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Supporting information for article:

Direct protein crystallization on ultrathin membranes for diffraction measurements at X-ray free-electron lasers

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Table S1 Parameters of the crystallization conditions tested for producing large crystals for XFEL experiments. Unless otherwise stated (SD) experiments were carried out in hanging drop (HD) geometry. Loaded chips were mounted on the lid of the ibidi® box and the reservoir of the chamber was charged with 1 or 0.5 ml of 0.6 M NaCl, 0.1 M NaAc, pH 4.5 (reservoir solution; referred to as "R" in the table). They were incubated at room temperature (20-22°C), at approximately 55% relative humidity. Sizes shapes and densities of crystals were assessed by light microscopy. Chips that were selected, mounted on the aluminium holder and exposed to the FEL beam are indicated by bold type, chip 48 was used for the synchrotron diffraction data collection.

Chip no.	**Window size [µm^2]**	**Si_xN_y layer thickness [nm]**	**Crystallization condition: protein concentration and precipitating agent composition**	**Volume deposited (µl)**
1	300 x 300	30	90 µl (50 mg/ml H_2O) + 10 µl "R"	20
2 (SD)	300 x 300	30	90 µl (50 mg/ml H_2O) + 10 µl "R"	20
3	400 x 400	30	50 mg/ml H_2O	20
4	400 x 400	30	90 µl (50 mg/ml H_2O) + 5µl "R"	20
5	500 x 500	85	100 mg/ml H_2O	20
6	500 x 500	85	90 µl (100 mg/ml H_2O) + 10 µl "R"	20
7	200 x 200	30	90 µl (50 mg/ml H_2O) + 10 µl "R"	5
8	200 x 200	30	90 µl (50 mg/ml H_2O) + 5µl "R"	5 vs 0.5 ml R
9	300 x 300	30	90 µl (50 mg/ml H_2O) + 10 µl "R"	5 vs 0.5 ml R
10	200 x 200	30	90 µl (50 mg/ml H_2O) + 10 µl "R"	20
11	200 x 200	30	90 µl (50 mg/ml H_2O) + 20 µl "R"	20
12	300 x 300	250	90 µl (50 mg/ml H_2O) + 10 µl "R" + 10 µl PEG 50%	20
13	200 x 200	250	90 µl (50 mg/ml H_2O) + 20 µl "R" + 5 µl 100% EG	20

14	400 x 400	30	50 mg/ml 50 mM NaAc	20
15	300 x 300	250	50 mg/ml 50 mM NaAc	20 vs 0.5 ml R
16	200 x 200	250	50 mg/ml 50 mM NaAc	20 vs 0.5 ml R
17	300 x 300	250	90 µl (50 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%	20 vs 0.5 ml R
18	400 x 400	250	90 µl (50 mg/ml H_2O) + 10 µl „R“ + 10 µl EG 1%	20 vs 0.5 ml R
20	200 x 200	250	50 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
21	300 x 300	250	50 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
22	400 x 400	250	50 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
23	200 x 200	250	90 µl (50 mg/ml H_2O) + 10 µl „R“ + 10 µl 50% PEG; fresh	20 vs 0.5 ml R
24	**300 x 300**	**250**	**90 µl (50 mg/ml H_2O) + 10 µl „R“ + 10 µl 50% PEG; fresh**	**20 vs 0.5 ml R**
25	400 x 400	250	90 µl (50 mg/ml H_2O) + 10 µl „R“ + 10 µl 50% PEG; fresh	20 vs 0.5 ml R
26	**200 x 200**	**250**	**90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh**	**20 vs 0.5 ml R**
27	**300 x 300**	**250**	**90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh**	**20 vs 0.5 ml R**
28	400 x 400	250	90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh	20 vs 0.5 ml R
29	400 x 400	250	70 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
30	**200 x 200**	**250**	**50 mg/ml 50 mM NaAc; fresh**	**20 vs 0.5 ml R**
31	300 x 300	250	50 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
32	400 x 400	250	50 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
33	**200 x 200**	**250**	**90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh**	**20 vs 0.5 ml R**

