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# Virology
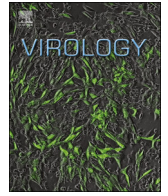
# Non-plaque-forming virions of Modified Vaccinia virus Ankara express viral genes

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Anna-Theresa Lülf [a,b], Astrid Freudenstein [a], Lisa Marr [a], Gerd Sutter [a,b,*], Asisa Volz [a,b]

[a] Institute for Infectious Diseases and Zoonoses, LMU University of Munich, Veterinaerstrasse 13, 80539 Munich, Germany
[b] German Centre for Infection Research (DZIF), Veterinaerstrasse 13, 80539 Munich, Germany

ABSTRACT

In cell culture infections with vaccinia virus the number of counted virus particles is substantially higher than the number of plaques obtained by titration. We found that standard vaccine preparations of recombinant Modified Vaccinia virus Ankara produce only about 20–30% plaque-forming virions in fully permissive cell cultures. To evaluate the biological activity of the non-plaque-forming particles, we generated recombinant viruses expressing fluorescent reporter proteins under transcriptional control of specific viral early and late promoters. Live cell imaging and automated counting by fluorescent microscopy indicated that virtually all virus particles can enter cells and switch on viral gene expression. Although most of the non-plaque-forming infections are arrested at the level of viral early gene expression, we detected activation of late viral transcription in 10–20% of single infected cells. Thus, non-plaque-forming particles are biologically active, and likely contribute to the immunogenicity of vaccinia virus vaccines.

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## 1. Introduction

Vaccinia virus (VACV) is the best-studied poxvirus and the prototype live virus vaccine used to eradicate human smallpox (for review see (Moss, 2013a)). VACV was among the first animal viruses to be cultured and purified (Maitland and Maitland, 1928; F. Parker and Nye, 1925; R.F. Parker and Rivers, 1935; Steinhardt et al., 1913). Early studies with virus preparations from the skin of infected rabbits (Smadel et al., 1939) or the chorioallantois membrane (CAM) of embryonated chicken eggs (Dumbell et al., 1957) already provided evidence that more total particles (also called "elementary bodies") are produced than infectious VACV virions. Indeed, a ratio of 10 total particles to 1 plaque-forming unit (PFU) was determined for purified preparations of VACV using a protocol of ultra-centrifugation through sucrose-gradient similar to the one still used for producing vaccine preparations (Joklik, 1962). A single study using semi-purified (removal of cellular debris from virus harvest via low-speed centrifugation) MVA and comparing EM counted particles to tissue culture infectious dose 50 (TICD50) determined 2–3-fold more total particles than the TICD50 (Guggenberger, 1989). This biological phenomenon is true for many animal viruses, and even when disregarding morphologically or biochemically

"incomplete" virions, 10 to 1 million virus particles are usually needed to initiate a single event of infection (Isaacs, 1957).

With a ratio of about 1:10 for CAM infections and tissue culture plaques, VACV has one of the highest ratios of virus infections to particles (Galasso and Sharp, 1963; Joklik, 1962). Interestingly, early experiments suggested that the greater fraction of the non-plaque-forming VACV particles enter cells and are infectious in tissue culture (Galasso and Sharp, 1964). Yet, it remained obscure whether these infections were able to initiate the virus life cycle, switching on gene expression and protein synthesis. Furthermore, the prevalence of non-plaque-forming particles in VACV vaccine preparations and their possible influence on immunogenicity or vaccine efficacy are largely unknown.

Today, more than three decades after eradicating smallpox as a natural disease, VACV is still needed as a reserve vaccine against smallpox due to the threat of bioterrorism (Moss, 2011). Moreover, the virus is successfully used as a vector for developing new vaccines against other diseases (Mackett et al., 1982; Panicali and Paoletti, 1982). However, the VACV vaccines used during the smallpox eradication campaign would be currently considered to have an unsatisfactory safety profile, and safer strains of VACV such as Modified Vaccinia virus Ankara (MVA) have been established for vaccine development (for review see (Moss, 2013b)).

MVA was obtained by extensive serial passaging of VACV strain Ankara in chicken embryo fibroblasts (CEF), resulting in DNA deletions and mutations at multiple sites of the viral genome (Antoine et al., 1998; Mayr and Munz, 1964; Meyer et al., 1991). MVA

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lost its ability to productively replicate in most mammalian cells, but maintained expression of all classes of viral and recombinant genes under conditions of non-permissive viral growth. Therefore MVA can serve as an exceptionally safe live viral vector vaccine (Sutter and Moss, 1992). Multiple recombinant MVAs are currently undergoing clinical testing for vaccination against various human infections including AIDS, tuberculosis, and malaria (for reviews see (Gilbert, 2013; Gomez et al., 2013)). Critically, recent candidate MVA vaccines are being developed for experimental immunization of humans against emerging infections such as highly pathogenic avian influenza virus H5N1, the Middle East Respiratory Syndrome, West Nile fever, or Ebola hemorrhagic fever (Ewer et al., 2016; Kreijtz et al., 2014; Volz et al., 2015, 2016).

As the clinical use of MVA vector vaccines expands more, vaccine preparations require non-clinical characterization for lot consistency, purity, infectivity, and recombinant gene expression. Our experience

and that of others have indicated that the biological properties of the purified MVA particles in these preparations are generally unknown. More fundamental questions pertaining to the regulation of host-restriction and gene expression, as well as practical questions such as MVA vaccine immunogenicity also remain unclear.

