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Extremely thin layer plastification for focused-ion beam scanning electron microscopy: an improved method to study cell surfaces and organelles of cultured cells

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Summary

Since the recent boost in the usage of electron microscopy in life-science research, there is a great need for new methods. Recently minimal resin embedding methods have been successfully introduced in the sample preparation for focused-ion beam scanning electron microscopy (FIB-SEM). In these methods several possibilities are given to remove as much resin as possible from the surface of cultured cells or multicellular organisms. Here we introduce an alternative way in the minimal resin embedding method to remove excess of resin from two widely different cell types by the use of Mascotte filter paper. Our goal in correlative light and electron microscopic studies of immunogold-labelled breast cancer SKBR3 cells was to visualise gold-labelled HER2 plasma membrane proteins as well as the intracellular structures of flat and round cells. We found a significant difference (p < 0.001) in the number of gold particles of selected cells per 0.6 µm² cell surface: on average a flat cell contained 2.46 ± 1.98 gold particles, and a round cell 5.66 ± 2.92 gold particles. Moreover, there was a clear difference in the subcellular organisation of these two cells. The round SKBR3 cell contained many organelles, such as mitochondria, Golgi and endoplasmic reticulum, when compared with flat SKBR3 cells. Our next goal was to visualise crosswall associated organelles, septal pore caps, of Rhizoctonia solani fungal cells by the combined use of a heavy metal staining and our extremely thin layer plastification (ETLP) method. At low magnifications this resulted into easily finding septa which appeared as bright crosswalls in the back-scattered electron mode in the scanning electron microscope. Then, a septum was selected for FIB-SEM. Cross-sectioned views clearly revealed the perforate septal pore cap of R. solani next to other structures, such as mitochondria, endoplasmic reticulum, lipid bodies, dolipore septum, and the pore channel. As the ETLP method was applied on two widely different cell types, the use of the ETLP method will be beneficial to correlative studies of other cell model systems and multicellular organisms.

Introduction

Preparation methods of cultured cells for focused-ion beam scanning electron microscopy (FIB-SEM) involving resins may include en bloc resin embedding, thin layer plastification (Bittermann et al., 2012; Kizilyaprak et al., 2014), ultrathin embedding (Belu et al., 2016) and minimal resin embedding of multicellular specimens (Schieber et al., 2017). Cell model systems being used in studies for FIB-SEM are bacteria, yeast and mammalian cells cultured on a substrate, for example glass cover slips, sapphire discs, Aclar or Thermanox cover slips. All these different cell types are being prepared for en bloc resin embedding. For example human umbilical vein endothelial cells (Jiménez et al., 2010); mammalian melanosome cells and T-cells (Murphy et al., 2011; Narayan et al., 2014); human pancreatic carcinoid cells (Villinger et al., 2012); fibroblasts...
and HeLa cells (Wierzbicki et al., 2013; Narayan et al., 2014; Perkovic et al., 2014); human cardiac telocytes (Cretou et al., 2014); yeast cells (Heymann et al., 2006; Wei et al., 2012); and bacteria (Narayan et al., 2014). After flat embedding the cultured cells are found at the bottom of the polymerised resin block. At the apical side of these cells a layer of at least 2–3 mm resin is present, whereas at the basal side there is hardly any resin layer present when the used growth substrate has been removed from the cell layer. For FIB-SEM, the resin block is positioned in the microscope in such a way that the basal side of the cells is facing up. Prior to ion milling a platinum (Pt) layer of about 500 nm is deposited onto the cell of interest. After ion milling, and as seen from top to bottom when imaging in the SEM mode, the block face will clearly show the Pt layer, the cells from basal side to apical side with sliced organelles and cell structures and a part of the 2–3 mm resin layer of the resin block. Although stunning results have been obtained from en bloc resin-embedded biological samples after FIB-SEM, the cells are always being viewed from their basal side. When cells are growing in layers on top of each other, it is difficult to find the cell of interest in the upper layer. Moreover, in en bloc resin-embedded samples it is impossible to visualise the cell surfaces as is done when viewing in conventional SEM, that is cells are being chemically fixed, dehydrated, critical point dried and viewed in a scanning electron microscope to visualise the cell surface.

As an alternative to en bloc resin embedding of cell cultures for FIB-SEM, Bittermann et al. (2012) developed a new method by introducing the thin layer plastification (TLP) method. In this TLP method only a thin layer of resin is left on top of the cells, allowing thick cells to bulge out. After polymerisation of the resin, the surface contours of the embedded cells can be seen in the SEM mode after which the intracellular structures can be visualised by FIB-SEM. In the work of Bittermann et al. (2012), endothelial cells clearly revealed a rough surface. This overall morphology of the endothelial cell culture made it possible to select the cell of interest for FIB-SEM. The TLP method has also successfully been applied on osteoclast cultures by Kizilyaprak et al. (2014). After chemical fixation, heavy metal staining, dehydration and resin infiltration of osteoclasts, these authors proposed different ways to keep the resin surrounding them. After heat polymerisation of the resin, the organisms were further processed by the use of electron microscopic imaging applied on three different model organisms: the zebrafish embryo Danio rerio, the marine worm Platynereis dumerilii, and the dauer larva of the nematode Caenorhabditis elegans. The zebrafish embryos and the marine worms were chemically fixed, whereas the larva of the nematode were high-pressure frozen. Thereafter, these three different organisms were further processed by the use of electron microscopic standards, and finally resin infiltrated. Depending on the size of the organism, excess of resin was blotted away on absorbent paper, filter paper, or Aclar by gently moving the samples with a toothpick around on their substrate to minimise the resin surrounding them. After heat polymerisation of the resin, the organisms were sputter-coated with gold. The minimal resin method developed by Schieber et al. (2017) facilitates in finding the region of interest on multicellular organisms as long as surface features are observable in the light microscope as well as in the scanning electron microscope.

After immunogold labelling of cell surface membrane proteins of cultured cells, gold particles can easily be found by SEM in back-scattered electron mode. However, in the work of Bitterman et al. (2012), Kizilyaprak et al. (2014), Belu et al. (2016) and Schieber et al. (2017) prior to ion milling a protective layer of 0.5–1 µm platinum was deposited on the region of interest. This platinum layer will prevent the visualisation of the gold-labelled proteins on the surface of the cells. To
improve the addressed drawbacks when applying the three different minimal resin embedding methods, we developed an extremely thin layer plastification (ETLP) method. In the ETLP method we used Spurr’s low viscosity resin, and removed excess of resin from the surfaces of the cells by blotting with Maccotte filter paper followed by centrifugation. Human breast adenocarcinoma cells, SKBR3 cells, were prepared according to this ETLP method and subjected to FIB-SEM. Without a protective platinum layer both the distribution of gold particles on the cell surface and the contents of the cell could be visualised. In addition, the combined use of the ETLP method and FIB-SEM easily allowed us to find and visualise the septal pore caps in selected hyphal walls of the plant pathogenic fungus *Rhizoctonia solani*. We will discuss currently used thin layer plastification methods on cell cultures for FIB-SEM, and address some variables that may influence the impact of the ETLP method.

