High-Resolution Crvo-EM Structure Determination of α-Synuclein – A Prototypical Amyloid Fibril

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18 Abstract

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20 The physiological role of α -synuclein (α -syn), an intrinsically disordered presynaptic neuronal protein, is believed 21 to impact the release of neurotransmitters through interactions with the SNARE complex. However, under certain 22 cellular conditions that are not well understood, α -syn will self-assemble into β -sheet rich fibrils that accumulate 23 and form insoluble neuronal inclusions. Studies of patient derived brain tissues have concluded that these inclusions 24 are associated with Parkinson's disease, the second most common neurodegenerative disorder, and other synuclein 25 related diseases called synucleinopathies. In addition, repetitions of and specific mutations to the SNCA gene, the 26 gene that encodes α -syn, results in an increased disposition for synucleinopathies. The latest advances in cryo-EM 27 structure determination and real-space helical reconstruction methods have resulted in over 60 in vitro structures of 28 α -syn fibrils solved to date, with a handful of these reaching a resolution below 2.5 Å. Here, we provide a protocol 29 for α -syn protein expression, purification, and fibrilization. We detail how sample quality is assessed by negative 30 stain transmission electron microscopy (NS-TEM) analysis and followed by sample vitrification using the Vitrobot 31 Mark IV vitrification robot. We provide a detailed step by step protocol for high resolution cryo-EM structure 32 determination of α -syn fibrils using RELION and a series of specialized helical reconstruction tools that can be run 33 within RELION. Finally, we detail how ChimeraX, Coot, and Phenix are used to build and refine a molecular model 34 into the high resolution cryo-EM map. This workflow resulted in a 2.04 Å structure of α -syn fibrils with excellent 35 resolution of residues 36 to 97 and an additional island of density for residues 15 to 22 that had not been previously 36 reported. This workflow should serve as a starting point for individuals new to the neurodegeneration and structural 37 biology fields. Together, this procedure lays the foundation for advanced structural studies of α -synuclein and other 38 amyloid fibrils. 39

40 Key Features:

- In vitro fibril amplification method yielding twisting fibrils that span several micrometers in length and are suitable for cryo-EM structure determination.
- High-throughput cryo-EM data collection of neurodegenerative fibrils, such as alpha-synuclein.
- Use of RELION implementations of helical reconstruction algorithms to generate high-resolution 3D structures of a-synuclein fibrils.
- Brief demonstration of the use of ChimeraX, Coot, and Phenix for molecular model building and refinement. 47

48 Keywords: cryo-EM | helical reconstruction | alpha-synuclein | amyloid proteins | neurodegeneration | vitrification



Graphical overview of \alpha-synuclein fibrilization and cryo-EM structure determination. α -synuclein protein expression and purification is followed by a fibrilization protocol yielding twisting filaments that span several micrometers in length and are validated by negative stain transmission electron microscopy (NS-TEM). The sample is then vitrified, followed by cryo-EM data collection. Real-space helical reconstruction is performed in RELION to generate an electron potential map that is used for model building.

56 Introduction and Background.

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58 Amyloid formation within neurons has been well documented to cause neurodegeneration in patients leading to a 59 variety of diseases including Alzheimer's (AH), Parkinson's disease (PD), Lewy Body disease (LB), and multiple 60 system atrophy (MSA) [1-3]. The formation of amyloids is due to protein aggregation resulting in helical. 61 filamentous assemblies with cross β -sheet quaternary structure (Figure 1) [4]. Amyloid filaments interact with 62 different cellular components such as membranes, cytoskeletal factors, and other filaments to form inclusion bodies 63 that disrupt cellular processes and ultimately lead to cell death [2]. These inclusion bodies are prominent in 64 postmortem brains of patients who have suffered from these neurodegenerative diseases, and early investigation of 65 inclusion bodies revealed the presence of filamentous a-synuclein (α -syn) [1,2]. α -syn is a small (14.4 kDa) 66 intrinsically disordered protein whose physiological role remains elusive. α -syn has the capability to bind to the 67 SNARE complex and associate with vesicles at the neuronal axon terminus providing evidence that it may have an 68 impact on neurotransmitter release, vesicle docking and vesicle trafficking [5-8]. However, upon misfolding, α -syn

69 first forms oligomeric aggregates that eventually undergo fibrilization, these fibrils display the highly ordered cross70 β-sheets classically found in amyloids [9,10]. These, in turn, form the extended filaments that cause

neuropathological changes in the brain and are specifically responsible for PD, LB, and MSA. Diseases caused by α -synuclein in this manner are called synucleinopathies [11].

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74 The high-resolution structure presented here of filamentous wild-type α -syn is of a helical filament composed of 2 75 protofilaments and each turn (or rung) of the filament is comprised of 2 copies (one per protofilament) of α -syn 76 facing nearly 180 degrees from each other (Figure 1). Between the monomers that make up each protofilament there 77 is a hydrophobic interface composed of residues 50-57, similar to previously solved structures of filamentous α -syn 78 [12-14]. This interface is stabilized by salt-bridges and pseudo screw symmetry, as previously reported [12,13]. For 79 α -syn, there are 7 different missense familial mutations commonly found in patients who have a higher disposition 80 for synucleinopathies (A30P, E46K, H50Q, G51D, A53E, A53T, and A53V) [15-21]. Interestingly, 6 of these 81 familial mutations lie within the core of the structure and may cause destabilization resulting in a variety of different 82 fibril morphologies. The presence of polymorphism has been demonstrated particularly well through the analysis of 83 *in vitro* α -syn fibrils. Fibril twist, crossover distance, packing arrangement, number of protofilaments, interface, 84 tertiary structure, etc. can vary greatly under different micro- and macro-environments. Many different 85 environmental factors such as pH, salt concentrations, temperature, quiescence, and post translational modifications 86 have an impact on fibril morphology—this has led to documentation of more than 60 in vitro structural polymorphs 87 of α -syn in the PDB [22,23]. These structural differences in the *in vitro* filaments can have direct effects on 88 nucleation rates, seeding propensities, and even cytotoxicity [23]. Unfortunately, the ties between these structurally 89 distinct in vitro polymorphs to those found in sarkosyl-insoluble brain-derived structures remains elusive. However, 90 evidence suggests that different polymorphs may influence pathologies [24-26]. This is demonstrated by the 91 difference in α -syn folds of the filaments extracted from patients diagnosed with MSA versus PD [27]. 92

93 The formation of the filaments responsible for synucleinopathies are propagated in brain tissue by primary 94 nucleation events in which α -syn monomer spontaneously undergoes structural changes resulting in nucleation. This 95 nucleation site can then recruit additional α -syn monomers to bind, thus elongate the fibril [28,29]. However, there 96 can also be secondary nucleation events in which preformed fibrils are introduced into the cellular environment as 97 "seeds" [30]. These seeding events are significantly more potent at fibril formation and elongation. Remarkably, 98 seeds from a particular polymorph have been shown to recruit wild-type α -syn, provide a structural template, and 99 form filaments expressing the polymorph of the seed regardless of whether the endogenous protein recruited is 100 pathogenic or not [31]. A consequence of this prion-like self-replication is that α -syn fibrils may move from cell-to-101 cell spreading cytotoxic polymorphs.

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103 The introduction of polymorphism has a multifactorial effect on clinical treatments of neurodegenerative diseases.

104 Our understanding of the implications associated with each polymorph on disease progression, pathology, and

105 patient outcomes is very limited. In addition, the differences in folding, packing, twists, etc. of each polymorph

106 introduces complexities in binding sites, affinities, and accessibility for a "one size fits all" drug for

107 synucleinopathies; this is further complicated by evidence that not only are there disease specific morphisms, but

108 evidence shows that each synucleinopathy can exhibit patient-to-patient heterogeneity [32]. Thus, to overcome these

109 challenges, explore new therapeutic targets, understand specific polymorph effects on neuropathology, and develop

110 therapies with patient-specific approaches, solving both patient-derived and in vitro amyloid polymorphs should be 111 explored.

112

113 Here, we describe a helical reconstruction workflow that we use to solve the structure of *in vitro* assembled 114 filamentous α -syn to a global resolution of 2.04 Å. We purify α -syn filaments from a reaction in which fibril seeding

115 material is combined with monomeric α -syn. The fibril seeding material provides a template for fibril elongation via

116 monomer addition over a 6-week incubation period at 37 °C with shaking at 200 rpm. The purified α -syn filaments

- 117 are then imaged using negative stain transmission electron microscopy (NS-TEM) to evaluate sample integrity and
- 118 fibril concentration on the grid. The sample is then applied to grids and plunge frozen, and the vitrified grids are
- 119 used for cryo-EM data collection. We provide a detailed protocol utilizing RELION to reconstruct a high-resolution cryo-EM electron potential map that is then used for building an atomic model of the fibril (Figure 1B, 1C, 1E). The
- 120
- 121 steps presented here may be applied to studies of various amyloid fibrils and accelerate cryo-EM structure 122 determination in the fields neurodegenerative research and medicine.
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Figure 1. Structural features of α -syn fibrils from cryo-EM structures. A. Cryo-EM structure of full-length α -126 syn fibril depicting two protofilaments (one in red; one in grey). B. Magnified view of α -syn fibril portraying 127 stacked rungs and filament twist. C. Cross-section of α -syn fibril electron potential map displaying two α -syn 128 monomers that make up each protofilament approximately 180 degrees from each other. D. Electron potential map 129 of individual β -sheet stacks twisting. E. Model depicting secondary structure of stacking β -sheets. F. Example of rise 130 measurement for P21 symmetry (Red) and C2 symmetry (Blue). G. Possible packing symmetry between 131 protofilaments for P2₁ symmetry (out of register) (Red) and C2 symmetry (in register) (Blue).

132 133

134 Materials and Reagents

135

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136 Biological materials

137 1. Plasmid with wild-type α -syn construct in *E. coli* BL21(DE3)/pET28a-AS [33].

139 Reagents

- 140 1. LB broth (Invitrogen, catalog number: 12780029)
- 141 2. Bacto Agar (Dot Scientific Inc., catalog number: DSA20030-1000)
- 142 3. Magnesium sulfate, MgSO₄ (Fisher Scientific, catalog number: 01-337-186)
- 143 4. Calcium chloride, CaCl₂ (Fisher Scientific, catalog number: BP510-500)
- 144 5. Sodium phosphate, NaH₂PO₄ (Fisher Scientific, catalog number: 01-337-702)
- 145 6. Potassium phosphate, KH₂PO₄ (Fisher Scientific, catalog number: 01-337-803)
- 146 7. Sodium chloride, NaCl (Fisher Scientific, catalog number: S271-500)
- 147 8. IPTG (Fisher Scientific, catalog number: BP1755-10)
- **148** 9. Tris-HCl (Fisher Scientific, catalog number: PRH5125)
- 149 10. EDTA (Fisher Scientific, catalog number: AAA1516130)
- 150 11. Kanamycin monosulfate (Thermo Scientific, catalog number: J61272.14)
- 151 12. SDS-PAGE gels (Bio-Rad, catalog number: 4561096)
- 152 13. SDS-PAGE Loading Dye (Bio-Rad, catalog number: 1610737)
- 153 14. Coomassie Brilliant Blue (TCI, catalog number: 6104-59-2)
- 154 15. BME vitamins (Sigma-Aldrich, catalog number: B6891-100mL)
- 155 16. Sodium azide (Sigma-Aldrich, catalog number: 19-993-1)
- 156 17. Studier trace metal mix (Sigma-Aldrich, catalog number: 41106212)
- 157 18. Ammonium Sulfate (Fisher Scientific, catalog number: A702-500)
- 158 19. Deuterium oxide, ²H₂O (Cambridge Isotopes Laboratories, catalog number: DLM-4-1L)
- 159 20. BioExpress Bacterial Cell Media 10X concentrate (U-¹³C, 98%; U-¹⁵N, 98%; U-D 98%) (Cambridge Isotopes Laboratories, catalog number: CGM-1000-CDN)
- 161 21. ¹⁵N-NH₄CI (Cambridge Isotopes Laboratories, catalog number: 39466-62-10)
- 162 22. ²H-¹³C-glucose (Cambridge Isotopes Laboratories, catalog number: CDLM-3813-5)
- 163 23. Sodium deuteroxide, NaO²H (Cambridge Isotopes Laboratories, catalog number: DLM-45-100)
- 164 24. 2% Uranyl Acetate (UA) (EMS, catalog number: 22400-2)

165 166 Solutions

- 167 1. Kanamycin Stock Solution (1000x, 40 mg/ml) (recipe below)
- 168 2. Kanamycin Stock Solution (1000x, 90 mg/ml) (recipe below)
- 169 3. Conditioning Plate (recipe below)
- 170 4. Pre-Growth Media (recipe below)
- 171 5. Wash Buffer (recipe below)
- 172 6. Growth Media (recipe below)
- 173 7. IPTG Stock Solution
- 174 8. Buffer A (recipe below)
- 175 9. Buffer B (recipe below)
- 176 10. TEN Buffer (recipe below)
- 177 11. Saturated Ammonium Sulfate Solution (recipe below)
- 178 12. Fibrilization Buffer (recipe below)
- 179 13. 1% Uranyl Acetate (recipe below)180

181 Recipes

182 1. Kanamycin Stock Solution (1000x, 40 mg/ml)

Reagent	Final Concentration	Amount	
Kanamycin monosulfate	40 mg/ml	0.4 g	
$^{2}\text{H}_{2}\text{O}$	n/a	10 mL	
Total	n/a	10 mL	
1. Completely dissolve kanamycin monosulfate in ² H ₂ O			

- Completely dissolve kanamycin monosulfate in ²H₂O
 Sterilize solution using a 0.22 μm syringe filter (GenClone) and 10 mL syringe (BD)
- 185 3. Aliquot 1000 uL stocks and store at -20°C until use.

Reagent	Final Concentration	Amount
Kanamycin monosulfate	90 mg/ml	0.9 g
² H ₂ O	n/a	10 mL
Total	n/a	10 mL
1. Completely dissolve kanamy	cin monosulfate in ² H ₂ O	
 Sterilize solution using a 0.2 Aliquot 1000 uL stocks and a 	2 μm syringe filter (GenClone) and store at -20°C until use.	l 10 mL syringe (BD)
3. Conditioning Plate		
Reagent	Final Concentration	Amount
$^{2}\text{H}_{2}\text{O}$	70%	700 mL
LB Broth	2%	20 g
Bacto Agar	1.5%	15 g
H ₂ O	n/a	Fill to 1000 mL
Total	n/a	1000 mL
 Combine reagents in a flask Allow the media to cool to ~ Pour ~25 mL of media per P 	and autoclave at 121°C, 15 PSI for 55°C, then add 1000 uL of the kan etri plate (100 mm), repeat for rem	at least 20 minutes. amycin stock solution (1000x, aining 1 L.
4. Pre-Growth Media		
Reagent	Final Concentration	Amount
² H ₂ O	70%	35 mL
LB Broth	2%	1 g
$H_{2}O$	n/a	Fill to 50 mL
1120	1	I III to 50 IIIE
Total	n/a	50 mL
Total 1. Combine reagents in a flask 2. Allow the media to cool to ~	n/a and autoclave at 121°C, 15 PSI for 55°C, then add 50 uL of the kanam	at least 20 minutes. aycin stock solution (1000x, 40
Total 1. Combine reagents in a flask 2. Allow the media to cool to ~ 5. Wash Buffer	n/a and autoclave at 121°C, 15 PSI for 55°C, then add 50 uL of the kanam	<u>50 mL</u> at least 20 minutes. bycin stock solution (1000x, 40
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, 8			L aboratoriog)	
0		2 Sterile filter solution using	1000 mL filtration system (Fisher)	Scientific)
9		2. Sterne mer solution using (root me maaron system (risher)	selentine)
) 7	7.	IPTG Stock Solution		
		Reagent	Final Concentration	Amount
		IPTG	0.5 M	1.2 g
		² H ₂ O	n/a	10 mL
		Total	n/a	10 mL
		1. Completely dissolve IPTG i	n ² H ₂ O	
		2. Sterilize solution using a 0.2	22 µm syringe filter (GenClone) and	10 mL syringe (BE
		3. Aliquot 1000 uL stocks and	store at -20°C until use.	
8	3.	Buffer A		
		Reagent	Final Concentration	Amount
		Tris-HCL	30 mM	4.73 g
		NaCl	30 mM	1.75 g
		H ₂ O	n/a	<u>1L</u>
		Total	n/a	1L
		1. Dissolve Tris-HCL and NaCl	in water while stirring.	
		2. pH buffer to 7.4 at 37°C using	g IM NaOH.	
ç	9.	Buffer B	E'm 1 Commentention	A
		Reagent	Final Concentration	Amount
		Iris-HCL	30 mM	2.36 g
		NaCl	1 M	29.22 g
		H2U Tetel		500 mL
		1 Dissolve Tris UCL and NaCl	n/a	300 mL
		2. pH buffer to 7.4 at 27°C using	In water while surring.	
		2. pri ourier to 7.4 at 57 C using	, 11vi 1vaO11.	
1	10	TEN Buffer		
	10.	Reagent	Final Concentration	Amount
		Tris-HCL	30 mM	4 73 σ
		NaCl	30 mM	1.75 g
		EDTA	0.1 mM	29.22 mg
		H ₂ O	n/a	1 L
		Total	n/a	11
		1. Dissolve Tris-HCL, NaCL a	nd EDTA in water while stirring	12
		2. pH buffer to 8.0 at 37°C usi	ng 1M NaOH.	
		1	2	
1	11.	Saturated Ammonium Sulfate So	olution	
-		Reagent	Final Concentration	Amount
		Ammonium Sulfate	saturated	~550 g
		H ₂ O	n/a	1 L
		Total	n/a	1L
		1. Add ammonium sulfate into w	vater while stirring.	
		2. Heat gently until all ammoniu	m sulfate is dissolved.	
		3. Cool to room temperature. Cr	ystals should form to indicate the so	lution is saturated.
		T	,	
1	12.	Fibrilization Buffer		
		Reagent	Final Concentration	Amount
		NaH ₂ PO ₄	50 mM	1.2 g

0.02%

Sodium Azide

40 ug

H ₂ O	20%	40 mL
$^{2}\text{H}_{2}\text{O}$	80%	160 mL
Total	n/a	200 mL

1. Add NaH₂PO₄, EDTA, 0.02% sodium azide solution into ²H₂O and H₂O.

2. pH to 7.4 at 37°C using 1M NaO²H.

