



Structural Investigation of Photosynthetic Membrane Using Small-Angle Scattering

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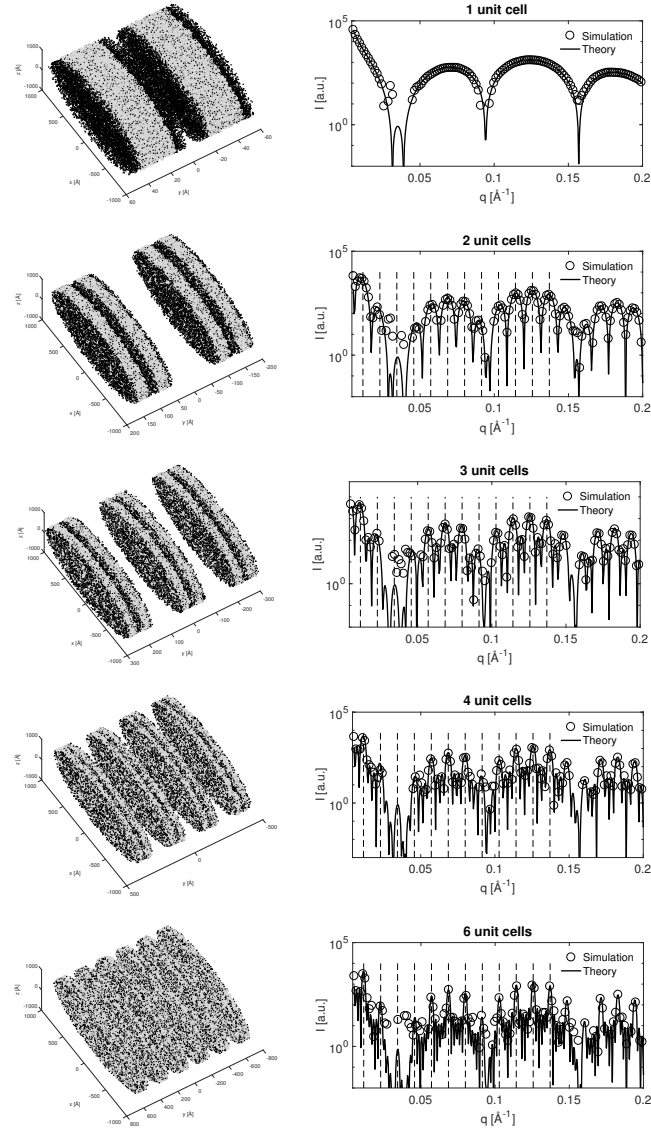


Figure 4. (left column) 3D point representations of the simulated structures going from 1 to 6 unit cells. Gray points represents the interior bilayer tail region and black points the exterior head group points. Note that the cylinder (y) axis is much smaller than the lateral axes for a unit cell. (right column) Comparing scattering simulations with the theoretical model described above. In this example 1-6 unit cells are simulated with parameters: $d_H = 10 \text{ \AA}$, $d_T = 15 \text{ \AA}$, $d_L = 50 \text{ \AA}$, $\rho_H = 0.2$, $\rho_T = -0.1$, $\rho_L = 0$, $d = 550 \text{ \AA}$ and $\eta_{cp} = 0$. N takes values 1-6 as indicated. We have done extensive model calculations to understand the influence of individual model parameters to guide the fitting procedure (not shown). The dashed lines indicate the position of the Bragg peaks of order 1-12 for an infinite perfect stack.

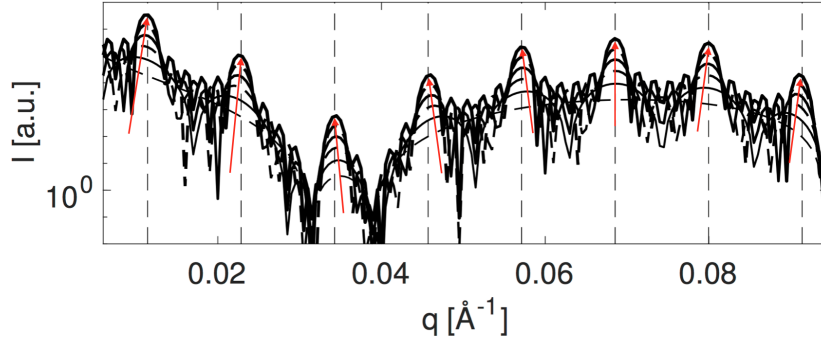


Figure 5. Direct comparison of theoretical scattering patterns corresponding to Figure 4. As the number of unit cells increases from 1 to 6 (plotted with increasing intensity) the manifestation of the Bragg peaks becomes increasingly pronounced. The red arrows indicate the curvature of the form factor governing the shift direction of the peaks for few unit cells.

In Figure 6 we compare model scattering patterns with and without the instrumental resolution smearing from the Bilby instrument. It is clear that for SANS data the resolution smearing is absolutely crucial to fully model the data as it has a pronounced effect on the resulting patterns. Further, we compare the model predictions for three values of the Caillé parameter going from a perfect flat sheet ($\eta_{cp} = 0$) to very stiff layers ($\eta_{cp} = 0.01$) and finally more undulated sheets ($\eta_{cp} = 0.1$). This progression leads to a gradual smearing of peak features from the high q end of the spectrum. The conclusion is that the appearance of the very distinct peak series around $q = 0.05$ - 0.08 which is clear in the experimental data in Fig. 9 will be a signature of a stiff and well-ordered membrane system and will pose a requirement to keep the Caillé parameter very low.

Another issue which needs to be considered is the effect of large length scale fluctuations, or polydispersity, on the experimental patterns. It is easy to physically justify the inclusion of polydispersity for the repeat and lumen distances as fluctuations in these parameters are immediately obvious upon inspection of TEM data and is quantified by the statistics shown in Figure 8. On the other hand, the local membrane thickness is hard to detect so we do not include polydispersity for this. An important feature when comparing the model calculations in Fig. 6 and the experimental curves in Fig. 9 is that the pure model displays a very deep form factor minimum around $q = 0.01$ - 0.03 Å^{-1} . However, from the experimental data it is clear that the peaks in this region still survive although to a varying degree. In Fig. 7 model calculations illustrating the effect of polydispersity on the repeat distance and lumen width are shown. The polydispersity is simply implemented as an sum across a Gaussian distribution, thus the repeat distance and lumen width each becomes associated with a standard deviation σ_D and σ_L . In the implementation of the double polydispersity

one can do the full double sum over the two distributions, but it turns out that one can split the two effects with negligible effect (tested, not shown), i.e. one can calculate the polydisperse form factor first (lumen polydispersity) and then use that in the sum over the structure factor distribution (repeat distance polydispersity) speeding up the fitting routine. As illustrated in Figure 7 lumen polydispersity smears out the deep form factor minimum and smears out structure factor features for ca. $q > 0.1 \text{ Å}^{-1}$. Repeat distance polydispersity on the other hand smears peaks slightly, but mostly for q -values higher than the deep form factor minimum which is hardly affected. It maintains the 3 peak features around $q = 0.3$ - 0.5 for quite large polydispersity values, but smears out features for higher q values. Not surprisingly, the effect of repeat distance fluctuations are basically the same as increasing the Caillé parameter. The combined effect of simultaneous variation of the overall repeat distance and the lumen basically smears all high q features leaving only broad 1st and 2nd order peaks.

3.4 Molecular constraints

The model described above is implemented in the WillItFit framework [44] allowing for instrument resolution effects to be included in the model fits following [45]. Also, care has been taken in the WillItFit development to implement trust region estimation, based on the profile likelihood strategy [44]. A final term to be included in the model is a background contribution I_b . Although a basic background subtraction of buffer scattering has been performed this does not include the contributions arising from any other biological material present in the samples. There are two main contributions to this, a general flat incoherent background and scattering from the cell wall, enclosing the cell. As was recently demonstrated the scattering from the latter is basically a q^{-2} power law [46], thus we implement the background contribution as a simple sum of two such terms

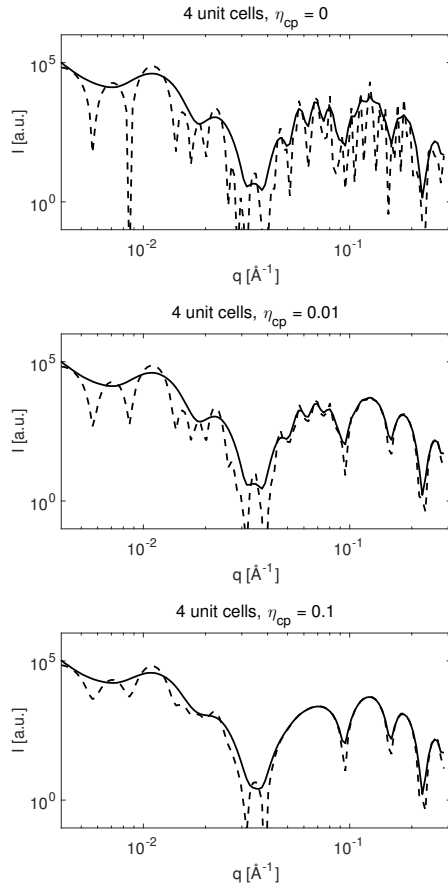


Figure 6. Comparing model scattering patterns with (full lines) and without the Bilby instrument resolution smearing (dashed lines). (top) With $\eta_{cp} = 0$. (middle) With $\eta_{cp} = 0.01$. (bottom) With $\eta_{cp} = 0.1$. Other parameters as simulations in Figure 4.

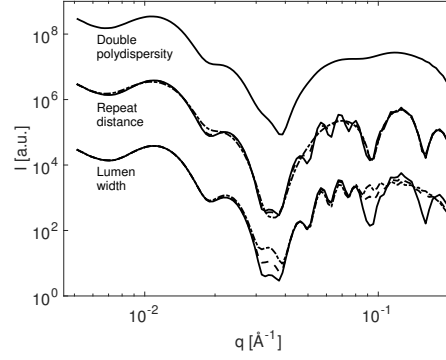


Figure 7. Effects of large length scale fluctuations on the scattering patterns. The lower group of curves shows the effect of increasing the lumen width polydispersity σ_L from $0 \rightarrow 10 \rightarrow 20$ Å. The middle curves shows the effect of increasing the repeat distance polydispersity σ_D from $0 \rightarrow 20 \rightarrow 50$ Å. The top curve show the effect of double polydispersity with $\sigma_L = 20$ and $\sigma_D = 50$. All curves have $N = 4$. Other parameters as simulations in Figure 4.

and add that to Eq. 1:

$$I_b = B + Cq^{-n} \quad (12)$$

where B, C are constants and where we require the power law exponent n to be close to 2.

The procedure for conducting the fits is not easily transcribed in a single sentence. The most general description would be to first determine the repeat distance which is fairly accurately determined from the higher order peaks around $q \approx 0.05$. Also, starting out with representing the bilayer as one large box allows a rough determination of the bilayer width, lumen width and associated contrasts as these parameters to a large extent control the peak ratios of the characteristic three peak pattern in this region. After that parameters are tweaked into place followed by a final global fit. The fit uses a combination of the Levenberg-Marquardt algorithm for quick adjustments of single parameters but more generally the grid search implementation of the algorithm which is implemented in the WilltFit framework [44]. The resulting fit parameters are listed in Tables 3 and 4.

3.5 Scattering length density calculations

Thylakoid membranes differs from other cellular membranes by three aspects: its protein content is significantly higher than other membranes, thylakoid fatty acids are largely non-saturated and thylakoid lipid head groups are predominantly non-charged galactosides [47, 48, 49, 50, 51, 52, 53]. Thus, using SLDs calculated for myelin [54] or artificial lipid membranes [55, 56] is unsuitable for modelling cyanobacterial or higher plant chloroplast scattering. We calculated average scattering length density values of cyanobacterial thylakoid membrane components to use as

molecular constraints or as best estimates for fitting parameters in our thylakoid membrane modelling. X-ray and neutron scattering length densities were calculated for four different entities: the cyanobacterial inter-thylakoid space and three integral thylakoid membrane components: membrane proteins, lipid headgroups and lipid tailgroups. To calculate the final thylakoid scattering length, these membrane components were averaged using a protein/lipid ratio of 0.7/0.3 [4]. Chlorophylls or cofactors were not considered in the thylakoid scattering length density calculations. The procedure for these calculations are outlined in the following and the results are listed in Tables 1 and 6.

3.5.1 Thylakoid membrane proteins

To avoid individual protein volume calculations and their multimerization, amino acid sequences of all unique subunits of *T. vulcanus* PSII, *S. elongatus* PSI, *S. cerevisiae* V-ATPase and *M. lamosus* cytochrome b_6f were subsequently joined into four long polypeptide sequences. Neutron SLDs of these polypeptides were individually calculated using the Biological Scattering Length Density Calculator with standard parameters and amino acid volumes [57] in 100 % D₂O. As photosynthetic complexes are large membrane-embedded proteins and cyanobacterial cell equilibration time in D₂O-media is maximally two hours, 0 % labile H-D exchange in thylakoid membrane-embedded proteins [58] was considered (Table S3). Individual polypeptide SLDs were average-weighted using protein molar ratios (1 PSII: 0.7 PSI: 0.7 *Cyt_{b6f}*: 0.5 ATPase [59]) and used in the thylakoid SLD calculations with a protein density of 1.33-1.35 g/mL [60].

Table 1. Absolute SLD values of cyanobacterial components. Values for aqueous compartments are calculated with a 60-85 % phycobilisome content.

	Neutron SLD 10 ⁻⁶ Å ⁻²	X-ray SLD 10 ⁻⁶ Å ⁻²
SLD _{headgroups}	1.83	13.4 (calculated) 11.9 (from fits)
SLD _{tailgroups}	1.33	11.2
SLD _{thylakoid membrane}	1.58	12.3
SLD _{lumen}	5.40-4.30	11.16-11.74
SLD _{inter-thylakoid space}	4.43-3.61	11.16-11.86
SLD _{D₂O}	6.4	9.47

3.5.2 Thylakoid membrane lipids and saccharides

Lipids constitute 10 - 28 % of cyanobacterial dry weight [5, 6]. Although major lipid classes in cyanobacteria are similar to plants and algae [61], the fatty acid compositions of their lipids differ [6], as cyanobacterial thylakoid membranes do not contain polyunsaturated fatty acids [50]. Following work on nanodiscs [56], lipid SLD was calculated with the NIST scattering length density calculator [62] separately for fatty acids (tailgroups) and sugars (headgroups), using fatty acid composition of *Synechocystis* sp. PCC 6803 [50, 63] and physical properties of individual fatty acids and sugars. In more detail, a weighted mixture of 16 and 18-C fatty acids was used for SLD_{tailgroup} calculations, and a weighted

mixture of galactose, sulfogalactose and phosphoglycerol was used in SLD_{headgroup} calculations (Tables S4-S7).

3.5.3 Thylakoid membrane final SLD

During the fitting of the SANS data it became clear that the neutron contrast does not allow us to distinguish between membrane head and tail regions, thus for the neutron model we describe the whole thylakoid membrane as a single scattering length density box. To calculate the average SLD_{thylakoid membrane} value of this box, SLD_{tailgroup} and SLD_{headgroup} were averaged with SLD_{protein} using a ratio 0.7/0.3 as mentioned above. The final SLD_{thylakoid membrane} value is then defined as a sum of 1/2 protein-averaged SLD_{tailgroup} and 1/2 protein-averaged SLD_{headgroup}, thus: $SLD_{thylakoid\ membrane} = 0.5 \cdot (1.832 \cdot 10^{-6} \text{ Å}^{-2} + 1.327 \cdot 10^{-6} \text{ Å}^{-2}) = 1.58 \cdot 10^{-6} \text{ Å}^{-2}$. This value was used throughout the analysis of the SANS data (Tables 1, S8, S9). In the case of X-ray scattering, the averaging is not performed, both SLD_{headgroup} and SLD_{tailgroup} are used individually.

3.5.4 Inter-thylakoid space SLD

For simplicity, we assume that inter-thylakoid space is only composed of phycobilisomes and heavy water. Using *Porphyridium cruentum* phycobilisome dimensions and phycobilisome packing density in low light [15] and the average inter-thylakoid space width of 590 Å, obtained from Table 2, we obtain that the phycobilisome volume fraction in the inter-thylakoid space varies from 68-100 %. Therefore, the average volume fraction of 60-85 % phycobilisomes and 40-15 % D₂O was used in SLD_{inter-thylakoid space} calculations (Table S9). To calculate SLD_{phycobilisome}, 25 unique protein chains of *Griffithsia pacifica* phycobilisome [19] were combined into a single polypeptide and calculated as in the previous section. Since phycobilisomes have a higher water accessibility than thylakoid membrane proteins, we assume that their labile H-D exchange is similar to globular proteins and that 0-90 % of exchangeable protein hydrogens are exchanged to deuterium, which effectively amounts to 0-27 % of all protein hydrogens exchanged to deuterium [64].

3.5.5 Lumen SLD

The exact lumen protein content is unknown [65], therefore lumen composition is assumed to be a mixture of plastocyanin and D₂O volume fractions, i.e. all lumen proteins in SLD_{lumen} calculations are accounted for as plastocyanin dissolved in D₂O. It is estimated that all luminal water is exchanged by the cytoplasmic water 100 times per second [66], therefore we assume that after three subsequent cyanobacterial resuspension cycles in 100 % D₂O, cyanobacterial lumen contains predominantly heavy water. We do not account for any spatial or temporal variation in the SLD_{lumen} due to illumination-induced ion transport and resulting lumen volume changes [20], as the cyanobacteria were not illuminated during the scattering measurements.

3.5.6 From SLD to contrast

Object scattering in a solvent only arises if the scattering density difference between the object and the solvent is non-zero. In this paper, we define the thylakoid membrane as 'the object' and the inter-thylakoid space as 'solvent', i.e.

we calculate thylakoid and lumen contrasts relatively to the inter-thylakoid space. For convenience and to minimize the number of fitting parameters, we arbitrarily define the $SLD_{tailgroup}$ as -1. That is, we assume that relatively to the inter-thylakoid space, the tailgroup scattering is lower and the contrast between inter-thylakoid space and tailgroups is set to 1 arbitrary unit. Lumen contrast is scaled accordingly by constants C_1 and C_2 , which are derived from fits and all numbers are subsequently mapped back to absolute values. The absolute value of $SLD_{tailgroup}$ is fixed to $1.58 \cdot 10^{-6} \text{ \AA}^{-2}$ and since we define contrast relative to the inter-thylakoid space, the $SLD_{inter-thylakoid\ space}$ is obviously 0. For the lumen the range of absolute values is $3.61\text{-}4.43 \cdot 10^{-6} \text{ \AA}^{-2}$ (neutron) and $11.16\text{-}11.9 \cdot 10^{-6} \text{ \AA}^{-2}$ (X-ray) (Tables 3, 6).

To obtain scattering contrasts of thylakoid and lumen, we subtract $SLD_{inter-thylakoid\ space}$ from $SLD_{thylakoid\ membrane}$ and SLD_{lumen} . This assumption has an underlying physical explanation. Firstly, $SLD_{inter-thylakoid\ space}$ cannot be calculated precisely in absolute scale due to lacking biological information; if so, then deriving absolute $SLD_{thylakoid\ membrane}$ and SLD_{lumen} values are prone to large errors. Secondly, scattering length densities relative to inter-thylakoid space (in arbitrary units) are obtained from scattering curve fittings (Figure 9, Tables 3 and 4) and can be converted into absolute scale under the assumption that thylakoid membrane and inter-thylakoid space composition are known. Thirdly, relative SLD_{lumen} comparison to $SLD_{inter-thylakoid\ space}$ allows calculating the SLD_{lumen} value on absolute scale. Combining information from X-ray and neutron fits and varying inter-thylakoid space composition, we estimate volume fractions of D_2O and protein (expressed as the 'relative plastocyanin') in the lumen (see below).

4. Results

4.1 Structural characterisation of cyanobacteria from TEM

To assess biological variation of cyanobacterial RD, we measured thylakoid repeat distance in N 'pseudograna' from three biological cyanobacterial replicates, grown under the same temperature and illumination conditions and fixated during cell exponential phase. RD distribution histogram (8) exhibits a large RD variation within three replicates (total σ_{RD} is $116.5 \text{ \AA}$, $df = 1668$). RD medians for replicates (Table 2, diamonds in Fig. 8) are within $530\text{-}630 \text{ \AA}$ range. From statistical analysis of normalized data, average RD between investigated cyanobacterial species is significantly statistically different ($p < 0.001$, $df = 1677$), but *post hoc* Tukey contrast test shows that the RD difference between different cyanobacterial species can be considered biologically insignificant. Therefore, we infer that the average thylakoid repeat distance is the same between 6803, 7942 and 7002 and is equal to the average repeat distance of the nine replicas of Table 2 yielding $590 \text{ \AA}$.

4.2 Structural characterisation of cyanobacteria using SANS

TEM studies revealed a large thylakoid RD variation inside cyanobacterial cells. Therefore the model derived in this study includes RD and lumen thickness uncertainties.

The model includes a large set of parameters, and in order to get reasonable fits, several assumptions were imposed in modelling: (1) SLD contrast of tailgroups was constrained to -1, (2) total membrane thickness (a sum of headgroups+tailgroups) was constrained to $20\text{-}60 \text{ \AA}$ [67, 68, 69], (3) lumen thickness was constrained to $45\text{-}300 \text{ \AA}$ [10, 70, 20, 23], (4) maximum repeat distance was constrained to $450\text{-}950 \text{ \AA}$ [7, 30, 24], (5) average number of thylakoids was constrained to 2-6 [10]. The triplicates of experimental SANS data for cyanobacteria are shown in Fig. 9. Clear maxima are observed between $q = 0.01\text{-}0.1 \text{ \AA}^{-1}$, the group of three maxima around $q = 0.03\text{-}0.05 \text{ \AA}^{-1}$ is best resolved for the first replica of 7002. Otherwise, scattering peaks are largely smeared. This supports the expectation that the polydispersity in the sample is relatively high and that instrument resolution needs to be accounted for in the analysis. Contrast variation series (Fig. S1) in 42 % and 21 % D_2O medium supports that scattering peaks occur from thylakoid membranes. Importantly, investigation of 21 % contrast shows background scattering, occurring from other cellular components - cell wall and plasma membrane. To account for background scattering, we included the background term from Equation 12 where n is determined from fits and varies from 1.95 to 2.5.

The fittings were performed for all biological replicates, measured in 100 % medium, and the best fits are presented in Fig. 9. It became clear, that lumen polydispersity alone was enough to explain the smearing of scattering curves, therefore RD polydispersity was not considered in the final as this is already reflected in the Caillé parameter as mentioned above. It also became clear, that including headgroup scattering as a separate parameter did not improve fittings, therefore the entire membrane thickness was fitted as a single box (i.e. only using protein-adjusted $SLD_{tailgroup}$).

The model fit results are plotted along with the experimental data in Fig. 9. As seen from the plots, model fit quality is satisfactory and the model fits capture almost all significant features of the data. The model parameters describing the average dimensions of thylakoid system are summarized in Table 3. Average thylakoid repeat distance of $600\text{-}700 \text{ \AA}$, number of layers in a 'pseudogranum' is $3.5\text{-}4.5$, average lumen thickness is $60\text{-}85 \text{ \AA}$ and average thylakoid membrane thickness is $33.8\text{-}42 \text{ \AA}$. Inter-thylakoid space height $d_{inter-thylakoid\ space} = RD - d_L - 4 \cdot d_T = 430\text{-}555 \text{ \AA}$, is in very close agreement with values obtained from (cryo-)TEM studies [7, 10, 20, 23, 16]. From cryoEM measurements, which exclude fixation-induced sample shrinkage, inter-thylakoid space height of 6803 is $580 \pm 130 \text{ \AA}$, and of 7942 - $450 \pm 30 \text{ \AA}$ [16]. Membrane fluctuations evaluated by η_{cp} from SANS models, is also of the same order of magnitude as the parameters A , used to describe the fluctuations of *Rhodospseudomonas viridis* thylakoid system [71].

4.3 Structural characterization of cyanobacteria using SAXS

The results of SAXS model fits are plotted on the experimental data in Fig. 10. As seen from the plots, model fit quality is reasonable and the model fits capture most significant features of the data. The overall system smearing is not accounted for in modelling and be believe this inherent

Table 2. Cyanobacterial repeat distances, measured from TEM images. All distances are in Å. CL are confidence limits.

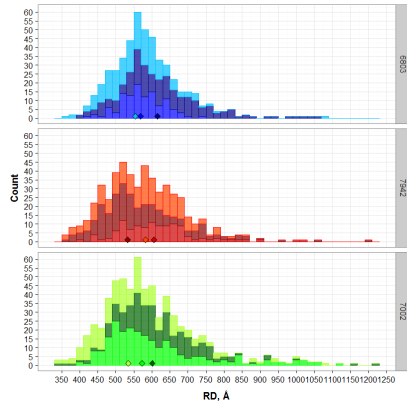
Strain	Replica	Number of pseudograna	Average RD	Median RD	Standard deviation	Lower CL	Upper CL
6803	1	114	574.1	568.1	10.91	552.7	595.5
	2	198	632.5	615.0	8.28	616.3	648.8
	3	189	556.9	554.2	8.48	540.3	573.6
7942	1	114	612.8	605.5	10.91	591.4	634.2
	2	207	565.7	532.6	8.10	549.8	581.6
	3	204	572.7	582.1	8.16	556.6	588.7
7002	1	242	615.8	572.6	7.49	601.1	630.5
	2	205	622	601.1	8.14	606.0	638.0
	3	204	556.7	534.6	8.16	540.7	572.7

Table 4. Individual fitting parameters for SANS profiles of cyanobacteria.

	1	2	3	1	2	3	1	2	3
$\Delta\rho_T$, a.u.	-1	-1	-1	-1	-1	-1	-1	-1	-1
$\Delta\rho_L$, a.u.	0.47±0.046	0.33±0.044	0.20±0.025	0.5±0.038	0.29±0.04	0.27±0.019	0.31±0.023	0.48±0.055	0.39±0.019
d_T , Å	13.83±0.60	17.84±0.91	19.02±0.43	20.17±0.5	17.14±0.93	18.08±0.34	19.85±0.50	23.26±1.17	19.60±0.44
d_L , Å	53.04±3.18	72.42±4.17	60.96±1.50	47.57±1.83	80.53±5.54	64.42±1.41	87.00±1.96	80±3.73	87±1.71
RD, Å	780.3±33.9	643.2±15.39	610.11±5.09	704.50±11.35	699.1±22.0	675.85±5.17	586.01±4.77	588.24±9.68	618.52±6.37
N	6±1.12	3±0.92	3±0.36	6±3.97	3±0.099	4.06±0.55	5.97±3.9	2±0.583	2.282±0.36
η_{CP}	0.029	0.02	0.02	0.0266	0.029	0.0292	0.02	0.018	0.0309
Background (B)	0.55±0.01	0.0044±0.0042	0±0.006	0.22±0.0072	0.005±0.004	0±0.0067	0.069	0.0015	0
Scale	6.6e-8±6.3e-9	1.4e-8±1.7e-9	1.9e-7±8.5e-9	6e-8±3.1e-9	9.7e-9±1.4e-9	2.4e-7±8.9e-9	5.8e-8±2.9e-9	1.8e-8±1.5e-9	1.6e-7±6.5e-9
σ_T , Å	17.61±4.11	7.30±13.88	8.56±3.20	20.83±2.02	13.28±12.64	13.92±2.03	16.65±2.80	15.23±6.03	19.30±1.98
η power law	2.55±0.018	2.543±0.00837	2.35±0.006	1.93±0.01	2.22±0.016	2.19±0.007	2.09±0.0081	2.534±0.0068	2.25±0.0069
Scale power law (C)	2.1e-5±1.6e-6	2.5e-5±9.8e-7	7.5e-4	4e-4±2.9e-6	4e-5±2.9e-6	9e-4±2.4e-5	0.000406±1.31e-5	0.000056±1.68e-6	0.00051±1.48e-5
χ^2	5.25	7.51	64.45	80.9	1.95	69.25	36.25	11.31	24.34

Table 3. Average structural thylakoid membrane parameters obtained from SANS model fitting.

