FEI Falcon
Direct Electron Detector

Best Practice Document

FEI Falcon Direct Electron Detector
Best Practice Application Guide

1. Introduction

The FEI Falcon Detector is based on direct electron detection using complementary metal oxide semiconductor (CMOS) technology. Due to the radiation damage and signal saturation, conventional charged-coupled devices (CCD) cannot be in direct contact with electrons. Therefore, CCD's rely on a scintillation layer to convert electrons to photons which can then be digitized. During the electron-to-photon conversion process the spatial resolution of the information is reduced. FEI's Falcon Detector avoids the intermediate light conversion step required for conventional scintillated CCD detectors and as a result the spatial resolution of information is retained and digitized. This can be observed in the Detector Quantum Efficiency (DQE) plots between a conventional CCD and FEI's Falcon Detector (Fig. 1).

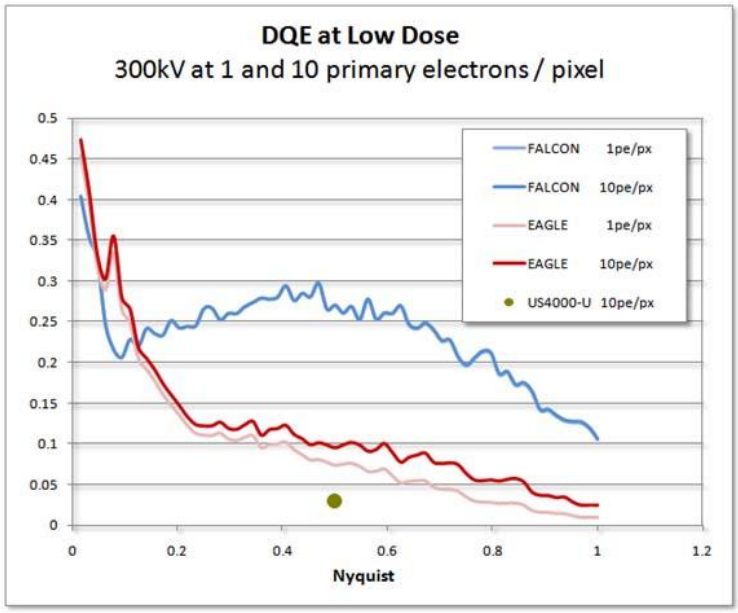


Figure 1. Detector Quantum Efficiency (DQE) graph showing the improvement of signal transfer using the FEI Falcon Detector compared to other conventional CCD's.

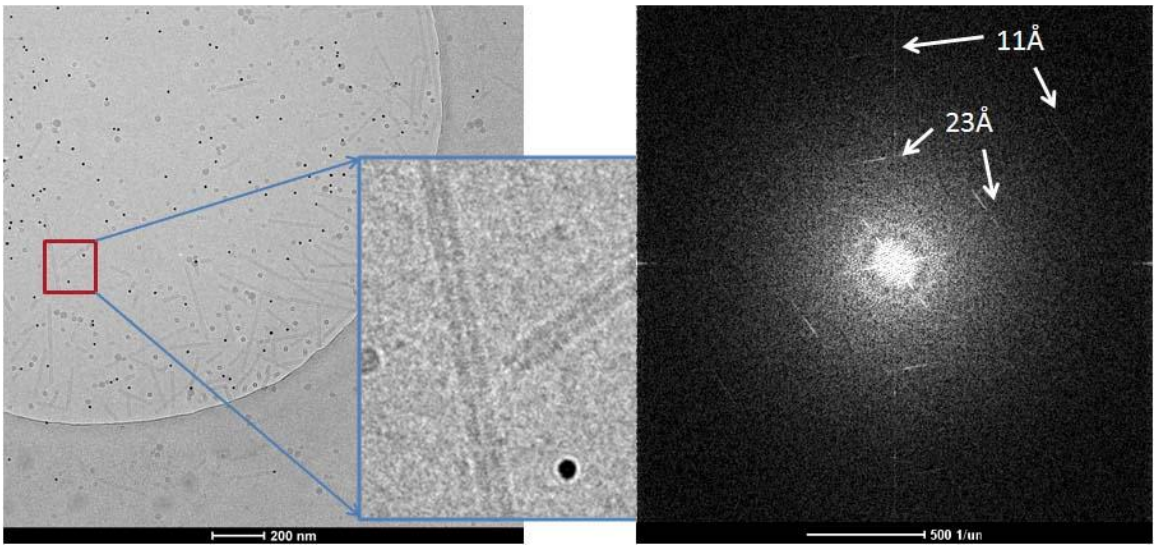


Figure 2. Vitreous sample of Tobacoo mosaic virus imaged under low-dose conditions using an FEI Falcon Dectector. The image is at lower magnification (larger pixel size) than conventional cryo-imaging but still retains the high frequency information (23 and 11.5 Å layer lines) as seen in the FFT.

