Chapter 2

Zebrafish as a Model for the Study of Host-Virus Interactions

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Abstract

Zebrafish (*Danio rerio*) has become an increasingly important model for in vivo and in vitro studies on host-pathogen interaction, offering scientists with optical accessibility and genetic tractability, and a vertebrate-type immunity that can be separated into innate and adaptive ones. Although it is shown in previous studies that few species of viruses can naturally infect zebrafish, the spring viraemia of carp virus (SVCV), a rhabdovirus that causes contagious acute hemorrhagic viraemia in a variety of cyprinid fishes, can infect zebrafish by both injection and static immersion methods in laboratory conditions. In addition, SVCV can infect zebrafish fibroblast cell line (ZF4 cells), together with the *Epithelioma papulosum cyprini* (EPC) cell line (EPC cells), a common cell line used widely in fish disease research. The infection and propagation of SVCV in zebrafish and especially in these cell lines can be employed conveniently in laboratory for functional assays of zebrafish genes. The zebrafish, ZF4 and EPC cell, and SVCV can serve as a simple and efficient model system in understanding host-virus interactions. In the present chapter, we provide detailed protocols for the host-virus interaction analysis based on zebrafish embryos, ZF4/EPC cells, and SVCV, including infection methods of zebrafish embryos and cell lines, analyses of immune responses by quantitative PCR (qPCR) and RNA sequencing (RNA-Seq), antiviral assays based on ZF4 and EPC cells, and the analysis of host-virus interaction using luciferase assays. These protocols should provide efficient and typical means to address host-virus interactions in a more general biological sense.

Key words Zebrafish, SVCV, Host-virus interaction, Immunity, ZF4, EPC

1 Introduction

Zebrafish (*Danio rerio*) is a species of vertebrates, which have both innate and adaptive immune systems, and the innate immunity can be investigated separately from the adaptive immunity which is found to be mature at least 3 weeks post-fertilization [1]. As a kind of fish with high fecundity (producing hundreds of embryos from one spawning pair), short spawning span, optical transparency of embryos and larvae, small size and rapid embryonic development, as well as the short life cycle, zebrafish has been an attractive model system to dissect host-pathogen interactions [2, 3]. In addition to the above features, zebrafish has the advantage of its well-

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annotated genome [4]. Transient gene knockdown and overexpression can be achieved in vivo by microinjection in one-cell zygotes with designed morpholinos (MOs) and synthetic mRNA or recombinant DNA, respectively [5–7]. Furthermore, recently developed genome-editing approaches based on zinc finger nucleases (ZFNs) [8], transcription activator-like effector nucleases (TALENs) [9, 10], and clustered regularly interspaced short palindromic repeats (CRISPR) [11, 12] can be applied reliably and efficiently in zebrafish to achieve permanent and specific gene manipulation, which makes zebrafish model much more attractive and suitable for biological research in a much wider sense.

Although fish viruses have been studied extensively in relation with aquaculture [13], few viruses are found to infect zebrafish naturally [3, 14]. It has been documented that zebrafish could be experimentally infected with viruses from other fish species, such as Infectious hematopoietic necrosis virus (IHNV) [15, 16], Infectious pancreatic necrosis virus (IPNV) [17], Infectious spleen and kidney necrosis virus (ISKNV) [18, 19], Nervous necrosis virus (NNV) [20], Snakehead rhabdovirus (SHRV) [21, 22], Viral hemorrhagic septicemia virus (VHSV) [23, 24], and Spring viraemia of carp virus (SVCV) [25–30]. Despite this, zebrafish is also used for human viral disease models, such as Herpes simplex virus (HSV) [31, 32], Hepatitis C virus (HCV) [33], Chikungunya virus (CHIKV) [34], and Influenza A virus (IAV) [35]. Notably, most of the infection methods used for the viruses described above are through injection in zebrafish embryos and also in adult fish. However, two viruses, SHRV and SVCV, can infect zebrafish through static immersion [21, 22, 25, 26, 30]. Overall, zebrafish model may serve as the only, or complement other, animal models in examining host-virus interaction and in conducting genetic and chemical or therapeutic screenings, which may facilitate the functional assay of target genes, and the development and testing of new antiviral strategies.

In addition to the wide and successful use of zebrafish model for in vivo studies, zebrafish cell cultures such as embryonic fibroblast cell line (ZF4) and liver cell line (ZFL) are also powerful tools for in vitro analyses, contributing efficiently and sufficiently to the investigation of host immunity and host-pathogen interactions [21, 26, 29, 36, 37]. SVCV can infect not only zebrafish, but also zebrafish cell line (e.g., ZF4) under laboratory conditions [26, 29]. Another important cell line, *Epithelioma papulosum cyprini* (EPC) cells, can be the suitable cells for SVCV propagation. Due to the easy culture and high transfection efficiency, EPC cells have been used widely in functional assay of fish genes and in the study of host-virus interaction, especially in functional assay of genes in zebrafish which also belongs to the Cyprinidae family as the fish from which EPC cells were derived [27, 28, 37, 38]. In combination with the in vivo assays in the zebrafish model system, ZF4 as

well as EPC cells, and SVCV have been used as in vitro host-virus interaction models for understanding host immune responses as well as host-virus association in laboratory.

In this chapter, we provide techniques and detailed protocols for the study of host-virus interaction and for the examination of host immune responses, by using zebrafish embryos, ZF4 and EPC cells, and SVCV as an infection virus. Our main aim is to present essential protocols for researchers to examine host-virus interactions, and to provide useful suggestions in performance of such studies, although some details such as the virus propagation as well as the infection method may not be applicable for other viral pathogens.

2 Materials

2.1 Zebrafish Embryos, Cells, and Virus Preparation

1. Zebrafish rearing facility.
2. Adult wild-type AB fish or specific gene manipulation fish.
3. Spawning tanks.
4. Incubator maintained at 28 °C.
5. Embryo water: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ in sterilized water, supplemented with 0.3 μg/mL methylene blue [39].
6. 100 × 20 and 35 × 10 mm Petri dishes.
7. 25 cm^2, 75 cm^2 flasks.
8. Cell culture plates (6-well and 12-well).
9. CO_2 incubator.
10. Zebrafish embryonic fibroblast cells (ZF4).
11. ZF4 cell culture medium: 1:1 mixed Dulbecco modified Eagle medium (DMEM) and Ham F12 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin.
12. *Epithelioma papulosum cyprini* (EPC) cells.
13. EPC cell culture medium: Minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin.
14. 0.25% Trypsin-EDTA.
15. Methylcellulose.
16. Paraformaldehyde.
17. Crystal violet.
18. Spring Viraemia of Carp Virus (SVCV).
19. Centrifuge tubes (2.0 mL and 50 mL).
20. Refrigerated centrifuge.