Fit parameter	6803	7942	7002
RD, Å	677.8	693.1	597.6
N	4	4.3	3.41
d_L , Å	62.13	64.26	84.66
d_T , Å	16.89	18.46	20.90
$\Delta\rho_L$, a. u.	0.334	0.3398	0.395
$\Delta\rho_T$, a. u.	-1	-1	-1
Deduced parameter			
$d_{inter-thylakoid\ space}$, Å	548.11	555.0	429.3
$d_{thylakoid\ membrane}$, Å	34	37	42
SLD_{lumen} , 10^{-6} Å^{-2}	4.29	4.30	4.41

**Figure 8.** Cumulative histograms from TEM measurements of repeat distances (RD) from all investigated species (three biological replicates).

instrumental smearing account for the differences around $q = 0.05\text{ Å}^{-2}$. Again, scattering contrast $\Delta\rho_T$ is constrained to -1 a. u and $SLD_{tailgroupX}$ absolute value is fixed to $11.2 \cdot 10^{-6}\text{ Å}^{-2}$. Contrary to neutrons, headgroup scattering was included as a separate parameter in SAXS modelling (therefore no $SLD_{thylakoidX}$ is calculated). Accordingly, thylakoid membrane thickness in SAXS model was a double sum of tailgroup and headgroup thicknesses. The model parameters describing the average dimensions of the thylakoid system are summarized in Table 5. Average $SLD_{headgroupX}$ value obtained from fits is slightly lower than purely theoretically calculated (11.9 vs. $13.4 \cdot 10^{-6}\text{ Å}^{-2}$, Table 1), but this difference is acceptable. Compared to earlier literature value of average $SLD_{thylakoidX} = 400\text{ electrons/nm}^3 = 7.27 \cdot 10^{-6}\text{ Å}^{-2}$ (50 % protein, 30 % lipid), $SLD_{headgroupX} = 450\text{ electrons/nm}^3 = 8.18 \cdot 10^{-6}\text{ Å}^{-2}$, $SLD_{tailgroupX} = 160\text{--}280$

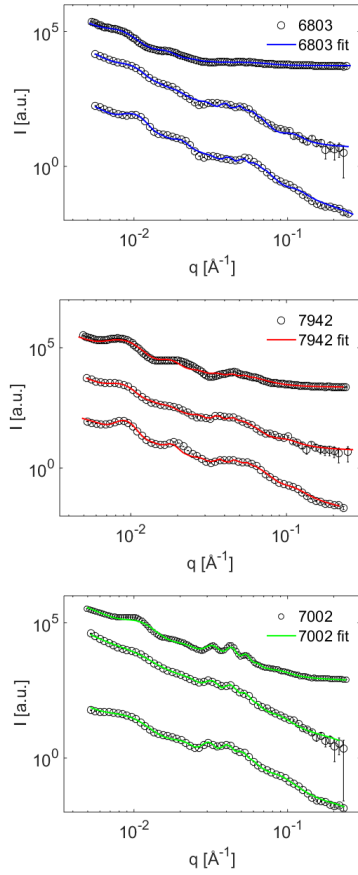


Figure 9. SANS data and model fits from Table 4. Replicas 1 - 3 arranged from top to bottom, curves are arbitrarily scaled for clarity.

electrons/nm³= $2.91 \cdot 5.09 \cdot 10^{-6} \text{ \AA}^{-2}$ [71], SLD_{X-ray} values derived in this article are higher - most probably due to a higher protein content in the thylakoid membrane, but largely comparable. Structural parameters obtained from X-ray fits: average thylakoid repeat distance RD is equal to 460-816 Å, the number of layers in a 'pseudogranum' is 2-3, average lumen thickness is 63 Å. Derived average thylakoid membrane thickness is 38.5-48 Å and the inter-thylakoid space height $d_{inter-thylakoid \text{ space}}$ is 350-710 Å. These values, although slightly higher, are generally comparable to our SANS measurements. As SAXS and SANS measurements were not performed simultaneously on the same bacterial samples in the same environmental conditions, the structural thylakoid values obtained from SAXS and SANS fittings shall not be directly inter-compared. However, as discussed in the following, we use the SAXS results to discriminate between possible SANS interpretation scenarios.

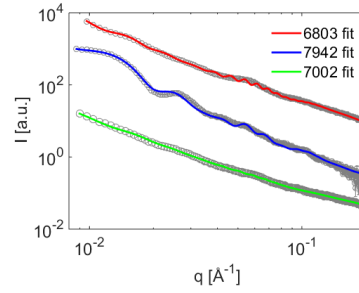


Figure 10. SAXS model fitting. Experimental datapoints with errorbars in grey, SAXS model fits in color.

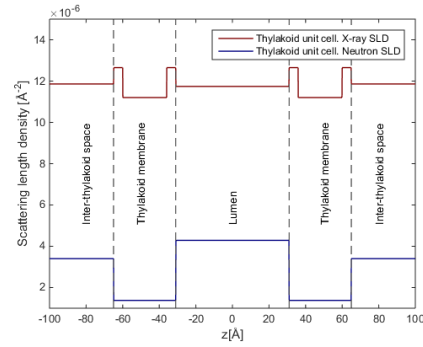


Figure 11. X-ray and neutron SLD profiles of a thylakoid unit cell. Inter-thylakoid space contains 85 % protein and 15 % of D₂O.

4.4 Lumen protein volume fraction

Absolute values of SLD_{lumenX} and SLD_{lumenN} have been estimated from relative average values obtained from fit-

Table 5. Structural thylakoid membrane parameters obtained from SAXS model fitting.

	6803	7942	7002
Fit parameters			
$\Delta\rho_H$, a. u.	1.32±0.044	1.07±0.07	1.20±0.08
$\Delta\rho_T$, a. u.	-1	-1	-1
$\Delta\rho_L$, a. u.	-0.02±0.0144	-0.47±0.0144	-0.035±0.0312
d_H , Å	6.0±0.2	4.65±0.25	6.95±0.45
d_T , Å	15.8±0.22	19.2±0.34	12.3±0.44
d_L , Å	62.5±2.75	63.7±2.71	63.8±12.63
RD, Å	815.8±4.63	462.8±2.61	748.1±30.8
N	2±0.23	3±0.28	2±1.03
σ_L , Å	18.0±2.5	15.0±3.28	19.5±9.53
η_{cp}	0.01	0.036	0.02
Background (B)	0.03±0.002	0.024±0.0043	0.020±0.0019
Scale (y)	1.6e-7±6.9e-9	1.42e-7±3.73e-9	7.57e-8±9.89e-9
n power law	2.37±0.0023	2.91±0.004	2.14±0.0037
Scale power law (C)	0.00058±5.32e-6	9.0e-5±1.45e-6	0.000573±8.32e-6
χ^2	51.91	54.12	10.84
Deduced parameters			
$d_{inter-thylakoid\ space}$, Å	709.7	351.4	645.8
$d_{thylakoid\ membrane}$, Å	43.6	47.7	38.5
SLD_{lumen} , 10 ⁻⁶ Å ⁻²	11.85	11.55	11.84
$SLD_{headgroup}$, 10 ⁻⁶ Å ⁻²	12.73	12.57	12.65

tings (X-rays: -0.175, neutrons: 0.34) using different inter-thylakoid space volume compositions - varying phycobilisome/water volume fractions, different labile H-D exchange percentage and D₂O/H₂O fraction inside inter-thylakoid space. The scattering length density profile of the thylakoid membrane with absolute values is depicted in Fig. 11 (also given in Table 6). Lumenal protein content, expressed as 'relative plastocyanin' was calculated from absolute values of SLD_{lumenX} and SLD_{lumenN} , solving the system of coupled equations (Eq. 13). Proportionality constants C_1 and C_2 were derived from fits, $SLD_{inter-thylakoid\ space}$ and SLD_{lumen} were calculated for several scenarios: with different protein/water volume fractions, for the case of neutrons also varying D₂O/H₂O volume fractions of total water composition, and with different protein labile H-D exchange percentages (w). Lumen protein volume fractions, derived for a number of stromal composition scenarios are given in Table 12. As X-ray and neutron SLDs provide complementary data, the case in which a lumen protein volume fraction calculated from X-ray data is the most similar to lumen protein volume fraction calculated from neutron data is the most likely. Such conditions are denoted in orange in Fig. 12. It is also assumed that $\frac{\phi_{D_2O}'}{\phi_{H_2O}'}$ of the lumen is either equal or lower than that of the inter-thylakoid space.

$$\begin{cases}
 SLD_{inter-thylakoid\ spaceN} = \frac{\phi_{D_2O}}{\phi_{H_2O}} SLD_{waterN} + \phi_{phycobilisomeN} SLD_{phycobilisomeN} \\
 SLD_{inter-thylakoid\ spaceX} = \phi_{water} SLD_{waterX} + \phi_{phycobilisomeX} SLD_{phycobilisomeX} \\
 SLD_{lumenN} = \frac{\phi_{D_2O}'}{\phi_{H_2O}'} SLD_{waterN} + \phi_{plastocyaninN} SLD_{plastocyaninN} \\
 SLD_{lumenX} = \phi_{water}' SLD_{waterX} + \phi_{plastocyaninX} SLD_{plastocyaninX} \\
 SLD_{lumenN} = C_1 \cdot SLD_{inter-thylakoid\ spaceN} \\
 SLD_{lumenX} = C_2 \cdot SLD_{inter-thylakoid\ spaceX} \\
 \phi_{phycobilisome} + \phi_{water} = 1 \\
 \phi_{plastocyanin} + \phi_{water} = 1
 \end{cases}
 \quad (13)$$

As observed from Table 12, lumenal protein volume fraction is lower than of inter-thylakoid space from both neutron and X-ray calculations. From comparing volume fractions of X-ray and neutron calculations for different scenarios (Table 12), our best estimate (dark green) is that lumenal protein content is 81-82 % and D₂O/H₂O is 0.9/0.1, protein H-D exchange is 30 %. This result suggests, that lumenal content is higher than protein volume fraction of thylakoid membrane, but lower than of the inter-thylakoid space. The second-best lumenal composition scenario (brown) is that lumenal protein occupies 75 % volume, D₂O/H₂O is 0.9/0.1 and protein labile H-D exchange is 50 %. Together, these two scenarios support the argument of Beebo et al. [66], that lumenal water is efficiently exchanged with cytoplasmic (inter-thylakoid space) water. From neutron fits, we also estimate that the exchange of labile H-D of lumenal proteins is 30-50 %, what equates to a total protein H-D exchange of 9-15 %. To our knowledge, this is the first attempt to quantitatively estimate the protein concentration inside the thylakoid lumen.

Correspondingly, the most feasible inter-thylakoid space composition is 85%/15% phycobilisome/water, where D₂O/H₂O fraction is 0.9/0.1 and 0 % labile H-D exchange (dark green). The second-best scenario (brown) requires an inter-thylakoid space composition of 80%/20% phycobilisome/water, where D₂O/H₂O fraction is 1.0 and 0 % labile H-D exchange. In principle, lower H-D exchange of phycobiliproteins compared to 'a relative plastocyanin' is very feasible, as phycobiliproteins are large multisubunit complexes, which do not necessarily get fully exchanged, whereas soluble lumenal proteins are generally smaller due to lumen size restrictions and predominantly globular, therefore we assume that their labile hydrogens can be exchanged to deuteriums more easily. However, we argue that the complete exchange of inter-thylakoid space water with D₂O, although

not disallowed, is not practically likely, therefore we reject this (brown) scenario.

Table 6. Absolute SLD values of inter-thylakoid space and lumen in units of $10^{-6} \text{ \AA}^{-2}$.

Protein/Water vol. fraction, %	D ₂ O/H ₂ O %	Labile H-D exchange, %	Neutron SLD	X-ray SLD
SLD_{inter-space}				
60/40	100/0	0	3.685	11.159
60/40	100/0	90	4.432	11.159
70/30	100/0	0	3.232	11.440
80/20	100/0	0	2.78	11.722
85/15	100/0	0	2.554	11.86
85/15	90/0	0	2.449	11.86
85/15	90/0	90	3.40	11.86
85/15	100/0	90	3.612	11.86
SLD_{lumen}				
60/40	100/0	0	4.400	11.16
60/40	100/0	90	5.402	11.16
70/30	100/0	0	4.962	11.40
80/20	100/0	0	3.188	11.63
85/15	100/0	0	2.885	11.75
85/15	90/0	0	2.745	11.75
85/15	90/0	90	4.021	11.75
85/15	100/0	90	4.303	11.75

5. Discussion

Having increased contrast by D₂O exchange, we used SANS to determine the transverse membrane structure by recording scattered intensity $I(q)$. Cells for this experiment were cultured as described above in water-based BG-11 or A+ medium, then transferred to 100 % deuterated medium, concentrated and measured immediately. We did not perform cell viability assays, but from other *in vivo* experiments with *B. subtilis*, more than 90 % cells remain viable over a period of 4 h in 25°C [46]. The same is expected for cyanobacteria and no scattering curve changes were ever observed during the timeframe of experiment, even after 24 h (SAXS) in 30°C or RT.

5.1 Previous scattering work on cyanobacteria

Imagine a one-dimensional crystal in the form of a stack of infinitely thin sheets separated by 100 Å. From Bragg's law such a stack would produce a series of Bragg peaks at positions $q_1 = 2\pi/100 \text{ \AA} = 0.0628 \text{ \AA}^{-1}$, $q_2 = 2 \cdot 2\pi/100 \text{ \AA} = 0.1257 \text{ \AA}^{-1}$, $q_3 = 3 \cdot 2\pi/100 \text{ \AA} = 0.1885 \text{ \AA}^{-1}$ and so on. If one now takes the third order peak and interprets it as a 1st order peak, then one must conclude that the peak originates from a structure with a repeated separation of $2\pi/0.1885 \text{ \AA}^{-1} = 33.33 \text{ \AA}$, but clearly no such structure exists in this one-dimensional crystal and the conclusion is erroneous. This simple example illustrates one of the problems with a number of previously published papers on photosynthetic membranes and in the present context, particularly papers on cyanobacterial membranes. For example in [31] Liberton *et al.* presented a series of SANS measurements which are very similar to those we present here, although our Bilby-data seem to be of slightly superior resolution. Thus, their data could straightforwardly be analysed using the presented framework. However, their analysis

involves a putative peak assignment where all peaks are interpreted as 1st order peaks in the manner just described. In Figure 13 we plot their reported peaks normalised to the the lowest q -peaks and it is clear that all peaks fall very close to an integer multiple of this peak illustrating that they are part of a lamellar peak series and not individual peaks originating from a range of independent substructures. Thus, any discussion relating individual higher order peaks to specific substructures becomes purely speculative and in our opinion erroneous. The conclusion is that to extract reliable information from scattering data, a full structural model has to be invoked, such as described in this paper.

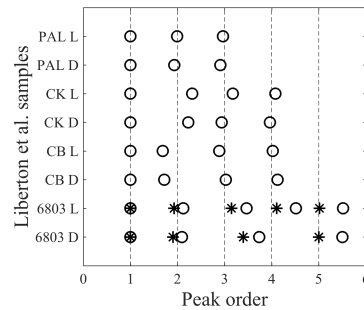


Figure 13. Peak positions reported in [31] normalized to the first order peak position (circles). Artificial rescaling of wild type data to match 5th order peak (asterisk). See text for details.

6. Conclusions

In conclusion, we have developed a straight-forward scattering model based on a double-bilayer membrane stack to study the ultrastructure of thylakoids inside cyanobacteria *in vivo*. Model fitting yields realistic values of thylakoid membrane, lumen thickness and RD. This model presents a rationale to explain the entire cyanobacterial scattering pattern as occurring from an ordered lamellar system. This model can be extended to study other organisms - diatoms or systems with functional photosynthetic deficiencies, thylakoid ultrastructure modifications (CURT1 variants) or organisms with larger number of thylakoid layers, such as grana stacks of higher plants. Further considerations, such as cyanobacterial thylakoid lumen change in relation to the light intensity and spectral characteristics, different ionic conditions and temperature, are needed to discuss the most likely mechanism of thylakoid ultrastructure adaptation to environmental factors.

On the basis of luminal scattering length density contrast fitting estimates, we propose an estimate of the luminal protein concentration. The combination of SLD calculations derived from SANS and SAXS scattering model fits results in the conclusion that protein content in lumen is smaller than in the inter-thylakoid space and is about 82 %. Further considerations, such as biological variation/deviation of the

[illegible]

Figure 12. Lumen protein volume fraction. Stromal composition is listed as rows, luminal composition as columns. Disallowed compositions depicted in red. All feasible luminal/stromal compositions depicted in orange, the best in green.

parameters when applying this scattering model to other multi-layered biological systems, needs to be discussed in the future.

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Supplementary Information

Detailed account of the contrast variation measurements, illumination spectra, SLD derivations. This material is available free of charge via the Internet.

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Table S1. A+ medium for 7002

Substance	MW, g/mol	Density, g/cm3	Weight per liter, g	Neutron SLD, *10 ⁻⁶ Å ⁻²	X-ray SLD, *10 ⁻⁶ Å ⁻²	Amount of mol in 1L	Weighted SLD, *10 ⁻⁶ Å ⁻²	
							Neutron	X-ray
Mg5O4*7H2O	246.4746	2.66 g/cm3	5	1.281	24.009	0.020286	0.025986451	0.487048158
NaCl	58.44	2.165 g/cm3	18	2.947	17.921	0.308008	0.907700205	5.519815195
KCl	74.5513	1.984 g/cm3	0.6	2.123	16.612	0.008048	0.017086221	0.133695858
NaNO3	84.9947	2.257g/cm3	1	4.862	19.074	0.011765	0.057203567	0.224413993
CaCl2*2H2O	1470.01	1.85 g/cm3	0.37	1.554	16.064	0.000252	0.00039114	0.004043292
Na2EDTA C10H14N2Na2O8	338.22	0.86 g/cm3	0.03	1.333	7.594	8.87E-05	0.000118237	0.000673585
K2HPO4	174.2	2.44 g/cm3	0.05	2.695	20.764	0.000287	0.000773536	0.005959816
FeCl3*6H2O	270.3	1.82 g/cm3	0.00389	1.141	15.685	1.44E-05	1.64206E-05	0.000225729
Trizma C4H11NO3	121.14	1.328 g/cm ³	1	0.807	12.328	0.008255	0.006661714	0.101766551
H3BO3	61.83	1.435 g/cm3	0.0343	1.606	12.668	0.000555	0.000890923	0.007027534
MnCl2*4H2O	197.91	2.01 g/cm3	0.00432	0.532	17.132	2.18E-05	1.16126E-05	0.000373959
ZnCl2	136.315	2.907 g/cm3	0.000315	3.19	22.865	2.31E-06	7.37153E-06	5.2837E-05
MoO3	143.95	4.69 g/cm3	0.00003	4.735	36.568	2.08E-07	9.86801E-07	7.62098E-06
CuSO4*5H2O	294.685	2.286 g/cm3	0.000003	1.401	19.551	1.02E-08	1.42627E-08	1.99036E-07
CoCl2*6H2O	237.93	1.924 g/cm3	0.000122	0.564	16.418	5.11E-07	2.88009E-07	8.38392E-06
H2O	18.01528	0.9970 g/cm3	21.934	-0.559	9.441	1.217522	-0.680594806	11.49462534
D2O	20.027	1.11 g/cm3	1085.58	6.393	9.455	54.20582	346.5378209	512.5160483
For 1 L media	978 mL	D2O						
	22 mL	H2O				Sums	55.78093	346.8740748
								530.4957864
H2O percentage:	2.249489	%						
							Final SLD	Neutron SLD
								X-ray SLD
								6.2185e-6 Å ⁻²
								9.5103 e-6 Å ⁻²

Table S2. BG-11 medium for 7942 and 6803

Substance	MW, g/mol	Density, g/cm3	Concentration, M	Neutron SLD, $\times 10^{-6} \text{ \AA}^{-2}$	X-ray SLD, $\times 10^{-6} \text{ \AA}^{-2}$	Amount mol in 1L	Weighted neutron SLD, $\text{ \AA}^{-2}$	Weighted X-ray SLD, $\text{ \AA}^{-2}$
NaNO3	84.9947	2.257g/cm3	1.76E-05	4.862	19.074	1.76E-05	8.56E-05	3.36E-04
K2HPO4	174.2	2.44 g/cm3	2.29E-04	2.695	20.764	2.29E-04	6.17E-04	4.75E-03
MgSO4*7H2O	246.4746	2.66 g/cm3	3.04E-04	1.281	24.009	3.04E-04	3.89E-04	7.30E-03
CaCl2*2H2O	1470.01	1.85 g/cm3	2.45E-04	1.554	16.064	2.45E-04	3.81E-04	3.94E-03
Citric acid monohydrate, C6H8O7*H2O	210.14	1.542 g/cm3	2.85E-05	2.162	13.764	2.85E-05	6.16E-05	3.92E-04
Ferric ammonium citrate, C6H8FeNO7	261.975	1.8 g/cm3	2.30E-05	2.872	15.433	2.30E-05	6.61E-05	3.55E-04
Na2EDTA, C10H14N2Na2O8	338.22	0.86 g/cm3	3.40E-06	1.333	7.594	3.40E-06	4.53E-06	2.58E-05
Na2CO3	105.9888	2.54 g/cm3	1.89E-04	4.521	21.332	1.89E-04	8.54E-04	4.03E-03
H3BO3	61.83	1.435 g/cm3	4.63E-05	1.606	12.668	4.63E-05	7.44E-05	5.87E-04
MnCl2*4H2O	197.91	2.01 g/cm3	9.15E-06	0.532	17.132	9.15E-06	4.87E-06	1.57E-04
ZnSO4*7H2O	287.5496	3.31 g/cm3	7.65E-07	1.387	28.782	7.65E-07	1.06E-06	2.20E-05
CuSO4*5H2O	294.685	2.286 g/cm3	3.19E-07	1.401	19.551	3.19E-07	4.47E-07	6.24E-06
CoCl2*6H2O	237.93	1.924 g/cm3	2.10E-07	0.564	16.418	2.10E-07	1.18E-07	3.45E-06
NaMoO4*2H2O	241.95	3.78 g/cm3	1.61E-06	3.141	30.887	1.61E-06	5.06E-06	4.97E-05
H2O	18.01528	0.9970 g/cm3	1.715599	-0.559	9.441	1.72E+00	-9.59E-01	1.62E+01
D2O	20.027	1.11 g/cm3	53.707	6.393	9.455	5.37E+01	3.43E+02	5.08E+02
For 1 L media	969 mL	D2O						
	31 mL	H2O				Sums	5.54E+01	3.42E+02
H2O percentage	3.1	%						
							Final SLD	Neutron SLD
								X-ray SLD
							6.1777e-6 $\text{ \AA}^{-2}$	9.4548e-6 $\text{ \AA}^{-2}$

Table S3. Thylakoid membrane protein SLD calculations

Organism	T vulcanus	S elongatus	Sacharomyces cerevisiae	Mastigocladus laminosus
PDB ID	4UB6	1JB0	3J9T	4H13
Number of unique protein chains	20	12	11	8

Neutrons . Protein SLD calculations

Fraction of exchangeable hydrogen	PSII	PSI	ATPSynthase V type	Cyt b6f	SLD Weighted average Å ²
0 % Exchange	1.89E-06	1.91E-06	1.83E-06	1.81E-06	1.8646E-06
10 % Exchange	2.01E-06	2.03E-06	1.96E-06	1.93E-06	1.9864E-06
20 % Exchange	2.13E-06	2.15E-06	2.08E-06	2.05E-06	2.1080E-06
50 % Exchange	2.49E-06	2.51E-06	2.47E-06	2.40E-06	2.4704E-06
80 % Exchange	2.85E-06	2.87E-06	2.85E-06	2.75E-06	2.8297E-06
90 % Exchange	2.97E-06	2.99E-06	2.98E-06	2.87E-06	2.9493E-06
100 % Exchange	3.08E-06	3.11E-06	3.10E-06	2.98E-06	3.0678E-06

X-rays . Protein SLD calculations

PSII	PSI	ATP synthase V type	Cyt b6f	SLD weighted average
1.25E-05	1.24E-05	1.25E-05	1.25E-05	1.2457E-05 Å ²

SLD weighted average = (1·SLD_{PSII}+0.7·SLD_{PSI}+0.7·SLD_{Cytb6f}+0.5·SLD_{ATPase})/2.9,
using ratios of Table 6, Antal *et al.* 2013.

A total 20-30% of protein hydrogen atoms are exchangeable with deuterium. Therefore, ‘50% Exchange’ means that 10-15% of total protein hydrogen atoms are exchanged to deuterium.

Table S4. *Synechocystis* thylakoid membrane lipid tailgroup SLD calculations

Synechocystis sp. PCC 6803 lipid composition (mol%): 37.4% MGDG, 20% DGDG, 28% SQDG and 13.7% PG (Sakurai2006). Total weighted sum for each fatty acid (mol%) from different lipids calculated as follows (example for 16:00 is given): 0.531·0.374+0.514·0.2+0.691·0.28+0.513·0.137 = 0.55810 = 55.81 mol%. Column ‘Corrected’ contains individual weighted sums divided by the total weighted sum 0.964 in order to obtain the final sum of 1.

Fatty acid	MGDG	DGDG	SQDG	PG	Weighted sum	Corrected	Neutron SLD, Å ²	X-ray SLD, Å ²
16:0	0.531	0.514	0.691	0.513	0.550818	0.571387967	-3.4532E-08	8.1493E-06
16:1Δ ⁸	0.054	0.078	0.078	0.034	0.060836	0.063107884	1.2184E-07	8.4925E-06
18:0	0.002	0.015		0.039	0.009037	0.009374481	-6.739E-08	9.0048E-06
18:1 Δ ⁸	0.097	0.046	0.123	0.161	0.099356	0.10306639	7.8055E-08	8.5200E-06
18:2 Δ ^{5,12}	0.117	0.106	0.102	0.212	0.119403	0.123862033	2.2365E-07	8.5202E-06
18:3 Δ ^{6,9,12} and 18:3 Δ ^{8,12,15}	0.199	0.241	0.006	0.041	0.12455	0.129201245	3.7767E-07	8.6266E-06
SUM	1	1	1	1	0.964	1		

		Neutron, Å ²	X-ray, Å ²
	Weighted fatty acid SLDs	7.1868E-8	8.3248E-6

Table S5. *Thermosynechococcus vulcanus* thylakoid membrane lipid tailgroup SLD calculations

Thermosynechococcus vulcanus lipid composition (mol%): 43.5% MGDG, 25.6% DGDG, 24.8% SQDG and 6.1 % PG (Sakurai2006). Total weighted sum for each fatty acid (mol%) from different lipids calculated as in the Table S4.

Fatty acid	MGDG	DGDG	SQDG	PG	Weighted sum	Neutron SLD, Å ²	X-ray SLD, Å ²
16:0	0.451	0.436	0.62	0.479	0.49078	-3.4532E-08	8.1493E-06
16:1Δ ⁸	0.155	0.151	0.039	0.107	0.12228	1.2184E-07	8.4925E-06
18:0	0.039	0.025	0.08	0.089	0.048634	-6.739E-08	9.0048E-06
18:1 Δ ⁸ and 18:1 Δ ¹¹	0.355	0.388	0.261	0.325	0.338306	7.8055E-08	8.5200E-06
SUM	1	1	1	1	1	Neutron, Å ²	X-ray, Å ²
					Weighted fatty acid SLDs	2.1080E-8	8.3583E-6

Table S6. *Synechocystis* thylakoid membrane lipid headgroup SLD calculations

Synechocystis sp. PCC 6803 lipid composition (mol%): 37.4% MGDG, 20% DGDG, 28% SQDG and 13.7% PG (Sakurai2006). Scattering length density of DGDG and MGDG is summed and calculated as for single galactose molecule. No glycosidic bond formation is taken into account.

Lipid headgroup	Fraction	Neutron SLD, Å ²	X-ray SLD, Å ²	
Galactose	0.574	1.6114E-06	1.4679E-05	
Sulfogalactose	0.289	2.1336E-06	1.7495E-05	
Phosphoglycerol	0.137	1.5610E-06	1.5202E-05	
SUM	1			
		Neutron, Å ²	X-ray, Å ²	
		Lipid headgroup SLDs	1.7554E-06	1.5564E-05

Table S7. *Thermosynechococcus vulcanus* thylakoid membrane lipid headgroup SLD calculations

Thermosynechococcus vulcanus lipid composition (mol%): 43.5% MGDG, 25.6% DGDG, 24.8% SQDG and 6.1 % PG (Sakurai2006). Scattering length density of DGDG and MGDG is summed and calculated as for single galactose molecule. No glycosidic bond formation is taken into account.

Lipid headgroup	Fraction	Neutron SLD, Å ⁻²	X-ray SLD, Å ⁻²
Galactose	0.691	1.6114E-06	1.4679E-05
Sulfogalactose	0.248	2.1336E-06	1.7495E-05
Phosphoglycerol	0.061	1.5610E-06	1.5202E-05
SUM	1		
		Neutron, Å ⁻²	X-ray, Å ⁻²
		Lipid headgroup SLDs	
		1.7378E-06	1.5409E-05

Phosphoglycerol has two exchangeable hydrogens in -OH, but ester bonds are formed in lipids between phosphoglycerol and fatty acids. Therefore no exchange is accounted for phosphoglycerol when deriving neutron SLD.

Table S8. Protein-adjusted SLD values of headgroups and tailgroups with different Protein/Lipid ratios.

Neutron SLDs for 10-15% (N10) and 0% (N0) total deuterium-hydrogen exchange as well X-ray SLDs (X) are calculated in different blocks.

