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BIO ONE BIOPRINTING PROTOCOL

PhotoAlginate®

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 PhotoAlginate® at 20 mg/mL (2% w/v) concentration using the BIO ONE. This document covers PhotoAlginate® preparation, bioprinting with cells and photocuring (with LAP photoinitiator).

Materials

- PhotoAlginate® with LAP kit (200 mg lyophilizate and 100 mg LAP photoinitiator)*
- 1X Phosphate buffer saline (PBS), no calcium.
- $CaCl_2$ Crosslinking Agent (optional)*
- 1000 µL positive displacement pipette and tips
- 5 mL amber centrifuge tubes
- 1.5 mL conical microcentrifuge tubes
- 0.22 µm sterile syringe filter
- 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 use with BIO ONE, with printhead and print bed maintained at room temperature, i.e. 20-25°C. Reconstitute PhotoAlginate® (Step 1) and prepare the LAP stock solution (Step 2) 1-2 days in advance of the subsequent steps.

1. Reconstitution of PhotoAlginate® at 5% stock solution

MATERIAL

PhotoAlginate® lyophilizate (200 mg vial)

1X cold PBS, no calcium

Positive displacement pipette and tips

15 mL syringe

Tip caps

DESCRIPTION

- Add 10 mL of 1X cold PBS to PhotoAlginate® vial to achieve a concentration of 5%.
- Use a shaker table or rotator plate at 2-10°C overnight or until PhotoAlginate® is fully solubilized.
- Transfer the PhotoAlginate® 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 is functional for 1 month 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 PhotoAlginate® 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, no calcium

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 LAP 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 3D bioprinter

Irradiance test report (comes with BIO ONE)

DESCRIPTION

- Select an irradiance level for curing PhotoAlginate®. We recommend using irradiances in 10–40 mW/cm^2 range.

- Determine the corresponding height for the selected irradiance using the irradiance test report. This value will be used to configure the photocuring protocol.
- Refer to **Figure 1** to understand the relationship between photocuring duration and construct stiffness. The data are based on oscillatory rheology tests performed on 200 µL of 2% PhotoAlginate® with 0.5% LAP, exposed to 405 nm light at 40 mW/cm². These results are comparable to the static exposure conditions using the BIO ONE photocuring protocols: "cure every print area" and "cure after complete print".
- Refer to **Table 1** for the calculated dose values (mJ/cm²) corresponding to different photocuring exposure times.

Table 1. Exposure times to achieve the relevant 405 nm light doses to cure PhotoAlginate® at 40 mW/cm².

Conditions	**Irradiance at 40 mW/cm²**			
Photocuring time (s)	10	15	20	30
Dose (mJ/cm²)	400	600	800	1200

DESCRIPTION (continuation)

To achieve an equivalent dose to the static photocuring protocols ("cure after complete print" or "cure every print area") when using the dynamic protocol ("cure full surface after complete print"), you must also measure the light beam diameter. Follow the instructions provided to accurately determine this value, which is essential for dose calculation:

- 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 exposure 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.

Notes:

- In the "Cure every print area" the photocuring is performed in each well individually before printing begins in the next well; "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.
- If you use different irradiances, adjust the doses linearly. For instance, if the irradiance is 20 mW/cm², it will take twice as long to deliver the same dose as at 40 mW/cm².
- Prolonged photocuring can cause cell death; therefore, adjust the photocuring duration based on the construct size. High mesenchymal stem cell viability (~90%) was observed when photocuring exposure time was set up to 30 s (~1200 mJ/cm²) in 5 µL PhotoAlginate® droplets at irradiance of 40 mW/cm². For detailed viability data, refer to: https://cellink.sharepoint.com/:w:/s/cellinkbioink/ET5aAsvuyvVKmI9FcYdpv30BptJeuxHX-RzGQRI9rAkYgw?e=cRFtAt.
- Extended photocuring times may prolong the printing process, which can negatively affect small droplets in high-throughput setups by increasing the risk of dehydration
- Photocuring 5 µL droplets for 10 s at 40 mW/cm² typically forms only a thin shell rather than fully gelling the entire droplet. For consistent gelation, it is recommended to use doses in the range of 600–1200 mJ/cm².

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 models.
- Adjust the parameters according to the selected irradiance and protocol, described on step 3.

Notes:

- For the protocol “cure every print area” or “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	**Print and photocuring settings**			
	Print protocol: • Droplets (single) • Models (center of the print) **Photocuring protocol:** • Cure every print area • Cure after complete print	**Print protocol:** • Droplets (array) **Photocuring protocol:** • Cure every print area • Cure after complete print	**Print protocol:** • Model (cover whole model) **Photocuring protocol:** • Cure every print area • Cure after complete print	**Print protocol:** • Droplets (single and arrays) • Models **Photocuring protocol:** • Cure full surface after complete print
LED height (cm)	5-8	5-8	5-8	5-8
Time (s)	10-30	-	-	-
Speed (mm/s)	-	1-10	1-10	1-10
Curing cycles	-	1-10	1-10	1-10
Curing lines	-	-	4-7	4-7

3) **Printhead**:
- Create a new bioink profile for PhotoAlginate® using the settings provided in **Table 3** and **4,** corresponding to droplet and model printing respectively.