Cryogenic sample preparation methods ensure that organic samples are observed in close-to-native state. As a consequence of this optimal fixing process, most "soft" materials are highly sensitive to the electron beam therefore limiting the exposure dose for imaging. For single particle acquisition (SPA) and Tomography applications, using the FEI Falcon Detector, images can be collected at a lower dose and digitized with an improved efficiency of information transfer compared to conventional CCD's, which limits the dose and possible beam damage done to the sensitive cryo sample (Fig. 2).

This best practice document is intended as a guide for calibration and proper operation of the Falcon Direct Electron Detector and all associated software.

Operation of the Falcon Detector

1. Prerequisites

Perform these procedures to prepare the TEM for successful operation of the FEI Falcon Detector

1) The correct alignments and FEG registers should be loaded for the high tension and extraction settings used for acquisition. For a guide to a quick TEM alignment check please refer to the online microscope help guide and/or *Appendix A*.

2) Perform Magnification Calibrations for the Falcon detector using FEI magnification calibration software (which is performed on the Orius CCD Camera).

3) The FEI Falcon Detector Dose Protector should be properly calibrated. See *Appendix B* for help or use the F1 help menu in the user interface.

4) Explore3D Tomography Software, EPU, and Low Dose should all be calibrated for proper image acquisition.

2. Prepare the Falcon Detector for Imaging

Acquiring a Gain Reference

1) For preparing the Gain reference, the sample should be removed from the stage. For Tecnai this can be achieved by retracting the side entry holder into the park position and for the Titan the autoloader cartridge can be unloaded from the stage and placed into an empty cassette slot position. Alternatively, one can locate an area within the grid that is devoid of sample, i.e. a grid square where the support film is missing or broken.

2) Make sure that the Falcon camera is inserted (CCD/TV Camera tab – the Falcon camera should be selected from the drop down menu and the insert tab should be active – yellow).

3) Set the illumination conditions to the precise conditions to be used during the experiment. For example of the most commonly used beam setting for low dose imaging please see *Appendix C*. The illumination conditions should result in parallel illumination and the beam should be spread over the entire flu screen (which results in the most accurate Flu screen current reading). For a Low Base Titan the screen current reading shouldn't exceed **1 nA** and for a high base Titan Krios the current shouldn't exceed **0.75nA**.

4) Select the Falcon Reference Image Manager in the Bias/Gain tab of the CCD/TV Camera OCX flap out window. Set the conditions to acquire **160** frames with **10** images to average (Figure 3) and check the 'Remove noise from image' box.

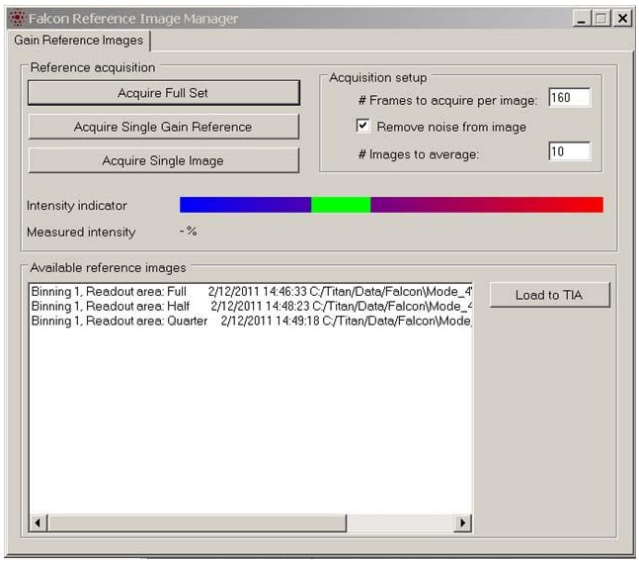


Figure 3. Falcon Reference Image Manager.

