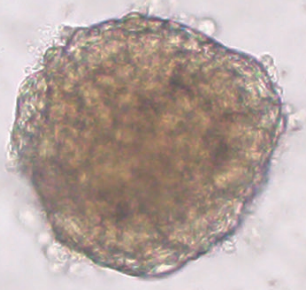
Automated 3D cell culture using Corning® 96-well spheroid microplates on the Fluent® Automation Workstation.

Application Note



AUTOMATED CELL SEEDING AND MEDIUM EXCHANGE FOR THE GROWTH OF INDIVIDUAL 3D CELL SPHEROIDS.

INTRODUCTION

Evidence over the past two decades has shown that *in vitro* three-dimensional (3D) cell models provide more biologically-relevant results than 2D monolayer models for cancer research. This is because cells grown in 3D cultures – like spheroids – more closely resemble the complex microenvironment of a tissue or solid tumor in the human body than 2D cultures. By maintaining physiological traits, such as the natural shape of the cells, inter-cellular communications, cell-matrix interactions, cell proliferation and differentiation, and drug metabolism, 3D models have proven to be useful tools for the screening of anti-cancer drugs.

Although 3D cell models are recognized to be more suitable for *in vitro* efficacy testing and analysis of drug penetration, the majority of high throughput cell-based screening is currently being conducted using 2D cell models. The main reason for this is that most available techniques for 3D spheroid generation and quantification are time consuming, tedious and show poor reproducibility.

This application note describes the automated production and growth of single spheroids in ultra-low attachment (ULA) Corning 96-well spheroid microplates on a Fluent Automation Workstation, using a Multiple Channel Arm™ (MCA) for cell seeding and medium exchange.

MATERIALS AND METHODS

Automation platform

Experiments were conducted on a Fluent 780 system that included, from left to right, an eight-channel air-displacement Flexible Channel Arm™ (Air FCA), a long Z Robotic Gripper Arm™ (RGA) able to reach below the Dynamic Deck™, and an MCA with an extended volume adapter to pipette up to 500 µl with 96 tips in parallel (Figure 1). The system is fitted with a vertical laminar flow HEPA hood with UV light to ensure a clean environment for sterile cell culture applications. Liquid handling tasks with the MCA were conducted with 150 µl filtered disposable tips. Sterile 150 ml reservoirs (INTEGRA Biosciences) were used for pipetting into sterile 96-well spheroid microplates featuring an ULA surface (Corning) with the MCA. Individual scripts for cell seeding and medium exchange with the MCA were developed using FluentControl™ software.

Cell culture

Human fibroblasts fetal lung cell line, WI38 (Sigma), and murine embryonal fibroblasts, NIH3T3 (Sigma), were expanded in 2D conditions in Dulbecco's modified Eagle's medium (DMEM) high glucose (Sigma) supplemented with 10 % fetal bovine serum (FBS) (Gibco), and 1 % penicillin-streptomycin (P/S) (Sigma). Cells were cultivated at 37 °C and 5 % CO_2, and medium was changed every second or third day.

