Murphy’s law—if anything can go wrong, it will Problems in phage electron microscopy

Hans-W. Ackermann1,2* and Kenneth L. Tiekotter2
1Department of Microbiology; Immunology and Infectiology; Faculty of Medicine; Laval University; Quebec, QC, Canada; 2Department of Biology; University of Portland; Portland, OR USA

The quality of bacteriophage electron microscopy appears to be on a downward course since the 1980s. This coincides with the introduction of digital electron microscopes and a general lowering of standards, possibly due to the disappearance of several world-class electron microscopists. The most important problem seems to be poor contrast. Positive staining is frequently not recognized as an undesirable artifact. Phage parts, bacterial debris, and aberrant or damaged phage particles may be misdiagnosed as bacterial viruses. Digital electron microscopes often seem to be operated without magnification control because this is difficult and inconvenient. In summary, most phage electron microscopy problems may be attributed to human failure. Journals are a last-ditch defense and have a heavy responsibility in selecting competent reviewers and rejecting, or not, unsatisfactory articles.

Introduction

Legend has it that Murphy’s law was formulated in 1949 by a Captain Edward A. Murphy, then at Edwards Air Force Base in California.15 There are several variants, all meant to express a perverse outcome. Murphy’s law certainly applies to bacteriophage electron microscopy. This type of investigation is a multi-step procedure that depends on expensive and complicated instruments, refined techniques, bacteriophages, and, not least, the investigator. Problems and errors beset every step. Artifacts in particular have received little attention and will be the focus of this article.

Negative staining of viruses, arguably the technically simplest and most important single method in virology, was introduced in 1956. Hall and later Brenner and Horne used phosphotungstic acid for contrasting plant viruses.8,12 This technique was extended in 1959 to coliphage T2.9 Viruses and their components stood out as white on a dark background with unprecedented clarity. Negative staining by phosphotungstic acid rapidly superseded shadowing for virus visualization. Uranyl salts and ammonium molybdate were introduced later. The standards of viral electron microscopy were set in the 1970s.11,20,21 Today, negative staining is done almost exclusively with uranyl acetate (UA) and phosphotungstate (PT) salts (Figs. 1 and 2) and has been applied to thousands of viruses. At present, at least 6,300 bacterial and archael viruses have been examined in the electron microscope after negative staining.6

Transmission electron microscopes (TEMs) fall into three categories. (1) Conventional or "manual," also called "analog" microscopes using mechanical devices for stage drive and aperture alignment and analog potentiometers or variable resistors for electronic controls. Images are recorded on photographic film or plates. (2) "Digital" microscopes using far fewer controls due to a computer-based system with digital potentiometers or variable resistors for electronic controls. Images are recorded on electronic media. (3) Hybrids or conventional microscopes equipped with a digital camera.
Since about 1985, "manual" electron microscopes were gradually replaced by "digital" instruments. These instruments were marketed almost simultaneously by three major competing companies that did so with meager or no instructions relating to contrast and image quality. An additional modification by microscope manufacturers was to change the focal length of the objective pole piece (eta or Z angle) to increase fidelity at the cost of reduced contrast. All these factors could explain a wave of poor electron microscopical images which, seemingly, has not abated. Unfortunately, this wave coincided with the death of several famous and highly skilled electron microscopists, such as E. Kellenberger in Switzerland, D.E. Bradley in Canada, and A.S. Tikhonenko in Russia, who could have stemmed the tide.

To assess this problem, one of us (HWA) analyzed 155 publications with phage micrographs originating in 28 countries, published between 2007 and January 2012 in over 50 journals. Two-thirds (109) were of poor quality, namely contrastless, unsharp, astigmatic, of small size and low magnification (below 150,000×). Only 46 could be considered good or acceptable. The adjectives "poor and good" are somewhat subjective and we apologize for this. The micrographs had been obtained with a wide selection of about 50 types or models of electron microscopes, most of which were "digital." The vast majority of articles presented several pictures. "Manual" microscopes were apparently disappearing fast. Clearly, poor electron microscopy is a global problem.

