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Corresponding Author:	Tenaya Vallery UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	tenayavallery@gmail.com
Order of Authors:	Tenaya K Vallery
	Joan A Steitz
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TITLE:

Quantitative Fluorescence In Situ Hybridization (FISH) And Immunofluorescence (IF) of Specific Gene Products in KSHV-Infected Cells

AUTHORS AND AFFILIATIONS:

Tenaya K. Vallery[1], Joan A. Steitz[2]

[1]Norfolk Academy, Norfolk, Virginia, USA

[2]Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale School of Medicine, New Haven, Connecticut, USA

Corresponding Author:

Joan A. Steitz (jsteitz@yale.edu)

Email Addresses of Co-Author:

Tenaya K. Vallery (tvallery@norfolkacademy.org)

KEYWORDS:

RNA FISH, IF, herpesviruses, quantification, nucleocytoplasmic shift, suspension cells, KSHV

SUMMARY:

We describe a protocol utilizing fluorescence in situ hybridization (FISH) to visualize multiple herpesviral RNAs within lytically infected human cells, either in suspension or adherent. This protocol includes quantification of fluorescence producing a nucleocytoplasmic ratio and can be extended for simultaneous visualization of host and viral proteins with immunofluorescence (IF).

ABSTRACT:

Mechanistic insight arrives from careful study and quantification of specific RNAs and proteins. The relative locations of these biomolecules throughout the cell at specific times can be captured with fluorescence in situ hybridization (FISH) and immunofluorescence (IF). During lytic herpesvirus infection, the virus hijacks the host cell to preferentially express viral genes, causing changes in cell morphology and behavior of biomolecules. Lytic activities are centered in nuclear factories, termed viral replication compartments, which are discernable only with FISH and IF. Here we describe an adaptable protocol of RNA FISH and IF techniques for Kaposi's sarcoma-associated herpesvirus (KSHV)-infected cells, both adherent and in suspension. The method includes steps for the development of specific anti-sense oligonucleotides, double RNA FISH, RNA FISH with IF, and quantitative calculations of fluorescence intensities. This protocol has been successfully applied to multiple cell types, uninfected cells, latent cells, lytic cells, time-courses, and cells treated with inhibitors to analyze the spatiotemporal activities of specific RNAs and proteins from both the human host and KSHV.

INTRODUCTION:

In their lytic (active) phase, herpesviruses hijack the host cell, causing changes in cell morphology and localization of biological molecules, to produce virions. The base of operations is the nucleus,

where the double stranded DNA viral genome is replicated and packaged into a protein shell, called a capsid[1]. To begin, the virus expresses its own proteins, hijacking host machinery and preventing expression of non-essential host genes, a process termed the host shutoff effect. The majority of this activity is localized to specific 4',6-diamidino-2-phenylindole (DAPI)-free nuclear regions called viral replication compartments, comprised of both host and viral proteins, RNAs, and viral DNA[2]. The cell is overhauled to provide space and resources for the replication compartments and thus assembly of viral capsids. Once the capsid exits the nucleus, how the capsid is enveloped in the cytoplasm to produce a membrane-bound viral particle, also known as a virion, is unclear. Understanding of the localization and spatial shifts of both host and viral biomolecules during the lytic phase provides deeper mechanistic insight into the arrangement of the replication compartment, host shutoff effect, the virion-egress pathway, and other processes related to herpesviral infection and replication.

Currently the best method to detect and study these changes is the visualization of proteins and RNAs in infected cells with immunofluorescence (IF) and fluorescent in situ hybridization (FISH), respectively. Use of a time-course with these techniques reveals the localization of biomolecules at key points of the lytic phase or simply, spatiotemporal data. FISH and IF complement other biochemical techniques, such as inhibition of a cellular process (e.g., inhibition of viral DNA replication), RT-qPCR (real-time polymerase chain reaction), RNA sequencing, Northern blots, mass spectrometry, western blotting, and analysis of viral DNA production, that may provide a more global picture of cellular activities.

We developed RNA FISH strategies to examine the RNA products from specific genes and a computational analysis that quantitatively calculates the nucleocytoplasmic ratio of a specific gene product. The sample preparation, modified from earlier publications by Steitz and colleagues[3,4], is relatively easy and can be used for both adherent and suspended cells. The protocol is also adaptable for simultaneous use of multiple RNA FISH strategies (double RNA FISH) or RNA FISH with IF strategies. Development of a specific FISH strategy is challenging, but suggestions to improve success are outlined. The data analysis described here is quantitative if fluorescent beads and strong markers of compartment boundaries are used and offers additional insight into the micrographs, insight that removes observation bias. The detailed protocol is designed for both latent and lytic cells infected by Kaposi's sarcoma-associated herpesvirus (KSHV) and can be used with uninfected cells or cells infected by other herpesviruses[5]. The methods of quantitation are applicable to studies on nucleocytoplasmic shifts or relocalization between subcellular compartments in most cells.

PROTOCOL:

1. Design of fluorescence in situ (FISH) anti-sense oligonucleotides to detect a specific herpesviral transcript

1.1) Select 25 to 40 nt segments from the sequence of RNA of interest and convert to be anti-sense. A successful FISH strategy may contain from one up to ten or more different anti-sense oligonucleotides. When selecting sequences, consider the following.

1.1.1) If the RNA of interest contains a unique repeat region, then capitalize on this feature and design an anti-sense oligonucleotide to target the repeat sequence.

NOTE: Tycowski and colleagues[5] provide an example of this strategy with rhesus rhadinovirus (RRV) polyadenylated nuclear (PAN) RNA.

1.1.2) If the RNA of interest contains a known protein-binding site or stem-loop structure, design oligonucleotides that avoid these regions.

1.1.3) Depending on the goals of the experiments, consider the intronic sequence and whether or not to design an anti-sense oligonucleotide for it.

1.2) Perform simple computational analyses on the selected anti-sense sequences to insure binding specificity and reduce aggregation of the anti-sense oligonucleotide.

1.2.1) The sequences must be approximately 50% GC-rich (high guanine and cytosine content) and have a melting temperature in the range of 60 to 70 °C.

1.2.2) Use a sequence analyzer tool to select sequences that do not self-dimerize or form hairpins with melting temperatures above 37 °C, the hybridization temperature.

1.2.3) Perform a NCBI BLASTn (National Center for Biotechnology Information Basic Local Alignment Search Tool for nucleotide alignments) search of the selected sequences against both the host and viral transcriptomes using the 'somewhat similar' setting. This search will identify unique anti-sense oligonucleotides that will not likely bind to other host or viral transcripts.

NOTE: If transcriptomes are not available, perform the BLAST search with the genomic sequences. It is ideal if the searches are performed on the sequences from the virus isolated from the infected cells used in the experiment because wild strains tend to diversify and contain a combination of sequences from different lab strains.

1.3) Order purified DNA oligonucleotides corresponding to the anti-sense sequence that has been verified computationally to be unique and likely to bind to the target RNA. No special modifications need to be introduced into the oligonucleotides.

1.4) Test the designed anti-sense oligonucleotides for binding specificity by FISH and Northern blot.

1.4.1) Using an uninfected cell-line from the same host species (e.g., 293T) and ideally the same cell type, conduct a transfection with a plasmid expressing the RNA of interest from a robust promoter (CMV, cytomegalovirus) and one with the empty vector (e.g., pcDNA3). Use a positive control for transfection such as co-transfection with a GFP (green fluorescent protein) plasmid (e.g., pmaxGFP) or the vector containing GFP.

NOTE: It is important to avoid cell-lines that have been immortalized using the herpesvirus Epstein-Barr virus (EBV) since there are sequence similarities between herpesviruses.

