Imaging the Cell Surface: Argon Sputtering to Expose Inner Cell Structures

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ABSTRACT Established microscopies such as Scanning Electron Microscopy (SEM) and more recent developments such as Atomic Force Microscopy (AFM) and X-ray Photo-Electron Emission spectroMicroscopy (X-PEEM) can only image the sample surface. We present an argon sputtering method able to progressively expose inner cell structures without apparent damage. By varying the sputtering time, the structure of cell cytoskeleton, vesicles, mitochondria, nuclear membrane, and nucleoli can be imaged. We compared images obtained with confocal fluorescence microscopy, transmission electron microscopy (TEM), SEM, and X-PEEM on similar samples after argon sputtering, then confirmed the similarity of reference intracellular structures, including cytoskeleton fibers, cell-cell and cell-substrate adhesion structures, and secretory vesicles. We conclude that the sputtering method is a new valuable tool for surface sensitive microscopies. *Microsc. Res. Tech. 63:115–121, 2004.*

INTRODUCTION

Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM) and chemical and spectroscopic investigations by X-ray PhotoElectron Emission spectroMicroscopy (X-PEEM) can only image and analyze the sample surface. Therefore, inner cell organelles such as the vesicular apparatus and mitochondria, or supramolecular assemblies such as the internal structure of plasmalemma, cell-cell junctions, the cytoskeleton, and the nuclear membrane cannot be visualized. We present here a novel method suited to expose inner structures in whole fixed cells by argon sputtering their top-most portion, making these structures available for surface-sensitive techniques. By varying the sputtering time, structures located at different depths can be exposed and imaged. We compared SEM and X-PEEM images with those obtained on similar cells using confocal fluorescence microscopy and TEM.

MATERIALS AND METHODS

The Caco-2 human colon carcinoma cells (Mahraoui et al., 1994) were plated at a density of 20×10^3 cells/cm^2, grown in Dulbecco's modified Eagle minimum essential medium (DMEM, GIBCO Labs, Grand Island, NY), supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO Labs), L-glutamine (2 mM), penicillin (50 IU/ml), and streptomycin (50 μg/ml). Cells were plated and allowed to grow for 1 to 3 days on 10×10 mm silicon wafers, followed by fixing with 2.5% glutharaldehyde in 0.1 M Millonig's phosphate buffer (MPB) at 4°C for 1 hour. After washing in MPB, cells were dehydrated in increasing acetone concentrations and then critical-point dried using liquid CO_2.

Samples were kept and transported in desiccators, and handled only in a dry nitrogen glove box. They were transferred to the Spectromicroscope for PHotoelectron Imaging of Nanostructures with X-rays (SPHINX) (Frazer et al., 2003) X-PEEM ultra-high vacuum preparation chamber for sputtering. The base pressure in this chamber is 1×10^{-10} Torr. Sputtering was performed by leaking Ar gas into the chamber to a constant pressure of 1.5×10^{-5} Torr, with an ion gun (SPECS model IQE 11/35) at 3 kV, mounted at a distance of approximately 40 cm from the sample surface. Argon sputtering of the cells was performed for 0–15 minutes depending on the desired cell structure to be exposed. After sputtering, the cells were sputter-coated with 200 Å gold for SEM analysis, and with 10 Å Pt/Pd for SPHINX analysis.

Scanning electron microscopy observation was carried out using the Stereoscan 240 scanning electron microscope (Cambridge Instr., Cambridge, UK).

SPHINX spectromicroscopy analysis was performed at the Wisconsin Synchrotron Radiation Center (SRC). SPHINX is an X-PEEM, described in detail in Frazer et al. (2003), and mounted on the undulator PGM beamline (12–240 eV photon energy) or on the HERMON beamline (62–1,200 eV).

