All the single cells: if you like it then you should put some virus on it

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All the single cells: if you like it then you should put some virus on it

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ABSTRACT Across a rich 70-year history, single-cell virology has revealed the impact of host and pathogen heterogeneity during virus infections. Recent technological innovations have enabled higher-resolution analyses of cellular and viral heterogeneity. Furthermore, single-cell analysis has revealed extreme phenotypes and provided additional insights into host-pathogen dynamics. Using a single-cell approach to explore fundamental virology questions, contemporary researchers have contributed to a revival of interest in single-cell virology with increased insights and enthusiasm.

KEYWORDS single cell, virology, virus, heterogeneity, host-pathogen

From environmentally abundant bacteriophage to endogenous retroviral elements in eukaryotic genomes, viruses shape ourselves and the world (1, 2). Viruses vary drastically in their replication strategies, the cells and hosts they infect, and the diseases they cause (3). An inherent property of many viruses is their existence as a swarm of closely related but genetically distinct minor sequence variants, referred to as quasispecies (4, 5). The genetic diversity explored through each generation of viral progeny production can produce expanded quasispecies diversity or reduction through natural selection (4, 5). The outcomes of viral infections reflect the dynamic interplay between highly heterogeneous viral progeny and the diverse cell populations they infect (6). Yet analysis of viral infections at a population level fails to assess the numerous interactions that occur when a virus infects an individual cell.

Delving into the heterogeneity of viral infection at the level of individual cells offers a more detailed perspective on the environment and the evolutionary trajectory of viral populations. To date, analysis of individual infected cells can address four main biological insights into viral infection dynamics. These include the analysis of viral progeny production, kinetics of virus release or gene expression, analysis of defective interfering particles (DIPs), and transcriptomic analysis using reverse transcription-quantitative PCR (RT-qPCR) or single-cell RNA sequencing (scRNA-seq) (Fig. 1). By using these varied approaches, the study of virus infections at the single-cell level can reveal information about viral fitness, evolution, and diversity (7–9).

Despite recent interest in single-cell virology, pivotal experiments on single cells were conducted over six decades ago (10–12). Early investigations regarding viral and cellular heterogeneity laid essential groundwork for current virology research. This review aims to bring the reader on a journey through time to examine the roots of single-cell virology, beginning in the 1950s, to modern day techniques and technologies that have enabled rapid expansion of the field.

THE EMERGENCE OF SINGLE-CELL VIROLOGY: PIONEERING INSIGHTS INTO VIRAL HETEROGENEITY

Single-cell virology has become an extremely powerful tool for exploring host-pathogen heterogeneity. Early investigations revealed variations across populations of phage

