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TITLE:

Single-cell multiplexed fluorescence imaging to visualize viral nucleic acids and proteins and monitor HIV, HTLV, HBV, HCV, Zika virus, and Influenza infection

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SUMMARY:

Presented here is a protocol for a fluorescence imaging approach, **m**ultiplex **i**mmunofluorescent **c**ell-based **d**etection of **D**NA, **R**NA, and **p**rotein (MICDDRP), a method capable of simultaneous fluorescence single-cell visualization of viral protein and nucleic acids of different type and strandedness. This approach can be applied to a diverse range of systems.

ABSTRACT:

Capturing the dynamic replication and assembly processes of viruses has been hindered by the lack of robust in situ hybridization (ISH) technologies that enable sensitive and simultaneous labeling of viral nucleic acid and protein. Conventional DNA fluorescence in situ hybridization (FISH) methods are often not compatible with immunostaining. We have therefore developed an imaging approach, **MICDDRP** (**m**ultiplex **i**mmunofluorescent **c**ell-based **d**etection of **D**NA, **R**NA and **p**rotein), which enables simultaneous single-cell visualization of DNA, RNA, and protein. Compared to conventional DNA FISH, MICDDRP utilizes branched DNA (bDNA) ISH

technology, which dramatically improves oligonucleotide probe sensitivity and detection. Small modifications of MICDDRP enable imaging of viral proteins concomitantly with nucleic acids (RNA or DNA) of different strandedness. We have applied these protocols to study the life cycles of multiple viral pathogens, including human immunodeficiency virus (HIV)-1, human T-lymphotropic virus (HTLV)-1, hepatitis B virus (HBV), hepatitis C virus (HCV), Zika virus (ZKV), and influenza A virus (IAV). We demonstrated that we can efficiently label viral nucleic acids and proteins across a diverse range of viruses. These studies can provide us with improved mechanistic understanding of multiple viral systems, and in addition, serve as a template for application of multiplexed fluorescence imaging of DNA, RNA, and protein across a broad spectrum of cellular systems.

INTRODUCTION:

While thousands of commercial antibodies are available to specifically label proteins via conventional immunostaining approaches, and while fusion proteins can be engineered with photo-optimized fluorescent tags for tracking multiple proteins in a sample[1], microscopic visualization of protein is often not compatible with conventional DNA fluorescence in situ hybridization (FISH)[2]. Technical limitations in simultaneous visualization of DNA, RNA, and protein using fluorescence-based approaches have hindered in-depth understanding of virus replication. Tracking both viral nucleic acid and protein during the course of infection allows virologists to visualize fundamental processes that underly virus replication and assembly[3-6].

We have developed an imaging approach, **m**ultiplex **i**mmunofluorescent **c**ell-based **d**etection of **D**NA, **R**NA, and **P**rotein (MICDDRP)[3], which utilizes branched DNA (bDNA) in situ technology to improve the sensitivity of nucleic acid detection[7-9]. In addition, this method utilizes paired probes for enhanced specificity. bDNA sequence-specific probes use branching preamplifier and amplifier DNAs to produce an intense and localized signal, improving upon previous hybridization methods that relied on targeting repeated regions in the DNA[9]. Infected cells in a clinical context often do not contain abundant viral genetic material, providing a commodity for a sensitive method for fluorescent nucleic acid detection in diagnostic settings. The commercialization of bDNA technology through approaches such as RNAscope[7] and ViewRNA[10] have filled this niche. The sensitivity of bDNA fluorescence imaging also has important utility in cell biology, allowing detection of scarce nucleic acid species in cell culture models. The vast improvement of sensitivity makes bDNA-based imaging methods suitable for studying viruses. A potential shortcoming, however, is that these methods focus on visualizing RNA or RNA and protein. All replicating cells and many viruses have DNA genomes or form DNA during their replication cycle, making methods capable of imaging both RNA and DNA, as well as protein, highly desirable.

In the MICDDRP protocol, we perform bDNA FISH for detection of viral nucleic acid using the RNAscope method, with modifications[7]. One of the major modifications to this protocol is optimization of protease treatment following chemical fixation. Protease treatment facilitates removal of proteins bound to nucleic acid to improve probe hybridization efficiency. Protease treatment is followed by incubation with branched oligonucleotide probe(s). After application of bDNA probe(s), samples are washed and subsequently incubated with signal pre-amplifier

and amplifier DNAs. Multiplexed in situ hybridization (ISH), labeling of multiple gene targets, requires target probes with different color channels for spectral differentiation[7]. Incubation with DNA amplifiers is followed by immunofluorescence (IF).

bDNA ISH imparts improvements in signal-to-noise by amplification of target-specific signals, with a reduction to background noise from non-specific hybridization events[7,11]. Target probes are designed using software programs publicly available that predict the probability of non-specific hybridization events, as well as calculate melting temperature (T_m) of the probe-target hybrid[7,11]. Target probes contain an 18- to 25-base region complementary to the target DNA/RNA sequence, a spacer sequence, and a 14-base tail sequence. A pair of target probes, each with a distinct tail sequence, hybridize to a target region (spanning ~50 bases). The two tail sequences form a hybridization site for the pre-amplifier probes, which contain 20 binding sites for the amplifier probes, which, in addition contain 20 binding sites for the label probe. As an example, a one kilobase (kb) region on the nucleic acid molecule is targeted by 20 probe pairs, creating a molecular scaffold for sequential hybridization with the preamplifier, amplifier, and label probe. This can thus lead to a theoretical yield of 8000 fluorescent labels per nucleic acid molecule, enabling detection of single molecules and vast improvements over conventional FISH approaches[7] (See **Figure 1A** for schematic of bDNA signal amplification). To set up probes for multiplexed ISH, each target probe must be in a different color channel (C1, C2, or C3). These target probes with different color channels possess distinct 14-base tail sequences. These tail sequences will bind distinct signal amplifiers with different fluorescent probes, thus enabling facile spectral differentiation across multiple targets. In the presented Protocol, **Table 4** in **Step 9,** provides further information on fluorescently labeling target probes. In addition, **Figures 2-3** provide examples of how we chose the appropriate Amplifier 4-FL (A, B, or C) (fluorescent probe & the final hybridization step) to achieve specific fluorescent labeling of multiple viral nucleic acid targets following HIV-1 and HTLV-1 infections.

We have demonstrated several applications of simultaneous fluorescence visualization of RNA, DNA, and proteins, observing critical stages of virus replication with high spatiotemporal resolution[3-5]. For example, simultaneous single-cell visualization of viral RNA, cytoplasmic and nuclear DNA, and protein have allowed us to visualize key events during HIV-1 infection, including following RNA containing cores in the cytoplasm prior to nuclear entry and integration of proviral DNA[3]. In addition, we have applied MICDDRP to characterize the effects of host factors and drug treatment on viral infection and replication[4,5]. In Ukah et al., we tracked reactivation of HIV-1 transcription in latency cell models treated with different latency-reversing agents to visualize HIV transcription and latency reversal[4]. In addition, MICDDRP can allow us to visualize phenotypic changes associated with antiviral inhibition attributed to small molecule treatment or host factor restriction. As a proof of concept to the robustness and broad applicability of our approach, we have demonstrated that we can use modifications of our protocol to efficiently label viral nucleic acid to follow infection not only in human immunodeficiency virus (HIV)-1, but also human T-lymphotropic virus (HTLV)-1, hepatitis B virus (HBV), hepatitis C virus (HCV), Zika virus (ZIKV), and influenza A virus (IAV). As the HIV-1 life cycle consists of both viral DNA and RNA species, we have performed the majority of our optimization of MICDDRP following HIV-1 replication kinetics. However, in addition, we have

demonstrated that we can track synthesis of different viral RNA transcripts of either or both sense (+) and antisense (-) strandedness in viruses such as ZIKV, IAV, HBV, and HCV to monitor viral transcription and replication[3-6]. The studies aim to improve the mechanistic understanding of several viral processes and serve as a guideline to implement this fluorescence imaging technology to a broad range of cellular models.

PROTOCOL:

1. Seed cells (Suspension *vs.* Adherent Cells) on coverslips or chamber slides (protocol presented uses coverslips).

1.1. Seeding of suspension cells

1.1.1. Prepare poly-D-lysine (PDL) coated coverslips (facilitates adherence of suspension cells to coverslip) by first incubating coverslips in ethanol (EtOH) for 5 minutes (sterilizes coverslips and removes any residue). Then wash 2x in phosphate buffered saline (PBS) before incubating coverslips in PDL (20 µg/mL) for 30 minutes (min) at room temperature (RT).

1.1.2. Remove PDL and wash 2x in PBS. Pellet cells (previously infected/treated based on desired imaging conditions) and resuspend 10^6 cells in 50 µL of PBS. Spot 50 µL of cells on glass (PDL-coated) and incubate at RT for 30 min.

1.2. Seeding of adherent cells

1.2.1. Culture cells on sterile coverslip placed in 6-well dish and infect cells with viral particles or treat with compound of interest. Allow cells to reach 50-70% confluency prior to sample preparation for imaging experiments.

2. Cellular fixation: To preserve cellular morphology for fluorescence imaging studies

NOTE: Keep 4% PFA and PBS at RT for at least 30 minutes before cellular fixation.

2.1. Aspirate the cellular media, wash cells on coverslips 3x in PBS, and fix cells in 4% paraformaldehyde (PFA) for 30 min. Aspirate PFA and wash cells in PBS 2x.

NOTE: Cells can now be dehydrated and stored, if the experimenter wishes to resume the experiment on another date. Fixed cells can be dehydrated in EtOH prior to storage.

2.1.1. Remove PBS following wash after cellular fixation and replace with 500 µL of 50% EtOH (v/v in water). Incubate at RT for 5 min.

2.1.2. Remove 50% EtOH and replace with 500 µL of 70% EtOH. Incubate at RT for 5 min.

2.1.3. Remove 70% EtOH and replace with 500 µL of 100% EtOH. Incubate at RT for 5 min.

2.1.4. Remove 100% EtOH and replace with fresh 100% EtOH.

NOTE: Dehydrated cells can be stored at -20 °C for 6 months. Seal plates with tape or parafilm prior to storage to prevent evaporation of EtOH.

2.1.5. Rehydrate cells to move onto multiplexed fluorescence labeling of cells/virus.

2.1.6. Remove 100% EtOH and replace with 500 µL of 70% EtOH. Incubate at RT for 2 min.

NOTE: Do not let cells dry out at any time. Always use enough solution to submerge all the cells.

2.1.7. Remove 70% EtOH and replace with 500 µL of 50% EtOH. Incubate at RT for 2 min.

2.1.8. Remove 50% EtOH and replace with 1x PBS.

NOTE: Prepare protease dilution, hybridization probes, and wash buffer in advance of protease treatment (**Step 4)** and target probe application (**Step 5**). Specific instructions on reagent preparation are presented at the beginning of each respective step.

[Place **Table 1** here]

3. Cell permeabilization: Increases access into the cell and cellular organelles for entry of large molecules (antibodies, nucleic acid hybridization probes)

3.1. Remove 1x PBS following cellular fixation or rehydration and replace with 500 µL of 0.1% (v/v) Tween-20 in 1x PBS. Incubate at RT for 10 min. Replace one by one. Wash once with 500 µL of 1x PBS and add fresh 1x PBS.

4. *Coverslip immobilization* on glass slide and *protease treatment* to remove nucleic acid binding proteins from fixed viral/cellular nucleic acid to improve hybridization efficiency

NOTE: Prepare the diluted Protease III (see specifics of reagent in **Table of Materials**) solution during cellular permeabilization. Let protease reach RT for 10 min before permeabilization.

