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1 **Revealing crystalline domains**
2 **in a mollusc shell “single-crystalline” prism**

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17
18
19 **Summary**

20 Biomineralization integrates complex processes leading to an extraordinary diversity of
21 calcareous biomineral crystalline architectures, in intriguing contrast with the consistent
22 presence of a submicrometric granular structure. Hence, gaining access to the crystalline
23 architecture at the mesoscale, *i. e.*, over a few granules, is key to building realistic
24 biomineralization scenarios. Here we provide the nanoscale spatial arrangement of the
25 crystalline structure within the “single-crystalline” prisms of the prismatic layer of a *Pinctada*
26 *margaritifera* shell, exploiting three-dimensional x-ray Bragg ptychography microscopy. We
27 reveal the details of the mesocrystalline organization, evidencing a crystalline coherence
28 extending over a few granules. We additionally prove the existence of larger iso-oriented
29 crystalline domains, slightly misoriented with respect to each other, around one unique
30 rotation axis, and whose shapes are correlated with iso-strain domains. The highlighted
31 mesocrystalline properties support recent biomineralization models involving partial fusion of
32 oriented nanoparticle assembly and/or liquid droplet precursors.

34 **Introduction**

35 Biocrystallisation involves extraordinarily complex and regular biochemical processes
36 whereby living organisms control the crystalline form and texture of their organo-mineral
37 components^{1,2}. Only considering calcium carbonate polymorphs in molluscs, corals or
38 sponges, a variety of sub-millimetric structures and shapes are observed, often related to
39 outstanding mechanical properties¹. These controlled biocrystallization pathways result not
40 only in the synthesis of otherwise unstable crystalline forms but also in hierarchical composite
41 structures with a variety of microstructures, of which mollusc shells are one of the most
42 striking representatives³. Different crystalline polymorphs forming various architectures are
43 observed depending not only on the species but also on the precise location within the
44 organism. This latter feature is perfectly illustrated by the famous pearl oyster *Pinctada*
45 *margaritifera*, which possesses an internal nacreous aragonite layer and an outer prismatic
46 calcite layer^{4,5}. The simultaneous crystallization of distinct calcium carbonate polymorphs
47 goes along with very different contents in organic compounds⁶, which obviously results in the
48 selection of different nucleation pathways⁷.

49 This architectural diversity at the micro-scale strongly contrasts with the systematic
50 occurrence in calcareous biominerals of crystalline spheroid units⁸⁻¹⁰ of apparent diameter in
51 the 50-500 nm range¹¹, coated by a visco-elastic (likely organic) cortex^{8,12}, and referred to as
52 granules. In addition, the irregular round shapes of the granules do not provide morphological
53 evidence for a crystalline organization. Nevertheless, the generic characteristic of this sub-
54 micrometric structure, which presents the capability to organize into various micro-scale
55 morphologies, justifies the need of studying the hierarchical arrangement of these sub-
56 micrometric 'building-blocks'. It is foreseen that the understanding of crystallization at this
57 mesoscale level, *i.e.*, over a few granules, is key to building realistic scenarios of bio-
58 crystallization¹³.

59 This issue is repeatedly addressed in the literature, where (indirect) evidences of a mesoscale
60 ultrastructure have been reported, typically in the form of sub-micrometer sized (coherent)
61 crystalline domains. Several bivalve mollusc shells containing many micrometre sized units,
62 which diffract x-rays like single crystals (and are therefore referred to as single-crystal like
63 material)¹⁴⁻¹⁶, present sub-micrometric crystalline domains of coherence lengths in the 250-
64 700 nm range, evidenced by high-resolution x-ray diffraction analysis^{14,17}. Domains of
65 comparable sizes were also imaged with 2D transmission electron microscopy¹⁸. Low
66 mosaicity, in the order of 1° or less between adjacent domains, was noted or inferred in some

67 biogenic crystals^{15,14,19} while largely misoriented crystalline domains (by a few tens of
68 degrees) were observed along the growth direction of mature crystalline units.^{5,20,21} Micro-
69 strain fluctuations, in the order of a few 10^{-3} with respect to the mean lattice spacing, were
70 reported as well^{14,17,18}. These structural fluctuations (orientations and strains) within the
71 crystal are attributed to the presence of occluded organic molecules.^{22,14,17,18} However, a
72 three-dimensional (3D) structural description, which would allow the visualization of the
73 spatial arrangement of those domains, is strongly missing. In this work, we exploit a newly
74 developed x-ray microscopy, 3D x-ray Bragg ptychography, to image the mesoscale
75 crystalline architecture of a *Pinctada margaritifera* calcite prism.

76 X-ray Bragg ptychography belongs to the so-called coherent diffraction imaging
77 microscopies. These methods aim at retrieving the sample scattering function from a set of
78 coherent intensity measurements, using numerical approaches to determine the phase of the
79 scattered amplitude (not accessible experimentally) and invert it back to real-space²³. The
80 resulting images contain quantitative sample information, such as material density and/or
81 crystalline displacements, with sub-beam-size spatial resolution. Since the late 90's²⁴, with
82 the advent of highly-brilliant synchrotron sources, various x-ray methods have been
83 proposed²⁵: among these, finite support coherent diffraction imaging and Fourier-transform
84 holography have been largely exploited, either in the forward scattering regime^{26,27} or in the
85 Bragg geometry^{28,29}. X-ray Bragg ptychography, inspired by electron beam ptychography³⁰
86 and combining approaches arising from Bragg coherent diffraction imaging³¹ and x-ray
87 (forward) ptychography,^{32,33} has been recently proposed for the 3D imaging of extended
88 crystalline material structured at the nanoscale.³⁴⁻³⁶ It takes advantage of the weak interaction
89 of x-rays with matter, allowing for structural investigations of a sample without specific
90 preparation. In the forward direction, *i. e.*, in the vicinity of the reciprocal space origin, the
91 sensitivity of x-ray ptychography is exploited for imaging the 3D density of materials such as
92 amorphous biominerals³⁷. In Bragg diffraction conditions, the internal periodicity of the
93 crystalline lattice produces highly intense Bragg peaks that contain information on the crystal
94 properties of a given crystal plane family^{36,38}. A Bragg ptychography measurement consists in
95 recording a set of 3D Bragg diffraction intensity patterns, resulting from the interaction
96 between a finite-sized coherent beam and the investigated object under Bragg conditions.
97 From the data set, a phase retrieval algorithm reconstructs numerically the 3D sample map,
98 which provides detailed information on the sample crystalline properties, such as crystalline
99 coherence, relative strain field and relative crystalline plane rotation, imaged with a spatial

100 resolution of few tens of nanometers.³⁶ The high-sensitivity of this crystalline microscopy
101 allows one to distinguish crystalline domains with lattice mismatch of a few 10^{-4} and/or
102 presenting misorientations of a few 10^{-2} degree. In the context of the biomineralization
103 question, the unique capabilities of x-ray Bragg ptychography represent assets allowing one to
104 shed new light on the mesoscale crystalline organization.

