Automated, 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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Introduction

Human mesenchymal stem cells (hMSCs) have recently emerged as a leading candidate in cellular therapies due to their ability to differentiate and mature into multiple lineages including adipocytes, chondrocytes, and osteocytes, their immunesuppressive properties, and distinct migratory and trophic effects during tissue repair and regeneration[1-3]. Early work with hMSCs incorporated two-dimensional (2D) cell culture, leading to a realization 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"[4-5], in addition to preclinical studies where intramyocardial transplantation of three-dimensional (3D) cultured MSCs into porcine models improved cell survival and integration[6-7]. This has increased the desire to fully understand the impact that aggregation has on hMSCs within spheroidal structures.

One such therapeutic area is the creation of complex tissues from preformed 3D hMSC spheroids. Here, large numbers of spheroids are typically required. Current methods commonly incorporate single or low spheroidal numbers per well. Therefore, high numbers of plates are used to generate the tissue density required, which can be expensive and labor intensive. Others methods that do create higher spheroid numbers allow spheroids with great variation in size, and also suffer from undesired "preaggregation". Multiple pore hanging drop microplates can eliminate these complications by offering a method to create large numbers of spheroids of consistent size from a single well of a 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 and remain in the plate while undergoing differentiation without further manual manipulation. Upon completion of the desired process, spheroids are easily dropped from the insert by touching off the insert to media filled wells below.

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.

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 shaking, 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, which 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, and is controlled by integrated Liquid Handling Control™ (LHC) Software. 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.

Hanging Drop Spheroid Formation

Normal Human Bone Marrow Derived hMSCs (Lonza, Basel, Switzerland) were thawed from cryopreservation, resuspended in complete mesenchymal stem cell growth media (MSCGM), and dispensed into three separate T-75 flasks at a concentration of 5000 cells/cm², per the vendor's recommended protocol. Cells propagated in the flasks for seven days while the cells reached a confluency of 80%. Cells were removed from the flasks and resuspended in either complete MSCGM or complete chondrogenic induction medium at a concentration of $8.5x10^4$ cells/mL in preparation for the hanging drop spheroid formation procedure (Figure 1). The RAD module of the MultiFlo FX was then used to dispense ten 400 µL aliquots of cell suspension to separate locations of each Elplasia micro-hole hanging insert within the 6-Well plate (Kuraray Catalog No. MPc 500 6) using the x-, y-, and z-axis offsets (Figure 2A) programmed into the LHC Software (Figure 2B). The dispensing pattern was optimized to guarantee cells were evenly distributed across the insert. The z-axis height was further optimized to ensure that the force of 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 75% volume of spent medium and replacing with fresh growth or differentiation medium.

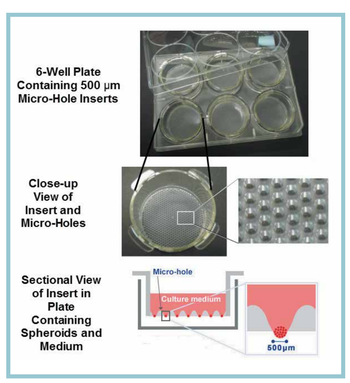
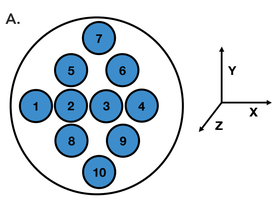


Figure 1. MPc 500 6 Micro-hole hanging drop insert design for spheroid formation.

A.



B.

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

Figure 2. Automated hMSC Dispensing. **(A)** Multi-Flo FX RAD module hMSC suspension dispense pattern; and **(B)** liquid handling control step offsets for RAD module dispensing of hMSC suspension.

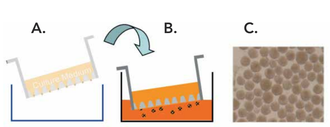


Figure 3. Spheroid transfer from insert to microplate. **(A)** Insert is lifted and medium is added to well; **(B)** bottom of insert is touched to medium in well; **(C)** spheroids move from insert holes to medium below.

Label-Free Imaging of hMSC Spheroids and Micro-Hole Inserts

Elplasia 6-well plates containing hanging drop inserts were placed into 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. Brightfield images were captured using a 2.5x objective and an 18 row by 12 column montage to image the entire insert in a label-free manner (Figure 4A).

