

1 **Heterologous Expression of Human Norovirus GII.4 VP1 Leads to Assembly of**  
2 **T=4 Virus-Like Particles**

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13 SHORT TITLE: Cryo-EM structure of GII.4 norovirus VLPs

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18 ABSTRACT

19

20 Human noroviruses are a leading cause of acute gastroenteritis, yet there are still no  
21 vaccines or antivirals available. Expression of the norovirus capsid protein (VP1) in  
22 insect cells typically results in the formation of virus-like particles (VLPs) that are  
23 morphologically and antigenically comparable to native virions. Previous structural  
24 analysis of norovirus VLPs showed that the capsid has a T=3 icosahedral symmetry  
25 and is composed of 180 copies of VP1 that are folded into three quasi-equivalent  
26 subunits (A, B, and C). In this study, we determined the cryo-EM VLP structures of  
27 two GII.4 variants, termed CHDC-1974 and NSW-2012. Surprisingly, we found that  
28 greater than 95% of these GII.4 VLPs were larger than virions and 3D reconstruction  
29 showed that these VLPs exhibited T=4 icosahedral symmetry. We found that the T=4  
30 VLPs showed several structural differences to the T=3 VLPs. The T=4 particles  
31 assemble from 240 copies of VP1 that adopt four quasi-equivalent conformations (A,  
32 B, C, and D) that form two distinct dimers, A/B and C/D. The T=4 protruding  
33 domains were elevated  $\sim 21\text{-\AA}$  off the capsid shell, which was  $\sim 7\text{-\AA}$  more than the  
34 previously studied GII.10 T=3 VLPs. A small cavity and flap-like structure at the  
35 icosahedral twofold axis disrupted the contiguous T=4 shell, a consequence of the D-  
36 subunit S-domains having smaller contact interfaces with neighboring dimers.  
37 Overall, our findings that old and new GII.4 VP1 sequences assemble T=4 VLPs  
38 might have implications for the design of potential future vaccines.

39 IMPORTANCE

40

41 The discovery that the GII.4 VLPs have a T=4 symmetry is of significance, since this  
42 represents the first known T=4 calicivirus structure. Interestingly, the GII.4 2012  
43 variant shares 96% amino acid identity with a current GII.4 VLP vaccine candidate  
44 sequence, which suggests that this vaccine might also have a T=4 symmetry. Our  
45 previous results with these GII.4 VLPs showed functional binding properties to  
46 antibodies and Nanobodies that were raised against T=3 (GII.10) VLPs. This suggests  
47 that the T=4 VLPs were antigenically comparable to T=3 particles, despite the  
48 obvious structural and size differences. On the other hand, these larger T=4 VLPs  
49 with novel structural features and possibly new epitopes might elicit antibodies that  
50 do not recognize equivalent epitopes on the T=3 VLPs. Further structural and binding  
51 studies using a library of GII.4-specific Nanobodies are planned in order to precisely  
52 investigate whether new epitopes are formed.

## 53 INTRODUCTION

54

55 Human noroviruses are members of the *Caliciviridae* family and are a leading cause  
56 of outbreaks of acute gastroenteritis. The virus has a positive sense, single stranded  
57 RNA genome of ~7.7 kbp. The genome is organized into three open reading frames  
58 (ORFs), where ORF1 encodes nonstructural proteins and ORF2 and ORF3 encode a  
59 major structural protein (termed VP1) and a minor structural protein (termed VP2),  
60 respectively. Noroviruses are genetically diverse and based on VP1 sequences have  
61 been classified into seven genogroups (GI-GVII), where GI, GII, and GIV cause  
62 infections in humans (1, 2). The GI and GII are further subdivided into numerous  
63 genotypes, with GII genotype 4 (GII.4) recognized as the most prevalent and  
64 clinically important strain (3, 4).

65

66 Recently, two human norovirus cell culture systems were developed (5, 6). However,  
67 mechanistic studies of norovirus structure and biology, such as interaction with the  
68 host receptor(s), remain challenging owing to difficulties in preparing large-scale  
69 virion preparations. Expression of ORF2 alone in insect or mammalian cells can result  
70 in the formation of virus-like particles (VLPs) that are antigenically and  
71 morphologically similar to native virions (2, 7-10). Expression of norovirus VLPs has  
72 permitted studies on host binding factors, interactions with norovirus-specific  
73 antibodies, and structural studies (2, 11-16). Indeed, histo-blood group antigens  
74 (HBGAs) and bile acids were shown to be important binding co-factors for human  
75 norovirus VLPs and/ or virions (17-22).

76

77 Structural analysis of GI.1 VLPs revealed that VP1 is separated into two distinct  
78 domains: a shell domain (S domain) that encloses the RNA and a protruding domain  
79 (P domain) that binds to co-factors, such as HGBAs and bile acids (8, 19, 21). A  
80 hinge region, which is typically composed of 10-14 amino acids, connects the S and P  
81 domains. The P domain has a  $\beta$ -barrel fold that is structurally conserved in the  
82 *Caliciviridae* family. Dimerization of the P domains forms arch shaped protrusions  
83 that are visible in electron microscopy images. The P domain is further subdivided  
84 into P1 and P2 subdomains, where the P2 subdomain is an insertion in the P1  
85 subdomain and is the most variable region on the capsid (8).

86

87 Structural studies have shown that caliciviruses have a common overall organization  
88 of T=3 icosahedral symmetry and are comprised of 180 copies of VP1 (7, 8, 23-25).  
89 Within the asymmetric unit, VP1 adopts three quasi-equivalent conformations, termed  
90 A, B, and C (8). The norovirus A and B subunits assemble into 60 dimers (termed  
91 A/B) at the quasi twofold axis, whereas the C subunits assemble into 30 C/C dimers  
92 that are located at the strict icosahedral twofold axis. For the GI.1 VLPs, the A/B  
93 dimers have a convex S domain conformation, whereas the C/C dimers have a flat S  
94 domain conformation (8). The conformational differences within these dimers likely  
95 facilitates the curvature of the virus particle to form a closed shell, such features are  
96 commonly seen in other T=3 icosahedral viruses (26). Interestingly, smaller norovirus  
97 VLPs (~25 nm in diameter) that are assumed to have a T=1 icosahedral symmetry  
98 have also been reported (22, 27); however to date, structures of these smaller VLPs  
99 have not been published.

100

101 Here, we show the cryo-EM structures of VLPs for two GII.4 variants that were  
102 identified in 1974 and 2012, termed CHDC-1974 and NSW-2012, respectively (15,  
103 28). We show that these VLPs have a T=4 icosahedral symmetry and are composed of  
104 240 copies of VP1. In order to form the T=4 icosahedral structure, VP1 adopts four  
105 quasi-equivalent conformations, termed A, B, C, and D, giving rise to two distinct  
106 types of dimers, termed A/B and C/D. The VLPs consisted of 60 A/B dimers and 60  
107 C/D dimers, with B, C, and D subunits located at the twofold axis, and the A subunit  
108 at the fivefold axis. As GII.4 VLPs are currently under consideration as vaccine  
109 candidates, our findings might have implications with respect to the likely antigenic  
110 properties of such assemblies.

## 111 MATERIALS AND METHODS

112

### 113 **VLP and virion preparation**

114 The NSW-2012 and CHDC-1974 VLPs (Genbank accession numbers JX459908 and  
115 ACT76142, respectively) were expressed in a baculovirus system as previously  
116 described (29-31). Briefly, the bacmid containing the recombinant VP1 gene was  
117 transfected in Sf9 insect cells. After incubation for five days, the culture medium was  
118 centrifuged for 10 min at 3,000 rpm at 4°C. The recovered baculovirus was  
119 subsequently used to infect Hi5 insect cells. At five days post infection, the culture  
120 medium was centrifuged for 10 min at 3,000 rpm at 4°C and then 1 h at 6,500 rpm at  
121 4°C. The VLPs in the supernatant were concentrated by ultracentrifugation at 35,000  
122 rpm (Beckman Ti45) for 2 h at 4°C and then further purified using CsCl equilibrium  
123 gradient ultracentrifugation at 35,000 rpm (Beckman SW56) for 18 h at 4°C. To  
124 remove the CsCl, the VLPs were pelleted for 2 h at 40,000 rpm (Beckman TLA55) at  
125 4°C and subsequently resuspended in PBS (pH 7.4). GII.4 virions in stool were also  
126 purified using this centrifugation technique, except for the CsCl gradient step.

127

### 128 **Negative stain electron microscopy**

129 The integrity of the VLPs was confirmed using negative stain electron microscopy  
130 (EM). The VLPs were diluted 1:30 in distilled water and applied to EM grids. The  
131 grids were washed with distilled water, stained with 0.75% uranyl acetate, and the  
132 excess uranyl acetate was removed with filter paper. Virion samples were applied to  
133 EM grids, washed with water, fixed with 4% glutaraldehyde, and then stained as  
134 above. EM images were acquired on a Zeiss 910 electron microscope at 50,000×  
135 magnification.

136

137 **Cryo-EM data sample preparation and data collection**

138 UNSW-2012 and CHDC-1974 VLPs (3  $\mu$ l) were applied to freshly glow discharged  
139 Quantifoil holey carbon support films (R1.2/1.3) and blotted for 18 seconds at 100%  
140 humidity and 10°C before being plunged in liquid ethane using an FEI Mark IV  
141 Vitrobot (Thermo Fischer Scientific). Vitrified specimens were imaged on a Titan  
142 Krios microscope (Thermo Fisher scientific) operated at 300 keV. NSW-2012  
143 micrographs were acquired with a K2 direct electron detector with Latitude S  
144 software (Gatan) at 64,000 $\times$  magnification corresponding to a pixel size of 2.27  $\text{\AA}$ /px,  
145 while CHDC-1974 micrographs were collected using a K3 direct electron detector at  
146 64,000 $\times$  magnification, corresponding to a pixel size of 1.375  $\text{\AA}$ /px.

147

148 **Cryo-EM data processing**

149 Initially, the movies containing 16 frames for NSW-2012 and 40 frames for CHDC-  
150 1974 were motion corrected using motioncor2 software (32) and defocus estimation  
151 was performed using ctfind 4.1 software (33). All further image-processing steps  
152 were performed using Relion 2.1 software for NSW-2012 and cryoSPARC software  
153 for CHDC-1974 (34, 35). An initial set of 1,000 particles was manually picked for 2D  
154 classification to produce averages suitable as references for automated particle  
155 picking. The autopicked particles were sorted in a 2D classification step and the best  
156 particles were used for calculation of an initial starting model, followed by 3D  
157 classification. A subset of particles that generated the highest resolution was selected  
158 for further refinement. The 3D refinement and post-processing of NSW-2012 from  
159 10,548 T=4 particles produced a final map at 7.3- $\text{\AA}$  resolution with icosahedral (I2)  
160 symmetry imposed (0.143 FSC cutoff). Smaller NSW-2012 T=3 particles were



161 manually picked and sorted in 2D classification. In total, 391 particles were selected  
162 for further 3D structure determination. Refinement of these smaller NSW-2012 T=3  
163 particles yielded a map of 15-Å resolution. For CHDC-1974, a subset of 42,485  
164 particles for refinement led to the calculation of a map at 6.1-Å resolution using the  
165 0.143 FSC cutoff. Cryo-EM T=4 VLP structures for CHDC-1974 (accession number:  
166 EMD-4549) and NSW-2012 (EMD-4550) were deposited at EMDB. The cryo-EM  
167 VLP structure for NSW-2012 with T=3 icosahedral symmetry is available on request.