34	300 x 300	250	90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh	20 vs 0.5 ml R
35	400 x 400	250	90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh	20 vs 0.5 ml R
36	**200 x 200**	**250**	**50 mg/ml 50mM NaAc; stored at 4°C**	**20 vs 0.5 ml R**
37	200 x 200	250	90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; stored at 4°C	20 vs 0.5 ml R
38	300 x 300	250	90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; stored at 4°C	20 vs 0.5 ml R
39	400 x 400	250	50 mg/ml 50mM NaAc; stored at 4°C	20 vs 0.5 ml R
40	200 x 200	250	50 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
41	300 x 300	250	50 mg/ml 50 mM NaAc; fresh	20 vs 0.5 ml R
42	**200 x 200**	**250**	**90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh**	**20 vs 0.5 ml R**
43	**300 x 300**	**250**	**90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh**	**20 vs 0.5 ml R**
44	**200 x 200**	**250**	**50 mg/ml 50 mM NaAc; stored at 4°C**	**20 vs 0.5 ml R**
45	300 x 300	250	50 mg/ml 50 mM NaAc; stored at 4°C	20 vs 0.5 ml R
46	200 x 200	250	90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; stored at 4°C	20 vs 0.5 ml R
47	400 x 400	250	90 µl (70 mg/ml H_2O) + 10 µl „R“ + 10 µl PEG 50%; fresh	20 vs 0.5 ml R
48	**300 x 300**	**250**	**90 µl (50mg/ml H_2O) + 20 µl „R” + 10 µl 25% EG**	**20**
49	100 x 100	30	5 nl (50 mg/ml H_2O) on top of 8 nl „R“	13 nl in each well

Table S2 Parameters of the diffraction data collected on EIGER X 16M detector at PX-I beamline, SLS on lysozyme microcrystals grown *in situ* on the silicon nitride membranes and preserved from dehydration by sandwich-type enclosure in between two chips.

Data collection	
Temperature (K)	293
Beamline	Swiss Light Source PXI X06SA
Wavelength (Å)	1.00
Flux of the full beam; transmission; exposure;	1.5e11 ph/s; 0.005; 0.1 s/frame
Total range collected per crystal (°); per frame	50; 0.1
Crystal-to-detector distance [mm]	200
Number of crystals merged	2
Phasing method	Molecular replacement (search model PDB: 2lyz)
Space group	$P4_32_12$
Unit cell parameter *a* (Å)	79.50
Unit cell parameter *b* (Å)	79.50
Unit cell parameter *c* (Å)	37.81
$\alpha = \beta = \gamma$ (°)	90
Resolution (Å)	50-1.57 (1.61-1.57)
R_{meas}	0.05 (0.847)
$\langle I/\sigma(I) \rangle$	13.74 (1.04)
Completeness	0.954 (0.952)
$CC_{1/2}$	99.9 (70.5)
Mosaicity (°)	0.033
Refinement	
Resolution (Å)	39.75-1.57
No. of reflections R_{work}/R_{free}	16960/849
R_{work}/R_{free}	0.1656/0.1819
No. of non-hydrogen atoms:	
Protein	1020
Ions	5
Water	65
Bond lengths RMS (Å)	0.005
Bond angles RMS (°)	0.77
Ramachandran favored (%)	99.25
Ramachandran allowed (%)	0.75
Ramachandran outliers (%)	0
Rotamer outliers (%)	0
Clashscore	1.00
B-factors (Å^2):	
Protein	30.44
Ions	42.11
Water	41.05
PBD ID	5NE0

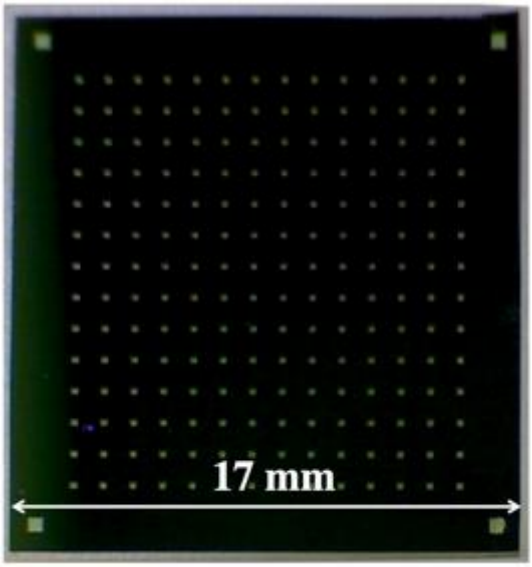

Figure S1 Microfabricated chip with Si_3N_4 windows (here: 200 x 200 µm^2) designed for serial femtosecond crystallography time-resolved experiments. Large (400 x 400 µm^2) membranes placed in the corners were knocked-out and served as alignment marks.

