Application Note

3D Cell Culture, Biomarkers, Cell Imaging, Cell-based Assays, Spheroid Imaging, Stem Cells

Automation and Image-Based Analysis of a Hanging Drop Micro-Hole Plate Model to Create and Differentiate 3D Mesenchymal Stem Cell Spheroids for Downstream Tissue Formation

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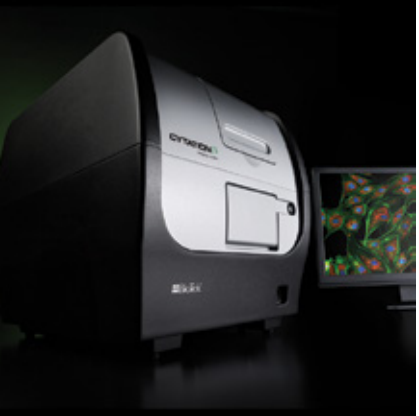

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Introduction

Human mesenchymal stem cells (hMSCs) have recently emerged as a leading candidate in cellular therapies due to their unique properties[1]. The promise of hMSCs, supported by over 300 clinical trials using this type of stem cell[2], is due to their ability to differentiate and mature into multiple lineages including adipocytes, chondrocytes, and osteocytes, in addition to their immunesuppressive properties, and distinct migratory and trophic effects during tissue repair and regeneration[3-5]. Despite the fact that early work with hMSCs incorporated two-dimensional (2D) cell culture on plastic tissue culture treated plates, a realization has occurred that this culture method alters the native phenotype of hMSCs. This has been shown by tests with self-assembled 500-10,000 cell hMSC aggregates revealing the ability to create an "in vivo-like microenvironment and better preserve MSC phenotype[6-7]. The benefits of aggregating hMSCs into spheroidal models has also been seen in preclinical studies where intramyocardial transplantation of three-dimensional (3D) cultured MSCs into porcine models improved cell survival and integration[8-9]. Due to these findings, there is an increasing desire to fully understand the impact that aggregation has on hMSCs within each spheroidal structure.

One such therapeutic area is the creation of complex tissues from preformed 3D hMSC spheroids. Due to the demands of this application, large numbers of spheroids are typically required. Current methods commonly incorporated for spheroid formation create single or low spheroidal numbers per well. Because of this, high numbers of plates are used to generate the density of tissue required, which can be expensive and labor intensive. Others that do create higher spheroid numbers allow spheroids with great variation in size to be created, and also suffer from undesired "pre-aggregation". The Elplasia multiple pore hanging drop microplate from Kuraray can eliminate these complications by offering a method to create large numbers of spheroids of consistent size from a single well of the 6-well plate. Using an insert design, cell suspension is added across the top of the insert where cells are free to fall into the 650 micro-holes in the insert. Spheroids form without further manual manipulation, which can then remain in the plate to undergo differentiation. Upon completion of the desired process, spheroids can easily be dropped from the insert by filling the wells below with appropriate media and touching off the insert to the media.

Here we demonstrate the ability to automate the steps necessary to place hMSCs into the microplate inserts, track spheroid formation, and monitor spheroids during differentiation into chondrocyte lineages. By incorporating automation, the simplicity and repeatability of cell dispensing and spheroid formation monitoring procedures can be improved compared to manual processing. Use of a non-contact liquid dispenser ensures that consistent cell numbers are dispensed evenly across each insert. Automated brightfield imaging allows cells and spheroids across the entire area of each insert to be visualized without manual intervention. Expression of normal mesenchymal cell protein markers in undifferentiated hMSC spheroids, as well as chondrocyte differentiation of spheroidal cells upon induction was confirmed through immunofluorescence. The combination of an appropriate high density plate model and automated processing offers an easy-to-use, robust method to create the high density numbers of hMSC spheroids needed for important therapeutic applications.

Materials and Methods

Materials

Cells

Poietics™ Normal Human Bone Marrow Derived Mesenchymal Stem Cells (Catalog No. PT-2501), Mesenchymal Stem Cell Growth Medium (MSCGM) BulletKit™ (Catalog No. PT-3001), and hMSC Chondrogenic BulletKit™ (Catalog No. PT-3003) were donated by Lonza (Basel, Switzerland).

