Application Note

The Thermo Scientific Nunclon Sphera surface supports formation of embryoid bodies from pluripotent stem cells

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Key Words
Nunclon Sphera, embryoid body, low cell binding, 3D cell culture, stem cell

Abstract
Keeping stem cells in an undifferentiated state is important for expansion and maintenance. However studying the process that stem cells follow to become body tissues requires them to differentiate. The formation of spheroids is an important milestone in the differentiation process. Here we introduce a new cell culture surface, Thermo Scientific™ Nunclon Sphera that supports the *in vitro* formation of stem cell spheroids. The Nunclon Sphera surface coating inhibits cell attachment to the culture dish by blocking the adsorption of extracellular matrix (ECM) proteins that usually mediate cell adhesion, thereby promoting cell-cell aggregation *in vitro*. The low binding property of Nunclon Sphera surface was demonstrated by low cell attachment of adherent cell lines (e.g. Vero, A549, and U937) as well as by the formation of embryoid bodies (EB) of both human embryonic stem cells (hESCs) and mouse embryonic stem cells (mESCs). EB formation in Nunclon Sphera dishes was compared to either non-treated or cell culture treated dishes. The integrity and consistency of the Nunclon Sphera surface was also showcased through the differentiation of cells derived from the embryoid bodies. These results demonstrate the advantage of the Nunclon Sphera surface for embryoid body formation in the process of pluripotent stem cell differentiation.

Introduction
Stem cells are often used in research due to their ability to become multiple different cell types. The formation of spheroids in suspension cultures is an intermediate step in producing different cell lineages from pluripotent stem cells and other progenitor cells. The variability seen in experiments using spheroid-forming cultures has been linked to medium composition and volume, cell density, and duration in culture. While these factors all affect the culture, the surface of the culture dish is one of the most critical factors contributing to the success of spheroid formation *in vitro*.

On cell culture surfaces where cell attachment is required, non-covalent interactions between extracellular proteins and the functional groups within the molecular structure of the surface bind the proteins to the culture surface. The cells then use these bound proteins as anchor points to attach to the surface substrate. In cultures where spheroids are desired, this cellular attachment must be prevented in order to promote the cell-cell aggregation required for spheroid formation. Although several different surfaces that offer properties of low adhesion are commercially available, spontaneous cell differentiation resulted from

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random cell attachment is still a challenge to many researchers. The novel Nunclon Sphera surface by Thermo Fisher Scientific effectively prevents protein adsorption and allows cells to grow in suspension with virtually no cell attachment, providing superior performance over other low binding surfaces.

Materials and methods

ECM Protein Adsorption

Nunclon Sphera 96-well plates and Nunclon Delta (standard cell culture-treated) 96-well plates were coated with 100 µL/well of either 24 µg/mL FITC labeled Bovine Collagen Type I or 20 µg/mL of TAMRA labeled Fibronectin in DPBS. The plates were incubated for 24 hours at 2-8°C or 16 hours at room temperature, respectively. The solution was aspirated and plates were washed 3 times with 200 µL/well PBST (0.05% Tween 20 in PBS). The fluorescence intensity was read at Ex495/Em525 (Collagen) or Ex543/Em570 (Fibronectin) on a fluorescent plate reader.

Stem Cell Line Cultivation

Human and mouse ESC lines (Hes-3, B6N #1) were maintained in the appropriate HyClone media containing 20% serum (mESC) or 10% serum replacement (hESC), non-essential amino acids, penicillin/streptomycin, and beta-mercaptoethanol. Cultures were incubated at 37°C and 5% CO_2. For passaging, the cells were released from the surface using HyQTase (HyClone).

Spheroid Formation

Stem cells were seeded into Nunclon Sphera, non-treated, and standard cell culture treated 6-well multidishes at a density of $6.3x10^4$ cells/well (hESC) or $4x10^4$ cells/well (mESC) in the appropriate HyClone media containing Rho-kinase inhibitor. Cells were incubated at 37°C and 5% CO_2. For hESCs, fresh media was added after 2 days, and media was replaced with fresh media after 3 days. For mESCs, media change was performed after 2 days. After 7 days in culture, cells were imaged, and the number of EBs and spheroid morphology was assessed. Cell viability was also determined using a NucleoCounter (Chemometec).

