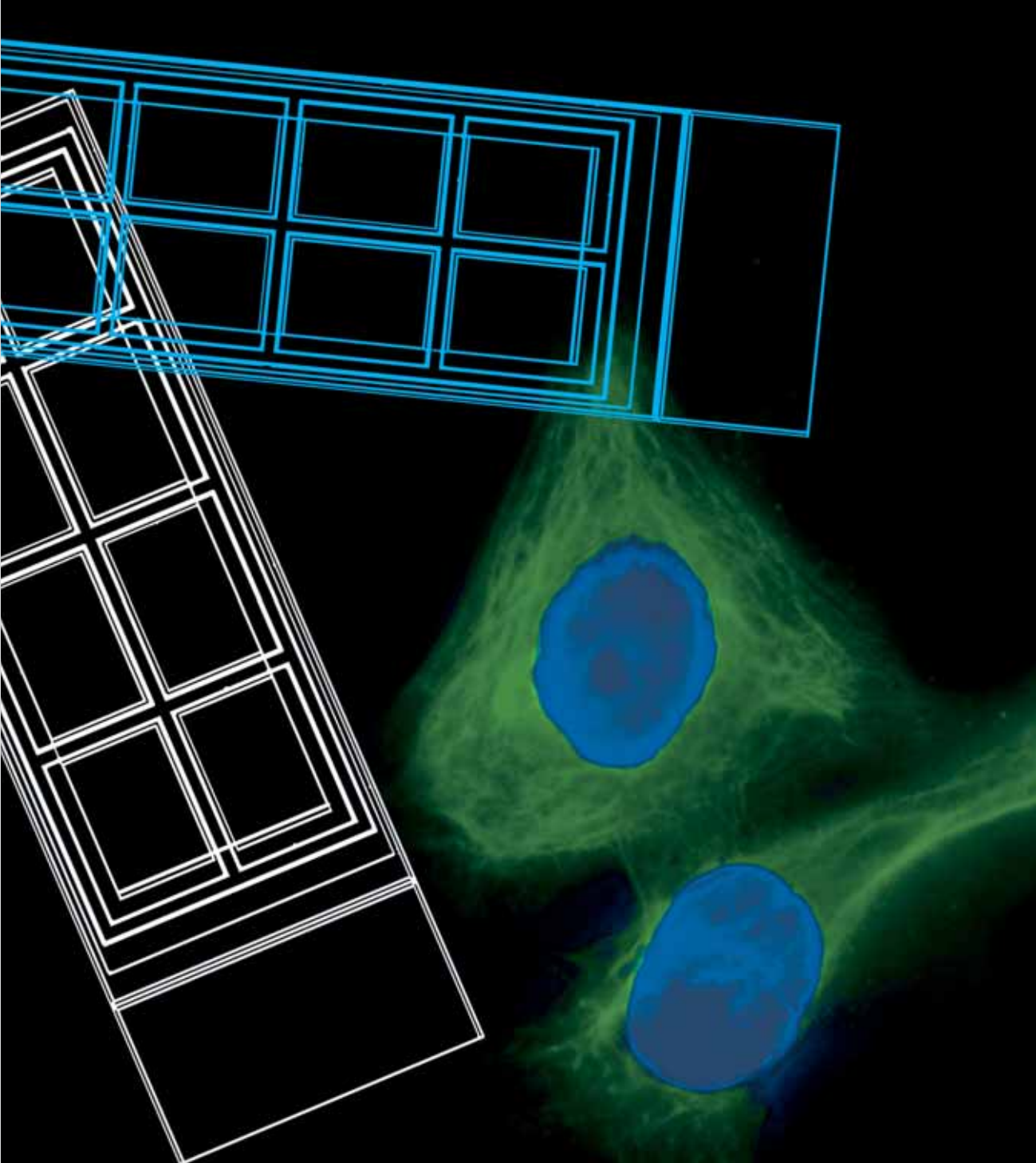


Application Note

Immunofluorescence staining on MultiwellSlides & SlideFlask



Introduction

Greiner Bio-One MultiwellSlides

The MultiwellSlide family is a new product line from Greiner Bio-One comprising slides which are subdivided into compartments. Offered in several different formats, the MultiwellSlides are ideally suited for cell culture testing, immunofluorescence and histological staining of cells as well as microscopic imaging. The slides are additionally featured in a broad range of materials to meet the high demands of current cell culture and microscopic applications. The MultiwellSlides consist of a 1-well, 2-well, 4-well and 8-well format (**Fig. 1**) as well as a unique flask format – the SlideFlask – to complete the slide portfolio. All formats are available in a variety of materials including lumox™, polystyrene, polyolefin, glass, coverglass I and coverglass II slides.



