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Protocol for FRESH extrusion 3D printing of Type-1 collagen hydrogels photocrosslinked using Ruthenium

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We use this protocol and it's working

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Abstract

The extrusion bioprinting of collagen material has many applications relevant to tissue engineering and regenerative medicine. Freeform Reversible Embedding of Suspended Hydrogels (FRESH) technology is capable of 3D printing collagen material with the specifications and details needed for precise tissue guidance, a crucial requirement for effective tissue repair. While FRESH has shown repeated success and reliability for extrusion printing, the mechanical properties of completed collagen prints can be improved further by post-print crosslinking methodologies. Photoinitiator-based crosslinking methods are simple and have proven effective in strengthening protein-based materials. The ruthenium and sodium persulfate photoinitiator system ($Ru(bpy)_3$/SPS) has been suggested as an effective crosslinking method for collagen materials. Herein, we describe the procedure our group has developed to combine extrusion-based 3D printing of type-1 collagen using FRESH technology with $Ru(bpy)_3$/SPS photoinitiated crosslinking methods to improve the strength and stability of 3D printed collagen structures.

Materials

- Corning® 50 mL centrifuge tubes
- Kimwipes® disposable wipers
- Allevi Plastic Syringes (5cc); Catalog No. SKU-PSYR5
- Eppendorf Centrifuge 5810 R 15amp
- 40 × 25mm glass dish
- 30 gage, 1-inch blunt end, lavender, Fisnar®; Catalog No. 8001104
- Allevi 3 systems, SN. 7c1057c
- Sundee glue tape
- Fisherbrand™ Premium Microcentrifuge Tubes: 2.0mL; Catalog No 05-408-138
- Aluminum foil
- Fisherbrand 10ml serological pipettes (2X)
- Eppendorf Centrifuge 5810 R 15amp
- Heidolph Instruments Vortex D-91126 Schwabach REAX top 541-10000-01-1
- 40mm x 25mm glass dish
- Fisherbrand™ Semimicro Spatula with One Tapered End, One Rounded End 14-374
- Sundee glue tape
- Heidolph Instruments Vortex D-91126 Schwabach REAX top 541-10000-01-1
- AmScope 3WX2 LED-6WD LED Spot Light with Two 6500K LED Attachments; Catalog Number YAG20201215
- 100-1000 µl pipette, Fisherbrand Elite NU09348 and pipette tips
- Drummond Scientific Co. Pipetaid XP
- Thermoscientific HERRAcell i150 Incubator

Software:

- Slic3r
- Repitier
- Autodesk Fusion, Shapr3D, or TinkerCAD

Protocol materials

1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4

Lifeink 240 Acidified Collagen Bioink **Advanced Biomatrix Catalog #**5267

Sodium Persulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6172

Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**224758

LifeSupport **Advanced Biomatrix Catalog #**5244

LifeSupport Preparation

10m 45s

1 Prepare the centrifuge by cooling the temperature to 4 °C

2 Prepare 35 mL of 1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4 , and refrigerate until temperature reaches 4 °C .

3 Retrieve the Advance Biomatrix LifeSupport, which is stored at room temperature.

4 Prepare an empty 50 mL conical tube for mixing LifeSupport. Separate the LifeSupport powder into two 1 g aliquots, each in a separate 50 mL conical tube.

5 To each of the 1g aliquots of LifeSupport, add 17.5 mL of 4 °C PBS.

6 For each conical tube, shake vigorously for 00:01:00 by 1m

6.1 Shaking horizontally along the length of the tube, and

6.2 Tapping the end of the tube against the edge of the table. Continue until the gelatin has been uniformly mixed, and there’re no longer large clumps clinging to the walls of the tube.

7 Place the conical tubes containing LifeSupport into a 4 °C refrigerator for 45m 00:45:00 . This step allows the LifeSupport to fully rehydrate, and ensures the mixture is at an adequate temperature.

8 Vortex each conical tube for 00:00:45 . 45s

9 Centrifuge the conical tubes at 2500 x g for 00:05:00 . 5m

10 Remove conical tubes from the centrifuge, open the caps, and pour off the supernatant. Absorb the remaining supernatant with Kimwipes (low-linting) to remove as much liquid as possible.

11 Recap and shake the conical tubes again. For each conical tube, shake vigorously for 00:01:00 by 1m

11.1 Shaking horizontally along the length of the tube, and

11.2 Tapping the end of the tube against the edge of the table. Continue until the gelatin has been uniformly mixed, and there are no longer large clumps clinging to the walls of the tube.

