Recombination between co-infecting herpesviruses occurs where replication compartments coalesce

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Running title:

\textbf{HSV-1 Recombination at colaescing replication compartments}

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Abstract

Recombination between co-infecting herpesvirus DNA genomes is an important process affecting viral population diversity and evolution. Each herpes simplex virus type 1 (HSV-1) replication compartment (RC) derives from a single incoming genome and maintains a specific territory within the nucleus. This raises intriguing questions about where and when co-infecting viral genomes interact. To study the spatiotemporal requirements for inter-genomic recombination, we developed an assay with dual-color fluorescence in situ hybridization enabling detection of homologous recombination (HR) between different co-infecting HSV-1 pairs. Our results revealed that when viral RCs enlarge towards each other, there is detectable overlap of genomes from each virus. Infection with paired viruses that allow visualization of HR correlates with increased overlap of RCs. Taken together, these findings suggest that HR events take place during replication of HSV-1 DNA and are mainly confined to the periphery of RCs when they coalesce.
Homologous recombination is considered a major driving force of evolution since it generates and spreads genetic diversity. In the case of HSV-1, a ubiquitous human pathogen that causes significant morbidity and mortality, evidence of homologous recombination can be found frequently, both in vitro and in clinical isolates. Here we designed an experimental system to detect where and when recombination takes place between viral genomes during infection. We found that recombination events occur after viral DNA replication during the late stages of infection, and is prevalent at the interface of expanding viral replication compartments. Overall, our results provide spatial and temporal information regarding the process of HSV-1 replication and recombination, and these observations have implications for understanding the recombination restrictions of other DNA viruses and cellular DNA.
Introduction

Herpes simplex virus type 1 (HSV-1) is a large double strand DNA virus. Like all other herpesviruses, viral gene expression, replication and capsid assembly all occur in the host nucleus of infected cells. Viral genomes enter the nucleus through the nuclear pore complex as naked DNA molecules (1), and these rapidly recruit several host and viral proteins to the viral genomes (2-10). Expression of the immediate early and early viral genes allows initiation of viral DNA replication (11). HSV-1 DNA replication proceeds at distinct foci within the nucleus known as replication compartments (RCs) (12, 13). The formation of the viral RCs was suggested to initiate from small pre-RCs (14, 15). Live cell imaging of viral DNA binding proteins suggested that the pre-RCs migrate toward nuclear speckles, sites of RNA processing, and come into contact with other pre-RCs, where they seem to coalesce into large mature RCs (16). On the other hand, direct visualization of the viral DNA suggested that each RC usually emerges from a single incoming genome (17, 18). Our previous study with Pseudorabies virus (PRV - a swine alphaherpesvirus) suggested that although viral replication compartments are found in close proximity, they retain distinct territories for each individual genome (18). Earlier experiments with HSV replicons also supported this notion (17). A recent study showed that viral genomes entering the nucleus are observed as condensed foci, and suggested that viral expression and DNA replication allow decondensation of these genomes and formation of RCs (19). Interestingly, some genomes remain highly condensed at the edge of newly developing RCs (19). Here, we visualised co-infecting HSV-1 genomes and confirmed that alphaherpesviruses RCs initiate from single genomes.

Recombination is considered to be a major driving force in evolution of most organisms, since it accelerates adaptation (20, 21). Viruses with double strand DNA genomes
need to adapt to the changing environment. Since the rate of mutation accumulation is lower for DNA viruses than that of viruses with RNA genomes (22, 23), it has been hypothesised that high rates of recombination can facilitate genetic adaptation (24). Indeed, homologous recombination (HR) among co-infecting HSV genomes is very frequently observed in both *in vitro* genetic assays (25-31) and in sequence analysis of clinical isolates (32-34).

Viral DNA recombination can be impacted by both viral and cellular proteins. Two viral proteins have been suggested to work as a complex to facilitate viral recombination and have been shown to catalyze strand exchange *in vitro*: the single strand binding protein ICP8 and an exonuclease UL12 (35). Single strand annealing was found to be a recombination mechanism upregulated during viral infection and thus is considered as the mechanism by which the viral recombinase induces recombination (36). While ICP8 is required for viral DNA replication, UL12 is not essential for DNA replication *per se*, although it is required for formation of infectious viral genomes that can be packaged into capsids (37). Recombination of HSV-1 genomes can be enhanced by DNA double stand breaks (DSB) (31, 38) and components of cellular DSB repair pathways are recruited to sites of viral DNA replication (4, 6, 8, 39, 40). Host proteins that are known to be involved in host HR, were found to support either viral DNA replication or production of infective viral progeny (4, 39, 41-43). These findings suggest that viral recombination and replication are processes that are closely related (44). While knowledge regarding the molecular aspects of HR has been accumulating over the last few years, little is known regarding spatiotemporal constraints on inter-genomic recombination.

