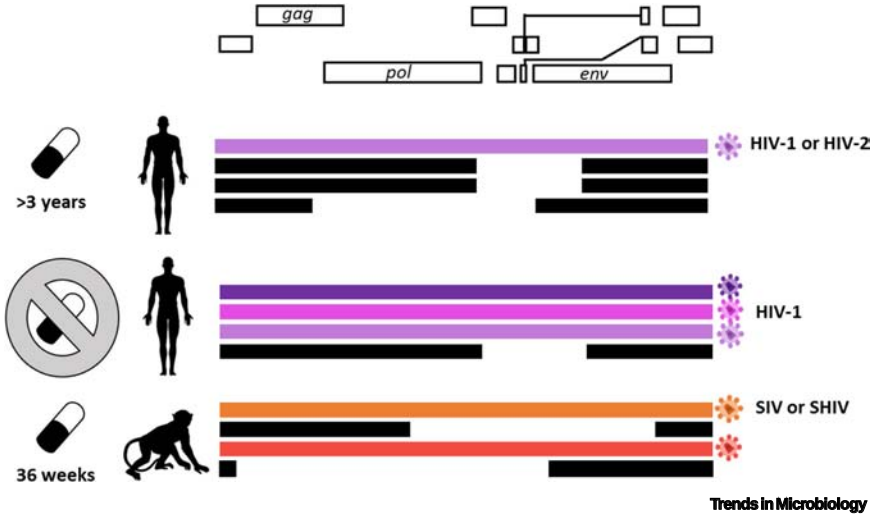

Figure 1. Comparison of Retroviral Reservoirs in Different Hosts and Treatment Contexts. The retroviral reservoirs in rhesus macaques infected with either simian immunodeficiency virus (SIV) or a chimera (SHIV) [4] as compared to humans infected with either HIV-1 [5–8] or HIV-2 [4]. The proportion of intact genomes found in the reservoir is similar between macaques infected with either SIV or SHIV [4], and is much higher in macaques than in humans after 3 years of antiretroviral therapy (ART) [5–8]. The macaque reservoir approximates the untreated or early post-treatment-initiation human HIV-1 CD4$^+$ T cell reservoir [6,8].

been employed to confirm this finding [10]. As such, the major differences in the intact reservoir found between macaques and humans delineated in the Bender study may be due to the fact that the reservoir in each host was observed at a different stage of decay. Follow-up analysis of the reservoir within macaques at >3 years post ART initiation is warranted.

While there seems to be striking differences between retroviral reservoirs in macaques and humans, the results of Bender *et al*. do not imply that the non-human primate model is inadequate for HIV-1 curative research. While a higher proportion of intact provirus was observed in macaques infected with either SHIV or SIV, the intact proviruses did not make up the majority of sequences observed in the reservoir. If the discrepancies in the proportions of intact virus between humans and macaques are indeed due to differences in the time at which the reservoirs were analysed, then this knowledge can be used when designing new non-primate studies. The value of an animal model is largely influenced by its ability to mimic human disease. Analysing the SIV or SHIV reservoir in macaques after they have been suppressed for 3 or more years would increase the likelihood that the reservoir in the non-human primate model is more reflective of the HIV-1 reservoir in humans on prolonged effective ART. While financially challenging, this would increase the usability and translatability of this model in preclinical trials of HIV-1 curative strategies.

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Forum

Imaging the Hepatitis B Virus: Broadcasting Live

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Although important breakthroughs in our understanding of the hepatitis B virus (HBV) life cycle have been made since the discovery of its main entry factor, the spatiotemporal dynamics of HBV–host interactions remains understudied. Here, we discuss recent advances and continuing challenges to image the HBV life cycle in live cells.

