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Mon, 10/21/2019 - 04:04

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barbierialessandro87

## 3D refinement not improving

Hello,

I am a new cistem user, basically first experiences on data-processing. Currently I am trying to perform 3D refinement based on some ab-initio classes but I get stuck, so I would need guidance, perhaps also conceptual.

My dataset requires the VPP settings and unfortunately circa a third of the 2,4k micrographs had poor/absent CTF-fitting. About the 1,6K micrographs left (>310k particles) almost 50% are slightly astigmatic, but maintaining good 1D plots, good CTF fitting. I was able to easily perform good particle picking and 2D classification runs. I can clearly see different orientations of my protein (known from literature) with micelles.

**Q1)** This loss of micrographs is due to the Volta PP settings?

**Q2)** Is there a way to fix it and recover micrographs lost? I tried slow CTF or other settings on a subset, but it employs too much and it doesn't give me anything good back (too close to focus?).

Despite there were apparently no changes between 2D runs between 'astigmatic' and 'regular' I later performed ab-initio run (80steps) using only the 50% with better CTF-fitting, no astigmatism (after several 2D classification runs > 50k particles) and among the 5 different classes, 3 were showing a very good profile and slightly different (but evident) conformations. I could see clearly the helices as tubes separated between each other and a CC of 0.77 when employing Chimera + PDB model from MDFF run.

**Q3)** I don't know if employing the 50% 'regular' for abinitio was a good thing or conceptually wrong, but since in my lab with relion they were experiencing some troubles with a reference model (suspect cause of flexibility) I wanted Cistem to calculate the initial 3D classes in some way.

**Q4)** Shouldn't be higher the initial CC or I am making an uncorrect consideration?

**Q5)** Estimating the resolution with Resmap of the 3 classes is reliable at this point?

After that I started to play with auto/manual refinement ('astigmatic' + 'regular'), with dozens of attempts, someone promising, but nothing really satisfying, perhaps due to my background limitations. I started with initial Res limit in the auto-refinement of 20A or later 16, 10, but I get only a worsening. When I employed 8,7,6 A as initial value, it started to produce something fine, but then playing with different smoothing factor I get completely different results.

Employing SF 0.5 I generally can see a gradual increasing of the FSC curve, while if I try 0.25 I can visually see the quality of the maps dropping down. No general change with SF 1. Some lecturers told me to use SF of 3.0 / 4.0 and employ a mask (done on Relion derived from the same density ) and apparently the density after that looks much better, with the helices assuming a jagged shape more than tubes. But at the same time I can clearly see that some parts are lost and not fitting the shape of the protein (in terms of CC I see a decreasing from 0.77 to 0.50) and in terms of FSC curve I don't see any increasing.

**Q6)** How many refinement runs are generally advisable? Generally we should start with autorefinement?

**Q7)** Which strategy I should follow? Perhaps it's a 'beginner' problem, but I would be glad if you could direct me in the right way. I can send you a small table with resumed all attempts, if necessary.

Many thanks in advance,

Alessandro





timgrant

Hi Alessandro,

Hi Alessandro,

Sorry for taking so long to reply.

1 & 2. If your micrographs are very close to focus, and then there may not be enough rings to fit the CTF. Do you think this could be the case? Do you see rings in the spectra?

3. There is nothing wrong with taking the best data for ab-initio, in fact it is probably a good idea.

4. Is the CC you are taking about here the CC with the model? In general CC is not a very good figure for comparisons, you might want to consider using an FSC instead.

5. I think Resmap is never particularly reliable as an estimation for absolute resolution, it can give you information about the relative resolutions, but often gives misleading absolute resolutions.

6. If you only get worsening results with 20A it implies that your initial model may not have been correct. What is the molecular weight of the particle? The smoothing factor shouldn't change anything unless apply likelihood blurring is selected. This is a little used option that you probably shouldn't turn on. The manual refine is really for doing more "experimental" things, and pushing the results when at high resolution. In general you should use the auto-refinement if you are not confident in what you are changing. It is always very important to pay attention that your resolution limit it less than your measured resolution. E.g. If you start with a resolution of 8A, and the final map is not better than say 5 the result is not necessarily believable, you should always quote your highest resolution ever used in any results / publications.

7. It is hard to give more advice without more details. What is the size of the protein, and how do the 2D classes look?

Tim







barbierialessandro87

Hi Tim,

Hi Tim,

No problem, I also needed some time to reply.

**R1&2)** Around 66% of micrographs show clearly rings, with a 50:50 ratio between 'circular' and 'elliptical'. In the 33 % left I can't see any ring and the 1D plot look like a 'scribble', despite often micrographs look good and particles are clearly visible. Could be the case of (close to focus)? If you have any idea how to proceed, please let me know. I guess I can't use them...

**R3,4,5)** Ok. Sometimes the FSC curve drops down very sharply...

**R6&7)** The molecular weight is 140 KDa, radius 60-72A. 2D classes look good to me and other people in my lab I've spoken with. I understood your point about auto/manual/smoothing/blurring.

*New:*

Probably I tried too many experiments, but I might not have employed an initial model correct after all, so maybe the abinitio classes are not perfectly fine for some reasons: the Abinitio run package included 50k particles and 5 output classes....the final best three classes possess around 12k particles each and are slightly different in terms of conformation, so:

**Q8)** Should I have employed results after just a single abinitio run ? (40 cycles x 1 start instead of 40 cycle x2?) (I have saved all intermediate steps if this helps)

**Q9)** Could be not enough in terms of particles number? Or could have happened that the number of output classes (5) was too much? The program seems to separate three slightly different classes but what if actually the correct one it's a mixture of them and the program was forced to arrive to that result? Do you think lowering to (3) results could help?

**Q10)** I guess I have to employ new volumes from literature, if there are any matching...are there any other options to correct what I have?

Many thanks in advance for your availability, sorry for plenty of questions, but I'd prefer to be ehaustive also to learn,

Best,

Alessandro



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