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CELLINK
A BICO COMPANY

HYBRID PRINTING PROTOCOL

# PCL-GelMA

This is a suggested procedure, please adjust according to your experimental needs. To maintain the sterility of the product, work under sterile conditions.

## Protocol aim

The aim of this protocol is to provide instructions for printing biocompatible and biodegradable 3D hybrid structures using CELLINK's polycaprolactone (PCL) and GelMA bioink on the BIO X platform equipped with a Thermoplastic Printhead (TPPH), a Temperature-Controlled Printhead (TCPH) and the 405 nm photocrosslinking module.

This protocol covers:
• Design of modular "puzzle-like" scaffolds and G-code generation.
• Printing of a PCL lattice scaffold.
• Dispensing of GelMA into the PCL scaffold and photocrosslinking.

Before starting, read the full protocol and plan PCL pre-heating (~90 min) and cell harvest/mixing with GelMA in advance.

# Safety and handling

- **Thermal hazards:** The Thermoplastic Printhead (TPPH) operates up to 250 °C. Avoid contact with hot metal parts and allow components to cool before handling when indicated.
- **Photocrosslinking module (405 nm):** Keep the BIO X door closed during exposure. If observation is required, use appropriate UV-blocking safety glasses. Minimize skin exposure.

*The product can be purchased in the CELLINK shop at https://www.cellink.com/shop/

# Materials needed

| Material / Component | Supplier | Product ID / SKU | Key specifications | Lot No. Record |
|---|---|---|---|---|
| **Biomaterials** | | | | |
| Polycaprolactone (PCL)* | CELLINK | TP60505 0001 | Mn:50,000 g/mol, Melting point: 60°C Storage: 2–10°C Not sterile, 50 g, pellet form | ☐ |
| GelMA Bioink* | CELLINK | IK30510 20303 | 10% (w/v) GelMA, High degree of methacrylation, premixed with 0.25% (w/v) LAP photo initiator, Supplied in 3 x 3 mL cartridges | ☐ |
| **Cells and media** | | | | |
| Cells of interest in suspension | | | Cells relevant for bone and cartilage tissue engineering applications | ☐ |
| Appropriate cell culture medium | | | Pre-warmed to 37 °C | ☐ |
| **Equipment** | | | | |
| 3D bioprinter* | CELLINK | BIO X | Equipped with photocrosslinking module (405 nm) | ☐ |
| Thermoplastic Printhead* | CELLINK | CL-PH-TPPH | For high-temperature extrusion of PCL (up to 250°C) | ☐ |
| Temperature-controlled Printhead* | CELLINK | CL-PH-TCPH | For hydrogel dispensing with thermal control | ☐ |
| External air compressor | California Air Tool | D161100 21361 | Air compressor compatible with BIO X (for PCL extrusion). | ☐ |
| Class II biological safety cabinet | | | Biological Safety Cabinet appropriate class for your cells experiments | ☐ |
| Incubator | | | (37 °C, 5 % $CO_2$) | ☐ |
| **Consumables** | | | | |
| Thermoplastic high-precision nozzle* | CELLINK | NZ0000 0015 | Metal, Conical, Size: #15 (30G), Inner Diameter: 150 µm | ☐ |
| Sterile conical nozzle* | CELLINK | NZ3270 005001 | Sterile, Plastic, Luer lock, Conical, Size: 27 Gauge, Inner Diameter: 200 µm | ☐ |
| Glass Petri dish* | CELLINK | OH0000 00012 | Cylindrical, Glass, Dimensions: 10x10x2 cm | ☐ |
| Sterile 3 mL syringes | | | Sterile 3 mL syringes with Luer lock connections. | ☐ |
| Luer lock adaptor | | OH0000 00010 | Sterile female/female Luer lock adaptor. | ☐ |
| UV-shielding cartridges | | CSO010 311502 | Sterile 3 cc UV-shielding cartridges with tip caps. | ☐ |
| Ruler | | | Ruler for measuring photocrosslinking distance. | ☐ |
| 6-well plates | | | | ☐ |
| 70 % ethanol | | | 70 % ethanol and lint-free wipes. | ☐ |

# Protocol

# Pre-print

## *1a. Model design*

**MATERIAL**

Computer with Blender and the CELLINK Blender tool: **Blender-Multi-Stack-Puzzle.blend** and README documents.

Implementing this strategy relies on a two-step computational workflow that bridges parametric design with high-resolution print preparation.

**DESCRIPTION**

1. Import your anatomical or generic CAD model (e.g. nose, cartilage block, or any STL model) into the supplied Blender project file.
2. Use the provided Blender script to generate:
    - Interlocking "puzzle" modules by slicing the model into stackable layers.
    - Internal gaps to support interlock perfusion and media layer diffusion.
3. Set the parametric values according to (**Table 1)** (example): rib thickness, spine thickness, offsets and clearances (1.0 mm in the validated configuration).
4. Export the resulting parts as STL files.

***Table 1**. Parameters for the Blender script configuration.*

| **Script parameter** | **Value in (mm)** |
|---|---|
| Rib thickness | 1.0 |
| Spine material thickness | 1.0 |
| Spine perimeter offset | 1.0 |
| Spine hole clearance | 1.0 |

**Note:** The "nose" and "cube" models are used as examples only. Any compatible STL that fits the BIO X print area can be used.

**Parametric puzzle generation in blender**

Cellink Bioprinting has prepared a Python-Blender Script for this protocol application that automates the design of a 3D puzzle model. It programmatically slices a 3D model into distinct layers and generates the interlocking features that allow the puzzle to be precisely reassembled after printing, transforming a biological design into a manufacturable, multi-party system engineered for maximum cell viability and easy-to-build construction. First, access the provided material in the links, and once it is open, go to the tab at the right of the upper bar and run the multi-stack blender script **(Figure 1).**

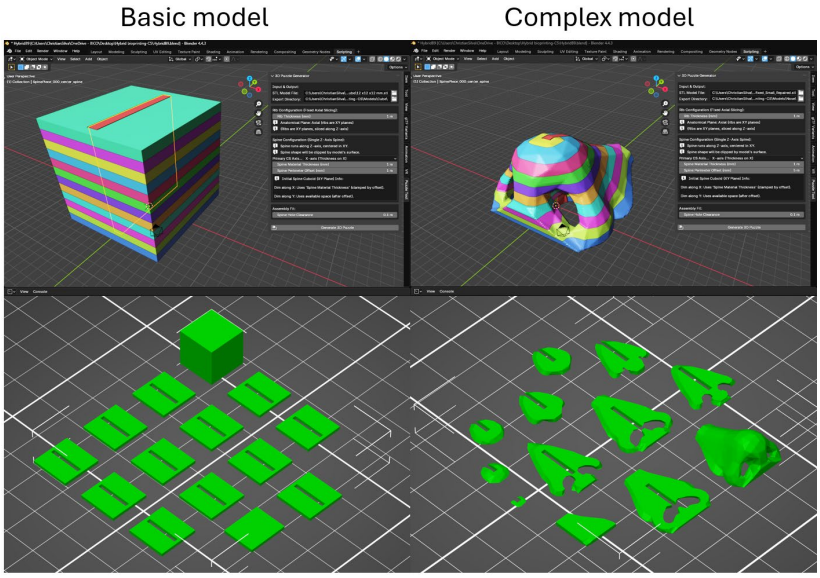


***Figure 1.*** *Stackable 3D puzzle strategy for large-scale biofabrication of simple and complex anatomical models. A Blender-based tool slices 3D geometries (e.g., a nose) into interlocking, stackable layers, enabling modular assembly and the formation of perfusable networks to enhance cell viability throughout the construct while maintaining an easy-to-build design.*

Ensure all your parameters are defined as in (**Table 1)** and **Figure 2**. This protocol was validated using the provided Blender Script and STL models, using different models may require adjustments.

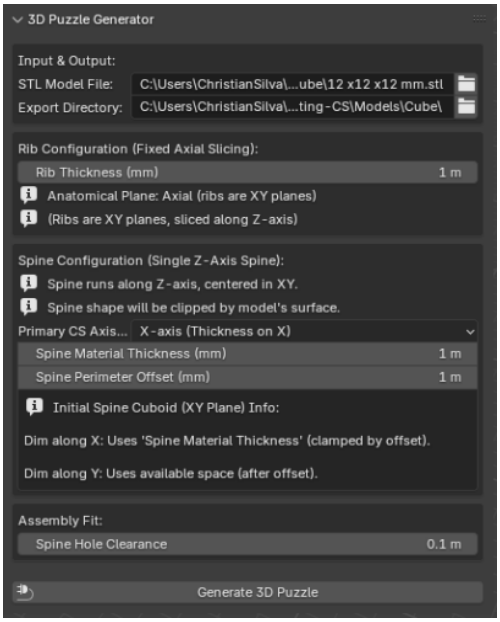


***Figure 2.*** *Parametric configuration panel for the stackable puzzle generator. Key values, such as slice thickness and inter-module gaps, are entered here to control the automated slicing of a 3D model into its final interlocking components.*

Once the pieces are generated, they can be passed through the G-code tool, to get the G-code to print the parts. After printing, they can be assembled in stack and used for the objective application as shown in **Figure 3** below.

