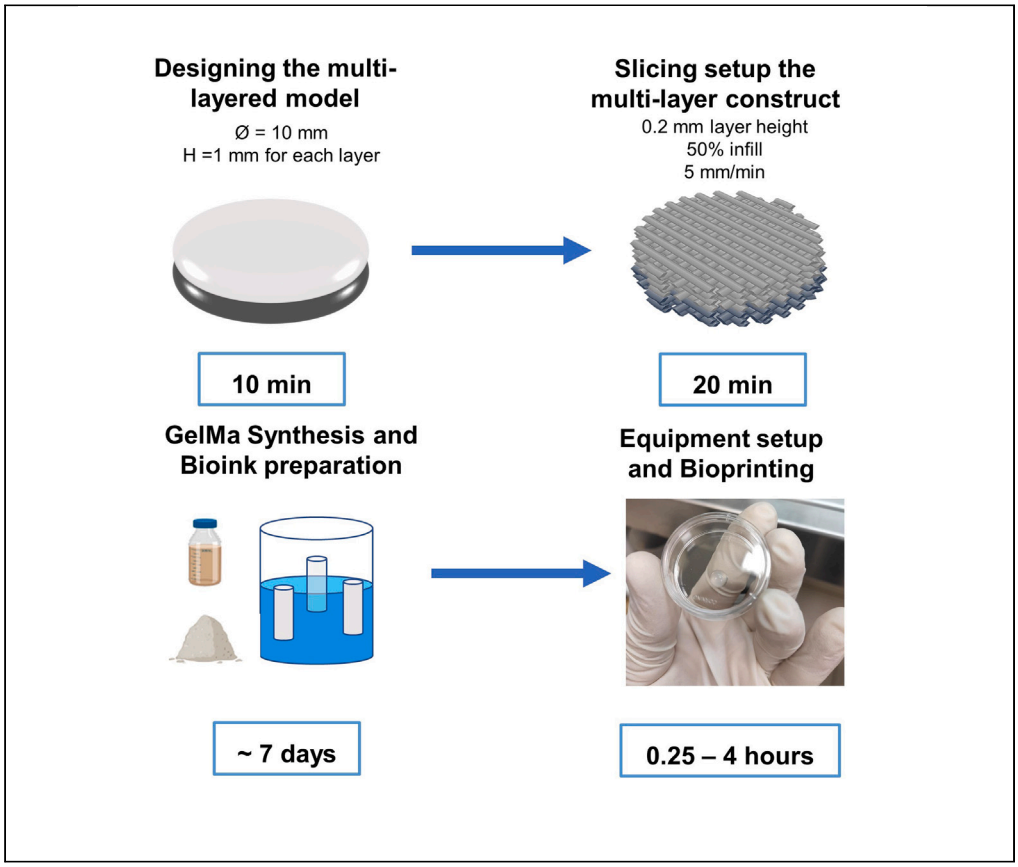
STAR Protocols

CellPress
OPEN ACCESS

Protocol

# Protocol for designing and bioprinting multi-layered constructs to reconstruct an endothelial-epithelial 3D model




3D bioprinting has opened new possibilities and elevated tissue engineering complexity. Here, we present a protocol to design a 3D model with two cell lineage layers (A549 and HUVEC) to recreate multi-cell constructs. We describe the steps for slicing the constructs, handling hydrogels, and detailing the bioprinting setup. These 3D-bioprinted constructs can be adapted to various cell models—from primary cell cultures to commercial cell lines and induced pluripotent stem cells (IPCs)—and applications, including drug screening and disease modeling.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Eduardo Henrique Backes, Laura Nicoleti Zamproni, Lina Maria Delgado-Garcia, Leonardo Alves Pinto, Robertha Mariana Rodrigues Lemes, Cynthia Silva Bartolomeo, Marimélia Aparecida Porcionatto

backes@ufscar.br (E.H.B.)
marimelia.porcionatto@unifesp.br (M.A.P.)

## Highlights

Software designing and setting up single- or multi-layered bioink 3D constructs

Optimized parameters for GelMA/Geltrex® diverse applications

Reproduce complex multi-layered biological microenvironments

Backes et al., STAR Protocols 4, 102467
September 15, 2023 
https://doi.org/10.1016/j.xpro.2023.102467

STAR Protocols

CellPress
OPEN ACCESS

Protocol

# Protocol for designing and bioprinting multi-layered constructs to reconstruct an endothelial-epithelial 3D model

Eduardo Henrique Backes,[1,2,3,4,7,8,9,*] Laura Nicoleti Zamproni,[1,2] Lina Maria Delgado-Garcia,[1,2] Leonardo Alves Pinto,[4] Robertha Mariana Rodrigues Lemes,[5] Cynthia Silva Bartolomeo,[6] and Marimélia Aparecida Porcionatto[1,2,*]

[1]Department of Biochemistry, Escola Paulista de Medicina, Universidade Federal de São Paulo, Sao Paulo, São Paulo 04044020, Brazil

[2]Laboratory of Molecular Neurobiology, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo 04039002, Brazil

[3]Department of Materials Engineering (DEMa), Universidade Federal de São Carlos, São Carlos 13565905, Brazil

[4]Graduate Program in Materials Science and Engineering, Universidade Federal de São Carlos, São Carlos 13565905, Brazil

[5]Department of Biological Sciences, Universidade Federal de São Paulo, Diadema 04021001, Brazil

[6]Department of Interdisciplinary Health Sciences, Universidade Federal de São Paulo, Baixada Santista 11015020, Brazil

[7]Present address: Universidade Federal de São Carlos, São Carlos, São Paulo 13565905, Brazil

[8]Technical contact: backes@ufscar.br

[9]Lead contact

*Correspondence: backes@ufscar.br (E.H.B.), marimelia.porcionatto@unifesp.br (M.A.P.)
https://doi.org/10.1016/j.xpro.2023.102467

## SUMMARY

**3D bioprinting has opened new possibilities and elevated tissue engineering complexity. Here, we present a protocol to design a 3D model with two cell lineage layers (A549 and HUVEC) to recreate multi-cell constructs. We describe the steps for slicing the constructs, handling hydrogels, and detailing the bioprinting setup. These 3D-bioprinted constructs can be adapted to various cell models—from primary cell cultures to commercial cell lines and induced pluripotent stem cells (IPCs)—and applications, including drug screening and disease modeling.**

**For complete details on the use and execution of this protocol, please refer to Cruz et al.[1]**

## BEFORE YOU BEGIN

This protocol describes in an unprecedented level of detail the bioprinting process of 3D multi-layered and -cell-bioinks constructs. By using and adapting the protocol, one can model different geometries, fabricate different bioinks and tune precisely the bioprinting process to achieve a high fidelity regarding the native tissue cytoarchitecture and microenvironment. Modeling the construct using basic shapes, the user can build a variety of geometries -such as cylinders, squares, prisms, cones, and tubes- to mimic diverse biological structures. In that context, it has been reported a wide range of *in vitro* 3D-bioprinted models such as stem cell niches, specific vascular and pulmonary-like structures, or complete artificial organ (kidney, cartilage, and artificial skin) reconstructions with high reproducibility.[1–6]