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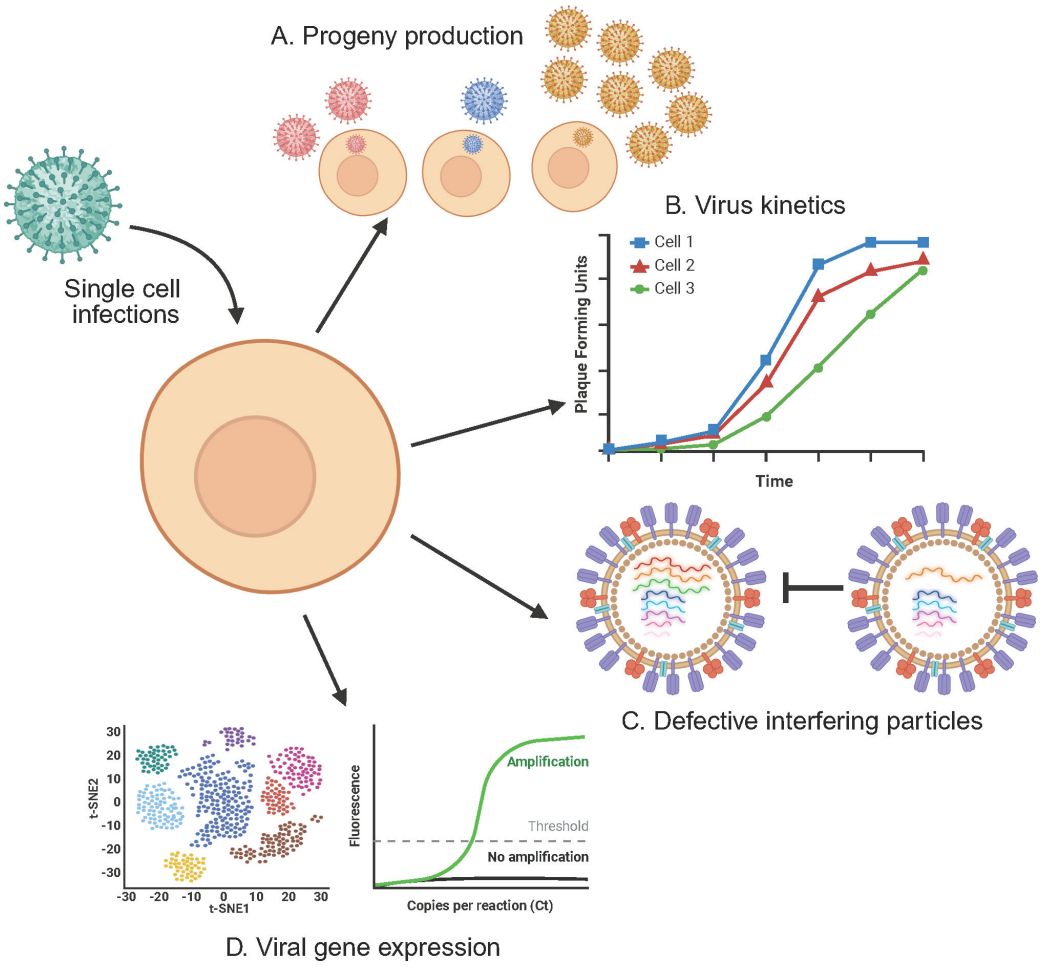

FIG 1 Biological insight from single-cell infections. Analysis of individual infected cells has measured four main biological parameters that provide insight into viral infection dynamics. (A) Progeny yields have the deepest history with a majority of published works focusing on the heterogeneity of virus progeny production at a single-cell level. (B) Single-cell kinetics of viral gene expression have led to significant insights into how quickly viruses replicate and how differences in inoculating dose matter. (C) Single-cell infection enables further dissection into the role that defective interfering particles play during virus infection. (D) Exploring differences in viral gene expression and host gene expression has been accomplished using both RT-qPCR and scRNA-seq, a technique that is revolutionizing single-cell virology. Figures created with biorender.com.

particles (13). These initial insights spurred further inquiry, notably by Delbruck in 1945, regarding diversity in the amount of bacteriophage progeny produced from individual bacteria, termed "burst size" (14). Delbruck observed that each bacterium would produce differing quantities of phage, and that the "burst size" calculated from bulk experiments was an average of a very heterogeneous population. Time and again, the power of single-cell virology would continue to recognize this heterogeneity in viral infections.

In 1952, Renato Dulbecco pioneered the plaque assay for animal viruses and promptly applied it to quantify virus production from individual infected cells (10, 15, 16). In 1954, Dulbecco and Vogt utilized chicken embryo cells infected with Western equine encephalomyelitis virus (WEEV) in suspension and employed a limiting dilution technique to generate tubes containing individual infected cells (10). The virus released into the supernatant from the individual cells was collected and titered at multiple time points post-inoculation. Their observations on WEEV infections echoed Delbruck's findings on bacteriophage burst size. A wide variability in the number of infectious virus particles, or plaque-forming units (PFUs), was observed, ranging from less than 10 to over 130 per individual cell. Unlike the burst size observed by Delbruck, where bacteria

are lysed releasing the assembled phage particles in a singular burst, Dulbecco and Vogt noted that WEEV-infected cells could continuously produce virus over time, and that it was not a singular release (10).

Apart from WEEV, Dulbecco and Vogt collaborated with the Lwoffs in 1955 to investigate the kinetics of poliomyelitis virus (PV) release from individual cells (11). Employing a method developed by De Fonbrune in 1949 (17), they submerged droplets of nutrient fluid under liquid paraffin oil on a glass coverslip (Fig. 2A). Cells were introduced to the nutrient droplets post-virus inoculation, pioneering the first in-drop infection assays. The ability to manipulate individual, microliter volume drops in the De Fonbrune chamber facilitated the sampling of the single infected cells over time to derive the kinetics of PV release. Lwoff et al. quantified that PV release was more rapid than WEEV with infectious virus first detected at five and a half hours post-infection. Interestingly, the cell that produced the virus first also produced the highest overall titer of 195 PFUs. While only a few cells were studied, the observed heterogeneity in the amount of virus liberated from individual cells was consistent with prior observations.

