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Abstract

The visualization of viral particles only became possible after the advent of the electron microscope. The first bacteriophage images were published in 1940 and were soon followed by many other publications that helped to elucidate the structure of the particles and their interaction with the bacterial hosts. As sample preparation improved and new technologies were developed, phage imaging became important approach to morphologically classify these viruses and helped to understand its importance in the biosphere. In this review we discuss the main milestones in phage imaging, how it affected our knowledge on these viruses and recent developments in the field.

Keywords: bacteriophage ; phage ; virus ; imaging ; structure ; microscopy

Earlier years (1940-1948)

Although the existence of viruses was known since the end of the 19th century, the true nature of the so called "*contagium vivum fluidum*", and whether it was liquid or particulated, remained unknown for many years [**1**]. Direct imaging of viral particles only became possible after the advent of the transmission electron microscope, allowing the determination of viral morphological characteristics. These machines were developed in the late 1930s by two different groups: one working at the Siemens & Halske laboratory company in Germany and another working at the University of Toronto in Canada. Ernst Ruska led the German team while James Hillier led the Canadian team, in development processes based on a concept that was already old by 1930, as mentioned by Hillier several years later. While Ernst Ruska developed a model for Siemens in Europe, Hillier and Prebus got a model working in America, and by the end of the thirties both teams had fully functional machines [**2,3**]. Helmut Ruska, Ernst's brother, was part of the team that used the German "hypermicroscope" to image a virus for the first time in 1938. The virus in question was ectromelia, a large DNA virus from the *Poxviridae* family, capable of infecting mice [**4**].

The first bacteriophage micrographs appeared on the literature in 1940, in two papers published at the same issue of the Naturwissenschaften journal. In one paper Helmut Ruska imaged infected bacterial cells and was able to show virus adsorption, cell lysis and resistant bacterial cells (Fig.1a). Phages were described as small round particles, and crystalloid structures were seen and hypothesized to be centers for genesis of viral proteins [**5**]. In the other paper Pfankuch and Kausche, also working at the Siemens & Halske laboratory, analyzed purified phage suspensions and described the viruses as small

rounded corpuscles that aggregate in higher concentrations [**6**]. Both papers mention particle destruction by electron irradiation. It is now believed that the phages seen at the time were T7 coliphages. Translated reprints of both articles were published in 2011 [**7**]. Following these publications, phage images spread in Europe causing excitement, reaching even Felix d´Herelle, one of the discoverers of these viruses. Helmut Ruska continued to be an important influence on phage imaging. In the early forties he described some phage particles obtained from bacterial lysates as being club-like, possessing distinct heads and tails (probably these were T4-like phages) ; reported at least four phage morphotypes ; proposed a morphological classification for viruses and even introduced the term "phage" as an abbreviation to the term bacteriophage [**7-10**].

Meanwhile Luria and Anderson used the commercial version of Hillier's microscope to analyze unstained *Escherichia coli* and *Staphylococcus* phages in New York. In their first paper on the subject it is mentioned that phage imaging can "*offer favorable possibilities for the identification of the virus particles through a study of the reaction between the individual particles and the bacterial cell under the microscope*" [**11**]. Interestingly, the imaging papers published by German authors including Ruska were mentioned, showing that despite the Second World War scientific information was still flowing from Europe to America. Luria and Anderson described coliphages as extremely constant particles composed of a round head and a much thinner tail, with the heads not being homogeneous in their composition but consisting of a pattern of granules. Adsorption and cell lysis were visually described, but due to the lack of knowledge on virus biology and genetics at the time, some of the speculations on their mechanisms have later proven to be wrong (such as mentioning that adsorption could happen by either head or tail, and that

phage reproduction might take place at the cell wall). Imaging of Staphylococcal phages was mentioned to be harder, but particles containing heads and tails, able to adsorb to the host cells, were also detected. In their conclusions Luria and Anderson highlighted the interest of finding constant and relatively elaborate structural differentiation of macromolecular entities, and mentioned that the correspondence between particle size determined from microscopy to that obtained from indirect methods of measurement was remarkable. They also hypothesized that electron microscopy could also have an impact for genetics, since genes are also macromolecular entities and had been indirectly measured before [**11**]. The phages described in this study were later classified as T2 (T-even type) [**7,12**].