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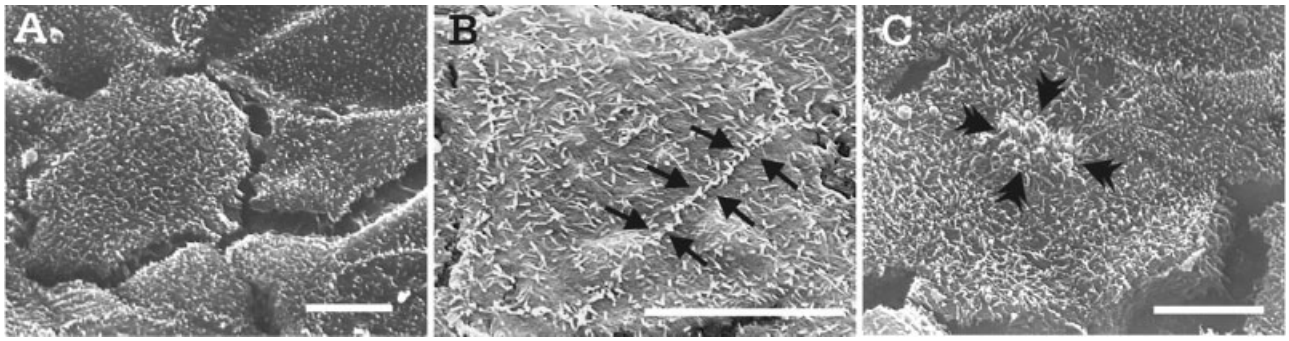

Fig. 1. Scanning electron micrographs of unsputtered Caco-2 cells. **A:** Numerous villous structures are present on cell surface in a non-confluent monolayer; **B:** Cell-cell contacts are shown (arrowheads); **C:** In differentiated cells, brush border microvilli are present at the apical pole (double-pointed arrows). Bars = 10 μm.