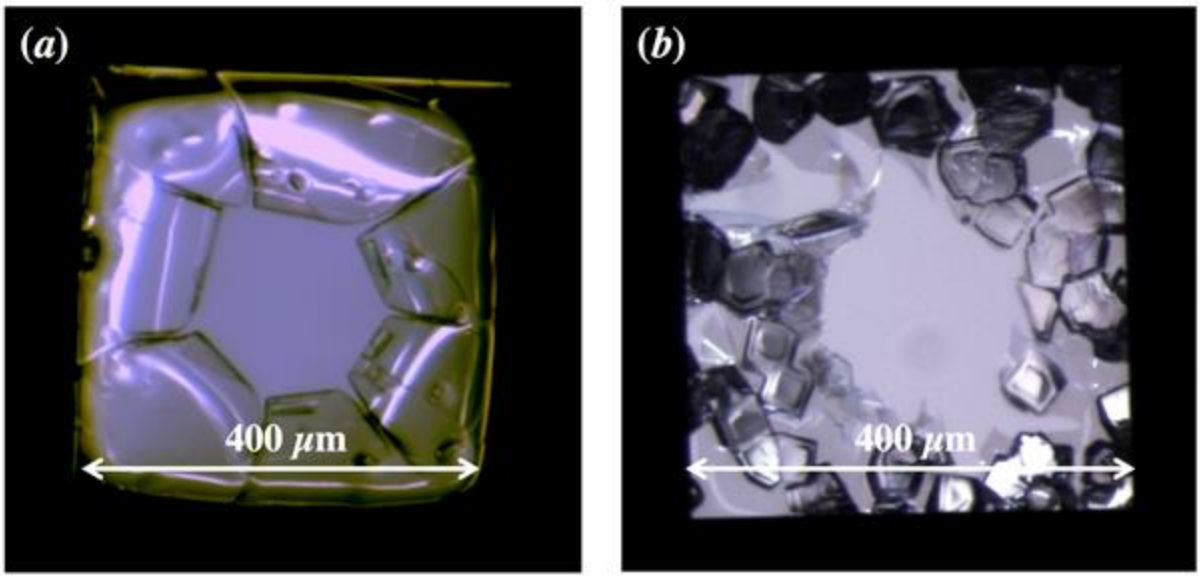

Figure S2 Inadequate crystallization conditions led to membrane damage. Thin membranes (30 nm) are prone to damage during (a) or after (b) crystallization – due to the tension created on the membrane.

