Analyzing biological drug effects in 3D

Fluorescence-based drug sensitivity testing using 3D tumor microtissues and the Infinite® M200 PRO monochromator-based multimode reader

Introduction

Cell viability assays are commonly used in cell biology and drug discovery to characterize cell responses to endogenous and exogenous factors or substances, such as cytotoxic drugs and environmental changes (1). Generally, cell viability is assessed using vital dyes, from which viability can be concluded either directly or indirectly. This method, although often used, is labor intensive and tedious. Automated alternatives include electric cell counters (1) and flow cytometers which, although accurate, are associated with sophisticated equipment and high assay costs, and require technical expertise. As a result, fluorescence-based cellular assays are becoming increasingly popular, due to their sensitivity and versatility.

The Infinite M200 PRO offers enhanced fluorescence intensity reading for cell-based and biochemical applications, with a range of features designed to improve sensitivity and inter-/intra-well reproducibility. Functions such as orbital shaking, temperature control and enhanced fluorescence bottom reading with the optimal reading (OR) function ensure excellent performance and reliability.

To further improve the predictive power of in vitro cell-based assays, cell models have to mimic more closely the three dimensional (3D) structure of organs and tissues in vivo (2). Scaffold-based 3D cell culture approaches often suffer high background fluorescence, due to autofluorescence from the scaffold biomaterials. Scaffold-free microtissues are therefore ideally suited to this application, offering tissue-like structures while allowing researchers to take advantage of embedded fluorescent reporter technology.

Combining Tecan's Infinite M200 PRO with InSphero's organotypic microtissue tumor model (which harbors fluorescent reporter proteins) provides a scalable system to assess drug sensitivity in complex 3D cell culture models. This allows long-term, tissue-based analyses – such as cell proliferation studies – offering reproducible measurements over time.

Materials and methods

Instrument

- Infinite M200 PRO Quad4 Monochromators™-based multimode reader

Microplates

- 96-well GravityPLUS™ hanging drop plate (InSphero AG)
- 96-well GravityTRAP™ clear with transparent optical bottom (InSphero AG)
- 96-well cell culture plate (TPP)
- 96-well black half-area clear bottom plate (Greiner® BioOne)

Reagents

- RPMI 1640 (PAA Laboratories)
- Fetal calf serum (PAA Laboratories)
- Penicillin/streptomycin (PAA Laboratories)
- HEPES (PAA Laboratories)
- PBS (PAA Laboratories)
- Trypsin (PAA Laboratories)
- DMSO (Sigma)
- Triton X-100
- compound
- CytoTox-ONE™ Homogenous Membrane Integrity Assay (Promega)

Cell culture and test set-up

Human colon carcinoma cells (HCT116) stably transfected with eGFP (Sirion Biotech GmbH) were grown to confluence in RPMI 1640 supplemented with 10 % fetal calf serum (FCS), 1 % penicillin/streptomycin and 25 mM HEPES at 37 °C under 5 % CO_2.

Single cell suspensions were prepared according to standard cell culture protocols. For monolayer production, 10,000 cells per well were seeded into a standard 96-well plate (TPP).

GravityPLUS plates were used for production of microtissues, as the well design of these plates allows droplets to be formed by top-loading of the cell suspension. The plates were then placed into an incubator at 37 °C in an atmosphere containing 5 % CO_2 for 3-4 days to allow for gravity-enforced cell assembling and microtissue formation.

Compact microtissues were transferred from the GravityPLUS hanging drops into GravityTRAP plates, which are designed to center the microtissues in a culture compartment with a flat bottom 1 mm in diameter.

The GFP signal in microtissues was determined every other day, correlated with diameter and compared to the GFP signal achieved by a confluent monolayer culture using the parameters in Table 1.

Measurement parameter	Instrument settings
Mode	Fluorescence intensity bottom
Excitation wavelength	480 (20) nm
Emission wavelength	514 (9) nm
Flash number	25
Integration time	20 µsec
Gain	100 (manual, preoptimized)

Table 1 Instrument settings for GFP readout on the Infinite M200 PRO.

HCT116eGFP microtissues, with an initial cell number of 500 cells, were treated for 72 h with Taxol®, staurosporine and chlorambucil as reference compounds at eight different concentrations. The IC_{50} value for each compound was determined measuring LDH (biochemical readout) and GFP signal (fluorescence readout).

The LDH assay was performed according to the manufacturer's protocol using the Infinite M200 PRO. Briefly, microtissues were washed once with PBS and lysed with 1 % Triton X-100 for 30 mins at room temperature. The lysate was then homogenized by repeated up and down pipetting and 20 µl of lysate was transferred into black half-area clear bottom plates. 1 % Triton-X100 was used as a background control, and serum containing growth medium as internal control, to account for technical variances between measurements.