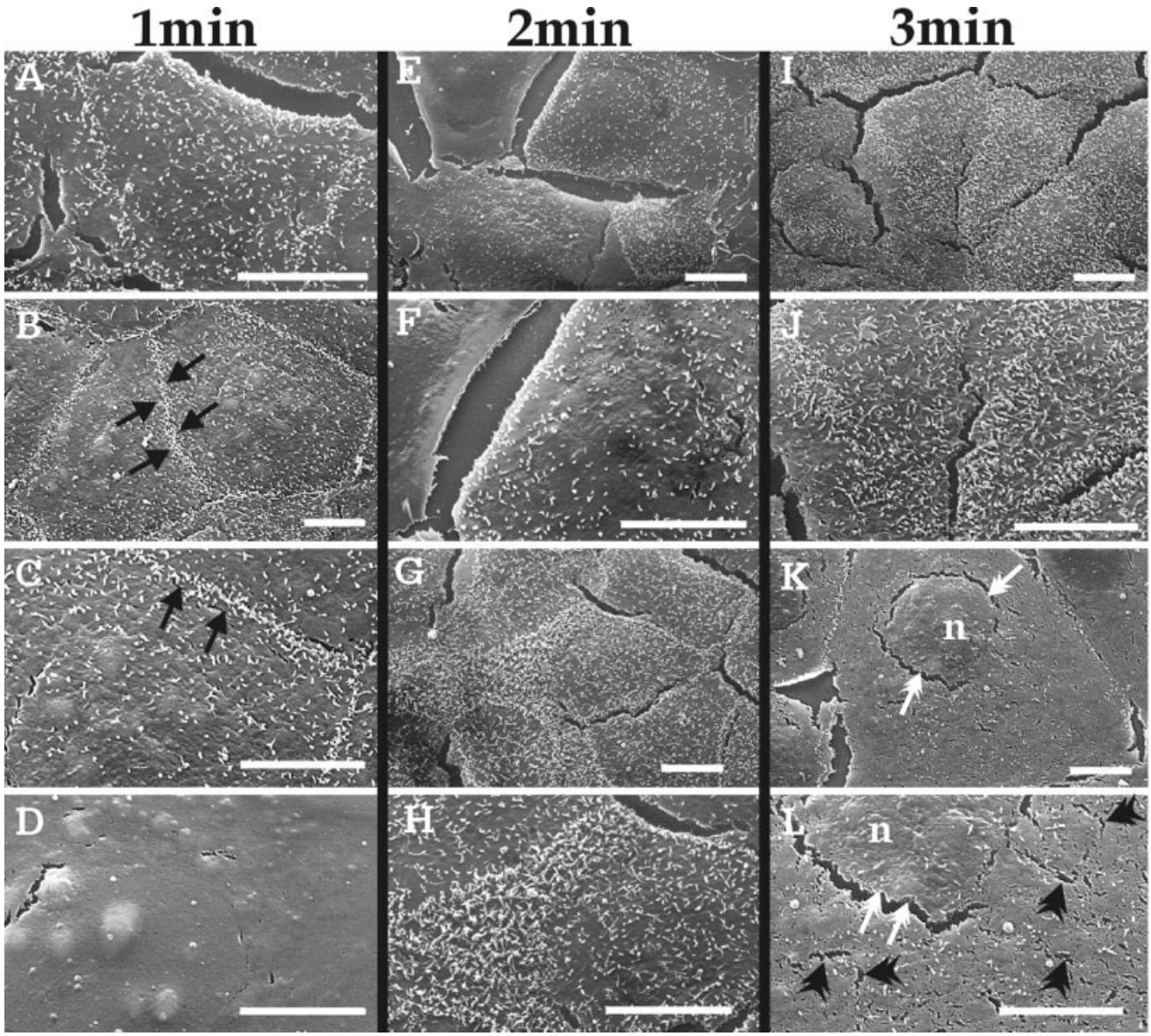

Fig. 2. Scanning electron micrographs of Caco-2 cells after 1, 2, and 3 minutes sputtering. **A–H:** After 1 and 2 minutes sputtering, the cell appearance is very similar to unsputtered controls; arrows point to cell-cell junction. **I–L:** After 3 minutes sputtering, some inner cytoplasmic structures, such as cisternae of endoplasmic reticulum (arrowheads) and nuclear envelope (double-pointed arrows), become visible only in flat cells (K and J). n: nucleus. Bars = 10 μm.

