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Development of a 96-well Assay for Assessing Cell Viability in Mouse Small Intestinal-Derived Organoids After Treatment with Cytotoxic Compounds

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Introduction

The intestinal epithelium is a single cell layer forming the lining of the gastrointestinal (GI) tract. It is a rapidly renewing tissue composed of multiple cell types that arise from intestinal stem cells at the base of the crypts. Recent advances have led to the development of a novel method for the long-term culture of isolated intestinal crypts in a three-dimensional (3D) extracellular matrix. This 3D model system of intestinal-derived organoid culture exhibits the key features of adult intestinal epithelium, including a crypt-villus morphology, a functional central lumen and all of the major cell types. As these cultures better represent the physiology of the intestinal epithelium than immortalized or transformed cell lines (e.g. Caco-2, MC38) and are not associated with the same costs and ethical considerations as in vivo experiments, organoid cultures may serve as useful screening tools during drug development. We have developed a 96-well assay to screen small molecule compounds for toxicity in mouse small intestinal-derived organoids. Crypts isolated from the small intestines of mice were successfully cultured in Corning Matrigel® and IntestiCult™ Organoid Growth Medium in a 96-well format. Organoids were seeded at several densities and treated with varying concentrations of drugs that cause GI toxicity (Irinotecan, 5-Fluorouracil (5-FU) and Flavopiridol) and one anti-diarrheal (Loperamide) for 24 - 72 hours, after which cell viability was assessed using CellTiter-Glo 3D. Optimal seeding density and treatment times were first established, after which inter-assay reproducibility and animal, operator, and intra-assay variability were assessed.

Materials & Methods

Isolation of Mouse Intestinal Crypts: Small intestines of C56/BL6 mice were dissected and washed several times in cold PBS. Intestinal fragments were incubated in 25 mL of Gentle Cell Dissociation Reagent (GCDR) [STEMCELL Technologies Inc. (STI)] on a rocking platform (20 rpm) at room temperature for 20 minutes, after which the fragments were allowed to settle by gravity and supernatants removed. Intestinal pieces were resuspended in 10 mL cold PBS + 0.1% BSA by pipetting up and down several times. The intestinal pieces were again allowed to settle by gravity and the supernatants were collected, passed through 70 µm filters and kept on ice (fraction 1). This process was repeated three times to generate fractions 2 - 4. The quality of each fraction was assessed using an inverted microscope and selected fractions were centrifuged. Crypts were then resuspended in cold DMEM/F-12 (STI), counted, aliquoted into 15 mL tubes in volumes containing 500 - 3000 crypts and centrifuged. Crypts were resuspended in a 50:50 mixture of complete IntestiCult™ Organoid Growth Medium (Supplement 1, Supplement 2 and 100 units/100 µg per mL penicillin/streptomycin) (STI) and Corning Matrigel® at 1,667 - 10,000 crypts/mL, and 50 µL/well of the suspension was pipetted into pre-warmed 24-well plates to form domes containing 80 - 500 crypts. The domes were solidified at 37°C for 10 minutes before complete Intesticult™ Organoid Growth Medium was added (750 µL/well). Cultures were incubated at 37°C, 5% CO_2, for 7 - 10 days before passaging, with medium changes 2 - 3 times per week. In some instances, organoids cryopreserved using CryoStor® CS10 were thawed and used to initiate cultures as described above.

Passaging of Mouse Intestinal Organoids: Every 7 - 10 days, organoids were passaged by treating cultures with 1 mL cold GCDR for 1 minute followed by mechanical disruption to break up domes. Suspensions were transferred to 15 mL conical tubes, incubated on a rocking platform at 20 rpm for 10 minutes at room temperature and then centrifuged. Pellets were washed once with 10 mL cold DMEM/F-12, resuspended in a 50:50 mixture of IntestiCult™ Organoid Growth Medium and Matrigel® and plated as described above. A split ratio of 1:6 - 1:8 was generally used.

