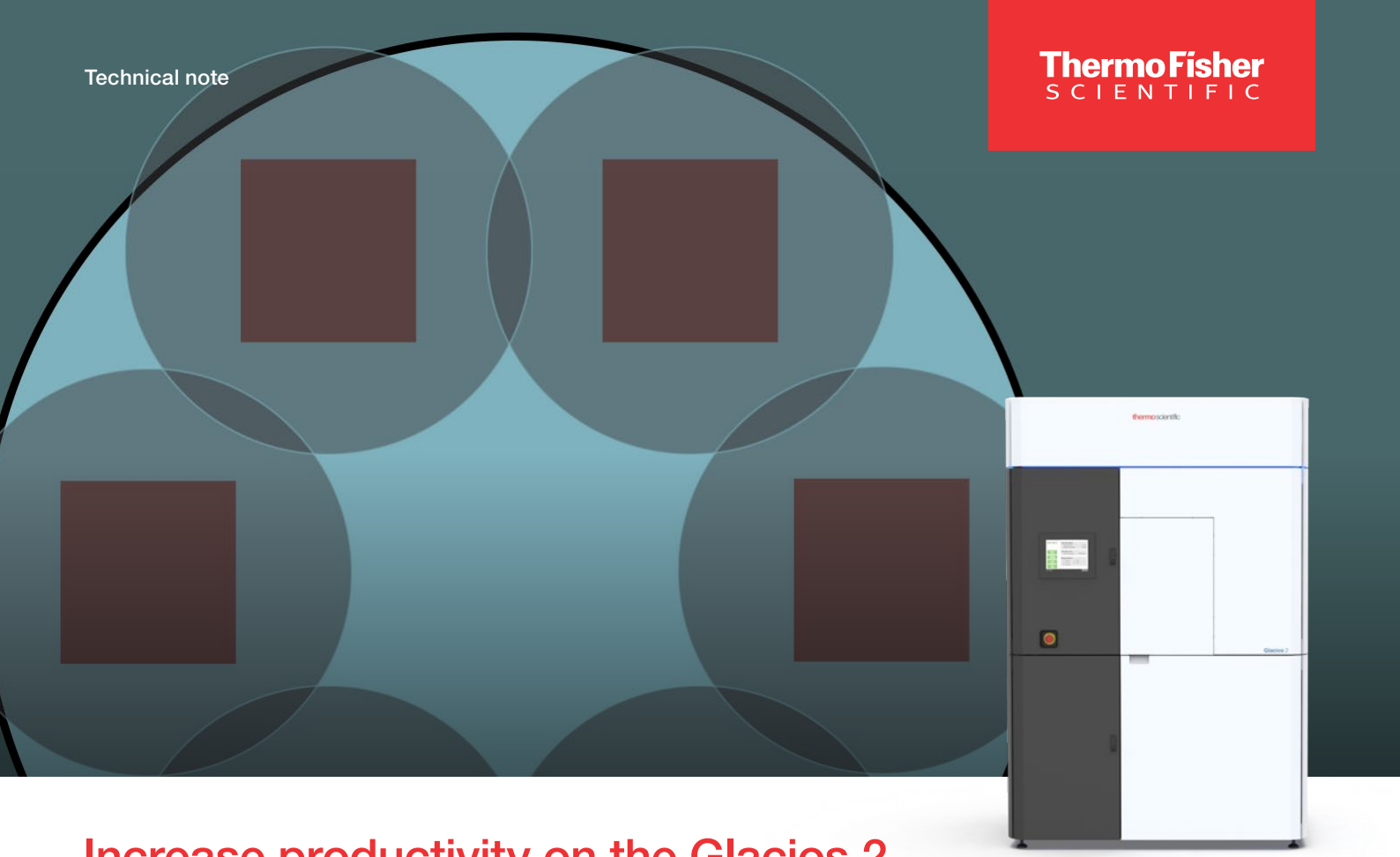
Technical note



Increase productivity on the Glacios 2 Cryo-TEM with fringe-free imaging

Electrons can be described as both particles and waves. In a microscope column, when electrons pass through a small opening like the condenser 2 (C2) aperture, they are diffracted by the edge of the aperture and the interference of waves results in Fresnel fringes (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 and 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 which can be recorded from a single hole (Fig. 2A).