Hanging Drop Spheroid Formation System

500 µm micro-hole hanging drop devices and 6-well plates (Catalog No. MPc 500 6), and 24-well micro-space cell culture plates, with a 700 µm depth (Catalog No. RB 500 700) were donated by Elplasia brands of Kuraray (Tokyo, Japan).

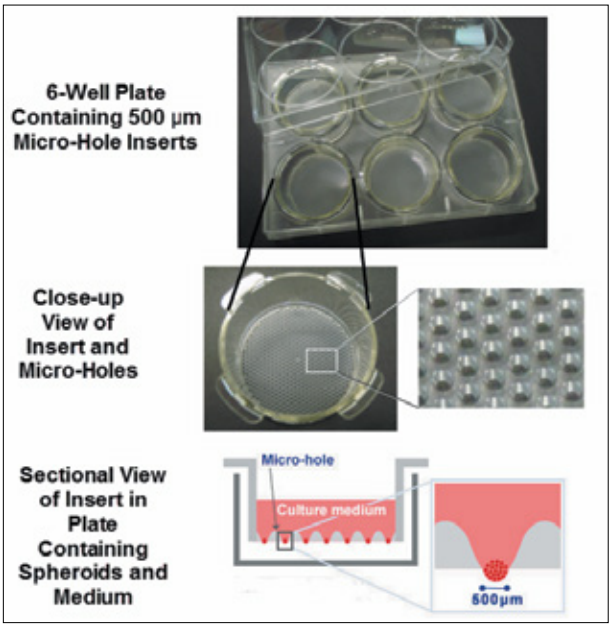

Figure 1. MPc 500 6 Micro-hole hanging drop insert design for spheroid formation.

Assay and Experimental Components

Goat anti-ITGB1/CD29 antibody (Catalog No. ED08199) was purchased from Everest (Oxfordshire, UK). Rabbit anti-CD44 monoclonal antibody [EPR1013Y] (Catalog No. ab51037), mouse anti-CD166 monoclonal antibody [8E12C7] (Catalog No. ab175428), rabbit anti-Collagen II polyclonal antibody (Catalog No. ab34712), donkey anti-goat IgG H&L (Alexa Fluor® 488) polyclonal antibody (Catalog No. ab150129), and donkey anti-rabbit IgG H&L (Alexa Fluor® 647) polyclonal antibody (Catalog No. ab150075) were purchased from abcam (Cambridge, UK). Goat anti-mouse IgG H&L (Alexa Fluor® 594) polyclonal antibody (Catalog No. A-11032) and Alexa Fluor® 488 Phalloidin (Catalog No. A-12379) were purchased from ThermoFisher Scientific (Waltham, MA). Hoechst 33342 (Catalog No. 14533) was purchased from Sigma-Aldrich (Saint Louis, MO).

Cytation™ 5 Cell Imaging Multi-Mode Reader

Cytation 5 is a modular multi-mode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, along with laser-based excitation for Alpha assays. The microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. With special emphasis on live-cell assays, Cytation 5 features shaking, temperature control to 65 °C, CO_2/O_2 gas control and dual injectors for kinetic assays. Integrated Gen5™ Microplate Reader and Imager Software controls Cytation 5, and also automates image capture, analysis and processing. The instrument was used to monitor spheroids in the inserts during the differentiation process using the brightfield channel, as well as capture fluorescent images to verify protein marker expression and chondrocyte differentiation.

MultiFlo™ FX with RAD™ Module

MultiFlo™ FX is a modular, automated reagent dispenser for 6- to 1536-well plates. Up to four independent reagents are dispensed in parallel without potential carryover. The choice of peristaltic or syringe pumps allows reagent conservation and unattended operation down to 500 nL. A wash module is available for use with 6- to 384-well plates. The RAD module allows liquid to be dispensed to individual wells of 6- to 384-well plates. The instrument was used to dispense hMSC suspension to the hanging drop inserts.

