

Practical N°1

The random conical tilt series, using a simulated tilted pair of micrographs of *Lumbricus terrestris* giant hemoglobin

PDF copy Practical01.pdf & annexe01.pdf

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Some informations :

For questions on this practical email to [Nicolas Boisset](#) and to [Ricardo Aramayo](#).

1) **To use the Web software :** you need to start an 8 bytes session: The JavaWed never incorporated the Random conical tilt particle picking and lacks most of the fonctionnalités required for a normal usage of display or interactive clustering, commonly used in the RCT reconstruction scheme.

2) **To get information about SPIDER operations:** open this Web link : http://www.wadsworth.org/spider_doc/spider/docs/operations_doc.html and keep it open during the practical.

3) You will need **ghostview** or an equivalent program to display postscript files on your computers. You will also need **gnuplot** to draw resolution curves.

3) For news about the **SPIRE** reconstruction engine, contact [Bill Baxter](#)

4) If you forgot it check the paragraph [Reminder about Fourier units](#).

Introduction

For this practical session, we will need some files kept in "[TP1.zip](#)" (click with left button of your mouse to download it or send me an [email](#) if you have trouble getting it), and we will need to define a working directory and several subdirectories:

1- Create the following subdirectories : type "**unzip datatp1.zip**".

This should create subdirectory TP1 with six subdirectories names: **micrographs, batch, doc,**

images, r2d, and r3d. If you cannot download the [TP1.zip](#) from this web site, send us an email, and we will send you the file as attached documents (size: 3.0 Mo).

4- For traveling from subdirectory to an other, you can type the following orders:

to see where you are type : "**pwd**"

to go to TP1 type : "**cd TP1**"

to go from TP1 to TP1/micrographs type : "**cd micrographs**"

to go from TP1/micrographs to TP1/images, type : "**cd ../images**"

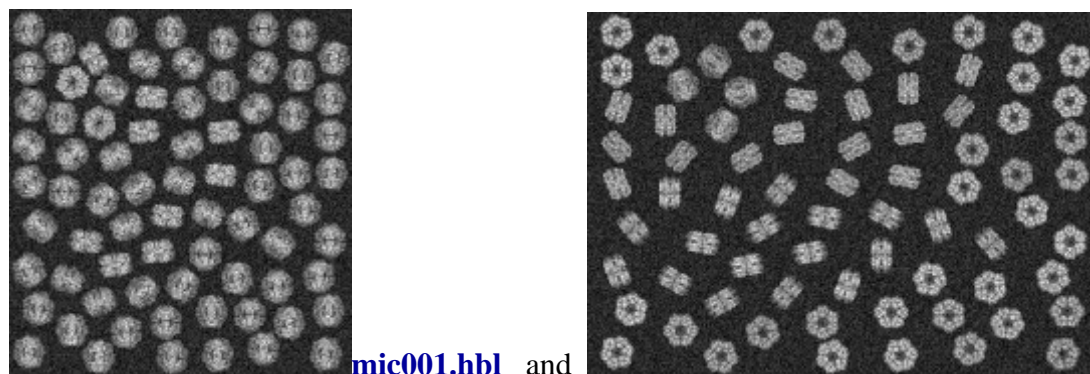
to go from TP1/images to TP1, type : "**cd ..**"

5- In this tutorial, we will use two softwares "**spider**" and "**web**". The spider version used here is the latest released by Joachim Frank "**VERSION: UNIX 14.16 ISSUED: 03/30/2006**". For information about this latest version of spider and web contact the Wadsworth Center for Laboratories and Research (Pr J. Franck), and visit their web site http://www.wadsworth.org/spider_doc/spider/docs/master.html.

1. Interactive particle picking

1.1 Load the two micrographs mic001.hbl et mic002.hbl in subdirectory TP/material:

The two micrographs are in raw 8 bytes format (rawmic001.hbl and rawmic002.hbl) in the subdirectory micrographs. To translate then into the real*4 spider format there is a conversion procedure. Go to the "batch" subdirectory and then type the order "spider fed/hbl b00". This order executes a series of spider operations stored into the text file or batch file "[b00.fed](#)". This batch file reads the 8bytes formats (rawmic001.hbl and rawmic002.hbl), and convert them into the spider format (mic001.hbl and mic002.hbl). the same batch also rescales the densities between values of Fmin=0 and Fmax=1 and computes images pic001.hbl and pic002.hbl, which are the reduced versions by a factor of 2 of the original micrographs.



mic001.hbl and **mic002.hbl**

These are synthetic images corresponding to what you could ideally get by cryoEM while recording the same specimen field tilted by 45° and 0°, respectively. Isolated particles are disposed in different - so called "preferential" - orientations within the vitreous ice layer. We will use this phenomenon while carrying out the "random conical tilt series" reconstruction scheme developed by Michael Radermacher ([Radermacher *et al.*, 1987, Three-dimensional reconstruction from a single-exposure, random conical tilt series applied to the 50 S ribosomal subunit of Escherichia coli. J. Microsc. 146:113-136.](#)

1.2 Interactive particle picking, using the Web Software

You must be in a 8_byte session in your linux machine to run the web software !!! This can be a problem depending the machine you use. you can try to use Web24 in a 24_byte session, but the background color of menus make this program unusable.

Start the web software typing : "**web hbl &**" (by default the program is looking for files ***.DAT**. You will have to change the default suffix by "***.hbl**").

In the menu "**Commands**", select option "**Tilted particles**".

The program asks the name of the (**untilted image**). change in the upper window "**../TP/micrographs/*.DAT**" by "**../TP/micrographs/*.hbl**" and click on option "**filter**". The list of images with the suffix "hbl" will appear in the window. Then, click on "**pic002.hbl**" then on "**OK**".

Then it asks for the name of the (**tilted image**). click on "**pic001.hbl**" then on "**OK**".

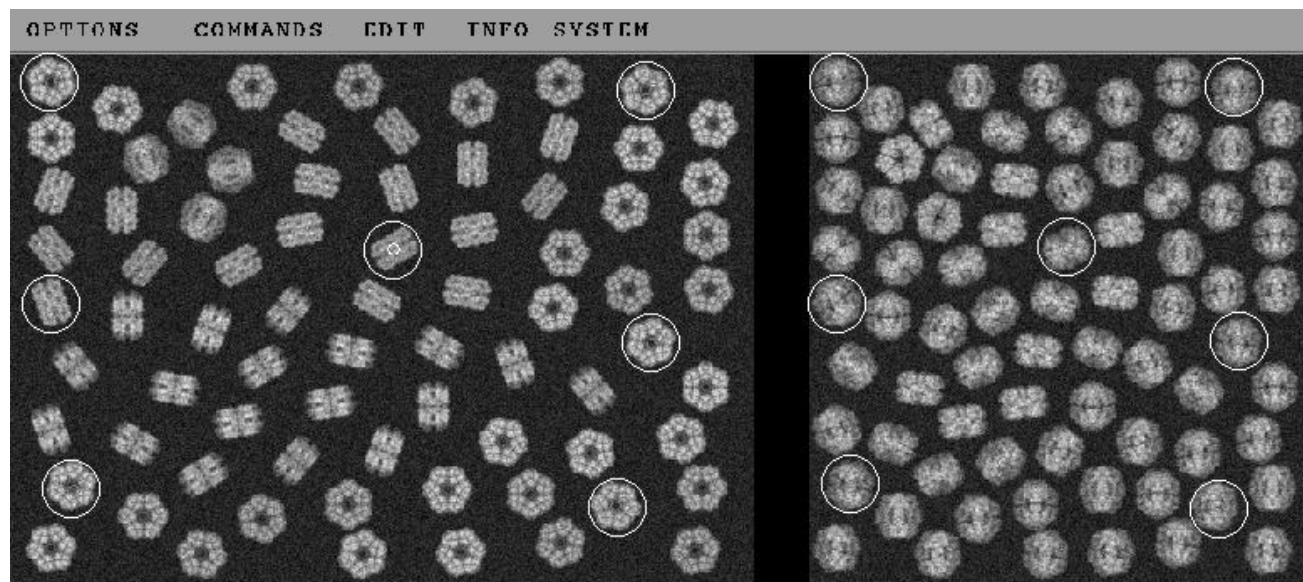
Then the program asks for a number that will be attributed to all results document files (**Doc file number**). type : "**001**"

then the reduction factor applied to the digitized micrographs (Reduction factor), here type "**2**", then click on "**Accept**".

You will now select one particle within the left-hand side of the screen by putting the arrow on the center of the particle and clicking the mouse.

Then you need to select the homologous particle within the right-hand side of the screen. You will repeat this pairwise selection for 5 to 10 particles that you will select all around the field (**see example below**):

(**Remark:** if you make a mistake and select the wrong particle, you can correct this mistake by clicking the right button of the mouse before starting again your selection (follow the orders given on the screen)).



Now click on the central button of the mouse to look at the main menu of the interactive selection :

Particle picking menu_popup

Key number: Offset:

Click on the first option "**Fit angles**"

The program will ask which of your selected particle will be used as the "origin" for calculs (**Key number for origin**), type "**1**" for instance.

A new menu appears :

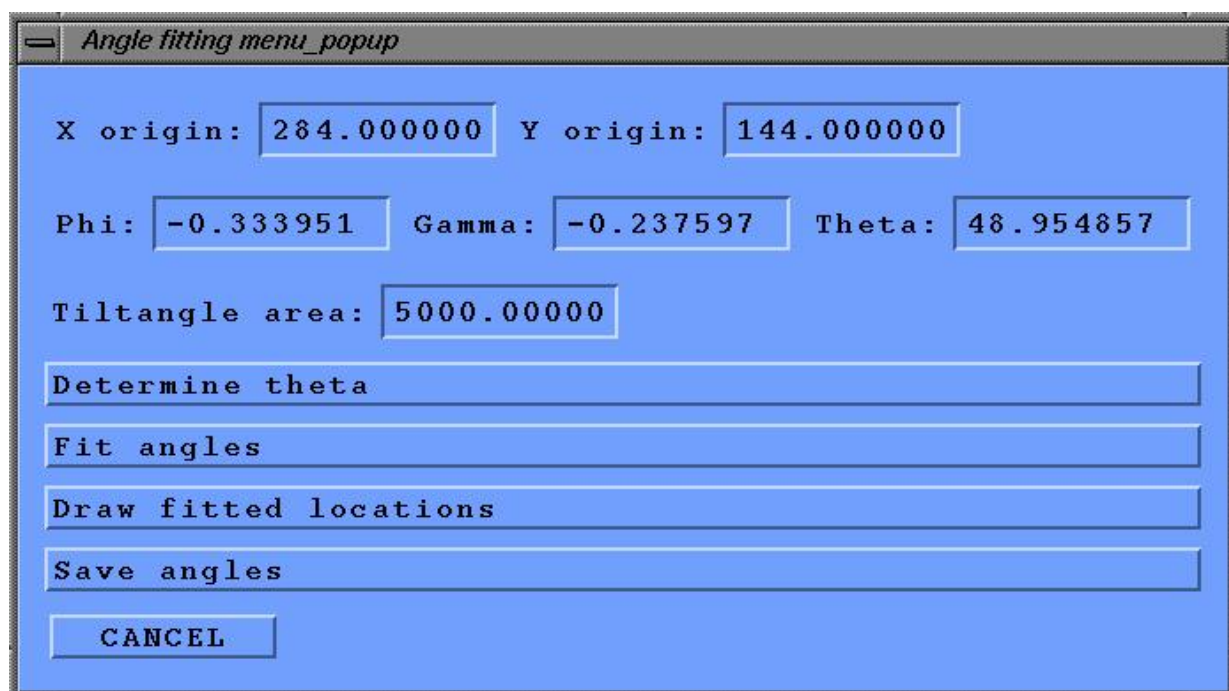
Angle fitting menu_popup

X origin: Y origin:

Phi: Gamma: Theta:

Tiltangle area:

Click successively on options "**Determine theta**", "**Fit angles**", and "**Save angles**".
The new Theta value appears in the menu:
(normally Theta is close to 48° and phi & gamma are close to 0. see below).



