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TITLE:

Localization and Quantification of Begomoviruses in Whitefly Tissues by Immunofluorescence and Quantitative PCR

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SUMMARY:

We describe an immunofluorescence and quantitative PCR method for the localization and quantification of begomoviruses in insect tissues. The immunofluorescence protocol can be used to colocalize viral and vector proteins. The quantitative PCR protocol can be extended to quantify viruses in whole whitefly bodies and virus-infected plants.

ABSTRACT:

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) are transmitted by whiteflies of the *Bemisia tabaci* complex in a persistent, circulative manner. Considering the extensive damage caused by begomoviruses to crop production worldwide, it is imperative to understand the interaction between begomoviruses and their whitefly vector. To do so, localization and quantification of the virus in the vector tissues is crucial. Here, using tomato yellow leaf curl virus (TYLCV) as an example, we describe a detailed protocol to localize begomoviruses in whitefly midguts, primary salivary glands, and ovaries by immunofluorescence. The method is based on the use of specific antibodies against a virus coat protein, dye-labeled secondary antibodies, and a confocal microscope. The protocol can also be used to colocalize begomoviral and whitefly proteins. We further describe a protocol for the quantification of TYLCV in whitefly midguts, primary salivary glands, hemolymph, and

ovaries by quantitative PCR (qPCR). Using primers specifically designed for TYLCV, the protocols for quantification allow the comparison of the amount of TYLCV in different tissues of the whitefly. The described protocol is potentially useful for the quantification of begomoviruses in the body of a whitefly and a virus-infected plant. These protocols can be used to analyze the circulation pathway of begomoviruses in the whitefly or as a complement to other methods to study whitefly-begomovirus interactions.

INTRODUCTION:

In the last decades, begomoviruses (genus *Begomovirus*, family *Geminiviridae*) have caused serious damage to the production of many vegetable, fiber, and ornamental crops worldwide[1]. Begomoviruses are transmitted in a persistent manner by the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae), which is a complex species containing over 35 cryptic species[2,3]. Begomoviruses may directly or indirectly affect whitefly physiology and behavior, such as fecundity[4], longevity[4], and host preference[5,6]. Furthermore, the transmission efficiency of a given begomovirus species/strain varies for different whitefly cryptic species even under the same experimental conditions[7–10], indicating that there is a complex interaction between begomoviruses and whiteflies. To better understand the mechanisms underlying whitefly-begomovirus interactions, localization and quantification of the virus in whitefly tissues are essential.

Tomato yellow leaf curl virus (TYLCV) is a begomovirus that was first reported in Israel but nowadays causes serious damage to tomato production worldwide[11,12]. Due to its economic importance, it is one of the best-studied begomoviruses[13]. Like other monopartite begomoviruses, TYLCV is a single-strand circular DNA virus with a genome size of about 2,800 nucleotides[14]. While still under debate, several lines of evidence support the replication of TYLCV in whiteflies[15–17]. Moreover, the interaction of TYLCV particles and whitefly proteins has been reported[6,18–20]. For virus transmission, whiteflies acquire TYLCV by feeding on virus-infected plants, virions pass along the food canal to reach the esophagus, penetrate the midgut wall to reach the hemolymph, and then translocate into the primary salivary glands (PSGs). Finally, virions are egested with saliva along the salivary duct into plant phloem[21]. Furthermore, several studies show that TYLCV is able to be transovarially transmitted from female whiteflies to their offspring[22,23]. In other words, to achieve productive transmission, the virus has to overcome cellular barriers within the whitefly to translocate from one tissue to another. During the crossing of these barriers, interactions between whitefly and virus proteins are likely to occur, probably determining the efficiency with which the viruses are transmitted.

Immunofluorescence is a commonly used technique for protein distribution analysis. The specificity of antibodies binding to their antigen form the basis of immunofluorescence. Due to the economic significance of TYLCV, monoclonal antibodies against the TYLCV coat protein have been developed, offering a highly sensitive way to localize the virus[24]. Quantitative PCR (qPCR) allows sensitive and specific quantification of nucleic acids. This technique is most

frequently based on the use of hydrolysis probe (e.g., TaqMan) or fluorescent dye (e.g., SYBR Green) detection. For hydrolysis probe-based qPCR, specific probes are needed, which consequently increase the cost. Fluorescent dye-based qPCR is simpler and more cost-effective, because labeled amplicon-specific hybridization probes are not required[25]. So far, several studies have used immunofluorescence and qPCR along with other methods to investigate the complex begomovirus-whitefly interactions. For example, Pan et al. performed qPCR and immunofluorescence analysis of the virus in whitefly tissues and found that the difference in ability to transmit tobacco curly shoot virus (TbCSV) between whitefly species AsiaII 1 and Middle East Asia Minor 1 (MEAM1) was due to the virus being able to efficiently cross the midgut wall of AsiaII1 but not MEAM1[8]. Similarly, while Mediterranean (MED) whiteflies can readily transmit TYLCV, they fail to transmit tomato yellow leaf curl China virus (TYLCCNV). Selective transmission was investigated using immunofluorescence detection of virus in the PSGs, which showed that TYLCCNV do not easily cross the PSGs of MED whiteflies[26]. Immunofluorescence colocalization of TYLCV CP and the autophagy marker protein ATG8-II in whitefly midguts shows that autophagy plays a critical role in repressing the infection of TYLCV in the whitefly[27].

Here, using TYLCV as an example, we describe a protocol for the localization of begomoviruses in whitefly midguts, PSGs, and ovaries by an immunofluorescence technique. The technique includes dissection, fixation, and incubation with primary and dye-labeled secondary antibodies. Fluorescence signals showing the location of viral proteins in the whitefly tissues can then be detected under a confocal microscope. More importantly, this protocol can be used to colocalize begomoviral and whitefly proteins. We further describe a protocol for the quantification of TYLCV using SYBR Green-based qPCR in whitefly midguts, PSGs, hemolymph, and ovaries, that can be used to compare the amount of virus in different whitefly tissue samples.

