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Atomic force microscopy 3D structural reconstruction of individual particles in the study of amyloid protein assemblies

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25 **Abstract**

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27 Recent developments in Atomic Force Microscopy (AFM) image analysis have made 3D

28 structural reconstruction of individual particles observed on 2D AFM height images a reality.

29 Here, we review the emerging contact point reconstruction AFM (CPR-AFM) methodology

30 and its application in 3D reconstruction of individual helical amyloid filaments in the context

31 of the challenges presented by the structural analysis of highly polymorphous and

32 heterogeneous amyloid protein structures. How individual particle level structural analysis

33 can contribute to resolving the amyloid polymorph structure-function relationships, the

34 environmental triggers leading to protein misfolding and aggregation into amyloid species,

35 the influences by the conditions or minor fluctuations in the initial monomeric protein

36 structure on the speed of amyloid fibril formation, and the extent of the different types of

37 amyloid species that can be formed, are discussed. Future perspectives in the capabilities of

38 AFM based 3D structural reconstruction methodology exploiting synergies with other recent

39 AFM technology advances are also discussed to highlight the potential of AFM as an

40 emergent general, accessible and multimodal structural biology tool for the analysis of

41 individual bio-molecules.

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Introduction

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48 Since the development of atomic force microscopy (AFM) in the mid-1980s [1], there have

49 been many notable advances in the capabilities of AFM. Developments leveraging AFM's

50 sensitivity to force in the pico-Newton range well below the rupture force of single chemical

51 bonds, high signal-to-ratio three-dimensional (3D) multimodal imaging, and capabilities for

52 studying soft materials and biological systems under ambient conditions in air or in fluid

53 chambers have together enabled imaging of biomolecules to high details [2]. AFM is now

54 emerging as an indispensable tool for the structural analysis of single, individual helical

55 amyloid filament structures [3,4].

56

57 Amyloid structures represent a class of filamentous, proteinaceous assemblies typically 5-20

58 nm in width and between few nanometres to several micrometres in length [5-8]. All amyloid

59 fibrils share a common core structural feature, the cross-β arrangement where β-sheets run

60 perpendicularly to the fibril axis [9-11]. However, variations in the precise packing

61 arrangements of the β-sheets result in multiple fibril structures, often referred to as

62 polymorphs, that can form from the same peptide or protein building blocks even under

63 identical conditions [12,13]. The individual polymorph structures of amyloid fibrils may underlie

64 the different biological responses amyloid elicit and the varied associations of different fibril

65 polymorphs with different neurodegenerative diseases [6,14]. To resolve the extent of amyloid

66 polymorphism, to understand the structure-function relationships of these complex

67 biomolecular assemblies and to develop effective therapeutics targeting specific polymorph

68 structures, one must be able to map the population of amyloid structures in complex mixtures

69 using a technique that can interrogate individual or rarely populated structures in addition to

70 those in the majority. Recent advances in AFM image analysis have made such endeavour

71 possible where 3D structural reconstruction can be carried out from just one individual

72 filament observed without the need to average across particles using AFM height topology

73 images [15-17].

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75 A range of current AFM analysis software tools and workflows for extracting structural

76 information from single biomolecules, for example Gwyddion [18], FibreApp [19],

77 BioAFMviewer [20], TopoStats [21] and NanoLocz [22], are based on detailed 2D analysis of AFM

78 height topology images. Here, we review the recent advances in 3D structural analysis of

79 individual helical amyloid filaments with contact point reconstruction AFM (CPR-AFM) [4,17].

80 We discuss the links and the synergies to existing analysis workflows, and the exciting future

81 outlooks for AFM as a powerful and unique emergent technology capable of resolving the

82 structure and dynamics of bio-molecules, one individual particle at time.

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85 **Challenges in the structural analysis of polymorphic amyloid assemblies**

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87 Amyloid fibrils are associated with disorders including Alzheimer's disease (AD),

88 Parkinson's disease (PD), type II diabetes and various systemic amyloidosis where the

89 accumulation and deposition of amyloid fibrils occur in affected tissues and organs [6,8]. In

90 some instances, amyloid assemblies can also play crucial roles in providing regular

91 physiological functions in a broad range of hosts, including bacteria, fungi, and mammals

92 [23,24]. These functional amyloid structures are capable of multiple roles in biology. For

93 example, they can provide structural support to maintain the integrity of biological structures

94 [25], promote adhesion for organisms to surfaces or to each other [26], facilitate the formation of

95   protective barriers or matrices to shield cells from environmental stresses [24], and contribute to

96   long-term memory storage [27].