168

### 169 **Fitting of the X-ray structures into the density maps**

170 Crystal structures of NSW-2012 P domain (4OOS) and CHDC-1974 P domain  
171 (5IYN) were fitted into the respective densities using the “fit in map” command in the  
172 UCSF Chimera software (36). Since a high-resolution GII.4 shell domain structure  
173 was unavailable, the GI.1 Norwalk virus S domain was extracted from the X-ray  
174 crystal structure (1IHM) and fitted into the GII.4 cryo-EM densities using UCSF  
175 Chimera software.

## 176 RESULTS AND DISCUSSION

177

178 The purpose of this study was to analyze GII.4 VLP architecture and compare these  
179 assemblies to the previously solved GI.1 and GII.10 VLP structures. The NSW-2012  
180 VP1 sequence had a single amino acid insertion at position ~394 (NSW-2012  
181 numbering) compared to CHDC-1974 (Fig. 1). Overall, NSW-2012 and CHDC-1974  
182 shared 89% amino acid identity, with most (45 of 54) amino acid substitutions  
183 occurring in the P domain. Negative stain EM images revealed that the VLPs  
184 exhibited characteristic norovirus morphology (Fig. 2). However, the diameter of  
185 these VLPs was measured to be ~52 nm, which suggested that the GII.4 VLPs were  
186 larger than GII.10 and GI.1 VLPs that had diameters of ~43 nm and ~38 nm,  
187 respectively.

188

### 189 **Cryo-EM structure of NSW-2012 T=4 VLPs**

190 The structure of the NSW-2012 VLPs was determined using cryo-EM and 3D  
191 icosahedral image reconstruction. The VLPs were mono-disperse in vitreous ice and  
192 appeared mostly homogenous in size (Fig. 3A). From the 364 images, 10,548 particles  
193 were used for image reconstruction and refined to 7.3-Å resolution (Fig. 3C).  
194 Unexpectedly, NSW-2012 VLPs were discovered to have T=4 icosahedral symmetry  
195 (Fig. 4). Our data revealed that these VLPs were composed of 240 copies of VP1,  
196 rather than 180 VP1 copies as in GI.1 and GII.10 VLPs. The inner diameter of NSW-  
197 2012 shell was measured as 32 nm, while the outer capsid diameter was 50 nm. The  
198 larger diameter of the T=4 VLPs corresponded to a capsid volume of ~12,724 nm<sup>3</sup>,  
199 which was ~2.1 times the volume of the GII.10 VLPs that had an inner diameter of 23  
200 nm, corresponding to a volume of ~5,985 nm<sup>3</sup> (7).

201

202 Another interesting structural feature of these NSW-2012 VLPs was a small cavity  
203 and flap-like structure on the contiguous shell (Fig. 5). This feature was associated  
204 with the S domain and found on opposing sides at the twofold axis. This feature may  
205 arise as a consequence of the C/D dimer having a stronger curvature, preventing the  
206 S-domain from forming the expected contacts with neighboring B and C-type  
207 subunits. Alternatively, there might be insufficient space to allow the D subunit S-  
208 domain to pack, such that it lies in the plane of the shell without causing steric  
209 collision with neighboring subunits.

210

211 Structural analysis of the T=4 VLPs indicated that VP1 adopted four quasi-equivalent  
212 conformations, termed A, B, C, and D. These four subunit conformations formed two  
213 distinct dimer classes: A/B and C/D (Figs. 4 and 6). The B, C, and D subunits  
214 alternated about the two-fold symmetry axis, while the A subunit was positioned at  
215 the fivefold axis (Fig. 4). The T=4 VLPs were composed of 60 A/B dimers and 60  
216 C/D dimers, which was distinct from the T=3 VLPs that have been shown to be  
217 assembled from 60 A/B and 30 C/C dimers. We also observed that both A/B and C/D  
218 dimers had a convex S domain conformation, which was in contrast to the GI.1 VLPs  
219 that consisted of both convex (A/B) and flat (C/C) dimers (8).

220

221 In order to better comprehend how VP1 assembled into the T=4 VLPs, the X-ray  
222 crystal structure of NSW-2012 P domain (4OOS) and GI.1 S domain (1IHM) were  
223 fitted into the VLP density map. We found that the NSW-2012 P domain dimer could  
224 be unambiguously positioned into the VLP density, with a cross correlation  
225 coefficient of 0.96 (Fig. 7A). This result indicated that the P domain dimers on the

226 T=4 VLPs had not undergone any major structural modifications. In the case of the S  
227 domain, the GI.1 S domain needed to be manually positioned into the density map.  
228 The GI.1 S domain fitted well into to A/B dimer and the C subunit, while the D  
229 subunit needed to be further repositioned (Fig. 7B). This additional fitting in the D  
230 subunit was necessary in order to occupy the elevated density of the flap-like regions.

231

232 Unfortunately, it was problematic to fit the hinge region, since the hinge region on the  
233 X-ray crystal structure of NSW-2012 P domain was excluded from the expression  
234 construct and the hinge region of the GI.1 VLPs was flattened (7, 8). Interestingly, an  
235 apparent point of contact was also observed between the S domain and the C-terminus  
236 of the P domain on the D subunit (Fig. 8). This may stabilize the convex conformation  
237 of the C/D dimer and possibly the T=4 VLPs. Indeed, the C-termini of VP1 on the  
238 GI.1 VLPs and the GII.4 P domain were found to be flexible (8, 37). Moreover, the C-  
239 terminus of VP1 was previously shown to be important for the size and stability of  
240 VLPs (38).

241

242 Another interesting feature that we observed with the T=4 VLPs was the raised P  
243 domains (Figs. 4 and 8). We found that the T=4 P domain was elevated  $\sim 21$ -Å off the  
244 shell by an extended hinge region, which was higher than the P domains on the GII.10  
245 VLPs, which were raised  $\sim 14$ -Å (7). The hinge region in NSW-2012 and GII.10 (7)  
246 were both  $\sim 10$  amino acids and mainly conserved (39). This result suggested that the  
247 raised P domains might be a structural feature of GII noroviruses, since the P domains  
248 on the GI.1 VLPs were essentially resting on the shell (8).

249

250 **Cryo-EM structure of NSW-2012 T=3 VLPs**

251 To determine the structure of a recent GII.4 isolate (NSW-2012), 10,548 particles  
252 were imaged by cryo-EM, and a 3D image reconstruction was calculated.  
253 Unexpectedly, this analysis revealed a previously unknown T=4 assembly. In this  
254 preparation however, a very small number of particles (391 particles) appeared  
255 smaller (~46 nm) than the ~50 nm VLPs (Fig. 4A). Reconstruction of these particles  
256 at ~15-Å resolution revealed the expected T=3 icosahedral symmetry (Fig. 9). At this  
257 resolution, the hinge region was not visible. Also, the cavity and flap-like structures  
258 that characterized the T=4 VLP were not present. Based on the T=3 geometry, these  
259 VLPs were likely composed of 180 copies of VP1, however the VP1 dimers or  
260 subunits were not clearly resolved. Overall, these results indicated that NSW-2012  
261 VP1 formed VLPs of which ~5% of the population (391 of 10,548) were T=3  
262 particles. This proportion was confirmed over several VLP preparations.  
263 Unfortunately, owing to the low numbers it remains challenging to improve on the  
264 achieved resolution, since only few T=3 VLPs were expressed and were not able to be  
265 separated from the T=4 VLPs using CsCl or sucrose gradient ultracentrifugation.

266

#### 267 **Cryo-EM structure of GII.4 CHDC-1974 T=4 VLPs**

268 To test whether assembly of VLPs with T=4 icosahedral symmetry is a property of  
269 VP1 from all GII.4 strains, we proceeded to determine the cryo-EM structure of VLPs  
270 produced by VP1 of CHDC-1974. The VLPs were mostly mono-disperse and  
271 homogenous in size (Fig. 10A). From 591 images, 42,485 particles were processed to  
272 calculate a reconstruction with a final resolution of 6.1-Å (Fig. 10B). The CHDC-  
273 1974 VLPs also had T=4 symmetry (Fig. 11) and their structure closely resembled  
274 that of NSW-2012 VLPs. Unlike NSW-2012 however, CHDC-1974 VP1 only  
275 assembled into T=4 VLPs and no T=3 VLPs were identified.

276

277 We found that CHDC-1974 T=4 VLPs were also composed of 240 copies of VP1 that  
278 formed the quasi-equivalent subunits A, B, C, and D and A/B and C/D dimeric  
279 capsomeres. The inner diameter of the shell was 32 nm, whereas the outer diameter of  
280 the capsid was 50 nm. As in NSW-2012 VLPs, cavity and flap-like structures were  
281 also present on the CHDC-1974 VLPs (Fig. 12). The CHDC-1974 A/B and C/D  
282 dimers showed a similar convex conformation as NSW-2012 dimers, although  
283 slightly less pronounced (Fig. 13). The X-ray crystal structure of CHDC-1974 P  
284 domain (5IYN) was easily fitted into the CHDC-1974 VLP density map (Fig. 14A).  
285 The GI.1 S domain also fitted into the A, B, and C subunits, whereas the GI.1 S  
286 domain was repositioned to occupy the D subunit density (Fig. 14B). Similar to  
287 NSW-2012 VLPs, a possible contact interface was observed between the S and P  
288 domains on the D subunit (Fig. 15). Lastly, we found that CHDC-1974 P domain was  
289 also lifted off the shell ~21-Å by an extended hinge region (Figs. 11 and 15).

290

291 Overall, these results showed that GII.4 VP1 sequences isolated over three decades  
292 apart remained structurally conserved. This could imply that other GII.4 VP1  
293 sequences would also form T=4 VLPs when expressed in insect cells, especially since  
294 these two sequences had only 89% amino acid identity.

295

### 296 **Previous binding studies using GII.4 capsids**

297 GII.4 VLPs and their corresponding P domains have been extensively examined for  
298 binding to co-factors (HBGAs and bile acid), antibodies, human milk  
299 oligosaccharides (HMOs), and Nanobodies (21, 31, 37, 39-41). NSW-2012 P domains  
300 were capable of binding numerous HBGA types and the VLPs cross-reacted with

301 Nanobodies and antibodies that were raised against other genotypes or GII.4 variants.  
302 However, the NSW-2012 VLPs did not bind bile acid and poorly bound HMOs  
303 (unpublished). Overall, these results suggested that T=4 VLPs were indeed  
304 antigenically relevant and capable of functioning similarly to GII.4 virions with  
305 respect to antigenicity and HBGA interactions.

