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Hoornweg, Tabitha Elina

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CHAPTER 4

Chikungunya virus requires an intact microtubule network for efficient genome delivery

Tabitha E. Hoornweg, Denise P.I. van de Pol, Izabela A. Rodenhuis-Zypert, Jolanda M. Smit

1 Department of Medical Microbiology, University Medical Center Groningen, University of Groningen, The Netherlands

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Abstract

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne virus, which rapidly spread around the globe thereby causing millions of infections. CHIKV belongs to the Togaviridae family and enters its host cell primarily via clathrin-mediated endocytosis. Upon internalization, the endocytic vesicle containing the virus moves through the cell and delivers the virus to early endosomes where membrane fusion is observed. Trafficking of endocytic vesicles is often facilitated by the microtubule and actin cytoskeleton. Consequently, an intact cytoskeleton was found important for the infectivity of many viruses. However, the involvement of microtubules in alphavirus infection was never thoroughly studied. Here, we show that microtubules are important during the early steps of CHIKV cell entry. By taking advantage of previously established real-time single virus tracking techniques, we showed that CHIKV exhibits two distinct intracellular trafficking patterns prior to membrane fusion. Whereas half of the CHIKV virions remained quite static during cell entry, the other half showed fast-directed movement. Only the fast-directed movement was found to depend on an intact microtubule network. Microtubule disruption did however not inhibit the extent of CHIKV fusion. Yet, it did affect the cellular location of membrane fusion and the release of the viral genome. Taken together, this study describes the role of microtubules in CHIKV cell entry.
Introduction

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne virus, which in the last decade caused millions of infections in the tropical and subtropical regions of the world\(^1\). CHIKV belongs to the alphavirus genus of the Togaviridae family. Its virions are enveloped spherical particles of approximately 70 nm in diameter. The CHIKV genome consists of a single positive-stranded RNA molecule of 11.8 kb in length, which is packaged by the CHIKV capsid (C) protein to form a nucleocapsid. The CHIKV nucleocapsid is in turn encapsulated by a host-cell derived lipid bilayer. In the lipid bilayer, 240 copies of the CHIKV transmembrane glycoproteins E1 and E2 are inserted. The mature virus particle has 80 spikes which are arranged in an icosahedral T=4 symmetry. Each spike consists of three E1/E2-heterodimers (reviewed in \(^2\)). Both E1 and E2 are important in the initial steps of infection. Whereas E2 is facilitates binding to the host cell, E1 contains the hydrophobic fusion-loop and mediates membrane fusion\(^2\).

Like most alphaviruses, CHIKV enters cells primarily via clathrin-mediated endocytosis (CME\(^3\)-\(^5\)). CME is a constitutive process that occurs in all mammalian cells\(^6\). It starts with the formation of a membrane invagination coated with clathrin molecules: the clathrin-coated pit. Cargo is sequestered into the pit, after which the pit grows and scissions from the plasma membrane in a dynamin-dependent manner. The vesicle subsequently loses its clathrin coat and delivers its cargo to the early endosome\(^7\). CME of CHIKV was found to be a rapid process; 50% of the CHIKV particles fused within 9 minutes post-addition of the virus to BS-C-1 cells\(^5\). During CME, CHIKV co-localized with clathrin for approximately 50 seconds. Subsequently, the clathrin signal was lost and the virus was delivered to the mildly acidic early endosome, where membrane fusion was observed.

Host organelles need to be actively transported through the cell, as molecular crowding and the dense cytoskeletal network prevents free diffusion of objects larger than 20 nm\(^8\). Intracellular transport is highly regulated and primarily occurs on actin filaments and microtubules\(^9,10\). Of these, actin is mainly involved in movements close to the plasma membrane. These movements are either mediated by newly polymerizing actin filaments propelling an organelle or by myosin motor proteins trafficking along the actin filaments\(^10,11\). Additionally, newly formed endocytic vesicles were found to be transported away from the plasma cortex in an actin polymerization-dependent manner\(^10,12\). During this transport, the vesicles often switch from an actin- to a microtubule-based movement. Microtubules generally provide long distance tracks for the transport of organelles to and from the perinuclear region\(^9,10\). The direction of the movement is governed by motor proteins traversing the microtubule; whereas in most cell types dynein transports cargo towards the perinuclear region, kinesins direct their cargo towards the periphery\(^9,10\).

