MODULE 1: Depositing fluorescent beads into paper scaffolds

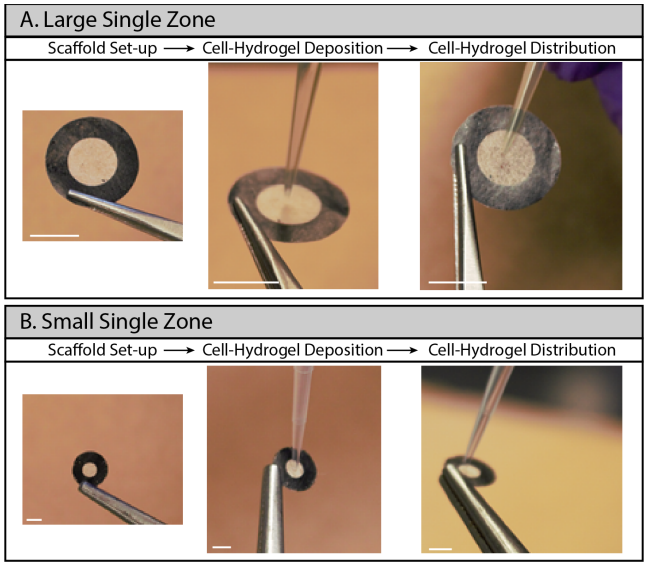

Figure 2. Demonstration of the deposition of cell-laden collagen into a A) Large Single Zone (3.14 mm^3 culture area) and B) Small Single Zone (0.22 mm^3 culture area). The scale bars represent A) 10.00 mm and B) 2.65 mm.

Materials:

1X DPBS (Gibco no. 14190-144)
1 M NaOH (see recipe)
10X phosphate buffered saline solution (PBS, See recipe)
70% ethanol (see recipe)
CellTiter-Glo 2.0 (CTG 2.0) reagent (Promega, no. G9241)
Collagen I, high concentration, rat tail (Corning, no. 354249)
DI water
Fluoresbrite YG Microspheres, 10.0 µm (Polysciences, no.18140)
High glucose DMEM with supplements (see recipe)
CellTracker Green CMFDA (Fisher Scientific, no. C2925)
T-150 culture flask of fluorescent HepG2 cells
Trypan blue, 0.4% aqueous solution (Sigma-Aldrich, no. T8154)
TrypLE Express (Gibco, no. 12605-028)

1.5 mL microcentrifuge tube (Fisher Scientific, no. 05-408-129)
5.75" Pasteur pipettes (Fisher Scientific, no. 13-678-6A)
10 cm plastic petri dish, untreated (Fisher Scientific, no. S33580A)

Aven 3rd hand with tweezers (Amazon, no. B006RB93IG)
Benchtop 15 mL centrifuge

Biosafety cabinet
Inverted microscope
Cell counter
Flatbed fluorescence scanner
Ice bucket
Inverted fluorescence microscope with camera
Microcentrifuge
Micropipettes and pipette tips
pH paper, pH 6.0-8.0 (Fisher Scientific, no. 13-640-510)
Plate reader capable of fluorescence and chemiluminescence readouts
Pre-sterilized small paper scaffolds

***Steps**:*

1. Transfer the pre-sterilized small single zones, as well as a 10 cm untreated Petri dish, into the tissue culture hood.

2. Calculate the total volume of fluorescent beads (V_{Beads}) needed to deposit 1,000 beads per zone with Eqns. 1 and 2, where C_{dep} is the concentration of beads deposited (2.00 x 10^3 beads/mL) and C_{beads} is the concentration of beads in the stock solution (4.55 x 10^7 beads/mL). For each small single zone (N_{zones}), 0.5 µL of suspension is required. A 1.5x factor in the deposition volume (V_{dep}) accounts for loss during trituration.

$$V_{Beads} = \frac{C_{dep} \times V_{dep}}{C_{beads}} \quad \text{Eqn. 1}$$

$$V_{dep} = 0.5\,\mu L \times N_{scaffolds} \times 1.5 \quad \text{Eqn. 2}$$

   Note. The 1.5x additional factor in the equation is to ensure there is enough volume when depositing and to account for loss during mixing. Triturate the solutions slowly to avoid introducing bubbles.

