

1 **Running head:** The algal Rubisco micro-compartment

2

3 **Title:** The Algal Pyrenoid: Key Unanswered Questions

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16

17 **Abstract**

18

19 The confinement of Rubisco into a chloroplast micro-compartment, or pyrenoid, is a
20 distinctive feature of many micro-algae, and possibly contributes to around 30 Pg of carbon
21 fixed each year. Our understanding of pyrenoid composition, regulation, and function,
22 however, are still fragmentary. The model pyrenoid of *Chlamydomonas reinhardtii* is
23 increasingly well-resolved under different regimes of light or inorganic carbon availability.
24 The emergence of molecular details in other lineages provides a comparative framework for
25 this review, and evidence that most pyrenoids function similarly, even in the absence of a
26 common ancestry. The objective of this review is to explore pyrenoid diversity throughout
27 key algal lineages and discuss whether common ultra-structural and cellular features are
28 indicative of common functional processes. By characterising pyrenoid origins in terms of
29 mechanistic and structural parallels, we hope to provide key unanswered questions which will
30 inform future research directions.

31

32 **Part A: Form Follows Function - Compartmentalisation Requirements For Operating A**
33 **CO₂-Concentrating Mechanism In A Single Cell**

34

35 Pyrenoids are permeable Rubisco-containing micro-compartments present in the chloroplast
36 stroma of many, but not all, algae operating a biophysical CO₂-concentrating mechanism
37 (CCM) (Badger *et al.*, 1998; Raven, 2010). CCMs enhance the CO₂ concentration near the
38 primary carboxylating enzyme, Rubisco, through the coordinated action of membranal
39 inorganic carbon (C_i) pumps, one or more carbonic anhydrases (CA), and generally, the
40 packaging of Rubisco into one, or multiple pyrenoids. Common in unicellular, colonial or
41 filamentous algae with examples in nearly all lineages except Chrysophytes (Lee, 2008;
42 Maberly *et al.*, 2009), pyrenoids are rarer in frond-forming seaweeds (pyrenoid-positive
43 examples include the sea lettuce *Ulva* or the edible red alga *Porphyra*). The prokaryotic
44 analogues of pyrenoids, carboxysomes, are an obligatory feature of cyanobacterial CCMs
45 (reviewed in Raven *et al.*, present issue), whereas pyrenoids are not obligatory. When present,
46 pyrenoids physically separate the site of CO₂-fixation from C_i accumulation by the CCM
47 machinery (primarily thought to be via plasmamembrane and chloroplast envelope, and
48 perhaps direct delivery to the pyrenoid). Such an aggregation of Rubisco enhances CCM
49 effectiveness, as demonstrated empirically through quantification of CCM-leakiness, and loss
50 of C_i accumulation affinity, when Rubisco is redistributed to the stroma in *Chlamydomonas*
51 mutants (Meyer *et al.*, 2012).

52 The advantages of a pyrenoid were initially quantified in unicellular green algae
53 through demonstrations that the concentration of internal C_i was 5-10X higher in pyrenoid
54 possessing algae than phylogenetically close species lacking the chloroplastic micro-
55 compartment (Morita *et al.*, 1998). Pyrenoids can therefore be viewed as an evolutionary
56 adaptation enhancing the performance of a basal CCM consisting only of C_i pumps and

57 CA(s), within the constraints of a single cell, without the need for multicellular specialisation.
58 The absence of a C_i impermeable boundary or shell around the pyrenoid nevertheless imposes
59 an additional compartmentalisation requirement: where to localise the CA that dehydrates C_i
60 to CO_2 such that leakage is minimised? Current models posit its localisation either to the
61 lumen of trans-pyrenoidal thylakoids or at the pyrenoid periphery. The seminal experiment
62 by Price and Badger (1989) demonstrating that in cyanobacteria, a CA must be packaged
63 alongside Rubisco into the micro-compartment to avoid short-circuiting the CCM, has yet to
64 be validated in eukaryotic algae.

65 The Rubisco matrix can be either naked or enclosed by starch plates forming a sheath,
66 together with peri-pyrenoidal protein complexes or parts of the chloroplast envelope, with
67 likely effects on CO_2 permeability. Membranes can provide a conduit between the stromal
68 pool of C_i and the heart of the pyrenoid, but C_i entry could also occur *via* proximal diffusion
69 or other channels. These peripheral elements, when present, should probably be viewed as
70 defining features of pyrenoids. The first part of this review will integrate recent developments
71 with information from the historical literature to update our understanding of the three major
72 pyrenoid components - a Rubisco matrix (common to all pyrenoids), thylakoid lamellae
73 traversing the matrix, and peripheral elements – and conclude with models on how these
74 could interact.

75

76 *Composition And Inner Architecture Of The Pyrenoid Matrix*

77 The word pyrenoid (from the Greek *pyrene*, stone or kernel-like) was coined by Schmitz
78 (1882) to describe highly refractive near-spherical inclusions in algal chloroplasts examined
79 through a light microscope. Schmitz observed pyrenoids in the majority of green algae he
80 studied, to a lesser extent in red algae and only occasionally in brown algae. The generalised
81 use of transmission electron microscopy (TEM) from the early 1950s onward greatly

82 facilitated the diagnosis of pyrenoid presence/absence, as the matrix of these micro-
83 compartments appears as uniformly electron-dense inclusions in the stroma. Arguably, it was
84 only with the advent of techniques capable of discerning Rubisco localisation (*e.g.* immuno-
85 gold labelling, indirect-immunofluorescence tagging or translational fusions with fluorescent
86 proteins) that pyrenoids were incontrovertibly established as the site of Rubisco localisation
87 (see Fig. 1).