Angle fitting menu_popup

X origin: 284.000000 Y origin: 144.000000

Phi: -0.333951 Gamma: -0.237597 Theta: 48.954857

Tiltangle area: 5000.00000

Determine theta

Fit angles

Draw fitted locations

Save angles

CANCEL

Click once on "**CANCEL**" to shut the menu Fit angles.
Then in the main menu click on "**ACCEPT**" to resume the interactive particle picking.

From now on, the program knows the first estimation of respective orientations of both micrographs, and it can help you for the pairwise particle picking. (*This is not the case in this Web version*) Hence, once you will pick the tilted-specimen particle in the left-hand side of the screen, **the cursor will automatically appear** on the right-hand side of the screen **on the corresponding untilted-specimen image**. You might have to correct slightly the position before clicking on the center of this second particle, but that's all . For this tutorial, you will need to select all the particles present in your screen before going any further. Normally, you should get 76 pairs of particles (otherwise you missed some).

You can now restart a more accurate estimation of the (Euler angles) respective orientations of the two micrographs now that you have all the coordinates of the particles to rely on, for fitting of phi, theta and gamma tilt angles. Therefore, you click once on the central button of the mouse to get the main menu, then you click on the option "Fit angles" etc... (see as above). Finally, you will get values close to the ones shown below.

Angle fitting menu_popup

X origin: 284.000000 Y origin: 144.000000

Phi: -0.272676 Gamma: -0.223645 Theta: 48.205406

Tiltangle area: 5000.00000

Determine theta

Fit angles

Draw fitted locations

Save angles

CANCEL

You can now stop the interactive selection of particles. For this you click one the central button of the mouse then you click on option "**STOP**".

To come out of the web software, you click on the menu "**SYSTEM**", and option "**EXIT**". All results of your selection are kept in the following document files :

<u>dcu001.hbl</u>	Doc file containing the coordinates of untilted-specimen images
<u>dct001.hbl</u>	Doc file containing the coordinates of tilted-specimen images
<u>dcb001.hbl</u>	Doc file containing the results of the angular determination

(**Remark:** Due to a bug in Web it is most likely **dcu001.DAT**, **dct001.DAT**, and **dcb001.DAT** that you might obtain with this version of Web)

The doc file **dft001.hbl**, which is also created only contains calculation intermediates and can be removed).

You can look at the content of these files by typing:

more dcu001.hbl (to visualize screen by screen. You need to press the space bar to move to the next screen).

or you can type **cat dcu001.hbl** (for a continuous visualization of the whole file without stopping)

You can also edit these files with an editor, depending on your machine different text editors might be available (try **vi** or **vim** or **nedit**, or **kate dcu001.hbl** (here you can modify the contain of the file).

At this stage you need to move these document files to the subdirectory TP1/doc. For this, type the following commands :

mv dcu001.hbl ../doc/.

mv dct001.hbl ../doc/.

mv dcb001.hbl ../doc/.

Or if you detected the "DAT" bug in Web

```
mv dcu001.DAT ../doc/dcu001.hbl
mv dcu001.DAT ../doc/dcu001.hbl
mv dcu001.DAT ../doc/dcu001.hbl
```

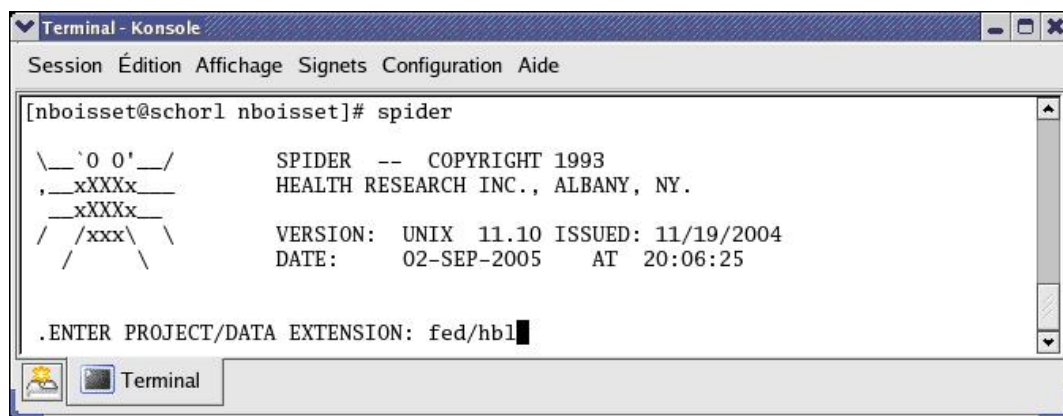
WARNING ! If you would encounter some problem during particle picking with the Web software, you could still do the rest of the tutorial, because I already put particle picking document files ([dcu001.hbl](#) [dct001.hbl](#) [dcb001.hbl](#)) in the "doc" subdirectory.

1.3 Boxing of isolated particles in individual images

One could interactively window all the images, but that would be too time consuming. Therefore, we will first do it interactively just for one image and then we will use a "do loop" to do the same treatment to all images in automatic mode (or batch mode).

Still in the subdirectory TP1/micrographs, you start the spider software, typing: **spider**
The software then asks for the project code and the data code. In both cases it should be a 3 letters code.

- The **project code** will be the suffix of a command file and the results file opened for this session. For example if the **project code is "fed"** the spider will open a results file "**results.fed.0**" which will contain the history of all the treatments done in this session (until you stop the spider using the command EN).
- The **data code** is simply the suffix of the images and document files opened by the spider program. In our case the **data code is "hbl"**, and by default every image and document file treated within this spider session (modified or created) will have the suffix "hbl" (ex: mic001.hbl).



```
Terminal - Konsole
Session Édition Affichage Signets Configuration Aide

[nboisset@schorl nboisset]# spider

  \_ '0 0' _/
  ,_xXXXx_
  ,_xXXXx_
  /_xxx\_ \

SPIDER -- COPYRIGHT 1993
HEALTH RESEARCH INC., ALBANY, NY.

VERSION: UNIX 11.10 ISSUED: 11/19/2004
DATE: 02-SEP-2005 AT 20:06:25

.ENTER PROJECT/DATA EXTENSION: fed/hbl
```

When the spider software is started, it asks the user for an OPERATION to do. The complete list of operations is accessible on the web at the following address, but the first operation to learn is "EN" to stop the spider software. To get a list of all spider operations and specific explanation on what they do and what input they need, activate this link, and keep it open in a specific window. http://www.wadsworth.org/spider_doc/spider/docs/operations_doc.html

Here we will use the operation "**UD**" for reading back the image coordinates from the document files. Then we will use the operation "**WI**" to extract each particle within 100x100

pixel images. (**Remark:** pixel is a contraction of "picture" and "element"). Check the links "UD" and "[WI](#)" in the spider manual to see how these operations work.

The document file dcu001.hbl contains the coordinates:

```
mercure 98% more dcu001.hbl
; hbl/hbl dcu001.hbl Tue Feb 13 16:49:44 2001
0001 6 1.000000 568.000000 288.000000 284.000000 144.000000 1.000000
0002 6 2.000000 62.000000 44.000000 31.000000 22.000000 1.000000
0003 6 3.000000 96.000000 644.000000 48.000000 322.000000 1.000000
0004 6 4.000000 940.000000 52.000000 470.000000 26.000000 1.000000
0005 6 5.000000 950.000000 428.000000 475.000000 214.000000 1.000000
0006 6 6.000000 896.000000 668.000000 448.000000 334.000000 1.000000
0007 6 7.000000 424.000000 600.000000 212.000000 300.000000 1.000000
```

line Number	image Number	X coord in mic002	Y coord in mic002	X coord in pic442	Y coord in pic442
-------------	-----------------	----------------------	----------------------	----------------------	----------------------

To read back the document files, you will use registers (marked **Xn**, with **10<n<999**), which allow to temporarily keep in memory some numerical values. In the following example the order "X10=1." temporarily stores the value of 1 in this register. Hence, if one answers X10 the next time spider will ask for an operation, the program will read back the value stored in X10 and put it on screen. In our example, we use this mean with operation UD to read back the values contained in line No.1 of file [dcu001.hbl](#). The values are stored in consecutive order in registers X11 to X16, for example the coordinates of the particles in X and Y on the micrograph [mic002.hbl](#) are respectively X12=568 and X13=288.

```
.OPERATION: x10=1.
x10=1.
.OPERATION: x10
x10
1.000
.OPERATION: ud x10,x11,x12,x13,x14,x15,x16
ud x10,x11,x12,x13,x14,x15,x16
.DOCUMENT FILE: dcu001
dcu001
.OPERATION: x12
x12
568.0
.OPERATION: x13
x13
288.0
.OPERATION: x14
x14
284.0
.OPERATION: x15
x15
144.0
.OPERATION: x16
x16
1.000
.OPERATION: [ ]
```

Now for the windowing, the operation [WI](#) is used and need answers to the following questions (see figure below). In this example, if one wants to extract an image from

[mic002.hbl](#) in a smaller image [unt00001.hbl](#) (100 pixels in X and 100 pixels in Y), for locating the exact position of the image, the programs asks for coordinates of the "upper left corner of the images" (therefore, we must subtract half of 100 pixels to the coordinates stored in the original document files, hence: **568-50=518** and **288-50=238**).

```
.OPERATION: wi
wi
.INPUT FILE: ../micrographs/mic002
../micrographs/mic002
../micrographs/mic002
(R ) 1100 780 CREATED 13-Feb-1901 AT 15:44:11 0 HEADER BYTES: 4400
.OUTPUT FILE: ../images/unt00001
../images/unt00001
. ENTER X & Y DIMENSIONS (NSAM,NROW): 100,100
100 100
../images/unt00001
(R ) 100 100 CREATED 02-SEP-2004 AT 18:44:53 N HEADER BYTES: 1200
.TOP LEFT COORDINATES: 518,238
518 238 0
.OPERATION: [ ]
```

Remark: we could also have used the values stored in the registers X12 and X13 by typing :

```
x12=x12-50.
x13=x13-50.
wi
../micrographs/mic002
../images/unt00001
100,100
x12,x13
```

Now, we can do these operations automatically to the rest of the images, using a command or "batch" file. See for instance the batches [b01.fed](#) and [b02.fed](#) which will be used for the windowing of the two micrographs. These batches read the document files ([dcu001.hbl](#) [dct001.hbl](#)) of the interactive particle selection and create images of 100 x 100 pixels corresponding to all the particles in subdirectory **TP1/images**. The tilted-specimen images are termed "**tilt[00001-00076].hbl**" and the untilted-specimen images are called "**unt[00001-00076].hbl**".

Warning ! if you edit these batches you will see that most of the time (except for b00.fed), I took care to write some lines of explanation or descriptive note (always preceded by a semicolon ";").

```
;-----!
; b01.fed/hbl: batch for boxing images from the 45 degree tilted specimen:
;-----!
```

Then, I declared my numerical **PARAMETERS**, **INPUT** file names, and **OUTPUT** file names, following a certain format, and using operations **FR L** or **FR G** to declare file names. I recommend that you follow the same format because (1) it is a good habit to get right from the start, and (2) because this format for editing your batches will allow you to use a new metaprogram called **SPIRE** and currently under the developpement of [Bill Baxter](#). This

metaprogram will help you to create a library of batches easy to modify from one project to the next, and it will allow you to create your own html based note book for each image processing experiment. For more explanation on this very interesting development, please contact directly [Bill Baxter](#) in Joachim Frank's laboratory at the Wadsworth Center.

```
; PARAMETERS
  x76=76          ; last image number
  x81=100.         ; image dimension
  x82=x81/2.       ; 1/2 image dimension
; INPUTS:
  fr 1
  [init_tilted_coords]../doc/dct001      ; tilted image coordinates
(from WEB)
  fr 1
  [tilted_micrograph]../micrographs/mic001 ; tilted micrograph
; OUTPUTS:
  fr 1
  [tilted_images]../images/tilt{*****x10} ; windowed, tilted images
  fr 1
  [new_tilted_coords]../doc/dwintilt      ; new tilted image
coordinates
; END BATCH HEADER
;-----!
```

Then, to start these batches, you just need to type :

```
> spider new/hbl b01
> spider new/hbl b02
```

To visualize them, you can go in this subdirectory (**cd ../images**), and start the web program, typing : **web hbl &**. Then you go into the **COMMANDS** menu and chose the option **Montage**. Then click on the name of the first image of the series "**tilt00001.hbl**" and then click on **OK**. A new window appears, asking for the number of images per line (** will automatically adjust this value to the width of the screen*), but you can also specify the number you want **10 Frames / ROW** and click on **ACCEPT**.

