



Viral and host heterogeneity and their effects on the viral life cycle

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Abstract | Traditionally, the viral replication cycle is envisioned as a single, well-defined loop with four major steps: attachment and entry into a target cell, replication of the viral genome, maturation of viral proteins and genome packaging into infectious progeny, and egress and dissemination to the next target cell. However, for many viruses, a growing body of evidence points towards extreme heterogeneity in each of these steps. In this Review, we reassess the major steps of the viral replication cycle by highlighting recent advances that show considerable variability during viral infection. First, we discuss heterogeneity in entry receptors, followed by a discussion on error-prone and low-fidelity polymerases and their impact on viral diversity. Next, we cover the implications of heterogeneity in genome packaging and assembly on virion morphology. Last, we explore alternative egress mechanisms, including tunnelling nanotubes and host microvesicles. In summary, we discuss the implications of viral phenotypic, morphological and genetic heterogeneity on pathogenesis and medicine. This Review highlights common themes and unique features that give nuance to the viral replication cycle.

Pleomorphic viruses

A population of viruses exhibiting irregularity of size, shape or protein copy number.

Receptor avidity

The strength or affinity of the interaction between a viral receptor-binding protein and its target ligand on a host cell.

The textbook depiction of the viral replication cycle^{1,2} contains four relatively uniform core steps: entry, replication, assembly and egress (FIG. 1a). Innovative studies have uncovered novel and heterogeneous strategies for accomplishing each of these steps. In this Review, we examine the phenotypic implications of both host and viral heterogeneity in shaping the viral replication cycle. We consider several aspects of heterogeneity in hosts, including availability and post-translational modifications (PTMs) of host receptors, and variability in antiviral defences. We also discuss heterogeneity within viruses, specifically how viral polymerase fidelity, virion morphology and egress mechanisms lead to a more nuanced view of viral infection (FIG. 1b).

Technological advances (TABLE 1) have been crucial for identifying how heterogeneity redefines viral replication. Human airway epithelial cell cultures^{3–5} are emerging as an important resource for studying the role of cellular heterogeneity during respiratory viral infections. These cultures maintain well-differentiated patient-derived primary airway bronchial surface epithelial cells at an air–liquid interface^{3–5}. These cultures comprise ciliated, non-ciliated, goblet and basal cells, thus recapitulating the complex multicellular environment of the lung⁵. Single-cell RNA sequencing (scRNA-seq)^{6–17} techniques reveal cell-to-cell variability in viral infections and provide important insights into the heterogeneity of transcriptional responses and viral growth. Live-cell imaging and advances in single-particle tracking have also increased our understanding

of heterogeneous viral infections of individual cells¹⁸. The study of pleomorphic viruses has also benefited from technological advances. Historically, cryoelectron microscopy has been used to resolve the structure of homogeneous virus particles through two-dimensional averaging and single-particle reconstruction. However, heterogeneous samples such as pleomorphic viruses cannot be resolved in this way. Cryoelectron tomography^{19–21} and subtomogram averaging eliminated this barrier in structural biology and now enable visualization of heterogeneous virus particles.

In this Review, we highlight the key findings of studies using the above techniques as well as other innovative studies as we dissect phenotypic heterogeneity at different steps of the viral replication cycle. Although we have restricted our discussion to recent examples relevant for human disease, we invite the reader to consider the implications of the heterogeneity discussed here on other viruses, such as environmental viruses and viruses with plant and bacterial hosts.

Entry

Heterogeneity in entry receptor usage is a well-established concept in virology. It has long been recognized that viruses may use several receptors to gain entry into different cell types¹. In this section, we highlight recent studies that reveal the importance of viral heterogeneity for receptor avidity and the importance of host heterogeneity in PTMs of receptors. Heterogeneity in host receptor preference and PTMs extends our

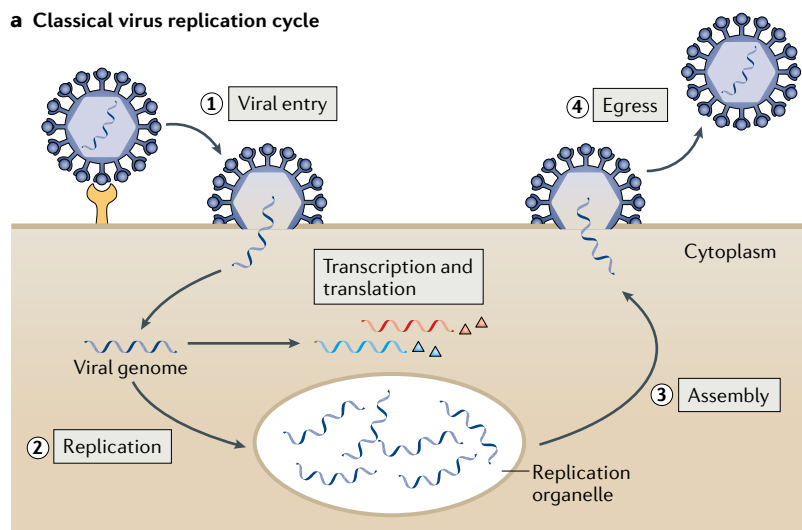
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a Classical virus replication cycle



