29th July 2005

Dear Dr Grigorieff

Your manuscript entitled "Quaternary structure of a mature Alzheimer's β-amyloid fibril" has now been seen by 4 referees, whose comments are attached. While they find your work of some potential interest, they have raised concerns which in our view are sufficiently important that they preclude publication of the work in Nature, at least in its present form.

Should further experimental data allow you to address these criticisms we would be happy to look at a revised manuscript (unless, of course, something similar has by then been accepted at Nature or appeared elsewhere). In the case of eventual publication, the received date would then be that of the revised paper.

Any revised manuscript should conform to our format instructions and publication policies, which can be found at www.nature.com/nature/authors/.

Please use the link below to submit a revised paper.

I should stress, however, that we would be reluctant to trouble our referees again unless we thought their comments had been addressed in full, and we would understand if you preferred instead to submit your manuscript elsewhere. In the meantime we hope that you find our referees' comments helpful.

Yours sincerely

Senior Editor, Nature

Reviewers comments:

Referee #1(Remarks to the Author):

In this paper, Sachse et al. report cryo-EM analysis of a form of Aβ(1-40) amyloid fibrils grown in vitro. The data appear to be reasonably interpreted and model is very pretty. Other amyloid fibrils have been analyzed by this technique to similar resolution. There are several reasons why this work stands out, however, as being highly significant in spite of that. First, because of the potential disease importance of Aβ fibrils compared to the SH3 and insulin fibrils previously reported by CEM. Second, because of the existence of a variety of data on the Aβ fibril by other methods, including ss-NMR, that provide models for the basic folding unit of Aβ within the fibril that can be married with the EM data to build an interested and provocative model of how amyloid filaments bundle to make a fibril.

The paper is succinctly written and I find no major flaws in interpretation or over-interpretations. Perhaps because of the very stimulating nature of the data and discussion, I do have a rather longish list of questions and comments that should be addressed by the authors.

1. I am glad the Goldsbury paper is cited in the first paragraph, but I think it should also be cited in the sentence beginning "Different mature fibril morphologies ..." . In fact the Goldsbury paper is much more effective, than the other papers cited in support of this statement, at demonstrating how varied are the fibril morphologies one can see by examining a single preparation of amyloid fibrils. This previsouly reported heterogeneity of Aβ fibrils also raises a key question, however: Just how representative of a typical fibril preparation are the very long, straight, and regular fibrils analyzed here? The image averaging used to create the models shown in this paper require an extremely regular, straight fibril, including a highly regular twisted repeat structure, in order to align the images. Only this

kind of fibril can be analyzed, and it could presumably be done even if the long straight fibrils were only present as 1% of the total

I think the authors need to make some statement about the percentage of the total Aβ fibrils in their preparations that are in the structural class of those analyzed here. A low representation does not take away from the value of this work; one analyzes what one can. But knowing the purity is important if we are going to try to reconcile past and future data on Aβ(1-40) fibrils using this model - perhaps certain macroscopic analyses will not agree with the model, for example, because it represents only a minor component. We therefore need to know if it is a major or minor species.

2. Most of the alternative fibril morphologies chronicled by Goldsbury et al. appear to be different modes of assembly of a similar, basic filament. It would be useful for the authors to address whether their model suggests anything about the basis for the assembly of the wide variation in fibril morphologies suggested by the Goldsbury paper.

3. The authors cite the Tycko paper discussing fibril conformational variants, and use in their model building the Tycko model derived from data collected on fibrils grown under agitated conditions. But according to my reading of their methods, the authors grow their fibrils without stirring, suggesting that they more resemble the "quiescent" fibrils that Tycko has not analyzed in detail. I think it is important that the authors acknowledge this (if it is true) and discuss the possibly limitations of their attempt to overlay the Tycko model of the Aβ hairpin onto their fibril cross-sectional density. More broadly, they need to discuss how the existence of multiple, self-propagating "conformations" of fibril affects their analysis and its significance.

4. Paul Fraser published a paper in the early-mid '90s claiming to show that the histidine residues at positions 13 and 14 are critical for fibril formation from protofibrils; a double Ala mutant forms only protofilaments. But in the model shown here it is not clear how the His residues could play such a central role. Is this because this particular fibril is only a minor species, or is there another way to explain the Fraser data in the context of the Sachse et al. model?

5. The authors state in their introduction that the ss-NMR data shows that Aβ folds into a U-shape when it engages the fibril structure. But I believe the published ss-NMR data does not show this U-shape conclusively; it is only assumed in the model building. Aβ could fold into a zigzag, or slightly bent, motif in the packed fibril and still satisfy the basic ss-NMR data, which only addresses the strand-strand register in the H-bonding direction, not in the side-chain packing direction. I believe the first and only published unequivocal demonstration that Aβ must fold back upon itself in the monomer to make the fibril comes from the Shivaprasad and Wetzel 2004 disulfide crosslinking paper. These experiments were also with quiescent fibrils, presumably more like what Sachse et al. analyze here.

6. The speculations on page six at the end of the top paragraph are interesting, but I don't see how the authors can be sure that the energetics of protofilament packing drives or controls fibril morphology. Could it not be the other way around, that local conformational differences in the folded peptide influence and control protofibril packing, rather than follow it? It all comes down to the question of how much energy is gained in the basic folding of Aβ on itself, and its polymerization into the single protofilament array, versus how much is contributed by the packing of these long filamentous units into a fibril. I don't think this is known, so I don't know how the authors can conclude that the peptide fold follows the lead of protofilament packing.

