Application Note

Stem Cell Research

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An Automated Walk-Away System to Perform Differentiation of 3D Mesenchymal Stem Cell Spheroids

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Introduction

Human mesenchymal stem cells (hMSCs) are multipotent and found in multiple areas of the body including bone marrow, skeletal muscle, dermis, and blood. The cells are known for their ease of isolation and ability to differentiate and mature into multiple lineages including adipocytes, chondrocytes, and osteocytes. hMSCs also play a critical role in adult tissue repair, therefore are of great interest in tissue engineering applications. For example, as adult cartilage cannot repair itself, chondrocyte-derived hMSCs may be used for cartilage repair applications, and in fact, transplantation of spheroidal chondrocytes is already being studied as a treatment for hip joint cartilage defects.[1] Initial hMSC experimentation involved two-dimensional (2D) cell culture in a monolayer. However, culturing the cells in this manner results in a loss of replicative ability, and differentiation capability over time.[2,3] A number of techniques to culture hMSCs in a three-dimensional (3D) format were then incorporated, such as pellet and micromass culture.[4,5] These methods better exemplified the differentiation process, but disadvantages included requiring large numbers of cells, difficult manual processing steps, and a high overall cost per method. Recently developed 3D cell culture technologies, which have the ability to create spheroids from smaller cell numbers in high density microplates, can overcome the limitations of earlier methods while still providing the necessary environment for proper stem cell differentiation.

Complete differentiation from multipotent hMSCs to final target lineages, such as chondrocytes, typically takes 14 to 28 days. With media exchanges required every 2 to 3 days, a manual process is not only tedious, but when working with nonattached cells, increases the risk of accidental spheroid removal. Automating the processing steps and incorporating a 3D magnetic bioprinting method frees researchers to perform other tasks and increases repeatability with little to no risk of spheroid loss. In this method (Figure 1), cells are first incubated with a biocompatible magnetic nanoparticle assembly consisting of gold, iron oxide, and poly-L-lysine, which magnetizes the cells without eliciting deleterious biological effect. The cells are then placed into a microplate well and levitated by placing a magnet above the well, where they aggregate and form extracellular matrix (ECM) within a few hours. After levitation, the magnet is removed, and the 3D aggregates are dissociated into a disperse cell suspension of single cells and small cell aggregates by gentle pipetting action. Cells are then transferred to a 384-well assay plate and a spheroid magnet is positioned below the plate for an appropriate incubation period, allowing the cells within each well to be patterned into a spheroid configuration. The magnetized spheroid can be held intact during regular media exchanges.

Here we demonstrate the validation of a combined solution to perform automated chondrocyte differentiation from 3D hMSC spheroids, where all instrumentation was contained within a laminar flow hood. A combination washer/dispenser with magnetic plate adapter was used for media exchanges, while an automated incubator maintained proper microplate environmental conditions between exchanges. Label-free cellular imaging under environment control was performed following media exchanges to confirm maintenance of spheroids during processing. Immunofluorescence following differentiation confirmed the effectiveness of the system for use with critical stem cell differentiation. The combination of 3D magnetic bioprinting, automated liquid handling and incubation, and image-based analysis provides easy-to-use, robust methods to optimize hMSC spheroid creation and differentiation processes.

