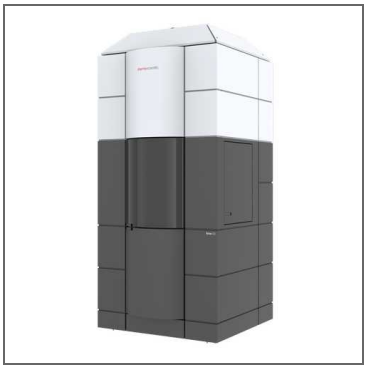

AUG 05, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.6qpvr3442vmk/v1

Protocol Citation: Josh Hutchings, Siyu Chen 2023. Quick guide to use paceTOMO for cryo-ET data collection from Titan Krios. **protocols.io** https://dx.doi.org/10.17504/protocols.io.6qpvr3442vmk/v1

Protocol status: Working
We use this protocol and it's working

Created: Aug 04, 2023

Quick guide to use paceTOMO for cryo-ET data collection from Titan Krios

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DISCLAIMER

ABSTRACT

This quick guide provides key minimal steps for preparing the Titan/SerialEM for tomogram data collection on lamella or in vitro specimens with K3 camera. paceTOMO routine is also included for a typical tomogram data collection session. Please note that this is not an exhaustive guide, but summarises order of key steps.

GUIDELINES

Useful resources:
SerialEM repository (Nexperion): https://serialemscripts.nexperion.net/
SerialEM mailing list: https://bio3d.colorado.edu/SerialEM/#List
All SerialEM help dialogues can be accessed from SerialEM directly

Last Modified: Aug 05, 2023

PROTOCOL integer ID: 85958

Keywords: cryo-EM, Titan, K3

SAFETY WARNINGS

Handle liquid nitrogen with gloves and face shields equipped all the time

BEFORE START INSTRUCTIONS

Check the following before the session is started:

- Titan LN2 tank at least 20% full.
- GMS3 should be open on the K3 PC. If not, open it (takes few minutes).
- Check that GMS3 is in Power-User mode (Help menu).
- Cross-grating grid should be on the stage.

Preparation before performing alignment

1 Load samples into the Titan. Slot 1 is reserved for cross-grating grid, which is used for daily alignment.

> **Note**
>
> When loading grid with lamella on to the cartridge, make sure dots are facing right and blue dot is point up to adjust the orientation of the lamella.

2 Do inventory in TEM UI once all temperatures shown on the screen are <100K. If not already, load the cross-grating grid onto the microscope.

3 Prepare

Software

serialEM	NAME
Win 17763.3287	OS
Regents of the University of Colorado	DEVELOPER

3.1 Start server from icon on the desktop

3.2 Make a new folder in X drive for data collection

3.3 Find a setting file that has been recently used and start from this file:
The master settings can be found either from other people or your own previous settings. Copy such setting file and paste it to the current data collection folder, then rename it to a unique, informative name. For example: 20230101_name_from_name_20221231_settings.txt

3.4 To launch

Software

serialEM	NAME
Win 17763.3287	OS
Regents of the University of Colorado	DEVELOPER

Open the Anaconda shell on the K3 PC desktop.
Use terminal with administrator privilege, then run the following command to start the software:

Command

start serialEM (Anaconda shell)

```
conda activate serialem
cd C:/Users/VALUEDGATANCUSTOMER/Documents/SerialEM_beta
SerialEM.exe
```

3.5 in serialEM, open the newly copied settings file as well as the Navigator. Load 580x from imaging states window

4 With flu-screen down (L1 from the control pad, or in GMS), find a completely empty square (save position as e.g. “hole”) and a square with intact film (save position as e.g. “film”).

Note

save position function can be found in TEM UI. Tab name is 'stage2'

5 Decide on CDS or non-CDS mode for the data collection session in GMS3 and check that the SerialEM camera set-up is consistent with that.
CDS mode mitigates some K3 gain artefacts but will increase the data acquisition time.

paceTOMO data collection workflow

6 Move stage to hole position.

7 Initial configuration of Record settings:

7.1 Change IS to desired low dose data collection settings (either 42k or 53k). Double click to activate the preset.

7.2 Note the C2 aperture (typically 70 or 100 mm can be used) - this will influence the flux. Check View setting magnification (generally 4800x or higher) and slit is inserted for Record mag.

7.3 Check Record beam centering on flu-screen and check flux in GMS3 with a one-second exposure.

> Note
>
> If changing any settings, click “continuous update” in SerialEM and perform changes. Make sure to uncheck this once done, also save settings and update IS.

