniques that incorporate fluorescent probes into viral components provide opportunities for understanding mRNA expression, interaction, and virus movement and localization. Other viral replication events inside a host cell can be exploited for non-invasive detection, such as single-virus tracking, which does not inhibit viral infectivity or cellular function. This review highlights some of the recent advances made using these novel approaches for visualization of viral entry and replication in live cells.**

## Introduction

Viruses constitute a major class of pathogens that account for a variety of ailments ranging from common cold, which leads to loss of productivity, to acute infections, such as influenza, which can be responsible for high-mortality epidemics and, in certain cases, pandemics [1,2]. Over the years, the need for rapid and specific viral detection has been realized. The major reasons for this drive have been the time consuming and expensive nature of traditional viral diagnostic techniques and the availability of several antiviral therapeutics. In the past decade, the rapidly evolving nature of some virus families has necessitated the use of adaptable diagnostic tools to identify the presence of newly emerging strains for treatment, because most antiviral drugs must be administered within 48 h of the onset of symptoms to be most effective [3].

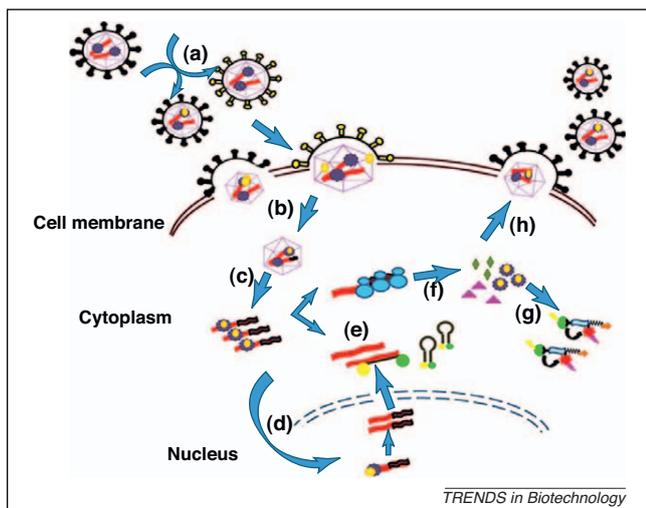
Until the 1980 s, viral detection was solely based on traditional viral isolation and culture techniques – plaque assay, hemagglutination assay, histological observations – which even today, remain gold standards in many

diagnostic laboratories [4]. However, these techniques are time-consuming, labor-intensive, and culture-based methods that are not suitable for viral classes that are difficult to cultivate in the laboratory environment. Several direct examination techniques, such as immunofluorescence, ELISA, and electron microscopy, have also been used for viral particle detection, although they suffer from poor sensitivity and specificity, which makes it difficult to interpret the results [5]. In the 1990 s, highly sensitive molecular methods based on amplification of nucleic acids using PCR took center stage, and quantitative PCR, which employs fluorescent probes, has shown greater success owing to the ease of standardization and higher sensitivity [6]. However, most of these above mentioned techniques serve primarily as detection tools and provide minimal information on infectivity. In addition, very few insights about the molecular mechanisms involved in viral replication and the pathogenetic events that occur within the cells can be provided by these methods. A detailed understanding of the molecular mechanisms involved in virus replication might not only lead to new and rapid detection methods, but could also help the design of targeted approaches to develop more powerful antiviral compounds.

Over the past several years, researchers have begun to realize several techniques for visualizing the viral life cycle inside living cells (Figure 1). The focus of these studies involves: viral entry mechanisms (Figure 1a,b) [7]; transport of viral core molecules through the cell to the nuclear region (Figure 1c,d) [8]; transport of viral nucleic acids in and out of the nucleus (Figure 1d) [9]; assembly and transport of newly synthesized viral components (Figure 1e–g) [10]; the cell-to-cell spread of newly synthesized viral particles (Figure 1h) [11]. These methods are mostly based on either the use of nucleic acid probes, protease activity probing, or viral coat/genetic core labeling [12–14]. Detection of a wide range of viruses, such as HIV, poliovirus (PV) and influenza virus has been achieved; even offering single molecule sensitivity [15–18]. This review highlights such modern techniques that are employed for the detection of viral infection in living cells.