4.1. Place a small drop of nail polish on a sterilized glass slide. Dry back (side with no cell layer) and place the edge of coverslip on the nail polish drop, with the side with the adhered cells facing upwards. Add few drops of PBS on the immobilized coverslip to prevent drying.

4.1.1. Draw a circle (about 3 mm away from the coverslip) around the perimeter of the coverslip now adhered to the slide using hydrophobic barrier pen (water-repellant pen that keeps reagents localized on cells).

4.2. Dilute Protease III in 1x PBS (100 µL/coverslip).

NOTE: Protease concentration may need to be adjusted depending on differences in cell types, probes, or target nucleic acid(s) through empirical optimization (See **Discussion**). For most efficient RNA/DNA labeling across different viral systems, we had success with the following dilutions presented in **Table 2** below with dilutions ranging from (1 to 2)-(1 to 15) (protease to 1x PBS).

[Place Table 2 here]

4.3. Decant the 1x PBS on the coverslip following immobilization and apply the diluted Protease III.

4.4. Incubate in a humidified oven at 40 °C for 15 min.

4.5. Decant protease solution and submerge slides in 1x PBS. Agitate with a rocking dish for 2 min at RT. Repeat wash with new 1x PBS.

4.5.1. For **only** DNA detection, wash samples three times with nuclease-free water for 2 min each, followed by incubation with 5 mg/mL RNase A diluted in PBS for 30 min at 37 °C.

4.5.2. Decant RNase A solution, and wash 3x for 2 min with ultrapure water. Continue with ISH of target probe(s).

NOTE: Hybridization buffer improves vDNA detection without affecting vRNA staining efficiency for the results presented (**Representative Results**). Dilute DNA Channel 1 (C1) probes 1:1 with hybridization probe. Dilute RNA C2 and C3 probes in hybridization buffer. The C2 and C3 probes used in our imaging studies are in 50X solutions (1:50; target probe **to** hybridization buffer).

4.6. Prepare hybridization buffer in nuclease-free water following the step-by-step procedure below:

4.6.1. In a 15 mL tube, add 700 µL of nuclease free-water, 300 µL of 50% (weight/volume (w/v)) dextran sulfate, 300 µL of 5 M NaCl, 125 µL of 200 mM sodium citrate (pH 6.2), and 375 mg (powder) of ethylene carbonate.

4.6.2. Mix well using a vortex to dissolve ethylene carbonate (ensure all powder is dissolved and clear solution).

4.6.3. Add 25 µL of 10% (volume/volume (v/v)) Tween-20 and enough nuclease-free water to complete 2.5 mL (2x solution).

NOTE: The recipe for the hybridization buffer presented can be found in **Table 3** below. Solution is stable for a week. Ensure sufficient mixing of Tween-20 detergent, while being careful to prevent bubbling of solution.

[Place **Table 3** here]

5. Incubation with DNA/RNA target hybridization probes: Target oligonucleotide probes bind to region(s) of interest, creating a molecular scaffold for pre-amplifiers, amplifiers, and fluorescent probes to bind.

NOTE: Warm DNA/RNA probes at 40 °C for 10 min (during Cell Permeabilization) and cool down to RT for at least 10 min, if no RNase-treatment is included. If RNase-treatment or further sample treatment is needed prior to target probe hybridization, warm probes accordingly. Spin down C2 and C3 probes after warming and dilute in hybridization buffer. After dilution, C2 and C3 probes can be briefly warmed. Warm 50x wash buffer (See **Table of Materials** for more detail) at 40 °C for 10-20 min and dilute to 1x in molecular biology grade water.

5.1. Incubate 200 µL of the hybridization buffer at 67 °C for 10 min prior to addition of hybridization probe(s). Dilute probe in hybridization buffer (recipe listed in **Table 2**). Add 50 µL/coverslip.

5.2. Incubate in humidified oven at 40 °C for 2 hours (h). Decant probes and submerge slides in 1x wash buffer. Agitate by rocking dish for 2 min at RT. Repeat wash with new 1x wash buffer.

6. Amplifier (Amp) 1-FL Hybridization: Addition of pre-amplifier that is complementary to the tail sequence of the target DNA/RNA probes (Step 5)

NOTE: Amplifiers should be at RT before use. Get each individual amplifier out of the fridge 30 min before use and leave on the bench at RT.

6.1. Remove slides from 1x wash buffer and tap/absorb to remove excess liquid.

6.2. Add 1 drop of Amp 1-FL on the coverslip. Incubate in humidified oven at 40 °C for 30 min.

6.3. Decant Amp 1-FL and submerge in 1x wash buffer. Agitate by rocking dish 2 min at RT. Repeat wash with new 1x wash buffer.

7. Amp 2-FL Hybridization: Incubation with signal amplifier with cognate recognition sequence to pre-amplifiers (Amp 1-FL)

7.1. Remove slides from 1x wash buffer and tap/absorb to remove excess liquid.

7.2. Add 1 drop of Amp 2-FL on the coverslip. Incubate in humidified oven at 40 °C for 15 min.

7.3. Decant Amp 2-FL and submerge in 1x wash buffer. Agitate by rocking dish 2 min at RT. Repeat wash with new 1x wash buffer.

8. Amp 3-FL Hybridization: Incubation with second signal amplifier

8.1. Remove slides from 1x wash buffer and tap/absorb to remove excess liquid.

8.2. Add 1 drop of Amp 3-FL on the coverslip. Incubate in humidified oven at 40 °C for 30 min.

8.3. Decant Amp 3-FL and submerge in 1x wash buffer. Agitate by rocking dish 2 min at RT. Repeat wash with new 1x wash buffer.

9. Amp 4-FL Hybridization: Fluorescent label and final hybridization step

NOTE: First, see Table 4 to choose the suitable Amp 4 (A,B, or C)- FL based on the channels of the target probe(s). Assess what Amp 4-FL is needed to label DNA/RNA of interest. An example is provided by the table below:

[Place **Table 4** here]

NOTE: For multiplexed FISH (multiple targets), choosing the correct Amp 4 (A, B, or C)- FL is critical for properly labeling your target of interest(s). Target probes (Step 5) have different color channels (C1, C2, or C3), which dictate their respective fluorescent label (Alexa 488, Atto 550, or Alexa 647), based on the Amp 4-FL chosen. Examples of choosing fluorophore combinations for multiplexed imaging are provided in the legends of **Figures 2-3**. As an additional example, selection of Amp 4B-FL will selectively label DNA C1 probes with Atto 550 and RNA C3 probes with Alexa 647.

9.1. Remove slides from 1x wash buffer and tap/absorb to remove excess liquid.

9.2. Add 1 drop of Amp 4-FL on the coverslip. Incubate in humidified oven at 40 °C for 15 min.

NOTE: Following step 9.2, keep samples covered, protected from the light.

9.3. Decant Amp 4-FL and submerge in 1x wash buffer. Agitate by rocking dish for 2 min at RT. Repeat wash with new 1x wash buffer. Wash with 1x PBS (2 min) and store in PBS.

10. Protein immunostaining: To label protein(s) of interest

10.1. Decant PBS and add 200 µL of blocking buffer (1% w/v BSA, 10% v/v FBS in PBS with 0.1% v/v Tween-20 (PBST)) to the coverslip. Incubate 1 h at RT.

10.2. Decant blocking buffer and apply 200 µL of primary antibody diluted in PBST + 1% w/v BSA. Incubate 1 h at RT.

10.3. Wash the slide twice with PBST for 10 min at RT with shaking.

10.4. Apply secondary antibody of choice for 1 h at RT in PBST + 1% w/v BSA.

10.5. Wash the slide with PBST for 10 min at RT with shaking.

11. Nuclear staining: Counter-stain nuclei following immunostaining

11.1. Decant PBST and apply DAPI or nuclear stain of choice for 1 min at RT.

11.2. Wash the slide twice with PBS for 10 min at RT with shaking.

12. Mounting

12.1. Place 1 drop of antifade solution (e.g., Prolong Gold) on new sterile glass slide (First, clean slide with EtOH and let dry to ensure no residues are on glass). With the same tip, spread the antifade solution drop to cover an area approximately the size of the coverslip.

NOTE: The antifade solution is very viscous and may be difficult to pipette. Cutting the tip off a 200 µL tip prior to pipetting may mitigate these issues.

12.2. Remove coverslip with cell sample from the slide and submerge in PBS to remove residual nail polish at the back using the forceps and PBS. Dry forceps and back of the coverslip using a Kimwipe.

12.3. Gently imbed coverslip in the drop of antifade solution, placing sample side (side with cell layer) of coverslip on drop).

12.4. Let samples dry overnight.

13. Imaging

13.1. Image with an epifluorescent microscope.

REPRESENTATIVE RESULTS:

A schematic of MICDDRP is depicted in **Figure 1**. Labeling of DNA and RNA is followed by immunostaining. The use of branching amplifiers increases signal, allowing detection of single nucleic acid molecules.

[Place Figure 1]

Application of MICDDRP to study the course of HIV-1 infection has been a useful tool in tracking viral replication kinetics in primary cells. As a proof of concept of this procedure, HIV-1 DNA, RNA, and protein are simultaneously labeled and visualized microscopically at the single-cell level (**Figure 2**). Two HIV-1 DNA genomes are visualized in a single cell, as they are actively transcribing viral RNA (vRNA). vRNA has been exported through the nuclear pore complex and viral protein is synthesized in the cytoplasm.

[Place Figure 2]

In addition, we have performed dual viral DNA (vDNA) and vRNA staining to follow HTLV-1 infection. For optimization of the nucleic acid labeling, we adhered closely to the vDNA/VRNA staining procedure developed for multiplexed fluorescence imaging of HIV infection. We have demonstrated that we can specifically label HTLV-1 DNA and RNA simultaneously.

[Place Figure 3-4]

To verify the specificity of the hybridization probes and to assess how the ISH labeling method impacts protein staining efficiency and overall background, we perform critical controls to ensure the highest level of rigor and reproducibility for the experiments. As an example, in **Figure 4A-D**, we verify the specificity of our HIV-1 and HTLV-1 vDNA and vRNA probes, as we show very little to no cross-reactivity between the probe sets across the two viruses. Despite labeling of two retroviruses with the potential for probe cross-reactivity, the HIV-1 probes are *only* specific to HIV-1 genetic material and not HTLV-1. The same trend is true for the HTLV-1 probes. In addition, in **Figure 4F**, we show that we can eliminate vRNA staining if we RNase-treat our cells during sample preparation. To assess the possibility of attenuation of protein staining efficiency due to ISH (protease treatment (Step 4 in Protocol & **Figure 1B**) and hybridization conditions, which can lead to ablation of epitope recognition or increased background signal, we demonstrate that protein staining efficiency for the nuclear speckle marker, sc-35, is comparable across conventional IF approaches and immunostaining during the MICDDRP protocol. In the conventional IF protocol, cells were permeabilized with 0.1% Triton X-100 for 15 minutes, rather than permeabilization with 0.1% Tween-20 for 10 minutes, which is used in MICDDRP protocol (Step 3 in Protocol & workflow in **Figure 1B**). Protein staining across both conditions (IF *vs.* MICDDRP) produced a signal several orders of magnitude greater than the control (MICDDRP with no primary antibody), further demonstrating the low background generated following this protocol and preservation of protein epitopes for efficient immunostaining. Image quantification of mean integrated fluorescence intensity of sc-35 signal per cell (**Figure 5E**) was performed as previously described[3]. For all imaging results shown, we ensured probe and antibody specificity, as well as assessed any possible perturbations or higher than normal background noise attributed to our ISH approach.