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236 13. 1% Uranyl Acetate (UA)237

Reagent	Final Concentration	Amount
2 % Uranyl Acetate	1% (v/v)	250 μL
H ₂ O	n/a	250 µL
Total	n/a	500 μL

1. Mix 1-part sterile water with 1-part 2% UA stain (EMS) and filter through a Spin-X centrifuge tube with a 0.22 μm filter (Costar).

241 <u>Laboratory Supplies</u> 242

- 243 1. 10 ml Syringe (BD, catalog number: 309604)
- 244 2. 0.22 um filter (GenClone, catalog number: 25-240)
- 245 3. 100 mm × 15 mm Petri dishes (Fisher Scientific, catalog number: S33580A)
- 4. 1000 mL filtration system (Fisher Scientific, catalog number: FB12566506)
- 247 5. 50 mL conical tubes (VWR, 525-1074)
- 248 6. 0.45 μm syringe filter (GenClone, catalog number: 25-246)
- 249 7. 1.7 mL centrifuge tubes (Denville, catalog number: C2170)
- 250 8. Parafilm (Bemis, catalog number: PM996)
- 251 9. 0.22 μm Spin-X centrifuge tube filter (Costar, catalog number: 8160)
- 252 10. 200 mesh carbon film, copper grids (EMS, catalog number: CF200-CU)
- 253 11. Whatman #1 filter paper (Whatman, catalog number: 1001-090)
- 254 12. Quantifoil R2/1 200 mesh, copper grids (Quantifoil Micro Tools GmbH, catalog number: Q210CR1)
- 255 13. Standard Vitrobot Filter Paper, Ø55/20mm, Grade 595 (Ted Pella, catalog number: 47000-100)
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257 <u>Equipment</u> 258

- 259 1. HiTrap Q HP anion exchange column (Cytiva, catalog number: 17115401)
- 260 2. Stirred cell concentrator (Amicon, catalog number: UFSC05001)
- 261 3. HiPrep 16/60 Sephacryl S100- HR gel filtration column (Cytiva, catalog number: 17119501)
- 262 4. 5424 R Microcentrifuge (Eppendorf, catalog number: 05-400-005)
- **263** 5. Grid holder block (Pelco, catalog number: 16820-25)
- 264 6. Plasma Cleaner (Harrick Plasma Inc., catalog number: PDC-32G)
- 265 7. Static dissipator (Mettler Toledo, catalog number: UX-11337-99)
- 266 8. Style N5 reverse pressure tweezers (Dumont, catalog number: 0202-N5-PS-1)
- 267 9. Talos L120C 120 kV transmission electron microscope (TEM) (Thermo Fisher Scientific), or equivalent
- 268 10. Cryo grid box (Sub-Angstrom, catalog number: SB)
- 269 11. Plasma Cleaner (Harrick Plasma Inc., catalog number: PDC-32G)
- 270 12. Vitrobot Mark IV vitrification robot (Thermo Fisher Scientific)
- 271 13. Titan Krios G3i 300 kV transmission electron microscope (TEM) (Thermo Fisher Scientific)
- 272 14. K3-GIF direct electron detector with energy filter (Gatan Inc., AMETEK)
- 15. High-performance computing (HPC) cluster with an EPYC Milan 7713P 64-core 2.0GHz CPU (AMD), 512 GB
 RAM, 4x RTX A5000 24GB GDDR6 GPU (NVIDIA), 2x 960GB Enterprise SSD, mirrored OS, 2x 7.68TB
 nVME SSD as 15TB scratch space, dual-port 25GbE Ethernet.
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276 <u>Software</u>

- 277 1. SBGrid (<u>https://sbgrid.org/</u>)
- 278 2. IMOD (<u>https://bio3d.colorado.edu/imod/</u>)

- 279 3. RELION (<u>https://relion.readthedocs.io/en/release-4.0/</u>)
- 280 4. MotionCor2 (https://emcore.ucsf.edu/ucsf-software)
- 281 5. Gctf (<u>https://sbgrid.org/software/titles/gctf</u>)
- 282 6. Topaz-filament (<u>https://github.com/3dem/topaz</u>)
- 283 7. UCSF ChimeraX (https://www.cgl.ucsf.edu/chimerax/)
- 284 8. Coot (<u>https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/</u>)
- 285 9. PHENIX (<u>https://phenix-online.org/</u>)

286 <u>Procedure and Results.</u>

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288 A. α-synuclein sample preparation

289 Expression and purification of α -syn protein is performed as reported previously [33]. The protein preparations and

fibrilization protocol presented here were developed for joint cryo-EM and NMR studies. Preparations include the use of isotopically labeled reagents that are critical for NMR experiments but are not necessary for cryo-EM. Thus,

the α -syn sample preparation protocol may be adapted for cryo-EM only studies by substituting isotopically labeled reagents with a standard equivalent reagent.

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295 α -synuclein protein expression

- **296** 1. Expression of wild-type α -syn is performed in *E. coli* BL21(DE3)/pET28a-AS.
- 297 2. Plate transformed cells onto conditioning plate, overnight at 37 °C.
- Inoculate a 50 mL pre-growth flask with a single colony from the overnight conditioning plate and incubate overnight at 220 rpm at 37 °C until OD₆₀₀ = ~3.
- Transfer cells into a 50 mL conical tube (VWR) using aseptic techniques. Centrifuge tubes at 5000 rpm for 5 minutes at 4 °C. Decant supernatant and wash with ~20 mL of cold wash buffer.
- 302 5. Resuspended cells with the growth media in 4x 1L baffled flasks, 250 mL each. At an OD₆₀₀ of ~1-1.2, induce
 303 α-syn over-expression with the addition of 1 mL of an IPTG stock solution. Incubate at 25 °C with shaking at 200 rpm.
- After overnight growth, collect cells and combine for harvesting (~15 hours post-induction). Centrifuge at 5000 rpm for 10 minutes at 4 °C. Decant the supernatant and wash the cell pellet with the wash buffer to remove residual growth media components.
- 308 7. Cell pellets may then be frozen and stored at -80 °C until use.309

310 α -synuclein protein purification

- Cells may be lysed via heat denaturation, as α-syn is thermostable and will be unaffected. Place 50 mL conical tubes (VWR) containing cell paste in boiling water (98°C) for 30 minutes. Cool cell lysate on ice. Clear the cell lysate by centrifugation at 5000 rpm for 10 minutes at 4 °C.
- 314 2. α -syn should then be precipitated via addition of a saturated ammonium sulfate solution on ice. Collect α -syn precipitate via centrifugation at 16900 rpm for 45 minutes at 4 °C and decanting the supernatant.
- 316 3. Equilibrate the HiTrap Q HP anion exchange column (Cytiva) with Buffer A.
- Resolubilize the α-syn precipitate with ~5 ml Buffer A. Make sure to filter the resolubilized α-syn using a 0.45 μm syringe filter (GenClone). Inject the resolubilized α-syn to bind to the QFF anion exchange resin (GE Healthcare Life Sciences, Marlborough, MA). Elute using a linear gradient of 0.03–0.6 M NaCl by increasing the proportion of Buffer B flow through the column. Collect fraction as they come off the column. In our hands, fractions containing α-syn monomer usually elute at about 0.3 M NaCl.
- 322 5. After completion, Run SDS-PAGE to check to determine which fractions (gel bands) contain α-syn. Take 20 µL
 323 samples from each fraction tube from 20% Buffer B to 40% Buffer B. Add 20 µL 2x SDS loading dye (Bio324 Rad) to each sample tube and heat at 90°C for 5 minutes. Run all samples on an SDS-PAGE gel (Bio-Rad). Use
 325 Coomassie Brilliant Blue stain (Sigma-Aldrich) to stain the gel. Examine the stained gel for α-syn over326 expression bands. Note that α-syn tends to run at an apparent size of 18 kDa. Pool these fractions.
- 3276. Concentrate the α-syn monomer solution using a stirred cell concentrator (Amicon) using a 3.5 kDa molecular328weight cut off filter to a final concentration of ~15 mg/mL. Prewet the concentrator with Buffer A before adding329α-syn solution to prevent loss of sample to the filter.
- For the 16/60 Sephacryl S-200 HR gel filtration column (GE Healthcare Life Sciences) with TEN Buffer, 5x column volume.
- 8. Inject 1 mL of the concentrated α-syn pool into the loop path of the 16/60 Sephacryl S-100 HR gel filtration
 column (Cytiva) and run the protocol at 0.5 mL/min until the fraction with an apparent mass of 15 kDa, at ~97 minutes.
- 9. Pool fractions, concentrate to ~15 mg/ml α-syn using a clean stirred cell concentrator (Amicon) and a 3.5 kDa
 molecular weight cut off filter. Prewet the unit and filter with TEN buffer before adding α-syn solution to
 prevent loss of sample to the filter.
- 338 10. Purified α -syn may then be frozen and stored in -80 °C freezer until use. 339

340 α -synuclein fibrilization

- Buffer exchange from the TEN buffer to fibrilization buffer. Add purified α-syn from above to a prewetted (with the fibrilization buffer) stirred cell concentrator (Amicon) and a 3.5 kDa molecular weight cut off filter. Dilute 10x with fibrilization buffer and concentrate down to the initial volume. Repeat 3 times to effectively remove TEN buffer and completely exchange to fibrilization buffer.
- Purified α-syn protein in above buffer was concentrated to 15 mg/ml using 3.5 kDa cut off stir cell concentrators and 0.5 ml aliquoted into clean, sterile 1.7 ml Eppendorf tubes (Denville).
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 3. Fibril formation may be seeded with ~50 ng of previously made mature α-syn fibril (in this case: sample used to determine the PDB ID: 2N0A fibril structure).
- 349 4. Seal the tubes with parafilm (Bermis) for the duration of the incubation.
 - 5. Incubate at 37 °C and shake at 250 rpm continuously for 3 weeks. The viscosity of the fibril solution will greatly increase over time.
 - 6. At the end of 3 weeks, add 100 μ L of fibrilization buffer and continue the incubation for 3 weeks under the same conditions.
 - 7. After a total of 6 weeks the fibrils at a protein concentration of \sim 13 mg/ml are ready for TEM analysis.

356 B. Negative stain

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357 Fibrilization can be characterized by thioflavin-T (ThT) assays, which leverage the fluorescence signal observed 358 when thioflavin-T binds to fibrils, a property not observed in the presence of purified protein monomers [34]. 359 Although this method is powerful and can even detail fibrilization kinetics, there are limitations in the 360 technique. Specifically, this assay can not specify whether fibrils are twisting, if the fibrils span several 361 micrometers in length, or are small fragments 10s of nanometers in length. For high-resolution cryo-EM 362 structure determination, fibrils should be both twisting and span several crossovers. Additionally, fibrils should 363 be concentrated to a point where several fibrils span the micrograph but are not crowded or overlapping. This 364 ensures there are enough individual particles for the reconstruction process. To determine if the fibrils possess 365 these qualities, we perform negative stain transmission electron microscopy (NS-TEM) with the following 366 procedure to test a range of sample concentrations. We found that a concentration of 6.5 mg/ml (i.e., 1:1 ratio of 367 sample to buffer) was best for our sample on the grid.

- Place the desired number of 200 mesh carbon film, copper EM grids (EMS) on a grid holder block (Pelco) and using a plasma cleaner PDC-32G (Harrick Plasma Inc.), or equivalent system, to glow discharge grids under a 100-micron vacuum for 30 seconds on low (Figure 2, step 1).
 Cut a piece of parafilm (Bemis) to about 2"x4" and demagnetize with a static dissipater (Mettler Toledo)
 - 2. Cut a piece of parafilm (Bemis) to about 2"x4" and demagnetize with a static dissipater (Mettler Toledo) (Figure 2, step 2).
 - 3. Retrieve one glow discharged EM grid using style N5 reverse pressure tweezers (Dumont), or similar tweezers (Figure 2, step 3).
 - 4. Spot two 50 μL drops of sterile, Nanopure water and two 50 μL drops of 1% UA on to the piece of parafilm (Bemis), ensure the drops do not touch (Figure 2, step 4).
 - 5. Apply 4 μL of the sample to the EM grid and allow the sample to incubate at room temperature for one minute (Figure 2, step 5).
 - 6. Blot away the liquid by touching the edge of the EM grid to a piece of filter paper (Whatman) (Figure 2, step 6).
 - 7. Wash the EM grid by touching the face of the EM grid to the 1st drop of water, then blot away the liquid as in step 6. Repeat, but this time wash with the 2nd drop of water (Figure 2, step 7).
 - 8. Pre-stain the EM grid by touching the face of the EM grid to the 1st drop of 1% UA, then blot away the stain as in step 6 (Figure 2, step 8).
 - 9. Stain the grid by holding the face of the EM grid to the 2nd drop of 1% UA for 15 seconds, then blot away the stain as in step 6 (Figure 2, step 9).
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 10. Allow the EM grid to dry for at least 5 minutes at room temperature before storing the grid in a grid box (Figure 2, step 10). Store the grid box in a desiccator or humidity-controlled room until imaging.
- 11. Repeat for additional sample dilutions to assess the sample conditions that may be best suited for cryo-EM analysis. We imaged the sample at a concentration of 13 mg/ml (undiluted), 6.5 mg/ml (2x dilution), and 2.6 mg/ml (5x dilution) and found that a concentration of 6.5 mg/ml showed the best sample distribution on the grid (Figure 3).
- Note: Since fibrilization conditions greatly impact the length of the fibrils, and thus sample distribution on
 the grid, it is important to test each sample by NS-TEM before sample vitrification and cryo-EM data
 collection.

396 12. Image grids on a Talos L120C 120 kV TEM, or equivalent microscope, at a pixel size of 1.58 Å and a total electron dose of 25 e⁻/Å².

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400 Figure 2. Negative stain protocol. Detailed steps for preparing negative stain grids of α-syn fibrils. The protocol 401 yields lightly stained fibrils allowing for the visualization twisting fibrils comprised of two protofilaments (Figure

402 3). The procedure is repeated spanning a range of fibril concentrations that are imaged by transmission electron

403 microscopy.



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Figure 3. Negative stain TEM analysis of α -synuclein fibrils. Representative micrograph of fibrils lightly stained with 1% UA. The fibrils are comprised of two protofilaments (arrows) and appear to be twisting with distinct crossover points (stars). Scale bar, 100 nm.

C. Sample vitrification

Basic sample vitrification for single particle analysis has become routine in the cryo-EM field. Here, we present a brief workflow of the vitrification process using the Vitrobot Mark IV (Thermo Fisher Scientific) with blotting conditions that yield grids suitable for cryo-EM data collection.

- 1. Using a plastic syringe (BD), add 60 mL of distilled water to the Vitrobot Mark IV water reservoir.
- 2. Turn on the Vitrobot Mark IV, set the chamber temperature to 20°C and the relative humidity to 95%.
- 3. Attach standard Vitrobot filter paper (Ted Pella) to the blotting pads and allow the system to equilibrate while to the conditions set in step 2 (~15 minutes).
- 4. Using a plasma cleaner (Harrick Plasma Inc.), or equivalent system, glow discharge R2/1 200 mesh, copper grids (Quantifoil).
- 5. Use liquid nitrogen (LN_2) to cool the Vitrobot foam dewar, ethane cup, and metal spider.
- 6. Once the setup has cooled, condense the ethane in the ethane cup. Be sure to monitor ethane and LN₂ levels throughout the vitrification process.
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 7. On the Vitrobot, set the wait time to 60 seconds and set the drain time to 0.5 seconds. For blot force and blot time it is usually necessary to test a range of parameters that work best. For these fibrils a blot time between 4 and 5 seconds, and a blot force of -1 to +2 worked well.
- 426 8. Using the Vitrobot tweezers, pick up a grid and attached the tweezers to the Vitrobot. Select *"continue"* on the screen to raise the tweezers and mount the foam dewar in place. Follow the prompts on the screen to bring the tweezers and dewar into position for sample application.
 - 9. Apply 4 μ L of the fibrils to the carbon side of the grid. Select *"continue"* to begin the wait time, then the system will automatically blot and plunge the sample into liquid ethane.
 - 10. Once the system has plunged the specimen into the cryogen, transfer the vitrified grid to a labeled grid box and store appropriately.
- 433 11. Repeat steps 7 to 9 for any additional grids. In addition to duplicate grids, it is always beneficial to test a range of blotting conditions and/or sample concentrations. Cryo-EM data was collected on a grid with a blot time of 4 seconds and a blot force of +2 at a protein concentration of ~6.5 mg/ml.

436 437 D. Cryo-EM data collection

438 Data collection parameters should be tailored to the resources available and thus, users should work closely 439 with EM facility staff to optimize the data collection parameters for their individual sample. Here, the data was 440 acquired on a Titan Krios G3i FEG-TEM (Thermo Fisher Scientific). The microscope is operated at 300 kV and 441 is equipped with a Gatan K3 direct electron detector (Gatan) and a BioQuantum energy filter set at 20eV 442 (Gatan). Correlated-double sampling (CDS) was used to collect dose fractionated micrographs using a defocus 443 range of -0.5 to -2.5 µm with increments of 0.25 µm. Micrographs were collected at a magnification of 444 105,000X with a pixel size of 0.834 Å and a total dose of 40 e⁻/Å² (1 e⁻/Å²/frame). On average ~250 movies 445 were collected per hour using EPU/AFIS (Thermo Fisher Scientific) acquiring 3 shots per hole and multiple 446 holes per stage movement. A representative micrograph at an estimated defocus of -2.0 µm shows twisting 447 fibrils suspended in vitreous ice (Figure 4). 448



Figure 4. Representative cryo-EM micrographs of \alpha-synuclein fibrils. Motion corrected micrograph of vitrified α -synuclein fibrils at an estimated defocus of -2.0 µm. The fibrils are comprised of two protofilaments (arrows) that are twisting at distinct crossover points (stars). Twisting fibrils are critical for high-resolution structure determination. Scale bar, 100 nm.