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**Figure 1.** Real-time imaging techniques aim to monitor the viral life cycle at different stages. These techniques include: **(a)** probing viral entry mechanisms by labeling viral coat or core proteins; **(b)** monitoring transport and uncoating of the viral core inside the host cell via labeled core proteins; determination of the cytoplasmic **(c)** or nuclear **(d)** localization of viral genome through associated labeled viral proteins; **(e)** detection of infection with viral-mRNA-binding MBs introduced externally; **(f)** visualization of the assembly and transport of newly synthesized viral components through genetically engineered labels or through externally introduced cell-permeable dyes; **(g)** monitoring infection progress using FRET proteins; and **(h)** monitoring the release of newly synthesized viruses and their intercellular spread via labeled core components.

### Nucleic-acid-based methods for visualization of viral infection

An in-depth understanding of mRNA expression, interaction, movement and localization provides information on cellular responses to different conditions. Live-cell imaging of mRNA provides the opportunity to study the dynamics of mRNA expression and localization, and can be an important tool for advancing disease pathophysiology, drug discovery, and medical diagnostics. In the field of virology, live-cell RNA imaging enables a detailed understanding of viral infection and pathogenesis [19]. The vast majority of viruses contain RNA genomes that play a crucial role by serving as templates for the transcription to make viral RNAs, which are later translated into proteins, and finally assembled into new virions [20]. The lack of suitable technology has been the key limitation in the past; however, live-cell RNA imaging has recently been realized using direct fluorescent labeling [21], the use of fluorescent probes [22], and sequence-specific mRNA recognition by GFP fusion proteins [23].

#### Fluorescently labeled RNA

A detailed understanding of viral entry into the host cell is not only essential for developing therapies to combat viral infection, but it also provides insights into fundamental viral infection processes [7]. The entry pathway of PV was recently characterized by live-cell imaging using dual-labeled virus particles [15]. This was accomplished by incorporating a membrane-permeable, non-toxic Syto82 dye into the newly synthesized viral RNA before packaging, followed by labeling of the viral capsid with an amine-reactive Cy5 dye. By combining biochemical inhibition assays with direct visualization of PV in the early infection cycle, the authors have confirmed that the release of viral

RNA is energy-, actin- and kinase-dependent. Similar imaging techniques are largely applicable to most non-enveloped viruses for which the cellular sites of genome release are still unknown, and the information obtained is valuable for designing inhibitors that are suitable for blocking viral entry [15].