The microtubule network has been implicated in the endocytic trafficking of multiple viruses, including SV40\(^13\), Influenza A virus\(^14,15\), Polio virus\(^16\), Ebola virus\(^17\) and Dengue Virus\(^18\). For example, upon caveolae-mediated endocytosis, SV40 was found to move
along microtubules towards the smooth ER. Microtubule disruption was found to block both the trafficking behavior as well as infection of SV40\textsuperscript{13}. Endocytic vesicles containing Influenza A virus (IAV) were targeted towards dynamic early endosomes in a microtubule-dependent manner\textsuperscript{15}. Additionally, the endosomal trafficking of IAV, as well as endosomal trafficking of dengue virus, was mediated by microtubules and disruption of microtubules was found to impair infection\textsuperscript{14,18}. Retrograde polio virus trafficking inside neurons and Ebola virus entry and fusion were also found to be dependent on microtubules, yet the exact vesicles in which these viruses were residing upon microtubule-dependent trafficking were not studied\textsuperscript{16,17}. Finally, also Japanese encephalitis virus\textsuperscript{19} and Crimean-Congo hemorrhagic fever virus\textsuperscript{20} require microtubules early in infection, yet the step at which microtubules function is unknown.

Here, we show that microtubules play an important role early in CHIKV infection. Detailed analysis of the intracellular trafficking behavior of CHIKV particles revealed two distinct patterns; half of the particles remained quite static whereas the other half were transported along microtubules prior to entry in early endosomes. Interestingly, while microtubule disruption inhibited CHIKV infectivity there was no effect on the extent of membrane fusion. Microtubule disruption however did change the cellular location of membrane fusion and impaired viral genome delivery. This suggests that trafficking along microtubules might direct the virus to an environment that is beneficial for CHIKV infection or that microtubules play an active role in nucleocapsid uncoating.

**Materials and Methods**

*Cells, inhibitors and plasmids.*

Green monkey kidney BS-C-1 cells (ATCC CCL-26) were cultured in Dulbecco’s modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Lonza), 25 mM HEPES, penicillin (100 U/ml), and streptomycin (100 U/ml). Green monkey kidney Vero-WHO cells (ATCC CCL-81) were maintained in DMEM (Gibco) supplemented with 5% FBS (Lonza), penicillin (100 U/ml), and streptomycin (100 U/ml). Baby hamster kidney cells (BHK-21 cells; ATCC CCL-10) were maintained in RPMI medium (Gibco) supplemented with 10% FBS (Lonza), penicillin (100 U/ml), and streptomycin (100 U/ml). All cells were cultured at 37°C under 5% CO\textsubscript{2}.

Nocodazole was purchased from Sigma-Aldrich. The compound was dissolved in DMSO and stored according to the manufacturer’s instructions. A standard MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay was used to test for cytotoxicity, as described previously\textsuperscript{5}. Microtubule disruption upon nocodazole treatment was visualized by staining for α-tubulin. For this purpose, cells were treated with nocodazole after which they were fixed by 4% PFA and permeabilized using 0.02% Triton X-100 in PBS. Subsequently, the samples were stained using mouse anti-α-tubulin (1:2000, Sigma) and rabbit anti-mouse AF647 (1:1000, LifeTechnologies).
The clathrin-LCa-eYFP plasmid was provided by X. Zhuang (Harvard University, Cambridge, MA, USA). The Rab5-wt-GFP plasmid was a gift from P. van der Sluijs (University Medical Center, Utrecht, The Netherlands).

**Virus production, purification, labeling and quantification.**

Virus production, purification and labeling procedures have been described previously⁵. Briefly, CHIKV strains LS3 and LS3-GFP²¹ were produced by electroporation of *in vitro*-transcribed RNA transcripts into BHK-21 cells. Virus working stocks were produced by subsequent passage in Vero-WHO cells.

For large scale production, BHK-21 monolayers were inoculated with CHIKV at an MOI of 4. At 24 h post-infection (hpi) the supernatant was harvested and cleared from cell debris by low-speed centrifugation. Subsequently, the virus was pelleted by ultracentrifugation, resuspended in HNE and purified by ultracentrifugation on a sucrose gradient (20 to 50% [wt/vol] in HNE). The 40%-to-45% section containing the virus was harvested, aliquoted, and stored at −80°C.