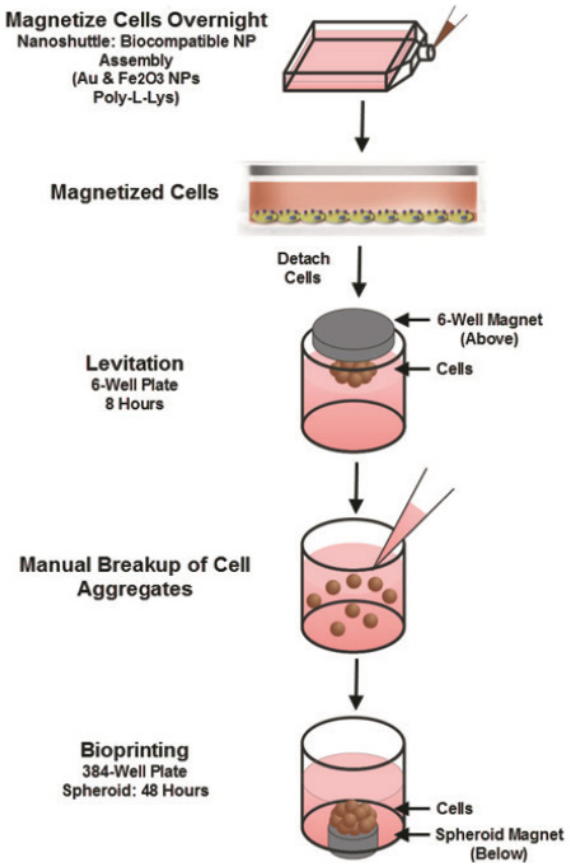

Figure 1. BiO Assay Kit protocol. The 384-Well BiO Assay Kit uses the NanoShuttle-PL nanoparticle assembly to magnetize cells. After incubation, cells are detached, resuspended in a cell-repellent plate, and magnetically levitated to aggregate and induce ECM. After breaking up the aggregates, single cells are transferred to a 384-well cell-repellent plate placed atop a 384-well magnet, where they are printed at the well bottom.

Materials and methods

Materials

Cells

Poietics Normal Human Bone Marrow Derived Mesenchymal Stem Cells (part number PT- 2501), Mesenchymal Stem Cell Growth Medium (MSCGM) BulletKit (part number PT-3001), and hMSC Chondrogenic BulletKit (part number PT- 3003) were donated by Lonza (Basel, Switzerland).

Antibodies and inhibitor compounds

Goat anti-ITGB1/CD29 antibody (part number ED08199) was purchased from Everest (Oxfordshire, UK). Rabbit anti-CD44 monoclonal antibody [EPR1013Y] (part number ab51037), mouse anti-CD166 monoclonal antibody [8E12C7] (part number ab175428), rabbit anti-Collagen II polyclonal antibody (part number ab34712), donkey anti-goat IgG H&L (Alexa Fluor 488) polyclonal antibody (part number ab150129), and donkey anti-rabbit IgG H&L (Alexa Fluor 647) polyclonal antibody (part number ab150075) were purchased from abcam (Cambridge, UK). Goat anti-mouse IgG H&L (Alexa Fluor 594) polyclonal antibody (part number A-11032) and Alexa Fluor 488 Phalloidin (part number A-12379) were purchased from ThermoFisher Scientific (Waltham, MA). Hoechst 33342 (part number 14533) was purchased from Sigma-Aldrich (Saint Louis, MO).

Assay and experimental components

The 384-Well BiO Assay Kit (GBO part number 781846, consisting of 2 vials NanoShuttle-PL, 6-Well Levitating Magnet Drive, 384-Well Spheroid and Holding Magnet Drives (2), 96-Well Deep Well Mixing Plate, 6-Well and 384-Well Clear Cell Repellent Surface Microplates), prototype 384-Well Ring Drive and additional Cell Repellent Surface 6-Well (GBO part number 657860) were generously donated by Nano3D Biosciences, Inc., (Houston, TX) and Greiner Bio-One, Inc., (Monroe, NC).

Agilent BioTek Cytation 5 cell imaging multimode reader

Cytation 5 is a modular multimode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield, and phase contrast. The instrument can perform fluorescence imaging in up to four channels in a single step. With special emphasis on live-cell assays, Cytation 5 features temperature control, CO_2/O_2 gas control, and dual injectors for kinetic assays, and is controlled by integrated Agilent BioTek Gen5 microplate reader and imager software. The software was also used for dual-masking and automated analyses.

Agilent BioTek BioSpa 8 automated incubator

The BioSpa 8 automated incubator links Agilent BioTek readers or imagers together with Agilent BioTek washers and dispensers for full workflow automation of up to 8 microplates. Temperature, CO_2/O_2 and humidity levels are controlled and monitored through the Agilent BioTek BioSpa software to maintain an ideal environment for cell cultures during all experimental stages.

Agilent BioTek EL406 combination washer dispenser

The EL406 offers fast, accurate media removal and plate washing capabilities through its dual-action manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps, with volumes ranging from 500 nL to 3,000 µL/well. A specialized magnet adapter (part number 7182104) and 384-well flat magnet (part number 7103017) were used to secure placement of the 3D spheroids during media transfer steps.