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E. Cryo-EM data processing of alpha-synuclein fibrils.

Cryo-EM structure determination of amyloid fibrils has revolutionized the fields of neuroscience and 457 neurodegenerative medicine, providing key structural details that were previously unattainable by other 458 methods. Here, we provide a data processing protocol, that is both detailed and reproducible, to serve as a 459 starting point for those new to cryo-EM and helical reconstruction workflows. The raw micrographs, gain file, 460 and the detector mtf file can be accessed at EMPIAR-12229, allowing users to work through the steps below 461 before applying the workflow to new experimental data. We must note that all data sets are unique and possess 462 their own challenges, but this workflow should greatly improve the user's ability to resolve amyloid fibril 463 structures. Finally, as with any software, it is best to first become accustomed to the program by completing the 464 appropriate tutorial datasets. We highly encourage readers to first complete the RELION single particle tutorial 465 (https://relion.readthedocs.io/en/release-4.0/SPA tutorial/index.html) before proceeding with the steps below 466 [35].

Creating a RELION Project

468 Create a directory that will house the entire RELION project. For simplicity call this directory *a* 469 *syn_data_processing*. Within this directory you should have two files titled *gain.mrc* and *k3-CDS-300keV*-

470	<i>mtf.star</i> , and a subdirectory titled <i>Micrographs</i> that contains all the raw movie frames in tiff format. These
471	files can be downloaded from EMPIAR-12229. Now that your directories are organized <i>cd</i> to the <i>a</i> -
472	syn data processing directory, this will serve as the RELION parent directory for all subsequent jobs.
473	Launch RELION by running <i>relion</i> & in the terminal. The "&" will allow RELION to run in the
474	background in case the terminal is needed for additional commands. As a final note, we have listed the
475	input files for each job based on our RELION project so there will be discrepancies in job numbers
476	between our project and yours. Thus, it is important to use the proper input path file for your project at each
477	step. For each step we have detailed where the input file comes from (i.e. the step the file was generated in)
478	to ensure successful reconstruction of the EMPIAR-12229 dataset.
479	Allocating computational resource when running RELION jobs.
480	RELION uses a <i>Compute</i> and <i>Running</i> tab to allocate computational resources based on user defined
481	parameters. These parameters are completely dependent on the resources available to each individual. Thus,
482	rather than detailing these parameters for each step we have outlined the <i>Compute</i> and <i>Running</i> parameters
483	that work well for our HPC cluster with slurm queueing system here. However, these parameters may not
484	work for your computational setup, and you may need to seek the advice of IT professionals at your
485	institute.
486	Compute:
487	Use parallel disk I/O? Yes
488	Number of pooled particles: 30
489	Skip padding? No
490	Pre-read all particles into RAM? No
491	Copy particles to scratch directory: Leave Blank
492	Combine iterations through disc? No
493	Use GPU acceleration? Yes
494	Which GPUs to use: Leave Blank
495	Running (GPU jobs):
496	Number of MPI procs: 5
497	Number of threads: 6
498	Submit to queue? Yes
499	Queue name: a5000
500	Queue submit command: sbatch
501	Standard submission script:////share/sbatch/relion_template_gpu.sh
502	Minimum dedicated cores per node: 1
503	Additional arguments: Leave Blank
504	Running (CPU jobs):
505	Number of MPI procs: 20
506	Submit to queue? Yes
507	Queue name: cpu
508	Queue submit command: sbatch
509	Standard submission script:////share/sbatch/relion_template_cpu.sh
510	Minimum dedicated cores per node: 1
511	Additional arguments: Leave Blank
512	In addition to the protocol below, a workflow diagram of the RELION GUI with parameters for each step
513	are provided (Figure 5).
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Figure 5. Helical reconstruction workflow for α -synuclein fibrils using RELION. Overview of each RELION job utilized to reconstruct α -synuclein fibrils to ~2.0 Å. Each job corresponds to the step number in section E.

540		-
519	1.	Import:
520		First, import the raw movie frames into RELION for data processing. Select the Import job, ensure <i>Raw</i>
521		input files is set to the location of the movie frames use the "*" argument to select all the tiff files in the
521		<i>input files</i> is set to the location of the movie names, use the argument to select an the fill these in the
522		directory, set the additional parameters below, and click the <i>Run</i> ! button.
523		Movies/mics:
524		Import raw movies/micrographs: Yes
525		Party in the test man of the test of t
525		Kuw input files. Micrographs/ .ujj
526		Optics group name: opticsGroup1
527		MTF of the detector: k3-CDS-300keV-mtf.star
528		Pixel size (Angstrom): 0.834
529		Voltaga (VV) 300
525		$\frac{1}{2}$
530		Spherical aberration (mm): 2.7
531		Amplitude contrast: 0.1
532		Beamtilt in X (mrad): 0
533		Reamtilt in Y (mrad): 0
E24		
554		<u>Utilers</u>
535		Import other node types? No
536		The output log will display 5,193 micrographs were imported.
537	2.	Motion Correction:
538		The raw movie frames from the previous ich (movies star) must now be aligned. The data was collected
220		The taw movie names nom the previous job (<i>movies.sun</i>) must now be aligned. The data was concreted
539		with a dose per frame of 1 e ⁷ A ² over 40 frames for a total dose of 40 e ⁷ A ² . Note, that the <i>EER</i>
540		<i>fractionation</i> parameter will be ignored by RELION since these images were collected on a Gatan K3
541		detector and are tiff files. Perform motion correction by using the MotionCor2 program [36]. Tell RELION
542		where the program is located via the MOTIONCOR? executable parameter Your computational setup will
542		where the program is located via the <i>MOTONOVOX exclusive</i> parameter. Four computational setup with
545		be different and MotionCor2 may be saved in a different location, so the executable path may be different.
544		In the terminal, run <i>which motioncor2</i> to determine the correct path for the program. Similarly, your
545		computational setup will dictate the number of GPUs available. Our setup includes multiple nodes that can
546		each run 4 GPUs concurrently. In the RELION GUI, use Which GPUs to use to indicate the GPUs available
5/7		for your setup leaving this block will automatically allocate the GPUIs Salast the Motion correction ich
547		for your setup, reaving this blank with automatically anocate the Gross Select the Wotton correction job,
548		set <i>Input movies SIAR file</i> to the <i>movie.star</i> file from step 1, set the following parameters and update any
549		paths or parameters that are specific to your computational setup, then click the <i>Run!</i> button.
550		I/O:
551		Input movies STAR file: Import/iob001/movies star
551		Final formes of new former in the second sec
552		First frame for corrected sum: 1
553		Last frame for corrected sum: -1
554		Dose per frame $(e'/Å^2)$: 1
555		Pre-exposure $(e^{-}/A^{2}) \cdot 0$
556		EED function (c) (c)
550		EEK fractionation. 52
557		Write output in float 16? Yes
558		Do dose-weighting? Yes
559		Save non-dose weighted as well? No
560		Save sum of nower spectra? Yes
500		Survey sources and $(2^{1/2})$ is the second secon
201		Sum power specira every (e/A ²): 4
562		Motion:
563		Bfactor: 150
564		Number of patches X. Y. 5. 5
565		Group frames 1
505		Group rumes. 1
500		Dinning Jacior. 1
567		Gain-reference image: gain.mrc
568		Gain rotation: 180 degrees (2)
569		Gain flip: Flip left to right (2)
570		Defect file: Leave blank
570		Las DELION's sum implementation? No
571		Use RELION'S OWN IMPLEMENTATION? INO
572		MOTIONCOR2 executable: /programs/x86_64-linux/motioncor2/1.3.1/motioncor2
573		Which GPUs to use: 0,1,2,3
574		Other MOTIONCOR2 arguments: Leave blank

575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 592 593 594 595 596 597 598 599 600	3.	This job will take several hours to run and will generate a <i>corrected_micrographs.star</i> file. If interested, you may open the <i>logfile.pdf</i> to visualize the results from the job. Under the <i>Finished Jobs</i> list click on the Motion correction job. This will update the <i>Current:</i> job display (located in the center of the GUI) to your Motion correction job and upload the results to the user interface. On the right side of the RELION GUI there is a drop-down menu called <i>Display:</i> that allows for the user to visualize outputs from the finished job. Click on the drop-down menu and select <i>Out: logfile.pdf</i> . A new window will appear with the results of the job. Subsequent <i>logfile.pdf</i> files from finished jobs can be opened this way. CTF Estimation: Now estimate the CTF values for the motion corrected micrographs from the previous step, these are stored in the <i>corrected_micrographs.star</i> file. Use Gctf to estimate CTF values [37]. In the terminal, run <i>which Gctf</i> to determine the correct executable path for your setup. In the RELION GUI, select the CTF estimation job, set <i>Input micrographs STAR file</i> to the <i>corrected_micrographs.star</i> file from step 2, set the following parameters and update any paths specific to your setup, then click the <i>Run!</i> button. <u>I/O:</u> <i>Input micrographs STAR file: MotionCorr/job002/corrected_micrographs.star</i> <i>Use micrograph without dose-weighting? No</i> <i>Estimate phase shifts? No</i> <i>Amount of astigmatism (Å): 100</i> <u>CTFFIND-4.1?</u> <i>No</i> <i>FFT box size (pix): 512</i> <i>Minimum resolution (Å): 30</i> <i>Maximum resolution (Å): 5000</i> <i>Maximum defocus value (Å): 5000</i> <i>Maximum defocus value (Å): 5000</i> <i>Maximum defocus value (Å): 5000</i> <i>Maximum defocus value (Å): 5000</i> <i>Maximus defocus value (Å): 5000</i>
600 601		Defocus step size (A): 500 Getf:
602		Use Getf instead? Yes
604		Gctf executable: /programs/x86_64-linux/gctf/1.06/bin/Gctf
0U4 605		Ignore searches parameters? Yes
605		Perform equi-phase averaging? Tes Other Cettorne: Lagua blank
607		Which GPUs to use: 0.1.2.3
608		The job results in a micrographs, ctf star file and a logfile ndf file. The logfile ndf contains a graphical
609		representation of the metadata related to micrograph defocus, astigmatism, max resolution, and figure of
610		merit values. These values will be used in the upcoming steps to filter the micrograph dataset.
611	4.	Subset Selection (Defocus Filter):
612		Extensive testing has shown that using stringent parameters during the micrograph curation steps allow for
613		a segment picking neural network that performs better than one trained on the entire data set. The following
614		steps will use CTF estimation results to curate a set of micrographs for manual picking. Those picks will
615		then be used to train the Topaz neural network [38]. Finally, a modified version of Topaz called Topaz- filement, that allows for nicking filementous structures, is ontimized on a small subset of micrographs
617		before applying the neural network to our entire dataset [38]
618		Open the <i>logfile.ndf</i> from the CTF estimation iob and use the values provided in this file to eliminate any
619		outliers or suboptimal micrographs. Filter the dataset based on defocus, astigmatism, max resolution, and
620		figure of merit values using a series of Subset Selection jobs. Select the Subset Selection job, input the
621		following parameters and update OR select from micrograph.star to the micrographs_ctf.star file from step
622		3, then click the <i>Run!</i> button.
623		
625		Select classes from job: Leave blank OR select from micrograph star: CtfFind/iob003/micrographs, ctf star
626		OR select from particles.star: Leave blank
627		<u>Class options:</u>
628		Automatically select 2D classes? No
629		Re-center the class averages? No
630		Regroup the particles? No

631		Subsets:
632		Select based on metadata value? Yes
633		Matadata labal for subsat salaction: vlnDafocusU
000		
634		Minimum metadata value: -9999
635		Maximum metadata value: 25000
636		OR: select on image statistics? No
637		OR split into subsets? No
638		Dunlicates
620		OB the line of March 1 and 1 a
039		OK: remove auplicates? No
640		This job reduces the number of micrographs from 5,193 to 4,858 based on a maximum defocus value of 2.5
641		μm (25,000 Å).
642	5.	Subset Selection (Astigmatism Filter):
643		Filter the micrograph subset from step A by the astigmatism values in the CTF estimation logfile ndf file
644		The the metograph subset from step + by the astignation values in the C T estimation <i>togite.pu</i> inc.
044		Select the Subset Selection job type, set OK select from micrograph.star to the micrographs.star file from
645		step 4, input the following parameters, then click the <i>Run!</i> button.
646		<u>I/O:</u>
647		Select classes from job: Leave blank
648		OR select from micrograph star: Select/iob004/micrographs star
640		OR select from mentioles start. Leave blank
049		OK seleci from particles.star. Leave blank
650		Class options:
651		Automatically select 2D classes? No
652		Re-center the class averages? No
653		Regroup the particles? No
654		
655		Select based on metadata value? Vos
055		Select based on metadala value? Tes
656		Metadata label for subset selection: rlnCtfAstigmatism
657		Minimum metadata value: -9999
658		Maximum metadata value: 700
659		QR: select on image statistics? No
660		OP: split into subsets? No
661		OK. Spit into Subsets? No
001		Duplicates:
662		OR: remove duplicates? No
663		This job reduces the number of micrographs from 4,858 to 3,390 micrographs.
664	6.	Subset Selection (Max Resolution Filter)
665		Further filter the micrograph subset from step 5 by the max resolution values from the CTF estimation
666		lagella adf file Salest the Subset Salestion ich set OP salest from micrograph star to the micrograph star
000		logine.paj me. Select me Subset Selection job, sel OK select from merograph.star to the micrograph.star
667		file from step 5, set the following parameters, and click the <i>Run</i> ! button.
668		<u>I/O:</u>
669		Select classes from job: Leave blank
670		OR select from micrograph.star: Select/iob020/micrographs.star
671		OR select from particles star: Leave blank
672		Chase entitions
672		
6/3		Automatically select 2D classes? No
674		Re-center the class averages? No
675		Regroup the particles? No
676		Subsets:
677		Select based on metadata value? Yes
679		Mote data labol for white a decision where the management of
078		Metadala label for subset selection. FinCifMaxResolution
679		Minimum metadata value: -9999
680		Maximum metadata value: 4
681		OR: select on image statistics? No
682		OR: split into subsets? No
683		Dunlicates:
601		OD, remains durbliggtor? No
004		UK. remove auplicates? No
685		This job reduces the number of micrographs from 3,390 to 910 micrographs.
686	7.	Subset Selection (Figure of Merit Filter):

~~-		
687		Lastly, filter the micrograph subset from step 6 by the figure of merit values from the CTF estimation
688		logfile adffile Select the Subset Selection ich set OP select from micrograph star to the micrograph star
000		toghte.paj me. Select me subset selection job, set OK select from micrograph.star to the micrograph.star
689		file from step 6, set the following parameters, then click the <i>Run!</i> button.
600		
090		
691		Select classes from job: Leave blank
602		
692		OR select from micrograph.star: Select/job021/micrographs.star
693		OR select from particles star: Leave blank
604		
094		Class options:
695		Automatically select 2D classes? No
606		
696		Re-center the class averages? No
697		Regroup the particles? No
600		
090		<u>Subsets:</u>
699		Select based on metadata value? Yes
700		
/00		Metadata label for subset selection: rinCifFigureOfMerit
701		Minimum metadata value: 0.065
702		
702		Maximum metadata value: 0.9
703		OR: select on image statistics? No
704		
704		OR: split into subsets? No
705		Duplicates:
700		
706		OK: remove duplicates? No
707		This job reduces the number of micrographs from 910 to 774 micrographs
700	0	
708	8.	Subset Selection (2 Sets of 20 Micrograph)
709		From the remaining 774 micrographs generate 2 sets of 20 micrographs. The first set of micrographs will
740		To the termining // three graphs generate 2 sets of 20 three organs. The first set of the organs with
/10		be used for manual picking and training the neural network. The 2^{nd} set of 20 micrographs will be used to
711		test and optimize the picking thresholds that will then be applied to the entire dataset. Select the Subset
7 4 2		est and optimize the preving an esholds that will then be appred to the entitle dataset. Select the Subset
/12		Selection job, set OR select from micrograph.star to the micrographs.star file from step 7, then click the
713		Run! button
713		
/14		<u>I/O:</u>
715		Select classes from job: Leave blank
715		
/16		OR select from micrograph.star: Select/job022/micrographs.star
717		OR select from particles star. Leave blank
717		on select from purificies.star. Leave blank
/18		Class options:
719		Automatically select 2D classes? No
715		Automatically select 2D classes: 100
/20		Re-center the class averages? No
721		Pearoun the narticles? No
/21		Regroup the particles? No
722		Subsets:
722		Salast hased on matadata value? No
725		Select bused on metadulia value: No
724		OR: select on image statistics? No
725		OP: split into subsets? Yes
725		OK. spin into subsets? Tes
726		Randomise order before making subset? No
727		Subset size: 20
727		Subset size. 20
728		OR: number of subsets: 2
720		Duplicates
725		Dupicates.
/30		OR: remove duplicates? No
731		This step results in two STAR files labeled micrographs split star and micrographs split? star Each star
731		This step results in two STAR files labeled metographs_spirit.star and metographs_spirit.star
/32		tile contains 20 micrographs.
733	0	Manual Picking
733).	
/34		Select the Manual picking job, set <i>Input micrographs</i> to the <i>micrographs split1.star</i> file from step 8, set
735		the additional parameters below then click the $Run/$ button A new window will appear with 20 rows (1 per
700		the additional parameters below, then enex the <i>Nan</i> : button. A new window with appear with 2010ws (1 per
/36		micrograph) with the micrograph name, a <i>pick</i> button, the number of picks, a <i>CTF</i> button, and the defocus
737		estimate for that micrograph Click on the <i>nick</i> button to launch a new window for the specified
720		estimate for that the organic check on the pick outon to rather a new window for the specified
/38		micrograph. Use the left mouse button and click at one end of a fibril, then click a 2^{na} time at the opposite
739		end of the fibril. This creates a line segment between the two end points defined by the user. The segments
7.00		and of the norm. This effects a mile segment between the two end points defined by the user. The segments
740		will be used for the particle extraction job in subsequent steps. Repeat this process until all the fibrils are
741		nicked Ensure segments do not overlap, and if fibrils contain curvature increase the number of segments
740		proved. Ensure segments to not overlap, and it norths contain our value interase the number of segments
/42		that make up the filament (Figure 6A). When done picking, right click on the micrograph and select Save