7. The authors point out that their model for a three-filament fibril suggests an asymmetric structure with two different filament folds present in a 2:1 ratio, and reference ss-NMR data (on agitated fibrils) as independent experimental support of this model. They should also reference the observations of Kheterpal et al (2001) who showed in limited proteolysis experiments on (quiescent) Aβ(1-40) fibrils that about 20% of the Aβ peptides in the fibril have an N-terminus that is protected from

proteolysis, while the majority of the Aβ possess N-termini that are readily cleaved.

8. Considering the 24-28 loop to be the head, the model shown in Fig. 3a can be summarized as being head-to-head/tail-to-head. Such arrangements introduce additional asymmetry into the model (in addition to the relative 'squareness' of the central filament cross-section). A simple, more symmetric model would be two successive 'head-to-tail' arrangements. Could the authors briefly explain in the paper why they feel the asymmetric packing arrangement is required by their data? This arrangement requires that the 24-28 loop be capable of interacting both with itself, at one interface, and with something in the open part of the hairpin, at the other interface. This is of course not ideal from the point of structural simplicity and symmetry, and seems not to be required by the data presented.

9. Can the authors tell us more about the conformational antibody? Have them imaged fibrils decorated with this antibody and know anything about the epitope? How does it compare to the previously described conformational anti-Aβ-fibril antibody of O'Nuallain and Wetzel (which should be cited)? Does it also recognize other, non-Aβ amyloid fibrils?

10. The authors should discuss why it is that the antibody 22C4 with the linear epitope in the 31-39 segment of Aβ can bind so well to their amyloid fibrils, when the Tycko model on which the Sachse model is based places all of these residues packed into H-bonded extended chain that would be expected to be inaccessible to antibody binding?

Referee #2(Remarks to the Author):

Title: Quaternary structure of a mature Alzheimer's beta-amyloid fibril

Authors: Sachse, Gellermann, Xu, Habicht, Hortschansky, Brodhun, Gotz, Diekmann, Horn, Grigorieff, & Fandrich

Summary: The electron density of beta amyloid (1-40) fibrils has been reconstructed from 1533 segments of cryo EM images, to achieve an electron density map at 21 A effective resolution. This is too low to resolve subunits, but instead shows a ribbon-like shape having a cross section of an expanded S. A second map shows the fibril decorated by Fab fragments. Into these electron densities the authors fit a model for the A beta hair-pin model, and a model for the hairpin bound to Fab fragments for the decorated fibril.

Comments:

The cryo-EM reconstructions are of great interest, and to the extent that they are correct, they place severe constraints on acceptable molecular models of the fibrillar state of A-beta (1-40). But the single model proposed here has serious difficulties. These include the following points:

1. Parallel vs. antiparallel molecules: The arguments in the final paragraph of page 4, continuing on page 5, that the beta sheet orientation is parallel and not antiparallel are flawed. First, asymmetric density in the longitudinal direction could arise from antiparallel fibrils. Second, the diffraction pattern of Fig. 2e IS consistent with antiparallel fibrils. With antiparallel fibrils, we would expect even layer lines to be stronger than odd layer lines, and this does seem to be the case for the 11th, 12th, and 13th and 14th layer lines marked with reciprocal distances. What about layer lines at low resolution ? Why are these unmarked ? The third argument of the authors, the asymmetric binding of the Fab, DOES indeed tend to rule out antiparallel, but as far as I can see, it is also incompatible with the parallel model presented by the authors (see point 4).

2. Dimensions of the electron density compared to the dimensions of the model:

At the top of page 4 it is stated that core regions of electron density are 3.5 nm wide. This is far too wide for two beta sheets. Another question of dimensions concerns the axial spacing of the lobes of electron density in Fig 2a for the Fab decorated fibril. What is this spacing and how do the authors reconcile the value with the interstrand separation of 4.7 A, which is surely smaller.

3. Asymmetry and bonding in the model: Fig. 3e suggests that the three proto-fibrils are not in contact with each other, or at least that the left-hand one is not in contact with the others. Is this so ? If they are in contact, then there must be two different types of bonds between the protofibrils: the left two interact between the beta-turn of the left hand A-beta molecule and the disordered N-terminus of the central molecule, whereas the central and right-hand molecules interact between their beta-turns. What would prevent these interactions from continuing at the outer wings of the fibrils ? That is, there is a serious self-assembly problem with this model, with nothing limiting lateral growth of the fibril.

4. Density discrepancy of the model: Fig. 2c represents the fibril cross section as three capsules, with a dense (dark) end and a undense (light end). Now if we superimpose the model of Fig. 3a onto these capsules, the beta-turn ends of the peptide are all in the undense regions. This does not seem to make sense. Also if the Fab binds to the C-terminal region of the chain, the middle Fab molecule seems to misplaced. That is, the Fab binding does not fit the model.

5. Polarity of the model: Fig. 3a leaves the highly apolar C-terminus of each A-beta molecule exposed to solvent, which seems unreasonable.