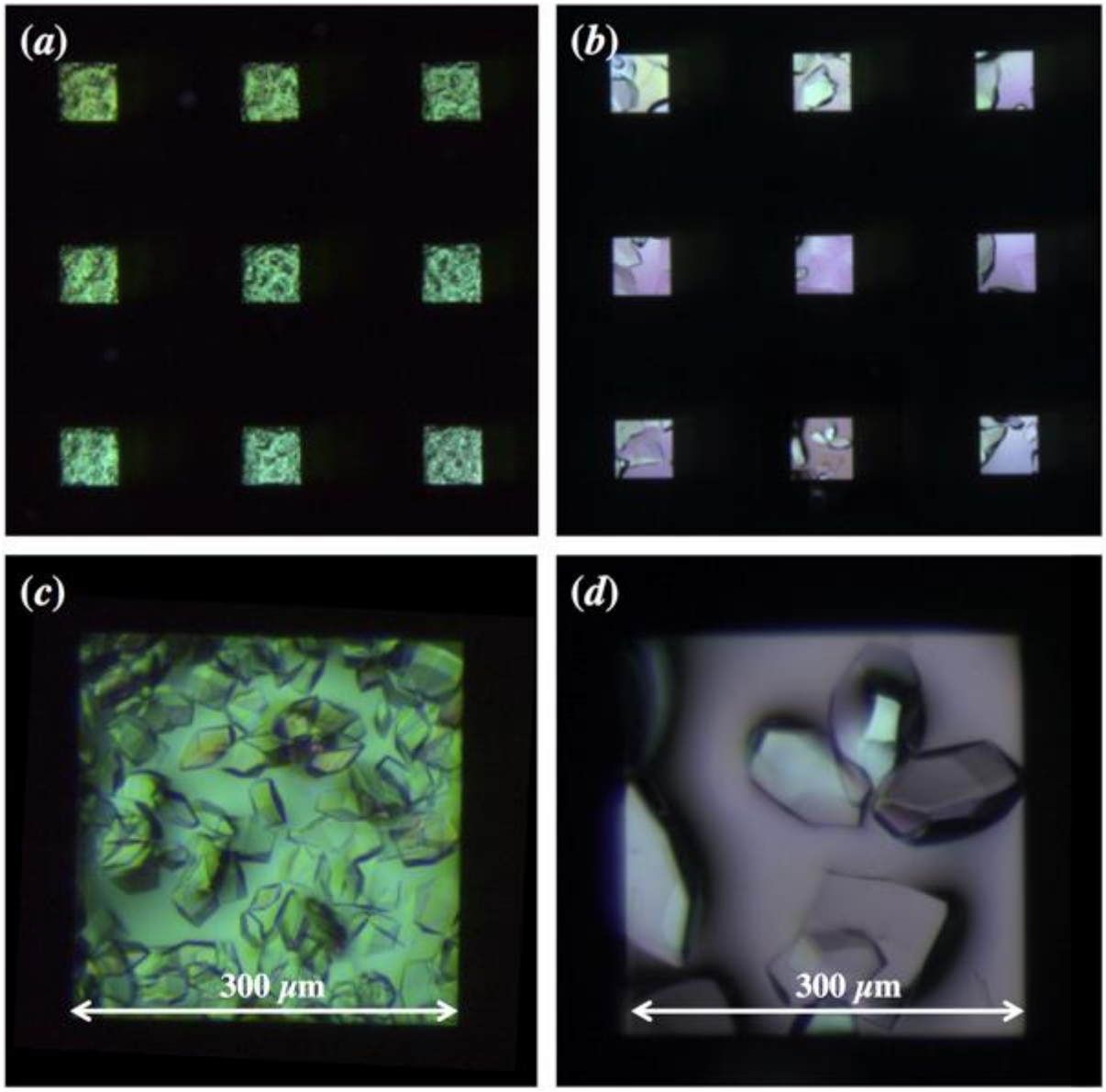

Figure S3 Protein crystals grown on Si_3N_4 membranes. (a) and (b): overview, (c) and (d): zoomed in view of selected wells. (a) and (c): Crystals grown at RT from mixture previously stored at 4°C overnight, chip 12, chemical composition: 90 µl 50 mg/ml H_2O + 10 µl R + 10 µl PEG 50%. (b) and (d): Crystals grown at RT from fresh solutions, chip 24: chemical composition: 90 µl 50 mg/ml H_2O + 10 µl R + 10 µl PEG 50%. The chip reference numbers refer to Table S1.