743 STAR with coordinates, close the micrograph and repeat the process for the remaining 19 micrographs. If 744 vou need to remove points, use the center button and click over an existing point to remove it. Ensure that 745 all the micrographs have an even number of picks (i.e. one start point and one end point per segment) and 746 that segments are centered over fibrils. When done picking from all 20 micrographs close the window to 747 finalize the job. 748 I/O: 749 Input micrographs: Select/job023/micrographs split1.star 750 Pick start-end coordinates helices? Yes 751 Use autopick FOM threshold? No 752 Display: 753 Particle diameter (Å): 100 754 Scale for micrographs: 0.2 755 Sigma contrast: 3 756 White value: 0 757 Black value: 0 758 Lowpass filter (Å): 20 759 Highpass filter (Å): -1 760 Pixel size (Å): -1 761 OR: use Topaz denoising? No 762 Colors: 763 Blue<>red color particles? No 764 The output log will list the total number of picks (start and end points). Here, we picked 414 particles (i.e. 765 207 segments) from 20 micrographs and the coordinates are saved to the manualpick.star file located in the 766 directory for this job. The total number of segments may vary due to differences in picking but ensure picks 767 are made on all 20 micrographs. 768 NOTE: The parameters in the *Display* tab are for visualization purposes only and do not impact 769 downstream processing steps. 770 NOTE: We observed that in some versions of RELION there is a bug that results in an empty coordinate 771 file from the Manual picking job. To bypass this error, simply select the Manual picking job from the 772 Finished jobs section and then click on the Continue! button. This will reopen the manual picking GUI 773 then, close the window; the coordinate file should now be updated with all the picks saved. There is no 774 need to repick particles or change any settings. 775 10. Particle Extraction (Manual Picks): 776 The manually picked segments must now be processed to extract particles for 2D classification. In 777 principle, this step will take user defined parameters to then cut the segments into individual particles for 778 downstream steps (Figure 6B). This is achieved by providing the number of unique asymmetrical units and 779 *helical rise* (\hat{A}) values in the helix tab. RELION will use these values to establish an interbox distance, i.e. 780 the spacing between each particle, that will separate overlapping 360-pixel boxes that traverse the length of 781 the segment (Figure 6B). Here, we have set the interbox distance to ~38.5 Å (4.82 Å x 8) to increase the 782 number of particles for training purposes. This value will be expanded later once auto-picking is complete. 783 Select the **Particle extraction** job, set *Micrograph STAR file* to the *micrograph split1.star* file from step 8, 784 set Input coordinates to the manualpick.star file from step 9, then click the Run! button. 785 I/O: 786 *Micrograph STAR file: Select/job023/micrographs split1.star* Input coordinates: ManualPick/job024/manualpick.star 787 788 OR re-extract refined particles? No 789 OR re-center refined coordinates? No 790 Write output in float16? Yes 791 Extract: 792 Particle box size (pix): 360 793 Invert contrast? Yes 794 Normalize particles? Yes 795 Diameter background circle (pix): -1 796 Stddev for white dust removal: -1 797 Stddev for black dust removal: -1 798 Rescale particles? No

799		Use autopick FOM threshold? No
800		Helix
201		Extract holized segments? Yes
001		Exhibiting the first segments (165
802		Tube diameter (A): 140
803		Use bimodal angular priors? Yes
804		Coordinates are start-end only? Yes
805		Cut halical tubas into segments? Yas
805 806		Currentui tutes into segments: 1es
800		Number of unique asymmetrical units: 8
807		Helical rise (A): 4.82
808		This job resulted in 5.919 particles extracted to a pixel size of 0.834 Å/pix with a box size of 360 pixels.
809		Differences in particle counts are due to differences in the number of segments picked during the manual
005		indice star A im for at least 4 000 nexticles at this store.
010		picking step. Ann for at least 4,000 particles at this stage.
811		NOTE: Amyloid structures have a consistent helical rise of ~4.8 A. This estimate is sufficient for this stage
812		of processing, as the helical rise will be optimized in subsequent steps.
813	11.	2D Classification (Manual Picks):
81 <i>1</i>		Although the particles were manually nicked and thus should be free from subortimal nicks or background
014		Autough the particles were manuarly picked, and this should be nee from suboptimal picks of background
815		noise, we prefer to perform a round of 2D classification to curate the particles that will be used to train the
816		Topaz neural network. Select the 2D classification job, set <i>Input images STAR file</i> to the <i>particles.star</i> file
817		from step 10, set the parameters below, then click on the <i>Run</i> ! button.
818		I/O·
010		Lunit images STAP flat Future t/ich020/2 auticles star
019		Imput images STAK file. Extract/Job029/particles.star
820		<u>CIF:</u>
821		Do CTF-correction? Yes
822		Ignore CTFs until first peak? No
823		Optimisation:
824		Vumber of classes: 20
024		Number of clusses. 20
025		Regularisation parameter 1. 2
826		Use EM algorithm? Yes
827		Number of EM iterations: 20
828		Use VDAM algorithm? No
829		Mask diameter (Å): 285
830		Mask individual particles with zeros? Yes
Q21		Limit machine F star to (4) 10
001		Limit resolution E-step to (A). 10
832		Center class averages? Yes
833		Sampling:
834		Perform image alignment? Yes
835		In-plane angular sampling: 2
836		Offset search range (niv): 5
000		Offset search range (pix). 5
037		Offset search step (pix). 1
838		Allow coarser sampling? No
839		Helix:
840		Classify 2D helical segments? Yes
841		Tube diameter (A) : 140
842		Do himodal angular searches? Yes
042		Lo olimona anguan scarcis res
043		Angular search range-psi (aeg). 0
844		Restrict helical offsets to rise: Yes
845		Helical rise (A): 4.82
846		Due to the small number of particles and the small number of classes this job should only take a couple of
847		minutes to run. The final classes can be visualized by clicking on the Display: dron-down menu and
848		selecting out: run it020 ontimiser star A RELION display GUI will annear check the box next to Sout
Q/Q		images on and solect un Class Distribution from the dren down many then slick Display to a slock of the solection of the sole
043		images on, and select rinclassDistribution from the drop-down menu, then click Display! to see the classes
850		sorted with the most populated classes at the top (Figure 6C). Close the window when done.
851	12.	Subset Selection (2D Classes for Topaz Training):
852		Next, use the Subset selection job to select the best classes to train the Topaz neural network. Set Select
853		classes from job to the run it020 optimiser.star file from step 11, set the additional parameters below. and
854		click the Run! button. This will launch a RELION display GUI. Check the box next to Sort images on: and

855	select <i>rlnClassDisribution</i> , then click the <i>Display</i> button. This will look identical to the previous step
856	where we visualized the classes but now use the left mouse button to select all the classes to move to the
857	next sten (Figure 6C). Once done right dick and select State selected allogses then close the dichlory
057	next step (Figure 6C). Once done, right check and select save selected classes, then close the display
858	Window.
859	
860	Select classes from job: Class2D/job030/run_it020_optimiser.star
861	OR select from micrograph.star: Leave blank
862	OR select from particles star: Leave blank
863	Class options:
864	Automatically select 2D classes? No
965	De acastar ha alars anarganos Vas
805	Re-center the class averages? Tes
000	Regroup the particles? No
867	Subsets:
868	Select based on metadata values? No
869	OR: select on image statistics? No
870	OR: split into subsets? No
871	Duplicates:
872	\overrightarrow{OR} · remove duplicates? No
873	This job resulted in 15 classes selected with 5 712 particles (Figure 6C, green hoves). Your values may be
874	slightly different at this sten due to differences in menuel niking, but the key is to select classes that among
074	slightly directing a mission of the more than a manual preking, but the key is to select classes that appear
0/5	normar in nature (Figure 6C, green boxes).
8/6 1:	3. Auto-Picking (Iopaz Iraining):
877	Use the curated particle stack to train a new Topaz neural network. It is critical that the executable path
878	within the <i>Topaz</i> tab directs RELION to the <i>topaz-filament</i> program [38]. The path here is to where <i>topaz</i> -
879	<i>filament</i> is located on our HPC cluster, but this may be different for your setup. If you are unsure where this
880	program is located, you may attempt to locate the program path by running the <i>which topaz-filament</i>
881	command from the terminal. Select the Auto-picking job. set <i>Input micrographs for autopick</i> to the
882	micrographs selected star file from step 9 in the Topaz tab set Particles STAR file for training to the
883	narticles star file from step 12 set the additional parameters below and modify the eventuable path to fit
005	your computing a stur the additional parameters below and modify the executable part to the
004	your computational setup, then check on the <i>Kun</i> ? button.
885	
886	Input micrographs for autopick: ManualPick/job024/micrographs_selected.star
887	Pixel size in micrographs (A): -1
888	Use reference-based template-matching? No
889	OR: use Laplacian-of-Gaussian? No
890	OR: use Topaz? Yes
891	Laplacian
892	This tab is ignored since we onted to use Topaz in the I/Ω tab
893	Tonaz.
801	Topuz,
004 005	Topuz executione. /programs/x00_0+-intux/system/s0gria_ontropuz-fitument
895	rariice diameter (A). 140
896	Perform topaz picking? No
897	Perform topaz training? Yes
898	Nr of particle per micrograph: 300
899	Input picked coordinates for training: Leave blank
900	OR train on a set of particles? Yes
901	Particles STAR file for training: Select/iob032/particles.star
902	Additional topaz arguments: Leave blank
903	References:
904	This tab is ignored since we onted to use Tonaz in the I/Ω tab
005	Autonialing:
903	Autopicking.
900	Use GPU acceleration? Tes
907	All other parameters on this tab are ignored since we opted to use Topaz in the I/O tab.
908	<u>Helix:</u>
909	This tab is ignored since we opted to use Topaz in the I/O tab.
910	This job results in a trained Topaz model titled <i>model_epoch10.sav</i> and is saved in the folder for this job.

911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931	 NOTE: Topaz training is not parallelized so the job will only use 1 MPI process. 14. Auto-Picking (Topaz Picking Optimization) The trained Topaz model will be applied to a subset of 20 micrographs to test how the model performs before it is applied to the entire dataset. For <i>topaz-fîlament</i> to pick segments, and not individual particles as in traditional single particle analysis, the additional flags for filament (<i>-f</i>) and threshold (-t) must be provided in the <i>Additional topaz arguments</i> box. Additionally, an integer value must be provided after the threshold flag. This threshold determines how many particles are picked. A lower threshold results in more particles, but if the threshold is too low, then the model will start picking noise. With any new trained Topaz neural network, we test a range of threshold values, typically from -6 to 0, to see which threshold works best (Figure 6D). Each threshold value will be its own job. Select the Auto-picking job, set <i>Input micrographs for autopick</i> to the <i>micrographs_split2</i> from job 8, in the <i>Topaz</i> tab set <i>Trained topaz model</i> to the <i>model_epoch10.sav</i> file from step 13, set the parameters below, and click the "<i>Run!</i>" button. To test additional thresholds, once the first Auto-picking job is complete, click on the job in the <i>Finished jobs</i> list and then click on the Auto-picking job to load the previous settings. Now, simply change the threshold that you would like to test. We tested thresholds -6, -5, -4, -3, -2, -1, and 0, and found that threshold -5 worked best for the dataset (Figure 6D). We have also included an extreme case with a threshold of -10 to better visualize bad picks that would be unsuitable for further processing (Figure 6D). <u>I/O:</u> <i>Input micrographs for autopick: Select/job023/micrographs_split2.star Pixel size in micrographs (Å): -1</i>
932	Use reference-based template-matching? No
933	OR: use Laplacian-oj-Gaussian? No OP: use Tenge? Ves
934	UK. use Topuz? Tes Lanlacian:
936	<u>Explacial</u> . This tab is ignored since we opted to use Topaz in the I/O tab
937	Tonaz.
938	Topaz executable: /programs/x86_64-linux/system/sbgrid_bin/topaz-filament
939	Particle diameter (Å): 140
940	Perform topaz picking? Yes
941	Trained topaz model: AutoPick/iob033/model_epoch10.sav
942	Perform topaz training? No
943	Additional topaz arguments: -f-t-5
944	References:
945	This tab is ignored since we opted to use Topaz in the I/O tab.
946	Autopicking:
947	Use GPU acceleration? Yes
948	All other parameters on this tab are ignored since we opted to use Topaz in the I/O tab.
949	Helix:
950	This tab is ignored since we opted to use Topaz in the I/O tab.
951	A picking threshold of -5 resulted in 688 segments (1,376 particles, i.e. end points) from 20 micrographs.
952	NOTE: Topaz picking is parallelized so multiple MPI processes can be run simultaneously, we typically run
953	20 MPI processes for this job. This setting can be found in the <i>Running</i> tab and is dependent on the
954	computational resources available.
955	•



Figure 6. Manual picking, 2D class selection, and auto-picking threshold determination. A.
Micrograph with examples of manually picked segments (step 9). Each "end" of the segment is selected by
the user (indicated by the stars). The endpoints are then linked by a line (indicated by an arrow), this region
will be divided into particles based on the user defined interbox distance. Each new color represents a new
segment that has been manually picked. B. Schematic of interbox distances (step 10). The filament that is
shown is a region that has been selected for particle picking. RELION will use a user defined "box" to
select as a particle. The interbox distance shown is the distance in which no overlap from previous boxes is
present (i.e., the region that is unique to each box). C. 2D Classes from manually picked particles (step 11).
The green boxes indicate the classes selected to use for neural network training (step 12). D. Micrographs
depicting trained neural network auto-picking results from different threshold values. As the threshold for
picking is decreased, the stringency in which the neural network determines whether the feature fits the
trained model is decreased—first resulting in an increase in picked particles, however, as the threshold
continues to decrease, the neural network starts to categorize "noise" as pickable particles.

15. Auto-Picking (Topaz Picking on the Entire Dataset)

The trained Topaz model and the optimized picking threshold are now applied to the entire dataset to select
segments for downstream processing. As detailed previously, upload the settings from the best picking job
(threshold -5), update Input micrographs for autopick to the micrographs.star file from step 4, and click the
<i>"Run!"</i> button.
7/0

975	<i>"Run!"</i> button.
976	I/O:
977	<i>Input micrographs for autopick: Select/job004/micrographs.star</i>
978	Pixel size in micrographs (Å): -1
979	Use reference-based template-matching? No
980	OR: use Laplacian-of-Gaussian? No
981	OR: use Topaz? Yes
982	Laplacian:
983	This tab is ignored since we opted to use Topaz in the I/O tab.
984	Topaz:
985	Topaz executable: /programs/x86_64-linux/system/sbgrid_bin/topaz-filament
986	Particle diameter (Å): 140
987	Perform topaz picking? Yes
988	Trained topaz model: AutoPick/job033/model epoch10.sav
989	Perform topaz training? No
990	Additional topaz arguments: -f -t -5

991		References:
992		This tab is ignored since we opted to use Topaz in the I/O tab.
993		Autopicking:
994		Use GPU acceleration? Yes
995		All other parameters on this tab are ignored since we opted to use Topaz in the I/O tab.
996		Helix:
997		This tab is ignored since we opted to use Topaz in the I/O tab.
998		This job results in 156,526 segments (313,052 particles, i.e. end points) from 4,858 micrographs.
999	16.	Particle Extraction (Large Box Size):
1000		For helical reconstruction methods, the helical twist and rise values are critical for crvo-EM data
1001		processing. The helical twist can be estimated from 2D class averages with large box sizes that span the
1002		fibril crossover distance (Figure 7A, 7B, 7D). Here, extract the particles to a box size of 864 pixels (~720
1003		Å) so we can estimate the crossover distance in subsequent steps. At this stage in the processing there is no
1004		need for high resolution information, so the box size is rescaled to 144 pixels (i.e. binning to a pixel size of
1005		5.004 Å/pixel). Alternatively, users may estimate the crossover distance from cryo-EM micrographs.
1006		typically those with higher defocus values are easier to visualize, or from negative stain TEM micrographs.
1007		However, extraction at a larger box size is still necessary to generate an initial reference for 3D
1008		reconstruction. Select the Particle extraction job. set <i>Micrograph STAR file</i> to the <i>micrographs.star</i> file
1009		from step 4, set <i>Input coordinates</i> to the <i>autopick star</i> file from step 15, set the additional parameters below.
1010		and click the " <i>Run!</i> " button.
1011		I/O:
1012		Micrograph STAR file: Select/iob004/micrographs.star
1013		Input coordinates: AutoPick/iob041/autopick.star
1014		OR re-extract refined particles? No
1015		OR re-center refined coordinates? No
1016		Write output in float 16? Yes
1017		Extract:
1018		Particle box size (pix): 864
1019		Invert contrast? Yes
1020		Normalize particles? Yes
1021		Diameter background circle (pix): -1
1022		Stddev for white dust removal: -1
1023		Stddev for black dust removal: -1
1024		Rescale particles? Yes
1025		Re-scale size (pixels): 144
1026		Use autopick FOM threshold? No
1027		Helix:
1028		Extract helical segments? Yes
1029		Tube diameter (A) : 140
1030		Use bimodal angular priors? Yes
1031		Coordinates are start-end only? Yes
1032		Cut helical tubes into segments? Yes
1033		Number of unique asymmetrical units: 15
1034		Helical rise (Å): 4.82
1035		This job results in 771,754 particles with an original box size of 864 pixels that is rescaled to 144 pixels at
1036		a pixel size of 5.004 Å/pixel.
1037		NOTE: The number of asymmetrical units was increased to 15, this results in an interbox distance of ~72 Å
1038		or ~25% of the small box size (360 pixels) that will be used for the final reconstruction.
1039		



Figure 7. Determining crossover distance, helical twist, and helical rise. A. An initial map depicts the crossover distance observed in twisting fibrils. The crossover distance is described as the length where the fibril turns 180° (red dotted line). Scale bar, 100 nm. B. The crossover distance can be measured (red line) from well aligned 2D classes where the twisting nature of the fibril is observed, this requires a box size that spans a distance that is close to or larger than the crossover distance for an accurate measurement to be made. Here, a box size of 864 pixels (720 Å) was used for initial crossover estimates. Poor 2D classes are mis-aligned or blurry preventing crossover distance measurements. C. The helical rise can be determined from 2D classes with a small box size (360 pixels) extracted at their original pixel size (0.834 Å/pix) that yield high resolution details (i.e. spacing of the β -sheets). The sigma contrast of the 2D classes must be adjusted to visualize the helical layer lines in reciprocal space. From the average power spectrum, a measurement (red line) can be made from the meridian to the highest intensity layer line, this measurement can be used to estimate the helical rise. D. The measurements made in B and C are used to calculate the helical rise and the crossover distance. Then, the crossover distance and helical rise are used to calculate the helical twist of the structure. The estimated helical parameters are used for subsequent 3D refinement steps.