105 **Results**

106 Our study was performed on the outer layer of a juvenile *Pinctada margaritifera* border shell
107 (Fig. 1). This organism was selected owing to the well-known apparent simplicity of its
108 calcitic prisms, each prism being described as a homogeneous single-crystalline like material
109 (Supplementary Figure 1), at least during the early stage of the prism growth³⁹. The prism
110 assembly displays a rather homogeneous orientation of the crystal lattice *c*-axis, which lies
111 roughly perpendicular to the prism surface, while the in-plane crystalline orientation varies
112 from one prism to the other. Some of the specific morphologies observed at different length
113 scales of the shell are shown in Figs 1A-D. At the shell border, where the thickness is
114 estimated to $0.5\text{-}2\ \mu\text{m}$ ⁵, the calcite prisms are easily recognized with a typical lateral size of
115 $30\ \mu\text{m}$, surrounded by their organic envelopes (Fig. 1B-C). The presence of disks - the
116 preliminary form of prisms - only visible at the very border of the shell, confirms that the
117 earliest stages of the prismatic growth are present in our sample⁵. In apparent opposition with
118 the prism crystalline homogeneity, atomic force microscopy in tapping mode (phase lag)
119 emphasizes, with a nanoscale resolution, the existence of a granular structure, which is
120 composed of rounded mineral units with apparent sizes estimated here to about $50\text{-}200\ \text{nm}$
121 (Fig. 1D). At slightly larger length scales, 3D images obtained with CARS (coherent anti-
122 Stokes Raman scattering) microscopy, a non-linear 3D optical microscopy method sensitive
123 to chemical bond vibrations (here the crystalline carbonate vibration mode) evidences spatial
124 fluctuations of crystalline carbonate density, with visible homogeneity along the prism
125 thickness (Supplementary Information and Supplementary Figure 2).

126 The 3D x-ray Bragg ptychography experiments were performed on a prism selected in the
127 vicinity of the preserved growth edge, using the experimental set-up of the ESRF ID13
128 beamline, schematically depicted in Fig. 2 (see also Methods). The sample, located on a
129 translation and rotation stage, is illuminated by a nano-focused beam (Supplementary Figures
130 3 and 4) under Bragg conditions, so that a given Bragg reflection can be investigated in
131 details (Fig. 2A, B). The chosen prism region is probed by shifting the sample transversely to
132 the beam along two translation directions, t_x and t_y . The Laue geometry employed here, which

133 gives access to a Bragg reflection in transmission, allows minimizing the beam footprint on
134 the surface, a condition that has been found to be of major importance for the successful
135 reconstruction of this complex material. The chosen 110 reflection probes the lattice planes
136 that are almost perpendicular to the prism thickness (Fig. 2B). The detailed characteristics of
137 the 3D intensity distribution produced by the illuminated volume are investigated by scanning
138 angularly the sample with respect to the incident angle ω , over an angular range of about 2°
139 (Fig. 2C and Supplementary Figure 5). In this way, the 2D far-field intensity patterns
140 successively extract information along the direction perpendicular to the Bragg vector. The
141 patterns are finally assembled to produce a 3D intensity volume, corresponding to a finite 3D
142 extent of the crystal reciprocal space, for each illumination position (Fig. 2D). While a single
143 3D diffraction intensity pattern encodes 3D information on the sample structure at a length
144 scale possibly smaller than the beam size, the whole set of spatially-dependent 3D intensity
145 patterns has to be simultaneously phased back^{34,40} to provide the 3D structural image over the
146 whole illuminated sample volume (see Methods). Note that the finite extents of the spatial and
147 angular scans (translation and rocking curve) impose that only a limited spatial field of view
148 is imaged and that only the crystal components that meet the Bragg conditions are probed.
149 Consequently, one has to keep in mind that the final image is a spatial truncation of the
150 sample, which possibly exhibits some empty parts that would correspond to misoriented
151 crystalline regions.

152 The retrieved 3D images are shown in Figs. 3 and 4. They feature a region close to the prism
153 center, schematically represented in Fig. 3A. The envelope of the full retrieved volume is
154 shown as a yellow-grey surface. Its finite extent along the prism surface reflects the equally
155 limited extent of the illuminated volume according to the measurement modalities, while
156 along the third direction ($\approx \mathbf{z}$), the limit corresponds to the prism thickness, estimated to about
157 $1.75 \mu\text{m}$ (see Methods and Supplementary Figure 6). The Bragg ptychography reconstruction
158 returns a complex-valued function whose phase (φ) holds information on the crystalline
159 properties^{31,34,41}. This retrieved phase φ , which is directly proportional to the displacements of
160 the (110) crystalline planes, was therefore further analyzed: its spatial derivatives are related
161 to the tilt and strain of the (110) crystal planes (see Pateras *et al.*³⁶ and Methods). Both tilt and
162 strain present abrupt spatial variations, revealing that even at this early growth stage, the
163 crystal is not (strictly speaking) single-crystalline but is composed of several iso-oriented
164 domains, slightly tilted with respect to one another, and of several iso-strain domains, slightly
165 strained with respect to the mean lattice parameter. Concerning the rotations, we unexpectedly