3. Calculate the volume of collagen needed to prepare both the bead suspension (V_{dep}) and blank scaffolds ($V_{dep,\ blank}$) with Eqns. 3 and 4. The 1.5 factor is to match Eqn. 2 and ensure there is enough collagen to deposit beads into all the scaffolds needed for the experiment.

$$V_{dep} = 1.5 \times \left(N_{scaffolds} \times 0.5\ \mu\text{L}\right) \quad \text{Eqn. 3}$$

$$V_{dep,\ blank} = 1.5 \times \left(N_{scaffolds} \times 0.5\ \mu\text{L}\right) \quad \text{Eqn. 4}$$

   Note. $V_{dep,\ blank}$ are scaffolds that only contain collagen. These blank scaffolds are needed during analysis.

4. Calculate the volume of each component needed to prepare a 1.2 mg/mL solution of collagen I with Eqn. 5 or 6.

$$V_{total} = V_{dep} + V_{dep,blank} \quad \text{Eqn. 5}$$

or

$$V_{total} = V_{stock\ collagen} + V_{PBS} + V_{NaOH} + V_{water} \qquad \text{Eqn. 6}$$

In these equations, $V_{stock\ collagen}$ is the volume of collagen stock (Eqn. 7), V_{PBS} is the volume of a 10X PBS solution (Eqn. 8), V_{NaOH} is the volume of a 1 M solution of sodium hydroxide (Eqn. 9), and V_{water} is the volume of deionized water (Eqn. 10).

$$V_{stock\ collagen} = \frac{(V_{total} \times 1.2\ mg/mL)}{C_{stock\ collagen}} \qquad \text{Eqn. 7}$$

$$V_{PBS} = \frac{V_{total}}{10} \qquad \text{Eqn. 8}$$

$$V_{NaOH} = V_{stock\ collagen} \times 0.025 \qquad \text{Eqn. 9}$$

$$V_{water} = V_{total} - (V_{stock\ collagen} + V_{PBS} + V_{NaOH}) \qquad \text{Eqn. 10}$$

Note. The exact concentration of the collagen stock is lot dependent.

5. Transfer V_{Beads} determined with Eqn. 1 to a 1.5 mL microcentrifuge tube and centrifuge at 1000 xg for 5 min.

6. Prepare the collagen suspension using the volumes calculated in Eqns. 7 - 10. First, combine the DI water, 10X PBS solution, and 1 M NaOH solution to an appropriate volume tube, mix by trituration, and then add the concentrated suspension of collagen I. Keep the prepared collagen solution on ice.

   Note. The collagen suspension is viscous, pipette slowly to ensure transfer of the appropriate volume.

7. Mix the newly prepared collagen suspension thoroughly by trituration. Place a small volume (< 5 µL) on a piece of pH paper. If the pH is not approximately 7.2, add 1 M NaOH until the correct pH is reached. Keep collagen on ice.

8. Aspirate the supernatant from the fluorescent beads.

9. Add V_{dep} calculated in Eqn. 3 to the fluorescent beads and mix until the suspension is homogenous.

   Notes. Mixing is a critical step to ensure the same number of beads/cells are deposited into each paper scaffold. Trituration, scraping the bottom and sides of the microcentrifuge tube, and mixing an XYZ-circular motion are all necessary to make a homogenous mixture. Once mixed, the collagen-bead suspension should appear yellow in color, like the beads.

   Take care to hold the tube by the top while mixing, as your hands will warm the collagen and accelerate gelation.

The mixing step is one of the first places to troubleshoot the deposition protocol if there is a large deviation between replicates.

10. Place 10 mL of fresh, 37 °C, 1X PBS into a 10 cm untreated petri dish.

11. Deposit the collagen-bead suspension into the paper scaffolds. For the small single zones, deposit 0.5 µL with the 2.5 µL micropipette. Place the pipette tip just above the center of the paper scaffold, slowly dispense the contents and allow it to wick into the paper and continue dispensing until you reach the first stop of the micropipette.