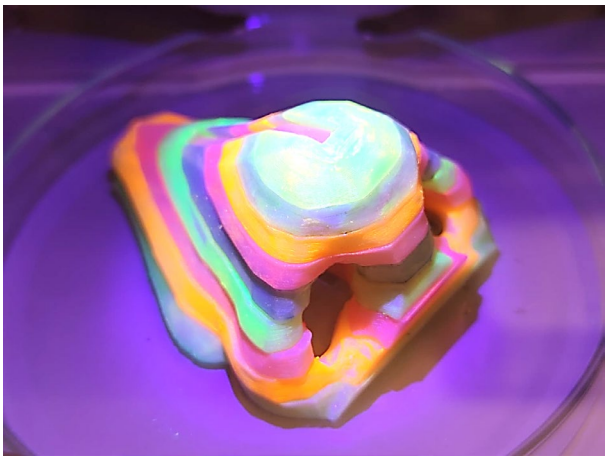

***Figure 3**. 3D-printed stackable components assembled post-fabrication into an interlocking 3D puzzle construct.*

# 1b. *G-code generation*

**MATERIAL**

G-code generation tool G-code tool and README and DNA Studio 4.

**DESCRIPTION**

1. Import each STL piece into the G-code tool.
2. Define the microarchitectural parameters (layer height, line spacing, infill pattern) according to your mechanical and diffusion requirements (e.g. grid spacing 100–1000 µm).
3. Generate PCL G-codes ensuring:
    - First-layer line spacing matches the desired void size.
    - Total scaffold height and footprint fit within the photocuring-reachable area of the BIO X (see Section 8 for GelMA printing).
4. Optionally visualize the final toolpath in DNA Studio to confirm that:
    - The scaffold fits inside the usable print area.
    - Paths do not collide with the printhead or door.
5. Save the final G-code files for PCL and for GelMA.

**Python Gcode Generation**

Once the 3D puzzle pieces are designed by using the Blender Tool, the G-code tools **(Figure 4)** are used to prepare them for G-code generation. This software is essential for defining the high-resolution microarchitecture within each piece. Features like parametric infill generation allow for precise control over the grid spacing required to achieve both the target biomimetic stiffness and to satisfy the 100-200 µm diffusion rule. The ability to visualize the final G-code toolpath within software like DNA Studio 4, further aids verifying and refining the printing strategy before fabrication.

**Design rationale: Engineering a biomimetic microenvironment**

Advanced bioprinting often demands 3D constructs with tightly defined microarchitecture—e.g., a precise grid line spacing below 1 mm—beyond the native resolution of standard DNA Studio. A parametric design plus the specialized G-code generation tool overcomes this limitation by optimizing geometry at the microscale (layer height, line spacing, infill pattern, infill density), which in turn promotes capillary wicking of bioinks and uniform filling throughout the lattice. Reference video.

To build clinically relevant constructs several millimeters thick to centimeters while preserving cell viability, we use a modular strategy: assemble the final piece from smaller, interlocking “puzzle-piece” modules (Chen et al., 2023; Williams et al., 2020). Purposeful clearances between modules create an interconnected network of perfusion

channels, allowing culture medium to penetrate the entire structure and mitigating the diffusion limits inherent to large scaffolds. The implementation and model generation are illustrated in the figure below. **(Figure 4)**.

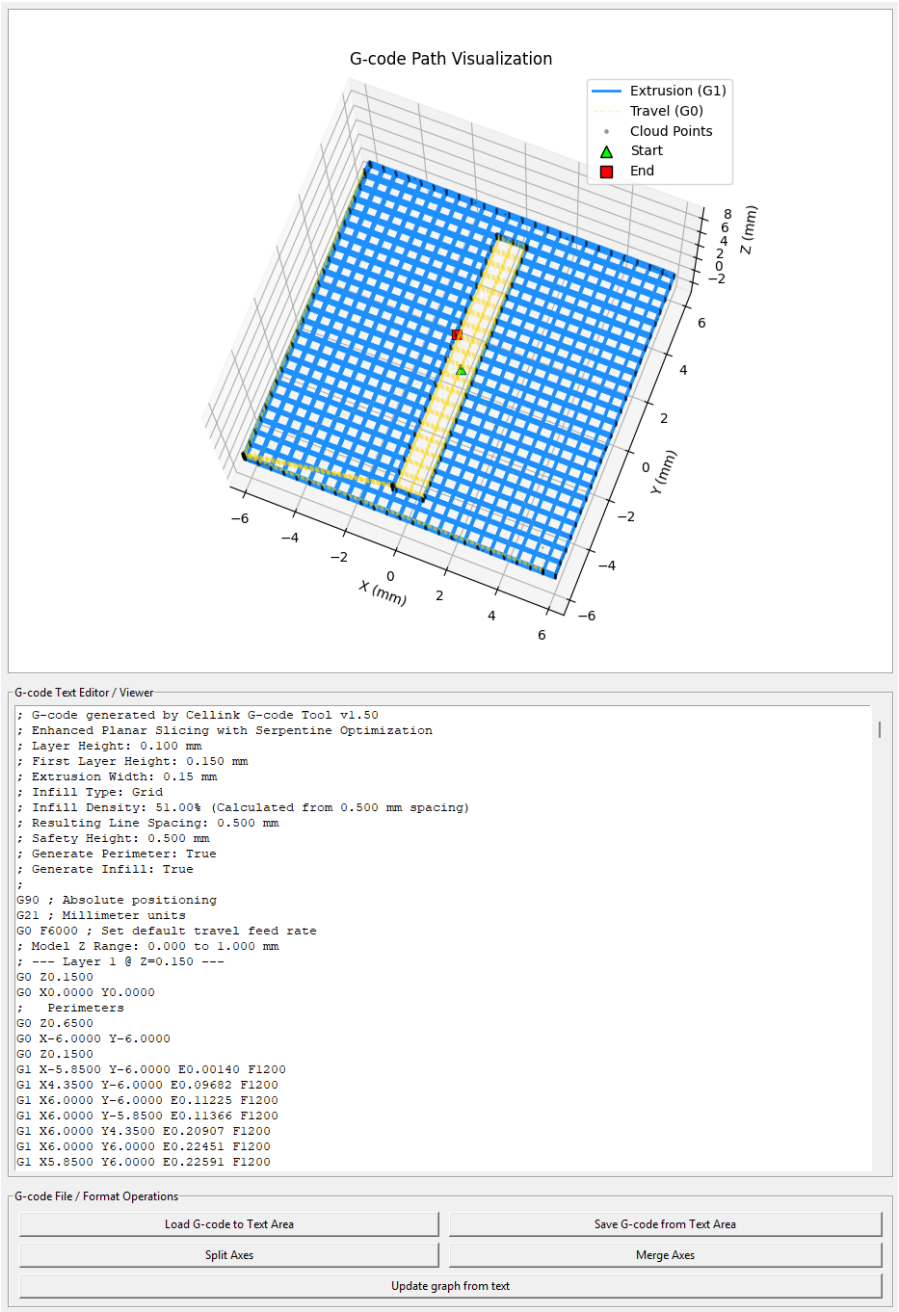


***Figure 4.*** *G-code tool for precise control of scaffold microarchitecture. This tool allows user-defined control of parameters like grid spacing to tune the scaffold's final mechanical and biological properties.*

In bioprinted scaffolds, the internal grid size and layer height are critical parameters. This microarchitecture, often a lattice of a structural polymer like PCL filled with a cell-laden hydrogel like GelMA, dictates both the mechanical stiffness of the scaffold and the efficiency of media diffusion.

The G-code, which contains machine-readable instructions for the bioprinter, is created by processing a 3D model file (STL) with the settings defined in the G-code tool generator. **Figure 5** shows the user interface where these specific parameters—such as layer height, infill density, and grid spacing—are applied to the STL model before generating the final instructions. This critical step translates the digital microarchitecture design into a precise physical fabrication plan, enabling the creation of scaffolds with systematically varied properties, as demonstrated by the final printed constructs shown in **Figure 6**. Link to the generated G-codes for the application: Hybrid Bioprinting Gcodes.

