Hepatic Steatosis Shifts Phase I Metabolism & Alters In Vitro Toxicant Susceptibility

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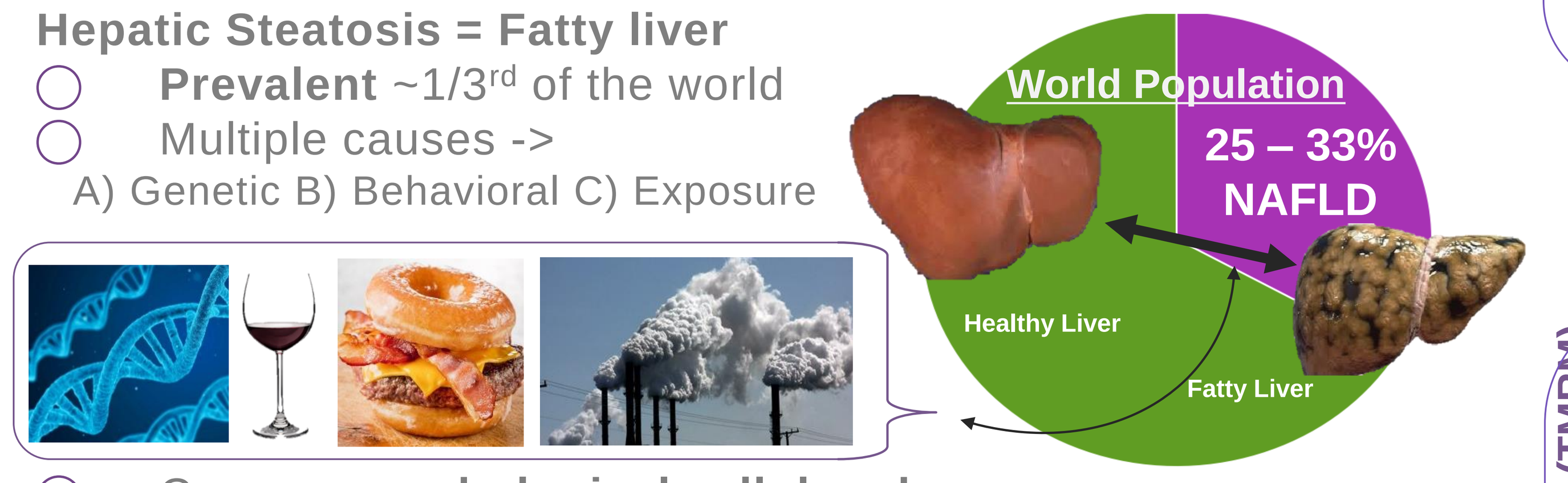
Abstract

Hepatic steatosis (fatty liver disease) is a pathological condition that can alter xenobiotic metabolism, and thereby, alter susceptibility to environmental toxicants. Here we used a metabolically competent human liver-derived cell line - HepaRG - to model steatosis for in vitro toxicity assessment. Cells were exposed to vendor-provided completed-media with added 1% BSA-complexed oleate and palmitate fatty acids to induce a steatotic state. An optimum free fatty acid (FFA) ratio of 1:2 oleate to palmitate fatty acid, total FFA concentration of 1 mM, and a 7-day FFA incubation time were identified experimentally and chosen to achieve measurable lipid accumulation with minimal toxicity. The hepatotoxic piscicide rotenone was selected to assess chemical toxicity in our steatotic model. Cell viability was measured 24h after exposure using Cell Titer Glow, an intracellular ATP, assay. The IC50's for rotenone were altered with lipid loading, shifting a naïve IC50 from 0.64 μM to 0.46 μM in a steatotic model. The altered cell viability becomes increasingly significant, as seen using a two-tailed students t-test where p<0.05, with increasing concentrations of rotenone. There was a reduction in expression of several cytochrome P450 (CYP) genes in the HepaRG cells in a steatotic state when measured by qPCR. For example, CYP3A4, the most active P450 enzyme in rotenone metabolism, expression was reduced. P450 activity is a major factor in limiting rotenone toxicity as rotenone metabolites are less active than the parent compound. These results suggest that our in vitro HepaRG steatosis model can be a useful tool for evaluating in vivo hepatic steatosis as a risk factor in chemical toxicity. Future addition of high content analysis of oxidative stress and mitochondrial dysfunction in the model may enhance its predictive capability for human hepatotoxicity susceptibility screening.

Introduction

Hepatic Steatosis = Fatty liver

- **Prevalent** ~1/3rd of the world
- Multiple causes ->
  A) Genetic B) Behavioral C) Exposure



- Causes **morphological cellular changes**
- Often **asymptomatic** & reversable
- Disease state (altered lipid metabolism + ↑ triglyceride retention) **may increase susceptibility** to environmental chemicals.

