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Characterization of Hepatic 3D Spheroids Using Multiphoton Fluorescence Microscopy and OMICS

Background

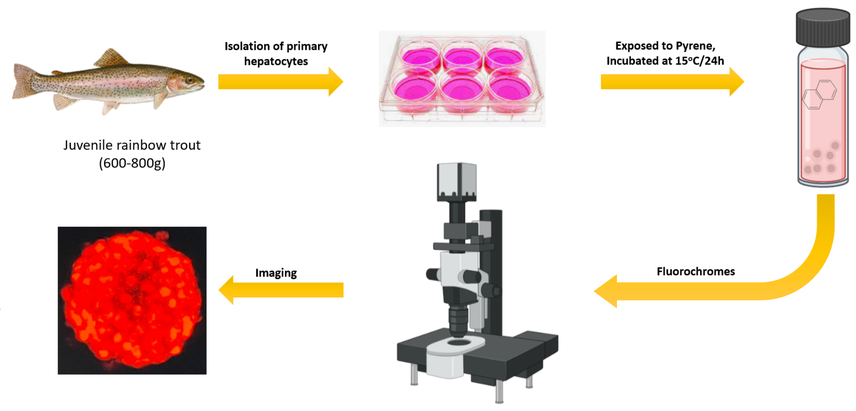
Traditional animal testing methods for hazard and risk assessment of chemicals have ethical and economical concerns. Non-animal methods implementing the 3Rs (refinement, reduction, and replacement) are therefore warranted. However, there are currently limited suitable *in vitro* bioassays available for assessing long-term toxicity in fish. The 3-dimensional (3D) hepatic spheroid model from rainbow trout (*Oncorhynchus mykiss*) appears to be a promising model as it maintains morphological, physiological and biochemical properties for weeks after their formation[1][2]. Being a novel model, a comprehensive morphological and physiological characterization is required. Multiphoton fluorescence microscopy (MFM) produces high-resolution pictures with deeper tissue penetration, more effective light detection, less phototoxicity, and improved spectral accessibility and flexibility[3].

Objectives

- To characterize the morphology of rainbow trout hepatic 3D spheroids.
- To optimize and visualize cellular and subcellular responses of hepatic 3D spheroids during exposure to a solvent control (Dimethyl sulfoxide, DMSO) and sublethal concentrations of pyrene, by using MFM.

Approach

- Spheroids were exposed to sublethal concentrations (25 and 75 nM) of pyrene for 24h, then visualized under MFM (Bruker Ultima IV) to detect viability, cytotoxicity (metabolic activity and cell membrane integrity), reactive oxygen species (ROS) activity and hypoxia using the appropriate fluorochromes. The fluorescence signals generated by MFM were split into a green and a red channel using two optical bandpass filters (CWL 525 nm/FWHM 70nm and CWL 593nm/FWHM 45nm) in front of photomultiplier tubes (PMTs).
- Metabolic activity of spheroids was also analyzed with the Alamar Blue assay at concentrations from 3 to 1000 nM of pyrene.



Results

Cytotoxicity - Metabolic activity

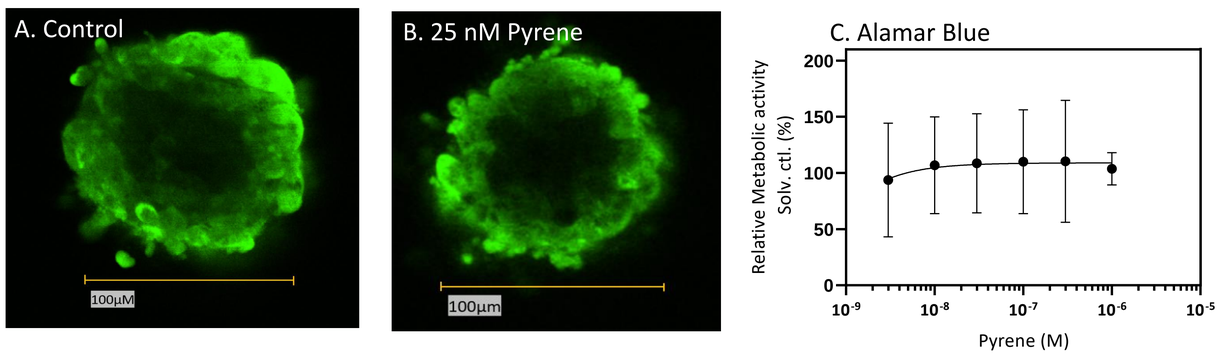


Figure 1. Spheroids from solvent control (A) and exposed to 25 nM pyrene (B) were stained with Fluorescein diacetate (37.5 µM) for 30 min. Images taken at the middle layer, transverse section (λ =800 nm). Metabolic activity was measured using Alamar Blue (C)[4]. Metabolic activity data was normalized towards the solvent control.

