

Video Article

Detection of the Genome and Transcripts of a Persistent DNA Virus in Neuronal Tissues by Fluorescent *In situ* Hybridization Combined with Immunostaining

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Abstract

Single cell codetection of a gene, its RNA product and cellular regulatory proteins is critical to study gene expression regulation. This is a challenge in the field of virology; in particular for nuclear-replicating persistent DNA viruses that involve animal models for their study. Herpes simplex virus type 1 (HSV-1) establishes a life-long latent infection in peripheral neurons. Latent virus serves as reservoir, from which it reactivates and induces a new herpetic episode. The cell biology of HSV-1 latency remains poorly understood, in part due to the lack of methods to detect HSV-1 genomes *in situ* in animal models. We describe a DNA-fluorescent *in situ* hybridization (FISH) approach efficiently detecting low-copy viral genomes within sections of neuronal tissues from infected animal models. The method relies on heat-based antigen unmasking, and directly labeled home-made DNA probes, or commercially available probes. We developed a triple staining approach, combining DNA-FISH with RNA-FISH and immunofluorescence, using peroxidase based signal amplification to accommodate each staining requirement. A major improvement is the ability to obtain, within 10 μ m tissue sections, low-background signals that can be imaged at high resolution by confocal microscopy and wide-field conventional epifluorescence. Additionally, the triple staining worked with a wide range of antibodies directed against cellular and viral proteins. The complete protocol takes 2.5 days to accommodate antibody and probe penetration within the tissue.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51091/>

Introduction

Herpes simplex virus type 1 (HSV-1) is a persistent human neurotropic virus, establishing a long-term latent infection in neurons of the trigeminal ganglia (TG) of the peripheral nervous system, from which it reactivates periodically to replicate and spread. The HSV-1 genome is a 150 kb dsDNA localizing in the nucleus of the host neuron where it remains as multicopy chromatinized plasmids, which do not integrate into the host-cell genome^{1,2}. During latency, the HSV-1 replicative cycle genetic program is strongly repressed, and gene expression is restricted to the latency-associated transcript (LAT) locus, from latency establishment to initiation of reactivation³. LAT produces a long 8.5 kb noncoding RNA processed into a major 2 kb stable lariat, and several miRNA⁴⁻⁷. HSV-1 latency is thus characterized by the presence of the viral genomic DNA, LAT RNA, and the absence of detectable replicative cycle proteins.

Animal models, predominantly mouse and rabbit, are experimental models recapitulating several features of latency in human. One of the main interests of those models is that they allow studying physiological aspects of HSV-1 latency in immunocompetent hosts. Over the past decades, many experimental tools, such as genetically modified viruses and mice, have been developed to study the physiology, genetics, and cellular biology of HSV-1 latency, from animal tissues. Until now, viral genomic DNA was detected and quantified by Southern blot and qPCR from dissociated TGs. However, there is currently no method available to detect HSV-1 genome by *in situ* hybridization on tissue sections⁸. Consequently, latency is routinely assessed on histological sections through the detection of LAT RNA by RNA *in situ* hybridization rather than viral genome detection. Because it has been impossible to characterize infected cells based on the presence of viral genomes, this technical limitation has been a major drawback to the analysis of many aspects of the host-virus interactions, such as the relationship between the viral genome and cellular and viral gene expression or the host cell-mediated immune response^{9,10}.

Most importantly, the cell-to-cell heterogeneity of the latent infection remains relatively unexplored and has been shown to be a key feature of latency in mice and in human sensory ganglion neurons implanted into SCID mice¹¹⁻¹⁷. Typically, it was shown by qPCR that the HSV-1 genome

copy number per cell varies from 5 to several hundreds. Although LAT appears as a key regulator of latency and reactivation, qPCR data on isolated neurons and *in situ* PCR indicated that only a subset of latently infected neurons, as low as 30%, expresses the LAT locus^{11,12,18-21}. How the host cell and the cellular environment within the tissue impact on the virus latency establishment and viral gene expression remains unclear. Here we describe a robust fluorescent *in situ* hybridization (FISH) method for the efficient detection of low-copy HSV-1 genomic DNA within animal neuronal tissue sections. This method has been designed and used by us to get access to high resolution microscopy imaging that is necessary to study the interaction of the viral genome with the host cell intra-nuclear components²². Additionally, we describe a multiple staining method for the simultaneous detection of the viral DNA with RNA and proteins, which is a unique tool to describe the virus-host interactions that regulate viral gene expression. The method can also be applied for a broad range of analyses requiring the detection of HSV-1 latent genome, such as quantifying infected neurons in large number of sections. A key step is to apply antigen retrieval treatment to make the viral DNA accessible to hybridization. Thus, this protocol might also be efficient to the detection of other dsDNA viruses, which are currently not detectable by conventional DNA-FISH approaches within animal tissues.

Protocol

This method was used in a study published previously²². For general background and description of conventional manipulation on ISH, IF and FISH, we suggest the following available literature²³.

1. Animal Infection

All procedures involving experimental animals conformed to ethical issues from the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in research, and were approved by the local Ethical Committee of UPR-3296-CNRS, in accordance with European Community Council Directive 86/609/EEC. All animals received unlimited access to food and water.

The method of mouse infection with HSV-1 described below has been used in studies previously published²⁴⁻²⁶.

1. Prepare the following solutions to prepare before starting:
HSV-1 virus stock solution
Anesthetizing solution - Ketamine-100 mg/kg and Xylazine-10 mg/kg
2. Take up 1 μ l of virus (10^6 pfu) into a 5 μ l glass microsyringe connected to a microsyringe pump device delivering 0.1 μ l/sec. **Note:** Resuspend the virus stock in a phenol red-free medium to see the limit between the red oil present in the capillary and the virus solution and to avoid air injection at the site of virus inoculation.
3. Lay the anesthetized mouse (Ketamine-100 mg/kg and Xylazine-10 mg/kg) on its back face up.
4. Position the anesthetized mouse head under a binocular stereo-microscope and insert the needle in the subepithelial layer of the left upper lip at the mucocutaneous border. Inject the virus solution in two steps (twice 0.5 μ l) at a speed of 0.5 μ l per 5 sec. **Note:** Respect a 10 sec pause between the two 0.5 μ l injections, to enable the viral suspension to absorb at the site of injection.
5. Place the mouse in a 37 °C incubator until waking.
6. Leave the mouse in the animal facilities to recover for the required time. **Note:** In the mouse model, HSV-1 induces a primary infection, called acute infection, which lasts less than 10 days at the site of inoculation and within several neuronal tissues including TGs, superior cervical ganglia (SCG), and dorsal root ganglia (DRG) depending on the inoculation site. Signs of primary infection progressively disappear and latency is considered fully established about 28-30 days post-infection (dpi) onwards. Neuronal tissues can be harvested usually from 4 dpi for studies on acute infection, and from 28 dpi for latency studies.

