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Abstract

Human papillomaviruses (HPV) are small, non-enveloped DNA viruses, which upon chronic infection can provoke cervical and head-and-neck cancers. Although the infectious life cycle of HPV has been studied and a vaccine is available for the most prevalent cancer-causing HPV types, there are no antiviral agents to treat infected patients. Hence, there is need for novel therapeutic entry points and a means to identify them. In this work, we have used high-content microscopy to quantitatively investigate the early phase of HPV infection. Human cervical cancer cells and immortalized keratinocytes were exposed to pseudoviruses (PsV) of the widespread HPV type 16, in which the viral genome was replaced by a pseudogenome encoding a fluorescent protein as reporter for successful infection. Using the fluorescent signal as readout, we measured differences in infection between cell lines, which directly correlated with host cell proliferation rate. Parallel multiparametric analysis of nuclear organization revealed that HPV PsV infection alters nuclear organisation and inflates promyelocytic leukemia protein body content, positioning these events at the early stage of HPV infection, upstream of viral replication. Time-resolved analysis revealed a marked heterogeneity in infection kinetics even between two daughter cells, which we attribute to differences in viral load. Consistent with the requirement for mitotic nuclear envelope breakdown, pharmacological inhibition of the cell cycle dramatically blunted infection efficiency. Thus, by systematic image-based single cell analysis, we revealed phenotypic alterations that accompany HPV PsV infection in individual cells, and which may be relevant for therapeutic drug screens.

Key words

human papillomavirus, pseudovirus, infection kinetics, high-throughput microscopy, live cell imaging, cell cycle, nucleus

Introduction

Papillomaviridae represent a large family of small non-enveloped DNA viruses. Several human papillomavirus (HPV) types are described as the primary cause of anogenital cancers and a growing number of tumors of the head and neck in humans (1). HPV16, the most prevalent and best-studied cancer-causing type, is the primary etiological agent of cervical cancer (1). The structure of HPV particles are spherical icosahedrons (T=7) with a diameter of 50-55 nm. The viral capsid is built up of 72 homo-pentameric capsomers of the major structural protein L1 and a variable number (12-72 copies) of the minor structural protein L2. This capsid surrounds the 8-kb circular double-stranded viral DNA (vDNA) (2).

As most DNA viruses, HPV requires access to the host cell nucleus for its replication (3). To do so, it will first enter and become transported in the cytoplasm (4). Viruses exploit different mechanisms to enter the nucleus, *e.g.,* by passing through nuclear pore complexes or by temporarily perturbing nuclear envelope (NE) integrity (5). In contrast with these active mechanisms, HPV has been shown to await mitotic nuclear envelope breakdown (NEBD) to gain access. A large-scale RNA interference (RNAi) screen uncovered a cluster of mitotic regulators as major drivers for HPV infection (6). This was further supported by experiments showing that pharmacological inhibition of cell cycle progression prevented HPV infection (6, 7). The current view is that after cellular entry, HPV will interact with cytosolic motor proteins to become transported to the trans-Golgi network, where it will reside until the onset of mitosis (4, 8). During mitosis, HPV will enter the nuclear space within a transport vesicle that will be disassembled several h after NE reformation by unknown mechanisms (9, 10). Inside the nucleus, the viral protein L2 and the vDNA colocalize within promyelocytic leukemia protein (PML) nuclear bodies (NBs) (11, 12). In contrast with other DNA viruses that dissolve PML NBs after entry, HPV demands intact PML NBs for an efficient replication and transcription (11, 13). It is assumed that the association of viral genomes with PML NBs

conceals them from innate immune sensors (13). Since mitosis leads to restructuring of PML NB components PML and Sp100, early mitotic events may be critical for vDNA localization in these domains and for initiating HPV viral transcription (7, 11, 12, 14).

Currently available vaccines provide up to 90% protection towards HPV infection with cancer-causing types HPV16 and HPV18, but also HPV6 and HPV11. However, vaccines are not equally accessible in all regions of the world and they are not designed for eliminating pre-existing infections and associated preinvasive lesions, leading to a persistence of this virus worldwide (15, 16). The development of therapeutic agents targeting active or latent infections is needed but has been so far very slow. And, despite promising preclinical results, clinical efficacy remains low (15). Identification of promising lead agents demands assays that report on efficiency and target specificity with high throughput. For their replication, papillomaviruses depend on the differentiation of keratinocytes in stratified epithelia (17, 18). This complicates their production and study. To resolve this, recombinant viral particles such as pseudoviruses (PsV) have been conceived that are highly immunogenic and have an identical capsid composition as the authentic wild type HPV but lack the replicative part of the vDNA (17–19). HPV PsV are primarily used for analyzing neutralization or infectious entry pathways, but can also be used as gene delivery vehicle (19, 20). Here, we have exploited HPV PsV harbouring a fluorescent reporter (EGFP) to monitor early infection kinetics in human cells. We show that automated microscopy allows accurate monitoring of infection kinetics, whilst providing spatial information that is not accessible by other means.

Materials and methods

Cell culture

HeLa cells (ATCC) and HaCaT cells (DKFZ, German Cancer Research Center, Heidelberg, Germany (21)) were cultured in DMEM high glucose with L-glutamine with and without pyruvate, respectively (Gibco, 41966-029 and Gibco 41965-039), supplemented with 10% fetal bovine serum (Gibco, 10270-106) and 1% penicillin/streptomycin (Gibco, 15140-122), according to standard procedures. For the HPV16 PsV production, 293TT cells (National Cancer Institute, Rockville, USA) were cultured in high glucose DMEM with L-glutamin and pyruvate (Gibco, 41966-029) supplemented with 400 µg/ml Hygromycin B (Merck-Millipore, 400052) and 10% fetal bovine serum (FBS). Proliferative capacity was monitored by cell counting with every passage and cultures were tested regularly for the most common mycoplasma infections using a PCR test kit (Bio-connect, PK-CA91-1024). For starvation, cells were switched to medium containing 0.1% FBS.