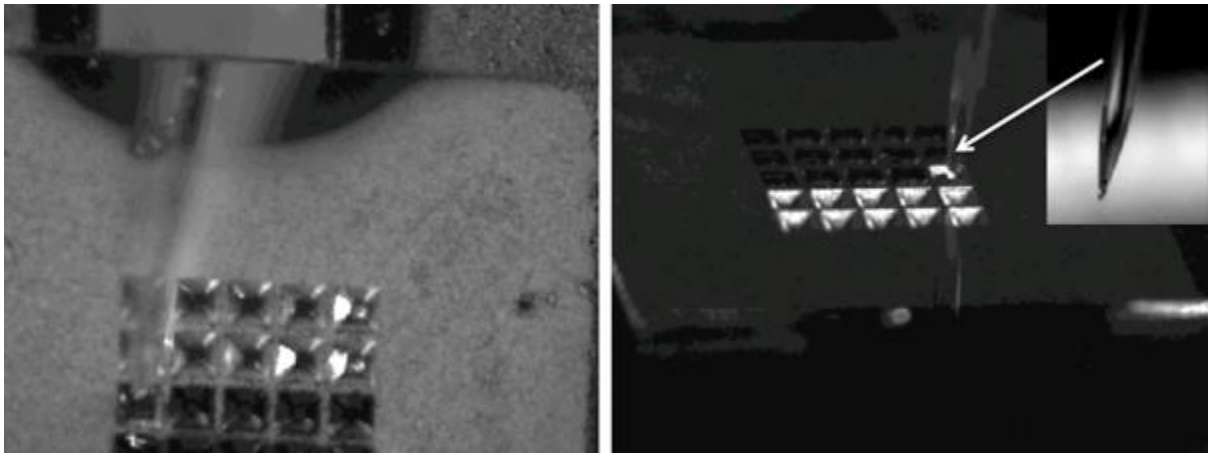

Figure S4 Microfabricated chip on the dew point stage as the nano-wells are filled with protein (crystallization) solution, with nanoliter accuracy. Left – top view; right – side view. The 5 x 5 well array is 2 mm x 2 mm in size. The automatic method was used to load each well with 15 nl of liquid. It took about 2 min. to load all 25 wells. See the Supplementary Movie for a demonstration.