**Materials and methods**

**Cell lines and culture conditions**

Human HER2-positive breast cancer cells, SKBR3 cells, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Gibco, Thermo Fisher Scientific Inc., Bleiswijk, the Netherlands) supplemented with 7.5% (v/v) FBS, 100 IU mL\(^{-1}\) penicillin, 100 mg mL\(^{-1}\) streptomycin and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO\(_2\). The SKBR3 cells were cultured either on carbon coated borosilicate glass coverslips (Menzel-Gläser, Thermo Scientific, Germany) or on carbon coated 8 × 8 mm Thermanox cover slips (LUX, Thermanox AG-3 strain CBS 346.84 was maintained on YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% dextrose, 2% agar; Oxoid, Hampshire, UK). Culture medium was autoclaved for 20 min at about 1.1 bar, 110 °C, and aseptically poured into 100 mm × 15 mm polystyrene Petri dishes (Sigma-Aldrich Chemie N.V., Zwijndrecht, the Netherlands). An autoclaved perforated polycarbonate track-etched (PCTE) filter with a diameter of 47 mm and 0.6 µm pore size (Poretics, Osmonics Inc., Minnetonka, MN, USA) was gently placed on the solidified YM agar as described by Wöstien et al. (1991). Hyphal cells were scraped from a slant culture and put on the PCTE filter. Thereafter, a second autoclaved PCTE filter was placed on top and the Petri dish was closed. The hyphal cells sandwiched between the two PCTE filters formed a colony of about 2–4 cm in diameter after 3 days at 25 °C.

**Immunolabelling of SKBR3 cells**

SKBR3 cells on Thermaxon substrate were fixed by adding a volume of 4% (w/v) formaldehyde (FA, from Paraformaldehyde, Sigma-Aldrich Chemie N.V.) in 0.1 M PHEM buffer pH 6.9 (Schliwa & Blerkom, 1981), equal to the volume of the culture medium in the Petri dish with the Thermaxon coverslips, resulting in a final concentration of 2% (w/v) FA. After 15 min the fixative/medium mixture was replaced by 4% (w/v) FA in 0.1 M PHEM buffer. The fixation was carried on for 2 h at room temperature (RT) and continued overnight at 4 °C. All following steps were done at RT. After washing six times 5 min with 0.15 M phosphate buffered saline (PBS) pH 7.4, free aldehyde groups were quenched with 100 mM NH\(_4\)Cl (Merck) in PBS for 10 min. Subsequently the SKBR3 cells were washed twice in PBS for 5 min each washing step and blocked for 15 min with 1% (w/v) BSA (Bovine Serum Albumin fraction V, Sigma-Aldrich Chemie N.V.) in PBS. To immunogold label the HER2 proteins on the surface of the SKBR3 cells, cells were incubated for 60 min with Herceptin® (Roche Nederland B.V., Woerden, the Netherlands), 10 µg mL\(^{-1}\) in PBS containing 1% (w/v) BSA. After three washes of 10 min with PBS containing 0.1% (w/v) BSA, SKBR3 cells were incubated for 60 min with polyclonal Rabbit anti-human IgG (code-number A 0424, Dako, Heverlee, Belgium), 4 µg mL\(^{-1}\) in PBS containing 1% (w/v) BSA. After three washes of 10 min with PBS containing 0.1% (w/v) BSA, the cells were incubated for 20 min with Protein-A coupled to 15 nm gold (CMC, Utrecht, the Netherlands), 1:60 diluted in PBS containing 1% (w/v) BSA. Finally, the cells were washed three times for 10 min and three times for 5 min with PBS.

**Postfixation**

After immunogold labelling the SKBR3 cells on Thermaxon coverslips were postfixed for at least 1 h at RT and for this study for 48 h at 4 °C in a modified Karnovsky fixative (Karnovsky, 1965), that is 2% (w/v) FA and 2.5% (v/v) glutaraldehyde (GA, Taab, Calleva Park Aldermaston, Berks, RG7 8NA, England) plus 0.025 mM CaCl\(_2\) (Merck) and 0.05 mM MgCl\(_2\) (Merck) in 0.08 M sodium cacodylate (BDH VWR Analytical, Radnor, PA 19087, USA), pH 7.4. After washing two times 5 min with 0.1 M sodium cacodylate buffer pH 7.4, SKBR3 cells were postfixed for 90 min on ice in 1% (w/v) osmium tetroxide (Agar Scientific Limited, Stansted Essex CM24 8DA, England) + 1.5% (w/v) potassium hexacyanoferrat (II) (Merck) in 0.065 M sodium cacodylate buffer pH 7.4, protected from light and on a rocking shaker table at 2 rpm (Duomax 1030 Shaker, Heidelberg-Instruments, Schwabach, Germany). Next, after washing four times 5 min with Milli-Q water at RT and two times 5 min with Milli-Q water at 30 °C, the cells were subjected to the TCH-OsO\(_4\)-UAc-Pb method (modified from methods described by Tapia et al., 2012 and Paridaen et al., 2013). In this method the cells were first incubated in freshly prepared 1% (w/v) thio-carbohydrazide (TCH, Merck) for 15 min at 30 °C. Note: After the 1% (w/v) TCH preparation at 60 °C, the TCH solution was kept at 30 °C in the dark, and before use filtered through a 0.22 µm Millipore filter (Merck Millipore, Darmstadt, Germany) at 30 °C. Second, the
cells were washed two times for 5 min with Milli-Q water at 30 °C and four times with Milli-Q water at RT, and further fixed with 1% (w/v) osmium tetroxide (Agar Scientific Limited) in Milli-Q water for 30 min on ice, protected from light and on a rocking shaker table at 2 rpm, and subsequently washed six times for 5 min with Milli-Q water at RT. Third, the cells were en bloc stained for 30 min at RT with 2% (w/v) uranyl acetate (SPI supplies, West Chester, PA 19381–0656, USA) in Milli-Q water, filtered through a 0.22 µm Millipore filter (Merck Millipore), protected from light and on a rocking shaker table at 2 rpm. Thereafter the cells were washed six times for 5 min with Milli-Q water and finally for 30 min at 60 °C en bloc stained with freshly prepared lead aspartate vial on ice, containing 1% (w/v) OsO₄ (Agar Scientific Limited) + 1.5% (w/v) potassium hexacyanoferrate (II) (Merck) in 0.065 M sodium cacodylate buffer, pH 7.4. After 90 min post-fixation on ice, protected from light and on a rocking shaker table at 2 rpm (Heidolph-Instruments), the colony parts were washed six times for 5 min with Milli-Q water, subsequently stained according to the TCH-OsO₄-UAc-Pb method (see Post-fixation), and thereafter dehydrated in an increasing series of ethanol (see Dehydration). The glass vials with hyphal samples were placed in a benchtop rotary mixer at 2 rpm (Pelco rotary R2 mixer, TED Pella, CA, USA). The hyphal samples were infiltrated according to the following percentages and infiltration times with Spurr’s resin mixture, containing 5.0 g ERL 4206, 3.0 g DER and 13.0 g NSA, and 0.2 g DMAE: 20% (v/v) for 1 h, 40% (v/v) overnight (O/N), 60% (v/v) for 8 h, 80% (v/v) O/N, 90% (v/v) for 8 h, 90% (v/v) O/N, fresh 100% for 8 h, fresh 100% O/N, fresh 100% for 8 h, fresh 100% O/N, fresh 100% for 8 h and fresh 100% O/N. Subsequently, the resin-infiltrated hyphal cells were subjected to the ETLP method (see ETLP method). Finally, the Spurr’s resin was polymerised at 70 °C (O/N).