The compartmentalization of co-infecting genomes at different RCs raises the question of where and when recombination takes place during the course of infection. To tackle
this question, we utilized a fluorescence in situ hybridization (FISH) based assay to
differentiate between de-novo synthesized variants of viral genomes. Our results
suggest that multiple recombination events occur at later stages of infection following
DNA replication and that recombination takes place at the interface between mature
RCs. We also found strong evidence of correlation between the number of RCs and
nuclear size, suggesting a possible spatial restriction on the number of viral genomes
that initiate replication.

Results

A FISH based assay designed to detect recombination events between co-
infesting viruses. Recombination among co-infecting HSV-1 strains is a frequent
event that can be detected by the progeny viruses released from infected cells (27-29, 31). To study the spatiotemporal constraints of these recombination events we
developed a FISH based experimental assay that enables visualization of two different
viral genomes within a co-infected nucleus. For this assay, we constructed a series of
viral isolates, isogenic to each other except for two unique tag sequences (YPET or
mCherry, yellow and red fluorescent proteins encoding genes, respectively) inserted
into various loci throughout the viral genome (Figure 1A and Table 1). We designed
two sets of fluorescent probes, one set for each tag sequence (Figure 1B). Each probe
set was conjugated to a distinct fluorophore (Cy3 or Cy5) to enable visual identification
of the genomes containing each tag sequence. We hypothesize that using different
mixtures of the viruses should lead to distinct patterns within the infected nucleus
(Figure 1C). As was shown previously for PRV (18), we expect that co-infection with
two HSV-1 viruses containing tag sequences at the same genomic locus cannot result
in a new recombinant genome containing both tags. Thus, each RC will react to a
single fluorophore, including at the contacting edges of proximate RCs (Figure 1C
I. Infection with a viral recombinant containing two tag sequences within one genome is expected to result in RCs stained with both fluorophores (dually-labelled RCs) (Figure 1C example IV). Infection with two viral recombinants containing tag sequences at different genomic loci could result in progeny genomes that either contain both tag sequences on a single genome or contain neither tag sequence. One of two types of spatial patterns could be expected to dominate under these conditions. Dually-labelled RCs (fully covered by both probe sets) imply that recombination takes place early during infection before the viral DNA replicates and RCs mature. In this situation, the reciprocal recombinant genome would contain no tag sequences and will generate RCs that are not detected since they are not covered by any of the probes (Figure 1C example II). Alternatively, partially overlapping RCs that fuse to each other at their periphery, suggest that recombination occurs later during the infection cycle following viral DNA replication (Figure 1C example III). Therefore, this FISH assay is designed to detect viral recombination events, with visual readouts for when and where it takes place.

Patterns of RC interactions dependent on HR. To identify recombination events, we co-infected Vero cells with different pairs of viruses listed in Table 1, as detailed below. All infections were carried out at MOI 20, to increase the likelihood of interactions between incoming viral genomes (27). Cells were either infected with one virus that contained both mCherry and YPET tag sequences (OK31) or were co-infected with two viruses, each carrying a different tag sequence (OK35 together with either OK25, OK32 or OK26). First, we collected the progeny viruses released from the infected cells at 24 hours post infection (HPI) and plated for single plaques. When the fluorescent protein genes are at different sites in the viral genomes, we found that co-infections can result in dual color (yellow) plaques under our infection conditions.
(Figure 1D), suggesting that progeny viruses can be the outcome of HR events (27).

To visualize HR events at the single cell level, cells were co-infected and fixed at 6HPI for hybridization with the appropriate fluorescent probes. Using confocal fluorescent microscopy, we imaged the viral RCs and identified four distinct patterns of interactions between the RCs (Figure 2A-D). The first interaction type is of two RCs that come into close contact but without evidence of mixing between the two RCs i.e. no visible co-localized pixels and no intersection between RCs margins (Figure 2A). These interactions were mostly observed when the tag sequences were at the same genomic locus (OK25 and OK35, Figure 2E). The second interaction type is of two RCs that come into close contact and clearly mix, as defined by the presence of dual color pixels at the site of contact or blurring of the contacting edges (Figure 2B). This was the most common interaction observed in all co-infections, particularly when the two tag sequences were located further from each other on the viral genome (OK26 and OK35, Figure 2E). In the third interaction type, one RC seems to be fully overlapped with part of a second RC that is usually much larger (Figure 2C). This interaction was most prevalent with co-infection of the OK32 and OK35 viruses (Figure 2E). The last interaction is a dual color RC, in which the entire area of the RC contains both fluorophores (Figure 2D). The relative frequencies of these events can be quantitated during single or dual infections (Figure 2E). As expected, the overlapping pattern accounted for almost 90% of RCs in cells with single infection of virus carrying both tag sequences (OK31), and was rarely detected during co-infections. We deduce that our assay can be used to readout recombination, since detectable HR events change the overall distribution of the interactions among the RCs. In all co-infection assays, partial overlap at the point of contact are more frequent, compared to entirely overlapping RCs. The rare occurrence of entirely overlapping RCs compared to the
expected high frequency of HR during HSV-1 co-infection (31) suggest that these RCs are unlikely to reflect most of the HR events. We therefore suggest that inter-genomic HR occur at points of physical interaction between co-infecting genomes, after viral DNA replication has initiated.