For this protocol, extra care was taken during the design, slicing and setup because the bioprinter used is RepRap-based and lacks some resources from more robust models. The protocol gives to the reader an opportunity of taking a deep look at all the steps involved during the bioprinting process

and adapt it according to its need. By tuning the slicing software correctly, fine deposition of material can be achieved. It also allows different infill patterns (grids, rectangular, triangle) and densities (especially for solid constructs). Other slicing modifications include the layer height change and needle gauge; those will require new settings that are automatically correct by the software (for example the mass flow correction). These conditions are especially important for 3D models with different cell layers.[7] For instance, in cancer metastasis studies, cell layers allow the identification of invasive properties, cell-cell interactions and paracrine signaling in organ-specific microenvironments.[8]

Here we encourage the reader to use as main cell matrix the Gelatin Methacrylate (GelMA) compound that is one of the first bioinks developed and has a vast amount of literature regarding its applications and tailoring properties.[9–11] By controlling the methacrylate degree and crosslinking level, GelMA offers the flexibility to adjust its mechanical and rheological properties, making it a versatile choice for applications in bioprinting -been the first choice for adipose and cartilage-like models. Methacrylation degree is obtained and modified through the synthesis steps, while the crosslinking level is regulated by controlling the intensity and exposition times during the photo crosslinking steps.[12,13] Furthermore, GelMA inherits RGD sites from gelatin, making it a biocompatible biomaterial. GelMA modifications have been proposed to recreate natural features and improve cell viability, such as adding Geltrex®, a basement membrane extract with Lamin, collagen IV and proteoglycan.[1,14–17]

Finally, we used a GelMA/Geltrex® mixture in combination with two different cell lines -alveolar epithelial (A549) and human umbilical vein endothelial cells (HUVEC)- to reconstruct an endothelial-epithelial 3D model; however, this set up can be adapted to create different constructs that aggregate complexity to cell culture systems advancing basic research and tissue engineering.

### Institutional permissions

This protocol does not include experiments on live vertebrates or higher invertebrates. All experiments conform to local and worldwide relevant regulatory standards. We remind readers the need to acquire permissions from their institutions.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Experimental models: Cell lines | | |
| Human umbilical vein endothelial cells | N/A | N/A |
| A549 | ATCC | CCL-185 |
| Software and algorithms | | |
| ImageJ | Cruz[17] | https://imagej.nih.gov/ij/ |
| Chemicals, peptides, and recombinant proteins | | |
| Dulbecco's modified Eagle medium nutrient mixture F-12, DMEM/F12 | Gibco | 12500062 |
| Fetal bovine serum, FBS | Gibco | 12657011 |
| Gelatin from porcine skin | Sigma-Aldrich | G2500, 9000-70-8 |
| Geltrex (LDEV-Free) | Gibco | 1413202 |
| Methacrylic anhydride | Sigma-Aldrich | 276685 |
| Penicillin/Streptomycin | Gibco | 15070063 |
| Photoinitiator | Sigma-Aldrich | 106797-53-9 |
| Trypsin/EDTA | Gibco | 15400054 |
| Live/dead kit cell imaging kit 488/570 | Thermo Scientific | R37601 |
| Equipment | | |
| 3D Bioprinter (Model Octopus) | 3D Biotechnology Solutions (3DBS) | http://www.3dbiotechnologiessolutions.com/octopus/ |
| Confocal microscope | Leica | TCS SP8 CARS |
| Universal testing Equipment | Instron | 5569 |

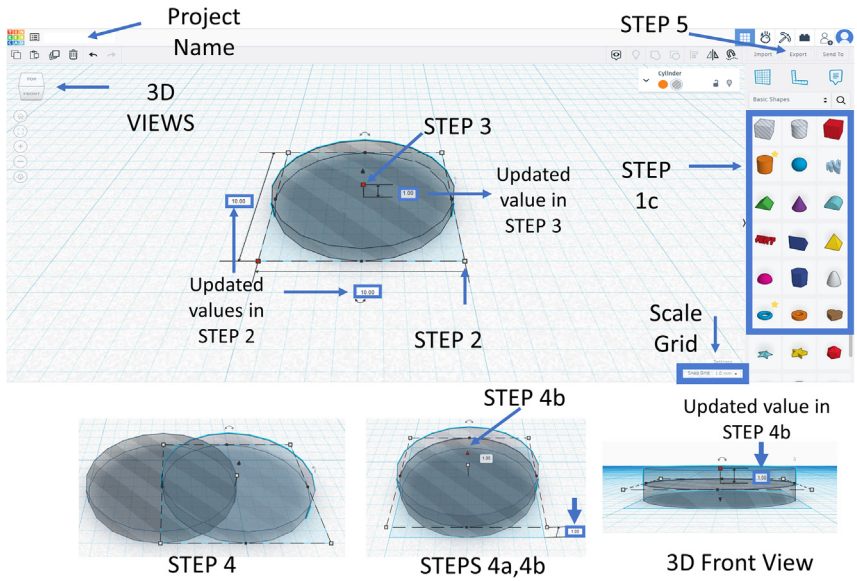



**Figure 1. Procedural steps from 1 to 5**

## STEP-BY-STEP METHOD DETAILS

### Major step one: Model design

Timing: 10 min

1. Download and install *TinkerCAD®* at https://www.tinkercad.com/.
   a. Create a new account or log in via the option of your preference.
   b. Click on "Create your first 3D Design" on the new page (Figure S1).
   c. Select basic shapes in the shapes menu and drag it to the table. The cylindrical form is the most used, and it is demonstrated here (Figure 1).
2. Select the white squares at the vertices of the cylinder to modify its length and width to 10.0.

   *Note:* The white squares become red when selected, as shown in Figure 1. The "millimeters" is the default scale, shown at the bottom right as "Snap Grid ". If necessary, it can be changed by clicking on "Edit Grid" and at the "Grid Properties" window, changing the item "Unit" to "Millimeter", and finally, "Update Grid".

3. Select the white square in the middle of the cylinder to modify its height to the value to 1 mm.

   *Note:* Since we intend to use Slicing software to split the geometry into different layers, the height chosen must be a multiple of the layer height. As pointed out by several authors, although thin layers might give better precision (0.1 mm), they can take longer, and without a supporting bath, the cells are more prone to die during longer periods, while thicker (> 0.25) layers might not adhere to the printing table and stick to the needle (for further information, we recommend the following ref. 18).

