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Author: PG, EP, KB and VK. Version: 1

BIO ONE BIOPRINTING PROTOCOL

PhotoCol®

This is a suggested procedure, please adjust it according to your experimental needs. Work under aseptic conditions.

Protocol aim

The aim of this protocol is to provide instructions for dispensing droplets and printing constructs with PhotoCol® at concentrations in 3-6 mg/mL range using the BIO ONE. This document covers PhotoCol® preparation, bioprinting with cells and photocuring (with LAP photoinitiator).

Materials

- PhotoCol® LAP kit (100 mg lyophilizate, 10 mL neutralization solution, 50 mL acetic acid solution and 100 mg LAP photoinitiator)*
- Ice bath
- 1000 µL positive displacement pipette and tips
- 5 mL amber centrifuge tubes
- 1.5 mL conical microcentrifuge tubes
- 0.22 µm sterile syringe filter
- pH paper
- NaOH or HCl for pH adjustments
- Cell suspension and cell culture medium
- 3 mL BD Plastipak™ Syringes with Luer-Lok™ Tip (Ref#309658)
- 15 mL syringe
- Tip caps*
- Female/female Luer lock adaptor*
- Conical bioprinting nozzles, 22-25G*
- BIO ONE 3D bioprinter*
- Photocuring LED aperture
- Well plate or Petri Dish

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

This protocol is optimized for BIO ONE with a single cooled printhead. To minimize nozzle clogging, pre-set the printhead temperature to 2°C before starting. Pre-chill all plastics that will contact the collagen solution and keep all solutions on ice. Ensure the PhotoCol® remains cooled on ice until it is loaded into the pre-cooled BIO ONE printhead. Reconstitute PhotoCol® (Step 1) and prepare the LAP stock solution (Step 2) 1-2 days prior to proceeding with the subsequent steps.

1. Reconstitution of PhotoCol® at 8 mg/mL stock solution

MATERIAL

PhotoCol® lyophilizate (100 mg vial)

20 mM acetic acid solution

Positive displacement pipette and tips

15 mL syringe

Tip caps

DESCRIPTION

- Keep all the material at 2-10°C.
- Add 12.5 mL of 20 mM acetic acid to PhotoCol® vial to achieve a concentration of 8 mg/mL.
- Use a shaker table or rotator plate at 2-10°C overnight or until PhotoCol® is fully solubilized.
- Transfer the PhotoCol® to a 15 mL syringe using a positive displacement pipette (avoiding air bubbles), cap it with a tip cap, and store it in the fridge.

Notes:

- The stock solution reconstituted with acetic acid remains functional for 2 months when stored at 2-10°C, however the crosslinking capacity may slightly decrease with time. To maximize reproducibility of results between experiments, it is always recommended to use freshly reconstituted PhotoCol® from the same production batch.
- The use of a magnetic stirrer can speed up the reconstitution process.

2. Preparation of LAP stock solution (40 mg/mL)

MATERIAL

Photoinitiator LAP (100 mg)

1x PBS

0.22 µm sterile syringe filter

5 mL amber centrifuge tubes

DESCRIPTION

- Add 2.5 mL of 1x PBS to the LAP vial.
- Mix on a shaker table or rotator plate at 2-10°C overnight or until PhotoCol® is fully solubilized.
- Sterile filter the LAP solution using a 0.22 µm syringe filter.
- Store the filtered LAP solution in a 5 mL amber centrifuge tube in the fridge.

3. Photocuring parameter setup

MATERIAL

BIO ONE

Irradiance test report (comes with BIO ONE)

DESCRIPTION

- Chose an irradiance to cure PhotoCol®. We recommend using irradiances between 10 – 40 mW/cm^2.
- Find the height for the desired irradiance on the “irradiance test report”. This value will be used to setup the photocuring protocol.
- Use Table 1 as a reference to determine the time required to reach the dose range at an irradiance of 40 mW/cm² to photocure 200 µL of PhotoCol® under a 405 nm light wavelength, following the "cure after complete print protocol (single droplet)." If you use different irradiances, adjust the doses linearly. For example, using a 20 mW/cm² irradiance, it would take 10 s to achieve the equivalent dose of 40 mW/cm².