Methods

Cell preparation and spheroid formation

hMSCs were thawed from cryopreservation, resuspended in complete MSCGM medium, and dispensed into three separate T-75 flasks at a concentration of 5,000 cells/cm^2, per the vendor's recommended protocol. Cells propagated in the flasks for seven days while the cells reached a confluency of 80%. A 600 µL volume of NanoShuttle-PL was then added to each flask and incubated overnight. Cells were removed from the flasks and added to the 6-well cell repellent plate at a concentration of 1.2×10^6 cells/mL in a volume of 2 mL/well. A 6-well magnet drive was placed atop the plate to levitate the cells, where they aggregated and induced ECM formation during an eight-hour incubation at 37 °C/5% CO_2. After incubation, cells and ECM were broken up and resuspended. A total of 5,000 cells were added to wells in a 384-well cell repellent microplate intended for 3D spheroid formation, while a total of 10,000 cells were added to wells in a 384-well cell culture microplate intended for 2D cell culture in a volume of 50 µL mesenchymal stem cell complete growth media. The process was replicated for a total of four microplates intended for each cell culture method. A magnet was placed under each 3D spheroid plate. All microplates were incubated at 37 °C/5% CO_2 for approximately 48 hours to allow the 2D cells to attach to the microplate well bottom, and to allow the 3D cells to aggregate into spheroids within each well.

hMSC confirmational imaging

Prior to initiating the differentiation process, immunofluorescent staining was performed on a subset of undifferentiated 3D cultured spheroids to confirm proper hMSC functionality via expression of common biomarker proteins using the procedure outlined in Table 1. Undifferentiated hMSC staining was also performed on 2D cultured cells using generally accepted staining methods. Expression of hMSC CD29, CD44, and CD166 surface antigen markers was assessed using the specific primary and secondary antibodies detailed in Table 2.

Table 1. Spheroid fixing and staining procedure.

No.	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% Tritonx100	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 at 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 at 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 at 4 °C until time of imaging

Table 2. Antigen primary and secondary antibodies.

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

After the 3D spheroids or 2D cultured cells were immunostained, they were imaged using a 20x objective or a 10x objective, respectively, using the fluorescence channels listed in Table 3.

Table 3. Fluorescent probe and Cytation 5 imaging channel setup.

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

Automated stem cell differentiation

After incubation to allow 2D cell attachment and 3D spheroid creation, the plates were placed into the BioSpa 8 at 37 °C/5% CO_2 for up to twenty days during the differentiation period. The BioSpa 8 method was programmed such that plates were automatically moved to the EL406 on day 0 and every three days subsequent to replace the respective media. The EL406 was fitted with a specialized magnet adapter and 384-well flat magnet to secure the 3D spheroids during liquid handling. A one-minute resting period allowed the spheroids to magnetically secure at the well bottom, after which EL406's aspirate pins removed 75% of the spent media from the wells of each microplate, and new growth media was added via the peripump to negative control wells for a total volume of 50 µL per well, while chondrocyte differentiation media was dispensed in the same manner to positive control wells. Following media exchange, the BioSpa 8 arm then automatically moved each plate from the EL406 to Cytation 5, where brightfield imaging was performed to confirm successful media exchange without loss of cells. The parameters listed in Table 4 were used to accurately focus on the hMSC spheroids and stitch together the montage tiles into a final image.

Table 4. Cytation 5 imaging parameters.

Brightfield Imaging Parameters	
Objective	4x
Imaging Channel	Brightfield
Image Focusing	Autofocus
Image Montage	3 × 2
Delay after Plate Movement	30 msec
Montage Autofocus Option	Only focus on center of montage
Tile Overlap	Columns: 197 µm/Rows: 300 µm

Expression of the collagen II protein, a prominent component of healthy cartilage[6] and a validation of chondrocyte differentiation[7], was determined using the antibodies in Table 5.

Table 5. Collagen II 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

Additionally, at Day 5, 10, and 20, one microplate each containing 2D cells and 3D spheroids was removed from BioSpa 8, and fluorescent immunostaining per the aforementioned procedure was performed to detect collagen formation. A placeholder microplate was substituted for each removed assay plate to maintain the robotic protocol.