> Note
>
> To center beam with beam shift: in TEM UI, find the tab of Direct alignment -> Beam shift -> Use multi-X & Y knobs to center it.

> Note
>
> Note the exact flux for future uses (eps, e/A^2/s), ideally 15 eps for non-CDS or 7.5 eps for CDS through sample.
> Note that K3 typically requires at least 50% more flux over vacuum to achieve suitable counts with samples loaded, e.g. 22-23 eps over vacuum for non-CDS will give 10-15 eps over samples. 11-12 eps over vacuum for CDS will give 7-8 eps over samples.

8 Update hardware dark reference:
In GMS3, find Camera tab > Prepare Dark Reference.
Takes ~10 seconds.

9 GIF tune:

9.1 Check beam centering on flu-screen and change spot size to achieve 50 e/p/s flux. Lift screen and unblank beam.

Note

To center beam with beam shift: in TEM UI, find Direct alignment-> Beam shift -> Use multi-X & Y knobs to center it.
When flux achieves 50 eps, do not update the settings in serialEM because such settings will only be used for GIF tune, not for data collection.

9.2 Perform full GIF tune in GMS3 with illumination settings intended for SerialEM data collection.

Note

If prompted that tuning gets worse, “keep anyway” and do not undo. This is currently a bug that prevents GMS3 from tunings GIF during the final steps.

10 Prepare gain reference if necessary. (typically needed once a week)

10.1 It is recommended to check if the reference is needed. To perform that in GMS3:
Take a dark reference
adjust the beam intensity go to to ~15eps
take an 1s, 20-frame exposure
in the Process tab, click autocorrelation and see if there is any pattern existing.

10.2 If gain reference is needed, select gain reference from GMS3 Camera drop-down tab and proceed with the prompts.

Note

For the first, linear gain, lower spot size (normally use spot size 1 or 2), check beam centering, withdraw slit in GMS3, unblank beam to reach ~1500 flux

Note

For the second, counting gain, accept default counts (15 eps for non-CDS, 7.5 eps for CDS). Revert back to original Record settings (hit Go to: Rec. in SerialEM) and adjust illumination for target counts.

Align the microscope with stable carbon/gold materials in t...

11 In TEM UI, move stage to foil position.

12 Check View magnification and centering on Flu screen.
If changing anything, tick and untick continuous update in serialEM

13 Find eucentricity with SerialEM Rough Eucentricity function. Can be found in the drop-down menu.

14 Switch to Record magnification and find focus with GMS3 (with Live > FFT window on). When FFT doesn't show any Thon rings, press L2 on the control panel to zero defocus.

15 Use SerialEM to autofocus to -2.5 um.

Note

in the drop-down menu, find Focus/Tune -> Set target to -2.5um

16 Correct astigmatism by CTF in SerialEM.

Note

Settings (Focus/Tune > Set CTF Aqcquire Params):
Exposure time: 2 sec; Binning: 0; Drift settling: 0 sec; 1 for full-view; Smallest defocus: -1.5 um. Amount to increase underfocus 0.5

17 Coma-free alignment by CTF in SerialEM.

Note

Settings (Focus/Tune > Set CTF coma-free params):
Beam tilt: 5 mrad; 0 not to do full 3x3 array; Threshold: 0.1 mrad.

18 Coma vs Image Shift calibration.

Note

Uses image settings from Set CTF Acquire Params above. Only change the exposure time to 10s when necessary.
Sometimes not enough Thon rings can be used for fitting and longer exposure time can help with the issue.
Beam tilt: 4 mrad.

When the task is done, put in the C2 aperture size: 70 or 100 (check Apertures tab in TEM UI).

19 Insert OA (objective aperture, 70 or 100 um) and check centering in diffraction mode (make sure the flu screen is DOWN).
Use roll wheel to get bright view and use rings to center OA.
Change magnification to meters when rings are too small to be visualized.

Note

To adjust the OA: in TEM UI, click Adjust in Apertures tab -> use multi X & Y to adjust until the entire ring is centered

Once done, correct astigmatism by CTF in SerialEM again.

20 Align SerialEM Record/Focus/View offsets:

20.1 Check “continuous update” in SerialEM for the low-dose IS.
in serialEM, copy Record setting parameters to Focus and Trial settings.
Lower down the flu-screen to check centering of Record/Focus/View settings.

20.2 Check image shift for Record/View settings using a feature such as a bead on the XG grid.
Untick “move stage for big mouse shifts” and retick once offset is set.