PROTOCOL:

1. Whitefly, virus, plants, and acquisition of the virus

1.1. Rear whiteflies (MEAM1) on cotton (*Gossypium hirsutum* cv. Zhemian 1793) in insect-proof cages in a greenhouse at 26 ± 1 °C with a 14:10 light:dark cycle and 60 ± 10% relative humidity.

1.2. Perform conventional PCR based on whitefly mitochondrial cytochrome oxidase I gene to determine the purity of the whitefly population.

1.2.1. Collect 20 adult whiteflies and transfer them into a PCR tube containing 30 µL of lysis buffer (10 mM Tris, pH = 8.4, 50 mM KCl, 0.45% [wt/vol] Tween-20, 0.2% [wt/vol] gelatin, 0.45% [vol/vol] Nonidet P 40, 60 g/mL Proteinase K).

1.2.2. Add 5–7 ceramic beads (2 mm in diameter) into each PCR tube and grind the samples to perform the tissue lysis.

1.2.3. Incubate all samples at 65 °C for 1 h followed by 10 min at 100 °C, and then centrifuge briefly. Use this supernatant as the template for PCR amplification.

NOTE: The incubation time at 65 °C can be increased if necessary.

1.2.4. Prepare the PCR reaction as follows: 1 U of Taq DNA polymerase, 2 µL of 10x buffer (with Mg^{2+}), 1.6 µL of dNTP mixture (2.5 mM), 0.5 µL of each primer (10 µM each), 2 µL of whitefly tissue lysate supernatant, and double distilled water to a total volume of 20 µL per reaction. The forward primer sequence is 5'-TTGATTTTTTGGTCATCCAGAAGT-3' and the reverse primer sequence is 5'-TAATATGGCAGATTAGTGCATTGGA-3'.

NOTE: A negative control where the DNA is substituted with nuclease-free water and a positive control with previously verified MEAM1 whitefly DNA should be included.

1.2.5. Perform PCR using the following cycling parameters: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50–55 °C for 30 s, and extension at 72 °C for 1 min, followed by 72 °C for 10 min.

1.2.6. Digest the amplified product with restriction enzyme Taq I. To do so, prepare the digestion mixture with 0.1 µL (1 U) of enzyme,1 µL of 10x Taq I Buffer, 1 µL of BSA, 5 µL of amplified product, and double distilled water to a total volume of 10 µL. Incubate at 65 °C for 1 h in a thermocycler.

1.2.7. Perform agarose gel electrophoresis of the samples by loading 5–7 µL of each digested product and a DNA ladder (100-2,000 bp) into the wells of a 1% agarose gel. Then, observe the DNA band sizes by gel-red staining in a gel documentation machine using UV light. Compare the band sizes of the digested products to the positive control to determine the purity of the whitefly population.

1.3. Agro-inoculate the infectious clone of TYLCV (GenBank accession number AM282874.1) (pBINPLUS-SH2-1.4A) into 3–4 true leaf stage tomato plants (*Solanum lycopersicum* L. cv. Hezuo 903).

1.3.1. Inoculate 10 mL of Luria–Bertani (LB) medium containing kanamycin (50 µg/mL) and rifampicin (50 µg/mL) with a single colony. Incubate at 28 °C with shaking at 200 rpm until the OD_{600} reaches ~1.5.

1.3.2. Harvest agrobacteria by centrifugation at 4,000 x *g* for 10 min. Discard the supernatant.

1.3.3. Resuspend pellets in 10 mL of infiltration buffer, consisting of 200 µM acetosyringone, 10 mM 2-(N-morpholino) ethane sulfonic acid (MES), and 10 mM $MgCl_2$. Incubate at room temperature (RT) for 1–2 h.

1.3.4. Infiltrate 2–3 plant leaves using the mixture from step 1.3.3 with a 1 mL needleless syringe. Maintain plants in a greenhouse at 26 ± 1 °C.

1.4. About 1 month later, perform a visual inspection and choose plants showing yellow curl leaf and stunt symptoms.

1.5. Transfer non-viruliferous whitefly adults onto TYLCV-infected tomato plants for 48 h for virus acquisition.

2. Dissection and collection of midguts, primary salivary glands (PSG), ovaries, and hemolymph of female whiteflies

2.1. Collect whiteflies using aspiration, and then place them on ice to put them in a coma. Add 1x PBS (phosphate-buffered saline) buffer onto a microscope slide, and then place whiteflies in the 1x PBS buffer for dissection under a stereo microscope.

2.2.1. For the midgut dissection, break the abdomen of the whitefly and pull out the midgut using a fine acupuncture needle (0.25 mm in diameter). Transfer the midgut to clean, 1x PBS.

2.2.2. For PSG dissection, break the body of the whitefly in the thorax near the head from the dorsal side. Next, insert a needle into the thorax to fix the whitefly, and use another needle to shake the whitefly until the two salivary glands are separated from the body. Then, transfer the PSGs to clean, 1x PBS.

2.2.3. For the ovary dissection, completely break the abdomen of the whitefly, and use a needle to separate the ovaries from other tissues. Then, remove excess tissues by pipetting.

2.2.4. For the hemolymph collection, add 10 µL of 1x PBS buffer onto a microscope slide, and then place a whitefly in the buffer. To obtain the hemolymph, gently tear a hole in the abdomen using a fine acupuncture needle, and slightly press the abdomen to release the hemolymph out into the buffer. Collect 8 µL of the liquid as the hemolymph sample.

3. Localization of TYLCV in whitefly midgut, primary salivary glands, and ovaries by immunofluorescence

NOTE: The procedure for the localization of TYLCV in the midguts is presented as an example. The method can be used for PSGs and ovaries.

3.1 Dissect the midguts in TBS buffer (10 mM Tris-HCl, 150 mM sodium chloride, pH = 7.5) as described in step 2.2.1. Collect 15–20 whitefly midguts, transfer them into a glass Petri dish (3.5 cm in diameter), and then remove excess TBS buffer.