HPV PsV production

HPV16 PsV were produced by co-transfecting 293TT cells with p16SheLL and pClneo-EGFP (or p8RwB) plasmids (Addgene plasmid #37320, plasmid #46949 and plasmid #48733, all a kind gift of Dr. John Schiller) (19, 22, 23), as described earlier (17). 293TT cells were plated in 144 mm plates at a density of 1.2×10^7 cells the day before transfection. Lipofectamine 2000 (Invitrogen, 11668019) was used for the transfection. Five h after the transfection, the medium was replaced by fresh medium. For pseudogenome labeling experiments, the growth medium was supplemented with 20 µM 5-ethylnyl-2'-deoxyuridine (EdU). 48 h post-transfection, cells were trypsinized and harvested along with the culture medium. Cells were centrifuged to form a cell pellet, after which the cell pellet was resuspended in PBS/9.5 mM MgCl2 and centrifuged. Again, the cell pellet was resuspended in PBS/9.5 mM MgCl2 but

now 0.35% Brij-58 (Sigma, P5884), 0.2% Benzonase (Merck-Milipore, 1016950001) and 0.2% DNase (Epicentre, E3101K) and 25 mM ammonium sulfate were added to lyse the cells. This mixture was incubated at 37°C in the incubator for 24 h on a rotator, for the capsids to mature. After 24 h incubation, the cell lysate was put on ice, and 0.17 volumes of NaCl were added. After centrifugation, the supernatant was collected in a low-binding tube and stored on ice. Virion buffer was added to resuspend the remaining pellet, where the supernatant, after centrifugation, also was added to the collected supernatant. For the purification, the supernatant was loaded on top of a 25% and 39% continuous iodixanol gradient (Optiprep: PROGEN Biotechnik, 1114542) in quickseal polyallomer centrifugation tubes (Beckman, 355870). Afterwards, ultracentrifugation of the gradients was carried out for 6.5 h at 41.000 RPM and 16°C (Beckman, SW41 rotor). Approximately 12 x 200 µl fractions were collected after ultracentrifugation by puncturing the bottom of the tubes.

Fractions were subjected to SDS-PAGE (NuPAGE™ Novex™ 4–12% Bis-Tris Protein Gels with MOPS running buffer, Thermo Scientific, J00047) and stained with Coomassie Brilliant Blue (Bio-Rad, 161-0400). To detect protein bands, the Coomassie stained protein gels were destained with 10% acetic acid. Protein concentration of these fractions was measured with the Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23227). Additionally, an infection test was performed by incubating HeLa cells with different concentrations of the purified HPV PsV. Infection rates were then determined by flow cytometry. Viral titers were expressed in terms of viral genome equivalents (VGE) and were determined based on the protocol of Biryukov (24). vDNA was extracted from capsids by adding Hirt DNA extraction buffer, proteinase K (Thermo Scientific, AM2548) and SDS buffer and incubated for 2 h at 37°C on a rotator. DNA was purified with the DNA Clean & Concentrator kit (Zymo Research, D4013) and the absolute number of VGE was quantified by qPCR using a standard curve that was established using a dilution series of the reporter plasmid pClneo-EGFP and

146 primers targeting the EGFP sequence: forward: 5'–ATGGTCCTGCTGGAGTTCG–3' and
147 reverse: 5'– TCATCAATGTATCTTATCATGTCTG– 3'.
148
149 *Compound treatments*
150 To block cell cycle progression, cells were treated with different pharmacological
151 compounds. To arrest cells throughout the S-phase, a double thymidine block was applied by
152 exposing cells twice to 1 mM of thymidine (Sigma, T1895) for 18 h, separated by 9 h of
153 growth in normal medium (25). As an alternative approach to block cells in early S-phase,
154 cells were incubated with 1 µg/ml of aphidicolin for 24 h (Sigma, A0781). As a more broad-
155 spectrum cell cycle blocker, mitomycin C was used (Sigma, M4287) at the lowest
156 concentration of 0.02 µg/ml for 24 h, to arrest cells in S-phase and at the G2/M border.
157
158 *Transfection*
159 As control for HPV PsV experiments, cells were transfected with the constituent pClneo-
160 EGFP plasmid (Addgene #46949) using Lipofectamine 2000 (Life Technologies, 11668027)
161 according to the manufacturer's instructions. For live cell imaging of PML NBs, cells were
162 lipofected with an expression plasmid encoding EYFP-PML under control of a
163 cytomegalovirus (CMV) promotor (gift from Dr. J Wiegant, University of Leiden, the
164 Netherlands).
165
166 *HPV PsV infection studies*
167 Cells were seeded 24 h before infection at 5×10^4 cells/well in 12-well glass bottom plates
168 (Cellvis, P12-1.5H-N), 2×10^4 cells/compartment in 4-well glass-bottom dishes
169 (CELLview™, Greiner) or 5×10^3 cells/well in 96-well µClear plates (Greiner Bio-One,
170 655090), and infected with HPV16 EGFP PsV at a MOI of 100 - 400 VGE/cell (also referred

to as VGE_{MID}), unless stated differently. The cells were incubated with the PsV for 2 h on a shaker at 37°C. Afterwards the medium of the cells was removed, and new medium was added. For live cell imaging, HPV PsV was added just prior to imaging and medium was left untouched throughout the imaging procedure.