4. Select the object and copy-paste it.
   a. Click on the surface of the new object, and by using the mouse's left button, drag it to fit the first object.
   b. Click at the dark arrow (pointed up) in the middle of the objects and lift one of the objects 1 mm above the other (by doing this, the option to insert the desired distance is enabled).

*Note:* If the user desires to build objects with another height, it is necessary to adjust the vertical translation to fit one object at the top of the other.

5. Select only the object below and click on export (right corner at the top of the screen).
   a. Select the option "The selected shape" and the panel "For 3D Print", select click on ".STL").
   b. Name the file as "Bottom".
   c. Select only the object above, repeat steps 5 and 5a and name it as "Top" file.
6. Close Tinkercad.

### Major step two: Slicing setup

Timing: 20 min

7. Download *Prusa Slicer* at https://www.prusa3d.com/page/prusaslicer_424/ and select "PrusaSlicer Windows Standalone".
   a. Install by double-clicking on the download file.
   b. Open PrusaSlicer and click on "Configuration Tab > Mode >" and select "Expert" to enable additional features.
8. Select the "Print settings" tab on the configuration menu (Figure S2).
   a. Select "Layers and perimeters" on the left menu.
   b. Configure the "Layer height" and "First layer height" as 0.2 mm.
   c. Configure "Vertical Shells" as 0.
   d. Configure "Horizontal shells", "Solid Layers", "Top", and "Bottom" as 0.
9. Go to the next subitem on the left menu and select "Infill" (Figure S3).
   a. Configure "Fill density" at 50% and "Fill Pattern" as "Rectilinear" at the "infill" panel.

   *Note:* Higher infill or cell concentrate can be used if the bioprinted construct requires a higher cell density. At this condition, each construct has around 50.000 cells.

   b. Configure "Combine infill every" to 1 at the "reducing printing times" panel.
   c. Configure "Solid Infill" and "Fill Angle" to 0 and 90, respectively, at the "advanced" panel.
10. Select "Skirt and Brim" on the left menu (Figure S4).
    a. Configure "Loops (minimum)" and "Brim width" at 0 in "skirt" and "brim" panels, respectively.
11. Select the "speed" on the left menu (Figure S5).
    a. Configure the speed of the "infill", "Support material", and "Bridges" to 5 at the "Speed for print moves" panel.

    *Note:* The cell deposition at higher speeds requires higher flow, which, in excess, leads to cell death and might also reduce shape fidelity.

    b. Configure "Travel" to 20 at the "Speed for non-print moves" panel.
    c. Configure "First layer speed" to 5 at the "Modifiers" panel. The cell deposition at higher speeds requires higher flow, which, in excess, leads to cell death.
12. Select "Advanced" on the left menu (Figure S6).
    a. Configure all variables to 0.4 at the "Extrusion width" panel.
    b. Save the configuration by clicking on diskette.
13. Select "Filament settings" and "Filament" on the left menu (Figure S7).
    a. Configure the "Diameter" as 12 mm on the "filament" panel. The 5 mL syringe has a 12 mm internal diameter; therefore, if a different syringe is used, it is necessary to change this value to obtain the right flow through the needle.
    b. Set the value as 0 for all the options on the "Temperature" panel.
    c. Save the configuration by clicking on diskette.

14. Select "Printer settings" and "General" on the left menu (Figure S8).
    a. Click on "Set" at the "Bed Shape" option in the "Size and coordinates" panel.
    b. At the new window, change the shape of the bed to X: "300", Y: "300", Origin X "100" and Origin Y "140" and click on "OK".
    c. Configure the "Max Print height" to "100" and "Z offset" to 1.2 at the "Size and coordinate" panel.

    *Note:* The "Z offset" was adjusted to 1.2 because the bioprinting takes place on a petri dish with a 1.4 mm height; each bioprinted layer height has 0.2 mm. This way, at the start, the needle faces the petri dish's surface, assuring higher precision and bioink adhesion at this surface.

    d. Configure the parameter "Extruders" to 2 at the "Capabilities" panel.
15. Select "Custom G-Code" on the left menu (Figure S9).
    a. At the "Start G-Code" panel, substitute the content to:

```
''G1 X0 Y70 Z1.4 F3000''
```

    *Note:* The "Start G-Code" command assures that the bioprinting process starts at this position, the same as the sliced file. At the "End G-Code" command the needle moves upwards and holds for 5 s before the initial position with the first extruder.

    b. At the "End of G-code" panel Substitute the content to:

```
''G1 Z25 F3000

G4 P5000

T0

G1 X0 Y70 Z1.4''
```

16. Select "Extruder 1" on the left menu (Figure S10).
    a. At the "Size" panel, set "Nozzle Diameter" as 0.4 mm.
    b. Set the value at "0" "for "Length" at the "Retraction" panel.
    c. Set the value at "0" for "Length" at the "Retraction tool when tool is disabled" panel.
    d. Proceed as steps 11a to 11c for the "Extruder 2" on the left menu. However, set the value of the "Extruder offset" at "85" for "X coordinate" at the "Position" panel.

⚠ CRITICAL: In this case, Extruder 2 is horizontally swift 85 mm from Extruder 1. By setting this condition, the slicing software automatically corrects the relative position of both extruders during the bioprinting process.

17. Return to the "Platter" at the "Configuration menu" and import the two "STL" files exported from TinkerCAD (Figure S11).
    a. Click "Yes" at the "Multi-part object detect" warning message.
18. Click on "Bottom" on the right menu to move both parts simultaneously and replace them at position "(X,Y):(0,70)" (Figure S12).

    *Note:* Certify that the "Bottom" file uses Extruder 1 and the "Top" file Extruder 2; otherwise, select at gear on the right of the option to change the order of the extruders.

