MODULE 3: Generating cell-laden calibration curves

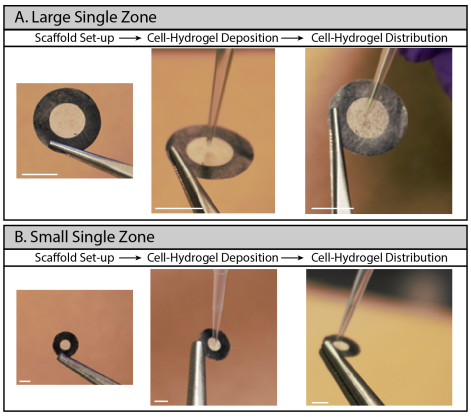

Figure 1. Demonstration of the deposition of cell-laden collagen into a A) Large Single Zone (3.14 mm^3 culture area) and B) Small Single Zone (0.22 mm^3 culture area). The scale bars represent A) 10.00 mm and B) 2.65 mm.

***Materials**:*

1X DPBS
10 cm untreated petri dish
96-well plate
High glucose DMEM with supplements
T150 flask of fluorescently labeled HepG2 cells (transfected or labeled)
TrypLE express

Pre-sterilized small single zones
15 mL conical tube
1.5 mL microcentrifuge tube (Fisher Scientific, no. 05-408-129)
96-well plate

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. In the Biosafety cabinet, detach the fluorescently labeled HepG2 cells from the plate using TrypLe express and count following standard culture techniques. Record the cell concentration as cells/ mL (C_{stock}).

2. Determine the volume of the stock cell suspension required ($V_{Cell\ stock}$) required to obtain 4.0×10^6 cells with Eqn. 72. This is enough stock to make 40 scaffolds containing 1.0×10^5,

$$V_{Cell\ stock} = \frac{4.0 \times 10^6,}{\left(C_{stock}\frac{cells}{mL}\right)} \quad \text{Eqn. 1}$$

3. Calculate the volume of collagen needed to prepare both the cell suspension ($V_{Cell\ dep}$) and blank scaffolds ($V_{dep,\ blank}$) with Eqns. 2 and 3.

$$V_{Cell\ dep} = (40 \times 0.5\ \mu L) \quad \text{Eqn. 2}$$
$$V_{dep,\ blank} = (5 \times 0.5\ \mu L) \quad \text{Eqn. 3}$$

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. 4 or 5.

$$V_{total} = V_{Cell\ dep} + V_{dep\ ,blank} \quad \text{Eqn. 4}$$

or

$$V_{total} = V_{stock\ collagen} + V_{PBS} + V_{NaOH} + V_{water} \quad \text{Eqn. 5}$$

In these equations, $V_{stock\ collagen}$ is the volume of collagen stock (Eqn. 6), V_{PBS} is the volume of a 10X PBS solution (Eqn. 7), V_{NaOH} is the volume of a 1 M solution of sodium hydroxide (Eqn. 8), and V_{water} is the volume of deionized water (Eqn. 9).

$$V_{stock\ collagen} = \frac{(V_{total} \times 1.2\ mg/mL)}{C_{stock\ collagen}} \quad \text{Eqn. 6}$$
$$V_{PBS} = \frac{V_{total}}{10} \quad \text{Eqn. 7}$$
$$V_{NaOH} = V_{stock\ collagen} \times 0.025 \quad \text{Eqn. 8}$$
$$V_{water} = V_{total} - (V_{stock\ collagen} + V_{PBS} + V_{NaOH}) \quad \text{Eqn. 9}$$

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

5. Transfer $V_{Cell\ stock}$ 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. 6 - 9. 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 the 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 cell pellet and add the volume of collagen calculated in Eqn. 2.

9. Place 1 mL of medium into 9 wells of the 12-well plate. Label the wells as 100k, 50k, 25k, 12.5k, 6.25k, 3.125k, 1.562k, 781, and 0 cells/zone.

10. Resuspend the cell pellet in Step 10 with the volume of collagen calculated in Step 5 ($V_{Cell\ dep}$). Mix the cell pellet by triturating 30 times while mixing in an XYZ-circular motion resulting in a homogenous mixture is formed.

    Notes. Mixing is a critical step to ensure the same number of 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. Take care not to introduce bubbles, as they could harm the cells. Mix gently as not to kill the cells.

    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 large deviation between replicates.

11. Deposit the collagen-cell 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. Deposit cells into four scaffolds.

Note. When depositing the cell-collagen suspension, orient the paper perpendicular to the bench surface and hold it in place using a single “third hand” device, as demonstrated Figure 1.

12. Allow the scaffold to dry for approximately 30 sec before transferring it to the appropriate well in the 12-well plate.

    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. Dilute the cell-laden collagen by mixing 10 µL of the cell suspension with 10 µL of fresh collagen in a fresh 1.5 mL microcentrifuge tube.

14. Repeat steps 10-13 until a cell density of 781 cells/zone is reached, a total of 7 times.

15. Deposit three zones with 0.5 µL of cell-free collagen. These zones will serve as a reference to account for the background fluorescence of the collagen.