This method can also be applied to study RNA viruses that may or may not include DNA templates for viral replication. For instance, strand-specific bDNA probes can be designed to monitor expression of sense (+) or antisense (-) strand RNA and different vRNA species in viruses such as HBV, HCV, IAV, and ZIKV. The visualization of different RNA species during the

course of infection can provide insight into the replication kinetics of various viral systems.

Following the time course of HBV infection, we can see that the amount of HBV pre-genomic RNA (pgRNA) and total HBV RNA increases as a function of time. In addition, we simultaneously immunostained a cellular host factor, MOV10 (**Figure 6**).

[Place Figure 6]

Imaging was performed *via* confocal microscopy using a 60x oil-immersion objective. The excitation/emission bandpass wavelengths used to detect DAPI, Alexa 488, ATTO 550, and Alexa 647 were set to 405/420-480, 488/505-550, 550/560-610, and 647/655-705 nm, respectively (**Figure 7-9**).

[Place Figures 7-9]

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic of MICDDRP and step-by-step workflow. (A) bDNA signal is amplified *via* branching preamplifier and amplifier DNAs to enhance detection of viral DNA (1) and RNA species (2). Oligonucleotide probes are hybridized in pairs (ZZ in schematic) to target(s) of interest, creating a scaffold for pre-amplifier (Amp 1-FL, **Step 6** in **Protocol**), amplifier (Amp 2- & 3-FL), and fluorescent probes (Amp 4, **Step 9** in **Protocol**). Consult **Table 4** for choosing appropriate Amp 4 (A,B, or C)-FL for multiplexed ISH. Labeling of nucleic acid is followed by immunostaining proteins of interest (3). (B) Thirteen main steps in MICDDRP protocol with estimation of time duration for each respective step.

Figure 2. MICDDRP of primary blood mononuclear cells (PMBCs) infected with HIV-1. PMBCs infected with HIV-1 (NL4.3) at a multiplicity of infection (MOI) of 2. Cells were fixed 48 hours post infection (hpi). (**A**) HIV-1 bDNA FISH probe (labeled with ATTO 550; red in Figure). This probe hybridizes to the template (3'<—5') vDNA strand to prevent crosstalk with sense (+) vRNA. (**B**) Unspliced HIV-1 RNA (labeled with Alexa 647; green in Figure). The HIV-1 vRNA probe hybridizes to viral transcripts transcribed in the 5'—>3' orientation. (**C**) Immunostaining of HIV-1 capsid (p24) protein (secondary antibody conjugated to Alexa 488; white in figure). (**D**) Merged image. Scale bar represents 5 µm. Nuclei were stained with DAPI (blue). To label HIV-1 DNA (DNA C1 probe) with ATTO 550 and HIV-1 RNA (RNA C3 probe) with Alexa 647, respectively, samples were incubated with Amp 4-FL '**B**' (Consult **Table 4** in **Protocol** to choose appropriate channel colors for hybridization probes).

Figure 3. Simultaneous labeling of HTLV-1 vDNA and vRNA in MT-2 cells. (**A**) HTLV-1 vDNA (labeled with ATTO 550; red in Figure) (**B**) Unspliced HTLV-1 sense (+) RNA (RNA C2 probe & labeled with Alexa 488; green in Figure)). (**C**) HTLV-1 HBZ antisense (-) vRNA (RNA C3 probe & (labeled with Alexa 647; white in Figure)). (**D**) Merged image. Scale bar represents 20 µm. Nuclei were stained with DAPI (blue). Amp 4-FL '**B**' was used (Consult **Table 4** in **Protocol**) to achieve multiplexed labeling of HTLV-1 ('**+**' & '**-**') RNA**.**

Figure 4. Control experiments to assess target probe specificity and background. Specificity of HIV-1 and HTLV-1 target probes were assessed following infection in lymphocytic cell lines (Uninfected Jurkat T cells, HTLV-1 infected cells (MT2), and HIV-1 infected cells (H9111B)). Scale bars represent 10 µm. (**A**) Cells were treated with HTLV-1 RNA probes. (**B**) Cells were treated with HIV-1 (+) RNA probe (**C-D**). Further demonstration of the specificity of HTLV-1 probes with no cross-reactivity with HIV-1. White arrows in Figure C denote HTLV-1 DNA (red). (**E-F**). vRNA staining (green) +/- RNase-treatment.

Figure 5. Comparison of protein staining efficiency following conventional IF *vs.* MICDDRP. The cellular protein, sc-35, a biomarker for nuclear speckles, was immunostained in Jurkat T cells. All scale bars represent 10 µm. (**A**) Uninfected Jurkat T cells underwent the MICDDRP protocol and were treated with HIV-1 DNA/RNA hybridization probes used in **Figures 2** & **4** above. During immunostaining, no primary antibody was added. (**B**). Uninfected Jurkat T cells underwent conventional IF, labeling sc-35 (white). Cells were permeabilized with 0.1% Triton X-100 for 15 minutes. (**C**). MICDDRP was performed on HIV-infected Jurkat T cells. vRNA is labeled green, vDNA is red, and sc-35 is white. (**D**). Close-up of vRNA, vDNA, and protein labeling (sc-35) in HIV-infected cell. (**E**) Quantification of mean integrated fluorescence signal per cell of sc-35 across different immunostaining conditions. The y-axis is on a logarithmic scale. The dotted line represents the background signal from uninfected cells that underwent the MICDDRP protocol where no primary antibody was added. No significant difference in signal following MICDDRP *vs.* conventional IF. Over 500 cells were sampled for quantification for reach respective condition.

Figure 6. HBV. Time course of HBV infection of 3E8 cells. Cells were infected with 300 HBV genomes per cell. Viral replication is shown at three time points (24, 48, and 72 hpi). pgRNA (labeled with ATTO 550; red in Figure), total HBV RNA (labeled with Alexa 647; green in Figure), and MOV10 (secondary antibody conjugated to Alexa 488; white in Figure). Nuclei are stained with DAPI in blue. Scale bar on merged images represent 10 µm. hpi, hours post-infection.

Figure 7. Time course of HCV infection of Huh-7.5.1 cells. Huh-7.5.1 cells were infected with hepatitis C virus (HCV) Jc1/Gluc2A at an MOI of 0.5. At the time intervals indicated, the cells were fixed and probed sequentially for sense (+) vRNA, antisense (-) vRNA and NS5A (HCV protein). Nuclei were stained with DAPI. Representative merged images from each time-point, showing (+) RNA in green (labeled with Alexa 647) (-) RNA in red (labeled with Atto 555), NS5A in white (secondary goat anti-mouse conjugated to Alexa 488), and nuclei in blue. Scale bars represent 10 µm. The lower images are enlarged cut outs from the corresponding time-point. hpi, hours post-infection.

Figure 8. Strand-specific bDNA FISH and immunostaining of A549 cells infected with influenza A virus. A549 cells infected with PR8 Flu A virus were fixed and probed for (**A**) IAV nucleoprotein (NP) RNA (labeled with Alexa 488; green in Figure), and (**B**) IAV polymerase protein (PB1) (secondary goat anti-mouse Atto 550; red in Figure) and nuclei were stained with DAPI (blue). (C) Merged image. Scale bar represents 10 µm.

Figure 9. Strand-specific bDNA FISH in Zika virus (ZIKV)-infected cells. Vero cells were infected with ZIKV at a MOI of 0.1. Cells were fixed at 48 hpi. (**A**) Cells were simultaneously stained for sense (+) vRNA (labeled with Alexa 488; green in Figure) and antisense (-) vRNA (labeled with Atto 550; red in Figure). Nuclei were stained with DAPI. In (A), the white box denotes a region with both (+) and (-) vRNA. The insets present a close-up of (+) vRNA (green) and the scarcer (-) vRNA species (red). (**B**) sense (+) vRNA (green). (**C**) antisense (-) vRNA (red). Scale bar in (**A**) represents 10 µm.

Table 1: Key reagents in MICDDRP protocol

Table 2: Protease III dilutions in PBS for viral nucleic acid hybridization

Table 3: Hybridization buffer list of reagents.

Table 4: Selection of Amp 4 (A, B, or C)-FL fluorescent probe for multiplexed ISH

DISCUSSION:

Simultaneous visualization of RNA, DNA, and protein often requires extensive optimization. Two commonly used methods are 5-ethynyl-2-deoxyuridine (EdU) labeling and DNA FISH. EdU labeling has been applied to visualize viral DNA and protein simultaneously, as EdU is incorporated in nascent DNA and subsequently labeled with azide-containing fluorescent dyes *via* click chemistry. EdU labeling can thus be used to monitor native virus replication kinetics of DNA viruses or viruses with DNA templates for replication[12]. A shortcoming of EdU labeling, however, is that in dividing cells, the replicating genome will incorporate EdU, generating high background and confounding image analysis. DNA FISH can circumvent these issues by directly hybridizing a nucleic acid probe to the respective target regardless of the cell cycle. However, conventional FISH often relied on high temperatures to achieve efficient probe hybridization, hindering immunostaining or even simultaneous RNA staining[13]. MICDDRP can potentially circumvent these issues providing robust simultaneous fluorescent labeling of DNA, RNA, and protein across a variety of cellular systems.

While we have demonstrated that we can label protein and nucleic acid simultaneously using our MICDDRP protocol, optimization was needed across different systems. The first major parameter that we had to optimize was protease treatment. We varied protease III concentration across the conditions. Optimization of protease treatment was empirical, as we used different dilutions to assess what yielded the greatest hybridization efficiency, without compromising immunostaining efficiency. Appropriate controls were performed side-by-side to assess probe specificity and changes to protein staining efficiency attributed to protease treatment. The next major parameters that needed optimization were probe design and probe hybridization.

Proper design of capture and amplifier probes are critical for achieving the sensitivity and specificity of bDNA technology. Software packages that predict the probability of non-specific hybridization events are available to improve probe design[7,11]. bDNA probes with the

accompanying pre-amplifier, amplifier, and fluorescent label probes can now be commercially purchased to ensure compatibility with bDNA imaging kits. Users can supply manufacturers with sequence information (~300-1000 base pairs) for the target region(s) in the form of a fasta file (text-based format for representing nucleotide sequence). Target probes are generated with > 90% sequence homology to the supplied sequence.

For DNA labeling, we have found that dilution of the probes in the hybridization buffer described in Step 5 of the Protocol improves DNA hybridization. When labeling both DNA and RNA, the RNA probe can be diluted in the hybridization buffer. DNA labeling in the absence of RNase cannot exclude the possibility that the observed nucleic acid includes RNA of the targeted strandedness. Temperature may also have to be adjusted for improving hybridization efficiency. Increasing temperature may affect protein staining efficiency, as increased temperatures may promote protein denaturation, ablating epitope recognition of the primary antibody. In our presented representative data, we have performed ISH at 40 °C.

Compared to conventional DNA FISH, MICDDRP provides an improved procedure for simultaneously labeling DNA, RNA, and protein to visualize *via* fluorescence microscopy. A potential limitation is that the selection of probe may affect the efficiency of hybridization and ability to quantitatively compare data between probes. This protocol has been effective across diverse cellular and viral systems in our hands with only minor optimization needed across varying conditions. Recent high-profile publications have utilized our approach to study HIV integration site selection[14] and HIV reverse transcription kinetics[15]. Future applications of MICDDRP could include visualization of viral nucleic acids concomitantly with nucleic acid sequences of specific cellular genes and cellular proteins.

