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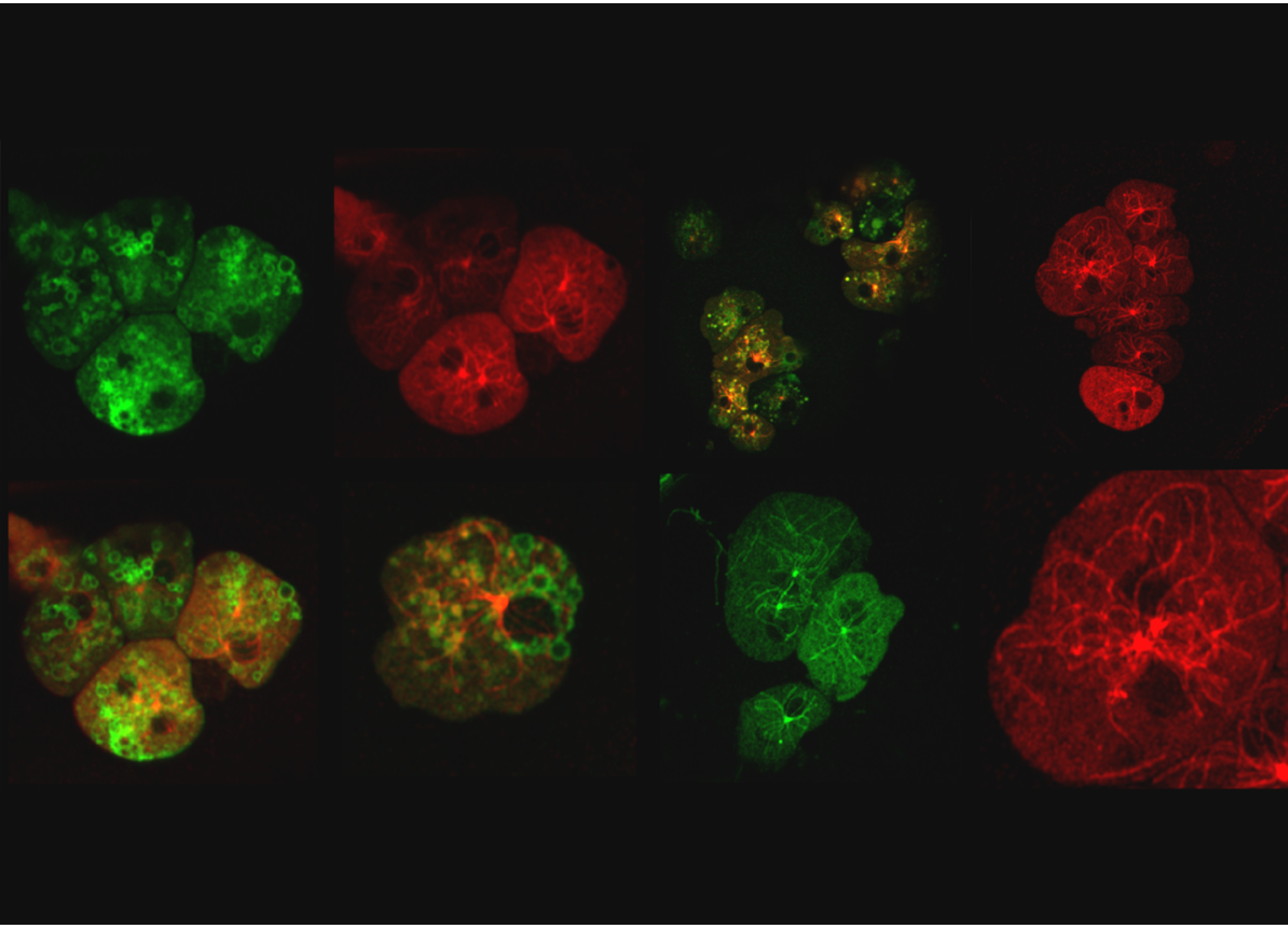