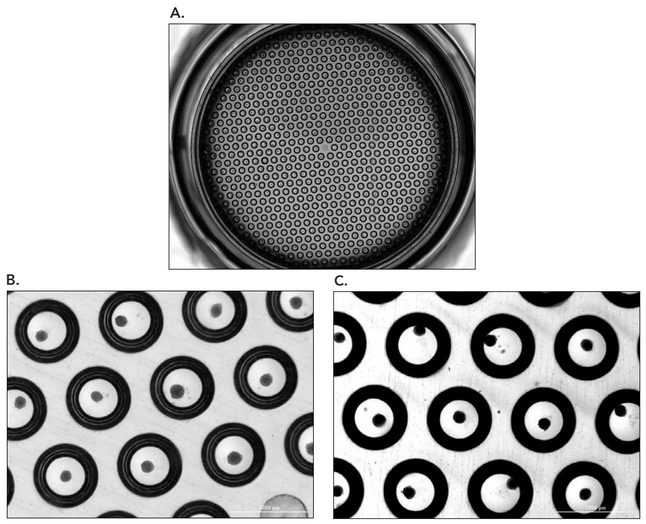


Figure 4. Spheroid monitoring. (A) Stitched images of 18 row by 12 column montage tiles exhibiting 6-well plate well, insert, and spheroids in micro-holes after 48 hour incubation. Zoomed 2.5x images of spheroids in growth medium **(B)**, or chondrocyte differentiation medium **(C)** after 14 day incubation.

By viewing the final stitched images, it was confirmed that spheroid formation was completed in 48 hours, and spheroids remained intact and were not removed during the 14 day process (Figures 4B and C).

hMSC Surface Antigen Expression Validation

Following spheroid formation, undifferentiated spheroids in growth media were transferred from the insert to the well of the 6-well plate per Figure 3. After spheroid transfer, a small number of spheroids were moved via manual pipetting to the wells of a 24-well micro-space plate with a 700 µm depth (Kuraray Catalog No. RB 500 700) where immunofluorescent staining was performed 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 immunostaining, the 3D spheroids or 2D cultured cells were imaged in Cytation 5 using a 20x objective or a 10x objective, respectively, to capture signal from the appropriate fluorescent probe.

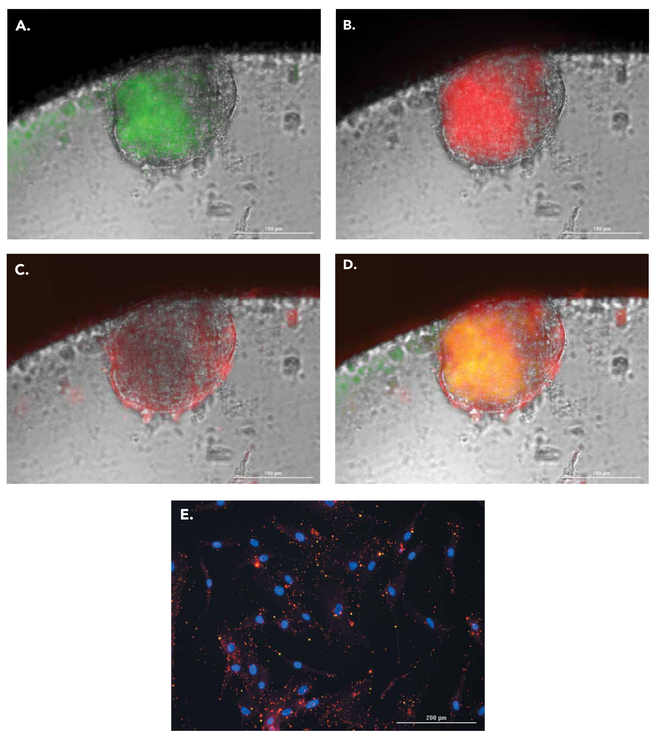


Figure 5. Image-based surface antigen expression validation. (A) 3D hMSC spheroid, GFP channel: CD29 expression identified by goat anti-ITGB1/CD29 1° Ab and donkey anti-goat IgG H&L (Alexa Fluor® 488) 2° Ab, brightfield channel: whole spheroid; **(B)** 3D hMSC spheroid, CY5 channel: CD44 expression identified by rabbit anti-CD44 monoclonal 1° Ab [EPR1013Y] and donkey anti-rabbit IgG H&L (Alexa Fluor 647) 2° Ab, brightfield channel: whole spheroid; **(C)** 3D hMSC spheroid, Texas Red channel: CD166 expression identified by mouse anti-CD166 monoclonal 1° Ab [8E12C7] and goat anti-mouse IgG H&L (Alexa Fluor 594) 2° Ab, brightfield channel: whole spheroid; **(D)** 3D hMSC spheroid, overlay of GFP, CY5, Texas Red and brightfield channels; **(E)** 2D hMSC cells, GFP, CY5, Texas Red channels, DAPI channel: Hoechst 33342 stained nuclei.