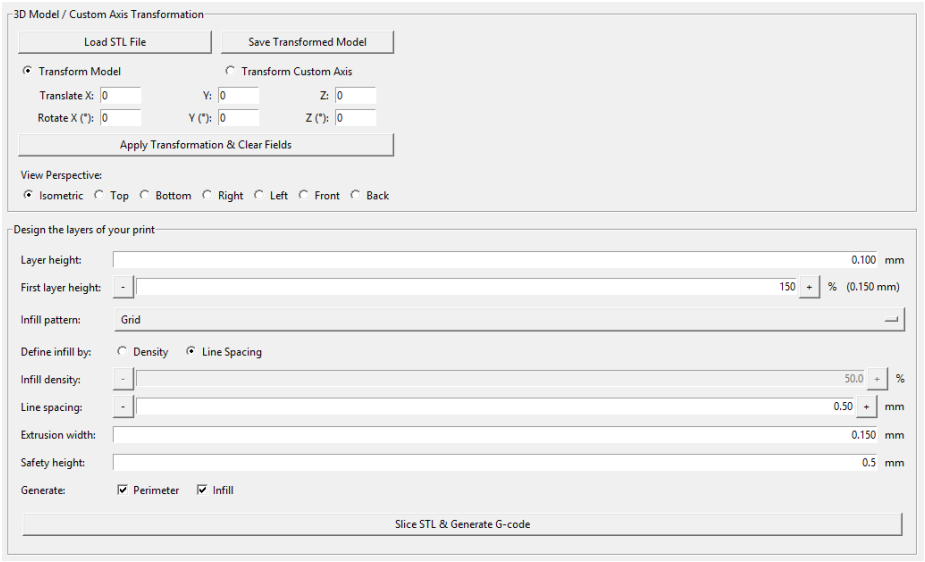


***Figure 5.*** *Set to process a 3D model file (STL) using the specified settings to generate the printable G-code.*

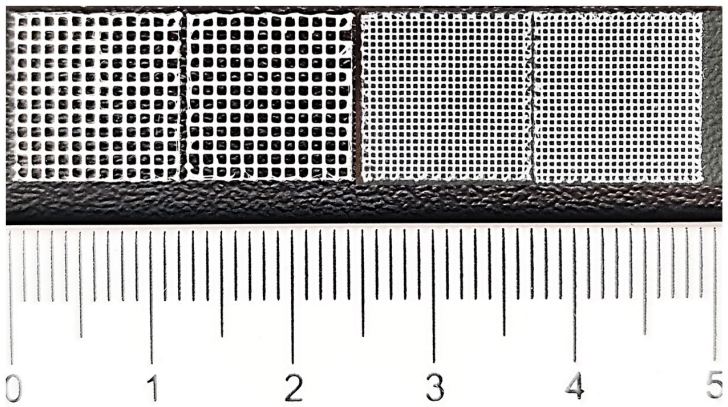


***Figure 6.*** *The resulting 3D printed PCL scaffolds demonstrate the fabrication of grids with varying void sizes, shown next to a ruler for scale in mm.*

# Printing

## 2. *Filling the cartridges with PCL and GelMA Bioink*

**MATERIAL**

PCL

Thermoplastic Printhead

GelMA Bioink

Temperature-controlled Printhead

**DESCRIPTION**

- Load the metal cartridge as recommended in the Thermoplastic Printhead User Manual.

Note: To ensure the most efficient and uniform heating of the PCL, fill a maximum of one-quarter of the cartridge (approx. 80 pellets). A smaller thermal mass allows the pellets to melt completely and homogenize within the recommended pre-heating time, preventing inconsistencies during extrusion.

- Load the plastic cartridge as recommended in the Temperature-controlled Printhead User Manual.

# 3. *Pre-heating*

**MATERIAL**

BIO X and TPPH

**DESCRIPTION**

- Once the Thermoplastic Printhead is attached to BIO X, start pre-heating at **200°C**. Heat for 60-90 min until the PCL is fully melted and homogenized.

  **Best practice for sequential prints**: The 90-minute pre-heating time is for a cold start. If you plan to perform multiple prints on the same day, you can keep the Thermoplastic Printhead at its 200°C setpoint between prints to eliminate this long pre-heating step for subsequent runs.

- Once the **Temperature-controlled Printhead** is attached to the BIO X, start pre-heating at **37°C**. Heat for 20 min until the GelMA is fully melted and homogenized. At the same time the **print-bed** heating starts to **30°C**, it is essential for ensuring the adhesion of the PCL to the Petri dish surface.

While waiting for the PCL and GelMA materials to be fully homogenized, prepare the model to print.

# 4. *Preparation of cell-laden bioink*

**Safety and aseptic handling**

- Follow institutional biosafety and chemical safety procedures; consult the SDS for every reagent before use.
- **PPE:** Wear a lab coat, safety glasses, and suitable chemical-resistant gloves (e.g., nitrile) always.
- **Biological materials:** Perform all steps involving GelMA, cells, and printed cell-laden constructs in a Class II (or appropriate) biological safety cabinet, consistent with the biosafety level for your cell type.
- **Waste disposal:** Dispose of PCL waste, used cartridges, nozzles, and cell-containing materials as chemical or biological waste according to institutional guidelines.

**Note:** This part of the protocol includes the critical step of incorporating **live cells** into **GelMA** prior to printing. Conduct **all** cell-handling and GelMA-mixing steps inside a sterile biological safety cabinet.

**MATERIAL**

- GelMA Bioink, pre-warmed to **37 °C** (from Step 3).
- **Cell suspension** in culture medium, prepared at **11×** the desired **final** cell concentration in the bioink *(10 parts bioink + 1-part cells = 11 parts total).*
- Two sterile **3 mL** syringes with Luer-lock connections.
- Sterile **female/female Luer-lock adaptor**.
- Sterile **3 cc UV-shielding cartridge** with tip cap.
- Nozzle 27G.

**DESCRIPTION**

1. **Prepare cell suspension**
   - Harvest cells per your SOP (detach, centrifuge, resuspend).
   - Adjust to **C_stock = 11 × C_final** (e.g., target **5×10⁶ cells/mL** → stock **5.5×10⁷ cells/mL**).
2. **Load syringes**
   - Draw **1.0 mL** liquid **GelMA** into a 3 mL Luer-lock syringe.
   - Draw **0.1 mL** of the **11× cell suspension** into a second 3 mL Luer-lock syringe.
   - *Scale volumes proportionally while maintaining the **10:1** (bioink: cells) ratio.*

3. **Mix bioink and cells**
    - *(Optional)* Pre-fill the Luer adaptor with a small amount of GelMA to reduce trapped air.
    - Connect the two syringes via the adaptor, avoiding bubbles.
    - Pass the material gently back and forth for **≥ 1 min (~20 cycles)** until the cell distribution is homogeneous.
    - *Minimize light exposure with LAP-containing inks.*
4. **Load the cartridge (do this before the QC check)**
    - Attach the mixed-ink syringe to a **UV-shielding cartridge** and **slowly** transfer the bioink, avoiding bubbles.
    - Cap the cartridge and **place it immediately in the TCPH/warming block at 25°C** to maintain printable viscosity.
    - *Avoid leaving the mixed bioink sitting in syringes at room temperature.*
5. **Quick visual check**
    - With the cartridge inside the temperature-controlled printhead, attach a sterile nozzle 27G and gently dispense small drop onto a microscope slide.
    - Inspect rapidly for large bubbles or clumps; aim for a uniform single-cell suspension.
    - Wipe the nozzle tip if needed.
6. **Proceed to printing**
    - Keep the cartridge at **25 °C** and continue with **Printhead Calibration** and the next printing steps **(Step 8)**.

# 5. *Printhead calibration*

**MATERIAL**
BIO X with TPPH (Tool 1), TCPH (Tool 3), glass Petri dish and ruler.

**DESCRIPTION**

1. **Mount Petri dish and clamps**
    - Place the glass Petri dish on the print bed and secure it firmly with three clamps to prevent any movement during printing as shown in the reference video.
2. **Determine photocuring-reachable area**
    - Using the BIO X interface, move the photocrosslinking module across the dish to identify the area that can be reached at the required LED height (≈ 6.0–6.5 cm).
    - Ensure your G-codes are positioned so the entire scaffold fits within this area.
3. **Manual calibration (DNA Studio or embedded UI)**
    - Set the reference point at the center of the usable print area with Tool 3 (TCPH).
    - Follow the system instructions to move the printhead along the Y-axis by the recommended offset (e.g. 13–14 mm).
    - For safety in the example, round up the recommended offset to **15 mm** to ensure adequate clearance to the bioprinter door. the TCPH and photocrosslinking module.
4. **Align Tool 1 (TPPH)**
    - Calibrate Tool 1 (TPPH) at the same reference point and offset as Tool 3.
    - Leave at least one empty head slot between Tool 1 and Tool 3 to minimize heat transfer from the 200 °C TPPH to the TCPH.