The De Fonbrune chamber method and in-drop infection assays were also employed by Wildy et al. in 1959 to explore herpes simplex virus type 1 (HSV-1) release from infected HeLa cells (12). From a total of 80 infected HeLa cells, the range of virus released was 1–65 pock units, with an average yield of 13 pock units, which we are considering an equivalent measurement to PFUs. This pioneering research, performed over 70 years ago, laid the groundwork for single-cell virology and the study of virus and host heterogeneity. With the advent of new technologies in the last decade, mainstream recognition of single-cell virology has resurged.

RESURGENCE OF SINGLE-CELL VIROLOGY

As the latter half of the 20th century unfolded, virology research gravitated toward increasingly intricate questions. Viral infections involve multiple cellular processes, which vary between cells (19). Analysis of diverse patterns of interconnected cellular activities within cellular populations, including the microenvironment, the cell state, and the likelihood of them being infected, was foundational in understanding the origin of cellular heterogeneity within diverse cell populations (19). Many innovative studies to disentangle population-level effects were propelled by the development of new molecular biology tools, a proliferation of diverse tissue culture models, and the refinement of monoclonal antibody technology (20). Remarkably, it was not until 2008 that single-cell virology came full circle with experiments reminiscent of those conducted in the 1950s. Notable among these pioneering endeavors were the assessments of progeny production and viral kinetics from single cells infected with vesicular stomatitis virus (VSV) (21, 22). These investigations revealed that like many RNA viruses, VSV produces heterogeneous populations due to the error prone nature of the RNA-dependent RNA polymerases. To measure virus production, baby hamster kidney (BHK) cells were inoculated with VSV expressing green fluorescent protein (VSV-GFP) at a low multiplicity of infection (MOI) of 0.01 to minimize the number of cells infected with more than one infectious virus particle. The use of VSV-GFP also facilitated sorting via fluorescence activated cell sorting (FACS) for positively infected cells and subsequent quantification of viral progeny which ranged from 50 to 8,000 PFUs/cell (21). Given the substantial range of virus production, researchers hypothesized that differences in progeny production may be attributed to variations in cell size or the cell cycle stages. However, the analysis of these two variables did not fully account for the observed heterogeneity, suggesting that other unknown factors were involved (21).

Echoing the endeavors of Dulbecco and Vogt, the kinetics of virus production from individual BHK cells infected with VSV were also explored (22). In contrast to the previous work by Zhu et al., infection of BHK cells was done at a high MOI of 5, resulting in progeny virus detected at markedly varied rates, between 4 and 12 hours post-infection (hpi) and ranging from 10 to 3,000 PFU per hour (21, 22). Moreover, increasing the MOI from 5 to 80 did not significantly alter the quantity of progeny produced from infected

