

Supplementary Information:

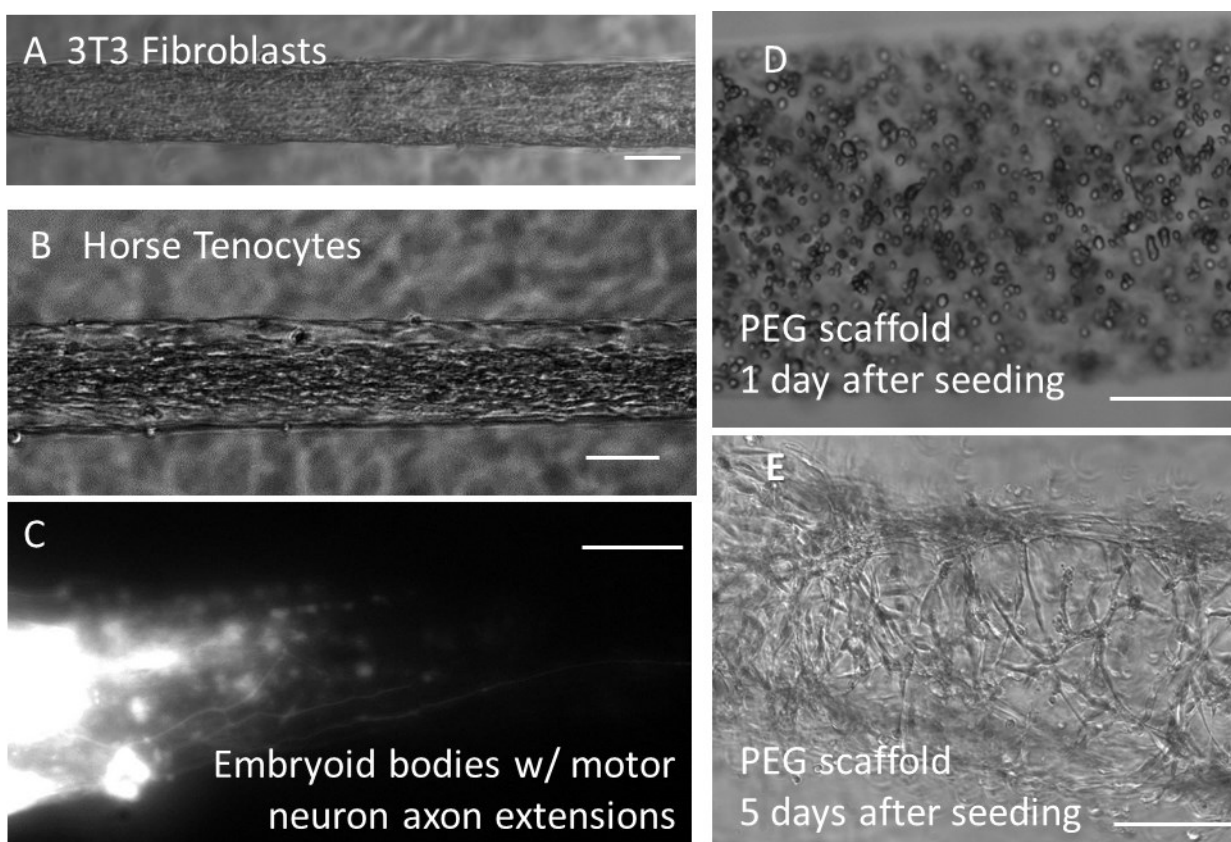


Figure S1. Demonstration of how the presented method may be expanded to a broad class of cells and hydrogels providing a unique approach to the formation of fascicle-like constructs. Phase contrast images of (A) 3T3 fibroblasts and (B) horse tenocytes seeded in fibrin. (C) Fluorescent image of GFP expressing motor neurons with axons extending from embryoid bodies seeded in fibrin. Phase contrast image of C2C12 cells seeded in PEG hydrogel 1 day (D) and 5 days (E) after seeding. Scale bars are 100 μm .

Supplementary Video 1. Contraction of muscle cells grown in fascicle-like construct using presented method.

Supplementary Method Information

Sacrificial Mold Requirement

In order for this method to work, the sacrificial mold material must be solid at the temperature at which the hydrogel solution is injected. If the sacrificial mold material is not solid, no cavity will be formed in which to inject the hydrogel solution. For practical implementation purposes, the ideal temperature for injection is room temperature (20 $^{\circ}\text{C}$).

Additionally, the sacrificial mold material must be liquid at a temperature below a level affecting cell viability, ideally below 37 °C. For implementation purposes, the material should have a viscosity low enough to inject into the seeding wells around the spacer pins without introducing inconsistencies and/or bubbles in the seeding well. If a bubble is introduced that touches the spacer pin, then when the spacer pin is removed, the cavity will include the geometry of the bubble.

