Three-dimensional cellular ultrastructure resolved by X-ray microscopy

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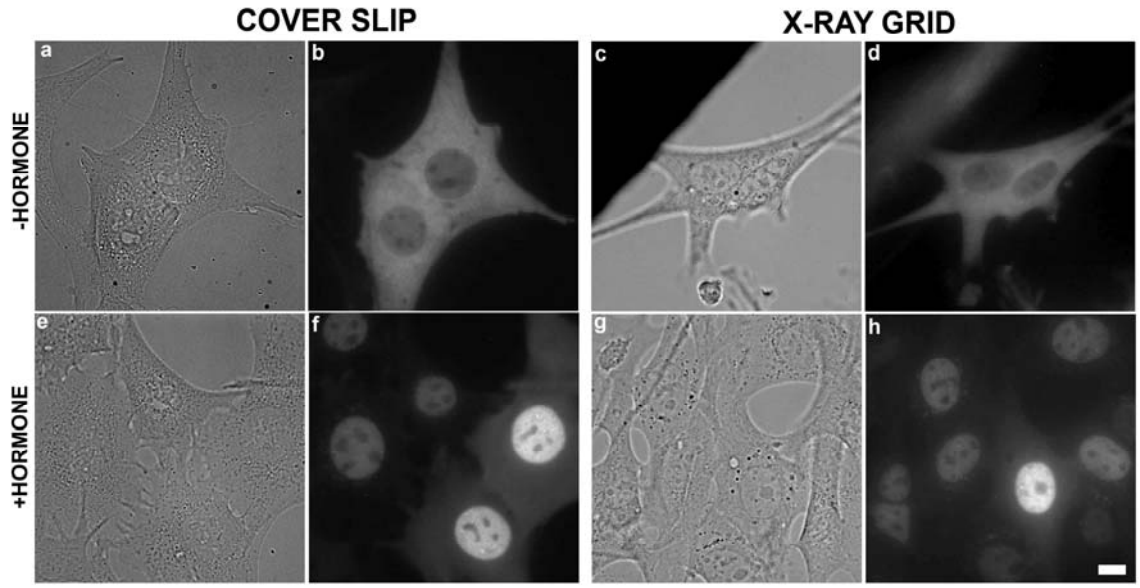
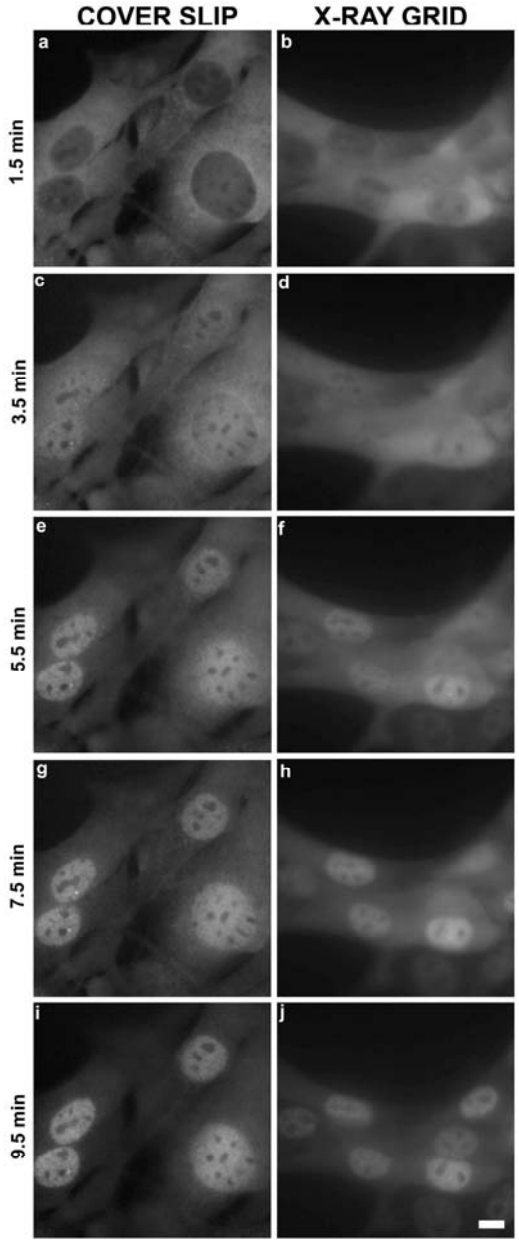
Supplementary Figure 1 – Cell morphology on cover slips and grids