3.2 Add 1 mL of 4% paraformaldehyde to fix midguts for 2 h at room temperature, and then remove the fixative solution. Pipette slowly to wash the midguts 3x in TBS for 2 min each.

3.3 Incubate the midguts in 1 mL of 0.5% Triton X-100 for 30 min to permeabilize. Then remove the permeabilization solution and wash the midguts 3x with TBST (1× TBS with 0.05% Tween 20) as described in step 3.2.

3.4 Add 1 mL of 1% BSA (bovine serum albumin prepared in TBST) to block the midguts. Perform the blocking step at RT for 2 h, and then remove the blocking solution and wash the midguts 3x in TBST.

3.5 Dilute the primary antibodies (MAb 1C4 against TYLCV coat protein) at 1:400 with TBST[24] and add the solution into the Petri dish to immerse the midguts. Incubate at 4 °C overnight in the dark. Discard the primary antibody solution and wash 3x in TBST.

3.6 Dilute the secondary antibodies (anti-mouse) at 1:400 with TBST and add the solution into the Petri dish to immerse the midguts. Incubate the dish for 2 h at RT in the dark. Discard the secondary antibody solution and wash the midguts 3x in TBST.

3.7 Transfer the midguts to a clear microscope slide. Aspirate as much TBST off the slide as possible. Add 1 drop of DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) to stain the nucleus, and then place the coverslip and seal with nail polish. Examine under a confocal microscope.

4. Quantification of TYLCV in whitefly midgut, primary salivary glands, hemolymph, and ovaries with qPCR

4.1. Dissect the whitefly midguts, PSGs, hemolymph, and ovaries in 1x PBS as described above.

4.2. Extract the DNA of the midguts, PSGs, hemolymph, and ovaries.

4.2.1. After dissection, wash the midguts, PSGs, and ovaries in clean, 1x PBS buffer 2–3x.

4.2.2. Collect 20 midguts or 20 PSGs in 5 µL of 1x PBS and transfer them into 25 µL lysis solution. Transfer each ovary in 5 µL of 1x PBS and then transfer into 25 µL lysis solution individually.

4.2.3. Grind midguts, PSGs, and ovaries samples as described in step 1.2.2.

4.2.4. Collect 8 µL of hemolymph with 1x PBS into a PCR tube, and then add 10 µL of lysis solution. Mix thoroughly.

4.3. Extract DNA from all samples as described in 1.2.3.

4.4. Prepare two separate qPCR master mixes (18 µL each) for TYLCV and the internal control (β-actin gene) as follows: 10 µL of SYBR Green mix, 0.8 µL of each primer (10 µM each), and 6.4 µL of double distilled water per reaction. For TYLCV, the forward primer sequence is 5′-GAAGCGACCAGGCGATATAA-3′ and the reverse primer sequence is 5′-GGAACATCAGGGCTTCGATA-3′. For β-actin, the forward primer sequence is 5′-TCTTCCAGCCATCCTTCTTG-3′ and the reverse primer sequence is 5′-CGGTGATTTCCTTCTGCATT-3′.

4.5. Dispense 18 µL of the master mix into vials of qPCR strip tubes or a 96 well plate. Then, add 2 µL of the DNA samples into each vial. Perform all reactions with at least three technical replicates and three biological replicates.

NOTE: Steps 4.3 and 4.4 should be performed on ice.

4.6. Close the qPCR tubes or seal the 96 well plates with adhesive plate seal.

4.7. Centrifuge to collect the liquid at the bottom of the qPCR tubes or 96 well plate.

4.8. Place the tubes or plate into the real-time thermal cycler and perform qPCR.

4.8.1 Perform the qPCR using the following cycling parameters: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s for primer annealing and elongation, 1 cycle at 95 °C for 15 s, ending with a melting curve step of 60 °C to 95 °C with an increment of 0.5 °C per 15 s.

4.8.2 Choose SYBR as the fluorophore dye and unknown as sample type.

4.9. Export the quantitative data to a spreadsheet format and analyze the relative quantity of viral DNA by the $2^{-\Delta\Delta CT}$ method[28].

REPRESENTATIVE RESULTS:

The MEAM1 whiteflies of the *B. tabaci* complex and TYLCV were used as an example here to describe the procedures. The overview of the immunofluorescence and viral quantification procedures described in this manuscript is shown in **Figure 1**. **Figure 2** shows representative results of immunofluorescence detection of TYLCV and DAPI staining in PSGs, midguts, and

ovaries, indicating that TYLCV accumulated more in the PSGs and midguts, and less in the ovaries. **Figure 3** shows the relative quantity of virus in the whitefly PSGs, midguts, hemolymph, and ovaries after whiteflies fed on TYLCV-infected tomato for 24, 48, and 72 h, indicating that the quantity of virus increased in different whitefly tissues with the increase of the acquisition access period.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the protocols presented in this paper. (**A**) Experimental procedures for immunofluorescence in whitefly midguts, PSGs, and ovaries. Tissues were collected from TYLCV-infected adult female whiteflies, followed by a series of fixation, permeabilization, blocking, incubations with primary antibodies that bind to a TYLCV coat protein, and fluorochrome-conjugated secondary antibodies that bind to the primary antibodies. Finally, samples were analyzed under a confocal microscope, allowing the acquisition of images. (**B**) Experimental procedure for the quantification of virus in the whitefly midguts, PSGs, ovaries, and hemolymph. Tissues were collected from TYLCV-infected adult female whiteflies, and then DNA extraction was performed. qPCR was performed on a real-time PCR instrument, allowing the acquisition of data that can be subsequently analyzed.