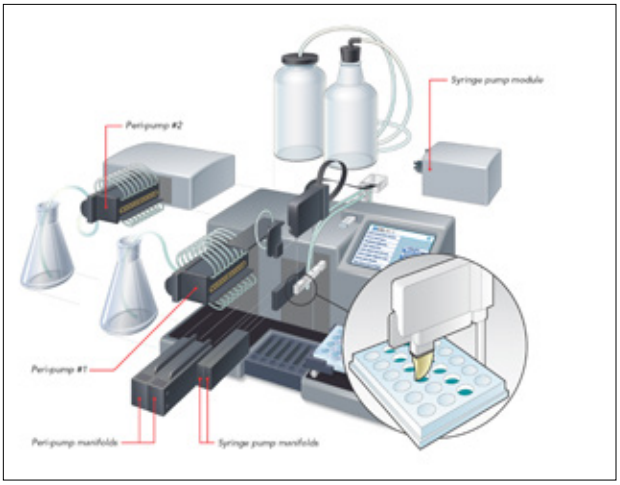

Figure 2. MultiFlo FX with RAD technology.

Methods

Cell Dispensing

hMSCs were thawed from cryopreservation, resuspended in complete MSCGM™ BulletKit™, and dispensed into three separate T-75 flasks at a concentration of 5000 cells per cm^2, per the vendors recommended protocol. Cells were allowed to propagate in the flasks for seven days while the cells reached a confluency of 80%. hMSCs were then removed from the flasks and resuspended in either complete MSCGM™ Medium or complete chondrogenic induction medium at a concentration of $8.5x10^4$ cells/mL. The RAD module of the MultiFlo FX was then used to dispense ten 400 µL aliquots of cell suspension to separate locations of each insert (Figure 3) using the x-, y-, and z-axis offsets programmed into the Liquid Handling Software (Table 1). The dispensing pattern was optimized to guarantee cells were evenly distributed across the insert. The z-axis height was further optimized to ensure that force of the cell suspension moving from the RAD module tip to the insert surface was low enough to maintain the hMSCs

on the top of the insert. Cells were also dispensed into a T-25 flask to act as 2D controls. Medium exchanges were performed every three days by removing ¾ volume of old medium and replacing with fresh growth or differentiation medium.

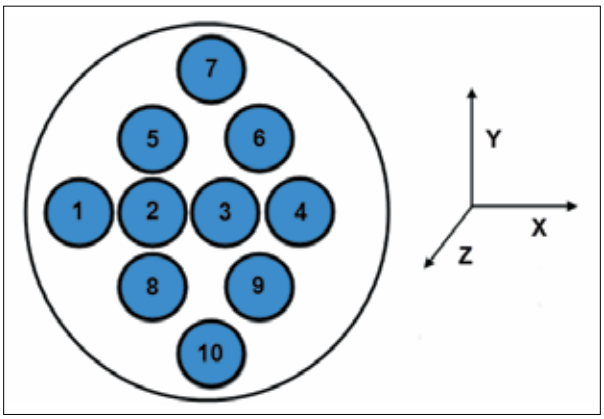

Figure 3. Multi-Flo FX RAD module hMSC suspension dispense pattern.

Axis Offset (steps)	1	2	3	4	5	6	7	8	9	10
X	-50	30	170	300	30	220	150	30	220	150
Y	0	0	0	0	50	50	99	-50	-50	-99
Z	260	260	260	260	260	260	260	260	260	260

Table 1. Liquid handling control step offsets for RAD module dispensing of hMSC suspension.

Brightfield Imaging of Spheroids within Insert Micro-holes

6-well plates containing hanging drop inserts were placed into the Cytation 5 every 4-6 hours to monitor spheroid formation and on a daily basis to confirm maintenance of spheroids within the inserts during the 14 day differentiation process following media exchanges. The parameters explained in Table 2 were used to accurately focus on the hMSC spheroids and stitch together the montage tiles into a final image.

