MOLECULAR DEVICES

APPLICATION NOTE

Assay apoptosis in magnetically bioprinted HepG2 spheroids

Cathy Olsen, PhD | Sr. Applications Scientist | Molecular Devices

Benefits

- **Magnetically bioprint spheroids in as little as 15 minutes**
- **Center spheroids in flat-bottom microplate wells for rapid 1-site imaging of results**
- **Assess apoptosis in spheroids in less than one hour with a homogeneous, fluorescent assay**

Introduction

Two-dimensional (2D) cell culture has been used for over a century to investigate a wide range of cellular processes. However, more recent studies have shown that responses in 2D systems can differ significantly from those seen *in vivo* (Duval et al., 2017). A variety of methods for transforming 2D cultures into 3D spheroids have been devised, but many of these require a long time to form spheroids that can be difficult to handle.

The 384-Well Bioprinting Kit from Greiner Bio-One offers an easier way to quickly form spheroids that can be kept in culture for up to three weeks. Cells growing in 2D culture are first treated with NanoShuttle™-PL, a nanoparticle assembly consisting of gold, iron oxide, and poly-L-lysine, to magnetize them. They are then trypsinized and transferred to a cell-repellent 384-well plate, which is placed atop a spheroid drive consisting of 384 magnets positioned below the wells of the plate that cause the magnetized cells to aggregate at the well bottom in the shape of the magnet over the course of 15 minutes to several hours. Afterwards, the plate is kept in an incubator, and the spheroids can be treated under a variety of experimental conditions.

Here, we show how HepG2 cells can be bioprinted using the magnetic 3D cell culture system from Greiner Bio-One (m3D). The resulting spheroids are treated with compounds that induce apoptosis and assayed using Molecular Devices EarlyTox™ Caspase-3/7-D NucView 488 Assay Kit, a homogeneous method that reduces hands-on time. Spheroids are imaged on the SpectraMax® i3x Multi-Mode Microplate Reader with SpectraMax® MiniMax 300 Imaging Cytometer and results analyzed with SoftMax® Pro Software, yielding quantitative results.

Materials

- 384-Well Bioprinting Kit (Greiner Bio-One m3D cat. #781840 or 781841), includes the following:
  - NanoShuttle-PL (2 600-µL vials)
  - 384-Well Spheroid Drive (1)
  - 384-Well Holding Drive (1)
  - Greiner Bio-One 384-Well Cell Culture Microplate with Cell-Repellent Surface with Flat bottom (2 plates; 781840 contains clear microplates and 781841 contains Black with µClear® bottom)
- HepG2 human hepatocellular carcinoma cell line (ATCC cat. #HB-8065)
- Eagle's Minimum Essential Medium (ATCC cat. #30-2003)
- BenchMark™ Fetal Bovine Serum (Gemini Bio-Products cat. #100-106)
- Penicillin-streptomycin (10,000 U/mL, ThermoFisher cat. #15140122)
- Staurosporine (Sigma cat. #S5921)
- Anisomycin (Sigma cat. #A9789)
- EarlyTox Caspase-3/7-D NucView 488 Assay Kit (Molecular Devices cat. # R8348)
- SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices cat. #i3x)
  - SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices cat. #MINIMAX 300)

Methods

HepG2 cell culture

HepG2 human hepatocellular carcinoma cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. In preparation for spheroid formation, cells were cultured to 80% confluence in a T-25 culture flask. They were then treated with NanoShuttle-PL as follows:

1. NanoShuttle-PL was allowed to stand at room temperature for at least 15 minutes.
2. NanoShuttle-PL was homogenized in its vial by pipetting up and down at least 10 times.
3. 200 µL of NanoShuttle-PL was added directly to the medium in the T-25 flask, and cells were incubated at 37°C overnight.

Spheroid formation

The next day, the magnetic spheroid drive was wiped down with 70% ethanol and placed in a biosafety cabinet to keep it sterile. HepG2 cells that had been incubated in NanoShuttle-PL overnight were trypsinized, then counted and resuspended to 20,000 cells per mL of culture medium, a density that would yield 1000 cells per well when seeded at a volume of 50 µL per well. A cell-repellent 384-well plate was placed on top of the 384-well spheroid drive. The cell suspension was seeded into the plate at 50 µL per well, and the cell plate was placed atop the magnetic drive for 15 to 30 minutes so that cells aggregated (printed) at the bottom of each well in the shape of the magnet.

After bioprinting, the plate was removed from the spheroid drive and transferred to an incubator. At day 2 and day 5 after bioprinting, the spheroids were imaged using the transmitted light (TL) channel of the SpectraMax MiniMax 300 Imaging Cytometer (settings are shown in Table 1). Plates were placed on the holding drive to center the spheroids in each well prior to imaging. This enabled imaging of a single spheroid in each well using one site per well.

