

## RESEARCH ARTICLE

# Bioprinting of a human neuron model for quantifying pollutant toxicity

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## Abstract

Pesticides are widely used in modern agriculture; however, growing evidence indicates that chronic exposure to these environmental contaminants contributes to the development of neurodegenerative disorders. This concern is amplified by the global rise in aging populations and the corresponding increase in the incidence of neurodegenerative diseases. Among the most widely used pesticides, organophosphates, particularly chlorpyrifos (CPF), are of significant concern due to their extensive agricultural use and well-established neurotoxic effects. Here, we present a 3-dimensional (3D) human neuron progenitor cell (hNPC) tissue model, fabricated using digital light processing-based bioprinting. This platform enables precise spatial control of cell density, geometry, and mechanical properties within a gelatin methacrylate scaffold, providing a tunable and biomimetic extracellular matrix. Using this system, we evaluated hNPC response to CPF exposure within a physiologically relevant 3D context. We further investigated CPF-induced neurotoxicity, specifically exploring acetylcholinesterase gene expression as a primary mechanistic etiology. Our findings demonstrate that our engineered 3D-bioprinted constructs offer a more physiologically relevant and cost-effective platform for studying pollutant-induced neurotoxicity than conventional two-dimensional and *in vivo* rodent models. This work establishes a scalable 3D-bioprinted system for mechanistic toxicology studies and advances predictive modeling of human neurological responses to environmental toxicants.

**Keywords:** Bioprinting; Human neuron tissue model; Pollutants; Toxicity; Chlorpyrifos

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## 1. Introduction

Pesticides are important to contemporary agricultural systems; however, mounting evidence implicates chronic exposure in the pathogenesis of neurodegenerative and neurodevelopmental disorders.<sup>1,2</sup> This concern is compounded by aging populations:

while advances in healthcare have extended human lifespan, they have also driven a parallel rise in neurodegenerative diseases.

Organophosphates are among the most used pesticides worldwide. Both acute and chronic exposure are toxic to the nervous system and can lead to neurological diseases.<sup>3</sup> Specifically, chlorpyrifos (CPF), an organophosphate pesticide, is of particular concern. It is one of the most recognized pesticides worldwide, used as an insecticide on corn, soybeans, wheat, and many fruit trees. It is highly effective against termites, fleas, earthworms, and more.<sup>4</sup> CPF's primary mechanism of toxicity is through irreversible inhibition of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of neurotransmitter acetylcholine. In recent years, epidemiological evidence has suggested that occupational or environmental exposure to CPF is implicated in Alzheimer's disease (AD).<sup>5,6,7</sup>

Mechanistic investigation of CPF-induced neurotoxicity has historically relied upon reductive two-dimensional (2D) *in vitro* culture systems and *in vivo* rodent models.<sup>8</sup> While these models have been informative, each carries inherent limitations that constrain their translation to humans. Conventional 2D cultures fail to recapitulate the complex organization of the brain, limiting investigation of cell–cell and cell–matrix interactions.<sup>9,10</sup> *In vivo* models preserve systemic physiological complexity, but species divergence reduces their relevance.<sup>11</sup> There is an urgent need for human-relevant *in vitro* models that recapitulate the cellular architecture and biochemical environment of neuronal brain tissue, within which the neurotoxicity trajectory of CPF exposure can be properly characterized.<sup>12</sup>

In this study, we utilized digital light processing (DLP)-based bioprinting to create cell-encapsulated hydrogel scaffolds. DLP allows for controlled shape, size, cell density, and mechanical properties.<sup>13,14</sup> The matrix material is gelatin methacrylate (GelMA), whose methacrylate functional groups enable a controllable, reproducible scaffold. By leveraging the unique properties of GelMA, we can enhance the structural and functional fidelity of the tissue models. Using these models, we evaluated the effects of CPF on human neuron progenitor cells (hNPCs) and investigated the primary degradation mechanism. The progenitor-like state was chosen over maturing the neurons because the hNPCs are particularly vulnerable to environmental toxins, and they can differentiate into multiple cell types, which enables assessment of toxin-induced alterations. Furthermore, we modulated the matrix's mechanical properties by varying stiffness to include soft or stiff tissue models. This biologically relevant feature of the neural microenvironment influences neural progenitor cell behavior, including proliferation,

differentiation, and cellular signaling. Changes in tissue mechanics have been implicated in neurodegenerative disorders,<sup>15,16</sup> and our platform provides a method to study these changes. This study advances our ability to investigate mechanisms of neurotoxicity using both known and emerging neurotoxins, helping to bridge the translational gap between traditional *in vitro* models and the complex neurons within the human brain. By providing a more biomimetic neural microenvironment, these models may improve the prediction of human responses to toxic exposure and support the development of more effective therapeutic strategies.