Here, we studied recombinant MVA, and found over 70% non-plaque-forming virions in standard MVA vaccine preparations from avian cell lines. Specifically, we were able to monitor the ability of these virions to infect different host cells and activate viral gene expression using reporter viruses expressing fluorescent proteins under the control of early and late viral promoters. The resulting single cell infections synthesized early, and to a lesser extent late gene products. For comparison we also analyzed the particle properties and infection progression of recombinant virus preparations based on the fully replication competent parental VACV Ankara strain CVA152.
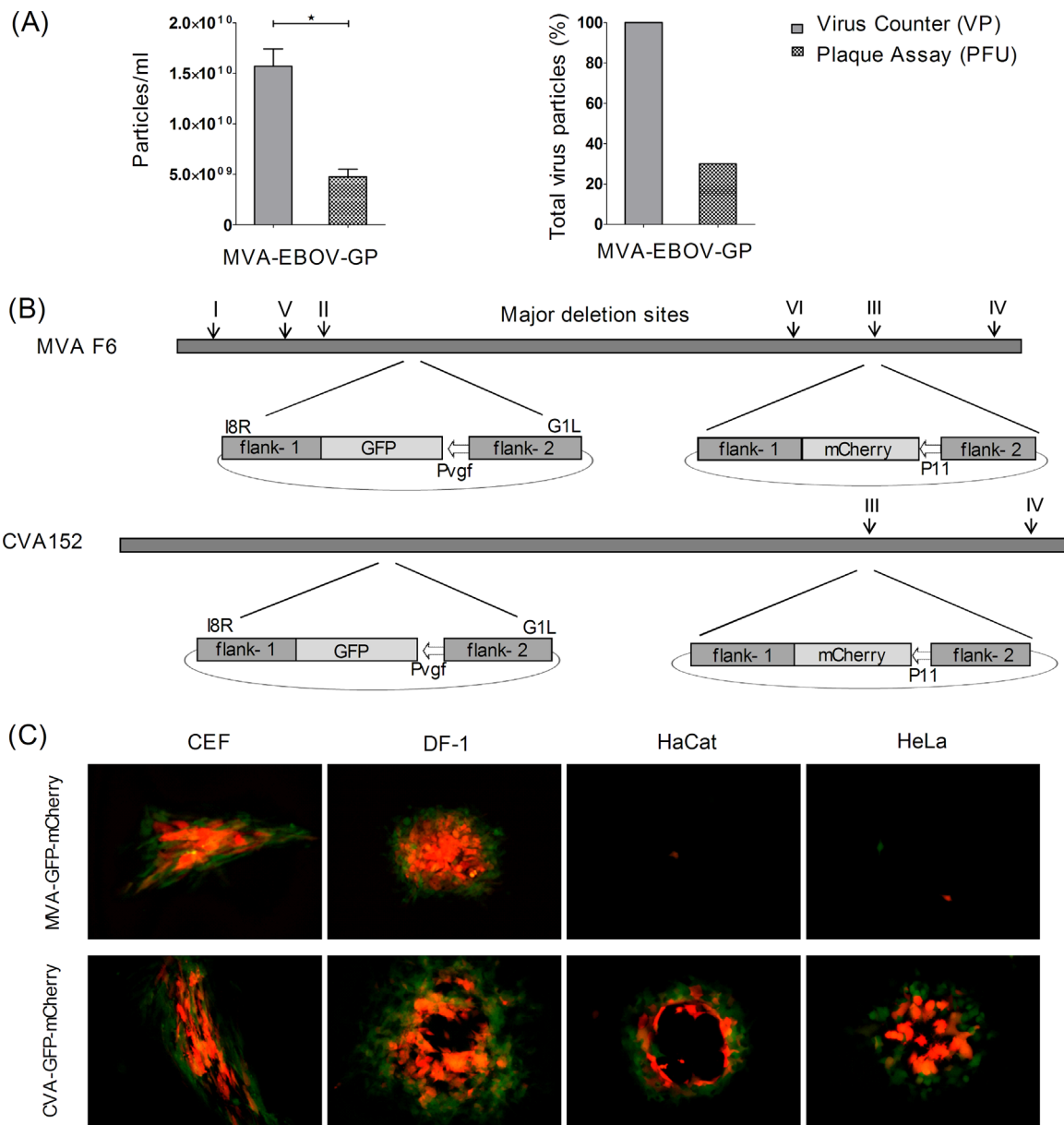


Fig. 1. Monitoring non-plaque-forming virus particles and gene expression with reporter fluorescent proteins. (A) Plaque forming units (PFU) and total virus particles (VP) in sucrose-cushion purified stock preparations of an MVA recombinant candidate (MVA-EBOV-GP) vaccine were quantified via plaque assays (PFU/ml) and virus Counter measurement (VirusCounter 3100-ViroCyt) (VP/ml). *P < 0.05. (B) Schematic diagram of the MVA F6 and CVA152 genome indicating the I8R/G1L site, used to insert GFP regulated by the early vaccinia promoter Pvgf and the deletion III site used to insert mCherry under the transcriptional control of the late vaccinia promoter P11. (C) Fluorescence imaging of CEF, DF-1, HaCat and HeLa cells infected with MVA-GFP-mCherry and CVA-GFP-mCherry at an m.o.i of 0.005 at 24 hpi.