Table 1. Exposure times to achieve the relevant 405 nm light doses to cure PhotoCol® at 40 mW/cm^2.

Conditions	**Irradiance at 40 mW/cm^2**			
Photocuring time (s)	2	5	10	20
Dose (mJ/cm^2)	80	200	400	800

Notes:

- “Cure after complete print” refers to the curing option in all individual wells or Petri dish after all printing is complete. “Cure full surface after complete print” refers to the curing option in a line scan pattern over the entire well plate or Petri dish after all the printing is complete. It can be performed for single or droplet array and for models at center of print or covering the whole model. Refer to the BIO ONE manual for all the parameters and combinations.
- Prolonged photocuring can cause cell death; therefore, adjust the photocuring duration based on the construct size. Avoid long exposure time: higher cell death was observed when photocuring for 20 s (~800 mJ/cm^2) compared to 10 s (~400 mJ/cm^2) or lower exposure time in 5 µL PhotoCol® droplets at irradiance of 40 mW/cm^2.
- Photocuring for just 2 s at 40 mW/cm² irradiance (~80 mJ/cm^2) can increase stiffness by approximately 50%, while 20 s increases it close to the plateau of stiffness.

DESCRIPTION (continuation)

To achieve equivalent dose when using the protocol cure after complete print (static) and cure full surface after complete print (dynamic), it is necessary to additionally measure the light beam diameter.

- Create a droplet printing protocol with the photocuring on and select a central well on a 96-well plate.
- Select “cure every print area” and select the height corresponding to the desired irradiance. Also, increase time to 20 s.
- Choose CELLINK START print protocol
- Attach the insulator and put a piece of white paper on top of the printbed.
- Proceed with the print, but do not add a well plate to the printbed.
- Run the autocalibration.
- During the fake print, press stop when it comes to the photocuring and use a ruler to measure the photocuring light beam diameter on the paper.
- Use the **Annex1** parameters for filling in the photocuring section on the software (**step 4**), searching for the corresponding irradiance and beam diameter.

4. Software setup

MATERIAL

BIO ONE 3D bioprinter

DESCRIPTION

Create a droplet or model print tab and navigate through:

1) **Surface**:
- Select the well plate and the wells to be printed in. If printing droplets, define if printing single or droplet arrays.

2) **Printer**:
- Toggle on photocuring and set the pre-photocuring retraction volume to 10 µL.
- The parameter range for each photocuring protocols are displayed in Table 2 for droplets and Table 3 for models.
- Adjust the parameters according to the selected irradiance and protocol, described on step 3.

Notes:

- For the protocol “cure after complete print”, the photocuring beam overlaps occurs in wells when the cone size exceeds the well plate diameter at a safe height (5-8 cm). This effect is pronounced in well plates with more than 48 wells. To ensure uniform exposure energy across the entire plate, avoid using the droplets printed at the edges of 384- (2 rows/lines), and 96-well plates (1 row/line).
- For the protocol “cure full surface after complete print” it is recommended to avoid using the droplets printed at AB and OP rows of 384-well plates.

Table 2. Recommended settings in DNA Studio Core for photocuring droplets or droplet arrays into well plates.

Parameters	Photocuring protocol		
	Cure after complete print (single droplet)	**Cure after complete print (droplet array)**	**Cure full surface after complete print (single droplet or droplet array)**
LED height (cm)	5-8	5-8	5-8
Time (s)	1-20	-	-
Speed (mm/s)	-	1-10	1-10
Curing cycles	-	1-10	1-10
Curing lines	-	-	4-7

Table 3. Recommended settings in DNA Studio Core for photocuring constructs into well plates or Petri dishes.