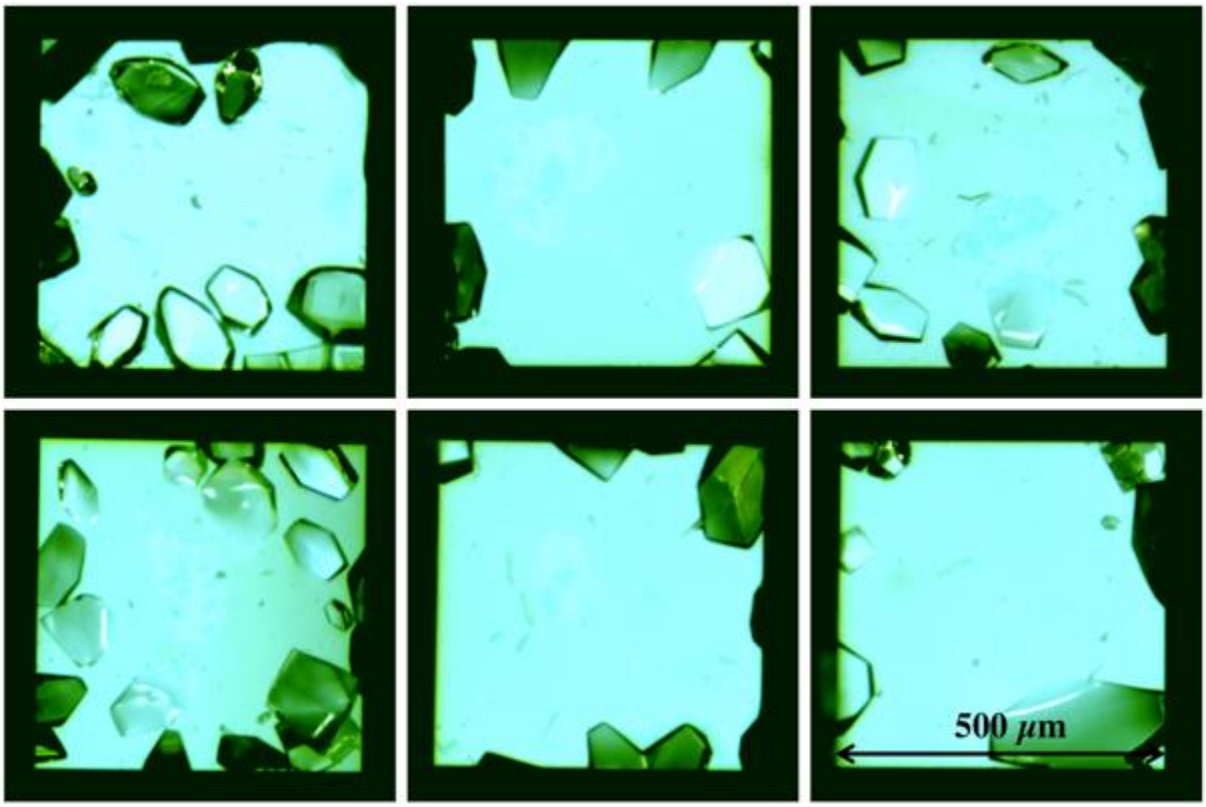

Figure S5 Protein crystals grown on Si_3N_4 membranes. Crystals are clustering in the corners and close to the edges of the membranes resulting in poor coverage of the windows. Growth from 20 µl of the solutions mixture (50 mg/ml H_2O lysozyme and 0.6 M NaCl, 0.1 M NaAc, pH: 4.5 in proportion 9:1) in HD geometry against 1 ml of reservoir solution. Growth observed after 17 h under light microscope.

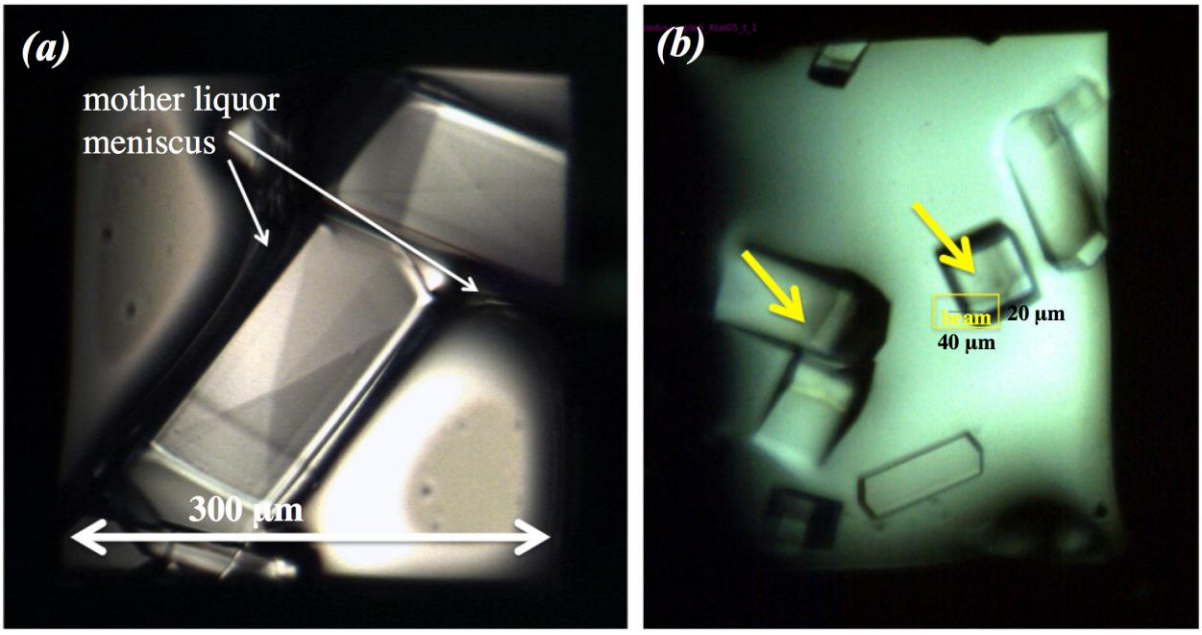

Figure S6 Protein crystals grown at RT from fresh solutions on 250 nm thick Si_3N_4 membranes: (a) observed in the sandwich 1 h after the enclosure showing minute amounts of mother liquor around the crystals. (No water rings were observed in the diffraction pattern); (b) microscope view of the sandwich placed on the goniometer of the SLS PX-I; data used for structure refinement are originating from the crystals indicated by arrows.