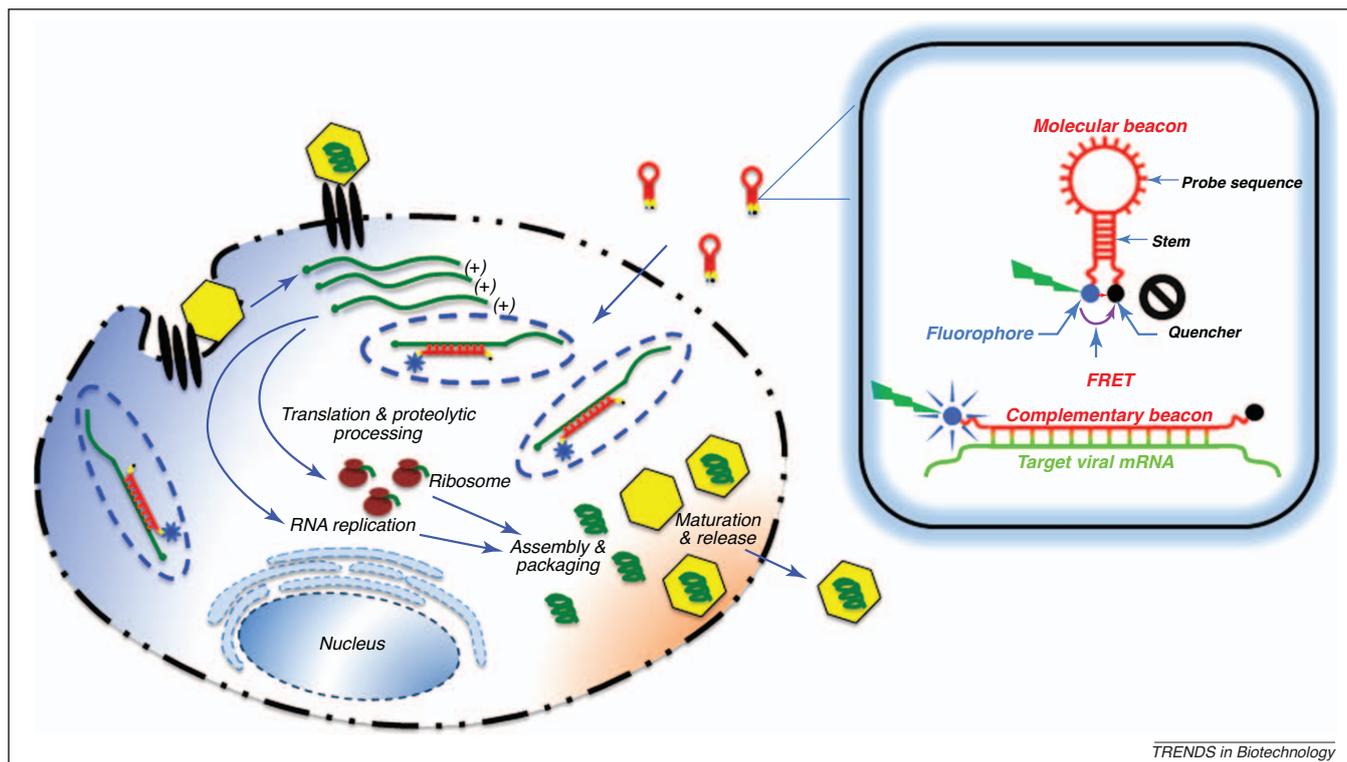
As an improvement to the dual-labeling method, multiply labeled tetravalent RNA imaging probes (MTRIPs) have been developed to provide accurate imaging of native mRNAs [18], particularly when delivered into live cells using streptolysin-O (SLO). An increase in the signal is achieved owing to binding of multiple probes to a single target RNA. The feasibility of tracking viral infection has been successfully demonstrated for paramyxovirus [18]. Although the signal to noise ratio is relatively modest, the small size of the MTRIPs and their ability to fluoresce at multiple wavelengths makes them a potential candidate for tracking viral RNA in living cells.

#### GFP-tagged mRNA

Intracellular mRNAs can be directly visualized while bound to GFP. To tag mRNA, GFP is fused with an RNA-binding protein and expressed along with the target mRNA. A motif on the target mRNA is used to associate with the RNA binding protein [24]. The coat protein of the bacteriophage MS2 has been identified as an ideal tag because it binds to a unique hairpin region in the genomic RNA with strong affinity [25]. Using this approach, simultaneous imaging of the HIV-1 structural protein Gag and the HIV-1 genome has uncovered the dynamics and functional interactions during the packaging of individual virions [26]. A related study that employs simultaneous fluorescent imaging has shown that a few Gag molecules are required to recruit viral RNA into the plasma membrane [27]. Both the reporter proteins and the target mRNA are genetically encoded; therefore, stable transgenic cell lines can be expressed and mRNA transport can be studied in detail. However, GFP tags might not have high multiplexing potential, owing to the limited availability of high-affinity RNA tags and the contribution of high background fluorescence [24]. Importantly, this approach is only suitable for studying the fundamental mechanisms of the viral life cycle, but not for clinical detection of viral infection.