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

Table 3. Recommended settings in DNA Studio Core used for dispensing 5 µL PhotoAlginate® droplets at 20 mg/mL through a 22G nozzle in a 96-well plate using the Droplet Print function on BIO ONE.

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

Table 4. Recommended settings in DNA Studio Core used for printing single layered structures using PhotoAlginate® at 20 mg/mL

Parameters	20 mg/mL (2%)
Well plate	6-well plate
Printbed temperature	Disabled
Nozzle	0.25 mm (25G)
Speed	10 mm/s
Printhead temperature	Disabled
Preflow volume	3.5 µL
Extrusion rate	1.0 µL/s
Retract volume	3.2 µL
Z-offset	0.1 mm
Extra preflow volume	2.5 µL
Infill extrusion multiplier	100%
Retract rate	5.0 µL/s
Extra retract	0 µL
Postflow stop time	0.5 s
Z-lift	2.0 mm

Notes:

PhotoAlginate® at 20 mg/mL (2%) has low viscosity allowing printing of droplets and single-layered constructs only.

5. PhotoAlginate® dilution and mix with LAP

MATERIAL

PhotoAlginate® 5% stock solution

PBS, no calcium

LAP stock solution (40 mg/mL)

Positive displacement pipette and tips

3 mL BD syringes with Luer lock connections

Tip caps

Microcentrifuge tubes

DESCRIPTION

- Transfer the specified volumes of PhotoAlginate®, PBS, and LAP into a microcentrifuge tube as outlined in **Table 5**.

- Mix gently via pipetting up and down using a positive displacement pipette.
- Transfer the solution using the positive displacement pipette to a 3 mL BD syringe.

Notes:

- Adjust the calculations if using different total volumes. The maximum filling level of the 3 mL syringes is 2.7 mL.
- Avoid using PhotoAlginate® final concentrations below 2% or LAP below 0.5%, as lower concentrations require significantly longer photocuring times which may affect the cell viability.
- Avoid light exposure when preparing and transferring the PhotoAlginate® to the syringes.

6. Mixing PhotoAlginate® with the cells

MATERIAL

PhotoAlginate® solution (prepared on step 5)

Cell suspension or cell medium

3 mL BD syringes with Luer lock connections

Female/female Luer lock adaptor

DESCRIPTION

- Dilute the cell suspension in cell medium according to the volume described on Table 6.

Note: If not mixing with cells, use cell medium or PBS according to **Table 5**.

- Mix the PhotoAlginate® solution with cell suspension or cell medium according to Table 6 volumes, 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 3 mL syringes with the Luer lock and divide the PhotoAlginate® 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.

Table 5. Preparation of PhotoAlginate® bioink at 2% with LAP at 0.5%.

Final PhotoAlginate® concentration	**20 mg/mL (2%)**
Total volume (µL)	1000
PhotoAlginate® 5% (µL)	400
LAP 40 mg/mL (µL)	125
Cell suspension (µL)	475

7. Preparation for bioprinting

MATERIAL

PhotoAlginate® bioink, prepared on step 6, 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 printhead. Rotate the plunger holder arm over the syringe plunger, then twist the syringe counterclockwise using the tabs to lock it securely in place.

8. Calibration and nozzle priming

MATERIAL

BIO ONE 3D bioprinter

PhotoAlginate® bioink in 3 mL BD syringe

Well plate or Petri dish

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 3** or **4**.
- If the system has been idle for an extended period, the PhotoAlginate® 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 PhotoAlginate®, 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 PhotoAlginate® prior to printing. Cells may sediment in the PhotoAlginate® if idle for extended periods. Remove the syringe from the printhead and flip back and forth a few times. Place it back into the printhead and repeat Step 7.

9. Printing and crosslinking

MATERIAL

BIO ONE 3D bioprinter

PhotoAlginate® bioink in 3 mL BD syringe

Photocuring LED aperture

$CaCl_2$ Crosslinking Agent (optional)

Well plate or Petri dish

DESCRIPTION

- Dispense droplets or print single layer structures with parameters according to **Table 3** or **Table 4**, in a well plate of choice.
- After printing, the photocuring will start as outlined on step 3 and 4.
- After photocuring, verify if sufficient crosslinking was achieved by testing resistance to pressure with a pipette tip. See **Figure 1** for the comparative stiffness of PhotoAlginate® constructs at different photocrosslinking conditions.
- Optional: additional crosslinking with $CaCl_2$ based crosslinking agent may be performed to add extra stiffness. Submerge the cell-laden constructs in the Crosslinking Agent for 30 seconds to 5 minutes depending on construct size, infill density and desired construct stiffness. Remove the Crosslinking Agent and rinse constructs with basal culture media once.