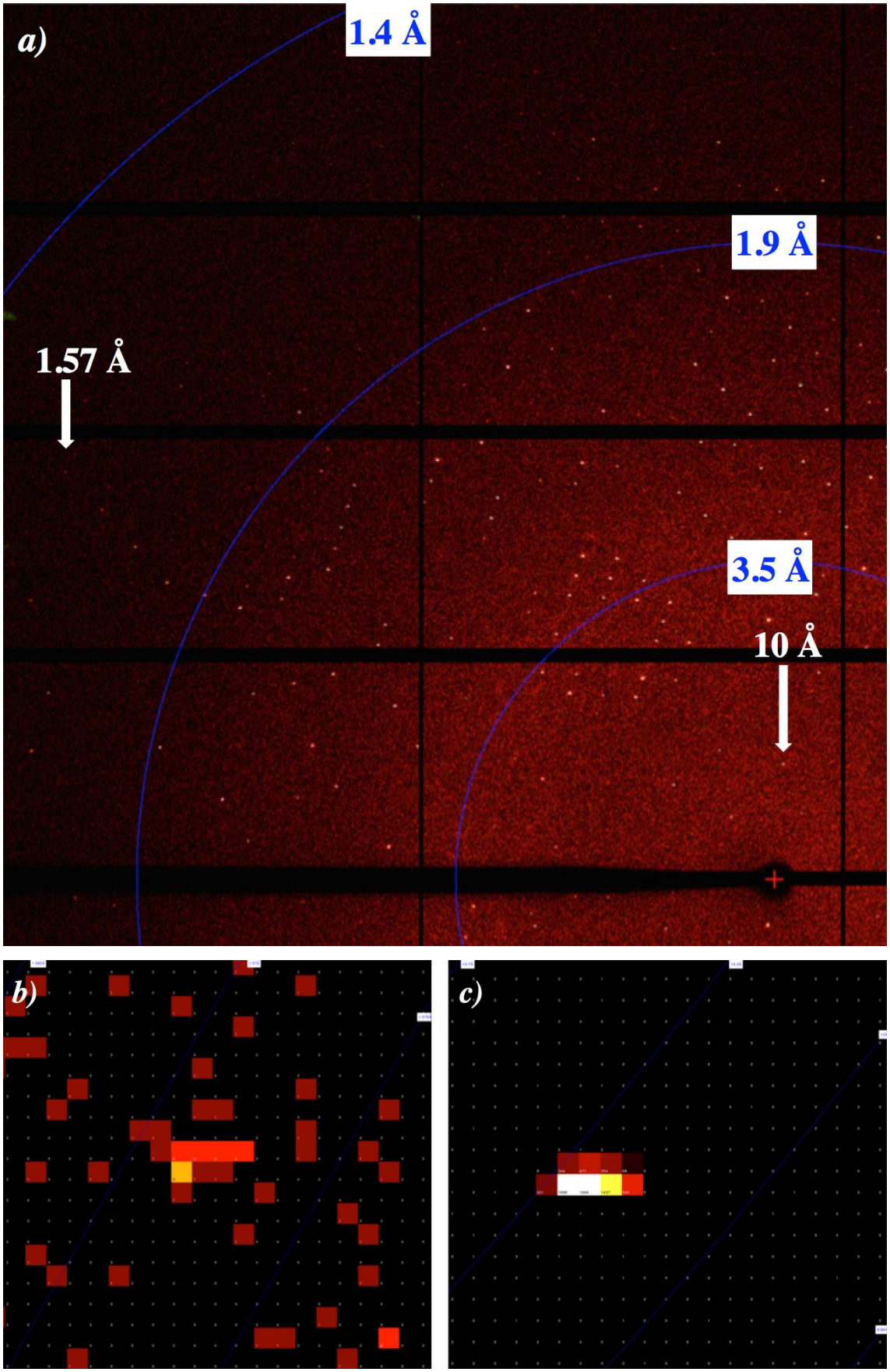
a)
1.4 Å
1.9 Å
1.57 Å
3.5 Å
10 Å
b)
c)

Figure S7 (a): Representative diffraction pattern of a lysozyme crystal between silicon nitride membranes. Only a quarter of the Eiger 16M detector is shown. Transmission for this testshot was 5%. (b): Close-up on a spot at 1.57 Å resolution (testshot with transmission 5%). Maximum counts value: 3. (c): Close-up on a spot at about 10 Å resolution (similar to Bragg peaks used at LCLS for the delay experiments), in a dataset collected at the Swiss Light Source (transmission 0.3%). Counts are up to about 2000.

