A microfluidic platform for real-time and *in situ* monitoring of virus infection process

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Microfluidic chip is a promising platform for studying virus behaviors at the cell level. However, only a few chip-based studies on virus infection have been reported. Here, a three-layer microfluidic chip with low shear stress was designed to monitor the infection process of a recombinant Pseudorabies virus (GFP-PrV) in real time and *in situ*, which could express green fluorescent protein during the genome replication. The infection and proliferation characteristics of GFP-PrV were measured by monitoring the fluorescence intensity of GFP and determining the one-step growth curve. It was found that the infection behaviors of GFP-PrV in the host cells could hardly be influenced by the microenvironment in the microfluidic chip. Furthermore, the results of drug inhibition assays on the microfluidic chip with a tree-like concentration gradient generator showed that one of the infection pathways of GFP-PrV in the host cells was microtubule-dependent. This work established a promising microfluidic platform for the research on virus infection. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4756793>]

I. INTRODUCTION

Viruses have long threatened the global health due to the rapid infection, fast dissemination, and difficulty in detection.^{1,2} It is fundamental to understand the virus infection process for effectively reducing the damage caused by viruses. For most virus detection techniques, such as polymerase chain reaction (PCR), western blot, flow cytometry, and transmission electron microscopy (TEM),^{3–7} the virus samples are often obtained from the infection system at a certain time point, which interrupts the virus infection process. These techniques cannot be applied to monitor the dynamic behaviors of virus infection in real time and *in situ*. Real-time and *in situ* techniques have played an important role in revealing viral infection mechanism.⁸ Single-virus tracking is a powerful real-time imaging technique to visualize the virus transport in live cells by labeling the virus with the fluorescent molecules.^{8–11} However, a large number of virus particles are needed to cause a productive infection which is a great challenge for the precious and rare samples.⁸ So, it is necessary to establish a miniaturized platform with low consumption of virus for the research on virus infection.

In recent years, microfluidic chip has been widely applied to cell biology^{12,13} due to the advantages of good biocompatibility, low reagent consumption, and high throughput. These researches make it possible to study the cell-based virus behavior on the microfluidic platform. Nevertheless, most virus researches based on the microfluidic chips have focused on the development of virus detection techniques, such as chip-based PCR^{14–16} and only a few works on virus

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infection have been reported. The microfluidic chips have been used to generate different concentrations of virus to infect cells,¹⁷ enhance the plaque formation to improve the detection sensitivity,¹⁸ produce retrovirus continuously,¹⁹ and increase the infection efficiency of adenovirus.²⁰ Additionally, the neuron-to-cell spread of Pseudorabies virus (PrV) has been observed by guiding the growth of neuron axon in the microfluidic channels.²¹ Although these studies have displayed the applications of microfluidic chip to the virus infection, the virus infection process in live cells has never been studied in real time and *in situ*, which is fundamental for virus researches.

One great advantage of microfluidic chip is the high throughput. The tree-like microstructure²² to generate different concentration gradients is a useful design to improve the throughput and has been used to study gene expression, cell response to stimuli and cell-based cancer drug screening.^{23–26} The high-throughput capability of this design also makes microfluidic chip an attractive platform for the study of virus infection mechanism.

Here, a three-layer microfluidic chip with low shear stress was developed to monitor the infection process of a recombinant PrV (GFP-PrV) in real time and *in situ*. PrV is a swine alphaherpesvirus with significant homology to the human pathogens herpes simplex virus type 1 (HSV-1), HSV-2, and varicella-zoster virus (VZV).²⁷ It has been well characterized and widely used as a model to elucidate the molecular biology of herpes viruses. The virus infection process was monitored by tracking the fluorescence intensity of GFP and determining the one-step growth curve. These results were compared with those obtained on traditional Petri dishes. Moreover, nocodazole, a drug which can disrupt the microtubules, was used to study the infection pathway of GFP-PrV in the host cells on a high-throughput microfluidic chip with a tree-like concentration gradient generator.

