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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.

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