2.2 Microinjection and Static Immersion Apparatus

1. Microinjector (e.g., Eppendorf, Femto Jet).
2. Mechanical xyz micromanipulator arm (e.g., Narishige, M-152).
3. Microloader pipette tips.
4. Stereo-microscope.
5. Borosilicate glass capillaries.
6. Pipette puller.
7. Phenol red.
8. Agarose.
9. Tricaine.
10. Dumont Watchmaker's forceps No. 5.
11. 100 × 20 and 35 × 10 mm Petri dishes.

2.3 Tools for qPCR and RNA-Seq Analyses

1. Trizol® reagents.
2. Trichloromethane.
3. Isopropanol.
4. Ethanol 75%.
5. Homogenizer.
6. Micro-pipette (e.g., Eppendorf) and tips (10 μL, 200 μL, and 1000 μL).
7. Microtubes (1.5 mL and 200 μL).
8. DNase I, RNase-free.
9. cDNA synthesis kit.
10. qPCR primers with Tm of 58–60 °C.
11. qPCR mix: SYBR Green dye, 50 U/mL Taq DNA polymerase, 0.4 mM each dNTPs, 6 mM $MgCl_2$, 40 mM Tris–HCl pH 8.4, 100 mM KCl, 20 nM fluorescein.
12. Thermal cycler for PCR.
13. Quantitative real-time PCR detection system.

2.4 Tools for Antiviral Assays

1. Plasmids that can express target genes in eukaryotic cells and the desired constructed plasmids (e.g., pcDNA3.1/myc-His(-) A and ptGFP1 [40]).
2. Plasmid purification kit.
3. Cell lines (e.g., ZF4 and EPC).
4. SVCV.
5. Electronical transfection system (e.g., Lonza, Amaxa® Nucleofector® II Device).
6. Electronical transfection reagents (e.g., Lonza, Amaxa™ Cell Line Nucleofector™ Kit V).

7. G418 sulfate.
8. ZF4 cell culture medium.
9. EPC cell culture medium.
10. Opti-MEM medium.
11. 0.25% Trypsin-EDTA.
12. Methylcellulose.
13. Paraformaldehyde.
14. Crystal violet.
15. Lipofectamine™ 2000 transfection reagent.
16. Cell culture plates (12-well, 24-well, and 48-well).
17. 25 cm^2, 75 cm^2 flasks.
18. Microtubes (1.5 and 2.0 mL).

2.5 Tools for Luciferase Assays

1. Constructed plasmids that express host gene as well as viral components.
2. Luciferase reporter plasmids (e.g., IFNφ1pro-Luc, pGL3-Basic, pRL-TK).
3. Cell lines (e.g., EPC).
4. SVCV.
5. Opti-MEM medium.
6. ZF4 cell culture medium.
7. 0.25% Trypsin-EDTA.
8. Lipofectamine™ 2000 transfection reagent.
9. Cell culture plates (24-well).
10. Luminometer (e.g., Promega, GloMax® 20/20).
11. Dual-Luciferase® assay system.

3 Methods

3.1 Zebrafish Embryos, Cells, and Virus Preparation

3.1.1 Preparation of Embryos for Infection

1. Prepare several spawning tanks (containing inner tank with holes that allow laid embryos falling through the holes and preventing adults from eating the embryos) with three to five adult pairs of the breeding wild-type AB zebrafish or specific gene manipulation zebrafish.
2. Collect the zebrafish embryos carefully and rinse with fresh water and transfer the embryos in standard Petri dishes with indicated embryo water.
3. Keep the dishes with embryos in an incubator at 28 °C, remove bad embryos (abnormal developmental embryos) and the remaining waste, and change the culture medium with fresh embryo water every day.

3.1.2 Cell Culture

1. Culture ZF4 cells in a ZF4 cell culture medium at 28 °C in an incubator with 5% CO_2 [36] and EPC cells in an EPC cell culture medium at 25 °C in an incubator with 5% CO_2. The detail methods for cell propagation are as the followings.
2. Maintain the cells in the culture medium as described above in 25 cm^2 or 75 cm^2 flasks.
3. When the cells in the flasks are about 80% confluent, remove the medium and add appropriate 0.25% Trypsin-EDTA (1 mL for 25 cm^2 flask and 2 mL for 75 cm^2 flask) and incubate for 3–5 min to detach the cells from culture flasks.
4. Flutter the flasks gently for detaching the cells completely, add appropriate fresh medium (~ 5 mL), and mix gently.
5. Remove the cell suspension to a 50 mL tube and count the total cell numbers by using hemocytometer.
6. Dilute the cell suspension with fresh medium to an appropriate amount (e.g., 2×10^5 cells in 500 μL plating medium for one well of the 24-well plate), and transfer cell culture into the plates or flasks for later assays such as transfection and infection.

3.1.3 Virus Propagation and Titer Determination for Infection

SVCV could propagate effectively in EPC cells and the virus titer could also be determined by plaque assay on EPC cells. Herein, we describe the detailed procedures used for SVCV propagation and titer determination.

1. Prepare the EPC cells in the 75 cm^2 flask to be about 90% confluent.
2. Thaw SVCV on ice (*see* **Note 1**) and dilute about 2 μL seed virus (*see* **Note 2**) in the 5 mL MEM medium without FBS.
3. Remove the medium of EPC cells and wash the cells once with FBS-free MEM. Remove the medium and gently add the diluted seed virus medium into the flask. Gently shake the flask every 15 min to make sure the virus contact with the cells, and incubate the cells at 25 °C in a CO_2 incubator for 1 h.
4. Remove the seed virus medium and add appropriate volume of normal MEM medium (~15 mL) for cell culture.
5. Check the cell status daily until the appearance of total cytopathic effect (CPE) rising to 80%, put the flask of cells in −80 °C and release the virus from the cells by the repeated freeze-thaw method for three times.
6. Collet the cell lysate and centrifuge at 12,000 × *g* for 20 min to remove the cell debris, transfer the supernatant to new tubes, and store at −80 °C for further analysis.
7. Prepare the EPC cells in 12-well plates for the determination of the virus titer (the cells in plates should be about 90% confluent).