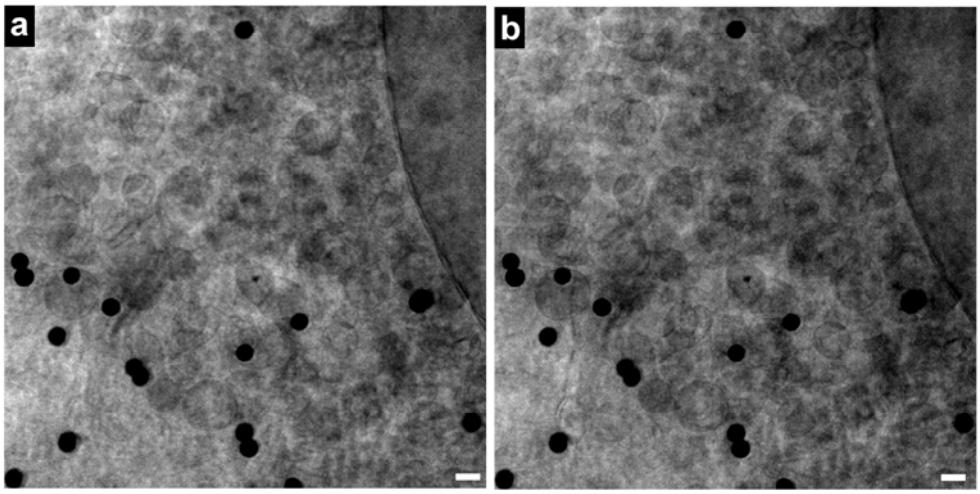
Mouse adenocarcinoma cells exhibit similar morphology whether grown on cover slips or on cellulose-nitrate-coated gold grids. Bright-field images (a,c,e,g) and fluorescence images (b,d,f,h) are shown. The fluorescent marker is a GFP-tagged glucocorticoid receptor which is cytoplasmic before addition of steroid hormone and nuclear upon hormone addition. Scale bar = 5 µm.

Supplementary Figure 2 – Cell physiology on cover slips and grids



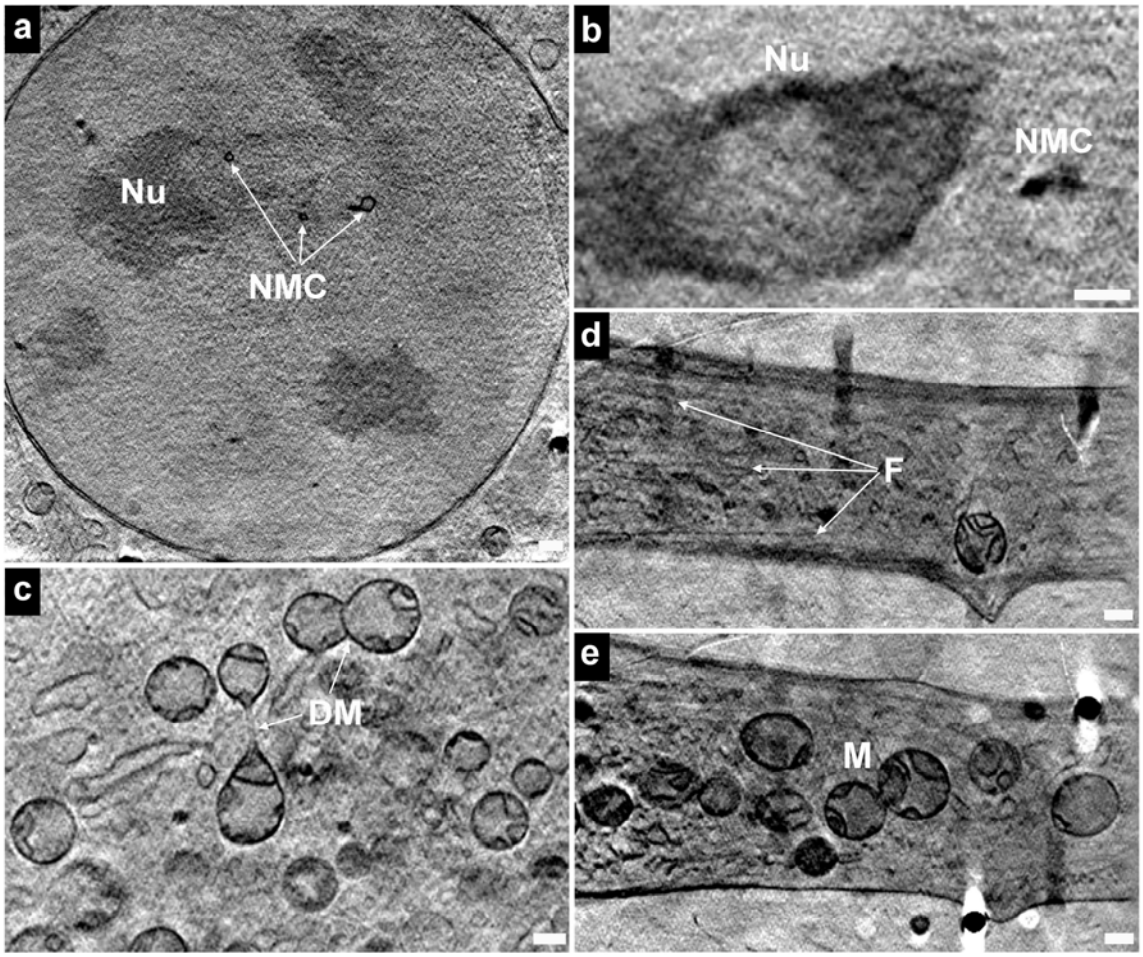
Mouse adenocarcinoma cells exhibit similar physiology whether grown on cover slips or cellulose-nitrate-coated gold grids. Normal physiology was assayed in live cells by visualizing nuclear import of a GFP-tagged glucocorticoid receptor. Steroid hormone was added to the cells at 0 min, and then fluorescent images of the cells were acquired over the next 9.5 min. The rate of import of GFP-tagged glucocorticoid receptor is similar under both growth conditions. Scale bar = 5 μm.