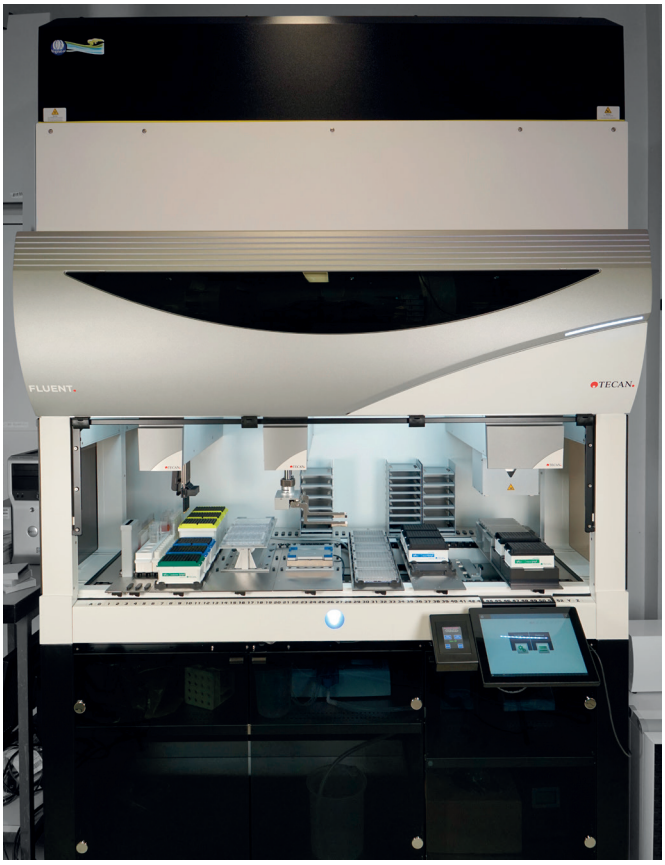


Figure 1: The Fluent Automation Workstation with the deck layout for cell culture applications.

Cell seeding

Cells were cultivated up to 80 % confluence before being detached with trypsin/EDTA (PAN Biotech) and suspended in fresh culture medium: DMEM high glucose, 10 % horse serum (HS) (GE Healthcare), and 1 % P/S for WI38 cells; DMEM low glucose, 6.25 % heat inactivated FBS, 3.75 % HS, 2 mM L-Glutamine (Sigma), 1 % P/S for NIH3T3 cells.

Cell seeding was conducted both manually and with the Fluent workstation. Cells were seeded at three different densities – 10,000, 25,000 and 50,000 cells/ml – in Corning 96-well spheroid microplates (Catalogue no. CLS4520). 100 µl of cell suspension was dispensed to give concentrations of 1,000, 2,500 and 5,000 cells per well (n=32). During manual cell seeding, a multichannel pipette (12 channels) and sterile reservoirs (Corning) were used to dispense the cell suspension into the 96-well spheroid microplates. Automated cell seeding was performed with the MCA using 32 tips per cell density. Cells were mixed three times to obtain homogenous cell suspensions, before being dispensed at the speed of 84 µl/s into the center of each well.

Spheroid formation and medium exchange

Microplates were centrifuged at 200 x g for 1 minute and incubated at 37 °C and 5 % CO_2 for 4 days to allow cells to aggregate into single spheroids (Day 0). The spheroids were cultivated for up to 14 days, changing the medium either manually or using the Fluent two times per week. During manual medium exchange, a multichannel pipette (12 channels) and sterile reservoirs were used for both aspiration and dispensing. 75 % of media per well was exchanged manually. Similarly, 75 % of media was exchanged per well using the MCA on the Fluent workstation, aspirating from the back, right corner of each well at the low aspiration speed of 1 µl/s to avoid disturbing the spheroids. Fresh medium was dispensed at the speed of 10 µl/s at the same position as aspiration to prevent any spheroid displacement from the center of the well. Automating this step with the MCA allowed medium exchange for all 96 wells in parallel.

Spheroid analysis

Both the morphology and size of the spheroids were analyzed under an Olympus IX81 microscope. During the culture period, bright-field images were recorded, and the spheroids' area was measured with ImageJ.

RESULTS

Manual and automated cell seeding led to the successful formation of single spheroids in Corning 96-well spheroid microplates (Figure 2). No difference in the morphology or dimensions of the spheroids was detected between the two methods at Day 0 (four days after seeding the cells), independent of the cell line used.

Previous results on WI38 cells cultivated up to 14 days in DMEM led to the generation of spheroids that showed a constant dimensions throughout the entire culture period. In accordance with these results, WI38 spheroids produced either manually or with the Fluent showed no increase in area over time. Moreover, no difference in dimensions was observed between the manual and automated methods, showing the Fluent platform is suitable for the culture of even small sized spheroids of 160 µm diameter (see Figure 2 – WI38 1,000 cells/well seeding density).

