An Automated Walkaway 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 of 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 14-28 days. With media exchanges required every 2-3 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, such as the 384-Well BiO Assay Kit and NanoShuttle-PL particles from nano3D Biosciences, frees researchers to perform other tasks and increases repeatability with little to no risk of spheroid loss.

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 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.

BioTek Instrumentation

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, 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 to 65 °C, CO_2/O_2 gas control and dual injectors for kinetic assays, and is controlled by integrated Gen5™ Microplate Reader and Imager Software. The software was also used for dual-masking and automated analysis.

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

EL406™ Combination Washer Dispenser: The EL406 offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ Manifold along with reagent dispensing through peristaltic or syringe pumps, with volumes ranging from 500 nL – 3000 µL/well. A specialized magnet adapter and 384-well flat magnet were used to secure 3D spheroid placement during media transfer.

2D and 3D hMSC Cell Preparation

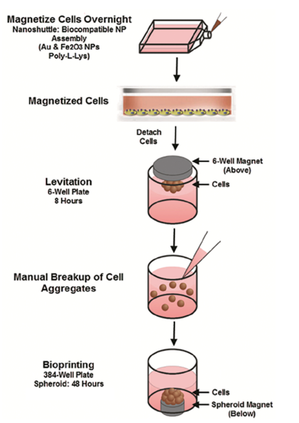

Figure 1. 384-Well BiO Assay™ Kit protocol

Normal Human Bone Marrow Derived hMSCs (Lonza, Basel, Switzerland) were thawed from cryopreservation, resuspended in complete mesenchymal stem cell complete growth media (MSCGM), and dispensed into three separate T-75 flasks at a concentration of 5000 cells/cm^2, per the vendor's recommended protocol. Cells propagated in the flasks for seven days until they 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 1.2x10^6 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 extra-cellular matrix (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 5000 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 MSCGM. 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.

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. A one-minute resting period allowed the spheroids to magnetically secure at the well bottom, after which EL406's aspirate pins removed the spent media from the wells, and new growth media was added via the peripump to negative control wells, 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, and montage tiles were automatically stitched into a final image. Expression of the collagen II protein as a validation of chondrocyte differentiation was assessed using specific primary and secondary antibodies. Additionally, at Day 5, 10, 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.

hMSC Confirmed Images

Immunofluorescent staining was performed on a subset of undifferentiated 3D cultured spheroids to confirm proper hMSC functionality via expression of common biomarker proteins using an optimized staining protocol. 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 specific primary and secondary antibodies. After the 3D spheroids or 2D cultured cells were immunostained, they were imaged in Cytation 5 using a 20x objective or a 10x objective, respectively, to capture signal from the appropriate fluorescent probe.

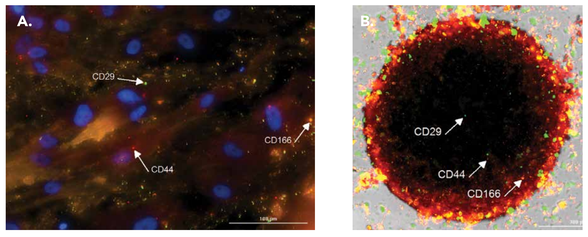

Figure 2. hMSC biomarker expression as imaged by Cytation 5. Arrows indicate immunofluorescence identification of protein expression to confirm proper cell function in **(A)** 2D cultured cells using 10x objective and; **(B)** 3D cultured spheroids using 20x objective. DAPI: Hoechst 33342 stained nuclei, GFP: CD29 expression identified by goat anti-ITGB1/CD29 1° Ab and donkey anti-goat IgG H&L (Alexa Fluor® 488) 2° Ab, Texas Red: CD166 expression identified by mouse anti-CD166 monoclonal 1° Ab [8E12C7] and goat anti-mouse IgG H&L (Alexa Fluor 594) 2° Ab, CY5: CD44 expression identified by rabbit anti-CD44 monoclonal 1° Ab [EPR1013Y] and donkey anti-rabbit IgG H&L (Alexa Fluor 647) 2° Ab.

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, confirming the presence of commonly expressed surface antigen markers. 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.

Post-Media Exchange Imaging

Following designated media exchange periods, brightfield imaging was performed to confirm that cells and spheroids remained intact during the aspiration and dispense procedure.

Figure 3. Post-media exchange imaging validation. Day 5, Day 10 and Day 20 3x2 montage brightfield images captured using a 4x objective of **(A-C)** 3D spheroids; and **(D-F)** 2D cultured cells demonstrating visible cell loss starting at Day 10.

As seen in Figures 3A-C, 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[6].

2D Chondrocyte Differentiation Confirmational Imaging

Differentiation from multipotent hMSCs to chondrocytes in 2D cultured cells was then examined by comparing cells cultured in differentiation media to those remaining undifferentiated in growth media. Expression of collagen II, a commonly used marker of chondrocyte differentiation, was assessed using a specific collagen II primary and fluorescently labeled secondary antibody.

