Cryo-EM, basic image processing course, IGBMC, ReNaFoBis, 24.6.2019, B. Klaholz

Deactivate wifi! Useful: mouse (middle button needed), for Mac's: adaptor for Ethernet cable and activate XQuartz; if display doesn't work anymore after some time try to logout and reconnect

To boot an USB drive => SanDisk extrem: - display the startup menu (make sure that your USB flash is connected on your computer, start (or restart) your computer, and press Esc button to display the startup menu) - choose "Boot Menu" or F9 => "Boot device" : sanDisk extrem => enter

Start up two TERMINALs: [[if needed: source ~/.bashrc (use "Alt n" to get the ~ sign) or source ~/.cshrc]] cd data-oleron/CryoEM/EM-course-Oleron-Imagic [go to your team's directory] module load imagic [to activate the path] [For information: to get a screenshot from your displayed images: Maj cmd 4 → selection; Maj cmd 3 → full screen] Capture screen shots under Mac: Control-Shift-4 → select region of interest with mouse Control-Shift-3 → full screen shot

Use CTRL Z and bg / fg to create batch job or bring it back Display in imagic: use " * " or "quit" to leave, better not CTRL C;

I. Illustration of the Fourier transformation

Files names are:	description:
checker_8	checkerboard array
checker_32	checkerboard array
disc	sharp disc
disc-smooth	smooth disc
square	square

Calculate Fourier transformation of these images: module load imagic

[shortcut for starting IMAGIC program]

IMAGIC-COMMAND: fft

i

** INC2DMENU (vs. 5-Apr-2017) welcomes you ** Mode of operation : FOURIER

Mode of Fourier operation: FT2D FORWARD FT2D REVERSE FT1D FORWARD FT1D REVERSE AMPLITUDES AMP PRETREATED PHASES POWER SPECTRUM REAL PART IMAGINARY PART HERMITIAN HERM TO REAL AUTO CORRELATION SELF CORRELATION Please specify option [] : FT2D FORWARD Input file, image loc#s [] : checker 8 Output file, image loc#s [] : checker 8-fft [give output file name] Use MPI parallelisation [NO] [hit return]

[do the same for the other images: produce files: checker_8-fft, checker_32-fft, disc-fft, disc-smooth-fft, square-fft

quit [or * or Ctrl C] when finished

display these files in IMAGIC:	
disp [under linux or within IMAGIC]	
Input image file, loc#s [checker_8] : checker_8	
Size of the display window [600,600] :	[hit return for default]
Type of cursor:	
CROSS SQUARE CIRCLE	
Please specify option [CROSS] :	[hit return for default]
Parameters to be changed:	
NO_CHANGES(=DISPLAY), SETTINGS, OPTIONS [NO]] : [hit return for default]
To go back on option settings:	
back	
To adjust scale of display:	
<mark>scale</mark>	
1	
To adjust grey values:	
gre [grey]	
sur [survey]	
2d	

Useful options:

grey [to adjust the dynamic range of the image] interactive 0,0 [full range] or for example -10,10 [limited range]

file [read in another file] filename

dev [device, size of display window] 600,1200

erase [removes displayed image, to display freshly another one]

profile [to make profile] Use cursor to position profile: NO Starting point (IMAGE coordinates X,Y): 1,1 End point (IMAGE coordinates X,Y): 65,65 [center of a 128,128 image, i.e. center of powerspectrum]

To start a second display: Ctrl Z bg [background, batch job] To quit the display: * [orl_quit______very importa-

^k [or] quit very important!!! (otherwise display problems)

reactivate a background job: fg [foreground], then stop it with * or quit

<u>disp</u>

Input image file, loc#s [checker_8] : checker_32 [next file name] etc.

