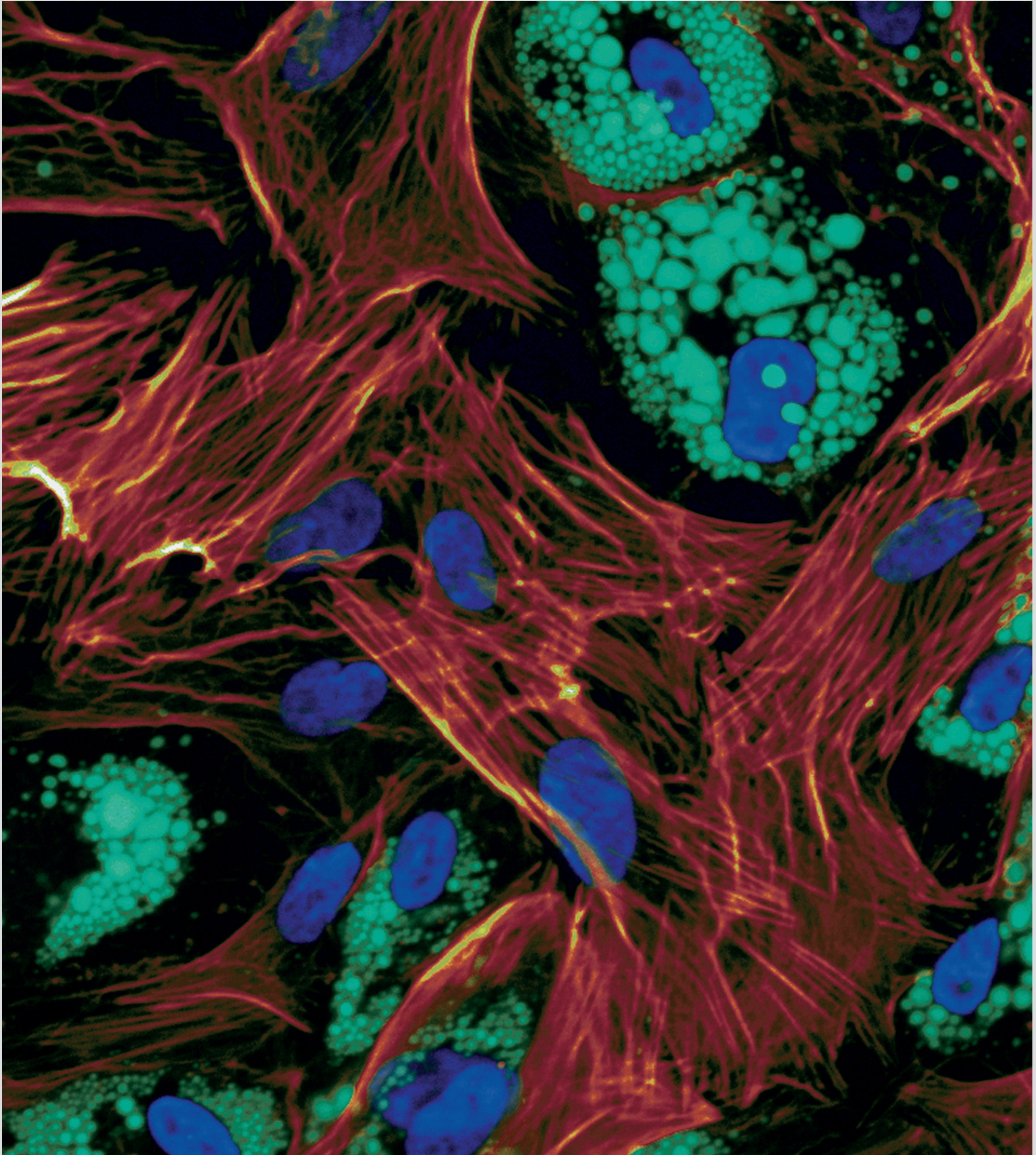
Application Note

Selection of Cell Culture Surfaces for the Adipogenic Differentiation of Human Mesenchymal Stem Cells (hMSC)



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Introduction

The *in-vitro* maintenance and propagation of vertebrate, insect or plant cells is a basic tool in life science research. Since the introduction of the first immortalised mammalian cell line in 1951[1], the establishment and progressive study of numerous immortalised cell lines helped distinguish cell biology as a fundamental specialised field within the realm of biology.
Cell biology finds multiple varied uses in applied technologies such as the analysis of signal transduction in drug candidate screening as well as the development and production of biopharmaceuticals and vaccines. Furthermore, the employment of *in-vivo* cell modeling as a first line screen can reduce the overall quantity of animal experiments needed to perform toxicological studies.

With general ease for proliferation and propagation, immortalised cells became a common tool in drug screening in the latter half of the 20th century. However, current increasing criticism of 2-dimensional immortalised cell lines as not being representative of realistic *in-vivo* conditions gives reason to speculate this limitation may have played a role in the failure of nearly 90% of recent drug candidates to become pharmaceutical drugs[2, 3].

As one alternative some investigators employ to overcome the drawback of immortalised cell lines and mimic a more native condition is the use of primary cells. Unlike immortalised cells, primary cells are isolated from tissue and are not passaged over years under *in-vitro* conditions. However, because primary cells are difficult to isolate and maintain, it can be very challenging to achieve homogenous cell batches with minimised lot to lot variations, a major requirement for reliable results in high-throughput screening.
The emergence of stem cell technologies in cell biology has brought about another fascinating alternative approach to drug discovery due to the potential significance of stem cell therapies for degenerative diseases in regenerative medicine[4]. The term 'Stem Cell' is loosely used to describe what actually comprises a variety of cell types with different properties and characteristics as depicted in table 1[2].

