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1 **High-speed fixed-target serial virus crystallography**

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## 21 **Abstract**

22 We have developed a method for serial X-ray crystallography at X-ray free electron lasers (XFELs),  
23 which allows for full use of the current 120 Hz repetition rate of the Linear Coherent Light Source  
24 (LCLS). Using a micro-patterned silicon chip in combination with the high-speed Roadrunner  
25 goniometer for sample delivery we were able to determine the crystal structures of a picornavirus,  
26 bovine enterovirus 2 (BEV2), and the cytoplasmic polyhedrosis virus type 18 polyhedrin. Total data  
27 collection times were less than 14 and 10 minutes, respectively. Our method requires only micrograms  
28 of sample and will therefore broaden the applicability of serial femtosecond crystallography to  
29 challenging projects for which only limited amounts of samples are available. By synchronizing the  
30 sample exchange to the XFEL repetition rate it further allows for the most efficient use of the limited  
31 beamtime available at XFELs and a significant increase in sample throughput at these facilities.

32

## 33 **Introduction**

34 X-ray crystallography has been the dominant method for the determination of high-resolution virus  
35 structures in the last 30 years. For non-enveloped viruses, numerous X-ray crystallographic structures  
36 have been solved, at resolutions of up to 1.4 Å<sup>1</sup>. Due to the large unit cell dimensions and limited size  
37 of the crystals, Bragg reflections from virus crystals are typically weak<sup>2,3</sup>. Thus, X-ray structure  
38 determination of virus crystals are ideally carried out at highly brilliant X-ray sources, allowing a large  
39 number of photons to be focused into a small spot, ideally matching the size of the virus crystals<sup>4</sup>. A  
40 further challenge for virus crystallography is radiation damage. Structure determination from  
41 biological macromolecules are usually carried out at cryogenic temperatures in order to reduce the  
42 detrimental effect of ionizing radiation on the diffraction properties of the crystals<sup>5-7</sup>. While finding  
43 appropriate conditions for cryo-protection is often straightforward for many protein crystals, this has  
44 remained a challenge for virus crystals since they only possess weak crystal contacts and a small  
45 increase in crystal mosaicity often results in overlapping reflections and a reduction of the measured  
46 resolution<sup>2,3</sup>. Thus so far relatively few virus structures have been determined at cryogenic  
47 temperatures<sup>1</sup> and much work is still performed at room temperature<sup>4</sup>.

48 A promising method for biological structure determination from virus crystals is Serial Femtosecond  
49 X-ray Crystallography (SFX) at X-ray Free Electron Lasers (XFELs) as it is well suited to room  
50 temperature data collection, overcoming the classical radiation dose limits by several orders of  
51 magnitude<sup>8,9</sup>. SFX has been a success story over the past 5 years<sup>10-13</sup>. By taking snapshots from tens to  
52 hundreds of thousands of nano- to micrometer-sized crystals, more than 80 structures of biological  
53 molecules have been solved using SFX to date. Whilst the first experiments dealt with static ground  
54 state structure determination, the method has also been very successfully extended to the time domain  
55 to investigate protein kinetics and enzyme reactions<sup>14,15</sup>. SFX therefore has not only the potential to

56 yield high-resolution structural information about the structure of the virus itself, it also offers the  
57 possibility of studying the dynamics, since many viral proteins undergo structural changes during their  
58 life cycles, for example due to protein interactions with cellular receptors and changes in pH during  
59 entry through the endosome<sup>16-18</sup>.

60 A remaining limiting factor for virus SFX in particular is efficient sample delivery, as typically only  
61 microgram amounts of virus crystals are available. Most SFX experiments today are carried out using  
62 gas dynamic virtual nozzles (GDVNs) and high-viscosity gel matrices typically requiring more than  
63 500 µg (~370 nL) of sample for a structure determination<sup>19-26</sup>.

64 First attempts of virus crystallography at XFELs were undertaken by Lawrence *et al.*<sup>27</sup>. In their  
65 experiment 10<sup>4</sup> micro-crystals of the Sindbis virus with unit cell dimension of ~700 Å were suspended  
66 in a slow-moving stream of agarose and delivered to the XFEL beam. Using this approach, diffraction  
67 patterns with up to ~40 Å resolution and a hit rate of 0.8%, defined by the number of images  
68 containing Bragg spots from an exposed crystal divided by the total number of collected images, could  
69 be obtained. Due to the technological challenges, especially regarding the amount of sample required,  
70 no virus structure has yet been determined at an XFEL.

71 A promising sample delivery approach for SFX – in particular if only small amounts of sample are  
72 available – is the use of solid sample supports, also referred to as fixed targets<sup>28-37</sup>. Here several tens to  
73 thousands of crystals are loaded onto a structured solid support and automatically raster-scanned  
74 through the X-ray beam. A major challenge for fixed-target experiments is fast and precise scanning of  
75 these supports and synchronization of predefined sample positions to the arrival of the XFEL pulses.

76 So far, such experiments have been performed in step-scanning mode, rendering them less competitive  
77 compared to other sample delivery methods due to the relatively long data collection times<sup>29,34,38</sup>.  
78 Another obstacle for fixed-target experiments with biological samples is the relatively high  
79 background scattering level, which is mainly caused by air scattering from the direct beam. Further  
80 contributions originate from scattering by non-sample material such as the surrounding mother liquor  
81 and other materials, such as Kapton or Mylar foil typically used as sealing materials to prevent the  
82 crystals from drying out.

83 We have developed a method for fixed-target serial crystallography at low background levels and a  
84 sample exchange rate of 120 Hz requiring only micrograms of sample. We demonstrate the  
85 applicability of our method for the determination of virus structures, shown by taking the example of  
86 an intact virus, BEV2, and a viral protein, polyhedrin of the cytoplasmic polyhedrosis virus type 18  
87 (CPV18).

88 BEV2 belongs to the virus family *Picornaviridae*, genus *Enterovirus*, and is a non-enveloped,  
89 positive-stranded RNA virus, 30 nm in diameter, which is endemic in some cattle and cattle

90 environments<sup>39-41</sup>. Unlike some other picornaviruses it is not a serious economic or animal health  
91 threat and is therefore a suitable model system for the investigation of the application of new  
92 technologies and the exploration of biological processes such as virus uncoating which are common to  
93 all enteroviruses. Enteroviruses (including BEV2) are stabilized by lipid cofactors such as  
94 sphingosine, which bind to a hydrophobic pocket of the VP1 capsid protein<sup>42</sup>. Potent binders to this  
95 pocket have potential as antivirals (by preventing uncoating) and so we are investigating the  
96 specificity of binding natural and synthetic moieties in this pocket. BEV2 crystals represent a  
97 challenging system for current SFX experiments, as they possess a large unit cell constant of 432 Å,  
98 and typically only a few micrograms of small crystals are available.