Cryo-Confocal Imaging with Airyscan

Improving Resolution and Signal-to-Noise in Cryo-Fluorescence Microscopy

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Cryo-Confocal Imaging with Airyscan

Improving Resolution and Signal-to-Noise in Cryo-Fluorescence Microscopy

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- Cryo-preparation by vitrification preserves cells in their native hydrated state.
- This structural preservation makes cryo-samples ideally suited for high resolution imaging with correlative cryo-fluorescence microscopy.
- High resolution imaging requires objectives with high numerical apertures (NA), often with immersion. These are not compatible with cryo-stages.
- Airyscan – a novel confocal detector – in combination with cryo-compatible long distance (LD) objectives allows imaging with high resolution and signal-to-noise.

Abstract

Studying the fine details of biological cells and tissues not only requires a microscope which can attain high resolution images, but also a sample preparation technique that preserves the structures in a close-to-life or near-native state. Rapid-freezing techniques allow to maintain the high water content of cells and immobilize the sample in a near-native state for cryo-imaging by fluorescence, electron or X-ray microscopy. A major limitation for correlative cryo-fluorescence microscopy is the current unavailability of cryo-immersion optics that could yield a higher numerical aperture. Combining ZEISS LSM 880 with Airyscan and cryo-fluorescent imaging utilizing the Linkam CMS 196 cryo-stage allows to obtain a significant increase in resolution and signal-to-noise (SNR) compared to standard confocal images under cryo-conditions. ZEISS Airyscan, with its novel confocal detection scheme, enables the user to record high-quality cryo-fluorescence data even without immersion optics.

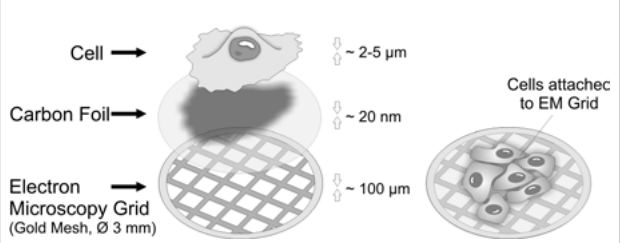

Figure 1 *Growing cells on electron microscopy grids. Various kinds of eukaryotic cells can be cultured on gold specimen supports for electron microscopy. Gold is preferred over copper in order to prevent cytotoxic effects by copper ions. The cells adhere to a carbon support film covering the whole gold EM mesh grid.*

Cryo-Preparation of cells

Biological objects such as cells are primarily composed of carbon compounds and up to 70 percent water. Studying the fine details of cells and tissues in their native aqueous environment not only requires a microscope which can attain high resolution images, but also a sample preparation technique that rules out the possibility of any structural changes. Vitrification is a cryo-preparation technique which allows to maintain the high water content of cells, by freezing them to a temperature of app. -190° C within a matter of milliseconds [1]. This fast-freezing process prevents the water molecules from crystallizing and forms a glass-like layer of amorphous ice that preserves cellular structures in a near-native state.

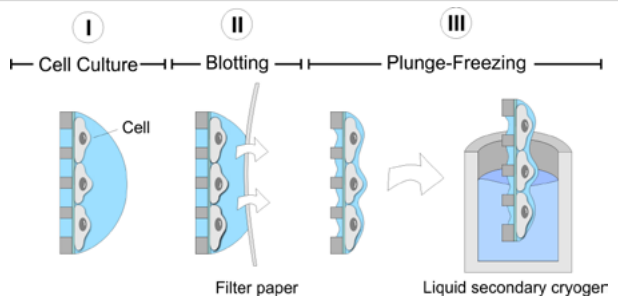

Figure 2 *Fast-freezing of cells on EM grids by plunge-freezing into a secondary cryogen (e.g. ethane). The rapid freezing process vitrifies the cells, preserving them in amorphous ice. Grids with cells are removed from the cell culture dishes and residual buffer or medium is blotted by a filter paper. The thin film of liquid remaining is then vitrified by rapid immersion into a secondary cryogen.*