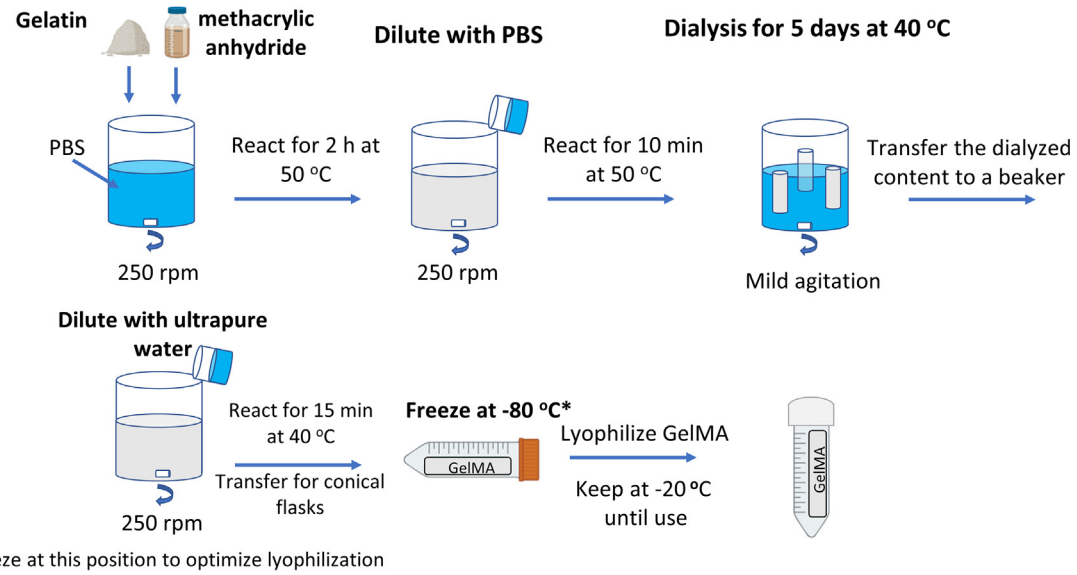



**Figure 2. GelMA synthesis protocol**

19. Click "View" and "Front" on the main menu to observe the stacked objects (Figure S13).
    a. Click "Preview" or "Slice now" buttons to slice the objects.
    b. Use the bar to visualize the split layer. If the layers are not properly sliced, check possible solutions at problem 1.
    c. Click on "Export G-Code" to export the G-code used to control the printer during the bioprinting process.
20. Open the exported file with the option "open with" and choose Notepad.
    a. Press CTRL + F to locate the following line "T1". T1 stands for extruder 2/s syringe.
    b. Add the following steps before "T1" and save the file:

```
''G1 Z25 F3000

G1 X-86.429 Y74.285 F3000

G1 E0.01

Z2.4 F3000''
```

*Note:* Check command positions to avoid contact with the petri dish while changing the needle for the other bioink (Figure S14).

## Major step three: Bioink synthesis

Timing: 7 days

21. Preheat 50 mL of PBS at 50°C using a heating plate (Figure 2 summarizes this procedure).
    a. Cover the top of the Becker with aluminum foil paper to avoid PBS evaporation (Methods video S1).
    b. Let it stabilize for at least 30 min under a fume hood. Complete the following steps in the fume hood.
22. Add 5 g of gelatin from porcine skin and stir it at 250 RPM until it forms a clear solution.
23. Add slowly (dropwise) 0.5 mL of methacrylic anhydride, MA (Methods video S2).

a. Transfer 0.5 mL to a beaker and add it using a plastic pipette.

*Note:* This concentration of MA results in Low GelMA, and higher concentrations can also be used (High GelMA 5 mL of MA, Medium GelMA 2–2.5 mL of MA, Low GelMA 0.5–1 mL of MA).

⚠ CRITICAL: MA is a hazardous material, and it is harmful if swallowed or inhaled and may cause damage to the skin if spilt. For further information, check the FISPQ. Acute Tox. 4 Inhalation - Acute Tox. 4 Oral - Eye Dam. 1 - Skin Irrit. 2 - Skin Sens. 1 - STOT SE 3. Personal safety items recommended: Eyeshields, Faceshields, Gloves, type ABEK (EN14387) respirator filter.

24. Cover the solution with aluminum foil and let it react at 50°C for 2 h.
    a. Monitor the temperature every 15 min to ensure it does not shift more than ± 2°C.

*Note:* A temperature shift higher than the specified might alter the methacrylation degree of GelMA.

25. Hydrate dialysis membrane in deionized water, about 2 pieces of 50 cm each.

*Note:* Test the membranes with deionized water for holes or leaks before use.

26. Preheat 50 mL of PBS and dilute the gelatin-MA solution.
    a. Let it stir for 10 min at the same conditions previously described.
27. With the aid of a glass funnel, pour the solution of gelatin-MA equally into the dialysis membranes.
    a. Close one of the ends before adding the gelatin-MA solution inside the membrane.

*Note:* Let left enough space for air before closing the membrane; the air makes the membrane float and aids homogenization.

28. Dialyze with distilled water at 40°C for 5 days with mild agitation (Methods video S3).

*Note:* Cover the Becker with aluminum foil paper to avoid evaporation. Approximately 2 L of distillate water is enough to remove the unreacted MA completely. Change the distilled water with preheated water twice daily and homogenize the GelMA by flipping it upside-down.

29. At the end of dialysis -5th day- remove the content of the membranes.
    a. Mix it with 100 mL of ultrapure water preheated at 40°C.

*Note:* If more solution is made, it is necessary to dilute it to 50%; in this case, the content of the dialyzed membranes was 100 mL.

    b. Agitate for 15 min with magnetic stirring.
    c. Transfer the solution of GelMA to 50 mL conical tubes and store at −80 for at least 2 days before lyophilization.

*Note:* Adjust the volume in each tube to approx. 25 mL and store them horizontally during freezing to optimize the lyophilization process.

30. Retrieve the flasks from −80°C, remove the taps and add the top of each conical flask tissue paper.
    a. Lyophilizate the reagent for 2 days (Figure S15).
    b. Store the freeze-dried GelMA at −20°C until use.

*Note:* The GelMA is no longer hazardous at this stage and can be handled as a gelatin.

### Major step four: Bioink formulation

**Timing: ~ 4 h before bioprinting**

31. Retrieve GelMA from −20°C.
    a. In a 5 mL flask with a cap, weigh 320 mg of prepared GelMA (8 wt.%).
    b. Mix it with 4 mL of PBS.
    c. Add 20 mg of photoinitiator (0.5% m/v) and dissolve it in a furnace at 37°C for at least 4 h or overnight (Figure S16).
    d. Cover the flask with aluminum foil, light exposure activates the photoinitiator.

*Note:* This concentration will be later diluted to 4 wt.% by adding an equal volume of Geltrex®. For mechanical tests, the GelMA can be prepared directly at 4 wt.%. The Geltrex® is stored at −20°C; therefore, it is recommended to transfer an aliquot to freeze at least 30 min before using it.

32. Filter the solution of GelMA using a syringe and a polypropylene 0.22 μm filter under a laminar flow cabinet.

*Note:* During filtering, part of the material is lost. Therefore, more solution needs to be prepared. Filter GelMA without letting it cool down, this compromises the filtering process.

33. Maintain the filtered GelMA at 37°C using a water bath until its use.

*Note:* Filtered GelMA stored in the fridge can be used within 2–3 days; GelMA mixed with Geltrex® need to be used as soon as possible.