88 Early biochemical analysis of pyrenoids isolated from green and brown algae found
89 that ~90% of the matrix was composed of biochemically active Rubisco, alongside a dozen or
90 so other unidentified proteins (reviewed in Meyer & Griffiths, 2013). For green algae, this
91 included the chaperone Rubisco Activase, where localisation to the matrix was confirmed by
92 immuno-cytochemistry (McKay & Gibbs, 1991a; Suess *et al.*, 1995). Non-green algae do not
93 code for Rubisco Activase, but express a CbbX protein instead (reviewed in Kroth, 2015),
94 which belongs to an unrelated AAA+ ATPase gene family with an Activase-like property
95 (Mueller-Cajar *et al.*, 2011). Whether CbbX also localises to non-green pyrenoids remains, as
96 yet, unknown. Pyrenoid compositional analysis was refined by mass spectrometry for
97 *Chlamydomonas* (Mackinder *et al.*, 2016), which in addition to Rubisco and Rubisco
98 Activase, identified EPYC1 (formerly known as LCI5, Miura *et al.*, 2004). EPYC1 was
99 particularly abundant in pyrenoids isolated from cells acclimated to CCM-active conditions
100 (*i.e.* grown under air-level CO₂) and is speculated to act as a linker that either recruits
101 Rubisco to the matrix or serves as an anchoring scaffold for the enzyme. Genes coding for
102 proteins with properties similar to those of EPYC1 are present in other pyrenoid-positive
103 algae (*e.g.* diatoms and haptophytes) (Mackinder *et al.*, 2016).

104 It is still unclear to what extent, if at all, Rubisco is arranged periodically within the
105 matrix. Resin embedding for TEM usually obliterates the native arrangement of the enzyme,
106 leaving pyrenoids to appear as amorphous. There have been, however, several reports of

107 para-crystalline structures of the matrix or a fraction thereof, *e.g.* in Chlorophytes (Bertagnoli
108 & Nadakavukaren, 1970) and Charophytes (Gärtner & Ingolić, 1989), in diatoms
109 (Holdsworth, 1968; Taylor, 1972), in dinoflagellates (Kowallik, 1969), in Haptophytes
110 (Leadbeater & Manton, 1971), and in red algae (McBride & Cole, 1972; Tsekos *et al.*, 1996).
111 These early studies concluded a possible cubic- or hexagonal-closed packing. Hexagonal-
112 closed packing was also identified in a recent study of the fine architecture of the
113 *Chlamydomonas* pyrenoid, using techniques that preserve the native molecular conformation,
114 by Engel and co-workers (2015). Although the analysis was limited to small areas of the
115 matrix and the alignment was not perfectly crystalline, it fitted models of periodically
116 arranged Rubisco linked directly by EPYC1 (Mackinder *et al.*, 2016).

117 Encouraged by early successes in isolating pyrenoids, comparative MS studies of
118 pyrenoid composition should now be undertaken on key representatives of all major
119 phytoplankton lineages, to identify additional components helping to aggregate Rubisco and
120 other factors that are commonly present. Understanding the fine mechanistic details will also
121 require crystallographic reconstructions of protein-protein interactions and validation through
122 the characterisation of mutants, which is now increasingly possible in the model system
123 *Chlamydomonas*, for which insertional mutant libraries covering more than 80% of all coding
124 genes are available (Li *et al.*, 2016).

125

126 *Function Of Pyrenoidal Tubules And Association With Carbonic Anhydrases*

127 Most, but not all pyrenoids appear to be traversed by at least one lipid bilayer, usually, but
128 not always, in continuity with the stromal network of thylakoids. These membranes are
129 assumed to play a role in the delivery of CO₂ to Rubisco. The complexity of the membrane
130 network traversing the Rubisco matrix, when present, has been used as a taxonomic marker
131 (*e.g.* in dinoflagellates, Dodge, 1968, or diatoms, Schmid, 2001). Fig. 2 illustrates the

132 diversity and complexity of this feature, as it appears in thin TEM sections, throughout key
133 algal lineages. The simplest is in the form of a single membrane bisecting the Rubisco matrix,
134 as in many *Chlorella* species (Ikeda & Takeda, 1995; see also recent example in Treves *et al.*,
135 2016) or diatoms (Schmid, 2001). Multiple, non-connecting parallel membranes, are common
136 in green algae, dinoflagellates and Euglena (Kusel-Fetzmann, 2008). More complex
137 morphologies have been observed in the unicellular red alga *Porphyridium cruentum*, where
138 a highly anastomosed network increases the surface area in contact with Rubisco (McKay &
139 Gibbs, 1991a). The pattern is somewhat reminiscent of the one found in the green alga
140 *Zygnema*, which computational 3D reconstructions revealed to match a gyroid cubic
141 organisation of photosynthetic membranes (Zhan *et al.*, 2017). The *Chlamydomonas* pyrenoid
142 is structurally the best resolved, following the work by Engel and colleagues (2015), building
143 on earlier studies (Sager & Palade, 1957; Ohad *et al.*, 1967). Here, pyrenoid-specific
144 membranes are formed by the fusion of stromal thylakoids into cylindrical membranes ~100
145 nm across, called tubules. These tubules are continuous with stromal thylakoids. When
146 extending into the pyrenoid matrix, tubules twist and turn at sharp angles to fuse into an
147 interconnected star-shaped network, or knot, at the pyrenoid centre (see SI animation).
148 Additionally, tubules contain within their lumen between two and eight mini-tubules, formed
149 “outside-in” as the thylakoid membranes coalesce. As a result, mini-tubules enclose their own
150 luminal phase, which is continuous with the chloroplast stroma. Mini-tubule dimensions are
151 sufficient for the transit of small molecules like Rubisco substrates and products but too small
152 for the shuttling of larger proteins, say a CA. There is no evidence yet that trans-pyrenoid
153 membranes in other algae also possess similar inner channels. We speculate that the central
154 star-shaped knot of tubules of the *Chlamydomonas* pyrenoid could play a role in situating and
155 anchoring the Rubisco matrix in a conserved chloroplastic locus.