We have previously shown that in U2OS cells, on average more HSV-1 genomes initiate expression per cell compared to infections of Vero cells (45). To test if the number of initiating genomes has an effect on the interactions between RCs, we repeated the FISH based experiments with infections of U2OS cells. We observed all four patterns of RCs interactions that we defined for infections of Vero cells (Figure 3). However, in U2OS cells, identifying more than one pattern in a single cell was more common than in Vero cells (Figure 3A and 3B), probably due to the higher number of RCs observed per cell in this cell type (see below, Figure 5A). The distribution of patterns observed for the different infections in U2OS cells (Figure 3D) was similar to the distribution detected in Vero cells (Figure 2E).

**Overlap between RCs is enhanced by HR.** Identifying patterns does not estimate the relative proportion of overlapping areas between RCs. Therefore, we developed a quantitative evaluation of the RC overlapping areas in each cell. We found that common pixel based co-localization methods do not provide an accurate estimate of the degree of overlap between HSV-1 RCs, probably due to the relaxed form of the replicating viral DNA (19). Similar to our results with infection of virus carrying the two tag sequences (OK31), two probes corresponding to adjacent loci on HSV-1 genomes showed limited co-localization at the pixel level (46). We applied a semi-automated object based method to quantify the degree of overlap for RCs in cells infected with viruses that have two distinct genomes (see Methods). For each cell nucleus, we independently segmented the RCs in each channel. The relative overlapping area was
calculated as the ratio between the overall area of the overlap and the total overall area occupied by viral DNA (total area of all RCs minus the total overlap area). The images analysed were collected from three infections carried out on different days from separate viral stocks. Over 200 cells from each cell type were analysed for each co-infection. The dual-tagged virus (OK31) had such a distinct and reproducible pattern, that only 120 Vero and 99 U2OS cells were analysed. We calculated the percentage of cells in which no overlap was identified (Figure 4A-B). Some of these cells resulted from RCs that were spatially distant, while others had interactions that did not result in any mixing between coalescing RCs. For both cell types, when the sequences are at the same site on the viral genome and therefore do not allow visualization of HR between RCs (OK25 and OK35, Blue columns), only about 25% of the cells had no measured colocalization. In U2OS cells, the possibility of HR reduced the number of cells without colocalization to ~8.5% (OK35 and either OK32 or OK26, yellow and orange columns respectively). In Vero cells, the possible visualization of HR had much lower effect, probably due to higher probability for no interactions between the RCs. These results suggest that when HR is possible to detect between co-infecting viruses, interactions between RCs are observed more frequently.

We analysed all the cells in which co-localization was observed. For both cell types we found a significant increase in the relative overlapping area for each nucleus for cells infected by viruses with tags in different loci (Figure 4C-D). The resolution of our FISH assay was not sufficient to detect significant differences between the two co-infections with viruses that have tags in different loci. To test that cellular parameters did not bias our measurements, we compared the relative overlapping area to the nuclear area (Figure 4E-F) or to the number of RCs per nucleus (not shown). We found no evidence of correlation between these two parameters to the relative overlapping
area in all infection conditions (Pearson correlation below 0.3 for each of the infections). We conclude that the observed significant increase in overlapping area indicates the readout of HR events. We speculate that the relative high background levels of overlapping areas (~20%) are due in part to noise of the assay and to non-HR events during viral replication.

**The number of RCs correlates with nuclear size.** The ability to distinguish between coalescing RCs in our assay provides an opportunity to quantitate the number of mature RCs within individual cells. We note that the OK31 infection was omitted from the calculation in this section due to our inability to distinguish between coalescing RCs in this infection. We therefore tested the association of the number of RCs to other parameters of infection. First, we observed that in U2OS cells more RCs were detected per individual nucleus. We detected an average of ~5.1 RCs per nucleus in U2OS cells compared to ~3.7 RCs per nucleus in Vero cells (1.375 fold increase, Figure 5A). Interestingly, a similar fold increase of ~1.38 was identified in nuclear area (Figure 5B). This led us to speculate the existence of a correlation between nuclear area and the number of RCs per nucleus. Indeed, the number of RCs correlates to the nuclear area for individual cells of both cell types (Pearson correlation: R > 0.538 for all infections, p < 0.0001 for all infections, Figure 5C-D). We hypothesize that the increase in the number of RCs per nucleus will result in a total of more viral DNA (RCs area) in larger nuclei. As expected, the nuclear area correlates to the total area of the RCs per nucleus (Pearson correlation: R = 0.530 for OK32 and OK35 co-infection on Vero and R > 0.625 for all other infections, p < 0.0001 for all infections, Figure 5E-F).

