Microfluidic-driven viral infection on cell cultures: Theoretical and experimental study

Elisa Cimetta,^{1,a),b)} Mauro Franzoso,^{1,2,a)} Marta Trevisan,^{3,a)}
 Elena Serena,^{1,2} Alessandro Zambon,^{1,b)} Stefano Giulitti,^{1,2,b)}
 Luisa Barzon,^{3,a)} and Nicola Elvassore^{1,2,a),b),c)}

¹*Department of Chemical Engineering, University of Padova, via Marzolo 9, I-35131 Padova, Italy*

²*Venetian Institute of Molecular Medicine, Padova, Italy*

³*Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, via Gabelli 63, I-35131 Padova, Italy*

(Received 22 December 2011; accepted 15 May 2012; published online 4 June 2012)

Advanced cell culture systems creating a controlled and predictable microenvironment together with computational modeling may be useful tools to optimize the efficiency of cell infections. In this paper, we will present a phenomenological study of a virus-host infection system, and the development of a multilayered microfluidic platform used to accurately tune the virus delivery from a diffusive-limited regime to a convective-dominated regime. Mathematical models predicted the convective-diffusive regimes developed within the system itself and determined the dominating mass transport phenomena. Adenoviral vectors carrying the enhanced green fluorescent protein (EGFP) transgene were used at different multiplicities of infection (MOI) to infect multiple cell types, both in standard static and in perfused conditions. Our results validate the mathematical models and demonstrate how the infection processes through perfusion via microfluidic platform led to an enhancement of adenoviral infection efficiency even at low MOIs. This was particularly evident at the longer time points, since the establishment of steady-state condition guaranteed a constant viral concentration close to cells, thus strengthening the efficiency of infection. Finally, we introduced the concept of effective MOI, a more appropriate variable for microfluidic infections that considers the number of adenoviruses in solution per cell at a certain time. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4723853>]

I. INTRODUCTION

The importance of performing efficient and controlled viral infections on mammalian cell cultures has long been crucial to optimize the gene transfer procedures for basic research and gene therapy.¹⁻⁴

The common denominator is the necessity of increasing viral infection efficiency while preserving viability and biological processes of the cultured cells. The use of adenoviruses, non-integrating viruses, preserve genomic integrity and offer reduced risks for human safety. Moreover, process automatization, low volumes of reagents, and reduced costs are desirable. Standard procedures for culture infection involve virus dilution in the media to defined concentrations, usually quantified by the multiplicities of infection (MOI), representing the number of viral particles per cell. Efficiency of transfection of some cell types may be low, thus requiring high MOIs potentially resulting in toxic side effects on the cells.

In parallel, the advent and ever-increasing use of microscaled technologies and microfluidic devices for lab-on-a-chip applications has led to relevant improvements in the study of complex

^{a)}E. Cimetta, M. Franzoso, M. Trevisan, L. Barzon, and N. Elvassore performed infection experiments.

^{b)}A. Zambon, S. Giulitti, E. Cimetta, and N. Elvassore developed microfluidic platform and cell culture integration.

^{c)}Electronic mail: nicola.elvassore@unipd.it.

biological systems.^{5–10} Examples of applications of microfluidic platforms have been extensively reviewed^{11,12} and point at the advantages deriving from the miniaturization, integration, and automation of biochemical assays. Recent literature reflects increased interest in adopting microfluidic devices in drug discovery process,^{13,14} molecular detection,¹⁵ and in clinical and medical research.¹⁶ In order to efficaciously control and exploit their potential, it is fundamental to understand the physics of mass-transport phenomena and of fluid flows at the microscale^{17,18} and the fabrication processes, and properties of typically used materials.^{19–21}

Despite the advantages and versatile applications deriving from microfluidic platforms, only few studies combining these devices and viral infections of cultured cells can be found in the literature. Examples include some applications of microfluidic bioreactors for the continuous production of retroviral vectors,²² or the dielectrophoretic capture and imaging of viral particles on microelectrodes.²³ A microscale platform was developed to detect and quantify virus growth and spread²⁴ and micropatterning has been used to characterize the *in vitro* propagation of viruses in cell arrays.²⁵ Cells were infected using virus gradients,²⁶ but the biological readout showed a low number of cells within the microchannels, and virus replication studies were performed on hepatocytes seeded within micro cell-culture chambers.²⁷ However, neither rational studies on the influence of perfusion nor a screening of the infection parameters were performed. Finally, most of these systems suffered some of the major limitations deriving from culturing cells within standard microfluidic channels such as lower growth rates, and the need for frequent changes of media during the preliminary phases.