One year later Luria, Delbrück and Anderson published another paper on phage imaging [**13**], mentioning in the introduction the revival of interest in phages and the advantage of using these organisms as models. Images were taken from crude or partially purified viral suspensions, and also from dried drops of bacterial and phage mixtures for studying interaction between both. Besides typical tailed phage visualizations, a rounded phage without tail was described. It was mentioned that differential centrifugation mechanically inactivated one of the tailed phages (as noted by broken tails in the micrographs). Different multiplicities of infection were tested, which showed an agreement between the numbers of visible adsorbed particles to infective titers obtained by titrations. Micrographs also confirmed the eclipse period, allowed the observation of several steps of the phage infection cycle, and showed long *E. coli* cells (mentioned as "*not unusual*" in young broth cultures of the strain used). It was seen that new viruses were liberated from the interior of the bacterial cell, but it was not possible to determine where inside the bacteria the viruses are produced (deep interior or inner surfaces). The absence of bacterial

components of size comparable to viruses released by lysed cells was used to explain why crude suspensions, differential centrifugation and filtrations can be used successfully for phage work. It was also noted, unexpectedly at the time as pointed out by the authors, that adsorbed particles remained at the cell surface. This was considered to be the finding of greatest consequence, and the most plausible theory chosen to explain was that only one particle enters the cell and then makes the bacteria impermeable to other viruses (an analogy to monospermic eggs fecundation was made, with the caution to mention that there was no conclusive data to fully support it). This imaging paper also helped to test and eliminate three theories concerning phages that existed at the time: 1) no phage aggregates were seen, contradicting an idea that some phages would normally bind to larger unspecific carriers (such as bacterial debris) ; 2) the homogeneity of particle size disproved that there was a reversible equilibrium between small and large viral particles ; 3) and the consistency in progeny morphology when the same host was infected with different phages debunked a proposition that bacterial cells could contain a precursor of the phage particle, which upon infection would be converted to viruses. These three theories were based on indirect measurements made by diffusion on differential filtration, by sedimentation rate in ultracentrifugation, or as an analogy to proteolytic enzymes and its precursors, and were all disproved by direct imaging on the electron microscope. There was also a discussion on the common practice at the time of considering viruses to be molecules, warning that "*such a terminology should not prejudice our views regarding the biological status of the viruses, which has yet to be elucidated*" [**13**].

Improvements on sample preparation (1948 onwards)

A next advance on phage imaging was the introduction of contrast to the samples. By using chromium vapor to cover the preparations, Wyckoff was able to obtain more information on height and shape of the particles. In 1948 he used the technique to study T coliphages, chosen for their distinct shapes and for the ease of working with their hosts when compared to opaque staphylococci or mucoid and capsular streptococci. Two papers were published. The first was based on imaging phage plaques on samples obtained from solid media, using the embedded replica technique [**14**] (Fig.1b). Elongated *E. coli* were seen on young cultures and plaque characteristics were described and shown to differ between phages. The second paper focused on micrographs prepared from liquid samples [**15**]. Purified T4 preparations were used to describe phage morphology, and infected liquid cultures used for showing cells undergoing lysis with phages within and around their limits. Variation on the structure of phage heads and their contents was mentioned, and a correlation between grainy content inside the head and stages of maturation was made. Bacterial contents release by lysis were described, and their "conversion" to phages in favorable instances was noted as the most impressive result of the paper, hinted to be crucial in understanding how phages multiply.

