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Live Cell Imaging of Hepatitis C Virus Trafficking in Hepatocytes

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Abstract

Standard fixed cell confocal microscopy is inherently limited in visualizing dynamic processes involving two- and three-dimensional movement. To overcome these limitations, live cell imaging approaches have been developed to study hepatitis C virus (HCV) entry, replicase protein trafficking, virion assembly, and egress. These studies have relied on fluorescent labeling of viral proteins by epitope tag insertion, genome labeling via nucleophilic dyes, or using lipophilic dyes to label the virion envelope. In this method review, we describe two approaches to study HCV virion trafficking in live cells. Lipophilic labeling of the envelope allows for study of the early events (through virion uncoating/fusion) in the HCV lifecycle. Tetracysteine (TC) tag insertion into the capsid protein permits study of virion assembly and capsid trafficking via binding of a fluorogenic biarsenical dye.

Keywords

Hepatitis C virus; Fluorescent labeling; Live cell imaging; Viral entry; DiD; Tetracysteine tag; TC-core

1 Introduction

HCV, a member of the *Flaviviridae*, is a small, single-stranded positive-sense virus of roughly 9600 nucleotides. Initial investigations into HCV protein dynamics focused on NS5A, in part because it is the only HCV gene identified that tolerates large insertions, such as GFP fusions. NS5A-GFP was shown to partially localize to replication compartments. Live cell imaging demonstrated two dynamic groups—larger, less motile foci, and smaller, motile puncta [1, 2]; such movement could be reduced by NS5A inhibitors [3].

Advances in cell culture systems and infectious viral strains have allowed for elucidation of all aspects of the viral lifecycle [4–7]. Live cell imaging of virion trafficking was hindered by the inability to generate infectious HCV containing fluorescent protein fusions with its structural genes. This hurdle has been cleared using two distinct approaches: lipophilic dyes to study HCV entry and small tetracysteine tags in the capsid to study HCV assembly and egress [8–10].

HCV is enveloped by a highly lipidated bilayer. For two other enveloped viruses, influenza A virus and dengue virus, protocols were developed wherein DiD, a membrane-permeable lipophilic dye, is used to label the envelope [11–14]. The fluorogenic DiD (1,1'-di-octadecyl-3,3',3'-tetramethylindodicarbocyanine) readily intercalates viral envelopes without compromising infectivity. These fluorescently labeled virions were then used to image viral entry. The limitation of this approach is that viruses, such as HCV, with poor

specific infectivity (i.e., 1 in ~2000 HCV virions produced in cell culture is infectious) cannot be studied with confidence because the vast majority of the particles visualized will not lead to a productive infection.

We overcame this limitation by taking advantage of the observation that infectious HCV is highly lipidated and has a different buoyant density than noninfectious HCV [15]. In our protocol, several rounds of purification ensure specific labeling of highly infectious virus with a specific infectivity of ~10 [8]. Characterization of the DiD-HCV particles showed that >95% of them colocalize with the capsid and E2, DiD-HCV could be immunodepleted with anti-E2 antibodies, and that DiD-HCV uncoating required the HCV receptor CD81 and acidic pH, as does bona fide HCV entry [8]. The study used these DiD-labeled particles to examine the requirements of HCV entry, using live cell imaging to investigate DiD-HCV association with its receptors, actin, its internalization pathway, and subsequent endosomal trafficking. Sainz et al. used DiD-HCV to study the roles and requirements of cellular proteins in regulating HCV entry [16]. Although this imaging protocol is highly reproducible, the specificity of DiD labeling and specific infectivity of the labeled inoculum should be confirmed for each preparation.

We and others adopted the TC tag system for labeling viral core proteins in an infected cell [9, 10]. TC motifs (CCPGCC) allow for binding of a small fluorescein derivative, FIAsh (fluorescein arsenical hairpin binder) or ReAsH (resorufin arsenical hairpin binder) [17]. The TC-core protein was first used to study the dynamics of core recruitment to lipid droplets [10]. Our group used a virtually identical TC-core HCV to characterize viral egress. The trafficking of TC-core with fluorescently labeled secretory components showed that HCV is released from the cell via various components of the secretory pathway, including the Golgi, recycling endosomes, and VAMP1 vesicles [9]. Importantly, processive TC-core movements require NS2, which is essential for virion assembly. Thus, there is confidence that motile TC-core is indeed an assembled nucleocapsid. The TC tag was also inserted into the nonstructural NS5A protein, looking at the requirements for its trafficking and localization [1]. Finally, the TC-core protein can be used to assay cell–cell spread, visualizing the transfer of TC-core puncta from producer to recipient cells [18].