Then you can do the same thing on the untilted-specimen images.

Remark: if you want to clear the screen before displaying a new images series, go in the **COMMANDS** menu and choose option **Clear**.

2. Alignment and centration of the 0° and 45° tilted-specimen images

2.1 Computing of the average and variance maps before alignment

- Start the spider software "**spider hbl**" and try the operation "**AS R**"

OPERATION: **AS R**

INPUT FILE TEMPLATE (E.G. PIC****): **../images/unt*******

ENTER FILE NUMBERS OR SELECTION DOC. FILE NAME: **1-76**

ALL, ODD-EVEN (A/O): **A**

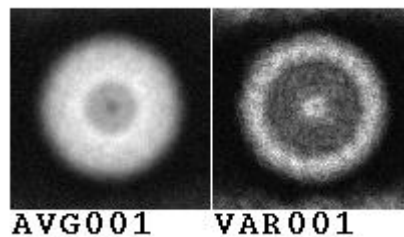
AVERAGE FILE: **avg001**
VARIANCE FILE: **var001**

The list of the 76 images appears with a message of the type :

```
** VARIANCE COMPUTATION BASED ON 76 IMAGES
CONTAINING 10000 ELEMENTS
TOTAL VARIANCE = 2224.0  TOTAL S.D. = 47.159
VARIANCE P.P. = 0.22240  S.D. P.P. = 0.47159
AVERAGE OFFSET = 0.00000E+00
VARIANCE OF AVERAGE IMAGE = 1.1959
```

OPERATION: **EN --> to quit spider.**

- To look at the images start the WEB software and within the menu **COMMANDS** start the option **image**.
- Click on the name of the average map **"avg001.hbl"** then click on **OK**.
- Click on the name of the variance map **"var001.hbl"** then click on **OK**.



2.2 Two-dimensional alignment of the untilted-specimen images

Here, as we have different orientations of the particle in your images, we want to align our images while restricting the influence of a reference image to a minimum. Therefore, we will use a "reference free" approach developed by Pawel Penczek ([Penczek, P., M. Radermacher, et J. Frank. 1992. Three-dimensional reconstruction of single particles embedded in ice. *Ultramicroscopy*, 40:33-53](#)). Operation "[AP SR](#)" takes in charge shift and rotation alignments in an iterative mode. When no improvement is measured from one cycle to the next the operation stops. For each cycle an output overall image is created, and an output document file is created, which contains rotation angle and X & Y shift to apply on each image. As this operation is "reference free", it has a random aspect in the way images are aligned, and it can stop after different numbers of cycles from one run to the next. Therefore I show you below how to align images in three steps.

Run the batch [b03.fed](#) by typing the following order (**remark**: you must be in subdirectory random/batch).

> spider new/hbl b03

First step, batch [b03.fed](#) applies a circular mask on the untilted-specimen images and performs the "[AP SR](#)" operation. The Unix orders "ls -l ../r2d/avs*" and "ls -l ../doc/davs*" are included at the end of the batch to show how many cycles were necessary to get an optimum alignment.

You can then go to the second step of the alignment and edit the batch [b04.fed](#) and if necessary modify the value of parameter indicating the number of cycles produced in the previous steps:

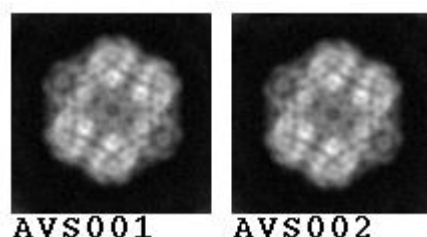
```

;-----!
; b04.fed/hbf : - check last cycle of reference free alignment with respect
;               - to horizontal and/or vertical orientation
;-----!

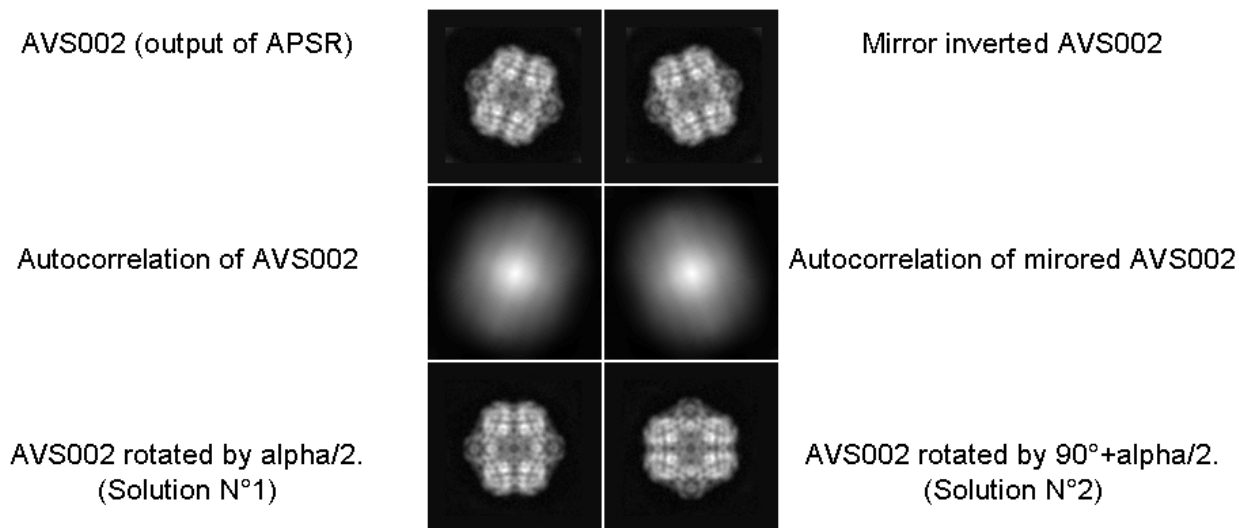
; PARAMETERS:
X61=2.      ; ending cycle number in the reference free alignment of b03.fed

```

Here $x61=2$. means that AP SR operation produced 2 cycles of alignment. If you got 4 or 5 cycles, modify parameter $x61$ accordingly, save your new version of the batch [b04.fed](#) with your text editor and run it with spider. As the AP SR operation is reference-free, the output image obtained after alignment might not be in the best orientation to conveniently apply symmetries, or just in the best orientation for pleasing our sense of esthetic (see example below the two global average maps obtained at the end of the two alignment cycles performed by AP SR).



In this context, the batch [b04.fed](#) applies a mirror inversion of the output image and using auto-correlation and angular cross-correlation functions, it finds the angle α necessary to align the output image to its mirror inverted counterpart. Then, depending on the overall shape and orientation of our output image, two solutions can be applied to get a "vertical" or "horizontal" final orientation : either applying $(\alpha/2)^\circ$ or applying $(\alpha/2)+90^\circ$. To check this, both options are computed and put into a montage spider image called [../r2d/MALIGN.hbl](#) (see image below) . Therefore, at the end of spider batch, you can use the Web software to display this image (submenu: **COMMAND/IMAGE** in the **web software**).



MALIGN001.hbl

The first row of this montage shows the output file and its mirror inverted copy. The second row of the montage shows their respective autocorrelation functions. Finally the last two images correspond to the two solutions of our alignment $(\alpha/2)^\circ$ and $(\alpha/2)+90^\circ$.

At this stage we can decide which of the two solutions we want to apply (here it is solution 2), and move to the next step of the alignment. Hence, we have to edit batch [b05.fed](#) and if necessary modify the values of parameters indicating the number of cycles produced in the previous steps:

```

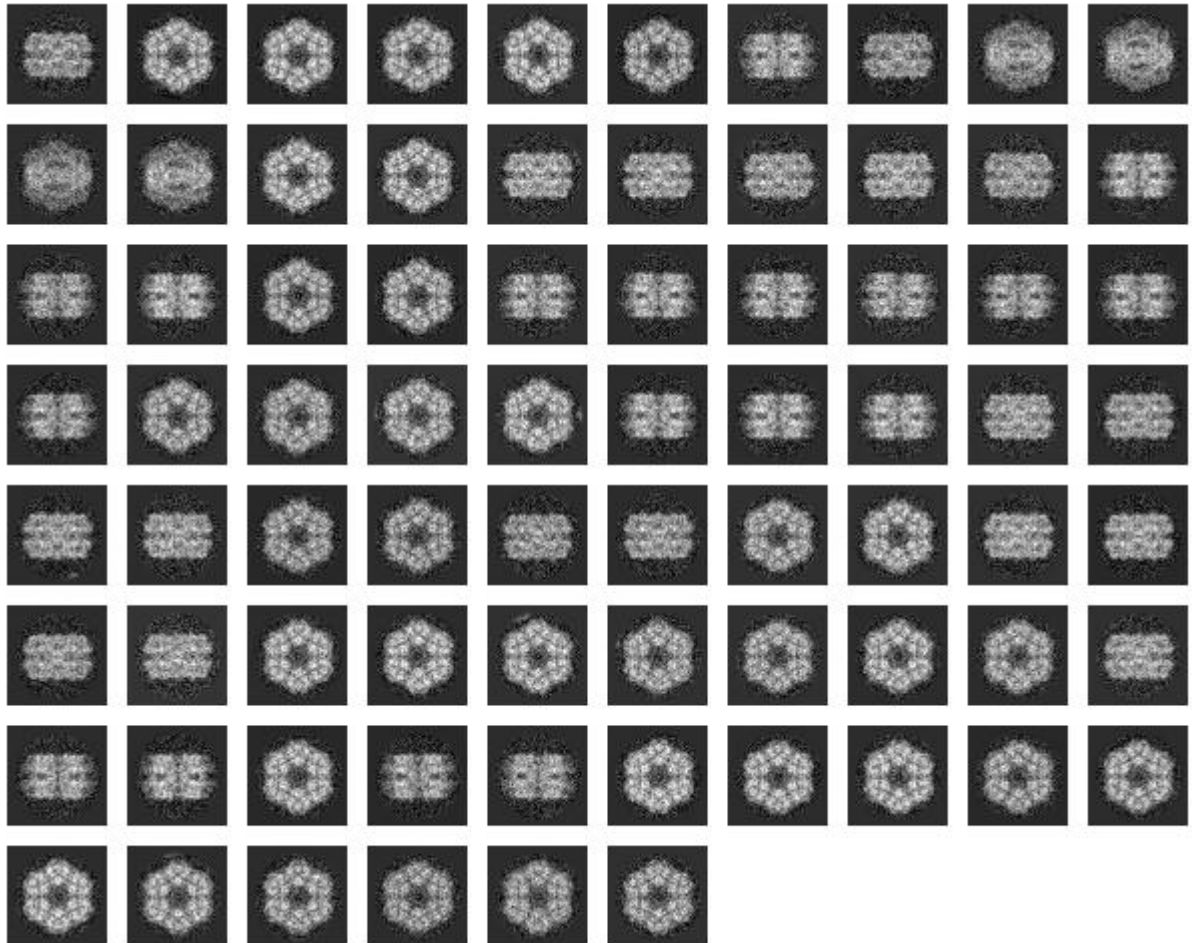
;-----!
; b05.fed/hbf : - after reference free alignment and visual checking
;               - apply solution No.1 or 2. to all original files
;-----!

; PARAMETERS:
X61=2.      ; ending reference free alignment cycle number
X10=2.      ; solution choosed No.1 or No.2 for final orientation of
particles

```

Here $x61=2$. means that AP SR operation produced 2 cycles of alignment. Once more, if you got 3, 4 or 5 cycles, modify parameter $x61$ accordingly. More important here, is the value you want to give to $x10$. Depending on which angle you choose $X10 = 1$ (for $\alpha/2^\circ$) or $X10 = 2$

(for $(\alpha/2)+90^\circ$). Then save your new version of the batch [b05.fed](#) with your text editor and run it with spider. The 76 images resulting from this final alignment step are kept under the name : [../images/cenu\[00001-00076\].hbl](#) and the rotation angles and X/Y translations applied to them are kept within a document file [../doc/dalu001.hbl](#) . Using web with menu COMMAND, option montage, should allow you to have something like this.