Parameters	Photocuring protocol		
	Cure after complete print (center of print)	**Cure after complete print (cover whole model)**	**Cure full surface after complete print**
LED height (cm)	5-8	5-8	5-8
Time (s)	1-20	-	-
Speed (mm/s)	-	1-10	1-10
Curing cycles	-	1-10	1-10
Curing lines	-	4-7	4-7

3) **Printhead**:
- Create a new bioink profile for PhotoCol® with the settings displayed in Table 4 and 5 for droplets and models respectively.

4) **Print page**:
- Ensure steps 5, 6, and 7 are completed before proceeding further.

Table 4. Recommended settings in DNA Studio Core used for dispensing 5 µL PhotoCol® droplets at 3, 4 and 6 mg/mL through a 22G nozzle in a 96-well plate using the Droplet Print function on BIO ONE. Do not heat the printbed while printing to minimize evaporation.

Parameters	3 mg/mL	4 mg/mL	6 mg/mL
Well plate	96-well plate	96-well plate	96-well plate
Printbed temperature	Disabled	Disabled	Disabled
Printhead temperature	2°C	2°C	2°C
Extrusion rate	20 µL/s	20 µL/s	20 µL/s
Extrusion volume	10 µL	10 µL	8 µL
Retract volume	5 µL	5 µL	3 µL
Z-offset	0.7 mm	0.7 mm	0.7 mm
Extra preflow volume	0 µL	0 µL	0 µL
Retract rate	20 µL/s	20 µL/s	20 µL/s
Postflow stop time	0.5 s	0.5 s	0.7 s
Z-lift between wells	30.0 mm	30.0 mm	30.0 mm

Table 5. Recommended settings in DNA Studio Core used for printing single layered structures using PhotoCol® at 3, 4 and 6 mg/mL.

Parameters	3 mg/mL	4 mg/mL	6 mg/mL
Well plate	6-well plate	6-well plate	6-well plate
Printbed temperature	25°C	25°C	25°C
Nozzle	0.25 mm (25G)	0.25 mm (25G)	0.25 mm (25G)
Speed	7 mm/s	10 mm/s	10 mm/s
Printhead temperature	2°C	2°C	2°C
Preflow volume	3.5 µL	3.5 µL	3.5 µL
Extrusion rate	0.7 µL/s	1.0 µL/s	1.2 µL/s
Retract volume	3.2 µL	3.2 µL	3.2 µL
Z-offset	0.1 mm	0.1 mm	0.1 mm
Extra preflow volume	1.7 µL	2.5 µL	2.7 µL
Infill extrusion multiplier	100%	100%	100%
Retract rate	5.0 µL/s	5.0 µL/s	5.0 µL/s
Extra retract	0 µL	0 µL	0 µL
Postflow stop time	0.5 s	0.5 s	0.3 s
Z-lift	2.0 mm	2.0 mm	2.0 mm

Notes:

- PhotoCol® at 3-6 mg/mL has low viscosity allowing printing of droplets and single-layered constructs only.
- There is a risk of dehydration when printing in multi-well plates due to longer printing time. Maintaining the printbed at 25°C enhances fidelity but also increases dehydration.

5. PhotoCol® neutralization and mix with LAP

MATERIAL

PhotoCol® 8 mg/mL stock solution

Neutralization Solution (NS)

LAP stock solution (40 mg/mL)

pH paper

HCl for pH adjustments

Positive displacement pipette and tips

Ice bath

3 mL BD syringes with Luer lock connections

Tip caps

Microcentrifuge tubes

DESCRIPTION

Keep all the material at 2-10°C and avoid light exposure

- Define the desired final concentration for the PhotoCol® bioink (See Table 6 for suggested concentrations). Adjust the calculations if using different total volumes.
- Transfer the PhotoCol®, NS and LAP to the microcentrifuge tube according to the volumes in Table 5, keeping it in an ice bath.
- Mix gently via pipetting up and down using a cool positive displacement pipette.
- Check if the pH of the mixture is in the 7.0- 7.5 range using the pH paper.