Here we provide methods to visualize HCV virions via both DiD and the TC tag. The procedures will detail virus concentration, DiD-labeling and purification for enhanced infectivity, TC-core HCV staining, imaging setup, and basic guidelines for live cell imaging and analysis of these fluorescent viruses (Fig. 1).

2 Materials

HCV stocks are generated in Huh-7.5 cells or related derivatives by standard protocols [19]. Ideally, viral stocks should have a starting TCID₅₀/mL (preconcentration) of at least 7×10^5 – 3×10^6 .

2.1 Virus Concentration: 40% PEG 8000

1. Double distilled water (ddH₂O).

2. Phosphate-buffered saline (PBS, 10×): 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄.
3. PEG 8000.
4. Dark-colored bottle (or bottle covered in foil).
5. Stir bar.
6. Magnetic stir plate.
7. Sterile filter unit: bottle top filter, bottle.
8. 100 mL graduated cylinder.
9. Aluminum foil.
10. High-speed centrifuge tubes with sealing cap.
11. High speed centrifuge.
12. 50 mL conical tubes.
13. 10% DMEM: DMEM, 10% fetal bovine serum, 1% nonessential amino acids, 1% penicillin–streptomycin.

2.2 DiD-HCV Labeling

1. 1.5 mL centrifuge tubes.
2. DiD lipophilic tracer or related lipophilic dyes, e.g., DiI, DiO (ThermoFisher Scientific).
3. Shaker/rotator.
4. OptiPrep (60%, w/v, solution of iodixanol in water; Sigma).
5. Sterile ddH₂O.
6. Gradient maker: 20 mL total volume (10 mL per side).
7. Open top polyallomer ultracentrifuge tubes (14 × 89 mm).
8. Ultracentrifuge, SW 40Ti rotor and swinging buckets.
9. Ring stand and clamp.
10. Sterile needle.
11. 24-well plate.
12. Amicon Ultra centrifugal filters (0.5 mL, Ultracel 100 k).
13. 10% DMEM: *See* Subheading 2.1.

2.3 Imaging Setup

1. 35 mm imaging dishes, 17 mm thickness (must have interlocking lids to prevent spillage).

2. Type 1 Collagen solution, 1%.
3. Sterile water.
4. 10% DMEM: *see above*.
5. Sterile PBS (for cell culture).
6. Imaging media: DMEM-F12 (no phenol red), 10% fetal bovine serum, 1% nonessential amino acids, 1% penicillin–streptomycin, 50 μ M HEPES.
7. Parafilm.

2.4 TC-Core HCV Labeling

1. Collagen-treated imaging dishes (*see Subheading 2.3*).
2. Cells infected with TC-core HCV (*see Note 1*).
3. Opti-MEM.
4. 1,2-Ethanediol (EDT): 100 mM, in DMSO (*see Note 2*).
5. FLaSH or ReAsH Biarsenical dye (ThermoFisher Scientific): 1.25 μ M final concentration, diluted in Opti-MEM (*see Note 3*).
6. 1 $\times$ BAL Wash Buffer: 100 $\times$ BAL buffer (ThermoFisher Scientific), Opti-MEM, 500 μ M EDT.
7. Imaging media: (*see Subheading 2.3*).
8. Parafilm.

2.5 Imaging

1. Olympus DSU Spinning Disc Confocal microscope with 100 $\times$ NA 1.45 oil objective, Hamamatsu back-thinned EM-CCD high speed/sensitivity camera.
2. Heated stage with imaging dish insert.
3. Slidebook software (Intelligent Imaging Innovations, Inc.).
4. Microscope immersion oil (for objective).
5. 70% Ethanol in a spray bottle.
6. Cling wrap.

2.6 Image Analysis

1. ImageJ (NIH).
2. RGB profiler plugin (Christophe Laummonerie).
3. Colocalization highlighter (ImageJ).
4. JACoP plugin [20].
5. Multiple kymograph plugin (ImageJ).

