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

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TITLE:

Double Labeling Immunofluorescence using Antibodies from the Same Species to Study Host-Pathogen Interactions

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KEYWORDS:

immunolabeling, double labeling, same host antibodies, host-pathogen interaction

SUMMARY:

Here, the protocol describes how to perform double labeling immunofluorescence using primary antibodies raised in the same species to study host-pathogen interactions. Also, it can include the third antibody from a different host in this protocol. This approach can be made in any cell type and pathogens.

ABSTRACT:

Nowadays, it is possible to find a wide range of molecular tools available to study parasite-host cell interactions. However, some limitations exist to obtain commercial monoclonal or polyclonal antibodies that recognize specific cell structures and proteins in parasites. Besides, there are few commercial antibodies available to label trypanosomatids. Usually, polyclonal antibodies against parasites are prepared in-house and could be more challenging to use in combination with other antibodies produced in the same species. Here, the protocol demonstrates how to use polyclonal and monoclonal antibodies raised in the same species to perform double labeling immunofluorescence to study host cell and pathogen interactions. To achieve the double labeling immunofluorescence, it is crucial to incubate first the mouse polyclonal antibody and then follow the incubation with the secondary mouse IgG antibody conjugated to any fluorochrome. After that, an additional blocking step is necessary to prevent any trace of the primary antibody from being recognized by the next secondary antibody. Then, a mouse monoclonal antibody and its specific IgG subclass secondary antibody conjugated to a different fluorochrome are added to the sample at the appropriate times. Additionally, it is possible to perform triple labeling

immunofluorescence using a third antibody raised in a different species. Also, structures such as nuclei and actin can be stained subsequently with their specific compounds or labels. Thus, these approaches presented here can be adjusted for any cell whose sources of primary antibodies are limited.

INTRODUCTION:

To study the interaction of the pathogen with the host cell at the cellular level provides essential information on the underlying causes of the disease since different groups— such as viruses, bacteria, and protozoa— can infect most host cell types[1–4]. It can also help develop and identify potential therapeutic targets that can slow or inhibit the growth of the pathogen. In live conditions, the produced antibodies are responsible for recognizing self-components, antigens from viruses, bacterial components or products, fungi, parasites, and others[5].

For this purpose, antibodies are widely used tools, mainly for understanding the location and function of cellular structures and proteins. Several studies using multiple antibody labeling demonstrate that additional blocking steps contribute to the specificity of the immunolocalization. In addition, most described protocols use specific commercial monoclonal antibodies, including antibodies from the same host species[6-14].

Usually, double labeling immunofluorescence uses two antibodies raised in different species to stain the cell structures of interest or the pathogens and the host cells to see the interaction between them. However, this can be a problem when no commercial monoclonal or polyclonal antibodies specific for some pathogens are available to perform the double labeling. Also, there are commercially available antibody conjugation kits, and it is possible to conjugate the primary antibodies directly to the fluorophore by a succinimidyl ester reaction[15]. The problem is that these kits are often expensive, and it is necessary to have enough antibodies to label them. Knowing this, we successfully developed a double immunofluorescence method using two different antibodies raised in the same species to study protein localization in *Trypanosoma brucei* [16]. However, for intracellular parasites, including *Trypanosoma cruzi*, this approach has not been demonstrated. Here, we show how to perform double labeling immunofluorescence to study intracellular *T. cruzi* parasites and the host cell using primary antibodies raised in the same species without cross-reactions. Besides this method, a triple immunofluorescence labeling has been established with the addition of the third antibody from a different species. These approaches help when the source of antibodies is limited and can be used in any cell type.

PROTOCOL:

1. Cell and parasite cultures

1.1. Grow LLC-MK2 (Rhesus Monkey Kidney Epithelial) cells from the American Type Culture Collection (CCL-7) in a 25 cm^2 cell culture flask containing in RPMI medium supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum) and antibiotics (100 U/mL Penicillin and 100 µg/mL Streptomycin) at 37 °C in 5% CO_2 [17].

1.2. Infect LLC-MK2 cells with *Trypanosoma cruzi* (Y strain) according to a previous protocol [18].

1.3. Collect the supernatant of the LLC-MK2 infected cells (5 mL) in a 15 mL cell culture conical centrifuge tube and centrifuge at 500 x g for 10 minutes and 22 °C to lower cell debris. Keep the tube for 10 minutes at 37 °C to allow trypomastigotes to swim to the supernatant.