5) Press the 'Screen Lift' to retract the fluscreen from the beam.

6) Select **Acquire Full Set**. This will include each of the cameras read out areas – Full, half, and quarter. If desired, select Acquire Single Gain Reference for only the current read out and binning.

Check the Accuracy and Quality of the Gain Reference

1) Once the gain reference task has completed, acquire a bias and gain corrected image using TIA (in the settings tab of the CCD/TV Camera) at the exact same illumination conditions used for acquiring the gain reference. Select the auto correlation button (located in TIA components, correlation, autocorrelation), which will provide an indication of the gain reference quality. There should be a sharp auto correlation peak in the center of the image indicating that the gain reference was acquired properly (Figure 4).

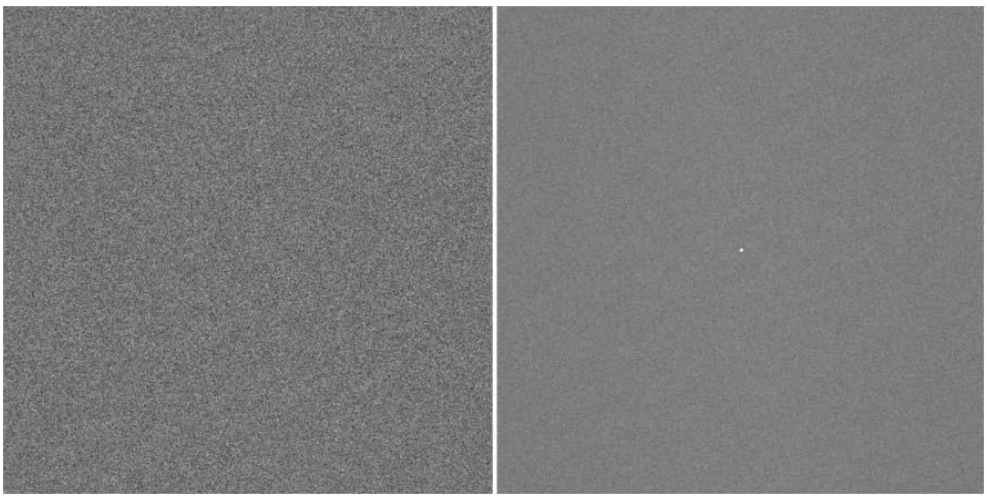


***Figure 4**. Gain Reference image taken with the Falcon detector and the subsequent auto correlation that displays a sharp peak indicating that the gain reference was done properly.*

2) The quality of the gain reference image can be further validated for a peak at the center. If a strong peak is at zero in the gain reference this could influence and potentially cause Auto Functions in FEI Tomography software and EPU to fail (the cross correlation converges on the peak at zero rather than the focus/eucentricity peak).

   a. After acquiring a gain reference, take two separate bias and gain corrected images with TIA under identical illumination conditions (simulating Auto Functions).

   b. Select the cross correlation button in the TIA user interface. This is located in the components, correlation, and then cross correlation. Enter the correct images into the cross correlation window and then proceed. You should see a separate window next to the images with the cross correlation.

   c. Observe the center of the cross correlation image. This can be done with the magnification icon in TIA. Alternatively, a line can be added that exactly crosses the center pixels of the cross correlation image (as shown in Figure 5). There should be no sharp cross correlation peak in the center of the image (Figure 5). In the unfortunate case a significant cross correlation peak at zero is present (Figure 6), in particular under low dose conditions, the number of Images to average in the gain reference should be increased and re-acquired.

3) The Bias/Gain reference information is stored in C:\Titan\Data\Falcon\Mode_4.