# 2. Results

## 2.1. Non-plaque-forming virus particles in recombinant MVA vaccines

To characterize the particle content of standard MVA vaccine preparations we selected a sucrose-cushion purified preparation of an MVA candidate vaccine producing the Ebola virus glycoprotein (MVA-EBOV-GP) obtained from clonal isolation by plaque purification. First, to obtain standardized counts of total particles we used a virus counter device (ViroCyt Virus Counter 3100) known to be used for quality control monitoring by vaccine manufacturers. Virus counting is based on flow cytometry using two different fluorescent dyes to stain and co-localize proteins and nucleic acids. For the MVA-EBOV-GP preparation we counted a total of $1.6 \times 10^{10}$ virus particles (VP)/ml (Fig. 1A). In comparison, infectious virus particles quantified by plaque titration resulted in $4.8 \times 10^{9}$ plaque-forming units (PFU)/ml, approximately 30% of the total virus particle count in this virus preparation.

The above data are in line with previous results from particle counts by electron microscopy, suggesting considerable fractions of non-plaque-forming particles are present in VACV preparations.

## 2.2. Monitoring the VACV life cycle with reporter viruses expressing fluorescent proteins

To investigate the biological properties of non-plaque forming VACV particles in more detail, we constructed the recombinant viruses MVA-GFP-mCherry and CVA-GFP-mCherry as reporter viruses (Fig. 1B). CVA-GFP-mCherry served to directly compare MVA to a fully replication-competent VACV, by using the Ankara strain CVA152 as a true ancestor virus of MVA. These recombinant viruses were designed to monitor the infection cycle by visualizing key steps in viral gene expression, detected through the synthesis of fluorescent marker proteins. Early GFP production should indicate virus entry and transcription of viral early genes; the detection of red fluorescent mCherry protein (late-mCherry) should signal the start of DNA synthesis and viral late gene expression.

The viruses were quality controlled by PCR analysis, confirming the stable integration of the GFP encoding sequences at the I8R/G1L intergenic site and mCherry sequences at deletion site III of both MVA and CVA152 genomes (Fig. 1B; Fig. S1; data not shown). Both recombinant viruses were genetically stable, and tested for growth, as well as production of recombinant GFP and mCherry proteins as detected by Western blot analysis (Fig. S2; data not shown). As expected, MVA-GFP-mCherry replicated efficiently in avian CEF and DF-1 cells but not in human HeLa or HaCat cells.

To test the activity of our reporter viruses after tissue culture infection, we examined the production of the fluorescent proteins GFP and mCherry in chicken (CEF and DF-1) or human cells (HaCat and HeLa) by fluorescence microscopy (Fig. 1C). At 24 h post infection (hpi), microscopy revealed foci in MVA-GFP-mCherry infected CEF and DF-1 cells; red fluorescent cells in the center were surrounded by green fluorescent cells, marking the early and late phases of the MVA infection. In contrast, in HeLa and HaCat cells we detected single green or red fluorescent cells, but no formation of plaques or foci with fluorescent cells.

Productive CVA-GFP-mCherry infections resulted in typical VACV plaques visible 24 hpi in all four cell cultures. Again red late-mCherry producing cells accumulated in the centers of large virus plaques, while green early-GFP synthesis was found around the periphery of the plaques. These results provided reasonable proof of principle for using these recombinant viruses to visualize various productive and abortive infection events in tissue culture.

## 2.3. Infections with MVA-GFP-mCherry reveal numerous single cell infections without plaque formation

Again using standard sucrose-cushion purified preparations of MVA-GFP-mCherry and CVA-GFP-mCherry, we determined total virus particle numbers by particle counting, and measured infectivity by plaque titration (Fig. 2A). In agreement with our data above this analysis also revealed at least 3-fold more total particles than PFU. In detail, Virus Counter measurements recorded $1.5 \times 10^{10}$ VP/ml for MVA-GFP-mCherry and $2.3 \times 10^{10}$ VP/ml for CVA-GFP-mCherry. In contrast, the plaque forming units as quantified by plaque titration amounted to $3.8 \times 10^{9}$ PFU/ml ($\sim$25% of the total particles) for MVA-GFP-mCherry and $3.5 \times 10^{9}$ PFU/ml ($\sim$15% of the total particles) for CVA-GFP-mCherry.

To monitor infection events in cell cultures permissive for productive virus replication, we analyzed the kinetics of GFP and mCherry expression upon MVA-GFP-mCherry infection in CEF and DF-1 cells (Fig. 2B). GFP and mCherry expression detected by fluorescence microscopy mostly revealed single green fluorescent cells at 12 hpi. At 14 hpi some of the single green cells shifted to red fluorescence, and adjacent cells started to show green fluorescence, forming foci of infected cells. MVA-GFP-mCherry formed more even and rounder plaques in DF-1 cells than the plaques in CEF, which were more irregularly shaped, corresponding to the well-known MVA plaque phenotypes in these cell cultures (Kremer et al., 2012). By 18 hpi we readily observed growing MVA-GFP-mCherry plaques in the cell monolayers, but the majority of the monitored single green fluorescent cells neither changed to red fluorescence nor developed into new virus plaques until 40 hpi.

