Journal of Visualized Experiments

Detection of the genome and transcripts of a persistent DNA virus in neuronal tissues by fluorescent in situ hybridization combined to immuno-staining

--Manuscript Draft--

Manuscript Number:	JoVE51091R2
Full Title:	Detection of the genome and transcripts of a persistent DNA virus in neuronal tissues by fluorescent in situ hybridization combined to immuno-staining
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	- Virology - Herpes Simplex Virus (HSV) - Latency - In situ hybridization - Nuclear organization - Gene expression - Microscopy - Neuroscience
Manuscript Classifications:	95.51: Life Sciences (General); 95.51.26: microbiology
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Abstract:	Single cell co-detection 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 10µm 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.

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April 24th 2013

Dear Editor,

Thank you for inviting us to submit our manuscript entitled: “Detection of persistent DNA virus genome and transcripts in neuronal tissue sections by fluorescent *in situ* hybridization combined to immuno-staining.” By Catez F., Rousseau A., Labetoulle M., and Lomonte P. This manuscript describes the method we recently developed and published in the PLoS Pathogens article "HSV-1 Genome Subnuclear Positioning and Associations with Host-Cell PML-NBs and Centromeres Regulate LAT Locus Transcription during Latency in Neurons". By Catez F, Picard C, Held K, Gross S, Rousseau A, Theil D., Sawtell N., Labetoulle M., and Lomonte P.

The impact of nuclear organization and nuclear domains on regulation of gene transcription has become a major issue in the study of eukaryote genes expression. The originality of the study, beyond the scientific novelties on transcriptional regulation of a viral genome through nuclear positioning, is that it uses fluorescent-based approaches to analyse, at the single cell level and in neuronal tissues, the behaviour of the genome of a parasite inside the cell. To do so, we have developed and applied a triple staining method based on DNA and RNA fluorescent *in situ* hybridization (FISH) combined to immuno-staining, to detect simultaneously, and in the same cell, a DNA locus, its RNA product, and different cellular proteins. This approach has, to our knowledge, never been used for the study of the regulation of a viral genome in tissue sections, and particularly in neuronal tissues from infected adult animal models, or from human samples.

This paper describes in a step-by-step protocol the method that enables the detection of HSV-1 DNA, HSV-1 transcripts, and either centromere loci or cellular proteins. The major aspect of the detection of the viral genome by FISH in tissues is the absolute requirement to perform an unmasking step (just like what is done in ISH to reveal protein epitopes). This unusual way of performing FISH to reveal DNA has been the key step that enabled us to overcome the difficulties that prevented many teams to successfully conduct FISH studies on infected tissues. Another key aspect of our protocol is the combination of nucleic acid staining (DNA and RNA) using direct and indirect labeling (biotin/streptavidin or digoxigenin/anti-DIG followed by TSA detection), in order to obtain optimal results and to be able to visualize three colored signals. The methodological details of

these key steps can only be provided through a step-by-step protocols including troubleshooting, which are not currently available in the literature. The article includes details and troubleshooting on the unmasking step and on how to perform the triple staining according to the performance and quality of the users' antibodies. Our method is robust, reproducible, allow the use of conventional high-resolution confocal microscopy, and have been validated in animal models, as well as in human tissues.

We believe that our protocol can be useful for researchers in the fields of Microbiology and Cell Biology. Certainly, the *in situ* detection of HSV-1 viral genomes during latency in mouse tissues represents a major breakthrough in the field of virology. Indeed, the lack of efficient *in situ* detection technique has been a major issue that considerably slowed-down the understanding of herpesvirology by hindering access to key cell biology data and information on virus-host cell interactions, including for other major human pathogens such as Epstein-Barr virus (EBV), human cytomegalovirus (CMV), varicella-zoster virus (VZV) or Kaposis' sarcoma virus (KSHV).

Thank you again for giving consideration to our manuscript.

Sincerely,

Patrick Lomonte.

Additional requested information:

Author contribution.

F. Catez and P. Lomonte designed the DNA-FISH protocol and performed staining experiments, M. Labetoulle designed animal infection protocol, A. Rousseau and M. Labetoulle performed animal experiments and sections, F. Catez and P. Lomonte wrote the manuscript.

Name of editor who assisted with submission process.

Nandita Singh

List of 6 peer reviewers, and up to 3 opposed reviewers.

- Pr. Roger Everrett. MRC, University of Glasgow, UK.
- Pr. Stacey Efstathiou. University of Cambridge, UK.
- Pr. Nancy Sawtell. Cincinnati Children's Hospital Medical Center, USA.
- Pr. Lynn Enquist. Princeton University, USA.
- Pr. Ian Mohr. New York University, USA.
- Pr. Thomas Stamminger. Erlangen University, Germany.

- No opposed reviewer.

Date of filming

Several of the contributors will not be available from July 22nd to end of August.

Detection of the genome and transcripts of a persistent DNA virus in neuronal tissues by fluorescent in situ hybridization combined to immuno-staining.

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KEYWORDS (6 to 12 key words)
Virology, Herpes Simplex Virus (HSV), Latency, *In situ* hybridization, Nuclear organization, Gene expression, Microscopy, Neuroscience

SHORT ABSTRACT
We established a fluorescent *in situ* hybridization protocol for the detection of a persistent DNA virus genome within tissue sections of animal models. This protocol enables studying infection process by co-detection of the viral genome, its RNA products and viral or cellular proteins within single cells.

LONG ABSTRACT
Single cell co-detection 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 10µm 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.

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 150kb 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 reactivation [3]. LAT produces a long 8.5kb non-coding RNA processed into a major 2kb stable lariat, and several miRNA [4-7]. 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 immuno-competent 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 sections [8]. 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 mice [11-17]. 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 components [22]. 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 previously [22]. For general background and description of conventional manipulation on ISH, IF and FISH, we suggest the following available literature [23].

I. 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 published [24-26].

1. Prepare the following solutions to prepare before starting:
HSV-1 virus stock solution
Anesthetizing solution - Ketamine-100mg/kg and Xylazine-10mg/kg

2. Take up 1µL of virus (10^6 pfu) into a 5µL glass micro-syringe connected to a micro-syringe pump device delivering 0.1µL/sec. **Note:** Re-suspend 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-100mg/kg & Xylazine-10mg/kg) on its back face up.

4. Position the anesthetized mouse head under a binocular stereo-microscope and insert the needle in the sub-epithelial layer of the left upper lip at the muco-cutaneous border. Inject the virus solution in two steps (twice 0.5µL) at a speed of 0.5µL per 5 sec. **Note:** Respect a 10 sec pause between the two 0.5µL injections, to enable the viral suspension to absorb at the site of injection.

5. Place the mouse in a 37°C incubator until wakening.

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.

II. MOUSE PERFUSION-FIX

1. Prepare the following solutions before starting:
50mL of physiological saline buffer
60ml of 1X PBS
150ml of freshly prepared 4% paraformaldehyde in 1X PBS
60ml 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 (part I step 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 20ml 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 6min. At that stage the mouse is ready for TG harvesting. **Note:** Set the pump to 10mL/min. A good perfusion is noticeable when the mouse tail stiffens up, lift up then fall again.

III. 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 24h. **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 24h before embedding.

4. Embed TGs in a single block in cryo-sectioning embedding medium and freeze at -80 °C.

5. Store blocks at -80 °C until sectioning.

IV. 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 to 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.

V. 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 30kb portions of HSV-1 genomes (cosmids number 14, 28 and 56 described in [20] 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 signal [27-29]. 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 2µg 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 3µL of 0.5M EDTA in the mixture and heating at 70 °C for

10min. Cool on ice.

5. Purify the probe on a G50 gel exclusion mini-column. Add 150µg 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 100µL 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 2µg of DNA template and 100µL of formamide, it is considered to be 20ng/µL. Store at -20 °C. The labeled probe can be prepared in large quantity and stored frozen for several months.