b Heterogeneous virus replication cycle

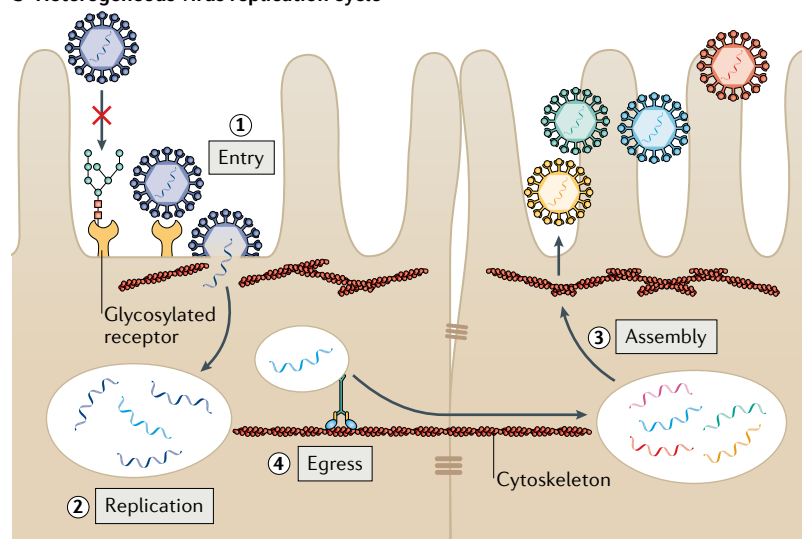


Fig. 1 | Heterogeneity in the virus replication cycle. a | Traditional depiction of the viral replication cycle with its series of defined, uniform steps, using human metapneumovirus (HMPV) as an example. HMPV virions bind to a target receptor and fuse with the host cell membrane (step 1). After fusion, viral RNA is replicated in cytoplasmic replication organelles (step 2) and viral proteins are produced and undergo assembly with the viral RNA (step 3). Homogeneous mature virus particles undergo egress through the cellular membrane (step 4). **b** | Evidence of substantial heterogeneity in the viral replication cycle is accumulating. Entry can be impacted by the presence of post-translational modification of receptors, including glycosylation (step 1). Upon entry, HMPV replication organelles can traffic along actin-containing nanotubes across tight junctions of the lung epithelium³ (step 2). Variation in assembly (step 3) and egress (step 4) lead to the maturation of heterogeneous virus particles from the cell. Similar heterogeneity has been observed in many different viruses.

understanding beyond dogmatic claims of viral tropism based on the presence of specific receptors on host cells. Glycosylation is perhaps the best-studied PTM relevant for viral entry; accordingly, we focus on this PTM and its implications for viral pathogenesis and host range.

Viral heterogeneity in receptor avidity. Viruses exploit abundant host receptors to enter into target cells, and recent studies have shown that receptor glycosylation

influences avidity. Haemagglutinin (HA) of human influenza virus binds ubiquitous α 2,6-linked sialic acid glycan moieties on the cell surface to initiate entry into target cells, and several different cellular receptors then contribute to the entry process²². A recent study demonstrated that weak virion affinity for sialic acid can lead to multiple transient association and dissociation events along the cell surface, enabling the virus to travel to regions actively undergoing endocytosis and enhancing uptake into an infected cell²³. Interestingly, the direction of virion motion on the cell surface depends on virion morphology: movement of spherical virus particles lacks directionality, but filamentous virions travel in a straight, directed manner²⁴. As discussed in the Assembly section, neuraminidase (NA) from influenza virus, which cleaves sialic acids and is required for motility²⁵, is highly polarized on the surface of filamentous virions²⁵. Given these observations, it is tempting to speculate that the density and distribution of HA and NA molecules on a virion and the respective affinity for sialic acid binding and release may influence the movement of viruses along cell surfaces and affect virus entry. It is worth noting that receptor avidity is not static for influenza viruses, as evolution of the HA protein increases²⁶ or decreases²⁷ the affinity for α 2,6-linked sialic acid. In recent years, H3N2 viruses and pandemic H1N1 viruses have caused more severe disease than previous strains^{28,29}, which may be linked to changes in receptor avidity for long-chain branched glycans³⁰. Further insight into the relationship between viral entry and virion morphology is needed to connect the role of pleomorphic viral populations in the infectivity and spread of viruses.

Host heterogeneity in receptor glycosylation. Receptor glycosylation varies between cell types and in different hosts, leading to differential receptor avidity during viral infection^{31–38}. The arenavirus Lassa virus preferentially enters cells that express α -dystroglycan (α -DG) modified with long-chain matriglycans³⁹. Like-glycosyltransferase (LARGE) glycosylates α -DG³¹. The extent to which LARGE modifies α -DG influences the avidity of Lassa virus for α -DG, and in the absence of these long-chain PTMs, Lassa virus alternatively uses TAM (Tyro3, Axl, Mer) receptors^{32,40}. Population genetics data indicate positive selection of certain LARGE alleles in West Africa, a region where Lassa haemorrhagic fever is endemic³¹. Therefore, population heterogeneity in α -DG glycosylation by LARGE may influence the prevalence and severity of Lassa fever.

Similar findings have been reported for hepatitis C virus (HCV)³⁴ (FIG. 2a). Glycosylation of human scavenger receptor class B type I (SR-BI), an HCV co-receptor^{41,42}, is thought to be mediated by UDP-glucose:glycoprotein glucosyltransferase 1 (UGGT1)³⁴. Silencing of UGGT1 or inhibition of N-linked glycosylation of SR-BI reduces its expression, and this diminishes HCV entry³⁴. SR-BI is also a receptor for high-density lipoprotein (HDL) and some individuals with high HDL levels carry the SR-BI variant T175A³³, which not only interferes with removal of HDL but also disrupts SR-BI glycosylation and does not support SR-BI-mediated HCV entry⁴³. Therefore, allelic variants that change receptor PTMs

may profoundly impact receptor avidity. Future studies will be necessary to determine whether such allelic variants influence disease outcomes.

Consequences of receptor heterogeneity for host range.