99 Cytoplasmic polyhedrosis viruses (CPVs) are found as parasites in many insects and cause significant  
100 losses in silkworm cocoon harvests<sup>43</sup>. These crystals, termed polyhedra, vary in size from hundreds of  
101 nanometers to several micrometers, depending on the CPV strain and typically contain up to several  
102 thousands of CPV particles<sup>43-46</sup>. CPV polyhedra were chosen as a well-established and robust model  
103 system for SFX data collection at cryogenic temperatures<sup>47</sup>.

## 104 105 **Results**

### 106 **High-speed fixed-target structure determination of BEV2 and CPV18**

107 Micro-crystals of BEV2 and CPV18 were measured by fixed-target SFX using the Roadrunner  
108 goniometer installed at the XPP instrument at the Linear Coherent Light Source (Supplementary Fig.  
109 1). The Roadrunner setup consists of high-precision x and y piezo-motor driven scanning stages  
110 mounted on a horizontal translation stage and a vertical rotation axis (Supplementary Fig. 2, also see  
111 Methods section for details). A high-magnification inline microscope allows visualization of samples  
112 and their support structure (Supplementary Fig. 3). The Roadrunner setup is capable of data collection  
113 at both room temperature and cryogenic temperatures.

114 To reduce air scattering of X-rays most of the path of the direct beam in air is enclosed in capillary  
115 shields, both upstream and downstream of the sample, reducing the free path of the direct beam in air  
116 to 20 mm (Supplementary Fig. 4). By streaming helium gas across the remaining unenclosed direct  
117 beam behind the sample, the number of photons scattered by air is further reduced (Fig. 1). Combining  
118 these two approaches, air scattering can be reduced by a factor of about 8.

119 Using micro-patterned chips made of single-crystalline silicon as substrate material, the background  
120 scattering signal caused by the support can be further reduced<sup>31</sup>. Dehydration of the crystals is  
121 prevented either by keeping them constantly in a stream of humidified gas<sup>32</sup> or by flash freezing and  
122 collecting data at cryogenic temperatures<sup>31</sup>. With this approach, sealing of the sample holder *e.g.* with  
123 Mylar foil, which would result in an increased background scattering level, is not required. For SFX  
124 data collection, the chips are mounted on the Roadrunner goniometer. For room-temperature

125 measurements, an empty chip is first mounted on the goniometer and the microcrystal suspension is  
126 then applied to the chip as described by Roedig *et al.*<sup>32</sup>. During loading and measurement, the chip is  
127 exposed to a continuous gas stream of controlled humidity, preventing the crystals from drying out<sup>32,48</sup>.  
128 For measurements at cryogenic temperatures, a preloaded and cryo-cooled chip is mounted on the  
129 goniometer and exposed to the cold gas stream of an open flow cryostat (Supplementary Fig. 5).

130 Our silicon chip (Fig. 2a) provides 22,500 pores for crystals. For sample loading, 2 – 3  $\mu$ L of sample  
131 suspension is pipetted onto the chip so that the amount of sample material used is typically in the  
132 range of a few micrograms, depending on the crystal sizes, desired coverage of the chip membrane and  
133 the amount of sample available<sup>31,32</sup>. The resulting arrangement of the crystals is a result of the pore  
134 pattern and allows for a highly efficient measurement strategy by shooting through all pores with the  
135 FEL pulses in a fully automated procedure (Fig. 2b,c). With the Roadrunner control software the scan  
136 points are defined by drawing a grid, which is graphically overlaid on the inline-microscope image  
137 (Supplementary Fig. 6). For data collection the coordinates of scan points are downloaded to the  
138 motion controller and the entire chip is scanned in a meander-scan manner (Fig. 2d,e, for details see  
139 Methods section). With this approach most of the crystalline material is used for the diffraction  
140 experiment and not wasted.

141 To achieve fixed-target data collection rates of 120 Hz we have developed an improved version of the  
142 so-called fly-scan. In a conventional fly-scan the sample is accelerated and then moved along a  
143 predefined trajectory at constant velocity. This approach would in principle allow data collection at  
144 120 Hz, but in most cases results in lower hit rates than obtained with our method since the X-ray  
145 pulses would not necessarily always hit through the pores where the crystals are located. To achieve  
146 higher hit rates – and thereby requiring significantly less sample – more precise motion control is  
147 required. Such an approach demands, in addition to velocity control, also phase control of the  
148 movement of the stages with respect to the arrival of the X-ray pulses. The synchronized movement  
149 assures that every X-ray pulse hits through a pore (Fig. 2c-e, also see Methods section, and  
150 Supplementary Figure 7).

151 In the case of BEV2 samples, which were measured at room temperature, diffraction images were  
152 collected at an FEL pulse rate of 30 Hz per line, resulting in an effective frame rate of up to 12.2  
153 images/s per chip, when taking the time for line switching into account (Table 1). A maximum hit rate  
154 of more than 9% for one chip and on average of about 5% was achieved for BEV2. The relatively low  
155 hit rates in this case are due to the fact that the density of the crystals on the chip was low. As only  
156 limited amounts of sample were available we aimed at making most efficient use of the available  
157 sample instead of optimizing hit rates. For room-temperature data collection, it was not possible to run  
158 at the full LCLS frame rate of 120 Hz since crystals in the neighboring compartments were already  
159 pre-damaged by the wings of the X-ray beam. When operating at 120 Hz and shooting at every  
160 position, diffraction could be only observed from the first crystal of a chip. By shooting every 4<sup>th</sup> hole

161 (40  $\mu\text{m}$  separation) in the horizontal direction and every 2nd row in the vertical direction (20  $\mu\text{m}$   
162 separation) no effects of pre-damage were observed (Fig. 2d). With a maximum speed of the  
163 horizontal scanning stage of 2.5 mm/s data collection at 60 Hz with 40  $\mu\text{m}$  separation or at 120 Hz  
164 with 20  $\mu\text{m}$  separation would have also been possible.

165 For CPV18, data collection was performed at cryogenic temperatures with the full LCLS repetition  
166 rate of 120 Hz, resulting in an average data collection rate of 33.6 images/s (Fig. 2e). From these  
167 images more than 70% were classified as a hit. In other runs, we were able to achieve hit rates of more  
168 than 90%. No pre-damage of the neighboring crystals by the wings of the X-ray beam could be  
169 observed for CPV18, which is probably due to reduced diffusion rates of free radicals and the resulting  
170 higher radiation tolerance of macromolecular crystals at cryogenic temperatures<sup>5-7</sup>.