The time point 24 hpi seemed suitable for determining the ratio of single infected cells to virus plaques in CEF and DF-1 after MVA-GFP-mCherry infection (Fig. 2C). Single fluorescent cells and virus plaques in 96-well cell monolayers were automatically quantified (Keyence Hybrid Cell Count Module, BZ-H3C). In both chicken cell cultures about 20–30% of all detected fluorescent events were identified as viral plaques. However, most of the infection events ($>$70%) were detected as single fluorescent cells. After imaging we fixed the infected cell cultures and performed a standard VACV-specific immunostaining assay to confirm the data from plaque counting using the Hybrid Cell Count analysis assay. An equal number and very similar pattern of virus plaques were detected (Fig. 2C). These results indicate a surprisingly high number of single cell infection events, which closely correlated with the calculated number of non-plaque forming particles in the MVA-GFP-mCherry preparation.

## 2.4. VACV reporter viruses reveal viral early gene expression in most non-plaque forming infections

To more precisely follow up the outcome of MVA-GFP-Cherry infections in DF-1, CEF, and HeLa cells grown in 6-well plates, we selected 30 single GFP positive cells at 12 hpi. We automatically monitored the selected infected cells in the monolayers via live cell imaging (Keyence Time Lapse Module) over a time period of 24 h (Fig. 3).

In DF-1 cells, 20% of the selected single infected green fluorescent cells resulted in the formation of plaques, 5% of the single cells proceeded to late gene expression as indicated by red fluorescence, and 75% of the infected cells remained green, i.e. in early gene expression. We could confirm these findings in CEF cells, where 16.9% of the selected cells formed plaques, 10.6% shifted to red fluorescence without plaque development, and 72.5% remained single green fluorescent cells. As expected, MVA-GFP-mCherry infection of human HeLa cells did not result in detectable virus plaques. However, the overall ratio of infected green cells arrested at the early stage of gene expression (83.9%) to infected red cells exhibiting late gene expression (16.1%) was similar to the proportion observed in the avian cell cultures (Fig. 3A).
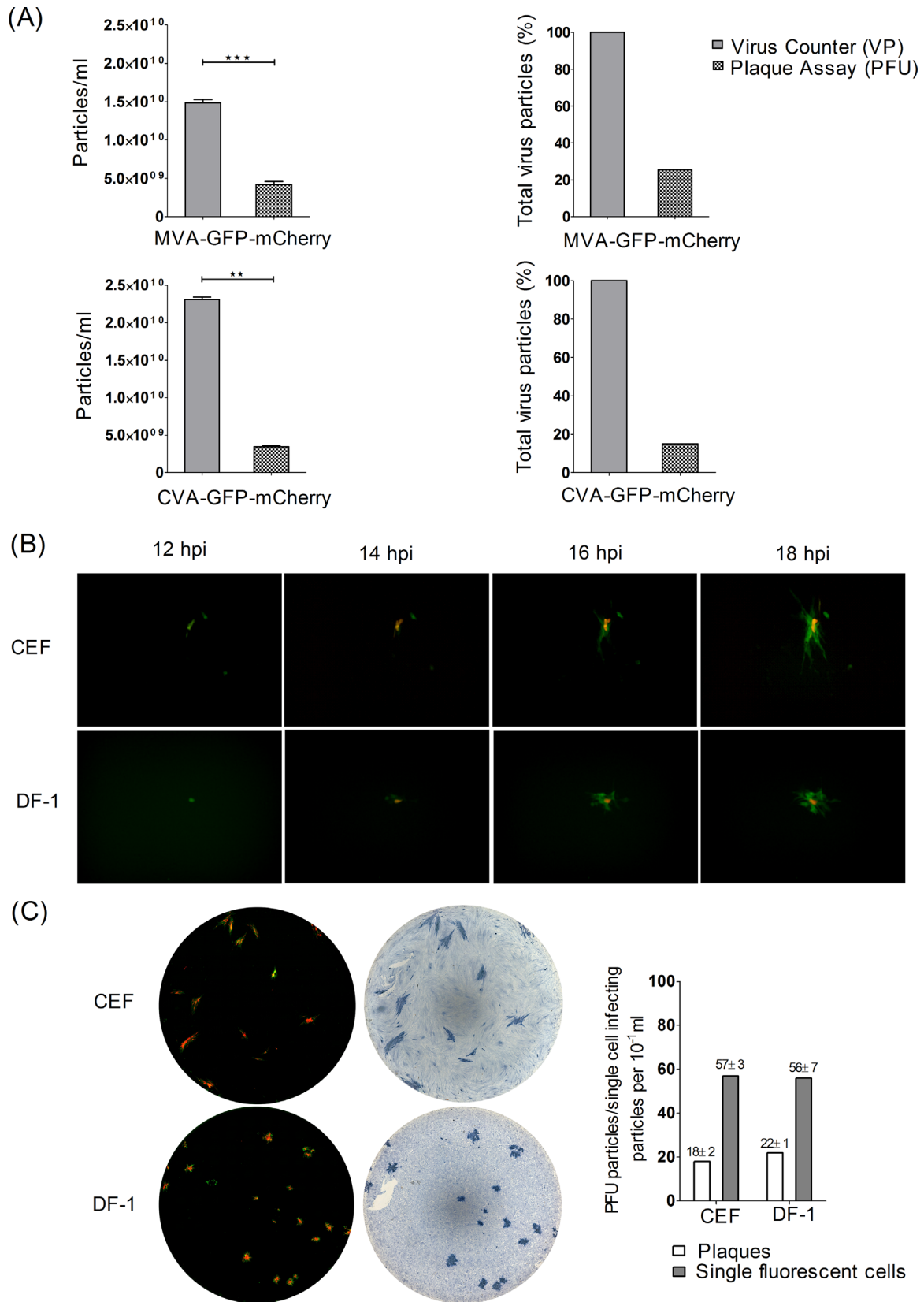
**Fig. 2.** Analysis of the MVA life cycle using the reporter virus. (A) Plaque forming units (PFU) and total virus particles (VP) in sucrose-cushion purified stock virus preparations of MVA-GFP-mCherry and CVA-GFP-mCherry were measured via plaque assays (PFU/ml) and virus Counter measurement (VirusCounter 3100-ViroCyt) (VP/ml). **P < 0,01; ***P < 0,001. (B) Time course of plaque development. CEF and DF-1 cells were infected with MVA-GFP-mCherry at an m.o.i. of 0.005. Infection events were monitored via fluorescence imaging in two hours increments. (C) CEF and DF-1cells grown in 96-well plates were infected with MVA-GFP-mCherry at an m.o.i. of 0.01. The wells were imaged via fluorescence microscopy at 24 hpi (left panel) and a VACV-specific immunostaining plaque assay was performed as a control (middle panel). Single infected fluorescent cells and plaques were quantified using Hybrid Cell Count Software (right panel).
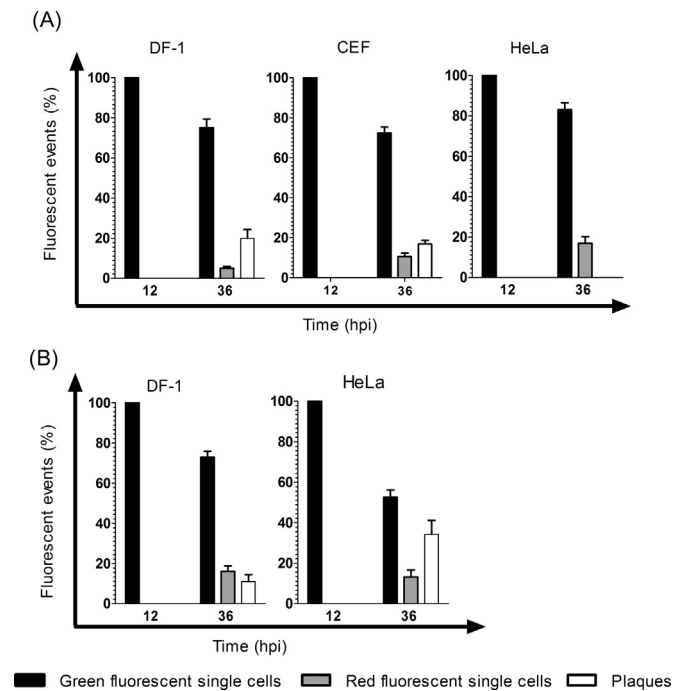
**Fig. 3.** Tracking single cell infection via live cell imaging. Single green fluorescent DF-1, CEF and HeLa cells, infected with sucrose-cushion purified viral stock preparation of MVA-GFP-mCherry (A) or sucrose-cushion purified viral stock preparation of CVA-GFP-mCherry (B), were selected at 12 hpi and monitored for 24 h in 30 min increments via Time Lapse Software. Each value represents the mean with SEM of at least three separate experiments.

