Optimization of 2D and 3D cell culture to study membrane organization with STED microscopy

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Abstract

Epithelial cells assemble into sheets that compartmentalize organs and generate tissue barriers. This is achieved by forming polarized membrane domains, which are connected by junctional complexes. While much is known about the organization of the basal membrane due to its easy accessibility by high and super-resolution microscopy, the apical and lateral membrane domains remain poorly characterized. Here we describe our methods to study the molecular organization of apical and lateral membrane domains by combining 2D and 3D epithelial cell culture with super-resolution STED microscopy. We show that inverted cell monolayers enable live cell imaging of the apical membrane with a resolution sufficient to resolve the densely packed micro-villi of human enterocytes. Furthermore, 3D cell culture enables us to resolve adhesion complexes in the lateral domain of kidney derived cells. We envision that these methods will help to reveal the supra-molecular structure of lateral and apical membrane domains in epithelial cells.

Keywords: membrane organization, STED, epithelial tissue, adhesion complexes, actin cortex, cell polarization

Supplementary material for this article is available online
(Some figures may appear in colour only in the online journal)

Introduction

Our organs and internal cavities are bordered by polarized epithelial tissues. The cells forming these tissues have evolved specialized membrane domains (apical, lateral, and basal) with unique compositions and structures to provide optimal biological function (figure 1(A)) [1, 2]. The basal membrane typically faces a collagen rich extracellular matrix (ECM) and anchors cells to the ECM via integrin rich focal adhesion complexes [3, 4]. Within the lateral membrane, cells form cell-cell adhesion complexes such as adherens junctions, desmosomes and tight junctions [5, 6]. The apical domain typically faces an internal fluid filled cavity and is highly folded to optimize secretion or resorption of molecules [7]. To learn how these membrane domains function, we need to understand how the underlying molecules self-organize into complex patterns such as adhesion complexes, micro-villi or a primary cilium.

Electron microscopy (EM) has been extensively used to study the ultrastructural organization of epithelial tissues [8–10]. In fact, most of our current text book knowledge about adhesion complexes and micro-villi on the apical membrane domain was generated by EM. However, with the development of super-resolution techniques our ability to localize up to three specific proteins simultaneously with a resolution down to the molecular scale in principle opens new opportunities to study the supra-molecular organization and dynamics of these membrane domains [11–14]. So far, the basal membrane and in particular the focal adhesion complexes have been most extensively...
R Maraspini et al. studied with super-resolution microscopy [12, 15–18]. A first landmark was the reconstruction of the nanoscale focal adhesion architecture by 3D-PALM [12]. Unfortunately, the other membrane domains (lateral and apical) are not easily accessible by super-resolution techniques. The lateral membrane is typically orientated along the low-resolution axis of the microscope. To access the apical domain, one must image through at least 10 µm of cell volume, which significantly reduces signal to noise ratio and resolution in super-resolution imaging.

Here, we present the optimization of 2D and 3D cell culture methods to enable super-resolution stimulated emission depletion (STED) imaging of the apical and lateral membrane domains of epithelial cells. Our 2D method allows for direct access to the apical membrane (brush border) of human enterocytes where we are able to resolve the organization of their micro-villi. In addition, our optimized 3D organotypic cell culture permits the study of adhesion complexes in the lateral membrane domain of MDCK cysts. Together, our methods will facilitate the characterization of epithelial membrane organization with super-resolution microscopy.