Switch between display windows to compare the images (do not move displays around such that they remain aligned with respect to each other)

Then display the corresponding FT's, files: checker_8-fft checker_32-fft Switch between these display windows to compare the images

With the same procee	lure, compare sharp and smooth discs and the square:
disc	disc-fft
disc-smooth	disc-smooth-fft
square	square-fft

When displaying the files **disc-fft** and **disc-smooth-fft** you can draw a profile of the spectrum: In the display command window:

Parameters to be changed:

NO_CHANGES(=DISPLAY), SETTINGS, OPTIONS [NO] :	profile
Use cursor to position profile [NO]:	[NO; hit return for
default]	
Starting point (IMAGE coordinates X,Y) [1,1] :	128,128 [centre would be
65,65]	-
End point (IMAGE coordinates X,Y) [128,128] :	70,70
Parameters to be changed:	
NO CHANGES(=DISPLAY), SETTINGS, OPTIONS [NO] :	[hit return for default]
Output device (X_WINDOWS, PS, FILE) [X_WINDOWS] :	[hit return for default]
Display settings:	
device	
600, 1200	
scale	
4	

b [back, is to get back to the command line inside the display options]

file

filename [allows loading another image; if to remove the previously displayed one use "erase"] grey

- -3,1 profile
- 128,128

70,70

Capture screen shots under Mac: Control-Shift-4 → select region of interest with mouse Control-Shift-3 → full screen shot

When interpreting the results, consider that the absolute scales on the y-axis can be different!

II. Pre-processing:

<u>a) Display a digitized micrograph / negative or CMOS camera image of single particles</u> <u>imaged by cryo-EM</u>

In your team directory:

module load eman1 boxer &

read in the file called **10719c3** or one of the files called **1.mrc**: File→ read Micrograph Process→ Median Filter 5x5 (makes a block convolution) **now better**? adjust grey values/contrast: **middle mouse button** change scale to **0.4** change box size to 96 or 128 [adjust box size to the particle size: should be ~2/3 of the image size; will be smaller than 128 if you use the **1.mrc** image from the CMOS camera; ideally, values of the type of 64, 96, 128, 256, 512, 1024, 2048, 4096 etc. should be used for fast Fourier transform calculations) select ~6-10 particles

Boxes \rightarrow Autobox, adjust parameters for a reasonable selection and let it select automatically;

afterwards, deselect some bad images manually

[*if you want to process the next steps with your own data:* Boxes→ Save Box

read in again the file called 10719c3, change box size to 128:

Boxes→ Resize Boxes: 128

Boxes \rightarrow Save Boxed Particles to file name 10719c3_128 Comment: for CTF correction write out into much larger boxes, e.g. 512

b) Calculate a power-spectrum:

ctfit

→ Open particle set

File name: 9_ptcl.hed

Adjust grey values to see the power-spectrum better (middle mouse button), adjust parameters to make the predicted spectrum fit with experimental spectrum, adjust defocus value to make the high-resolution peaks fit (not the first peak and first zero which contain information from the particle itself, e.g. secondary structure elements)

```
Compare with power-spectra from other defocus values: file names: 7_ptcl 1_ptcl 10_ptcl
```

[[alternative program, not installed at the moment: findctf2d &

File \rightarrow Open Micrograph file name: 10719c3 Moving the mouse over the image indicates the resolution: edge=Nyquist frequency! (needs to have put 200kV and 3Å for the pixel size into CTF \rightarrow Edit Microscope Settings) Increase image size: Tools \rightarrow Zoom \rightarrow 200%

An outer mask can be put with the left mouse, and an inner mask with the right mouse button Find out the defocus value of the micrograph: $CTF \rightarrow Find CTF$]]

