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Gridded Aclar: preparation methods and use for correlative light and electron microscopy of cell monolayers, by TEM and FIB–SEM

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Summary

Aclar, a copolymer film with properties very similar to those of tissue culture plastic, is a versatile substrate to grow cells for light (including fluorescence) and electron microscopic applications in combination with both chemical fixation and cryoimmobilization. In this paper, we describe complete procedures to perform correlative light and electron microscopy using Aclar as substrate for the culture of cell monolayers to be finally embedded in plastic. First, we developed straightforward, efficient and flexible ways to mark the surface of the Aclar to create substrates to locate cells first at the light microscopy and then the electron microscopy level. All the methods enable the user to self-design gridded Aclar pieces, according to the purpose of the experiments, and create a large number of substrates in a short time. Second, we confirmed that marked Aclar supports the normal growth and morphology of cells. Third, we validated the correlative light and electron microscopy procedure using Aclar. This validation was done for the high-resolution analysis of endothelial cells using transmission electron microscopy and focused ion beam–scanning electron microscopy in combination with the use of fluorescence, phase contrast and/or bright field microscopy to map areas of interest at low resolution. The methods that we present are diverse, easy to implement and highly reproducible, and emphasize the versatility of Aclar as a cell growth substrate for diverse microscopic applications.

Introduction

Electron microscopy (EM) of cells cultured *in vitro*, frequently growing as monolayers, provides valuable ultrastructural information that can be useful to understand cellular biology. Human umbilical vein endothelial cells (HUVECs), grown as a cell monolayer mimicking an endothelium, are an experimental model that can be used to address several aspects of the endothelial cell biology (Striker *et al.*, 1980). In current projects using HUVECs we found that EM studies often need to be performed on specific cells present in a population, because not all of them exhibit simultaneously the characteristic of interest. Correlative light and electron microscopy (CLEM) is the method to identify specific areas in a cell culture by screening at low resolution (i.e. with light microscope) and to re-localize them at EM level. Cell monolayer embedding in plastic (e.g. epon) after chemical or cryo-fixation is an approach commonly used during specimen preparation for EM that renders blocks (containing the cells) that can be further sectioned. To perform CLEM in combination with this preparative method, position marks, to map the cells of interest by light microscopy (LM) (prior to processing for EM) and to trace the cells back in the epon block, are required. For this, cell monolayers have been grown so far (1) on gridded glass cover slips (as those supplied nowadays by MatTek Corporation (Ashland, MA, U.S.A.) or Bellco Glass (Vineland, NJ, USA); see Polishchuk *et al.*, 2000 for an example) when they would undergo chemical fixation or (2) in the case of cells to be high-pressure frozen, on sapphire discs carbon coated with the pattern of a finder grid (McDonald, 2009), or clamped to finder grids (Leica Microsystems, Rijswijk, The Netherlands) just before starting the sample screening by LM (Verkade, 2008). In these cases, a critical step during sample preparation is to remove the glass cover slips or sapphire discs from the embedding plastic without leaving traces of the support material (glass or sapphire) on the block.

Aclar® 33C (Honeywell, Morristown, NJ, USA) is a transparent and chemically stable plastic film made from fluorinated-chlorinated resins. It shares many properties with ordinary polystyrene tissue culture plastic and has been successfully used to culture monolayers of diverse mammalian cell types (Kingsley & Cole, 1988; Masurovsky & Bunge,

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1989; Meyer *et al.*, 1998; Jiménez *et al.*, 2006), including HUVECs (Jiménez *et al.*, 2009). Aclar film is flexible (even at liquid nitrogen temperatures), is easy to handle, can be punched or cut to the desired shape and size, remains flat, does not show autofluorescence, is inert to chemicals commonly used in specimen preparation and can be easily peeled off the polymerized epon or lowicryl leaving a perfectly smooth and flat surface with cells embedded in the polymer. Not surprisingly Aclar has proven to be a convenient cell growth substrate for light and electron microscopic applications, in combination with both chemical fixation (Masurovsky & Bunge, 1968, 1989; Kingsley & Cole, 1988) and cryoimmobilization by high-pressure freezing (Jiménez *et al.*, 2006). Interestingly, because Aclar is relatively soft, it is possible to scribe its surface (Masurovsky *et al.*, 1971). This combination of characteristics predicts that Aclar might be very well suited for CLEM, which is explored in this paper.

In this paper, we show straightforward procedures to perform CLEM with cell monolayers grown on Aclar and finally embedded in epon. Three different approaches to create position marks (grid patterns) on the surface of Aclar are described in detail. In the first method, a needle is used to physically engrave patterns by hand. In the second method, a carbon coater is used to 'print' grids on the surface of Aclar. The third, state-of-the-art, method is based on the use of a focused ion beam (FIB) tool integrated with a scanning electron microscope for the mechanical, physical engraving of marks in Aclar. Special attention is given to the practicability of the approaches to create multiple grid patterns at high rate. With the exception of the focused ion beam–scanning electron microscope, the required equipment is standard and, therefore, Aclar marking procedures can be easily implemented at EM labs. Next, the validation of the CLEM approach with HUVECs grown on gridded Aclar is illustrated with several examples. Both (manually or mechanically) engraved and carbon-printed Aclar enable the low-resolution screening of (immunolabelled) samples by fluorescence, phase contrast and bright field LM in order to identify cells of interest and to map their location. All types of marked Aclar allow tracing back the areas of interest on epon blocks for transmission electron microscopy (TEM) analysis of sections, whereas only the physically engraved Aclar is compatible with the re-localization of regions of interest done by focused ion beam-scanning electron microscopy (FIB–SEM). The methods that we present are diverse, simple and highly reproducible and emphasize the versatility of Aclar as a substrate to grow cells for analysis by varied microscopic approaches.

Materials and methods

Procedures to mark Aclar for CLEM

Aclar® 33C is obtained as 20 × 26 cm films (51 μm thick) from Electron Microscopy Sciences (#50426–10; Fort Washington, PA, U.S.A.). Aclar pieces have to be cut from the supplied films according to the marking method to be applied. This can be best done under a stereomicroscope, on a slightly soft surface (e.g. a polyethylene plate) covered with a piece of standard Cartesian graph paper (as reference for distances), using a GEM® blade (#71972; Electron Microscopy Sciences), previously cleaned with benzine, and assisted by a ruler to get straight edges. Aclar pieces are subsequently cleaned with acetone and left to dry in a dust-free environment before marking. All marking methods render (relatively large) pieces of Aclar containing many grid patterns and therefore several smaller 'custom-made' pieces of marked Aclar can be finally obtained after splitting. Accordingly, and depending on the purpose of the experiment, a single piece of Aclar with multiple patterns can be housed in a culture dish and eventually separated in smaller pieces during or after sample processing. Manipulation of Aclar with gloves during all the procedure is advised. Fine forceps should be used to hold Aclar (always at the edge of the piece).

Method of marking Aclar by needle-mediated grid engraving. The basis of this method is that Aclar can be physically scribed because it has a soft surface. In order to standardize this procedure a pattern to mark the Aclar and a sharp object, easy to handle and able to make very thin marks, were required. In our hands, the thin needle permanently attached to a 1 mL insulin syringe (#309309; Becton Dickinson, Franklin Lakes, NJ, U.S.A.) turned out to be a very suitable marking object. Patterns could be easily self-designed with a graphics editing program and subsequently printed. One example to engrave Aclar is illustrated in Fig. 1(A) and is next explained step by step.