### Major step five: Multi-layered bioprinting

**Timing: ~30 min to 4 h**

*Cell retrieval and resuspension*

34. Retrieve -HUVEC- cells from the incubator, remove the media, and resuspend the cells using a Trypsin/EDTA solution as follows (Figure S17):
    a. Incubate the cells in 1 volume of 0.25% Trypsin/EDTA warm-solution for 5 min. For instance, 1 volume was considered as 5 mL of 0.25% Trypsin/EDTA.
    b. Inactivate the Trypsin/EDTA solution with the same media volume 1:1 (DMEM/F12 supplemented with 10% (v/v) Fetal Bovine Serum and 1% (v/v) Penicillin/Streptomycin). For instance, 1 volume was considered as 5 mL of DMEM/F12 supplemented with 10% of Fetal Bovine Serum and 1% of Penicillin/Streptomycin.
35. Retrieve the filtered GelMA or GelMA/Geltrex® and transfer it to laminar flow cabinet.
36. Remove the supernatant of the cells-trypsin-DMEM-12 solution (Methods video S4).

⚠ CRITICAL: Perform this step carefully to avoid aspirating the cells from the tube. Remove first using a 1000 μL pipette and then a 100 μL pipette.

37. Add the filtered GelMA or GelMA/Geltrex® into the -HUVEC- cells and dissociate the pellet with a slow up-down move.

⚠ CRITICAL: Perform this step carefully to avoid forming bubbles in the cell-bioink solution (problem 2). Cell aggregates or cell clumps can be formed, and in these cases, it is advised to use cell strainers before this step (Methods video S5).

38. Transfer the cells-bioink (HUVEC-bioink) solution to a 5 mL sterile syringe (Methods video S6).
    a. Pipette the solution inside the syringe body.
    b. Carefully insert the syringe plunger.
    c. Remove all the bubbles formed during the process.

*Note:* The user can use a *3-way* adapter or a syringe *luer cap* to facilitate the cell-bioink solution transfer.

39. Add a 22G needle (without a bevel) to the syringe with the cell-bioink solution.

*Note:* Some distributor sells this kind of needle as an irrigation needle. Another alternative is the use of plastic conical syringes.

40. Repeat steps 34–39 for -A549 epithelial- cells to obtain the A549-bioink solution.
41. Transfer the syringes with cell-bioink (HUVEC-bioink and A549-bioink) solutions to the refrigerator for 5 min before starting bioprinting.

*Cell bioprinting*

42. Sterilize bioprinter and mounting parts using 70% (v/v) alcohol and UV for 15 min.

*Note:* If possible, perform the bioprinting inside a flow chamber cabinet or reinforce safety precautions. The bioprinter used in this protocol has 710 × 465 × 600 mm (L x X x H), and due to this size, the bioprinting was performed in a culture room (Figure S18).

43. Turn on the bioprinter and connect it to the computer using a USB cable (Figure S19).
    a. Open the software to control the printer *Pronterface*.
    b. Click on the "House" box or digit "G28" in the command line to set zero for all coordinates.

*Note:* Most *RepRap printers* require zeroing before printing, and without this step, the equipment does not recognize the reference coordinates.

44. Retrieve the syringes with both cell-bioink from the refrigerator (Step 41).
45. At the left syringe holder, insert the syringe with cell-bioink solutions (HUVEC).

⚠ CRITICAL: The bioprinting was done outside a lamellar flow but in a controlled room. Therefore, we recommend only removing the needle protection when inserting a sterile petri dish below it. The petri dish used in this protocol has 1.4 mm of thickness; at this height, the needle faces the petri dish's surface.

46. Adjust its height to 1.4 mm using the following command line:

```
''G1 X0 Y70 Z1.4 F3000''
```

    a. Click on "Send" to send the command to the bioprinter (Figure S20).

*Note:* The bioprinter used in this protocol has a rotating screw that is used to precisely adjust the need of the petri dish surface (Methods video S7).

47. Extrude the bioink until it starts flowing from the needle to the petri dish.
48. Configure the "length" as "0.1" mm for better precision during this step.

*Note:* Use higher values for the first approach of the plunger to the syringe. Use the reverse to put away the plunger from the syringe.

49. Set up the next syringe at right holder-A549-bioink- by lifting the nozzle and setting the extruder 2 to the X-offset position. Use the command line to send the following commands:
    a. Lift the syringe 25 mm by using the command:

```
''G1 Z25 F3000''
```

*Note:* After every command line is necessary to click the button "send".

    b. Translate the X-axis to the right syringe using the command:

```
''G1 X-85 F3000''
```

    c. Active the extruder 2 by using the command:

```
''T1''
```

    d. Move Z to touch the petri dish by using the command:

```
''G1 Z1.4 F3000''
```

50. Repeat step 47.
51. Lift the Extruder 2 and return to the position of step starting position (Extruder 1, HUVEC bioink) by using the following commands line:
    a. Lift the syringe 25 mm by using the command:

```
''G1 Z25 F3000''
```

    b. Translate the X′ axis to the left syringe using the command:

```
''G1 X0 F3000''
```

    c. Active the extruder 1 using the command:

```
''T0''
```

    d. Move Z to touch the petri dish by using the command:

```
''G1 Z1.4 F3000''
```

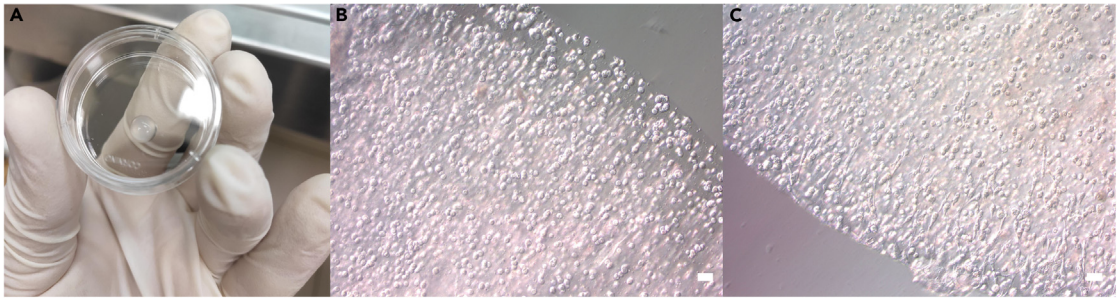



**Figure 3. Visual aspect of a 3D bioprinted scaffold**
(A) Representative image of a bioprinted scaffold.
(B and C) (B) Optical micrographs of the bioprinting construct right after the bioprinting process, and (C) after 7 days under culture. Magnification 10×, scale bar 100 μm.

52. Load the Gcode file saved in step 20 of the Major step two section.
    a. Click on "Load" and select it.
53. Start the bioprinting process by clicking on "Print".

*Note:* Each bioprinting cycle takes approximately 2–3 min (Methods videos S8 and S9).

⚠ CRITICAL: Expose the 3D HUVEC/A549 bioprinted construct to 5 min UV (2 mW/cm$^2$) without the petri dish lid after each printing cycle. The photo-crosslinking efficiency depends on exposure time, the lamp's intensity, and the distance of the light source to the sample. In this case, we performed UV intensity measurements using an ultraviolet meter.

