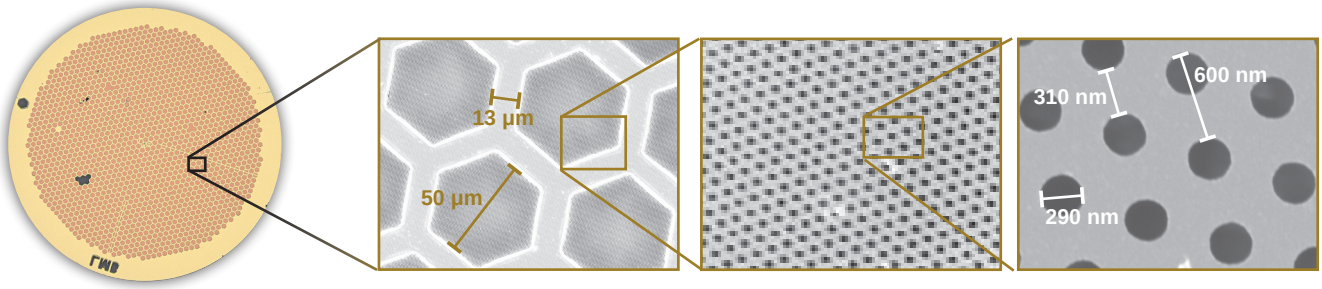
Improving every image with HexAuFoil® sample supports

Best practice suggestions for cryoEM protocols utilising HexAuFoil® sample supports, as discussed in Naydenova and Russo, Ultramicroscopy, 2022.

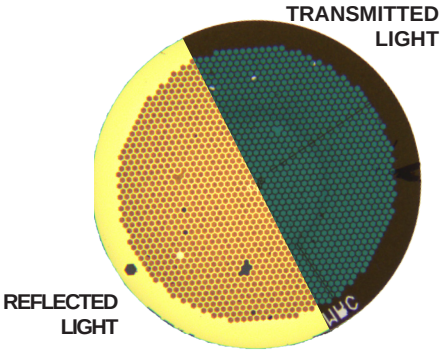
Appearance and handling of HexAuFoil® sample supports

HexAuFoil® sample supports consist of a holey gold foil on a hexagonal gold mesh grid. The grid hexagons are approximately 50 µm in size with ~10-12 µm diameter bars. The gold foil has 290 nm diameter holes arranged in a hexagonal array with 310 nm spacing, resulting in a 600 nm repeat, as illustrated below.



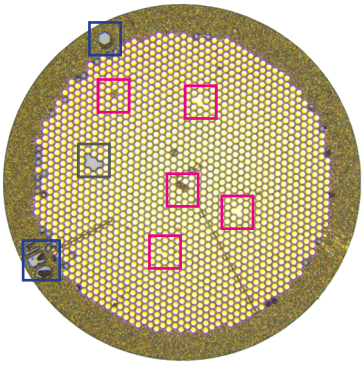
HexAuFoil® sample supports should be handled in the same way as other gold films or traditional carbon foils, and are similarly robust. As with other foils, care should be taken when handling HexAuFoil® sample supports with tweezers and during plunge freezing. If the foil is damaged in these processes, the stability of the support may be severely degraded. We recommend collecting data only from hexagons where the foil is uniform and intact.

When inspected by eye, HexAuFoil® sample supports should appear shiny gold similar to UltrAuFoil® grids. However, when using a microscope to check for foil integrity, the appearance will change depending on the type of illumination selected. Under **reflected light**, the grid bar side will be **light yellow**, and the foil side will be shiny with a brown tint (LHS in image). In contrast, if HexAuFoil® are viewed using **transmitted light**, plasmon resonance from the regularly-arranged small holes will make the foil appear **blue** (RHS in image). The film side of the grid may occasionally appear blue at the edge of the grid and over grid bars for similar reasons.



Fiducial marks

The grids include a number of fiducial marks to aid with orientation. There are 2 fiducial marks on the rim of the grid which are visible by the naked eye (differing slightly from those illustrated here). In addition, there are a further 6 on grid fiducial marks to aid with orientation. The largest on grid mark, bottom left in the illustration, is free of foil to facilitate microscope alignments and flux measurements.