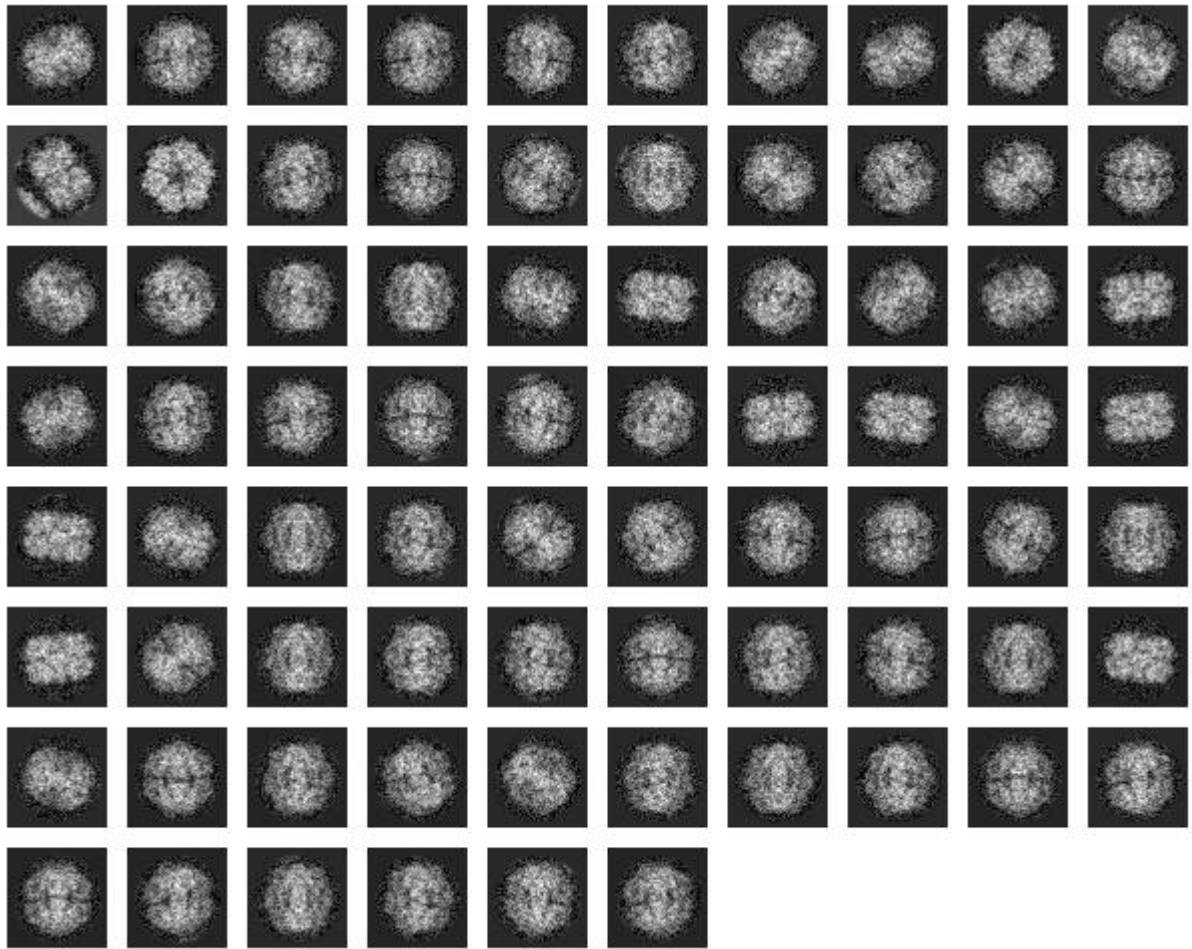


Remark: If you have 90° rotated images compared to these ones, then it means that you choosed the wrong final rotation angles. In this case you should remove file [../doc/dalu001.hbl](#) from your subdirectory TP1/doc, modify the value of X10 in batch [b05.fed](#) and run it again with spider.

2.3 Centration of the 45° tilted-specimen images

Here, we want to center all the images so that their centres of gravity correspond to the exact center of the images, but we want to keep the images in their original orientation. Therefore, we only use the operation [AP SA](#) which performs only half of the reference free alignment (only the shift alignment, check also [AP RA](#) if you have time as there is an example of alignment batch using AP RA and APSA). Hence, look at the batch [b06.fed](#), and run it in interactive mode with the following order : `spider new/hbl b06`

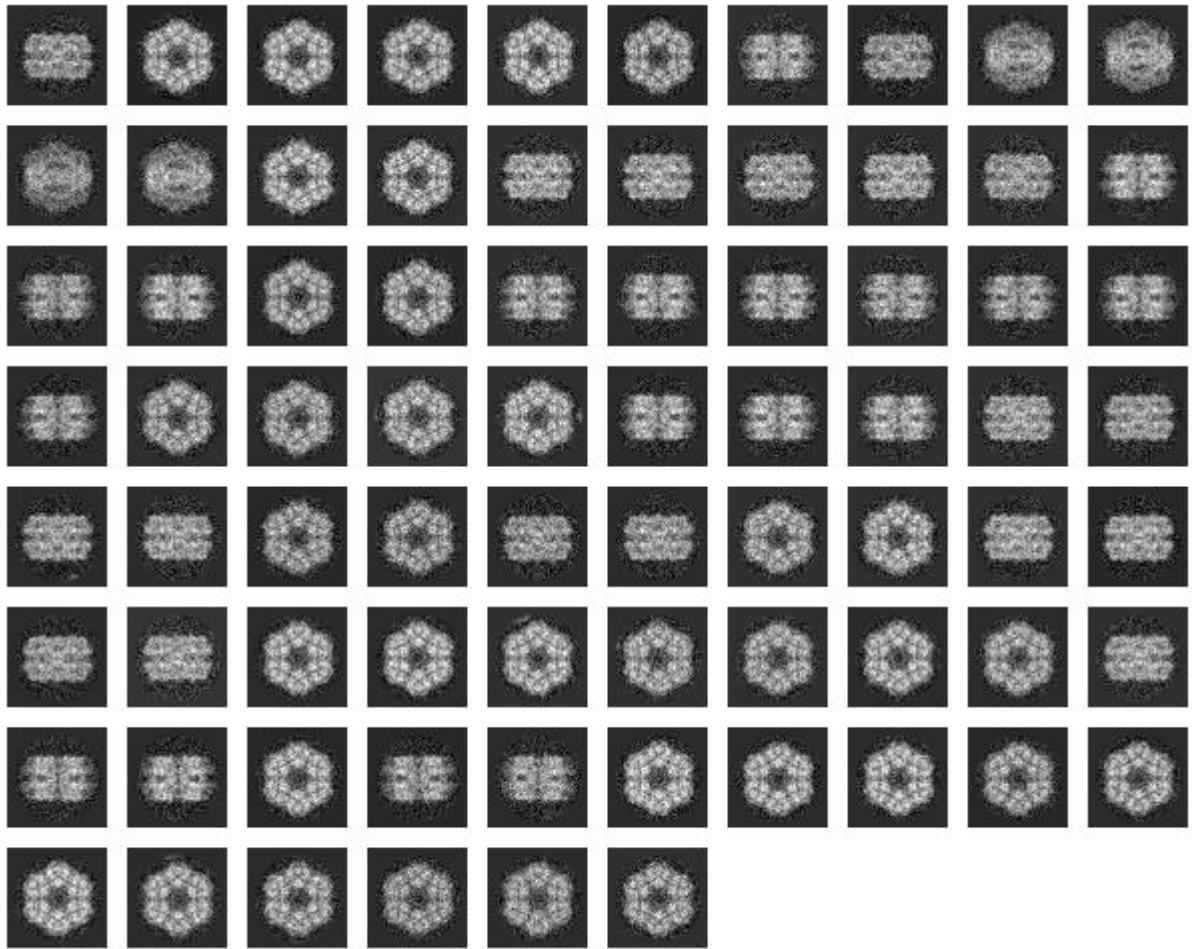
The images obtained after this centering step are kept under the name : [../images/cent\[00001-00076\].hbl](#).



Remark: these images correspond to several random conical-tilt series whose Euler angles ϕ , θ , and ψ were determined during the interactive particle picking (ψ = azimuthal orientation of the tilt axis in our micrographs, θ = tilt angle), and during the rotational alignment on the untilted-specimen images (ϕ). The batch [b07.fed](#) reads these angles and stores them in a document file [../doc/dang005.hbl](#). **Run this batch with the order: spider new/hbl b07.**

3. Classification of the untilted-specimen images using Multivariate statistical analysis

We must now sort our untilted-specimen images in order to produce homogeneous image classes. In our case, this could be done visually, but in practice, one must rely on an automatic statistical procedure, as several thousands of images with a low signal-to-noise ratio must be sorted.



3.1 Correspondence analysis

For the analysis, we want to use only the portion of the images corresponding to the particles. In our present course we will just apply a circular mask, using operation [MO](#), but as you might work on all kinds of shapes, I show you below a way to create custom made masks that will follow the outer boudaries of your particle. If you have time, you can try it, otherwise just go further down to " [Running correspondence analysis](#)".

Custom made mask:

The first operation to compute a mask that will roughly follows the outer shape of the global of your particle is to compute the total average map of your aligned particles. This was done at the end of batch [b06.fed](#), which created the map [../r2d/avgu001.hbl](#). From this map we will create a binary image (containing 1. and 0. as densities), we use operation [TH M](#), and then operation [FQ NP](#) to smooth out and filter this binary image. Finally [TH M](#) is used once more to create a new binary image from its smoothed out copy. (For example, look below and try to reproduce this mask interactively to create your own mask within subdirectory **TP1/r2d**: Watch out when using TH M operation as the Threshold density will change greatly the size and shape of the output binary file.

Creation of a mask :

```
cd ../r2d
```

```
spider hbl
```

```
.OPERATION: TH M
```

```
.IMAGE TO BE THRESHOLDED FILE: ../r2d/avgu001
```

```
.OUTPUT FILE: scr001
```

```
.BLANK OUT (A)BOVE OR (B)ELOW THRESHOLD? (A/B): B
```

```
.THRESHOLD: 0.8 -----> Watch out for this value !
```

```
.OPERATION: FQ NP
```

```
.INPUT FILE: scr001
```

```
.OUTPUT FILE: scr002
```

```
1 - LOW-PASS, 2 - HIGH-PASS
```

```
3 - GAUSS LOW-PASS, 4 - GAUSS HIGH-PASS
```

```
5 - FERMI LOW-PASS, 6 - FERMI HIGH-PASS
```

```
7 - BUTER LOW-PASS, 8 - BUTER HIGH-PASS
```

```
.FILTER TYPE (1-8): 3
```

```
.FILTER RADIUS: 0.08
```

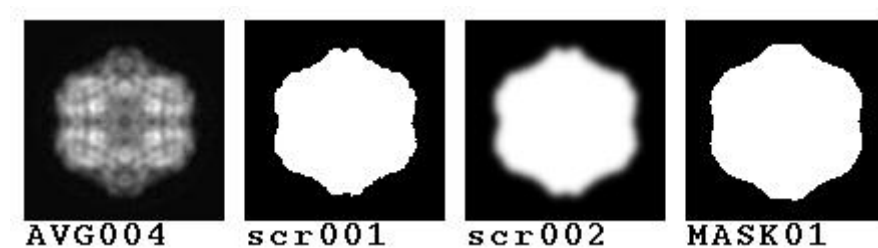
```
.OPERATION: TH M
```

```
.IMAGE TO BE THRESHOLDED FILE: scr002
```

```
.OUTPUT FILE: ../r2d/mask01
```

```
.BLANK OUT (A)BOVE OR (B)ELOW THRESHOLD? (A/B): B
```

```
.THRESHOLD: 0.1 -----> Watch out for this value !
```



Depending on which Threshold values and how strongly you smooth out the first binary file, you can expand or shrink the mask. Two operations were also developed to do the same effects, and you can also try them. They are called [DI](#) to dilate and [ER](#) to erode any binary map. However, they tend to form "squarish" shapes, if used intensively.