156 Two lines of evidences support the notion that tubules may serve a similar function
157 across a wide range of algal lineages. The first pertains to a CCM critical CA: in
158 *Chlamydomonas* and in the marine diatom *Phaeodactylum tricornutum*, this CA is localised
159 to the lumen of tubules (Karlsson *et al.*, 1998; Blanco-Rivero *et al.*, 2012; Kikutani *et al.*,
160 2016). It is pivotal to the functioning of the CCM (mutants have a high-CO₂ requiring
161 phenotype) but sets an additional requirement for a C_i transporter without which the stromal
162 pool of C_i could not be fed to the luminal CA for conversion to CO₂. *Chlorella* also has a CA
163 associated with trans-pyrenoidal thylakoids (Villarejo *et al.*, 1998), but whether it is luminal
164 or even essential to the CCM has yet to be demonstrated. A second line of evidence is the
165 biochemical nature of pyrenoidal tubules, which is distinct from stromal thylakoids. O₂-
166 evolving Photosystem II (PSII) are absent from these membranes in green algae (McKay &
167 Gibbs 1991a), red algae (Mustardy *et al.*, 1990) and diatoms (Pyszniak & Gibbs, 1992). This
168 is maybe an evolutionary adaptation to minimise oxygen production in the vicinity of
169 Rubisco, and hence potential for oxygenation, but the mechanism by which PSII is excluded
170 has yet to be investigated. Light harvesting antennae of PSII and their accessory pigments are
171 also excluded from pyrenoids, as shown by localisation experiments of phycobilisomes and
172 phycoerythrin in red algae (McKay and Gibbs, 1990a; Tsekos *et al.*, 1996).

173 Universality of the above arrangement is challenged by tubule-less pyrenoids. Stalked
174 pyrenoids that bulge from the chloroplast into the cytosol in a sac-like structure are frequently
175 not traversed by membranes. In these pyrenoids, the Rubisco matrix is almost fully enclosed
176 by the chloroplast envelope, and in species with a secondary or higher order chloroplast, by
177 additional lipid-bilayers. Tubule-less pyrenoids are common in Phaeophytes, dinoflagellates
178 (Dodge, 1973), and Chlorarachniophytes (Ishida *et al.*, 1999). In most instances however,
179 part of the Rubisco matrix is at least tangentially in contact with stromal thylakoids. Tubule-
180 less pyrenoids are also observed to a limited extent in red algae, green algae and diatoms. If

181 we discount the possibility that published micrographs simply failed to capture rare
182 membranes, it will be important to localise the closest CA, and determine whether Ci
183 accumulation is stromal in these species. Part of the answer will also come from better
184 imaging of pyrenoids, either through increased use of 3D sectioning and reconstruction
185 microscopy (*e.g.* focused ion beam or serial block face scanning-electron microscopy; see SI
186 animation) or through confocal imaging of pyrenoid-specific proteins.

187

188 *Pyrenoid Matrix Peripheral Structures*

189 The Rubisco matrix of green algal pyrenoids is often surrounded either partially, or almost
190 entirely, by starch. Red algae and algae that inherited a red algal chloroplast through
191 secondary endosymbiosis can also have their pyrenoid encased by starch, but only when it is
192 stalked (Ford, 1984). This can easily be explained by differences in site of starch synthesis
193 and deposition: it is stromal in “greens” but cytosolic in “reds”. The close spatial relationship
194 between starch and pyrenoids, even when situated in different cellular compartments, is
195 perhaps indicative of a positive role for carbohydrate deposition in the CCM. In green algae,
196 starch formation around the pyrenoid is controlled by light and the state of CCM induction
197 (Kuchitsu *et al.*, 1988; Ramazanov *et al.*, 1994; Lin & Carpenter, 1997; Borkhsenius *et al.*,
198 1998), which in turn also determine the maximal packaging of Rubisco to the pyrenoid
199 matrix (Mitchell *et al.*, 2014; Tirumani *et al.*, 2014). A starchless *Chlorella* mutant with
200 naked pyrenoid has been used to question the role of the starch sheath in the CCM (del Pino
201 Plumed *al.*, 1996), but *Chlamydomonas* mutants with partial or no starch sheath have a high-
202 CO₂ requiring phenotype (Thyssen *et al.*, 2003). There is therefore a pressing need to clarify
203 the relationship of starch and the CCM and to further investigate the distinct nature of
204 pyrenoidal and stromal starch granules (Izumo *et al.*, 2007).

205 Calvin Benson Basham Cycle (CBBC) enzymes other than Rubisco are absent from
206 the *Chlamydomonas* pyrenoid matrix (Suess *et al.*, 1995). McKay & Gibbs (1991b) found
207 phosphoribulose kinase (PRK), which operates just upstream of Rubisco, in stromal
208 inclusions of pyrenoid tubules (which may in fact represent mini-tubules *sensu* Engel *et al.*,
209 2015), and proposed that this provided a means for exchanging CBBC metabolites between
210 pyrenoid and stroma. However, PRK forms a dimer of >70 kDa, which is well above the
211 estimated size-exclusion of mini-tubules, qualifying the localisation of this enzyme to the
212 pyrenoid. The co-purification of Fructose-1,6-bisphosphatase with *Chlamydomonas*
213 pyrenoids and their starch sheath (in SI Mackinder *et al.*, 2016) suggests that the CBBC may
214 nevertheless operate in close proximity to the pyrenoid. Identifying the CBBC location in
215 relation to the starch synthesis pathway would also clarify the role of the different starch
216 forms in algae with chloroplastic starch.