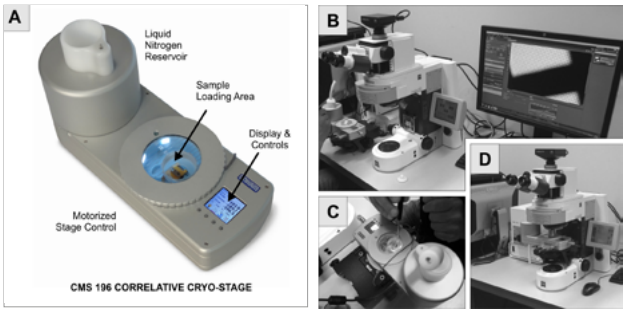

Figure 3 *Setup of the cryo-correlative sample stage (CMS196, Linkam Scientific) on a widefield imaging system (ZEISS Axio Imager). (A) Cryo-stage with integrated motorised XY system and dewar for liquid nitrogen. (B) ZEISS Axio Imager with detached cryo-stage on carrier. (C) Cryo-sample manipulation. (D) Configuration for imaging.*

Prior to vitrification, cells are cultivated on electron microscopy (EM) grids coated with thin carbon films (Fig. 1). The grids are made out of a non-toxic material (typically gold). Immediately before freezing, the residual growth medium or buffer is removed by blotting with a filter paper, leaving behind a thin liquid film covering the cells on the EM grid. The EM grid is then rapidly immersed into a secondary cryogen (e.g. ethane or propane), leading to vitrification of the cells [1] (Fig. 2). Once frozen, the sample needs to remain at liquid nitrogen temperature at all times.

Specimen stage for imaging cryo-samples

One of the key challenges for imaging cryo-specimens is that the sample remains extremely cold and free of contamination at all times. It is essential to ensure that the temperature of the frozen cells does not rise above the devitrification temperature of app. -140° C. Above this temperature a phase transition from vitreous to cubic ice occurs, that can cause subtle distortions within the cellular ultrastructure, thus rendering the specimen unusable for high-resolution techniques such as cryo-electron microscopy. The ultra-low temperatures pose a challenge for the imaging system as conventional immersion media and optics cannot be used.

For imaging cryo-samples a dedicated sample stage is required that replaces the standard stage of the light microscope (Fig. 3). Such a cryo-stage must be able to keep the sample vitrified and at the same time provide capabilities to safely handle and transfer frozen-hydrated samples. In addition it must be mechanically stable to allow imaging of vitrified samples with optical microscopy while keeping them free

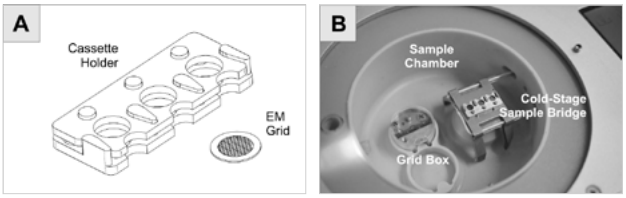

Figure 4 *CMS 196 sample chamber and EM grid mounting. (A) EM-grid cassette holder. (B) Holder attached to motorized cryo-stage within the Linkam CMS196 stage.*

of contamination at all times. The stage used here is the Linkam CMS196 cryo-correlative stage. It is used in combination with ZEISS LSM 880 and Airyscan and allows to perform confocal cryo-fluorescence microscopy.

Vitrified samples on EM grids are mounted inside the cryo-stage specimen chamber into a holder (Fig. 4) which is placed on a motorized sample bridge, kept at app. -180° C. The Linkam cryo-stage is then attached to ZEISS LSM 880 with Airyscan for cryo-confocal imaging. Objectives used for grid screening and overview imaging were ZEISS LD EC Epiplan-Neofluar 20×/0.22 and 10×/0.3. For confocal imaging and recording of z-stacks a ZEISS LD EC Epiplan-Neofluar 100×/0.75 objective was used.