### 171 **Image quality and background analysis**

172 The collected images contained X-ray diffraction to a resolution of 2.3  $\text{\AA}$  for BEV2 and 2.4  $\text{\AA}$  for  
173 CPV18, respectively. An example diffraction image (Fig. 3a) obtained from a BEV2 crystal illustrates  
174 the high quality of the diffraction patterns obtained using the Roadrunner goniometer. We analyzed  
175 the averaged background signal of the measured diffraction images (Fig. 3b) and compared it to that of  
176 an SFX experiment where – in contrast to our fixed-target approach – a liquid jet was used for sample  
177 delivery (Fig. 3c). The azimuthally averaged background signal as a function of resolution clearly  
178 shows that in the diffraction images measured with our fixed-target setup, background is dominated by  
179 air scattering, which is most prominent at resolutions lower than 10  $\text{\AA}$  (Fig. 3d). The chip itself  
180 consists of single-crystalline silicon and therefore does not contribute to any background signal. The  
181 absence of a water ring for room-temperature data collection reveals the efficient removal of mother  
182 liquor during sample loading. In typical liquid jet experiments, the averaged background signal shows  
183 a strong water ring around 3.0  $\text{\AA}$ .

### 184 **Structure determination**

185 Summary information regarding data collection and structure refinement for both samples are given in  
186 Supplementary Table 2. The structure of BEV2 was solved using the diffraction data obtained from 5  
187 chips, with a total of about 446 crystal hits in less than 14 minutes of scanning time. For CPV18 the  
188 complete structure could be solved with the data obtained from only one single chip, collected in less  
189 than 10 minutes. Unfortunately, the resolution was limited by the dimensions of the detector for the  
190 given detector distance in this case. Structure refinement (based on prior models) yielded high-quality  
191 electron density maps (Fig. 4) with model R-values of  $R_{\text{work}}/R_{\text{free}} = 23.3/25.7\%$  for BEV2 and  
192  $11.3/14.5\%$  for CPV18. The BEV2 structure was determined as part of an investigation of the  
193 specificity of the hydrophobic pocket in VP1 for different fatty acids. The structure revealed that  
194 despite co-crystallising the virus with lauric acid, the pocket factor present in the particles was  
195 indistinguishable from that observed in native particles, which is well modelled as sphingosine (Fig.

196 4d), explaining biophysical observations that lauric acid has essentially no effect on the stability of the  
197 virus particles. The CPV18 structure is similar to recently published structures of isolated crystals of  
198 CPV18<sup>49,31</sup>. A part of the electron density map is shown in Supplementary Figures 8 and 9.

## 199 **Discussion**

200 Using our micro-patterned silicon chip in combination with the Roadrunner goniometer we were able  
201 to determine the structure of the BEV2 virus particle co-crystallized with lauric acid at room  
202 temperature from microgram amounts of sample only.

203 To our knowledge, this is the first time the structure of a virus particle has been determined at an X-  
204 ray Free Electron Laser by means of serial crystallography. The electron density maps obtained  
205 provide a high level of detail, sufficient to demonstrate that lauric acid cannot displace sphingosine  
206 from the VP1 pocket, which is the major target for the design of anti-enterovirus compounds.

207 We could further show that the method is also applicable to data collection at cryogenic temperatures,  
208 where hit rates of more than 70% were achieved and the structure of CPV18 crystals was solved from  
209 the measurement of one chip loaded with about 4 µg of protein with a data-collection time of less than  
210 ten minutes.

211 The periodic arrangement of the crystals on our chip in combination with the Roadrunner goniometer  
212 allows for very effective use of beam time. With data collection rates of 120 Hz during a line scan,  
213 combined with hit rates of more than 70%, we were able to obtain up to 29.6 indexable diffraction  
214 patterns per second. Sample loading onto the chip is very efficient and no precious crystalline material  
215 is lost. The method has been shown to be more reliable than liquid jet experiments, which often suffer  
216 from clogging of the nozzles and settling of the crystals, leading to substantial downtimes during the  
217 experiments.

218 A further benefit of our method is the ultra-low sample consumption, which requires orders of  
219 magnitude less sample compared to current liquid jet methods at XFELs and also significantly less  
220 compared to room temperature experiments at synchrotrons. The synchrotron structure<sup>4</sup> of the apo  
221 form of the BEV2 capsid was determined at 2.1 Å resolution based on the measurement of 28 crystals  
222 of a cubic edge length of about 50 µm, which amounts to a total crystal volume of 3.5 nL. Our work is  
223 based on data collected from 446 much smaller crystals with a cubic edge length of only about 8 µm  
224 corresponding to a total volume of 228 pL. Co-crystallisation of BEV2 with lauric acid limits the  
225 achievable crystal size and renders these crystals far too small for a conventional synchrotron structure  
226 determination. The obligatory use of the XFEL therefore not only provided the high intensity X-ray  
227 pulses required to generate strong enough diffraction to solve the ligand-bound virus structure, but  
228 also reduced the total sample amount used for structure factor calculation by 15-fold. It is notable that  
229 high-quality phases and hence electron density map could be derived from amplitudes assembled from

230 so little material, and such a low multiplicity XFEL data set assembled from only 324 crystals. We  
231 attribute this to the high quality of the data obtained from this experimental setup, to the advances in  
232 data processing methods, and in part to the 5-fold non-crystallographic symmetry.

233 In the current setup the X-ray scattering background is dominated by air scattering from the short  
234 remaining beam path in humidified air or cold nitrogen gas, respectively. By further reducing the path  
235 of the primary beam in air and by replacing air or nitrogen with helium we aim to significantly reduce  
236 the background level in future experiments to achieve higher resolution data from even smaller  
237 crystals. A larger chip design with up to 200,000 micro-pores in combination with faster scanning  
238 stages will allow longer data collection runs at frame rates of up to 1 kHz, which will result in even  
239 more efficient use of beamtime both at XFELs and synchrotron facilities.

## 240 **Methods**

### 241 Sample preparation

242 Bovine enterovirus type 2 (BEV2) was produced and purified as described previously for BEV type  
243 1<sup>39</sup> and crystallized in nanoliter drops<sup>50</sup>. The obtained cubic-shaped crystals had a typical dimension of  
244 8  $\mu\text{m}$  in each direction. Further details regarding BEV crystallization are given in Supplementary Note  
245 1. An image of the BEV2 crystals is shown in Supplementary Figure 10. CPV18 polyhedrin crystals  
246 were prepared as described in reference <sup>31</sup>.

### 247 Chip design and fabrication

248 The chip design is illustrated in Figure 2. The chips are made from single-crystalline silicon by UV  
249 lithography and have overall dimensions of 2.5 x 4  $\text{mm}^2$  with a thickness of 0.1 mm. The inner  
250 membrane part with an area of 1.5 x 1.5  $\text{mm}^2$  is thinned down to a thickness of 10  $\mu\text{m}$  and provides a  
251 hexagonal dense pattern of pores with diameters between 4  $\mu\text{m}$  and 8  $\mu\text{m}$  and a 10  $\mu\text{m}$  periodicity  
252 (inset Figure 2a). The chips are glued to plastic pins, which can be mounted on conventional magnetic  
253 caps routinely used in macromolecular crystallography.

