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Establishment of viral infection and analysis of host-virus interaction in Drosophila Melanogaster

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Dear Dr. Alisha DSouza,

We thank editors and referees for insightful and constructive comments for our paper entitled "Establishment of viral infection and analysis of host-virus interaction in Drosophila Melanogaster" (JoVE58845).

We have answered the questions one by one, and polished the paper by native speakers for a better flowing narrative. Per editor's suggestion, we re-highlighted the steps required for video:

On behalf of all co-authors, we would like to submit this revised manuscript. And we hope all these revisions and modifications will be able to address the concerns of the editors and referees, significantly improve the quality of the paper to be published in JoVE.

Please do not hesitate to contact me if you have any concerns or questions.

Sincerely yours,

Lei PAN

TITLE:

Establishment of Viral Infection and Analysis of Host-Virus Interaction in *Drosophila melanogaster*

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KEYWORDS:

Drosophila melanogaster, Virus, Infection, Innate Immunity, Antiviral Response, Nanoinjection

SUMMARY:

This protocol describes how to establish viral infection *in vivo* in *Drosophila melanogaster* using the nano-injection method and basic techniques to analyze virus-host interaction.

ABSTRACT:

Virus spreading is a major cause of epidemic diseases. Thus, understanding the interaction between the virus and the host is very important to extend our knowledge of prevention and treatment of viral infection. The fruit fly Drosophila melan*ogaster* has proven to be one of the most efficient and productive model organisms to screen for antiviral factors and investigate virus-host interaction, due to powerful genetic tools and highly conserved innate immune signaling pathways. The procedure described here demonstrates a nano-injection method to establish viral infection and induce systemic antiviral responses in adult flies. The precise control of the viral injection dose in this method enables high experimental reproducibility. Protocols described in this study include the preparation of flies and the virus, the injection method, survival rate analysis, the virus load measurement, and an antiviral pathway assessment. The influence effects of viral infection by the flies' background were mentioned here. This infection method is easy to perform and quantitatively repeatable; it can be applied to screen for host/viral factors involved in virus-host interaction and to dissect the crosstalk between innate immune signaling and other biological pathways in response to viral infection.

INTRODUCTION:

Emerging viral infections, especially by arboviruses, such as *the* Chikungunya virus[1], the Dengue virus, the Yellow fever virus[2] and the Zika virus[3], have been a huge threat to public health by causing pandemics[4]. Thus, a better understanding of virus-host interaction has become

increasingly important for epidemic control and treatment of viral diseases in humans. For this goal, more appropriate and efficient models must be established to investigate the mechanisms underlying virus infection.

The fruit fly, *Drosophila melanogaster (D. melanogaster)*, provides a powerful system to investigate virus-host interaction[5,6] and has proven to be one of the most efficient models to study human viral diseases[7-9]. Highly conserved antiviral signaling pathways and incomparable genetic tools make flies a great model to produce significant results with real implications for human antiviral studies. In addition, flies are easy and inexpensive to maintain in the laboratory and are convenient for large-scale screening of novel regulatory factors[6,10] in the virus and the host during infection.

Four major highly conserved antiviral pathways (*e.g*., the RNA interference (RNAi) pathway[11], the JAK-STAT pathway[12], the NF-κB pathway, and the autophagy pathway[13]) are well studied in *Drosophila* in recent years[6]. The RNAi pathway is a broad antiviral mechanism that can suppress most kinds of virus infection[6,14]. Disruption of this pathway by mutation in genes like *Dicer-2 (Dcr-2)* or Argonaute 2 *(AGO2)* can lead to increased virus titer and host mortality[15-17]. The JAK-STAT pathway has been implicated in control of infection by a virus from the *Dicistroviridae* family and the *Flaviviridae* family in insects, *e.g*., Drosophila C virus (DCV) in flies[16] and West Nile virus (WNV) and Dengue Virus in mosquito[18,19]. The *Drosophila* Toll (homologous to the human NF-κB pathway) and Immune deficiency (IMD) pathways (similar to the human NF-κB and TNF pathway) are both involved in defending virus invasion[20-22]. Autophagy is another conserved mechanism involved in the regulation of viral infection, which is well characterized in *Drosophila*[23,24]. Thus, identification of novel regulatory factors of these pathways and dissecting crosstalk between these antiviral signaling and other biological pathways, such as metabolism, aging, neural reaction and so on, can be easily set up in the *Drosophila* system.

Although most well-established viral infectious models in *Drosophila* are induced by RNA viruses, infection by the Invertebrate iridescent Virus 6I (IV-6) and Kallithea viruses have shown the potential for study of DNA viruses in flies[25,26]. Moreover, the virus can also be modified to allow infection of *Drosophila*, such as the *influenza virus*[9]. This has greatly expanded the application of the *Drosophila* screening platform. In this procedure, we use DCV as an example to describe how to develop a viral infectious system in *Drosophila*. DCV is a positive-sense single stranded RNA virus of approximately 9300 nucleotides, encoding 9 proteins[27]. As a natural pathogen of *D. melanogaster*, DCV is considered as a suitable virus to study host physiological, behavioral and basal immune response during host-virus interaction and co-evolution[28]. Additionally, its rapid mortality rate following infection in wild type flies makes DCV useful to screen for resistant or susceptible genes in the host[29].

However, there are several aspects of concern when studying viral infections in *Drosophila*. For example, symbiotic bacteria *Wolbachia* have an ability to inhibit a wide spectra of RNA virus proliferation in *Drosophila* and mosquito[30-32]. Recent evidence shows a possible mechanism in which *Wolbachia* blocks Sindbis virus (SINV) infection through the upregulation of methyltransferase Mt2 expression in the host[33]. Additionally, the genetic background of insects is

also critical for viral infection. For instance, the natural polymorphism in the gene, *pastrel (pst)*, determines the susceptibility to DCV infection in *Drosophila*[34,35], whilst the loci of *Ubc-E2H* and *CG8492* are involved in Cricket paralysis virus (CrPV) and Flock house virus (FHV) infection, respectively[36].