217 Finally, evidence is emerging in *Chlamydomonas* that there is yet another layer to
218 pyrenoids, in the form of a network of proteinaceous complexes residing outside the starch
219 sheath (~440 kDa, encoded by two genes, *lcib* and *lcic*). Crystallisation of the two monomers,
220 as well as the finding of a functional homologue in the diatom *P. tricornutum* (Jin *et al.*,
221 2016), confirmed that these proteins had a typical CA fold, although no CA activity was
222 found in *Chlamydomonas*. It therefore remains open to debate what purpose this complex
223 serves. A true CA in the stroma, as mentioned above, would short-circuit the CCM. It is
224 tempting to speculate that LCIB-LCIC, subject to tight regulation, could be active only when
225 CO₂ concentrations are in excess of other C_i species, and the CA-moiety operates uni-
226 directionally from CO₂ to bicarbonate, acting to recapture CO₂ leaking from the pyrenoid.
227 Immuno-gold labelling of LCIB and LCIC revealed deposition in pockets rather than forming
228 a continuous ring around the pyrenoid (Yamano *et al.*, 2010) and it must be clarified whether
229 these coincide with the starch plate interfaces and thylakoid tubule entry points, where

230 leakage of CO₂ is likely to be maximal. Alternatively, the complex could serve as a non-
231 catalytic structural barrier, or even play a positional role in situating the pyrenoid in the
232 chloroplast, as suggested by pyrenoid-mislocalisation phenotypes in mutants with aberrant
233 localisation of LCIB (Yamano *et al.*, 2014).

234

235 **Part B: Pyrenoid Plasticity And Dynamics Across Cell Divisions**

236 In addition to the diversity across different algal species highlighted above, the pyrenoid is
237 also highly plastic, changing in terms of morphology and composition in response to different
238 cues, both endogenous and externally derived. This section focuses on the former, and
239 specifically assesses the way in which pyrenoid morphology, structure and composition
240 change as a function of cell-cycle progression. Additionally, the way in which the pyrenoid is
241 accommodated through the process of cytokinesis and cell division is also considered. At all
242 stages, considerations are not restricted to the model alga *Chlamydomonas reinhardtii*, and a
243 wide range of algal species are used to assess the existence of commonalities, and inform
244 evolutionary considerations.

245

246 *Pyrenoid Dynamics And The Cell Cycle*

247 Progression through the cell cycle necessitates significant changes to the physiology and
248 functioning of an algal cell, given the extensive preparations that must be undertaken before
249 division can successfully occur. Many of these significant changes, and their effect on the
250 pyrenoid, are often lost to studies using asynchronous cell populations, and thus many of the
251 associations between cell cycle stage and pyrenoid/CCM functionality remain under-explored.
252 This section will consider how the composition and structure of the pyrenoid changes as the
253 cell progresses through the stages of the cell cycle, and specifically during cytokinesis and
254 the act of cell division.

255 Previous studies assessing the activity of the CCM over time have shown it to vary
256 with the cell cycle (Sültemeyer, 1997) but whether this is due to variation in pyrenoid
257 function or the activity and abundance of other CCM components remains outstanding.
258 Considerations of different algal species have produced contrasting results. The localisation
259 of Rubisco to the pyrenoid during the cell cycle of the green alga *Dunaliella tertiolecta*,
260 suggest that aggregation of Rubisco was independent of cell cycle stage, and was more likely
261 to be associated with the active growth phase (Lin & Carpenter, 1997). By contrast, results
262 observed in the brown alga *Scytosiphon lomentaria* highlighted the formation of new Rubisco
263 aggregates separate from existing pyrenoids during mid-S phase (Nagasato *et al.*, 2003).
264 Disruption of the cell cycle using specific pharmacological agents further clarified the
265 relationship between changes in pyrenoid morphology and specific cell cycle events:
266 blocking DNA replication using aphidicolin inhibited the formation of new pyrenoids,
267 whereas the disruption and blocking of the process of mitosis using nocodazole resulted in an
268 increased size of Rubisco aggregates compared to untreated cells. Addition of
269 chloramphenicol resulted in no new occurrence of pyrenoids, despite the successful
270 completion of mitosis and cytokinesis, suggesting that these aggregates were a product of
271 newly-synthesised Rubisco.

272 This study points to the role of distinct cellular events in shaping pyrenoid
273 composition, morphology and structure during specific cell cycle events. Overall however,
274 there is a relative paucity of data analysing the impact of the cell cycle on pyrenoid dynamics,
275 and the efforts that are present throughout the literature have been restricted to a limited
276 number of algal species. More work is needed to assess the effect of cell cycle on pyrenoid
277 structure and composition, and by extension, CCM function. There is also a need to
278 characterise such putative cell cycle dependencies at a molecular level - recent evidence
279 suggests that as much as 80% of the *Chlamydomonas* transcriptome displays a strong

280 periodicity in cells where the cycle has been synchronized under a standard dark-light cycle
281 (Zones *et al.*, 2015). Thus studies linking the ultrastructural changes observed to cell cycle
282 dependent changes in transcriptional output would be highly instructive in furthering our
283 understanding of the processes driving pyrenoidal dynamics throughout the cell cycle.