The infectious virus titer was determined by a standard plaque assay using Vero-WHO cells. Plaques were counted 2 days after infection. The number of genome equivalents were determined by reverse transcriptase PCR (RT-PCR) followed by quantitative PCR (qPCR), as described previously²². For detection of (+)-sense and (-)-sense viral RNA template specific primers were used during the reverse transcriptase step. Preceding the qPCR, the viral RNA was removed by treatment with 10 units of RNAse A (Fermentas) for 1h at 37°C.

For microscopy, CHIKV was labeled with the lipophilic fluorescent probe DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt; Life Technologies), as described previously ⁵. DiD-labeled virus was stored at 4°C in the dark and was used within 2 days.

**Single-particle tracking of DiD-labeled CHIKV.**

Single-particle tracking experiments were performed as described previously⁵. Briefly, BS-C-1 cells were seeded into 8-well Lab-Tek II chambered coverglass slides (Nunc) to obtain 50 to 70% confluence on the day of tracking. When indicated, 1.25 × 10⁶ BS-C-1 cells were transfected with 5 µg of plasmid DNA by electroporation before seeding.

Cells were washed three times with phenol red-free MEM. Subsequently, phenol red-free MEM supplemented with 1% glucose was added to the cells in presence or absence of 10 µM nocodazole. When nocodazole was added, cells were pre-incubated for 2 h to allow disruption of the microtubules. Just before microscopy, GLOX was added to prevent phototoxicity²³. Cells were mounted on the Leica Biosystems 6000B microscope and kept at 37°C throughout the whole experiment. DiD-labeled CHIKV was added *in situ*, and image series were recorded at 1 frame per s for 25 to 30 min. To localize the nucleus and plasma membrane of the cell, DIC snapshots were taken before and after the imaging.

Image processing and analysis were carried out using ImageJ (NIH) and Imaris.
Trajectories were generated using the ‘particle tracking’ function of Imaris by pairing peaks per frame to previously established trajectories according to proximity and similarity of intensity. For the generated trajectories, the ‘particle tracking’ function of Imaris calculated the particle velocity. The fluorescence intensity during a trajectory was quantified using an in-house macro based on the ‘particle analyzer’ plugin of ImageJ. A sudden (within 1 to 2 seconds) >2-fold increase in fluorescence intensity was defined as membrane fusion. Only particles smaller than 40 arbitrary units (a.u.) were considered individual particles and were selected for further analysis. Additionally, as the thickness of the cell close to the nucleus is larger than one focal plane, we excluded particles that bound to the cell in this area from our analysis\(^5\). Upon simultaneous tracking of the virus and either clathrin or Rab5, co-localization was assessed by eye.

**Microscopic fusion assay.**
The membrane fusion capacity of CHIKV in the presence and absence of 10 µM nocodazole was estimated by a microscopy-based fusion assay. For this purpose, BS-C-1 cells were seeded into 8-well Lab-Tek II chambered coverglass slides (Nunc) to obtain a sub-confluent monolayer the next day. Cells were washed three times with serum-free, phenol red-free MEM (Gibco). Subsequently, phenol red-free MEM containing 1% Glucose was added in presence or absence of nocodazole and cells were incubated for 2 h at 37°C. Next, DiD-labeled CHIKV was added to the cells at MOI 20 after which cells were incubated at 37°C for another 30 min in order to allow viral fusion. Unbound virus was removed by washing three times with serum-free, phenol red-free MEM after which fresh phenol red-free MEM containing 1% glucose was added.

Microscopic analysis was done with the Leica Biosystems 6000B instrument. Fields were randomly selected in the differential interference contrast (DIC) settings, after which snapshot in both the DIC and DiD channel were taken. Per experiment a total of 20 random snapshots were taken. Snapshots were analyzed using the ‘particle analyzer’ plugin of ImageJ. The total area of fluorescent spots was quantified in a.u. for each snapshot and averaged per experiment.

**Flow cytometry analysis of infection.**
Flow cytometry analysis was used to assess the effects of nocodazole on CHIKV infection. For this purpose, 10 µM nocodazole was diluted in BS-C-1 medium containing 2% FBS. Cells were pre-incubated with nocodazole for 2 h, after which CHIKV LS3-GFP was added to the cells at an MOI of 20. At 1.5 hpi, fresh BS-C-1 medium containing 10% FBS in presence or absence of 10 µM nocodazole was added. At 10 hpi, cells were washed with PBS, trypsinized and fixed with 4% paraformaldehyde (PFA). Subsequently, cells were analyzed by flow cytometry.