Here, we develop a microfluidic platform that can be easily and reversibly coupled to cell cultures, that allows performing multi-parametric experiments and exerting a precise control over the soluble extracellular microenvironment, thus increasing the efficiency of infection. Our microfluidic device is used for the optimization of the process of cell infection through an approach that combines mathematical modeling with the experimental validation. On one hand, mathematical models evaluate the transport phenomena and the dominating regimes within a defined system, while experiments, on the other, analyze static and perfused microfluidic-driven infection processes, validating the modeled conditions and demonstrating that our microfluidic platform allows increasing the infection efficiency when compared to static conditions, even at the lowest MOIs.

Infections are usually carried out in standard culture plates at defined MOIs and since the efficiency of infection is proportional to the virus adsorbed on the cellular membrane, the minimization of the total volume of viral suspension is crucial to favor the contact between viral particles and adhering cells. Viral particles are uniformly dispersed in the solvent, and their transport from the bulk of the liquid to the cell surface is purely driven by brownian-like diffusion. However, in microfluidic experimental setup, mass transport of particles is driven by both diffusion and convection phenomena. In particular, diffusion has a driving force represented by a difference in concentration (Δc), while convection results from a bulk velocity of the fluid. Consequently, convection gives an additional contribution enhancing the transport of viral particles to the cells, thus increasing the efficiency of infection.

It will be crucial to define a method to compare the results of static and microfluidic-perfused conditions in terms of infection efficiency. In order to have fair comparison between static and perfused conditions, a proper experimental design has been proposed to maintain the same concentration, MOI, and total volume of medium. This experimental design will allow to highlight the influence of different intrinsic properties of the hydrodynamic regimes (static and perfused) on infection efficiency. Mathematical modeling will allow to analyze the theoretical variations of the ratio of virus fluxes in static and perfused conditions and derives the optimal operative variables such as flow rates and infection times.

II. MATERIALS AND METHODS

A. Cell culture

Mouse embryonic fibroblasts (MEFs) were purchased from Chemicon and were cultured in 79% Iscove's modified Dulbecco's medium (IMDM, Invitrogen), 20% foetal bovine serum (FBS, Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Human foreskin fibroblasts

(HFFs) were supplied by Dr. L. Barzon from the University of Padova and were cultured in 89% Dulbecco's modified eagles medium (DMEM, Sigma-Aldrich), 10% FBS (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Passaging of both cultures was performed with Trypsin 0.025%-EDTA (Invitrogen) and cells were either re-plated on culture flasks for further expansion or seeded on glass coverslips, both coated with 0.66% A-type pork gelatin (Sigma-Aldrich).

B. Microfluidic platform

The multilayered microfluidic platform (overall dimensions: 75×50 mm) was designed for an easy interface with the cell system, and fabricated using lithographic techniques and molded in poly-dimethylsiloxane (PDMS).²⁸

The platform (Figure 1(A)) comprised: (i) a supporting glass slide with a PDMS slab carved to accommodate the cell culture coverslip, (ii) a membrane-based vacuum system for its reversible sealing, and (iii) the microfluidic channels (width $\times$ height 0.2×0.1 mm) delivering fluids to the cultured cells. The circular channel creating the suction sealing the two layers, faces the PDMS slab in (i) and is thermally (and irreversibly) bonded to the upper microfluidic layer (iii). The assembled platform formed a 16×16 mm culture chamber, area in which the cultured cells were exposed to the fluid streams from the microfluidic channels. The height of the chamber could be varied as needed; for all the experiments presented here we used 0.5 mm high membranes. The platform was entirely optically transparent, allowing in-line observations of the cultures by easy interface with standard microscopes (Figure 1(B)). The micro-perfusion apparatus (Figure 1(C)) was composed of the multilayered microfluidic platform, two syringe pumps (PHD, Harvard Apparatus, Holliston, MA), and a vacuum control system (membrane pump H35M and digital vacuum sensor, Vuototecnica, Italy). All connections between components were made using Tygon tubings (0.5 mm ID, 1.5 mm OD, Cole Palmer, USA).