Table 1: Stem Cell Types and their properties [2]

Type of Cells	Potentiality	Self-renewal	Origin
Embryonic Stem (ES) Cells	Pluripotent	Unlimited	Inner cell mass of blastocysts
Induced Pluri Potent Stem (iPS) Cells	Pluripotent	Unlimited	Genetic alteration of somatic cells by trans-fecting specific genes
Fetal Stem Cells	Pluripotent	Limited	Discarded of unused fetus
Cord Blood Cells	Multipotent	Limited	Umbilical cord
Non-embryonic (adult or tissue) Stem Cells	Multipotent	Limited	Adult tissues such as brain, muscle, bone, fat, ...
Progenitor Cells	Unipotent	Very Limited	Adult tissues and organs
Cancer Stem Cells	Unipotent / multipotent	Limited	Tumor tissues

Along with human embryonic stem cells (hESC), known for unlimited potential in self-renewal, plasticity, and prospective use in human stem cell therapy, many other stem cell types such as adult stem cells, induced pluripotent stem cells (iPS), fetal stem cells and cancer stem cells are of interest to derive therapeutic and investigative use (table 1). Adult hematopoietic stem cells have already been in use for years as a stem cell therapy for cure of leukemia[5], and, as a fertile source of hematopoietic stem cells, cord blood has been collected and banked in recent years due to potential use in future treatments for hematopoietic and genetic disorders.
The frequently challenging requirements of *in-vitro* culture can restrict the use of stem cells in high-throughput screening. In addition to critical legal and ethical questions, human embryonic stem cells are especially complicated to maintain and propagate in the large quantities required for high-throughput screening. For this reason, use of animal-derived embryonic stem cells, adult human stem cells, induced human stem cells (iPS) or human cancer stem cells may offer more realistic approaches for drug discovery. iPS and cancer stem cells for example, can be better associated with a specific disease by using the affected tissue as an investigative source to thereby align a more target-oriented approach for drug discovery.
For *in-vitro* growth or differentiation of stem cells, it is necessary to utilise the appropriate cell culture media and surface chemistry to successfully achieve the specific intent. Depending on the aim of stem cell research, the requirements for cell culture surfaces can dramatically differ. For maintaining non-differentiated embryonic stem cells, e.g. feeder layers are generally recommended, whereas when optimal differentiation is desired, surfaces specifically treated for cell culture or biological coated surfaces are more often advocated. A summary of different cell culture surfaces manufactured by Greiner Bio-One and their field of application in stem cell research is given in table 2.

Table 2: Standard cell culture surfaces for stem cell applications

Surface	Description	Recommended for:
CELLSTAR®	Physical surface treatment	Feeder layer + embryonic stem cells; Hematopoietic / mesenchymal stem cells
Suspension	Non-treated polystyrene	Embryonic stem cells / Cultivation of embryonic bodies
CELLCOAT®	Biological coating	Differentiation of stem cells; Growth of differentiated neuronal stem cells
Advanced TC™	Advanced chemical surface treatment	Similar to biological coatings like CELLCOAT® - but non-biological

Optimising the cell culture media for stem cell cultures is basis of many research projects. However when it comes to determining recommendations for use of the best cell culture surface to achieve differentiation or non-differentiation of stem cells, relatively few examinations have been undertaken to establish the most suitable surfaces.

Additionally, very little information can be found in published literature. Therefore the intent of this experiment was to analyse the influence of various commercially available cell culture surfaces on the differentiation of human mesenchymal stem cells into adipocytes within 384 well clear bottom microplates (figure 2) frequently used for high-content and high-throughput screening.

The selected cell culture model of adipogenesis offers a variety of methods for analysing differentiation, from classical high-throughput screening cell based assays to more recently emerged advanced instrumentation to conduct high-content imaging and analyses. The variety of analytical methods available and ease of technical implementation indicated human mesenchymal stem cells as a good starting point for the examination of cell culture surfaces.

