Video Article

RNAi Screening for Host Factors Involved in *Vaccinia* Virus Infection using *Drosophila* Cells

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Abstract

Viral pathogens represent a significant public health threat; not only can viruses cause natural epidemics of human disease, but their potential use in bioterrorism is also a concern. A better understanding of the cellular factors that impact infection would facilitate the development of much-needed therapeutics. Recent advances in RNA interference (RNAi) technology coupled with complete genome sequencing of several organisms has led to the optimization of genome-wide, cell-based loss-of-function screens. *Drosophila* cells are particularly amenable to genome-scale screens because of the ease and efficiency of RNAi in this system [1]. Importantly, a wide variety of viruses can infect *Drosophila* cells, including a number of mammalian viruses of medical and agricultural importance [2,3,4]. Previous RNAi screens in *Drosophila* have identified host factors that are required for various steps in virus infection including entry, translation and RNA replication [5]. Moreover, many of the cellular factors required for viral replication in *Drosophila* cell culture are also limiting in human cells infected with these viruses [4,6,7,8, 9]. Therefore, the identification of host factors co-opted during viral infection presents novel targets for antiviral therapeutics. Here we present a generalized protocol for a high-throughput RNAi screen to identify cellular factors involved in viral infection, using *vaccinia* virus as an example.

Video Link

The video component of this article can be found at https://www.jove.com/video/2137/

Protocol

Part 1: RNAi in 384 Well Plates

1. Standardization of the reagents used for screening is essential. We test individual lots of Schneider's media, fetal bovine serum, and staining reagents. Then we aliquot and use the same lot for the entire screen.
2. For any assay, it is best to test several different cell lines to determine which is most amenable to your biological read-out. We typically use the S2-DRSC line.
3. Grow *Drosophila* cells at 25°C in complete Schneider's media.
    a. Schneider's media
    b. 10% heat inactivated FBS
    c. 1X L-glutamine
    d. 1X Pen/strep antibiotics
4. *Drosophila* cells are semi-adherent and are passaged without trypsinization every four days by pipetting the cells off the flasks and diluting the cells 1:10 into a fresh flask. Cells should be in log phase for experiments.
5. Plate-to-plate and day-to-day variability are minimized through the use of automated liquid handling for all liquid additions to multi-well plates. We use a WellMate (Matrix).
6. Prepare WellMate tubing.
    a. Spray hood and WellMate with 70% ethanol.
    b. Remove tubing from packaging and spray with 70% ethanol.
    c. Insert tubing cassette in dispensing position on the WellMate.
    d. Prime tubing with 25 mL of 70% ethanol.
    e. Sterilize tubing in ethanol for 15 minutes, then prime with another 25 mL of ethanol.
    f. Rinse tubing by priming with 50 mL of sterile PBS.
7. Dislodge cells from flask and count. Pellet cells so that you have 25% more than needed (300xg, 5 min). The number of cells seeded per well must be optimized according to assay length. When conducting automated image analysis, a single monolayer with no cell clumping is ideal for image segmentation.
8. Resuspend pelleted cells in serum-free Schneider's media at $1.7x10^6$ cells/mL.

9. We perform the screen in 384 well plates pre-aliquoted with a commercially available library of dsRNA at a concentration of 250ng/well. Several wells of each plate are left empty for controls, which are added manually. We use dsRNA to luciferase as a negative control. We use dsRNA against Thread (dIAP) as a control for robust RNAi. Knock down of this anti-apoptotic factor results in dramatic cell death and is a quick visual way to assess the quality of the knock down. Lastly, we use a dsRNA against the viral reporter, in this case beta-galacosidase, as a positive control to validate that we can inhibit viral infection using our assay read-out. If spotting dsRNA into plate at the time of cell seeding, aliquot 1 µl dsRNA into the corner of the well to allow for easier visualization, then centrifuge dsRNA onto bottom of well (300xg, 1 min) before adding cells.
10. Use WellMate to add 10 µL cells prepared above to each well.
11. Spin cells onto plates by centrifuging 300xg, 1 min.
12. Incubate in 25°C incubator for 45 minutes.
13. Add 20 µL complete Schneider's media and centrifuge 300xg, 1 min.
14. Place plates in humidified Tupperware containers lined with water-soaked paper towels. The volume of a 384 well is small; thus, evaporation can have large effects on the biology, which in turn can lead to artifactual changes in the read-outs of the edge wells. To overcome this, plates should be maintained under high humidity.
15. Incubate for three days to allow for robust knockdown. There is no need to change the media or open humidified containers during this time.