In summary, the model does not fit well into the experimental electron density. My suggestion to the authors on this crucial point of their paper is to present their electron density, which is their main finding, and then attempt to interpret it with a range of models, including linear peptides, and models with more than 3 peptides per layer. Even so, I do not see how they can get around the fact that the electron density is quite symmetrical, although the Fab binding pattern is asymmetrical. This is a dilemma.

Some minor points:

1. Introductory paragraph, first sentence: the evidence that amyloid fibrils represent 'a fundamental structural state of the polypeptide chain' seems weak at this point. Why start with a controversial assertion ?

2. In the final paragraph it is claimed that CEM can provide high resolution information about fibrils. What information is this ? And in the final sentence, it is stated that this work may provide a basis for rational approaches to clinical presentations ? Please give an example or drop the assertion, which seems to be too great a reach.

Referee #3(Remarks to the Author):

This paper reports an elegant study of in vitro grown A-beta amyloid fibrils, using cryo EM to obtain a 3D reconstruction and fitting of the beta hairpin peptide structure previously obtained by solid state NMR. A second reconstruction of the fibril with antibody decoration confirms that there are 3 protofilaments and gives some indication of the polarity of peptide packing. A second, fibril-specific antibody is used to probe the epitope in an Alzheimer brain. The experimental work is very impressive. I have the following comments on the interpretation and presentation of the work:

The sites of antibody labelling do not appear to be consistent with the authors' interpretation of the peptide packing. The 2 outer protofilaments are labelled towards the C terminal end of the peptide, in accordance with the specificity of the antibody, but the middle protofilament is labelled at the bend region, not at the C

terminus. A model in which the middle peptide is flipped over (about an in-plane axis normal to the peptide long axis) would make the labelling more consistent. I am not convinced that the density variations within each protofilament are reliable at this resolution. Why should the density be higher at the peptide termini?

The features of the antibody labelled map are much coarser than those of the peptide alone. No information is given about the amount of data used for this map or the resolution obtained. Did the labelling affect the helical parameters or order of the fibrils? This information should be provided, at least in the methods section. Could the camelid antibody be used to check the labelling result?

The fitted model cannot be evaluated by a comparison of the surface views in figure 3 b and c. This is not very useful. Moreover, it hides the problem that there is a density peak in an empty region of the model. The density cross sections of the observed and model structures must be compared. This is particularly important in view of the discrepancy mentioned above.

I do not agree with the statements on page 6, par 2 that the in vitro fibrils are obviously similar to tissue deposited fibrils. Congo red binds to any amyloid fibril, and the ultrastructure of in vivo fibrils is too poorly resolved to make any detailed comparison. In vitro grown fibrils exhibit a wide variety of morphologies. In this case, the fibrils were grown at pH 9 (why?), which is not in the physiological range.

The description of the methods and results is not always easy to follow. I dont understand what is meant by helix dislocations and how they could be accounted for by the alignment procedure (image processing methods). Also, the tissue section staining is unclear - the plaques are visible but what shows that they are stained by the antibody?

The resolution criterion of 0.143 correlation: This criterion should be noted in the text where Fourier ring correlation is mentioned (bottom of page 3) and not only hidden in the methods section. Since most cryo EM papers still use the 0.5 criterion, the resolution claimed could mislead some readers. It would be useful to give the resolution at 0.5 correlation in the methods, for comparison to other work.

Referee #4(Remarks to the Author):

Grigorieff MS

The authors propose a fanciful atomic model for a "mature" Alzheimer's beta-amyloid fibril based on interesting image analyses of the cross-sectional structure of selected electron cryo-micrographs of A-beta(1-40) fibrils and complexes with a Fab antibody which are interpreted in terms of a published model for the A-beta amyloid peptide based on solid state NMR data. If the Alzheimer's A-beta amyloid fibrils had the ordered three-stranded helical structure proposed here, this structure would most likely have been determined years ago. Experimental data on a great variety of amyloid fibrils formed by unrelated proteins and peptides indicate that they share a similar one-dimensionally periodic cross-beta backbone structure with variable lateral structures. The authors note "that individual fibrils differ in diameter, length and cross-over distance" but no quantitative data on the variations is presented
Understanding the structural organization of A-beta amyloid fibrils will require information about these intrinsic variations.

The cross-sectional map (Fig.2b) was obtained by averaging image data from 1533 segments of 105 nm length cut from 52 straight fibrils. This averaging evidently involves stretching or shrinking individual images to fit the authors' 295 nm long Procrustean helical bed. It would be illuminating if the authors would present a histogram of the ~1090 cross-over lengths actually measured in their data set. The assumption made in the averaging is presumably that there is a smooth 180o rotation of the fibril cross-section between cross-overs, which provides the defined tilts

requires for reconstructing the cross-sectional image. It is not evident that this image reconstruction requires imposition of artificially regular helical periodicity. In the illustration of the idealized side view of the 295 nm period three-stranded helical fibril in Fig. 2a, it would be appropriate to shorten one cross-over and lengthen the other by the standard deviation of the cross-over length distribution

Furthermore, as noted in the "Image processing" section: "Analysis of alignment parameters and the known spatial relationship of the segments revealed helix dislocations". Data on the nature and frequency of such dislocations would be illuminating. If noticeable helical dislocations occur, is it not possible that there are more subtle variations in helical twist between cross-overs? The fact that the distance between cross-overs is variable obviously means there are at least long range variations in the helical twist.