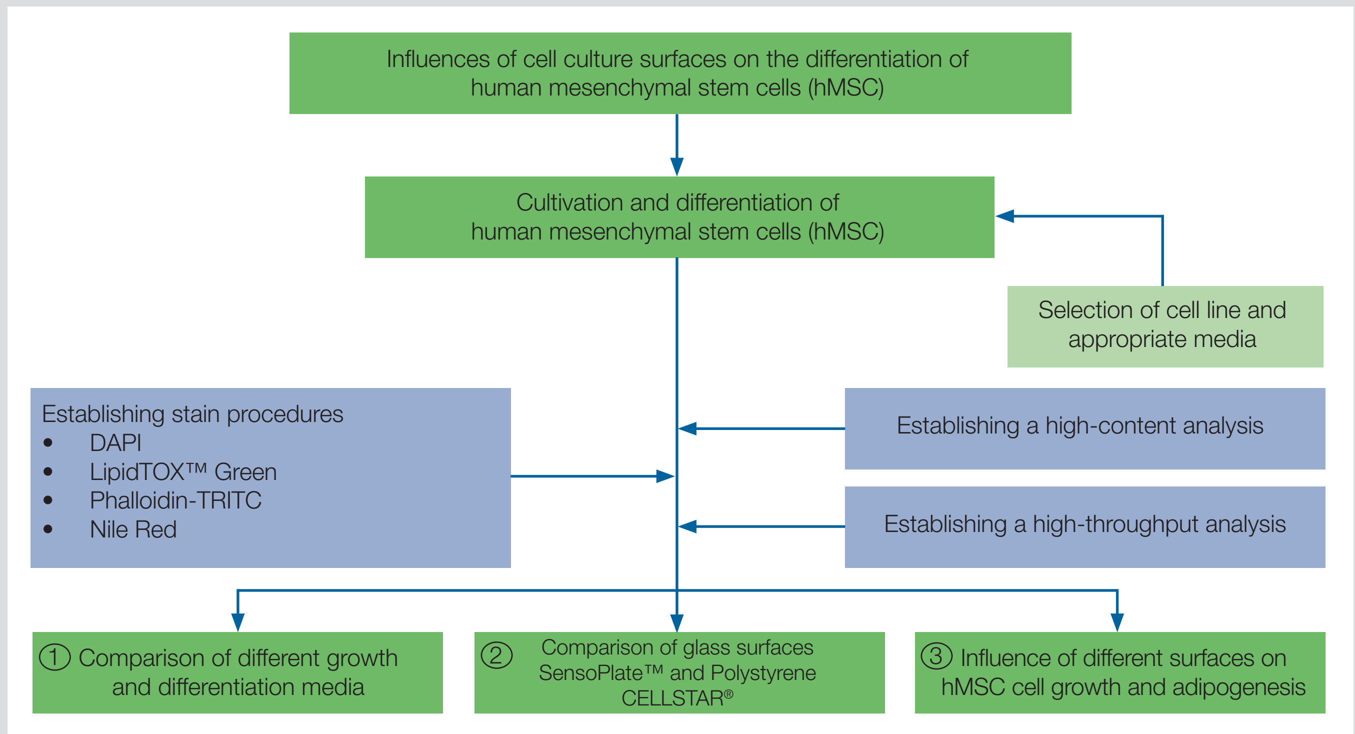

Figure 1:
Adipogenesis of human mesenchymal stem cells: Experimental design

Material and Methods

The goal of the tests was to analyse the influence of cell culture surfaces on the growth and differentiation of hMSC. The experimental design of **figure 1** summarises the project and the strategies.

Cell line:

For the comparison of cell culture substrates, a human adipose derived stem cell line was used (StemPro®, Invitrogen, Carlsbad, USA, #R7788-110). The pluripotency of the cells was examined by testing their ability to differentiate into adipocytes, chondrocytes and osteocytes[6].

Cell culture media:

Cells were grown and differentiated using different media and media combinations (**table 3**). The most suitable media combination was used for the final analysis of adipogenesis on different cell culture surfaces. Two growth media (growth media I and II) and three differentiation media (differentiation media I-III) were prepared in such way that each differentiation media was combined with each growth media.

Growth media I (MesenPRO RS™ growth media, #R7788-110, InVitrogen, Carlsbad, USA) and differentiation media I (Stem Pro™ Adipogenesis Supplement media, #R7788-110, Invitrogen, Carslbad, USA) were prepared according to the supplied protocol[7]. Growth media II and differentiation media II and III were based on information given in the literature [8, 9, 10]

Table 3: Composition of growth and differentiation media

	Growth Media I	Growth Media II	Differen-tiation Media I	Differentiation Media II (Low Insulin Concent-ration)	Differentiation Media II (High Insulin Concent-ration)
MesenPro RS™ Growth Media	100 ml	-	-	-	-
StemPro® adypocyte differentiation basal media	-	-	90 ml	-	-
Growth supplement	2 ml	-	-	-	-
StemPro® Adipogenesis Supplement	-	-	10 ml	-	-
FCS/FBS	-	10 %	-	-	-
DMEM	-	1000 ml	-	-	-
Dexamethasone	-	-	-	1 µM	-
1-Methyl-3-isobutylxanthine (IBMX)	-	-	-	115 µM	-
Insulin	-	-	-	1,74 µM	10 µM
Indomethacin	-	-	-	20 µM	200 µM
L-Alanyl, L-Glutamine	1 ml	2 %	-	2%	2 %
Antibiotic / Antimycotic (100x)	1 ml	1 %	1 ml	1 %	1 %

Cell culture surfaces:

All tests were conducted in 384 well black clear bottom microplates (**figure 2**), the most frequently used format in high-throughput and high-content screening.
Different biological coatings (CELLCOAT®), surface treated microplates (CELLSTAR®) as well as different advanced chemical surface treatments (Advanced TC™) were compared. A summary of all surfaces tested is given in **table 4**.

Figure 2:
384 well clear bottom microplates / µClear® film bottom 190 µm (#781090, Greiner Bio-One, Germany)

Table 4: Surfaces tested for the growth and adipogenesis of human mesenchymal stem cells; Assay volume and number of cells per well

Description All plates were 384 Well, black with clear bottom (µClear®)	Assay Volume	Cells / Well
Standard cell culture surfaces		
CELLSTAR® TC, Greiner Bio-One (#781091)	50 µl	500
Competitor 2, standard cell culture treated	50 µl	500
Advanced cell culture surfaces		
Advanced TC™, Greiner Bio-One (#781986)	50 µl	500
Competitor 2, advanced cell culture surface	50 µl	500
Competitor 1, amine functionalized	50 µl	500
Biological coatings		
CELLCOAT® PDL, Greiner Bio-One (#781946)	50 µl	500
CELLCOAT® Collagen I, Greiner Bio-One (#781956)	50 µl	500
Competitor 1, PDL coated	50 µl	500
Competitor 1, Collagen I coated	50 µl	500
Non-coated glass bottom		
SensoPlate®, Greiner Bio-One (#781892)	50 µl	5.000

Quantification of adipogenesis

Two approaches were selected to analyse the adipogenesis of human mesenchymals stem cells: a high-content screening approach based on microscopic imaging and a high-throughput screening approach based on fluorescence measurements within a microplate reader.

High-content screening approach

Differentiation of hMSC into adipocytes was examined on CELLSTAR® cell culture surfaces (#781091, Greiner Bio-One, Frickenhausen, Germany) and glass bottom surfaces (#781892, SensoPlate™, Greiner Bio-One, Frickenhausen, Germany). For microscopic analysis, adipocytes were stained with LipidTOX™ green (#34475, Invitrogen, Carlsbad, USA), a specific fluorescent dye which binds selectively to lipid vesicles formed during adipogenesis. LipidTOX™ green can be excited at 395 nm and emits light at 405 nm.

DAPI (#D8417, Sigma-Aldrich, St. Louis, USA) and Phalloidin-TRITC (#P1951, Sigma-Aldrich, St. Louis, USA) were used to visualise the nuclei and the cytoskeleton, respectively (figure 3). Analysis of adipogenesis was performed using fluorescence microscopy (Leica) and the publicly available software program ImageJ (ImageJ Download[11]). Cell number was determined by counting DAPI stained nuclei. Adipogenesis was determined by analysis of the area stained by LipidTOX™ green. The ratio of lipid vesicle surfaces versus cell number was used to calculate the grade of differentiation (Formula 1). Additionally a high-content screening system (Operetta, Perkin Elmer, Hamburg, Germany) was used to create confocal images.