Supplementary Figure 3 – Image comparison before and after the tilt series



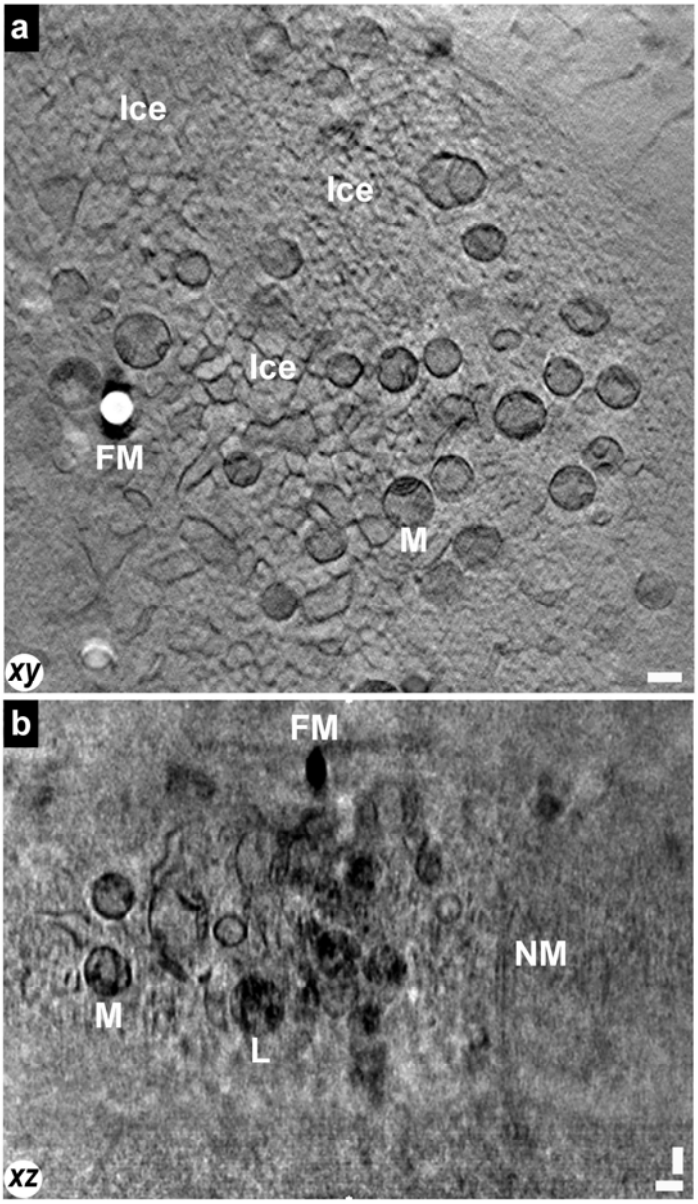
X-ray irradiation during the tilt series does not alter the cellular ultrastructure detectable by X-ray microscopy. Shown is a raw X-ray image at 0° tilt angle acquired before (a) and after (b) the complete tilt series of 121 angles (These are raw images from the same cell as that reconstructed in Fig. 2a). Black spheres are 270 nm gold-shell fiducial markers used for aligning the tilt series. Note that the images acquired before and after the tilt series are virtually indistinguishable. A cross correlation of these two images yields a correlation coefficient of 0.90. Pixel size is 9.8 nm. Scale bar = 0.39 μm.

