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A high-throughput drop microfluidic system for virus culture and analysis

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High mutation rates and short replication times lead to rapid evolution in RNA viruses. New tools for high-throughput culture and analysis of viral phenotypes will enable more effective studies of viral evolutionary processes. A water-in-oil drop microfluidic system to study virus–cell interactions at the single event level on a massively parallel scale is described here. Murine norovirus (MNV-1) particles were co-encapsulated with individual RAW 264.7 cells in 65 pL aqueous drops formed by flow focusing in 50 μm microchannels. At low multiplicity of infection (MOI), viral titers increased greatly, reaching a maximum 18 h post-encapsulation. This system was employed to evaluate MNV-1 escape from a neutralizing monoclonal antibody (clone A6.2). Further, the system was validated as a means for testing escape from antibody neutralization using a series of viral point mutants. Finally, the replicative capacity of single viral particles in drops under antibody stress was tested. Under standard conditions, many RNA virus stocks harbor minority populations of genotypic and phenotypic variants, resulting in quasispecies. These data show that when single cells are encapsulated with single viral particles under antibody stress without competition from other virions, the number of resulting infectious particles is nearly equivalent to the number of viral genomes present. These findings suggest that lower fitness virions can infect cells successfully and replicate, indicating that the microfluidics system may serve as an effective tool for isolating mutants that escape evolutionary stressors.

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1. Introduction

RNA virus populations exist as quasispecies as a result of high mutation rates and short replication times. High mutation rates provide a powerful mechanism to evade host immunological defences and other evolutionary selection pressures. Rapid evolution allows viruses to persist both within individuals and within a population. Moreover, new viral species can emerge with greater pathogenic potential. Viral quasispecies theory proposes that new viral species emerge when selective pressures confer an evolutionary advantage upon minor alleles within a population (Lauring and Andino, 2010; Vignuzzi et al., 2006). In this context, most experimentation with viral quasispecies under bulk cell culture conditions will be dominated by major alleles. A high throughput cell culture system, in which millions of infections can occur in parallel could be a useful tool for maintaining minor alleles in a quasispecies and studying their responses to selective pressure.

A large body of prior work suggests that microfluidic technology based on soft lithography and polydimethylsiloxane (PDMS) devices may be the key to achieving the next level of cost effectiveness in high-throughput assays with significant advantages over existing high-density microtiter plate-based methods (Guo et al., 2012; Kintses et al., 2012; Theberge et al., 2010). Previous studies

Abbreviations: CV, Coxsackie virus; MNV-1, murine norovirus 1; MOI, multiplicity of infection; pfu, plaque forming unit; polydimethylsiloxane, PDMS; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TCID, tissue culture infectious dose.

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have demonstrated the ability of water-in-oil drop microfluidic devices to generate large numbers of stable, monodisperse drops and their potential usefulness as an ultra-high throughput culture system for single eukaryotic cells (Agresti et al., 2010; Clausell-Tormos et al., 2008; Mary et al., 2011; Mazutis et al., 2013). This technology has been improved to include biocompatible surfactants, a gas permeable oil-phase, and methods for drop rupture and cell recovery post-encapsulation (Edd et al., 2008).

In the present study, this work is extended, describing a high-throughput culture system for Murine Norovirus 1 (MNV-1) in drops. MNV-1 is closely related to human noroviruses, and serves here as a model for a rapidly evolving quasispecies. The murine virus can be cultured effectively in murine macrophages and dendritic cells, including the mouse macrophage cell line RAW 264.7, and thus is often used as a model to study human norovirus biology (Karst et al., 2003; Wobus et al., 2004, 2006). A better understanding of noroviruses could help to define evolutionary features of this species and aid in the development of new therapeutics. A microfluidic flow focusing dropmaker device is employed here to co-encapsulate individual cells with MNV-1 particles in stable 65 pL drops of aqueous solution in fluorinated oil. The results show that RAW 264.7 cells can be cultured in these drops for a period of time sufficient to support viral infection and replication. The results further demonstrate antibody neutralization in drops, and show that mutations in the MNV-1 capsid protein confer antibody escape phenotypes in drops similar to those observed in bulk. Finally, evidence is presented that infection of RAW 264.7 cells in drops at low MOIs may result in a higher fraction of viral particles infecting successfully and replicating. It is proposed that this feature of the drop-based infection system makes it especially useful for investigating viral evolution, as lack of competition among viral particles in drops may lead to greater genetic diversity in the replicating fraction, and thus many more possible evolutionary trajectories.