We speculated that the increase in total RCs area could also result from the possibility that RCs may expand faster to a larger size in larger nuclei. We therefore compared the mean RC area (per cell) to the nuclear area (Figure 5G-H). We found much weaker
correlation between these parameters (Pearson correlation: < 0.5 for U20S cells and < 0.3 for Vero cells in each of the infections). These results suggest that the increase in RC area in larger nuclei results from higher numbers of RCs rather than increased RC size. Taken together, our results suggest that nuclear size is a limiting factor for the number of incoming genomes that are able to initiate replication.

**Triple-color FISH indicates that inter-genomic recombination occurs at edge of coalescing RCs.** We speculated that if there are RCs that originate from a single recombinant genome and contain both tag sequences, there must be reciprocal recombinant RCs that contain none of the tags and are undetectable in our FISH assay (Figure 1C II). To determine whether these RCs exist, we designed an additional probe conjugated to a third fluorophore corresponding to the genomic HSV a’ sequence, a repetitive sequence found in four copies within the HSV-1 genome (47). This probe stains all HSV-1 viral DNA regardless of the tag sequence inserted. Both Vero and U2OS cells were co-infected with two isolates containing tags at different genomic loci, fixed and hybridized to all three probes as previously described. We inspected over 600 RCs from each cell type and found that 99.6% stained by the HSV non-specific probes reacted to either of the two specific probe sets (example in Figure 6). The existence of at least one tag sequence for all RCs suggests that RCs are not generated from recombinant genomes. This result further supports the hypothesis that recombination occurs at the edge of mature coalescing RCs at the later stages of infection and is coupled with replication. Similarly, all areas at the interaction between the mature RCs respond to both the specific probe sets in contrast to our prediction in Figure 1C III. This suggests that areas containing recombinant DNA do not arise from a single recombination event but rather from multiple events along the contact front of
replicating RCs. In other words, the overlapping areas contain a mixture of genomes with one tag, both or none of them.

**Discussion**

Recombination among co-infecting herpesviruses is fundamentally important for understanding viral evolution and pathogenesis (24). It is also important to consider recombination when developing vectors for vaccination and oncolytic viral therapy, since evidence for recombination between a vaccine strain and a wild type strain has already been found in herpesviruses (48). Here, we developed a unique experimental system to identify the spatiotemporal constraints of inter-genomic recombination. Our results corroborate the hypothesis that each RC initiates from a single incoming genome. We showed that the possibility to visualize HR increases the overlapping areas between RCs, although the experimental system also detects overlapping signals unrelated to HR. We found that most viral recombination events occur at the edges of developing RCs where they coalesce with others. Finally, we suggest that areas in which overlapping RCs are detected, result from multiple independent recombination events.

We have infected cells with different combination of viral isolates: OK35 & OK25 where tags are inserted into the same genomic locus, OK35 & OK32 containing tags on separate genomic loci spaced approximately 72 Kbp apart, OK35 & OK26 spaced approximately 105 Kbp apart and OK31 containing two tag sequences on a single viral genome. In our analysis, we have not detected any significant differences between the two combinations of the separate genomic loci (i.e. OK35 & OK32 and OK35 & OK26), in agreement with the hypothesis that recombination events occur between replicating genomes in either concatemeric or circular state (26).
We observed four recognizable patterns of interactions between adjacent RCs: i. no mixing, ii. partial mixing, iii. one within another and iv. complete overlap (Figure 2 and 3). From our previous results with PRV (18), we expected that the no mixing of RCs would be the dominant interaction following co-infection with two viruses carrying the tag sequences at the same location in the viral genomes (OK25 and OK35). We found that this readout is most commonly observed in this co-infection, although to our surprise the majority of RCs (~66%) showed some degree of mixing. Comparison with the images obtained in the Kobiler et al. paper suggested that there is no major difference between the images from the two alphaherpesviruses co-infection, although robust quantitative analysis of PRV images was not carried out.