For the time-chase experiments, cells were treated with 10 µM nocodazole diluted in BS-C-1 medium for either for the complete course of infection (-2 – 10 hpi); pre-entry (-2 –
0.5 hpi; or post-entry (0.5 – 10 hpi). CHIKV LS3-GFP was added at an MOI of 20. At 0.5 hpi the virus inoculum was removed, cells were washed once with PBS and bound, but non-internalized viral particles were removed from the plasma membrane using a high salt high pH shaving buffer (1M NaCl, pH 9.5) for 2 min, as described before\textsuperscript{24}. Subsequently, cells were washed one more time with PBS and fresh BS-C-1 medium containing 10% FBS was added in the presence or absence of 10 µM nocodazole. Infection was continued until 10 hpi, after which cells were washed with PBS, trypsinized, fixed and subsequently analyzed by flow cytometry.

**Cell fractionation.**
Virus genome delivery was estimated by use of a cell fractionation protocol followed by PCR-based quantification of the viral genomic (+)-strand RNA. Cells were pretreated for 2 h with 10 µM nocodazole in BS-C-1 medium containing 2% FBS, after which they were infected for 1 h at an MOI of 10. Subsequently, cells were washed once with PBS, bound virus particles were removed using high salt high pH shaving buffer (1M NaCl, pH 9.5) for 2 min after which cells were washed twice with PBS, trypsinized and harvested. In order to fractionate the cells, cells were treated with ice cold fresh digitonine buffer (10 mM TRIS pH 7.5; 10 mM NaCl; 0.15 mM spermine; 0.5 mM spermidine; 1 mM EDTA and 100 µg/ml digitonine) for 10 min at 4°C\textsuperscript{25–27}. Subsequently, samples were centrifuged at 1000 g for 5 min at 4°C. Both the supernatant (cytosolic fraction) as well as the pellet (vesicular fraction) were adjusted to 0.5% Triton X-100. Debris was removed by briefly centrifuging the samples at 10,000 rpm using a MiniSpin Microcentrifuge (Eppendorf). Subsequently, samples were subjected to RT- and qPCR.

To control for efficient cell fractionation, the fractionation of GAPDH, a cytosolic marker, and LAMP-1, a vesicular marker, were assessed by Western blot. For this purpose, fractionated samples were loaded onto precast 10% Mini-PROTEAN\textsuperscript{®} TGX™ gels (BioRad). Gels were blotted using the Trans-Blot Turbo Transfer system (Biorad) and Trans-Blot Turbo Mini PVDF Transfer Packs (Biorad). PBS containing 0.1% Tween20 and 4% milk powder was used to avoid unspecific binding. Mouse anti-GAPDH (1:1000; Merck Millipore) and mouse anti-LAMP1 (1:500; BD Biosciences) were used as primary antibodies. Goat anti-mouse-AP (1:5000; Southern Biotech) was used as a secondary antibody. Protein bands were visualized using NBT/BCIP (Roche).

**Results**

**An intact microtubule network is important early in CHIKV infection**

To study whether microtubules play a role in CHIKV infection, we first determined the effect of the microtubule-depolymerizing agent nocodazole on CHIKV infectivity. For this purpose, BS-C-1 cells were treated for 2 h with 10 µM nocodazole prior to infection with CHIKV. Incubation of the cells for 2 h with nocodazole was sufficient to disrupt the
microtubule network (Figure S1A). Infection was allowed for one round of replication (10 h) in the presence of the inhibitor. Upon microtubule disruption, the number of infected cells was inhibited by more than 70% (Figure 1A). The inhibition of infection could not be attributed to nocodazole-induced cytotoxicity, as treatment with nocodazole was well-tolerated for periods up to 18 h after addition of the compound (Figure S1B). Subsequent MFI analysis of the infected cell population revealed that there is no significant effect on CHIKV protein translation, which suggests that once the cell is infected the translation/replication machinery is not affected (Figure S2). To more carefully assess at which stage of infection CHIKV requires an intact microtubule network, time-of-addition experiments were performed. For this purpose, BS-C-1 cells were either treated with nocodazole at the ‘pre-entry’ (from 2 h prior to infection to 0.5 hpi) or ‘post-entry’ (from 0.5 h to 10 hpi) stages of infection or for the duration of one complete replication cycle (from 2 h prior to infection to 10 hpi) (Figure 1B). At 0.5 hpi, non-internalized CHIKV particles were removed by washing with a high-pH high-salt buffer, as described before24. CHIKV infectivity was

