PART I :
SINGLE PARTICLE ANALYSIS

SEMC Appion workshop series

July 29-30, 2020

NATIONAL CENTER FOR
CRYOEM ACCESS & TRAINING

New York Structural
Biology Center

SIMONS ELECTRON
MICROSCOPY CENTER

NMR
CoMD/
NMR

X-ray
NYX
@NSLS-II

Protein Production
COMPPÅ

SCHEDULE

JULY 29-30, 2020 2PM-4PM

PRACTICAL WORKSHOP WHERE STUDENTS WILL WORK IN PAIRS ON A TEST DATASET. AFTER EACH LECTURER GIVES AN OVERVIEW OF THE TOPIC THE ATTENDEES WILL HAVE HANDS-ON TIME TO USE THE INTERFACE.

1. Workshop intro

- Overview of Leginon/Appion
- Krios workflow
- Leginon targeter

2. Micrograph evaluation / CTF Estimation

3. Object Selection

4. Stack creation / Particle Alignment

[3 (cont). Object Selection with templates after initial class averages]

5. Other features: Pro tips / Direct Detector Tools / Import/Export Tools

6. Closing

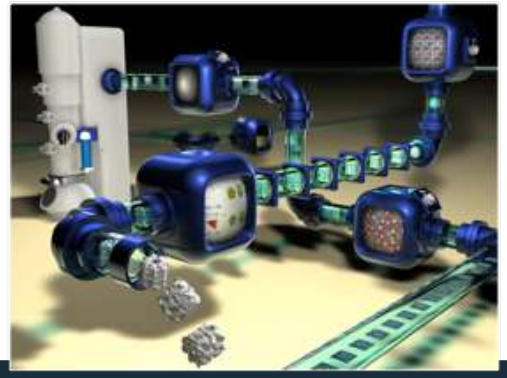
HOW DO WE USE AUTOMATION?

NRAMM

The overall mission of NRAMM is to develop, test and apply technology for automating and streamlining cryo-electron microscopy (cryoEM) for structural biology.

Specimen preparation

Image acquisition



Data processing

HOW DO WE USE AUTOMATION?

Leginon is a system designed for automated collection of images from a transmission electron microscope.

Appion is a "pipeline" for processing and analysis of EM images.

SIMONS ELECTRON MICROSCOPY CENTER

Acknowledgements

Some of this work was performed at the National Center for CryoEM Access and Training (NCCAT) and the Simons Electron Microscopy Center located at the New York Structural Biology Center, supported by the NIH Common Fund Transformative High Resolution Cryo-Electron Microscopy program (U24 GM129539,) and by grants from the Simons Foundation (SF349247), NYSTAR, and the NIH National Institute of General Medical Sciences (GM103310), Agouron Institute (F00316), NIH OD019994 and RR029300 and NY State Assembly.

SEMC OPERATIONS TEAM

EM Directors

EMG@nysbc.org

Staff

SEMC APPION DEVELOPMENT TEAM

Anchi Cheng

Sargis Dallakyan

Swapnil Bhaktar

Shaker Krit

Alex Noble

Tomography

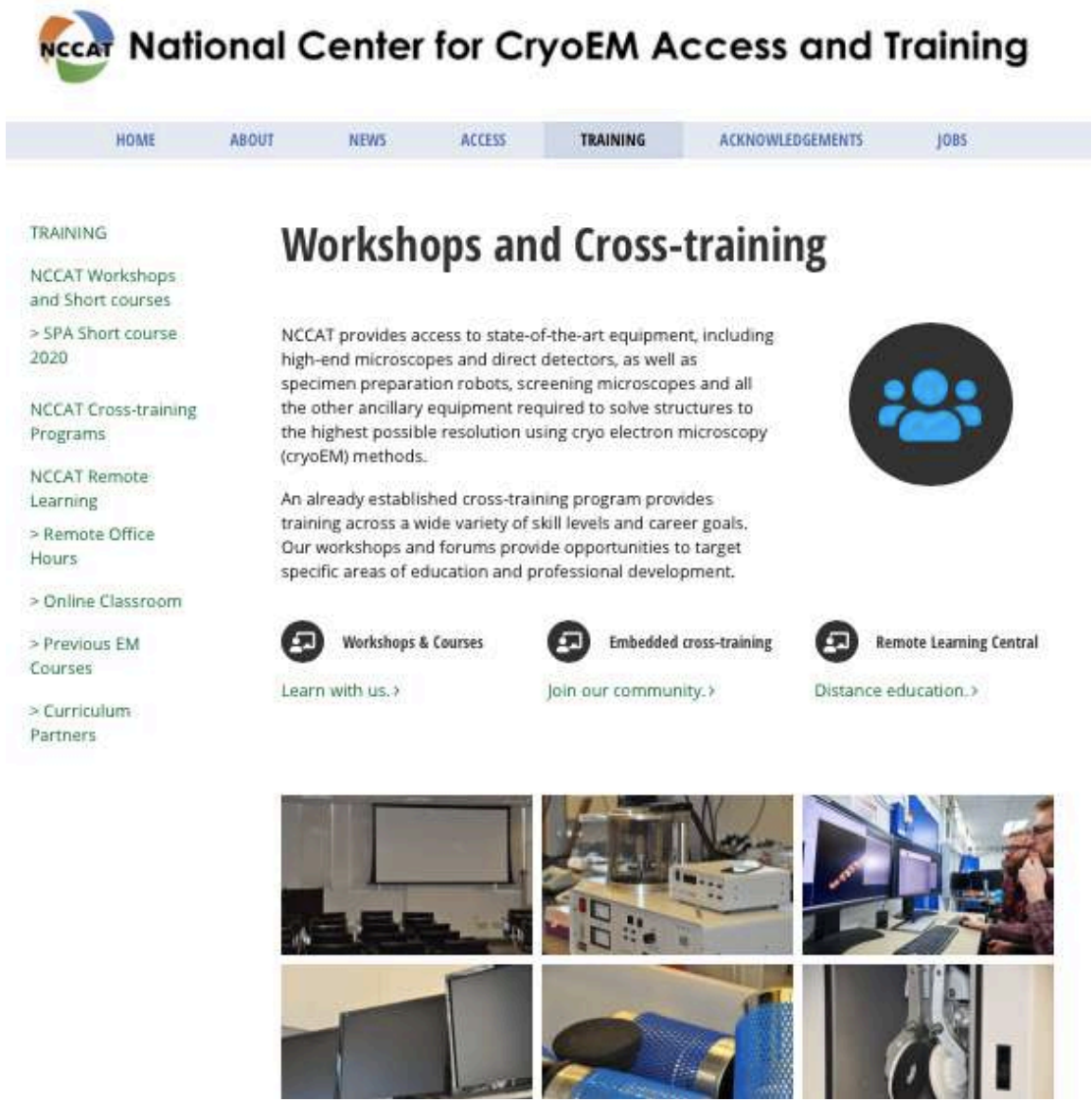
SEMC TRAINING

Type	Frequency	Title	Description
feedback	yearly	General user meeting	State of the center
knowledge	yearly	SEMC EM Course	Theory behind EM *Graduate level course*
computation	quarterly	SEMC Appion workshop	Data processing workshops
access	monthly	SEMC New User Orientation	Facility use and Safety Training Sample preparation [neg-stain & cryo] *Leginon intro/use of screening microscopes*
troubleshooting	weekly	User Project Discussion Meetings	*Appointments: Tue @3pm, Thurs @3pm & @3:30pm*
instrumentation	daily	Advanced Leginon use / 1-1 training appointments	Training for independent use of the microscopes and other SEMC resources

NCCAT TRAINING DOCUMENTATION

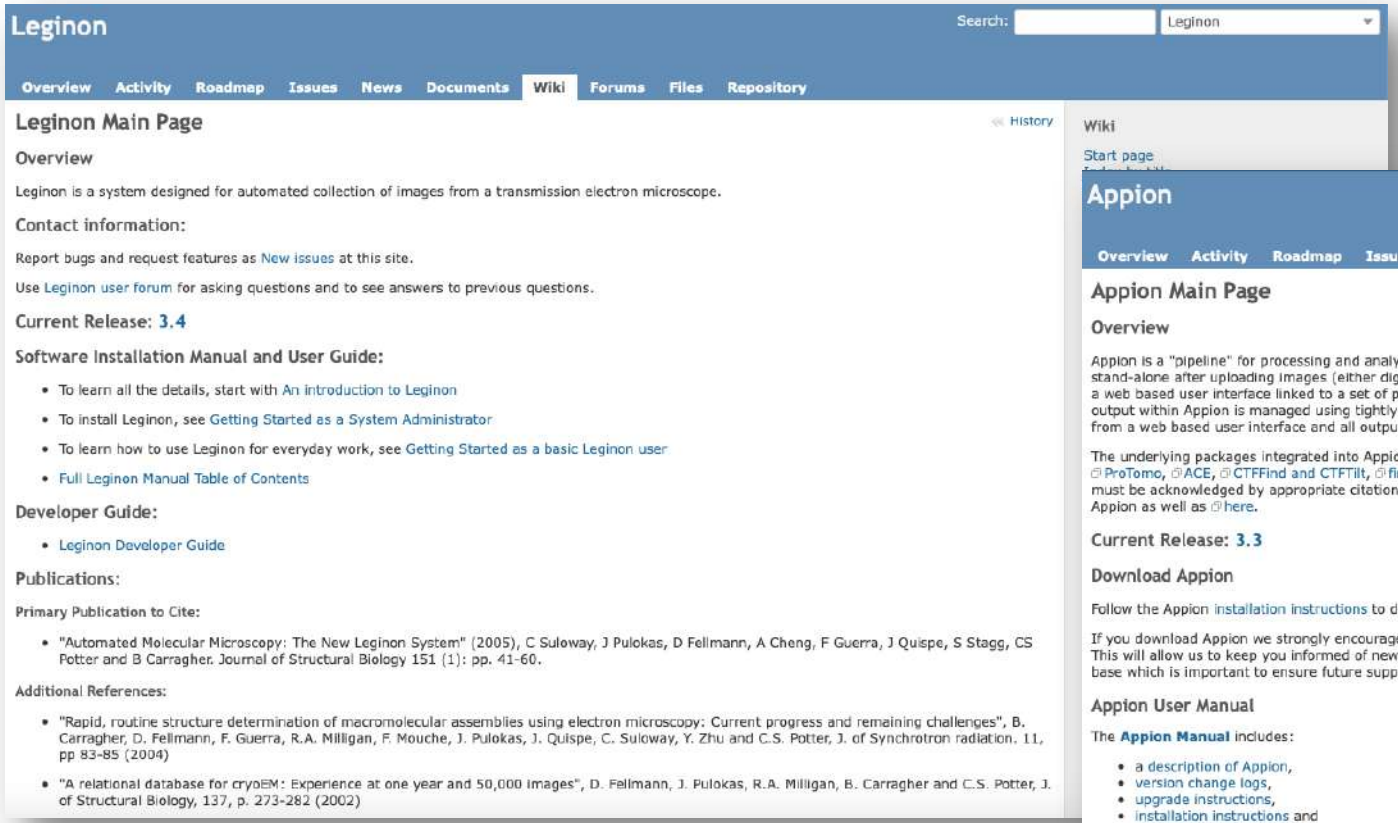
https://nccat.nysbc.org/activities/nccat-cross-training/

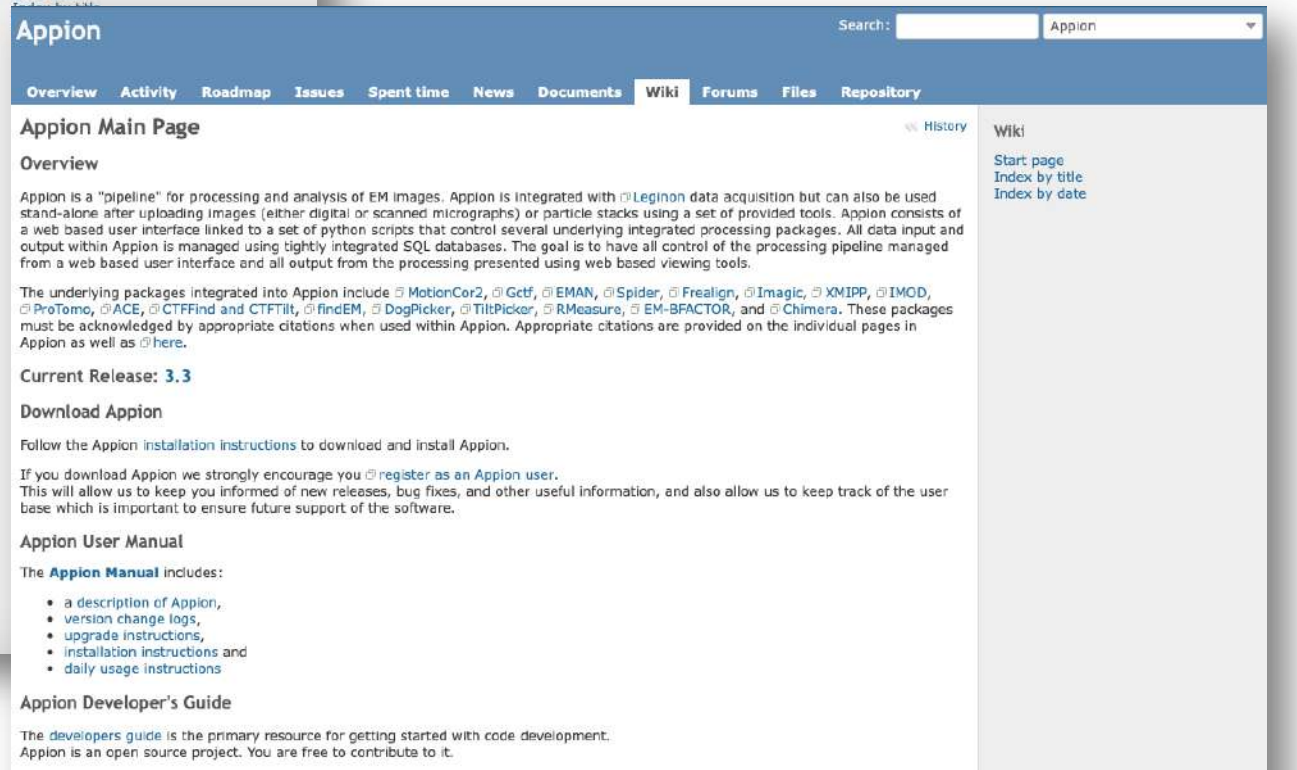
https://nccat.nysbc.org/activities/nccat-remote-learning/



LEGINON & APPION TRAINING DOCUMENTATION

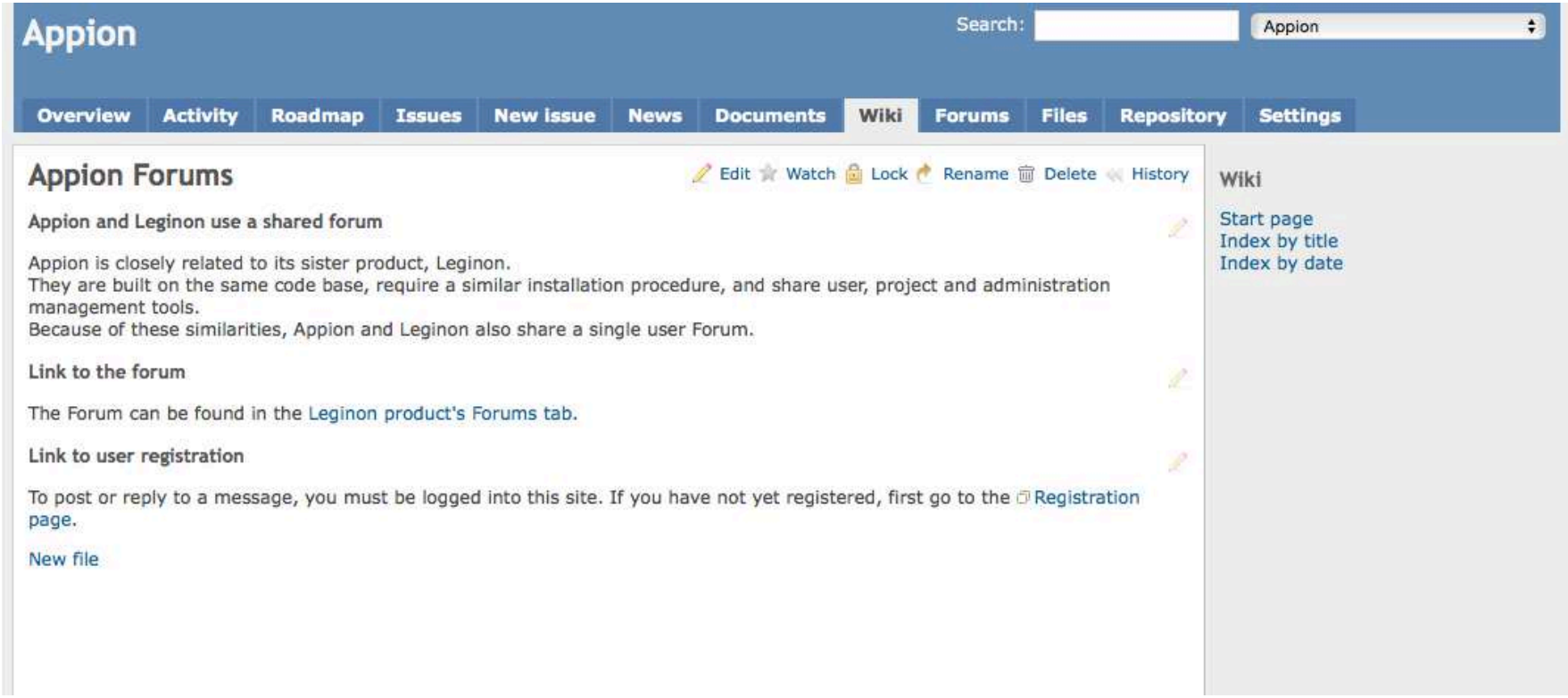
leginon.org | appion.org





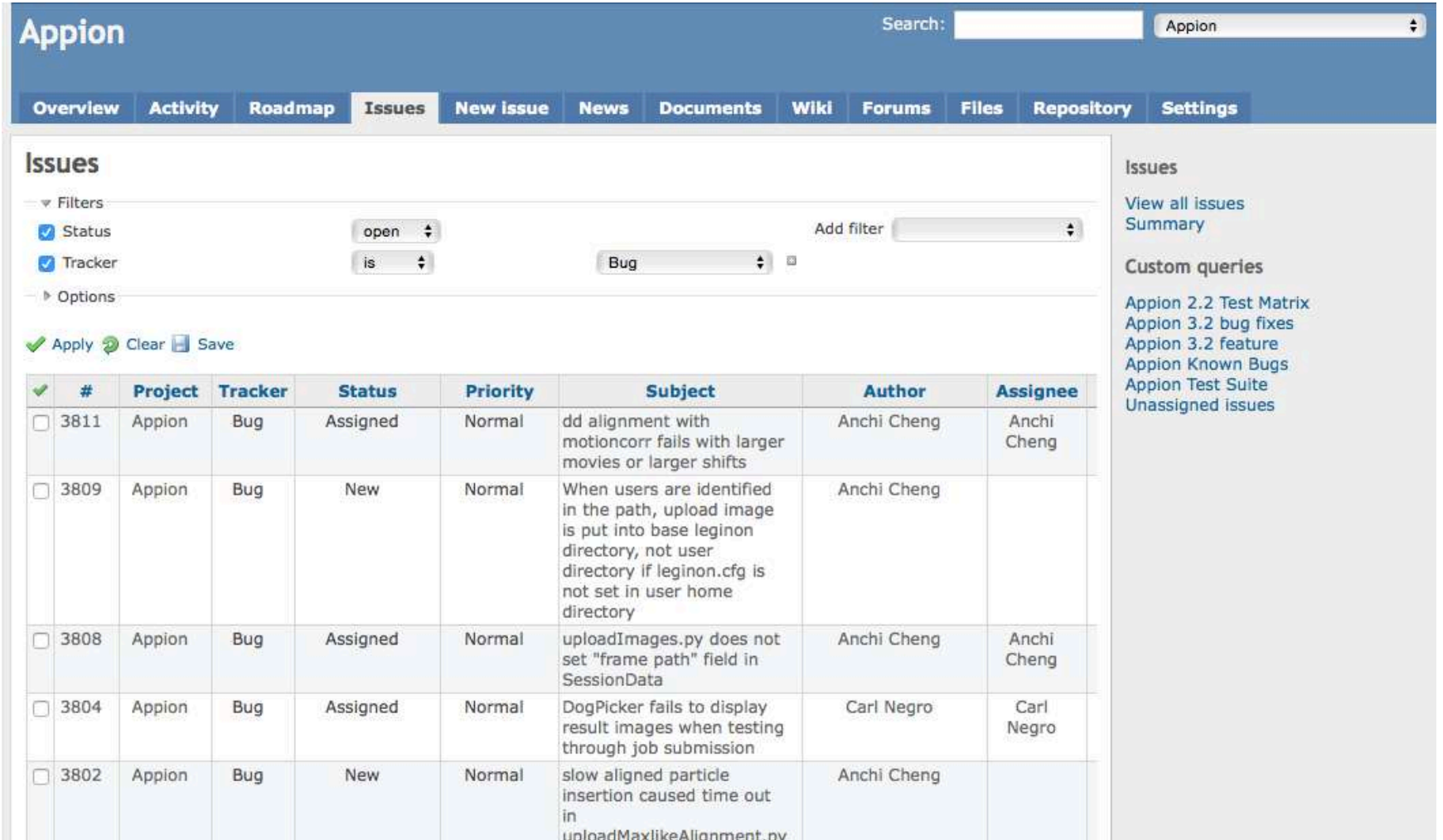
APPION.ORG

Register an account



APPION.ORG

http://emg.nysbc.org/redmine/projects/nysbc-semc-user-group/issues

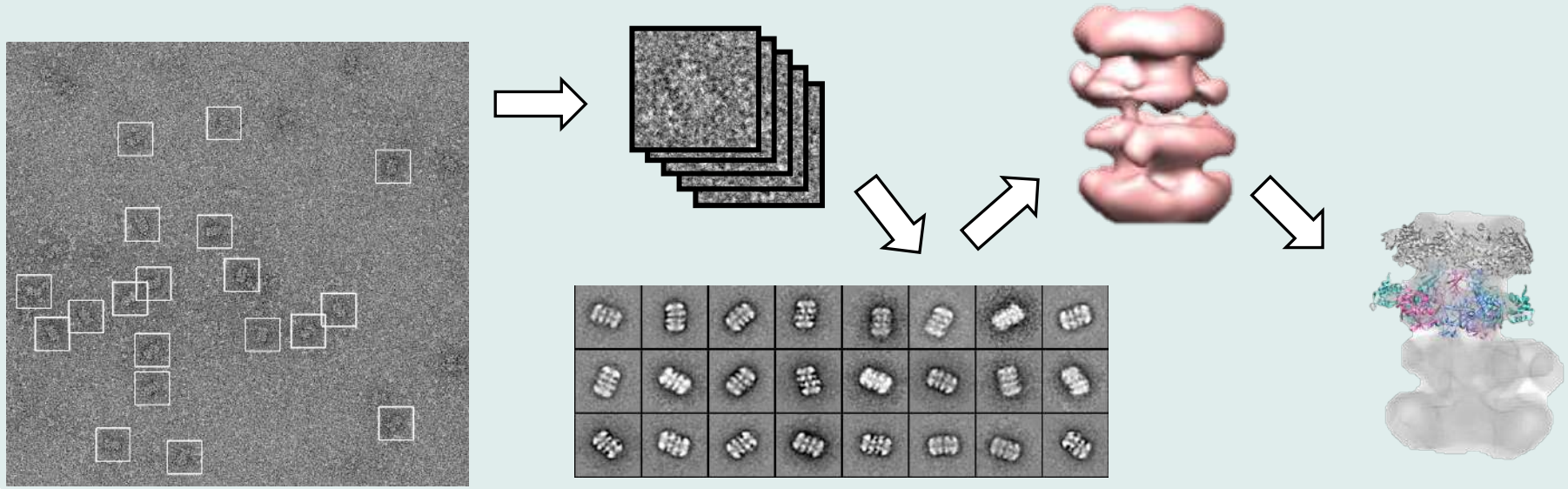


APPION WORKSHOPS

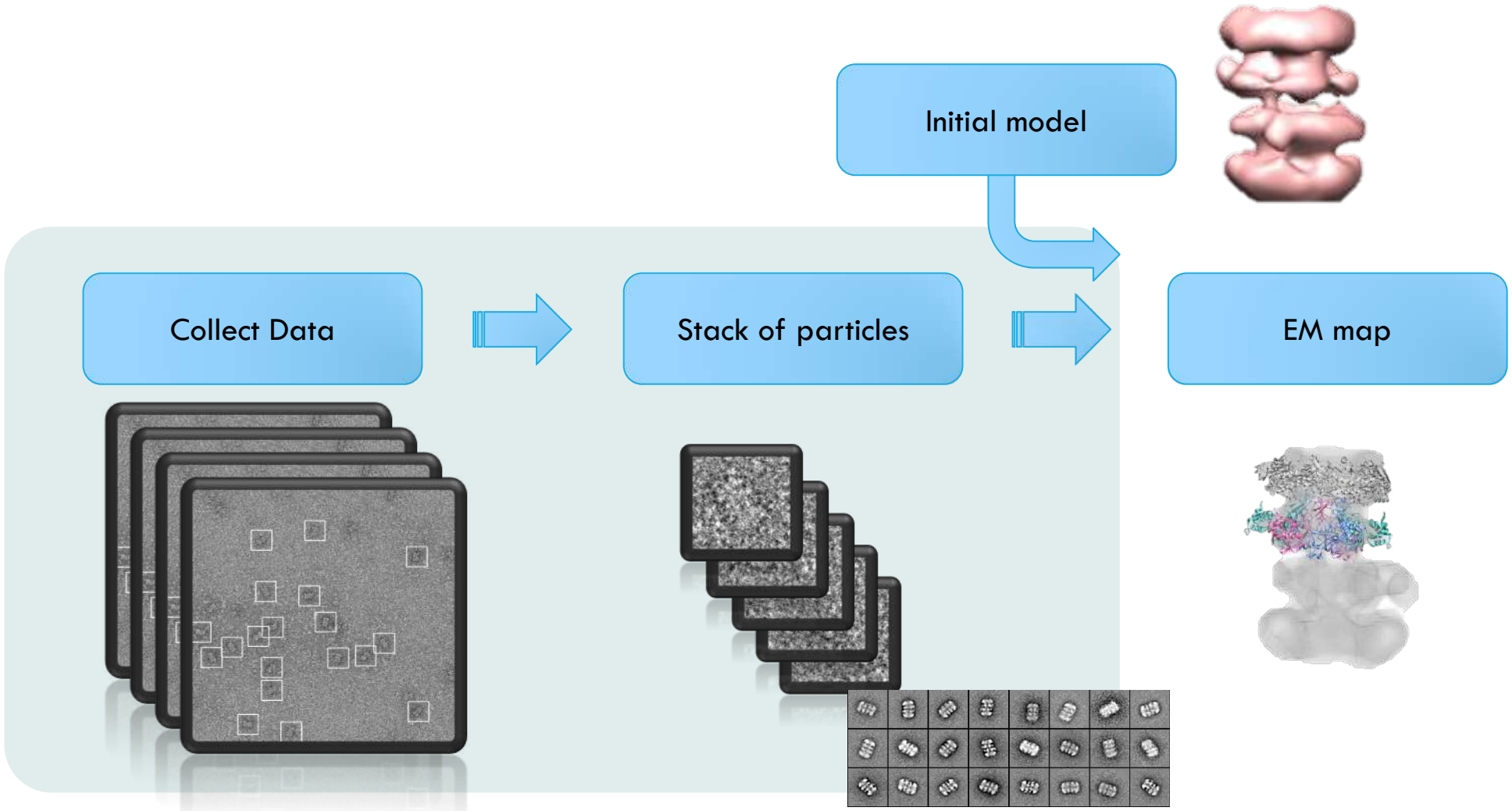
Single particle

Tomography

Part I	Part II	Part III
Tools to generate a particle stack from micrographs and obtain an initial assessment of the sample.	**3D reconstruction, refinement and validation.**	Tomography tools.



