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Structure and physiology of giant DNA viruses

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Although giant viruses have existed for millennia and possibly exerted great evolutionary influence in their environment. Their presence has only been noticed by virologists recently with the discovery of *Acanthamoeba polyphaga mimivirus* in 2003. Its virion with a diameter of 500 nm and its genome larger than 1 Mpb shattered preconceived standards of what a virus is and triggered world-wide prospection studies. Thanks to these investigations many giant virus families were discovered, each with its own morphological peculiarities and genomes ranging from 0.4 to 2.5 Mpb that possibly encode more than 400 viral proteins. This review aims to present the morphological diversity, the different aspects observed in host–virus interactions during replication, as well as the techniques utilized during their investigation.

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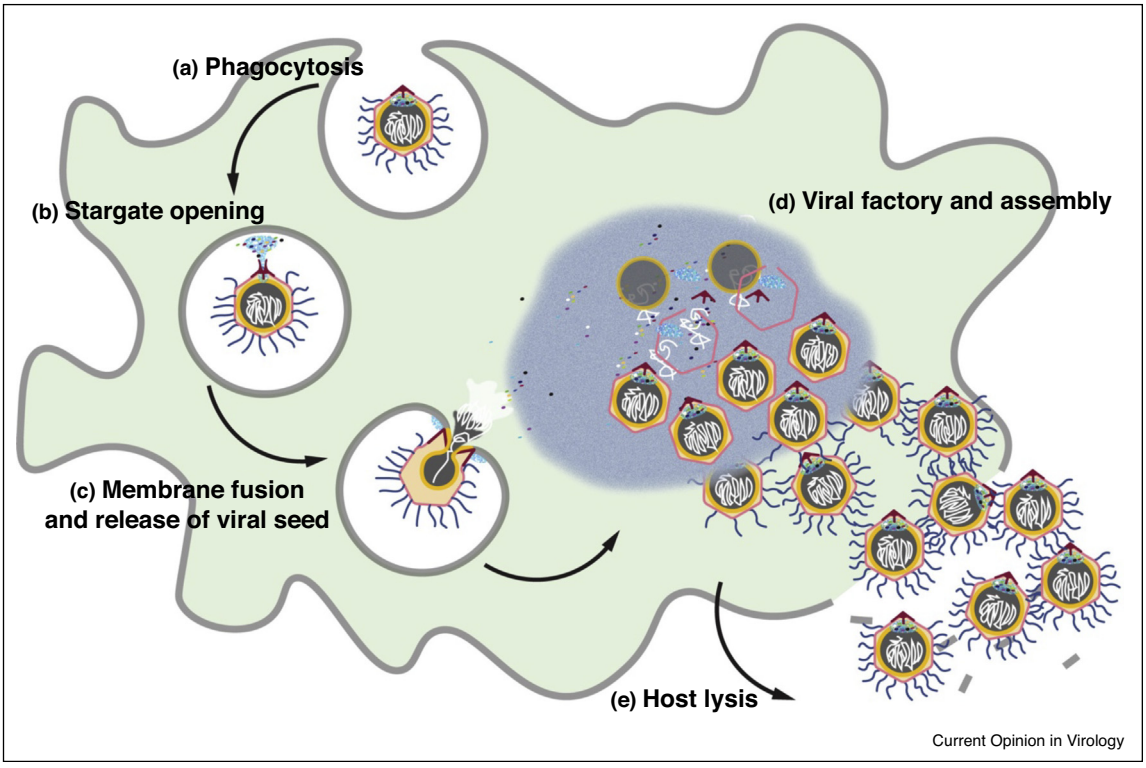
What are giant viruses?