III. Processing of real experimental data

The basic steps of a structure determinati	on of single	particles:
a) pre-process the data: bandpass-filter		
b) centering / alignment		
c) multivariate statistical analysis (MSA) and	d classification	on
d) angle assignment		
e) 3D reconstruction		
1) display particle set:		
disp [under linux or within IMAGIC]		
Input image file, loc#s [] : CMOS	_ctf-append	_500
Size of the display window [600,600] :		[hit return for default]
Type of cursor:		
CROSS SQUARE CIRCLE		
Please specify option [CROSS] :		[hit return for default]
Parameters to be changed:		
NO_CHANGES(=DISPLAY), SETTINGS,	OPTIONS []	NO] : [hit return for default]
To start a second display:		
Ctrl Z		
2) bandpass-filter		
i	[shor	tcut for starting IMAGIC program]
IMAGIC-COMMAND : prep-image	-	
** INC2DMENU (vs. 5-Apr-2017) welcome	es you **	
Mode of operation : PR	EPARE IM	AGES
Input file, image loc#s : CM	1OS_ctf-app	end_500
Output file, image loc#s : Cl	MOS_ctf-app	pend_500-bp
The image will be filtered. Please specify:		
Low frequency cut off (0:low-pass) [0.02]	· 0.025	[roughly narticle size, nivel size: 3Å.
Nyquist 6Å]		[louging paralete size, price size, err,
Remaining low-freq. transmission [0.0]	: 0.1	[leave 10% of low frequencies]
High frequency cut off (0: high-pass) [0.9]	: 0.5	[high frequency cut off]
The image will be marked by a sirale Place	aspecify	
the mask radius (nixels or fraction of inner r	adius)	
If you specify a drop-off it will be a soft mas	sk.	
Mask radius drop off (0: no mask) [0.8.0.0]	· 0 000	lkoon maximum to the adge of
a circular areal	. 0.777	INCOP maximum to the cuge of
Desired new sigma \Box · 3		[normalise the variance to 3 sigma]
Remove (dust) outliers [NO]	[hit return	for default]
Invert the image densities [NO]	: [hit r	eturn for default]
Use MPI parallelisation [NO]	: NO [hit	return for default]

Display the filtered version of the particles for comparison

3) calculate the total sum of the particle images which will serve as a reference for particle centering

IMAGIC-COMMAND : sum-images

** SUMMER (vs. 17-May-2016) welcomes you **

Mode of summing: TOTAL SUM SOME SUM CONDITIONAL SUM MOVIE SUM CHECKERS SUM ODD EVEN SUM SEQUENCE SUM Please specify option [TOTAL SUM] : [hit return for default] Input file, NO loc#s [] : CMOS ctf-append 500-bp Output file, image loc#s [] : CMOS ctf-append 500-bp sum Variance : **NO** Display the file CMOS ctf-append 500-bp sum 4) particle centering: **IMAGIC-COMMAND**: ali-dir ** ALIDIR (vs. 12-Oct-2016) welcomes you ** What is to be aligned: **IMAGES MOVIE FRAMES** Please specify option [IMAGES] [hit return for default] : Overall direct alignment modes available: HORIZONTAL VERTICAL TRANSLATIONAL ROTATIONAL BOTH (ROT&TRANS) BRUTE FORCE Please specify option [] : TRANSLATIONAL Correlation functions available: CCF MCF Please specify option [] : CCF Max shift (pixels/fraction of radius) 0.3 [e.g. 30% of the image size] CMOS ctf-append 500-bp Input file, image loc#s [] : Output file, image loc#s []: CMOS ctf-append 500-bp cent1 Using which reference: INPUT REFERENCE FROM FILE CREATE REFERENCE Please specify option [INPUT REFERENCE FROM FILE] : **INPUT REFERENCE FROM FILE** Input reference file, ONE image loc []: CMOS ctf-append 500-bp sum Mask reference before alignment: CIRCULAR MASK RECTANGULAR MASK NO MASK Please specify option [] : NO MASK Give this reference a number (1,2,...) [0] [hit return for default] : Options to filter the reference(s): NO FILTER LOWPASS Please specify option []: LOWPASS [filtering the reference; try also NO FILTER] High-frequency cut-off [] : [e.g. 10% of the Nyquist frequency] 0.1 Refine reference iteratively []: **NO** Full output? []: YES Maximum allowable (radial) shift is ... pixels. IMAGE #-ITER ANGLE XSHIFT YSHIFT CCC 1 1 0.00 -1.14 3.81 0.1768 2 1 0.00 -2.32 0.08 0.1810 etc.