6. Manual tracking plugin (ImageJ).

3 Methods

All work with infectious virus (with the exception of live cell imaging) is done in BSL2 cell culture hoods. Materials coming into contact with infectious virus should undergo proper decontamination procedures as detailed in institutional biosafety committee (IBC) protocols. Live cell imaging is done with an interlocking imaging dish (*see* Subheading 2.3), then wrapped with Parafilm. The prepared imaging dish is then transported to imaging facilities in a sealed Styrofoam container to prevent infectious virus spills. Imaging can be performed in a BSL2 microscopy facility in the USA. A bottle of 70% ethanol should also be kept at the imaging facility in case of accidental spills.

3.1 PEG Concentration

1. Combine 40 mL ddH₂O and 10 mL 10× PBS in a dark-colored bottle. Add 40 g PEG 8000. Shake to combine, then add stir bar. Mix on magnetic stir plate until powder has dissolved (30–45 min) (*see* Note 4). Refrigerate until no bubbles remain (*see* Note 5).
2. Using 5 mL ddH₂O, prewet the filter. Pour the PEG solution into the graduated cylinder, being careful to avoid bubbles. Note the volume of PEG, then pour into the filter unit. Add enough ddH₂O to the graduated cylinder to bring the total volume up to 95 mL (The final volume will be 100 mL). Swirl the graduated cylinder to dissolve any residual PEG solution, then pour into the filter unit. Cover filter unit with foil and apply vacuum to filter the PEG (*see* Note 6).
3. Once filtered, wrap the bottle of 40% PEG 8000 in foil and store at 4 °C (*see* Note 7).
4. In high speed centrifuge tubes, combine virus stock and 40% PEG 8000 at a 4:1 ratio (bringing the final PEG concentration to 8%). Cap and invert 8–10 times to mix. Cover in aluminum foil and incubate at 4 °C overnight.
5. Centrifuge tubes at $9000 \times g$ for 20 min, 4 °C (*see* Note 8).
6. Pipette off all but roughly 15 mL of the supernatant (*see* Note 9). Resuspend the pellet and transfer to a 50 mL conical tube.
7. Spin down again, $9000 \times g$ for 10 min, 4 °C.
8. Pipette off all supernatant. Resuspend in serum free DMEM at 1/100th of initial concentration. Wrap in foil and store at 4 °C (*see* Note 10).

3.2 DiD Labeling

1. Prechill the rotor and buckets.
2. In a 1.5 mL centrifuge tube, combine 1 mL of concentrated virus with 5 µL DiD dye. Incubate on a rotator or shaker, protected from light, for 90 min at 4 °C.

3. Using a gradient maker, prepare a 10–60% OptiPrep gradient in the ultracentrifuge tubes—1 tube per 1 mL of labeled, concentrated virus. Leave roughly 2–3 cm space at the top of the tube. Chill until use.
4. Load 1 mL of labeled virus onto the top the ultracentrifuge tube, being careful not to disturb the gradient. Spin tubes in the ultracentrifuge at $142,000 \times g$ for 16.5 h at 4 °C (*see* Note 11).
5. After spinning, secure the tube in a ring stand. Puncture the bottom of the tube with a needle and separate into 1 mL fractions using a 24-well plate (*see* Note 12).

3.3 Infectivity and Labeling Specificity of DiD

1. Remove two 50 μ L aliquots from each fraction. One aliquot is used to determine density, then TRIzol-LS extracted for RNA. These RNA extracts are assayed for RNA levels via qPCR. The second aliquot is titered for infectious virus (*see* Note 13). Determine the specific infectivity of each fraction, measured as the ratio of RNA level to infectious virus particles (*see* Notes 14 and 15).
2. After selecting the fraction with lowest (best) specific infectivity, use an Amicon filter to remove the OptiPrep solution: Add 0.5 mL of the selected fraction to each Amicon filter. Spin at $14,000 \times g$ for 20 min at 4 °C. Invert the filter into a new tube and spin at $2000 \times g$ for 2 min at 4 °C. Pool the concentrated virus and resuspend in 1 mL 10% DMEM (or alternate concentration of FBS).

3.4 Imaging Setup

3.4.1 DiD Imaging Setup

1. Prepare collagen imaging dishes (in sterile tissue culture hood): coat dishes with 500 μ L of collagen solution for 3–4 h at 37 °C. Aspirate excess collagen from plate and rinse twice with sterile water. Sterilize imaging dishes via overnight exposure to UV light in the tissue culture hood.
2. The day before imaging, seed cells in 10% DMEM so that they are roughly 70% confluent at the time of imaging (*see* Note 16). Just prior to imaging, wash with PBS and replace with 500 μ L of cold imaging media (*see* Note 17).
3. Add 100 μ L DiD labeled virus to imaging dish and incubate on ice for 1 h, protected from light (*see* Note 18). Replace media with 500 μ L fresh imaging media (optional). Wrap the edge of the dish in Parafilm. Cells should be immediately transported to the imaging facility for visualization.