VI. 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 co-detect RNA and protein, as described in sections 7 to 9.

1. Prepare the following solutions before starting:
0.5% Triton X-100 in 1X PBS
2X SSC and 0.2X SSC buffer
100mM pH6.0 Citrate buffer (10X stock solution) and 10mM pH6.0 Citrate buffer (working solution)
2X hybridization buffer (see step 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. Re-hydrate 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 3 times 10min 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 200mL of 10mM sodium citrate buffer (pH 6.0). Place the tray in a larger container filled with 500mL of distilled water. This setting allows for a better control of heating pulses. Before placing the slides in the tray, pre-heat the buffer in the microwave oven until the buffer reaches boiling (around 8 min at 800W).

4. Place the slides in the pre-heated citrate buffer-containing tray, and verify that they are completely covered with buffer. Heat for about 20s until the buffer reaches boiling. Caution, **DO NOT** let the buffer over boil, which might damage the tissue. Cool down at room temperature for 2min. Repeat the heating cycle 6 times (7 heating cycles total)*. Cool down 2min 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 15min, then in a methanol:acetic acid mix (3:1) for 15min (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 10min incubation in 70%, then twice 10min in 100% ethanol. Let dry at room temperature for 10min. Keep dry until probing.

7. Prepare the probing solution as follow: prepare in advance a 20mL stock of 2X hybridization buffer containing 20% dextran sulfate (MW 500 000), 2X Denhardt's solution, 4X SSC*. Aliquot the solution as 500µL in microtubes, and store at -20 °C. For 1 slide (coverslip of 22x50mm), mix 90ng of HSV-1 Cy3-labeled probe (30ng of probe for each cosmid), and complete the volume to 40µL with formamide. Add 40µL of 2X hybridization buffer. Mix well by pipetting up and down several times.

Note*: To prepare the 2X hybridization buffer, mix 4g of MW 500 000 dextran sulfate in 10mL of distilled water. It makes a viscous mix that dissolve upon 3 to 4 hours at 70 °C. Mix regularly to help dissolve. Add 4mL of 20X SSC, 400µL of 100X Denhardt's solution. Complete to 20mL and mix well using a vortex.

8. Drop 80µL of probing solution onto the dried sections. Cover with a 22x50mm 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 2h 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 5min. 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 5min. 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 three times with 2X SSC at 37 °C for 5min, and three times with 0.2X SSC at 37 °C for 5min. Wash once with 2X SSC at room temperature for 5min and proceed with DNA staining and mounting (see part X 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 sequences [22].

VII. 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 2mM RVC
0.5% Triton X-100 in 1X PBS containing 2mM RVC
3% H_20_2 in distilled water.
70%, 80%, 95% ethanol, molecular biology grade.
4X RNA hybridization solution (see step 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 vector [30], or another vector for *in vitro* transcription of the LAT locus, as previously described in [26]. Briefly: Linearize 10µg of pSLAT-2 with HindIII and purify the digested vector with a DNA purification kit. Synthesize a single strand ribo-probe from 2µg 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 260nm using a spectrophotometer. Dilute the probe at 50ng/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. Re-hydrate the sections in 1X PBS containing 2mM Ribonucleoside Vanadyl Complex (RVC)* for 10 min. Incubate the sections for 20 min with 0.5% Triton X-100 in 1X PBS containing 2mM RVC to permeabilize the tissue.

Wash 3 times 10min with 1X PBS, 2mM RVC. To quench any endogenous peroxidase activity, incubate the section in 3% H_2O_2 for twice 10min, and then wash once with 1X PBS, 2mM RVC.

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

4. Incubate twice 10min 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, 5min 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 pre-hybridization step in 50% formamide – 2X SSC can be used (see below in part IX 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 10mL stock of a 4X RNA hybridization solution containing 8X SSC, 20X Denhardt's solution, 4mM EDTA, 40% dextran*. Aliquot the solution as 500µL in microtubes, and store at -20 °C. For 1 slide prepare 80µL of solution (coverslip of 22x50mm), by mixing 20ng of biotinylated LAT riboprobe, 40ng of yeast tRNA, 2mM RVC, and complete to 20µL with water. Add 20µL of 4X RNA hybridization solution, and 40µL of formamide (50% final concentration). For easier pipetting and mixing, it is recommended to pre-warm 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 4g of dextran sulfate (MW 500 000) in 3mL of distilled water and 4mL of 20X SSC. It makes a viscous mix that dissolves upon 3 to 4 hours at 70 °C. Mix regularly to help to dissolve. Add 2mL of 100X Denhardt's solution, and 80µL of a 0.5M EDTA solution. Complete to 10mL with water and mix well using a vortex. Caution: use only RNAse/DNase free products.

6. Denature the probe 10min at 75 °C. Meanwhile, place the slides on a slide incubator set to 65 °C. Drop 80µL 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 non-specific 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 1mL of 50% formamide in 2X SSC, pre-warmed at 65 °C. Caution, do not let the section dry. Wash twice 10min at 65°C in 50% formamide in 2X SSC and twice 10min 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 10min. 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 (part VI. Step 2).

VIII. 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 part VIII. Step 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 (parts VI.1 and VI. Step 2).

3. After unmasking, incubate the sections with 1X PBS containing 3% NGS for 1h.

4. Incubate with the primary antibody diluted in 1X PBS containing 3% NGS for 24h. 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 48-72h of incubation might be required

for low affinity primary antibodies such as IgM.

5. On day 2, wash three times 10min with 1X PBS.

6. **Protocol with antibody post-fixation**: incubate 1h with the secondary antibody coupled with a green fluorescent dye, at 1/200 in 1X PBS containing 3% NGS. Wash three times 10min with 1X PBS. Post-fixation is performed with 2% PFA in 1X PBS for 10min*. Wash three times 10min with 1X PBS.

Protocol with TSA detection: incubate 1h with the HRP-coupled secondary antibody at 1/250 in 1X PBS containing 3% NGS. Wash three times 10min with 1X PBS. Proceed with tyramide detection according to the manufacturer instructions. As indicated above (part VII. Step 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 10min reaction time. Wash three times 10min 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 (part VI. Step 3). Duration of immuno-DNA-FISH is typically 3 days, with the first antibody incubated overnight.

IX. 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 part IX. Step 3.

1. Prepare the following solution before starting:
50% formamide in 1X PBS containing 2mM 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 (part VII. Step 2).

3. **Case 1**, the immunofluorescence staining works after ethanol dehydration: proceed with RNA-FISH as indicated above in parts VII. Step 3 to VII. Step 9. Then proceed to step IX.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 1.5h in a pre-hybridization solution containing 50% formamide, 1X PBS, and 2mM RVC. Drain the pre-

hybridization solution and drop 80µL of hybridization solution as indicated in part VII. Step 4 and VII. Step 5. Then proceed with RNA-FISH hybridization and detection as indicated above in parts VII. Step 6 to VII. Step 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 part VI. Step 2 for details). Then, follow the immuno-DNA-FISH protocol as indicated above in parts VIII. Step 2 to VIII. Step 6.

X. Slide mounting and Imaging

1. Prepare the following solution before starting:
Hoechst 33342 at 0.5µG/mL in 1X PBS

2. Stain nuclei for 10min with DAPI or Hoechst 33342* at 0.5µg/mL in 1X PBS for 10min. Wash three times 10min 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 80µL of mounting medium containing an anti-fading agent on one end of the slide. Cover the sections with a high optical quality coverslip (n°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 x40 or higher magnification oil immersion objective, with high numerical aperture (for example x40 N.A 1.1, x60-x63 N.A 1.4, x100 N.A 1.3) and an excitation light source of at least equivalent to a 100W 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 RESULT

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 0.01M pH 6.0 Citrate buffer (that we used in all our studies), 1X PBS, 1mM EDTA, 0.1M 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 architecture [33-35], 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 co-detection of HSV-1 genome and viral or cellular RNAs and proteins. **Figure 4** illustrates the co-detection of HSV-1 genome together with the HSV-1 LAT RNA (**Figure 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.