Gelatin dissolved in PBS or culture media meets these requirements when dissolved at certain concentrations. Gelatin dissolved at 0.5%, 1.5%, 5 %, 20%, and 30% w/v was used to test for appropriate concentration levels to meet the following criteria: melting temperature below 37 °C, solid at room temperature (20 °C), and implementable viscosity. Melting below 40 °C is a hard requirement for gelatin because the strength and viscosity gradually weaken upon prolonged heating above 40 °C.

A gelatin concentration of 30% did not melt at 37 °C, and could not be injected around the spacer pin. Concentrations of 0.5% and 1.5% were liquid at 37 °C, but do not solidify at room temperature. At these lower concentrations, no cavity was formed upon removal of the spacer pin.

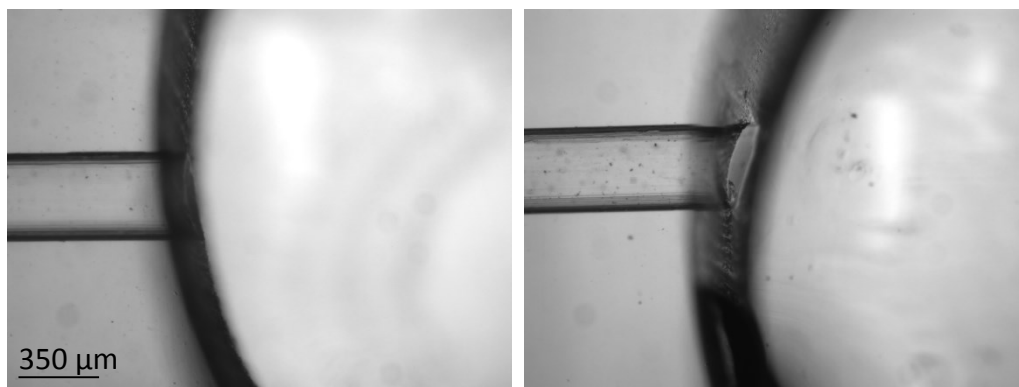


Figure S2. 0.5% (left) and 1.5% (right) gelatin concentrations resulted in no cavity remaining after spacer pin removal. The cavities visible are those inside the PDMS permanent mold.

Concentrations of 5% and 20% met the temperature requirements. After filling the seeding wells, and removing the spacer pins, cavities remained within the gelatin.

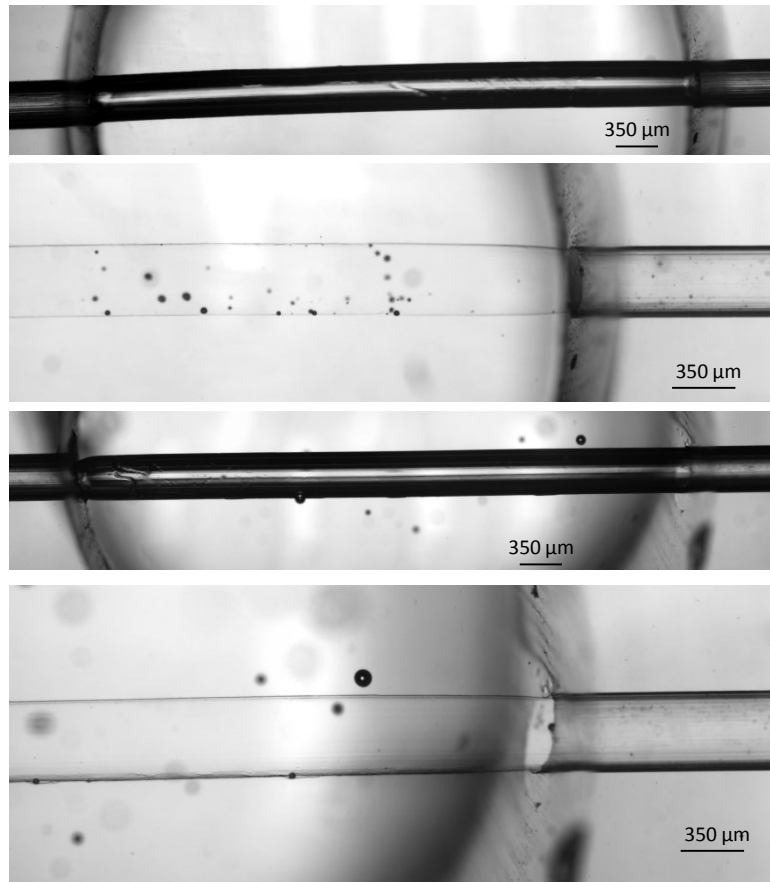


Figure S3. 5% (top 2), and 20% (bottom 2) gelatin concentrations in cavities remaining after spacer pin removal (1st and 3rd from top). Upon injection of another fluid, these cavities are filled (2nd and 4th from top).