## 2. Methods

### 2.1. Cell culture

Human induced pluripotent stem cell-derived neural progenitor cells were obtained from StemCell Technologies (200-0620, Canada) and were cultured using STEMdiff™ Neural Progenitor Medium kit (05833, StemCell Technologies, Canada) with 100 U/mL penicillin–streptomycin (Sigma-Aldrich, United States). This combination is referred to as the medium throughout this study. Before plating the cells, Matrigel (Corning, United States) was used to coat the plates overnight. Media were changed daily, and cells were passaged every three days by dissociating with Accutase (Sigma-Aldrich, United States) and seeding at 1:3 onto new plates. Cells were incubated at 37 °C under 5% CO<sub>2</sub>.

### 2.2. Bioink composition

The bioink consisted of 10% (w/v) GelMA, 0.6% (w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphinate (Sigma-Aldrich, United States), Dulbecco's phosphate-buffered saline (Thermo Fisher, United States), and cells. GelMA was synthesized according to previously published protocols.<sup>17</sup> Briefly, gelatin (Sigma-Aldrich, United States) was dissolved in a 0.25 carbonate-bicarbonate buffer solution. Methacrylic anhydride (Sigma-Aldrich, United States) was added at a 0.095 mL/g ratio and reacted for 1 hour at 50 °C. GelMA was purified by dialysis in Milli-Q water using 12–14 kDa tubing (Spectrum Labs, United States) for one week at 37 °C. It was then collected, lyophilized, dissolved in Dulbecco's phosphate-buffered saline to a 20% (w/v) stock solution, and filtered (Millipore Sigma, United States) to ensure sterility.

### 2.3. Bioprinting

Bioprinting was conducted using an in-house DLP bioprinter equipped with a 365 nm light-emitting diode light source at 6.8 mW/cm<sup>2</sup> for soft scaffolds and 10.8 mW/cm<sup>2</sup> for stiff ones. Polydimethylsiloxane sheets were

sectioned and mounted onto glass slides to form the bioink reservoir, while methacrylated glass coverslips served as the printing substrate, prepared using an established protocol.<sup>13</sup> A brain-shaped mask 250 mm in height and 500 mm in diameter was used for all scaffolds unless otherwise specified. The bioprinting process was as follows: hNPCs were collected by lifting and centrifuged for 5 minutes at 200 g; cell concentration was determined using Trypan Blue exclusion (15250061, Thermo Fisher, United States). Bioink was added to the cells, and 5 mL of bioink was placed into the bioink reservoir. Soft samples were created at a light intensity of 136 mJ/cm<sup>2</sup> (6.8 mW/cm<sup>2</sup>), and stiff samples at 324 mJ/cm<sup>2</sup> (10.8 mW/cm<sup>2</sup>), then immediately placed in the media. Total cell density was 50 million/mL. Soft samples were exposed for 20 seconds; stiff ones were exposed for 30 seconds.

## 2.4. Young's modulus

Young's modulus was quantified using CellScale (Canada)'s MicroTester. Samples underwent three compression cycles using a 0.3048 mm diameter stainless steel beam and platens. Custom MATLAB (version R2026a) scripts processed force and displacement data to calculate the overall compressive modulus.

## 2.5. Live/Dead staining

Cells were stained with ApexBio (Taiwan)'s Live/Dead kit (K2081). Calcein-acetoxymethyl ester (excitation: 490 nm, emission: 515 nm) was prepared at a working concentration of 2 mM to stain viable cells, and propidium iodide (excitation: 535 nm, emission: 617 nm) was prepared at a working concentration of 1.5 mM to stain dead cells. Bioprinted scaffolds were stained for 30 minutes in the incubator, then immediately washed and imaged (Keyence BZ-X, United States).

## 2.6. Real-time quantitative polymerase chain reaction

The hNPCs were collected from bioprinted scaffolds by dissociating the hydrogel with TRIzol (Thermo Fisher, United States) and mechanical pipetting. Total RNA was extracted using Direct-zol RNA MicroPrep Kit (R2062, Zymo Research, United States) following the manufacturer's protocol. To ensure RNA purity, the 260/280 nm ratios were characterized using a plate reader (Tecan, Switzerland), and only ratios above 1.7 were used. Protoscript FirstStrand cDNA synthesis kit (E6300, New England BioLabs, United States) was used to synthesize cDNA from the extracted RNA. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with Luna Universal Master Mix (M3003, New England BioLabs, United States) in a QuantStudio 3 machine

(ThermoFisher, United States). Raw data were analyzed using the Ct threshold method against the housekeeping gene *GAPDH*, and fold change was compared between dosed and control groups. All primers used were purchased from Integrated Data Technologies (United States) and designed using NCBI's Primer-BLAST tool:

- |        |                  |         |        |                               |
|--------|------------------|---------|--------|-------------------------------|
| (i)    | <i>PAX6</i>      | forward | primer | sequence:                     |
|        |                  |         |        | 5'-TGTCCAACGGATGTGTGAGT-3'    |
| (ii)   | <i>PAX6</i>      | reverse | primer | sequence:                     |
|        |                  |         |        | 5'-TTTCCCAAGCAAAGATGGAC-3'    |
| (iii)  | <i>Nestin</i>    | forward | primer | sequence:                     |
|        |                  |         |        | 5'-AACAGCGACGGAGGTCTCTA-3'    |
| (iv)   | <i>Nestin</i>    | reverse | primer | sequence:                     |
|        |                  |         |        | 5'-TTCTCTTGTCCCGCAGACTT-3'    |
| (v)    | <i>SOX2</i>      | forward | primer | sequence:                     |
|        |                  |         |        | 5'-TACCAGCATGTCCTACTCGCAG-3'  |
| (vi)   | <i>SOX2</i>      | reverse | primer | sequence:                     |
|        |                  |         |        | 5'-GAGGAAGAGGTAACCACAGCG-3'   |
| (vii)  | $\beta$ -amyloid | forward | primer | sequence:                     |
|        |                  |         |        | 5'-CCTTCTCGTTCCTGACAAGTGC-3'  |
| (viii) | $\beta$ -amyloid | reverse | primer | sequence:                     |
|        |                  |         |        | 5'-GGCAGCAACATGCCGTAGTCAT-3'  |
| (ix)   | <i>ACHE</i>      | forward | primer | sequence:                     |
|        |                  |         |        | 5'-GTTCTCCTTCGTGCCTGTGGTA-3'  |
| (x)    | <i>ACHE</i>      | reverse | primer | sequence:                     |
|        |                  |         |        | 5'-ATACGAGCCCTCATCCTTCACC-3'  |
| (xi)   | <i>BACE1</i>     | forward | primer | sequence:                     |
|        |                  |         |        | 5'-GTGAGGTTACCAACCAGTCCTTC-3' |
| (xii)  | <i>BACE1</i>     | reverse | primer | sequence:                     |
|        |                  |         |        | 5'-CGTGGATGACTGTGAGATGGCA-3'  |
| (xiii) | <i>GAPDH</i>     | forward | primer | sequence: 5'-GAGTCA           |
|        |                  |         |        | ACGGATTTGGTCGT-3'             |
| (xiv)  | <i>GAPDH</i>     | reverse | primer | sequence:                     |
|        |                  |         |        | 5'-TTGATTTTGGAGGGATCTCG-3'    |

## 2.7. Toxin dosing

Stock solutions of toxin compounds were prepared in dimethyl sulfoxide (DMSO) or deionized water, selected based on compound solubility, and stored at -20 °C until use. CPF (32212, Restek, United States) was dosed into the scaffolds at 500 ug/L concentration. Benzo[a]pyrene (BaP; 50-32-8, MedChemExpress, United States) was dosed into the scaffolds at 100 ug/L. hNPC tissue models were constructed in 96-well plates with 3-6 replicates each. All toxins were dosed into scaffolds directly after printing. Samples were incubated for three or five days at 37 °C with 5% CO<sub>2</sub>. The drugs were dissolved in the medium and added to the wells to a total volume of 200  $\mu$ L. Side wells without samples were filled with phosphate-buffered saline to prevent evaporation. All drug-dosed wells contained less than 0.5% DMSO, and the control conditions contained the same DMSO amount as the dosed samples.

## 2.8. Cell viability assay

The viability of hNPC cells in the three-dimensional (3D) bioprinted tissues was assessed using CellTiter-Glo (G7570, Promega, United States). CellTiter-Glo was added in a 1:1 ratio with cell media and incubated away from light. For 3D samples, incubation time was increased to one hour to allow the buffer to penetrate the sample, while 2D cells were incubated for 10 minutes. Samples were then measured using a Tecan plate reader in a quartz well plate using a 1-second integration time.

## 2.9. Statistical analysis

GraphPad Prism (version 11.1.0) was used to perform statistical analysis. The data are presented as the mean, and error bars are the standard deviation, unless otherwise specified. Comparisons were performed with Student's *t*-test or one-way analysis of variance, and significance was determined if *p*-values were less than 0.05.

## 3. Results

Throughout this study, we utilized hNPCs. A 3D GelMA-based hNPC scaffold was bioprinted with an in-house DLP bioprinter. As shown in [Figure 1A](#), a digital micromirror device projected a defined mask using ultraviolet light onto the bioink reservoir, dictating the scaffold pattern. After light irradiation, the hNPCs were encapsulated in GelMA and cultured in the incubator to proliferate, migrate, and interact with their environment ([Figure 1B–E](#)). To ensure the hNPCs grow well in the scaffold, we measured their viability on days 0, 3, and 7. We found that their growth profile reflects robust proliferation throughout the week ([Figure 1F](#)).

After 48 hours of incubation under normal culture conditions, the scaffolds received CPF dosing for three days. This mimics physiological scenarios in which CPF can penetrate the epidermis and enter tissues or organs.<sup>18</sup> CPF-treated scaffolds received a broad spectrum of potential neurotoxic responses, including 5, 20, and 500 µg/L per scaffold in fresh media. The lower concentrations (5, 20 µg/L) were chosen to assess subtle or early cellular effects, while 500 µg/L was included to evaluate accumulation under long-term exposure ([Figure 2A](#)). As expected, the lower CPF concentrations showed higher cellular viability (\*\* *p* ≤ 0.01).