284

285 *Pyrenoid Dynamics During Cell Division*

286 Pyrenoid morphology and dynamics have been explored extensively during the process of
287 mitotic cell division. Such a cell division necessitates the equable distribution of parental
288 contents to daughter cells and poses problems for cells whether containing a single pyrenoid
289 or multiple pyrenoids. Griffiths (1970) broadly divided pyrenoid containing algae into two
290 groups based on the behaviour of the pyrenoid during the process of mitosis. One group
291 encompassed species wherein the pyrenoid divides either prior to or concomitant with the cell
292 division, whereas the other consisted of algae where the pyrenoid disappears during division,
293 and reforms *de novo* in daughter cells. There is perhaps one other possibility not considered
294 by Griffiths, which is potential *de novo* pyrenoid formation in parental cells, followed by
295 distribution to daughter cells. Surveys of the available literature provide supporting evidence
296 for the existence of each of these processes, in different algal species, and will be explored
297 below.

298

299 *De Novo Pyrenoid Formation - Before And After Cell Division*

300 One of the first reports utilising electron microscopy to study the pyrenoid examined the
301 green colonial alga *Scenedesmus quadricauda* and identified the disappearance of the
302 pyrenoid in parental cells, followed by reappearance in daughter cells, suggesting dissolution
303 and *de novo* formation (Fig. 3A) (Bisalputra & Weier, 1964), a phenomenon supported by
304 more contemporary reports (Vítová *et al.*, 2008). Experiments conducted in another green

305 alga, *Tetracystis excentrica* obtained similar results, with “regression and dissolution” of the
306 pyrenoid prior to cell division observed (Brown & Amott, 1970). A similar phenomenon was
307 also observed in *Euglena gracilis*, which possesses a secondary chloroplast, with
308 disappearance of the pyrenoid prior to cell division, followed by reformation in daughter cells
309 (Osafune *et al.*, 1990). Other algae appear to lack the dissolution mechanism - in *Volvulina*
310 *steinii* a single daughter cell inherits the parental pyrenoid, implying *de novo* formation
311 following cell division in the other (Fig. 3B) (Nozaki *et al.*, 1987). Other species, such as
312 *Scytosiphon lomentaria* form a second pyrenoid *de novo* in the parental chloroplast prior to
313 cell division, with the two pyrenoids now present in the chloroplast then being equally
314 distributed among daughter cells upon division (Fig. 3C) (Nagasato & Motomura, 2002).
315 Whilst the observation of *de novo* pyrenoid formation across a range of algal species supports
316 the existence of this mechanism as a means of ensuring pyrenoidal continuity across cell
317 divisions, there is some ambiguity surrounding the exact nature of the bodies forming *de*
318 *novo* in some of these strains. In her landmark 1970 study, Goodenough observed a number
319 of dense bodies that superficially appear similar to the pyrenoid, although ultimately
320 discounted the notion that they might represent new pyrenoids. Irrespective, it is apparent that
321 if such a premise of *de novo* pyrenoid formation is correct, it inevitably raises numerous
322 questions, perhaps most notably with regards to the location in which they form. Specifically,
323 why do they form there and are there any features of that particular location, ultrastructural or
324 otherwise, that are permissive, conducive or essential to pyrenoid formation? Addressing
325 such questions through comprehensive studies of a diversity of different algae will allow
326 physical and structural features of the chloroplast to be correlated with *de novo* pyrenoid
327 formation and the processes underpinning biogenesis.

328

329 *Pyrenoid Fission*

330 The process of pyrenoid fission during mitosis is comparatively well established. Electron
331 microscopy based experiments in *Chlamydomonas* established that both the pyrenoid and
332 chloroplast in this algal species divide by fission (Fig. 3D) (Goodenough, 1970). In this study,
333 a marked increase in pyrenoidal mass prior to cell division was observed, concomitant with a
334 lateral elongation perpendicular to the plane of the furrow driving chloroplast fission;
335 subsequent narrowing of the furrow and further elongation ultimately results in a roughly
336 even partitioning of the pyrenoid, and the formation of two daughter pyrenoids from the
337 original parent. This phenomenon has similarly been observed in a wide array of different
338 algal species, including *Porphyridium cruentum* (Gantt & Conti, 1965), *Porphyridium*
339 *purpureum* (Schornstein & Scott, 1982), *Pleurochrysis haptonemofera* (Hori & Inouye, 1981)
340 and *Isochrysis galbana* (Hori & Green, 1985). In addition to these red algae and haptophytes,
341 division of the pyrenoid in this way has also been observed and confirmed in the brown algal
342 species *Cylindrocapsa germinella* (Sluiman, 2004), as well as *Splachnidium rugosum* and
343 *Scytothamnus australis* (Tanaka et al., 2007). Thus, the process of fission and its role in
344 ensuring equitable distribution of pyrenoids appears to be a common phenomenon present
345 across many evolutionarily distinct clades of pyrenoid possessing algae. In context of this
346 apparent conservation of pyrenoidal fission across a diverse range of algal strains, an
347 interesting question arises from consideration of the morphological diversity in body plans
348 that exists among these species - whereas some are polarised (as for *Chlamydomonas*
349 *reinhardtii*), others (such as *Porphyridium purpureum*) are radially symmetric, and thus,
350 despite conservation of the process by which pyrenoidal division occurs, the mechanisms
351 underpinning such divisions might be differentially regulated.