In-Vitro Model

Methods available : LINK

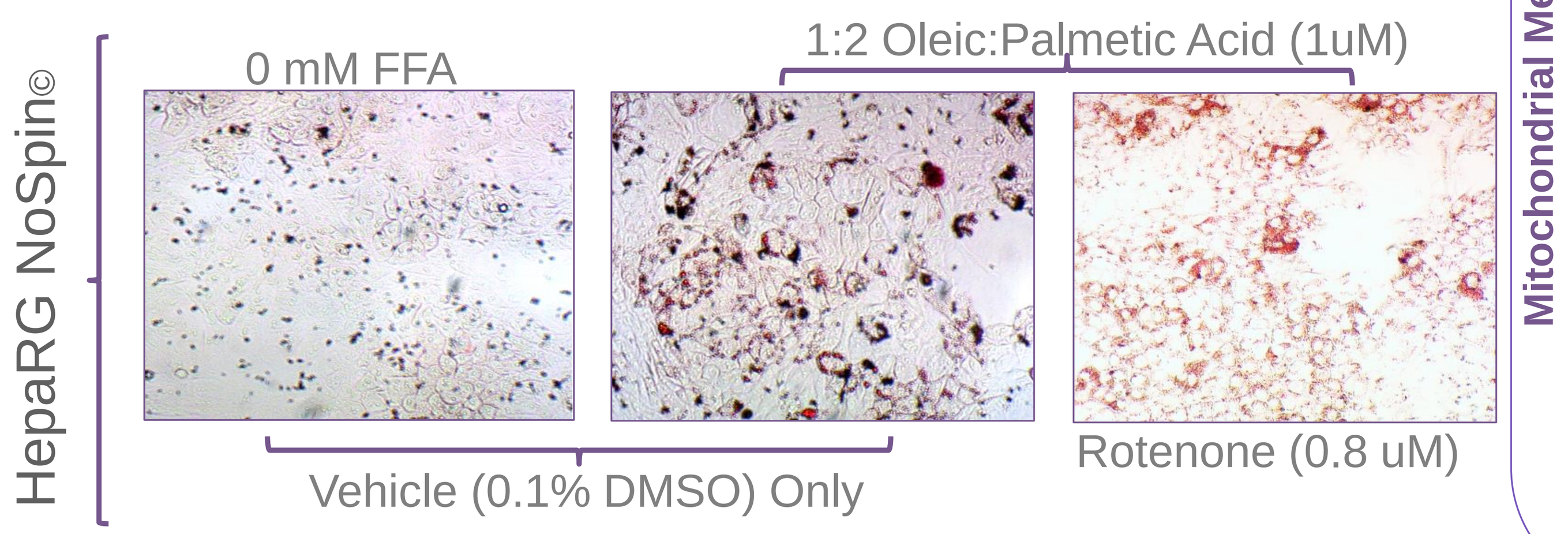


Figure 1. Establishing significant fat build-up in HepaRG cells. Oil red O staining indicated intracellular accumulation of lipid droplets after free-fatty acid [**FFA**] exposure in the media for 1 week. The addition of 0.8 uM rotenone, a known hepatotoxicant and mitochondrial respiratory chain complex I inhibitor, significantly increased fat retention due to decreased fatty acid metabolism.

U.S. Environmental Protection Agency
Office of Research and Development
Center for Computational Toxicology & Exposure
Biomolecular & Computational Toxicology Division
Alternative Experimental Toxicology Modeling Branch