1.4. Collect the supernatant in a new conical tube and centrifuge at 2500 x g for 15 minutes at 22 °C. Discard the supernatant and resuspend the pellet containing the parasites in complete RPMI medium to determine cell density by counting cells in a Neubauer chamber.

2. Control immunofluorescence protocol

NOTE: Once fixed, it is possible to store plates containing coverslips at 4 °C in 1x PBS (pH 7.2) for one week. To be stored, it is important that the cells have not gone through the permeabilization step.

2.1. Settle LLC-MK2 cells (2 x 10^4) in 24 well plates containing UV sterilized rounded coverslips in RPMI media for 16 hours.

2.2. For infected cells, add a supernatant containing *T. cruzi* (item 1.4) to each well in proportion (MOI 10:1) and leave for 6 h of infection. Wash coverslips containing infected and non-infected cells five times with PBS solution and fixed with 2% paraformaldehyde in 1x PBS (pH 7.2) for 10 min at room temperature (RT).

2.3. Wash the coverslips three times for 5 minutes each with 1x PBS (pH 7.2).

2.4. Permeabilize the coverslips with 0.2% IGEPAL CA-630 in 1x PBS (PH 7.2) for 10 min at RT.

2.5. Wash the coverslips three times for 5 minutes each with 1x PBS.

2.6. Incubate coverslips for 30 min at RT with the blocking solution (2% BSA in 1x PBS, pH 7.2).

2.7. Incubate coverslips for 30 min at RT either with mouse monoclonal anti-hnRNPA1 (dilution 1:200) or with mouse polyclonal anti-TcFAZ (dilution 1:100) antibodies diluted in blocking solution.

2.8. Wash the coverslips three times for 5 minutes each with 1x PBS.

2.9. Incubate the coverslips for 30 min at RT with goat anti-mouse IgG F (ab')2 (H+L) conjugated to Alexa Fluor 488 (1:600) together with phalloidin conjugated to Alexa 594 (1:300) to stain actin filaments (F-actin) in the host cell diluted in the blocking solution.

2.10. Wash three times with 1x PBS (pH 7.2) for 5 min each.

2.11. Apply a small amount of antifade mounting reagent with DAPI medium to the surface of the slide.

2.12. Using forceps, gently tilt the coverslip in the mounting medium to prevent bubbles from forming. Once dry, seal the coverslip if desired.

3. Double labeling immunofluorescence protocol using monoclonal and polyclonal antibodies raised in the same host

3.1. Repeat steps 2.1 to 2.6 described above.

3.2. Incubate coverslips containing infected and non-infected cells with in-house mouse polyclonal anti-TcFAZ antibody (1:100) diluted in blocking solution for 30 min at RT.

3.3. Wash the coverslips three times for 5 minutes each with 1x PBS.

3.4. Incubate coverslips for 30 min at RT with goat anti-mouse IgG F (ab')2 (H+L) conjugated to Alexa Fluor 647 (1:600) diluted in the blocking solution.

3.5. Wash the coverslips three times for 5 minutes each with 1x PBS.

3.6. Perform the second blocking step with AffiniPure rabbit anti-mouse IgG (H+L) diluted (0.12 mg/mL) in blocking solution for 30 min at RT.

3.7. Wash the coverslips three times with 1x PBS (pH 7.2) for 5 min each. Then incubate with mouse monoclonal anti-hnRNP A1 IgG2b antibody (1:200) for 30 min at RT.

3.8. Wash the coverslips three times for 5 minutes each with 1x PBS.

3.9. Incubate the coverslips for 30 min with goat anti-mouse IgG2b antibody conjugated to Alexa Fluor 488 (1:600) and with phalloidin conjugated to Alexa 594 (1:300) diluted in the blocking solution.

3.10. Wash the coverslips three times for 5 minutes each with 1x PBS.

3.11. Repeat steps 2.8 to 2.10 described above.

4. Triple labeling immunofluorescence protocol with the addition of the third polyclonal antibody from different host species

NOTE: For the additional labeling, note the IgG subclasses, antibody isotypes, and follow the order of antibodies: 1. mouse polyclonal, 2. rabbit polyclonal, 3. second block, and 4. mouse monoclonal. Consider the type of lasers available in the confocal microscope to choose the correct fluorophore-conjugated secondary antibody.