96-Well Plate Assay: Organoids were harvested as described above and seeded at ~100 - 200 crypts (1:6 - 1:12 split ratio) per well in a 2:3 mixture of Matrigel® and complete IntestiCult™ Organoid Growth Medium in 96-well plates. Matrigel® was solidified, 100 µL of complete IntestiCult™ Organoid Growth Medium added to each well and the plates incubated at 37°C, 5% CO_2 for three days. On day 3, media was removed and fresh media containing varying concentrations of drug was added at four replicates per drug dose. The drugs used included the GI toxic compounds 5-FU (anti-proliferative), Irinotecan (topoisomerase inhibitor) and Flavopiridol (CDK inhibitor), and the anti-diarrheal Loperamide. Drugs (purchased from Sigma) were solubilized in DMSO and added to cultures to ensure the final DMSO concentration did not exceed 0.1%. Cultures were incubated with drugs for 24 - 72 hours with fresh drug being added every 24 hours. After treatment, 100 µL of CellTiter-Glo® 3D (Promega) was added to each well and the Matrigel® disrupted by mechanical disruption. The contents were vigorously mixed on a plate shaker for 5 minutes, incubated at room temperature for 25 minutes, transferred to opaque-walled plates and the luminescence recorded using a SpectraMax M5 plate reader.

IC50 Determination: The concentration of 50% inhibition (IC_{50}) of mouse small intestine organoid growth for each drug was calculated by plotting the log of the drug concentration against the percent of maximum viability using GraphPad Prism® 5. To generate a curve fitting these data points the following equations were used:

log(inhibitor) vs. normalized response – variable slope: $Y=100/[1+10^{((LogIC_{50}-X)*HillSlope)}]$

log(inhibitor) vs. response (3 parameters): $Y=bottom + (top-bottom)/[1+10^{(X-LogIC_{50})}]$

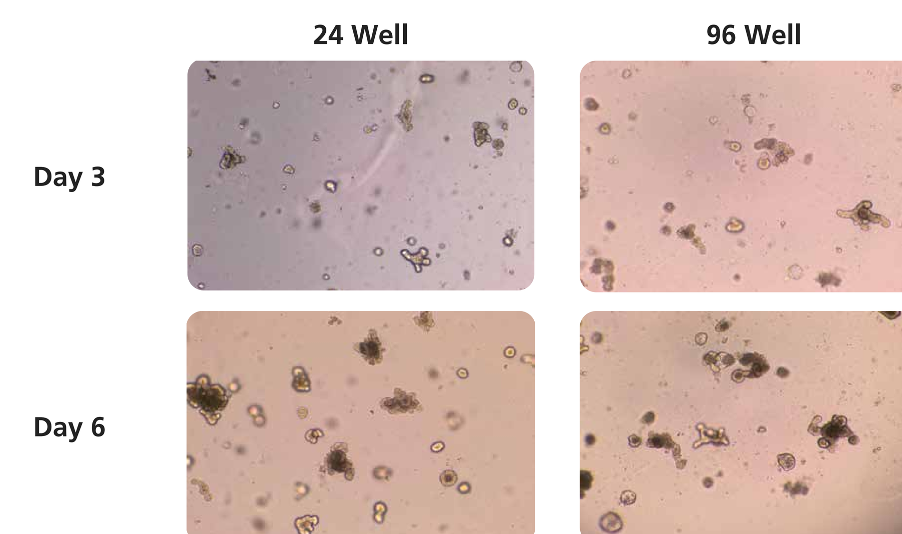
log(inhibitor) vs. response – variable slope (4 parameters): $Y=bottom + (top-bottom)/[1+10^{((LogIC_{50}-X)*HillSlope)}]$

Percent Coefficient of Variation (%CV) Determination: The %CV was calculated by dividing the sample standard deviation by the mean of replicates and multiplying by 100. $\%CV = \sigma / \bar{X}$ where: σ = Standard Deviation = $\sqrt{[\Sigma(X_i-\bar{X})^2/(n-1)]}$ and $\bar{X}$ = Average = $\Sigma X_i/n$

FIGURE 1: Schematic of 96-well mouse small intestine organoid cell viability assay