To prepare the pattern (Fig. 1A1, pattern illustrated at real size):

(1) In Adobe Photoshop (we use version 9.0.2) create a high-resolution (1200 dpi) blank document (2 × 4 cm) with a white background, in psd format.
(2) In vertical, draw a straight thin (1-pixel thick) black line across the canvas. Duplicate this line four times in independent layers. Zoom in, align the five lines and distribute them equidistant in 1 mm (i.e. leaving 250 μm distance between adjacent lines). Merge the layers and duplicate the new layer three times in independent layers. Align the four sets of lines and arrange them to fit, equidistant, in 1 cm (i.e. leaving 2 mm distance between adjacent sets).
(3) Following a similar procedure, create thin horizontal lines. In the example there are eight sets of five horizontal lines distributed in two groups.
(4) Add thick external lines to serve as reference to help during Aclar splitting after marking.
(5) Print at 1200 dpi. Adjust the printer settings to ensure that the thin lines are also printed as thin as possible.

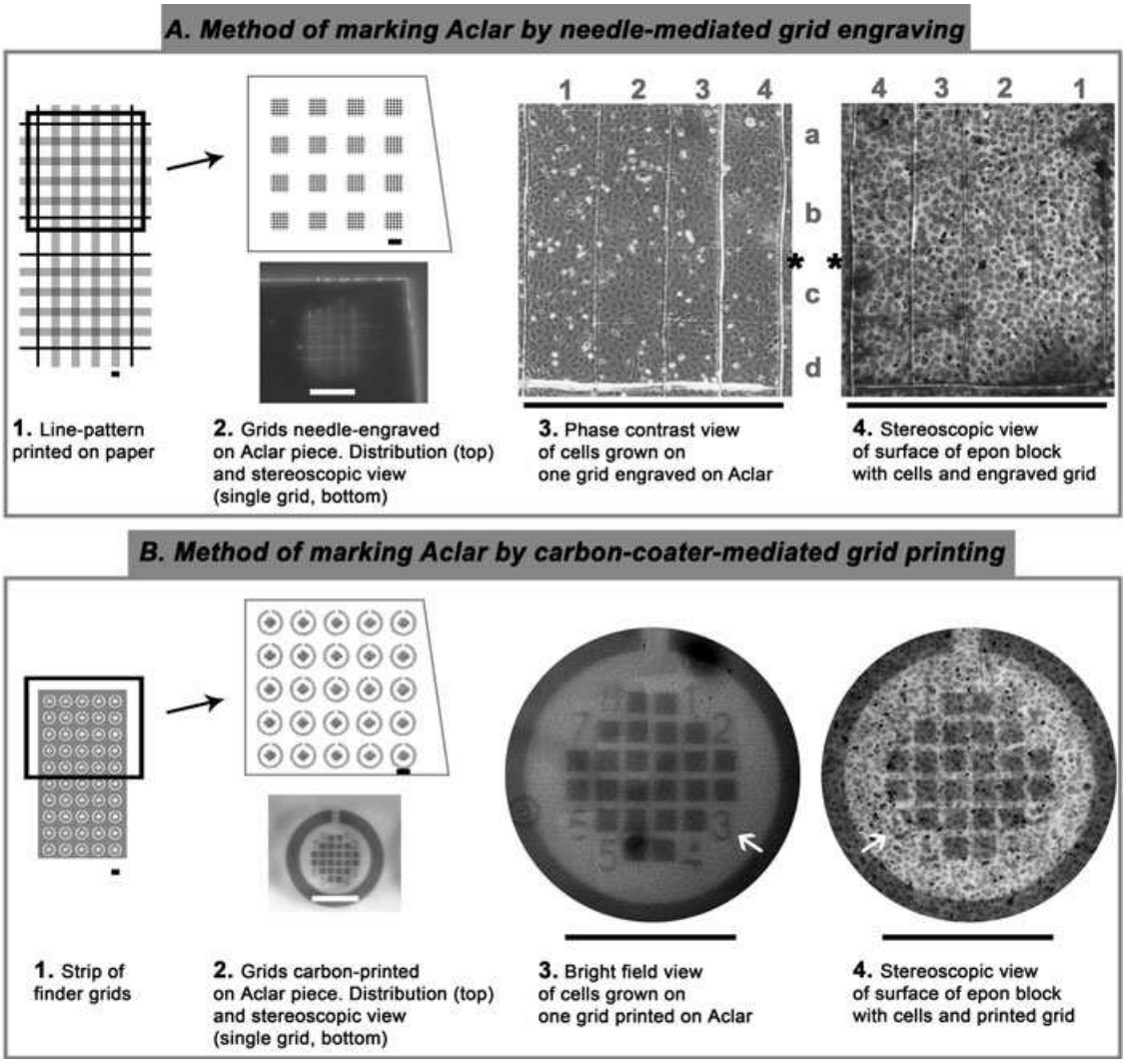

Fig. 1. Procedures to mark Aclar and visualization of grid patterns in Aclar and in epon blocks. (A) In the method to engrave grids with a needle, a printed line-pattern (1) is used as reference to mark several grids on Aclar (2). The grid patterns and the cells, that grow normally on this Aclar, are visible at LM level (3) enabling the mapping of areas of interest. The marks engraved in Aclar serve as a mould during embedding in epon and result in the patterns found in the surface of the polymerized epon, that also contains the embedded cells (4). (B) In the method to print grids, a strip of finder grids (1) put on the Aclar is used as a filter during carbon coating and allows obtaining several carbon imprints on Aclar (2). This marked Aclar also supports the normal growth of cells, and marks are visible at LM level (3). After epon polymerization, carbon imprints stay on the surface of the block (4). Note the mirrored image of the marks in Aclar as compared to the epon blocks (asterisks and arrows point to the same position). Scale bars: 1 mm.

(6) Cut the printed pattern (2 × 4 cm).

(i) *In the example two patterns, each good for marking 16 grids, are obtained. Every grid pattern is composed of 4 × 4 squares (250 μm side). Note that, in Fig. 1(A1), the design of the pattern cannot be well discerned by eye due to the limited resolving power of the human eye.*

To engrave the Aclar:

(1) Cut and clean a 2 × 4 cm piece of Aclar following the advices given before.

(2) Put the printed pattern on a stand at the base of a stereomicroscope (in our case, a Leica MZ6 equipped with ring-lights), centre the Aclar piece on the printed pattern and tape the Aclar and paper to the stand with small pieces of Scotch tape at the corners (to avoid movements during marking).

(3) Magnify the image to clearly discern the single lines while still seeing the complete printed pattern.

(4) Put a ruler on the top horizontal line and, without pressing, touch the Aclar gently with the tip of the needle to mark 4 small (1 mm) lines corresponding to the grid patterns. Move the ruler gently to the next horizontal line and mark.

(5) When all the horizontal marks are done, rotate the stand by 90° and repeat the procedure to engrave the perpendicular lines.

(6) Subdivide the engraved Aclar as suitable.