SINGLE PARTICLE ANALYSIS

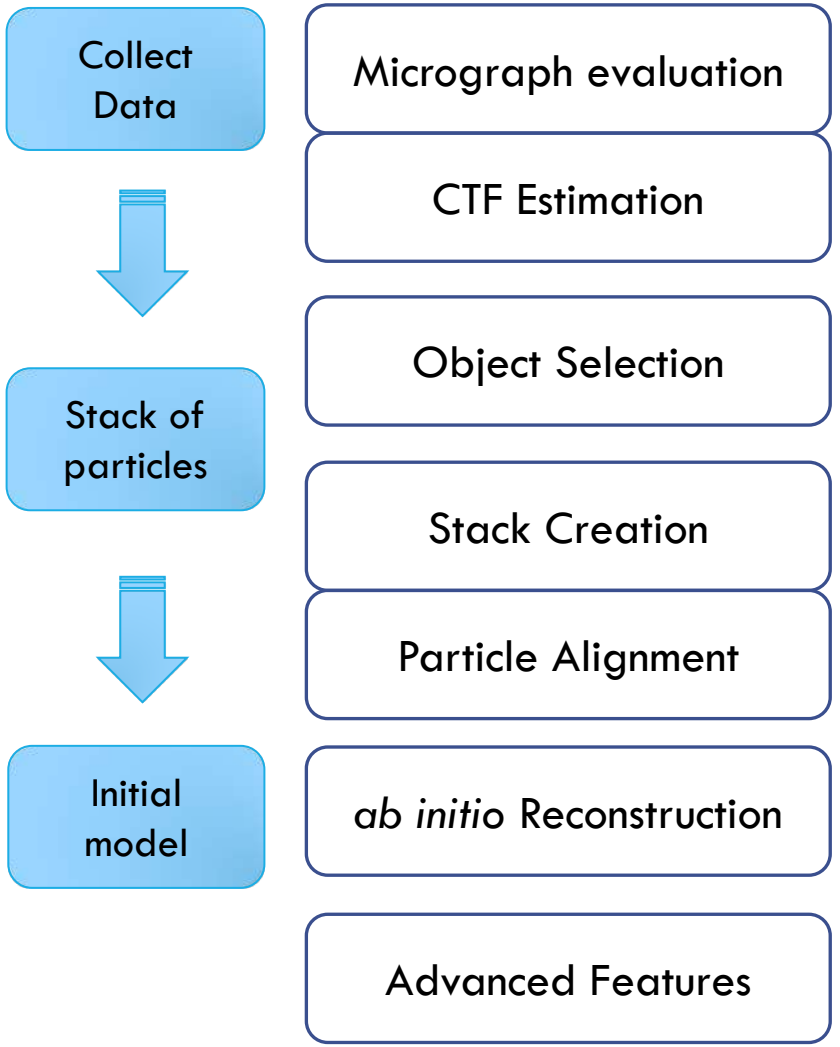


Schedule

July 29-30 2pm-4pm

Practical workshop where students will work in pairs on a test dataset. After each lecturer gives an overview of the topic the attendees will have hands-on time to use the interface.

SINGLE PARTICLE ANALYSIS



DATA ACQUISITION AND PROCESSING PLATFORMS

EM instrumentation

FEI Titan Krios#1 / #2 / #3
#4 / #5 / #6
GIF/K3

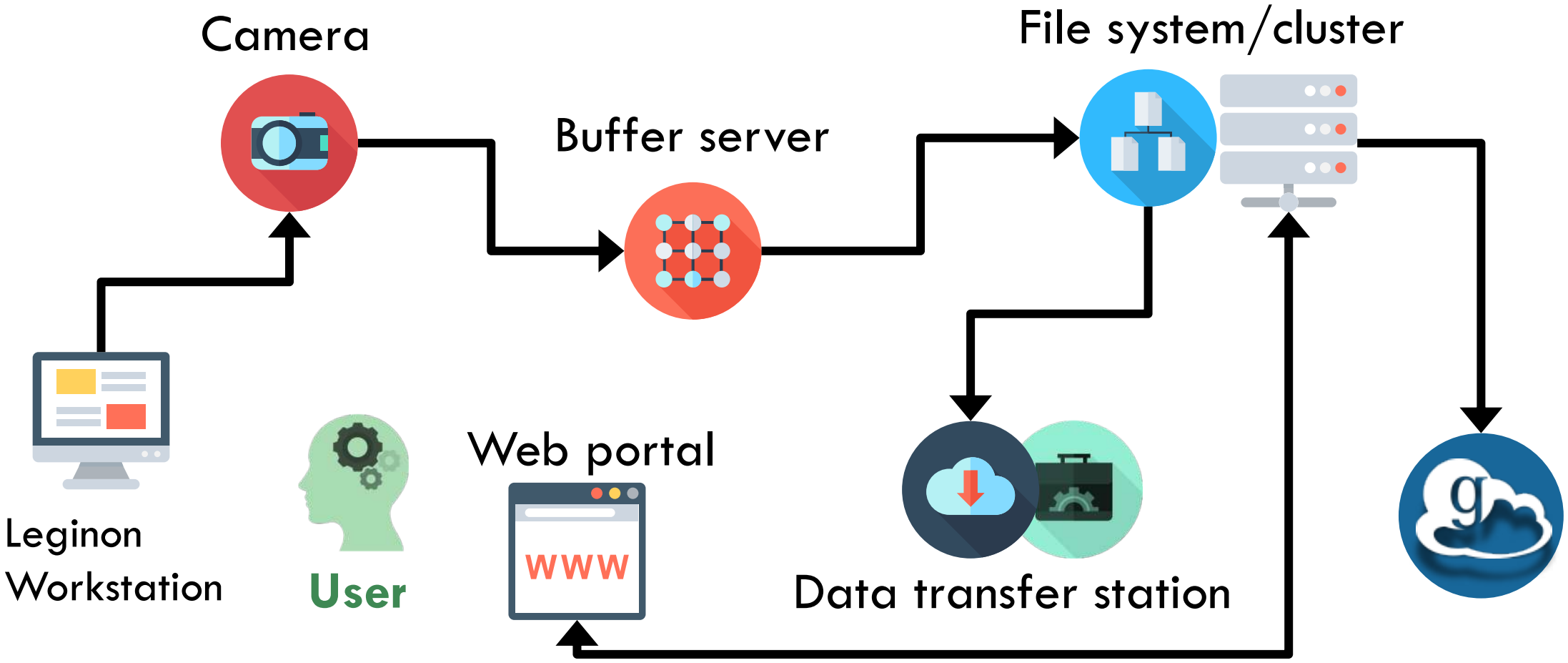
FEI Tecnai F20
DE20
TVIPS 4K CMOS

FEI Tecnai Biotwin
TVIPS 4K CMOS

JEOL 1230
Gatan US4000 CCD

FEI Helios 650
Quorum cryostage

ON THE FLY DATA PIPELINE



EXAMPLE: SINGLE-PARTICLE WORKFLOW

During EM session

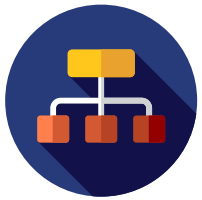
After EM session

Leginon session

- Setting up workflow
- Data acquisition
- Workflow Optimization *if needed*

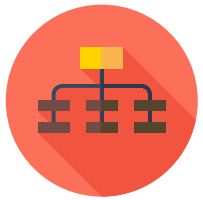
Appion session

- Frame alignment
- CTF estimation
- Particle picking
- Micrograph/ Particle curating



SEMC computing

- Micrograph/ Particle sorting
- Initial 2D classification
- Initial model generation
- 3D refinement

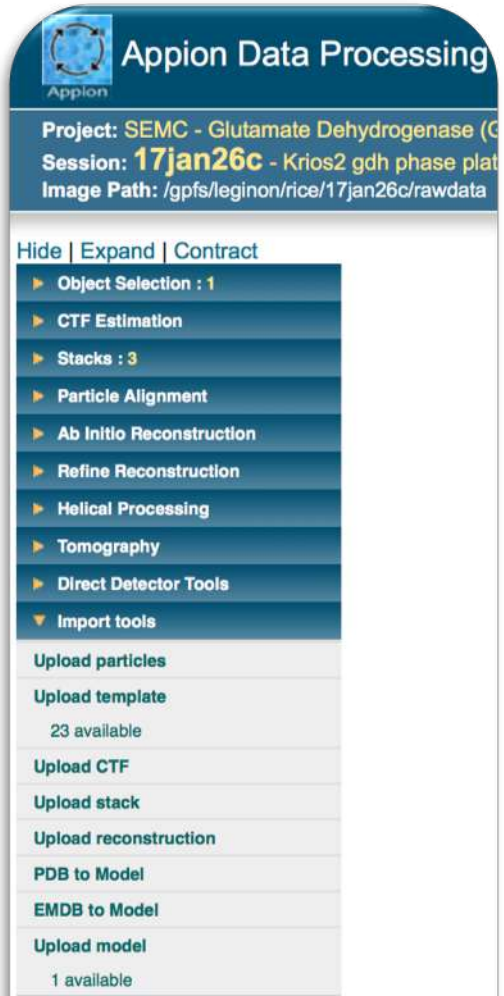


Home institution computing/cluster

- 2D classification
- 3D classification
- 3D refinement
- Model building

PROCESSING WORKFLOW

During EM session



Appion session

Frame alignment

CTF estimation

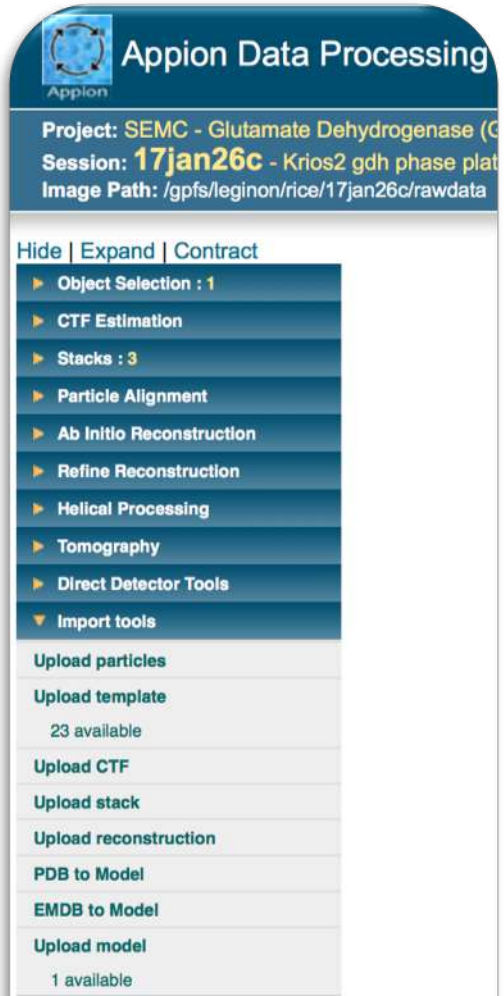
Particle picking

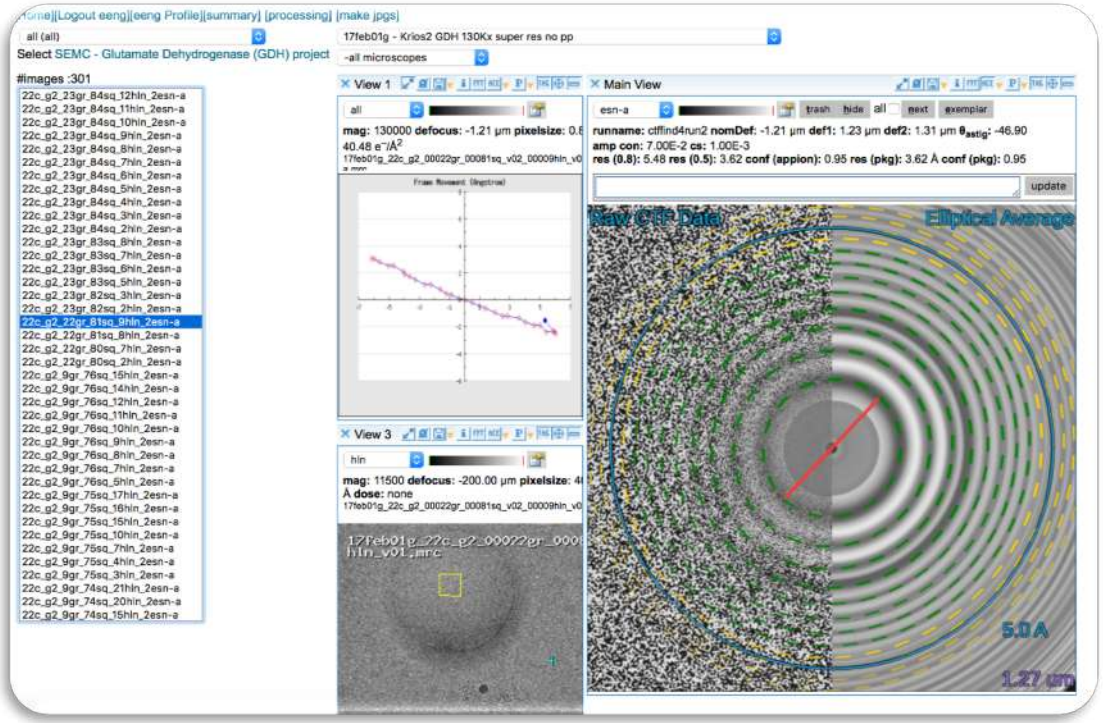
Micrograph/
Particle curating

PROCESSING WORKFLOW

During EM session

Leginon / Appion web interface

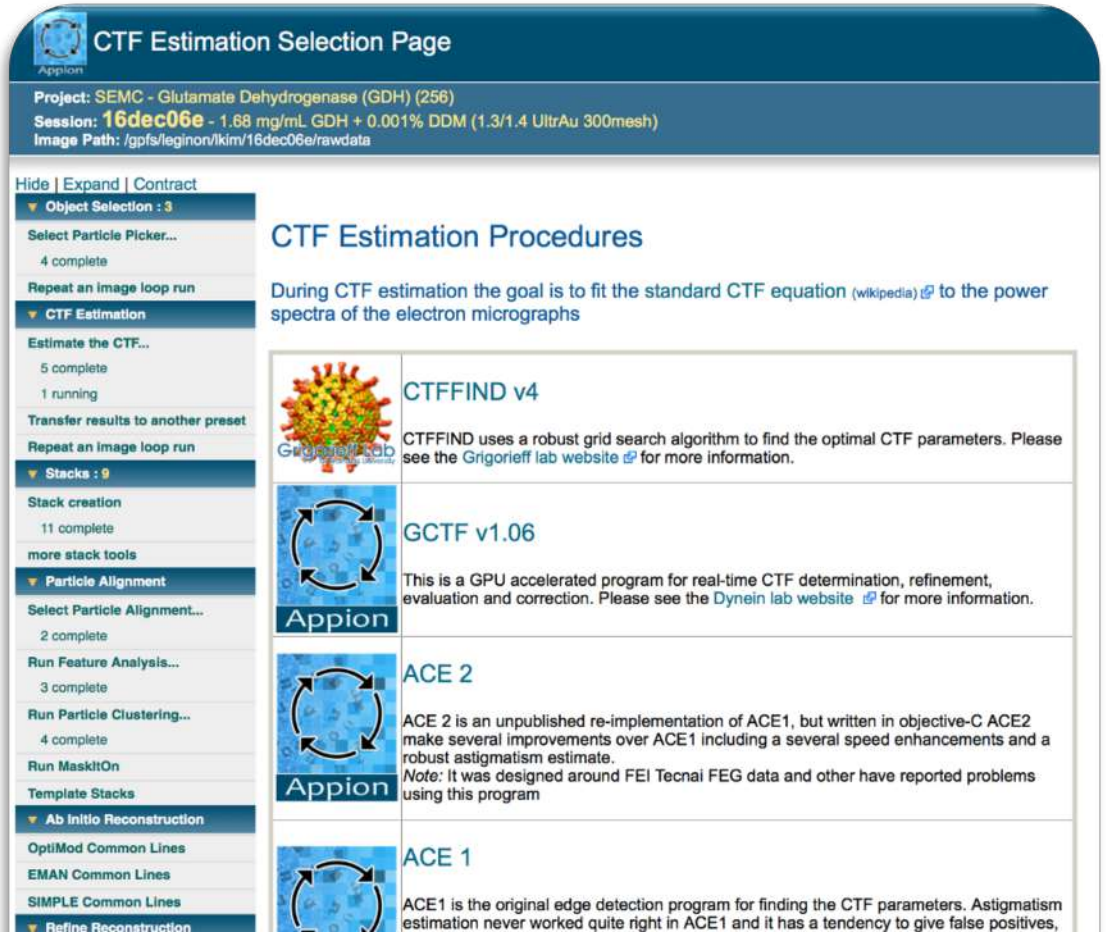




PACKAGES OFFERED

After EM session

Appion web interface



WHO DOES THE PROCESSING WORKFLOW?

After EM session

SEMC computing

Micrograph/
Particle sorting

Initial 2D classification

Initial model
generation

**Home institution
computing/cluster**

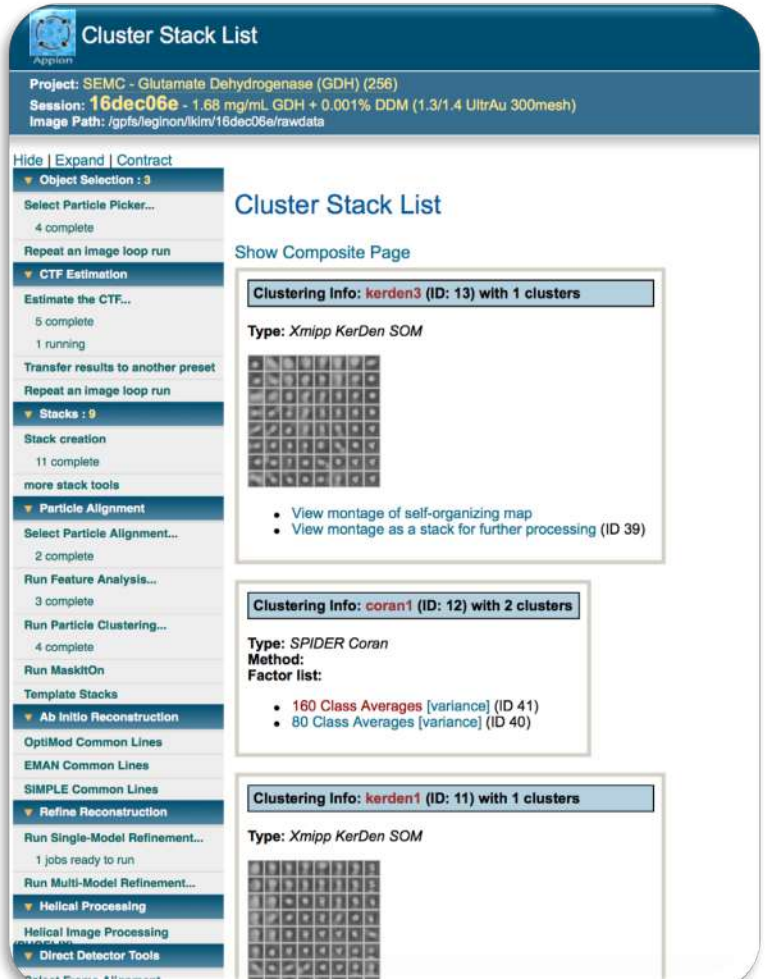
2D classification

3D classification

3D refinement

Model building

Appion web interface



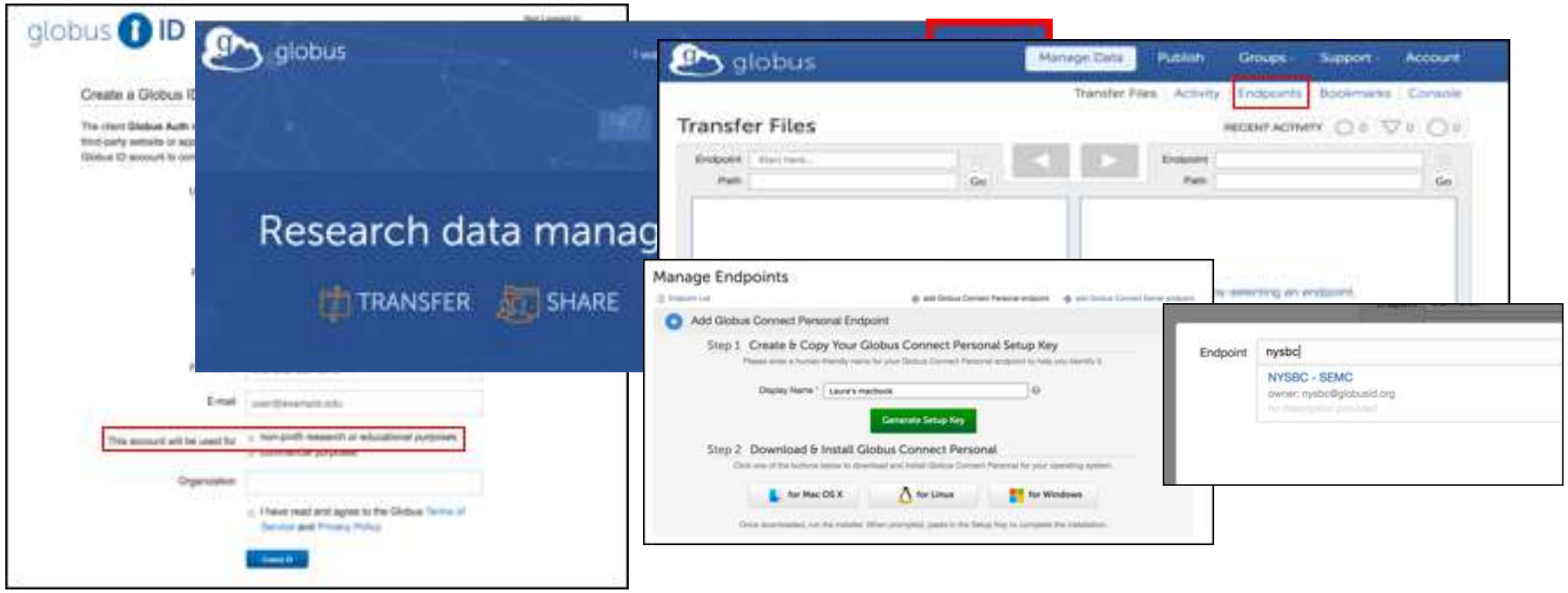
REMOTE DATA TRANSFER

Create an account at globusid.org

Login at globus.org

Transfer files

Remote data transfer



REMOTE DATA TRANSFER

Remote data transfer

/beegfs/frames/[username]/[session name]/

/beegfs/leginon/[username]/[session name]/

/beegfs/appion/[username]/[session name]/

SCHEDULE

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5. Other features: Pro tips / Direct Detector Tools / Import/Export Tools