The authors carried out "two truly independent reconstructions" by parting the whole data set in two halves. It would be illuminating to show the two independent reconstructions and the variance map between them. Furthermore, since the helical twist is variable, it would be interesting to sort the image data in two halves according to whether the cross-over length is shorter or longer than average to see if there is any correlation between the cross-sectional image and the tightness or looseness of the helical twist.

The reconstructed cross-sectional image of the 22C4-Fab complex with selected A-beta amyloid fibrils in Fig. 2b is impressive. The statistics on this reconstruction are not clear. Were the same number of fibrils and segments used in the image analysis as for the unlabeled fibrils? It would be illuminating to show the same information about cross-over length distribution and independent reconstructions suggested for the unlabeled fibrils. Are the distances quoted between bound Fab-fragments just the axial component of the separations or is it a measure in the plane of the image? The identification of the diffraction pattern in Fig. 2e is unclear. It is identified as from "an image of 22C4-Fab bound fibrils" but it does not look like an averaged diffraction pattern. Is it from a single cryo-image or is it from a negatively stained specimen? If the 5.3-6.7 nm reflections marked correspond to interpretable structural features in the image(s?) a filtered image should display these features. Illustration of some cryo-images of Fab labeled fibrils would be appropriate. The fanciful side view of the antibody labeled fibril in Fig. 2a has the antibody lumps all in register, but it would seem more likely that these molecules would be somehow staggered on the three protofilaments.

All the images selected for analysis presumably have the three-protofilament structure. How common is this form? The mass per length (mpl) measurement on "quiescent" fibrils from Tycko's lab (Petkova et al. 2005), which appear to have been formed under conditions similar to those in this study, show a broad distribution ranging from less than 2 to more than 5 A-beta(1-40) molecules per 4.7A cross-beta repeat period, with a maximum at 3-3.5 molecules per repeat. The experimental uncertainty in these measurements was large due to limited sampling and poor counting statistics. The Brandeis Structural Biology Laboratory has a long history of constructive involvement with the BNL STEM facility where mpl measurements on homogeneous populations of fibrous structures can be made with a standard deviation of a few percent

Would it not substantially enhance the impact of this paper if accurate mpl measurements were made on the distribution of fibrils formed under the conditions used in this study?

The molecular interpretation of the cross-sectional images in Fig. 2b is unavoidably fanciful, since there is insufficient hard data to define an atomic model. The surmise that the three elongated segments correspond to projected views of Tycko's hairpin A-beta molecular model is plausible, but there are four possible orientations of the model molecule in each segment. The antibody labeling does appear to rule out some of the possible combinations, but distinction of head to tail, head to head, tail to tail, up strand or down strand are ambiguous. The interpretation of small differences in image density is hazardous since as shown by

the antibody images, disorder can obscure features. A set of possible projected models like Fig. 3a could be generated that might help to guide further experiments. It is hard to see scientific value in Figs. 3b-d.

Fig. 4 could be included in the supplemental material, but micrographs of this fibril-specific antibody bound to fibrils would be appropriate for inclusion.

11th November 2005

Dear Dr Grigorieff

Your revised manuscript entitled "Quaternary structure of a mature Alzheimer's β-amyloid fibril" has now been seen again by 4 referees, whose comments are attached. As you will see, one of our reviewers has chosen to reveal his identity to you. In the light of their advice we have decided that we cannot offer to publish your manuscript in Nature.

While the reviewers find your work of interest, and two support publication in principle, the others raise concerns about the strength of the novel conclusions that can be drawn at this stage. We feel that these reservations are sufficiently important as to preclude publication of this study in Nature.

I am sorry that we cannot be more positive on this occasion but hope that you find our referees' comments helpful when preparing your paper for resubmission elsewhere.

Yours sincerely

Senior Editor, Nature

Reviewers Comments:

Referee #1(Remarks to the Author):

Grigorieff and coworkers have very diligently addressed the criticisms and I have no further concerns about this very interesting paper. In particular, the analysis showing that these represent the dominant form of fibrils under these conditions clarifies my major concern.

Referee #2(Remarks to the Author):

Title: Quaternary structure of a mature Alzheimer's beta-amyloid fibril

Authors: Sachse, Gellermann, Xu, Habicht, Hortschansky, Brodhun, Gotz, Diekmann, Horn, Grigorieff, & Fandrich

Summary: The electron density of beta amyloid (1-40) fibrils has been reconstructed from 1533 segments of cryo EM images, to achieve an electron density map at 21 A effective resolution. This is too low to resolve subunits, but instead shows a ribbon-like shape having a cross section of an expanded S. A second map shows the fibril decorated by Fab fragments. Both the Abeta fibril and the Fab-decorated Abeta fibril are asymmetric. The authors note that this asymmetry

cannot be explained by three identical protofibrils.

Into these electron densities the authors fit three protofibrils, each a stack of A beta hair-pin models. At the start of their discussion, the authors use appropriately cautious language for this fitting of molecular models to low-resolution electorn density: 'We find that a fully extended chain and the four-strand protofilament model do not fit easily into the density of our reconstruction. By contrast, a better fit can be obtained when U-turn-like structures...are considered.' And, 'However, there are two possible placements of the peptide. Although the present electron density map cannot distinguish between these possibilities...'