3.4.2 TC-Core HCV Labeling and Imaging Setup

1. Prepare collagen-treated dishes and cell monolayers as in Subheading 3.4.1.
2. Prewarm Opti-MEM, $1 \times$ BAL wash buffer, and imaging media. For staining, wash cells with Opti-MEM. Aspirate, then replace with 400 μ L FIAsH or ReAsH dye labeling solution. Incubate 30 min at 37 °C (*see* Note 19).

3. Remove dye and wash cells for 5 min at 37 °C with the 1× BAL wash buffer. Repeat three more times. Rinse cells for 5 min at 37 °C with Opti-MEM.
4. Replace with imaging media (*see* Note 20). Wrap the edge of the dish in Parafilm and incubate cells at 37 °C until ready to image. Cells should be visualized within 2 h of staining.

3.5 Live Cell Imaging

1. *See* Note 21 for safety considerations.
2. Preheat stage. If uncovered, set to 39° (so that center of the dish is 37 °C). If enclosed/incubator type set-up, then 37 °C is sufficient.
3. Image acquisition is dependent on the microscope used. Our setup uses Slidebook software for image acquisition, with intensification set to 255 (maximum). Live cell imaging is carried out by acquiring sequential static images of labeled virus and cellular markers of interest.

3.5.1 DiD Detection—DiD is detected with a Cy5 filter set (excitation: 635/20; emission: 685/40); DiO is detected with a EGFP filter set (excitation: 480/25; emission: 525/40); DiI is detected with the Cy3 filter set (excitation: 555/28; emission 617/73). DiD images are usually taken every 5–10 s. At 100–200 ms exposure times, 100 frame time courses are readily achievable (*see* Note 22).

3.5.2 TC-Core Detection—FlAsH is detected with an EGFP filter set (excitation: 480/25; emission: 525/40). ReAsH can be visualized with a DSReD filter set (excitation: 565/25; emission 620/60). Biarsenical dyes photo-bleach quickly during live cell imaging. For confocal imaging, 100–200 ms every 1–10 s maximizes data capture while minimizing photobleaching. Nonspecific labeling of organelles (e.g., mitochondria) can often occur. This can be alleviated by increasing EDT concentration or using additional wash steps.

3.6 Image Analysis

1. Images/videos are processed using ImageJ. Channels can be separated, then processed via the “subtract background” tool, followed by the “smooth” function. Channels can then be merged and contrast adjusted (especially for DiD/TC-core puncta, as they tend to be less bright than cellular markers).
2. Colocalization: For individual frames, colocalization between the HCV particle and other cellular/viral proteins can be determined using several plugins.

RGB profiler plugin can be used to generate an intensity profile of each channel along a selected line.

Colocalization highlighter plugin: For simple colocalization analysis, split channels can be analyzed for colocalization of particles given a set threshold.

JACoP: A more sophisticated plugin, this allows analysis via various methods, including Pearson’s and Manders coefficients.

3. Montages/kymographs: Montages can be created from either single or multiple channels via the “Make Montage” command. Kymographs are produced with the “Multiple kymograph” plugin.
4. Calculating distance: Following generation of a kymograph, the “Manual tracking” plugin can be used to calculate the distance from origin traveled as well as velocity (distance vs time).