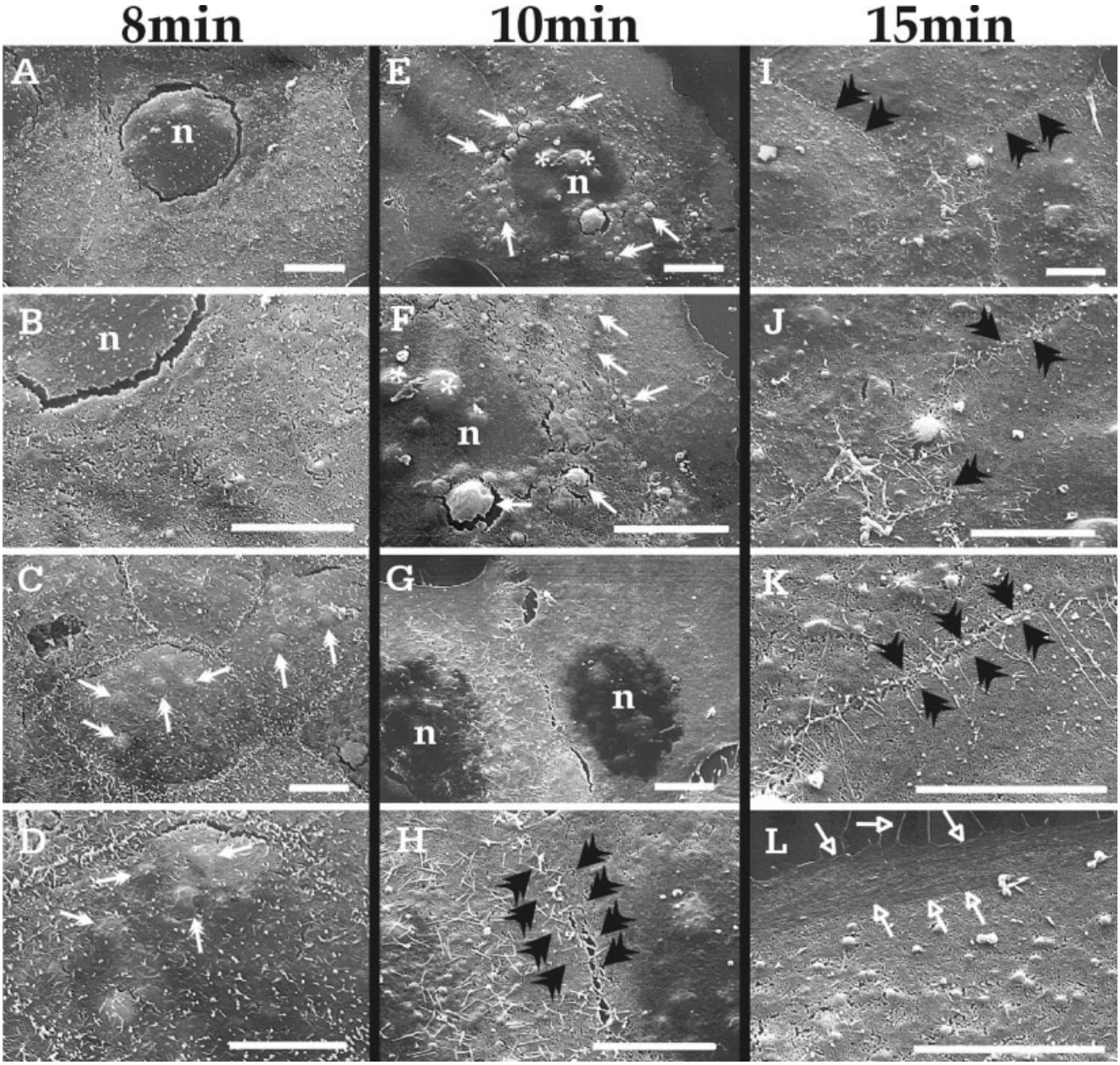

Fig. 3. Scanning electron micrographs of Caco-2 cells after 8, 10, and 15 minutes sputtering. **A–D:** After 8 minutes sputtering, the exposure of endoplasmic reticulum in flat cells is more evident (A, B); some cytoplasmic organelles (arrows) and cytoskeletal structures become visible beneath the cell surface of thick differentiated cells (C, D). **E–H:** After 10 minutes sputtering, cytoplasmic organelles and vesicles (arrows) and nucleoli (asterisks) are completely exposed (E, F) as well as cytoskeleton fibers (G, H); in H, arrowheads point to cell-cell junction. **I–L:** After 15 minutes, the cytoskeletal network at cell-cell junction (arrowheads in I–K) and at the cell-substrate adhesion site (arrowheads in I–K), as well as secretory vesicles (open arrows in L), are well visible. n: nucleus. Bars = 10 μm.

For immunofluorescence labeling and confocal laser scanning microscopy observations, Caco-2 cells grown on coverslips were washed with phosphate buffer saline (PBS), fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton-X 100 in PBS for 5 minutes, and then labeled with fluorescent staining. Actin microfilaments were stained with fluorescein (FITC)-conjugated phalloidin (Sigma Chem. Co., St Louis, MO). Cell-substrate plaques were labeled by mAbs to human vinculin (Sigma Chem. Co), revealed by tetramethylrhodamine-isothiocyanate (TRITC)-conjugated anti-mouse IgG. For Golgi apparatus and endoplasmic reticulum staining, cells were incubated with TRITC-conjugated wheat germ agglutinin (WGA-TRITC; Sigma) for 1 hour at room temperature. Fluorescently labeled samples were imaged by a confocal LEICA TCS 4D microscope (Leica, Heidelberg, Germany) equipped with an argon/kripton laser and 40 × 1.00–0.5 or 100 ×

