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Author: EZ, VK. Version: 1

Neutralization and Printing Protocol

ColMA Solution

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 neutralization and subsequent printing of the neutralized ColMA solution. This document covers the bioprinting of ColMA droplets with embedded cells and cell post-seeding on printed ColMA constructs. ColMA can be efficiently photocrosslinked through photoinitiator-activated polymerization. The photocrosslinking was optimized for ColMA solution diluted with cell suspension in 10/1 volume ratio. Note that changing the ratio will change the photocrosslinking time. For photocrosslinking, a vial of photoinitiator (PI), e.g. LAP or Irgacure 2959, is available for purchase separately.

Materials needed

- ColMA Solution (20 mL at 5 mg/mL)*
- Photoinitiator*
- Collagen Buffer (5 mL)*,
- Ice bath
- 1x PBS
- 1M NaOH
- Container for mixing (15 mL Falcon tube or 5 mL Eppendorf tube)
- 0.22 µm sterile syringe filter
- Amber cartridges, 3 cc*
- BIO X* or INKREDIBLE-series* 3D Bioprinter
- Temperature-controlled Printhead (optional)
- Sterile conical bioprinting nozzles*
- Cells + culture medium
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptors*

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

Protocol for neutralization

During the whole procedure keep all materials on ice. Cool down the ColMA solution after every component is added. Be aware to always keep the photoinitiator (PI) protected from light.

Step	Title	Material	Description
1	ColMA preparation	- ColMA solution - Collagen Buffer - Ice bath	- Place the vial of ColMA Solution and Collagen Buffer on ice to keep them cool. - C_S is the concentration of the original ColMA solution (5 mg/mL). - Record the desired final volume (in mL) of the bioink (V_{INK}). - Record the desired final ColMA concentration after neutralization (C_F). Note: C_F and C_S cannot be the same, otherwise the solution would not be neutralized (refer to Table 1).
2	Calculations for neutralization and photoinitiator (PI) addition	- Calculator (optional)	- Volume of needed ColMA Solution: $$V_{ColMA}\ (mL) = \frac{C_F \times V_{INK}}{C_S}$$ - Volume of Collagen Buffer: $$V_{CB}\ (mL) = V_{ColMA} \times 0.154$$ - Mass of PI to dissolve in V_{CB}: $$m_{PI}\ (mg) = V_{INK} \times 2.5$$ - Volume of 1M NaOH: $$V_{NaOH}\ (mL) = V_{ColMA} \times 0.02$$ - Volume of 1x PBS to reach C_F: $$V_{PBS}\ (mL) = V_{INK} - V_{ColMA} - V_{CB} - V_{NaOH}$$
3	Neutralization	- 1x PBS - 1M NaOH - Container for mixing - Ice bath - Photoinitiator - 0.22 µm sterile syringe filter	- Dissolve the PI (m_{PI}) in the Collagen Buffer (V_{CB}) to achieve a 0.25% final concentration of PI in the solution. Sterile filter the resulting solution using a 0.22 µm filter. Note: Always protect the PI and PI-containing solution from light. - Mix V_{ColMA} and V_{CB} in a sterile container with a suitable volume capacity by

			vortexing or pipetting up and down. Be extra careful with keeping the solution cool once the V_{CB} is added as ColMA may self-assemble if heated. - Add V_{NaOH} to the mixing container. Note: The natural material oxidation after the bottle opening may affect the material pH. Therefore, if using previously opened bottle, it is recommended to proceed by adding V_{NaOH} by smaller volume steps, until the color of the solution corresponds to a pH between 6.9-7.3 (refer to Figure 1). Note down the volume used. Cool on ice. - Add V_{PBS} to the mixing container and homogenize by pipetting up and down or by vortexing.

Table 1. Preparation of ColMA biomaterial with different concentration.

V_{INK}, mL	C_F, mg/mL	C_S, mg/mL	V_{ColMA}, µL	V_{CB}, µL	m_{PI}, mg	V_{NaOH}, µL	V_{PBS}, µL
1	4	5	800	123	2.5	16	61
	3		600	92		12	296
	2		400	62		8	530