Receptor glycosylation can also influence the host range of a virus (FIG. 2b), as is the case for many pathogens associated with human pandemics, including influenza virus and at least two coronaviruses: severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV). SARS-CoV and the newly emerged SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19), both use angiotensin-converting enzyme 2 (ACE2) as a receptor^{44,45}, whereas MERS-CoV uses dipeptidyl peptidase 4 (DPP4) for entry into host cells⁴⁶. For both MERS-CoV and SARS-CoV, receptor glycosylation leads to a species barrier. Bat, camel and human DPP4 orthologues, which are not glycosylated, support infection of MERS-CoV, but glycosylated DPP4 orthologues from other species do not^{36,37}. Glycosylated DPP4 does not support MERS-CoV entry in cells expressing mouse, ferret, hamster and guinea pig DPP4 orthologs^{36,37}. Removal of DPP4 glycosylation fully restores permissivity of cells expressing mouse DPP4 and partially restores permissivity of cells expressing hamster and guinea pig DPP4 to MERS-CoV infection^{36,37}. SARS-CoV efficiently replicates in a wide range of animals, most prominently humans, bats and palm civets (reviewed elsewhere³⁸). A species barrier exists in mice and rats, however³⁸. This barrier has been mapped to two mutations in the ACE2 receptor, one of which introduces a glycosylation site in rat ACE2 that sterically hinders SARS-CoV infection. The newly emerged SARS-CoV-2 also uses ACE2 as its receptor⁴⁵ and considerable interest in the receptor avidity of SARS-CoV-2 is already apparent⁴⁷. Whether glycosylation or other PTMs in ACE2 or in the receptor-binding protein, spike (S), have influenced the emergence of this pandemic virus should be explored.

Host range is heavily influenced by the glycan specificities of influenza A viruses (IAVs) in different hosts. Most epithelial cells in the human respiratory tract preferentially express long-chain α 2,6-linked sialic acid

glycans, whereas avian gut cells express α 2,3-linked sialic acids³⁵. Correspondingly, human-origin IAV strains have higher affinity for long-chain α 2,6-linked sialic acids, which predominate in the human respiratory tract, and avian-origin IAV strains have higher affinity for α 2,3-linked sialic acids^{35,48,49}. As we have already seen, affinity for specific sialic acid linkages can change over time³⁰, with consequences for disease severity. As we continue to study and learn from past pandemics, the impact of PTMs on receptor avidity and host range must be explored.

Replication

There is an exquisite balance between viruses and hosts during viral replication, and clinical outcomes are further influenced by interactions between viruses and the host immune response. Accordingly, we see extensive viral and host phenotypic heterogeneity during this step. Error-prone viral polymerases^{50–52} and genetic reassortment contribute to viral phenotypic heterogeneity. Relative to DNA viruses, RNA viruses show exceptionally high replication error rates that provide genetic versatility in different fitness landscapes⁵³. In addition to the accumulation of random mutations, low-fidelity viral polymerases are prone to ‘slipping’ during replication, increasing heterogeneity in viral transcripts⁵⁴. Host heterogeneity can also alter viral replication. Here, we examine how viral and host heterogeneity manifest during viral replication, specifically the diversity of host immune responses in individual cells, defective viral genomes (DVGs) and viral population diversity.

Inverse relationship between viral replication and host immunity within a cell.

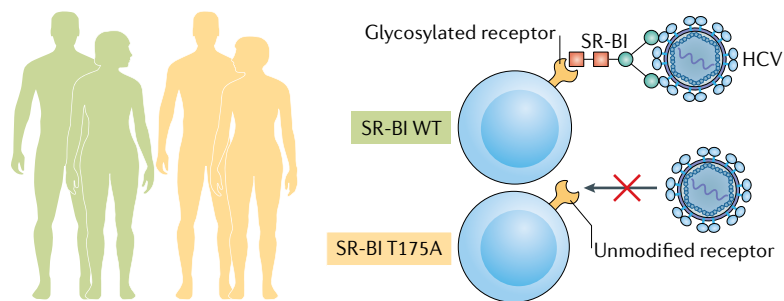
scRNA-seq has been used to examine the viral RNA burden across single cells. This methodology revealed cell-to-cell variation in viral RNA abundance for IAVs, dengue virus, poliovirus, West Nile virus and Zika virus^{6,9,11,12} ranging from <0.1 to 50% of the cellular transcriptome¹². Viral infections trigger antiviral defences upon activation of interferon and downstream interferon-stimulated genes⁵⁵. However, scRNA-seq demonstrated that robust activation of interferon- β (IFN β) mRNA transcripts is found in only a few cells infected with IAVs or West Nile virus^{6,8,12}. Defective IAV particles activate interferon expression in bulk cells⁵⁶, yet individual infected cells rarely induce interferon production^{16,57}. West Nile virus-infected cells exhibit several expression patterns of individual interferon-stimulated genes across single cells⁶. Interferon-stimulated gene expression ranged from strongly negatively correlated to weakly positively correlated with viral RNA abundance⁶. It is likely that use of scRNA-seq in a wide range of cell types will spur the detection of novel antiviral gene candidates with therapeutic potential^{6,11}. Whether these observations indicate active suppression of interferon or a failure to activate interferon should be explored further.

The scRNA-seq methodology has been indispensable in the study of incomplete or semi-infectious influenza virus particles. The IAV genome consists of eight viral RNA segments that are not always expressed in an infected cell^{13,58,59}. Failure to express all eight segments

Table 1 | Emerging technologies to study viral heterogeneity

Step of replication cycle	Technology	Application	Refs
Entry	Glycan microarray	Receptor specificity and avidity	30
Replication	Single-cell RNA sequencing	Cell-to-cell replication variability	6–17
	Single-cell RNA labelling		59
	Rare sequence detection and single-virion genomics	136,137	
Assembly	Cryo-electron tomography	Pleomorphic virion morphology	19–21
	Small-molecule labelling		86
	Live cell tracking	Intracellular particle tracking	18
Egress	Air–liquid interface cultures of the human epithelium	Cell-type specific entry or egress mechanisms	3–5

