Video Article

Monitoring Plasmid Replication in Live Mammalian Cells over Multiple Generations by Fluorescence Microscopy

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Abstract

Few naturally-occurring plasmids are maintained in mammalian cells. Among these are genomes of gamma-herpesviruses, including Epstein-Barr virus (EBV) and Kaposi's Sarcoma-associated herpesvirus (KSHV), which cause multiple human malignancies [1-3]. These two genomes are replicated in a licensed manner, each using a single viral protein and cellular replication machinery, and are passed to daughter cells during cell division despite their lacking traditional centromeres [4-8].

Much work has been done to characterize the replications of these plasmid genomes using methods such as Southern blotting and fluorescence *in situ* hybridization (FISH). These methods are limited, though. Quantitative PCR and Southern blots provide information about the average number of plasmids per cell in a population of cells. FISH is a single-cell assay that reveals both the average number and the distribution of plasmids per cell in the population of cells but is static, allowing no information about the parent or progeny of the examined cell.

Here, we describe a method for visualizing plasmids in live cells. This method is based on the binding of a fluorescently tagged lactose repressor protein to multiple sites in the plasmid of interest [9]. The DNA of interest is engineered to include approximately 250 tandem repeats of the lactose operator (LacO) sequence. LacO is specifically bound by the lactose repressor protein (LacI), which can be fused to a fluorescent protein. The fusion protein can either be expressed from the engineered plasmid or introduced by a retroviral vector. In this way, the DNA molecules are fluorescently tagged and therefore become visible via fluorescence microscopy. The fusion protein is blocked from binding the plasmid DNA by culturing cells in the presence of IPTG until the plasmids are ready to be viewed.

This system allows the plasmids to be monitored in living cells through several generations, revealing properties of their synthesis and partitioning to daughter cells. Ideal cells are adherent, easily transfected, and have large nuclei. This technique has been used to determine that 84% of EBV-derived plasmids are synthesized each generation and 88% of the newly synthesized plasmids partition faithfully to daughter cells in HeLa cells. Pairs of these EBV plasmids were seen to be tethered to or associated with sister chromatids after their synthesis in S-phase until they were seen to separate as the sister chromatids separated in Anaphase[10]. The method is currently being used to study replication of KSHV genomes in HeLa cells and SLK cells. HeLa cells are immortalized human epithelial cells, and SLK cells are immortalized human endothelial cells. Though SLK cells were originally derived from a KSHV lesion, neither the HeLa nor SLK cell line naturally harbors KSHV genomes[11]. In addition to studying viral replication, this visualization technique can be used to investigate the effects of the addition, removal, or mutation of various DNA sequence elements on synthesis, localization, and partitioning of other recombinant plasmid DNAs.

Video Link

The video component of this article can be found at https://www.jove.com/video/4305/

Protocol

1. Engineering of Cells with Visible Plasmids

1. Use standard restriction digestion or homologous recombination techniques to introduce a DNA fragment containing approximately 250 copies of the lactose operator sequence (LacO) into the plasmid to be visualized. Our plasmid was a BACmid of approximately 170 kbp and contained the entire genome of Kaposi's Sarcoma-associated Herpes Virus (KSHV) and encoded resistance to hygromycin [12].
2. Introduce LacO-containing plasmid DNA into mammalian cells by transfection with Lipofectamine 2000. We transfected HeLa and SLK cells in 60 mm dishes for 4 hr with 10 µg purified plasmid DNA with 25 µl Lipofectamine 2000, 0.5 ml OptiMEM, and 3.5 ml DMEM.
3. Change the culture medium to 5 ml DMEM with 10% fetal bovine serum and incubate cells for 48 hr.
4. Plate the cells at low density in large dishes and culture cells in medium containing an antibiotic selection for the introduced plasmid; we selected cells in DMEM with 10% fetal bovine serum with 300 µg/ml hygromycin for 3 weeks.
5. Use pieces of sterile, trypsin-soaked filter paper to transfer hygromycin-resistant colonies to new culture dishes.

6. When transfected clones have grown to fill the dish, isolate DNA for restriction digestion and Southern blotting to ensure that the polymer of the LacO plasmid is homogenous and of the expected size.
7. If the plasmid has not been engineered to express a fusion of LacI, infect suitable clones with a retrovirus encoding the lactose repressor (LacI) fused to a red fluorescent protein, such as LacI-tdTomato. We use tdTomato rather than proteins that fluoresce closer to green to minimize photoxicity that would occur from multiple exposures over long times[13].
8. Once the LacI fusion protein is introduced, culture the cells in the presence of 200 µg/ml isopropyl-b-D-thiogalactoside (IPTG) to block the fusion proteins from binding DNA.
9. Culture the cells for at least 3 days to allow expression of the LacI-tdTomato protein.
10. Screen clones (by washing away the IPTG and viewing cells as described below) for those with optimal levels of LacI-tdTomato that reveal distinct signals with minimal background levels of unbound fusion protein.