Correlative Cryo-Fluorescence Imaging

Cryo-fluorescence imaging [2] is a relative new discipline, which combines optimal sample preservation by cryo-preparation methods with the advantages of fluorescence labelling of biological structures. It is becoming more and more popular in correlative microscopy, where fluorescence imaging is used to identify areas or events of interest within cells,

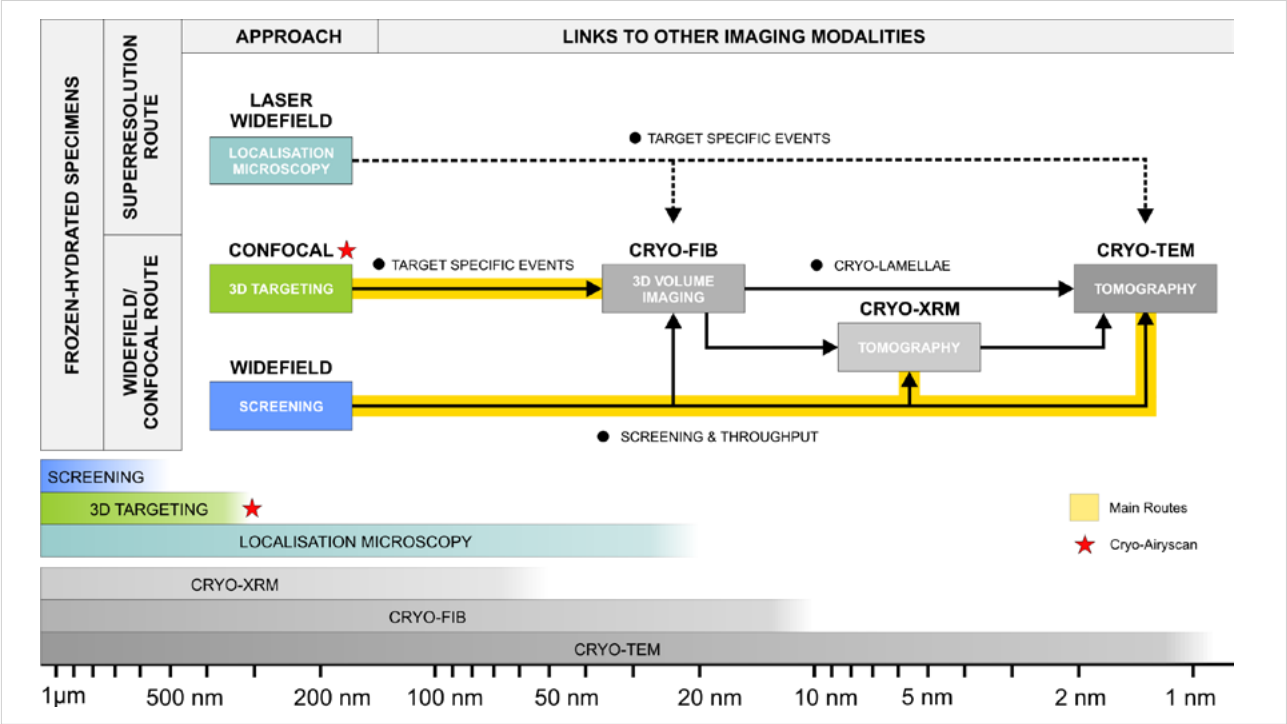

Figure 5 *Correlative routes in cryo-fluorescence microscopy.*

which are then examined in detail by electron microscopy [see ↗: *Reference Guide to Correlative Sample Preparation*]. A major advantage of correlative cryo-imaging workflows over resin embedding workflows is that no compromise between ultrastructural or fluorophore preservation is made. Cryo-conditions offer superior structural preservation and enhanced photostability during imaging [3].