Figure 2: Immunofluorescence detection of TYLCV in the primary salivary glands, **midguts, and ovaries of female adult whiteflies after a 48 h virus acquisition access period**. Whiteflies were allowed to feed on TYLCV-infected tomato plants for 48 h, and then 20 PSGs, 20 midguts, and 20 ovaries were dissected and collected. Next, samples were fixed in 4% formaldehyde, permeabilized in 0.5% Triton X-100, blocked in 1% BSA, and then incubated with mouse anti-TYLCV monoclonal antibodies (MAb 1C4 against TYLCV coat protein)[24] and goat anti-mouse secondary antibodies conjugated to a red fluorescent dye (i.e., Dylight 549). Nuclei were stained with DAPI (blue). Immunofluorescence signals were examined under a confocal microscope.

Figure 3: Quantification of TYLCV DNA in the midguts, **hemolymph**, **primary salivary glands, and ovaries in MEAM1 whiteflies.** Adult fed on TYLCV-infected tomato plants for 24, 48, and 72 h, and then the whitefly midguts, hemolymph, PSGs, and ovaries were dissected and collected. Next, extraction of DNA and qPCR were performed for the midguts (**A**), hemolymph (**B**), PSGs (**C**), and ovaries (**D**). Data were firstly normalized to the whitefly *β-actin* DNA, and then the relative quantity of virus obtained were again normalized to that at 24 h. NV: Non-viruliferous. Data are presented as the mean ± SEM of relative quantity of virus.

DISCUSSION:

Here we describe a protocol for the localization and quantification of a begomovirus in the tissues of its whitefly vector by immunofluorescence and qPCR. Dissection represents the first step to localize and quantify the virus in whitefly tissues. The whitefly body is about 1 mm in length, which means that the tissues are extremely small, and it is difficult to dissect them. Besides, there are strong connections between the tissues. For example, the ovaries are

tightly connected with the bacteriocytes, making them difficult to isolate. Here, considering the characteristics and locations of the different tissues, we describe different approaches for dissection. However, a lot of practice is needed to rapidly and accurately dissect these tissues. In addition, it is best to use fresh whiteflies, because dead insects would increase the difficulty of dissection. Furthermore, postdissection, tissues must be cleaned to avoid contamination.

The immunofluorescence technique is based on the use of specific antibodies. A suitable working concentration for each antibody is very important in imaging, because a high concentration of antibodies reduces the signal-to-background ratio and a low concentration may not provide sufficient signal. Here, we used a dilution of 1:400 for both primary antibodies and second antibodies. These antibodies should be divided into small aliquots and kept at -20 °C to avoid repeated freezing and thawing. The antibody dilutions should be prepared right before use. When the protocol described here is used to colocalize viral and whitefly proteins, it is important that the primary antibodies against viral and whitefly proteins are produced in animals of different species. Additionally, the fluorophores of the secondary antibodies should be chosen carefully to prevent bleed-through between channels. Furthermore, in view of the small size of whitefly tissues, they should always be washed under the microscope to avoid loss. Although immunofluorescence is highly sensitive, it is relatively more time-consuming and costly when compared to other virus location methods such as Fluorescence in situ Hybridizations (FISH). In addition, antibodies against begomoviruses may not be available for purchase.

The key to the successful application of qPCR for the quantification of the virus in whitefly tissues lies in the design of suitable primers and selection of endogenous reference genes. Rodríguez et al. described the criteria for designing qPCR primers in detail[25], which also applies in the design of virus primers. In this study, β-actin was selected as the reference gene for virus quantification in whitefly tissues. However, in some cases (e.g., RNA genes), other endogenous genes should be used as reference genes if they are suitable for the experimental design[29]. Also, to obtain stable and accurate results, whiteflies of same developmental stage should be used, because whiteflies of different developmental stages possess differential capabilities to acquire and transmit viruses[22]. Moreover, while we used 20 PSGs or midguts as one sample, we used a hemolymph collected from one whitefly as one sample for qPCR analysis. This is due to the fact that the collection of the hemolymph is time-consuming and the DNA of the hemolymph samples is vulnerable to degradation if not processed in a timely manner. This protocol can also be used to quantify the amount of virus in whitefly whole body and virus-infected plants.

In summary, we describe an efficient and sensitive protocol to localize and quantify begomoviruses in whitefly tissues by immunofluorescence and qPCR, respectively. This protocol can also be adapted for the localization and quantification of other begomoviruses. Furthermore, the protocol of immunofluorescence can be used to colocalize viral and whitefly proteins.

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DISCLOSURES:

The authors have nothing to disclose.