97

98   Recent rapid advances in amyloid structural biology methodologies have resulted in detailed

99   maps of amyloid fibril structures [14]. These advances have also revealed a key characteristic of

100   amyloid assembly in their capacity for displaying structural polymorphism, wherein protein

101   or peptide precursors of identical amino acid sequence can assemble into a variety of distinct

102   fibril structures, often called polymorphs. Amyloid formation, even under identical

103   environmental conditions, may result in highly complex and heterogeneous samples [12]. The

104   high degree of amyloid sample heterogeneity has been observed regardless of if the fibrils

105   were formed in vitro or purified from human patients' tissues [3,28,29]. The limited availability

106   of analysis workflows to quantify and analyse the nuanced variation between structural

107   features of amyloid polymorphs poses further challenge. This has rendered traditional

108   structural biology methods such as X-ray crystallography unable to resolve the detailed

109   structures of amyloid. Instead, advances in solid-state nuclear magnetic resonance

110   spectroscopy (ssNMR) and cryogenic transmission electron microscopy (cryo-EM) have

111   resulted in structural understanding of amyloid fibrils at near-atomic details, including

112   amyloid fibrils purified from post-mortem human brain tissues [28,30]. In particular, the

113   'resolution revolution' of cryo-EM saw drastic improvement in the resolution of single-

114   particle 3D reconstructions of biological structures such as amyloid fibrils in the past half-

115   decade. What was previously a low to moderate resolution technique, cryo-EM is now

116   capable of providing near-atomic resolution maps of amyloid cores typically at 3Å or better

117   [12,31].

118

119   While cryo-EM and ssNMR offered valuable insights into the core architecture of *in vitro*

120   assembled or *ex vivo* amyloid fibrils purified from human patient tissues, they provide static,

121   averaged snapshots of one or few highly populated polymorphs in typically highly complex

122   amyloid populations. Therefore, for polymorphous amyloid fibrils, methods that can

123   interrogate individual or rarely populated structures in addition to average structures of those

124   in the majority have become important if key questions on the polymorph structure-function

125   relationships, the environmental conditions that trigger the pathway leading to misfolding and

126   aggregation, and the influences by the conditions or minor fluctuations in the initial

127   monomeric protein structure on the speed of fibril formation and the types of abnormal

128   species formed, are to be resolved. Super-resolution microscopy (SRM) has shown potential

129   to resolve individual amyloid filaments in aqueous environments, and the resolution

130   necessary for individual filament structural characterisations can be reached through the use

131   of transiently bound fluorescent dyes and deconvolution algorithms [32]. Crucially, SRM is

132   capable of allowing dynamic processes such as fibril elongation to be observed on an

133   individual filament level [32,33]. Resolving complex structures *in situ* in cells or in tissue can be

134   achieved through cryogenic electron tomography (cryo-ET) [34]. In terms of amyloid structures,

135   cryo-ET presents an important opportunity in that it is capable of resolving individual

136   filament structures *in situ* in tissue sections, which reveal information that can potentially be

137   used for localisation and spatial correlation studies. However, similar to cryo-EM, cross-

138   particle averaging by subtomogram averaging methods must currently be carried out from the

139   low signal-to-noise ratio cryo-ET tomograms to generate averaged structural maps that are of

140   sufficiently high resolution to be used in template matching analysis to known filament

141   structures [35]. Thus, neither SRM nor cryo-ET can resolve sufficient structural details at

142   individual filament level from a single observation to allow quantitative comparative

143   structural analysis or structural analysis of rare species. These challenges and limitations have

144 stimulated development of an AFM based individual particle level structural analysis

145 approach.