2. Mouse Perfusion-fix

1. Prepare the following solutions before starting: 50ml of physiological saline buffer
60 ml of 1x PBS
150 ml of freshly prepared 4% paraformaldehyde in 1x PBS
60 ml of 20% sucrose in 1x PBS.
2. Prepare the perfusion tubing: connect the needle to a capillary, which is connected to a peristaltic pump.
3. Anesthetize the mouse as described above (step 1.2) and lay it on its back on a dissection tray, attached by the four legs with pins.
4. Using scissors cut the skin from the belly up to the throat*. Tear away the thin tissue layer covering the organs. Open the rib cage by cutting the ribs on one side of the sternum. Remove the skin by tearing off. Move away from each other the right and left parts of the rib cage to reveal the heart. **Note:** Pay attention not to incise the guts, lungs and big vessels (carotid arteries and jugular veins), which will make perfuse-fixation impossible.
5. Insert the needle in the left ventricle*. Incise the right atrium with the scissors. Proceed with exsanguination by injecting 20 ml of physiological saline in the blood vessels through the heart. Let the blood flow in the tray. **Note:** During this procedure, pay attention not to puncture through the other side of the heart.
6. Perfuse with 150 ml of 4% PFA in 1x PBS during 15 min* (Caution: PFA is toxic, manipulate under fume hood). Perfuse 60 ml of 20% sucrose in 1x PBS during 6 min. At that stage the mouse is ready for TG harvesting. **Note:** Set the pump to 10 ml/min. A good perfusion is noticeable when the mouse tail stiffens up, lift up then fall again.

3. TG Harvesting

1. Prepare the following solution before starting:
20% sucrose in 1x PBS

2. Cut the head at the level of the neck. Cut the tip of the nose just behind the incisors in order to reveal the nose cavity. Incise the palate in two with scissors and move away each side of the palate. **Note:** The TGs appears just beneath the palate, as two white and oblong masses of 2-3 mm in length located on the right and left side and connected to the trigeminal nerve.
3. Cut the trigeminal nerve branches on each side of the TGs to release the TGs from the brainstem. Remove the TGs with surgical pliers and keep them in a 20% sucrose sterile solution for 24 hr. **Note:** Use two different recipients for the left and right TGs, to avoid confusion between the left TG (infected) and the right TG (not or weakly infected). Incubate the TGs in 20% sucrose in 1x PBS for 24 hr before embedding.
4. Embed TGs in a single block in cryosectioning embedding medium and freeze at -80 °C.
5. Store blocks at -80 °C until sectioning.

4. Cryosection Preparation

1. Cut the TGs lengthways as 10 µm sections on a -20 °C cryostat, and place them on slightly heated (30° C) Superfrost slides. Let the slices dry for 5-10 min then freeze and store at -80 °C until use. **Note:** Up to 4-5 sections can be placed on a single slide, if large number of sections is to be processed at the same time. We proceed as follow: serial sections are deposited onto 3 series of slides labeled A1, B1, and C1, then A2, B2, C2 and so on. Hence, each slide series (A, B, and C) can be processed for different staining (*in situ* hybridization, FISH, *in situ* PCR, LCM) and data obtained using the different techniques can be compared knowing that slides carrying the same number correspond to the same region of the TG.

5. HSV-1 Probe Labeling

The protocol described hereafter for the detection of HSV-1 genome by DNA FISH has been successfully used with two types of probes. The first is a home-made Cy3-labeled fluorescent probe which is appropriate for the fine analysis of nuclear organization within individual cells, by high magnification fluorescent microscopy. The second is a commercially available biotinylated probe, which can be combined with peroxidase-based signal amplification to provide a bright signal. The latter is appropriate for identification and quantification of virus containing neurons at low magnification in whole section, and for the analysis of the HSV-1 genome patterns. End-users should evaluate which approach fits best the goal of their study. The commercially available probe is listed in the reagent section, and the preparation of the home-made probe is described below.

1. Prepare the following solutions before starting:
HSV-1 genome containing vectors (see step 1)
70% ethanol, molecular biology grade.
2. Prepare cosmids containing 30 kb portions of HSV-1 genomes (cosmids number 14, 28, and 56 described in reference²⁰ using purification columns dedicated to large vectors. **Note:** Other type of libraries containing HSV-1 genomes, such as bacterial artificial chromosomes should work as well, as long as the probe covers a large portion of the HSV-1 genome to produce sufficient signal²⁷⁻²⁹. In our hands, probes made from the cosmid vector backbone did not produce any signal, and were used as negative control. Thus entire cosmid vectors containing HSV-1 sequence were used in our study. It is also possible to cut out the HSV-1 sequence and use it to prepare the probe.
3. Label 2 µg of each cosmid with Cy3-dCTP using a nick-translation kit according to the manufacturer guidelines. **Note:** Perform labeling with a reaction mix containing only Cy3-dCTP and no unlabeled dCTP.
4. Stop the reaction by adding 3 µl of 0.5 M EDTA in the mixture and heating at 70 °C for 10 min. Cool on ice.
5. Purify the probe on a G50 gel exclusion minicolumn. Add 150 µg of salmon sperm DNA to the probe and precipitate the probe by ethanol precipitation. The DNA pellet should be pink due to Cy3 incorporation. Wash the pellet with 70% ethanol and remove as much ethanol as possible with a pipette. Do not let the pellet dry.
6. Dissolve the pellet with 100 µl of deionized formamide (Caution: formamide is toxic. Manipulate under fume hood). The probe concentration cannot be reliably measured at this point. The probe quantities mentioned within the text refer to the quantity of the template DNA used to make the probe. The protocol being based on 2 µg of DNA template and 100 µl of formamide, it is considered to be 20 ng/µl. Store at -20 °C. The labeled probe can be prepared in large quantity and stored frozen for several months.

6. DNA-FISH

Figure 1 shows an overview of the main steps of the DNA-FISH protocol, and how to perform DNA-FISH as part of a multiple staining experiment to codetect RNA and protein, as described in Protocols 7-9.

1. Prepare the following solutions before starting:
0.5% Triton X-100 in 1x PBS
2x SSC and 0.2x SSC buffer
100 mM pH 6.0 citrate buffer (10x stock solution) and 10 mM pH 6.0 citrate buffer (working solution)
2x hybridization buffer (see Protocol 4)
2. On day 1, place the slides on a slide holder at room temperature, and let the sections dry for 10 min. Circle the sections with a hydrophobic pen. Rehydrate the sections in 1x PBS for 10 min. Incubate the sections 20 min with 0.5% Triton X-100 in 1x PBS to permeabilize the tissue. Wash 3x 10 min with 2x SSC, and keep in 2x SSC until the unmasking buffer is heated (see below).
3. For unmasking, prepare a glass slide tray (20 slides capacity) filled with 200 ml of 10 mM sodium citrate buffer (pH 6.0). Place the tray in a larger container filled with 500 ml of distilled water. This setting allows for a better control of heating pulses. Before placing the slides in the tray, preheat the buffer in the microwave oven until the buffer reaches boiling (around 8 min at 800 W).