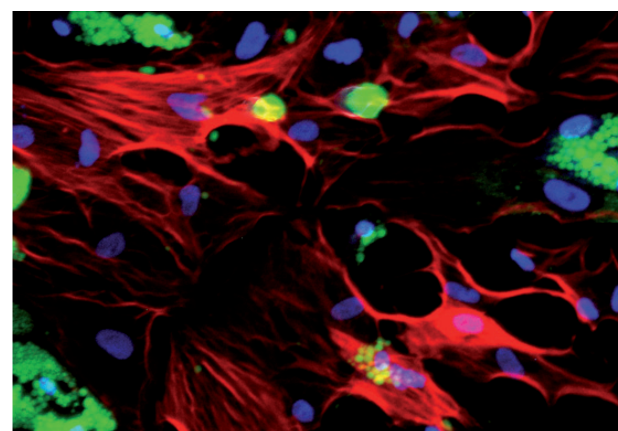

Figure 3:
Fluorescence microscopy of differentiated human mesenchymal stem cells. (Leica, Wetzlar, Germany, 40 x magnification)
Green = LipidTOX™ green, staining of lipid vesicles
Red = Phalloidin TRITC, staining of the cytoskeleton
Blue = DAPI staining fo the nuclei

ImageJ analysis: Comparison non-treated glass vs. CELLSTAR®

$$F_{(differentiation)} = \frac{A_{(LipidTOX™ green)}}{N_{(DAPI)}}$$

$F_{(differentiation)}$ = Grade of differentiation
$A_{(LipidTOX™ green)}$ = Surface stained by LipidTOX™ green
$N_{(DAPI)}$ = Number of cells defined by DAPI staining

Staining Procedures

Staining solutions were prepared and staining procedures performed according to standard protocols.

DAPI:	10 µg / ml
Phalloidin-TRITC:	5 µg / ml
LipidTOX™ green:	Stock solution diluted 1:200 according to the supplied protocol

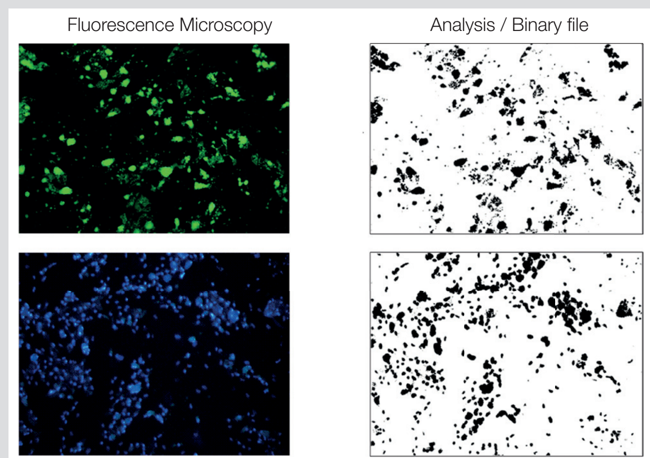

Figure 4:
ImageJ analysis of adipogenesis. For determining cell number and lipid surfaces fluorescence microscopy pictures were transformed for analysis in binary files. Subsequently the ImageJ [11] analysis software was applied.

Influence of cell culture surfaces on adipogenesis

To determine the influence of a surface on the differentiation of human mesenchymal stem cells, the high-throughput screening approach was applied. As the optics of microplate readers are not able to detect the weak LipidTOX™ green signals, it was necessary to employ an alternative stain. Because it specifically stains lipid vesicles and vacuoles in addition to displaying a strong signal detectable by microplate readers, Nile Red (#PT-7009, AdipoRed™ Assay Reagent, Lonza, Basel, Switzerland) was found to be a perfect replacement. The excitation wavelength of Nile Red is 485 nm with an emission wavelength at 572 nm.

Cell growth was analysed using the CyQUANT® cell proliferation assay kit (#C7026, Invitrogen, Carlsbad, USA). The CyQUANT® dye binds to the nucleic acids of cells and based on a standard curve with known cell numbers the fluorescence signal can be set in relation to the cell number.

As nucleic acids from proliferating and non-proliferating cells are stained the CyQUANT® is an indication for the total cell number. The readout was conducted at 480 nm / 520 nm in a microplate reader.

Human embryonic stem (hESC) cells were seeded in 384 well microplates with different cell culture surfaces (**table 4**). After 8 days of inoculation the growth media was replaced by differentiation media. Sample plates were measured after 4, 7, 11, 14 and 21 days (**figure 5**).

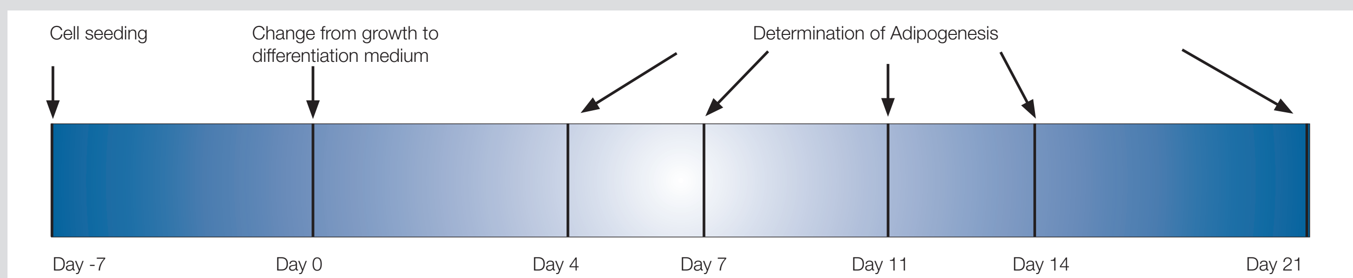

Figure 5:
Experimental design of the cell culture surface testing
Cells were seeded in 384 well clear bottom microplates and expanded for 7 days. Differentiation was started by changing from growth to differentiation media. Adipogenesis was determined after 4, 7, 11, 14 and 21 days.