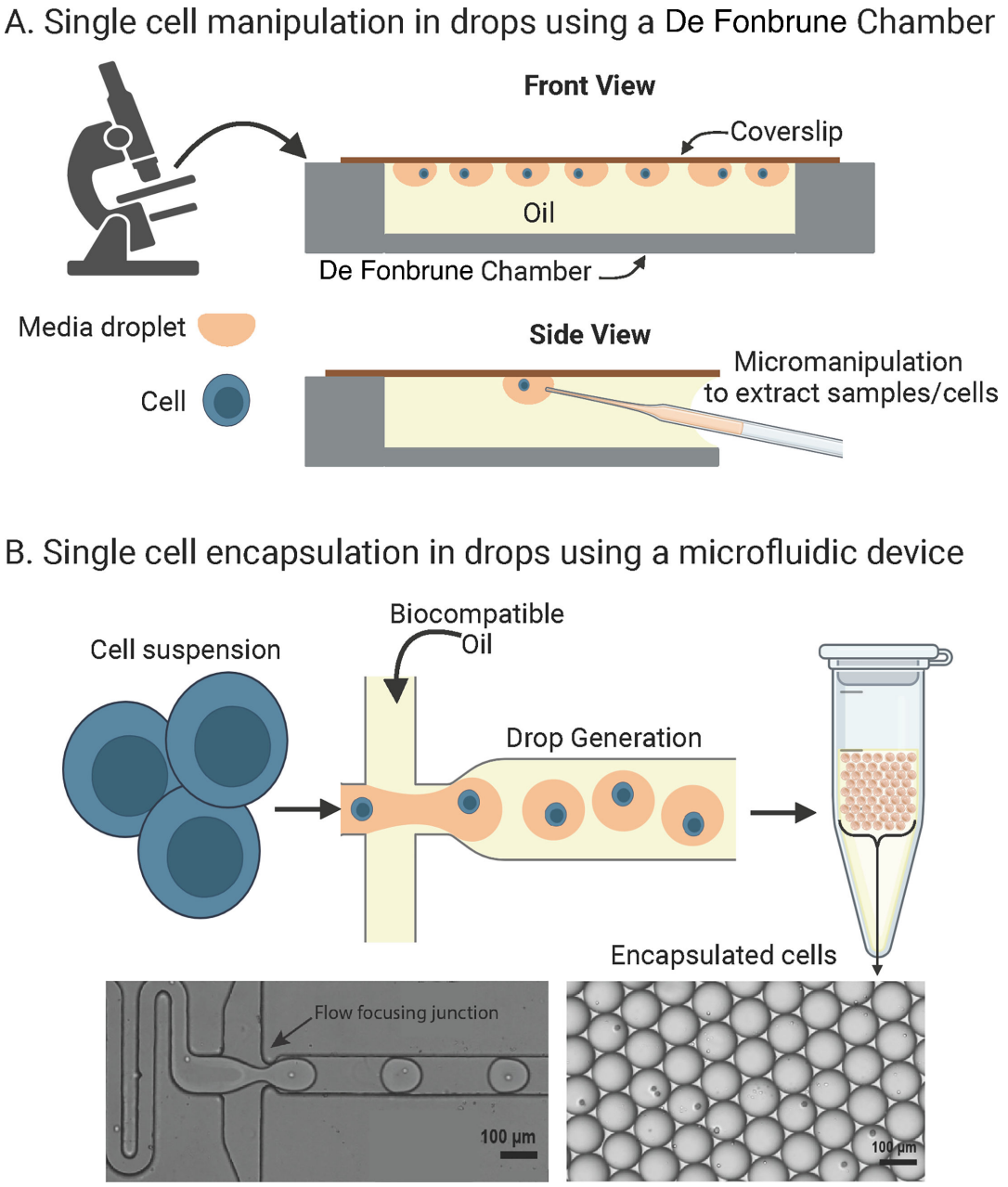

FIG 2 How it started…How it's going. Drop-based methods for single-cell virology assays. (A) Early single-cell virology studies were performed using the De Fonbrune chamber to encapsulate and hold infected cells within media droplets. A schematic of the De Fonbrune chamber is displayed [adapted from early illustrations by Lwoff et al. (11) with permission of the publisher]. The De Fonbrune chamber (gray) holds a coverslip (brown) spotted with single cells (blue) encapsulated in media microdroplets (orange). The coverslip, with cells, is submerged in oil (yellow) to enable viewing on a dissecting scope for extraction of single-cell virus samples through the oil via micromanipulation via the open face as shown in the side view. (B) Drop-based microfluidics is a high-throughput method that can be used for encapsulation of single infected cells (blue) in media (orange). Droplets are generated using a polydimethylsiloxane (PDMS) device by co-flowing a biocompatible oil (yellow). Both a schematic of this process and microscopy images adapted from Loveday et al. (18) of droplet formation and encapsulated cells are presented. Figures created with biorender.com and adapted from Loveday et al. (18) (https://creativecommons.org/licenses/by/4.0/).

cells. However, the kinetics of virus production were not measured at the higher MOI, leaving uncertainty as to whether the virus was produced at a faster rate. In the decade that followed, multiple single-cell studies covering a diverse range of different viruses, including PV, hepatitis C virus, human immunodeficiency virus, dengue virus, foot and mouth disease virus, influenza A virus (IAV), and others were performed and have been previously reviewed elsewhere (8, 9, 23–25).

Analysis of individual infected cells primarily focused on RNA viruses containing a single genome segment. However, it remained unclear if multi-segmented genomes, such as IAV, exhibited similar trends in terms of progeny production and replication. With eight individual negative-sense RNA genome segments, the replication and packaging of IAV is a more complex process (26).