Results

**Inverted filter mounting to access the apical membrane with STED**

Epithelial cell lines self-organize into continuous 2D sheets when seeded on a flat substrate in *in vitro* culture (figure 1(B)). During this process, cells polarize and orient their apical membranes away from the substrate (i.e. the cover-glass). This orientation impedes access to the apical domain when using a typical inverted STED microscope. In order to circumvent this problem, and optimize optical access to the apical membrane, we implemented an inverse mounting of tissue. Inverse mounting of epithelial tissue was accomplished through the adoption of the Transwell filter culture method, resulting in the apical side of the cells being in closer proximity to the cover-glass. Transwell filters are polycarbonate membranes containing pore sizes in the micrometer range. When cells are seeded on filters the developing epithelial tissue separates two compartments (figure 1(B)); in this way cells have access to fluids and nutrients from both the apical and basal sides and therefore...
develop more organotypic phenotypes compared to cultures on glass or plastic [19]. This configuration is typically used to study electrical resistance or molecular transport through tissue [20, 21]. We adopted this method for our needs by orienting the apical domains of epithelial cell lines downwards towards the cover-glass. To this end we constructed a cell culture chamber which permits us to adjust the distance between the Transwell filter and the cover-glass to less than 5 \( \mu m \) (figures (B) and (C)). Figure 1(C′) shows a XZ slice of a live MDCK-II monolayer stained with SIR-actin and imaged with 3D-STED microscopy using 60× water immersion objective. When using highest NA objectives (oil immersion) to obtain maximum signal and resolution, resolution enhancement along the Z-axis is highly dependent on the sample distance to the cover glass, due to spherical aberrations caused by refractive index mismatching. Therefore, in aqueous samples the usable range for oil immersion objectives with 3D STED microscopy is limited to a distance around 5 \( \mu m \). Taken together, our inverted filter mounting provides optimal access to image the apical membrane of epithelial tissues using 3D STED microscopy with water and oil immersion lenses. The image shows that the distance of the apical membrane is close enough to achieve a clear resolution enhancement in the Z-direction also with oil immersion objectives.

### Apical micro-villi characterization by 2D and 3D STED-microscopy

First, we implemented our adjustable filter mounting technique to image the distribution of micro-villi on the apical membrane of a human intestinal enterocyte cell line (Caco2) and a canine kidney tubule cell line (MDCK-II) after chemical fixation. Both cell lines have already been characterized by EM [22, 23]. The apical membrane is folded into microvilli, which are protrusions of actin filament bundles that emerge from the cortical actin beneath the apical membrane (terminal mesh, figure 1(A′)). In the case of a fully differentiated intestinal epithelium, the microvilli are very densely packed to form the so call brush border, which is essential for nutrient absorption [24]. The dimension of a single villus ranges between 1–3 \( \mu m \) in length and are around 100 nm in diameter [8].

After culturing confluent Caco2 and MDCK-II monolayers for 14 d on Transwell filters, we fixed the tissue with PFA and stained f-actin with a probe suitable for STED-microscopy (phalloidin-StarRed). After mounting the Transwells in our adjustable chamber, we used conventional diffraction limited confocal microscopy and 2D-STED microscopy to characterize the apical membranes. We found that due to their tight packing, villi could not be resolved with diffusion limited confocal microscopy (figure 2(A)). With STED the central actin-bundle in each villus was clearly resolved. The representative STED image in figure 2(A) shows that the villi are packed in islands or groups with channel-like voids in between. In each island the villi form an ordered hexagonal array, as revealed by 2D auto-correlation analysis (figure 2(A′)) and the inter-villi distance histogram (figure 2(A′′)) with a mean spacing of 176 nm. To check if the chemical fixation had an influence on microvilli organization we compared images obtained from live cell imaging of SiR-actin stain Caco2 tissue to PFA fixed and phalloidin samples (figure 2(B)). While the phalloidin staining produced brighter staining, the average villi spacing was similar to live cell images, indicating that no significant changes to the cellular structure during fixation could be resolved. Comparison of our data against previous TEM data ([25], figure 1) revealed that villi spacing in the TEM images was \( \approx 30\% \) smaller than in our images. The reason for this discrepancy is not clear, but sample shrinking during EM preparation could be a reason.