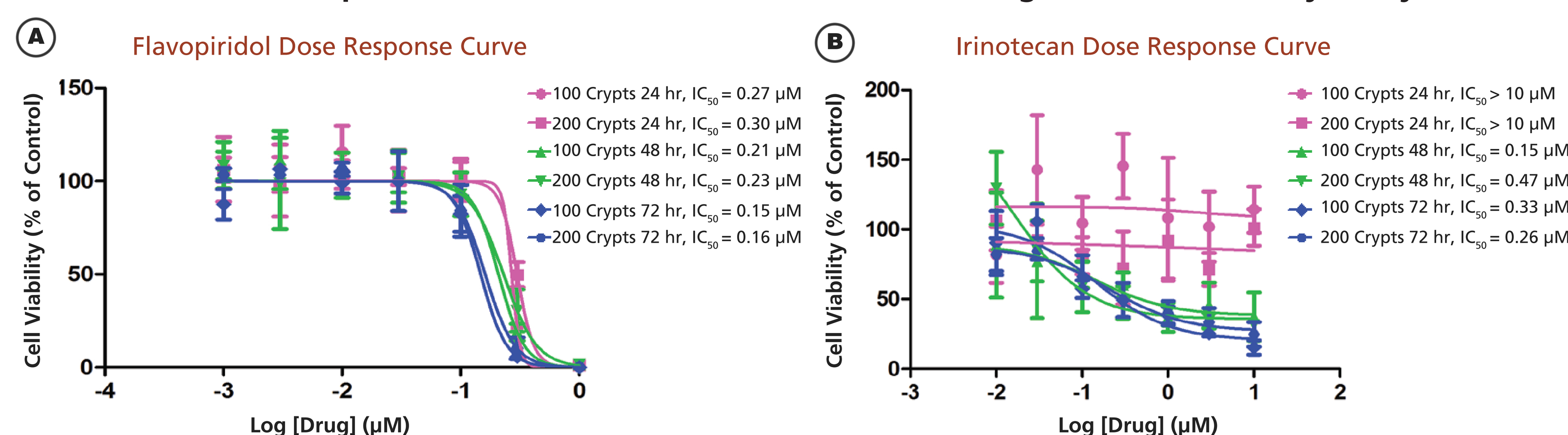
Results

FIGURE 2: Mouse small intestine organoid growth can be scaled down to a 96-well format



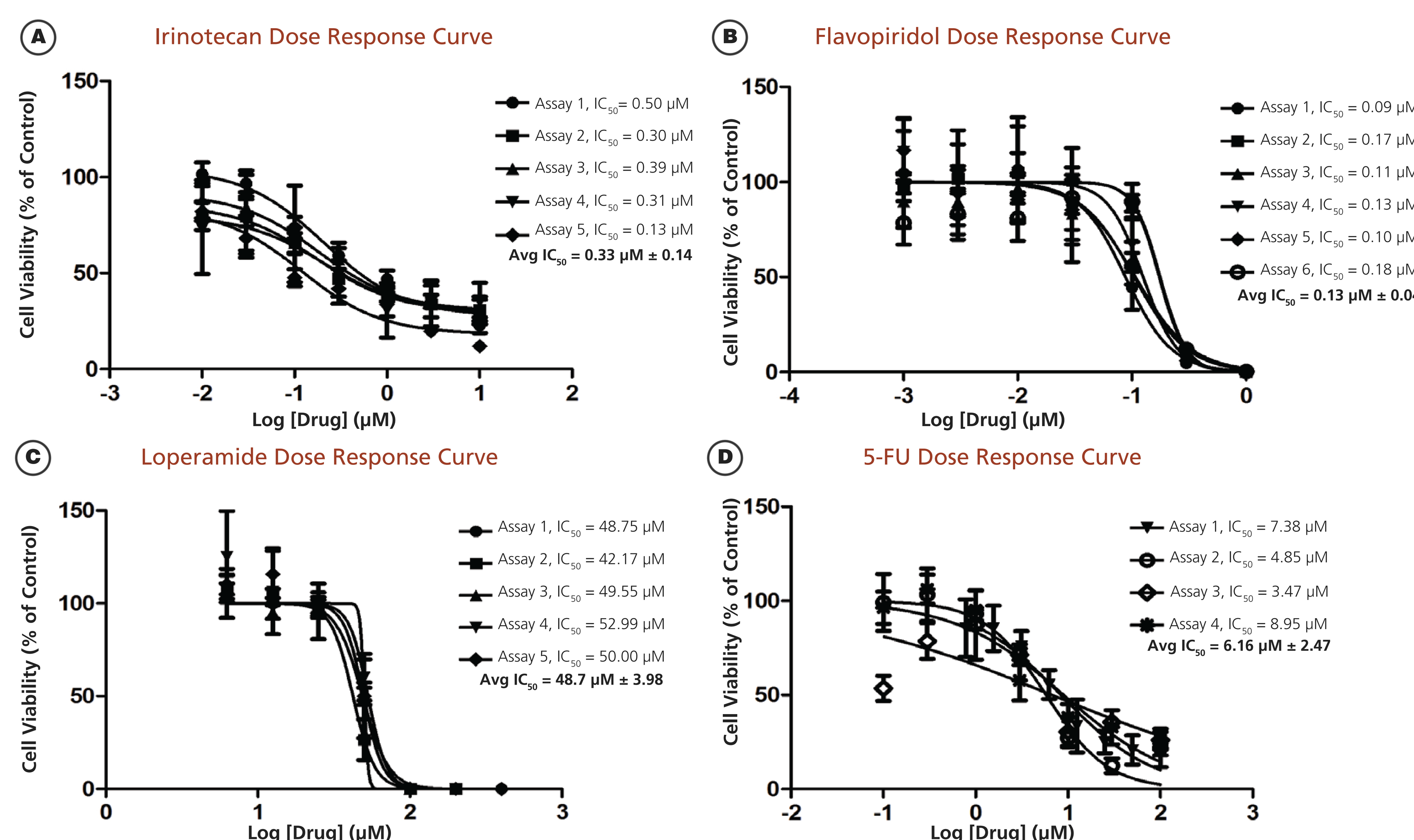
Morphology of mouse small intestine organoids grown in 96-well and 24-well tissue culture treated plates for 3 and 6 days. Morphology of organoids grown in a 96-well format is comparable to organoids grown in 24-well plates.