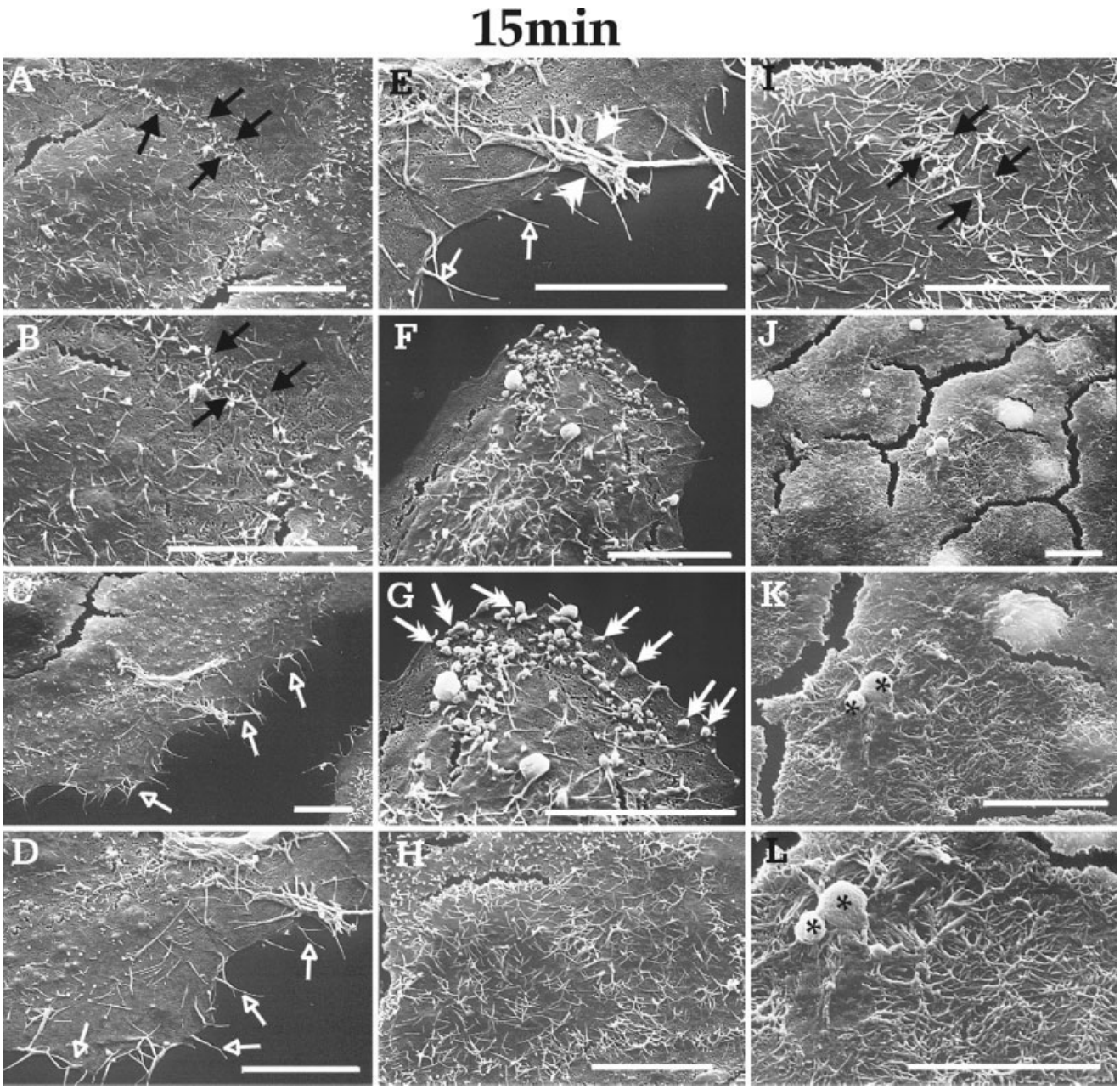

Fig. 4. Scanning electron micrographs of Caco-2 cells after 15 minutes sputtering. **A, B:** Details of cytoskeleton fibers at cell-cell junction (arrows). **C–E:** Details of cytoskeleton fibers at the cell-substrate adhesion site (open arrows); in E, arrowheads point to thick packed fibers. **F,G:** Details of exposed cytoplasmic organelles and secretory vesicles (double-pointed arrows). **H–L:** Details of exposed cytoskeletal network in thick differentiated cells; arrows in I) point to cell-cell junction; in K and L, cytoplasmic organelles connected to cytoskeleton fibers are shown (asterisks). Bars = 10 μm.

1.3–0.6 oil immersion lenses. The excitation and emission wavelengths employed were 488 and 510 nm, for FITC-labeling, and 568 and 590 nm, for TRITC-labeling. The images were recorded by pseudo-color representation.

For TEM observations, cells were fixed for 1 hour at 4°C with 2.5% glutharaldheyde in 0.1 M Millonig's phosphate buffer (MPB) containing 2% sucrose, and post-fixed for 1 hour at 4°C with 1% OsO_4 in the same buffer. Specimens were dehydrated in increasing ethanol concentrations and embedded in Spurr epoxy resin (Agar Scientific LTD, Stansted, Essex, UK). Ultrathin sections were stained with uranyl acetate and lead citrate, then observed by a Philips CM12 transmission electron microscope operating at 80 kV.