6. Closing

Start your web browsers

nccatweb.nysbc.org

Login: nworkshop

Password: appion2020

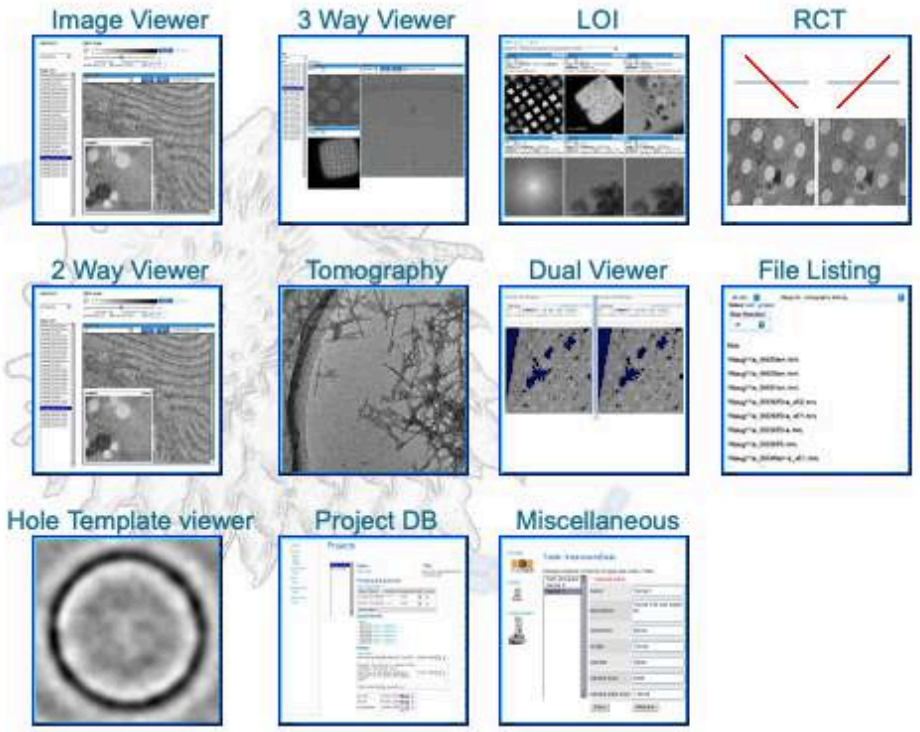
APPION & LEGINON WEBTOOLS

nccatweb.nysbc.org

[Logout nworkshop][nworkshop Profile]

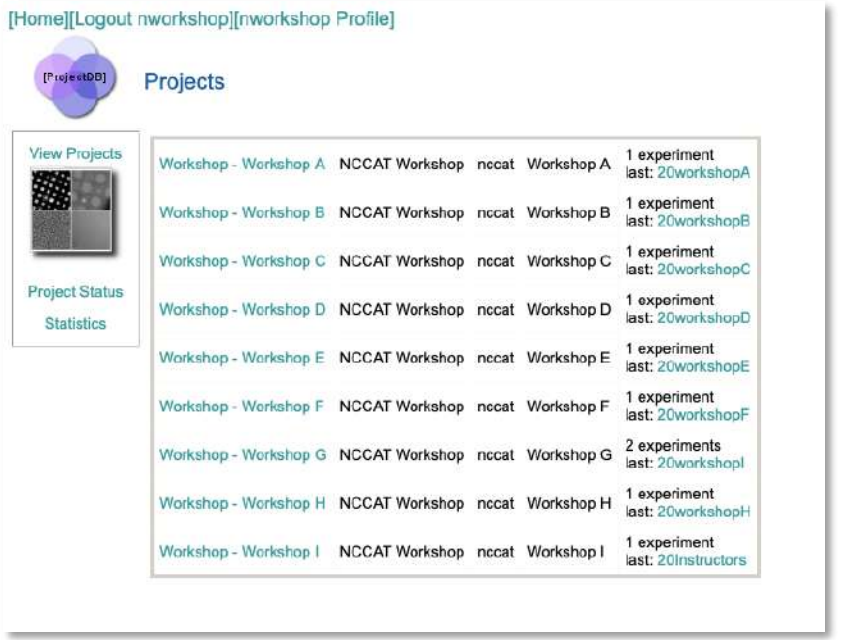
Appion and Leginon DB Tools

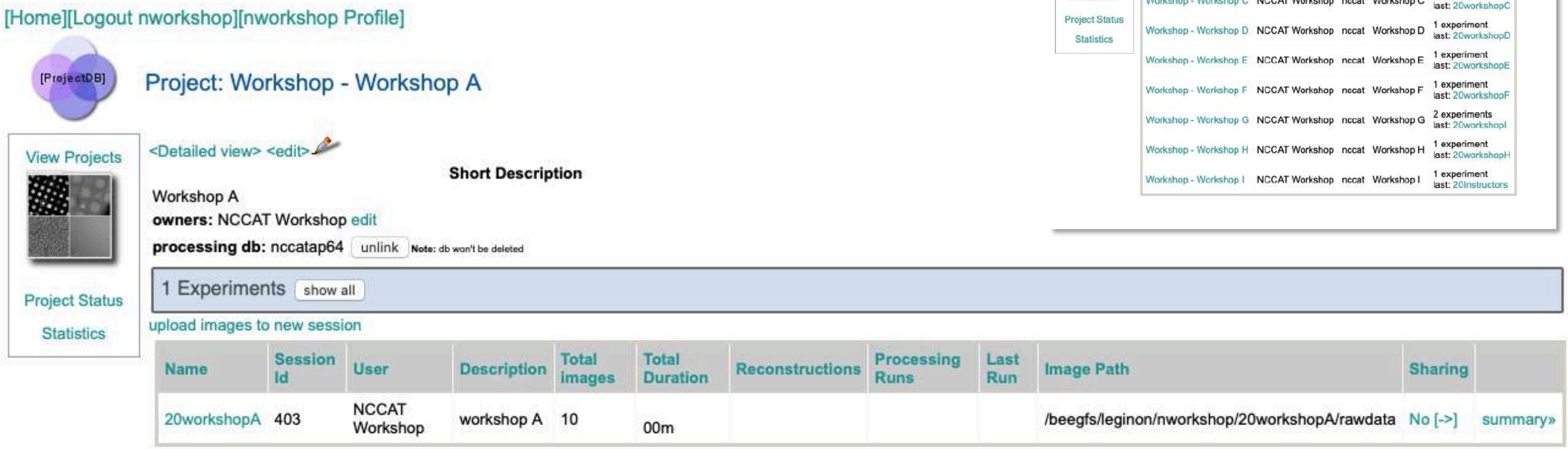
[test Dataset]



APPION & LEGINON WEBTOOLS

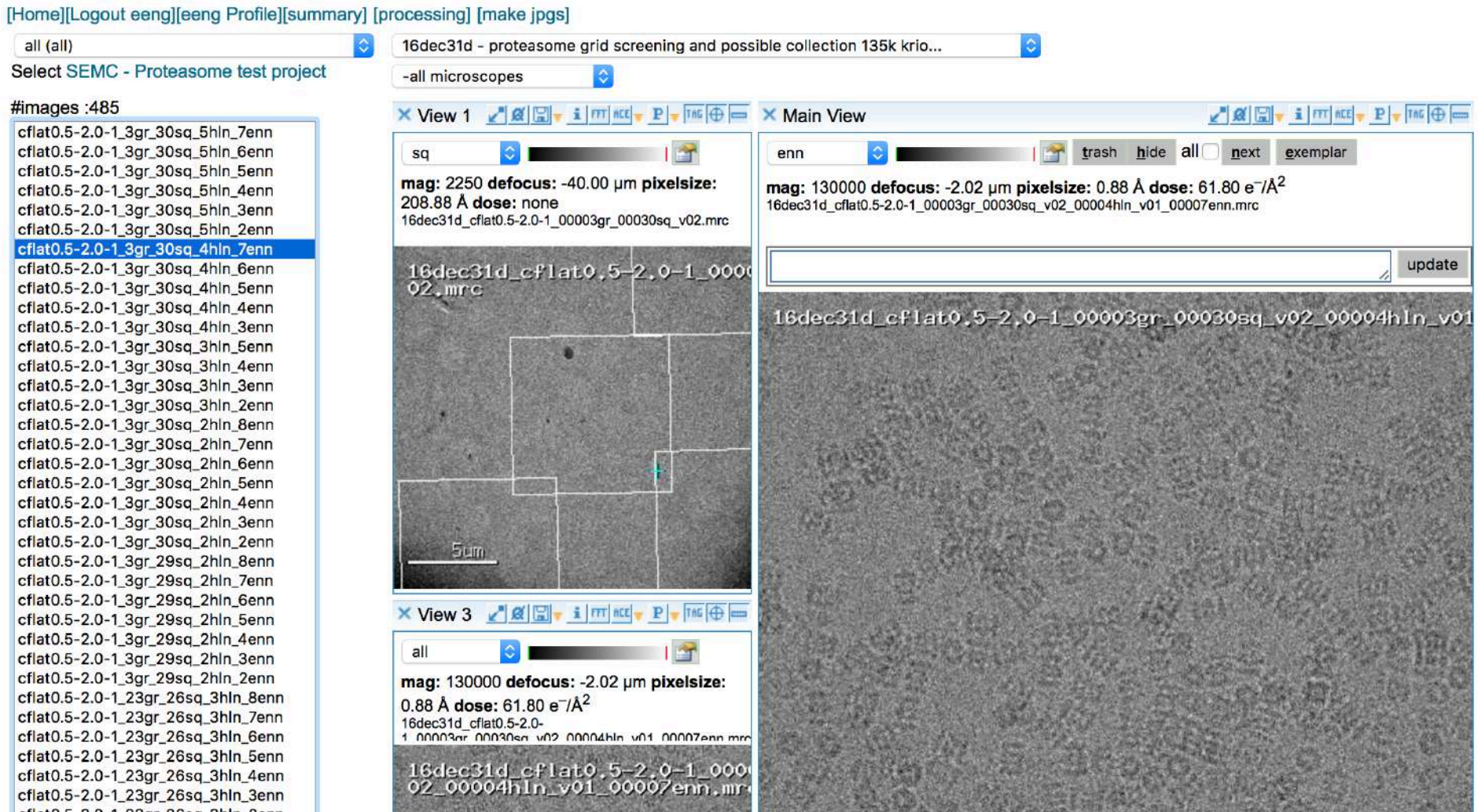
nccatweb.nysbc.org





APPION & LEGINON WEBTOOLS

nccatweb.nysbc.org



APPION & LEGINON WEBTOOLS

nccatweb.nysbc.org

SEMC Operations
Krios1
proteasome 29k k2 versus falcon!!!! (krios1 k2)

Session
18apr26e

TFS
Titan Krios

Gatan
K2 Summit
Direct Detector

Imaging Summary

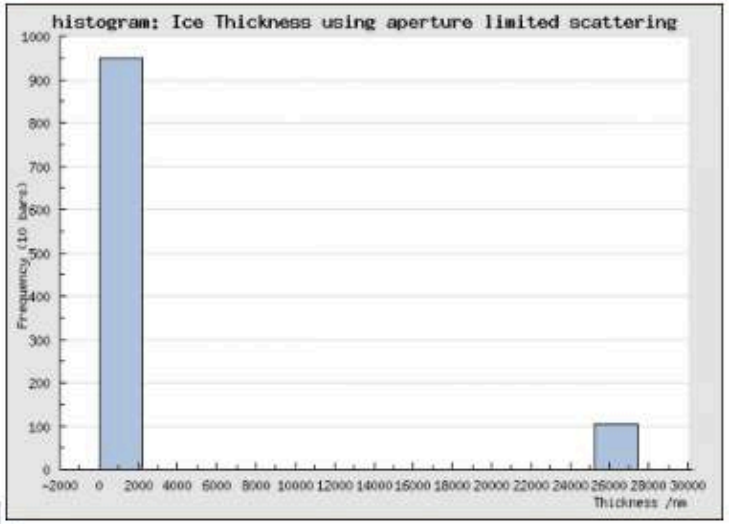
Preset label	Mag (X)	Dose ($e^-/Å^2$)	Pixel size (Å)	Dimensions	Binning	Image count
gr	100		3,327.6	1024x1024	4	24
sq	400	0.002	246.8	927x959	4	59
hln	1300	0.024	72.8	924x924	4	277
enn2	22500	46.473	1.073	3710x3838	1	952
enn2-a	22500	46.473	1.073	3710x3838	1	952
enn2-a-DW	22500	46.473	1.073	3710x3838	1	952
enn	29000	60.677	0.832	3710x3838	1	105
enn-a	29000	60.677	0.832	3710x3838	1	105
enn-a-DW	29000	60.677	0.832	3710x3838	1	105

Total images: 3531 **Duration:** 17 hrs, 36 min

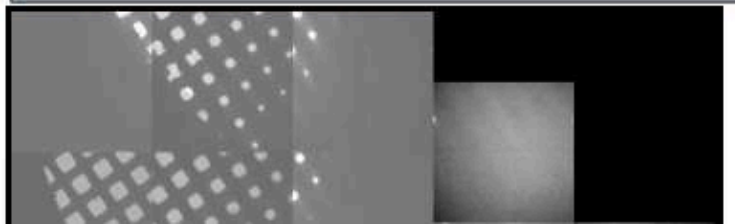
Defocus summary

	nb	min	max	avg	stddev
defocus1	1057	-0.28 µm	3.95 µm	1.75 µm	0.58 µm
defocus2	1057	-0.10 µm	4.01 µm	1.81 µm	0.61 µm
angle_astigmatism	1057	-89.891	89.370	-34.363	33.837
extra_phase_shift	1057	0	0	0	0
resolution_80_percent	1057	4.26 Å	100.00 Å	16.05 Å	24.24 Å

Ice thickness summary



Atlas



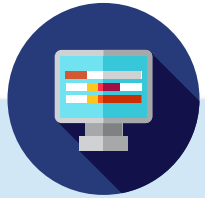
COMPUTATIONAL RESOURCES

Web resources

nccatweb.nysbc.org

emg.nysbc.org/ redmine

nccat.nysbc.org



Cluster resources

NCCAT workq cluster

On site resources

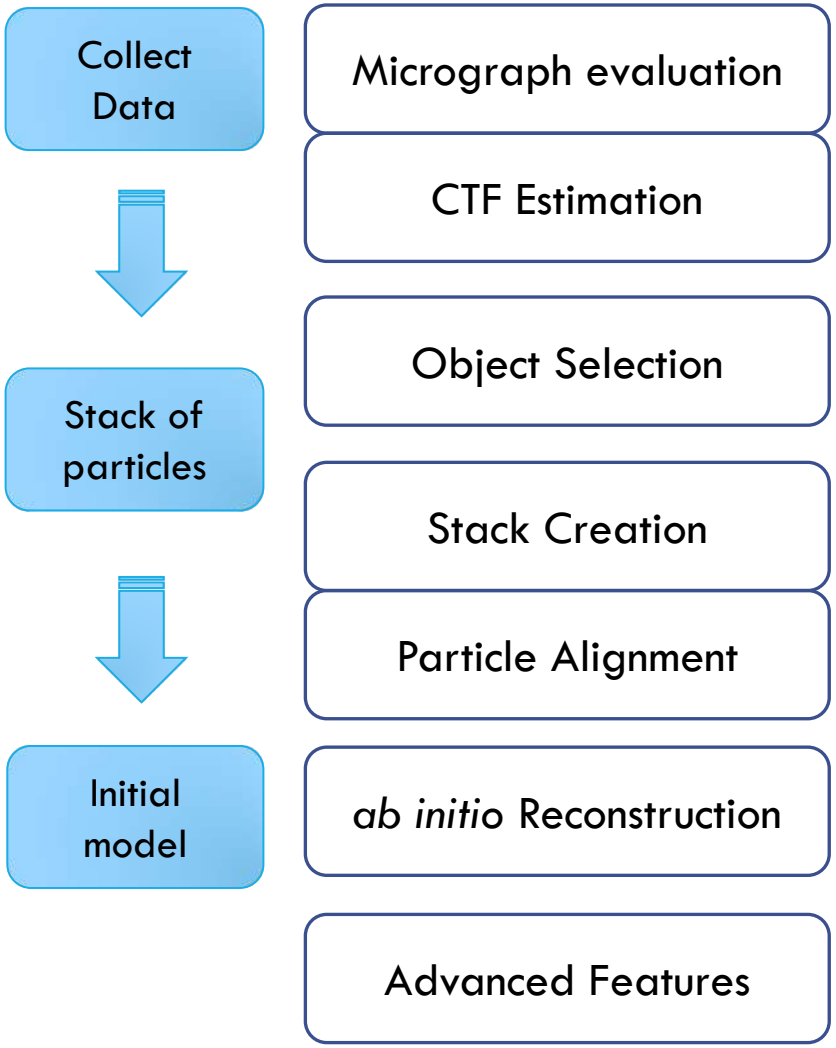
EM session user

Leginon / support workstations

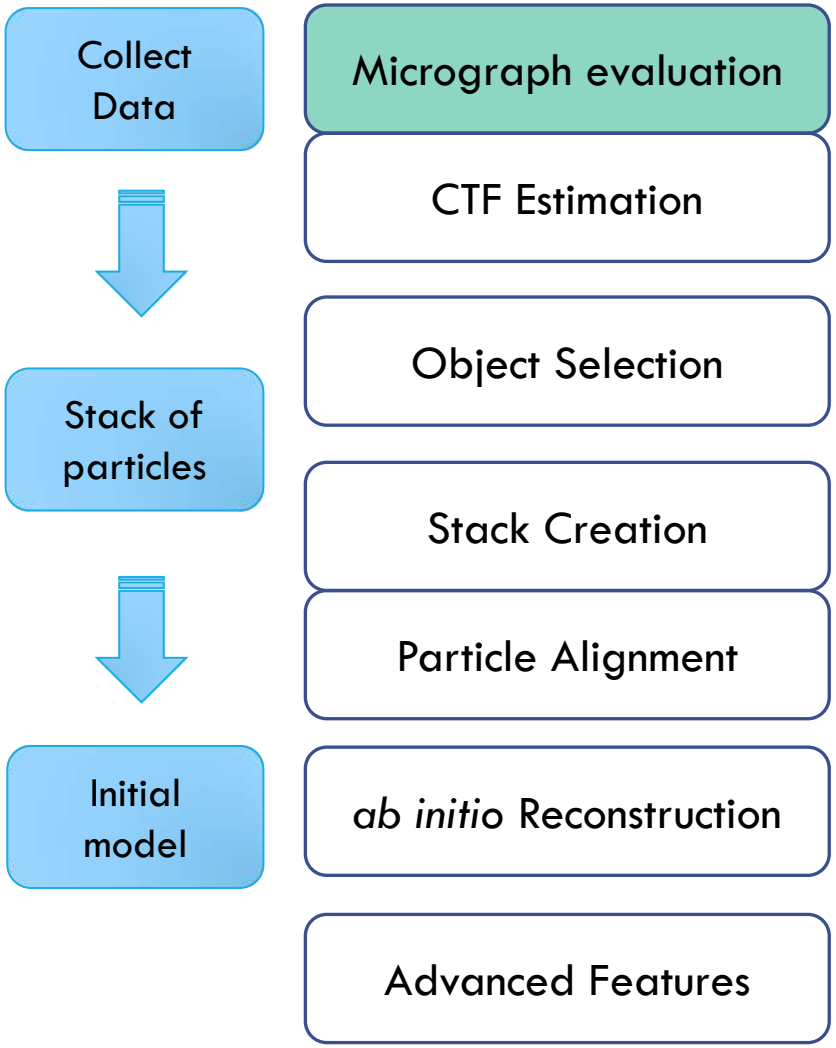
GPU workstations

Data transfer computers

SCHEDULE


Collect Data
Micrograph evaluation
CTF Estimation
Object Selection
Stack of particles
Stack Creation
Particle Alignment
Initial model
ab initio Reconstruction
Advanced Features

SCHEDULE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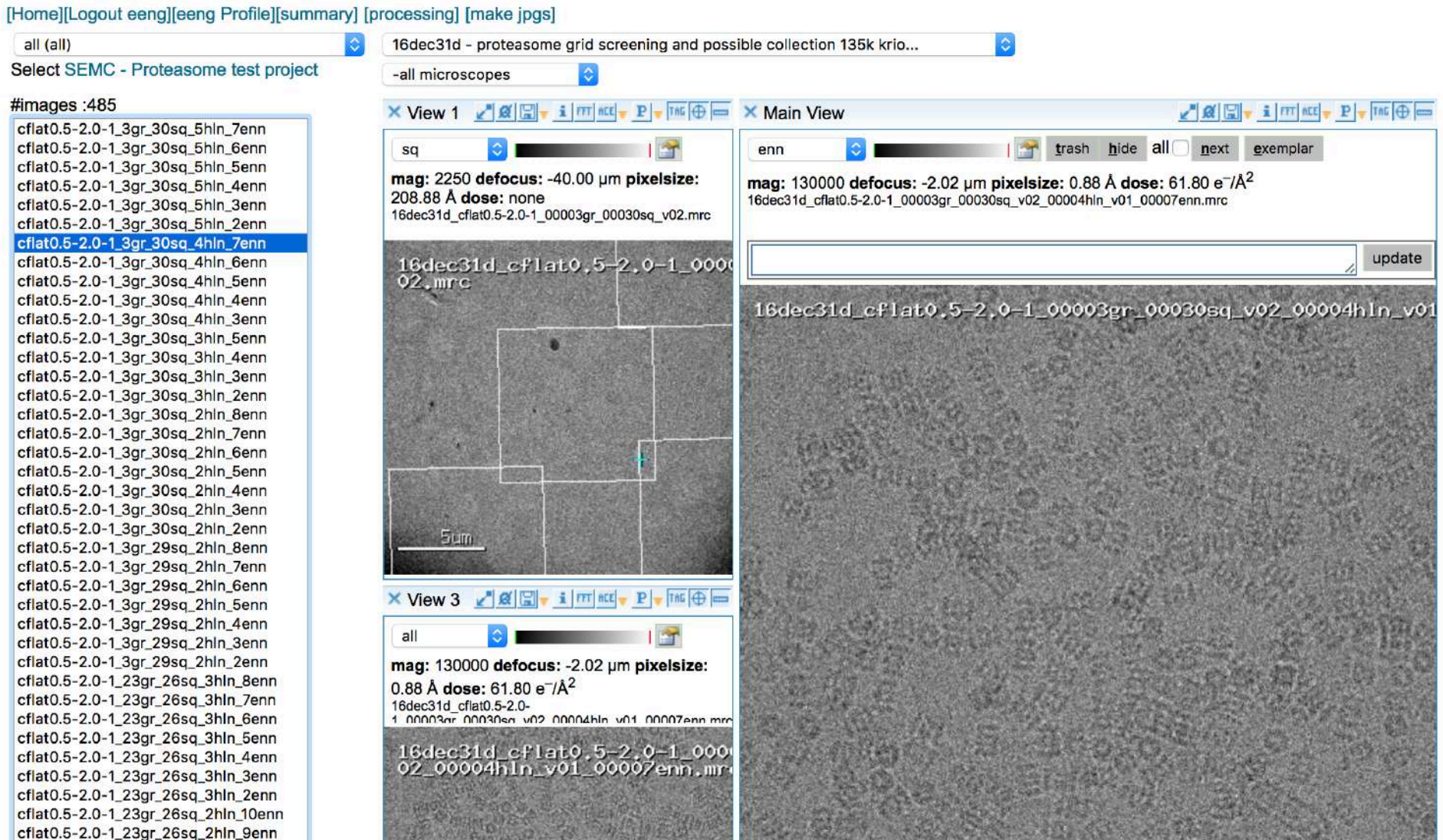
MICROGRAPH EVALUATION