166 observe that the iso-oriented domains display a strongly anisotropic tilt distribution, over an
167 angular range of about 1.2 degree, which occurs solely around a single rotation axis, named δ
168 and found to be almost collinear to \mathbf{y} , an axis that lies along the prism surface (Fig. 3B). We
169 note that this anisotropic rotation distribution obtained from the reconstruction is in
170 agreement with the peculiar shape of the Bragg intensity, which presents a linear spread
171 perpendicular to the mean 110 Bragg vector direction (Fig. 2D). On the contrary, an isotropic
172 distribution of the crystal plane tilts, around a mean direction, would result in a Bragg vector
173 distribution describing a cone on the Ewald sphere. A series of 2D cross-sections through the
174 rotation map is shown in Fig. 3B. The visualization of the 3D spatial arrangements of the iso-
175 oriented domains, in Figs. 3B, D-G, is facilitated with a surface color rendering, where each
176 iso-oriented domain external surface is drawn using a color that encodes its respective
177 rotation angle, according to the color-scale of Fig. 3B. The discrepancy observed between the
178 retrieved volume shape and the illuminated cylindrical volume, indicates some missing parts
179 in the probed region. Most likely, these correspond to misorientations, which could not be
180 captured owing to the limited amplitude of the rocking curve. The same image analysis has
181 been made on the strain map, which evidences iso-strain domains of total strain amplitude ϵ
182 of about $2.5 \cdot 10^{-3}$ (Fig. 3H-K). For both tilt and strain domains, a striking result is the
183 elongated shape of the iso-oriented and iso-strain crystalline domains that extend all along the
184 prism thickness (Fig. 3E,I). In the transverse direction, where the apparent extent of the
185 domains is artificially limited by the experimentally accessible field of view, the domain
186 transverse size is estimated at least to about 400 nm. Finally, the 3D visualization of both
187 strain and rotation maps makes possible the comparison between the iso-oriented and iso-
188 strain domain shapes, which exhibit some strong but partial correlations: while one of the iso-
189 oriented domains (in *blue*) fully corresponds to one of the iso-strain domains (in *orange*), the
190 large iso-oriented domain (in *orange*) is actually composed of two different iso-strain
191 domains (in *blue* and *green*). The third tiny iso-oriented domain (in *green*) appears as part of
192 one of the last iso-strain domain (in *green*).

193 In addition to the crystal plane tilt and strain, the phase that is retrieved with the 3D Bragg
194 ptychography reconstruction allows the visualization of the crystalline coherence within the
195 iso-oriented domains (see Methods). The 2D maps plotted in Fig. 4 correspond to 2D cuts
196 through the distribution of the retrieved φ , extracted solely for the iso-oriented domain of Fig.
197 3D. The abrupt changes in the colored representation of φ correspond to a phase shift of about
198 4.2 rad, *i. e.*, a displacement of 0.16 nm (or 2/3 of the (110) lattice spacing), located at the

199 transition between the iso-strain domains of Fig. 3I and J. They evidence the existence of a
200 finite coherence length, which manifests itself as an iso-color area, with typical dimensions of
201 about 400 nm.

202 A second ptychography reconstruction, which corresponds to an investigation performed
203 within the same prism, in a region nearby the one shown here (Supplementary Figure 7)
204 exhibits similar crystalline features: spatial distribution of crystal plane tilt and strain, single
205 (and same) rotation axis, extent of the iso-oriented and iso-strain domains over the prism
206 thickness and finite crystalline coherence length. In complement to these 3D microscopy
207 analyses, an incoherent x-ray micro-diffraction set-up was used to perform an extended
208 spatial investigation of the 006 Bragg peak, on the whole area of another prism
209 (Supplementary Figure 3). The obtained 3D intensity patterns, which were found to be
210 systematically elongated along a fixed direction (over an angular range of about 1°) confirms
211 the existence of the distribution of crystal plane tilts around a single rotation axis, whose
212 direction is contained into the (**xy**) plane, that is the prism surface. These microdiffraction
213 results are in full consistency with the ptychography reconstruction.

214 **Discussions**

215 The produced ptychography images result from the inversion of a single Bragg reflection,
216 whose extent was restricted by the limited signal dynamics. Therefore, the 3D images detail
217 structural information on the 110 crystalline planes only, with mesoscale spatial resolution.
218 This implies that local imperfections of the crystalline lattice, *i. e.*, near the atomic level
219 (vacancies, inclusions), cannot be resolved and that the full strain tensor is not accessible. We
220 note that the later one is not an intrinsic limit²⁵ and significant resolution improvements are to
221 come thanks to the on-going advent of several extremely brilliant synchrotron sources, which
222 should deliver increased coherent flux and allow for improved measurement strategies.⁴² A
223 first conclusion drawn from the presented experiments is the confirmation of a mesoscale
224 arrangement of the granules into larger crystalline domains. The herein 3D images of the
225 crystalline properties allowed us to identify two typical length scales: (*i*) a shorter one, of
226 about 400 nm (*i. e.* larger than the granule size) corresponding to the typical size of the
227 coherent crystalline domains, in agreement with previous observations on several
228 species^{14,17,18,43} and (*ii*) a second one, most likely slightly larger, corresponding to the extent
229 of domains which exhibit the same crystalline plane tilt and strain (the iso-oriented and iso-
230 strain domains). We note that mosaicity of comparable angular spread (about or less than a
231 degree) was already measured in thick, *i. e.* older, biogenic marine crystal units.^{14,15,19}

232 Comparable micro-strain fluctuations (in the order of a few 10^{-3}) have already been reported
233 as well.^{14,17,18} The presence of a crystalline mosaicity and strain distribution, already observed
234 at the most early growth stage of the prisms, indicates that structural disorder is a core part of
235 the growth mechanism. The additional and new information that is brought with this work
236 concerns the spatial organization of those sub-micrometric domains, a step forward made
237 possible by the Bragg ptychography microscopy. In some of our observations (Figs. 3E,I,J
238 and Supplementary Figure 7), the iso-oriented and iso-strain domains clearly expand over the
239 whole prism thickness, which is the prism growth direction according to the model of layered
240 growth mode^{10,44}. The orientation distribution (or rotational disorder) of the calcite planes
241 presents a clear anisotropic behavior, with the rotation occurring around a single axis,
242 contained into the prism surface. Moreover, the rotation axis direction is constant all over the
243 prism. We note that the existence of the rotation axis and the determination of its direction,
244 that requires the 3D measurement of the Bragg diffraction pattern (with coherent or
245 incoherent x-rays) has not been evidenced previously, to the best of our knowledge. Finally,
246 the similarity between the iso-oriented and iso-strain domain shapes demonstrates a common
247 origin of these structural features, although the latter do not fully correlate in space.