Part 2: Infecting with *Vaccinia* Virus

1. Sterilize multi-channel aspirator in Quatricide for 15 minutes, then rinse in 70% ethanol.
2. Prepare WellMate as described above.
3. Prepare Schneider's media with 2% FBS for infection by diluting complete media 1:5 in serum free media.
4. Filter crude virus stock through 0.8 um syringe filter to remove cellular debris or use purified virus.
5. Dilute *vaccinia* virus in 2% serum Schneider's media to obtain multiplicity of infection (MOI) of 2.
6. Remove PBS from WellMate tubing, and prime with diluted virus until the tubing is free of air bubbles.
7. Use sterilized multi-channel aspirator and vacuum manifold to gently remove media from the plates of dsRNA-treated *Drosophila* cells.
8. Dispense 30 µL virus per well onto plates using the WellMate.
9. Centrifuge plates 1 minute at 300xg.
10. Sterilize WellMate tubing. Prime with 25 mL of 10% bleach, wait 10 minutes, and prime with another 25 mL 10% bleach.
11. Prime tubing with 50 mL water followed by 50 mL 70% ethanol. Return tubing to its packaging.
12. Return plates to humidified Tupperware container. Incubate at 25°C for 48 hours.

Part 3: Staining Plates

1. Fix plates in 15 µL 4% formaldehyde/PBS for 10 minutes. Liquid is removed using a multi-channel aspirator and vacuum manifold at each step. Once plates have been fixed, sterile equipment and reagents are no longer necessary.
2. Remove fix and use WellMate to add 30 µL PBST (PBS + 0.1% Triton-X). Incubate 10 minutes and repeat. Operate WellMate as described above, except tubing does not need to be sterilized.
3. To detect *vaccinia* infection we use a β-galactosidase reporter driven by an early/late *vaccinia* promoter.
4. Remove liquid and incubate cells in block (PBST with 2% BSA) for 10 minutes.
5. Dilute primary antibody in block, remove liquid and add 15 µL antibody to each well using a multi-channel repeat pipettor (Matrix).
6. Spin down plates (300xg, 1 min), cover with a clear sticker, and incubate at 4°C overnight.
7. Remove primary antibody, and use the WellMate to wash wells in PBST 3 times, 10 minutes each.
8. Dilute fluorescently labeled secondary antibody (FITC) and nuclear stain in block, and add 15 µL to wells using a multi-channel repeat pipettor.
9. Incubate plates 1 hour at room temperature, protected from light.
10. Wash wells in PBST 3 times, 10 minutes each using the WellMate.
11. Seal plate with clear sticker, cover, and store at 4°C up to three weeks.

Part 4: Imaging and Image Analysis

1. Use an automated microscope at 20X magnification to image plates.
2. Capture images of total nuclei (DAPI) and infected cells (FITC). Optimize exposure time for each channel individually in order to maximize dynamic range.
3. Fine-tune the automatic focus function by taking a Z stack of images, spaced 1um apart, that span the planes above and below the expected plane of focus. Once the optimal focal plane is located, adjust autofocus settings accordingly.
4. Image the entire plate, capturing at least three images per well in both the Hoechst channel (DAPI) and the virus channel (FITC). Choose sites from several different regions within one well in order to capture images that represent the well as a whole.
5. Use MetaXpress software to write a journal to segment the image and use Count Nuclei modules to calculate the total number of cells positive for nuclear staining (total cells) and infection (FITC) for each plate.
6. Calculate percent infection (100*FITC positive/total cells) and log transform.
7. To identify candidates we use a robust Z score based on the median and interquartile range of each plate. This method is insensitive to outliers and nonsymmetric data, and thus provides greater power in identifying even weak or moderate hits 10. For each plate, the median is subtracted from the log-transformed data. Then the resulting values are divided by 0.74 times the interquartile range (the difference between the values of the 75^{th} percentile and the 25^{th} percentile). The 0.74 factor is used to approximate a standard normal distribution.
8. The robust Z score is a measure of distance in standard deviations from the median of the plate. For example, a robust Z-score of 2 indicates the % infection of the well is ~2 standard deviations from the plate median or $p<0.05$.
9. Analyze cell number to identify cytotoxic candidates. Calculate the robust Z score of nuclear counts. Candidates with a robust Z<-2 in duplicate screens are considered cytotoxic.