The partial mixing of RCs was the most common interaction observed among the different co-infection conditions tested (Figure 2E). This is in part due to the categorization of all partial mixing interactions into one single pattern, regardless of the proportion of mixing. To overcome this problem, we developed an image analysis code that calculates the relative area of mixing. We found a significant increase in the relative area of mixing when co-infections were carried out with viruses in which HR can result in the mixing of the colors. These measurements were significant both at the single cell level (Figure 4C and D) and at the single RC level, indicating that a significant proportion of the mixing we observe is due to HR. On the other hand, even when HR cannot result in signal mixing (i.e. OK25 and OK35 co-infection), we observed RCs and cells in which high proportion of mixing occurred (Figure 4E and F). Similar findings were found in the PRV co-infection assays (18). These results suggest that some color mixing can occur without recombining both tag sequences into the same genome, or that there are high levels of non-HR that take place during viral replication. We note that non-HR events were observed previously both in HSV-
1 replication (49) and in other herpesviruses (50). These explanations are not mutually exclusive, and probably both contribute to the mixing of colors observed without HR.

The interaction in which one RC is fully mixed into a larger RC (Figure 2C) can be explained by different mechanisms. First, it could originate from a small RC that coalesces with a larger one. Second, the smaller RC could interact with two larger RCs from both sides of the small RC that eventually unite to a single larger RC. A third mechanism could involve the recently observed condensed viral genomes at the edge of RCs (19). Sekine et al. showed that at 3HPI several incoming genomes remain condensed at the periphery of an enlarging RC. These condensed genomes might be silent throughout the lytic infection or may serve as templates for recombination at later time points (which will result in our observed third interaction). Further experiments are required to distinguish between these different possibilities.

Our experimental system provides the opportunity to calculate accurately the number of functional RCs within a nucleus, since the separation by color increases our ability to differentiate between coalesced RCs. We were able to detect an increase in the average number of RCs detected in U2OS nuclei compared to Vero nuclei. This corroborates our previous finding that in U2OS cells on average a higher number of incoming viral genomes per cell initiate expression and replication compared to Vero cells (45). These observations also further support the emerging view that individual RCs initiate from single incoming genomes (18).

We previously demonstrated that the number of HSV-1 genomes replicating per cell can influence the outcome of infection (51) and that host conditions prior to infection can also affect the outcome of infection at the single cell level (51, 52). Here, we define correlations between the nuclear area and the number of detectible RCs, similar to the
correlation between cell size (as measured by flow cytometry) and the number of replicating genomes (51). Since our images represent snapshot during the infection process, we cannot distinguish between the possibilities that larger nuclei allow a larger number of incoming viral genomes to initiate replication or that the nuclei in which more genomes initiated replication, expand faster. The use of new methods to visualise incoming genomes using click chemistry (19, 53) should be able to resolve these two hypotheses. We note, that U2OS cells are known to have larger nuclei and indeed have more RCs per cell than Vero cells, suggesting that the larger nuclei support more incoming viral genomes. On the other hand, U2OS are known to be missing parts of the intrinsic immunity (54-56) and therefore that could explain the difference in number of genomes.

In conclusion, our results support a model in which incoming viral genomes establish RCs at distinct sites in the nucleus. The vast majority of RCs initiate from a single genome and only at later stages coalesce but maintain a detectable separation between RCs. These areas of interactions between the expanding RCs are predicted to be the site in which HR occurs in tight association with viral DNA replication. Our results suggest that in these sites, recombination is a frequent event, although not all areas of interactions between the RCs are supportive of close contact that can lead to recombination. Further research will shed light on the differences between these types of RC interactions.
Materials and methods

Cell culture

African green monkey kidney cells (Vero, ATCC CCL-81) and human female osteosarcoma cells (U2OS cells ATCC HTB-96) were grown in Dulbecco’s Modified Eagle Medium (DMEM X1; Gibco), supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 1% Penicillin (10,000 units/ml) and Streptomycin (10 mg/ml; Biological Industries, Israel).

Viruses

All viral recombinants are derivatives of herpes simplex type 1 strain 17. Each viral recombinant contains one or two tag sequences for specific staining by FISH. To facilitate isolation, both tag sequences are expression constructs for fluorescent proteins. The red fluorescent protein mCherry driven by the human cytomegalovirus promoter (CMVp) and the yellow fluorescent protein YPET driven by the simian virus 40 promoter (SV40p). Tag sequences were inserted into the viral genome by homologous recombination. Viral DNA was co-transfected along with a plasmid containing the tag sequences flanked by sequence homologies to the viral site of insertion (synthetically generated by GenScript, Piscataway, NJ, USA). Recombinant viruses were isolated from the progeny by plating lysate from transfected Vero cells and picking fluorescent plaques using an epi-fluorescent microscope. Viral stocks were prepared by growing purified plaques for each recombinant virus on Vero cells. The viral recombinants were validated by PCR. Viral titers were measured by plaque assay. An additional viral recombinant containing both tag sequences was isolated by crossing the recombinant OK26 to the previously described OK11 (57) and selecting
for plaques containing two fluorescent proteins by plaque assay. All viral recombinants constructed for this paper are described in Table 1