Dehydration

After rinsing with Milli-Q water the samples were dehydrated at room temperature in an increasing series of ethanol (Merck), and each step two times 5 min, 30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), 96% (v/v), only the step with 100% ethanol was done six times 5 min. Anhydrous ethanol was made by adding together 100 mL 96% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), 96% (v/v), 100% for 8 h and fresh 100% O/N. Subsequently, the resin from the back side of the Thermaxon coverslip and the R. solani colony parts were placed in a benchtop rotary mixer at 2 rpm (Pelco rotary R2 mixer, TED Pella, CA, USA). The hyphal samples were infiltrated according to the following percentages and infiltration times with Spurr’s resin mixture, containing 5.0 g ERL 4206, 3.0 g DER and 13.0 g NSA, and 0.2 g DMAE: 20% (v/v) for 1 h, 40% (v/v) overnight (O/N), 60% (v/v) for 8 h, 80% (v/v) O/N, 90% (v/v) for 8 h, 90% (v/v) O/N, fresh 100% for 8 h, fresh 100% O/N, fresh 100% for 8 h, fresh 100% O/N, fresh 100% for 8 h and fresh 100% O/N. Subsequently, the resin-infiltrated hyphal cells were subjected to the ETLP method (see ETLP method). Finally, the Spurr’s resin was polymerised at 70 °C (O/N).

Spurr’s resin

To make a resin mixture with a lower viscosity and a longer pot life, we used Spurr’s low viscosity resin, hereafter referred as Spurr’s resin (EMS, Hatfield PA 19440, USA; Spurr, 1969) containing: 5.0 g ERL 4221 (replacement for ERL 4206), 3.0 g DER and 13.0 g NSA, and mixed for 10 min with a stirrer in a fume hood. Thereafter, 0.1 g DMAE was added and mixed once more for 10 min.

Spurr’s resin infiltration and polymerisation

Anhydrous ethanol was mixed with Spurr’s resin to infiltrate the samples. To this end the Spurr’s resin mixture contained ERL 4221, DER and NSA without DMAE to infiltrate: 50% (v/v) two times 2 h, 75% (v/v) for 2 h, and 75% (v/v) O/N. Thereafter, the cells were infiltrated three times 2 h each with fresh 100% Spurr’s resin containing DMAE. Subsequently, the resin infiltrated cells were subjected to the ETLP method (see below paragraph ETLP method). Finally, the Spurr’s resin was polymerised at 70 °C (O/N).

Rhizoctonia solani hyphae preparation for FIB-SEM

After 3 days the R. solani colony cultured between two PCTE filters on YM agar in a Petri dish was chemically fixed with 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 16 h at 4 °C. After fixation, the upper PCTE filter was removed and the colony was washed two times for 5 min with 0.1 M sodium cacodylate buffer. Subperipheral colony parts of about 7 × 10 mm were removed from the colony with a scalpel, and transferred to a screw cap glass vial on ice, containing 1% (w/v) OsO₄ (Agar Scientific Limited) + 1.5% (w/v) potassium hexacyanoferrate (II) (Merck) in 0.065 M sodium cacodylate buffer, pH 7.4. After 90 min post-fixation on ice, protected from light and on a rocking shaker table at 2 rpm (Heidolph-Instruments), the colony parts were washed six times for 5 min with Milli-Q water, subsequently stained according to the TCH-OsO₄-UAc-Pb method (see Post-fixation), and thereafter dehydrated in an increasing series of ethanol (see Dehydration). The glass vials with hyphal samples were placed in a benchtop rotary mixer at 2 rpm (Pelco rotary R2 mixer, TED Pella, CA, USA). The hyphal samples were infiltrated according to the following percentages and infiltration times with Spurr’s resin mixture, containing 5.0 g ERL 4206, 3.0 g DER and 13.0 g NSA, and 0.2 g DMAE: 20% (v/v) for 1 h, 40% (v/v) overnight (O/N), 60% (v/v) for 8 h, 80% (v/v) O/N, 90% (v/v) for 8 h, 90% (v/v) O/N, fresh 100% for 8 h, fresh 100% O/N, fresh 100% for 8 h, fresh 100% O/N, fresh 100% for 8 h and fresh 100% O/N. Subsequently, the resin-infiltrated hyphal cells were subjected to the ETLP method (see ETLP method). Finally, the Spurr’s resin was polymerised at 70 °C (O/N).