LEGEND TO FIGURES

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 co-detection of RNAs and proteins are schematically represented. Critical steps are indicated by warning signs. DNA-FISH main steps are re-hydration, 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 immuno-detection. These will impact on the RNA-FISH procedure for the triple staining, and on the detection strategy of immuno-fluorescence.

Figure 2. Detection of HSV-1 latent genome after antigen unmasking. **A.** TG sections from 28dpi 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 perform 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 AlexaFluor 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 epi-fluorescence microscope. Scale bar is 5µm.

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 5µm. **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 10µm.

Figure 4. Co-detection 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 AlexaFluor 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 5µm.

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 immuno-histochemistry. 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 system [38]. 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: VZV [41]; HCMV [42,43]; EBV [44-46]; KHSV [47-49], 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 RNA [22]. The use of DNA-FISH and co-detection 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 sub-types, 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 neurons [22]. 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 mice [13-17]. 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 co-detection 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 pre-hybridization. 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 immuno-fluorescence 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-co-detection of reporter constructs (β-galactosidase, fluorescent proteins…) that are commonly used to analyze promoter activity from viral genomes.

ACKNOWLEDGMENT

We thank N. Sawtell (Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA), S. Efstathiou (University of Cambridge, UK) and James Hill (LSU Health Sciences Center, New Orleans, USA) for providing samples from HSV-1-infected mice and rabbits, respectively, and for reagents; H. Masumoto (Kazusa DNA Research Institute, Chiba, Japan) and S. Khochbin (Institut Albert Bonniot, Grenoble, France) for helpful discussions.

This work was funded by grants from the Centre National de la Recherche Scientifique (CNRS) (ATIP program, to PL, http://www.cnrs.fr), the French National Research Agency (ANR) (ANR-05-MIIM-008-01, CENTROLAT, http://www.agencenationale-recherche.fr), the FINOVI Foundation (http://www.finovi.org/:fr:start), the LabEX DEVweCAN (ANR-10-LABX-61) of Université de Lyon, within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the ANR (http://www.agence-nationale-recherche.fr), l'Association pour la Recherche contre le Cancer (ARC-7979 and ARC-4910, http://www.arc-cancer.net), la Ligue Nationale Contre le Cancer (LNCC, http://www.ligue-cancer.net), and INCa (EPIPRO program, http://www.e-cancer.fr). FC and PL are CNRS researchers.

DISCLOSURE

The authors declare that they have no competing financial interests.

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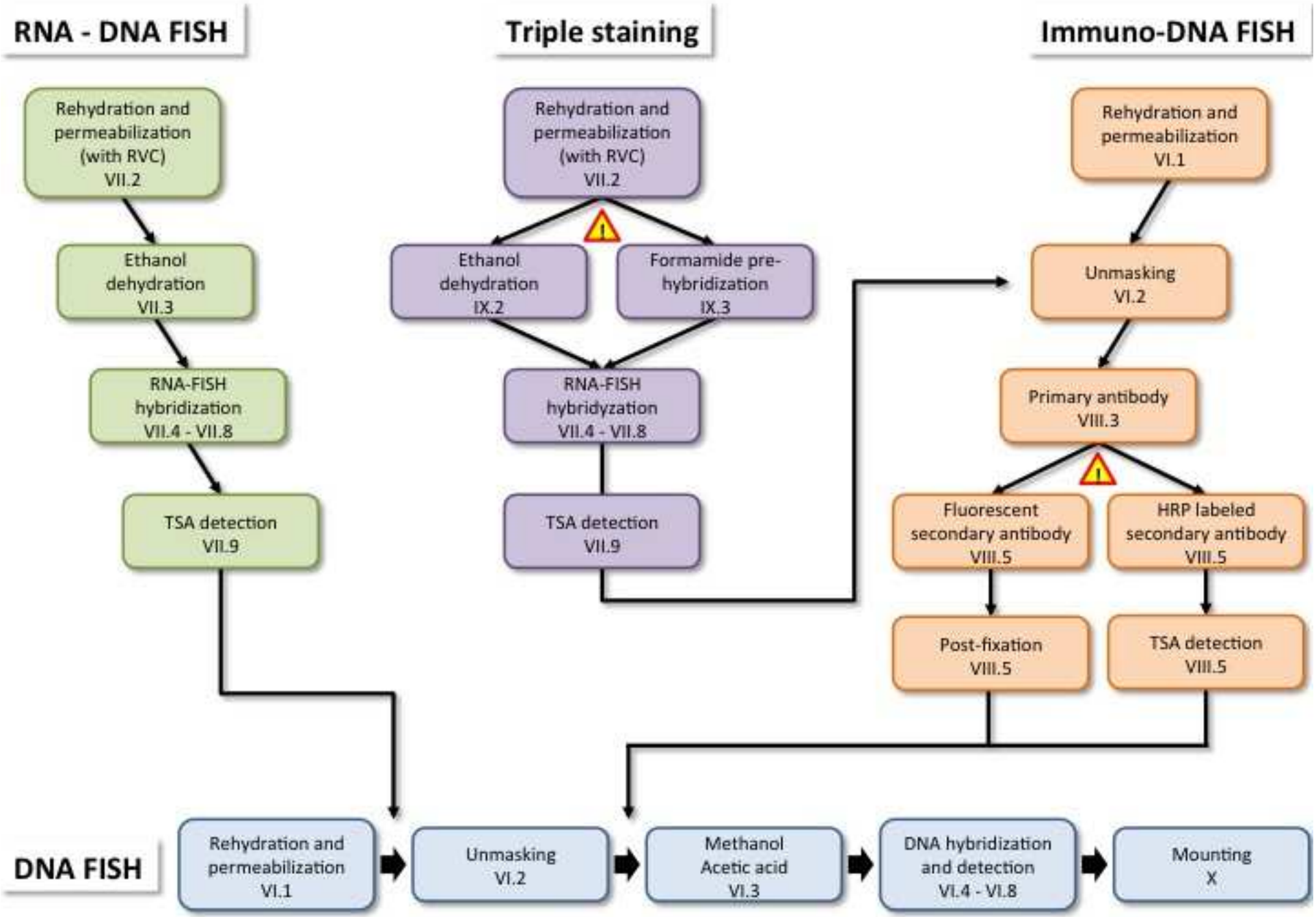