FIDUCIAL VISIBLE BY EYE

ADDITIONAL FIDUCIAL TO AID ORIENTATION IN MICROSCOPE

FOIL-FREE FIDUCIAL FOR SCOP ADJUSTMENT AND FLUX MEASUREMENT

HexAuFoil

Sample preparation

Plasma Cleaning

HexAuFoil® grids should be plasma-cleaned or glow-discharged to increase their wettability prior to use. In general, for best results, they will need glow discharging for significantly longer than other sample supports, such as UltrAuFoil® holey gold supports. However, the gold foil is not volatile when glow-discharged or plasma-treated, so the grids may be subjected to more extensive plasma treatments than standard carbon foils, without any risk of degrading the surface. A good rule of thumb is to approximately double the plasma-cleaning/glow-discharge times compared to UltrAuFoil® grids. A table of suggested settings is provided below for some common glow discharge instruments (as originally published in Naydenova and Russo, 2022).

PARAMETER	FISCHIONE 1070	EDWARDS S150B	TEDPELLA EASIGLOW
ATMOSPHERE	9:1 Ar:O_2	Residual air	Residual air
PROCESS PRESSURE	21 mTorr	150 mTorr	0.39 mBar (290 mTorr)
POWER/CURRENT	40 W	30 mA	25 mA
EXPOSURE TIME	120-180 s	60 s	90 s

SOME SUGGESTED PLASMA CLEANING SETTINGS

Vitrification Settings

In general, HexAuFoil® grids can be directly substituted into your vitrification protocol, with the exception that blot times should be extended compared to similar samples on a standard larger hole grid. For example, if you currently blot for 10 seconds, with HexAuFoil® grids, you should initially try a 15 second blot. We recommend applying sample to the foil side of the grid and blotting from the same or both sides, but this is sample dependent, and any variation can be used.

Typical settings for vitrification with a HexAuFoil® grid on some common plunge freezing instruments are given below by way of example (taken from Naydenova and Russo, 2022).

PARAMETER	MANUAL PLUNGER	VITROBOT MKIV	LEICA GP2
TEMP	4°C	4°C	4°C
RELATIVE HUMIDITY	100 %	100 %	100 %
WAIT TIME	0 s	0 s	0 s
FORCE SETTING	n/a	10	n/a
BLOT TIME	15 s	5 s	5 s
DRAIN TIME	0 s	0 s	0 s
VITRIFICATION MEDIA	Ethane	Ethane	Ethane
VITRIFICATION TEMP	93 K	93 K	93 K

SOME SUGGESTED VITRIFICATION SETTINGS

Microscope alignment, calibration, and data collection

We strongly recommend very careful alignment of eucentric height and calibration of image shift immediately before data collection to ensure the best results. Due to the high consistency and dense design data collection time can be halved even including the additional time taken in set up.

Software

EPU

EPU has been updated to handle HexAuFoil® hexagonal mesh grids with increasing functionality from 3.1 onwards.

SerialEM

SerialEM is also able to automatically identify HexAuFoil holes, if using version Testing-4.1 or later.

Data collection settings

Sample data collection settings are provided below, from a Titan Krios. Aberration-free image shift (AFIS), fringe-free illumination with no specimen tilt is preferred to take full advantage of the dense hexagonal array. Data collection is optimised with a single exposure per hole, one entire hole per image. Use magnifications corresponding to pixel sizes around 0.5–0.8 Å/pix on the detector to achieve this. The beam should be concentric with the hole with diameter ~500 nm, ightly larger than the 290 nm diameter holes, but small enough to avoid exposing adjacent holes. With a 600 nm centre to centre distance, and ±100 nm error in beam position, beam diameters up 700 nm are acceptable.

Larger beam sizes

Users can use a larger beam diameter (1µm), but does require some holes to be skipped, to avoid double exposure. This adaptation was used in Naydenova, Jia and Russo, Science, 2020, and some example settings are provided below (from a Glacios)

Alignment and Calibration

The regular hole lattice may be used for beam diameter and image shift calibrations. In addition, during processing, the edges of gold foil provide a built-in magnification calibration using the gold lattice reflections (2.347 Å). As with UltrAuFoil® holey Gold supports, a separate grid with an amorphous foil (normally carbon) will be required for other alignments including coma and astigmatism.

Data processing

In general, data processing protocols for HexAuFoil® grids are unchanged from data collected on traditional sample supports. However, the following changes to standard protocols should be considered:

1. The presence of the gold foil in every micrograph provides a built-in magnification calibration using the gold lattice reflections (2.347 Å). These edges are also high-contrast fiducials for motion correction.

2. The lace of electron beam induced movement of the vitreous ice means only whole-micrograph motion correction without subdivision into patches is necessary.

3. Wint no movement other than stage drift, bayesian polishing can still be used to confirm the absence of correlated particle movements within each hole but is not necessary.

