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Bacteriophage imaging: past, present and future

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1 **Title:** Bacteriophage imaging: past, present and future

2

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**Abstract**

20  
21 The visualization of viral particles only became possible after the advent of the electron  
22 microscope. The first bacteriophage images were published in 1940 and were soon followed  
23 by many other publications that helped to elucidate the structure of the particles and their  
24 interaction with the bacterial hosts. As sample preparation improved and new technologies  
25 were developed, phage imaging became important approach to morphologically classify  
26 these viruses and helped to understand its importance in the biosphere. In this review we  
27 discuss the main milestones in phage imaging, how it affected our knowledge on these  
28 viruses and recent developments in the field.

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

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#### 40 **Earlier years (1940-1948)**

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

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

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

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

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

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

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

130 **Improvements on sample preparation (1948 onwards)**



131 A next advance on phage imaging was the introduction of contrast to the  
132 samples. By using chromium vapor to cover the preparations, Wyckoff was able to obtain  
133 more information on height and shape of the particles. In 1948 he used the technique to  
134 study T coliphages, chosen for their distinct shapes and for the ease of working with their  
135 hosts when compared to opaque staphylococci or mucoid and capsular streptococci. Two  
136 papers were published. The first was based on imaging phage plaques on samples obtained  
137 from solid media, using the embedded replica technique [14] (Fig.1b). Elongated *E. coli* were  
138 seen on young cultures and plaque characteristics were described and shown to differ  
139 between phages. The second paper focused on micrographs prepared from liquid samples  
140 [15]. Purified T4 preparations were used to describe phage morphology, and infected liquid  
141 cultures used for showing cells undergoing lysis with phages within and around their limits.  
142 Variation on the structure of phage heads and their contents was mentioned, and a  
143 correlation between grainy content inside the head and stages of maturation was made.  
144 Bacterial contents release by lysis were described, and their “conversion” to phages in  
145 favorable instances was noted as the most impressive result of the paper, hinted to be  
146 crucial in understanding how phages multiply.

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

154 the affirmation that phages were much more complex than previously thought, while the  
155 second gave strength to the idea that tails serve an important purpose to the phage life  
156 cycle. The same technique was used shortly after to reexamine T-phages, in a larger effort  
157 to compare all these phages in similar conditions. Preservation of tails during freeze drying,  
158 possibility of artifacts generated by air drying, true three dimensional forms, the number of  
159 facets of phage heads, and particle dimensions were all discussed. The particle dimensions  
160 obtained by air drying were considered to be unreliable when compared to freeze drying  
161 [17].

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

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

### 179 **Complementary imaging approaches**

180 Besides negatively stained TEM samples, other techniques were developed  
181 and used to image phages over the years. Direct observation of particles by TEM does not  
182 provide much insight on the phage life cycle or interaction with the host. For those  
183 purposes, pelleting of infected bacteria from liquid cultures and their subsequent fixation  
184 and drying was used, with the possibility of embedding the samples in polymers for ultrathin  
185 sectioning [22]. Based on worries about studying virology without the access to an electron  
186 microscope, a technique to visualize phages in a bright field light microscope was developed  
187 [23]. It was made possible by staining phages with flagella stain, a procedure that increases  
188 the particle size and make phage heads increase to the limits of detection of light  
189 microscopy. Obviously, the particles became deformed and no fine details could be seen,  
190 making the use of the technique limited. Nevertheless, it allowed crude phage imaging. The  
191 use of scanning electron microscope (SEM) has also been applied to phage imaging. Phage  
192 P1, capable of infecting *Shigella*, was used as model to test parameters related to sample  
193 preparation and visualization by SEM (Fig.1d). The paper describes the best conditions for  
194 SEM sample preparation, and suggests a correlation of SEM and TEM images to study virus  
195 life cycles [24,25].

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

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

### 207 **Techniques for three-dimensional structural determination**

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

223 Cryo-electron microscopy (cryo-EM) is a technique that does not need fixing  
224 and staining during sample preparation, and structures can be determined without the need  
225 to form crystals, making it an interesting alternative to X-ray crystallography. Recent  
226 advances in the cryo-EM field increased the resolution of the technique to near atomic  
227 levels. In 2010 the structure of human adenovirus was solved by X-ray and by cryo-EM at  
228 similar resolutions [35-36], making it relevant for viral studies. Three dimensional  
229 determination of structures is possible by algorithmic means, resulting in cryo-EM  
230 tomography and single particle cryo-EM. For example, the asymmetric structure of the  
231 phage MS2 attached to its receptor has been determined by cryo-EM tomography [37]. The  
232 capsid structure of the *Salmonella* phage epsilon 15 was analyzed by single particle cryo-EM  
233 at a level of detail close to X-ray crystallography, in near-native solution conditions [38].  
234 Cryo-EM was also used to solve the structure of the T4 baseplate-tail tube complex, in pre  
235 and post host attachment states, helping to understand sheath contraction in atomic details  
236 [39]. The structure of the FLiP (Flavobacterium-infecting, lipid-containing phage) virion, a  
237 boreal lake ssDNA phage with limited sequence similarity to other known viruses, was also  
238 solved by cryo-EM technique [40]. The determination of particle structure helped to  
239 understand its evolutionary relationship to other viruses by complementing sequence based  
240 approaches. The capsid structure of the phage Sf6 has also been determined by cryo-EM  
241 [41].

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

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

### 253 **Significance of microscopy in phage ecology and environmental studies**

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

268 Besides phage morphology and host interaction, electron microscopy has also  
269 been used to study phage diversity in environmental samples. Seawater samples were  
270 prepared and fixed for direct observation on an electron microscope, without the use of an  
271 enrichment process for phage isolation. Even without taking extra steps to grow the phages  
272 before analysis, various phage particles with distinct morphologies were found, as well as  
273 phage particles bound to bacterial cells [50]. An estimative of  $10^3$  to  $10^4$  viruses per milliliter  
274 of sea water was made, but it was noted by the authors that it is probably lower than the  
275 real number due to phage loss during sample preparation. The presence of so many phages  
276 led to the speculation of their importance in microbial ecology. Phages of marine origin  
277 previously obtained by isolation in bacterial hosts were analyzed by TEM of negatively  
278 stained samples. Seventy five phages were imaged and divided into twelve different groups  
279 based on morphological similarities, showing new structures and high structural diversity  
280 [51]. The ecological importance of phages got even more evident after environmental  
281 aquatic samples were analyzed again by TEM. Water samples from different locations were  
282 analyzed directly, without an enrichment step, and this time the sample preparation process  
283 minimized phage loss. Different phages, either as free particles or attached to bacterial cells,  
284 were visualized. Phage counts varied between  $10^4$  to  $10^8$  particles per milliliter of water,  
285 depending on sampling location and time of the year, revealing that phage abundance in the  
286 environment was higher than previously thought. The impressive number of phages in  
287 unpolluted water samples led to speculations about the so far overlooked importance of  
288 phages for keeping bacterial populations in balance on the environment, and also of the  
289 impact of these viruses in genetic transfers in natural prokaryote populations [52]. An  
290 alternative imaging technique was developed for counting environmental viruses, based on  
291 staining the samples with a fluorochrome specific for nucleic acids and directly counting the