**Note**: that the combination of TPPH and TCPH is not compatible with automatic calibration **(Figure7, Figure 8)**. Therefore, the most reliable method for calibrating those printheads is to perform manual calibration.

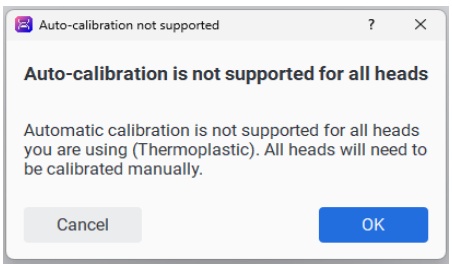


***Figure 7.*** *DNA Studio Software Prompt Indicating the Need for Manual TPPH Calibration*

Another important consideration is that when using the thermoplastic printhead and the temperature-controlled printhead in combination with the photocrosslinking light from the BIO X print box, the usable printing area is reduced **(Figure 9)**. This is because the crosslinking Module Tool cannot reach certain sections of the Petri dish—specifically the area at the front. As a result, this portion cannot be utilized for printing. To address this, ensure that your scaffold is adjusted to fit within the reachable area, and pay special attention to the calibration of both printheads.

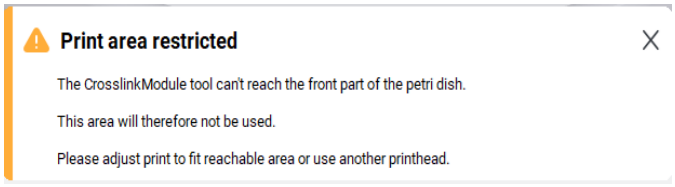


***Figure 8.*** *System Notification of the Reduced Print Area for the combination of TPPH and TCPH.*

To begin the calibration, it is important to note that the workflow differs depending on whether you are calibrating the BIO X using the embedded user interface or DNA Studio on a computer.

**DNA Studio printhead calibration**

The first step is to calibrate the thermoplastic printhead, TPPH (Tool 1). This is done by setting a Y-offset value (in mm) along the Y-axis from the center of the Petri dish. To simplify the manual calibration process, mark a small dot on the back face of the Petri dish—the side opposite to where printing occurs. This reference point helps ensure accurate alignment.

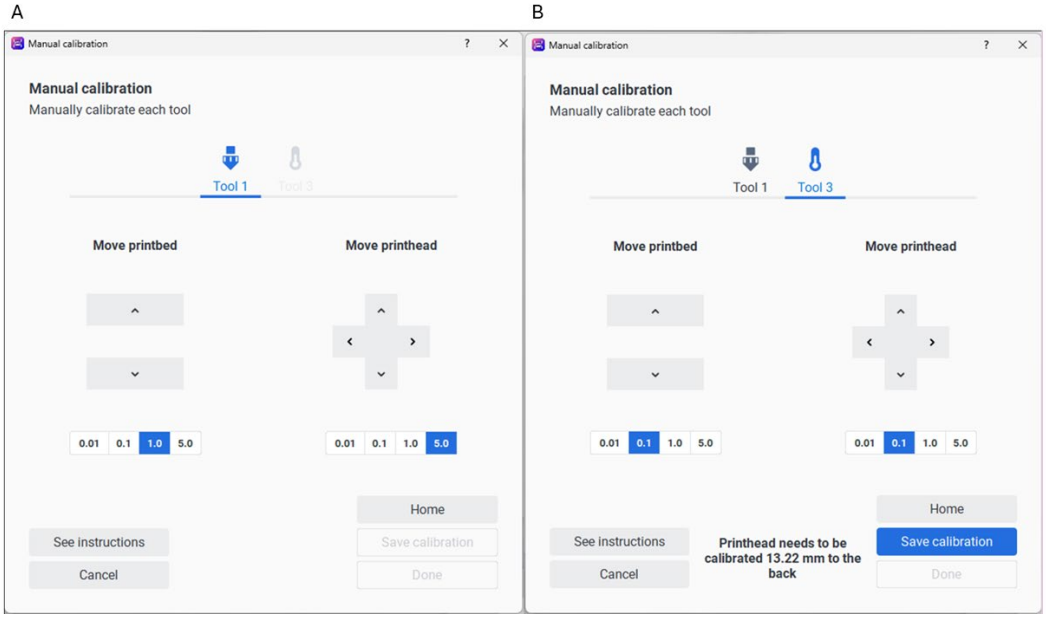


***Figure 9.*** *Manual calibration of TPPH and TCPH in DNA Studio. (A) Manual calibration interface for Tool 1 (TPPH). (B) System-guided manual calibration of Tool 3 (TCPH), indicating the required Y-axis offset for the temperature-controlled printhead.*

The required Y-offset is a system-generated value, determined during the initial calibration of the temperature-controlled printhead. While this value is calculated for the specific model size (e.g., 13.22 mm), **(Figure 9)**, it represents the minimum clearance required. To ensure process reliability, it is best practice to apply a conservative safety margin. Therefore, we recommend rounding this figure up to a more practical value, such as 15 mm. This increased offset ensures sufficient clearance for the printheads' full range of motion, mitigating the risk of process failures caused by a potential collision with the bioprinter's door along the Y-axis.

Following the same principle, the second printhead, TCPH (Tool 3) must be calibrated at the same reference point as Tool 1, according to the recommended Y-offset provided by the system.

**Note**: that there is one printhead slot between Tool 1 and Tool 3 to ensure there is no temperature compromise from the thermoplastic printhead at 200°C to the temperature Controlled printhead at lower temperature.

**Embedded User Interface calibration:**

When using the embedded software on the BIO X bioprinter, the touchscreen interface allows calibration directly with the Tool 3 -TCPH temperature-controlled printhead **(Figure 10)**. This simplifies the process, since Tool 3 is

the printhead with the specific requirement of ensuring enough space for the photocrosslinking process, particularly important when working with GelMA, which will be crosslinked with 405 nm photocrosslinking light.

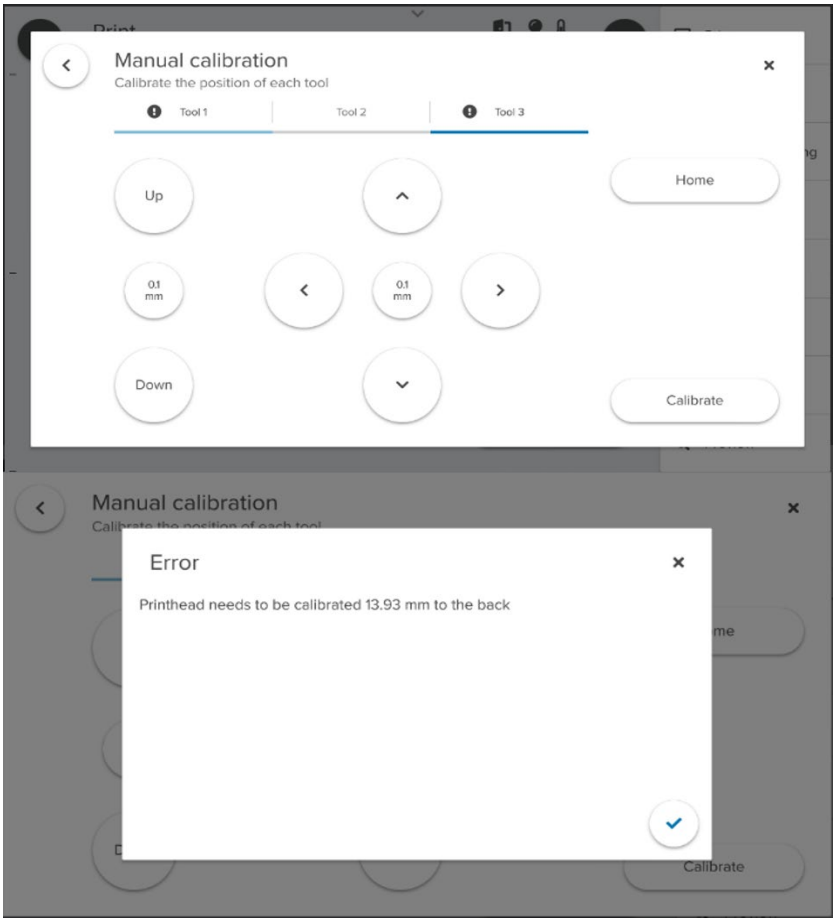


***Figure 10.*** *System-guided manual calibration via the embedded touchscreen interface.*

Calibrating Tool 3 for the first time at the center of the Petri dish will define the required Y-axis offset to stay within the secure photocrosslinking area for the model being used. Simply follow the recommendations provided by the user interface, which will instruct you to move the printhead a certain distance along the Y-axis. For example, if the system suggests 13.93 mm, it is recommended to round this up to 15 mm for additional safety. Perform the calibration of Tool 3 at this point.