(i) *In the example 2 pieces of Aclar (approx. 1.5 × 1.5 cm, with an oblique side for orientation) with 16 grids each (Fig. 1A2, top) are obtained. Every engraved grid consists of 4 × 4 squares (approx. 250 μm side; Fig. 1A2, bottom). By consensus we assign a number to every grid pattern (1 to 16, beginning up on the left), and an alphanumeric location to each particular quadrant in an engraved grid (Fig. 1A3; a-d, 1–4 locations).*

Method of marking Aclar by carbon-coated-mediated grid printing. This method is based on the fact that Aclar can be carbon coated. To standardize this procedure a pattern to carbon-print many grid patterns simultaneously on the surface of Aclar was necessary. We found the finder grids of Leica (#16707896) to be an excellent pattern. Apart from the fact that they contain a numeric arrangement that facilitates the CLEM procedure, many grids (100 pieces) are supplied in a single strip. In Fig. 1(B) one example to carbon-print Aclar is shown. Step by step:

(1) Cut and clean a 1.5 × 3 cm piece of Aclar.
(2) Tape a piece of Whatman filter paper, larger than the Aclar, to a Petri dish lid (upside down). Under a stereomicroscope, put the Aclar piece on the filter paper and subsequently centre a piece of finder-grids strip on the Aclar; in the example (Fig. 1B1, pattern illustrated at real size) half of a supplied strip, containing 50 finder grids, is used. Tape the corners of strip and Aclar to the filter paper.
(3) Prepare the carbon coater (in our case, a Cressington 208carbon; Cressington Scientific Instruments Ltd., Watford, U.K.) for standard coating. Put the Aclar assembly on the stand of the coating chamber and begin a cycle of carbon evaporation.
(4) Proceed to coat. As a rule, carbon imprint should be clearly visible by eye on the Aclar after coating. Aclar overheating should be avoided. In order to do so, short pauses (e.g. 15 s) between consecutive coat pulses (e.g. 15 s) during one evaporation cycle might help.
(5) Stop evaporation cycle. Demount the Aclar assembly. If grains of carbon are found on the Aclar, clean it again with acetone.
(6) Subdivide the printed Aclar as suitable.

(i) *In the example two pieces of Aclar (approx. 1.3 × 1.3 cm, with an oblique side for orientation) with 25 patterns each (Fig. 1B2, top) are obtained. By consensus we assign a number to every grid pattern (1 to 25, beginning up on the left). Every printed grid includes 24 squares (approx. 125 μm side;* Fig. 1B2, *bottom) and a numeric arrangement (1 to 8) centred in a circle. A strip of finder grids can be used through multiple evaporation cycles.*

Method of marking Aclar by FIB-mediated grid engraving. A FIB is able to remove material at nano- and micro-scale, a process called milling. This capability has been used to create patterns in Formvar- and carbon-coated TEM grids in order to localize nanoparticles placed on the grids (Nowak *et al.*, 2008). In our study, we explored the possibility to use a FIB to mill grid patterns on the surface of Aclar. The FIB that we used is combined with a scanning electron microscope (FEI Nova 600 NanoLab DualBeam; FEI Company, Eindhoven, The Netherlands), which allows machining the material with the FIB and examining it with the scanning electron microscope. As the FIB is operated by scan coils, the radiated area can be controlled precisely, enabling one to mill self-designed patterns.

To mark Aclar, the applied acceleration voltage of the ion beam was 30 kV and the current was set at 20 nA. Shortly after the milling, the SEM image appeared severely distorted, suggesting that the grid lines were not straight. However, using a higher electron current quickly compensated the ion beam induced charge and revealed the straight pattern. Therefore, it can be concluded that, despite the fact that Aclar is an electrical insulator, charging did not influence the milling process done by the FIB. Furthermore, because the process is machine controlled, the milled lines were always more regular than those manually engraved with a needle. One example, step by step:

(1) Cut and clean a 1 × 1 cm piece of Aclar.
(2) Attach the corners of the Aclar to the sample holder by placing it on small pieces of double-sided carbon tape (#G263; Agar Scientific, Essex, U.K.) previously stuck to the holder.
(3) Introduce the sample in the FIB–SEM chamber and visualize the Aclar piece with the scanning electron microscope. Align the sample and tilt it to bring the surface perpendicular to the FIB.
(4) Proceed to mill the Aclar following the self-designed pattern. Using a 30 kV, 20 nA ion beam, we milled 16 patterns (4 × 4 squares) with different width and depth of the lines (widths 0.5, 1, 2 or 3 μm and depths 2, 5, 10 or 15 nm). Each grid pattern was assigned a number that was milled with the ion beam on the Aclar, next to the corresponding grid.
(5) After marking, remove the sample from the microscope and separate the Aclar gently from tape.
(6) Subdivide the marked Aclar if desired.

(i) *In the example, one piece of Aclar (approx. 1 × 1 cm, with an oblique side for orientation) with 16 grids distributed as in* Fig. 1(A2) *(top) is obtained. Every pattern milled on Aclar consisted of 4 × 4 squares (approx. 200 μm side) and had a number (given following the same criteria as for the needle-engraved Aclar) milled next to a corner. Squares were assigned a location as in the case of the needle-engraved Aclar.*

Cell culture on gridded Aclar

Depending on the experiment we housed the Aclar in regular polystyrene culture dishes or well plates (Corning Inc. Life Sciences, Acton, MA, U.S.A.) or glass-bottom culture dishes (uncoated, non-gridded; #P35G-0–20-C; MatTek Corporation). Because Aclar pieces can float in fluids, it is necessary to attach them to the culture vessels before cell seeding. Aclar sticking by adhesive capillary forces is not effective in our long-term cell culture. Most of the Aclar pieces will detach and float in the culture medium in the course of the experiment. Aclar can be attached to polystyrene applying focal heat as previously detailed (Jiménez *et al.*, 2006). In this way, Aclar and polystyrene melt together and Aclar pieces stick to the vessel. Another possibility is to use Matrigel (Matrigel^{TM} Basement Membrane Matrix Growth Factor Reduced; #356230; BD Biosciences, Bedford, MA, U.S.A.) as 'glue'. This approach is strongly advised, especially when Aclar has to be attached to glass-bottom culture dishes (e.g. when sample screening at LM level is to happen with laser scanning microscope). In this case the application of focal heat is less effective to stick Aclar because glass does not melt. To handle Matrigel, the guidelines given by the manufacturer have to be strictly followed. Matrigel aliquots have to be stored at −20°C. To attach Aclar pieces using Matrigel, culture dishes (or well plates) are pre-cooled and left on ice and aliquots (15 μL) of Matrigel are thawed and kept on ice. Next, a pre-cooled micropipette tip is inserted in the Matrigel, which will enter the tip by capillarity. Under a stereomicroscope and using a cold illumination system (Leica KL 1500 LCD), tiny droplets of Matrigel are applied, by gentle touches with the tip, on an area of the dish smaller than the piece of Aclar to be attached. The Aclar piece, held with a forceps by its edge, is then placed on the Matrigel droplets that should spread without overflowing the Aclar surface. This procedure is repeated for every Aclar piece to be attached to a particular dish. Subsequently, the dish is removed from the ice and incubated 30 min at 37°C on a warm plate to ensure the gelling of Matrigel. Finally the Aclar-containing dish has to be sterilized, either by microwaves (Jiménez *et al.*, 2006) or preferably by ultraviolet light.

For all the experiments presented in this paper we used HUVECs. Umbilical cords for isolation of the cells were obtained from the Department of Obstetrics and Gynecology, Diakonessen Hospital, Utrecht, The Netherlands, with the informed consent of the parents. HUVECs were isolated and cultured exactly as described before (Jiménez *et al.*, 2009). Aclar pieces were always attached to the culture vessel using Matrigel and were sterilized by ultraviolet light. For every experiment, cells were seeded on gridded Aclar (pieces marked by one of the three methods explained before; therefore containing 16 or 25 grid patterns) and also on regular (non-marked) Aclar in order to control if cell growth was affected by the presence of marks. Cells were grown on Aclar till 4 days after they reached confluence.

Experimental conditions, sample processing and CLEM procedures

CLEM strictly based on cell morphology. HUVECs are primary cells with a well-defined phenotype but it is not unusual to find cells showing morphological irregularities (e.g. larger size, as in the case of senescent cells) in a particular culture. To define these abnormalities at high resolution, we cultured HUVECs in gridded Aclar housed in polystyrene well plates as explained in the previous section. Because Aclar is transparent, cells can be perfectly monitored during culture by phase contrast light microscopy. Furthermore, marks (engraved and printed) on Aclar proved to be visible with phase contrast illumination and also after retracting the condenser phase contrast ring (i.e. with bright field illumination) (Fig. 1A3 and B3). Therefore, gridded Aclar pieces showing regions of interest could be selected and these regions were mapped at LM level as follows. Using a phase contrast light microscope (Leica DMIL) equipped with a HiPlan 10×/0.25 Ph1 and a HiPlan 20×/0.40 Ph1 objective lenses, an S 55/0.35 condenser and a Leica EC3 camera pictures of the samples were taken, including the cells and the marks in the Aclar. The position of the areas of interest was annotated, including the number of the grid pattern and the alphanumeric location of the corresponding quadrant.