17. 2D Classification (Large Box Size)

58Classify the particles to remove junk particles and to estimate the crossover distance. Select the 2D59classification job, set Input images STAR file to the particles.star file from step 16, set the additional60parameters below, then click on the "Run!" button.61<u>I/O:</u>62Input images STAR file: Extract/job042/particles.star63CTF:64Do CTF-correction? Yes65Ignore CTFs until first peak? Yes66Optimisation:67Number of classes: 5068Regularisation parameter T: 269Use EM algorithm? Yes70Number of EM iterations: 2071Use VDAM algorithm? No72Mask diameter (Å): 710

1073 1074 1075 1076 1077 1078 1079 1080 1081 1082		Mask individual particles with zeros? Yes Limit resolution E-step to (Å): -1 Center class averages? Yes Sampling: Perform image alignment? Yes In-plane angular sampling: 2 Offset search range (pix): 5 Offset search step (pix): 1 Allow coarser sampling? No Helix: Charter of 2D helicher and 2 M
1083		Classify 2D helical segments? Yes
1004		Tude diameter (A). 140 De himodal angular sogrehes? Ves
1085		Angular search range psi (deg): 6
1080		Angulur seurch runge-psi (deg). 0 Restrict helical offsets to rise: Ves
1088		Helical rise (Å): 4.82
1089		This job results in the run $it020$ ontimiser star file that contains the 2D class averages. This file can be
1090		viewed using the <i>Display</i> : dron-down menu on the right side of the GUI
1091		NOTE: Sometimes it can be helpful to determine the helical rise of the filament rather than assume 4.8 Å as
1092		the starting point. To do this, users can utilize 2D classifications and use measurements of the average
1093		power spectra in Fourier space to calculate the estimated rise. To perform this analysis, use a box size of
1094		360 pixels and high-resolution data (0.834 Å/pix), as this allows for more detail to be visualized in the 2D
1095		classes (specifically the β -sheet rungs). To do so, use the Particle Extraction job to extract particles to
1096		their original pixel size. Use the parameters as instructed in step 16, but ensure that <i>Particle box size</i> is set
1097		to 360 and that <i>Rescale particles</i> is set to <i>No</i> . Once particle extraction is complete, run a 2D Classification
1098		job as described in step 17. Ensure <i>Input images STAR files</i> is set to the correct <i>particles.star</i> file from the
1099		Farticle Extraction job, and <i>Mask alameter</i> is set to 500. When the job is done, open the average power spectra by selecting the output if 020, ontiminant from the display output. Enter on increased Signa
1100		<i>Contrast</i> value in the top box of the RELION display GIU (we used 1 for our data) (Figure 7C). If the user
1102		fails to increase the Sigma Contrast, the average power spectra will not be visible (Figure 7C). Once the 2D
1103		classes are displayed right click on a class and select <i>Show Fourier amplitudes (2X)</i> . This will open an
1104		image of the average power spectra. Make a measurement from the meridian to either layer line with the
1105		strong intensity (Figure 7C). This can be done by clicking and holding the center button on the mouse. Use
1106		the following formula to calculate the rise: rise $(Å) = (\underbrace{box \ size \ (pix)}) * 2 *$
1107		$(A = 1)^{A}$ (Figure 7C, 7D). For non-analysis and the figure of the size $(A = 1)^{A}$ (Figure 7C, 7D).
1107		pixel size $\left(\frac{1}{\text{pix}}\right)$ (Figure /C, /D). For new experimental data, if the rise is substantially different then
1100		in a helical rise of 4.84 Å that will be refined in later steps (Figure 7C)
1110	18	Subset Selection (2D Classes for Initial Man)
1111	10.	Select 2-3 good classes that will be used to generate an initial 3D volume. Select the Subset Select job set
1112		Select classes from iob to the run it020 optimiser star file from step 17, set the parameters below, then
1113		click the <i>Run!</i> button. A RELION display GUI will appear, reverse sort the class averages by
1114		rlnClassDistribution (as described in step 11) and select 2 class averages (Figure 8A, green boxes). To
1115		measure the crossover distance right click on a 2D class and select Show original image. A new window
1116		will appear, using the center button click and drag to measure the distance between two crossovers (Figure
1117		7B). The distance in pixels is displayed over the image and in the terminal (Figure 7B). Multiply the
1118		measured distance by the current pixel size of 5.004 Å/pixel to calculate the distance in angstroms (Figure
1119		7D). Here, we estimated a crossover distance of 120 pixels or 600 A (Figure 7B). When done, close the
1120		original image. Repeat the process for any additional 2D classes you want to measure. Lastly, in the
1121		window with all the 2D classes, right click and select Save STAR with selected images, then close display
1122 1122		
1123		<u>VU.</u> Salact classes from job: Class 2D/job044/mm_it020_optimiser star
1125		OR select from microoranh star: Leave blank
1126		OR select from particles.star: Leave blank

1127 Class options: 1128 Automatically select 2D classes? No 1129 Re-center the class averages? Yes 1130 Regroup the particles? No 1131 Subsets: 1132 Select based on metadata values? No 1133 OR: select on image statistics? No 1134 OR: split into subsets? No 1135 **Duplicates:** 1136 OR: remove duplicates? No 1137 This job results in a *class* averages.star file containing the 2 selected classes. 1138 19. Initial Map Generation Using relion helix inimodel2d 1139 Generate an initial map from the selected 2D classes in step 18 using the *relion helix inimodel2d* program 1140 [39]. The following steps must be completed in the terminal. First create two directories to keep our data 1141 organized. In the terminal, navigate to the RELION project directory, this is the directory that contains all 1142 the RELION subdirectories, and enter the commands *mkdir inimodel* and *mkdir ini4refine*. This will create 1143 two directories one that will house the initial volumes and a second that will contain the rescaled volumes 1144 that will be used for refinement steps. Documentation on relion helix inimodel2d can be found at 1145 https://relion.readthedocs.io/en/release-4.0/Reference/Helix.html. For convenience, we have detailed each 1146 argument below, alternatively running relion helix inimodel2d with no additional arguments will detail all 1147 available arguments for the program. Before running the command, ensure that the input STAR file (--i) 1148 and the output root name (--o) are updated to your specific project, then run the command from the 1149 terminal. The following command generates an initial volume with an estimated crossover distance of 750 1150 Å (Figure 8F). 1151 relion helix inimodel2d --i Select/job045/class averages.star --angpix 5.004 --mask diameter 300 --svm 2 1152 --iter 10 --search shift 70 --search angle 15 --search size 10 --j 20 --crossover distance 750 --o 1153 inimodel/Select045 CO750 1154 The arguments run with relion helix inimodel2d are detailed below. 1155 --i input STAR file with 2D classes 1156 --angpix pixel size in angstroms 1157 --mask diameter size in angstroms of circular mask around 2D classes 1158 --sym order of symmetry in 2D slices 1159 --iter number of iterations to run 1160 --search shift distance in angstroms to search translations perpendicular to helical axis 1161 --search angle degrees to search in-plane rotations 1162 --search size \pm number of pixels to fix best crossover distance 1163 --*i* number of threads 1164 --crossover distance distance in angstroms between 2 crossovers 1165 --o output root name 1166 The program generates several files, and the initial 3D volume is saved with the suffix 1167 class001 rec3d.mrc which can be opened in ChimeraX for visualization [40-42]. Since the initial 1168 crossover distance is an estimate, we prefer to generate several initial maps for a round of 3D refinement to 1169 see what best fits our experimental data (Figure 8B-8G). Although our initial estimate for crossover 1170 distance was 600 Å, we found that an initial map with a crossover distance of 750 Å is best for this dataset 1171 (Figure 8C, 8F). You may test additional crossover distances as we typically do with new experimental 1172 datasets. To do so, change the --crossover distance and --o arguments of the command above to generate 1173 additional maps of varying crossover distances with appropriate output root names (Figure 8B-8G). 1174 20. Rescale Initial Map Using relion image handler 1175 The initial maps generated in the previous step must be rescaled because the 3D refinement steps will be 1176 performed with a smaller box size (360 pixels) at the original pixel size (0.834 Å/pixel). Use 1177 relion image handler to rescale the maps. For a list of all possible arguments simply run the program in 1178 the terminal with no additional arguments. The command below was used to rescale the 750 Å crossover 1179 map. Before running the command ensure the MRC input file (--i) and the MRC output file (--o) reflect 1180 your project. For convenience, the arguments used to run relion image handler are detailed below. 1181 relion image handler -- i inimodel/Select045 CO750 class001 rec3d.mrc -- angpix 5.004 -- rescale angpix 1182 0.834 -- new box 360 -- o ini4refine/Select045 CO750 box360.mrc

1183		<i>i</i> input MRC file of the initial map
1184		angpix pixel size in angstroms of the input file
1185		rescale_angpix scale input map to this new pixel size in angstroms
1186		new_box resize the input map to this box size in pixels
1187		o output name of resized map
1188		Repeat this step for any additional maps that will be tested. Ensure that the input file (i) and output file (
1189		o) are updated to reflect the maps being rescaled (Figure 8B-8G).
1190	21.	Subset Selection (2D Classes for Refinement)
1191		Select additional classes from the 2D Classification job (step 17) to ensure there are enough particles for
1192		additional processing. Repeat the Subset Selection job as in step 18 but now select all the good classes for
1193		further processing (Figure 8A).
1194		<u>I/O:</u>
1195		Select classes from job: Class2D/job044/run_it020_optimiser.star
1196		OR select from micrograph.star: Leave blank
1197		OR select from particles.star: Leave blank
1198		<u>Class options:</u>
1199		Automatically select 2D classes? No
1200		Re-center the class averages? Yes
1201		Regroup the particles? No
1202		Subsets:
1203		Select based on metadata values? No
1204		OR: select on image statistics? No
1205		OR: split into subsets? No
1206		Duplicates:
1207		OR: remove duplicates? No
1208		Here, we selected 21 classes with 413,249 particles saved in the particles.star file (Figure 8A).



Figure 8. Initial model generation. A. Classes selected from all 2D classes. All classes shown in A are the classes selected (job 21) from the classes rendered from the trained neural network auto-picking job on all micrographs (step 17). The green boxes indicate the two classes selected for initial model generation (step 18). B-G. Initial maps for the crossover distances 550-800 Å. One showing the cross-section of the refined filament (cross-section location shown by the black crossbar) and the other depicting the entirety of the filament. The commands used to generate (step 19) and rescale (step 20) the initial models are shown.

22. Particle Extraction (Small Box Size):

1218		Select the Particle Extraction job, set <i>Refined particles STAR files</i> to the <i>particles.star</i> file from step 21,
1219		set the additional parameters below, and click the Run! button.
1220		<u>I/O:</u>
1221		Micrograph STAR file: Leave blank
1222		Input coordinates: Leave blank
1223		OR re-extract refined particles? Yes
1224		Refined particles STAR file: Select/job046/particles.star
1225		Reset the refined offsets to zero? Yes
1226		OR re-center refined coordinates? No
1227		Write output in float16? Yes
1228		Extract:
1229		Particle box size (pix): 360
1230		Invert contrast? Yes
1231		Normalize particles? Yes
1232		Diameter background circle (pix): -1
1233		Stddev for white dust removal: -1
1234		Stddev for black dust removal: -1
1235		Rescale particles? No
1236		Use autopick FOM threshold? No
1237		Helix:
1238		Extract helical segments? Yes
1239		Tube diameter (Å): 140
1240		Use himodal angular priors? Yes
1241		Coordinates are start-end only? Yes
1242		Cut helical tubes into segments? Yes
1243		Number of unique asymmetrical units: 15
1244		Helical rise (Å): 4 82
1245		The 413.249 particles were re-extracted to a box size of 360 pixels and a pixel size of 0.834 Å/pixel. The
1246		particles are stored in the <i>particles.star</i> file.
1247 2	23.	3D Auto-Refine (Fixed Symmetry)
1248		The particle set from step 22 and the rescaled initial map generated in step 20 will be subjected to a round
1249		of 3D refinement. First, take the estimated helical rise and calculate the initial twist for the estimated
1250		crossover distance using the following formula: $twist = \frac{rise \times 180}{crossover distance}$ (Figure 7D). The rise is
1251		estimated to be 4.82 Å and the crossover distance was estimated to be 750 Å, so the initial twist value is
1252		1.16°. Finally, apply a negative value to the initial twist based on the assumption that fibrils typically
1253		display a left-handed helical form and as supported by atomic force microscopy studies [13]. Select the 3D
1254		Auto-Refine iob, set <i>Input images STAR file</i> to the <i>particles star</i> file generated in step 22, set <i>Reference</i>
1255		man to the rescaled initial volume generated in step 20 in our case this file was named
1256		Select045 CO750 hox360 mrc set the parameters below and click the Run/ button
1257		I/O:
1258		Input images STAR file: Extract/iob047/narticles star
1259		Reference map: ini4refine/Select045 CO750 box360 mrc
1260		Reference mask (ontional): Leave blank
1261		Reference:
1262		Ref. man is on absolute grevscale? No
1263		Initial low-pass filter (Å): 10
1264		Symmetry: C1
1265		CTF:
1266		Do CTF-correction? Yes
1267		Jonore CTFs until first neak? No
1268		Ontimisation.
1269		Mask diameter (Å): 220
1270		Mask individual narticles with zeros? Yes
1271		Use solvent-flattened FSCs? No
1272		Auto-sampling
/-		пись випринд.

1273		Initial angular sampling: 3.7 degrees
127/		laitia officia usuga (niv): 5
12/4		Initial offset range (pix). 5
1275		Initial offset step (pix): 1
1276		Local searches from auto-sampling: 1.8 degrees
1277		Palay symmetry: Lasya blank
1277		Ketax symmetry. Leave blank
1278		Use finer angular sampling faster? (No)
1279		Helix:
1200		De heliest reconstruction? Vos
1200		Do netical reconstruction? Tes
1281		Tube diameter – inner, outer (A): -1, 140
1282		Angular search range – rot tilt nsi (deg): -1 15 10
1202		
1283		Range factor of local averaging: -1
1284		Keep tilt-prior fixed: Yes
1285		Apply helical symmetry? Yes
1205		The first for the second
1280		Number of unique asymmetrical units: 15
1287		Initial twist (deg), rise (A): -1.16, 4.82
1288		Central 7 length (%): 25
1200		De la construcción (70, 20
1289		Do local searches of symmetry? No
1290		The job results in a map with a global resolution of 3.66 Å (Figure 9A, 9B, blue). Repeat this step for any
1291		additional initial maps and crossover distances that you would like to test. We tested crossover distances of
1202		additional inflat inflat in the state of 200 ($E_{\rm state}$ 0.4 0.0). There takes a state of the takes are state of the state of 200 ($E_{\rm state}$ 0.0).
1292		550, 600, 650, 700, 750, and 800 A (Figure 9A, 9B). These tests showed that three maps resolved to a
1293		resolution of 3.66 Å. The map generated from the 750 Å crossover distance was selected because the
1294		backhone density was best resolved, and the man showed side chain densities for some residues (Figure 9B
1205		buckbone density was best resolved, and the map showed side chain densities for some residues (righte 5D),
1295		blue). Additionally, the map showed clear separation of the β -strands along the helical axis.
1296	24.	3D Classification (Symmetry Search)
1297		During the Subset Selection (objected 21), we selected all the 2D classes that resembled amyloid fibrils
1200		During the Subset Selection job (step 21), we selected an the 2D classes that resembled anytoid norms.
1298		Being less stringent after 2D classification means that heterogeneity most likely exists in our dataset. By
1299		using 3D classification, we can further sort the heterogeneity that may exist in the particle set and improve
1200		the quality of the reconstruction. Use the 2D reconstruction from step 22 as an initial storting point to then
1300		the quarty of the reconstruction. Ose the 3D reconstruction from step 25 as an initial starting point to then
1301		sort particles into 4 classes. Use the <i>Do local searches of symmetry</i> tool to search a range of helical
1302		parameters that best fit the dataset. Select the 3D Classification job, set <i>Input images STAR file</i> to the
1202		f_{1} with data star file from step 23 set <i>Pafaranca</i> map to the number of f_{1} along 0.01 unfil must from step 23 set
1303		<i>Tun adda.star</i> the nom step 25, set <i>Reference map</i> to the <i>tun null</i> ^T <i>Classoon_unfut.mic</i> nom step 25, set
1304		the additional parameters below, and click the <i>Run!</i> button.
1305		I/O:
1306		Input images STAP file: Pafine3D/iab060/mm data star
1300		Input images STAK file. Refine SD/100009/11/1 autustur
1307		Reference map: Refine3D/job069/run_half1_class001_unfil.mrc
1308		Reference mask (optional): Leave blank
1200		
1209		Kelerence:
1310		<i>Ref. map is on absolute greyscale? No</i>
1311		Initial low-pass filter (Å): 4.5
1212		
1512		Symmetry: C1
1313		<u>CTF:</u>
1314		Do CTF-correction? Yes
1215		Len our CTEs until first nearly No
1515		Ignore CIF's until first peak? No
1316		Optimization:
1317		Number of classes: 4
1218		Pagularization parameter T: 1
1310		
1319		Number of iterations: 20
1320		Use fast subsets (for large data sets)? No
1321		Mask diameter (\hat{A}) · 220
1021		
1322		Mask individual particles with zeros? Yes
1323		<i>Limit resolution E-step to (Å): -1</i>
1324		Sampling:
1005		Definition of the second secon
1325		Perform image alignment? Yes
1326		Angular sampling interval: 3.7 degrees
1327		Offset search range (nix): 5
1220		Offset search (ange (ph)), b
1270		Ojjsel search slep (plx): 1