4.1. Repeat steps 2.1 to 2.6 described above.

4.2. After steps 3.2 to 3.5 (washing step), start a new incubation with rabbit polyclonal antibody in blocking solution for 30 minutes at RT.

4.3. Wash the coverslips three times with 1x PBS (pH 7.2) for 5 minutes each.

4.4. Incubate the coverslips with goat anti-rabbit antibody IgG conjugated to Alexa 647 (1:600) in blocking solution for 30 minutes.

4.5. Wash the coverslips three times for 5 minutes each with 1x PBS.

4.6. Perform a blocking step with AffiniPure rabbit anti-mouse IgG (H+L) diluted (0.12 mg/mL) in blocking solution for 30 min at RT.

4.7. Wash the coverslips three times with 1x PBS (pH 7.2) for 5 minutes each and incubate with any mouse monoclonal IgG subclass antibody diluted in blocking solution for 30 minutes.

4.8. Wash the coverslips three times for 5 minutes each with 1x PBS.

4.9. Incubate the coverslips for 30 minutes with a specific pair of goat anti-mouse IgG subclass antibody conjugated to Alexa 594 (1:600) in blocking solution for 30 minutes.

4.10. Wash the coverslips three times for 5 minutes each with 1x PBS.

5. Confocal imaging acquisition

5.1. Analyze the immunofluorescence samples using a confocal microscope, with a 63X oil immersion objective and detect the fluorescence with a photomultiplier tube (PMT) and Hybrid detector (HyD).

NOTE: We used the setup from the Multiuser Laboratory of Confocal Microscopy - LMMC, Ribeirão Preto Medical School, University of São Paulo.

5.2. Acquire all confocal images with separated channels. Perform image processing using Adobe Photoshop.

REPRESENTATIVE RESULTS:

Here, we show how to study host-parasite interactions by immunofluorescence when the source of antibodies is limited due to the unavailability of commercial antibodies that recognize specific structures and proteins in trypanosomatids.

Among trypanosomatids, *T. cruzi* has one of the most complex life cycles involving various development stages between vertebrate and invertebrate hosts [19]. During the *T. cruzi* life cycle, at an early stage of mammalian infection, metacyclic trypomastigotes invade cells through a process that involves a wide variety of molecules present in parasites and the

vertebrate host cells [19,20]. To study these processes, our laboratory routinely produces in-house polyclonal antibodies against proteins of *T. cruzi*, *T. brucei,* and *Leishmania sp* [16, 21-23] to use together with commercial mouse monoclonal antibodies and/or with rabbit antibodies.

In **Figure 1,** confocal microscopy images show the results with the control experiments of infected and non-infected cells, highlighting the specificity of the antibodies in the host cell and the internalized parasite. The mouse polyclonal antibody (anti-TcFAZ) raised in our lab recognized only *T. cruzi* giant protein in the FAZ at the parasite flagellum but not in the host cell (**Figure 1A**). The distribution of the heterogeneous nuclear ribonucleoprotein A1[24] in the nuclei was observed using a commercial mouse monoclonal anti-hnRNP A1 antibody that recognizes only the host mammalian cells but not the parasite (**Figure 1B**). Also, host and parasite nuclei and the parasite kinetoplasts were stained with DAPI, and host F-actin was stained with Phalloidin conjugated to Alexa 594 (**Figure 1**). These control results show the specificity of the antibodies, and then, we can run the double labeling immunofluorescence protocol (**Figure 2**).

In **Figure 2,** double labeling immunofluorescence shows the protein distributions in the host and the parasite analyzed by confocal microscopy. No cross-reactions occur between antibodies using this methodology. The efficiency of the second blocking step using purified rabbit anti-mouse IgG is enough to impair the nonspecific labeling by the secondary antibodies. This labeling allows studying the interaction between the parasite and the host proteins and their behavior during infection.