Results and Discussion

Influence of cell culture media on growth and differentiation of hMSC → see figure 1, ①

Different media combinations were tested using the Nile Red staining protocol. The goal of the test was to determine the most suitable media combination for subsequent surface tests. Hereby the grade of differentiation of hMSC into adipocytes was the main criteria. Highest differentiation was achieved by using Growth media I (MesenPRO™ RS growth media) and differentiation media I (StemPro® Adipogenesis Supplement). Good differentiation grades were also achieved by using a combination between growth media I (MesenPRO™ RS growth media) and differentiation media III (high insulin concentration). The lowest differentiation was detected using growth media II in combination with the differentiation media II (media with lower Insulin concentration).
The combination of growth media I (MesenPRO™ RS growth media) and differentiation media III (media with high insulin concentration) was selected to conduct the experiment within this application note. For an industrial high-throughput screen, the combination of growth media I (MesenPRO™ RS growth media) and differentiation media I (StemPro® Adipogenesis Supplement) may offer higher suitability because of its ability to induce a higher differentiation grade (**figure 6**)

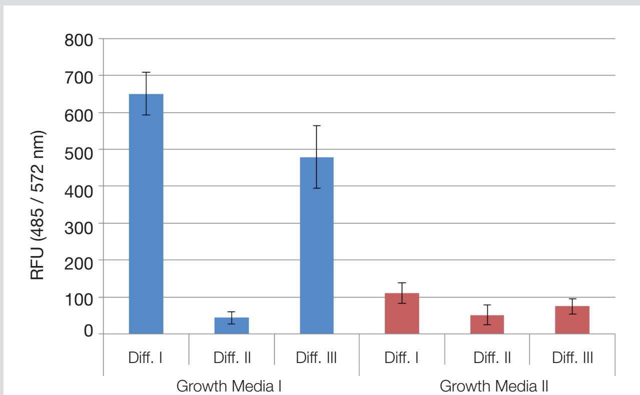

Figure 6:
Adipogenesis of hMSC induced by different combinations of growth and differentiation media

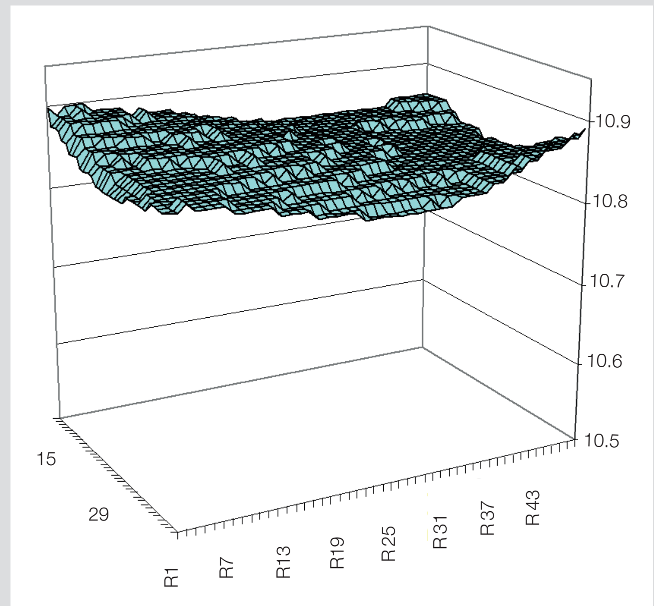

Figure 7:
Plate bottom profile of a 384 well SensoPlate™ (#781892, Greiner Bio-One, Frickenhausen, Germany)
Mean planarity of the 175 µm glass bottom < 77 µm

High-content analysis of cells grown on glass (SensoPlate™) and polystyrene film bottom microplate surfaces (µClear® CELLSTAR®) → see figure. 1, ②

In a high-content screening application the selection of the best microplate can be a very important consideration. It is generally desirable to use a cover glass thickness for best compatibility with instrumentation. Greiner Bio-One manufactures two microplate designs that are compatible with imaging optics. To determine the best suitability of these microplates for high-content analysis, both of the microplate types were used for the experiment.
Glass bottom microplates, like the SensoPlate™ from Greiner Bio-One, are the gold standard for high magnification and high resolution microscopy because of their excellent optical properties. The SensoPlate™ is generally offered in a black pigmented polystyrene frame with a borosilicate glass bottom. In addition to being terminally sterilised, the SensoPlate™ is manufactured under clean room class 1000 conditions to guarantee the highest level of cleanliness.

The glass bottom features high intra-/inter-well flatness (figure 7), its 175 µm in thickness for a perfect fit with standard microscope objective lenses, and offers a refractive index of $\eta e_{(\lambda = 546)}$ 1.52.

µClear® is a Greiner Bio-One tradename used to represent black or white microplates with a transparent 190 µm thin polystyrene film bottom. The µClear® film is suitable for sterilisation and different cell culture treatments, resulting in a variety of biological and non-biological cell culture surfaces (table 4) compatible for a wide range of different cell types. The 190 µm thickness is perfect for microscopic objective lenses which are adapted to 175 µm glass cover slips.