The particular ways to establish the virus-host interaction in flies, must be chosen according to research purposes such as a high-throughput screen for host cellular components in *Drosophila* cell lines[37,38], oral infection to study gut-specific antiviral response[22,39,40], needle pricking[41,42] or nano-injection by passing epithelial barriers to stimulate systemic immune responses. Nano-injection can precisely control the viral dose to induce a controlled antiviral reaction and a physiological lesion[43], thus guaranteeing high experimental reproducibility[44]. In this study, we describe a nano-injection method to study virus-host interactions in *Drosophila*, highlighting the importance of the flies' background effects.

PROTOCOL:

NOTE: Before starting experiment, the cell lines and fly stocks used must not be contaminated by other pathogens, especially for viruses such as DCV, FHV, Drosophila X virus (DXV), and Avian nephritis virus (ANV). Ideally, RNA sequencing or a simpler PCR-based identification are used to detect the contamination[10,45]. If contamination occurred, the cell lines and fly stocks should not be used any more until they are decontaminated completely[46].

1 Virus and Fly Preparation

1.1 Virus propagation and collection

NOTE: *Drosophila* S2* cells are used for DCV propagation and titration. The S2* cell line is derived from a primary culture of late stage *D. melanogaster* embryos. This cell line has been well described previously[47].

1.1.1 Grow S2* cells in Schneider's Drosophila Medium at 25 °C without additional CO_2, as a loose, semi-adherent monolayer in a 10 cm cell culture dish, at a density of 1 x 10^7 viable cells/mL. Use complete medium for S2* cells: Schneider's Drosophila Medium containing 10% heat inactivated FBS, 100 units/mL Penicillin and 100 µg/mL Streptomycin.

Note: Healthy cells should show a round shape and exhibit homogenous size.

1.1.2 Quickly thaw the DCV stock from -80 °C in a 25 °C water bath. Infect S2* cells with DCV at MOI = 0.01 immediately by directly adding 1 mL of virus solution to the cell culture dishes (growth area: 55 cm^2) with 10 mL of complete medium for S2* cells.

1.1.3 Incubate cells at 25 °C for 3 to 5 days. When the virus is ready for collection, the cell morphology is abnormal (cell shape looks blur) and the culture medium is full of black particles indicative of cell debris.

1.1.4 Collect the whole cell culture in a 15 mL tube by pipetting up and down and freeze at -80 °C (for up to a year).

NOTE: DCV can be stored for prolonged periods of time at -80 °C with minimal loss of infectivity, for up to one year.

1.1.5 Thaw the cell culture in a 25 °C water bath with constant shaking and then centrifuge at 6,000 x g, for 15 min at 4 °C. Collect the supernatant in a sterile 15 mL tube and vortex. Aliquot up to 200 µL of virus solution per tube.

NOTE: Virus aliquots can be stored for short periods of up to 3 days at 4 °C and for up to 1 year at -80 °C. Repeated freeze-thaw cycles should be avoided. It is always better to use all the aliquot at once.

1.1.6 Pick 5 random virus-containing tubes to determine the average virus tissue culture infective dose (TCID50) by a cytopathic effect (CPE) assay (1 unit of $TCID_{50}$= 0.7 plaque forming unit [PFU]).

NOTE: CPE refers to structural changes in host cells caused by viral invasion. The infecting virus causes lysis of the host cell or when the cell dies without lysis due to an inability to reproduce[48].

1.1.6.1 On day 1, collect prepared S2* cells by pipetting. Count the cell number. Centrifuge cells for 300 x g for 4 min and discard the supernatant. Dilute the cells to the density of 1 x 10^6 cells/mL with complete medium for S2* cells as described in step 1.1.1.

1.1.6.2 Seed 1 x 10^5 S2*cells (100 µL) per well to the flat-bottom 96-well-plate (~30% confluency).

1.1.6.3 Perform serial 10-fold dilutions of the DCV stock with complete medium for S2* cells. Add 50 µL dilutions into the well. Add 50 µL of culture medium without virus to the well classified as the negative control well.

NOTE: For each dilution rate, 8 wells were used. As the typical DCV yield is around 10^8-10^9 PFU/mL, the dilution should at least cover ~10^{-5}-10^{-10}.

1.1.6.4 On day 4, observe CPE with a bright-field microscope with a 20X or 40X objective. Classify a well in which the cells look blurry and the medium is full of fragments as a "positive well", and a well in which the cell morphology is normal as “negative well”.

1.1.6.5 Mark CPE positive or negative wells with + or –, respectively. Calculate the virus $TCID_{50}$ by the Reed and Muench method[49].

1.1.7 Dispose of all contaminated materials and gloves in the decontamination pan. If the virus cannot reach the desired $TCID_{50}$, concentrate 100 mL of virus solution by centrifuging at 68,000 x g for 1 h at 4 °C.

NOTE: Depending on the aim of the experiment, a range of doses from 10^2 to 10^6 TCID50 units can be used for virus infection. Typically, we use 3 x 10^6 TCID50 units.

1.1.8 Discard the supernatant and re-suspend in 1 mL of Tris-HCl buffer (10 mM, pH 7.2). Aliquot 20 µL of virus solution per tube and store at -80 °C. Pick 5 random tubes to determine the average virus $TCID_{50}$ again.

1.2 Fly preparation for infection

NOTE: Symbiotic bacteria *Wolbachia* can suppress many kinds of RNA virus infection in insects[30-33,41]. Thus, it is essential to purge *Wolbachia* from flies before studying host-virus infection *in vivo*[33].

1.2.1 Prepare fly food containing tetracycline by adding 400 µL of 50 µg/mL tetracycline (in 70% ethanol) to 4 g of fresh standard cornmeal fly food (1 L of food contains 77.7 g of cornmeal, 32.19 g of yeast, 10.6 g of agar, 0.726 g of $CaCl_2$, 31.62 g of sucrose, 63.3 g of glucose, 2 g of potassium sorbate and 15 mL of 5% $C_8H_8O_3$). Mix thoroughly and place the food at 4 °C overnight to evaporate ethanol.

NOTE: A high dose of ethanol may affect fly fertility, so do NOT omit this step.

1.2.2 Warm the food to room temperature and put newly eclosed adult flies (20 females and 10 males) in the vial. Breed the flies at 25 °C, 60% humidity under a normal light/dark cycle.