Immunofluorescence staining

For HPV infection studies, cells were fixed with 4% paraformaldehyde (freshly made) for 25 min followed by 3 x 5 min wash step with PBS (Life technologies, 14190-169). After permeabilization in 0.3% Triton X-100 (Sigma, X100-500ml), cells were washed again 3 x 5 min with PBS before staining with DAPI (1 µg/ml) for 15 min. Plates were kept in PBS-NaN_3 at 4°C pending microscopic imaging. For cell cycle arrest, the protocol was similar as above, but cells were only stained with DAPI. For PML detection, cells were blocked in 50% FBS after 30 min permeabilization and a rabbit anti-PML antibody (Santa Cruz, sc5621, 1/250) was added for one hour. After 3 x 5 min wash step with PBS, a secondary CY3-labeled donkey anti-rabbit antibody (Jackson, 711-165-152,1/600) was added for 30 min. Afterwards, cells were washed again 3 x 5 min with PBS and stained with DAPI similar as above. Click-iT EdU Alexa Fluor™ 647 imaging kit (Invitrogen, C10340) was used to visualize EdU-labeled pseudogenomes. Cells were incubated for 30 min at room temperature with the Click-iT reagent after blocking and before the primary antibody was added.

Automated widefield microscopy

Cells were seeded in 12-well glass bottom plates or 96-well µClear plates. When using the latter, the outer wells were excluded to avoid edge effects (leaving the inner 60 wells). Imaging was performed on a fully automated Nikon Ti Eclipse inverted widefield fluorescence microscope, equipped with a Perfect Focus System and LED-based illumination

source. For the HPV infection study, at least three wells were used as technical replicates for a given condition. Per well, 32 regions were acquired using a 10×/0.30 Plan Fluor dry lens or 24 regions when using a 20×/0.75 Plan Fluor dry lens. Illumination was optimized to guarantee minimal intensity variation (<5%) in the field of view, and we verified that the randomized acquisition of large image data sets levelled out any putative remaining bias. 405 nm, 488 nm and 561 nm LED illumination was used for excitation of DAPI, EGFP and CY3, respectively. Detection was done through a quadruple dichroic using 395/25 nm, 470/24 nm and 555/25 nm band pass filter, respectively, with a DS-Qi2 CMOS camera.

Super-resolution by optical realignment confocal microscopy

To define co-localization of EdU spots with PML spots in EdU labeled HPV PsV infected HeLa cells, images were made with a Nikon CSU-W1-01 SoRa spinning disk confocal microscope, mounted on a Nikon Ti Eclipse body, equipped with a Perfect Focus System. Images were made using a 60×/1.20 Plan Apo water lens. 640nm (excitation of EdU), 561 nm (excitation of PML) and 405 nm (excitation for DAPI) diode lasers and 447/60 nm, 640/75 nm and 692/40 nm bandpass emission filters were used. Images were acquired with a Kinetix sCMOS camera.

Live cell imaging

For live cell imaging, cells were seeded in 4-well glass-bottom dishes 24 h before imaging. Two h prior to imaging, cells were stained with Silicon-Red Hoechst (SiR-DNA, Spirochrome, sc007) after which medium was replenished. Time-lapse imaging was performed on a Perkin Elmer Ultraview Vox dual spinning disk confocal microscope, mounted on a Nikon Ti body, equipped with a Perfect Focus System II and a microscope incubator equilibrated at 37°C and 5% CO_2. Recordings were made every 10 min, using a

20×/0.75 Plan Achromat dry lens. 640 nm (excitation of SiR-DNA), 561 nm (excitation of RFP) and 488 nm (excitation of EGFP) diode lasers and 525/50 nm and 705/90 nm bandpass emission filters were used. Detection was done on a Hamamatsu C9100-50 camera. Image acquisition was done using the Volocity software. Per well, 10 fields of view were monitored and used as technical replicates, meaning that 40 different regions were imaged every 10 min and this for 48 h.

Flow Cytometry

For flow cytometry analysis of HPV PsV infection, cells were trypsinized and resuspended in PBS, after which they were fixed in freshly made 4% paraformaldehyde for 20 min at room temperature in the dark. After centrifugation and washing, cells were resuspended in PBS with 1% EDTA and 1% FBS for flow cytometry analysis using the CytoFLEX Flow Cytometer (Beckman Coulter). GFP was excited using a 488 nm laser and emission was detected using the 525/40 filter. Control cells were included to set the correct gates for removing doubles and debris (Suppl. Fig. S1). FlowJo (BD Biosciences) was used to export the data to CSV files, which were further analyzed with R.

Image analysis

Image analysis was performed in FIJI image analysis freeware (26). An in-house developed image analysis pipeline (https://github.com/DeVosLab/CellBlocks) was used to detect nuclei in HTM and live cell imaging in the nuclear counterstain channel (DAPI or SiR-DNA) (27–29). To quantify morphological (shape features) and textural (Haralick features derived from the Gray Level Co-occurrence Matrix (30)) characteristics of individual nuclei, their regions of interest were first detected using a trained convolutional neural network as implemented in

the StarDist plugin. A Laplacian operator was used to selectively enhance spots in the PML channel, prior to their detection with a user-defined threshold, which was set per cell type.

For tracking cells through time, we made use of the TrackMate plugin (version 6) (31). Since at the time of this work, nuclei detection within TrackMate was less tailored towards dense and overlapping nuclei, we pre-processed the images by exploiting StarDist-mediated nucleus detection (27) and conversion of the detected nuclear ROI into indexed spots of 2 µm radius. Within TrackMate, we limited the linking distance during tracking to 20 µm, allowed gap closing of max. 20 µm over 2 frames and allowed track splitting within a distance of 20 µm. The combination of StarDist-mediated nuclear segmentation and TrackMate-based tracking improved tracking accuracy and allowed relating nuclear morphotextural changes to infection status and lineage (https://github.com/VerschuurenM/StarTrack).