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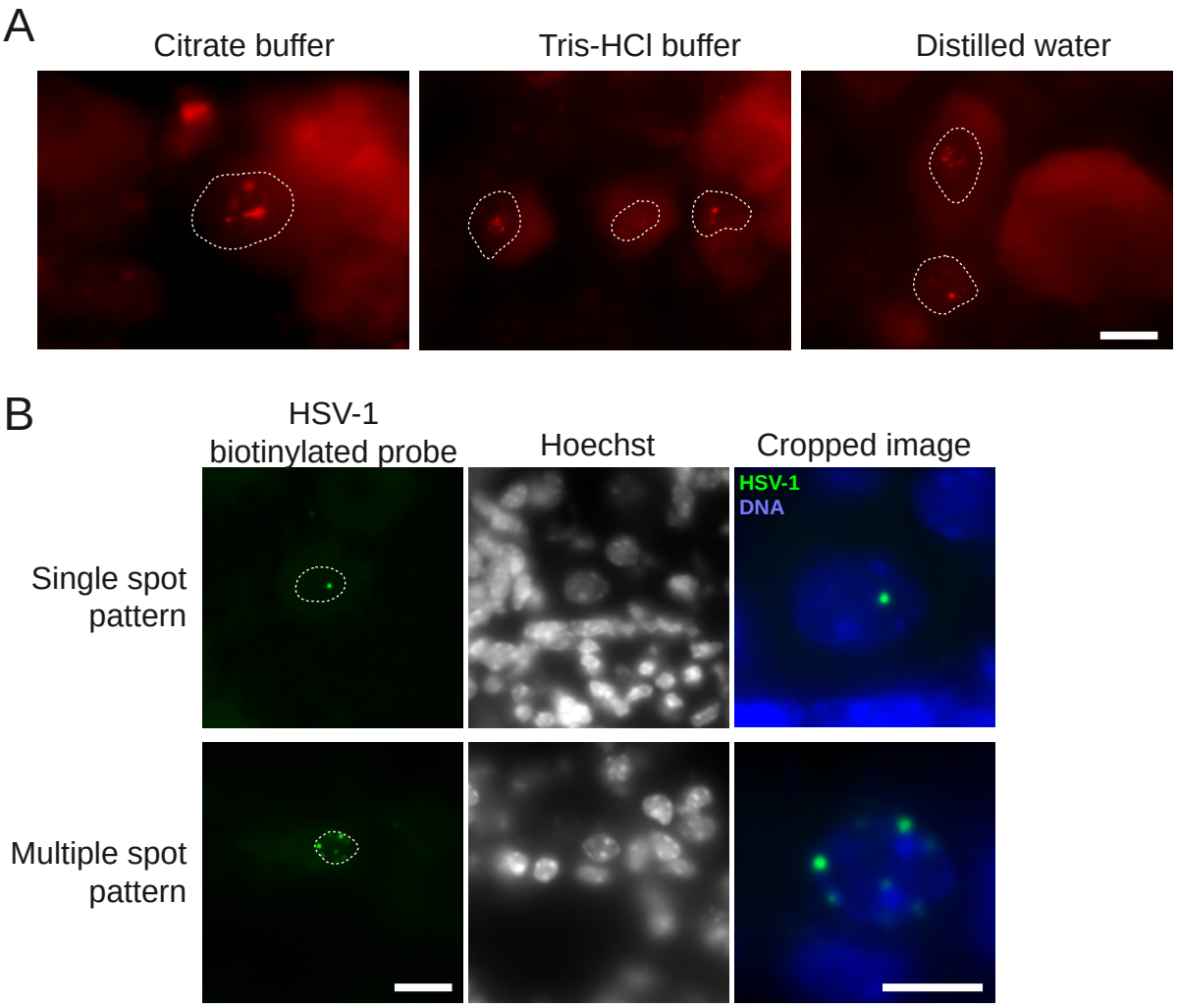

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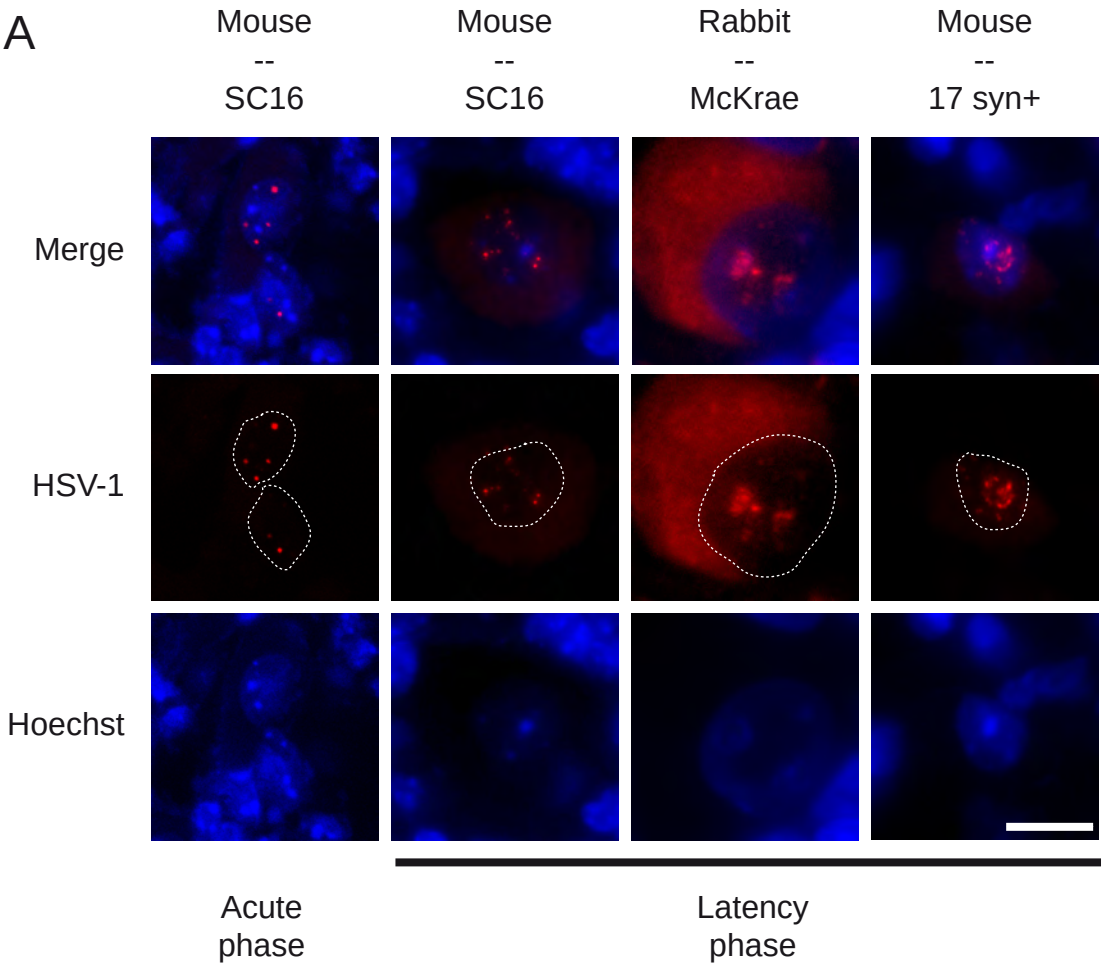