So far, so good. But now the authors select biochemical data of others to support a particular molecular model, while ignoring other biochemical information, such as the extreme unlikelihood of 'leaving the [highly apolar] C-terminus solvent-exposed and not buried by inter-protofilament interactions.' They go on to conclude (bottom of page 7), 'Taken together, these data show that the combination of CEM with complementary structural techniques can provide novel high-resolution information about ...amyloid fibrils.' In fact, the ms does not provide high-resolution information. It provides a model based on low resolution information, and notes aspects of the model that are consistent with other experiments. They also include an unsupported sentence at the end of the paragraph saying '...such data may provide a basis for novel structure-based drug design...'

What would make this paper stronger: First, remove unsupported claims and take care not to exaggerate the assurance of the models offered (including in the introductory paragraph). Second, provide some independent (spectroscopic ?) data that their fibrils contain two conformers. Third, provide mass per length measurements. I agree strongly with Referee 4 that such measurements would greatly enhance the assurance of the proposed models.

Some minor points:

1. At the end of page 3, the average cross over distance is cited to 4 significant figures. Surely the measurement is not this precise.

2. Page 5, second paragraph, line 3: 'principle' should be 'principal'.

Referee #3(Remarks to the Author):

The authors have clarified most of my questions, but there is one point about the image processing that I find completely baffling. How can they find helical dislocations of 180 degrees in their projections of the continuous helix? The projections of a segment at 0o or rotated by 180o about the helix axis are identical! This does not make any sense. There is a serious error either in the analysis or in the communication of what was actually done. Aside from this problem, I think the paper is worthy of publication. The reconstructions are fairly low resolution and do not restrict the fitting to a unique atomic model, but the combination of 3D density, peptide structure, antibody binding and correlation with tissue labelling makes it a strong experimental story.

Referee #4(Remarks to the Author):

The manuscript on A-beta amyloid fibril structure that you have transmitted to Nature is not suitable for publication in its present form becuse of a fundamental question regarding the way asymmetry in the fibril cross-section has been imposed by the data processing. You are presumably aware of the mistake that Unwin and Klug made in imposing non-existent asymmetry in their image analysis of the TMV stacked-disk structure, and the work by Diaz-Avalos and myself demonstrating the

symmetric bipolarity of the disk in this structure . Our work has established simple criteria for deciding if asymmetric features in an EM image average are significant above the noise level in the data. It seems unlikely that you have taken a very active part in the physically unrealistic data manipulation involved in enhancing asymmetric features in the image reconstruction of the A-beta fibril cross-section. Whoever carried out the image analysis and wrote the paper, the results are not in your best interest.

It is easy to recognize the approximate level of noise in the image averages obtained in this study. The two pairs of independent averages of the A-beta cross-section (one based on odd and even samples and the other on cross-overs shorter or longer than average)and the pair of antibody-labeled cross-sections, which are included in response to my queries, provide an objective measure of similarities and differences. The maps are contoured with a six step grey scale (suggesting that the standard deviation is the order of one sixth the maximum density). It is evident that the difference between any pair is not uniformly zero. The integral of the differences must be zero since the maps are scaled the same, but there are a number of positive and negative difference features at the one step grayscale level and perhaps some features at the two step level. The ratio of the integral of the absolute value of the differences (or the square root of the integral of the squared differences) relative to the integral of the average provides a measure of the noise. By eye, I would judge this noise level to be at least 20%. A quantitative measure could be easily made by computing the differences. The displayed maps all have approximate two-fold symmetry. For the A-beta maps, a 180 degree rotation about the center will lead to superposition within the same outline. With the antibody labelled cross-section, if the arbitrary flipping had not been imposed in the image averaging, the one noteable assymetric feature would almost certainly be symmetrized. The difference between the two-fold average and the asymmetric image appears likely to be about the same as the difference between any two independent asymmetric averages - i.e. the noise level. This does not establish that the cross section has dihedral symmetry; it would indicate that the available image data cannot discriminate between a dihedrally symmetric and asymmetric cross section.

What the authors of this paper have done goes beyond what Unwin and Klug did in aligning images of individual particles to maximize the the correlation of the antisymmetric component of their Fourier transforms, which was reasonable on the presumption that the structure might be polar, as I had predicted in 1963 - what was missing in their analysis of the stacked-disk structure was an objective assessment of the noise in the images. What has been done in this paper is based on the assertion at the bottom of pg. 8 that "the helix alignment procedure consistently picked up groups of consecutive segments with orientations that were rotated 180 degrees around the helix axis compared with orientations expected for a continuous helix". In simple terms, the last five words mean that the helix ribbon should have a "dark" side and a "light" side. Thus, if the dark side is on the left in one loop of the helix, it should be on the right in the next loop. If the dark crest is on the same side in consecutive loops, then that was presumed that there must have been a "dislocation" involving a "cut and paste" operation that switched the dark crest to the wrong side. To correct for these presumed dislocations, the offending 105nm segments "were included in the 3D reconstruction with the determined orientation" - that is, they were flipped by 180 degrees. If the two sides of the helical ribbon were the same, the two sides of the projected image would be related by an axial mirror line. The one selected cryo image shown in fig. 2b appears mirror symmetric about its axis, but the computationally averaged image in the background of the top frame of fig. 2a has lost this mirror symmetric appearance after the cut and paste manipulation of the averaging procedure. The three stranded structure modeled in this paper is presumed to have an asymmetric structure - the zig end of the zig-zag is darker than the zag end. But the atomic models presented in fig.3g all presume that the zig and zag lobes correspond to identical Tycko hairpins that can be arranged in four possible combinations, two of which would be dihedrally related and the other two in parallel orientations. If this were true, the densities of these lobes should be the same - in contrast to the enforced asymmetry. To my mind, the most unreasonable aspect of the cut and paste operation involved in the imposition