a Host-level determinants: HCV example



b Species-level determinants: MERS-CoV example

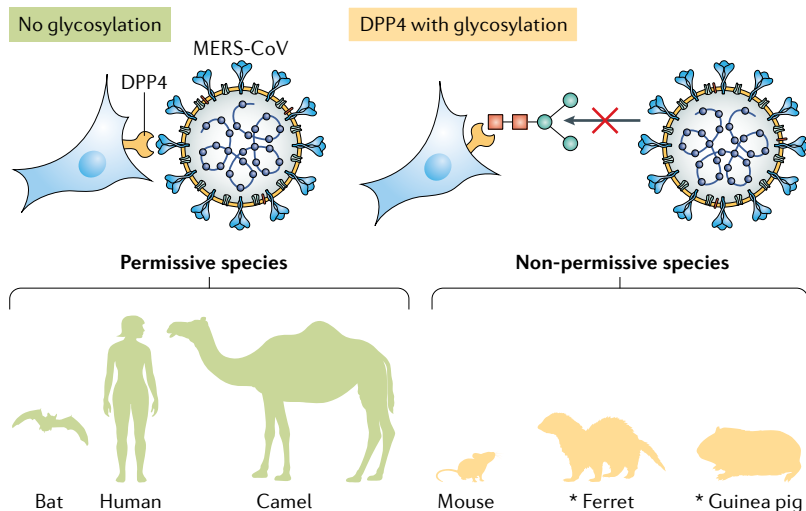


Fig. 2 | Heterogeneity in glycosylation of entry receptors. **a** | Differences between individual hosts can affect viral entry, such as heterogeneous host populations (left) that carry differentially modified viral receptors. In the example shown, hepatitis C virus (HCV) has different affinities for variants of its co-receptor, scavenger receptor class B type I (SR-BI). The glycosylated wild-type (WT) T175 variant (top) binds HCV, and the unmodified T175A variant found in human SR-BI (bottom) does not. **b** | Furthermore, species-level determinants of viral entry exist. Middle East respiratory syndrome coronavirus (MERS-CoV) differs in its affinity for different variants of the receptor dipeptidyl peptidase 4 (DPP4). Humans, camels and bats carry unmodified DPP4 (left), which binds MERS-CoV, whereas the glycosylated DPP4 variants found in mice, ferrets and guinea pigs (right) do not support infection. *Additional determinants exist.

impairs viral mRNA transcription and the productivity of infection^{12,13,17,60,61}. More recently, one study coupled single-cell transcriptomics with long-read PacBio sequencing, determining that two-thirds of cells were infected with IAV variants with mutations and deletions that lead to higher levels of immune activation than the wild-type variant⁸. Sequencing viral genes in individual cells captures heterogeneity that has so far been overlooked and provides a unique perspective on viral species present during infection.

Defective viral genomes are drivers of replicative heterogeneity. Most RNA viruses produce DVGs upon replication at high multiplicity of infection⁷. DVGs are truncated forms of the viral genome that lack essential genes and cannot replicate in the absence of a helper virus with complementing components. Two main types of DVGs exist, deletion and copy-back DVGs^{7,62}.

Deletion DVGs lack parts of the genome and hundreds of different variants of these have been identified from a single infection⁷. By contrast, copy-back DVGs are regulated by sequence-specific ‘break points’ and ‘rejoin points’ at which the polymerase detaches and rejoins the template, and thus are not stochastic in origin⁶². For unknown reasons, Sendai virus DVGs are unable to interact with host factors involved in viral packaging and budding, limiting replication of these variant genomes in the absence of full-length genome complementation^{63,64}. Evading or counteracting the host antiviral response is critical for productive infection and efficient replication. Yet DVG production decreases infectivity by competing with full-length genomes and robustly triggering the host antiviral response. DVGs also affect temporal variation of the host cell transcriptome⁶¹. The role of DVGs during infection remains unclear but may lie in providing diversity to viral populations with high recombination or reassortment rates, or in offering a link to the development of persistent infections.

Viral population diversity and implications for host fitness. Cross-species transmission is a major driver of viral evolution and diversity, with greater genetic heterogeneity maximizing the likelihood of host jumping. RNA virus families such as Rhabdoviridae and Picornaviridae that undergo frequent host switching are substantially less co-evolved with their hosts than double-stranded DNA viruses, which infrequently switch hosts⁶⁵. This suggests that the genetic plasticity of RNA viruses allows host switching with the potential cost of reduced fitness in the original host; however, there are noteworthy exceptions to this theory, including the HCV and HIV-1 examples described at the end of this section. Here, we highlight recent examples of genetic heterogeneity in different viruses and examine how they impact host fitness and host jumping.

Population heterogeneity facilitates host jumping in different ways. In the arthropod-borne chikungunya virus, acquisition of the A226V mutation in the envelope protein E1 expands vector competence from the tropical mosquito vector *Aedes aegypti* to the Asian tiger mosquito *Aedes albopictus*, an invasive mosquito tolerant of both tropical and temperate climates^{66,67}. However, the E1-A226V mutation requires lineage-specific epistatic mutations, with the Asian chikungunya virus lineage unable to adapt to *Ae. albopictus*⁶⁸. Different rabies virus (RABV) lineages also exhibit differential propensities for host jumping⁶⁹. Dog-adapted and fox-adapted RABV lineages both show comparable rates of population heterogeneity during serial passage, yet only dog-adapted RABV efficiently replicates in both the domestic dog and the European red fox⁶⁹. These observations suggest that adaptation within certain host species can increase viral variants capable of host switching. In addition, the acquisition of certain genetic changes through viral evolution can influence the success of a given strain in a new host.

The importance of host-adaptive mutations on cross-species jumping has been examined most often for zoonotic viruses that successfully jump into human

Multiplicity of infection

The ratio of the number of infectious units of a virus to the number of cells.

Epistatic mutations

Mutations whose effects are dependent upon the presence or absence of one or more mutations in other genes.

hosts, often spurring severe outbreaks that occasionally lead to widespread pandemics. Wild birds are the primary reservoir for zoonotic IAVs⁷⁰, and the virus can spread from birds to a wide range of hosts, which can promote viral heterogeneity and occasional spillover events to humans, as observed for H5N1, H7N9, H9N2, H6N1 and H10Nx strains. Bats are believed to harbour more zoonotic viruses than any other animal, including RABV, Hendra virus, Nipah virus, Ebola virus, Marburg virus, hantaviruses and coronaviruses (reviewed elsewhere^{71,72}). Spillover events of these viruses to human hosts tends to cause severe morbidity and mortality⁷¹. However, spillover of bat viruses to humans often involves an intermediate host^{70,73,74}. The exact reasons for the involvement of intermediate hosts remain unclear, but include barriers to infection⁷¹ such as receptor specificities described in the Entry section. Nevertheless, viral families with a wide host-tropism can accommodate viral diversity to facilitate host jumping from animal reservoirs to humans.