NOTE: Typically, it takes 3-4 days for the flies to lay enough eggs. Too many eggs inhibit larvae development and decrease tetracycline efficiency.

1.2.3 Collect newly eclosed adult flies (within 3~5 days) under a light flow of CO_2, and repeat step ~1.2.1-1.2.2. Typically, at least 3 generations are required for the completely elimination of *Wolbachia*.

1.2.4 After 3 generations with tetracycline treatment, collect 5 flies under a light flow of CO_2 for the *Wolbachia* infection test. Grind flies for 1 min with 250 µL of double-distilled water (ddH_2O) and a few 0.5 mm sterile ceramic beads in a 1.5 mL tube using a homogenizer. Add another 250 µL of 2x buffer A (0.2 M Tris-HCl, pH 9.0; 0.2 M EDTA; 2% SDS) to the sample and vortex.

NOTE: Since SDS will impede the bead movement, 1x buffer A cannot be directly used to grind flies. If the flies have red eyes, remove heads before grinding, as this may influence the color of DNA product.

1.2.5 Freeze the samples at -80 °C (keep for up to one year). Quickly thaw the DCV stock from -80 °C in a 25 °C water bath until dissolved. Incubate the samples at 70 °C for 30 min.

1.2.6 Add 190 µL of 5 M KOAc, vortex and incubate on ice for 15 min or longer.

1.2.7 Centrifuge samples at 13,000 x g for 5 min at room temperature, collect the supernatant and transfer them to new tubes.

1.2.8 Repeat step 1.2.7 to obtain DNA with better quality.

1.2.9 Add 750 µL of isopropanol to each sample and gently invert a few times by hand. Incubate at room temperature for 5 min.

1.2.10 Centrifuge samples at 13,000 x g for 5 min at room temperature and discard the supernatant. The white genomic DNA pellet will stay at the bottom of the tube.

1.2.11 Add 1 mL of 70% ethanol to the pellets, centrifuge at 13,000 x g for 5 min, and discard the supernatant. Air-dry the DNA until it becomes transparent.

1.2.12 Dissolve DNA in 100 µL of ddH_2O, titrate DNA concentration by UV-Vis absorption spectrophotometry and dilute the DNA to 100 ng/µL.

1.2.13 Use 200 ng of DNA for the PCR reaction (in a 20 µL system, see the **Table of Materials**). Perform PCR by the following program: 95 °C for 15 min; 36 cycle of 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min; and a final extension step (72 °C for 1 min) in a thermal cycler.

NOTE: Based on the Wolbachia 16S rRNA, use the following primers: 5' TGAGGAAGATAATGACGG 3' (forward) and 5' CCTCTATCCTCTTTCAACC 3' (reverse). For wsp, the primers were 5' CATTGGTGTTGGTGTTGGTG 3' (forward) and 5' ACCGAAATAACGAGCTCCAG 3' (reverse).

1.2.14 Analyze PCR products with 1% agarose gel electrophoresis. The PCR product size of *Wolbachia* 16S rRNA and *wsp* is around 200 bp and 600 bp, respectively.

1.2.15 Rear *Wolbachia* free fly stock on standard cornmeal fly food. Collect 3-5 day newly eclosed male flies for infection.

NOTE**:** Tetracycline treatment can also affect intestinal microbiota composition, which subsequently influences antiviral responses in the host. We recommend that all flies should receive the same tetracycline treatments. Keep *Wolbachia*-free flies growing under standard conditions for at least 3 generations to restore gut microbiota.

1.3 *Pastrel* genotyping

NOTE: The presence of the S or R allele of the *pst* gene in the genetic background has been reported to affect DCV infection in *Drosophila*[34,35]. It is necessary to check the *pst* genotype, especially for DCV infection. There are a few SNPs in *pst* that can affect virus infection, the major SNP is 512C/T. The temperature gradient PCR is a quick method to identify *pst* genotype.

1.3.1 Extract fly genomic DNA as described above (step 1.2.4 - 1.2.15).

1.3.2 Use 100 ng of DNA as the template, the 512C primer, 5' CAGCATGGTGTCCATGAAGTC 3' (forward) and 5' ACGTGATCAATGCTGAAAGT 3' (reverse) to detect the R allele, the 512T primer, 5' CAGCATGGTGTCCATGAAGTT 3' (forward) and 5' ACGTGATCAATGCTGAAAGT 3' (reverse) to detect the S allele.

1.3.3 Set the Tm (melting temperature) gradients as follows: 54 °C, 54.7 °C, 55.5 °C, 58 °C, 59.7 °C, 62.2 °C, 63.7 °C, and 64 °C (45 s at each temperature).

NOTE: 30 cycles of PCR chain reaction are recommended (20 µL system).

1.3.4 Visualize the 100-bp PCR product by 2% agarose gel electrophoresis.

2 Viral Infection in *Drosophila*

2.1 Infect flies by nano-injection and perform survival assays.

2.1.1 Pull the capillaries to prepare injection needles, back-fill the injection needle with oil and assemble the injector as previously described[50].

2.1.2 Anaesthetize flies on the pad under a light flow of CO_2. Inoculate flies by intra-thoracic injection of 50.6 nL of virus solution (100 PFU, diluted with Tris-HCl buffer, pH 7.2). Inject at least 3 vials of flies (20 flies per vial).

NOTE: The injection is time-consuming (generally 100 flies per hour) and DCV replication is very rapid, so it is very important to write down the exact time on the tube once finishing each vial (20 flies). Buffer only injection is set as a mock control. Usually, male adult flies are preferred, as results are more stable and reproducible than when using females since hormonal variations during mating and reproduction may influence the readout of females[51].

2.1.3 Grow the injected flies at 25 °C, 60% humidity under a normal light/dark cycle. Supply flies with fresh food daily and record the number of dead flies.

2.1.4 Perform at least 3 biological replicates and plot the survival curve.

2.1.5 For the statistical analysis of survival data, generate Kaplan-Meier survival curves by statistical software. Perform log-rank test to calculate the P values. On the survival table, enter information for each subject. The software then computes percent survival at each time, and plots a Kaplan-Meier survival plot.

