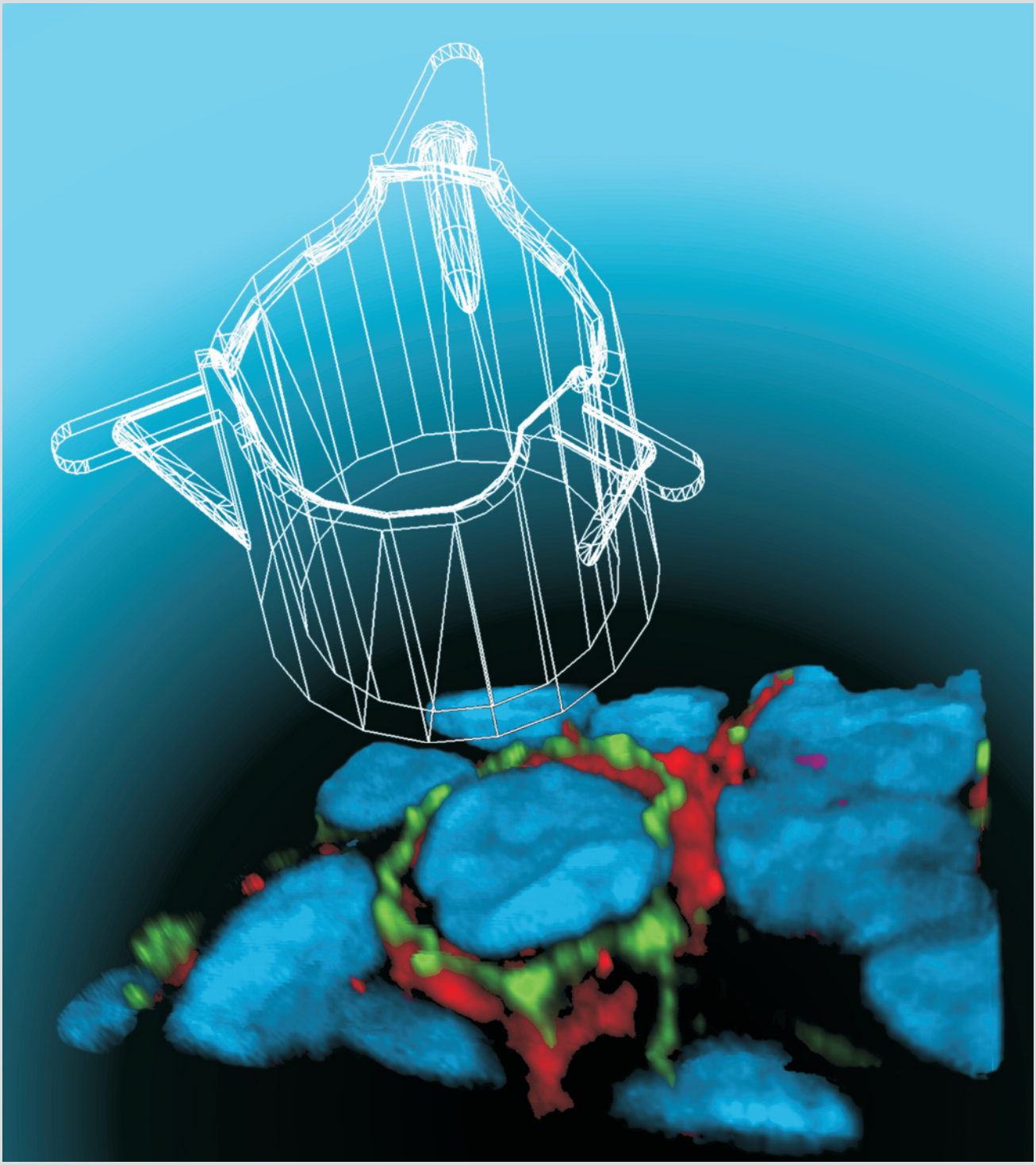
Application Note

The reconstruction and immunocytochemical characterisation of polarised epithelia in ThinCert™ cell culture inserts



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Introduction

The establishment of biologically relevant *in vitro* approaches is becoming increasingly important as objections to animal experimentation continue to rise. One of the challenges facing the composition of such models is to reconstruct epithelia or tissues in such a way that would preserve their native biological features and functions; to include the occurrence of polarisation, multi-layered growth, barrier function and vectorial transport.

