Dear Editor,

You will find hereafter our answers to the reviewers’ comments concerning our manuscript JoVE51091R1. We would like to thank all the reviewers for their comments/suggestions, which were very helpful in improving the quality of the manuscript.

Sincerely,

Patrick Lomonte

**Editorial comments:**

\*Protocol text:

1) There are a few steps that could use more detail to help viewers complete the protocol. Please review your protocol steps and add more details accordingly. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some of these steps include, part 1 (step 2, what is the route of injection (while the information is in the table it should also be placed in the protocol text) and step 5, what is the specific required time the animals are left (how many days?).

Answer: Changes have been made according to the reviewers’ comments.

\*Please remove copyright and trademark symbols from table of reagents/materials and ensure that all font in the table is consistent.

Answer: All copyright and trademark symbols have been removed. Font has been checked.

Editor modified the formatting (converted to Word) of the manuscript to comply with JoVE instructions for authors, please maintain the current formatting throughout the manuscript. You can find the updated manuscript under "file inventory" and download the Microsoft Word document. Please use this updated version for future revisions.

**Reviewers' comments:**

**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.