248 The structural information that we are bringing along provides new elements that have to be
249 accounted for in the calcareous biomineralization scheme, a question that we briefly discuss
250 in the following. At large length scale, recent results indicate that the growth of the prismatic
251 structure (prisms size and shapes) may originate from simple physical effects, related to grain
252 growth theory.⁴⁵ On the contrary, at the nano and meso-scales, it is generally accepted that
253 calcareous biominerals, and more specifically mollusc shells, cannot be produced within the
254 classical crystalline nucleation and growth scheme, owing to their mesocrystal structure
255 (nanoparticle assembly)¹³ and to the likely occurrence of an amorphous precursor, which
256 would explain the observed round granules and molten morphologies⁴⁶. Based on bio-inspired
257 syntheses and micro-structural observations, several bio-crystallization scenarios have been
258 proposed, some of which are consistent with our observations. The observed iso-oriented and
259 iso-strain domains of assembled granules could be explained by a mesocrystal formation
260 scheme, which involves colloidal organic-mediated arrangement of crystalline nanoparticles
261 of common crystallographic orientation.⁴⁷ The iso-strain domains could be induced by the
262 presence of previously observed occluded organics molecules, as seen in biogenic^{18,48} and
263 synthetic biomaterials.⁴⁹ Additionally, the proposed oriented attachment of crystalline primary
264 nanoparticles during mesocrystal formation⁴⁷ can preserve the crystalline coherence between

265 nanoparticles if they fuse together. Mesocrystalline growth could therefore agree with our
266 observations in *Pinctada* prisms, of coherent crystalline domains larger than the granule size,
267 as well with other reported observations^{14,43}. However, the round shape of the granules
268 suggests a slightly different mechanism where mesoscale structuring occurs through an
269 assembly and subsequent crystallization of amorphous particles⁵⁰. An attractive scenario has
270 been currently developed, in which these solid amorphous granules result from the solid
271 transformation of a liquid precursor, the so-called PILP (polymer induced liquid precursor)
272 process⁴⁶. Such a scenario would explain the observed crystalline coherence by the
273 propagation of a crystallization front through the inter-connected granules and would agree as
274 well with the likely absence of porosity in our structural reconstruction (observed at least in
275 the limit of our resolution level, Supplementary Figure 8). Note that this scenario would also
276 be consistent with TEM observations performed on another calcareous biocrystal¹⁰. Finally,
277 our observations of a reduced rotational disorder, both in disorder dimension (observation of a
278 single rotation axis, contained in the prism plane) and in disorder amplitude (no rotation
279 observed along the prism thickness) calls for a specifically oriented growth mode. Extrinsic
280 preferential orientation can be invoked, through the growth along organic fibers⁵¹ or sheets⁵²,
281 for instance, while the fluctuations in the organic molecule density could produce strain
282 fluctuations within the same iso-oriented domains^{17,18}. A gradual shift in orientation of the
283 crystal lattice has been reported in films produced by PILP, attributed to dehydration
284 shrinkage stresses during crystal solidification constrained by the substrate⁵³: similar effects
285 could be at play during the uniaxial growth of the prism. These could be confirmed by the
286 detailed investigations of the rotational disorder spatial evolution, as a function of the distance
287 to the crystallization centers, if any⁵. It is remarkable, that the herein observed rotation axis (*i.*
288 *e.*, the rotational disorder axis) lies in the growth layer plane⁴⁴. The possibility to conclude on
289 a definite biomineralization mechanism, at least for *Pinctada* prisms, would certainly require
290 additional structural investigations involving a larger field of view. Future works will aim at
291 identifying and investigating regions near the mineralization fronts, where we expect to
292 observe structural differences with respect to the two models. Indeed, assuming that the
293 regions near the mineralization front correspond to a not fully completed crystallisation
294 process, one may expect the coherence domains to be of smaller extent than the crystalline
295 domain for the oriented-attachment model while the crystalline coherence should scale with
296 the size of the crystalline domain, for the liquid-like precursor model. Still, our 3D description
297 of the crystalline architecture brings a set of new arguments in favor of recently reported
298 models, namely the oriented attachment and partial fusion of a mesoscale assembly scenario⁴⁷

299 and the PILP intermediate model⁵⁴, which both appear able to account for the herein observed
300 complexity of the mesoscale arrangement of the granules produced by biomineralization.

301

302 **Conclusions**

303 To conclude, we have revealed the complexity of the crystalline biomineral at the mesoscale,
304 by providing a 3D spatial description of the crystalline network with a nanometric resolution.

305 The wealth of information that we brought with this recently developed imaging method
306 demonstrates that new findings on the understanding of the biomineralization process are
307 within reach.

308

309 **Methods**

310

311 **Sample preparation.** The juvenile *Pinctada margaritifera* shells were grown at the
312 IFREMER hatchery at the biological station of Taravao-Vairao (Tahiti). They were cultivated
313 in an optimal and controlled sanitary environment to avoid any contamination during growth.
314 Once selected, they were preserved in ethanol/sea-water solution and transferred to the
315 geology laboratory in Orsay (France). Before the x-ray experiment, they were cut into small
316 pieces of about 1 mm² and glued on the top of a metallic tip. This process ensures the
317 integrity of the investigated part of the shell is preserved.