Results and discussion

hMSC confirmational imaging

Proper hMSC function was validated by confirming the presence of commonly expressed surface antigen markers. As seen in Figure 2, fluorescent signals corresponding to CD29, CD44, and CD166 surface antigen markers were detected in 2D cultured cells and 3D cultured spheroids. Signal from bound primary and fluorescently labeled secondary antibodies appear as punctuate spots within each image, indicating distinct areas of antigen expression within 2D or 3D cultured cells.

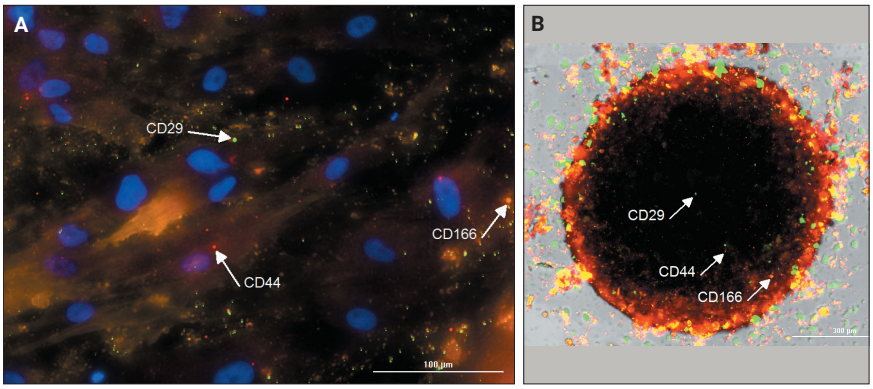

Figure 2. hMSC biomarker expression as imaged by Cytation 5. Arrows indicate immunofluorescence identification of protein expression to confirm proper cell function in (A) 2D cultured cells using 10x objective and; (B) 3D cultured spheroids using 20x objective. DAPI: Hoechst 33342 stained nuclei, GFP: CD29 expression, Texas Red: CD166 expression, CY5: CD44 expression.

Automated stem cell differentiation

During designated media exchange periods, brightfield imaging was performed to confirm that cells and spheroids remained intact during the aspiration and dispense procedure. As seen in Figures 3A to 3C, 3D spheroids are confirmed to remain intact in the wells during media exchanges over the entire twenty-day incubation. The same can be said for 2D cultured cells up to 10 days of incubation (Figures 3D and 3E). However, after 10 days of culture in the plates, visible cell loss is witnessed following media exchange (Figure 3F). This observation confirms previous research findings that 2D cultured cells lose integrity, detach, and become non-viable following ten days of incubation.[8]

Chondrocyte differentiation in 2D cultured cells was then examined by comparing cells cultured in differentiation media to those remaining undifferentiated in growth media. Per Figure 4, initial chondrocyte differentiation (Figure 4D) is seen within five days of incubation, and rapidly peaks at ten days (Figure 4E), compared to no differentiation in cells cultured in growth media (Figures 4A to 4C). After ten days, loss of viability occurs in all 2D cultured cells; and in the differentiated cells, the collagen II fluorescent probe is leached into the surrounding media (Figure 4F). This confirms the limitations associated with incorporating 2D differentiated hMSCs in long-term studies.