Two exposure durations, three and five days, were evaluated to investigate both early and prolonged effects of CPF exposure on the neural samples. The three-day time point was selected to assess acute cellular responses and initial toxicity, while the five-day time point allowed evaluation of transcriptional and cellular responses after continued exposure. Given the cytotoxic effects observed

following CPF exposure, the gene expression profiles at Day 5 were interpreted in the context of the surviving cell population and may reflect a transcriptional stress response to continued CPF exposure rather than homeostatic physiological adaptation. Comparing multiple exposure durations enabled characterization of the temporal progression of CPF-induced neurotoxicity within the 3D microenvironment, and follows published precedent.<sup>19</sup> Human CPF exposure occurs primarily through dietary intake, typically as low-dose exposure across the lifespan.<sup>20</sup> To better reflect translational relevance, the experiments moving forward used a five-day exposure period.

Initial studies utilized a cellular density of 15 million cells per mL. However, native neural tissue is characterized by a higher cellular density and extensive cell–cell interactions; the final tissues were fabricated with 50 million cells per mL. The 50 M/mL density showed higher viability compared to the lower density, with a significant (\* *p* ≤ 0.05) increase at Day 3 ([Figure 2B](#)). We visually observed this using Live/Dead staining in [Figure 2C](#). This high cellular density better recapitulates the *in vivo* neural microenvironment.<sup>21</sup>

By varying 3D-printing parameters, we created two groups: a soft hNPC group (2 kPa) and a stiff group (4 kPa) ([Figure 3A](#) and [3B](#)). Overall, samples dosed for three days survived significantly better than samples dosed for five days. This trend was observed in both the stiff and soft groups. The stiffer groups had higher cellular viability than the soft groups ([Figure 3C](#)).

To elucidate cellular mechanisms underlying their responses to CPF, we evaluated established hNPC proliferation and prognosis markers. We investigated neuronal markers *PAX6*, *Nestin*, and *SOX2*. We found that the stiffer model upregulated *PAX6* and *Nestin* compared to the soft model, which upregulated *SOX2* ([Figure 3D](#)). Furthermore, we investigated three AD-associated markers, βeta amyloid, *BACE1*, and *ACHE* ([Figure 3E](#)). The soft group upregulated these markers more than the stiff group, indicating two different responses to CPF injury.

To further investigate CPF toxicity in hNPCs, we incorporated the physiological degradation mechanism into our model. CPF enters the body and is metabolized by the liver into two components: CPF and CPF-oxon (CPF-o). The bioprinted tissue model does not include hepatic metabolism; therefore, we further included the metabolized form, CPF-o, in our experiments. CPF and CPF-o affect hNPCs through different mechanisms: CPF relies on oxidative stress, whereas CPF-o relies on inhibition of AChE activity.<sup>22</sup> We explored the qPCR markers associated with AD in both CPF- and CPF-o-dosed samples ([Figure 4A–E](#)). However, compared with their non-dosed controls,

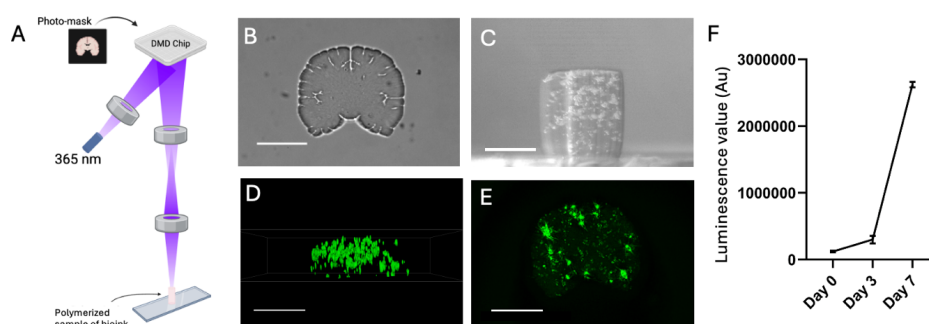


**Figure 1.** Human neural progenitor cell (hNPC) constructs were created using digital light processing (DLP) bioprinting. (A) Schematic of DLP bioprinting setup. (B) Acellular model of the scaffold (top view). (C, D) Brightfield (C) and confocal (D) side images of hNPC distribution throughout the bioprinted sample. (E) hNPC-laden tissue after seven days in culture with visible axons sprouting. Scale bar: 250  $\mu\text{m}$ ; magnification: 10 $\times$ . (F) hNPC growth profile in three dimensions at day 0, 3, and 7.

