MS: JoVE62219

Double labeling immunofluorescence using antibodies raised in the same host species to study host-pathogen interactions

Dr. Nam Nguyen
Manager of Review
JoVE

Dear Dr. Nguyen,
We are pleased to provide a revised manuscript and a point-by-point rebuttal that addresses all of their comments.
We have made major and minor edits (highlighted in red font) requested by the reviewers.
We hope that you will now consider our manuscript suitable for publication in the JoVE.
Sincerely,

Munira M. A. Baqui

We would like to thank all reviewers for their detailed and very helpful comments.
We have addressed all of these comments as described below.
Sincerely,
Munira

Response to Reviewers' comments

Reviewer #1 Comments for the Author:

Major Concerns:

1. No demonstration that the blocking step is necessary

Blocking step is widely applied in multi-labeling protocols. However, the adaptations of this block depend on the type of sample and the antibodies being used. Based on previous references sent by you (Refs 6-14), we have included in the text explanation about the use of the protocol with and without blocking (lines 289-298). We believe that citing these published articles (Ref 5-13) justify the use of the second blocking. We have also included a cartoon demonstrating our sequential labeling procedure to use in samples infected with pathogens (Figure 3).

2. Failure to allow to compare with other protocols.

The authors' introduction to the immunolabeling technique begins (rightly enough) at a very elementary level: "antibodies are widely used tools, mainly for understanding

the location and function of cellular structures and proteins". Since JoVe is about methods, and the reader will want to compare the different methods available, I assumed that the authors would tell us something about what is known about the use of two antibodies from the same host species. A quick search brings up papers from as early as 1985, including one in JoVe:

Ansorg A, Bornkessel K, Witte OW, Urbach A. J Vis Exp. 2015 Apr 22;(98):52551. Immunohisto-chemistry and multiple labeling with antibodies from the same host species to study adult hippocampal neurogenesis.

Tóth ZE, Mezey E. J Histochem Cytochem. 2007 Jun;55(6):545. Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species.

Ma B, Winkelbach S, Lindenmaier W, Dittmar KE. Acta Histochem. 2006;108(4):243 Six-colour fluorescent imaging of lymphoid tissue based on colour addition theory.

Buchwalow IB, Minin EA, Boecker W. Acta Histochem. 2005;107(2):143 A multicolor fluorescence immunostaining technique for simultaneous antigen targeting.

Nakamura A, Uchihara T. J Neurosci Methods. 2004 May 30;135(1-2):67 Dual enhancement of triple immunofluorescence using two antibodies from the same species.

McCormick J, Lim I, Nichols R. Cell Tissue Res. 1999 Aug;297(2):197 Neuropeptide precursor pro-cessing detected by triple immunolabeling.

K S Shindler 1, K A Roth 1996 Double immunofluorescent staining using two unconjugated primary antisera raised in the same species J Histochem Cytochem 1996 Nov;44(11):1331.

Wang BL, Larsson LI. Histochemistry. 1985;83(1):47. Simultaneous demonstration of multiple antigens by indirect immunofluorescence or immunogold staining. Novel light and electron microscopical double and triple staining method employing primary antibodies from the same species.

Thank you for the references related to immunofluorescence using two antibodies from the same species. The articles mentioned by the reviewer described protocols that worked very well in cells and tissues and surely will enrich our discussion. Since we are attending a Methods Collection titled "Current methods to study parasite-host interactions and immunomodulation", we are not comparing protocols, but we have made a small comparison with our protocol and other different methods available (lines 289-298).

Our protocol can detect pathogen and host cell proteins by immunofluorescence in the same time. This protocol will help laboratories that work with parasites to overcome the obstacle of having few commercially available antibodies.

3. What the authors did was to give one reference to an irrelevant article (Moreira et al 2017) and one to a book that is not widely available (ref. 8). They now state "Thank you for pointing this out, we have corrected the references". But Ref. 7 is unchanged, and still wrong.

Thank you for pointing this out. We have deleted Ref. 7 (Moreira et al, 2017). Also, the book chapter from my group (Moreira et al, 2017) is full available on Researchgate.net: (https://www.researchgate.net/publication/317107919_Use_of_antibodies_from_the_same_host_species_in_double_labeling_immunofluorescence_on_trypanosome_cytoskeleton).