Centre for Integrative Biology, IGBM	IC, France, 2019
[[optional: 5) repeat steps 3 and 4 with the pre better]] final file containing centered images is CMOS_c	e-centered images in order to center them even tf-append_500-bp_cent1 (or _cent2)
display the files: CMOS_ctf-append_500-bp CMOS_ctf-app bp_cent2) to check the success of the centering	pend_500-bp_cent1 (CMOS_ctf-append_500-g
6) create a mask for the area to be considered du IMAGIC-COMMAND : test-	ring multivariate statistical analysis (MSA)
** TESTIM (vs. 6-Feb-2017) welcomes you **	
Output filename, image loc#s []:msarImage dimensions X,Y [96, 96]:IMAGIC data formats you can choose:84,84PACK INTG REAL COMP RECO	nask 4 [hit return for default]
Please specify option [REAL] : Currently, you can choose:	[hit return for default]
Please specify option [] : DISC Disc radius (pixel or fraction of inner radius) []:	0.75 [to be adjusted to particle size]
Display the file msamask	
7) multivariate sta tistical analysis (MSA): IMAGIC-COMMAND :	msa-run
** MSA (vs. 11-Oct-2016) welcomes you ** Choose mode of operation: FRESH_MSA REFINE Please specify option [FRESH_MSA] :	[hit return for default]
MSA distances: EUCLIDIAN CHISQUARE MODULATION Please specify option [MODULATION]	: [hit return for default]
Input (= output) file (aligned "images") [] : Input MSA mask file [msamask] : Eigenimages output file [] : Use default answers for other MSA options [] : Number of iterations (<65) [] : Number of eigenimages (< 70) [] :	CMOS_ctf-append_500-bp_cent1 [hit return for default] eigenim NO 25 40
Normalized for results file, NO ext. [msa] :Use MPI parallelisation [NO]: NO	0.8 [hit return for default] [hit return for default] [hit return for default] [hit return for defaults]

Display the file eigenim

8) hierarchical ascendant classification:

IMAGIC-COMMAND :	m	sa-class(ify)
** CLASSIFY (vs. 13-Dec-2016) welcom	nes you **	
Input to be classified:	-	
IMAGES PIXEL-VECTORS SEC	QUENCES	
Please specify option [IMAGES] :		[hit return for default]
Classification options:		
HAC HYBRID		
Please specify option [] :	H	AC
Input (=output) file (treated by MSA)[]:	С	MOS ctf-append 500-bp cent1
Fraction of images to be ignored [0] :		[hit return for default]
Active eigenimages for classification [] :	3(
Use default classification options [YES]	:	[hit return for default]
What number of classes do you wish [] :	100 [t	otal particle number divided by number of
members per class (usually 10-20, or 3-5	with high	-contrast images)]
Name of output results files []:	cl	asses0_100
9) form class averages:		
IMAGIC-COMMAND : msa-sum		
** CLASSUM (vs. 23-Jan-2017) welcom	es you **	
Input images to be summed []:	C	MOS_ctf-append_500-bp_cent1
Rootname of input classification files []:	cl	asses0_100
Output class averages []:	cl	assums0_100
Downweight small classes [NO] :		[hit return for default]
Fraction of worst class members to ignore	[0]:	[hit return for default]
Mode of summing statistics:		
NONE VARIANCE S-IMAGE I-IMAG	ìΕΓΓ	
Please specify option [NONE] :		[hit return for default]
Display the file classums0_100 (and keep	it display	red, use Ctrl Z)
10) band-pass filter the class averages:		
IMAGIC-COMMAND : band-pass		
** INC2DMENU (vs. 5-Apr-2017) welcom	nes vou *'	<i>د</i>
Input file, loc#s []:	classums	0 100
Output file, image loc#s [] :	classums	0 ⁻ 100-bp
The image will be band-pass filtered.		
Please specify		
Low frequency cut off [] :	0.05	[remember about Nyquist
frequency]		
Remaining low freq. transmission [0.005]:	0.005	[hit return for default]
High frequency cut off [] :	0.7	t J
ASQ filter the images too [NO]:		[hit return for default]
Use MPI parallelisation [NO]	:	[hit return for default]