hMSCs were confirmed to be functional by visualization of CD29, CD44, and CD166 antigen expression in 3D (Figure 5A-D) and 2D cultured cells (Figure 5E) via immunofluorescence. This finding validates the use of the micro-hole insert to perform hMSC spheroid formation.

3D Chondrocyte Differentiation Validation

Collagen II, one of the prominent components of healthy cartilage and produced by healthy chondrocytes, is a common marker of chondrocyte differentiation from hMSCs. Following the 14 day incubation period and differentiated spheroid transfer, validation of differentiation into the chondrocyte lineage for hMSCs aggregated into 3D spheroids was carried out through immunostaining as previously described and the incorporation of a collagen II specific primary antibody and fluorescently labeled secondary antibody.

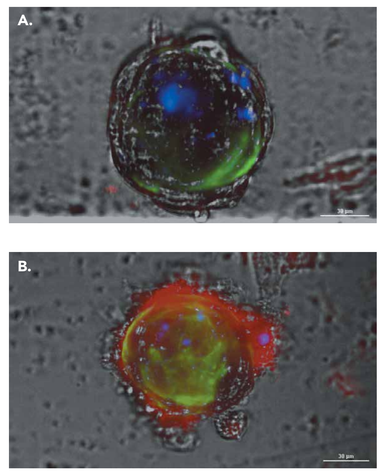


Figure 6. 3D chondrocyte differentiation imaging. 3D image-based chondrocyte validation performed by capturing single images of hMSC spheroids incubated in **(A)** growth medium, or **(B)** complete chondrocyte differentiation medium using a 20x objective. DAPI channel: Hoechst 33342 stained nuclei, GFP channel: Alexa Fluor 488 phalloidin, CY5 channel: collagen II protein expression identified by rabbit anti-Collagen II polyclonal Ab and donkey anti-rabbit IgG H&L (Alexa Fluor 647) Ab, 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 6A) or chondrocyte differentiation medium (Figure 6B), 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. Detailed object masks were applied around the spheroids in each image (Figure 7A). This allows the signal in each imaging channel to be quantified only from the spheroid, and not from other portions of the image, thus increasing the robustness of the validation method.

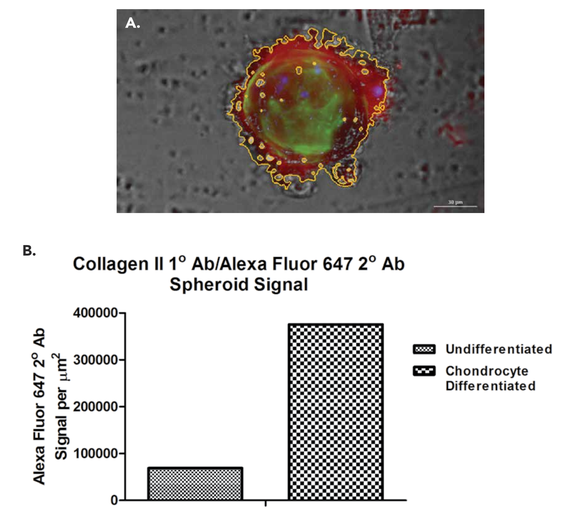


Figure 7. 3D chondrocyte differentiation cellular analysis and quantification. (A) Object mask applied to hMSC spheroid incubated 14 days in chondrocyte differentiation medium. **(B)** Fluorescent signal captured in CY5 channel from undifferentiated and differentiated spheroids normalized to spheroid area.

The total CY5 signal intensity quantified within the object mask for each test spheroid 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 µm² values in Figure 7B 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

1. 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 application. Stem cell differentiation can also be performed while spheroids remain in the inserts.

2. 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.

3. Cytation 5's brightfield and fluorescent imaging capabilities 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.

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