NOTE: Do NOT add additional yeast into the vial. Compared with mock controls, DCV-infected flies occasionally have ascites and are clumsy, which make them vulnerable to the sticky yeast. It

is better to record more time points in the preliminary experiment to find out a time frame at which the massive death will happen for infected flies. Generally, the infected flies rarely die within the first two days, even for the *Dcr-2* mutant lines. For the *Wolbachia* free w^{1118} (Bloomington 5905) fly infected with 100 PFU of DCV, this time window is around 3-5 days post infection. Fruit flies that die within 0.5 h post injection hours should not be counted as a fatality because they may be killed by needle injury not by viral infection.

2.2 DCV load measure by CPE assay

Note: Viral load is measured similarly to the titration of the virus stock (Step 1.1.6) but requires additional sample preparation (step 2.2.1-2.2.4).

2.2.1 On day 1, infect male flies as described in step 2.1.1-2.1.2. Group injected flies in groups of 20 and keep them growing on fresh fly food for the desired time (typically for 24 h or 3 days). Provide flies with fresh food daily.

2.2.2 On day 2, seed *Drosophila* S2* cells in a 10 cm cell culture dish to the density of approximately 5 $x10^6$ to $1x10^7$ cells/mL as described in step 1.1.1.

2.2.3 On day 3, collect 5 flies (under a light flow of CO_2) and grind them in a 1.5 mL centrifuge tube for 30 s with 200 µL of Tris buffer and ceramic beads using homogenizer. Store the samples at -80 °C or immediately proceed to the next step.

2.2.4 Thoroughly vortex the sample. Use a 1 mL syringe and 0.22 µm filter to filter the supernatant to a new 1.5 mL tube. Proceed with the titration, as described in steps 1.1.6.1-1.1.6.5.

2.3 DCV load measured by qRT-PCR

2.3.1 On day 1, infect flies as described above. Group injected male flies (20 flies/group) and grow on fresh fly food for desired time, typically for 24 h or 3 days. Provide flies with fresh food daily.

2.3.2 On day 3, collect 5 flies under a light flow of CO_2 in a 1.5 mL tube with 200 µL of lysis buffer and 20 ceramic beads (diameter = 0.5 mm), grind the samples by homogenizer with high speed for 30 s. Add 800 µL of additional lysis buffer to each tube and store the samples at -80 °C or immediately conduct RNA extraction and qRT-PCR.

2.3.3 Prepare RNase free tips, tubes and deionized, diethylpyrocarbonate (DEPC) treated and 0.22 µm membrane-filtered water. Add 200 µL of chloroform (≥99.5%) into the sample and vigorously shake for 15 s by hand. Incubate for 5 min or longer at room temperature until the water phase and the organic phase are clearly separated.

NOTE: Chloroform is extremely hazardous and must be handled with care. Do NOT vortex the sample, which will increase the risk of DNA contamination.

2.3.4 Centrifuge samples at 13,000 x g for 15 min at 4 °C.

2.3.5 Collect 400 µL of supernatant and transfer the supernatant to new tubes. Mix with the same volume isopropanol (≥ 99.5%), gently invert the samples for 20 times by hand, and then precipitate for 10 min or longer at room temperature.

NOTE: Precipitation at -20 °C will increase RNA yield.

2.3.6 Centrifuge samples at 13,000 x g for 10 min at 4 °C. White RNA pellets are visible at the bottom of the tube.

2.3.7 Discard the supernatant and add 1 mL of 70% ethanol (ethanol in DEPC water) and invert few times to wash the RNA.

2.3.8 Centrifuge samples at 13,000 x g for 5 min at 4 °C. Discard the supernatant and dry RNA for 5-10 min; RNA should become transparent.

2.3.9 Add 50 µL of DEPC water to the sample, pipette for few times and vortex.

2.3.10 Take 3 µL of RNA for titrating the RNA concentration. Quantify the RNA at 260 nm. Normally, the RNA concentration extracted by this protocol is approximately 100-500 µg/µL, which is suitable for cDNA synthesis.

2.3.11 Use 2 µg of RNA for cDNA synthesis. Dilute the sample to 100 µL with ddH_2O and store at -20 °C.

2.3.12 Perform qRT-PCR according to the manufacturer's instructions and run qRT-PCR.

Note: For cDNA synthesis of DCV, random primers worked much better than Poly A primer in our hands. DCV mRNA expression is normalized to endogenous ribosomal protein 49 (rp49) mRNA. Oligonucleotide primers used in this part: rp49 5’ AGATCGTGAAGAAGCGCACCAAG 3’ (forward) and 5’ CACCAGGAACTTCTTGAATCCGG 3’ (reverse); DCV, 5’ TCATCGGTATGCACATTGCT 3’ (forward) and 5’ CGCATAACCATGCTCTTCTG 3’ (reverse); vago, 5’ TGCAACTCTGGGAGGATAGC 3’ (forward) and 5’ AATTGCCCTGCGTCAGTTT 3’ (reverse); virus-induced RNA 1 (vir-1) 5’ GATCCCAATTTTCCCATCAA 3’ (forward) and 5’ GATTACAGCTGGGTGCACAA 3’ (reverse).

REPRESENTATIVE RESULTS:

Results of this section are obtained after DCV infection of *D. melanogaster*. **Figure 1** shows the flow chart of viral infection in *Drosophila*. Flies are injected intra-thoracically, and then the samples are collected for the measurement of the viral $TCID_{50}$ and the genome RNA level (**Figure 1**). Virus infection can induce cell lysis and CPE is observed at 3 days post infection (**Figure 2A**). The virus load measured by the CPE assay is in line with that measured by qPCR (**Figure 2B**). As shown in **Figure 3**, *Wolbachia* inhibits DCV infection in *Drosophila*. *wol-16s rRNA* and *wsp* primers are used to detect *Wolbachia* presence (**Figure 3A**) and *Wolbachia*-free flies show significantly

decreased survival rate after DCV infection (**Figure 3B**). **Figure 4** shows how to verify *pst* polymorphism by gradient PCR using specific primers. **Figure 5** shows dose dependent effects of DCV infection in the wildtype fly. The survival rate in **Figure 5A** and the virus load at 3 days post infection is shown in **Figure 5B**. **Figure 6** demonstrates that DCV infection can activate the Dcr2/RNAi and JAK/STAT antiviral signaling pathways in the host[52]. The expression level of the reporter gene *vago* expression of Dcr2/RNAi pathway is elevated 3 days post infection (**Figure 6A**). The JAK-STAT pathway is also activated upon DCV infection, which is indicated by the expression level of reporter gene *vir-1* (**Figure 6B**). **Figure 7** shows that the Dcr2/RNAi pathway is critical for antiviral infection in *Drosophila*[52]. *Dcr-2* mutant flies have a decreased survival rate (**Figure 7A**) and an increased virus load (**Figure 7B**) after DCV infection.