Focusing on phage tails and the controversy concerning their role in the life cycle of phages at the time, Fraser and Williams used the freeze-drying technique to prepare T3 and T7 phages (believed to be tailless until then) for microscopy [**16**]. The technique was used for its minimal preparative distortion, and purified phages were freeze-dried for comparative analysis to air dried samples. Freeze-drying made clearer that phages are not spherical but geometrical, and short appendages ("*stubby tails*") were detected on the phages that were thought to be tailless at the time. The first result was taken as support of

the affirmation that phages were much more complex than previously thought, while the second gave strength to the idea that tails serve an important purpose to the phage life cycle. The same technique was used shortly after to reexamine T-phages, in a larger effort to compare all these phages in similar conditions. Preservation of tails during freeze drying, possibility of artifacts generated by air drying, true three dimensional forms, the number of facets of phage heads, and particle dimensions were all discussed. The particle dimensions obtained by air drying were considered to be unreliable when compared to freeze drying [**17**].

By the end of the fifties the introduction of negative staining to viral electron microscope samples greatly improved the quality and clarity of the preparations [**18**]. It was quickly applied to phage samples and helped to describe the phage structural components such as head, tail sheath and tail fibers in details [**19**] (Fig.1c). These samples were prepared by negative staining using the phosphotungstate method, and the microscopy results combined with biochemical analysis helped to better understand the phage particles. In the following year, negative staining was used to study 22 different phages in details, leading to morphological grouping and description of a subunit structure for heads and tails [**20**]. Besides coliphages, viruses that infect other bacterial genera such as *Staphylococcus*, *Streptococcus*, *Pseudomona*s and *Brucella* were visualized. Contrast differences were noted in phage heads and attributed to the presence or absence of DNA. Morphology was suggested to be an aid to the already confusing phage taxonomy, and one truly tailless phage was mentioned. After these studies negative staining of phage preparations and their analysis by transmission electron microscopy (TEM) became the most common practice to determine the phage structure and particle size in the following decades. A phage survey

made in 2007 revealed that at least 5568 phages had been examined by negative stained TEM samples from 1959 to 2007 [**21**].

Complementary imaging approaches

Besides negatively stained TEM samples, other techniques were developed and used to image phages over the years. Direct observation of particles by TEM does not provide much insight on the phage life cycle or interaction with the host. For those purposes, pelleting of infected bacteria from liquid cultures and their subsequent fixation and drying was used, with the possibility of embedding the samples in polymers for ultrathin sectioning [**22**]. Based on worries about studying virology without the access to an electron microscope, a technique to visualize phages in a bright field light microscope was developed [**23**]. It was made possible by staining phages with flagella stain, a procedure that increases the particle size and make phage heads increase to the limits of detection of light microscopy. Obviously, the particles became deformed and no fine details could be seen, making the use of the technique limited. Nevertheless, it allowed crude phage imaging. The use of scanning electron microscope (SEM) has also been applied to phage imaging. Phage P1, capable of infecting *Shigella*, was used as model to test parameters related to sample preparation and visualization by SEM (Fig.1d). The paper describes the best conditions for SEM sample preparation, and suggests a correlation of SEM and TEM images to study virus life cycles [**24,25**].

In the early nineties the scanning tunneling microscopy (STM) and atomic force microscopy (AFM) approaches were also applied for phage imaging. In 1990 coliphages T7 and fd were visualized by STM after coating with a thin metal layer and deposition on a flat non-conductive substrate, allowing imaging with some cost to resolution [**26**]. Two years

later, in 1992, a paper was published describing T4 phage imaging by AFM, taking advantage of the fact that the technique allows imaging of non-conductive samples. Images shown intact viral particles, either isolated or on aggregates, and damaged particles with DNA streaming out from the viral heads [**27**]. More recently a force distance based AFM approach was used to image single phages extruding from living cells. Biochemically sensitive tips were used to image *E. coli* infected with filamentous phages, providing direct visualization of phage assembly and localization on host cells [**28**].