The taxonomic classification of giant viruses (GVs) has been a topic of constant debate, partially due to the difficulty of reconciling diverging characteristics of viruses that have been called 'giant'. Initially, these viruses were inserted in the nucleo-cytoplasmic large DNA viruses (NCLDV) a so-called monophyletic group established before discoveries including *Phycodnaviridae*, *Iridoviridae*, *Poxviridae*, *Asfarviridae*, and *Ascoviridae*. In 2012, a new order Megavirlaes was proposed, and grouped viruses that possessed particle and/or genome size considerably larger than other viruses (>300 nm) and nearing the proportions of small bacteria (e.g. *Haemophilus influenzae*, with 1.8 Mbp) [1,2]. Later in 2019, the International Committee on Taxonomy of Viruses proposed another form of classification that would group GVs with other dsDNA viruses that possess a vertical jelly roll protein fold for the major capsid proteins (Realm Varidnaviria). Other taxonomic changes were also included, such as changing their inclusion in the NCLDV group to the new phylum Nucleocitoviricota, and the substitution of the Megavirales order for the Imitervirales order under the novel Megaviricetes class. Using the mimiviruses as an example the current classification establishes the following organization: Varidnaviria (Realm), Bamdfordvirae (Kingdom), Nucleocytoviricota (Phylum), Megaviricetes (Class), Imitervirales (Order), Mimiviridae (Family). Even with the establishment of this classification, the phylogeny debate will most likely continue due to the existence of GVs that are little understood and/or that possesses unique attributes, such as mollivirus and pacmanvirus. The continuous discoveries regarding GVs, exacerbated by the advances of molecular techniques such as metagenomics analysis that allows for the identification of new species in a vaster array of data when compared to the conventional methodologies [3,4], defers a cohesive and accurate phylogenetic classification from being realized.

Replication cycle of different giant viruses

Varying considerably in particle morphology and composition, GVs do share a number of similarities in their replication cycles (see Figure 1). The initial step of infection consists of particle internalization by the phagocitic host, in most cases amoebas, through phagocytosis. Triggering of said process has been associated with larger particle sizes (>500 nm) [5], and/or with fibrils present in some GV surface, whose polysaccharide composition is recognized by amoeba surface receptors. Among the constituting carbohydrates, *N*-acetylglucosamine (GlcNAc) and (mainly) mannose were highlighted as being related to such recognition. Mannose saturation experiments during infection

Figure 1



Proposed replication life cycle of Samba Virus in *Acanthamoeba castellanii*.
Entry of the virion into the host is mediated via phagocytosis **(a)**. Inside the phagosome, opening of the stargate seal complex is triggered and a complement of viral proteins is released **(b)**. By an unknown mechanism, the released proteins aid in the fusion of the sac of the virus with the host membrane, allowing release of the nucleocapsid into the cell **(c)**. A viral factory is established in the cytoplasm, where the viral dsDNA genome is replicated, and viral proteins are translated ultimately leading to assembly of viral progeny **(d)**. The newly formed virions fill the host cell, leading to lysis and release **(e)**.

resulted in reduction of cell adherence by the viruses [6,7•]. Some GVs are smaller than 500 nm but still enter the cell through phagocytosis, having developed strategies to bypass the minimum size requirement. One such example are GVs within the *Marseilleviridae*, that have ~250 nm icosahedral particles. These viruses either group together forming a large viral aggregate, or are available in the environment inside vesicles >1000 nm in size, allowing them to be effectively phagocytized [7•,8–10]. It's worth mentioning that there are other cell invasion mechanisms among the Megaviricettes that do not involve phagocytosis, with one example being the marine viruses from the *Phycodnaviridae* Family [11,12]. This family comprises viruses with sizes ranging between 100−220 nm that mainly infect algae [12]. Although some members of this family enter the cell through endocytosis, in a similar manner to other giant viruses, others such as those from the *Chlorovirus* genus have a remarkably different entry pathway that resembles those of bacteriophages [11,12]. One such example is the *Paramecium bursaria* chlorella virus (PBCV-1) whose infection occurs when the virus adheres to the cell wall with a long spike structure present at one of the capsid vertex first establishing contact with the host surface and external fibers helping the virus attachment [11]. It is believed that the spike penetrates the cell wall using the associated enzymes activity degrade it, thus allowing the fusion of the viral and the cell membrane permitting the genome injection while leaving an empty shell at the cell surface [11,12].