Figure 1. Microtubules are required early in CHIKV infection. (A) Flow cytometry analysis of CHIKV infection in presence of nocodazole. BS-C-1 cells were pretreated for 2 h with 10 µM nocodazole and infected with CHIKV LS3-GFP for 10 h. Percentage of infection is normalized to the positive control. Data represents two individual experiments in triplicate; error bar represents SD. (B) Flow cytometry-based nocodazole time-chase assay of CHIKV infection. Nocodazole (10 µM) was added during the complete course of infection (-2 – 10 hpi), pre-entry (-2 – 0.5 hpi) or post-entry of the virus (0.5 h – 10 hpi). CHIKV LS3-GFP was added at 0 h, at 0.5 hpi non-internalized virus was removed using a high-pH-high-salt shaving buffer, and incubation was continued for another 9.5 h. Data represents four individual experiments in duplicate. Error bars represent SD. (C) Detection of negative-sense RNA by qPCR as described in Materials and Methods. Negative-sense RNA is normalized to the non-treated control. Data represents two individual experiments in triplicate; error bars represent SD.
quantified by flow cytometry at 10 hpi. We observed 54% inhibition of infection when cells were treated with nocodazole for the complete duration of the experiment. Importantly, 57% inhibition of infection was detected when nocodazole was only present at the early stages of infection (-2 h – 0.5 hpi). Addition of nocodazole after 0.5 hpi perturbed CHIKV infection by 26%, implying that microtubules are mainly required during the early steps of CHIKV infection. To confirm this finding, we next evaluated the presence of negative-sense viral RNA copies upon infection in presence and absence of nocodazole. Figure 1C shows that microtubule disruption indeed reduces the number of negative-strand RNA copies with more than 75% at both low and high MOIs.

**CHIKV exhibits two types of trafficking behaviour upon endocytosis**

The observation that microtubule disruption only has effect in the first 0.5 hpi is suggestive for a role of microtubules in CHIKV cell entry. To study this in more detail, we next examined the intracellular trafficking behaviour of CHIKV particles prior to fusion by live cell imaging of DiD-labelled CHIKV particles, as described before. The DiD probe was selected because of its self-quenching properties at a high surface density. Consequently, membrane fusion can be observed as a sudden increase in fluorescence intensity due to dilution of the probe in the target membrane. DiD was incorporated into the CHIKV membrane to such an extent that the fluorescence was largely quenched yet uniformly labelled single virus particles could still be discerned. Single particle tracking of DiD-labelled CHIKV was performed in the kidney epithelial cell line BS-C-1. These cells were selected since epithelial cells are considered natural target cells during CHIKV infection. Furthermore, the relative flatness of these cells increases the chance of capturing complete viral trajectories from virus-cell binding to viral fusion in the epi-fluorescence microscopy setup applied. The fast nature of CHIKV cell entry prevented us to use confocal microscopy as the current time resolution is too limited for accurate recording of fast complex movements.

In order to track the cell entry of CHIKV virions in BS-C-1 cells, DiD-labelled CHIKV was added *in situ* and images were recorded at 1 frame per second for a total of 25 min. CHIKV exhibited two different types of trafficking behaviour during cell entry (Figure 2A & 2B). In the first type of trafficking, virions remained relatively immobile at first, after which they suddenly exhibited a fast and directed movement. Next, the virus moved relatively slowly again until viral fusion occurred (Figure 2A left panels). The fast-directed movement is visible as a distinct increase in velocity when the particle velocity was plotted against the time (Figure 2A right panels). A movie showing this virus trafficking behaviour is added as supplementary data (Movie S1). Other particles remained relatively static till the moment of membrane fusion (Figure 2B left panels). Consequently, considerably shorter distances were covered by the virions. Indeed, when viral velocity was plotted against time no distinct peaks were observed (Figure 2B right panels). Movie S2 shows an example of this kind of trafficking behaviour.
Figure 2. Trajectory analysis of CHIKV cell entry. (A) Depicts two single virus particles exhibiting fast-directed movement before fusion. (B) Two representative particles that remain quite static during cell entry. Left panel (A and B) shows images of a cell obtained with DIC optics. On top of the DIC image the trajectories of two single virus particles are projected. Trajectories are depicted color-coded, with purple representing the start and red representing the end of the trajectory. Fusion is indicated by a white star. Scale bar represents 25 µm. In the middle panels the trajectories are 5 times enlarged (scale bar represents 5 µm) for visual purposes. Right panel shows the relative velocity (in black) and fluorescence intensity (in red) of a single virus particle over time. A sudden increase in fluorescence intensity indicates the moment of fusion. The data correspond to the trajectories depicted in the left and middle panels of A and B. (C) Analysis of the percentage of fusion-active virus particles showing fast-directed and static trafficking during cell entry. In total 29 individual trajectories were analyzed. (D) Time of fusion analysis for particles exhibiting fast-directed or static movement. A total of 29 particles were analyzed, of which 14 showed fast-directed movement and 15 remained static. (E) BS-C-1 cells were transfected with clathrin-YFP (left panel) or Rab5-GFP (right panel) and subjected to dual-color single particle tracking using DiD-labelled CHIKV. A total of 9 and 10 trajectories showing fast-directed movement in BS-C-1 cells transiently expressing clathrin-YFP or Rab5-GFP were used, respectively.

