Automation of the full workflow for 3D cancer spheroid assays with CellXpress.ai

Oksana Sirenko, Angeline Lim, Astrid Michlmayr, Emilie Keidel, Felix Spira, Krishna Macha, Cathy Olsen | Molecular Devices, LLC

Introduction

Finding efficient drug combinations to treat cancer patients is critical for therapy success. Accordingly, there is a critical need to develop methods for efficiently testing drug efficacy to discover new therapeutic targets. 3D cancer models are highly valuable tools for cancer research and drug development, however, the complexity of performing 3D assays remains a hurdle for the wide adoption of these methods for compound screening.

We developed cell culture automation methods using the CellXpress.ai™ Automated Cell Culture System. The instrument enables full automation of 2D or 3D assays for prolonged complex workflows. The system provides automated plating, passaging, media exchanges, organoid monitoring, along with compound treatment and endpoint assays. In this study, we describe automated cell culture process and end-point assays that enable the scaling up of complex 3D cell-based assays and compound screening.

As an example of 3D protocol, we automated culture and imaging of colorectal cancer 3D spheroids formed from HCT116 cell lines in U-shape low attachment plates. HCT116 cells were expanded in 2D, then spheroids were formed after automated dispensing of cell suspension into U-shape 96 or 384 plates. After 48h, spheroids were treated with several anti-cancer compounds at multiple concentrations for 3–5 days then stained and imaged. Cell plating, compound additions, media exchange, and staining were performed automatically by the CellXpress.ai system. During culture, spheroids were monitored using transmitted light with analysis of phenotypic changes, including inhibition of growth or spheroid disintegration. Endpoint assay spheroids were stained with a combination of Hoechst nuclear stain and viability dyes Calcein AM and EtHD then imaged and analyzed for spheroid size and live-dead cell scoring. In addition, we measured ATP content by using the CellTiter-Glo assay. Luminescent read-outs were done by using SpectraMax® iD3 Multi-Mode Microplate Reader. We observed a concentration-dependent decrease in ATP content, inhibition of spheroid growth, and cell death in response to anti-cancer compounds and evaluated effective concentrations for compound effects.

Automated cell culture processes powered by imaging have the potential to bring 3D biology to another level, allowing increased throughput and reproducibility, and enabling a variety of high-throughput drug discovery and precision medicine applications.

Methods

Automation of organoid workflow

The powerful new CellXpress.ai Automated Cell Culture System automates the entire cell culture process with an integrated incubator, liquid handler, and image-based decision-making. This hands-off automated solution manages demanding feeding and passaging schedules by monitoring the development of cell cultures with periodic imaging and analysis, and leverages machine learning to initiate passaging, endpoint assay, or troubleshooting steps.

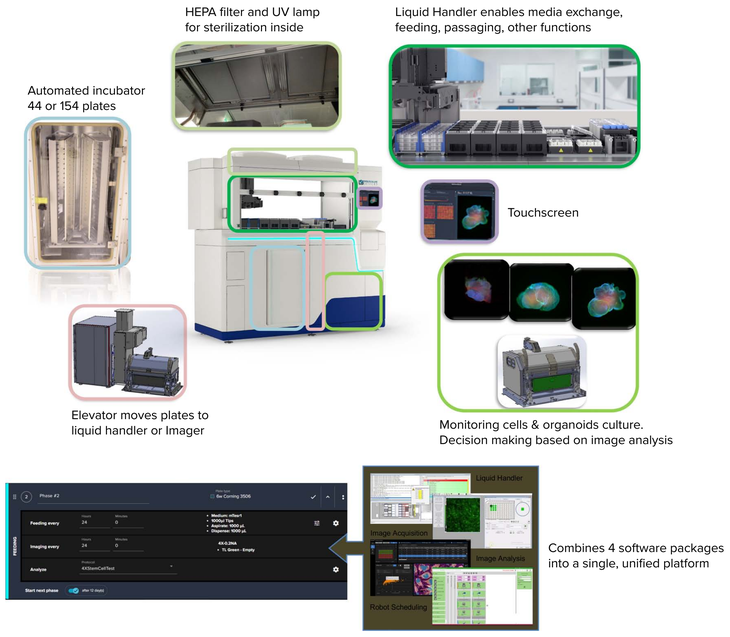


Figure 1. CellXpress.ai components and functionality

Cell culture protocols

3D spheroids were formed from HCT116 colorectal cells (ATCC) using U-shaped ultra-low attachment 96-well plates (Grainer). Cell culture steps including seeding, media exchange, compound addition and staining, imaging, and analysis were done using the CellXpress.ai system. Spheroid imaging was done on the CellXpress.ai system during the culture in transmitted light (TL) using 4X magnification. Endpoint assay imaging was done under 10x magnification using fluorescent imaging (FL) and analysis for organoid size and intensities of viability markers was based on staining with Calcein AM, MitoTracker and Hoechst nuclear stain. Analysis in TL was done using the SYNAP model while analysis in FL was done using the custom module editor (CME) analysis protocol.