FIGURE AND TABLE LEGENDS:

Figure 1. Flow chart of *Drosophila* infection and virus load analysis.
Establishing *Drosophila* infection by nano-injection. The schema chart shows that flies are infected intra-thoracically and that virus load are measured by the CPE assay and qRT-PCR.

Figure 2. DCV load analysis.
The virus load measured by the CPE assay is in line with that measured by qPCR. (**A**) S2* cells are cultured at 25 °C for 3 days with a different virus dilution (50 µL). 1 Unit $TCID_{50}$ of used virus is 4.29×10^9 (equivalent to 3×10^9 PFU/mL). First panel, 10^{-3} dilution; second panel, 10^{-7} dilution; third panel, 10^{-10} dilution; forth panel, w/o infection. Cells in panel one and two show the typical CPE positive phenotype (white arrows indicate cell debris), and cells in panel three and four show the typical negative phenotype. The scale bar is indicated in the figures. (**B**) Measurement of DCV load by qPCR. Perform serial 10-fold dilutions of DCV and infect the S2* cell line. The total RNA of infected cells is extracted and qPCR is performed to measure the DCV RNA level. Gradient viral infectious load (determined by CPE assay) in cells is matched and positively related to the measurement by qPCR (r^2=0.999).

Figure 3. Viral Infection of flies with or without *Wolbachia*.
Wolbachia infection contributes to DCV resistance in *Drosophila*. (**A**) The Wolbachia presence is detected by *wol-16s rRNA* and *wsp* primers. G1, G2 and G3 represented Ore^r flies (*Oregon-R*) (Bloomington 5) treated by tetracycline for one, two and three generations respectively. The PCR product size is around 200 bp (*wol-16s rRNA*) and 600 bp (*wsp*). (**B**) *Wolbachia* free flies are more sensitive to DCV infection. 3-5 day old male adult flies are injected with 100 PFU of DCV. *Wolbachia* free flies (solid lines) show significantly reduced survival than *Wolbachia* protected flies (dash lines) upon DCV infection. Two different wildtype lines, Ore^r and w^{1118}, are used for the survival assay and show similar result. All error bars represent standard error (s.e.) of at least three independent tests; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns, not significant. Kaplan–Meier (B).

Figure 4. *Pastrel* genotyping by gradient PCR.

pst polymorphism is verified by gradient PCR. Gradient PCR is performed to distinguish *pst* R (512C) and S (512T) allele in the *Drosophila* adult. Tm gradients are a: 54 °C, b: 54.7 °C, c: 55.5 °C, d: 58 °C, e: 59.7 °C, f: 62.2 °C, g: 63.7 °C, h: 64 °C.

Figure 5. Survival rate and DCV titer of *Ore*^{r} flies infected with a different dose of DCV.
Ore^{r} flies are injected with three different doses of DCV, 10 PFU/fly, 50 PFU/fly and 100 PFU/fly. (**A**) The flies' survival rate is significantly lower when infected with higher infectious doses. (**B**) The DCV RNA level in the whole body of indicated flies is measured by qRT-PCR at the indicated time and normalized to that of 10 PFU/fly inoculation group. All error bars represent standard error (s.e.) of at least three independent tests; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns, not significant. Kaplan–Meier (A) or One-way anova (B).

Figure 6. Dcr2/RNAi and JAK/STAT antiviral signaling pathway activated after DCV infection.
Ore^{r} flies were infected with 100 PFU of DCV. Vago (**A**) and vir-1 (**B**) mRNA expressed in the whole body are measured by qRT-PCR at indicated time points post infection. The change of expression is normalized to that of basal level before infection. All error bars represent standard error (s.e.) of at least three independent tests; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns, not significant. One-way ANOVA (A, B).

Figure 7. Dcr-2 mutant fly sensitive to DCV infection.
Dcr-2 mutant flies and its genetic control w^{1118} flies were infected with 100 PFU of DCV. (**A**) Survival rates of *Dcr-2* deficient flies and wild-type (w^{1118}) flies post DCV infection. (**B**) DCV RNA level in the whole body of indicated flies is measured by qRT-PCR 2 days post infection and normalized to that of w^{1118}. All error bars represent standard error (s.e.) of at least three independent tests; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns, not significant. Kaplan–Meier (A) or student's T-test (B).

DISCUSSION:
In this article, we present a detailed procedure on how to establish a viral infectious system in adult *Drosophila melanogaster* using nano-injection. The protocols include the preparation of appropriate fly lines and virus stock, infection techniques, the evaluation of infectious indicators and the measurement of the antiviral response. Although DCV is used as an example of a viral pathogen, tens of different kinds of virus have been successfully applied for study in the Drosophila system. In addition, hundreds of regulatory factors, either of the virus or of the host, have been identified through large-scale screening in flies. Thus, this method is highly adaptable and does not limit to DCV/*Drosophila* interaction.

To successfully propagate and harvest high titer DCV, a few precautions must be taken. Cells should be healthy, cultured at a suitable density and harvested on time. Reducing cell culture volume does not significantly affect virus titer. Typically, 10^7 cells infected by DCV at MOI=0.01 will finally yield approximate 10^8-10^9 $TCID_{50}$ of DCV, which can meet the demands of most *in vivo* and *in vitro* experiments. The CPE assay and qRT-PCR assay are described in this protocol for the measurement of virus load. The CPE assay is somehow tricky. The appropriate density of cell inoculation and an experienced standard of positive/negative CPE discrimination allow a quick

and successful CPE assay. Thus, we provide an easy qRT-PCR assay here to help decide the cut-off dilution point in CPE assay before mastering the CPE assay.