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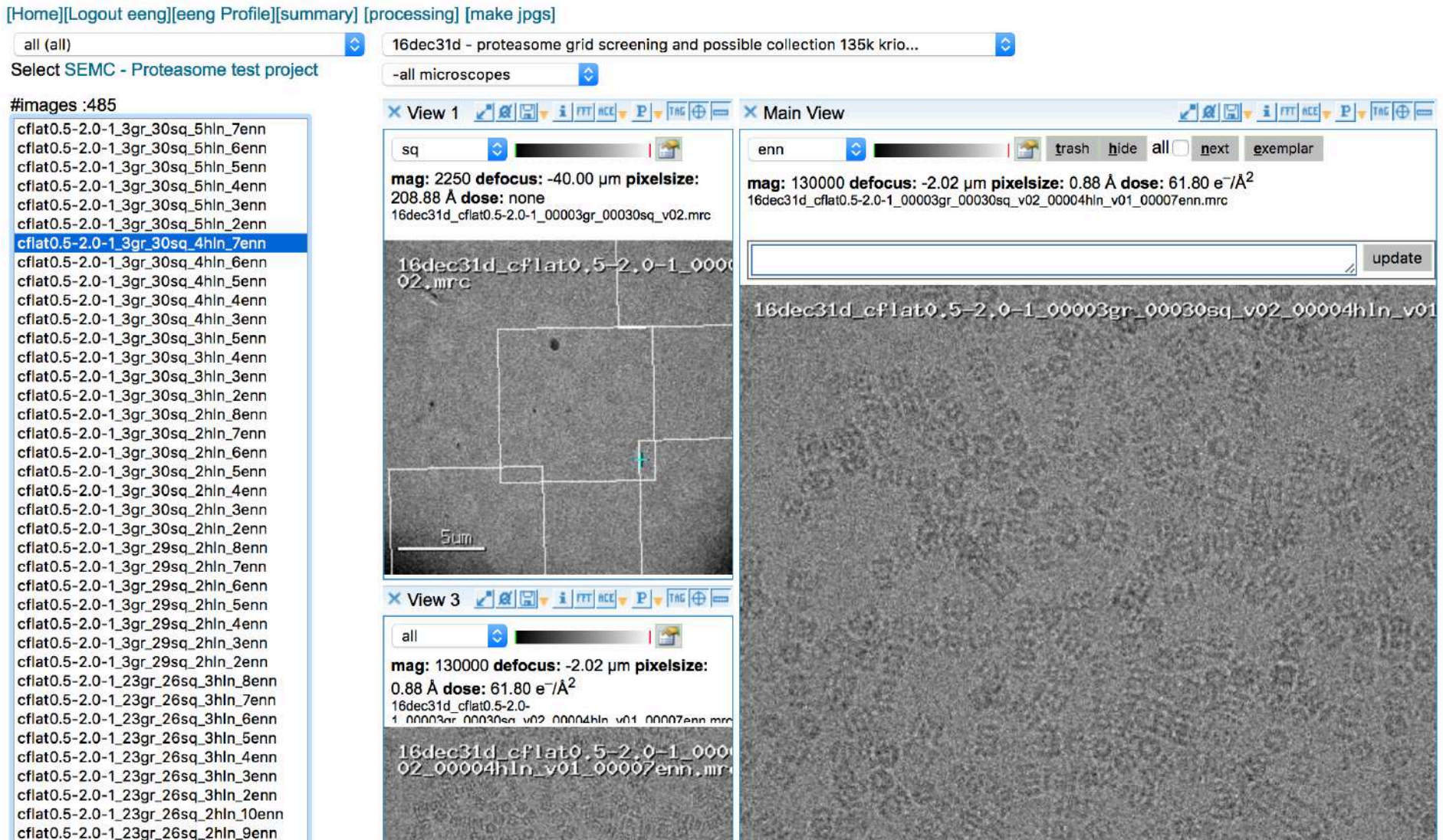

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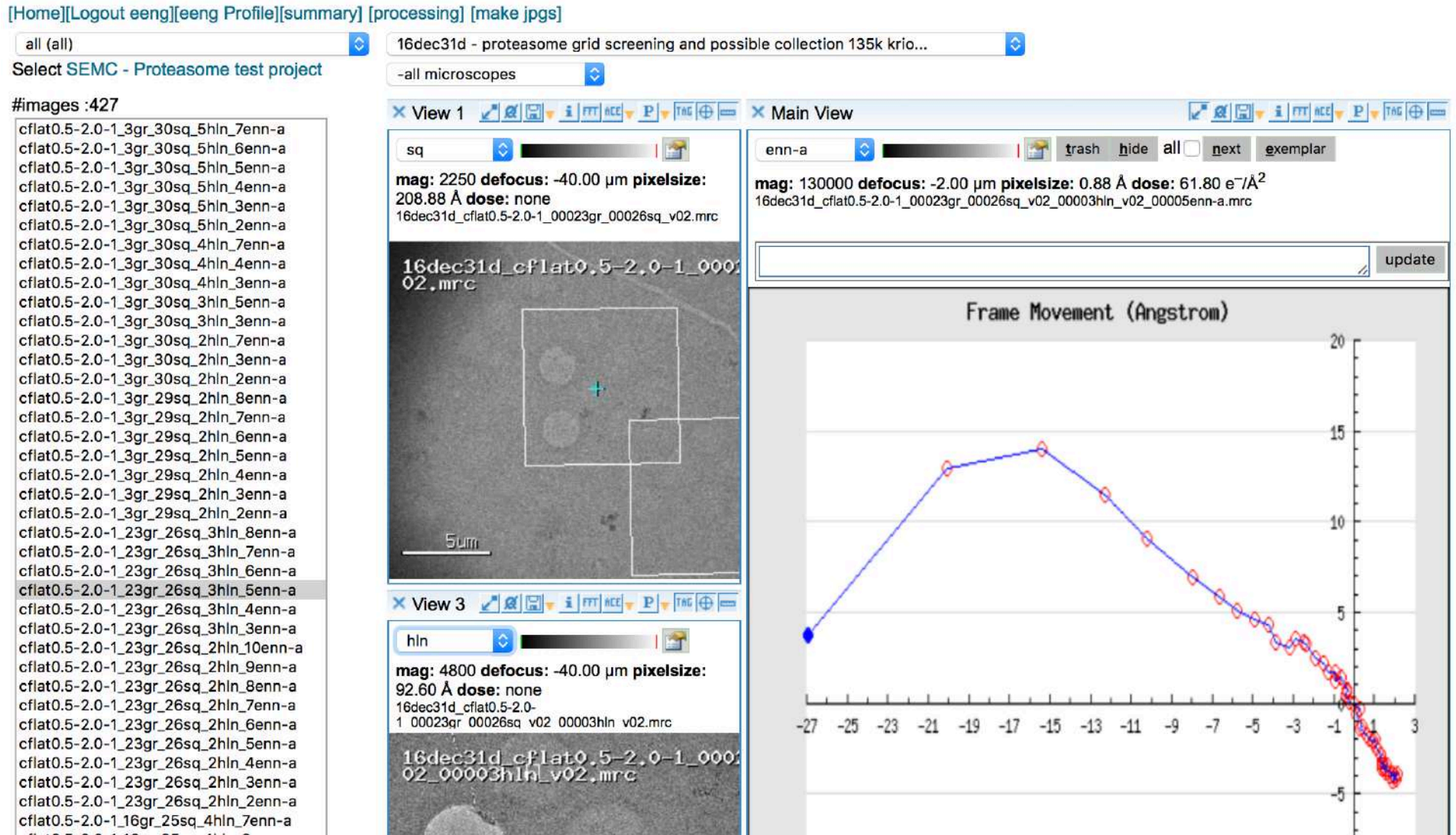
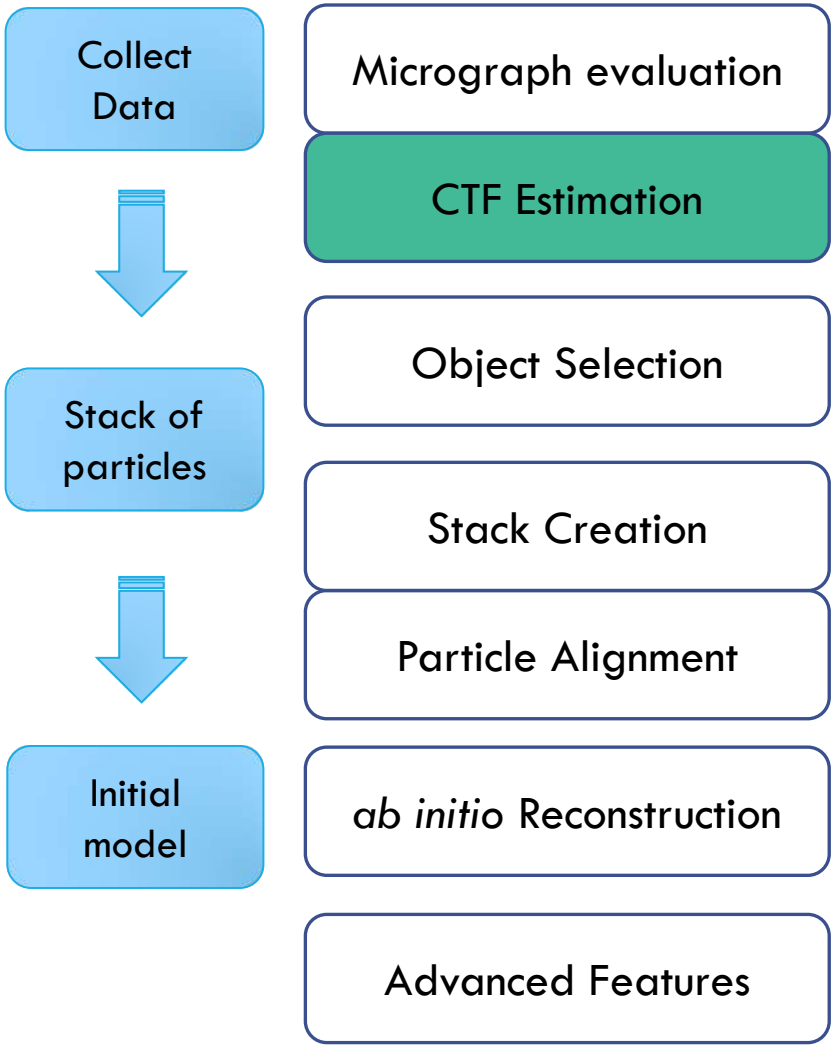


SCHEDULE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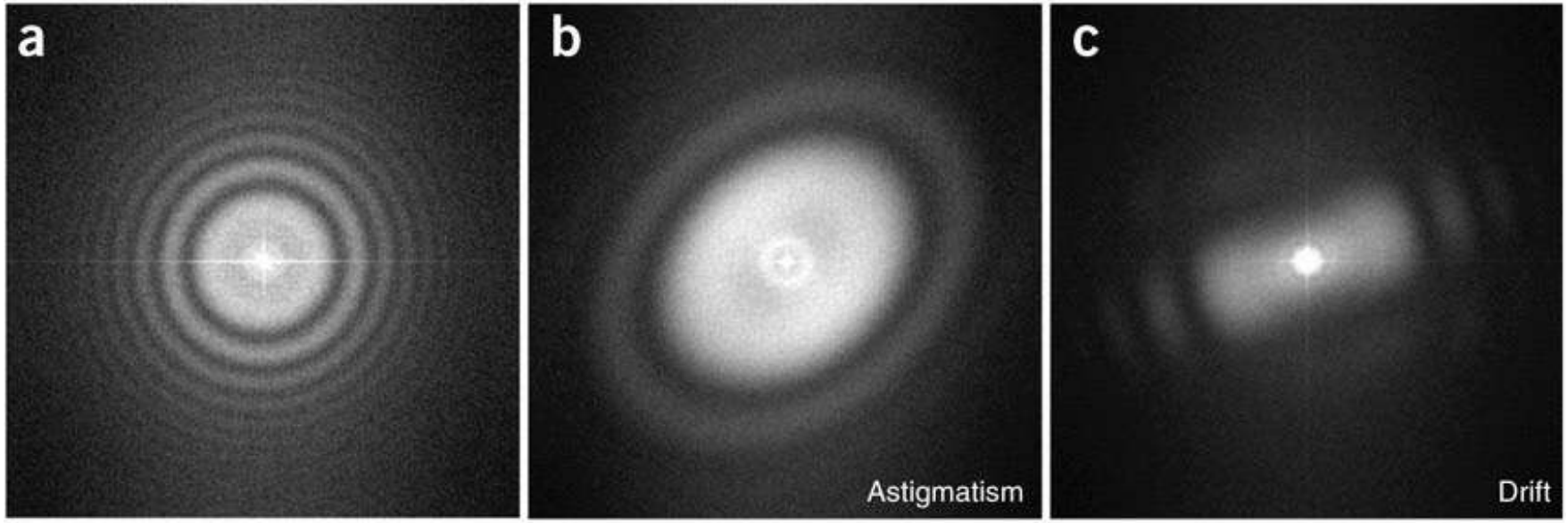
CTF

Contrast**T**ransfer**F**unction

CTF is the function which modulates the amplitudes and phase of an electron beam in a TEM

A recorded image can be regarded as a CTF degraded true object and allows for CTF correction to restore that image

CTF: WHY IS MONITORING THE CTF IMPORTANT IN OUR DATA COLLECTION?



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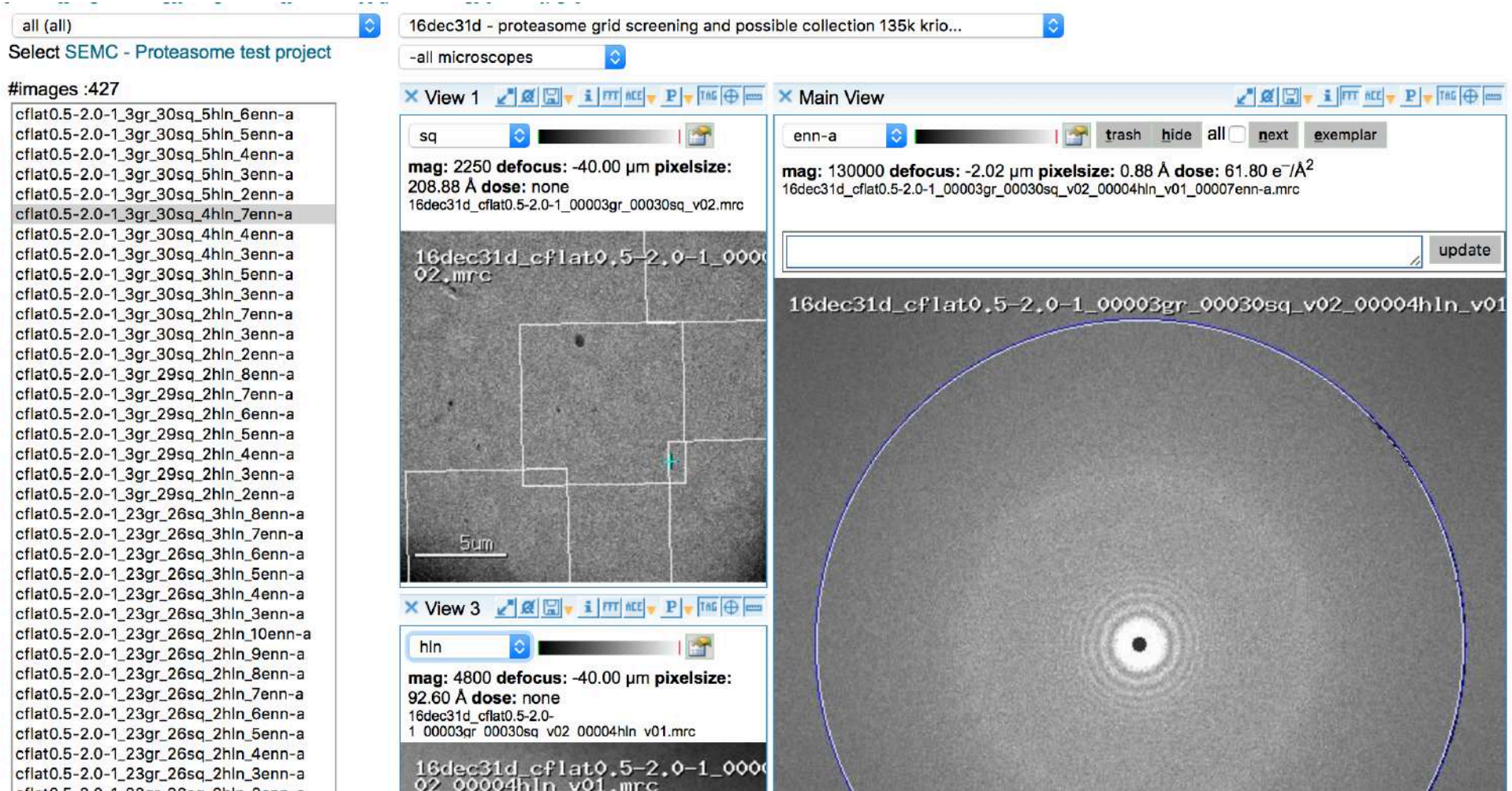

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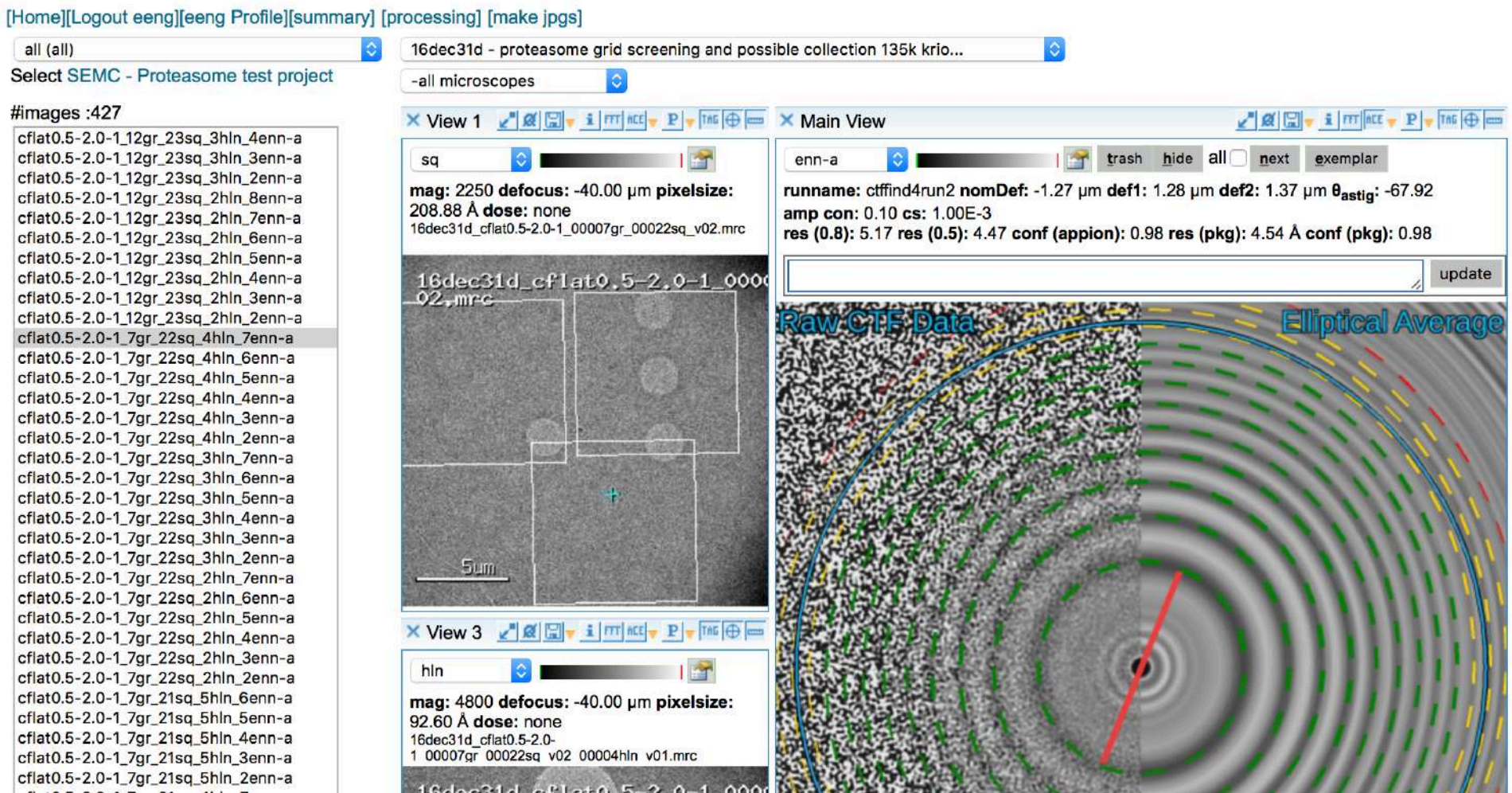

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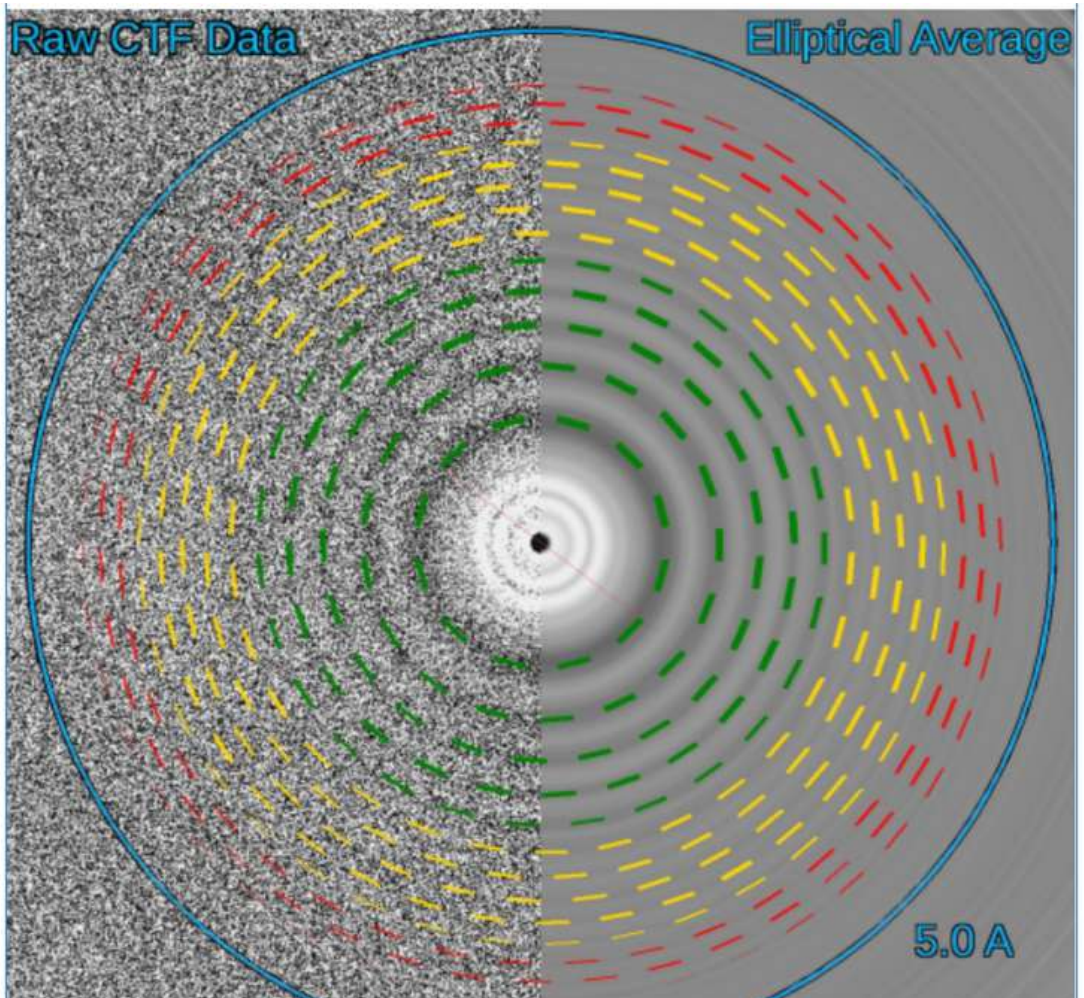