> Note
>
> To adjust the image shift, use record(preview) to find a feature in the field of view -> take view image -> center view using right mouse-> SET SHIFT under “offsets for …” in serialEM

Once done, uncheck “continuous update”, save settings and update IS.

Aquire multiple atlases and screen the sample quality

21 With the cross-grating loaded, switch to 135x Atlas IS.

22 Set up full montage (6x9 atlas) in serialEM drop-down menu. Run MapGrids macro from script editor.

> Note
>
> Adjust the following number according to the sample slots:
> Loop x index; – index start from 1, then loop for x times. So x equals to the number of grids you are planning to check
> i = $index + y; – y should be the slot number of your first grid - 1 since index starts from 1

23 Inspect each atlas and decide on overall grid quality. Reloading grids will require registration alignment to account for small shifts/rotations during reloading.

Note

Registration alignment procedure:
Add at least two points (on something remarkable, like a crack or large piece of ice) far from each other on the original Atlas.
Mark each of these points as Registration Points using the button at the top left of the navigator window. They should now have an indication of which registration and which point they are in the “Reg.” column (e.g. 1R1, 1R2)
Increase the registration from 1 to 2.
At the atlas mag, visit each navigation point from the original navigator: select the old navigation point, click “Go to XYZ”, take a Record image. Locate the feature in the new image, and add it as a point in the new image. Again, select “Registration point” as before, and make sure the number matches the point in the previous registration (1R1 should match 2R1 and so on).
After repeating for all registration points, in Navigator menu select “Transform Items” to transform the atlas and any old points from the old registration to the new one. You can now proceed normally.

Change back to 135x first for all of the following alignments

24 Optional step: if lamella is being imaged and paceTOMO is not used, pre-tilt determination is required ahead of time.

24.1 Tilt stage to +40’ and take a Record image.
Use shift+drag-left-click to measure top-to-bottom lamella length (appears in log window).
Tilt stage to -40’ and take a Record image.
Use shift+drag-left-click to measure top-to-bottom lamella length (appears in log window).
Whichever gives the longest distance is the direction of the pre-tilt (e.g. +/– 9 to 18’).
Tilt stage to desired pre-tilt and stay at this angle for subsequent steps.
*for PACEtomo routine, change the tilt back to 0° since the script calculates the tilt by itself.

25 Obtain square/lamella detailed montage/maps using los-does presets (42k or 53k)

25.1 Insert objective aperture and check slit is inserted.
Switch to LD data collection IS (the same used for XG calibrations above) and do shift-to-marker with View using one or two square/lamella maps.

Note

To align 4800x with 580x:
In the atlas, find a feature and click 'go to XYZ' in IS
Take a view (should be 4800x), add a green marker on the same feature. If not able to find it, use flu-screen to roughly identify it first then take a view again.
From drop down menu, use Navigator -> shift to marker, tick 2nd options for both tabs (should be 580x to 4800x)
When set, go to XYZ again and take view to confirm the alignment

25.2 Load each square/lamella map in the Navigator and add a point in the centre of the square/lamella for montage view if desired.
Set each points to “Acquire” and select “new file at item” with montage settings (provide an indexable name, e.g. squarev1.mrc).

Note

2x3 at 4800x is normally sufficient for lamella.
Set binning to 1 for more detailed overviews.

25.3 in serialEM drop down menu, select Navigator > Acquire at items. No tasks checked except for montage/map collection.
Make sure Z height is applied by unchecking the mark.
Use LD View for montage and use stage movements.
Save Navigator when done.

26 Inspect detailed square/lamella montages for data collection.

26.1 Under Camera setup in serialEM, check exposure and frame time depending on desired total dose.

Note

For 120e/A2 total dose, use the flux recorded previously (for example, 5.01eps to determine total exposure time:
120/37/5.01 = 0.647s, 37 is the planned total frame number
In this scenario 8 frames per tilt gives 120/37/8=0.4e/A2/frame dose. Gatan suggests 0.6, so 8-frames/tilt is a good starting point.
Use 8 frames to determine fractionated time: 0.649/8 = 0.081s per frame

26.2 In the same tab, set folder for frames to be saved to.
Also set file saving options: tif (LZW compression), set filename as current open file (minus extension), sequential number starting from 0, tilt angle in name.

27 Use sacrificial area on the specimen to check defocus (with target something like 4-5 um) and View/Record image shift before queueing up for data collection.
Use vacuum area next to data collection area to re-check beam centering and ZLP.