RESULTS AND DISCUSSION

SEM images of un-sputtered Caco-2 cells are shown in Figure 1. Their characteristic microvillar structure

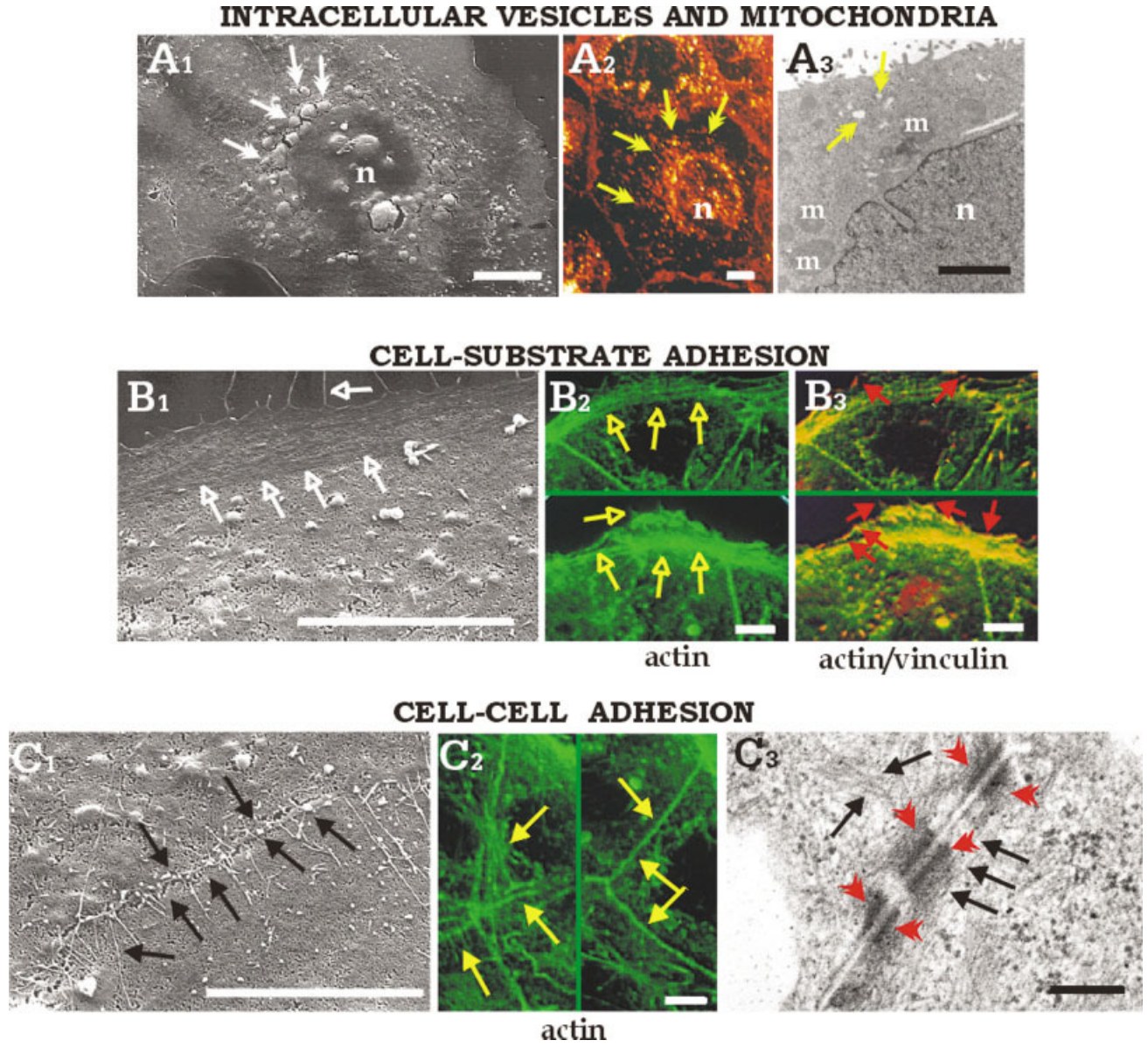

Fig. 5. Scanning electron micrographs of Caco-2 cells after 15 minutes sputtering (A_1, B_1, C_1) compared with images obtained by confocal microscopy (CLSM, A_2, B_2, B_3, C_2) on whole cells and transmission electron microscopy (TEM, A_3, C_3) on ultrathin sections. **A:** Exposure of intracellular organelles (white arrows) similar in size and distribution to WGA-stained vesicles (yellow arrows) and mitochondria (m) observed by CLSM (A_2) or TEM (A_3). **B:** Exposure of microfilament network at the cell-substrate adhesion site (open white arrows) similar in distribution to actin fibers (open yellow arrows) and substrate adhesion plaques (red arrows) observed by CLSM (B_2, B_3); in detail, in B_2 the actin fiber-deriving signal (green) is reported, while in B_3, the double staining actin (green)/vinculin (red) is shown, with the yellow hue indicating the colocalization of the two fluorescent signals. **C:** Exposure of microfilament network at the cell-cell adhesion structures similar in distribution to actin fibers observed by CLSM (yellow arrows, C_2) or by TEM (black arrows, C_3) at the cell-cell junctions (red arrowheads). Bars = 10 μm, except in A_3 (2 μm) and C_3 (200 nm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