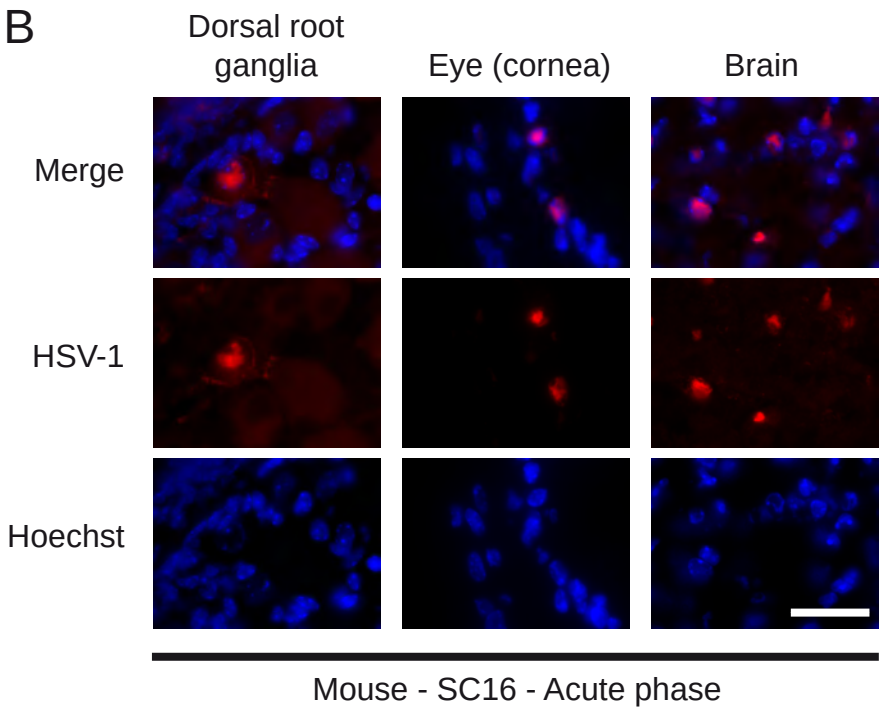

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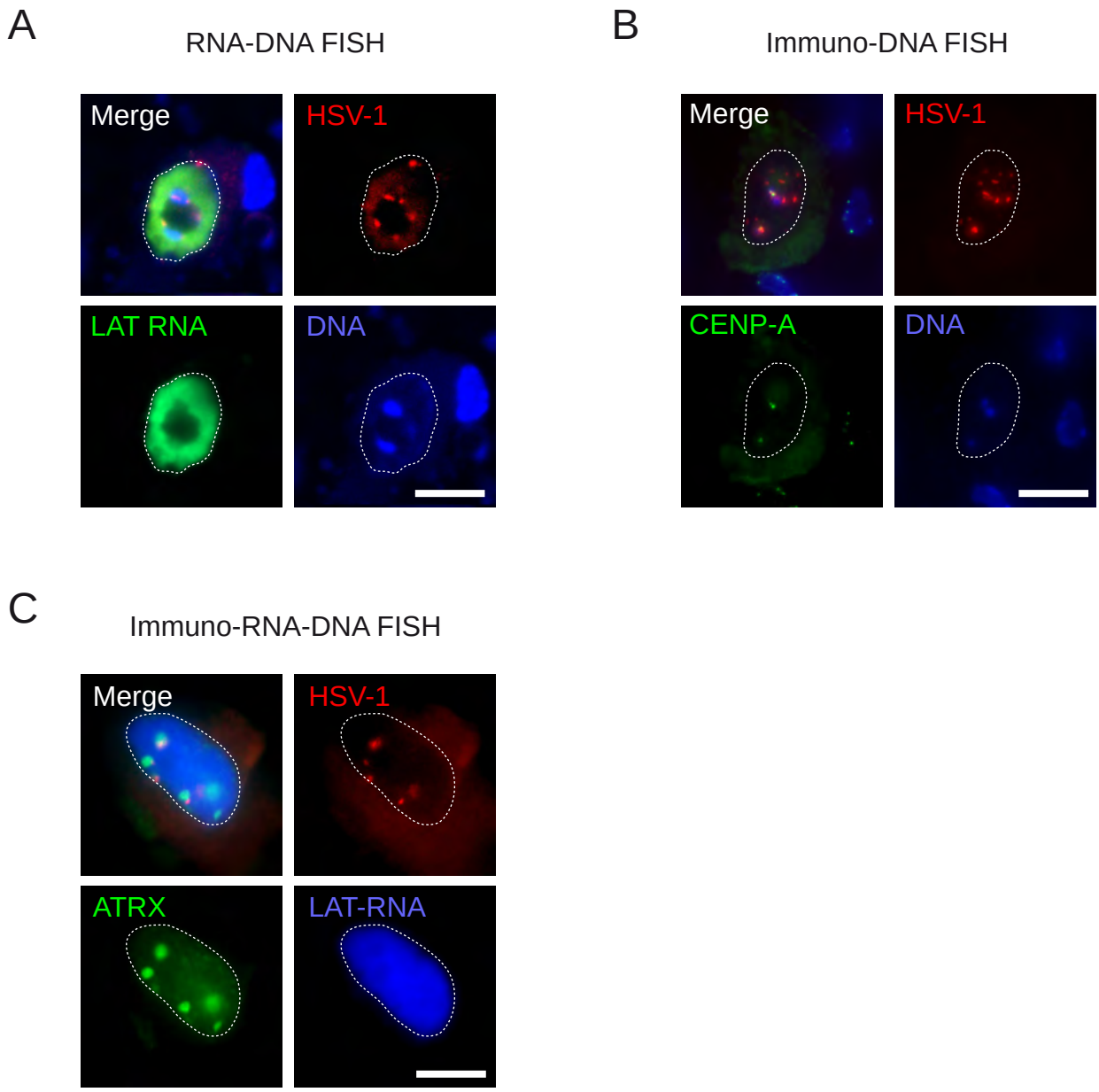

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REAGENTS			
Reagents	**Company**	**Reference**	**Note**
Balb/c mice	Janvier, France		6 week-old females
HSV-1 strains	SC16 strain (wild type)		See Labetoule, M. et al. (2003) Invest Ophthalmol Vis Sci 44: 217–225, for details on HSV-1 strain and virus stock preparation.
Ketamine hydrochloride	Sigma	K2753	Intraperitoneal injection of a solution containing Ketamine (100mg/kg) and Xylazine (10mg/kg)
Xylazine hydrochloride	Sigma	X1251	
Paraformaldehyde (PFA)	Sigma	158127	Suspend 4g of PFA in 90mL of water. Add 50µL of 1N NaOH, and heat at 60°C in a water bath with agitation. PFA dissolves in about 30min. Add 10mL of 10X PBS. This solution can be prepared in advance and stored at -20 °C in 5mL tubes. Caution. Manipulate under a fume hood.
Physiological Saline	Sigma	07982-100TAB-F	
1X PBS, pH 7.4 (sterile)	Life Technologies	10010-015	
Sucrose	Sigma	84100	Prepare a 20% sucrose solution in 1X PBS.
Cryosectionning embedding medium - Tissue-Tek OCT Compound -	SAKURA	4583	
Large vector DNA purification kit	Qiagen	12462	To purify Cosmid or BAC vector containing HSV-1 genome and store at -20°C
Nick translation kit	Roche Applied Sciences	10 976 776 001	
Cy3-dCTP	GE Healthcare	PA53021	Protect from light
0.5M EDTA	Sigma	E6758	
G50 Mini spin column	GE Healthcare	27-5330-01	
Salmon sperm DNA 10mg/mL	Invitrogen / Life Technologies	15632-011	
Ethanol molecular biology grade	Sigma	87047	Prepare a 70% solution
Salmon sperm DNA	Invitrogen / Life Technologies	15632-011	
Formamid Molecular biology grade	Sigma	F9037	Caution. Manipulate under fume hood.
HSV-1 biotinylated commercial probe	Enzo Life Sciences	ENZ-40838	
ImmEdge hydrophobic pen	Vector Laboratories	H-4000	
20X Saline Sodium Citrate (SSC)	Sigma	S6639	Prepare a 2X SSC solution in ddH_20.
Triton X-100	Sigma	T8787	Prepare a 10% stock solution in water and store at +4°C. Prepare the 0.5% solution in 1X PBS right before use.

10mM sodium citrate pH 6.0	Sigma	S1804	Prepare a 100mM stock solution (10X). Weigh 10,5g of citric acid (MW 210.14. Caution, irritant and toxic, wear appropriate mask and gloves), and dissolve in 400mL water. Adjust pH at 6.0 with 1N NaOH (caution, irritant, wear gloves). Adjust to 500mL with distilled water. Dilute 10 times in distilled water before use.
Acetic Acid	Sigma	320099	
Methanol, molecular biology grade	Sigma	322415	
Dextran sulfate - MW 500 000	Euromedex	EU0606-A	
Denhardt's solution (100X)	Euromedex	1020-A	
Rubber Cement "FixoGum"	Marabut	290110000	
DNA purification kit - Qiaquick PCR purification kit -	Qiagen	28104	
T7 *in vitro* transcription kit	Ambion / Life Technologies	AM1314	
Biotin-16-UTP	Roche Applied Sciences	11388908910	
RNA purification mini-column	Qiagen	73404	
Ribonucleoside Vanadyl Complex	New England Biolabs	S1402S	
H_2O_2	Sigma	H3410	Prepare a 3% solution in distilled water. Store at +4 °C and protect from light.
Yeast tRNA	Invitrogen	15401011	prepare a 10mg/mL solution in RNAse free water
Normal Goat Serum	Invitrogen	PCN5000	
Primary antibodies	Any supplier		The following primary antibodies were used in the result section: anti-mouse CENP-A (rabbit mAb C51A7, Cell Signaling Technologies), and anti-ATRX H-300 (Santa Cruz Biotechnology)
Secondary fluorescent antibodies	Invitrogen / Life Technologies		The fluorescent secondary antibodies routinely used in our protocol are AlexaFluor labeled goat antibodies (IgG H+L). The antibody used in the result section is an anti-rabbit goat antibody labaled with AlexaFluor 488 (reference A11001)
Tyramide Signal Amplification (TSA) kit - Streptavidin + AlexaFluor 350 (blue fluorescence)	Invitrogen / Life Technologies	#T20937	TSA kits are also available from Perkin Elmer
Tyramide Signal Amplification (TSA) kit - Streptavidin + AlexaFluor 488 (green fluorescence)	Invitrogen / Life Technologies	#T20932	
Hoechst 33342	Invitrogen / Life Technologies	H3570	Prepare a 0.5µg/mL solution in 1X PBS immediatly before use. Discard the remaining solution.
22x50mm coverslip. n°1.5 glass.	Electron Microscopy Sciences	72204-04	

