Methods for characterizing the individual filament structures of amyloid peptide assemblies using atomic force microscopy

Afsheen Shahbaz, Samantha L. Weetman, Claudia Chitty, Wei-Feng Xue*

School of Natural Sciences, University of Kent, CT2 7NJ, Canterbury, UK;

Correspondence to *W.F.Xue@kent.ac.uk

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ABSTRACT

A wide range of distinctive helical filamentous amyloid structures self-assembled from monomeric peptide or protein building blocks are found both in nature and in human disease states. Amyloid nano-fibrils are also being developed and synthetically made as novel peptide-based nanomaterials. In humans, accumulation of a range of amyloid structures, for example those formed from the amyloid-beta peptides in Alzheimer's disease, play a crucial role in the pathology of neurodegenerative and metabolic diseases. The diverse range of amyloid structures found is a manifestation of the amyloid structural polymorphism phenomenon. This is where different filament structures are assembled even from the same peptide or protein precursors. Due to the structural diversity of amyloid fibrils that can be found even in the same sample or the same disease state, an experimental method that allows structural analysis of individual amyloid filaments is required to understand the relationships between the polymorphic structures and the biological and physicochemical properties they elicit. Here, a method with a detailed protocol to analyze the structures of individual amyloid filament assemblies by topological atomic force microscopy (AFM) imaging and Contact-Point Reconstruction AFM (CPR-AFM) image analysis is described. This approach to resolve the 3D shapes of amyloid polymorphs, one individual fibril at a time, allows mapping of the polymorphic landscapes of amyloid assemblies. It serves as an inexpensive, fast and effective experimental tool for individual filament level structural analysis, and offers new, exciting opportunities in elucidating population distributions of heterogeneous amyloid samples, rare amyloid structures within the populations, and structural variations between or within individual filaments. These are all key parts to experimental developments in therapeutic discovery and novel bio-nanomaterials applications.

1. INTRODUCTION

Amyloid fibrils are insoluble filamentous protein structures which can accumulate in different tissues or organs in humans. They are hallmarks for a wide range of human diseases including several neurodegenerative diseases (Dobson et al., 2020; Eisenberg & Jucker, 2012; Ke et al., 2020). For example, the deposition of specific amyloid-based structures, such as amyloid plaques formed from amyloid-beta (Aβ) peptides and neurofibrillary tangles (NFTs) of tau found in Alzheimer's Disease (AD), and Lewy bodies associated with Parkinson's Disease formed from alpha-synuclein, underlie the pathology of distinct human diseases. However, not all amyloid structures are associated with pathology, some play essential roles in normal or even vital physiological processes, and they are often termed as 'functional'. The presence of functional amyloid structures has been reported in a wide range of organisms, spanning from prokaryotes to higher eukaryotes. For example, many bacteria including *Escherichia coli* and *Salmonella* produce amyloid nanofibrils known as curli, which is a key component of biofilm formation. These nanofibrils provide bacteria with structural integrity, protection from environmental stress, and facilitate adhesion to substrates (Chapman et al., 2002). In mammals, PMEL is a pigment-cell-specific protein that forms amyloid fibrils to provide scaffolding for melanin polymerization inside melanosomes (Hee et al., 2017). Numerous secretory peptides and proteins are stored in membrane bound granules as functional amyloid deposits prior to their release into the bloodstream, where they subsequently adapt their functional conformations. For instance, growth hormone (Jacob et al., 2016), parathyroid hormone (Gopalswamy et al., 2015) and glucagon (Horvath et al., 2023) have been reported to be stored as amyloid fibrils in pituitary secretory granules under specific conditions, creating a storage reservoir of these hormones. The diversity of disease association and biological function

highlights the context dependent nature of amyloid formation, where controlled assembly leads to utility and dysregulated assembly is associated with pathology.

Unlike globular proteins, which usually result from the folding of polypeptide chains into a single well-defined 3D-structure, amyloid-forming peptides and proteins of the same sequence can fold and assemble into a diverse range of distinct structures. This phenomenon, called structural polymorphism (Iadanza et al., 2018; Lutter et al., 2021; Meinhardt et al., 2009; Seuring et al., 2017), has been shown to be prevalent in the assembly of amyloid fibrils. Importantly for disease-associated amyloid fibrils, each specific fibril polymorph, even assembled from the monomeric building blocks with the same amino acid sequence, can be related to a different pathology (Li & Liu, 2023; Shi et al., 2021). Moreover, structural polymorphism may result in amyloid structures that exhibit diverse physiological properties, leading to differences in toxicity, cell-to-cell transmission, and immunogenicity (Lutter et al., 2021; Lutter et al., 2019). While pathologically-associated amyloid structures arise from uncontrolled protein misfolding and aggregation, functional amyloid structures are thought to be purposefully assembled by their host organisms to perform specific biological functions. However, functionally relevant amyloid fibrils are not necessarily structurally uniform or devoid of structural polymorphism. For example, a cryogenic electron microscopy (cryo-EM) study of PMEL amyloid cores from a human melanoma cell line revealed two distinct polymorphic forms, highlighting the structural diversity that can exist within a functional amyloid (Yanagisawa et al., 2025). Thus, a full structural analysis of polymorphous disease-related or functional amyloid populations requires a methodological approach capable of resolving the structure of individual fibrils within complex heterogeneous mixtures. Experiments that allow the identification and characterization of the precise molecular and structural determinants of specific polymorphic amyloid species that relate to certain biological

effects, are therefore, particularly valuable for both therapeutic interventions and biomaterials innovation. Structural analyses of individual amyloid filaments by AFM offer such an opportunity to understand the relationships between the polymorphic structures of amyloid, including heterogeneous structural populations and rare species therein, and the biological and physicochemical properties they elicit.