**The Poor Picture Syndrome**

Electron microscopes. The most frequent types of transmission electron microscopes (TEMs) used in phage research are produced by JEOL (Japanese Electron Optical Laboratories), Hitachi and FEI, a successor of Philips (Eindhoven, The Netherlands), followed distantly by Carl Zeiss, Germany. One also finds a few old AEM (England) and Tesla (Czechia) instruments. The prevalence of JEOL and Hitachi instruments may be attributed to their relatively low cost. The most popular types are the JEOL 1200 EX, the Hitachi H-1700, and three instruments of the Philips family (FEI CM 100, Technai Spirit or Morgagni). A few "manual" microscopes, for example the Philips EM 300, persist and seem to be, because of their high quality, the objects of a cult among inconditionals of electron microscopy: FEI-Tecnai electron microscopes appear to be the most highly evolved TEMs and have indeed produced excellent micrographs, whereas, for example, the JEOL 1200 is often associated with poor pictures. This impression is superficial. Indeed, good and poor pictures have been produced with JEOL, Hitachi and Philips-FEI instruments. In conclusion, electron microscopes are above all "operator-sensitive." Poor pictures must generally be attributed to poor maintenance and untrained users. There is no cure but know-how.

**The dirty picture.** Quite often, specimens are not purified in any way and investigators examine crude lysates. This is almost certain to go wrong. Crude lysates contain huge amounts of impurities, notably proteins (Figs. S4 and S19). This results in contrastless, flat images and the presence of bacterial debris and even complete bacteria. The debris may mimic certain types of bacteriophages (Table 2). Images of nonpurified specimens should not be accepted for publication. Purification can be achieved by centrifugation in sucrose or CaCl₂ density...
gradients; unfortunately, this technique is limited to a small number of specialized laboratories. Instead, we recommend to sediment phages in Eppendorf tubes followed by two washes in buffer or even tap water. Using fixed-angle rotors, phages can be sedimented in as little as 1 h at 25,000 g. It is not necessary to stain phages for long minutes or to fix them. Staining is almost instantaneous and fixation is merely a complication. While purification is mandatory for any real investigation, crude lysates can be examined for quick checks of phage presence and identity. It is also possible to examine phages extracted from a lysed agar surface. It is possible to examine phages extracted from a lysed agar surface. For this, a drop of staining solution is deposited on the agar and the Petri dish is agitated gently. The drop is then touched with a grid and excess liquid is drawn off.

The technically deficient picture. The root causes of unsharp, “fuzzy” images (Figs. S5 and S6) are poor microscope maintenance and misalignment of column components. Both focusing and astigmatism correction can go wrong and produce unsharp images. Much has been said in manuals of electron microscopy (e.g., in ref. 7) about focusing and astigmatism and this does not need to be repeated. Digital microscopes allow the investigator to use the FFT (Fast fourier transform) function as a measure of astigmatism (Fig. 3). This function is available in FEI, Hitachi, and JEOL instruments and, for example, the widespread AMT, Gatan, and OlympusSis cameras. If fuzziness and astigmatism persist, the electron microscope should be checked by the installer. Test specimens for resolution checks and conventional astigmatism correction (“holey” grids) are commercially available and a must for the serious microscopist. Unsharp images are generated by:

(1) Misalignment of the whole EM column and/or the objective aperture.
(2) Object drift due to a specimen support film that breaks, does not adhere to the grid, or has not been stabilized with carbon, thus causing the substrate to charge and float.
(3) Discharges in the electron gun altering the focus.
(4) Over- or under-focusing by the user (Fig. S7).
(5) Astigmatism, generally caused by a dirty object holder or objective lens diaphragm, resulting in fuzziness and fine parallel lines instead the normal grain of micrographs (Fig. S8).

Contrast. Poor contrast (Figs. S5 and S6) seems to be a pervasive, if not the main problem of “digital” electron microscopy (EM). Indeed, many “digital” micrographs are lamentably dark and poorly contrasted. This is not an intrinsic limitation of “digital” microscopes or cameras, but rather due to inappropriate parameters or complete misunderstanding of the dynamic signal range during image acquisition.

In “manual” instruments, poor contrast can be overcome by apertures, high-contrast films, or elongating the focal distance of the objective lens, resulting in fuzziness and fine parallel lines instead the normal grain of micrographs (Fig. S8).

