Ultra-thin resin embedding method for scanning electron microscopy of individual cells on high and low aspect ratio 3D nanostructures

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Abstract

The preparation of biological cells for either scanning or transmission microscopy requires a complex process of fixation, dehydration and drying. Critical point drying (CPD) is commonly used for samples investigated with a scanning electron beam, while resin-infiltration is typically used for transmission electron microscopy. CPD may cause cracks at cellular surface and a sponge-like morphology of non-distinguishable intracellular compartments. Resin-infiltrated biological samples result in a solid block of resin, which can be further processed by mechanical sectioning. Here, we propose a method for removing resin excess on biological samples before effective polymerization. In this way the cells result to be embedded in an ultra-thin layer of epoxy resin. This novel method highlights in contrast to standard methods the imaging of individual cells not only on nanostructured planar surfaces but also on topological challenging substrates with high aspect ratio 3D features by SEM.

Keywords

Scanning electron microscopy, epoxy-based resins, cells, chemical fixation, ultrastructures.

1. Introduction

Understanding the interaction of cells with biomaterials has become a very broad field of research spanning over various methods of investigation and applications such as implants(Recum and Kooten, 1996), neuroimplants(Wang et al., 2005), biofilm formation(Pavithra and Doble, 2008), stem cell differentiation(Burdick and Vunjak-Novakovic, 2008), and can lead to *e.g.* cell differentiation, adhesion and affinity towards the matierial. Standard techniques such as fluorescence microscopy(Axelrod, 2001), surface plasmon microscopy(Toma et al., 2014) or electron microscopy(Friedmann et al., 2011; Santoro et al., 2014b; Wrobel et al., 2008) are powerful tools for the investigations of biological samples yet, but generally not allowing to achieve resolution in all three space dimensions at the nanoscale(Alivisatos et al., 2013).

It seems of foremost importance to establish an artifact-free preparation and visualization on the single cell level of any kind of surface. The imaging of cellular outgrowth with nanometer

resolution with scanning electron microscopy is established since decades and recently allowed a full 3D volume reconstructions of cells and tissues(Bushby et al., 2011; Hoffpauir et al., 2007; Holzer and Cantoni, 2011; Villa et al., 2013). Nevertheless, traditional preparation techniques such as critical point drying (CPD) and air drying have some major limitations. Firstly, these two methods are notapplicable to every type of sample. Secondly, CPD and air drying induce typically volume shrinking artifacts in particular for confluent cells' monolayers(Schroeter et al., 1984) and, at last, only the sample surface is visible. For these reasons, alternative approaches have been carried out such as HDMS(Braet et al., 1997) biological sample preparation. salicylic acid. These methods are very efficient for SEM investigations of whole cells with volume preservation. In contrast, techniques such as thin resin plastification have been used for visualization of intracellular compartments with SEM(Bittermann et al., 2012; Kizilyaprak et al., 2014). Thin resin plastification method allows the preservation of intracellular structures but the draining of the resin is not sufficient to visualize the effective cell adhesion on the substrate. Cell interiors and the substrate-cell interaction is typically imaged by transmission electron microscopy or scanning electron microscopy, which requires cells to be embedded in a resin block and losing surface information, but gaining highly resolved access to cellular ultrastructures, in particular for slices of cells. This bulk like resin embedding favors a volume preservation of the cell avoiding structural and morphological artifacts due to the fact that the intracellular water component is basically replaced first with an intermediate liquid medium and , finally, with resin. In addition, mixtures of heavy metals-based compounds (*i.e*. osmium tetroxide, uranyl acetate) can be used to stain intracellular cell membranes and proteins prior to embedding, favoring contrast enhancement during the image acquisition. By using the additional technique of a focused ion beam, one is able to slice perpendicularly to the substrate surface through a sample and, achieving a tomographic imaging of the cell surface interface(Bushby et al., 2011; Drobne, 2013; Friedmann et al., 2011; Heymann et al., 2006; Knott et al., 2008; Lešer et al., 2009). A more complex scenario opens when cells interact with 3D nanoengineered substrates. Materials and geometries adopted during the nanostructures development can involve very challenging processes aimed to the investigation of 3D nanostructure-cell interaction and visualization of the actual interface. Many attempts have been carried out recently to characterize the tight adhesion between cell and 3D nano and microstructures with TEM and SEM with and

without cross sectioning techniques (Angle et al., 2015; Bonde et al., 2014; Fendyur et al., 2011; Santoro et al., 2014a, 2014b; Spira and Hai, 2013).

