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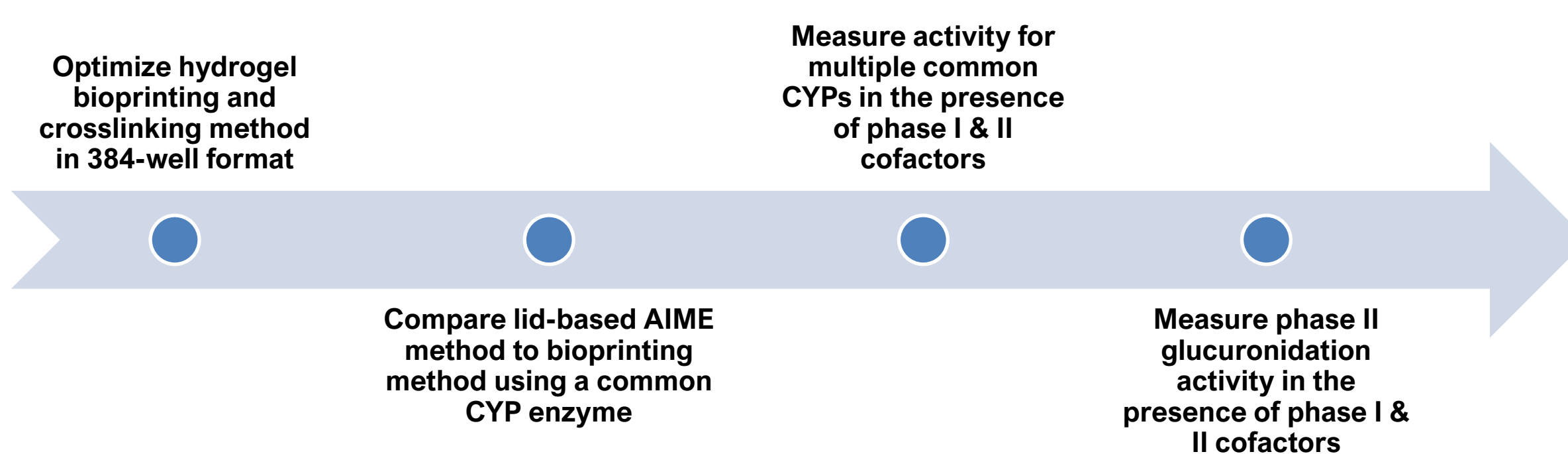
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Introduction

Acceptance and use of *in vitro* data for hazard identification, prioritization, and risk evaluation is limited in part by uncertainties associated with xenobiotic metabolism. Most *in vitro* systems lack the biotransformation capabilities of intact in vivo systems. This raises the possibility of overestimating the hazard of compounds that are rapidly bioinactivated *in vivo* or underestimating the hazard of those that are transformed to more active metabolites. The Alginate Immobilization of Metabolic Enzymes (AIME) is a lid-based method for retrofitting bioassays with metabolic competence. However, accessibility and throughput of the AIME platform is limited by proprietary custom materials and a complex workflow.

The objective of this study was to address limitations of the lid-based AIME method through the incorporation of automated 3D bioprinting, with the goal of depositing S9-encapsulated microspheres directly into standard 384-well plates and including requisite cofactors for phase I and II hepatic metabolism. Further refinement of this assay system has the potential to expand high throughput screening capabilities in a robust, accessible way to incorporate *in vitro* xenobiotic metabolic competence.

Study Overview

- Optimize hydrogel bioprinting and crosslinking method in 384-well format
- Compare lid-based AIME method to bioprinting method using a common CYP enzyme
- Measure activity for multiple common CYPs in the presence of phase I & II cofactors
- Measure phase II glucuronidation activity in the presence of phase I & II cofactors

Alginate Immobilization of Metabolic Enzymes (AIME)

The **Alginate Immobilization of Metabolic Enzymes (AIME)** platform utilizes rat hepatic S9 fraction to evaluate phase I xenobiotic metabolism with minimal cytotoxic and assay interference effects compared to free S9 assays.