Data analysis

Data analysis and visualization was done in RStudio (32). All plots were made using the ggplot2 package. In point plots, both independent experimental replicates and technical replicates are shown to illustrate the underlying variability, unless stated otherwise. But the difference between both replicate types was taken into account for statistical analysis. The exact p-values of all statistical tests can be found in Suppl. Table 1. In HTM experiments, infected cells were identified by setting a user-defined intensity threshold on the raw EGFP intensity or the EGFFP intensity normalized to the average of control cells per independent experimental replicate (Suppl. Fig. S2). Based on this, the ratio of infected cells to the total number of cells was calculated. Cell cycle analyses were based on the integrated DAPI intensity which was normalized to the average of control cells per biological replicate. To determine the time- and concentration dependency of HPV PsV infection within HeLa and HaCaT cells, a linear mixed effects model with independent experimental replicate as a

random factor and technical replicate (well for fixed assays, field for live cell imaging) as nested factor was used (*lme4::lmer*) combined with the Tukey method to control the family-wise error during multiple comparisons. Similar statistical models were used for comparing nuclear morphotextural metrics, alterations in cell cycle profiles and PML occupancy within each cell type. Principal component analysis was done using 17 nuclear morphotextural descriptors (area, perimeter, circularity, roundness, solidity, minimum diameter, maximum diameter, aspect ratio, inverse difference moment, contrast, energy, entropy, homogeneity, variance, shade, prominence and correlation). A student T-test was used to compare the doubling time of HeLa and HaCaT cells, after having validated requirements for normality and homoscedasticity. A non-parametric Kruskal Wallis test was used to compare the differences in mitotic window time between the first and second mitosis of EGFP-positive and -negative HeLa cells. Pairwise post-hoc tests were done using Benjamini-Hochberg correction. Non-parametric Kruskal Wallis test was also used to compare the average coefficient of variance between synced and scaled intensities over time. For live cell imaging, raw EGFP intensities were normalized by subtracting the average EGFP intensity in non-infected (CTRL) cells in each frame and replicate. Infected cells were identified as cells that had a normalized nuclear EGFP intensity higher than 5 times the standard deviation of normalized nuclear EGFP intensities in CTRL cells. Only cells that displayed an EGFP signal above threshold for more than 60 min were retained. An estimation of the number of apoptotic cells per frame was based on user defined area, intensity and circularity thresholds that needed to be attained for more than 60 min. To quantify HPV PsV infection kinetics in cells for a defined time period after infection, only cells were tracked that could be identified in the first time point. A logistic function (Equation 1) (33) was fitted to the normalized nuclear EGFP intensities over time using the Levenberg-Marquardt algorithm implemented in the *forestmangr:nls_table* function. The estimated parameters were used to quantify infection

kinetics (normalized EGFP intensity), with a the maximal intensity, b the steepness of the curve and c the time at the inflection point of the curve.

$$Norm\, I_{EGFP} = \frac{a}{1+e^{-b(t-c)}} \quad (1)$$

Results

High-throughput microscopy reveals cell type-dependent HPV PsV infection kinetics

The premise of this work was that microscopy could serve to automatically quantify the HPV infection ratio and the modulation thereof in adherent human cells with high throughput and spatiotemporal resolution. To test this, we incubated cells with HPV PsV containing a pseudogenome that encodes an EGFP reporter under the control of the CMV early promoter for a direct fluorescent readout. We infected two cell types with relevance for HPV pathology, namely HeLa human cervix carcinoma cells (a common transformed cell model) and HaCaT human keratinocytes (natural HPV host cells) and quantified the number of EGFP-positive cells with automated widefield microscopy (Fig. 1a). Cell counting revealed that HaCaT cells showed a significantly longer population doubling time than HeLa cells (Fig. 1b). We evaluated the relative fraction of EGFP-positive cells (infection ratio) 48 h post-infection (PI) with different HPV PsV VGE/cell (10 – 40 VGE/cell (VGE_{LOW}), 100 – 400 VGE/cell (VGE_{MID}), and 1000 – 4000 VGE/cell (VGE_{HIGH})). A consistent, titer-dependent increase in infection ratio was measured with microscopy for both cell types, which correlated well with that measured by flow cytometry (Fig. 1c, d). The absolute infection ratio values differed between cell lines, which may be due to differences in their metabolism and proliferation rate. To obtain a better view on the kinetics, we next performed an experiment in which we scored the infection ratio as a function of time up to 96 h PI. For both cell lines we found the infection ratio to level at 48 h PI using both microscopy and flow cytometry (Fig. 1e, f), indicating that the difference between both cell types is mainly determined by the early phase of infection – befitting the non-replicative nature of the PsV. Note that for microscopy, we

deliberately applied a stringent gating strategy, which excluded cells with very faint EGFP signal but prevented autofluorescent debris from introducing false positives. HaCaT cells displayed higher background signal in the green channel than HeLa cells, resulting in a slightly increased (> 0) infection ratio in the negative control condition. Based on our analyses, we conclude that automated microscopy allows sensitive detection of time- and titer-dependent infection ratios and reveals differences between cell types, whilst allowing spatial validation.

HPV PsV infection alters nuclear shape and inflates nuclear PML body content

The major asset of microscopy versus flow cytometry is that it can be used to simultaneously measure infection status and phenotypic information of individual cells. We exploited this advantage to query nuclear morphological and textural changes in both cell types. A biplot of a principal component analysis on 17 morphotextural features revealed a clear separation between both cell types and cell-type dependent differences in infection status (Fig. 2a). Interestingly, within the population of cells that was infected with HPV PsV, we also noted marked cell-type dependent differences between the ones that were EGFP-positive and those that were EGFP-negative. For example, EGFP-positive HaCaT cells (+HPV PsV EGFP+) displayed a significantly decreased nuclear area and circularity (Fig. 2a). In addition, a significant decrease in homogeneity and concomitant increase in contrast were observed for both cell types, suggesting an altered chromatin condensation state in EGFP-positive HPV PsV infected cells (+HPV PsV EGFP+) (34). Reasoning that this might reflect a change in cell cycle, we measured the cell cycle phase distribution of both cell types using the integrated DAPI intensity as a proxy for DNA content (Fig. 2b). We found a modest, but significant increase (1.22 and 1.12 fold change for HeLa and HaCaT cells, respectively) in the proportion

of cells in G2/M phase and concomitant decrease in the G1 phase fraction compared to EGFP-negative cells (+HPV PsV EGFP-).