Our approach tries to establish a novel embedding method, which brings the excellent properties of resin embedding of the TEM preparation to a single cell level by removing excess material and can be consequently imaged by a SEM and eventually prepared for FIB sectioning. This resin embedding approach of single cells becomes in particular relevant when high-aspect ratio spanning structures are analyzed and very thin and fragile cellular compartments need to be prevented from any damage.

(Duan et al., 2012; Hai et al., 2010; Lešer et al., 2009; Lin et al., 2014; Santoro et al., 2013; Spira et al., 2007; Tian et al., 2010; Wierzbicki et al., 2013; Xie et al., 2012, 2013).

Our new sample preparation procedure renders a determination of the exact contact area between surface and cell membrane possible.

2. Material and methods

2.1 Cell culture

2.1.1 Substrates preparation

The patterned samples were fabricated from a silicon wafer by depositing 100 nm Si_3N_4 and 100 nm SiO_2 by plasma-enhanced chemical vapor deposition (PECVD, Sentech, Germany). The pattern was generated by means of electron-beam lithography (EBPG 5000+ from Leica) followed by reactive ion etching (AMR RIE system from Oxford). The structures were etched to an overall depth of 100 nm. The molds contained a pattern with lines and spaces with a width of 400 nm and a length of 4 µm.

All Si, Si/SiO_2 (oxide thickness of 100 nm), and $Si/Si_3N_4/SiO_2$ substrates were cleaned with acetone for 15 minutes in a sonicator, 2-propanol (Merck, Darmstadt, Germany) and bi-distilled water in order to remove any possible organic residues from the substrate surface. Then, the samples were processed for surface activation in a plasma chamber (Plasma surface technology - Pico, Diener electronic Company) using 1.4 mbar as pressure and a power of 200 W for 2 minutes. 3D gold mushroom-shaped microstructures were fabricated as shown in our former studies(Santoro et al., 2014a, 2014b). Afterwards, samples were prepared for cell culture, performing a sterilization under UV light (HS type, HERA Safe Company) for at least 30

minutes. In addition, samples were coated with 10 mg/ml poly-*D*-lysine (PDL) diluted in Grey's Balance Salt Solution (GBSS, Life Technologies, Darmstadt, Germany) at 4º C overnight. Finally, samples were rinsed two times with GBSS shortly before cells' seeding.

2.1.2 **Neuronal culture**

Primary cortical neuronal cultures were prepared by removing cortices from Wistars rat embryos at embryonic day 18. Then, cortices were incubated with 0.25% trypsin/EDTA (Life Technologies) in a Petri dish (diameter of 35 mm) for 5 minutes at 37°C. After the incubation, the solution consisting of tissue and trypsin/ EDTA was transferred in to a 2 mL plastic tube. The tissue was let to settle at the bottom of the tube and the overlying trypsin/ EDTA was removed. Neurobasal® media (Life Technologies) was supplemented with 1% (vol/vol) B27 (Invitrogen, Darmstadt, Germany), 0.25% (vol/vol) L-glutamine (Invitrogen) and 0.1% (vol/vol) gentamycin antibiotic. One mL of supplemented Neurobasal® media at room temperature was added and, then, the tube was gently swirled by hand. This procedure was repeated 3 times. Finally, the supernatant was exchanged with 1 mL of fresh Neurobasal® media, and tissues were triturated until the cells were completely dissociated. A sample of cells re-suspension (10 µL) was extracted, diluted in 20 µL of supplemented Neurobasal® media and 10 µL of trypan blue. This solution was placed in a cytometer (Neubauer chamber) for counting live and dead cells. After counting, the remaining cells were re-suspended in a final volume for being cultured on the substrates. The media was replaced completely 2 hours after seeding time. Every second day, half of the media was exchanged with freshly-prepared warm (supplemented) Neurobasal® media.