Next we aimed to resolve the villi organization in 3D by combining two STED donuts (XY and Z) thereby generating a close to isotropic detection spot [26]. For this measurement we used fixed phalloidin stained 2-weeks old monolayers of MDCK-II cells. To minimize spherical aberrations and maximize resolution with a 100× oil objective, we used 2,2′-thiodiethanol (TDE) as mounting medium between the cells and the glass-slide [27]. In figure 2(C) we represent XYZ stacks of the apical membrane using a colormap for the Z-position where villi can be clearly traced in 3D, which is also shown in the XZ slice below. Interestingly, the 3D images revealed that villi in MDCK-II cells where often connected at the tip, forming tent like structures. See the supplementary movie (stacks.iop.org/JPhysD/53/014001/mmedia) for a 3D rendering of the apical membrane. This configuration has recently been shown to result from inter microvilli adhesion and it is implicated in self-organization of a densely packed brush border [25].

**Live cell STED microscopy reveals dynamics of microvilli**

Next, we attempted to use our mounting method to study the dynamics of microvilli with live cell STED microscopy. For this we stained MDCK-II monolayers with the cell permeable, far-red fluorescent actin probe SIR-actin [28]. First, we checked the cell morphology and f-actin staining with a large field of view wide-field microscope (figure 3(A)). After confirming that the staining was successful and the cell monolayer was intact, we applied 2D-STED microscopy. To reduce photobleaching and photo-toxicity we reduced the STED intensity to 50 mW, which provided a spatial resolution of 60 nm and allowed us to discriminate single microvilli (figure 3(A′)). Using this configuration, we recorded XZT stacks with a time interval of 15 s (figure 3(B)). The time-series revealed that microvilli move as clusters on the apical membrane. To quantify this movement, we used particle image velocimetry (PIV) analysis [29]. PIV is based on image cross-correlation analysis of sub-sequent frames to calculate local displacement vectors between each time window. To correct for the long-range movement of the whole tissue, we used rigid body registration of the time-series before the PIV analysis. The resulting vector displacement map is shown in figure 3(C). In line with the visual impression of the dynamics, the vectors show a local flow field of villi domains on the apical membrane. Interestingly, the flow velocity was highest in the center of the apical domain, which contains an actin depletion zone around the primary cilium (figure (C′)). Overall, the combination of live cell STED imaging and PIV analysis provided
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a detailed map of the dynamics of microvilli on the apical membrane domain and could be useful tool to study microvilli dynamics in response to flow of luminal fluids [30].

3D cell culture STED resolves cell-cell adhesion complexes in the lateral domain

Finally, we aimed to also access the lateral membrane domain with STED. Unfortunately, when grown as monolayers, the lateral membrane of polarized cells is typically oriented along the optical and hence low-resolution axis of the microscope (figure 1). In this configuration a resolution below 100 nm is very difficult to achieve even with 3D-STED. To circumvent this problem, we developed a protocol to grow epithelial cysts directly on the cover glass. Epithelial cysts develop from single cells seeded in collagen rich hydrogels after 3–4 d (figure 4(A)) [31]. Cysts are formed by a single layer of cells where all the cells polarize their apical membrane towards the internal fluid filled lumen and the basal membrane toward the surrounding matrix. The most important feature for our application is that around the equator of the cysts lateral membranes lie parallel to the imaging plan, resulting in the optimal orientation to achieve maximum resolution with 2D-STED imaging (figure 4(B)). The major remaining challenge to image horizontal lateral membranes was to bring them close enough to the cover glass. Typically, cysts are grown suspended into the solid 3D matrix of collagen, which positions them too far away for super-resolution imaging. We solved this problem