12 Vortex each conical tube for 00:00:45 45s

13 Centrifuge the conical tubes again at 2500 x g for 00:05:00 5m

14 Pour off or absorb any remaining supernatant with a Kim wipe.

15 Prepare a 40 × 25mm glass dish.

16 Uncap the first conical tube, and invert the tube into the glass dish so that it's upside down with the open end inside the glass dish. Tap the tube up and down vertically until the gelatin LifeSupport dislodges and fills the glass dish.

17 Remove the conical tube from the dish. Using a small spatula, scoop the remaining LifeSupport from the walls of the conical tube, being careful not to introduce new air bubbles. Any LifeSupport that is scooped out of the conical tube should be placed on top of and in the center of the LifeSupport that is sitting in the glass dish.

18 Carefully but forcefully tap the glass dish flat against a table to get the LifeSupport to spread out and fill the base of the dish. If small air bubbles form during this process, use the spatula to carefully scoop them out, and then tap the LifeSupport against the table again to fill the void.

19 Uncap the second conical tube, and carefully scoop the LifeSupport out, careful not to create air bubbles. Place the LifeSupport from the second tube on top of the LifeSupport

from the first tube. Again, tap against the table to disperse the LifeSupport evenly. Use the spatula to remove any large air bubbles that form.

20 LifeSupport should achieve a vaseline-like consistency, with no flow of material from side-to-side when tilted.

21 The LifeSupport can be stored at 4 °C for up to 01:00:00 but should be used as quickly as possible to obtain the best results

1h

Ink Preparation

5m

22 Commercially available 0 g syringes designed for Allevi 3 systems 3D printers were used to hold and disperse LifeInk 240 during 3D printing. Prepare the 5cc syringes by removing the plunger cap from the plunger and placing it at the bottom of the syringe until it is in contact with the syringe neck.

23 Using a syringe female-female luer lock adapter, transfer 4 mL of Lifeink 240 Acidified Collagen Bioink **Advanced Biomatrix Catalog #**5267 to the 5cc syringes.

24 To eliminate air bubbles and gaps in the ink, cap the syringe, then centrifuge the syringe at 4 °C and 2000 x g for 00:05:00

5m

25 To remove any final air bubbles, use an empty syringe with a 30 gauge needle. Insert the needle into the plunger cap and suck out any air pockets. You may also use a spatula to move the plunger cap down into the empty space, while simultaneously letting air escape out the side of the plunger cap.

26 Store the prepared syringe at 4 °C until printing.

3D Print File Preparation

27 3D files can be designed using standard computer-aided-design (CAD) software. Popular options include AutoCAD, Solidworks, Autodesk Fusion, Shapr3D, etc. Any CAD software that can adequately meet your design needs and can export files as .stl or .obj is acceptable.

27.1 3D files were designed in Autodesk Fusion, Shapr3D, or TinkerCAD. Files were exported in .stl file format.

28 After 3D files have been created, they need to be “sliced.” Slicing is the process by which the 3D file is translated into a G-code file. G-code is a software programming language used to control 3D machines, such as CNC machines and 3D printers. The Allevi 3 3D Bioprinter used in this process accepts G-code files. Popular slicing software includes UltiMaker Cura, PrusaSlicer, Slic3r, Repetier, etc. Files for this protocol were sliced using Slic3r and Repetier.

28.1 To slice a 3D CAD file, open Repetier.

28.2 Select “Config”, and select the standard printer configuration.

28.3 Click “Load,” then upload your .stl 3D file.

28.4 Under “Object Placement,” assign extruder 0 to the “Object Group 1.”

28.5 Click “Slicer.” From the dropdown menu titled “Slicer:” select “Slic3r.”

28.6 If desired, select “Configuration” to choose your specific slicing parameters (see below), then save and return to the Repetier window.