#### Molecular beacons

For some viruses, such as hepatitis A virus (HAV), the replication process is relatively slow and non-lytic; as such, cultivation in the laboratory can take up to several weeks for maximum virus production [28]. This presents a significant challenge to detect infectious viruses that rely on the visualization of cytopathic effects using the mammalian cell culture method. Therefore, rapid methods are needed to understand better the replication cycle and for early detection of viral infection. Molecular beacons (MBs) are single-stranded oligonucleotide probes that form a stem-loop structure with a fluorophore at the 5' end and a quencher at the 3' end (Figure 2) [29–31]. Upon excitation of the fluorophore, energy is transferred to the quencher through a process called fluorescence resonance energy transfer (FRET), which results in loss of fluorescence



**Figure 2.** Schematic representation of the MB design and its application in live-cell tracking and imaging of viral RNA. The MBs form a closed hairpin structure in the absence of a complementary target. The proximity of the quencher (3') to the fluorophore (5') results in a loss of fluorescence signal. In the presence of a complementary viral RNA target, the MB opens up; this can be directly measured as a fluorescent signal. MBs are introduced into live cells to visualize viral RNA. Hybridization with complementary target RNA results in a direct fluorescent signal which is observed at different stages of viral infection.

signal. However, in the presence of a complementary target, the fluorophore and the quencher move apart in response to a spontaneous conformational change, thus allowing the MB to fluoresce as a direct indicator of target binding (Figure 2). The spontaneous hybridization that occurs between the beacon and the target is highly specific and can be used to differentiate even a single base pair mismatch [32–34]. Using this technique, a combined cell culture–MB assay has been reported for the detection of HAV by visualization of the viral RNA in infected cells after permeabilization [28].

The use of MBs to provide a dynamic snapshot of the translocation of PV RNA has also been demonstrated, which indicates the potential to conduct transient studies inside infected cells [35]. Similar success has been achieved by probing the intracellular transport of influenza virus mRNA in infected cells [36]. Results indicate that influenza virus mRNA transport is energy-dependent, by utilization of the cellular TAP/p15 transport pathway, with the participation of the viral NS1 protein and cellular RNA Polymerase II (RNAP-II). The possibility of studying the spread of bovine respiratory syncytial virus (bRSV) in living cells over a 7-day period has also been demonstrated [37]. Unfortunately, no real-time information was provided as the MBs were introduced at different post-infection time points, rather than immediately following infection.

#### Improved MBs for imaging viral replication in live cells

Even though MBs have enabled live-cell imaging, some of the major challenges in using traditional MBs for *in vivo*

imaging include their modest half-life (~50 min) owing to nuclease degradation and the lack of a non-invasive intracellular delivery technique [38,39]. The stability of MBs can be improved significantly by modifying the oligonucleotide backbones using 2'-O-methyl RNA bases and 2'-O-methyl-phosphorothioate internucleotide linkages [40]. To provide non-invasive delivery of MBs across the plasma membrane, the pore-forming bacterial exotoxin, SLO, has shown promise as a potential delivery tool. However, some studies have reported rapid nuclear localization of cargoes. Moreover, it can only be used in *ex vivo* cellular assays [41]. The HIV-1-derived TAT peptide is a cell-penetrating carrier that has received attention as a possible vector for the delivery of oligonucleotides across the cell membrane, in a seemingly energy-independent manner [42–44]. The TAT-based delivery method does not interfere with specific targeting or produce hybridization-induced fluorescence of the MBs [45]. This novel delivery method, when combined with nuclease resistant MBs, can provide real-time monitoring of coxsackievirus B6 virus replication in living cells, as well as intercellular spreading of infection [14]. These results suggest that the TAT-modified, nuclease-resistant MBs might be very useful for understanding the dynamic behavior of viral replication and for therapeutic studies of antiviral treatments.