of asymmetry in the image averaging is that it postulates switching from the parallel in-register cross-beta hydrogen bonding established by SSNMR to an occasional anti-parallel link, at least for the central strand, every time one of the fancied "dislocations" is identified. I expect that the measured frequency of these dislocations could be quantitatively accounted for by random noise that makes one or the other side side of the helix loops darker in a head or tail sequence of random coin flips. The manipulations involved in the image processing were not evident in the original manuscript and were detailed in this revised version to explain the nature of the "dislocations". When I queried the nature of the dislocations,I had expected localized changes in twist which are physically possible, and evident in some published A- beta amyloid fibril micrographs. I have detailed the unreasonableness of the cut and paste flipping since this kind of nonsense could be an embarrassment for you.

At this stage, I do not think Nature should consider another revision of this MS even though there are evident biologically interesting aspects to the fibrils analyzed in this study. I urge you to take charge of writing a more detailed report which omits the nonsense in this MS and presents more complete and objectively analyzed data. A straight forward averaging of the 52 A-beta and 54 Fab labeled fibril images will necessarily produce more dihedrally symmetric appearing cross-sections. Quantitative analysis of the noise in the images would establish if dihedral symmetry is possible - but at the available resolution, such symmetry could not be established. Thus, there may be a rather large number of possible models that fit the data. Two of the postulated three-stranded models in fig.3g have dihedral symmetry for the outer pair of protofilaments. It seems to me that four stranded models cannot be ruled out from the image data - but mass per length measurements could make such a distinction, and might produce some surprises, like the recent measurements on strains of yeast Sup35p prion amyloid fibrils by Diaz-Avalos et al. from my lab. The observations on the 12-13nm fibrils are interesting, since they appear a bit thin to be two thirds of a 21nm twisted ribbon.

The demolition of defunct amyloid fibril models detailed in fig. 3b-e is in bad taste. Prof. Pottle at Yale half a century ago told his English Lit students that such attacks on moribund targets constituted "beating dead woodchucks". I was sorry to see Max Perutz's fanciful posthumous amyloid model among the dead woodchucks. His model, even though it has little connection with reality (and no connection with A-beta), is a monument to his ability to focus on fundamental unsolved problems even as he was dying. He continues as an inspiration to all of us who knew and admired him.

I wish you better luck with your collaborators in the future.

12th December 2005

*Please ensure you delete the link to your author home page in this e-mail if you wish to forward it to your coauthors

Dear Dr Grigorieff

Thank you for your letter asking us to reconsider our decision on your manuscript entitled "Quaternary structure of a mature Alzheimer's β-amyloid fibril". After careful consideration we have decided that we would be willing to consider a revised version of your manuscript. We are planning to ask further advice from our reviewers on the concerns raised by Dr *******, and, as per your suggestion, involve an additional referee.

When revising your paper:

* include a point-by-point response to our referees and to any editorial suggestions

* ensure it complies with our format requirements for Letters to Nature as set out in our Guide to Authors (see www.nature.com/nature/authors/gta/index.html for more details)

* state in a cover note the length of the text, methods and legends; the number of references; number and estimated final size of figures and tables; and the number of Nature pages you estimate your paper will fill (with figures reduced to their minimum size).

* ensure that all communications, including your revised paper and the subject line of your email message, are marked with your Nature reference number

We hope to receive your revised paper as soon as possible.
Please use the following link to submit your revised manuscript.

We look forward to hearing from you soon.

Yours sincerely

Senior Editor, Nature

17th January 2006

Dear Dr Grigorieff

Your manuscript entitled "Quaternary structure of a mature Alzheimer's β-amyloid fibril" has now been seen again by two of our original referees and an independent expert in the field (the comments are attached). In the light of their advice we have decided that we cannot offer to publish your manuscript in Nature.

While the reviewers find your work of interest, they continue to raise issues, of technical nature, which cast doubt on the strength of the conclusions that can be drawn at this stage. We feel that these reservations are sufficiently important as to preclude publication in Nature. Your work seems best suited to a journal that caters for the specialist and that is able to spare the space for a detailed discussion of your results and their implications.

I am sorry that we cannot be more positive on this occasion but hope that you find our referees' comments helpful when preparing your paper for resubmission elsewhere.

Yours sincerely

Senior Editor, Nature

Reviewers Comments:

Referee #3(Remarks to the Author):

In the current revision, the authors have removed unsupported claims, and have added arguments to support their analysis. I am not expert in the critical question of noise analysis, and cannot comment on the reliability of the reconstructions.

From the start I have been doubtful of the authors' molecular interpretation of their reconstructions, because they do not obey conventional ideas about self-assembly. In the current revision, the authors hypothesize that the apparent three protofilaments with different Fab binding sites are the result of a 2:1 mixture of molecular conformers. Even if two conformers are present, it is hard to imagine what sort of different bonds there can be between them that lead to the proposed arrangement. How can one Fab bind end on to the central unit, and two others bind side on to the two peripheral units ? So I remain skeptical of the interpretation.