Spheroid Imaging Parameters	
Objective	Meiji 2.5x
Imaging Channel	Brightfield
Imaging Focusing	Autofocus
Image Montage	18x12
Delay After Plate Movement	1000 msec
Montage Autofocus Option	Only focus on center of montage
Tile Overlap	Columns: 1000 µm/rows: 1000 µm

Table 2. Cytation 5 hanging drop insert imaging parameters.

hMSC and Chondrocyte Differentiation Marker Immunofluorescence Staining and Imaging

Following spheroid formation (hMSC antigen expression) or differentiation (chondrocyte differentiation validation), spheroids were transferred from the insert to the well of the 6-well plate below. This was accomplished by filling the well with 2 mL of appropriate growth or differentiation medium and touching off the bottom of the insert onto the medium (Figure 4).

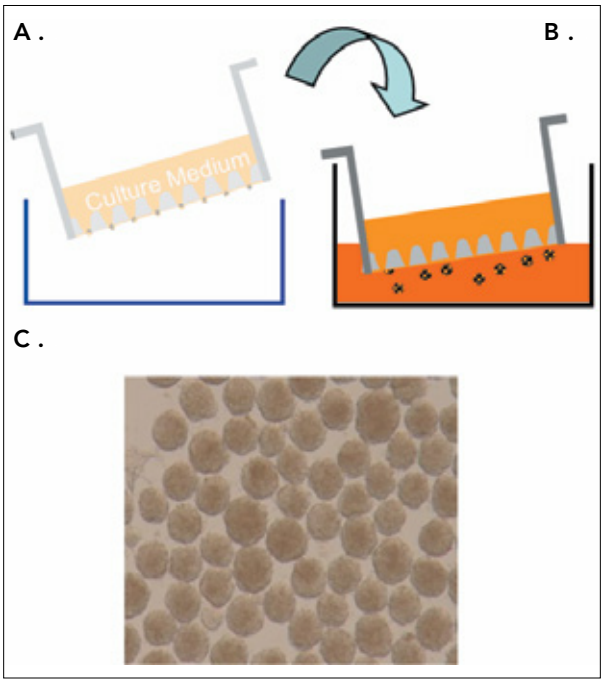

Figure 4. **Spheroid transfer from insert to microplate.** (**A.**) Insert is lifted and medium is added to well. (**B.**) Bottom of insert is touched to medium in well. (**C.**) Spheroids move from insert holes to medium below.

After spheroid transfer, a small number of differentiated or undifferentiated spheroids were moved via manual pipetting to the wells of the 24-well micro-space plate where immunofluorescent staining was performed with undifferentiated 3D cultured hMSCs and differentiated chondrocyte spheroid models using the procedure outlined in Table 3. Staining of undifferentiated hMSCs was also performed on cells cultured in the T-25 flask in a 2D manner. Expression of hMSC CD29, CD44, and CD166 surface antigen markers was assessed using the specific primary and secondary antibodies detailed in Table 4. Expression of the collagen II protein as validation of chondrocyte differentiation was determined using the antibodies explained in Table 5.

Number	Step	Explanation	Iteration/Incubation time
1	Wash	Aspirate media and add 1 mL PBS	Incubate 5 minutes following each addition/Repeat 2x
2	Fixation	Aspirate PBS and add 1 mL 4% paraformaldehyde	60 Minutes
3	Permeabilization	Aspirate 4% paraformaldehyde and add 1 mL 0.2% Triton-x100	60 Minutes
4	Wash	Aspirate media and add 1 mL PBS	
5	Blocking buffer addition	Aspirate PBS and add 1 mL 1% BSA / 5% goat serum in PBS	60 Minutes
6	Primary antibody preparation	Dilute primary antibody according to specifications	
7	Primary antibody addition	Aspirate PBS and add 1 mL diluted primary antibody	Overnight @ 4 °C
8	Wash	Aspirate primary antibody and add 1 mL PBS	Repeat 3x
9	Secondary antibody preparation	Dilute secondary antibody according to specifications	
10	Secondary antibody addition	Aspirate PBS and add 1 mL diluted secondary antibody	5 Hours @ RT
11	Wash	Aspirate secondary antibody and add 1 mL PBS	Repeat 3x
12	Imaging Preparation	Aspirate final wash and add 1 mL PBS	Store @ 4 °C until time of imaging