II. MATERIALS AND METHODS

A. Chip design and fabrication

The three-layer microfluidic chip for virus infection (overall dimensions: about 30 mm × 20 mm × 6 mm, Fig. 1(a)) comprised three layers: (i) a glass slide as the substrate; (ii) a piece of thin polydimethylsiloxane (PDMS, GE Toshiba Silicones Co., Ltd., Japan) membrane

FIG. 1. (a) The schematic diagram of the three-layer microfluidic chip for virus infection. The chip comprised: (i) a glass slide as the substrate; (ii) a PDMS middle layer with eight cell culture chambers (diameter × height: 1 mm × 200 μm) and microfluidic channels (width × height: 200 μm × 40 μm); (iii) a PDMS upper layer with microfluidic channels (width × height: 200 μm × 40 μm). (b) The schematic diagram of the microfluidic chip for drug inhibition with a concentration gradient generator in the upper layer and six cell culture chambers in the middle layer. (c) A photograph of the three-layer microfluidic chip for virus infection (length × width × height: ~30 mm × 20 mm × 6 mm). (d) The schematic diagram of the side view of the three-layer microfluidic chip. It clearly showed the step flow in the cell culture chamber.

(height: $200\ \mu\text{m}$) with microfluidic channels and eight parallel cell culture chambers (diameter $\times$ height: $1\ \text{mm} \times 200\ \mu\text{m}$) as the middle layer; (iii) a covered PDMS with microfluidic channels as the upper layer. The dimensions of all microfluidic channels were the same (width $\times$ height: $200\ \mu\text{m} \times 40\ \mu\text{m}$). The microfluidic chip for drug inhibition (Fig. 1(b)) was composed of a tree-like concentration gradient generator in the upper layer and six cell culture chambers in the middle layer.

All patterns were transferred from masks to silicon wafers by the standard soft photolithography technique. The positive photoresist AZ 50XT (AZ Electronic Materials USA Corp, USA) was spin-coated onto a silicon wafer to obtain a $40\ \mu\text{m}$ -thick film. After exposure with a mask under UV light, the photoresist was developed in the developer (1:2 v/v AZ400K/H₂O) to obtain the patterns.

A brief procedure of chip fabrication was illustrated as follows. The degassed mixture of PDMS monomer and cross-linking catalyst (mass ratio of 10:1) was spin-coated onto the mould to obtain the middle layer with the height of $200\ \mu\text{m}$ at the speed of 600 rpm for 60 s. The upper layer was obtained by pouring the mixture onto the mould. After the mixture cured at 75°C for 3 h, the solid polymers were peeled off. The cell culture chambers in the middle layer were punched by flat needles. Then, the upper layer, the middle layer, and the glass substrate were irreversibly bonded in sequence after treated by oxygen plasma. All chips and tubes were sterilized by an autoclave before cell culture.

The fluid velocity distributions of the step flow in the cell culture chambers with different heights were simulated by COMSOL MULTIPHYSICS 3.5 a software.

B. Cell culture

African green monkey kidney (Vero) cells were obtained from China Center for Type Culture Collection (Wuhan University, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen). After suspension, Vero cells (1.0×10^6 cell/ml) were injected into the microfluidic chip and then the fresh culture medium was perfused at a flow rate of $20\ \mu\text{l/h}$. After cultured on the microfluidic chip at 37°C with 5% CO₂ for 24 h, the cells were imaged on an inverted microscope (TE2000-U, Nikon, Japan) equipped with a CCD camera (Retiga 2000 R, Qimaging, Canada) and then were counted manually.

