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Reprinted from *American Laboratory* November/December 2016

Automating 3-D Cell Culture and Screening by Flow Cytometry and High-Content Imaging

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For over half a century, researchers have used 2-D cell culture methods, though the physiological relevance of these models has frequently been in doubt, largely because monolayer culture systems cannot replicate in vivo conditions. By way of contrast, 3-D cell cultures help elucidate more about cell morphology, motility, polarity and response to stimuli. The spheroidal structure of many 3-D cultures creates diffusion gradients that mimic solid tumors, and therefore provides cancer researchers with a more useful drug response model. Three-dimensional cell cultures also survive longer—3-D aggregates can be cultured for up to at least four weeks, compared to about one week for 2-D cultures.[1]

Limitations of 3-D cell culture methods

Despite their many benefits, 3-D cultures can be more expensive, complicated and cumbersome than 2-D cultures, often necessitating a significant investment in time and materials; further, there is no model that is appropriate for all applications. The most widely used 3-D cell culture methods are cell aggregates called spheroids, solid scaffolds and hydrogel-based matrices. Most of these cannot be easily automated or scaled for high-throughput screening, though innovative uses of automation, as well as evolving technologies and better 3-D assays, are helping to overcome these limitations. Automation has been used to develop 3-D cultures that demonstrate excellent consistency and, through cellular analysis by complementary methods, have generated a more complete picture of drug response.

Figure 1 – Biomek FX^P Automated Workstation.

Automating 3-D spheroid cultures

Spheroids naturally simulate solid tissues, and typically contain exposed and buried cells, proliferating and nonproliferating cells and well-oxygenated and hypoxic cells.[2] This makes them particularly useful in cancer research.

In separate experiments, spheroid formation was automated using two methods: Perfecta3D Hanging Drop Plates (3D Biomatrix, Ann Arbor, Mich.) and ultra-low attachment (ULA) plates (Corning Incorporated, Corning, N.Y.). Each method offers unique advantages as well as challenges such as

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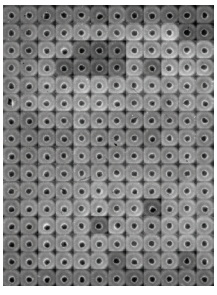

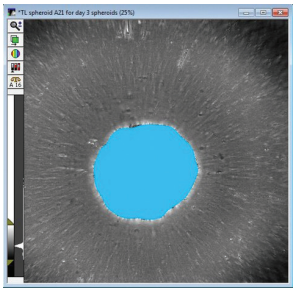

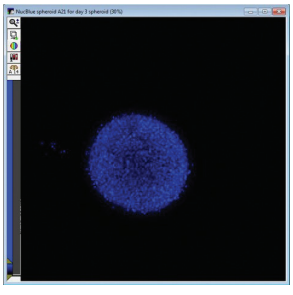

b

		D3 Avg (n=192)	D3 %CV	D4 Avg (n=32)	D4 %CV
Shape	Shape Factor	0.84	4.0%	0.81	0.8%
	Elliptical Form Factor	1.11	7.7%	1.06	3.5%
Size	Perimeter (µm)	1423	4.2%	1828	1.5%
	Area (µm^2)	134529	5.9%	216216	2.8%
	Volume (µm^3)			5.09X10^7	2.7%
	Nuclei			529	9.0%

Figure 2 – a) Montage of 192 day-3 spheroids from ULA plates, imaged at 10× using brightfield with the ImageXpress Micro Confocal High Content Imaging System (Molecular Devices, Sunnyvale, Calif.). Spheroids were analyzed for circularity (shape factor, elliptical form factor) and size (perimeter and area) to ensure consistency across wells. Thirty-two wells were then stained for 24 hours with NucBlue Live ReadyProbes Reagent (Thermo Fisher Scientific, Waltham, Mass.) so that cell nuclei could be counted and spheroid volume determined. b) All measurements at both time points showed excellent consistency, with %CV values of <10%.

unstable drops, the creation of air bubbles and the possibility of aspirating the spheroids during media exchanges. Using the Biomek FX^P Automated Workstation[3] (*Figure 1*; Beckman Coulter Life Sciences, Indianapolis, Ind.), consistent spheroid formation was achieved (*Figure 2*) with both methods by automating steps that can be laborious and prone to human error. Spheroids were screened for apoptosis induction via high-content imaging and flow cytometry (*Figures 3* and *4*). The latter required the dissociation of spheroids into single-cell suspensions, and automation enabled significantly increased throughput for this labor-intensive step.

Use of either plate type for screening requires reliable spheroid formation, treatment and analysis at high throughput. Automating the workflow on a Biomek workstation achieved this consistency with minimal bench time, with optimized pipetting techniques showing significant improvements over manual cell plating and spheroid transfer (data not shown).