is evident. Figures 2–4 show SEM images of the cell surface appearance revealed by increasing the sputtering times up to 15 minutes. At very short times (Fig, 2A–H) the cell appearance is very similar to un-sputtered control cells (Fig. 1), while by increasing the sputtering time, several inner cytoplasmic structures become visible, such as endoplasmic reticulum, nuclear membrane and nucleoli, cytoplasmic organelles, secretory vesicles, and cytoskeleton fibers.

In Figure 5, we show a comparison of the SEM micrographs of intracellular structures exposed after 15 minutes sputtering with images obtained by conventional microscopies, including confocal fluorescence microscopy and TEM. In particular, in Figure 5A_1 intracellular organelles are exposed, similar in size and distribution to: (1) the secretory vesicles specifically labeled and observed by confocal microscopy (Fig. 5A_2), and (2) the mitochondria, as visualized by TEM (Fig.

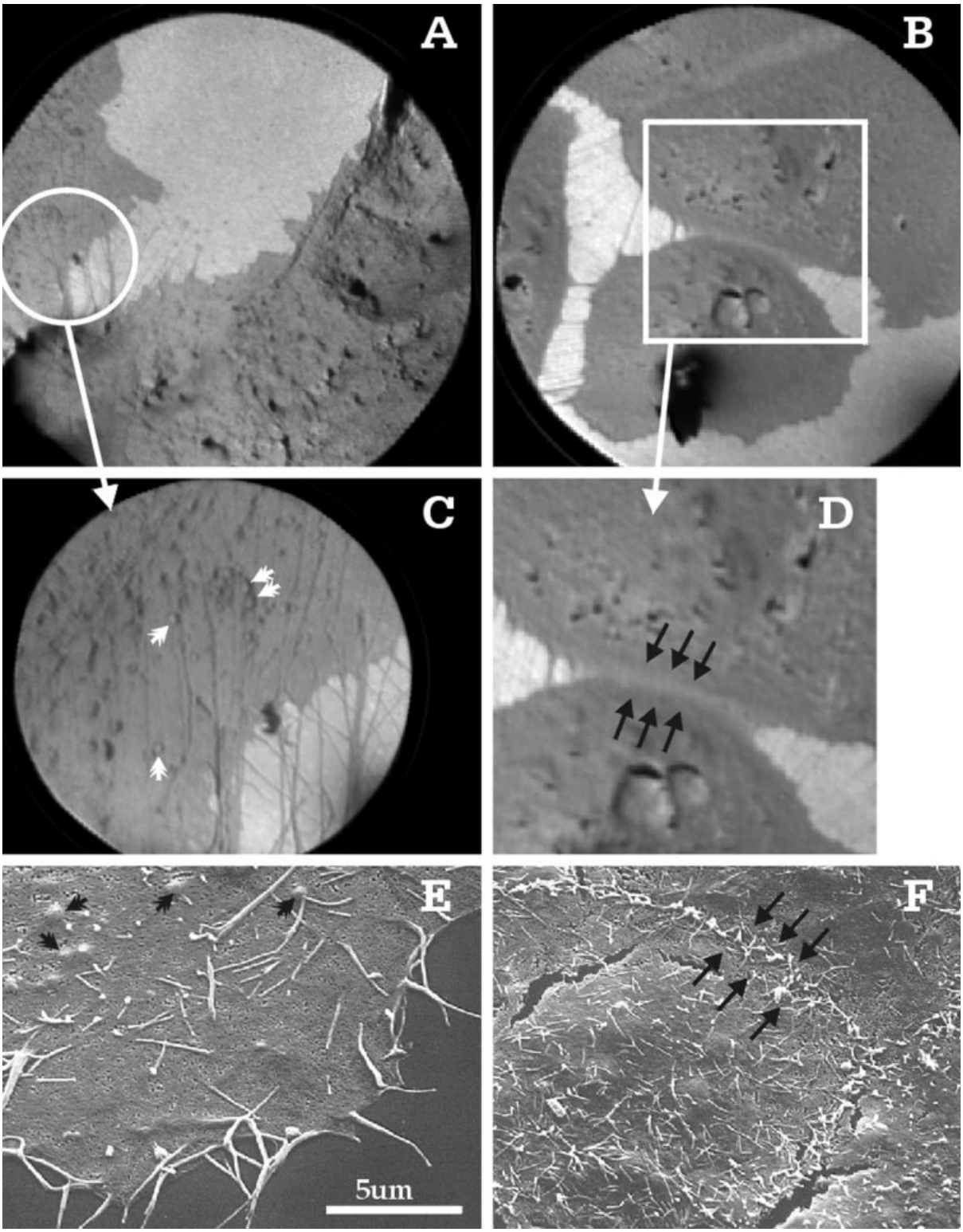

Fig. 6. SPHINX images of Caco-2 cells after 15 minutes sputtering compared to SEM images. Actin microfilament network at cell-substrate (**A, C, E**) and cell-cell (**B, D, F**) adhesion sites and exposed cytoplasmic vesicles (white arrows) imaged by SPHINX (A–D) and SEM (E, F). Bars = 5 μm.

$5A_3$). Actin fibers at the cell-substrate adhesion sites, imaged after 15 minutes sputtering are shown in Figure $5B_1$ and compared with confocal microscopy images of specifically labeled actin microfilaments (Fig. $5B_2$), and of vinculin, a cytoskeletal component of substrate adhesion plaques (Fig. $5B_3$). A sputtering time of 15 minutes served also to expose actin fibers involved in the formation of cell-cell adhesion structures (Fig. $5C_1$), as easily identifiable for comparison with the confocal (Fig. $5C_2$) and TEM (Fig. $5C_3$) images.

The SPHINX images of Caco-2 cells after 15 minutes sputtering are shown in Figure 6A–D and compared to the images obtained by SEM (Fig. 6E and F). This sputtering time was sufficient to expose several fibers of the microfilament network at the cell-substrate adhesion sites, as well as some intracytoplasmic vesicles (double-pointed arrows).