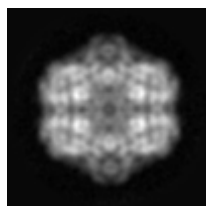
Reminder about the correspondence between Fourier units and real space distances:

The spider software uses Fourier units which are independant of the size of the image. As some operations will ask you some radius for filtering in Fourier space, this little paragraph reminds you how to deal with Fourier units :

- in reciprocal distances,
- in absolute Fourier units ($0 < R_f < 0.5$) with Nyquist Frequency = 0.5,
- or in pixel numbers.

Lets take the image ../r2d/avgu001.hbl used to create the MSA mask. Knowing that this images has a size of NSAM = 100 pixels and a pixel size of 3.5 Å. You can execute a low-

pass filtering of this image at $1/35 \text{ \AA}^{-1}$ resolution, and a high-pass filtering at $1/25 \text{ \AA}^{-1}$ resolution.



NSAM = 100 (image of 100 x 100 pixels)

Ra = Radius in reciprocal Angstroems ($1/\text{\AA}$)

PS = 3.5 A (pixel size = 3.5 \AA)

Rf = Radius in Absolute Fourier units ($0 < Rf < 0.5$)

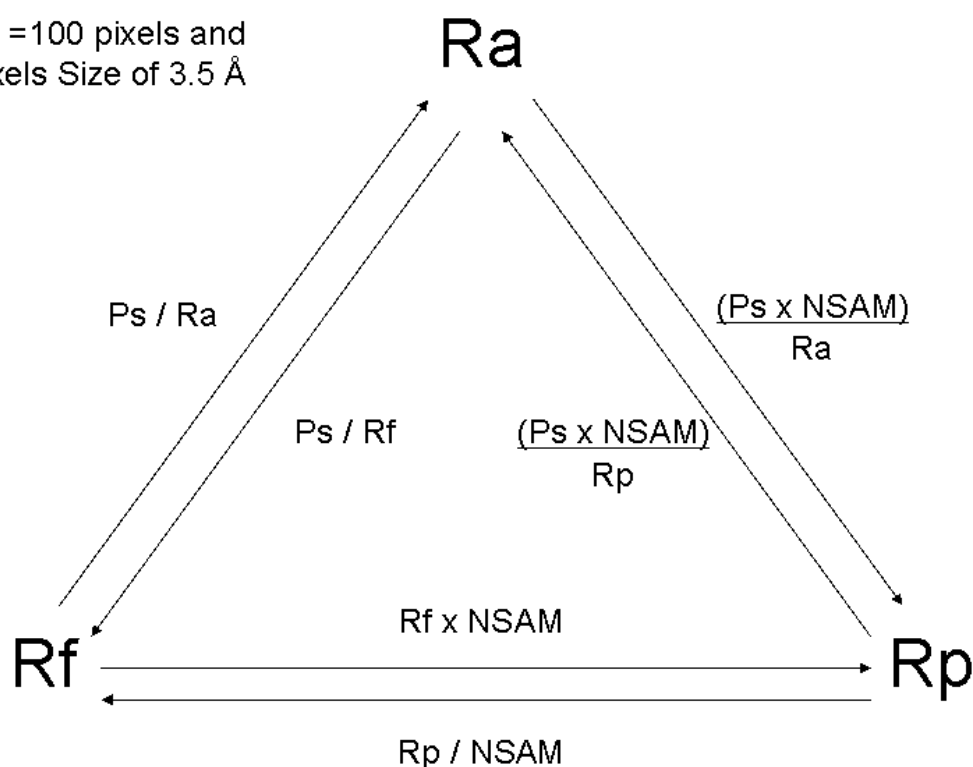
Rx = Radius in Fourier space with

Rp = Radius in pixels

./r2d/avgu001.hbl

Always remind these conversion rules in spider :

If NSAM = 100 pixels and
PS = Pixels Size of 3.5 \AA



In these experimental conditions :

$Ra = 35 \text{ \AA} \rightarrow Rp = (3.5 \times 100) / 35 = 10 \text{ pixels} \rightarrow Rf = 3.5 / 35 = 0.10 \text{ Fourier units}$

$Ra = 25 \text{ \AA} \rightarrow Rp = (3.5 \times 100) / 25 = 14 \text{ pixels} \rightarrow Rf = 3.5 / 25 = 0.14 \text{ Fourier units}$

.OPERATION: **FQ NP**

.INPUT FILE: **./r2d/avgu001**

.OUTPUT FILE: **scr001**

1 - LOW-PASS, 2 - HIGH-PASS

3 - GAUSS LOW-PASS, 4 - GAUSS HIGH-PASS

5 - FERMI LOW-PASS, 6 - FERMI HIGH-PASS

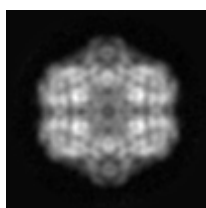
7 - BUTER LOW-PASS, 8 - BUTER HIGH-PASS

.FILTER TYPE (1-8): **5**

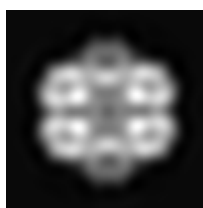
.FILTER RADIUS: **0.1** <-----Radius in Rf

.TEMPERATURE (0=CUTOFF): **0.02**

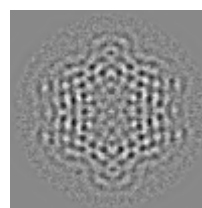
```
.OPERATION:      FQ NP
.INPUT FILE:    ../r2d/avgu001
.OUTPUT FILE:   scr003
1 - LOW-PASS,      2 - HIGH-PASS
3 - GAUSS LOW-PASS, 4 - GAUSS HIGH-PASS
5 - FERMI LOW-PASS, 6 - FERMI HIGH-PASS
7 - BUTER LOW-PASS, 8 - BUTER HIGH-PASS
.FILTER TYPE (1-8): 6
.FILTER RADIUS: 0.14 <-----Radius in Rf
.TEMPERATURE (0=CUTOFF): 0.02
```



Original image
../r2d/avgu001.hbl



Low_pass filtered at 1/35 Å⁻¹
../r2d/scr001.hbl



High-pass filtered at 1/25 Å⁻¹
../r2d/scr003.hbl

Running correspondence analysis

The multivariate statistical analysis is now done by a single operation [CA S](#). It will ask you for a prefix name (for instance [coran1](#), and it creates four output files corresponding to: the sequential file (SEQ), the image coordinates file (IMC), the pixel coordinate file (PIX), and the eigen values file (EIG). The output file names will then be formed by a combinaison of the prefix and of the suffix, as shown below :

[coran1_SEQ.hbl](#)
[coran1_IMC.hbl](#)
[coran1_PIX.hbl](#)
[coran1_EIG.hbl](#)

cd ../r2d

spider hbl

.OPERATION: **CA S**

.ENTER IMAGE FILE TEMPLATE: **../images/cenu*******

.ENTER FILE NUMBERS OR SLECTION DOC FILE: **1-76**

.MASK FILE: **../r2d/mask01**

.NUMBER OF POINTS IN MASK: 5785 <----- (remark: here the mask contains 5785 pixels)

.NUMBER OF FACTORS : **7**

.CORAN, PCA, OR SKIP ANALYSIS (C/P/S) : **C**

.ADDITIVE CONSTANT: **0.2**

.OUTPUT FILE PREFIX~: **../r2d/coran1**

Here, one could imagine that within a multi-dimensional space (with 5785 dimensions) each image can be represented by a single point whose coordinates are the densities contained in

each of its pixels. The correspondence analysis start from this situation and defines a new distance based on **chi square** metric rather than Euclidian distances. This change relates directly the image disposition to the variations among the images, also termed "**trends**". Then, correspondence analysis will look for a new coordinated system relying on orthogonal axes corresponding to biggest trends within our image population. These new axes, also termed "**factorial axes**" or "**eigen vectors**" are independent as they are orthogonal, and they are sorted by decreasing order.

Projection maps:

We can project the 76 images within 2D planes defined by two of the 7 factorial axes. Intuitively, one can see that two closely related images will be projected nearby each other on the projection plan, while two images very different will be projected far apart on the map.

> spider hbl

Results file: **results.hbl.1**

.OPERATION: **CA SM**

.(I)MAGE OR (P)IXEL: **I**

.OUTPUT FILE PREFIX~: **../r2d/coran1**

.NUMBER OF HORIZONTAL PATCHES: **1**

.ENTER 2 FACTOR NUMBERS FOR MAP (e.g: 1,5): **1,2**

.(S)YMBOL, (A)SSIGN SYMBOL, (C)LASS, (D)OC, (I)D: **S**

.ENTER 1 CHAR. SYMBOL FOR ACTIVE IMAGES: **o**

.PREPARE POSTSCRIPT MAP? (Y/N): **Y**

.NUMBER OF SD OR <CR>=2.3: **3**

.1=FLIP #1/ 2=FLIP #2/ 3=FLIP 1+2/ <CR>=NO FLIP: <CR>

.POSTSCRIPT OUTPUT FILE: **../r2d/map12.ps**

.TEXT SIZE FOR AXIS AND DATA : **12,12**

.ENTER X AXIS OFFSET: <CR>

.ENTER NEW LOWER, UPPER AXIS BOUND or <CR>: <CR>

.ENTER NEW AXIS LABEL UNITS /LABEL or <CR>: <CR>

.ENTER LABEL NOetc or <CR> TO CONTINUE: <CR>

.ENTER Y AXIS OFFSET: <CR>

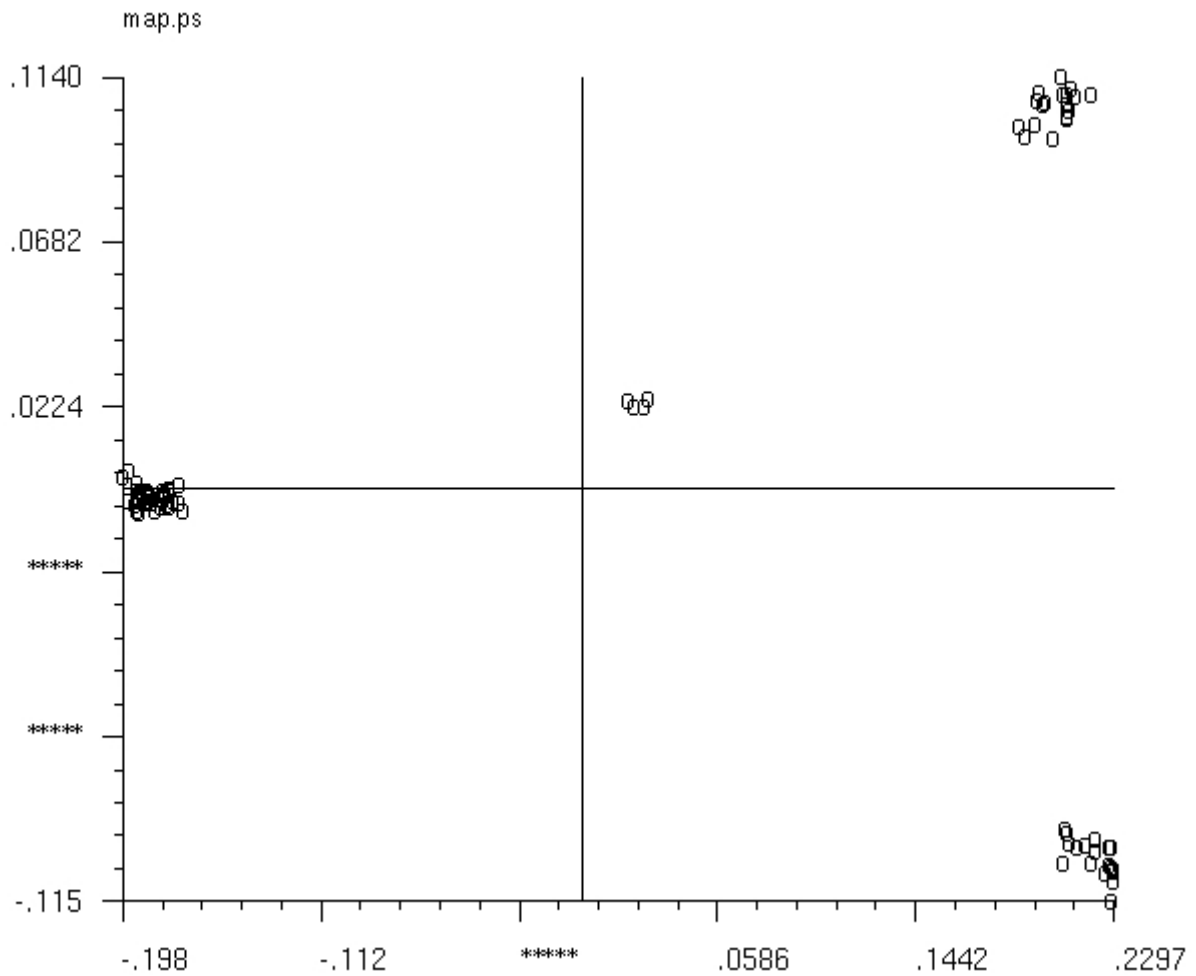
.ENTER NEW LOWER, UPPER AXIS BOUND or <CR>: <CR>