Cell culture inserts with porous membrane supports are widely accepted tools for the reconstruction of functional epithelia and tissues *in vitro*. In cell culture inserts, high density cell populations may be achieved with nourishment from two different areas: the surface that faces the porous membrane support as well as the one that faces away. This dual nutrient access, along with the presence of a solid substrate that may carry components of the ECM[1], has proven to be an indispensable stimulus to establish polarity in cultivated epithelia (Chambard et al., 1983; Guguen-Guillouzo and Guillouzo, 1986; Saunders et al., 1993).

In the experiments presented, epithelia were cultivated on ThinCert™ cell culture inserts using Madin-Darby canine kidney cells (MDCKII) and colorectal adenocarcinoma cells (CACO-2). Applying fluorescence immunocytochemistry the cell adhesion protein E-Cadherin and the tight junction proteins Claudin-1 and ZO-1 were localised to the basolateral compartment and the apical rim of the cell membrane, respectively. The localisation of these proteins to sides where they would also occur under *in vivo* conditions (see **Fig. 1**) clearly demonstrates the suitability of ThinCert™ cell culture inserts for the cultivation of epithelia and the achievement of cellular polarity *in vitro*. Within this application note, detailed protocols for the cultivation of epithelial cells and their molecular characterisation by fluorescence immunocytochemistry are provided using ThinCert™ cell culture inserts.

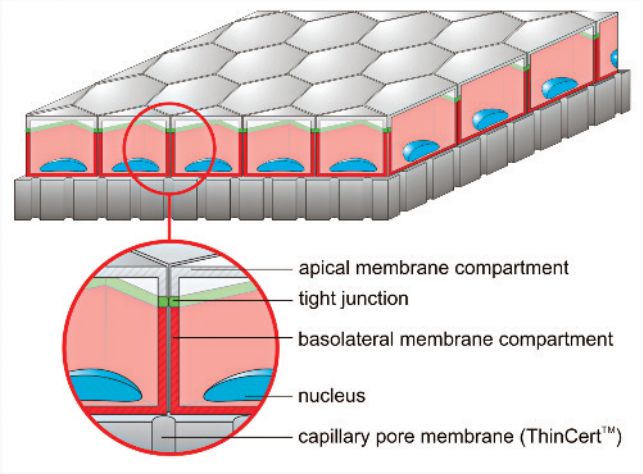

Figure 1
When cultivated on porous membrane supports (ThinCert™) epithelial cells form a dense cell layer. The formation of tight junctions at the apical rim of the lateral cell membrane and the establishment of basolateral and apical membrane compartments prove this cell layer to be an epithelium with its characteristic features.

Material and Methods

Coating of cell culture inserts

For all the experiments described here, 24 well ThinCert™ cell culture inserts with 0.4 µm pores and transparent or translucent PET membranes were used. The inserts were placed in the wells of a CELLSTAR® 24 well cell culture plate. 60 µl of an aqueous fibronectin solution (50 µg/ml) was applied to each cell culture insert. After 2 h incubation at room temperature, the fibronectin solution was removed and the cell culture inserts were rinsed three times with PBS.