At molecular level, all amyloid fibrils are defined by the cross β-sheet architecture in which the peptide chains are aligned in an in-register β-sheet conformation with the β-strands running perpendicular to the fibril axis. This creates an extensive hydrogen network parallel to the fibril axis (Sunde et al., 1997). Subsequently, amyloid fibril polymorphs, although sharing the common cross-β architecture in the fibril cores, differ in the precise spatial arrangement of their β-strands. For example, amyloid fibril cores can be formed from different segments of the same polypeptide sequence, which contributes to varied fibril core structures arising from a single sequence (Sawaya et al., 2021). Additionally, alternative splicing of mRNA (Liu & Gong, 2008) or alternate cleavage of a precursor protein can result in the formation of various peptide sequence isoforms (Hampel et al., 2021), which further complicates amyloid populations. Some other factors that also contribute to polymorphism include posttranslational modifications (PTMs), environmental factors such as pH, and availability of co-factors. For instance, cryo-EM studies have shown that phosphorylation of α-synuclein at Tyr^{39} and phosphorylation or O-GlcNAcylation at Ser^{87} of α-synuclein can cause formation of two structurally distinct fibrils, which differ in their conformation compared to fibrils without PTMs (Hu et al., 2024; K. Zhao et al., 2020). Similarly, it was shown that phosphorylation at Ser^{8} of amyloid-beta (Aβ) results in less compact conformations at the N-terminus of Aβ in the aggregates (Rezaei-Ghaleh et al., 2016). Cryo-EM studies have shown that subtle changes in pH can induce structurally distinct fibrils of α-synuclein (Frey et al., 2024). Similarly, the use

of anionic cofactors such as fatty acids, sulphated glycosaminoglycans, heparin or RNA for *in vitro* synthesis of tau filaments leads to the formation of structurally distinct fibrils compared to those observed in patients (Abskharon et al., 2022; Lovestam et al., 2022; Zhang et al., 2019). An important example of a group of related amyloid-forming peptide sequences capable of assembling into a diverse range of polymorphic amyloid fibril structures, is provided by the Aβ peptides. The Aβ peptides are formed by the cleavage of the transmembrane amyloid precursor protein (APP). During the amyloidogenic pathway, APP is sequentially cleaved by β- and γ-secretases, at the N- and C- termini respectively, resulting in the formation of Aβ40 (40-amino acid peptide) or Aβ42 (42-amino acid peptide) (Hampel et al., 2021) as well as other peptide lengths (J. Zhao et al., 2020). Misfolding, assembly and aggregation of extracellular Aβ peptides cause their accumulation in senile plaques, which are one of the major contributors of AD. The two-residue difference between Aβ40 and Aβ42 at the C-terminus cause significant differences in the aggregation kinetics and fibril structures of two peptides (Meisl et al., 2014). Aβ42 fibrils extracted from AD patients have shown the formation of two major polymorph structures upon analysis by cryo-EM (Yang et al., 2022), whereas ss-NMR revealed that both Aβ40 and Aβ42 display considerable structural polymorphism (Qiang et al., 2017). Individual filament structural analysis by CPR-AFM has shown that the Aβ42 peptide sequence is capable of assembling into a continuum cloud of different fibril structures and the polymorphic Aβ42 amyloid populations are sensitive to the solution environment during assembly (Aubrey et al., 2023). This highlights the importance of structural analysis at the individual filament level in understanding the extent of polymorphism and heterogeneity of amyloid populations, and demonstrates the value of AFM topology imaging together with the CPR-AFM image analysis methodology.

Here, the CPR-AFM methodology for individual filament level 3D structural analysis by AFM topology imaging is described with an in-depth step-by-step protocol for the analysis of amyloid fibril structures. To analyze amyloid structures and their structural polymorphism, Atomic Force Microscopy (AFM) has recently emerged as a powerful technique because it allows in-depth structural analysis at an individual particle level. How physiological and environmental conditions affect and contribute to the extent of amyloid polymorphism, now widely observed *in vitro* and in patient-derived samples, is not fully understood. One contributing factor is the lack of structural information at the individual fibril level. It is of paramount importance to identify the different polymorphs in heterogeneous amyloid populations and to establish their impact on amyloid aggregation and the biological effects of these complex populations in disease progression. Structural analysis techniques, such as X-Ray fiber diffraction and solid-state nuclear magnetic resonance (ss-NMR) (Ke et al., 2020), have provided key insights into amyloid fibril structures. In recent years, cryo-EM have revolutionized our structural understanding of amyloid fibrils at near-atomic resolution (Ke et al., 2020). However, these techniques can only characterize the entire population or the dominant species of amyloid aggregates within the populations, and thus only provide maps of population-averaged amyloid core structures. In contrast to population-averaged structural analysis methods, AFM enables the extraction of independent structural data from individual fibrils. In AFM topology imaging, the sample surface is scanned by a probe with a sharp tip attached to a cantilever. The movement of the cantilever and the interactions between the probe tip and the sample create a detailed topographical image of the molecular surfaces with a high signal-to-noise ratio (Lutter et al., 2020), allowing individual amyloid fibrils to be visualized and their surface envelopes 3D-reconstructed using contact-point reconstruction (CPR-AFM) based image analysis (Xue, 2025). Integrative approaches combining AFM image analysis with cryo-EM data have further enhanced structural analysis of individual polymorphic amyloid

fibrils (Lutter et al., 2022). The individual filaments observed in AFM topology images and 3D reconstructed by CPR-AFM, including rare filament species, can be subsequently matched and identified using cryo-EM density maps. The workflow described here presents an exciting new opportunity for novel amyloid-targeting therapeutics as well as amyloid-based nanomaterials research applications, and it moves structural analyses towards an integrative structural biology approach where one 3D structure can be obtained from one single observation of one individual particle.