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

### 299 **Moving away from electrons: helium ion microscopy**

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



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

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

### 332 **Conclusions and perspectives**

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

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

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

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

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382 **Conflict of interests**

383 There is no conflict of interests between the authors.

384

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400 **References**

- 401 [1] Lustig A, Levine A. One hundred years of virology. *J Virol* 1992;66:4629–31.
- 402 [2] Oransky I. James Hillier. *Lancet* 2007;369:1162.
- 403 [3] Kruger DH, Schneck P, Gelderblom HR. Helmut Ruska and the visualisation of viruses.  
404 *Lancet* 2000;355:1713–7.
- 405 [4] von Borries B, Ruska E, Ruska H. Bakterien und Virus in übermikroskopischer Aufnahme.  
406 *Klin Wochenschr.* 1938;17:921–51938;17:921–5.
- 407 [5] Ruska H. Über die Sichtbarmachung der bakteriophagen Lyse im Übermikroskop.  
408 *Naturwissenschaften* 1940;28:45–6.
- 409 [6] Peankuch E, Kausche GA. Isolierung und übermikroskopische Abbildung eines  
410 Bakteriophagen. *Naturwissenschaften* 1940;28:46.
- 411 [7] Ackermann H-W. The first phage electron micrographs. *Bacteriophage* 2011;1:225–7.
- 412 [8] Ruska H. Morphologische Befunde bei der bakteriophagen Lyse. *Arch Gesamte*  
413 *Virusforsch* 1942:345–87.
- 414 [9] Ruska H. Versuch zu einer Ordnung der Virusarten. *Arch Gesamte Virusforsch*  
415 1943;2:480–98.
- 416 [10] Ruska H. Ergebnisse der Bakteriophagenforschung und ihre Deutung nach  
417 morphologischen Befunden. *Ergeb Hyg Bakteriol Immunforsch Exp Ther* 1943, p. 437–98.
- 418 [11] Luria SE, Anderson TF. The identification and characterization of bacteriophages with  
419 the electron microscope. *Proc Natl Acad Sci U S A* 1942;28:127–130.1.

- 420 [12] Sharma S, Chatterjee S, Datta S, Prasad R, Dubey D, Prasad RK, et al. Bacteriophages  
421 and its applications: an overview. *Folia Microbiol (Praha)* 2017;62:17–55.
- 422 [13] Luria SE, Delbrück M, Anderson TF. Electron Microscope Studies of Bacterial Viruses. *J*  
423 *Bacteriol* 1943;46:57–77.
- 424 [14] Wyckoff R. The electron microscopy of developing bacteriophage. I. Plaques on solid  
425 media. *Biochim Biophys Acta* 1948;2:27–37.
- 426 [15] Wyckoff R. The electron microscopy of developing bacteriophage. II. Growth of T4 in  
427 liquid culture. *Biochim Biophys Acta* 1948;2:246–53.
- 428 [16] Fraser D, Williams RC. Details of frozen-dried T3 and T7 bacteriophages as shown by  
429 electron microscopy. *J Bacteriol* 1953;65:167–70.
- 430 [17] Williams RC, Fraser D. Morphology of the seven T-bacteriophages. *J Bacteriol*  
431 1953;66:458–64.
- 432 [18] Brenner S, Horne RW. A negative staining method for high resolution electron  
433 microscopy of viruses. *Biochim Biophys Acta* 1959;34:103–10.
- 434 [19] Brenner S, Streisinger G, Horne RW, Champe SP, Barnett L, Benzer S, et al. Structural  
435 components of bacteriophage. *J Mol Biol* 1959;1:IN9-IN21.
- 436 [20] Bradley DE, Kay D. The Fine Structure of Bacteriophages. *J Gen Microbiol* 1960;23:553–  
437 63.
- 438 [21] Ackermann HW. 5500 Phages examined in the electron microscope. *Arch Virol*  
439 2007;152:227–43.

- 440 [22] Bradley DE. Ultrastructure of bacteriophage and bacteriocins. *Bacteriol Rev*  
441 1967;31:230–314.
- 442 [23] McCamish J, Mickelson JC. Staining of bacteriophages for light microscopy. *Appl*  
443 *Microbiol* 1971;21:149.
- 444 [24] Wendelschafer-Crabb G, Erlandsen SL, Walker DH. Conditions critical for optimal  
445 visualization of bacteriophage adsorbed to bacterial surfaces by scanning electron  
446 microscopy. *J Virol* 1975;15:1498–503.
- 447 [25] Broers AN, Panessa BJ, Gennaro JF. High-resolution scanning electron microscopy of  
448 bacteriophages 3C and T4. *Science* 1975;189:637–9.
- 449 [26] Keller RW, Dunlap DD, Bustamante C, Keller DJ. Scanning tunneling microscopy images  
450 of metal-coated bacteriophages and uncoated, double-stranded DNA. *J Vac Sci Technol A*  
451 *Vacuum, Surfaces, Film* 1990;8.
- 452 [27] Kolbe WF, Ogletree DF, Salmeron MB. Atomic force microscopy imaging of T4  
453 bacteriophages on silicon substrates. *Ultramicroscopy* 1992;42–44:1113–7.
- 454 [28] Alsteens D, Trabelsi H, Soumillon P, Dufrêne YF. Multiparametric atomic force  
455 microscopy imaging of single bacteriophages extruding from living bacteria. *Nat Commun*  
456 2013;4.
- 457 [29] Wikoff WR, Duda RL, Hendrix RW, Johnson JE. Crystallization and preliminary X-ray  
458 analysis of the dsDNA bacteriophage HK97 mature empty capsid. *Virology* 1998 Mar  
459 30;243(1):113-8.