306

### 307 **GII.4 VLPs in vaccine trials**

308 VLP vaccines against norovirus that are currently in clinical trials use a combination  
309 of GI.1 VLPs and modified GII.4 VLPs (termed GII.4c) (42, 43) Negative stain EM  
310 images of these GII.4c VLPs showed the typical norovirus morphology. However, the  
311 size determination and the structure are not available. Interestingly, GII.4c and NSW-  
312 2012 shared 94% amino acid identity with most substitutions (28 of 31) located in the  
313 P domain (Fig. 1). Therefore, it is tempting to speculate that GII.4c VP1, which  
314 closely matched NSW-2012 VP1 sequence, might also form T=4 VLPs particles. In  
315 both VLPs, VP2 is not present.

316

317 To test whether GII.4 virions are T=3 or T=4 assemblies, we used negative stain EM  
318 of authentic GII.4 virions. These virion images revealed a smaller diameter of ~44 nm  
319 compared to the T=4 VLPs (~50 nm) (Fig. 16). This size corresponds to the diameter  
320 determined for the NSW-2012 T=3 VLPs and indicates that GII.4 virions likely  
321 exhibit T=3 icosahedral symmetry.

322

323 In general, studies have shown that norovirus-specific antibody titers were raised after  
324 vaccination with VLPs, but the levels of protection were not strongly improved  
325 compared to placebo groups (44). This might indicate that vaccination leads to

326 production of neutralizing antibodies against epitopes on T=4 VLPs that are not  
327 accessible on T=3 virions (i.e., when challenged) and that therefore vaccine efficacy  
328 could be lowered by a sub-optimal antigen. Clearly, further studies are needed in  
329 order to determine whether the GII.4c VLPs form T=4 particles and if the T=4 VLPs  
330 are antigenically identical to T=3 VLPs.

331

### 332 **Summary**

333 We have shown that upon heterologous expression of human norovirus GII.4 VP1 in  
334 insect cells, VLPs are formed that adopt T=4 icosahedral symmetry. This is at odds  
335 with the likely T=3 symmetry virions encoded by this virus. There are two important  
336 consequences of this outcome. Firstly, the assembly of GII.4 T=4 VLPs may impact  
337 on results from previous studies in which it was assumed that GII.4 norovirus VLPs  
338 were morphologically similar to virions. Secondly, the cavity and flap-like structures  
339 on the T=4 could elicit antibodies that are not capable of recognizing T=3 virions.  
340 Further structural and binding studies using a library of GII.4-specific Nanobodies are  
341 planned in order to investigate these novel epitopes.



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352 FIGURE LEGENDS

353

354 **Figure 1. Amino acid sequence alignment of GII.4 VP1.** NSW-2012 (JX459908),  
355 CHDC-1974 (ACT76142), and GII.4c (42) VP1 amino acid sequences were aligned  
356 using ClustalX. The S domain (orange), hinge region (green), P1 subdomain (light  
357 blue), and P2 subdomain (navy) were labeled accordingly. Compared to CHDC-1974,  
358 NSW-2012 and GII.4c VP1 had a single amino acid insertion (arrow) at position 394  
359 (NSW-2012 numbering). The S domain and hinge region were mainly conserved,  
360 whereas most amino acid substitutions were located in the P2 subdomain.

361

362 **Figure 2. EM images of GI and GII VLPs.** Negative stain EM images of norovirus  
363 VLPs show the characteristic norovirus virion morphology (50,000× magnification).  
364 The diameter of the CHDC-1976 and NSW-2012 VLPs was ~52 nm, whereas the  
365 diameter of GI.1 West Chester (AY502016.1) and GII.10 Vietnam026 (AF504671)  
366 VLPs were ~38 nm and ~43 nm, respectively (45, 46). The bar represents 100 nm.

367

368 **Figure 3. NSW-2012 cryo-EM data processing.** (A) A representative cryo-EM  
369 micrograph of NSW-2012 VLPs at 64,000× magnification. The blue and green arrows  
370 show examples of VLPs measuring ~46 nm and ~50 nm, respectively. The scale bar  
371 represents 100 nm. B) 2D classification of GII.4 NSW-2012 T=4 VLPs. (C) Gold  
372 standard FSC plot of the icosahedral reconstruction of NSW-2012 indicates a  
373 resolution of 7.3-Å.

374

375 **Figure 4. Cryo-EM reconstruction structure of NSW-2012 T=4 VLPs.** The left  
376 side shows NSW-2012 VLPs have a T=4 icosahedral symmetry (symmetry axis

377 labeled 2, 3, and 5). These VLPs were composed of 240 copies of VP1 and VP1  
378 adapted four quasiequivalent conformations (A, B, C, and D) that gave rise to two  
379 distinct dimers (A/B and C/D). At the icosahedral twofold axis, the B, C, and D  
380 subunits were alternating, while the A subunits are positioned at the fivefold axis. The  
381 right side shows a cutaway section of these VLPs and indicates that the inner and  
382 outer diameters are 32 nm and 50 nm, respectively. The P domains are elevated  $\sim 21$  Å  
383 off the S domain.

384

385 **Figure 5. NSW-2012 T=4 VLPs shows several new structural features.** The cavity  
386 and flap-like structures are observed at the twofold axis and are found on opposing  
387 sides. The cavity and flap-like structures are associated with the S domain on the D  
388 subunit.

389

390 **Figure 6. NSW-2012 T=4 VLPs are formed with 60 A/B and 60 C/D VP1 dimers.**  
391 The A/B and C/D dimers show an equivalent convex conformation of the S domain.  
392 An additional connection was also observed between the D subunit of the S and P  
393 domain.

394

395 **Figure 7. The X-ray crystal structures of NSW-2012 P domain and GI.1 S**  
396 **domain were fitted into the T=4 VLP density map.** (A) The X-ray crystal structure  
397 of NSW-2012 P domain (4OOS, cartoon) could be fitted into the A/B and C/D P  
398 domain dimers, indicating little conformational change. (B) The X-ray crystal  
399 structure of the GI.1 S domain (1IHM, cartoon) fitted into the A/B and C/D S domain  
400 dimers. However, the cavity and flap-like structures on the D subunit suggests a large  
401 conformational change compared to typical T=3 particles.

402

403 **Figure 8. A close-up view of NSW-2012 C/D dimer.** The fitted X-ray crystal  
404 structures of the GI.1 S domain (cartoon) and the GII.4 P domain (cartoon) into the  
405 cryo-EM map shows the how the hinge region connects the S and P domains. Also,  
406 the new connection between the S domain and the C-terminus of the P domain is  
407 shown. The asterisk represents the missing hinge region on the X-ray crystal  
408 structures that connects of the S and P domains for the C subunit (blue) and D subunit  
409 (red).

410

411 **Figure 9. Cryo-EM reconstruction structure of NSW-2012 T=3 VLPs.** (A) 2D  
412 classification of 1780 manually picked particles. The first class (red circle) shows  
413 particles of a smaller diameter, compared to averages of other classes. These smaller  
414 particles were used for further refinement. (B) FSC plot of the icosahedral  
415 reconstruction of T=3 VLPs indicates a resolution of 15-Å. (C) The left side shows  
416 NSW-2012 exhibiting T=3 icosahedral symmetry (symmetry axis labeled 2, 3, and 5).  
417 These VLPs were composed of 180 copies of VP1 that forms A/B and C/C dimers.  
418 The cutaway section (right) shows the inner and outer diameter of the particle, which  
419 measured 24 and 46 nm, respectively. At this resolution the hinge region could not be  
420 resolved, but the large gap between S and P domain indicates that the P domains are  
421 raised up from the shell.

422

423 **Figure 10. CHDC-1974 cryo-EM data processing.** (A) A representative cryo-EM  
424 micrograph of CHDC-1974 VLPs at 64,000× magnification. The scale bar represents  
425 100 nm. (B) 2D classification of CHDC-1974 particles. (C) FSC plot of the

426 icosahedral reconstruction of CHDC-1974 indicates a resolution of 6.1-Å at 0.143  
427 cutoff.

428

429 **Figure 11. Cryo-EM structure and analysis of CHDC-1974 VLPs.** The image on  
430 the left side shows that CHDC-1974 VLPs has a T=4 icosahedral symmetry  
431 (symmetry axis labeled 2, 3, and 5) and was composed of 240 copies of VP1. The  
432 VP1 exhibited four quasiequivalent conformations (A, B, C, and D) that gave rise to  
433 two distinct dimers (A/B and C/D). At the icosahedral twofold axis, the B, C, and D  
434 subunits were alternating, while the A subunit was located around the fivefold axis.  
435 The right side shows a cutaway section of these VLPs and indicates that the inner and  
436 outer diameters are 32 nm and 50 nm, respectively. The P domains are elevated ~21 Å  
437 off the S domain.

438

439 **Figure 12. CHDC-1974 T=4 VLPs shows several new structural features.** The  
440 cavity and flap-like structures are observed at the twofold axis and are found on  
441 opposing sides. The cavity and flap-like structures are associated with the S domain  
442 on the D subunit.

443

444 **Figure 13. CHDC-1974 T=4 VLPs are formed with 60 A/B and 60 C/D VP1**  
445 **dimers.** The A/B and C/D dimers show an equivalent convex confirmation on the S  
446 domain. Also, the additional connection between the D subunit of the S and P domain  
447 was found on the CHDC-1974 T=4 VLPs.

448

449 **Figure 14. The X-ray crystal structures of CHDC-1974 P domain and GI.1 S**  
450 **domain were fitted into the VLP density map.** (A) The X-ray crystal structure of

451 NSW-2012 P domain (5IYN, cartoon) easily fitted into the A/B and C/D P domain  
452 dimer densities. (B) The X-ray crystal structure of the GI.1 S domain (1IHM, cartoon)  
453 fitted into the A/B and C/D S domain dimers. However, the cavity and flap-like  
454 structures on the D subunit suggests a large conformational change from typical T=3  
455 particles.

456

457 **Figure 15. A close-up view of CHDC-1974 C/D dimer.** The fitted X-ray crystal  
458 structures of the GI.1 S domain (cartoon) and the GII.4 P domain (cartoon) into the  
459 cryo-EM map shows the how the hinge region connects the S and P domains. Also,  
460 the new connection between the S domain and the C-terminus of the P domain is  
461 shown. The asterisk represents the missing hinge region on the X-ray crystal  
462 structures that connects of the S and P domains for the C subunit (blue) and D subunit  
463 (red).

464

465 **Figure 16. EM images of GII.4 virions.** Negative stain EM images of GII.4 virions  
466 show that the virions exhibit a smaller diameter (~44 nm) than GII.4 VLPs expressed  
467 in insect cells that were ~52 nm (see Figure 2).