Figure 1: Confocal Microscopy showing the localization of the *Trypanosoma cruzi* giant protein and host hnRNP A1 antibodies in non-infected (NI) and infected (INF) LLC-MK2 cells with *T. cruzi*. (**A**) *T. cruzi* giant protein (TcFAZ) localized in *T. cruzi* flagellum is labeled with mouse polyclonal anti-TcFAZ antibody and visualized with the goat anti-mouse IgG secondary antibody conjugated to Alexa 488 (green). (**B**) Host nuclear hnRNP A1 in LLC-MK2 is labeled with mouse monoclonal anti-hnRNP A1 IgG2b antibody and visualized by goat anti-mouse IgG2b antibody conjugated to Alexa 488 (green). Phalloidin conjugated to Alexa 594 stains F-actin (red). Nuclei and kinetoplasts are stained with DAPI (blue). Scale bar = 5 µm. All experiments were made in biological triplicate. White arrows indicate intracellular parasites.

Figure 2: Double labeling immunofluorescence shows no-cross reaction between antibodies raised in the same host species in non-infected (NI) and infected (INF) LLC-MK2 cells with *T. cruzi* analyzed by Confocal Microscopy. Giant protein localized in the flagellum attachment zone (FAZ) is labeled with mouse polyclonal anti-TcFAZ antibody and visualized by goat anti-mouse IgG antibody conjugated to Alexa 647 (red). Host nuclear hnRNP A1 is labeled with mouse monoclonal anti-hnRNP A1 IgG2b antibody and visualized by goat anti-mouse IgG2b antibody conjugated to Alexa 488 (green). Phalloidin conjugated to Alexa 594 stains F-actin (grey). Nuclei and kinetoplasts are stained with DAPI (blue). Merged images are shown as indicated. Inset corresponds to the enlarged area of the host nucleus. Bar=5 µm. All experiments were made in biological triplicate. The white arrow shows the presence of the parasite near the host cell nucleus.

Figure 3: Schematic illustration of sequential double immunostaining with primary antibodies derived from the same species. Diagram shows the order of the antibodies to ensure the efficiency of this protocol.

DISCUSSION:

Here, we present a protocol to perform double immunolabeling in *Trypanosoma cruzi* infected cells using two different antibodies from the same host species. To study, with more detail, the implications of the infection, structures in the host cell such as the nucleus or cytosolic organelles can be labeled using this protocol. Also, it can be used in the post-embedding thin section immunogold labeling method. This approach helps to overcome the obstacle of having few antibodies available to study trypanosomatids and other parasites.

Additionally, our protocol showed an intracellular parasite is labeled together with the host cell, two eukaryotes in the same immunofluorescence, different from those protocols realized in different kinds of tissues[7-14]. Our protocol shows that no epitope of the primary polyclonal antibody can be recognized by the second secondary antibody (step 3.9), ensuring that no cross-reactions occur between the antibodies (**Figure 3**). The success of this methodology is due to the efficiency of the blocking step (step 3.6) using purified rabbit anti-mouse IgG. A similar protocol described by Ansorg et al. (2015) has two additional blocking, where the first uses serum from the same host as the primary antibody and the second is realized with unconjugated Fab-fragments[6]. Also, without the second blocking, false-positive labeling can occur, as explained by these authors [6].

Furthermore, for triple immunolabeling, the third antibody of a rabbit host species can be added before the second blocking step described above in the protocol (step 4.2). Also, it is possible to use more than one monoclonal antibody since they have different IgG subclasses. For this, the corresponding secondary antibodies should be highly adsorbed to minimize cross-reactivity between them. The use of different IgG subclasses allows the utilization of the same host antibodies without cross-reaction. These protocols work well, but control groups are necessary to avoid false-positive results. The specificity of IgG subclasses and their properties makes it possible to do double and triple labeling. It has been reported that the IgG subclasses differ in the complement activation and Fc receptors in the inter-heavy chain disulfide bonds, hinge region amino acids, molecular mass (kDa), and the relative abundance (in percentage) in response to proteins, saccharides, and allergens [25]. They have slight differences in the hinge structure amino acids, influencing the stability and flexibility of each IgG subclass. IgG2 has the shortest and even more rigid hinge of all IgG subclasses due to a CH2 region with one amino acid deletion and an extra inter-heavy chain disulfide bridge [25].

In summary, the protocol described here to study host-pathogen interactions presents a basic and elaborated technique adapted to any combination of antibodies. This approach makes immunofluorescence cost-effective and can help when the source of antibodies is limited. The protocol can simultaneously detect pathogens and the host cell proteins in infected host cells; it can also be applied to any cell type and free-living organisms.

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DISCLOSURES:

The authors declare no competing or financial interests.