Automating 3-D cell cultures using hydrogels

Used as substrates for 3-D cell culture, hydrogels can be made from natural mixtures (e.g., collagen and alginate) or from synthetic molecules.[4] The most frequently used extracellular matrix-based hydrogel is Matrigel (Corning Life Sciences), a reconstituted basement membrane preparation extracted from mouse sarcoma. Naturally derived hydrogels are inherently biocompatible and bioactive.[5] Due to the presence of endogenous factors, they promote numerous cellular functions, which can be useful for supporting viability, proliferation and development of many cell types.[5]

Synthetic hydrogels (e.g., polyethylene glycol-based gels) can be used for 3-D applications that are unsuitable for naturally derived matrices. Biologically inert synthetic gels provide greater control over material and biological properties used to create structural support for a variety of cell types.

To determine how colon cancer cells grown in Matrigel respond to apoptosis inducers, the workflow was automated on a Biomek FX^P Automated Workstation with numerous temperature-controlled Peltier positions to maintain Matrigel in liquid form and heat the assay plate to induce gel polymerization. Automation enabled use of 384-well plates rather than a 96-well format. This resulted in significant cost savings by reducing the amount of Matrigel required, as consistent positioning of the pipette tip

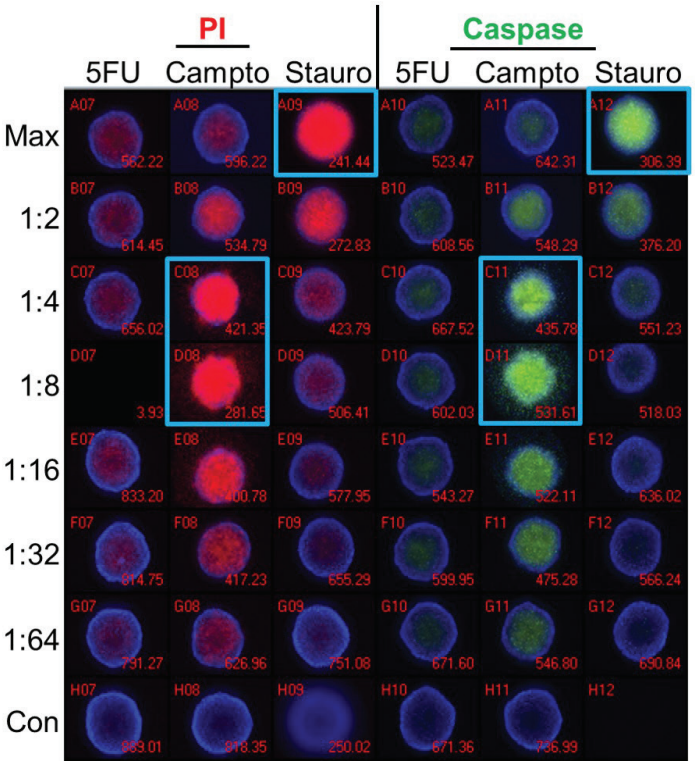

Figure 3 – Spheroids created using hanging drops were treated with 5-fluorouracil (5FU), camptothecin (Campto), and staurosporine (Stauro) at the indicated dilutions for 24 hours, and stained for apoptosis markers for analysis by imaging. Control spheroids (Con) were treated with DMSO alone. Wells with maximal staining by propidium iodide (PI) or activated caspase substrate are identified by blue boxes. Staurosporine shows a traditional dose response while the highest level of staining was seen at the 1:4–1:8 dilutions of camptothecin. 5-Fluorouracil treatment resulted in no significant staining of spheroids.

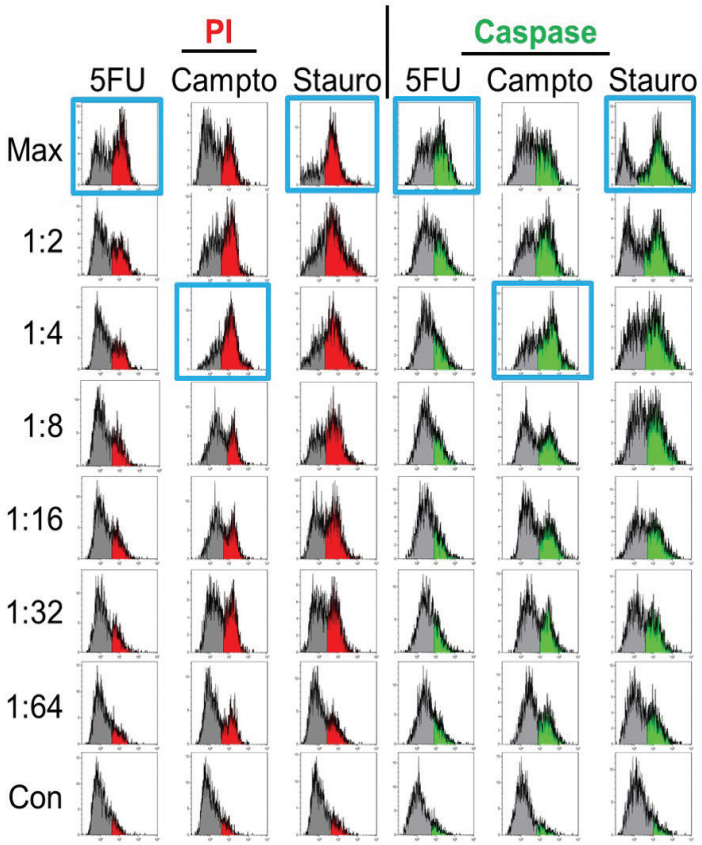

Figure 4 – Spheroids treated identically as in Figure 3 were dissociated and stained for apoptosis markers and analyzed by flow cytometry. Maximal responses (blue boxes) correlated with imaging results for staurosporine and camptothecin, but 5-fluorouracil treatment showed positive staining only by flow cytometry, illustrating the value of multiple analyses.