C. Virus infection and one-step growth curve

All experiments about virus infection were performed in DMEM supplemented with 2% FBS (named maintenance medium). Vero cells were exposed to GFP-PrV at 37°C for 1 h and then the citrate buffer (40 mmol/l sodium citrate, 10 mmol/l KCl, 135 mmol/l NaCl, $\text{pH} = 3.0$) was added to inactivate the extracellular viruses. The maintenance medium was continuously supplied through the whole experiment. The microfluidic chip was kept in an online cell culture system (INUBG2-PI) connected with a confocal microscope (Andor Revolution XD). The images were captured every 15 min by an EMCCD (Andor iXon DV885K Single Photon Detector) and analyzed by ANDOR IQ software. Meanwhile, the effluents from the microfluidic chip were collected at certain time points to obtain the one-step growth curve of GFP-PrV by determining virus titers. Vero cells were infected at a multiplicity of infection (MOI) of 10 which represents the average number of viral particles per cell. High MOI was used here to ensure that each cell could be infected.

D. Drug inhibition

Vero cells were cultured on the microfluidic chip for 24 h and then $10\ \mu\text{g/ml}$ nocodazole and the maintenance medium were perfused into the chip through the two inlets respectively to generate different concentrations of nocodazole: 0, 2, 4, 6, 8, $10\ \mu\text{g/ml}$. After 1 h pretreatment of Vero cells with nocodazole, GFP-PrV was injected into the chip rapidly and incubated with Vero cells for another 1 h (MOI = 10). Then $10\ \mu\text{g/ml}$ nocodazole and the maintenance medium were added at a flow rate of $20\ \mu\text{l/h}$ and perfused continuously during the whole experiments.

The effluents between 0 and 24 h of post infection (hpi) from the six channels were collected, respectively, to test the inhibition effect of nocodazole by determining virus titers.

III. RESULTS AND DISCUSSION

A. Step flow in the three-layer microfluidic chip

In order to monitor the virus infection process, we designed a three-layer microfluidic chip (Fig. 1(a)) to reduce the shear stress which might cause detrimental effects on cells.^{28,29}

In the three-layer microfluidic chip, the height of cell culture chamber was much higher than that of microfluidic channel, and the inlet and the outlet were in different layers, so a step flow (Fig. 1(d)) could be achieved.³⁰ Assuming that the medium is a steady and incompressible fluid, the velocity distributions of the step flow at different chamber heights of 40 μm (the same height as the microchannels), 200 μm , 400 μm , and 600 μm were simulated at a flow rate of 20 $\mu\text{l/h}$. The surface and arrow plots of the middle plane in x axis were shown in Figs. 2(a)–2(d). The surface plots illustrated that the shear stress in the cell culture chamber of the three-layer microfluidic chip (blue region) was lower than $2.0 \times 10^{-3} \text{ dyn/cm}^2$ (Fig. 2(e)). The arrow plots showed that the step flow in the three-layer microfluidic chip had a sub-vector in the vertical direction. For the 2D parabolic flow, the shear stress at the wall between parallel plates can be determined by the following formula:

$$\tau = \frac{6\mu Q}{h^2 w},$$

where μ is viscosity ($\text{kg}/(\text{m} \cdot \text{s})$), Q is flow rate (m^3/s), h is height (m), and w is width (m).³¹ For the step flow, the shear stress was reduced much more than the 2D flow due to the vertical

FIG. 2. (a)–(d) The velocity distributions of the step flow in the middle plane of x axis when the chamber heights were 40 μm , 200 μm , 400 μm , and 600 μm , respectively ($Q = 20 \mu\text{l/h}$). (e) The shear stress of the step flow at $z = 20 \mu\text{m}$. When the chamber height was 40 μm , the shear stress in the chamber (square dots) was incapable displayed which showed that the shear stress was higher than 0.18 dyn/cm^2 . The shear stress in the chambers of the three-layer microfluidic chip was lower than $2.0 \times 10^{-3} \text{ dyn/cm}^2$. (f) The shear stress at the point ($x, y, z = 1/2$ chamber diameter, $1/4$ chamber diameter, 20 μm) of the cell culture chamber in the 2D flow (round dots) and the step flow (square dots).