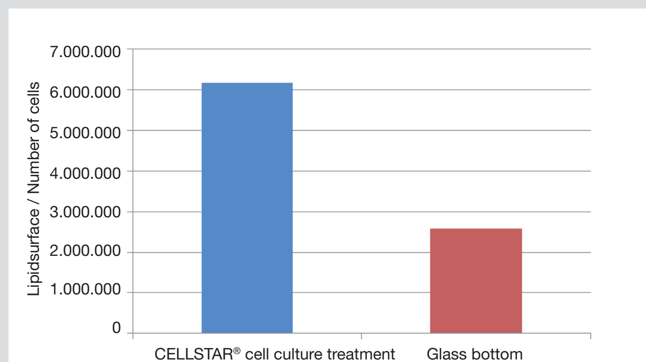

Figure 10:
Differentiation of hMSC on cell culture treated surfaces (CELLSTAR®) and glass bottom microplates (SenoPlate™) after 14 days

The adipogenesis of hMSC on glass (#781892, Greiner Bio-One, Frickenhausen, Germany) and cell culture treated polystyrene µClear® film (#781091, Greiner Bio-One, Frickenhausen, Germany) was examined in the high-content screening approach based on microscopic fluorescence imaging (figure 8 / 9). Adipogenesis of human mesenchymal stem cells on polystyrene film bottom microplates was highly reliable and required low cell numbers for inoculation (table 4). Human mesenchymal stem cells differentiated on glass after inoculation with higher cell numbers, but on a lower level than on polystyrene.

The morphology of the cytoskeleton of non-differentiated cells is similar on glass and on polystyrene, but the cytoskeleton of differentiated cells on polystyrene surfaces show a more globular morphology than the cytoskeleton of cells differentiated on glass (figure 9 / 10). The explanation for this observation is the lower grade of cell differentiation (figure 11) on the glass substrate. In order to enhance the cell differentiation on glass a biological coating may be helpful.

With 40 fold magnification, the image quality obtained with the polystyrene µClear® film surface was similar to the image quality obtained using the glass surface. Because the polystyrene µClear® microplates enabled more rapid and higher level of hMSC adipogenesis differentiation (figure 9), coupled with their availability in a wide range of cell culture surfaces (table 4), renders the µClear® film microplates the perfect selection for an industrial high-content screening approach.

However for specialised applications such as Fluorescence Correlation Spectroscopy (FCS), high magnifications (64-fold and higher), and 3D imaging, the glass bottom SensoPlate™ offers the best choice.

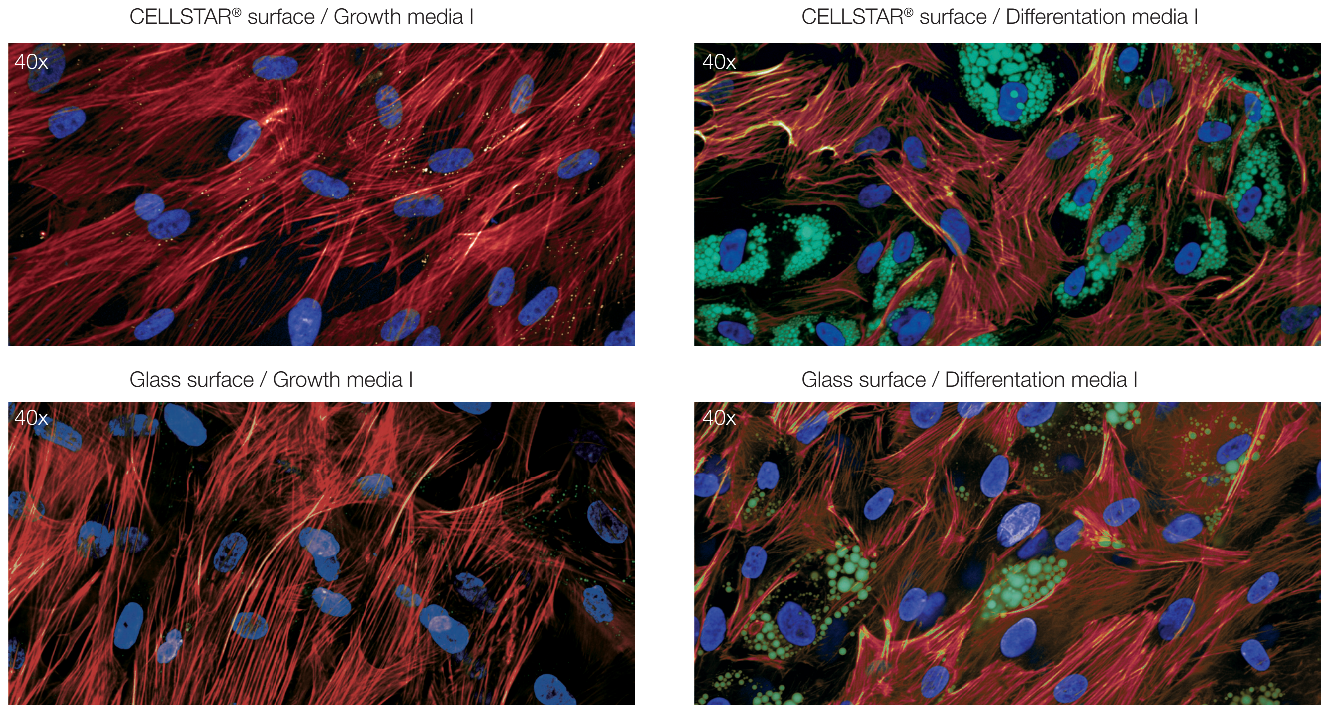

Figure 8:
Adipognesis of human mesenchymal stem cells on 384 well polystyrene film (#781091, CELLSTAR® cell culture treated, µClear® bottom, Greiner Bio-One, Frickenhausen, Germany) and glass bottom microplate surfaces (#781892, SensoPlate™, Greiner Bio-One GmbH, Frickenhausen, Germany).
Operetta, Perkin Elmer, Hamburg, Germany, 40x magnification