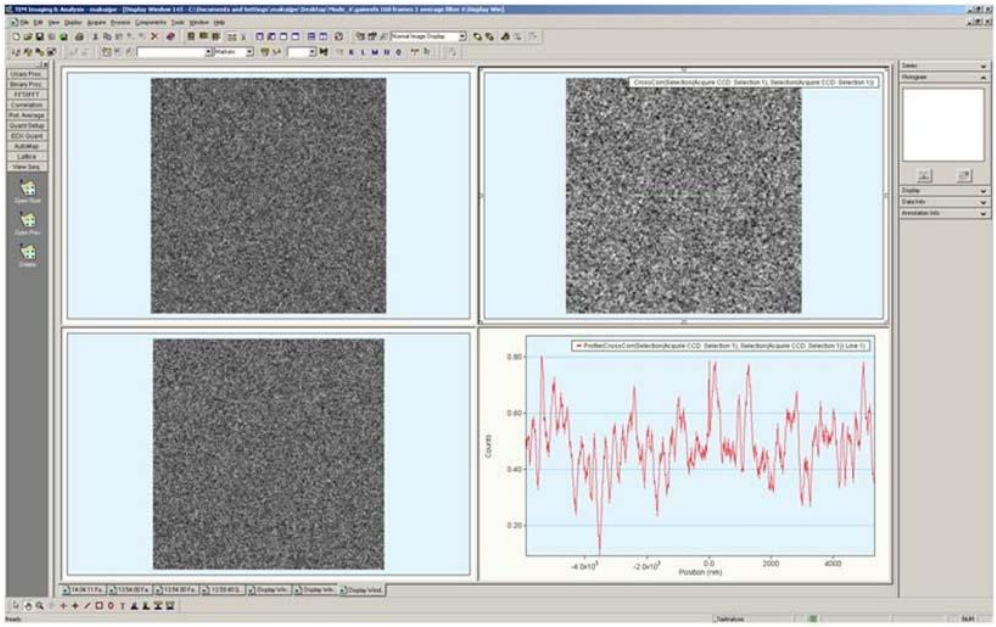


Figure 5*. Two bias-gain corrected image taken with the Falcon detector and the subsequent cross correlation that displays no sharp peak at zero indicating that the gain reference was done properly.*

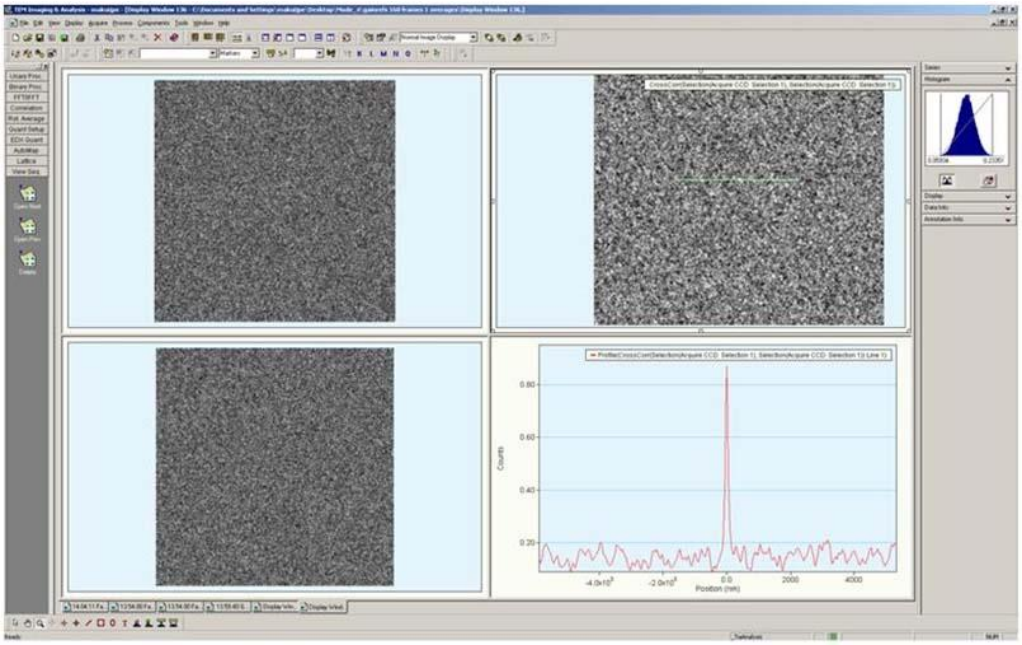


***Figure 6**. Two bias-gain corrected image taken with the Falcon detector and the subsequent cross correlation that displays a sharp peak at zero indicating that the gain reference was done with too few averaging of images.*