To verify whether the morphotextural changes were driven by the overexpression of EGFP, we analysed the same metrics after lipofection of the cells with the constituent pClneo-EGFP reporter plasmid. In contrast with cells treated with HPV PsV, the EGFP signal of lipofected cells showed a significantly weaker linear relationship and correlation with the selected metrics (Suppl. Fig. S3). Although the EGFP intensity range was larger for cells transfected with pClneo-EGFP and we cannot exclude side effects of the lipofection reagent, these results suggest that the observed nuclear reorganization is more likely associated with another aspect of HPV PsV infection than sheer EGFP expression.

PML protein is crucial for HPV infection as it has been reported to surround the viral genome (10, 13, 35). We wondered whether HPV PsV would induce the same cellular reflex, and therefore quantified the number of PML NBs 24 h and 48 h PI by staining for PML protein. As we have only stained for one constituent protein of PML NBs, we will use the looser term PML foci. We found that the relative fraction of the nuclear area that was occupied by PML foci (*i.e.,* the PML spot occupancy) at 24 h and 48 h was significantly increased in EGFP-positive cells (+HPV PsV EGFP+) compared to EGFP-negative (+HPV PsV EGFP-) and non-infected cells (-HPV PsV) for both cell types, suggesting an all-over increase in nuclear PML content (Fig. 3a). Live cell imaging of HeLa cells expressing EYFP-PML confirmed this accrual of PML foci in HPV PsV-RFP infected cells (Fig. 3b). To confirm that HPV PsV genomes reside inside PML foci, we infected HeLa cells with EdU labeled HPV PsV. This revealed that indeed some – but not all – of the EdU signals overlap with PML foci (Fig. 3c). Therefore, we conclude that HPV PsV infection alters nuclear shape in a cell-type dependent manner and triggers PML foci accrual.

Live cell imaging reveals mitotic requirement for HPV PsV infection and cellular variability in subsequent kinetics

To scrutinize the time- and cell cycle-dependence of HPV PsV infection, we performed live cell imaging experiments in which we monitored HeLa cells for 48 h PI at 10 min intervals. A consistent occurrence of mitosis throughout the imaging time window and a population doubling time close to that of cells grown under standard culture conditions (~20 h) confirmed close-to-physiological settings. Despite image acquisition settings being titrated to minimize phototoxicity, we noted a limited number of dying, apoptotic cells as well as a gradual decrease of the SiR-DNA staining quality at longer time intervals (> 24 h, Fig. 5f).

When determining the infection ratio over time, we found that on average 20% of the cells became EGFP-positive within the 48 h time span, which is in line with the widefield measurements reported above (Fig. 4a). When monitoring the average EGFP intensity at the population level, we found a strong time-dependent increase in infection that was clearly different from that of non-infected cells (Fig. 4b). Non-infected cells also showed a gradual increase in intensity, albeit at much lower level, due to autofluorescence and the accumulation of apoptotic cell debris at later time points. After correction for this non-specific background, we obtained a sigmoidal curve reflecting the population level infection kinetics (Fig. 4b), starting at approximately 18 h PI and reaching a plateau at around 36 h PI. However, we noted a marked heterogeneity in both the timing and the rate of the EGFP signal increase between individual cells (Fig. 4c). Part of this heterogeneity was due to differences in cell cycle status. Indeed, in line with earlier findings (6, 7), we noted that signals only started to increase in cells that had undergone successful mitosis (Fig. 4d). Hence, we tracked individual cells that became EGFP-positive and synchronized them to the first time point in which they could be distinguished as one of two daughter cells during mitosis (roughly corresponding to late telophase) (Fig. 4e). This showed that the EGFP intensity starts to increase at around 2 h

post-mitosis. Yet, even when synchronized, the intercellular heterogeneity in EGFP intensity was still clearly visible. To verify whether this was due to the differences in cell cycle length between individual cells, we normalized the EGFP intensity profiles to the time span between two consecutive mitoses (Fig. 4f). However, this scaling did not significantly reduce the variation (coefficient of variation of the EGFP intensity per time point for synced or synced and scaled data: 9.3 ± 12.0 resp. 7.8 ± 9.3; p = 0.8, Kruskal Wallis). Heterogeneity was even present between pairs of daughter cells. Visual assessment revealed that about 10 % of the mitoses only one of the two daughter cells became EGFP-positive, but also those mitoses with two EGFP-positive daughter cells demonstrated substantial variability (Fig. 4g, j). While the temporal evolution of daughter cell EGFP fluorescence was quite similar (as reflected by a high correlation coefficient, Fig. 4h), their dynamic range diverged significantly (as evidenced by the ratio of their maximum intensities, Fig.4i). This may be due to an asymmetric distribution of the number of pseudoviral units, as also suggested by the variable number of EdU spots between daughter cells (Fig. 4k).

HPV PsV infection influences cell cycle dynamics

Having in hand the complete temporal track record of HPS PsV infection, we wondered whether we could use this information to unveil a causal relationship with the observed differences in nuclear organisation. In line with our widefield observations, we found a decrease in nuclear area in EGFP-positive cells compared to EGFP-negative cells (Fig. 4l). This suggests that HPV PsV infection induces a nuclear compaction rather than favouring cells with smaller nuclei. Since the observed difference was more limited than that captured with widefield microscopy, we wondered whether cell cycle changes contributed to the observed decrease in nuclear area. To this end, we measured the mitotic window, *i.e.,* the time between pro- and late telophase (Fig. 4m). While we found no significant difference for the

first mitosis between EGFP-positive and EGFP-negative cells, the mitotic window of the second mitosis was significantly extended in EGFP-positive cells. This indicates that HPV PsV infection occurs stochastically but prolongs the mitotic window of the host cell.