2.1.3 HL-1 culture

3D gold mushroom-shaped microstructures and control coverslips were washed and sterilized as mentioned earlier. Substrates were coated with fibronectin in 0.02% Bacto TM gelatin (Fisher Scientific) for 1 hour, before cells' seeding. The HL-1 cells were cultured in a T-25 flask until confluency and dissociated with 1 mL of 0.25% trypsin/ EDTA for 5 minutes at 37ºC. The cells were re-suspended in 5 mL of Claycomb culture medium supplemented with 10% concentration (vol/vol) of fetal bovine serum (Life Technologies), 100 µg/mL penicillin/streptomycin (Life Technologies), 0.1 mM norepinephrine and 2 mM L-glutamin[35].The solution consisting of cells and media was transferred in a plastic 15 mL tube and centrifuged for 5 minutes at 1700 rpm. A

cell pellet formed at the bottom of the tube. Supernatant liquid was removed and the cell pellet was carefully re-suspended by adding 1 mL of warm media pipetting up and down. 2% of cells at confluency was placed on each substrate and media was added to reach a final volume of 1.5 mL. The cell culture media was replaced completely every day with fresh one.

2.2 Sample preparation for scanning electron microscopy/focused ion beam

After 4 DIV *(days in vitro)* neuronal cells and HL-1 cells were washed two times with warm phosphate buffer saline solution (PBS) and chemically fixed with a solution of 3.2% glutaraldehyde dissolved in PBS at pH 7.4 for 15 minutes at room temperature(Collins et al., 1977; Czeschik et al., 2015).Thereafter, samples were washed three times with PBS and, then, three times with bi-distillated water so as fixatives' residues could be completely removed. Gradually, water was replaced with an intermediate medium (ethanol) starting from a concentration of 10% up to 50% (vol/vol). At this time, the incubation time was 5 minutes for each solution. Then, samples were infiltrated in sequence with a solution of 70% ethanol (vol/vol) for 15 minutes; 90% 95%, and 100% (vol/vol) ethanol were exchanged three times and let for incubation for 5 minutes (each time). In the end, samples were stored in 100% ethanol in a sealed dish at 4ºC.

Here, we present two different preparation methods of cells for SEM. The first method is CPD based on CO_2 phase transition. The intermediate medium is exchanged to liquid CO_2, and, by temperature increase, the samples is finally dried.

In particular, samples were transferred into the chamber of a critical point drying machine (CPD 030, BAL-TEC, Balzers, Liechtenstein) ensuring that the samples were continuously immersed in 100% ethanol. The system was slowly cooled down to 10°C (typically 1°C/step). The ethanol was gradually replaced with liquid CO_2. This consist of typically 10 ethanol/CO_2 exchange repetitions. Then, the chamber's temperature was increased by 1°C/step until reaching a temperature of about 40°C. At the critical temperature and pressure, the liquid CO_2 turns into the supercritical state, the samples in the chamber completely dry off and, finally, the supercritical CO_2 can be released *via* a control valve.

The second method consists of epoxy-based resin infiltration. First, we prepared the different components of the embedding polymer. 12.5 mL solution of Epon embedding medium was mixed solely with 20 mL dihydro-3-(tetrapropenyl)furan-2,5-dione (DDSA) for 5 minutes using a graduated plastic pipette (Fig. 1A). In parallel, a solution consisting of 17.5 mL Epon

embedding medium and 15 mL methyl-5-norbornene-2,3-dicarboxylic anhydride (MNA) was prepared in a plastic tube as shown in Figure 1B. Afterwards, the two solutions were poured at the same time into a plastic container and 1.3 mL of 2,4,6-tris(dimethylaminomethyl)phenol (DMP30) was added. At this step, the color of the solution is typically red to orange (Fig. 1C). The solution is stirred for about 1 hour at room temperature until the solution's color turns into yellow, as exemplary shown in Figure 1D. Finally, the epoxy solution can be directly used for infiltration or, stored in plastic syringes at -20°C and used on a later stage, after being thaw out for at least 10 minutes at room temperature.