8. Dilute the virus at 1:10, 1:100, 1:1000 and so on with the no-FBS MEM to a volume of 400 μL, and plate the virus dilution in 12-well plates, and then perform as described in Subheading 3.1.3.
9. Remove the virus dilution medium, add 800 μL MEM (with 0.5% methylcellulose and 10% FBS) per well and incubate at 25 °C.
10. Check the cell growth and death everyday using a microscope. When the complete plaque shows up (about 48 h, *see* **Note 3**), fix the cells with a final concentration of 10% paraformaldehyde for 1 h.
11. Remove the mixture and gently wash the cells with fresh water and then stain the cells with 0.5% crystal violet for 2–3 h.
12. Remove the crystal violet medium, gently wash the cells with fresh water, and count the number of plaques. Determine the virus titer by calculating the amount of viruses using the formula: 2.5 × dilution ratio × numbers of plaques emerged at the dilution = total PFU (plaque forming units) of 1 mL virus medium (PFU/mL). Three individual experiments should be performed and data recorded as mean ± standard error (SE).

3.2 Infection with Virus

Zebrafish embryos as well as larvae can be infected with SVCV through static immersion and microinjection [25, 26, 30]. Basically, it is more convenient to infect the embryos by static immersion, whereas the infection by microinjection can be complicated but extremely efficient (the 50% lethal dose is ~2 PFU and 8 PFU for 30- and 54-h post-fertilization (hpf) embryos, respectively) [30]. Although recent genome-editing approaches like ZFNs, TALENs, and CRISPR can generate permanent gene manipulation embryos, transient gene knockdown techniques like MOs still play important roles in such studies [5]. However, MOs knockdown of zebrafish embryos is efficient for not more than 1 week post-fertilization, and zebrafish become susceptible to SVCV until they can swim and open the mouth and gill slits for respiratory movements (about 4 ~ 5 dpf) [5, 30]. It is the fact that only a few viruses can successfully infect zebrafish by immersion, making the infection by microinjection an important method in many research approaches. Herein, we provide the protocols of infecting zebrafish embryos by microinjection and immersion with SVCV, respectively. A typical work plan for infecting zebrafish embryos with SVCV by microinjection is shown in Fig. 1.

3.2.1 SVCV Infection in Zebrafish Embryos by Microinjection

1. Collect and culture zebrafish embryos as described in Subheading 3.1.1. Separate the 48 hpf zebrafish embryos into about 100 per dish for microinjection.

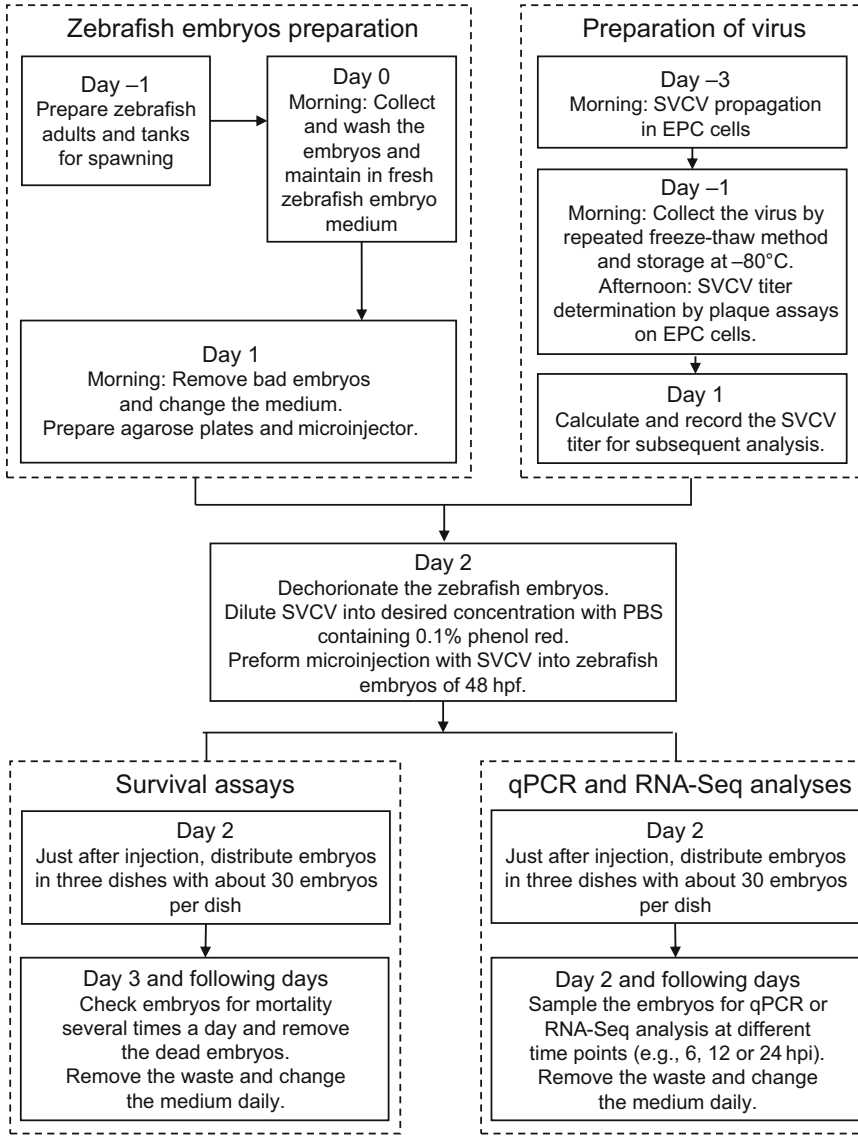

Fig. 1 Typical work plan for infection of zebrafish embryos with SVCV by microinjection. *hpf* hours post-fertilization; *hpi* hours post-infection

2. Manually dechorionate all embryos with Dumont Watchmaker's forceps No. 5 and transfer the embryos into new dishes.
3. Prepare 50 mL embryo water with 200 μg/mL tricaine and move the embryos for microinjection into the dish and incubate for not more than 5 min.
4. Transfer the embryos from the dish with tricaine to a new dish filled with 3% agarose, leaving as little water on the surface of