352

353 *FtsZ And A Role For the Ancestral Contractile Machinery?*

354 The question remains however as to how exactly such a process might occur: increasing
355 evidence highlights a role for plastid division proteins such as FtsZ. Originally descended
356 from cyanobacterial cell division proteins (Miyagishima & Kabeya, 2010), the GTPase FtsZ
357 assembles into a ring-like structure on the stromal surface of the chloroplast prior to division.
358 Through a poorly understood mechanism, FtsZ, along with components on the cytoplasmic
359 face of the chloroplast, then generates the contractile force required for membrane
360 constriction, and eventually, fission (Osawa *et al.*, 2008). Recent work by Hirakawa *et al.*
361 demonstrated the function of FtsZ proteins in the secondary plastid of chlorarachniophytes,
362 and in *Bigeloviella natans*, both FtsZD-1 and FtsZD-2 formed a ring-like structure that
363 bisected the midpoint of a bilobate pyrenoid found in the secondary chloroplast of this
364 species (Hirakawa & Ishida, 2015). This ring was constitutively present at this region and
365 was associated with a shallow furrow that penetrated the pyrenoid. Intriguingly, qPCR
366 analysis of gene expression was not suggestive of an involvement with the actual act of
367 plastid division *per se*, instead being upregulated following cell division, perhaps suggesting
368 a role in determining pyrenoid positioning following establishment of the daughter
369 chloroplast.

370 The study highlights the role that FtsZ proteins have in affecting pyrenoid
371 morphology. Such results are further supported by studies of algae possessing primary
372 plastids, namely *Scenedesmus quadricauda* where immuno-electron microscopic approaches
373 identified FtsZ structures localised around pyrenoids (Vítová *et al.*, 2008). Whilst it was
374 unclear whether such structures were rings or a hitherto unobserved spherical arrangement, it
375 highlighted the existence of FtsZ proteins not directly associated with the stromal
376 chloroplastic membrane, and is suggestive of a functional role for these proteins in pyrenoid
377 morphology, possibly coordinating the division of the chloroplast with the pyrenoid.
378 Intriguingly, Vitova *et al.* noted that FtsZ levels did not differ between untreated cells and

379 cells in which DNA replication had been inhibited. Thus, control of activity, rather than
380 expression levels, might be the key factor delimiting FtsZ activity to specific stages of the
381 cell cycle. The question remains however as to how FtsZ activity might be temporally
382 delimited to the period immediately leading up to cell division.. There is significant evidence
383 that phosphorylation has the capacity to affect the functionality of structural components
384 involved in cell and plastidial division. Phosphorylation has long been known to reversibly
385 control the localisation of various microtubule associated proteins with the actin cytoskeleton
386 in the cytoplasm (Ozer & Halpain, 2000). Phosphorylation modulates interactions between
387 bacterial cell division components, notably FtsZ and FipA (Sureka *et al.*, 2010) in
388 mycobacteria. The strong conservation of these components across both algal and prokaryotic
389 lineages suggests that in similar systems, activity of FtsZ can be modulated by
390 phosphorylation. Previous work had highlighted the strong dependence of the
391 phosphorylation state of thylakoid proteins on the stage of the cell cycle in *Chlamydomonas*
392 (Marcus *et al.*, 1986), thus raising the possibility that dynamic and reversible chemical
393 modification of FtsZ might play a role in delimiting its mechanical effects on pyrenoid
394 morphology to specific periods of the cell cycle.

395

396 *Evolutionary Routes To Diversity In Accommodating The Pyrenoid*

397 It appears that there exists substantial diversity between algal species as to how the pyrenoid
398 is accommodated during the process of cell division. Mounting evidence suggests a
399 functional role for the cyanobacterially derived family of FtsZ proteins and indeed, their
400 ancestral nature is consistent with the observation of pyrenoidal fission across a wide range of
401 evolutionary disparate algal species. The existence of others modes of ensuring successful
402 propagation of pyrenoids to daughter cells raises intriguing possibilities - the fact that some
403 plastid division proteins are depleted in certain algal species (Miyagishima *et al.*, 2014) raises

404 the question as to whether loss of FtsZ might have prompted the diversification of pyrenoid
405 accommodation strategies during cell division from fission to other methods, such as
406 dissolution followed by *de novo* formation. Why this loss might occur is unclear, and indeed,
407 it would perhaps be considered disadvantageous given the evident capacity for such a system
408 to automatically couple pyrenoid division to plastid and cellular division. Analyses
409 examining the presence or absence of particular FtsZ proteins across different algal strains
410 employing different pyrenoid division processes would be particularly timely, allowing
411 differences in presence/absence to be related to the phenotype observed.

412

413 **Conclusion – Integrating pyrenoid composition and dynamics**

414 Notwithstanding independent origins, that will be clarified when the detailed molecular
415 compositions can be compared across algal lineages, pyrenoids appear to deliver saturating
416 concentrations of CO₂ to Rubisco on a limited set of functional elements. Identifying
417 interactions within the Rubisco matrix and between the matrix and tubules and peripheral
418 elements should now be a major priority for the CCM research community. Fig. 4 illustrates
419 three possible modes of high-level pyrenoid biogenesis and regulation. Interacting inter-
420 dependencies (Fig. 4a) depicts a model in which major component are under independent
421 genetic control. The recruitment of Rubisco to and the situation of the matrix within the
422 chloroplast requires a tightly regulated interplay between all three components. Proof of
423 concept could be the finding of mutants which retain tubules and/or pyrenoid-peripheral
424 elements in a locus where the pyrenoid would normally form, even when the Rubisco matrix
425 is lost. Hierarchical or “Russian nesting doll” model (Fig. 4b) assumes a unidirectional and
426 sequential formation (and dispersion when the CCM is repressed), starting with the
427 deposition of Rubisco around a conserved anchoring site, with secondary deposition of
428 peripheral elements. The hybrid model (Fig. 4c) integrates the two previous ones and

429 accounts in particular for the observation that a CO₂-acclimation independent fraction of
430 Rubisco is always retained in the pyrenoid (as in *Chlamydomonas reinhardtii*, Borkhsenius
431 *et al.*, 1998; Mitchell *et al.*, 2014). Simpler models can be derived for naked pyrenoids and
432 pyrenoids not surrounded by peripheral elements.