Upon the initial uptake, particles engulfed into phagosomes 'escape' from this inhospitable cell compartment via fusion between a viral lipid membrane and the vesicular membrane, thus delivering either a portion of the viral content to the host cytoplasm, or in some cases, the entire virion. GVs are generally sealed by a protein complex with morphology and release mechanisms that vary considerably among families. Capsids within *Mimiviridae* are sealed by a stargate, located at a unique vertex of the icosahedral particle [13••]. *Pandoraviridae*, *Cedratviridae*, and *Pithoviridae* families present non-isocahedral GVs with an apical pore portal, which can vary in number and morphology. Singular pores, or two pores located at opposite extremities, are sealed by a cork-like structure [14–17]. Other families possess unique seal structures

Table 1

Summary of known structural giant and biological giant viruses' features

Families	Capsid morphology	Average particle size	Fibrils	Number of layers	Genome release portal	Known hosts	Average replicative cycle length	Viral factory	Morphogenesis and virion release	References
Mimiviridae	Icosahedral	650−1500 nm	Lineage A Mimivirus and Tupanvirus Fibrils cover the whole capsid except for the stargate region.	7 layers.	Stargate	Amoeba (Acanthamoeba/ Vermamoeba vermiformis, Dyctiostelium discoideum); algae; zooflagellates; corals (possibly). The original host is unknown.	12 hours	Mature viral factory, formed by an association of initial small replicative centers. Electron-dense and delimited region.	Particle assembly occurs at the center and periphery of the viral factory. After capsid assembly, the genome is packed by a mechanism yet to be described. Viral particles are liberated by cellular lysis.	[7•,13••,35,49, 56,60–62]
Marseilleviridae	Icosahedral ∼10 nm thick	180−250 nm particle diameter	∼12 nm fibrils with globular tips. Covers the whole capsid.	One icosahedral layer encircling the nucleocapsid.	Not described	Amoeba (Acanthamoeba) The original host is unknown.	24 hours	Large mature VF, occupying 50% of the amoeba cytoplasm; Electron-dense region	The endosomes are recruited to the periphery of the VF, originating the membranes that surround the nucleocapsid. The newly assembled particles are reunited in vesicles and liberated through cellular lysis.	[7•,8,9,10, 15,63]
Pandoravidae	Oval	1 μm length and 0,5 μm diameter	Fibril matrix composing the second layer of the particle.	3 layers. The outermost layer with ∼25 nm diameter. The middle layer (∼25 nm) is composed of a fibril matrix, marked by a dark coloration. The third layer (∼20 nm) is turned directly to a lipid membrane. Pandoravirus massiliensis: The particle exterior is surrounded by polysaccharides similar to those found on plants cellulose.	Apical pore in one extremity.	Amoeba (Acanthamoeba) The original host is unknown.	10–15 hours	Composed of multiple small VF, with the first one being derived from the nucleus. The recruitment of mitochondria increases proportionally to the number of VFs formed and virions assembled. Region electron-lucent.	DNA packaging occurs parallelly to the capsid assembly, in the opposite region to the apical pore. Viral particles are liberated through the lysis of the amoebas, although some species of this family are liberated through exocytosis.	[14,17,15, 25,64]

Table 1 (*Continued*)

Families	Capsid morphology	Average particle size	Fibrils	Number of layers	Genome release portal	Known hosts	Average replicative cycle length	Viral factory	Morphogenesis and virion release	References
Pithoviridae	Oval	1,35−1,65 length and 750–850 nm width	X	4 layers. The outermost layer presents minimum density. Below it, the second layer is embedded in the integumentary matrix, above layers 3 and 4. This last one is considered putative and establishes an interface with the membrane that surrounds the viral genome. The particle center is encircled by a lipid membrane	An apical pore is present in one of the extremities and is closed off by cork.	Amoeba (Acanthamoeba) The original host is unknown.	10–20 hours	The matures VF is formed by the fusion of small replicative centers. Electron-dense region.	Particles are assembled in the periphery of the VF. The recently formed particles can possess a rectangular morphology due to the external matrix that envelopes them not having been fully synthesized. In another moment, some particles already in the oval morphology can have its striated membrane assembled, achieving the final stage of maturation. The mature particles are released through cellular lysis.	[15,19,53]
Cedratvirus*	Oval	750 nm–2 μm length and 0,4–0,6 μm width	X	The external layer is considerably thick and composed of parallel lines.	2 apical pores present in both extremities of the capsid closed off by two corks.	Amoeba (Acanthamoeba) The original host is unknown.	24 hours	One large VF. Region Electron-lucent Mitochondrial recruitment.	Particle assembly occurs in two steps: the closing of the capsid in the center of the VF, and the thickening of its walls in the periphery of the VF. The mature particles are released through cellular lysis.	[16,22,65]
Mollivirus*	Spherical	500−600 nm diameter	Covered by two to four layers of fibrils(with varying lengths), separated by a distance of 25 nm.	The 10 nm thick external layer is disposed of in two interspersed 30−40 nm lanes tangent to the capsid surface. The inner layer is composed of a 12−14 nm thick fibril matrix. The interior of the particle is covered by a lipid membrane.	The portal structure is located in a 160−200 nm diameter circular sulk.	Amoeba (Acanthamoeba) The original host is unknown.	10 hours	The nucleus acts as a scaffold for the VF, resulting in the loss of nucleus morphology.	Particle assembly is headed by a membrane prototype that interacts with the plane pole (seemingly membranous structure) in its center. This pole orients the spatial organization of the membrane that comprises the particles. The internalization of the particle content is regulated by the interaction with another structure, located at the opposite end of the plane pole. The viral particles are released by exocytosis. Replication cycle is not lytic.	[15,19,66,67]