2. BEFORE YOU BEGIN

Timing: 1–2h

1. Preparation of support surface substrate. Typically, mica is used as the support surface substrate of choice. It provides a flat hydrophilic surface suitable for the adsorption of a wide range of amyloid fibrils. It can deliver a clean image background for high resolution imaging and is relatively inexpensive and easy to source. Assembly of mica on AFM specimen discs should be done in advance of the specimen preparation (**Figure 1a-c**). While a preferred mica disc size is mentioned in the protocol, discs of different sizes or shapes can also be used, and the protocol can be adjusted accordingly depending on the disc size and the AFM instrument.
    a. Cut a small (3x3–5x5 mm) piece of double-sided tape and attach it to a 15 mm diameter AFM specimen disc (Agar Scientific).

b. Attach a 9.9 mm diameter muscovite mica disc (Agar Scientific) firmly on the upper side of the tape (**Figure 1c**).

c. Mica discs mounted on AFM specimen discs can be stored prior to use in a container lined with filter paper (e.g. a Petri dish).

d. Note: Highly oriented pyrolytic graphite (HOPG) can also be used as a support surface substrate. In contrast to the negatively charged and hydrophilic mica surfaces, HOPG surfaces are more hydrophobic in character than mica. Consequently, in some cases, the hydrophobic HOPG surface adsorbs certain amyloid types more effectively than mica. However, HOPG may also effectively adsorb unwanted non-amyloid components in the samples, which could result in a reduction in the cleanness and quality of the image background. Often, a trial of both types of surface substrate is needed to determine the best one to use. HOPG can be glued to a specimen disc and subsequently cleaved in a similar manner to mica.

2. Amyloid sample preparation. Pre-assembled amyloid samples should be diluted to the required concentration prior to specimen preparation. This can be achieved by diluting the sample, preferably with sterile-filtered MilliQ water. Alternatively, sterile-filtered sample buffer or dilute HCl (e.g. at pH 2.0) can be tested and used. Thus, both the pre-assembled amyloid and appropriate diluent should be prepared in advance.

3. Preparation of nitrogen gas source. For individual filament structural analysis by AFM topology imaging, the amyloid fibrils can be imaged in air. Gentle drying of the mica discs containing deposited fibrils should be performed using a stream of pure nitrogen gas from a pressurized gas cylinder, delivered through a narrow-tipped tube or nozzle to ensure controlled, even, and gentle gas flow over the sample surface.

3. KEY RESOURCES TABLE

REAGENT or	SOURCE	IDENTIFIER
Software and Algorithms		
Trace_y (version 7)	Xue, Structure 33 (2025), 363-371, doi.org/10.1016/j.str.2024.11.007 (Xue, 2025)	https://github.com/wfxue/Trace_y
MATLAB (release 2024a)	MathWorks	https://www.mathworks.com/products/matlab.html
Nanoscope Analysis (v3)	Bruker	https://www.bruker.com/en/products-and-solutions/microscopes/materials-afm.html
Alternative to Nanoscope Analysis: Gwyddion (2.68)	David Nečas and Petr Klapetek, Central European Journal of Physics 10 (2012) 181–188, doi.org/10.2478/s11534-011-0096-2 (Necas & Klapetek, 2012)	https://gwyddion.net
Other		
Bruker MultiMode 8 scanning probe microscope with Nanoscope V controller †	Bruker	https://www.bruker.com/en/products-and-solutions/microscopes/materials-afm.html
AFM Probe -ScanAsyst silicon nitride tip, nominal tip radius 2 nm, nominal spring constant 0.4 N/m ††	Bruker	https://www.brukerafmprobes.com/p-3726-scanasyst-air.aspx
Mica (muscovite) Discs 9.9 mm diameter	Agar Scientific	https://www.agarscientific.com/afm-mica-discs
AFM Specimen Discs 12 mm diameter	Agar Scientific	https://www.agarscientific.com/specimen-discs

† Alternative instrumentation: any scanning probe microscope capable of AFM operations in gentle tapping or force-distance curve-based topology imaging modes.

†† Alternative cantilever probes with comparable nominal spring constant and tip radius: for example, NuNano SENSE70 (https://www.nunano.com/store/sense-70, can be purchased together with SEM images of each probe tip), and MikroMasch SelfAdjust-Air (https://www.spmtips.com/afm-tip-selfadjust-air-for-scanasyst-in-air).

4. MATERIALS AND EQUIPMENT

The following materials and equipment are recommended in addition to those listed in the key resources table.

- Low protein binding Eppendorf tubes
- AFM specimen discs with mica discs attached
- Micropipettes
- PVC (insulation) tape
- Double-sided tape
- Scissors
- Precision forceps
- Covered containers or Petri dishes with lids
- Filter paper
- Nitrogen gas cylinder with pure N_2 gas
- Magnifying glass
- Sterile filtered MilliQ water

5. STEP-BY-STEP METHODS DETAILS

5.1 AFM Sample Preparation

Timing: typically 15 min per sample but will depend on the chosen incubation length.

The following steps are recommended to prepare specimen with amyloid fibrils for AFM imaging in air on circular specimen discs. The protocol can also use mica attached to any support such as a glass slide.