### 254 Pre-orientation of the chips

255 With the extremely high X-ray intensity per FEL pulse, Bragg reflections arising from the silicon chip  
256 material can easily damage the detector. Hence it is essential to know the exact angular orientation of  
257 the chips with respect to the incident X-ray beam in order to avoid these Bragg reflections. As a  
258 reference mark, the magnetic caps carrying the chips were modified by removing some material at the  
259 lower rim of the caps as shown in Figure 2a. All chips glued to the plastic pins were then oriented and  
260 fixed in such a way that the chip surface was always parallel to the face of the magnetic caps.

### 261 Sample loading

262 Sample loading is performed by applying 2-3  $\mu\text{L}$  of crystal suspension to the upper side of the chip.  
263 Additional mother liquor is then removed by soaking with a wedge of filter paper attached to the lower  
264 side of the chip (see reference <sup>31</sup> for details). The chip allows for data collection at both room  
265 temperature (BEV2) and cryogenic temperatures (CPV18). For room-temperature data collection the  
266 samples are loaded onto the chips directly at the experimental setup. Similar to recently performed  
267 synchrotron experiments<sup>32</sup>, a humidified gas stream with adjustable relative humidity was used to  
268 prevent the crystals from drying out during loading and data collection<sup>48,51</sup>. For BEV2 data collection  
269 the relative humidity was set to 96%. The experimental setup can be also used for data collection at  
270 cryogenic temperatures. The major difference is simply the replacement of the humidity stream used  
271 for room temperature data collection by a cold nitrogen gas stream.

### 272 Roadrunner goniometer

273 For the experiment, we have designed a special goniometer, the main part of which is a fast piezo-  
274 motor driven x,y translation stage for fast raster scanning of the chips carrying the samples. A  
275 technical overview drawing of the Roadrunner goniometer is provided in Supplementary Figure 1. The  
276 setup consists of three major components, a high-resolution inline sample-viewing microscope, the  
277 high-precision goniometer itself, and a post sample beam pipe unit, all mounted on a common support  
278 frame structure. With outer dimensions of 250 mm along the beam direction, a width of 400 mm and a  
279 height of 515 mm the entire setup is compact and can be therefore easily installed at different  
280 experimental endstations such as XPP (as in the case presented here), the new MFX endstation at  
281 LCLS, or other X-ray sources.

282 The first element in the X-ray beam path, the inline sample-viewing microscope, is shown and  
283 described in more detail in Supplementary Figure 3 and the corresponding figure caption. It provides a  
284 high-resolution image of the samples mounted on the goniometer and is used for precise alignment of  
285 the chips with respect to the X-ray beam. The X-ray beam passes through a molybdenum collimator  
286 tube inserted into the hole of the objective lens with an inner diameter of 0.35 mm. The capillary is  
287 utilized to prevent X-ray damage to the microscope lenses. It extends to only 3 mm from the sample  
288 position to reduce air scattering along the beam path.

289 The micro-patterned silicon chip carrying the samples is mounted on a high-precision goniometer axis.  
290 A technical drawing of the goniometer and a detailed description of its functionality is provided in  
291 Supplementary Figure 2. Main element is the high x,y precision stage for scanning of the chips  
292 synchronized to the time structure of the X-ray pulses. The x,y scanning stage is controlled by a DMC-  
293 4080 motion controller from Galil. The motion controller is capable of synchronizing the two axes of  
294 the scanning stage to the repetition rate of the LCLS beam at 120 Hz. The synchronization is done on a  
295 line-by-line basis to insure that each X-ray pulse hits the center of the holes in the silicon chip. This is  
296 accomplished by providing the motion controller with the start point of each line, the number of scan  
297 points (number of holes), the angular orientation of the line and the repetition rate of the LCLS beam.  
298 The synchronization scheme is illustrated and described in more detail in Supplementary Figure 7.  
299 Upon start, the controller moves the scanning stages to a defined position before the first scan point  
300 and sends a trigger signal to the LCLS control system. This trigger signal induces a defined sequence  
301 of TTL signals to be sent from the LCLS control system to the motion controller to allow the scanning  
302 stage to reach a constant speed and the position of the chip pores to be in phase with the arrival of the  
303 X-ray pulses at the pre-defined beam position. Once the starting point of the grid is reached, the pulse  
304 picker opens and the X-rays are hitting the crystals located in the pores of the chip. At each scan point  
305 the current position is read out by the controller and any error is instantaneously injected into the  
306 control loop and compensated for in order to prevent accumulation of the errors. After a predefined  
307 number of pulses (equal to the number of pores selected) has been reached, the pulse picker closes and

308 the scanner decelerates before switching to the next line of the chip. This sequence is repeated for all  
309 rows of the chip in a meander-scan like manner.

310 After interacting with the sample the direct undiffracted beam is guided into a beam pipe, which is  
311 shown and described in Supplementary Figure 4. By enclosing the beam in a beam pipe all X-rays  
312 scattered by air are absorbed in the walls of the tube and thereby do not contribute to background  
313 scattering on the detector.

314 Roadrunner control system and software

315 Alignment of the goniometer setup, control of individual motors, pre-alignment of the individual  
316 chips, definition of the scan grid, and data collection is controlled by a custom software written in the  
317 Python programming language with the underlying control system TANGO. The software provides a  
318 Graphical User Interface (GUI) for easy and efficient operation. A screenshot of the Roadrunner GUI  
319 together with a more detailed functionality of the software is provided in Supplementary Figure 6.  
320 Style and functionality of the GUI are adapted from GUIs typically used at protein crystallography  
321 beamlines. The software is available for free download (see section “accession codes”).

322 Data collection

323 Measurements were conducted on the XPP instrument at the Linear Coherent Light Source (LCLS) at  
324 SLAC under experiment number LH90. An X-ray energy of 9.5 keV was chosen for the experiment as  
325 it provides a good compromise between detector efficiency and pulse intensity on one hand and X-ray  
326 absorption by the silicon chips on the other. The X-ray beam size at the sample was  $3 \times 3 \mu\text{m}^2$ . X-ray  
327 pulse energies were attenuated to 40% of the full flux. A photograph of the Roadrunner setup installed  
328 at the XPP instrument at LCLS is shown in Supplementary Figure 5.