- 460 [30] Abrescia NGA, Cockburn JJB, Grimes JM, Sutton GC, Diprose JM, Butcher SJ, et al.  
461 Insights into assembly from structural analysis of bacteriophage PRD1. *Nature* 2004;432:68–  
462 74.
- 463 [31] Skinner MM, Zhang H, Leschnitzer DH, et al. Structure of the gene V protein of  
464 bacteriophage f1 determined by multiwavelength x-ray diffraction on the selenomethionyl  
465 protein. *Proc Natl Acad Sci U S A*. 1994;91(6):2071-2075.
- 466 [32] Welsh LC, Marvin DA, Perham RN. Analysis of X-ray diffraction from fibres of Pf1  
467 Inovirus (filamentous bacteriophage) shows that the DNA in the virion is not highly ordered.  
468 *J Mol Biol*. 1998;284(5):1265-1271.
- 469 [33] Rissanen I, Pawlowski A, Harlos K, Grimes JM, Stuart DI, Bamford JKH. Crystallization  
470 and preliminary crystallographic analysis of the major capsid proteins VP16 and VP17 of  
471 bacteriophage P23-77. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2012;68:580–3.
- 472 [34] Reddy HKN, Yoon CH, Aquila A, et al. Coherent soft X-ray diffraction imaging of  
473 coliphage PR772 at the Linac coherent light source. *Sci Data* 2017;4.
- 474 [35] Reddy VS, Natchiar SK, Stewart PL, Nemerow GR. Crystal Structure of Human  
475 Adenovirus at 3.5 Å Resolution. *Science*. 2010;329(5995):1071-1075.
- 476 [36] Liu H, Jin L, Koh SBS, et al. Atomic structure of human adenovirus by Cryo-EM reveals  
477 interactions among protein networks. *Science*. 2010;329(5995):1038-1043.
- 478 [37] Dent KC, Thompson R, Barker AM, Hiscox JA, Barr JN, Stockley PG, et al. The asymmetric  
479 structure of an icosahedral virus bound to its receptor suggests a mechanism for genome  
480 release. *Structure* 2013;21:1225–34.



- 481 [38] Jiang W, Baker ML, Jakana J, Weigle PR, King J, Chiu W. Backbone structure of the  
482 infectious epsilon15 virus capsid revealed by electron cryomicroscopy. *Nature*  
483 2008;451:1130–4.
- 484 [39] Taylor NMI, Prokhorov NS, Guerrero-Ferreira RC, Shneider MM, Browning C, Goldie KN,  
485 et al. Structure of the T4 baseplate and its function in triggering sheath contraction. *Nature*  
486 2016;533:346–52.
- 487 [40] Laanto E, Mäntynen S, De Colibus L, Marjakangas J, Gillum A, Stuart DI, et al. Virus  
488 found in a boreal lake links ssDNA and dsDNA viruses. *Proc Natl Acad Sci* 2017:201703834.
- 489 [41] Zhao H, Li K, Lynn AY, Aron KE, Yu G, et al. Structure of a headful DNA-packaging  
490 bacterial virus at 2.9 Å resolution by electron cryo-microscopy. *Proc Natl Acad Sci*  
491 2017;114:3601–6.
- 492 [42] Stevens GB, Krüger M, Lатыchevskaia T, Lindner P, Plückthun A, Fink HW. Individual  
493 filamentous phage imaged by electron holography. *Eur Biophys J* 2011;40:1197–201.
- 494 [43] Peralta B, Gil-Carton D, Castaño-Díez D, et al. Mechanism of Membranous Tunnelling  
495 Nanotube Formation in Viral Genome Delivery. *PLoS Biol.* 2013;11(9).
- 496 [44] Chaikeratisak V, Nguyen K, Khanna K, Brilot AF, Erb ML, Coker JKC, et al. Assembly of a  
497 nucleus-like structure during viral replication in bacteria. *Science (80- )* 2017;355:194–7.
- 498 [45] St-Pierre F, Endy D. Determination of cell fate selection during phage lambda infection.  
499 *Proc Natl Acad Sci* 2008;105:20705–10.
- 500 [46] Zeng L, Skinner SO, Zong C, Sippy J, Feiss M, Golding I. Decision Making at a Subcellular  
501 Level Determines the Outcome of Bacteriophage Infection. *Cell* 2010;141:682–91.

- 502 [47] Trinh JT, Székely T, Shao Q, Balázs G, Zeng L. Cell fate decisions emerge as phages  
503 cooperate or compete inside their host. *Nat Commun* 2017;8.
- 504 [48] Van Valen D, Wu D, Chen YJ, Tuson H, Wiggins P, Phillips R. A single-molecule Hershey-  
505 chase experiment. *Curr Biol* 2012;22:1339–43.
- 506 [49] Allers E, Moraru C, Duhaime MB, Beneze E, Solonenko N, Barrero-Canosa J, et al. Single-  
507 cell and population level viral infection dynamics revealed by phage FISH, a method to  
508 visualize intracellular and free viruses. *Env Microbiol* 2013;15:2306–18.
- 509 [50] Torrella F, Morita RY. Evidence by electron micrographs for a high incidence of  
510 bacteriophage particles in the waters of Yaquina Bay, Oregon: Ecological and taxonomical  
511 implications. *Appl Environ Microbiol* 1979;37:774–8.
- 512 [51] Frank H, Moebus K. An electron microscopic study of bacteriophages from marine  
513 waters. *Helgoländer Meeresuntersuchungen* 1987;41:385–414.
- 514 [52] Bergh Ø, Børshem KY, Bratbak G, Haldal M. High abundance of viruses found in aquatic  
515 environments. *Nature* 1989;340:467–8.
- 516 [53] Hara S, Terauchi K, Koike I. Abundance of viruses in marine waters: Assessment by  
517 epifluorescence and transmission electron microscopy. *Appl Environ Microbiol*  
518 1991;57:2731–4.
- 519 [54] Hermes KP, Suttle CA. Direct counts of viruses in natural waters and laboratory cultures  
520 by epifluorescence microscopy. *Limnol Oceanogr* 1995;40:1050–5.
- 521 [55] Naicker K, Durbach SI. Epifluorescent microscopy to evaluate bacteriophage capsid  
522 integrity. *Biotechniques* 2007;43:473–6.

- 523 [56] Ward BW, Notte JA, Economou NP. Helium ion microscope: A new tool for nanoscale  
524 microscopy and metrology. *J Vac Sci Technol B Microelectron Nanom Struct* 2006;24:2871.
- 525 [57] Joens MS, Huynh C, Kasuboski JM, Ferranti D, Sigal YJ, Zeitvogel F, et al. Helium Ion  
526 Microscopy (HIM) for the imaging of biological samples at sub-nanometer resolution. *Sci*  
527 *Rep* 2013;3.
- 528 [58] Leppänen M, Sundberg L-R, Laanto E, de Freitas Almeida GM, Papponen P, Maasilta JJ.  
529 Imaging Bacterial Colonies and Phage-Bacterium Interaction at Sub-Nanometer Resolution  
530 Using Helium-Ion Microscopy. *Adv Biosyst* 2017:1700070.
- 531 [59] Zhu Y, Dürr H. The future of electron microscopy. *Phys Today* 2015;68:32–8.
- 532 [60] Schneider JP, Basler M. Shedding light on biology of bacterial cells. *Philos Trans R Soc B*  
533 *Biol Sci* 2016;371:20150499.
- 534 [61] Takayuki U, Scheuring S. Applications of high-speed atomic force microscopy to real-  
535 time visualization of dynamic biomolecular processes. *Biochim Biophys Acta*  
536 2018;1862:229–40.
- 537 [62] Bettiol AA, Mi Z, Watt F. High-resolution fast ion microscopy of single whole biological  
538 cells. *Appl Phys Rev* 2016;3.
- 539 [63] Simpson DA, Ryan RG, Hall LT, Panchenko E, Drew SC, Petrou S, et al. Electron  
540 paramagnetic resonance microscopy using spins in diamond under ambient conditions. *Nat*  
541 *Commun* 2017;8.