4. Place the slides in the preheated citrate buffer-containing tray, and verify that they are completely covered with buffer. Heat for about 20 sec until the buffer reaches boiling. Caution, **DO NOT** let the buffer over boil, which might damage the tissue. Cool down at room temperature for 2 min. Repeat the heating cycle 6x (7 heating cycles total)*. Cool down 2 min and transfer the slides in 2x SSC for 5min.
Note*: This is one of the most critical steps. The optimal number and duration of heating cycles should be determined empirically, and may vary upon the type of tissue or the type of probe used. The microwave, tray, container and volume of buffer in the tray and water in the container should be kept identical for reproducibility of unmasking. Excessive boiling could result in tissue loss and damaged cells. For each heating pulse, the appearance of boiling is carefully watched, and heating should be stop at first signs of boiling. Once set up, unmasking appears robust and reproducible. **Figure 2A** shows that HSV-1 genome can be detected by unmasking with different buffers, indicating that unmasking conditions can be further explored. Citrate-based unmasking was found to consistently provide good FISH signal, and tissue preservation, with sections from various laboratory and animal models.
5. Incubate the slides in a methanol:acetic acid:PBS mix (3:1:4) for 15 min, then in a methanol:acetic acid mix (3:1) for 15 min (Caution: acetic acid is corrosive. Manipulate under fume hood and use adequate protection. Prepare this solution right before use).
6. Dehydrate the section through successive 10 min incubation in 70%, then 2x 10 min in 100% ethanol. Let dry at room temperature for 10 min. Keep dry until probing.
7. Prepare the probing solution as follow: prepare in advance a 20 ml stock of 2x hybridization buffer containing 20% dextran sulfate (MW 500,000), 2x Denhardt's solution, 4x SSC*. Aliquot the solution as 500 µl in microtubes, and store at -20 °C. For 1 slide (coverslip of 22 mm x 50 mm), mix 90 ng of HSV-1 Cy3-labeled probe (30 ng of probe for each cosmid), and complete the volume to 40 µl with formamide. Add 40 µl of 2x hybridization buffer. Mix well by pipetting up and down several times.
Note*: To prepare the 2x hybridization buffer, mix 4 g of MW 500,000 dextran sulfate in 10 ml of distilled water. It makes a viscous mix that dissolve upon 3-4 hours at 70 °C. Mix regularly to help dissolve. Add 4 ml of 20x SSC, 400 µl of 100x Denhardt's solution. Complete to 20 ml and mix well using a vortex.
8. Drop 80 µl of probing solution onto the dried sections. Cover with a 22 mm x 50 mm glass coverslip, and verify that the probing solution spreads over the entire surface of the coverslip. Caution: there should be no bubbles. Presence of bubbles decreases hybridization efficiency. Seal the coverslip using rubber cement, and let it dry. Keep the slides in the dark at room temperature for at least 2 hr for optimal hybridization signal throughout the slide.
Note: Rubber cement is convenient as it dries quickly, is waterproof, and protects the sample from drying during hybridization. It is also easy to peel off to remove the coverslip after hybridization. Nail polish is an alternate efficient, but less convenient option.
9. Proceed with denaturation by placing the slides on an 80 °C slide incubator for 5 min. Alternatively, place the slides on a metal tray and place the tray in an 80 °C water bath (the tray should float). Then quickly transfer the slides onto a metallic tray placed on ice. Leave for 5 min. Transfer the slides at 37 °C (slide heater or incubator) for overnight hybridization.
Note: We do not recommend shorter hybridization, which results in weak or no signal.
10. On day 2, remove the rubber cement with forceps, while maintaining the slide onto the heater to keep the section at 37 °C. Remove the coverslip gently with the tip of a scalpel blade.
11. Wash 3x with 2x SSC at 37 °C for 5 min, and three times with 0.2x SSC at 37 °C for 5 min. Wash once with 2x SSC at room temperature for 5 min and proceed with DNA staining and mounting (see Protocol 10 below).
Note: This method allows for the detection of cellular genomic targets, by using corresponding probes into the probing solution together with HSV-1 specific probes as published for centromeric and pericentromeric sequences²².

7. Dual RNA-DNA FISH

See **Figure 1**, green boxes for an overview.

For multiple staining procedures including an RNA-FISH step, it is generally advised to first perform RNA detection as such target is sensitive to degradation by RNase and chemicals. Additionally, DNA-FISH procedure includes treatments that reduce the efficiency of other staining. For RNA-FISH, we chose an enzyme based detection approach (Tyramide Signal Amplification (TSA) using biotinylated probes and peroxidase (HRP) coupled streptavidin). TSA is based on a fluorescent tyramide substrate (see reagent table for details), which is covalently linked to the tissue by a peroxidase enzymatic reaction. The RNA-FISH signal is thus preserved during DNA-FISH.

1. Prepare the following solutions before starting:
 Vector for *in vitro* transcription of LAT locus (pSLAT-2)
 1x PBS containing 2 mM RVC
 0.5% Triton X-100 in 1x PBS containing 2 mM RVC
 3% H₂O₂ in distilled water.
 70%, 80%, 95% ethanol, molecular biology grade.
 4x RNA hybridization solution (see Protocol 4)
2. At least one day before the RNA-FISH experiment, prepare the RNA FISH probe. To detect the HSV-1 Latency Associated Transcript (LAT), prepare a biotinylated ribo-probe from the pSLAT-2 vector³⁰, or another vector for *in vitro* transcription of the LAT locus, as previously described in reference²⁶. Briefly: Linearize 10 µg of pSLAT-2 with HindIII and purify the digested vector with a DNA purification kit. Synthesize a single strand ribo-probe from 2 µg of digested pSLAT-2 with a T7 *in vitro* transcription kit in the presence of biotin-16-UTP*. Purify the probe with an RNA mini-column and quantitate at 260 nm using a spectrophotometer. Dilute the probe at 50 ng/µl in DNase/RNase free water and store at -80 °C, in small aliquots to avoid freezing-thawing cycles.
Note*: The biotin-16-UTP:UTP ratio must be empirically determined. We found a 40:60 ratio to provide probes efficiently detecting LAT by RNA-FISH.
3. On day 1, place the slides on a slide holder at room temperature, and let the sections dry for 10 min. Circle the sections with a hydrophobic pen. Rehydrate the sections in 1x PBS containing 2 mM Ribonucleoside Vanadyl Complex (RVC)* for 10 min. Incubate the sections for 20 min with 0.5% Triton X-100 in 1x PBS containing 2 mM RVC to permeabilize the tissue. Wash 3x 10 min with 1x PBS, 2 mM RVC. To quench any endogenous peroxidase activity, incubate the section in 3% H₂O₂ for 2x 10 min, and then wash once with 1x PBS, 2 mM RVC.

Note*: Throughout the RNA-FISH procedure, ribonucleoside vanadyl complex (RVC) is added to buffers and solutions to prevent RNA degradation.

4. Incubate 2x 10 min in 70% ethanol. At this stage, sections can be stored in 70% ethanol at -20 °C for several weeks. Dehydrate the sections by incubation in 80%, 95%, and 100% ethanol, 5 min each. Let the section dry for at least 10 min.

Note: In some cases, ethanol treatment can be deleterious for detection of other targets. An alternate protocol using a prehybridization step in 50% formamide - 2x SSC can be used (see below in Protocol 9 for details on triple staining). However, ethanol treatment is the most versatile approach for RNA-FISH and provides the lowest background.