Evaluation of compound effects by ATP assay

Analysis of ATP content (CellTiterGlo, Promega), as well as other viability and apoptosis assays done with plate readers, are routinely used for 2D and 3D cancer assays. We used the SpectraMax iD5 plate reader to measure ATP content in spheroids treated with anti-cancer compounds.

High-content imaging and analysis

For high-content analysis of phenotypic changes, fluorescent images were acquired on the ImageXpress® Confocal HT.ai High-Content Imaging System (Molecular Devices) using MetaXpress Software. For intestinal organoids, Z-stack images were acquired with the 4X or 10X objectives using confocal mode. MetaXpress or IN Carta® Image Analysis Software was used for all analysis.

Results

Automation of 3D spheroid/tumoroid assay

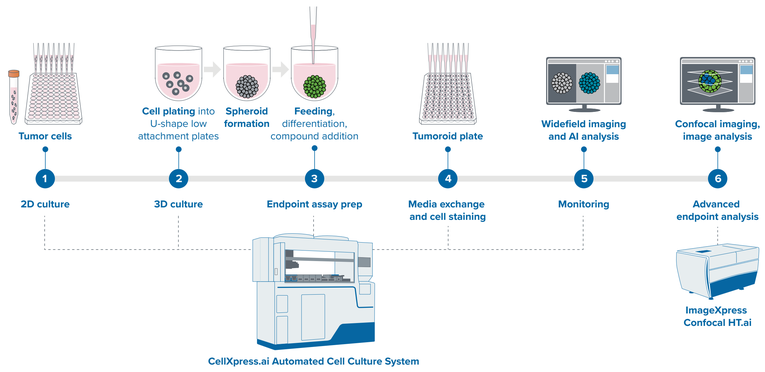
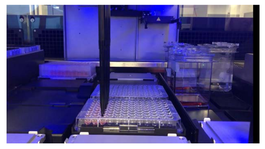


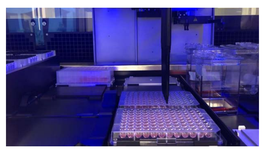
Figure 2. Schematic diagram of automated spheroid culture

1. Cells plated into U-ULA plates
2. Formation of spheroids
3. Media exchange
4. Reagent addition
5. Monitoring, imaging

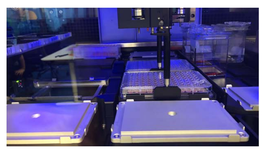
- Workflow is suitable for cancer spheroid assays
- Cardiac organoids, neuro-spheroids
- U-shape ULA plates, culture with media exchange compound treatment (as a reagent), staining and imaging



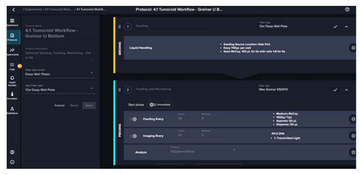
- Cell suspension in media pipetted from deep well reservoir
- Seeded 100µL 10,000 cells into 96well U-bottom plates (Grainer)



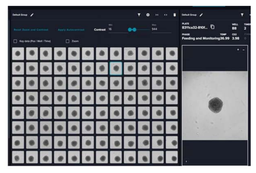
- Removal of media
- Add fresh media
- Protocol set for repeated media exchange every “X” hours



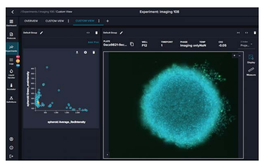
- Move to incubator for 48h
- Monitor spheroid formation with imaging



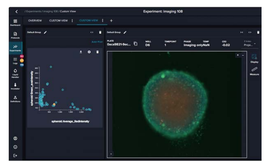
- Full workflow protocol set in “phases” defined by user:
- Plating, feeding/monitoring, staining, imaging
- Each step defines main attributes (source and destination plates, pipetting protocols, volumes, media, imaging settings, analysis protocols
- Main steps can be modified by fine-tuning parameters, that allow further optimization
- Decisions for the process can be made automatically based on imaging analysis



- Periodic imaging of plate is defined by user
- On-the-fly image analysis done by using pre-trained deep-learning based model, or customized protocol
- Data analytics tools allow to view growth of organoids over time
- Staining organoids and washes were done by using “media exchange” protocol
- FL imaging of spheroids was done after treatment with compounds and staining with Calcein AM, MitoTracker and Hoechst nuclear stain.



- Spheroids Cells imaged using CellXpress with the DAPI, FITC and Texas Red channels, 10X or 4X objective. Image analysis was performed with CME (InCarta).



Hoechst – Blue
Calcein AM – Green
EtHD – Red

Figure 3. Steps for the spheroid culture and analysis workflow.

Brief description of spheroid protocol

Protocol was started from cell suspension with media placed into a deep-well reservoir (we used 12-well reservoir). Cell suspension was dispensed into the 96-well U-bottom low-attachment plates (Greiner or Corning). Plates were placed into the incubator and incubation continued with imaging every 12h or 24h using 4x or 10x magnification in TL. Image analysis was run on the fly, detecting spheroids and evaluating size and density.