Next, we analyzed infections with the replication-competent CVA-GFP-mCherry in DF-1 and HeLa cells using the same experimental setting (Fig. 3B). In DF-1 monolayers 10.1% of the selected single infected cells developed into typical plaques, 17.6% of the single cells displayed red fluorescence without formation of plaques, and 72.3% of the infected cells remained green. These results were comparable to those obtained with MVA-GFP-mCherry in DF-1 cell cultures. Interestingly, in HeLa cells replication-competent CVA-GFP-mCherry formed about three-fold more plaques than seen in DF-1 cell infections. Overall, these data demonstrated that most tissue culture MVA or CVA infection events remain arrested at the level of viral early gene expression.

## 3. Discussion

Here, we tested the composition and biological activities of virus particles present in standard MVA vaccine preparations. For the first time we used new reporter viruses expressing the fluorescent markers GFP and mCherry under the control of an early and late viral promoter. Over the past few years VACV biology has greatly benefited from advances in genetic engineering and fluorescent imaging techniques. Recombinant viruses with fluorescently tagged viral proteins have been elegantly used to elucidate the cell-to-cell spread and intracellular movement of VACV particles (Frischknecht et al., 1999; Hollinshead et al., 2001; Ward and Moss, 2001). In addition, fluorescent reporter viruses serve in screening experiments for antiviral drugs (Dal Pozzo et al., 2008). Here, our reporter viruses successfully monitored VACV infection progression of both plaque-forming and non-plaque-forming virions and identified active transcription of viral early and late genes.

In infection research recombinant MVA viruses serve as candidate vaccines compatible with clinical use and industrial-scale production (for review see Volz, 2016 Adv Virus Res, in press). The data quality required before initiating clinical trials include evaluation of the phenotypic properties of the vaccine virus

preparations (European Medicines Agency. 2010. Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines. EMA/CHMP/VWP/141697/2009).

Sucrose-gradient purified preparations of conventional VACV are known to comprise about 10-fold more total particles than infectious particles, e.g. as counted by electron microscopy and measured by plaque tests (Joklik, 1962). Also in this study, using an industry-standard virus counter and our regular protocol for MVA titration (Kremer et al., 2012), we found a majority (>70%) of non-plaque-forming virions in standard MVA vaccine sucrose-cushion purified preparations from CEF or DF-1 cells, avian cell lines that are consistently used with MVA to amplify and purify normal titers from cell cultures.