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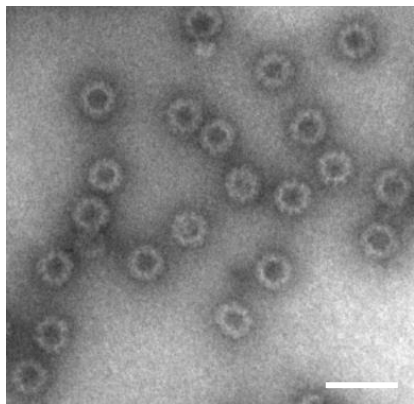
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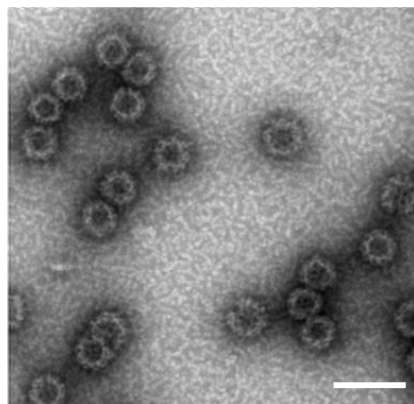
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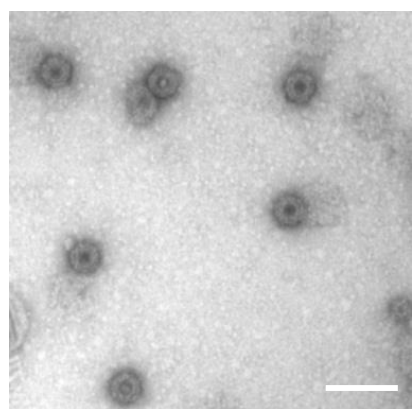
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CHDC-1974	1	.....T.....I.....	60
GII.4c	1	.....T.....	60
		*****	
NSW-2012	61	GGFTVSPRNAPGEILWSAPLGPLNPLYSHLARMYNGYAGGFVQVILAGNAFTAGKVI	120
CHDC-1974	61	.....S.....S.....I.....	120
GII.4c	61	.....I.....	120
		*****	
NSW-2012	121	FAAVPPNFPTEGLSPSQVTMFPHIVVDVRQLEPVLIPDPVRNRFYHYNQSNPTIKLIA	180
CHDC-1974	121	...I.....I.....AH.S.L...	180
GII.4c	121	.....I.....	180
		*** ***** *	
NSW-2012	181	MLYTPLRANNAGDDVFTVSCRVLTRPSPDFDFIFLVPPTVESRTKPFVSPVLTVEEMTNS	240
CHDC-1974	181	.....T..I.....S..	240
GII.4c	181	.....T..I.....	240
		***** *	
NSW-2012	241	RFPIPLEKLFSGPSSAFVVPQNGRCTTDGVLGTTQLSPVNICTFRGDVTHITGSRNYT	300
CHDC-1974	241	.....Y.....A...N.....RVGI..HD..	300
GII.4c	241	.....G.....A.TQE..	300
		***** ** *	
NSW-2012	301	MNLASQNWNDYDPTTEEIPAPLGTPDFVKGVIQGVLTQTTRTDGSTRGHKATVYTGSADFAP	360
CHDC-1974	301	...V...N.....L.....A...A...S...VH.T.	360
GII.4c	301	.....N.....G.....S...VH.T.	360
		*** ***** ** *	
NSW-2012	361	KLGRVQFETDTRDFEANQNTKFTPVGVIQDGGTTHRNEPQQWVLPYSYGRNTHNVHLAP	420
CHDC-1974	361	...S...T...NN..QTG.....DH-.Q.....N...TSG.....	419
GII.4c	361	...S...S...SN...TG.....V...S...Q.....D...DS.....	420
		*** ** * * ***** * ***** *	
		↑	
NSW-2012	421	AVAPTFPGEQLLFFRSTMPGCSGYPNMDLDCLLPQEWVQYFYQEAPASDVALLRVNP	480
CHDC-1974	420	.....N.....SH.....	479
GII.4c	421	.....N.....H.....	480
		*****	
NSW-2012	481	DTGRVLFECKLHKSGYVTVAHGTGQHDLVIPPNGYFRFDSWVNQFYTLAPMNGTGRRRAV	540
CHDC-1974	480	.....I.....PY.....S...K.VL	539
GII.4c	481	.....L	540
		***** *	