However, DCV is a very potent virus for the adult fly and *Drosophila* cell line. Only 100 PFU/fly inoculation can kill 50% of wild flies within 5 days. An excess infection dose may shadow the significance between the wild type fly and potential susceptible lines. It is important to apply at least two infection doses when initiating a new screen. Since the massive death of *Drosophila* may happen in a very short time window because of the high lethality of DCV, recording the death number more frequently in preliminary experiments is strongly recommended. After DCV infection, some flies occasionally have ascites syndrome. Notably, flies that die within 0.5 h post infection should be excluded, which might be caused by inappropriate needle injection injury.

The infectious strength of DCV is also influenced by many host factors, which should be considered before starting an experiment. *Wolbachia* is a vertical transmit endosymbiont that has great impact on virus infection in flies[32]. Tetracycline treatment in fly food is a common used method to eliminate *Wolbachia* infection. However, this method also alters intestinal microbiota composition, which may in turn affect antiviral response[53]. A few studies have emphasized the importance of genetic variance on virus infection[36], such as *ubc-e2h*, *cg8492* and *pst*. For example, most wild type fly lines have *pst* S allele and are sensitive to picorna-like virus, while most mutant lines we tested from Bloomington stock center have *pst* R allele, thus having resistant phenotypes. It is very important to rule out the impact of *pst*, especially when screening out a resistance gene comparing with wild type control[29].

Besides nano-injection to induce systemic virus infection, other methods are available to study virus infection in flies. *Drosophila* cell lines have proven to be a high-throughput method to screen out virus infection related host cellular component[37,38]. However, an *in vitro* system is always accompanied with a high rate of false positives, when confirming *in vivo*. DCV can also be applied to infect Drosophila orally, whereas it can only trigger a restricted and milder infection. Only 20% of larvae were infected within 12 h and 14% mortality was observed, with just 25% mortality in 20 days in adult flies[22]. Pricking flies with 0.15-mm-diameter pins is another way to set viral infection but it cannot ensure the accurate infection dose and quantitative repeatability. Overexpressing viral proteins by genetic manipulation in flies is a good way to study molecular interactomes *in vivo*[7]. However, it may not portray the reality of physiology and pathology in the host upon infection. Meanwhile, the nano-injection method also has drawbacks, as it is not the natural route of infection and can directly stimulate a strong system immune response just after infection, which bypassed the cuticle, the first antiviral defense line. In sum, the specific research purpose and available experimental condition are the deciding factors in the choice among different methods.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