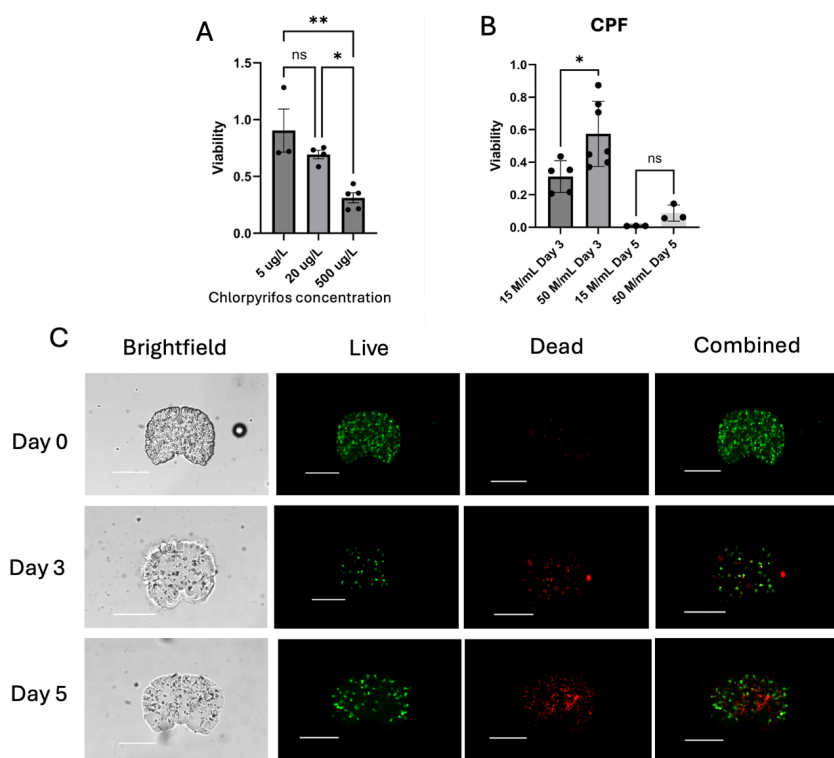


**Figure 2.** Parameter dependence of the bioprinted human neural progenitor cell (hNPC) tissue models. (A) Concentrations of 5, 20, and 500  $\mu\text{g/L}$  chlorpyrifos (CPF) were dosed into the samples. (B) Cell densities of 15 million/mL and 50 million/mL were printed and dosed with CPF for 3 and 5 days. (C) Live/Dead staining of CPF-dosed bioprinted samples at day 0, 3, and 5. Scale bar: 250  $\mu\text{m}$ ; magnification: 10 $\times$ . Note: ns = not significant, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ .

all three AD-associated markers were significantly upregulated in CPF-O-dosed samples. *Nestin*, a cellular stress marker, increased significantly by 20-fold. Similarly,  $\beta$  amyloid demonstrated a 4-fold significant increase, indicating that more  $\beta$  amyloid plaques were deposited. *ACHE* was significantly downregulated, indicating that the CPF-O-dosed hNPCs did not break down acetylcholine as effectively as CPF. It is important to note that the mRNA

expression does not directly reflect the enzyme activity. Comparing the CPF-dosed samples with their non-dosed control, all three AD-associated markers had no upregulation. From a viability perspective, CPF caused a larger viability decrease than CPF-o (Figure 4F). This greater viability loss may reflect generalized cellular stress or oxidative injury leading to broader toxicity rather than more selective neurodegenerative pathways. Although