1.4.2) Perform FISH as described in section 3 on both sets of cells with the anti-sense oligonucleotides as described in this protocol. Deduce the successful candidates by comparing FISH experiments with individual, pairs, or sets of the anti-sense oligonucleotides. Use a positive control for the FISH protocol such U2 snRNA (small nuclear RNA) FISH, present at 500,000 copies per human cell nucleus[6] (**Table 1**).

1.4.3) The fluorescent signal should be specific and strong in the cell containing the RNA of interest. Design additional anti-sense oligonucleotides to strengthen the signal and remove anti-sense oligonucleotides that bind nonspecifically from consideration. Signal strength must be above background and autofluorescence.

1.4.4) Test binding specificity by Northern blot.

2. Oligonucleotide and cell preparation

2.1) Following manufacturer's instructions, use terminal transferase to label the anti-sense oligonucleotides with dioxigenin(DIG)-dUTP or, if strongly binding, directly with a fluorescent nucleotide like Alexa Fluor 594-5-dUTP. After labeling, additional purification is not necessary. Store labeled oligonucleotides at -20 °C up to several years and in tin foil if directly labeled to prevent photobleaching.

CAUTION: The labeling solution contains a toxic material, potassium cacodylate. Handle labeling reactions with gloves.

NOTE: To conserve resources, several different anti-sense oligonucleotides can be labeled in one reaction. This protocol utilizes 3'-end labeling. Internal labeling is challenging because the chemical group (e.g., DIG or the fluorophore) gets caught or is unable able to enter the active site of a DNA polymerase. An experiment with two different RNAs can be performed using directly-labeled anti-sense oligonucleotides and anti-DIG immunofluorescence with a different fluorophore (e.g., FITC (fluorescein) or Alexa Fluor 488 with Alexa Fluor 594).

2.2) Adhere the cells to the eight-chamber slides.

NOTE: Eight-chamber slides allow several simultaneous experiments while minimizing precious resources like antibodies. An alternative to eight-chamber slides is a six-well tissue culture plate with standard coverslips (22 mm x 22 mm) that are both sterile. A similar arrangement is possible with circular coverslips and a 24-well tissue culture plate. For both, increase the volumes mentioned in this protocol by 10–15x (e.g., 1.75 mL hybridization solution), and 4x (e.g., 600 µL hybridization solution) respectively.

2.2.1) For adherent lytic cells, use 1x trypsin/PBS at 37 °C and 5% CO_2 for 10 min to suspend cells and dilute to 60% confluency.

NOTE: Adherent cell lines used in FISH experiments included 293T, iSLK.219[7], and iSLK-BAC36 cells[8].

2.2.2) Apply 200 µL of cell suspension to each chamber of the sterile eight-chambered slides and allow seed growth for 12–24 h at 37 °C and 5% CO_2. Adjust as necessary for slow- or fast-growing cells and for cells that are easily damaged by trypsin.

NOTE: The objective is to have evenly spaced cells firmly attached to the slide. Consider inducing lytic phase after adhesion if the lytic cells are fragile. Conclusions drawn from experiments with iSLK cells are limited[9].

2.2.2) For lytic suspension-cells, pre-treat eight-chamber slides with 1:10 poly L-lysine for 5 min under the tissue culture hood. Then leave the slides to dry overnight at room temperature or 1 h at 65 °C. Incubate 800 µL of lytic cells at a concentration of 1 x 10^6 cells/mL with the chambered slides for 30 min to 1 h at 37 °C and 5% CO_2.

NOTE: Suspension-cells will settle in a monolayer, sticking to the poly L-lysine and thus excess cells are not a concern in comparison to adherent cells. Lytic cells that form grape clusters, if possible, should be separated by gentle vortexing or chemical means. Admittedly, the authors have not had much success with such recommendations in the case of lytic BJAB-RRV-GFP cells. If suspension-cells do not adhere well, consider increasing either the time or concentration of the poly L-lysine incubation.

3. Fixation, Immunofluorescence (Optional), Hybridization, and Visualization of Viral RNAs

3.1) Remove media and excess cells. Throughout this protocol, use vacuum suction to remove solutions and gentle micropipetting to add solutions.

NOTE: The strength of a vacuum can be reduced by placing a 200 µL micropipette tip over the glass Pasteur pipette. Replace the micropipette tip between wash steps to prevent contamination. Each wash step must be performed quickly because it is imperative that the cells never dry out.

3.2) Immediately, fix the cells with pre-chilled 4% formaldehyde/PBS (phosphate buffered saline) on ice for 30 min. Wash the cells three times with 200 µL 1x PBS cooled to 4 °C and incubate for 5 min at room temperature or on ice.

3.2.1) Permeabilize the fixed cells with 200 µL of pre-chilled 0.5% Triton-X/PBS (phosphate buffered saline) for 10 min on ice or 750 µL of pre-chilled 70% ethanol at 4 °C for 1 h (min) to 7 d (max).

NOTE: Collect protein, total RNA, and genomic DNA samples at the point of fixation to ensure consistency between images and biochemical assays. All washes throughout this protocol are performed in the same manner unless otherwise specified. 70% ethanol loosens the glue between the chambers and the slide, which eases later separation, and also provides a significant pause in the protocol. Nonetheless, use paraffin film around the chamber slide to reduce evaporation and check the level of the ethanol in each chamber about every 8 h. 70% ethanol also flattens the cells, making a crisper image, while Triton-X does not dehydrate the cells and change the dimensions of the cell.

3.3) Remove chambers carefully to prevent cracking the slide. If the experiment includes immunofluorescence (IF) of a viral or host protein with a polyclonal primary antibody, perform the IF as described below before proceeding to RNA FISH. If the immunofluorescence uses a monoclonal primary antibody, then perform immunofluorescence as described in step 3.3.1 after step 3.11.

NOTE: Use a fresh removal device or one with very little leftover adhesive provided by manufacturer and gently ease the chambers off to prevent the slide from cracking. Using 70% ethanol as the permeabilizing reagent for 4 h greatly reduces the likelihood of cracking. In the case of a crack, continue the protocol on chambers not affected by the crack and be mindful of the higher oxidation- rate of imperfectly sealed slides (i.e. decreased storage- life).

3.3.1) Rinse cells with pre-chilled 1x PBS and block with pre-chilled 4% BSA (bovine serum albumin)/1x PBS for 30 min at 4 °C.

NOTE: The use of BSA throughout this protocol limits nonspecific labeling.

3.3.2) Remove blocking solution and incubate the cells with 1:200 or another polyclonal primary antibody in 0.1% BSA/1x PBS for 1 h at 4 °C. Then wash three times with 1x PBS.

NOTE: An antibody[10] for detection of SSB/ORF6 (viral single-stranded DNA binding protein) was used at 1:200 dilution.

3.3.3) Incubate the cells with a secondary antibody with fluorophore compatible with the FISH-detecting antibody for 1 h at 4 °C. Wash three times with 1x PBS. Then fix with 4% formaldehyde/1x PBS for 10-15 min and permeabilize with either Triton-X or 70% ethanol as previously described before proceeding to FISH. Cover slide with tin foil to preserve fluorescent signal and prevent photobleaching.

3.4) Wash the cells with 2x SSC (saline sodium citrate) once and then apply 45 µL of hybridization solution consisting of 50% formamide, 10% dextran sulfate, 2x SSC, 0.1% BSA, 500 µg/mL salmon sperm DNA, 125 µg/mL *E. coli* tRNA, and 1 mM vanadyl ribonucleoside complexes. Incubate for 1 h at 37 °C in a humidity chamber that can be a 150 mm Petri dish with moistened sterile wipes.

NOTE: Prepare fresh hybridization solution at least an hour before use. Dissolve the dextran sulfate in water first, vortexing frequently and incubating in a 37 °C water bath.

