

Use of Laser Capture Microdissection Together With *In Situ* Hybridization and Real-Time PCR to Study Distribution of Latent Herpes Simplex Virus Genomes in Mouse Trigeminal Ganglia

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Summary

The herpes simplex virus (HSV) frequently establishes a latent state in neurons, which can then be reactivated from the infected neurons. Quantifying the single-cell viral load is essential for understanding latency and reactivation of this virus. In this chapter the methods of laser capture microdissection and quantitative real-time polymerase chain reaction with *in situ* hybridization have been combined to determine the HSV copy number per neuron in latently infected trigeminal ganglia. The distribution of latent herpes simplex genomes at the individual cell level has been determined and the relationship of the number of latent genomes to the expression of latency-associated transcripts established.

Key Words: LCM; PCR; *in situ* hybridization; herpes simplex virus; trigeminal.

1. Introduction

Herpes simplex virus (HSV) characteristically establishes a lifelong latent state in neurons. The existence of latent genomes in sensory ganglia was first inferred from the ability of virus to be reactivated from infected ganglia that no longer harbor infectious virus, and subsequently confirmed on the cellular level by the detection of latency-associated transcripts (LATs), abundant non-polyadenylated RNA transcripts found in the nucleus of neurons of latently infected individuals (1–4). There have been conflicting reports regarding the role of LAT expression in the establishment of the latent state, or reactivation

of virus from latency (5–8). The ability to quantify the viral burden at the level of single cells is essential for understanding many aspects of latency and reactivation (9).

The estimated total number of latent genomes per ganglion reported in the literature varies widely, in part depending on the method used to calculate that number, with estimates ranging from 3500 genomes per ganglion determined by semiquantitative Southern blot (10) or semiquantitative polymerase chain reaction (PCR) (11), to 10^5 genomes determined by other PCR-based methods (12,13). Combining *in situ* hybridization (ISH) with PCR techniques, and making the assumption that only cells that are LAT-positive by ISH contain HSV genomes, Hill et al. (14) calculated the presence of 17–34 genomes per LAT-positive cell (14) in latently infected rabbit trigeminal ganglia (TG). In a more comprehensive analysis, using semiquantitative PCR to determine the number of genomes in neurons dissociated from latently infected fixed TG, Sawtell (9) determined that 20% of the neurons in a TG infected by the Syn17 strain of HSV contained HSV genomes, and that the number of latent genomes ranged from less than 10 to more than 1000 per neuron (9). TG infected with KOS-strain HSV contained substantially fewer latent genomes per cell than TG infected by Syn17 (15).

LATs are detected by ISH in only 0.1% to 3% of the neurons in latently infected ganglia (16), but more sensitive *in situ* PCR techniques demonstrate that latent genomes are present in neurons that are LAT-positive by ISH, as well as LAT-negative by ISH (13,17,18). The relationship between the number of latent genomes in an individual neuron to the expression of LATs detectable by ISH has not previously been investigated. One possibility is that expression of LATs to a level detectable by ISH is a function of the number of latent HSV genomes in the cell. According to this hypothesis, LATs would be detected when the number of latent genomes exceeded a threshold value. The alternative possibility is that LAT expression from latent HSV genomes depends on the presence of cell-specific factors within the host cell. According to this alternate hypothesis, the number of latent HSV genomes in LAT-positive and LAT-negative cells would display a similar distribution. In order to test the two hypotheses, we combined the methods of laser capture microdissection (LCM) and quantitative real-time PCR with ISH to determine the number of genomes per neuron in latently infected ganglia, in order to establish the distribution of latent genomes in TG at the single-cell level, and to examine the relationship of the number of latent genomes to the expression of LATs detectable by ISH.

From a methodologic perspective, these experiments illustrate the utility of LCM in defining parameters of viral infection of tissue on a single-cell basis. The details of the method employed are described below.

2. Materials

2.1. Virus

The laboratory strain of wild-type HSV-1, KOS, propagated and prepared from KOS-infected African green monkey kidney (vero) cells, was used in these experiments.