Extremely thin layer plastification (ETLP) method

The extremely thin layer plastification (ETLP) method included the following preparation steps: (1) The SKBR3 cells on Thermaxon coverslips and the R. solani colony parts were once more infiltrated with freshly made 100% Spurr’s resin. After 2 h of infiltrating, the Thermaxon coverslips with cells up and R. solani colony parts were placed on a PCTE filter supported by a Whatman filter paper (Sigma-Aldrich Chemie N.V., Qualitative circles, #1001090, 90 mm diam.) and a single-fold paper hand towel. To speed up the removal of the excess of Spurr’s resin from the back side of the Thermaxon coverslip and the R. solani colony part, the PCTE filter had been punctured with a thin needle to make about 40 holes in an area of about 10 × 10 mm. The samples were left in the fume hood for 10 min, after which the Spurr’s resin had been soaked up by the Whatman filter paper and the single fold paper hand towel. This excess of resin was found around the samples (Fig. 1A). (2) The SKBR3 cells on Thermaxon coverslips with cells up and the R. solani colony parts supported by a piece of approximately 8 × 8 mm PCTE filter (not shown) were transferred onto a Mascotte filter paper sheet of 7 × 13.5 cm (Mignot & De Block B.V., The
Fig. 1. Preparation steps of the Extremely Thin Layer Plastification (ETLP) method to remove excess of Spurr’s resin from cultured cells. (A) Two Theranox coverslips with cells facing up on the PCTE filter with Spurr’s resin around the Theranox coverslips removed from the back site of the substrate. The excess of resin on top of the cells is still present. (B) Theranox coverslips with cells facing up on a clean Mascotte filter paper with resin showing a convex meniscus. (C) Clean Mascotte filter paper on top of the cells to remove Spurr’s resin from the cell surfaces. (D) After repeated sandwiching between clean parts of the Mascotte filter papers, lesser resin will be removed from the cells. (E) Whatman filter paper (fp) was used as a utensil to swab over the Mascotte filter paper (M) to remove residual resin from the Theranox coverslip with cells (T) sandwiched between two clean Mascotte filter papers. (F) Theranox coverslip with cells positioned face up on a clean Mascotte filter paper prior to transfer into micro tubes. Note: There is no resin with a convex meniscus present as in (A) and (B). (G) A Theranox coverslip with cells in a microtube with a screw cap containing a small ball of Mascotte filter paper at the bottom. Inset shows a close up of a side view of the Theranox coverslip with cells on Mascotte filter paper at the bottom of the tube. (H) Microtubes with Theranox coverslips with cells transferred into an Eppendorf centrifuge and centrifuged for 10 min at 956 RCF at 25 °C. (I) Theranox coverslip with cells facing up placed on the back side of a green flat embedding mould. (J) After resin polymerisation the Theranox coverslip with cells can be used for FIB-SEM. The figure shows a part of the Theranox coverslip with groups of cells as seen in the scanning electron microscope.
Mascotte Company, Eindhoven, the Netherlands) (Fig. 1B). After 2 min the samples were placed on a clean part of the Mascotte filter paper sheet about 1 cm below the previous place, which showed excess of Spurr’s resin. (3) After another 2 min the samples were put again on a new part of the Mascotte filter paper sheet about 1 cm below the previous place. A second clean Mascotte filter paper sheet of $7 \times 13.5$ cm was gently put on top of the cells to remove the excess of resin from the surfaces of the cells by soaking up Spurr’s resin (Fig. 1C). After 2 min the upper Mascotte filter paper sheet was removed by lifting the bottom right corner, pulled backwards while leaving the samples on the lower Mascotte filter paper sheet. If necessary, a forceps was used to hold the samples in place during the removal of the upper Mascotte filter paper sheet. Step #3 was repeated at least twice until most of excess of Spurr’s resin was taken up by the Mascotte filter paper sheet (Fig. 1D). (4) Thereafter, the samples were placed on a clean Mascotte filter paper sheet of $7 \times 13.5$ cm, and covered with another clean Mascotte filter paper sheet of $7 \times 13.5$ cm alike step #3. Thereafter a Whatman filter paper was used as a utensil to remove as much resin as possible from the surfaces of the cells by gently swabbing by wrist action 10 times from left to right over the upper Mascotte filter paper sheet which covered the samples (Fig. 1E). Afterwards, to remove the upper Mascotte filter paper, the samples were lifted with clean forceps from the lower Mascotte filter paper sheet, and put back on a clean part of the Mascotte filter paper sheet, about 1 cm below the previous place. Thereafter a clean part of the upper Mascotte filter paper sheet was put on top of the cells and by wrist action gently swept with Whatman filter paper 10 times as before. This step #4 was repeated several times to remove the excess of Spurr’s resin from the cells. Thereafter, the upper Mascotte filter paper sheet was carefully removed (Fig. 1F).

**Note:** We always used clean areas of the upper and lower Mascotte filter paper sheets. (5) Then, the samples were transferred with a clean fine forceps (SC forceps, Dumostar, Fine Science Tools GmbH, Heidelberg, Germany) into a screw cap microtube 2 mL (Sarstedt AG & Co. Nürnberg, Germany) containing at the bottom a small paper ball of Mascotte filter paper to absorb the remaining resin during centrifugation (Fig. 1G). For the *R. solani* colony parts, the PCTE filter was replaced by a Thermaxov coverslip which was used as a support to place the colony part in the screw cap vial (not shown). (6) The tubes with samples were placed in a centrifuge with a swing-out rotor (Eppendorf Centrifuge 5417R, Eppendorf Nederland B.V., Nijmegen, the Netherlands) in such a way that during centrifugation the Thermaxov coverslip was in a horizontal position in the tube with cells facing up and centrifuged for 10 min at 30000 × *g* (Equal an RCF value of 956), at 25 °C (Fig. 1H).

(7) After removing the tubes with samples from the centrifuge, the Thermaxov coverslips with cells facing up were taken out with a fine forceps and placed onto the back side of a green flat embedding mould which is resistant to Spurr’s resin (Agar Scientific Limited, Fig. 1I), and was put into a Greiner square Petri dish $120 \times 120$ mm (Greiner Bio-One GmbH, Friefsenhain, Germany) covered by the lid. (8) Subsequently, the green flat embedding mould with samples was transferred to a Memmert oven (Memmert GmbH+Co. KG) set at 70 °C to let the Spurr’s resin polymerise overnight. (9) Thereafter, each Thermaxov coverslip with SKBR3 cells facing up was put on an aluminium stub with a carbon adhesive (Agar Scientific Limited). Whereas the resin-polymerised *R. solani* colony part was gently removed from the Thermaxov support and put on a bare aluminium stub. (10) For better conductivity, carbon tape (Agar Scientific, the Netherlands) was put on the long sides of the Thermaxov coverslips and the supporting carbon adhesive, and on the long sides of the colony part and the bare aluminium stub. (11) To prevent sample charging, the samples were rotary sputter coated with 8 nm carbon according to the supplier’s manual (Leica ACE600 sputter coater, Leica Microsystems, Vienna, Austria) and viewed with a scanning electron microscope (Fig. 1J).

**Immunoscanning electron microscopy**

SKBR3 cells were HER2 immunogold labelled as described earlier in section Immunolabelling of SKBR3 cells, followed by postfixation and *en block* staining with heavy metals (see Postfixation). After washing eight times with Milli-Q water, the samples were transferred to specimen baskets (Plastic capsules D13xH18 mm fine mesh base, #16702734, Leica, Vienna, Austria) from which the height was modified to 9 mm, and placed in a 24-well plate (Costar, Corning Incorporated, Corning, NY, 14831, USA). Wells were filled with an increasing series of ethanol, 1.5 mL per well. The baskets containing the samples were during dehydration transferred from well to well. SKBR3 cells in 100% ethanol were further processed for scanning electron microscopy in the following way. The specimen baskets containing the Thermaxov coverslips with cells were quickly transported to a Leica CPD300 critical point drying apparatus (Leica Microsystems, Vienna, Austria) with a CPD-chamber that contained first a 1/2-height-Teflon filler and on top a four numbered wells 1/2-height-Teflon sample holder (pivot dimension $15 \times 21$ mm; mesh size 0.5 mm; replacing 50% of chamber volume), filled with anhydrous ethanol. The level of the ethanol was about 2 mm higher than the top of the four numbered wells sample holder. Once all the baskets containing the Thermaxov coverslips with cells were put into the sample holder, a covering mesh lid (mesh size 0.5 mm) was placed, and the chamber was further filled with anhydrous ethanol. After closing the CPD-chamber the samples were critical point dried according to the Leica CPD-300 manual with liquid CO2 as the transitional fluid. We used the following programme settings: Automatic On; Stirrer 100%; CO2 in, with speed slow and a delay of 120 s: exchange with speed 5 and cycles 12; CO2 out; heat slow and gas out with speed 40%. The total CPD processing time was about 2 h and 10 min. The dried specimens were mounted on aluminium stubs containing a carbon adhesive, and were subsequently
coated with 4 nm platinum by the use of a Leica ACE600 sputter coater according to the Leica ACE600 manual (Leica Microsystems, Vienna, Austria). Hereafter the SKBR3 cells were visualised with a scanning electron microscope (XL30-FEG, FEI Company as part of Thermo Fisher Scientific Inc., Eindhoven, the Netherlands) at an acceleration voltage of 5 kV with a WD of 3.1—3.5 mm, or at an acceleration voltage of 15 kV with a WD of 5 mm.