**Fluorescent probes**

A set of 20 fluorescent probes was designed to correspond to each one of the two tag sequences. The probes for the CMVp:mCherry and sequences SV40p:YPET were conjugated on their 5' end to the fluorescent dyes cy3 and cy5 respectively. An additional probe was designed to stain HSV1 viral DNA non-specifically. This probe corresponds to the viral a' sequence and conjugated to the fluorescent dye Alexa Fluor 488 on its 5' end. Fluorescent probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The probes were dissolved in TE buffer to a stock concentration of 10 μM. Probes from each set were pooled together in equal ratios and kept in -20°C before hybridization. Probes sequences are detailed in Table 2.

**Fluorescent in situ hybridization**

Cells were seeded and grown to confluence inside 12 well plates underlined with a glass coverslip. Cells were infected at MOI 20 with 200 μl inoculums. The cells where incubated on ice for 1 hour following the addition of the inoculums. The inoculums were then removed and replaced with DMEM+10%FBS. The infected cells were incubated on 37°C for 6 hours. Cells where then fixed, permeabilized and hybridized as in (58) with minor adjustments as follows: Denaturing of DNA was carried out in 95°C instead of 75°C for 2 minutes because of the relative high GC content of HSV1 DNA. Washing in SSCX0.1 was carried out in 63°C instead of 60°C. The fluorescent probes were diluted into the hybridization mix to final concentration of 75 nM for each probe set. Since the probes were directly labeled, all stages regarding antibody staining and post-fixation were omitted from the protocol. Coverslips where then
mounted to glass slides using Fluoroshield mounting medium containing DAPI (Abcam, Cambridge, MA, USA), sealed with nail polish and inspected under a confocal microscope.

**Microscopy**

Viral plaques were visualized under a Nikon Eclipse Ti-E epifluorescence inverted microscope. Single cell analysis by FISH assay was performed using a Nikon Eclipse Ti microscope equipped with Yokogawa CSU X-1 spinning disc confocal system.

**Image analysis**

Nuclei segmentation: Nuclei were automatically segmented from the DAPI channel as follows. Gaussian smoothing ($\sigma = 2$) was applied to the image followed by Otsu thresholding (59) to partition the image to nuclei / background regions. The nuclei segmentation was refined by morphological operators: opening (width = 3 pixels) to exclude small excesses; closing and an additional opening (width = 20 pixels) to unify regions that belong to the same nucleus; Filling holes; considering only connected regions of area exceeding 900 pixels - the minimal nucleus size. The output of this stage is a set of masks, each corresponds to a single nucleus.

RC segmentation: RCs were segmented from each fluorescent channel independently. Three groups of intensities were observed within each nucleus: background - at intensities close to those outside the nucleus; intermediate - above the background levels; and bright - which are the replication centers. First, Gaussian smoothing was applied ($\sigma = 2$) to the image. Second, background statistics were calculated (mean intensity, standard deviation) from the pixels outside the nuclei masks. A threshold of 2.5 standard deviations above the background mean intensity level was calculated. To exclude pixels with background intensities from further analysis, the threshold was
applied for each nucleus mask. Otsu thresholding was then applied to the remaining pixels, pixels below the threshold were pooled and a new threshold of two standard deviations above the mean was calculated. The pixels above these thresholds were defined as the RCs. The segmentation refined by applying morphological operators: opening - excluding small excess, closing - filling gaps, and opening again - disconnecting independent replication centers; all with a square kernel of 3 pixels width. Last, were holes filled to define the RCs. Parameters were optimized by visual assessment of RC segmentation accuracy.

Manual selection of nuclei: Region for statistical analysis were manually selected based on the accurate segmentation of RCs. Regions of accurate RC segmentation were selected independently for each channel, the intersection (implying accurate segmentation on both channels) was used for quantifications. The program outputs required measurements such as nuclei area, number and area of RCs in each channel of each nucleus and the size of overlapping area for each RC. The data were then transferred into Microsoft excel for subsequent processing and statistical analysis.

Code availability: The Matlab source code used for analysis is publically available at https://github.com/assafzar/RecombinationHSV1.