Finally, repeat the same calibration step with the Tool 1 - TPPH, thermoplastic printhead, ensuring that both printheads are aligned to the same reference point.

# 6. *PCL printing*

**MATERIAL**

BIO X

Petri dish

TPPH

**DESCRIPTION**

Before starting any print, prime the nozzle following the *Nozzle Priming Procedure* described below.

**Nozzle priming procedure**

To ensure consistent material flow and prevent over-extrusion during the printing process.

- High-Pressure Initial Prime: Set the external compressor to a high-pressure range of 200–700 kPa.
- Activate Extrusion: Enable the extrusion and monitor the printhead. Continue only until the material exits the nozzle as a stable, continuous filament.
- Adjust to Operating Pressure: Immediately reduce the pressure to the operational range (7–200 kPa) before initiating the print.

**Warning:** Failure to lower the pressure will result in severe over-extrusion and may compromise the integrity of the construct.

If the nozzle is clogged, please refer to the accompanying video demonstration for instructions on how to clear it using a wire.

**Safety Notice**

- **Thermal Hazard:** Use extreme caution when working near the printhead. The nozzle tip reaches high temperatures and can cause severe burns upon contact.
- **PPE:** It is recommended to use heat-resistant tools or gloves when performing maintenance near the nozzle.

Once the nozzle is correctly primed:

- Set the first layer height at 100% of the nozzle diameter, (150 µm), the rest of the layers can be set at 66% of the first layer height, (100 µm).
- Start with a printing temperature of 200°C and the pressure of 200 kPa to 700 kPa using external compressor as reference in (**Table 2)**.

**First layer calibration**

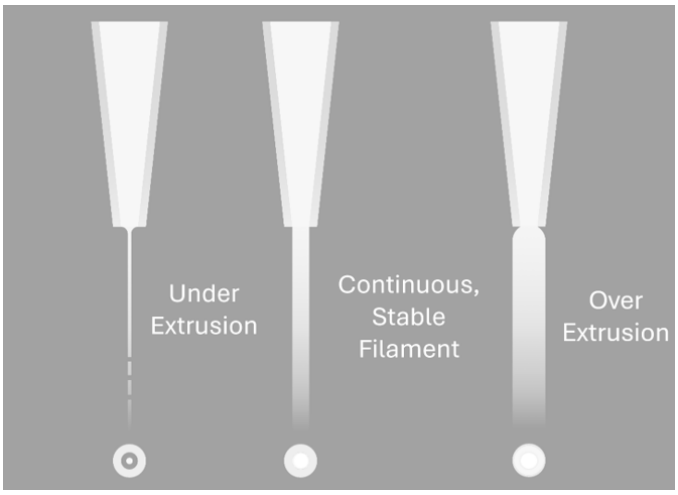


***Figure 11**. Nozzle fidelity and characterization*

To achieve optimal print fidelity and ensure accurate construct geometry **(Figure 11)**, it is essential to calibrate extrusion by characterizing the relationship between pressure and speed. The primary goal is to extrude a continuous, stable filament whose measured width precisely matches the nozzle's internal diameter. This calibration is performed by setting a constant travel speed and systematically adjusting the pressure until a continuous filament is achieved. Once a stable pressure is found, evaluate the filament diameter at different travel speeds to understand how they interact. An ideal filament should not be significantly wider than the nozzle diameter (indicating over-extrusion) nor so thin that it breaks (indicating under-extrusion). This process is especially critical for producing the finest and most consistent first layer possible, which is fundamental for print adhesion and overall quality. For a visual guide on what an ideal first layer should look like, please refer to the provided video.

**Notes**

- Due to the need to fuse filaments of successive layers together, it is recommended to use a layer height that is smaller than the nozzle diameter. This is to allow the filaments to fuse and to account for shrinkage of the layers during the cooling process.
- If the extruded filament becomes discontinuous during a print, key parameters such as speed, pressure, and layer height can be adjusted in real time. Access the *Utilities* menu while the print is active and modify these settings in small increments until a stable, continuous line is achieved.
- If PCL is extruding inconsistently, the tip may be dirty. Wipe the tip with steel wool to remove the excess polymer. If the PCL is turning brown, the temperature is too high. Lower the temperature or replace the PCL in the cartridge. Re-start from step 1, then flush the nozzle with new PCL from the printer overview page or the utilities menu. If necessary, run a Pressure/Speed calibration Gcode scripts to reoptimize the printing characteristics, (speed and pressure).

***Table 2.*** *Bioprinting parameters for PCL through a 150 µm nozzle and GelMA dispensing through a 27G nozzle (200 µm) on a Petri dish surface using the Model Print function and G-code on BIO X.*

| Printing parameters | PCL | GelMA |
|---|---|---|
| **Surface** | Petri dish | Petri dish-PCL |
| **Print bed temperature** | 30°C | 30°C (during printing), 15°C (during- photocrosslinking) |
| **Printhead temperature** | 200°C | 25°C |
| **Speed** | G-code defined.<br><br>(7 mm/s to 20 mm/s) | G-code defined (7 mm/s to 20 mm/s) |
| **Pressure** | 200 kPa-700 kPa (external compressor) | 15-32 kPa |
| **Photocrosslinking time** | - | 10 s |
| **Z-offset** | 0.150 mm | 0.6 mm |
| **Nozzle** | Thermoplastic high-precision nozzle # 15 (0.150 mm) | 27G |

A stable printing surface is essential for print quality. Before starting, use three clamps to firmly secure the petri dish to the print bed. This prevents shifting during printing, which can cause layer misalignment and print failure.

**Printing sequence**

Fabricate each scaffold individually. Always print the complete PCL frame first, following the parameters established in (**Table 2**).

**Examples of G-code files**

The following pre-generated G-code files are provided to help you perform initial experiments and calibration. They can be downloaded here: Hybrid Bioprinting G-codes and README.

*Table 3. Summary of Pre-Generated G-code Files using PCL.*

| File Name | Material(s) | Layer Z-Height | Line Spacing | Layers | Purpose |
|---|---|---|---|---|---|
| Single Test Only PCL Centered 100um - 1x1.gcode | PCL only | 0.1 mm | 1.0 mm | 5 | Less dense PCL scaffold for calibration |
| Single Test Only PCL Centered 100um-0.5x0.5.gcode | PCL only | 0.1 mm | 0.5 mm | 5 | Dense PCL scaffold for calibration |
| PCL Centered 100um-0.5x0.5 with GelMA-Photocuring-Pause-G4S60.gcode | PCL + GelMA | 0.1 mm / 0.6 mm | 0.5 mm | 5 + inter-layer pause + 1 | Hybrid dense scaffold, Void Size: 0.5 x 0.5 mm² Includes G4S60 inter-layer pause |
| PCL Centered 100um-1x1 with GelMA-Photocuring-Pause-G4S60.gcode | PCL + GelMA | 0.1 mm / 0.6 mm | 1.0 mm | 5 + inter-layer pause + 1 | Hybrid less dense scaffold, Void Size: 1.0 x 1.0 mm² Includes G4S60 inter-layer pause |

# 7. *Solidification*

**MATERIAL**

BIO X

Petri dish

**DESCRIPTION**

**Final PCL solidification**

After the print is complete, allow the PCL construct to cool and solidify for at least **30 seconds**. The structure is sufficiently cooled when it appears fully opaque and is rigid to the touch. Do not handle it before this point to prevent deformation. This allows the structure to fully solidify and cool down, which is essential for its stability before proceeding with GelMA dispensing.

**Preparation for Hydrogel Dispensing**

Following PCL solidification, prepare for the GelMA dispensing and after that for photocrosslinking step by cooling the print bed to **15°C**. Hold at this temperature for **1 to 5 minutes**. This pre-chill the entire scaffold, which is critical to ensure the subsequent thermal gelation of the photocrosslinked hydrogel.

**Notes**

- Troubleshooting Warping: If the structure contracts or warps during the printing process, additional cooling time between layers is required. To resolve this, modify the G-code to add a **60-second** (1 min) pause after each layer by inserting the command G4 S60 at the end of each layer's code.

# 8. *GelMA printing*

**MATERIAL**

BIO X

Petri dish

**DESCRIPTION**

Dispense the cell-laden GelMA into the PCL scaffold voids using one of the example G-codes. While the G-code runs, **manually adjust the extrusion pressure** based on visual feedback to maintain a continuous filament. The required pressure will typically be within the **15-32 kPa** range when using the 27G nozzle, as specified in (**Table 2**).

For in-depth guidance on fine-tuning print parameters, managing viscosity with temperature adjustments, and advanced troubleshooting for this material, please refer to the dedicated GelMA Bioink Protocol.

