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Branching of cytoplasmic intermediate filaments can be demonstrated using STEM tomography of high-pressure frozen keratinocytes

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Intermediate filaments (IFs) constitute together with microtubules (MTs) and microfilaments (MFs) the three main structural elements of the cytoskeleton in metazoans. The large protein family of the IFs is encoded by approximately 65 genes in the human genome [1]. The fibrous proteins are located in the cytoplasm and the nucleus. In contrast to MTs and MFs which break easily by shear stress, IFs are very flexible rodlike structures buffering the mechanical stress in the cells.

It is still not clear how the single intermediate filaments are assembled to one another. The aim of the project is to investigate, whether the filaments are branched.

In previous projects we were able to show branches using SEM tomography of triton extracted cells [2, 3] (Figure 1 and 2). It is, however, not clear, whether the natural state of the filament network is changed due to the harsh extraction procedure. For this reason we investigated the filament network using high pressure freezing, freeze substitution and STEM tomography, a protocol generally accepted to induce fewer artifacts [4]. STEM tomography was further optimized by using a parallel beam alignment [5] and the bright field signal that provides sharper images in the depth of the sample [6].

Here, keratinocytes were seeded on carbon coated and glow discharged sapphire discs (3 mm in diameter, 160 µm thick) for 48 hours (37°C, 5% CO2). The cells were then high pressure frozen (Wohlwend Compact 01) and freeze substituted [4]. Afterwards, 475 nm thick sections were cut by an ultramicrotome (Leica Ultracut) and images were acquired using a Titan (FEI, 300 kV) using the STEM mode. The specimens were tilted from -72° to +72° with 2° increment. The reconstruction of the single images into a 3D construct was performed using the IMOD software.

Figure 3 represents a virtual (computed) 10 nm thick section of a STEM tomogram of a 475 nm thick microtome section (measured in the electron microscope). The green arrows depict the leaflets of the membrane bilayer, the yellow arrow points to a microtubule. The red arrows point to branches of intermediate filaments. We assume that these are real branches, since the thickness of the virtual section is in the range of the thickness of an intermediate filament and there is not enough space in the virtual section for two intermediate filaments to cross without touching each other.

This data demonstrate that branches in intermediate filaments can not only be observed in triton extracted samples (Figures 1 and 2), but also in high pressure frozen and freeze substituted cells, as shown in Figure 3. Since this method is considered to be the gold standard for preventing artifacts, we conclude that the branches are real structures and not preparation artifacts.

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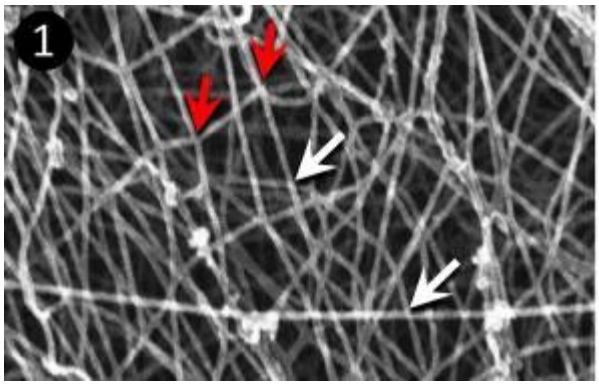

Figure 1. shows one image out of an SEM tilt series of intermediate filaments in a cell prepared by triton extraction. From one image it cannot be decided whether the filaments are branching or just crossing each other.

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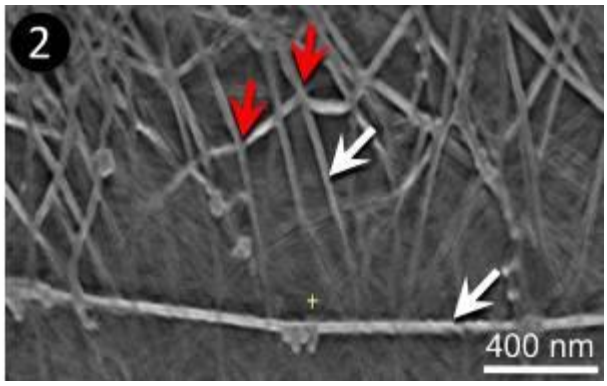