ACKNOWLEDGMENTS:
This work was supported in whole or in part by the National Institutes of Health (R01 AI121315, R01 AI146017, R01 AI148382, R01 AI120860, R37 AI076119, and U54 AI150472). We thank Dr. Raymond F. Schinazi and Sadie Amichai for providing cells infected with influenza A Virus.

DISCLOSURES:
The authors have nothing to disclose.

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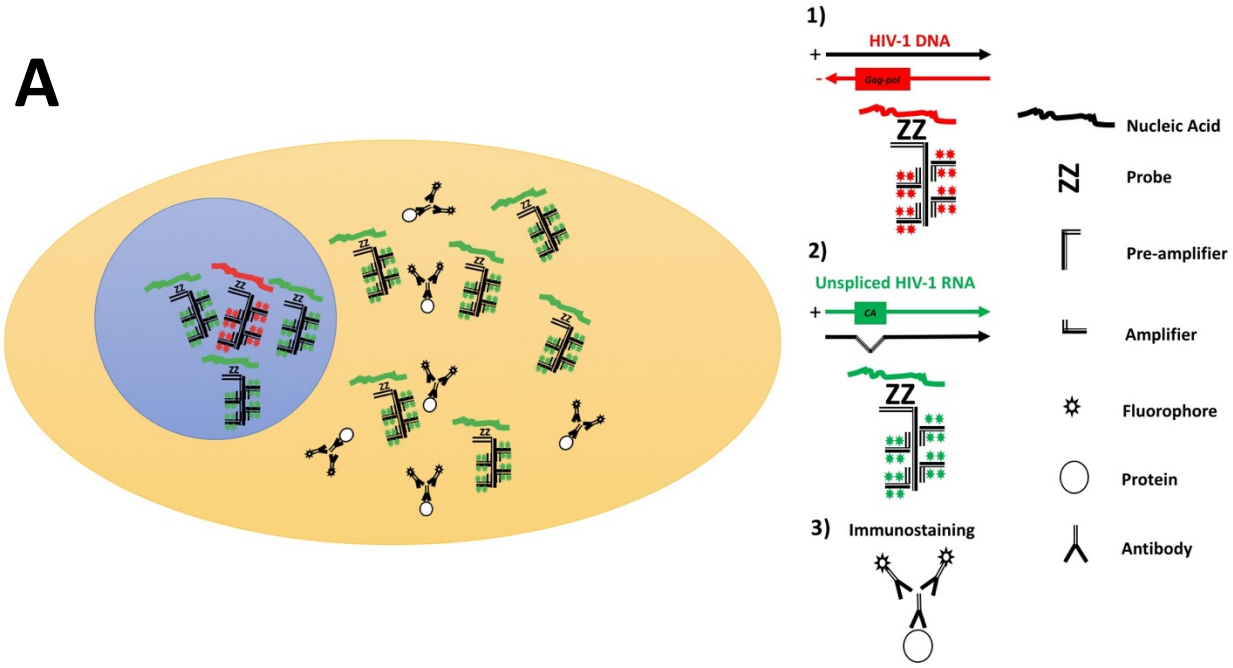
Figure 1

Click here to access/download;Figure;JoVE-Figure1-MICDDRP-schematic.pdf



B Multiplexed fluorescence imaging step-by-step:

1) Plate/seed cells on coverslip
2) Fixation
3) Cell permeabilization
4) Protease treatment
5) DNA/RNA hybridization
6-9) Signal amplification (Amplifiers 1-4)
10) Immunostaining
11) Counter-stain nuclei
12) Mount
13) Image