Two major routes for imaging frozen hydrated samples are currently emerging (Fig. 5): One involves the routine screening of frozen-hydrated samples, where light microscopy is used to localize a feature or region of interest prior to investigation at higher magnifications by electron microscopy. The second route aims at maximizing resolution in cryo-fluorescence microscopy by applying single molecule localisation techniques (e.g. PALM, STORM) [2,4] to vitrified specimens. This route utilizes the fact that cryo-preservation maintains the high water content of cells while immobilizing the sample in a near-native state, rendering the specimen unadulterated by chemical fixation techniques.

So far, a major bottleneck for imaging frozen-hydrated specimens by cryo-fluorescence microscopy is the lack of immersion optics that would allow a higher numerical aperture. At these low temperatures conventional immersion media would freeze. Hence, only air objectives can be used for cryo-imaging, limiting the available numerical apertures to below NA 1.

Besides the mere finding of the feature of interest, localizing it precisely within a cell is a challenging task that is frequently compared to “searching for the needle in a haystack”. In some cases a coarse localisation by screening the vitrified sample with a widefield microscope may be sufficient (see Fig. 5 “screening route”; e.g. when a certain phenotype must be identified), but a precise localisation remains a major task in cases where a feature must be localized to the subcellular level for subsequent investigations by FIB or TEM (see Fig. 5 “3D targeting route”). Such a precise localisation can be required, for instance, for detecting whether a certain label is localized outside or inside a membrane.

Cryo-Imaging with LSM 880 and Airyscan

Confocal laser scanning microscopes are renowned for their optical sectioning capability, a feature enabled by utilizing a pinhole that rejects out-of-focus light. Closing the pinhole improves lateral resolution, but also causes less light to reach the detector leading to reduced signal-to-noise ratios (SNR). In a typical experiment the pinhole will not be smaller than one Airy Unit (AU), sacrificing resolution for better SNR.

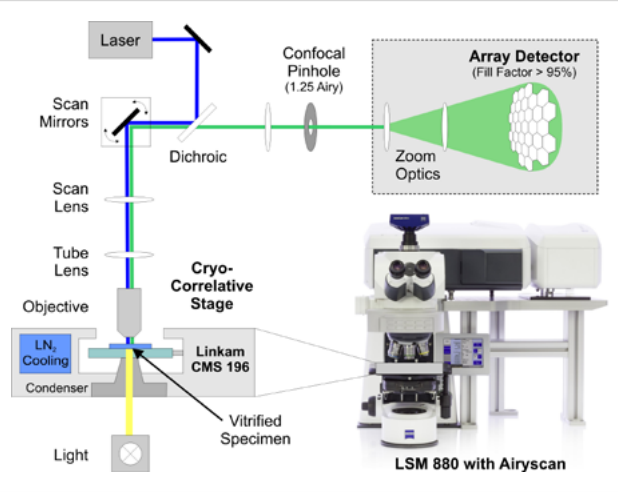

Figure 6 *Imaging setup and beam path for cryo-confocal microscopy.*

The Airyscan detection module allows the spatially-resolved detection of fluorescence light otherwise rejected by the pinhole in a standard confocal system. The Airyscan detector consists of a Gallium Arsenide Phosphid (GaAsP) photomultiplier tube (PMT) array of 32 elements, which are arranged in a compound eye fashion. In this way each detector element receives light that is displaced from the optical axis by defined distances. Imaging 1.25 Airy units (AU) on the detector array allows performing sub-airy sampling with each individual detector element, since every element acts as a pinhole of 0.2 AU. A pinhole set to 0.2 AU in a standard confocal will capture only 5% of the light compared to Airyscan. The majority of the resolution improvement results from the significant increase in SNR.

For confocal cryo-fluorescence imaging of vitrified cells on electron microscopy grids, the vitrified sample is mounted to a cryo-stage attached to ZEISS LSM 880 with Airyscan (Fig. 6). Frozen-hydrated cells are illuminated by a scanned focused laser beam and the emitted fluorescence signal is sent to the Airyscan detector through the confocal pinhole aperture, adjusted to 1.25 AU.