1. Sample dilution. Optimal monomer equivalent concentration for AFM specimen preparation depends on many factors such as the monomer peptide/protein size, amino acid sequence, solution conditions (e.g. pH, ionic strength etc.) and surface substrate used. Typically, as a starting point, a monomer equivalent concentration of 1-10 mM (alternatively 1-10 mg/mL) for short peptide sequences below 10 amino acid residues or 10-100 μM for larger peptides/proteins such as amyloid formed from Aβ peptides or tau sequences. If sample dilution is required, dilute the amyloid sample solution with an appropriate diluent (for example sterile filtered MilliQ water) to achieve the required final concentration in low protein binding Eppendorf tubes.

    a. Note: Sterile filtered MilliQ water is the preferred diluent for achieving a clean imaging background. In general, low ionic strength solutions are preferred as the diluent since salt crystals may otherwise form during the incubation due to evaporation, resulting in a reduction in the quality of the image data.

    b. Note: Muscovite mica used as surface substrate is a silicate material and its surface is negative charged at neutral pH. Consequently, some amyloid structures may be electrostatically repelled by the negatively charged mica surface. Thus, brief exposure to dilute HCl solution (i.e. at pH 2.0) used as a sample diluent can be an effectively strategy to reduce the mica surface charge density during incubation, thereby reducing the potential repulsive interactions between the amyloid fibrils and the mica surface, and increasing the adsorption. While amyloid fibrils are sufficiently chemically stable for a brief incubation at low pH, extended incubation

should be avoided if deposition is carried out at low pH as it may disrupted amyloid structures assembled at neutral pH. In such cases, control experiments should be carried out to ensure no structural changes are observed during the incubation at low pH.

2. Cleave mica. The top layer of the mica support surface substrate already attached to an AFM specimen disc should be cleaved immediately prior to sample deposition. First, gently scrape the edges of the mica disc with flat-edged metal forceps so that the layers at the disc edge become loose. Place a piece of PVC tape firmly on the mica disc and then carefully remove it. The tape will subsequently cleave the mica surface by taking the top layers off the mica disc (**Figure 1d-e**). This process must be repeated until uniform removal of an entire top mica surface from the disc is achieved. To assure that the freshly cleaved mica surface remains free of contamination, keep the disc in a closed container.

3. Sample deposition. After dilution to the appropriate sample monomer equivalent concentration, quickly deposit a 10-20 μL droplet of the sample using a micropipette onto the freshly cleaved mica surface (**Figure 1f**) and incubate it for 5-10 minutes at room temperature. During the incubation, keep the samples in covered containers to avoid contamination. As with sample concentration, the ideal amount of the amyloid sample and the incubation time can be varied depending upon the experiment and type of amyloid specimen being prepared.

4. Washing and gentle drying of the specimen disc. After the incubation, the mica surface with adsorbed amyloid fibrils should be washed prior to gentle drying with nitrogen gas (**Figure 1g-h**). Use sterile filtered MilliQ water or the diluent used in previous steps if it contains low ionic strength (e.g. sterile filtered dilute HCl). Hold the AFM specimen disc

at a 10–45° angle with the precision forceps and quickly wash it with 100-500 ul sterile filtered wash solution. Next, while keeping the specimen disc at the same angle, quickly apply a gentle and fine stream of nitrogen gas to dry the mica disc. The nitrogen gas nozzle should be ensured to not come into contact directly with specimen mica surface.

5. Short-term storage of the AFM sample specimen. Once prepared, keep the AFM specimen discs in a covered container or petri dish lined with filter paper prior to AFM topology imaging in air (**Figure 1i**).

5.2 AFM Topographic Imaging

The following steps describing AFM topographic imaging refer to the ScanAsyst imaging mode (Bruker's force-distance curve-based topographic imaging mode) on a Bruker MultiMode 8 scanning probe microscope with Nanoscope V controller. However, alternative instrumentation with any scanning probe microscope capable of AFM operations in a force-distance curve-based topographic imaging mode or a gentle tapping/intermittent contact imaging mode can be used. Before imaging, ensure that the AFM scan head with the cantilever probe is correctly mounted and the laser is accurately aligned and ready for scanning. This can be carried out following the instrument manufacturer's instructions. The steps below describe image data acquisition.

1. Preparing the instrument for topology imaging. The amyloid samples can be imaged in air using a gentle force-distance curve-based imaging mode where the imaging force can be controlled and kept as low as possible. A Multimode 8 SPM with a Nanoscope V controller operating the Peak-Force tapping mode in the ScanAsyst in Air experiment can be used. A cantilever probe made of a soft cantilever spring with a low nominal spring

constant and a sharp tip with a small nominal tip radius, for example the Bruker ScanAsyst probes (silicon nitride tips with nominal tip radius of 2 nm and nominal spring constant of 0.4 N/m, Bruker), should be used.

2. Use the precision forceps to place the prepared AFM specimen disc onto the sample holder/sample stage. The forceps should be carefully handled so that the mica disc surface containing the adsorbed amyloid sample does not come into direct contact.

3. The scan size can be adjusted according to the sample and experimental requirements. This can be typically 10x10, 4x4, 2x2 or 1x1 μm areas.

4. The sample surface can be scanned with user-defined pixel sampling according to sample/experiment requirements. For example, images ranging from 512 x 512, 1024 x 1024, 2048 x 2048 or higher pixel counts can be acquired. For the downstream individual filament level structural analysis, a pixel density of at least 0.5 pixel/nm (e.g. 2x2μm scanned 1024x1024 pixels) is recommended, but a lower pixel density (e.g. 0.25 pixel/nm) can also be used for scouting and initial experiments.