Note: adjust the pH if needed by adding more NS or HCl.

- After neutralization, transfer the solution using the positive displacement pipette to a cool 3 mL BD syringe. Keep it on ice and begin the next step immediately.

Note: If the neutralization solution is chilled for too long, the salts can precipitate. Keep the solution at room temperature until it becomes homogeneous again.

Table 6. Preparation of PhotoCol® at different concentrations.

Final PhotoCol® concentration	**3 mg/mL**	**4 mg/mL**	**6 mg/mL**
Total volume (µL)	1000	1000	1000
PhotoCol® 8 mg/mL (µL)	375	500	750
Neutralization solution (µL)	30	40	60
LAP 40 mg/mL (µL)	62.5	62.5	62.5
Cell suspension (µL)	532.5	397.5	127.5

Note: The final concentration of LAP in the bioink is 0.25%.

6. Mixing PhotoCol® with the cells

MATERIAL

Neutralized PhotoCol® solution

Cell suspension or cell medium

3 mL BD syringes with Luer lock connections

Female/female Luer lock adaptor

Ice bath

DESCRIPTION

- Keep all the material at 2-10°C and avoid direct light exposure.
- Dilute the cell suspension in cell medium according to the volume required for each PhotoCol® final concentration according to Table 4 and keep it on ice.

Note: If not mixing with cells, use cell medium according to Table 4 and keep it on ice.

- Mix the PhotoCol® solution with cell suspension or cell medium according to Table 4, taking care not to introduce air bubbles to the mixture.
- In brief, prepare a cell suspension with the desired number of cells. It is recommended to connect two cooled 3 mL syringes with the Luer lock and divide the PhotoCol® between the two syringes. Disconnect the two syringes and pipette the cell suspension into one of the syringes very gently while pulling on the plunger to create room for the cell suspension in the syringe. Remove any air introduced into the syringe

and connect the two syringes again with the Luer lock. Gently mix back and forth between the syringes until the mixture is homogeneous. If detecting any air bubbles during mixing, disconnect the syringes and evacuate the air. Mix until homogeneous. The number of mixing cycles will depend on the cell type and bioink volume. Work fast and avoid heating the syringes with your hands to prevent self-assembly of the PhotoCol® bioink.

Note: The maximum filling level of the 3 mL syringes is 2.7 mL.

7. Preparation for bioprinting

MATERIAL

PhotoCol® bioink in 3 mL BD syringe

Conical bioprinting nozzles, 22-25G recommended

DESCRIPTION

- Cap the syringe with a bioprinting nozzle of choice, 22-25G recommended.
- Attach the thermal insulator to the cooling block, by inserting it from below and rotating counterclockwise.
- Place the syringe into the pre-cooled printhead pre-set to 2°C. Rotate the syringe plunger holder arm over the plunger and twist the syringe by the tabs counterclockwise to ensure it is locked in place.

8. Calibration and nozzle priming

MATERIAL

BIO ONE 3D bioprinter

PhotoCol® bioink in 3 mL BD syringe

Well plate

DESCRIPTION

- Place a well plate on the printbed and perform automatic calibration.
- Right before each print, prime the nozzle by extruding a couple of drops. If any material has gelled at the tip of the nozzle, ensure it is fully extruded prior to starting a print.

Notes:

- Perform calibration each time a new syringe is placed in the printhead or if the well plates are replaced.
- Before starting the print, test the flow of the bioink using the Test extrude button with the recommended starting parameters in Table 2 or 3.
- If the system has been idle for an extended period, the PhotoCol® in the nozzle can dry or gel causing it to clog. If this occurs, purge the nozzle by extruding 10 to 50 µL of the PhotoCol®, or until the gelated part is extruded. If the clog cannot be removed, replace it with a new nozzle. Always ensure the nozzle is fully primed with PhotoCol® prior to printing. Cells may sediment in the collagen if idle for extended periods. Remove the syringe from the printhead and flip back and forth a few times. Place back in the printhead and repeat Step 7.