Material

Item	Manufacturer	Cat.-No.
Alexa Fluor 488 goat anti rabbit IgG antibody	Invitrogen GmbH	A11008
Alexa Fluor 546 goat anti mouse IgG antibody	Invitrogen GmbH	A11003
Rabbit Anti Claudin-1 antibody	Zytomed Systems GmbH	RP153
Mouse Anti E-Cadherin antibody	Becton Dickinson GmbH	610181
Rabbit Anti ZO1 antibody	Invitrogen GmbH	40-2300, PAD: ZMD.437
CELLSTAR® 24 well cell culture plate	Greiner Bio-One GmbH	662 160
DakoCytomation Fluorescent Mounting Medium	Dako Deutschland GmbH	S3023
DAPI, dilactate	Sigma-Aldrich Chemie GmbH	D9564
DMEM medium	Biochrom AG	F0435
Fetal calf serum	Invitrogen Life Technologies	10270-106
Fibronectin	TeBu-Bio GmbH	2004
Formalin	Sigma-Aldrich Chemie GmbH	HT5014
L-alanyl-L-glutamine	Biochrom AG	K0302
MEM-amino acids, 50x	Biochrom AG	K0363
PBS	Biochrom AG	L1825
RPMI medium	Biochrom AG	F1295
ThinCert™ 24 well cell culture insert with 0.4 µm pores and transparent membrane	Greiner Bio-One GmbH	662 641
ThinCert™ 24 well cell culture insert with 0.4 µm pores and translucent membrane	Greiner Bio-One GmbH	662 640
Triton® X100	Sigma-Aldrich Chemie GmbH	T8787
Water (tissue culture grade)	Sigma-Aldrich Chemie GmbH	W3500

[1] ECM: extracellular matrix

Cell culture

Cell cultures were prepared and maintained according to standard cell culture procedures. For the propagation of MDCKII cells DMEM medium with 10% fetal calf serum (FCS) and 4 mM L-alanyl-L-glutamine was used. CACO-2 cells were cultivated in DMEM medium supplemented with 20% FCS, 4 mM L-alanyl-L-glutamine and 1x MEM-amino acids.

For the establishment of an epithelial cell layer $5x10^3$, $25x10^3$, $5x10^4$ or $4x10^5$ cells (MDCKII or CACO-2) were sown into each cell culture insert and cultivated for 24, 48 and 72 h in a cell culture incubator (37°C, 5% CO_2).

Immunocytochemistry[2]

Unless otherwise specified, all steps were carried out at room temperature. Prior to fixation, the cell culture medium was removed from both cell culture inserts and cell culture plate wells. 500 µl 4% formalin solution was added to each insert[3]. The fixation was carried out for 5 min on ice, followed by two washes with 500 µl PBS per insert. Cells were permeabilised for 25 min using 500 µl 0.5% Triton/PBS per insert, followed by two washes with 500 µl PBS per insert. For the blocking of non-specific protein binding sites, 500 µl 10% FCS/PBS was applied to each insert and incubated for 1.5 h. Thereafter, the inserts were washed three times 5 min with 500 µl PBS. After removing the washing solution, 100 µl primary antibody solution in 1% FCS/PBS was applied to each insert, and an incubation of 1 h was performed[4].

For a detailed description of the applied antibodies (origin, specificity and working concentrations), see **Tab. 1**. After incubation with the primary antibodies, the inserts were washed three times for 5 min with 500 µl PBS. 100 µl of the secondary antibody solution in 1% FCS/PBS was applied to each insert and incubated for 1 h at room temperature. For details on the secondary antibodies used in this protocol see **Tab. 1**. Alexa 488 coupled secondary antibodies were used to detect the ZO1 and Claudin-1 specific primary antibodies. Alexa 546 was used to detect the anti E-Cadherin primary antibody. After incubation with the secondary antibodies, two additional washing steps with 500 µl PBS per insert, 5 min incubations, were performed. For the subsequent nuclear staining, each insert was incubated for 5 min with 100 µl DAPI solution (10 µg/ml). Thereafter, the inserts were washed two times for 5 min with 500 µl PBS. The insert membranes were detached from the insert housings using a scalpel (see **Fig. 3**) and mounted onto microscopy slides using fluorescence mounting medium.

