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| CENTRE DE GENETIQUE et de PHYSIOLOGIE MOLECULAIRE ET CELLULAIRE **UMR 5534**  Domaine Scientifique de la Doua - 16, rue R. Dubois, Bâtiment G. Mendel - 69622 Villeurbanne Cedex - France |

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. Labetoulleperformed 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.