3.5) Calculate to have a suggested concentration of 25 µM oligonucleotides in 35-uL hybridization solution per chamber. Adjust concentration of anti-sense oligonucleotide as needed. Add distilled water to the oligonucleotides to bring the denaturation-volume to 10 µL. Remove the pre-hybridization solution and then add hybridization solution containing the labeled oligonucleotides to the cells.

NOTE: Following the labeling reaction, the oligonucleotides are stored in the quenched solution containing 0.18 M potassium cacodylate, 23 mM Tris-HCl, 0.23 mg/mL BSA, 4.5 mM $CoCl_2$, 18 mM EDTA, 2.7 mM K-phosphate, and 6.8 mM KCl, 45 µM 2-Mercaptoethanol, 0.02% Triton X-100, and 2% glycerol. The concentrations are high enough that dilution with water will bring the denaturation solution to concentrations near to 1x TE (10 mM Tris-HCl and 1 mM EDTA), a standard oligonucleotide denaturation buffer.

3.6) Denature the DIG- and/or Alexa Fluor 594-labeled oligonucleotides at 95 °C for 5 min. Then add 35 µL fresh hybridization solution per intended chamber to the denatured oligonucleotides. If performing double FISH, both sets of anti-sense oligonucleotides may be denatured and hybridized together.

3.7) Incubate overnight in the humidity chamber at 37 °C with tin foil to protect the fluorophore-labeled oligonucleotides.

NOTE: Incubation should be at least 10 h and not more than 24 h.

3.8) The next day, wash the cells twice with 2x SSC for 10 min at 37 °C and then twice with 1x SSC for 10 min at 25 °C.

3.9) Fix the cells with pre-chilled 4% formaldehyde/1x PBS for 10-15 min on ice. Then wash the cells with PBS three times and permeabilize for 1 h with pre-chilled 70% ethanol or for 10 min with pre-chilled 0.5% Triton-X/1x PBS at 4 °C.

3.10) Incubate the cells with 1:200 anti-DIG FITC in pre-chilled 0.1% BSA/1x PBS for 1 h at 4 °C. Remove the antibody solution and wash three times with 1x PBS.

3.11) Fix with pre-chilled 4% paraformaldehyde/1x PBS for 10–15 min at 4 °C and then wash three times with 1x PBS. If performing immunofluorescence for a host or viral protein with a monoclonal primary antibody, permeabilize the cells and then perform the IF protocol outlined in step 3.3.1. Otherwise proceed to the DAPI staining.

3.12) Incubate the cells with 0.4 µg/mL DAPI in pre-chilled 0.5% Triton-X/1x PBS for 15 min on ice and then wash three times with 1x PBS.

3.13) Mount slides with fluorescent beads (optional) and a mounting medium. Then seal the coverslip to the slide with clear nail polish.

3.14) Using a confocal microscope, collect images of the samples within an hour to a week of performing the protocol at 630x magnification. Apply multiple coats of nail polish to seal the cover slip and to prolong fluorophore life by reducing the rate of oxidation.

NOTE: Do not use a DAPI-containing mounting medium. When collecting the images, include the scale bar on each image for later quantification. Fluorescent beads serve as controls of fluorescence intensity between slides and sample preparations[11]. Acquire images at the midsection of the cell for two dimensional (2D) quantification in step 4.

4. Quantification of FISH and IF images to highlight subcellular localization and to determine nucleocytoplasmic ratio of fluorescence

4.1) Perform image analysis on an assembled stack of the various fluorescent stained and merged images to insure consistency. Set the scale of the image analysis software using the scale bar included when the images were collected.

4.2) To quantify fluorescence intensity across several channels and in reference to the nuclear DAPI stain, use a line tool and a plot-profile function. Then indicate the line permanently on a copy of the image using markers that do not obstruct or influence the viewer's judgment.

4.2.1) Establish criteria to guide where the line is drawn such as a trace that captures a diversity of topographical features, peaks and valleys, along a central axis or a line that does not traverse supersaturated areas.

NOTE: These line traces depict raw fluorescence in a cell and thus are limited to comparisons of the locations of a stain, not intensity. To compare intensities of the same stain between slides, treatments, or preparations, add a fluorescent bead to the slide as an internal control during step 3.13. The fluorescent bead must be added during the mounting process and detected with the same settings on the excitation laser and photomultiplier tube (confocal).

4.3) To quantify a shift in subcellular localization, calculate nucleocytoplasmic ratios of cells undergoing different treatments.

4.3.1) Measure the area and raw fluorescence intensity of both the nucleus and cytoplasm using the nuclear DAPI stain to set the inner boundary. Include nuclear and cytoplasmic controls such as a nuclear RNA (e.g., KSHV PAN RNA) and cytoplasmic RNA (e.g., host GAPDH mRNA). Moreover, calculate background intensity for three cell-like areas and average the values per pixel or μm^2.

NOTE: Intensity values tend to lack units and so the term 'units' is used.

4.3.2) Normalize both nuclear and cellular raw intensity values by first determining the average background for the same area and then subtracting that individualized value from the raw intensity of the area.

4.3.2.1) For example, a nucleus of a lytic B-cell has an area of 133.4 μm^2 and a raw intensity of 75976 units while the background intensity for the same fluorescent signal was determined to be 0.67 units per μm^2. The normalized nuclear intensity would be:

$$\text{Ex: Normalized Nuclear Intensity} = \left(75976 \text{ units} - \left(0.67 \frac{\text{units}}{\mu m^2} * 133.4 \ \mu m^2\right)\right)$$

4.3.3) Enter the values in the following equation.

$$\log\left(\frac{\left[\frac{\text{Normalized Nuclear Intensity}}{\text{Normalized Cellular Intensity} - \text{Normalized Nuclear Intensity}}\right]}{\left[\frac{\text{Nuclear Area}}{\text{Cellular Area} - \text{Nuclear Area}}\right]}\right)$$

NOTE: This calculation controls for changes in subcellular area. Lytic induction and drug treatments can enlarge the nucleus or change the size of the cell, respectively.

4.3.4) To interpret the results, create a box-whisker plot. An equal distribution of the fluorescent signal would be close to zero, whereas a nuclear distribution would favor a positive ratio value and a cytoplasmic distribution would trend toward a negative ratio value.

REPRESENTATIVE RESULTS:

The FISH and IF methods detailed in this manuscript are shown in **Figure 1** along with the quantification of results by line traces of fluorescent intensity. The results presented here are semi-quantitative and offer insight into localization, rather than into comparisons between intensities of different fluorescent stains because experiments did not include a fluorescent bead in the slide preparation. **Figure 1** also reveals that the cytoplasmic and nuclear areas and their ratios are different for latent and lytic KSHV-infected cells. Thus, area is controlled in the nucleocytoplasmic ratio showcased in **Figure 2**. **Figure 2** validates the calculation detailed in this manuscript for a nucleocytoplasmic ratio with the use of a nuclear control, the viral polyadenylated nuclear (PAN) RNA, and a cytoplasmic control, the host GAPDH mRNA. **Figure 3** reveals that when KSHV DNA replication is inhibited in the lytic phase by the use of either phosphonoacetic acid (Doxy + PAA) or cidofovir (Doxy + Cido), the early ORF59-58 transcript shifts to a predominantly cytoplasmic localization. The micrographs and the two quantification methods in **Figure 3** support this result and reveal that PAN RNA localizes to specific nuclear sites despite inhibition of viral DNA replication and the change seen for early ORF59-58 transcript.