Another major limitation to conventional MBs for FRET-based applications is the use of organic fluorophores that have low resistance to photodegradation, which makes them unsuitable for long-term *in vivo* imaging [46]. Semiconductor nanocrystals known as quantum dots

(QDs) have gained a lot of attention for viral imaging applications, owing to their excellent photostability, brighter fluorescence than organic fluorophores, and their ability to be used in multiplex detection schemes [46,47]. Recent studies have reported the development of nuclease-resistant MBs using QD and Au NPs as the FRET pair [48–50], [44]. These MBs have been used for real-time *in vivo* viral detection via TAT peptide delivery. The new FRET pair (Au-NP-MB) resulted in a sevenfold increase in fluorescent signal upon target binding, which makes it suitable for exploring the real-time molecular mechanisms that are essential for viral pathogenesis [51].

### Fluorescence labeling methods for visualization of viral infection

In addition to probing viral RNA for real-time monitoring of viral infection, other viral replication events inside a host cell can be exploited for non-invasive detection. One popular and powerful method involves single-virus tracking in living cells. Single-virus tracking incorporates live imaging of cells infected with recombinant viruses that express fluorescently tagged structural viral proteins and cellular structures of interest [52]. This method enables the dynamic visualization of the virus maturation pathways at a single virus-particle level (Figure 1). A crucial requirement of this technique involves labeling of viruses and cellular structures with a sufficient number of fluorophores for detection, without inhibition of viral infectivity and cell functions. Two general strategies exist for labeling viruses: the fusion of a target viral protein with a fluorescent protein (FP), or direct chemical labeling with a small fluorescent dye [52].

#### FP tagging

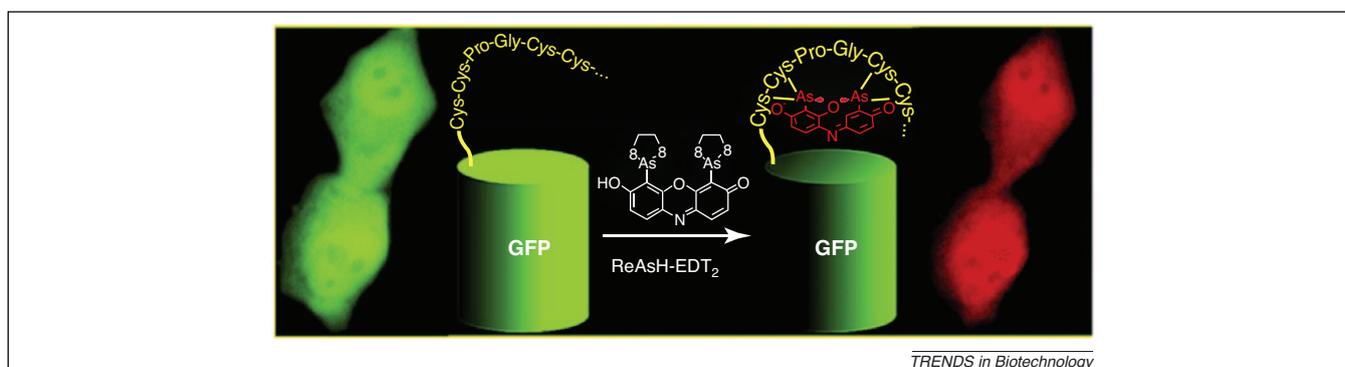
To incorporate a FP, the DNA sequence of the FP is inserted into the open reading frame of the target viral protein; labeling then occurs during virus replication in host cells. FP labeling has been used to study the dynamics of virus entry [15,52,53] to distinguish between complete and subviral particles after membrane fusion [54], and to visualize the replication cycle of viruses [55]. Unfortunately, the study of viral assembly is often difficult using FPs as they are usually overexpressed in infected cells, which

results in a considerable amount of background signal. However, various approaches including instrumentation (two-photon laser scanning microscopy, time-resolved fluorescence microscopy), chemical methods (autofluorescence quenching) and molecular approaches (different mutants, promoters, varying ratio of wild type and FP) can be used to increase signal to noise ratio [56].