The optimized platform consists of custom 384-well microplate peg lids with encapsulated S9-alginate microspheres attached to solid supports.

- Pre-stamp peg lid in Matrigel
- Stamp in S9-alginate
- Stamp in BaCl₂ solution ($BaCl_2$ solution)
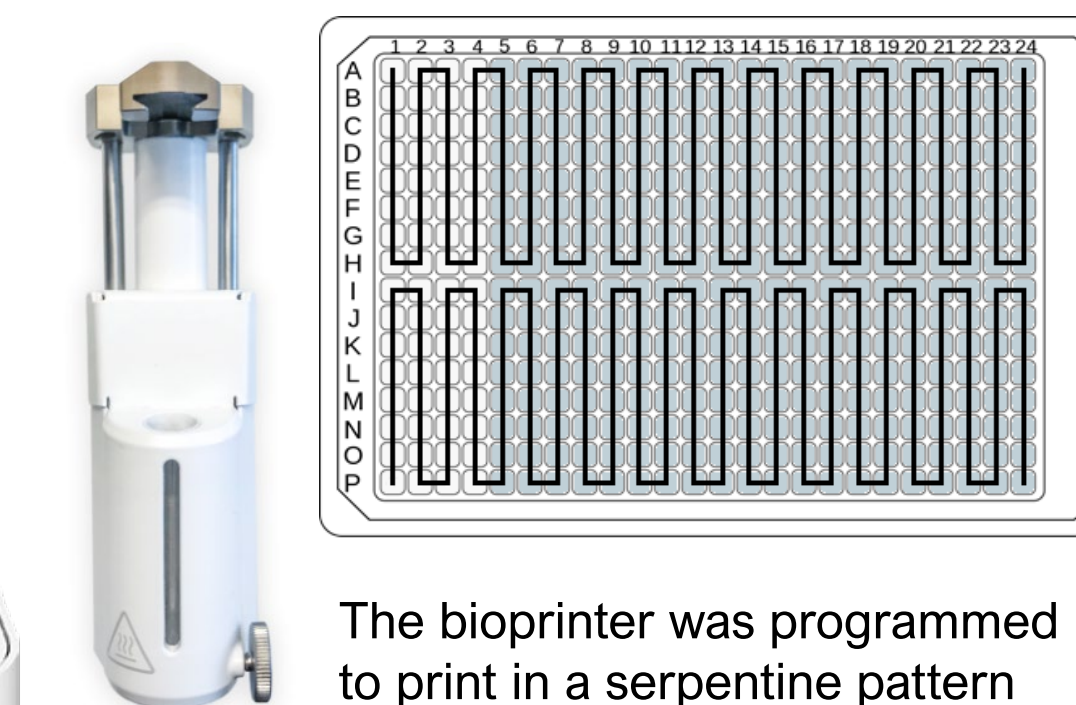- Stamp in PLL solution
- Ready for assay

Overview of BIOX 3D Bioprinter

The **BIOX 3D Bioprinter** has the ability to quickly deposit S9-alginate microspheres directly into standard 384-well microplates.