Figure 1: MultiwellSlides from Greiner Bio-One
From left to right: 8-wellSlide^{coverglass I}, 8-wellSlide^{coverglass II}, 8-wellSlide^{polystyrene}, 8-wellSlide^{lumox™}, 8-wellSlide^{glass}, 8-wellSlide^{polyolefin}

The polystyrene housing which is fixed on the slides forms different culture chambers dividing the slide in multiple growth surfaces (**Fig. 2A + Fig. 2B**). This allows a simultaneous cultivation of various cells on one slide as well as the parallel testing of the same cell line for different conditions on the same slide. The housing can be removed of lumox™ and polyolefin slides allowing the conservation of stained cells by embedding with mounting media.

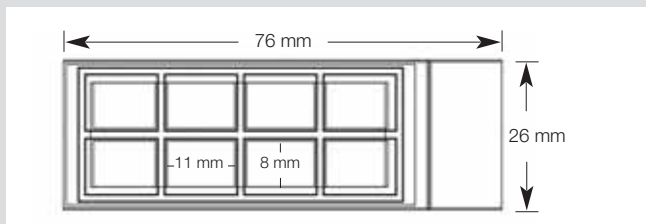


Figure 2A: Overview image of 8-wellSlide lumox™ and glass

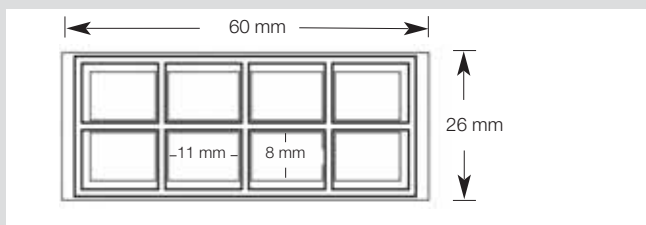


Figure 2B: Overview image of 8-wellSlide polystyrene, coverglass I and II

Cultivating cells directly on the slides eliminates need for labour intensive transfer of cells from larger format tissue culture flasks to slides for testing and microscopic imaging and expedites endpoint analysis.

In addition, the small growth area per well – especially in the 8-well option of the MultiwellSlides – reduces consumption of cost intensive reagents; therefore expenses for staining protocols can be significantly reduced while maintaining a high quality result.

Microscope imaging is rapid and easy to perform because cells within different chambers on the slide remain at the same focus level. Laborious exchanging of slides and the resulting necessary readjustment of focus level after each sample can be avoided with the new slide system.

A writing area is present on the MultiwellSlides with lumox™, glass and polyolefin slide to enable sample identification.

MultiwellSlides^{lumox™} offer a surface with reduced autofluorescence, advantageous for fluorescence application and imaging. A removable polystyrene housing enables cells to be easily embedded in aqueous- and xylene-based mounting media for staining conservation. The slides are resistant against numerous organic solvents.

MultiwellSlides^{polyolefin} offer a clear optical plastic surface with excellent properties for fluorescence applications. The upper polystyrene housing is removable and cells can be embedded in mounting media.

MultiwellSlides^{glass} offer a clear optical surface for cell culture and imaging applications. The upper housing is non-removable; imaging can be done easily right after staining.

MultiwellSlides^{coverglass} enable a high magnification imaging of immunofluorescence and histological staining and are additionally suitable for confocal microscopy. Two versions are available with different coverglass thickness (130 µm for coverglass I and 170 µm for coverglass II).

MultiwellSlides^{polystyrene} complete the slide family with standard plastic surfaces providing a useful tool for cell culture and direct imaging after staining.

