APPLICATION NOTE

Fringe-Free Imaging (FFI)

1. Introduction

1.1. Fringe-free imaging

Electrons can be described as both particles and waves. In a microscope column, when electrons pass through a small opening like a condenser 2 (C2) aperture, they are diffracted by the edge of the aperture (Fig. 1A). The upper pole piece of the objective lens (OBL) produces an image of the C2 aperture. This image does not always coincide with the sample plane, which is focused by the lower OBL pole piece and is imaged on a camera/fluorescent screen. This leads to simultaneous imaging of the focused sample and the C2 aperture, which is out of focus. When the C2 aperture is imaged out of focus, wave interference at the edge of the condenser beam appears as Fresnel fringes (Fig. 1B). Fresnel fringes reduce the useful beam area for data acquisition and limit the number of images that can be recorded from a single hole (Fig. 1C).

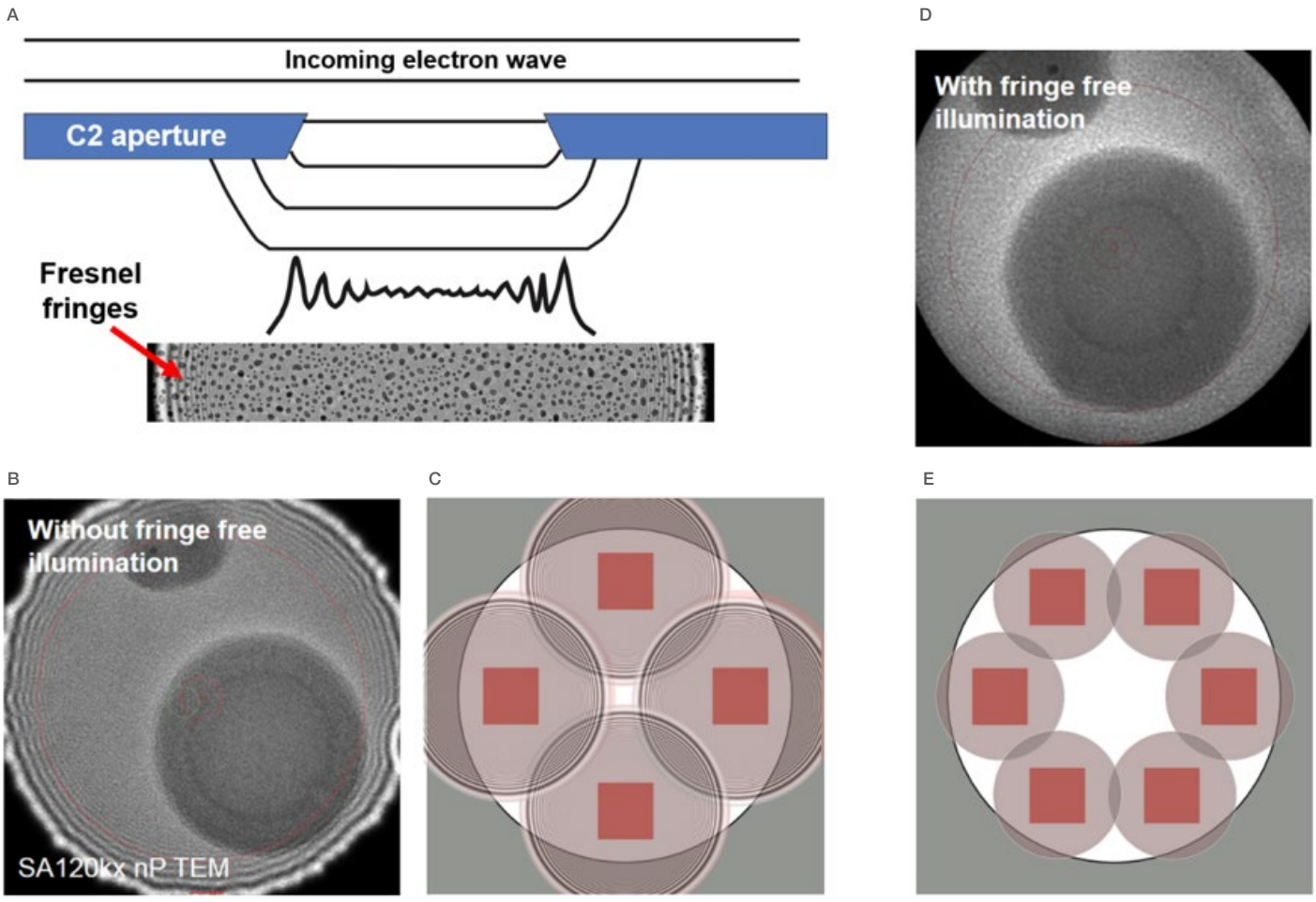

Figure 1. Fringe-free imaging. A) C2 aperture diffracts the electron beam and generates Fresnel fringes. B) Beam image where the C2 aperture is out of focus. Multiple Fresnel fringes are visible. C) Fresnel fringes reduce the number of images that can be recorded per hole. D) Beam image when the C2 aperture is in focus. No Fresnel fringes are visible. E) Fringe-free imaging allows more images to be taken per hole.

To minimize the presence of Fresnel fringes in the recorded image, the stage position needs to be adjusted to bring the specimen to the image plane of the C2 aperture produced by the OBL upper pole piece. The lower OBL pole piece strength is adjusted so that the sample is focused on the camera imaging plane. With this setup, both the C2 aperture and the sample will be in focus and no, or very few, Fresnel fringes will be visible in the image recorded by the camera (Fig. 1D). This allows reduction of the beam size and more images can be acquired from a single hole (Fig. 1E).