1329		Perform local angular searches? No
1330		Allow coarser sampling? No
1331		Helix:
1332		Do helical reconstruction? Yes
1333		Tube diameter – inner outer $(Å)$: -1 140
1334		Angular search range – rot tilt nsi (deg): -1 15 10
1335		Range factor of local averaging: -1
1336		Kaap tilt-neior firad: Vas
1227		Apply holigal symmetry? Vas
1220		Apply neucui symmetry ? Tes
1220		Number of unique asymmetrical units. 15 I_{i} is the state of the
1339		Initial twist (deg), rise (A): -1.14, 4.82
1340		Central Z length (%): 25
1341		Do local searches of symmetry? Yes
1342		Twist search – Min, Max, Step (deg): -0.9, -1.2, 0.01
1343		<i>Rise search – Min, Max, Step (A): 4.75, 4.95, 0.01</i>
1344		The job runs for 20 iterations, sorting the particle set into 4 classes and optimizing helical parameters at
1345		each iteration. A cross section of the 3D volumes can be visualized by displaying the
1346		run_it020_optimiser.star file in RELION. Alternatively, the 4 MRC files generated in this job
1347		(run_it020_class001.mrc, run_it020_class002.mrc, etc.) can be opened in ChimeraX for easier
1348		visualization of the 3D maps. Class 3 was the best 3D volume with a helical twist of -1.12° and a helical
1349		rise of 4.84 Å (Figure 9C, green box).
1350	25.	Subset Selection (3D Class for Additional Processing)
1351		Use the Subset selection job to select the best class from the 3D classification job in step 24. Ensure Select
1352		classes from job is set to the run it020 optimiser.star file that was generated in step 24. Set the parameters
1353		below and click the <i>Run!</i> button. Refer to step 12 for how to display, select, and save classes in a Subset
1354		selection job.
1355		I/O:
1356		Select classes from job: Class3D/job077/run_it020_optimiser.star
1357		OR select from micrograph star: Leave blank
1358		OR select from particles star: Leave blank
1359		Class ontions:
1360		Automatically select 2D classes? No
1361		Recenter the class averages? Yes
1362		Re-center the cluss averages: Tes
1262		Subasta:
1264		Subsets. Select based on metadata values? No
1265		OP, select on image statistics? No
1303		OR. select on image statistics? No
1267		OK. spill into subsets? No
1207		Duplicates:
1308		UK: remove duplicates? No
1309		Class 3 was selected in this job and the data was saved to the <i>particles.star</i> file that contained all 129,940
1370		particles for that class (Figure 9C).
1371		





Figure 9. 3D Refinement of different crossover distances and 3D classification. A. Cross-sections, resolution, and calculated twist and rise of each initial models after 3D refinement (550-800 Å) (step 23). Red and Blue squares indicate respective electron potential maps for B. B. Cross-section of the electron potential maps refined with 600 Å (Red) and 750 Å (Blue) crossovers. Scale bar, 25 Å. C. 3D Classifications from 750 Å crossover initial model (step 24). Green box indicates selected 3D class used for further refinement (step 25).

1379		
1380	26	3D Auto-Refine (Symmetry Search)
1381	20.	Select the 3D auto-refine job undate <i>Input images STAR file</i> to the <i>particles star</i> file from step 25 and the
1382		Reference man to best 3D man from the 3D classification in step 24 in our case this was
1383		run it020 class003 mrc but this may be different for your project. Then set the parameters below and click
1384		the Run/ button
1385		
1386		<u>vo.</u> Innut imagas STAP file: Select/iob080/particles star
1387		Reference man: Class 3D/iob077/mm_it020_class003 mrc
1288		Reference map. ClassoD/j000///lan_llo20_class005.mrc
1280		Deference:
1300		Reference. Ref. map is on absolute gravscale? No
1201		Initial low pass filter (Å): 4.5
1307		Symmetry: C1
1392		CTE:
130/		CIT: Do CTE convection? Vas
1205		Do CIF-correction? Tes
1206		Optimisation:
1207		$\frac{Optimisation}{Mask diamatan} (\hat{A}): 220$
1308		Mask individual particles with zeros? Ves
1300		Use solvent flattened ESCs? No
1400		Auto sampling:
1400		<u>Auto-sampling</u> .
1401		Initial angular sampling. 5.7 degrees
1402		Initial offset stan (pix): 1
1403		Initial offset step (pix). I
1404		Local searches from auto-sampling. 1.0 degrees
1405		Keiax Symmetry. Leave Diank
1400		Use Jiner angular sampling Juster? (NO)
1407		<u>nellx.</u> De holioglassonstruction? Ver
1400		Do netical reconstruction: les Tube digmeter inner outer $\binom{1}{2}$, 1,140
1409		Tube alameter – Inner, outer (A): -1, 140 Angulan aggreb hange – vet tilt pei (deg), $1, 15, 10$
1410		Angular search range – rol, ill, psi (deg)1, 15, 10
1411		Kange Jacior of local averaging: -1
1412		Keep tilt-prior fixed: les
1413		Apply helical symmetry? Yes
1414		Number of unique asymmetrical units: 15
1415		Initial twist (aeg), rise (A): -1.11, 4.84
1410		Central Z length (%): 25
1417		Do local searches of symmetry? Yes
1418		Iwist search – Min, Max, Step (deg): $-0.9, -1.3, 0.01$
1419		$Kise \ searcn - Min, \ Max, \ Step \ (A): 4.75, 4.95, 0.01$
1420		The optimized herical parameters converged to a herical twist of -1.11° and a rise of 4.84 A. The resolution
1421		without masking is 3.23 A. The <i>run_class001.mrc</i> file can be downloaded and opened with ChimeraX to
1422		visual the 3D volume (Figure 10, step 26).
1423		



Figure 10. Results of 3D refinements and post-processing steps. The 3D refinements and their corresponding post-processed maps of our processing pipeline are depicted here. The step number, resolution, twist, rise, and mask percentages are displayed for each electron potential map. A description as to whether the electron potential map display is a result of a 3D refinement job or post-processing job is

1429 1430 1431		displayed at the top of the figure. The processing workflow incrementally improves maps quality and resolution, resulting in a final map at 2.04 Å resolution.
1432	27.	Mask Creation (80% Mask)
1433		Helical reconstruction is prone to loss of resolvability as the volume reaches the edge of the box. Thus
1434		masking encompasses a central portion of the fibril and excludes the ends of the fibril. The mask can be as
1435		small the as the <i>Central Z length</i> established in the 3D auto-refine job. However, at this stage in processing
1436		we may benefit from a larger mask to ensure we have sufficient signal for the CTF refinement steps. Open
1437		the <i>run class001.mrc</i> file from step 26 in ChimeraX. Ensure that the volume step is set to 1 then lower the
1438		volume threshold until noise starts to appear in the solvent space. Note this threshold and set this as the
1439		<i>Initial binarization threshold</i> for the Mask creation job. A value of 0.00096 worked well for this project.
1440		Update the <i>Input 3D map</i> to the <i>run class001.mrc</i> generated in the step 26. Set the additional parameters
1441		below then click the <i>Run!</i> button.
1442		I/O:
1443		Input 3D map: Refine3D/job081/run class001.mrc
1444		Mask:
1445		Lowpass filter map $(Å)$ 15
1446		Pixel size $(A) - 1$
1447		Initial binarization threshold: 0.00096
1448		Extend binary map this many pixels: 5
1449		Add a soft-edge of this many pixels: 5
1450		Helix:
1451		Mask a 3D helix? Yes
1452		Central Z length (%): 80
1453		In ChimeraX, open the <i>mask.mrc</i> file and the <i>run_class001.mrc</i> file from step 26. Ensure both maps are set
1454		to a step size of 1, set the mask threshold to 0.99 to visualize the mask volume, and for easier visualization
1455		lower the mask opacity to 50% (Figure 11A). Inspect the mask and map, when viewing the central cross-
1456		section of the map ensure the entire proteinaceous volume is within the mask. If there are no issues, then
1457		proceed to the next step. However, if the map is not completely encompassed by the mask, lower the Initial
1458		binarization threshold value and rerun the job by clicking the Continue! button. Repeat this process until
1459		the mask is satisfactory (Figure 11A).
1460		



1461
1462Figure 11. Mask central Z length coverage. A. A mask (gray) covering 80% of the map (purple) along the
fibril axis (step 27), used during CTF refinement steps. B. A mask (gray) covering 25% of the map (purple)
along the fibril axis (step 44), used in the final post-processing job (step 45). C. Filament after applying
real-space symmetrization (step 47) to the edge of the box using the *relion_helix_toolbox* program. Scale
bars, 25 Å.1467

28. Post-Processing

The post-processing job will recalculate the global resolution with masking, and it will automatically estimate and apply a B-factor to sharpen the map, further improving the quality of the map. Select the **Post-processing** job, set *One of the 2 unfiltered half-maps* to the *run_half1_class001_unfil.mrc* file from step 26, set *Solvent mask* to the *mask.mrc* file from step 27, set *MTF of the detector (STAR file)* to the *k3-CDS-300keV-mtf.star* file that is supplied with EMPIAR-12229. Set the remaining parameters below then click the *Run!* button. I/O:

- 1476 One of the 2 unfiltered half-maps: Refine3D/job081/run_half1_class001_unfil.mrc
- 1477Solvent mask: MaskCreate/job086/mask.mrc1478Calibrated pixel size (Å) -1
- 1478Calibrate1479Sharpen:

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- 1480 Estimate B-factor automatically? Yes
- 1481 Lowest resolution for auto-B fit (Å): 10
- 1482 Use your own B-factor? No
- 1483 Skip FSC-weighting? No
- 1484 MTF of the detector (STAR file): k3-CDS-300keV-mtf.star
- 1485 Original detector pixel size: -1
- 1486The job estimated a b-factor of -97, and the processed map is saved as *postprocess.mrc*. The job also1487calculated a resolution of 2.97 Å with masking and the volume is saved as *postprocess_mask.mrc* (Figure148810, step 28).
- 1489 29. Bayesian Polishing (Round 1)

1490		The next steps will aim at improving the quality of the particles to further improve the resolvability of the
1491		map. The poising will use motion corrected micrographs and particle positions to improve motion
1492		correction on a per-particle basis. Select the Bayesian polishing job, set the <i>Micrographs (from</i>
1493		MotionCorr) to the corrected_micrographs.star file from step 2, set the Particles (from Refine 3D or
1494		<i>CtfRefine</i>) to the <i>run_data.star</i> file from step 26, set the <i>Postprocess STAR file</i> to the <i>postprocess.star</i> file
1495		from step 28, set the remaining parameters below, and click the <i>Run!</i> button.
1496		<u>I/O:</u>
1497		Micrographs (from MotionCorr): MotionCorr/job002/corrected micrographs.star
1498		Particles (from Refine 3D or CtfRefine): Refine3D/job081/run data.star
1499		Postprocess STAR file: PostProcess/iob088/postprocess.star
1500		First movie frame: 1
1501		Last movie frame: -1
1502		Extraction size (nix in unbinned movie): -1
1502		Pa sagla size (pix in unbinneu movie)1
1503		Ne-scale size (pixels)1
1504		
1505		<u>Irain:</u>
1506		Irain optimal parameters? No
1507		Polish:
1508		Perform particle polishing? Yes
1509		<i>Optimized parameter file: Leave blank</i>
1510		OR use your own parameters?
1511		Sigma for velocity (Å/dose): 0.2
1512		Sigma for divergence (Å): 5000
1513		Sigma for acceleration (Å/dose): 2
1514		Minimum resolution for B-factor fit (A) : 20
1515		Maximum resolution for B -factor fit (A) -1
1516		The job will save the particles to the <i>shiny star</i> file. The improvements of the particle positions can be
1517		found in the logfile off file
1518	30	3D Auto-Refine (Pseudo Screw Symmetry)
1510	50.	Up to this point we have apply applied belies a summative to the 2D reconstruction. We will now address
1515		of to this point we have only applied hencal symmetry to the 5D reconstruction. We will now address
1520		additional symmetry that may be present to further improve the quality of the reconstruction. Previous
1521		studies have shown that amyloid fibrils exist with varying degrees of symmetry. For two protofilament
1522		fibrils, we observe either C2 symmetry, where two protofilaments are identical and in register, as
1523		commonly observed in Tau fibrils, or we observe pseudo-screw symmetry (P2 ₁), where two protofilaments
1524		are identical but out of register, as observed in α -synuclein fibrils (Figure 1G, 1F) [12,13,43]. To
1525		understand this difference in symmetry, it is necessary to manually inspect the reconstruction to determine
1526		the best symmetry for the dataset. This can be done by using ChimeraX to analyze the 3D volume from
1527		either the run class001.mrc file from step 26 or the postprocess.mrc file from step 28. Here, we determined
1528		that pseudo-screw symmetry exists within our dataset. To apply this symmetry, we will continue to set the
1529		Symmetry parameter to C1, but we will divide the helical rise in half and subtract the helical twist from
1530		180°. By doing so, we can impose pseudo-screw symmetry to our reconstruction. Select the 3D auto-refine
1531		job, set <i>Input images STAR files</i> to the <i>shiny star</i> file generated in step 29, set <i>Reference man</i> to the
1532		run half1 class001 unfil mrc file from job 26, set the additional parameters below, then click the Run
1533		hutton
1534		I/O:
1535		Innut images STAP file: Polish/ioh001/shim.star
1526		Polononoo man: Polino2D/ioh021/min half1_alass001_unfil muo
1530		Defense magic (entional), Leave blank
1537		Deference mask (optional). Leave blank
1530		<u>Reference:</u>
1539		Rej. map is on absolute greyscale? No
1540		Initial tow-pass filter (A): 4.5
1541		Symmetry: C1
1542		<u>CTF:</u>
1543		Do CTF-correction? Yes
1544		Ignore CTFs until first peak? No
1545		Optimization:

4540		
1546		Mask diameter (A): 220
1547		Mask individual particles with zeros? Yes
1548		Use solvent-flattened FSCs? No
1549		Auto-sampling:
1550		Initial angular sampling: 3.7 degrees
1551		Initial offset range (nit) - 5
1001		Initial offset range (pix). 5
1552		Initial offset step (pix): 1
1553		Local searches from auto-sampling: 1.8 degrees
1554		Relax symmetry: Leave blank
1555		Use finer angular sampling faster? (No)
1556		Helix.
1557		Do holical reconstruction? Var
100		Do neucla reconstruction: Les $(k) = 1.140$
1000		Tube alameter – Inner, outer (A): -1, 140
1559		Angular search range – rot, tilt, psi (deg): -1, 15, 10
1560		Range factor of local averaging: -1
1561		Keep tilt-prior fixed: Yes
1562		Apply helical symmetry? Yes
1563		Number of unique asymmetrical units: 15
1563		Number of and a symmetrical and (3) , 100.445, 2.42
1504		Imitiat (Wist) (aeg), rise (A). 179.445, 2.42
1565		Central Z length (%): 25
1566		Do local searches of symmetry? Yes
1567		Twist search – Min, Max, Step (deg): 179.24, 179.65, 0.01
1568		Rise search – Min. Max. Step (\mathring{A}) : 2.2.2.6.0.01
1569		The unmasked reconstruction improved from 3.23 Å (step 26) to 3.00 Å and is stored in the
1505		The dimaster recensultation improved from 5.25 4 (step 20) to 5.00 A and is stored in the
1570		run_classoor.mrc me (rigue 10, step 50). The symmetry parameters reneed pseudo-screw symmetry and
15/1		were optimized to a twist of 1/9.45° and a rise of 2.42 A.
1572	31.	Post-Processing
1573		Run a Post-processing job to see how masking the solvent region improves the resolution and how
1574		automated sharpening can improve the map quality. Select the Post-processing job, set <i>One of the 2</i>
1575		unfiltered half-mans to the run half class(0) unfil mrc file from step 30 set the Solvent mask to the
1576		mask was file from ston 27, soft the additional parameters below then aligh the <i>Burd</i> button
1570		<i>max.mc</i> the non step 27, set the additional parameters below, then enck the <i>Kun</i> ² button.
15//		<u>I/O:</u>
1578		One of the 2 unfiltered half-maps: Refine3D/job093/run_half1_class001_unfil.mrc
1579		Solvent mask: MaskCreate/job086/mask.mrc
1580		Calibrated pixel size (\mathring{A}) -1
1581		Sharpen:
1582		Estimate R-factor automatically? Vas
1502		Estimate D'fuctor automatically: les
1202		Lowest resolution for auto-B fit (A): 10
1584		Use your own B-factor? No
1585		Skip FSC-weighting? No
1586		<i>MTF of the detector (STAR file): k3-CDS-300keV-mtf.star</i>
1587		Original detector pixel size: -1
1588		Use Chimera's to visualize the improvements to the <i>nostprocess</i> masked mrc map. The GS-FSC $_{0.142}$ for this
1500		man with marking improved from 2.00 Å and alternative interval did abain domain of the second
1505		The point of the set
1590		throughout the map (Figure 10, step 31).
1591	32.	CTF Refinement (Anisotropic Magnification, Round 1)
1592		The next three steps will utilize the CTF refinement job to improve the CTF fits for the particle set. The
1593		three jobs perform corrections for 1) anisotropic magnification, 2) asymmetrical and symmetrical
1594		aberrations, and 3) recalculates per-particle defocus and per-micrograph astigmatism. Together these steps
1595		improve CTF fits that translate into improvements in the reconstruction. The first job will correct for
1596		anisotronic magnification Select the CTF refinement ich set the Particles (from Refine 2D) to the
1507		unsouropic magnification. Select the Control and State a
1221		run_uuu.suur me nom step 50, set tue <i>Postprocess STAR jue</i> to tue <i>postprocess.star</i> me from step 51, set
1228		the parameters below, then click the <i>Run!</i> button.
1599		<u>I/O:</u>
1600		Particles (from Refine3D): Refine3D/job093/run_data.star
1601		Postprocess STAR file: PostProcess/job095/postprocess.star