Techniques for three-dimensional structural determination

Although some viruses have been crystallized and studied by X-ray diffraction techniques, phage particles are often complex in structure and for that reason do not form ordered crystals that could be used for whole virion structural determination. Besides, most phage particles are near the size limit of biological structures that can be determined by this approach. Nevertheless, X-ray crystallography techniques have also been used for phage structural analysis. Phage HK97 was the first tailed phage to have its capsid structure determined by crystallography. Empty heads of the phage were produced by expressing capsid proteins in *E. coli*, and after purification they were successfully crystallized and measured by X-ray diffraction [**29**]. In 2004 the structure of the membrane-containing phage PRD1 was determined by X-ray crystallography [**30**]. As with other types of proteins, crystallization and X-ray diffraction have been used to study several individual phage particle components, such as the gene V from phage f1, fibers from phage Pf1 and the major capsid proteins of phage P23-77 [**31-33**]. In 2017 soft X-ray diffraction, an X-ray tomography approach that can be applied to samples without prior crystallization, has been used to image coliphage PR772, opening new possibilities for studying phage structures [**34**]**.**

Cryo-electron microscopy (cryo-EM) is a technique that does not need fixing and staining during sample preparation, and structures can be determined without the need to form crystals, making it an interesting alternative to X-ray crystallography. Recent advances in the cryo-EM field increased the resolution of the technique to near atomic levels. In 2010 the structure of human adenovirus was solved by X-ray and by cryo-EM at similar resolutions [**35-36**], making it relevant for viral studies. Three dimensional determination of structures is possible by algorithmic means, resulting in cryo-EM tomography and single particle cryo-EM. For example, the asymmetric structure of the phage MS2 attached to its receptor has been determined by cryo-EM tomography [**37**]. The capsid structure of the *Salmonella* phage epsilon 15 was analyzed by single particle cryo-EM at a level of detail close to X-ray crystallography, in near-native solution conditions [**38**]. Cryo-EM was also used to solve the structure of the T4 baseplate-tail tube complex, in pre and post host attachment states, helping to understand sheath contraction in atomic details [**39**]. The structure of the FLiP (Flavobacterium-infecting, lipid-containing phage) virion, a boreal lake ssDNA phage with limited sequence similarity to other known viruses, was also solved by cryo-EM technique [**40**]. The determination of particle structure helped to understand its evolutionary relationship to other viruses by complementing sequence based approaches. The capsid structure of the phage Sf6 has also been determined by cryo-EM [**41**].

Combining cryo-EM with other techniques has provided detailed insight on phages and their interactions with the host. An *E. coli* filamentous phage (f1.K) was imaged by the combination of cryo-microscopy with the concept of in line electron holography, resulting in the first electron hologram of an individual phage particle [**42**]. By using the

combination of immuno-labelling, negative staining, cryo-EM and cryo-electron tomography (cryo-ET), it was possible to understand how PRD1, a lipid containing tailless phage, delivers its genome to the bacterial host across the cell envelope [**43**]. Furthermore, fluorescence microscopy has been combined with cryo-EM to study the replication of the phage 201f2-1 on *Pseudomonas chlororaphis*. The assembly of a nucleus-like structure that separates viral DNA from the cell cytoplasm was described, showing that at least this phage is able to use compartmentalization inside the host cell for virus replication [**44**].

Significance of microscopy in phage ecology and environmental studies

Imaging has also been applied to research phage "behavior", using the lytic/lysogenic outcome of phage lambda infections as a model. Single cell fluorescence microscopy has been used to study infection results, showing that the fate of infected cells correlates with variations in cell size. Larger cells had increased frequency of lysogenic outcome [**45**]. Using a more detailed, single virus approach, it was demonstrated that the cell fate after infection can be explained by the combination of individual viral "decisions" that occur at the subcellular level [**46**]. More recently, a four-color fluorescence system has been designed to study single cells, single phages and single viral DNA at the same time. When combined with computational models, it has helped to observe subcellular behaviors like phage cooperation for lysogenization, competition during lysis, and even confusion between both pathways [**47**]. Fluorescence microscopy has also been used to show DNA translocation from phages to hosts in a single molecule resolution [**48**], and a fluorescence in situ hybridization protocol has been adapted for studying phage infections on a single cell level [**49**].