Context

Over 250 million people worldwide are chronically infected with HBV, significantly increasing risks of fibrosis, cirrhosis, and ultimately hepatocellular carcinoma. While an efficient vaccine

Table 1. Requirements for Studying the Spatiotemporal Dynamics of Single Viral Particles

	Requirements	Goals	Example of strategies to be further developed
Labeled particles	Small tag	Preserve particle integrity	Capsid-specific small molecules Genetic code expansion-based tags coupled with click chemistry Ligase-specific fluorescent peptides (sortase, LplA, ...)
	Biologically inert	Avoid aspecific interactions and prevent impact on particle infectiveness	
	Bright and photostable	Improve signal-to-noise detection, lower phototoxicity and cytotoxicity	
Cellular models	Expressing fluorescent proteins and markers	Visualize proteins of interest while preserving their function and expression levels	Primary cells 3D organoids Mouse models Human liver slices
	Three-dimensional	Better recapitulate the complex organization of epithelium architecture	
	Primary source	Obtain more physiological outcome	
Imaging	High speed	Capture rapid virus–cell interactions	Spinning disk Lattice light-sheet Intravital imaging Live 3D super-resolution
	Low photobleaching	Allow for longer imaging windows	
	High sensitivity	Detect single particles	
	High resolution	Better assess nanodomain organization	

exists, no treatment is available to fully eliminate the virus and, thus, HBV remains a global health threat (see HBV biology at-a-glance [1]). The HBV life cycle is complex, and very few tools were available to study the molecular mechanisms involved in virus–host interactions until recently. A synthetic myristoylated PreS1 peptide (myr-PreS1) derived from the HBs antigen of HBV, and currently under clinical trial [2], allowed for the identification of the sodium taurocholate cotransporting polypeptide receptor (NTCP) as a major entry factor for the virus, providing new cellular tools for researchers [3]. Although significant efforts to dissect molecular mechanisms involved in the HBV life cycle are ongoing, studies investigating the spatiotemporal dynamics of HBV–host interactions at the subcellular scale remain noticeably scarce. The visualization of HBV entry has been previously nicely reviewed [4], and here, we focus on recent developments and challenges associated with live-cell imaging approaches for studying the HBV life cycle.

Cellular Models for Imaging

The overexpression of NTCP in HepG2 cells provides a highly permissive cellular model for studying the full HBV life cycle *in vitro*. This cell line opens new avenues of research to unravel HBV–host interactions at the biochemical, molecular, cellular, and immunological levels. For live imaging, fluorescently-tagged proteins could be relatively easily expressed in these cells, allowing the realization of detailed studies investigating the dynamics of the molecular events occurring during HBV entry, replication, assembly, or budding. Yet, these HepG2-NTCP cells remain partially resistant to HBV, and the recent identification of the epidermal growth factor receptor (EGFR) as a second host entry factor may provide further strategies to improve the current cellular HBV models [5]. In the future, primary hepatocytes, organoids, or liver explants should be further implemented to gain more physiological insights into these processes, although technical limitations remain to be overcome beforehand (see Table 1).

Live Cell Imaging of HBV Cell Entry

For successful live imaging of viral cell entry, a major challenge to overcome resides in our ability to obtain fluorescently tagged HBV particles (Table 1). Indeed, HBV is particularly difficult to genetically modify because of the overlapping open reading frames composing its small 3.2 kb genome. Fluorescent yeast-derived HBsAg-expressing bionanocapsules were engineered to monitor HBV entry, but their biological origin and large size (≈ 117 nm) may not fully recapitulate the dynamic properties of bona fide HBV virions [6]. Another study used 'HBsAg particles' labeled with small fluorescent dyes (lipophilic or amine-reactive) to investigate the spatiotemporal dynamics of HBV entry into COS-7 cells. Over 20% of the tracked HBsAg particles were mobile, in an actin-dependent manner [7]. Although this study led the way to HBV live imaging, the importance of these observations must be balanced with the fact that COS-7 cells do not express NTCP and are not permissive to HBV infection. More recently, a detailed protocol was proposed by Konig and Glebe to image in live cells the early interactions between a PreS1 HBV envelope domain and host cells [8]. On one hand, they generated an HBV-permissive HepG2 cell line expressing NTCP fused to green fluorescent protein (GFP) in the C terminal. On the other hand, they used a fluorescently labeled myr-PreS1 as a surrogate for virus–receptor interactions. Although