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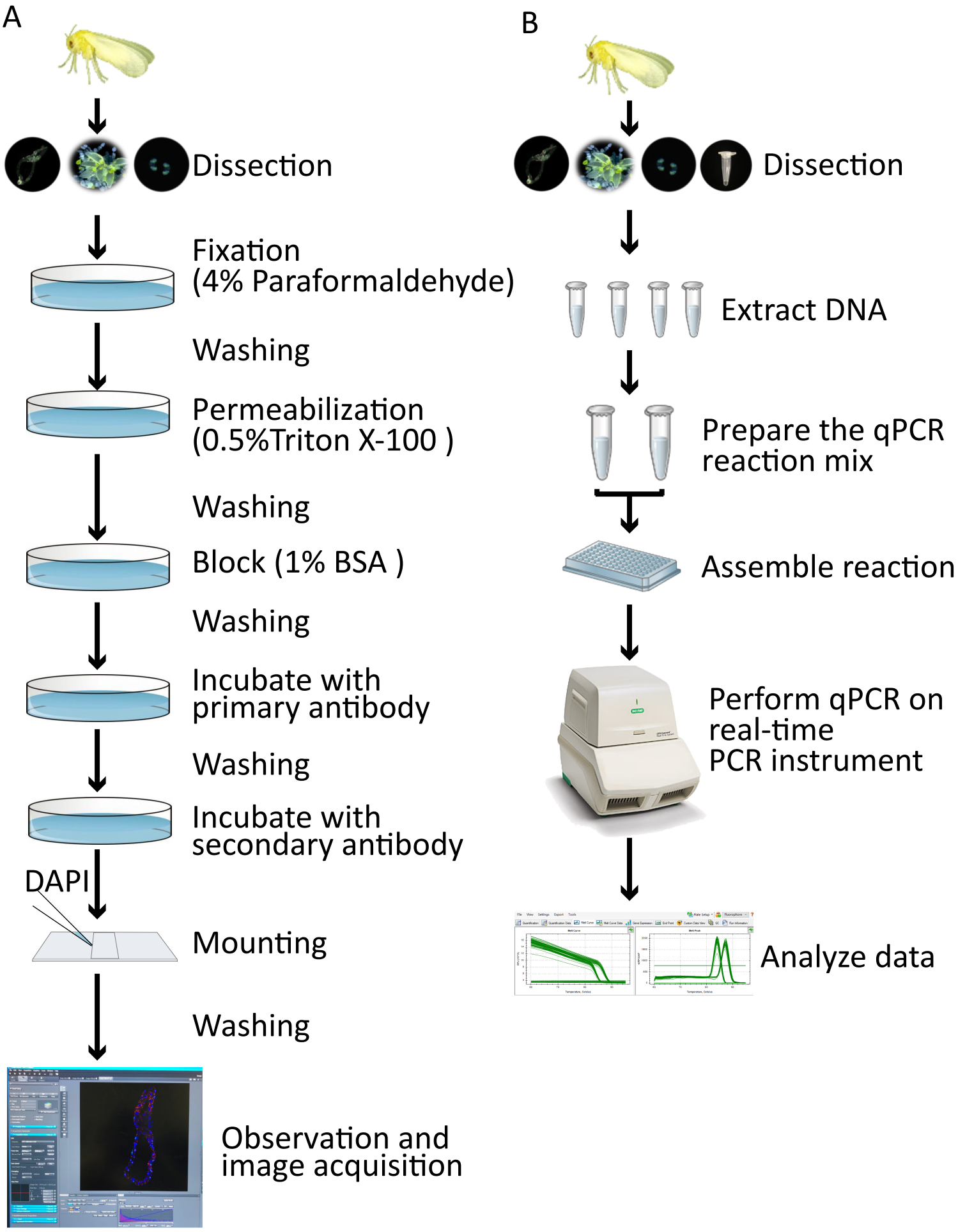