Table 1 (*Continued*)

Families	Capsid morphology	Average particle size	Fibrils	Number of layers	Genome release portal	Known hosts	Average replicative cycle length	Viral factory	Morphogenesis and virion release	References
Faustoviridae	Icosahedral with $T = 277$.	External capsid = ~260 nm diameter. Inner core = ~160–190 nm diameter. 200–240 nm	Long and thick fibers distributed along the inner capsid.	Two layers organized in an external capsid ($T = 277$) and an inner core ($T = 16$).	Not described	Vermamoeba vermiformis (VV) is the only known host.	18–24 hours.	The nucleus loses its morphology. Viral factory surrounded by mitochondria.	The assembly of the inner core occurs simultaneously with the genome packaging. Later the external capsid is synthesized and interacts with the nucleocapsid through putative encroaching regions. The mature particles are released through cellular lysis.	[7•,15,20,23]
Pacmanvirus*	Icosahedral with $T = 309$	175 nm	X	A membrane covers the particle's core.	Absence of capsid opening. Virus escapes phagosome with intact virion and interacts with the mitochondria.	Amoeba (Acanthamoeba) The original host is unknown.	8 hour	Mature viral factory	Not described.	[21]
Orpheovirus *	Oval	900–1100 nm length and ~500 nm diameter.	Fibril layer covering the whole virion.	Virion is composed of 5 layers, with the last one in direct contact with the core.	Ostiole is located in one of the particle's extremities.	Solely infects Vermamoeba vermiformis	30 hours.	One large VF. Region electron-lucent Mitochondrial recruitment.	Initially, semi-circular structures suffer expansion, and then are filled with internal viral content. Virion liberation occurs through cellular lysis and exocytosis.	[18•,46]

such as the Orpheovirus having an osteole-like portal [18•], and *Molliviridae* having a yet to be named structure [19]. Alternatively, members of the *Marseilleviridae* and the Pacmanvirus families have no known capsid opening [8,10,20,21]. Members of the *Marseilleviridae* have particles enveloped by one or more membranes. These membranes are derived from the host endoplasmic reticulum and drive fusion with the phagosome allowing the entire virion to enter the cytoplasm [7•,8,10]. In early stages of infection, Pacmanvirus particles were observed near mitochondria suggesting possible interactions with the organelle, even in the absence of obvious membrane fusion [21]. A summary of the above information is shown in Table 1. After the genome is transferred to the cytoplasm, the host cell undergoes varying degrees of reorganization that culminates in the formation of a viral factory. A common modification is the recruitment of organelles such as endosomes and mitochondria to the periphery of the viral factory. In most GVs, the viral factory forms near the intact nucleus [7•,8,10,15,21,22]. The size and number of factories are also a point of divergence among GV families with most producing only one viral factory. The viral factories from *Mimiviridae*, *Marseileviridae*, *Pithoviridae*, *Faustoviridae*, and Pacmanvirus families are characterized by a single large, notably electron-dense structure when mature [7•,8,10,15,20,21,23]. On the other hand, the viral factories of the *Cedrativiridae*, *Pandoraviridae*, *Orpheuvirus*, and *Molliviridae* were observed to be electron-lucent [17,18•,22,24,25]. In both *Pandoraviridae* and *Molliviridae* the nucleus structure is disrupted and this serves as a scaffold for the formation of multiple small initial replication centers [14,17,19]. In Mollivirus, these small centers fuse together forming a large mature viral factory [19]. *Pandoravidae* is peculiar in that these small replication centers remain separated and thus form multiple small viral factories [14,17]. It is yet to be determined the role of membranes in the formation and maintenance of viral factories [25–30].