The final embedding was carried out by replacing the 100% ethanol in which the samples were immersed, prepared as described earlier, with the yellow epoxy-based polymer. This procedure was gradually performed starting with a solution of ethanol/resin in a 3 to 1 proportion incubated for 3 hours at room temperature followed by two solutions in 2 to1 and 1 to1 proportions incubated for 3 hours and overnight, respectively. Then, solutions with ethanol/resin proportions of 1 to 2 and 1 to 3 were incubated for 3 hours and, in the end, the samples were infiltrated only with pure epoxy resin (Fig. 1E). Considering that most of the chemical components are highly toxic, the complete embedding procedure was done in a hood under laminar flow conditions. The final step of our resin embedding method is the removal of excess viscous resin surrounding the sample (Fig. 1F). This is achieved by quickly splashing down ethanol on the back of the sample first and, afterwards, on the substrate's side where the cells adhered. As reference, we used a 5 mL plastic pipette to splash 5 mL of 100% ethanol at every repetition. The distance of the pipette from the substrate was about 1 cm. We carried out experiments testing the effect of different numbers of repetition onto the final cell morphology. For this purpose, we performed splashing for 1, 5, 10, 30 times. The resin polymerization was achieved by heating the resin-embedded samples for 12 hours at 60º C.

2.3 Scanning electron microscopy imaging and focused ion beam sectioning

The samples were coated with a thin layer of iridium (15-30 s deposition time, 15mA current) *via* sputtering deposition (K575X Sputter Coater, Quorum EMITECH, Ashford, UK). Then, each sample was mounted on a typical electron microscopy stub using a carbon adhesive tape or liquid silver paste. Samples were observed either with a single scanning electron beam (Magellan 400, FEI, Oregon, U.S.A and 1550VP, Zeiss, Oberkochen, Germany) or, complementarily, with an electron and ion beam in a dual beam machine (Helios 600i NanoLab Dual-beam, FEI,

Oregon, U.S.A). Images were acquired using a detector for secondary electrons (SE) or back-scattered electrons (BSE). The final beam acceleration was obtained by fixing a voltage between 3kV and 10 kV, and a current of 21 pA.

In addition, we performed a transversal cross sectioning and we fixed the milling parameters as presented in our former studies (Santoro et al., 2014b). Briefly, the target area was covered with a protective layer of platinum. The layer of platinum was deposited in two steps. During the first step, a 0.5 µm thick layer of platinum was deposited *via* electron beam induce deposition (EBID) fixing the machine's stage at 0°. In addition, the sample was tilted to 52° and 0.3 - 0.5 µm of platinum were deposited *via* ion beam induced deposition (IBID). A first cross section was created by an ion beam milling procedure (at 30 kV and 0.74 nA) fixing a milling depth of 1 µm (nominally valid for silicon). Finally, fine polishing was performed at 30 kV with a current of 80 pA. The SEM imaging of the resulting section was performed using an 'in-lens' detector for both SE and BSE.

3. Results and discussion

Our main purpose is the minimization of volume shrinkage artifacts during electron microscopy preparation protocol for individual cells adhering on planar and high aspect ratio 3D-nanostructures. In addition to preservation of cells' components, we aim to develop a reliable resin embedding method for investigating cells' spreading and adhesion on to the 3D nanostructures. In our investigation, we compare the effects of critical point drying and resin embedding preparations by SEM investigations.

After finding an optimal ultra-thin resin thickness for external structure preservation, we compared resin embedded neuronal cells to neuronal cells prepared *via* an optimal CPD procedure(Santoro et al., 2014b).

In Fig. 1G, we schematically represent the effect of the two tested methods, with particular attention to the deformation of the cell membrane domain attaching the culture substrate, such as a substrate with 3D features *(i.e.* cylindrical or mushroom-shaped 3D nanostructures).