318

319

320 **X-ray Bragg diffraction experiments.** The x-ray Bragg diffraction experiments were
321 performed at the ID13 beamline of the European Synchrotron Radiation Facility. The
322 monochromatic coherent beam of wavelength λ (energy bandwidth $\Delta\lambda/\lambda \approx 10^{-4}$) was focused
323 down to the sample position (see Supplemental Information). The sample, fixed on a metallic
324 tip was mounted onto a three-axis piezo-electric stage, itself placed on the top of a hexapod.
325 The laboratory frame (x,y,z) was defined with respect to the sample surface: **x** and **y** are along
326 the prism surface, with **y** along the shell growth direction, and **z** is perpendicular to the prism
327 surface. The sample was translated into the focal plane of the lenses, using an optical
328 microscope with a 1 μm depth of focus. A 2D Maxipix (486 x 516 pixels, 55 μm width)
329 detector was placed on a rail, allowing the adjustment of the diffraction angle and detector-
330 to-sample distance. The 3D diffraction patterns were built by stacking the 2D intensity
331 patterns obtained while scanning the incidence angle ω along the so-called "rocking curve", in
332 step of $\delta\omega$. The beam-to-sample position was adjusted by translating the sample along the t_x
333 and t_y directions.

334

335 For the ptychography experiment, the beam was tuned to 15 keV ($\lambda = 0.83 \text{ \AA}$), illuminating a
336 set of crossed silicon refractive lenses of 25 μm effective aperture and 0.01 m focal length. A
337 pair of slits was placed in front of the lenses in order to restrict the illumination area. Its
338 aperture was set to 40 x 60 μm^2 along the horizontal (H) and vertical directions (V),
339 respectively, ensuring fully spatially coherent illumination. Before the sample investigation
340 itself, a full characterization of the complex-valued illumination function was performed³⁵.
341 The transverse beam size at the sample position was hereby estimated to 0.08 x 0.1 μm^2 (H x
342 V, full width at half-maximum). For the shell imaging, the Bragg ptychography experiment
343 was performed in Laue geometry on the transmitted 110 calcite diffraction peak, where the
344 transmission geometry was chosen in order to minimize the beam footprint on the sample. For
345 this diffraction angle, the longitudinal coherence condition across the sample is ensured up to
346 a sample thickness of about 1.9 μm . This condition is verified in our experiment where the
347 prism thickness has been estimated to 1.75 μm (from both scanning electron microscopy and
348 reconstruction results, see Supplemental Information). The diffraction signal was collected at
349 a Bragg angle $2\theta_B$ of 19.2° while the detector-to-sample distance (2.6 m) ensured that
350 coherence and sampling conditions are fulfilled. The five-dimensional ptychography data set
351 was obtained by recording the 2D intensity patterns while scanning the sample across the
352 beam (along t_x and t_y) with steps of 45 nm. It provides an overlapping of approximately 70%
353 between two successive positions of the 9 x 9 point mesh. This strong spatial redundancy is
354 needed to ensure the convergence of the phase retrieval inversion process. After each mesh,
355 the incidence angle ω was modified ($\delta\omega = 0.007^\circ$) before a new mesh was performed. A total
356 of 286 points along the rocking curve was needed to cover the whole Bragg diffraction peak

357 extent (about 2°). The acquisition time, limited to 0.8 s per frame led to a maximum of
358 intensity of approximately 30 photons/pixel. In total, the acquisition time, including motor
359 motions, electronic reading of the detector and data transfers, took 9 hours corresponding to a
360 total illumination time of about 5.2 hours. For the reconstruction shown in the Supplemental
361 Information, the whole scan was slightly reduced: the rocking curve was limited to 230 points
362 and the acquisition time was limited to 0.5 s per frame. The whole scan took approximately
363 4.7 hours for a total illumination time of 2.6 hours.

364
365 For further analysis, the 3D intensity patterns were registered as a function of the momentum
366 transfer \mathbf{q} , where $\mathbf{q} = \mathbf{k}_f - \mathbf{k}_i$ and $\mathbf{k}_{i,f}$ is the incident (resp., exit) wave vector ($|\mathbf{k}_{i,f}| = 2\pi/\lambda$). A
367 relevant 3D frame for \mathbf{q} is given by the detection frame (q_1, q_2, q_3), where q_1 and q_2 are along
368 the detector plane (q_1 being additionally contained in the diffraction plane) and q_3 is aligned
369 the rocking curve direction.

370
371 The Bragg micro-diffraction experiments (further detailed in the Supplemental Information)
372 were performed in Bragg geometry on the same set-up as the one used for ptychography,
373 during a different beam time. The 12.36 keV incoming radiation ($\lambda = 1\text{\AA}$) was impinging onto
374 a partially illuminated Fresnel zone plate focusing element, resulting in a focal spot size of 0.3
375 $\times 0.2 \mu\text{m}^2$ (H x V, full width at half-maximum). The Bragg diffraction patterns were collected
376 in the vicinity of the 006 calcite Bragg reflection ($2\theta_B = 20.2^\circ$), in a reflection geometry,
377 leading to an elongation of the beam footprint onto the sample up to $1.7 \times 0.2 \mu\text{m}^2$. The
378 detector was placed 2 m away from the sample position. The 3D patterns were collected with
379 $\delta\omega = 0.1^\circ$ over the prism area, using translation steps of 5 and 1 μm along x and y ,
380 respectively.

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382
383 **3D Bragg ptychography phase retrieval.** The inversion of the five-dimensional data set was
384 performed with our 3D Bragg ptychography algorithms adapted from our 2D development⁴⁰.
385 In order to preserve the signal information, the phase retrieval algorithm was directly
386 performed in the detection frame, which is non-orthogonal and results in defining a non-
387 orthogonal direct space obtained from Fourier conjugation relations³⁵. The final 3D image
388 was transferred into the orthogonal laboratory frame.

389 The inversion was performed with an optimized procedure, extensively tested on noisy
390 numerical data, mimicking as much as possible the experimental conditions (probe, sampling,
391 scanning, intensity dynamical range, *etc.*), and detailed in the Supplemental Information. All
392 along, the error-metric derived from a Gaussian likelihood was used to account for the
393 photonic shot noise in the intensity measurements. A regularization term was introduced in
394 the criterion in order to favor thickness-limited solution. The thickness of the shell sample
395 was first refined directly from the inversion method. Indeed, we observed on numerical tests,
396 that the lower error metric value corresponded to the best agreement between the
397 regularization thickness and the true shell thickness. This was obtained with 100 cycles of
398 ordered-subset algorithm iterations, initiated with a sample structure of random values
399 (amplitude and phase). The best agreement was found for a sample thickness of 1.75 μm .
400 Once the regularization thickness was optimized, 100 cycles of truly converging conjugated
401 gradient algorithm were run, resulting in the presented reconstructions.