We next assessed how frequent the two types of trafficking behaviour occurred during CHIKV cell entry. Forty-eight percent of the CHIKV particles exhibited fast-directed movement before fusion whereas the other 52% remained static during cell entry. This indicates that both trafficking behaviours are equally common (Figure 2C). Moreover, no substantial differences in the time to membrane fusion were observed (Figure 2D). Half of the viral particles that exhibited fast-directed movement (n=14) fused within 8 min post-infection (mpi). The virions that remained static during entry fused slightly earlier; half of these particles (n=15) fused within 7 mpi (Figure 2D).

**CHIKV fast-directed movement occurs after clathrin-mediated internalization yet before delivery to the early endosome**

We next aimed to determine at what stage of the CHIKV cell entry pathway fast-directed movement occurs. We and others previously published that CHIKV mainly enters mammalian cells via CME3–5. After internalization, the viral particles are delivered to Rab5-positive early endosomes, from which CHIKV fusion primarily occurs5,28. In order to assess whether the fast-directed movement occurs before or after internalization, we transfected BS-C-1 cells with clathrin-YFP and performed dual-colour single particle tracking using DiD-labelled CHIKV. In total 9 CHIKV trajectories displaying fast-directed movement in clathrin-YFP-transfected BS-C-1 cells were used. In all these trajectories, fast-directed movement was seen after co-localization of CHIKV with clathrin (Figure 2E left panel). These results indicate that fast-directed movement occurs after virus internalization and thus is an intracellular movement. We next zoomed in on the endocytic stages post CME, namely endocytic and endosomal trafficking. For this purpose, we tracked DiD-labelled CHIKV in BS-C-1 cells transfected with the early endosomal marker Rab5-GFP. Rab5-GFP was used as the vast majority of particles fuse from within Rab5-positive early endosomes5. Furthermore, based on our previous work we estimated that posterior to CME, CHIKV resides in a clathrin- and Rab5-negative endocytic vesicle for on average 14 s5. Thereafter, the virus is delivered to the Rab5-positive early endosome, where it resides for on average 38 s till membrane fusion occurs. Focusing on the trajectories
Figure 3. CHIKV fast-directed movement through the cell is mediated by microtubules. (A) Two representative CHIKV trajectories in nocodazole-treated cells, similar as described in the legend to Fig 1 A & B. (B) Analysis of the percentage of virus particles showing static or fast-directed movement in nocodazole-treated cells. A total of 36 individual trajectories were analyzed. (C) Microscopic fusion assay in BS-C-1 cells pretreated with nocodazole, as described in Materials and Methods. The graph represents four individual experiments. Error bars represent standard deviations (SD). (D) Analysis of the location of fusion. For untreated cells, 15 ‘static’ particles and 14 particles showing ‘fast-directed movement’ were analyzed. In nocodazole-treated cells, 34 particles were analyzed.
exhibiting fast-directed movement, we observed that in 9 out of 10 trajectories fast-directed movement occurred just before the co-localization between CHIKV and Rab5. In 1 trajectory fast-directed movement was observed when the virus was already co-localizing with the Rab5-positive early endosome (Figure 2E right panel). These results demonstrate that CHIKV fast-directed movement occurs after CME, but before delivery of the virus to the early endosome. Consequently, during fast-directed movement, CHIKV likely resides in a clathrin- and Rab5-negative endocytic vesicle.