FIGURE and TABLE LEGENDS:

Figure 1: Lines traces of fluorescent intensity reveal subtleties in fluorescence in situ hybridization (FISH) of KSHV transcripts and immunofluorescence (IF) of KSHV replication

compartments. (**A–B**) Confocal images of TREx RTA (tetracycline inducible viral replication and transcription activator protein) BCBL-1 cells[12] that have been induced into the lytic phase for 24 h with doxycycline (Doxy). Scale bar indicates 10 µm. (**A**) Fluorescence in situ hybridization (FISH) for viral RNAs (green) and immunofluorescence (IF) for viral single-stranded DNA binding protein (ORF6/SSB) (red), a component of KSHV replications compartments, reveal that viral transcripts localize in the cytoplasm, nucleus, and in nuclear foci outside ORF6/SSB enriched areas, also known as replication compartments. The anti-SSB antibody[10] was diluted to 1:200 in 0.4% BSA/1x PBS and detected with 1:500 anti-rabbit Alexa Fluor 594 secondary antibody in 0.4% BSA/1x PBS. All anti-sense oligonucleotides used throughout this study are provided in **Table 1**. The detection of ORF59-58 mRNA includes both the bicistronic and monocistronic transcripts. However, in KSHV-infected JSC-1 cells, the monocistronic mRNA is at least 18-fold less abundant than the bicistronic transcript and likely contributes only a minor portion of the total fluorescent signal observed[13]. Moreover one of the PAN RNA oligonucleotides (SB88) can also detect the viral transcript for K7. The signal from a detection of K7 will not be as significant compared to the signal detecting KSHV PAN RNA, which is present at nearly 80% of all polyadenylated RNA in a lytic KSHV-infected cell[14]. Additionally one of the four anti-sense oligonucleotides (tkv13) in the detection of the K8.1 mRNA is able to bind to multiple isoforms of K8.1 and other isoforms of nearby open reading frames (ORF). The FISH signal from only oligonucleotide tkv13 is insufficient (data not shown). The combined hybridization of the four oligonucleotides and the binding of them on the same transcript likely provides the observed strong signal. White lines flanking cells in (A) depict the line path of fluorescence intensities for the FISH and IF signals, plotted in (C). (**B**) Digitally zoomed images of cells in (A) flanked by white lines. For simplicity, the blue DAPI channel is omitted. (**C**) The plots show the relative fluorescent intensities for each stain along the same line: αSSB (red), viral transcripts (green; transcript indicated on plot), and DAPI (blue). Shaded areas indicate DAPI-reduced regions that correspond to viral replication compartments or SSB/ORF6-enriched areas. (**D**) The ratio of nuclear area to cellular area changes and thus the fluorescence intensity ratio used throughout was normalized for area. (**E**) Nuclear and cellular areas measured for TREx RTA BCBL-1 cells with and without undergoing lytic activation. Statistically significant changes are seen compared to uninduced cells. The box and whisker plots represent the 10 and 90 percentiles. Figure reprinted with slight modifications from Vallery, Withers, and colleagues[15] under a Creative Commons Attribution license.

Figure 2: Control nuclear and cytoplasmic FISH strategies validate the calculation-method of the nucleocytoplasmic ratio. (**A**) FISH for the host GAPDH mRNA (red) and for the viral polyadenylated nuclear (PAN) lncRNA (green) and DAPI nuclear staining (blue) are positive FISH controls for the calculation- method determining the nucleocytoplasmic ratio. Host GAPDH mRNA is a canonical target of the KSHV's host shutoff effect and is degraded upon lytic induction as shown here. (**B**) Fluorescence intensities along a line indicated by white lines flanking lytic cells in (A). DAPI (blue), PAN RNA (green), and GAPDH mRNA (red). Shaded areas are as defined in **Figure 1**. (**C**) Quantification of the fluorescence intensities of cells represented by (A) (n = 150 for each GAPDH sample, n = 75 for the ORF59-58 or K8.1 samples) were performed for three biological replicates of cells shown in **Figure 2** and **Figure 3**. P-values: >0.05 (ns), <0.05 (*), <0.005 (**), and <0.0005 (***). (**D**) Representative Northern blot of RNA from TREx RTA BCBL-1 cells 24 h after Doxy. The box and whisker plots represent the 10 and 90 percentile. Figure reprinted with

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Figure 3: Line traces and calculation of nucleocytoplasmic ratios reveal a strong shift to the cytoplasm for the early lytic ORF59-58 transcript upon inhibition of viral DNA replication. TREx RTA BCBL-1 cells were treated for 24 h with no drug (Unind), doxycycline only (Doxy), or with doxycycline and one inhibitor of herpesviral DNA replication, phosphonoacetic acid (Doxy + PAA) or cidofovir (Doxy + Cido). Panels (**A–C**) show data from samples collected from three biological replicates. (**A**) qPCR values for viral intracellular DNA during inhibition of viral DNA replication were normalized to the quantity of promoter DNA of the host-cell GAPDH gene. (**B**) Northern blot (left) and quantification (right) show total RNA levels during inhibition of viral DNA replication. Uninduced levels of all RNAs were undetectable. (**C**) Representative FISH images for viral ORF59-58 transcripts (green) and PAN RNA (red) upon inhibition of viral DNA replication. DAPI (blue) was the nuclear stain. (**D**) Quantification of the fluorescence intensities of cells represented by (C) (n = 75 each) was done on biological triplicates. (**E**) Fluorescence intensities along lines drawn across cells indicated by white lines in (C) are shown: DAPI (blue), PAN RNA (red), and ORF59-58 mRNA (green). P-values: >0.05 (ns), <0.05 (*), <0.005 (**), and <0.0005 (***). The sequences of all oligonucleotides in this study are provided in **Table 1**. The box and whisker plots represent the 10 and 90 percentiles. Figure reprinted from Vallery, Withers, and colleagues[15] under a Creative Commons Attribution license.

Table 1: All oligonucleotides used in the analyses of this publication. Table 1 was reproduced with permission from the American Society for Microbiology under a Creative Commons Attribution license from Vallery et al.[15].

DISCUSSION:

The protocol described in this manuscript can be adapted to different cell types and includes steps for double RNA FISH and RNA FISH with IF using both monoclonal and polyclonal primary antibodies. Although prepared slides are typically imaged with a confocal microscope, imaging can be performed with a STED (stimulated emission depletion) microscope after modifications of increased antibody concentration and a different mounting medium. For enhanced analysis of individual cells, samples prepared with this protocol may also be sorted, imaged, and analyzed by a cell sorter or flow cytometer with modest changes, as shown by Borah and colleagues[16]. This protocol however cannot be adopted for live cell imaging.

The quantification methods detailed eliminate observation bias and serve to validate a potential nucleocytoplasmic shift. The nucleocytoplasmic ratio also pinpoints when a biomolecule adjusts from being evenly dispersed to localizing in a specific subcellular compartment. The results presented here are semi-quantitative while the protocol outlines ways to strengthen the quantification. The strength of the nucleocytoplasmic ratio and line traces (step 4) depend on the use of fluorescent beads as intensity controls (step 3.13) and the use of clear subcellular markers, such as one for nuclear Lamin A/C. At this time, a clear boundary-marker does not exist for KSHV viral replication compartments. Regardless, this calculation can be extended to other subcellular compartments with the use of appropriate markers.