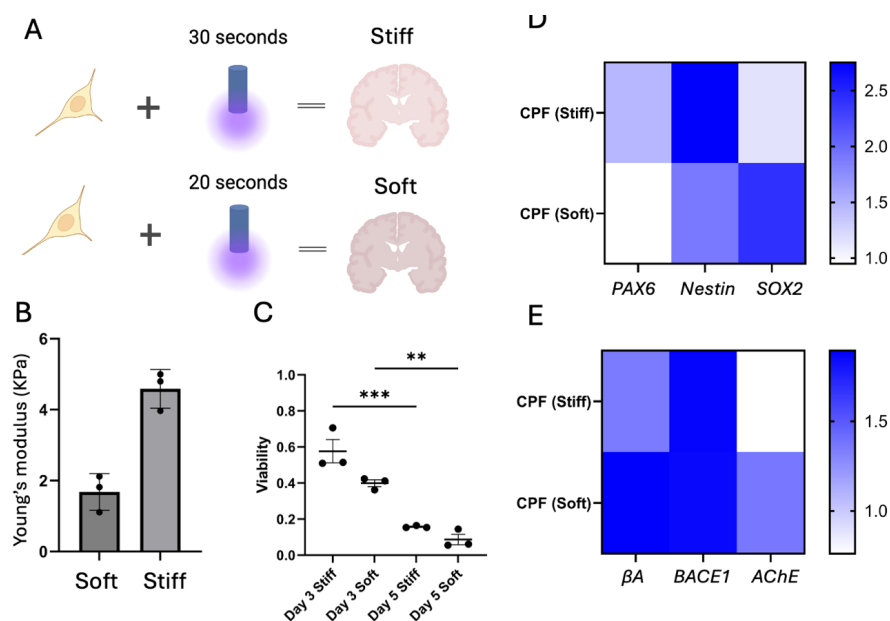


**Figure 3.** Characterization of bioprinted stiff versus soft human neural progenitor cell (hNPC) neuron tissue models. (A) Bioprinting diagram: To create a stiff sample, we used 30 sec of light exposure. To create a soft sample, we used 20 sec of light exposure. (B) Mechanical stiffness of stiff and soft models, as shown by Young's modulus. (C) Stiff samples with chlorpyrifos (CPF) dosing on days 3 and 5, compared with soft samples. (D) Real-time quantitative polymerase chain reaction results of neuronal markers *PAX6*, *Nestin*, and *SOX2*. (E) Real-time quantitative polymerase chain reaction results of Alzheimer's disease-associated markers  $\beta$  amyloid, *BACE1*, and *ACHE*. Error bars represent standard deviation; \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

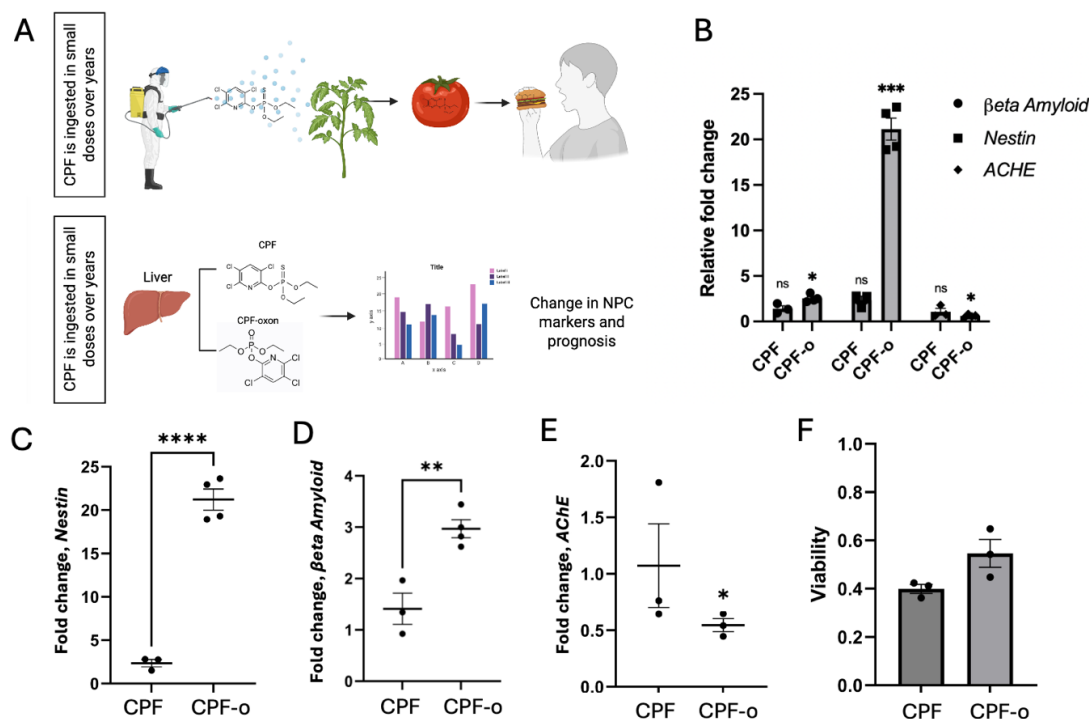


**Figure 4.** Mechanistic studies of chlorpyrifos (CPF) and its metabolite CPF-oxon. (A) Diagram of human CPF ingestion over time and its effect on the body. (B) Real-time quantitative polymerase chain reaction (RT-qPCR) of Alzheimer's disease-associated markers comparing CPF and CPF-oxon normalized to non-CPF-dosed samples. (C, D, E) RT-qPCR comparing CPF- and CPF-oxon-dosed samples with (B) *Nestin*, (C)  $\beta$  amyloid, and (D) *ACHE* markers. (F) Cellular viability of the bioprinted neuron tissue after CPF and CPF-oxon dosing. Note: ns = not significant, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ .

the parent compound CPF appeared more potent, CPF-o showed a stronger AD-associated transcriptional response despite its lower overall toxicity. These results strongly suggest that the two compounds engage in fundamentally different cytotoxic and neurotoxic mechanisms, and that the CPF-o metabolite drives the AD-relevant pathology.

To further test our neuron toxicity model, we utilized a second environmental pollutant, BaP. BaP is a polycyclic aromatic hydrocarbon that is prevalent in cigarette smoke, charcoal-based foods, car exhausts, and waste.<sup>23</sup> Occupational exposure typically occurs through dermal contact or inhalation, and due to its high lipophilicity, BaP can cross the blood–brain barrier. This exposure has become associated with cognitive impairments and neurodegenerative-like diseases.<sup>24,25</sup> Using our established model, we dosed BaP into both stiff and soft samples for 3 and 5 days. From a viability perspective, we observed that the stiffer samples survived better at day 3 compared to the soft samples, with similar viability rates at day 5 (Figure 5A). Using RT-qPCR, we found that BaP-dosed samples significantly upregulated *SOX2* and downregulated *ACHE*. We visualized degradation in viability using Live/Dead staining (Figure 5D). Although we did not further investigate BaP's degradation mechanism in our model, the same workflow as CPF can be used to elucidate it.