R1: Model Characterization

Steatotic Cells – Fluorescence

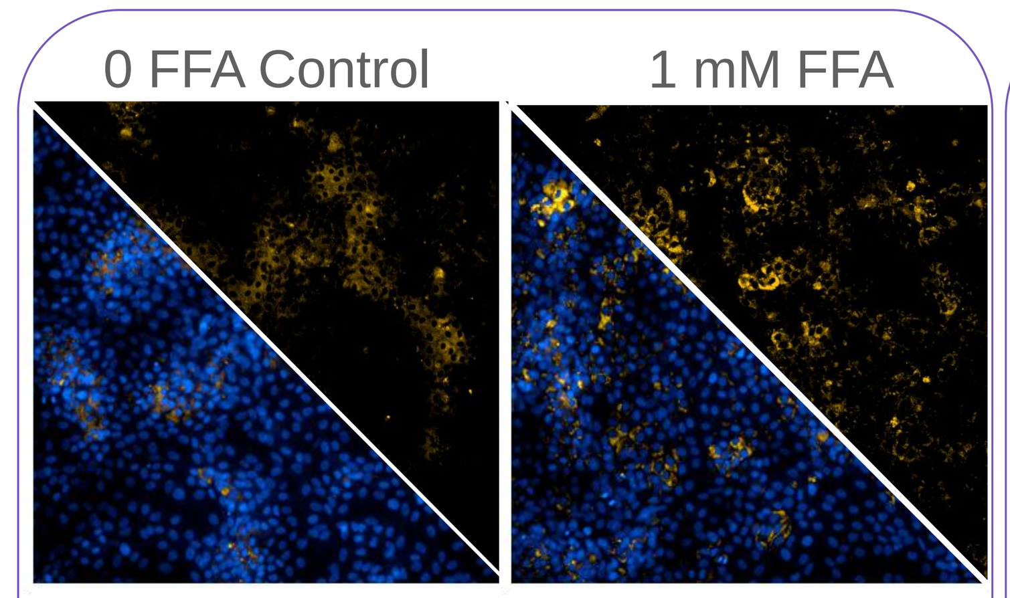


Figure 2. Visual comparative morphological change in steatosis. Hoechst [HO] nuclear stain and Nile Red [NR] triglyceride stain following 48h exposure to media containing 1 mM of a 1:2 oleate:palmitate free-fatty acid [FFA].

Lipid Accumulation – Spot Count / Cell

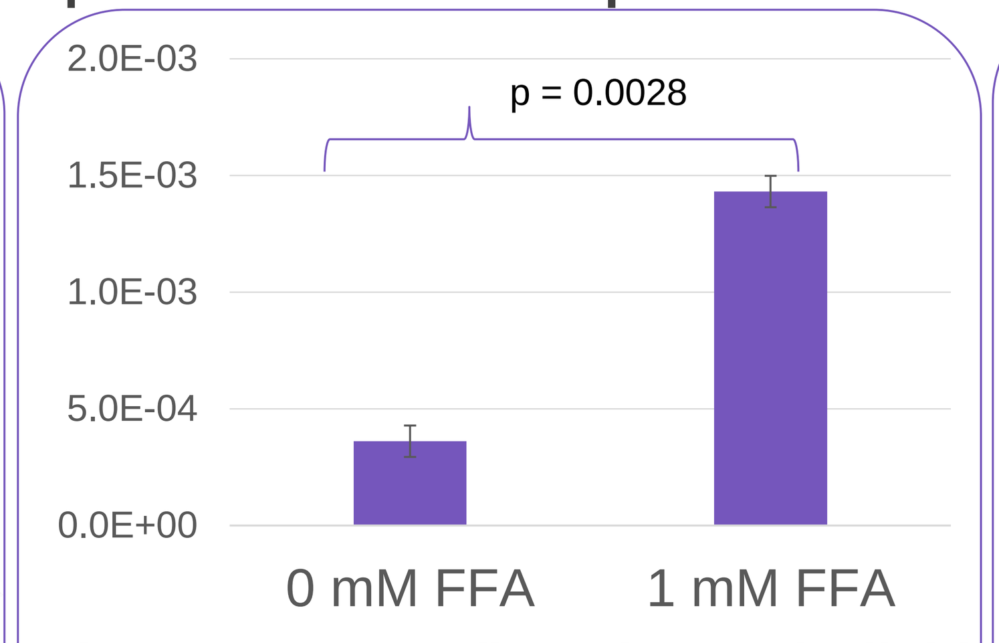


Figure 3. Confirmation of lipid accumulation with NR staining. Lipid accumulation due to 1 week FFA exposure in media was assessed by measuring fluorescence of lipid-bound Nile Red dye normalized to cell count determined by Hoechst staining.