329 For measurements performed at room temperature, it was observed that, with the aforementioned  
330 procedure, only the first shot of each line yielded useful diffraction data, probably due to pre-damage  
331 of the subsequent crystals by the wings of the X-ray beam. It was therefore necessary to increase the  
332 displacement of subsequent pulses in order to prevent damage of the crystals caused by the previous  
333 X-ray pulse. For this reason the beam shutter was used to chop the repetition rate of the laser to 30 Hz,  
334 shooting only every 4<sup>th</sup> hole in the chip (Figure 2d). In addition, during the scan only every 2<sup>nd</sup> line of  
335 the chip membrane was scanned. In this way an effective acquisition rate of up to 12.2 images/s was  
336 achieved for room-temperature measurements.

337 At cryogenic temperatures in each line the chip was translated with a speed of 1.2 mm/s so that the  
338 displacement of subsequent pulses matched the distance between two neighboring holes in the chip  
339 (Figure 2e). This way a maximum data acquisition rate of 120 Hz could be achieved within a line.  
340 After the end of a line was reached, the chip moved to the next line and scanned in reverse direction.

341 This allowed scanning of the entire chip membrane with about 19,000 collected detector frames in less  
342 than 10 minutes, resulting in an effective data acquisition rate of 33.6 images/s (see Table 1).

#### 343 Data processing / structure refinement

344 The large unit cell and the resulting small spot separation on the detector for the BEV2 crystals present  
345 a major challenge for current FEL data processing software. Furthermore, the experimental parameters  
346 have to be very well defined and to be kept constant during such a diffraction experiment. Diffraction  
347 images considered hits were isolated from the XTC streams using *cctbx.xfel*<sup>52,53</sup> according to previous  
348 protocols with adjustment of the integration windows for foreground and background subtraction and  
349 passed into the data processing pipeline *cppxfel*<sup>54</sup>. The technical challenges of indexing the BEV2  
350 diffraction patterns stimulated the development of the TakeTwo algorithm<sup>55</sup>, which was then applied  
351 to both the BEV2 and CPV18 samples. Integration, initial orientation matrix refinement and post-  
352 refinement were carried out similarly to previous publications<sup>47,54</sup>. Geometry was refined initially with  
353 *cctbx.xfel* and then further refined using the geometry algorithm in *cppxfel* using the spot predictions  
354 from the indexing solutions and the nearest peak pixel value. For BEV2, a  $2 \times 2$  foreground  
355 integration window was used to match the spot size, and care was taken to ensure the background  
356 subtraction region did not overlap with a neighboring spot. After geometry refinement, the accuracy of  
357 spot prediction allowed interpolation between pixels to be used. After post-refinement, the BEV2 data  
358 were reintegrated with the updated orientation matrix to more accurately predict the spot positions. For  
359 CPV18, the integration window was  $5 \times 5$  due to the larger spot size.

360 For the BEV2 samples 446 detector frames out of 8,812 collected images from 5 different chips were  
361 classified as possible hits (see Table 1). Out of these, 352 indexed diffraction patterns could be  
362 obtained, of which 324 diffraction patterns were included in the final dataset and used for structure  
363 refinement. Structure refinement (based on prior models) yielded model R-values of  $R_{\text{work}}/R_{\text{free}} =$   
364  $23.3/25.7\%$  for BEV2 (to  $2.3 \text{ \AA}$ , data were measurable to  $2.0 \text{ \AA}$  resolution but the resulting map was  
365 only marginally improved and the statistics were significantly worse). Since the BEV2 data were  
366 derived from only 324 crystals and had a multiplicity of only 2 and the merging statistics were  
367 correspondingly poor ( $R_{\text{split}} 0.486\%$ ,  $CC_{1/2} 0.746$ ). We therefore performed two tests to determine if the  
368 amplitudes contained enough information to determine the high-resolution structure in the absence of  
369 accurate phase information.

370 Firstly, to test whether the amplitudes were sufficiently accurate and complete to support phase  
371 determination, an initial map was calculated from phase determination, an initial map was calculated  
372 from phase information using the known BEV coordinates truncated to  $5 \text{ \AA}$ . Density modification,  
373 non-crystallographic symmetry (NCS) averaging and gradual phase extension was performed from  $5$   
374  $\text{ \AA}$  to  $2.5 \text{ \AA}$ , providing an interpretable map with clear side chain density, into which atoms were  
375 rebuilt<sup>56</sup> (Supplementary Fig. 11, which also shows the relationship between the phases derived from

376 phase extension and those derived from an averaged map derived from phases obtained from the  
377 synchrotron data). The atomic coordinates were rebuilt into the map derived from phase extension  
378 to remove bias from the model reported by Axford *et al.*<sup>4</sup>, using CNS<sup>57</sup> with strict NCS constraints.  
379 The result was an excellent map, with largely successful phase recovery (Supplementary Fig. 11).

380 Secondly, we performed molecular replacement starting from a distantly related virus (FMDV type  
381 A22). The level of sequence identity between the capsid protein of these two viruses was only  
382 19.5%. Test phases derived from the suitably placed capsid of FMDV A22 were combined with  
383 amplitudes from BEV2 (to a resolution of 2.3 Å). As expected the initial map showed significant bias.  
384 This map was then refined by cyclic density modification and NCS averaging and resulted in a high-  
385 quality electron density map (Supplementary Fig. 12).

386 For the CPV18 sample, 13,424 diffraction images out of 19,028 collected images were regarded as  
387 hits, all from one single chip. Images were indexed using the multiple lattice version of the TakeTwo  
388 algorithm, producing 16,739 indexing solutions. Up to 5 diffraction patterns could be indexed on a  
389 single image due to multiple hits (Supplementary Fig. 13). Finally, 9,293 patterns were included in the  
390 final dataset. For structure refinement of CPV18, phases were introduced from PDB code 4OTS as a  
391 template file and the structure was refined using Phenix<sup>58</sup>. The CPV18 data were measured with high  
392 redundancy (>100 fold) and were of very high quality ( $R_{\text{split}}$  9.2%,  $CC_{1/2}$  0.993). Structure refinement  
393 (based on prior models) yielded model R-values of  $R_{\text{work}}/R_{\text{free}}$  11.3/14.5 % for CPV18 (to 2.4 Å).  
394 Further data evaluation details are summarized in Supplementary Table 2. Part of the electron density  
395 map for CPV18 is shown in Supplementary Figures 10 and 11.

396 **Accession Codes:** Solved structures were deposited in the Protein Data Bank (PDB) under PDB  
397 IDs 5MQU and 5MQW for BEV2 and CPV18, respectively. The Roadrunner control software is  
398 available under doi 10.5281/zenodo.571598 (direct link: <http://doi.org/10.5281/zenodo.571598>).