Microtubules are required for CHIKV fast-directed movement, but not for membrane fusion

To investigate whether the fast-directed movement resembles transport along microtubules, we next performed tracking experiments in cells pre-treated with 10 µM of nocodazole. At these conditions, the microtubule network is completely disrupted (Figure S1A), yet no cytotoxicity was observed in the standard MTT assay (Figure S1C). Figure 3A shows two representative examples of CHIKV trajectories in nocodazole-treated cells. In both trajectories no fast-directed movements were observed. Furthermore, when plotting the velocity of the virus particle over time no sudden peaks in velocity were apparent (Figure 3A right panels). In total 36 CHIKV trajectories were recorded in nocodazole-treated cells. Fast-directed movements were observed in only 3 (8%) of these trajectories (Figure 3B). Thus, treatment with nocodazole was found to inhibit the occurrence of fast-directed movements over 80%.

Microtubule-dependent movements during endocytosis have been described for multiple viruses, and were repeatedly associated with fusion and infection\textsuperscript{13,14,17,18}. Interestingly, although nocodazole treatment impaired CHIKV fast-directed movement, membrane fusion could still be observed (Figure 3A). To more directly assess whether microtubule disruption affects CHIKV fusion, we employed a microscopic fusion assay\textsuperscript{5,23}. In this assay DiD-labelled CHIKV is allowed to enter and fuse within BS-C-1 cells for 30
min. Subsequently, unbound virus is washed away and 20 random images are taken using both DIC and DiD optics. The total extent of DiD fluorescence is taken as a measure of CHIKV fusion. In correspondence to our single particle tracking results, no impairment of CHIKV fusion was observed using the microscopic fusion assay (Figure 3C).

Microtubule disruption did however affect the location of CHIKV fusion (Figure 3D). Whereas under normal infection conditions the majority of the static particles fuse in the periphery, 64% of the particles that are transported via fast-directed movement fuse close to the perinuclear region. Upon nocodazole treatment, virtually no perinuclear fusion is seen. Consequently, the location of CHIKV fusion in nocodazole-treated cells largely resembles the location of fusion of static CHIKV particles.

**Nocodazole impairs CHIKV genome release**

Although microtubule-dependent movement during cell entry might be important in CHIKV infectivity, an alternative hypothesis is that microtubules aid in nucleocapsid delivery and/or uncoating. To investigate this possibility, we employed a cell fractionation protocol followed by the quantification of the CHIKV genomic RNA (gRNA) using qPCR. Cells were fractionated by use of digitonine treatment, which specifically permeabilizes the plasma membrane25. After fractionation, a cytosolic fraction containing the gRNA of the CHIKV particles that efficiently released their genome, and a vesicular fraction containing the gRNA of the unfused virions were obtained and used for qPCR. First, we checked the fractionation efficiency in three independent experiments by Western Blot. A representative blot is shown in Figure S3. As expected, the vast majority of both the vesicular marker LAMP-1 and the cytosolic marker GAPDH were found in the corresponding fractions. Upon nocodazole treatment, a 2-fold decrease in the quantity of cytosolic CHIKV gRNA was observed compared to that of the non-treated control (Figure 4). These results suggest that microtubules are indeed important in efficient release of the CHIKV genome.

**Discussion**

This study shows that microtubules play an important role during the early steps of CHIKV infection. Using real-time single virus tracking we showed that half of the CHIKV particles traffic along microtubules during cell entry. Microtubule-mediated trafficking occurred after clathrin mediated-endocytosis but before the delivery of the particles to the early endosome. Disruption of the microtubule network did not influence the membrane fusion efficiency of the virus yet altered the cellular location of fusion. Furthermore, microtubule disruption was found to impair CHIKV genome delivery.

Our findings suggest that the location of fusion may be important for efficient CHIKV genome delivery. Whereas the CHIKV fusion events of particles with a ‘static’ trafficking behaviour and in nocodazole-treated cells were almost exclusively seen in the periphery, the majority of particles that showed microtubule-dependent trafficking fused in the
perinuclear region. Trafficking towards the perinuclear region may thus be required for efficient genome delivery and infection. Indeed, two types of early endosomes were identified that differ in their motility and maturation kinetics. Rapidly maturing early endosomes are predominantly observed in the perinuclear region of the cell and delivery of cargo to these organelles was found microtubule-dependent. During endosomal maturation the membrane composition alters and it is possible that the rapidly maturing early endosomes in the perinuclear region have a different membrane composition than slowly maturing early endosomes. Taken together, microtubule-dependent trafficking of CHIKV may direct the virus to an organelle with a membrane composition that is favourable for CHIKV fusion pore formation and thus genome delivery.