Fig. 7 shows cryo-fluorescence confocal versus Airyscan images of *Dictyostelium discoideum* cells coexpressing mRFP-alpha-Tubulin [5] and VatM-GFP [6]. The mRFP-alpha-Tubulin fusion protein allows to visualize the cellular microtubule system (Fig. 7A,B). Compared to confocal images the cryo-Airyscan images are less noisy and allow to visualize fine tubular arrays. VatM-GFP labels a subunit of the vacuolar H+-ATPase. This multi-subunit enzyme is found primarily in

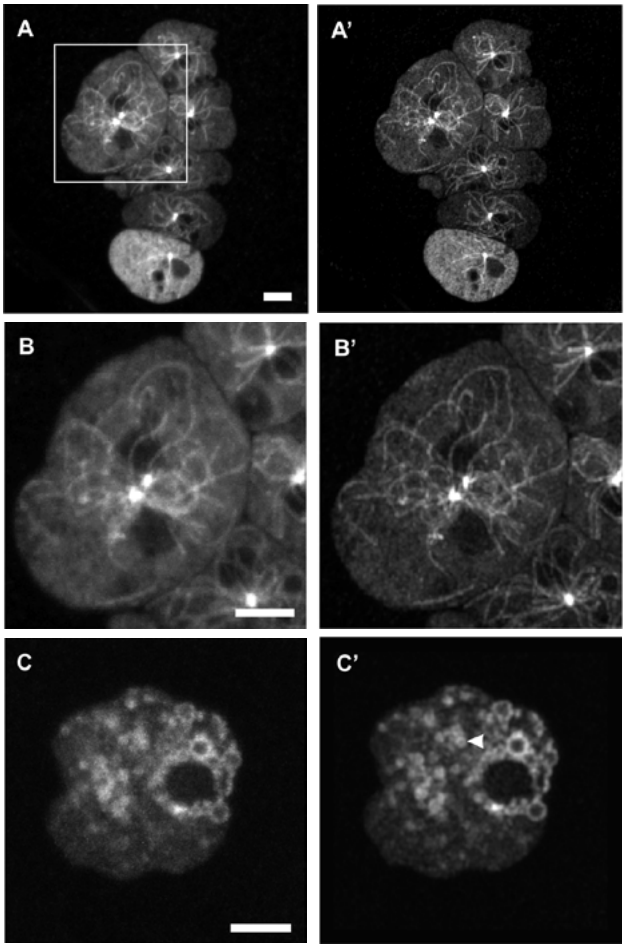

Figure 7 *Comparison between cryo-confocal (A-C) and cryo-Airyscan (A'-C') images.* Dictyostelium discoideum *cells expressing mRFP-alpha-tubulin (Microtubules (A,B)) and VatM-GFP (V-ATPases (C)). Scale bars: 5 µm. Sample courtesy of Dr. Günther Gerisch, MPI of Biochemistry, Martinsried.*

membranes of the contractile vacuole complex, where it energizes fluid accumulation by this osmoregulatory organelle and also in membranes of endolysosomes, where it serves to acidify the endosomal lumen. The confocal Airyscan image (Fig. 7C) clearly benefits from the improved SNR and resolution, as evidenced by small endosomal vesicles whose lumen only becomes visible in the Airyscan image (Fig. 7C', white arrowhead).

Fig. 8 shows virus protein-induced vesicular and tubular structures in the nucleus of plunge-frozen porcine epithelial-like embryonic kidney cells. The fluorophore-tagged protein pUL34-GFP [7, 8] is the nuclear membrane anchor of the nuclear egress complex of the Herpesviridae and is involved in expanding the nucleoplasmic reticulum. With Airyscan subtle tubular structures in the nucleus of the frozen-hydrated cells become clearly visible as a result of the improved SNR (arrows in Fig. 8B).