#### Chemical labeling

Viral structures can be chemically labeled in purified form and used for viral entry, transport and assembly; and can also be labeled using specific dyes after infection to study replication [52]. Chemical labels can be covalently attached or non-covalently associated with the target protein. For example, capsids of purified, non-enveloped viruses can be covalently labeled with amine-reactive dyes (e.g. Alexa) [52], whereas the outer membrane of purified enveloped viruses can be labeled with lipophilic dyes and analogs [57]. Although labeling of viral structural proteins enables the tracking of individual virus particles in living cells, there are certain limitations. First, because of the smaller structure of the viruses, a limited number of fluorescent chemical labels can be attached to a virus particle without causing self-quenching effects or affecting viral infectivity [57]. Second, because the cells also fluoresce, it can be difficult to distinguish between the signal from a single virus particle and the background fluorescence of a cell; however, the use of red fluorescent dyes, such as Cy5, Alexa 647, and 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate and red fluorescent proteins, such as mCherry, can be used to negate the background signal problem, because the cell autofluorescence background levels tend to be weaker at longer excitation wavelengths.

Metabolic labeling can be used to label viruses during viral replication. This approach uses cell-permeable biarsenical dyes, which can specifically bind to viral proteins that contain tetracysteine (TC) peptide sequences. A small TC tag that contains two pairs of cysteines held in a hairpin configuration (e.g. CCPGCC) can be genetically engineered into the protein of interest [58], which then specifically reacts with membrane-permeable biarsenical compounds that selectively fluoresce when covalently bound to the



**Figure 3.** Method for site-specific fluorescent labeling of recombinant proteins that contain TC peptide sequences, using cell-permeable biarsenical dyes, in living cells. The sequence Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa is a non-cysteine amino acid, is genetically fused to or inserted within the protein, where it can be specifically recognized by a membrane-permeant fluorescein derivative with two As(III) substituents, FAsH or ReAsH, which fluoresces only after the arsenics bind to the cysteine thiols. Affinity *in vitro* and detection limits in living cells are optimized with Xaa-Xaa = Pro-Gly, which suggests that the preferred peptide conformation is a hairpin rather than the previously proposed  $\alpha$ -helix. Reproduced with permission from [58].

cysteine pairs (Figure 3). This genetic tag is relatively small and simple; thus, it can be placed into target proteins with minimal disruption. Furthermore, the cysteine-based structure can form even under detergent-based denaturing conditions, which indicates that the dye-binding sequence does not require extensive structure for activity [58]. Therefore, the TC tag is competent to bind biarsenical dyes almost immediately after translation and can fluoresce rapidly.