FIG. 1. Platform design and experimental setup. Panel (A): The platform comprised (i) a supporting glass slide with a PDMS slab carved to accommodate the cell culture coverslip (f), (ii) a membrane-based vacuum system for the reversible sealing of (i), and (iii) the microfluidic channels, 0.2×0.1 mm ($w \times h$), delivering fluids to the cultured cells (inlets in (a) and outlets in (b)). The assembled platform formed a $16 \times 16 \times 0.5$ mm culture chamber (c) where cells were exposed to the fluid streams. The top layer embedded connections to the vacuum system (d) and to a pressure-monitoring auxiliary service (e). Panel (B) reports an image of the assembled platform, which was entirely optically transparent, operated flowing a color tracer (fluorescein) in 2 of the 8 channels. Panel (C): The micro-perfusion apparatus was essentially composed by the multilayered microfluidic platform, two syringe pumps, and a vacuum control system. The interface with a fluorescence microscope equipped with an environmental chamber is shown.

A brief description of the experimental procedures follows. Before assembling and coupling to the cell cultures, all components, connections, and tubings were rinsed with water and then sterilized via autoclave treatment. Tubings were then rinsed with sterile culture medium and incubated for at least 1 h prior to the platform assembly. This preconditioning of the tubings' walls reduced the potential loss of viral particles due to undesired adsorption. Sterile 3 ml syringes to be connected to the platforms outlets were filled with 500 μl of sterile PBS, to avoid the elastic effect of air, and connected to the microtubes exiting the platform. Sterile 3 ml syringes to be connected to the platforms inlets were filled with culture medium. The open cell chamber was covered with 1 ml of culture medium and the syringe-pumps activated to stabilize the fluid flow and eliminate any residual bubble. Finally, the glass coverslip with the cultured cells was coupled to the lower layer, the entire platform assembled and the vacuum system ensuring hydraulic sealing activated. The multiple inlet and outlet channels allowed creating highly compartmentalized fluid regions within the culture chamber, thus increasing the throughput of the system potentially consenting to test several levels for a variable (i.e., virus MOIs) at a single time (Figure 2).

C. Fluid dynamics modeling

The Navier-Stokes equations for incompressible fluids were numerically solved using the finite elements method implemented in COMSOL Multiphysics (Burlington, MA). The 2D domain of the culture chamber was geometrically modeled and a non-structured mesh was automatically generated with triangular elements. Subsequent grid refinements were required to ensure independency of the solution from the spatial discretization. No-slip boundary conditions were used for the chamber and microfluidic conduits walls, a fixed velocity for the inlet channel and finally zero pressure for the outlet. The fluid properties viscosity and density were taken from the literature.²⁹

To obtain concentration profiles within the chambers, the mass balance equations for a convective-diffusive regime were solved again using COMSOL Multiphysics software (Burlington, MA, USA). Fluid velocity profiles were obtained from the Navier-Stokes solutions. Defined concentrations were used as boundary conditions at the different inlets, convective flux at the outlets and insulation/symmetry elsewhere. The diffusion coefficient for the adenoviral vector (AdV) was calculated from the Stokes-Einstein equation.³⁰ The diffusion coefficient of a virus particle, approximated by a 90 nm hydrodynamic diameter, was assumed to be $6.0 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$.

Fundamental assumptions of our modeling approach follows. Focusing on the resistances within the media compartment, we assumed that the virus adsorption was much faster (steady-state assumption) than the mass transport. We also assumed that all intracellular phenomena

FIG. 2. Model validation. Panel (a) reports representative results of the mathematical modeling showing concentration maps within the culture chamber. For a defined molecular species with its diffusion coefficient and fixed systems geometrical specifications, increases in the fluid flow rate change the shape of the compartment. Transport phenomena span from diffusion- to convection-dominated regimes following increases in flow rate. Panel (b) shows merged fluorescent images reconstructing the entire culture chamber, acquired during the experimental runs performed using parameters equal to the modeled ones.

related to viral protein expression such as virus internalization, virus decay, and protein production, were not affected by the velocity profile in the media compartment. Within these assumptions, the calculated virus molar flux at cell membrane could be considered directly related to the efficiency of infection. The ratio between virus molar fluxes of dynamic and static conditions is defined as the theoretical relative efficiency, Φ_r . In this work, we compared Φ_r as a function of different parameters such as MOI, time of infection, and hydrodynamic regime. In particular, hydrodynamic regime was described through the dimensional Péclet number defined as $Pe = vH/D$, where v is the velocity, H is a characteristic length (chamber height in our case), and D is the diffusion coefficient. This dimensionless variable identifies flow rate conditions at defined geometrical constraints into specific diffusional or convective transport regimes.