.ENTER NEW AXIS LABEL UNITS /LABEL or <CR>: <CR>

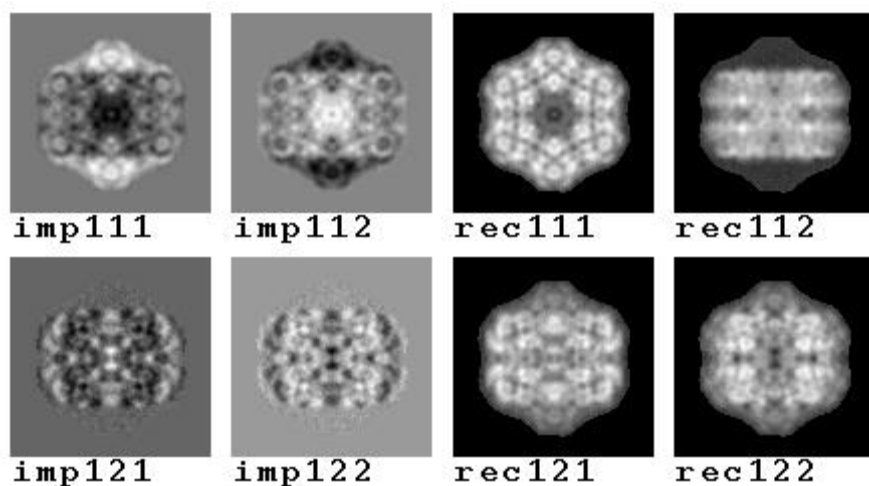
.ENTER LABEL NOetc or <CR> TO CONTINUE: <CR>

.OPERATION: **en**

Remark: All these steps that you just carried out in interactive mode are summerized in the batch [b08.fed](#). In this batch the projection map is created as a postscript file called : **../r2d/map.ps**. If you can open it on your machine, you should see a map similar as the one below :



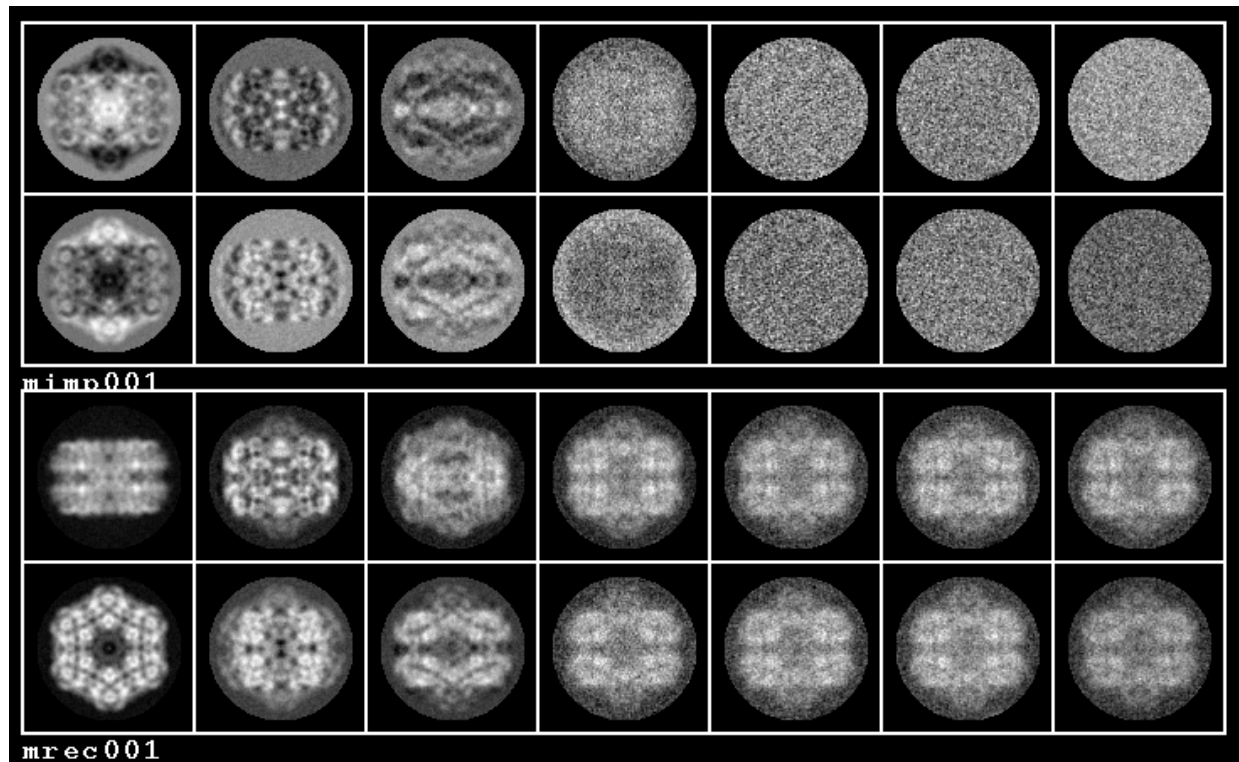
To understand which trends correspond to factorial axes 1 and 2, we keep track of the extreme values on each axis (*e.g.*, Axis (1) [-0.2045 : 0.2424], and Axis (2) [-0.11 : 0.12]) and we can use operations [CA SRD](#) and [CA SRA](#) to create **importance** and **reconstitution** images for factorial axes 1 and 2.



The importance images show which pixels vary along negative and positive portions of **axis 1** (**imp111** & **imp112**) and of **axis 2** (**imp121** & **imp122**).

The reconstitution images show how should look an original image if it would be projected at the negative and positive edges of each axis. Here one can see that the **negative part of axis 1 (rec111.hbl)** corresponds to **hexagonal top** views, while the **positive part of axis 1 (rec112.hbl)** corresponds to **rectangular side** views. The meaning of axis 2 is more difficult to guess (**rec121.hbl et rec122.hbl**), but it is clearly related to a variation concerning the rectangular side views.

The batch [b09.fed](#) explores the same way the meaning of all 7 factorial axes. We can look at the at the resulting montages [../r2d/MIMP001.hbl](#) and [../r2d/MREC001.hbl](#).



What can you say about the meaning of axis 3 and the remaining axes ?

3.2 Automatic clustering of the images

At this stage, we are going to use the simplified representation of our image set which are the coordinates of our 76 images on the 7 factorial axes, to characterize automatically the different image classes. For this, we will use the Hierarchical Ascendant Classification (HAC), which progressively merges the 76 points (corresponding to our 76 images in our new 7 axes factorial space). The merging is done in an ascending direction. First the two closest points (most similar images) are merged in a single point. The mass and location of this new point correspond to the global mass and center of gravity of the two original points. The aggregation is then resumed with the nearest next points until all the points are progressively merged in a single one. The "history of aggregation" is kept in a file called "dendrogram" (from the greek dendros = roots). Indeed, each merging of two points is represented by a vertical step whose height is related to the total "distances" and "masses" of the merged points.

Lets see on a practical level how to run the HAC. Start the spider software and type the following orders:

```
>spider hbl
```

```
.OPERATION: cl hc
.CORAN/PCA FILE (e.g. CORAN_01_IMC~) FILE: ../r2d/coran1_IMC
.FACTOR NUMBERS: 1-7
.FACTOR WEIGHT: 0
. CLUSTERING CRITERION (1-5): 5  <-- here we used Ward's merging criterion
(No.5) but you can try others 1 or 2 for instance.
DO YOU WANT DENDROGRAM POSTSCRIPT PLOT? (Y/T/N): N
.DO YOU WANT DENDROGRAM DOC FILE? (Y/N): Y
.DOCUMENT FILE: ../doc/dhac001
.OPERATION: en
```

Now explore the results file created during this interactive spider session:

```
> cat results.hbl.4
```

```
147 135 141 18 18.0.61      *
148 143 146 36 36.0.75      *
-----> Threshold value between 0.75 & 7.1 (for example: 3.0)
149 129 145 22 22.7.1       ****
150 147 149 40 40.15.       *****
151 148 150 76 76.2.03E+02 *****
NODE INDEX SENIOR JUNIOR SIZE DESCRIPTION OF THE HIERARCHY CLASSES
```

Here the highest step of the dendrogram (creating two major classes) has a height of **203**.

The second highest step of the dendrogram (creating a third class) has a height of **15**. and the third highest step (creating a fourth class) has a height of **7.1**
Finally, all the following steps have a height of **0.75** only.

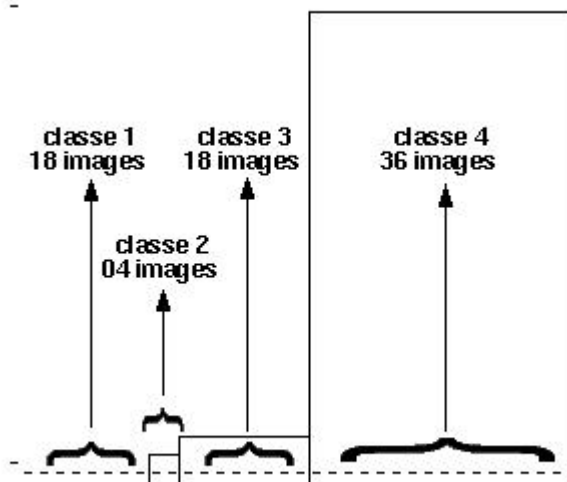
From these results, you can decide to truncate the dendrogram between its third and fourth steps (with a threshold of 3.0 for instance). This will sort four image classes.

Restart spider and run again the HAC to create a truncated dendrogram file **psdndplot.ps** that you will be able to visualize using the Web program and the **COMMAND "Show Contour file"**.

```
.OPERATION: cl hc
.CORAN/PCA FILE (e.g. CORAN_01_IMC~) FILE: ../r2d/coran1_IMC
.FACTOR NUMBERS: 1-7
.FACTOR WEIGHT: 0
. CLUSTERING CRITERION (1-5): 5
.DO YOU WANT DENDROGRAM PLOT FILE? (Y/T/N):T
.ENTER PLOT CUTOFF: 3.
. DENDROGRAM FILE: ../r2d/psdndplot -----> postscript file containing the truncated
dendrogram
.DO YOU WANT DENDROGRAM DOC FILE? (Y/N): n
.OPERATION: en
```

To see how many images are contained in each class, you can use operation "CL HD" and specify the threshold value rescaled between 0 and 1. Here, you just divide the absolute threshold value of (3.0) by the highest step value of the dendrogram (203.). Results are stored in a new document file (here ../doc/dcount001.hbl), but you can also read them directly on the screen (see below).

```
.OPERATION: x11=3/203
.OPERATION: cl hd
.Threshold (0-1): x11
.Dendrogram document FILE: ../doc/dhac001
.DOCUMENT FILE: ../doc/dcount001
OPENED DOC FILE: ../doc/dcount001.hbl
1.          18.
2.          4.
3.          18.
4.          36.
Total number of objects = 76   Number of classes = 4 .
```



In our case, the resulting truncated dendrogram postscript file **../r2d/psdndplot.ps** looks as shown above.