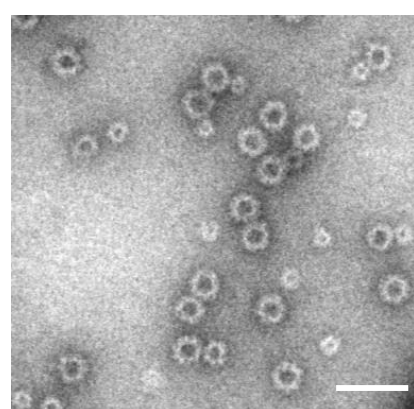
GII.4 NSW-2012



GII.4 CHDC-1974

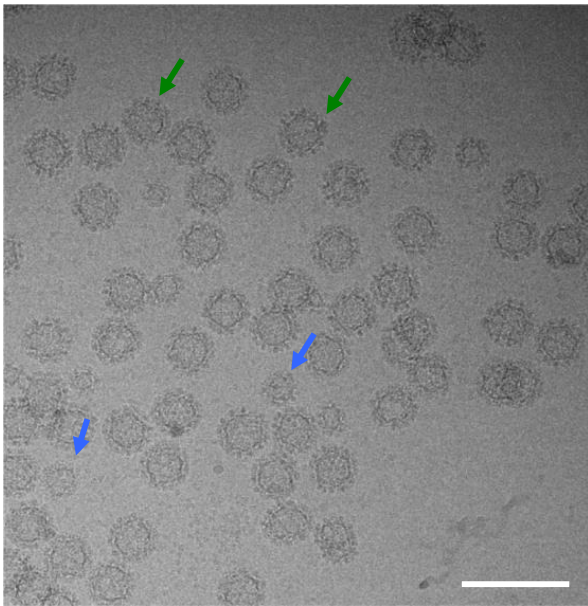


GI.1 West Chester

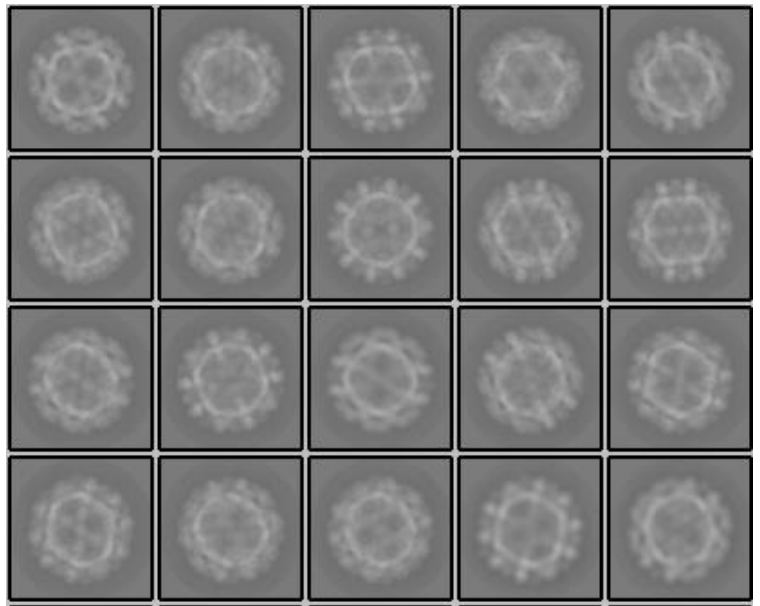


GII.10 026-Vietnam

A



B



C

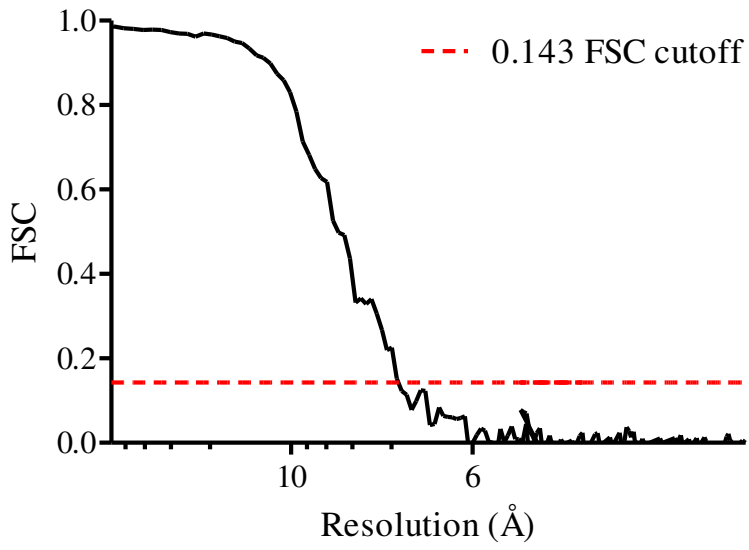


Figure 4

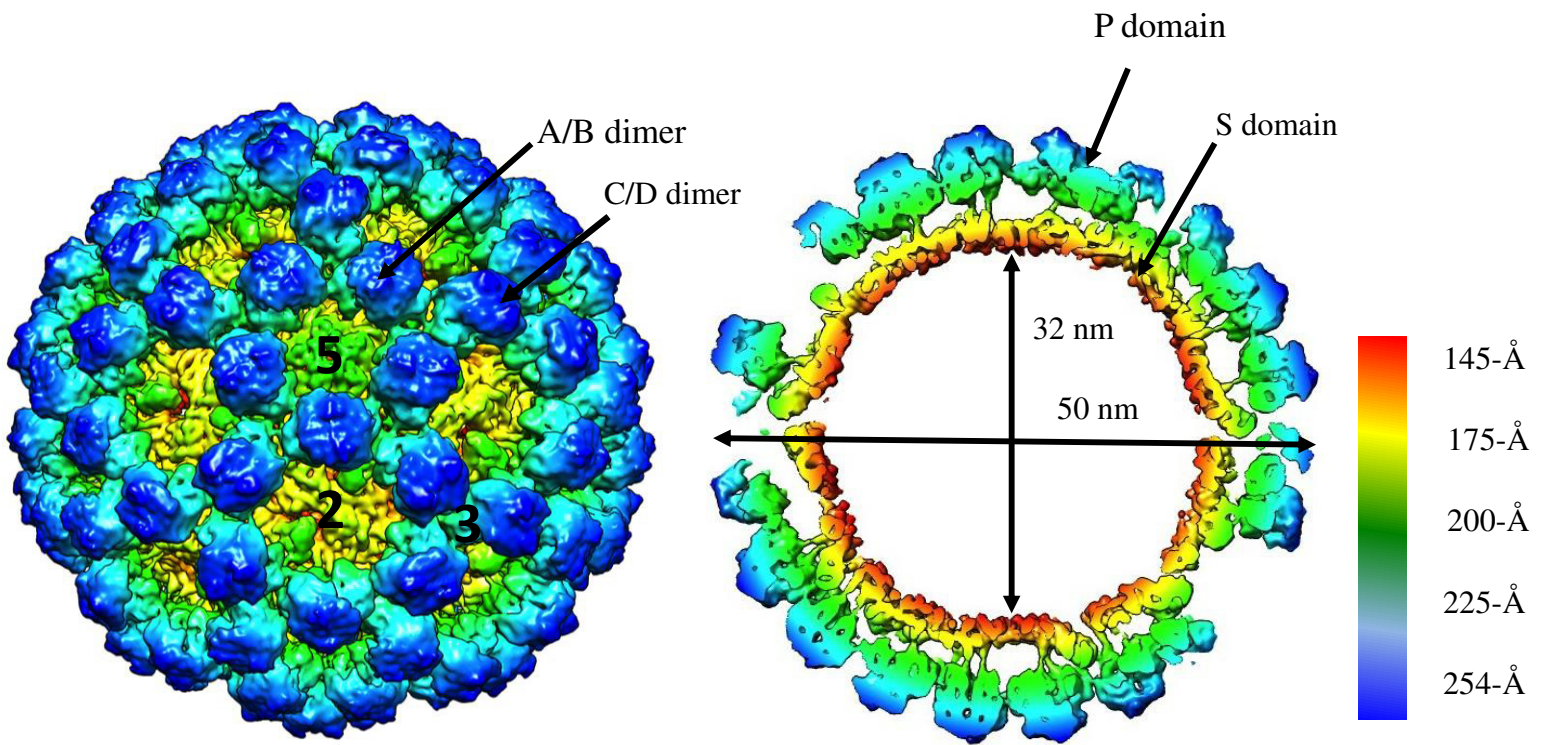
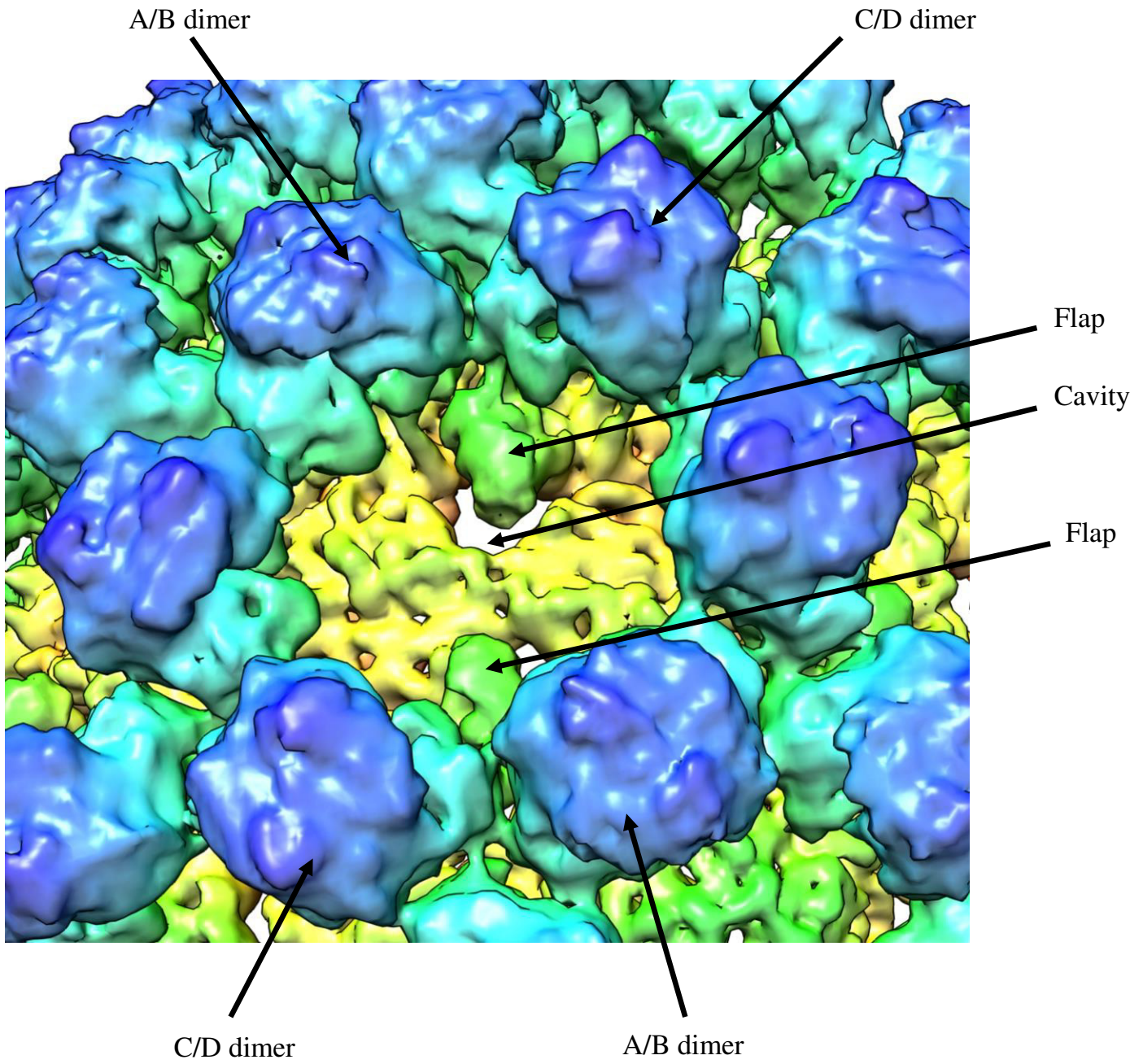
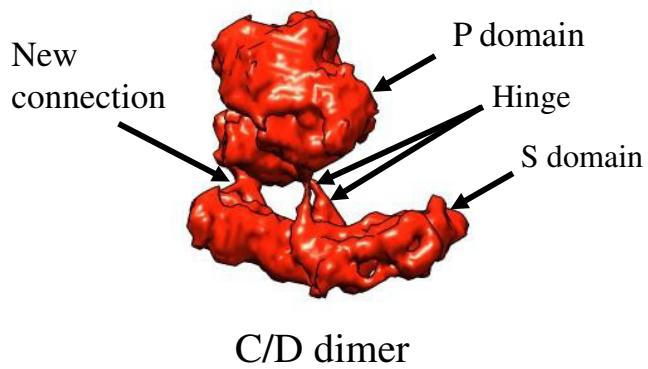
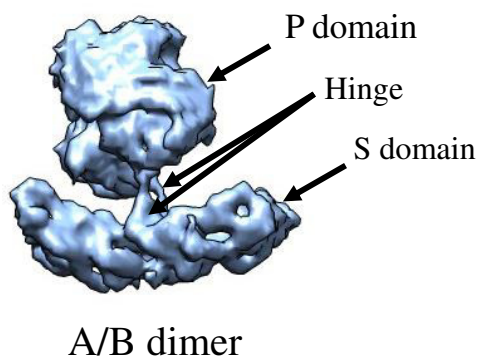


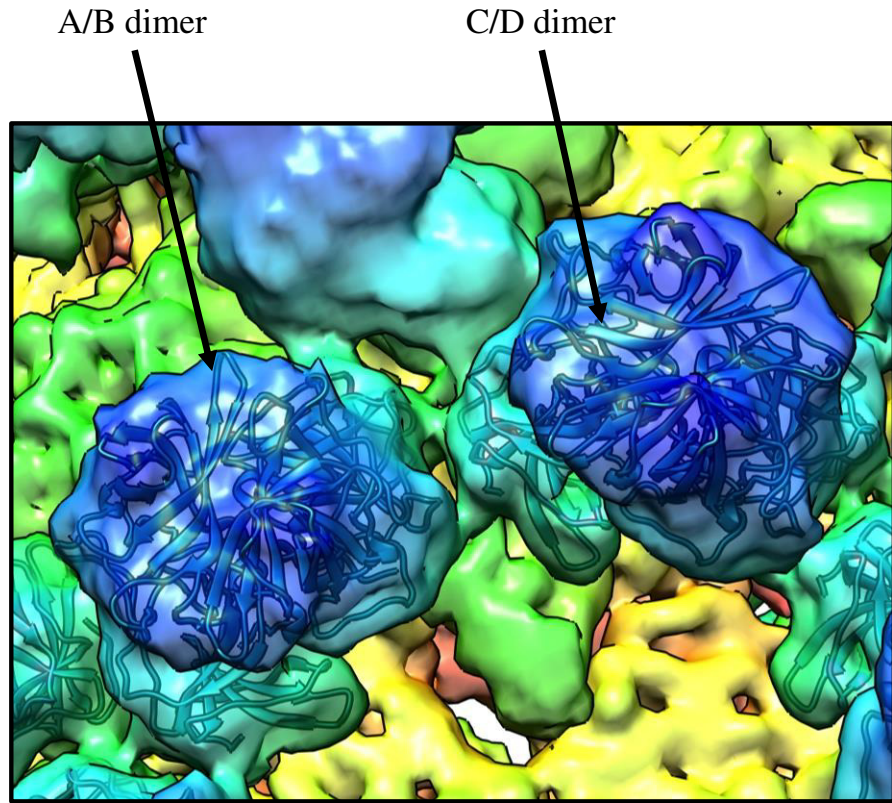
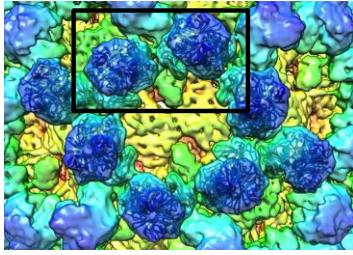
Figure 5