Figure 2. STED microscopy of f-actin on the apical membrane of fixed Caco2 and MDCK-II cells (A) confocal and STED image of a 2-weeks old Caco2 monolayer. Fixed with PFA and stained with phalloidin-StarRed. (A′) and (A″) STED resolved the hexagonal packing of microvilli into ordered arrays with a mean villus–villus distance of 176 nm. (B) Comparison of microvilli between live cell images and PFA fixed cells. Live cells where stained with SiR-actin fixed cells where stained with phalloidin-StarRed. (B′) Line profiles of regions indicated by green line show no significant difference in villi spacing. (C) 3D STED imaging with a close to isotropic PSF (≈100 nm) of phalloidin stained MDCK-II monolayers. Colormap indicates depth. Note that single villi (1 µm long and 100 nm thick) are perfectly resolved in all dimensions, which is also shown in the XZ section. See supplementary movie-1 for the complete 3D stack.
by coating the cover-glass with laminin prior to cell seeding. When MDCK-II and Caco2 cells are seeded on laminin they adhere but do not spread on the surface. Subsequent addition of 5% to 10% Matrigel to the culture medium promoted development of 3D cysts which were directly attached to the surface. To image the distribution of E-cadherin based adherens junctions and f-actin we fixed 4 d old adherent MDCK-II cysts and immuno-labelled for 2 color STED (Alexa594, StarRed).

To minimize spherical aberrations with the 100× oil objective we tried refractive index matching using TDE. Unfortunately, TDE caused severe deformation of the tissue, most likely due to osmotic gradients, even when applied in many dilution steps.

However, using a 90% PEG200 buffered aqueous solution improved penetration depth and had no effect on tissue morphology. With this method were able to resolve the distribution of single E-cadherin clusters in relation to f-actin in the lateral membrane. We found that E-cadherin clusters were rarely larger than 200 nm and were often elongated. F-actin did not clearly co-localize with the E-cadherin clusters but rather formed filaments surrounding E-cad. This observation is in line with recent 3D-STORM data, which have been acquired on inclined lateral membranes in epithelial monolayers [32]. We note that special care should be taken to ensure proper fixation of membrane proteins in super-resolution imaging, which requires addition of glutaraldehyde [33, 34]. Overall, our combination of 3D cell culture and STED opens the door to systematically study the structure of adhesion junctions and the cortex in the lateral membrane domain.

Figure 3. Live cell STED microscopy of f-actin on the apical membrane of MDCK-II cells (A) wide-field image of a live MDCK-II monolayer expressing mNeonGreen-ZO1 and stained with SIR-actin. (A’) Confocal and STED image of SIR-actin in this monolayer. With STED single villi can be resolved. (B) Time-series of villi resolved with live-cell STED. See supplementary movie-2 for the dynamics. (C) Particle image velocimetry analysis of the STED time-series. Due to the high spatial resolution of STED local movements of villi can be resolved. (C’) At the center of the apical domain velocities of villi movements are the largest. (C’’) The velocity histogram indicates confined movement of villi most likely due to the connection of the underlying terminal actin meshwork.
Conclusions

We have optimized two cell culture methods to study the apical and lateral membrane with super-resolution STED microscopy. First, we adopted Transwell filter mounting of epithelial monolayers to bring the apical membrane close to the microscope cover-glass. Combining this with 2D and 3D-STED allowed us to characterize the packing of microvilli on the apical membrane of Caco2 and MDCK-II cells. We also used this approach to show that live cell STED imaging can be used to infer the dynamics and flow of villi within the apical domain. Finally, we combined adherent 3D-cell culture with STED microscopy to access the organization of cell-cell adhesion complexes in the lateral membrane domain. Since the 3D positioning of cell-cell interfaces in this method allows to image large adhesion complexes at different orientations with respect to the optical axis, it may be possible to generate supra-molecular 3D models by particle averaging methods. This would provide access to the organization of the extremely dense packed junctional plaques of desmosomes, gap-, adherens- and tight junctions, which in conventional EM appear as dark clouds and are not yet accessible by cryo-EM. Taken together, our results show that the organization of all epithelial membrane domains can now be systematically studied with close to molecular resolution using STED microscopy.

Material and methods

2D transwell filter cell culture

Previous to seeding, MDCK-II (00062107, Public Health England) or Caco2 (ATCC CRL-2102, clone C2BBe1) cells were resuspended from a 2D monolayer by addition of 0.25%
Tryptsin EDTA (Gibco, ref. 25200-056) for 10 min at 37 °C 5% CO₂.

