Fish Liver Microtissues for Aquatic Tox: Integrating Morphological & Molecular Responses for *In Vitro* Assessment of Environmental Pollutants

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Alternative Toxicity Testing Approaches

What do we do when a new, potentially toxic material needs to be tested?
Alternative Toxicity Testing Approaches

Select Target Organs & Cells

High-throughput Screens

Complex Cell Models
3D Models for Environmental Toxicology

- 2D cells do not accurately reflect the response of *in vivo* tissues
  - Increasing need for advanced screening tools for aquatic toxicology
- 3D cell culture acts as a bridge between monolayer *in vitro* assays and *in vivo* exposures
  - Balancing increased throughput with increased tissue complexity

*In Vivo* → *In Vitro*
Aromatic Hydrocarbon Toxicity

- Polycyclic aromatic hydrocarbons (PAHs) persist in sediment and can accumulate in lipids
  - Many are carcinogenic and EPA priority pollutants
  - Metabolic activation by Cytochrome P450 enzymes can cause cell death, reactive oxygen species, and DNA adducts

![Metabolism of Aromatic Hydrocarbons](image)

**Metabolism of Aromatic Hydrocarbons**

**Naphthalene** → **Benzo(a)pyrene** → **Additional Enzymes** → **P-450** → **Carcinogenic Metabolites**

**Carcinogenic Metabolites**

- Benzo(a)pyrene 7,8 diol-9,10 epoxide
- Naphthalene-1,2-epoxide
- 1,2-Naphthoquinone
Fish Liver Microtissue Formation

- Microtissues formed with PLHC-1 fish liver cells
  - Self-assemble through cell-cell adhesion and cytoskeletal forces
  - Method applied to many cell types and known to increase hepatocyte differentiation
- Can be assessed using both fluorescent and histological techniques
Microtissue Characterization

- Microtissues are stable and viable for at least 2 weeks
- Markers of liver differentiation stable or increasing over time
Cytochrome P450 1A (Cyp1a) Expression

- Cyp1a metabolizes polycyclic aromatic hydrocarbons (PAHs)
  - Generates both detoxified and reactive metabolites
  - Specific biomarker upregulated in response to PAH exposure
Microtissue Exposure to Benzo(a)pyrene

- 3D cell culture allows for more prolonged and complex exposures
  - Microtissues have extended window of exposure
  - Added complexity of multiple exposures
- Metabolic activation of PAHs can cause delayed effects
  - May go undetected following acute, single exposure exposures
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Sensitive Cyp1a Induction After B(a)p Exposure

- Response to a 24hr benzo(a)pyrene exposure
  - Highly sensitive increase in *cyp1a* gene expression
  - Dose dependent increase in Cyp1a protein *in situ* with three-dimensional protein induction

![Cyp1a Gene Expression](image)

![Cyp1a Protein Expression](image)

Scale bars 50μm
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\textit{Cyp1a Protein Expression}

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image</th>
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<tbody>
<tr>
<td>DMSO</td>
<td>![Image]</td>
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<tr>
<td>100nM b(a)p</td>
<td>![Image]</td>
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<tr>
<td>1( \mu )M b(a)p</td>
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<tr>
<td>5( \mu )M b(a)p</td>
<td>![Image]</td>
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Scale bars 50\( \mu \)m
Continued Cellular Changes During Recovery

- Continued adverse effects after recovery period
  - 2D monolayer cells proliferate, while 3D microtissues do not
  - Gene expression of *cyp1a* falls following b(a)p removal

![Cyp1a Gene Expression Graph](image)

![Cyp1a Protein Expression Images](image)

Scale bars 50µm
Continued Cellular Changes During Recovery

- Continued adverse effects after recovery period
  - Cell death and spheroid morphological change continue to increase

Scale bars 50µm
Cyp1a Induction Unaltered By Previous B(a)p Exposure

- Cyp1a gene or protein expression equally induced after second 24hr exposure to b(a)p
Changes in Microtissue Architecture After Repeated B(a)p Exposure

- Repeated exposure to benzo(a)pyrene shows survivability
  - Survival of spheroid core after high exposures
  - Disrupted spheroid architecture and morphology

Scale bars 50µm  
Hematoxylin & Eosin
Effect of Multiple B(a)p Exposures

100nM b(a)p  5μM b(a)p

5μM b(a)p  100nM b(a)p

5μM b(a)p

Cyp1a Nuclei

Cyp1a Nuclei

Sublethal

Dose

First Exposure

Second Exposure
Summary

• Single benzo(a)pyrene exposures elicit sensitive and specific responses in fish liver microtissues

• Repeated exposure results in tissue-level changes to microtissue architecture without altering the induction of Cyp1a

• Microtissues can be used as a sensitive tool to assess environmentally relevant aquatic exposures
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