CYP Gene Expression

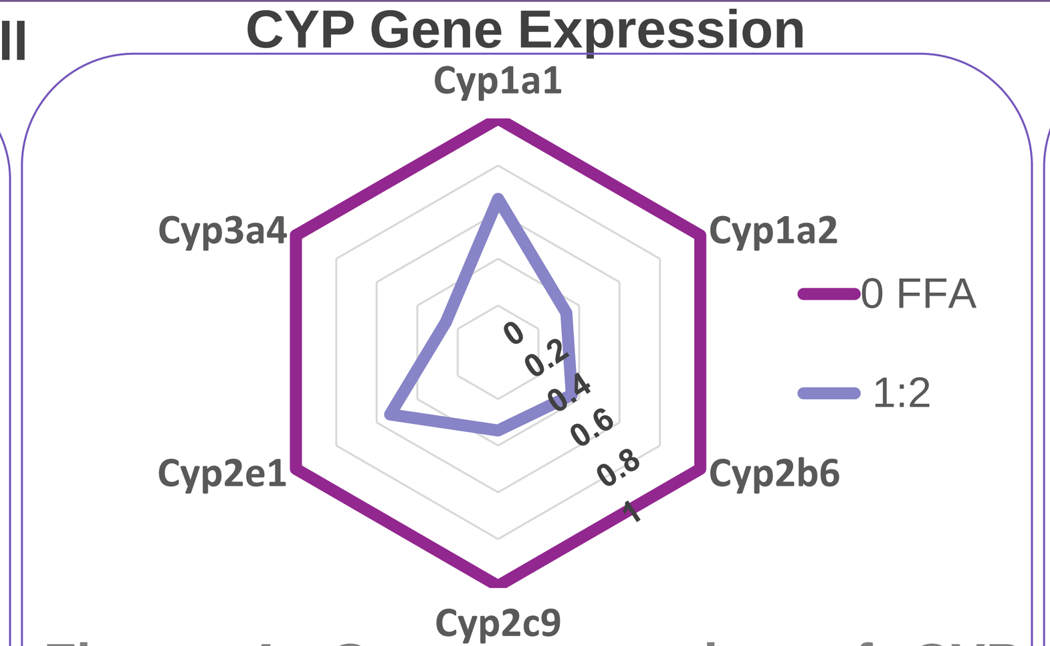


Figure 4. Gene expression of CYP enzymes indicating general reduction in metabolism due to hepatic steatosis in HepaRG. Real-time qPCR measured 6 common CYP enzymes active in human liver. Metabolic enzyme expression following incubation 1 mM of 1:2 oleate:palmitate FFA.

Metabolomic Activity

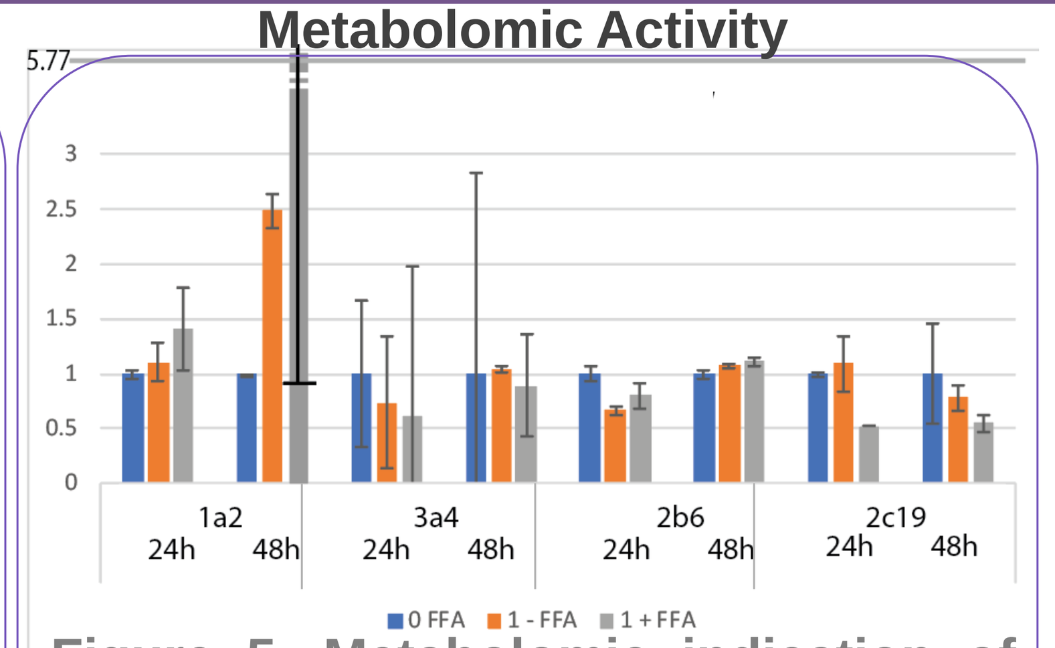


Figure 5. Metabolomic indication of CYP enzymatic activity demonstrates alteration in metabolism due to hepatic steatosis in HepaRG. HPLC measurement of 3 CYP activity levels using targeted substrates. Metabolic enzyme activity measured following a 1h substrate incubation.