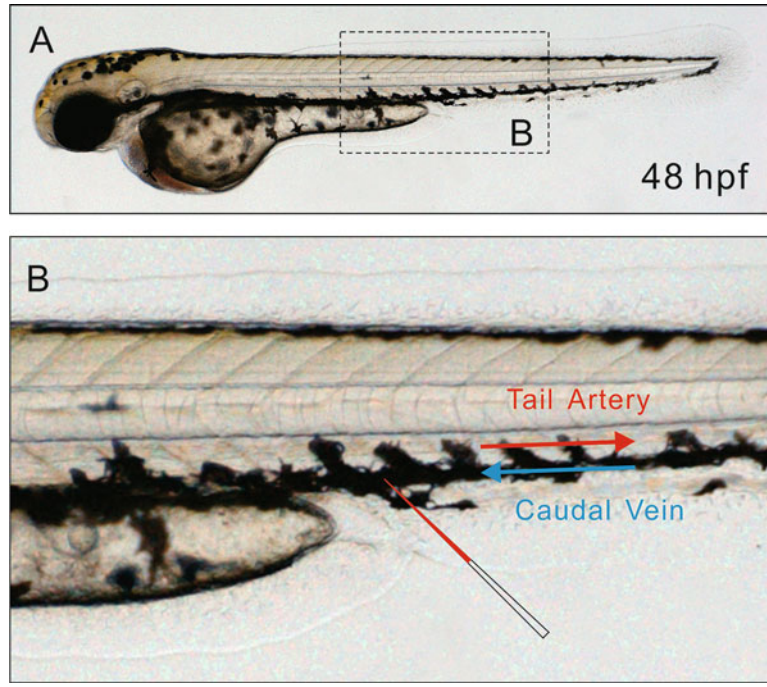

Fig. 2 Microinjection sites of zebrafish embryos. The figure provides the commonly used method for microinjection of SVCV into a 48 hpf zebrafish embryo (**a**). The position of the tail artery and caudal vein of the zebrafish embryo are shown, and the microinjection site is directed with a schematic red needle (**b**)

agarose as possible for aligning the embryos in a same direction.

5. Thaw SVCV on ice and dilute the SVCV titer to reach your desired dose with PBS containing 0.1% phenol red (usually 10^7 PFU/mL).
6. Load 3 ~ 5 μL SVCV dilution into a pulled capillary pipette using a microloader tip and place on the micromanipulator (*see* **Note 4**). Adjust time and pressure of the microinjector to obtain an injection volume of around 2 nl.
7. Locate the capillary needle at the caudal vein and microinject the SVCV dilution with a single pulse with the phenol red to visualize the injection (Fig. 2). Remove the embryos that are poorly injected.
8. Rinse and transfer the embryos into new dishes containing fresh embryo water and maintain at 25 °C (*see* **Note 5**).
9. Separate the embryos into two groups, one for survival experiments and another for gene induction measurements; each group includes three dishes with 30 embryos per dish.

The embryos should be checked daily and dead embryos should be removed, with fresh embryo water added.

10. For control group, the embryos should be microinjected with the same volume of PBS containing 0.1% phenol red at the same position and treated under the same condition.

3.2.2 SVCV Infection in Zebrafish Embryos by Static Immersion

1. Separate the zebrafish embryos into about 100 per dish for infection. At 4 dpf, remove as much embryo water as possible to collect the zebrafish larvae, most of which could swim by then.
2. Immerse the larvae into 10 mL embryo water containing 10^6 PFU/mL SVCV. Control larvae should be exposed to an equal volume of PBS in 10 mL embryo water.
3. Maintain the larvae for 12 h at 25 °C.
4. Wash the larvae carefully with fresh embryo water, transfer the larvae into new dishes of fresh embryo water, and maintain at 25 °C.
5. Separate the infected larvae for two groups, one for survival experiments, and another for gene induction measurements; each group includes three dishes with 30 larvae per dish. The larvae should be monitored daily and dead larvae should be removed, with fresh embryo water added.

3.2.3 SVCV Infection in Zebrafish Cells

Compared with the zebrafish embryos as a model to investigate host-virus interaction in vivo, zebrafish cell lines, like ZF4 and ZFL cells, are also important materials for understanding host-virus interaction in vitro, especially in gene induction measurements and antiviral assays [27–29, 37]. The following section provides the protocols for SVCV infection in ZF4 cells as an example.

1. Transfer the ZF4 cells from the flasks to 6-well plates as described in Subheading 3.1.2. Usually, 1×10^6 cells per well with 2 mL medium should be set up. Then, incubate the cells at 25 °C overnight (*see* **Note 4**).
2. Prepare SVCV for infection in the following day. The specific multiplicity of infection (MOI) of virus to each well must be calculated before infection. Use the formula: (the number of cells) × (desired MOI)/(virus titer (PFU/mL)) = total mL of virus suspension needed to reach your desired dose. For example, you have SVCV with a titer of 1×10^8 PFU/mL and ZF4 cells with 1×10^6 per well, and the desired MOI is 10. Then, the formula is: (1×10^6 cells) × (10 MOI)/(1×10^8 PFU/mL) = 0.1 mL or 100 μL. Therefore, the total volume of virus suspension needed is 100 μL.
3. Dilute the desired volume of SVCV with no FBS added DMEM/F12 medium to a total volume of 2 mL.

4. Remove the culture medium in 6-well plates and add SVCV dilution into the well and incubate at 25 °C for 1 h, with gentle shaking of the plates every 15 min to make sure the virus contacting with the cells.
5. Remove the virus dilution and add 2 mL DMEM/F12 medium with 10% FBS per well for cell culture.
6. Monitor the cell status daily and sample the cells at various time points like 6 or 24 h post-infection (hpi) for further analyses such as gene expression analysis and antiviral assay.

3.3 qPCR and RNA-Seq Analyses

Quantitative real-time PCR (qPCR) is a simple and efficient method to examine the host response such as the induction of immune-related genes under the infection of virus. The following section describes the methodological details for qPCR and RNA-Seq analyses.