Blocking cell cycle progression blunts HPV PsV infection

Having confirmed the dominant requirement for mitosis with live cell imaging, we exposed cells to a variety of cell cycle blockers prior to infection, reasoning that it would negatively regulate infectivity (Fig. 5a). Progression from G1 to S phase was blocked with aphidicolin (APH) or double thymidine (THY) incubation, while a more broad-spectrum (S and G2/M) block was applied using mitomycin C (MIT) treatment. Serum starvation was tested to force cells into early G1-phase. However, cell cycle profiling revealed that this latter treatment did not achieve the desired effect in HeLa cells (Fig. 5b). Although the double THY treatment led to cell cycle arrest, the timing requirements were difficult to combine with live cell imaging procedures after HPV infection (Fig. 5c). Therefore, based on their consistent impact on the cell cycle profile and compatibility with live cell imaging, we focused on the effect of APH and MIT on HPV PsV kinetics. The time-dependent increase in nuclear count that was observed in control cultures, was completely abolished upon APH and MIT treatment (Fig. 5d, f). MIT treatment also caused a strong increase in the number apoptotic cells at later time points (Fig. 5e, f). For APH, the identification of apoptotic cells was more variable as the treatment led a decrease in nuclear intensity and an increase of the average nuclear size (Fig. 5f).

Upon APH and MIT treatment, a stark drop in the ratio of infected cells compared to the non-treated HPV PsV infected cells was observed (Fig. 6a). Unexpectedly, at different time points, EGFP-positive cells could still be detected. When scrutinizing individual cell cycle kinetics, we noticed that, despite an unequivocal negative impact of both treatments on the number of

444 cells, a small increase in the number of nuclei during the first h of imaging was still present,
445 especially in MIT-treated cells (Fig. 5d). Exactly this persistent increase in the number of
446 cells, due to leaky mitoses, was responsible for the minimal infection rates. At the population
447 level, the reduced ratio of infected cells also translated into strongly decreased EGFP
448 intensities over time (Fig. 6b). Yet, due to the incomplete action of the pharmacological
449 agents, an increase in EGFP signal was still observed. To gain insight into the infection
450 kinetics, we fitted a logistic function to the average curves of cells that became EGFP-positive
451 (Fig. 6c). APH-treated cells showed an earlier inflection point than non-infected cells, in line
452 with the progressive cell cycle inhibition of APH and higher leakiness during the early phase
453 of the experiment.
454
455

Discussion

In this work, we have used automated high-throughput microscopy to determine early HPV PsV infection kinetics with high efficiency and specificity. To our knowledge, this is the first time that infection ratios have been automatically quantified in a time-independent manner. Only one other study used live cell imaging, but quantification was done manually (9). And while other studies have made use of fixed cells to quantify HPV infection by applying either flow cytometry or microscopy (36–38), we compared both techniques, and illustrated the power of microscopy for spatiotemporal mapping and revisiting of individual cells. Automated microscopy enabled accurate determination of HPV PsV infection ratios in a concentration-, time- and cell type-dependent manner, and it allowed relating changes in cell cycle and nuclear morphology to the early, non-replicative phase of infection.

Despite the frequent use of HeLa cells in relevant HPV infection studies (6, 8), it is important to note that they contain multiple copies of integrated HPV18 DNA, which may bias their response to PsV infection (39). That is why we have directly compared with the HaCaT cell line, which does not contain such sequences (21). We found a lower infection efficiency in HaCaT cells, but with mitosis being a prime requirement for HPV infection, we attribute this difference to their longer population doubling time (6, 7). As pre-binding HPV PsV with extracellular matrix (ECM) has been shown to significantly increase the infection efficiency in HaCaT cells, proliferation may not the only determinant (20).

Using a multiparametric analysis of nuclear morphotextural descriptors, we could discriminate both cell types as well as their infection status. We also found marked differences between EGFP-positive and EGFP-negative HPV PsV infected cells. We speculate that this heterogeneity in cellular response may be caused by differences in the efficiency with which the PsV particles reach the nucleus. Despite the high PsV titer, only part of the pseudoviral particles is infectious (17, 22), and of those that are infectious, not all

will be equally successful in cell and nuclear entry owing to a variety of intracellular barriers and defense mechanisms (40). We hypothesize that in EGFP-negative cells, PsV were not able to enter the nucleus and therefore did not induce nuclear rearrangements. While we cannot irrefutably rule out that there is an influence of the EGFP expression as well, our results with lipofection suggest it is not the prime elicitor of the observed changes. Various viruses have been found to alter nuclear morphology and size upon infection (41). This may be driven by direct interactions with the nuclear lamina, nuclear actin and other nucleoskeletal components that control nuclear shape (42), but it may also be caused by chromatin rearrangements. Nuclear shape changes are a hallmark for high-grade lesions caused by a chronic HPV infection and are the basis for Pap smear diagnostic tests (43, 44). And textural features, reflecting chromatin condensation, have been linked to HPV positivity before as well (45). Strikingly, we now document nuclear rearrangements during the very early phase of infection, independent of viral replication. This may explain some discrepancies. For example, *in vivo* HPV infections elicit nuclear enlargement, while we found a decrease in nuclear area *in vitro*. This may be due to the short incubation time (48 h) and non-replicative nature of the PsV, but it might also be a (partial) reflection of a shift in the cell cycle status of HPV PsV infected cells. For, we noticed a shift towards G2/M, which we believe may be linked to the prolonged mitotic window (and consequent size reduction). The use of G2/M specific markers (e.g. cyclin B1 (46)) or real-time cell cycle indicators (47) could help to validate and dissect these changes more accurately. Although PsV do not contain the viral genome, the central region of the minor capsid protein L2, which is still present in PsV, mediates the tethering of vDNA to mitotic chromosomes (48), and might be responsible for this prolongation.