Table 3. **Spheroid Fixing and Staining Procedure.**

Antigen Marker	Primary Antibody	Dilution	Secondary Antibody	Dilution
CD29	Goat anti-ITGB1/CD29 antibody	1:100	Donkey anti-goat IgG H&L (Alexa Fluor® 488) polyclonal antibody	1:200
CD44	Rabbit anti-CD44 monoclonal antibody [EPR1013Y]	1:100	Donkey anti-rabbit IgG H&L (Alexa Fluor® 647) polyclonal antibody	1:200
CD166	Mouse anti-CD166 monoclonal antibody [8E12C7]	1:100	Goat anti-mouse IgG H&L (Alexa Fluor® 594) polyclonal antibody	1:200

Table 4. Antigen primary and secondary antibodies.

Differentiation Marker	Primary Antibody	Dilution	Secondary Antibody	Dilution
Collagen II	Rabbit anti-Collagen II polyclonal antibody	1:100	Donkey anti-rabbit IgG H&L (Alexa Fluor® 647) polyclonal antibody	1:200

Table 5. Collagen II primary and secondary antibodies

Following completion of the immunostaining procedure, the spheroids or 2D cultured cells were imaged by the Cytation 5 using a 20x objective to image 3D spheroids or a 10x objective to image 2D cultured cells. The imaging channels listed in Table 6 were used to capture the signal from the appropriate fluorescent probes.

Channel	Fluorescent Probe
DAPI	Hoechst 33342
GFP	Alexa Fluor 488
Texas Red	Alexa Fluor 594
CY5	Alexa Fluor 647

Table 6. Fluorescent antibody and probe Cytation 5 imaging channel setup.

Results and Discussion

hMSC Spheroid Formation Monitoring using Brightfield Imaging

By initially defining the imaging focal height as the position in the z-axis of the bottom of the inserts, and incorporating auto-focus, the imager is able to accurately focus at the level of the spheroids in the hanging drop (Figure 5). Brightfield images were then captured using a 2.5x objective to kinetically monitor the aggregation of hMSCs into spheroids in a label-free manner. Initial spheroid formation was seen after 10 hours (Figure 5A) and was completed by 48 hours (Figure 5B).

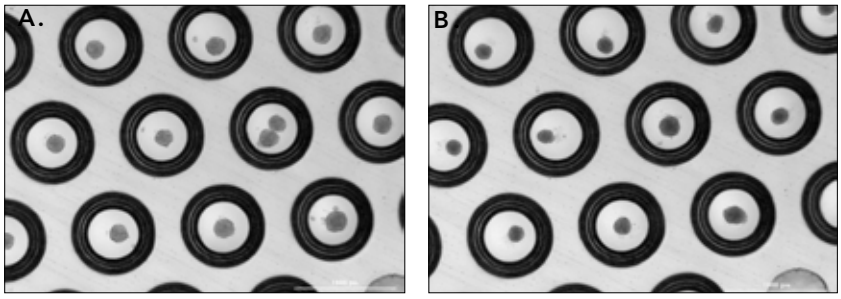

Figure 5. **Spheroid formation imaging.** Single tile 2.5x images of spheroids in individual holes of the hanging drop insert captured after (**A.**) 10; or (**B.**) 48 hours.

The individual tiles in the 18 row by 12 column montage were then stitched together to create a final single image in which all holes and spheroids can be viewed together. A custom tile overlap length of 1000 µm for rows and columns was selected to ensure accurate stitching (Figure 6).