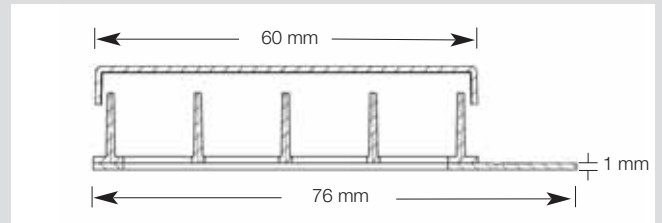


Figure 3A: Cross section image of 8-wellSlide lumox™ and glass

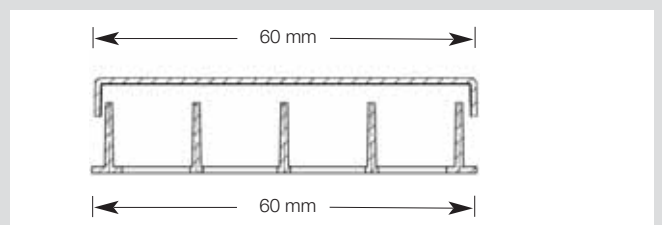


Figure 3B: Cross section image of 8-wellSlide polystyrene, coverglass I and II

The products fulfil all requirements for adherent cell culture with treated surfaces to improve attachment of adherent cell types. All products are sterile, free of DNase/RNase, pyrogens and are non-cytotoxic. A polystyrene lid maintains the sterile conditions inside the culture chamber during cultivations within an incubator.

As a valuable tool for immunofluorescence and imaging applications, the 8-wellSlides^{coverglass} and SlideFlask^{lumox™} were used in this report to perform immunocytochemical staining of cytokeratins with two different epithelial cell lines as well as collagen I staining on dedifferentiated primary porcine chondrocytes. The following paragraphs provide a short scientific background about these characteristic markers.

The biology of cytokeratins

Cytokeratins are typical markers for epithelial cell differentiation [1] and have been widely used in tumor diagnosis [2]. They belong to a family of intermediate filament proteins which are cytoplasmic cytoskeletal structures found in most vertebrate cells [3]. The at least 29 known cytokeratins are expressed in epithelial tissue in certain combination of polypeptides of the acidic (type I cytokeratin) and basic (type II cytokeratin) subfamilies [4]. Cytokeratins are known to participate in the maintenance of the structural integrity of the cell [5].

In this application report examples of two epithelial cell lines (human epithelial cells and MDCK cells) have been used to perform cytokeratin staining to illustrate the possibilities of the MultiwellSlides for immunocytochemical cell characterisation.

Characteristic markers for chondrogenic dedifferentiation

Cultivated chondrocytes derived from cartilage are known to lose their chondrogenic phenotype during long term culture *in vitro* [6]. While monolayer culture is still a basic experimental approach used in cellular and molecular biological studies of chondrocytes, it is often disregarded that cells do not maintain their characteristic phenotype and lose their ability to produce proteoglycans and change collagen synthesis from type II to type I [7]. Collagen II is an extracellular matrix protein which endows the cartilage with its tensile strength while collagen I is a protein mostly present in skin, tendon and bone. The decrease of collagen II and increase in collagen I synthesis are clear indications of chondrogenic dedifferentiation observed with primary chondrocytes in monolayer culture.

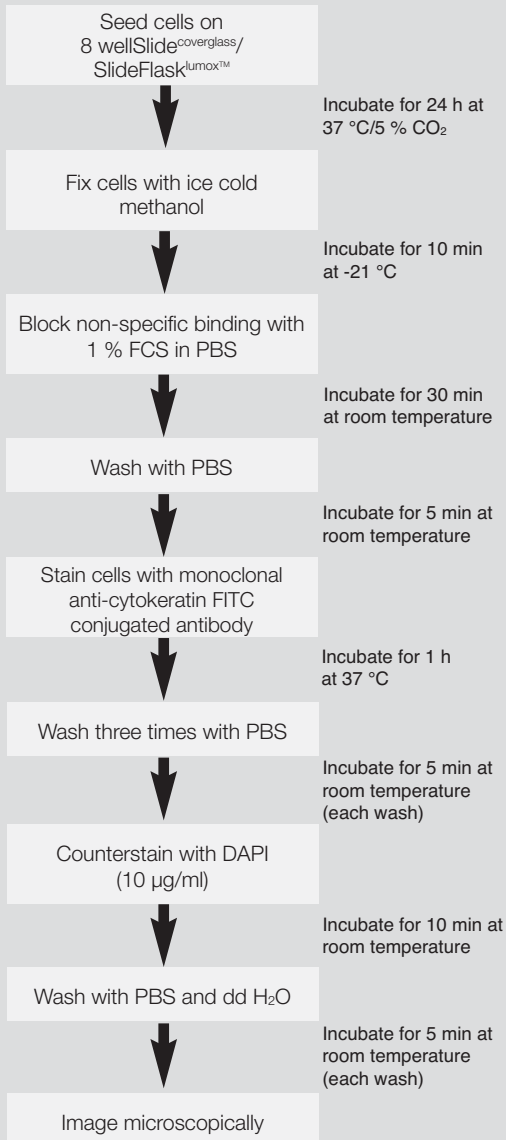
Collagen I was detected in the following experiment with chondrocytes cultivated for three weeks in monolayer culture using an immunocytochemical protocol.