R2: Altered Susceptibility + Toxic Fingerprint

Profiling Toxicity via Mechanistic Indicators

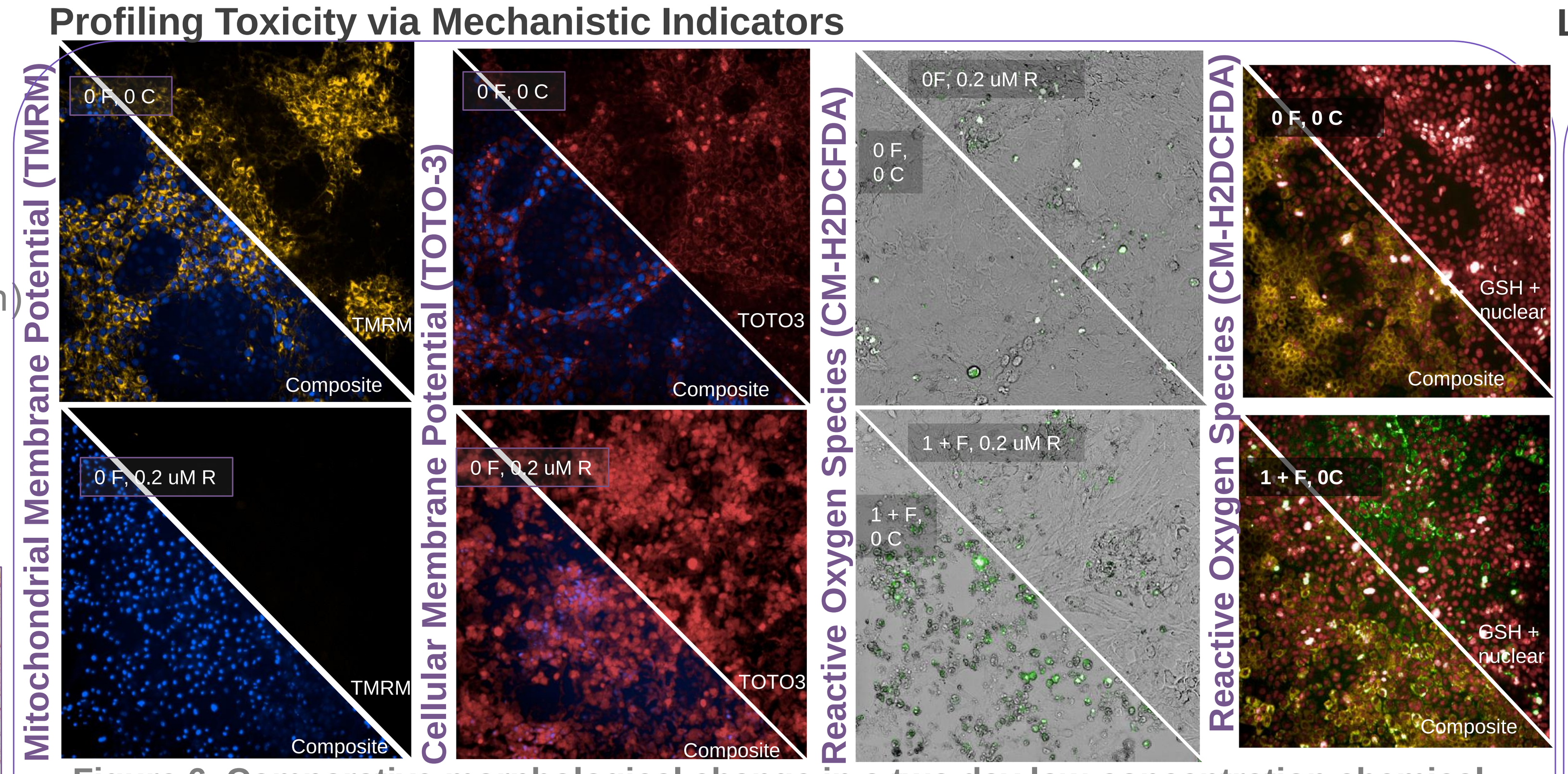


Figure 6. Comparative morphological change in a two day low-concentration chemical exposure. No FFA exposure, HO nuclear stain and: TMRM (mitochondrial membrane potential) / TOTO-3 (cellular membrane potential); or -/+ FFA & -/+ 0.2 uM rotenone for 48h in brightfield and CM-H2DCFDA (reactive oxygen species); or +/- FFA, using Draq5 nuclear stain, mBCI (GSH), and YOYO-1 (mitochondrial membrane potential).