The DNA replication and virion assembly occur in the virus factory. However, particle assembly and encapsidation of the genome occur in different steps, depending on the GV family. For example, in pandoraviruses DNA packaging is located in the opposite extremity of the apical pore, and occurs as the capsids are being assembled. Conversely, genome packing in mimiviruses happens only after the complete capsid formation, through a yet unknown process/feature. It was observed that DNA is packaged via one of the facets [17,25,31].

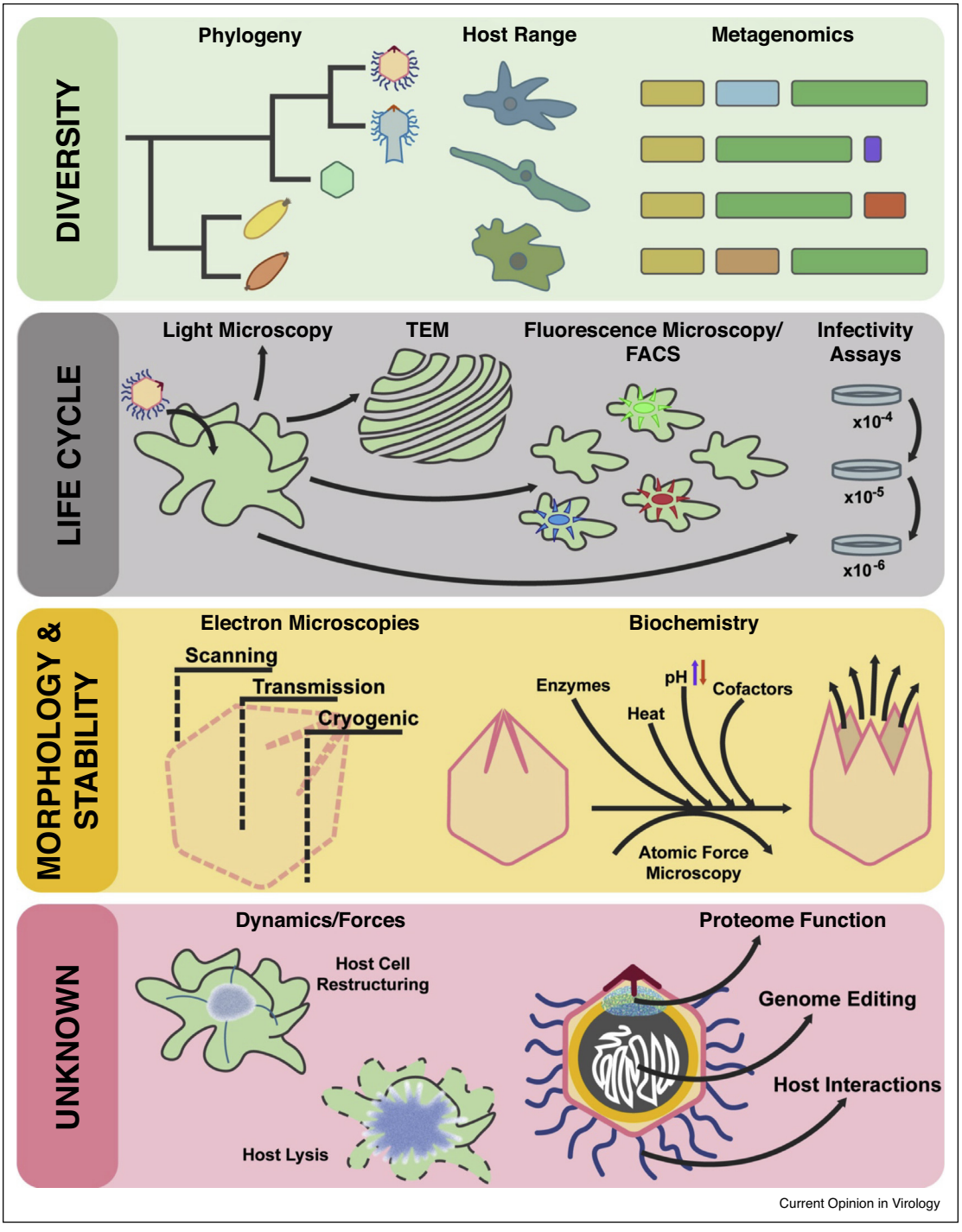
The last step of the life-cycle is the release of newly assembled viral progeny. In the vast majority of giant viruses, this occurs via cell lysis. In the final stage of *Pithoviridae* infections, the viral factory is composed of both mature and incomplete particles, and saturation within the amoeba cytoplasm causes lysis [15]. In *Marseilleviridae* assembled capsids are organized inside vesicles [7•,8,10]. Exocytosis is another means of viral release used by molliviruses [19]. Although it is believed that the endosome recruited during viral factory formation can act in the release of the viral progeny enabling exocytosis [10], it is yet to be confirmed if such release mechanism is present in other GVs families.