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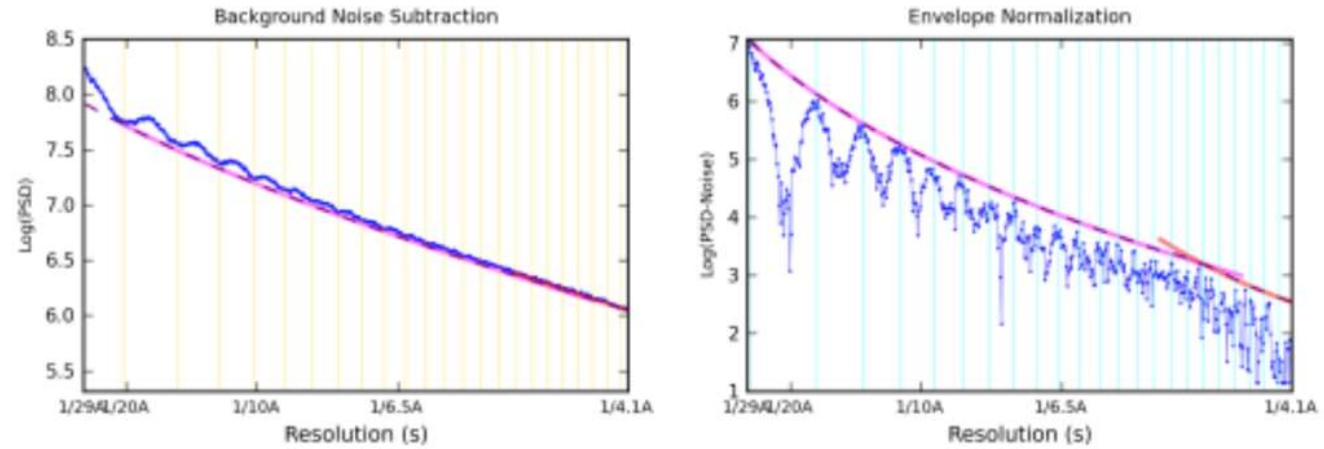
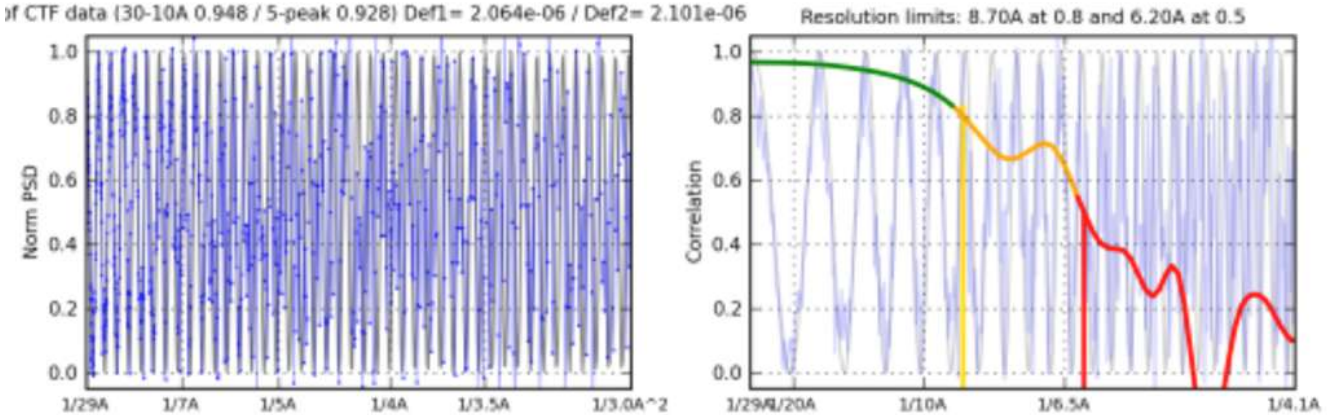
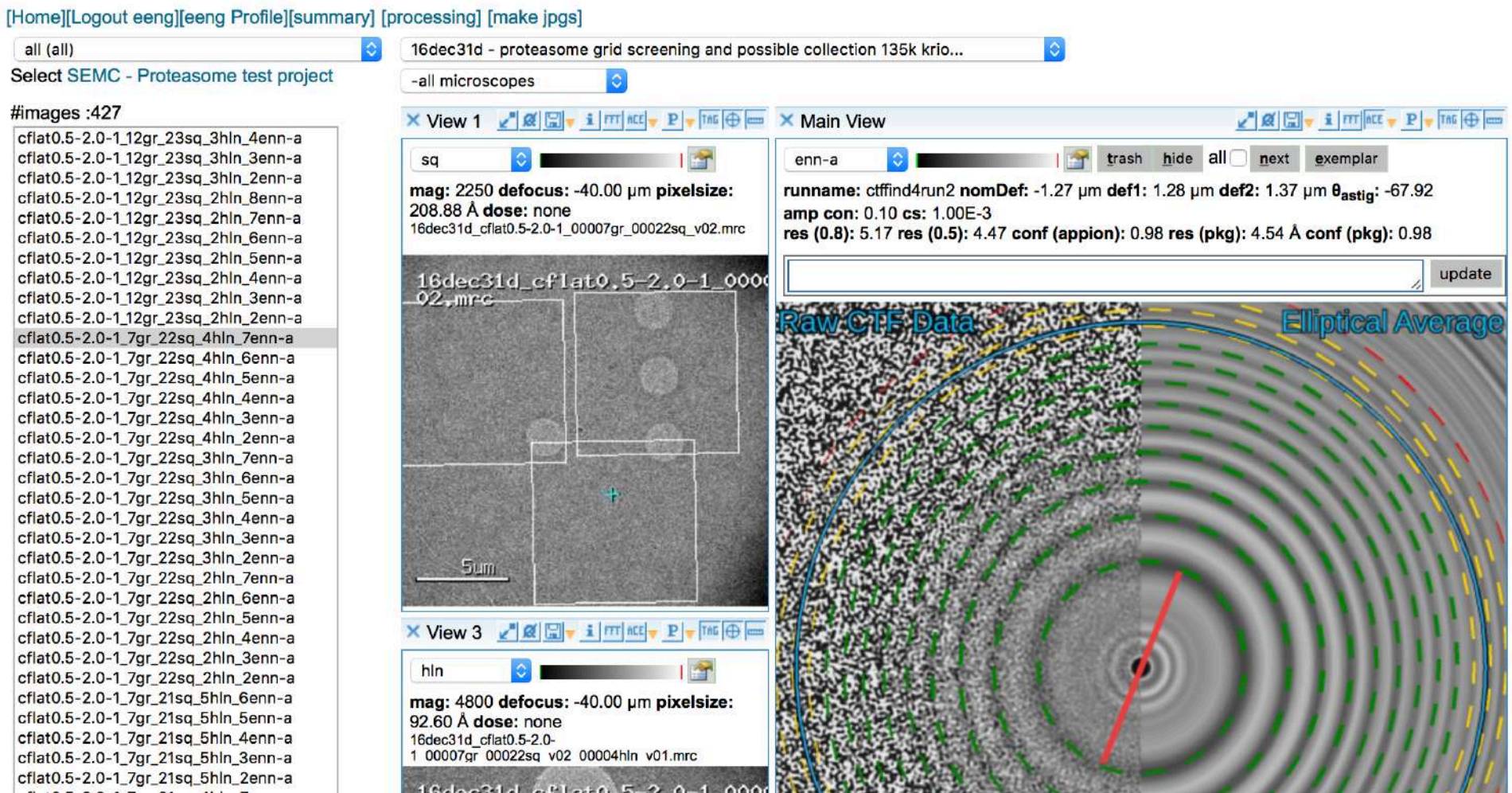
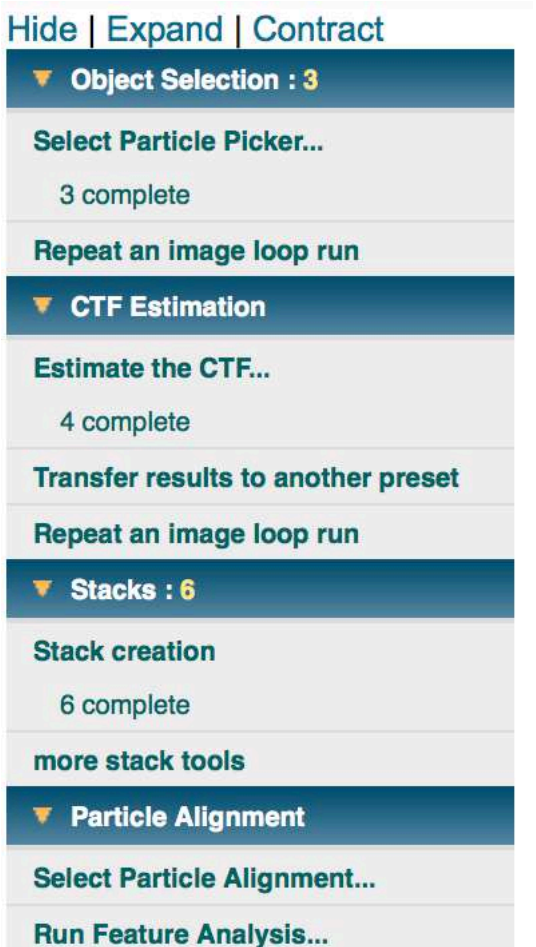




CTF: WHY IS MONITORING THE CTF IMPORTANT IN OUR DATA COLLECTION?



CTF: HOW TO RUN A JOB.



nccatweb.nysbc.org

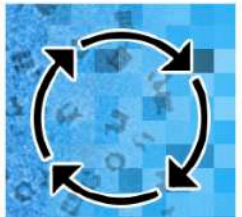
Login: nworkshop

Password: appion2020

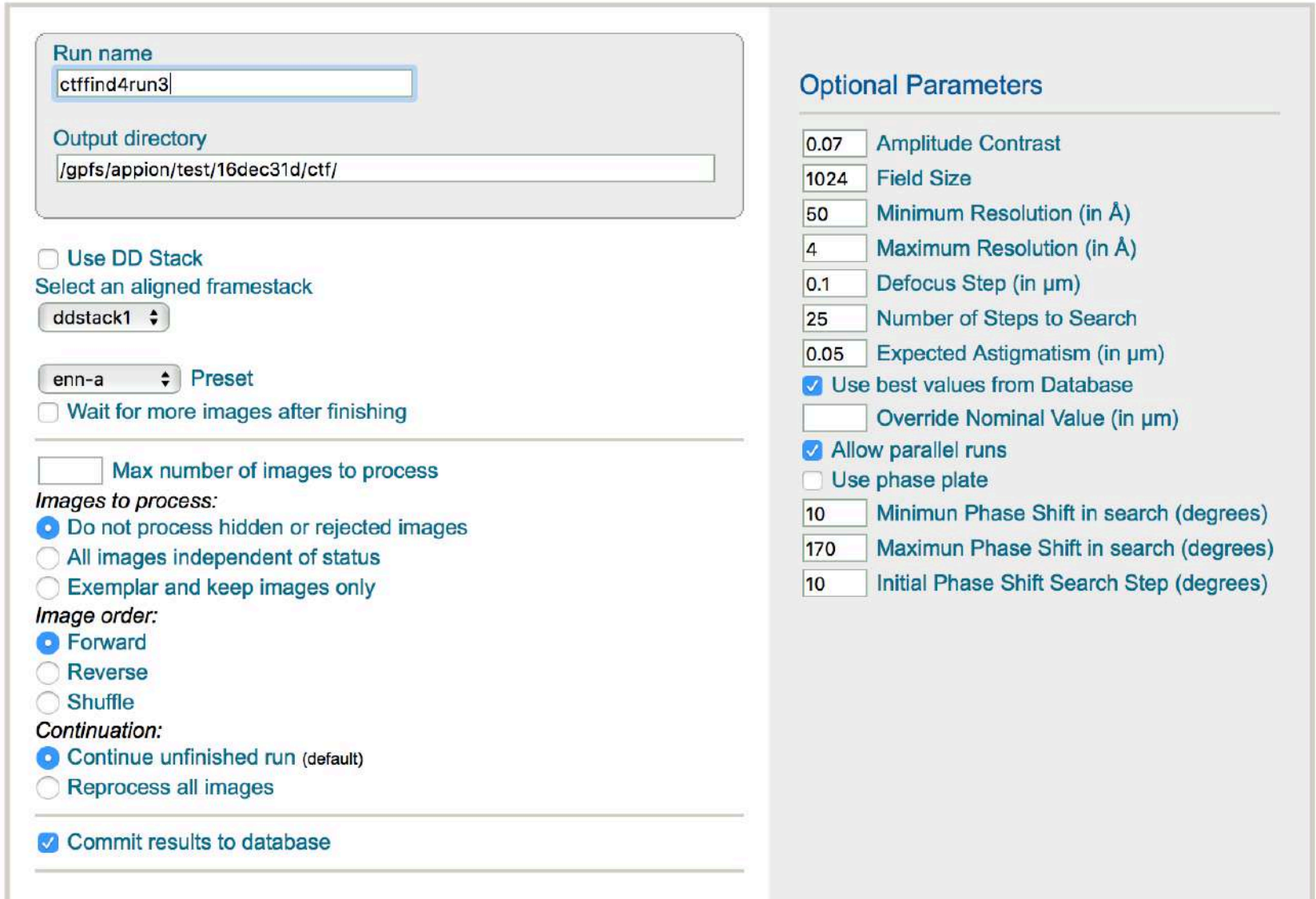
CTF: HOW TO RUN A JOB.

CTF Estimation Procedures

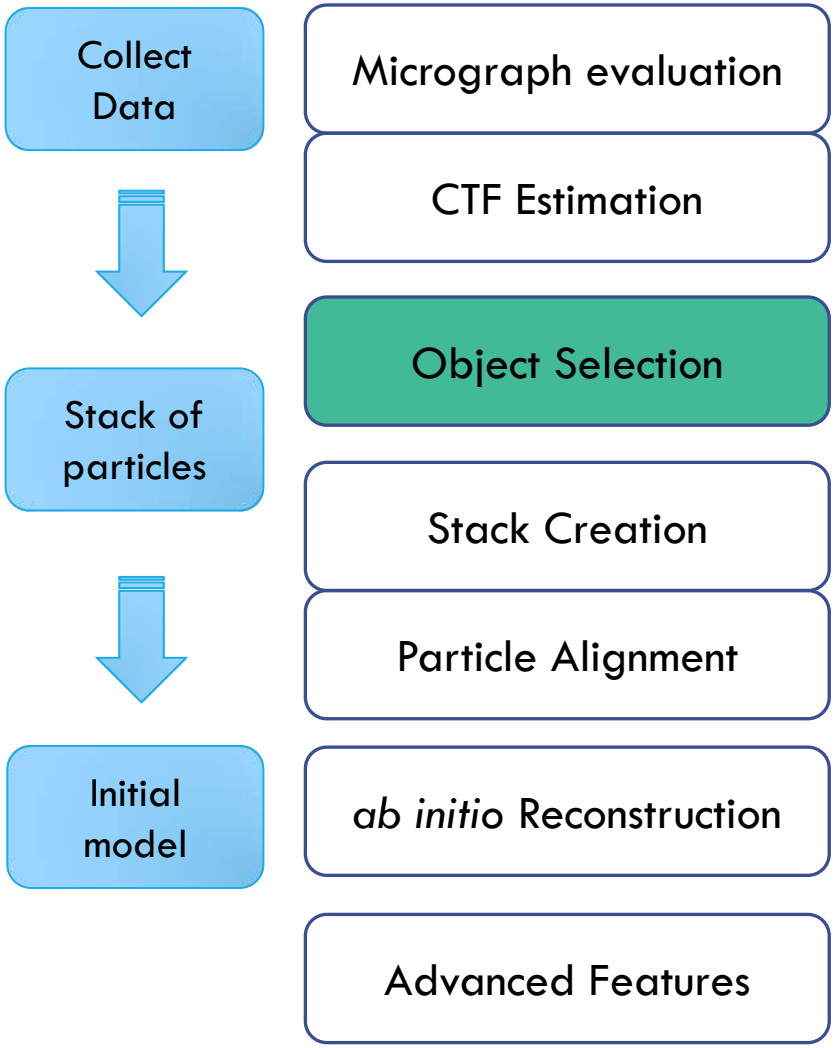
During CTF estimation the goal is to fit the standard CTF equation (wikipedia) to the power spectra of the electron micrographs

	CTFFIND v4 CTFFIND uses a robust grid search algorithm to find the optimal CTF parameters. Please see the Grigorieff lab website for more information.
	GCTF v1.06 This is a GPU accelerated program for real-time CTF determination, refinement, evaluation and correction. Please see the Dynein lab website for more information.
	**ACE 2** ACE 2 is an unpublished re-implementation of ACE1, but written in objective-C ACE2 make several improvements over ACE1 including a several speed enhancements and a robust astigmatism estimate.

CTF: HOW TO RUN A JOB.



SCHEDULE


Collect Data
Stack of particles
Initial model
Micrograph evaluation
CTF Estimation
Object Selection
Stack Creation
Particle Alignment
ab initio Reconstruction
Advanced Features

PARTICLE PICKING

1. **Initial Picking**

   DoG Picking

   - Unbiased and rough

   Manual Picking

   - Time consuming, possible bias, can be more accurate

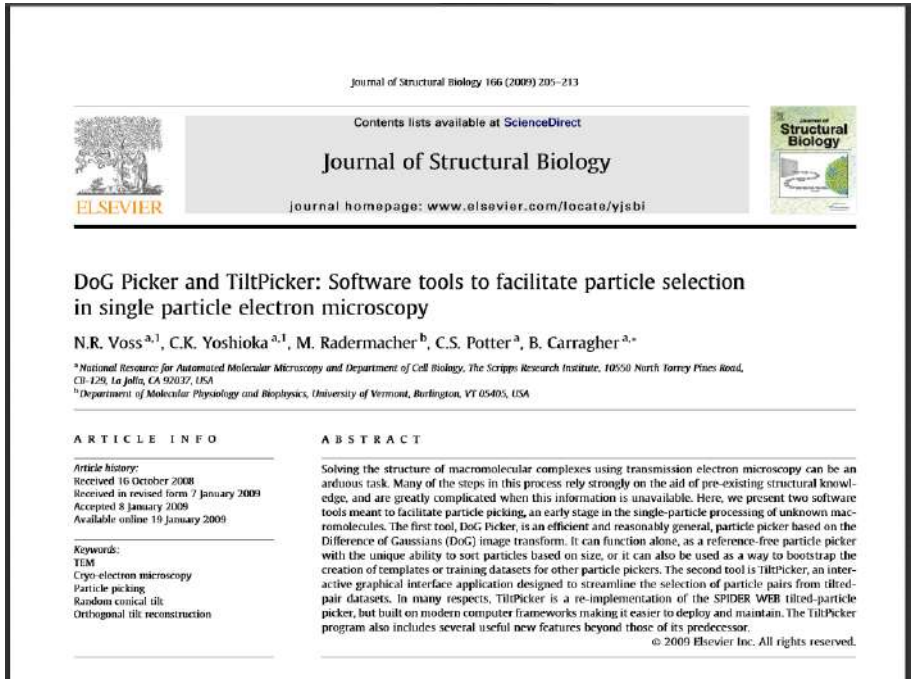
2. Make a stack, 2D alignment, generate good class averages

3. **Template Picking**

   - Based on generated class averages

PARTICLE PICKING

Difference of Gaussian (DoG) Picker



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DoG Picker and TiltPicker: Software tools to facilitate particle selection in single particle electron microscopy

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ABSTRACT

Solving the structure of macromolecular complexes using transmission electron microscopy can be an arduous task. Many of the steps in this process rely strongly on the aid of pre-existing structural knowledge, and are greatly complicated when this information is unavailable. Here, we present two software tools meant to facilitate particle picking, an early stage in the single-particle processing of unknown macromolecules. The first tool, DoG Picker, is an efficient and reasonably general, particle picker based on the Difference of Gaussians (DoG) image transform. It can function alone, as a reference-free particle picker with the unique ability to sort particles based on size, or it can also be used as a way to bootstrap the creation of templates or training datasets for other particle pickers. The second tool is TiltPicker, an interactive graphical interface application designed to streamline the selection of particle pairs from tilted-pair datasets. In many respects, TiltPicker is a re-implementation of the SPIDER WEB tilted-particle picker, but built on modern computer frameworks making it easier to deploy and maintain. The TiltPicker program also includes several useful new features beyond those of its predecessor.

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PARTICLE PICKING

Difference of Gaussian (DoG) Picker

Run name
dogrun1

Output directory
/gpfs/appion/test/15workshopH/extract

Preset: upload
☐ Wait for more images after finishing

Max number of images to process
Images to process:
◉ Do not process hidden or rejected images
○ All images independent of status
○ Exemplar and keep images only
Image order:
◉ Forward
○ Reverse
○ Shuffle
Continuation:
◉ Continue unfinished run (default)
○ Reprocess all images

☑ Commit results to database

Particle Diameter:
Particle diameter for filtering *(in Angstroms)*

Peak thresholds:
0.7 Minimum threshold
1.5 Maximum threshold
1500 Max number of particles allowed per image

Filter Values:
15 Low Pass *(in Angstroms; 0 = off)*
0 High Pass *(in Angstroms; 0 = off)*
2 Median *(in pixels; 0 = off)*
4.0 Pixel Limit *(in Standard Deviations; 0 = off)*
4 Binning *(power of 2)*
☑ Plane regression

Defocal pairs:
☐ Calculate shifts for defocal pairs

Advanced options:
1.0 Maximum peak area multiple
1.5 Minimum peak overlap distance multiple
Peak extraction type
◉ Center of mass *(default)*
○ Maximum position
☐ Pick only doubles
☐ Invert image density
K-factor *(sloppiness)*

Multi-scale dogpicker:
Number of Slices *(number of sizes)*
Size Range *(in Angstroms)*

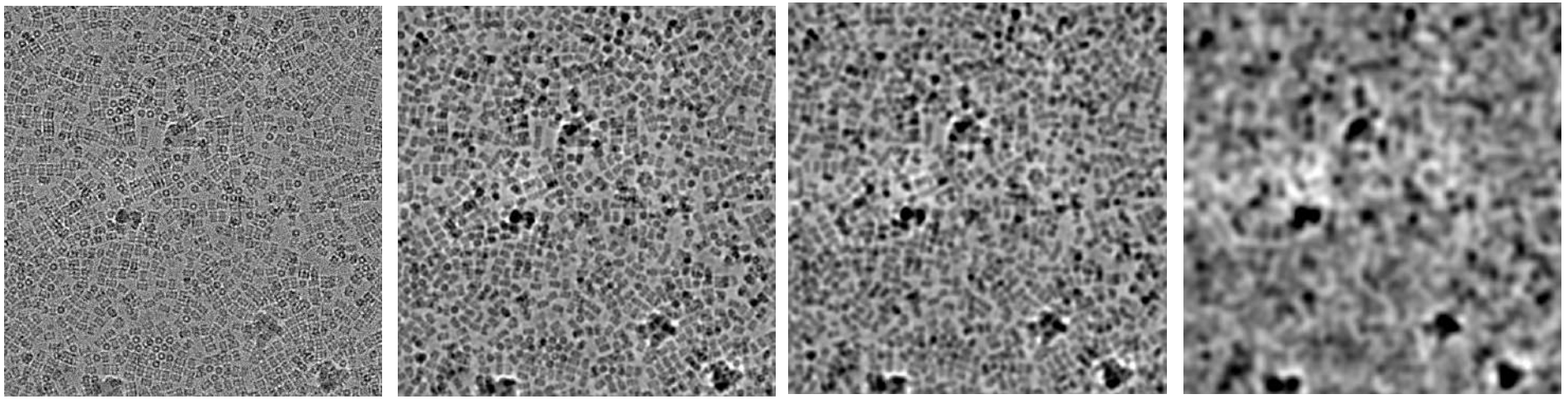
☐ Test these settings on image: mrc file name

Just Show Command

PARTICLE PICKING

Difference of Gaussian (DoG) Picker

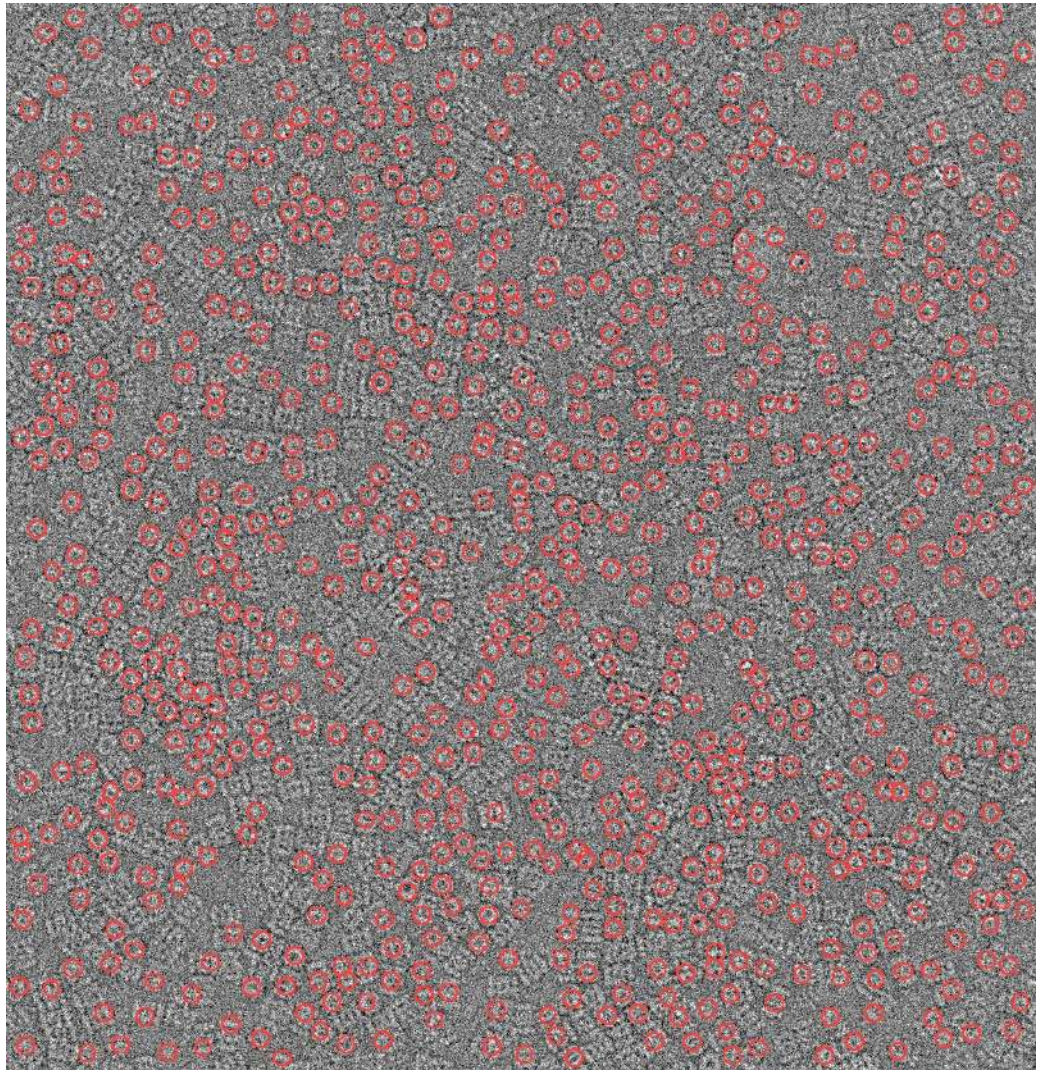
Low Pass Filtering



0 Å 20 Å 40 Å 60 Å

PARTICLE PICKING

Difference of Gaussian (DoG) Picker



Particle Diameter:
80 Particle diameter for filtering (in Angstroms)