Manual medium exchange led to the loss of between 15 and 31 % of NIH3T3 spheroids throughout the culture period (Table 1). The variation in the number of spheroids lost was dependent on their size, with a higher percentage of smaller spheroids being removed during the aspiration step of manual medium exchange. In comparison, no spheroids were lost during automated medium exchange with the Fluent platform (Table 1). It is also relevant to note that automated medium exchange was repeated five times on 12 wells containing spheroids made of both cell types, demonstrating the reproducibility of the automated process.

	Time points					
	d0	**d1**	**d3**	**d7**	**d10**	**d14**
5,000 cells/S, m	n=32	n=30	n=30	n=30	n=27	n=27
2,500 cells/S, m	n=32	n=30	n=30	n=29	n=26	n=26
1,000 cells/S, m	n=32	n=27	n=25	n=25	n=22	n=22
5,000 cells/S, a	n=32	n=32	n=32	n=32	n=32	n=32
2,500 cells/S,a	n=32	n=32	n=32	n=32	n=32	n=32
1,000 cells/S,a	n=32	n=32	n=32	n=32	n=32	n=32

Table 1: Total number of NIH3T3 spheroids (S) remaining in Corning 96-well spheroid microplates after each manual (m) and automated (a) medium exchange, after a period of up to 14 days (d).