FIGURE 3: Protocol optimization of the mouse small intestine organoid cell viability assay



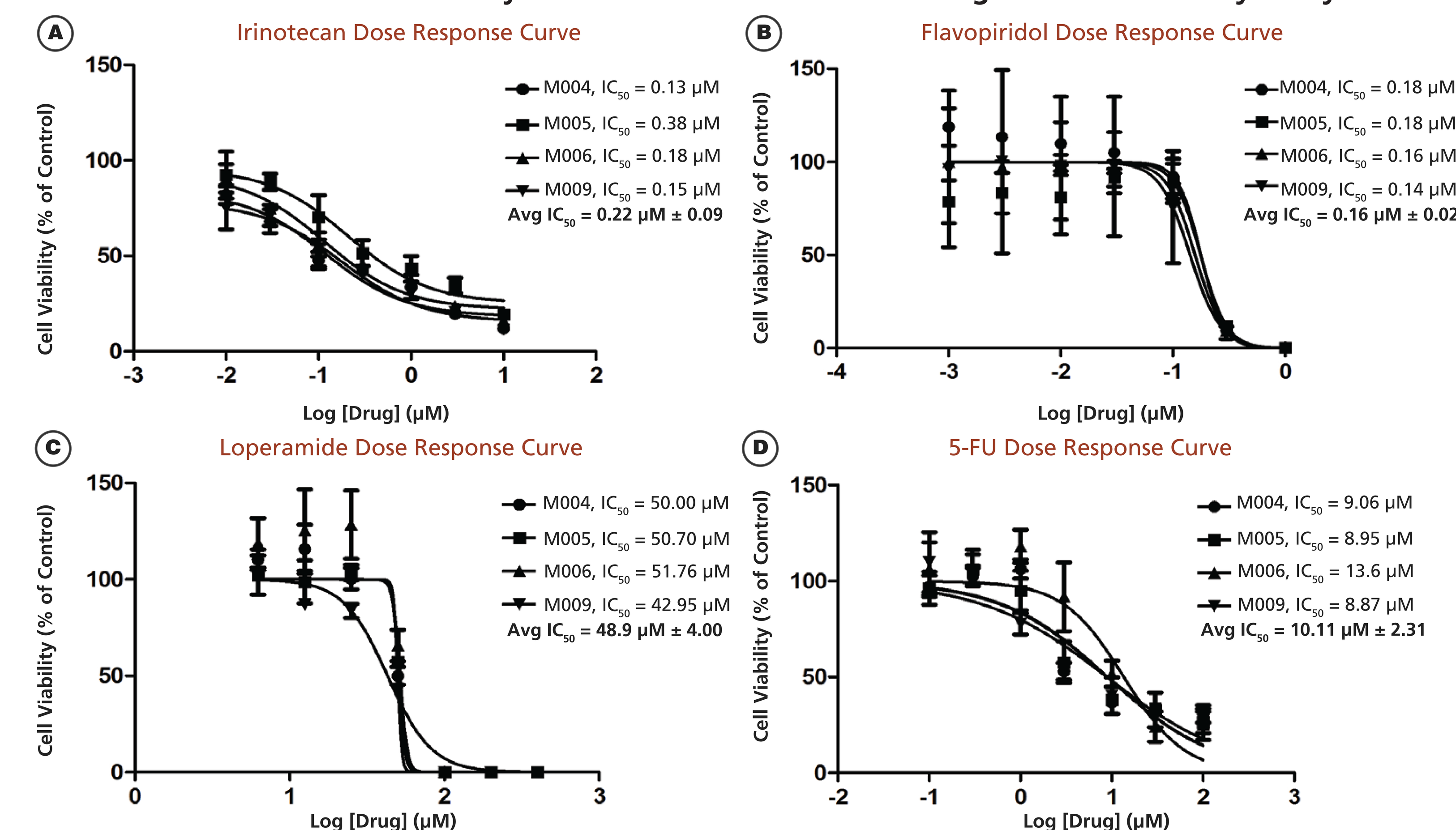
Different treatment periods (24 - 72 hr) and initial seeding densities (100 or 200 crypts/well) were assessed using Flavopiridol and Irinotecan as model toxic drugs. **(A)** IC_{50} values for Flavopiridol dose response curves were very similar for 24, 48 and 72 hr cultures at the different seeding densities; however, the variability between technical replicates was lower in cultures seeded at 100 crypts/well [median %CV = 13 (100 crypts/well), and 28 (200 crypts/well)]. **(B)** IC_{50} values for Irinotecan dose response curves were similar for 48 and 72 hr cultures at both seeding densities. While there was little difference in the variability between technical replicates at the different seeding densities, there was lower variability between technical replicates at 72 hr (median %CV = 18) versus 24 (median %CV = 24) and 48 hr (median %CV = 26). The viability of solvent control cultures increased up to 72 hr, suggesting that cultures were not overgrown at this time point. Due to slightly higher sensitivity and lower variability, optimal assay conditions were determined to be an initial seeding density of 100 crypts/well and a treatment time of 72 hr.