Supplementary Figure 4 – Additional X-ray images of mouse adenocarcinoma cells



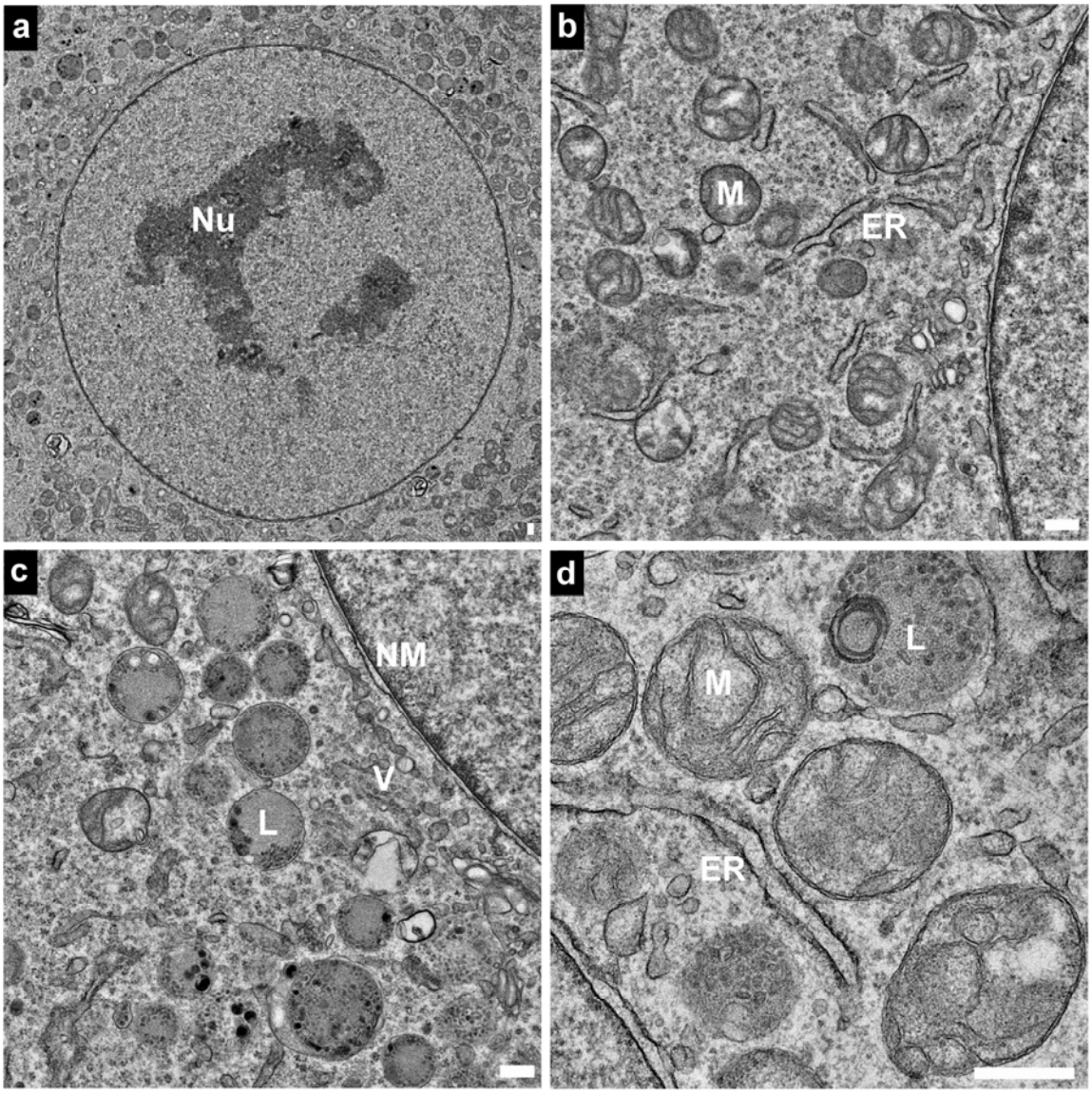
Nuclei consistently revealed nucleoli (a) (Nu), sometimes with clear substructures (b), plus nuclear membrane channels (NMC) visible here in cross section (a,b). A few cytoplasmic images provided evidence for dividing mitochondria (c) (DM) and filaments (d) (F), which were more prevalent in superficial slices of long cytoplasmic extensions (d), but less so in deeper slices (e). Pixel size and slice thickness are 19.6 nm. Scale bar = 0.39 μm.