3.3.1 qPCR and RNA-Seq Analyses Using Embryos

1. Prepare the primers for qPCR, and the target genes may be immune-related genes, such as interferon, Toll like receptors (TLRs), RIG-I like receptors (RLRs) genes, and so on. The primers for housekeeping genes like GAPDH and β-Actin should also be included.
2. Culture and infect zebrafish embryos with SVCV as described in Subheading 3.2.1.
3. At appropriate time post-infection (e.g., 6, 12, 24 hpi, and so on), collect the embryos (~10 embryos) and transfer them into RNase-free tubes and wash with water.
4. Discard the water and add 1 mL Trizol® reagents for total RNAs extraction. The methodological details are as the followings:
   (a) Homogenize embryos with Trizol using a homogenizer.
   (b) Add 200 μL trichloromethane into the tube containing homogenized embryos with vigorous mixing for 1 min, then incubate at room temperature for 5 min.
   (c) Centrifuge the suspension at 12,000 × *g* for 10 min at 4 °C.
   (d) Transfer the supernatant gently (~400 μL) into a new tube.
   (e) Add 600 μL isopropanol into the tube and mix gently, then incubate at room temperature for 5 min.
   (f) Centrifuge at 12,000 × *g* for 15 min at 4 °C.
   (g) Discard the supernatant and add 1 mL of 75% cold ethanol with the gentle inverting of the tube several times, and then centrifuge at 12,000 × *g* for 10 min at 4 °C.

(h) Discard the liquid and dry the RNA precipitation (usually place the tube at room temperature for 15 min with the lid open).

(i) Add appropriate volume (~30 μL) of RNase-free water to dissolve the RNA precipitation. Determine the quality as well as the concentration by using a spectrophotometer (*see* **Note 6**).

5. Use appropriate amount of total RNA (~1 μg) for cDNA synthesis. Prior to cDNA synthesis, total RNA should be treated with RNase-free DNase I to remove trace amount of DNA as described by the manufacturer.
6. Use the DNase I treated RNA for cDNA synthesis using a cDNA synthesis kit on a thermal cycler for PCR following the instructions of the manufacturer.
7. Perform qPCR on a quantitative real-time PCR detection system with amplifying target genes (e.g., TLRs) as well as housekeeping gene (e.g., GAPDH) transcripts. The gene expression pattern can be presented as fold change relative to the control group, which can be referred to a previous publication [41]. Additionally, the purified RNA could also be used for RNA-Seq analysis to obtain an extensive understanding of the gene transcription under virus infection.

3.3.2 qPCR and RNA-Seq Analyses Using Cell Lines

1. Culture and transfer the cells (e.g., ZF4) into 6-well plates, and on the following day, infect the cells with SVCV at desired MOI as described in Subheading 3.2.3.
2. At different time points post-infection (e.g., 6, 12, 24 hpi, and so on), remove the medium and add 1 mL Trizol® reagents for total RNA extraction. Completely lyse the cell pellet by pipetting the liquid several times.
3. Extract total RNA and synthesize cDNA as described in Subheading 3.3.1.
4. Perform qPCR to analyze the gene expression pattern in a quantitative real-time PCR detection system using the synthesized cDNA, and the RNA-Seq assay could also be used to achieve a full understanding of gene transcription in cells under virus infection.

3.4 Antiviral Assays

To examine the function of genes in zebrafish antiviral response, it is a simple and effective method to overexpress genes in cell lines for further antiviral assays or for checking the induction of downstream molecules [27–29, 37]. The following protocol provides the methods for antiviral assays in vitro by using stably transfected ZF4 cells (ZF4 cells stably transfected with ptGFP1-NOD2 plasmid as an example) and transiently transfected EPC cells (EPC cells transiently transfected with pcDNA3.1-MDA5 as an example)

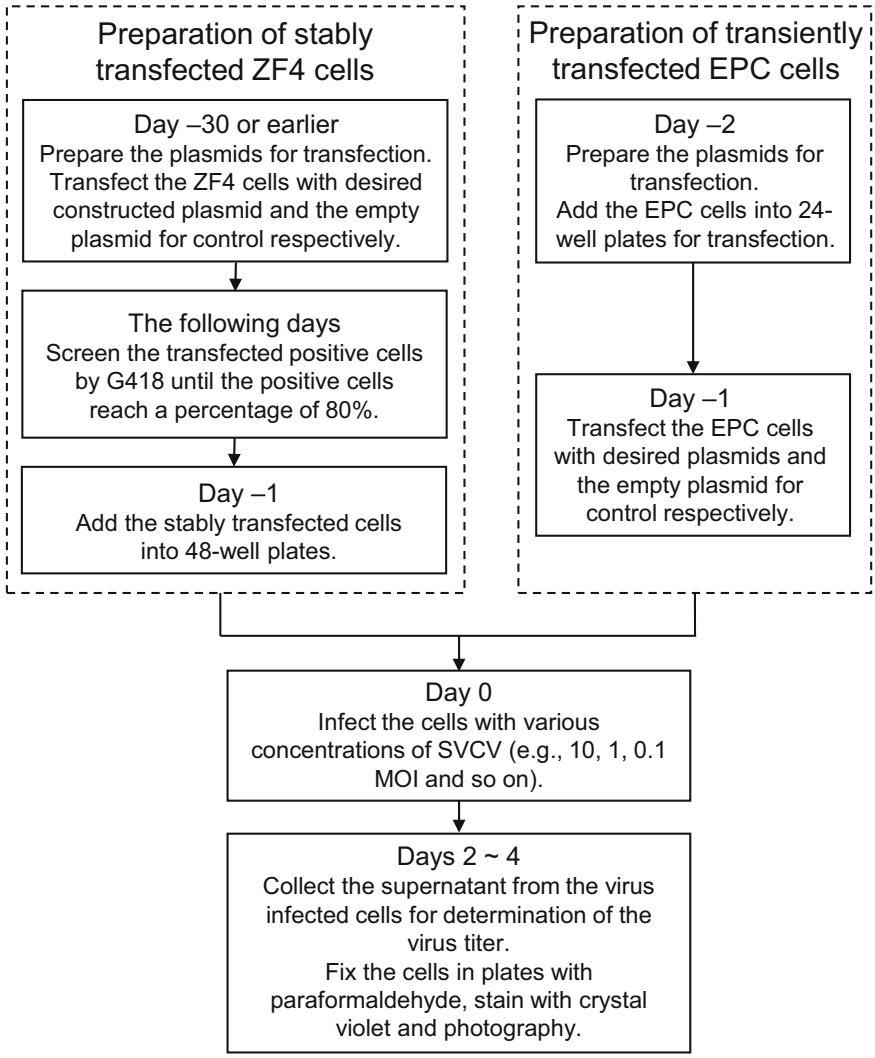

Fig. 3 Typical work plan for antiviral assays using ZF4 and EPC cells. *MOI* multiplicity of infection

respectively [27, 28]. A typical work plan of antiviral assays using stably transfected ZF4 cells as well as transiently transfected EPC cells against SVCV infection can be referred to in Fig. 3.