Afterwards, cells were chemically fixed with aldehydes, post-fixed with OsO_4, and tannic acid-mediated osmium impregnated (one incubation with tannic acid followed by one incubation with OsO_4; to increase sample contrast) exactly as reported (Jiménez *et al.*, 2009). Alternatively, thiocarbohydrazide (1% in ultra-pure distilled water; 15 min, at room temperature) was used instead of tannic acid. After dehydration, Aclar pieces were immersed in a drop of fresh epon, on a polyethylene plate, and divided with a GEM blade under a stereomicroscope. Using ring-light (and tilting the Aclar piece when necessary) the single grid patterns were located. Patterns containing regions of interest were cut (always with an oblique side for orientation) and epon-embedded as described before (Jiménez *et al.*, 2009) in independent blocks for *en face* sectioning. When epon was polymerized, the blocks were trimmed, under a stereomicroscope, with a GEM blade to completely expose the Aclar pieces. Using fine forceps a corner of the Aclar piece was then lifted up and the Aclar was subsequently peeled off the block. This left behind a perfectly smooth surface containing both embedded cells and marks (Fig. 1A4 and B4), with mirrored positions with respect to the original ones in the Aclar as seen by phase contrast or transmitted light (Fig. 1A3 and B3). Blocks were then prepared for TEM or FIB–SEM purposes.

For TEM analysis, blocks were trimmed with the oblique side for orientation leaving just a quadrant containing cells of interest. Cells were then serial-sectioned *en face*. Sections (80-nm-thick) were collected on Formvar-carbon-coated

copper slot grids, left non-post stained and examined with a Tecnai-12 (FEI Company) operating at 100 kV equipped with a side-mounted Megaview II camera (Olympus Soft Imaging Systems, Münster, Germany). Using the information of the phase contrast pictures, cells of interest were located (at low magnification) and further analysed at high magnification.

For FIB–SEM analysis, blocks were trimmed leaving not only a quadrant but the complete grid pattern containing the cells of interest in a short epon block. This block was attached onto the scanning electron microscope holder using carbon cement. To prevent electrical charging when radiated with the electron beam, epon was covered as much as possible with the cement and the sample was further sputter coated with 3 nm platinum/palladium (in a Cressington 208HR). Samples were then visualized in the FEI Nova 600 NanoLab DualBeam. Note that only blocks with engraved lines were used for FIB–SEM studies. The reason for this is that, with a scanning electron microscope, topological features of the sample (the lines in our case) can be visualized. However, the carbon imprint will be masked by the platinum/palladium coating. Using a 2 kV electron beam in secondary electron (SE) and in back scatter electron (BSE) modes, the grid lines were clearly visible on the surface of the block. This enabled the re-localization of the squares containing cells of interest. With the sample perpendicular to the electron beam and increasing the voltage to 20 kV, BSE contrast from the cells was obtained and in this way, cells of interest were traced back. With the milling capabilities of the FIB, cross-sections of samples can be made (De Winter *et al.*, 2009; Hekking *et al.*, 2009). First, a trench was milled, with a high current (5 nA), just in front of the area of interest. Then, the side of the trench to be visualized was polished at much lower current (0.3 nA). Images of the cross-section were taken in BSE mode as contrast in the samples is generated by the heavy metals (osmium in our case). The acceleration voltage of the electron beam was kept low (2 kV) to reduce the interaction volume of the beam. This increased both the spatial (~10 nm) and depth (estimated to be 50–100 nm) resolution (De Winter *et al.*, 2009; Hekking *et al.*, 2009).

CLEM with low-resolution cell identification based on fluorescent labels. Aclar does not exhibit any autofluorescence (Kingsley & Cole, 1988). Therefore, it might be used to identify cells labelled with fluorescent tags for CLEM purposes. To validate this procedure, we performed two types of experiments. In both cases, cells were grown as explained before on gridded Aclar pieces mounted on glass-bottom dishes to allow for direct sample analysis with a confocal laser scanning microscope (model LSM 5 Pascal; Carl Zeiss B.V., Sliedrecht, The Netherlands). This microscope was equipped with an Argon laser (25 mW; 488 nm line used operating at 17% power) and a Helium–Neon laser (1 mW; 543 nm, used at 26% power). Imaging was done using a 10×/0.3 NA Plan-Neofluar objective lens for grid pattern overviews and a 40×/1.2 NA C-Apochromat water immersion objective lens for (high magnification) quadrant visualization. The pinhole was set to 1 Airy unit. The working distances of the mentioned objective lenses are 5.2 and 0.28 mm, respectively. In our experiments we used dishes with a glass thickness #0 (i.e. 0.085–0.13 mm thick), that added to the thickness of the mounted Aclar (0.051 mm) resulted in samples (maximally 0.181 mm thick) within the working distance of the objective lenses.

In one approach, cells were incubated with low-density lipoprotein (LDL) labelled with fluorescent Oregon green (OG-LDL) in culture medium for 45 min to study the uptake of LDL by HUVECs. After removing the non-internalized OG-LDL by washing, the cells were fixed with 4% paraformaldehyde in 0.2 M HEPES buffer pH 7.3, 30 min at room temperature. Cells were then rinsed with phosphate buffered saline (PBS) and fluorescence was monitored with the LSM 5 Pascal microscope using the 488 nm line of the Argon laser for excitation as specified above. Patterns were screened, and pictures of the squares containing cells of interest were taken with fluorescence (to visualize labelled cells) and transmitted light (to see marks, engraved or printed) modes. The position of the regions of interest was annotated, including the number of the grid pattern and the alphanumeric location of the corresponding square. Afterwards, phase contrast images of these regions were usually taken (as explained in previous section) in order to obtain extra information on the morphology of the cells.

In another set of experiments, caveolin was detected by combined immunofluorescence and pre-embedding immunogold labelling. For this, HUVECs were fixed with 4% paraformaldehyde as explained above. After washing with PBS, samples were blocked, quenched and permeabilized in a one-step incubation with a cocktail containing 0.5% bovine serum albumin (BSA), 0.045% cold water fish gelatin, 50 mM NH_4Cl and 0.1% saponin in PBS, for 30 min at room temperature. The same cocktail was used to dilute primary and secondary antibodies. Next, cells were incubated 1 h at room temperature with 2.5 μg mL^{-1} rabbit α-caveolin (BD Biosciences, San Jose, CA, U.S.A.), thoroughly washed with PBS, and subsequently incubated 1 h at room temperature with a mixture of protein A conjugated to 5-nm-gold (Department of Cell Biology, Medical School, Utrecht University; The Netherlands) and goat α-rabbit antibodies conjugated to Alexa Fluor 555 (Molecular Probes, Eugene, OR, U.S.A.). The rationale for using this mixture is that both α-rabbit antibodies and protein A can bind the α-caveolin antibodies, because these were obtained in rabbit. The α-rabbit antibodies, conjugated to a fluorescent tag, will make possible to detect caveolin expression first by fluorescence microscopy. The protein A, conjugated to gold, will allow localizing caveolin by EM analysis after proper sample processing. Cells were then washed with PBS,

fluorescence was monitored by laser scanning microscope (in this case using the Helium–Neon laser), and regions containing cells of interest were mapped as detailed in the previous paragraph.