146

147 AFM is capable of a broad spectrum of imaging applications spanning from live cells to

148 individual molecules [36]. It has also been applied to probe dynamics [37-39] and molecular

149 responses to mechanical force [40] in air and liquid environments [41-43]. The physics of AFM

150 relies on the interaction between the sample surface and the probe, which comprises of a

151 molecularly sharp conical tip attached to a cantilever spring. As the AFM tip scans across the

152 sample in the x/y plane, high signal-to-noise ratio z-height topography images, with sub-

153 Ångstrom noise levels, can be generated [44]. AFM imaging has been previously used to

154 characterise the size distributions [45-49] and the morphological features [50-52] of amyloid

155 populations by morphometric and dimensions analysis on an individual filament level.

156 Recently, the CPR-AFM method has been developed to extract the 3D information encoded

157 in the height topology images and the necessary information to reconstruct 3D surface

158 envelope models of individual helical amyloid fibrils [3,4,15-17].

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161 **CPR-AFM and 3D reconstruction of individual helical amyloid filaments**

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163 In essence, the key conceptual idea utilised in the CPR-AFM algorithm is the realisation that

164 useful 3D information is encoded in the two-dimensional (2D) AFM height images,

165 essentially as 3D point clouds [4] (**Figure 1**). A point cloud is a discrete set of data points in

166 space that is not gridded in the way pixels or voxels are gridded in conventional images. For

167 AFM height image data, the pixel values representing the z-coordinates are recorded when

168 the sample surface comes into contact anywhere on the probe tip. The recorded centre

169   coordinates of the tip, therefore, do not represent the actual contact points. This is commonly

170   referred to as the tip-sample convolution artefact [53]. The post-experiment CPR-AFM

171   algorithm estimates the 3D coordinates of the actual contact points in 3D space, effectively

172   moving the recorded tip positions off the pixel grid to subpixel locations [17]. Advantageously,

173   the spatial resolution of the information encoded in the data may, therefore, be higher than

174   the estimates based on the distance between the pixels on the image grid for globular or

175   cylindrical filament structures where the actual contact points are on average more closely

176   spaced than the pixels of the original height image [17]. The resulting 3D contact point cloud

177   can be subsequently used to reconstruct the 3D surface envelope of the molecular surface that

178   has interacted with the tip during imaging. In addition, for helical structures such as amyloid

179   fibrils, the 3D surface envelope of the whole filament can be reconstructed since the

180   molecular surfaces around the whole of the filament cross-sections can be inferred using tip-

181   sample contact points from a single side of the helical filament due to the helical symmetry

182   [4,17].

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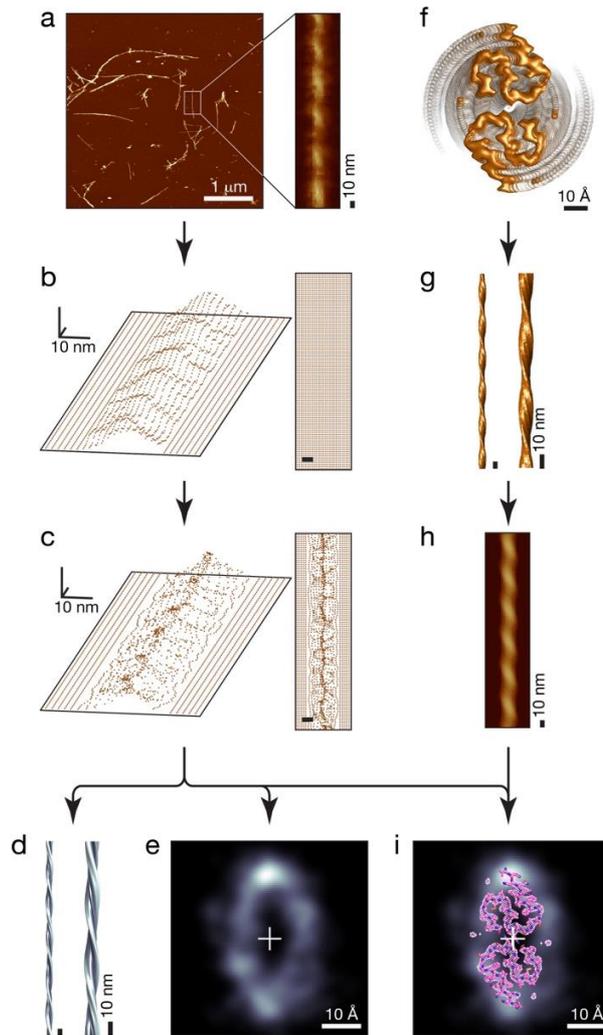
Figure 1: *Workflow summary of individual helical filaments structural analysis through 3D contact point reconstruction. a) Selection of an example individual filament segment to be analysed from an AFM height topology image. The example image of amyloid fibrils formed in vitro from human Aβ42 is shown with a 1 μm scale bar, and a 180 nm segment of the isolated individual filament is shown with scale bar indicating 10 nm. b) The helical axis is estimated for the selected fibril. The pixel z-height values of axis aligned image are shown in the 3D plot and the 2D plot shows the original pixel grid of the image. The scale bars indicate 10nm in all axes directions. c) CPR-AFM estimates the tip-sample contact points as seen in the 3D plot and moves the recorded values off the pixel grid as seen in the 2D plot. The scale bars indicate 10nm in all axes directions. d) 3D surface envelope model of the filament from a) with a 2x zoomed-in view reconstructed from the 3D contact point cloud from c). The scale bars indicate 10nm. e) Contact point density map of the filament cross-section. The scale bar indicate 10Å. f) Cryo-EM derived map of type II Aβ42 amyloid fibril purified from human patients (EMD-15771 [54]). The scale bar indicates 10Å for the cross-sectional view. g) Helical axis aligned and extended*