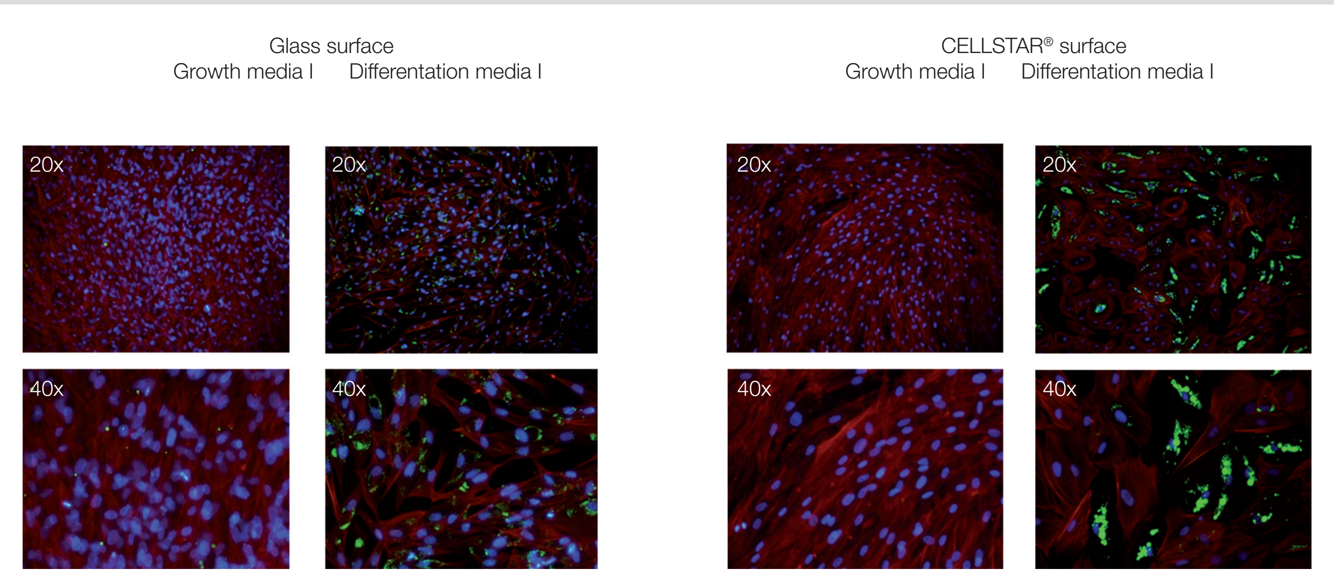

Figure 9:
Adipogenesis of human mesenchymal stem cells on a 384 well polystyrene film bottom (#781091, CELLSTAR® cell culture treated, µClear® bottom, Greiner Bio-One, Frickenhausen, Germany) and glass bottom microplate surfaces (#781892, SensoPlate™, Greiner Bio-One, Frickenhausen, Germany).
Leica, Wetzlar, Germany, 20x / 40x magnification

Surface comparison → see figure 1, ③

After establishing the advantage of a µClear® film bottom for standard high-content screening applications using magnifications up to 40-fold it was necessary to elucidate which surface treatment performs best in the adipogenesis assays.

According to the experimental setup described in materials and methods, hMSC cells were seeded and differentiated on various cell culture surfaces and their influence on cell growth (CyQuant®) and differentiation (Nile Red) was determined.

Although cell growth and differentiation was detected on all tested surfaces, the Advanced TC™ surface (# 781986, Greiner Bio-One, Frickenhausen, Germany) displayed the highest cell growth and differentiation rate (**figure 12, figure 14**). Advanced TC™, a non-biological surface, illustrated even slightly higher differentiation levels than the protein coated surfaces. Within the protein coated surfaces, the Poly-D-Lysine (PDL) coating outperformed the Collagen I coating (**figure 11, figure 13**).

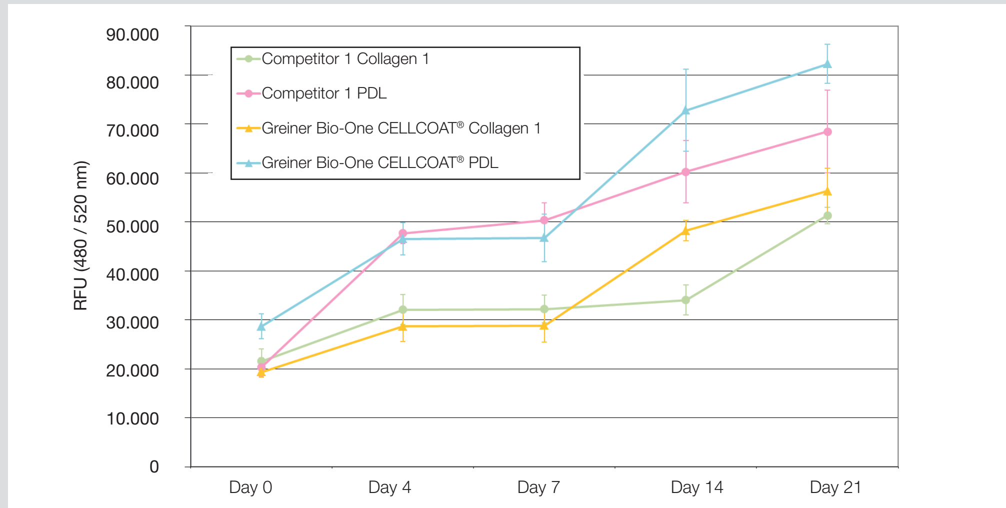

Figure 11:
Cell Growth of human mesenchymal stem cells on biological surfaces. Cell growth was analysed using the CyQuant® proliferation assay kit.