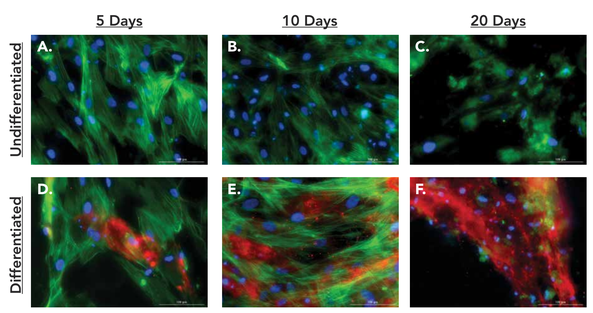

Figure 4. 2D cultured hMSC chondrocyte differentiation over time. Day 5, Day 10 and Day 20 images of **(A-C)** undifferentiated cells; and **(D-F)** differentiated cells, captured using 10x objective. DAPI: Hoechst 33342 stained nuclei, GFP: Alexa Fluor 488 phalloidin stained actin filaments, CY5: Collagen II expression identified by rabbit anti-Collagen II polyclonal 1° Ab and donkey anti-rabbit IgG H&L (Alexa Fluor 647) 2° Ab.

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-C). 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.

3D Chondrocyte Differentiation Confirmational Imaging

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-C, 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-F). 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.

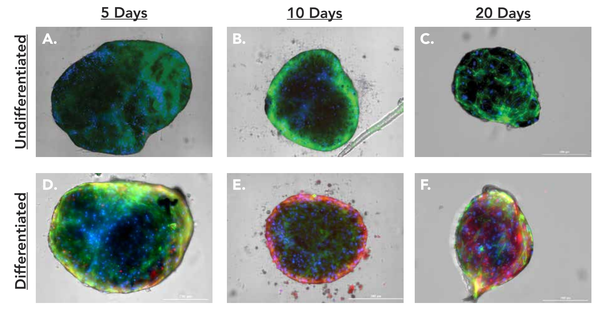

Figure 5. 3D cultured hMSC spheroid chondrocyte differentiation over time. Day 5, Day 10 and Day 20 images of **(A-C)** undifferentiated spheroids; and **(D-F)** differentiated spheroids, captured using 20x objective. DAPI: Hoechst 33342 stained nuclei, GFP: Alexa Fluor 488 phalloidin stained actin filaments,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

Quantification of Chondrocyte Differentiation via Cellular Analysis

Images of 3D spheroids were captured on multiple z-planes using the z-stacking capabilities in the Gen5 software (Figure 6A-C). A final projected image was then created by Gen5 to increase the clarity of the different stained cellular areas and collagen II protein (Figure 6D).

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

Using the z-stacked image, Gen5 software automatically pre-processed 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 7A.

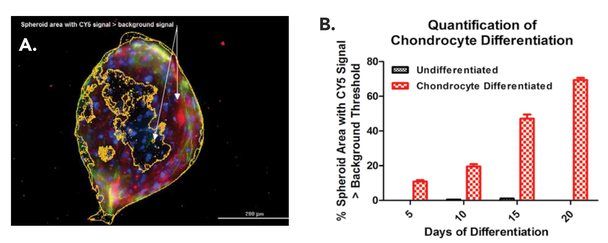

Figure 7. Automated dual-mask analysis. (A) Primary mask placed around the entire spheroid, and secondary mask placed around discontinuous areas of increased CY5 signal. **(B)** Change in CY5 signal compared to background threshold over time.

The percent of CY5 area coverage, indicating greater differentiation and collage 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 7B 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.

Conclusions

1. The 384-Well BiO Assay Kit and NanoShuttle-PL particles manufactured by nano3D Biosciences provide a simple and robust method to create biomimetic hMSC spheroids that can be differentiated into lineages such as chondrocytes.
2. Use of 3D cultured hMSC spheroids may be more appropriate than 2D cultured cells in long-term differentiation studies.
3. Through incorporation of the BioSpa 8 and magnetic adapter on the EL406, the differentiation process can be automated to simplify and increase the repeatability of included procedures.
4. Automation and differentiation confirmation can be performed using brightfield and fluorescent imaging with the Cytation 5.
5. The combination of appropriate cell models, assay methodology, and automated liquid handling, imaging and analysis create a robust method to carry out differentiation of 3D cultured stem cells.

1. Körsmeier, K.; Claßen, T.; Kamminga, M.; Rekowski, J.; Jäger, M.; Landgraeber, S. Arthroscopic three-dimensional autologous chondrocyte transplantation using spheroids for the treatment of full-thickness cartilage defects of the hip joint. *Knee Surg Sports Traumatol Arthrosc.* **2016**, *24*(6), 2032–2037. 2. Park, E.; Patel, A.N. Changes in the expression pattern of mesenchymal and pluripotent markers in human adipose-derived stem cells. *Cell Biol Int.* **2010**, *34*, 979–984. 3. Baer, P.C.; Griesche, N.; Luttmann, W.; Schubert, R.; Luttmann, A.; Geiger H. Human adipose-derived mesenchymal stem cells *in vitro*: evaluation of an optimal expansion medium preserving stemness. *Cytotherapy.* **2010**, *12*, 96–106. 4. Chang, C.H.; Lin, H.Y.; Fang, H.W.; Loo, S.T.; Hung, S.C.; Ho, Y.C.; Chen, C.C.; Lin, F.H.; Liu, H.C. Chondrogenesis from immortalized human mesenchymal stem cells: comparison between collagen gel and pellet culture methods. *Artif Organs.* **2008**, *32*(7), 561-566. 5. Ahrens, P.B.; Solursh, M.; Reiter, R.S. Stage-related capacity for limb chondrogenesis in cell culture. *Dev Biol.* **1977**, *60*(1), 69–82. 6. Maguire, T.; Novik, E. Methods in Bioengineering : Alternative Technologies to Animal Testing; Artech House methods in bioengineering series; Artech House: Boston, MA, 2010.