1602		Fit:
1603		<i>Estimate (anisotropic) magnification? Yes</i>
1604		Minimum resolution for fits (A) : 30
1605		This job estimated a magnification anisotropy of 0.31% and stored the refined particles to the
1606		particles ctf refine.star file.
1607	33.	CTF Refinement (Asymmetrical and Symmetrical Aberrations, Round 1)
1608		Use the refined particles from the previous job to correct for asymmetrical and symmetrical aberrations.
1609		Select the CTF refinement job, set <i>Particles (from Refine3D)</i> to the <i>particles_ctf_refine.star</i> file from step
1610		32, set <i>Postprocess STAR file</i> to the <i>postprocess.star</i> file from step 31, set the parameters below, then click
1611		the <i>Run!</i> button.
1612		<u>I/O:</u>
1613		Particles (from Refine3D): CtfRefine/job096/particles_ctf_refine.star
1614		Postprocess STAR file: PostProcess/job095/postprocess.star
1615		<u>Fit:</u>
1616		Estimate (anisotropic) magnification? No
1617		Perform CTF parameter fitting? Yes
1618		Fit defocus? Per-particle
1619		Fit astigmatism? Per-micrograph
1620		Fit B-factor? No
1622		Fil phase-shijl? No
1622		Estimate definitions? No
1624		Estimate 4 order aberrations? No Minimum resolution for fits (\hat{A}) : 20
1625		The refined particles are stored in the <i>particles</i> of <i>rafine star</i> file and the results of the job can be
1626		visualized by opening the logfile ndf
1627	34	CTE Refinement (Recalculate Defocus and Astigmatism Round 1)
1628	51.	Next recalculate defocus values on a per-particle basis and astigmatism on a per-micrograph basis. Select
1629		the CTF refinement job, set <i>Particles (from Refine3D)</i> to the <i>particles ct, refine star</i> file from step 33, set
1630		<i>Postprocess STAR file</i> to the <i>postprocess.star</i> file from step 31, set the additional parameters below, then
1631		click the <i>Run!</i> button.
1632		I/O:
1633		Particles (from Refine3D): CtfRefine/job097/particles ctf refine.star
1634		Postprocess STAR file: PostProcess/job095/postprocess.star
1635		<u>Fit:</u>
1636		Estimate (anisotropic) magnification? No
1637		Perform CTF parameter fitting? No
1638		Estimate beamtilt? Yes
1639		Also estimate trefoil? Yes
1640		Estimate 4 th order aberrations? Yes
1641		Minimum resolution for fits (A) : 30
1642		The refined particles are saved to the <i>particles_ctf_refine.star</i> file and are now ready for 3D refinement.
1643	35.	3D Auto-Refine (CTF Refined Particles, Round 1)
1644		Generate a new 3D volume with the refined particles. Select the 3D auto-refine job, set <i>Input images STAR</i>
1645		<i>file</i> to the <i>particles_ctf_refine.star</i> file from step 34, set <i>Reference map</i> to the <i>run_half1_class001.mrc</i> file
1040		from step 30, set the parameters below, ensure that the helical parameters are updated to the optimized twist
1647		and rise values from step 50 (these are found in the output log from step 50), then click the <i>Run!</i> button.
1640		<u>I/U:</u> Input imagas STAP file: CtfPafina/iob002/narticles_atf_rafina star
1650		Reference man: Refine3D/ioh003/run half1_class001_unfil mrc
1651		Reference map. RefinesD/j000/5/ un_nuj1_class001_unj1.nrc
1652		Reference:
1653		Ref. map is on absolute grevscale? No
1654		Initial low-pass filter (Å): 4.5
1655		Symmetry: C1
1656		<u>CTF:</u>
1657		Do CTF-correction? Yes

1658		Ignore CTFs until first neak? No
1650		Ontimization
1059		$\frac{Optimization}{Optimization} = \chi^2 + 220$
1000		Mask alameter (A): 220
1661		Mask individual particles with zeros? Yes
1662		Use solvent-flattened FSCs? No
1663		Auto-sampling:
1664		Initial angular sampling: 3.7 degrees
1665		Initial offset range (nix).
1666		Initial offset runge (pix). S
1000		Initial offset step (pix): 1
1667		Local searches from auto-sampling: 1.8 degrees
1668		Relax symmetry: Leave blank
1669		Use finer angular sampling faster? (No)
1670		Helix:
1671		Do belical reconstruction? Yes
1672		Tube diameters inner outer $(\hat{\lambda})$: 1.140
1672		Tube unimeter - inner, outer (A), -1, 140
1073		Angular search range – rot, tilt, psi (aeg): -1, 15, 10
1674		Range factor of local averaging: -1
1675		Keep tilt-prior fixed: Yes
1676		Apply helical symmetry? Yes
1677		Number of unique asymmetrical units: 15
1678		Initial twist (deg) rise (A) : 179 448 2 42
1679		Contral 7 Ionato (40): 25
1600		Central Length (70), 25
1000		Do local searches of symmetry? Tes
1681		<i>Iwist search – Min, Max, Step (deg): 1/9.24, 1/9.65, 0.01</i>
1682		<i>Rise search – Min, Max, Step (A): 2.2, 2.6, 0.01</i>
1683		After CTF refinement, the resolution of the unmasked 3D reconstruction improved from 3.00 Å to 2.38 Å
1684		(Figure 10, step 35). The helical parameters did not change, and converged to a twist of 179.45° and a rise
1685		of 2.42 Å. The 3D map is saved to the <i>run_class001 mrc</i> file and can be opened in ChimeraX for
1686		visualization
1607	26	
1007	50.	
1688		Apply the mask from step 2/ to recalculate the FSC and sharpen the map. Select the Post-processing job,
1689		set One of the 2 unfiltered half-maps to the run_half1_class001_unfil.mrc file from step 35, set Solvent
1690		<i>mask</i> to the <i>mask.mrc</i> file from step 27, set the additional parameters below, and click the <i>Run!</i> button.
1691		I/O:
1692		One of the 2 unfiltered half-maps: Refine3D/iob099/run half] class001 unfil mrc
1693		Solvent mask/ MaskCreate/ioh086/mask mrc
1604		Calibrated pixel size (Å)
1094		
1695		Sharpen:
1696		Estimate B-factor automatically? Yes
1697		<i>Lowest resolution for auto-B fit (Å): 10</i>
1698		Use your own B-factor? No
1699		Skip FSC-weighting? No
1700		MTF of the detector (STAR file): k3-CDS-300keV-mtf star
1701		Drivingl detector pixel size 1
1701		The D factor was estimated to 55 and emplied to the man. The resolution of the modeled man immerved
1702		The B-factor was estimated to -55 and applied to the map. The resolution of the masked map improved
1703		from 2.89 A to 2.31 A (Figure 10, step 36). The 3D map was saved to the <i>postprocess_masked.mrc</i> file and
1/04		can be visualized in ChimeraX.
1705	37.	Bayesian Polishing (Round 2)
1706		Perform one more cycle of polishing and CTF refinement before a final round of 3D refinement and
1707		postprocessing (step 29-36). Select the Bayesian polishing job. set <i>Micrographs (from MotionCorr)</i> to the
1708		corrected microroranhs star file from step 2 set Particles from Refine 3D or CtfRefine to the run data star
1709		file from sten 35 set Postnacoss STAR file to the nostnacoss star file from sten 26 set the additional
1710		me nom step 55, set i ospioless start file to the posipioless star me nom step 50, set the additional
1710		parameters below, and click the <i>kun!</i> button.
1/11		<u>I/O:</u>
1712		Micrographs (from MotionCorr): MotionCorr/job002/corrected_micrographs.star
1713		Particles from Refine 3D or CtfRefine: Refine3D/job099/run_data.star

1714		Postprocess STAR file: PostProcess/job100/postprocess.star
1715		First movie frame: 1
1716		Last movie frame: -1
1717		Extraction size (pix in unbinned movie): -1
1718		Re-scale size (pixels): -1
1719		Write output in float 16? Yes
1720		Train:
1721		Train optimal parameters? No
1722		Polish:
1723		Perform particle polishing? Yes
1724		Ontimized parameter file: Leave blank
1725		OR use your own narameters?
1726		Sigma for velocity (Å/dose): 0.2
1720		Sigma for divergence (Å): 5000
1728		Sigma for acceleration (Å/dose): 2
1720		Signa for acceleration (A/aose). 2 Minimum resolution for P factor fit (\hat{A}) : 20
1729		Minimum resolution for D-factor fit (\hat{A}) , 1
1721		Maximum resolution for D-factor fit (A): -1 The policibod particles are stared in the chimustan file
1731	20	2D Arte Definition (Definition Description Description)
1/32	38.	3D Auto-Refine (Polished Particles, Round 2)
1733		Use the polished particles from step 3/ and perform a round of 3D refinement. Select the 3D auto-refine
1734		job, set <i>Input images STAR files</i> to the <i>shiny.star</i> file from step 37, set <i>Reference map</i> to the
1/35		<i>run_half1_class001_unfil.mrc</i> file from step 35, set the parameters below, and click the <i>Run!</i> button.
1/36		
1/3/		Input images STAR file: Polish/job101/shiny.star
1/38		Reference map: Refine3D/job099/run_half1_class001_unfil.mrc
1/39		Reference mask (optional): Leave blank
1740		Reference:
1741		Ref. map is on absolute greyscale? No
1742		Initial low-pass filter (Å): 4.5
1743		Symmetry: C1
1744		<u>CTF:</u>
1745		Do CTF-correction? Yes
1746		Ignore CTFs until first peak? No
1747		Optimization:
1748		Mask diameter (Å): 220
1749		Mask individual particles with zeros? Yes
1750		Use solvent-flattened FSCs? No
1751		Auto-sampling:
1752		Initial angular sampling: 3.7 degrees
1753		Initial offset range (pix): 5
1754		Initial offset step (pix): 1
1755		Local searches from auto-sampling: 1.8 degrees
1756		Relax symmetry: Leave blank
1757		Use finer angular sampling faster? (No)
1758		Helix:
1759		Do helical reconstruction? Yes
1760		Tube diameter – inner outer $(Å)$: -1 140
1761		Angular search range – rot tilt $nsi (deg): -1$ 15 10
1762		Range factor of local averaging: -1
1763		Keen tilt-nrior fixed: Ves
1764		Apply helical symmetry? Yes
1765		Number of unique asymmetrical units: 15
1766		Initial twist $(d\rho\sigma)$ rise (\hat{A}) : 179.440.2.42
1767		Contral 7 longth (%). 25
1768		Do local sources of symmetry? Yes
1769		$Twist search = Min Max Sten (deg) \cdot 170 24 170 65 0.01$
1,02		1 wist search – 14111, 1414A, Step (ueg). 1/7.24, 1/7.05, 0.01

1770		Rise search – Min, Max, Step (Å): 2.2, 2.6, 0.01
1771		After a 2^{nd} round of polishing the unmasked map did not improve in resolution staving at 2.38 Å (Figure
1772		10 sten 38) Next we will see if there is an improvement in the marked reconstruction
1772	20	To, step 50). Ivex, we will see it diele is an improvement in the masked reconstruction.
1775	39.	Post-Processing
1//4		Select the Post-processing job, set <i>One of the 2 unfiltered half-maps</i> to the <i>run_half1_class001_unfil.mrc</i>
1775		file from step 38, set the <i>Solvent mask</i> to the <i>mask.mrc</i> file from step 27, set the additional parameters
1776		below, and click the <i>Run!</i> button.
1777		I/O:
1778		\overline{One} of the 2 unfiltered half-maps: Refine3D/iob102/run half1_class001_unfil mrc
1779		Solvent mask: Mask Create/inh086/mask mr
1700		Galikusta al anisa (k) 1
1700		Calibratea pixel size (A) -1
1/81		Sharpen:
1782		Estimate B-factor automatically? Yes
1783		Lowest resolution for auto-B fit (\mathring{A}) : 10
1784		Use your own B-factor? No
1785		Skin FSC-weighting? No
1786		MTF of the detector (STAR file): k3-CDS-300keV-mtf star
1700		Original dataton visal size:
1700		Original delector pixel size1
1788		The resolution of the masked reconstruction increased slightly from 2.31 A to 2.27 A (Figure 10, step 39).
1789		The 3D map is stored in the <i>postprocess_masked.mrc</i> file and can be visualized in ChimeraX.
1790	40.	CTF Refinement (Anisotropic Magnification, Round 2)
1791		Perform a final round of CTF refinements as in steps 32-34. Select the CTF refinement job, set <i>Particles</i>
1792		(from Refine 3D) to the run data star file from step 38, set Postprocess STAR file to the postprocess star file
1793		from step 39 set the parameters below then click the <i>Run</i> / button
170/		
1705		$\frac{VO}{D}$
1795		Particles (from ReginesD): RefinesD/job102/run_aata.star
1796		Postprocess SIAR file: PostProcess/job103/postprocess.star
1797		<u>Fit:</u>
1798		Estimate (anisotropic) magnification? Yes
1799		Minimum resolution for fits (A) : 30
1800		The refined particles are stored in the <i>particles ctf refine star</i> file and will be used in the next step.
1801	41	CTE Refinement (Asymmetrical and Symmetrical Aberrations Round 2)
1802		Select the CTF refinement ich set Particles (from Refine3D) to the particles eff refine star file from step
1002		Select the CTT remember 100, set Taractes from Repueblo to the paractes cig-repuestar the non-sep
1005		40, set <i>Postprocess STAR fue</i> to the <i>postprocess.star</i> the from step 59, set the parameters below, then click
1804		the <i>Run</i> ! button.
1805		<u>I/O:</u>
1806		Particles (from Refine3D): CtfRefine/job104/particles ctf refine.star
1807		Postprocess STAR file: PostProcess/iob103/postprocess.star
1808		Fit:
1809		
1810		Darform (TE normotor fitting? Vos
1010		Terform City parameter Juting: Tes
1011		Fil defocus? Per-particle
1812		Fit astigmatism? Per-micrograph
1813		Fit B-factor? No
1814		Fit phase-shift? No
1815		Estimate beamtilt? No
1816		Estimate 4 th order aberrations? No
1817		Minimum resolution for fits (\hat{A}) : 30
1010		The new fields were written out to the new fields, at refue star file and will be used in the next star
1010	40	The particles were written out to the <i>particles</i> $\frac{1}{2}$ $\frac{1}$
1020	42.	CIF Keineineni (Kecalculate Delocus and Astigmatism, Kound 2)
1820		Select the CIF retinement job, set <i>Particles (from Refine3D)</i> to the <i>particles_ctf_refine.star</i> file from step
1821		41, set <i>Postprocess STAR files</i> to the <i>postprocess.star</i> file from step 39, set the additional parameters below,
1822		then click the <i>Run!</i> button.
1823		<u>I/O:</u>
1824		Particles (from Refine3D): CtfRefine/job105/particles ctf refine.star
1825		Postprocess STAR file: PostProcess/iob103/postprocess star
-0-0		1 ospioeos surrigino, 1 osti i ocessigo i osposipi ocessistui

1826		Fit-
1020		<u>Internet de la constructional de la constructiona </u>
1027		Estimate (anisotropic) magnification? No
1828		Perform CIF parameter fitting? No
1829		Estimate beamtilt? Yes
1830		Also estimate trefoil? Yes
1831		<i>Estimate</i> 4 th order aberrations? Yes
1832		Minimum resolution for fits (A) : 30
1833		The refined particles are stored in the <i>particls</i> ctf refine star file and are ready for 3D refinement.
1834	43	3D Auto-Refine (CTE Refined Particles Round 2)
1025	чу.	Dur a 2D reference in the articles, Round 2D reference in the set lumit images STAD
1035		Run a 5D remientent using the CTF remied particles. Select the 5D auto-remie job, set <i>mput images STAK</i>
1830		file to the particles_ctf_refine.star file from step 42, set Reference map to the run_half1_class001_unfil.mrc
1837		file from step 38, set the additional parameters below, and click the <i>Run!</i> button.
1838		<u>I/O:</u>
1839		Input images STAR file: CtfRefine/job106/particles ctf refine.star
1840		Reference map: Refine3D/job102/run half1 class001 unfil.mrc
1841		Reference mask (optional): Leave blank
1842		Reference:
1042		Part man is on absolute annuagle? No
1043		Kej, map is on absolute greyscale? No
1844		Initial low-pass filter (A): 4.5
1845		Symmetry: C1
1846		<u>CTF:</u>
1847		Do CTF-correction? Yes
1848		Ignore CTFs until first peak? No
1849		Ontimization
1850		Mask diamator (Å): 220
1050		Musk utameter (A). 220
1051		Mask individual particles with zeros? Tes
1852		Use solvent-flattened FSCs? No
1853		Auto-sampling:
1854		Initial angular sampling: 3.7 degrees
1855		Initial offset range (pix): 5
1856		Initial offset step (pix): 1
1857		Local searches from auto-sampling. 18 degrees
1858		Relax symmetry: Larva blank
1950		Here and the second frequency of the second se
1059		Use inter angular sampling juster: (100)
1860		<u>Helix</u>
1861		Do helical reconstruction? Yes
1862		Tube diameter – inner, outer (A): -1, 140
1863		Angular search range – rot, tilt, psi (deg): -1, 15, 10
1864		Range factor of local averaging: -1
1865		Keen tilt-prior fixed. Yes
1866		Annly helical symmetry? Yes
1867		Number of unique asymmetrical units: 15
1007		Number of unique asymmetrical units. 15
1868		Initial twist (deg), rise (A): 1/9.449, 2.42
1869		Central Z length (%): 25
1870		Do local searches of symmetry? Yes
1871		<i>Twist search – Min, Max, Step (deg): 179.24, 179.65, 0.01</i>
1872		Rise search – Min, Max, Step (Å): 2.2, 2.6, 0.01
1873		The resolution of the unmasked map increased slightly from 2.38 Å to 2.35 Å (Figure 10, step 43). This
1874		result suggests that any additional rounds of polishing or CTF refinement will not vield meaningful gains in
1875		man quality and are thus not necessary
1075	11	map quanty and are thus not necessary. Maste Creation (259/ Maste)
1077	44.	$\frac{1}{12} \frac{1}{12} \frac$
1070		In the <i>Helix</i> tab of the 3D auto-refine job we set the <i>central Z length</i> to 25% of the particle box. This
18/8		central region is where searching for helical symmetry occurs and is also the region where real-space
1879		helical symmetry is imposed. In the previous Mask creation job from step 27, the mask length was set to
1880		80% of the central axis to ensure enough signal was available for CTF refinements. Now, in the final stages
1881		of processing we can reduce the mask size to a <i>central Z length</i> of 25% as was used in the 3D