2. Development of the Imaging System

Our imaging system consists of a Zeiss Axiovert 200M equipped with a motorized stage and Plan-Aochromat 63x/1.4 oil objective. Fluorescence excitation light is provided by the "neutral white" LED of a Colibri system. Emission is detected by a CascadeII:1024 EMCCD camera. This is a cooled, back-thinned camera with a gain register for amplifying signals; the dark current is 0.061 electrons per pixel per second, and the quantum efficiency is greater than 0.90. The system is controlled by Axiovision 4.8 software, including the SmartExperiments module. For detection of tdTomato we use a Zeiss filter set 75HE for which 85% of the passed light can excite the fluor and 95% of the emitted light will pass the emission filter.

1. Assemble an imaging system with an inverted microscope and an EMCCD camera for sensitive detection of signals, a LED light source for specific excitation and fast shuttering, a motorized stage and focus arm, and software control for automated acquisitions of images over long time intervals with multiple z-stacks. The system should sit on an air table to be isolated from vibration.
2. Assemble a stage-top incubator to maintain cells at 37 °C, 5-10% CO_2 in a humidified chamber. Allow sufficient time for the system to come to a stable temperature to minimize movement artifacts during the experiment.
3. Use a heated collar for the objective to prevent the objective from siphoning heat from the sample.

3. Visualization of Labeled DNA

1. Plate cells in a 35 mm glass-bottom dish at a density of less than 10% confluence, which will allow pairs of cells to divide into colonies of 8-16 cells without overlapping. Do this at least 24-48 hr before imaging so that the viable cells have time to divide.
2. One to three hr before the start of imaging, rinse the plate 3 times with culture medium which lacks both selective drugs and IPTG. This procedure washes away non-viable cells and allows LacI fusion proteins to bind LacO sites in the plasmids.
3. Replace the culture medium with 2 ml DMEM with 10% fetal bovine serum and 25 mM HEPES.
4. Replace the top of the culture dish with CultFoil stretched in a mounting ring for a 35 mm dish. This cover will inhibit evaporation of the culture medium, which would increase salt concentration over time and kill the cells.
5. Use differential interference contrast (DIC) imaging to view the cells and determine the focal plane of the top and bottom of the cells in multiple fields of view. In each field, move to a focal plane approximately 1/3 of the way down from the top of the cell. Save the x, y, z coordinates in the list of saved stage positions.
   Note: Plasmid signals may not be visible through the eyepieces when cells are illuminated by LEDs of the wavelength appropriate for the LacI fusion protein. Weak signals may only be visible with the use of the EMCCD camera, which allows integration of the signal over time and thus effectively amplifies weak signals.
6. In the microscope control software, define a z-stack of images to be performed at each saved stage position. Choose a z step size of 0.35-0.50 µm, which will allow you to approximate Nyquist sampling in the z dimension[14]. Include "extra" slices above and below the planes in which the cells are located; these slices allow detection following cell movements during the cell cycle, and are also used in post-acquisition processing (deconvolution) of the image stacks. This inclusion will result in a stack of 40-60 images, depending on the thickness of the cells.
7. In the microscope control software, define a time series experiment to collect a bright field image of the z-stacks at 30 min intervals (for tracking cell divisions and migrations over long times) and fluorescence images at 10-60 min intervals (for visualizing plasmids within cells). Do not use DIC imaging during the experiment, as it requires more light than standard bright field imaging, which can expose the cells unnecessarily to phototoxicity over the course of long experiments.
8. Start the software-controlled experiment. Ensure that the system is not disturbed (bumped, exposed to excess room light, etc.) during the experiment.
9. If necessary, replenish the water in the humidification system during the experiment.

4. Post-acquisition Processing of Images

1. Review the images for each z-stack and time point. Crop out any unneeded areas of the images to reduce the computer processing time in the next step.
2. Use a deconvolution algorithm to re-assign signal intensity to the pixel of origin in each z-stack[15]. While this deconvolution can be based on a theoretical point spread function for your imaging system, it is preferable to measure the point spread function of your particular system by imaging fluorescent microspheres, and use this measurement in the deconvolution algorithm. We measured the point spread function of our imaging system and applied a constrained iterative maximum likelihood algorithm in AxioVision software (AxioVision software description, Zeiss).
3. Examine the images to track the intensity changes and movements of plasmids during the cell cycle and the partitioning of plasmids to daughter cells.