Mechanical extrusion with the syringe pump printhead (pictured below) provided the most precise and consistent printing between wells.
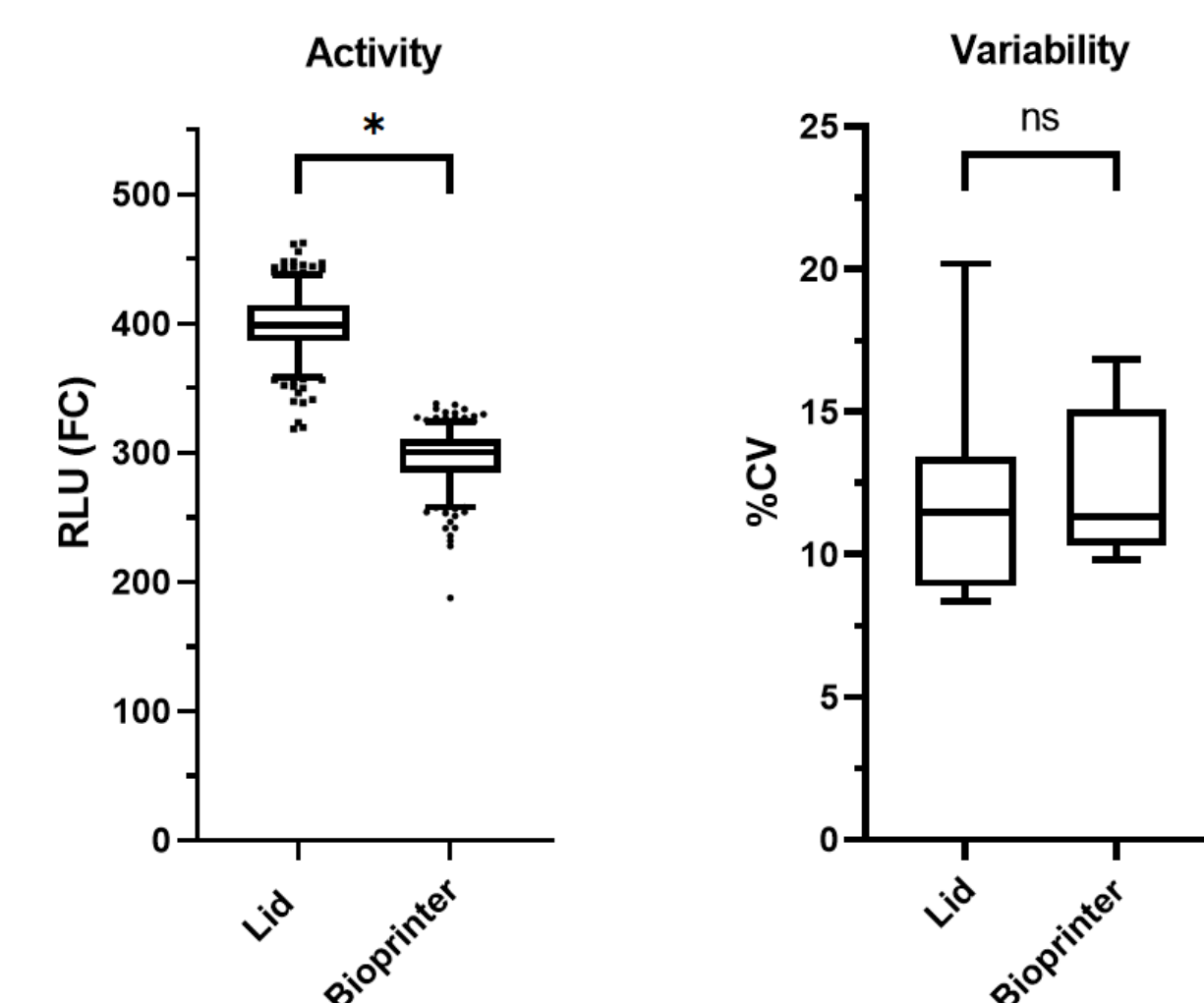

The bioprinter was programmed to print in a serpentine pattern (pictured above).