3.4.1 Antiviral Assays by Stably Transfected ZF4 Cells

1. Prepare the construct plasmids for transfection. The plasmid can be commercial plasmid (e.g., pcDNA3.1/myc-His(-) A), and can also be those constructed by researchers, which can express efficiently the target protein in cells. Herein, we choose ptGFP1 vector for overexpression, which is modified from the commercial plasmid pTurboGFP-N vector (Evrogen), and contains two sets of CMV promoter and SV40 3′ UTR to drive the expression of target gene product and GFP as separate proteins rather than as a fusion protein [40].

2. Construct the target gene (e.g., NOD2) sequence into ptGFP1 vector using molecular cloning technology, and confirm the constructed plasmid by sequencing (*see* **Note 7**).
3. Purify the plasmid by using the commercial plasmid purification kit and measure the purified plasmid concentration by a spectrophotometer.
4. Add the ZF4 cells into a new 25 cm^2 flask as described in Subheading 3.1.2 and culture the cells for 1 ~ 2 days.
5. Transfect the cells with the 2 μg constructed ptGFP1-NOD2 plasmid by using the Amaxa Nucleofector II transfection system (Lonza). Briefly, harvest the cells as described in Subheading 3.1.2 by using 0.25% Trypsin-EDTA and transfer the cells into a new tube and centrifuge at 400 × *g* for 10 min to pellet the cells. Discard the supernatant and resuspend the cells with a 100 μL room-temperature cell line Nucleofector® solution and add a 2 μg constructed ptGFP1-NOD2 plasmid. Transfer the cell/plasmid suspension into the certified cuvette with the cap closed and select Nucleofector® program T20 for transfection. Add ~500 μL medium into the cuvette and gently transfer the cells into a new 25 cm^2 flask with 5 mL medium for culture in an incubator.
6. Remove the medium with the dead cells in the following day, and add 5 mL medium containing 1000 μg/mL G418 sulfate for screening of transfected positive cells.
7. Change the medium with G418 per 3 ~ 5 days and culture the cells with screening for 2 ~ 4 weeks till the transfected positive cells reach a percentage of about 80% (the ptGFP1 plasmid could express GFP separately in transfected cells which could determine as a marker and be examined under a fluorescence microscope). Then, maintain the cell with medium containing 200 μg/mL G418.
8. Harvest the stably transfected ptGFP1-NOD2 cells and transfer into 48-well plates with 1.5 × 10^5 cells per well. The cells stably transfected ptGFP1 empty vector is passed separately and set as control. Incubate the plates of cells at 25 °C.
9. Infect the cells with various concentrations of SVCV (e.g., 10 MOI, 1 MOI, 0.1 MOI, and so on), as described in Subheading 3.2.2.
10. Monitor the cells daily under a microscope till the target cells represent some different status relative to the control cells (e.g., the cells stably transfected ptGFP1-NOD2 cells represent fewer CPE compared to the control cells under the same infection concentration of SVCV), and the time point can be normally 4 days after infection.

11. Collect the supernatant from the target cells and the control cells with the same infection concentration for titer determination by using EPC cells, and fix the plates of ZF4 cells with paraformaldehyde, followed by staining with crystal violet as described in Subheading 3.1.3 and then record by photography.
12. Representative antiviral assay using ZF4 cells stably transfected with ptGFP1 (vector control) or ptGFP1-NOD2 with the infection of SVCV is shown in Fig. 4.

3.4.2 Antiviral Assays by Transiently Transfected EPC Cells

Zebrafish, as a freshwater fish belonging to the Cypriniformes, has a close relationship with common carp (*Cyprinus carpio*) also belonging to this order. It is possible to express zebrafish genes in EPC, which is a cell line from carp [27–29, 37, 38]. In addition, the cell line of EPC has a much higher transfection efficiency and susceptibility to SVCV infection compared to zebrafish cells like ZF4 and ZFL. To some extent, the EPC cell line can be an easy and efficient tool for understanding the function of zebrafish genes in host-virus interaction in vitro. The following section provides the protocols for antiviral assays in transiently transfected EPC cells.

1. Prepare the construct plasmids (e.g., pcDNA3.1-MDA5 for overexpressing MDA5, and empty pcDNA3.1 plasmid for control) for transfection as described in Subheading 3.4.1.
2. Transfer the EPC cells into 24-well plates with 1×10^6 cells per well and incubate the cells at 25 °C overnight.
3. Transfect the cells with 1 μg pcDNA3.1-MDA5 or pcDNA3.1 empty plasmid respectively by using Lipofectamine™ 2000 according to the manufacturer's instructions. Briefly, the methodological details for the transfection of a well in a 24-well plate are as the followings:
   (a) Dilute the plasmids in 50 μL Opti-MEM® and mix gently.
   (b) Dilute appropriate amount (~ 1 μL) of Lipofectamine™ 2000 in 50 μL Opti-MEM® medium and incubate for 5 min at room temperature.
   (c) Combine the diluted plasmid with diluted Lipofectamine™ 2000, mix gently and incubate for 20 min at room temperature.
   (d) Add the 100 μL complex to each well and mix gently by shaking the plate back and forth.
   (e) Change the medium after 4 ~ 6 h incubation at 25 °C.
4. At 24 h post-transfection, infect the transfected EPC cells with various concentrations of SVCV (e.g., 1 MOI, 0.1 MOI, 0.01 MOI, and so on) as described in Subheading 3.2.2.