The major hurdle for the protocol detailed in this manuscript is the development of FISH strategies for specific transcripts (step 1). Success relies on the abundance and binding strength of anti-sense oligonucleotides. Specificity to particular transcripts is made even more difficult by the presence of overlapping open reading frames (ORFs) in viral genomes. Thus, viral transcripts often have sequence similarity[17] with other viral transcripts from the same genomic region, especially in case of herpesviruses. Often development of a FISH strategy must take advantage of more abundant transcripts. To troubleshoot a lack of a FISH signal, users should perform the FISH protocol with the U2 snRNA FISH strategy to confirm that techniques in human cells and preparation of reagents are adequate. Likewise, the KSHV PAN RNA FISH strategy can confirm lytic activation in KSHV-infected cells. To troubleshoot binding by the anti-sense oligonucleotides, the authors recommend developing several anti-sense oligonucleotides. If all fails, a commercial option is available as demonstrated by the use of the GAPDH FISH strategy in **Figure 2** and by Vallery, Withers, and colleagues[8].

Stronger algorithms to define the cellular and subcellular boundaries would further eliminate quantification bias. Some analytical image processing software can set boundaries for the cell, nucleus, and more, but require definitive markers. Unusual cell morphologies such as viral replication compartments are difficult for such software—a challenge for future development. Moreover the quantification methods described here are limited to one optical slice of a cell (2D image analysis). While 3D image acquisition is possible[18], future development of a quantitative 3D image analysis may provide further insight into spatiotemporal regulation of viral replication compartments.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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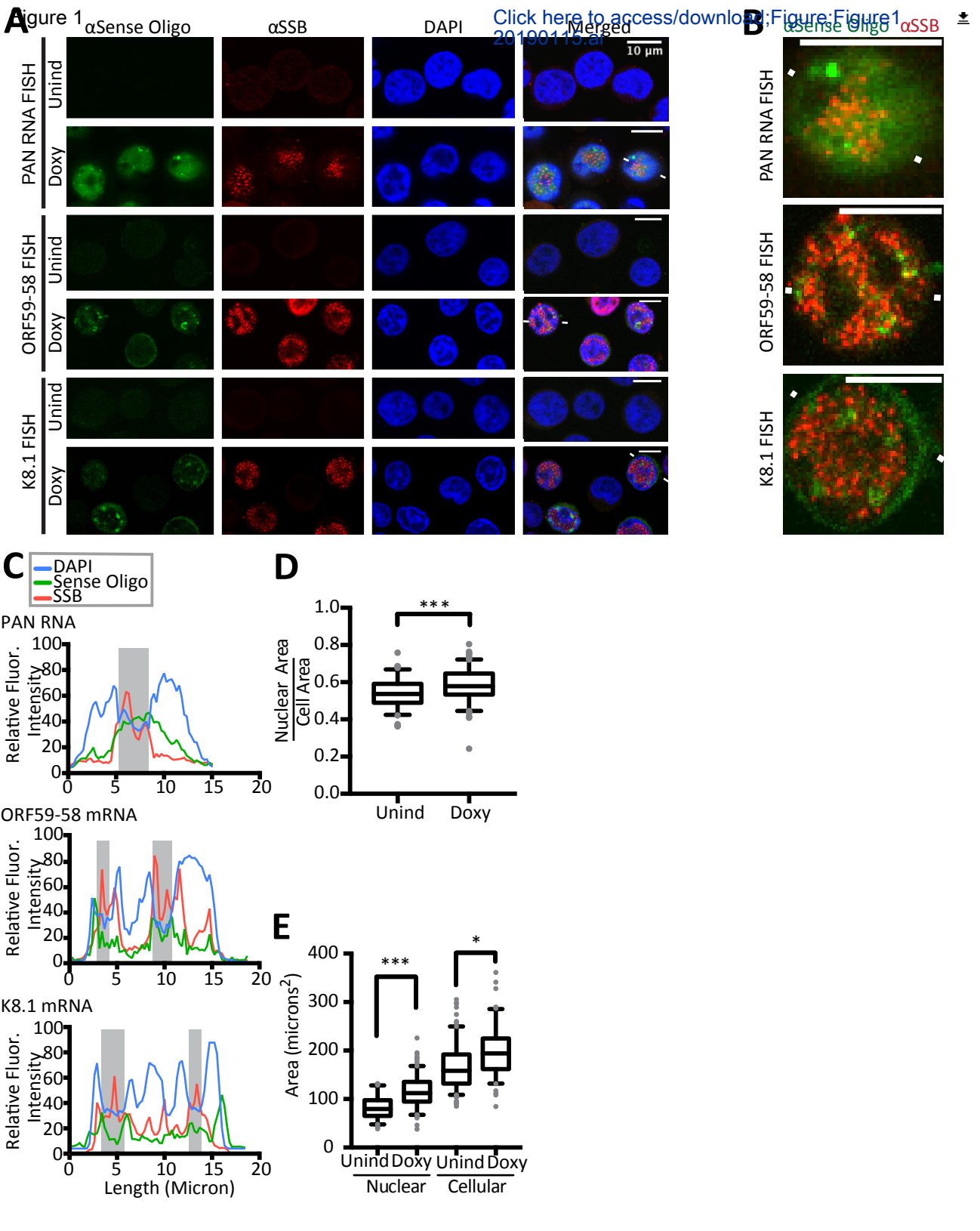