In theory, FFI can be achieved using any microscope by driving the stage motor to change sample height and adjusting the defocus of the sample. However, this means that the sample will no longer be at eucentric height anymore. Thermo Fisher Scientific FFI modification is a mechanical stage adjustment where the whole stage is lowered to the required Z-height using stage wedges. The tilt axis and eucentric height are retuned to work optimally at that stage location. This allows seamless acquisition of single-particle and tomography data with FFI.

1.2 FFI increases productivity

The smaller beam size enabled by FFI means more images from a single hole can be recorded. This allows more efficient use of the grid surface available for imaging. In addition, the increase in exposure areas per hole enhances acquisition speed by reducing the number of times stage movement (including settling time) and autofocusing must be performed during the data acquisition run. The exact throughput enhancement depends on several variables like grid hole size and spacing, camera type, and magnification. The combination of FFI with Aberration-Free Image Shift (AFIS) offers the highest throughput increase when compared to a standard data acquisition scheme (Fig. 2).

1.3. TEM system compatibility

FFI is a standard feature on every Thermo Scientific™ Krios™ G4 Cryo-TEM. Earlier Krios Cryo-TEM models (G1, G2, G3i) need to undergo modifications in order to operate in FFI mode. The Krios Cryo-TEM G1 is only FFI compatible with the plan 3 Autoloader systems. FFI modifications are not available on Thermo Scientific™ Glacios™ Cryo-TEM or Thermo Scientific™ Arctica™ TEM systems. FFI can only be used in nanoprobe (nP) mode.

1.4. FFI installation and calibration summary during a field upgrade

FFI requires physical adjustment of the stage position (~60 µm displacement) and correction of the Autoloader sample handover position. Stage height is adjusted using this procedure:

- Preconditions achieved: nP mode and parallel illumination, 96kx magnification in TEM or 165kx EFTEM, beam diameter set to ~600 nm
- Objective lens excitation increased until Fresnel fringes are not visible
- Image focused by lowering stage Z-height
- Stage wedges adjusted to redefine eucentric height

After stage height adjustment these alignments need to be performed:

- Align nanoprobe
- Align HM-TEM>Basic SA, Basic Mh
- Condenser> focus + calibrate µP, focus + calibrate nP
- If needed: HM-Stem, Stem µP, Phase Plate µP and nP, dyn con., df µP and nP
- Redo magnification calibration
- Recalibrate dose protector
- Redo AFIS alignments
- Redo EPU and tomography (SerialEM/Leginon) calibrations

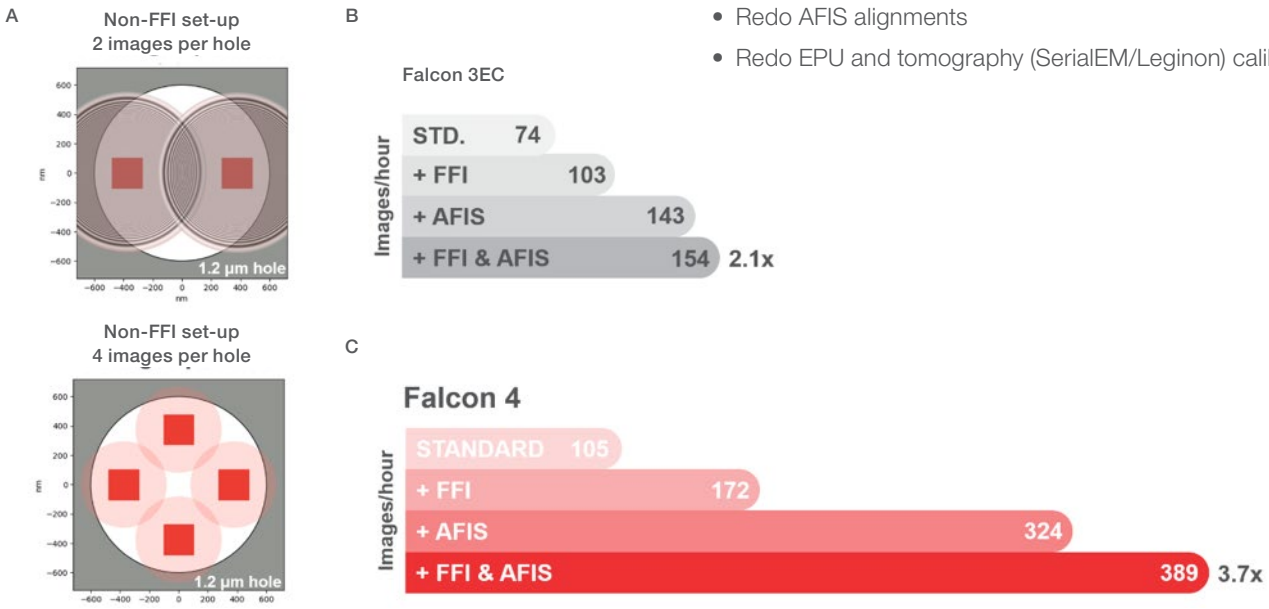

Figure 2. Example of how FFI increases data acquisition throughput. A) Experimental setup for r 1.2/1.3 grids. Non-FFI setup allows only 2 exposure areas versus 4 for FFI setup using a pixel size of 0.5 Å/pix. B-C) Throughput rates for Falcon 3EC (B) and Falcon 4 (C) cameras during standard conditions and in combination with FFI and AFIS.

2. Practical considerations

If the TEM system has FFI modifications implemented and the relevant calibrations have been completed, then it is ready to use straight away. However, FFI has several differences when compared to a normal TEM illumination that are discussed below.

2.1. FFI beam appearance in TEM mode

When the stage height is readjusted to meet the FFI requirements, the electron beam has a different appearance when condensed to a spot. It is not as small and round anymore (Fig. 3). By selecting a smaller C2 aperture, the spot can be made smaller and more similar in appearance to a non-FFI system. However, the appearance of the beam does not influence the performance of the system in TEM mode. Condenser astigmatism is now best optimized by adjusting the stigmators to create a round beam in parallel mode instead of optimizing the spot shape by condensing the beam.