Peak thresholds:
0.4 Minimum threshold
1.5 Maximum threshold
1500 Max number of particles allowed per image

4 Binning (power of 2)

Filter Values:
15 Low Pass (in Angstroms; 0 = off)
0 High Pass (in Angstroms; 0 = off)
2 Median (in pixels; 0= off)
4 Pixel Limit (in Standard Deviations; 0 = off)
☑ Plane regression

Defocal pairs:
☐ Calculate shifts for defocal pairs

Image Contrast:
○ White Particles (i.e. negatively stained)
◉ Black Particles (i.e. ice embedded)

Peak extraction type:
◉ Center of mass (default)
○ Maximum position

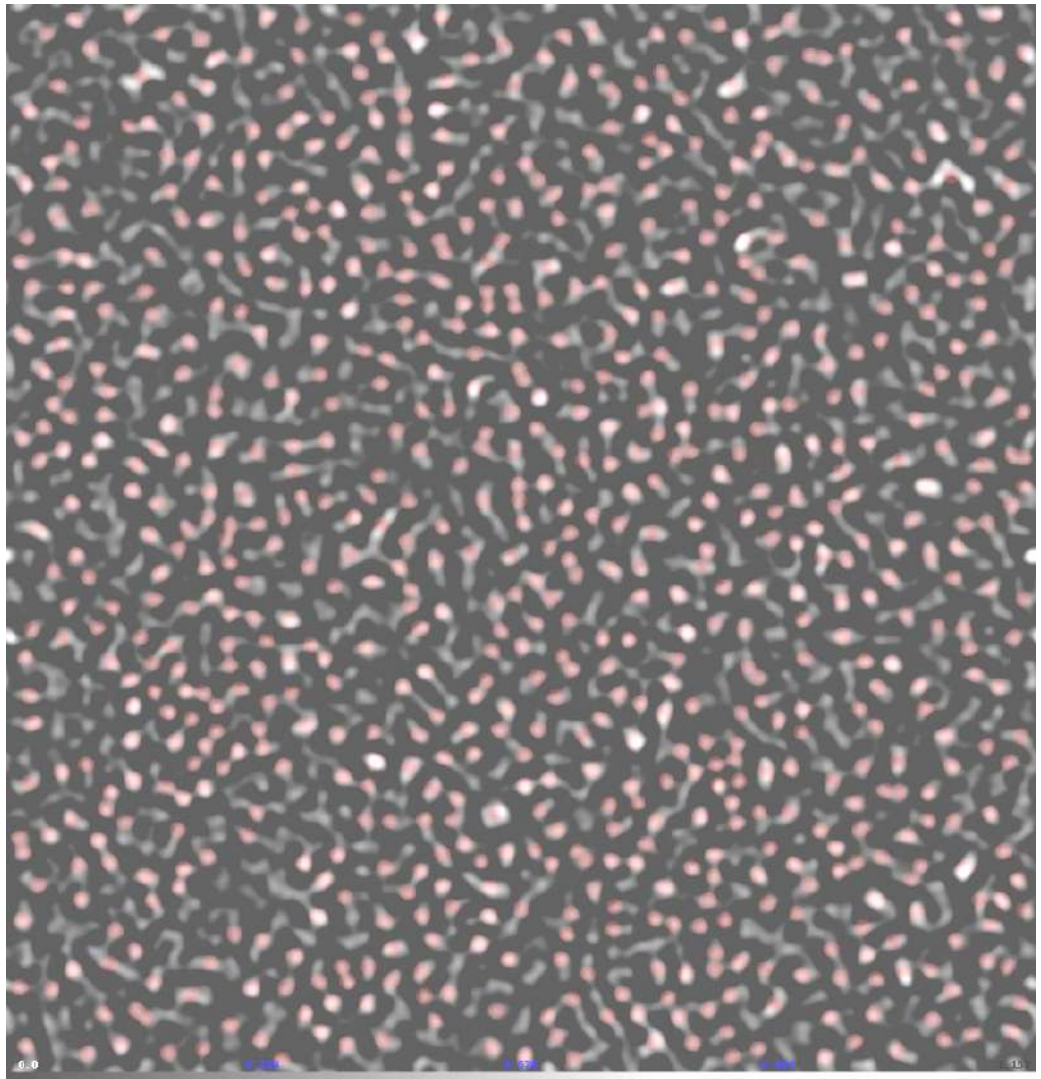
☐ Do NOT create summary peak pairs
☐ Pick only doubles
1.2 Maximum peak area multiple
1.5 Minimum peak overlap distance multiple
1.2 K-factor (slopiness)

Multi-scale dogpicker:
Number of Slices (number of sizes)
Size Range (in Angstroms)

PARTICLE PICKING

Difference of Gaussian (DoG) Picker

DoG Map



Particle Diameter:
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0.4 Minimum threshold
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Filter Values:
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2 Median (in pixels; 0= off)
4 Pixel Limit (in Standard Deviations; 0 = off)
☑ Plane regression

Defocal pairs:
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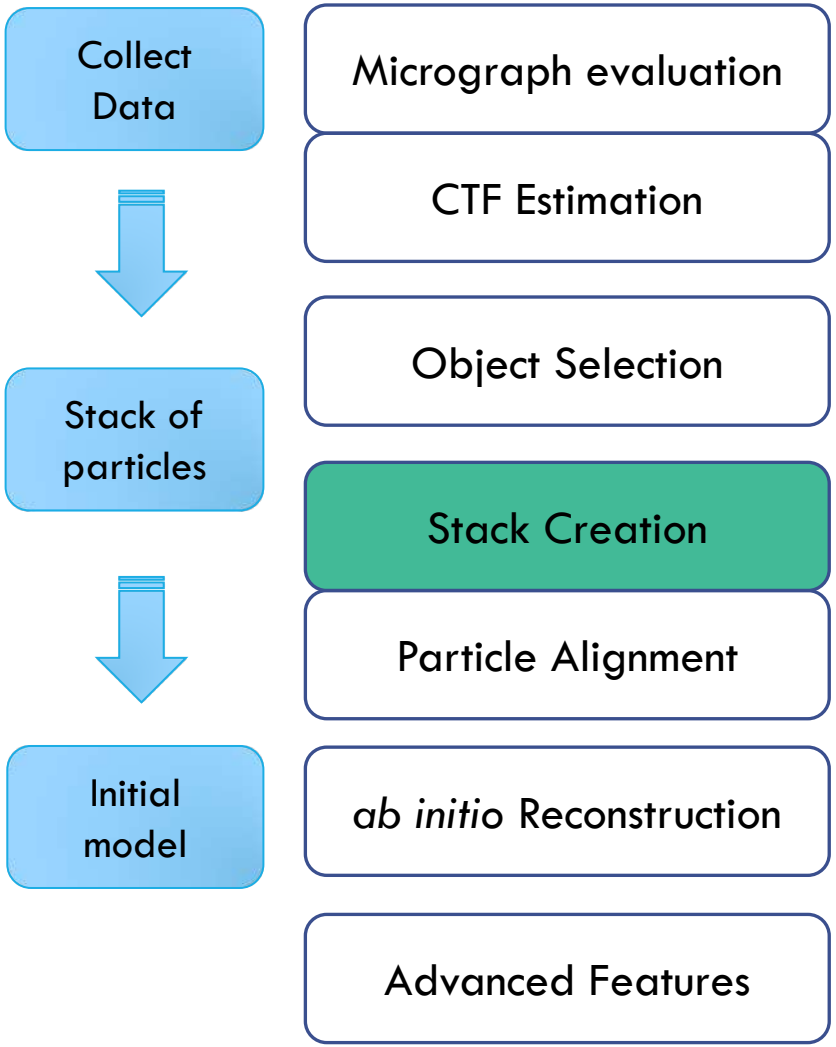
○ White Particles (i.e. negatively stained)
◉ Black Particles (i.e. ice embedded)

Peak extraction type:
◉ Center of mass (default)
○ Maximum position

☐ Do NOT create summary peak pairs
☐ Pick only doubles
1.2 Maximum peak area multiple
1.5 Minimum peak overlap distance multiple
1.2 K-factor (slopiness)

Multi-scale dogpicker:
Number of Slices (number of sizes)
Size Range (in Angstroms)

SCHEDULE


Collect Data
Stack of particles
Initial model
Micrograph evaluation
CTF Estimation
Object Selection
Stack Creation
Particle Alignment
ab initio Reconstruction
Advanced Features

STACK CREATION AFTER PARTICLE PICKING

Box size: should be at least 1.5 X particle size

Should be a multiple of small primes (2,3,5)

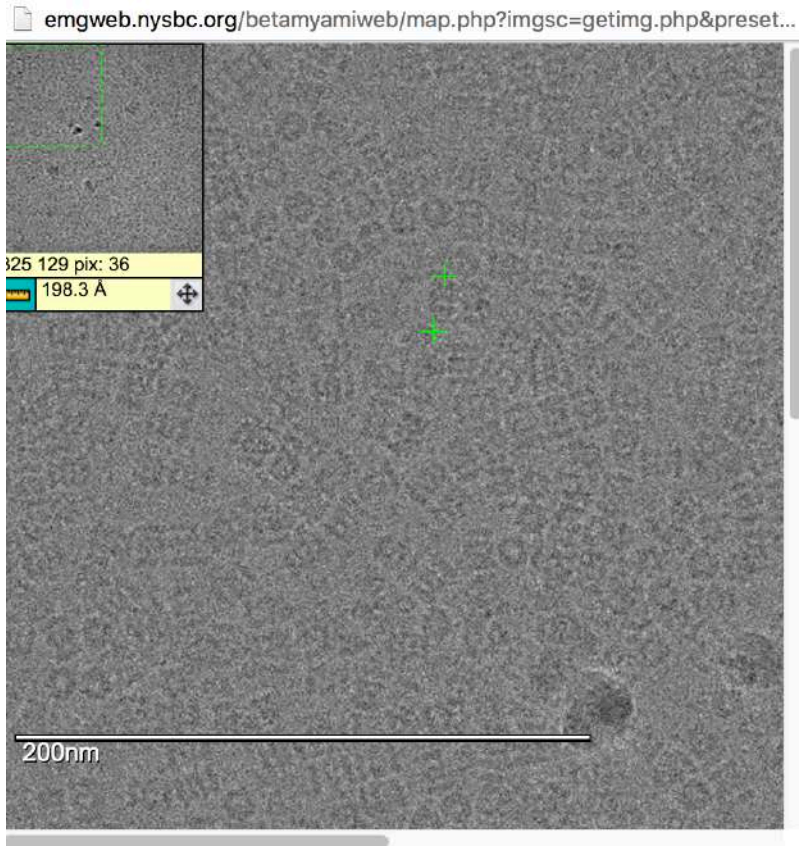
Measure approximate particle size in Leginon using Ruler tool

Convert to pixels

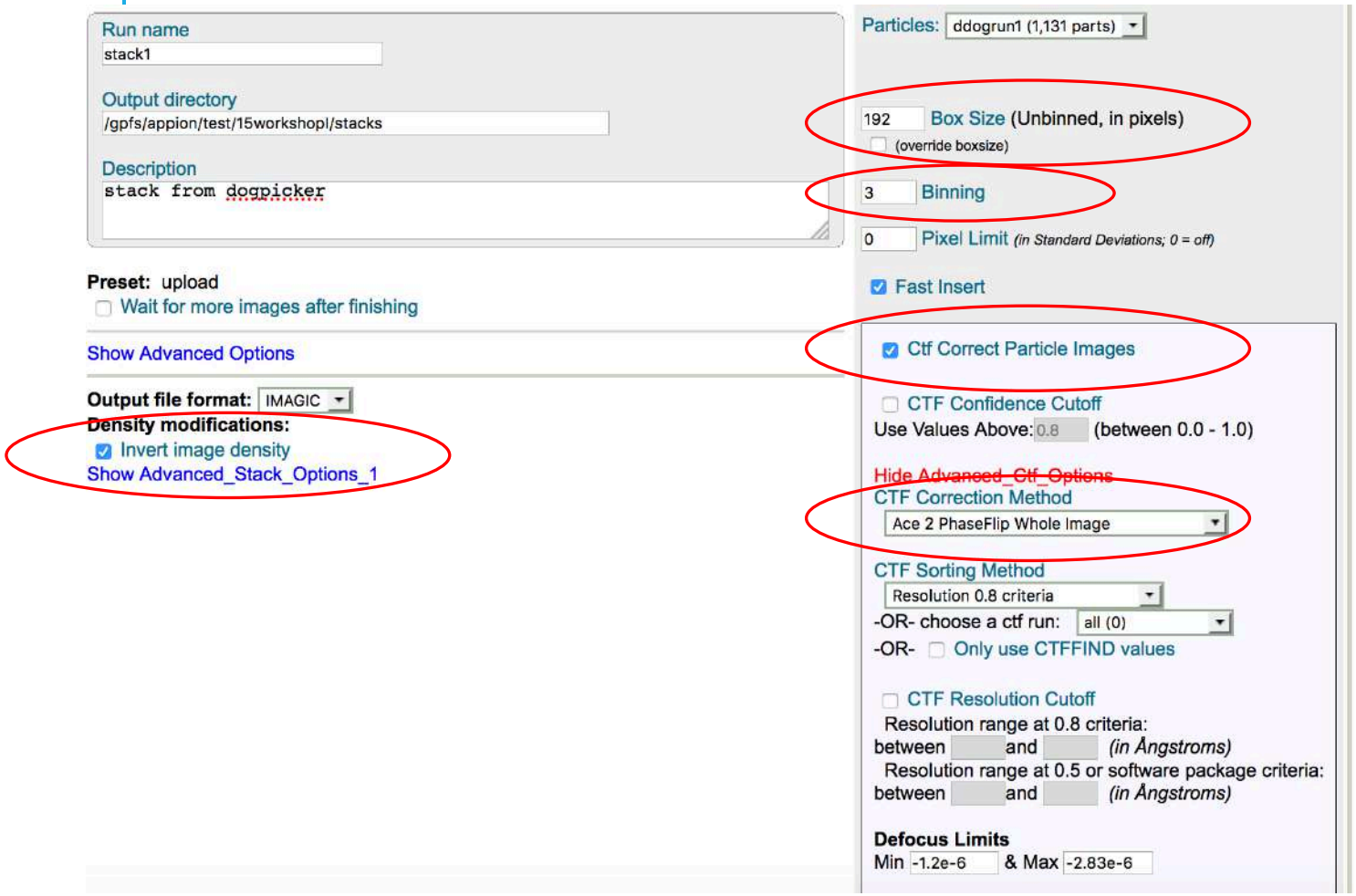
Today's sample:

- Diameter ~ 200A
- Pixel size ~1.5A

Box size 192 pixels



STACK CREATION FOR TEMPLATES / QUICK ANALYSIS



Initial Stack: After dogpicking, goal is to make templates

Bin as much as possible

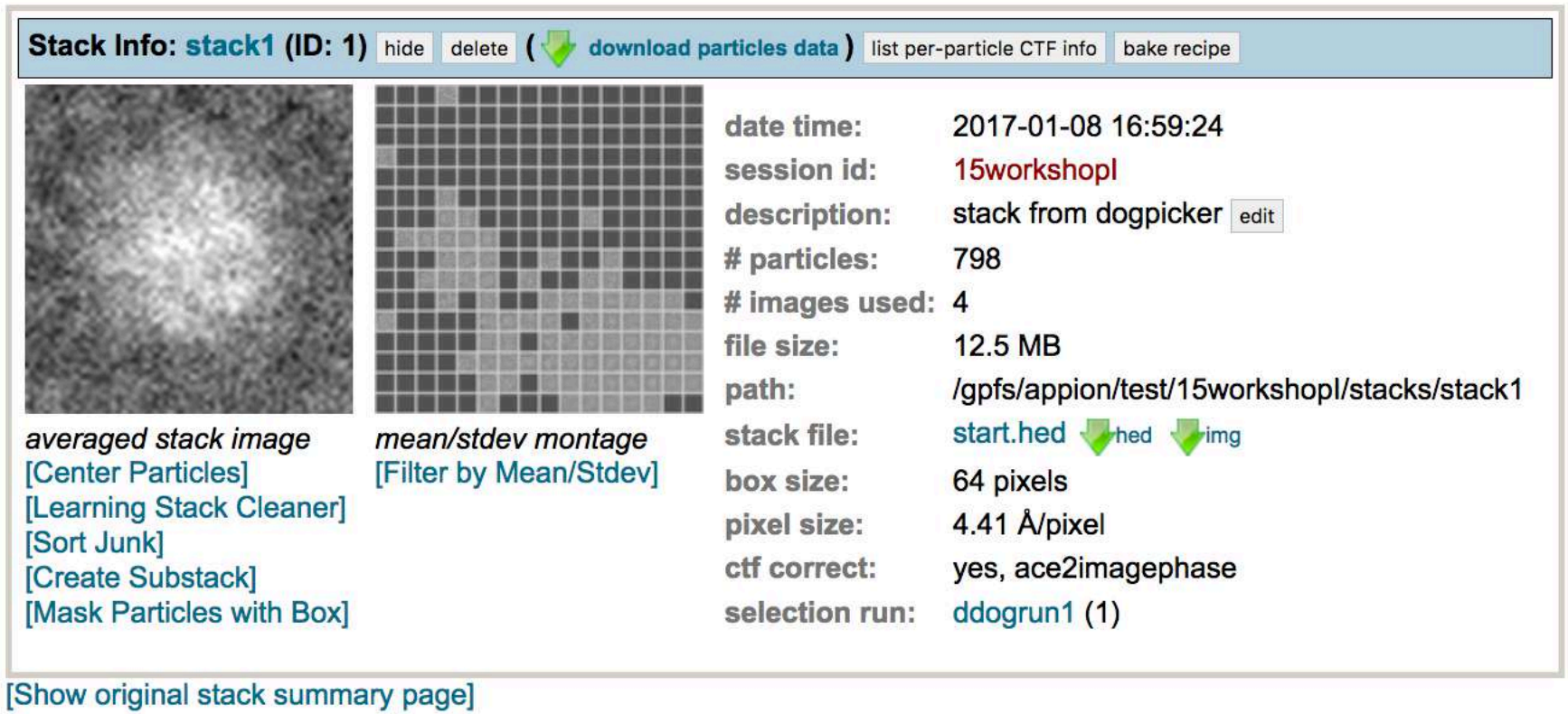
- Ideally boxes 72 pixels or fewer in dimension

CTF correct by phase flipping

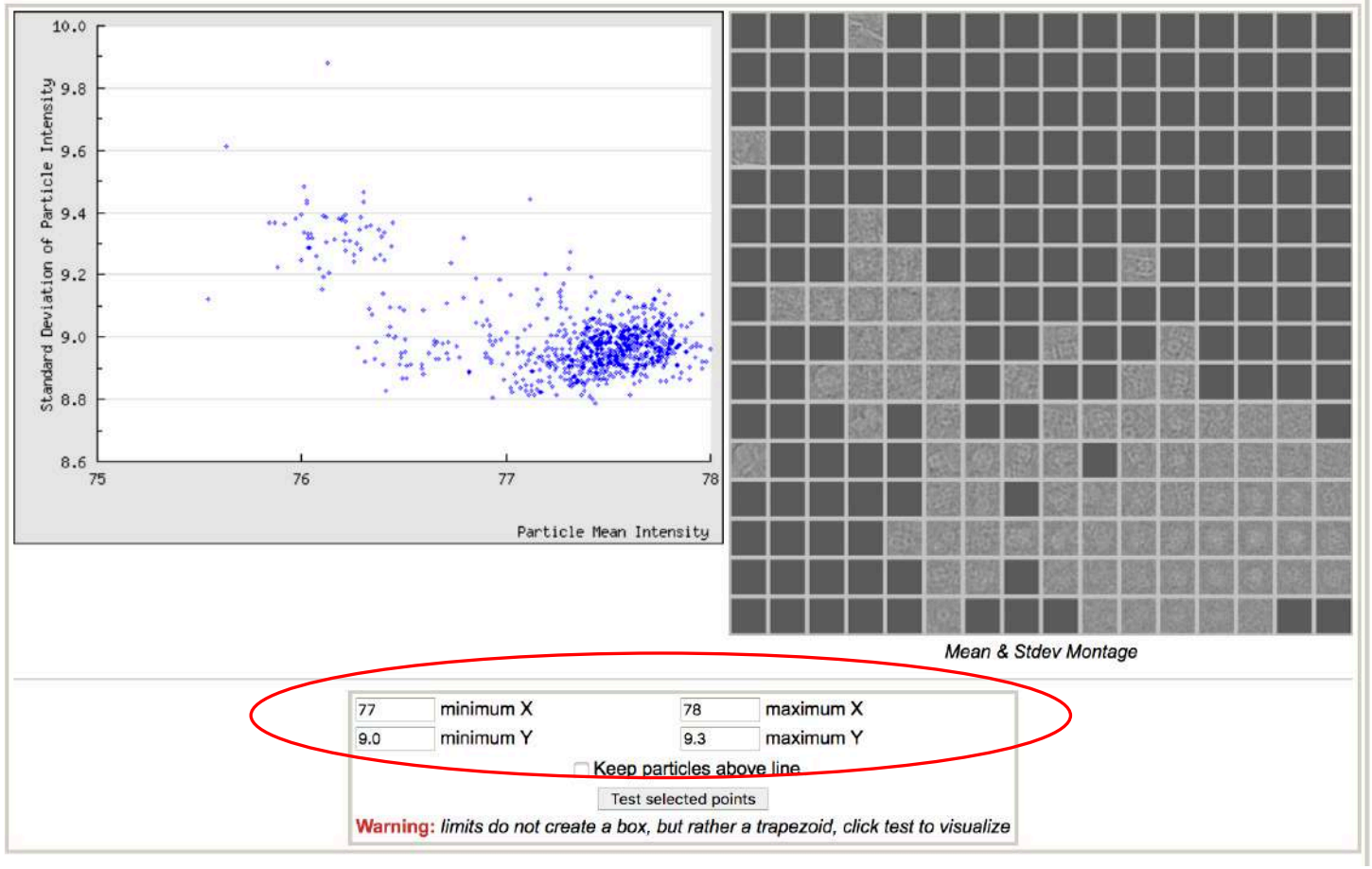
Invert image density for Cryo!