Referee #2(Remarks to the Author):

I did not previously understand what was being done about dislocations, because the idea of 180o dislocations in the structure seemed so improbable. According to the methods (page 9), the crossover repeat is 135 nm and dislocations are found on average once every 176 nm. This appears to mean that there is a 180o flip in almost every crossover repeat.

The MSA analysis (eigen image 2) could arise from asymmetry or perhaps from slight misalignments. In any case projection matching can not be expected to give a 100% reliable assignment for such a marginally detectable feature. If a minor fraction of the segments were seen to flip, I would suggest omitting those segments in calculating the map. But if I have understood correctly, there is a dislocation in almost half the segments! Such a physically unreasonable model would need stronger evidence. I suspect I am still not following the argument, because the wording on page 9 implies that fewer dislocations were detected for the antibody bound structure, but the figure is one in 156 nm, making them even more frequent than in the unlabeled structure. The iterative helical method of Egelman has generally been applied to helical structures with clearly visible subunits, rather than to this type of structure, which is effectively a continuous helix in the resolution range available. What is the effect of the segment length on the apparent number of dislocations? What about doing 2 separate reconstructions from flipped and unflipped segments? Do they each show the asymmetry?
I think the comparison of models to the data is very important. There are many speculative models for amyloid structure in the literature and and not much real 3D data.

In summary, I think that the issue of dislocations and the implications of their presence or absence for the structural model must be clarified in order to make the paper acceptable.

Referee #5(Remarks to the Author):

This manuscript describes a cryo-EM study of fibrils formed by the 40-residue beta-amyloid peptide (Ab40) associated with Alzheimer's disease. The authors present a reconstruction of the electron density in Ab40 fibrils that exhibit a pronounced twist with approximate 135 nm cross-over distance. The fibril consists of three ribbon-like subunits, arranged to give an S-like cross-section. From the density itself in Ab40 fibrils and from the structure of fibrils after binding the 22C4-Fab, the authors conclude that the three subunits are asymmetric, with the central subunit being the most different of the three. The cryo-EM results are also consistent with parallel beta-sheet structures (not antiparallel beta-sheets). The authors show that a U-shaped peptide conformation previously proposed by Petkova et al. fits the electron density of the fibril better than several alternatives.

Finally they show that the B10AP antibody that binds their synthetic Ab40 fibrils also binds to amyloid plaques in post mortem Alzheimer's brain tissue.

This is an interesting paper, but several of the conclusions drawn from the cryo-EM data represent confirmation of aspects of Ab40 structure that have already been established by other measurements. These aspects include the U-shaped peptide conformation and the parallel beta-sheet structure (established by solid state NMR and EPR several years ago, and supported by subsequent mutagenesis, cross-linking, and hydrogen/deuterium exchange data from several groups, including Wetzel, Riek, Meredith, and Fernandez), as well as the existence of three subunits in Ab40 fibrils that have pronounced twist (established by scanning transmission electron microscopy data from Petkova et al., Science 2005). The 2:1 asymmetry was suggested by Petkova et al., based on observed splittings of NMR signals, but not firmly established. The main new result in this paper is therefore the overall shape of the electron density, which suggests how the U-shaped peptides may interact. Unfortunately the cryo-EM data can not resolve the molecular-level details, so various possible peptide orientations and hence modes of interaction (see Fig. 3g,h) can not be distinguished, and the differences in molecular structure of the three subunits can not be determined.

My overall assessment is that, while this is a valuable contribution to the amyloid structure field, there is not enough new information in this paper to justify publication in Nature.

Regarding technical issues raised by another reviewer:
1. The other reviewer apparently questions whether the structural asymmetry, particularly between the two "outer" subunits in Ab40 fibrils, is statistically significant. The authors claim to have checked this and verified that their conclusions are significant. They refer to supplemental Figure 1 as a demonstration of statistical significance. In supplemental Figure 1, I don't understand why the apparent "noise level" in part E is higher than in part C. It seems to me, if the authors are using the same contour levels in parts E and C, the "noise level" (away from the center of the depicted area) should be the same. Some explanation seems necessary here.
2. The other reviewer questions how "dislocations" can occur, as described in the Methods section of the main manuscript. This is also unclear to me. I think it's unlikely that 180 degree rotational dislocations about the helix axis really occur in the Ab40 fibrils. Perhaps these apparent dislocations are an artifact of the reconstruction method (e.g., perhaps the fibrils do not really have the assumed helical symmetry?).

April 4, 2006

Title: "Quaternary structure of a mature Alzheimer's beta-amyloid fibril reconstructed from cryo electron microscopy images"

Author(s): Sachse et al.

Dear Dr. Fändrich,

The expert who served as editor for your manuscript has obtained reviews from two reviewers, whose reports are attached. After careful consideration, the Editor has decided that we cannot accept the manuscript. However, since the reviewers thought the work was of interest and the Editor concurs, we would be willing to consider one resubmission that constructively addresses all of the concerns raised in the critiques. Remember that the paper would have to satisfy both the reviewers and the Editor, and new criticisms could arise during the review, so there is no guarantee of success, and we will be unable to consider further resubmissions.

For now, we must consider your manuscript to be rejected. Once a paper has been rejected, it may not be resubmitted through an Academy Member.

Thank you for your submitting to PNAS.