Parameter	Transmitted light	Fluorescence
Optical configuration	MiniMax	
Read mode	Imaging	
Read type	Endpoint	
Wavelength	Transmitted light	Fluorescence Excitation wavelength: 456 nm Emission wavelength: 541 nm
Plate type	384 Well Greiner blk/clrbtm*	
Read area	[Select wells to be imaged]	
Well area settings	1 site	
Image acquisition settings	Exposure: 7 ms Focus adjustment: 100 µm	Exposure: 11 ms Focus adjustment: 100 µm

*Plate type as listed in SoftMax Pro Software

Table 1. Settings used to acquire images of spheroids on the MiniMax cytometer. Settings are shown for transmitted light (TL) images collected of spheroids on days 2 and 5 after printing. Fluorescence settings shown were used to image compound-treated spheroids assayed with the EarlyTox Caspase-3/7-D NucView 488 Assay Kit.

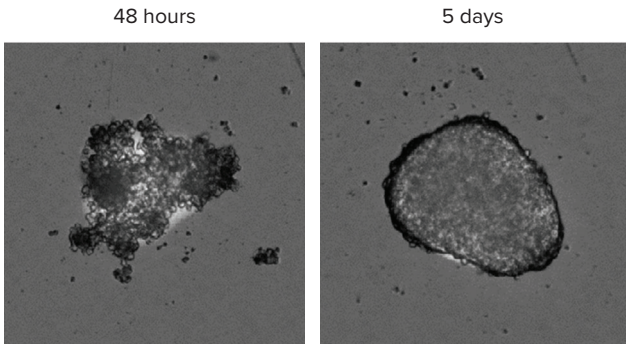

Figure 1. Spheroids imaged in the TL channel of the MiniMax cytometer. Images of the same well were taken 48 hours or five days after spheroid bioprinting.

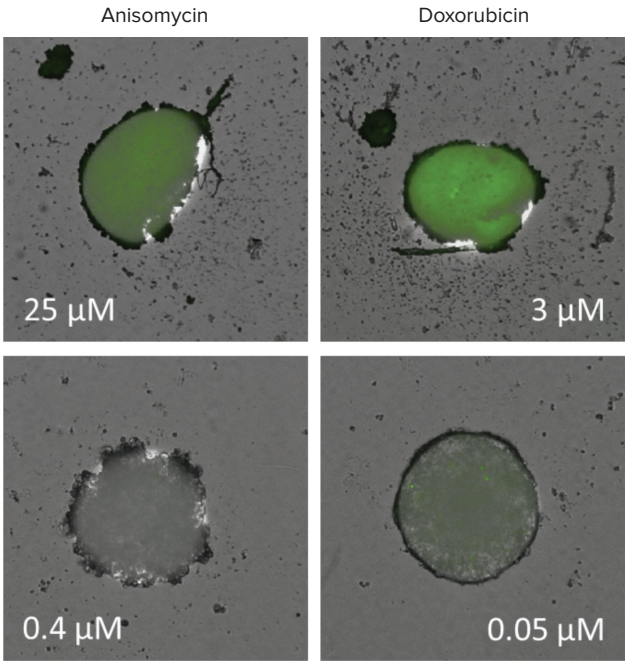

Figure 2. Overlaid TL and fluorescence images. Spheroids were treated with high (top row) or low (bottom row) concentrations of anisomycin or doxorubicin and assayed with the EarlyTox Caspase-3/7-D NucView 488 Assay Kit. Images taken in the TL and green fluorescent channels were overlaid.

Compound treatment

Anisomycin and doxorubicin, compounds known to induce apoptosis in HepG2 cells, were diluted in culture medium to make working solutions to add to the spheroids. Anisomycin working solutions were made from 25 µM down to 0.01 µM, in a 1:3 dilution series. Doxorubicin working solutions were made from 3 µM down to 0.0001 µM, in a 1:3 dilution series. On day 5 after spheroid bioprinting, the plate containing spheroids was placed on the holding drive to keep spheroids on the well bottoms while medium was removed from the wells and replaced with working solutions of anisomycin or doxorubicin. Cells were treated with compounds for four days. After compound treatment, they were assayed for apoptosis.

Apoptosis assay

The EarlyTox Caspase-3/7-D NucView 488 Assay Kit was used to determine the level of apoptotic activity in each spheroid after four days of compound treatment. The 1 mM stock of NucView 488 Caspase-3 Substrate in DMSO was diluted to 7.5 µM in culture medium and added to the spheroids. Cells were incubated at room temperature for 15-30 minutes, protected from light.