2. Materials and methods

2.1. Viruses and antibodies

Chemicals were purchased from Sigma–Aldrich (St. Louis, MO). The plaque-purified MNV-1 clone (GV/MNV1/2002/USA) MNV-1.CW3 (Thackray et al., 2007) (referred herein as MNV-1) was used at passage 6 for all experiments. Recombinant MNV1 viruses containing P-domain point mutants G300K, T301I, V378F, A381F, A382P, A382R, A382K, D385G, D385E, and L386F were generated as previously described (Kolawole et al., 2014). Virus titers were determined by plaque assay, or by tissue culture infectious dose (TCID) assay, as described previously (Arias et al., 2012; Gonzalez-Hernandez et al., 2012; Hwang et al., 2014). One plaque forming unit (pfu) corresponded to 0.7 TCID50 units.

The isotype control IgG directed against Coxsackievirus B4 (CV) (clone 204-4) was purchased from ATCC (Manassas, VA; HB 185). The neutralizing anti-MNV-1 mAb A6.2 (IgG2a isotype) was isolated previously and binds to the MNV-1 P domain (Katpally et al., 2008; Taube et al., 2010; Wobus et al., 2004). Both hybridomas were grown in Bioreactor CELLine CL 1000 flasks (Sigma–Aldrich) at the Hybridoma Core, University of Michigan, and purified over a HiTrap protein A column (GE Healthcare Bio-Sciences, Piscataway, NJ) as described (Kolawole et al., 2014).

2.2. Microfluidic device design and fabrication

Polydimethylsiloxane (PDMS) devices were fabricated using replica molding with SU8 photoresist as the mold master (McDonald et al., 2000). Devices are rendered more hydrophobic by coating them with Aquapel (Rider, MA, USA). Aquapel injection into the devices is followed by drying the devices by blowing air through the channels and baking at 65 °C for 15 min. The microfluidic flow focusing dropmaker device used here had a junction with a square cross-section of 50 μm and a constriction 32 μm immediately downstream from the junction.

2.3. Cell culture

RAW 264.7 (murine macrophage lineage) cells were purchased from ATCC and maintained as described previously (Taube et al., 2010; Wobus et al., 2004) in Dulbecco's Modified Eagle's Medium with 4 mM L-glutamine, 100 μg/mL penicillin, 100 U/mL streptomycin, 10 mM HEPES, 10% fetal bovine serum. Cells were adapted to suspension culture in spinner flasks for drop encapsulation experiments. Culture medium for suspension cells was supplemented with 7.5% sodium bicarbonate.

2.4. Viral infection and neutralization in bulk culture

RAW 264.7 suspension cells were centrifuged for 5 min at 3000 rpm and re-suspended in fresh medium at 8×10^6 cells/mL. Virus was diluted to 200 μL in PBS before being brought to 2× final desired concentration in suspension growth medium. 1 mL of cell suspension and 1 mL of virus suspension were mixed in a single well of a 12-well dish containing a sterile stir bar and incubated on stir plate in a 37 °C incubator, 5% CO_2, for 24 h. Cell lysates were harvested by 2 rounds of freeze/thaw.

For bulk neutralization studies using multiple MOIs, virus was diluted to 200 μL in PBS and preincubated with mAb A6.2 for 30 min at 37 °C. The virus-antibody mixture was then diluted to 2× the final desired concentration in growth medium prior to mixing with 1 mL of cell suspension. After 1 h, the inoculum was removed and cells were washed twice with ice-cold PBS. Media was added and cells were incubated at 37 °C for 24 h. Virus titers were determined by TCID50 after 2 rounds of freeze/thaw.

To obtain a neutralization curve, overnight cultures of adherent RAW 264.7 cells in 6-well plates (1×10^6/well) were infected with MNV-1 (MOI = 0.05) on ice. Virus was diluted and preincubated with antibody as described above before being added to the cell monolayers. After 1 h, the inoculum was removed and cells were washed twice with ice-cold PBS. Growth medium was added and cells incubated at 37 °C for 24 h. Virus titers were determined by plaque assay after 2 rounds of freeze/thaw. Neutralization was plotted as a function of antibody concentration; the data was fit according to the equation $y = a + b^{*}c^{n}/(c^{n} + x^{n})$, where a is pfu/mL expected as the antibody:virus ratio approaches infinity, b is pfu/mL expected at an antibody concentration of 0, c is the half-maximal antibody:virus ratio, and n is the Hill coefficient.