433 Though now published over 50 years ago, Bisalputra and Weier were correct in their
434 declaration that ‘to understand the function of such organelles as the pyrenoid, developmental
435 studies are necessary’ (Bisalputra & Weier, 1964). Though perhaps not completely correct in
436 their categorisation of the pyrenoid, the notion they put forth is as timely then as it is now. To
437 truly comprehend the dynamic, malleable structure that is the pyrenoid, and by extension, its
438 role and place in the existing CCM paradigm, approaches that explore the variability in this
439 sub-cellular micro-compartment, both across the cell cycle and across a range of algal species
440 are required.

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Figure Legends

Figure 1: Pyrenoid diagnostic in *Chlamydomonas reinhardtii*

(a) The pyrenoid matrix and surrounding starch sheath are easily identifiable in unicellular green algae, as illustrated by the model alga *Chlamydomonas*, using only light microscopy (here, enhanced with Nomarski interference contrast). (b) In electron microscopy, the pyrenoid appears as electron dense matrix traversed by trans-pyrenoidal thylakoids (tubules), surrounded by slightly spaced starch plates, indicating that the carbohydrate deposition does not fully encapsulate the Rubisco matrix. (c,d) Definitive proof of preferential Rubisco targeting to the pyrenoid requires additional methods, like electron microscopy of immunogold-labelled Rubisco (c) or confocal imaging of fluorophore-tagged Rubisco (d). [All images by MTM; transformational plasmid used in (d) as per Mackinder *et al.*, 2016]

Figure 2: Examples of morphological diversity of micro-algal pyrenoid matrix and associated network of tubules

(a) Green algae, with examples taken from Cladophorales and Siphonocladales (after Hori & Ueda, 1975), and *Chlamydomonas reinhardtii* (“star shaped” tubules); (b) Red algae (after Gantt & Conti, 1965; Ford, 1984); (c) Chlorarachniophytes (after Ishida *et al.*, 1999); (d) Diatoms (after Bedoshvili *et al.*, 2009); (e) Dinoflagellates (after Dodge, 1973). Legend: **dots** = pyrenoid matrix (mainly composed of Rubisco); **thick lines** = stromal thylakoids when outside the pyrenoid matrix or trans-pyrenoidal thylakoids (tubules) when traversing the pyrenoid matrix; **hatched boxes** = peri-pyrenoidal starch plates, stromal in green algae and cytosolic in non-green algae; **dashed lines** = membranal delimitation between chloroplast

and cytosol.

Figure 3: Speculative models of pyrenoid biogenesis integrating all three major components.

(a) Interacting inter-dependencies depicts a model in which major components are under independent genetic control. The recruitment of Rubisco to and the situation of the matrix within the chloroplast requires a tightly regulated interplay between all three components. Proof of concept could be the finding of mutants which retain tubules and/or pyrenoid-peripheral elements in a locus where the pyrenoid would normally form, even when the Rubisco matrix is lost. **(b)** Hierarchical or “Russian nesting doll” model assumes a unidirectional and sequential formation (and dispersion when the CCM is repressed), starting with the deposition of Rubisco around a conserved anchoring site, with secondary deposition of peripheral elements. **(c)** The hybrid model integrates the two previous ones and accounts in particular for the observation that a CO₂-acclimation independent fraction of Rubisco is always retained in the pyrenoid (as in *Chlamydomonas reinhardtii*, Borkhsenius *et al.*, 1998; Mitchell *et al.*, 2014). Note that simpler models can be derived for naked pyrenoids and pyrenoids not surrounded by peripheral elements.

Figure 4: The diversity of mechanisms in different algal species ensuring pyrenoidal continuity across mitotic cell divisions.

(a) Apparent dissolution of the pyrenoid in the parent cell, followed by *de novo* formation in each daughter cell upon cytokinesis and completion of cell division e.g. *Scenedesmus quadricauda*. **(b)** Inheritance of the pyrenoid by a single daughter cell, with *de novo* pyrenoid formation in the other e.g. *Volvoxella steinii*. **(c)** *De novo* pyrenoid formation preceding

cytokinesis and cell division, with the two pyrenoids in the parent cell then distributed equally between daughter cells e.g. *Scytosiphon lomentaria*. **(d)** Fission of the parental pyrenoid leading to its equitable distribution between daughter cells e.g. *C. reinhardtii*.