As measured in the SPHINX and SEM micrographs, the cell-cell fibers range between 0.1–0.4 μm in diameter, while cell-substrate fibers range between 0.2–0.3 μm. The cell-cell adhesion fibers are 0.26 ± 0.09 μm (mean ± standard deviation) before sputtering and 0.22 ± 0.043 μ m after sputtering (Student $t = -0.08$, not significant), while the cell-substrate fibers are 0.28 ± 0.04 μ m in diameter before sputtering and 0.14 ± 0.026 μ m after sputtering ($t = 5.23$, highly significant). Since the cell-substrate adhesion fibers are significantly thinner than the ones observed on the unsputtered cells, we infer that after sputtering we observe exposed naked actin fibers, while membranes, membrane proteins, and possibly parts of the fibers themselves have been removed.

CONCLUSIONS

We presented the first tests of a cell-sputtering approach to reveal the cytoskeleton and intracytoplasmic organelles of eukaryotic cells. The sputtering efficacy in exposing inner cell structures was evaluated by comparing SPHINX images with TEM, SEM, and confocal fluorescence microscopy images, acquired on similar parallel samples. The size and distribution of the fibers and intracellular vesicles are consistent in all used microscopy approaches. With this new sputtering method, future studies on the elemental composition and the oxidation state of elements in the cytoskeleton are made possible.

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REFERENCES

Frazer BH, Girasole M, Wiese LM, Franz T, De Stasio G. 2003. Spectromicroscope for the PHotoelectron Imaging of Nanostructures with X-rays (SPHINX): performance in biology, medicine and geology, Ultramicroscopy (in press).

Mahraoui L, Rodolosse A, Barbat A, Dussaulx E, Zweibaum A, Rousset M, Brot-Laroche E. 1994. Presence and differential expression of SGLT1, GLUT1, GLUT2, GLUT3 and GLUT5 hexose-transporter mRNAs in Caco-2 cell clones in relation to cell growth and glucose consumption. Biochem J 298:629–633.