2.5. Virus experimentation in drop culture

To infect single cells in drops, suspension-adapted cells were centrifuged for 5 min at 3000 rpm and re-suspended in fresh medium before transfer to a 3 mL luer-lock syringe containing a sterile stir bar. Virus was diluted to the desired density in suspension culture medium and transferred to 1 mL syringes. Both cells and virus were added to syringes at 2× the desired final concentration. 1% of a block co-polymer surfactant of perfluorinated polyethers (PFPE) and polyethyleneglycol (PEG) surfactant (Ran Biotechnologies, Beverly, MA) (Holtze et al., 2008) was solubilized in Novec HFE 7500 oil (3M, St. Paul, MN) and transferred to a 10 mL luer-lock syringe. Syringes were driven by pumps (Harvard Apparatus, Holliston, MA) and fitted with 26½ gauge needles and 0.38 mm polyethylene tubing. Flow rates into the microfluidic devices were 4 mL/h for oil/surfactant, and 1 mL/h for cells and virus. In antibody

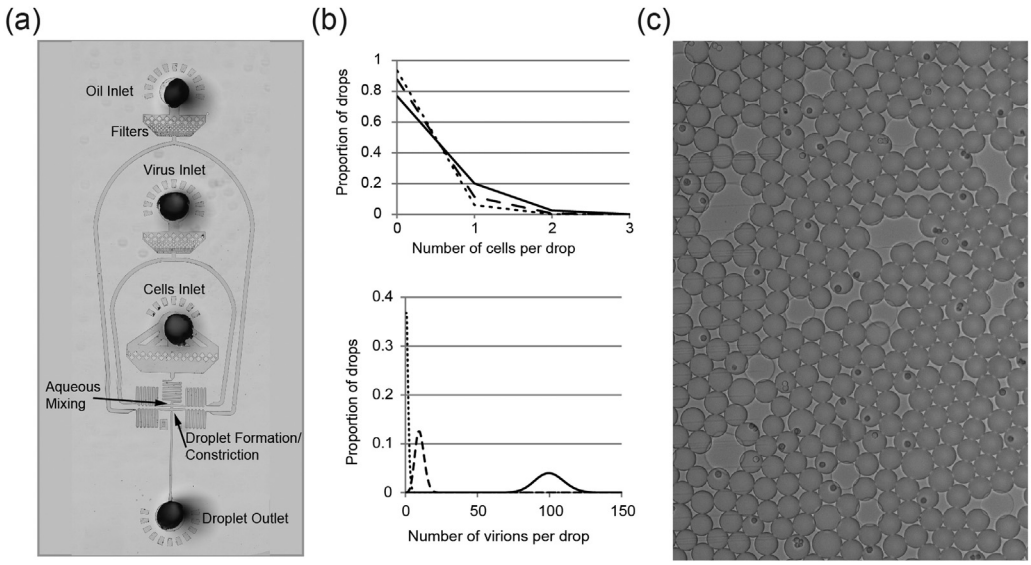

Fig. 1. Microfluidic system. (a) Droplet formation chips contain 3 inlet ports (one for oil/surfactant and two for aqueous solutions) and one outlet port for droplet collection. The aqueous inlets flow together immediately prior to exposure to oil. Droplets leaving the outlet are collected into a tube for incubation. (b) Upper panel: predicted distribution of cells in 50 μm drops as a function of syringe density. Dotted line: 2 million cells/mL, dashed line: 4 million cells/mL, solid line: 8 million cells/mL. Lower panel: predicted distribution of viral particles per drop as a function of density. Dotted line: 3×10^5 pfu/mL, dashed line: 3×10^6 pfu/mL, solid line: 3×10^7 pfu/mL. (c) RAW 264.7 cells encapsulated in 50 μm droplets.

neutralization experiments, virus was pre-incubated for 30 min at 37 °C with mAb A6.2 in 200 μL PBS prior to dilution to final densities corresponding to MOI = 0.01, 0.1, or 1 per drop in suspension cell medium. Drops were collected in 1.5 mL tubes and incubated at 37 °C, in 5% CO_2 before rupture. Emulsions were broken by 2 rounds of freeze/thaw; aqueous fractions were transferred to 1.5 mL tubes for storage and analysis. Neutralization curve fitting was performed as described above.