5. Prepare the probing solution as follow: prepare in advance a 10 ml stock of a 4x RNA hybridization solution containing 8x SSC, 20x Denhardt's solution, 4 mM EDTA, 40% dextran*. Aliquot the solution as 500 µl in microtubes, and store at -20 °C. For 1 slide prepare 80 µl of solution (coverslip of 22 mm x 50 mm), by mixing 20 ng of biotinylated LAT riboprobe, 40 ng of yeast tRNA, 2 mM RVC, and complete to 20 µl with water. Add 20 µl of 4x RNA hybridization solution, and 40 µl of formamide (50% final concentration). For easier pipetting and mixing, it is recommended to prewarm the 4x RNA hybridization solution at 75 °C. Mix well by pipetting up and down several times.

Note*: To prepare the 4x RNA hybridization solution, mix 4 g of dextran sulfate (MW 500,000) in 3 ml of distilled water and 4 ml of 20x SSC. It makes a viscous mix that dissolves upon 3-4 hr at 70 °C. Mix regularly to help to dissolve. Add 2 ml of 100x Denhardt's solution, and 80 µl of a 0.5 M EDTA solution. Complete to 10 ml with water and mix well using a vortex. Caution: use only RNase/DNase free products.

6. Denature the probe 10 min at 75 °C. Meanwhile, place the slides on a slide incubator set to 65 °C. Drop 80 µl of probe solution onto the sections and quickly place a coverslip onto the drop. Caution: there should be no bubble. Presence of bubbles decreases hybridization efficiency. Incubation has to take place in a humidified chamber since the coverslip is not sealed. Use either a slide incubator that can hold water (see material table), or prepare a humidified chamber within a sealed box and place it in a 65 °C hybridization oven.
 7. Incubate overnight at 65 °C. **Note:** LAT RNA-FISH is carried out at 65 °C to prevent nonspecific binding of the probe, which is observed at 37 °C. Many other RNA-FISH probes should result in good signal at 37 °C.
 8. On day 2, before commencing washing, prepare the detection reagents according to the manufacturer instructions of the TSA detection kit. Detection is performed with a commercial TSA detection kit including a horseradish peroxidase (HRP) coupled streptavidin and a green or blue fluorescent substrate.
 9. Keep the slides on the slide heater at 65 °C. Carefully remove the coverslip and quickly add 1 ml of 50% formamide in 2x SSC, prewarmed at 65 °C. Caution, do not let the section dry. Wash twice 10 min at 65 °C in 50% formamide in 2x SSC and 2x 10 min in 2x SSC at 65 °C. Wash once more with 2x SSC and place the slide on a 37 °C slide heater.
 10. Proceed with the detection step according to the TSA detection kit manufacturer instruction. The concentration of HRP-streptavidin, the concentration of fluorescent substrate and the time of reaction must be empirically determined*. For LAT RNA detection, we routinely used the following condition: HRP-streptavidin diluted at 1/500, fluorescent reagent at 1/100 for blue fluorescence and 1/500 for green fluorescence, and reaction time of 10 min. The signal can be quickly checked with an inverted fluorescent microscope without mounting, before proceeding with DNA-FISH.
- Note*:** The amplification protocol will be design according to the main goals of the experiment. For example, to finely localize the target RNA, it is advised to use short reaction time. In contrast, to quickly identify and count positive cells at low magnification, reaction time can be increased to generate a bright signal.
11. For DNA-FISH, follow the procedure indicated above, starting from the unmasking step (step 6.2).

8. Immuno-DNA FISH

See **Figure 1**, purple boxes for an overview.

Similarly to RNA-DNA-FISH, it is advised to perform first the immunofluorescence, since DNA-FISH is likely to denature proteins and prevent their detection by antibodies. The quality of immunofluorescence signal is highly dependent on the antibody characteristics, and several antibodies should be tested whenever possible. Epitope unmasking is performed once before the immunofluorescence to improve both the protein detection and DNA-FISH. To preserve the immunofluorescence signal on the sample during the DNA-FISH procedure it is necessary to covalently link it to the tissue. We present here two approaches that provided good results in our hands, antibody post-fixation and tyramide based detection (see step 8.5). The choice should be driven by preliminary tests for each target/antibody pair.

1. Prepare the following solutions before starting:
1x PBS containing 3% normal goat serum (NGS).
2% PFA in 1x PBS (dilution from the 4% stock solution)
2. On day 1, the first steps are identical to DNA-FISH, up to the unmasking step (steps 6.1-6.2).
3. After unmasking, incubate the sections with 1x PBS containing 3% NGS for 1 hr.
4. Incubate with the primary antibody diluted in 1x PBS containing 3% NGS for 24 hr. Lower incubation time can be used to shorten the protocol, although we found that an overnight incubation usually results in a stronger signal. Up to 48-72 hr of incubation might be required for low affinity primary antibodies such as IgM.
5. On day 2, wash 3x 10 min with 1x PBS.
6. **Protocol with antibody post-fixation:** incubate 1 hr with the secondary antibody coupled with a green fluorescent dye, at 1/200 in 1x PBS containing 3% NGS. Wash three times 10 min with 1x PBS. Post-fixation is performed with 2% PFA in 1x PBS for 10 min*. Wash 3x 10 min with 1x PBS.

Protocol with TSA detection: incubate 1 hr with the HRP-coupled secondary antibody at 1/250 in 1x PBS containing 3% NGS. Wash three times 10 min with 1x PBS. Proceed with tyramide detection according to the manufacturer instructions. As indicated above (step 7.9), reagent dilution and reaction time must be empirically determined. In most cases, our protocol has been based on a 1/500 dilution of the fluorescent substrate and a 10 min reaction time. Wash 3x 10 min with 1x PBS.

Note *: Post-fixation is a critical step in immuno-FISH, and requires careful set-up. The stronger the post-fixation the better the IF signal, and the lower the DNA-FISH efficiency.

7. Proceed with DNA-FISH from the methanol-acetic acid step (step 6.3). Duration of immuno-DNA-FISH is typically 3 days, with the first antibody incubated overnight.

9. Dual DNA-RNA FISH Coupled with Immunofluorescence

See **Figure 1**, orange boxes for an overview.

RNA-FISH is performed first, followed by immunofluorescence, and lastly DNA-FISH. If immunofluorescence is detected by tyramide reaction, it is key to quench completely the HRP activity from the RNA-FISH step with H_2O_2 , and to verify that quenching is efficient. This is done by using one slide as a "no primary antibody" control.

Because ethanol-based dehydration is deleterious for some solvent-sensitive proteins, this step of RNA-FISH can inhibit immunofluorescence. If so, RNA-FISH can be performed with an alternate protocol, as detailed below in step 9.3.

1. Prepare the following solution before starting:
50% formamide in 1x PBS containing 2 mM RVC
2. On day 1, prepare the RNA-FISH probe and proceed as for dual RNA-FISH, up to the H_2O_2 quenching step (step 7.2).
3. **Case 1**, the immunofluorescence staining works after ethanol dehydration: proceed with RNA-FISH as indicated above in steps 7.3-7.9. Then proceed to step 9.6 below.
4. **Case 2**, the immunofluorescence staining is prevented by ethanol treatment: Place the slides in a humidified chamber on a slide heater set to 65 °C and incubate for 1.5 hr in a prehybridization solution containing 50% formamide, 1x PBS, and 2 mM RVC. Drain the prehybridization solution and drop 80 μl of hybridization solution as indicated in steps 7.4 and 7.5. Then proceed with RNA-FISH hybridization and detection as indicated above in steps 7.6-7.9 (day 2 and 3).
5. On day 2, after RNA-FISH is completed, proceed with immuno-DNA-FISH starting with the unmasking step (see step 6.2). Then, follow the immuno-DNA-FISH protocol as indicated above in steps 8.2-8.6.