402
403
404 **Image analysis.** The 3D complex-valued solution resulting from the inversion process is the
405 sample scattering function ρ , which for a crystal can be expressed by⁴¹

$$\rho = |\rho| \exp(i\varphi)$$

407 where the phase φ is related to the crystalline properties through

$$408 \quad \varphi = \mathbf{G}_{110} \cdot \mathbf{u}$$

409 with \mathbf{u} the crystalline displacement field and \mathbf{G}_{110} the Bragg vector of the investigated
410 reflection ($G_{110} = 2.525 \cdot 10^4 \mu\text{m}^{-1}$). Hence, the knowledge of φ gives access to the projection
411 u_{110} of the displacement field onto the Bragg vector. From this quantity, one can access
412 several crystalline properties. As an example, with respect to crystalline coherence, u_{110} is
413 continuous in a coherent crystalline domain, while it presents a change (*e. g.*, a shift) in the
414 vicinity of a crystalline defect. Furthermore, the crystalline plane rotations around the two
415 directions perpendicular to \mathbf{G}_{110} can be extracted directly from the derivative of u_{110} with
416 regards to these two axes. In the same way, the derivative of u_{110} with regards to the
417 coordinate collinear to \mathbf{G}_{110} gives access to a relative strain ε (derivations of the complete
418 expressions can be found elsewhere³⁶).

419 For all investigated prisms, it was found that the two accessible crystalline plane rotations
420 were proportional to each other (see also the experimental diffraction pattern distributions
421 shown in the Supplemental Information, which are all elongated in the plane perpendicular to
422 the Bragg vector, *i. e.*, the (q_2, q_3) plane). Hence, the lattice orientation was expressed directly
423 with respect to the reduced coordinate δ , which measures the crystal rotation around the axis
424 perpendicular to the intensity distribution plane.

425

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Supplemental Figures 1 to 8 are provided as Supplementary Information.

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Author Contributions

VC, JDa and MB conceived the experiment with contributions from PGo, CC and PGu. PGo, MA and VC developed the inversion code. VC, MB, PGo, CC and JDa performed the experiments at ID13, ESRF. The 3D ptychography reconstructions were performed by FM with inputs and discussion from VC and PGo. JDu performed the white light microscopy experiment and performed and analyzed the CARS experiments. JNo performed the AFM experiments. FM, VC, JDa, CC and JNo wrote the manuscript with contributions from all co-authors.

Author Information

The authors declare no competing financial interest.

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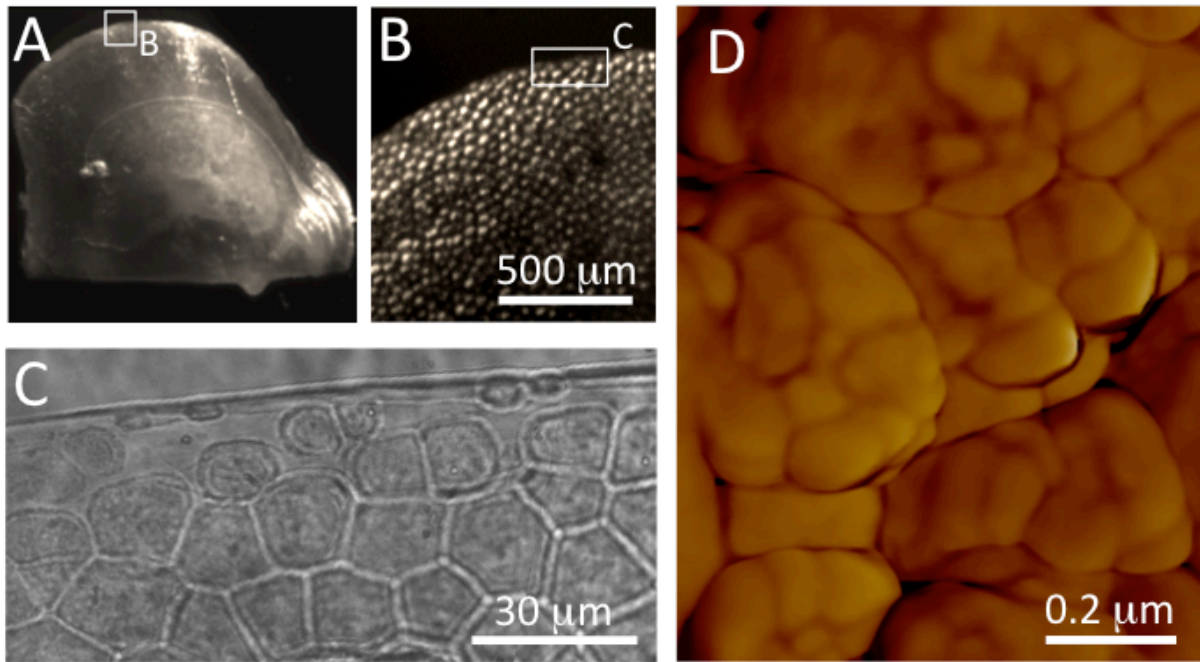
Correspondence should be sent to V. Chamard, virginie.chamard@fresnel.fr.

Code availability

Matlab routines for 3D Bragg ptychography reconstruction from an experimental data set are available on demand by contacting the corresponding author.