20 µl CytoTox-ONE substrate was added, followed by vortexing and a 10 min incubation at room temperature in the dark. Afterwards, 10 µl of stop solution was added, and the fluorescence intensity was measured using the parameters in Table 2.

Measurement parameter	Instrument settings
Mode	Fluorescence intensity bottom
Excitation wavelength	560 (20) nm
Emission wavelength	590 (9) nm
Flash number	25
Integration time	20 µsec
Gain	74 (manual, preoptimized)

Table 2 Instrument settings for Promega's CytoTox-ONE readout on the Infinite M200 PRO.

The experimental data was exported automatically by the i-control™ software to Microsoft Excel® for further analysis. The data was plotted with XLfit and shown as triplicates +/- SD, n=3 replicates with a fitting curve (dose response one side). Red lines indicate calculated IC_{50} values (Figure 5).

Figure 1 Infinite M200 PRO with GCM.

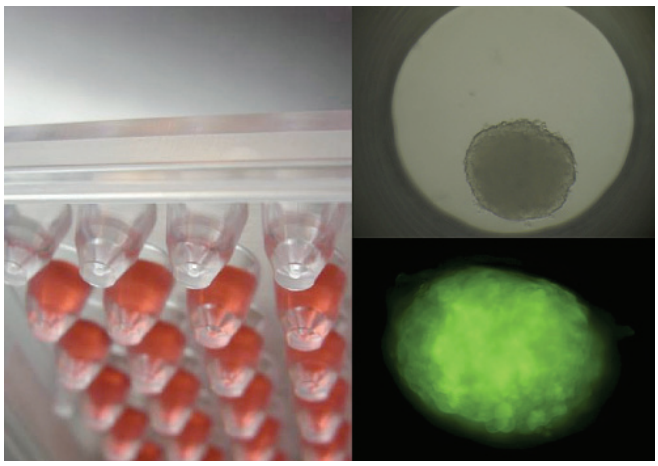

Figure 2 InSphero's GravityTRAP assay platform allows fluorescence measurements directly in the plate. Due to the special design of the GravityTRAP, the Infinite M200 PRO is able to detect the whole fluorescence signal; the beam diameter and detector slit were found to be optimal for the GravitiyTRAP plate (compared to microplate readers from other instrument providers).

Results and discussion

The microtissue format enables higher 3D biological complexity and leads to an increased fluorescence signal compared to monolayer cultures. HCT116-eGFP colon cancer cells grown to confluence in monolayer cultures displayed a 10-fold reduction in signal intensity compared to microtissues initiated with 100 cells after 3 days in culture (Figure 3).

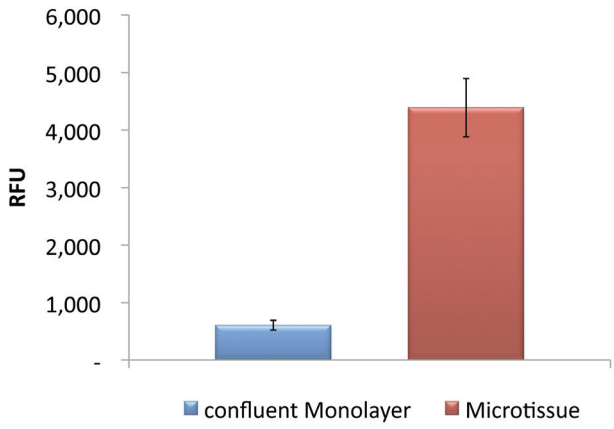

Figure 3 Signal intensity of confluent HCT116-GFP monolayer and microtissues inoculated with 100 cells per microtissues after 3 days in culture.

To compare whether the increase in signal intensity over time correlates with the increase in size, HCT-eGFP microtissues were initiated with 100 cells per microtissue, and fluorescence intensity and size were measured over 12 days in culture. As can be seen in Figure 4, fluorescence intensity correlated with increasing size over this time period.