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204   In the current workflow for 3D surface envelope reconstruction of individual helical amyloid

205   filaments (see [4] with implementation in https://github.com/wfxue/Trace_y, **Figure 1**), a semi-

206   automatic filament tracing algorithm first isolates an individual user-selected helical filament

207   and estimates its central helical axis to sub-pixel accuracy. The tip geometry is then estimated

208   by comparing the observed filament image dilation caused by the finite size of the cantilever

209   probe tip and the expected dilation from a rounded cone tip model with a featureless

210   cylindrical approximation of the filament. The algorithm aims to find a tip radius estimate by

211   matching the calculated dilation resulting from the tip model to the observed dilation. Pre-

212   measured tip geometry parameters can also be used in this step (e.g. service provided by Nu

213   Nano Ltd, Bristol, UK for SEM analysis of AFM probes). The estimated tip-sample contact

214   points of the top of the filament in contact with the tip are then extracted as a 3D point cloud

215   using the CPR-AFM algorithm. The point cloud is subsequently aligned to the central helical

216   axis. The helical handedness of the filament twist can be established through the tilt direction

217   of the striation pattern of the filament. The helical pitch can be estimated by analysing the tilt

218   angle of the striation pattern directly in the filament image or its Fourier transformed 2D

219   power spectrum. The helical symmetry parameters are then applied to the axis-aligned

220   contact point cloud and a 3D surface envelope model of the individual filament can be

221   reconstructed using the point cloud as previously described [17]. This comprehensive workflow

222   make detailed analysis of individual filaments possible even when segments as short as one

223   or two helical pitch in length is observed once in an AFM height image [17].

224

225   For the structural analysis of polymorphous amyloid fibril populations, the individual

226   filament level 3D reconstruction approach opens up several important opportunities. For

227   example, this approach offer the capacity to delve into the structural variations of complex

228   amyloid populations [12]. Individual filament level structural analysis has shown promise in the

229   quantification of sample heterogeneity, and the mapping of the polymorph distribution of

230   amyloid fibrils and its sensitivity to assembly conditions [3,15]. It has enabled quantitative

231   measurements of structural parameters of individual filaments such as cross-sectional radius,

232   area and shape, and twist periodicity, handedness and pitch, allowing the objective

233   discrimination of different fibril polymorphs [15]. It has also allowed for the assessment of

234   structural variations within individual fibrils [12]. By integration with Cryo-EM datasets

235   available in the Electron Microscopy Data Bank (EMDB), quantitative structural analysis of

236   individual amyloid filaments formed in vitro from a tau sequence has identified that these

237   filaments closely resemble disease-associated paired helical filaments (PHFs) isolated from

238   brain tissues of patients [16,55]. More recently, individual filament level 3D reconstructions

239   were applied the *in vitro* assembly of the human 42-amino acid amyloid beta peptide (Aβ42),

240   revealing the sensitivity of the fibril polymorph distribution to small changes in the assembly