Similar "noninfectious" particles have been found with many mammalian viruses, but the biological activities associated with these particles have largely been ignored (Marcus et al., 2009; Pierson and Diamond, 2012). Further characterizing these non-plaque-forming particles in MVA vector vaccine preparations seems relevant due to their potential role in inducing innate or adaptive immune responses (Lopez, 2014).

When testing sucrose-cushion purified preparations of MVA-GFP-mCherry or CVA-GFP-mCherry in tissue culture infections we detected an unexpectedly high number of single fluorescent cells. Such monitoring of the infection events shows that non-plaque-forming particles also activate viral gene expression, although most of these single cell infections are restricted to synthesizing early gene products.

The de novo synthesis of early viral RNAs suggests that these non-plaque forming infections trigger innate immune signaling, as previously described for NFκB activation following VACV early transcription (Martin and Shisler, 2009; Willis et al., 2011). Notably, our time lapse experiments allowed us to precisely follow the development of single viral plaques or the fate of single infected cells by observing the synthesis of GFP and mCherry (see video clips S1-S3).

Regardless of the stage of viral gene expression (early or late) and the permissiveness for productive infection, many of the remaining single fluorescent cells underwent morphological changes, including phases of contraction with retractions of cell projections followed by cell rounding, membrane blebbing, and disintegration or detachment from the cell monolayer. Since the results from experiments with replication-competent CVA-GFP-mCherry confirmed the data seen for MVA-GFP-mCherry, we are confident about the general validity of these findings for infections with particles from different strains of VACV and possibly for infections with virions from other orthopoxviruses.
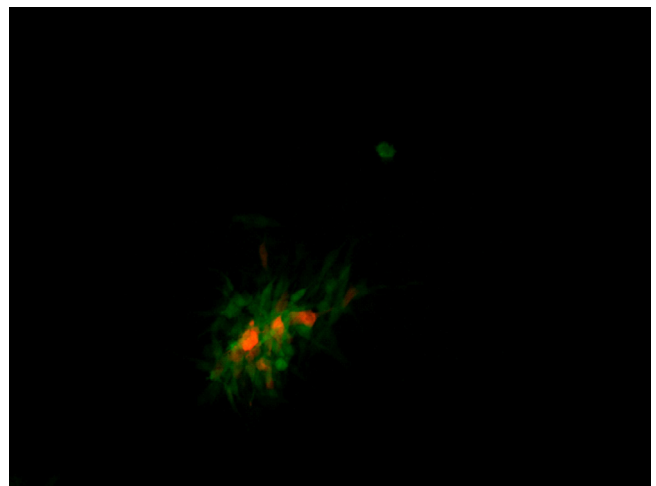


**Video S1.** Time Lapse video of recombinant MVA-GFP-mCherry infection in DF-1 cells from early until late gene expression as detected by green and red fluorescent gene expression in multiple cells. Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.virol.2016.09.006.

**Video S2.** Time Lapse video of recombinant MVA-GFP-mCherry infection in a single DF-1 cell demonstrating early gene expression as identified by green fluorescence. Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.virol.2016.09.006.
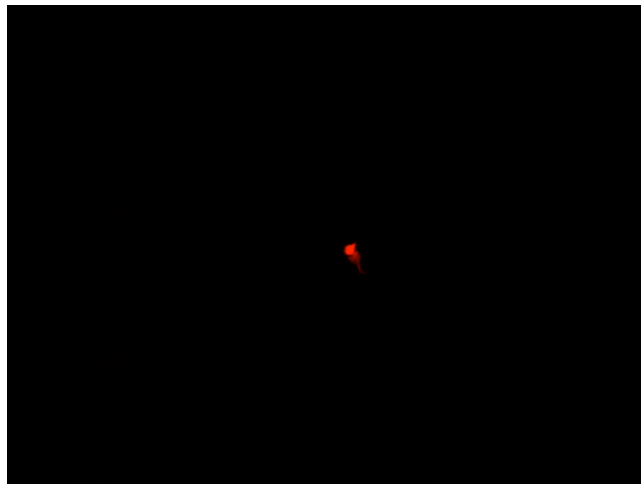


**Video S3.** Time Lapse video of recombinant MVA-GFP-mCherry infection in a single DF-1 cell demonstrating late gene expression as identified by red fluorescence. Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.virol.2016.09.006.

The mechanistic reason(s) for the substantial portion of abortive infection events still remain unclear. One possibility is that VACV preparations commonly contain fractions of "defective" virions unable to initiate a full cycle of viral gene expression and productive virus replication. We examined the virus particles in our stock preparations by electron microscopy and detected only mature brick-shaped virions with normal morphology (data not shown). Yet, this apparent absence of malformed particles does not formally exclude the possibility of mutations blocking later stages of infections. Alternatively, the biology of the host cell is essential for the replication of VACV and it is tempting to speculate that simply the condition of the infected host cell determines the outcome of an infection. The activation of host cell signaling pathways e.g. involving the MAPKs (mitogen-activated protein kinases) ERK (extracellular signal-regulated kinase) and JNK (Jun N-terminal kinase) has been shown to be critical for productive infection and VACV spread (Andrade et al., 2004; de Magalhaes et al., 2001; F. Wang et al., 2004). Moreover, the activation of these host signaling pathways is specifically modulated in the favor of virus replication by various poxvirus regulatory proteins (de Magalhaes et al., 2001;

Schweneker et al., 2012; Torres et al., 2016; G. Wang et al., 2006). These findings are used in the development of oncolytic poxviruses that better grow in tumor cells where such pathways are malignantly activated (Bell and McFadden, 2014). Our observation of CVA-GFP-mCherry infection resulting in enhanced plaque formation in HeLa cells might be based on the preferential productive replication of this virus in tumor cells (see Fig. 3B).