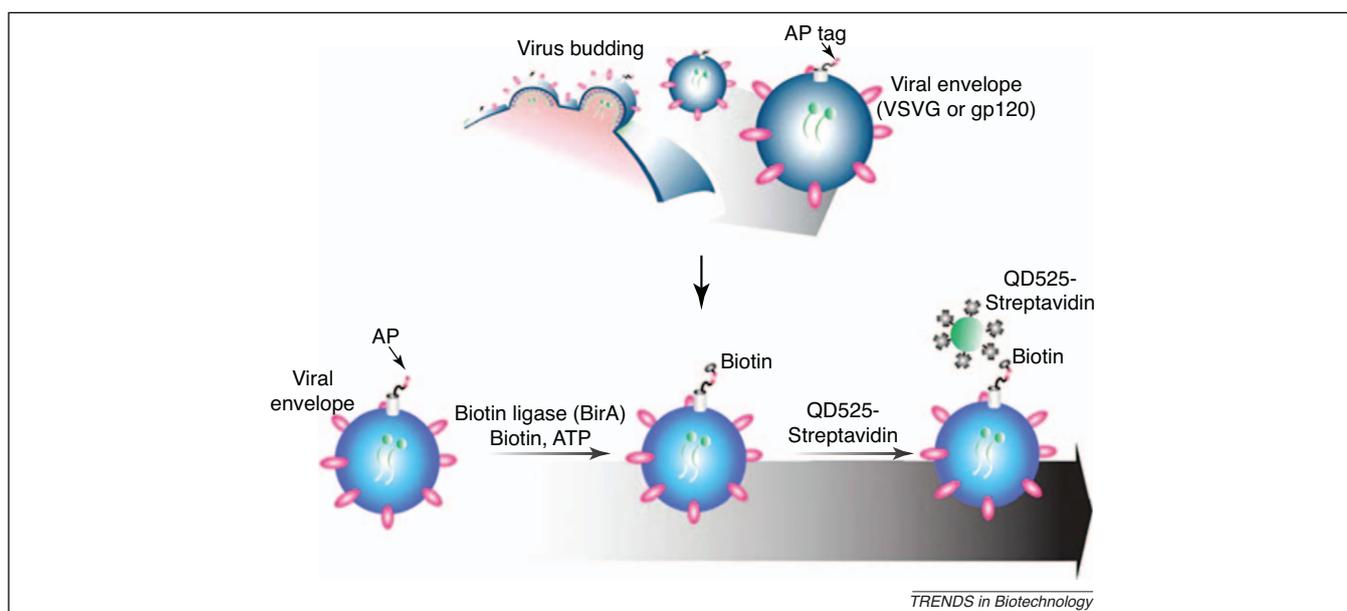
An additional advantage is the availability of two colors of biarsenical reagents, FAsH and ReAsH, which fluoresce green and red, respectively. This allows for examination of accumulation of nascent protein by labeling the existing target protein in the cell with one color, and then labeling newly synthesized protein with the other [58]. This approach has been used to examine the localization of HIV-1 Gag inside cells, and to identify the trafficking patterns of newly synthesized Gag by fluorescently labeling HIV-1 Gag [59]. As an additional example, the challenge of generating replication-competent fluorescent influenza A virus is its segmented genome; this has been overcome by introducing the TC biarsenical labeling system into influenza virus to label viral protein fluorescently in the virus life cycle [60]. With biarsenical labeling, the engineered replication-competent virus allows the real-time visualization of nuclear import of the NS1 protein, which is involved in multiple functions during virus infection. These results have established biarsenical labeling as an important method to study viral protein–host factor interactions during virus infection.

Good photostability of fluorophore labels is desirable for the continuous tracking of individual viruses. QDs are ideal for single-virus trafficking and imaging because they exhibit remarkable photostability (retain 97% of initial intensity compared to 55% for organic dyes at the end of 3 min illumination) [61] and brightness (molar extinction coefficient is 10–100× that of organic dyes) [62]. Successful

examples of tagging viruses with QDs include: (i) the use of 2–3 antibody layers (a virus-specific primary antibody, followed by a biotin-labeled secondary antibody and a streptavidin-conjugated QD) [47,63]; (ii) encapsulation of QDs in viral capsids [64]; and (iii) covalent linkage of QDs to viral capsids [65,66]. However, these labeling schemes probably affect the normal properties of viruses, as well as their interactions with the host [52]. Thus, a new method to label live and membrane-enveloped viruses site-specifically using QDs has been developed [12]. The strategy is to incorporate a 15-amino-acid biotin acceptor peptide tag onto the surface of a virion, followed by modification of the peptide tag with biotin ligase, to introduce the biotin moiety to the viral surface. By means of the tight interaction between biotin and streptavidin ( $K_d = 10^{-13}$  M), the addition of streptavidin-conjugated QDs allows the site-specific labeling of viral particles with QDs (Figure 4). The authors were able to study the kinetics of internalization of the recombinant lentivirus enveloped with vesicular stomatitis virus glycoprotein into the early endosomes [12], and to perform live-cell imaging of viral trafficking to the Rab5<sup>+</sup> endosomal compartments. The utility of this technique has also been used to study the molecular mechanisms of viral entry. The enhanced brightness of QD-labeled HIV particles has enabled the detection of single virions on the surface of target cells that express either CD4/CCR5 or DC-SIGN [12], and detailed tracking of viral internalization. These studies have demonstrated the potential of QD-based probes for visualization of viral and host structure interactions.