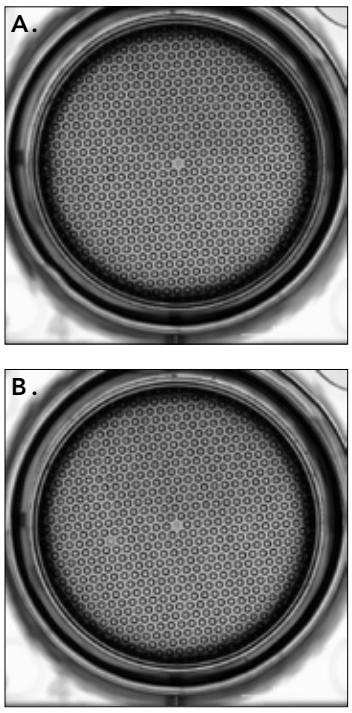

Figure 6. **Whole insert brightfield images.** Stitched images of 18 row by 12 column montage tiles exhibiting 6-well plate well, insert, and spheroids in micro-holes after (**A.**) 10; and (**B.**) 48 hour incubations.

The imaging procedure was repeated after each successive medium exchange. By viewing the captured 2.5x image tiles, it was confirmed that spheroids remained intact and were not removed during the 14 day process (Figure 7).

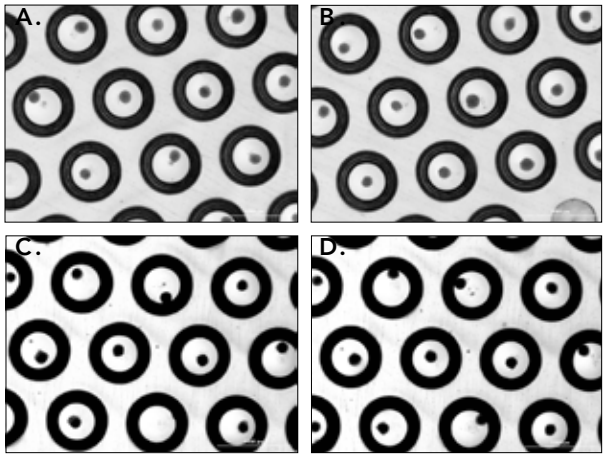

Figure 7. **Spheroid monitoring during hMSC differentiation.** Single tile 2.5x images of spheroids in growth medium after (**A.**) 7 and (**B.**) 14 day incubations, or chondrocyte differentiation medium after (**C.**) 7 and (**D.**) 14 day incubations.

Confirmation of hMSC Surface Antigen Expression in 2D and 3D Cell Models

By performing the immunofluorescent procedure detailed in Table 3 and adding the primary and secondary antibodies listed in Table 4, confirmation of commonly expressed hMSC surface antigen markers could be performed as a method to validate proper cell function when the cells were in a 3D spheroid configuration (Figure 8).

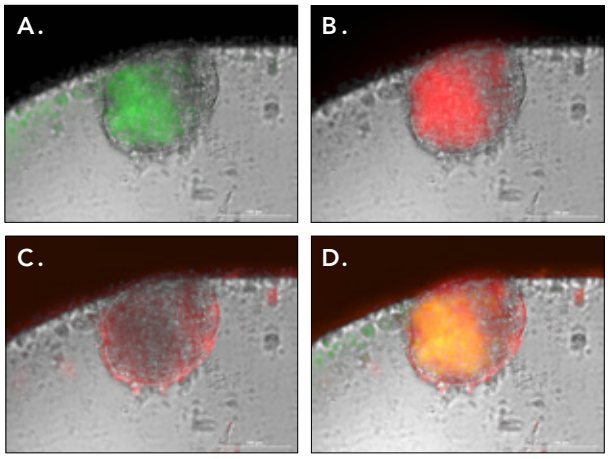

Figure 8. **3D hMSC surface antigen imaging.** 3D image-based surface antigen expression validation performed by capturing single images of hMSC spheroids using a 20x objective. (**A.**) GFP channel: CD29 expression, brightfield channel: whole spheroid; (**B.**) CY5 channel: CD44 expression, brightfield channel: whole spheroid; (**C.**) Texas Red channel: CD166 expression, brightfield channel: whole spheroid; (**D.**) overlay of GFP, CY5, Texas Red and brightfield channels.