2.2. In Situ Hybridization

1. Precoated slides from Surgical Path (Richmond, IL).
2. 1X Standard saline citrate (SSC): 150 mM NaCl and 15 mM sodium citrate.
3. Hybridization buffer: 50% formamide, 5X SSC, and 40 µg/mL salmon sperm DNA.
4. Genius buffer III: 1X detection buffer (Roche), 50 mM MgCl₂, 1 mM levamisole (Sigma, St. Louis, MO). Levamisole was added before using.
5. The colorimetric substrate was made fresh by adding 87.5 µL nitroblue tetrazolium (Roche) and 87.5 µL of 5-bromo-4-chloro-3-indolylphosphatetoluidium (Roche) to 25 mL Genius buffer III.

2.3. PCR Analysis

1. PCR lysis buffer was made fresh before using. The lysis buffer contained 1X PCR buffer without MgCl₂ (Perkin-Elmer, Mountain View, CA), 1% Tween-20, and 0.4 mg/mL proteinase K.
2. Real-time PCR mixture for detection of the HSV-1 UL44 gene contained 900 nM of primer (forward primer 5' GAT GCC GGT TTT GGA ATT C 3'; reverse primer 5' CCC ATG GAG TAA CGC CAT ATC T 3'), 250 nM probe (5' FAM-ACC CGC ATG GAG TTG CGC CTC –TAMRA 3') (Synthegen, Houston, TX), and 1X TaqMan universal mix (Applied Biosystems, Foster City, CA).
3. 0.5 M EDTA, pH 8.0.
4. 4 M Lithium Chloride.
5. Standard sodium citrate chloride (SSC).
6. Phosphate-buffered saline.
7. Thermoplastic film-coated PCR caps (Capsure™, Arcturus, Mountain View, CA).
8. Gene AMP 5700 Sequence Detector (Applied Biosystems, Foster City, CA).

3. Methods

3.1. Animal Model

To establish herpes simplex virus latency in mouse trigeminal ganglia, 4–8-wk-old female Balb/c mice were inoculated with wild-type HSV-1 virus, KOS. 5×10^6 p.f.u. of virus in 5 µL of PBS was administered to each eye by corneal scarification under anesthesia. Thirty to forty days after infection, the animals were sacrificed and both trigeminal ganglia removed quickly and embedded in Cryo-Gel (Instrumedics, Hackensack, NJ). Tissue blocks were stored at –70°C until used.

3.2. Probe Generation and In Situ Hybridization

3.2.1. Generation of Probe

1. The antisense riboprobe against latency-associated transcripts (LAT) was generated from a plasmid containing the BamHI fragment of HSV (**19**).
2. The plasmid was linearized with EcoRI and transcribed in vitro with T7 RNA polymerase using digoxigenin-UTP to label the transcript.
3. Linearized template DNA (1 μg) was incubated with 4 μL DIG-nick translation mix in a final volume of 20 μL (the volume was filled with distilled H_2O) at 15°C for 90 min, and the reaction paused by placing on ice.
4. The distribution of labeled products was then checked on a 1.5% agarose gel. The ideal product size is between 200 bp and 600 bp, and the incubation time should be adjusted as needed to achieve the proper product size.
5. Once the reaction was completed, 1 μL 0.5 M EDTA (pH 8.0) was added to the reaction tube (for 1 μg DNA) and the reaction mixture heated to 65°C for 10 min to stop the reaction.
6. Carrier DNA (100 μg) from sheared salmon egg DNA (SSD) was added to 1 μg of template DNA.
7. After precipitation with 0.1 volume of 4 M LiCl and 2.5–3.0 vol of ice-chilled absolute ethanol, labeled probes were resuspended in hybridization buffer at about 100 ng/ μL .