Testing Llama antibodies to label HER2 proteins

To test Llama antibodies for immunolabelling of HER2 proteins on the surface of the cells, the immunofluorescence labelling was done on HER2-positive SKBR3 cells as described by Kijanka et al. (2017). In short, SKBR3 cells were incubated with 3 µg mL⁻¹ Llama antibodies VHH 11A4 (QVQ, Utrecht, the Netherlands) in blocking buffer, that is a mixture of cold water fish gelatin and acetylated bovine serum (CFG: Gelatin from cold water fish skin 45% in water, Sigma-Aldrich Chemie N.V.; BSA-c: acetylated Bovine Serum Albumin 10% in water, Aurion). Thereafter, the SKBR3 cells were incubated with secondary antibodies Rabbit anti-VHH (R1216, BAC B.V., Naarden, the Netherlands) and tertiary antibodies Goat anti-Rabbit IgG/Alexa 488 (Thermo Fisher Scientific Inc., Molecular Probes, Invitrogen, Waltham MA, USA; and described in Panchuk-Voloshina et al. 1999). The negative control was incubated with blocking buffer and was further incubated with secondary and tertiary antibodies. The nuclei were stained with DAPI (Roche, Almere, the Netherlands). Fluorescence imaging was performed on an LSM 700 commercial scanning confocal microscope (Carl Zeiss MicroImaging, Jena, Germany) equipped with a Plan-Apochromat 63×1.40 NA Oil DIC (WD = 0.19 mm), using 405 nm (DAPI) and 488 nm (Alexa488) laser lines and laser powers <5%.

SKBR3 cells for CLEM

The SKBR3 cells were grown in a 12-well plate (Costar, Corning Incorporated, Corning, NY, 14831, USA) on carbon-coated glass coverslips and fixed in a final concentration of 2% (w/v) FA in PHEM buffer, pH 6.9. After 15 min the fixative/medium mixture was replaced by 2 mL 4% (w/v) FA in PHEM buffer, pH 6.9. After 2 h fixation at RT the cells were stored in the same fixative overnight at 4 °C. The fixed SKBR3 cells were washed with 0.15 M PBS, and free aldehyde groups were quenched with 100 mM NH₄Cl (Merck) in PBS for 10 min at RT, followed by two washes of 5 min with PBS. All next steps of the immunolabelling procedure were carried out at RT in droplets on a sheet of parafilm (Sigma-Aldrich Chemicals B.V.). To avoid evaporation a wet tissue paper was placed aside the droplets. Droplets and wet tissue paper were covered with the lid of a square culture dish 120 × 120 mm (Greiner). Coverslips with SKBR3 cells facing up were placed in droplets of PBS. Thereafter, to prevent non-specific labelling, the cells were incubated for 15 min in 100 µL blocking solution, containing 0.225% (v/v) CFG + 0.1% (v/v) BSA-c in PBS. Thereafter to detect the HER2 plasma membrane proteins, the SKBR3 cells were incubated with 30 µL of 3 µg mL⁻¹ Llama antibodies VHH 11A4 (QVQ, Utrecht, the Netherlands) in blocking solution for 1 h. After three washes of 10 min with 10× diluted blocking solution the cells were incubated for 1 h with 30 µL of 20 µg mL⁻¹ Rabbit anti-VHH purified serum (BAC B.V., Naarden, the Netherlands) in blocking solution. After three washes of 10 min with 10× diluted blocking solution the cells were incubated for 20 min with Protein A coupled to 15 nm gold (CMC, Utrecht, the Netherlands), 1:60 diluted in blocking solution. Then the SKBR3 cells were washed six times for 5 min each with 10× diluted blocking solution. Hereafter all steps were protected from light. The SKBR3 cells were incubated for 60 min with 30 µL of 10 µg mL⁻¹ Goat anti-Rabbit IgG/Alexa488 (Thermo Fisher Scientific Inc., Molecular Probes, Invitrogen, Waltham MA, USA) in blocking solution. Subsequently, the cells were washed three times for 10 min with PBS, and washed three times for 5 min with PBS. Finally, the coverslips with SKBR3 cells facing up were placed in a 12-well plate filled with fixative solution, and fixed overnight at 4 °C with 4% (w/v) FA in 0.1M Na/Na phosphate buffer, pH 7.4, also known as a modified Sörensen’s buffer (Sörensen, 1909).

After rinsing with 0.1M Na/Na phosphate buffer, fluorescence imaging was performed with an EVOS® FL digital inverted microscope (Westover Scientific, Inc., AMG, Seattle, U.S.A.). The cells were imaged in bright field with the following objectives and % light settings: Plan PH2 4×/0.13 NA phase-contrast (26% light), Plan Fluor 10×/0.3 NA (38% light) and Plan Fluor 20×/0.5 NA (58% light). The fluorescent signal of Alexa488-labelled cells was imaged with the following objectives, % light, and exposure times settings: Plan Fluor 10×/0.3 NA (60% light, 250 ms), and Plan Fluor 20×/0.5 NA (50% light, 500 ms), and with solid state light assembly filter settings: excitation 470 nm and emission 525 nm (GFP filter).