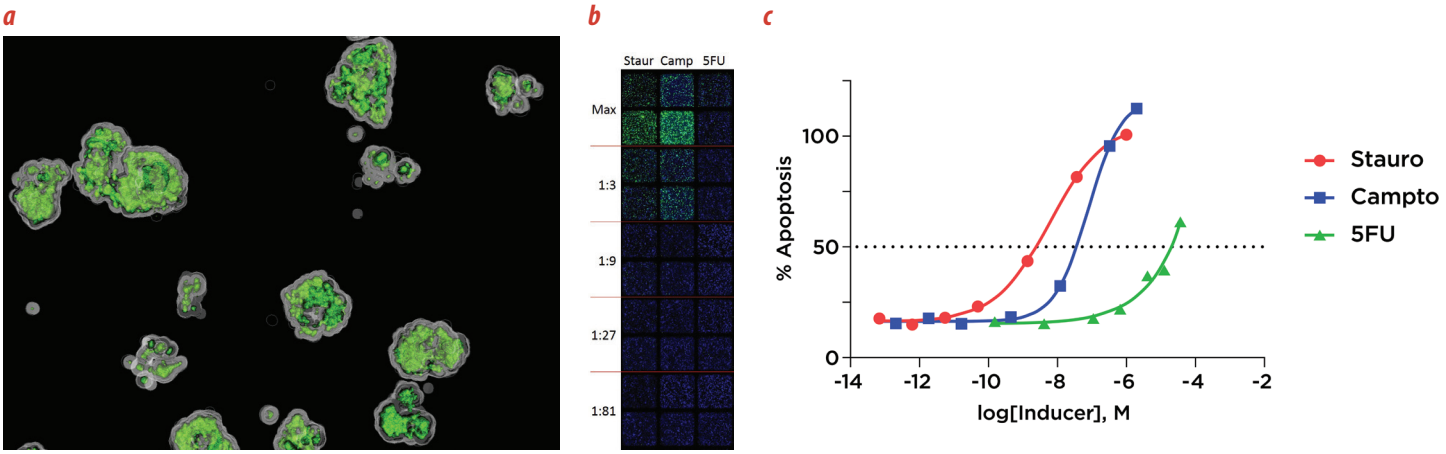

Figure 5 – a) Image acquired using the ImageXpress Micro Confocal High Content Imaging System illustrating 3-D Matrigel colonies stained with a fluorescent caspase 3/7 substrate as a marker of apoptosis. b) Montage of 2-D projections of Z-stacks acquired at 4× magnification with the ImageXpress Micro Confocal system. Replicate wells were treated with dilutions of 10 µM staurosporine, 20 µM camptothecin and 375 µM 5-fluorouracil (top five dilutions shown) and stained for cell nuclei (blue) and apoptosis (green). c) Dose-response curves generated from imaging data for each compound.

0.3 mm above the well bottom allowed the gel to spread over the entire well. Manual pipetting required contact with the well bottom (potentially scratching and/or affecting transfer volume) or the side of the well, which can cause gel to adhere to the side, thus failing to cover the well. The only way to achieve consistent well coverage through manual transfer was to double the Matrigel volume to 50 µL, thereby doubling experiment costs.

Another advantage to automated plating was the ability to use slow aspirate, dispense and mix speeds to avoid generating bubbles in the Matrigel solution. A small additional volume of Matrigel was aspirated into the pipette tip to avoid introducing a bubble when dispensing to the assay plate. Automated plating consistently resulted in fewer bubbles than manual cultures, despite the use of narrow pipette tips to access the 384-well plates.

While Matrigel is only one scaffold type employed to study 3-D cultures, it can be used to measure susceptibility of cancer cells to apoptosis inducers in a more physiologically relevant system than 2-D culture. Automation of the plating, treatment and staining reduced time at the bench—as well as reagent costs—to achieve consistent cell growth and measurable dose response curves (*Figure 5*).

Conclusion

Although 3-D methods have yet to surpass use of 2-D models on a large scale—and will probably never replace 2-D cultures completely—their usefulness in supporting cell growth, tissue morphogenesis, stem cell differentiation and drug discovery is well-recognized. Automation of 3-D cell cultures can provide the consistent spheroids required for screening, while automating sample processing for imaging and analysis removes barriers to gathering the data required for a more complete understanding of spheroid drug responses. Likewise, gel-based 3-D cultures can be useful for many types of studies, though challenges to using Matrigel assays are significant. Automation's positional control and optimized pipetting, coupled with temperature regulation, can overcome some of these obstacles.

References

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