A



B

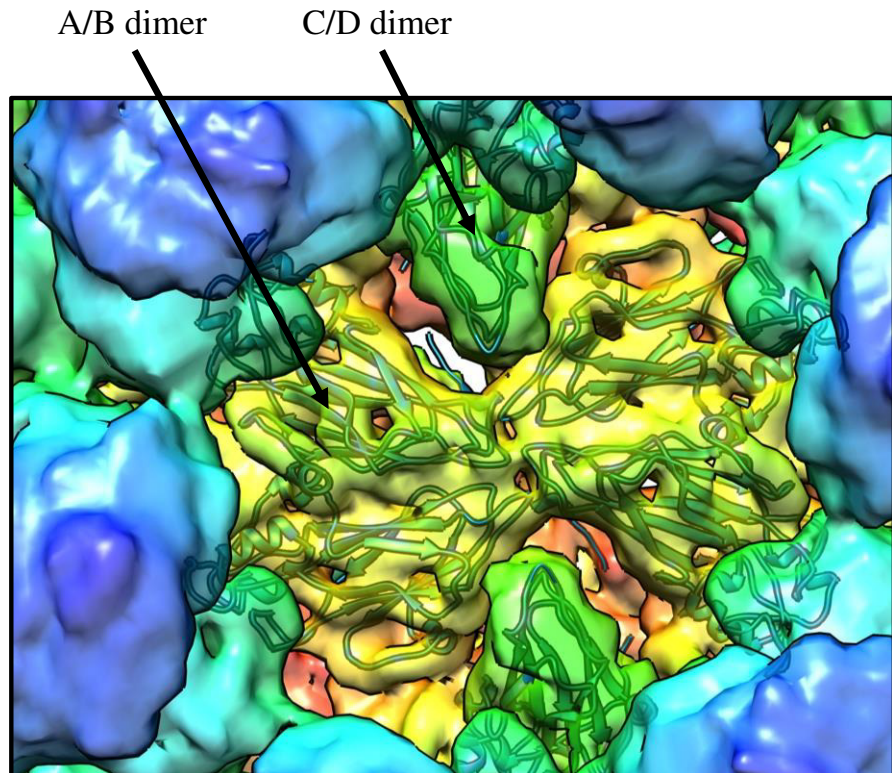
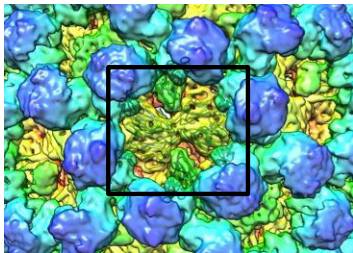
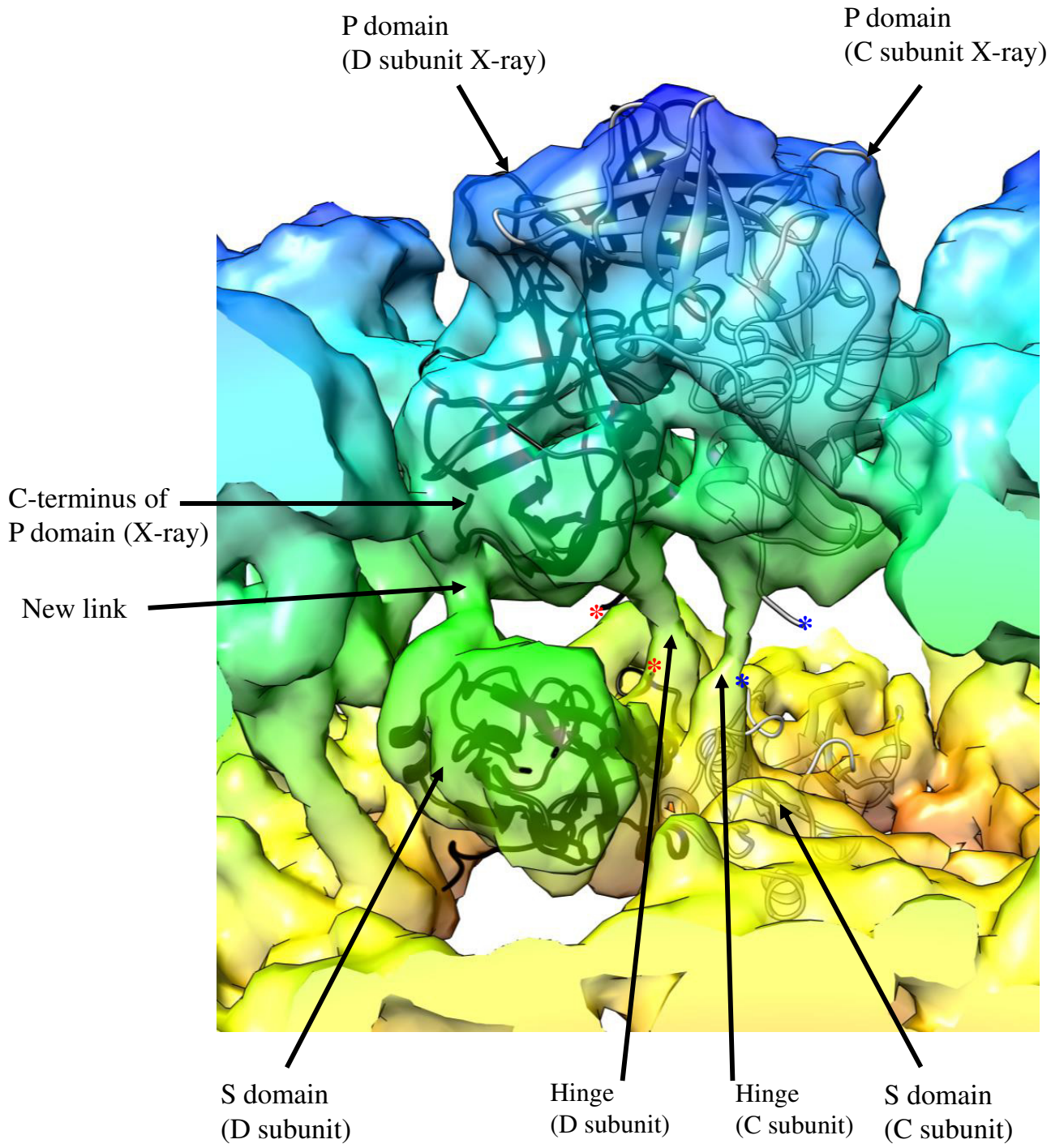
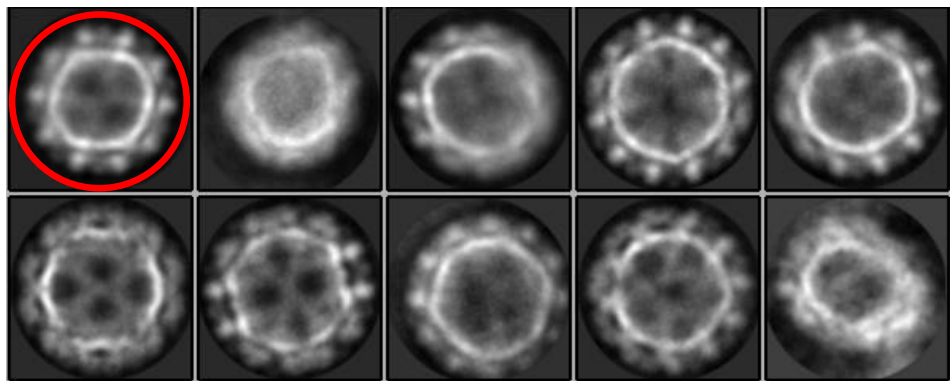