To correlate after imaging the IF-light microscopic regions of interest by FIB-SEM, the same coverslips with immunolabelled SKBR3 cells up were quickly transferred to 7 mL screw cap glass vials (Aluglas B.V., Uithoorn, the Netherlands) containing 1.5 mL modified Karnovsky fixative (as described in Postfixation), and fixed at 4 °C overnight. After fixation, the coverslips with cells were kept in the vials during the following procedure. The cells were first washed two times for 5 min with 0.1 M sodium cacodylate buffer pH 7.4 at RT, then fixed for 90 min with 1% (w/v) OsO₄ (Agar Scientific) + 1.5% (w/v) potassiumhexacyanoferrat (II) (Merck) in 0.065 M sodium cacodylate buffer, pH 7.4 on ice, protected from light and on a rocking shaker table at 2 rpm (Heidelberg-Instruments). Thereafter the fixed cells were washed six times for 5 min with Milli-Q water, and subsequently heavy metal stained according to TCH-OsO₄-UAc-Pb method, thereafter
Focused-ion beam scanning electron microscopy of SKBR3 cells and fungal cells

After the ETLP method the stubs with SKBR3 cells as well the stubs with R. solani fungal cells were placed in the chamber of a dual beam microscope (Scios, FEI Company as part of Thermo Fisher Scientific Inc., Eindhoven, the Netherlands). The cells were viewed at an angle of 0° with a focused e-beam in secondary electron mode with an Everhart-Thornley detector at 2.0 kV or 3.5 kV and 0.4 nA, and 7 mm work distance (WD). Once the cell of interest was chosen, the eucentric height was determined. The stub was gradually tilted to 52°, and the cell of interest was first viewed in SE mode at 2.0 or 3.5 kV, 0.2 nA or 0.4 nA, magnification of 3500×, and an e-image was taken with a line speed of 3 μm s⁻¹ with 1536 × 1024 resolution. To change from e-imaging to ion-imaging the magnification was set to a low magnification of 500×. With the focused ion beam (FIB) a snap shot of the neighbouring cells, not the cell of interest, was taken at a low magnification of 500× with a 100 ns dwell time, a low resolution of 768 × 512, an acceleration voltage of 30 kV, a beam current of 50 pA, and a WD of 6.8 mm. To prevent damage of the cells created by the FIB, the ion-image was focused in the reduced area mode, subsequently put off, and immediately another snapshot was taken. Thereafter the position of the cell of interest was restored, and at a magnification of 3500× a trench was made by ion milling at 30 kV and 5 nA beam current, and milling debris was removed from the block face by making a cleaning cross section at 30 kV and 0.5 nA beam current. The OptiPlan BSE T1 detector was chosen with area 0.2 nA or 0.4 nA to take images with a dwell time of 5 μs or 8 μs with a resolution of 1536 × 1024 or 3072 × 2048.

Analysis and statistics

To determine the number of gold particles on the surface of the selected cells, a raster with 10 columns and 7 rows, cell size 2 × 2 cm, was placed on the scanning electron microscopic photograph (21.4 × 14.3 cm) of the surface of the selected SKBR3 cells. Per cell of the raster the number of gold particles was counted. In total 70 cells of the raster were used. The data were put into a Microsoft Excel 2010 sheet (Microsoft, Redmond, WA 98052, U.S.A.). The data were given as number of gold particles per 0.6 μm² membrane surface of the SKBR3 cell, and were expressed as mean ± standard deviation. Thereafter the Student’s t-test was applied with the following settings: array 1 with the 70 raster cells with number of gold particles on the membrane surface of a selected flat SKBR3 cell, array 2 with the 70 raster cells with number of gold particles of a selected round SKBR3 cell, two-tailed distribution, and two-sample unequal variance. A p value of ≤ 0.001 was considered as statistically significant.

Results

CPD and SEM of Immunogold-labelled SKBR3 cells

Scanning electron microscopy of HER2 immunogold-labelled and critical-point dried SKBR3 cells revealed different SKBR3 cell morphologies. In the cell culture, round cells were present next to differently sized flat cells. These flat cells showed different cell protrusions on their cell surface, thin finger-like structures and membrane flaps (Fig. 2A). At higher magnification the immunogold-labelled HER2 proteins were found to be on the cell surface and on the cell surface protrusions. In back-scattered electron microscopic mode (BSE-mode), the gold particles appeared as bright small dots on the cell surface and on cell surface associated structures (Fig. 2B).

ETLP method and SEM of SKBR3 cells immunogold-labelled

The SKBR3 cells prepared accordingly to the ETLP method clearly showed after SEM the various cell morphologies present in the cell culture. As indicated by the following numbers we observed round (1) and elongated (2) cells, cells just after cytokinesis (3), flat (4), and very flat (5) cells (Fig. 2C). Even at a low magnification of 1000× the polymerised extremely thin Spurr’s resin layer followed the contours of the cell surface and the cell surface protrusions of SKBR3 breast cancer cells. At 35 000× magnification, that is pixel size 1.93 nm, the HER2 immunogold-labelled cells clearly displayed the gold particles on the cell surface and on the cell surface protrusions (Fig. 2D).

Testing Llama antibodies to label HER2 proteins

SKBR3 cells were incubated with Llama antibodies VHH11A4, thereafter incubated with secondary and tertiary antibodies. When the primary antibody incubation was omitted, no green fluorescence signal was observed at the plasma membrane, whereas the nuclei were blue stained by the DAPI (Fig. 3A). This indicated that the secondary and tertiary antibodies did not cause a specific binding. Hence, the Llama
EXTREMELY THIN LAYER PLASTIFICATION

Fig. 2. Scanning electron microscopy of HER2 immunogold-labelled human breast cancer SKBR3 cells after critical point drying or after the ETLP method. (A) A low magnification of a part of the cultured and critical point dried SKBR3 cells showing different cell morphologies. (B) Part of the surface of an immunogold-labelled and critical point dried selected cell revealing cell surface extended structures and the distribution of the gold particles as small bright dots locating the HER2 plasma membrane proteins as seen in BSE mode. (C) A 52° tilted view at low magnification of a part of the cultured SKBR3 cells after the ETLP method, showing different cell morphologies, such as round (1) and elongated (2) cells, cells just after cytokinesis (3), flat (4) and very flat (5) cells. (D) A 52° tilted view of a part of the surface of an immunogold-labelled selected cell after the ETLP method, revealing cell surface extended structures and the distribution of the gold particles as small bright dots locating the HER2 plasma membrane proteins as seen in BSE mode.

antibodies VHH 11A4 specifically bound to the HER2 proteins on the plasma membrane (Fig. 3B).

CLEM of immunolabelled-SKBR3 cells

After immunolabelling and fluorescent light microscopy (IF-LM), the same breast cancer SKBR3 cell of interest imaged by IF-LM could be traced back by FIB-SEM. HER2 immunofluorescently labelled SKBR3 cultured cells showed cells with various fluorescence-intensity signals. For FIB-SEM purposes, a group of cells with round and flat cells was selected (Figs. 3C, D). After sample preparation for FIB-SEM, first a round cell was selected (Fig. 3E). After ion milling the round SKBR3 cell displayed the presence of a nucleus at the basal side of the cell. Further, many mitochondria and other subcellular structures were present between the nucleus and the apical side of the cell (Fig. 3F). Note: Figure 3(F) at 15 000× magnification, that is pixel size 9 nm, had been composed by merging the top and bottom images of the round cell by the use of Adobe Photoshop. After ion milling, the selected flat SKBR3 cell displayed at higher magnifications the cell organelles like mitochondria, a part of the nucleus, and also the distribution of the HER2 immunogold-labelled proteins on the cell surface (Fig. 3G). To visualise the distribution of the gold particles on the surface of the round cell, this selected round cell was imaged prior to ion milling (Fig. 3H). Prior to ion milling, the distribution of the gold particles on the surface of the selected flat cell was also visualised (Fig. 3I). At 40 000× magnification, that is pixel size 1.69 nm, a selected area of the round cell as in Figure 3(F) was chosen to show that, after heavy metal staining and the ETLP method, detailed images of the mitochondria with their cristae, the endoplasmic reticulum and the Golgi apparatus with flat cisternae and vesicles can be acquired (Fig. 3J). In another selected and ion milled cell at 35 000× magnification, that is pixel size 1.93 nm, also filamentous structures were observed (Fig. 3K).