Material and Methods

Item	Manufacturer	Cat.-No.
Monoclonal anti-Pan cytokeratin FITC conjugated antibody	Sigma Aldrich Chemie GmbH	F3418
Collagen I antibody	Acris Antibodies GmbH	AF 5610-1
Cy3-conjugated goat anti mouse IgG	Jackson ImmunoResearch Europe Ltd.	115-165-003
Antibody background reducing reagent	Dako Deutschland GmbH	S3022
DAPI, dilactate	Sigma Aldrich Chemie GmbH	D9564
DMEM medium	Biochrom AG	F0435
DMEM/Hams's F12	PAA Laboratories GmbH	E15-813
Fetal calf serum	Invitrogen GmbH	10270-106
Penicillin Streptomycin	Biochrom AG	A2213
PBS	Biochrom AG	L1825
8-wellSlide ^{coverglass I}	Greiner Bio-One GmbH	9618 0802
8-wellSlide ^{coverglass II}	Greiner Bio-One GmbH	9619 0802
SlideFlask ^{lumox™}	Greiner Bio-One GmbH	9615 0001

Cytokeratin staining

Cells have been seeded on 8-well Slides^{coverglass}/SlideFlask^{lumox}TM. After 24 h of incubation at 37 °C and 5 % CO₂, cells were fixed on slides with ice cold methanol for 10 min at -21 °C. Non-specific binding was blocked with 1 % FCS in PBS for 30 min at room temperature. After one washing step with PBS, cells were incubated with monoclonal anti-Pan Cytokeratin FITC conjugated antibody in background reducing agent at the dilution recommended by the manufacturer for 1 h at 37 °C. Non-bound antibody was washed away by three washing steps with PBS (5 min, room temperature). The nucleus of the cells were counterstained with DAPI (10 µg/ml in PBS) for 10 min at room temperature. Slides were washed once with PBS and rinsed with water afterwards.



Collagen I staining

Porcine chondrocytes were isolated from porcine knee cartilage applying standard collagenase digest procedures. Cells were seeded on 8-well Slides^{coverglass} using DMEM/Ham's F12 medium. Fixation of the cells for collagen I staining was performed in accordance to the cytokeratin staining protocol. Collagen I specific monoclonal mouse antibody was incubated (diluted in background reducing agent) for 24 h at 4 °C. Non-bound antibody was removed by three washing steps with PBS (5 min, room temperature). Cy3-conjugated goat anti-mouse antibody diluted in background reducing agent was incubated for 1 h at 37 °C. After three washes with PBS (5 min, room temperature) slides were counterstained with DAPI (10 µg/ml in PBS) for 10 min at room temperature. Slides were washed once with PBS and rinsed with water afterwards.