Compound addition: 48 hours after plating, 50µl of media was removed and 2x concentration of compounds was added in the volume of 50µl. Compounds were added using “different media” protocol.

Compounds were pre-diluted in the 96-well deep-well block, then each compound was added from a single column of the block into 4 or several columns of the 96-well plate. Each compound was added as a separate step that required making a plate map for each compound and then triggering a compound addition step.

Staining spheroids: After 3 days (or other time for compound treatment) organoids were stained using a premixed solution of 3 viability dyes. Staining was done as a media exchange step. Important: to avoid cross-contamination, tips need to be discarded during this step. Staining incubation is done in the incubator for one hour. Imaging protocol can be tested during this time.

Washing step (one step seems sufficient) is done using media exchange (feeding) phase, media was exchanged with PBS.

Spheroids imaging is done using a pre-defined protocol with DAPI, FITC, TexasRed, or other channels. 10–15 steps, 10–15 µm apart, offset 50–100, Z-stacks around focus, and best focus projection. CME analysis protocol was used to define spheroid sizes and intensities with different channels. Changes in spheroids area and average intensities, and ratios of live/dead average intensities were used to evaluate compound effects. 10x magnification allowed for some nuclei resolution, but counting individual cells is not recommended for accurate analysis. For phenotypic characterization/cell counts an ImageXpress confocal high-content imaging instrument is recommended (see below).

Note: this protocol can be used for creating cardiac, liver, or neuro spheroids.

Results

Spheroid culture and analysis

A Screen-shot of compound treatment

B Effects of anti-cancer compounds

- Staining: Hoechst, Calcein AM (live, green), EtHD (dead, read)
- Image analysis: Custom module editor in INCarta software: define spheroids via Find blobs using Hoechst stain mask, then measure average intensities in Calcein AM and EtHD, use ratio to evaluate Live/Dead cell content.

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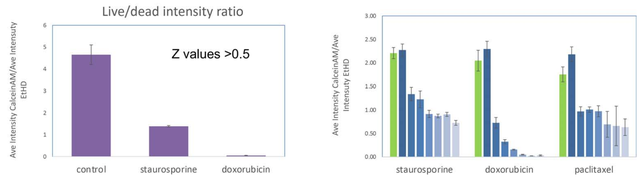

Figure 4. A, B Representative images (10X) of organoid culture taken after treatment with indicated compounds and staining. C. Graphical presentation of spheroid analysis: Ratios of the Ave Intensities of spheroids for Calcein AM and EtHD, maximum concentrations, and across concentration range. Averages and STDEV calculated from quadruplicates.

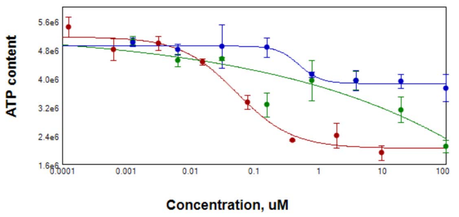

Figure 5. After compound treatment for 3 days, spheroid samples were tested for ATP content using CellTiterGlo reagent for 3D samples. EC_{50} for Staurosporin (red) was 0.05µM, for Paclitaxel (blue) 0.5µM. Data for Doxorubicin (green) were ambiguous due to the possible contribution of compound into Lumi signal.

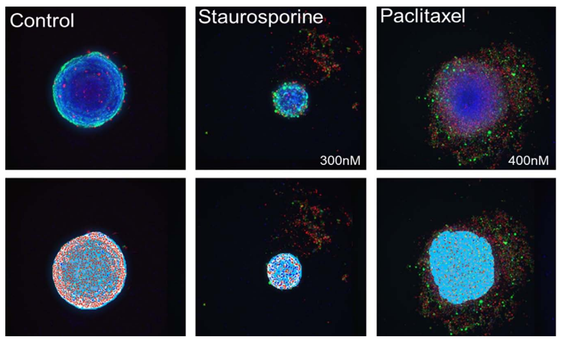

Figure 6. Confocal images of spheroids taken after treatment with compounds and staining with Calcein AM, MitoTracker and Hoechst nuclear stain. Cells were imaged using the ImageXpress HT.ai imaging system with the DAPI, FITC and Texas Red channels, confocal, 10X objective. Red: MitoTracker; Green: Calcein AM; Blue: DAPI

Summary

- There is a great need to develop more effective patient-specific approaches for cancer treatment.
- Organoid technologies are considered a game-changer in disease modeling and drug screening since they better resemble tissue structure and functionality and show more predictive responses to drugs.
- CellXpress.ai Automated Cell Culture System automates the entire cell culture process, from cell culture to assay set-up, screening, and data analysis.
- Automated cell culture processes have great potential to bring 3D biology to another level, increasing throughput and reproducibility, and enabling a variety of high-throughput drug discovery and precision medicine applications.

MOLECULAR DEVICES