In “manual” instruments, poor contrast can be overcome by apertures, high-contrast films, or elongating the focal distance of the objective lens, resulting in fuzziness and fine parallel lines instead the normal grain of micrographs (Fig. S8). Contrast can be improved in the darkroom by means of fast developers, polychromatic papers and optical filters. Unfortunately, this is very difficult with “digital” electron microscopes where contrast must be adjusted before or after image acquisition. CCD digital technology offers a wide range of gray scale values. They can be selected to favor the mid-tonal range, thus reducing the extreme dark and white values. For example, a 12-bit image comprises 4,096 levels from black to white. If the software is set to adjust the predominant intensity to a middle

Figure 2. Vibrio parahemolyticus phage KFP40. The phage is very similar to coliphage T4, but has a longer head and a larger genome. Negative staining with phosphotungstate. Tail fibers are unfolded.
tonality, all values will be adjusted accordingly. The mid-tones typically yield low-contrast images. Contrast levels should therefore be set to exclude a given percentage of black and white outliers. After image acquisition, the histogram camera software allows for adjustments of gamma (a function of luminance), contrast, and brightness. The observer is left to find his or her best combination by trial and error. Contrast may also be improved by third-party software (e.g., Adobe Photoshop or Image J; http://rsbweb.nih.gov/ij/). This seems often to be misunderstood or ignored. Poorly contrasted digital images should be a thing of the past.

Farming out. The high cost of electron microscopes and the specialized knowledge needed to operate them generated a questionable, even damnable development: the “farming out” of investigations to central laboratories or institutions carrying out examinations for a fee. Anything might go wrong during this procedure. It might be acceptable if phage researchers themselves have access to the electron microscope used or the technician that carries our investigations, is properly qualified or backed by an experienced phage researcher. Unfortunately, this seems to be rarely the case and has resulted in situations when examinations are performed by incompetents without instructions or personal interest in the subject, or simply unused to magnifications above 200,000x. The investigating laboratories sometimes charge outlandish fees for essentially worthless data. This should be resisted. We propose fees of $50 US for access to an electron microscope and $75 US for an investigation aided by a technician or a scientist.

The journals. Murphy’s law also rules the final step of publication. Indeed, a good micrograph can be ruined by a journal that reduces it to postage stamp size or darkens it beyond recognition. Part of this may be attributed to the now frequent procedure of outsourcing manuscript assembly to distant offices, e.g., in South East Asia. Only protests will help here and sometimes do.

Staining Artifacts

Positive staining. This is the most frequent artifact in virus electron microscopy. Uranyl salts cause both negative and positive staining, while phosphotungstates and molybdates cause negative staining only (Table 1). The principal incriminated stain is uranyl acetate (UA). In positive staining (Figs. S9 and S13), virus particles themselves are stained and then may appear black on a white background. This is due to the strong affinity of uranyl ions to DNA and seen in any viruses with compact masses of DNA, e.g., phage heads and adeno- or herpes-viruses. It never occurs in filamentous or ssRNA phages. Positively stained phage heads are invariably shrunken by about 10–15% and show neither capsomers nor transverse edges. Phage tails are not stained and appear as shadows. Consequently, these viruses can rarely be identified by EM. Their dimensions are perfectly useless and...
other hand, UA acts as a fixative and tractile tails are 2 nm larger in UA than in PT and less sharply defined. Typically, con- 

Table 1. Stains and staining artifacts

| Stain            | Uranyl acetate                                      | Phosphotungstate                      |
|------------------|-----------------------------------------------------|---------------------------------------|
| Properties       | Acidic, optimal pH 4–4.5, stable over years, remains sterile, radioactive, precipitates, produces negative and positive staining | Neutral, optimal pH 7, negative staining only |
| Advantages        | High contrast, longevity of grids up to 10 years, good for resolution of tail striations and virus coats at low magnification, acts as a fixative | Good for screening, ease of interpretation |
| Problems          | Difficulties in identifying normal structures       | None                                  |
| Artifacts         | Positive staining, head shrinkage, swelling of tails, crystals and precipitates | Contamination by bacteria, very hydrophilic, longevity of grids 1 mo |

should not be published. If positive staining is generally an undesirable artifact, it has however two important applications. It allows (A) the visualization of phage heads in sectioned bacteria and (B) facili- 

tates the quantification of aquatic viruses. The reasons for positive staining are unknown. Both negative and positive staining may occur in adjacent areas of the same EM grid (Figs. S10, S12 and S13). The addition of protein to UA solutions seems to alleviate the incidence of positive staining.