2.6. Isolation of infected cells from drop culture

Virus (0.01, 0.1, or 1 pfu/drop) and cells (8×10^6 mL^{-1}) were co-encapsulated in 50 μm drops and incubated for 4 h at 37 °C. Drops were ruptured with 20% 1H,1H,2H,2H-perfluoro-1-octanol (PFO; Sigma–Aldrich) in the oil phase, and cells were pelleted at 800 rpm. The aqueous fraction containing cells was transferred to a fresh 1.5 mL tube. Cells were centrifuged for 5 min at 1200 rpm, and supernatant was aspirated. Cells were washed twice in PBS before resuspension in 2 mL fresh growth medium and plating in a single well of a 12-well tissue culture plate. The plate was incubated for 20 h at 37 °C in 5% CO_2. Cells were lysed by 2 rounds of freeze/thaw.

2.7. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

MNV1-specific primer/probe sequences (Forward: GTGCGCAACACAGAGAAACG, Reverse: CGGGCTGAGCTTCCTGC, and probe: FAM/CTAGTGTCTCCTTTGGAGCACCTA/TAMARA) (Taube et al., 2009) were used in combination with Takara One Step (Clontech, Mountain View, CA) reagent to measure genome copies of viral samples. qRT-PCR was performed on an ABI StepOnePlus (Life Technologies, Carlsbad, CA) real time PCR machine using the following thermal cycling parameters: 5 min at 42 °C and 10 s at 95 °C, 40 cycles of 5 s at 95 °C and 34 s at 60 °C. A titered MNV-1 viral stock and pT7MNV3′RZ plasmid (Chaudhry et al., 2007) quantified on a Qubit 2.0 fluorometer using PicoGreen were used as standards for pfu/mL and genome copies/mL analysis, respectively.

3. Results

3.1. System description

Microfluidic devices were designed to achieve controlled addition of single cells and viral particles in monodisperse 50 μm drops. On this scale, low flow rates (~1 mL/h) can generate millions of drops in less than an hour, and large numbers of independent single-cell infections that occur in parallel. Devices were designed to mix suspended cells and viral particles immediately upstream of a flow-focusing feature for drop formation in oil with 1% surfactant (Fig. 1a). Channels were 50 μm in diameter and yielded monodisperse drops with calculated volumes of 65 pL. Cells, viral particles, and oil/surfactant were placed in separate syringes and driven by syringe pumps through tubing into the device. Drops were collected from the outlet port in 15 mL conical tubes. Previous work suggests that the probability λ of a certain number of particles (cells or viral particles) per drop can be described by a Poisson distribution, where x is the average number of cells per drop (Fig. 1b) (Clausell-Tormos et al., 2008; Mazutis et al., 2013). When mixing suspended cells with growth medium alone, we found that a density of 8×10^6 cells/mL (4×10^6 cells/mL after 2× dilution upstream of drop formation) yielded the highest proportion of single cells in occupied drops (data not shown). Fig. 1c shows a representative view of 50 μm drops made at this cell density. Approximately 14% of drops contain at least one cell, and approximately 11% contain one cell. Most drops containing multiple cells contain cells in close contact with one another, suggesting that cell–cell interactions account for the higher than expected frequency of multiple cell-containing drops. The lower-than-expected fraction of drops containing cells can be attributed to cells becoming trapped upstream in a filter feature of the device. To achieve the highest proportion of single cell infections, subsequent drop infections were performed using a syringe density of 8×10^6 cells/mL.

3.2. Cell viability and infection in 50 μm drops

First, cell viability of permissive RAW264.7 cells and the ability of MNV-1 to infect these cells under the droplet conditions were

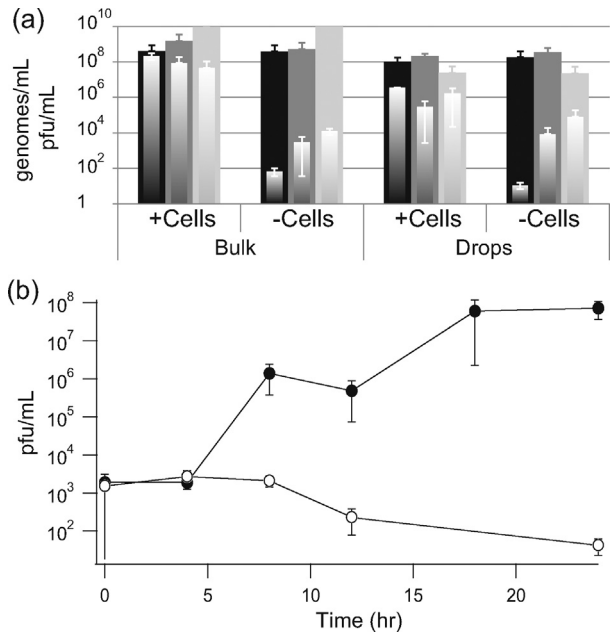

Fig. 2. Comparison of viral replication in droplets and bulk culture. (a) Viral titer and genome copy analysis of MNV-1 virus incubated with RAW 264.7 cells in bulk and in 50 μm droplets at varying MOI: 0.01 (black), 0.1 (gray), and 1.0 (light gray). Genomes/mL presented as solid bars in background of pfu/mL presented as gradient bars. (b) Comparison of MNV1 titers following encapsulation with (black) and without (white) cells and incubation at 37 °C for the indicated periods of time. Error bars show SEM for 3 independent experiments.