10. Slide Mounting and Imaging

1. Prepare the following solution before starting:
Hoechst 33342 at 0.5 $\mu\text{g}/\text{ml}$ in 1x PBS
2. Stain nuclei for 10 min with DAPI or Hoechst 33342* at 0.5 $\mu\text{g}/\text{ml}$ in 1x PBS for 10 min. Wash 3x 10 min with 1x PBS.
Note *:Caution. If one of the detection systems uses a fluorescent blue dye, do not use DAPI or Hoechst. Instead, use a fluorescent DNA dyes with an emission spectra within the far-red wavelength such as Topro3.
3. Drain as much liquid as possible from the slide. Drop 80 μl of mounting medium containing an antifading agent on one end of the slide. Cover the sections with a high optical quality coverslip ($n \approx 1.5$ glass). Let the coverslip go down slowly by maintaining it at one end with forceps, in order to let the mounting medium spread over the section without bubbles.
4. Seal with nail polish and store at 4 °C in a dark slide box.
5. Direct observation of DNA-FISH signal of latent HSV-1 genomes requires a 40X or higher magnification oil immersion objective, with high numerical aperture (for example 40X N.A 1.1, 60X-63X N.A 1.4, 100X N.A 1.3) and an excitation light source of at least equivalent to a 100 W mercury lamp. We advise using a high-efficiency transmission microscope such as those provided by manufacturers in the last 5 years (see table of equipment). Confocal microscopy provides tools to collect images with lower background due to auto-fluorescence of the tissue, such as thin sectioning or spectral imaging.

Representative Results

After several months of extensive testing, we discovered that heat-based chemical unmasking made latent HSV-1 genome available for fluorescent *in situ* hybridization. During the process, we tried various unmasking procedures, and only heat-based treatments (*i.e.* heating the sections up to sub-boiling temperature in a microwave oven) appeared efficient. We then tested several salt buffers that are routinely used in immunohistochemistry (IHC) and electron microscopy to retrieve epitopes^{31,32}, including 0.01 M pH 6.0 Citrate buffer (that we used in all our studies), 1x PBS, 1mM EDTA, 0.1 M Tris-HCl pH 7.4 and distilled water. While EDTA buffers tend to damage the tissue, all other buffers were suitable to HSV-1 detection, which appears as single or multiple spots within the nucleus of neurons (**Figure 2A**, Note that these tissues are highly auto-fluorescent, which appears in some images as a homogeneous signal in the cytoplasm). In our hands, citrate buffer appeared to constantly provide a good signal without damaging the tissue and thus was chosen for our routine protocol.

The use of directly labeled Cy3-DNA probes (made from parts of HSV-1 genome cloned in cosmids) provides robust and reproducible results. However, it requires having access to HSV-1 genome libraries. We thus verified that our protocol would work for anyone having access only to commercial probes. **Figure 2B** shows HSV-1 latent genome detection by DNA-FISH using a pan-HSV-1 biotinylated probe obtained from Enzo Biochem. Along these lines, we tested whether the DNA-FISH protocol could potentially be used by scientists using other HSV-1 animal models. **Figure 3A** illustrates the detection of HSV-1 genome on samples from mouse and rabbit, infected with three commonly used strains, SC16, 17syn+ and McKrae, at either acute or latent stage of infection, within sections of trigeminal ganglia. In all cases HSV-1 genomes show as a spotty signal, of brightness and intensity that varies from cell to cell. Finally, we extended the applicability of our protocol to the replicative cycle of HSV-1, by performing DNA-FISH on tissues from mice undergoing a general herpes infection. In these animals large and bright aggregates of HSV-1 genomes could be detected in various tissues including brain, spinal cord, eyes and dorsal root ganglia (**Figure 3B**).

Many aspects of HSV-1 latency are still poorly understood, such as how HSV-1 gene expression is regulated through its interactions with the nuclear architecture³³⁻³⁵, whether a specific subset of neurons are preferred host-cell for latency establishment and reactivation^{13,14,36,37}, or how

immune surveillance takes place within the ganglia according to virus load or virus gene expression^{9,10}. The protocol described here will help tackle these questions, by codetection of HSV-1 genome and viral or cellular RNAs and proteins. **Figure 4** illustrates the codetection of HSV-1 genome together with the HSV-1 LAT RNA (**Figures 4A and 4C**), with cellular proteins such as the centromeric protein CENP-A (**Figure 4B**, IF followed by PFA post-fixation), or the chromatin and PML-NB associated protein ATRX (**Figure 4C**, IF followed by TSA based detection). **Figure 4C** illustrates data from a triple staining experiment showing HSV-1 DNA (red), its RNA product LAT (blue) and a candidate regulatory protein, ATRX (green). The combination of our DNA-FISH protocol and direct or enzyme based detection of RNA-FISH and immunofluorescence signal, represent a versatile and widely applicable set of tools to explore *in situ* the virus-host relationship at the cell and tissue level.