28.7 Ensure that “Extruder 1:” selection lists your extruder that the bioink will be extruded from.

28.8 Click “Slice with Slic3r.” Wait for the Slic3r window to pop up.

28.9 Click “Export G-code...” Save the Gcode. You will upload it to the Allevi 3 Bioprinter in the next section.

29 **Slicing Parameters:**

For our purposes in designing a nerve guide conduit, we used the following parameters:

50% Infill (This prevents the object from being too dense and overfilled)
Rectilinear Infill (This is the pattern of infill inside your print)
150% Overlap (This ensures that each individual layer and line is properly connected to the last, allowing for cohesion of layers during crosslinking)
No Perimeter (Typically slicing software will allow you to print additional perimeters around your design with a different set of parameters than the rest of the design. We avoided this for two reasons: 1) Additional perimeters were not factored into our design specifications, thus adding perimeters after the fact would increase the size and deform the shape. 2) In our extensive testing, we found that perimeters, with our particular collagen bioink, tended to shear and separate from the rest of the print)
0.10mm Layer Height (Setting the layer height shorter than the inner diameter of the needle allowed us to force additional vertical overlap, thus creating a well-bonded and cohesive object)
0.17mm Extruder Width (The extruder width is traditionally set at or slightly larger than the inner diameter of the extruder nozzle. In this situation, the inner diameter of our needle was 0.15mm, but through experimentation and testing, we identified 0.17mm to be the best extruder width)
6 mm/s print speed (This is the tool speed at which the extruder moves along it's path. For our purposes, we identified 6mm/s to be the best print speed, both for print quality and to prevent excessive drag interference.)

Allevi 3 System Preparation

30 Allevi 3 systems should be turned on and connected to a WiFi server without interruption during the printing process.

31 Make sure working room environmental conditions read to between 17-22 °C with minimal surface vibrations for optimal printing.

32 Using an empty 40 × 25mm glass dish, center the dish on the print stage then mark the edges of the dish onto the print stage with a marker. Using an empty 5cc syringe with a 1 inch, 30 gauge needle attached, position the needle into the printer head and lock in place with the air compression hose.

33 On the Allevi interface, we use the directional controls (x,y, and z-axis) to position the tip of the needle to the bottom center of the 40 × 25mm glass dish. We position the tip of the needle so that there is only 0.1mm z-spacing between the needle tip and the glass dish surface. If the position of the needle tip relative to the glass dish is correct and acceptable to the print dimensions, then using the Allevi interface move the print stage on the z-axis down from the needle tip by 25 mm. Remove the empty syringe from the print head and the glass dish from the print stage.

34 Set the printer head parameters on the interface screen to the following: Ink temp 4 °C, air pressure of 50psi.

35 Collect the prepared 5cc of LifeInk from the 4 °C fridge. Place into the syringe slot on the printer head and securely attach and lock the air compression hose to the top of the syringe.

36 Calibrate the extrusion properties of the ink by manually extruding the ink through the length of the needle and observing the rate of droplet formation at the needle tip. Manually extrude several times to clear the line of air bubbles. Make sure to wipe the needle tip with Kim wipe after testing to ensure no residual dried ink will interfere with droplet formation and ink flow.

37 Upload the Gcode file for the desired print to the Allevi server using the Allevi interface. Ensure the Gcode is selected in the interface screen before printing.

38 Collect the prepared lifesupport dish from the 4 °C fridge. Add double-sided Sundee glue tape to the bottom of the dish, then place the dish on the printer stage so that the edges of the dish align with the marks on the stage. Ensure the dish is adhered tightly to the stage.

39 Press the print button on the interface to begin printing.

Crosslinking

40 **For 25/250 mM Ru/SPS Concentration:**

1. Prepare two 2.0ml microcentrifuge tubes by wrapping them in aluminum foil to prevent light leakage.
2. Label one tube Ru and the other SPS.
3. To each, add 1 mL of
   1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4 .
4. To the tube labeled Ru, add 0.0187 g of
   Tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**224758
   . Close the tube and ensure it is adequately wrapped in aluminum foil.

5. To the tube labeled SPS, add 0.0595 g of

   Sodium Persulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6172 .

   Close the tube and ensure it is adequately wrapped in aluminum foil.

For each tube, vortex for one minute to ensure that the mixture is uniformly distributed

41 **For 50/500 mM Ru/SPS Concentration:**

1. Prepare two 2.0ml microcentrifuge tubes by wrapping them in aluminum foil to prevent light leakage.
2. Label one tube Ru and the other SPS.
3. To each, add 1 mL of

   1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4 .
4. To the tube labeled Ru, add 0.0374 g of

   Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**224758

   . Close the tube and ensure it is adequately wrapped in aluminum foil.
5. To the tube labeled SPS, add 0.1191 g of

   Sodium Persulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6172 .

   Close the tube and ensure it is adequately wrapped in aluminum foil.
6. For each tube, vortex for one minute to ensure that the mixture is uniformly distributed.