Empirical calculations were also performed (fluxes were evaluated as a function of time, diffusion coefficient, volumes) and used as a comparison to validate the mathematical modeling (data not shown). Cell densities, MOIs, viral concentrations, and infection times were kept constant at their optimized values.

In addition, during the preliminary design and development phases, experimental validation of the modeled fluid compartmentalization in multichannel platform was performed using fluorescein dye as a tracer. Figure 2 shows the good agreement between model prediction and experimental analysis allowing model prediction of small diffusing particles. Supplementary Figure S1 reports additional data quantifying the fluorescence levels of the experimental images (directly correlated to concentration values).³¹ These plots can be compared to the analogous concentration curves obtained by the modeled concentration maps.

III. INFECTION PROTOCOLS

A. Static condition

AdVs carrying the EGFP transgene were used at different MOI to infect multiple cell lines, both in static or in perfused conditions. Briefly, AdV is based on the Ad5 genome and lacking the E1 and E3 regions was constructed by homologous recombination in *E. coli* using AdEasy vector system (Qbiogene, Carlsbad, CA). In this vector, human cytomegalovirus promoter was used to drive expression of green fluorescent protein. AdEGFP was propagated in E1-complementing HEK 293 cells, purified by cesium chloride density centrifugation, and titrated by TCID50 cpe endpoint assay according to the AdEasy production protocol. Viral vector stocks were stored at 5.0×10^9 pfu/ml concentration in 10% glycerol at -80°C until use.

The infection efficiency was evaluated at different time-points post-infection quantifying the EGFP expression on the live samples via image analysis. Cells were seeded on gelatin coated coverslips 24 h before infection; the volume of the viral high-titer stock solution to be used was calculated for any given cell density and experimental MOIs. The viral stock solution was thawed and aliquots prepared and diluted to the final volume with the required culture medium. Cell cultures were then incubated (37°C , 5% CO_2 , 95% humidity) with the viral solutions for defined times. Cells were rinsed with warm PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Gibco) and re-incubated with culture medium. Post-infection incubation time varied depending on the experiment. In time-course runs, cells were re-incubated for up to 3 days. Images were acquired 48 h post infection.

B. Microfluidic perfused conditions

All of the above described procedures were followed, with the sole difference that the viral suspension was loaded in 3 ml syringes and connected to the assigned inlet channels. Particular attention had to be paid at calculating the exact viral particles number which would ensure correspondence between the static and perfused infections.

C. Measurement of the infection efficiency

At the established time points, cell cultures were incubated with Hoechst 33342 (Invitrogen) nuclear dye. After this assay, images of randomly chosen positions were acquired

(microscope Leica DMI 6000-B) on both fluorescence channels: blue for Hoechst marking all cell nuclei and green for the cytoplasmic EGFP signal expressed by the infected cells only. In order to obtain comparable set of data, the exposure, gain, and intensity values should be the same in every image. Quantification of these results was performed via image analysis on paired fluorescence pictures (blue and green channels). A custom developed script listing the command lines was implemented and run in MATLAB. Briefly, this script organized images in pairs, converted them in grayscale, enhanced contrast and finally converted them in binary format. Further processing allowed removing cell-debris, a potential source of quantification errors. The binary image of the nuclei was used to automatically count the total number of cells. After that, a pixel by pixel subtraction between the two binary images produced a new matrix creating the final image showing only the nuclei of successfully infected cells. Automated counting led to the obtainment of the number of infected cells and of the global efficiency of infection (number of infected cells over total number of cells).