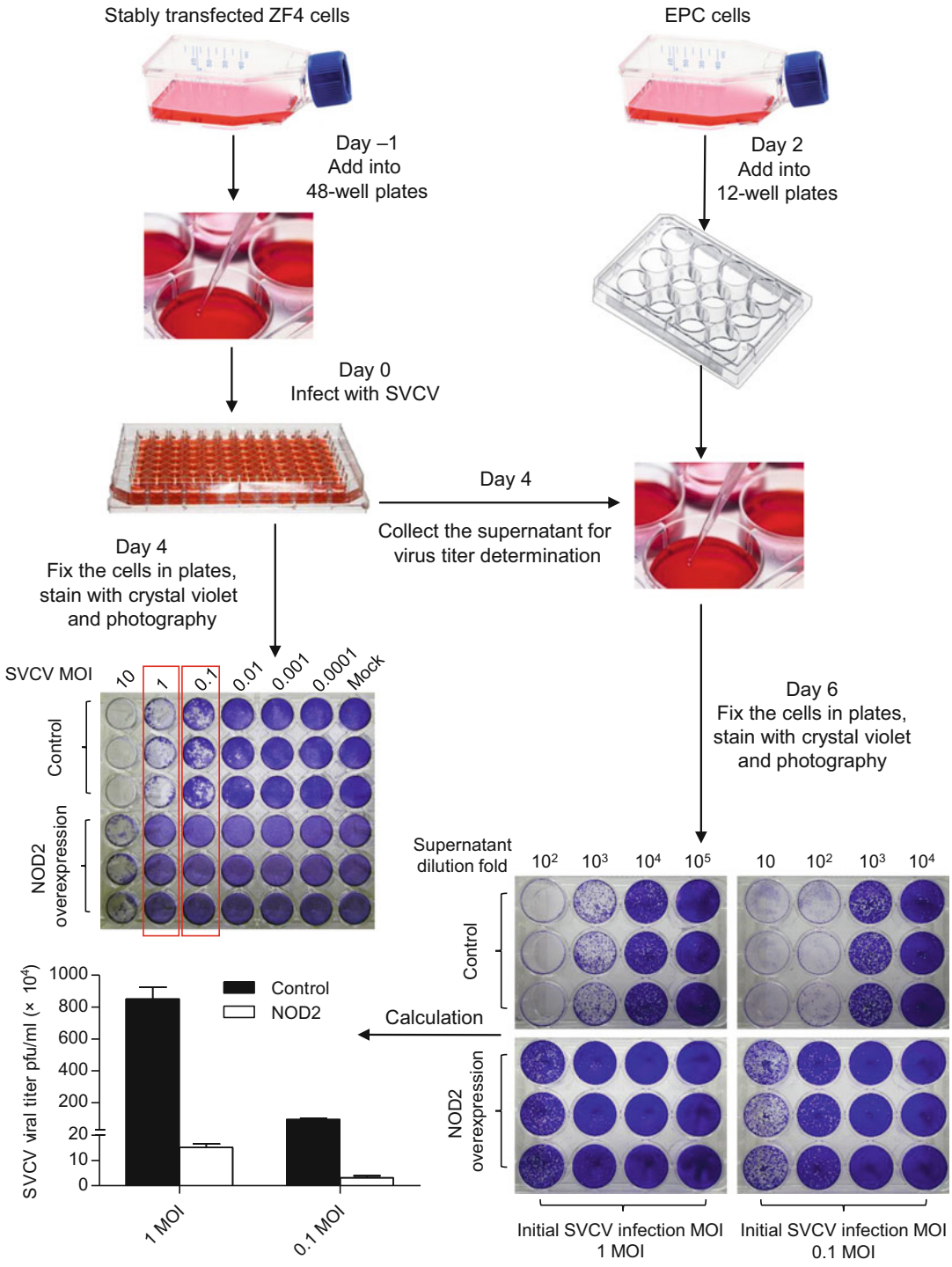

Fig. 4 Antiviral assays of zebrafish NOD2 in ZF4 cells. ZF4 cell line stably transfected with ptGFP1 (vector control) or ptGPF1-NOD2 was transferred into a 48-well plate and infected with tenfold-diluted SVCV for 4 d. The supernatants from stably transfected ZF4 cells with initial SVCV infection at MOI of 1 and 0.1 (marked out with red boxes) were collected and titrated on EPC cells in 12-well plates. The 48-well plates were then fixed with 10% paraformaldehyde, stained with 0.5% crystal violet, washed and photographed. About 2 d after seeding the supernatant dilution on EPC cells in 12-well plates, the plaque can be identified directly with naked vision, then fixed, stained and photographed as described above, and the virus titer can be calculated. Date were expressed as mean ± SE

5. Monitor the cells under a microscope daily till the target cells (cells transfected with pcDNA3.1-MDA5) exhibit obvious different status relative to the control cells (cells stably transfected with pcDNA3.1), such as cells have fewer CPE compared to the control cells under the same infection concentration of SVCV, and the time point for the result should normally be 48 h or less after infection (*see* **Note 2**).
6. Collect the supernatant from the target cells and the control cells with the same infection concentration for titer determination by EPC cells, and fix the plates of EPC cells with paraformaldehyde, followed by staining with crystal violet as described in Subheading 3.1.3 and then record by photography.

3.5 Luciferase Assays for the Study of Host-Virus Interactions

As described above, EPC cells can be transfected with plasmids at high efficiency, and could be used successfully in expressing zebrafish genes even in the promoter assays of zebrafish genes, such as the induction of IFNφ1, IFNφ2, IFNφ3, and IFNφ4 promoters [37]. In addition to the high susceptibility of EPC cells under SVCV infection, EPC cells can be effective tools for understanding host-virus interaction even at the molecular level. We provide a method using EPC cells to investigate the interaction of zebrafish proteins and SVCV proteins. The following section is the details of a method based on luciferase assay to investigate that the N protein of SVCV suppresses zebrafish IFNφ1 production by targeting MAVS as an example.

3.5.1 Luciferase Assay Under Direct Virus Infection to Study Host-Virus Interaction

1. Construct the IFNφ1 promoter reporter plasmid (IFNφ1pro-Luc) using pGL3-Basic luciferase reporter vector, and the plasmid encoding zebrafish MAVS (pcDNA3.1-MAVS) in the pcDNA3.1 vector.
2. Add the EPC cells into 24-well plates with 1×10^6 cells per well and incubate the cells at 25 °C overnight.
3. Transiently transfect the cells with 250 ng IFNφ1pro-Luc, 25 ng pRL-TK plus 250 ng pcDNA3.1-MAVS or pcDNA3.1 (control) using Lipofectamine™ 2000 as described in Subheading 3.4.2.
4. At 6 h post-transfection, cells were infected with SVCV at various concentrations (e.g., 1 MOI or 0.1 MOI) (*see* **Note 8**), and the cells without SVCV infection should be set as mock.
5. At 48 h post-transfection, collect the cells and prepare for luciferase assay by Dual-Luciferase® according to the manufacturer's instructions as the followings:
   (a) Dilute 5 × Passive Lysis Buffer (PLB) into 1 × PLB using distilled water, and mix well.
   (b) Remove the medium from culture cells and gently apply 800 μL PBS to rinse the bottom of the well once.