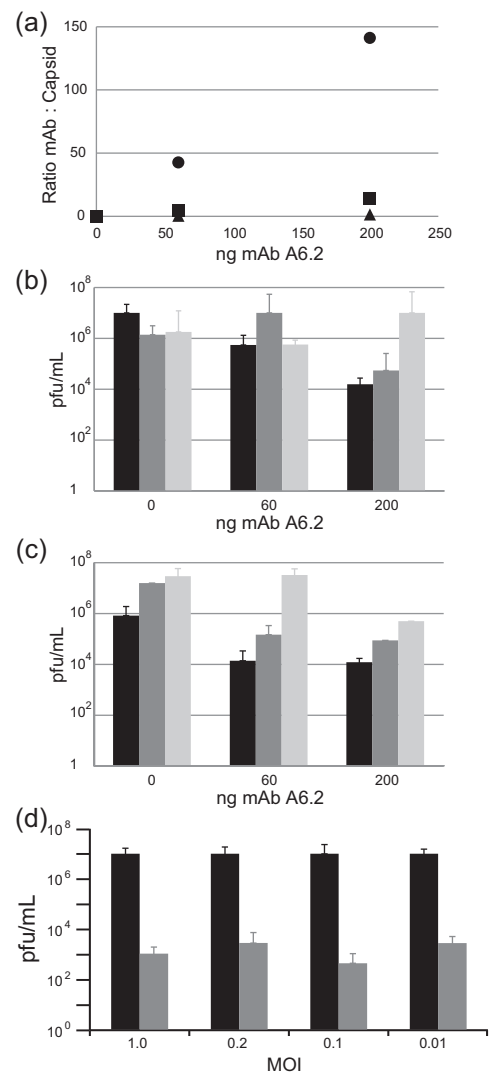

Fig. 3. Antibody neutralization with MOI variation. (a) Calculated relationship between ng mAb and ratio of mAb/VP1 capsid protein over a series of MOIs: 0.01 (●), 0.1 (■), and 1.0 (▲). Neutralization of MNV-1 in droplets (b) and in bulk (c) at MOIs of 0.01 (black), 0.1(gray) and 1.0 (light gray) and 0, 60, or 200 ng/mL of antibody mAb A6.2. Error bars show SEM for 3 independent experiments.

determined. MNV-1 replication in RAW 264.7 cells is detectable ~8 h following infection in bulk culture (Wobus et al., 2004), though cell viability may be maintained for as long as 24 h. To determine the overall health of cells, RAW 264.7 cell viability was evaluated in 50 μm drops and bulk suspension culture. After 24 h incubation, drops were ruptured by the addition of 20% PFO, followed by gentle vortexing and centrifugation. Cells in the resulting aqueous layer were examined by trypan blue exclusion assay. Cells incubated in bulk culture at 4×10^6 cells/mL final density for 24 h were 90.7 ± 1.0% viable, while cells encapsulated in drops at the same density for 24 h were 91.4 ± 0.8% viable (data not shown).

Next, viral replication in drop-encapsulated cells was compared to replication in bulk culture over a range of MOIs. After 24 h incubation, drops were ruptured and aqueous lysates were analyzed for viral replication. Viral replication was evident at all MOIs tested in both bulk- and drop-based infections (Fig. 2a). In both the drop and bulk experiments in which virus was incubated with cells, analyses indicated that the ratio of infectious particles to viral genomes was approximately 1:100. This is consistent with previously published results showing that approximately 1% of MNV-1 genomes form a plaque (Baert et al., 2008) (Fig. 2a). In contrast, when viral particles were incubated in drops alone, there were much higher numbers of genome copies than infectious particles. This can be attributed to non-degraded viral RNA remaining in drop solutions. To examine the production of infectious particles over time in drops, a time-course was conducted with a standard inoculum (MOI 0.01). In the presence of cells, new infectious viral particles emerged at 8 h, reaching a maximum at 18 h post-encapsulation. In the absence of cells, a decrease in infectious virus was observed over this period (Fig. 2b). These data provide evidence that MNV-1 replication capacity and kinetics are similar in both bulk and drop contexts.