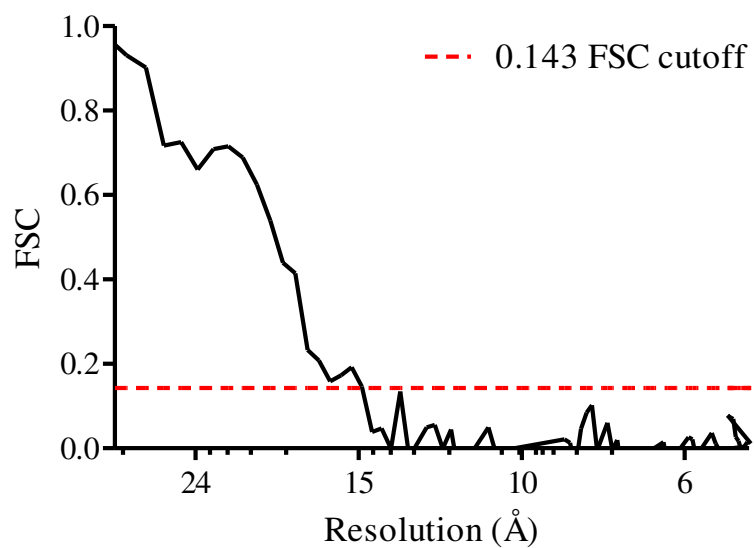
Figure 8



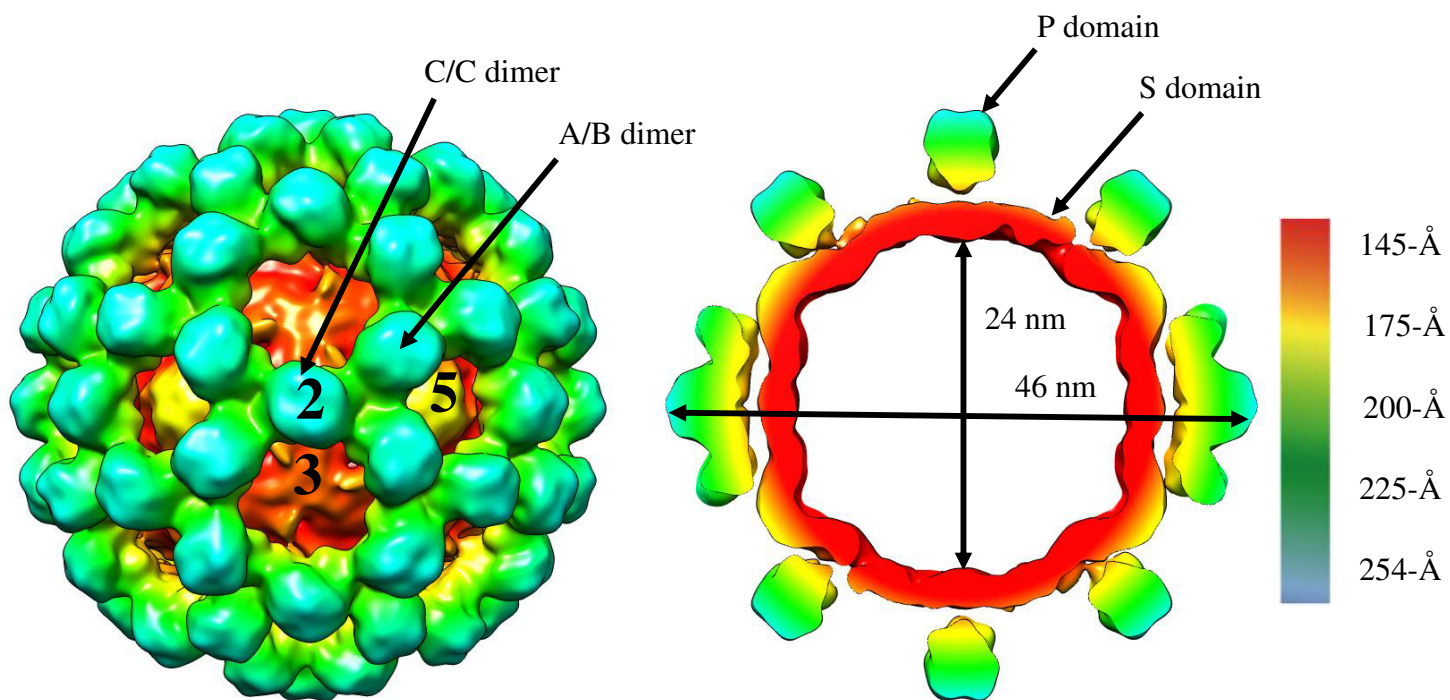
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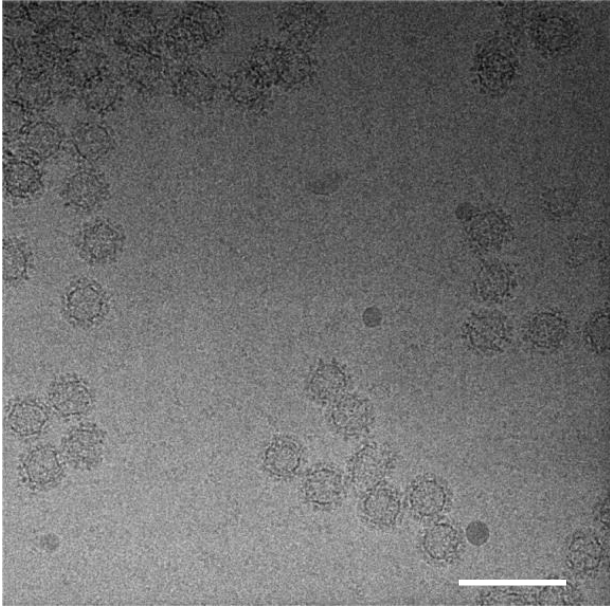
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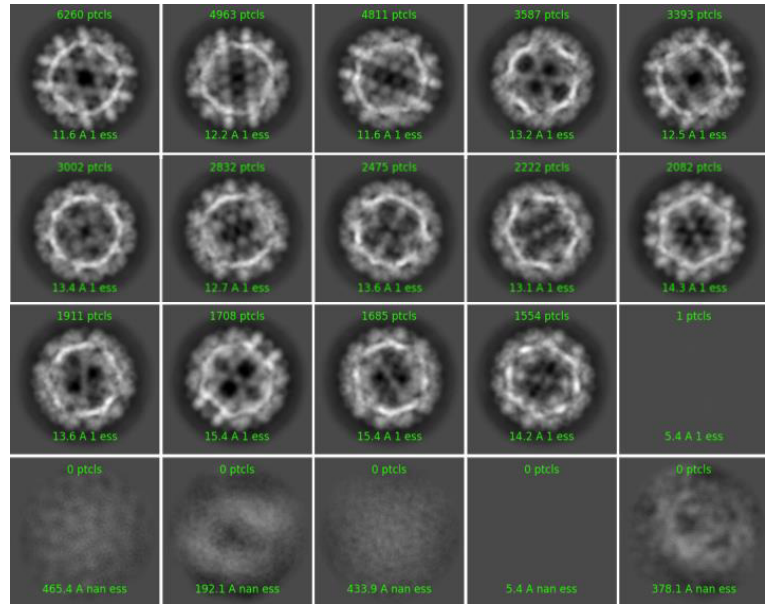
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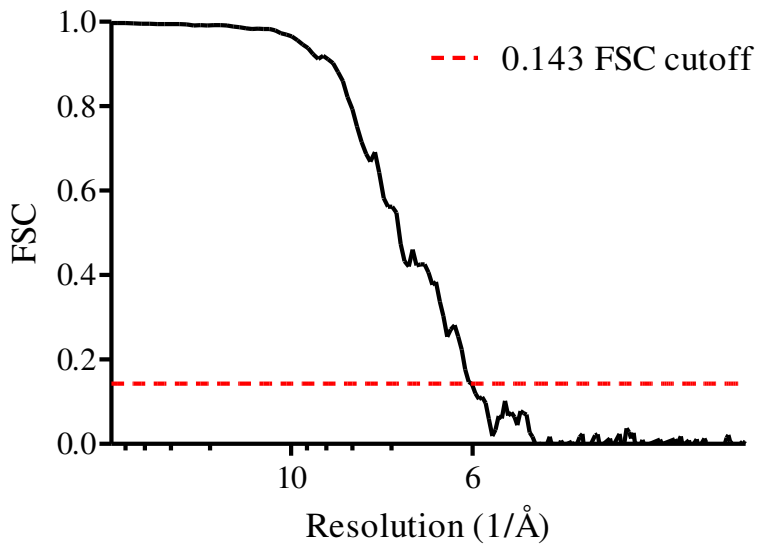


Figure 11

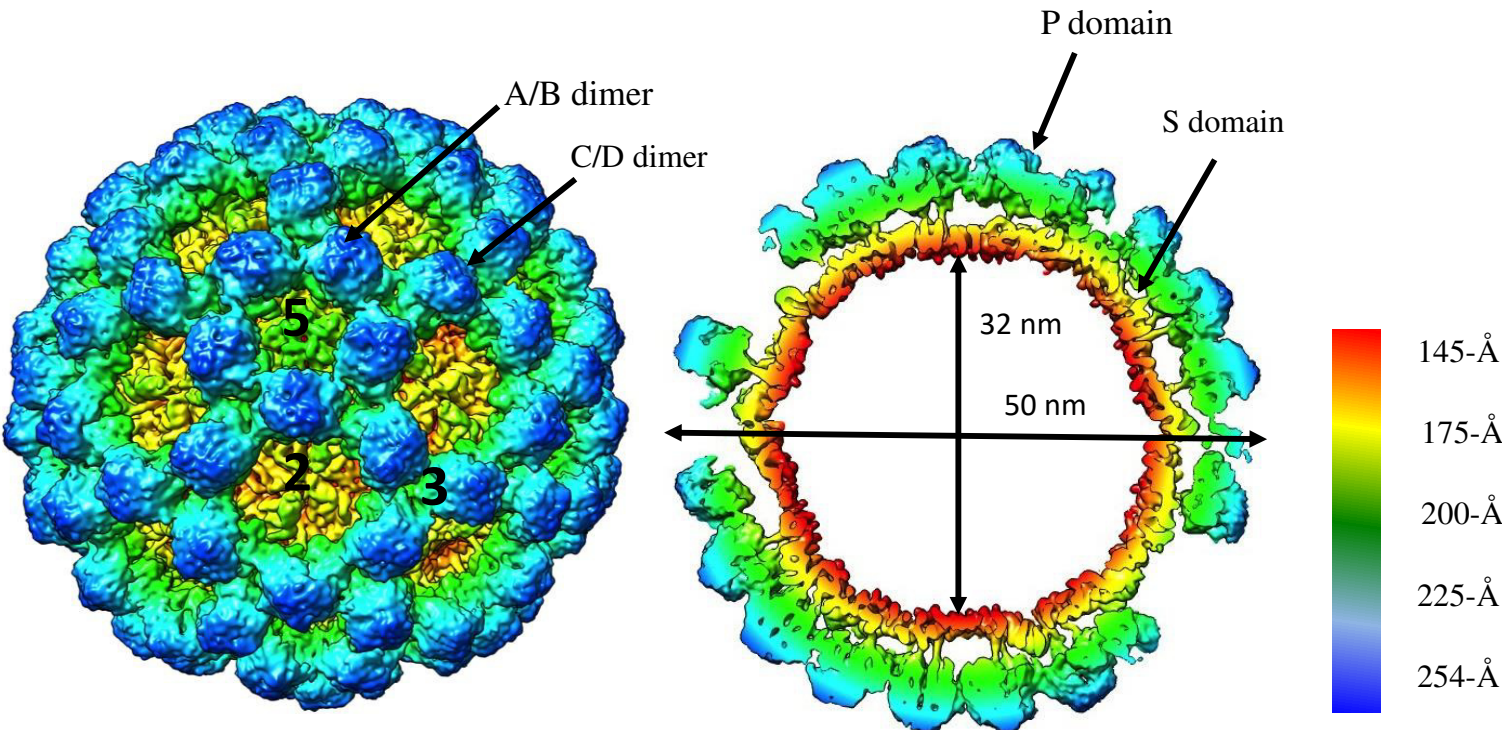
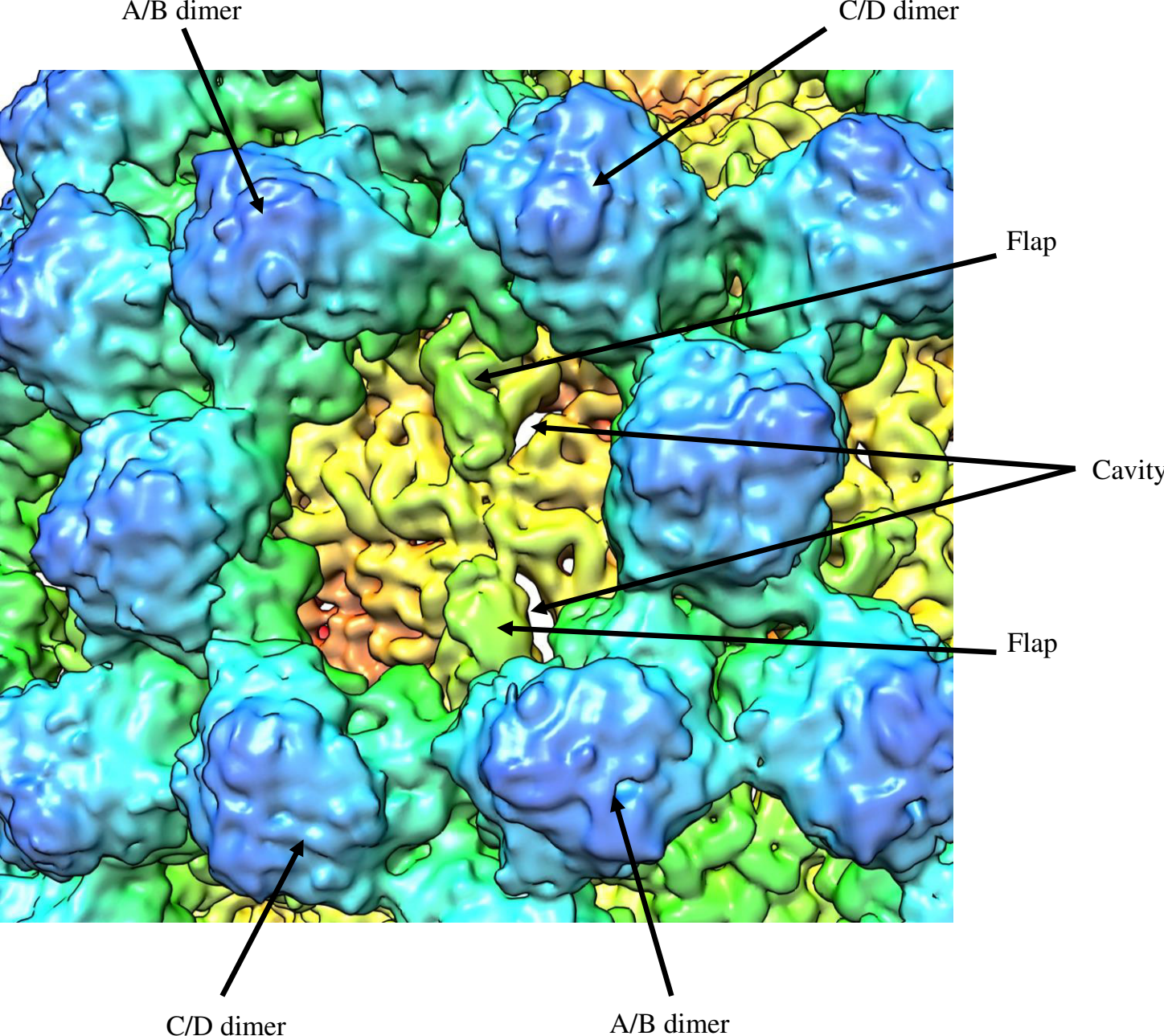
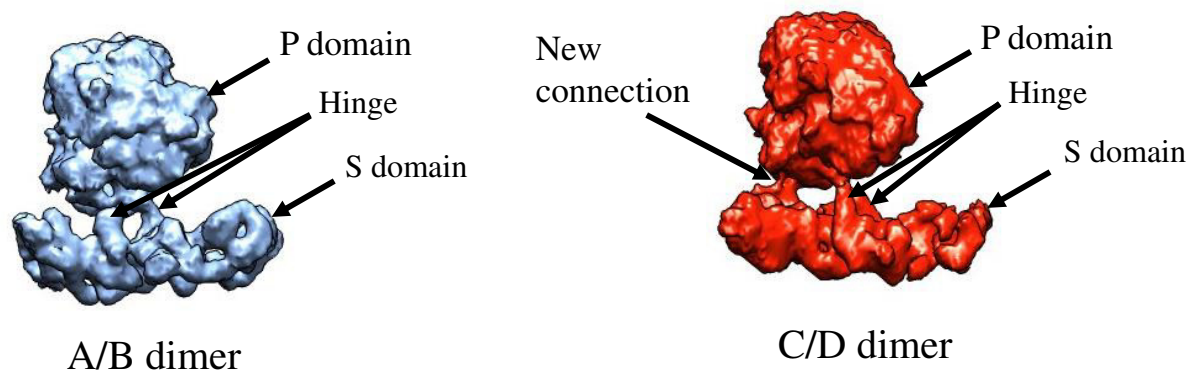
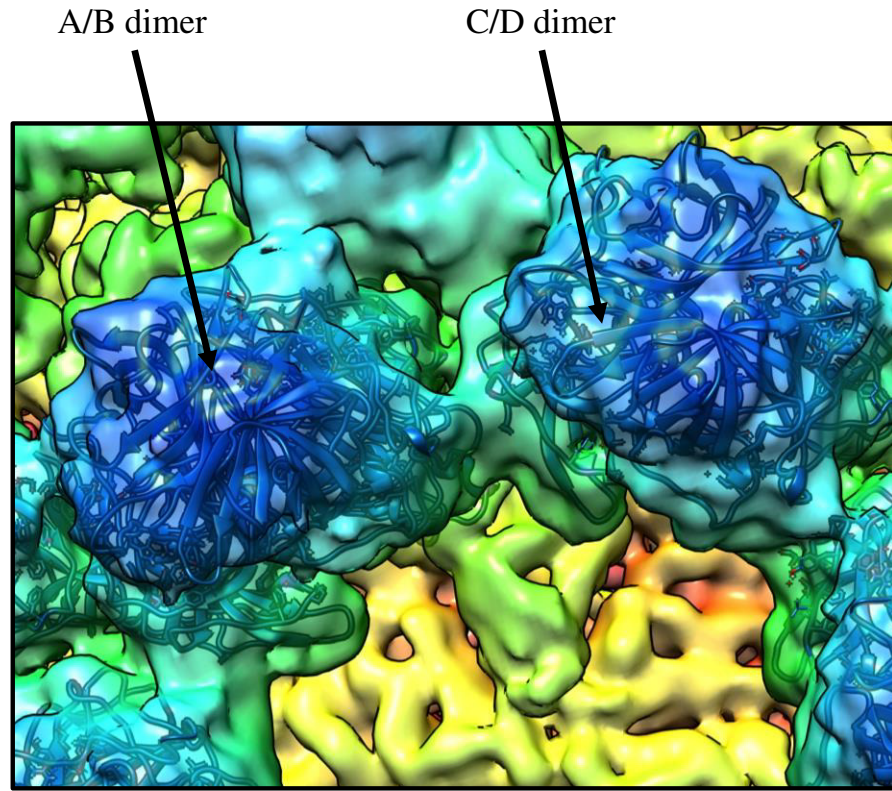
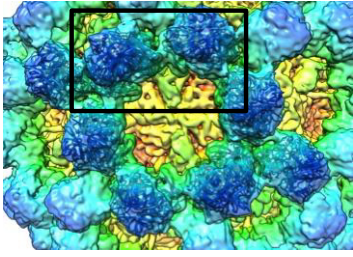