3.1 Ultra-thin resin layer embedding procedure for single cells cultured on 3D nanostructures.

In the first part of our study, we optimized a thin-layer resin embedding procedure in order to reduce shrinkage artifacts, which could alter the cells' structures. Our method mainly consists of the excess resin's removal before polymerization in contrast to standard procedures, where the resin is first polymerized and, then, mechanically removed. In order to achieve that, we splashed ethanol on the sample several times in quick sequential order, so that cells are still impregnated with liquid resin and no resin left overs remain on the substrate. The number of rinsing repetitions is a very critical parameter since high amount of ethanol could penetrate into the cell and introduce artifacts during the resin polymerization process. In order to find an optimal resin thickness, we quantified the effective resin's thickness as a function of the rinsing repetitions' number, (final ethanol rinsing volume) as shown in Fig. 2. Considering a nominal ethanol volume of 5 mL for each repetition, we tested final ethanol volumes of 5 mL, 25 mL, 50 mL, and 150 mL on an overall substrate area of 1.2 cm^2 using planar Si/SiO_2 substrates with primary cortical neurons.

In case of the lowest amount of ethanol (only one splashing repetition equals to an overall volume of 5 mL), the layer of residual resin is relatively thick (about 1 μm) and it was not even possible to clearly identify the neuronal cell body perimeter and neurites while using a maximum acceleration voltage of 10 kV, a current of 21 pA and a secondary electron detection.

In contrast, using a nominal ethanol volume of 25 mL the layer thickness of residual resin decrease to 500 nm. Under these conditions, it was possible to clearly identify single cells on the substrate and, thus, several regions of interest for imaging. Finally, we found an optimal resin thickness of about 5 nm in correspondence of an ethanol volume of 50 mL (Fig. 2). In this case, small neurites, filopodia or neurites branching without cracks were clearly observed. In contrast to that, when 150 mL of ethanol were used for resin removal, neuronal cell membrane and neurites exhibited damages and typical cracks due to the quick evaporation of ethanol and a lack of resin embedding the cell. In several cases, we even observed a partial or total detachment of cells from the culture substrate.

In addition to SEM investigation, we performed FIB transversal sectioning to prove that our sectioning method, previously adopted for CPD-based cells(Santoro et al., 2014a, 2014b) is also suitable for thin-layer embedded cells. As shown in Fig. 2 (insets), the typical sectioning

curtaining artifacts are not present, if cross sections are polished with a voltage of 30 kV and a current of 80 pA.

3.2 Effective comparison of critical point *vs.* ultra- thin resin layer embedding of single cells.

In the second part of our study, we directly compared the effects of critical point drying and thin-layer resin embedding preparation while using planar culture substrates and high aspect ratio 3D nanostructures.

We investigated planar substrates with HL-1 cells and primary rat cortical neurons. For cells prepared with CPD, it is clearly observable that the cell membrane has a porous morphology and this porosity can cease to a consistent damage (Figs. 3A,C). In contrast, thin-layer resin embedding leads to reduced artifacts as shown in Figs. 3B,D. In this case, the cell membrane appears continuous and, furthermore, cell structures such as cell-cell attachment points (*i.e.* gap junctions) are perfectly preserved as shown for HL-1 cells (Fig. 3B). Similar conditions are present in thin-layer embedded primary neurons where cell body and neurites do not show any structural damage or cracks (Fig. 3D) in contrast to strongly-damaged CPD based samples (Fig. 3C).