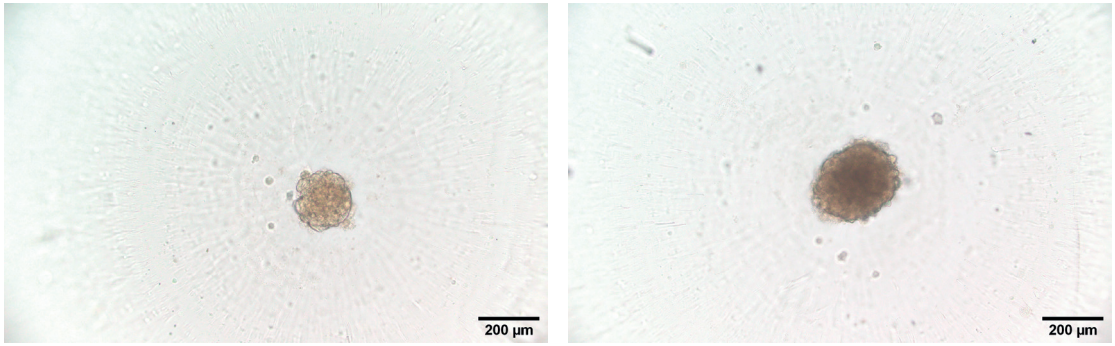
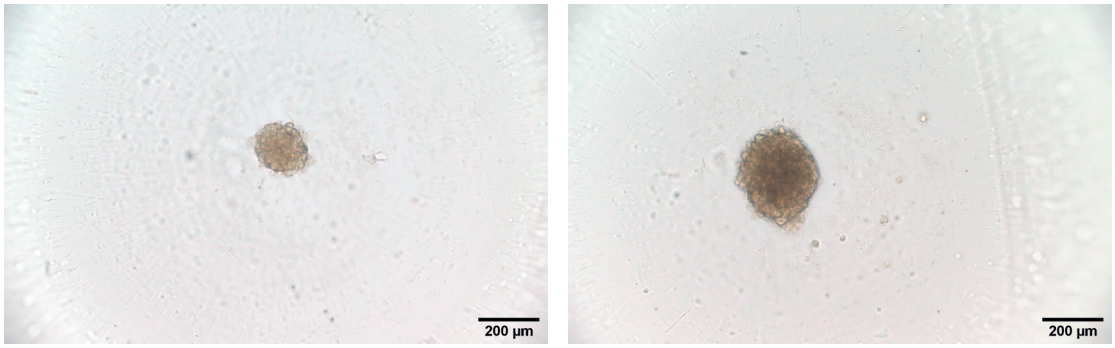
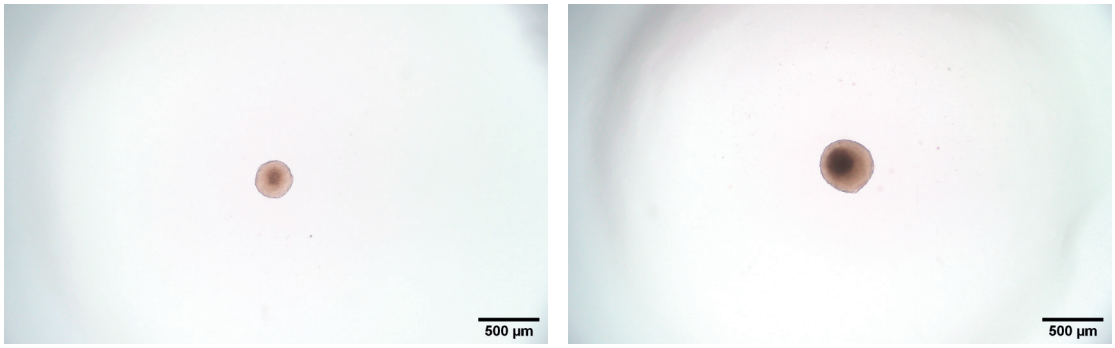
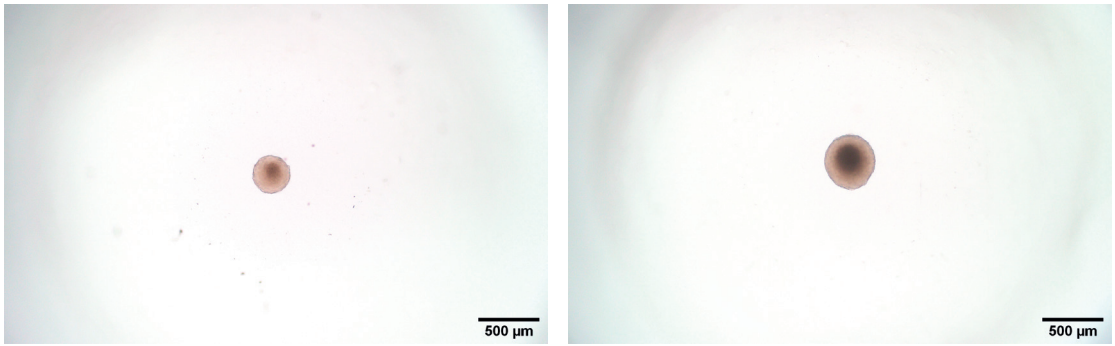
Cell type/ seeding method	Seeding density per well	
	1,000	5,000
WI38/manual		
WI38/automated		
NIH3T3/manual		
NIH3T3/automated		

Figure 2: Images of single spheroids taken four days after manual and automated cell seeding (Day 0) in the 96-well spheroid microplates. Scale bar, 200 µm for WI38 and 500 µm for NIH3T3 cells.

Furthermore, to ensure that cell culture automation does not affect the spheroids' growth rate, the size and morphology of NIH3T3 spheroids were monitored during the culture period. Results showed that both manual and automated medium exchange methods led to similar dimensions for NIH3T3 spheroids at each time point, and they continued to propagate and increase in area over time at all initial cell densities (Figure 3). It is important to note that no contamination issues were observed throughout the manual or automated culture period, proving the operating conditions were sterile.

CONCLUSIONS

This application note describes the successful automation of cell seeding and medium exchange during single spheroid formation in Corning 96-well spheroid microplates on the Fluent Automation Workstation. Automating these cell culture steps enables fast cell seeding and reproducible medium exchange for 96 wells in parallel, leading to the conservation of all grown spheroids in the wells. Importantly, no contamination issues were observed during the experiment, proving the system ensures a clean and sterile environment for cell culture applications.