- Initiate printing on BIOX
- Crosslink on liquid handler
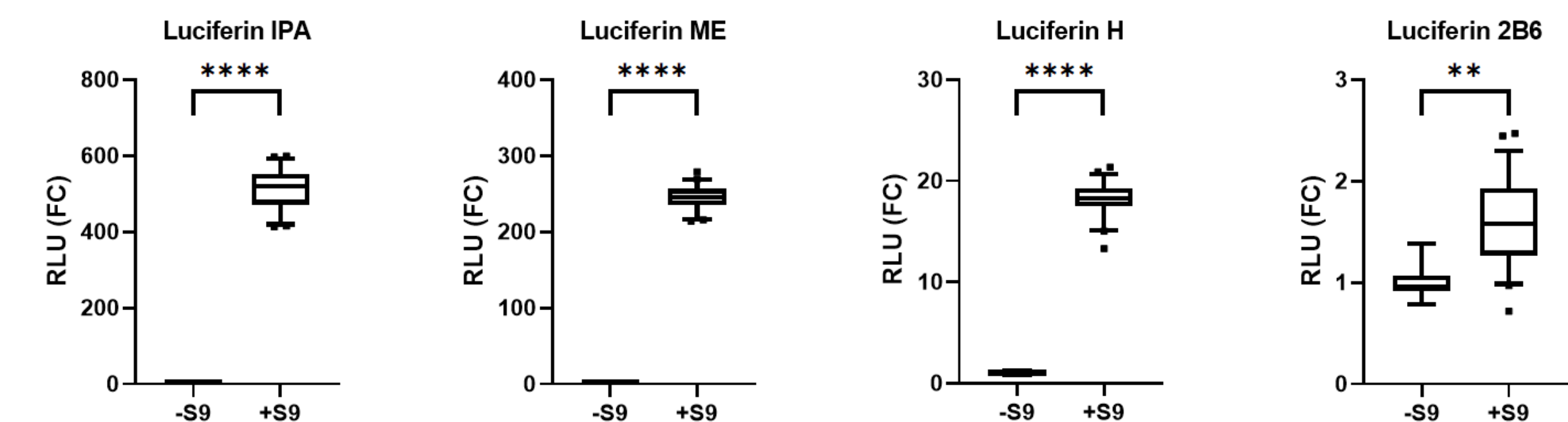- Ready for assay

AIME Method Comparison

Comparison of lid-based and 3D bioprinter AIME methods. Promega P450-Glo™ substrate Luciferin-IPA was used to measure CYP3A activity with rat S9-alginate microspheres in a 384-well format using lid-based (Lid) and 3D bioprinter (Bioprinter) AIME methods. Assay medium was supplemented with phase I (NADPH) cofactor and then added to either an S9-alginate dipped lid or an S9-alginate printed microplate for two hours. Values were normalized to plate-based controls and plotted across experiments (n = 4). Unpaired, one-way t-tests showed a significant difference between methods (* = $P < 0.05$) and no difference (ns = no significance) between coefficients of variation (%CV) for either method.


Phase I CYP Enzyme Activity

Luciferin Substrate	Human CYP	Rat CYP
IPA	CYP3A4	CYP3A1
ME	CYP1A2	CYP1A2
H	CYP2C9	CYP2C6
2B6	CYP2B6	CYP2B1

Evaluation of phase I CYP enzyme activity using 3D bioprinter AIME method. Promega P450-Glo™ substrates Luciferin-IPA, -ME, -H, and -2B6 were used to measure CYP activity with (+S9) and without (-S9) rat S9-alginate microspheres in a 384-well format. Assay medium was supplemented with phase I (NADPH) and phase II (UDPGA, GSH, PAPS) cofactors, and then added to an S9-alginate printed plate for two hours. Values were normalized to plate-based controls and plotted across experiments (n = 3-5). Unpaired, one-way t-tests showed significant differences between -S9 and +S9 (**** = $P < 0.0001$, ** = $P < 0.01$).