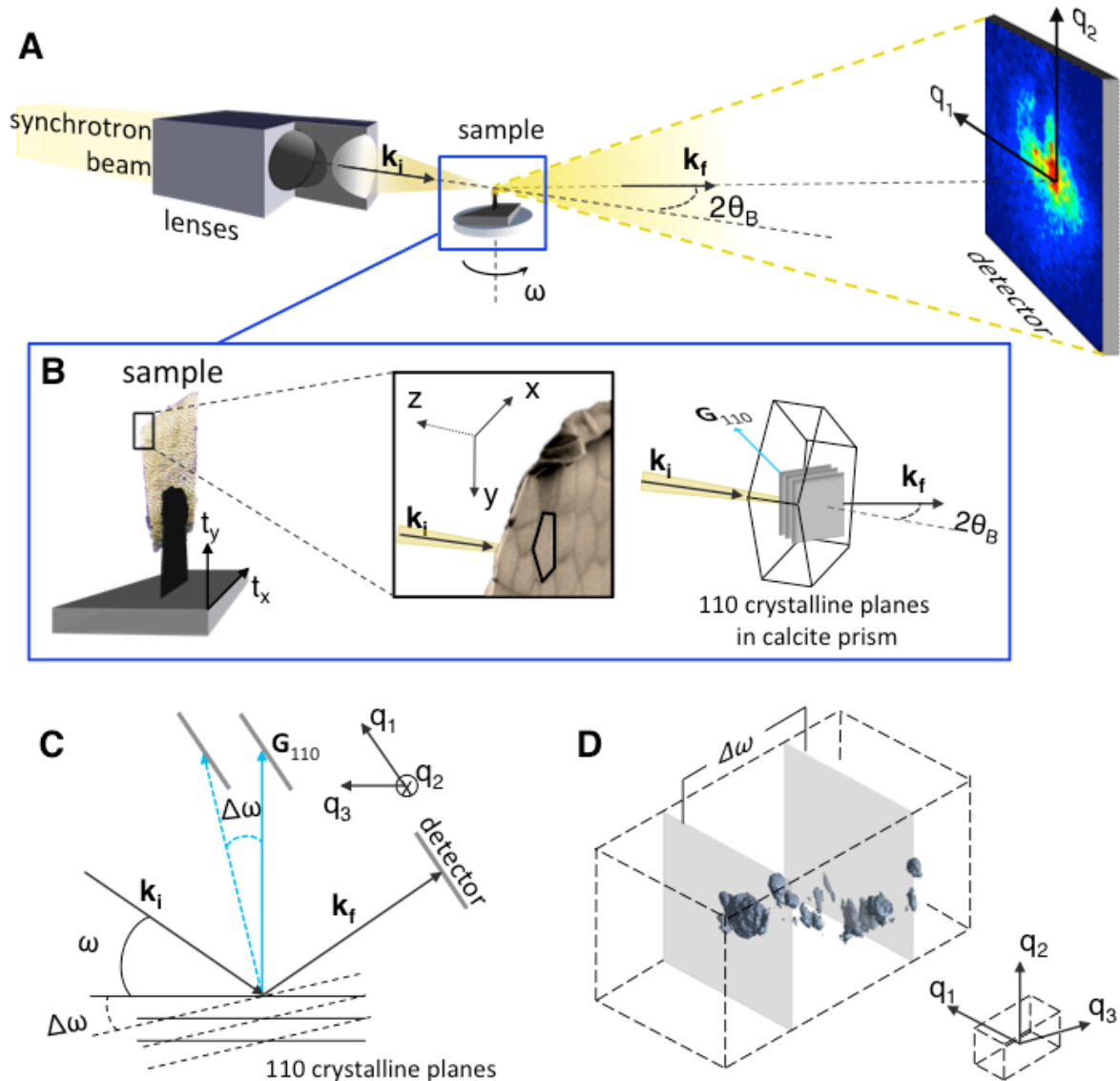
Data availability statement

The experimental data that support the plots within this paper are available on demand by contacting the corresponding authors.



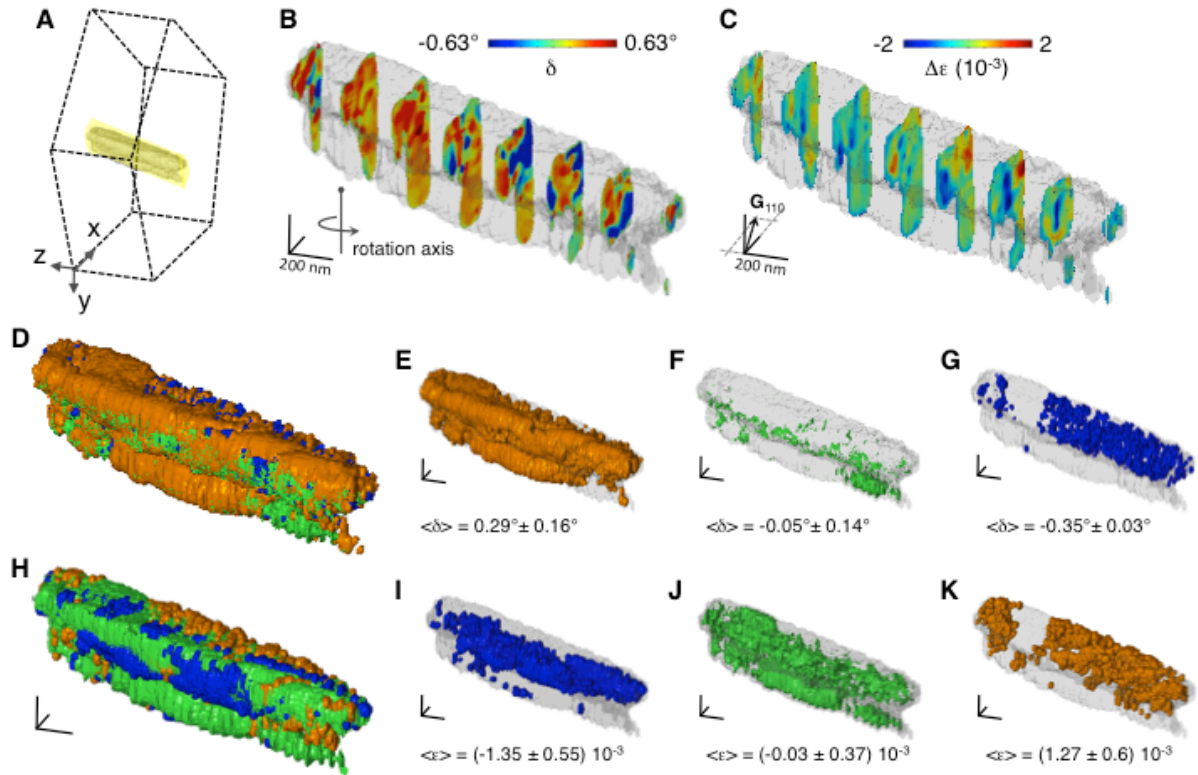
602

603 **Fig. 1: Structure of *Pinctada margaritifera* shell at different length scales.** (A-C) Optical
 604 micrographs of a juvenile *Pinctada margaritifera*. (A) The whole shell. (B) Zoom-in view
 605 showing the prism assembly in the vicinity of the growth border. (C) The shell growth border.
 606 One clearly identifies calcite prisms, delimited by thick organic walls, as well as isolated
 607 disks, the preliminary form of the prisms. (D) Atomic force microscopy images (phase
 608 contrast) obtained on the surface of a *Pinctada margaritifera* prism showing the granule
 609 assembly, a common structure of many calcifying organisms.