Halos. UA positive staining is often accompanied a gray halo around the virus capsid that increases with the time of irradiation (Figs. S12 and S13). The halo has unsharp margins and resembles an envelope. Its origin and significance are unknown.

UA crystals and precipitates. UA tends to crystallize on the grid, often starting with viruses as crystallization origin. Crystals come in many varieties. Some are small (Fig. S14) and feathery or appear as long black needles. Others are flat, large sheets (Figs. S15 and S16). One also observes, albeit rarely, membraneaceous precipitates around phage particles. Viruses within crystals often appear as brilliant white struc- 

tures of abnormally large size (Fig. S17).

Swollen proteins. As an acidic stain (pH 4 to 4.5), UA causes proteinic structures to swell. Compared with PT, the walls of UA-stained empty phage capsids and tails appear as relatively thick and less sharply defined. Typically, con- 

tracile tails are 2 nm larger in UA than in PT. Tail length is not affected. On the other hand, UA acts as a fixative and preservative, so that UA-stained specimens can be kept over years. Phage heads are better preserved in UA than in PT.

Artifacts caused by phosphotungstate. Surprisingly, these are few. Compared with UA, and depending on the phage, heads tend to be rounded and are sometimes broken and empty.

Impurities, Sources of Errors, and Fake Viruses

From the medium. Unwashed prepara- 

tions contain proteins, sugars, and cell debris (below). They yield dirty, poorly contrasted, even opaque images with little structural details (Fig. S4). PEG (poly- 

ethylene glycol), which is frequently used to concentrate phages, produces similar effects. PEG will collect anything: DNA, proteins, cell debris, phages and phage debris. Fortunately, PEG is is easily 

to be removed by washing. In one recent case, four washes were needed to obtain a clean preparation. CsCl, used for phage puri- 

fication, may persist after incomplete 

dialysis. CsCl forms flat crystals, but does not interfere with staining. Arborescent or flat NaCl crystals are frequent in lysates from halophilic bacteria which require NaCl for growth, e.g., Vibrio and Halobacteri-

a. Again, bacteriophages may act as nuclei of crystallization (Fig. S18). NaCl crystals may obscure phage particles, but do not alter the quality of staining. In lysates from bacteria prepared with meat- 

based media, e.g., of clostridia, one may observe occasional fibers of irritated muscle and bundles of double-walled rings which resemble phage tails (Fig. S41) and may be attributed to self-assembly under the action of phospholipase C.

From bacteria (Table 2). Bacteria con- 

tribute proteins, DNA, ribosomes, cyto- 

plasm, capsular material, pili, flagella, and fragments of plasma membrane and cell wall. Proteins and cytoplasm may interfere with the staining process, but are not a source of error. However, this is the case with the other cellular component that one may find in a lysate. Their presence depends very much on the phage host. For example, slime and capsular material are present in huge amounts in lysates of Acinetobacter and Xanthomonas and cell wall debris abound in those of Pseudomonas.

Slime and capsular material normally appear as rounded elements, but can be distorted by centrifugation and then superficially resemble filamentous viruses (Inoviridae) (Figs. S20 and S21) or even tailed phages (Fig. S27). Similarly, pili and debris of flagella may be mistaken as filamentous phages and short debris of flagella, of 100 to 200 nm length, may be confused with contractile phage tails. Occasionally, a fragment of a pilus still attached to a piece of plasma membrane may suggest the presence of a tailed phage. These elements should not be much of a problem as they are easily identified at magnifications above 150,000×.

Chloroform, sometimes used for sterilization of lysates, is dangerous as it may produce a thick smear of particles thought to be lipopolysaccharide (LPS). This goo interferes with staining and can make observations impossible. The amount of smear probably depends on the bacterium and seems to be parti- 

cularly high in lysates of Brucella spp and Cronobacter sakazakii (Fig. S22).