5. Bring the tip close to the AFM specimen disc. The approach of the tip can be visualized either manually by using a magnifying glass or through a dedicated view in the microscope interface. To prevent damage to the probe or the sample, the probe tip must be treated carefully, and it must remain slightly above the sample surface until probe engagement is initiated in the instrument.

6. Engage the cantilever probe and begin image acquisition. The precise spot being imaged can be adjusted as required to find areas where fibrils are sufficiently well-populated while remaining well-separated and not clustered. It is recommended to acquire two separate images for each scanned spot; one trace and one retrace image. As every scan line is scanned twice in opposite directions either by default or by user setup, this generates two images that can be helpful for comparison in subsequent analysis.

7. Once image acquisition is complete, ensure the images are saved before withdrawing the tip and retracting the probe.

5.3 Initial Processing of the Topographic Images

Once the raw AFM data is acquired, the topographical height images must be baseline-flattened prior to any structural analyses. This is because raw AFM data is not usually levelled to any reference height. The flattening process removes any scanner bow and sample tilt effects from the image, and references the z-height of the molecularly flat mica surface to zero. This could be achieved by using software supplied by the instrument manufacturer (e.g. Bruker Nanoscope Analysis (v3) software for Bruker SPM systems). Alternatively, general AFM image processing software such Gwyddion (Necas & Klapetek, 2012) can also be used. The following is a recommended workflow for flattening the image background.

1. Initial image visualization and background flattening. To perform the initial baseline flattening and to visualize the overall image, apply a first-order polynomial (line) fit to each scan-line.
2. Masking foreground filaments from the background. The final baseline subtracted from the scanlines in the flattening procedure should be calculated with the filaments in the foreground masked. The mask used can be manually adjusted so that all filament particles are excluded from the baseline. Subsequently, calculate and subtract a second- or a third-order polynomial fit to the baseline until a defined image with a clean background is obtained.
3. Initial visualization and contrast adjustment. In the AFM height images, height variations of the sample surface can be mapped to a color scale, where different colors correspond

to specific height values. This allows clear visualization of the surface topography. The default color scale, usually shades of black, brown, orange and yellow, can be adjusted manually to a suitable contrast setting so that amyloid filaments with their striation patterns from the helical twist are more clearly visible from the background, facilitating the subsequent filament picking, tracing, and 3D structural reconstruction.

5.4 Individual Filament 3D Reconstruction

This section describes the quantitative image analysis workflow for individual filament level structural analysis using the open access software Trace_y (Xue, 2025). Here, the structural information from individual helical fibrils captured in topographical AFM images are extracted as the Trace_y software enables the reconstruction of the 3D surface envelopes of individual fibrils without cross-filament averaging. Therefore, the structural information independently extracted from each fibril can then be used to analyze the sample population polymorph distribution, to quantify the sample heterogeneity, and to discover any rare species or subpopulations.

Trace_y version 7 is currently publicly available to download from https://github.com/wfxue/Trace_y. Trace_y can be run with the command-line interface (CLI) or a Graphical User Interface (GUI) within the MATLAB command window (MATLAB release 2024a or newer is required for version 7 of Trace_y). Alternatively, a stand-alone application with the GUI is also available (MATLAB Runtime Libraries 2024a is required for the stand-alone application). Both CLI and GUI methods are very similar with minor advantages for each in different aspects. The GUI version is user-friendly and beginner-friendly, with a “Functions” window containing a suggested workflow (**Figure 2**). Use the GUI version

by clicking through the menu items in the Functions window, the input parameters can be adjusted for each workflow step in the adjacent box. Each workflow step can be subsequently run by clicking the "Run" button underneath the function's description box. Using Trace_y with the CLI presents a more customizable approach, in which the commands for Trace_y functions, along with any non-default input parameters, can be typed directly into the MATLAB Command Window. The CLI version still retains many of the same visual elements as the GUI version, but there is no single Functions window where the entire workflow is easily accessible.

The detailed protocol described below will mainly focus on using the GUI version; additional notes will highlight the main differences on how to execute the comparable function from the CLI with example commands indicated by ">>". More detailed input options for the CLI commands and input parameters can be found in the source code files of each function in the Github homepage for Trace_y.

Timing: Typically, 1-10 min per filament depending on the image resolution and filament size.

1. Launching Trace_y application. Make sure the correct version of MATLAB Runtime Libraries is installed on the computer running Trace_y, then double-click the Trace_y application to start. If running Trace_y from source code in the MATLAB command window, make sure your working directory is set to the extracted Trace_y folder containing the file Trace_y.m. To start the GUI version, type in the command window:

   >> Trace_y

This will open the Functions window containing a suggested workflow, and the image display window in front of the MATLAB windows. Alternatively, to start Trace_y with the CLI type in the command window:

>> Trace_y('-m');

2. Import an AFM topology image file. In the functions window (**Figure 2**), click "Import AFM image data". The CLI equivalent uses the following command:

>> img = ImportAFMImageData;

where "img" can be set to any variable name referring to the image. Both methods will open a standard file-selection window to choose the image file to import; compatible file extensions include .spm (Bruker format), .csv, and .txt (file format compatibility will be extended in future releases). The image will automatically become visible in the GUI image display window (**Figure 2**). In the CLI, the AFM image variable "img" will subsequently be listed in the MATLAB workspace; to display it, use:

>> img.DispImage;

   a. Note: Image data in channel 1 is imported by default. To use a different channel, the image data can be loaded in the CLI using the command:

      >> img = ImportAFMImageData(channel_number)

      For example, setting channel_number to 3 will import channel 3 into the CLI; setting channel_number to 'all' will import all channels, in which case the variable "img" will be a stack in the form of an array of images. To use the GUI version with a non-default channel, first save the imported channel data from the CLI version in the MATLAB Workspace. Then, in GUI version, click "Load Trace_y file (.trcy)" and select saved file.