	Designation	Origin	Coupled fluoro-phore	Detected protein	Working concentration [dilution]
Primary antibodies	anti E-Cadhe-rin antibody	mouse	-	human E-Cadherin	2.5 µg/ml [1:100]
	anti ZO1 antibody	rabbit	-	human Zona occludens 1 protein	2.5 µg/ml [1:100]
	anti Claudin-1 antibody	rabbit	-	human Claudin-1 protein	~10 µg/ml [1:100]
Secondary antibodies	Alexa 546 anti mouse IgG antibody	goat	Alexa 546	mouse IgG	8 µg/ml [1:250]
	Alexa 488 anti rabbit IgG antibody	goat	Alexa 488	rabbit IgG	8 µg/ml [1:250]

Table 1: Key features of the applied antibodies

1. Remove cell culture medium from insert
2. Fix cells for 5 min on ice with 4% formalin, use 500 µl per insert
3. Wash insert twice with PBS, use 500 µl per insert
4. Permeabilise cells for 25 min with 0.5% Triton in PBS, use 500 µl per insert
5. Wash insert twice with PBS, use 500 µl per insert
6. Block unspecific binding sites for 1.5 h with 10% FCS, use 500 µl per insert
7. Wash inserts three times for 5 min with PBS, use 500 µl per insert
8. Incubate cells for 1 h with primary antibody in 1% FCS, use 100 µl per insert
9. Wash inserts three times for 5 min with PBS, use 500 µl per insert
10. Incubate cells for 1 h with secondary antibody in 1% FCS, use 100 µl per insert
11. Wash inserts two times for 5 min with PBS, use 500 µl per insert
12. Incubate cells 5 min with DAPI in PBS (10 µg/ml), use 100 µl per insert
13. Wash inserts two times for 5 min with PBS, use 500 µl per insert
14. Detach membrane from the insert housing using a scalpel
15. Mount membrane onto a microscopy slide, use fluorescence embedding medium

Figure 2
Flow chart. This guide is for quick reference only. Familiarise yourself with details before performing the assay. All volumes are adjusted to fit 24 well inserts and have to be modified if other inserts are used.

[2] For a flow chart of the immunocytochemistry protocol see **Fig. 2**
[3] At this stage, solution was not added into the lower compartment (well of the plate). For fixative to remain in the upper compartment (insert), it is necessary to keep the lower compartment dry to ensure a liquid bridge does not form between the membrane underside and the plate well bottom. This also applies to all subsequent steps where solution is transferred solely into the upper compartment.
[4] Alternatively, some primary antibody incubations were performed overnight at 4°C.

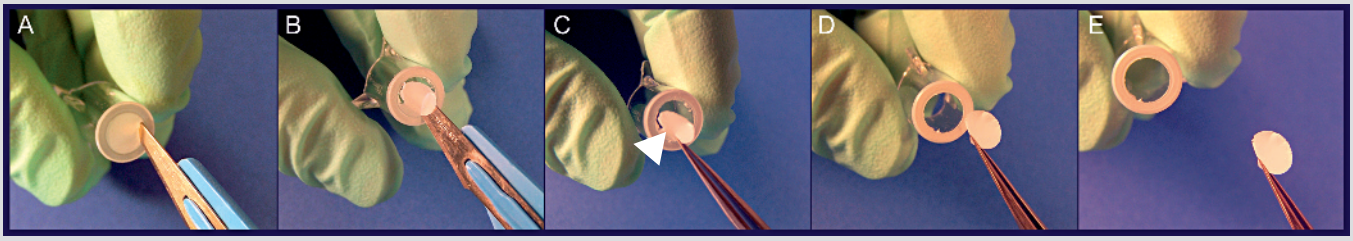

Figure 3
For the detachment of the capillary pore membrane from the insert housing a scalpel is plunged into the membrane adjacent to the insert housing (A). The membrane is cut out moving the scalpel alongside the inner edge of the housing (B), thereby leaving a small segment attached to the membrane (arrowhead in C). Finally, using tweezers, the membrane is torn off the housing (C-E). The membrane is now ready for further processing such as mounting onto a microscopy slide, sectioning or other applications.

Microscopy

Images were captured with a Zeiss Axioplan 2 wide field fluorescence microscope. Z-scans were acquired with a Zeiss Cell Observer with Apotome applying two times averaging. Pictures were processed with the AxoVision 4.5 software (Zeiss) and mounted using Photoshop 6.0 (Adobe). Details of the applied filter sets are given in **Tab. 2**.