Cell wall and plasma membrane frag- 

ments (Figs. S23 and S26) may be misdiagnosed as enveloped pleomorphic viruses, novel viruses, or tailless phages. This error is easily explained. Upon bacteriophage lysis, bacteria fall into pieces, many of which are round and have indeed the size of plasmaviruses or cysto- 

viruses (70–80 nm). The latter are char- 

acterized by an envelope surrounding an isometric capsid. When superposed over each other, some cellular elements exhibit what seems to be an external and internal

©2012 Landes Bioscience. Do not distribute.
components (Fig. S25). Cell wall fragments carrying on adsorbed phage tail may be taken at low magnification for tailed phages (Figs. S26).

These membrane fragments are much more than an inconsequential nuisance because phage counts in seawater and freshwater are increasingly often based on fluorescent microscopy. As it happens, bacterial DNA can associate with membrane fragments during lysis and will stain with fluorescent dyes. The fluorescent membrane fragments are approximately the size of phage heads and appear as tiny green dots simulating phages. So far, investigations of phage prevalence in the environment seem to have neglected this potentially serious source of errors.18

From Bacteriophages (Tables 2 and 3)

Bacterial viruses produce a wide variety of abnormal particles (Table 3). Nearly all of them have been observed in tailed phages, although the filamentous inoviruses sometimes give rise to double-length particles. Tailed phages are classified into three families according to tail structure: Myoviridae with contractile tails, Siphoviridae with long, noncontractile tails and Podoviridae with short tails. Nature and frequency of “freaks” or “monsters” vary with the complexity of phages. Some aberrations have a genetic basis, others reflect errors of assembly, and still others result from propagating phages on the wrong substrate, e.g., a medium containing amino acid analog. The subject has been reviewed elsewhere in some detail. Phage T4 and its relatives have a particular propensity to produce aberrant particles. Some sources of error (and Mr Murphy’s and the electron microscopist’s delight) are:

(1) Abnormally long tails (Fig. S28). They are found in very numerous siphoviruses, are extremely rare in myoviruses, and have never been reported in podoviruses. Normal tails may appear short when the head partly covers the tail (Figs. S29 and S30).

(2) Isolated contracted tails, which have been interpreted by inexperienced observers as complete tailed phages and even novel species of viruses.

(3) Polyheads (Figs. S31, S32 and S34) made of polymerized capsid protein, may be regular or irregular.

(4) Proheads, recognizable by their small size and wavy outline (Fig. S33), may be mistaken as complete isometric viruses or phage debris.

(5) Motted heads and polyheads (Figs. S34 and S35); they seem products of faulty phage synthesis.

(6) Freak particles with two tails, two tail sheaths (Figs. S36 and S37) or even two heads. The latter were observed in Lactococcus lactis phages of the c2 species (Fig. S38).

(7) Myoviruses mimicking as siphoviruses after loss of their tail sheath (Fig. S38).

(8) Head-size variants (Fig. S40), notably in phages with prolate heads.

Table 2. Errors and misinterpretations

| Feature or observation | Interpreted as |
|------------------------|---------------|
| Halo around positively stained head | Envelope or double capsid |
| Isometric head | Icosahedron |
| Empty head | Spherical phage, novel virus family, also qX74 |
| Head or tail size variant | Presence of two different viruses |
| Broken tail in siphoviruses | Presence of two different viruses |
| Prohead, broken-off tail | Novel type of virus, also mimics when standing on an end |
| Contracted tail sheath | Novel type of virus, also mimics when standing on an end |
| Protruding tail tube | Tail fiber |
| Polyhead | Novel filamentous virus |
| Polytail | Inovirus |
| Flagellum | Inovirus |
| Pilus | Inovirus |
| Slim filament | Inovirus |
| Membrane vesicle | Cystovirus, pleomorphic phage, novel virus family |