1882 1883 1884 1885		reconstruction steps. As in step 27, you may need to open the <i>run_class001.mrc</i> file from step 43 in ChimeraX to determine the appropriate <i>Initial binarization threshold</i> for the reconstruction. A value of 0.0011 worked for well for us. Select the Mask creation job, set <i>Input 3D map</i> to the <i>run_class001.mrc</i> file from step 43, set the parameters below, then click the <i>Run!</i> button.
1886		<u>I/O:</u>
1887		Input 3D map: Refine3D/job107/run_class001.mrc
1888		Mask:
1889		Lowpass filter map (A) 15
1890		Pixel size $(A) - 1$
1891		Initial binarization threshold: 0.0011
1892		Extend binary map this many pixels: 5
1893		Add a soft-edge of this many pixels: 5
1894		Helix:
1895		Mask a 3D helix? Yes
1896		Central Z length (%): 25
1897		The mask is saved to the mask.mrc file and will be used in the next step (Figure 11B).
1898	45.	Post-Processing
1899		Apply the latest mask from step 44 to the final reconstruction from step 43 to recalculate the resolution and
1900		B-factor. Select the Post-processing job, set One of the 2 unfiltered half-maps to the
1901		run_half1_class001_unfil.mrc file from step 43, set Solvent mask to the mask.mrc file from step 44, set the
1902		additional parameters below, then click the Run! button.
1903		<u>I/O:</u>
1904		One of the 2 unfiltered half-maps: Refine3D/job107/run half1 class001 unfil.mrc
1905		Solvent mask: MaskCreate/job109/mask.mrc
1906		Calibrated pixel size $(A) - I$
1907		Sharpen:
1908		Estimate B-factor automatically? Yes
1909		Lowest resolution for auto-B fit (A) : 10
1910		Use your own B-factor? No
1911		Skip FSC-weighting? No
1912		MTF of the detector (STAR file): k3-CDS-300keV-mtf.star
1913		Original detector pixel size: -1
1914		The final masked map has a resolution of 2.04 Å and a B-factor of -40 (Figure 10, step 45). The map
1915		displays well resolved side chain densities as expected for a map at ~ 2 Å resolution.
1916		NOTE: We observe a spike in the FSC plot at ~ 2.4 Å (the repeating unit) in both our reconstruction and in
1917		several published structures (Figure 12) [22,27,44]. This spike is alleviated with masking, but it is a
1918		common feature observed in amyloid structures that resolve to high resolution. Additionally, other helical
1919		structures, such as tad pili, also display a similar spike due to the strong signal at the repeating unit of ~ 4.9
1920		Å [45].
1921		



1922Spatial frequency (A*)Spatial frequency (A*)1923Figure 12. Comparison of Fourier Shell Correlation (FSC) plots of α-synuclein maps deposited to the1924EMDB resolving to below 2.3 Å. The unmasked FSC plots (calculated FSC from deposited half maps,1925orange) for the deposited maps display a FSC spike at a spatial frequency of 0.4 Å⁻¹ (~2.4 Å). The masked1926FSC plots (author provided FSC, blue) dampen this feature.

1927		
1928	46.	Local resolution
1929		Calculate a local resolution map to understand the differences in resolution across the map. Select the
1930		Local resolution job, set One of the 2 unfiltered half-maps to the run half1 class001 unfil.mrc file from
1931		step 43, set User-provided solvent mask to the mask.mrc file from step 44, set the additional parameters
1932		below, then click the <i>Run!</i> button.
1933		<u>I/O:</u>
1934		One of the 2 unfiltered half-maps: Refine3D/job107/run half1 class001 unfil.mrc
1935		User-provided solvent mask: MaskCreate/job109/mask.mrc
1936		Calibrated pixel size (Å): 0.834
1937		ResMap:
1938		Use ResMap? No
1939		Relion:
1940		Use Relion? Yes
1941		User-provided B-factor: -40
1942		MTF of the detector (STAR file): k3-CDS-300keV-mtf.star
1943		The job results in a <i>histogram.pdf</i> file that contains a graph of the local resolution within the provided
1944		mask. The relion locres.mrc file can be opened in ChimeraX along with the postprocess.mrc file from step
1945		45 to color the surface of the map by resolution (Figure 13). Please see the "Analyzing the results" section
1946		in the RELION local resolution documentation page for details on handling these maps in ChimeraX
1947		(https://relion.readthedocs.io/en/latest/SPA_tutorial/Validation.html).
1948		





Α

Figure 13. Local resolution map of α -syn fibril from cryo-EM data. A. Local resolution map of filamentous α -syn depicting loss of resolution towards the end of the fibril, with the best resolution located along the central portion of the map. B. Cross-section of the local resolution map of filamentous α -syn

1953 1954		showing the best resolved regions of the map are located along the fibril core and protofilament interface.
1055		A7 Real-Space Symmetrization (Ontional)
1956		As stated previously real space symmetry is applied to only the central 25% of the reconstruction and the
1057		molecular model is built into this central region (Figure 1E). However, in some cases it is beneficial to
1958		extend the symmetrization to the edge of the box. For example, to better visualize the crossover distance we
1959		generate a map with real-space symmetry imposed to the edge of the box, then we align several models in
1960		ChimeraX to generate a multi-map volume that spans close to 1000 Å (Figure 1A). This process allows for
1961		easier visualization of the crossover distance when making figures. To impose real-space symmetry run the
1962		relion_helix_toolbox command in the terminal. Before running the command, cd to the job directory for
1963		step 45.
1964		relion helix toolboximposei postprocess masked.mrco postprocess masked sym.mrc
1965		cyl outer diameter 220 angpix 0.834 rise 2.42 twist 179.45 z percentage 0.25
1966		The arguments used in the command above are as follow:
1967		impose apply real-space helical symmetry
1968		<i>i</i> input file
1969		o output file
1970		cyl_outer_diameter outer diameter of the cylindrical mask
1971		angpix pixel size in angstroms
1972		rise helical rise in angstroms
1973		twist helical twist in degrees
1974		z_percentage central z-length
1975		
1976	F.	Model building and validation for alpha-synuclein fibrils.
1977		There are many methods for building molecular models. Here we used PDB 6H6B as a starting point, the model
1978		was fit into the EM map using ChimeraX, then one subunit was rebuilt and refined in Coot. The monomer
1979		model was subjected to a round of real-space refinement in Phenix. Then, ChimeraX was used to fit additional
1980		refined subunits into the map to generate a multimer model. The multimer model was subjected to final round of
1001		real space refinement in Dhenix. We encourage users of this protocol to review tutorials and manuals for

real-space refinement in Phenix. We encourage users of this protocol to review tutorials and manuals for
 ChimeraX, Coot, and Phenix before proceeding with model building [40-42,46,47]. During the modeling
 process users should use our refined model PDB 9CK3 as a reference. An overview of the entire modeling and
 validation workflow is provided for reference (Figure 14).

1985





Figure 14. Model building and validation protocol for α-synuclein fibrils. Step by step protocol for
 building, refining, and validating a α-synuclein fibril molecular model. This protocol uses ChimeraX, Coot, and

1989 Phenix in an iterative fashion to improve the molecular model.

1991	1.	Download PDB 6H6B by running open 6h6b from the ChimeraX command line [12]. This model covers
1992		residues 38-95 of the α -synuclein protein and contains 10 monomers displaying the amyloid fold.
1993	2.	Open the final <i>postprocess.mrc</i> file from section G step 45 and under <i>Volume viewer</i> set <i>step size</i> to 1.
1994	3.	In ChimeraX, use the Fit tool to place PDB 6H6B into the central region of the postprocess.mrc file. You
1995		may need to rotate the model to correctly fit the model into the map.
1996	4.	Run the command below from the ChimeraX command line to trim the ends of the map for easier
1997		visualization of the central region. Then continue fitting until the model is well placed in the map.
1998		view orient; clip front - 30 back 30
1999	5	NOTE: Chipping can be turned off by running <i>clip off</i> from the ChimeraX command line.
2000	5.	line. We will use Coot to build and refine one subunit and add additional subunits later
2002	6	Save the file as a PDB ensure that <i>Save relative to model</i> is checked and in the dron down menu select the
2003	0.	postprocessed map.
2004	7.	In Coot, go to File \rightarrow Open Coordinates and select the PDB file saved in step 6. The go to File \rightarrow Open
2005		Map and select the postprocessed map from section G step 45.
2006		NOTE: We encourage new users to review the Coot tutorial to become familiar with the software before
2007		proceeding (https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/web/tutorial/tutorial.pdf).
2008	8.	On the right-hand side is the modeling toolbar, click Map, then click Estimate to set the map weight, then
2009		click Ok.
2010		NOTE: Click the arrow at the bottom of the modeling toolbar and select <i>Icons and text</i> to add the name of
2011	0	each tool to the modeling toolbar.
2012	9.	Go to Refine \rightarrow All-atom Refine to improve the fit of the map to the model. When the refinement is done
2013		Click Accept to save the refined atom positions. The refinement will impose geometry restraints and
2014		NOTE: Use the mouse scroll wheel to adjust the man contour level as necessary throughout this process
2015	10	The density generated in this protocol allows for modeling of additional residues not resolved in PDB
2010	10.	6H6B so we need to build additional regions of the model. Go to leucine 38 located at the n-terminus
2018		from the modeling toolbar click <i>Add Terminal Residue</i> and click on leucine 38, this will add an alanine
2019		residue to the n-terminus. From the modeling toolbar click <i>Simple Mutate</i> , then click on alanine 37, a
2020		window will appear listing all the amino acids, click Val (V) to change alanine 37 to a valine. From the
2021		modeling toolbar click <i>Real Space Refine Zone</i> , then click on valine 37 and valine 40, this will refine the
2022		region between these two residues and improve the fit of the model to the map, then click Accept to save
2023		the refined atom positions.
2024	11.	Repeat step 10, to add glycine 36 to the n-terminus, and lysine 96 and lysine 97 to the c-terminus.
2025	12.	Next, build residues 15 to 22 into a well resolved island of density located near the n-terminus. Go to the
2026		island of density and rotate the density so the fibril core is oriented towards the top of the screen, this will
2027		help minimize the number of movements needed to place the strand into place. Go to Calculate \rightarrow Other
2028		Modelling lools \rightarrow Place Strand Here, set Estimated number of restaues in strand to 8 and click Go. A
2029		the fit of the strand into the man
2030	13	Use the <i>Simple Mutate</i> tool to change the alapine strand to the correct residues (V15, V16, A17, A18, A19)
2032	15.	E20. K21. T22). Then use the <i>Real Space Refine Zone</i> tool to further improve the fit of the strand.
2033		NOTE: The n-terminus (i.e. island of density) folds back towards the fibril core adjacent to residues 36 to
2034		44, with residue 15 closest to the fibril core.
2035	14.	Click on Display Manager, you will see that there are two molecules, one is the PDB that was imported and
2036		the second is the new strand that was created. We need to renumber the residues in the new strand, merge
2037		the molecules and fix the chain ID. Go to Edit \rightarrow Renumber Residues, under Renumber Residue Range of
2038		Molecule select the newly generated strand, under Start Residue select N-terminus, in the Apply Offset box
2039		provide an integer value to correct for the difference in residue number for the residue that should be valine
2040		15, then click <i>Apply</i> . For example, if the value on the n-terminus of the strand is labeled as V40 then the
2041		onset should be -25 to set the value to residue 15. Click on the n-terminus value of the strand to verify the numbering is correct.
2042	15	numbering is context. To merge the molecules Go to Edit \rightarrow Marga Molecules under Annand/Insert Molecule(s) solat the strend
2043	13.	and under into Molecule select the original PDR from the dron-down menu, then click Marga
2045	16	Change the chain IDs so both fragments are labeled as chain A Go to <i>Edit</i> \rightarrow <i>Change Chain IDs</i> under
2046	10.	Change Chain ID in Molecule select the file that contains both fragments (i.e. the recently merged
		5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5

molecule), under *From Chain ID* select either chain, under *Using Residue Selection* select *Whole Chain*, under *To Chain ID* set this value to *A*, then click *Apply New Chain ID*. Repeat the process if the second fragment is labeled anything other than chain A. The fragments should now be one molecule labeled as chain A with a dotted line showing the missing residues from residues 23 to 36 that are not resolved.

- 2051 17. Refine the new molecule that spans residues 15-22 and 37-98. Go to *Refine* → *All-atom Refine*, if the atoms are well positioned click *Accept*. If not, manually adjust misplaced atoms by dragging the atoms into place and then click *Accept*.
 2053 18. Save the coordinates, go to *File* → *Save Coordinates*, under *Select Molecule Number to Save* select the
 - 18. Save the coordinates, go to *File → Save Coordinates*, under *Select Molecule Number to Save* select the molecules that was refined in step 17, click *Select Filename* and save the file to the desired location.
 19. Open Phenix and setup a new project.
- 2056 19. Open Phenix and setup a new project.
 2057 NOTE: We encourage new users to review the Phenix tutorial, specifically the real space refinement tutorial, to become familiar with the software before proceeding (<u>https://phenix-online.org/documentation/reference/real_space_refine.html</u>)
- 2060 20. Under the cryo-Em section select the *Real-space refinement* job. Provide the PDB file from Coot as the 2061 model file and the postprocessed file as the map file. Set Resolution as determined in the final RELION 2062 postprocessing job, in this case the resolution is 2.04 Å. Under the *Refinement Settings* tab, in addition to 2063 the default settings ensure Use secondary structure restraints and Ramachandran restraints is checked, set Nproc to 4, click Rotamers and under Fit select outliers and poormap, then click Run. Upon completion, 2064 2065 the validation report shows that the model statistics are favorable. The *Rotamer outliers (%)* will be slightly 2066 elevated due to a salt bridge that forms between lysine 80 and glutamic acid 46, causing lysine 80 to be a 2067 rotamer outlier that is supported by the data. 2068
 - 21. In ChimeraX, open the refined model and the postprocessed map. Open the refined model again and now two models are available. Select the second model and use the *Fit* tool to place the second monomer into the opposing protofilament. Repeat the process of opening the refined model and fitting it into a new region of the map. For PDB 9CK3 we built a dodecamer model.
 - 22. Once the desired number of subunits are fitted into the map, run *combine* from the ChimeraX command line to merge the subunits into one model. The command should provide a unique chain ID to each subunit.
 - 23. Repeat step 6 to save the model relative to the postprocessed map.
 - 24. In Phenix, repeat real space refinement as in step 20 with the additional parameter *Ncs constraints* selected. The final validation report shows excellent model statistics with only lysine 80 as a rotamer outlier, as expected. This step can be repeated, if necessary. The model is now ready for structure analysis.

Additional Validation.

This protocol or parts of it has been used and validated in the following research articles:

- Dhavale, et al. [33]. Structure of alpha-synuclein fibrils derived from human Lewy boy dementia tissue. Nature Communications. <u>https://doi.org/10.1038/s41467-024-46832-5</u>.
- Montemayor et al. [48]. Flagellar Structures from the Bacterium Caulobacter crescentus and Implications for Phage φ CbK Predation of Multiflagellin Bacteria. Journal of Bacteriology. <u>https://doi.org/10.1128/jb.00399-20</u>.
- Sanchez et al. [49]. Atomic-level architecture of Caulobacter crescentus flagellar filaments provide evidence for multi-flagellin filament stabilization. bioRxiv. https://doi.org/10.1101/2023.07.10.548443.

• The cryo-EM structure in the manuscript has been validated by our submissions to the PDB (9CK3) and EMDB (EMD-45639). <u>https://doi.org/10.2210/pdb9CK3/pdb</u>.

2091 <u>Discussion.</u>

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2092 The fibrilization conditions presented here are specific to one form of *in vitro* assembled α -synuclein fibrils. 2093 Extensive optimization of protein purification and fibrilization conditions, testing buffer conditions and incubation 2094 parameters, may be necessary to generate different in vitro forms. The cryo-EM helical reconstruction methods 2095 presented here assume that fibrils are both twisting and are of sufficient length to determine the crossover distance 2096 for helical twist estimates. There are cases where fibrils may not twist and thus this workflow would not be 2097 amendable to such samples. Finally, structure determination of patient derived fibrils is of high interest, but 2098 extraction of fibrils from patient tissue is outside of the scope of the work presented here. Though in theory, the data 2099 processing methods presented here should be applicable to these samples. 2100

Cryo-EM data processing is dependent on the sample, data collection instrumentation and parameters used, and
 computational hardware and software. What we have presented here should provide users with the necessary details

- 2103 for cryo-EM structure determination of a range of amyloid fibrils. We used this approach to generate a cryo-EM map
- 2104 and atomic model of *in vitro* assembled α -synuclein fibrils; and atomic models were deposited in the Protein Data 2105 Bank (PDB) under accession 9CK3. Cryo-EM maps, including the final map, half-maps, and mask were deposited
- 2106 in the Electron Microscopy Data Bank (EMDB) under accession EMD-45639.
- 21072108 The work presented here, including sample preparation, NS-TEM, cryo-EM data collection, cryo-EM data
- 2109 processing, and molecular model building serves as a starting point for individuals new to cryo-EM structural
- 2110 analyses of amyloid proteins. For cryo-EM structure determination, new samples will pose their own unique set of
- challenges, but by first completing the data processing workflow in section E with the EMPIAR dataset under
- 2112 accession EMPIAR-12229, new users will be more adept at troubleshooting new issues.
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2129 2130 Data deposition.

2131 The atomic model was deposited in the Protein Data Bank under accession 9CK3. Cryo-EM maps were deposited in

- the Electron Microscopy Data Bank under accession EMD-45639. The raw micrographs, gain file, and detector
- 2133 MTF file are available on the EMPIAR-12229 database under accession EMPIAR-12229.
- 2134
- 2135 Competing Interests. The authors declare no competing interests.

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