Analysis and statistics

As there was a difference between the fluorescence signal of the flat cell and the round cell (Fig. 3C), the number of gold particles on these cells was determined (Figs. 3H, I). After analysis and statistics the round SKBR3 cell contained in a raster of 10 × 7 (see Materials and methods) a total of 396 gold particles, per 0.6 µm² cell surface with on average 5.66 ± 2.92 gold particles, whereas the flat SKBR3 cell contained a total of 172
Fig. 3. LM, SEM and FIB-SEM views of HER2 immunolabelled SKBR3 cells. (A), (B) SKBR3 cells labelled without and with Llama VHH 11A4 as primary antibodies to locate HER2 proteins on the plasma membrane of SKBR3 cells. The negative control only shows blue-stained nuclei and some weak green dots between the nuclei (A). Labelled VHH 11A4 cells clearly show after IF-LM blue nuclei and green fluorescence staining of the plasma membrane, indicating that VHH 11A4 is suitable for preembedding labelling of the SKBR3 cells (B). Bars = 25 µm. (C) After IF-LM, the different SKBR3 cell morphologies show different fluorescence intensities. Bar = 100 µm. (D) Light phase-contrast microscopic image of a region of interest (ROI, yellow circle) before preparation for FIB-SEM. Bar = 100 µm. (E) Cells prepared after the ETLP method for FIB-SEM and viewed in SE mode, showing the ROI as seen in the (C), (D) with small round cells and larger flat cells. (F) A part of a selected HER2 immunogold-labelled round SKBR3 cell after ion milling with a focused-ion beam scanning electron microscope, showing, for example organelles like mitochondria (arrows) and a part of the nucleus (Nu) with the nuclear envelop (NE) in BSE mode. (G) A part of a selected HER2 immunogold-labelled flat SKBR3 cell after ion milling, revealing the distribution of the gold particles on the cell surface as bright dots (see dots in the circles), as well as organelles like mitochondria (arrows) and a part of the nucleus (Nu) with the nuclear envelop (NE) in BSE mode. (H) Higher magnification of a part of the cell surface of the round cell as seen in (F) with the distribution of gold particles. Bar = 1 µm. (I) Higher magnification of a part of the cell surface of the flat cell as seen in (G) with the distribution of gold particles. Bar = 1 µm. (J) Higher magnification of the round cell as seen in (F) with mitochondria (Mi), endoplasmic reticulum (ER), and the Golgi apparatus among other cellular structures in BSE mode. (K) A part of a selected SKBR3 cell after the ETLP method and FIB-SEM, revealing filamentous structures (arrow) next to a part of a nucleus (Nu), mitochondria (Mi), and Golgi (G).
gold particles per 0.6 µm² cell surface with on average 2.46 ± 1.98 gold particles. The number of gold particles per 0.6 µm² cell surface of the round cell and of the flat cell was put into a bar graph. This bar graph clearly showed that, for example the round SKBR3 cell contained 14 cell surfaces of 0.6 µm² each with 5 gold particles, whereas the flat SKBR3 cell had only 6 cell surfaces of 0.6 µm² each with 5 gold particles (Fig. 4). Though the standard deviations of the data set of the flat cell and that of the round cell overlapped, they have a significant pairwise Student’s t-test with p ≤ 0.001. Hence, the round SKBR3 cell had significantly more gold particles per 0.6 µm² cell surface than the flat SKBR3 cell, which corresponded with the difference in the fluorescence high and low signals between these two selected cells.

Discussion

This study investigated an alternative to the current used minimal resin embedding methods how to remove as much resin as possible from the surface of cultured cells. Our extremely thin layer plastification (ETLP) method is a combination of staining cells with heavy metals, infiltration of the cells with a low viscosity resin, removal of resin from the cell surface with Mascotte filter paper followed by centrifugation, and finally resin polymerisation. This resulted into a thin layer of polymerised resin of about several nm thin, covering the used resin human breast cells and fungal cells. Because of this thin layer of polymerised resin the gold particles on the surface of the HER2 gold-labelled SKBR3 breast cancer cells could clearly be visualised as well as the presence of stained organelles in a selected SKBR3 cell after focused-ion beam electron microscopy (Figs. 3F, G). In addition, after the ETLP method and FIB-SEM, dolipore septa and associated organelles, such as perforate septal pore caps (Bracker & Butler, 1963; Müller et al., 2000a, b; Van Driel et al., 2008), could easily be found in cells of the plant pathogen filamentous fungus Rhizoctonia solani (Figs. 5D–H). The importance of these findings is that the ETLP method can be used on different cell types to study not only their cell surfaces, but also the subcellular ultrastructure. For reproducible results of resin removal from the cell surface the
Fig. 5. Hyphae of the filamentous fungus *Rhizoctonia solani* after the ETLP method and focused-ion beam scanning electron microscopy (FIB-SEM). (A) In SE mode the region of interest is marked by the yellow circle. (B) Region of interest as shown in BSE mode. The arrows pinpoint the location of the crosswalls, also known as septa, of the filamentous fungus. (C) The region of interest as shown in (A), (B) of a part of a hypha of *Rhizoctonia solani* after FIB-SEM in BSE mode. At this low magnification the figure clearly shows the dolipore septum (circle). (D) Higher magnification of (C) that shows a part of the fungal cell with, among others, the two perforate septal pore caps (arrows) flanking the dolipore septum (S), and lipid bodies (LB). (E)–(H) A series of a part of a fungal cell as in (D) imaged at the spot of a dolipore septum. Only in (H) the septal pore channel has been longitudinally sliced revealing cytosolic contents from one side to the other side of the septal pore caps (arrows) and the dolipore (D, i.e. septal swelling) via the septal pore channel. Bar = 500 nm.
used combination of Spurr’s low viscosity resin (Spurr, 1969). Mascotte filter paper and centrifugation was thus necessary.