3.2.2. In Situ Hybridization

1. Cryostat sections (10 μm) were sectioned from the snap-frozen trigeminal ganglia tissue blocks and mounted on precoated slides from Surgical Path (*see Note 1*).
2. Tissues were fixed with 4% paraformaldehyde for 30 min at room temperature.
3. For wax sections, sections were dewaxed by incubating slides at 60°C for 30 min, followed by two washes in xylene for 5 min, two washes of 100% ethanol for 2 min, two washes of 95% ethanol for 2 min, one wash with water for 2 min and the final two washes of PBS for 5 min.
4. Dewaxed sections were postfixated with 4% paraformaldehyde for 10 min at room temperature.
5. Fixed tissues were washed twice with PBS containing 0.1% active DEPC for 5 min each to protect RNA from degradation.
6. After equilibration with 5X SSC for 5 min at room temperature, tissues were hybridized with 7.5 ng of digoxigenin-labeled antisense probe in 15 μL hybridization buffer.
7. Sections were covered with cover slips, sealed with rubber cement, and incubated for 1 h or overnight at 56°C in a humidified chamber.
8. Sections were washed with 2X SSC for 5 min at RT, followed by 10 min in 2X SSC prewarmed to 65°C, by 10 min in 0.1X SSC prewarmed to 65°C, and by 2 min in PBS at room temperature.
9. After blocking with PBS containing 5% normal goat serum for 15 min at room temperature, sections were incubated for another 2 h at room temperature or 4°C

overnight with alkaline phosphatase conjugated anti-digoxigenin antibody (1:5000 dilution in blocking solution).

10. After three washes in PBS for 2 min each, sections were equilibrated with Genius buffer III for 5 min at room temperature.
11. Final detection was made by incubating slides with color substrate containing 5-bromo-4-chloro-3-indolylphosphatetoluidium-nitroblue tetrazolium (BCIP-NBT) for about 30 min or the time needed.
12. Sections were washed with tap water for 5 min, after which they were ready for laser capture.

3.3. Microdissection of Virus-Infected Cells

3.3.1. Dehydration of Slides

The dehydration step is critically important for the laser capture experiment (19). However, slides must not be allowed to dry prior to the final dehydration step (see **Note 2**). Sections that had undergone ISH were dehydrated by two changes of 95% ethanol for 2 min, two changes of 100% ethanol for 2 min and two changes of xylene for 5 min before being air-dried.

3.3.2. Laser Capture Microdissection

1. The PixCell LCM™ (Arcturus) system was used to view and capture individual LAT-positive or LAT-negative neurons from the slides.
2. Individual neurons were captured with either the 15- μ m or 30- μ m laser beam (depending on the size of the cell) to thermoplastic film-coated PCR caps (**Fig. 1**).
3. The amplitude applied ranged between 30 mW and 50 mW for 44–66 ms as suggested by Arcturus (PixCell Laser Microdissection System Instruction Manual, Arcturus, 2002, p 16).
4. The cap with captured tissue on it was placed in a microcentrifuge tube for further DNA analysis (see **Notes 3** and **4**).

3.4. Real-Time PCR Analysis

1. Single LAT-positive or -negative neurons captured on the cap were dissolved in 20 μ L of PCR lysis buffer at 37°C overnight (see **Note 5**).
2. Proteinase K was heat-inactivated in boiling water for 10 min before the PCR reaction was performed.
3. Lysate (10 μ L) was added to 40 μ L real-time PCR mixture to amplify the HSV-1 UL44 gene sequence in order to quantify HSV-1 genomes present in each LAT-positive or LAT-negative neuron.
4. The real-time PCR reaction included 2 min incubation at 50°C and 10 min of incubation at 95°C for denaturation and 50 cycles of PCR (95°C for 15s and 60°C for 1 min) in a GenAmp 5700 Sequence Detector.
5. Each PCR run contained two blank controls, serially diluted highly purified KOS DNA as standard, and the experimental samples run in duplicate.