542 [64] Liu TL, Upadhyayula S, Milkie DE, Singh V, Wang K, Swinburne IA, et al. Observing the  
543 cell in its native state: Imaging subcellular dynamics in multicellular organisms. *Science*  
544 2018;360 eaaq1392.

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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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570 im Übermikroskop. *Naturwissenschaften* 1940;28:45–6. Permission obtained from Springer

571 *Nature*, license number 4343490675464. B) Wyckoff R. The electron microscopy of  
572 developing bacteriophage. I. Plaques on solid media. *Biochim Biophys Acta* 1948;2:27–37.

573 DOI: 10.1016/0006-3002(48)90005-5. Permission obtained from Elsevier, license number

574 4325171419142. C) Brenner S, Streisinger G, Horne RW, Champe SP, Barnett L, Benzer S, et  
575 al. Structural components of bacteriophage. *J Mol Biol* 1959;1:IN9-IN21. DOI:

576 10.1016/S0022-2836(59)80035-8. Permission obtained from Elsevier, license number

577 4325180199035. D) Wendelschafer-Crabb G, Erlandsen SL, Walker DH. Conditions critical for

578 optimal visualization of bacteriophage adsorbed to bacterial surfaces by scanning electron

579 microscopy. *J Virol* 1975;15:1498–503. Permission obtained from the American Society for

580 Microbiology, license number 4325191273206. E) Leppänen M, Sundberg L-R, Laanto E, de

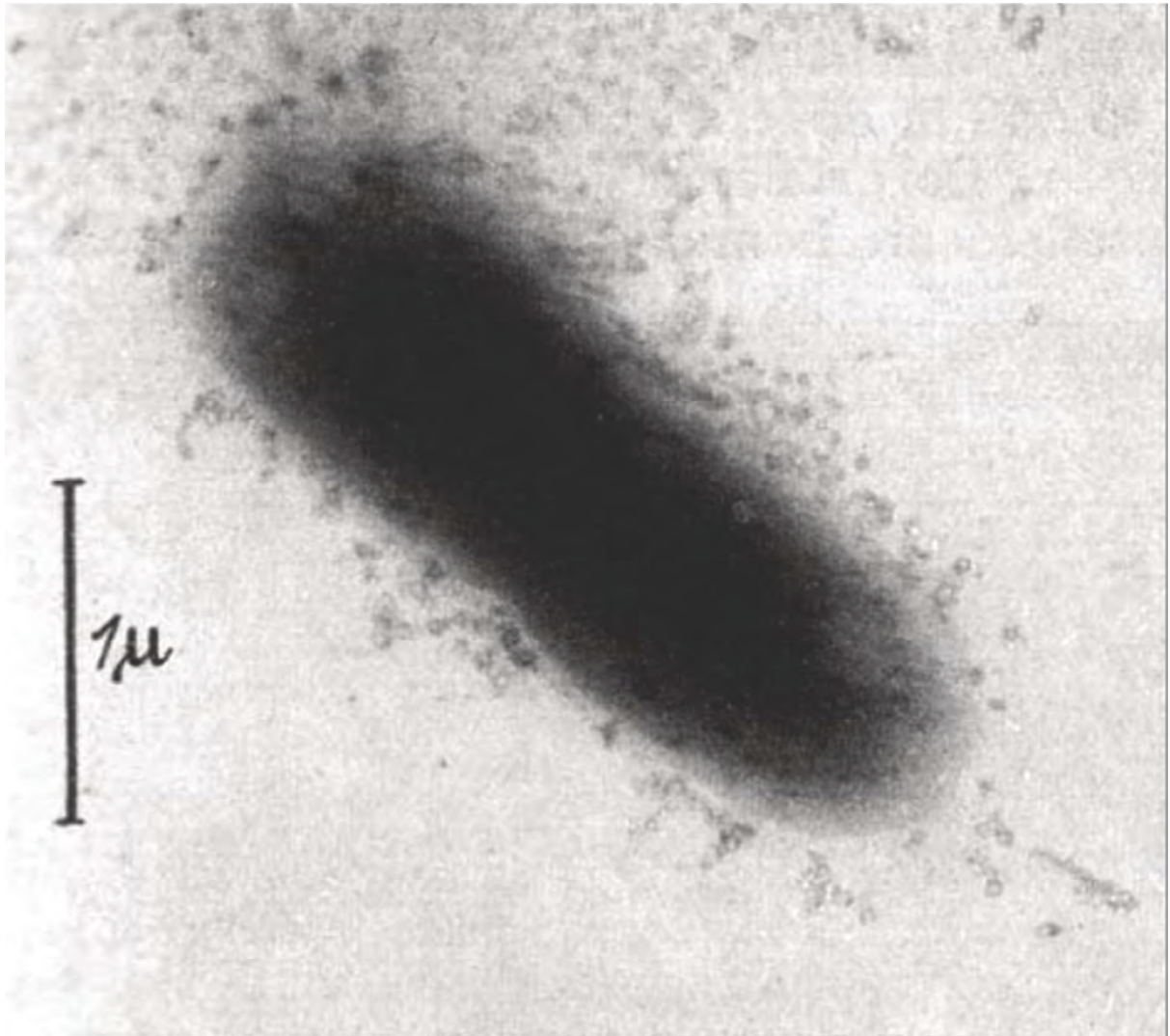
581 Freitas Almeida GM, Papponen P, Maasilta IJ. Imaging Bacterial Colonies and Phage-

582 Bacterium Interaction at Sub-Nanometer Resolution Using Helium-Ion Microscopy. Adv  
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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.