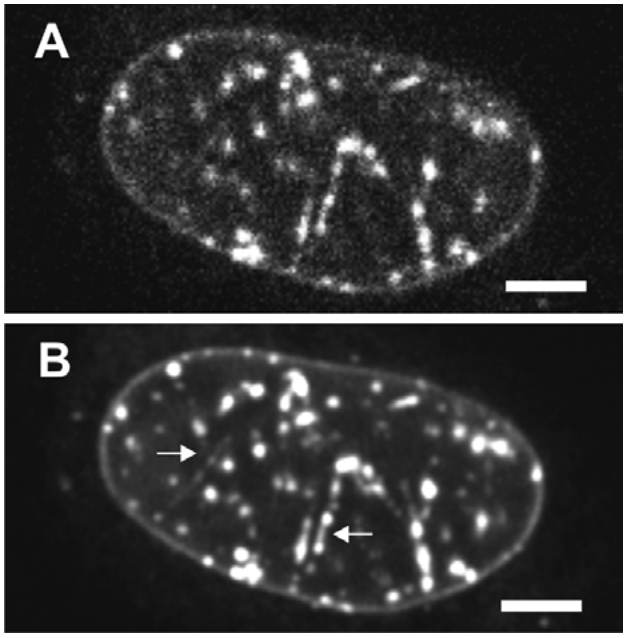

Figure 8 *Comparison between cryo-confocal (A) and cryo-Airyscan (B) image. Nucleus of a porcine epithelial-like embryonic kidney cell stably coexpressing pseudorabies virus (PrV) nuclear egress complex components pUL31 and pUL34-GFP. Tubular structures in the nucleus (white arrows in (Fig.8 B). Scale bars: 4 µm. Sample courtesy of Prof. Kay Grünewald, University of Oxford.*

In this communication we demonstrate, for the first time, cryo-confocal Airyscan imaging of vitrified specimens. The data proves that under cryo-conditions, even without immersion optics, a significant increase in resolution and SNR can be obtained with Airyscan compared to standard confocal imaging. Fig. 9 displays results obtained from point spread function (PSF) measurements of 100 nm bead data sets (Alexa 488) under cryo-conditions. The corresponding full width at half maximum (FWHM) for cryo-confocal (1 AU) (Fig. 9, blue curve) and cryo-Airyscan measurements (Fig. 9, red curve) indicates an improvement in resolution by a factor of 1.4.

Cryo-Airyscan allows to record 3D information (z-stacks) from frozen-hydrated specimens. This enables the user to target subcellular features for subsequent imaging by other imaging modalities, such as cryo-FIB volume imaging [9] or electron cryo-tomography [10] (see Fig. 5 “3D targeting route”).

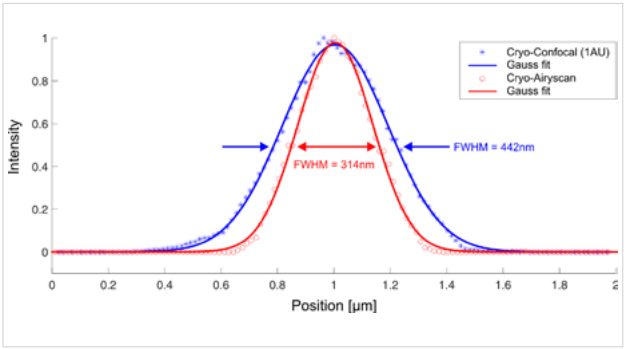

Figure 9 *Point Spread Function (PSF) measurements of 100 nm bead data sets (Alexa 488). Full width at half maximum (FWHM) for cryo-confocal (1AU) and cryo-Airyscan measurements. Gaussian intensity distribution (blue curve: cryo-confocal; red curve: cryo-Airyscan).*

Requirements

- Confocal laser scanning microscope ZEISS LSM 800 or LSM 880 with Airyscan
- Linkam CMS196 cryo-correlative stage
- Equipment for vitrification and handling of vitreous specimens

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