Lipid Accumulation – NR → Boxplot of Lipid Intensity by FFA

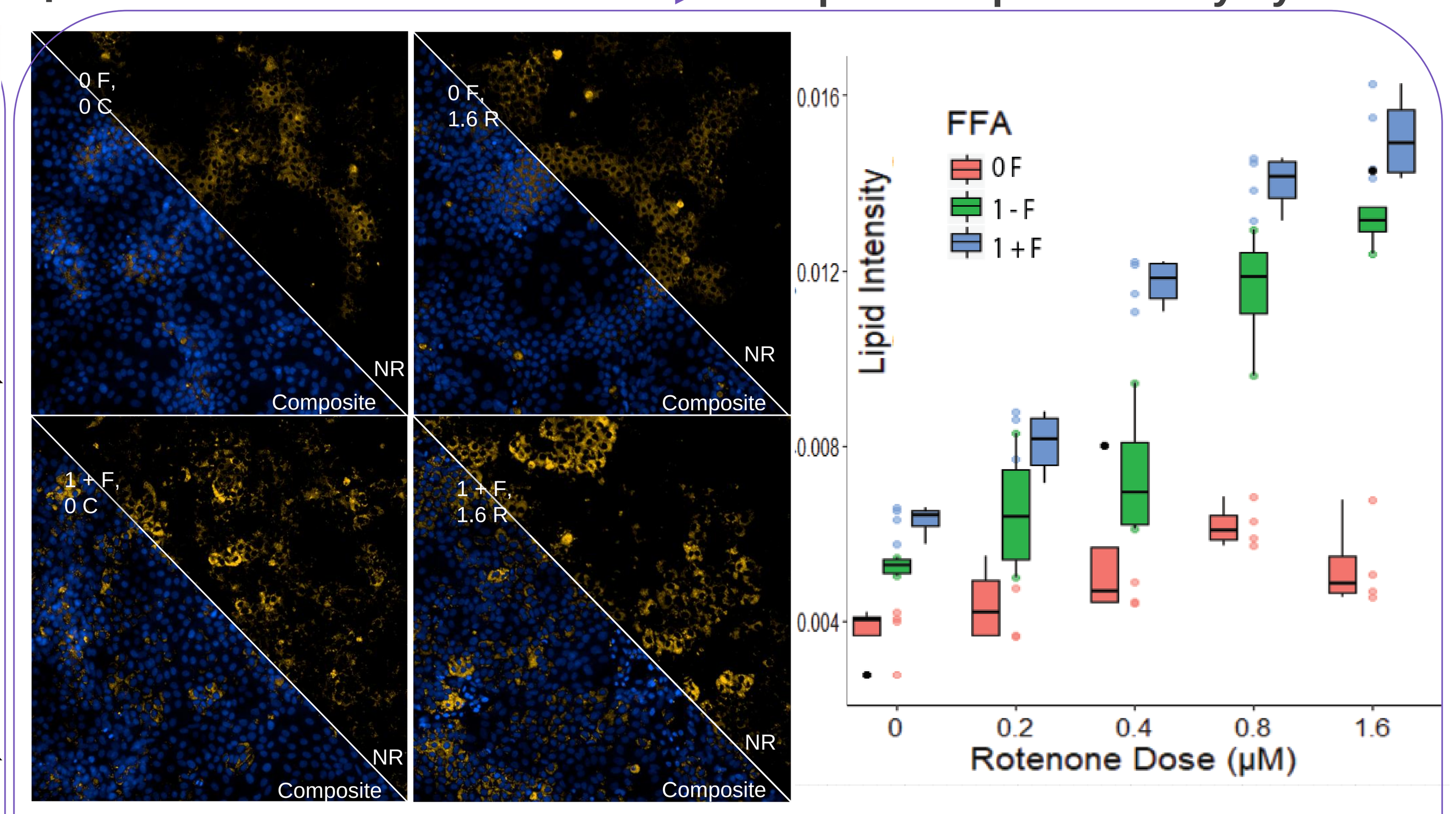


Figure 7. Comparative morphological change in one day high-concentration chemical exposure. Using the Opera Phenix analyzer and Harmony software, fluorescent stains are reduced to values on the cellular level. Further analysis using R, R studio, and the Tidyverse suite result in quantitative output confirming visual and complementary assay outcomes.

Fatty liver changes liver metabolism.

Toxicants are metabolized differently by the 1/3rd of the world exhibiting fatty liver.