Mounting medium with anti-fading agent - Vectashield -	Vector Laboratories	H-1000	Another conventional product is Fluoromount G from electron microscopy Science
Superfrost glass slides	FisherScientific	12-550-15	

EQUIPMENT

Equipment / material	**Company**	**Reference**	**Note**
Needle for infection			Glass micropipette hot drawn. Home made.
Dissection equipement	Moria, France		Microsurgical scissors and forceps
Peristaltic pump	Cole Palmer Instruments		Easyload Masterflex
Micro-syringe pump device (Nano Pump	kdScientific	KDS310	
Cryostat	Leica France	CM 1510-1	
-80 °C freezer	Sanyo		Ultra Low -80°C
Domestic microwave oven			
Dry block heater	Eppendorf	022670204	
Incubator Slide moat	Boekel Scientific	240000	
Coplin Jar	Dominique Dutscher	68512	
Staining glass container	Dominique Dutscher	68506	
Fluorescent microscope	Zeiss		The images presented in the result section were collected with a Zeiss AxioObserver with objective x40 LD NeoFluor N.A 0.6, and x100 PlanApochromat N.A 1.3. Filter set #38, #43 and #43. HXP 120 fluorescence light source. Photometrics CoolSNAP HQ2 CCD camera. Signal will be more easily observed on a recent high efficiency microscope such as Zeiss AxioImager/AxioObserver series, Nikon Ti-E/Ni-E series or Leica DM/DMI6000 series

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Author(s): Frédéric Catez, Antoine Rousseau, Marc Labetoulle, and Patrick Lomonte

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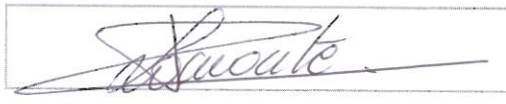
Signature:  Date: 24/04/2013

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Reviewer #1:
Manuscript Summary:
This paper describes a complex, involved and technically very impressive suite of methods for detecting HSV-1 genomes by DNA FISH in latently infected mouse neurones, and how this technique can be combined with RNA FISH and conventional immunofluorescence for protein detection. The method achieves a long sought after goal, that of detecting HSV-1 DNA in latently infected cells. The methods are described in a detail that is sufficient for others to harness the technique to their own studies, and it may have more general applicability to other latent viral infections. Given the amount of development that must have been necessary to achieve success, it is unlikely that others hoping to use this method would easily be able to do so from what might be expected of the methods section of a

normal paper. Therefore a dedicated and detailed methods paper such as this is both desirable and justified.

Because this is the first description of a successful detection method for HSV-1 DNA in latently infected cells, it is not possible to comment on the validity of the experimentation - although the example images provided strongly support the case that the method works. Overall, the methods are well described, and the 'Representative Results' and 'Discussion' sections are well written. However, there are sections of the text that have many minor language issues, particularly in the initial parts, and there are many typographical errors (there is also one in Fig. 1 - hybridyzation). I would recommend that the authors go through the text thoroughly to correct these errors, if necessary arranging for a native English speaker to do so. There are far too many for a reviewer to list.

Answer: We thank the reviewer for his positive comments. English and spelling have been corrected throughout the text.

There are a number of minor issues that could also be addressed.
Line 32: It would be better to say 'in part' rather than 'mostly'.
Answer: The text as been modified as suggested.
Line 102: does 'injector device' require further definition? This would be unclear to many.
Answer: The type of device used is a micro-syringe pump device supporting 5µl glass micro-syringes. The sentence has been added.
Line 118: The text should give a bit more detail on times of establishment of latency, etc. At present it reads a bit like the infections are done, then soon after the ganglia are harvested. Therefore a little more detail is required for those not closely involved in HSV research.
Answer: A brief description has been added on part I. step 5 to clarify this point.

Reviewer #2:

Manuscript Summary:

Catez et al describe in detail an important methodology that allows for the detection of HSV1 genomes in neuronal tissue sections. This technique is a key breakthrough in studying HSV1 latency and needs to be disseminated to a wide variety of investigators. Step by step details are explained well. This will be extremely useful to the field. The following statements need to be modified in the text:

Major Concerns:

line 54: change to "the initiation of reactivation"

Answer: The text has been modified as suggested.

line 56: change to "the absence of detectable replicative cycle proteins"

Answer: The text has been modified as suggested.

line 57 & 58: As stated, this gives the impression that these models recapitulate a complete latency cycle of HSV1--meaning they are faithful to what is observed in its human host. In fact this is not the case. It must be stressed that these models, while useful and having contributed to our understanding of latency and reactivation, do not fully recapitulate the pathogenesis of HSV1 disease in humans, the only natural host. For example, reactivation in humans is not evaluated by explanting trigeminal ganglia to find infectious virus and does not require hyperthermic induction.

Answer: The reviewer is correct, and we, of course, are aware that mice are not human and do not recapitulate all the aspects of latency in the human host. We changed the sentence in order to stress this point.

line 70- 71: Please revise to read: "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 mice". Also: references to Bertke et al., J. Virol., 85: 6669-77 AND Zerboni et al J Virol. 87:2791-802 in addition to ref 11,12 must be included here.

Answer: The text has been modified as suggested.

line 544: Actually, LAT RNA is also expressed in productively-infected cells, so the statement as written is incorrect. Instead, it would be accurate to state that latently-infected neurons express LAT but do not express detectable levels of productive cycle genes.

Answer: The text has been modified as suggested.

The author's must point out in the discussion that their 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 mice. In support of this, they must reference the following papers:
Bertke et al., J. Virol., 85: 6669-7
Bertke et al, J. Virol., 87:6512-6
Kobayashi et al, J Vis Exp. 2012 Apr 2;(62). doi:pii: 3823. 10.3791/3823.
Kim et al., PLoS Pathog. 2012 Feb;8(2):e1002540.
Zerboni et al J Virol. 87:2791-802
Answer: The applicability of the technique to other models is indeed relevant. This point has been added to the second paragraph of the discussion.

Minor Concerns:
line 49: should read "The HSV-1 genome is a..."
Answer: The text has been modified as suggested.

line 50: should read, "remains as a multicopy plasmid"
Answer: The text has been modified as suggested.

line 52: should read, "the HSV-1 replicative cycle genetic program..."
Answer: The text has been modified as suggested.

line 65-66 change to, "Because it has been impossible to characterize infected cells based on the presence of viral genomes"
Answer: The text has been modified as suggested.

line 102: Instead of "suck", perhaps the author's could say, "Take up"
Answer: The text has been modified as suggested.

Additional Comments to Authors:
N/A

Reviewer #3:

Manuscript Summary:

The manuscript by Catez et al. submitted to JOVE describes a protocol to detect latent HSV-1 genomes in neuronal tissue sections. The authors pioneered in using the described protocol to detect latent HSV-1 genomes in tissue sections which widened the toolkit available to study HSV-1 latency in vivo. The provided protocol is mostly detailed and will be of great use to the community. However, more emphasis should be put on the mechanism behind the key step of this protocol ("heat-based unmasking") and how it might affect subcellular structure and epitope composition.

Major Concerns:

1) The title as well as the short abstract imply a protocol to detect various persistent DNA viruses. However, the authors do not demonstrate the detection of any other virus than HSV-1. The title should be limited to HSV-1, if the authors do not provide data of the protocol's usefulness for the detection of other DNA viruses.

Answer: As the terms virus and genome are singular and not plural, we do not believe that the title gives the feeling that the protocol described in this article has been tested on several persistent viruses. However, we believe that this approach can be extended to other viruses as mentioned in the discussion part. Nonetheless, we added the word “a” in front of “persistent DNA virus” in the title and the short abstract in order to avoid this confusion. We also changed the position of the words in the title, which becomes: “Detection of the genome and transcripts of a persistent DNA virus in neuronal tissues by fluorescent in situ hybridization combined to immuno-staining”. Moreover, this comment is opposite to that of the reviewer 4 who advocates that the title could be less specific.