Phase II Glucuronidation Activity
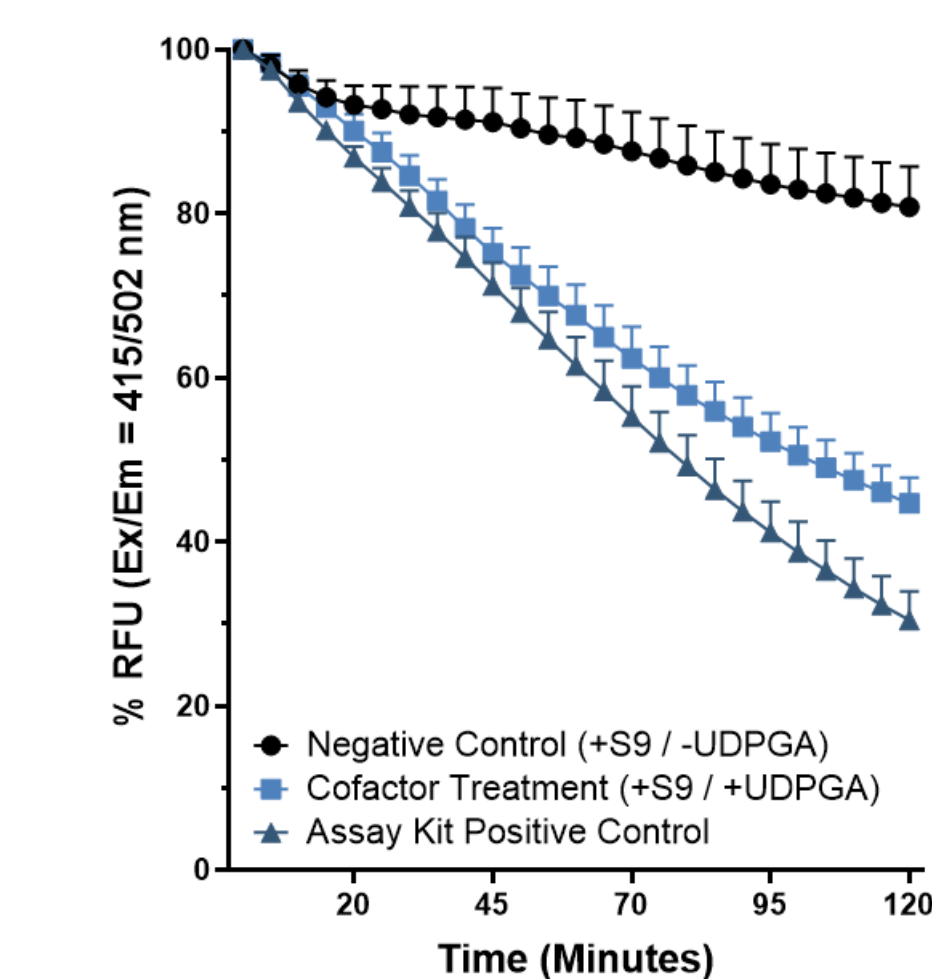
Evaluation of phase II glucuronidation activity using 3D bioprinter AIME method. The BioVision UGT Activity Assay utilizes a highly fluorescent uridine diphospho-glucuronosyltransferases (UGT) substrate that monitors a drop in fluorescence emission as the substrate is converted into a non-fluorescent glucuronide conjugate. UGT substrate was used to measure glucuronidation with rat S9-alginate microspheres in a 384-well format. Assay medium supplemented ± phase I (NADPH) and phase II (UDPGA, GSH, PAPS) cofactors was added to an S9-alginate printed plate and fluorescence measured in kinetic mode for 120 min at 37°C. A UGT Positive Control (-S9) was run for comparison. Values were normalized to T = 0 min and plotted across experiments (n = 3). Statistically significant glucuronidation of the UGT substate ($P < 0.0001$) was observed compared to negative control.

Conclusions

- The lid-based AIME method was successfully adapted to an automated bioprinter method.
- The bioprinter method expands the functional capacity for hepatic phase I (CYPs) and phase II (UGTs, SULTs, GSTs) metabolic enzymes.
- Future proof-of-concept application of this new approach method is an important step toward incorporating metabolic competence into high-throughput *in vitro* assays.

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