WIP: Nuclear Features – Toxicity Marker & % Responder

Rotenone Exposure - ATP IC50

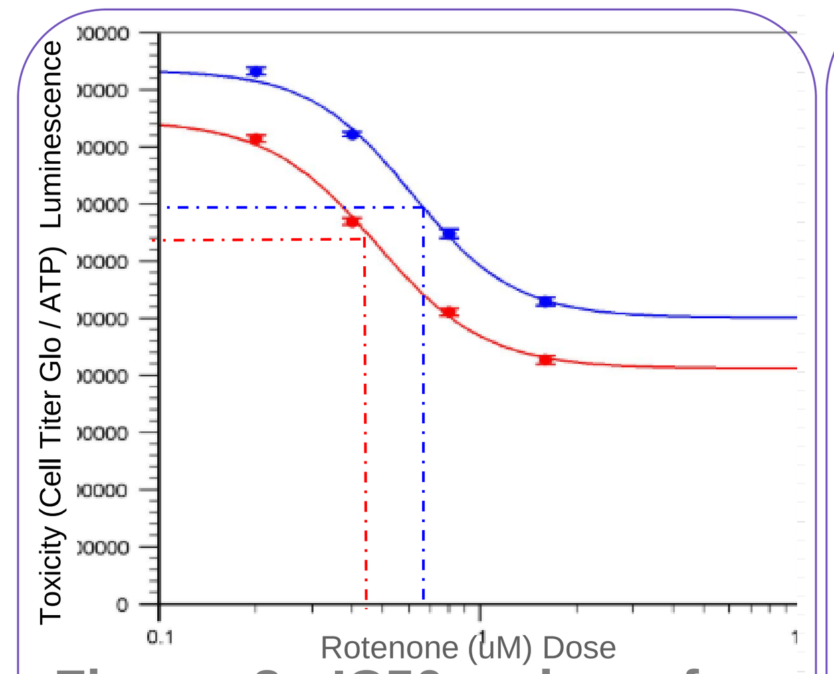


Figure 8. IC50 values for rotenone-mediated toxicity decrease due to hepatic steatosis. Data points derived from an ATP-proxy viability assay were fit to a non-linear least-squares model and IC50 values were determined (www.ic50.tk).

Total Cells / "Responders"

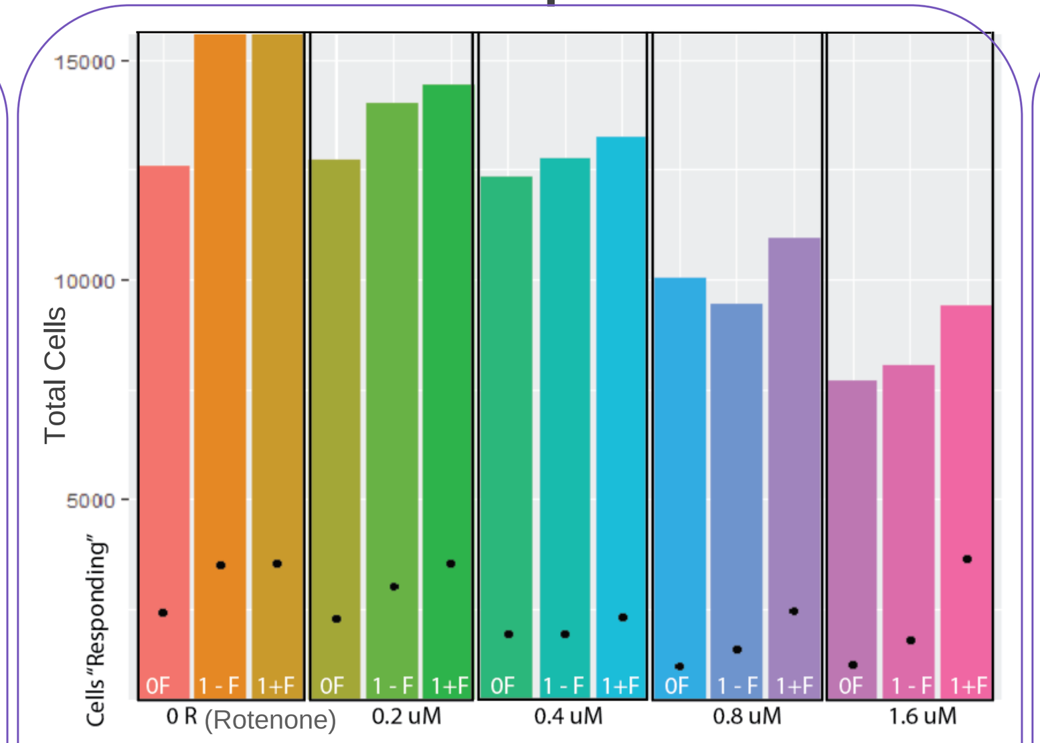


Figure 9. Percent Responder Toxicity Identification. Comparing total cell count per condition to the count of cells with a nuclear intensity above 2 standard deviations above control average illustrates differential response in toxicity due to fatty incubation alongside rotenone exposure (0 – 1.6 uM, 48h).

Comparative IC50s

	CTG	LDH	Obj#
0F	0.62	0.83	0.80
1 - F	0.46	0.61	0.54
1 + F	0.48	0.57	0.62

Figure 10. Parallel Assays & Resulting IC50 Calculations. IC50 results from 3 assay types examining different biological processes in response to rotenone illuminates mechanisms of toxicity invoked with rotenone exposure. (CTG/LDH from same plate, Obj count from later experiment.)