Representative Results

Maximum intensity projections of z-stacks acquired in a representative experiment are shown in **Figure 3**. Typical plasmid signals are present in 3-8 slices of the z-stack, depending on whether they represent single or clusters of plasmids. In the case of EBV- and KSHV-derived plasmids, the signals move slowly within the cell until the cell reaches mitosis. The cells themselves migrate over the course of the experiment. They also become spherical and detach from the dish during mitosis. The cell cycle for healthy HeLa and SLK cells takes approximately 24 hr; cells of these types that take more than 30 hr to complete the cell cycle should be considered unhealthy. Daughter cells typically attach near each other and go on to divide at the same time in the next cell cycle. Only cells that can be shown to complete a cell cycle should be analyzed.

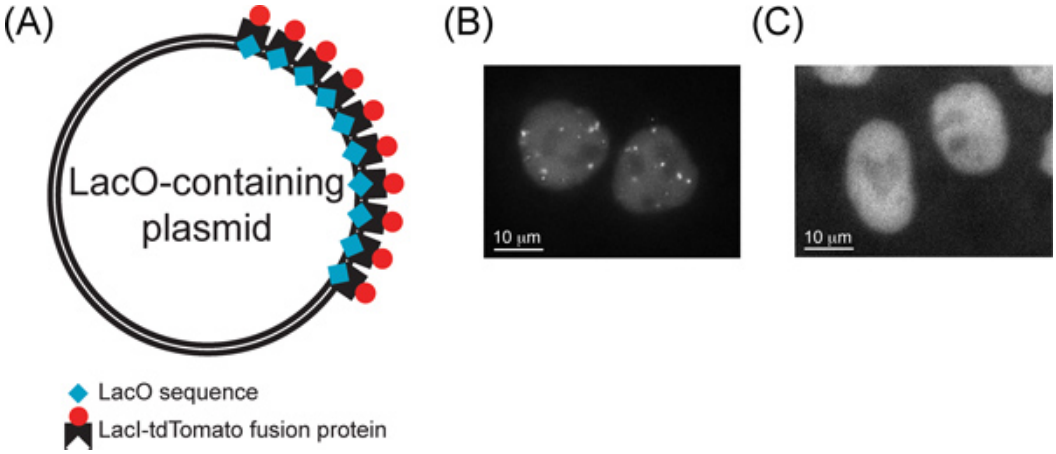

Figure 1. Scheme for making plasmids visible. (A) Tandem repeats of the lactose operator sequence (LacO) are cloned into the plasmid of interest. The lactose repressor protein (LacI) binds specifically to these sequences; fusion to a fluorescent protein recruits the fluorescent tag to the plasmid of interest. (B and C) The nucleus of a HeLa cell expressing LacI-tdTomato is fluorescent due to the nuclear localization signal on the fusion protein localizing it to the nucleus. KSHV genomes encoding LacO sequences recruit many copies of the fluorescent protein and stand out as bright signals over the background intensity of the nucleus in the absence of IPTG (B), while the LacI- fluorescent proteins are prevented to bind to its recognition LacO sequence in the presence of IPTG (C). These images are made computationally to include multiple z-planes in which the different signals reside.

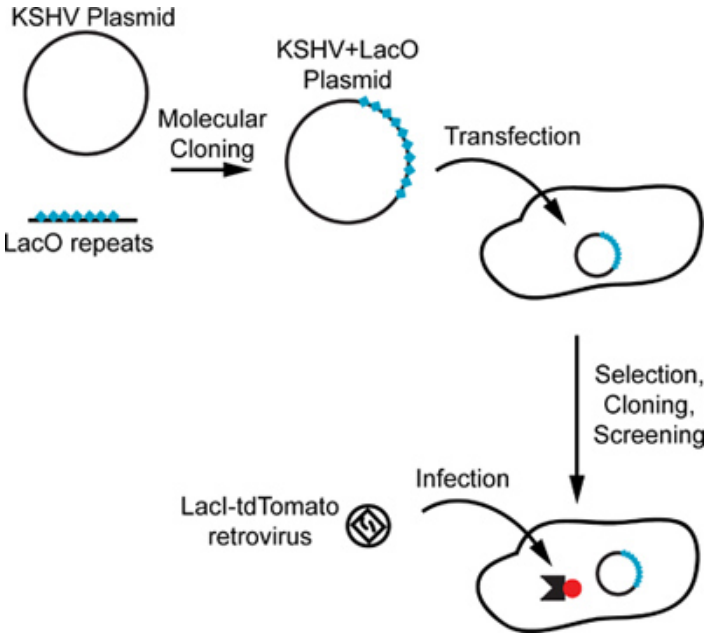

Figure 2. Diagram of the plasmid visualization procedure. First, tandem LacO repeats are cloned into the plasmid of interest. That plasmid is introduced into cells by transfection. Clones of transfected cells are selected and screened, and then infected with a retrovirus expressing a fluorescent LacI fusion protein.