54. Retrieve the 3D HUVEC/A549 construct, cover it with a petri dish lid and store it in the refrigerator for 5 min.

⚠ CRITICAL: This bioink is very soft, and 5 min exposure to UV melts the non-gelified part a little. Therefore, this step aims to improve construct stability before adding cell media.

55. Insert DMEM-F12 media at 37°C from the side of the petri dish and incubate the HUVEC/A54 bioprinted constructs in an incubator for 24 h (5% $CO_2$, humidity of 90% and 37°C).

*Note:* Each petri dish allows 3–4 HUVEC/A549 bioprinted samples; since the syringe axes are fixed, at every finished bioprinting, we only must move the petri dish into a different position to distribute new bioprinted samples (Figure 3).

56. The next day, with the aid of a sterile spatula, retrieve the scaffolds and transfer them to either a 24-well plate and add 1000 μL or to a Transwell® and add 500 μL to the bottom chamber of the well (5% $CO_2$, humidity of 90% and 37°C).
    a. Change culture medium every 2–3 days.

*Note:* Pipette gently as far as possible from the construct to reduce its decomposition. It is normal in long-term cultures that the constructs may degrade so that they can be decomposed if not properly crosslinked or sucked during pipetting. If the construct can be removed, check problem 3.

*Note:* Transwell® inserts can be used to recreate the air-liquid barrier, transfer the sample with care using a spatula and, if necessary, use a 100 μL pipette to aid scaffold positioning inside the insert.

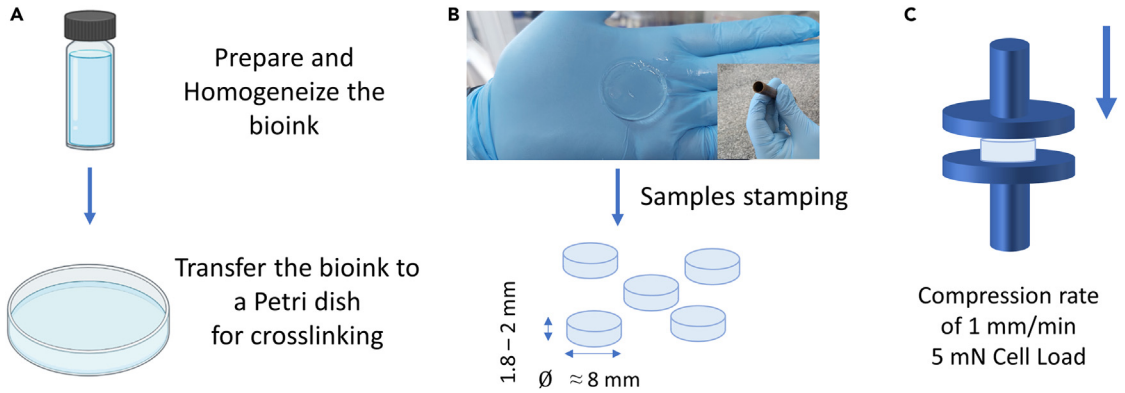



**Figure 4. Scheme of the procedure to fabricate samples for mechanical characterization**

(A–C) (A) Preparation of bioink and transfer to a petri dish, (B) Stamp crosslinked samples with a puncture, and (C) Insert the sample into the equipment and test according to the protocol defined.

## Major step six: Functional assays

Timing: ~1 h

*GelMA mechanical characterization*

57. Prepare a solution of 2 mL of 4 wt.% GelMA and photoinitiator as previously described (Figure 4).
58. With a 1 mL pipette, dispense the solution onto a 35 mm petri dish.

*Note:* The solution needs to be translucid and without bubbles. This size of petri dish was chosen because 2 mL originates samples with 1.8–2 mm thickness.

59. Let it cool down at room temperature for 30 min for gelification.

*Note:* This procedure is not adopted during bioprinting because the bioink becomes too viscous and hinders bioprinting. On the other hand, one can test the bioinks in a more stable condition, reducing errors related to measuring and during testing.

60. Expose the sample to UV for 5 min. Perform this step without the lid (Figure S21).

*Note:* GelMA acquires a slightly light grey aspect due to the gelification and crosslinking process.

a. With the aid of a spatula, remove the photocured sample (Methods videos S10 and S11).
b. Stamp the samples with a puncture (Ø = ~ 8 mm).

*Note:* Perform this step by holding the sample in hand (Figure 4B).

c. Measure 3 points for diameter and height using a Digital Pachymeter.
d. Calculate the average to insert into the mechanical test machine software.

⚠ CRITICAL: Be careful not to smash the samples during this process. The diameter will pretender lower variation due to the stamping process.

61. Insert the sample between the plates and test it on 1 mm/min compression mode.
    a. Set zero compression force, touch the sample with the plate.

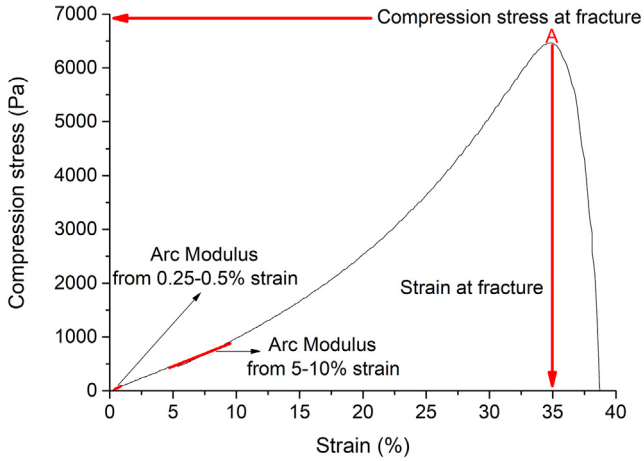



**Figure 5. Representative stress x strain curve of a 4% GelMA hydrogel**
Point "A" stands for the compression deformation at fracture.

b. Apply a Preload force of 10 mN.

⚠ CRITICAL: The preload applied is very important to reduce the reproducible results since if you start testing your sample without it, sometimes even a tiny gap difference may interfere with results. Be careful during the approach of the plate to the sample. Proceed with the test until 90% of the cell load or the sample fracture. Test at least five samples for each composition and analyze the data using Blue Hill software (compression modulus at different strains, compressive modulus, and strain at fracture, Figure 5).

*Viability assay*

Timing: ~1 h

62. Retrieve HUVEC/A549 construct samples.
    a. Transfer them to a confocal dish with the aid of a spatula.
63. Wash the sample 1× with PBS (RT).
64. Dilute the Live/dead reagent at a proportion of 1:10 (v/v) with media.