Sincerely yours,

Senior Editor
Proceedings of the National Academy of Sciences
pnas@nas.edu

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Editor's Comments

The two referees have each made comments identifying a number of problems with the manuscript in its current form. The first referee would like to see the X-ray diffraction data included, questions the conditions used to form the filaments and is baffled by the structure, suggesting further experiments. The second referee does not believe the model can explain the observations, suggests making additional measurements of the mass-per-unit-length of the filaments, and has a variety of criticisms about the presentation and the way the work is related to or cites earlier work. I believe it is best to reject the paper from PNAS in its present form and advise the authors to do more work at least in the presentation but preferably also in the experiments before resubmitting, or to submit the paper to a more specialised journal.

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Reviewer #1:

Suitable Quality?:
Yes

Sufficient General Interest?:
Yes

Conclusions Justified?:
Yes

Clearly Written?:
Yes

Procedures Described?:
Yes

Supplemental Material Warranted?:
Not Applicable

Comments (Required):
The manuscript by Sachse et al describes the cryo-electron microscopy and single particle helical processing of amyloid-like fibrils composed of Abeta1-40.

The authors should make clear that the morphology of amyloid fibrils depends on different growth conditions. Presumably these particular preparation conditions were chosen for their possibilities for further processing of the fibrils?

X-ray diffraction data is mentioned, but not shown. This is very important data that contributes to the structural interpretation and therefore should either be included or at least included in supplementary data. The authors mention the meridional at 4.76 A, but no mention is made of an expected equatorial. Was this present? This is necessary to regard the structure as "cross-beta".

The resulting structure is baffling. The S-shaped cross section is very difficult to understand. If this structure is made up of hairpins, why would they arrange themselves like this, with different contact surfaces between the "hairpins". I wonder if treatment of these fibrils (heating, freezing) might induce some unravelling. It would be interesting to see if the "protofilaments" are just associated together (via the turns) or whether they are more intimately linked via a extended strands. One could envisage the exchange of b-strands between the protofilaments? Certainly further discussion of the nature of the morphology is required.

I do not feel qualified to comment on the use of the antibody to examine the structure of tissue amyloid. I wonder how specific the antibody is to different amyloid fibril morphologies? It seems to me to be very important to show this data if the authors are arguing that the fibrils in vivo and in vitro are structurally identical. As I said before, many different morphologies of Ab40 fibrils can be formed depending on conditions. Clearly, the conditions used to form fibrils in vitro are not very "physiological!"

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Reviewer #2:

Suitable Quality?:
No

Sufficient General Interest?:
No

Conclusions Justified?:
No

Clearly Written?:

No

Procedures Described?:
No

Supplemental Material Warranted?:
Yes

Comments (Required):
In this paper the authors use electron cryomicroscopy to compute a cross-section through amyloid filaments made in vitro from the Alzheimer Abeta(1-40) peptide. They interpret the cross-section in terms of the packing of a hairpin model of the peptide. However the resolution achieved in the map (~2nm) is too low to allow any detailed axial or other internal features to be discerned. The model building is therefore highly speculative and not very convincing (see points 6-9 below).

The description is is quite hard to follow in places and the following points need attention.

1. Abstract and Introduction p.3. It would be useful to mention that there are other amyloid filaments present in Alzheimer's disease, namely the tau filaments in neurofibrillary tangles. Also the synuclein filaments in another neurodegenerative disease, Parkinson's disease. These are surely more relevant than non-disease related filaments, such as apomyoglobin or polyamino acids.

2. Abstract l.6. It is totally unclear what is meant by "... three protofilaments with a 2:1 ratio."

3. p.4. Meaning of last sentence obscure.

4. p.5 Fig.2a,b. What are the black traces superimposed on the histograms?

5. p.5 last line. What is the significance of the segment length of 105nm and why is it different from the average cross-over spacing?

6. p.6 middle paragraph, Fig.2g. It seems quite implausible that a single hairpin, in which the main-chain strands will be ~1nm apart, can adequately fill the cross-section of a protofilament, which is quoted to be about 3.5 nm across. It also seems implausible that a model in which all the hairpins in the central protofilament (green) point in the same direction can account for the strongly 2-fold appearance of the overall cross-section, as the red and blue strands would be making completely different interactions with the central green strand. Have the authors considered models in which the hairpins in the central protofilament are stacked alternately, thus creating equivalent interfaces for the outer strands? The sentence (l.16) "Due to the....." is very obscure.

7. p.8, l.14. Is there good agreement between the U-turn like peptide and the cross-section (point 6 above)?

8. p.9 l.5 and Fig.4. Many other packings would lead to a cross-beta structure. As the authors point out (l.9), the resolution of the reconstruction makes it hard to justify one particular model. Fig.4 in its present format is not very useful as the "cross-beta" structure is hard to see and in any case uninformative.

9. Have the authors considered making mass-per-unit-length measurements, which would give some independent idea of how many peptides might be packed in the cross-section?

10. p.11 Image processing. What was the initial reference for the iterative reconstruction? How many segments were chosen initially and how many were finally included? Why was the subunit repeat of 4.76A imposed? It could well be a multiple

of this and in any case is not really needed for the reconstruction? What was the electron dose and defocus range and were any corrections made for CTF?

11. p.13 l.5/6 Does not make sense.

12. p.18 Fig.2a legend. How was the variable cross-over spacing dealt with in the manual aligment?