Non-treated SLD values taken from Tables S3, S4 and S6		SLD Neutrons, Å ⁻²	SLD X-rays, Å ⁻²
	Tailgroups	7.1868E-08	8.3248E-06
	Headgroups	1.7554E-06	1.5564E-05
10% total hydrogen exchange	Protein 10	2.4704E-06	NA (identical to 1.2457E-05)
0% total hydrogen exchange	Protein 0	1.8646E-06	1.2457E-05

HYDROGEN EXCHANGE: 10-15% of total hydrogens are exchanged			
	Protein/Lipid ratio	SLDAdjustedHeadgroupN10, Å ⁻²	SLDAdjustedTailgroupN10, Å ⁻²
100% lipids	0	1.7554E-06	7.1868E-08
	0.2	1.8984E-06	5.52E-07
	0.4	2.0414E-06	1.0313E-06
	0.5	2.1129E-06	1.2711E-06
	0.6	2.1844E-06	1.5110E-06
	0.7	2.2559E-06	1.7508E-06
	0.8	2.3274E-06	1.9907E-06
100 % proteins	1	2.4704E-06	2.4704E-06

HYDROGEN EXCHANGE: 0% of all hydrogens are exchanged			
	Protein/Lipid ratio	SLDAdjustedHeadgroupN0, Å ⁻²	SLDAdjustedTailgroupN0, Å ⁻²
100% lipids	0	1.7554E-06	7.1868E-08
	0.2	1.78E-06	4.30E-07
	0.4	1.7991E-06	7.8896E-07
	0.5	1.8100E-06	9.6823E-07
	0.6	1.8209E-06	1.1475E-06
	0.7	1.8318E-06	1.3268E-06
	0.8	1.8428E-06	1.5060E-06
100 % proteins	1	1.86E-06	1.86E-06

X-rays			
	Protein/Lipid ratio	SLDAdjustedHeadgroupX, Å ⁻²	SLDAdjustedTailgroupX, Å ⁻²
100% lipids	0	1.5564E-05	8.3248E-06
	0.2	1.49E-05	9.15E-06
	0.4	1.4321E-05	9.9777E-06

	0.5	1.4011E-05	1.0391E-05
	0.6	1.3700E-05	1.0804E-05
	0.7	1.3389E-05	1.1217E-05
	0.8	1.3078E-05	1.1631E-05
100 % proteins	1	1.2475E-05	1.2475E-05

Table S9. Inter-thylakoid space X-ray and neutron SLD derivation using different phycobilisome/D₂O volume fractions and phycobilisome H-D exchange.

Inter-thylakoid space SLD was derived as the weighted sum of phycobilisome and D₂O SLDs. 25 unique polypeptides of *Griffithsia pacifica* (PY6P) from (Zhang 2017) were combined to a single sequence. Neutron SLD calculated using biological SLD calculator for different H-D exchanges.

Neutrons			
Protein/D ₂ O volume ratio	D ₂ O/H ₂ O volume ratio in inter-thylakoid space.	Phycobilisome H-D exchange %	Neutron SLD, Å ⁻²
Phycobilisome SLD			
1/0	1/0	100%	3.258E-6
1/0	1/0	90%	3.12E-6
1/0	1/0	80%	2.982E-6
1/0	1/0	0%	1.875E-6
1/0	0.9/0.1	90%	3.12E-6
1/0	0.8/0.2	80%	2.76E-6
1/0	0.8/0.2	50%	2.428E-6
1/0	0.5/0.5	50%	2.22E-6
1/0	0.5/0.5	0%	1.875E-6
Inter-thylakoid space SLD			
0.6/0.4	1/0	0%	3.685E-6
0.6/0.4	1/0	90%	3.2325E-6
0.7/0.3	1/0	0%	4.104E-6
0.7/0.3	1/0	90%	3.94E-6
0.75/0.25	1/0	90%	2.78E-6
0.8/0.2	1/0	0%	3.6656E-6
0.8/0.2	1/0	80%	2.55375E-6
0.85/0.15	1/0	0%	2.5018E-6
0.85/0.15	0.8/0.2	80%	3.09102E-6
0.85/0.15	0.9/0.1	90%	3.40135E-6
0.85/0.15	1/0	90%	3.612E-6
0.9/0.1	1/0	0%	2.3275E-6
0.9/0.1	1/0	90%	3.448E-6

X-ray SLD calculated of inter-thylakoid space in NIST calculator for 25 unique *Griffithsia pacifica* (PY6P) phycobilisome subunits, C₃₅₂₁₆H₅₅₃₀₀N₉₇₁₆O₁₀₇₂₉S₃₁₁ with the density of 1.35 g/mL.

X-rays	
Protein/D2O volume ratio	X-ray SLD, Å ⁻²
0.5/0.5	10.877E-6
0.6/0.4	11.1586E-6
0.7/0.3	11.4402E-6
0.8/0.2	11.7218E-6
0.85/0.15	11.8626E-6
0.9/0.1	12.0034E-6
1	12.285E-6

Table S10. Lumen X-ray and neutron SLD derivation using different plastocyanin/D₂O volume fractions and plastocyanin H-D exchange.

Amino acid sequence of mature *Synechocystis* sp. (strain *PCC 6803* / *Kazusa*) plastocyanin (UniProtKB P21697) was used. Lumen SLD was derived as the weighted sum of plastocyanin and D₂O SLDs. Neutron SLD calculated using biological SLD calculator for different H-D exchanges.

Neutrons			
Protein/D2O volume ratio	D2O/H2O volume ratio in inter-thylakoid space.	Plastocyanin H-D exchange %	Neutron SLD, Å ⁻²
Plastocyanin SLD			
1/0	1/0	100%	3.021E-6
1/0	1/0	90%	2.90E-6
1/0	1/0	80%	2.779E-6
1/0	1/0	0%	1.808E-6
1/0	0.9/0.1	90%	2.791E-6
1/0	0.8/0.2	80%	2.2585E-6
1/0	0.8/0.2	50%	2.294E-6
1/0	0.5/0.5	50%	2.112E-6
1/0	0.5/0.5	0%	1.808E-6

X-ray SLD calculated in NIST calculator for C₄₅₆H₇₀₃N₁₁₉O₁₄₄S₃ (UniProtKB P21697) *Synechocystis* sp. (strain PCC 6803 / Kazusa) plastocyanin with the density of 1.35 g/mL.

X-rays	
Protein/D2O volume ratio	X-ray SLD, Å ³
0.5/0.5	10.872E-6
0.6/0.4	11.1526E-6
0.7/0.3	11.4332E-6
0.8/0.2	11.7138E-6
0.85/0.15	11.8541E-6
0.9/0.1	11.9944E-6
1	12.275E-6

Table S11. Absolute values for inter-thylakoid and lumen SLDs

Neutrons

	SLD I-I space abs scale	SLD I-I -SLDmembrane	DiffSLDrel scale	LumenAbsScale	D2O/H2O	H-D exchange of labile hydrogens
60% *0%-H exchanged protein + 40%*100% D2O	3.685	2.105	0.7157	4.4007	100%	0%
60% *90%-H exchanged protein + 40%*100% D2O	4.432	2.852	0.96988	5.40168	100%	0%
70% *0%-H exchanged protein+30%* 100% D2O	3.2325	1.6525	0.56185	3.79435	100%	0%
70% *90%-H exchanged protein+30%* 100% D2O	4.104	2.524	0.85816	4.96216	100%	90%
75% *0%-H exchanged protein+25%* 100% D2O	3.94	2.36	0.8024	4.7424	100%	90%
80% *0%-H exchanged protein + 20%*100% D2O	2.78	1.2	0.408	3.188	100%	0%
80% *100%-H exchanged protein + 20%*100% D2O	3.8864	2.9064	0.78418	4.670576	100%	100%
85% *0%-H exchanged protein+15%* 100% D2O	2.55075	0.97575	0.31098	2.884825	100%	0%
85% *50%-H exchanged protein+15%* 80% D2O	2.525	0.745	0.2553	2.5783	50%	50%
85% *80%-H exchanged protein+15%* 80% D2O	3.09102	1.51102	0.51375	3.6047668	80%	80%
85% *0%-H exchanged protein+15%*90% D2O	2.44935	0.86935	0.29558	2.744929	90%	0%
85% *90%-H exchanged protein+15%* 90% D2O	3.40135	1.82135	0.61925	4.030698	90%	90%
85% *90%-H exchanged protein+15%* 100% D2O	3.612	2.032	0.69088	4.30388	100%	90%
90% *0%-H exchanged protein+10%* 100% D2O	2.3275	0.7475	0.25415	2.58165	100%	0%
90% *90%-H exchanged protein+10%* 100% D2O	3.448	1.868	0.63512	4.08312	100%	90%
100% 100%-H exchanged protein	3.258	1.678	0.37652	3.82852	0%	100%
	SLDmembrane	1.58	SLDLumen rel scale	0.34		

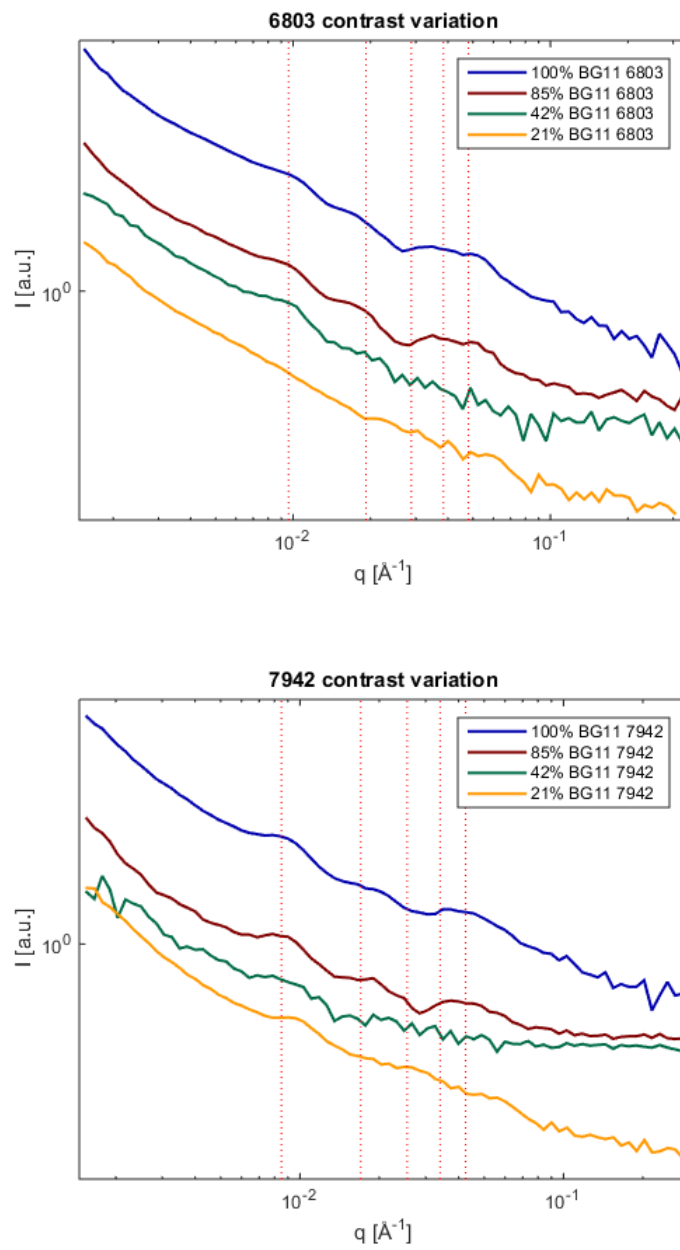
X-rays

LUMEN	X-rays		Plastocyanin/Water
Phycobilisome/Water	SLDi-t ab Delta	LumenRelIScale	LumenAbsScaleX
50% protein 50% H2O	10.877	-0.323	0.056525
60% protein 40% H2O	11.1586	-0.0414	0.007245
70% protein 30% H2O	11.4402	0.2402	-0.042035
80% protein 20% H2O	11.7218	0.5218	-0.091315
85% protein 15% H2O	11.8626	0.6626	-0.115955
90% protein 10% H2O	12.0034	0.8034	-0.140595
100% protein	12.285	1.085	-0.189875
75% protein 25% H2O0	11.581	0.381	-0.066675
Plastocyanin/Water			
	SLD X-rays		
50% protein 50% H2O	10.872		
60% protein 40% H2O	11.1526		
70% protein 30% H2O	11.4332		
80% protein 20% H2O	11.7138		
85% protein 15% H2O	11.8541		
90% protein 10% H2O	11.9944		
100% protein	12.275		

Table S12. Lumen protein volume fraction. X-rays and neutrons.

INTER-THYLAKOID SPACE				6.471+0.310%	6.471+0.21%	6.471+0.24%	6.471+0.180%	6.704+0.278%	6.704+0.27%	6.704+0.236%	6.704+0.21%	6.704+0.210%	6.704+0.180%	6.86070+0.2595%	4.0081+0.240%	3.8161+0.224%	2.8271+0.21%	8.86071+0.227%
				lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen	lumen protein volume fraction X-ray + QCD Neutron - lumen
Varstein/VQ20	Q20/Q20%	Labile H-D exchange %																X-ray
0.6*1.875+0.4*6.4	60%/4%	100%	0%	0.59488389	0.571228571	0.501706399	0.435387631	0.44740817	0.416256787	0.389044776	0.365274664	0.334522587	0.237677387	-0.04811228	-0.574653031	-1.832549505	0.854770242	
0.6*1.11+0.4*6.4	60%/0%	100%	80%	0.05048338	0.285294386	0.20251844	0.227404381	0.122772542	0.096557011	0.090344776	0.084703942	0.077397536		-0.262684	-0.57236865	-1.332680772	-0.071886199	0.694770242
0.7*1.875+0.3*6.4	70%/0%	100%	0%	0.77126512	0.744471428	0.620866462	0.567432491	0.655561777	0.609148959	0.579044776	0.552321807	0.490216571		0.45023978	0.72005374	-0.317878465	-1.082118337	0.887534255
0.7*1.11+0.3*6.4	70%/70%	100%	50%	0.42552244	0.410811249	0.360810409	0.313184607	0.254605294	0.236938887	0.223444776	0.207934798	0.190412678		0.00249306	-0.342686254	-0.89477721	-2.547425743	0.887534255
0.75*1.12+0.2*6.4	75%/12%	100%	50%	0.605259537	0.4706	0.416599649	0.36097561	0.300105419	0.187222525	0.239044776	0.269504709	0.148821248		0.06423445	-0.217857198	-0.842431378	-1.255455445	0.728911282
0.8*1.875+0.2*6.4	80%/0%	100%	0%	0.95057704	0.917774286	0.836226385	0.694477352	0.863774384	0.803577132	0.751254776	0.705156951	0.645700554		0.768363128	0.588142707	0.311176041	-0.331683168	0.770308268
0.8*1.158+0.2*6.4	80%/70%	100%	100%	0.51182133	0.484122143	0.433983438	0.376161925	0.354782788	0.30061961	0.328484776	0.285630771	0.265222567		0.124398804	-0.189704092	-0.771788706	-2.166554435	0.770308268
0.85*1.875+0.1*6.4	85%/12%	100%	0%	1.602030861	1.506330774	0.881201331	0.765499782	0.987789397	0.800407218	0.864584176	0.796017308	0.723607546		0.874118314	0.747208191	0.532631039	0.653553436	0.811705274
0.85*1.22+0.15*2.92	85%/75%	50%	50%	1.153133393	1.091914286	0.95902133	0.832251162	1.073017508	0.99820725	0.93044776	0.876368996	0.802888494		1.002812999	0.980027282	0.756537619	0.42889604	0.811705274
0.85*1.76+0.15*4.8680	85%/12%	80%	80%	0.873188414	0.78681667	0.701438695	0.608718931	0.720643548	0.470477327	0.626595376	0.588555112	0.538817556		0.573850545	0.569482567	0.006744485	-0.847483663	0.811705274
0.85*1.875+0.15*3.04	85%/15%	90%	0%	1.081701083	1.044006	0.912702777	0.795962439	1.018181654	0.945088151	0.883304776	0.795851544	0.709151544		0.932833724	0.820601892	0.634823768	0.21667205	0.811705274
0.85*1.895+0.15*5.704	85%/15%	90%	90%	0.704270169	0.679726	0.597086826	0.518160061	0.577889118	0.537053827	0.502504776	0.47180241	0.432081879		0.397238796	0.311309325	-0.297011887	-1.362118831	0.811705274
0.85*1.12+0.1*6.4	85%/12%	100%	90%	0.620613323	0.599777145	0.53625345	0.45668895	0.480986871	0.447499202	0.416244776	0.392890583	0.35961039		0.278747186	0.00210913	-0.503398416	-1.11485148	0.811705274
0.9*1.875+0.1*6.4	90%/0%	100%	0%	1.130023676	1.000957143	0.958180578	0.831522618	1.071887491	0.997257304	0.932044776	0.875088004	0.801424538		1.001406499	0.946266679	0.754090577	0.418875	0.851022281
0.9*1.12+0.1*6.4	90%/10%	100%	90%	0.885871017	0.846196574	0.58440251	0.50447038	0.564247977	0.517186764	0.48384776	0.444282111	0.416030961		0.373101519	0.18859669	-0.342673464	-1.49350495	0.851022281
1*1.258	100%/10%	100%	100%	0.783218053	0.734708571	0.645289837	0.559912899	0.648811102	0.599005113	0.559844776	0.525638013	0.481386037		0.477907645	0.232088143	-0.15669832	-1.124405941	0.935896234

Figure S1 Contrast variation measurements



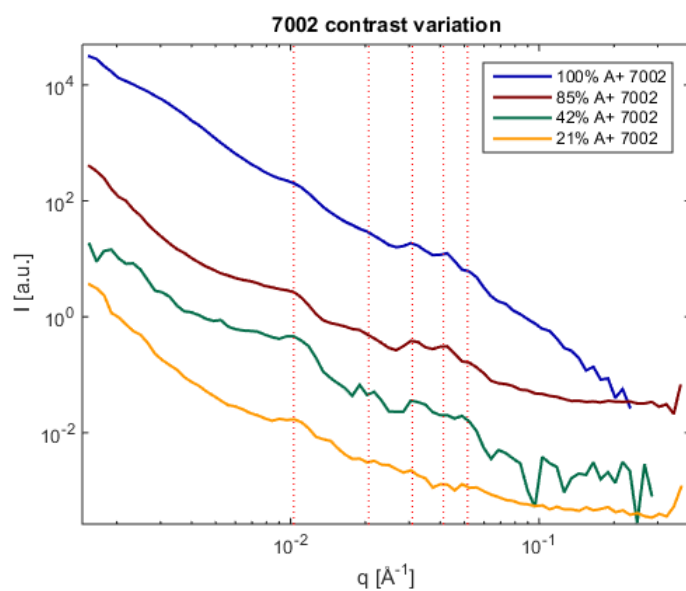
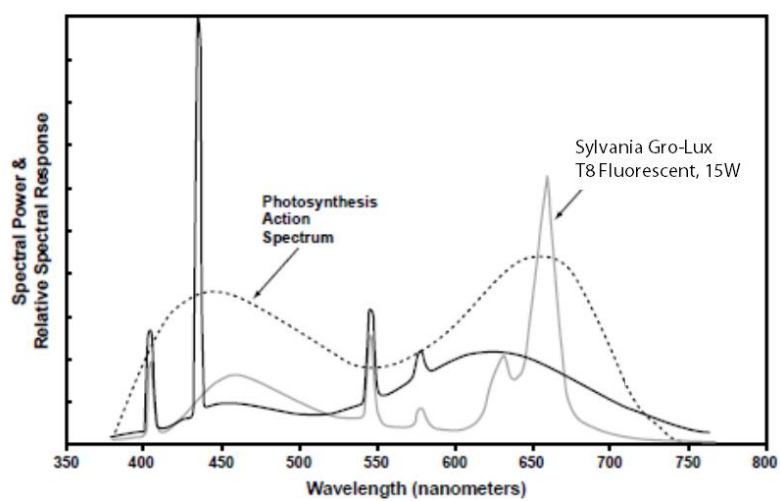
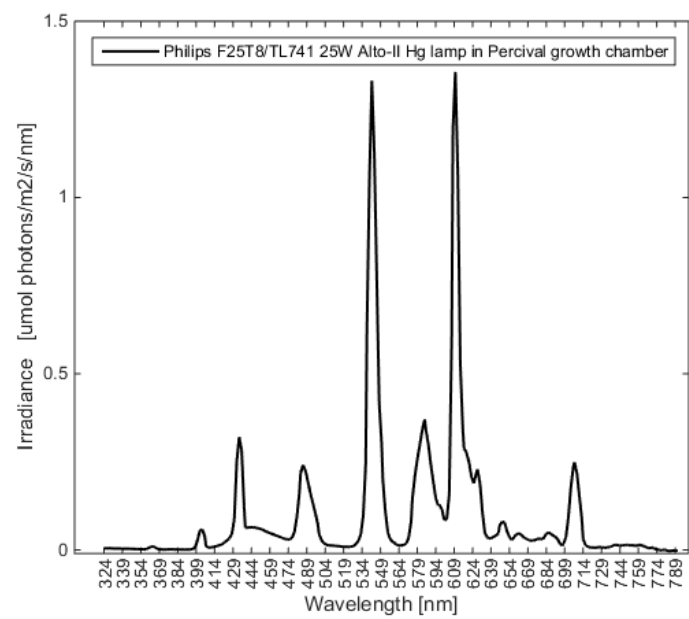


Figure. S2 Illumination spectra

Illumina standard light source



Percival



Manuscript 2

Light-induced thylakoid dynamics: the structural approach

ILLUMINATION-INDUCED THYLAKOID MEMBRANE DYNAMICS OF HIGHER PLANTS

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Introduction

Investigating and understanding chloroplast thylakoid dynamics in relation to illumination is of a key importance to increase photosynthetic yield and plant biomass, as light-induced adjustments of thylakoid ultrastructure are proposed to regulate photosynthetic efficiency^{1a}. Light-induced thylakoid dynamics have been investigated by fluorescence microscopy^{2,3}, electron microscopy^{4–16}, atomic force microscopy¹⁷, light scattering^{5,8,18} and small angle scattering^{19–24} methods. Despite a considerable amount of literature on the topic, the underlying mechanism still remains controversial, as the underlying biochemistry, which governs these ultrastructure changes is not well understood. Indeed, two seemingly irreconcilable dynamical processes are observed experimentally: illumination-induced decrease of thylakoid stacking repeat distance, SRD (the height of granal thylakoid plus the height of the inter-thylakoid space across to the next granal thylakoid), or SRD expansion.

The current understanding of illumination-induced thylakoid dynamics in granum is visualized in Fig. 1: at least eight ultrastructural outcomes have been observed. The experiments observing light-induced lumen and thylakoid stacking repeat distance shrinkage^{16,4–7,10,12–15,21,22,25–27}, have been recently contradicted with experimental observations, suggesting a medium/high light illumination-induced increase of lumen height^{8,10,17}. Furthermore, many more ‘dark-light’ dynamical outcomes have been documented: grana margin expansion and a simultaneous shrinkage of grana core^{6,7,28}, a decrease in grana diameter^{3,16} and changes of the distance between two adjacent thylakoids (inter-thylakoid space height)^{8,29} have been observed upon leaf illumination *in vivo* (Fig. 1). In accordance with literature analysis, our findings support the claim that thylakoid ultrastructural changes and the direction of the change (SRD expansion or shrinkage) can be affected by ion concentrations^{30,31}, light quality¹⁷ and previous plant growth condition¹².

To get a comprehensible and method-independent overview of thylakoid dynamics, we here report the use of complementary ultrastructure-tracking techniques: microscopy and small angle scattering. As chlorophyll naturally fluoresces, the confocal laser scanning microscopy (CLSM) on a plant leaf section enables following large-scale chloroplast dynamics in medium resolution. Complementary transmission electron microscopy (TEM) technique reveals a nanometer resolution and is an excellent tool to investigate local thylakoid membrane features such as membrane, lumen, and inter-thylakoid

gap thicknesses. However, TEM has a practical limit of the number of analyzed samples and requires sample cryo/chemical fixation, what may introduce putative artefacts. The use of small angle neutron scattering (SANS) method avoids such artefacts. Also, scattering probes a large sample area *in vivo* in Ångstrom-scale resolution and yields the averaged ultrastructure *in vivo*.

This paper aims to re-examine the previously published findings on light-induced thylakoid dynamics in the context of plants containing a high number of grana layers. In the first part of the paper we explain thylakoid membrane scattering *in vivo*. We analyse scattering curves of leaves *in vivo* by applying the model of Jakubauskas *et al.* (Manuscript 1) provides the average thylakoid membrane, lumen thicknesses and the stacking repeat distance between thylakoids in grana stacks.

In the second part of the paper we combine scattering, TEM and CLSM techniques to investigate the thylakoid dark-to-medium light transition of selected shade-tolerant mono- and dicot plant species with high grana stacks, exemplified by *Ficus elastica*, and compare it to thylakoid ultrastructure changes in plants with a ‘typical’ thylakoid arrangement, exemplified by *Arabidopsis thaliana*.

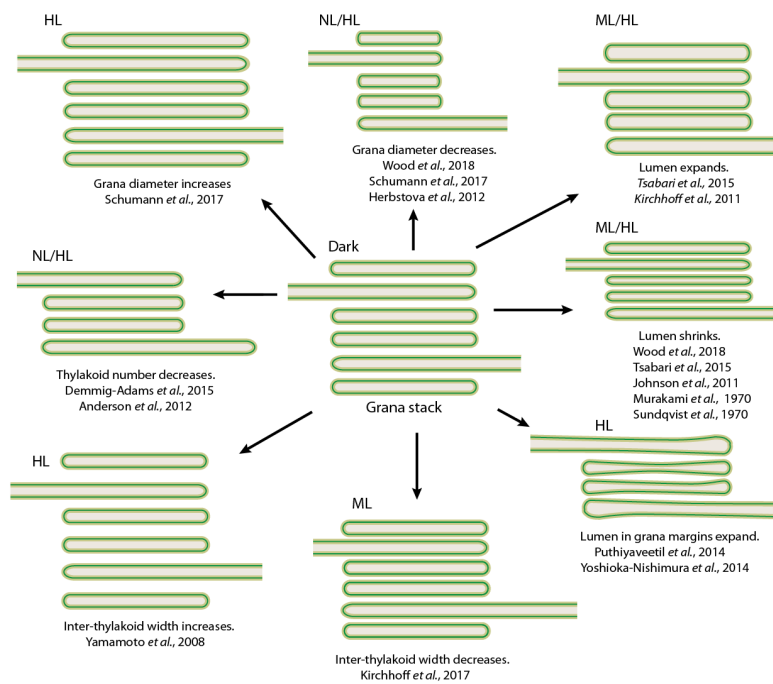


Fig. 1 Granal thylakoid ultrastructure changes between dark-light conditions. HL – high light, ML – medium light, NL – natural light.

Table 1. Quantitative SRD changes. Stacking repeat distance of various plants in darkness and after illumination in given conditions. Green – illumination-induced SRD increase, orange – illumination-induced SRD decrease. Numbers given in brackets is lumen thickness in Å. Numbers given with a star is thickness of individual thylakoid in Å. D ReAd – dark-readapted, ChemF – chemical fixation, HPF – high pressure freezing, FS – freeze-substitution, SANS – small angle neutron scattering, ft-c – foot candles, +ATP – with 1 mM ATP added.