It may be worth noting that the filled cavities of the 5% concentration seeding wells are slightly larger in diameter than the 20% concentration seeding wells. This is likely due to the increase in gelatin stiffness at higher concentrations.

The most important difference between 5% and 20% is the difference between their viscosities and how this effects injection in the seeding well. The warm liquid 20% gelatin is much more difficult to work with and results in more inconsistencies/bubbles when filled into the seeding wells than the 5% concentration. The 5% gelatin does not liquefy for several minutes and is easily injected via 200 μl pipette tip. Conversely, the 20% gelatin liquefies readily at room temperature, and even quicker while injecting into the seeding well leaving numerous bubbles and requiring careful removal of the pipette tip so as not to remove the gelatin along with the tip.

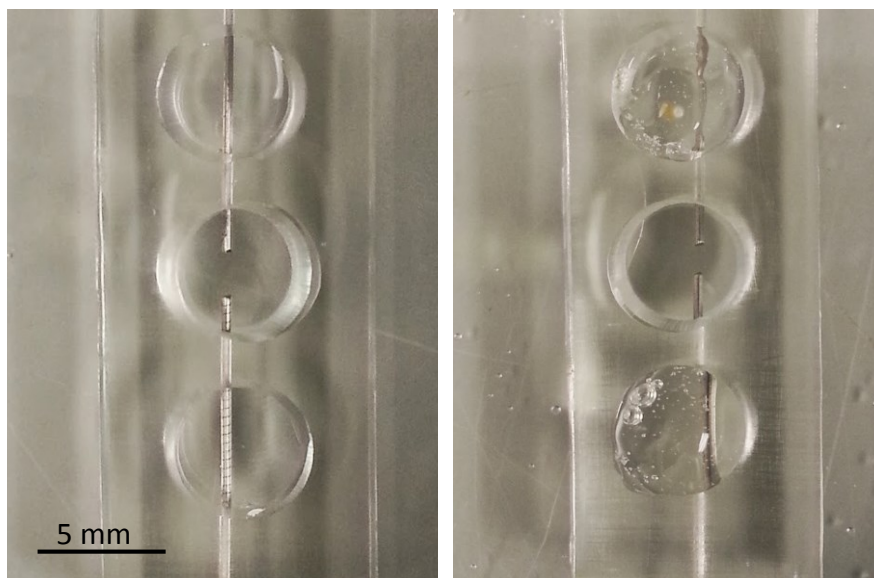


Figure S4. 5% (left) and 20% (right) gelatin concentrations seeded into the top and bottom seeding wells of each device. The 5% gelatin seeding wells are clear and without bubbles. The 20% gelatin seeding wells contain bubbles and have inconsistent texture resulting from tip removal.

In conclusion, when gelatin dissolved in an aqueous solution is used as the sacrificial mold material, w/v concentrations between 5% and 20% should be used. Within this range, the melting point is between 20 °C and 37 °C. This allows for a solid sacrificial mold when seeding and release of the sacrificial mold when incubating. Additionally, concentrations at the lower end of this range are easier to work with, however gels at this concentration are less stiff affecting resulting hydrogel geometry slightly.

Gelling reagent release from sacrificial mold

A significant advantage to using the sacrificial mold comes from being able to dissolve additional reagents within the sacrificial mold material. These reagents are released into the final gel solution as the sacrificial mold melts. It is particularly useful to dissolve a gelling reagent into the sacrificial mold material. To form a fibrin gel strip using this method, first thrombin is dissolved into the sacrificial mold, next fibrinogen solution is injected into the cavity formed by removing the spacer pin by hand from the sacrificial mold, and then the thrombin is released into the fibrinogen solution as the sacrificial mold melts. This process creates a uniform strip of fibrin spanning the seeding well.

An alternative method is to not include thrombin in the sacrificial mold and mix the thrombin and fibrinogen solution immediately before injecting. There are 2 significant drawbacks to using this alternative method arising from the quick onset of gelation once thrombin is mixed with fibrinogen; 1) there is only time enough to fill a single channel after mixing, and 2) the fibrin strip formed will be non-homogeneous. The first issue, that there is only time for one channel to be filled for each mixing, adds greater deviation from one strip to

the next because of the slight differences in mixing and increased time difference between injections. This time difference is especially important for larger experiments with many strips. Additionally, from a practical standpoint, this adds time and tedium to the process. Conversely, dissolving thrombin in the sacrificial mold allows for rapid filling of many channels. The second issue, that the fibrin strip is non-homogenous, results from the gel forming as it is being injected. These nonuniformities may generate randomly distributed stress concentrations affecting cell behavior.

