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An improved unroofing method consisting of tearing off the cell membrane with adhesive mesh instead of vitreous ice section (cryo-section) enabled us to view membrane cytoskeleton panoramically with extremely high contrast in native state. The mesh pretreated with Alcian blue was placed on cells, and a part of dorsal plasma membrane was transferred onto the mesh while floating the mesh by pouring the buffer solution \cite{1}. Such membrane fragments contained enough cytoskeleton and also suitable thickness for observation in cryo-electron microscopy (cryo-EM). The actin filaments in native state extended as a stream with a smooth contour, which appeared to be overlapped each other among actin filaments, microtubules and smooth endoplasmic reticulum (smooth ER) as shown in Fig. 1. Upon increasing the magnification, each actin filament revealed a short periodicity derived from globular actin (G-actin) that assembled to form filaments (Fig. 1 inset). Furthermore, it should be noted that many microtubules extended with a wavy curvature, but not in straight (Figs. 1). Unlike chemically fixed samples, microtubules spread out more than 3 \( \mu \) as far as we can trace without fragmentation. This is true for actin filaments in cytoplasm beneath the plasma membrane. Actin filaments spread out smoothly in a wavy manner without branching and fragmentation in the native state. Upon fixing with 1 \% glutaraldehyde, actin filaments showed linear shape with fragmentation like fixed microtubules. The average thickness of actin filaments and microtubules \textit{in situ} were 7.2 \pm 0.7 nm and 21.2 \pm 1.0 nm, respectively in native state. As a result of using the tearing off method to expose the inside of cells instead of cryo-section, smooth endoplasmic reticulum (Smooth ER) was detected successfully in cells while forming intricate network with lacy sheet and tubular structure among cytoskeleton. This is the first time observation of smooth ER in ordinary cells in cryo-EM (Figs. 1), though distribution of smooth ER is well known in muscle and adrenal cells. It is very significant on thinking the function that smooth ER network was located at the cytoplasm just beneath the plasma membrane. Judging from the image contrast, the smooth ER appears to be very thin envelope like a sarcoplasmic reticulum and to be very soft. Although smooth ER network was found in the periphery of the plasma membrane in present study, such network may penetrate deeper cytoplasm close to nuclear region while forming complicated network within a cell. Smooth ER network were found easily in native cells unroofed by tearing method. But it was difficult to observe complete network of smooth ER in samples fixed chemically and/or unroofed vigorously by sonication. However, when mild sonication developed more recently is applied to freeze etching EM, smooth ER was detected as well (Fig. 2). Even in this case, smooth ERs were shrunk and fragmented more or less by fixation and sonication effect when compared with native state (compare Fig. 2 with Fig. 1). In freeze etching EM, however, the contrast of sample was raised so much due to the shadowing of platinum. Therefore, it is easy to trace the continuity of smooth ER three
dimensionally in cytoplasm. Smooth ER described in Fig. 2 seems to be a part of the intricate network remained after sonication. Probably, complicated network of smooth ER may be wrapping many actin bundles and microtubules.

Clathrin-coats and caveolae were observed as well on the cytoplasmic surface of plasma membrane like a freeze etching replica electron microscopy (freeze etching EM). Unroofing was also applicable to immuno-labeling method in Cryo-EM. Antibody labeling of IQGAP1, that is one of effecter proteins facilitating network formation of actin filaments, was localized alongside actin filaments. IQGAP 1 showed spatial specificity together with antigenic specificity. Freeze etching EM confirmed morphological findings provided by Cryo-EM complementarily.

References:
[3] The reported results were obtained as part of the JST-SENTAN program (J.U.). Beginning in April 2015, the AMED-SENTAN program has promoted this development project (16hm0102003h0005) (J.U.), and it was also supported in part by a Grant-in-Aid for Challenging Exploratory Research (26650049) from JSPS (J.U.)

Figure 1. Cryo-electron micrograph of native membrane cytoskeleton beneath the cell membrane exposed by improved unroofing. Actin filaments and microtubules (arrows) spread out as a stream without fragmentation in wavy manner, which appear to be flexible. Smooth ER (SER) network is distinctly observed. Actin filaments, microtubules and smooth ER overlap each other depending on the thickness of sample. Short periodicity of actin filaments appeared clearly on raising magnification (inset).

Figure 2. Freeze etching electron micrograph showing membrane cytoskeleton. Stress fiber (SF) including actin filaments and microtubules are found on the cytoplasmic surface of plasma membrane. Smooth ER (painted with green color) is intermingled with cytoskeleton while forming intricate network. Both tubular and sheet region of smooth ER are shrunk and deformed by sonication and fixation, when compared with native hydrated samples in Fig.1. MT: microtubule.