After mapping regions of interest for both OG-LDL uptake and caveolin distribution, cells underwent further chemical fixation, post-fixation, osmium impregnation and epon embedding as explained in the previous section. This extra fixation and *en block* osmium impregnation after immunolabelling was done in order to improve the ultrastructural preservation and contrast of the cells for EM. Finally, blocks were prepared for TEM or FIB–SEM analysis exactly as reported above.

Results and discussion

Aclar® 33C provides an excellent substrate to grow a wide variety of mammalian cell types for light and electron microscopic analysis, combined with sample processing by chemical or cryo-fixation (Masurovsky & Bunge, 1968, 1989; Kingsley & Cole, 1988; Jiménez *et al.*, 2006). In addition, Aclar film is cheap. The versatility of this plastic and its advantages compared to other substrates frequently used to culture cell monolayers for CLEM, as glass or sapphire discs, form an ideal starting point to develop protocols to perform routine CLEM with an alternative substrate.

The reported methods to mark Aclar are simple, effective and flexible

We have described in detail three methods to mark Aclar. Two of them engrave, whereas the third carbon-prints patterns on the surface of Aclar. In one engraving method the marks are done by hand (using a needle). The other two procedures are mechanical (done using a FIB or a carbon coater). All methods are easy to perform. Using equipment usually present at EM labs, Aclar can be needle engraved or carbon printed. Obviously, implementation of the FIB-mediated engraving method is still limited, because a FIB–SEM is not a standard instrument yet. The three methods are very efficient because they render relatively large pieces of Aclar containing numerous grid patterns in a short time. In Fig. 1 the basics of the marking methods is illustrated. In practice, when we print grids with a carbon coater, we perform the procedure with a complete strip of finder grids (twice the pattern shown in Fig. 1B1), producing 100 finder grid patterns like the one shown in Fig. 1B2 (bottom) in about 20 min (note that most of this time is required to pump air out of the coating chamber). In the case of Aclar engraved by needle we use a printed pattern that contains twice as many grid patterns as the one shown in Fig. 1A1, and it usually takes 10 to 15 min to scribe 64 grids as that illustrated in Fig. 1A2 (bottom). The marking procedure using the FIB enables one to mark mechanically under controlled conditions, i.e. it is possible to mill grids with different width and depth of the lines. In the 'Material and methods' section, we explained that we milled 16 types of patterns with line-width of 0.5, 1, 2 or 3 μm and line-depth of 2, 5, 10 or 15 nm. As the milling time is roughly linear with the volume to be milled, the total milling time varied between 6 s (for grids with line-width = 1 μm, and line-depth = 2 nm) and 2 min and 13 s (line-width = 3 μm, line-depth = 15 nm). In any case, preparing one Aclar piece with 16 different grid patterns by FIB took 25 min, including pumping time.

One of the main advantages of using engraved Aclar is the flexibility of the procedures to prepare it, i.e. the user can design the individual grid patterns and determine their distribution on the Aclar. For example, the distance between the lines can be increased or decreased with respect to the one proposed in our example (Fig. 1A) according to the size of the cells. In the same way, the number of and distance between patterns present in a single piece of Aclar can be adapted according to the requirements of the experiment. The finder grid-printing on Aclar as explained in this paper is less flexible, because the (strips of) finder grids are to be used as designed by the manufacturer. A common feature is that all three methods produce Aclar pieces, with numerous patterns on (Fig. 1A2 and B2, top), that can be housed in a single culture vessel. In this way, one experimental condition can be applied to multiple grid patterns at once. This can be very useful for example in the case of experiments where expensive drugs are added to the cells. Because Aclar is easily cut (with blade or scalpel, for example), once the regions of interest have been mapped at LM level, smaller Aclar pieces containing the desired number of patterns can be separated and distributed for different purposes (e.g. part for EM processing, part for immunofluorescence, etc.). In addition, one Aclar piece can provide patterns to prepare multiple epon blocks to perform CLEM as we explained above. Obviously, gridded Aclar can be also used for purposes other than CLEM where specific cells have to be localized, like for example in microinjection experiments.

All the marking methods render grid patterns that allow normal cell growth, mapping of areas of interest at LM level and re-localization on epon blocks

Endothelial cells, as HUVECs, grown *in vitro* are an experimental model widely used to study normal endothelial cell biology and mechanisms underlying the vascular pathology (Striker *et al.*, 1980). To perform these types of studies HUVECs have to be grown to a cell monolayer in 'cobblestone' in order to mimic the tight endothelium *in vivo*. For every experiment using gridded Aclar, cells were seeded in parallel also on regular (non-marked) Aclar in order to control if the presence of marks influenced cell growth. We could confirm that HUVECs grow on gridded Aclar, marked by any of the reported methods, at the same rate as HUVECs seeded on regular Aclar. Furthermore, cell monolayers grown

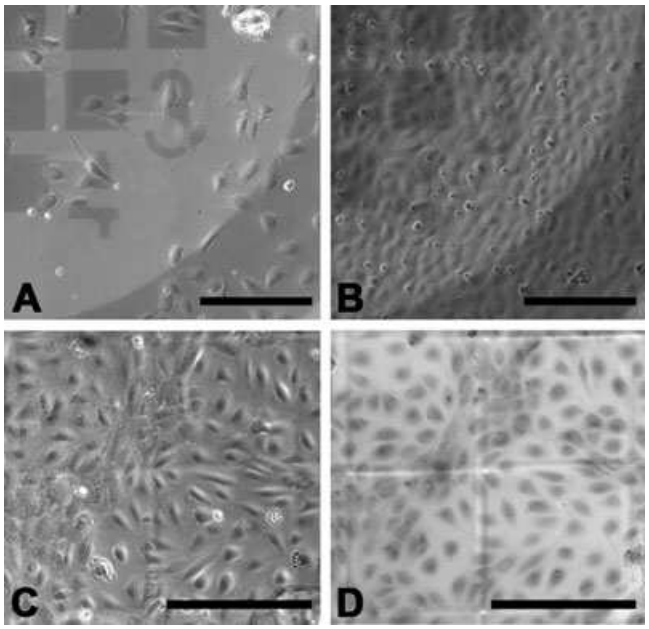

Fig. 2. (A, B) Carbon imprints on Aclar seen by phase contrast LM. The imprints are more easily discernable when cells are sparse (A) than when they form a tight monolayer (B). (C, D) Grid engraved by FIB. Even patterns made up of very thin and shallow lines can be observed with phase contrast illumination in the Aclar (C) or under a stereomicroscope in the surface of epon (D). Scale bars: A and B, 250 μm; C and D: 200 μm.

on marked Aclar acquire the characteristic 'cobblestone' morphology of HUVECs (see examples in Figs 1A3, 2B, 3C or 5A) as they do on regular Aclar and also on glass or polystyrene culture vessels (our unpublished observations). Therefore, marked Aclar is a suitable substrate to grow cell monolayers.

To perform CLEM, position marks are essential. As a first requirement, these marks need to be visible at LM level to allow the mapping of the regions of interest. We found that both the engraved and the carbon-printed marks were well visible under phase contrast and transmitted light (see examples in Figs 1A3, B3, 2A, B, 3B, C or 5A). Obviously, the patterns obtained with carbon coater (Fig. 2A) or FIB (Fig. 5A) are more regular than those obtained manually with a needle (Fig. 3C). However, all three methods render patterns useful to map the position of the cells of interest. Using the FIB, grid lines with controlled depth and width were engraved. This way we could determine that lines milled as thin as 0.5 μm and as shallow as 5 nm are visible with phase contrast microscope (Fig. 2C). However, in our opinion the use of grid patterns that are more clearly visible is convenient, especially when cells grow tight on the lines (compare the lines in Fig. 5A, 1 μm wide and 2 nm deep, with those in Fig. 2C). Using patterns engraved with FIB we could also conclude that too thick and too deep lines can cause the cells to grow into the 'grooves' (data not shown). Taking into consideration the latter, our choice is for grids made up of 1-μm-wide and 2-nm-deep lines. Preparation of a piece of Aclar with 16 of these patterns would take 12 min, including pumping time of the FIB–SEM. Using carbon-printed grids, the imprint is easily seen with phase contrast when cells are sparse (Fig. 2A). The same imprint seems to be weakened, as seen by phase contrast, by the presence of confluent cells on (Fig. 2B). However, visualization of the marks by transmitted light shows that the imprint stays intact during cell culture (Fig. 1B3).