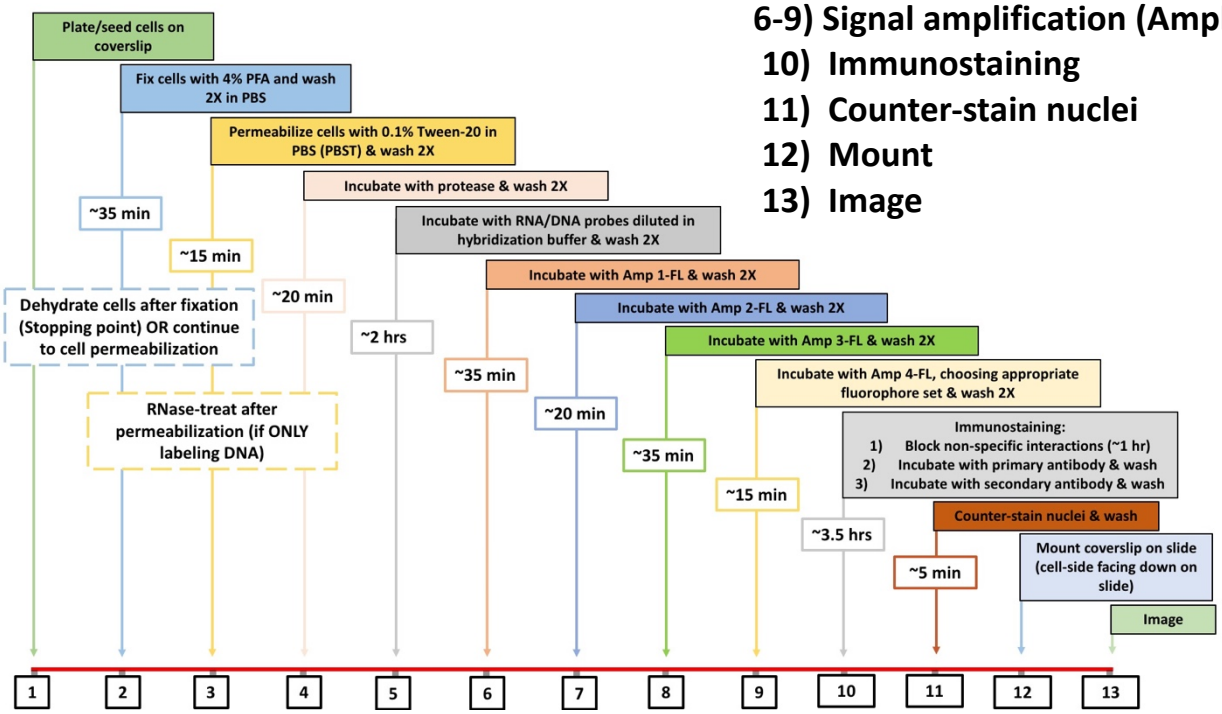


Figure 2

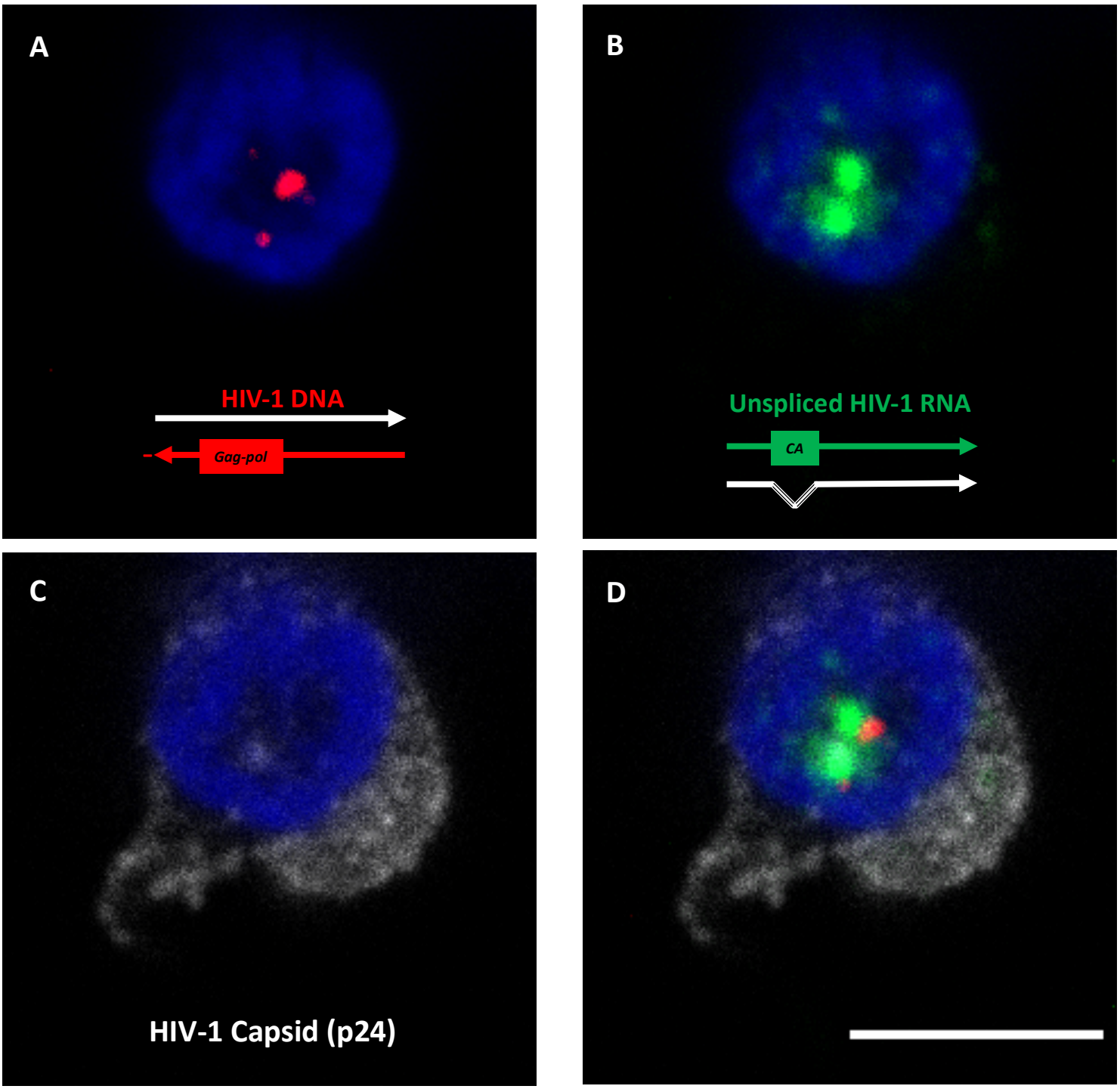


Figure 3

Click here to access/download;Figure;Figure3-HTLV-JoVE-V2.pdf

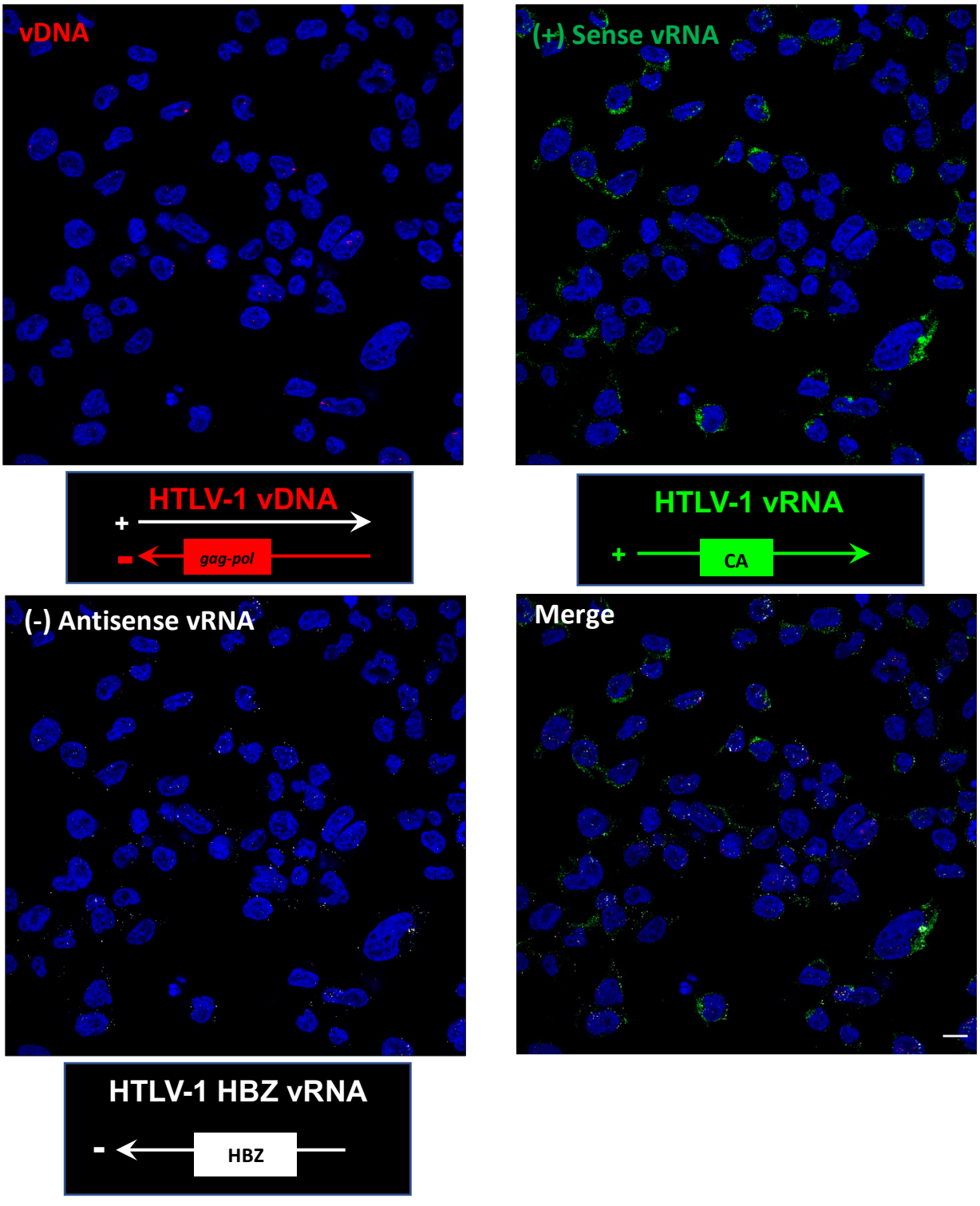


Figure 4

Click here to access/download;Figure;JoVE-Figure4-control-09022020.pdf

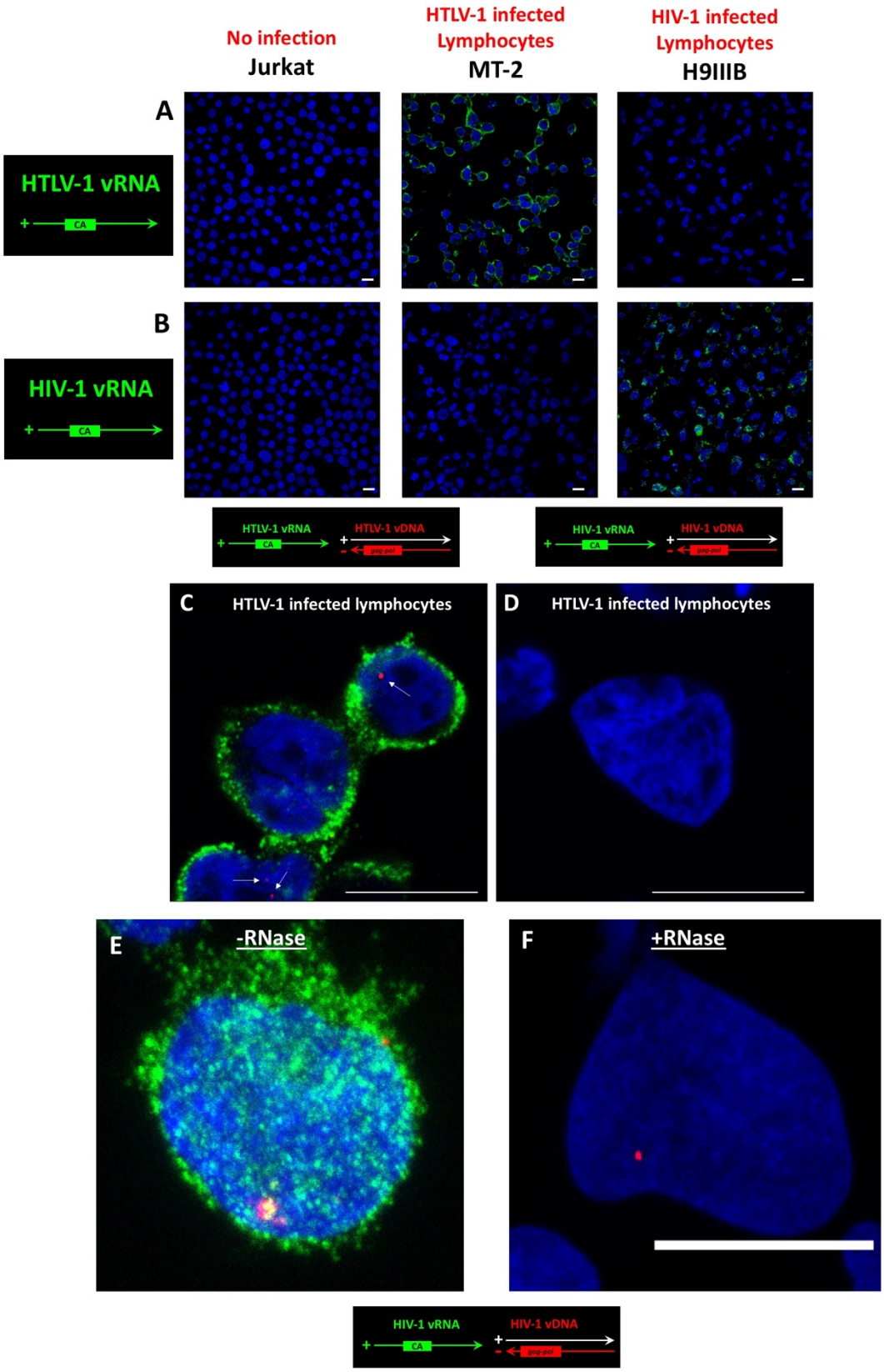


Figure 5

Click here to access/download;Figure;JoVE-Figure-5-control-09022020.pdf

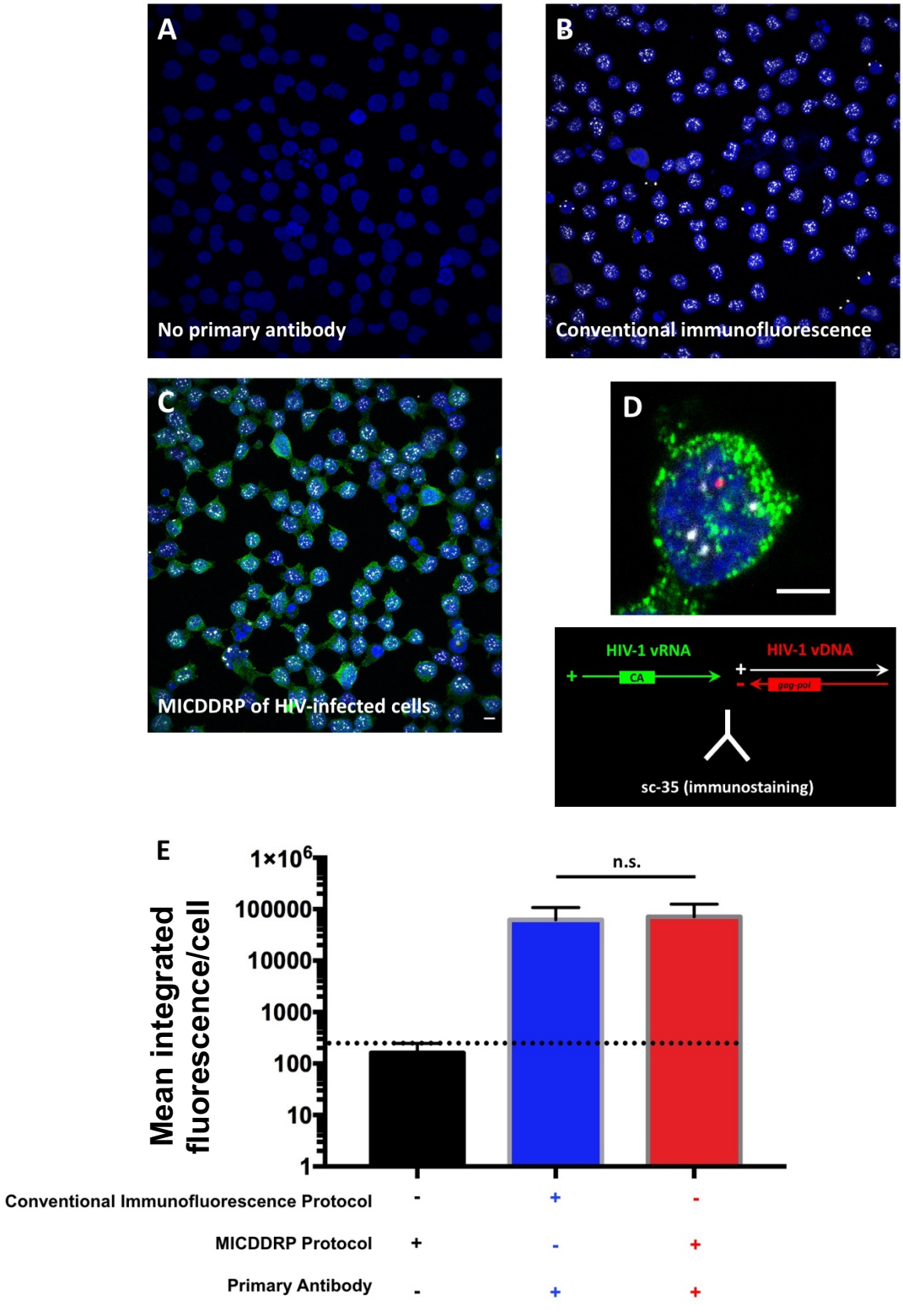


Figure 6

Click here to access/download;Figure;Figure6-HBV-timecourse.pdf