Results

Optimum cell culture conditions for the formation of tight junctions

In order to determine the optimum cell culture conditions for the formation of epithelium-like cell layers, MDCKII cells were cultivated on ThinCert™ cell culture inserts at various seeding densities for different time periods. Cell cultures were fixed and stained for E-Cadherin and the tight junction protein Claudin-1 according to the above-mentioned protocol.

Seeding $5x10^3$ cells per insert and keeping them 24 h in culture did not allow the cells to reach confluence and Claudin-1 expression above background levels (**Fig. 4**, left column, lower panel). Although confluence was reached after 48 h Claudin-1 expression was still low at this time (**Fig. 4**, left column, center panel). After 72 h the cells grew to a maximum density and began to express high levels of Claudin-1 (**Fig. 4**, left column, upper panel).

With higher initial seeding densities ($25x10^3$ and $5x10^4$ cells per insert) confluence and expression of Claudin-1 were achieved after only 24 h in cell culture (**Fig. 4**, center and right column). Under all conditions, the localisation of E-Cadherin to the cell membrane correlated with the appearance of Claudin-1 (**Fig. 4**, all columns). It is noteworthy, that with an increasing cell density also the ratio between the nuclear and cytoplasmic volumes increased (**Fig. 4**, all columns). This phenomenon correlates with cells and nuclei forming a small base area and achieving a tall appearance (not shown), thus indicating the cell layer obtained a high prismatic morphology.

In summary it may be recommended to seed cells at a high density ($5x10^4$ cells per insert and above) to achieve a rapid formation of tight junctions *in vitro*.

Fluorophores	Excitation wavelength	Emission wavelength	Wavelength transmitted through beamsplitter
DAPI (4',6-diamidino-2-phenylindole)	335-383 nm	420-470 nm	> 395 nm
Alexa Fluor 488	455-495 nm	505-555 nm	> 500 nm
Alexa Fluor 546	533-558 nm	570-640 nm	> 570 nm

Table 2: Features of the used fluorescence filters sets

Establishment of polarity in cultivated epithelial cells

In another series of experiments MDCKII or Caco-2 cells were seeded at a high initial density ($4x10^5$ cells per insert), cultivated for 48 h and stained simultaneously for the tight junction proteins Claudin-1 or ZO1 and the basolateral cell adhesion protein E-Cadherin. Nuclei were counterstained with DAPI. Image series along the z-axis[5] of the stained cell layers were acquired using a Zeiss Cell Observer with Apotome. Individual images were aligned to a z-stack, and virtual x-z- and y-z-cross sections along lines of interest (see **Figs. 5 and 6**) were generated using the AxoVision 4.5 software (Zeiss).

[5] Here, the z-axis corresponds to the apical-basal-axis of the cultivated epithelium.

The cultivation and analysis of MDCKII and CACO-2 cells according to this protocol revealed the formation of tight junctional complexes in the upper (apical) part of the lateral cell membrane (**Figs. 5 and 6**). These tight junctions seal the cell membrane, thus generating apical and basolateral membrane compartments. Whereas E-Cadherin was detectable in the entire basolateral membrane compartment, the apical membrane compartment was devoid of E-Cadherin in all cells (**Figs. 5 and 6**). In contrast to MDCKII cells CACO-2 cells formed a less regular network. Projections of the staining patterns of Claudin-1, E-Cadherin and DAPI along the z-axis did not align as well as those observed with MDCKII cells. This phenomenon is mainly due to the rather irregular than prismatic pattern of the CACO-2 cell layer. However, the analysis of the Claudin-1 and E-Cadherin distribution along the z-axis clearly indicated the formation of tight junctions in the apical part of the lateral cell membrane and the separation of the membrane into apical and basolateral compartments within these cells (**Fig. 6B**).