Besides phage morphology and host interaction, electron microscopy has also been used to study phage diversity in environmental samples. Seawater samples were prepared and fixed for direct observation on an electron microscope, without the use of an enrichment process for phage isolation. Even without taking extra steps to grow the phages before analysis, various phage particles with distinct morphologies were found, as well as phage particles bound to bacterial cells [**50**]. An estimative of 10^3 to 10^4 viruses per milliliter of sea water was made, but it was noted by the authors that it is probably lower than the real number due to phage loss during sample preparation. The presence of so many phages led to the speculation of their importance in microbial ecology. Phages of marine origin previously obtained by isolation in bacterial hosts were analyzed by TEM of negatively stained samples. Seventy five phages were imaged and divided into twelve different groups based on morphological similarities, showing new structures and high structural diversity [**51**]. The ecological importance of phages got even more evident after environmental aquatic samples were analyzed again by TEM. Water samples from different locations were analyzed directly, without an enrichment step, and this time the sample preparation process minimized phage loss. Different phages, either as free particles or attached to bacterial cells, were visualized. Phage counts varied between 10^4 to 10^8 particles per milliliter of water, depending on sampling location and time of the year, revealing that phage abundance in the environment was higher than previously thought. The impressive number of phages in unpolluted water samples led to speculations about the so far overlooked importance of phages for keeping bacterial populations in balance on the environment, and also of the impact of these viruses in genetic transfers in natural prokaryote populations [**52**]. An alternative imaging technique was developed for counting environmental viruses, based on staining the samples with a fluorochrome specific for nucleic acids and directly counting the

particles on an epifluorescence microscope. Although this technique does not provide any structural details, its simplicity in sample preparation and equipment requirements made it the most common technique for enumerating viruses from the environment [**53-54**]. This method revealed that TEM analysis is not only more time consuming for this purpose but also tends to underestimate viral abundance. A variation of the technique, consisting of stained particles treated with Dnase I, has been applied for indirect evaluation of phage capsid structural deformity [**55**].

Moving away from electrons: helium ion microscopy

The scanning helium ion microscope (HIM) is a recent advance in imaging. Instead of using electrons, imaging is based on the use of a positively charged helium ion beam [**56**]. Helium beam allows higher image resolution (close to 0.5nm), larger depth of focus and dispenses conductive coating of biological samples, this last advantage being important for imaging of fragile sub-nanometer structures and for avoiding artifacts or masking generated by coating. Biological samples, including a bacterium, were imaged by HIM for the first time in 2013 [**57**]. In 2017 the nanoscale imaging capacity of the HIM was used to investigate plaques formed by T4 infection on *E. coli* bacterial lawns in order to test the applicability of helium ion microscopy to phage-host interaction studies [**58**] (Fig.1e). The samples were directly prepared from pieces of double layered agar containing the bacteria lawn with phage plaques, so the imaging could be made on viral plaques as they naturally occurs. Various stages of T4 infection could be seen by imaging different spots within and around a plaque, since the infection spreads radially from the ground zero, with no cells in the center and newly infected cells on the edges. It was possible to obtain high resolution images of burst cells, cells with multiple phages attached, phages with normal

morphology and phages with already contracted tails. Icosahedral head shape, widening in the tail end due to the baseplate structure, and tail fibers attached to the bacterial cell wall were also visualized in detail. A large number of elongated *E. coli* cells, also mentioned in older phage imaging papers [**13,14**], were also seen, hinting that these mutants may be more common than previously thought. Another characteristic of the HIM was also tested in the samples mentioned above. By increasing the ion current, it is possible to mill (cut) the material at specific locations. Cross sections of bacterial cells, of phage particles and removal of agar substrate pieces were all demonstrated by the milling process. Comparing to other imaging techniques, HIM imaging appears to be more straightforward to use and provides the opportunity to image whole colonies or plaques or other types of complex microbial samples directly on their substrate, with sub-nanometer resolution, with no need for metal coating [**58**].

We are continuing to apply helium ion microscopy to study phages and phage-bacteria interactions. We have developed protocols to avoid agar collapse during preparations, and gained experience with different types of organisms. In Fig.2 we present a few images as examples of our latest phage-bacteria imaging. Sample preparation was made according to Leppänen et al 2017 [**58**].