For large sets, can choose only exemplar images

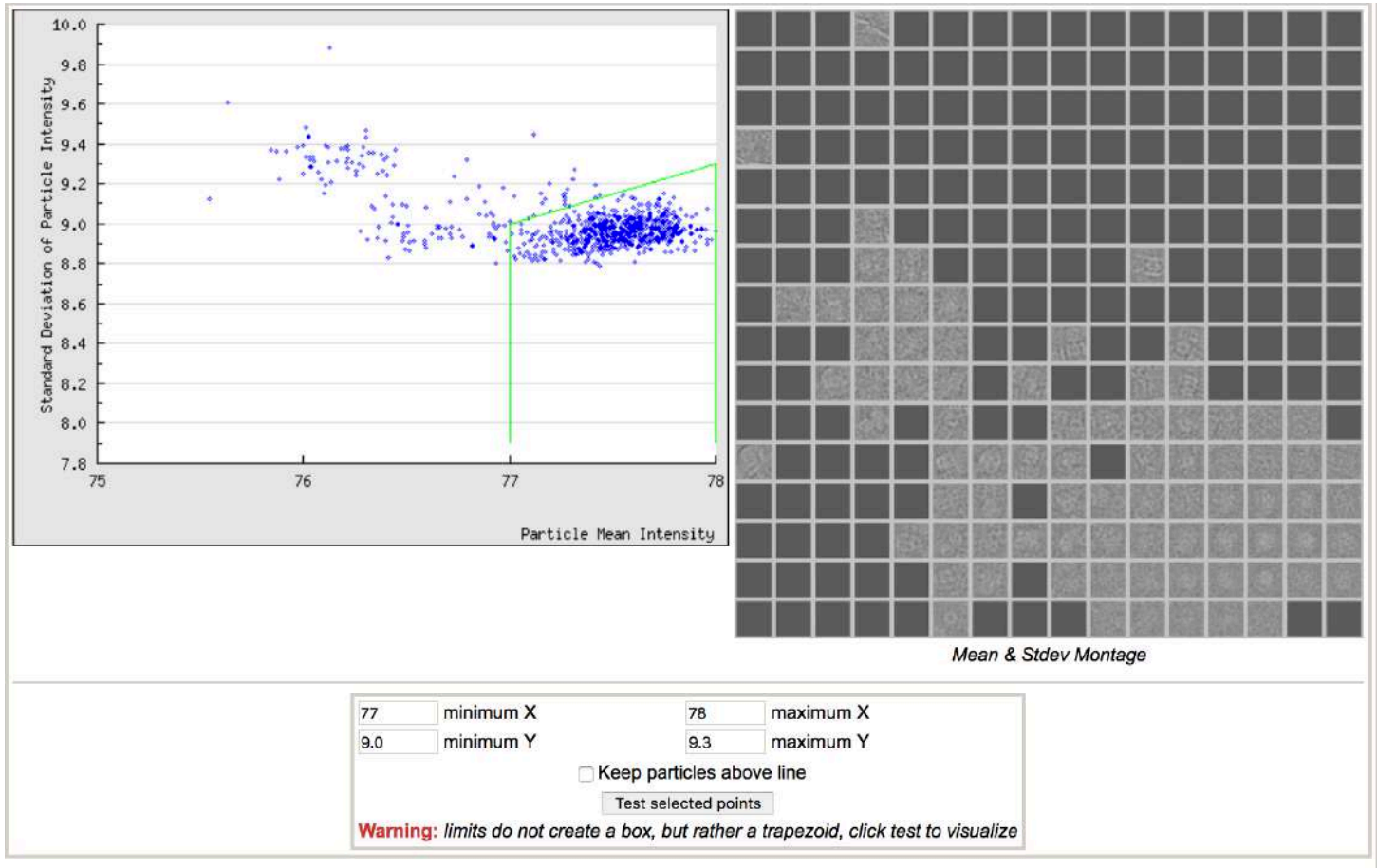
EXAMINE STACKS



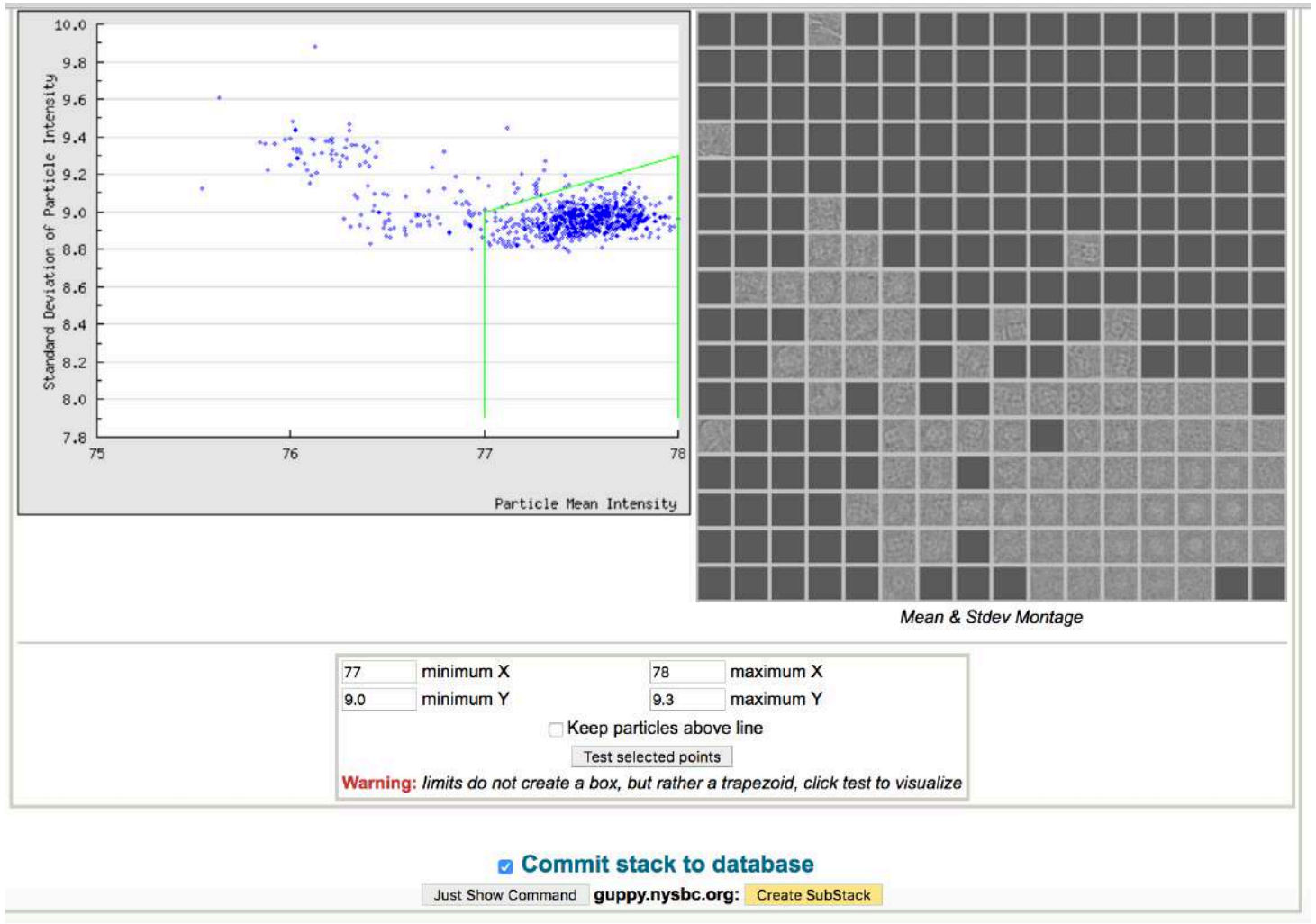
CLEAN UP BY MEAN / SD FILTER



TEST POINTS TO DRAW TRAPEZOID



CREATE SUBSTACK

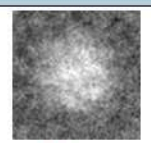


Hide | Expand | Contract

- Object Selection : 2
  - Select Particle Picker...
    - 2 complete
  - Repeat an image loop run
- CTF Estimation
  - Estimate the CTF...
    - 4 complete
  - Transfer results to another preset
  - Repeat an image loop run
- Stacks : 5
  - Stack creation
    - 7 complete
  - more stack tools
- Particle Alignment
  - Select Particle Alignment...
    - 4 complete
  - Run Feature Analysis...
    - 2 complete
  - Run Particle Clustering...
    - 8 complete
  - Run MaskItOn
  - Template Stacks
- Ab Initio Reconstruction
  - OptiMod Common Lines
    - 2 complete
  - EMAN Common Lines
  - SIMPLE Common Lines
- Refine Reconstruction
  - Run Single-Model Refinement...
  - Run Multi-Model Refinement...
- Helical Processing
  - Helical Image Processing (PHOELIX)
- Direct Detector Tools

[Show original stack summary page]

Stack Info: stack1 (ID: 1) hide delete (download particles data) list per-particle CTF info bake recipe



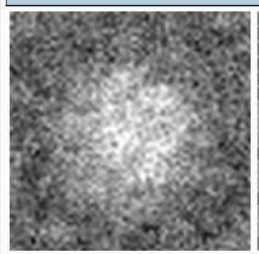
average

[Sort Junk]

date time:	2017-01-08 16:59:24
session id:	15workshopI
descript:	stack from dogpicker edit
size:	798 / 12.5 MB
path:	/gpfs/appion/test/15workshopI/stacks/stack1/start.hed hed img
box/apix:	64 pixels / 4.41 Å/pixel

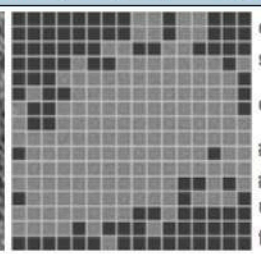
substack —>

Stack Info: stack1-meanfilt1 (ID: 2) hide delete (download particles data) list per-particle CTF info



averaged stack image

[Center Particles]
[Learning Stack Cleaner]
[Sort Junk]
[Create Substack]
[Mask Particles with Box]



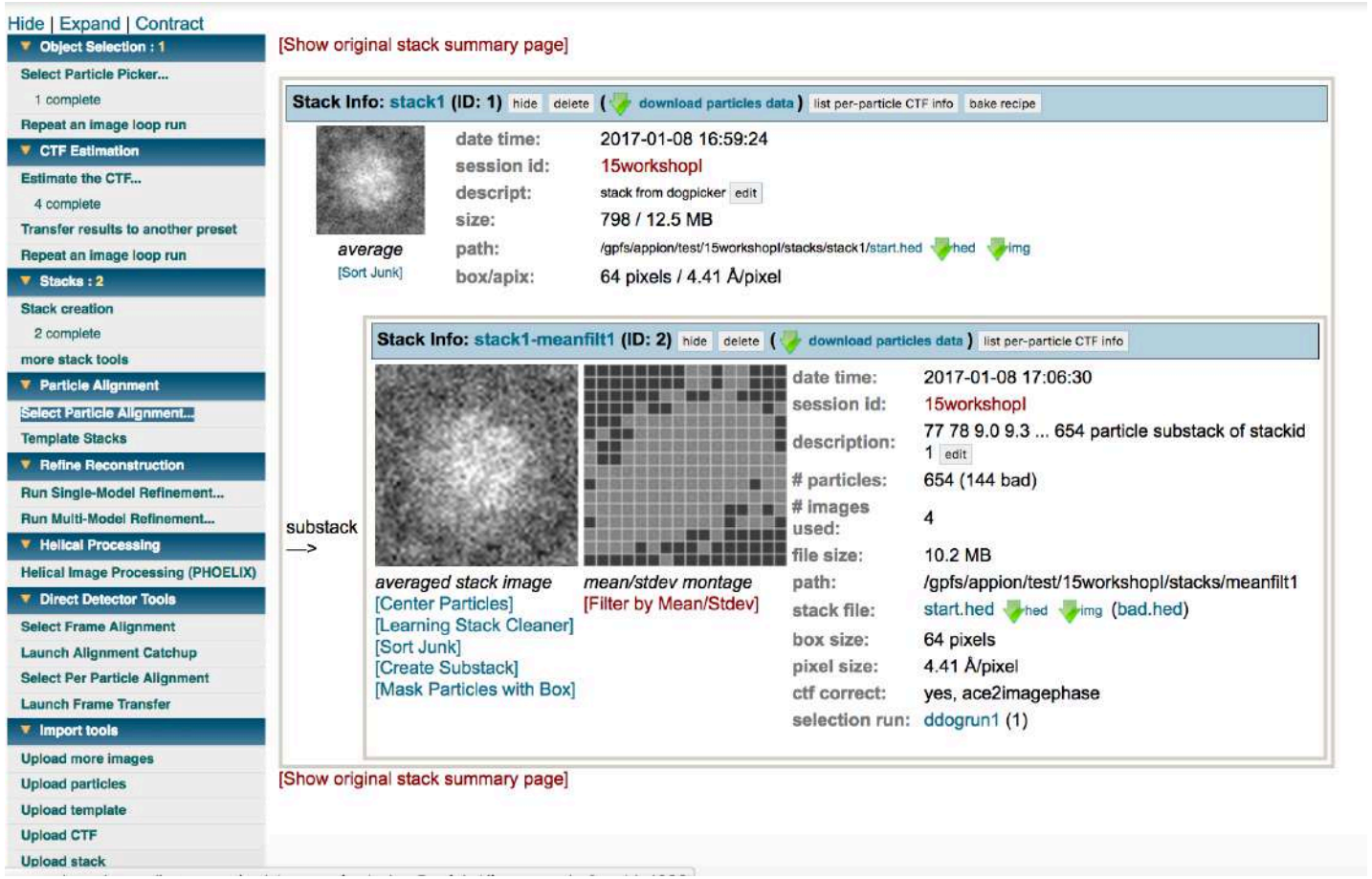
mean/stdev montage

[Filter by Mean/Stdev]

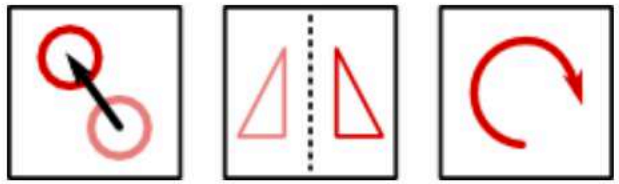
date time:	2017-01-08 17:06:30
session id:	15workshopI
description:	77 78 9.0 9.3 ... 654 particle substack of stackid 1 edit
# particles:	654 (144 bad)
# images used:	4
file size:	10.2 MB
path:	/gpfs/appion/test/15workshopI/stacks/meanfilt1
stack file:	start.hed hed img (bad.hed)
box size:	64 pixels
pixel size:	4.41 Å/pixel
ctf correct:	yes, ace2imagephase
selection run:	ddogrun1 (1)

[Show original stack summary page]

PARTICLE ALIGNMENT AND CLASSIFICATION



Particle Alignment Procedures



Particle alignment consists of shift, rotation, and mirror transformations for all particles in a stack to a common orientation or template. The reference free methods are great methods to create a templatefor particle picking or reference-based alignments.

Xmipp Maximum Likelihood Alignment

this method is the most robust, but takes some time to complete. It uses the Xmipp ml_align2d program to perform alignments.

This method is unbiased and very thorough, but also the slowest of the methods (~days). While it produces excellent templates, it only does a course search (integer pixels shifts and large angle increments), so it is best to use ref-based alignment to get better alignment parameters

Xmipp 2 Clustering 2D Alignment

this method builds a hierarchical classification of particles It uses the Xmipp 2 cl2d program to perform alignments. It is a relatively fast method that aligns and classify the images at the same time. The method starts by estimating a few classes that are further subdivided till the desired number of classes is reached. Every time an image is compared to the class averages it is aligned before-hand. **NOTE: in Xmipp 2.4 the alignment parameters are not saved in the database, and therefore this method cannot be used for RCT / OTR reconstructions.**

Xmipp 3 Clustering 2D Alignment

this method builds a hierarchical classification of particles It uses the Xmipp 3 cl2d program to perform alignments. It is a relatively fast method that aligns and classify the images at the same time. The method starts by estimating a few classes that are further subdivided till the desired number of classes is reached. Every time an image is compared to the class averages it is aligned before-hand.

Relion 2.0 GPU-Powered 2D Maximum Likelihood Alignment

this method is the most robust, but takes some time to complete. It uses the Relion Refine 2d program to perform alignments.

Like Xmipp Maximum Likelihood (from the same author), this method is unbiased and very thorough, but also the slowest of the methods (~days). While it produces excellent templates, it only does a course search (integer pixels shifts and large angle increments), so it is best to use ref-based alignment to get better alignment parameters

Relion 2D Maximum Likelihood Alignment

this method is the most robust, but takes some time to complete. It uses the Relion Refine 2d program to perform alignments.

Like Xmipp Maximum Likelihood (from the same author), this method is unbiased and very thorough, but also the slowest of the methods (~days). While it produces excellent templates, it only does a course search (integer pixels shifts and large angle increments), so it is best to use ref-based alignment to get better alignment parameters

Note: does not upload alignment parameters to database. Thinks it is Xmipp Max Like run when finished eventhough upload will not work.

IMAGIC Multi Reference Alignment (MRA)

this method uses the IMAGIC m-r-a command to align your particles to the templates within a specified template stack

Iterative Stable Alignment and Clustering (ISAC)

Initial version. More information about ISAC is available from: sxisac - SPARX

Iterative MSA/MRA

Fast & easy 2D ref-free alignment by iterative MSA/MRA. Classification can be performed using a topology-representing network or IMAGIC MSA. Multi-reference alignment can be performed using IMAGIC or EMAN.

Spider Reference-based Alignment

first you select template and then this method uses the Spider AP MQ command to align your particles to the selected templates. Multiprocessing additions has made this extremely fast (~1 hour).

This method is broken on SPIDER version 18 or newer, see bug report

Xmipp Reference Based Maximum Likelihood Alignment

similar to reference-free but you select templates first. It uses the Xmipp ml_align2d program to perform alignments.

Still untested as to how much bias the reference gives you, but this may be useful in some cases. Also, when no particles align to a particular template, it goes black and unused in further iterations.

Spider Reference-free Alignment

this method uses the Spider AP SR command to align your particles.

WARNING: this method is very quick (~few minutes), but also very sloppy and does not always do a great job. The only way to obtain decent results is to run several times and compare the results.

Ed's Iteration Alignment

this method uses the Spider AP SR and the Spider AP SH commands to align your particles through multiple iterations of ref-free and ref-based alignments.

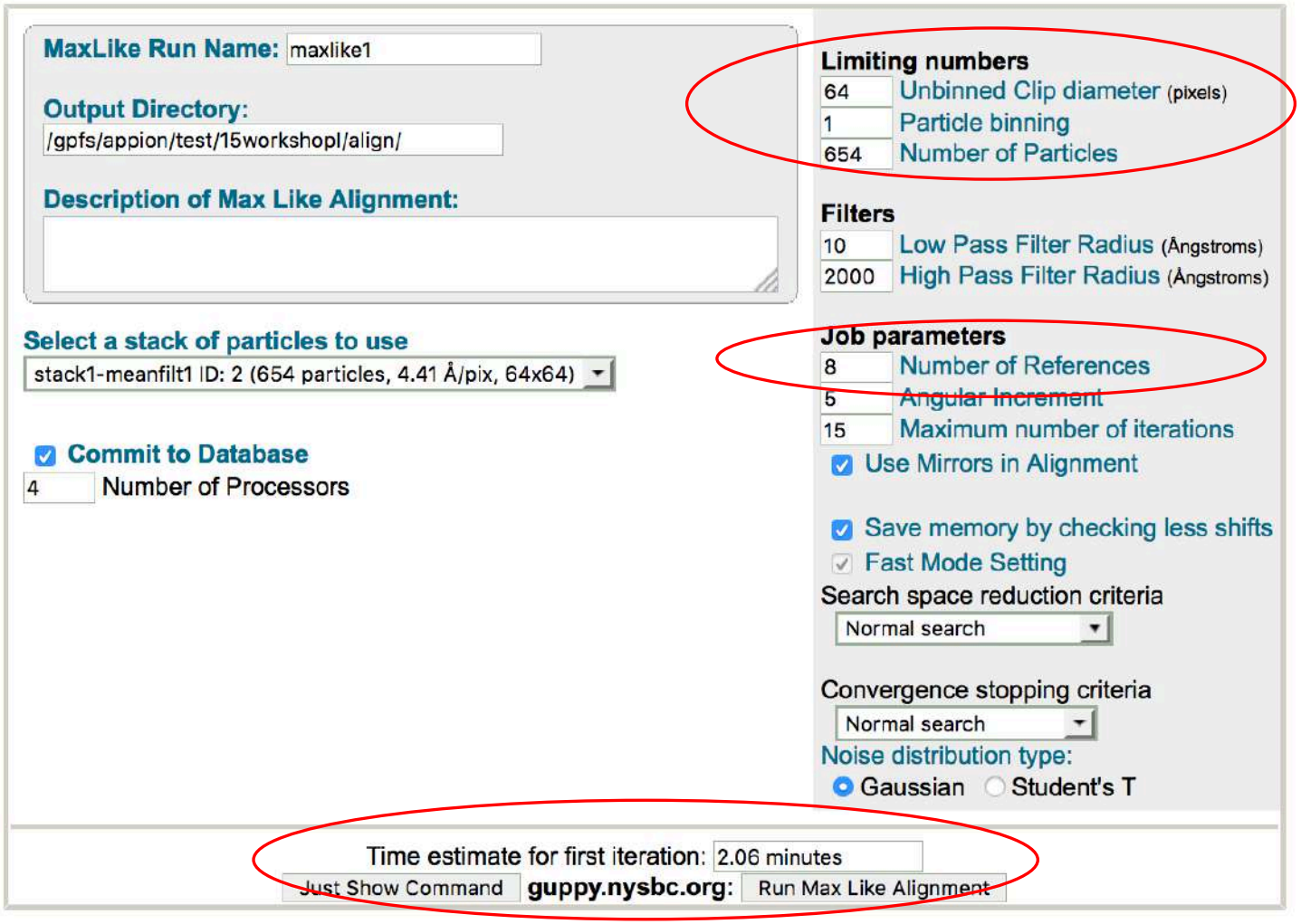
WARNING: report all problems to Ed Brignole

EMAN Refine 2d Reference-free Alignment

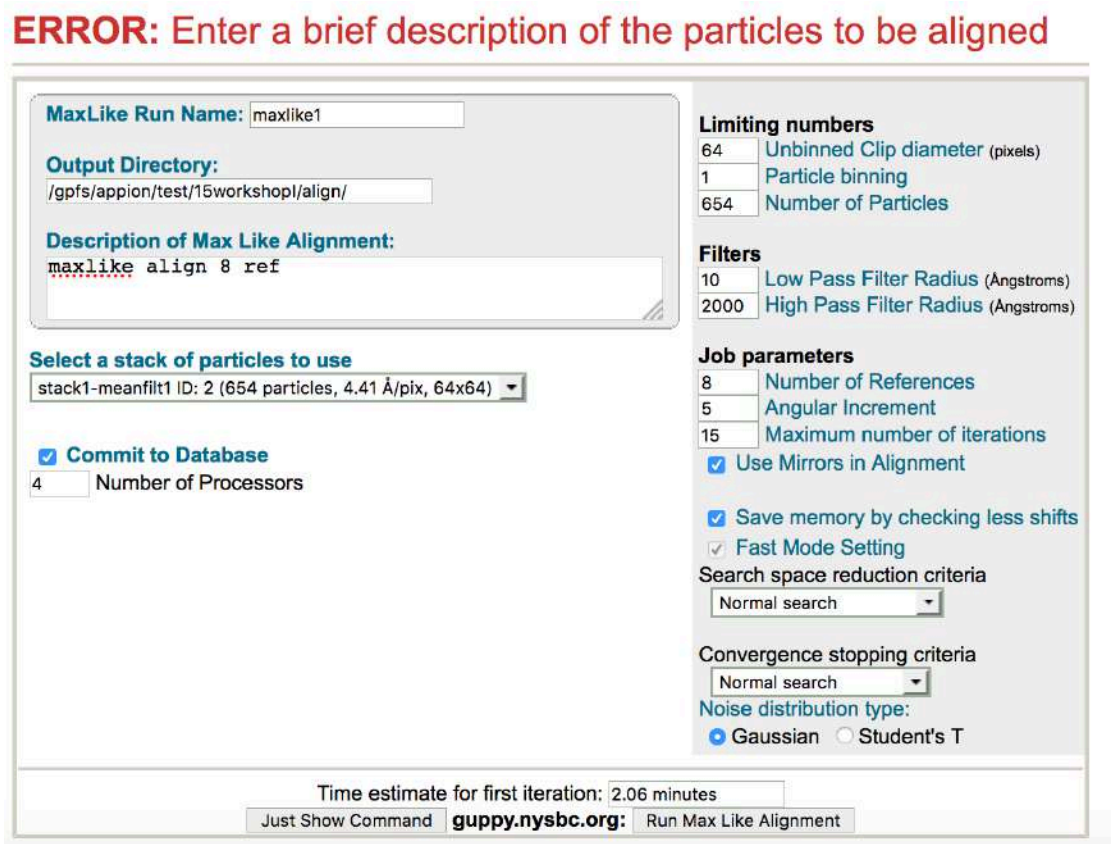
Fast and easy 2D ref-free alignment using EMAN's refine2d.py program