3.3. An MNV-1 neutralizing mAb inhibits viral replication in drops

To expand the utility of the microfluidic system for virological approaches, the ability of the MNV-1-neutralizing monoclonal antibody (mAb) A6.2 to inhibit viral replication in drops was evaluated. Performing neutralization studies in drops presented a number of novel challenges. In bulk neutralization protocols, antibody and virus are often pre-incubated together, added to adherent cells, and then unbound virus is washed away after an incubation period. In drops, cells can be co-encapsulated with an antibody/virus complex at a given ratio, but after drop formation, the antibody remains in the drop medium. A second complication arises from the definition of MOI in the context of single cell experiments. When antibodies are added based on ng/mL, this creates large discrepancies between the number of antibodies/epitope at higher antibody concentrations for any given MOI (Fig. 3a). First, neutralization in drops and in bulk was compared at 3 MOIs when adding A6.2 at fixed concentrations in terms of ng/mL. To best replicate drop-like conditions, virus/antibody complexes were added to small suspension cultures with a starting cell density of 4×10^6 cells/mL for 24 h. TCID50 analysis (Fig. 3b and c) showed that in bulk, viral titers are reduced

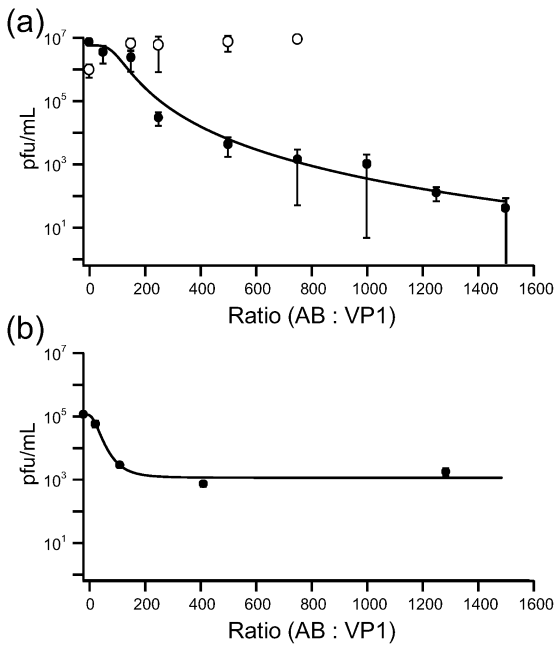

Fig. 4. Droplet-based neutralization using antibody/epitope ratios. (a) MNV-1 neutralization by A6.2 over varying MOI with constant ratio of 500 mAb/VP1 protein. (b) Concentration-dependent neutralization of MNV-1 encapsulated with cells in droplets for 24 h. Monoclonal Ab A6.2 (●) or isotype control antibody (○) were preincubated with MNV-1 for 30 min at 37 °C prior to encapsulation with cells. A6.2 neutralization data was fit according to the equation $y = a + b^{*}c^{n}/(c^{n} + x^{n})$. See Section 2 for a description of the variables. For data plotted in (b), $a = 8.7 \times 10^{-2}$, $b = 5.9 \times 10^{6}$, $c = 97$, and $n = 4.1$; for (c) $a = 1.0 \times 10^{3}$, $b = 1.3 \times 10^{5}$, $c = 44$, $n = 3.7$.

by roughly two orders of magnitude by mAb A6.2 at 200 ng/mL regardless of MOI. In contrast, the same concentration of antibody neutralized MNV-1 very effectively in drops at MOI of 0.01, but had no effect at MOI of 1, and mAb A6.2 was only neutralizing at 200 ng/mL at MOI 0.1. In contrast, when constant antibody/epitope (MNV-1 VP1 protein, 180/virion) ratios were used, similar neutralization was observed regardless of starting MOI (Fig. 3d). Therefore, all subsequent neutralization experiments were conducted using antibody/epitope ratios rather than total antibody concentration.