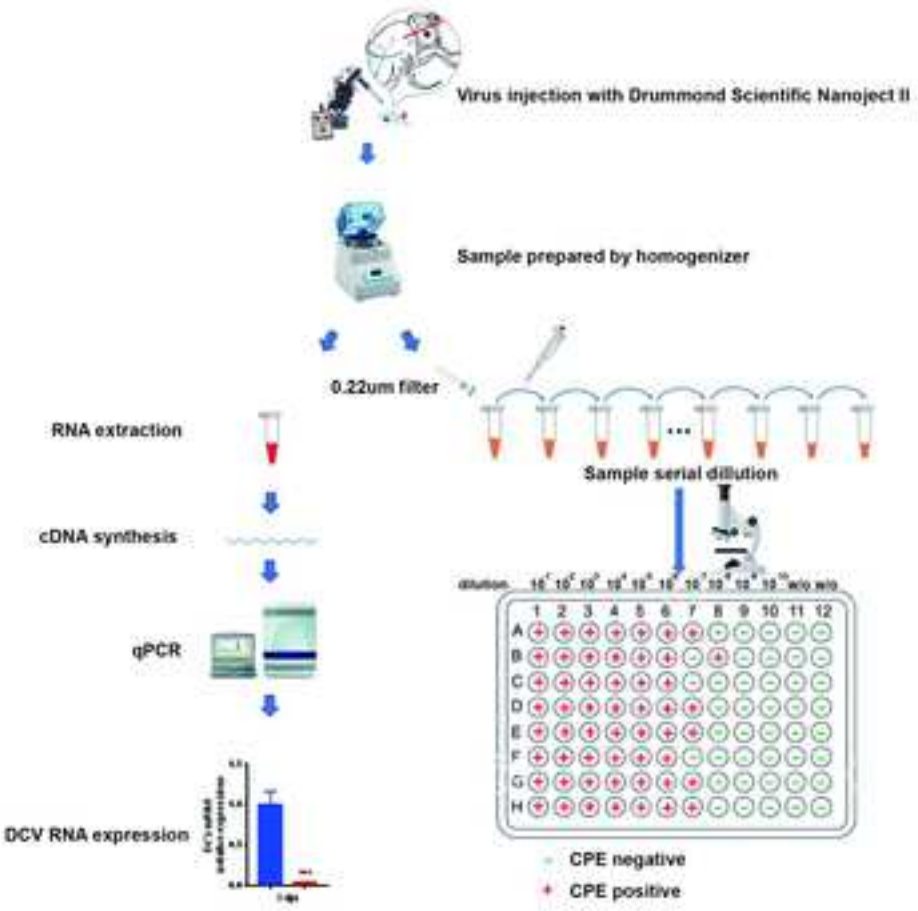

Figure 2

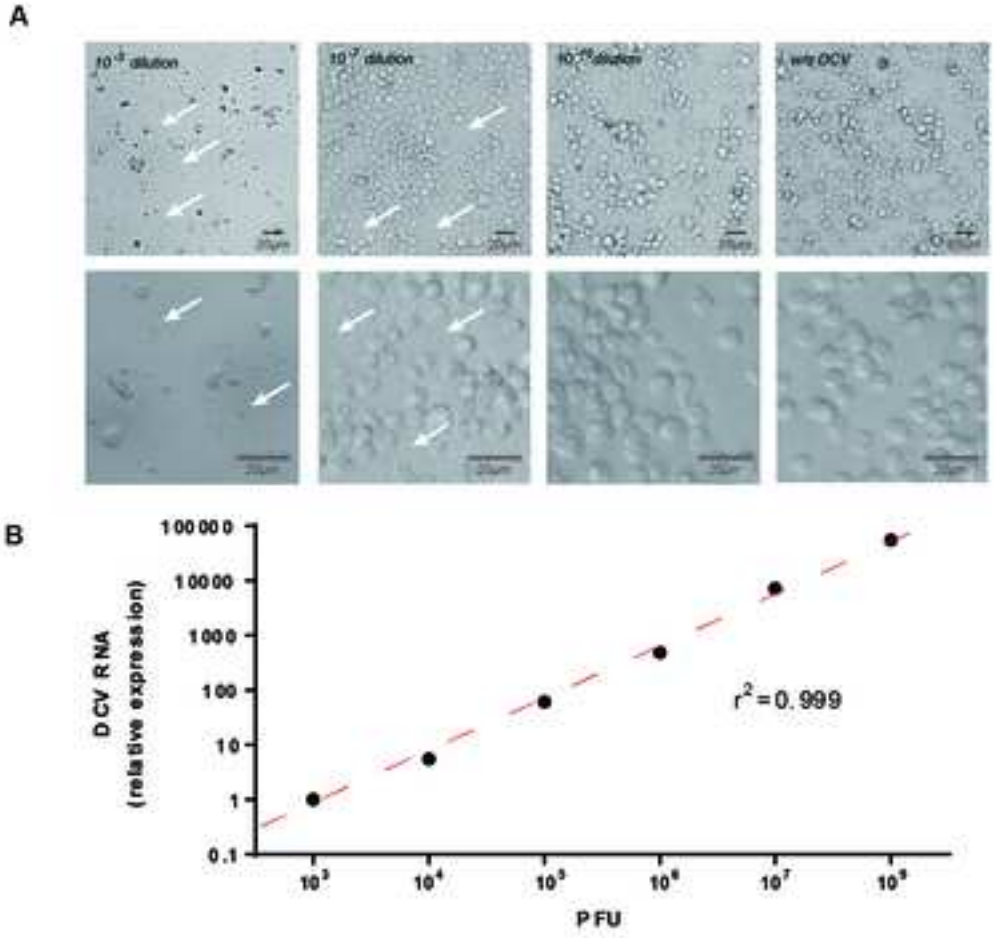

Figure 3

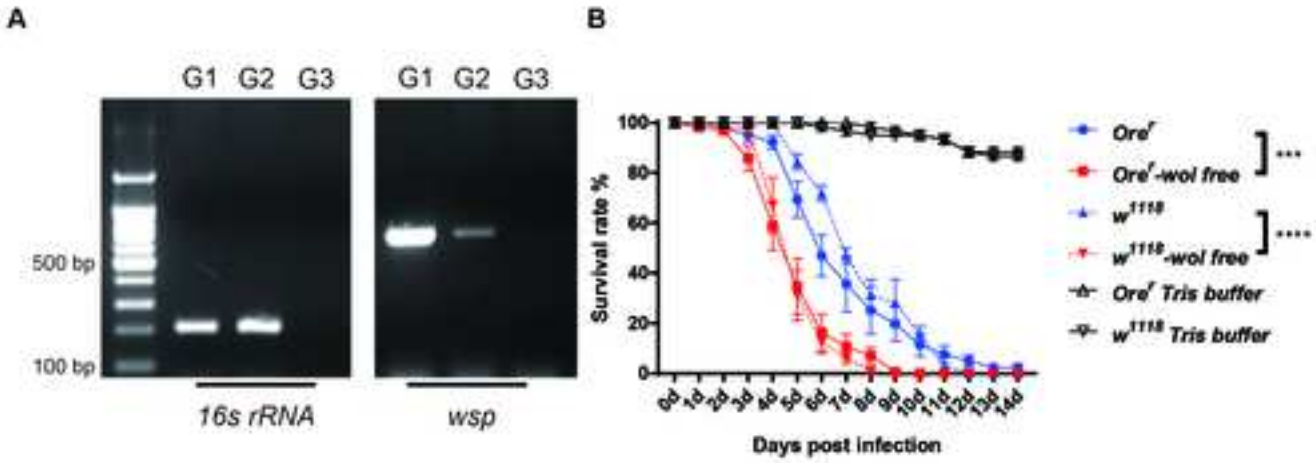
A
G1 G2 G3
G1 G2 G3
500 bp
100 bp
16s rRNA
wsp
B
Survival rate %
Days post infection
Ore^r
Ore^r-wol free
w^1118
w^1118-wol free
Ore^r Tris buffer
w^1118 Tris buffer
***
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Figure 4