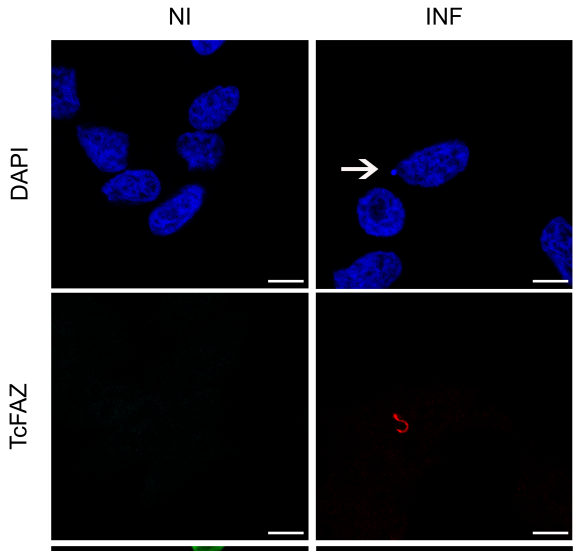
Minor Concerns:

4. L.77. *T. brucei*. L. 256. *T. cruzi*. These species have rather different life cycles: which are we talking about?

Based on the knowledge acquired with free-living *Trypanosoma brucei* through our protocol, we expanded the possibility of labeling other parasites, including *T. cruzi* in its intracellular form. We want to highlight that is possible to label multiple antigens in different parasites and their life cycles.

5. The TcFAZ labeling in Fig. 2 is so faint that it needs to be indicated with an arrowhead.

The arrow is only showing the location of the nucleus and kinetoplast of the parasite labeled with DAPI (blue). I believe that the "faint staining" mentioned by the reviewer is because of the PDF file. The original picture (TIF file) is very fine as you can see below.



Reviewer #2 Comments for the Author:

Major Concerns:

None

Minor Concerns:

The term Assay was questioned by the Reviewer 1, however the substitution of the word "assay" for "image" is not valid in the context used. The labelling procedure precedes image acquisition. I would suggest using procedure or protocol instead of image (lines 105, 149. 180)

Done

155 should read 0.12 mg/ml not 0,12 mg/ml

Done

Reviewer #3 Comments for the Author:

Major Concerns:
No.

Minor Concerns:
No

Reviewer #4 Comments for the Author:

Major Concerns:

In the title and in several parts of the manuscript the authors indicate that antibodies were raised in the same host species to study host-pathogen interactions. The word "host" should be removed when referring to the species used to generate the antibodies. It is confusing because it is not related to the host cells used to study host-pathogen interactions. In addition, antibodies against any antigen of a pathogen can be raised in species that are not necessarily the natural hosts of that pathogen. In this regard, I would suggest to replace the phase "the same host species" by simply "the same species" in the title and all over the text.

Minor Concerns:

Line 2: In the title, replace "same host species" by "same species"

Done

Line 28: Replace "same host species" by "same species"

Done

Line 36: Eliminate the words "so many", as in fact, commercial antibodies against trypanosomatids are practically unavailable.

Done

Line 38: Replace "belong to the same host" by "were produced in the same species"

Done

Line 40: Replace "same host species" by "same species"

Done

Line 45: Replace "the mouse" by "a mouse"

Done

Line 46: Replace " conjugated to a fluorochrome" by "conjugated to a different fluorochrome"

Done

Line 48: Replace "using the third antibody from a different host" by "using a third antibody raised in a different species"

Done

Lines 64-65: Replace "lead to antigen recognition specificity" by "lead to the recognition of a specific antigen"

Done

Line 65: Eliminate the word '"mainly", as all the mentioned techniques are widely used in molecular and cellular biology.

Done

Line 66: Replace "immunofluorescence" by "immunofluorescence analysis" or "immunofluorescence microscopy"

Done

Lines 68-69: Replace "antibodies from different host species" by "antibodies raised in different species"

Done

Line 73: Delete the word "antibodies" (repeated)

Done

Line 74: Delete "with different fluorochrome" (redundancy)

Done

Line 77: Delete "host"

Done

Line 80: The method should be referred as immunofluorescence analysis (IFA) or immunofluorescence microscopy. Also, please delete "host"

Done

Line 81: Here the authors should mention that Trypanosoma cruzi (an obligate intracellular parasite) has been used to describe the protocol.

Done

Line 83: Please replace "host" by "species"

Done

Line 96: Correct verbal tense (centrifuge instead of centrifuged)

Done

Line 220: Some scientific journals do not allow the manipulation of microscopy images using Adobe Photoshop. Instead, brightness and contrast can be adjusted using the original acquisition software of confocal microscope, while providing the original raw data in the submission. Please consider deleting this comment.

Thank you for your concern. We did not make manipulations on the images. We only use Adobe Photoshop to assemble the figures and intensify the brightness for better visualization.

Line 225: Rephrase: "is limited because commercial antibodies are unavailable to recognize..."

We rephrase this sentence (line 224-225).

Line 280: Please delete "host".

Done

Line 300: Replace: "to ensure no false-positive results" by "to avoid false-positive results".

Done

Line 309: Please refer properly to the method (immunofluorescence microscopy).

Done