component vector of the fluid. When the height of cell culture chamber increased from $40\text{ }\mu\text{m}$ to $200\text{ }\mu\text{m}$, the shear stress of the 2D flow at the position of one quarter of the chambers decreased 25 times according to the formula, from 0.44 to $1.76 \times 10^{-3}\text{ dyn/cm}^2$, while the shear stress of the step flow decreased to $7.49 \times 10^{-4}\text{ dyn/cm}^2$ (Fig. 2(f)). Taking into account the fabrication accessibility, we optimized the chamber height as $200\text{ }\mu\text{m}$.

In addition, the three-layer microfluidic chip would facilitate the form of confluent cell monolayer because the chamber height of the three-layer chip was much higher than that of one-layer designs.^{32,33} When the cells were continuously loaded, the synergistic effect of the gravity and the obstruction of the chamber wall would make more and more cells settle down, so the cell density in the chamber could be regulated to maximum in a few seconds and the cell culture time could be greatly reduced (Fig. 3). This design is also useful for the studies based on the confluent cell monolayer.

B. Fluorescence monitoring

The recombinant PrV, which was generated by inserting a GFP expression cassette into PrV genome at the thymidine kinase gene through the homologous recombination (unpublished), was used in this study. The virus infection process in continuous 24 hpi was monitored in real time and *in situ* (as shown in the movie in the supporting information) by tracking the fluorescence intensity of GFP which was correlated with the replication of GFP-PrV genome. As shown in Fig. 4(a), the morphology of Vero cells was still normal and no fluorescence appeared at 1 hpi. The fluorescence of GFP was detected at 3 hpi (Fig. 4(b)) and the intensity gradually increased until 15 hpi (Fig. 4(f)). Distinct cytopathic effect (CPE) appeared at about 9 hpi (Fig. 4(d), the cell in the white circle). The infected cells gradually became round and swollen (e.g., the cell in the white circle in Figs. 4(g)–4(i)). The information of CPE and virus replication could be extracted simultaneously on the microfluidic platform by monitoring the cell morphology and the fluorescence intensity of GFP.

To distinguish the details of virus infection process, the fluorescence intensity of group cells during the infection was analyzed (Fig. 5(a), square dots). The fluorescence intensity of group cells on the chip began to rise at about 3 hpi and showed a linear growth between 3 and 15 hpi, which indicated that PrV viral proteins could be detectable at 3 hpi²⁷ and then were progressively accumulated. Then the increase rate of the fluorescence intensity became slow until a maximum was reached at about 20 hpi. The results of the assays on Petri dishes (Fig. 5(a), round dots) were similar as those obtained on microfluidic chips which indicated that the behaviors of GFP-PrV infection in the host cells could hardly be influenced by the microenvironment in the microfluidic chip.

The virus infection chip with eight independent cell culture chambers was designed for parallel experiments. The similar increase tendency of the fluorescence intensity on the eight cell culture chambers (Fig. 5(b)) was obtained which showed that microfluidic chip could be used to improve the throughput of virus research.

FIG. 3. Vero cells cultured on the three-layer microfluidic chip for 1 h (a) and 17 h (b).

FIG. 4. The CPE of Vero cells and the GFP expression during the infection process of GFP-PrV on a microfluidic chamber (MOI = 10). (a)-(i) Merged images of the phase contrast image and the fluorescence image at 1, 3, 6, 9, 12, 15, 18, 21, 24 hpi, respectively. Two cells were highlighted to illustrate the CPE caused by GFP-PrV (enhanced online). [URL: <http://dx.doi.org/10.1063/1.4756793>]

Single-cell analysis is important for revealing the differences among cells because even genetically identical cells may have variations in observed behaviors.^{34,35} Hence, the fluorescence intensities of infected single cells and a typical single cell were analyzed in Figs. 5(c) and 5(d), respectively. The fluorescence intensities of single cells began rise at 3 hpi, showed a linear growth between 3 and 15 hpi and grew to a maximum after 15 hpi, which was the same as that of group cells (Fig. 5(a)). The results showed that there were no significant differences of observed behaviors among infected cells in our study which was consistent with the hypothesis that all cells should be infected simultaneously when the MOI is high enough. These results supplied a powerful support for further studies of virus infection mechanism on the microfluidic platform.