Display the file classums0_100-bp (and keep it displayed, use Ctrl Z, bg to put the job into background)

Before starting the next step: rm my_ordered* (this avoid some bugs)

11) Assigning angles without a reference, based on common lines / angular reconstitution

IMAGIC	C-COMN	AND:	_` .		ang-rec	(angular-reconstitution)
** EULI	ER (vs. 2	29-Apr-201	7) welcor	nes you **		
Pointgro	up symr	netry:	2			
	1	C2	2			
03	5	C4	4			
C5	2	C6	6			
C7	7	C8	8			
C9	9	CIO	10			
CII	11	C12	12			
CI3	13	CI4	14			
CI5	15	Cl6	16			
CI7	17	C18	18			
C19	19	C20	20			
C21	21	C22	22			
CN	Ν	D2	222			
D3	32	D4	422			
D5	52	D6	622			
D7	72	D8	822			
D9	92	TETRA	GONAL	23		
O (CUI	BIC) 4	32 IC	OSAHEI	DRAL 532		
NONE		.· гі	C1			
Please sp	becity of	otion []:	CI Det	[CI Point-§	group sym	metry for an asymmetric object
Minimai	stay-aw	ay from eq	uator (Be	ta=90) [0.0]:	[nit retur]	n for defaultj
Option fo NEW C1_STA SINOG	or angul ARTUP RAM	ar reconstit ANCHO SE SIN	ution: DR_SET CLF_SEA VE_CORF	RCH RELATION		
PREDI	CT SIN	ECORR P	EĀKS			
Please sp	becify of	otion [] :			C1 STA	RTUP
Input (cl	assum) i	mages, NC	loc#s [] :	:	classums	0 100-bp
Loc# of	THREE	(classum)	images to	be used [] :	2; 3; 9 [c	hoose 3 different views; separate
			•		location	numbers by ";"]
Output (s	selected) image file	[]: I	my ordered		[hit return for default;
1		sele	ected clas	s averages w	ill be put	into a new file called my ordered]
				0	•	·
Output s ASQ filt	inogram er the si	s, NO loc# nogram line	s [my_sin es [YES] :	no] : E [hit re	[YES, hi eturn for c	t return for default; sinogram file] lefault; amplitude square-root
filtering]	.		- -		
Linear m Output s	inecorr	ius for sino file, NO loc	grams [] #s [my_s	: 0.7 ine] :	[depends [hit retu	s on particle size] rn for default; sinogram correlation
IIIC] Wontad	monlar	norom and :	n coarde l	5 01 .	[hit water-	un fou dofoult
wanteu a Minimur	n inter d	nucientent l	n starti [[3.0] . [30] ·	[hit rotu	rn for defaulti
Full oute	ii iiitti-t	noulta IN	way angle	, [30.] . VEC	[mit retu	
r un outp	ut of the	L'ICSUILS [IN	UJ.	ILS		

Are the relative angles clearly bigger than $\sim 40^{\circ}$? If not, select another set of 3 views and start again