We are now going to keep in document files the list of images corresponding to each class:

```
.OPERATION: x11=3/203 <-- same threshold value
.OPERATION: cl he
.Threshold: x11
.Dendrogram document FILE: ../doc/dhac001
.Enter template for selection doc: ../doc/dcla***
```

```
OPENED DOC FILE: ../doc/dcla001.hbl
Group number   Number of elements
```

1	18
OPENED DOC FILE: ../doc/dcla002.hbl	
2	4
OPENED DOC FILE: ../doc/dcla003.hbl	
3	18
OPENED DOC FILE: ../doc/dcla004.hbl	
4	36
Total number of objects = 76 Number of classes = 4	

Finally, we will compute the average and variance maps of each class as shown below:

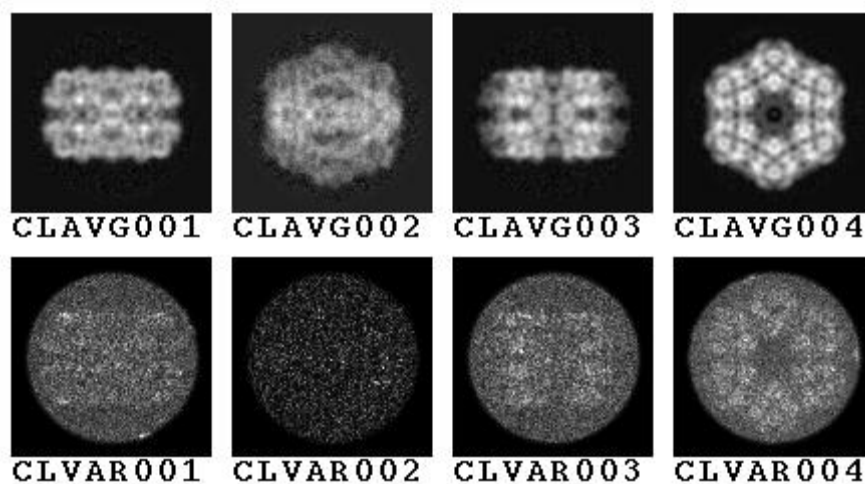
```
.OPERATION: AS DC
.INPUT FILE: ../images/cenu****
.DOCUMENT WITH FILE NUMBERS FILE: ../doc/dcla001
.ALL, ODD-EVEN (A/O): A
.AVERAGE FILE: ../r2d/clavg001
.VARIANCE FILE: ../r2d/clvar001
```

```
.OPERATION: AS DC
.INPUT FILE: ../images/cenu****
.DOCUMENT WITH FILE NUMBERS FILE: ../doc/dcla002
.ALL, ODD-EVEN (A/O): A
.AVERAGE FILE: ../r2d/clavg002
.VARIANCE FILE: ../r2d/clvar002
```

```
.OPERATION: AS DC
.INPUT FILE: ../images/cenu****
.DOCUMENT WITH FILE NUMBERS FILE: ../doc/dcla003
.ALL, ODD-EVEN (A/O): A
.AVERAGE FILE: ../r2d/clavg003
.VARIANCE FILE: ../r2d/clvar003
```

```
.OPERATION: AS DC
.INPUT FILE: ../images/cenu****
.DOCUMENT WITH FILE NUMBERS FILE: ../doc/dcla004
.ALL, ODD-EVEN (A/O): A
.AVERAGE FILE: ../r2d/clavg004
.VARIANCE FILE: ../r2d/clvar004
```

One can see below the average and variance maps of our four classes. In this situation, we can see that classes 1 and 3 correspond to two types of rectangular side views, while class 4 corresponds to the hexagonal top view, and the small class 2 (only 4 images) to an intermediate undefined orientation. This last small class will not be used for the rest of the project.



Remark : All these classification steps can be carried out automatically, using the batch [b10.fed](#). However, **the new Web software allows now a fast computation of the truncated dendrogram and of the corresponding average images. Therefore, the exploration of automatic classifications is now done almost instantly ! To try this option, you need to overcome a small bug in Web, which currently seems to open by default only images with a ".DAT" suffix. The batch [b16.fed](#) will help you to rename the files necessary for trying the Web interactive clustering program.** Then you only need to start the program web :

Web hbl &

Then choose Command "**Dendrogram**"

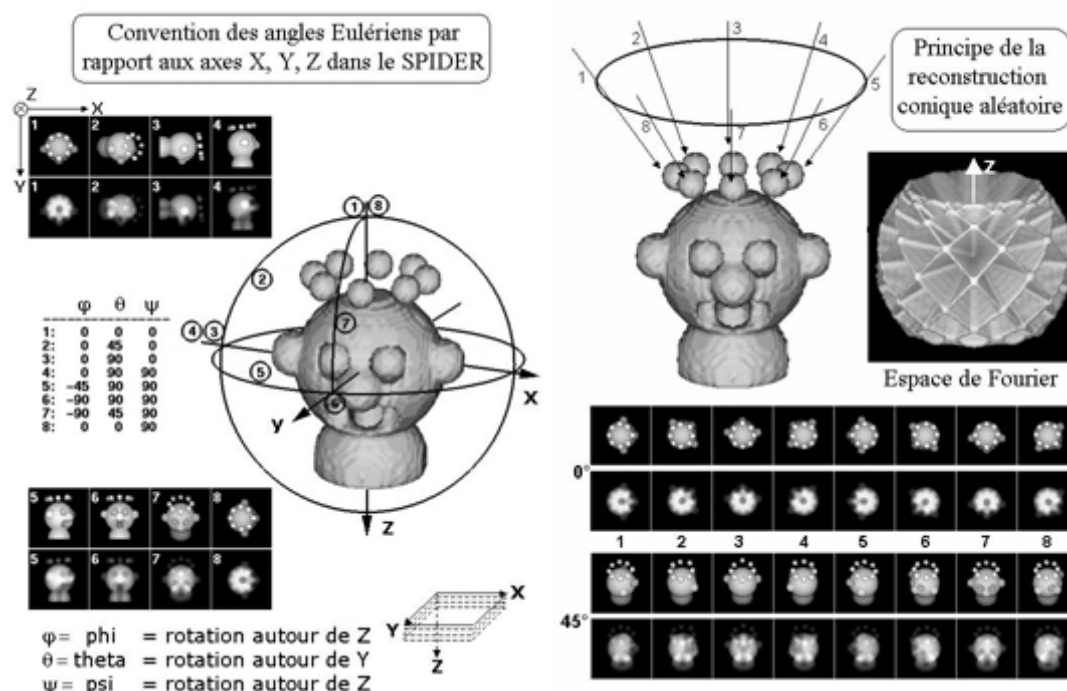
The program will ask for the **dendrogram doc file** -> **../doc/dhac001.hbl**

A new window will open asking you to pick a Threshold value between 0 and 1 for truncating the dendrogram. If you want to compute the averages corresponding to each class, you will need to **activate the option "Show Averaged images"** and **enter the template of the original images subjected to this classification** (in your case, you can type **../images/cenu0001.hbl**), and **click on "ACCEPT"**. The histogram and corresponding average maps will instantly appear on your screen. To get a new view click on the central button of your mouse and change the Threshold value from 0.5 to 0.05, 0.005, and finally to 0.0005. You will see that the lower you get the more smaller classes you will get. And you can also see that if you use a very small threshold (0.0005) you will get several averages looking alike, indicating that you are cutting too low and are artificially creating classes which are in fact identical. In our case the threshold of 0.005 (if you used Ward's merging criterion) seems the more appropriate as it distinguishes the two side views, the hexagonal and the intermediate views into 4 classes as shown above.

4. Three-dimensional reconstruction of the particle

We have now three well defined image classes on the untilted-specimen micrograph, and we know that their corresponding images windowed from the tilted-specimen micrograph produce three random conical tilt series of projection of our particle. As we have now the list of images contained in each class and we know their Euler angles (ϕ , θ , ψ) specifying

their orientation in space. With all these informations we can compute the 3D reconstruction of our particle.



The above figure gives the Euler angular conventions with respect to axes X, Y, and Z within spider, and it shows the basic principle of the random conical tilt-series 3D reconstruction approach. As shown in this figure, the limitation of the tilt that one can impose to the grid in the microscope induces a direct limitation of the Euler angle theta. Hence, within Fourier space (where each 2D projection corresponds to a central slice orthogonal to the projection axis) some areas remain empty. This phenomenon is responsible for the "missing cone artifact", as the empty regions correspond to two opposite cones pointing along the Z axis. To prevent this missing cone artifact, **we will use the symmetries of the particle, and we will merge the different series of images to compute a global or "merged" 3D reconstruction volume.**

4.1 Creation of the symmetry document file

To take into account the symmetries of the molecule, one must create two types of document files, describing the symmetries when the molecule is oriented in its top view (6-fold symmetry axis parallel to the Z axis), or in its side view (6-fold symmetry axis parallel to the Y axis). The batch [b11.fed](#) computes these files named [../doc/d6top.hbl](#) & [../doc/d6side.hbl](#) and which correspond to the different Euler rotation angles which allow to superpose the volume with its according to a D6 point-group symmetry.

4.2 Computation of three distinct 3D reconstruction volumes

For the 3D reconstruction itself, we will use the [BP RP](#) operation which is the SIRT algorithm (Simultaneous Iterative Reconstruction Technique) (See [Penczek, P., M. Radermacher, et J. Frank. 1992. Three-dimensional reconstruction of single particles embedded in ice. Ultramicroscopy, 40:33-53](#)). The batch [b12.fed](#) can be used to run these operations, but you

should at least use once the BP RP operation in the interactive mode to better understand its function and adjust the values of some parameters. Here three volumes corresponding to classes number 1, 3, and 4 will be computed. They are termed **../r3d/vcla001.hbl**, **../r3d/vcla003.hbl**, et **../r3d/vcla004.hbl**.

Remark: If BP RP is too slow on your machine, or if you are on a hurry, check the end of batch [b12.fed](#) , where the use of operation "[BP 3F](#)" is described.

These volumes can be visually checked using the Web software :

COMMAND "**Image**" can visualize a series of 2D slices within the volume along axes X, Y, or Z.

COMMAND "**Surface**" computes a surface rendering of the volume corresponding to a specific threshold value, and in a specific orientation. The [user manual of web](#) can be checked to better understand how these operations work, but we will see here a quick preview.

Start the web software typing : "[web hbl &](#)"

Within the menu "**Commands**", select option "**Surface**".

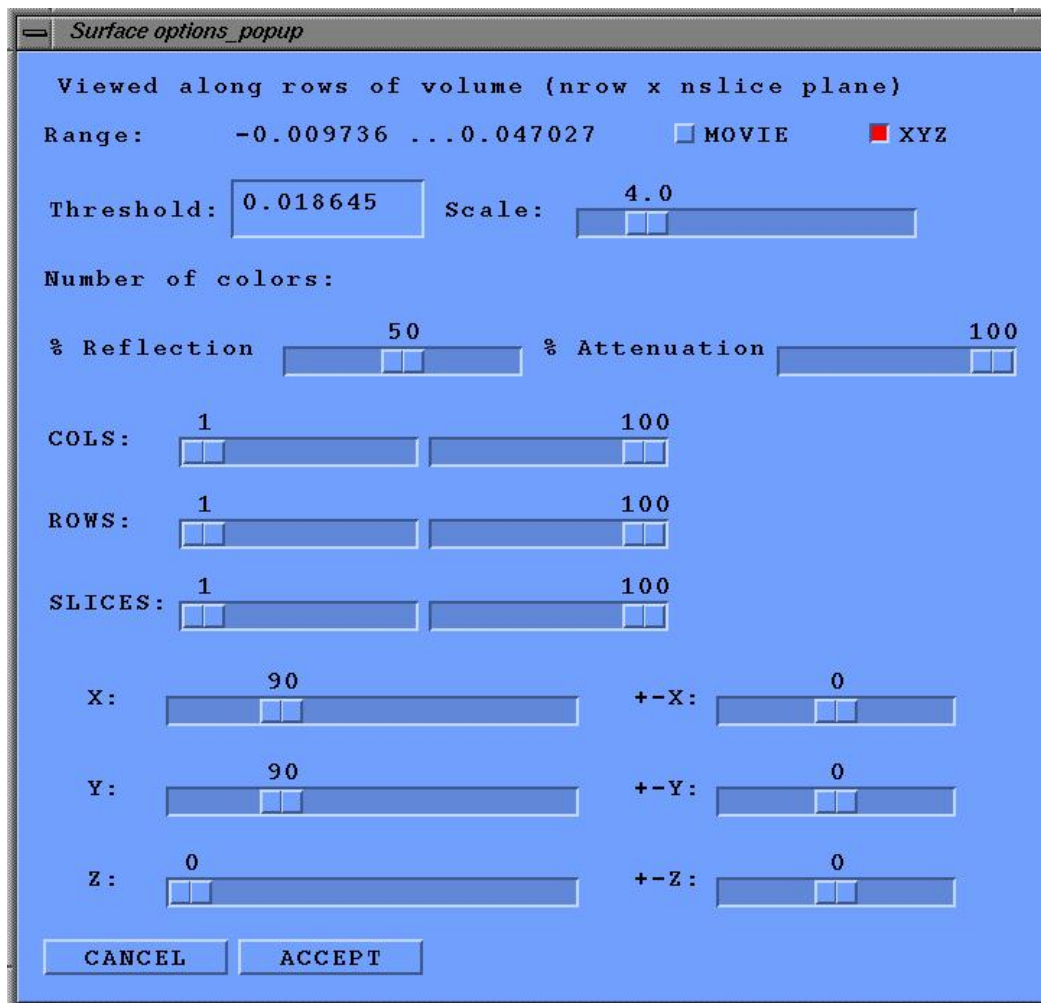
The program will ask for the name of the volume. Click on "**vcla001.hbl**" then on "**OK**".