9. Printing

MATERIAL

BIO ONE 3D bioprinter

Well plate

DESCRIPTION

- Dispense droplets or print single layer structures with parameters according to Table 2 or Table 3, in a well plate of choice.
- After printing the last droplet or construct, pause the process before starting the photocuring.

Notes:

- The values are only a reference of starting parameters. The actual values needed to print will vary depending on the preparation procedures, including cell density, cell medium composition, etc. Refer to Parameter Guidelines & Print Troubleshooting - MyCELLINK - Knowledge Center for adjusting printing parameters and improving print quality.
- If printing does not begin right away, it is most likely because the printhead and/or printbed have not yet reached the temperature set-point.

10. Crosslinking

MATERIAL

BIO ONE 3D bioprinter

Photocuring LED aperture

Well plate with constructs

DESCRIPTION

- PhotoCol® is initially crosslinked through thermal crosslinking: on the print page, toggle the printbed temperature to 37°C and maintain it for approximately 10-20 minutes, depending on the droplet or construct size.
- Put the lid back on the well plate during thermal crosslinking to avoid droplet or construct dehydration.
- Periodically verify sufficient crosslinking by assessing opacity or testing resistance to pressure with a pipette tip.
- After thermal crosslinking, press the pause button again to resume printing and proceed with photocuring as outlined in Table 1. See **Figure 1** for the comparative stiffness of collagen constructs at different concentrations with and without photocrosslinking.
- After photocuring, add the desired medium to the constructs and place it in incubator.
- Incubate the constructs in cell culture medium in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.

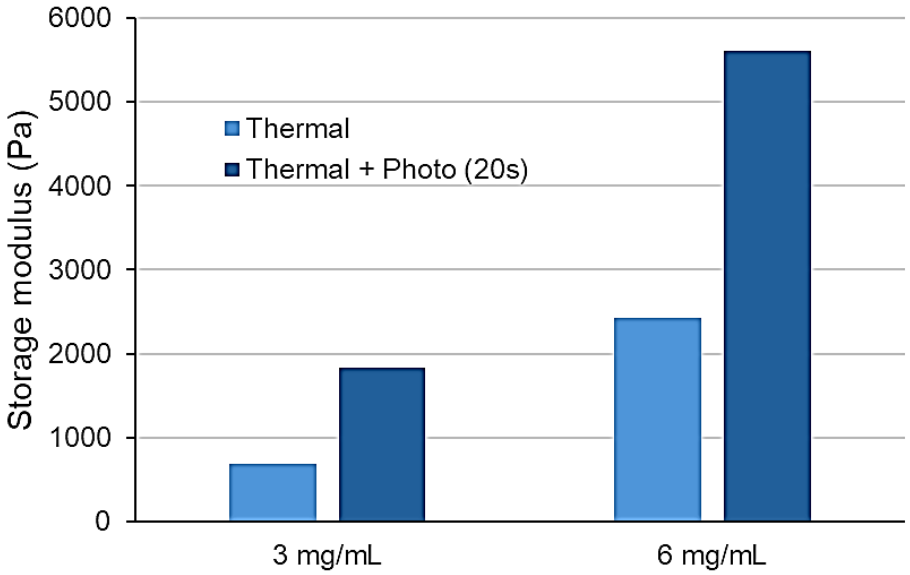

Figure 1. PhotoCol® stiffness at different concentrations comparing only thermal crosslinking with additional photocrosslinking. Oscillation rheology tests were performed on a Discovery Hybrid Rheometer, HR-10, TA instruments, and 20 mm plate geometry. Thermal crosslinking was done at 37°C for 5 min, followed by in situ photocrosslinking with exposure at 405 nm (0.25% LAP as a PI), for 20 s and at 40 mW/cm^2 irradiance.