To evaluate heterogeneity during IAV infection, Heldt et al. quantified progeny production via plaque assay and correlated these data to genomic viral RNA (vRNA) abundance (27). A limiting dilution approach isolated 430 infected cells, facilitating the sampling of viral progeny with titers ranging from 1 to 970 PFUs/cell across, covering three orders of magnitude. The range is comparatively lower than what had been observed in other RNA viruses. Interestingly, a majority of cells only released between 1 and 75 PFUs and close to 40% of cells producing no PFUs at all. vRNAs were detected in all the cells analyzed, demonstrating that while viral genomic material was present, it did not always lead to the production of progeny virions.

Over the ensuing decade, substantial technological strides would begin to unravel pivotal questions regarding the heterogeneity observed during single-cell infections. Single-cell studies also moved beyond examining progeny production to evaluate additional dynamics during the virus lifecycle.

TECHNOLOGICAL STRIDES PROPEL THE GROWTH OF SINGLE-CELL VIROLOGY

Single-cell analyses discussed thus far have relied on differences in progeny production from individual cells to measure heterogeneity (10–12, 21, 22). However, there are many types of viral assemblies produced from infected cells. The most notable of these are DIPs, which can impede intracellular viral growth and result in observed differences in progeny production. Discovered by Von Magnus in the 1950s (28) and further explored by Huang and Baltimore in the 1970s (29, 30), DIPs typically encode only a portion of the viral genome yet harbor normal viral structural proteins, reproducing solely when co-infected in the presence of a helper virus (29, 31).

Understanding the fundamental interplay between DIPs and infectious particles at a population level presents challenges. Consequently, single-cell infections have been used to dissect the role of DIPs during infection. In 1980, Sekellick and Marcus tested the hypothesis that a single interfering particle could entirely hinder the production of progeny virions (32). To test this hypothesis, they co-infected Vero cells with 8.5 PFUs of wild-type (wt) VSV and either 0, 1, or 16 VSV DIPs. Individual cells were sorted manually into individual tubes and analyzed for progeny virion production. Consistent with prior research, viral progeny production from VSV infected Vero cells without DIP co-infection was highly variable with over 90% of infected cells producing progeny with an average yield of 2,790 ± 1,250 PFUs/cell. Co-infection with 1 or 16 VSV DIPs reduced the average yield to 1,090 ± 1,760 and 370 ± 820 PFUs/cell, respectively (32). Alongside the overall decrease in yield, the number of cells that produced no or very little (1–100 PFUs/cell) virus significantly increased. Of the 160 cells analyzed following co-infection with VSV and a single DIP, 48% of cells produced no virus, and 13% of cells produced less than 100 PFUs. Surprisingly, co-infection with a higher number of DIPs resulted in fewer cells producing little or no virus than expected but shifted cells toward lower virion production. The single-cell analysis of VSV DIPs unveiled two to four times more interference than observed in larger cell populations. These observations led to the conclusion that VSV DIP interference is an all-or-nothing event. Furthermore, it was observed that a single DIP elicited greater interference of VSV replication than multiple DIP co-infections.

The advancement of single-cell virology in the 21st century reignited interest in DIP dynamics during infection to better understand their influence on viral gene expression kinetics, population dynamics, and transmission (31). Integrating recombinant viruses that express fluorescent proteins enabled the analysis of multiple additional parameters when cells were co-infected with various MOIs of DIPs (9). One example was the use of VSV expressing red fluorescent protein (VSV-RFP), which allowed for the analysis of viral gene expression kinetics during the virus lifecycle (33). VSV-RFP co-infection with

various DIP MOIs found that progeny production was inhibited at a greater rate than RFP expression, with some cells producing no measurable PFUs, but remained RFP positive.

To explore additional kinetic parameters, cells co-infected with VSV-RFP at a high MOI of 30 and DIPs at MOIs of 0, 0.1, 1, or 10 were seeded onto a microfluidic device that contained ~2,500 microwells. This enabled isolation of single cells and imaging of RFP expression from individual cells over time in a more high-throughput manner. Co-infection with DIPs at an MOI of 10 increased the latent time, representing the time between adsorption and the first infectious virus produced, threefold and delayed rise time, the time during which the cell produces the virus, up to 50%. The higher DIP inoculations also reduced overall viral yields by 100-fold. These findings diverge from some observations made in 1980 by Sekellick and Marcus (32) and could be attributed to differences in the production of DIPs analyzed at their respective times.