241   conditions, and subtle changes in assembly conditions can exert significant influence over the

242   resulting distribution of polymorphs [3]. Importantly, quantitative structural analysis on an

243   individual filament level has discovered rare species in the *in vitro* formed Aβ42 amyloid

244   population that resemble amyloid polymorphs seen *ex vivo* in samples purified from human

245   patents' brains [3]. These results collectively underscore the exciting potential of individual

246   particle level 3D reconstruction techniques in advancing the understanding of heterogeneous

247 polymorphous amyloid fibril populations and their biological impacts by offering insights

248 into the structural diversity, polymorphism distributions, and potential disease-associated

249 properties, as well as bridging the gap between the structures of *in vitro* and *in vivo*

250 assembled amyloid.

251

Future outlook for AFM based 3D structural reconstruction

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254 Structural analysis of individual helical amyloid filaments by CPR-AFM offers a powerful

255 approach allowing one 3D structural reconstructions for each one individual helical filament

256 segment observed on an AFM height image, and offers a unique opportunities in addressing

257 the key questions and challenges in amyloid structural biology. One fundamental question

258 revolves around the extent of possible structural diversity of amyloid fibril populations, and

259 how the population level properties change under different *in vivo* conditions associated with

260 specific disease states [12,14]. Structural characterisation of individual fibrils in complex and

261 heterogeneous amyloid populations [56] opens the door to the analysis and comparison of

262 population distribution and its dynamics. Understanding the connection between population-

263 level and single fibril-level properties is essential for deciphering the relationships between

264 specific polymorphs or structural properties and the phenotypic behaviours and biological

265 consequences of amyloid [5,8,14]. Resolving filament assembly mechanisms and structural

266 polymorphism arising within individual amyloid fibrils is also a critical frontier of

267 investigations. Different polymorphs have distinct physical properties, and these differences

268 likely translate into variations in biological activities. Even when formed from identical

269 precursors and coexisting within the same population or even the same filament particle,

270 individual fibril polymorphs may exhibit varying rates of elongation, fragmentation, surface

271 activities and cytotoxic potential [32,47]. Prions and prion-like amyloid fibrils that can propagate

272   between cells form strains that may manifest different biological activities, phenotypes and

273   localisations by its assembly [57-61]. AFM based individual filament level analysis may

274   rationalise the structure-function relationship between specific fibril polymorphs within a

275   heterogeneous population 'cloud' and their strain-specific propagation and behaviours. This

276   is of particular importance as certain fibril polymorphs may react differently to potential

277   inhibitors, leading to the development of "resistant" strains over time. Individual filament

278   level analysis may also contribute to the understanding of the time evolution of complex

279   amyloid population distributions during assembly by linking structural and quantitative

280   polymorph distribution information to the dominant intermediate structures seen by cryo-EM

281   [62,63]. Furthermore, understanding the factors that control the formation of dominant amyloid

282   polymorph structures, the roles of rare amyloid species, and the extent of structural variations

283   and population heterogeneity is crucial for gaining a comprehensive understanding of the

284   roles played by amyloid molecular populations in biology. Thus, population level insights

285   based on individual filament level structural analysis is essential for discerning whether some

286   amyloid aggregates act as causes or consequences of disease and how other amyloid

287   assemblies serve specific functions in physiological processes.

288

289   The 3D reconstruction approach for helical amyloid filaments has also the potential for

290   implementations in structural analysis of other types of helical structures or biological

291   structures with other symmetries. For example, the approach can be used to facilitate

292   individual filament level structural studies of helical DNA, cytoskeletal and collagen

293   filaments. Future developments may allow for the 3D reconstruction of bio-structures with

294   different symmetries, such as icosahedral viral capsids.