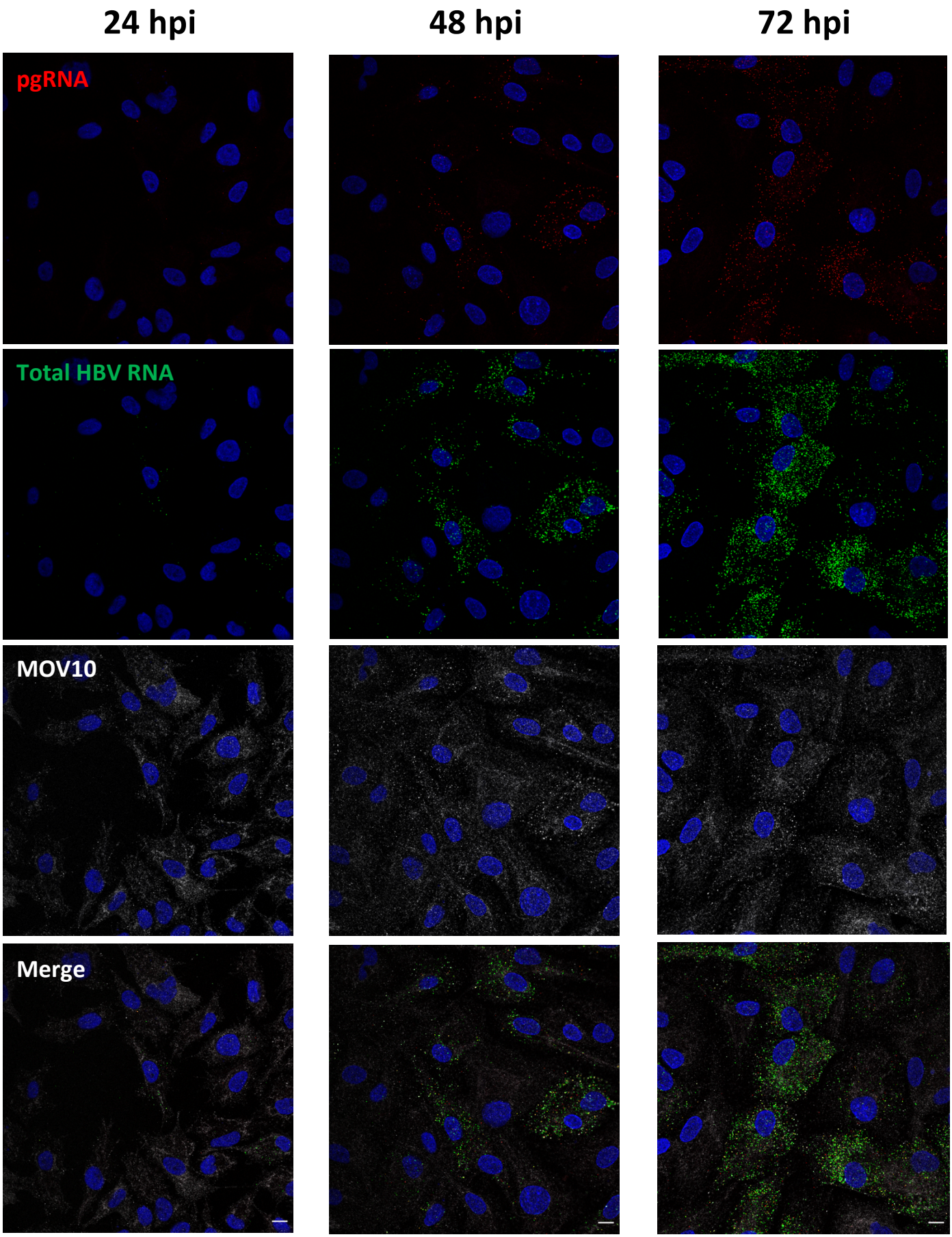


Figure 7

Click here to access/download;Figure;Figure7-HCV-time-course.pdf

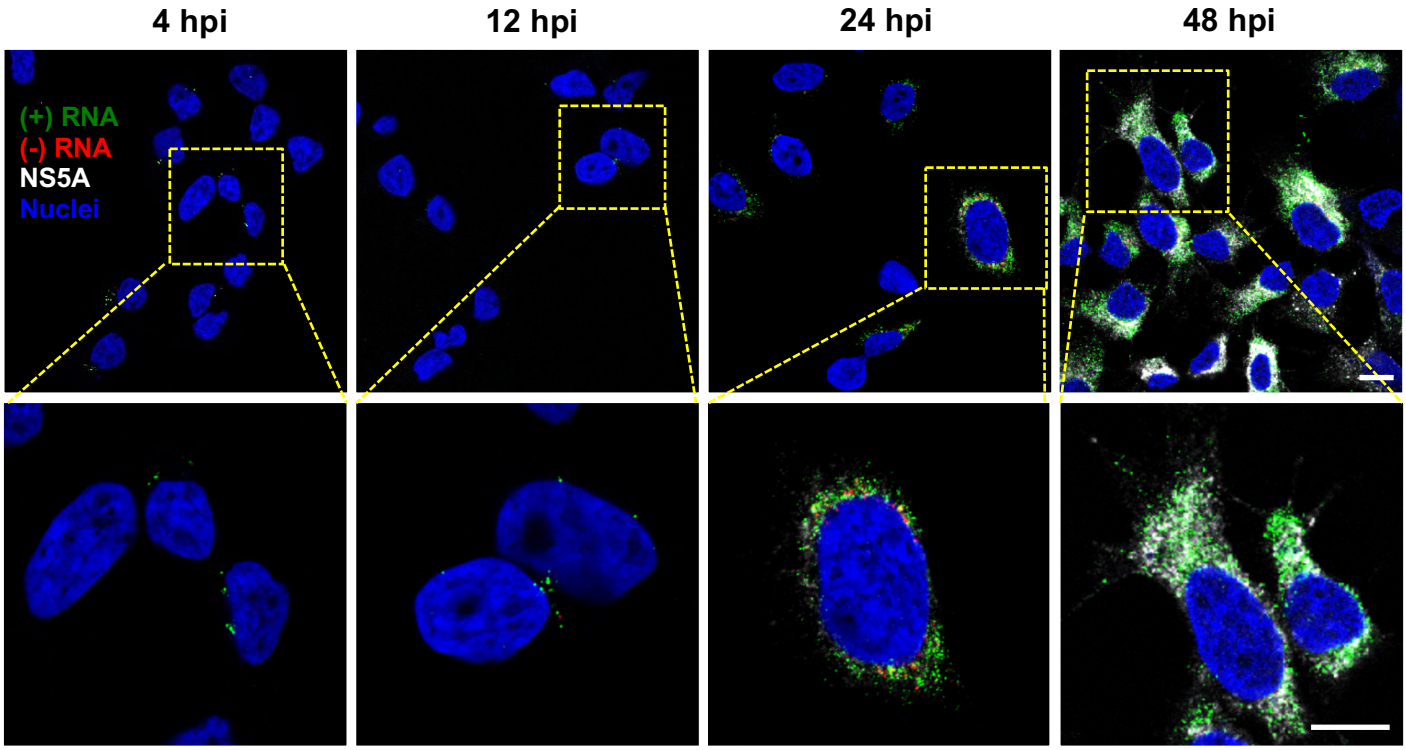


Figure 8

Click here to access/download;Figure;Figure8-flu.pdf

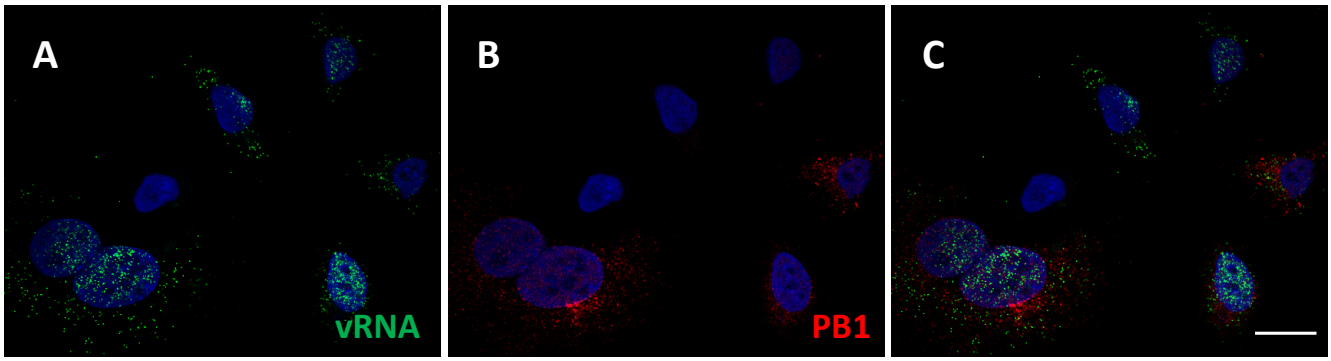


Figure 9

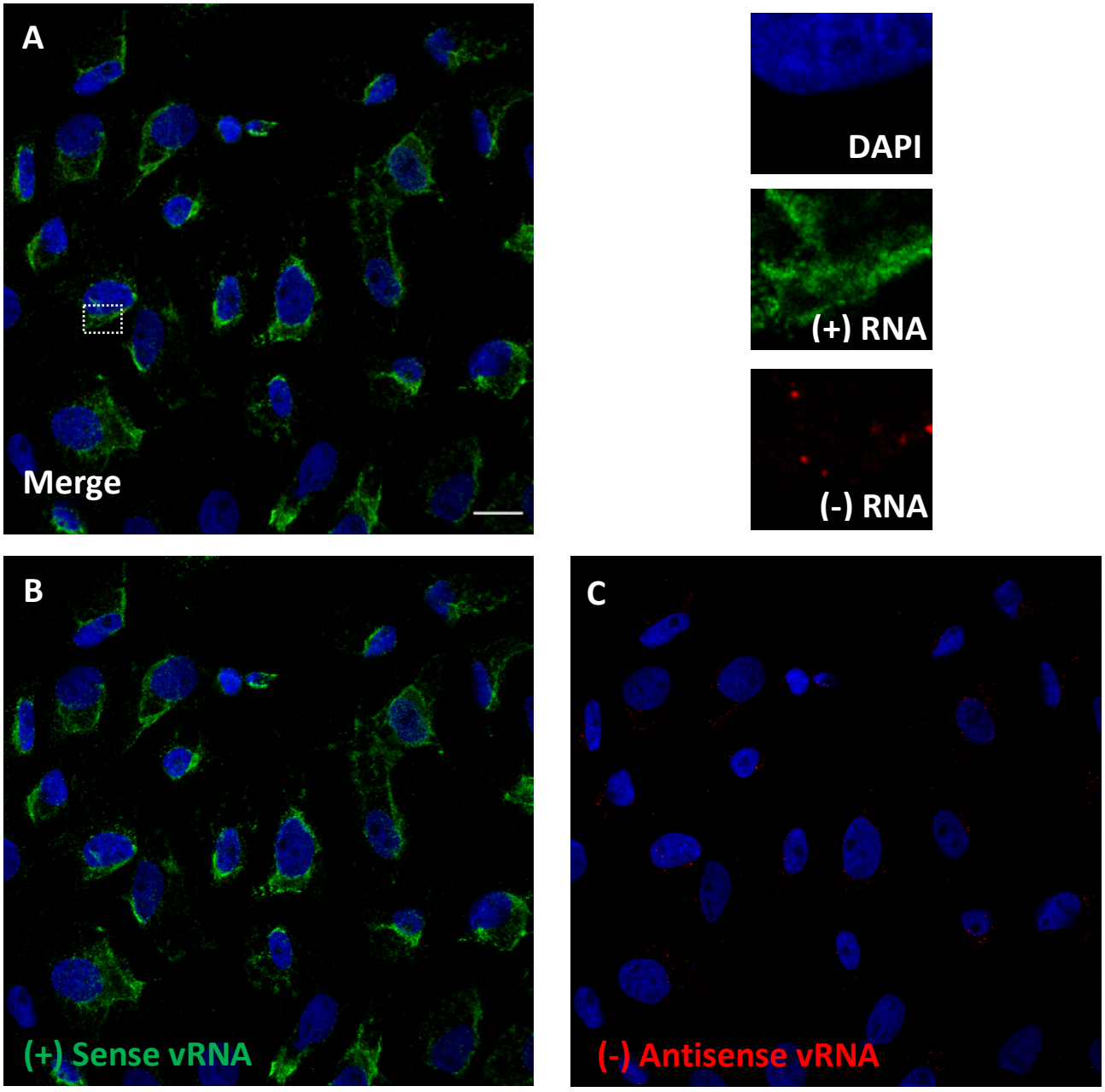


Table 1

Click here to access/download;Table;JoVE-reagent-setup-Table1-Protocol.xlsx

Reagents
Protease solution
Target oligonucletodie probe(s)
Wash buffer
Hybridization buffer