Differentiation

After EB formation, cells were transferred to gelatin-coated 12-well Nunclon Delta multidishes, cultured in the appropriate media with Rho-kinase inhibitor, and allowed to grow for 7 days with media changes every 2-3 days (mESC) or for 14 days with media changes every 3-4 days (hESC). Cells were then analyzed by immunocytostaining using markers for the three germ layers: alpha fetoprotein (AFP) for endoderm, beta-tubulin for ectoderm, and smooth muscle actin (alpha-SMA) for mesoderm.

Results and discussion

The adsorption of ECM proteins to the Nunclon Sphera surface is extremely low, resulting in low attachment of the adherent cells

Common ECM proteins are known to mediate cell attachment to culture surfaces. In order for adherent cells to form spheroids in suspension, the culture vessel must encourage the aggregation of cells through cell-cell binding by preventing ECM binding to the plastic surface. In this study, the binding of both Collagen I and Fibronectin on the culture surface was demonstrated by the fluorescence intensity following overnight incubation of solution with the fluorescence labeled ECM proteins (**Figure 1**). Unlike the standard cell culture-treated surface, the Nunclon Sphera surface has minimal binding interactions with the ECM proteins, consequently discouraging the cells from attaching to the surface.

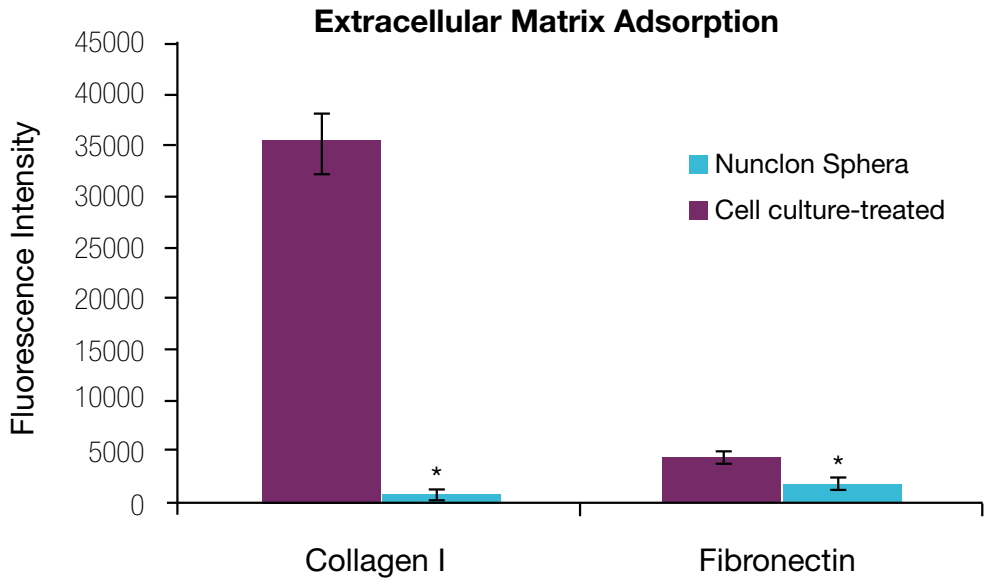

Figure 1. The adsorption of Collagen I and Fibronectin to the Nunclon Sphera surface is extremely low compared to the standard cell culture-treated surface (*, Student's T test, $p<0.01$).

In order to verify that adherent cells show little attachment to the Nunclon Sphera surface, the number of attached cells was examined in cell culture dishes with the Nunclon Sphera surface. The Nunclon Sphera surface showed virtually no cell adhesion for all cell lines tested (VERO, A549, and differentiated U937), whereas the cell culture treated and the non-treated polystyrene surfaces resulted in significant cell adhesion (data not shown). This suggests that neither the cell culture treated surface nor the non-treated surface is suitable for spheroid culture. The low adhesion properties of the Nunclon Sphera surface demonstrate the feasibility of this surface for spheroid cell culture applications.