10. Wells that have a robust Z score for percent infection of >2 or <-2 in duplicate screens ($p<0.0025$) without cytotoxicity are considered potential candidates from the primary screen.
11. Positive candidates are validated using independent reagents. dsRNAs are generated against the genes of interest from another region of the mRNA and tested as above.
12. Those genes for which two independent dsRNAs have the same phenotype can be considered for further study.

Part 5: Representative Images and Interpretation of Infection Rates

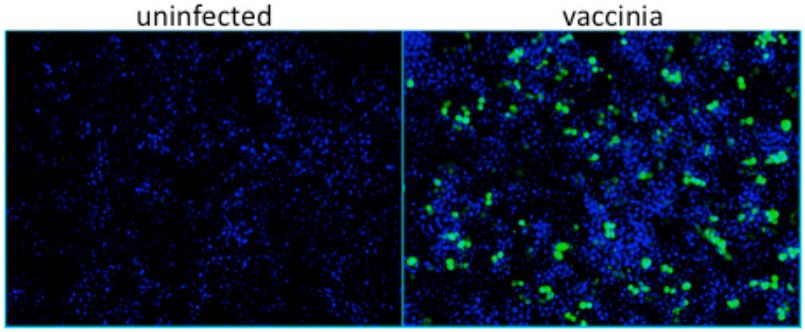

Figure 1. Uninfected wells do not show any staining for *vaccinia* virus proteins, while a representative infected well contains cells staining positive for *vaccinia*-expressed beta-galactosidase (βgal) protein, as measured by immunofluorescence microscopy. Virus=green, nuclei = blue.

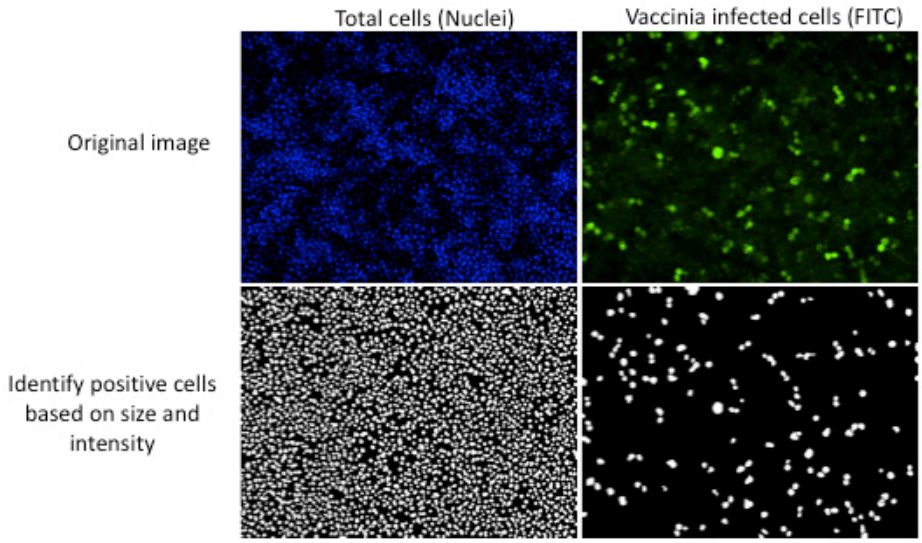

Figure 2. Automated image analysis software quantifies infection in each image using parameters set by the user. In a representative image, the total number of cell nuclei and the number of cells expressing viral antigen are counted based on size and the intensity of staining above the local background staining.

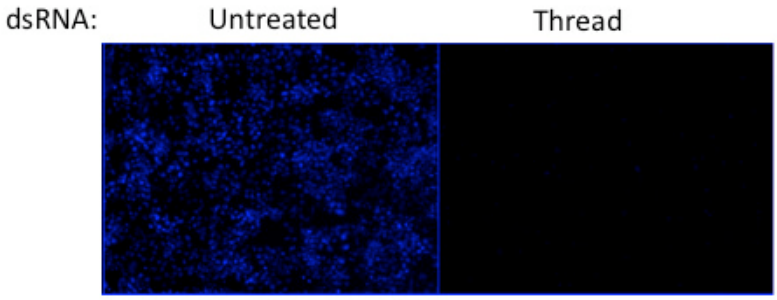

Figure 3. Knockdown of thread (dIAP) serves as a positive control for robust RNAi depletion of target genes. Knockdown of this anti-apoptotic factor leads to dramatic cell death. Nuclei=blue.