**Note on Calibration:** Before performing a full hybrid print with valuable cells, it is highly recommended to run a "GelMA-Only Calibration" G-code file. This allows you to determine the ideal extrusion pressure for your specific setup without wasting a PCL scaffold.

To assist with this process, several pre-generated G-code files can be downloaded here: Hybrid Bioprinting G-codes.

***Table 4.*** *Summary of Pre-Generated G-code Files using GelMA*

| File | Material(s) | Layer Z-Height | Line Spacing | Layers | Purpose |
|---|---|---|---|---|---|
| Centered only GelMA at 600 um.gcode | GelMA only | 0.6 mm | 0.5 mm | 1 | Top layer 0.6 mm height GelMA test, *use this to find the ideal pressure for dispensing GelMA on top of a pre-printed PCL scaffold* |
| Centered only GelMA at the base-Z0.gcode | GelMA only | 0.0 mm | 0.5 mm | 1 | Base layer 0.0 mm height GelMA test, *use this to find the ideal pressure for dispensing GelMA directly onto the petri dish.* |
| PCL Centered 100um-0.5x0.5 with GelMA-Photocuring-Pause-G4S60.gcode | PCL + GelMA | 0.1 mm / 0.6 mm | 0.5 mm | 5 + inter-layer pause + 1 | Hybrid dense scaffold, Void Size: 0.5 x 0.5 mm² Includes G4S60 inter-layer pause |
| PCL Centered 100um-1x1 with GelMA-Photocuring-Pause-G4S60.gcode | PCL + GelMA | 0.1 mm / 0.6 mm | 1.0 mm | 5 + inter-layer pause + 1 | Hybrid less dense scaffold, Void Size: 1.0 x 1.0 mm² Includes G4S60 inter-layer pause |

# 9. *Photocrosslinking*

After dispensing the GelMA, stabilize the hybrid scaffold via photopolymerization. The following sections describe the primary and alternative workflows for photocrosslinking.

**Photocrosslinking using the BIO X embedded user interface (primary workflow)**
Configure the photocrosslinking process directly on the BIO X touchscreen.

1. **Enable photocrosslinking:** In the printer settings, enable the photocrosslinking module that will be used (e.g., Module 2, 405 nm). This ensures the light source is active for the print job **(Figure 12, Panel A)**.
2. **Set crosslinking parameters:** Set the key parameters for the temperature-controlled printhead (Tool 3) as follows **(Figure 12, Panel B-C)**:
    - **Photocrosslinking time:** 10.0 s
    - **Height:** 6.5 cm
    - **Intensity:** 50% **(Figure 13)**
3. **Initiate printing and curing:** Start the print. The system will automatically perform the photocrosslinking step as configured. A summary of the settings can be viewed in the Utilities menu before starting the print **(Figure 12, Panel D)**.
4. **Ensure thermal gelation:** Following the 10-second photocuring, cool the BIO X print bed to 15°C and hold for at least 60 seconds to ensure the construct is fully thermally gelled.
5. **Complete crosslinking:** Allow the structure to sit for at least one minute after the light source has turned off to ensure the crosslinking process is complete.
6. **Transfer the construct:** After curing, aseptically transfer the scaffold to a designated well, in our example case, a 6-well plate containing 2 mL of pre-warmed culture medium. Return the plate to an incubator (37°C, 5% $CO_2$). Repeat this fabrication and transfer cycle for all subsequent scaffolds.

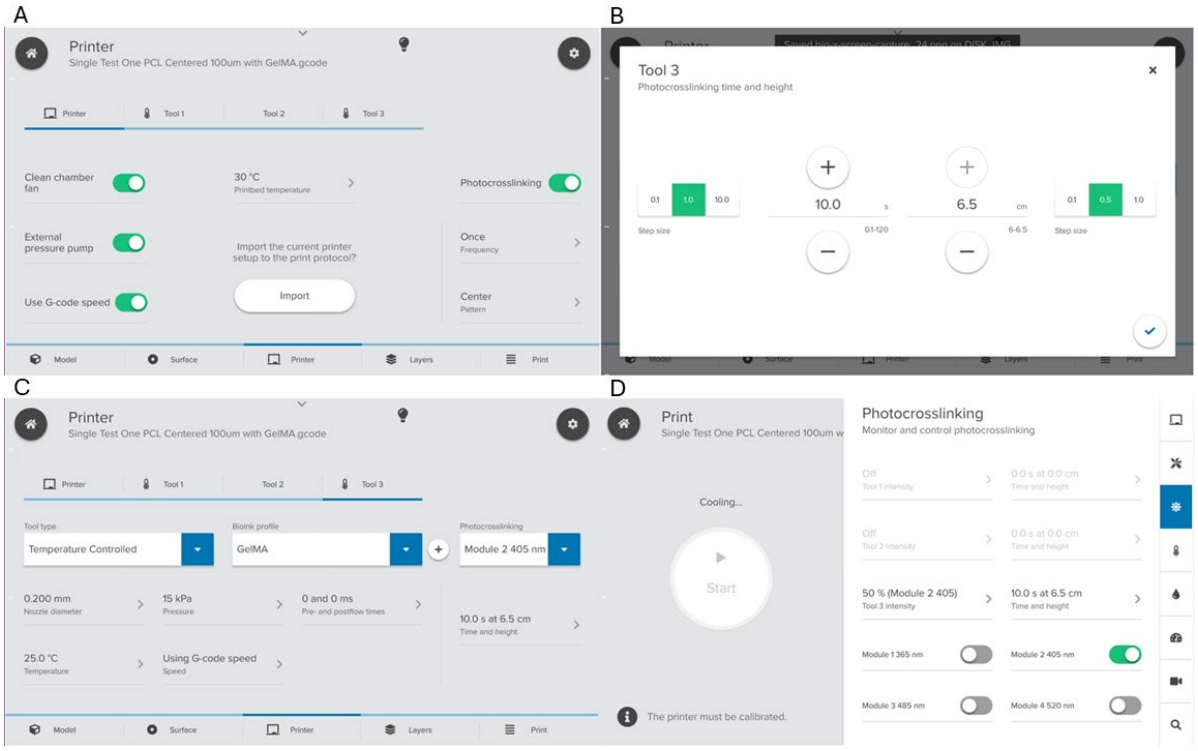


***Figure 12.*** *Configuration of the photocuring protocol for the temperature-controlled printhead (Tool 3). Panel A shows the printer configuration used to enable photocrosslinking; panels B–C show the key settings used to initiate and control the photocrosslinking process on Tool 3; and panel D shows a summary of the photocrosslinking parameters in the Utilities menu before starting the print.*

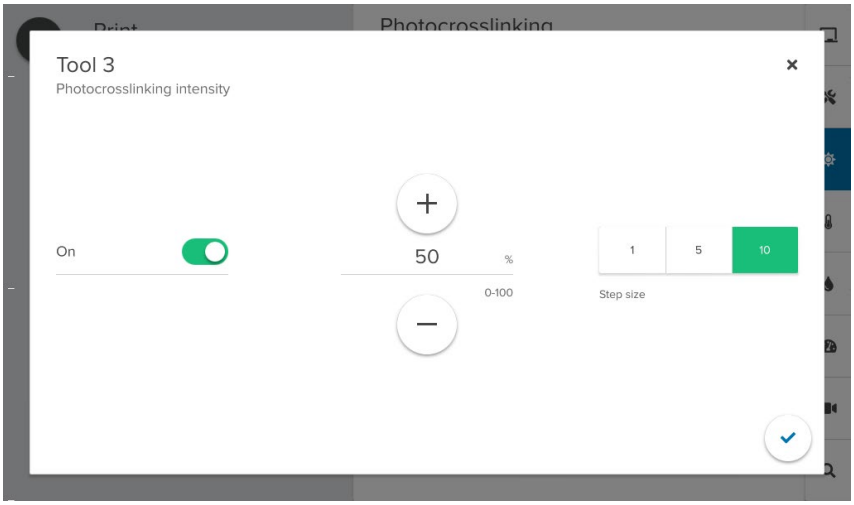


*Figure 13 Manual photocuring: Intensity setting to 50% intensity.*

**Manual photocrosslinking from the Utilities menu (optional workflow)**

If manual curing is preferred after the print is complete, follow these steps.

1. Position the UV source: open utilities → move on the bio x interface. position the photocuring source (photocuring module 2, 405 nm) above the PCL-GelMA scaffold.
2. Set working distance: set the working distance to 6.0 cm, measuring from the scaffold's top surface to the inner edge of the print box light module **(figure 14)**. use a ruler for accuracy.
3. Set intensity: select the photocrosslinking module and set the intensity. for this protocol, use the default intensity of 50%, which is pre-calibrated to deliver approximately 20 mw/cm² at this distance **(figure 13)**.
4. Activate curing: activate the photocuring module for 10 seconds. use a stopwatch to ensure precise timing.
5. Proceed to transfer after curing is complete, proceed with the thermal gelation and transfer steps as described in (steps 4-6).