Other Notes

Prepare in 1X PBS

Dilute in hybridization buffer

Washes following target hybridization steps

Recipe presented in Table 3

Table 2

Click here to access/download;Table;JoVE-Table2-protease-dilutions-Protocol.xlsx

Probe Targets	Protease Dilution in 1X PBS (Protease III to 1X PBS)
HIV-1 DNA/RNA	1 to 5
HTLV-1 DNA/RNA	1 to 5
HBV pgRNA and total HBV R	1 to 15
IAV RNA	1 to 15
ZIKV RNA	1 to 2

Table 3

Reagent	Stock Concentration	Additional Notes
Nuclease-free water	NA	
Dextran sulfate	50% (w/v)	Very viscous
Sodium chloride	5 M	
Sodium citrate (pH 6.2)	200 mM	Store at 4 °C
Ethylene carbonate	NA	Powder
Tween-20	10% (v/v)	

Table 4

Click here to access/download;Table;JoVE-Table4-Amp4-options-Protocol.xlsx

	A	B	C
DNA Channel 1 (C1)	488	550	550
DNA/RNA Channel 2 (C2)	550	488	647
RNA Channel 3 (C3)	647	647	488

Name of Material/ Equipment	Company	Catalog Number
4% PFA		
50% dextran sulfate	Amreso	198
50X wash buffer	ACD Bio	320058
6-well plates		
Amplifier 1-FL	ACD Bio	
Amplifier 2-FL	ACD Bio	
Amplifier 3-FL	ACD Bio	
Amplifier 4-FL	ACD Bio	
Anti-HCV NS5a antibody	Abcam	ab13833
Anti-HIV-1 p24 monoclonal antibody	NIH AIDS Reagent Program	3537
Anti-Mov10 antibody	Abcam	ab80613
Anti-PB1 antibody	GeneTex	GTX125923
Bovine serum albumin		
Cell media with supplements		
Coverslips		
DAPI	ACD Bio	
Dulbecco's phosphate buffered saline (1X PBS)	Gibco	14190250
Ethylene carbonate	Sigma	E26258
Fetal bovine serum (FBS)		
Fisherbrand colorfrost plus microscope slides	Fisher Scientific	12-550-17/18/19
HCV-GT2a-sense-C2 probe	ACD Bio	441371
HIV-gagpol-C1	ACD Bio	317701
HIV-nongagpol-C3	ACD Bio	317711-C
HybEZ hybridization oven	ACD Bio	321710/321720
ImmEdge hydrophobic barrier pen	Vector Laboratories	H-4000
Nail polish		
Nuclease free water	Ambion	AM9937
Poly-d-lysine (PDL)		
Probe diluent	ACD Bio	300041

Prolong gold antifade	Invitrogen	P36930
Protease III	ACD Bio	322337
RNAscope® Probe- V-Influenza-H1N1-H5N1-NP	ACD Bio	436221
RNase A	Qiagen	
Secondary antibodies		
Slides		
Sodium chloride		
Sodium citrate, pH 6.2		
Tween-20		
V-HBV-GTD	ACD Bio	441351
V-HBV-GTD-01-C2	ACD Bio	465531-C2
V-HCV-GT2a probe	ACD Bio	441361
V-HTLV-HBZ-sense-C3	ACD Bio	495071-C3
V-HTLV1-GAG-C2	ACD Bio	495051-C2
V-HTLV1-GAG-POL-sense	ACD Bio	495061
V-Influenza-H1N1-H5N1-NP	ACD Bio	436221
V-ZIKA-pp-O2	ACD Bio	464531
V-ZIKA-pp-O2-sense-C2	ACD Bio	478731-C2

	Comments/Description
For DNA hybridization buffer	
Consult Amp-4 table in protocol	
Mouse monoclonal; works with HCV genotypes 1a, 1b, 3, and 4	
Rabbit polyclonal	
Antibody against flu protein	
Blocking reagent for immunostaining	
Media appropriate for cell model	
Nuclear stain (RNAscope kit from ACD Bio)	
No calcium and magnesium	
Use specific FBS based on what serum secondary antibody was raised in (e.g goat FBS)	
Precleaned	
HCV(+) sense RNA probe	
HIV-1 cDNA probe	
HIV-1 RNA probe	
For immobolizing coverslip to slide prior to protease treatment	
Coat coverslips in 20 µg/mL of PDL for 30 minutes	
For diluting RNA C2 or C3 probes	

For DNA hybridization buffer
For DNA hybridization buffer
For DNA hybridization buffer and PBS-T
Total HBV RNA
HBV pgRNA probe
HCV(-) sense RNA probe
HTLV-1 (-) sense RNA probe targetting HBZ
HTLV-1 DNA probe
HTLV-1 (+) sense RNA probe
IAV RNA probe
Zika(+) sense RNA probe
Zika(-) sense RNA probe

Rebuttal (Revisions for JoVE Submission; Due date: September 14, 2020)

Responses to Reviewers' Comments

*We thank the reviewers for their thorough evaluation of our manuscript. We hope we have addressed their concerns with our responses and edits to the text and figures. Point-by-point responses are italicized below each respective comment. All revisions in the revised text is highlighted via 'Track Changes' or 'Comments'. Tables embedded in the **Protocol** have been removed from the text and instead included as separate .xls files. Desired placement of tables in the text of the **Protocol** is specified.*

Editorial Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

   Response: *We have thoroughly proof-read the manuscript, ensuring no spelling or grammatical issues.*

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

   Response: *We made sure to ensure to these formatting requirements.*

3. Please provide an email address for each author.

   Response: *Email addresses for each author are now included on the title page. Previously, we just included the emails of the first authors and corresponding author.*

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

   Response: *The Summary was revised to be within the word limit with a concise description of the protocol.*

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

   Response: *The Abstract is now within the word limit and states the goals of our protocol to use multiplexed fluorescence imaging to study a broad spectrum of cellular systems.*

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
   For example: Nikon C2 confocal microscope, Leica Sp8 confocal microscope, HybEZ, ACD Bio, Advanced Cell Diagnostics, etc

Response: *All commercial language was removed from the main text and only included in the Table of Materials.*

7. We cannot have non- numbered steps, subheadings, headings, paragraphs in the protocol section. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

   Response: Formatting has been adjusted in the **Protocol**. The numbering nomenclature is now following 1.1 to 1.1.1 to 1.1.2, as stated in the comment above.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

   Response: All text in the protocol is in the imperative tense.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

   Response: Further detail was added to the protocol steps to answer "how" each step is performed with additional detail on rationale for each step.

10. Please use complete sentences throughout providing all volumes, concentration, etc.

    Response: Complete sentences are used throughout the **Protocol.**

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

    Response: This comment has been addressed.

12. When do you introduce viral particles to the cells?

    Response: We added when to add viral particles to the cells in **Step 1** of the **Protocol.** Since there are differences in infection protocols across different viruses and since this protocol can be broadly applied to different cellular systems (without viruses), we specified to infect/treat cells during cell seeding of adherent cells or prior to seeding on poly-d-lysine coated coverslips for suspension cells. The specifics of infection/treatment vary upon experimental design.

13. Adherent cells: Cell number used for step 1?

    Response: In **Step 1,** we specified that we aim for 50-70% cellular confluence prior to fixation in 4% PFA.

14. Buffer preparation can be moved to a table and uploaded separately as .xlsx file. This table can be referenced wherever applicable.

    Response: Buffer setup has been added as Table 1. We uploaded the table as an excel file (.xls) and reference the table appropriately. In the body of the **Protocol**, we specified where we wanted Table 1 placed. (eg. [Place **Table 1** here])

15. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions per step.

    Response: Shorter steps were combined to contain multiple actions.

16. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

    Response: This Table explaining selection of Amp 4-FL (fluorescent probe selection for multiplexed labeling) is now uploaded as an xls file similar to the Reagent Setup Table (Table 1). All four Tables were uploaded as .xls files and specified where to add in the **Protocol**.

17. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.

    Response: The text in the **Protocol** now follows the specifications stated above.

18. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next.

    Response: Steps that we want to highlight for filming have highlighted in yellow.

19. Notes cannot be filmed.

    Response: No notes are now highlighted in yellow.

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

    Response: All figures and content presented are original data not previously published.

21. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
    a) Critical steps within the protocol
    b) Any modifications and troubleshooting of the technique
    c) Any limitations of the technique
    d) The significance with respect to existing methods
    e) Any future applications of the technique

    Response: We discussed in depth critical steps that may require optimization (protease treatment, probe design, and target probe hybridization. In addition, we discuss limitations to the technique being attributed to differences in probe hybridization efficiency across different probe sets. In addition, improving hybridization may come at the cost of impaired immunostaining efficiency (higher temperatures for probe annealing and target denaturation may affect protein epitope stability for antibody recognition). We also highlighted benefits of our method over conventional FISH and other nucleic acid labeling methods (Edu labeling). Future applications of this method include application to a broad array of cell culture models.

22. Please do not abbreviate the journal titles in the references section.

    Response: We ensured we are following proper citations in the reference section. We are using the JoVE citation output in EndNote.

Reviewer 1:

Comments: The authors provided a very well detailed protocol of their innovative Multiplex Immunofluorescent Cell-based Detection of DNA, RNA and Protein (MICDDRP) approach to achieve simultaneous detection of RNA, DNA and proteins at a single cell level. They took advantage of the existing branched DNA (bDNA) in situ hybridization technology to obtain a highly sensitive and easily detectable nucleic acid staining and further improved the technique in order to allow simultaneous detection of DNA, RNA and proteins, which would be otherwise unachievable with the commercialized products. This technique will prove particularly useful to study viral life cycle and viral infection dynamics. As a proof of concept, the authors provided representative results where they successfully detected DNA, RNA and proteins in single cells infected with a broad range of RNA and DNA viruses (i.e HIV-1, HTLV-1, HBV, HCV, ZKV and IAV).

Overall, the method proposed by the authors is extremely interesting and smart. They abundantly proved the versatility and solidity of the technique by showing representative results on different cell types infected with six different viruses.

However, the protocol could use few minor adjustments in order to further facilitate method reproducibility.

Major Concerns:

1. The addition of a schematic and accurate workflow depicting the main steps of the protocol will provide an overview of the protocols and largely facilitate its implementation. The authors could then refer to the figure throughout the text to guide the reader into the method application. This figure will be a great addition to the already provided figure 1 where the authors provided the method concept.

Response: We would like to thank **Reviewer 1** for the thorough and thoughtful feedback on improving the readability and clarity of the **Protocol**. The addition of a **step-by-step workflow** to complement our schematic (**Figure 1A**), which we reference throughout the text with the purpose of helping orient the reader in this lengthy protocol, is now included as **Figure 1B.** We highlight the 13 major steps, stating when to perform optional steps (Cellular dehydration (for storage) and RNase-treat), as well as how long each step may take.