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Figure 1. Schematic representation of dual colour FISH assay. (A) A series of viral recombinants with two unique tag sequences (designated red and green) inserted into various loci of the parental genome. Illustration of the insertion sequences (colour boxes) and viral genomes (black line with repeats marked in gray boxes). Illustrations are not to scale. (B) Two sets of probes conjugated to distinct fluorophores, each set corresponds to one tag sequence. (C) Illustration of expected results, in which the gray nucleus contains viral RCs originating from genomes with one tag sequences (either red or green), two tag sequences (yellow) or no tag sequence (black). The expected results in case of infection with tag sequences on the same locus of two co-infecting genomes (I), with tag sequences on separate loci in two co-infected genomes where recombination takes place before DNA replication (II) or following DNA replication (III) and with two tag sequences in the same genome (IV). (D) Representative images of viral plaques initiating from progeny viruses collected from Vero cells co-infected with different viral recombinants as marked above each image. Scale bar is 100 µm.

Figure 2. Observed patterns of RC interactions. (A-D) Representative images of Vero cells infected with viral isolates containing two genomic tags, each corresponding to a set of fluorescent probes. The probe sets are imaged separately in red and green (I and II) and merged (III). Four observed patterns of interaction between RCs are represented: (A) RCs containing different tag sequences come into contact without overlap (orange arrowhead), imaged from cells infected by two viral recombinants containing tag sequences on the same genomic locus (OK25 and OK35). (B) RCs containing different tag sequences overlap at their periphery (white arrowhead), imaged from cells infected with two viral recombinants containing tag sequences on separate genomic loci (OK32 and OK35). (C) RC containing one tag sequence overlap completely within a larger RC with the second tag (black arrowhead) imaged from cells infected with two viral recombinants containing tag sequences on separate genomic loci (OK26 and OK35). (D) Entirely overlapping RCs, imaged from cells infected by a viral recombinant containing two tag sequences on the same genome in separate loci (OK31). DAPI nuclear stain is presented in gray. Scale bar is 5µm. (E) Percent of each of the observed RCs interactions out of total RCs interactions. Manually counted at the different co-infection conditions. >50 cells and >100 RCs interactions per infection type were counted.

Figure 3. Patterns of RC interactions on U2OS cells. (A-C) Representative images of U2OS cells infected with either (A) OK25 and OK35, (B) OK32 and OK35 or (C) OK31 viral recombinants. The probe sets are imaged separately in red and green (I and II) and merged (III). Arrowheads are colour coded as in figure 2. DAPI is presented in gray. Scale bar is 5µm. (D) Percent of each of the observed RCs interactions out of total RCs interactions. Manually counted at the different co-infection conditions. >50 cells and >100 RCs interactions per infection type were counted.
Figure 4. HR enhances overlap between viral genomes. Vero cells (A, C, E) and U2OS cells (B, D, F) were infected with the viral isolates OK35 & OK25 where tags are inserted into the same genomic locus (blue), the viral isolates OK35 & OK32 (yellow) or OK35 & OK26 (orange) containing tags on separate genomic loci and the viral isolate OK31 containing two tag sequences on a single viral genome (gray). (A and B) Each column represents the percentage of cells showing no overlap out of the total number of cell analysed for each infection condition. *P < 0.05, ***P < 0.001; by Z.score proportion test. (C and D) Each column represents the average relative overlapping area calculated from all cells (with detectable overlap) infected under the same condition. Error bars represent standard error of the means. *P < 0.05, ** P < 0.01; ***P < 0.001; by t test. (E and F) Individual cells were plotted to compare the relative overlapping area to the nuclear area. All analyses were conducted on images generated from three independent experimental repeats done on different days with viral stocks prepared and tittered separately.

Figure 5. Nuclear size correlates with the number of RCs per cell. Cells where co-infected with the viral recombinants OK35 & OK25 (blue), OK35 & OK32 (yellow) and OK35 & OK26 (orange). Comparing the mean number of RCs per cell (A) and the mean nuclear area (B) between Vero (bright coloured columns) and U2OS (dark coloured columns). Error bars represent standard error of the means. (C-H) Individual Vero (C, E, G) and U2OS (D, F, H) cells were plotted to compare the number of RCs per cells (C, D), the total area of RCs per cell (E, F) and the average area of each RCs (G, H) to the nuclear area. A trend line (colour coded as above) was calculated using the ordinary least squares (OLS) method is presented for each infection condition. All analyses were conducted on images generated from three independent experimental repeats done on different days with viral stocks prepared and tittered separately.