While previous studies on VSV aimed to unravel how DIPs impede overall virus production, the *de novo* diversity of DIP expression and production at a single-cell level remained unexplored. Variations in DIP production from individual cells likely represent another dimension of heterogeneity contributing to differences in viral replication and production (31). Previous analysis of single cells infected with IAV revealed extreme heterogeneity in virus production and vRNA expression (27). Integration of additional techniques such as scRNA-seq facilitated a deeper dissection of viral RNA heterogeneity and the generation of *de novo* DIPs during replication cycles (34). Using a limiting dilution approach, Kupke et al. infected single Madin Darby canine kidney (MDCK) cells with IAV at a high MOI of 10. This allowed for co-infection and potential expansion of DIPs already present within the inoculum. Both full length (FL) and defective interfering (DI) vRNAs and mRNAs were identified and quantified via RT-PCR and scRNA-seq. The initial analysis focused on quantifying DI vRNA for the three polymerase segments (PB2, PB1, and PA). Examination of 39 individual cells revealed that segment 3 (PA) produced more DI vRNA than the other two segments. Expanding the analysis to a total of 185 individually infected cells, those yielding more than 60 PFUs/cell exhibited fewer DI vRNAs with a DI to FL ratio of approximately 1.3 or less, while cells producing less than 60 PFUs/cell had a higher ratio of approximately 3.4 or less, suggesting that accumulation of DI vRNAs may contribute to IAV production heterogeneity (34).

The production of DI genomes is only one outcome of the heterogeneity inherent to IAV replication. To identify additional factors contributing to IAV heterogeneity, whole transcriptome analysis was conducted on single infected cells using Illumina-based next-generation sequencing technology (34). This scRNA-seq approach was complemented with progeny virus production to correlate transcriptomic changes and virus yields. Overall, virus titers ranged from 1 to 1,100 PFUs/cell. Transcriptomic analysis provided data from 86 of the single infected cells revealing that host mRNA levels were low when compared to controls, possibly due to viral suppression of host gene expression. Detection of DI mRNAs, which would result in defective protein expression, was higher in cells with lower overall virus yields. Notably, outliers included cells with undetectable DI mRNA and low virus production, as well as cells with high DI mRNA levels and high virus production, suggesting additional factors contributing to viral heterogeneity.

During single-cell infections and subsequent sequence analysis, there emerged 16 *de novo* DI vRNAs and 25 new deletion junctions in progeny virions, distinct from the DI vRNAs present in the initial inoculum. This accumulation of DIPs during infection can significantly influence the overall fate of infected cells (31). High levels of DIPs can be immunostimulatory (35) and affect the capacity of infected cells to produce viable progeny virions (31). Overall, using a multifaceted approach highlights the complexity of viral heterogeneity within individual infected cells. In addition, technological advancements, such as the use of microfluidic devices and scRNA-seq, enabled deeper insights into the dynamics of infection at the single-cell level.

UNRAVELING VIRAL DYNAMICS: HIGH-THROUGHPUT ANALYSIS OF INDIVIDUAL INFECTED CELLS

A limitation to the prior experiments has been the number of cells that can be readily manipulated and analyzed. The development of technologies that could be adapted to analyze greater numbers of cells was sorely needed. Analysis of single-cell infections has relied heavily on using limiting dilutions, micromanipulation, and FACS to isolate individual infected cells. While FACS can process thousands of cells quickly, a low MOI inoculum to ensure single infectious particles per cell results in a low number of infected cells that must also express sufficient viral antigen for detection and isolation (9). Limiting dilution approaches also result in low cell yields for analysis (9). The advent of microfluidic-based platforms aimed to increase throughput and allowed for kinetic analysis through image analysis of virions that express fluorescent proteins (33, 36). Microfluidic approaches present an opportunity for higher throughput analysis of single-cell infections. The use of different microfluidic technologies to study single-cell virology has recently been comprehensively reviewed (37–39).