Cytotoxicity - Cell membrane integrity

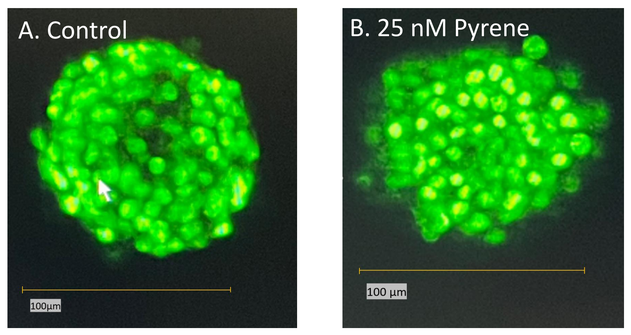


Figure 3. Spheroids from solvent control (A) and exposed to 25 nM pyrene (B), stained with Sybr Green (50 nM) for 15 min. Images taken at the middle layer, transverse section (λ =980 nm).

Hypoxia

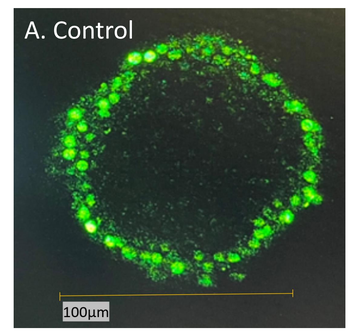


Figure 4. Unexposed spheroids stained with Image-iT green hypoxia reagent (5 µM) for 1hr. Images taken at the middle layer, transverse section (λ =800 nm).

Cytosolic Reactive Oxygen Species (ROS) activity

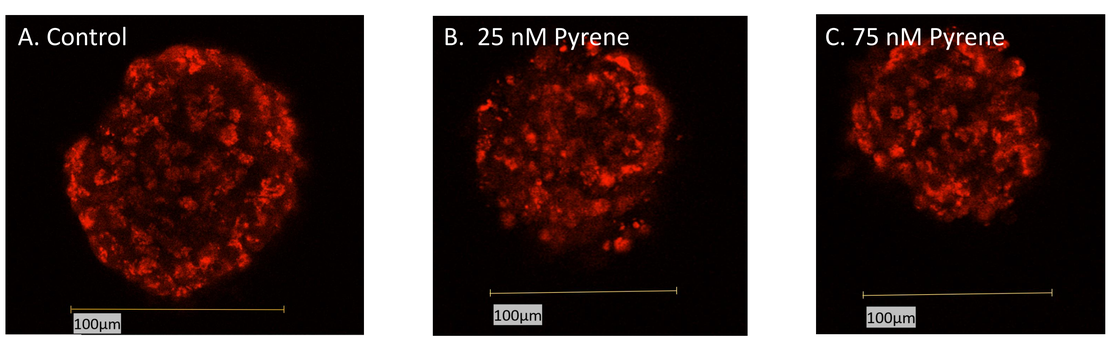


Figure 2. Spheroids from control (A) and exposed to 25 nM (B) and 75 nM (C) pyrene were stained with H_2DCFDA (1 µM) for 30 min. Images taken at the middle layer, transverse section (λ =780 nm).

Main findings:

- Pyrene showed no cytotoxic effects at tested concentrations → also supported by the Alamar Blue assay.
- Pyrene showed no alteration in cytosolic ROS at the tested concentrations.
- Pyrene displayed no alteration in cell membrane integrity at tested concentrations.
- No hypoxia was observed at the core of the spheroid, only at the outer layer → suggesting spheroids maintain their morphological and physiological integrity, as well as viability after 8-25 days in culture.

Conclusions and Prospects

- To investigate cytotoxicity, cell membrane integrity, ROS activity and hypoxia in spheroids, MFM seems to be an appropriate qualitative method.
- MFM images suggested that none of the pyrene concentrations tested had cytotoxic effects on the spheroids.
- There was no apparent hypoxia in the spheroids' core, supporting previously published data on fish spheroids[5].
- The use of MFM will further be refined to investigate morphological structures and physiological properties of the 3D hepatic rainbow trout spheroids to elucidate their potential use in toxicity assessment.
- Experimental transcriptomic and metabolomic data is currently being analyzed to unravel the spheroids physiological complexity and potential use in chemical toxicity studies.

References & Funding

[1] Baron, M.G., *Ecotox.* 21, 2419-2429(2012); [2] Hultman, M.T., *Environ toxicol chem* 38,1738-1747 (2019); [3] Wang, X., *Technol cancer res tret* 21, 1533-1541 (2022); [4] Eilenberger, C., MethodsX 5, 781-787 (2018); [5] Langan, L.M., Front Pharmacol, 947 (2018).

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