Figure 12





A



B

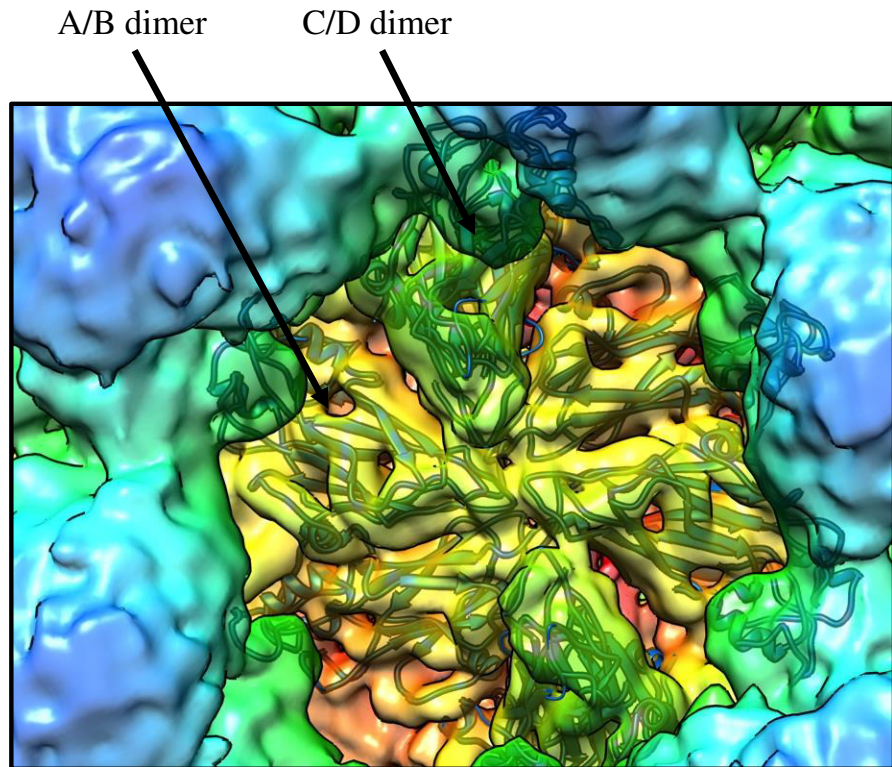
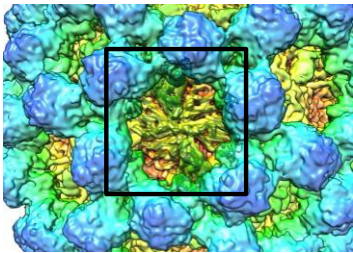




Figure 15

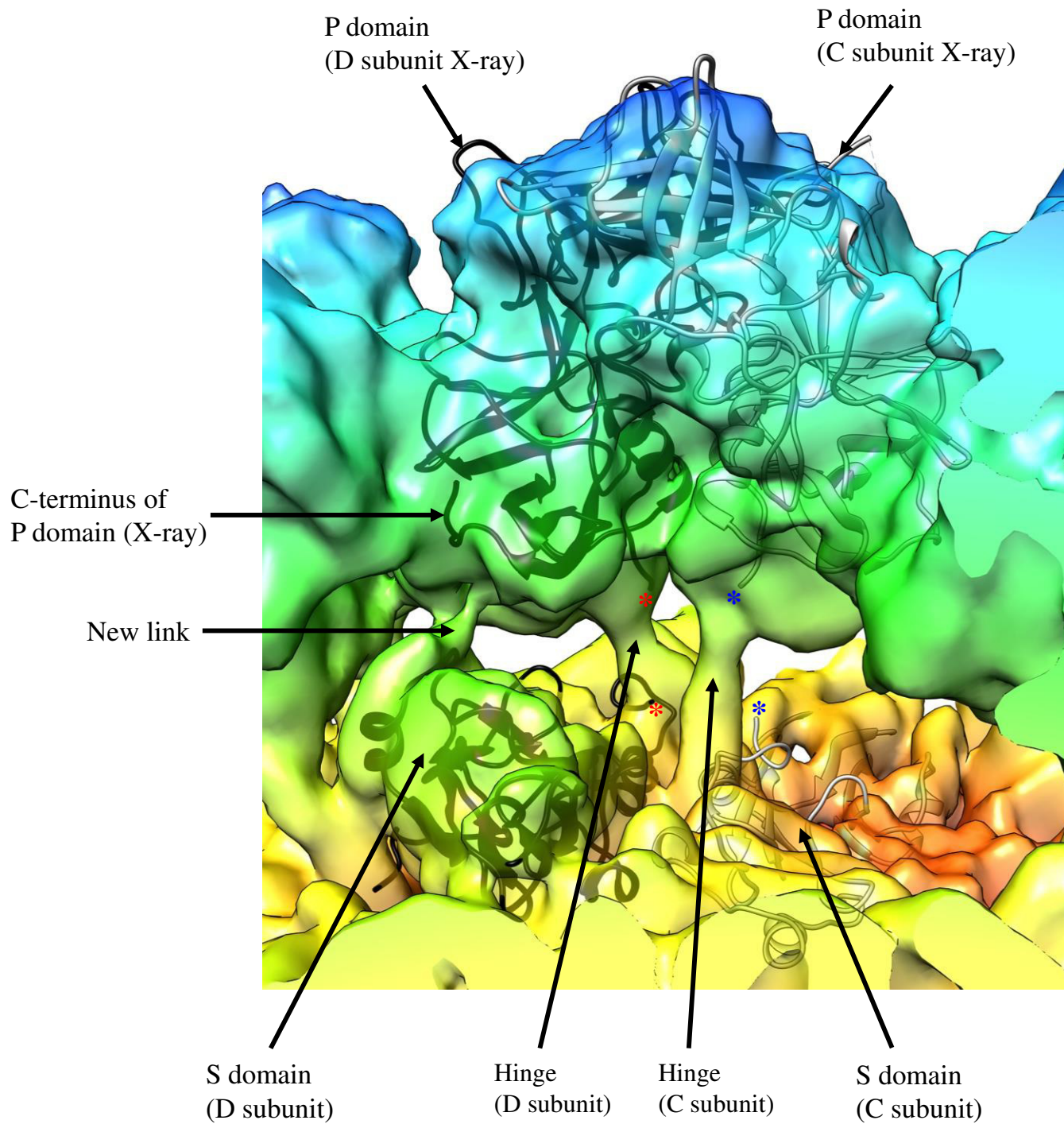


Figure 16