Conclusions and perspectives

From its origins almost eighty years ago to today, phage imaging has improved immensely (Fig.1) and helped to understand much of these intriguing and important organisms. The earlier years of the electron microscope development resulted in James Hillier and Ernst Ruska sharing the 1960 Albert Lasker Award for Basic Medical Research for their contribution to the development of the first electron microscopes, and Ernst Ruska

receiving a Nobel Prize in 1986 for his fundamental work in electron optics and for the design of the first electron microscope [**2,3**]. It is also possible to see in the first Luria, Delbruck and Anderson phage imaging papers [**13**] their interest in basic molecular biology that led to the shared Nobel Prize in 1969 related to replication mechanisms and genetic structure of viruses. In 2017, Dubochet, Frank and Henderson were awarded a Nobel Prize in chemistry for developing cryo-EM, a method that has had a significant impact in high-resolution imaging and consequently in three-dimensional structure determination of biomolecules and viruses. Electron microscopy in all its variations and other imaging techniques were crucial for better understanding phages, from structural details to interaction with hosts and diversity. Imaging has contributed to the knowledge that phages are the most abundant organisms in the biosphere, are crucial in regulating global biochemical cycles, have had an important role as models for molecular biology studies and are a viable alternative to treat bacterial diseases by the use of phage therapy.

It is hard to imagine how imaging techniques will improve in the next decades, and what knowledge will be gained from their use. However, it can be expected that there will always be attempts to improve existing equipment and technologies, and to create new ones. From a technical point of view, advances in the ability to see in more detail at molecular or atomic resolution, at shorter time scales, and close to native conditions may be the main motivations [**59**]. From a biological point of view, there is a high interest in single-cell live imaging, which can also be applied in combination to single-virus and single-molecule imaging. It has been advancing in relation to time resolution (changes detected in milliseconds) and sensitivity (detection of few photons per pixel), but still requires the use of light microscopy and fluorescent labels [**60**]. Latest developments in AFM include the High-

Speed AFM, which allow the following of single molecules dynamics in real time, with potential to be applied to viruses [**61**]. Mega-electron-volt (MeV) ion beams have been recently used for imaging cells, and the capacity of penetrating through several microns of biological tissue with little deflection (thus maintaining spatial resolution) can also be useful for viral infection studies [**62**]. Current advances in state of the art microscopy are based on the use of quantum mechanics for photoionization microscopy. A quantum magnetic resonance microscope approach was recently used to image copper complexes in solution, by a non-invasive and non-interfering process that could perhaps in time be applied to living cells [**63**]. A combination of adaptive optics to lattice light-sheet microscopy (AO-LLSM) was used to visualize cellular processes tri-dimensionally recently [**64**]. Its non-invasive imaging of events at different scales has potential to be adapted to the study of phage infected cells. As technology gets more advanced sample preparation steps and sample modifications prior to imaging may decrease, optimally leading to analysis of samples close to their native state by higher definition approaches.

382 **Conflict of interests**

383 There is no conflict of interests between the authors.

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560 **Figure legends**

561

562 **Figure 1:** Comparison between selected milestones in phage imaging.

563 A) First phage electron micrograph published (1940). B) First phage electron micrograph
564 published in which contrast was used in sample preparation (1948). C) One of the first
565 negative stained phage electron micrographs published (1959). D) One of the first scanning
566 electron microscope phage electron micrographs published (1975). E) First scanning helium
567 ion microscope phage image published (2017).

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585

586 **Figure 2:** Phage and bacteria interaction images obtained with helium ion microscopy.

587 A) *E. coli* cells infected with T4 phage. B) *E. coli* cells on the edge of a T4 plaque, growing as
588 cell islands with long cells apparently scanning the surface. C) Higher magnification of the
589 previous picture showing a lysed long cell (in white) and another one with several white
590 patches on its cell wall. White-grayish spots on the cell wall might indicate endolysin activity
591 from within. D) Details of susceptible *Flavobacterium columnare* cells infected with the FCL-
592 2 phage. Note the high number of dead cells on the field and four rounded cells, probably
593 losing its characteristic morphology before bursting.