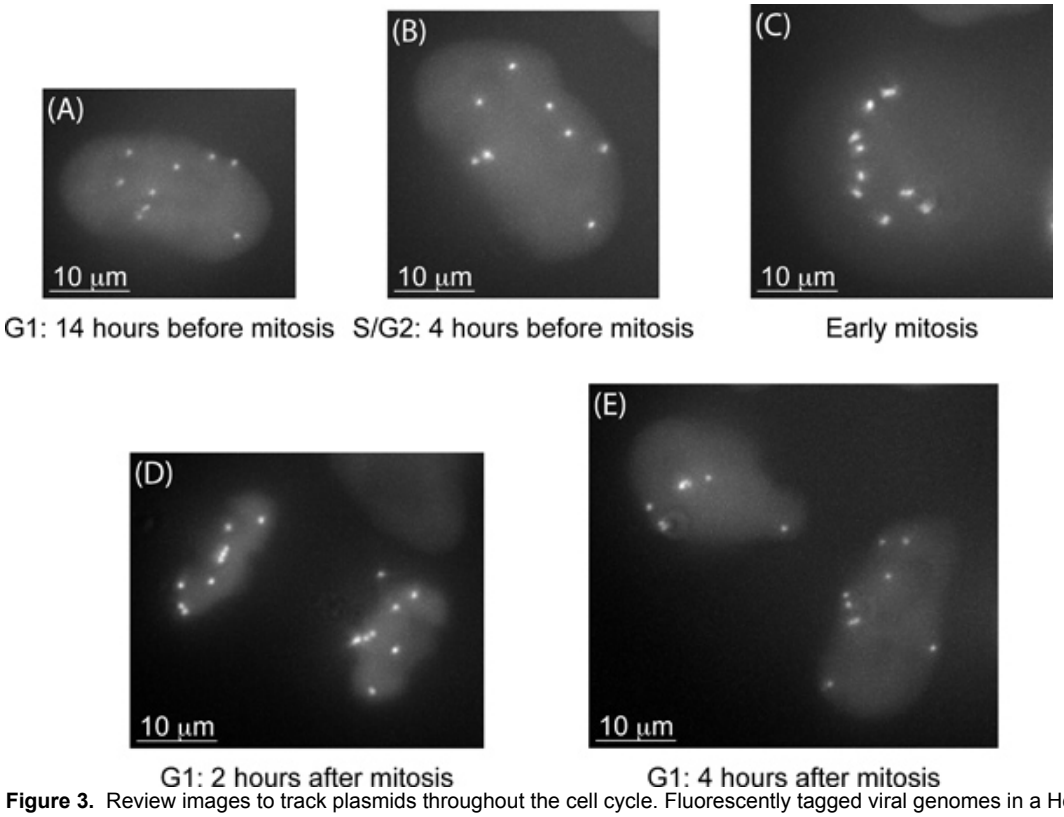

Figure 3. Review images to track plasmids throughout the cell cycle. Fluorescently tagged viral genomes in a HeLa cell (A) are synthesized in the S-phase (B) and partitioned to daughter cells during cell division (C-E) and can be followed for multiple generations. Shown are maximum intensity projections of z-stacks acquired of a HeLa cell cycle at five time points in a representative experiment. These images are made as in **Figure 1B and C**.

Discussion

The method described here can be used to follow plasmids in live mammalian cells over time spanning multiple generations. By limiting exposure to excitation light, we have used these techniques to follow cells from newly-divided pairs through colonies of 16-32 cells, representing at least 72 hr and 3 cell divisions. These experiments provide information that cannot be obtained from static assays such as quantitative PCR, Southern blotting, and FISH.

It is essential to screen clones of cells for plasmids with a homogenous structure, as rearrangements can occur when using plasmids with repetitive sequences such as the tandem LacO repeats. In addition, it is useful to optimize retrovirus infection and screen clones of infected cells for an optimal signal intensity. Cells expressing too much LacI protein will have high background fluorescence in the nucleus, and those expressing too little will have weak signals. Finally, it is important to look at cell morphology and choose healthy cells when marking fields of view to use during their visualization.

This technique can be used to visualize nearly any DNA molecule, provided it can be engineered to include tandem repeats of the LacO sequence. Examples include cellular chromosomes, naturally occurring and artificial plasmids, and viral genomes packaged within virions. In experiments similar to that depicted in **Figure 3**, we found that our KSHV-derived plasmids appear to be partitioned randomly during cell division. A logical extension of the technique is to tag one DNA molecule as a LacO/LacI pair with one fluorescent protein, and another DNA molecule in the same cell as a Tetracycline Operator/Tetetracycline Repressor pair with another fluorescent protein. Experiments are limited by the amount of excitation light the cells can withstand before experiencing cytotoxic effects.

Disclosures

No conflicts of interest declared.

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