Whereas population heterogeneity widens the host range of some viruses, viral diversity in a single host is associated with persistent infections in a single host. Considerable within-host diversity and population structure are observed for HCV and HIV-1 (REFS^{75–78}). Viral diversity in HCV infection can enhance liver cirrhosis necessitating transplantation⁷⁹, with minor variants persisting in the new liver following transplantation⁷⁶. A hallmark of HIV-1 infection is compartmentalization in different anatomical sites, triggering diversification into distinct, independently evolving subpopulations^{75,78,80–83}. HIV-1 *env* gene compartmentalization in cerebrospinal fluid is linked to HIV-associated dementia^{84,85}, suggesting that genetic diversification of HIV-1 and pathogenesis and disease progression might be related.

From these examples, it is clear that population heterogeneity can have vastly different outcomes for viral fitness in different hosts. The examples illustrated here include a wide range of genetically plastic RNA viruses that nonetheless exhibit very different propensities for host jumping. It is clear that population heterogeneity affords extreme flexibility in a virus to either co-adapt to a specific host or to host switch.

Assembly

Heterogeneity in virion structure, composition and morphology impact biological processes^{86–90}. For viruses with segmented genomes, a full-length genome must be packaged with high accuracy by properly folded virion components to ensure formation of fully infectious virions. For many viruses, highly regular particles form with readily defined morphology and identifiable patterns of symmetry that cannot accommodate more than one copy of the viral genome. Recent reviews have discussed heterogeneity in structural protein maturation, such as the high proportion of immature virions produced during flavivirus infection^{91–93}. Here, we focus our discussion on newly described and historically difficult to resolve structures of pleomorphic viruses and variations in virion glycoprotein levels. Pleomorphic virus particles are more flexible than viruses with regular morphology,

producing a highly heterogeneous mixture of virion particle sizes. These heterogeneous structures have relaxed constraints on genomic packaging and, in some cases, do package more than one genome copy^{94,95}. Accordingly, we also discuss low-fidelity packaging of segmented viral genomes.

Semi-infectious particles. Many viruses exhibit a particle to plaque-forming unit (PFU) ratio greater than 1, suggesting that not all viral particles are equally infectious. Particle to PFU ratios vary among virus family and type, ranging from 1 or 2 for Semliki Forest virus to as high as 10^7 for some HIV-1 variants⁹⁶. Segmented viruses have complex packaging requirements in which a single copy of each genomic segment must be packaged to produce an infectious virion. Packaging of the segmented bunyavirus, Rift Valley fever virus, is thought to be random, producing virions lacking one or more segments and leading to infected cells failing to express all viral proteins⁹⁷. Meanwhile, packaging of IAVs is tightly coordinated to incorporate one copy of each segment into the majority of virions⁹⁸. Despite this quality control, approximately 20% of IAV particles package fewer than eight genomic segments⁹⁹, and a large number of infected cells fail to express IAV proteins from all eight segments or to replicate each segment equally⁵⁸. Viruses either lacking gene segments or unable to fully translate or replicate all eight viral gene segments are referred to broadly as ‘semi-infectious particles’⁵⁸. The origin of naturally occurring semi-infectious particles is unknown and could be a consequence of incomplete genome packaging, failed nuclear import of viral RNA segments, abortive replication or epigenetic silencing of viral RNA segments. Recently, one study demonstrated that the fitness cost of infection with semi-infectious IAV particles is reduced through complementation by incoming virions from neighbouring infected cells⁸⁷. Semi-infectious particles are observed in IAV populations in vitro and in vivo⁸⁷, and may drive viral heterogeneity within a host and, potentially, between hosts. Therefore, the possibility of complementation of IAV genomic segments in humans and its relationship to IAV transmission should be explored.

Pleomorphic particles. Influenza virus, HIV, rubella virus, pneumoviruses and paramyxoviruses such as measles virus are all pleomorphic^{19,100–103}. Rather than a strictly uniform virion structure, these viruses are highly variable in length and/or diameter and versatile in genomic packaging capacity^{94,104,105}. Characterization of pleomorphic structures has been historically challenging, but these particles are now being resolved through cryo-electron tomography^{19–21,103}. This method was recently used to resolve the helical glycoprotein arrangement of rubella virus¹⁹.

Low-fidelity assembly is intricately interwoven with host adaptation, escape from antibodies or antiviral drugs and pathogenesis. IAVs are highly heterogeneous in structure, with a single cell capable of producing a diverse array of particles, including spherical, ovoid and bacilliform particles and filaments of varying length and diameter¹⁰². The filamentous morphology of IAVs

Plaque-forming unit (PFU). The concentration of viruses capable of lysing host cells and forming a visible plaque, per unit volume.

has been comprehensively reviewed recently¹⁰². Disruption of the actin cytoskeleton selectively impairs the budding of filamentous, but not spherical, virions¹⁰², implicating distinct mechanisms underlying the formation of each type of virion. Human clinical IAV isolates are typically filamentous in morphology, but many laboratory strains, which are usually produced in embryonated eggs, produce exclusively spherical particles¹⁰². Further investigation of the host and viral determinants of virion morphology will be necessary to determine the factors underlying these observations.

Virion morphology may have implications for pathogenesis. In influenza virus, HA is enriched over NA in longer filamentous particles⁸⁶, shifting the ratio of HA to NA molecules per virion and potentially impacting entry kinetics, NA activity and cleavage of sialic acids during budding. Differences in the HA to NA ratio on influenza virus particles could also influence sensitivity to NA antivirals⁸⁶, and possibly antibody epitope accessibility. Interestingly, efficient transmission of H1N1 2009 pandemic influenza viruses correlated with NA activity and filamentous virion morphology^{88–90}. Further investigation of the mechanism underlying these observations is necessary. Alternatively, the glycoprotein arrangement on filamentous particles may explain differences in HA and NA activity. As described in the Entry section, NA is polarized on filamentous virions²⁵. Whether these morphological differences impact pathogenesis remains an active area of investigation.