WIP: Interrogating CYPs with Targeted Chemicals

Fluorescence HO / NR – Acetaminophen Exposure

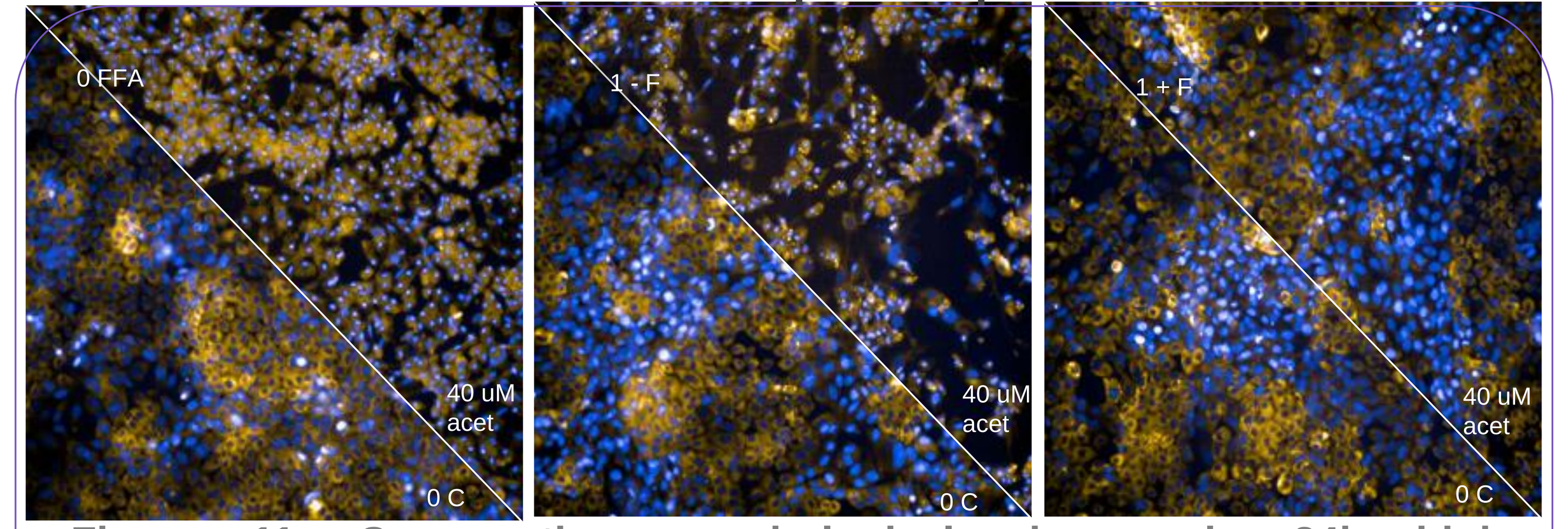


Figure 11. Comparative morphological change in 24h high concentration chemical exposure. Examining nuclear morphology indicates potential use of nuclear size and fluorescent intensity as proxy measures of cell viability. Textural features (not shown) are likely revelatory as indicating sub-lethal toxicity responses. As steatosis alters disproportionately particular CYPs, exposures to chemicals metabolized by different enzymes may have different outcomes in the steatotic state.

Initial Training Set

CYP	Toxic Before	Toxic After	Never Toxic
1a2	Clozapine	b[a]p	Melatonin
2a6	Coumarin Nifedipine	Metronidazole Phenacetin	Cisapride Montelukast
2b6	Efavirenz	Ticlopidine cyclophosph.	Buproprion
2c8	Paclitaxel Troglitazone	isotretinoin amiodarone	loperamide rosiglitazone
2c9	Fluvastatin	Ibuprofen phenytoin	Glimepiride warfarin
2c19	chloramphenicol clomipramine	Nelfinavir	primidone warfarin
2d6	fluvoxamine metoclopramide	mexiletine, phenacetin	diltiazem propranolol
2 e1	cisplatin	acetaminophen benzene	chlorzoxazone sevoflurane
3a4/5	Docetaxel	Dapsone Flutamide	midazolam testosterone

Figure 12. Panel of evaluated CYP-targeting chemicals. A selection of 50+ chemicals via literature review and will be assessed for mechanistic profile.

Conclusion and Future Directions

- Steatotic HepaRG: viable but have altered CYP metabolism.
- This state alters the hepatoxicity of some chemical exposures, underscoring the importance of assessing hepatic steatosis as a **common** risk factor for chemical toxicity.
- Further identification patterns in toxicity shifts with specific CYPs given a steatotic state via:
  - Targeting specific CYPs may reveal variation in cellular features via high-content imaging/analysis, especially the capacity to use GIS topographical measures in understanding cellular morphology, especially in terms of **Haralick features**
  - Mitochondrial- / Cellular membrane- potentials coupled with GSH measures may indicate additional parameters of mechanistic toxicity using processes similar to depicted herein.

Greatest thanks to Denise MacMillan and the chemistry core at the EPA for HPLC ID of CYP activity! Same to my collaborators / mentor! Literally couldn't do it alone.