To demonstrate these drawbacks, four conditions were tested. For every condition, 5% w/v gelatin in PBS was used for the sacrificial mold, and 5 mg/ml fibrinogen in PBS was injected. In the first condition, thrombin was dissolved in sacrificial molds. For the second condition, 120 μ l of fibrinogen solution was mixed with thrombin via pipetting resulting in a solution of 5 mg/ml fibrinogen and 4 U/ml thrombin, and this solution was injected into the sacrificial mold cavity. The third condition was the same as the second, except 20 μ l was used instead of 120 μ l. The fourth condition was a control with no thrombin. All four conditions were immediately warmed in a 37 °C incubator after injection of the fibrinogen solution.

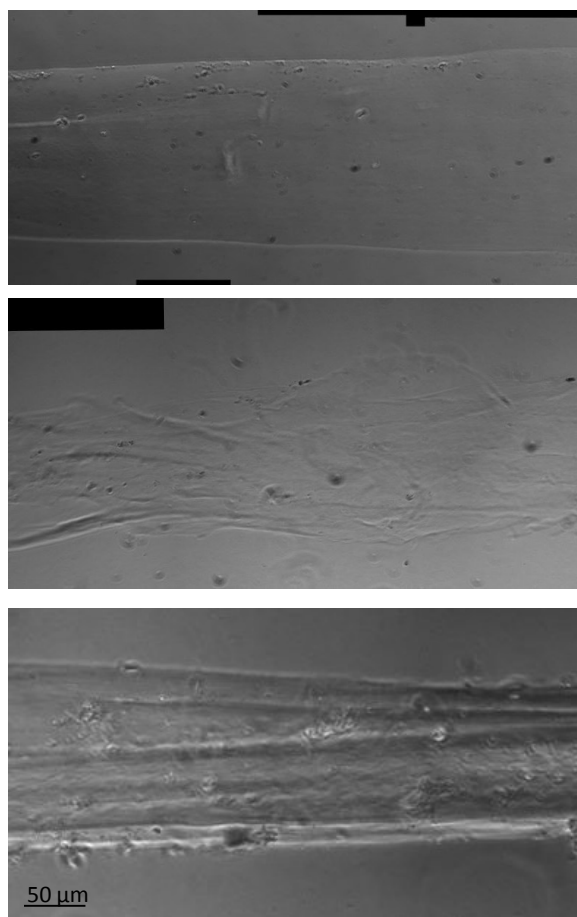


Figure S5. Strips of fibrin formed using different methods to incorporate thrombin. Thrombin dissolved in the gelatin sacrificial mold (top), thrombin mixed with fibrinogen for 120 μ l of solution (mid), and thrombin mixed with fibrinogen for 20 μ l of solution (bottom).

The control condition with no thrombin formed no fibrin strip as expected. The condition incorporating thrombin in the sacrificial mold formed strips that were visibly uniform. The fibrin strips generated by first mixing the thrombin and fibrinogen before injection were visibly nonuniform.

Producing Longer Constructs

Sagging of the tissue may occur for longer constructs; however this is not a significant limitation. The tissue is only slightly denser than the surrounding media. Tissues have been made that are 1 cm long with no noticeable sagging as shown in Fig. S6. Sagging has not been significant enough for us to even consider the issue at this scale. It may become noticeable at the 10 cm scale. However, if the seeding well is simply made deeper to accommodate whatever sagging occurs, then the tissue will not contact any surface, and the axial tension will still exist.

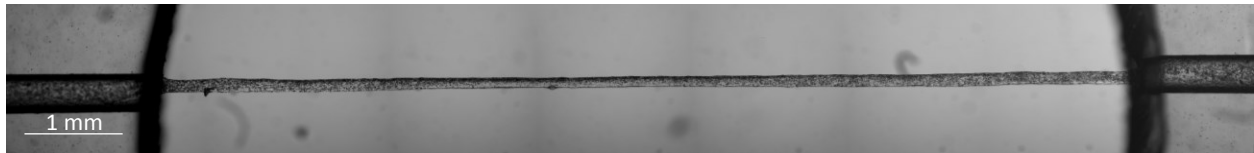


Figure S6. A 10 mm long tissue construct made with cardiac cells. There exists no noticeable sagging of the tissue.