Signal from binding of specific CD29, CD44, and CD166 antigen antibodies, in addition to fluorescently labeled secondary antibodies, was visualized in Figure A-C, as well as the overlaid images in Figure 8D. This finding confirmed that common surface antigen markers are expressed by hMSCs after aggregation into a 3D spheroid model.

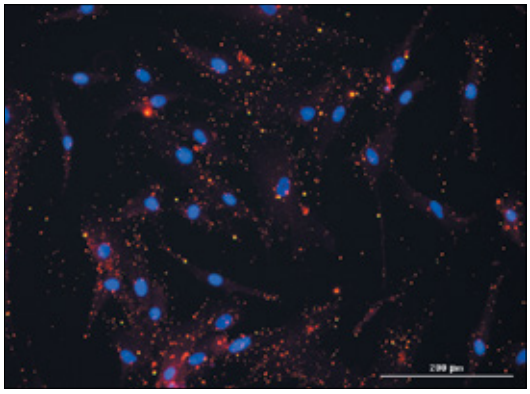

Figure 9. **2D hMSC surface antigen imaging.** 2D image-based surface antigen expression validation performed by capturing single images of hMSCs using a 10x objective. GFP channel: CD29 expression, CY5 channel: CD44 expression, Texas Red channel: CD166 expression, DAPI channel: Hoechst 33342 stained nuclei.

The same immunostaining procedure was carried out using hMSCs cultured in 2D with growth medium (Figure 9). Fluorescent signal once again is observed in the GFP, CY5, and Texas Red channels indicating expression of CD29, CD44, and CD166 antigens by 2D cultured hMSCs. Signal from bound antibodies appears as punctuate spots in 2D compared to images captured from 3D spheroids. Distinct areas of antigen expression can be visualized due to the fact that cells in 2D are in a monolayer. Cells in hMSC spheroid models are tightly aggregated together. Therefore signal appears over large areas of the total spheroid.

3D Chondrocyte Differentiation Validation

Collagen II, one of the prominent components of healthy cartilage[10] and produced by healthy chondrocytes, is a common marker of chondrocyte differentiation from hMSCs[11]. Following the 14 day incubation period, validation of differentiation into the chondrocyte lineage for hMSCs aggregated into 3D spheroids was carried out through immunostaining and the incorporation of a collagen II specific primary antibody.

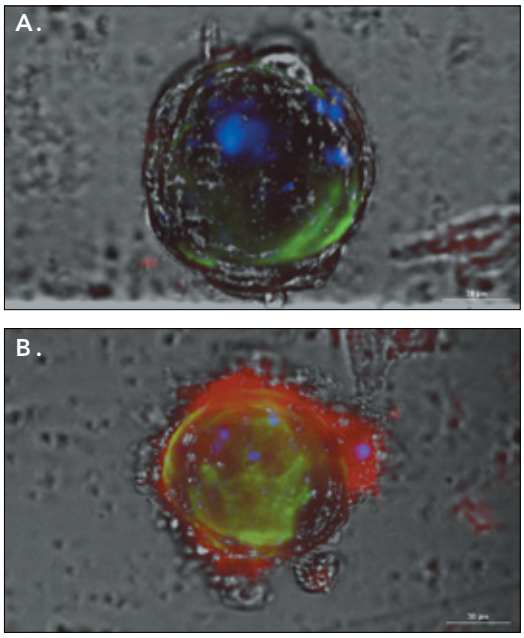

Figure 10. **3D chondrocyte differentiation imaging.** 3D image-based chondrocyte validation performed by capturing single images of hMSC spheroids incubated in (**A.**) growth medium, or (**B.**) complete chondrocyte differentiation medium using a 20x objective. DAPI channel: Hoechst 33342 stained nuclei, GFP channel: Alexa Fluor 488 phalloidin, CY5 channel: collagen II protein expression, brightfield channel: whole spheroid.