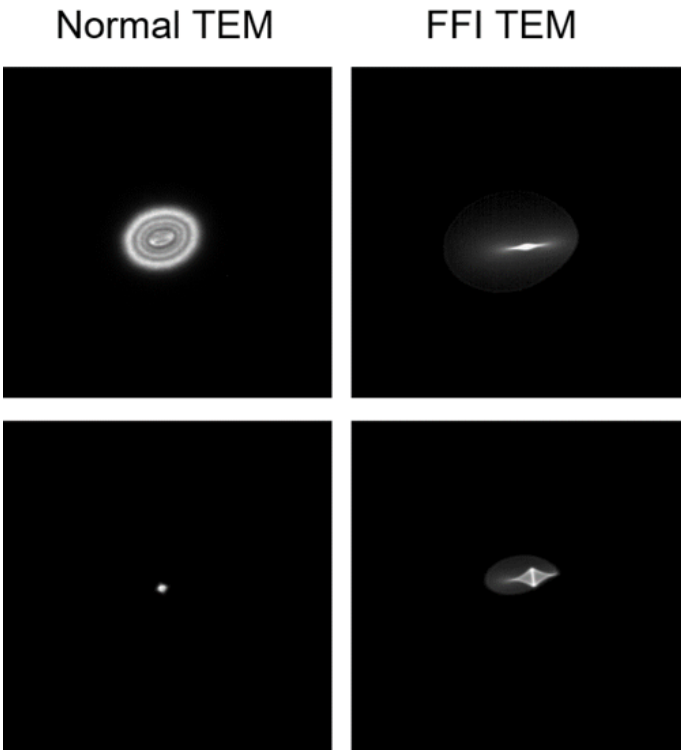

Figure 3. Beam appearance changes in normal and FFI TEM modes

2.2. Magnification range and defocus

FFI alignments are done at medium-high SA magnifications (96kx) corresponding to a beam diameter of around ~600 nm. When the beam diameter is varied by adjusting the condenser lenses, this also affects the focus of the C2 aperture and therefore the quality of FFI with several more fringes appearing in the image. Beam diameter changes are needed when working at different magnifications, and thus, working at lower or higher magnifications will result in slightly more fringing. This will not limit the use of FFI for magnifications that are typically utilized for single-particle analysis (SPA) experiments with a narrow beam diameter.

A similar effect will occur when applying an additional defocus with the objective lens. Such a defocus will change the focus of the C2 aperture and introduce some fringes around the edge of the beam. However, defocus values typically used in SPA experiments (1-4 µm) have a limited effect on fringing and will not hinder the FFI application.

2.3. Beam cut-off

On systems with an anti-contaminator aperture (e.g., Krios G3i), the beam can be cut-off at low magnifications below 5kx.

2.4. Beam tilt and shift pivot points

On systems with FFI configuration, the recommended way to adjust beam tilt pivot points for TEM experiments is to work with a parallel beam and change the magnification to allow displaying the full beam on the flu-cam viewer. Pivot points are then adjusted by minimizing the beam movement during the alignment. In systems where the Volta Phase Plate is used, the beam tilt pivot points cannot be adjusted perfectly anymore, since the perpendicular correction alignment for the beam tilt pivot points has been disabled to prevent the inadvertent misalignment of the beam shift pivot points. If the beam tilt pivot points are very far off, the advice is to iterate between beam tilt pivot points and rotation center alignments or to redo the beam shift pivot points in the phase plate alignments.

2.5. Probe appearance in STEM mode

In STEM mode the spot on FFI systems looks slightly hazier compared to non-FFI systems. In addition to this, the sensitivity of the condenser stigmators is reduced, so the stigmators might report higher values after stigmation. The probe alignments and STEM are still conducted in the same way. However, differences in semi-convergence angles need to be expected as compared to non-FFI systems. This is all normal behavior and regardless of these differences the Krios STEM resolution specifications will still be met.

References

Nakane, T. et al. Single particle cryo-EM at atomic resolution. Nature, 2020, doi.org/10.1038/s41586-020-2829-0

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Notes

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