b. Note: In addition to the suggested “Import AFM image data” workflow option, which can automatically detect the file format, other import options available. These include: “Import Bruker Nanoscope file (.spm)” or, in CLI:

>>img = ImportBrukerSPM;

which imports Bruker Nanoscope data files (.spm). “Import CSV text file (.csv)” or, in CLI:

>> img = ImportCSV;

which imports AFM image data in a csv or other delimited text file (.txt). If a generic delimited text file is imported, it is important to check the pixel order, because in some AFM image processing software for e.g. when exporting delimited text data using Gwyddion (Necas & Klapetek, 2012)) the pixel row order is reversed compared to Trace_y.

c. Note: The image should be flattened, with scanner bow and sample tilt effects from the image removed and the z-height of the molecularly flat mica surface to referenced to zero. This can be checked in Trace_y using “Display scan line” function (**Figure 3**), or in CLI use:

>> img.DispLine;

3. Find a filament of interest. Use the zoom and pan tools in the image display window to explore and search the image for a filament of interest.

4. Trace a filament to estimate its central helical axis. In the GUI functions window, click the “Trace filament” function. Options are available to adjust the default input parameters before clicking “Run”. The “Apparent width / pixels” is the apparent width of the filament as it appears in the image in pixels, and the “Height threshold / nm” is the z-height threshold in nm, typically set above the background noise level to be ignored by

the tracing algorithm. It is usually sufficient to leave the parameters at their default values. In the CLI, use:

```
>> img = img.TraceFilamentM(apparent_width, z_threshold);
```

In both versions, make sure the zoom or pan tools are unselected and then left-click to manually add points on the filament segment to be traced (right-click to remove points). A minimum of two points, a starting point and an end point, outlining the filament segment to be analyzed, must be defined before tracing (example in **Figure 2**). Additional points along the filament segment can also be added, this will increase the accuracy of the semi-automatic tracing algorithm by creating a template line that is more closely matched to the filament. Once the template line is satisfactory in indicating the filament to be analyzed, click the "Trace" button that appears at the bottom left corner of the image box. When the tracing algorithm has finished the helical axis estimation, a new results window will appear with the initial filament properties such as the central line height profile and its Fourier transform. Use this to assess the regularity of the peaks and troughs indicative of the helical properties of the filament. This assessment is required at this stage as the central-line height profile can be used to estimate the cross-sectional symmetry parameter. An initial guess of this parameter must be entered in the next step to reconstruct the 3D surface envelope. Importantly, it should be noted that the estimated cross-sectional symmetry parameter used in Trace_y refers only to the number of different peaks that occur within one helical pitch length in the repeating pattern of the height profile, and the value of this parameter may not correspond directly to the symmetry of the structure.

a. Note: If the height profile is irregular or without a clear indication of a repeating pattern at this step, it would indicate an irregular or non-helical structure.

Consequently, a 3D helical filament envelope cannot be fully reconstructed for this filament. At this stage, press “Delete Filament” to remove it from the dataset.

b. Note: It is important to establish objective rules for filament selection. Typically, this will involve i) a segment must have striations indicative of a helical structure, with at least two clear repeats; ii) the filament should be well separated from the others; iii) only one section per filament should be traced and analyzed (unless structurally distinct sections are evident); and iv) the segment should represent the longest possible section of the filament with a clear repeating pattern.

5. Starting the 3D structural reconstruction workflow. The structural reconstruction analysis of the selected individual filament can be initiated by selecting “Reconstruct helical 3D model” or, in CLI:

>> img = img.MakeHelicalFilamentModel(index, handedness, symmetry_estimate);

This function requires inputs including the index number of the traced filament, the helical twist handedness (“left” or “right”), and a symmetry parameter estimate. Optional input parameters can be adjusted but they are typically left at their default values. These include “Refine tip radius estimate”, a slow iterative process that estimates the AFM tip radius by minimizing the difference between the experimental image data and the simulated image of the reconstructed filament at different tip radius values. The “Smoothness” and “Refine” parameters control the cross-sectional and longitudinal sampling rates for displaying the tip-sample contact point cloud (Lutter et al., 2020; Xue, 2025) as a 3D surface envelope. These affect surface coordinate interpolation and the smoothness of the visualized 3D model. The parameters are defined by the number of cross-sectional points or subpixels sampled: a value of 1 corresponds to the original pixel density, 2 corresponds to twice the density, and so on (e.g. 4 equals four sub-divisions

per pixel). The CLI version also includes additional tip data parameter options to customize the tip radius and the tip side-angle.

6. Peak picking. In the subsequent pop-up window, select the peaks to be used as local reference points for helical reconstruction (**Figure 4**). The peaks in the height profile represent specific coordinates for local convex features that are prominent tip-sample contact points. Therefore, they are used as local reference points to pinpoint helical repeats during the helical 3D reconstruction process. Suggested peak locations based on different data smoothing models can be toggled by selecting different peaks on the 1D power spectrum. The peaks can also be defined or modified manually. To progress to the next step, press “Done”.

7. Initial evaluation of the tip radius and deconvolution. The next pop-up window shows the initial deconvolution and tip radius estimation, using an initial cylindrical structure (Lutter et al., 2020). This step is also useful to gauge the quality of individual pixels in the image. In this window, select the lines of pixels to be included in the final 3D reconstruction. The main aim here is to capture as much structural information about the filament as possible whilst minimizing the amount of “unreliable” pixels. These are displayed with an increasing degree of shade, indicating progressively less reliable 3D coordinates. Typically, for amyloid fibrils less than 10 nm in width captured on topology scans with 0.5-1 pixels/nm, we find selecting 3-5 lines of pixels is optimal. The subsequent 3D reconstruction process will typically take a few minutes on a personal computer of average specs, and the run time will depend on the length of the filament, the pixel resolution of the images and the specs of the computer used.