4. Final high-resolution reconstructions can be used to extrapolate the structure factors of the molecule to those of the undamaged structure, at zero electron dose (see over page).

	ATLAS		GRID SQUARE		HOLE/EUCENTRIC		DATA ACQUISITION		AUTOFOCUS	
MICROSCOPE AND BEAM DIAMETER	Krios G3i 0.5 µm	Glacios 1 µm	Krios G3i 0.5 µm	Glacios 1 µm	Krios G3i 0.5 µm	Glacios 1 µm	Krios G3i 0.5 µm	Glacios 1 µm	Krios G3i 0.5 µm	Glacios 1 µm
MAGNIFICATION	135 x	84 x	740 x	700 x	33,000 x	45,000 x	165,000 x/ 133,000 x	155,000 x	215,000 x	
DEFOCUS	- 1000 µm	- 1000 µm	- 50 µm	- 50 µm	5 µm	- 20 µm	3.5 µm	As required	N/A	
SPOT SIZE	8		4		7		6		6	
ILLUMINATED AREA	900 µm^2		402 µm^2		10 µm^2		449 nm^2/ 650 nm^2		400 nm^2	
C2	50	150	70	50	70	50		50	70	

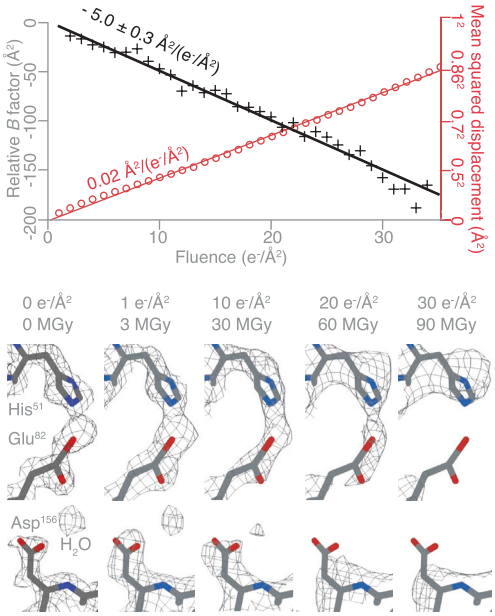
EXAMPLE DATA COLLECTION SETTINGS

HexAuFoil

Extrapolating structure factors to zero dose for reconstructions

Due to the elimination of specimen movement, final high-resolution reconstructions can be used to extrapolate the structure factors of biological samples to those of the undamaged structure, at zero electron dose.

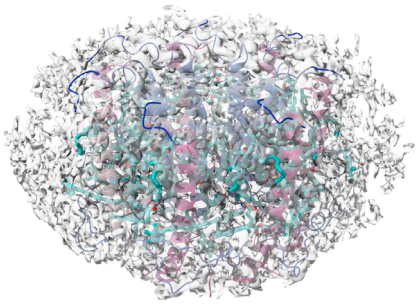
Software, called 0dose, is available to extrapolate structure factors to zero dose, written by Dr Chris Russo, the inventor of HexAuFoil grids. Use of 0dose is described in Naydenova, Jia and Russo, Science, 2020. It is open source, and available to download at https://www.mrc-lmb.cam.ac.uk/crusso/resources.html.



Further information

Further information about the development and use of HexAuFoil grids can be found at:

- Naydenova and Russo. Integrated wafer-scale manufacture of electron cryomicroscopy specimen supports. Ultramicroscopy 232: 113396 (2022)
- Naydenova, Jia, Russo. Cryo-EM with sub-1 Å specimen movement. Science 370: 223-226 (2020)



Light-harvesting 2 complex: PDBID 6ZXA, EMDB entry 11516. Structure solved with data collected using HexAuFoil® grids. As described in Gardiner *et al* (Sci. Adv.,2021)

Publications using HexAuFoil® grids specimen supports for Cryo-EM data collection:

- Naydenova et al. Structure of the SARS-CoV-2 RNA-dependent RNA polymerase in the presence of favipiravir-RTP. Proc. Natl Acad. Sci 118: e2021946118 (2021)
- Gardiner et al. The 2.4 Å cryo-EM structure of a heptameric light-harvesting 2 complex reveals two carotenoid energy transfer pathways. Sci. Adv. 7: e4650 (2021)
- Qian et al. 2.4-Å structure of the double-ring Gemmatimonas phototrophica photosystem. Sci. Adv. 8: eabk3139 (2022)