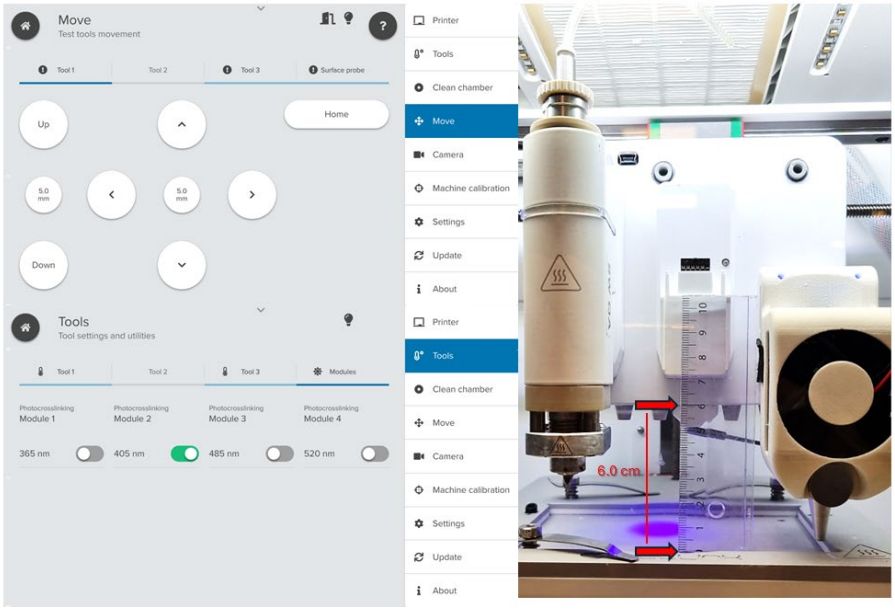


*Figure 14 Manual photocrosslinking setup: Utilities → Move; UV source positioned 6.0 cm above the PCL-GelMA scaffold; Photocuring Module 2 (405 nm) activated for 10 s (~20 mW/cm²).*

**Photocrosslinking using DNA Studio (alternative workflow)**

For users prefer a desktop software workflow, configure the photocrosslinking parameters in DNA Studio before sending the print job to the bioprinter.

1. **Configure parameters in DNA Studio:** In the photocrosslinking panel, define the curing parameters as follows **(Figure 15; Table 2)**:
    - **Light source:** Module (405 nm)
    - **Intensity:** 50%
    - **Height above surface:** 6.5 cm (This minimum height is required to ensure clearance for the thermoplastic printhead).
    - **Time:** 10 s
    - **Curing pattern:** Set to *Cure after complete print* or *after last layer*.
2. **Send the printer and initiate:** Send the G-code to BIO X and begin the print. The printer will execute the pre-configured photocrosslinking step automatically.
3. **Complete the protocol:** After the automated curing is finished, proceed with the thermal gelation and transfer steps as described in (steps 4-6).

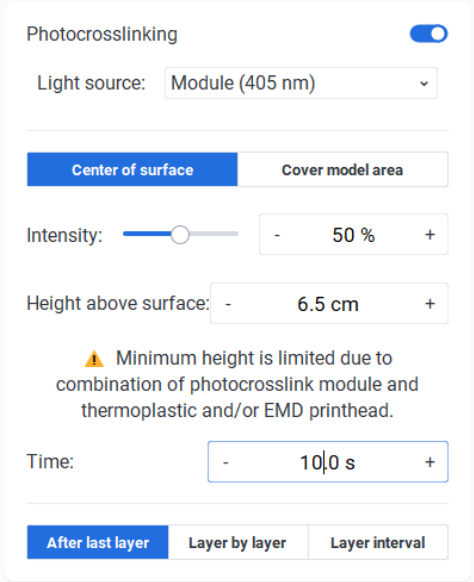


***Figure 15*** *Configuration of photocrosslinking parameters in the DNA Studio Software.*

**Best practice for long-term consistency:** The intensity of the photocrosslinking LED module can decrease with age. To ensure long-term reproducibility, it is recommended to periodically (e.g., every 6 months) measure the power density (mW/cm²) of the 405 nm light source using a suitable power meter. If the output has decreased, adjust the exposure time to deliver the same total energy dose.

# 10. *Procedure completion and cleanup*

Follow these steps after the final photocrosslinking is complete to ensure the equipment is properly shut down, cleaned, and materials are stored correctly. The procedure is ordered to perform hot tasks first, followed by a full system cooldown and final cleaning.

**Immediate post-print procedure**

1. **Purge thermoplastic nozzle (while hot):** To prevent clogging, it is best practice to clean the nozzle while it is still hot. Immediately after finishing the print, purge a small amount of PCL through the nozzle to clear it of any residual material.

**Printer shutdown**

1. **Cool down heaters:** After purging the nozzle, use the BIO X software interface to turn off the heating for all components. Set the target temperatures for the Thermoplastic Printhead (T1), the Temperature Controlled Printhead (T3), and the Print Bed to "OFF" or room temperature.
2. **Wait for cooling:** Allow the printheads and print bed to cool down completely to a safe handling temperature before proceeding. This may take several minutes.
3. **Remove cartridges:** Once cooled, carefully remove the PCL and GelMA cartridges from their respective printheads.
4. **Power off:** Power down the BIO X bioprinter using the main power switch.

**Printhead and workspace cleaning**

1. **Final nozzle and printhead cleaning (when cool):** after the printhead has cooled, carefully wipe any external polymer residue from the nozzle tip using a lint-free cloth. For stubborn residue, the nozzle can be removed and cleaned more thoroughly according to the Thermoplastic Printhead User Manual or section 11.
2. **GelMA cartridge disposal:**
    - Disconnect the used GelMA cartridge from the air pressure adapter.
    - Dispose of the used cartridge and nozzle according to your institution's biological waste procedures if it has been in contact with cells.

**Note:** Do not attempt to clean and reuse disposable cartridges or nozzles that have contained cell-laden bioink, as this poses a substantial risk of cross-contamination.

**Workspace:** Wipe down the print bed and surrounding surfaces with 70% ethanol to maintain a clean environment.

**Material storage**

1. **Unused PCL pellets:**
    - Store the PCL pellets in their original container inside a sealed bag in the fridge at **2-10°C**, as recommended by the manufacturer and Printing Protocol CELLINK PCL.
    - Ensure the container and the bag are tightly sealed to protect the polymer from moisture, which can cause degradation over time.
2. **Unused GelMA Bioink:**
    - Return any unused, unopened GelMA Bioink cartridges to refrigerated storage at **4°C**.
    - Keep the bioink protected from light to prevent premature crosslinking of the LAP photo initiator. The orange, UV-shielded cartridge is designed for this purpose.

**Notes:**
The procedures described in this section cover standard post-print shutdown, basic cleaning, and material storage after routine printing operations.
For first-time use of metal cartridges, material changes, removal of stubborn thermoplastic residues, or situations requiring complete disassembly and deep cleaning of the Thermoplastic Printhead, refer to Section 11: Thermoplastic Printhead and Metal Cartridge Cleaning Procedure.

# 11. *Thermoplastic Printhead and metal cartridge cleaning*

This section expands on the basic post-print cleaning described in Section 10 and provides detailed procedures for advanced cleaning, disassembly, and maintenance of the Thermoplastic Printhead, including metal cartridges, nozzles, and plungers. These procedures apply both before first use and after printing and are essential for removing machining residues, preventing cross-contamination between materials, and ensuring consistent extrusion performance.

**Pre-Use Cleaning of Metal Cartridges (New Components)**

Before first use, metal cartridges must be thoroughly cleaned to remove residues from the machining process (e.g., oils, chemicals, and debris).

1. Soak the cartridge in water.
2. Scrub thoroughly using dishwashing detergent and a suitable brush.
3. Rinse with 70% ethanol.
4. Perform a final rinse using deionized water.
5. Allow the cartridge to dry completely before assembly.