INITIAL FAST ALIGNMENT BY MAXIMUM LIKELIHOOD

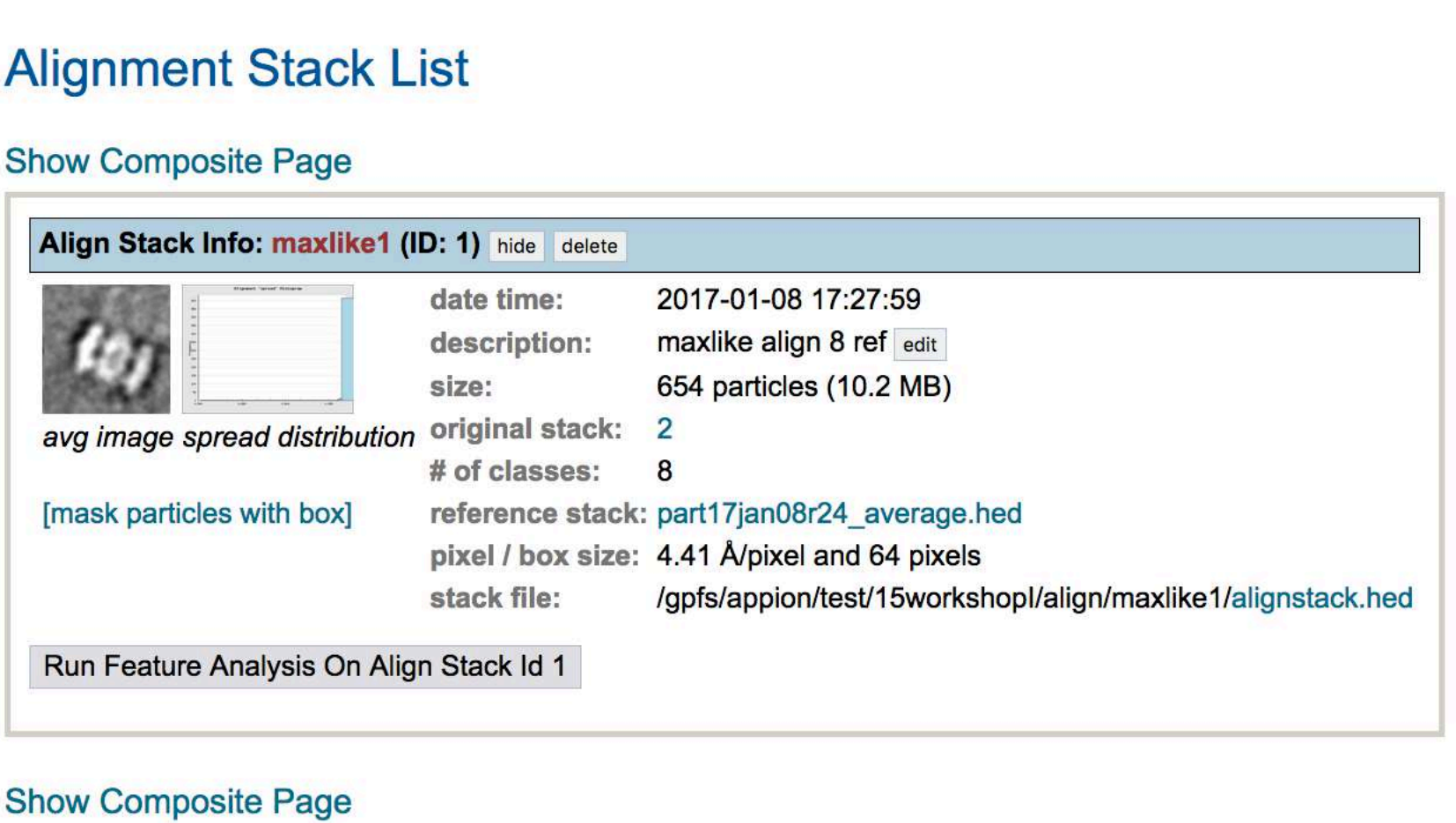
- Binned Stack
- Few References (classes)
- Decent No. Processors
- Monitor time estimate
- If too long
  - Bin more
  - More CPU's
  - Fewer Classes



OOPS, NEED TO ADD DESCRIPTION



DISPLAY ALIGNED STACKS

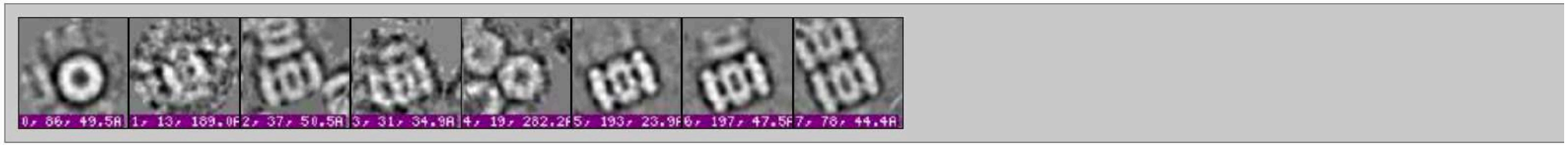


EXAMINE CLASSES

stack: /gpfs/appion/test/15workshopI/align/maxlike1/part17jan08r24_average.hed
#images: 8
from: 0 to: 7 binning: 1 quality: jpeg 50 info: scale bar: Load
Selection mode: exclude

Selected images:
Select: View Raw Particles | View Aligned Particles | Create SubStack | Create Templates | Include Particles | Create Template Stack | Run Common Lines

Excluded images:
Exclude: Create SubStack



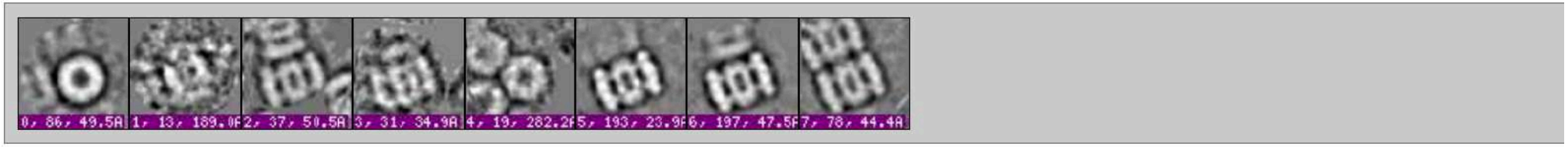
Note: With this particle, we basically have only 2 classes: a top view and a side view

GO TO SELECT MODE

stack: /gpfs/appion/test/15workshopI/align/maxlike1/part17jan08r24_average.hed
#images: 8
from: 0 to: 7 binning: 1 quality: jpeg 50 info: scale bar: Load
Selection mode: select

Selected images:
Select: View Raw Particles | View Aligned Particles | Create SubStack | Create Templates | Include Particles | Create Template Stack | Run Common Lines

Excluded images:
Exclude: Create SubStack

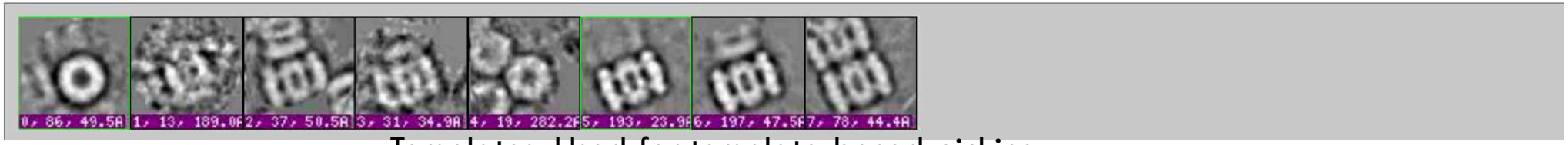


PICK CLASSES FOR TEMPLATES

stack: /gpfs/appion/test/15workshopI/align/maxlike1/part17jan08r24_average.hed
#images: 8
from: 0 to: 7 binning: 1 quality: jpeg 50 info: scale bar: Load
Selection mode: select

Selected images: 0,5
Select: View Raw Particles | View Aligned Particles | Create SubStack | Create Templates | Include Particles | Create Template Stack | Run Common Lines

Excluded images: 1,2,3,4,6,7
Exclude: Create SubStack

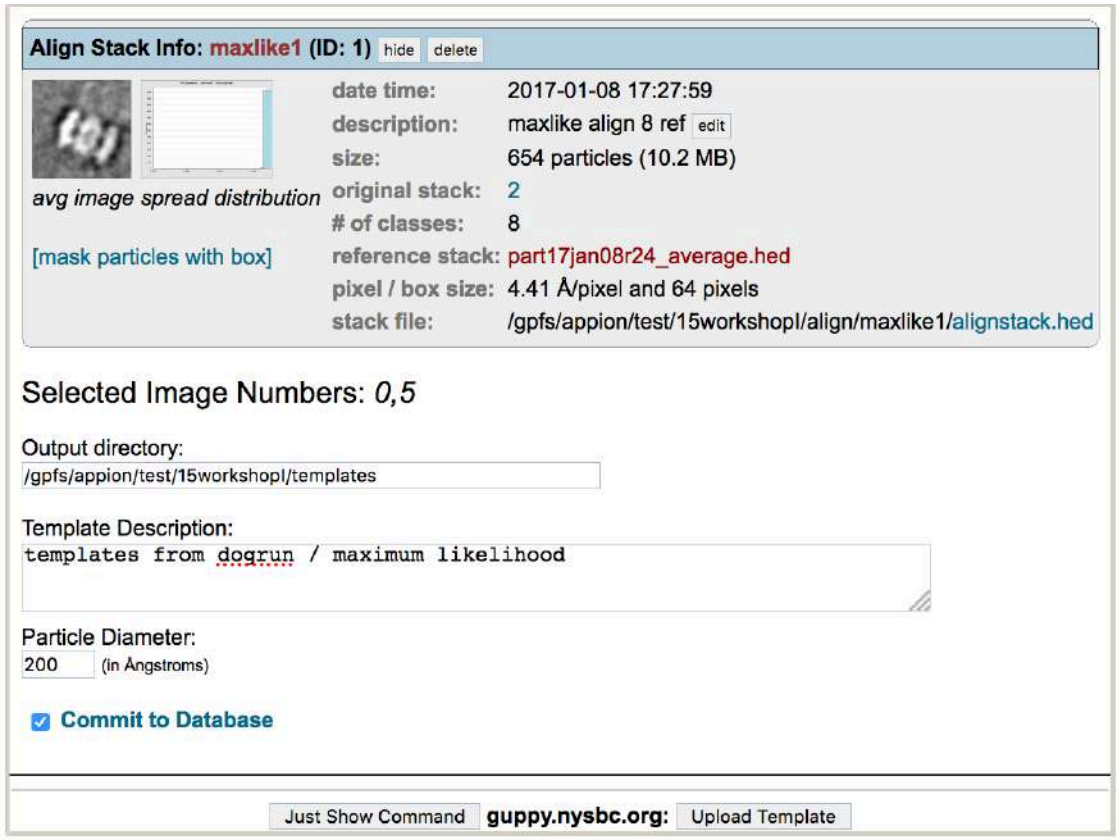


Templates: Used for template-based picking

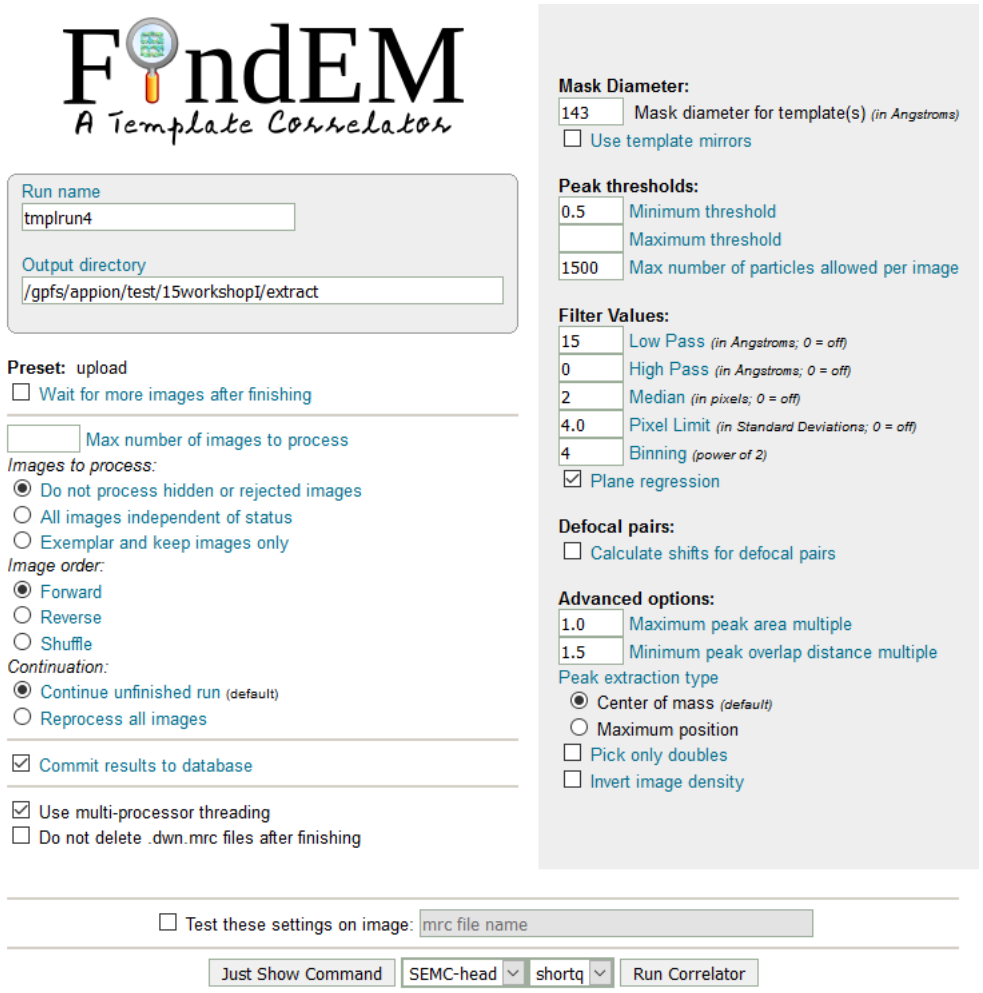
Template Stack: Used for ab initio reconstruction

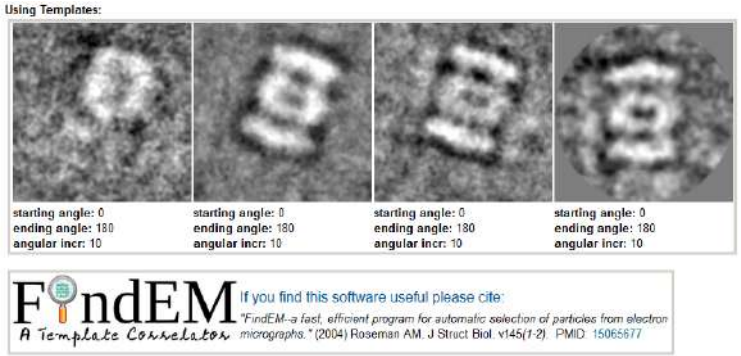
Substack: Make a new stack using only particles from good classes

UPLOAD TEMPLATES

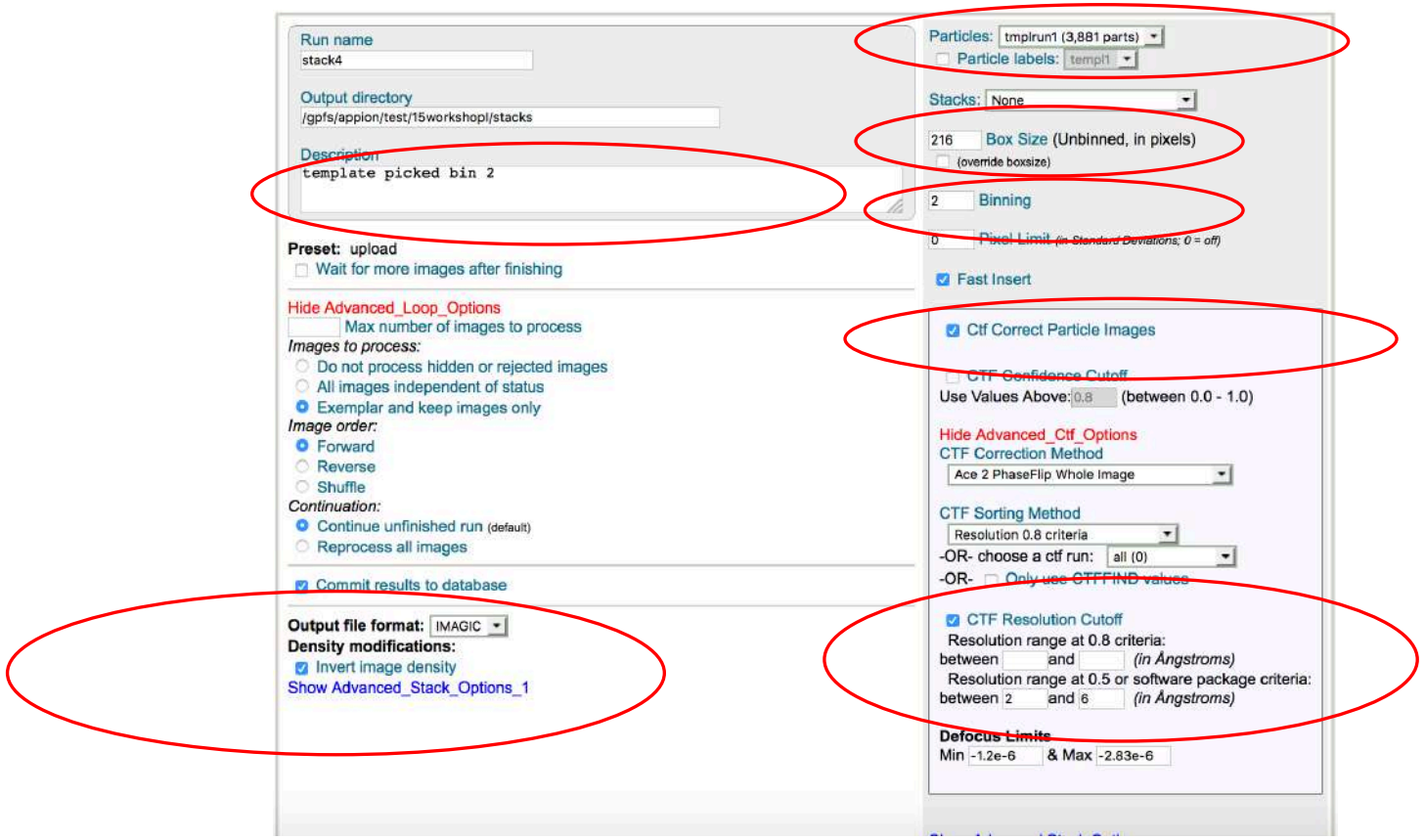


PARTICLE PICKING: TEMPLATE PICKER

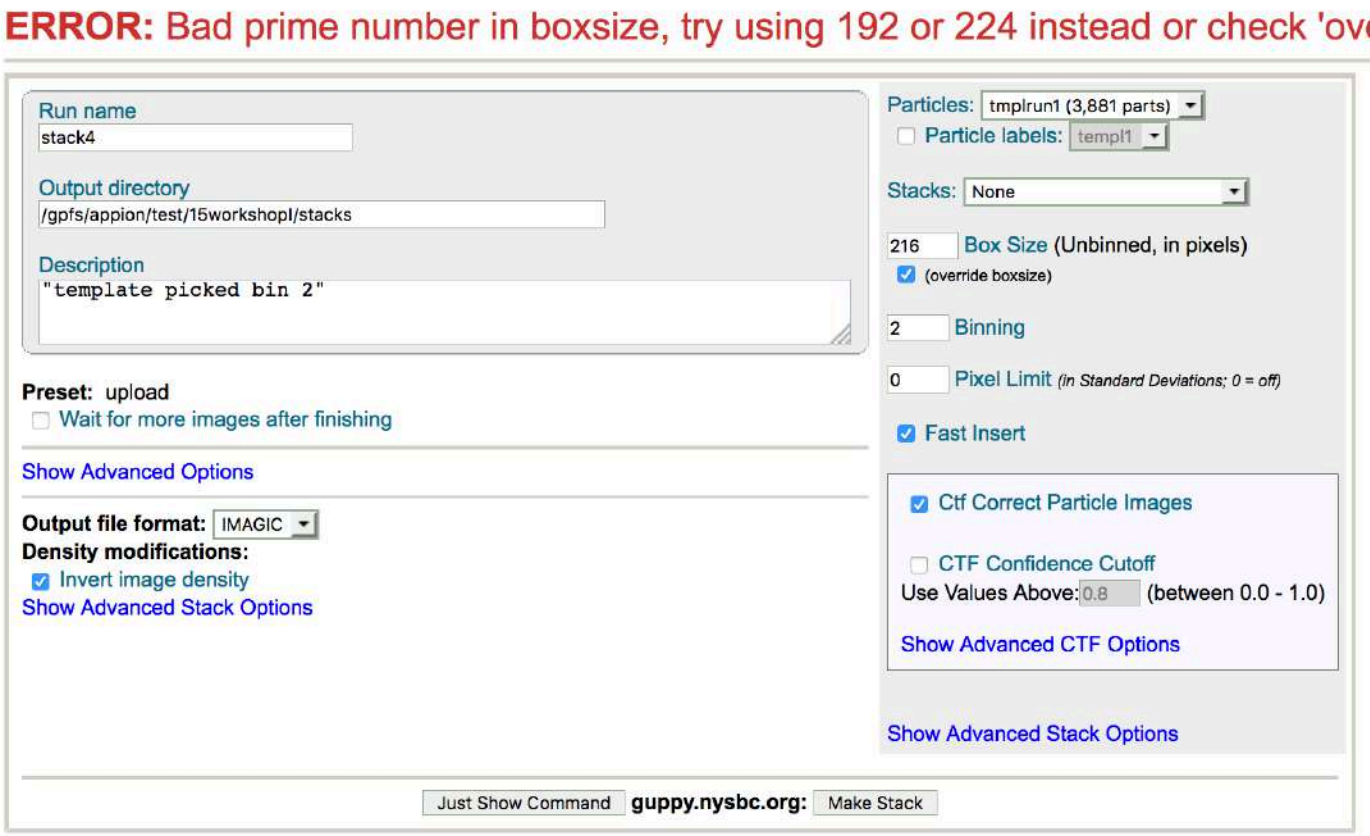




MAKE LARGER STACK FROM TEMPLATES

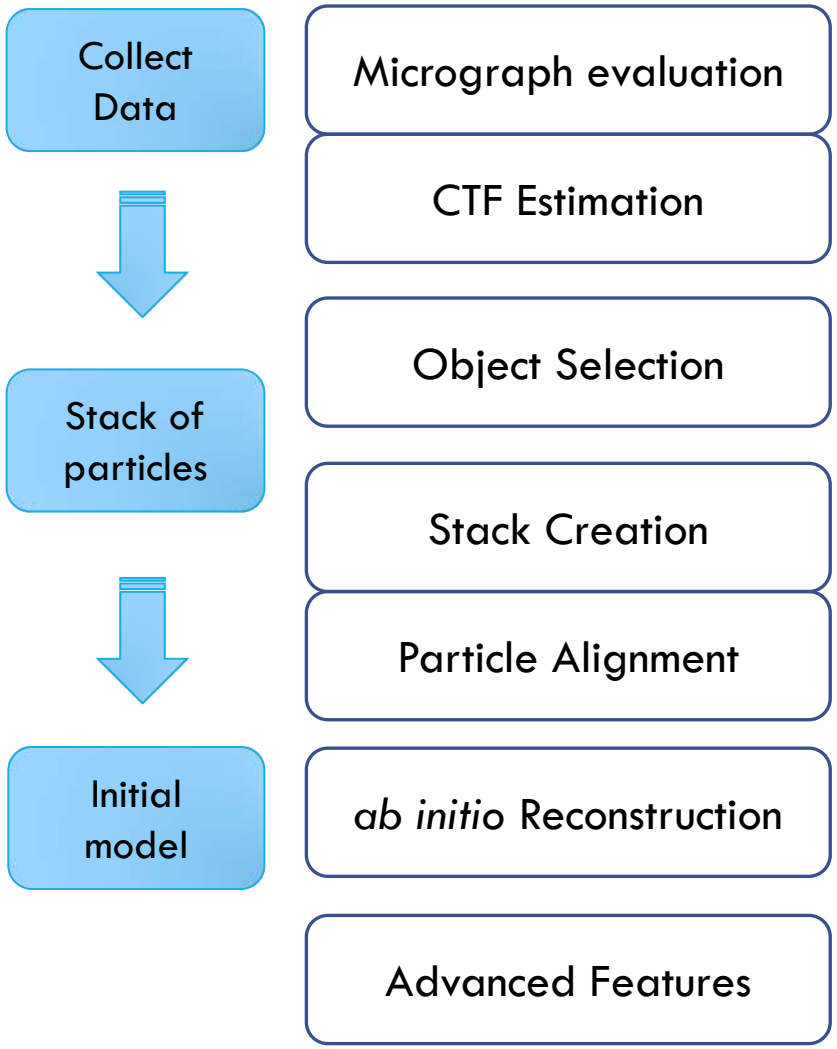


OVERRIDE SIZE IF NECESSARY



216/2=108
108/2=54
54/2=27
27=3*3*3

SCHEDULE


Collect Data
Micrograph evaluation
CTF Estimation
Object Selection
Stack of particles
Stack Creation
Particle Alignment
Initial model
ab initio Reconstruction
Advanced Features

SCHEDULE

JULY 29-30, 2020 2PM-4PM

PRACTICAL WORKSHOP WHERE STUDENTS WILL WORK IN PAIRS ON A TEST DATASET. AFTER EACH LECTURER GIVES AN OVERVIEW OF THE TOPIC THE ATTENDEES WILL HAVE HANDS-ON TIME TO USE THE INTERFACE.

1. Workshop intro

- Overview of Leginon/Appion
- Krios workflow
- Leginon targeter

2. Micrograph evaluation / CTF Estimation

3. Object Selection

4. Stack creation / Particle Alignment

[3 (cont). Object Selection with templates after initial class averages]

5. Other features: Pro tips / Direct Detector Tools / Import/Export Tools

6. Closing