Measles virus assembly also exhibits phenotypic heterogeneity, which may have consequences for clinical disease progression. During assembly, the measles virus matrix (M) protein binds the inner surface of the plasma membrane and the cytoplasmic tails of the viral fusion (F) glycoprotein and the attachment glycoprotein haemagglutinin (H)¹⁰⁶, and is believed to coordinate assembly¹⁰⁷. In rare cases, mutation or deletion of the F or H glycoprotein cytoplasmic tails accelerates measles virus dissemination through cell monolayers by the formation of syncytia and through the brain of patients suffering from subacute sclerosing panencephalitis^{106,108,109}, a highly lethal, progressive neurological disorder resulting from persistent measles infection. Interestingly, measles virus vaccine strains with mutations in the M protein have higher avidity for the H cytoplasmic tail, but are defective for syncytia formation. Further investigation of this apparent link between the avidity of M protein for the glycoprotein cytoplasmic tails and the role of syncytia formation in disease progression is needed.

Future studies on the role of low-fidelity assembly in the pathogenesis of other pleomorphic viruses is warranted, but lacking. It stands to reason that low-fidelity assembly may promote dissemination of other pleomorphic viruses.

Egress

Pathogens are faced with a litany of obstacles in the extracellular environment of a host. Viruses are routinely confronted with antibody neutralization, pattern recognition receptors and the downregulation of the cell surface receptors required for entry. Viruses have, in turn, developed strategies to improve cell-to-cell spread and

the chance of entry. Here, we examine a few alternate modes of cell-to-cell spread that blur the lines between entry and egress.

Tunnelling nanotubes promote rapid entry and egress.

A growing body of evidence suggests that viruses can bypass the extracellular environment altogether during subsequent rounds of infection. For many viruses, this process involves subversion of the actin–myosin cytoskeletal system through tunnelling nanotubes (TNTs). TNTs are actin-rich filamentous cellular projections that facilitate intercellular exchange of cargo such as organelles, proteins and ions¹¹⁰. TNTs are sometimes referred to as nanotubular bridges or immunological or virological synapses, depending on the context. Interestingly, major histocompatibility complex (MHC) class I molecules are among cargo transferred along recently dispersed immunological synapses, suggesting a role for TNTs in antigen presentation¹¹¹. Two distinct classes of TNTs have been reported that differ in size, composition and function^{110,112}. Thin nanotubes comprise filamentous actin (F-actin) and facilitate unidirectional cargo transfer^{110,112}. Thick nanotubes have a diameter greater than 0.7 µm, comprise both F-actin and tubulin, and are capable of bidirectional cargo transfer^{110,112}. The latter class, thus far reported only in macrophages, permits cargo exchange between cells tens to hundreds of microns apart^{111,113}. TNTs form either through actin polymerization or upon disassembly of a synapse¹¹¹ with lifetimes ranging from several minutes to over an hour¹¹⁰.

A broad swath of pathogenic viruses reportedly hijack TNTs, including retroviruses, herpesviruses, vaccinia virus, flaviviruses, human metapneumovirus (HMPV), measles virus and IAVs^{3–5,113–123}. The mechanism through which each of these viruses traverses a nanotubular bridge appears to be distinct, although the use of the actin–myosin cytoskeletal machinery for trafficking along a TNT is common for many^{3,4,114,115}. Retroviruses specifically traffic along the exterior surface of TNTs in a fashion that retains the classical egress step of budding from a host cell and receptor-mediated entry into the next cell. Murine leukaemia virus and HIV-1 bud from an infected cell and traffic unidirectionally along the outer surface of TNTs^{113,114,116}. Receptor-mediated binding of the murine leukaemia virus envelope glycoprotein, Env, stimulates outgrowth of a long, stable bridge between infected and uninfected cells, triggering apparent invagination through endocytosis¹¹⁶. TNTs are intrinsically more stable in human T cells, however, and intercellular dissemination of HIV-1 to uninfected T cells through TNTs is more efficient than that of cell-free virions^{113,117,118}.

In stark contrast to the use of TNTs by retroviruses, HMPV, IAVs and measles virus commandeer nanotubular bridges for direct transfer of viral genomic RNA across tight junctions in the host epithelium^{3–5,119}. HMPV phosphoprotein actively induces nanotube formation through actin polymerization and restructuring to promote cell-to-cell spread³. Intriguingly, HMPV and measles virus viral RNA and proteins traverse TNTs in lung epithelial cell lines and differentiated airway epithelial cell cultures^{3,5,119}, suggesting that direct delivery of viral RNA into target cells occurs in the absence of classically

Syncytia

A multinucleate cell arising from fusion of several cells.

described modes of virion egress and receptor-mediated entry. This mode of viral dissemination protects viruses from antibody neutralization during infection³.

TNTs thus seem to be a viable and attractive alternative mode of entry and egress for numerous viruses (FIG. 1b). Given that a diverse set of viruses have evolved mechanisms to traffic along TNTs, it is presumed that TNTs are widely used during viral infection. However, studies of viral trafficking along TNTs remain restricted to a small subset of cell types, frequently immortalized cell lines, some of which may not be representative of clinical disease. Given the considerable diversity of TNTs reported across cell types, further studies of TNT formation and use in relevant models of viral pathogenesis are warranted.

The use of TNTs for delivery of viral RNA to neighbouring cells by HMPV and measles virus has enormous implications for viral dissemination and suggests that TNT cargo is more diverse than previously appreciated. Do most viruses traffic along the exterior of TNTs, as HIV-1 does, or are intercellular TNTs widely used to evade circulating antibodies and pattern recognition receptors on cell surfaces? The answers to these questions will undoubtedly reflect the heterogeneous nature of TNTs.