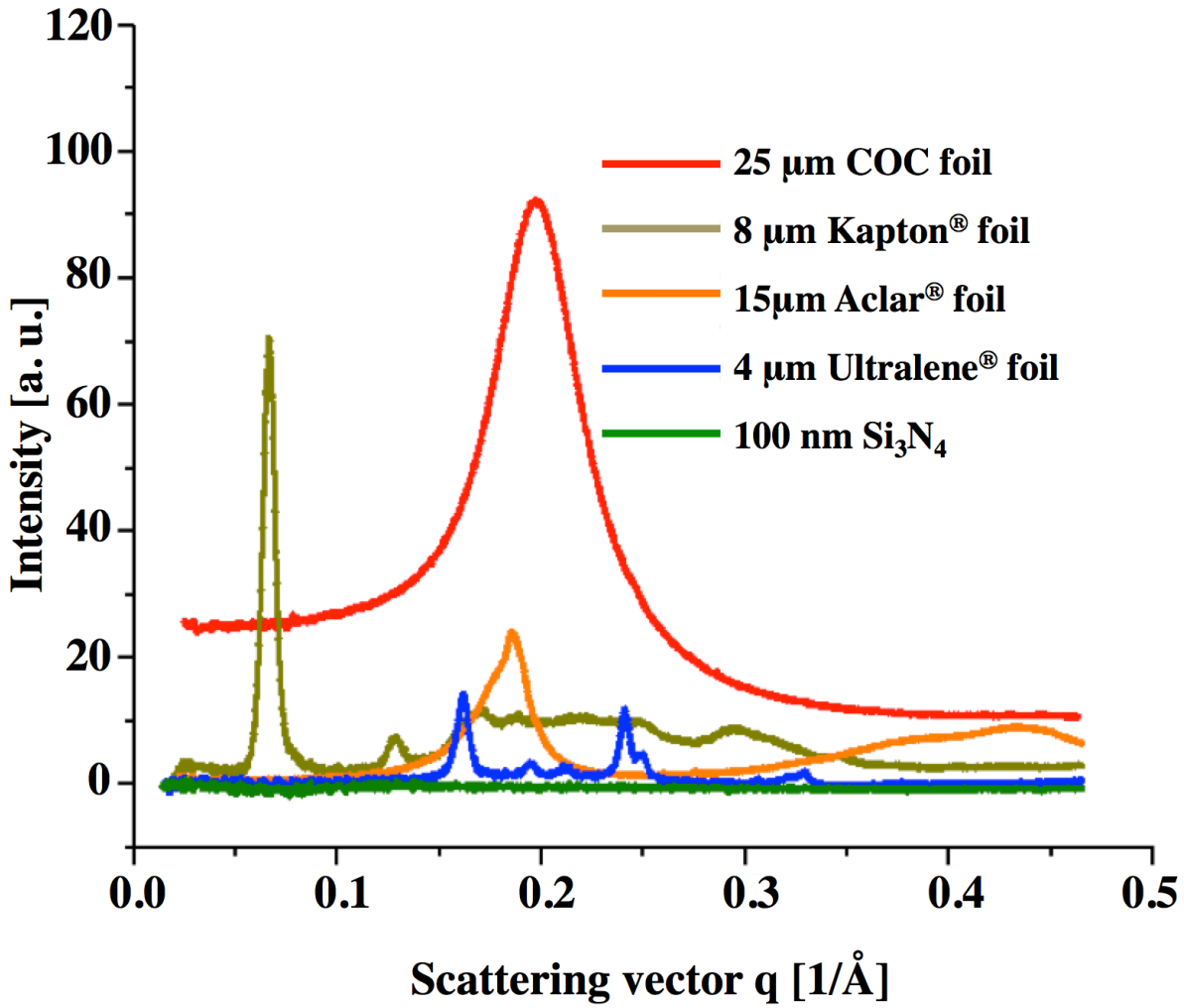

Figure S8 Comparison of the Si_3N_4 background signal to commonly used, commercial ultrathin support materials. Intensity of X-ray scattering from investigated samples has been collected at 12.7 keV photon energy, 1 s exposure and full flux (4e11 ph/s), detector distance 400 mm. Data recorded at PX-I beamline, SLS, Switzerland.