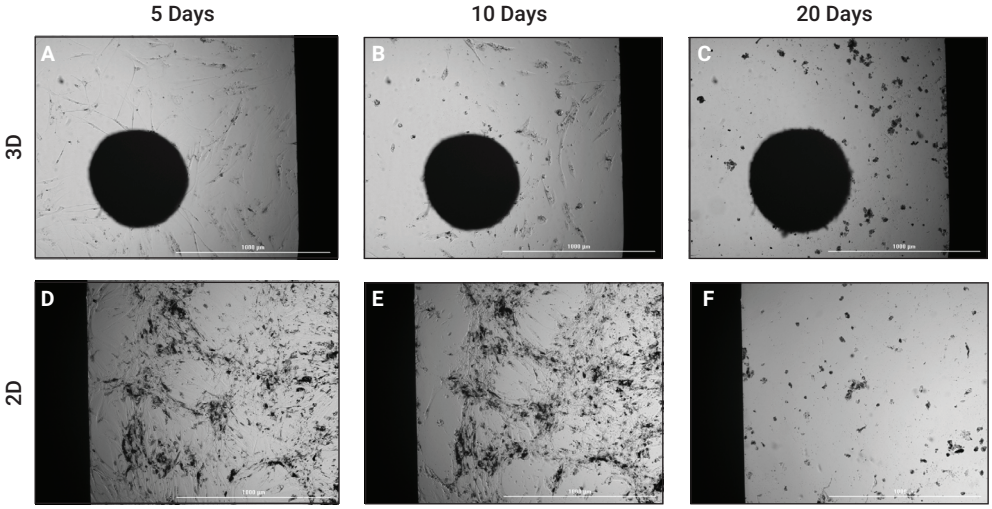

Figure 3. Postmedia exchange imaging validation. Day 5, Day 10, and Day 20 3 × 2 montage brightfield images captured using a 4x objective of (A to C) 3D spheroids; and (D to F) 2D cultured cells demonstrating visible cell loss starting at Day 10.

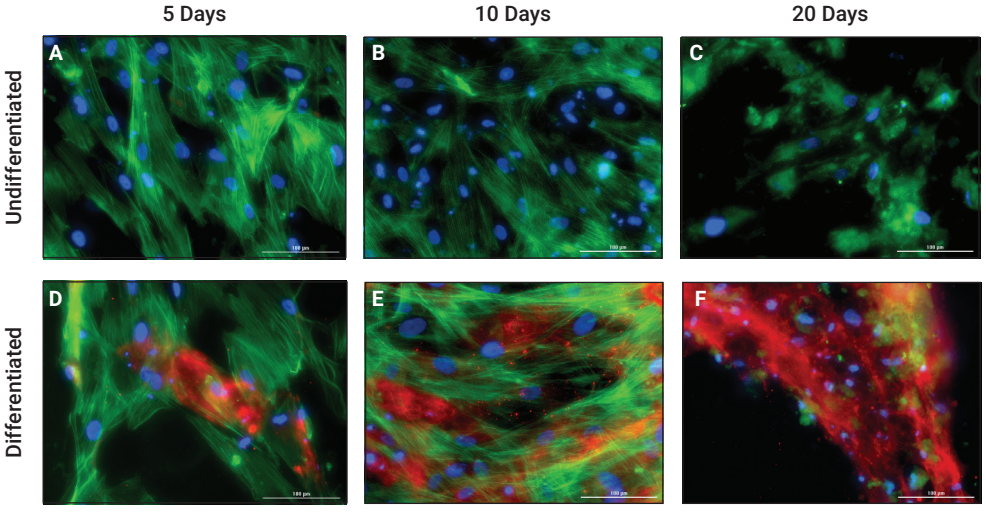

Figure 4. 2D cultured hMSC chondrocyte differentiation over time. Day 5, Day 10, and Day 20 images of (A to C) undifferentiated cells; and (D to F) differentiated cells, captured using 10x objective. DAPI: Hoechst 33342 stained nuclei, GFP: AlexaFluor 488 phalloidin stained actin filaments, CY5: Collagen II expression.

In the same manner, chondrocyte differentiation in 3D cultured spheroids was examined by comparing spheroids cultured in differentiation media to those remaining undifferentiated in growth media. Per Figures 5A to 5C, no discernible collagen expression is seen in undifferentiated spheroids, while a steady increase in collagen expression over time is seen in differentiated spheroids (Figures 5D to 5F). This confirms the suitability of 3D cultured and differentiated hMSC spheroids for long-term studies. Additionally, the differentiated spheroid images were overlaid at individual z-planes (Figure 6) to improve image clarity and enable quantification of differentiation.