Frequency of Acquiring a Gain Reference

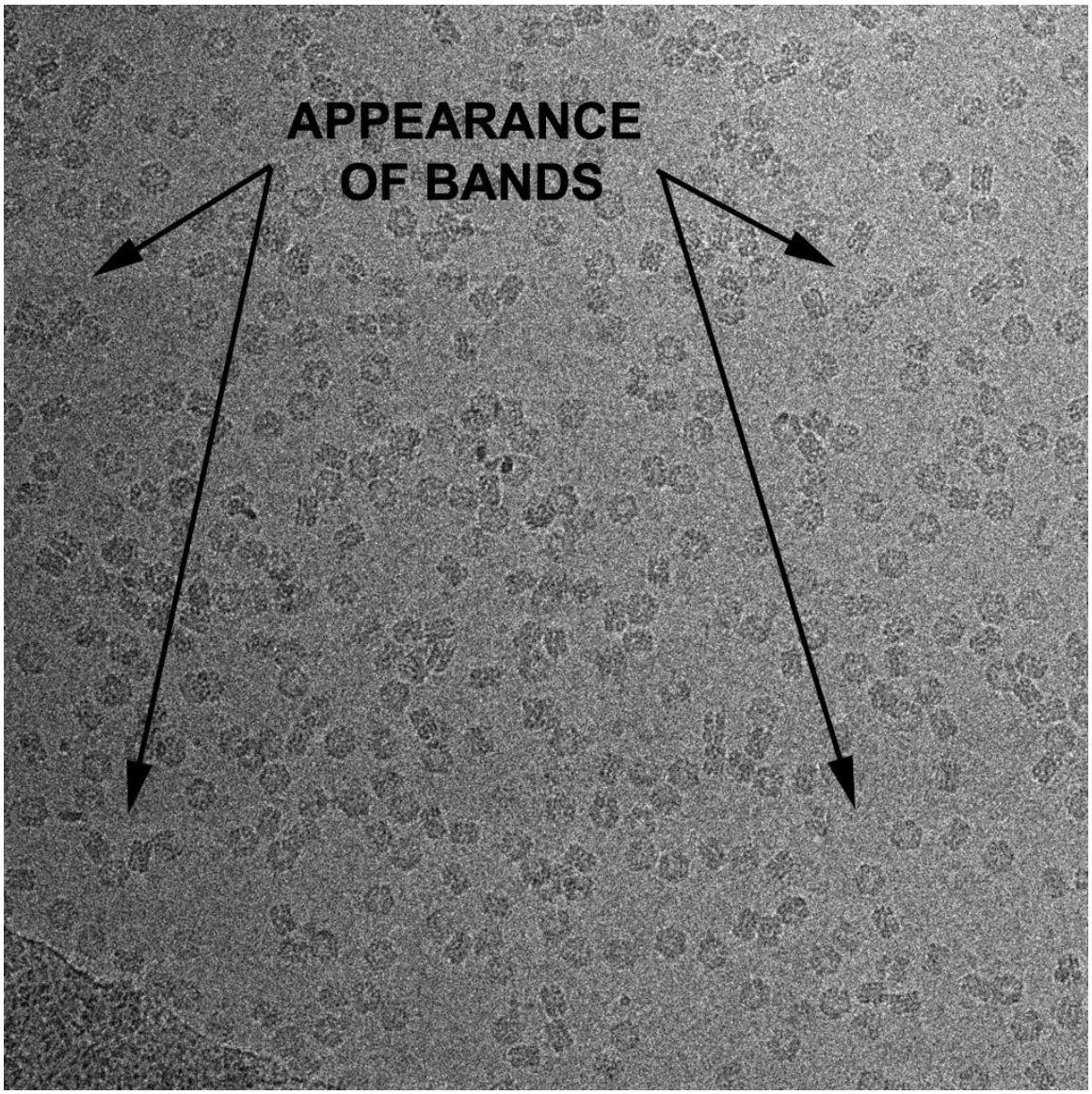
It is wise to acquire an image and check the quality of the gain reference before each session by collecting an image, absent of the sample, at the desired magnification and dose. Perform an auto-correlation or a central peak check as described above. Or, if the desired experimental illumination conditions need to be changed then it is ***absolutely necessary*** to acquire a new set of gain reference images.

