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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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** (multiplex immunofluorescent cell-based detection of DNA, RNA and protein), 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 sample¹, microscopic visualization of protein is often not compatible with conventional DNA fluorescence in situ

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The authors have nothing to disclose.

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^{3,4,5,6}.

We have developed an imaging approach, **multiplex immunofluorescent cell-based detection of DNA, RNA, and Protein (MICDDRP)**³, which utilizes branched DNA (bDNA) in situ technology to improve the sensitivity of nucleic acid detection^{7,8,9}. 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 DNA⁹. 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 RNAscope⁷ and ViewRNA¹⁰ 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 modifications⁷. 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 differentiation⁷. 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 approaches⁷ (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, Figure 2 and Figure 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^{3,4,5}. 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 DNA³. 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 reversal⁴. 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^{3,4,5,6}. 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. Seeding of suspension cells

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).

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.

2. Seeding of adherent cells

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.

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.

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. Remove 50% EtOH and replace with 500 µL of 70% EtOH. Incubate at RT for 5 min.
3. Remove 70% EtOH and replace with 500 µL of 100% EtOH. Incubate at RT for 5 min.
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.

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

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

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.

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

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

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.
 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).
2. Dilute Protease III in 1x PBS (100 μ L/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).

3. Decant the 1x PBS on the coverslip following immobilization and apply the diluted Protease III.
4. Incubate in a humidified oven at 40 °C for 15 min.
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.
 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.
 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).

6. Prepare hybridization buffer in nuclease-free water following the step-by-step procedure below:
 1. In a 15 mL tube, add 700 μ L of nuclease free-water, 300 μ L of 50% (weight/volume (w/v)) dextran sulfate, 300 μ L of 5 M NaCl, 125 μ L of 200 mM sodium citrate (pH 6.2), and 375 mg (powder) of ethylene carbonate.
 2. Mix well using a vortex to dissolve ethylene carbonate (ensure all powder is dissolved and clear solution).
 3. Add 25 μ L 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.

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.

1. Incubate 200 μ L 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 μ L/cover slip.
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.

1. Remove slides from 1x wash buffer and tap/absorb to remove excess liquid.
2. Add 1 drop of Amp 1-FL on the coverslip. Incubate in humidified oven at 40 °C for 30 min.
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)

1. Remove slides from 1x wash buffer and tap/absorb to remove excess liquid.
2. Add 1 drop of Amp 2-FL on the coverslip. Incubate in humidified oven at 40 °C for 15 min.
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

1. Remove slides from 1x wash buffer and tap/absorb to remove excess liquid.
2. Add 1 drop of Amp 3-FL on the coverslip. Incubate in humidified oven at 40 °C for 30 min.
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:

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 Figure 2 and Figure 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.

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

1. Decant PBS and add 200 μ L 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.
2. Decant blocking buffer and apply 200 μ L of primary antibody diluted in PBST + 1% w/v BSA. Incubate 1 h at RT.
3. Wash the slide twice with PBST for 10 min at RT with shaking.
4. Apply secondary antibody of choice for 1 h at RT in PBST + 1% w/v BSA.
5. Wash the slide with PBST for 10 min at RT with shaking.

11. Nuclear staining: Counter-stain nuclei following immunostaining

1. Decant PBST and apply DAPI or nuclear stain of choice for 1 min at RT.
2. Wash the slide twice with PBS for 10 min at RT with shaking.

12. Mounting

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 μ L tip prior to pipetting may mitigate these issues.

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.
3. Gently imbed coverslip in the drop of antifade solution, placing sample side (side with cell layer) of coverslip on drop).
4. Let samples dry overnight.

13. Imaging

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.

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.

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.

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 described³. 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).

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, Figure 8 and Figure 9).

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 replication¹². 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 staining¹³. 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 preamplifier, 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 selection¹⁴ and HIV reverse transcription kinetics¹⁵. Future applications of MICDDRP could include visualization of viral nucleic acids concomitantly with nucleic acid sequences of specific cellular genes and cellular proteins.