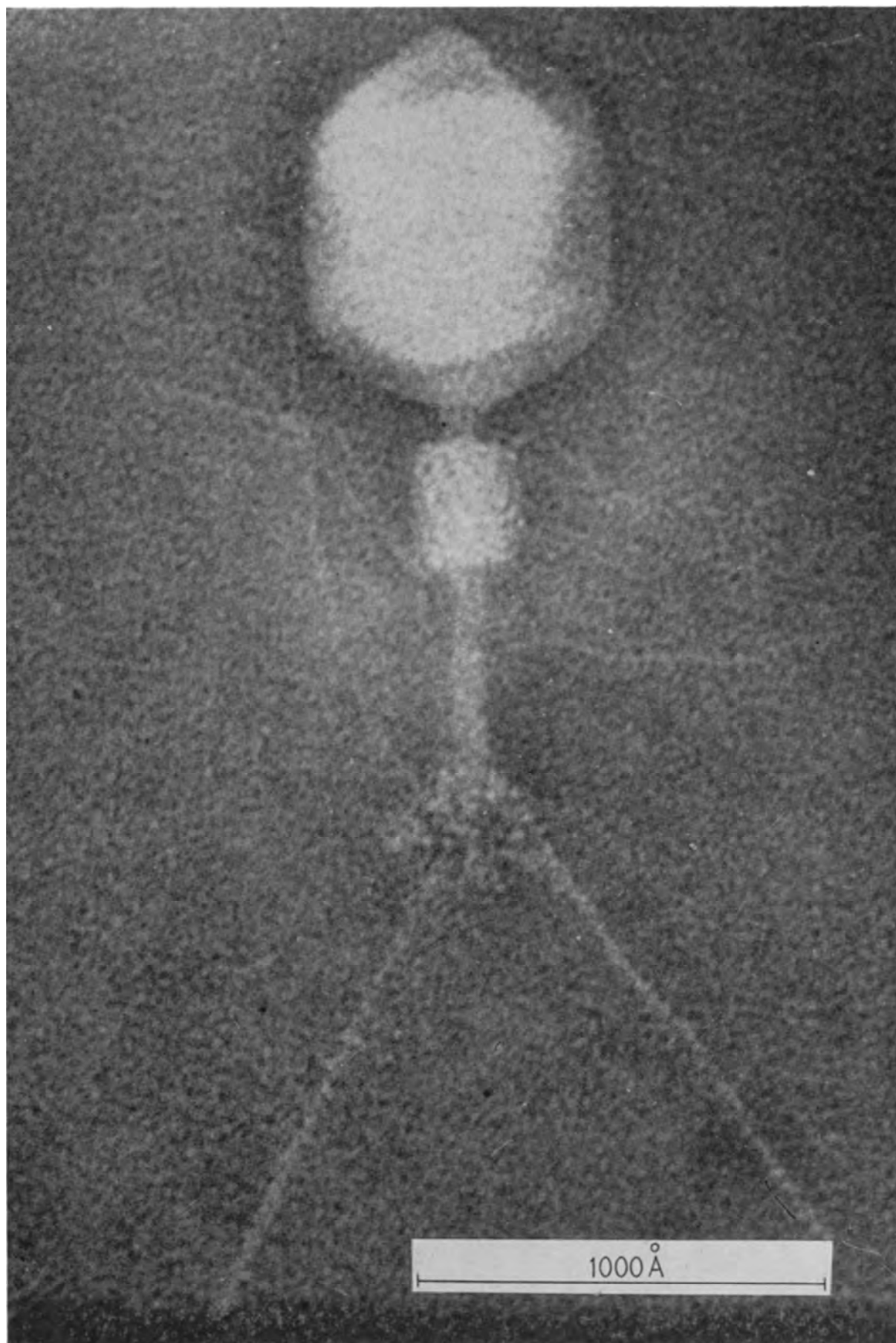


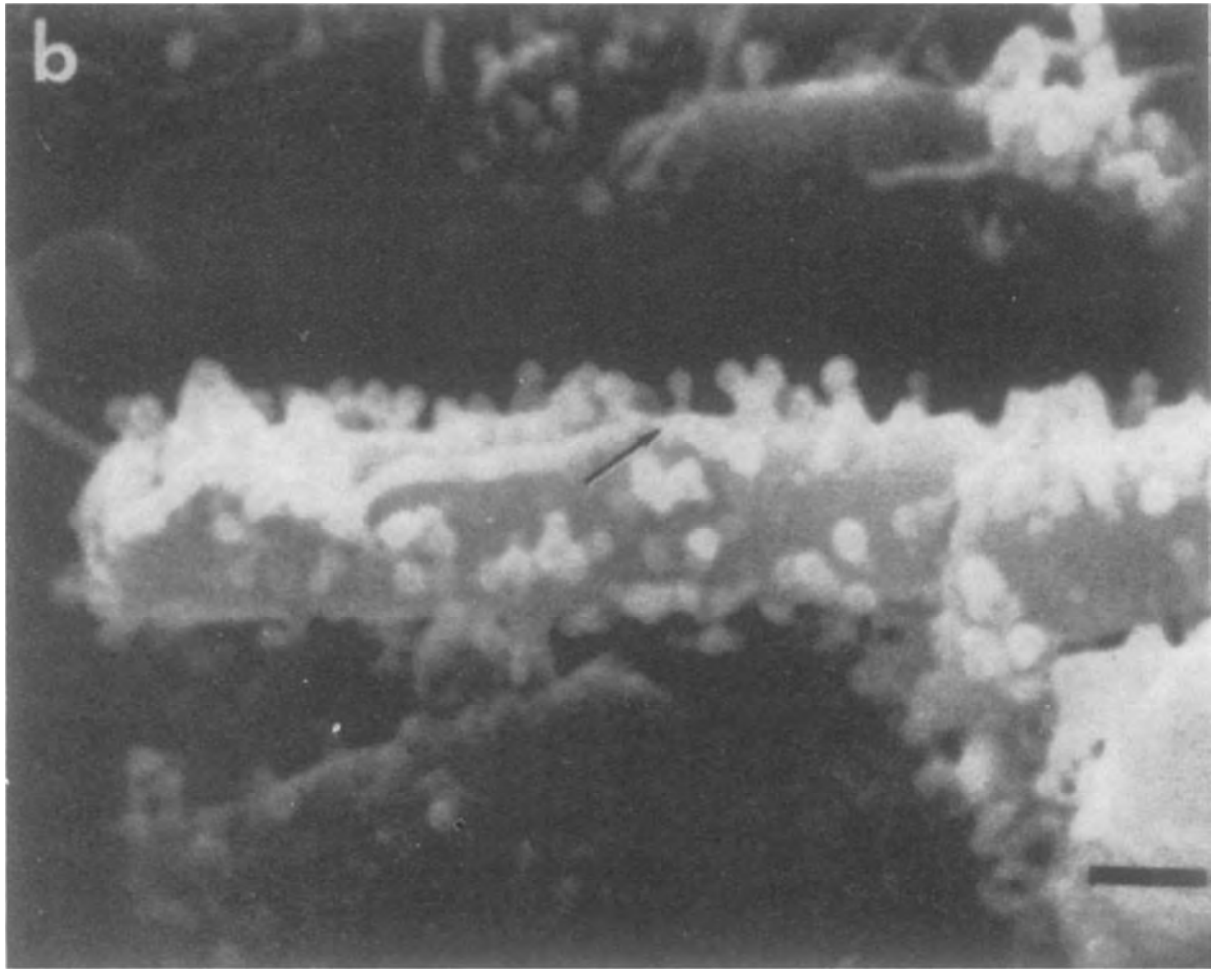
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