A second essential requirement is that the position marks have to be visible on the plastic blocks once the samples are embedded. After epon polymerization and proper trimming, the Aclar pieces can be easily peeled off the block. Under a stereomicroscope we observed patterns (both engraved and carbon printed) on the surface of the block. The carbon imprint was completely transferred from the Aclar to the surface of the epon (Fig. 1B4). Also the physical marks in the Aclar were clearly observed as they had served as a mould for the grids engraved in the epon (Fig. 1A4). Therefore, areas of interest could be traced back and, after trimming, sectioned by ultramicrotomy for TEM analysis. Analysis by SEM of a surface allows the visualization of topological features of the sample. We could establish that the engraved patterns are visible under SE and BSE modes (at 2 kV) and that upon increasing the voltage to 20 kV, cells were clearly visible in BSE mode (Fig. 5B and C). Therefore engraved Aclar can in principle also be used to do CLEM using FIB–SEM analysis. In addition, with FIB–SEM, complete grid patterns can be studied without the need of trimming off any squares.

Validation of CLEM procedures for TEM and FIB–SEM analysis

When the cells to be studied have a well-defined morphology at LM level, the use of specific markers is not necessary to localize them. However, in other cases specific markers are needed to pinpoint the cells of interest. Because Aclar does not exhibit any autofluorescence (Kingsley & Cole, 1988) and in view of the results described before, it should be possible to use Aclar for CLEM applications in which cells of interest are localized by fluorescent probes. To validate the CLEM method in combination with fluorescence detection, we performed two types of experiments. In one, cells were incubated with OG-LDL, and positive cells were localized and mapped directly after fixation. In the other, cells were first fixed and then underwent immunolabelling for caveolin, a caveolar marker, before being screened.

Elevated serum LDL cholesterol levels have been associated with an increased risk to develop cardiovascular diseases, as atherosclerosis (Glass & Witztum, 2001). In our current research we are interested in studying the different mechanisms of LDL uptake by human endothelial cells. In pilot experiments to set up experimental conditions we found that after incubation with LDL, some HUVECs present in a culture took up LDL in the cytoplasm whereas others did

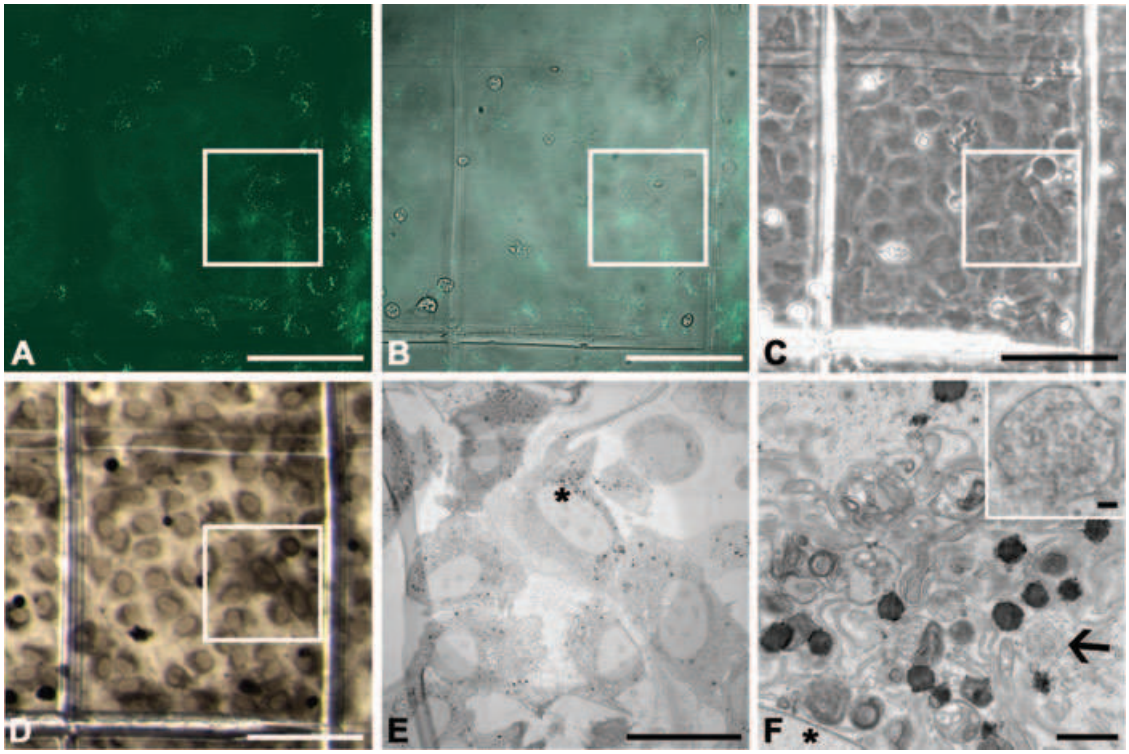

Fig. 3. Validation of CLEM procedures for TEM analysis: OG-LDL uptake by HUVECs. Cells positive for OG-LDL are localized by laser scanning microscopy in fluorescence mode (A) that combined with transmitted light mode (B) provides the information necessary to map the position of the cells of interest. (C) Imaging under a phase contrast light microscope gives valuable extra information on the shape of the cells of interest. (D) The mapped areas can be easily found on the surface of the epon block that can be subsequently trimmed and sectioned *en face*. (E) Under TEM, cells of interest are first localized at low magnification. (F) The analysis at higher magnification reveals that cells positive for OG-LDL are rich in endosomes/lysosomes and lipid droplets. Squares in A–D correspond to quadrant 'd4' shown in Fig. 1(A3) or (A4) and to the same area imaged in (E). Asterisks in (E) and (F) point out the nucleus of the same cell. The arrow in (F) points to the multivesicular body showed in detail in the inset. Note that some figures were electronically flipped in order to facilitate recognition of the cells of interest through the complete procedure. Scale bars: (A)–(D), 100 μm; (E), 30 μm; (F), 1 μm; (F) inset, 100 nm.

not. In order to characterize the cell subpopulation positive for LDL at high-resolution level, we performed CLEM. To this end, cells grown on marked Aclar were incubated with OG-LDL and fixed after uptake. Fluorescence, assessed by laser scanning microscopy, was found as discrete spots in the cytoplasm of some cells (Fig. 3A). The location of these cells was mapped for TEM analysis. Transmitted light allowed the visualization of position marks (Fig. 3B) that were later imaged by phase contrast together with the cells (Fig. 3C). With the mapping information, regions of interest were traced back in the epon blocks (Fig. 3D), and after proper trimming and *en face* sectioning, the cells of interest were found back under the TEM (Fig. 3E). This way, we could determine that cells which internalized OG-LDL were rich in lipid droplets and members of the endosomal/lysosomal system (Fig. 3F). LDL plays an essential role as vehicle to deliver cholesterol to the cells for membrane growth and maintenance. In mammalian cells, LDL incorporation in endosomes happens via a high-affinity pathway mediated by LDL-receptor (Brown & Goldstein, 1976). Upon degradation of LDL in the lysosomes, cholesterol in excess can be stored in esterified form in lipid droplets (Brown & Goldstein, 1986). Therefore, our observations are not surprising but provide insights in one of the mechanisms of LDL internalization in HUVECs that should be further explored.