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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 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 μ m. 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 4C 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 μ m. (A) Uninfected Jurkat T cells underwent the MICDDRP protocol and were treated with HIV-1 DNA/RNA hybridization probes used in Figure 2 & Figure 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.

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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 μ m. 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.

Reagents	Other Notes
Protease solution	Prepare in 1X PBS
Target oligonucleotide probe(s)	Dilute in hybridization buffer
Wash buffer	Washes following target hybridization steps
Hybridization buffer	Recipe presented in Table 3

Table 2:

Protease III dilutions in PBS for viral nucleic acid hybridization.

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 RNA	1 to 15
IAV RNA	1 to 15
ZIKV RNA	1 to 2

Table 3:

Hybridization buffer list of reagents.

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:

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

	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

Materials

Name	Company	Catalog Number	Comments
4% PFA			
50% dextran sulfate	Amreso	198	For DNA hybridization buffer
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		Consult Amp-4 table in protocol
Anti-HCV NS5a antibody	Abcam	ab13833	Mouse monoclonal; works with HCV genotypes 1a, 1b, 3, and 4
Anti-HIV-1 p24 monoclonal antibody	NIH AIDS Reagent Program	3537	
Anti-Mov10 antibody	Abcam	ab80613	Rabbit polyclonal
Anti-PB1 antibody	GeneTex	GTX125923	Antibody against flu protein
Bovine serum albumin			Blocking reagent for immunostaining
Cell media with supplements			Media appropriate for cell model
Coverslips			
DAPI	ACD Bio		Nuclear stain (RNAscope kit from ACD Bio)
Dulbecco's phosphate buffered saline (1X PBS)	Gibco	14190250	No calcium and magnesium
Ethylene carbonate	Sigma	E26258	
Fetal bovine serum (FBS)			Use specific FBS based on what serum secondary antibody was raised in (e.g goat FBS)
Fisherbrand colorfrost plus microscope slides	Fisher Scientific	12-550-17/18/19	Precleaned
HCV-GT2a-sense-C2 probe	ACD Bio	441371	HCV(+) sense RNA probe
HIV-gagpol-C1	ACD Bio	317701	HIV-1 cDNA probe
HIV-nongagpol-C3	ACD Bio	317711-C	HIV-1 RNA probe
HybEZ hybridization oven	ACD Bio	321710/321720	
ImmEdge hydrophobic barrier pen	Vector Laboratories	H-4000	
Nail polish			For immobilizing coverslip to slide prior to protease treatment
Nuclease free water	Ambion	AM9937	
Poly-d-lysine (PDL)			Coat coverslips in 20 µg/mL of PDL for 30 minutes
Probe diluent	ACD Bio	300041	For diluting RNA C2 or C3 probes
Prolong gold antifade	Invitrogen	P36930	
Protease III	ACD Bio	322337	
RNAscope® Probe- V-Influenza-H1N1-H5N1-NP	ACD Bio	436221	
RNase A	Qiagen		

Name	Company	Catalog Number	Comments
Secondary antibodies			
Slides			
Sodium chloride			For DNA hybridization buffer
Sodium citrate, pH 6.2			For DNA hybridization buffer
Tween-20			For DNA hybridization buffer and PBS-T
V-HBV-GTD	ACD Bio	441351	Total HBV RNA
V-HBV-GTD-01-C2	ACD Bio	465531-C2	HBV pgRNA probe
V-HCV-GT2a probe	ACD Bio	441361	HCV(−) sense RNA probe
V-HTLV-HBZ-sense-C3	ACD Bio	495071-C3	HTLV-1 (−) sense RNA probe targetting HBZ
V-HTLV1-GAG-C2	ACD Bio	495051-C2	HTLV-1 DNA probe
V-HTLV1-GAG-POL-sense	ACD Bio	495061	HTLV-1 (+) sense RNA probe
V-Influenza-H1N1-H5N1-NP	ACD Bio	436221	IAV RNA probe
V-ZIKA-pp-O2	ACD Bio	464531	Zika(+) sense RNA probe
V-ZIKA-pp-O2-sense-C2	ACD Bio	478731-C2	Zika(−) sense RNA probe