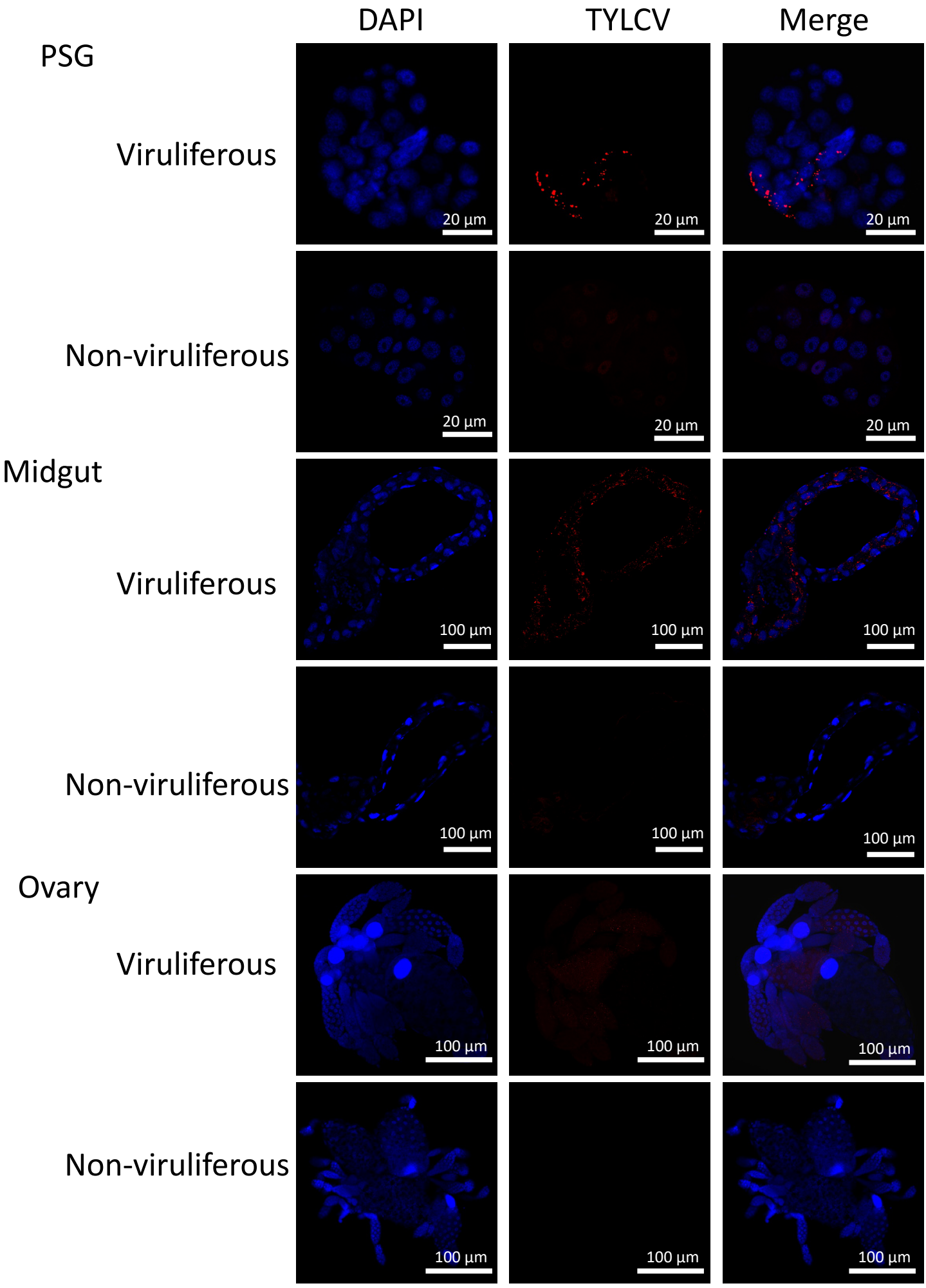

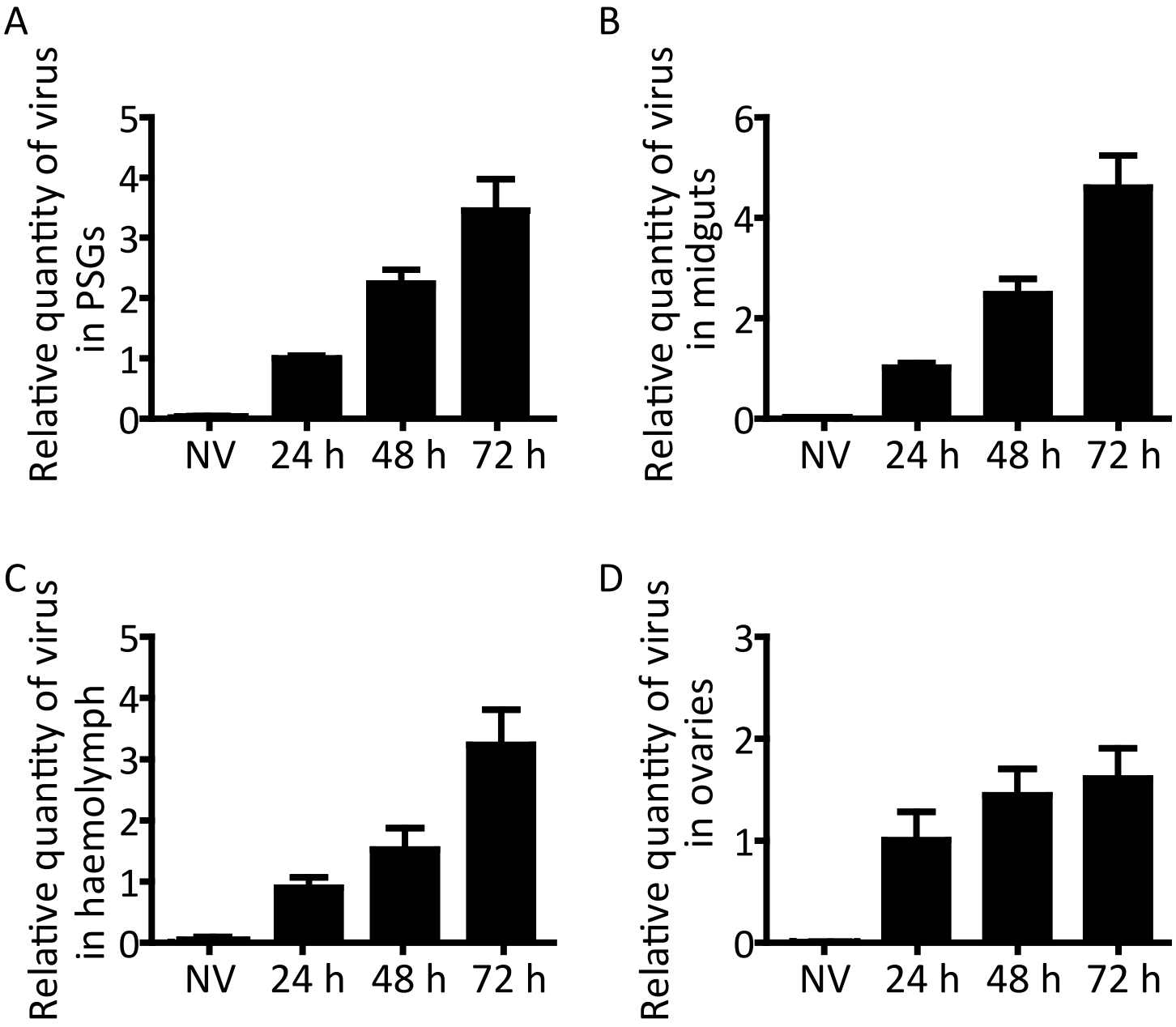

Name of Material/Equipment	Company	Catalog Number	Comments/Description
4% Paraformaldehyde	MultiSciences	F0001	
4',6-diamidino-2-phénylindole (DAPI)	Abcam	ab104139	
Bovine Serum Albumin (BSA)	MultiSciences	A3828	
CFX Connect Real-Time PCR Detection System	Bio-RAD	185-5201	
Confocal microscopy	Zeiss	LSM800	
Dylight 549-goat anti-mouse	Earthox	E032310-02	Secondary antibody
Monoclonal antibody (MAb 1C4)			Primary antibody
Phosphate Buffered Saline (PBS)	Sangon Biotech	B548119-0500	
Stereo microscope	Zeiss	Stemi 2000-C	
TB green premix Ex Taq (Tli RNase H Plus)	TaKaRa	RR820A	qPCR master mix
Thermocycler	Thermofisher	A41182	
Tissuelyzer	Shaghai jingxin	Tissuelyser-48	
Triton-X-100	BBI life sciences	9002-93-1	
Tween 20	BBI life sciences	9005-64-5	

October 16, 2019

Dear Editors, JoVE,

Re: JoVE60731
Localize and quantify begomoviruses in whitefly tissues by immunofluorescence and quantitative PCR

Enclosed please find a revised version of the above manuscript. We appreciate the valuable comments and suggestions from the editor and reviewers. We thank you for considering the manuscript to be published.

In the revised manuscript, we have addressed all of the editors' and reviewers' comments point-by-point. For easy review, the issues raised by the reviewers are given first in black, followed by our responses in blue, which can be found in the subsequent pages.

Overall, we have done our best to address all of the issues raised by the editor and reviewers. We believe that the reviewers' comments and your suggestions have improved the manuscript significantly. We hope that this revised manuscript is now acceptable for publication in JoVE.