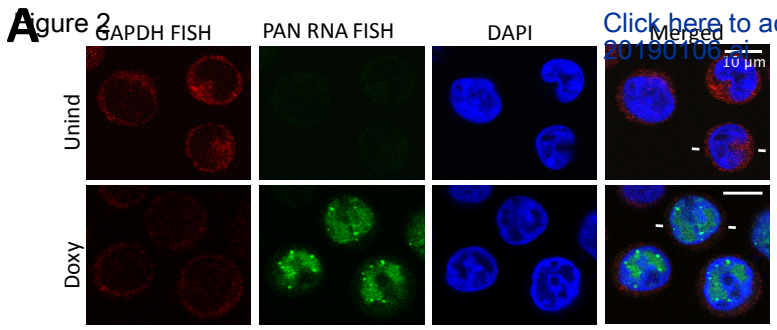

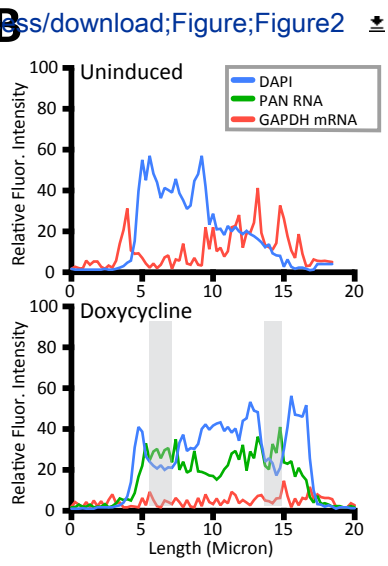

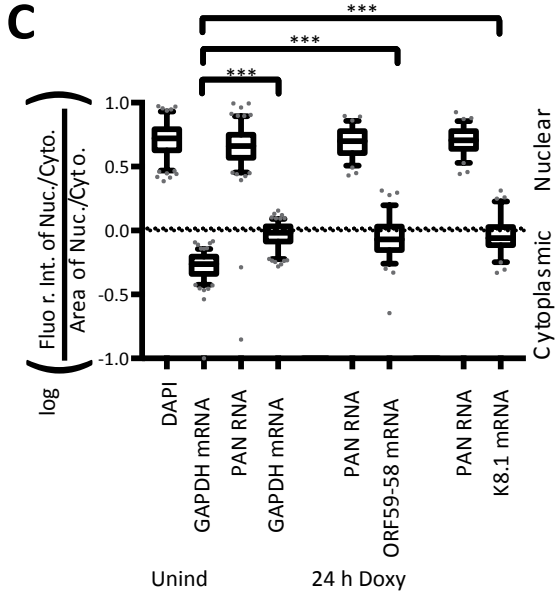

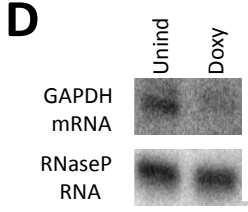

Figure 3

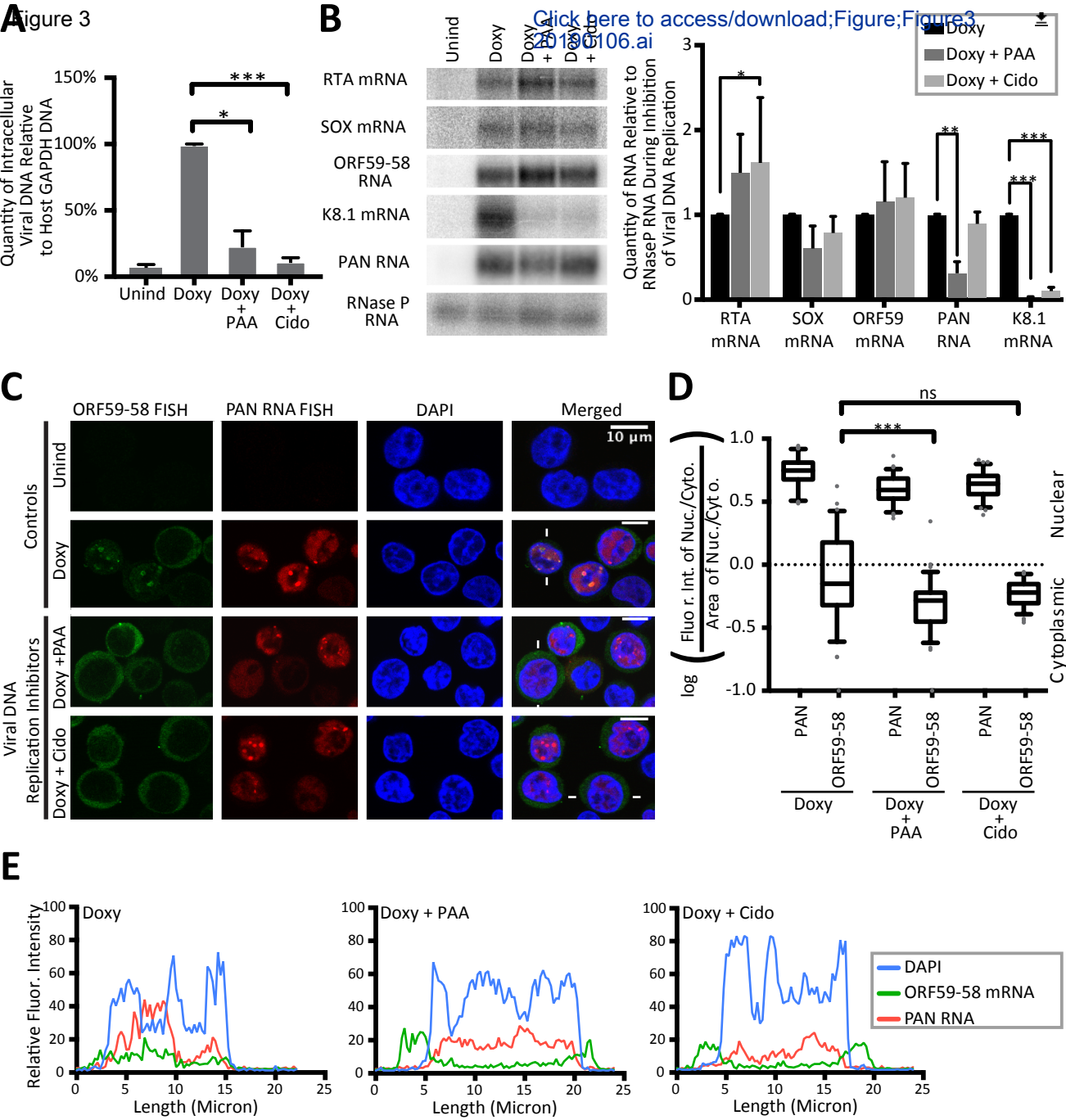

Table of Oligonucleotides

Click here to access/download;Table;Table of Oligos JoVE 2019.xlsx

Northern Oligos		
Oligo No.	Gene	Sequence
KORF50	KSHV RTA/ORF50	CGCATTGCGGTGGTTGAAATTGCTGG
JBW249	KSHV SOX/ORF37	TAACCTGACACCACCAAACACACGGTCCAC
tkv379	KSHV ORF59-58	TGGAGTCCGGTATAGAATCGGGAACCT
tkv13	KSHV K8.1	AAGGCATAGGATTAGGAGCGCCACAGGGATTTCTGTGCGAAT
SB2	KSHV PAN RNA	ACAAATGCCACCTCACTTTGTCGC
Rnase P	Human RNase P	TGGGCGGAGGAGAGTAGTCTG
FISH Probes		
Oligo No.	Gene	Sequence
SB2	KSHV PAN RNA	ACAAATGCCACCTCACTTTGTCGC
SB85	KSHV PAN RNA	CGCTGCTTTCCTTTCACATT
SB88	KSHV PAN RNA	GTGAAGCGGCAGCCAAGGTGACTGG
tkv13	KSHV K8.1	AAGGCATAGGATTAGGAGCGCCACAGGGATTTCTGTGCGAAT
tkv14	KSHV K8.1	TGATATTAAGGCATCGGTCAGTTCTGTGGTGGCCTGGA
tkv15	KSHV K8.1	GTAAGGTTACGCTTTATCCCTACACACCGACGGTTTACCC
tkv16	KSHV K8.1	GGACAAGTCCCAGCAATAAACCCACAGCCCATAGTATG
tkv376	KSHV ORF59-58	TAATGTGTTCATTGACCCTCCTGATT
tkv377	KSHV ORF59-58	GCCGATCCGTGCACTTGCACTACTCCGGTT
tkv378	KSHV ORF59-58	AAGGCTATGCCAGCGTCGAGTACATTCGCA
tkv379	KSHV ORF59-58	TGGAGTCCGGTATAGAATCGGGAACCT
tkv380	KSHV ORF59-58	AAAGAGTGTGAACGAGTACAGGGCCTT
tkv381	KSHV ORF59-58	AAACACTGCTGACGCGCAGATCCATTCC
tkv382	KSHV ORF59-58	TACCTGTGTACTATTGGCGGCGCCTGATACAC
tkv383	KSHV ORF59-58	GGGTCGAGATTCAGCTAATTAGGCGAAAACTCCACAGG
Stellaris	GAPDH	Premade by Stellaris
qPCR Primers		
tkv458	GAPDH Promoter	CTGCACCACCAACTGCTTAG
tkv459	GAPDH Promoter	GTCTTCTGGGTGGCAGTGAT
tkv319	KSHV ORF39 (gM)	GTGAGGTGCTTCGCTGAGTT

tkv320	KSHV ORF39 (gM)	CCTGGGTCAAGCTGTTGTTT

Table of Materials

Name of Material/Equipment	Company	Catalog Number
AlexaFluor594-5-dUTP	Life Technologies	C1100
anti-DIG FITC	Jackson Lab Immunologicals	200-092-156
Anti-Rabbit Secondary AlexaFluor594 Monoclonal Antib	Invitrogen	A-11037
Anti-SSB Antibody	N/A	N/A
BLASTn	NIH NCBI	N/A
Dextran Sulfate	Sigma Aldrich	D8906
DIG-Oligonucleotide Tailing Kit	Sigma Roche	#03353583910
Eight-Chamber Slides	Nunc Lab Tek II	#154453
Formamide	Sigma Aldrich	F9037
GAPDH Probes	Stellaris	SMF-2019-1
ImageJ	NIH, Bethesda, MD	N/A
OligoAnalyzer	IDT	N/A
pcDNA3	Invitrogen	A-150228
pmaxGFP	Amaxa	VDF-1012
Poly L-Lysine	Sigma Aldrich	P8920
Terminal Transferase	Sigma Roche	#003333574001
Vanadyl Ribonucleoside Complexes	NEB	S1402S
Vectashield	Vector Laboratories, Inc.	H-1000

Comments/Description
Goat
Ref. Chiou et al. 2002
Free Sequence Alignment Software
Molecular Biology Grade
2nd Gen
Blue seal promotes surface tension but separation by clear gel is also available.
Molecular Biology Grade
Compatible with protocol, Quasar 670
Free Image Analysis Software, [http:rsb.info.nih.gov/ij/]
Free Oligonucleotide Analyzer
DAPI within the mounting media scatters the light and reduces contrast.