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antibodies. *Journal of Histochemistry and Cytochemistry*. **41** (8), 1273-1278 (1993).
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Figure 1

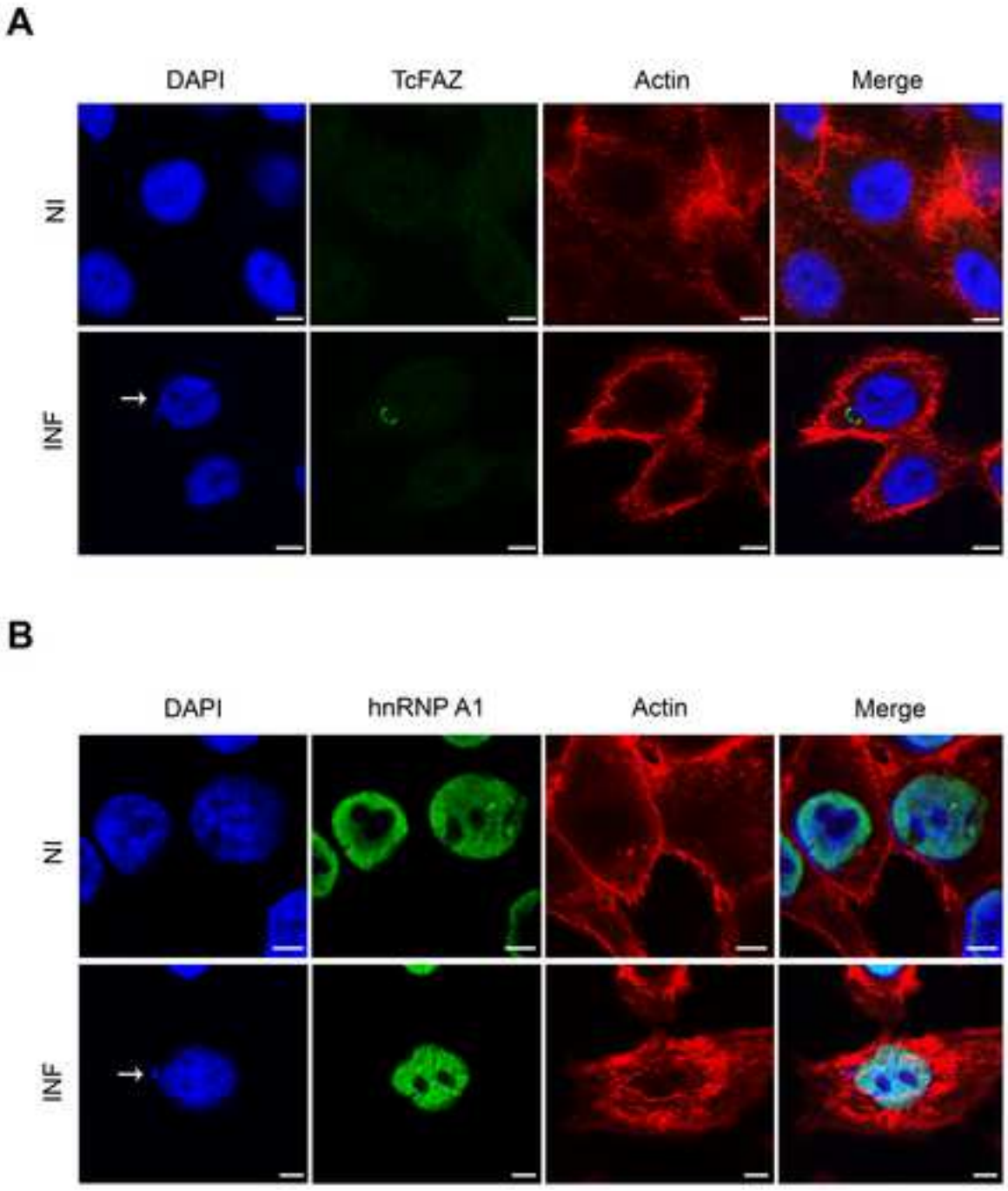


Figure 2

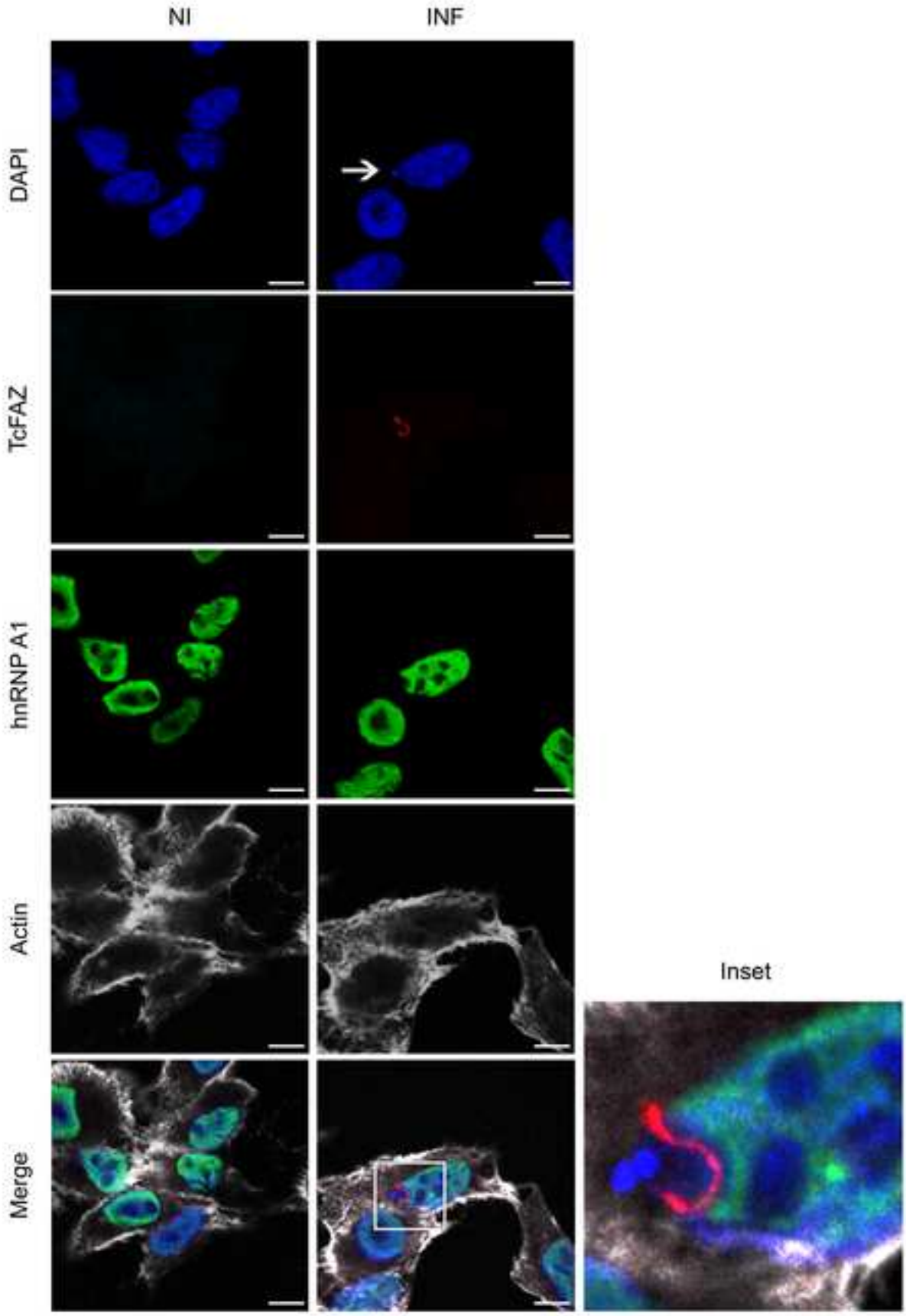
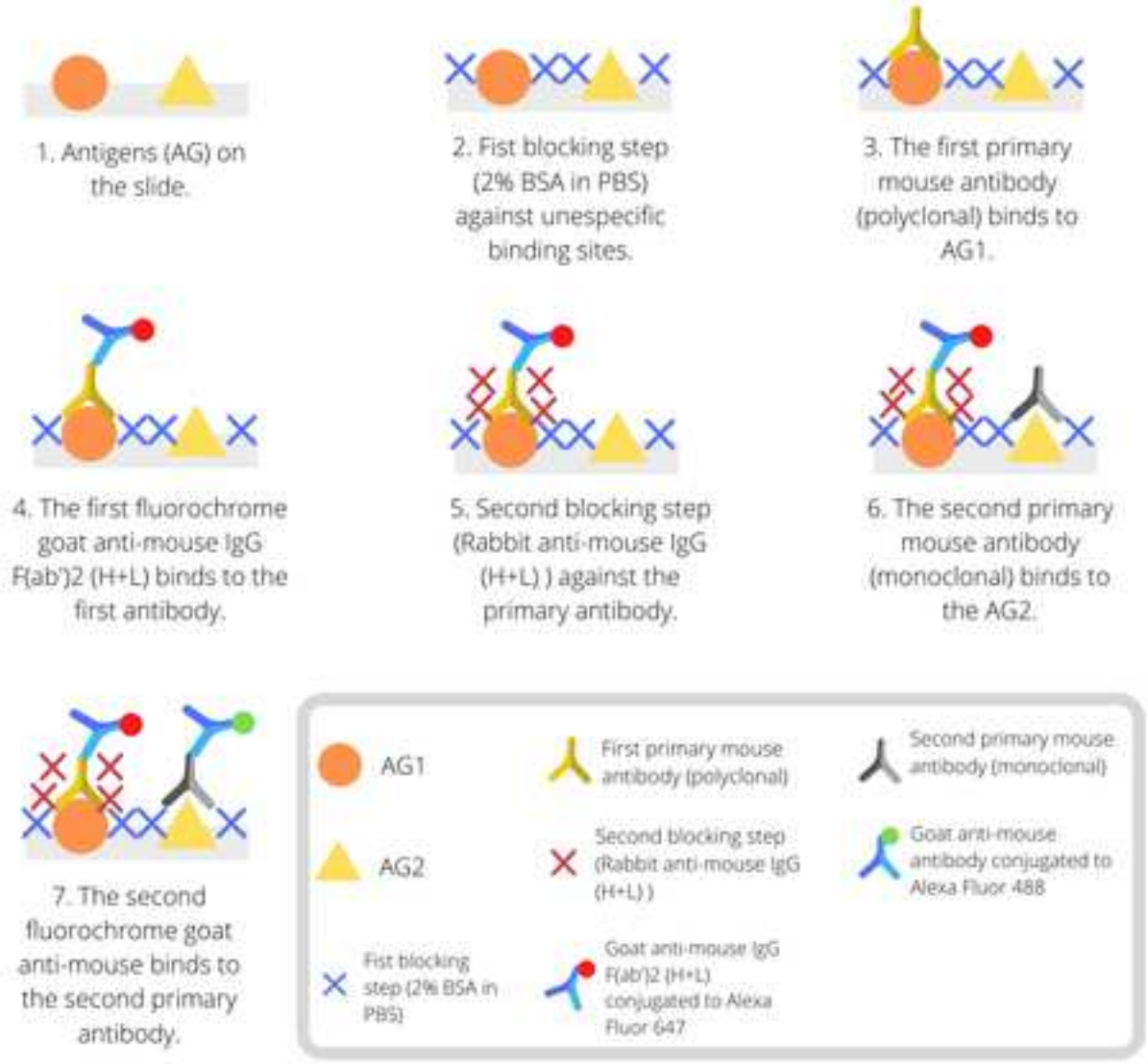


Figure 3



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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).

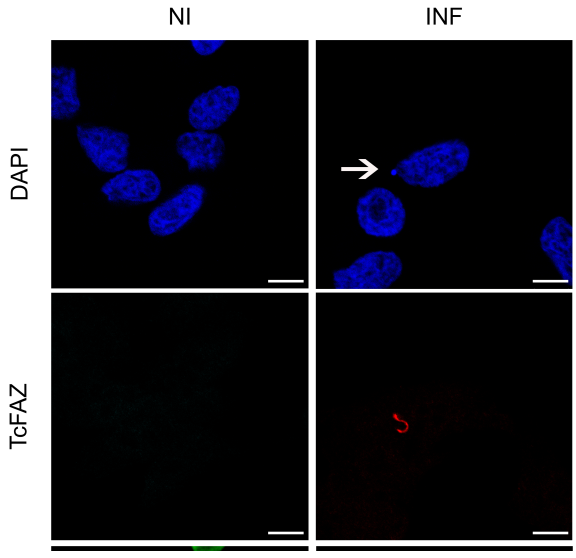
Minor Concerns:

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