295

296 Structural reconstructions of individual helical filaments using 3D contact point clouds

297 extracted from AFM height images by CPR-AFM [4,17] unequivocally demonstrated that useful

298 3D reconstructions of biological macromolecules are possible from AFM data. It further

299 demonstrated for this type of soft material structures that 3D surface envelopes of

300 intermediate spatial resolution around ~10Å (estimated by Fourier Ring Correlation, FRC,

301 and the ½-bit information criterion) [4,17], a resolution range that is useful for quantitative and

302 comparative structural analysis, and molecular identification based on template matching and

303 model fitting approaches, can be achieved on individual particle basis where one single

304 observation can lead to one useful 3D model. Factors based on the current limits of the

305 fundamental physics of AFM hardware such as the probe tip radius, scanner and environment

306 stability influence the quality of the structural data generated, and subsequently, the

307 resolution limit of the 3D reconstruction. Synergies between CPR-AFM and three other

308 recent developments in AFM technologies: High-Speed AFM (HS-AFM), localisation AFM

309 (L-AFM) and simulation AFM (S-AFM) may increase the resolution of reconstructed 3D

310 models and provide an exciting outlook for AFM as an emerging general structural biology

311 method with the crucial individual particle 3D reconstruction capabilities (**Table 1**).

312

313 ***Table 1:*** *Summary of AFM methods that enable or could improve 3D reconstruction of individual bio-*

314 *macromolecules.*

315

AFM method		Capability	Significance for 3D reconstruction of individual bio-macromolecules	Relevant References
AFM	(Conventional) Atomic Force Microscopy	Topological height imaging through different modes of operation, including contact, non-contact, tapping and force-distance curve-based modes.	Encodes 3D structural information of the scanned surface in topological height images.	Different AFM modes are reviewed in [2], and the widely used Gwyddion software is described in [18]
HS-AFM	High-Speed AFM	Allows image data	Synergy with CPR-	Reviewed in [64,65],

		acquisition at a temporal resolution of ~100 ms	AFM could allow for increased contact point cloud dataset size, thereby improving potential spatial resolution, and for acquisition of dynamic 3D contact point clouds, thereby allowing for analyses of structural dynamics.	and example application for amyloid research in [38,39]
L-AFM	Localisation AFM	Generates 2D structural maps of molecular surfaces at near-atomic resolution	Synergy with CPR-AFM could improve the 3D spatial localisation of contact points thereby improved potential spatial resolution of 3D reconstructions.	Theory and NanoLocz software implementation described in [22,66]
S-AFM	Simulation AFM	Generates simulated AFM height images from existing experimental structural maps or predicted molecular models	Enables an integrative structural biology approach for individual bio-macromolecules by linking AFM with existing data (e.g. from cryo-EM or NMR) or structural predictions (e.g. AlphaFold2)	BioAFMviwer implementation described and reviewed in [20,65], application example for amyloid research in [16]
CPR-AFM	Contact Point Reconstruction AFM	Generates 3D contact point clouds from AFM height images that can be used for 3D structural reconstruction	Enables 3D reconstruction of individual bio-macromolecules' surface envelopes, (e.g. helical filaments)	Example application for amyloid structures and the Trace_y software implementation described in [4,17]

316

317 The development of HS-AFM throughout the 1990s and early 2000s have significantly

318 enhanced the temporal resolution capabilities of AFM [64,65]. With improvements such as

319 integrating short cantilevers, faster scanners, and improved feedback mechanisms, a temporal

320 resolution of sub-100ms can be reached with currently HS-AFM instruments [64,65]. This

321 advance has allowed directly visualisation and analyses of the dynamics of biological

322 processes. For individual particle 3D reconstructions, video frames from data acquired by

323 HS-AFM could improve CPR-AFM analysis by providing increased point cloud dataset size

324 for 3D reconstructions and provide dynamic 3D point clouds that can be used to analyse

325 time-dependent structural evolutions in the sub-second regime. The LAFM post-experimental

326 image analysis method [66] is highly complementary to HS-AFM. Building on the localisation

327   analysis principles of SRM, the L-AFM algorithm is capable of enhancing the spatial

328   resolution of 2D images to ~5Å range [22,66]. L-AFM can be used in two different ways. The

329   first approach involves creating localisation maps from multiple molecules recorded in one or

330   several HS-AFM video frames to observe the particle averaged time- or environment-

331   dependent conformational changes. This process typically requires information from 50 or

332   more observations of the same type of molecules. The second approach involves creating

333   localisation maps of the same individual molecules imaged by HS-AFM over time. For 3D

334   structural reconstructions, applying the 2D localisation step of both of the L-AFM approaches

335   in 3D on the contact point clouds extracted by CPR-AFM from data acquired by HS-AFM