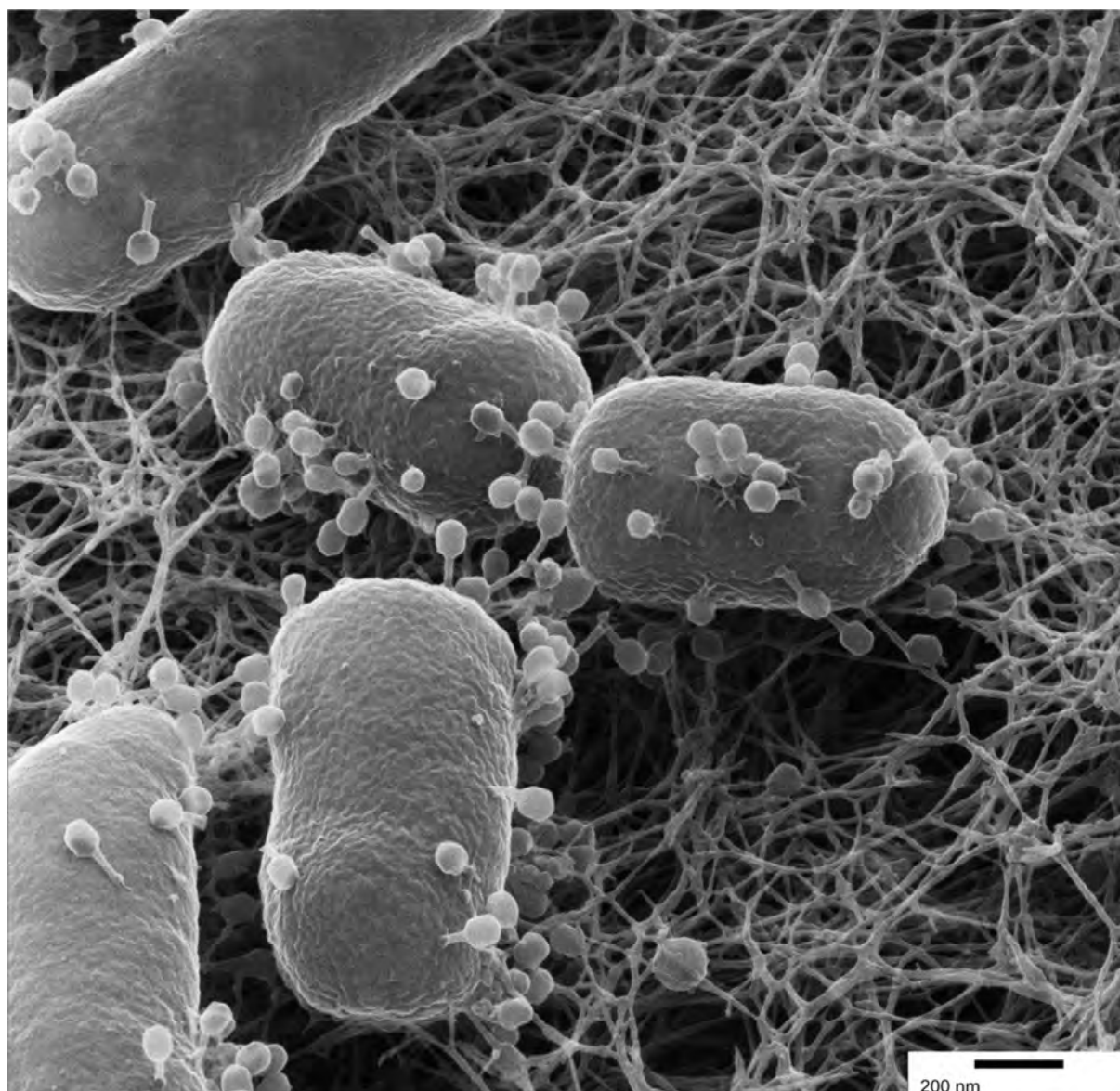
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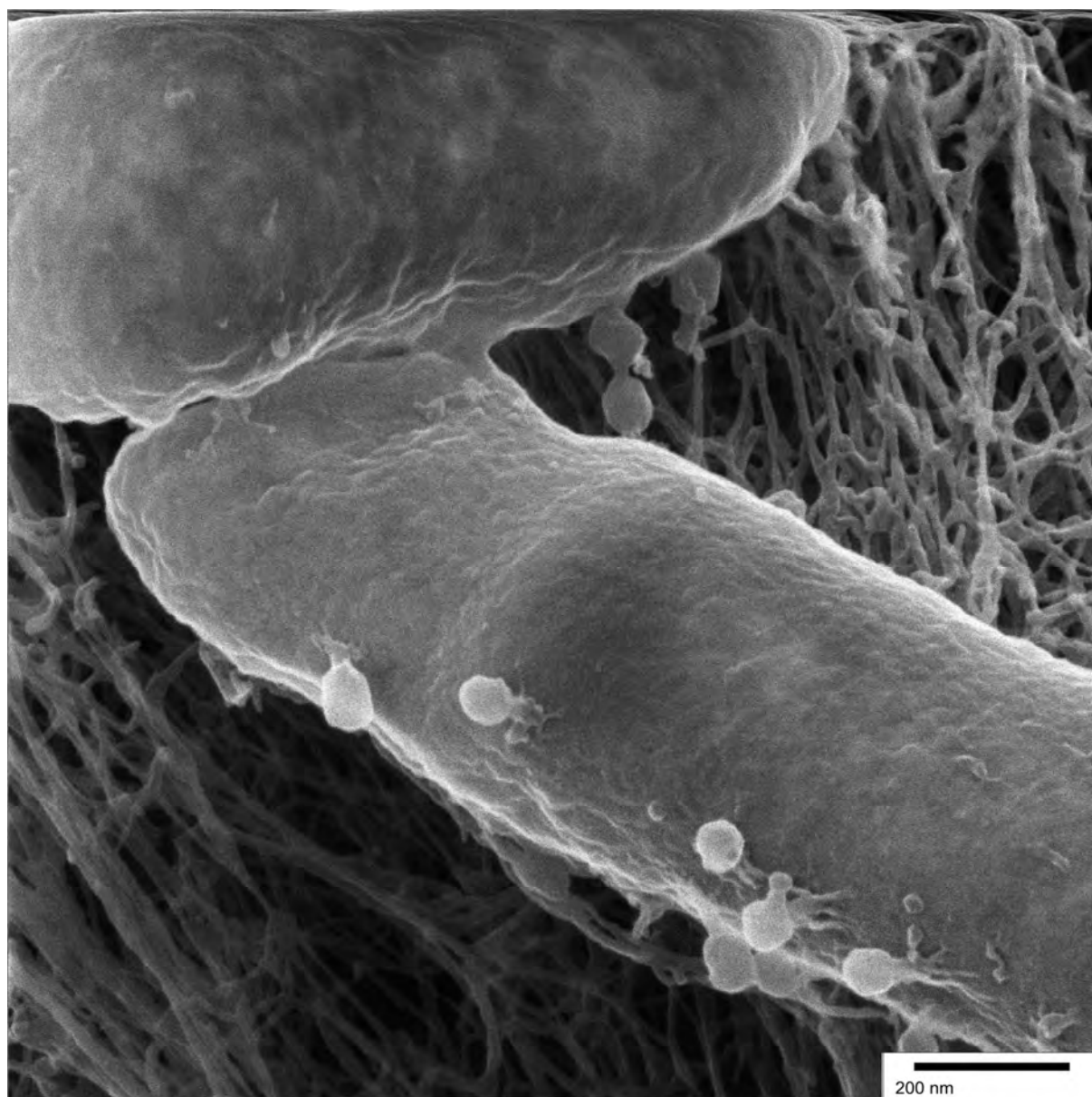