399 **Contributions:** PR, TP, PF, JM, AW, and AM designed the experiment. PR, GS, KH, TSW, RD,  
400 MW, and IV were involved in sample preparation. PR, TP, GS, KH, JM, PF, RD, BR, MS, SN, DSD,  
401 RAM, CD, AW, and AM participated in data collection. PR, HMG, ASB, IDY, TMC, NKS, JR, EEF,  
402 DIS analyzed the data. PR, HMG, DIS, AW, and AM wrote the manuscript.

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- 538
- 539

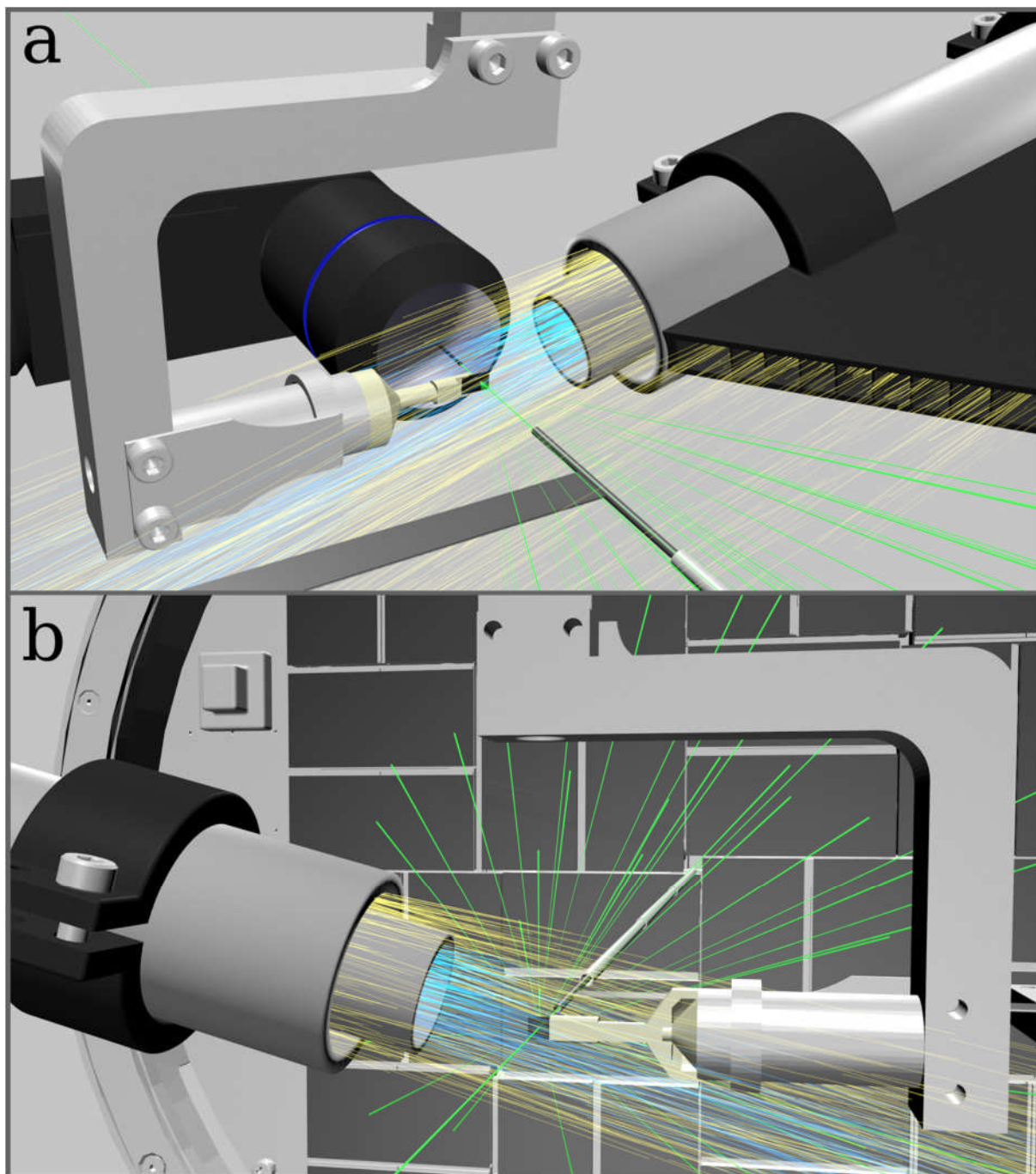
540 Table 1: Data collection parameters and hit rates for individual runs. For spot-finding parameters see  
541 Supplementary Table 1.

542

Run number	Sample	Temperature Conditions	Number of images	Acquisition time [s]	Effective acquisition rate [images/s]	Number of hits*	Hit rate [%]	Indexed
294	BEV2	RT	2280	230.7	9.9	159	7.0	124
296	BEV2	RT	180	14.8	12.2	17	9.4	16
298	BEV2	RT	1950	178.0	11.0	76	3.9	69
301	BEV2	RT	2015	180.7	11.2	150	7.4	113
303	BEV2	RT	2387	214.4	11.1	44	1.8	30
47	CPV18	100 K	19028	566.1	33.6	13424	70.5	16739

