High-pressure freezing of cell cultures cultivated on PEN-film µ-slides

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To find suitable substrates for live-cell imaging followed by high-pressure freezing and thin section electron microscopy, we have tested particular multi-well slides which are originally used for laser microdissection (No. 81831; ibidi GmbH, Germany). The slides contain 18 wells with a volume of 30 µl each, which can be covered by a common lid forming a closed chamber. The bottom of each well is made of a thin plastic film of polyethylene naphtalate (PEN). The thickness of the film is about 2 µm and allows the use of microscopic objectives with short working distance and high numerical aperture together with immersion fluids. The transparency of the film is somehow reduced by particulate inclusions which should facilitate laser microdissection. However, the film has a relatively low mass which might facilitate freezing of cells grown on this particular support.

We have grown native HeLa SS6 cells and cells expressing a viral protein fused to a fluorescent protein at its carboxy-terminus on PEN-film substrates of µ-slides. Pre-coating of the film with poly-L-lysine or collagen increased the adherence of cells and prevented their loss during preparation for electron microscopy. Imaging of cells was possible for some hours using a live cell imaging system (Nikon TE2000) equipped with a climate chamber. With most lenses we have used conventional bright-field imaging for visualization of cells because imaging with phase or interference contrast increased the granular background structure of the film. However, at higher power (objectives 60-100x) interference contrast allowed suitable imaging (Fig. 1A). Fluorescence microscopy of fusion protein was also possible at a reasonable quantum yield (Fig. 1B).

After live cell imaging cells were high-pressure frozen by using the HPF01 compact high-pressure freezer (Wohlwend Engineering, Switzerland). Small rectangular pieces (1.5x1.5 mm) of the film were cut out from the wells by turning the slide upside down and perforating the film from below using a sharp and peaked scalpel. Pieces of film were transferred into aluminum platelets (0.1 or 0.05 mm depth) filled with hexadecene. The assembly was covered by a flat aluminum platelet and transferred into the sample holder for freezing. Frozen cells were stored in liquid nitrogen before they were freeze-substituted in an aluminum bloc [1] placed in a deep freezer. For freeze-substitution films were removed from the platelets and from surrounding hexadecene by using sharp forceps. Films were put into little baskets [2] and covered with a lid. Baskets were then transferred into pre-cooled (-90 °C) substitution fluid composed out of 0.5% osmium tetroxide, 0.05% uranyl acetate and 5% water in acetone. After 3 days at -80° C, temperature was slowly increased (about 4,5 °C/h) to -5° C by transferring the aluminum bloc from the deep freezer to room temperature and substitution fluid was changed against pure acetone. Samples were infiltrated with Epon/acetone and embedded in Epon. After polymerization at 60° C in an oven, samples were sectioned using an ultramicrotome. Images were taken with a Tecnai12 microscope and a MegaviewIII CCD camera at 120 kV. Cellular preservation was satisfactory in many of the

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samples, i.e. absence of significant ice crystal damage (Fig. 2). However, sometimes mechanical cracks were observed, most probably introduced during post-fixation treatment of the film. Note that the PEN-films are somehow flexible even at liquid nitrogen temperature.

As a conclusion µ-slides equipped with a PEN-film substrate are suitable for light microscopy of fluorescent cells and for high-pressure freezing followed by freeze-substitution and resin embedding. Currently, we are developing procedures for correlative microscopy, which should allow re-localization of cells in transmission electron microscopy after live-cell imaging using µ-slides.

1. Steinbrecht, Microsc. Res. Techn. **24** (1993), p488.
2. Hohenberg et al., J. Microsc. **175** (1994), p34.

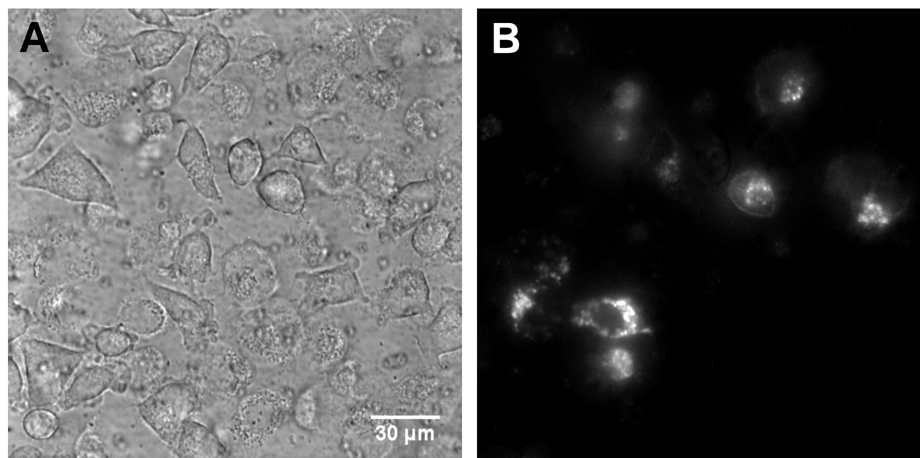

Figure 1. A Light microscopy of HeLa cells on PEN-film (µ-slide). **B** Fluorescence microscopy of the same cells as in A displaying fluorescence of a viral fusion protein.

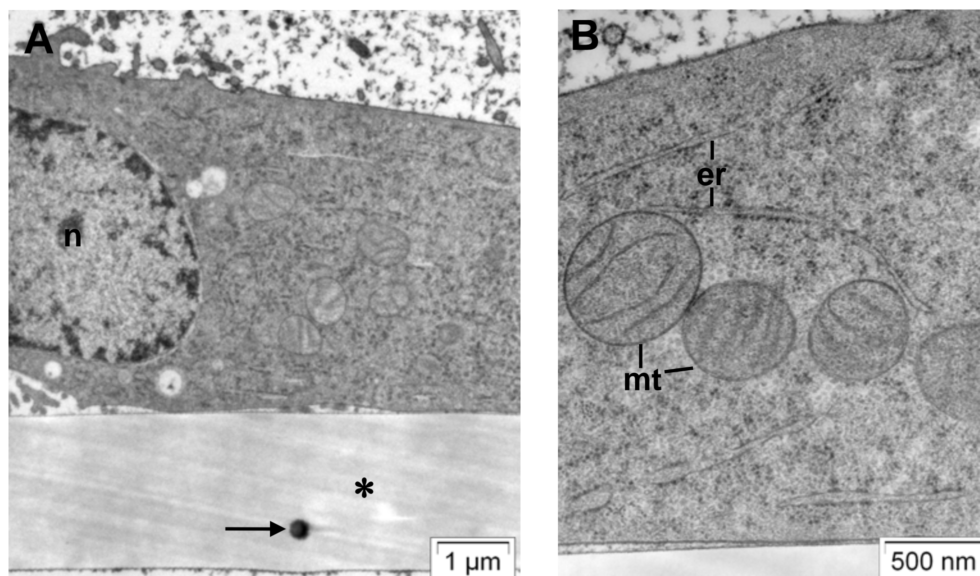

Figure 2. A Cross-section through a HeLa cell grown on a PEN-film support (*). **B** Detail of a HeLa cell showing satisfactory preservation of ultrastructure, e.g. the endoplasmic reticulum (*er*) and mitochondria (*mt*). (*n*) nucleus; (*arrow*) dense inclusion in the film.

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