Cloaking of viruses in host microvesicles. Given the vast heterogeneity in viral replication, transmission of communities of many virus particles en masse may complement defective or incomplete genomes. Recent work has revealed that picornaviruses package multiple viral particles into a cellular microvesicle to promote the spread of many heterogeneous virions together¹²⁴. Aggregation of enveloped viruses, such as vesicular stomatitis virus, can also facilitate the transmission of viral communities¹²⁵. Intriguingly, for many RNA viruses, the minimal infectious unit is the viral genome rather than a fully encapsidated and/or enveloped virion. Although they may improve replication efficiency, proteins packaged by an RNA virus into a virion are often not essential for replication, with the exception of nucleoprotein complexes for negative-sense RNA viruses. Historically, viruses have been classified as one of two binary types, enveloped or non-enveloped, but recent studies have indicated surprising overlap in this classification.

Many enteroviruses, such as hepatitis A virus (HAV), were historically believed to only encapsidate their genome and undergo lytic egress as non-enveloped particles. Although this mode of egress may still predominate, recent work describes a duality in viral particle composition in which quasi-enveloped HAV particles acquire an envelope from extracellular vesicles and shed non-lytically from the host cell¹²⁶. Enveloped HAV biogenesis resembles that of exosomes in multivesicular bodies as they are decorated with host exosomal markers, including CD9 and DPP4¹²⁷. Assembly of enveloped HAV also depends on at least two components of the endosomal sorting complex (ESCRT), VPS4B and ALIX¹²⁶.

Microvesicle cloaking has emerged in recent studies as a common egress strategy for many viruses. Other members of the Picornaviridae family exhibit particle duality as well, but the mechanism of envelopment

during enterovirus assembly is distinct from that of hepatoviruses¹²⁸. Poliovirus and Coxsackievirus B3 (CVB3) are shed non-lytically through the autophagy pathway^{128,129}. Poliovirus, CVB3 and rhinovirus are all found in phosphatidylserine-containing lipid vesicles, and the infectivity of these enveloped particles is higher than that of canonical non-enveloped particles¹²⁴. The authors of this study used innovative imaging approaches, including total-internal reflection fluorescence with super-resolution microscopy and direct stochastic optical reconstruction microscopy, to demonstrate that viral particles are packaged in microvesicles in clusters, thereby artificially increasing the multiplicity of infection¹²⁴. Thus, it seems that the classically non-enveloped viruses of the Picornaviridae family can subvert different host processes to achieve non-lytic egress. In addition to HAV, the classically non-enveloped enteroviruses, norovirus and rotavirus, both use microvesicle cloaking for similarly enhanced infectivity¹³⁰.

Enveloped viruses such as IAVs may also hijack microvesicle assembly¹³¹. The protein composition of the IAV virion includes over 300 host proteins, and many of these are known exosome markers, such as the tetraspanin CD9 (REF.¹³¹) identified in enveloped HAV¹²⁷ (FIG. 3). Microvesicle assembly has therefore emerged as a common mode of egress for many virus families, from the canonically non-enveloped picornaviruses to enveloped viruses such as influenza.

Given that enveloped picornaviruses show higher infectivity than their non-enveloped counterparts, cloaking a virus in a microvesicle may have huge implications for entry and egress beyond particle morphology and infectivity. These viral vesicles can be passively taken up by neighbouring cells through processes such as clathrin-mediated endocytosis, bypassing the requirement for a cellular receptor for entry. In the case of picornaviruses, non-lytic egress presents a mechanism for rapid dissemination through tissues without alerting nearby cells to the presence of a pathogen upon lysis. Continued investigation of such possibilities remains paramount to our understanding of viral entry and egress and the development of antivirals to target these processes.

Implications for medicine

Viral heterogeneity poses a difficult challenge for medicine. Adequate medical interventions are still lacking for many viral infections, particularly the ones discussed in this Review. Vaccines are available for several of the pathogens described here, but the emergence of novel viruses such as SARS-CoV-2 or zoonotic IAV strains is also a major public health concern. Viral heterogeneity influences many facets of disease, so here we highlight some elements worthy of deeper contemplation.

Antivirals and vaccines in the face of heterogeneous entry mechanisms. Current technologies make it easier than ever before to screen thousands of compounds for efficacy against viral infection and rapidly identify potential new therapeutic candidates. Nevertheless, these results should be interpreted with caution. A given virus may exhibit extraordinary diversity in genomic content and

Minimal infectious unit

The minimal concentration of virus particles required to initiate infection.

Exosomes

Extracellular vesicles produced in the host cell carrying proteins and RNA that are released into the extracellular space and can fuse with neighbouring cells.