Organism	Entity	SRD Dark, Å	SRD light, Å	Illumination	Light color, time	Method	Reference
<i>A. thaliana</i> Col0	Leaf degassed with buffer	168±4 (46)	186±4 (88)	500 µmol photons m ⁻² s ⁻¹	White, 30 min	FS	Kirchhoff <i>et al.</i> 2011 ⁸
	Attached leaf	175	233±8	2000 µmol photons m ⁻² s ⁻¹	White, 30-120 min	HPF	Tsabari <i>et al.</i> 2015 ¹⁰
	Attached leaf in N ₂ atmosphere	175	140±6	2000 µmol photons m ⁻² s ⁻¹	White, 120 min	HPF	Fig. 2a of Tsabari <i>et al.</i> 2015 ¹⁰
	De-enveloped chloroplasts in buffer	252±2	316±7 (+ATP) 275±4 (-ATP)	2000 µmol photons m ⁻² s ⁻¹	White, 120 min	ChemF	Chauartzman <i>et al.</i> 2008 ⁹
	Isolated thylakoids in buffer	200±100	213±100	150 µmol photons m ⁻² s ⁻¹	640 nm, 5 min	HPF	Clausen <i>et al.</i> 2014 ¹⁷ , Partial unstacking under PSII-light, no effect under PSI-light
	Leaf grown in LL	*241, D ReAd: *241	*229	1000 µmol photons m ⁻² s ⁻¹	White, 30 min, Dark readaptation 10 min	ChemF	Schumann <i>et al.</i> 2017 ¹²
	Leaf grown in NL	*223, D ReAd: *269	*231	1000 µmol photons m ⁻² s ⁻¹	White, 30 min, Dark readaptation 10 min	ChemF	Schumann <i>et al.</i> 2017 ¹²
	Leaf grown in NatL	*204, D ReAd: *259	*289	1000 µmol photons m ⁻² s ⁻¹	White, 30 min, Dark readaptation 10 min	ChemF	Schumann <i>et al.</i> 2017 ¹²
<i>A. thaliana</i> stn7	Leaf grown in HL	*211, D ReAd: *266	*257	1000 µmol photons m ⁻² s ⁻¹	White, 30 min, Dark readaptation 10 min	ChemF	Schumann <i>et al.</i> 2017 ¹²
	Isolated thylakoids in buffer	184±40	220±50	150 µmol photons m ⁻² s ⁻¹	640 nm, 5-30 min	HPF	Clausen <i>et al.</i> 2014 ¹⁷ , Partial unstacking under PSII-light, no effect under PSI-light
Spinach	Leaf as is, 4 °C	162	190	2000 µmol photons m ⁻² s ⁻¹	White, 1h	ChemF	Fig S1 of Yoshioka-Nishimura <i>et al.</i> 2014 ⁶
	Leaf as is	163.1±8.4	147.6±7.0	1500 µmol photons m ⁻² s ⁻¹	White, 1h	ChemF	Fig. 4 of Yamamoto <i>et al.</i> 2013 ⁷
	Leaf infiltrated with D2O	229; D ReAd: 223-227	220-224	1700 µmol photons m ⁻² s ⁻¹	White, 5-10 min	SANS	Ünneper <i>et al.</i> 2014 ³²
	Leaf	206±24	192±27	200 µmol photons m ⁻² s ⁻¹	White, 60 min	ChemF	Wood <i>et al.</i> 2018 ¹⁶
	Isolated chloroplasts in buffer	212±8; D ReAd: 214±4	144±9	290 µmol photons m ⁻² s ⁻¹ (900 ft-c)	Red (600-700nm), 3 min	ChemF	Murakami <i>et al.</i> 1970 ³³
	Isolated chloroplasts in buffer	196±4	144±3	290 µmol photons m ⁻² s ⁻¹ (900 ft-c)	Red (600-700nm), 3 min	ChemF	Murakami <i>et al.</i> 1970 ³³
	Isolated chloroplasts in buffer	164±23	146±18	70 klux (6500 ft-c)	Red, 2 min	ChemF	Sundquist <i>et al.</i> 1970 ³⁴
	Isolated chloroplasts in buffer	215±19	175±24	70 klux (6500 ft-c)	Red, 30 s	ChemF	Sundquist <i>et al.</i> 1970 ³⁴
	Isolated chloroplasts in buffer	(250)	(150)	350 µmol photons m ⁻² s ⁻¹	Red (635nm), 5 min	ChemF	Johnson <i>et al.</i> 2011 ²⁷
Maize	Isolated thylakoids in buffer	296; D ReAd: 294-298	262-288	150-1700 µmol photons m ⁻² s ⁻¹	White, 200 s	SANS	Nagy <i>et al.</i> 2013 ²²
	Leaf	*101±7	*88±8	1400 µmol photons cm ⁻² s ⁻¹	Red (650±7.5 nm), 60 min	ChemF	Mustardy <i>et al.</i> 1976 ¹
Pea	Leaf	*101±7	*96±8	1400 µmol photons cm ⁻² s ⁻¹	Far Red (707±8.5 nm), 60 min	ChemF	Mustardy <i>et al.</i> 1976 ¹
	Isolated thylakoids in buffer	195±4	from 152±4 to 172±3	1-37 µmol photons m ⁻² s ⁻¹ (50-2000 lux)	White, 60 min	ChemF	Miller <i>et al.</i> 1972 ³⁵
		330-355	271-280	330-1000 µmol photons m ⁻² s ⁻¹	White, 30 min	SANS	Possett <i>et al.</i> 2012 ²¹

<i>Ulva</i>	Algae cells in vivo	150	109±8	290 µmol photons m ⁻² s ⁻¹ (900 ft-c)	Red (600-700nm), 2 min	ChemF	Murakami <i>et al.</i> 1970b ¹⁸
<i>Porphyra</i>	Algae cells in vivo	198±10	144±6	290 µmol photons m ⁻² s ⁻¹ (900 ft-c)	Red (600-700nm), 2 min	ChemF	Murakami <i>et al.</i> 1970b ¹⁸

Table 2. Qualitative thylakoid ultrastructure changes between dark-adapted and illuminated plants. LL – low light, NL – natural light, HL – high light. Rest as in Table 1.

Organism	Entity	Dark	Light	Illumination	Light color, time	Method	Reference
Spinach	Isolated thylakoids in buffer		Unstacking under HL <i>in vitro</i>	500-2000 µmol photons m ⁻² s ⁻¹	White, 10-60 min	Digitonin fractionation	Khatoun <i>et al.</i> 2009 ²⁶
	Attached leaf	Avg. grana number per chloroplast lower	Avg. grana number per chloroplast higher	300-1500 µmol photons m ⁻² s ⁻¹	Sunlight, 30-180 min	ChemF	Rozak <i>et al.</i> 2002 ¹¹
		Avg. thylakoid number per grana higher	Avg. thylakoid number per grana lower	300-1500 µmol photons m ⁻² s ⁻¹	Sunlight, 30-180 min	ChemF	Rozak <i>et al.</i> 2002 ¹¹
<i>A. thaliana</i> Col 0 and aLhcb2 mutant	Leaf	Avg. grana number per chloroplast lower	Avg. grana number per chloroplast higher	150 µmol photons m ⁻² s ⁻¹	Growth light, 60 min	ChemF	Anderson <i>et al.</i> 2012 ²⁷
		Avg. thylakoid number per grana higher	Avg. thylakoid number per grana lower	150 µmol photons m ⁻² s ⁻¹	Growth light, 60 min	ChemF	Anderson <i>et al.</i> 2012 ²⁷
<i>A. thaliana</i> Col 0	Leaf grown in LL	No significant changes	No significant changes	1000 µmol photons m ⁻² s ⁻¹	White light, 30 min	ChemF	Schumann <i>et al.</i> 2017 ¹²
	Leaf grown in NL	No significant changes	No significant changes	100 µmol photons m ⁻² s ⁻¹	White light, 30 min	ChemF	Schumann <i>et al.</i> 2017 ¹²
	Leaf grown in HL	Irreversible stacking under 10 min of re-darkening	Grana unstacking, increase in grana height	1000 µmol photons m ⁻² s ⁻¹	White light, 30 min	ChemF	Schumann <i>et al.</i> 2017 ¹²
	Isolated thylakoids in buffer	Thylakoid stacking higher	Thylakoid stacking lower	500-1000 µmol photons m ⁻² s ⁻¹	White light, 20-60 min	Digitonin fractionation	Yamamoto <i>et al.</i> 2008 ²⁹
Barley	Leaves	Enlarged spherical chloroplasts	Ellipsoid chloroplasts	130 µmol photons m ⁻² s ⁻¹	White light, 180 min	HPF-FS	Pfeiffer <i>et al.</i> 2005 ¹³
		Small grana stacks, unstacked thylakoids. Lumen swollen	Typical grana/stroma thylakoid arrangement	130 µmol photons m ⁻² s ⁻¹	White light, 180 min	HPF-FS	Pfeiffer <i>et al.</i> 2005 ¹³
<i>Monstera deliciosa</i>	Leaves grown in LL	Grana stacked	Grana unstacking	1500 µmol photons m ⁻² s ⁻¹	White light, 20 min	ChemF	Demmig-Adams <i>et al.</i> 2015 ¹⁴
	Leaves grown in LL	Avg. thylakoid number per grana higher	Avg. thylakoid number per grana increases	1500 µmol photons m ⁻² s ⁻¹	White light, 20 min	ChemF	Demmig-Adams <i>et al.</i> 2015 ¹⁴
	Leaves grown in HL	Avg. thylakoid number per grana lower	Avg. thylakoid number per grana decreases	1500 µmol photons m ⁻² s ⁻¹	White light, 20 min	ChemF	Demmig-Adams <i>et al.</i> 2015 ¹⁴

Methods

Samples

For scattering experiments, variegated variants of *Ficus elastica* (hereby: *Ficus*), *Epipremnum aureum* (hereby: *Epipremnum*), *Alocassia macrorrhizos* (hereby: *Alocassia*), *Dieffenbachia* (hereby: *Dieffenbachia*), *Chlorophytum comosum* (hereby: *Chlorophytum*) plant species were kept in the deep shade ($0 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, with 10 min exposure to indoor light every day) at room temperature for 2 weeks to stimulate large grana formation. For the last two days before measurement, plants were dark-adapted. For TEM, CLSM, chlorophyll fluorescence and 77K experiments, these plants were grown in $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ambient light and dark-adapted. *Arabidopsis thaliana* Col 0 (hereby: *Arabidopsis*) plants for SANS studies were grown at 21°C with 12 hour light/dark cycle under $110\text{--}140 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and dark-adapted for 3 days in room temperature before measurement. *Arabidopsis thaliana* Col0 plants for TEM, CLSM and 77K were grown in $21/18^\circ\text{C}$ with 8/16 hour light/dark cycle under $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and dark-adapted for 2 days before measurements.

Light intensity conversions

With a conversion factor 0.322, 900 ft-c red light is equivalent to $290 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 6500 ft-c is equivalent to $2096 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, with a conversion factor of 0.0185³⁸, 2000 lux of sunlight is equivalent to $37 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ³⁹.

Chlorophyll fluorescence measurements

Chl emission spectra were measured in 77 K directly on a leaf blade ($0.8\text{--}1.7 \text{ mg Chl/cm}^2$) and from isolated plastids (plastids were isolated as in Kowalewska *et al.*⁴⁰ in 20 mM HEPES-NaOH (pH 7.5) 330 mM sorbitol, 15 mM NaCl and 4 mM MgCl_2 containing buffer and diluted to $10 \mu\text{g Chl/ml}$ concentration) using modified Shimadzu RF-5301PC spectrofluorometer where excitation and emission beams were supplied by optical fibers, as in Mazur *et al.*⁴¹. F_0 and F_m values of *in vivo* Chl fluorescence was measured at room temperature using Dual-PAM100 fluorimeter, maximum quantum yield of PSII was calculated as $F_v/F_m = (F_m - F_0)/F_m$. Variable fluorescence (F_v) reflects the PSII photochemistry indicated by the redox state of Q_A , and the F_v/F_m ratio informs about the maximum quantum yield of PSII in the dark-adapted state. The fast kinetics curves of chlorophyll fluorescence were registered simultaneously with F_0 and F_m values. The basic pattern of fluorescence induction curve is denoted as the 'OJIP' rise. The different inflection points of the fluorescence curve correspond to: J – the peak concentrations of quinones $Q_A^- Q_B^-$ and $Q_A^- Q_B^-$, I – the concentration change of $Q_A^- Q_B^{2-}$ and P – the peak concentrations of $Q_A^- Q_B^{2-}$ and PQH_2 . This conceptually means that all reaction centers are open at O and all are closed at P, if the light pulses are saturating (then $P = F_m$). Chl a and b content was measured according to Lichtenthaler⁴².

Small angle neutron scattering

Small angle neutron scattering was performed with 'BILBY' instrument in a time-of-flight mode (ANSTO, Sydney, Australia)⁴³. Experimental parameters: measuring time 60 min, q range 0.0035-0.26 Å⁻¹ (neutron wavelength 2-20 Å), resolution of 7%. Dark-adapted D₂O-infiltrated leaf samples were measured in quartz sample holders filled with D₂O in this sequence: 3 μmol photons m⁻² s⁻¹ ambient light, illuminated with 500 μmol photons m⁻² s⁻¹ with KL1500 LED cold white light for 1 hour with a pre-illumination period of 20 min and re-measured in 3 μmol photons m⁻² s⁻¹ ambient light after 12 h dark readaptation.

Transmission electron microscopy

Samples for TEM were prepared as in Rudowska *et al.*² by cutting pieces of about 3 mm² area from the middle of the green and yellow/white parts of the leaf. Plant material was fixed in 2.5% (w/v) glutaraldehyde in 5 mM cacodylate buffer (pH 7.4) for 2 h, washed in the buffer and placed in a 2% (w/v) OsO₄ at 4 °C in 50 mM cacodylate buffer (pH 7.4) for about 12 h. The specimens were dehydrated in a graded acetone series and embedded in a low viscosity epoxy embedding medium and cut on a Leica UCT ultramicrotome. Ultrathin sections of 90 nm were stained with uranyl acetate and examined with a JEM 1400 electron microscope (Jeol, Japan) in the Nencki Institute of Experimental Biology of the Polish Academy of Sciences, Warsaw. The number of grana in a plastid, the total grana height, grana diameter and stacking repeat distance were measured for both dark-adapted and illuminated (500 μmol photons m⁻² s⁻¹ for 50 min) *Arabidopsis thaliana* Col 0 and *Ficus elastica* leaves.

Confocal laser scanning microscopy

CLSM samples were prepared using a modified tape method⁴⁴ which enables isolation of single mesophyll cells layer and analysis of thylakoid network structure *in vivo* in chloroplasts localized in the living cell. CLSM samples were kept between the cover and the base glass without additional CO₂ access. Chloroplasts were visualized with the help of Nikon A1 MP microscope as described in Kowalewska *et al.*⁴⁰ with the z axis step set at 60 nm. Numerical parameters of fluorescence stacks and 3D models were calculated using the MeasurementPro package of Imaris 8.4.2.

Results

Characterization of grana morphology using transmission electron microscopy.

The TEM images of the green parts of dark-adapted *Dieffenbachia*, *Ficus*, *Epipremnum* leaves contain chloroplasts, having several wide grana of a diameter between 596 and 857 nm (Fig. 2A, B). Each grana stack is composed of numerous thylakoid layers and can reach the height in the range of 460 - 791 nm (Fig. 2C). Our findings are consistent with previous results; a very similar thylakoid ultrastructures were observed in low-light adapted shade-tolerant plant *Alocassia*⁴⁵. The green parts of dark-adapted *Chlorophytum* have a 'typical' thylakoid ultrastructure: the two distinct granal and stromal thylakoid regions, and grana of lower height - around 264 and 181 nm, respectively (Fig 2B, C).

The TEM studies of the white-yellow (variegated) parts of the same leaves (Fig. 2A middle column) show that the plastids in variegated leaf parts contain no developed and ordered photosynthetic membranes. Only various-sized membrane vesicles, plastoglobules, disorganized membrane lamella or tubules are observed in all plant species. The plastids from white parts of *Chlorophytum* leaf also contain small patches of prolamellar bodies in addition to vesicles.

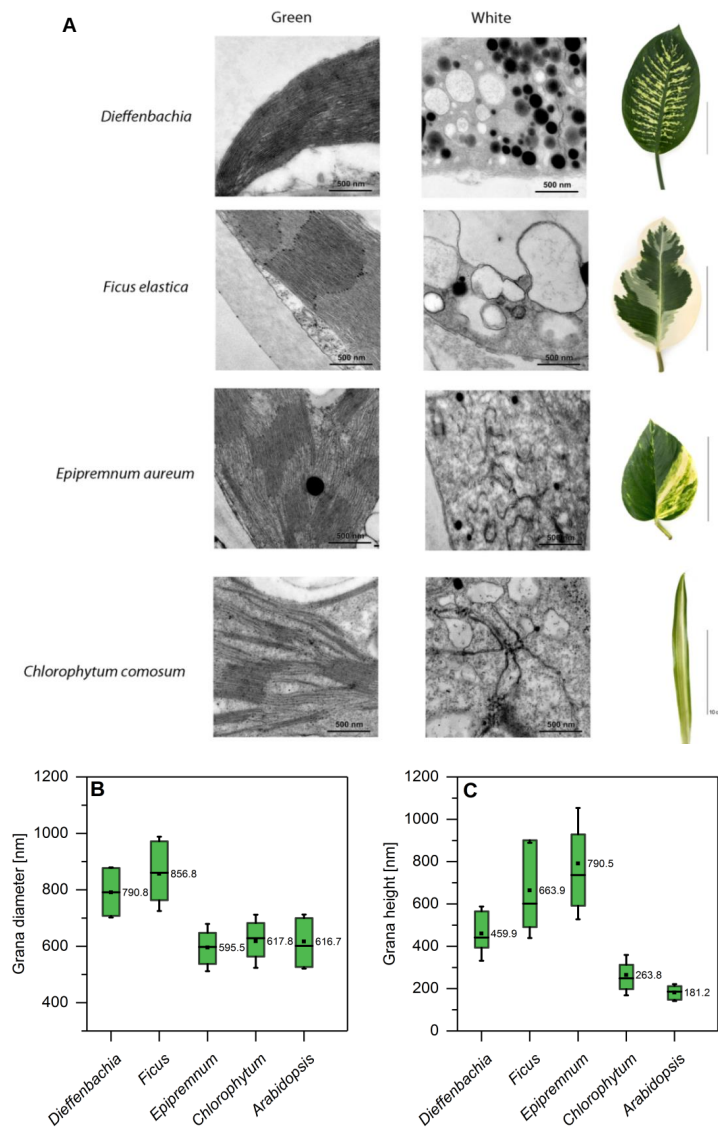


Fig. 2 **A**: Electron micrographs of green part (green) and variegated (white) parts of the shade-tolerant and 'normal' higher plant leaves. An extensive multi-layered granal ultrastructure is observed in green leaf parts. Vesicles and lipid droplets are observed in variegated plant parts. **B**, **C**: Grana diameter and height measurements ($n = 20$).

Green parts of leaf blades are photosynthetically functional and have high chlorophyll a content

The chlorophyll content in the green part of the leaves of all species analyzed is between 1-1.71 mg/cm² Chl *a* and 0.35-0.63 mg/cm² Chl *b*. This gives the following chlorophyll *a/b* ratios of 2.5 for *Ficus*, 2.7 for *Epipremnum*, 2.8 for *Chlorophytum* and 3 for *Dieffenbachia*. Plant cells have Chl *a*/Chl *b* ratios of 3.2-4.0 when exposed to high irradiance and 2.5-2.9 when grown under low irradiance⁴⁶. Therefore, measured Chl *a* / Chl *b* values indicate that plants are low-light adapted and have a high amount of chlorophyll bound to enlarged light harvesting complexes.

PSII photochemistry *in vivo* was estimated by measuring maximal quantum yield (F_v/F_m) in dark-adapted plant leaves. F_v/F_m has typical values of: 0.79 (*Dieffenbachia*), 0.76 (*Ficus*), 0.823 (*Epipremnum*), 0.765 (*Chlorophytum*), indicating the functionality of PSII well in agreement with previous measurements⁴¹.

77K fluorescence emission spectra of the green leaf parts *in vivo* and of isolated chloroplasts/plastids exhibit two main peaks. The wavelengths at 683 and 695 nm are associated with the PSII whereas the band around 740 nm corresponds to PSI. This peak is remarkably variable among the different species, ranging from 734 nm to 745 nm⁴⁷. Both the green parts of the leaves and the isolated chloroplasts (Fig 3A, B) exhibit a characteristic chlorophyll fluorescence pattern of developed chloroplasts with PSII, PSI and their associated antenna fluorescence peaks.

The Chl *a* fluorescence transient curves of dark-adapted leaves are shown on the logarithmic time scale from 10 μs up to 0.4 s (Fig. 3C). Fluorescence curves of green parts of leaf blades of all examined species exhibited typical features of OJIP rise. This indicates that electron transport through PSII takes place, being a marker of the photochemical activity of examined plants.

White/yellow parts of leaf blades are photosynthetically inactive

77K chlorophyll fluorescence spectra from both variegated leaf parts and isolated plastids exhibit low intensity peaks, corresponding to the fluorescence of PSII and PSI antennae (Fig. 3A,B). The spectrum of the white part of a *Chlorophytum* leaf (Fig. S4) is comparable to a spectrum of etioplast after its illumination for 1 hour from Kowalewska *et al.*⁴⁰.

In vivo chlorophyll fluorescence of variegated parts do not exhibit the characteristic J and I intermediate steps (Fig. 3C) and there is no curve flattening after the J point in variegated parts, indicating the lack of oxygen evolving activity. In variegated parts of *Chlorophytum* the shape of the fast kinetics curve is similar to that observed in developing *Chlorophytum* plant (Fig. S4). Overall, although signs of presence of PSs are indicated by the 77K fluorescence-emission measurements, the photosynthetic transfer chain is impaired in white/yellow parts of a variegated leaf and these parts are photosynthetically inactive.

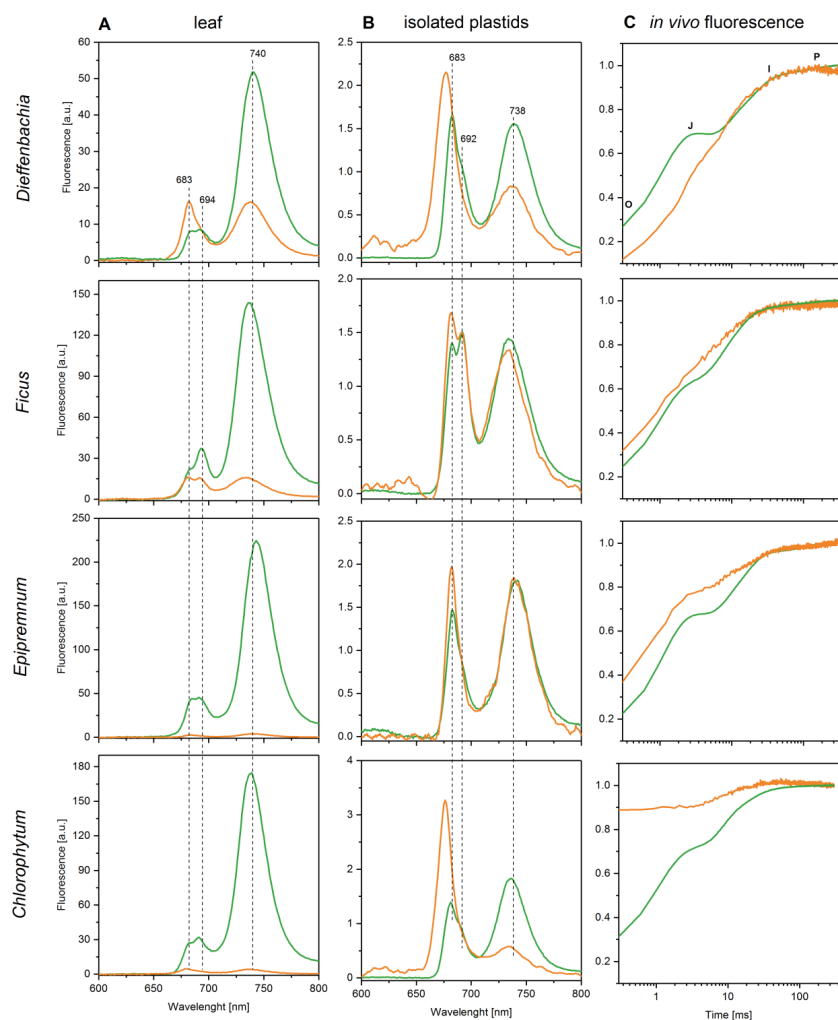


Fig. 3 A: 77K Chl emission spectra of green (green) and variegated (orange) leaf parts. B: 77K Chl emission spectra of plastids isolated from green (green) and variegated (orange) plant parts. C: *In vivo* Chl fluorescence kinetics of a green leaf part exhibits a normal OJIP curve (green) and no photosynthetic electron transport in a variegated leaf part (orange).

In vivo measurements on plant leaves using small angle neutron scattering

Small angle neutron scattering has previously been applied to investigate thylakoid ultrastructure and dynamics in plant leaves²⁶. Here we present an approach of higher resolution, using time-of-flight SANS (ToF SANS). By measuring a window of different energy neutrons in time-of-flight, small angle neutron scattering mode allows a simultaneous investigation of a wide range of the scattering vector q , $q = 4\pi/\lambda \sin\theta$. The higher resolution of time-of-flight setup allows resolving individual scattering peaks better. Overall, ToF SANS enables simultaneously following the behavior of several scattering peaks in a wide q range without the need of different instrumental settings, what considerably shortens measuring time and is important in following thylakoid ultrastructure changes upon illumination.

As exemplified by *Ficus*, the first three diffraction peaks (q_{1-3}) are observed at $q = 0.0279$, 0.0558 , and $0.0837 \text{ \AA}^{-1}$. The ratio of these diffraction peaks to the first scattering peak q_1 is 1 : 2 : 3 (Fig. 4A, red lines), what corresponds to stacked lamellae, where scattering peaks originate from a single granal thylakoid stacking repeat distance (SRD). As expected, scattering from white parts of the *Ficus* plant leaf (Fig. 4A, brown line) or from etiolated plant leaves (maize and barley – Fig. S1) do not exhibit lamellar scattering features in the entire q range. The amount of PLBs present in etiolated leaves (Fig. S6) is insufficient to give a detectable coherent scattering, therefore etiolated leaves can be used as a background if no variegated equivalent of the green leaf part is available. This has been done for the case of *Arabidopsis*.

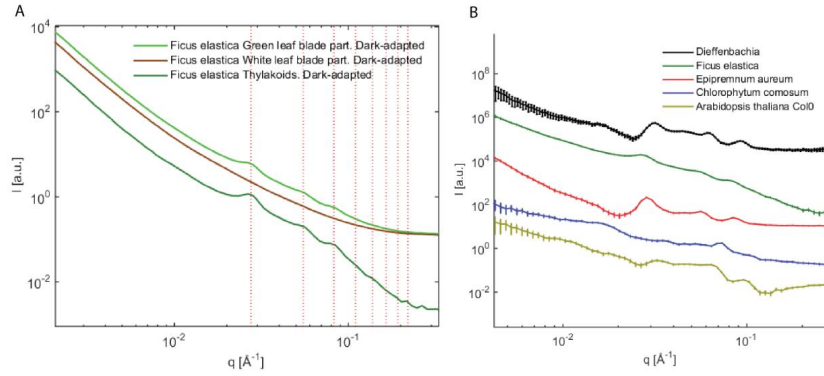


Fig. 4. A: SANS curves of white and green parts of *Ficus* leaves. Red dotted lines indicate q_{1-3} positions of peaks from *Ficus* thylakoid scattering; $q_1 = 0.0279 \text{ \AA}^{-1}$. B: SANS curves from dark-adapted D_2O infiltrated leaves, where scattering from white leaf part is subtracted, leaving only scattering of a thylakoid network. The curves are arbitrary y-spaced.

To investigate scattering of thylakoid membranes, we expand the early experimental work and seminal data analysis of Kreutz and Menke⁴⁸, where they have used variegated leaves to obtain X-ray scattering of thylakoids *in vivo*. Specifically, we subtracted the white/yellow part scattering from the green part scattering of the same leaf and thereby obtained scattering originating from the thylakoid network *in vivo* for *Ficus*, *Dieffenbachia*, *Epipremnum*, *Chlorophytum* and *Arabidopsis* (Fig. 4B). The scattering of *Arabidopsis* with white leaves was not measured, therefore etiolated maize leaf scattering (Figs. S1, S5) was used as background to obtain the *Arabidopsis* thylakoid scattering.

We can unequivocally confirm, that the neutron scattering of green leaf parts occur from the lamellar structure of the thylakoid membrane network in the entire observed q range and not from the ordered cellulose located in plant cell walls. The value of the most dominant scattering peak q_1 is 0.0279-0.031 Å⁻¹ (225-202 Å, Fig. 4B and Table 4), consistent with previous thylakoid SRD measurements from the control and D₂O-infiltrated leaves (205-254 Å)⁴⁹ and is within the range of the published TEM data (Table 1 and references therein).

From the appearance of scattering curves, the scattering peaks are much more evident and defined in the scattering curves of dark-adapted shade-tolerant plants (Fig. 4B) than in dark-adapted *Chlorophytum* or *Arabidopsis* plants. We explain this observation with the effect of a structure factor as a high stack with more ordered lamellar layers would have a much more pronounced lamellar scattering signal, compared to a lower stack with disordered layers.

We extended the applicability of the scattering model from Manuscript 1 to the SANS data of higher plants. The results of the model fit for *Ficus* and *Arabidopsis* are plotted along the experimental data in Fig. 5. As seen, the preliminary model fits capture all significant features of the data and a fit quality is visually satisfactory. The model parameters describing the average dimensions of a thylakoid system are summarized in Table 3. Both the SRD, lumen height and thylakoid membrane thickness are within the experimentally observed range. However, lumen thickness polydispersity σ_{lumen} and goodness-of-fit parameter χ^2 are not yet optimal, what unequivocally indicates the need of a further fitting refinement.

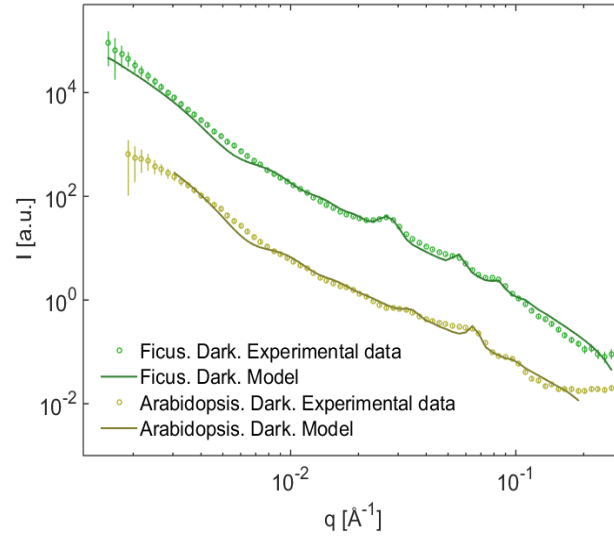


Fig. 5. Experimental SANS data (points) and model fittings (lines) for *Ficus* and *Arabidopsis*.

Table 3. Structural thylakoid parameters derived from SANS models, depicted in Fig. 5.

Parameter	<i>Ficus</i>	<i>Arabidopsis</i>
$SLD_{tailgroups}$, a. u.	-1	-1
SLD_{lumen} , a. u.	-0.26 ± 0.11	-0.05 ± 0.145
SRD , Å	225.5 ± 3.7	191.87 ± 3.6
<i>Lumen height</i> , Å	50 ± 7.3	64.3 ± 5.5
σ_{lumen} , Å	25 ± 25	5 ± 23.6
<i>Thylakoid membrane thickness</i> , Å	37.35 ± 4.4	47.7 ± 4.4
<i>No. of thylakoids in granum</i> (N)	5	5
<i>Caillé parameter</i> (η_{cp})	0.02	0.02
<i>Goodness-of-fit parameter</i> (χ^2)	18.23	8.06

Dark-light-dark thylakoid dynamics evaluated by thylakoid stacking repeat distance, SRD, changes

Small angle neutron scattering was subsequently measured on the same plant leaf sample in low light (Fig. 6, green curves), illuminated for 20 min + 1 hour with $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 6, yellow curves) and in low light after 12-h dark adaptation (Fig. 6, black curves). The scattering from dark-adapted and illuminated white parts does not differ (Fig. S3), therefore it is possible to use the same 'white leaf part' scattering to subtract it from dark-adapted and illuminated leaf scattering.