Endothelial cells are rich in caveolae (Palade, 1961), specialized invaginations of the plasma membrane that are coated with caveolin. Caveolae are involved in transcytotic transport through the endothelium (Simionescu, 1983) and have been related to LDL transcellular transport in animal models (Vasile *et al.*, 1983). Therefore, LDL internalization by caveolae has to be considered as a possible mechanism of LDL uptake by human endothelial cells. HUVECs grown to form a 'cobblestone' have a well-defined phenotype and show membrane labelling for caveolin all over the cytoplasm (our unpublished observations). Occasionally, cells isolated from a determined umbilical vein show abnormalities, e.g. they can locally reach the post-confluent state (Fig. 4A) without the typical 'cobblestone' morphology. By implementing CLEM in combination with immunolabelling for caveolin (detected by combined immunofluorescence and pre-embedding immunogold labelling) we could define

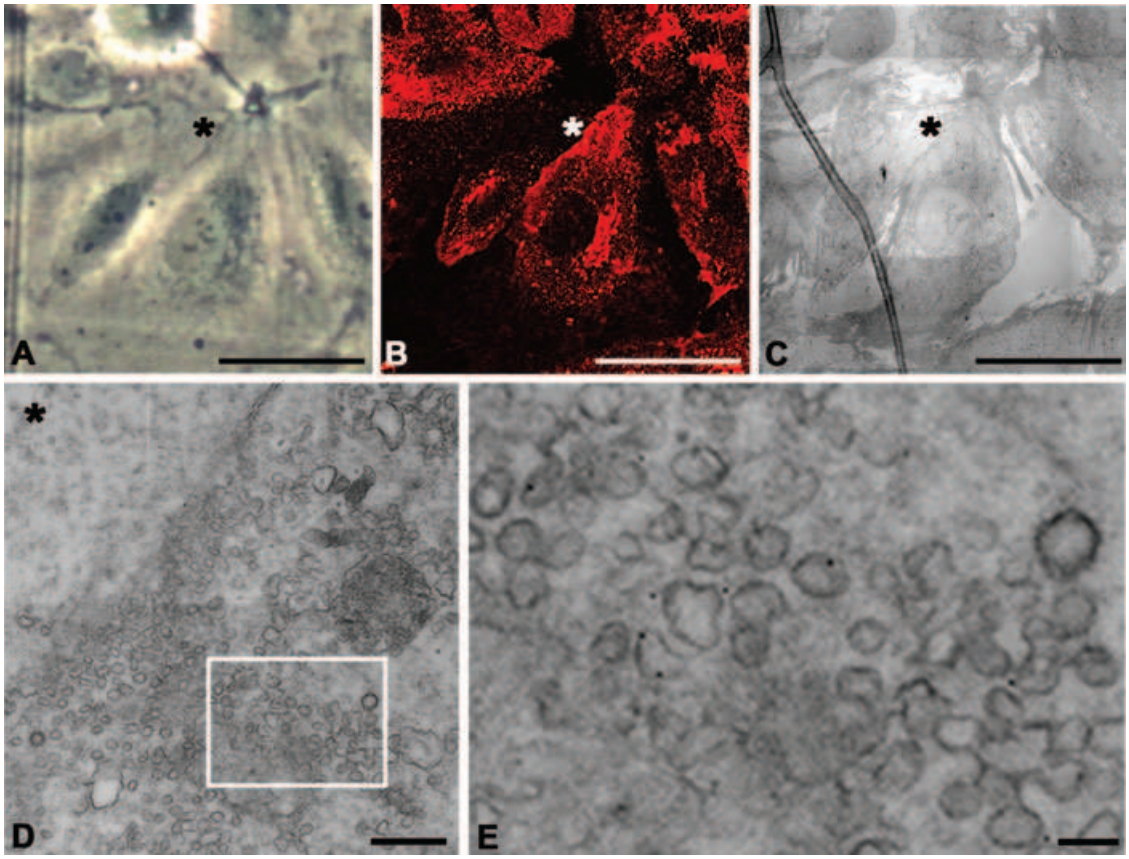

Fig. 4. Validation of CLEM procedures for TEM analysis: Combined immunofluorescence and pre-embedding immunogold labelling to determine caveolin distribution in HUVECs. Some cells that do not acquire the 'cobblestone' morphology (A) seem to have areas of the cytoplasm (asterisk) depleted of caveolin (B). Tracing back these cells at TEM level (C) allows confirming the absence of caveolae at these areas (D, asterisk). Comparing (B) and (D) it is also obvious that cells showing a strong immunofluorescence are also rich in caveolae (D) labelled for caveolin (E; close-up of the area marked by the rectangle in D). Asterisks at (A)–(D) point out the same cell. Scale bars: (A)– (C), 50 μm; (D), 500 nm; (E), 100 nm.

that these cells show an irregular distribution of caveolin labelling at LM level (Fig. 4B). By re-localizing these cells at TEM level (Fig. 4C) we could link immunofluorescence to immunogold labelling (Fig. 4D, E) and confirm that caveolae were abnormally absent at large areas of some cells (Fig. 4D). These observations are important to develop quality controls in the cell culture, namely to learn to recognize when a cell culture can be invalid for experimental purposes involving caveolae. Furthermore, they clearly show that the use of Aclar is compatible with immunofluorescence and immunogold labelling.

Aging, a risk factor for the development of atherosclerosis, has been associated to endothelial dysfunction and senescence (Brandes *et al.*, 2005). HUVECs are primary cells with a limited lifespan and as the number of population doublings increases, cells finally enter replicative senescence and stop dividing. One feature of senescent endothelial cells, including HUVECs, is their large size (van der Loo *et al.*, 1998; Eman *et al.*, 2006). We seeded HUVECs, cultured through several passages, on marked Aclar. Large cells, present among normal cells, were localized and mapped at LM level (Fig. 5A). Afterwards samples were processed for FIB–SEM analysis. Using the SE and BSE modes as explained before, marks and cell shapes could be visualized on the surface of the epon blocks (Fig. 5B,C), allowing for a direct comparison with the phase contrast images (Fig. 5A). This made the re-localization of the cells of interest with FIB–SEM possible. Furthermore, cross-sections of the endothelial layer were milled (Fig. 5D, E), which allowed comparison of large and normal HUVECs at high resolution (Fig. 5F, G). In this way we could determine that a wide peripheral area of the large cells is virtually depleted of organelles. In addition, clear junctional areas between large and normal cells could be visualized (Fig. 5G). The functional significance of these observations needs to be further addressed. This approach clearly shows, to our knowledge for the first time, the applicability of CLEM using the FIB–SEM. The *XY*-resolution of the FIB–SEM in biological systems currently ranges between 5 and 10 nm and therefore this CLEM approach offers excellent possibilities for obtaining ultrastructural 3D information of large volumes (range $\sim 10 \times 10 \times 10\ \mu m^3$) (De Winter