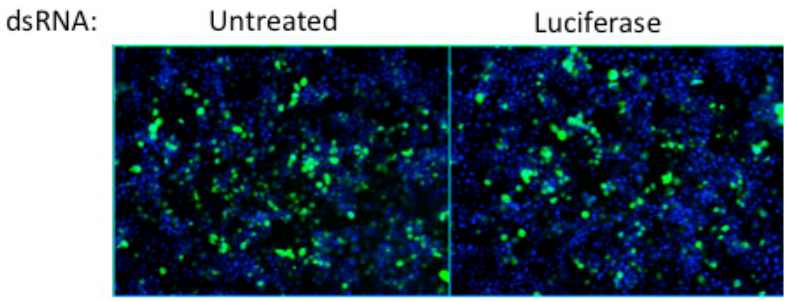

Figure 4. Knockdown of luciferase serves as a negative control for the effect of double-stranded RNA treatment on infection. Depletion of luciferase has no effect on infection relative to cells left untreated. Virus=green, nuclei = blue.

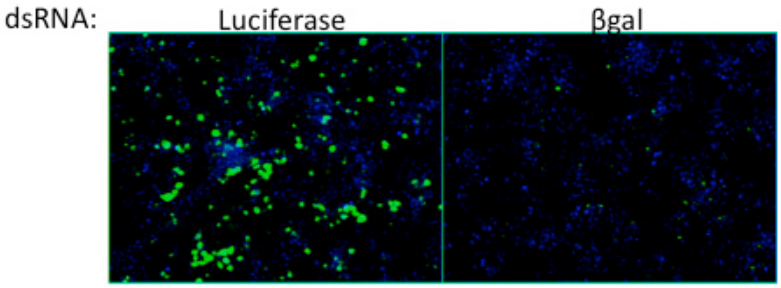

Figure 5. Knockdown of βgal protein serves as a positive control for a reduction in infection, since the assay uses βgal protein levels as a read out of infection. Depletion of βgal results in a decrease in the percentage of infected cells. Virus=green, nuclei = blue.

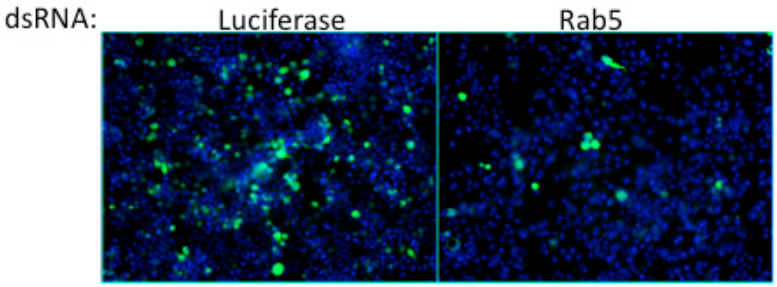

Figure 6. Knockdown of cellular factor Rab5 results in a decrease in the percentage of infected cells. Since Rab5 is known to participate in endocytosis, this factor likely contributes to *vaccinia* virus entry. This represents an example outlier from the screen. Virus=green, nuclei = blue.

Discussion

Genome-wide RNAi screening in *Drosophila* provides a robust and efficient method for examining the cellular component of viral infection. A number of mammalian viruses infect *Drosophila* cells, which can be used to identify components of the host intrinsic immune response, and host protein factors required for viral replication that may represent novel therapeutic targets. Before performing a screen, the assay must be carefully optimized for cell type, cell number, and infection level, and must be well controlled, including both positive and negative viral and cellular targets[11]. It is important to ensure sufficient separation between positive and negative controls prior to screening to maximize the dynamic range of the assay. Once the screen has been performed, candidates can be divided into functional categories to determine which host mechanisms contribute to infection. For mammalian viruses such as *vaccinia*, the contribution of mammalian homologs by RNAi should be determined as part of secondary analysis. Taken together, these robust screening methods will allow us to gain insight into complex mechanisms by which viruses interact with their host cells.

Disclosures

No conflicts of interest declared.

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