There are two major infectious forms of VACV particles: the mature virions (MV) and the extracellular enveloped virions (EV) (Moss, 2013a). MVs are infectious virions surrounded by a lipoprotein membrane that accumulate within the cytoplasm of VACV infected cells (Condit et al., 2006). Since MV are the most abundant virion form present in vaccine preparations, the data obtained in our experiments might be essential for the quality control of MVA based vaccines. Both infectious forms, MV and EV, have distinct biological properties and activities: EV are responsible for efficient cell-to-cell spread and contribute to transmission within the host, while the MV play a key role in the transmission between the host animals (Blasco and Moss, 1992; Cudmore et al., 1995; Moss, 2012). Further experiments are needed to clarify on a possibly distinct role of EV infections on gene expression and plaque formation in different host cell substrates.

In conclusion, our experiments confirm that infection with non-plaque-forming particles of VACV results in gene expression. Despite the inability of these "defective" virus particles to induce plaque formation, these particles are still able to switch on early gene expression. Interestingly, already early work by Galasso and Sharp (1964) suggested that "non-replicating" VACV particles enter cells and exert biological activities. More recently, UV-inactivated recombinant VACV is used to express early genes as nonreplicative vector in tumor immunotherapy (Zajac et al., 2003). Thus, viral and recombinant early transcripts might serve to efficiently trigger innate immune responses and antigen production by recombinant vaccines similar to processes described for other vaccine-induced activations of immunity. In addition, these findings indicate that the choice of early or early-late promoters is an appropriate approach to most practically activate vaccine-induced immunity. Finally, our results further support the ability of MVA-based vaccines to stimulate a balanced interplay between innate and adaptive immunity as the key to robust protection against a selected pathogen.

## 4. Materials and methods

### 4.1. Cells

DF-1, HaCat and MA-104 Jena cells were cultured in VLE Dulbecco's MEM (Biochrom GmbH) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (SIGMA), 1% Penicillin-Streptomycin (SIGMA) and 2% Hepes-buffer (SIGMA). HeLa cells were maintained in Minimum Essential Medium Eagle (MEM) (SIGMA) containing 10% HI-FBS and antibiotics as described above. Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old chicken embryos (SPF eggs, VALO, Cuxhaven) and subsequently grown in MEM supplemented with 1% Penicillin- Streptomycin, 1% MEM non-essential amino acid solution (SIGMA) and 10% HI-FBS. All cells were maintained at 37 °C in a 5% $CO_2$ atmosphere.

### 4.2. Generation of recombinant viruses

Recombinant MVA-GFP-mCherry was generated by homologous recombination as described previously (Kremer et al., 2012). MVA F6 (Meyer et al., 1991) served as the parental virus for generating MVA-GFP, used as the backbone virus for constructing MVA-GFP-mCherry. Vector plasmid pLW73 was used to direct the insertion of GFP, under the transcriptional control of vaccinia virus promoter

Pvgf, into the G1L/I8R insertion site of the viral genome. Pvgf (5′-GTTTATATTACTGAATTAATAATATAAAATTCCCAATCTTGTCATAAA-3′; underlined A indicates transcriptional start site as described by Broyles et al., 1991.), a natural vaccinia virus promoter, controls the early expression of VACV ORF C11R mRNA that was recently reported to belong to the temporally first class of early genes (immediate-early class or E1.1 subclusters) (Assarsson et al., 2008; Yang et al., 2010). Plasmid pIIIgptex served as the vector for inserting mCherry, placed under the vaccinia virus promoter P11, into Deletion III. Recombinant MVA was obtained by clonal isolation in plaque purifications using five to eight passages in CEF following the plasmid transfections. Finally, recombinant viruses were amplified in CEF. Screening for expression of the fluorescent proteins was used to identify and isolate recombinant viruses. Quality control experiments were performed using standard methodology (Kremer et al., 2012).

The genetic identity and genetic stability of the reporter virus were assessed via PCR analysis of genomic viral DNA. The replicative capacity of the recombinant virus was confirmed by one-step and multiple-step growth experiments in CEF, DF-1, HeLa and HaCat cells. CVA-GFP-mCherry was constructed in a similar manner to MVA-GFP-mCherry, but the fully replication-competent virus CVA152 (Waibler et al., 2009) was used as the parental virus and the propagation was carried out in MA-104 cells. CVA152 is a direct ancestor virus of MVA and its genome sequence is identical to MVA at the insertion sites Deletion III and I8R/G1L. Sucrose-cushion purified virus stocks of MVA-GFP-mCherry and CVA-GFP-mCherry were generated by ultracentrifugation through a 36% sucrose cushion, followed by resuspension in 10 mM Tris buffer pH 9 using standard procedures as described in (Kremer et al., 2012) and (Joklik, 1962).