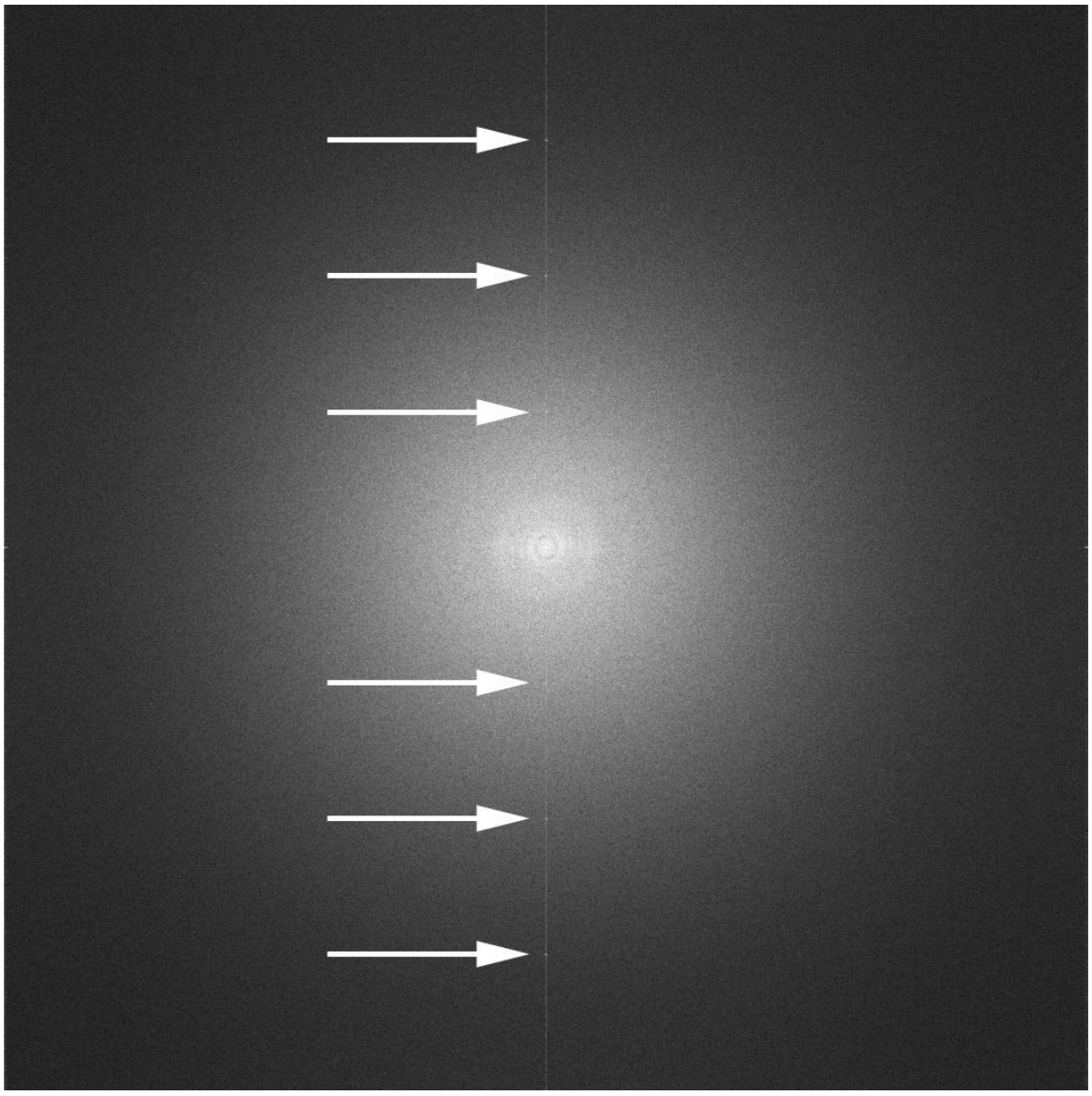
3. Acquiring Images with the FEI Falcon Detector

Low-Dose Imaging with the FEI Falcon Detector

In general, collecting low-dose images using the Low-Dose OCX is the same for the Falcon as it is for any other CCD camera. It should be inserted before acquiring an image. For quick searching and focusing in live mode use the Orius camera or the flu-screen/flu-cam. The user can also use the click-center feature option in TIA and the tomography software to navigate over the grid and the tomography auto focus routine for focusing.