Figure 1

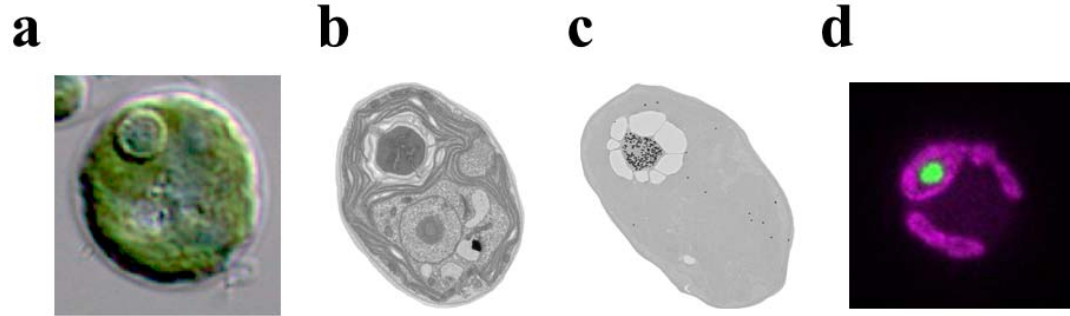


Figure 2

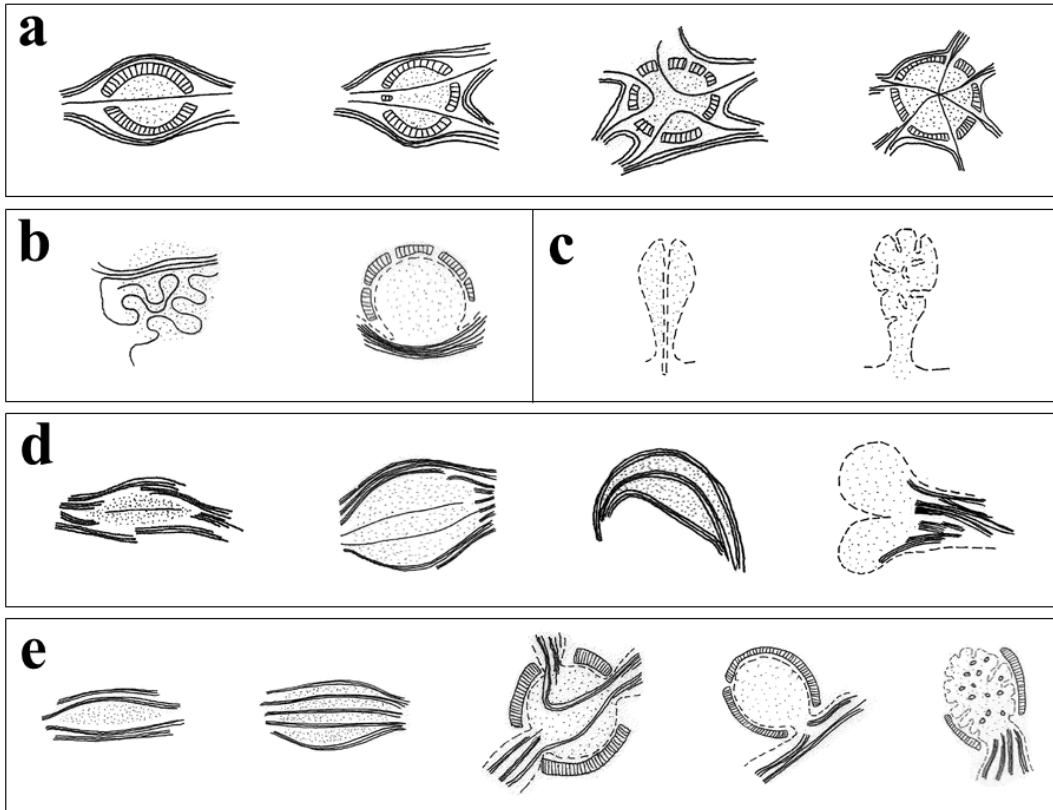


Figure 3

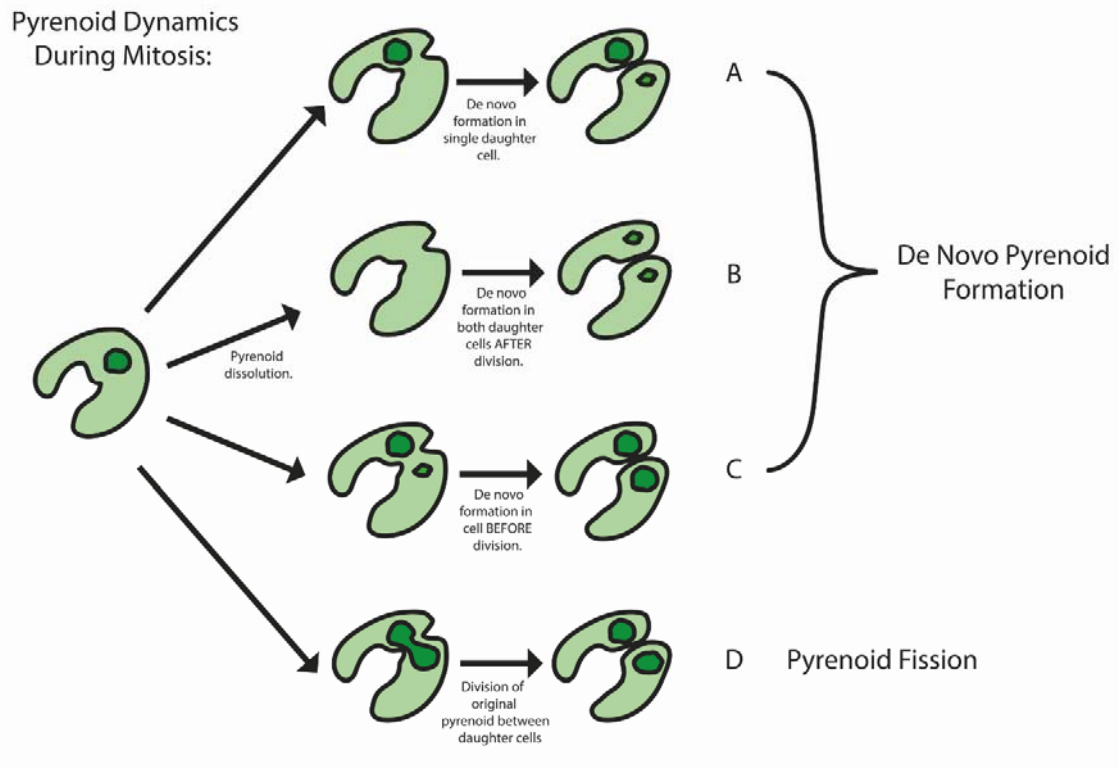


Figure 4