### 4.3. Quantification of virus

#### 4.3.1. Plaque assay

Viral plaque forming units (PFU) were determined via standard plaque assays, as described in Kremer and coworkers (Kremer et al., 2012). Briefly, confluent CEF cell monolayers in 6-well plates were infected with serial 10-fold dilutions of the viruses. After 2 h of incubation at 37 °C, the cells were washed once with phosphate buffered saline (PBS) and incubated with fresh medium containing 2% HI-FBS at 37 °C for two days, followed by a vaccinia specific immunostaining. Here, the infected cells were fixed in acetone-methanol (1:1) for 5 min, washed with PBS and incubated for 60 min with a polyclonal rabbit anti-vaccinia virus antibody. After an additional washing step (PBS), the cells were incubated with peroxidase-conjugated AffiniPure goat anti-rabbit secondary antibody for 60 min. Plaques were visualized using TrueBlue peroxidase substrate solution (KPL), counted and calculated as described previously (Kremer et al., 2012). All virus titrations were performed in duplicate and repeated at least three times.

#### 4.3.2. Total virus particle counts

Total virus particles were estimated using ViroCyt VirusCounter 3100 following the manufacturer's instructions (ViroCyt, Bloomfield, CO). Briefly, the sucrose-cushion purified virus samples were diluted 1:200 and 1:400 in ViroCyt sample dilution buffer. Subsequently, 300 μl of each sample were incubated with 150 μl Combo Dye solution, a combination of two fluorescent dyes specific for proteins and nucleic acids (Stoffel et al., 2005). After 30 min of light-protected incubation at room temperature, the virus particles were quantified in the virus counter. Simultaneous detection of both fluorescent signals using the virus counter's dual channel flow cytometer was measured as one virus particle. Intersample washes, followed by a cleanliness control run were performed between each sample analysis, and medium from uninfected CEFs was used as a negative control. Total virus particle concentration was recorded as virus particles per ml (VP/ml) and determined for at least three times per virus sample.

### 4.4. Fluorescent microscopy

Fluorescent images were obtained using an inverted fluorescence microscope (Keyence BZ-X700) with a x20 Plan Fluor NA 0.45 Ph1 objective and a z-section setting. Emission of GFP filter (excitation wavelength 470/40 nm, absorption wavelength 525/50 nm) and TexasRed filter (excitation wavelength 560/40 nm, absorption wavelength 630/75 nm) were used for detecting green and red fluorescence. Raw imaging data were processed and analyzed with BZ-X Analyzer Software.

### 4.5. Hybrid cell count

The ratio of single infected fluorescent cells to plaque-forming fluorescent units was assessed during MVA-GFP-mCherry infection in permissive cells by culturing CEF and DF-1 cells in 96-well plates. Each well was infected at an m.o.i. of 0.01 and received 100 μl of inoculum. After 30 min incubation at 4 °C (cold start), cells were washed with PBS three times and incubated at 37 °C with 200 μl fresh medium containing 2% HI-FBS. 24 h after infection multiple fluorescent and bright-field images of the infected wells were acquired using a 20x objective, and then stitched together via the Keyence Merge function. Afterwards a vaccinia specific immunostaining of the captured wells was performed, as described above (Plaque assay). Bright-field images of these wells served as controls. The total single fluorescent cell count was quantified using single extraction function of the Hybrid Cell Count Software (Keyence Hybrid Cell Count Module, BZ-H3C) and total foci were counted manually.

### 4.6. Live cell imaging

For live cell imaging, DF-1, CEF and HeLa cells cultured in 6-well plates, were infected with a sucrose-cushion purified viral stock preparation of MVA-GFP-mCherry or a sucrose-cushion purified viral stock preparation of CVA-GFP-mCherry at an m.o.i. of 0.005 via cold start as described above. After three washing steps with PBS, 2 ml fresh medium containing 2% FBS was added and the cells were incubated at 37 °C. At 12 hpi about 30 single green fluorescent cells were selected for each separate experiment. The development of the single infected cells was observed in 30 min increments for a further 24 h using Keyence Time Lapse Module, BZ- H3XT. Time lapse analysis was performed at 37 °C with 5% $CO_2$ concentration using an incubation chamber (Tokai Hit, Incubation Systems for Microscopes) to allow 24 h recording. All time lapse experiments with recombinant MVA and CVA viruses were performed at least three times.

### 4.7. Western blot analysis

Chicken embryo fibroblasts were infected at an m.o.i. of 10 with MVA-GFP-mCherry and CVA-GFP-mCherry in the absence or presence of cytosine arabinoside (AraC, 1 mg/ml). MVA wild type or mock-infected cells served as controls. Total cell extracts were prepared at 24hpi. After separating by 10% SDS-PAGE proteins were analyzed via Western Blots using anti-GFP rabbit fraction antibody (life technologies, 1:250) and anti-mCherry rat fraction antibody (kindly provided by Elisabeth Kremmer, Helmholtz Zentrum München, 1:10 dilution) as primary antibody. For detection of GFP and mCherry via MicroChemisystems (biostep), anti-rabbit HRP-conjugated antibody (Sigma, 1:5000 dilution) and goat anti-rat HRP-conjugated antibody (BioLegends, 1:2000) were used as secondary antibodies.

## 4.8. Statistical analyses

The differences in virus particle titres were assessed for statistical significance with GraphPad Prism version 5 software (GraphPad software, San Diego, USA); using the Mann-Whitney $U$ test and P-values less than 0.05 were considered to be statistically significant.

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## Appendix A.  Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.09.006.

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