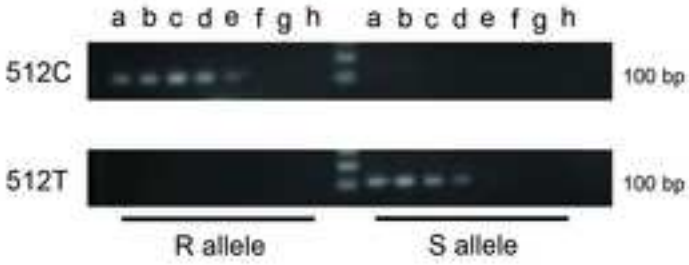


Figure 5

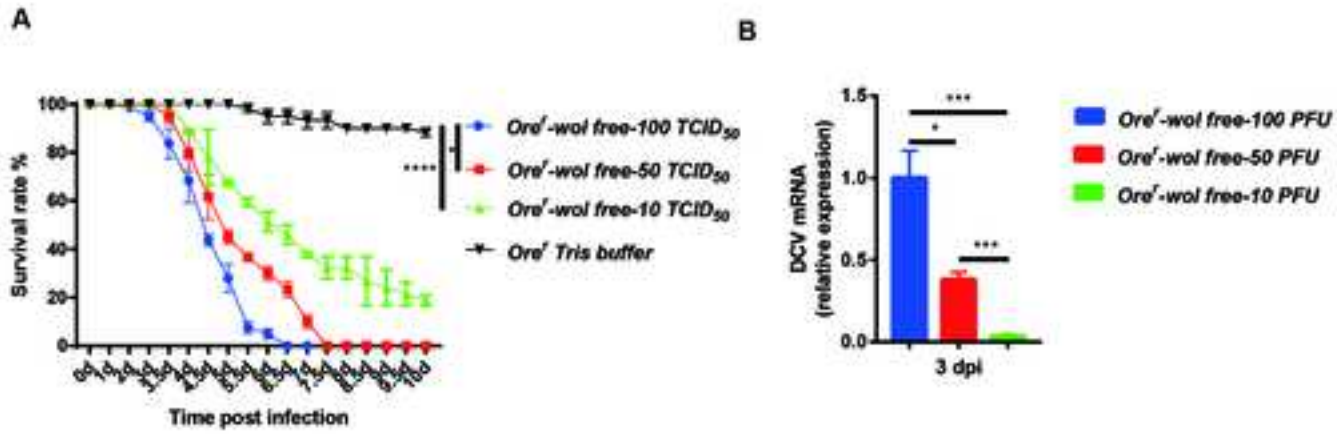


Figure 6

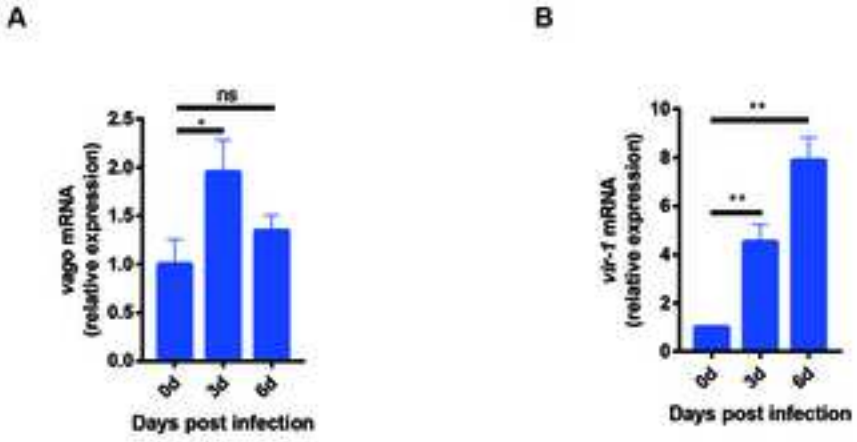
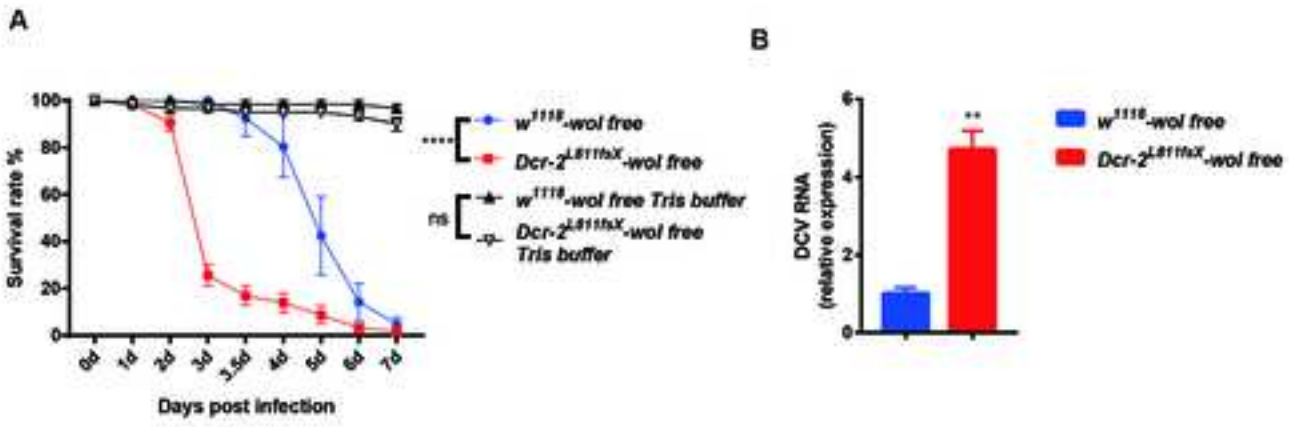


Figure 7



Name of Material/ Equipment	Company	Catalog Number
0.22um filter	Millipore	SLGP033RS
1.5 ml Microcentrifuge tubes	Brand	352070
1.5 ml RNase free Microcentrifuge tubes	Axygen	MCT-150-C
10 cm cell culture dish	Sigma	CLS430167
100 Replacement tubes	Drummond Scientific	3-000-203-G/X
15 ml tube	Corning	352096
ABI 7500 qPCR system	ABI	7500
Cell Incubator	Sanyo	MIR-553
Centriguge	Eppendof	5810R
Centriguge	Eppendof	5424R
Chloroform	Sigma	151858
DEPC water	Sigma	95284-100ML
Drosophila Incubator	Percival	I-41NL
FBS	Invitrogen	12657-029
flat bottom 96-well-plate	Sigma	CLS3922
Fluorescence microscope	Olympus	DP73
Isopropyl alcohol	Sigma	I9516
Lysis buffer (RNA extraction)	Thermo Fisher	15596026
Lysis buffer (liquid sample RNA extraction)	Thermo Fisher	10296028
Microscope	Olympus	CKX41
Nanoject II Auto-Nanoliter Injector	Drummond Scientific	3-000-204
Optical Adhesive Film	ABI	4360954
Penicillin-Streptomycin, Liquid	Invitrogen	15140-122
qPCR plate	ABI	A32811
Schneider’s Insect Medium	Sigma	S9895
statistical software	GraphPad Prism 7	
TransScript Fly First-Strand cDNA Synthesis SuperMix	TransScript	AT301
Vortex	IKA	VORTEX 3

Comments/Description
Cell culture
qPCR
RNA extraction
RNA extraction
Rearing Drosophila
Cell culture
Cell culture
RNA extraction
TRIzol Reagent
TRIzol LS Reagent
Nanoject II Variable Volume (2.3 to 69 nL) Automatic Injector with Glass Capillaries (110V)
qPCR
Cell culture
qPCR
Cell culture
RNA extraction
RNA extraction

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