To measure the dose-dependent neutralizing effects of A6.2 on MNV-1 in drops, individual cells were co-encapsulated with virus/antibody complexes at a MOI of 0.01 (1 particle/drop), and a range of final antibody/epitope ratios (Fig. 4a). MNV-1 titers were reduced by approximately 2 orders of magnitude at 250 mAb/VP1. These data agree with bulk measurements of neutralization in adherent RAW 264.7 cells (Figs. 3c and 4b). Neutralization in bulk required only 130 mAb/VP1 (60 ng/mL at MOI = 0.05) to achieve a reduction in titer of 2 orders of magnitude, and showed an apparent saturation close to this ratio, while neutralization in drops could be resolved at ratios up to 1500 mAb/VP1. Neutralization was dependent on specific interaction between MNV-1 and A6.2, because an isotype control antibody directed against human Coxsackie B4 virus capsid protein did not neutralize at identical antibody/epitope ratios. The titration curve for neutralization can be fit to an exponential function with a Hill coefficient greater than 1, possibly indicating a cooperative, multi-hit neutralization mechanism (Klasse and Sattentau, 2002) (Fig. 4a and b).

3.4. MNV-1 point mutant evaluation in drops

MNV-1 escape from A6.2 was assessed recently in bulk for a set of point mutants in the MNV-1 capsid P-domain predicted to disrupt antibody binding based on detailed structural mapping of the antibody-capsid complex (Kolawole et al., 2014). Point mutant resistance to A6.2 neutralization was evaluated in the drop-based microfluidic system in order to validate its potential as a high throughput screening system. Monoclonal antibody A6.2 neutralization was assessed for 20 single point mutants and one double point mutant in the A′-B′ and E′F′ loops in the P domain of MNV-1. Neutralization of MNV-1 was assayed at mAb/VP1 ratios of 0, 50, 250, 500, and 1000 (0.23, 1.15, 2.3, and 4.6 nM, respectively, when tested at MOI 0.1), and levels of neutralization were determined for each point mutant (Fig. 5, Supplementary Fig. 1). Similar to studies in bulk, E′–F′ loop mutations V378F, A382P, A382R, and L386F were confirmed as escape mutants (Kolawole et al., 2014). Furthermore, the double mutant D385G/V378F, was resistant to antibody neutralization, consistent with results of viral passaging studies in the presence of this antibody (Kolawole et al., 2014). Importantly, point mutants that were neutralized or not by mAb A6.2 in bulk neutralization assays were confirmed in our drop-based neutralization assays (Fig. 5), demonstrating that neutralization in a drop-based culture system is similar to bulk culture.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.12.003.

3.5. Evaluation of virus replication in the absence of competition

Evolutionary studies benefit from the maintenance of genetic diversity in populations under examination. In the absence of competition from other viruses, viral capsid mutants with reduced fitness might still be able to infect single cells successfully in drops. It was determined whether the ratio of pfu to genomes under these conditions would be closer to 1:1 than to 1:100 in order to test this hypothesis. To isolate viruses that had infected cells successfully, single cells were co-encapsulated with MNV-1 at MOIs of

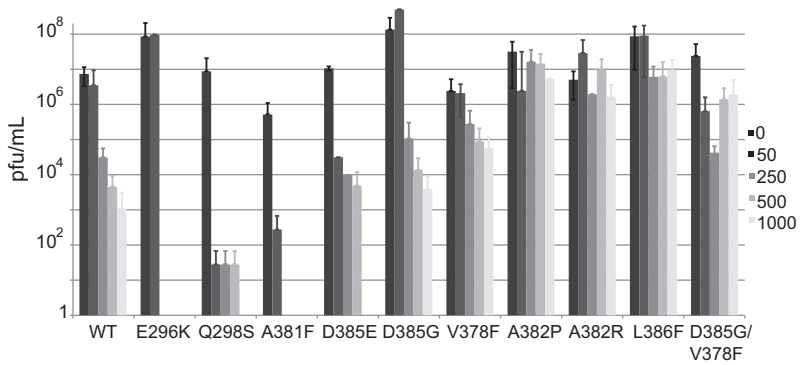

Fig. 5. Evaluation of MNV-1 point mutants. MNV-1 escape from A6.2 assessed in droplets for a set of point mutants in the MNV-1 capsid P-domain. Neutralization was assayed over varying mAb/VP1 ratios. Error bars show SEM for 3 independent experiments.

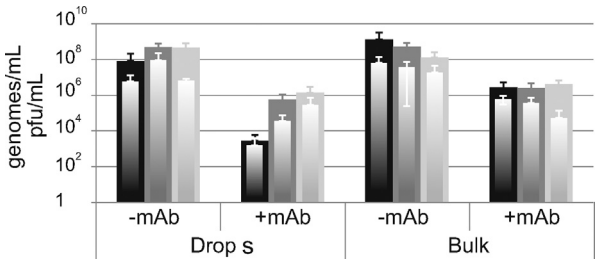

Fig. 6. Virus replication in the absence of competition. Titer and genome copy number analysis of MNV-1 virus incubated with cells in bulk and in 50 μm droplets for 4 h prior to washing cells and plating for 20 h. Bulk and droplet samples were evaluated at MOIs of: 0.01 (black), 0.1 (gray), and 1.0 (light gray). +mAb indicates 150 A6.2 monoclonal antibodies per VP1 epitope. −mAb indicates no-antibody control. Genomes/mL presented as solid bars in background. pfu/mL presented as gradient bars. Error bars show SEM for 3 independent experiments.