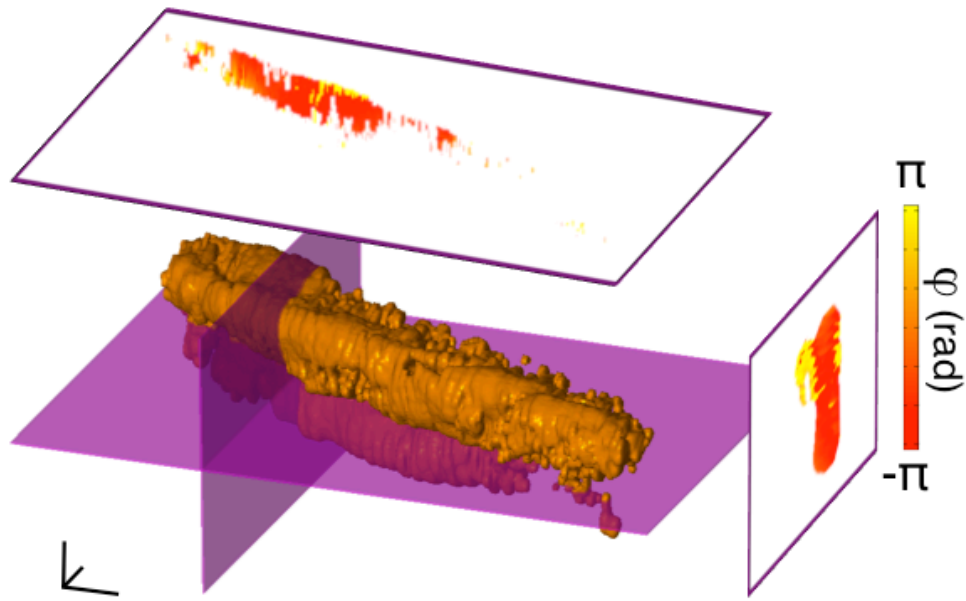
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611 **Fig. 2: 3D Bragg diffraction ptychography set-up.** (A) The sample is placed in the focus of
 612 a coherent x-ray nano-beam and oriented in Bragg diffraction condition in Laue geometry.
 613 The far-field intensity patterns are collected by a 2D detector. (B) (*left*) Details of the sample
 614 positioning together with the ptychographic translations (t_x and t_y), whose spatial
 615 displacements cover the region of interest. (*middle*) Zoom-in view of the sample where the
 616 selected prism is highlighted. The shell thickness is about $1\ \mu\text{m}$ at the border. The (x, y, z)
 617 orthogonal laboratory frame is related to the sample, with x - and y -axes along the surface of
 618 the prism and z -axis along the growth direction of the prism. The t_x and t_y ptychography
 619 scanning directions are collinear and anti-collinear to the x - and y -axes, respectively. (*right*)
 620 Chosen 110 Bragg reflection with respect to the prism. The G_{110} Bragg vector lies in the
 621 (x, z) plane. (C) Successive acquisitions of 2D diffraction patterns, obtained by scanning the
 622 sample along the incidence angle ω in steps of $\delta\omega$, result in the exploration of the 3D intensity
 623 distribution arising from the chosen crystalline family planes. The intensity is registered as a
 624 function of $\mathbf{q} = \mathbf{k}_f - \mathbf{k}_i$, in the 3D detection frame $(\mathbf{q}_1, \mathbf{q}_2, \mathbf{q}_3)$. (D) Schematic view of the 3D
 625 detection frame and iso-surface rendition of the 3D intensity distribution integrated over all
 626 illumination positions. Two detection planes, separated by n steps along the ω scan, are
 627 shown in grey.



628

629 **Fig. 3. 3D Bragg ptychography reconstruction.** (A) Schematic view of the reconstructed
 630 volume within the prism (in yellow-gray), embedded into the probed region (yellow cylinder).
 631 (B) and (C) Crystalline rotation (δ) and relative strain (ϵ) variation maps shown on several
 632 planes along the reconstructed volume, corresponding respectively to the rotation of the (110)
 633 crystalline planes around the rotation axis shown in (B) and to the relative crystalline
 634 displacement along \mathbf{G}_{110} . Iso-oriented and iso-strain regions, *i. e.*, regions presenting the same
 635 rotation or strain value, are evidenced. (D) Same as (B) shown as an isosurface rendering
 636 (with respect to δ values) of all iso-oriented crystalline domains. The colors encode the
 637 respective rotation angles δ according to the color-scale in (B). The total retrieved volume
 638 corresponds to the one shown in A. (E-G) Single iso-oriented crystalline domains, for three
 639 rotation angles δ indicated in the figure. (H) Same as (C) shown as an isosurface rendering
 640 (with respect to ϵ values) of all iso-strain domains. (I-K) Single iso-strain domains, for three
 641 relative strain ϵ indicated in the figure. The length scale is indicated in (B-K) and represents
 642 200 nm.



643

644 **Fig. 4: Crystalline coherence in the iso-oriented domain.** 2D cuts of the retrieved phase
 645 map φ extracted for the iso-oriented domain of Fig. 3E. This quantity is related to the
 646 crystalline coherence: abrupt changes in the phase value correspond to breakdown of the
 647 crystalline continuity. In average, the phase shift is about 4.2 radians, corresponding to a
 648 displacement of about 0.16 nm, *i. e.* $\approx 2/3$ of the (110) lattice spacing. The indicated length
 649 scale represents 200 nm.

650