Table 3. Malformations in tailed phages

| Particle or feature | Head full | Characteristics | Found in |
|---------------------|-----------|-----------------|---------|
| Isometric | + or - | Abnormally small | M |
| Elongated | + or - | Abnormally long, short, or large (very rare) | M, S, P |
| Mottled | - | Spherical or oval, serrated outline | M, S, P |
| Polyhead | - | Specks of unknown material in head |
| Multilayered tube | T4 only | |
| Polyhead tube | - | a. Regular simple tube |
| Polyhead tube | - | b. Multilayered tube |
| Polyhead tube | - | c. Polyhead tube |
| Polyhead tube | - | d. Mottled tube |
| Polyhead tube | - | e. Irregular (“crummy”) |
| Multiple tails | + or - | Up to four | M, S |
| Tail insertion | + or - | Lateral insertion, elongated heads only | M |
| Double head | + or - | Two heads linked by tail fragment (very rare) | S |
| Normal | + or - | Abnormally long or (rarely) short | M, S |
| Polytail | - | Thin, hollow tube | M, S |
| Tail | Polysheath | Sheath material with or without core | M |
| Composite tail | - | Contracted sheaths assembled end-to-end | M |
| Composite tail | + or - | Abnormally long tail with two sheaths | M |

M, Myoviridae; P, Podoviridae; S, Siphoviridae; +, yes; -, no.
producing particles with short or isometric capsids (9).

Bacteriophage Tail Structures

(9) Broken tails, suggesting the presence of podoviruses or isometric phages instead of spho- or myo-viruses.13

(10) Virus-like particles (Figs. S41 and S43) and true, but deformed phages (Fig. S44).

The presence of abnormal or damaged particles indicates a contamination by other phages or the presence of lysoytic phages produced by the phage host. The observer has to decide whether there are different phage populations or aberrant particles, e.g., a spectrum of phage tails of different length. The latter indicates the presence of a malformation. The matter requires prolonged observation at magnifications above 150,000×.

Magnification Control

Exact magnification depends first on the adjustments made on installing an electron microscope. It must be controlled later by means of test specimens (e.g., beef liver catalase crystals or T4 phage tails) and true, but deformed phages (Fig. S44).

The presence of abnormal or damaged particles indicates a contamination by other phages or the presence of lysoytic phages produced by the phage host. The observer has to decide whether there are different phage populations or aberrant particles, e.g., a spectrum of phage tails of different length. The latter indicates the presence of a malformation. The matter requires prolonged observation at magnifications above 150,000×.

Magnification Control

Exact magnification depends first on the adjustments made on installing an electron microscope. It must be controlled later by means of test specimens (e.g., beef liver catalase crystals or T4 phage tails) and true, but deformed phages (Fig. S44).

The presence of abnormal or damaged particles indicates a contamination by other phages or the presence of lysoytic phages produced by the phage host. The observer has to decide whether there are different phage populations or aberrant particles, e.g., a spectrum of phage tails of different length. The latter indicates the presence of a malformation. The matter requires prolonged observation at magnifications above 150,000×.
19. Suttle CA, Fuhrman JA. Enumeration of virus particles in aquatic or sediments samples by epifluorescence microscopy. In: Manual of Aquatic Virus Ecology, Wilhelm S, Weinbauer SM, Suttle CA, eds. American Society for Microbiology and Oceanography. (ASLO), Waco, TX, 2009; 145-155. doi:10.4319/mave.2010.978-0-9845591-0-7-175

20. Tiekotter KL, Ackermann H-W. High-quality virus images obtained by TEM and CCD technology. J Virol Methods. 2009; 159:87-92. PMID:19442850; http://dx.doi.org/10.1016/j.jviromet.2009.03.004

21. Tikhonenko AS. Ultrastructure of bacterial viruses. Izdadelstvo Nauka, Moscow, 1968; Plenum Press, New York, NY, 1970; ISBN-13 978-0306304217.

22. Williamson KE, Helton RR, Wommack KE. Bias in bacteriophage morphological classification by transmission electron microscopy due to breakage or loss of tail structure. Microsc Res Tech. 2012; 75:452-457; doi:10.1002/jemt.21077.

23. Zwillenberg LO, Knapp W. Rapid electron microscopy sampling of single bacteriophage plaques. Experientia 1966; 22:483-4; PMID 533609; http://dx.doi.org/10.1007/BF01818884