By comparing the fluorescent signal in the CY5 channel from the bound collagen II primary and Alexa Fluor 647 labeled secondary antibody from hMSC spheroids incubated in growth (Figure 10A) or chondrocyte differentiation medium (Figure 10B), a visual change in protein expression is witnessed. The visual difference in fluorescence can then be quantified using the cellular analysis capabilities in the Gen5 software. By applying the parameters listed in Table 7, detailed object masks can be applied around the spheroids in each image (Figure 11A). This allows the signal in each imaging channel to be quantified only from the spheroid, and not from other portions of the image, increasing the robustness of the validation method.

Spheroid Cellular Analysis Parameters	
Imaging Channel	Brightfield
Threshold	10,000
Background	Light
Split Touching Objects	Unchecked
Fill Holes in Masks	Checked
Min. Object Size	75 µm
Max. Object Size	150 µm
Advanced Analysis Parameters	
Rolling Ball Diameter	200 µm
Image Smoothing Strength	5 Cycles of 3x3 Average Filter
Evaluate Background On	5% of Lowest Pixels

Table 7. **Whole spheroid cellular analysis parameters.**

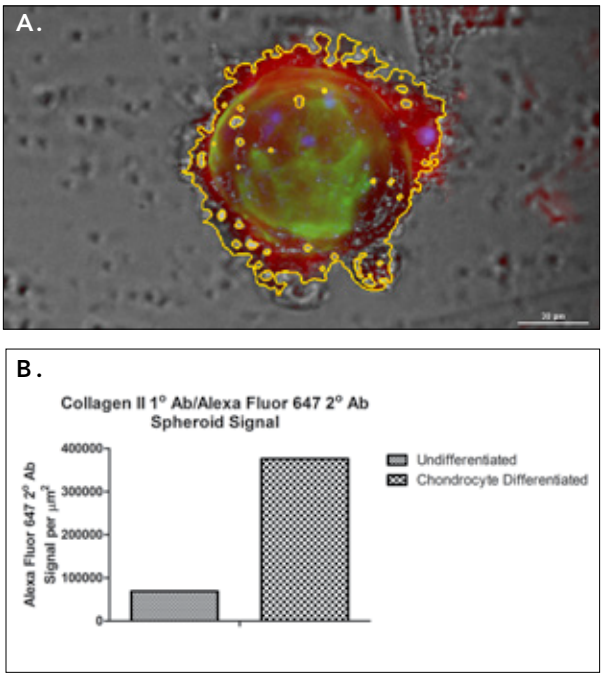

Figure 11. **3D chondrocyte differentiation cellular analysis and quantification.** (**A.**) Object mask applied to hMSC spheroid incubated 14 days in chondrocyte differentiation medium. (**B.**) Fluorescent signal captured in CY5 channel from undifferentiated and differentiated spheroids normalized to spheroid area.

The total CY5 signal intensity quantified within the object mask for each test spheroid (Object Int[CY5 628, 685]) was divided by the area of the spheroid within the same object mask (Object Area) using the following formula:

Object Int[CY5 628, 685]/Object Area

This allows the fluorescent signal from the bound antibodies to be normalized to any changes in the area

of the aggregated spheroids being tested. The final signal per μm^2 values in Figure 11B illustrate that collagen II expression is greatly increased in spheroids incubated in chondrocyte differentiation medium, validating that 3D hMSC spheroids can be differentiated into chondrocytes or other desired lineages using the hanging drop micro-hole insert method.

Conclusions

The 500 µm micro-hole hanging drop devices and 6-well plates represent a dependable method to simplify the process of creating large numbers of human mesenchymal stem cell spheroids for downstream tissue generation applications. Stem cell differentiation can also be performed while spheroids remain in the inserts. By incorporating automated liquid dispensing using the MultiFlo™ FX RAD module, consistent cell suspension dispensing can be achieved in a repeatable manner increasing the creation of similarly sized spheroids and reducing the time required for manual processing. The brightfield and fluorescent imaging capabilities then allow for real time kinetic monitoring of spheroids within the hanging drop inserts, in addition to qualitative and quantitative assessment of stem cell and differentiation markers before incorporation into final downstream applications.

References

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