2. Some step of the protocol could benefit of more clarity:

   - As a general comment: it will be useful to add a short description below every main step title explaing the rationale behind it and adding the material needed and any note or tips for the user at that point (i.e reactive should be at room temperature, or samples can be stored at -20°C) i.e: Step 1 Cell Fixation: this step allow to preserve cellular morphology
   Note: 4% PFA and PBS should be a brought at RT before applying on the cells

   Response: We added more detail and descriptions to each of the main steps, addressing the rationale for each step, as well as how to execute each step with greater clarity.

3. - Step 2 and 3 Dehydrate and Rehydrate cells:
   o I assume that those steps are optional and of use only if it is not possible to carry on the experiment right away. If this is the case, this should be state in the protocol as a note.
   o Every "note" or "Important" should be placed below the step title (or at the end of the paragraph if the author prefer) but there should be consistency throughout the protocol

   Response: This is a very good point since dehydration/rehydration of cells is an optional step (stopping point). We emphasized this point with a “Note” and added these steps as substeps following cellular fixation in **Step 2.**

4. - Line 145 to 154: this" Note" should be either divided and placed below every concerned step or the author should add a "Reagents preparation" or "Setup" section to include all solutions and material that should be prepared in advance or that could be prepare in batch and frozen (if any) (Hybridization buffer could also be on this section)

   Response: We made the revisions above, and this will provide readers with a Table (**Table I**) for setup of critical reagents and added additional detail on reagent preparation, as a ‘**Note’**, in each respective step.

5. - Step 5 Coverslip immobilization:
   o Line 165: although it may seem logical, for sake of clarity the authors should specify that the cell layer should face up when fixing the coverslip

   Response: We explicitly state what side the cell layer should be when immobilizing the coverslip before protease treatment in **Step 4,** and again clearly state what side the cellular layer should be when mounting the coverslip on a slide prior to imaging.

6. - Step 6 Protease treatment:
   o If possible, the authors should add some detail on how to dilute proteases, although they state that it will need optimization, would it be possible to add some tips on how to optimize the amount of protease and what to aim for during optimization?

   Response: We added additional 'tips' for diluting the protease in the **Protocol** (**Step 4)**, and in addition, added more detail in the **Introduction** and **Discussion** on how to optimize protease digestion to improve hybridization efficiency without compromising protein staining.

7. - Step 9 Probe application
   Probe acronyms are cited during the protocol already from the beginning of the text (line 150) without being introduced or explained
   o The authors may consider adding a paragraph with some information on the probes used and if possible tips on how design probes and combine them. Indeed a section at the beginning of the protocol explaining how to select a probe, how to combine them in case of multiplexing, which probes are available (in term of fluorophore) will be a highly valuable addition to the paper.
   o Crucial steps as Hybridization, addition of preamplifier and amplifiers should be a part of a workflow.

   Response: *We removed any probe acronyms. This originally served as an example, where we wanted to demonstrate how we can specifically use the HIV probes in this case to label viral nucleic acid and choose the appropriate fluorophore pairs for multiplexed DNA/RNA imaging. In addition, we added details on the theory of bDNA imaging, probe design (description of target probes, pre-amplifiers, amplifiers, and fluorophores), and how to choose the appropriate fluorophore combinations for multiplexed in-situ labeling. These details were specifically added to the* ***Introduction****, and we provide examples of how to choose fluorophore combinations in* ***Step 9*** *of the* ***Protocol*** *and within the* ***Figure Legends*** *of* ***Figures 2-3,*** *where we state what channel target probes were used for multiplexed imaging in HIV and HTLV-1 infections and how to choose the fluorescent probe (Amp 4-FL). We also stated that three color channels are available with three different fluorophores (Alexa 488, Atto 550, and Alexa 647)*

8. - Step 10-13 Amp 1-4 FL Hybridization:
   o The authors should clarify the role of the 4 amplifier and make clear how to use them (together in combination? In different slides?) although this may appear naïve, for a new user it could be very helpful.

   Response: *As stated in our response to 'Major Concern #7' above, we added detail on the role of the 4th amplifier (fluorescent probe) and described how to utilize these probes for multiplexed imaging in greater detail. In addition, we added examples in two of our Figures on how to appropriately choose the fluorescent probes. Additional details were added in the Note after Table 4.*

Minor Concerns:

1. - Figures:
   Figure 2 to 7 are very nice and provide strong evidence on the solidity of the proposed

technique
o For sake of clarity, the authors may consider adding the Probe name /channel and amplifier used in the legend.

Response: *This was a great suggestion, and we added the channel name for the DNA and RNA probes for HIV and HTLV-1 and the amplifier fluorescent probe in the* ***Legends*** *of* ***Figures 2-3.***

Reviewer 2:

Comments: The article by Shah et al., lays out a detailed protocol to perform the RNAscope hybridization technique to label viral nucleic acids in combination with immunofluorescence to label proteins. As a frequent user of this particular technique, I strongly believe this article would be a go to guide for anyone planning to use this approach.

The protocol is presented in a logical manner and thus easier for anyone to understand. In addition, every step described has the appropriate volumes mentioned. Apart from a few major points mentioned below, this is a well-described protocol paper and a technique that would be useful for a number of studies.

Major Concerns:

1. Section 7 (line 188) in the article was a bit confusing. What do the authors mean by Re-Fixation?

   Response: *This line/step was removed for clarity. We found that fixing twice may improve in some cases hybridization efficiency in certain cases. However, its contribution was marginal and did not have an effect on the representative imaging presented in this manuscript.*

2. Section 8 (Line 219-220). The hybridization buffer is incubated with the slides? Please mention slides in this line. Also please mention this step is required only for vDNA staining.
   Similarly in section 9 (line 226-227), is the prehybridization step at 67 degrees required when staining for both vRNA and vDNA? Please clarify and If this step (section 8) would result in the loss of vRNA signal.

   Response: *The hybridization buffer is incubated separately. The slides merely serve as a support for the coverslips, where the coverslips are adhered to slides with the cell side facing up through most of this protocol. The slides allow easy transfer of the coverslips in between hybridization steps and washes. Incubating the slides affects the adhesiveness of the nail polish, and the coverslips are more prone to following off and getting misplaced during the wash steps (wash with gentle agitation). We also clarified that the hybridization buffer can be used for both DNA/RNA staining. Target probes can be diluted in this buffer as appropriate. This step has not resulted in the loss of RNA signal, but we have found that other hybridization buffers used in more conventional FISH methods can affect RNA staining efficiency.*

3. For figures 2-7, what are the background signals observed with the probes? uninfected samples? Please show images or mention about the background signal obtained in the main text.

   Response: To address this concern, we included proper controls (**Figures 4-5**) to show the background signals associated with the hybridization probes and protein staining efficiency in

MICDDRP compared to conventional immunofluorescence approaches. To summarize, we demonstrate that our probes are highly specific (no cross-reactivity between HIV & HTLV) and that MICDDRP does not reduce protein staining efficiency or lead to higher levels of background compared to conventional immunofluorescence.

Minor Concerns:

1. A point worth mentioning in the discussion is the suitability of this staining protocol when using lentiviral transduced cell lines.
   For instance, the HIV-1 probes used might generate higher background even in uninfected population when a lentiviral construct is used to stably express a particular protein of interest.

   Response: *This is a very valid and important point. To validate the specificity and lack of cross-reactivity between our target probes, in our newly added* ***Figure 4,*** *we show that the HIV-1 probes are highly specific to HIV and not any host DNA/transcripts, as well as not cross-reactivity with HTLV-1 (another retrovirus).*

2. Are there references that have already used this protocol? It might improve the value of this article if they suggest some references that have already used this approach to address important questions.

   Response: *Within the* ***Introduction****, we highlight some research manuscripts from our lab that have used this protocol to study viral infection (Puray-Chavez et al. 2017, Nat. Comm., Ukah et al. 2018, Viruses, Liu et al. 2019, Viruses). In addition, we cited two recent high-profile publications that have used this approach and referenced our original study (Puray-Chavez et al. 2017) to track HIV integration site selection (Francis et al. 2020, Nat. Comm.) and HIV reverse transcription kinetics (Dharan et al. 2020, Nat Microbiol.). These two respective citations are referenced in the* ***Discussion****.*

Reviewer 3:

Comments: The authors demonstrate simultaneously fluorescence imaging of DNA, RNA and proteins of viral origin in fixed cells. Sensitivity of RNA-FISH (using commercial RNA-Scope probes, ACD Bio) and Immuno-fluorescence is demonstrated. There are two key characterizations that improve the suitability of the method for simultaneous NA/ protein imaging. 1. Standardization of Protease dilution ratio with PBS for different viruses and viral DNA/RNA combinations. 2.Usage of Hybridization buffer to dilute probes (which is standard in most FISH protocols) The authors show imaging across various viruses and some protein /RNA/DNA combinations in each case. In general the method seems to be broadly applicable.

Major Concerns:

1. The usage of the protease enzyme in samples before FISH and immuno-staining would cause loss of the proteins in the cells. By controlling the protease treatment, they are able to limit some of this loss but it might be an issue when low levels of proteins are present (eg. early in the virus infection cycle). There has been no attempt to estimate the level of such loss due to the initial protease treatment.

Response: As stated above, we addressed the issue of the effects of protease treatment and *in-situ* hybridization affecting protein staining efficiency in **Figure 5** by assessing differences in staining of nuclear speckle marker, sc-35, using MICDDRP compared to conventional IF. However, as suggested by Reviewer 3, it would also be a good control to assess labeling and detection of a less abundant protein. We have however, been able to detect viral protein during early infection time points, suggesting sensitivity is not heavily affected. Overall, our method does not appear to impair protein staining efficiency for the immunostaining we have performed to date, and in addition, MICDDRP does not impart high levels of background signal.

2. Negative controls for many cases is not shown. While the RNAscope probes are very specific, immunofluorescence post protease treatment can result in higher backgrounds.

Response: *As stated above, negative controls were added to* ***Figures 4-5****, demonstrating the target probes are highly specific and impart low to no discernable differences in background (no hybridization to uninfected cells). In addition, we demonstrate that RNase-treatment removes the vRNA signal following labeling of HIV nucleic acid. In* ***Figure 5****, we also quantified the levels of background following MICDDRP when we did not add a primary antibody. The levels of background were orders of magnitude lower that protein signal obtained through conventional IF or immunostaining following our MICDDRP protocol (****Figure 5E****).*

Minor Concerns:

1. The image sets for HTLV (figure -3) and ZIKV (figure - 7) shows signals for different kinds of nucleic acids only(RNA/DNA FISH) but no IF. It would be great if some more simultaneous IF/RNA FISH is shown.

Response: *We would have added images with protein staining for HTLV-1 and ZIKV if we had it. During our development of this protocol, we used HTLV-1 to validate the specificity of our HIV-1 probes to demonstrate no cross-reactivity and low background imparted by our protocol. For ZIKV imaging, we were interested in tracking (+) and (-) sense vRNA products. We added imaging for HTLV-1 and ZIKV to demonstrate that through this protocol we can not only label DNA and RNA but also label RNA of different strandedness. This is especially important when tracking replication kinetics of RNA viruses. Unfortunately, while the addition of imaging with IF would be a helpful additional figure, these images are not currently available.*

Reviewer 4:

Comments: The method paper by Shah et al applied their fluorescence imaging approach, multiplex immunofluorescent cell-based detection of DNA, RNA, and protein (MICDDRP), that they developed earlier for HIV-1 (Ref #3) for other viral detection. The method is presented in an appropriate way and well written and can be accepted in it's current form.

Major Concerns:
None

Rebuttal (Revisions for JoVE Submission; Due date: September 14, 2020)

Minor Concerns:
None