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Author(s):	**Tenaya K Vallery**

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Name:	Tenaya K Vallery
Department:	Science Department
Institution:	Norfolk Academy, Norfolk, VA, USA
Title:	Teacher, Chemistry and Co-Director, Engineering, Design, and Innovation Fellows Program
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We would like to thank the editors and the reviewers for their timely and insightful critique of our manuscript. Each point raised was carefully considered and addressed as detailed in the following response. Our responses are indicated in bold below each reviewer comment.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
Upon reexamination, we did find a few errors (see lines 138, 148, 230, 231, 268).
1) Please avoid personal pronouns “you”, “your”, etc
Lines 97, 105, and 108 have been changed to remove personal pronouns.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples are listed below.
1) 1.1.1,1.1.2, 1.2.3,1.4.3: Cite references that describe these steps in detail.
References 5, 6, 7, and 9 on the new bibliography reveal more details about the steps 1.1.1, 1.1.2, 1.2.3, ad 1.4.3.
2) 1.4.1: mention plasmid used and cite reference for its use.
Plasmids used in this study include pcDNA3 and pmaxGFP. Both plasmids were commercially obtained and a search of the literature did not yield a publication associated with the creation of the plasmids. Thus a citation was not included and we added the plasmids to the Table of Materials.
3) 1.4.2: Reference the steps describing FISh later in the protocol.
Reference to Step 3 was included in line 147 of Step 1.4.2.
4) 2.2.1: Which cell lines? How were the cells cultured? What are the environmental conditions. Mention trypsin volume added (with relative culture volume), duration of trypsin incubation duration
Mention of adherent 293T cells and the adheret KSHV-infected iSLK.216 and iSLK-BAC16 cell lines was included along with references 7 and 8 and a note for the iSLK cells. Specifics of the trypsin digest were also provided. See lines 189 and 196-7.
5) 3.3: which antibodies? Mention concentrations?
Mention of one example antibody [anti-SSB (abbreviation provided)], its concentration, and citation were included in lines 259-260. This antibody corresponds to Figure 1, the legend of which contained the information included in lines 259-260.
6) 3.13: mention magnification.
Magnification was included in line 324.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages,

please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) Notes cannot be filmed and should be excluded from highlighting.
Highlighting was removed from Notes in the Protocol section.
2) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.
We confirmed that the highlighted protocols did not include graphical user interface and command line scripting. Highlighting that included a calculation of the oligonucleotide concentration was removed in Step 3.5 (lines 280-282).

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
We reexamined the discussion for inclusion of the five mentioned topics. We found the following lines that addressed the topics or in certain cases, augmented the discussion to include deeper insight into the topics.

1. **Modifications to the protocol outlined in the manuscript are discussed in the first and second paragraph of the discussion. Troubleshooting is discussed in the third paragraph.**
2. **Limitations of the technique are discussed in lines 501-505 and 514-516. We also added a limitation of static imaging (lines 485) and the 2D image analysis (lines 517-520).**
3. **Significance with respect to existing methods is included in lines 483-485 and added in line 518.**
4. **Future applications are mentioned in lines 495 and 515-520.**
5. **Critical steps within the protocol are now more explicitly indicated in lines 492 and 500).**

• **Figure/Table Legends:**
1) Fig 1 D,E, 2C, 3A,B,D: Define the box edges and whiskers.
Definition of the box edges to 10 and 90 percentile was added to each figure.

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Full journal names were added.

• **Table of Materials:**Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as antibodies and their RRIDs.
We reviewed the Table of Materials and found that all materials other than software were listed with name, company, and catalog number in separate columns in the xlsx file. Open source software such as BLAST and ImageJ do not have catalog numbers and thus we believe we have complied with this request. We also included mention of the secondary antibody used to detect the anti-SSB antibody and referenced the lab-made anti-SSB antibody, which is not commercially available and thus will also not have a catalog number.

• Please define all abbreviations at first use.
We reviewed the abbreviations throughout the manuscript. At first mention, we provided definition of the following abbreviations: RT-qPCR, GC-rich, NCBI BLASTn, CMV, GFP, snRNA, PBS buffer, BSA, SSC, FITC, DAPI, TREx RTA, and STED. We did note that the title of the manuscript does contain one abbreviation (KSHV) without a definition. Due to word limit, we cannot provide the abbreviation. But it is included in the abstract (line 43) and the introduction (line 85). Otherwise, we considered the following to be commonly known: DNA, RNA, abbreviations for nucleotides (e.g. dUTP), and GAPDH.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
Upon reexamination, we did find use of "u" rather than "µ" (line 280).

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The figures were previously published by the Journal of Virology. In their permission statement (see uploaded re-print permission document), Journal of Virology requires full credit on the first page of the PDF, which includes the reprinted work. Considering this requirement, we examined the JoVE author guidelines for guidance on how to structure the first page of submitted manuscript to comply with this requirement and could not find explicit mention. Therefore we ask the editors to include the citation on the first page of the PDF version of the article and have included the full credit on the first page of the prepared manuscript (see lines 19-23). This addition satisfies the criteria of Journal of Virolog's full credit while maintaining JoVE's citation style. Here we have also included the full citation to facilitate our request.

Vallery, T. K., Withers, J. B., Andoh, J. A. & Steitz, J. A. Kaposi's Sarcoma-Associated Herpesvirus mRNA Accumulation in Nuclear Foci Is Influenced by Viral DNA Replication and Viral Noncoding Polyadenylated Nuclear RNA. *J Virol.* 92 (13), doi:10.1128/JVI.00220-18, (2018).

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The authors describe a general technique for visualizing transcriptional activity in KSHV-infected cells, using a combination of fluorescence in situ hybridization (FISH) and immunofluorescence. The title and abstract are appropriate for this methods article, and this technique should be of interest to researchers wishing to directly observe KSHV (or other herpesvirus) life cycle in infected cells.

Overall, the paper is very well written: the materials and equipment needed are clearly listed, as are the steps listed in each procedure clearly explained.

Major Concerns:
N/A

Minor Concerns:
In the first protocol, the steps (1.1-1.2.x) describing the design of optimal FISH oligonucleotides is certainly expected and appropriate for a regular printed methods paper - but for JoVE, this reviewer is not sure how this work can be demonstrated in the video presentation.
We agree with Reviewer #1. Steps 1.1-1.2.x will not be featured in the video presentation. Steps 2.2 and 3.x are the main focus of the video presentation.

Procedure for sample preparation and fluorescence labeling is detailed and clearly explained. However, there would need some consideration and explanation for image analysis parts.
We have included further mention of image analysis and reference 11 (see next two points). We hope that these edits deepen the discussion of the image analysis.