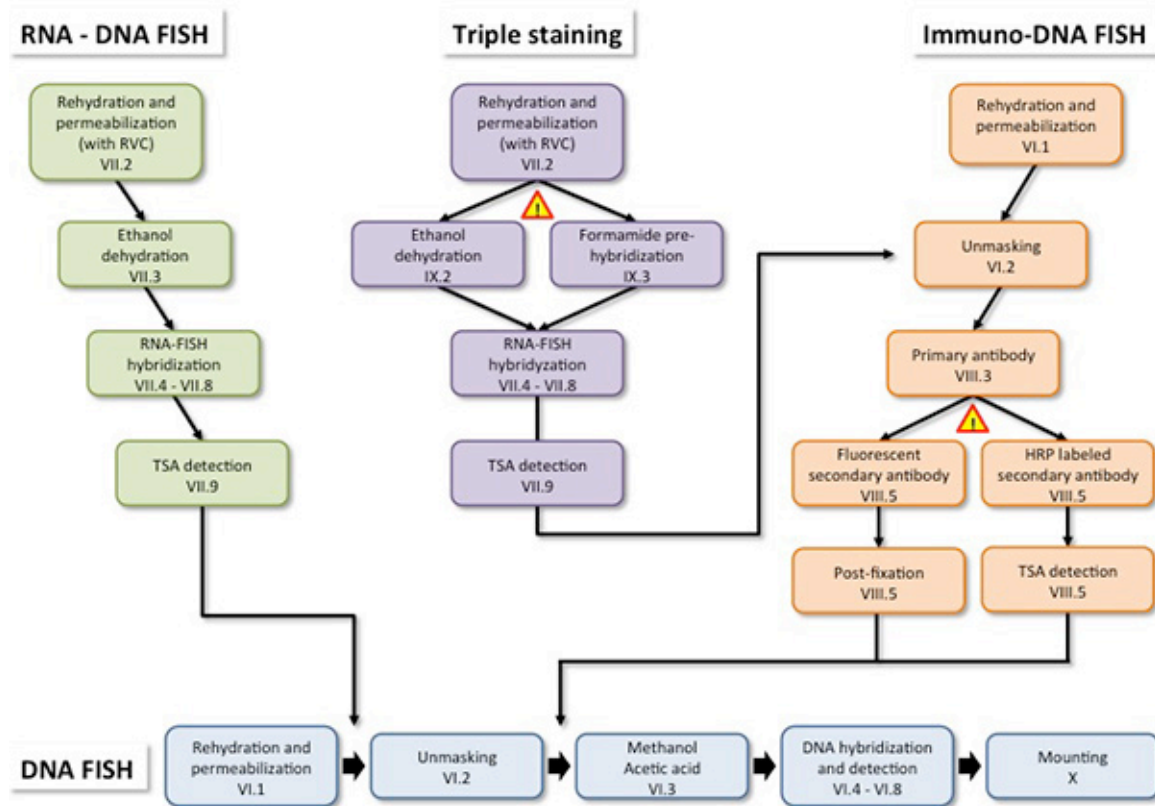


Figure 1. Overview of the DNA-FISH protocol and integration into multiple target staining. The main steps of the DNA-FISH protocol and the connection with protocols for codetection of RNAs and proteins are schematically represented. Critical steps are indicated by warning signs. DNA-FISH main steps are rehydration, permeabilization, unmasking, methanol-acetic acid treatment and hybridization. Within the procedure, unmasking is a critical step, which needs to be carefully set-up and respected. The two other critical steps depend on which technical procedures are compatible with the antibodies used for immunodetection. These will impact on the RNA-FISH procedure for the triple staining, and on the detection strategy of immunofluorescence. [Click here to view larger image.](#)

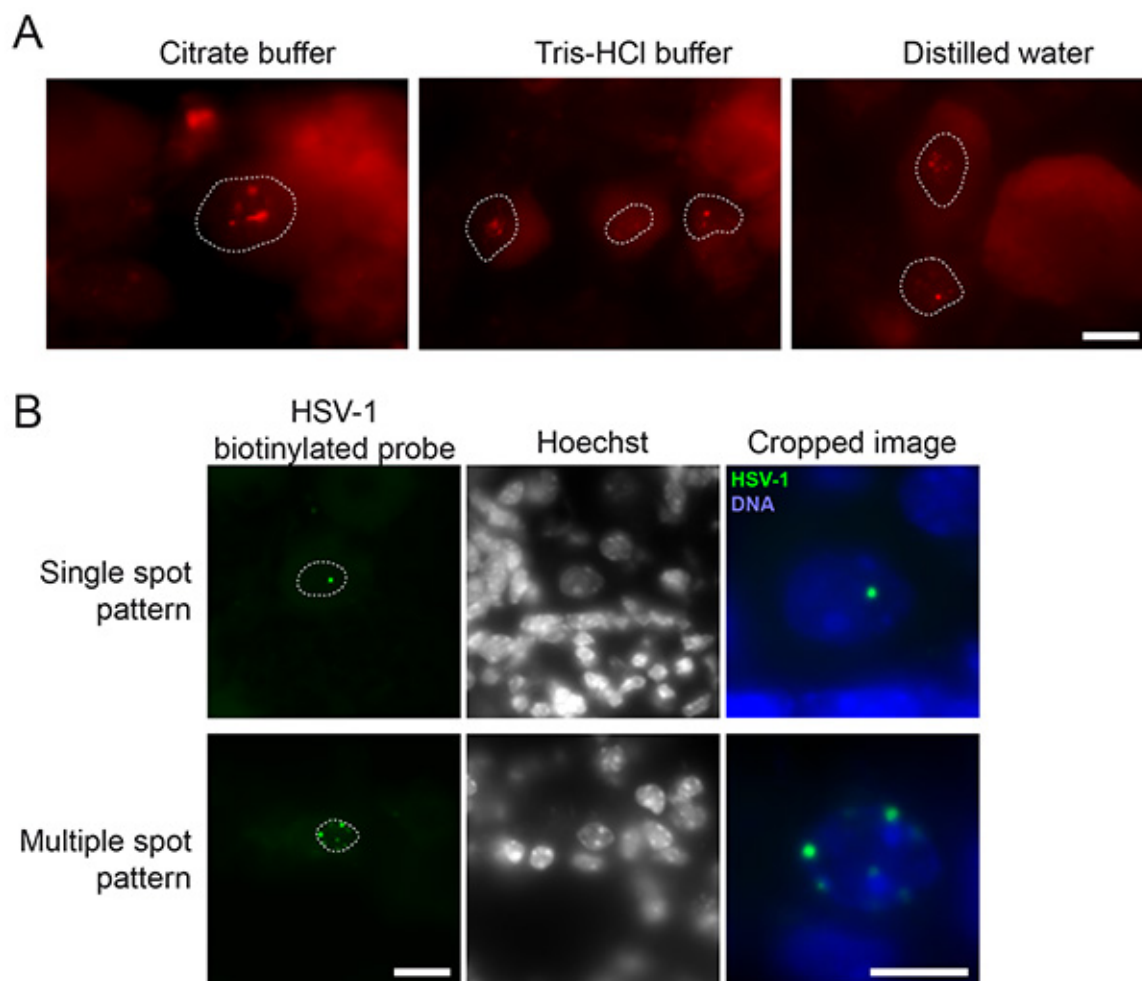


Figure 2. Detection of HSV-1 latent genome after antigen unmasking. **A.** TG sections from 28 dpi infected mice were prepared as indicated in the protocol section. TG sections were processed for DNA-FISH as indicated in **Figure 1**, and the heat-based unmasking was performed using the buffer indicated on top of each image. The outline of the nucleus is depicted as a dashed line. The signal observed in the cytoplasm is due to auto-fluorescence of the tissue. **B.** TG sections prepared as in **A.** were processed for DNA-FISH using a commercially obtained biotinylated HSV-1 probe. The hybridized probe was detected using TSA and an Alexa Fluor labeled substrate (green). Nuclei were counterstained with Hoechst 33352 (blue). An enlarged cropped image is shown in the right column. Two types of HSV-1 genome pattern are shown to illustrate a typical HSV-1 intranuclear localization. All images were collected on a wide-field epifluorescence microscope. Scale bar is 5 μ m. [Click here to view larger image.](#)