Another option is that post CHIKV fusion microtubules are needed for the release and uncoating of the nucleocapsid. Banerjee et al. recently reported that both intact microtubules and actin filaments were involved in uncoating of the influenza A virus (IAV) genome. IAV fuses from late endosomal compartments after which the IAV capsid becomes exposed at the cytoplasmic side of the endosome. The IAV capsid was found to carry unanchored ubiquitin chains, thereby mimicking misfolded protein aggregates. These ubiquitin chains recruit components of the aggresome processing machinery, including the microtubule motors dynein and dynactin and the actin motor myosin II to the IAV capsid. While linked to the IAV capsid, these molecular motors are thought to generate opposing forces via which they break apart the IAV capsid. Also for HIV, microtubule motor proteins, such as dynein and kinesin, were shown to be important for nucleocapsid uncoating. Furthermore, data from Bernard et al., suggests that microtubules are required at a post-fusion step of CHIKV infection in HEK cells. Future studies should elucidate whether the microtubule and perhaps actin cytoskeleton are involved in CHIKV uncoating.

Although microtubules were previously implicated in alphavirus infection, the exact involvement of microtubules appears to differ. The infectious cycle of Sindbis virus and Venezuelan Equine Encephalitis Virus were found to be independent of microtubules, while for SFV microtubules were found to be important for intracellular trafficking of viral replication complexes. Although trafficking of the alphavirus replication complexes could be blocked by microtubule disruption, the effect of microtubule disruption on virus production was however limited. In contrast to SFV, CHIKV replication complexes remain on the cytosolic side of the plasma membrane for the entire duration of infection, and thus microtubules are not involved in intracellular trafficking of CHIKV replication complexes. Here, we show that microtubules are important during the early steps of CHIKV infection.

In conclusion, this study enhances our understanding on the interactions of CHIKV with the cytoskeleton by visualizing the complex trafficking patterns during CHIKV entry. Based on our data, we conclude that microtubules are required for efficient CHIKV genome delivery into the cytosol. Further studies are required to pinpoint via which mechanism microtubules affect the delivery of the CHIKV genome.
References

1. WHO. Chikungunya. at <http://www.who.int/mediacentre/factsheets/fs327/en/>
Figure S1. Nocodazole treatment controls. (A) Representative pictures of the α-tubulin staining in absence (left) and presence (right) of 10 µM nocodazole. Scale bar: 25 µm. (B, C) Cell viability of BS-C-1 cells upon treatment with nocodazole. BS-C-1 cells were treated overnight (B) or 2.5 h (C; corresponding to treatment duration for the fusion assay and tracking), after which cell viability was assessed using standard MTT assay. For both conditions two experiments were performed in triplicate. Error bars represent SD.
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Figure S2. Translation of viral proteins. Flow cytometry analysis of translation efficiency in presence of nocodazole. BS-C-1 cells were pretreated for 2 h with 10 µM nocodazole and infected with CHIKV LS3-GFP for 10 h. Mean fluorescence intensity (MFI) of the infected population is normalized to the positive control. Data represents two individual experiments in triplicate; error bar represents SD.

Figure S3. Cell fractionation control. WB analysis of cell fractionation by digitonine. GAPDH was used as a cytosolic marker, whereas LAMP-1 was used as a marker for the vesicular fraction. The cytosolic fraction is indicated by C and the vesicular fraction by V. Three independent fractionations were performed; image shows one representative blot.

Supplementary Movie Legends

NB: the supplementary movies are not available on hardcopy. Please wait until paper is published.

Video S1. CHIKV trajectory showing fast-directed movement. Movie showing a the trajectory of a single virus particle displaying fast-directed movement before fusion. The trajectory is depicted color-coded, with purple and red representing the start end of the trajectory, respectively. Recording was performed at 1 frame/s. Playback time is 15 frames/s. Virtual time and the color code for time are shown in the right down corner.

Video S2. Trajectory of a CHIKV particle remaining relatively static during entry. The trajectory is recorded and depicted as Video S1. Playback time is 30 frames/s.

Video S3. Two examples of CHIKV trajectories in nocodazole-treated cells. The trajectory is recorded and depicted as Video S1. Playback time is 15 frames/s for A and 10 frames/s for B, respectively.
PART II