Figure 2. however, is a computed section of the reconstructed tomogram of the SEM tilt series. The red arrows depict real branches, whereas the white arrows depict filaments crossing without touching each other

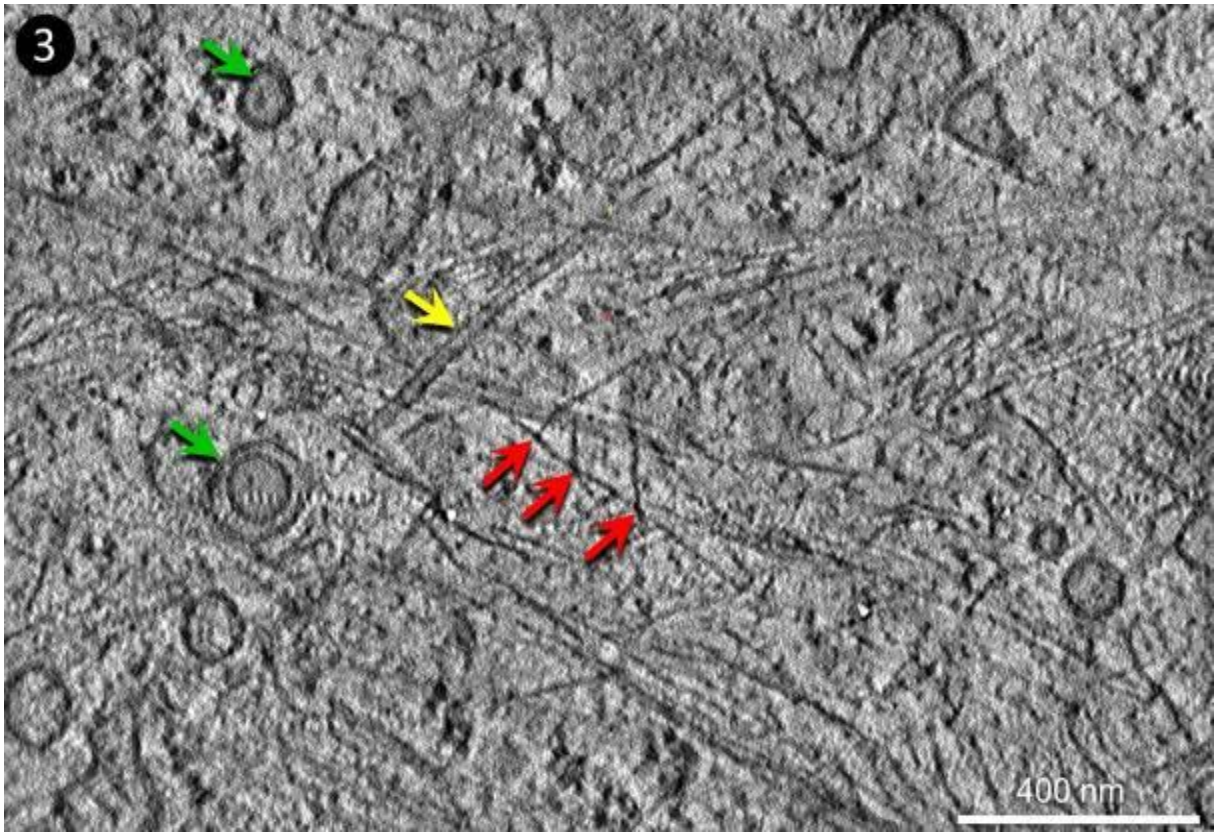

Figure 3. shows a 10 nm thick virtual section of a bright field STEM tomogram of a 475 nm thick epon section of a keratinocyte prepared by high pressure freezing and freeze substitution. Beside membranous structures (green arrows), where the two leaflets of the bilayer are well resolved, microtubules (yellow arrow) and intermediate filaments are visible. We conclude that the red arrows point to real branches of intermediate filaments, since the thickness of the virtual section is in the range of the thickness of an intermediate filamen