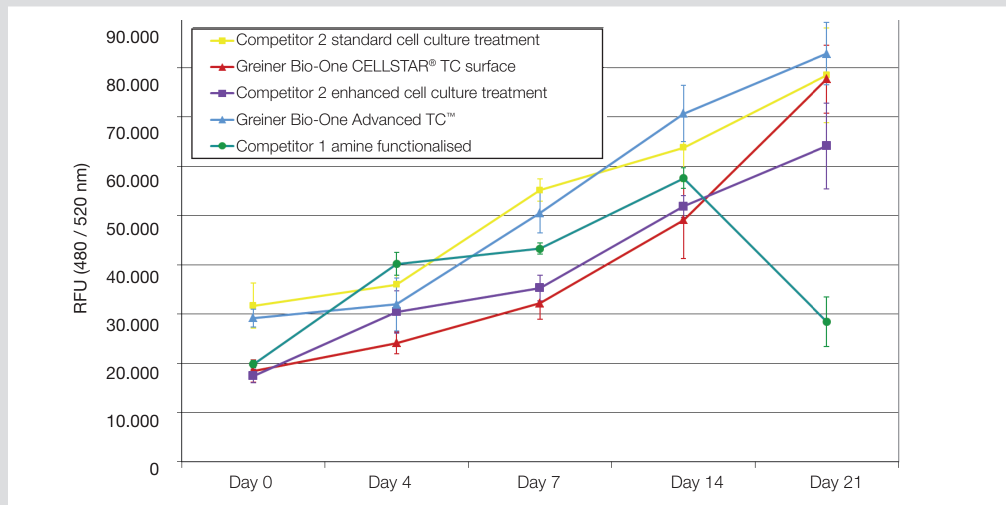

Figure 12:
Cell Growth of human mesenchymal stem cells on non-biological surfaces. Cell growth was analysed using the CyQuant® proliferation assay kit.

The effectiveness of the Advanced TC™ surface can likely be attributed to its ability to support interactions of the stem cells with the surface, enhancing performance in differentiation. In contrast to protein coated surfaces, the Advanced TC™ surface is sterile and can be stored at room temperature for longer time periods to better facilitate planning of high-throughput screening campaigns.

Using optical lenses with 40-fold magnification, the Advanced TC™ µClear® surface was the best choice for the selected assays. The adipogenesis of human mesenchymal stem cells is an ideal approach for high-throughput or high-content screening in order to detect compounds that induce or inhibit adipogenesis.

Despite the fact that differentiation of human adipose derived stem cells may be considered as an exemplary application, this experiment gives clear indication for the performance of the Advanced TC™ surfaces. Further studies of the Advanced TC™ surface are planned with other stem cell types such as induced pluripotent (iPS) or embryonic stem cells (ESC) to examine additional performance for cell growth and differentiation.

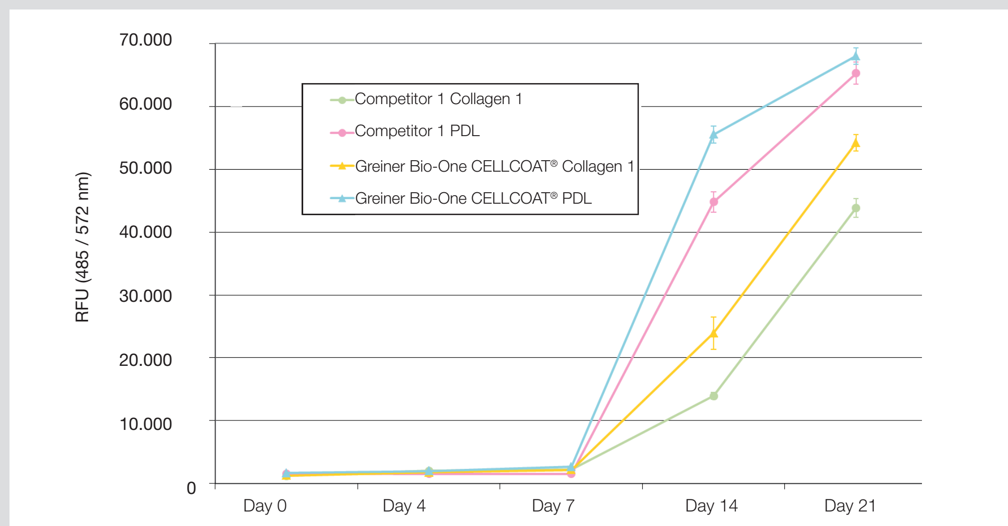

Figure 13:
Adipogenesis of human mesenchymal stem cells on biological surfaces. Adipogenesis was analysed with the AdipoRed™ Assay Reagent.

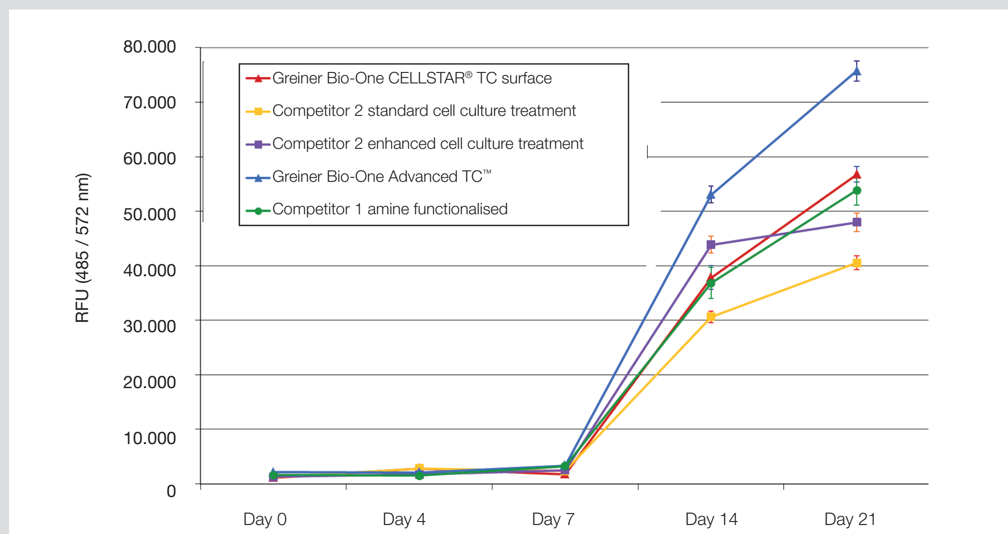

Figure 14:
Adipogenesis of human mesenchymal stem cells on non-biological surfaces. Adipogenesis was analysed with the AdipoRed™ Assay Reagent.

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Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA

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