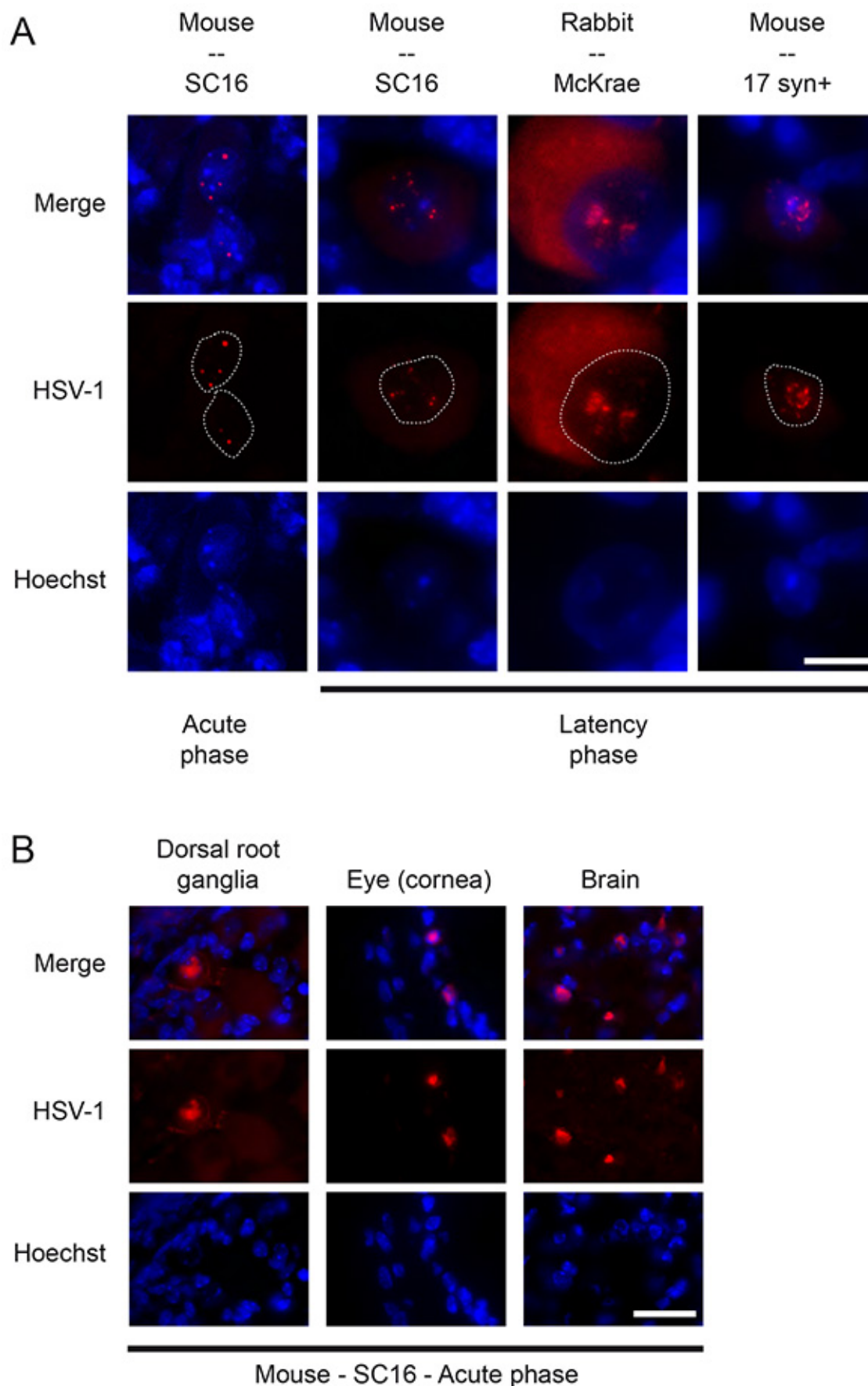


Figure 3. Detection of HSV-1 genome in several models. **A.** The standard DNA-FISH protocol was applied to TG sections from various origins. SC16 infected mouse TG sections were prepared as described in the protocol section. 17syn+ infected mouse sections and McKrae infected rabbit sections were provided by collaborators. Images were collected on a wide-field epifluorescence microscope. Scale bar is 5 μ m. **B.** SC16 infected mice undergoing a general herpes infection were sacrificed at 6 dpi and several tissues were collected, frozen and sectioned. The standard DNA-FISH protocol was applied as indicated in **Figure 1**. Images were collected on a wide-field epifluorescence microscope. Scale bar is 10 μ m. [Click here to view larger image.](#)