28 Add points for details of interest in the navigator. Save it when done.

paceTOMO data collection workflow

29 Under Low Dose control in serialEM, ensure Focus position on tilt axis is 0, i.e. the same area as Recording region.

30 Decide on camera and frames settings:
Focus, Trial and Preview exposures can be set for ~ 1 e/A2, e.g. ~0.2s if dose is around 5e/A2/s
View and Preview should have the same binning, normally 4x.
Decide on Record exposure and number of frames, ideally at least ~ 0.4 e/A2/frame.
Ensure the View defocus offset is 50 microns, rather than 150+ microns.
Record file saving options should have same root as open file (minus extension), with tilt angle and navigator item number included.

31 Under Camera Setup, change the frames folder to a dedicated folder for PACE and ensure dose

fractionation settings are as desired.

Note

Important: avoid changing this in the same PACE session as it creates a conflict with the PACE directory later.

Make another directory dedicated to PACEtomo targets
Check Tilt Axis Offset under the "Tasks > Eucentricity" tab in serialEM is set to the calibrated value, normally -2.2 microns. This is important for a stable defocus across the tilt series.

31.1 Check desired settings in the PACEtomo.py and PACEtomo_selectTargets.py scripts.
No need to change most of the things. Check to make sure:
zeroExpTime is set to 0, unless hybrid scheme is desired.

In PACEtomo.py:
startTilt and max tilt can be changed later.
minDefocus, maxDefocus can be -3 to -5 but can also modify later
delayIS and delayTilt should be 0.5 or even 1
taOffsetPos is something like 0.8, based on Josh's benchmarking on our Titan1 to deal with systematic defocus increase at high tilt

In PACEtomo_selectTargets.py:
Guidance is true for guided anchormap routine
beamDiameter should be something like 1.8 for 42x and suggested dose
maxTilt is ~60° for a 37-tilt tomogram

31.2 Inspect each lamella/square montage and decide on the number of needed PACE groups. Think diligently about how to arrange TS and where to place the sacrificial focus/tracking area - preferably something distinct. One can use Navigator Points on montages.

Note

Lamella can often be covered with one PACE group. But for 20+ micron length lamella, consider breaking into two PACE groups to limit 10+ micron beam shifts. Grid squares may need multiple PACE groups.

31.3 For each PACE group, add Navigator points for intended acquisition areas. As always, think diligently above the beam diameter and spacing of acquisition areas.

Note

To be considered one group, the points must be added in the same cycle of Add Points / Stop Adding in the Navigator.

The first point in the group must be the focus/tracking area and have enough features, e.g. a mitochondrion, a multi-lamella body, bacterial cell, etc.

31.4 Select this first point in group and move stage to XYZ in navigator. Autofocus to -4 µm here. Make sure "move stage for big mouse shifts" is unticked.
Verify grouping by clicking 'collapse' in the Navigator and checking the desired number of acquisition points, including the focus/tracking position.
With the first item in the group highlighted, run PACEtomo_selectTargets from the editor and follow the prompts.

Note

First, give the PACE root a logical and consistent name for the lamella/square, e.g. "L1" or "Sq1", with a PACE group identifier if collecting multiple groups on the same lamella/square, e.g. "L1_P1" or "Sq1_P1".
After that, the script will guide you to finalize the targeting positions of all points in this group.
One can add more targets when all targets are refined following the guidance.

31.5 In the targets GUI, assign at least three additional focus positions with mouse middle-clicks in vacant areas, hit "measure geometry" and wait. Note the estimated pre-tilt and rotation for this group.

31.6 Put in the number for tilt angles and steps based on the estimated pre-tilt.

Note

Note the number put in is negative to the estimation: the start tilt should be the opposite sign of the estimated pre-tilt and rounded to a multiple of the step (e.g. estimate pre-tilt of -10 would have a start tilt of +9 for a tilt step of 3).
Make sure the min/max tilts account for the pre-tilt (e.g. +/- 48 from a 12 pre-tilt would be -36 to 60).

Once done, Save the targets file and close it.

Note

The Navigator should now list all the queued areas for this group. The first point (focus/tracking) should automatically be set to Acquire ("A") with the target text file next to it.

31.7 Repeat above steps for each PACE group.

32 Pre-run checklist:
Focus area 0 microns away.
Untick "stage shift for large mouse shifts".
OA inserted and Slit is in

32.1 Go to "Navigator > Acquire at Items" to run the PACEtomo script at each group. Turn off defocus cycling/focus and make sure "Manage Dewars" is always checked.