IV. RESULTS

A. Model validation

The capability of the platform to generate well-defined concentration compartments was first modeled and then validated using fluorescein as a dye tracer. The results of the mathematical modeling are shown in Figure 2(a), where the sole culture chamber is represented for ease of visualization. The shape of the compartment can be precisely defined by simply tuning the fluid flow rate. At the lowest flow rates, transport by diffusion and convection competes determining a feather-like shaped concentration pattern. For increasing flow rates, convection becomes the dominant transport phenomena and leads to the formation of sharp compartments. Figure 2(b) reports the results of the experimental validation, performed using the same coefficients and geometrical specifications applied for the mathematical modeling. The extremely close resemblance between the experimental concentration patterns and the modeled ones validated the model predictions and the systems performance. A quantification of this observation is available in supplementary Fig. S1.³¹

B. Modeling of the cell infection process

Figure 3 summarizes representative results of the computational modeling of the infection process. Again, Φ_r is the theoretical relative efficiency of microfluidic perfused versus static infection. The curves in panel 3(a) are parametric in Pe and reported as a function of the square root of time. The horizontal line at $\Phi_r = 1$ highlights the threshold at which molar fluxes (and thus infection efficiencies) of perfused and static processes are equal, thus allowing to identify the parameters characterizing the variables-space where perfused-microfluidic ($\Phi_r > 1$) or standard static ($\Phi_r < 1$) infection conditions are favored.

For example, given the systems geometrical constraints and the duration of the infection process, increases in fluid flow rate (which directly translate into increases in Pe number), will favor perfused-microfluidic processes, which will result in higher infection efficiencies. *Vice versa*, at the lowest flow rates where convection gives no significant contribution to the overall transport of viral particles from the bulk of the liquid to the cell surface, standard static infections prove to be more efficient. Panel 3(b) plots the values of Φ_r as a function of Pe for a fixed time ($t=90$ min) of infection. Again, it is evident how for increasing flow rates, perfused-microfluidic infections lead to higher yields with a trend plateauing for Pe higher than 200 (corresponding to a $1\text{ }\mu\text{l/min}$ flow rate and $8\text{ }\mu\text{m/s}$ linear velocity). Finally, panel 3(c) plots the times at which $\Phi_r = 1$ for $Pe \geq 100$, showing again how for increasing Pe perfused-microfluidic infections could lead to higher yields than standard static processes exposing the cells to potentially harmful viruses for shorter times.

C. Cell infection

Several experiments have been performed in order to optimize the procedures and parameters characterizing the infection process, both in static and microfluidic culture. Cell line, cell

FIG. 3. Computational modeling of the infection process. Φ_r is the theoretical relative efficiency of microfluidic perfused versus static infection. Panel (a) reports Φ_r curves parametric in Pe and as a function of the square root of time. The horizontal line at $\Phi_r = 1$ (equality of perfused and static molar fluxes) separates the variables space where perfused-microfluidic ($\Phi_r > 1$) or standard static ($\Phi_r < 1$) infection conditions are favored. Panel (b) plots Φ_r as a function of Pe at a defined time ($t = 90$ min) of infection. Finally, panel (c) plots the times at which $\Phi_r = 1$ for $Pe \geq 100$.

density, cell passage, virus MOI, duration of the exposure to the viral solution (incubation time), and Pe number, were among the screened variables. From these preliminary experiments, we established optimal values and ranges: cell seeding density was kept constant at 100 cells/mm^2 , MOI was varied from 10 to 100, Pe levels for microfluidic cultures were 10 and 100, and infection times spanned from 90 min to 12 h.

In reporting some of the most significant findings on HFF cells, we highlight how: (a) increases in the incubation time led to increases in the infection efficiency (Figure 4(a)) (further

FIG. 4. Static infections on HFF cultures. HFF were plated at a 100 cells/mm² density, and all infections started 24 h after seeding. MOIs were: 50 in panel (a), 100 in panel (b), and 100, 200, 400, respectively, for the data points in panel (c). In panel (a), the plotted data points demonstrate how longer incubation times of cell cultures with the viral suspension led to increases in the infection efficiency. In panel (b), increases in the viral suspension volume (at a given MOI) led to reduced infection efficiencies; in parallel, panel (c) demonstrates that no significant changes in infection efficiencies were measured for increases in the viral suspension volume at constant viral particles concentration.