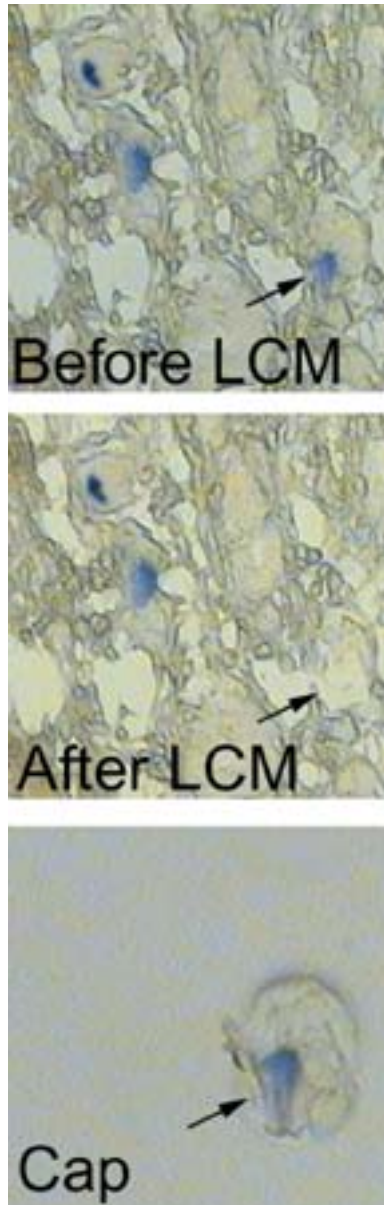


Fig. 1. LCM of individual neurons for real-time PCR. A single LAT-positive neuron is shown before LCM (arrow, top) and the same section after LCM (arrow, middle). The lifted cell on the cap is shown at the bottom (arrow, higher magnification). The ISH signal for LAT appears blue in this micrograph of ISH performed with a digoxigenin-labeled probe, and two additional LAT-positive neurons are seen. (Reprinted with permission from *J. Neurovirol.* **8**, 204–210.)

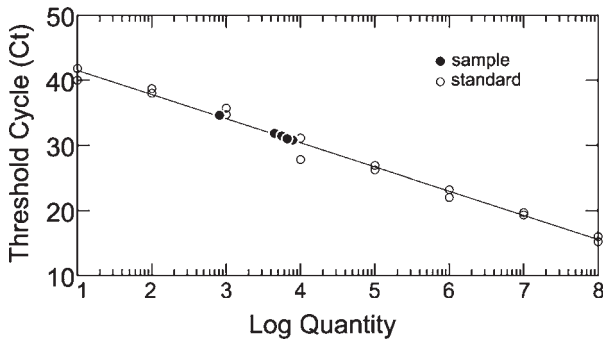


Fig. 2. Real-time PCR quantitation of HSV genomes. Purified HSV-1 DNA was amplified by PCR of UL44; the threshold cycle (Ct) is plotted against the amount of input DNA. (Reprinted with permission from *J. Neurovirol.* **8**, 204–210.)

6. The number of genomes in each sample was determined by comparing the threshold cycle (Ct) value to a standard curve obtained using KOS DNA.
7. The Ct value was defined, as the amplification cycle required observing a significant difference between emission intensity of the reporter dye in the sample and the emission intensity of the controls without DNA.
8. A standard curve was generated using known amounts of HSV KOS DNA purified by three rounds of phenol-chloroform extraction and ethanol precipitation (*see Note 6*).
9. The DNA amount was determined by UV spectrophotometer, and the DNA was serially diluted in nuclease-free water such that 1 μL of sample contained 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 or 10 copies of HSV-1 DNA.
10. Mouse genomic DNA (100 ng, Clontech, Palo Alto, CA) was added into each sample to mimic the DNA complexity of the virus-containing cell sample.
11. Real-time PCR amplification of HSV-1 DNA was linear over a range from 10 to 10^8 copies of HSV DNA (**Fig. 2**).

4. Notes

1. Precoated slides from Surgical Path gave the authors the best results in successfully lifting the tissues.
2. The sections should not be dried completely during the process of *in situ* hybridization. This has proven to be crucial to lift tissues successfully in LCM step (Dr. Kening Wang, NIH, personal communication).
3. The upgraded PixCell™ system is beneficial for viewing dehydrated and unmounted tissue. This is particularly important in finding positive cells with weak signal (as described in detail on the Arcturus web site, http://www.arctur.com/products/pixcell_ii_upgrade.htm).
4. A single laser firing is usually sufficient to obtain complete capture of tissue. Occasionally, multiple hits may be required to lift a cell with larger amount of BCIP-NBT precipitates.

5. The minimum volume needed to cover the surface of cap is 20 μ L. One should stop pushing the cap into the microcentrifuge tube at first resistance. Otherwise, leakage of solution may occur.
6. The copy numbers exist in highly purified KOS DNA were calculated based on the knowledge that HSV-1 is about 152,000 bp long and the molecular weight of one base pair is around 664 Da.

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