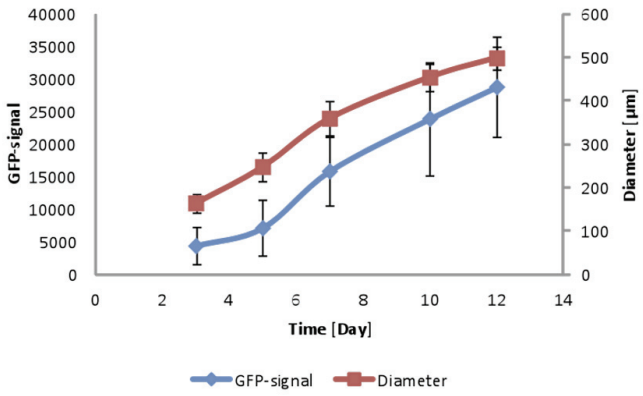

Figure 4 Correlation of the growth (measured by fluorescence intensity and increase in diameter) over 12 days in culture. Microtissue formation was initiated with 100 cells.

To assess the feasibility of using fluorescence intensity as a marker of drug sensitivity, IC50 values were determined either by measuring GFP-fluorescence intensity or tissue LDH levels via fluorescence (3). Both assays resulted in similar dose response curves, as shown for staurosporine (Figures 5). Calculating the IC50 values of the three reference compounds, no major differences were detected between the fluorescent and biochemical techniques (Table 3).

Compound	IC_{50} (LDH)	IC_{50} (RFU)
Taxol	0.30 µM	0.11 µM
Staurosporine	0.04 µM	0.03 µM
Chlorambucil	555.18 µM	306.00 µM

Table 3 IC_{50} values calculated using either an intra-tissue LDH content or relative fluorescence intensity.

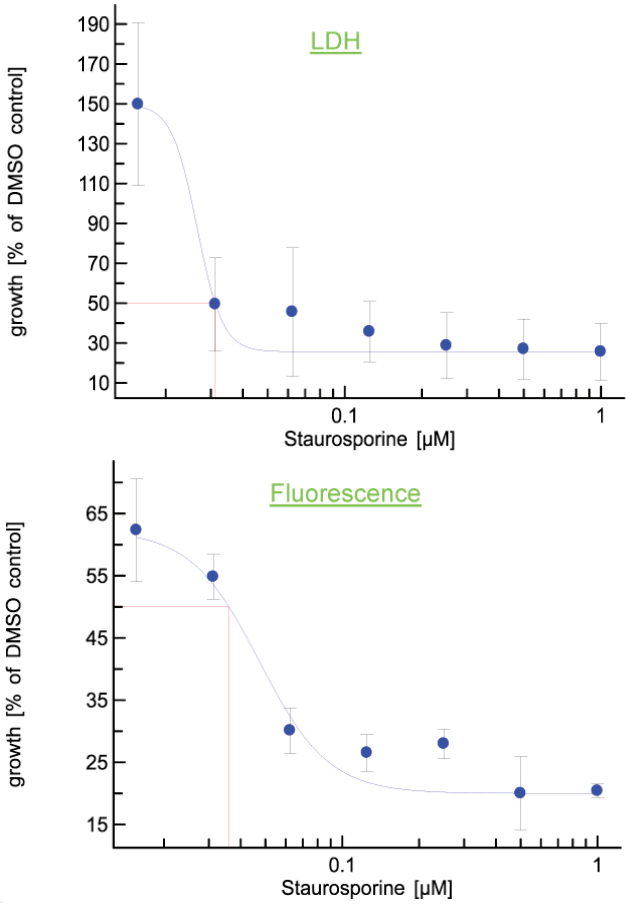

Figure 5 Dose response curve of HCT116-eGFP microtissues (initial 500 cells/MT) treated for 72h with staurosporin using biochemical (top) and fluorescent (bottom) techniques.

Conclusion

The results presented in this technical note clearly demonstrate that combining the Infinite M200 PRO multimode reader with InSphero's microtissue model provides a powerful tool to assess drug sensitivity in more complex 3D cell culture models. Its non-invasive analytical capabilities have shown similar sensitivity to biochemical assays. The signal intensity of even small cell populations in the microtissue model is highly elevated as compared to confluent monolayer cultures, leading to a higher dynamic range for the 3D model. Due to its optimized optical properties the Infinite M200PRO was found to be the instrument of choice for 3D cell culturebased measurement using the technology developed by InSphero.

Acknowledgements

Transgenic HCT116-eGFP cells were generated and provided by Siron Biotech GmbH in Munich, Germany.

Literature

1) Hynes et al. Journal of Biomolecular Screening 8(3); 2003)
2) Kelm et al. Trends in Biotechnology 86(2), 2004
3) Drewitz et al. Biotechnology Journal 6(12), 2011

Austria +43 62 46 89 33 **Belgium** +32 15 42 13 19 **China** +86 21 2206 3206 **Denmark** +45 70 23 44 50 **France** +33 4 72 76 04 80 **Germany** +49 79 51 94 170
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