#### Viral proteases as the probing target

For many viruses, the introduction of thousands of virus particles is required to infect a single cell, which makes it difficult to track an infectious versus non-infectious virus particle [67]. Viruses, such as picornaviruses, retroviruses, and caliciviruses, however, produce a polyprotein that is



**Figure 4.** Strategy for the site-specific labeling of enveloped viruses with QDs. Biotin acceptor peptide tag is incorporated onto the surface of viruses. The concentrated acceptor peptide-tagged viruses are biotinylated by adding biotin ligase, ATP, and biotin. Incubation with streptavidin-conjugated QDs allows the site-specific labeling of QDs to the biotinylated surface of viruses. Reproduced with permission from [12].

cleaved into individual proteins by virus-specific proteases. Thus, viral proteases could be a prime target for the detection of viral infection because within various viral families, the proteolytic cleavage event proceeds in a defined manner for production of infectious virus particles.

Immediately upon infection, the RNA genome is translated into a single polypeptide, which is subsequently cleaved by viral proteases to generate mature viral proteins, with high efficiency and specificity. Currently, to monitor the proteolytic event inside a host cell, one must engineer an FP pair that is linked by the target protease sequence of interest. Proteolytic cleavage of the target sequence can therefore be detected based on changes in the FRET using high-resolution live-cell imaging [68]. Several FRET reporter cell lines that express various FP pairs, such as CFP-YFP [69] and EGFP-DsRed2 [70], have been designed. FRET-based assays have been used for the rapid detection (within 7.5 h) of very low doses of infectious enteroviruses ( $\leq 10$  PFU) [69]. In addition, the FP pair can be used to probe the dynamic distribution of enterovirus protease in living cells [70]. Although most of the FRET analyses have been performed using fluorescence microscopy, flow cytometry has recently been used to provide automated analysis of fluorescent cells for rapid detection of viral infection and enumeration of infected cells [71]. However, the biggest challenge of this method is the development of a stable clone that expresses the fluorescent substrate for each protease, which is a time-consuming and intensive task because considerable optimization is required for the development of each stable clone. An alternative would be to deliver a synthetic FRET substrate that carries the specific proteolytic site of each protease into living cells using cell-penetrating peptides [72].

#### QD based FRET probes

One major limitation of conventional FRET substrates is the use of organic fluorophores or FPs as the label, which exhibit low resistance to photodegradation. As described in the earlier sections, QDs have the potential to circumvent these limitations owing to their excellent physical and optical properties. Recently, FRET-based bioassays have been successfully demonstrated using QDs as the energy donor and fluorescent dyes or quenchers as energy acceptors [72]. In addition, QDs attached to FPs have been successfully delivered into cells by using cell-penetrating peptides, such as TAT [73]. This novel delivery method, when combined with protease-specific FRET pairs, could provide a powerful means for rapid, real-time detection of viral protease activity in living cells, with high specificity and sensitivity. The feasibility of this approach has recently been demonstrated by generating a QD-modified, protease-specific protein module that can be used as a FRET substrate for probing *in vivo* HIV-1 protease activity [74].

#### Conclusion

Viruses are a major health threat and pose a great diagnostics challenge because of their ability to mutate rapidly. Traditional methods for detection of viral infection that rely on viral isolation and culture techniques might continue to be the standards used for detection of infectious viral particles. However, the emerging live-cell imaging

techniques highlighted in this review can potentially offer an improved understanding of virus-host interaction that is suitable for early detection and drug discovery. We believe further investigation of viral infection at the cellular level using non-invasive visualization methods based on advanced molecular probes could significantly improve our understanding of viral functions as well as clinical treatments.

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