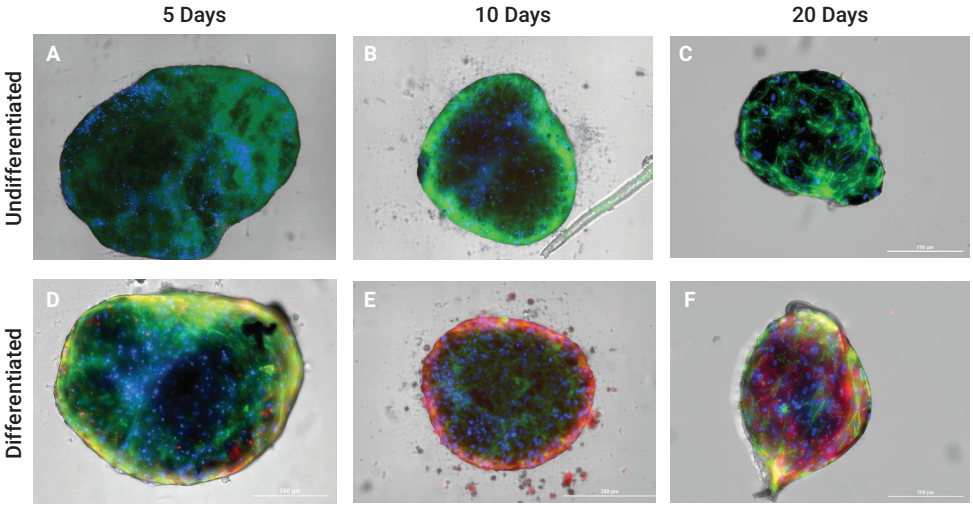

Figure 5. 3D cultured hMSC spheroid chondrocyte differentiation over time. Day 5, Day 10, and Day 20 images of (A to C) undifferentiated spheroids; and (D to F) differentiated spheroids, captured using 20x objective. DAPI: Hoechst 33342 stained nuclei, GFP: AlexaFluor 488 phalloidin stained actin filaments, CY5: Collagen II expression.