One important limitation of our work is that we have made use of an EGFP reporter gene under the control of a CMV promoter. Known as a highly active promoter (49, 50), the

transcription might be very different from that of HPV promoters. The early phase of HPV16 replication depends on the expression of the viral transcription factor E2 that regulates the early p97 promoter, upstream of the regulatory viral oncoproteins E6 and E7, ensuring survival of HPV infected cells, while the late phase of HPV16 replication is marked by the activation of the late p670 promoter, ensuring DNA replication and virion formation (51, 52). This temporally regulated transcription of HPV-driven promoters, in combination with differential regulation by host transcription factors (52, 53) may not only affect transgene expression, but could in theory also affect consequent nuclear morphology changes. Although a comparison with lipofected cells suggested that the observed morphotextural changes were not due to the expression of EGFP, infection with quasivirions (QsV) or (modified) HPV wild-type virus should allow dissecting the impact of different viral promoters and regulators.

Using HPV PsV, we found a significant increase in PML body content 24 h PI, showing it reflects an early response that is independent of viral replication. This confirms previous studies showing that, following completion of mitosis, PML is recruited to the nucleus to assemble around HPV genomes before the release from the transport vesicles (10, 13). Yet, it is remarkable that a similar response occurs for PsV that do not contain viral information and it raises the possibility that nucleation is sheerly achieved by means of the capsid proteins.

By synchronizing single cells to their first mitosis after infection, we found that EGFP signal increased over time only from that point onwards, proving the essential role of mitosis for infection (6, 7). However, even between two daughter cells the signal changes showed significant heterogeneity. While we cannot exclude variation in transcriptional activity, we assume this is primarily due to stochastic differences in viral load. This is also supported by the fact that cells that become infected have a similar (1st) mitotic window than cells that do not, and it aligns with the variability in EdU labeled HPV PsV between daughter cells. A

more direct means to assess this causal relationship could be to perform experiments with labeled pseudovirus and/or in situ hybridization.

Having confirmed the crucial demand for mitosis, we performed cell cycle blocking experiments as a proof of concept. A drastic drop in HPV PsV infected cells was seen for cells that were treated with APH or MIT. Despite the eight h cell cycle blocking treatment before the start of live cell imaging, there were still cells able to divide and to become infected. This may be due to the brevity of the chemical treatment as longer incubation times have been reported (6, 54). However, with the current procedure, we already quantified a time-dependent cytotoxicity, pointing to the limits of this approach.

To summarize, we have established a scalable microscopy approach to automatically quantify and analyze HPV PsV infection kinetics at the single cell level. We revealed intercellular heterogeneity in infection kinetics and cell-type dependent changes in nuclear organization and cell cycle profile. Systematic functional (*e.g.,* CRISPR panel or compound library) screens that build on this work will help unveil the molecular nodes that promote the early phase of HPV infection and vDNA settlement and transcription. In a next phase, replacing PsV by QsV or wildtype viruses, should allow screening for drugs that block replication and could be of use for treating infected patients.

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Authors' contributions

WDV designed the study and acquired funding; FM and MS carried out PsV, cell biology and microscopy experiments; MV and MB developed software; FM and MV analysed image data; NC was responsible for flow cytometry and PD for ultracentrifugation work; FM and WDV wrote the manuscript; all authors critically revised and approved the manuscript.

Competing interests

The authors declare no competing interests.

Data availability

All results are provided in the manuscript and accompanying supplemental files. Image analysis code is available on GitHub (https://github.com/VerschuurenM/StarTrack; https://github.com/DeVosLab/CellBlocks).

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Figure legends

Figure 1 Automated microscopy allows accurate determination of HPV PsV infection kinetics and reveals cell-type dependent infection ratios. **a)** General workflow; **b)** HeLa cells have a significantly shorter population doubling time than HaCaT cells ($n_{tech} = 6$) (error bars = SD) ($p < 0.05$, t-test); **c)** Representative images of HeLa and HaCaT, illustrating the titer-dependent increase in EGFP-positive cells (yellow arrows showing rare, weakly EGFP-positive cells). Bar plots represent the percentage of EGFP-positive cells as a function of viral titer. There is a significant titer-dependent increase in infection ratio within both cell types (HeLa and HaCaT) and for both modalities (flow and microscopy) ($n_{bio} = 1$, $n_{tech} = 3$) (error bars = SD) (different symbols indicate significantly different conditions ($p < 0.05$), linear mixed effects model); **d)** Correlation plot reveals good correlation ($R > 0.90$) between flow and microscopy; **e)** Representative images of infected HeLa and HaCaT cells at 48, 72 and 96 h PI with a MOI of 100-400 VGE/cell (yellow arrows showing rare EGFP-positive cells). Infection levels reach a maximum at around 48 h PI for both cell types and both modalities ($n_{bio} = 1$, $n_{tech} = 3$) (different symbols indicate significantly different conditions ($p < 0.05$), linear mixed effects model); **f)** Correlation plot reveals good correlation ($R > 0.90$) between flow and microscopy.

Figure 2 HPV PsV infection induces nuclear rearrangements as well as cell cycle alterations in HeLa and HaCaT cells. **a)** Principal component analysis discriminates both cell types and EGFP-positive and -negative cells based on morphological and textural DAPI descriptors. Of these descriptors, the nuclear circularity, nuclear area, homogeneity and contrast are shown for non-infected (- HPV PsV) and infected (+ HPV PsV) EGFP-negative or EGFP-positive cells (different symbols indicate significantly different conditions); **b)** HPV PsV infected EGFP-positive cells display a significant enrichment of cells in G2/M phase and a significant

decrease of the proportion of cells in G1 phase for both cell types (n_{bio} = 3, n_{tech} = 3) (error bars = SD) (different symbols indicate significantly different conditions; $p < 0.05$, linear mixed effects model).