Notes:

- Verify that the photocuring LED aperture is installed properly
- If printing multiple well plates, divide the process into two tabs: one for printing and one for photocuring. On the first tab, toggle off the photocopying and complete the print. Then, move the well plate to an incubator and proceed with printing the second plate, and so on. After thermal crosslinking is performed on all plates, place them back on the BIO ONE, open the second tab, enable the photocuring option, and adjust settings according to Table 1. To prevent the system from touching the current droplets or constructs, remove the syringe. Navigate to the Printhead tab, increase the extrusion rate to a maximum of 100 µL/s, set the extrusion volume to 1, and retract to 0. Ensure the plunger is raised before starting the process. This bioink profile can be saved as a protocol tailored for two-step photocuring. Continue printing until photocuring is complete and move to the next plate until all plates are photocured. Add medium and incubate.

Annex1. Parameter setting to achieve matching doses among the “cure full surface after complete print” (static) and “cure after complete print” (dynamic) protocols at 40 mW/cm^2 (A) and 12 mW/cm^2 (B).

A) Irradiance (40 mW/cm^2)						
Cure full surface after complete print					**Cure after complete print**	
Beam diameter (mm)	Speed (mm/s)	No of Cycles	Curing lines	Dose (mJ/cm^2)	Time (s)	Dose (mJ/cm^2)
17	1.02	1	6	797.0	20	800
	2.03	1		400.5	10	400
	4.06	1		200.2	5	200
	10	1		81.3	2	80
18	2	2	5	799.9	20	800
	2	1		399.9	10	400
	4	1		200.0	5	200
	10	1		80.0	2	80
19	1.78	2	4	799.2	20	800
	1.78	1		399.6	10	400
	3.56	1		199.8	5	200
	8.89	1		80.0	2	80
20	1.02	1	4	798.9	20	800
	2.04	1		399.5	10	400
	4.07	1		200.2	5	200
	10	1		81.5	2	80
21	1.13	1	4	803.2	20	800
	2.27	1		399.8	10	400
	4.54	1		199.9	5	200
	10	1		90.8	2	80
22	1.77	1	7	800.8	20	800
	3.54	1		400.4	10	400
	7.09	1		199.9	5	200
	10	1		141.7	4	80
23	2.02	1	7	799.2	20	800
	4.04	1		399.6	10	400
	8.07	1		200.0	5	200
	10	1		161.4	4	80

B) Irradiance (12 mW/cm^2)						
Cure full surface after complete print					**Cure after complete print**	
Diameter (mm)	Speed (mm/s)	No of Cycles	Curing lines	Dose (mJ/cm^2)	Time (s)	Dose (mJ/cm^2)
27	1.83	2	7	718.6	60	720.0
	1.66	1		396.1	33	396.0
	3.22	1		204.2	17	204.0
	7.83	1		84.0	7	84.0
28	1.92	2	6	661.0	55	660.0
	1.6	1		396.6	33	396.0
	3.11	1		204.0	17	204.0
	7.55	1		84.0	7	84.0

29	1.88	2	6	720.4	60	720.0
	1.71	1		396.0	33	396.0
	3.32	1		204.0	17	204.0
	8.06	1		84.0	7	84.0
30	1.04	1	5	585.8	49	588.0
	1.54	1		395.6	33	396.0
	2.99	1		203.7	17	204.0
	7.25	1		84.0	7	84.0
31	1.13	1	5	586.5	49	588.0
	1.67	1		396.8	33	396.0
	3.25	1		203.9	17	204.0
	7.89	1		84.0	7	84.0
32	1.21	1	5	587.1	49	588.0
	1.79	1		396.9	33	396.0
	3.48	1		204.2	17	204.0
	8.46	1		84.0	7	84.0
33	1.28	1	5	589.7	49	588.0
	1.91	1		395.2	33	396.0
	3.7	1		204.0	17	204.0
	8.99	1		84.0	7	84.0