543

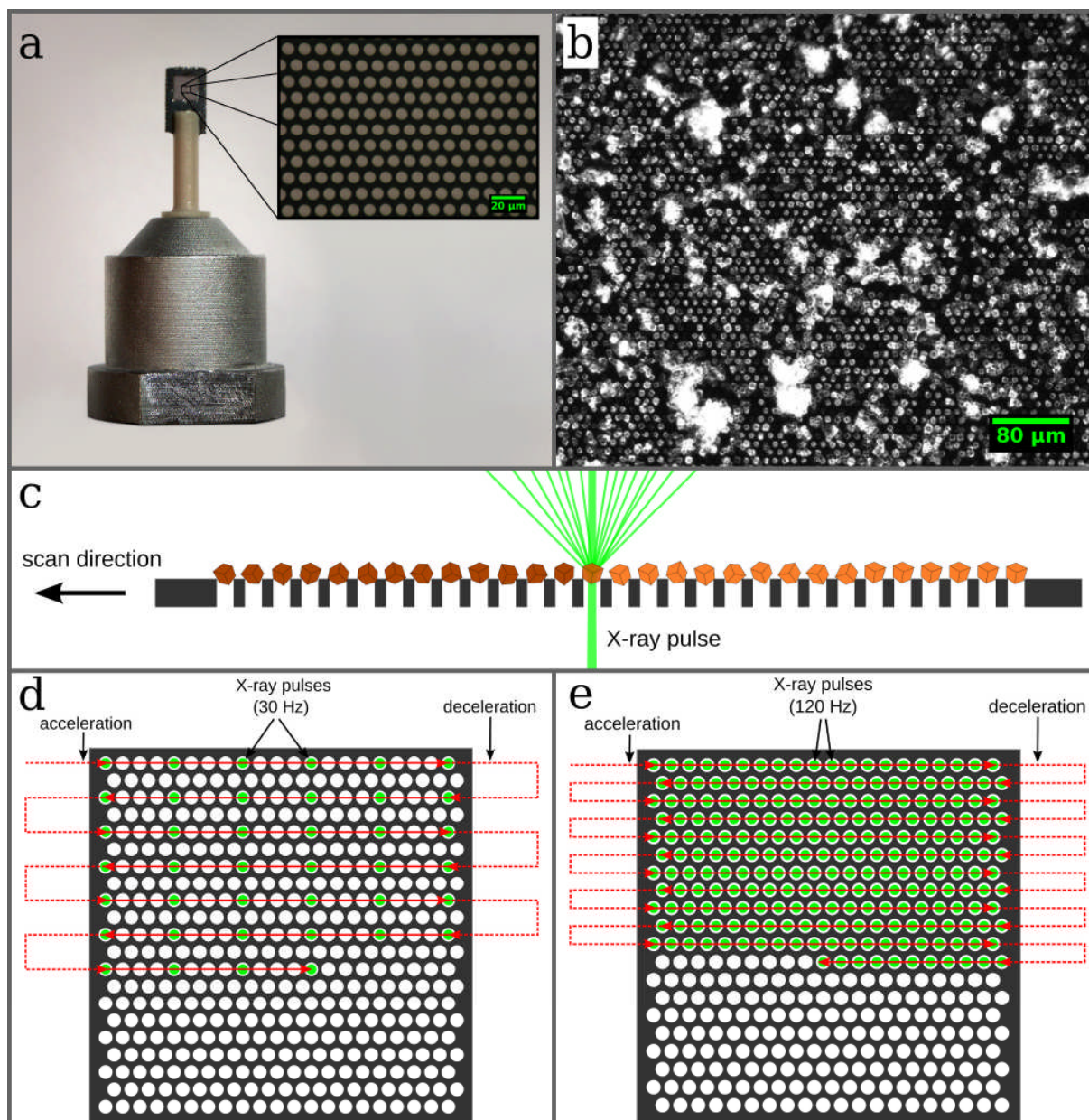
544 \*Images containing equal to or more than 50 (BEV2) and 20 (CPV18) strong spots were considered as a hit, respectively.



545

546 Figure 1: Low background experimental setup for fast fixed-target SFX experiments

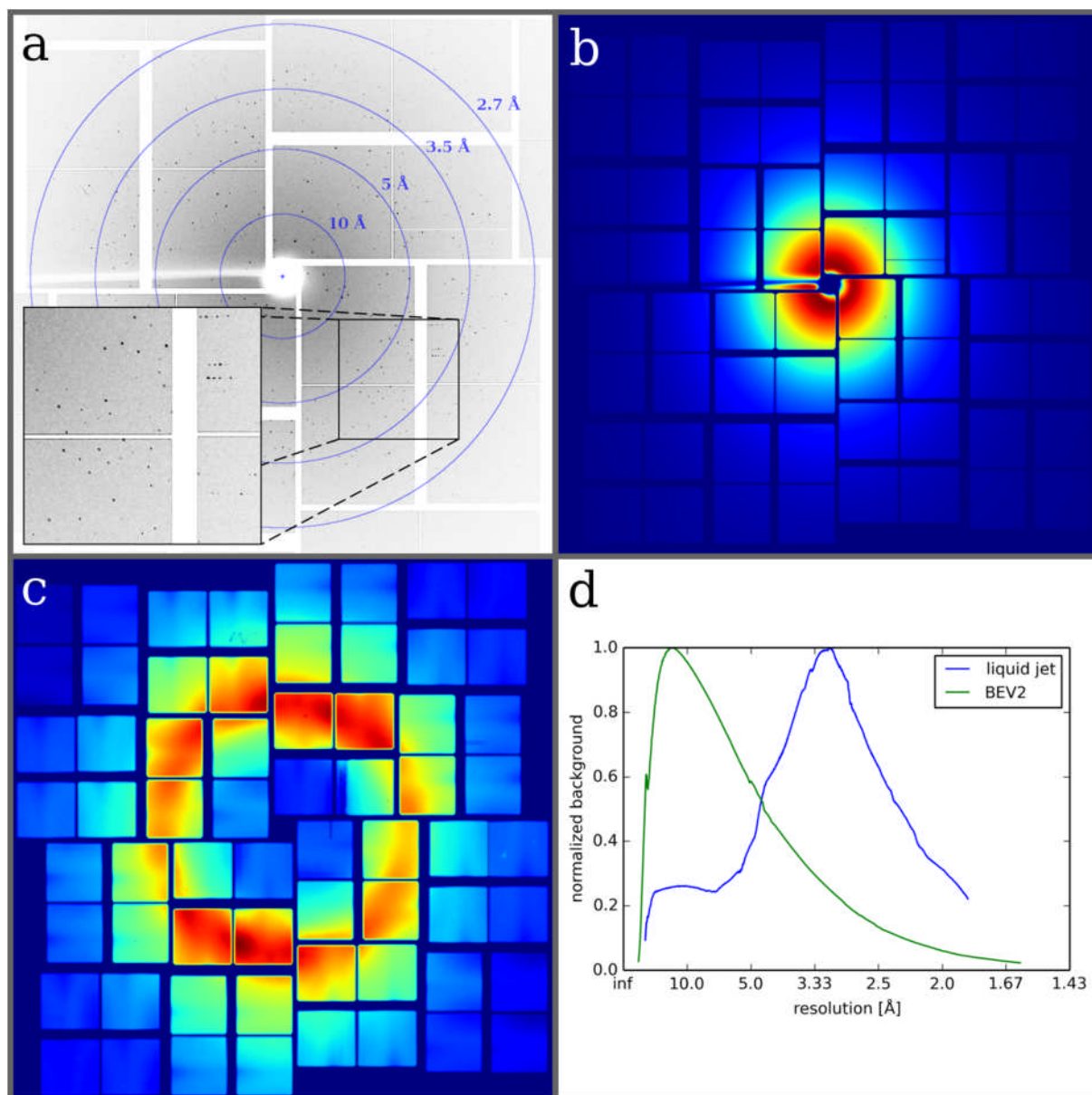
547 (a) Frontview: The silicon chip is raster scanned through the X-ray beam (green) while maintained in a  
 548 continuous stream of humidified air (blue). A helium sheath flow (yellow) is used to confine the  
 549 humidity stream and to reduce air scattering. Air scattering is further reduced by helium injection  
 550 along the beam path. An inline microscope is used for proper chip alignment and definition of the  
 551 scanning grid. (b) Backview: X-ray diffraction caused by the sample crystals is recorded with a CS-  
 552 PAD. After hitting the sample, the primary beam is enclosed by a molybdenum tubule and additional  
 553 steel tubules, which further absorb air-scattered photons. The inline microscope and gas streams are  
 554 not shown for better clarity.



