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## Imaging the Hepatitis B Virus: broadcasting live

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### Keywords

Live cell imaging; spatiotemporal dynamics; intracellular trafficking; virus entry; high-resolution microscopy

### Abstract

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 the hepatitis B virus (HBV), significantly increasing risks of fibrosis, cirrhosis, and ultimately hepatocellular carcinoma. While an efficient vaccine 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 co-transporting 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 remains noticeably scarce. The visualization of HBV entry has been previously nicely reviewed [4], and here, we will focus on recent developments and challenges associated to live cell imaging approaches to study the HBV life cycle.

## Cellular models for imaging

The overexpression of NTCP in HepG2 cells provides a highly permissive cellular model to study 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 fluorescently tag the HBV particle (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 ( $\approx 117$  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 in 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 König & Glebe to image in live cells the early interactions between a PreS1 HBV envelop domain and host cells [8]. On one hand, they generated a HBV-permissive HepG2 cell line expressing NTCP fused to GFP in 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 to give 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 co-internalized 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]. All together, these studies provide novel spatiotemporal information regarding the interactions between a 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 to label 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 non-infectious subviral particles (SVPs), deprived of a viral DNA, are highly over-represented (> 1000-fold). Ideally, one would need to co-label 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 ( $\approx$  2.5 times brighter than 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 incorporated 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 co-distribution 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 a 18-amino acids 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 non-specific labeling. Another original method was proposed, consisting of the detection of 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 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 is 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 to monitor 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 (in complement to other approaches) is in reach and should represent an attractive approach in the coming years, while technical barriers get alleviated.

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**Table 1.** Requirements to study the spatiotemporal dynamics of single viral particles

	Requirements	Goals	Example of strategies to be further developed
<b>Labeled particles</b>	Small tag	Preserve particle integrity	<ul style="list-style-type: none"> <li>- Capsid-specific small molecules</li> <li>- Genetic code expansion-based tags coupled with click chemistry</li> <li>- Ligase-specific fluorescent peptides (sortase, LplA, ...)</li> </ul>
	Biologically inert	Avoid aspecific interactions and prevent impact on particle infectiveness	
	Bright & photostable	Improve signal-to-noise detection, lower phototoxicity and cytotoxicity	
<b>Cellular models</b>	Expressing fluorescent proteins and markers	Visualize proteins of interest while preserving their function and expression levels	<ul style="list-style-type: none"> <li>- Primary cells</li> <li>- 3D organoids</li> <li>- Mouse models</li> <li>- Human liver slices</li> </ul>
	Three-dimensional	Better recapitulate the complex organization of epithelium architecture	
	Primary source	Obtain more physiological outcome	
<b>Imaging</b>	High speed	Capture rapid virus-cell interactions	<ul style="list-style-type: none"> <li>- Spinning disk</li> <li>- Lattice light-sheet</li> <li>- Intravital imaging</li> <li>- Live 3D super-resolution</li> </ul>
	Low photobleaching	Allow for longer imaging windows	
	High sensitivity	Detect single particles	
	High resolution	Better assess nanodomain organization	