Techniques for characterization of giant viruses: current progress and gargantuan challenges

GVs are just how they sound—giant. Unfortunately, we know very little about the molecular organization of these particles at the atomic level and many outstanding questions remain. In what way is the seal complex opening governed? To what is the great stability of the particles owed? Do fibers impart advantages during infection? How is genome packing coordinated? The relationship between GVs and their hosts raises further questions. Is host invasion coordinated with high specificity and receptor recognition? What determines the host range? How does resistance in hosts arise? How is lysis synchronized? Are there selective pressures on giants and their hosts? Are they evolving to become more complex or more simplistic? Here we review current techniques for investigating GV structure, genome, life-cycle, and explore potential challenges and limitations to the available technology (see Figure 2).

The genomic age has revolutionized biology. Progressively, the limitations of sequencing have been reduced and efforts to sequence whole genomes have improved scaling in several orders of magnitude [32]. Advancements in methodology, a higher capacity for computation, and better data management have led to drastically lower costs, time for assembly, and the creation of national repositories to make genomic data public and readily available for mining. While this has shed unprecedented light on both the diversity and conservation of organisms within and across biomes, ***not all of the data is easily interpreted and there are major gaps in our understanding.*** GVs genomes are large and complex with many containing over 1000 open reading frames [33,34]. Mining of GVs genomes has resulted in challenging long-standing dogmas. For example, GVs nearly meet the requirements of life, lacking only ribosomes [35], and have illustrated the potential for a fourth domain [36]. Through metagenomics analysis the identification of many relevant genes related to metabolism and macromolecules assembly pathway was possible [3,4]. Yet, enigmatic regions are present in virtually every GV genome contained within modern data bases. So-called hypothetical proteins, morons, and ORFans are spread throughout GVs genomes, occupying between ~40−90% of metagenomic sequences, and are particularly problematic to interpret when novel viruses emerge [37]. This is an especially important area to consider here as many new GVs are being discovered at an astonishing rate, highlighting the burden of viral metagenomic dark matter. The majority of GVs genomes do not have any significant nucleotide sequence identity to known

Figure 2



Overview of current techniques utilized in studying Giant Viruses and the outstanding areas requiring new approaches to study.
Cartoon representation of phylogenic analysis (tree not representative of actual ancestry), range of host organisms, as well as metagenomics are used to understand and classify GVs and their evolutionary relationships (diversity panel). The replication of GVs inside their host organisms is currently being studied using light, fluorescence, and transmission electron microscopies, as well as by using infectivity assays (life cycle panel). Because of variation in capsid shape and features, the morphologies of GVs are investigated using electron microscopic approaches (scanning, transmission, and cryogenic). The stability of the capsids and their seals (if present) are studied through biochemical treatments as well as atomic force microscopy to observe the conditions necessary for opening of the virions (morphology and stability panel). Limitation in current techniques reduces the ability to fully study some aspects of GV replication such as the dynamics of viral factory formation and the forces behind host cell lysis. Additionally, the ability to understand the synergy/function of the released proteome, and the inability to easily edit the genome of GVs to ultimately uncover the full mechanics of virion/host interactions are still limited by available techniques (unknown panel).