(c) Remove the PBS and add 100 μL 1 × PLB into each well and shake the culture plates at room temperature for 15 min on a shaker.

(d) Transfer the lysate into a 1.5 mL tube and centrifuge at 12,000 × *g* for 30 s in a centrifuge. Transfer the cleared lysates into a new tube.

(e) Prepare appropriate volume of Luciferase Assay Reagent II (LAR II) (20 μL per assay) and dilute 50 × Stop & Glo® Substrate into 1× with Stop & Glo® buffer to an adequate volume (20 μL per assay).

(f) Programme the luminometer to perform a 2 s premeasurement delay, followed by a 10 s measurement period for each assay.

(g) Transfer 4 μL cell lysate into the luminometer tube containing 20 μL LAR II and mix by pipetting for 3 ~ 5 times. Place the tube in the luminometer, and initiate reading and record the firefly luciferase activity measurement.

(h) Add 20 μL 1× Stop & Glo® Substrate into the luminometer tube and pipet for 3 ~ 5 times to mix. Place the tube in the luminometer, initiate reading, and record the *Renilla* luciferase activity measurement.

(i) Discard the reaction tube and proceed to the next assay.

6. Analyze the results of luciferase assays: relative luciferase activity = the value of firefly luciferase activity/the value of *Renilla* luciferase activity. The relative luciferase activity of cells transfected with pcDNA3.1 (control) or pcDNA3.1-MAVS under the infection of SVCV or without SVCV infection (mock) could be calculated and analyzed, in turn identify whether SVCV infection could affect zebrafish MAVS-mediated IFNφ1 promoter activation.

3.5.2 Luciferase Assay with Virus Components to Study Host-Virus Interaction

The section in Subheading 3.5.1 provides a method to investigate whether a virus could affect the host immune response by luciferase assays (e.g., zebrafish MAVS-mediated IFNφ1 promoter activation could be inhibited by SVCV infection). However, the components that affect the host immune response are still unknown. Herein, we describe a method based on luciferase assay to identify the components of virus which suppresses host immune response.

1. Prepare the plasmids such as IFNφ1pro-Luc, pRL-TK, pcDNA3.1-MAVS, and the plasmids expressing components of SVCV (e.g., pcDNA3.1-N, N protein of SVCV).
2. Add the EPC cells into 24-well plates with 1 × 10^6 cells per well and incubate the cells at 25 °C overnight.

3. Transiently transfect the cells with 250 ng IFNφ1pro-Luc, 25 ng pRL-TK, 250 ng pcDNA3.1-MAVS plus pcDNA3.1-N or pcDNA3.1 (control) using Lipofectamine™ 2000 as described in Subheading 3.4.2.
4. At 48 h post-transfection, harvest the cells and perform luciferase assay by Dual-Luciferase® as described in Subheading 3.5.1.

4 Notes

1. It is important to store your virus at −80 °C and threw on ice before using it; higher than the temperature may cause the loss of virus titer.
2. EPC cells are very sensitive to SVCV infection, and SVCV propagation could cause mass mortality of the cells. If the cells die faster following the seeding of virus, the virus may not propagate enough. Thus, it is important to use less seeding virus (e.g., 1 ~ 2 μL from the SVCV medium with a titer of 1×10^7 pfu/mL) in order to let the cells survive long enough (usually 2 ~ 3 days after infection with the seeding virus) and make the virus propagate sufficiently to reach a high titer when harvesting.
3. It is necessary to check the cell growth situation frequently to make sure the complete plaque in cells reach appropriate size which can be easily identified and counted with direct observation of naked eyes. If we fix the cells earlier, the plaque size would be too small to be identified, whereas if we fix the cells later, plaques would fuse together, resulting in difficulties to distinguish one plaque from another. We usually choose 48 hpi as the time point to fix the cells. In addition, appropriate density of plaques in wells of 12-well plates is important for the accurate determination of the virus titer (about 50 ~ 150 plaques per well should be sufficient enough). Thus, it is necessary to set various dilutions (such as 1:100, 1:1000, 1:10,000, and so on) to obtain a much more precise data of the virus titer.
4. If there are many zebrafish embryos (more than 200) needed to be microinjected with SVCV, which will take long time (more than 1 h), it is recommended to load some more microinjection capillary needles with SVCV suspension (depending on the number of embryos you need to microinject) and place them on ice whenever possible, change the microinjection capillary needle every 30 min in case that the infectivity of the virus may diminish when left too long in the microinjection capillary needle at room temperature.

5. Since the SVCV infectivity could diminish when the temperature becomes too high, it is recommended to culture the embryos or cells infected with SVCV at a relatively lower temperature. In case that low temperature could also influence the embryonic development, we suggest taking 25 °C for maintaining the embryos or cells that are infected with SVCV.
6. It is important to examine the quality as well as the concentration of the extracted total RNA, especially for RNA-Seq analysis in which highly pure and nondegraded RNA is required. The samples with low quantity of RNA can influence the subsequent cDNA synthesis and qPCR for gene expression analysis. The extracted RNA should be stored at −80 °C for long-time storage.
7. It is of great importance to confirm the constructed plasmids by sequencing since one single base shift could cause the failure in the expression of target genes. In addition, we noticed that in some circumstances, the constructed plasmids, even confirmed by sequencing, could not express the target protein after transfection into the cells. It is recommended that in the preliminary experiment, just transfect the plasmids into the cells and harvest the cells at 24 hpt for Western blotting analysis with the use of appropriate antibody (e.g., antibody for the target protein) to examine whether the target protein could be correctly expressed in cells.
8. Since SVCV infection could cause the cell death of EPC cells, a relatively high infection concentration could result in the complete death of the cells in a very short time span (less than 48 h), leaving no cells for the subsequent luciferase assay. It is recommended to perform a preliminary experiment to determine the appropriate quantity of viruses for infection, which could successfully infect the cells but not cause massive death of cells in 48 h.

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