discussion will be presented in the following sections). (b) An inverse-relationship correlation was established between viral suspension volume (at a given MOI) and infection efficiency: increases in the first led to decreases in the latter (Figure 4(b)); however, no changes in infection efficiencies were measured for increases in the viral suspension volume at constant viral particles concentration (Figure 4(c)). (c) For a given infection time, observation of the cell cultures over 3 days demonstrated how the efficiency increased over the first two days and reached a plateau by day 3 (data not shown). Additional material is presented in supplementary Figure S2.³¹ Fundamental relations were established between variables, to allow comparisons between the different

culture and infection conditions. In particular, to ensure constant virus concentration in static and microfluidic infections, MOIs must be translated into concentrations as follows:

$$c_v = \frac{MOI \cdot N_{Cells_{Stat}}}{Vol_{Stat}} = \frac{MOI \cdot N_{Cells_{\mu Fl}}}{Vol_{\mu Fl}} \quad (1)$$

and

$$Vol_{\mu Fl} = Q \cdot t, \quad (2)$$

where c_v is the virus concentration in culture medium, MOI is the number of viral particles per cell, N_{Cells} is the total number of cells exposed to the viruses, and Vol is the total volume of fluid used in the experiment. Q is the fluid flow rate and t is the duration of the infection process. Subscripts *Stat* and *Fl* refer to static and perfused-microfluidic infections, respectively. It is important to underline how the possibility of compartmentalizing fluids within the microfluidic platform would divide the culture area in 4 sections, each containing 1/4 of the total number of cells and exposing them to different MOIs; this thus need to be taken into account in evaluating virus concentrations and other variables. Rearranging the above equations, we can calculate the volume of the viral suspension to be used in standard conditions as a function of the fluid flow rate, corresponding to the chosen Pe value, of the microfluidic process:

$$Vol_{Stat} = \frac{N_{Cells_{Stat}}}{N_{Cells_{\mu Fl}}} \cdot Q \cdot t. \quad (3)$$

FIG. 5. Comparison between static and microfluidic infections at different Péclet numbers for two cell types. Cell density was kept constant at 100 cells/mm²; MOI was 100, and infection time 90 min. Experiments were performed at $Pe = 10$ and $Pe = 100$. Panels (a) and (c) refer to HFF cultures, (b) and (d) to MEF. Data were obtained via image analysis of cell cultures 48 h post infection. Panels (c) and (d) graph the modeled profiles for Φ_r (the theoretical relative efficiency of microfluidic perfused versus static infection) and allow comparison with representative experimental results. Empty markers are for $Pe = 10$ and filled markers for $Pe = 100$. ** $p < 0.001$. * $p < 0.01$.

We planned the experimental runs following these variables constraints and obtained the results shown in Figure 5. Cell seeding density was constant at 100 cells/mm².

Experiments were performed at $Pe = 10$ and $Pe = 100$. Panels 5(a) and 5(c) refer to HFF cultures, 5(b) and 5(d) to MEF. Image acquisition was performed 48 h post infections on cells treated at MOI 100 for 90 min. At lower Péclet, static infections led to significantly higher infection efficiencies when compared to the corresponding microfluidic perfused ones for both cell lines, while differences were strongly reduced at the highest Pe value. This is an expected trend described by our model, as can be seen in the bottom graphs of Figure 5 (panels 5(c) and 5(d)), where the relative efficiency of microfluidic perfused versus static infection is reported as a function of the square root of time. Here, Φ_r resulted in significant higher values at $Pe = 100$ when compared to the ones at $Pe = 10$ for both cell lines. In particular, the highest Φ_r values were detected on MEFs, a result that led us to the use of MEFs for the following experiments. The relative infection efficiencies are in very good agreement with the theoretical ones. Higher Pe always led to higher infection efficiencies (measured by the theoretical relative efficiency factor) and, under the same conditions, MEFs showed higher infection efficiencies if compared to HFF. Regarding HFF, the value at higher Pe is overestimated by the model. It is worth to remind that the model describes the ideal case in which the delivery of viral particle is mainly limited by the transport phenomena within the liquid domain.

If our hypothesis fails, an additional step normally faster than transport phenomena, such as virus adsorption or internalization, could negatively affect the overall infection efficiency.

To further explore this issue we performed additional experiments, whose results are presented in Figure 6. First of all, we analyzed the effect of increasing infection times at low MOIs.