2) The authors show that "heat-based unmasking" is essential in detecting latent HSV-1 genomes. However, little explanation is provided to how it works. Moreover, it is unclear what effects the heating might have on the sample (ultra-)structure. The authors should provide controls to show that their protocol does not change the nuclear ultrastructure or destroy especially structural protein epitopes.

Answer: Heat based epitope unmasking (or antigen retrieval) is a well-known, almost universally used technique, practiced by pathologist for IHC on FFPE and frozen tissues. It has been developed over the past two decades and was shown to preserve the morphology of the tissues at the scale of light microscopy. The point raised by the reviewer is indeed important. Thus, a sentence has been added in the discussion to clarify this point and to invite the end-user to include appropriate controls.

The main objective of antigen retrieval techniques is precisely to modify protein epitopes to make them available for detection by antibodies. Heat based epitope unmasking is thought to break the cross linkage formed by formaldehyde as already indicated in the discussion. The technique has actually been developed on this principle. To provide the reader with the corresponding information we added a sentence and a reference in the discussion.

3) The authors should provide guidance on how to optimize the protocol for a certain experimental setting (species, tissue, virus) to widen its applicability.
Answer: Once epitope unmasking was set-up, we did not need to modify the protocol to make it work on different virus strains or on different animal tissues. Because we are convinced that fixation and cross linkage breaking are key in the detection of the virus, we added a sentence at the end of the first paragraph of the discussion.

4) The authors base their probe preparation on a HSV-1 cosmid library. However in the last decade or so, several HSV-1 bacterial artificial chromosomes were constructed (to name a few: Tanaka et al. 2003, Gierasch 2006, Nagel 2008) which are widely used and distributed in the community as they allow easy virus mutagenesis and reconstitution. The authors should discuss if BACs are suitable as it would make the protocol applicable for a wider readership.
Answer: This is indeed an important comment. A sentence has been added to the protocol, in part V. step 1, and the table of reagent has been modified.

5) The quality of the included pictures is generally not high which might be due to the use of widefield microscopy on thick samples. Confocal microscopy could have provided much better image quality.
Answer: Most of our analyses did not require confocal microscopy, such that the majority of the data currently available are wide-field images.

6) Figure 4B appears to be already published in Catez et al., 2012, PLoS Path.. The Authors should mention this in the manuscript.
Answer: We thank the reviewer for noticing this. We changed the image with another one from the same experiment.

7) The manuscript should be corrected for wording and grammar.
Answer: Corrections have been made throughout the text.

Minor Concerns:
Lines 108 : This reviewer failed to understand the description of this step without watching the video.
Answer: We will be very cautious to show the exact procedure in the video. We also changed the sentence.

Line 186: Technically there should be no difference between preparing samples for wide-field or confocal microscopy, or the authors did not make clear why this is specifically mentioned here.
Answer: Indeed, the sentence was misleading. It has been modified.

Line 209: Formamide is toxic. If chemical warnings are given as in line 247-248 for acetic acid, it should be done for all dangerous chemicals used in this protocol.
Answer: Warnings have been added for Formamide and PFA and in the table of reagent.

Line 360: signal? Did the authors mean substrate?
Answer: Indeed, it has been corrected.

Line 372: Better: inverted fluorescence microscope
Answer: The text has been modified as suggested.

Line 414: A short explanation what TSA detection is and how it works should be given
Answer: We modified part VII. first paragraph accordingly.

Line 460: Examples of far-red DNA dyes should be given that are proven to work with the protocol.
Answer: The text has been modified as suggested.

Line 470-473: This sentence should be re-phrased.
Answer: The sentence has been modified.

Line 473: Mentioning a 100 W light source seems arbitrary. Light output at the objective strongly depends on the used optics and the used lighpath just to mention a few. Also different bulb techniques have different output efficiencies. Camera sensitivities also differ widely.
Answer: The sentence has been modified, and refers to a standard microscopy equipment. We added a sentence regarding the light transmission efficiency. However microscope manufacturers do not provide quantified light transmission efficiency, and editorial policy prevents the use of commercial names. We added more details in the table of equipment to provide the reader with sufficient information.

Line 473-475: The sentence is imprecise. The authors should further here if they wish to discuss the pros and cons of widefield vs. confocal microscopy in their experimental settings.
Answer: The authors only wish to provide the reader with a way to deal with autofluorescence. Autofluorescence does not prevent the observation of the signal not the analysis of the data. This matter is beyond the scope of the protocol.

Line 492-493: see Major concern 4).
Answer: "Cosmids" has been replaced by "libraries"

Line 499: Figure 3 A only shows the acute phase for mice infected with SC16. Not for the other settings.

Answer: The text has been modified to match the figure.

Line 503: The "aggregates of HSV-1" genomes are quite likely replication compartments (de Bruyn Kops and Knipe, 1988).
Answer: Yes indeed. The data come from acute phase (6dpi) infected mice, therefore it is likely that the patterns that are visible in this figure could be replication compartments. However, unlike what was described by de Bruyn Kops and Knipe (1988) in cultured cells; we did not observe multiple aggregates within the same neuron, but rather one big nucleoplasmic spread signal.

Line 534-535: No reference is given here. Moreover, the authors should discuss the mechanism of unmasking and the effects of heat-treatment more carefully. See also Major concerns 2) and 3).
Answer: A reference has been added. See answer on major comments 2 and 3.

Line 581-586: See Major concern 2). The authors should discuss reasons why certain antibodies might bind and others not. Does heat-based denaturation of structural epitopes play a role? A troubleshooting guide on this subject would help the reader. (Also, a more extensive list of validated antibodies that are working with this procedure would be very informative.)
Answer: As for any antibody-based technique, the quality of the signal depends on the primary antibody and the epitope it recognizes. We clearly state this point in the discussion. We added the name of validated antibodies from our studies published last year.

Line 588: This reviewer doubts that neither much enzymatic activity is detectable after heat-treatment nor that fluorescent proteins will still work sufficiently. The authors should provide data for such claims or explain their reasoning.
Answer: We modified the sentence to indicate that the authors meant immuno-detection. The suggestion of the reviewer has not been tested yet.

Line 619: SC16 is missing. M Strain?
Answer: “M strain” was a typo and was removed. SC16 has been added to figure 3B.

Figure 1: The chapter numbering is not roman as in the text.
Answer: The figure has been modified accordingly, as well as within the text.

Figure 2: The image quality is low. See also major concern 5). Nuclear outlines should be included also in 2B.
Answer: See answer to major concern 5. The nuclei have been outlined in Figure 2B

Figure 3: 3A: Scale bars are missing. Why is there so much background in the rabbit/McKrae setting? 3B: scale of the cornea sample should be equivalent to the other two samples or inserts should be included. Nuclear outlines should be marked as done in 2A.

Answers:

- Scale bar has been added in Figure 3A.
- In the rabbit cryosections, the higher background is due to a higher auto-fluorescence of the neurons cytoplasm. Because the auto-fluorescence is within the cytoplasm, it does not prevent the DNA-FISH signal detection.
- As requested, the image of the cornea sample has been changed by one taken at the same scale as the two other images of the figure.
- Nuclei have been outlined in Figure 3A.

Figure 4: The image quality especially of 4A is weak. Nuclear volumes should be marked.

Answer: As requested, the nuclei have been outlined.

Additional Comments to Authors:

N/A

Reviewer #4:

Manuscript Summary:

The authors have developed a method for co-detection of low-copy viral DNA by DNA-FISH, viral or cellular RNA transcripts by RNA-FISH, and immunostaining with various antibodies in animal tissues after infection with HSV1. While their protocol has been developed for detection of HSV in a mouse model, their method is a highly versatile approach that can be used for various applications besides viral infections and mice.

Major Concerns:

For someone who is familiar with ISH, this protocol would be fairly straightforward. For someone who has never performed ISH or IF, this protocol would be very difficult to follow successfully because quite a few details are missing. The protocol seems to be written for someone who is already very familiar with ISH and IF.