The Nunclon Sphera surface supports embryoid body formation

Next, the EB formation on Nunclon Sphera surface was examined. **Figure 2** shows the morphology of the EBs formed on three different culture surfaces. While the regular cell culture treated surface resulted in heterogeneous culture of the hESCs with spontaneous differentiation, both the Nunclon Sphera surface and the non-treated surface promoted good quality EB formation by the hESCs. The hESCs were cultured in serum-free media, and due to the lack of certain serum proteins for attachment, EB formation under these conditions can occur even with sub-optimal culture surface conditions. Because of this, even the non-treated surface performed adequately for hESC EB formation. On the other hand, mESCs were cultured in serum-containing media. They failed to form EBs on either the standard cell culture treated or the non-treated surface. Only the Nunclon Sphera surface was able to support EB culture of the mESCs with good morphology. This result is quantified in **Figure 3A**, showing the number of EBs formed on each surface over a 7-day incubation. Human ESCs formed more EBs on the Nunclon Sphera and non-treated surfaces than that on the cell culture treated surface, while mESCs formed EBs only on the Nunclon Sphera surface. **Figure 3B** shows the viability of cells with no significant differences between surfaces, indicating that the Nunclon Sphera surface coating does not negatively impact the growth of cells in culture. Taken together, the data suggests that Nunclon Sphera is a superior culture surface for EB applications despite the different culture conditions and the variability of different cell types.

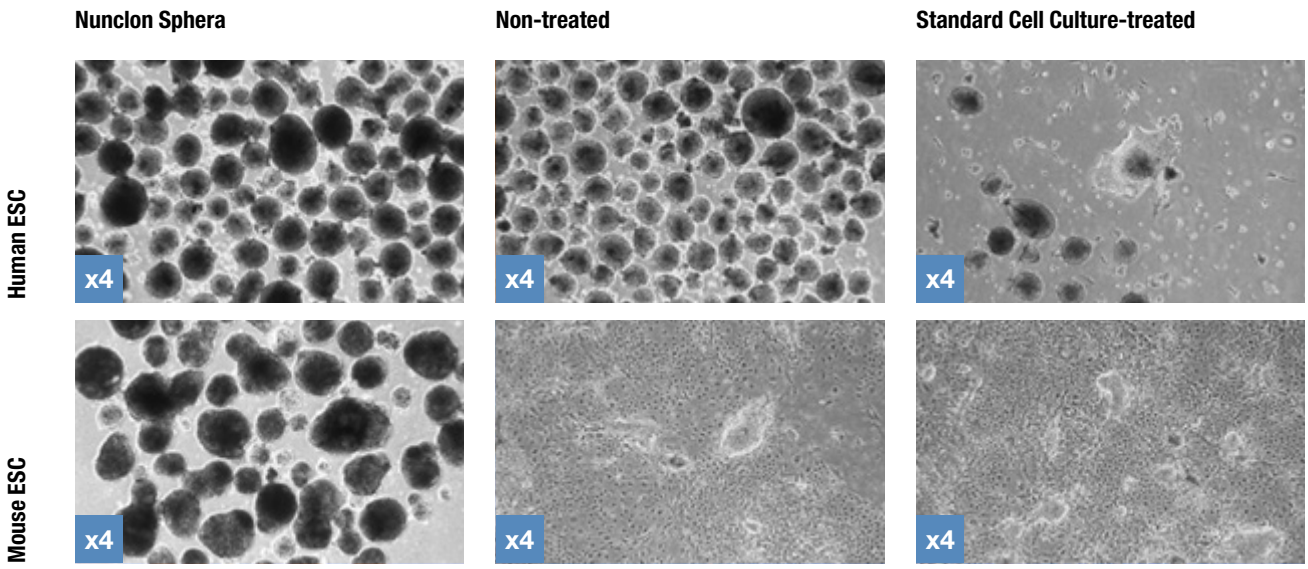

Figure 2. Morphology of the EB by hESCs and mESCs cultured on Nunclon Sphera, non-treated, and standard cell culture treated surfaces.