FIGURE 4: Inter-assay reproducibility in the mouse small intestine organoid cell viability assay



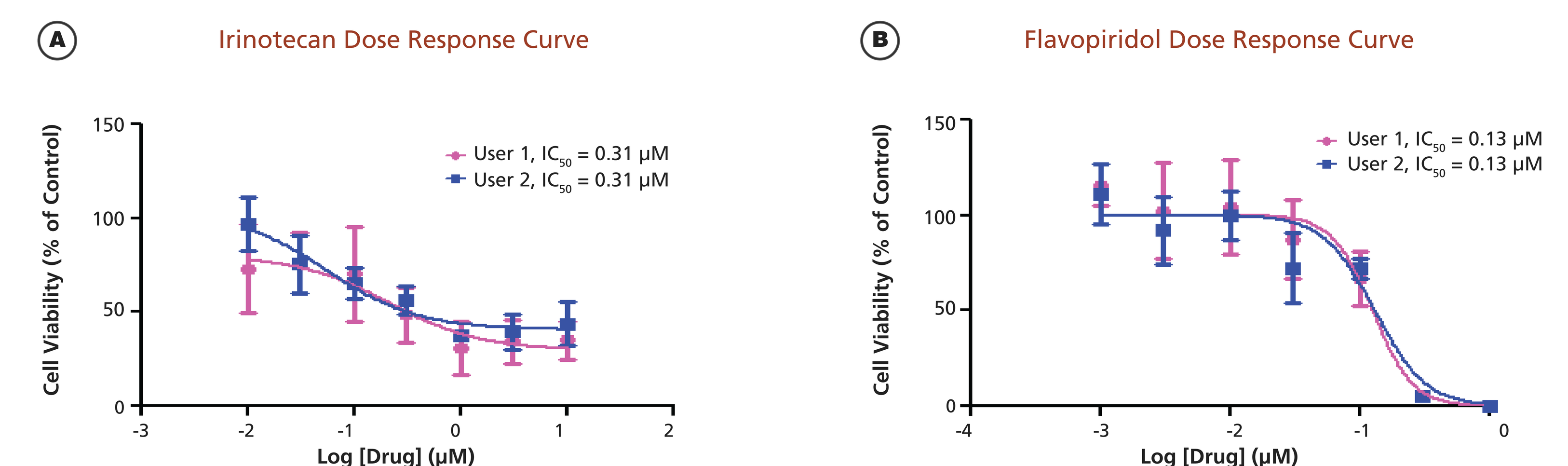
Mouse small intestine organoids were seeded at ~100 crypts/well and treated with various concentrations of Irinotecan **(A)**, Flavopiridol **(B)**, Loperamide **(C)** and 5-FU **(D)** for 72 hours. At the end of the incubation period cell viability was assessed using CellTiter-Glo® 3D and treated wells were compared to solvent control wells to generate dose response curves. IC_{50} values from independent assays performed using organoids from the same animal were compared.