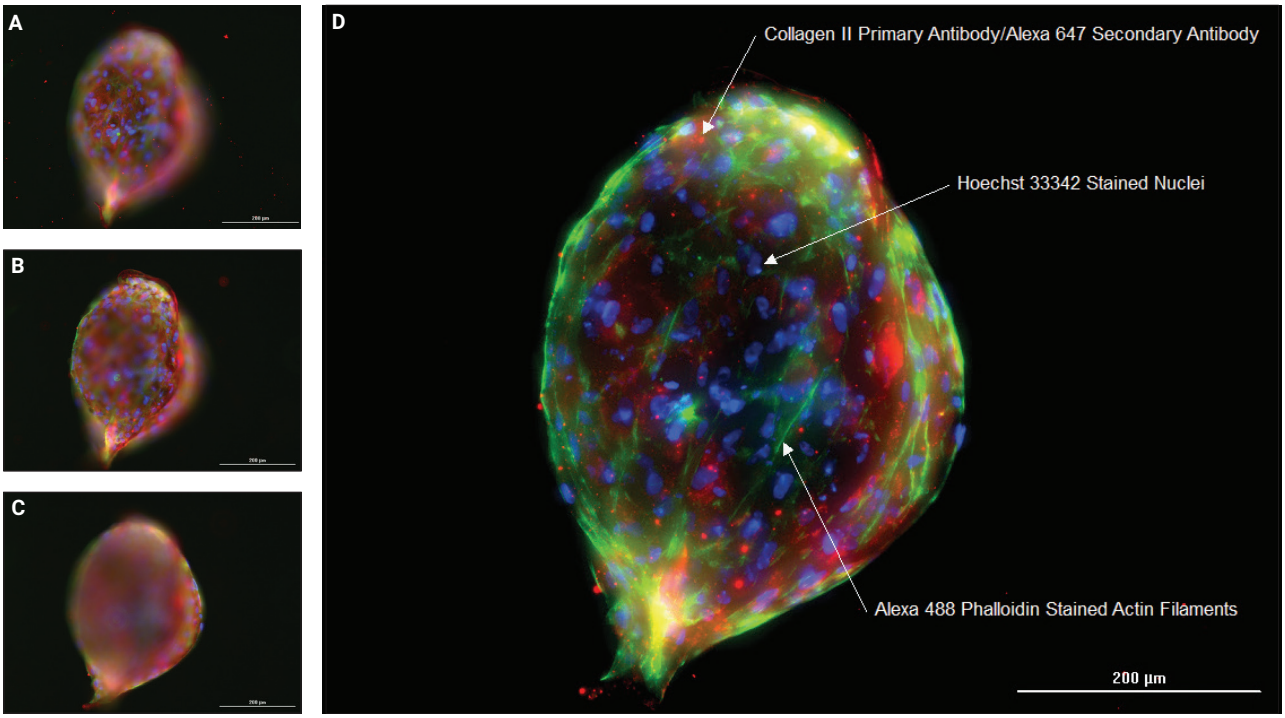

Figure 6. Z-stacking and projection of 3D spheroid images. (A to C) Images captured at individual z-planes. (D) Final z-projected image of chondrocyte differentiated hMSC spheroid. Arrows indicate nuclei, collagen, and protein expression. DAPI: Hoechst 33342 stained nuclei, GFP: AlexaFluor 488 phalloidin stained actin filaments, CY5: Collagen II expression.

Quantification of chondrocyte differentiation via cellular analysis

Using the z-stacked image, Agilent BioTek Gen5 software automatically preprocessed the samples to remove excess background signal and prepare the image for quantitative analysis. Primary cellular analysis criteria were applied to place an object mask around the entire spheroid. Secondary analysis criteria were then used to automatically mask areas within the spheroid where the CY5 signal from collagen II antibody labeling was greater than background threshold levels as indicated by the arrows in Figure 7.

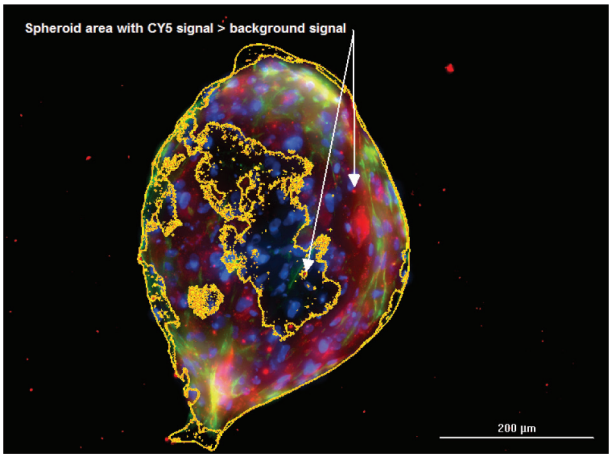

Figure 7. Automated dual-mask analysis, with primary mask placed around the entire spheroid, and secondary mask placed around discontinuous areas of increased CY5 signal.

The percent of CY5 area coverage, indicating greater differentiation and collagen II expression can then be calculated as a ratio of the secondary mask to the primary mask, expressed as a percentage. The final percentage values in Figure 8 indicate a significant increase in collagen II production in chondrocyte differentiated hMSC spheroids compared to undifferentiated hMSC spheroids, thus validating that 3D cultured hMSC spheroids can be successfully differentiated into chondrocytes.

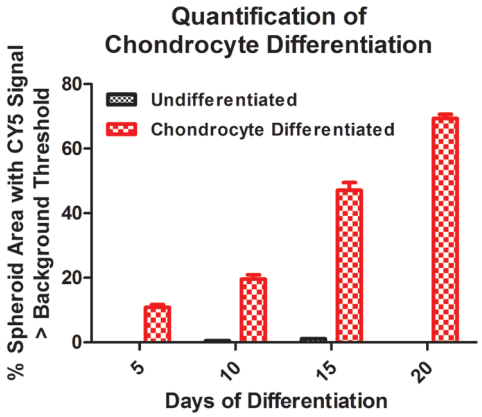

Figure 8. Change in CY5 signal compared to background threshold over time.

Conclusion

The 384-Well BiO Assay Kit and NanoShuttle-PL particles manufactured by nano3D Biosciences, combined with Greiner Bio-One Cell-Repellent Surface 6-Well and 384-Well Microplates, provide a simple and robust method to create biomimetic hMSC spheroids. Through incorporation of the Agilent BioTek BioSpa 8 and magnetic adapter on the Agilent BioTek EL406, the differentiation process can be automated to simplify and increase the repeatability of included procedures. Automation and differentiation confirmation can then be performed using brightfield and fluorescent imaging with the Agilent BioTek Cytation 5. The combination provides a proven method to carryout differentiation of 3D cultured stem cells.

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