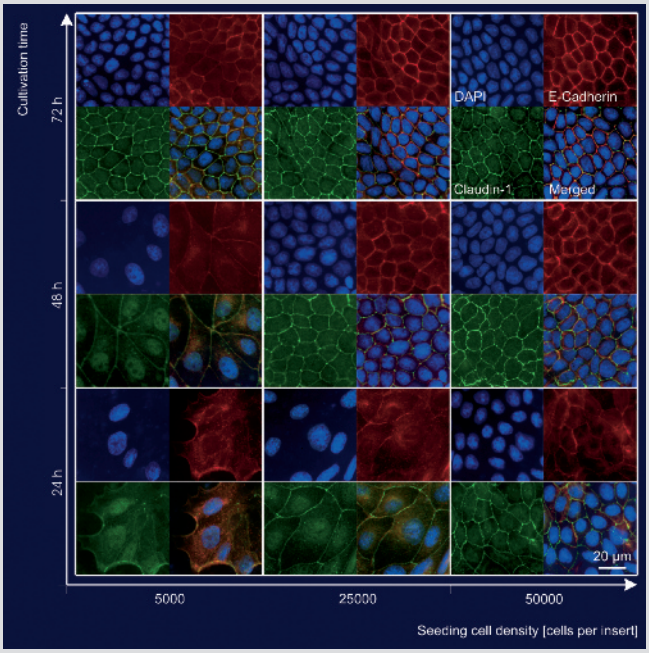

Figure 4
Influence of the initial seeding density and the cultivation time on the expression of Claudin-1 and E-Cadherin. With a low initial seeding density ($5x10^3$ cells per insert) up to 72 h of cell culture are required before Claudin-1 expression and the localisation of E-Cadherin to the cell membrane become detectable (left column). With higher seeding densities ($25x10^3$ and $5x10^4$ cells per insert) Claudin-1 expression and the localisation of E-Cadherin to the cell membrane become detectable after only 24 h in cell culture (center and right column).