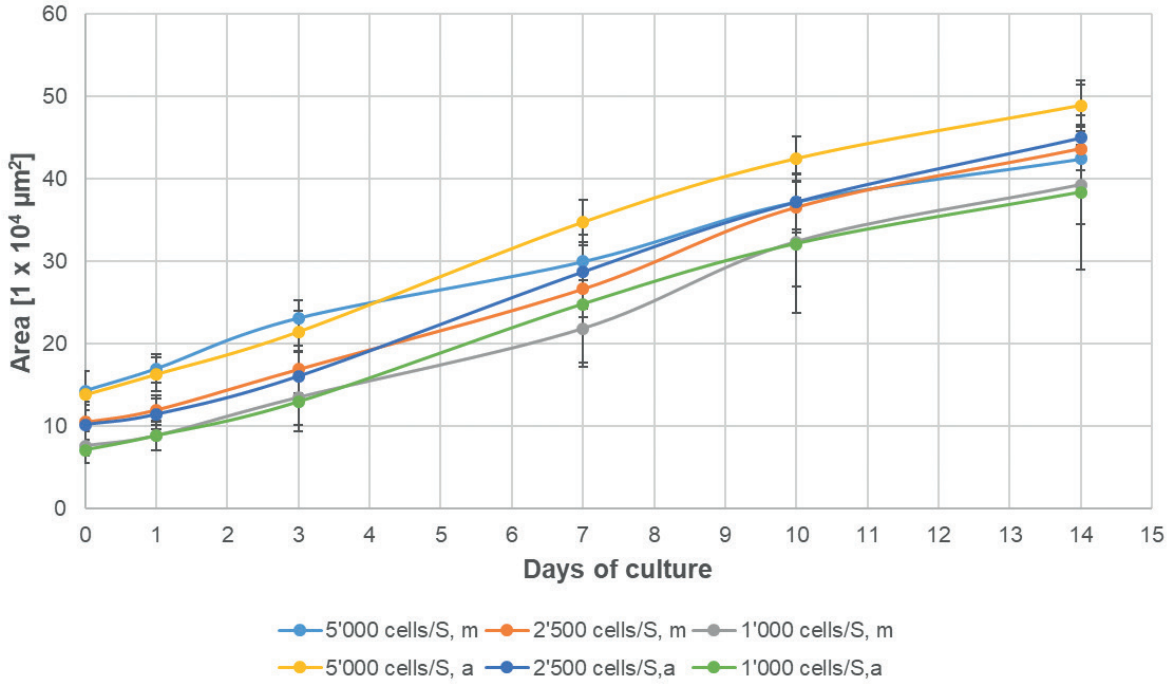


Figure 3: Kinetic proliferation of NIH3T3 spheroids (S) produced manually (m) and through automation with the Fluent platform (a) at different cell densities, with medium exchange conducted up to 14 days of culture after Day 0. The spheroids' area values, measured with ImageJ, are shown for the automated and manual spheroids per time point.

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About the author

Dr Epifania Bono is a research associate at ZHAW in Waedenswil. She completed her degree in biology, specializing in molecular biology, at the University of Palermo in Italy. During her PhD, Epifania focused on cell biology and molecular biology at the University of Palermo, and then worked at the Institute of Biomedicine and Molecular Immunology 'A.Monroy' in Palermo. Since January 2007, Epifania works at ZHAW in the Cell Biology and Tissue Engineering team. Her current scientific research focuses on cell culture technology, tissue engineering, and bioprinting for the development and application of biomaterials, as well as cell and tissue characterization in manual and automated cultivation systems.

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Australia +61 3 9647 4100 **Austria** +43 62 46 89 330 **Belgium** +32 15 42 13 19 **China** +86 21 220 63 206 **France** +33 4 72 76 04 80 **Germany** +49 79 51 94 170
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