Changes of scattering curves between dark/illuminated/dark-adapted measurements correspond to thylakoid dynamics *in vivo*. The values of q_1 for dark-adapted and illuminated plants are given in Table 4. From raw data analysis, there is a small increase in q_1 value of lamellar peak for plants with high grana stacks, indicating that thylakoid repeat distance decreased upon illumination. The situation is slightly different in *Chlorophytum* and *Arabidopsis* plants, which have a typical grana organization - illumination-induced SRD decrease is more pronounced for these species (Fig. 6 and Table 4). Most importantly, scattering peaks of illuminated plants are broader than those of dark-adapted plants. This has been observed for all investigated plants and is most pronounced for *Chlorophytum* and *Arabidopsis*. Broadening of scattering peaks putatively indicates that thylakoid membrane system loses its order upon illumination.

The SRD changes during 12-h dark-readaptation were more diverse and therefore less conclusive. The q_1 peak position of *Dieffenbachia*, *Epipremnum* (Fig. 6) and *Alocassia* (Fig. S7) plants decreased after 12-h dark adaptation, indicating a stacking repeat distance increase in the darkness. For *Ficus* and plants with typical grana structure (*Chlorophytum* and *Arabidopsis*), q_1 peak position slightly increased. This indicates that species-dependent thylakoid rearrangement takes place *in vivo* also after release of light excitation pressure.

Overall, a scattering signal of low intensity can potentially yield an ambiguous interpretation regarding dynamic changes if investigated *per se*. Therefore, scattering experiments of two characteristic plant species *Ficus* (large grana) and *Arabidopsis* (typical grana) are complemented with CLSM and TEM experiments performed in the same illumination conditions.

Table 4. SRD of the first order lamellar peak derived from SANS.

Plant	q_z Dark-adapted ($\text{\AA}^{-1}$), SRD ($\text{\AA}$)	q_z Illuminated ($\text{\AA}^{-1}$), SRD ($\text{\AA}$)
<i>Dieffenbachia</i>	0.0315, 199	0.033, 190
<i>Ficus elastica</i>	0.0279, 225	0.0285, 220
<i>Epipremnum aureum</i>	0.0285, 220	0.0285, 220
<i>Alocassia macrorhizos (S.I.)</i>	0.0305, 206	0.0305, 206
<i>Chlorophytum comosum</i>	0.036, 174	0.037, 169
<i>Arabidopsis thaliana Col0</i>	0.0327, 192	0.035, 179

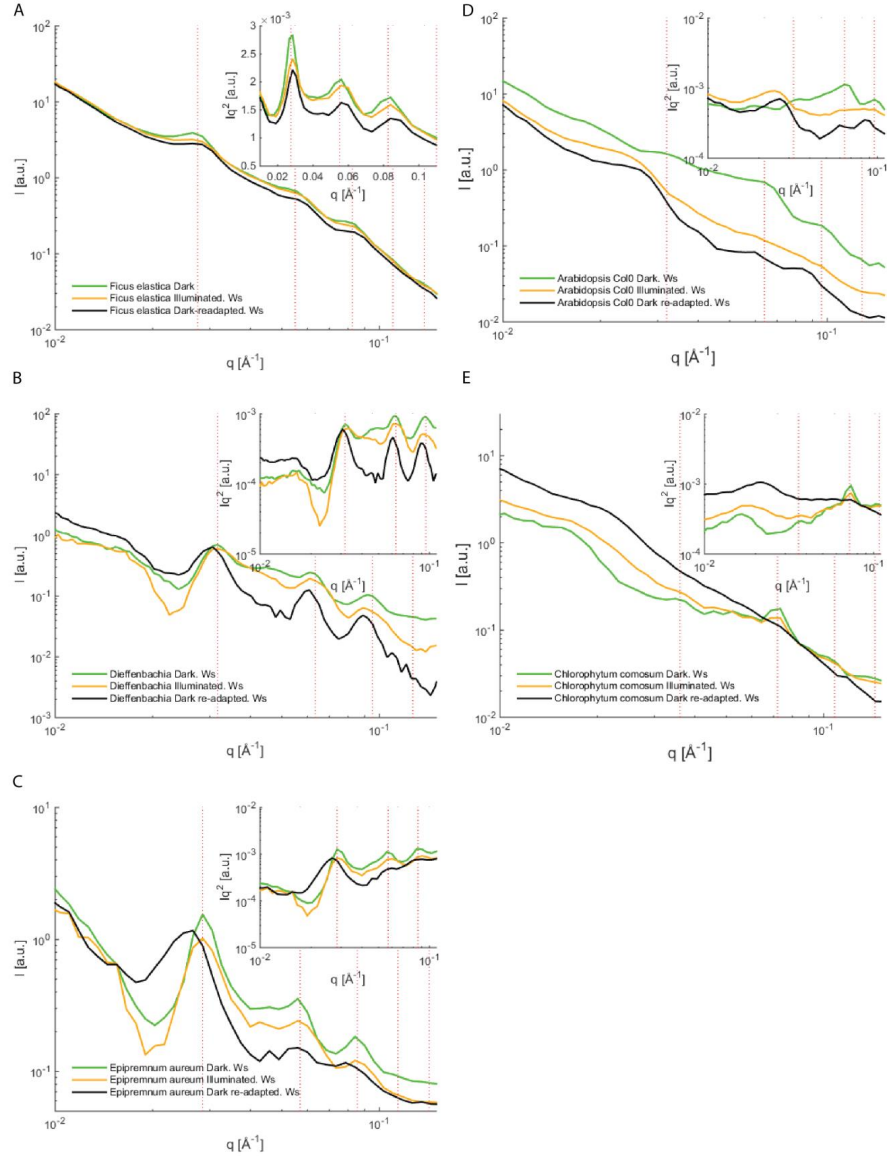


Fig. 6. SANS curves of thylakoid membrane scattering from leaves of A: *Ficus*; B: *Dieffenbachia*; C: *Epipremnum*; D: *Arabidopsis Col0*; E: *Chlorophytum* leaves. Curves: green – dark-adapted leaf, yellow – illuminated leaf, black – 12 h dark-readapted leaf. All insets: Kratky plots of zoomed-in region with peaks. Light-induced SRD shrinkage is inferred from peak movement to the right. Arbitrary y-spacing. Red dotted lines indicate positions of q_{1-5} peaks.

CLSM study of thylakoids shows a reduced dynamics of *Ficus* thylakoid network in the light, compared to *Arabidopsis*

The distribution of grana inside chloroplasts as well as the shape of the entire thylakoid network in the chloroplast is analyzed in dark-adapted and illuminated plants based on chlorophyll autofluorescence and rendered isosurface (Fig. 7A, red regions). CLSM measurements of 12 h dark-readapted plants have not been performed, since detached plant leaves are not viable for this period of time in such experimental setup.

Similarly to previously reported observations^{3,16}, we observe that light exposure influences the dimensions of the *Arabidopsis* thylakoid network *in vivo*. The thickness of the registered fluorescence in z-axis of illuminated chloroplasts corresponding to the total height of the thylakoid network is depicted by arrows in the last row of Fig. 7A decreases around two times compared to dark-adapted chloroplasts (Fig. 7B), indicating that illuminated *Arabidopsis* chloroplasts become more 'flat'. A 25% decrease in volume of the thylakoid network (Fig. 7C) and 25% decrease of the total thylakoid network area (Fig. 7D) in the light indicate a more compact thylakoid network arrangement upon illumination (Fig. 7A, columns 1-2 and Fig. 7B-D). A small increase in thylakoid network volume and area has been observed when chloroplasts were placed under laser light for 4 minutes, what shows that illumination with laser light *per se* does not cause a significant shrinkage (Fig. 7A columns 3-4 and Fig. 7C-D). In case of *Ficus*, light-induced thylakoid network shrinkage was less pronounced (Fig. 8).

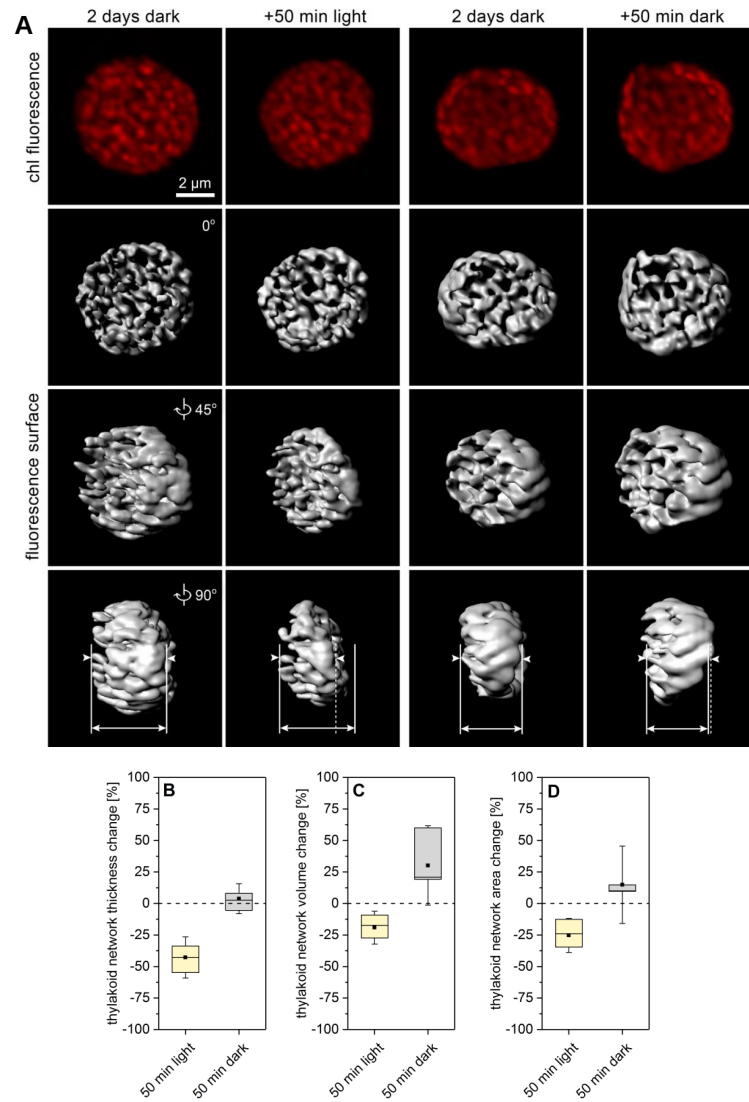


Fig. 7: A: Representative chlorophyll fluorescence and fluorescent surface reconstruction images of dark-adapted and illuminated *Arabidopsis* Col0 grana stacks in different angles. Total grana height is indicated as vertical bars in the last vertical line; B: Quantitative thylakoid network thickness change (n=25-30); C: Quantitative thylakoid network volume change (measured from 5 independent experiments); D: Quantitative thylakoid network area change (measured from 5 independent experiments).

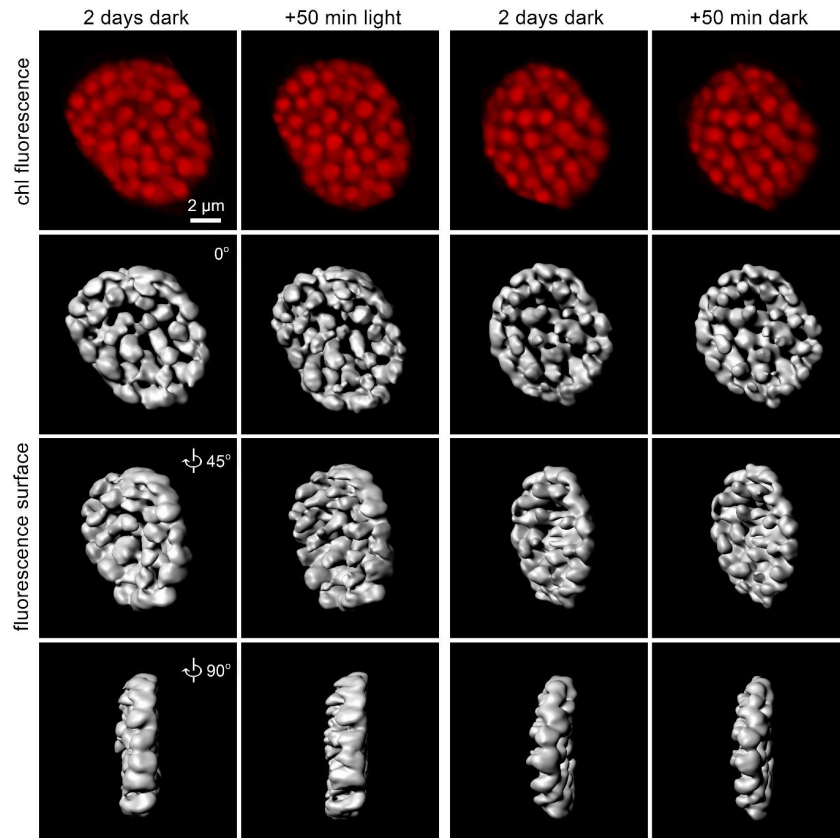


Fig. 8 Representative chlorophyll fluorescence and fluorescent surface reconstruction images of dark-adapted and illuminated *Ficus* grana stacks in different angles.

TEM study of thylakoids shows smaller SRD shrinkage in *Ficus* granum in the light, compared to *Arabidopsis*

When a dark-adapted *Arabidopsis* leaf is illuminated, a production of starch granules is observed, showing that photosynthesis is functional during the experiment (the bottom row of Fig. 9A). TEM measurements show that the number of individual grana stacks in the chloroplast increases upon illumination (Fig. 9B) with white light of $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Furthermore, the total granum diameter (Fig. 9C) and height (Fig. 9D) decrease upon

illumination. This observation is in line to other published results^{12,15} and indicates that individual grana probably undergo dichotomical splitting.

The thylakoid stacking repeat distance was measured from micrographs of chemically-fixed dark-adapted and illuminated plants. The decrease of stacking repeat distance from around 182 to 153 Å indicates that thylakoids and lumen shrink upon illumination (Fig. 9E). A similar behavior is observed in spinach leaves¹⁶.

The change of a *Ficus* thylakoid network ultrastructure upon illumination is similar, although less prominent than in *Arabidopsis* plants (Fig. 10A-E). A number of thylakoid layers in granum (Fig. 10B) and grana diameter (Fig. 10 C) remain constant upon illumination and a slight shrinkage of both grana height (Fig. 10 D) and SRD (Fig. 10 E) is observed.

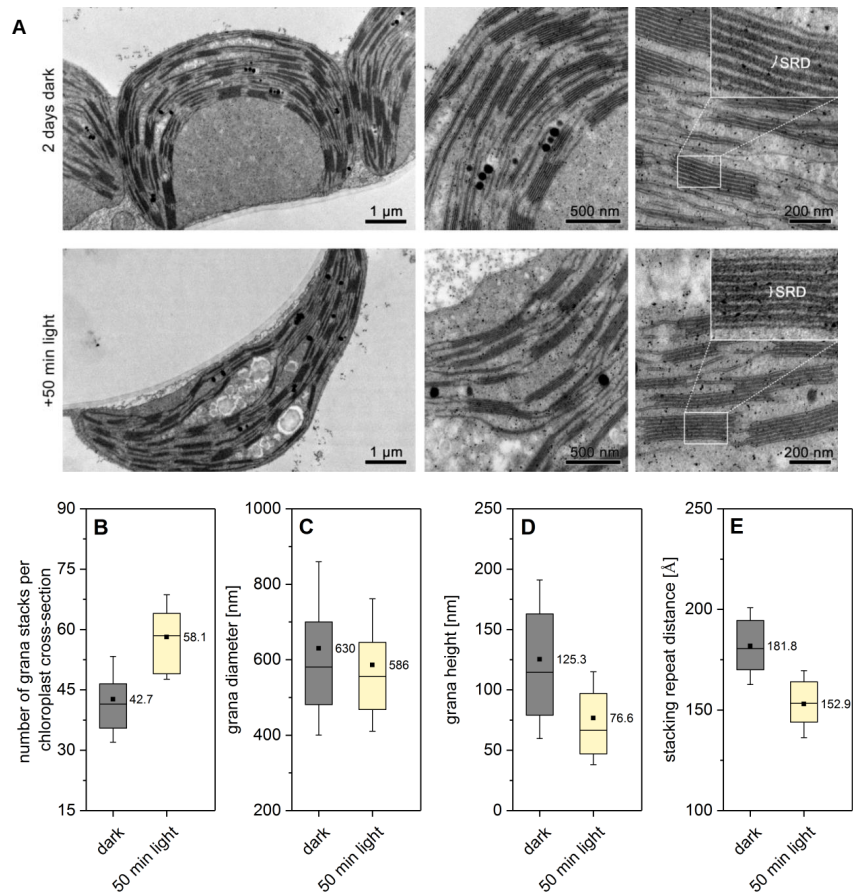


Fig. 9. A: Representative TEM images of dark-adapted and illuminated *Arabidopsis* Col0 grana stacks in different magnifications. The SRD is depicted in the inset. B: The boxplots of the number of grana per chloroplast cross-section; C: the grana diameter; D: the total height of granum; E: the SRD in dark-adapted and illuminated plants. Average values are depicted with a black square, median values depicted with a horizontal black line. SD is depicted by whiskers (n = 110).

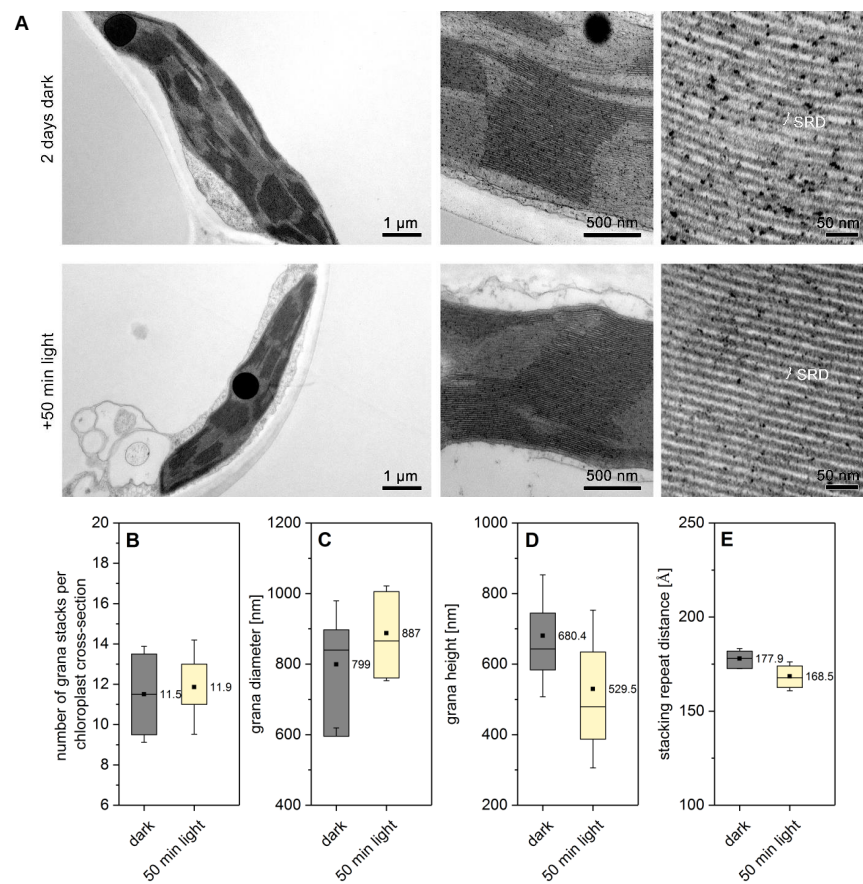


Fig. 10. A: Representative TEM images of dark-adapted and illuminated *Ficus* grana stacks in different magnifications. The SRD is depicted in the right panel. B: The boxplots of the number of grana per chloroplast cross-section; C: the grana diameter; D: the total height of granum; E: SRD in dark-adapted and illuminated plants. Average values are depicted with a black square, median values depicted with a horizontal black line. SD is depicted by whiskers (n = 20).

Discussion

Energy in the chloroplasts is stored as a transmembrane proton gradient (proton motive force, pmf), which drives ATP synthesis and is required to maintain functional photosynthesis^{50,51}. The proton motive force is a sum of two components – the transmembrane difference of protons between lumen and stroma (ΔpH) and the membrane potential ($\Delta\psi$) - the electrical field (charge) difference across the thylakoid membrane⁵². Proton influx into the lumen triggers the consequent net Cl^- anion influx into lumen, which is balanced by the outflux of counter-ions K^+ and Mg^{2+} into the stroma^{30,31}. This ion redistribution changes the net negative charge of stromal thylakoid membrane surface, inter-thylakoid repulsion forces and thus can induce changes in thylakoid stacking. Thylakoid stacking is also influenced by protein-protein interactions⁵³, protein phosphorylation^{16,54,55} and protein expression⁵⁶.

A summary of the current understanding of thylakoid dynamics upon dark-to-light transition, expressed in the stacking repeat distance, SRD changes, is given in Fig. 1, Tables 1 and 2. Published data provide an ambiguous picture of thylakoid dynamics in grana. Experiments observing light-induced lumen and thylakoid stacking repeat distance shrinkage^{4–7,10,12–16,21,22,25–27}, have been recently contradicted with experimental observations, suggesting a medium/high light illumination-induced increase of lumen height^{8,10,17}. Furthermore, many more ‘dark-light’ dynamical outcomes have been documented: grana margin expansion and a simultaneous shrinkage of grana core^{6,7,28}, a decrease in grana diameter^{3,16} and changes of the distance between two adjacent thylakoids (inter-thylakoid space height)^{8,29} have been observed upon leaf illumination *in vivo*. It has also been shown, that individual thylakoids can become thinner, less undulated more intrinsically ordered in light, compared to darkness^{13,33,57,58}. Even a simultaneous lumen shrinkage and expansion is observed in the same thylakoid, where expansion is localized in grana margins and shrinkage occurs in granal core²⁸. Furthermore, significant differences are observed in dark-to-light thylakoid dynamics on the same plant species, as dark-light structural changes depend on illumination conditions before dark-adaptation¹².

This infers that electrostatic ion redistribution is an important factor in governing thylakoid ultrastructure changes. Overview of previous experimental work shows that predominantly isolated thylakoids, saturating light intensities of different wavelengths and short measuring times were employed in ultrastructure/dynamical investigations of thylakoid membranes using the recent advent of cryo-microscopy and scattering techniques.

Initially, we show that white leaf parts are photosynthetically inactive, contain no ordered thylakoid structures and do not exhibit ordered scattering in the entire q range. For this we apply the ‘white leaf part subtraction’ methodology⁴⁸ and obtain scattering solely from thylakoid membranes. The ability to perform a proper background subtraction greatly expands previous work using leaf scattering *in vivo*^{26,48}, as a simultaneous investigation of several scattering peak orders (q_{1-5}) becomes possible with increased resolution.

Overall, our mathematical model captures many features of the scattering curve and yields reasonable values for lumen and inter-thylakoid membrane thickness, but the thylakoid number in granum N cannot be resolved yet. Also, the scattering length density of the

tailgroup, $SLD_{tailgroup}$ is constrained to -1 a. u. and the calculation of absolute SLD values is in progress. However, most importantly, a wide low intensity diffuse peak at $q = 0.01-0.02 \text{ \AA}^{-1}$ in scattering curves, which has been earlier arbitrarily attributed to a stromal thylakoid repeat distance⁵⁹, can be accounted for by applying the stacked lamellae model. Therefore only the granal unit cell is sufficient to explain thylakoid scattering, making the stromal unit cell²¹ a redundant entity. To compare our scattering results of *Arabidopsis* with literature data, we simulate scattering curves of dark adapted (Fig. 11A, green) and illuminated (Fig. 11A, yellow) thylakoids using according ultrastructure values from Kirchhoff *et al.*⁸. It is evident that the experimentally observed scattering curves (Fig. 6E) are of an inferior quality compared to the simulated non-polydisperse thylakoid curves, what supports our argumentation about the large *a priori* thylakoid system disorder in *Arabidopsis* thylakoids *in vivo*. Looking from a scattering perspective, *Arabidopsis* is an inferior model system than *Ficus*.

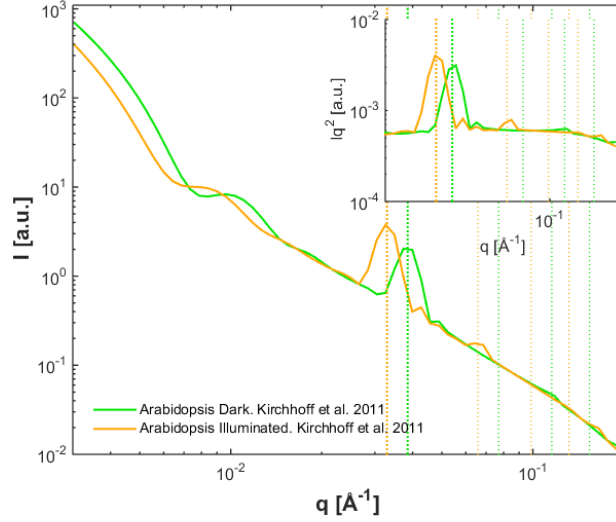


Fig. 11. Modelled scattering curves with *Arabidopsis* thylakoid ultrastructure parameters from⁸, with $N = 5$, $SLD_{tailgroup} = -1 \text{ a. u.}$, $SLD_{lumen} = 0.02 \text{ a. u.}$

A simultaneous change of several correlated thylakoid scattering peaks upon illumination allows connecting them to a change of thylakoid state and thus observing thylakoid dynamics *in vivo*. In the second part of this paper we have investigated thylakoid dynamics of plants with two thylakoid ultrastructures: extreme shade-tolerant, high grana plant *Ficus* and sun-tolerant, typical granal-stromal thylakoid arrangement plant *Arabidopsis*. In contrast to the short-term scattering experiments with saturating light intensities^{21,22,26}, we decided to perform non-invasive measurements on D₂O-infiltrated plant leaves *in vivo* and use moderate white light illumination of $500 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for 1 hour on dark-adapted plants. In

our understanding, a prolonged dark adaptation before the experiment and a moderate illumination with pre-illumination, which allows achieving thylakoid membrane system equilibrium, is more applicable as the first step of thylakoid ultrastructure investigations, since it separates two distinct ‘dark-’ and ‘light-adapted’ states and mimics non-destructive conditions. The investigation of short-term illumination-induced thylakoid ultrastructure adaptations is foreseen as the next step, when the modelling is utile.

Small angle neutron scattering experiments of the same leaf sample indicate a small, but discernible illumination-induced SRD decrease (shrinkage) and SRD increase during dark re-adaptation. Our observation that SRD shrinkage is less apparent for plants with a high number of thylakoid layers in granum is in line with previous TEM observation, where a highly-stacked granum of *Monstera deliciosa* does not undergo a significant (vertical) unstacking after its illumination with white light of $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 20 minutes⁶⁰. This indicates, that the findings of Kirchhoff *et al.*⁸ are not applicable for higher plants with extensive grana stacking. Indeed, despite the discrepancies of mathematical model, it is clear, that the experimental neutron scattering observations of dark-adapted and illuminated plant, presented in this paper (Fig. 6 and 11B) do not follow the ‘SRD increase in light’ trend. Instead, we observe a slight illumination-induced SRD decrease, what is otherwise well in line with the results from other neutron scattering^{21,32,58}, CLSM and TEM experiments (all Table 1).

Together with the fact that scattering signal intensity decreases in light^{22,61}, this could indicate a decrease of thylakoid system order, especially in the end membranes, or/and grana undergoing dichotomical splitting. This observation cannot be unequivocally confirmed by scattering modelling, as neither the exact N nor its decrease can be determined so far. Furthermore, the observation of light-induced increase in thylakoid disorder can be putatively linked to state transitions, similarly as observed for *Chlamydomonas*⁵⁸, or NPQ^{62,63}, but this requires a further clarification using modelling. Lastly, SRD of illuminated plants with a typical grana ultrastructure did not increase to original values after 12 hour dark re-adaptation, what can have several explanations: no thylakoid rearrangement occurs during dark re-adaptation or thylakoid system is damaged by a prolonged illumination and therefore cannot re-adapt due to its increased disorder.

To explain our experimental findings, we suggest a hypothesis of illumination-induced behavior of plants with two different thylakoid ultrastructures – ‘high’ and ‘typical’ grana arrangement, expressed by a number of layers N . If N is high, illumination induced SRD decrease is small and the overall grana ultrastructure does not change. On the contrary, if N is low, illumination induced SRD decrease is larger and individual granum undergoes dichotomical splitting with a possible decrease of thylakoid order in end membranes.