4 Notes

1. TC-Core HCV is produced by inserting a tetracysteine tag (CCPGCC) into the HCV core protein after amino acid 3 [9]. In vitro transcribed TC-Core HCV RNAs are electro-porated into cells and allowed to propagate for 48 h following electroporation, after which they can be seeded into imaging dishes. Imaging is performed at roughly 72 h post-electroporation. Alternatively, stocks of HCV TC-core virus can be prepared beforehand and used to infect cells; TC-core imaging can be performed using these infected cells.
2. Keep at room temperature with desiccator. Stock is usable for 2–3 weeks.
3. Prepare in tissue culture hood. Prepare only what is needed for staining. Optionally, the solution can be spiked with 10 μ M EDT.
4. After 15–20 min, check to make sure that no clumps of PEG remain. If so, shake until clumps have broken up.
5. To maximize titer, it is best to chill reagents before adding them to HCV. Refrigeration during this step is not required, but saves time at the end of the process.
6. The PEG solution is quite thick. In order to speed up the process, make sure all connections are as tight as possible.
7. Stored at 4 °C and protected from light, PEG solution is stable for up to 4 days. However, we find it best to prepare the solution the same day as concentrating the virus.
8. It is essential not to disturb the pellet at this point. It will look white, somewhat fluffy, and run the length of the tube (often to the lip of the bottle).
9. Do not aspirate the supernatant, as it will clog the tube.
10. Pellet can be resuspended in alternate media compositions (serum-free) depending on the desired experiment. One can also resuspend in less media for a higher concentration, depending on the initial titer of virus (For DiD labeling, the TCID₅₀/mL of the concentrated virus should be roughly 3×10^8). At this stage, the pellet is very compact and difficult to resuspend. We recommend storing the concentrated virus in the conical tube so as not to lose any of the pellet. Additionally, a resting period of 30–60 min will allow the all the bubbles to dissipate in the resuspension so as to utilize all of the concentrated virus for the next stage.

11. Subsequent steps with DiD labeled virus are done in low light so as to preserve fluorescence.
12. Initial high density fractions may clog the puncture site. Wider gauge needles may help with this problem. Be aware that larger holes will lead to uncontrolled fractionation at lower densities. We prefer to use a 25 gauge needle (5/8 in. in length).
13. Protocols for qPCR and titering via limiting dilution are standard in the field [21, 22].
14. The specific infectivity of the virus should ideally be somewhere in the range of 10 (particles or RNA copies per infectious virus).
15. Additionally, a squash may be performed to ensure specific labeling of the virus, wherein DiD labeled particles are immunostained for components of the virion (e.g., core, E2) [8].
16. Often, cells have been previously transfected or transduced with a fluorescently tagged protein of interest to study cotrafficking. Alternatively, there are many commercially available organelle-specific dyes. These are labeled according to the manufacturer's instructions.
17. The imaging media can be made in bulk, but it is best to spike with HEPES the day of imaging. The cold imaging media is necessary to prevent endocytosis of the virus during the ice incubation phase.
18. Calculate the volume and/or resuspension of Amicon-purified virus based on titer of the fraction. An multiplicity of infection (MOI) of 0.5–1 will provide enough DiD particles for successful imaging. Higher MOIs enable a larger *n* value per experiment.
19. To minimize reagent requirements, use a silicone well insert. The insert is placed in the well after washing with Opti-MEM and before adding the dye. If using these inserts, incubate at 37 °C with 50 µL dye solution for 5 min. Replace the dye solution 3×, for a total of 20 min incubation.
20. If using the well inserts, add 500 µL warm imaging media around the insert to maintain temperature.
21. Always use gloves when handling the dishes at the imaging facility. However, make sure that such gloves do not come in contact with common use equipment (It is often useful to place cling wrap on the keyboard to minimize glove changing).

Following imaging, gather up used gloves, cling wrap, etc. to dispose of in the proper biohazard container. Wipe down everything with ethanol.
22. The HCV entry process occurs within 2 h of the temperature shift to 37 °C. The majority of DiD-labeled HCV particles internalize within 30 min of this shift. Roughly 50% of particles have undergone uncoating by 85 min post-temperature shift [8].

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A DiD-HCV Labeling

B TC-Core HCV Labeling

Fig. 1.

Workflow for the labeling and imaging of fluorescently labeled HCV. **(a)** DiD-labeled HCV: HCV is first PEG-concentrated, then labeled with the lipophilic dye, DiD. Following iodixanol gradient purification, each fraction is assayed for specific infectivity. The fraction with the best (lowest) specific infectivity is then concentrated/purified via Centricon filtration. One day prior to imaging, cells are plated onto imaging dishes. Before infecting, imaging media is added and then cells are incubated with DiD-HCV on ice. A series of images are then acquired every 5–10 s, with an exposure time of roughly 100 ms for DiD. Resulting images are then processed and analyzed with ImageJ. **(b)** TC-Core Labeled HCV: Cells are either infected with TC-Core virus or transfected with *in-vitro* transcribed TC-Core HCV RNA. The day before imaging, cells are seeded onto imaging dishes. Just before imaging, TC-Core is labeled with either FIAsh or ReAsH, then washed and replaced with imaging media. TC-core labeled HCV is then imaged every 1–10 s (at 100 ms exposure). Following acquisition, images are processed and analyzed with ImageJ plugins