Figure 3 HPV PsV infection inflates nuclear PML NB content. **a)** Left: Representative images of control HeLa and HaCaT cells and cells infected with HPV PsV for 24 h or 48 h, stained for PML. The overlay shows EGFP signal (green), the nuclear outlines (in cyan) and segmented PML foci (spots) (white) (yellow arrows indicate EGFP-positive cells with larger PML content, contrast in the PML channel has been globally adapted per cell type for visualization purposes). Right: Bar plots of PML spot occupancy, showing a significant increase at 24 h and 48 h PI in EGFP-positive cells compared to EGFP-negative and non-infected cells for HeLa and HaCaT cells (n_{bio} = 3, n_{tech} = 6) (error bars = SD) (different symbols indicate significantly different conditions; $p < 0.05$), linear mixed effects model); **b)** Live cell imaging of EYFP-PML transfected HeLa cells confirm the increase in number and size of PML protein foci in HPV16-RFP PsV infected cells; **c)** HeLa cells infected with EdU labeled HPV PsV for 24 h show co-localization between some EdU pseudogenomes and PML foci (arrowheads) (EdU contrast has been globally adapted for clarity).

Figure 4 Live cell imaging reveals the requirement of mitosis for successful HPV PsV infection but significant intercellular heterogeneity in the subsequent kinetics. **a)** Ratio of infected, EGFP-positive cells over 48 h of time span for non-infected and HPV PsV infected cells (n_{bio} = 3, n_{tech} = 10) (error bars = SD); **b)** HPV PsV infection kinetics over time. In black, the EGFP intensity is shown per nucleus of cells tracked from the beginning of the recording. The orange curve shows the normalized EGFP intensity measurements (corrected for non-specific background) per nucleus for HPV PsV infected cells (n_{bio} = 3, cell based) (error bars

= SE); **c)** Representative montage of HPV PsV infected HeLa cells showing the variability in timing and rate of EGFP signal between individual cells; **d)** Bar plot showing the percentage of infected cells per round of mitosis, indicative for the need of mitosis for HPV PsV infection to occur and gradual decrease in positivity with additional divisions (n_{bio} = 3, n_{tech} = 10, average per biological replicate) (error bars = SD); **e)** Tile plot with EGFP intensity of individual cells, synchronized to the first mitosis illustrates the variability of signal increase over time; **f**) Tile plot of the same cells in which the intensities have been normalized to the time window of the first and second mitosis (expressed as a function of virtual cell cycle time) still reveals heterogeneity between individual cells; **g)** Synchronised EGFP intensity traces illustrate the variability between daughter cells (shown as pairs)**; h)** Pearson correlation between the EGFP intensity evolution of daughter cells over time ($n_{cell\ pairs}$ = 10); **i)** Ratio of the maximum EGFP intensity of daughter cells ($n_{cell\ pairs}$ = 10); **j)** Montage of 2 daughter cells and synchronized EGFP intensity trace corresponding to the dotted rectangle in panel g (red dot indicates corresponding parameters in panel h and i). While the upper daughter nucleus (indicated by orange arrowhead) displays EGFP signal in between 2 and 5 h after mitosis, the lower nucleus (red arrow) only becomes positive 10 h after mitosis and remains much weaker; **k)** Variations in viral load, visible as red spots in the DAPI stained nucleus, between daughter cells infected with EdU labeled HPV PsV (EdU contrast has been globally adapted for clarity); **l)** Subtle area differences between cells that express EGFP after HPV PsV infection compared to cells that remain EGFP-negative (n_{bio} = 3; 22, 16 and 11 cells respectively); **m)** HPV PsV EGFP-positive cells display a significant increase in the time span (mitotic window) of the second mitosis PI as compared to HPV PsV EGFP-negative cells (n_{bio} = 3; 22, 16 and 11 cells respectively) (different symbols indicate significantly different conditions; $p < 0.05$), Kruskal-Wallis test, using Pairwise post-hoc tests with Benjamini-Hochberg correction) (error bars = SD).

Figure 5 Impact of cell cycle blockers on infection kinetics. **a)** Workflow for measuring the impact of cell cycle inhibition on HPV PsV infection kinetics and the different used cell cycle inhibitors and their effect on the different stages of the cell cycle. Cells were seeded 24 h prior assay. The treatment with MIT or APH was done 8 h before live cell imaging, while HPV PsV were added just before the start of the 48 h during imaging; **b)** Cell cycle profiles of HeLa cells remain unaltered despite starvation. Starving of HeLa cells by applying 0,1% FBS instead of 10% FBS for different time points (48 h and 72 h), did not result in early G1 arrest (n_{bio} = 1, n_{tech} = 6); **c)** Cell cycle profiles of treated cells. Thymidine (THY) and aphidicolin (APH) treatment led to a prominent G1-peak, while mitomycin C (MIT) treatment, blocks cells in G2 (n_{bio} = 1, n_{tech} = 6); **d**) APH and MIT cause a stagnation in the number of cell nuclei, while non-infected and HPV PsV infected cells display a consistent increase (n_{bio} = 3, n_{tech} = 10) (error bars = SD); **e**) The percentage of apoptotic cells after MIT treatment increases over time during live cell imaging. The low percentage of apoptotic cells after APH treatment is attributed to their inaccurate detection due to a decreasing SiR-DNA intensity and nuclear expansion (total cell count > 600 at t0) (error bars = SD); **f)** Representative images of nuclei of the different conditions showing a decrease in SiR-DNA intensity over time, as well as overt changes in cell density (yellow arrows indicate apoptotic cells, red arrows indicate rare EGFP-positive cells after mitosis in MIT-treated cells).

Figure 6 Cell cycle arrest lowers HPV PsV infection. **a)** APH- and MIT-treated cells display a reduced percentage of infected cells (n_{bio} = 3, n_{tech} = 10) (error bars = SD); **b)** APH- and MIT-treated cells show lower EGFP intensity (total cell count > 600 at 0h) (error bars = SE); **c)** Logistic functions fitted to the average EGFP intensity of infected cells over time reveal that the few APH-treated EGFP-positive cells have an earlier inflection point. This is due to

leaky mitosis at the early phase of live cell imaging (n_{CTRL} = 5, $n_{HPV\ PsV}$ = 671, $n_{HPV\ PsV\ +\ \mathbf{APH}}$ = 21, $n_{HPV\ PsV\ +\ \mathbf{MIT}}$ = 196) (error bars = SE).