Also look at sinograms: display file my_sino and sinogram correlation: my_sine

12) 3D reconstruction **IMAGIC-COMMAND**: true ** TRUE_3D (vs. 11-May-2017) welcomes you ** Mode of 4D operation: ALL IN ONE 3D **3D MEMBERSHIP IN HEADER** SEQUENTIAL ASSIGNMENT RANDOM 3D MEMBERSHIP MULTIPLE RANDOM ASSIGN SPLIT 3D MEMBERSHIP FOURIER SHELL CORRELATION Please specify option [ALL IN ONE] [hit return for default] Pointgroup symmetry to be used: C1 1 C2 2 Please specify option [] : **C1** Use default 3D reconstruction options [YES] [hit return for default] : Input 2D (classum) images, loc#s [] : my ordered Source of Euler angles: ANGREC HEADER VALUES PLT FILE MRA HEADER VALUES Please specify option [ANGREC HEADER VALUES] : [hit return for default] Update input headers (3D reference) [YES] : [hit return for default] Output 3D rec. filename, loc#s []: 3d 0-1 [file which will contain the 3D reconstruction, sections by sections after weighted back-projection] Output file for reprojections, NO loc#s [] : 3d 0-1-reproj [reprojections according to the same Euler angles as the input images] Output file for error projections, NO loc# [] : 3d 0-1-err [difference between reprojection and input image, i.e. reflects amount of error] Spherically mask the reconstruction [] : YES Radius of the mask [] : 0.75 Hamming window factor [] : 0.6 Object size as fraction of image size []: 0.7 Also create a normalized 3D volume [NO] : [hit return for default] MPI parallelisation: ONLY 3D BOTH NO MPI Please specify option [NO MPI] : [hit return for default]

Now display the files my_ordered and 3d_0-1-reproj for comparison, do they look similar, i.e. is the angle assignment correct?

13) Add more views to the angular reconstitution to improve the structure: IMAGIC-COMMAND : ang-rec

** EULER (vs. 29-Apr-2017) welcomes you **

2

Pointgroup symmetry: C1 1 C2

...

Please specify option []: **C1** Option for angular reconstitution: **NEW IMAGE** ANCHOR SET C1 STARTUP SELF SEARCH SINOGRAM SINE CORRELATION PREDICT SINECORR PEAKS Please specify option [C1 STARTUP]: **NEW IMAGE** Option of NEW: FRESH ADD REMOVE PROJ Please specify option [ADD] ADD Input (classum) images, NO loc#s []: classums0 100-bp Location number(s) wanted []: 4:5 [select one image or a series of images] Output (ordered) image file [my ordered]: [hit return for default] Output sinograms, NO loc#s [my sino]: [hit return for default] ASQ filter the sinogram lines [YES] : [hit return for default] Linear mask radius for sinograms []: 0.7 [as before] Output sinecorr file, NO loc#s [my sine]: [hit return for default] Wanted angular increment in search [5.0]: [hit return for default] Full output of the results [YES] : NO

14) 3D reconstruction with more views

IMAGIC-COMMAND: true

** TRUE 3D (vs. 11-May-2017) welcomes you ** Mode of 4D operation: ALL IN ONE 3D **3D MEMBERSHIP IN HEADER** RANDOM 3D MEMBERSHIP SEQUENTIAL ASSIGNMENT MULTIPLE RANDOM ASSIGN SPLIT 3D MEMBERSHIP FOURIER SHELL CORRELATION Please specify option [ALL IN ONE] [as before] : Pointgroup symmetry to be used: ... Please specify option [C1] : [as before] Use default 3D reconstruction options [YES]: [as before] Input 2D (classum) images, loc#s [my_ordered]: [as before] Source of Euler angles: ANGREC HEADER VALUES PLT FILE MRA HEADER VALUES Please specify option [ANGREC HEADER VALUES] : [as before] Output 3D rec. filename, loc#s [3d 0-1] : 3d 0-2 Output file for reprojections, NO loc#s [3d 0-1-reproj] : 3d 0-2-reproj Output file for error projections, NO loc# [3d 0-1-err]: 3d 0-2-err Mask the reconstruction [YES] : [as before] Radius of the mask [0.75] : [as before] Hamming window factor [0.6] : [as before] Object size as fraction of image size [0.7]: [as before] Also create normalized 3D volume: [No] MPI parallelization: [NO MPI]