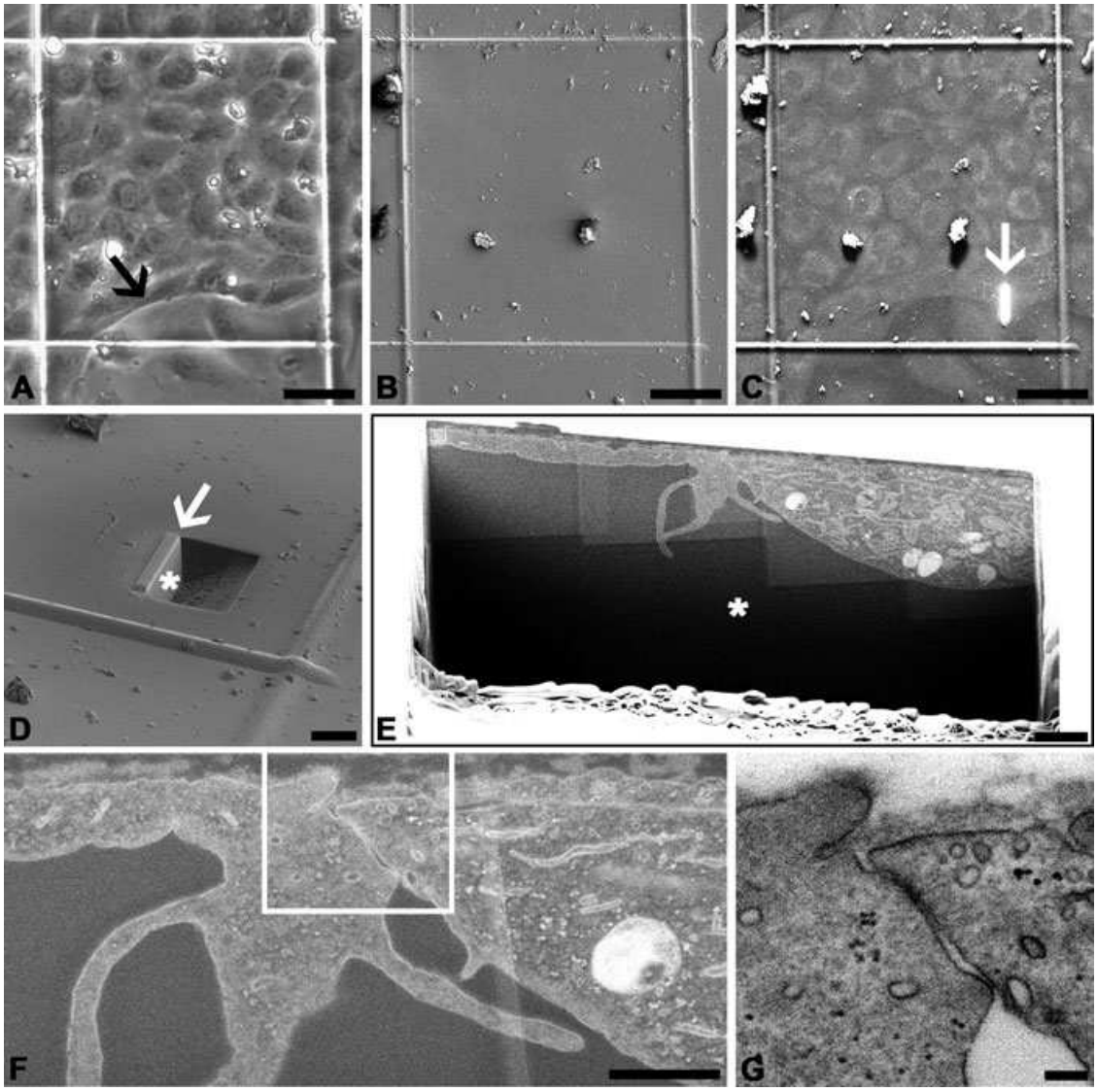

Fig. 5. Validation of CLEM procedures for FIB–SEM analysis. (A) After several passages some HUVECs become larger as seen in this area of engraved Aclar imaged with phase contrast light microscope (arrow). The lines present in the epon block can be visualized in the FIB–SEM in SE (B) and BSE (C) modes. Using BSE contrast at 20 kV it is also possible to see the cells embedded in epon (C) and in this way, cells of interest can be re-localized. After milling a trench in the block (D), cells can be cross-sectioned with FIB and imaged in BSE mode (E–G, at 2 kV). Inversion of contrast (G) renders images similar to those obtained from thin sections under TEM thereby facilitating interpretation. Large cells show few organelles in their periphery (E–G, cell on the left). Note that the arrows in (C) and (D) point to a platinum/carbon strip deposited on the area of interest to be milled and that the asterisks in (D) and (E) show the same wall of the trench. Scale bars: (A)– (C), 50 μm; (D), 10 μm; (E), 2 μm; (F), 1 μm; (G), 200 nm.

et al., 2009; Hekking *et al.*, 2009) at now specific sites of interest.

Gridded Aclar might also be used for CLEM in combination with sample cryoprocessing

In this paper, we have shown the validation of the CLEM using Aclar with chemically fixed samples. In ultrastructural studies in which preservation of cellular fine structure as close as possible to the native state is a prerequisite, cryofixation (by e.g. high-pressure freezing) combined with freeze-substitution and plastic embedding would be the processing method of choice. Aclar is not only a valid substrate to grow cells for high-pressure freezing and freeze-substitution purposes (Jiménez *et al.*, 2006), but also a convenient one because it overcomes practical difficulties encountered during the handling and processing of cells grown on other commonly used substrates, like sapphire discs (Jiménez *et al.*, 2006) and can be successfully used to freeze samples without addition of any cryoprotectant (Jiménez *et al.*, 2009). Engraved Aclar should prove to be a

suitable substrate to perform CLEM in combination with high-pressure freezing/freeze-substitution, as physical marks stay permanently on the Aclar surface. *A priori* Aclar marked by carbon coating might also be used for this kind of applications because we have observed that carbon imprints on Aclar withstand long-term incubations in acetone, a commonly used freeze-substitution agent. In any case, if the user aims to do CLEM with Aclar combined with cryoprocessing, the next practical suggestions may be taken into consideration. First, gridded Aclar should be punched to a proper shape and size (e.g. 1.5-mm-diameter discs, containing just one grid pattern) to fit the high-pressure freezing specimen carrier. This will diminish the manipulation time necessary for sample preparation prior to cryofixation. In the case of engraved grids, it will be extremely useful to scribe a mark for orientation beside the grid (an asymmetric sign, like an 'L'). And second, Aclar should be attached to the culture vessel using the Matrigel method and sterilized by ultraviolet light. This way, the surface of even very small pieces of Aclar will stay completely flat.

Final considerations

We have illustrated different ways to successfully perform CLEM, using TEM and FIB–SEM, with HUVECs grown on different types of gridded Aclar with some concrete examples. Obviously, other technical combinations to do CLEM are also possible. We showed that Aclar attached to glass-bottom dishes can be screened by laser scanning microscopy using Argon and Helium–Neon lasers. This means that marked Aclar can be also used to correlate *in vivo* fluorescence video microscopy (of e.g. live cells transfected with green fluorescent protein constructs) with EM, a technique that makes possible to study dynamic cellular processes (Polishchuk & Mironov, 2001). It should be noted that high power laser applications (as two-photon excitation microscopy) are not compatible with the use of Aclar, because the high pulse laser intensity induces local damage in the Aclar that perturbs imaging (Thibaud *et al.*, 2005). Alternatives for LM imaging other than the use of a laser scanning microscope are also possible – for example, an inverted microscope equipped with light fluorescence and appropriate objective lenses. We used Aclar in combination with immunofluorescence and immunogold labelling, but protocols involving the use of diaminobenzidine can be also applied because Aclar is compatible with this chemical (Rees *et al.*, 1976). We grew HUVECs to a post-confluent monolayer in order to mimic a tight endothelium. But the CLEM procedures can be equally applied to sparse cultures.

The technical versatility of Aclar together with the fact that it can be used to culture a wide range of cell types (Kingsley & Cole, 1988; Masurovsky & Bunge, 1989; Jiménez *et al.*, 2006) account for our statement that gridded Aclar is an excellent and convenient substrate to perform CLEM of cell monolayers.

Acknowledgements

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