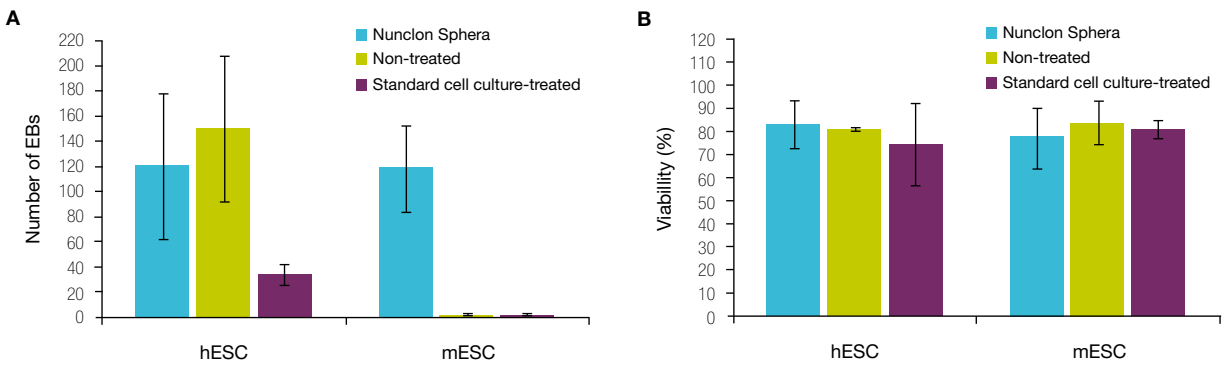

Figure 3. Quantitative examination of EB formation. (A) hESCs form EBs on both the Nunclon Sphera and the non-treated surfaces. mESCs only form EBs on the Nunclon Sphera surface. (B) The surface treatments have no impact on cell viability in culture.