42 **For 75/750 mM Ru/SPS Concentration:**

1. Prepare two 2.0ml microcentrifuge tubes by wrapping them in aluminum foil to prevent light leakage.
2. Label one tube Ru and the other SPS.
3. To each, add 1 mL of

   1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4 .
4. To the tube labeled Ru, add 0.0561 g of

   Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**224758

. Close the tube and ensure it is adequately wrapped in aluminum foil.

5. To the tube labeled SPS, add 0.1786 g of
   Sodium Persulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6172 .
   Close the tube and ensure it is adequately wrapped in aluminum foil.
6. For each tube, vortex for one minute to ensure that the mixture is uniformly distributed.

43 **Determining the Volume to add to different dish sizes:**
Calculate the volume of the dish you used for printing, and multiply the volume of the dish by 2%. This will give you the volume of each solution you need to pipette into the dish for crosslinking.

For Example: 40 × 25mm glass dish

$$V = h\pi r^2$$
$$V = (25mm)\pi(20mm)^2$$
$$V = 31.42ml$$

44 **Visible Light Crosslinking- Wavelength and Power Density:**
We used an AmScope 3WX2 LED-6WD LED Spot Light with Two 6500K LED Attachments that was positioned approximately 75mm away from the sample.

The light produced by this system was 425nm, with a power density of $53.3mW/cm^2$.

Crosslinking the collagen Print

1h 10m

45 Place a container of 500 mL of
1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4 in an incubator or water bath at 37 °C until it comes to temperature.

46 Place the 40 × 25mm glass dish containing the LifeSupport and completed
Lifeink 240 Acidified Collagen Bioink **Advanced Biomatrix Catalog #**5267
collagen print in an incubator at 37 °C for 00:45:00 .

45m

47 Prepare two 10 mL serological pipettes and a waste container. Set aside for later use.

48 Remove the dish from the incubator. Verify that the LifeSupport has melted to form a non-viscous liquid and that the Lifeink 240 Acidified Collagen Bioink **Advanced Biomatrix Catalog #**5267 print has turned an opaque white color. If not, place it back into the incubator and monitor it until a change is observed. If so, proceed to the next step.

49 Pipette 10 mL of LifeSupport out of the dish and place in the waste container. Be careful not to disturb the Lifeink 240 Acidified Collagen Bioink **Advanced Biomatrix Catalog #**5267 bioprint with the pipette.

50 Pipette 10 mL of 37 °C 1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4 into the dish containing the LifeSupport and Lifeink 240 Acidified Collagen Bioink **Advanced Biomatrix Catalog #**5267 bioprint. Be careful not to disturb the Lifeink 240 Acidified Collagen Bioink **Advanced Biomatrix Catalog #**5267 bioprint with the pipette.

51 Repeat steps 49 & 50 approximately 20 times, or until no gelatin remains in the dish or on the print.

52 If gelatin remains in the dish or on the print, place the dish back into the 37 °C incubator for 00:10:00, and then repeat steps 5 & 6 until no LifeSupport **Advanced Biomatrix Catalog #**5244 remains. 10m

53 If using the AmScope 3WX2 LED-6WD LED Spot Light with Two 6500K LED Attachments: Place the 40 × 25mm glass dish on a stage with both LED lights suspended over it such that all the LED light will be concentrated within the dish. This should be approximately 75mm above the dish if using the same dish size. Turn the brightness control slider up to 10 (max). Do not turn on the LED light at this time. If using another light source: adjust the setting of the light source to emit 425nm light at $53.3 mW/cm^2$

54 Pipette 628.4 µL of the Sodium Persulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S6172 solution into the 40 × 25mm dish. Discard the pipette tip.

55 Pipette 628.4 µL of the Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**224758 solution into the 40 × 25mm dish.

56 Use the pipette to gently draw liquid in and out, thereby mixing the solutions together in the dish. Complete this procedure approximately 20 times to ensure adequate mixing. Take caution not to disturb the collagen print.

57 Turn on the LED light. Expose the dish to the light for 00:15:00 . 15m

58 Use a spatula to remove the collagen bioprint from the dish. The crosslinking process is now completed.

59 Alternatively, you may also use a pipette to draw all of the solution out of the dish, which may make removing the print easier.

60 Store your completed bioprint suspended in 1X Phosphate Buffered Saline Solution **Catalog #**BP2438-4 or deionized water at 4 °C until ready for use.