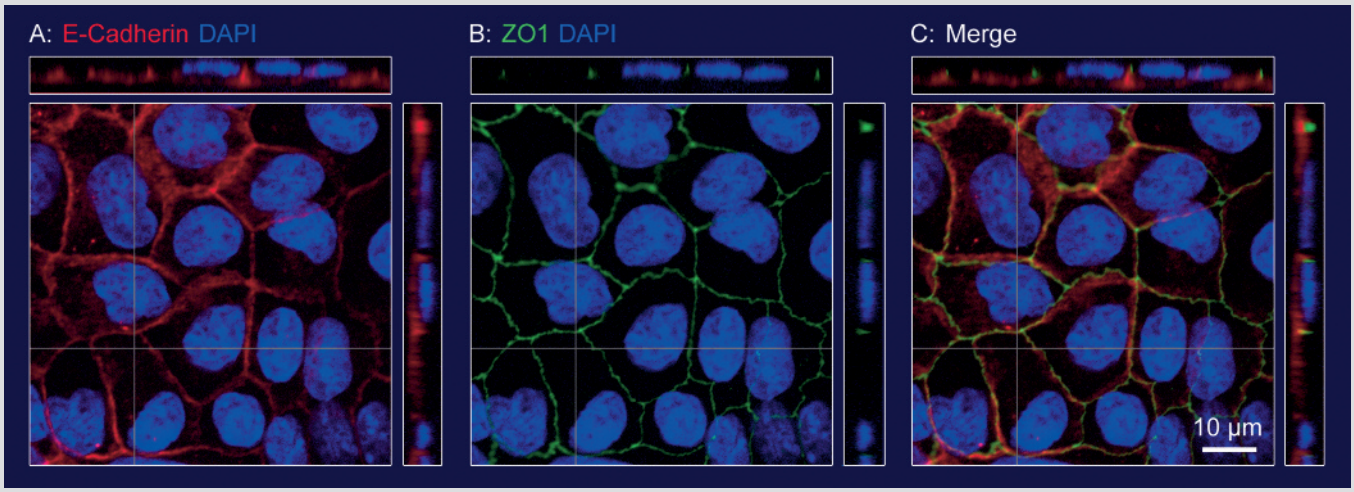

Figure 5
Microphotographs of MDCKII cells that were processed for fluorescence immunocytochemistry against E-Cadherin (red channel) and ZO1 (green channel). Nuclei were stained with DAPI (blue channel). Serial photographs were acquired along the z-axis of the cell layer (from apical to basal) and aligned to form a stack of pictures (z-stack). The large quadratic images in A-C represent one individual photograph that has been chosen from an apical level of the z-stack. Using the Axio Vision 4.5 software, the z-stack was sectioned along two planes, each of them being indicated by a gray line. Sectioning along the horizontal line produced the x-z-cross section depicted on top of each panel, sectioning along the vertical line produced the y-z-cross section shown at the right margin of each panel.

In the x-z-cross section as well as in the y-z-cross section the ZO1 positive belt of tight junctions appears in an apical domain of the lateral cell membrane (B, C). This domain clearly marks the site at which a basolateral compartment with E-Cadherin expression is separated from an apical compartment without E-Cadherin expression (A, C).

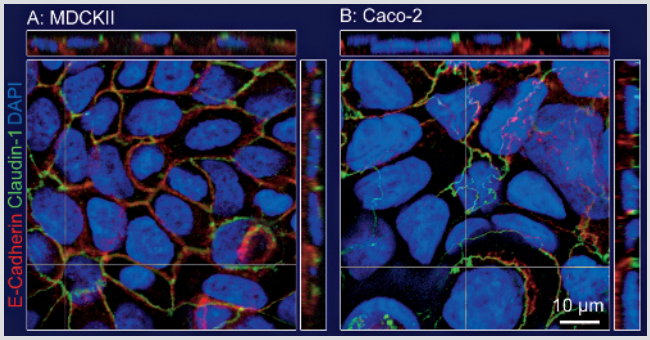

Figure 6
MDCKII (A) and CACO-2 cells (B) were processed for fluorescence immunocytochemistry to detect E-Cadherin (red channel) and Claudin-1 (green channel). Nuclei were stained with DAPI (blue channel). For a detailed explanation of the image acquisition and processing procedures see legend to Fig. 5.
In both, MDCKII (A) and CACO-2 cells (B) Claudin-1 immunoreactivity is localised to an apical domain of the lateral cell membrane. E-Cadherin immunoreactivity is only found in the basolateral membrane compartment below the Claudin-1 positive belt of tight junctions.

Conclusion and Discussion

In these experiments, evidence is provided that MDCKII and CACO-2 cells establish a polarised epithelium when cultivated on ThinCert™ cell culture inserts. Thus, the formation of ZO1 and Claudin-1 positive tight junctions was observed in an apical domain of the lateral cell membrane, whereas E-Cadherin expression was detected in the basolateral membrane compartment below.

In general ThinCert™ cell culture inserts were found to be excellent tools for fluorescence immunocytochemical stainings. The overall low autofluorescence of the ThinCert™ membrane assures low background signals[6]. In some cases enhanced light scattering may be observed with translucent membranes. If so, special care should be taken to work with optimised immunocytochemistry protocols, to yield highly specific fluorescence signals. No differences in the performance of translucent and transparent membranes were observed during the course of these experiments.

In this study, specific protein localisation was exploited to demonstrate epithelial polarisation on ThinCert™ cell culture inserts. Similarly, epithelial polarisation was depicted in Kopplow et al., 2005 by showing the specific localisation of transporter proteins to basolateral or apical membrane compartments. In addition, functional aspects of epithelial polarisation on ThinCert™ cell culture inserts have been described, such as the vectorial transport (Kopplow et al., 2005; Letschert et al., 2005) and the polarised endocytosis (Mettlen et al., 2006). In summary, ThinCert™ cell culture inserts prove to be excellent tools for the reconstruction of epithelia and the restoration of their biological functions *in vitro*.

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[6] For this also compare **Fig. 5** where x-z- and y-z-cross sections include parts of the ThinCert™ membrane.

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