Supplementary Figure 5 – Artifacts in X-ray tomography



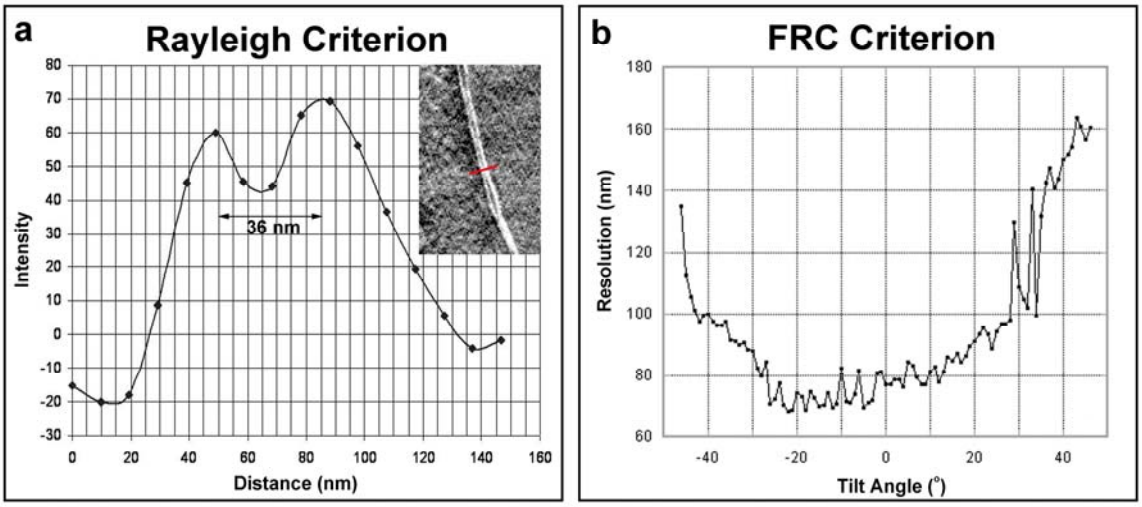
Ice formation, due to poor cryo-preservation, was the most common defect observed in the X-ray tomograms. The ice appeared as a lattice-like structure that disrupted organellar and vesicular structures in the vicinity (a). xz sections of the X-ray tomograms were also subject to the missing wedge artifact that arises from a lack of images beyond the tilt limits. This restriction can make it difficult to properly resolve the tops and bottoms of some structures. For example, a complete xz cross section from a well preserved cell reveals that the double nuclear membrane is not well resolved beyond the middle third of the cross section (b). Fiducial markers (FM) used for the tilt series alignment also obstruct random sites in the image (a,b). Pixel size and slice thickness are 19.6 nm. Scale bar = 0.39 μm.

Supplementary Figure 6 – TEM images of mouse adenocarcinoma cells



Transmission electron microscope images of the mouse adenocarcinoma cell line reveal similar ultrastructural features as those detected by X-ray tomography. Heterochromatin is absent in nuclei, although nucleoli (Nu) and a double nuclear membrane (NM) are evident (a,c). The cytoplasm (c,d) contains mitochondria (M), lysosomes (L), endoplasmic reticulum (ER) and vesicles (V). Higher magnification views reveal cristae in the mitochondria and dense inclusions in the lysosomes (d). Scale bar = 0.39 μm.