Panel 6(a) shows infection efficiencies measured following exposure of cell cultures (MEFs) to adenoviruses at MOI 10 for times ranging from 90 min to 12 h, both in static and microfluidic perfused culture. Additional material is available in Figure S3.³¹

The results highlighted how the use of microfluidics allowed obtaining higher efficiencies for longer incubation times. This is due to the fact that, while in static infections the concentration of viral particles surrounding a cell decreased with time, resulting in plateauing efficiencies, the steady state that was established perfusing the cultures ensured the maintenance of a constant concentration of viruses around cells and further increases in the infection efficiency. Finally, we introduced the new concept of “effective MOI”: as the standard MOI is the total number of viral particles per cell which varies with time as viruses are transported to cells, the effective MOI was defined as the number of viral particles surrounding a cell at a certain time. This value is considered constant in perfused conditions, according to the establishment of the

FIG. 6. Comparison between static and microfluidic infections for different infection times and at effective MOI. The results presented in panel (a) were obtained by exposing the cells to the viral suspension at MOI 10 for times ranging from 90 min to 12 h, both in static and microfluidic perfused culture. The use of microfluidics allowed obtaining higher efficiencies for longer incubation times. In panel (b), infections were performed at an effective MOI of 100 for 12 h and led to significantly higher efficiencies in microfluidic infections compared with those reached in static conditions. $**p < 0.001$.

steady state. Now, the two MOIs are related through the fluid volumes used for the experiment (in turn determined by the chosen Pe number):

$$MOI_{total} = MOI_{effective} \frac{V_i}{V_{ch}}, \quad (4)$$

where V_i is the viral suspension volume used for cell infection during a single experiment and V_{ch} is the volume of the cell chamber. The total and effective MOI are equal in static conditions, where V_i corresponds to V_{ch} , and different in experiments with perfusion, where $V_i > V_{ch}$. Experiments performed using the effective MOI produced the results shown in Figure 6(b). Exposing cells to the viral suspension at an effective MOI of 100 for 12 h led to favored efficiencies for the microfluidic perfused processes, resulting in significantly higher infection efficiencies than those reached in static conditions.

V. DISCUSSION AND CONCLUSIONS

In this study, we present a rational approach to the issue of viral infection of cell cultures, comparing theoretical modeling and experimental evidence. An accurate analysis of the phenomenological behavior of an infection process on a cell culture, explored the effects of transport (diffusional and convective) in static and microfluidic-perfused conditions. Rationalization of the infection steps and limiting phenomena acting on the system highlighted the pros and cons of both conditions. Static conditions, for example, represent the standard procedure and are thus routinely performed with well established techniques; however, they tend to utilize high MOIs in order to ensure high infection efficiencies, are diffusion-dependent and regulated by unpredictable and continuously varying kinetics (since virus concentration in the cells surroundings decreases uncontrollably due to virus degradation, consumption, and internalization by the cells). Perfused infections, on the contrary, can be precisely controlled and the persistence of a steady state renders the system more stable and predictable. At the steady state, the virus concentration at the cell membrane is constant and maintained at the established optimal level, thus allowing the use of lower MOIs to obtain higher infection efficiencies while reducing the risk of exposing cultures to a hostile environment.

In addition, we developed a relationship between molar fluxes of viral particles and infection efficiencies, with molar fluxes determined by the systems parameters and variables (geometry, flow rates, etc.). Such variables have been translated into correlation terms that take into account the necessity of having comparable entities for static and perfused conditions. Dimensionless forms, where applicable, were favored. This approach led us to a more accurate experimental plan, where only one variable at a time was varied in parallel static and perfused infections; the obtained results were thus directly comparable.

Our static controls were performed using standard multiwell plates, fitting the glass coverslips used as culture substrates. We want to point out how this choice, over that of statically operating the microfluidic platforms, allowed us to use the same total volumes reached in the perfused experiments; this is of paramount importance in sight of obtaining infection efficiency data that could be compared between the two conditions. Flow rate choices in the microfluidic experiments were translated in corresponding infection volumes in static controls ($V = Q \cdot t$), and a variable such as MOI could then be independently changed. All together, these results show how the microfluidic technology can be used for rational designing an infection process with high intrinsic efficiency without the risk of viral associated-cytotoxic derived by high MOI static treatment.

ACKNOWLEDGMENTS

We thank Ca.Ri.Pa.Ro., F.S.E., and Ministero della Salute for funding.

¹B. Kaspar, J. Lladó, N. Sherkat, J. Rothstein, and F. Gage, *Science* **301**, 839 (2003).