## 4. Discussion

### 4.1. Creation of a physiologically relevant human neuron progenitor cell tissue model through the Modulation of bioprinting parameters

Conventional 2D cellular models do not reveal complex cell–cell interactions in a 3D architecture.<sup>26,27,28</sup> Using our bioprinting platform, we created a physiologically relevant high-cell-density hNPC model that can represent cellular injury response to toxins. Mechanical stiffness is an important consideration, as it dictates cellular fate and drives migration and differentiation.<sup>29</sup> To represent soft or stiff conditions, we engineered the 3D-bioprinted models to match physiological stiffnesses (softer samples around 2 kPa and stiffer samples around 4 kPa). Using our platform, we can engineer parameters such as stiffness by modulating printing settings. Before biological characterization, we performed Live/Dead staining and proliferation assays to ensure hNPCs grew effectively within the biomaterial.

### 4.2. Chlorpyrifos-induced injuries: Mechanistic insights from a human neural progenitor cell model

To probe cellular expression after CPF-induced injury, we performed RT-qPCR. In the stiff hNPC CPF-dosed group, *PAX6* and *Nestin* were higher compared to the soft group. *PAX6*, a transcription factor involved in hNPC

development, neuronal differentiation, and proliferation, was examined following CPF exposure. Consistent with previously reported findings,<sup>30</sup> *PAX6* expression was upregulated after neuronal injury, suggesting a potential role in tissue repair. This difference in *PAX6* upregulation may reflect the greater regenerative capacity of stiffer hNPC tissue, which retains a more plastic and proliferative state conducive to injury-driven repair.

*Nestin*, an intermediate filament protein expressed in hNPCs, was significantly upregulated (\*  $p \leq 0.05$ ) in CPF-dosed samples relative to controls. This increase indicates injury-induced activation of neural progenitor populations, consistent with *Nestin*'s established role as a marker of progenitor cell mobilization in response to injury. The upregulation observed suggests that CPF exposure induced a cellular stress state that triggers repair mechanisms. Like *PAX6*, the stiffer samples upregulate *Nestin* significantly compared to the softer samples.

*SOX2* is a transcription factor crucial for the maintenance of neural progenitor cell identity. The stiff CPF-dosed and non-CPF-dosed samples showed similar *SOX2* expression, indicating that CPF did not cause differentiation and that neural progenitor cells maintained their identity throughout the process. In the soft samples, *SOX2* was upregulated, suggesting a stiffness-related change in stemness programs that may reflect reduced differentiation cues or an attempt to maintain progenitor identity.

*Nestin*, *SOX2*, and *PAX6* are markers associated with hNPCs and represent various aspects of progenitor biology, including proliferation, remodeling, stemness, and neural lineage commitment states. Previous studies have shown that matrix mechanics regulate the expression of these markers, highlighting how biomechanical cues influence hNPC fate.<sup>31,32</sup> The differential expression of these genes following CPF or neurotoxin exposure may reflect distinct transcriptional responses driven by a soft or stiff environment. When interpreted alongside cell viability, these changes provide insight into how matrix stiffness may influence hNPC responses to toxin stress and highlight the importance of personalized models for studying disease. We also investigated three AD-associated markers in both stiff and soft samples. In our soft samples, we found a non-significant upregulation of *ACHE*, indicating that acetylcholine is broken down more compared with the stiffer samples. Furthermore, we found a non-significant upregulation of  $\beta$  amyloid accumulation, indicating early-stage AD development.  $\beta$  amyloid accumulation has been implicated in the etiology of AD.<sup>33, 34</sup> *BACE1*, a  $\beta$ -secretase<sup>35</sup> which is involved in plaque formation in the brain, was upregulated in both groups.