One technique being developed is the use of drop-based microfluidics (Fig. 2B). Drop-based microfluidics can be used to compartmentalize individual cells within aqueous, picoliter volume droplets separated by immiscible oil (40). These drops can be generated and analyzed at a rate of thousands per second, while achieving a 1,000-fold reduction in volume compared to microtiter wells. Microfluidic advancements facilitated rapid and high-throughput analyses, applied to the directed evolution of microorganisms (41), protein expression profiling from single cells (42), digital droplet PCR (43), and scRNA-seq (37, 44). Such technology serves as a potent tool for encapsulating controlled numbers of individual host cells and viruses, offering a swift, precise, and targeted means of acquiring single-cell infection data (37, 44).

While the initial application of drop-based viral infections within single cells dates back to the 1950s (11, 12), the experiments were limited in both scope and throughput. The use of high-throughput droplet microfluidics to study virus infection used RAW 264.7 cells, a murine macrophage cell line, to study murine norovirus (MNV-1) (45). This system was used in a number of different studies to detect genomes using in-drop PCR analysis (46), co-infection and recombination (47, 48), and fitness landscapes obtained from next-generation sequencing (49). The use of MNV-1, an extremely stable virus due to a proteinaceous capsid (50), and macrophage cells cultured in suspension, was highly compatible with the aqueous nature of drop-based microfluidics (45–47, 49).

Recent research has expanded the application of drop-based microfluidics to analyze single-cell infections from adherent cell lines infected with IAV, an enveloped virus (18). Infections of A549, a lung carcinoma cell line, and MDCK cells with seasonal IAV were followed with cell encapsulation into droplets. IAV replication and production measured from encapsulated cells using RT-qPCR and plaque assays resulted in no significant difference between infections performed in bulk and in drops from 0 to 24 hpi (18). However, to quantify progeny production of viral RNA content, drops must be broken to access the necessary content for analysis. To address this and quantify viral progeny from each individual cell, technical advances were made to separate and quantify viral progeny following amplification of viral genomes using an optimized in-drop RT-qPCR assay (51). By utilizing an RT-qPCR approach, the progeny virions were not limited to measurements of infectious virus only and could also capture DIPs and other semi-infectious particles that exist within the IAV population (52). From these data, the distribution of IAV H3N2 and H1N1 genomes from individually infected A549 cells was determined to be highly heterogeneous, spanning three orders of magnitude from 10^1 to 10^4 copies of M gene RNA released from individual cells (53). The average progeny production, based on genome quantification, was determined to be 710 particles from H3N2 and 360 particles from H1N1. In addition, a Gini coefficient, which is a statistic used to measure disparities in income inequality (54), was used to analyze the resulting heterogeneity in virus production (53, 55). It was determined that only 10% of cells produce 60% and 70% of H1N1 and H3N2, respectively (53). These data align with recently published data

quantifying the number of HA and NA segments via scRNA-seq. In this instance, 6.5% of infected cells were found to generate over 50% of the counted progeny virus (56).

Microfluidics harnesses fluid flow through microchannels to create highly controllable and reproducible biological assays (37, 57). However, the liquid state of drops necessitates cells to be isolated in suspension and does not facilitate the use of differentiated primary cell types for single-cell infection studies. Micron-scale hydrogels, or microgels, produced via drop-based microfluidics offer a solid scaffold to enhance primary cell differentiation and stabilize fragile cellular extensions for use in microfluidic devices (58–61). These microgels establish a reproducible 3-D growth environment closely resembling the *in vivo* conditions of many cell types and can be collected for downstream assays, such as single-cell infection models (60–62).

Recent work underscores the utility of microgels by investigating infection dynamics of HSV-1 primary mouse neurons that were cultured and matured in microgels, resulting in extended axons (63, 64). Infection of the mature neurons with HSV-1 was performed using a microfluidic co-flow device in which the microgels and virus are simultaneously encapsulated into drops. Infections were performed with a recombinant dual fluorescent HSV-1 reporter. Immediate early gene expression correlated with YFP fluorescence, while detection of the minor capsid protein, fused to RFP (VP26-RFP), indicated viral assembly (64).