A new window then pops up (see below). Chose the following options :

XYZ

Scale = 4.0

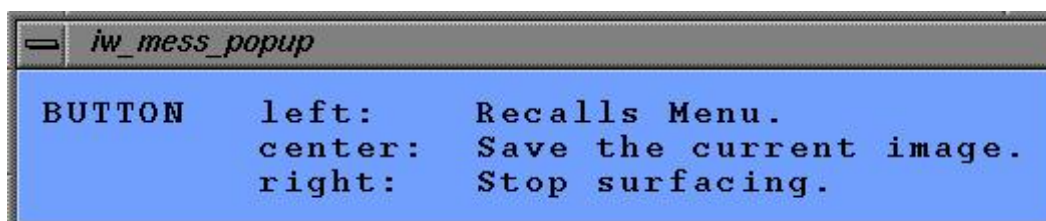
You can also change the threshold value for the surface rendering "**Threshold**" This value must remain between the actual minimum and maximum densities of the volume (reminded in the line "**range :**" at the top of the page (here minimum = -0.009736 & maximum = 0.047027)).



To change the orientation of the volume, change the values of X, Y, and Z at the bottom of the screen.

Remark: here **X = 90, Y = 90, & Z = 0** will put the observer on top of the volume and looking down along the Z axis.

Once the surface rendering is computed, you can start again by clicking on the left button of the mouse. You can also save the image by clicking the central button of the mouse, or stop the computation of surface representations and come back to the main menu by clicking on the right button of the mouse (see below).

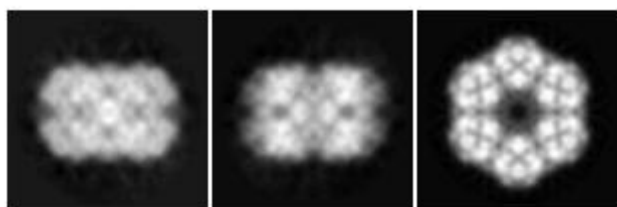


4.3 determination of the respective positions of the three volumes in real space

A method to understand the orientation of the volumes in real space is to project them on a plan to create a pseudo cryoEM image. This can be done using operation "**PJ 3**".

.OPERATION: **PJ 3**
.THREED FILE: **../r3d/vcla001**
.PROJECTION SAMPLE DIM.: **100**
.OUTPUT FILE: **scr001**
.AZIMUTH ANGLE (PHI): **0.0**
.TILT ANGLE (THETA): **0.0**

Here the azimuth angle corresponds to a rotation of the object around the Z axis (within the plan of the specimen grid), and the tilt angle corresponds to the real tilt angle imposed on the specimen grid within the microscope. In our example, phi and theta are set to zero, which corresponds to a projection of our volume along the Z axis in its original orientation, as if it were resting on the electron microscope grid. If you compute similar projections with the other volumes **vcla003.hbl** and **vcla004.hbl**, you will obtain this type of images, which are directly comparable to the average maps of our automatic clustering on the untilted-specimen images (section 3.2).

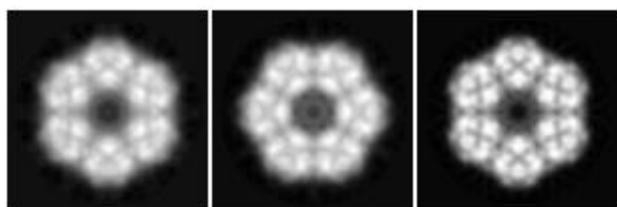


To rotate a volume in space, we will use the operation "**RT 3D**" :

.OPERATION: **RT 3D**
.INPUT FILE: **vcla001**
.OUTPUT FILE: **VOOUT001**
.Phi, Theta: **90.,90.** **by convention phi = rotation around Z, then theta = rotation around Y**
.Psi: **-90.** **and finally psi = rotation around Z again.**

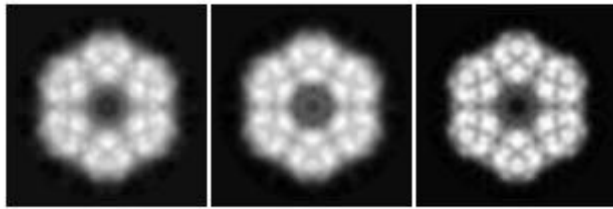
(This example corresponds to a 90° rotation around the X axis)

If you turn the two first volumes of 90° around the X axis, and "reproject" them along the Z axis as previously (Azimuth/phi and tilt/theta = 0), you will produce this new type of projections. In this series, the first and third volumes are in the same orientations.



To align the second volume in a similar orientation, you must apply an additional 30° rotation

around the Z axis.



The batch [b13.fed](#) computes these series of rotations and the corresponding montages of 2D projections shown above ([mpro\[001-003\].hbl](#)).

If you are completely lost in your alignment, the spider software can help you to find the best matching orientation. This is done stochastically from an original orientation, and the result found is not always the optimal orientation but corresponds to a local minimum searched by random jumps... Therefore, you should run this orientation search several times and always using different starting positions. The correlation coefficient printed at the end of the search provides an evaluation of the orientation search (*e.g.*, if the correlation is around 0.3 it is really a bad orientation, but if it is larger than 0.85 it is rather good).

```
.OPERATION: OR 3Q X11,X12,X13,X14
.Reference 3D FILE: ../r3d/vcla004
.Second FILE: ../r3d/vcla001
.Radius of the mask: 41.
.Phi, Theta: 90.,90.                                <--- Starting position for the orientation
search
.Psi: -90.
```

```
Iteration #      1 Distance = 0.5315237   r= 0.4684763064118211
FUNIQ - new parameters  90.0000  90.0000  90.0000
!                               !
!                               !               <--- iterations
V                               V
Iteration #      182 Distance = 0.1053701   r= 0.8946299150353252
```

The Euler angles found by minimization procedure

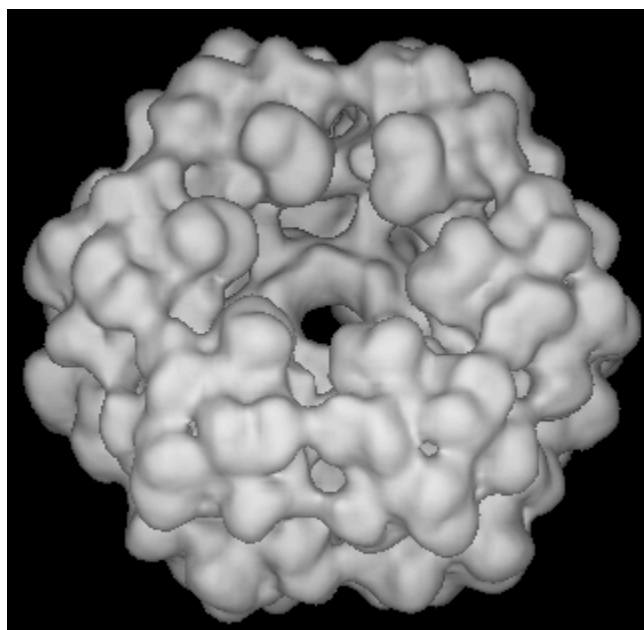
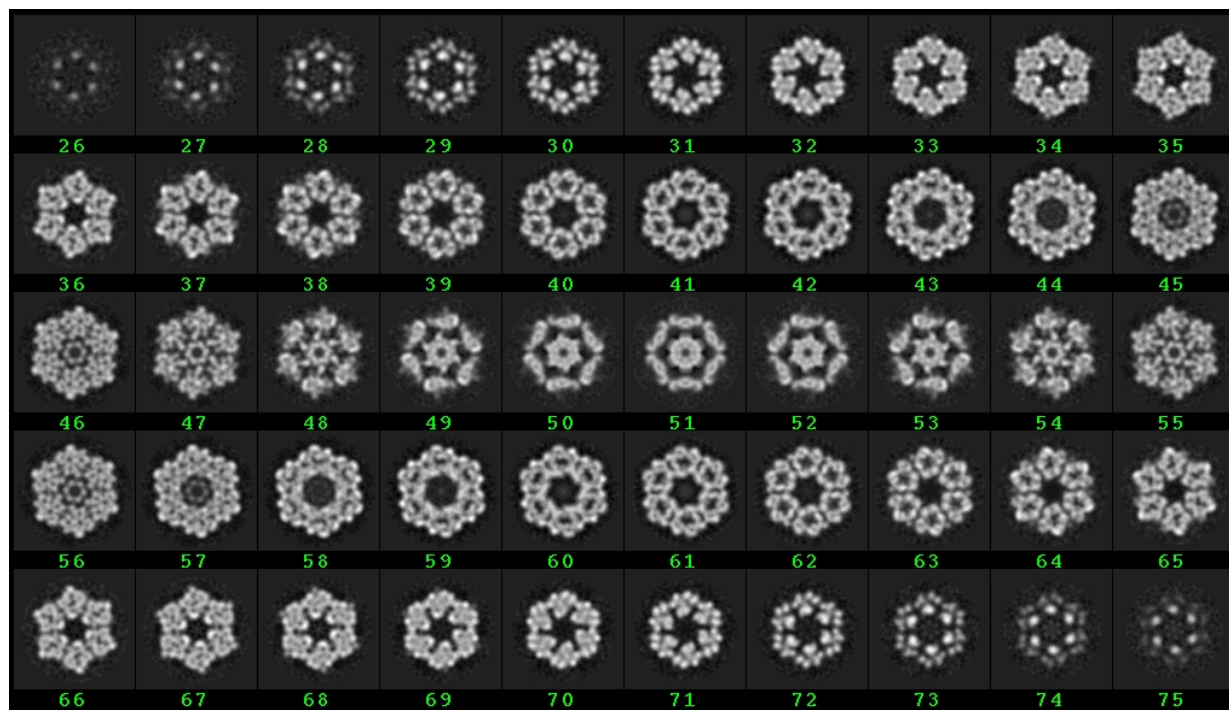
```
Phi,    theta,    psi and function value
90.0001  89.9993  -90.0005    0.8946275    <--- these values are stored in registers
X11, X12, X13, et X14
```

The batch [b14.fed](#), starts several orientation searches and the resulting values in document file [../doc/dalv001.hbl](#).

4.4 Merging of the three volume

Rather than adding the three reconstruction volumes aligned in real space, a more elegant solution is to compute a new global volume after modifying the Euler angles assigned to the images to take into account the results of the orientation search. As it is a delicate step, it is safer to use the batch mode to keep track of what you do. The batch [b15.fed](#) execute the following treatments for each image series, (1) a modification of Euler angles, (2) a copy of the images under a new name and with consecutive numbers [../images/cent\[10001-](#)

10072].hbl, (3) a copy of the new Euler angles with consecutive numbers in a new angular document file [../doc/dang010.hbl](#), and (4) computation of the global or merged volume [../r3d/vtot001.hbl](#).



Finally, we hope this rapid tutorial helped you in your first 3D reconstruction of a single particle, and we wish you good luck for your own research projects !

**Friendly yours,
Nicolas.**

(Please send me your comments by email at "Nicolas.Boisset@impmc.jussieu.fr" or "Ricardo.Aramayo@impmc.jussieu.fr" to help me improve this tutorial).