Answer: The protocol described here aims at providing the scientific community with a method to detect latent HSV-1 virus in mouse tissues. It requires basic knowledge and technical skills in ISH, IF and microscopy, molecular biology and also in animal handling, tissue sectioning, and virology. We did not include a detailed description of each step, because we feel it would make the description less clear and extremely long, and that it is best to refer the reader to appropriate literature. We added a sentence at the beginning of the protocol description to refer the reader to additional literature.

Minor Concerns:

1. The clarity of the procedure could be improved by careful proof-reading and addition of details, but the approach is scientifically sound, very useful, and provides high quality technical methods. While ISH is sometimes considered to be "old school," this technique is essential for certain scientific inquiries. The authors provide an improved adaptation of ISH that allows a researcher to obtain simultaneous information on DNA, RNA, and protein co-localization in animal tissues, which is very important when working in animal models to reduce the numbers of animals used while collecting the most data possible from animal studies. There are no similar methods that allow the researcher to identify DNA, RNA, and proteins within the structure of the cells and tissues. The methods presented are also useful and adaptable to many other applications besides viral infections.

Answer: We thank the reviewer for this comment as it took us several years to develop this protocol and we are happy that colleagues appreciate the efforts put in developing an additional approach that could help to the understanding of latent infection. We have proof-read and corrected the text to improve clarity.

2. The s is rather long and cumbersome but very specific...maybe too specific since this method is so versatile.

Answer: Reviewer 3 made the opposite comment. We indeed do not think that we need to change the title, but we just added the word « a » before « persistent DNA virus » and we

reordered the wording. The title becomes: ""Detection of the genome and transcripts of a persistent DNA virus in neuronal tissues by fluorescent in situ hybridization combined to immuno-staining".

3. With all of the solutions that must be prepared in advance, it seems that a section for solution preparation is needed.
Answer: Considering the variety of methods presented, and that most solutions are prepared shortly before they are used, we feel that it would be difficult to make a section for solution that would be easy to read. However, it is indeed common practice to list reagents ahead. To facilitate the preparation of the reagents, we listed the reagents that can be prepared ahead of time and stored, at the beginning of each part.

4. The English is difficult to read, commas are over-used, punctuation is often used improperly, and there are many mistakes in tense, plurality, and spelling. It would be prudent to have an editor correct the English and punctuation. Some of the mistakes are included in the specific line comments below, but there are many more.
Answer: We indeed re-checked the text following and added the corrections suggested by all 4 reviewers.

5. As I read through the protocol, I made many comments on who is the manufacturer, where was the reagent obtained, and which kit was used. Although there is a list of reagents provided, sometimes it's not completely clear which reagent or kit from the list applies to which step in the protocol. It would be helpful to refer to the name or manufacturer of the reagent within the text of the protocol. It would also be helpful if the reagents were listed in some kind of order, either in the order they appear in the protocol or alphabetically. Saline is used in II.5 but is not listed in the reagent list. OCT is in the reagent list but referred to as "cryo-sectioning embedding medium" in II.3 - this might be confusing for someone trying to follow this protocol if they are unfamiliar with the reagents. The commercial HSV probe from Enzo is not listed in the reagent list. The authors should go through the reagent list again to make sure that all are listed.
Answer: We thank the reviewer for this comment, since the reagent table is an important source of information to reproduce the experiments. Several references have been modified or added: PFA, sucrose, large vector DNA purification kit, ethanol, Triton X-100, rubber cement, normal goat serum, TSA kits, Vectashield.
We agree that for some reagents or equipments, it is easier to communicate using the commercial name or name of manufacturer. However, it is JoVE policy not to include commercial names within the main text.

6. I find it interesting that the authors reference so many miRNA papers, although they only mention miRNA in the background and it's unrelated to this protocol. They do, however, discuss "?how HSV-1 gene expression is regulated through its interactions with the nuclear

architecture?" and "?whether a specific subset of neurons are preferred host-cells for latency establishment and reactivation?", yet they fail to reference Roizman, Christie, or Margolis, all of whom have pioneered methods (including FISH/IF) to co-localize HSV DNA, RNA and proteins to explore in situ the virus-host relationship.
Answer: We refer to only 2 papers describing processing of the LAT into miRNAs, for the purpose of general information. We added the required references for the other topics.

Additional Comments to Authors:
Line 102: "Suck" is not a technical term.
Answer: It has been changed to "take-up"

Line 107: "Ketamin" should be "Ketamine." Step #2 is not clear - "maintain it with the left hand attached to the dissection paddle?" - what is a dissection paddle? why is the left hand attached to it?
Answer: Text has been modified as requested. Part I. section 2 has been modified. Dissection paddle has been changed by dissection tray.

Line 116: "?for the required time" - what is the required time?
Answer: The sentence has been modified to indicate appropriate time to reach HSV-1 latency.

Line 122: Is the 4% paraformaldehyde prepared fresh or purchased pre-mixed? Perhaps a protocol should be provided to prepare the PFA, since it is not simply a matter of dissolving it in PBS, as stated in the reagent list.
Answer: PFA preparation is now fully described in the reagent table.

Line 128: How is the mouse attached by the four legs?
Answer: By standard procedure using pins. The text has been modified.

Line 132: "rig" should be "rib"
Answer: This has been corrected.

Line 142: PBS was prepared in Step 1, not physiological saline. Which one is perfused in Step 5, saline or PBS? While they may serve the same purpose, they are not the same thing, and this may lead to confusion.
Answer: Preparation of physiological saline has been added in Step 1.

Line 151: "Harversting" should be "harvesting"
Answer: This has been corrected.

Lines 175-180: This is not necessarily true, as different neurons in the TG are different sizes. While these slides may represent the same region of the TG, you cannot rely on the same neurons being present in 3 consecutive sections. In addition, the nucleus will not be present in all of those sections.
Answer: We agree with the reviewer comment. The main purpose of serial sectioning being to run different staining on sections from the same region of the TG. We modified the text accordingly.

Lines 194-211: There is no step for cutting the cosmid. Is the entire cosmid labeled, not cut with restriction enzymes first? This could produce non-specific signal if the cosmid backbone binds to cellular or viral sequences. Step 5 - "the probe is considered to be?" is the actual probe concentration not assayed? It's just assumed to be that concentration?
Answers:
- Indeed, the cosmids are not cut for nick-translation labeling. We used whole cosmid vectors to generate probes in a more convenient manner, as gel purification of large DNA fragments is difficult. We ran control experiment with empty cosmid in parallel. We added a sentence after part V. step 1 to clarify this point.
- Regarding the probe concentration, it cannot be reliably measured at this point, because of the fluorescence and the presence of formamide. Consequently, we use the quantity of DNA template used to make the probe as our reference. The protocol being based on 2µg of DNA template and 100µL of formamide, it is considered to be 20ng/µL. The text has been modified accordingly.

Line 222: "unmasking setting" - is that the heat setting?
Answer: Yes, this is the pre-heating step. We modified the text to clarify this point.

Line 304: This is a personal opinion - I think since this is a methods paper, all of the methods should be included here instead of "as previously described in?" If most of the individual procedures were described elsewhere, there is really no point in publishing a consolidated methods paper if the authors are simply going to refer the reader to other sources.
Answer: We agree that referring to a previous publication was not in the "spirit" of a method paper. We modified and extended the description of the ribo-probe preparation.

Line 359: Which commercial kit is used?
Line 367: Which manufacturer?
Line 416: Which manufacturer?
Answer: For lines 359, 367 and 416, the reference to the reagent table has been clarified. Editorial policy does not allow including commercial name and trademarks in the main text.

Line 463: A novice may not know where to find an anti-fading agent or understand which is best to use. It would be best to state which mounting agent is used by the authors. Vectashield is listed in the reagent list, but why not just say Vectashield here too?

Answer: As mentioned above, editorial policy does not allow including commercial name and trademarks in the main text. We modified the table of reagent to clarify this point.