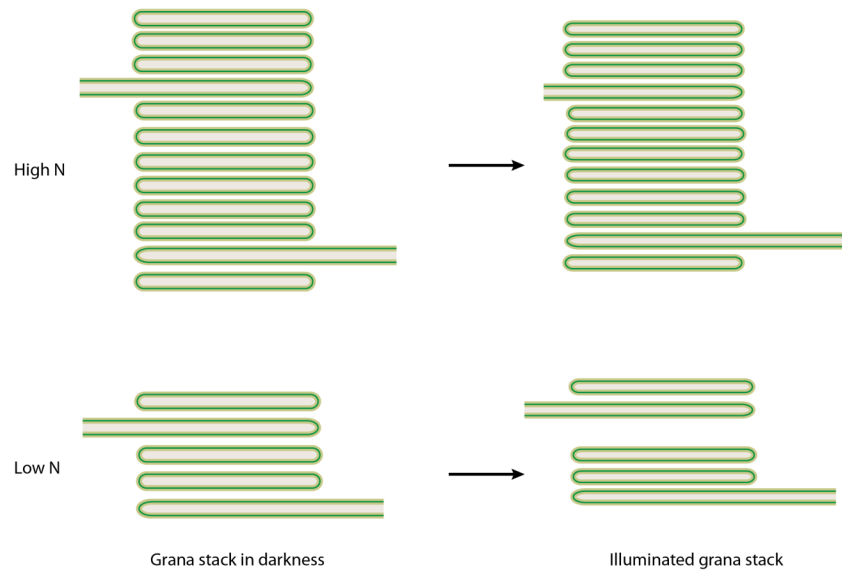


Fig. 12. A hypothesis of illumination-induced thylakoid system changes system in plants with a 'high' and 'typical' grana ultrastructure.

Conclusions

In this paper we extend the capacity of mathematical model of in Jakubauskas *et al.* (Manuscript 1) to analyze small angle neutron scattering and extract structural parameters of higher plant thylakoids. We also demonstrate that thylakoid dynamics of dark-adapted shade-tolerant plants with extensive grana stacking is significantly suppressed compared to sun species. The stacking repeat distance remains unchanged upon dark-to-light transition of shade-tolerant plant *Ficus elastica*, but is reduced in sun plant *Arabidopsis thaliana*. Complementary CLSM and TEM results on these plant species yield to an overall conclusion that the thylakoid system in illuminated *Arabidopsis* leaves becomes more compact and does not significantly change in illuminated *Ficus* leaves, what supports the results obtained from scattering experiments *in vivo*. As a proof of principle, small angle scattering with a posterior mathematical modelling can be used in combination with TEM to investigate plant species where CLSM - another *in vivo* method to investigate dynamics - is not suitable due to resolution restrictions.

Perspectives

In the context of plant biology, a functional non-invasional scattering method together with the ability to extract structural parameters of the thylakoid system from different plants and mutants enables systematically investigating light-induced changes in granal stacking *in vivo* - with the ultimate function of invoking underlying biochemical processes and key actors, such as function of certain proteins, ion distribution. In the context of biophysics, this mathematical model described a complex biological system and allows following its dynamics paves, what the way to the future applications of scattering methods in biology, and is a good example of interdisciplinary research collaboration. The next experimental step is to investigate spectral quality and temperature effects on thylakoid dynamics in wild type plants and photosynthetic mutants. Future research endeavors entail systematic experiments with outdoor-grown plants, naturally varying night/day illumination conditions and resulting thylakoid flexibility during short-term acclimation and in different temperatures.

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Supplementary figures

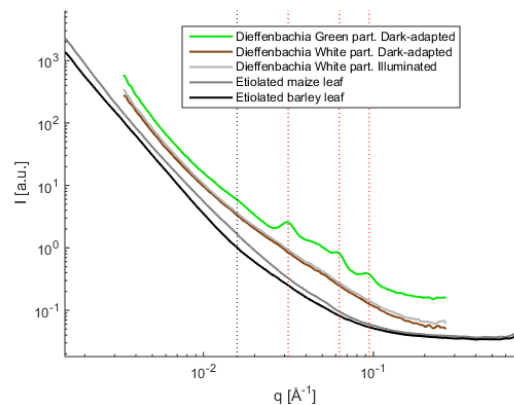


Fig. S1. Original neutron scattering curves. Etiolated leaves exhibit only background scattering, therefore scattering curves of etiolated leaves are suitable as background subtraction.

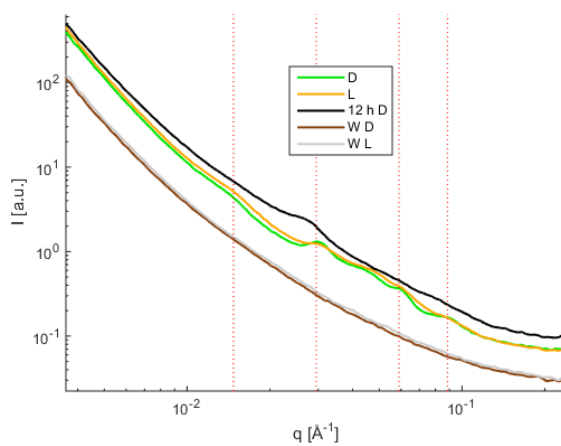


Fig. S2. *Dieffenbachia* SANS curves. The scattering of white part of the leaf does not change upon illumination.

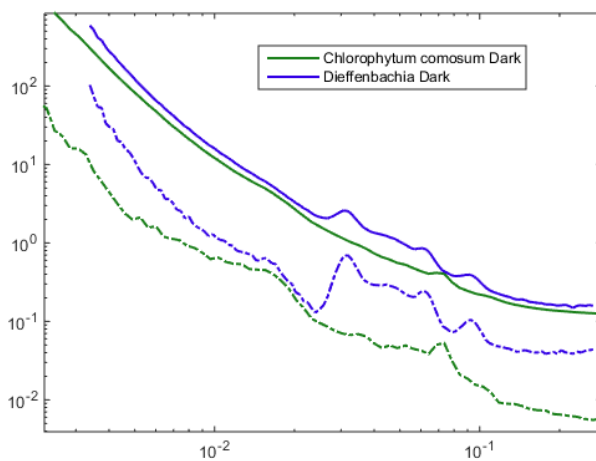
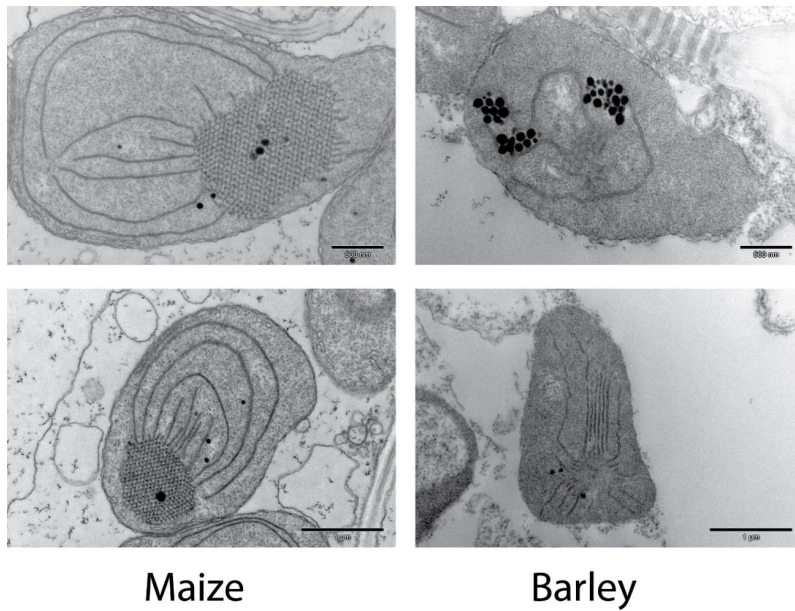


Fig. S3. SANS curve subtraction. Original scattering curves – solid lines. Scattering of green – white part is depicted as dotted lines.



Maize

Barley

Fig. S6. Plasmids from the etiolated leaf of maize and barley. Both PLBs and parallel prothylakoids are observed inside etioplasts.

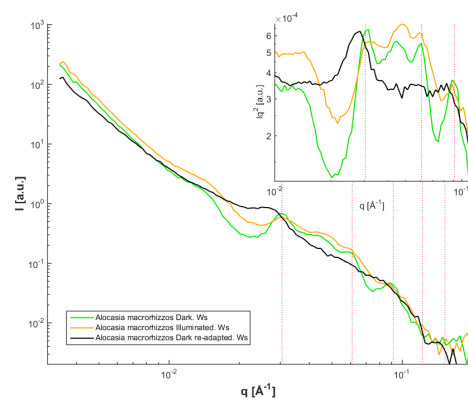


Fig. S7. *Alocassia macrorhizos* scattering curves.

Manuscript 3

Small angle scattering on photosynthetic membranes - a critical review

Small Angle Scattering on Photosynthetic Membranes - a critical review

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ABSTRACT

Keywords: SAXS SANS TEM Thylakoids Modelling

1 INTRODUCTION

The lamellar nature of thylakoids was confirmed in the pioneering works of Menke in 1940 [1, 2] who studied the inner structure of chloroplasts using a range of microscopy techniques. From microscopical studies it became evident that photosynthetic membranes are not randomly dispersed within the cell, but organise into highly complex ultrastructures. Figure 1 depicts representative images of the photosynthetic membrane systems: from remarkably intricate network structure of the prolamellar body found inside developing etioplasts to individual cyanobacterial thylakoids and stacked grana thylakoid arrays in higher plants. Structural regularity in transmission electron microscopy (TEM) images suggests that structural information can be extracted from X-ray or neutron diffraction and scattering methods. In this review we focus on small angle scattering techniques: small-angle X-ray (SAXS) and neutron (SANS) scattering. The first scattering studies on photosynthetic membranes were performed in 1953 by Finean *et al.* [3] and has continued ever since. There are currently about 40 works published on scattering from photosynthetic systems - photosynthetic bacteria, diatoms, algae and higher plants.

Small angle scattering enables investigating structures of 10-1500 Å in near-*in vivo* conditions and is widely applied in biology and soft matter science. Small-angle scattering complements various microscopic techniques, such as as transmission electron microscopy (TEM) [4, 5, 6, 7], scanning electron microscopy (SEM) [8, 9], cryo-EM [10, 11], cryo-electron tomography (ET) [12, 13, 14, 15], atomic force microscopy (AFM) [16, 17, 18, 19, 20]) and live cell imaging [21, 22]. In plant sciences, small angle scattering has been used to investigate structure and dynamic changes of thylakoid membrane systems of plants [3, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45], protists [46, 47, 33], diatoms [38, 48], photosynthetic bacteria [49, 50, 51, 52, 53, 54, 55], algae [38, 48, 56], light harvesting complexes [57, 58, 59], phycobiliproteins [60, 61], higher-plant prolamellar bodies (PLB) [62, 63] or plant cell wall cellulose microfibrils [64]. The aim of applying small angle scattering in biological sciences is to investigate ultrastructural changes of the biologic system and correlate them with underlying physiological processes *in vivo*. This paper critically reviews the current state of small angle scattering applied to photosynthetic membrane systems with a three-fold agenda: 1. small-angle scattering results on photosynthetic membrane systems are presented, 2. scattering results are correlated with microscopy, critical points of result interpretation and method limitations are discussed, 3. development

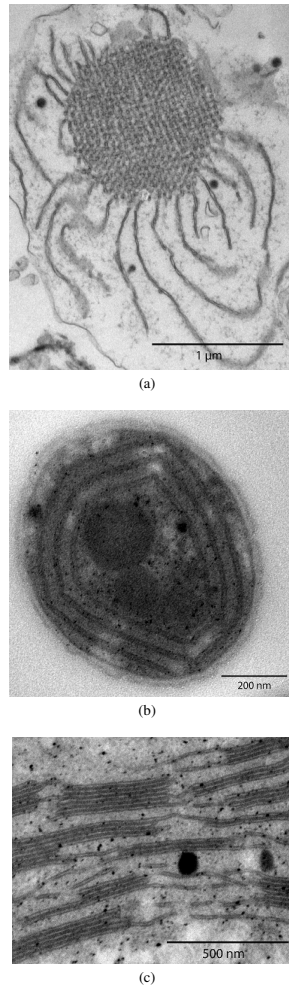


Figure 1: Structures found in photosynthetic membrane systems. a) Highly organised maize prolamellar body; b) Concentric *Synechocystis* sp. PCC 6803 thylakoids; c) Grana stacks of *Arabidopsis* Col0, interconnected by stromal thylakoids.

of the small angle scattering method and its data analysis is envisioned in the field of plant sciences. The review is organized as follows: small-angle scattering terminology and relevant background is introduced in Section 2. Scattering results from plant, cyanobacterial and diatom thylakoid membranes: changes in osmotica and illumination are critically evaluated in Section 3. Finally, an outlook is presented in Section 4.

2 SMALL-ANGLE X-RAY AND NEUTRON SCATTERING

2.1 The small-angle scattering experiment

Concepts described below hold both for X-rays and neutrons, however certain differences arise due to the different physical nature and interactions of photons and neutrons with the sample material (Table 1). Electrons (X-ray scattering) or nuclei (neutron scattering) of sample material interact with an incoming radiation and deflect X-ray photons or neutrons from their original path. The electrons or nuclei become sources of elastically scattered secondary waves, which are registered by a detector (Fig. 2). Contemporary detection systems produce a 2-dimensional output as shown in Fig. 3 a,b). If a sample scatters isotropically, the 2-dimensional pattern is centrosymmetric. For analysis, the 2-D pattern is typically radially averaged and collapsed to the 1-dimensional curve (Fig. 5 a) showing scattering intensity as a function of the scattering vector magnitude:

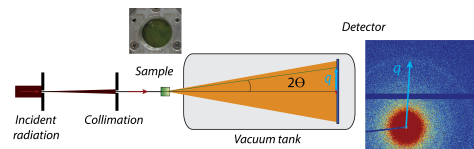


Figure 2: General experimental setup. Incident radiation is collimated and penetrates the sample (green box). Scattering arising in 2θ direction and the resulting scattering vector q is depicted in light blue. Beamstop (red) blocks primary radiation.

$$q = |q| = \frac{4\pi}{\lambda} \sin \theta \quad (1)$$

where λ is the wavelength of the incident radiation (for neutrons the de Broglie wavelength) and θ is half the scattering angle. For non-isotropic systems, sector averaging can be employed to yield 1-dimensional curves, but in some cases analysis is performed directly on the 2-dimensional dataset. By combining Eq. 1 with Bragg's law: $n \lambda = 2 RD \sin \theta$ ($n = 1, 2, 3, \dots$), a direct relation between q and a real-space sample dimension RD is obtained:

$$RD = \frac{2\pi n}{q} \quad (2)$$

The fundamental inverse relation between angles and distances is defined in Eqs. 1 and 2: larger angles probe smaller distances and vice versa. The integer n from Bragg's law appearing in Eq. 2 is called the 'peak order' and indicates that a certain distance RD , repeating in the material, gives rise to a series of peaks in the reciprocal space, ideally one for each value of $n = 1, 2, 3, \dots$. For an ordered lamellar stack, these peaks are placed equidistantly. For more complex structures, different peak positions reflect other crystallographic symmetries and are analysed in more detail. Once the thylakoid sample scattering intensity pattern is recorded and corrected for background contributions, structural sample parameters, including a thylakoid stacking repeat distance (RD in Eq. 2, which is defined as the height of granal thylakoid plus the height of the inter-thylakoid space across to the next granal thylakoid), lumen height and thylakoid membrane thickness can be extracted from the scattering pattern by means of modelling, as shown in unpublished Manuscripts 1 and 2 of Jakubauskas et al.

2.2 Scattering length density and contrast

Analogue to scattering of visible light where differences in refractive index give rise to the air-material contrast, differences in scattering length density enable to 'see' material constituents with X-rays and neutrons. Scattering length density (SLD) describes the degree of interaction between sample molecules and incoming radiation (b, c) and is used to quantify the scattering power of different molecular components. For interacting X-rays, contrast ($\Delta\rho$) arises from variations in the electron density of the material, and for neutrons the

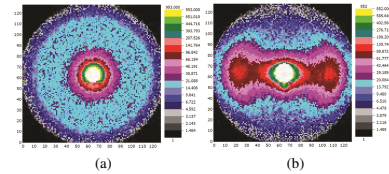


Figure 3: (a) Isotropic 2-dimensional scattering pattern from non-aligned system. (b) Non-isotropic 2-dimensional scattering pattern from magnetically aligned system.

contrast arises from different atom nuclear structures. Effectively, a larger contrast between the sample and medium ($\Delta\rho = SLD_{sample} - SLD_{medium}$) yields a larger registered scattering intensity (Eq. 3). Calculating precise SLDs for biological system components is important for modelling (see below), but is difficult, since the exact protein and lipid composition of the membrane, their volume fractions, protein H-D exchange degree, membrane-associated water content and solvent composition need to be known. Calculated SLD values (taken from the PhD thesis of D. Jakubauskas) of thylakoid membrane components, which are used in modelling, are given in Table 2.

The different physical nature of the two methods is advantageous and complementary in ultrastructural analysis - SAXS and SANS enable extracting different structural information of the same sample. As illustrated in Fig. 4(a) X-ray scattering power of atoms increases roughly linearly with atomic number (heavier atoms scatter stronger), while for neutrons the variation is less systematic and differs significantly between different atom isotopes [65]. This isotope difference is particularly important for biological samples, since the exchange of some sample hydrogen (H) atoms with the heavy-hydrogen isotope deuterium (D) allows fine-tuning the neutron scattering contrast; the method is called contrast variation. If SLD of the surrounding medium is equal to the SLD of a specific molecular component, no scattering is observed from that component. In its simplest form, mixing H₂O and D₂O-based buffers in specific ratios enables to enhance or diminish the contrast $\Delta\rho$ of different cellular components [66, 67] (Fig. 4(c)). To exemplify, contrast variation technique allows an individual investigation of either lipid or protein components in

a complex biological membrane system. In order to match out membrane lipids 5-20 % D₂O (v/v) containing solutions are used, and 40 - 45 % D₂O (v/v) containing solutions are used to match out protein components of the membrane (Fig. 4(d)).

2.3 Small angle scattering models

The most elaborate (to date) small angle scattering model on a complex biological system *in vivo* of Nickels *et al.* fit the entire scattering curve of *Bacillus subtilis* bacterium and elucidated its membrane thickness [68]. A thylakoid system *in vivo* and its model, presented in Manuscript 1, is much more complex. Previous interpretations of small angle scattering data on photosynthetic membranes are limited to the estimation of the average thylakoid membrane repeat distance from the observed peak position by directly applying Eq. 2. This method provides an approximation of the average spacing between thylakoid membranes and is used to follow system behaviour in changing conditions: e.g. illumination intensity, temperature, pH or different ionic strength (see below). However, a number of factors are not accounted for in this approach: luminal or membrane bilayer thicknesses, the number of thylakoid layers, the degree of system orientation and instrument resolution effects.

Therefore, more sophisticated analyses of scattering data on photosynthetic systems are required. Theoretical calculations of Kirkensgaard *et al.*, based on simulated scattering patterns, suggested a possible route [37] for further modelling. The general equation for the scattering intensity of particles in solution is:

$$I(q) = \Delta\rho^2 \Phi_p V_p P(q) S(q) \quad (3)$$

Here $\Delta\rho^2$ is the contrast of the particles relative to the solution, Φ_p the volume fraction of the particles in the solvent and V_p the particle volume. The terms $P(q)$ and $S(q)$ are named the *form factor* and *structure factor*, respectively. The form factor describes the scattering registered from an individual particle (its form), and the structure factor describes the interactions between these particles.

We extend these descriptions. For a grana stack, the form factor is the scattering from a single thylakoid lamella (two bilayers, lumen and inter-thylakoid space) and the structure factor describes the stacking order of lamellae in the

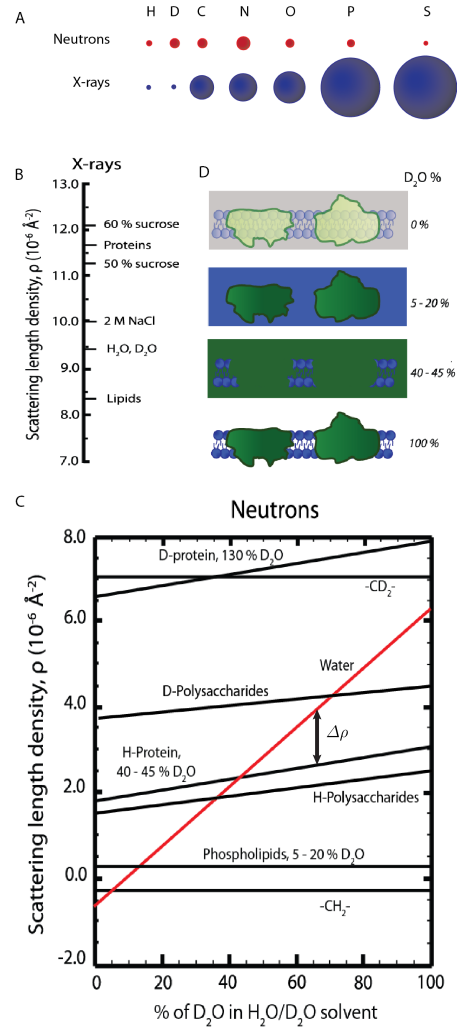


Figure 4: (a) Scattering length comparisons for X-rays and neutrons for selected elements. (b) SLD of X-rays and neutrons. (c) Dependence of neutron scattering length density of lipid and protein moieties on D₂O amount in the sample. (d) Contrast variation technique, where photosynthetic membrane is visualized in different D₂O buffers. The signal from lipid (20 - 25 % D₂O) or protein components (40 - 45 % D₂O) is 'masked out'. The total scattering signal is enhanced in 100 % D₂O, as indicated by more intense colors than in 0 % D₂O.

grana stack, the number of layers, in-plane undulations *etc.* Instrument resolution, inherent sample disorder and polydispersity affect the 'clarity' of scattering peaks and have to be accounted for. The overall goal of modelling thylakoid scattering curve is to construct a mathematical model that incorporates these parameters and properly reproduces the experimentally measured scattering curve without undue overparametrization. This has been done for cyanobacteria and higher plants (Manuscripts 1 and 2).

2.4 Small angle scattering complements microscopy

Both electron microscopy and small angle scattering are widely used techniques in thylakoid ultrastructural investigation, method practicalities are compared below.

Electron microscopy method visualizes the sample in Å-resolution in direct space, but artefacts due to sample preparation are common: fixation, dehydration and image contrast (Table 3). Therefore, probably the simplest way to obtain structural information from the sample containing ordered structures without an extensive sample preparation is to measure its X-ray or neutron scattering. Besides requiring a minimal sample preparation, scattering methods enable a relatively quick system analysis under near-native conditions, the method allows following sample dynamics. The investigation of sample dynamics *in situ* is only possible using scattering methods, as electron microscopy requires sample immobilization and light super-resolution microscopy techniques do not provide sufficient resolution [21, 22]. However, small-angle scattering reflections of the sample *in vivo* are registered in a reciprocal space: experimental scattering data needs to be converted to a direct space by means of a specific mathematical model, which has an underlying biological interpretation. As discussed above, a ready-to-use mathematical model is rarely available.

Furthermore, obtaining sharp Bragg peaks on photosynthetic membranes in physiological conditions (large protein content, required H-D contrast adjustments, measurements in room temperature and high osmolarity) is a tedious task. Large system inhomogeneity additionally smears peaks, and burdens precise structural parameter calculations. Centrifugation, controlled drying [23, 25, 34] or external magnetic field [70, 30, 38, 71, 40] are used to increase internal order of the sample during the scattering experiment. Cryo-microscopy

techniques allow preserving an *in vivo*-like environment, but the investigation of thick samples (*e. g.* the entire grana stack [72]) is yet impossible due to method restrictions and the generally insufficient contrast of membranes.

Electron microscopy allows investigating an ensemble of individual sample features in Å resolution, but the statistical analysis of ultrastructures from TEM micrographs requires choosing a number of well-preserved structures from the sample volume of 5^{-7} mm^3 order. To compare, a relatively large sample volume of $0.1\text{--}1 \text{ mm}^3$ is probed by X-rays or neutrons simultaneously and a statistical medium-resolution structure of the total-volume averaged particle is obtained. Microscopy methods are therefore complementary to scattering in accounting for sample heterogeneity, as minute differences of individual structure are clearly observed.

Overall, small angle scattering and microscopy methods complement each other and their parallel usage is advocated. Complementary investigation of the same sample with both techniques provides its detailed structural information - both in direct and indirect spaces - accounting for sample inhomogeneities (electron microscopy) and following sample behaviour *in vivo* (scattering).

3 THYLAKOID MEMBRANES

3.1 Synopsis of key works on thylakoid membrane scattering

The first SAXS experiment on isolated osmium-fixed *Aspidistra* chloroplasts [3] indicated the existence of a structure with a repeat distance of 250 Å, a similar RD was measured by Kratky *et al.* for *Allium porrum* chloroplast pellet [23]. Kreutz *et al.* measured X-ray scattering of the thin layer of dried chloroplast pellet from *Antirrhinum majus* [24, 25, 28]. Isotropic scattering curves with peaks corresponding to the repeat distances of 177–248 Å, which putatively occurred from the ordered-layered thylakoid structure had been obtained [26, 29]. A wide variation of repeat distance values from early experiments has been explained by different sample preparations and different degrees of thylakoid membrane swelling [73]. Common to all early works is that scattering was used to investigate the composition and internal structure of an individual thylakoid membrane and not thylakoid membrane stacking.

A systematic work trying to explain thylakoid membrane scattering has been conducted by Sadler *et al.* [46, 30], when he isolated chloroplasts from *Euglena gracilis* and spinach, aligned thylakoid pellet by centrifugation, partial dehydration or external magnetic field and measured X-ray or neutron diffraction: four orders of diffraction peaks having a lamellar periodicity of 165–170 Å were observed [46]. These peaks are now considered to arise from a thylakoid membrane bilayer (form factor peaks) [35, 36, 37]. Complementing X-rays with neutron scattering experiments in a magnetic field, Sadler *et al.* suggested a realistic thylakoid ultrastructure model with a thylakoid repeat distance of 240–250 Å (*Euglena*) or 170–190 Å (spinach), and thylakoid membrane thickness of 50 Å [32, 33]. These values are comparable with the current (cryo-)electron microscopy measurements [12, 11]. Overall, the main outcomes of early scattering investigations were: protein, lipid and pigment arrangement in the thylakoid membrane [73, 32], thylakoid membrane thickness [74] were suggested, and the understanding that both the inter-thylakoid space and thylakoid lumen are hydrophilic compartments was coined [33].

Small-angle scattering and modelling work on thylakoid membranes has been continued from 1998, when Javorfi *et al.* observed light-induced thylakoid membrane thickness changes [35, 75]. Holm *et al.* applied a hydrated lipid bilayer model of Wiener *et al.* [76, 77] and created mathematical models of granal and stromal thylakoid unit cells, fitted the experimental data with the mathematical model and calculated thylakoid repeat distances in granal and stromal one-dimensional unit cells from scattering patterns [36]. Although structurally acceptable, these models yield a different lumen thickness for stromal and granal thylakoids [36], what is currently challenged by cryo-EM results [12]. The most important results on thylakoid scattering, osmolarity and illumination-induced thylakoid changes are discussed in detail below.

3.2 The origin of scattering peaks

Scattering peaks are not observed in etiolated leaf samples with otherwise unaffected cellular components [23, 26, 41], what suggests that ordered scattering occurs from thylakoid membranes. Although the scattering was previously ascribed to stromal thylakoid membranes [38], the current understanding is that the q value of the first scattering peak (q_1) originates

from the thylakoid stacking repeat distance in granum [40, 41]. A weak intensity and dispersion of this scattering peak is explained by a high degree membrane disorder in a stack of swollen thylakoid membranes, what results in a range of different stacking repeat distances and therefore peak broadening.

The published explanations of the origin of other scattering peaks (q_{2-5}) are inconsistent. The interpretation of Liberton *et al.* treats all scattering peaks as 1st order Bragg peaks, and various repeat distances are ascribed for cyanobacteria [78]. Instead, we demonstrate that all scattering peaks can be explained by employing a model of stacked thylakoid lamellae from Jakubauskas *et al.* (Manuscript 1), which is a more physically satisfactory explanation. If the experimentally observed (Fig. 5, circles) scattering peak positions from Liberton *et al.* [78] are plotted together with peak predictions calculated from the Bragg's equation (Fig. 5, diamonds) with the lowest q value taken as the first order peak and treating the cyanobacterial membrane system as a stack of thylakoid lamellae, data points fit this explanation. Thus, correlations of Liberton *et al.* and Li *et al.* [78, 54], where the individual peaks are interpreted to originate from specific distances in the membrane system are challenged, as all scattering peaks originate from the single fundamental repeat distance of the lamellar stack.

Our explanation also applies for other photosynthetic organisms (Table 4). If the scattering curves from different photosynthetic organisms are looked in entirety (Fig. 5), a lamellar scattering pattern is observed. q_1 peak position of Fig. 5 is depicted as a big and q_{2-5} positions as smaller diamonds, they correspond to q values of experimentally observed peaks. Table 4 summarizes $q_1 - q_5$ values from the published data - nearly equidistant peak ratio is observed for all organisms, confirming our interpretation. Accounting for a low number N of thylakoids in a stack (for *Chlamydomonas* $N=3$ [79]) and inherent thylakoid polydispersity, a low intensity of scattering peaks (Fig. 5 a) is expected.