Below is an image that was acquired using different illumination conditions than what was used for acquiring the gain reference. You will observe bands in the image and sharp peaks in the FFT.





Appearance of bands of different grey level (Image) and corresponding Fourier transform components (dots in the FFT, arrows)

Appendix A: TEM System Alignment Check

A well aligned illumination system (gun and condenser system) is critical for the optimized performance of the Falcon detector.

1) Load the most recent and appropriate TEM alignments and FEG register for the active high tension.
2) Check and align the C2 aperture. Center the C2 by alternating the current (intensity) and adjusting it until the beam opens concentrically. (NOTE: For Titan Krios make sure that the C3 is switched ***off*** prior to centering the C2 aperture. This can be done in the 'beam settings' OCX, selecting 'Free Ctrl', and then selecting 'C3 Off' in the flap out. Once the C2 is aligned return to TEM mode).
3) If necessary, do the full gun alignment procedure in the 'Alignments' OCX. Follow the step-by-step instructions in the alignment procedures.
4) Check if the condenser alignment needs to be adjusted. This can be seen by selecting the Auto Zoom function (in the beam setting flap-out) and if the beam size on the viewing screen is roughly the same after switching magnification then it is aligned well. If not then you must realign the condenser system. Also, after pressing the normalize all button the beam should stay in the same position. If not this is an indication of a misaligned condenser system.
5) Check if the displayed illuminated area reading is accurate at 0um and 2um using a x-grating sample or known quantifoil.
6) Check if the output reading of parallel beam (when c2 and c3 are working together) is really parallel. This can be seen in diffraction (ca. 800mm). Press the eucentric focus and check if central spot (or gold rings) are sharp within most of the parallel range, at least until ~ 10um illuminated area it should be reasonable parallel (C2 of 150um). If illuminated area or parallel range is not aligned well then proceed to the condenser tab and align preparation, condenser zoom, and focus + calibration.
7) Select and align the beam tilt pivot point in 'Direct Alignments' and minimize beam wobbling.
8) Select and align the rotation center in 'Direct Alignments' and make the beam movement concentric.
9) Select and align the Coma free alignments for high resolution imaging including pivot points

Appendix B: Falcon Dose Protector Status

Introduction to the Falcon Dose Protector

The Falcon detector is more sensitive to electron-beam damage than scintillated CCD and therefore should not be overexposed with an excessive amount electron dose. A dose protection system has been implemented in the software which protectors the Falcon detector from potentially damaging situations. In addition, it protects the camera in unsupported modes.

Unsupported Operating Modes

There are a number of operating modes on the microscope that will not allow recording of signal on the CMOS camera. The exact behavior will depend on the main screen position and the mode. In some cases it is obvious that the user has consciously selected a truly incompatible mode and in that case the camera will be retracted. In other cases the mode switch may be due to an overshoot with magnification or activating diffraction mode unintentionally. In that case only the blanking is done.

As long as the main screen is down, no action will be taken. When the screen is raised, the blank system of the protector will be activated and the CMOS camera will be retracted (if needed). Once a suitable delay time (to allow the CMOS camera to retract fully) has elapsed, the blanking is undone.

CMOS use is thus not possible in:

EFTEM (retract)

LM (blank)

All diffraction modes LAD, D (blank)

Lorentz mode (retract)

STEM (retract)

On Titan the Probe and Free Control modes are also excluded, images can only be recorded in the Parallel mode.

User Interface

Falcon Protector Status displays the state of the detector. If the detector is safe with the current microscope settings then the display will read '*Safe, Protector Enabled*'. This means that the protector is actively keeping track of the detectors safety and no actions are taken to prevent imaging.

If the microscope is in an unsafe mode for the Falcon detector then the Safety status will display 'Unsafe' in red letters followed by an explanation of why it is in an unsafe mode, for instance the dose calibrations have not been done ('Unsafe. Falcon protection active, beam current/ not calibrated.')

The **Hardware** tab shows the status of the post-specimen beam blanker, the Forced blanking will be either 'Off' or 'On". The Falcon status, which includes if the Falcon detector is 'inserted' or

'retracted,' and the last known inserted status. Also displays if the fluscreen is 'inserted' or 'retracted'.