**Printhead Disassembly and Material Removal (While Warm)**

1. Remove the printhead from the printhead mount.
    - Use one hand to secure the top of the printhead mount.
    - With the other hand, slide the printhead upward to avoid damaging the motor of the printhead holder mount.
2. While the printhead is still warm, remove the nozzle using the supplied wrench.
3. Reinstall the printhead into the mount and reheat the Thermoplastic Printhead.
4. Wait approximately **30-60 minutes** to ensure that the leftovers of the thermoplastic granulate is fully melted.
5. Apply air pressure of up to **700 kPa** to extrude residual material until only air is expelled **(Figure 16).**

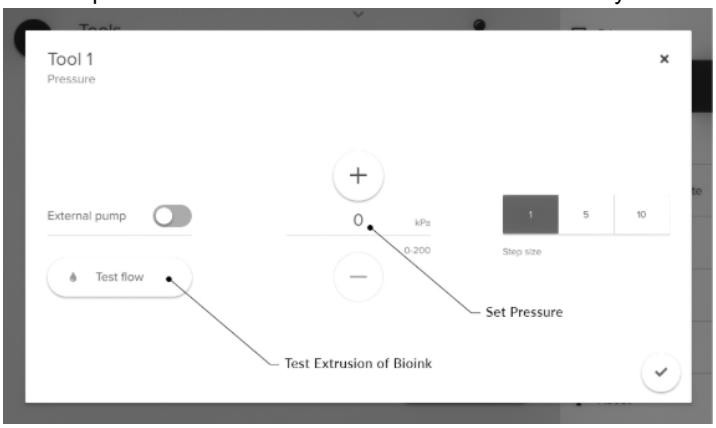


***Figure 16*** *Set pressure and test flow of melted granulated thermoplastic.*

6. Remove the printhead from the mount again.
7. Unscrew the top cap of the cartridge.
8. Insert the plunger retrieval tool gently into the cartridge and locate the threaded hole in the plunger.
9. Rotate the retrieval tool clockwise at least five turns to securely engage the plunger.
10. Firmly pull upward to remove the plunger.

**CAUTION:**

The plunger and residual thermoplastic material may be extremely hot. Allow sufficient cooling before handling directly.

**Cartridge Removal and Cooldown**

1. Disable heating via the **Tools** option in the **Utilities** menu.
2. Wait until the Thermoplastic Printhead has cooled to room temperature.
3. Remove the cartridge once fully cooled.
4. Use the wrench to loosen the cartridge.

**Critical Safety Precautions for Cleaning**

**WARNING: RISK OF CHEMICAL EXPOSURE AND INJURY**

1. Always perform chemical cleaning procedures in a certified fume hood.
2. Always wear appropriate PPE:
    - Chemical-resistant gloves (nitrile for alcohols; butyl for strong solvents such as acetone)
    - Safety goggles
    - Lab coat
3. Consult the Safety Data Sheet (SDS) for each solvent prior to use.
4. Never use flammable solvents (e.g., acetone, IPA) directly in an ultrasonic cleaner unless it is explicitly rated as explosion-proof.
5. Never heat components until all residual solvent has completely evaporated.

**Mechanical Pre-Cleaning (Optional – For Stubborn Residue)**

For heavy internal buildup, mechanical pre-cleaning may be performed prior to chemical cleaning.

**Recommended Tools:**

- Variable-speed drill or rotary tool
- Bottle brush with brass or stiff nylon bristles
  *(Avoid the use steel bristles, as they may damage internal metal cartridge surface)*

**Procedure:**

1. Ensure the cartridge is completely cool.
2. Secure the cartridge in a bench vise with soft jaws.
3. Attach the bottle brush to the drill.
4. Set the drill to low speed (100–300 RPM).
5. Carefully insert and move the brush back and forth inside the cartridge.
6. Wipe out loosened debris with a clean cloth before chemical cleaning.

**Chemical Cleaning (Solvent Soak)**

This is the primary method for dissolving thermoplastic residues.

1. Select a solvent-compatible container (e.g., borosilicate glass).
2. Place the nozzle, cartridge, and plunger into the container.
3. Submerge fully in the appropriate solvent:
    - **PLA:** Acetone
    - **PCL:** Ethyl acetate
      *(Dichloromethane or chloroform are effective but not recommended for routine laboratory use due to safety concerns.)*
4. Seal the container tightly.
5. Allow parts to soak for **8–24 hours**, until residues are fully dissolved.
6. Periodically agitate the container to improve solvent penetration.
7. Remove parts using tweezers and proceed to final rinsing.

**Ultrasonic Deep Cleaning**

Ultrasonic cleaning is recommended for first use or when switching between materials with different additives.

**Indirect Ultrasonic Cleaning (Flammable Solvents)**

1. Fill the ultrasonic bath with water and add a small amount of detergent.
2. Place the solvent (e.g., acetone) and parts into a sealed borosilicate glass beaker.
3. Submerge the beaker in the water bath.
4. Run the ultrasonic cleaner for **15–30 minutes** at **40–50 °C**.

**Direct Ultrasonic Cleaning (Non-Flammable Solutions)**

1. Fill the ultrasonic bath with a non-flammable ultrasonic cleaning solution.
2. Heat to **40–60 °C**.
3. Place parts in a mesh basket and submerge.
4. Run for **15–30 minutes**.

**Final Rinsing, Drying, and Inspection**

1. Rinse all components thoroughly with Isopropyl Alcohol (IPA).
2. Perform a final rinse with deionized water.
3. Dry completely using compressed air or air-drying in a clean environment.
4. Inspect all parts to confirm they are free of residue before storage or reuse.

# Troubleshooting guide

This table provides quick-reference solutions for common issues that may arise during the PCL-GelMA hybrid bioprinting process. Refer to this guide to quickly diagnose and resolve problems during calibration and printing.

***Table 5.*** *Troubleshooting guide.*

| Observed problem | Potential cause(s) | Recommended solution(s) |
|---|---|---|
| **PCL FILAMENT QUALITY** | | |
| **Filament is discontinuous, inconsistent, too thin, or breaks (Under-extrusion).** | 1. **Parameter mismatch:** The extrusion pressure is too low for the set printing speed. Adjust print height is too far from the surface.<br>2. **Dirty/clogged nozzle:** Polymer residue on the nozzle tip is obstructing the flow.<br>3. The set pressure is not guaranteed by the pressure supply. Check and adjust the **compressor manometers** to ensure the delivered pressure matches or exceeds the value set in DNA Studio. | 1. **Real-time adjustment:** While the print is active, access the **Utilities menu**. Increase the pressure in small increments (e.g., 5-10 kPa) or adjust print height until a stable, continuous filament is achieved.<br>2. **Clean nozzle:** Pause the print. Carefully wipe the nozzle tip with steel wool to remove any built-up polymer. If the problem persists, flush the nozzle with fresh PCL from the Utilities menu.<br>3. Clogged Nozzle: If the nozzle is clogged, please refer to the accompanying video demonstration for instructions on how to clear it using a metal wire.<br>**Safety Notice**<br>Thermal Hazard: Use extreme caution when working near the printhead. The nozzle tip reaches high temperatures and can cause severe burns upon contact.<br>PPE: It is recommended to use heat-resistant tools or gloves when performing maintenance near the nozzle. |
| **Filament is significantly wider than the nozzle diameter (Over-extrusion).** | **Parameter mismatch:** The extrusion pressure is too high for the set printing speed. Adjust print height is too close to the surface. | **Real-time adjustment:** While the print is active, access the **Utilities menu**. Decrease the pressure in small increments or either adjust print heigh until the extruded filament width matches the nozzle diameter. |
| **PCL MATERIAL CONDITION** | | |
| **PCL extrudes with a brown or yellowish discoloration.** | **Temperature too high:** The PCL is being heated above its degradation point, causing it to scorch. | **Lower temperature:** Lower the printhead temperature in 5°C increments. If the PCL in the cartridge is already discoloured, replace the cartridge with fresh PCL to avoid printing degraded material. |
| **Extruded PCL filament is brittle and snaps easily when handled, lacking its usual flexibility.** | **Thermal degradation of the polymer:** The PCL's molecular chains have been broken down, severely compromising its mechanical properties. This is typically caused by:<br>1. **Overheating:** The printhead temperature is set too high.<br>2. **Prolonged heating:** The PCL has been kept molten at the printing temperature for an excessive amount of time (e.g., several hours), leading to gradual degradation. | 1. **Immediate action (purge and replace):** Discard the degraded PCL from the cartridge. If the entire cartridge has been heated for a long time, replace it with a fresh cartridge of PCL pellets.<br>2. **Preventative action (parameter adjustment):**<br>• **Verify and reduce temperature:** Confirm that the printhead temperature is set correctly (e.g., 200°C). If the filament is also coloured, this is a strong sign of overheating; lower the temperature in 5°C increments for future prints.<br>• **Minimize idle heating time:** Plan your printing session to avoid leaving the PCL molten in the printhead for extended periods before or between prints. |
| **PRINTED STRUCTURE FIDELITY** | | |
| **The printed structure warps or contracts inwards as it builds multiple layers.** | **Insufficient cooling:** The heat from newly deposited layers is causing lower, still-warm layers to deform. | **Add pause between layers:** Modify the G-code to introduce a cooling pause. At the end of the G-code for each layer, add the command G4 S60. This will pause all activity for 60 seconds, allowing the layer to cool and solidify before the next one is printed. |