organisms. Adding to the complexity is the discovery that some GV transcripts need splicing, requiring transcriptomic as well as metagenomic data for interpretation [38]. Approaches used to probe the proteomic landscape of GVs face similar limitations as GV fit into a category of >500 viral proteins, occupying a space filled by less than 8% of the entire virome [39]. Of these proteins, many do not match known databases, or have known functions. More than 50% of virion particles for Samba and Tupanvirus are classified as 'unknown' [13••,60]. Even more astonishing is that >90% of Pandovirus [14] and newly discovered 'Yaravirus' are comprised almost entirely of ORFans [40•]. Advances in computational modeling to predict protein structures from amino acid sequences such as Alpha Fold [41] holds the promise of assigning structure and therefore also inferring function to unveil hidden secrets of viral metagenomic dark matter. Additionally, *in silico* methods are also emerging as a way to examine GV structure and assembly. Yet, these methods are not quite ready for routine use. For a time at least, experimentally derived or confirmed structures and/or functions of giant virus proteins will remain necessary. Lastly, genetic manipulations within giant viruses or their hosts is not commonplace and amoebas are polyploid [42,43], hindering the standard 'knock out the gene and see what happens approach' widely used in classic bacteriophage genetics and other fields. Although, advances in CRISPR-mediated gene editing have allowed some initial studies in *Dictyostelium* [44••,45] it may be some time before this becomes a widespread approach in giant virus research. As a result, we cannot understand the structure and function of the majority of GV proteins using metagenomics, proteomics or predictions alone.

So what are these particles made of and how do we identify key players located within GV virions? Imaging infected cells through the use of TEM and live fluorescence microscopy has led to a thorough understanding of cellular ultrastructure and general infection processes [9,13••,46]. Imaging individual particles with SEM, or the use of dyes tagging specific biomolecules such as proteins, fibers, lipids, and nucleic acids has also been very useful describing general virion architecture [47,48]. More recently, advances in cryo-electron microscopy have allowed imaging of GVs in a native like state producing unprecedented views of a variety of viral assemblies including icosahedral GVs such as Mimivirus [49], *Marseilleviridae* [50], CroV [51], and Medusavirus [52], as well as the non-icosahedral and gargantuan large *Pithovirus sibericum* [53]. Cryo-EM is revolutionizing the details we can see within GVs and recently near-atomic views of complexes such as the PBCV-1 virion [54] have been achieved. Since a holistic approach taking into account the entire genome/particle/cell is difficult at best, a divide and conquer attack has also been explored. Recombinant expression in *Escherichia coli* has been successful for purification, structural, and biochemical studies for individual GV proteins, such as the ChoanoV1 rhodopsin-like protein VirR [55•], enzyme R135 in Mimivirus that helps with viral entry [56], and R141 a Mimivirus 4,6-dehydratase [57], and others [58,59].

However, these current methods are insufficient to conclusively map out where every individual protein resides within GV particles or how they all work together synergistically *in vivo*. Since the dawn of metagenomics we have become accustomed to relying comfortably on applying homology methods towards unraveling novel biology. This is a fantastic approach as we often have a basis for comparison and can place new species into context of the greater whole of the biological world surrounding us. However, the complexity and novelty of giant viruses effectively bring us '***back in time***' to an era decades earlier, where the link between genomic information and the function of any given gene product was not readily available. We now have the need to develop new methodologies to traverse this frontier and we have a long road ahead to fully unlocking all of the mysteries of GVs.

Conclusions

The virology field has helped life sciences researchers to understand more complex events in Biology, using viruses as simpler models to display such cellular events. Not merely that, scientists were pushed to utilize techniques to understand the biochemical composition, structural arrangement and life cycle of viruses, with the electron microscopy being the highlight. The discovery of giant viruses is a 'wake up call' to virologists as it showed us that basic research is an ever-changing challenge and that we will be surprised for a long time with new findings on our road to understanding the structure and biology of giant viruses.

Conflict of interest statement

Nothing declared.

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