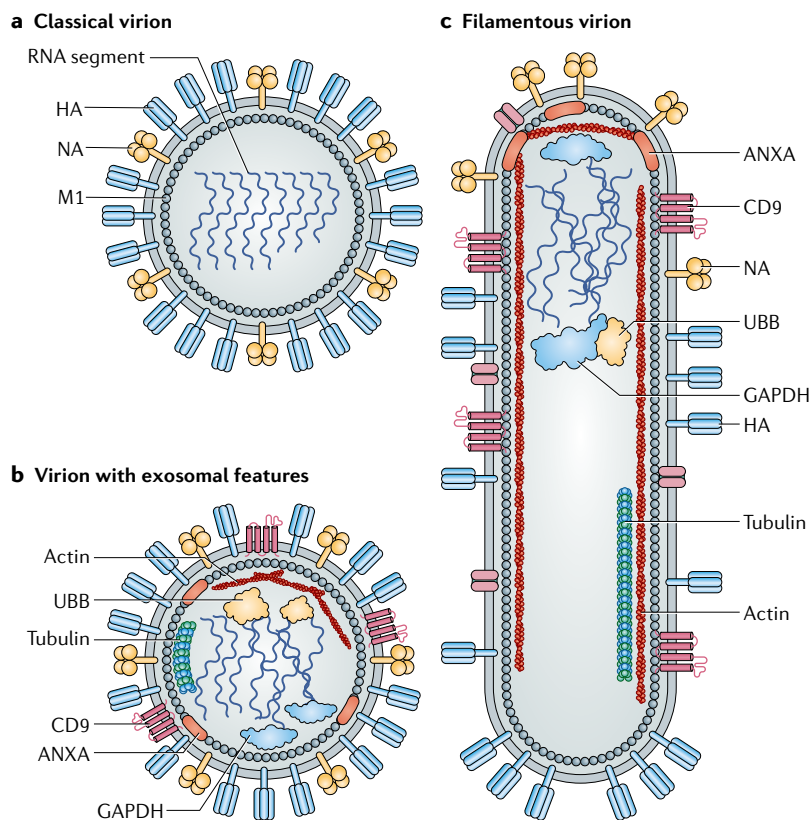


Fig. 3 | Heterogeneity in influenza virus particle morphology. **a** | Traditionally, all pleomorphic influenza virus particles were thought to comprise viral components only. This traditional influenza virus particle is shown, displaying spherical morphology and consisting of haemagglutinin (HA), neuraminidase (NA), matrix (M1) and viral genomic material. **b** | Careful studies of virions have revealed a revised architecture consisting of an influenza virion that encases several components of host exosomes¹³¹. **c** | Influenza virus also assumes a filamentous morphology of variable length with polarized NA proteins. ANXA, annexin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UBB, ubiquitin.

particle morphology, so candidate therapeutics must be pan-protective against a heterogeneous viral population. Consideration should be given to the cell type and virion morphology as well as the diversity in entry and egress processes. Many antiviral therapies identified to date are entry inhibitors, yet we have seen how diverse mechanisms of entry and egress enable rapid dissemination through tissues. The use of TNTs for delivery of viral genomes to a neighbouring cell bypasses receptor-mediated entry altogether. Therefore, entry inhibitors may prove ineffective against such infections. TNTs may also facilitate viral escape from antibody neutralization, thus weakening the effectiveness of vaccination. Such infections may progress faster than a classical infection would, as rapid dissemination to cells hundreds of microns apart can readily occur^{111,113}. Post-entry therapeutics or combination therapies with nanotube inhibitors might prove to be more effective against such viral diseases.

Dissemination of viral genomic material. A diverse set of viruses are cloaked in host microvesicles. Cloaked picornaviruses have higher infectivity than traditionally described non-enveloped particles and may disseminate more efficiently. Cloaked particles may have

other functions in addition to spreading virus particles; in the case of influenza virus, they may complement semi-infectious virus particles lacking one or more gene segments. Given that picornaviruses reportedly package multiple viral particles per microvesicle to achieve dissemination of many virions en masse¹²⁴, one could envision that microvesicles promote co-infection. Interestingly, microvesicle cloaking does not increase genetic complementation or population diversity during co-infection with multiple CVB3 variants¹³². This finding suggests that packaging of CVB3 into host microvesicles is highly selective. Considering that genetic reassortment is commonly reported at high multiplicity of infection during influenza virus co-infection¹³³, further investigation will be necessary to determine whether the selectivity observed in CVB3 microvesicles applies to other viruses. The mechanism of such high-fidelity packaging in microvesicles is nonetheless an exciting new area of research.

Heterogeneity in virion assembly may promote severe disease. Pleomorphic particles have been reported for decades, but the effect of heterogeneous assembly mechanisms on viral pathogenesis remains understudied. An intriguing function in mucus layer penetration and clearance has been proposed for long-filament IAV particles, which are defective for genomic packaging⁸⁶. Such a role for low-fidelity assembly of IAV particles would have clear implications for pathogenesis and may be a viable target in the development of future therapeutics. Alternatively, the production of pleomorphic virions in a host can result in viruses with different HA to NA ratios (see FIG. 3 for an illustration). Altered HA to NA levels on a virion could impact antibody binding and may, therefore, influence vaccine efficacy.

Synergy among polymicrobial communities. Although outside the scope of this Review, the contribution of diverse polymicrobial communities to the morbidity and mortality of heterogeneous viruses is a public health dilemma in need of deeper investigation. Recently, a complex interplay between HIV-1 and *Mycobacterium tuberculosis* co-infection and TNT formation was described. Active tuberculosis in HIV-1-infected patients is associated with elevated HIV-1 infection of macrophages found in the lungs and pleural effusions. Active tuberculosis skews human monocyte differentiation towards an anti-inflammatory M2 macrophage pathway and blood monocytes treated with *M. tuberculosis*-conditioned medium are more permissive to HIV-1 infection¹³⁴. *M. tuberculosis* infection stimulates TNT production in human macrophages, promoting HIV-1 dissemination into macrophages¹³⁴. This particular phenotypic diversity likely contributes to *M. tuberculosis*-mediated exacerbation of HIV-1 morbidity and mortality. Further study of bacterial and viral co-infection will undoubtedly reveal additional layers of phenotypic heterogeneity in these complex systems.

Conclusions

As new technologies emerge, our appreciation of phenotypic heterogeneity in the viral replication cycle grows. From alternate egress and entry pathways to variations in

genome assembly and virion morphology, these diverse replication mechanisms challenge how we classify viruses and what is considered an infectious particle. Here, we have highlighted the use of nanotubular bridges in cell-to-cell spread, exploitation of the microvesicle secretory pathway to cloak virions and promote infection, and how low-fidelity replication can drive viral heterogeneity. By redefining the canonical viral replication cycle, we will be better equipped to develop antiviral therapies against the many nuances of viral replication and address adaptive evasion strategies. Future studies examining the relationship between viral heterogeneity and disease

severity are particularly needed to help refine specific antiviral targets and biosensors. Investigation of heterogeneity in infections with emerging pathogens such as SARS-CoV-2 are particularly essential for combating emerging viral threats. Studies already indicate heterogeneity in SARS-CoV-2 entry mechanisms in different cell types, which ultimately determines susceptibility to antivirals like chloroquine and its derivatives¹³⁵. The gravity of the COVID-19 pandemic underscores the critical role of such studies in guiding global health policies.

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