the myr-PreS1 peptide lacks the morphological characteristics and heterogeneous composition of a complete enveloped HBV particle, this approach has the advantage of giving access to important information regarding the internalization dynamics of an analog of the antiviral drug Myrcludex B. In this study, live imaging analyses highlighted that myr-PreS1 was attaching only to NTCP-positive cells and that it cointernalized with NTCP in a temperature-dependent manner. Further mechanistic insights indicated recently that PreS1-dependent NTCP oligomerization facilitates successful HBV internalization (but not attachment) [9]. Together, these studies provide novel spatiotemporal information regarding the interactions between an HBV surface peptide and its viral receptor at the surface of target cells.

To address the dynamic behavior of complete infectious HBV particles, innovative strategies for labeling the virions should be elaborated to overcome current technical limitations (Table 1). Indeed, a major drawback of all imaging approaches aiming at studying virus entry is the incapacity to determine whether the tracked particles correspond to actual infectious viruses. In the case of HBV, <10% of the core-positive HBV particles are likely to correspond to infectious viruses, while noninfectious subviral particles (SVPs), deprived of a viral DNA, are highly over-represented (>1000-fold). Ideally, one would need to colabel HBsAg, HBc, and viral DNA on single viral particles in live cells, without affecting their infectiveness – a technically challenging goal to add to the current limitations of our models (Table 1).

HBV Core Dynamics

A stable cell line was recently derived from the HBV-producing HepAD38 cells, expressing a fluorescent mNeonGreen protein (≈ 2.5 times brighter than enhanced green fluorescent protein (EGFP)) in the N terminus of the HBV core linked by a flexible GGSGGSGGS sequence (mNG-HBc; Addgene plasmid #122202). This cell line, called HepAD38 mNG-HBc, secretes infectious HBV particles that incorporate both wild-type and fluorescent core proteins [10]. Three-dimensional live-cell imaging of HepAD38 producer cells showed that HBV mNG-HBc clusters, which may correspond to virus-assembly sites, were enriched in DNAse I proteins. These spatiotemporal observations highlighted the transient and dynamic codistribution of the DNAse I-HBc complex, although further mechanistic studies would be useful to determine how DNAse I and other host proteins are enriched at HBV assembly sites.

Although mNG-HBc-containing viruses remain infectious, the large tag (27 kDa) may still affect HBV genome packaging and virus assembly. Two other strategies using smaller tags have been proposed. First, insertion of an 18-amino acid tetracysteine tag allowed the generation of replication-competent fluorescent particles that fully retained infectiveness [11]. This method has been successfully used to tag other viruses, displaying good imaging properties (high labeling efficiency and brightness and low photobleaching), but the arsenic-derived labeling reagents showed some cytotoxicity and nonspecific labeling. Another original method was proposed, consisting of the detection of the HBV core using fluorescent small molecules that bind specifically to the core [12]. This capsid-specific molecular tag, linked to a permeable dye, would likely represent the least intrusive way to label the HBV core (<2 kDa) to perform live-cell imaging under the most physiological conditions.

Concluding Remarks

In conclusion, the eagerness of the community to thoroughly decipher the HBV–host interactions resulted in exciting breakthroughs that are fostering research development. Studying HBV in live cells had been challenging for years, but the novel biological tools and advanced imaging technologies available now are speeding-up the pace of research in the field. Yet, researchers are still facing technical barriers to follow HBV infectious particles without perturbing their biochemical properties (Table 1). Moreover, HepG2 cells cultured as a 2D monolayer currently represent the most suitable model for monitoring HBV infection in live cells so far, but future work using primary human hepatocytes, organoids, or fresh human liver explants will be required to better assess the HBV spatiotemporal dynamics under more physiological conditions. Together, unveiling the molecular mechanisms involved in the HBV life cycle using live-cell imaging strategies (to complement other approaches) is in reach and should represent an attractive approach in the coming years, while technical barriers are alleviated.

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