555

556 Figure 2: Design of the micro-patterned silicon chip and data collection strategy.

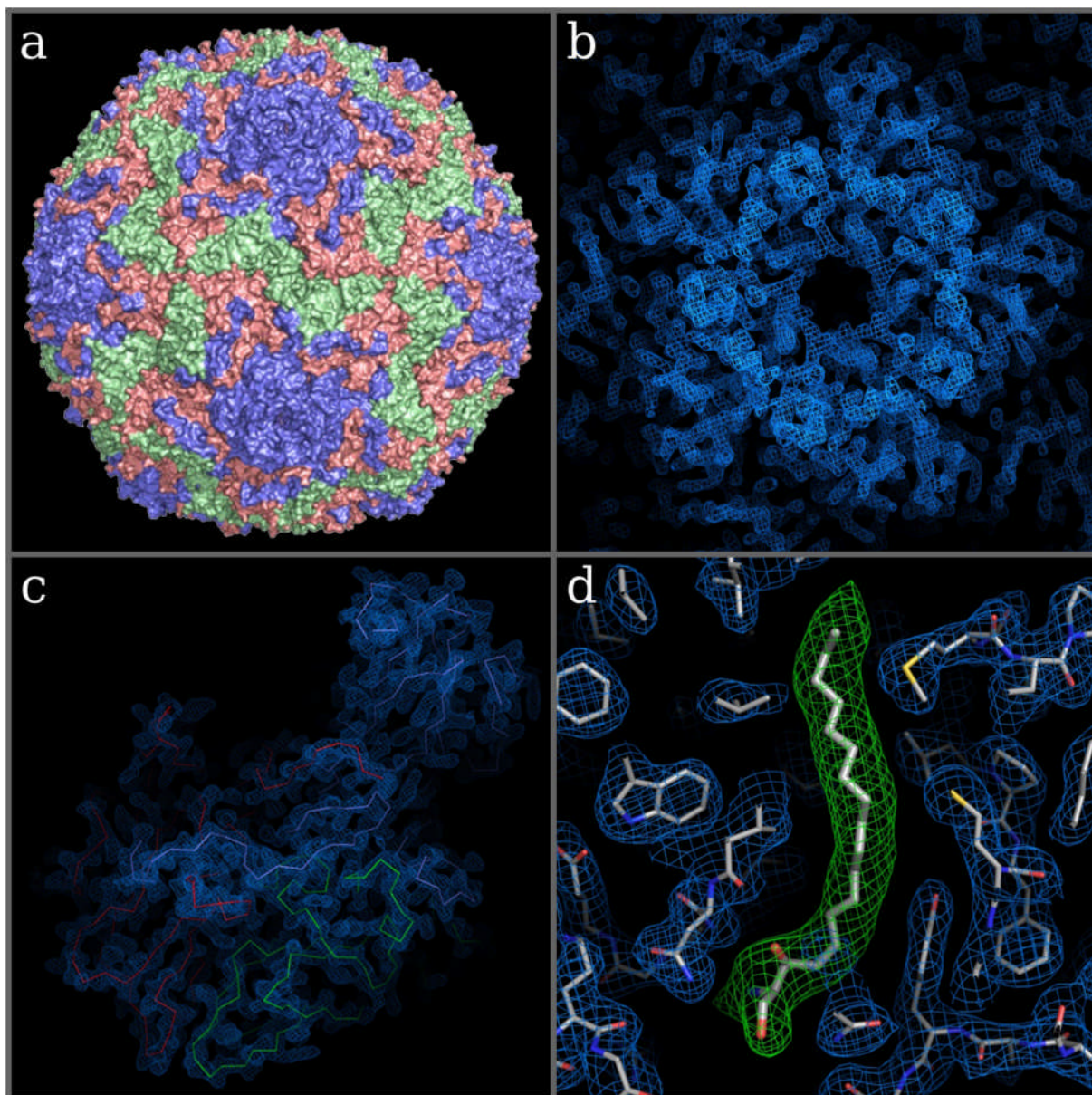
557 (a) The chip is attached to a plastic rod for the purpose of thermal isolation. The membrane part within  
 558 the outer frame consists of micropores with diameters of typically  $4\ \mu\text{m} - 8\ \mu\text{m}$ , which are arranged in  
 559 a triangular grid (a, inset). (b) This part acts as a sample holder for more than 20,000 microcrystals,  
 560 which largely organize themselves according to the pore pattern. (c) After loading, the microcrystals  
 561 are scanned through the X-ray beam. By shooting through the micropores in the chips the interaction  
 562 of the X-rays with any support material is further minimized. Subfigures (d) and (e) illustrate the  
 563 scanning strategies for measurements performed at room temperature and cryogenic temperatures,  
 564 respectively.



565

566 Figure 3: Exemplary BEV2 diffraction pattern and comparison of background scattering levels  
 567 achievable with different sample delivery methods.

568 (a) Diffraction image of BEV2 microcrystals obtained at the XPP instrument at LCLS using the micro-  
 569 patterned silicon chip as a sample holder. The low background contribution of the chip results in high-  
 570 resolution diffraction data with high signal-to-noise ratios. (b) Due to the efficient removal of  
 571 successive mother liquor during sample loading, no water ring is observed in the averaged background  
 572 image of the chip. (c) For comparison, an averaged background image from a typical SFX liquid jet  
 573 experiment with CPV 17 crystals<sup>47</sup> is shown. (d) The azimuthally averaged radial distribution of both  
 574 images is plotted as a function of resolution. The residual background of the chip is mainly caused by  
 575 air scattering, which is dominant at much lower resolutions than the water ring caused by a liquid jet.  
 576 Both curves are normalized since measurements were performed under different experimental  
 577 conditions and therefore a direct comparison was not possible.



578

579 Figure 4: Overall structure of BEV2 and corresponding high-resolution electron density maps.

580 (a) Surface representation of BEV2 particle as viewed towards an icosahedral 2-fold axis. VP1, VP2  
 581 and VP3 are shown in blue, green and red, respectively. (b-c) Electron density maps after one cycle of  
 582 5-fold real space averaging using the phases calculated from the current refined model showing the  
 583 electron density around the 5-fold in b and for a biological protomer in c. (c) C-alpha traces of VP1-3,  
 584 colored as in a. (d) A close-up view of the electron density for protein residues around the pocket  
 585 factor binding site of VP1 (blue mesh and thinner sticks) and density for the pocket factor (thicker  
 586 sticks show a sphingosine fitted to the density, while the green density is for a simulated annealing  
 587 omit map).