1, 0.1, or 0.01 in the presence or absence of A6.2 (150 Ab:VP1) (Fig. 6). Four hours post-encapsulation, droplets were burst and cells were recovered and washed before plating. After 24 h, culture supernatant and cell lysates were analyzed for virus production and genome replication. In parallel, the same experiment was performed using an equivalent number of cells in suspension culture in the absence of drop encapsulation. Viral replication in drops, measured by both number of genomes (qRT-PCR) and titer (TCID50), was relatively insensitive to MOI of the initial infection in the absence of antibody. In the presence of antibody in drops, there was a sharp dependence on starting MOI, both in terms of the number of genomes and infectious particles produced. In particular, when single cells were encapsulated with single viral particles (MOI = 0.01), the number of genomes detected after replication was almost identical to the number of infectious particles (pfu/genomes = 0.5). In contrast, when the experiment was performed in bulk, the expected reduction in genomes and in titer due to antibody neutralization was observed, and the effect was relatively insensitive to starting MOI. This data suggests that in drops, the culling effect of antibody neutralization is especially pronounced when the initial MOI is low, and that drop-based culture at low MOI may be an effective method to isolate variants that can escape.

4. Discussion

The goal of this work was to establish a high-throughput culture system for viruses that can be used to study viral quasispecies. Large numbers of independent infections have the potential to eliminate the dominance of major allele phenotypes that typify bulk viral culture, maintaining minor alleles in the population for further study. The results presented here show that key aspects of viral culture and antibody neutralization can be conducted effectively in microfluidic drops. In particular, cells survive long enough within the drops to support viral infection and replication, and antibody neutralization in drops mirrors findings observed under bulk conditions. Viral capsid variants that confer antibody escape in bulk culture conditions were confirmed to replicate in drops, even in the presence of high antibody concentrations. These experiments support the conclusions that amino acid mutations in the E′F′ loop of the MNV-1 P domain are critical for escape from mAb A6.2 neutralization.

To evaluate the potential for this system to isolate minor alleles that can escape evolutionary pressure, the number of infecting viruses (determined by TCID50) and the number of viral genomes (determined by qRT-PCR) present after infection was compared in drops and in bulk. Viruses and cells together for four hours to permit viral entry, then washed away viral particles that remained in the culture medium. This protocol selects for virions that are able to enter cells, and at low MOI eliminates competition from other, competing virions. Under antibody stress at MOI = 0.01 slightly greater than 0.01% of the original population (approximately 2300 pfu) could be isolated as apparent escape mutants.

A comparison of genetic diversity between successfully infecting MNV-1 populations in drop-based and bulk conditions is beyond the scope of this study. However, it was expected that drop-based infections at low MOI can permit the proliferation of alleles that might otherwise be subject to negative selection. In bulk viral infections where the number of available cells to infect is limited, certain viral phenotypes will be subject to positive selection. Over time, these are expected to dominate the population, limiting both the number of minor alleles and opportunities for evolution in the face of selective pressure. In a drop context, when single viral particles are incubated with single cells, the minor alleles may be able to proliferate due to an absence of competition for resources. This may lead to a greater apparent viability of the aggregate population, and may generate broader initial genetic diversity for subsequent challenge with selective pressures. It is anticipated that microfluidic methods are significant for the study of viral evolution because they provide unique capabilities to study individual genetic trajectories without confounding effects.

In addition to drop-based culture, the high-throughput and inexpensive nature of this technology raises the potential for future development of functional virological assays and high-throughput screening applications (Cimetta et al., 2012; Hattersley et al., 2013; Yin and Marshall, 2012). More advanced capacities of microfluidic drop systems include fluorescence-based sorting, injection, merging, splitting, and sampling of individual drops. Future studies of evolutionary processes could include millions of parallel passages coupled to genetic analysis of viral populations that are successful or unsuccessful at escaping selection pressures. The work presented here represents validation of drop microfluidic culture for viruses, and a potentially useful tool for the isolation and analysis of minor components of viral quasispecies.

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