Imaging was performed on a SpectraMax MiniMax 300 Imaging Cytometer using the 541 nm green fluorescence channel (settings are shown in Table 1). Images were collected and analyzed using SoftMax Pro Software. To analyze the spheroids, the Set Size and Intensity setting was used, with minimum width set to 200 µm and maximum width set to 1000 µm. Intensity Above Background was set to 200. These settings ensured that only spheroids, and not other cells or debris, were analyzed by the software.

Results

Printed HepG2 spheroids formed rounded, compact structures that could be maintained in culture for several days, offering flexibility in the scheduling of compound treatment. Images taken of spheroids 48 hours or 5 days after printing show the progression of the cellular arrangement (Figure 1).

Treatment of spheroids with anisomycin or doxorubicin induced apoptosis in a concentration-dependent manner. The EarlyTox Caspase-3/7-D NucView 488 Assay Kit was used to fluorescently stain the nuclei of apoptotic cells green, which was easily detected on the MiniMax cytometer (Figure 2). Spheroids were analyzed for total fluorescence intensity, indicating the amount of total apoptotic activity (Figure 3).

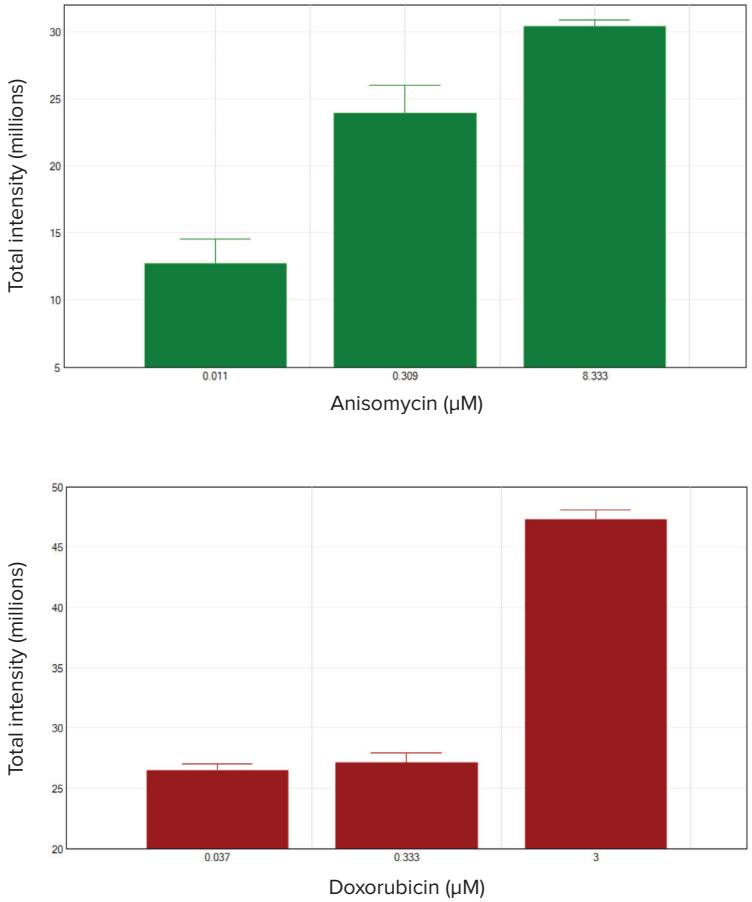

Figure 3. Analysis of apoptosis in anisomycin- and doxorubicin-treated spheroids. After assaying with the EarlyTox Caspase-3/7-D NucView 488 Assay Kit and imaging on the MiniMax cytometer, total fluorescence intensity of spheroids was plotted vs. compound concentration in SoftMax Pro Software. Low, intermediate, and high concentrations of compound are represented here.

Conclusion

Spheroid bioprinting is a rapid way to aggregate cells into three-dimensional structures that offer better biorelevancy than cells cultured in monolayers. HepG2 cells grown in a monolayer culture were magnetized and bioprinted using the m3D system, and the resulting spheroids were assayed for apoptosis using the EarlyTox Caspase-3/7-D NucView 488 Assay Kit. The SpectraMax i3x reader with MiniMax cytometer enabled efficient imaging of spheroids in one site per well, as well as quantitative analysis of apoptosis with SoftMax Pro Software.

Reference

Duval K, Grover H, Han LH, Mou Y, Pegoraro AF, Fredberg J, and Chen Z. 'Modeling physiological events in 2D vs. 3D cell culture.' *Physiology* (2017); 32(4): 266-277.

Contact Us

Phone: +1.800.635.5577
Web: www.moleculardevices.com
Email: info@moldev.com
Check our website for a current listing of worldwide distributors.

The trademarks used herein are the property of Molecular Devices, LLC or their respective owners. Specifications subject to change without notice. Patents: www.moleculardevices.com/productpatents
FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

©2018 Molecular Devices, LLC
8/18 2198A
Printed in USA

MOLECULAR DEVICES