The **Calibration** tab shows the current status of each of the three calibrations

Falcon Dose Protector Calibrations

The Falcon camera is designed for low-dose, low intensity beam settings therefore the 'dose protector' prevents excessive overexposure on the detector. The calibration procedures have three steps, the first two resulting in persistent calibrations stored in the registry; the third (beam current) not being persistent. For the Titan a caveat exists: the condenser system and gun must be well aligned. If not, the alignment must be done and that may take some time (and possibly another specimen – cross-grating – if the illuminated area calibration for Microprobe is severely wrong). When the defocus is severely off from the eucentric focus position also the measurement could not be reliable, therefore a limited deviation in focus is allowed from the eucentric focus position. Before starting the calibrations the user should first press the eucentric focus button and then perform the following steps when requested.

Initial Set-up

1) Select and center the 150 µm C2 aperture
2) Bring the sample to eucentric height and focus (press the eucentric focus button and if calibrated correctly should bring the objective lens in focus at the eucentric height).
3) Select the Falcon Tab in the Workset (if not not present then create a new tab called Falcon and place 'Falcon Protector', 'Falcon Protector Status', and 'CCD/TV Camera' from the 'User workspace layout).
4) Select Illuminated area from the drop down menu and click the calibrate tab. Follow the instructions for proper calibration. If properly aligned then the software will acknowledge that the illuminated area is calibrated, if not then it will read 'not calibrated'. See below for troubleshooting help if the calibration doesn't succeed.
5) Once illuminated area is calibrated then select focused beam and current. Again, follow the instructions for alignment.
6) Once Focused beam and current is calibrated then select 'Beam Current'.

Illuminated Area Calibration

The procedure differs for Tecnai and Titan. On Tecnai we determine the C2 setting for five beam sizes that the user must match, first focused beam and under- and overfocus to the 40 mm circle for the largest C2 aperture, then under- and overfocus to the 40 mm circle for a smaller C2 aperture.

A check is made on:

- The linearity of the match.
- Consistency of the beam currents measured with the different aperture sizes.

For Titan we check two illuminated-area settings (for Microprobe and Nanoprobe), for a focused beam and for an overfocused beam that matches the 40 mm circle. Only overfocus is used because underfocus is not always achievable. For the focused beam the absolute value of the indicated illuminated area must < 500nm, for the overfocused beam the ratio between the calculated illuminated area (working back from 40 mm by the magnification) and the indicated illuminated area, corrected for the focused-beam offset, must be between 0.25 and 4.

Focused beam and current

During the focused beam and procedure all spot sizes are focused and their current is measured. For the Titan an exception is made for spot size 1 which is not supported (large deviations plus frequent clipping problems because of X-ray safety). For Tecnai we measure the C2 offset, while for Titan we check that the illuminated area for focused spots other than spot 3 does not deviate significantly from 0.

Beam current

The beam current calibration is performed at the start of each microscope session and whenever the Protector detects that a signification optics value (like gun tilt) has changed. This procedure simply means that a standard low magnification is chosen; the largest C2 aperture and spot 3 are selected. The user must make sure nothing blocks the beam and the beam current is measured.

Frequency of calibration

Whenever illumination changes are involved the dose calibration has to be redone. This occurs when any FEG settings are changed or when the microscope alignments were redone. When changing the high-tension, gun lens settings or extraction voltage, the focus beam and beam current (step 2 and 3) have to be performed again.

Appendix C: Commonly Used Low Dose Imaging Parameters

Below is a quick guide to describe the most commonly used imaging conditions for low dose experiments. It is divided into two parts, Cryo-Electron Tomography and Single Particle Acquisition.

Cryo Electron-Tomography

1) 300kV, Gun lens 3, 3950 extraction voltage, TEM Mode
2) C2 Aperture Inserted (50 -150 um), Objective Aperture inserted (30 – 100 um)
3) Low Dose Mode activated
    a. Exposure Tab – Target dose of 1 e/A2 on a properly calibrated flucam, 1 second integration time (s), binning 1, full read out area.

Single Particle Acquisition

1) 300kV, Gun lens 3, 3950 extraction voltage, TEM Mode
2) C2 Aperture Inserted (50 -150 um), Objective Aperture inserted (30 – 100 um)
3) Low Dose Mode activated
    a. Exposure Tab – Target dose of 10 e/A2 on a properly calibrated flucam, 1 second integration time (s), binning 1, full read out area.