²J. Luo, M. Kaplitt, H. Fitzsimons, D. Zuzga, Y. Liu, M. Oshinsky, and M. During, *Science* **298**, 425 (2002).

- ³F. C. Marini, Q. Yu, T. Wickham, I. Kovesdi, and M. Andreeff, *Cancer Gene Ther.* **7**, 816 (2000).
- ⁴S. Vorburger and K. Hunt, *Oncologist* **7**, 46 (2002).
- ⁵H. Andersson and A. van den Berg, *Lab Chip* **4**, 98 (2004).
- ⁶E. Cimetta, C. Cannizzaro, R. James, T. Biechele, R. T. Moon, N. Elvassore, and G. Vunjak-Novakovic, *Lab Chip* **10**, 3277 (2010).
- ⁷E. Cimetta, E. Figallo, C. Cannizzaro, N. Elvassore, and G. Vunjak-Novakovic, *Methods* **47**, 81 (2009).
- ⁸N. Jeon, S. Dertinger, D. Chiu, I. Choi, A. Stroock, and G. Whitesides, *Langmuir* **16**, 8311 (2000).
- ⁹T. Keenan and A. Folch, *Lab Chip* **8**, 34 (2007).
- ¹⁰G. M. Whitesides, *Nature (London)* **442**, 368 (2006).
- ¹¹D. Breslauer, P. Lee, and L. Lee, *Mol. BioSyst.* **2**, 97 (2006).
- ¹²S. Haeberle and R. Zengerle, *Lab Chip* **7**, 1094 (2007).
- ¹³P. S. Dittich and A. Manz, *Nat. Rev. Drug Discovery* **5**, 210 (2006).
- ¹⁴Y. Wen and S.-T. Yang, *Expert Opin. Drug Discovery* **3**, 1237 (2008).
- ¹⁵B. D. Piorek, S. J. Lee, J. G. Santiago, M. Moskovits, S. Banerjee, and C. D. Meinhart, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 18898 (2007).
- ¹⁶M. Toner and D. Irimia, *Annu. Rev. Biomed. Eng.* **7**, 77 (2005).
- ¹⁷D. J. Beebe, G. A. Mensing, and G. M. Walker, *Annu. Rev. Biomed. Eng.* **4**, 261 (2002).
- ¹⁸T. Squires and S. Quake, *Rev. Mod. Phys.* **77**, 977 (2005).
- ¹⁹J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. Schueller, and G. M. Whitesides, *Electrophoresis* **21**, 27 (2000).
- ²⁰S. K. Sia and G. M. Whitesides, *Electrophoresis* **24**, 3563 (2003).
- ²¹G. M. Whitesides, E. Ostuni, S. Takayama, X. Jiang, and D. E. Ingber, *Annu. Rev. Biomed. Eng.* **3**, 335 (2001).
- ²²H. N. Vu, Y. Li, M. Casali, D. Irimia, Z. Megeed, and M. L. Yarmush, *Lab Chip* **8**, 75 (2008).
- ²³D. Akin, H. Li, and R. Bashir, *Nano Lett.* **4**, 257 (2004).
- ²⁴Y. Zhu, J. W. Warrick, K. Haubert, D. J. Beebe, and J. Yin, *Biomed. Microdevices* **11**, 565 (2009).
- ²⁵E. E. Endler, K. A. Duca, P. F. Nealey, G. M. Whitesides, and J. Yin, *Biotechnol. Bioeng.* **81**, 719 (2003).
- ²⁶G. Walker, M. Ozers, and D. Beebe, *Sens. Actuators B* **98**, 347 (2004).
- ²⁷T. R. Sodunke, M. J. Bouchard, and H. M. Noh, *Biomed. Microdevices* **10**, 393 (2008).
- ²⁸Y. Xia and G. Whitesides, *Annu. Rev. Mater. Sci.* **28**, 153 (1998).
- ²⁹E. Cimetta, M. Flaibani, M. Mella, E. Serena, L. Boldrin, P. De Coppi, and N. Elvassore, *Int. J. Artif. Organs* **30**, 415 (2007).
- ³⁰R. Bird, W. Stewart, and E. Lightfoot, *Transport Phenomena* (Wiley, 1960).
- ³¹See supplementary material at <http://dx.doi.org/10.1063/1.4723853> for images of infected cells.