C. One-step growth curve

One-step growth curve is a basic and direct evidence to quantitatively investigate the growth kinetics of virus.³⁶ It is a time-consuming and laborious work due to the long-term sample collection and the titration of numerous samples. Microfluidic chip can be used to simplify the sample collection process, as the effluents from the chip can be easily harvested. The one-step growth curve of GFP-PrV infection on our chip was shown in Fig. 6. GFP-PrV was in a period of rapid growth between 12 and 36 hpi (burst period) and then the production of viral progeny reached a plateau. In the burst period, the titers of GFP-PrV on microfluidic chips were lower than those on Petri dishes at the same time point. The difference might be caused by the virus adsorption to PDMS¹⁹ because the surface of PDMS could adsorb proteins.³⁷ The virus titers at the plateau of infection on microfluidic chips and on Petri dishes were equivalent which indicated that the microfluidic chip was able to produce virus progeny efficiently.

D. Drug inhibition on virus infection

Furthermore, we also concerned the transport pathway of viral particles in the host cells on the microfluidic chip. Herein, nocodazole, a drug which can disrupt the microtubules, was used

FIG. 5. (a) The fluorescence intensity of group cells during the GFP-PrV infection on microfluidic chips and on Petri dishes ($n = 16$). (b) The fluorescence intensity of group cells during the GFP-PrV infection on eight cell culture chambers in a chip. (c) The fluorescence intensity of single cells ($n = 11$). (d) The fluorescence intensity of a typical single cell. (MOI = 10)

to study the infection pathway of GFP-PrV. To improve the throughput, the tree-like concentration gradient generator was combined with the microfluidic chip in the upper layer. As shown in Figs. 7(a)–7(f), the number of cells expressing GFP reduced with the increase of the concentration of nocodazole. The maximal inhibition ratio of about 98% was obtained with 10 $\mu\text{g/ml}$ nocodazole by determining virus titers (Fig. 7(g)). It indicated that one of the infection pathways of GFP-PrV was microtubule-dependent, which was comparable with previous studies.^{38–40} However, even at

FIG. 6. The one-step growth curve of GFP-PrV infection on microfluidic chips and on Petri dishes ($n = 3$). (MOI = 10)

FIG. 7. Nocodazole inhibition of GFP-PrV infection on the microfluidic chip (MOI = 10). (a)-(f) The images of Vero cells infected by GFP-PrV with different concentrations of nocodazole 0, 2, 4, 6, 8, 10 µg/ml. The number of cells expressing GFP reduced with the increase of the concentration of nocodazole. (g) The inhibition effect of different concentrations of nocodazole ($n = 5$).

the maximal concentration of nocodazole, the virus infection could not be inhibited completely which suggested that GFP-PrV might infect the host cells through other pathways.³⁸

IV. CONCLUSIONS

A microfluidic chip has been developed for real-time and *in situ* monitoring of GFP-PrV infection process. The fluorescence intensity increase tendency of GFP and the one-step growth curve during the infection on microfluidic chips were similar as those obtained on Petri dishes. It was proved that the microenvironment in the microfluidic chip had little influence on the infection behavior of GFP-PrV in the host cells. Moreover, the results of drug inhibition assay performed on the microfluidic chip with a concentration gradient generator indicated that one of the infection pathways of GFP-PrV in the host cells was microtubule-dependent. Our work provided the basis for further studies of virus infection on microfluidic chip. The new platform would also be applied to virus transfection, drug screening, and so on.

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