    Spread the collagen, moving in a circular motion from the center toward the wax borders. Ensure there are no 'dry' spots that contain no collagen. Once the volume has been spread evenly across the scaffold, dispense to the second stop of the pipette.

    Note. When depositing the bead-collagen suspension orient the paper perpendicular to the bench surface and hold it in place using a single "third hand" device, as demonstrated Figure 2.

12. Allow the scaffold to dry for approximately 30 sec before transferring it to the Petri dish prepared in Step 10.
    Note. Selecting an appropriate and consistent drying time is extremely important for obtaining reproducible datasets. If the scaffolds gelate for too long, they can dry out and reduce the overall cell viability. If the scaffolds do not gelate enough, the collagen might not fully crosslink, causing the beads/cells to fall out of the scaffolds.

    The collagen is sufficiently dry when it is no longer shiny in appearance, but the cell-containing region is clearly still wet. Watch the first few scaffolds closely and choose a consistent drying time based on your observations. The small scaffolds require approximately 30 sec - 1 min to dry.

13. Repeat Steps 11 and 12 until 20 scaffolds have been deposited with fluorescent beads.

14. Deposit three zones with 0.5 µL of bead-free collagen. These scaffolds will serve as blanks to compare seeded fluorescence intensity and account for ECM autofluorescence.

15. Using the inverted microscope, check each scaffold to ensure the beads remained in the scaffold before continuing.

    Note. The beads look like black dots under the microscope. The dots should be evenly distributed throughout the scaffolds with little-to-no beads in the wax boarder. Check the Petri dish to determine if beads fell out of the scaffolds. Small numbers of beads in the Petri dish PBS is normal. If there are equal numbers of beads in the dish and the paper scaffolds, then the gelation time was too short.

16. Incubate the scaffolds for 4 h at standard culture conditions.

Measuring the fluorescent beads in paper scaffolds

1. In the tissue culture hood, transfer the scaffolds to a fresh 10 cm untreated Petri dish containing 10 mL of 1X PBS. Carefully aspirate PBS, so scaffolds are in the same focal plane.

2. Collect brightfield and fluorescence images of each scaffold using the flatbed scanner, gel imaging system, or fluorescence microscope.

   Notes. If using the Fluoresbrite YG microspheres, the excitation and emission wavelengths are 440 and 485 nm, respectively.

   For microscope capture, ensure the exposure and focus is the same for each scaffold.

Analyzing the fluorescence images

1. Export images and analyze them with desired analysis software (e.g., Fiji or ImageJ).

2. Inspect each scaffold to ensure there is a uniform distribution of fluorescence signal, and there are no regions of high and low (or no) fluorescence intensity, indicating a non-uniform spread during the deposition process. Visible differences in fluorescence are not acceptable, and the procedure (Protocols S2.1 a and b) must be repeated.

3. Quantify the distribution of beads by obtaining a 3x3 matrix of fluorescence intensity on each scaffold. Calculate the average, standard deviation, and percent relative standard deviation (%RSD) of each scaffold. The %RSD within each scaffold should be less than 15%.

4. Quantify the average fluorescence intensity of the entire culture region in each scaffold to ensure reproducible deposition of beads. On a scaffold, make a circle object that is just smaller than the size of the cell culture zone. Using this circle, obtain the average intensity for all 20 scaffolds. Calculate the average, standard deviation, and %RSD of the dataset. The %RSD should be below 20%.

   Note. Use the same-sized circle when obtaining intensity measurements

5. To ensure the fluorescence intensity measurements are from the beads, calculate the signal-to-noise ratio. On a scaffold, make a circle object that is just smaller than the size of the cell culture zone, then calculate the average intensity of the bead containing

scaffolds and blank scaffolds. Divide the average fluorescence intensity of the bead-containing scaffolds by the average fluorescence intensity of the blank scaffolds. This value should be greater than 3.

6. Repeat this protocol on various days. The training set is complete when less than 20% %RDS is achieved on three separate deposition trials.