Considering the efficiency of resin preparation in comparison to CPD on planar substrates, we investigated a more complex engineered substrate consisting of high and low aspect ratio 3D gold nanostructures (mushroom-shape) with cardiomyocyte-like HL-1 cells adhering on them. In our former studies, cylinder-like nanostructures induce lower wrapping states than 3D mushroom-like nanostructure corresponding also to higher membrane deformation in case of mushroom-like 3D nanostructures(Santoro et al., 2014a). Therefore, we investigate two extreme scenarios with respect to aspect ratio and dimensions for a direct comparison of CPD and thin-layer resin preparation methods. On the one hand, we considered the effect of the critical point drying procedure of HL-1 cells on 3D mushroom-shaped nanostructures with low aspect ratio (Figs. 4A,B). In this case, the CPD itself can induce high surface tension during the drying procedure while the cell membrane is under a relative high deformation condition due to the shape of the nanostructure. The cells membrane is extremely porous (Fig. 4B) and in some parts cracks are visible. On the other hand, we evaluated the efficiency of the ultrathin-layer resin embedding procedure (50 mL ethanol volume used for splashing) of HL-1 cells cultured on high aspect ratio 3D mushroom-like nanostructures (Figs. 4C, D). Here, the cell membrane is under

higher deformation conditions and, thus, likely to result in a structural collapse while wrapping cylindrical nanostructures. The chances of a crack or complete collapse of free-standing cellular projections depend on the length and lateral dimensions of the cellular outgrowth. The defect rate increases in particular in cases of early stage engulfment processes of the 3D nanoelectrodes. Filopodia can span large distances in the micrometer regime from the mushroom cap to the surrounding sample surface and therefore collapse easily in case of CPD preparation. This affects largely affects the quality of the electron microscopy investigations and, eventually, the analysis of the cell membrane deformation as a response to the 3D nanostructures. Thin-layer resin embedded cells on high aspect ratio mushroom-like nanostructures appeared to be integer and forming a continuous assembly around the 3D nanostructures, as representative shown in Figs. 4,D. Here, 3D nanostructures wrapped at the edge of the cell form an extremely tended morphology left completely intact by the embedding procedure.

3.3 Preliminary investigations of ultra-thin resin layer embedded cells on 3D nanostructures with focused ion beam sectioning.

In our former studies we optimized a procedure for performing a transversal sectioning of CPD-prepared single cells with FIB inducing almost no curtaining effects(Santoro et al., 2014a, 2014a). In those cases, cells attend to maintain a sponge-like morphology (Santoro et al., 2014a, 2013). CPD is restrictively limited to the observation of cell parts in direct contact with the planar substrate and with 3D nanostructures. In particular for 3D nanostructures, the deformation induced to intracellular structures is not observable in CPD-prepared cells. In these preliminary investigations, we only focused on defining a suitable sectioning method of ultra-thin resin embedded cells with FIB. Considering that we did not include any heavy metals compounds in our preparation, intracellular structures were not visible. We created transversal sections of primary neurons on planar substrates (Fig. 5A), HL-1 cells on 3D mushroom-like nanostructures (Fig. 5B) and primary neurons on grooved-like nanostructures (Fig. 5C-D). We found that the resin embedding does not alter the effective cell attachment on the different types of substrates. Moreover, in some cases (Figs. 5A,C) we were able to visualize some intracellular structures without using any contrast enhancement agent.

4. Conclusions

We optimized an embedding preparation procedure in order to have a SEM investigation of single cells or cell- cell interaction with an ultra thin-resin embedding and cells' structures preservation. Typically, resin blocks are commonly investigated by TEM rather than SEM after sectioning. This occurs because of the resin's thickness, which is often in the millimeter range. Our innovative finding grants the possibility to observe resin embedded individual cells on planar glass, silicon/silicon oxide substrates and 3D engineered substrates (low and high 3D mushroom-shaped nanostructures) directly with SEM. In particular, we have shown that the proposed resin infiltration procedure preserve the entire cellular volume as well as small cellular features (*i.e.* neurites, cell-cell attachment points). Moreover, preliminarily, we investigated the possibility to section ultra-thin resin embedded cells with FIB. For future experiments, it would be of great interest to perform sequential cross sectioning of resin-embedded individual cells aiming at a high resolution, artefact-free 3D reconstruction with contrast enhancement agents. In particular, additional staining with heavy metal ions can lead to an enhanced contrast and make different intracellular structures better observable.

Chemicals. Unless otherwise noted, all the chemical were purchased by Sigma Aldrich (Munich, Germany).

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