Compare again new files my_ordered and 3d_0-2-reproj

15) Make forward projections

(could be used as references for a multiple-reference alignement, here only for comparing forward projections of 3d 0-1 and 3d 0-2)

IMAGIC-COMMAND: threed-for ** FORWARD 3D (vs. 1-Feb-2017) welcomes you ** Input 3D image file [] : 3d 0-1 Output file for forward projections []: 3d 0-1-22 Threshold 3D density value [-99999] : [hit return for default] Use default interpolation (WIDENING) [YES]: [hit return for default] Choose Euler angle option: PLT FILE **ORTHOGONAL** ANOTHER INTERACTIVE **SPIRAL** TETRAHEDRON TOMOGRAPHY **STEREO** UNIFORM ICOSAHEDRON ASYM TRIANGLE RANDOM Please specify option [ASYM TRIANGLE] : [hit return for default] Point-group symmetry to be used: C1 C2 1 2 Please specify option [C1] : [hit return for default] Option to chose Euler angles: EQUIDIST RANDOM Please specify option [EQUIDIST] : [hit return for default] Minimum angular distance between projections [3.0] [hit return for default] Also generate mirror projections [NO] : [hit return for default] Option for Euler angle alpha: ZERO ROTATE Please specify option [ZERO] : [hit return for default] Wanted angular increment in search [] : 45 Full output of all parameters [] : NO Use MPI parallelisation [] : NO

Do the same for 3d 0-2;

Compare files 3d 0-1-22 and 3d 0-2-22, did the quality of the reconstruction improve?

If time allows: refine the structure by using forward projections as references: run m-r-a (multi-reference-alignment), then msa and classification and 3D reconstruction (beginning of the iterative procedure)

IMAGIC-COMMAND : m-r-a ** MRALIGN (vs. 13-Feb-2017) welcomes you ** MRA options: FRESH ADD NEW REFS LOCAL SEARCH Please specify option [FRESH] 4D options: ALL REFERENCES OWN REF3D REFERENCES Please specify option [] : ALL REFERENCES Methods available: ALIGNMENT CORRELATION ONLY Please specify option [] : ALIGNMENT Alignment modes available: HORIZONTAL VERTICAL TRANSLATIONAL ROTATIONAL BOTH (ROT AND TRANS) BRUTE FORCE Please specify option [] : BOTH (ROT AND TRANS) Options available: ROTATION FIRST TRANSLATION FIRST Please specify option [] : TRANSLATION FIRST [gives better centering] Correlation functions available: CCF MCF Please specify option []: CCF Input file, image loc#s [] : CMOS ctf-append 500-bp Output file, loc#s [] : ali-1 [will be the file with particles aligned now also in rotation] Original (pre-treated) file, loc#s [] : CMOS ctf-append 500 **WARNING: Check if input and original images have the same band-pass filter. The output images will have the filter of the ORIGINAL images Reference file, loc#s [] : 3d 0-3-24 Options to filter the reference(s): LOWPASS FILTER NO FILTER BANDPASS FILTER CUT OFF HIGH FREQUENCIES Please specify option []: LOWPASS FILTER High frequency cut-off []: 0.1 Max shift (compared to original images) []: 0.2 Min, max rot. angle (compared to originals) []: -180,180 Precision for rotational alignment: LOW MEDIUM HIGH INTERACTIVE Please specify option [] : MEDIUM Min,max radius for rot alignment [] : 0.0.0.7 Number of alignment iterations [] :5 Full output of all parameters [] : NO Use MPI parallelisation [] : NO Next steps: do msa-run, msa-classify, msa-sum using the ali-1 file as input; run ang-rec to assign angles but now with the option ANCHOR SET (use for this your new classums and the

forward projections 3d 0-2-22)

16) display a 3D reconstruction: compare with previous refinement cycle etc. and iterate