The fluorescent protein expression revealed complex outcomes of HSV-1 infection. Neurons inoculated with 1, 10, or 100 PFUs/drop exhibited variability in the onset of YFP detection. Expression of YFP was detected at later time points (4.9 ± 2.0 hpi) following inoculation with 1 PFU/drop than was observed following inoculation with 100 PFUs/drop (3.3 ± 1.7 hpi). This suggests that HSV-1 gene expression occurs at a faster rate when cells are inoculated with a higher dose of infectious virus. Detection of RFP, which indicates progression of viral replication and correlates with viral assembly, was also highly variable, with an average of only 2.6% and 6.5% of YFP-positive cells becoming RFP positive following inoculation with 1 or 10 PFU/drop, respectively. At the highest inoculating dose of 100 PFUs/drop, 55.7% of YFP-expressing cells also expressed RFP, and the average onset time was 9.4 ± 2.5 hpi. The differences in RFP expression demonstrate a non-linear effect of inoculating dose on productive replication. While many cells were infected in all conditions, only the highest inoculating dose demonstrated extensive productive replication. These findings are consistent with reports of low rates of productive replication observed with scRNA-sequencing from infected human fibroblasts (65).

Overall, the inoculating dose influenced the extent and frequency of progression of HSV-1 infection at a single-cell level. The use of microgels also expands the capacity to perform single-cell infections with a growing number of relevant cell types, including various primary cells. This work also demonstrates the power of drop-based microfluidic approaches for high-throughput culturing and assaying of single infected cells.

No single-cell virology review is complete without highlighting scRNA-seq. Emerging just over a decade ago, scRNA-seq has revolutionized our capacity to delve into transcriptomic heterogeneity across both host and pathogen (7, 55, 65–67). For further insights into single-cell virology utilizing scRNA-seq, we encourage readers to explore the recent reviews in the literature (7, 8, 25, 68, 69).

While scRNA-seq has enhanced knowledge of changes in the transcriptome during virus infection, these changes are not always fully reflective of changes to the proteome (70). In recent years, single-cell proteomics (SCP) has been used to analyze thousands of proteins within individual cells, deepening the understanding of cell-to-cell interactions, particularly in cancer research, biomarker discovery, and developmental biology (71, 72). A number of recent reviews highlight the different approaches that are used to study spatial proteomics at single-cell resolution (70, 72–77). SCP remains an emerging field for understanding heterogeneity during viral infection. Applying a single-cell proteomics approach would enable further analysis of protein level changes, such as post-translational modifications, that are likely highly heterogeneous

during virus infection. Bulk multi-omic analysis, which includes phosphorylation events and metabolic changes, has been assessed during severe acute respiratory syndrome coronavirus 2 and Crimean-Congo hemorrhagic fever virus infection (78, 79). These changes resulted in rewired virus-host protein-protein interactions and influenced innate immune responses. Expanding these analyses using SCP would further the knowledge of cellular and viral protein modifications during infection. In addition, SCP analysis of small molecule interactions could improve understanding of potential antivirals used for therapeutics (80).

By leveraging high-throughput methods, researchers have gained the ability to analyze vast numbers of single infected cells with unprecedented speed and accuracy. This has allowed for the identification of minor genetic variants within viral populations and the tracking of their emergence over time (37, 44). Additionally, advancements in sequencing technologies facilitated the exploration of viral gene expression heterogeneity, revealing intricate patterns of diversity within viral populations (7, 8, 25, 68–70). As sequencing technologies continue to evolve, they hold immense promise for unraveling the complexities of viral biology and informing strategies for combatting infectious diseases in the future.

ONE IS NOT THE LONELIEST NUMBER

Single-cell virology, though rooted in early observations of viral heterogeneity, has recently witnessed a resurgence driven by technological advancements. Pioneering experiments in the 1950s laid the groundwork for quantifying viral progeny production from individual infected cells. These early studies revealed substantial variability in virus yields that were later observed in other, more complex, experimental designs. Decades later, multiple studies have further elucidated the intricate dynamics of viral replication and release of virus at the single-cell level.

The development of microfluidic platforms has also significantly enhanced the throughput and precision of single-cell infection studies. Drop-based microfluidics and micron-scale hydrogels offer novel approaches for culturing and assaying single infected cells, expanding the repertoire of cell types amenable to study. Further integration with high-throughput imaging and sequencing technologies promises further insights into viral biology.

In summary, single-cell virology stands at the forefront of infectious disease research, offering unparalleled opportunities to unravel the complexities of viral infections and inform public health interventions in an ever-changing world.

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