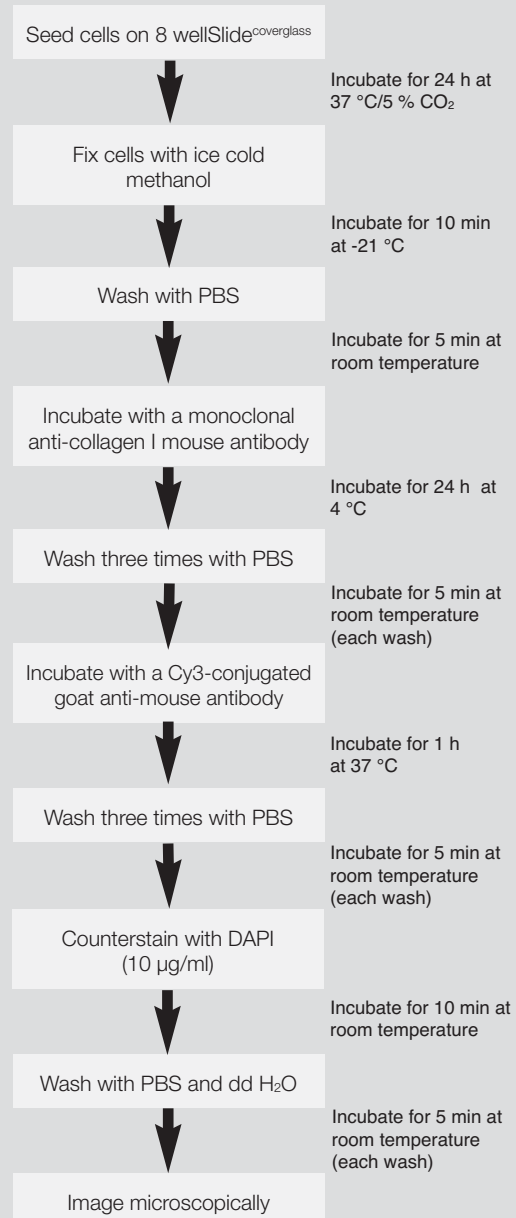


Figure 4: Working Protocol Cytokeratin staining

Figure 5: Working Protocol Collagen I staining

Results

Cytokeratin staining of human epithelial cell line and MDCK cells

Figure 6 shows a fluorescence image of the MDCK cells taken at a magnification of 100x. The green channel demonstrates that the cytokeratins within epithelial cell cytoskeleton can clearly be visualised at this magnification when utilising 8-wellSlides^{coverglass}. The DAPI counterstain shows the orientation of the nucleus in the blue channel. Similar images were taken with the human epithelial cells. **Figure 8** shows the staining on the SlideFlask^{lumox}™ in a magnification of 40x. Both the MDCK as well as the human epithelial cell line reveal positive staining for cytokeratins, characterising both as epithelial cells.

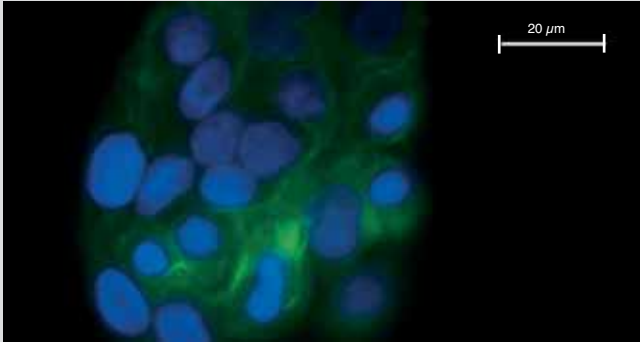


Figure 6: Cytokeratin/DAPI staining of MDCK cells on 8-wellSlide^{coverglass} I with 100x magnification

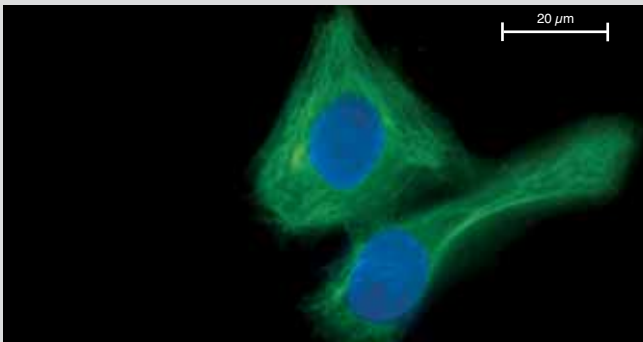


Figure 7: Cytokeratin/DAPI staining of human epithelial cell line on 8-wellSlide^{coverglass} II with 100x magnification