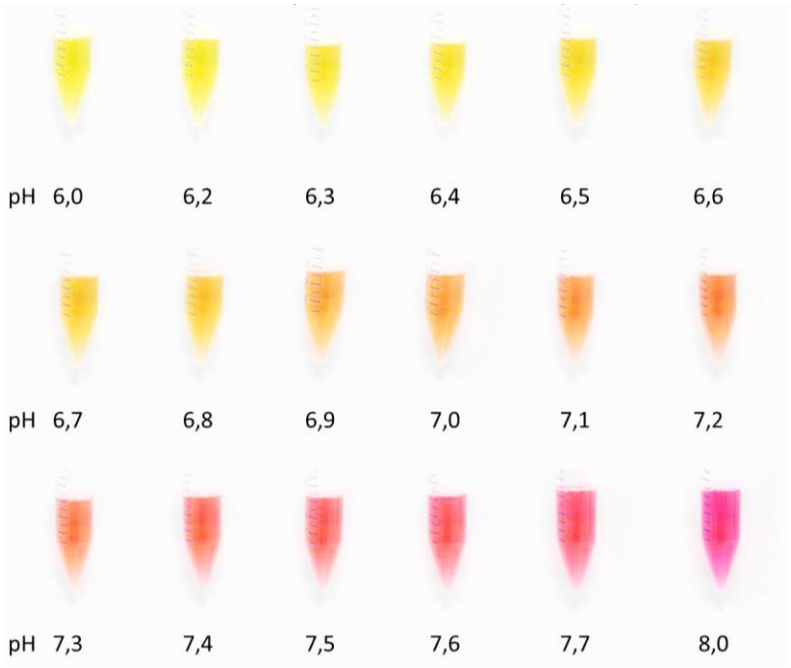

Figure 1. Illustration of color correspondence to pH [1].

[1]https://bit.ly/3lmRoCv

Protocol for printing of neutralized ColMA solution

Make sure to follow the neutralization protocol above prior to following the printing protocol. This printing protocol works best using the BIO X equipped with the Temperature-controlled Printhead. If using the INKREDIBLE+ system, the printing procedure should be performed fast to prevent the solution from warming and gelling in the cartridge during the experiment.

Step	Title	Material	Description
1	Prepare ColMA for mixing	- Neutralized ColMA solution - 3 mL syringe	- Cool down ColMA on ice for 10 min to make sure it remains in the liquid state. - Transfer the solution into a 3 mL syringe using the following procedure: remove a syringe plunger → cap the syringe → pour the ColMA solution in the syringe → insert the plunger → flip the syringe → release the tip cap to evacuate the air.
2	Mix ColMA with cells	- Cell suspension in a syringe - Cooled ColMA solution - Female/female Luer lock adaptor	If cells will be post-seeded on printed constructs, move directly to step 3. Mix ten parts of ColMA solution with one part of cell suspension without introducing air bubbles to the mixture. For detailed instructions see the *Mixing cells Protocol*. - Attach the ColMA solution syringe to the syringe with cell suspension using a female/female Luer lock adaptor. - Carefully mix the solution with the cell suspension by gently pushing them back and forth between the syringes. Note: Suggested cell suspension density is $5x10^6$ cells/mL to $10x10^6$ cells/mL. Note: To avoid an air gap when mixing the solution and the cell suspension, carefully pre-fill the Luer lock adaptor with ColMA solution before attaching the syringe with the cell suspension.
3	Load the cartridge	- Cartridge, 3 cc - ColMA mixed with cells - Sterile Conical Bioprinting nozzles, 25G	- Transfer the cell-containing solution to the cartridge and cap it. - If using the BIO X, pre-cool the printhead to 5°C. If using the INKREDIBLE-series, cool down the cartridge on ice if needed. Note: The printing temperature can be increased to 10°C or 15°C, but this will reduce the time available for ColMA printing process prior to its inadvertent self-assembly. - Place the cartridge in the printhead and cap with a bioprinting nozzle of choice.

4	Printing	- Bioprinter (BIO X or INKREDIBLE series)	- Print droplets with the desired size in a mold or well plate.
5	Crosslinking	- 365/405 UV module	CoIMA can be mildly crosslinked via thermal gelation at 37°C, though for the best mechanical properties photocrosslinking using the 365 or 405 nm UV module is recommended. If using both, begin with thermal crosslinking. - Thermal crosslinking (optional): warm the construct to 37°C until gelation occurs, approx. 10-15 min. The BIO X heated print bed or incubation can be alternatively used. - Photocrosslinking: set the distance of 3 cm from UV module to the sample, turn on the module and keep it on for 30 s. Note: The crosslinking time must be adjusted based on the construct thickness. Remember that UV overexposure might damage the cells.
6	Cell post-seeding	- Cell suspension	If cells were mixed with CoIMA solution prior to printing, move directly to step 7. - Dispense the cell suspension in the middle of the printed hydrogel. Suggested cell suspension density: $20x10^3$ cells/cm^2 to $50x10^3$ cells/cm^2 (a highly concentrated cell suspension is suggested for use in a volume not exceeding 10 µl).
7	Incubation	- Cell culture medium	- Add the desired medium to submerge the constructs and place in incubator. Note: Ensure that the bioprinted constructs are crosslinked and do not dissolve in media. - 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.