Thank you kindly in advance for your consideration.

Yours sincerely,
Dr. Xiao-Wei Wang
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E-mail: xwwang@zju.edu.cn
Tel: (86) 571-88982435

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Authors' response: Done.

2. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
Authors' response: Done.

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points throughout.
Authors' response: Done.

4. Please ensure the Introduction to include all the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Authors' response: Done.

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: TaqMan, SYBR Green, etc.
Authors' response: Done.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Authors' response: Done.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if

necessary.
Authors' response: Done.

8. Please include volume and concentration of all the solutions, buffers, reagents used.
Authors' response: Done.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Authors' response: Done.

10. 1.1- 1.2: There are missing actions between these two steps. At what stage do you isolate DNA? How is this done? What is the input for PCR? How is the PCR performed? Reaction set up, primers used, thermocyler conditions etc.
Authors' response: We are sorry that our description was insufficiently clear. We have added detail description for these missing actions. Please see lines 116-147.

11. 1.3: How is the agro-inoculation performed?
Authors' response: We are sorry that our description was not clear enough. We have added detailed description for the agro-inoculation. Please see lines 148-160.

12. 1.4: What symptoms are observed?
Authors' response: We are sorry that our description was not clear enough. We have added detailed description for the symptoms of plant. Please see lines 161-162.

13. 1.5: How long do you leave the flies for?
Authors' response: We have added the time of virus acquisition. Please see line 164.

14. 3.4: Which primary antibody is used in this case? Against what protein? Citation for using this?
Authors' response: We are sorry that our description was not clear enough. We have added the information for the primary antibody. Please see lines 203-204.

15. 4.3: Please label the primers as forward and reverse. Do you use any Fly specific primers as well?
Authors' response: We have changed the words and added detailed description for another whitefly specific primers. Please see lines 129-130 and 232-234.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Authors' response: we have highlighted the steps that we think should be visualized in video.

17. For the result section, please include a control to show that these primers/antibodies are specific to the virus. Please include all necessary controls.
Authors' response: Done. Please see Figure 2,3.

18. Please do not abbreviate the journal titles in the references section.
Authors' response: Done.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript describes protocols to localize viruses in whiteflies using microscopy and qPCR. The methods are of interest for everyone working on vector-borne pathogens. Overall, the quality of the paper is very high, however, some edits are needed.
Authors' response: Thank you for your comments on the manuscript. We have checked our manuscript carefully, and the point-by-point response was as follows.

Major Concerns:
Line 98: You never said before that the virus "infects" the insect, you just discussed the virus as a persistent circulative virus. Based on the results of the paper, I believe the study was performed with the persistent propagative virus. This has to be noted somewhere in this paragraph, otherwise it doesn't really make sense to use the word infect.
Authors' response: Thank you for your advice. We have added corresponding description for the virus. Please see lines 66-69.

Line 114: Which PCR? Please give primers name
Authors' response: Thank you for your advice. We have added detail description for the PCR and primers. Please see lines 116-147.

Line 187: please clarify that those are two different PCR mixes
Authors' response: Done. Please see line 229.

Line278: This idea here is misleading. Since begomoviruses are DNA viruses, the stability of the expression of a gene is of no importance. What is important to emphasize it that if the virus is a RNA virus, then the endogenous gene has to be carefully selected
Authors' response: Sorry for the mistake, we have rephrased the sentence. See line 322.

Minor Concerns:
Some minor edits are needed, I will send the PDF to the editor

Line 56: :
Authors' response: Done. Please see line 53.

Line 59: Do they really influence? Do you mean "are more or less competent vectors of the same begomovirus"?
Authors' response: According to previous studies, the transmission efficiency of a given begomovirus species/strain varies for different whitefly cryptic species even under the same experiment condition. We are sorry that our description was not clear enough. We have rephrased the sentence. Please see line 56-58.

Line 58: host preference.
Authors' response: Done. Please see line 55-56.

Line 58: physiology and behavior.
Authors' response: Done. Please see line 55.

Line 64: that was
Authors' response: Done. Please see line 62.

Line 71: are
Authors' response: Done. Please see line 72.

Line 74: the
Authors' response: Done. Please see line 75.

Line 75: the
Authors' response: Done. Please see line 76.

Line 76: I find the end of this sentence somewhat awkward. I would stop the sentence here and start a new sentence "During the crossing of these barriers, interactions between whitefly and virus proteins could occur, probably determining the efficiency with which viruses are transmitted." Or something like that.
Authors' response: Thank you for your advice. We have rephrased the sentence. See lines 77-79.

Line 81: the
Authors' response: Done. Please see line 83.

Line 82: to localize.
Authors' response: Done. Please see line 84.

Line 92: being able to
Authors' response: Done. Please see line 84.

Line 93: do you need TYLC here?
Authors' response: We are sorry that our description was not clear enough. We have rephrased the sentence. Please see line 95-96.

Line 98: Either, here it is "shows" or "showing"
Authors' response: we have changed "show" to "shows". Please line 100.

Line 98: s
Authors' response: Done. Please see line 100.

Line 102: Delete "Through" and "antibodies"
Authors' response: Done. Please see line 104.

Line 103: delete "and then"
Authors' response: Done. Please see line 105.

Line 114: which PCR are we talking about, please name the primers or what the primers target
Authors' response: Thank you for your advice. We have added detailed description for the PCR and primers. Please see lines 116-147.

Line 128: either the midgut, or midguts
Authors' response: we have changed "midgut" to "the midgut". Please see line 171.

Line 128: Here and for the PSG and ovaries, you can turn around the terms: For gut dissection,.. For PSGs dissection, ... For the ovaries dissection....
Authors' response: Thank you for your advice. We have rephrased the sentence. See lines 171, 174, 178, 181.

Line 128: the
Authors' response: Done. Please see line 171.

Line 129: using a
Authors' response: Done. Please see line 172.