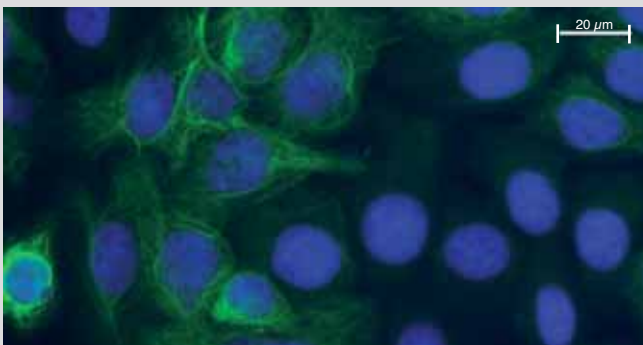


Figure 8: Cytokeratin/DAPI staining of human epithelial cell line on SlideFlask^{lumox}™ with 40x magnification

Collagen I staining of dedifferentiated porcine chondrocytes

Figure 9 shows a fluorescence image of chondrocytes transferred to 8-wellSlides^{coverglass} to perform immunocytochemical staining after having been cultivated in monolayer culture for three weeks. As a marker for chondrogenic dedifferentiation, collagen I could be clearly detected in cultivated porcine chondrocytes, visualised by the red fluorescence. Counterstaining with DAPI shows the nucleus. Both fluorescence images were taken at 63x magnification.

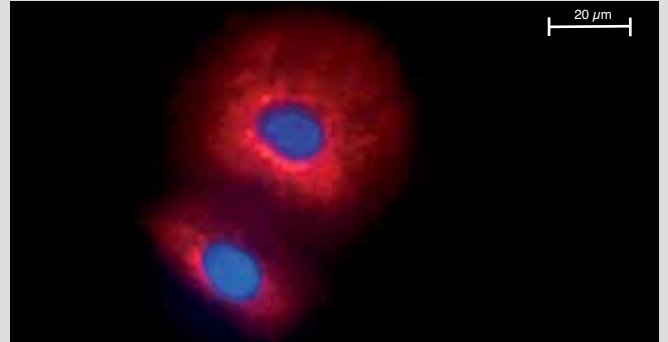


Figure 9: Porcine chondrocytes stained for collagen I/DAPI on 8-wellSlide^{coverglass} I with 63x magnification

Discussion

Immunocytochemical staining was established on Greiner Bio-One MultiwellSlides, with both epithelial cell lines demonstrating a well-defined cytoskeletal protein stain. DAPI staining was clearly visualised at 100x magnification on 8 well-Slides^{coverglass} as well as 40x on SlideFlask^{lumax}.

With a small growth area per well, the Greiner Bio-One 8-well Slides allow substantial savings by significantly reducing the amount of cost intensive antibodies for staining. As little as 80 µl of antibody dilution were sufficient to cover the bottom of the well in the described experiment. Additionally, as cells can be grown directly on the slides, there is no need for time-consuming transfers from a larger culture format, such as tissue culture flask to a slide format for staining and imaging. The entire staining protocol, including methanol fixation, could be performed directly in the wells, thereby allowing simultaneous staining of different epitopes and cells on a single slide. Staining could be visualised with high magnification (up to 100x) in red, green and blue fluorescence and imaging with an inverted microscope could easily be performed with the polystyrene housing remaining on MultiwellSlides^{coverglass I and II}.

Dedifferentiation of porcine chondrocytes was detected on a basis of the expression of collagen I after three weeks in monolayer culture. The fact that the staining for collagen II as a chondrogenic marker was nearly undetectable (data not shown) strongly supports the assumption that the cells have been dedifferentiated in monolayer culture. These data reinforce the currently accepted model of differentiation of primary chondrocytes when cultivated in monolayer culture. To avoid this phenomenon three dimensional cultivation techniques have been established with great success.

Conclusion

Fluorescence staining of cytokeratins on the human epithelial cell lines and MDCK cells as well as collagen I staining on primary porcine chondrocytes could easily be established on Greiner Bio-One MultiwellSlides. The 8-wellSlides^{coverglass} and the SlideFlask^{lumax} proved to have excellent suitability for microscopy at 100x magnification under oil and at 63x and 40x magnification respectively. Other MultiwellSlides products made of polystyrene, polyolefin and glass are optimal for use up to 40x magnification. Because of the ease of handling, high suitability for microscopic applications and reduction of cost intensive reagents through utilisation of a small growth area Greiner Bio-One MultiwellSlides offer a valuable tool for cell culture applications and microscopy.

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Revision: March 2008 - 073 102

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