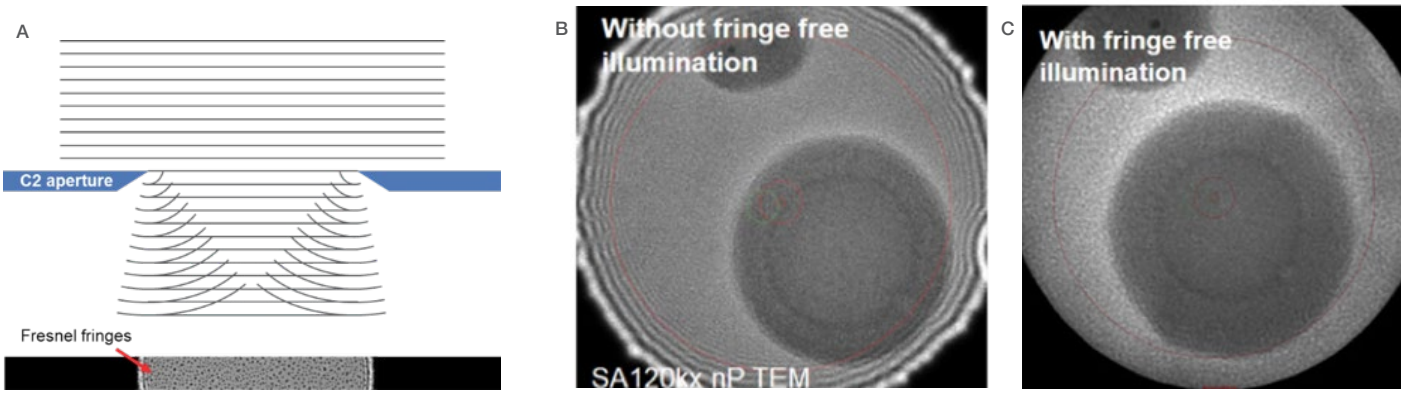


Figure 1. Fringe-free imaging. A) C2 aperture diffracts the electron beam and generates Fresnel fringes. B) Beam image with C2 aperture out of focus. Multiple Fresnel fringes are visible. C) Beam image with C2 aperture in focus. No Fresnel fringes are visible.