Our ETLP method is a refinement of the resin removal methods of Bittermann et al. (2012), Kizilyaprak et al. (2014), Belu et al. (2016), and Schieber et al. (2017). These methods have different ways to remove resin from the top of the cultured cells or from multicellular organisms. Different methods of thin layer plastification (Table 1) show the different preparation steps of cultured cells and multicellular organisms. The removal of excess resin can be done in different ways: let the sample stand upright on a filter paper (Bittermann et al., 2012; Kizilyaprak et al., 2014), hang in an Eppendorf tube (Bittermann et al., 2012; Kizilyaprak et al., 2014), centrifuge (Bittermann et al., 2012; Kizilyaprak et al., 2014; this study), splash with ethanol (Belu et al., 2016), put the sample on the top of a pin (Schieber et al., 2017), use absorbent or filter paper (Schieber et al., 2017), move the sample around under the laying substrate, such as filter paper or Aclar (Schieber et al., 2017), or use the combination of PCTE filter on filter paper and Mascotte filter paper (this study). The present ETLP method differs from previous studies how excess of resin is removed from the cell surfaces, and shows an alternative way for the ethanol splashing onto cells as described in the ultrathin embedding method of Belu et al. (2016).

Some limitations of the current ETLP study are that the cultured human breast cancer SKBR3 cells will be affected by the several preparation steps during this ETLP method. These cells have large protrusions, which might be pressed onto the cell surfaces after blotting with Mascotte filter paper. Further, the degree of SKBR3 cell confluency may also affect whether or not most excess of the Spurr’s resin will be removed. SKBR3 cells cultured to 90–100% confluency showed 99% of the SKBR3 cells with a thin resin layer of only several nanometers. In contrast, at 50% confluency, about 30% of the cells had a thick layer of resin as if they were processed according to the TLP method of Bitterman et al. (2012). In addition, glass coverslips, Aclar and Thermaxo can be used as a substrate for cultured cells. The use of Thermaxo is to be preferred, because the electron beam has less effect on the Thermaxo, whereas during focusing the Aclar will melt and the cells will get out of focus. Moreover, blotting with Whatman paper or single-fold hand towel paper did not result into sufficiently removing the excess of Spurr’s resin from the cells. Only the combination of Mascotte filter paper and centrifugation resulted into the efficient removal of excess of Spurr’s resin from the resin infiltrated cells.

For FIB-SEM purposes, a thin layer plastification method hinges not only on efficiently removing of excess resin from the resin infiltrated cells, but also on heavy metal staining of the cell membrane and the cell organelles. Since its introduction, the OTO method (Seligman et al., 1966) to enhance membrane contrast of, for example nuclear envelopes, endoplasmic reticulum and mitochondria has been modified. Further membrane enhancement has been achieved by postfixation of aldehyde fixed tissue with reduced osmium tetroxide, followed by tannic acid or thiocarbohydrazide, osmium tetroxide and en bloc staining with uranyl acetate and lead aspartate (Tapia et al., 2012; Paridaen et al., 2013). The staining with heavy metals as in the OTO method not only results into enhanced membrane contrast, but also in cells being more conductive, hence more stable in the e-beam (Friedman & Ellisman, 1981; Jem 140).

### Table 1. Different methods of thin layer plastification with focus on the removal of excess of resin from the surface of cells or multicellular organisms for FIB-SEM.

| Methods of thin layer plastification | I | II | III | IV |
|------------------------------------|---|----|-----|----|
| Cultured cells                     | * |    |     |    |
| Multicellular specimens ZE, PL, CL|    | * |     |    |
| Chemical fixation                  |    | * |     |    |
| Immunogold labelling               |    | * |     |    |
| Dehydration                        |    | * |     |    |
| Ethanol                            |    | * |     |    |
| Ethanol ZE, ethanol/aceton PL      |    | * |     |    |
| Resin                              |    |    | * |    |
| EMbed 812                          |    |    |    | * |
| EPON mixed with different components | |    |    | * |
| Dacron                               |    |    |    | * |
| Spurr’s resin                      |    |    |    | * |
| HPF-FS CL                          |    |    |    | * |
| Excess of resin removed by         |    |    |    | * |
| Stand upright on a filter paper    |    |    |    | * |
| Hang in the opening of an Eppendorf tube | |    |    | * |
| Centrifuge                         |    | * |     |    |
| Splashing with ethanol             |    |    | * |    |
| Pin, tooth pick ZE, CL             |    | * |     |    |
| Absorbent paper ZE, PL             |    | * |     |    |
| Filter paper ZE, PL, Aclar PL, CL  |    | * |     |    |
| PCTE plus filter paper             |    |    | * |    |
| Mascotte filter paper              |    |    | * |    |
| Resin polymerisation               |    |    |     | * |
| at 60 °C                           |    | * |     |    |
| at 70 °C                           |    |    | * |    |
| Sputter coating                    |    |    |     | * |
| Thin layer of platinum             |    | * |     |    |
| Thin layer of iridium              |    | * |     |    |
| Thin layer of gold                 |    | * |     |    |
| Thin layer of carbon               |    | * |     |    |

Note: Method I: Thin layer plastification by Bittermann et al. (2012); Kizilyaprak et al. (2014). Method II: Ultrathin resin embedding by Belu et al. (2015). Method III: Minimal resin embedding by Schieber et al. (2017). Method IV: Extremely thin layer plastification by Donselaar et al. (this study). ZE = Zebrafish Embryos; PL = Platynereis larvae; CL = C. elegans larvae.
Jongeboed et al., 1999). High conductivity of cells without a thick 0.5–1 μm platinum layer is of paramount importance when these cells are exposed to ion milling and viewed in back-scattered electron mode. This is needed for 2-dimensionally cultured cells on a substrate, like the SKBR3 cells, but conductivity is even more important when cells are growing in a three-dimensional structure like hyphae of filamentous fungi.

As an alternative to correlative studies by the use of TEM and SEM, the combined use of the ETLP method and FIB-SEM allows the visualisation of a large number of cells, which is important to obtain statistical reliable results. In addition to SKBR3 cells, the ETLP method can also be applied to immunogold-labelled plasma membrane proteins of a variety of cultured mammalian cells and this extends the use of correlative microscopic studies. Moreover, the ETLP method can be applied to evaluate the responses of cells after the addition of new compounds with potential therapeutic functionalities. The great difference with the current evaluation methods of such components will be that not just the effect on a selected marker or its signal transduction pathway can be evaluated, but also the integral responses of the cells, and this is closer to the in vivo situation. Different SKBR3 cell morphologies, like flat cells and round cells, not only differ in high and low fluorescence labelling (Fig. 3C) or the number of gold particles on the cell surface (Figs. 3H, I), but also reveal a different cellular organisation like, for example the amount of mitochondria and their location in the cell (Figs. 3F, G).

In conclusion, the significance of the ETLP method described here will be universally applicable to study the ultrastructure of many cultured cell types or multicellular organisms by FIB-SEM. Hence, the method will find application in research fields, such as oncology, cell biology, and microbiology.

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