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FRESH BIOPRINTING PROTOCOL

PhotoCol

This is a suggested procedure, please adjust it 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 the bioprinting of complex 3D structures with PhotoCol® (Methacrylated Type I Collagen from Advanced BioMatrix) using FRESH printing method. It covers the steps of pre-print procedures, printing, and post-print crosslinking. Changing the parameters in the protocol might change printing conditions such as pressure and speed. This protocol was optimized for the pneumatic Temperature-controlled printhead using the BIO X.

Materials needed

- PhotoCol®* (Kit 2,3,4* also includes 20 mM acetic acid, Neutralization Solution and a photoinitiator)
- LifeSupport™*
- 1X PBS
- Well plate or Petri dish
- Temperature-controlled printhead*
- BIO X*, BIO X6* 3D bioprinter
- Sterile filter, 0.22 µm
- Eppendorf tube 1.5 mL
- Positive displacement pipette
- Cell suspension in cell culture medium
- Cartridge, 3cc
- 22G Conical bioprinting needle (1-inch length)

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

Protocol

This protocol is adjusted for printing scaffolds at the final PhotoCol® concentration of 6 mg/mL. For other concentrations, recalculations need to be made but the same protocol can be followed. To avoid premature collagen self-assembly, we recommend keeping the collagen, all reagents (including cell culture medium) and all consumables in the fridge prior to printing. Once compacted, LifeSupport™ should be kept in a fridge at 4°C and used within 12 hours. The ambient temperature should not exceed 23°C during handling or printing.

1. Preparing PhotoCol® 10 mg/mL stock solution

MATERIAL

PhotoCol® (lyophilized methacrylated Type I collagen)

20 mM acetic acid

DESCRIPTION

- In a separate container, weigh the desired mass of PhotoCol® for stock solution preparation. Store the remaining PhotoCol® material in a fridge.
- Add the corresponding volume of 20 mM acetic acid to the PhotoCol® container to achieve the target concentration of 10 mg/mL.

Note. For example, to prepare 3 mL of 10 mg/mL stock solution, add 3 mL of 20 mM acetic acid to 30 mg of lyophilized Photocol®.

- Mix on a shaker table or rotator plate at 2-10°C overnight or until fully solubilized.

2. Preparing LifeSupport™ bath

MATERIAL

LifeSupport™

1X PBS

Well plate or Petri dish

DESCRIPTION

- Add 40 mL of cold 1X PBS (4°C) to LifeSupport™ tube (sterile powder).

Note: 2 g of sterile LifeSupport™ corresponding to 15 mL of LifeSupport™ bath. For detailed directions, visit https://www.cellink.com/wp-content/uploads/2022/03/FluidformDirectionsforUseVersion6-rev-Sep-2021.pdf.

- Vortex for 1 minute.
- Put the tube into the fridge (4°C) for 15 minutes.
- Centrifuge for 5 minutes at 400 *g*.
- Gently pour off or aspirate the liquid supernatant.
- Grab the tube by the cap, hold it horizontally and gently tap it against a palm 15 times.
- Shake the tube containing dislodged LifeSupport™ vigorously for 10 seconds. Shake along the length of the tube.
- Centrifuge for further 5 minutes at 400 *g*.
- The LifeSupport™ should now be compacted at the bottom of the centrifuge tube. Gently pour off or aspirate any remaining liquid supernatant to leave only the compacted LifeSupport™ in the bottom of the tube.
- Transfer the resulting LifeSupport™ bath with a sterile spatula into well plates or Petri dish and store it in a fridge until use.

3. Preparing for printing

MATERIAL

Temperature-controlled printhead

BIO X or BIO X6 bioprinter

Photoinitiator (PI)

Neutralization Solution (NS)

Sterile filter, 0.22 µm

PhotoCol® 10 mg/mL stock solution

Eppendorf tube 1.5 mL

Positive displacement pipette

Cell suspension in cell culture medium of choice

Cartridge, 3cc

22G Conical bioprinting needle (1-inch length)

DESCRIPTION

- Place the Temperature-controlled printhead in the fridge at least 30 minutes before printing.
- Prepare the cell suspension, place in the fridge while preparing the PhotoCol® solution.
- Weigh the necessary amount of PI to achieve a desired concentration of PI in the final bioink.

Note: LAP is commonly used PI, and the suggested concentration is 0.25% (w/v) (see Table 1).

Table 1. Suggested values for the preparation of 1 mL of 6 mg/mL PhotoCol® bioink.

V_{bioink}	$C_{final\ bioink}$	$C_{stock\ solution}$	$V_{stock\ solution}$	V_{NS}	m_{PI}	$V_{cell\ suspension}$
1 mL	6 mg/mL	10 mg/mL	600 µL	45 µL	2.5 mg	355 µL

- Dissolve the PI in the NS.

Note: Always prepare some extra solution to compensate losses during filtration and transfer from one container to another.

- Sterile filter the NS/PI solution using a 0.22 µm filter.
- To prepare 1 mL of 6 mg/mL PhotoCol® solution for printing, transfer 600 µL of PhotoCol® 10 mg/mL stock solution into a sterile Eppendorf using a positive displacement pipette. Keep the reagents cold by keeping the tubes on ice.
- Add 45 µL of cold NS/PI solution to the PhotoCol® and use the same pipette to homogenize the resulting solution. Place the tube on ice while setting up the BIO X.
- Take the Temperature-controlled printhead from the fridge and mount on the BIO X or BIO X6, set the temperature to 5°C and for the print bed 10°C, this is to guarantee the LifeSupport™ bath stability.

Note: Make sure the ambient temperature in the lab is maintained at 21-23°C, otherwise you may use ice packs inside the printing chamber to prevent the printing area from overheating.

- Add 355 µL of cool cell suspension with desired cell density and pipette the solution up and down until complete homogenization. Load a cartridge with the bioink using a pipette. Cap the cartridge with a printing needle and place it in the printhead.

4. Printing

MATERIAL

BIO X or BIO X6 bioprinter

Well plate or Petri dish previously filled with LifeSupport™ bath

Cartridge with the PhotoCol® bioink

DESCRIPTION

- Place the well plate or Petri dish previously filled with LifeSupportTM bath and place it on the print bed. Print constructs using suggested parameters:
    - pressure at 8-10 kPa.
    - speed at 3.5 mm/s.

Note: If printability is not as desired, adjust the pressure and/or speed to up/down to extrude more/less material at different speeds.

5. Incubation and crosslinking

MATERIAL

Cell culture medium

BIO X or BIO X6 bioprinter with LED modules of choice for photocuring

DESCRIPTION

- Keep the constructs for 10 min at room temperature to ensure initial collagen self-assembly prior to the melting of the supporting bath.
- Incubate the constructs for 30 minutes at 37°C (5% CO_2 and 95% relative humidity) for further self-assembly of PhotoCol® and LifeSupport™ melting.

Note: Large volumes may require longer times for the supporting bath to fully melt.

- Photocure each construct using the LED module. Time will depend on a chosen PI and its concentration.

Note: It is recommended to use the 405 nm photocuring module for 30 seconds instead of 365 nm if possible, when photocuring PhotoCol® with LAP photoinitiator. Overexposure at the 365 nm wavelength might damage the cells.

- Remove melted LifeSupport™ by replacing it with warm cell media to avoid handling the printed construct. For example, if you printed into a 6-well plate, this can be done by carefully aspirating 2 mL of melted LifeSupport™ out and adding 2 mL of warm cell media. Repeat this process until most of the support bath has been replaced by media.