*Note:* Although the Live/dead protocol states adding the reagent at the top of the sample, we noticed that the dilution does not comprise the analysis and might help cell survival during analysis. Depending on the setup conditions, each image can take 2–10 min to be acquired. If the test is done using Transwell®, use a spatula to remove the samples from Transwell® to optimize the Live/dead reagent.

65. Add 100 μL of the Live/Dead solution and incubate at 37°C for 10 min.

⚠ CRITICAL: Protect samples from light until analysis, and seal them with parafilm to avoid drying.

66. Analyze them using 488 (green) and 594 (red) nm lasers for Calcein AM (488, alive) and Propidium Iodide (594, dead) at a confocal microscope.
67. Determine the cell viability.
    a. Split the image into green -viable- and red -dead- channels.
    b. Calculate the area occupied by each channel.

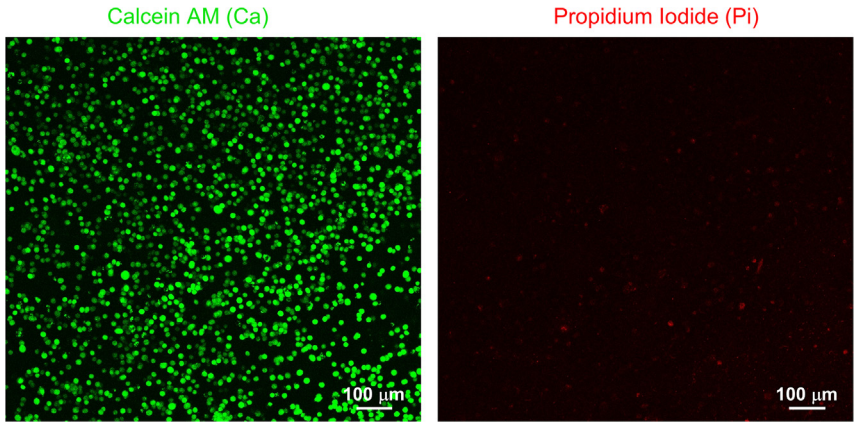



**Figure 6. Live/dead assay after 1 day of culture**
Scale bar 100 μm.

***Note:*** ImageJ® is one of the most accessible software to calculate image properties (Figure 6).

## EXPECTED OUTCOMES

By bioprinting 1 mL of GelMA/Geltrex® bioink and selected cell lines using the cited modeling conditions -a cylinder of 2 mm height and 1 mm diameter with 50% cell-bioink infill- it is possible to fabricate ~25 endothelial-epithelial 3D-HUVEC/A549- constructs.

The GelMA/Geltrex® mixture provides adequate viscosity during all bioprinting processes. The addition of Geltrex® to the HUVEC- and A549-bioink enhanced cell viability for longer periods (>50 days) at standard cell conditions (37°C incubator, 5% $CO_2$). Using this GelMA/Geltrex® mixture we demonstrated that the bioink supported neural cell survival and modulated neural differentiation.[1] Human induced pluripotent stem cells underwent neural maturation for 54 days and showed neuronal markers of differentiation -Tubulin beta-3 chain, TUBB3-. Furthermore, a pilot study in our laboratory using 3D-HUVEC/A549-constructs to recreate a pulmonary barrier with SARS-CoV-2 cell infection suggested that the 3D model improved main virus infection properties -entrance, replication, and inflammatory response- when compared with A549 or HUVEC sole transwell-scaffold models. The 3D model had similar reproducibility of air-liquid barrier properties when compared to the traditional A549 sole transwell-scaffold model (E.H.B., unpublished data).

This multi-layered 3D construct provides a robust model that can be tuned according to the user's demands on cell density, scaffolds infill and shape, and additional mechanical properties. The construct is suitable for the most common cell lines used for research and bioengineering for fabricating multi-layered bioinks to recreate either simple one-cell type models or more complex two- and three-cell type models. In addition, it can be combined with a variety of molecular biology, biochemistry, and pharmacological methodologies.

## LIMITATIONS

The bioprinting workflow can be time-consuming due to several parts involved in this process, as modeling the 3D construct, slicing according to equipment and printing conditions, sterilization of all the printing mountings parts, and also the printer if it is not located exclusively at the cell culture room; therefore all these details need to be taken into advice to assure that the bioprinting process occurs without printing problems or contaminations.

GelMA bioink is very easy to handle; however, it is quite sensitive to temperature, and if not properly crosslinked, it can decompose during longer culture times.

When using primary or human-induced pluripotent stem cells, it is necessary to disperse them in the bioink as kind as possible and longer printing times can lead to cell death.

## TROUBLESHOOTING

Most of the problems discussed below are related to the bioinks preparation; however, some problems can emerge during slicing and the bioprinting process; therefore, we encourage training it several times before trying it with cells.

### Problem 1

Slicing problems

The layers should present empty space between the sliced mesh (Figure S22).

### Potential solution

Revise the slicing configurations and check if "infill density" and "extrusion width" configurations are correct.

### Problem 2

Bubbles

If, during the process of dispersion of the cell-bioink bubbles is formed, the bioprinting process cannot be done properly. Bubbles are stress-concentrating points and might damage cells.

### Potential solution

Centrifuge cell-bioink solution at 300 g for 5 min at 25°C–30°C and proceed with slow up-down pipetting movement. Primary cells are more sensitive to centrifuge than cell lines.

### Problem 3

Non-gelified GelMA

It might be related to crosslinking efficiency if the constructs are not stiff enough to be removed from the petri dish -improper gelified GelMA- (Methods video S12).

### Potential solution

Prepare a new batch of GelMA. Before using this bioink again, perform crosslinking tests on a new GelMA batch, exposing it to a higher crosslinking degree (closer to the light source or increasing the curing time). After this procedure, insert the sample in the fridge for 5 min to consolidate the gelification process and try to handle the constructs.

### Problem 4

Petri dish dragged by the needle

If the distance between the petri dish surface and the needle is too short or the printing table is misaligned, it can cause this problem.

### Potential solution

Adjust the distance of the needle with the petri dish (Methods video S7). Check printing table alignment.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eduardo H. Backes (backes@ufscar.br).

### Materials availability

This study generated a new unique material, GelMA, and the procedure for its fabrication is described in Major Step Three.

### Data and code availability

The codes generated during this study are only applied to 3DBS Printer (Model Octopus) and might be available upon request.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102467.

## ACKNOWLEDGMENTS

This work was supported by the São Paulo Research Foundation (FAPESP), grant numbers 2016/19084-8, 2018/12605-8, 2020/05289-2, 2020/13742-2, 2020/06153-7, and 2021/14327-8; Coordination for the Improvement of Higher Education Personnel (CAPES), financial code 001; and National Council for Scientific and Technological Development (CNPq), grant numbers 406258/2022-8, 311026/2022-2, and 465656/2014-5.