FIGURE 5: Inter-animal variability in the mouse small intestine organoid cell viability assay



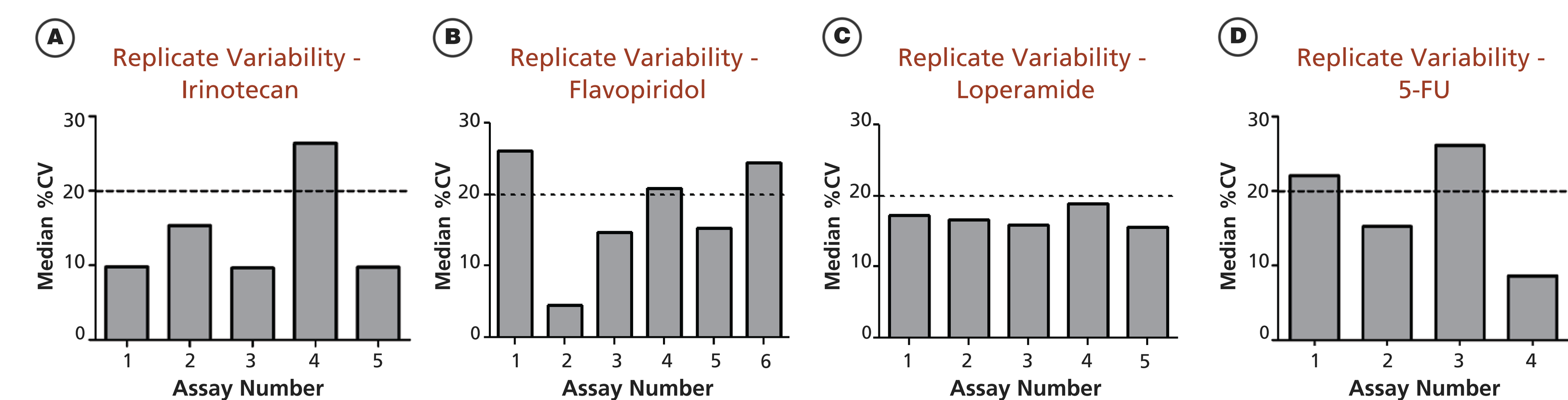
Mouse small intestine organoids from four animals were seeded at ~100 crypts/well and treated with various concentrations of Irinotecan **(A)**, Flavopiridol **(B)**, Loperamide **(C)** and 5-FU **(D)** for 72 hours. At the end of the incubation period, cell viability was assessed using CellTiter-Glo® 3D and treated wells were compared to solvent control wells to generate dose response curves. IC_{50} values for each animal were compared.

FIGURE 6: Inter-operator variability in the mouse small intestine organoid cell viability assay



Mouse small intestine organoids were seeded by at ~100 crypts/well and treated with various concentrations of Irinotecan **(A)** and Flavopiridol **(B)** for 72 hours. At the end of the incubation period, cell viability was assessed using CellTiter-Glo® 3D and treated wells were compared to solvent control wells to generate dose response curves. IC_{50} values from assays carried out independently by two different operators using the same batch of organoids were compared.

FIGURE 7: Replicate reproducibility in mouse small intestine organoid cell viability assays



Mouse small intestine organoids were plated in cell viability assays at 4 replicates per drug dose. The percent coefficient of variation (%CV) of replicates per dose was calculated for Irinotecan **(A)**, Flavopiridol **(B)**, Loperamide **(C)** and 5-FU **(D)**, and the median %CV per experiment is shown. The median %CV was under 20% in most experiments.

Conclusions

- Growth of mouse small intestine organoids can be scaled down to a 96-well format
- Optimal seeding density (~100 crypts/well) and treatment time (72 hr) were established
- Inter-assay, inter-animal and inter-operator variability in IC_{50} values was low
- Replicate reproducibility was good (median %CV under 20% in most experiments)
- 96-well mouse small intestine organoid cell viability assay may serve as a useful novel tool for investigating GI toxicity of candidate therapeutics in biologically relevant conditions during drug development

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