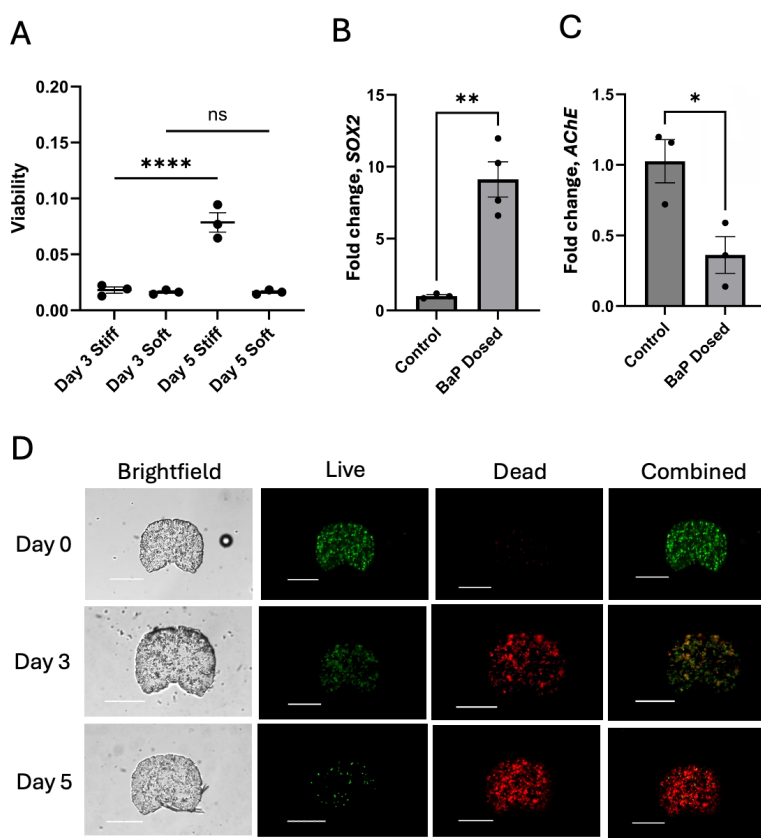


**Figure 5.** Application of the human neuron progenitor cell (hNPC) tissue model for environmental toxicology screening using benzo[a]pyrene (BaP). (A) Viability of BaP-dosed stiff and soft samples at day 3 and day 5. (B) Real-time quantitative polymerase chain reaction (RT-qPCR) of the SOX2 marker in control samples compared to BaP-dosed samples. (C) RT-qPCR of AChE in control samples compared to BaP-dosed samples. (D) Live/Dead staining of BaP-dosed samples at Day 0, 3, and 5. Scale bar: 250 μm; magnification: 10×.

Note: ns = not significant, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*\*  $p \leq 0.0001$ .

Paired with injury markers, AD-associated markers provide a broader view of both the stiff and soft models. Our data suggest that CPF harms hNPCs, and that injury mechanisms vary by stiffness. To ensure that cellular death does not interfere with measured expression, we measured the threshold count of *GAPDH*, which was not significantly different between dosed and not dosed groups (Figure A1). The soft group of hNPCs attempts to maintain progenitor identity and regulate self-renewal by upregulating *SOX2*, whereas the stiffer group activates cytoskeletal remodeling and mounts a structure-based injury response through *Nestin*. Further upregulation of AD-associated markers suggests the softer group is more susceptible to AD-associated injury than the stiffer group.

#### 4.3. High-throughput bioprinting platform for potential neurotoxin screening

We investigated a second class of pollutants, polycyclic aromatic hydrocarbons, focusing specifically on BaP. BaP was applied to both stiff and soft samples, followed by

incubation for 3 and 5 days. The stiffer samples survived better compared to the soft samples, particularly at day 5. Looking into the soft hNPC sample response to injury, *SOX2* was significantly upregulated (\*\*  $p \leq 0.01$ ), indicating injury triggered its cellular repair state. *AChE* expression was significantly downregulated (\*  $p \leq 0.05$ ) in the BaP-dosed sample.<sup>36</sup>

#### 4.4. Future work

Future work can focus on developing a more physiological and relevant model by incorporating additional cell types, including astrocytes. The interplay between hNPCs and astrocytes is particularly important for understanding injury and injury-related pathways. Our model and findings should be interpreted as a base model, intended to investigate the effects of compounds known to access the brain, and to be improved by adding more cell types, a blood–brain barrier, and dynamic culture. Ultimately, we aim to use this platform to screen a range of pollutants for their potential neurotoxic effects.

## 5. Conclusion

This study presents a precise, robust 3D bioprinting platform that incorporates hNPCs within biomimetic hydrogels, replicates key mechanical features of physiological tissue, and elucidates CPF-induced neurotoxicity. The model supports longitudinal studies of cellular viability, toxicity, and transcriptomic changes that traditional 2D models cannot. It serves as a toxicological *in vitro* neurotoxicity platform that can elucidate the cellular response to injury, specifically to CPF and other pollutants. While further integration of additional cell types is needed, this model represents a valuable step toward understanding the relationship between environmental pollutants and neurodegeneration.

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## Conflict of interest

The authors declare no competing interests.

## Author contributions

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## Ethics approval and consent to participate

None.

## Consent for publication

None.

## Availability of data

The data that support the findings of this study are available upon reasonable request.

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## Appendix

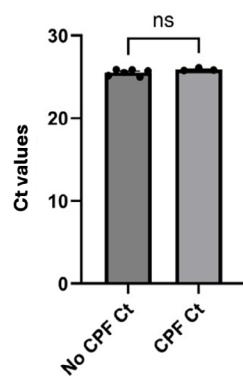


**Figure A1.** Ct values extracted from real-time quantitative polymerase chain reaction of the *GAPDH* housekeeping gene show no significant difference between no-chlorpyrifos (CPF) and CPF-dosed samples