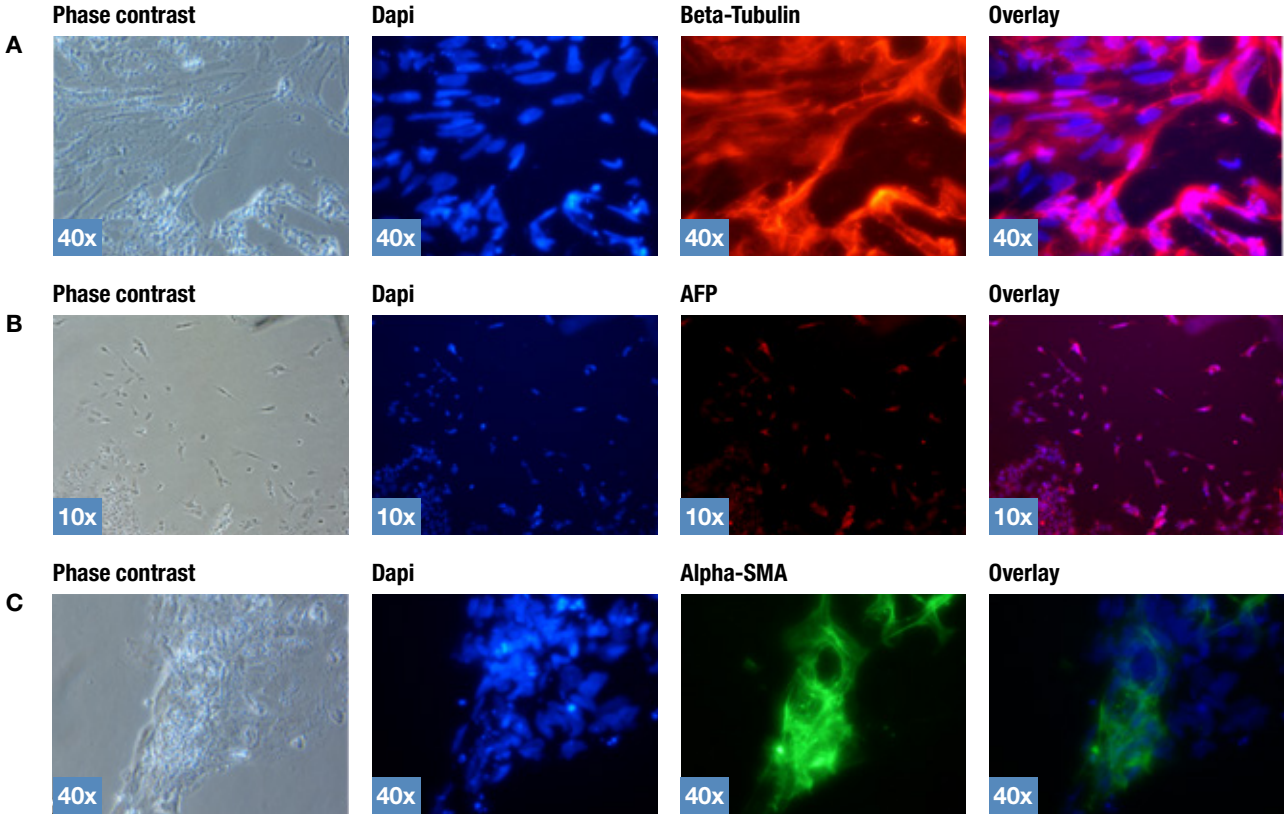

Figure 4. Photomicrographs of cell differentiation using hESCs derived from EBs formed on the Nunclon Sphera surface. The hESCs were transferred from Nunclon Sphera surface to gelatin-coated 12-well Nunclon Delta multidishes in appropriate differentiation media. Immunocytochemistry was performed to examine induced germ layer differentiation: (A) beta-tubulin for ectoderm; (B) AFP for endoderm; and (C) alpha-SMA for mesoderm. DAPI counterstaining was performed to visualize the presence of cell nuclei, and phase contrast images are shown to visualize cells.

The quality of the EB was assessed by the induced differentiation of 3 germ layers

The most important facet of stem cell research is that the hESC can differentiate into any cell in a human body, and preserving this ability is important for any materials used in hESC research. For the purposes of this study, it is critical to ensure that the EBs formed on the Nunclon Sphera surfaces maintain the capacity to differentiate into all three germ layers. **Figure 4** shows photomicrographs of the results of induced differentiation, clearly demonstrating that the hESCs derived on Nunclon Sphera surface can differentiate into cells of all three germ layers (endoderm, ectoderm, and mesoderm).

Conclusions

The Nunclon Sphera surface consistently demonstrates minimal ECM protein binding, extremely low cell attachment, and good formation and viability of EBs in both mESC and hESC. These results indicate that the Nunclon Sphera surface is an excellent choice for EB formation in stem cell culture.

- Minimal adsorption of ECM proteins and extremely low cell adherence highlight the low binding properties of the Nunclon Sphera surface.
- The Nunclon Sphera surface effectively supports the formation of EBs in different stem cell lines, providing a consistent culture system for spheroid growth.
- The Nunclon Sphera surface coating has no adverse effects on stem cell survival or their ability to differentiate.

Ordering Information

Thermo Scientific Cat. No.	Description	Units per bag	Units per case
174925	Microwell 96U-Well Plate, Round Bottom, Well Volume 300 µL	1	8
174927	Microwell 96F-Well Plate, Flat Bottom, Well Volume 400 µL	1	8
174929	Microwell 96U-Well Plate, Round Bottom Bulk Pack	1	8
174930	Multidish 24-Well, Culture Area 1.9 cm^2	1	7
174931	Multidish 12-Well, Culture Area 3.8 cm^2	1	7
174932	Multidish 6-Well, Culture Area 9.6 cm^2	1	7
174943	Dish 35 MM, Culture Area 8.8 cm^2	5	20
174944	Dish 60 MM, Culture Area 221.5 cm^2	5	20
174945	Dish 90 MM, Culture Area 256.7 cm^2	5	20
174951	T25 Cell Culture Flask, Culture Area 25 cm^2	6	18
174952	T75 Cell Culture Flask, Culture Area 75 cm^2	4	24

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