3.3 Scattering experiments on thylakoids

RD values from TEM and SANS of the same sample shall not be compared. Repeat distance values obtained from SANS experiments are higher than obtained from TEM measurements of the same sample (Fig. 6) [41, 55]. As discussed in Unnep *et al.*, to improve neutron scattering signal leaves

need to be D₂O-infiltrated, what changes thylakoid organization in the leaf. Slightly expanded thylakoid membranes with D₂O-infiltrated lumen produce a higher contrast and thus can outdominate in scattering, what yields to a larger average RD of the scattering peak [52]. On the other hand, TEM sample preparation involves fixation and dehydration, what invokes sample shrinkage. Therefore RD values *in vivo* can be larger than in TEM micrographs.

Isolation impacts thylakoid ultrastructure. Isolation upconcentrates thylakoid membranes in the sample and reduces scattering from other plant cell components (cell wall, ER membranes), what makes the sample more pure and the interpretation of the scattering curve easier. However, an osmotic environment of an isolated sample differs from the thylakoid environment *in vivo*. Therefore measurements on isolated and *in vivo* thylakoids are not equivalent - thylakoids swell in hypotonic [80, 81], and shrink in hypertonic solutions [82, 71, 41]. As shown by scattering experiments [3, 23, 24, 25, 41, 71, 44], different sample treatments yield different thylakoid repeat distances and increased thylakoid disorder [32] due to osmolarity and ionic force-related changes. RD change due to osmotic effects is also observed for diatom *Pheodactylum tricornutum* and *Chlamydomonas* cells *in vivo* [48, 71, 56, 52].

Scattering can improve the biochemical knowledge of a thylakoid system. Due to relatively easy and fast measurements, scattering method can be used to improve thylakoid or chloroplast isolation procedures - with the aim to find buffers where thylakoid membranes closely resemble situation *in vivo* [52]. For example, from scattering experiments NaCl is suggested to be a better osmoticum than sorbitol for thylakoid isolation [41, 52]. It was also shown that the nature of buffering compound (tricine, MES, potassium phosphate) [52] or addition of 200-250 mM monovalent salts [44] has no effect on repeat distance [52], but the increase of Mg²⁺ concentration or decrease of pH of the isolation medium [32, 71, 43] lowers RD. Scattering can be used to investigate the action of thylakoid membrane proteins (ion channels, transporters) and their effect to the overall thylakoid ultrastructure in different osmotic and ionic environments [44].

3.4 Scattering experiments on plant leaves

To avoid thylakoid ultrastructure change due to isolation, scattering experiments on intact plant leaves have been performed. In the first SAXS experiment, *Allium porrum* (leek)

leaves were stacked perpendicularly to the X-ray beam to suppress cell wall scattering. Thylakoid repeat distance of 250 Å was elucidated [23, 26]. Measuring scattering of both green and variegated snapdragon leaf parts [83] allowed subtracting background scattering and obtaining scattering solely from thylakoid membrane stacks [29]. Scattering in q 0.06-0.1 Å⁻¹ (60-100 Å) region correspond to form factor peaks of a single thylakoid membrane [29]. SANS measurements of the yellow part of *Shefflera arboricola* leaf or red bracts of *Euphorbia pulcherrima*, where thylakoid stacking is either absent or disordered, also did not exhibit scattering peaks [52]. By extending the methodology of Kreutz and Menke to SANS experiment on D₂O-infiltrated leaves with high grana, we obtained a thylakoid scattering curve *in vivo* (Fig. 7, dark green). An improved scattering with up to five orders of lamellar peaks visible (Fig. 7, green diamonds) is obtained (Manuscript 2), compared to 1-3 orders if the background subtraction is not performed [41].

3.5 Illumination-induced thylakoid dynamics

Illumination-induced thylakoid ultrastructure changes are versatile: both shrinkage and expansion of thylakoid repeat distance has been observed in different ionic environments [80]. Numerous TEM experiments suggest that thylakoid membranes both *in vivo* and *in vitro* contract in response to illumination, this shrinkage is dark-reversible, its degree depends on light intensity [84, 85, 86, 87, 88, 89, 90, 91, 92].

Scattering can also follow thylakoid stacking repeat distance changes - experiments on isolated thylakoids show that thylakoid repeat distance decreases in a matter of minutes when illuminated with white light intensities up to 1000 μmol photons m⁻² s⁻¹ and is restored, when the light is turned off [38, 40, 71]; the higher is the applied light intensity, the larger and quicker is the RD decrease [40]. If illumination intensity on isolated thylakoids is increased to 2500 μmol photons m⁻² s⁻¹, thylakoid damage occurs and dark-readaptation after shrinkage is no longer observed [71]. Changes of isolated thylakoids with uncouplers are not observed, what suggests that proton transport is involved in thylakoid membrane dynamics of higher plants [40].

Compared to isolated systems, light-induced thylakoid dynamics *in vivo* is more versatile and organism dependent. Several structural outcomes have been observed in plant

leaves: thylakoid expansion [11, 93, 91], thylakoid shrinkage [41, 92, 93, 90, 87, 45], simultaneous thylakoid shrinkage and expansion [94, 91]. Thylakoid shrinkage was observed in algal *Porphyra* and *Ulva* cells *in vivo* [85]. Light-induced increase of thylakoid repeat distance was been observed in *Chlamydomonas* [95], diatoms [48] and cyanobacterial phycobillosome mutant cells [78], but no RD change was observed in *Synechocystis* sp. PCC 6803 cells [78, 39]. We also show that thylakoid repeat distance does not change in leaves with large grana stacks (Fig. 8). This suggests that thylakoids *in vivo* are much more adaptive and sensitive to light spectral quality and intensity.

As discussed above, current SANS data analysis, which is based on peak position readings, cannot infer lumen height or thylakoid membrane thickness or their illumination-induced changes. Therefore, elucidations of dark-light-dark thylakoid dynamics based on scattering data [78] are so far speculative. Suitable mathematical models, similar to the ones suggested in Manuscripts 1 and 2, which investigate the entire scattering curve behaviour *en masse*, rather than the behaviour of individual peaks, are of high importance to investigate lumen changes and thylakoid dynamics in general.

Furthermore, inelastic neutron scattering (INS) technique can be used to study dynamics of individual membranes. INS investigation of dark-adapted and illuminated cyanobacterial thylakoid membrane dynamics show that the dark-adapted thylakoid membrane is softer before its illumination with white light (NB: OD_{750nm} of cyanobacterial cells used in this illumination experiment was 36.5–41.6) [53]. From SANS, thylakoids in *Chlamydomonas* cells are suggested to exhibit the same behaviour [95]. However, experiment with a fluorescence probe suggests that spinach thylakoid membrane fluidity is lower in dark-adapted than in low-light-illuminated state [90], what calls for a more detailed investigation.

To conclude, the observation of thylakoid (lumen) shrinkage or expansion is only the single facet of thylakoid dynamics *in vivo* and shall not be the sole experimental purpose, as it depends and is probably governed by numerous environmental factors. It has been extensively demonstrated, that thylakoid ultrastructure and degree of thylakoid dynamics *in vivo* depends on the organism [39, 52, 96, 78], its 'previous-growth-history' [97, 96], illumination spectral quality [98, 97, 56, 99, 54, 100] and illumination intensity [71, 40, 94, 90]. Understanding the

interplay these factors will yield a much more comprehensive picture.

As scattering enables registering Å-order changes and requires no fixation, it shall be routinely used to evaluate the thylakoid ultrastructure changes in order to investigate the biochemical processes and actors, which drive thylakoid dynamics. Despite the experimental limitations of the large scale scattering facilities, we actively invite scattering community and biologists to investigate thylakoid ultrastructures from different plant species and functional photosynthesis mutants, in different temperatures and light qualities, such as daily-fluctuating natural light or prolonged dark adaptation etc.). Now, that the mathematical model to explain thylakoid scattering is available, systematic scattering studies are of high necessity in order to understand the physiological effects of illumination to thylakoid ultrastructure.

4 OUTLOOK

The advent of new experimental facilities (Max IV, European Spallation Source or European XFEL) opens new horizons for biological scientific community. Scattering technique will ultimately enable to investigate, follow and model near-atomic ultrastructural changes of complex biological systems in their native environment in near-second range. In complement to 'static' microscopy techniques and together with the fact that comprehensive mathematical models explaining scattering data from complex systems are underway, an advent of new discoveries using scattering methods on complex biological system dynamics is expected.

From hosting predominantly hard-matter physicists and crystallography-oriented users, the number of users having purely experimental biological background (participating in X-ray and neutron beamtimes mainly as proposal co-applicants) is increasing. Though biologists, or other soft matter scientists, are actively involved in sample preparation before the experiment or in the user laboratories, they, due to a largely different educational background, restrain from actively taking part in physical measurements, leaving it 'to the physicists'. Primary interest of physicists, however, is the functionality of the instrument, data interpretation and related matters [101, 102]. This approach leaves many grey areas in experimental design and is not an optimal. Many biologically important parameters and conditions, which are of key importance to the biological

relevance and success of the experiment, *i. e.* a spectral composition of the ambient illumination and its intensity, evaluating plant fitness or dark-adaptation conditions, are not considered during the actual measurement.

To increase a synergistic interaction between physicists and plant scientists, this review provides a theoretical minimum of scattering knowledge for experimental biologists, and invites them to participate in the experimental design, to consider and raise critical technical questions before the experiment: 'How can we reproduce and improve this published data with your instrument?', 'What is the q range of interest?', 'What is the required resolution?', 'Is the absolute intensity calibration required?' or 'What additional instruments would be required in the beamline and sample preparation laboratories?' and checking for small practical details, *e.g.* 'What is a routinely used add-on light source in the facility and is the light fiber of a sufficient length?', 'Is a BG-28 light filter, cooling ultracentrifuge and sterile pipette tips present in the facility?', 'Is a dark room possible to use and how to perform a dark measurement of light-sensitive etioplast samples?'. These questions are important to consider beforehand, as these physical parameters are critical to the biological success, but changing them during the timeframe of experiment is largely infeasible or even impossible. A solid experimental pre-preparation requires bringing less equipment and materials to the experimental facility and to some degree eliminates shortcomings, foreseen in the large-scale scattering facility during the experiment.

Likewise, this review also calls for action from the scattering community to accommodate and support users with a biology background. As boldly expressed by the ESS support scientist Zoë Fischer, 'Since a proposal life cycle for scattering facilities is so long, if the experiment is not working, biologists are not going to come back here again'. Therefore, if the biological complexity of a scattering experiment shall be increased (plants measured in varying temperatures, adapted to different spectral qualities, in NPQ conditions), the availability of biological laboratories with a suitable GMO classification and containment levels, functional sterile benches and plant growth chambers, constantly calibrated instruments and a sufficient supply of standard laboratory items and reagents shall be ensured - which is, unfortunately, as the author has experienced, not always taken for granted. Furthermore, if experiments are performed without users on-site, a stringent

requirement of a qualified biologically-trained personnel in the large-scale scattering facility, which is potent to handle biological material, if precise instructions are given, is of high necessity. If these issues are considered, an advent of new discoveries using scattering methods on complex biological systems is expected.

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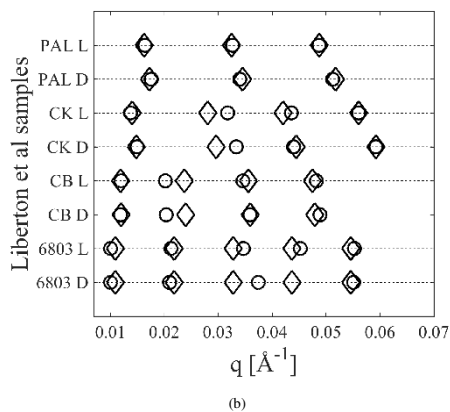
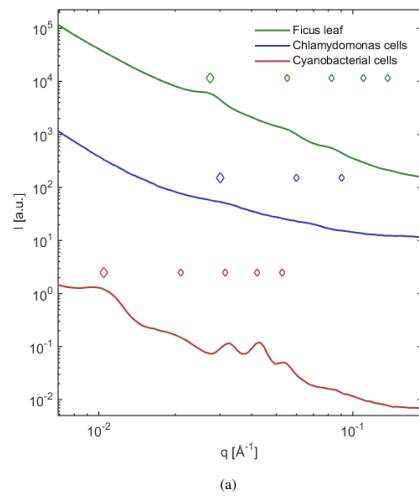


Figure 5: a) *In vivo* q positions of scattering peaks (my own data from BILBY); b) peak positions of cyanobacteria from Liberton *et al.* [78]. Circles are data values as reported, diamonds are the q values, calculated using the Bragg equation (Eq. 1) with the lowest q value as 1st order peak, in some cases moved by a few percent.

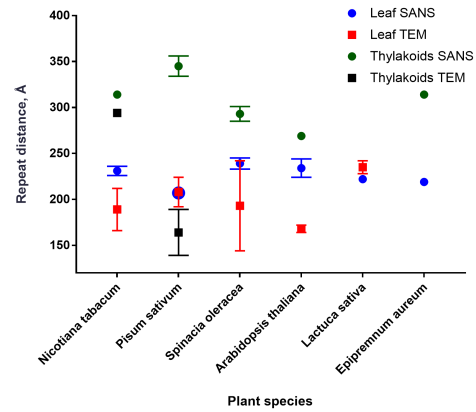


Figure 6: Comparison of repeat distances obtained by SANS and TEM for isolated thylakoids and plant leaves.

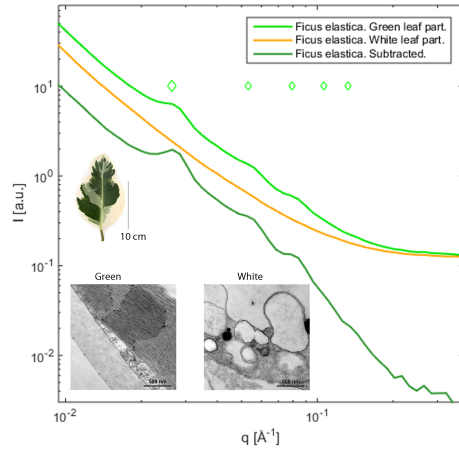


Figure 7: White leaf (yellow curve) scattering subtraction from the green leaf part (green curve) yields thylakoid system scattering (dark green curve). q_1 position is indicated as a big diamond, q_{2-5} positions as smaller diamonds. Inset: TEM pictures of corresponding leaf parts.

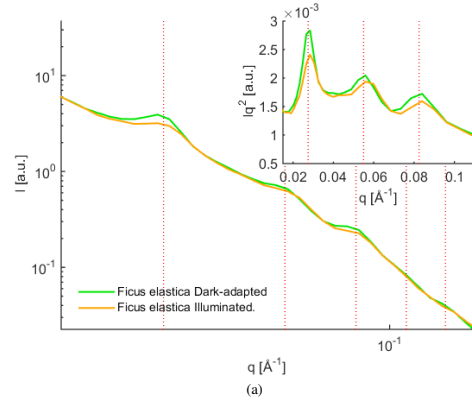


Figure 8: Scattering of dark-adapted and illuminated leaf, inset: Kratky plot.

Table 1. Differences and practicalities of SAXS and SANS experimental techniques Adapted from [59].

	SAXS	SANS
Interacting field	Electrons	Nuclei
Incident beam wavelength, Å	1.0-1.6	2.0-25.0
Flux of the source (particles/s/mm ²)	Medium to high (10^8 - 10^{11})	Very low to low (10^5 - 10^8 - 9)
Coherent scattering length density, 10^{-12} cm	H: 0.28, D: 0.28	H: -0.374, D: 0.667
Sample volumes required in 1-2 mm pathlength cell	20-30 μ L	150-500 μ L
Radiation damage to the sample	Low for laboratory sources, high for synchrotron sources	Low
Structural information extracted for an individual moieties in multicomponent systems	No (electron density average of the entire sample)	Yes (lipid, nucleic acid, protein can be investigated separately)
Contrast variation use	Rare	Common
Resolution	Low-Medium	Low-Medium
Experimental facilities	Laboratory and synchrotron radiation sources	Large facilities only

Table 2. Scattering length densities of thylakoid components. Taken from the PhD thesis of D. Jakubauskas

	Neutron SLD, $\text{\AA}^{-2}$	X-ray SLD, $\text{\AA}^{-2}$
Lipid headgroups. Plants	$1.73 \cdot 10^{-6}$	$8.98 \cdot 10^{-6}$
Lipid tailgroups. Plants	$1.36 \cdot 10^{-6}$	$1.12 \cdot 10^{-5}$
Lipid headgroups. Cyanobacteria	$1.79 \cdot 10^{-6}$	$1.32 \cdot 10^{-5}$
Lipid tailgroups. Cyanobacteria	$1.29 \cdot 10^{-6}$	$1.10 \cdot 10^{-5}$
Thylakoid proteins (10 % H-D exchange)	$2.43 \cdot 10^{-6}$	$1.21 \cdot 10^{-5}$
Chloroplast average	$5.35 \cdot 10^{-6}$	$9.98 \cdot 10^{-6}$
Stroma average	$6.36 \cdot 10^{-6}$	$945 \cdot 10^{-6}$
D ₂ O	$6.393 \cdot 10^{-6}$	$9.455 \cdot 10^{-6}$
H ₂ O	$-5.610 \cdot 10^{-6}$	$9.469 \cdot 10^{-6}$

Table 3. Comparison of scattering and microscopy techniques [69].

Feature	Scattering	Electron microscopy
Space	Reciprocal (inverse)	Real (direct)
Resolution	Small sample details not visible	Small sample details visible
Local structure details	Cannot be extracted	Can be extracted
Results	Representative of the entire sample average Result interpretation is ambiguous	Unique, but may not represent the entire sample Average structures are hard to obtain
Preparation/Experimental artifacts	Scarce in <i>in vitro</i> experiments Sample shall not degrade/change during measurement	Artifacts inherent with chemical fixation Artifacts scarce for cryo-fixation methods Overall cryoEM contrast is lower

Table 4. Measured repeat distances for various organisms.

Organism	q ₁ , $\text{\AA}^{-1}$ (Å)	q ₂ , $\text{\AA}^{-1}$ (Å)	q ₃ , $\text{\AA}^{-1}$ (Å)	q ₄ , $\text{\AA}^{-1}$ (Å)	q ₅ , $\text{\AA}^{-1}$ (Å)	q ₆ , $\text{\AA}^{-1}$ (Å)	Reference
<i>Synechocystis</i> sp. PCC 6803 WT	0.01 (627)	0.021 (300)	0.0374 (168)	0.0452 (139)	0.055 (144)		[78]
			0.036 (175)	0.043 (146)	0.059 (106)	0.11 (57)	[53]
<i>Halomicronema hongdechloris</i>	0.0097 (647)	0.021 (299)	0.042 (150)	0.056 (112)			[54]
<i>Pheodactylum tricornutum</i>	0.037 (170)	0.052 (121)	0.065 (97)				[48]
<i>Spinacia oleracea</i>	0.0258 (244)	0.0508 (123.6)	0.078 (80.5)				[41]
<i>Chlamydomonas reinhardtii</i>	0.033 (190)	0.055 (114)					[56]

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Appendices

Appendix 1. WillItFit? code

Model information file

```
E:\DoubleBilayerStackLumenAndRepeatDistancePolydisperse2\ModelInfo.h 1
/*****
 *
 * Model name: DoubleBilayerStackLumenAndRepeatDistancePolydisperse
 * Author    : D. Jakubauskas and J.J.K. Kirkensgaard.
 * Email     : jjkk@nbi.dk
 * Structure factor follows Nallet et al, 1993 J. de Physique II 3, 487-502
 *****/

// List of headers to be included on compilation
#include "Constraints2.h" //Empty file, since everything is defined in Model.h file
#include "Model.h"
#include "OutputData.h"
```

Model code file


```
1 double Model(double q, double * Parameters, double * Constraints, double Contrast, struct Protein
  ProteinStructure, struct UserDefined * UserDefinedStructure)
2 {
3     /// Declarations
4     // Parameters describing the membrane heads
5     double HeightOfHeads;
6     double ScatteringLengthOfHeads;
7     HeightOfHeads = Parameters[3];
8     ScatteringLengthOfHeads = Parameters[0];
9
10
11     // Parameters describing the membrane tails
12     double HeightOfTails;
13     double ScatteringLengthOfTails;
14     HeightOfTails = Parameters[4];
15     ScatteringLengthOfTails = Parameters[1];
16
17     // Parameters describing lumen
18     double HeightOfLumen;
19     double ScatteringLengthOfLumen;
20     HeightOfLumen = Parameters[5];
21     ScatteringLengthOfLumen = Parameters[2];
22
23
24     //Technical definitions
25
26     double Roughness;
27     double Scaling;
28     double Background;
29     double ScalingExtraTerm;
30     ScalingExtraTerm = Parameters[13];
31     double ScalingExtraTerm2;
32     ScalingExtraTerm2 = Parameters[18];
33
34 // double XRoughness;
35 // double XScaling;
36 // double XBackground;
37
38 // double NRoughness;
39 // double NScaling100;
40 // double NBackground100;
41 // double NScaling0;
42 // double NBackground0;
43
44 double ConcentrationOfSample;
45
46 //Get technical parameters
47
48 XScaling = Parameters[10];
49 XBackground = Parameters[9];
50
51
52 NScaling100 = Parameters[12];
53 NBackground100 = Parameters[11];
54
55
56
57 // Declarations of partial sums
58 double Intensity;
59 Intensity = 0.0;
60
61 double PartialSumOfGrana;
62
63
64
65 ///////////////
66 //Structure factor declarations
67 ///////////////
68
69 double RepeatDistance;
70 double EtaCP;
71 double Nstack;
72
```

```

73
74     RepeatDistance = Parameters[6];
75     Nstack = Parameters[7];
76     EtaCP = Parameters[8];
77
78
79
80 // Technical
81
82     int ii;
83     double SiLoop;
84     double Si;
85     double EulerConstant;
86     double alphaN;
87     //double Miu;
88     //int l;
89
90 /// Structure factor calculation from ii = 1 to ii = Nstack
91     EulerConstant = 0.57721;
92
93     Si = 0;
94
95
96 // Polydispersity parameters
97     int NumberOfSteps;
98     int NumberOfStepsdC;
99     int i;
100
101     // D is RepeatDistance
102     double MeanD;
103     double MinD;
104     double MaxD;
105     double DStepsize;
106     double SigmaD;
107     double PrefactorD;
108     double WeightD;
109     double Dvalue_tmp;
110     MeanD = RepeatDistance;
111     SigmaD = Parameters[14];
112     //D polydispersity loop
113     if (SigmaD != 0){
114         PrefactorD = 1.0 / (SigmaD * sqrt(2 * pi));
115         NumberOfSteps = 9;
116         MinD = MeanD - 3.0 * SigmaD;
117         MaxD = MeanD + 3.0 * SigmaD;
118         DStepsize = (MaxD - MinD) / (1.0 * NumberOfSteps);
119     }
120     else {
121         PrefactorD = 1.0;
122         WeightD = 1.0;
123         NumberOfSteps = 1;
124         MinD = MeanD;
125         DStepsize = 1.0;
126     }
127
128
129
130
131
132 //Form factor declarations
133 //Form factor declarations
134
135
136     // Lumen
137     double MeandC;
138     double MindC;
139     double MaxdC;
140     double dCStepsize;
141     double SigmadC;
142     double PrefactordC;
143     double WeightdC;
144     double dCvalue_tmp;
145     MeandC = HeightOfLumen;

```

```

146     SigmadC = Parameters[15];
147     //dC - lumen thickness
148     if (SigmadC != 0){
149         PrefactorDC = 1.0 / (SigmaD * sqrt(2 * pi));
150         NumberOfStepsdC = 9;
151         MindC = MeandC - 3.0 * SigmadC;
152         MaxdC = MeandC + 3.0 * SigmadC;
153         dCStepsize = (MaxdC - MindC) / (1.0 * NumberOfStepsdC);
154     }
155     else {
156         PrefactorDC = 1.0;
157         WeightdC = 1.0;
158         NumberOfStepsdC = 1;
159         MindC = MeandC;
160         dCStepsize = 1.0;
161     }
162
163 // Define box geometry
164
165     double a;
166     double b;
167     double c;
168     double d;
169
170 // Declarations of form factor amplitudes
171
172     double AmplitudeOfHeads;
173     double AmplitudeOfTails;
174     double AmplitudeOfLumen;
175     double AmplitudeOfGrana;
176
177 PartialSumOfGrana = 0;
178
179     for (i = 0; i < NumberOfStepsdC; ++i) {
180
181         // Model geometry calculations
182         dCvalue_tmp = MindC + i * dCStepsize;
183         if (SigmadC != 0){WeightdC = exp(- pow(MeandC - dCvalue_tmp, 2) / (2.0 * pow(SigmadC, 2)));}
184
185
186         a = dCvalue_tmp/2 + 2 * HeightOfTails + 2 * HeightOfHeads; // lumen/2 + head + 2*tails + head
187         b = a - HeightOfHeads; //lumen/2 + head + 2*tails
188         c = dCvalue_tmp/2 + HeightOfHeads; // lumen/2 + head
189         d = dCvalue_tmp/2; //lumen/2
190
191
192         /// Compute form factors of the different parts
193         /// for 1 unit cell
194
195         AmplitudeOfHeads = 2/q * ScatteringLengthOfHeads * ( sin(q*a) - sin(q*b) + sin(q*c) - sin
(q*d) );
196         AmplitudeOfTails = 2/q * ScatteringLengthOfTails * ( sin(q*b) - sin(q*c) );
197         AmplitudeOfLumen = 2/q * ScatteringLengthOfLumen * ( sin(q*d) );
198
199         //summing things - combine the different parts of the form factor
200
201         AmplitudeOfGrana = AmplitudeOfHeads + AmplitudeOfTails + AmplitudeOfLumen;
202
203         PartialSumOfGrana += WeightdC * pow(AmplitudeOfGrana, 2);
204
205     } // end of polydispersity loop
206
207
208
209 //Model computation
210 //Model computation
211 //Model computation
212
213     //
214     double ExtraTerm;
215     double ExtraTerm2;
216
217     double Npowerlaw;

```

```

218     double Npowerlaw2;
219
220 // power law extra terms
221
222     Npowerlaw = Parameters[16];
223     ExtraTerm = pow(q,-Npowerlaw);
224
225     Npowerlaw2 = Parameters[17]; //not necessary
226     ExtraTerm2 = pow(q,-Npowerlaw2); //not necessary
227
228
229
230     for (i = 0; i < NumberOfSteps; ++i) {
231         SiLoop = 1;
232
233         // Model geometry calculations
234         Dvalue_tmp = MinD + i * DStepsize;
235         if (SigmaD != 0){WeightD = exp(- pow(MeanD - Dvalue_tmp, 2) / (2.0 * pow(SigmaD, 2)));}
236
237         /// Caille structure factor
238         if ( Nstack <= 1) {
239             SiLoop = 1;
240
241         } else {
242             for (ii = 1; ii <= Nstack-1; ++ii) {
243                 alphaN = EtaCP / pow( 2 * pi, 2) * (log(pi * ii) + EulerConstant);
244                 SiLoop += 2.0 * (1.0 - ii/Nstack) * cos(q * Dvalue_tmp * ii)* exp(-q*q*Dvalue_tmp*
245                 *Dvalue_tmp*alphaN);
246             }
247         }
248     }
249
250 }
251
252     Si += WeightD * SiLoop * PartialSumOfGrana ;
253
254 } // end of polydispersity loop
255
256     Intensity = 1/(q * q) * Si ;          // Lorentz factor * form factor * structure factor
257
258     /// Compute roughness, scaling and background //check
259     if (Contrast >= 0.0 && Contrast <= 100.0) {
260
261         Background = NBackground100;
262         Scaling = NScaling100;
263
264     } else {
265         Background = XBackground;
266         Scaling = XScaling;
267     }
268
269
270 ////////////////
271 // Final scaling and background subtraction
272 ////////////////
273
274     Roughness = 5.0; //can be set to 0; 5 is a good value to nanodiscs according to Martin Cramer Pedersen (
275     (2018, personal communication)
276
277     Intensity = Intensity * Scaling * exp(- pow(q * Roughness, 2)) + Background + ScalingExtraTerm*ExtraTerm
278     + ScalingExtraTerm2*ExtraTerm2; //added roughness
279
280     return Intensity;
281 }
282

```

Model output file

```
1 void OutputData(double ChiSquare, double QMin, double QMax, struct Parameter * Parameters, int
  NumberOfParameters,
2     struct Dataset * Data, int NumberOfSpectra, char cardfilename[128], struct Protein
  ProteinStructure,
3     struct UserDefined UserDefinedStructure, char SampleFilename[256])
4 {
5     /// Declarations
6     // Declare dummy variables used in function
7     int i;
8
9     // Variables describing the output file
10    FILE *fp;
11    const char *filename;
12    filename = "Results.wif";
13
14    /// I/O
15    // Create the output file
16    fp = fopen(filename, "w+");
17
18    // Print filenames to file
19    fprintf(fp, ".card-file: \n");
20    fprintf(fp, "%s \n", cardfilename);
21
22    fprintf(fp, "\n");
23    fprintf(fp, "Datafiles: \n");
24
25    for(i = 0; i < NumberOfSpectra; ++i){
26        fprintf(fp, "%s \n", Data[i].Filename);
27    }
28
29    fprintf(fp, "\n");
30
31    // Print location of sample file
32    fprintf(fp, "Sample info-file: \n");
33    fprintf(fp, "%s \n", SampleFilename);
34
35    fprintf(fp, "\n");
36
37    // Print fit quality to file
38    fprintf(fp, "Final chisq = %g \n", ChiSquare);
39
40    fprintf(fp, "\n");
41
42    // Print range of q to file
43    fprintf(fp, "Lower limit on q = %g \n", QMin);
44    fprintf(fp, "Upper limit on q = %g \n", QMax);
45
46    fprintf(fp, "\n");
47
48    // Print parameters and properties of parameters to file
49    fprintf(fp, "Parameters: \n");
50    fprintf(fp, "Name:          Value:          Error: \n");
51
52    for(i = 0; i < NumberOfParameters; ++i){
53        if (Parameters[i].iParameter == true) {
54            fprintf(fp, "%-25s  %-15f  %-15g \n", Parameters[i].Name, Parameters[i].Value, Parameters[i].
55                .Error);
56        } else {
57            fprintf(fp, "%-25s  %-15f  Fixed \n", Parameters[i].Name, Parameters[i].Value);
58        }
59    }
60
61    fprintf(fp, "\n");
62
63    // Close file and end program
64    fclose(fp);
65 }
```

Appendix 2. PLB isolation protocol

Buffers

Adjust pH carefully with 2 M KOH; only 2-3 drops are enough!