Seeding 2D monolayers on Transwell filters (Corning 3460), 400 µl of a 0.5 million cells per ml were transferred on the external side of a standing transwell filter. Cells were left to adhere at 37 °C in a sterile environment for 30 min. Afterwards, each transwell filter was mounted into a 1-well plate with 1 ml of culture media on the bottom side and 500 µl of media on the internal side. MDCK-II cells were cultured in (MEM (Gibco, ref. 41090-028), 5% v/v FBS (South America Gibco, ref. 10270106), 1% v/v NeAA (Gibco, ref. 11140-050), 1% v/v Sodium pyruvate (Gibco, ref. 11360-039), 1% v/v Glutamax (Gibco, ref. 35050-038), 1% v/v Penstrep (Gibco, ref. 25200-056)); Caco2 were cultured in (MEM (Gibco, ref. 41090-028), 5% v/v FBS (South America Gibco, ref. 10270106), 1% v/v NeAA (Gibco, ref. 11140-050), 1% v/v Sodium pyruvate (Gibco, ref. 11360-039), 1% v/v Glutamax (Gibco, ref. 35050-038), 1% v/v Penstrep (Gibco, ref. 25200-056)).

**Adherent 3D cell culture**

Cells were resuspended from a confluent monolayer into a single cell suspension as explained above. In order to produce adherent 3D cysts, the surface of glass coverslips (thickness 0.17 mm) was coated with a solution of laminin 0.5 mg ml⁻¹ for 1 h at 37 °C 5% CO₂. Afterwards, a suspension of 16.000 single MDCK-II or Caco2 cells per cm² was seeded on the coated surface in the respective culture media complemented with 5% Matrigel (Corning MG matrix, ref. 356231). Cells were cultured for 4 to 5 d in 37 °C 5% CO₂ until cysts reached 30 to 40 µm in diameter.

**Cell fixation**

Fixation was performed in the same way for both monolayers on transwell filters and for adherent 3D cysts, 4% w/v PFA pH 7.25 in PBS for 10 min followed by a quenching step using a solution of 300 mM glycine, 0.3% v/v Triton X 100 in PBS for 15 min and unspecific binding sites were blocked with 2% w/v BSA, 0.1% v/v Triton X 100 for at least 45 min. F-actin was stained with phalloidin labelled with Abberior StarRed (STRED-0100-20UG) diluted in blocking buffer; cells were incubated for 1 h at room temperature and protected from light.

For live cell imaging of f-actin, monolayers were stained with SiR-actin (Spirochrome in the SiR-actin kit) and 2 µM SiR-actin probe. Before imaging, media was exchanged to culture media supplemented with 10 µM Verapamil (provided by Spirochrome in the SiR-actin kit) and 2 µM SiR-actin probe. Before imaging, media was exchanged to culture media supplemented with 10 µM Verapamil.

**Confocal and STED imaging**

Confocal and STED imaging, on fixed and live samples, was performed using an Abberior 3D-2 Color-STED system (Abberior Instruments, Goettingen) with a 100 x /1.4 NA oil or a 60 x /1.2 NA water objectives (Olympus). Star580 was imaged with a pulsed laser at 560 nm, and excitation of Abberior Star Red and SiR-actin probe was performed at 640 nm [35]. The depletion laser for both colors was a 775 nm, 40 MHz pulsed laser (Katana HP, 3W, 1 ns pulse duration, NKI Photonics). The optimal combination of excitation intensity, STED power and pixel dwell time where established by minimizing the onset of strong photo bleaching.

**Image processing and PIV analysis**

To reduce high frequency noise, STED images were mildly deconvolved using the Richardson-Lucy algorithm [29]. The PSF was approximated by a 2D/3D Gaussian with a FWHM that was determined from independent bead measurements at the respective STED power. To avoid deconvolution artefacts, we used a low number of iterations (<8). Using these conditions, we see a significant improvement in the signal-to-noise ratio, but little to no change in resolution.

Particle image velocity analysis was performed with the MATLAB toolbox PIVlab [36]. Interrogation area size was set to steps of 64, 32 and 16 pixels and the resulting vector field was smoothed.

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