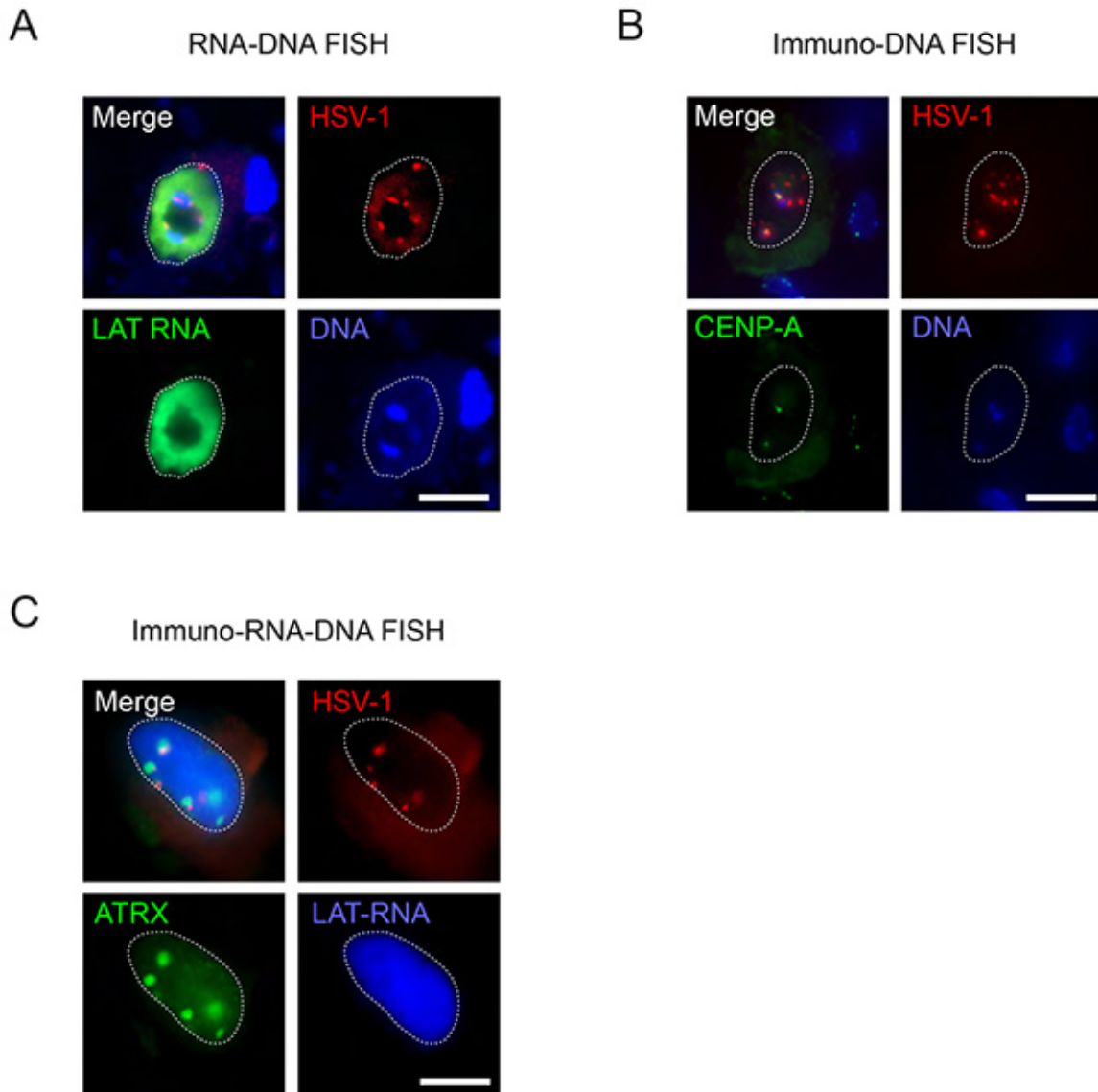


Figure 4. Codetection of HSV-1 genomic DNA and RNAs and proteins on single sections. SC16 infected mouse TG sections were processed for RNA-DNA FISH, immuno-DNA FISH or the triple staining as indicated in **Figure 1**. **A.** RNA-DNA FISH using a HSV-1 genome Cy3 labeled probe (red) and a RNA-LAT biotinylated ribo-probe (green). LAT RNA probe was detected using TSA and an Alexa Fluor 488 labeled substrate. Nuclei were counterstained with Hoechst 33352 (blue) **B.** Immuno-DNA FISH using an anti-CENP-A antibody (green) and a HSV-1 genome Cy3 labeled probe (red). The antibodies were post-fixed 10min with 1% PFA in PBS before running DNA-FISH. Nuclei were counterstained with Hoechst 33352 (blue). **C.** Immuno-RNA-DNA-FISH using a RNA-LAT biotinylated ribo-probe (blue), an anti-ATRX antibody (green) and a HSV-1 genome Cy3 labeled probe (red). The LAT RNA probe was detected using tyramide detection and a blue fluorescently labeled substrate, and the anti-ATRX and secondary antibodies were detected by tyramide detection and a green fluorescently labeled substrate. All images were collected on a wide-field epifluorescence microscope. Scale bar is 5 μ m. [Click here to view larger image.](#)

Discussion

The protocol described here allows the detection of HSV-1 latent genome within neurons of mouse neuronal tissue sections. Our understanding of the pathways regulating viral gene expression has been limited by the lack of method to detect HSV-1 genomic DNA *in situ* within neuronal tissues. Information on genome copy number and proportion of infected neurons came mainly from PCR analysis on dissociated neurons^{11,12}. In elucidating the role the host-cell nuclear architecture on HSV-1 latency, we set to determine the localization of latent HSV-1 genome by DNA-FISH, within the nucleus of neurons of latently infected mice. We have tested a wide variety of DNA-FISH protocols and tissue treatments, and found heat-based "epitope unmasking" as an essential step of virus DNA-FISH detection. Such treatment is routinely used to reveal protein epitope within paraffin embedded sections for immunohistochemistry. Although it is almost universally used in pathology, it is not conventionally used in DNA-FISH. While many studies support that this technique preserves the morphology of the tissue at the scale of light microscopy, the end-user should include appropriate controls to validate this point in his particular biological system³⁸. In our hands, other unmasking procedures such as protease treatments did not allow HSV-1 DNA detection, while heat-based unmasking using various buffers consistently made HSV-1 latent genomic DNA available to FISH probes (**Figure 2**). Heat-based unmasking is thought to eliminate part of the crosslinks between proteins, indicating that HSV-1 DNA is tightly associated to proteins. This is consistent with the recent demonstration that HSV-1 latent and lytic genome is

associated with cellular histones^{2,39,40}. Because the genomes of other herpesviruses are associated with histones: VZV⁴¹, HCMV^{42,43}, EBV⁴⁴⁻⁴⁶, KHSV⁴⁷⁻⁴⁹, the protocol described here might be applied to detect the genomes of these herpesviruses as well as many others, but probably also to detect other persistent nuclear viruses such as papillomaviruses, hepatitis B virus, and retroviruses. Interestingly, the use of the current protocol on different experimental settings (tissues from infected mice and rabbits, sections prepared by different laboratories, and use of different HSV-1 strains) did not require additional set-up for detection of HSV-1 genome. To apply the protocol to other biological systems, we anticipate that fixation procedure and antigen retrieval techniques could be areas of further development.

The classical approach in evaluating HSV-1 latency is to detect the presence of LAT RNA combined to the absence of detection of lytic cycle gene products in neurons. However, studies based on *in situ* PCR and qPCR on isolated neurons from mouse models of latency indicated that the number of infected neurons (*i.e.* HSV-1 genome positive neurons) was two to three time higher than the LAT expressing neurons^{12,18}. Using RNA-DNA-FISH, it was confirmed that in our mouse model 20-30% of HSV-1 DNA positive neurons are also positive for LAT RNA²². The use of DNA-FISH and codetection of viral and cellular DNA, RNA and protein components will provide a new set of tools to characterize the pathways regulating HSV-1 latent gene expression. In addition, HSV-1 latency is known to be a heterogeneous phenomenon as it takes place in a wide variety of neuron subtypes, and it is characterized by heterogeneity in genome copy number and in LAT RNA expression^{12,22}. A major benefit of DNA-FISH is to provide access to single cell analysis within the complexity of the tissue, and thus to take into account the heterogeneity of HSV-1 latency. For example, we have linked the expression of LAT RNA with the abundance of HSV-1 genome in individual neurons²². In addition, this technique may also be applicable to evaluating the status of viral genomes using *in vitro* cell culture models as well as animal models utilizing human ganglion implanted into SCID mice¹³⁻¹⁷. A future application of our DNA-FISH protocol will be the possibility to characterize HSV-1 latency through the number of HSV-1 genome positive neurons. This could be performed using biotinylated probes and tyramide-based detection, which produces a signal strong enough to be detected at low magnification. Such application is made possible by the very low background generated by the tyramide detection system. The Cy3 labeled HSV-1 probes described in this protocol could be used as well however requires observation at higher magnification, which would be highly time-consuming to read large number of sections.

Perhaps the major qualities of the DNA-FISH protocol described here are versatility and robustness, which makes it compatible with the codetection of both RNA and protein. Indeed we found that all treatments required to detect RNA or protein, or both, do not alter the quality and brightness of the DNA-FISH signal. RNA-FISH can be performed before DNA-FISH, using either ethanol dehydration or formamide-based prehybridization. We recommend the ethanol-based protocol, which results in less background with TSA detection reagents. The formamide-based protocol should be used when RNA-FISH is followed by immunofluorescence with antibodies that do not work on ethanol treated samples. When performing immuno-DNA-FISH, covalent linking of the immunofluorescence signal can be performed by tyramide-based detection (which amplifies the signal), or by post-fixation to preserve the details of the protein localization pattern. To optimize post-fixation, the immunofluorescence protocol should first be set-up to get a strong signal, and the strongest post-fixation procedure allowing good DNA-FISH signal should be determined. This will provide a range of conditions within which the best compromise between immunofluorescence preservation and DNA-FISH signal can be obtained. As for any other antibody-based detection method, the quality of the signal and the best protocol is highly dependent on the antibody itself. Our protocol is no exception and we have observed that some antibodies work very well with any of the protocol described here (for example anti-PML mAb clone 36.1) and some others need significant testing. Overall, we successfully detected most of our intended target (an exception was SP100 protein), including cytoplasmic and membrane proteins, and nuclear proteins associated with various nuclear domains (chromatin -HP1-, centromeres -CENP-A, CENP-B-, PML nuclear bodies -PML, Daxx, ATRX-). On the basis of our testing we anticipate that our DNA-FISH protocol be compatible with the immuno-codetection of reporter constructs (β -galactosidase, fluorescent proteins...) that are commonly used to analyze promoter activity from viral genomes.

Disclosures

The authors declare no competing financial interests.

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