Line 130: reading the rest of the protocol, you just put the tissues in PBS until you are ready to continue. I would remove the words "for washing"
Authors' response: Thank you for your advice. We have deleted the words "for washing". See lines 173.

Line 131: the
Authors' response: Done. Please see line 175.

Line 131: dorsal or ventral?
Authors' response: We are sorry that our description was not clear enough. We have rephrased the sentence. Please see lines 174-175.

Line 134: same comment as before with washing

Authors' response: Done. Please see line 177.

Line 136: either the ovary, or ovaries
Authors' response: we have changed "ovary" to "the ovary". Please see line 178.

Line 138: I don;t think you dissect the hemolymph, I believe it is for hemolymph collection
Authors' response: Thank you for your advice. We have rephrased the sentence. Please see lines 181.

Line 143: by immunofluoresce?
Authors' response: Done. Please see line 186.

Line 160: what dilution?
Authors' response: the primary antibodies were diluted by TBST (1× TBS with 0.05 % Tween 20) at 1:400. We have rephrased the sentence. Please see line 203-204.

Line 165: in the dark
Authors' response: Done. Please see line 205.

Line 178: 20 midgut or 20 PSGs
Authors' response: Done. Please see line 222.

Line 187: this is two mixes right, one for actin the other for TYLCV, please clarify
Authors' response: We are sorry that our description was not clear enough. We have rephrased the sentence. Please see lines 229.

Line 194: each vial
Authors' response: Done. Please see line 236.

Line 205: analyze the relative quantity of viral…
Authors' response: Done. Please see line 247.

Line 209: The overview of the immunofluorescence and viral quantification procedures described in this manuscript
Authors' response: Done. Please see line 251-252.

Line 216: different whitefly tissues
Authors' response: Done. Please see line 258.

Line 242: Data are
Authors' response: Done. Please see line 287.

Line 274: whitefly tissues depends
Authors' response: Done. Please see line 319.

Line 284: This is due to the fact that
Authors' response: Done. Please see line 329.

Line 286: if not processed in a timely manner
Authors' response: Done. Please see line 331.

Reviewer #2:
Manuscript Summary:
The manuscript by Ban et al. presents a detailed protocol to localize and quantify the begomovirus TYLCV (a typical emergent plant virus) in tissues of the whitefly Bemisia tabaci (a supervector of plant viruses) using immunofluorescence and qPCR. The increasing interest on emergent viruses and their vectors makes this work timely and surely will get the attention of a high number of scientists and students. in general, technical details are sufficiently described. The manuscript is well written and I have only a number of minor points for the authors to consider, listed below.
Authors' response: Thank you for your positive comments on the manuscript.

Major Concerns:
NONE.

Minor Concerns:
L46. I suggest to substitute "quantity" for "amount", to avoid quantification/quantity in the same sentence.
Authors' response: Done. Please see line 43.

L54. …many vegetable, fiber and ornamental crops worldwide.
Authors' response: Done. Please see line 51-52.

L55. I suggest to delete "circulative" as far as the circulative/propagative issue is a matter of debate.
Authors' response: Thank you for your suggestion, we have deleted "circulative".

L58. …affect fecundity, longevity and host preference of whiteflies.
Authors' response: Done. Please see line 55-56.

L60. Add another reference after references 6,8: Fiallo-Olivé E, Pan LL, Liu SS, Navvas-Castillo J (2019) Transmission of begomoviruses and other whitefly-borne viruses: dependence on the vector species. Phytopathology (in press).
Authors' response: Thank you for your suggestion, we have added the references. Please line 58 and references section.

L60. Change "These" to "This".
Authors' response: Done. Please see line 58.

L64. … in Israel which nowadays causes serious damage to tomato production worldwide.
Authors' response: Done. Please see lines 62-63.

L65. Add another reference after reference 9: Navas-Castillo J, Moriones E (2000) Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. Virus Research 71: 123-134.
Authors' response: Thank you for your suggestion, we have added the references. Please line 63 and references section.

L71. …virions are…
Authors' response: Done. Please see line 72.

L88. …complex begomovirus-whitefly.
Authors' response: Done. Please see line 90.

L90. Change "Aisa" to "Asia".
Authors' response: Sorry for the mistake, we have changed the word. Please see line 94.

L92. …due to the capacity of the virus to efficiently cross…
Authors' response: We have rephrased the sentence. Please see line 94.

L108. Change "quantity" to "amount".
Authors' response: Done. Please see lines 110.

L140. Please, explain how the hole was cut.
Authors' response: Done. Please see lines 182-184.

L182. Clarify what kind of grinder was used. A manual grinder?
Authors' response: We are sorry that our description was not clear enough. We have rephrased the sentence. Please see lines 121-122.

Reviewer #3:
Manuscript Summary:
This manuscript describes protocols for localization and quantification of begomoviruses within whitefly tissues. These techniques are important since clarification of spatial and temporal virus localization is the major step to understand begomovirus-whitefly interactions and in the future to device a novel strategy to control serious begomoviral diseases. These techniques are also worth reporting since whiteflies are very tiny and handling of them is not at all easy.
Authors' response: Thank you for your comments on the manuscript.

Major Concerns:
none
Minor Concerns:
This manuscript is generally well written. But the present Reviewer considers that description of FIGURE LEGENDS for Figure 2 and 3 should be more detailed. The reason is that other parts of this manuscript are "general" description, but FIGURE LEGENDS for Figure 2 and 3 describe information for "specific" experiments shown in these figures. For example, the Authors describe "mouse anti-TYLCV monoclonal antibodies" (line 233). Authors should give more information (a reference or commercial information) so that readers can repeat the same experiments only from the given information.
Authors' response: Thank you for your suggestion. We have added more detail about the experiments in the legends of Figure 2 and 3.

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Author(s):	Fei-Xue Ban, Tian-Yan Yin, Qi Guo, Li-Long Pan, Yin-Quan Liu, Xiao-Wei Wang

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