Supplementary Figure 7 – Resolution measurements of the X-ray tomograms



Rayleigh (a) and Fourier Ring Correlation (b) resolution criteria. An image of the nuclear membrane with intensities inverted (inset in a) was used to measure the smallest detectable separation of the double nuclear membrane. The red line in the inset shows the region of measurement that yielded the intensity profile shown. A mean background intensity was subtracted before plotting. The two peaks of the double membrane are separated by 36 nm. Unlike the Rayleigh criterion which is calculated from high contrast features in an xy slice, the FRC criterion is calculated from the entire tomogram and for all contrast ranges. The resolution achieved at each tilt angle is shown (b). Because this method is sensitive to the thickness of the specimen, the highly tilted images have the worst resolution, while those with lower tilt angles show a best resolution of ~70 nm. While a good comparative measure of the quality of a tomogram, the FRC analysis reflects all the imperfections in the tomographic data, such as the restriction to a limited tilt range, the inclusion of adjacent areas in the tomographic reconstruction, variations in focus due to specimen thickness, and the ever-present noise.

Protocol

Protocol for partially coherent X-ray microscopy

To obtain synchrotron beam time, potential users must submit a proposal that is reviewed by an international scientific committee (http://www.helmholtz-berlin.de/user/photons/beamtime/index_en.html). Deadlines for proposal submission are the end of March or August for beam time starting respectively the following August or February. Once a proposal is approved and beam time is allocated, short term visitors can reserve a room at the Helmholtz Zentrum Berlin guest house located within walking distance of the microscope. The synchrotron facility includes a basic biology laboratory, which is equipped with a CO_2 incubator, laminar flow hood, several light microscopes, a custom made plunge freezer and other smaller equipment necessary for basic cell culture.

Custom-designed gold grids (specified as IFR-1) can be purchased from Gilder Grids, UK. The grids are coated by hand with a cellulose nitrate film. This is done by spreading about 0.5 µl of the cellulose nitrate solution on a water surface, laying the grids on top of the surface using a forceps, and then laying the paper side of a piece of parafilm on top of the grids. Next, the parafilm with grids on it is removed by hand and inverted onto a paper towel, allowing the grids to dry coated side up. When dried, the coated grids are removed from the parafilm paper with a forceps and placed in a petri dish. Note that successful imaging with this X-ray microscope has also been accomplished with either lacey or non-lacey formvar or carbon coated grids. Different cell types may prefer different coatings for optimal growth.

The coated grids are placed with a forceps coated side up in petri dishes containing cell growth medium. A drop of cell suspension is added with a sufficiently high cell density such that a reasonable number of cells settle on each grid. The cells are incubated overnight to allow them to adhere to the cellulose nitrate. On the next day, gold beads (to be used as fiducial markers for the tomographic reconstruction) are added to the petri dishes at a sufficient density such that each cell will have at least 5-10 beads surrounding it. As described in the Online Methods, we added 2 µl of a suspension of custom made gold beads 270 nm in diameter to the petri dishes. However, other gold beads of different sizes are also suitable. These can be purchased from companies such as BBInternational, Cardiff, UK. The concentration and amount of beads to be added must be determined empirically by imaging cells on a light microscope. To allow the beads to settle, they should be added about one hour before freezing the cells.

The preceding approach can be adapted to accommodate non-adherent cells. A dense suspension of these cells is mixed with fiducial markers and then the cells and beads are allowed to settle together onto the coated grids. With non-adherent cells this can be done on the same day as the freezing. In addition to various mammalian cells, the current X-ray microscope has also imaged yeast and algae. A limiting factor is the thickness/height of the specimen, which should be less than 15 µm.

The plunge freezing protocol used in this study is described in the Online Methods, but variations in this approach are possible and may even be necessary depending on the type of cell examined, in particular the time for blotting is critical. It is also possible to freeze cells with different equipment at another location and then ship the frozen grids to the Helmholtz Zentrum Berlin using a dry shipper commonly used in both electron microscopy and protein crystallography.

In all cases, the frozen grids are transferred under cryo conditions to a cryo-holder, and then the holder is inserted into the tilt stage of the X-ray microscope. Scanning of a grid to identify cells of interest may take up to an hour, dependent on the cell density. Once a cell is selected, collection of a tomographic tilt series typically requires about 60-90 min due to the read-out time of the camera whereas the total exposure time is much smaller. Several tomographic tilt series can normally be acquired in one eight hour session on the X-ray microscope.

Tomograms can be reconstructed from the tilt series by different software packages designed for cryo electron tomography. The most common approach uses fiducial markers (usually gold beads) to align the 2D images before doing a 3D reconstruction to obtain the tomogram. Software packages such as IMOD (http://bio3d.colorado.edu) and Bsoft (http://bsoft.ws) are freely available for both tomographic reconstruction and visualization. Other packages such as Chimera (http://www.cgl.ucsf.edu/chimera/) and Amira (http://www.amira.com - commercial) can also be used for visualization and interpretation.