336   could improve the spatial resolution of the reconstructed surface envelopes in 3D space. In

337   particular, individual particle 3D reconstructions could be carried out if the same individual

338   molecules is imaged by HS-AFM over time. S-AFM involves emulating the experimental

339   AFM scanning process and the interaction of the AFM probe tip with biomolecules of

340   interest computationally to generate simulated AFM topographic images [16,20,65]. Simulations

341   are typically carried out by simulating rigid body interactions between the AFM tip and the

342   biomolecules of interest [16,20], although the biomolecules' response to the probing force may

343   also be taken into account in future developments [67]. This approach enables integrative

344   structural biology methodologies that allow direct linkage between molecular models or

345   atomic/near-atomic resolution structural maps (e.g. from cryo-EM or NMR data) with

346   experimentally acquired AFM images [3,16], and can be used to identify structures observed on

347   AFM height images by model fitting and template matching techniques. Machine learning

348   and AI methods could also make significant contributions into bio-AFM applications, from

349   data analysis to autonomous software and hardware operation. For example, the AlphaFold 2

350   algorithm [68] has excelled in predicting the 3D structures of globular proteins and these

351   predictions could be matched to AFM data through S-AFM [65]. Furthermore, S-AFM could

352 aid AFM based 3D reconstruction and molecular identification by generating synthetic

353 training datasets for neuronal network methods, using well-labelled structural data or

354 molecular models from public databases such as the Protein Data Bank (PDB) [20] or the

355 EMDB [3,16]. Additionally, machine learning and AI techniques can help with quantifying

356 population heterogeneity, discerning distinct subpopulations and structural classifications

357 within heterogenous molecular populations distributions mapped through individual

358 molecular structural analysis (e.g. [3]), thereby shedding light on the complex relationships

359 between specific structures or polymorphs and the biological effects and consequences of

360 these structures. Hence, the integration of AI and machine learning methods could further

361 advance AFM based 3D structural analysis on individual molecular level.

362

363

364 **Conclusions**

365

366 In conclusion, an AFM based individual particle level 3D structural reconstruction method

367 has already shown its potential to address key challenges presented by polymorphous and

368 heterogeneous amyloid molecular populations and complex amyloid structure-function

369 relationships [3,12,15,16,56]. AFM is already an indispensable tool for the analysis of a range of

370 bio-structures including DNA/RNA [21,41,69], membrane proteins [37,70], virus particles [71,72],

371 cytoskeletal filaments [73,74] and cell surfaces [75], in multimodal imaging modes that can also

372 incorporate optical, confocal, IR, Raman and nano-mechanical information. Future

373 developments in improving the individual particle 3D structural reconstructions capabilities

374 of CPR-AFM and in furthering its synergies with other AFM methods such as HS-AFM, L-

375 AFM and S-AFM present an exciting area of development that will undoubtedly lead to a

376 unique capability for AFM in 3D reconstruction of individual dynamic bio-macromolecule

377   structures under ambient aqueous conditions. These advances together with the broad range

378   of existing multimodal bio-AFM applications will open up exciting future opportunities for

379   AFM, forming the profile of a general, accessible, unique structural biology tool for the

380   analysis of individual bio-molecules, and taking a step closer to the structural biologists'

381   dream of being able to obtain one high detailed, dynamic and multimodal 3D structural map

382   from one single observation of one individual molecule.

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Perspectives

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389 – AFM is emerging as an indispensable tool for the structural analysis of single, individual

390 polymorphous helical amyloid filament structures through recent advances in AFM based 3D

391 structural reconstruction

392 – The key conceptual idea utilised in the 3D contact point reconstruction (CPR-AFM)

393 algorithm is the realisation that useful 3D information is encoded in the 2D AFM height

394 images as 3D point clouds.

395 – Future developments in improving the individual particle level 3D structural

396 reconstructions capabilities of CPR-AFM by furthering its synergies with other AFM

397 methods such as HS-AFM, L-AFM and S-AFM present an exciting area of development.

398 These together with machine learning, computer vision and AI analysis methods in new

399 software advances will undoubtedly lead to a unique capability for AFM in 3D reconstruction

400 of individual dynamic bio-macromolecule structures under ambient aqueous conditions.

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