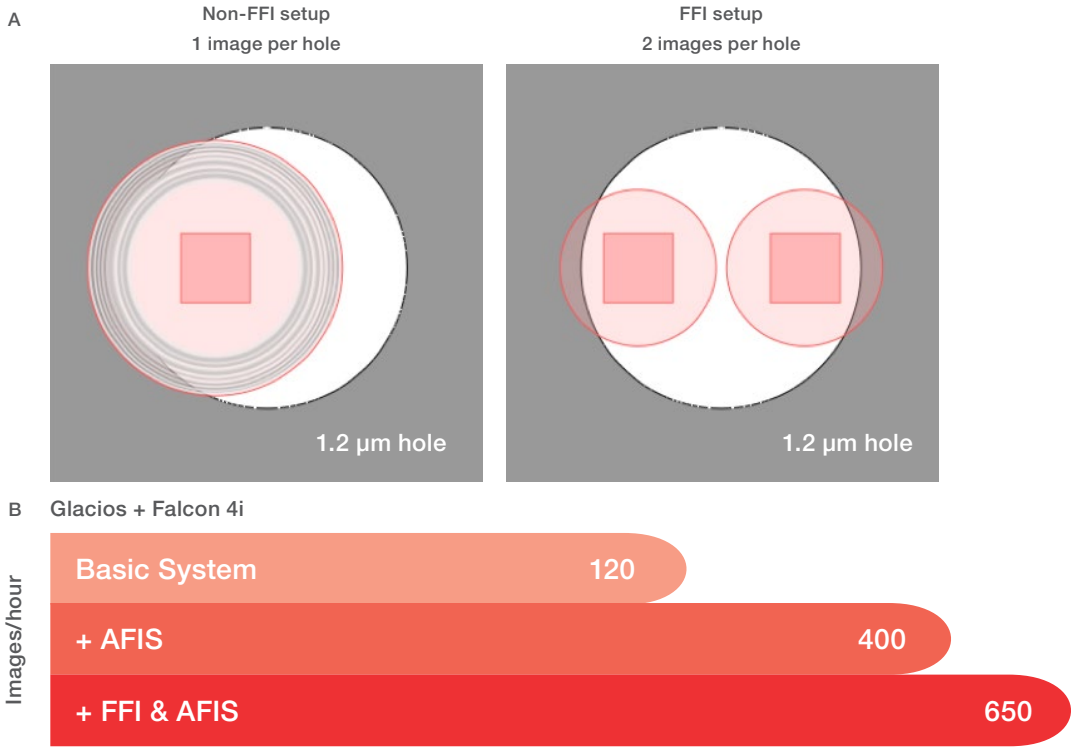


Figure 2. Example on how FFI increases data acquisition throughput. A) Experimental setup for R 1.2/1.3 grids. Non-FFI setup (30 µm C2 aperture) allows only one exposure area while FFI setup (20 µm C2 aperture) allows two. Both are using a pixel size of approximately 0.75 Å/pix. B) Throughput rates for Falcon 4i camera during standard conditions and in combination with FFI and AFIS.

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. 1C). This allows reduction of the beam size and more images to be acquired from a single hole (Fig. 2A).

The smaller beam size produced by FFI generates more images from a single hole that 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).

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. The FFI modification to the Thermo Scientific™ Glacios 2™ Cryo-TEM is a mechanical stage adjustment: the whole stage is lowered to the required Z-height using stage wedges. The tilt axis and eucentric height are re-tuned to work optimally at that stage location. This allows seamless acquisition of single particle and tomography data with FFI.

Compatibility

FFI only works in nanoprobe (nP) TEM imaging mode. It does not work with the microprobe (µP) because the mini-condenser lens affects the image position of the C2 aperture and does not allow for FFI.

FFI alignments on the Glacios 2 Cryo-TEM are done at medium-high SA magnifications, with a magnification that results in a pixel size of approximately 0.9Å/pixel. Different magnification ranges (LM, Mi, Mh) cannot be used with FFI because they do not satisfy the conditions for FFI. Nonetheless, applications such as single particle analysis and tomography are not affected by this as they require medium-high SA magnifications that work well with FFI.

When additional defocus is applied with the OBL, such defocus will also 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 to 4 µm) have limited effect on fringing and will not hinder the FFI application.

Methods

If the TEM system has FFI modifications implemented and the relevant calibrations have been completed, then it is ready to use immediately. However, FFI has several differences when compared to normal TEM illumination.

With FFI on the Glacios 2 Cryo-TEM system, which uses two-condensers, the diameter of the electron beam cannot be changed in nanoprobe mode by varying the excitation of the C2 lens because the plane of the C2 lens is imaged on the sample and thus has negligible effect on the beam. (In microprobe mode, the beam behaves as expected). The diameter of the beam in nanoprobe mode can only be changed using a different C2 aperture size. Instead of the diameter, the position of the beam may be affected when modulating intensity in nanoprobe mode. In practice, when operating at low to intermediate SA magnifications (e.g., for hole centering), working in nanoprobe mode is impractical and we advise using microprobe mode. Setting up parallel illumination remains a crucial step for high-resolution data collection in nanoprobe mode. This is done in the same way as on a non-FFI two-condenser system by focusing the beam on the back-focal (objective aperture) plane of the objective lens (in diffraction mode) by varying the C2 lens excitation.

The fact that the beam cannot be focused onto a spot in FFI mode influences the way direct alignments are performed. Specifically, when doing the direct alignment manually, the following alignments are impacted:

- C2 aperture: The recommended way to center the C2 aperture on a system with FFI is to switch from imaging mode to diffraction mode, in which you can condense and spread the beam and center the C2 aperture using a similar protocol as in the traditional imaging mode.
- Beam shift: The recommended way is to change the magnification until the beam edge is visible.
- Beam tilt and shift pivot points: Similarly, the recommended way is to change the magnification to display the full beam on the flu cam viewer, then adjust pivot points by minimizing the beam movement during the alignment.

An automated procedure is available on the Glacios 2 Cryo-TEM. For detailed instructions, please contact **thermofisher.com/em-contact**.

Conclusions

FFI removes fringes at the edge of the beam and increases productivity when using the Glacios 2 Cryo-TEM. Under FFI conditions, the beam behaves differently than in the non-FFI mode. The most prominent difference is that the electron beam cannot form a probe by varying the excitation of the C2 lens. A new method of manual optics alignment is needed and briefly described in this document. Finally, STEM mode is not compatible with FFI.

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