We are grateful for the technical support of the Confocal Microscopy Unit, INFAR/UNIFESP. We thank the Prof. Luiz A. Pessan (DEMa/UFSCar) for the mechanical analysis.

## AUTHOR CONTRIBUTIONS

E.H.B. conceptualized the study, performed the experiments and the analyses, wrote the original draft, and reviewed and edited the document, table, figures, and videos. L.N.Z., L.M.D.G., R.M.R.L., L.A.P., and C.S.B. performed experiments and analyses, wrote the original draft, and reviewed and edited the document, table, figures, and videos. M.A.P. conceptualized the study, advised the execution of the experiments and analyses, and reviewed and edited the final document.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

1. Cruz, E.M., Machado, L.S., Zamproni, L.N., Bim, L.V., Ferreira, P.S., Pinto, L.A., Pessan, L.A., Backes, E.H., and Porcionatto, M.A. (2023). A Gelatin Methacrylate-Based Hydrogel as a Potential Bioink for 3D Bioprinting and Neuronal Differentiation. Pharmaceutics *15*, 627. https://doi.org/10.3390/pharmaceutics15020627.

2. Cui, X., Breitenkamp, K., Finn, M.G., Lotz, M., and D'Lima, D.D. (2012). Direct Human Cartilage Repair Using Three-Dimensional Bioprinting Technology. Tissue Eng. Part A *18*, 1304–1312. https://doi.org/10.1089/ten.tea.2011.0543.

3. Yu, Y., Zhang, Y., Martin, J.A., and Ozbolat, I.T. (2013). Evaluation of Cell Viability and Functionality in Vessel-like Bioprintable Cell-Laden Tubular Channels. J. Biomech. Eng. *135*, 91011. https://doi.org/10.1115/1.4024575.

4. Lee, V., Singh, G., Trasatti, J.P., Bjornsson, C., Xu, X., Tran, T.N., Yoo, S.-S., Dai, G., and Karande, P. (2014). Design and Fabrication of Human Skin by Three-Dimensional Bioprinting. Tissue Eng. Part C Methods *20*, 473–484. https://doi.org/10.1089/ten.tec.2013.0335.

5. Tasoglu, S., and Demirci, U. (2013). Bioprinting for stem cell research. Trends Biotechnol. *31*, 10–19. https://doi.org/10.1016/j.tibtech.2012.10.005.

6. Mandrycky, C., Wang, Z., Kim, K., and Kim, D.-H. (2016). 3D bioprinting for engineering complex tissues. Biotechnol. Adv. *34*, 422–434. https://doi.org/10.1016/j.biotechadv.2015.12.011.

7. Datta, P., Dey, M., Ataie, Z., Unutmaz, D., and Ozbolat, I.T. (2020). 3D bioprinting for reconstituting the cancer microenvironment. NPJ Precis. Oncol. *4*, 18. https://doi.org/10.1038/s41698-020-0121-2.

8. Zhu, W., Holmes, B., Glazer, R.I., and Zhang, L.G. (2016). 3D printed nanocomposite matrix for the study of breast cancer bone metastasis. Nanomedicine. *12*, 69–79. https://doi.org/10.1016/j.nano.2015.09.010.

9. Mouser, V.H.M., Melchels, F.P.W., Visser, J., Dhert, W.J.A., Gawlitta, D., and Malda, J. (2016). Yield stress determines bioprintability of hydrogels based on gelatin-methacryloyl and gellan gum for cartilage bioprinting.

Biofabrication *8*, 035003. https://doi.org/10.1088/1758-5090/8/3/035003.

10. R Ibañez, R.I., do Amaral, R.J.F.C., Reis, R.L., Marques, A.P., Murphy, C.M., O'Brien, F.J., and O'Brien, F.J. (2021). 3D-Printed Gelatin Methacrylate Scaffolds with Controlled Architecture and Stiffness Modulate the Fibroblast Phenotype towards Dermal Regeneration. Polymers *13*, 2510. https://doi.org/10.3390/polym13152510.

11. Wang, Y., Ma, M., Wang, J., Zhang, W., Lu, W., Gao, Y., Zhang, B., and Guo, Y. (2018). Development of a photo-crosslinking, biodegradable GelMA/PEGDA hydrogel for guided bone regeneration materials. Materials *11*, 1345–1348. https://doi.org/10.3390/ma11081345.

12. Klotz, B.J., Gawlitta, D., Rosenberg, A.J.W.P., Malda, J., and Melchels, F.P.W. (2016). Gelatin-Methacryloyl Hydrogels: Towards Biofabrication-Based Tissue Repair. Trends Biotechnol. *34*, 394–407. https://doi.org/10.1016/j.tibtech.2016.01.002.

13. Yue, K., Trujillo-de Santiago, G., Alvarez, M.M., Tamayol, A., Annabi, N., and Khademhosseini, A. (2015). Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. Biomaterials *73*, 254–271. https://doi.org/10.1016/j.biomaterials.2015.08.045.

14. Hazur, J., Detsch, R., Karakaya, E., Kaschta, J., Teßmar, J., Schneidereit, D., Friedrich, O., Schubert, D.W., and Boccaccini, A.R. (2020). Improving alginate printability for biofabrication: establishment of a universal and homogeneous pre-crosslinking technique. Biofabrication *12*, 045004. https://doi.org/10.1088/1758-5090/ab98e5.

15. Ibanez, R.I.R., do Amaral, R.J.F.C., Simpson, C.R., Casey, S.M., Reis, R.L., Marques, A.P., Murphy, C.M., and O'Brien, F.J. (2022). 3D Printed Scaffolds Incorporated with Platelet-Rich Plasma Show Enhanced Angiogenic Potential while not Inducing Fibrosis. Adv. Funct. Mater. *32*, 2109915. https://doi.org/10.1002/adfm.202109915.

16. De Melo, B.A.G., Jodat, Y.A., Cruz, E.M., Benincasa, J.C., Shin, S.R., and Porcionatto, M.A. (2020). Strategies to use Fibrinogen as Bioink for 3D Bioprinting Fibrin-Based Soft and Hard Tissues. Acta Biomater. *117*, 60–76. https://doi.org/10.1016/j.actbio.2020.09.024.

17. Zamproni, L.N., Mundim, M.T.V.V., and Porcionatto, M.A. (2021). Neurorepair and Regeneration of the Brain: A Decade of Bioscaffolds and Engineered Microtissue. Front. Cell Dev. Biol. *9*, 649891. https://doi.org/10.3389/fcell.2021.649891.

18. O'Connell, C., Ren, J., Pope, L., Li, Y., Mohandas, A., Blanchard, R., Duchi, S., and Onofrillo, C. (2020). Characterizing bioinks for extrusion bioprinting: printability and rheology. Methods Mol. Biol. https://doi.org/10.1007/978-1-0716-0520-2_7.