- 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.

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.
- Ionic crosslinking with $CaCl_2$ alone is insufficient to fully polymerize the construct.
- Verify that the photocuring LED aperture is installed properly.

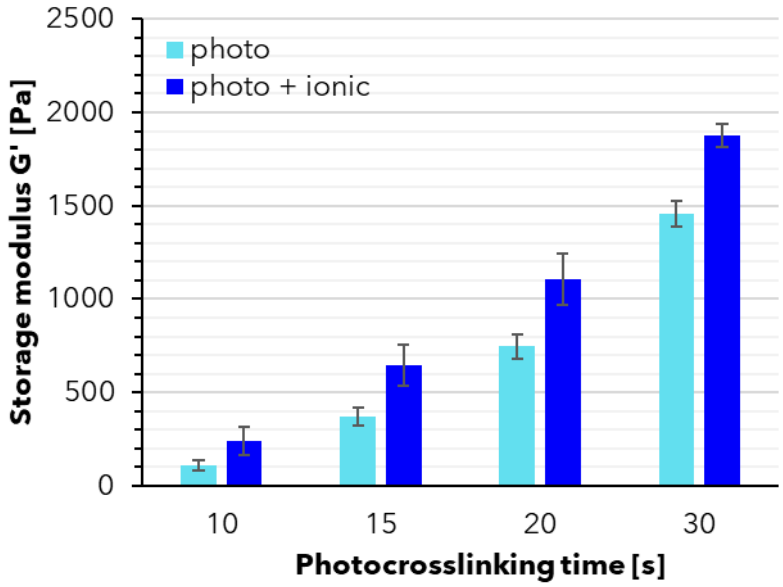

Figure 1. PhotoAlginate® stiffness dependance on photocrosslinking time, including comparison between photocrosslinking only and with additional ionic crosslinking. Oscillation rheology tests were performed on a Discovery Hybrid Rheometer, HR-10, TA instruments, and 20 mm plate geometry. In situ photocrosslinking was performed with exposure at 405 nm (0.5% LAP as a PI), for 10-30 s and at 40 mW/cm^2 irradiance, followed by incubation with $CaCl_2$ for 5 minutes.

Annex1. Parameter setting to achieve matching doses among the “cure every print area” and “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) irradiance.

A) Irradiance (40 mW/cm^2)						
Cure full surface after complete print					**• Cure every print area • 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	1.35	2	6	1204.4	30	1200
	1.02	1		797.0	20	800
	1.35	1		602.2	15	600
	2.03	1		400.5	10	400
18	1.33	2	5	601.4	30	1200
	2	2		799.9	20	800
	1.33	1		601.4	15	600
	2	1		399.9	10	400
19	1.18	2	4	1205.6	30	1200
	1.78	2		799.2	20	800
	1.18	1		602.8	15	600
	1.78	1		399.6	10	400
20	1.36	2	4	1198.4	30	1200
	1.02	1		798.9	20	800
	1.36	1		599.2	15	600
	2.04	1		399.5	10	400
21	1.51	2	4	1202.1	30	1200
	1.13	1		803.2	20	800
	1.51	1		601.1	15	600
	2.27	1		399.8	10	400
22	1.65	2	7	1204.4	30	1200
	1.77	1		800.8	20	800
	1.65	1		602.2	15	600
	3.54	1		400.4	10	400
23	1.79	2	7	1201.4	30	1200
	2.02	1		799.2	20	800
	1.79	1		600.7	15	600
	4.04	1		399.6	10	400
B) Irradiance (12 mW/cm^2)						
Cure full surface after complete print					**• Cure every print area • 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	1.09	2	7	1206.4	100	1200
	1.64	2		801.8	66.7	800
	1.09	1		603.2	50	600
	1.64	1		400.9	33.3	400
28	1.05	2	6	1197.3	100	1200
	1.59	2		798.2	66.7	800

	1.05	1		598.6	50	600
	1.59	1		399.1	33.3	400
29	1.13	2	6	1198.5	100	1200
	1.69	2		801.4	66.7	800
	1.13	1		599.3	50	600
	1.69	1		400.7	33.3	400
30	1.02	2	5	1194.5	100	1200
	1.52	2		801.6	66.7	800
	1.02	2		597.3	50	600
	1.52	1		400.8	33.3	400
31	1.1	2	5	1205	100	1200
	1.66	2		798.5	66.7	800
	1.1	1		602.5	50	600
	1.66	1		399.2	33.3	400
32	1.18	2	5	1204.1	100	1200
	1.78	2		798.3	66.7	800
	1.18	1		602.1	50	600
	1.78	1		399.1	33.3	400
33	1.26	2	5	1198.1	100	1200
	1.89	2		798.7	66.7	800
	1.26	1		599.1	50	600
	1.89	1		399.4	33.3	400