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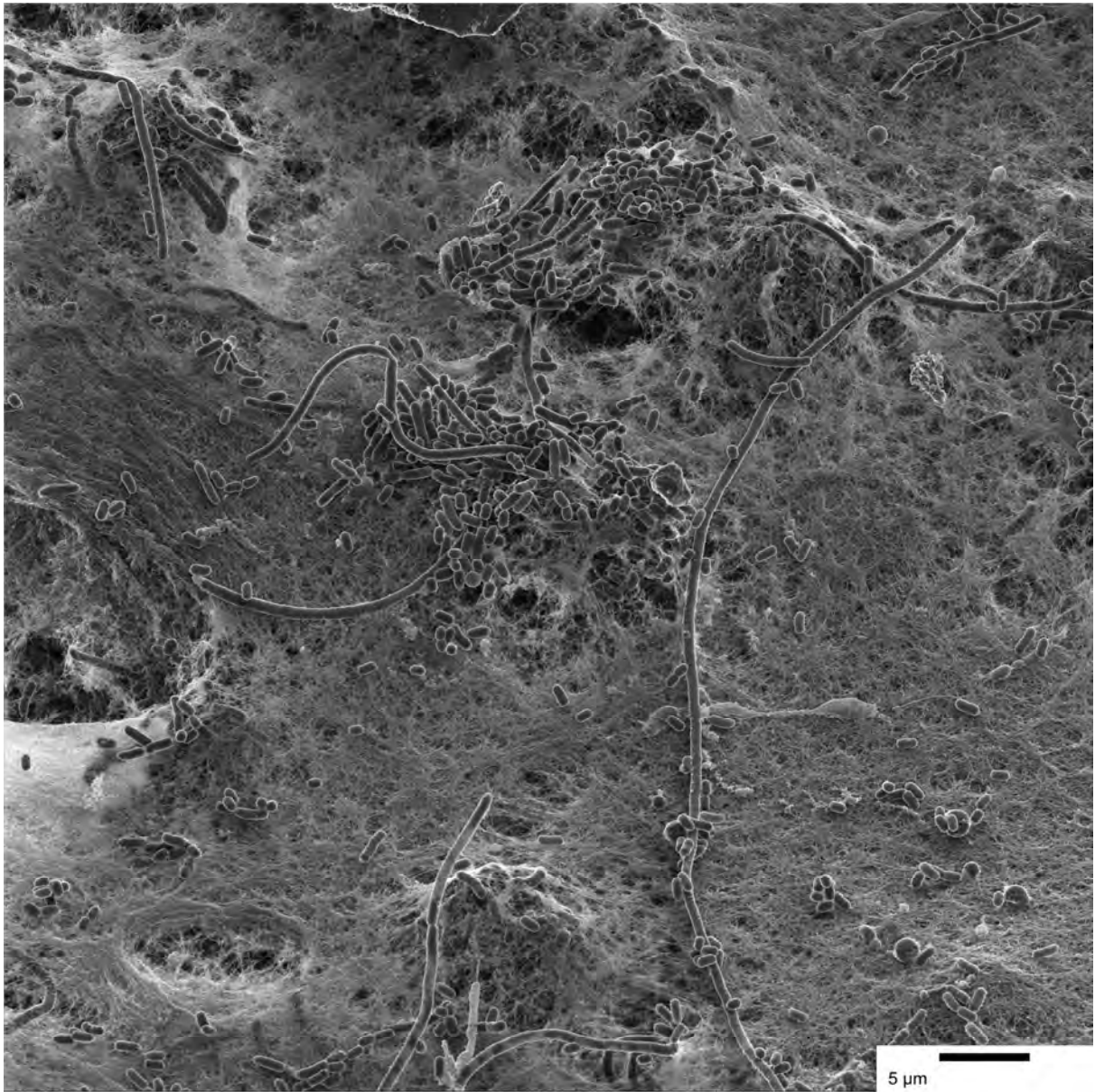


