**Refining a flexibly fitted homology model of subunits Rpb1 and Rpb2 in the map of elongating Pol III (EMD-3178)**

The homology model was built based on the crystal structure of RNA polymerase I (PDB ID: 4C3I). Long flexible loops were trimmed from the homology model (Core\_selection\_4c3i\_AB\_trimmed.pdb). Each chain of the model was then rigidly fitted using Chimera *Fit-in-Map* tool, and a couple of sub-domains that were clearly outside the density were also rigidly fitted by selecting sub-domains and allowing only the selected region to move while fitting (Disable *Move whole molecules*). 5 iterations of Flex-EM were run to fit subdomains into density (ribfind cutoff: 30). The flexibly fitted model is **4c3i\_AB\_flexfit\_5.pdb**

A map segment corresponding to these subunit was extracted using Chimers Segger tool (**emd\_3178\_core\_regions.mrc**).

**Refinement using Refmac5**

**1. Inspect model in Coot**

(a) Open Coot, and load the model and map:

Model: **4c3i\_AB\_flexfit\_5\_na\_shifted.pdb**

Map: **emd\_3178\_core\_regions\_shifted.mrc**

The \_shifted models and maps are moved to a grid with origin 0 (for Coot and Refmac to be consistent in placing the atomic model in map).

· Fix nomenclature error warning -> Click Yes

· If you don’t see the map, but you do see it listed in the Display Manager, then it could be that the contour level is too low – try increasing the map contour level (e.g. using the scroll wheel).

· It is often useful to display the box that encapsulates the map:

Draw -> Cell & Symmetry -> Show Unit Cells.

Then zoom in/out until you see the whole box (using the “m” and “n” keys).

· Increase the map radius in order to display more of the map (e.g. try 70 Å in this case):

Edit -> Map Parameters -> Map Radius. [if Coot becomes unresponsive drop this to 30 Å]

· When zoomed out, it is often useful to display the model as a CA trace (via the Display Manager).

**2. Refinement Preparation**

(a) Before refining the model, first investigate whether the map should be blurred/sharpened for refinement.

· Open the CCP-EM interface.

· Select the “MRC to MTZ” task. Provide the map, and specify the resolution (3.9 Å). Now run the job.

· Once the job has finished, look at the output plots. Is there an argument for blurring/sharpening the map for refinement?

· If you click on the “Coot” button at the top-left of the interface, Coot will open with the array of blurred/sharpened maps loaded, along with the model (if the model was provided as an input to the “MRC to MTZ” task).

Over-sharpening adds noise and/or suppress larger/low-resolution features. On the other hand excessive blurring removes high-resolution features like side-chain densities.

**(3) Refinement**

(a) Now try to refine the model using Refmac5, blurring the map using a B-factor of 50 Å.

· In the CCP-EM interface, select the Refmac5 task.

· Provide the map, your fitted model, and specify the resolution (3.9 Å).

· Open the “Refinement options”, change Refmac cycles to 200 and type “50.0” into the “Sharpen / blur” field.

· Now run the job (this should take between 40-60 minutes, depending on computing power).

While this is running, set up another run with modified parameters

(b) Refinement with modified parameters.

Now let’s see if we can improve the model by adjusting refinement parameters. We want to improve the fit-to-data (as judged by the FSC), without overly negatively affecting the geometry (agreement with prior knowledge). As can be seen in the Results page corresponding to the previous refinement run, the weight is ~0.00019. In order to improve fit-to-density, we need to increase this weight.

· Clone the previous Refmac5 refinement job in CCP-EM (double click on the job, and then select “Clone” from the top left of the window).

· In “Refinement options”, untick “Auto weight” and specify a higher weight of 0.001. Ensure that the “Sharpen / blur” field is still set to “50.0”. Make sure to hit Enter, or otherwise click away from the field, in order to ensure that the interface registers the parameter value change.

· Run the job.

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This run will take 40-60 minutes and carry on with the other tutorial(s) while waiting for them to finish

Once it has finished, compare the refinement statistics from this job and the original one.

· Click on the “Coot” button in the upper-left portion of the window. Look at the Ramachandran plot from this refinement run, and compare with the original.

· Which of the models do you prefer – the one with auto-weight, or the one with weight 0.001? Compare the models from both runs (see below).

(c) Inspect the refinement statistics.

· Once the job has finished, click on the “Results” tab. Look at the refinement statistics table, as well as the graphs.

· Does refinement seem to have converged? Were 200 cycles sufficient?

In practice one starts with fewer cycles (say 50) and extend the run until convergence.

· Is there evidence that refinement has improved the model, or made it worse? Consider statistics representing fit to the data, as well as geometric quality.

· What is the major contributing factor to the improvement of refinement statistics between Start and Finish? Is it surprising that the refinement statistics have improved? (Hint: consider the refinement protocol, particularly the treatment of atomic B-factors – look at the commands passed to Refmac5, which are listed at the top of the “Refmac refine (global)” logfile)

(d) Visual inspection.

Click on the “Coot” button at the top-left of the interface. Coot will open with the map and both the model before and after refinement loaded and displayed. The model before refinement will be coloured yellow, and the one after refinement green. If you cannot see the map then change the contour level (e.g. using the scroll wheel), and increase the map radius (e.g. to 70 Å).

· Inspect the models. Can you see any evidence of changes in the model, or improvements to local model quality?

· Zoom out so that you can see the whole model(s). Open the Display Manager. Change the representation of both models to “Colour by B-factors - CAs”. Now display/undisplay each of the two models in turn, and consider the colouring of the two models. What does this tell you about the differences between the biological assemblies of the two models (i.e. the structure under refinement versus the model of the homologous macromolecule)? Does this reflect anything about the relative resolutions of the maps underlying the two models?

· Compare the Ramachandran plots corresponding to the two models (in the Validate menu). Are any differences due to overall trends or individual residues, and are they substantial or minor?

**(4) Manual Intervention and Validation**

Unfortunately, not all issues can be solved automatically, in general. It is often necessary to inspect and manually correct the model in Coot in between rounds of refinement.

Since local resolution varies throughout the map, it is very useful to visualise multiple maps simultaneously when critiquing the model.

· Select the “MRC to MTZ” task. Provide the map, the PDB file corresponding to the output of the latest refinement run (this will be called “~/ccpem-project/Refmac5\_X/refined.pdb” or similar), and specify the resolution (3.9 Å). Now run the job.

· Once the job has finished, click the “Coot” button in the upper-left corner of the window.

· Inspect the model and map, in order to identify any discrepancies. There are various tools in Coot to help with this, notably (but not exhaustively):

o Validate -> Ramachandran Plot

o Validate -> Rotamers

o Validate -> Density fit analysis

o Display Manager -> Colour by B-factors

In Coot, go to residue 361A. You may notice that the residue backbone is out of the map density and can be fixed to fit into density.

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