Figure 6. Replication compartments contain at least one tag sequence. Representative images of U2OS cells infected with two viral isolates containing tag sequences at different genomic loci (OK35 and OK32). The cells were hybridized with two probes conjugated to three distinct fluorophores. I. Detection of the OK35 tag sequence is shown in red. II. Detection of the OK32 tag sequence is shown in green. III. Detection of the viral ‘a’ sequence (detect HSV DNA non-specifically) is shown in blue. Overlays of two and three colours are shown in panels IV and V, respectively. DAPI is presented in gray. Scale bar is 5 µm.
| Viral recombinant | Tag sequence | Locus of insertion | Insertion start point |
|-------------------|--------------|--------------------|-----------------------|
| OK25              | SV40p:YPET   | Between the ORFs UL3 and UL4 | 11736                |
| OK26              | SV40p:YPET   | Between the ORFs UL55 and UL56 | 116153               |
| OK32              | SV40p:YPET   | Between the ORFs UL37 and UL38 | 84252                |
| OK31              | SV40p:YPET   | Between the ORFs UL55 and UL56 | 116153               |
| OK31              | CMVp:mCherry | Between the ORFs UL37 and UL38 | 84252                |
| OK35              | CMVp:mCherry | Between the ORFs UL37 and UL38 | 11736                |

Table 1. List of viral recombinants used in this work
| SV40p:YPET | CMVp:mCherry | HSV-1 a’ sequence |
|------------|-------------|------------------|
| CTTGCATCTCAATTAGTCAGCAACC | CTTAAGCCCATATATGGAGTTCCGC | CCCCGGCTCTCCCCCCCGCT |
| GCTACGGCCTGCACTGCTTCGCCAG | TAACTTACGGTAATGGCCCGGCTTG | |
| TCCGCCCATCTCCTGCCCATCGCT | AAGCACCCTGCCCATGGACGTCAT | |
| ATTTATGCAAGGCGAGGCGCCCT | GACGTATGTTCCCATAGTAACGCA | |
| CCTCTGAGCTATTCCAGAAGTGAGGAG | GACCTTCCATTGACGTCAATGGGTG | |
| GAGGCTAGGCTTTTGCAAAAGC | CGTAAACTGCCCACCTTGGCAGTAC | |
| GACACAACAGTCGTAATTAGGCTAG | ATGGCGGTAGAGCGGGTTTGAACGTG | |
| CTGTCACCAGGAGTGGTCCTATCC | CCAAAATGTCAAACTCCGCC | |
| CGACGCTACCTACGGAAGCTGAACC | ATGGGCCGTAGAGCGGGTTTGAACGTG | |
| GTGCTTGCGCCACCCCTTGTGACCA | GCAGAGCTGGTTTAGTGAACGTG | |
| ACCCATGAAAGCGACGACTTTCTT | GTAACATGGCCCTCATCAAGGAG | |
| AACATCTGGGCCACAAGCTGGAGT | GTGGATACCGGGTTTGTACAGCAG | |
| CATCACCAGCGACAAGCAGAAGAA | GTCCCTCAGTTTGACGTACGGCTC | |
| CCCATCGGCGACGGTTCCGCCTGC | GTGGTACCAGCCTGGACGAAGACCTCTC | |
| CCTGACAACACTACCTGAGCTACCAG | GGACGGCGATTCTCAATACAGGGT | |
| TTCAAGGACCCAACGAAAGCGGG | CCCGTATGCAGAAGAGCAATGG | |
| CAGACTGTGGCTTCTAGTGACCAG | CCTGAGGCGGATGTAAGCCCGAGG | |
| GACCTTGGAGGCTGCACCTCCACT | CGGCGGCTAACAGTAAACATCAAG | |
| GAAATGCTGGCATTGTGCTGAGT | GTTCCACCCCATGACGTCAATGG | |
| GGAAGACAATAGGCGAGCAGTGCCTG | CATGACTATACATGGTACGG | |

**Table 2.** List of fluorescently labelled probes for FISH.
Figure 1
Figure 2

|   | A - minimal overlap | B - periphery overlap | C - Full overlap within larger RC | D - Full overlap |
|---|----------------------|------------------------|-----------------------------------|-----------------|
| OK25*OK35 | 33.0                 | 60.4                   | 6.6                               | 0               |
| OK32*OK35 | 28.7                 | 50.4                   | 15.7                              | 5.2             |
| OK26*OK35 | 18.4                 | 71.1                   | 8.8                               | 1.8             |
| OK31     | 0                    | 6.4                    | 5.7                               | 87.9            |
### Figure 3

**A** – minimal overlap

**B** – periphery overlap

**C** – Full overlap within larger RC

**D** – Full overlap

|       | A - minimal overlap | B - periphery overlap | C - Full overlap within larger RC | D - Full overlap |
|-------|---------------------|-----------------------|-----------------------------------|------------------|
| OK25*OK35 | 36.0               | 59.6                 | 4.4                              | 0                |
| OK32*OK35  | 16.5               | 73.3                 | 9.7                              | 0.6              |
| OK26*OK35  | 23.5               | 69.9                 | 4.6                              | 2.0              |
| OK31       | 0.6                | 8.0                  | 4.0                              | 87.4             |
Figure 4
Figure 5