In this reviewer's mind, the protocol for imaging and subsequent image analysis is less clear. Confocal microscopy and "image stacks" suggest 3D acquisition, but the line traces and area

measurements appear to be for 2D (e.g, planar) images. Are the image stacks "flattened" to a single 2D projection, or can an arbitrary confocal slice through the midsection of a cell be used for image analysis? Please clarify.
We thank the reviewer for pointing out this inconsistency. We included clarification of the image analysis as being 2D (line 331) and included a call for development of a similar quantitative analysis for 3D images (lines 518). We also added a reference that uses 3D imaging of viral replication compartments (reference 18; Chen et al. 2017 J. Virol.).

Fluorescent microbeads can indeed be used as a control to compare intensities between different sample preparations, but this should be mentioned earlier in the protocol. In addition, important to keep in mind other factors, such as nonspecific labeling, autofluorescence and photobleaching - which can lead to cell-to-cell variations in fluorescence intensity and inadvertently bias the measurement of fluorescence distribution. It is important to discuss such variations in text.
We mention the use of fluorescent beads in the introduction (line 82) but also added mention of fluorescent beads in the mounting step. We also added a new reference to facilitate use of fluorescent beads as a fluorescence intensity control (Step 3.13, lines 322 and 329-331, and reference 11). Additionally, we included specific mention of the other noted factors in lines 155-156, 166, 256, and 267.

Reviewer #2:
Manuscript Summary:
This manuscript describes a protocol utilizing fluorescence in situ hybridization to visualize multiple RNAs from KSHV within lytically infected human cells. This is a well written protocol that describes a useful technique for analyzing viral RNAs. However, the authors have not identified or dealt with the issue that herpesvirus genomes are complex with multiple overlapping RNA transcripts derived from different promoters that all terminate at a common polyadenylation site. Primers derived from ORFs proximal to the polyadenylation site will hybridize to all transcripts from the different promoters in polycistronic loci. The text needs to be rewritten to describe this issue and the terminology used for the transcripts detected in the analyses needs to be broadened in a way to indicate that multiple transcripts may be detected in the assay. These transcripts are all derived from the same strand therefore the antisense primers will detect multiple transcripts. Obviously overlapping transcripts derived from genes on the different strands will not be detected by the same antisense primer.

Major Concerns:
Line 365 - since the primers will hybridize to multiple different transcripts, see below, the word "specific" could be deleted.
We removed the word, “specific.”
Table of oligonucleotides - most of these primers are not specific to the specified genes see Bruce et al, 2017, Pathogens 6, 11 Table 2 and Figure 3 for a map of overlapping transcripts and proposed promoters and transcription terminators. The presence of overlapping RNA

transcripts is a major problem for identifying gene expression for herpesviruses. This problem should be clearly described with all of the caveats that result from it.

We agree with reviewer #1 and have added mention of this difficulty in the discussion section (lines 501-505). We also included the reference suggested by the reviewer (reference 17).

Tkv379 - this primer will detect different transcripts -a bicistronic ORF59/58 transcript and a monocistronic ORF58 transcript. This should be indicated for clarity.

We agree with the reviewer and have included mention of this in the legend for Figure 1 (lines 412-422). Our original publication (reference 15) addressed this issue, but we added similar discussion to the manuscript along with reference 13. However we disagree about the significance and believe that we are not misleading the readership. In KSHV-infected JSC-1 cells, this mRNA is at least 18-fold less abundant than the bicistronic transcript and likely contributes only a very minor portion of the total fluorescent signal observed (reference 13; Majerciak et al. Journal of Virology 2006). Moreover this oligonucleotide (tkv379) is one of eight sequences used in the ORF59-58 FISH strategy. The mention of the potential detection of the monocistronic mRNA and relative concentration of the monocistronic mRNA compared to the bicistronic mRNA in the legend of Figure 1 will bring this combined detection to the attention of the readership.

JBW249 - this primer will detect 4 transcripts, ORF34/35/36/37, ORF 35/36/37, ORF36/37 and ORF37

Since this primer is used in RT-qPCR with primer JBW480, detection of the aforementioned transcripts will not occur as productive PCR amplification requires both primers and we pre-tested all RT-qPCR primers to verify a single amplicon by gel electrophoresis of the completed reaction.

Tkv13 - this will detect a number of transcripts for different isoforms of K8.1 and will also detect a number of transcripts for different isoforms of K3 as well as the bi/tri cistronic transcript for ORF50(RTA) which contains the coding sequence for K8 isoforms and K8.1 isoforms.

We included a disclosure in the legend of Figure 1, the first appearance of the K8.1 FISH strategy (line 418). However we would like to note that despite tkv13's ability to bind to multiple isoforms within this genomic region, the oligonucleotide by itself cannot provide enough fluorescent signal, suggesting that the strength of the K8.1 FISH signal is the combined fluorescence of the four K8.1 oligonucleotides and possible co-localization on the same mRNA molecule.

SB2 - this primer will detect PAN RNA as well as the K7 RNA

We believe the reviewer is referring to SB88, rather than SB2. SB2 is well downstream of K7 whereas SB88 does overlap with the K7 transcript. However since PAN RNA is present at about 500,000 copies per lytic cell and also 80% of all polyadenylated RNA in a lytic KSHV-infected cell, we consider the detection of K7 to be insignificant compared to signal of KSHV PAN RNA. We included mention of this in legend for Figure 1 and referenced a new citation (reference 14; Sun et al. 1996 J. Virology).

The other primers should also be checked for specificity for different KSHV genes.

Line 377 - change "a viral transcript" to "viral transcripts"

The suggested change was made (line 426).

Line 389 - since the PAN primers also can hybridize to the ORFK7 transcript, this line should be revised

See above note. We believe that mention of this occurrence in Figure 1 is sufficient.

Line 393 - Change "PAN RNA" to "PAN/K7 RNA" or something to indicate that the primers are not specific to PAN

See above note. We believe that mention of this occurrence in Figure 1 is sufficient.

Line 394-395 - Indicate somehow that the ORF59/58 primers and the K8.1 primers do not hybridize specifically to the ORF59/58 bicistronic or the K8.1 monocistronic transcripts.

See above notes. We believe that mention of this occurrence in Figure 1 is sufficient.

Line 401-402 - same goes here, could be either the bicistronic or monocistronic transcript.

See above note. We believe that mention of this occurrence in Figure 1 is sufficient.

Line 409-410 - same here bicistronic or monocistronic

See above note. We believe that mention of this occurrence in Figure 1 is sufficient.

Line 413-414 - same here, multiple transcripts

See above note. We believe that mention of this occurrence in Figure 1 is sufficient.

Line 419 - should change to "used in the analyses in this publication"

We made the change (line 475).

Line 440 - should add a discussion of problems associated with detected specific transcripts in the complex genomes of herpesviruses in which multiple transcripts overlap with polycistronic transcripts.

We agree with Reviewer #2 and added discussion of the overlapping ORFs (lines 501-505).

Minor Concerns:

Line 242 - is the formamide buffered or treated in any way? Special ordering characteristics?

The formamide is not treated such as deionized. Throughout the study, molecular biology grade formamide was used. We included mention of this in the Table of Materials.

Line 366 - Need references and more description of the TREx RTA BCBL-1 cells so that all readers can understand the biology of the system.

At first mention of the cells, we included reference 12, which covers the design and provides details on TREx RTA BCBL-1 cell culture, and also some more description of the cells in lines 403-404.

Line 374-375 It is not clear that Fig 1C has multiple columns, why is the "first column" called out.

We removed mention of the first column (line 426).

Line 375-376 - shouldn't this say digitally zoomed images of nuclei.

B cells notably have a larger nucleus than most cells and the cytoplasm often appears as a small ring around the cell as seen in all Figures of this manuscript. This cell morphology is important in their role as an antibody-factory. This relative morphology is maintained during herpesvirus-infection, both latent and lytic. So the digitally zoomed images are of the whole cell.

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