8. Evaluation of the quality of the resulting 3D model (**Figure 5**). There are many factors to consider when judging the quality of the reconstructed 3D model. The most straightforward factor to check should be the twist handedness. The comparison of the

AFM image data with the simulated AFM image based on the 3D model provides a clearer indication of whether the striation pattern runs in the same direction. Comparison of the tilt directions for the main peaks in the 2D Fast Fourier Transform (2D-FFT) image should also indicate the twist handedness. However, these plots can be skewed by the presence of other nearby filaments, and they are sensitive to background and noise in the image. The second factor to consider is the cross-sectional symmetry. The appearance of the 3D model can be compared with the original AFM image data, and should be checked to ensure consistency with the AFM image. The 2D-FFT plots should also be compared. The tilt angle is indicative of apparent cross-sectional symmetry order, the more tilted the plot looks away from a central vertical line, the higher the symmetry parameter it is likely to require. In addition, other outputs to consider when judging the quality of the 3D model are the cross-section contact-point density, and the "Image-model RMSD" and "Image-model correlation distance" values, which are reported in the log shown in the main functions window or the MATLAB Command Window. These outputs are particularly helpful to consider if comparing the model output of one filament with a refined model of the same filament, or between filament models reconstructed from within the same image. In a good quality model output, the cross-section contact point-cloud will show an envelope with less scatter and less diffuse density. Lower "Image-model correlation distance" and "Image-model RMSD values" generally indicate a better model when comparing possible alternative models, and typically, a similar level of values across "good" 3D models reconstructed from within the same image should be expected. It is worth noting, however, that the RMSD can be heavily influenced by the number of lines of pixels selected for use in 3D reconstruction, hence reducing this value should not be prioritized if it compromises the quality of other factors in the model output.

9. Refining the contact point cloud and the 3D model. To make changes to the filament 3D model the “Reconstruct helical 3D model” function can be re-run by inputting the same filament index number. This will overwrite the previous model output. Alternatively, a new trace of the filament can be made for comparison without overwriting an existing model. A fresh trace of the filament can be analyzed and the resulting model compared with previous models to test alternative input parameters. For example, major changes can be explored by re-running the 3D reconstruction with alternative handedness or symmetry estimate inputs. If the striation pattern in the simulated image tilts in the opposite direction compared to the original AFM data, the handedness input should be changed. If the 2D-FFT plot of the data appears more tilted away from the image center than the simulated 2D-FFT plot, then the symmetry estimate parameter should be increased. Conversely, if the simulated 2D-FFT plot is overly tilted relative to the data, the symmetry estimate should be decreased. The remaining steps, including modifications to the peak/trough selection in the height profile or increasing/decreasing the number of pixel lines used, will also affect the appearance of the 3D model, the cross-sectional contact-point density, and the Image-model RMSD and the Image-model correlation distance values.

10. Repeat previous considerations to evaluate and compare the quality of different potential models. In particular, if the original estimation of the symmetry parameter is unclear from the central line profile view alone, reconstructed models, simulated images from the models, and their 2D-FFT power spectra images resulting from different symmetry parameter values should be constructed and compared to decide its optimum value (Lutter et al., 2020). In the CLI, use:

```
>> img = img.DeleteFilament(filament_index);
```

to delete any alternative filament models that are deemed lower quality according to criteria discussed in the previous steps.

11. Modelling the next individual filament. To reset the view for selecting the next filament to trace, click “Explore features” or, in the CLI:

    >> img.ExploreFeatures;

    This will display the AFM image with the contours of previously traced filaments highlighted. The previous steps to trace and reconstruct a 3D model can then be repeated for a new filament. To maximize the completeness of subsequent population-level analyses, this process should be reiterated until every filament with a clear helical pattern and at least two regular repeats has been traced and analyzed. Currently, 3D reconstruction of at least ~100 individual filaments for each sample is recommended for quantitative assessment of its heterogeneity and population distribution of fibril structures (Aubrey et al., 2020; Aubrey et al., 2023). If feasible, however, larger sample sizes will result in more detailed quantitative analyses and help with identifying rare species that may be populated under a few percentage units (Aubrey et al., 2023).

12. Saving progress. In the GUI version, select the “Save session Trace_y file (.trcy)” option. In the CLI version, the MATLAB Workspace containing img (or any chosen variable name) can simply be saved. This will save all filament traces and 3D model outputs in one file that can be reloaded. Note that when using the GUI, closing the image display window will clear the image, and the active Trace_y session will end.

13. Exporting results. The function “Export filament results (.csv)” or, in the CLI:

    >> img.CompileFilamentResults;

    will compile all resulting data for every annotated filament within the .trcy or .mat file into a table for further analysis. **Table 1** provides a description of the outputs in the exported table. The morphometric parameters listed in Table 1 and the cross-sectional

contact-point density map that can be exported from the results can be used to calculate a helical structural based distance metric d_ξ (described in (Aubrey et al., 2023)). This metric allows agglomerative clustering of the reconstructed 3D models for estimating whether any pairs of 3D reconstructed fibril models belong to the same polymorph class.

Table 1: Description of the individual filament level information summarised in the result table that can be exported from Trace_y.