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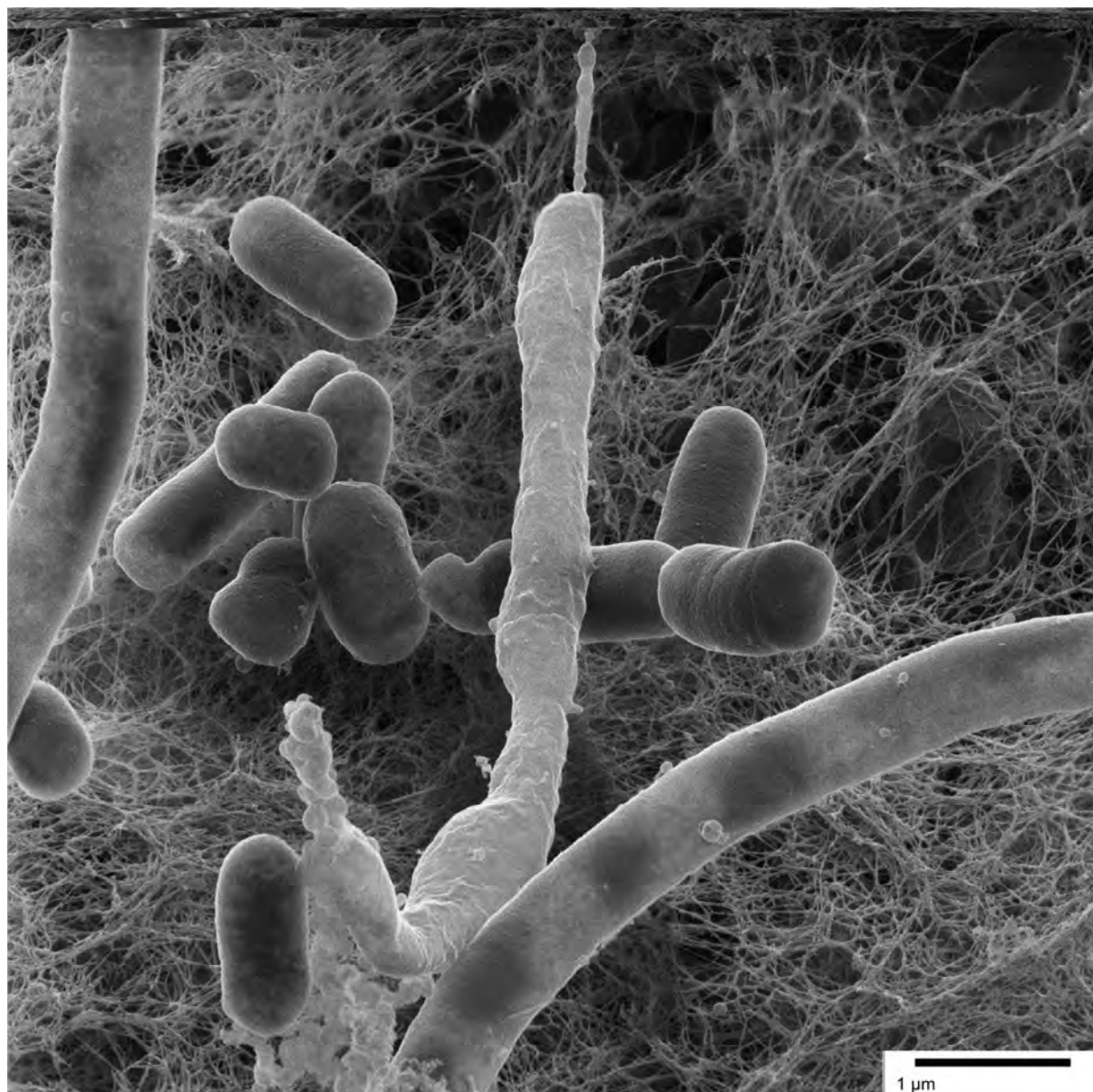


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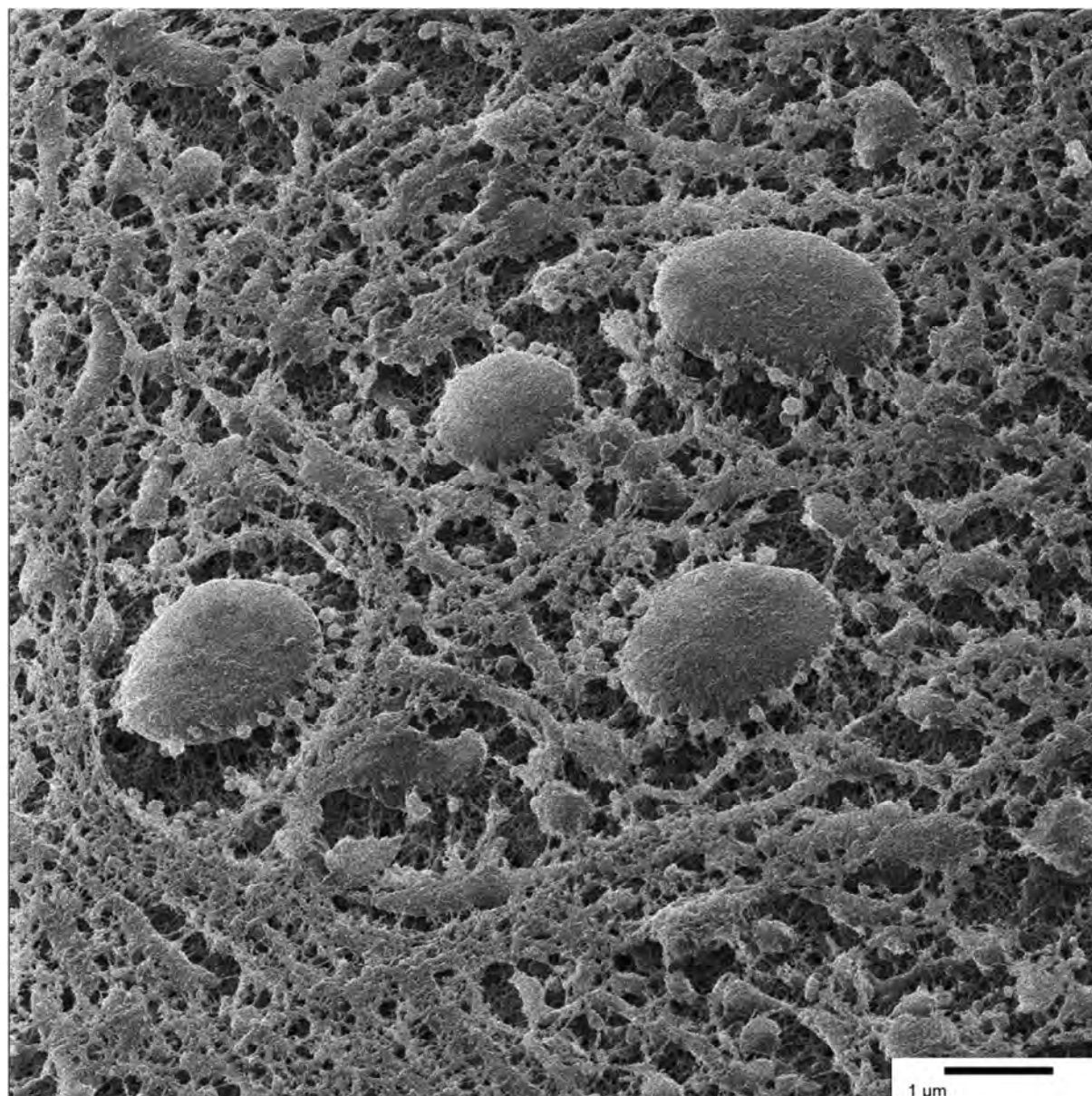


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