Isolation buffer; **pH=8.0 (KOH)**. 3 L needed

0.5 M sucrose (171.2 g/L)
20 mM Tricine (3.85 g/L)
10 mM HEPES (2.38 g/L)
50 mM KCl (3.73 g/L)
0.66 g/L Cysteine added just before use
0.42 g/L NaF can be added - if preserving phosphorylated state is important (not for PLB isolation)

Sucrose-free (0 %) buffer; pH=7.6 (KOH). 1 L needed

20 mM Tricine (3.85 g/L)
10 mM HEPES (2.38 g/L)
50 mM KCl (3.73 g/L)
1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.203 g/L)
1 mM EDTA disodium salt hydrate (0.372 g/L)

50 % Sucrose (50 %) buffer; pH=7.6 (KOH). 1 L needed

1.47 M sucrose (503.2 g/L)
20 mM Tricine (3.85 g/L)
10 mM HEPES (2.38 g/L)
50 mM KCl (3.73 g/L)
1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.203 g/L)
1 mM EDTA disodium salt hydrate (0.372 g/L)

60 % Sucrose (60 %) buffer; pH=7.6 (KOH). 200 mL needed

1.7 M sucrose (116.3 g/200 mL)
20 mM Tricine (0.717 g/200 mL)
10 mM HEPES (0.477 g/200 mL)
50 mM KCl (0.746 g/200 mL)
1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.040 g/200 mL)
1 mM EDTA disodium salt hydrate (0.0744 g/200 mL)
+ 0.3 mM NADPH (1.75 mg/7 mL). I omit this step.

Storing buffer; pH=7.6 (KOH). 50 mL needed

50 % sucrose (25.15 g/50 mL)
20 mM Tricine (0.179 g/50 mL)

10 mM HEPES (0.119 g/50 mL)
50 mM KCl (0.1865 g/50 mL)
+ 0.3 mM NADPH (1.75 mg/7 mL). I omit this step.
1 tablet of cOmplete protease inhibitor cocktail

0.3 mM NADPH stock = 1.25 mg/5 mL or 1.75 mg/7 mL As detailed below, I did not use additional NADPH in my isolations.

For sucrose gradients:

Use 50 % buffer with 0.3 mM NADPH and 0 % buffer to make 42 % and 46 % solutions.
Use 50 % buffer without 0.3 mM NADPH and 0 % buffer to make 37 % solution. For a single isolation, make 90 mL of 50 % buffer with 22.5 mg NADPH.

Make solutions (enough for 6 gradient tubes):

32 mL of 46 % solution from 29.5 mL 50 % with NADPH and 2.5 mL 0 %
62 mL of 42 % solution from 52.1 mL 50 % with NADPH and 10 mL 0 %
62 mL of 37 % solution from 45.9 mL 50 % without NADPH and 16.1 mL 0 %

Buffer yield for one isolation:

3 L of isolation buffer
150-200 mL of 0 % buffer
150 mL of 50 % buffer
2x7 mL of 60 % buffer
5-10 mL of storing buffer

Materials

Everything needs to be ice-cold and performed in the darkness. Light exposure destroys paracrystalline PLB structure.

Grinding

I used ice-cold pestle and mortar with addition of some sea-sand and manual work. Polytron or Turax grinders can be also used if probes are available.

For grinding - use 295 mL of isolation buffer (be precise) and 50 g of leaves at a time. Total slurry volume is $8 \times 295 \text{ mL} = 2360 \text{ mL}$ (around that).

If using Polytron/Turax, do two rounds of grinding in a max speed, 4 seconds long. If your probe is small or not sharp, it will immediately get clogged. Buy a suitable one or proceed with a mortar/pestle option. I have contacted KINEMATICA in 2016 and they suggested using 20 mm diameter dispersing aggregate PT-DA 20/2WMEC-F193 or PT-DA 20/2WEC-F193 (suitable for Polytron PT 10/35 GT laboratory homogenizer) for this leaf-grinding purpose, it costs around 1500 DKK.

Warring blender is not an option in PLB isolation, because it is very inefficient.

I have used mortar/pestle with a belief that PLBs will be more intact due to less intense grinding. However, such manual isolation is way longer. Then the proteases start working - to avoid it, I made sure everything was ice cold all the time. Rather than to grind for a longer time you can filter of the isolation medium after the short time of grinding and then continue to grind with a new medium added.

Rotors

All centrifugation done with acceleration/deceleration of 5.

Cold Sorvall RC-6 centrifuge F10 35/SLC-3000 rotor. Amount of holes - 6. Tube type 3141-0500. Tube volume 500 mL. Max speed 17700 g/15000 rpm

SLA-1500 rotor. Amount of holes - 6. Tube type 3141-0250. Tube volume 250 mL. Max speed 134155 g/15000 rpm

F13-14x50cy rotor (Sorvall RC-6+). Amount of holes - 14. Tube type 430829 Corning. Tube volume 50 mL. Max speed 15500 g

Cold Beckmann Optima XPN-80 ultracentrifuge

SW28 rotor (Sorvall RC-6+). Amount of hangers - 6. Tube type 344058. Tube volume 38.5 mL. Max speed 141000 g/28000 rpm

Plants

Early vigour maize seeds (I used var. 'Ambition' from Limagrain). Treat seeds with fungicide if not done beforehand - otherwise the seedlings will get mouldy. I used either mesurol pre-coated seeds or used anti-fungal powder Thuram (a teaspoon of fungicide for 300 mL of seeds). Spread the seeds on vermiculite, add some vermiculite on top. Soak with water extensively. Seeds shall be covered in vermiculite, but shall be in max 0.5 cm depth. Maize seeds are grown 2 days at 20 °C followed by 7 days at 27 °C in high humidity (75-80 %) dark chambers. I used 600 mL seeds grown in 4 plastic trays (56·26·4 cm), this gives 5-8·50 g leaf material. The start of growth in 20 °C improves germination (Eva Selstam), but I omitted this; grew constantly in 27 °C. Watering once after 4 days is required.

Protocol

Leaf harvesting and grinding

Only the first and second true leaves are harvested (plucked one by one) leaving long stalks behind. It is important to keep the same type of growth regime, since the unit cell length changes a bit with age. The age/structure of the etioplast and PLB also change from the tip of the leaf to the bottom. In a grass and barley leaf, the tip is mature and the bottom of the leaf is still developing. *Maize PLBs are a bit more uniform inside the entire leaf length - me.* Thus it is not good to harvest the bottom of the leaves. You have to discard the lower third of the first and second leaves to get out a uniform set of PLB.

A package of 50 g leaves is wrapped in aluminium foil and stored on ice until all leaves are harvested. *8 packages was an optimistic option for me, I usually obtained 6. This is enough.*

For grinding - use 295 mL of isolation buffer (be precise, use measuring cylinder) and 50 g of leaves at a time. Cut the leaves with scissors in small bits and use 2 spoons of clean sea sand. If doing so, always use 2 layers of Miracloth for filtration, this will capture sand. Add ice cold isolation buffer to some leaves (bit by bit) and grind manually. Be quick. Use a big mortar and a pestle with a big handle as well as cryo-gloves from resin. Pour isolation buffer for filtration, add more leaves and a bit more buffer. Cool the mortar with LN₂ between grindings if needed. You will get a total slurry volume of 8x295 mL = 2360 mL (around that). 3 L of isolation buffer shall be enough for 1 isolation.

PLB isolation

Filter the obtained leaf slurry through 5 layers of cheesecloth/nylon membrane mesh and 2 layers of Miracloth. Centrifuge 3300g for 10 min in a large rotor and save the pellet in 40 mL isolation buffer. Stir a bit to resuspend the pellet. Repeat centrifugation with other part of leaf slurry if needed.

Centrifuge both lots divided into two tubes (I use 50 mL Corning orange-caps) in a small rotor at 4340 g for 10 min. Remove supernatant. Stir the pellets with 0.5 mL isolation buffer and dilute with 5 mL of 0 % buffer in each tube to osmotically break intact etioplasts. Carefully stir for 2 min and add 3 mL 50 % buffer in each tube to restore sucrose concentration.

Dilute with isolation buffer up to 20 mL and centrifuge 20 min in 6000 g.

This is how I finish the protocol. I discard supernatant, wash the pellet with small amount of storage buffer, load them into SAXS capillary, put it inside a closed ice box and immediately go to measure SAXS. For SANS, use D₂O-based storage buffer, wash the pellet 2 times, discard buffer. Then resuspend thoroughly in D₂O-based storage buffer and flash-freeze in LN₂. Use black Eppendorfs and wrap them in 2 layers of aluminium foil each.

If not applicable, then standard protocol is continued here. After 6000 g centrifugation for 20 min, supernatant is discarded. Each pellet is stirred with 4-5 mL of 60 % buffer. The membrane slurry is sucked into a 10 mL disposable plastic syringe through a thin (0.6 mm diameter 2 cm long) needle. Membrane slurry is squeezed in and out 4 times. This breaks a connection between PLBs and PTs.

After squeezing, load membrane slurry UNDER the sucrose gradient. Avoid bubbles! The sugar concentration must be higher than 46 % to make membranes stay on the bottom of sucrose gradient tubes. Therefore: make a total volume of a membrane slurry equal to 12-14 mL by adding 60 % buffer BEFORE LOADING. Centrifuge in SW 28 in 110000 g/25000 rpm for 1 h.

6 sucrose gradients are premade from 10 mL 37 %, 10 mL 42 % and 5 mL 46 % buffers (see below how).

After ultracentrifugation, PLB membranes will be found in the interface between 42 % and 37 % sucrose layers. Suck off this PLB band with a thin-end glass Pasteur pipette. Dilute this PLB solution with equal amount of 0 % sucrose buffer and centrifuge at 8100 g for 10 min. Obtained pellet is the best PLB fraction. The supernatant can be centrifuged again at 7000 g for 20 and used as a less-good PLB fraction.

Save these PLB pellets and stir them with a storing buffer containing NADPH. E. Selstam obtained 2.4-5 mL of a total PLB pellet, aliquoted them by 500 μ L in dark Eppendorf tubes and flash-froze them in LN₂. For protein and pigment determination in PLB sample, see below.

Making sucrose gradients

Pipette 10 mL of 37 % sucrose buffer to each tube. Then use a 10 mL syringe equipped with a 8 cm long needle with a 0.8 mm wide hole and apply the following 42 and 46 % sucrose buffers below each other - in the order 37 %, 42 %, 46 %. Do it slowly and be Careful not to mix the layers, as by accidentally doing so you waste a lot of money on NADPH! For applying the membrane slurry under the gradient use the needle of the same width, not the small one used for squeezing. Avoid bubbles at all times!!!

Protein quantification

Protein is analysed with BCA method Pierce. BSA is used as a standard protein. E. Selstam has treated the PLB membranes with 0.1 M KOH in order to saponify the membrane lipids (1 h in 80 °C). It is also possible to solubilize the lipids with SDS. You have to treat the BSA standard the same way as the PLB sample.

Pigment analysis

Pigment analysis: The pigment analyses is made in 80 % acetone, however the storing buffer has too high sucrose concentration to make a uniform clear solution. Thus the sucrose buffer needs to be exchanged.

Dilute 200 μ L of PLB membranes to 1 mL with 0 % buffer or water. Then centrifuge full speed in Ependorf tabletop centrifuge for 10 min. Remove supernatant. Stir up the pellet in 200 μ L 0 % buffer and add 800 μ L of 80 % acetone. Use quartz cuvette. E PChlide = 31.1 mL $\cdot$ μ mol PChlide $\cdot$ cm⁻¹. Measure Absorption at 626 nm (signal) and 730 nm (background).

General comments from Eva Selstam and me

All handling of plants and membranes is done in a faint green safe light. Use black boxes to transform plants if necessary. I recommend locking the door when doing the isolation.

Isolation of PLBs with preserved cubic structure is not easy. I failed with approx. 25

% of the isolations. I tried to find out the why, but still I do not now. You need to work quickly and with cold solutions. If you use pestle and mortar, be mentally prepared for a lot of manual work in the cold and darkness. Also - act quickly to avoid the PLB damage by proteases. Make 6 tubes with sucrose gradients during the 20 min centrifugation step.

It is critical to keep higher pH high during the leaf grinding, since a pH lower than 7 will irreversibly destroy PLBs. I can recommend to raise the pH in the grinding buffer from 7.6 to 8.

In PLB isolations we use NADPH for stabilisation of the POR-protochlorophyllide-NADPH complex (POR-PChlide-NADPH). In some work from the 1980s Ryberg-Sundqvist postulated that PLB was preserved in the presence of NADPH. So we use NADPH to make the best possible for a preserved PLB. However, I am not 100 % convinced that it is necessary to add, but do not want to risk the PLB or start to try out without NADPH. *I did not add extra NADPH in my buffers and PLBs were fine - added by me.*

The reason why I am not 100 % convinced is:

1. The NADPH is binding to the complex, it can not be removed by washing in NADPH-free media. You can remove it by adding salt to the media, then NADPH leaves the site on the POR-Complex. (This means, NADPH already in POR in your leaves will stay there during your isolation);

2. It is possible to destroy the PLB structure by salt treatment - and then get the structure back if salt is removed without adding NADPH. Thus, as long as the oligomer complex of POR is intact, PLB is formed. The destruction of PLB by moderately high salt concentrations is due to shielding membrane surface charges and not by the release of NADPH from the POR.

PT on PLB: In the isolation process, there is a step where I squeeze the membrane slurry through a fine needle, to remove/detach the PT from the PLB. In order not to destroy the paracrystalline PLB structure we gradually weakened this step. For the X-ray/neutron diffraction I think it does not matter if there are some short PTs left on PLBs. The PTs will be part of the scattering. Some PLB isolating research groups use sonication to get rid of the PTs, we avoided that.

Normally I stored the PLB membrane (in 50 % sucrose buffer) preparations in -20 ° C. During transportation they were on dry ice. *I have always stored them in -80 ° C and shipped them to Australia in 10 kg of dry ice - added by me.*

It is not fun to make 60 % buffers. It is also very difficult to pipette such a viscous solution in the dark. To make things easier in the dark, I always had the 10 ml 60 % buffer (the amount I needed for each isolation) ready in a tube, in the -20 ° C storage. When planing an isolation I just took one tube, added NADPH and let the NADPH dissolve by itself. Do not shake, then NADPH forms lumps. In the dark it was then easy to have control of the amount of 60 % buffer that I was using. If there was some left-overs after the resuspension for the sucrose gradient centrifugation, it was used as

storing buffer, to save NADPH.

Appendix 3. Cyanobacterial RD statistics

Raw data

```
> shapiro.test(datacyano$RD[datacyano$Category=='6803' & datacyano$Replica=='1'])
```

Shapiro-wilk normality test

```
data: datacyano$RD[datacyano$Category == "6803" & datacyano$Replica == "1"]  
W = 0.9935, p-value = 0.8767
```

```
data: datacyano$RD[datacyano$Category == "6803" & datacyano$Replica == "2"]  
W = 0.9577, p-value = 1.249e-05
```

```
data: datacyano$RD[datacyano$Category == "6803" & datacyano$Replica == "3"]  
W = 0.9237, p-value = 2.269e-08
```

```
> shapiro.test(datacyano$RD[datacyano$Category=='7942' & datacyano$Replica=='1'])
```

Shapiro-wilk normality test

```
data: datacyano$RD[datacyano$Category == "7942" & datacyano$Replica == "1"]  
W = 0.9298, p-value = 1.516e-05
```

```
data: datacyano$RD[datacyano$Category == "7942" & datacyano$Replica == "2"]  
W = 0.8932, p-value = 5.636e-11
```

```
data: datacyano$RD[datacyano$Category == "7942" & datacyano$Replica == "3"]  
W = 0.9914, p-value = 0.2695
```

```
> shapiro.test(datacyano$RD[datacyano$Category=='7002' & datacyano$Replica=='1'])
```

Shapiro-wilk normality test

```
data: datacyano$RD[datacyano$Category == "7002" & datacyano$Replica == "1"]  
W = 0.8654, p-value = 9.573e-14
```

```
data: datacyano$RD[datacyano$Category == "7002" & datacyano$Replica == "2"]  
W = 0.9236, p-value = 7.778e-09
```

```
data: datacyano$RD[datacyano$Category == "7002" & datacyano$Replica == "3"]  
W = 0.9321, p-value = 3.904e-08
```

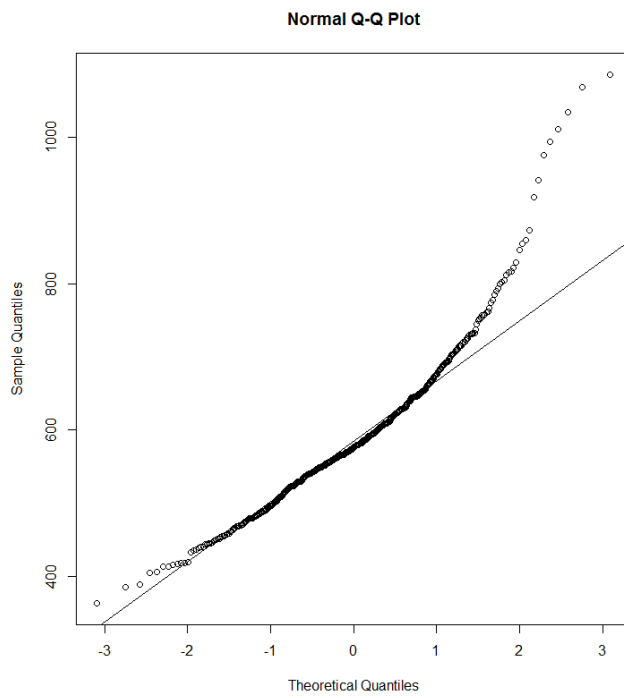
lsmeans output

```
> mod.lsmeans <- lsmeans::lsmeans(mod1, specs = c("Category","Replica"))  
> mod.lsmeans
```

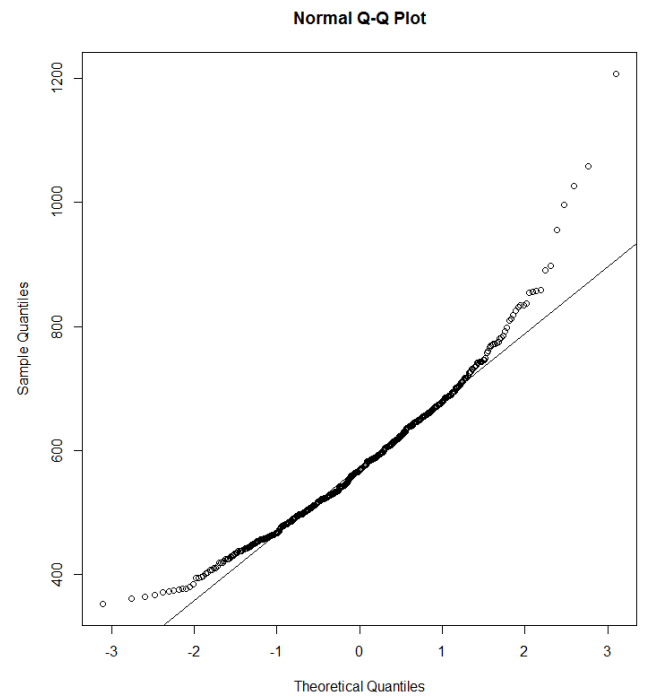
Category	Replica	lsmean	SE	df	lower.CL	upper.CL
6803	1	574.1123	10.914563	1668	552.7046	595.5200
7942	1	612.8377	10.914563	1668	591.4300	634.2454
7002	1	615.7887	7.491195	1668	601.0955	630.4818
6803	2	632.5374	8.281828	1668	616.2936	648.7813
7942	2	565.6762	8.099787	1668	549.7894	581.5631
7002	2	621.9866	8.139202	1668	606.0225	637.9507
6803	3	556.9211	8.476721	1668	540.2950	573.5472
7942	3	572.6480	8.159127	1668	556.6448	588.6512
7002	3	556.6844	8.159127	1668	540.6812	572.6876

confidence level used: 0.95

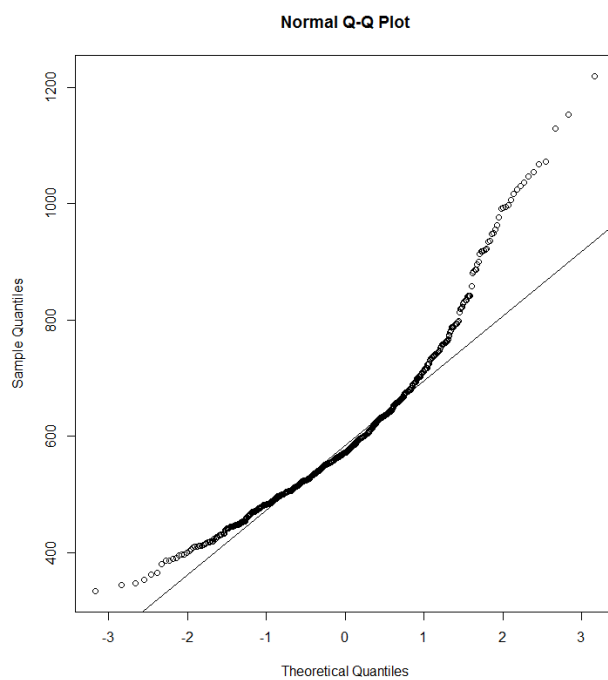
Raw data Q-Q plots



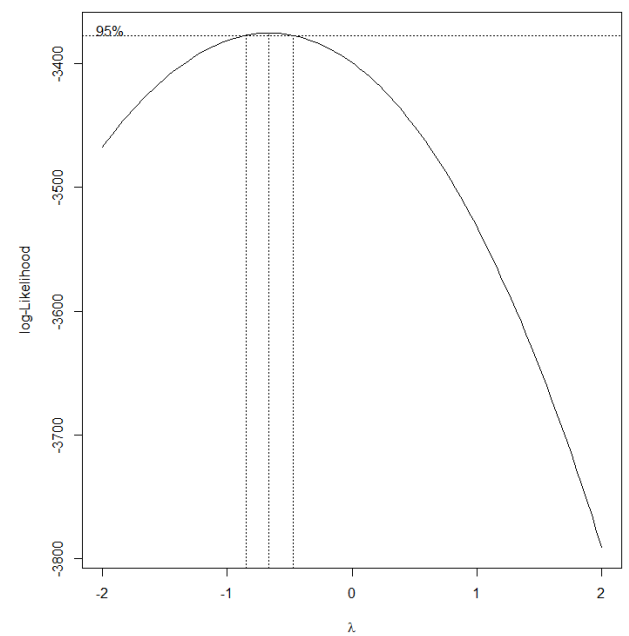
6803



7942



7002



Box-Cox lambda estimation

Box-Cox

```
> lambda <- Cox2[1, "Box.x"]
> lambda
[1] -0.6666667
```

Model

```
lme.cyanosBC <- lme(RD_box ~ Category-1, random =~1|Replica,data=datacyano)
> lme.cyanosBC
```

```
Linear mixed-effects model fit by REML
Data: datacyano
Log-restricted-likelihood: 7528.633
Fixed: RD_box ~ Category - 1
Category6803 Category7942 Category7002
1.478410 1.478018 1.478340
```

```
Random effects:
Formula: ~1 | Replica
(Intercept) Residual
StdDev: 0.0005802694 0.002674601
```

```
Number of Observations: 1677
Number of Groups: 3
```

```
> summary(lme.cyanosBC) ##p values
```

```
Linear mixed-effects model fit by REML
Data: datacyano
AIC BIC logLik
-15047.27 -15020.15 7528.633
```

```
Random effects:
Formula: ~1 | Replica
(Intercept) Residual
StdDev: 0.0005802694 0.002674601
```

```
Fixed effects: RD_box ~ Category - 1
      Value Std.Error DF t-value p-value
Category6803 1.478410 0.0003560206 1672 4152.597 0
Category7942 1.478017 0.0003551683 1672 4161.457 0
Category7002 1.478340 0.0003510794 1672 4210.842 0
Correlation:
      Ct6803 Ct7942
Category7942 0.890
Category7002 0.897 0.899
```

```
Standardized within-Group Residuals:
      Min      Q1      Med      Q3      Max
-3.690522575 -0.630468594 0.001438863 0.644311590 3.148658336
```

```
Number of Observations: 1677
Number of Groups: 3
```

Model GLHT

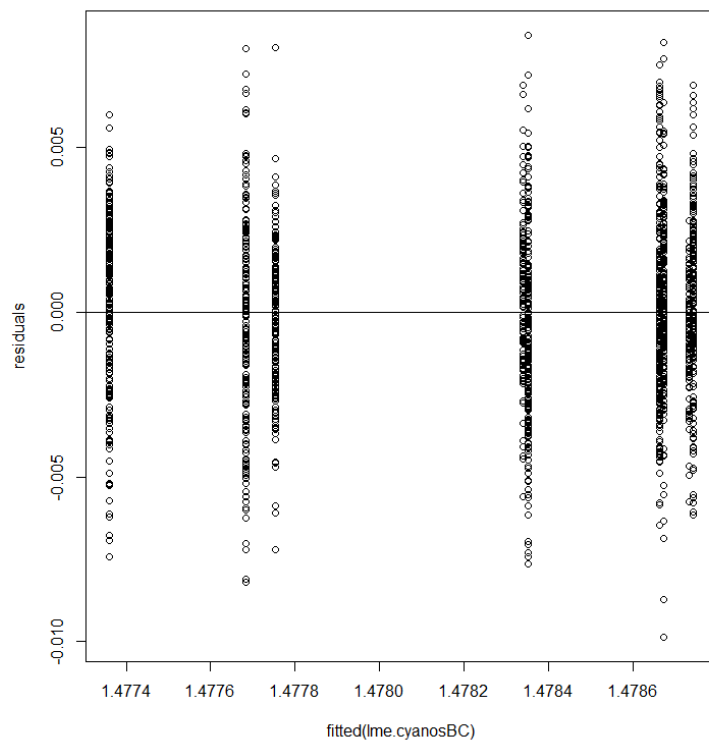
```
> (sumBClme <- glht(lme.cyanosBC, linfct = mcp(Category = "Tukey" ))) ##RD difference
s between groups
```

General Linear Hypotheses

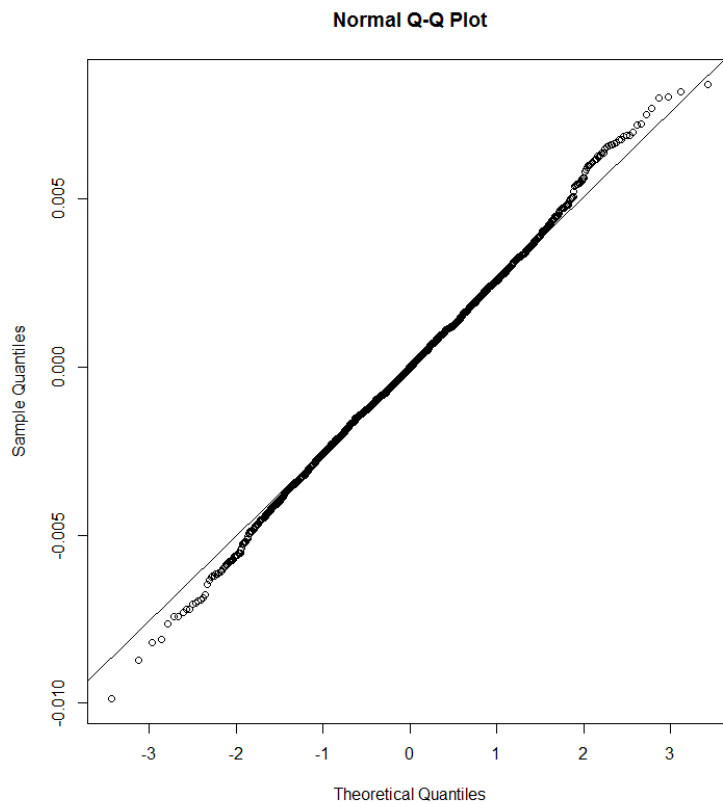
Multiple Comparisons of Means: Tukey Contrasts

```
Linear Hypotheses:
      Estimate
7942 - 6803 == 0 -3.926e-04
7002 - 6803 == 0 -7.014e-05
7002 - 7942 == 0 3.224e-04
```


Model plots



Residuals vs. model-fitted residuals



Model Q-Q plot