Output:	Description:
Image file name	The filename of the original image data file.
Filament index number	The number assigned to the filament segment when it was first traced.
Contour length / nm	The length of the filament contour following its estimated central axis
End-end distance / nm	The length of a straight line from one end of the filament segment to the other end.
Mean height / nm	The mean z-height value of the central-line height profile. This is approximately the filament diameter.
Mean peak height / nm	The mean z-height value of the local maxima.
Mean trough height / nm	The mean z-height value of the local minima.
Main FFT periodicity / nm	The spatial frequency of the most prominent periodic peaks, as evaluated by Fast Fourier Transform of the central-line height profile.

Mean cross over distance / nm	The mean distance between each pair of adjacent peaks.
Tip radius estimate / nm	Estimated tip radius for the filament segment image. Since the tip may become blunted whilst scanning or if the tip is consistently contacting the sample filament at a specific angle, this value is specific to the filament. This value could also be an optional user input.
Twist handedness	User input. The handedness of the helical twist, either "left" or "right".
Directional periodic frequency / nm^{-1}	The handedness (-1 for left, +1 for right) x (1/ helical pitch). The helical pitch length is the (cross over distance) x (the apparent symmetry estimate).
Estimated apparent cross-sectional symmetry	User input. This is based on the number of peaks in the central-line profile identified to be within a single repeat.
Mean cross-sectional area / nm^2	The mean area of interpolated cross-sections perpendicular to the filament helical axis.
Mean cross-section radius / nm	The rotational mean distance between the filament helical axis and the edge of the cross-section perpendicular to the filament helical axis.
Max cross-section radius / nm	The maximum distance between the filament helical axis and the edge of the cross-section perpendicular to the filament helical axis.

Minimum cross-section radius / nm	The minimum distance between the filament helical axis and the edge of the cross-section perpendicular to the filament helical axis.
2nd polar moment of cross-sectional area / nm^4	The second polar moment (polar moment of inertia) of the cross-section perpendicular to the filament helical axis.
Model-data RMSD / nm	Root-mean-square deviation between the simulated image based on the reconstructed 3D model and the original image data.
Model-data Correlation distance	Correlation distance (1-correlation coefficient) between the simulated image from the reconstructed 3D model and the original image data.

6. SUMMARY AND OUTLOOK

In summary, the methods we described here highlight how AFM imaging, combined with Contact-Point Reconstruction AFM (CPR-AFM) image analysis using Trace_y software, can reveal the structural details of individual amyloid fibrils within complex, heterogeneous and polymorphic amyloid populations. The unique ability of AFM topographic imaging to routinely resolve structural details of individual particles at nanometer resolution in heterogeneous populations, without the need for cross-particle averaging, makes it an ideal and particularly powerful technique to employ alongside cryo-EM single-particle analysis. Such integrative approach combining information from AFM-based individual filament level structural analysis with the high spatial resolution of cryo-EM derived structural maps, typically averaged over hundreds of thousands of images, can resolve the distribution of structures in complex

populations (Lutter et al., 2022). Further advances in automation, image analysis algorithms, artificial intelligence (AI) approaches, and correlative workflows will expand the applicability of CPR-AFM methodology. The protocols and methods described here could be applied to 3D reconstruction across a huge range of amyloid structures and helical filaments in general. Moreover, by providing 3D coordinates of tip-sample contact points corrected from tip-sample convolution artifact, CPR-AFM will support future developments in integrative structural biology workflows, combining not only with cryo-EM based information but also with diverse structural, biophysical, and computational approaches. This will deepen our understanding of the structure-function relationship in functional, plant-derived, disease-associated, and synthetic fibrils assembled from a wide range of peptides and proteins, in the contexts of agriculture, next-generation biomaterials, microbial adhesion, and therapeutics targeting neurodegeneration. Thus, vital insights gained from the detailed structural analysis of individual amyloid fibrils by CPR-AFM will enable the development of new strategies for diagnosing and monitoring amyloid-related diseases, and informing design and development of targeted, polymorph-specific therapeutics.

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FIGURE LEGENDS

Figure 1. Workflow for AFM sample preparation. (a) Mica surface substrate and AFM specimen discs that could be used for deposition of amyloid samples. (b)-(c) Preparation of an AFM specimen disc with a mica disc attached using double-sided tape. (d)-(e) Cleaving the mica using a piece of PVC tape. (f) Deposition and incubation of the sample solution on the freshly cleaved mica surface. (g) Washing the mica surface with sterile-filtered MilliQ water. (h) Gentle dying of the specimen surface with a stream of N_2 gas. (i) The specimen is ready for AFM imaging in air.

Figure 2. The main GUI windows of Trace_y (https://github.com/wfxue/Trace_y). An example image of amyloid fibrils formed *in vitro* from Aβ42 (Aubrey et al., 2023) loaded into Trace_y is shown together with the filament information of a traced and 3D analyzed left-hand twisted filament segment example.

Figure 3. The "Display Scan Line" display in Trace_y that could be used to check whether scanner bow and sample tilt are adequately removed, and the z-height of the molecularly flat mica surface is well adjusted to zero by baseline flattening. The example AFM image is the same as the example in Figure 2.

Figure 4. The peak picking user interface in Trace_y that is used to find as local maxima as reference points to pinpoint the helical repeats during the helical 3D reconstruction process. The central-line height profile of the example filament highlighted in Figure 2 is show together with the suggested peak locations in the height profile and in the image. Fourier transform of the central-line height profile is also shown.

Figure 5. Individual filament 3D reconstruction result output display from Trace_y. The 3D reconstructed surface envelope model of the example filament highlighted in Figure 2 is shown together with the image data, the simulated image based on the 3D model, 2D Fourier transform images of data and simulated image for comparison, and the cross-sectional contact-point density map.