16. Using the inverted microscope, check each scaffold to ensure there are cells in the scaffold before continuing.

    Note. The deposited cells look like clear spheres under the microscope, they should be packed throughout the scaffold's “culture area.” Also, look at the medium for any cells that may have fallen out of the scaffolds. Some cells in the media is normal. If there are equal numbers of cells in the medium as there are in the paper scaffolds, then the gelation time was too short.

Measuring the fluorescence intensity of the paper scaffolds

1. Transfer the cell-containing scaffolds to a fresh clear bottom 96-well plate with 100 µL of 1X DPBS. Using a pair of tweezers, carefully place the scaffolds on the surface of the PBS, so they float at the air-liquid interface.

   Note. The DPBS is used to ensure the scaffolds stay hydrated while preparing other scaffolds and the monolayer cells.

2. Aspirate the medium from the monolayer 96-well plate and the DPBS from the 3D 96-well plate just before analysis. If fluorescence measurements need to be taken sequentially (i.e., microscope, well plate reader), aspirate medium/DPBS from the plates one at a time.

   Note. When aspirating from the paper scaffold-containing wells, do not disturb the paper scaffold floating at the air-liquid interface. A pipette can be used to remove DPBS slowly and gently

3. Measure the fluorescence intensity of each scaffold- and monolayer-containing well, and blank scaffolds or wells, with the flatbed scanner, well plate reader, or a fluorescence microscope. Collect a white light and fluorescent image of each well.

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

Measuring the relative luminescence units per cell in the calibration curve paper scaffolds with the CellTiter-Glo 2.0 assay

1. Aspirate any residual culture medium or DPBS from each well after measuring fluorescence and replace with 100 µL of 1X DPBS. Add 100 µL of 1X DPBS to blank scaffolds and wells.

2. Add 100 µL of the CTG 2.0 reagent to each cell-containing scaffold and well. Add 100 µL of CTG2.0 to blank scaffolds and wells.

3. Place the well plate on an XY orbital shaker and shake at room temperature for 15 min at about 750RPM.

   Note. Ensure the medium is thoroughly mixed. Observe the scaffolds and well plates with an inverted microscope to ensure all the cells are lysed.

4. Transfer 100 µL of the cell lysate solution to an opaque white 96-well plate. Analyze on a well plate reader with a luminescence readout.

   Notes. If bubbles are present, use a 22G needle to pop them prior to analyzing the plate in the plate reader. To best visualize bubbles, Tilt plate 30° toward you and inspect along the top edge of each well.

   We measure luminescence by capturing all photons in the range of 300-850 nm.

Analyzing the fluorescence of the paper scaffolds to construct a calibration curve

1. Export images from step 3 above and analyze 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 must be repeated.

3. Quantify the distribution of cells 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 ($\bar{I}$) of the entire seeded region in each scaffold to ensure reproducible deposition of different cell densities. 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 three replicate scaffolds. Calculate the average ($\bar{I}$), standard deviation, and %RSD of the dataset. The %RSD should be below 20%.

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

5. To ensure the fluorescence intensity measurements are from the cells, calculate the signal-to-noise ratio. On a blank scaffold, make a circle object that is just smaller than the size of the cell culture zone to calculate the average intensity ($\bar{I}_b$). Divide the average fluorescence intensity of the cell-containing scaffolds (Step 4) by the average fluorescence intensity of the blank scaffolds. This value should be greater than 3 at all cell densities.

6. To construct a calibration curve based on fluorescence intensity, subtract the average blank intensity ($\bar{I}_b$) from the each of the measured cell-containing scaffold intensities (I) using Eqn. 10 to obtain the background subtracted intensity (I_{BS}).

   $$I_{BS} = I - \bar{I}_b \qquad \text{Eqn. 10}$$

7. Calculate the average background subtracted ($\bar{I}_{BS}$) signal and standard deviation for each cell density.

8. Construct a calibration curve plotting $\bar{I}_{BS}$ vs deposited cell number. Perform a linear regression line for the dataset and calculate the correctness of this fit (R^2 value).

9. Ensure the %RSD at each cell density is ≤20% and an R^2 ≥0.975 is achieved on three separate depositing trials.

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Analyzing the fluorescence of the paper scaffolds to construct a calibration curve

1. Export the luminescence data collected above.
2. Subtract the average background (I_b) from each of the cell samples.
3. Calculate the background subtracted average (I_{BS}), standard deviation, and %RSD for each of the cell densities.
4. Invert the average background correct luminescence signal (I_{BS}) with Eqn. 11.

$$Inverted\ signal = \frac{1}{I_{BS}} \qquad \text{Eqn. 11}$$

5. Plot the calibration curve as $\frac{1}{I_{BS}}$ vs $\frac{1}{cell\ density}$, perform a linear regression line to the dataset, and calculate the correctness of this fit (R^2 value).

   Note. The data for the CellTiter-Glo 2.0 assay is transformed to account for the turnover rate of the luciferin to oxyluciferin by the luciferase enzyme used in this assay; this plot affords a single linear range from 781-100,000 cells per zone.

6. Ensure the %RSD at each cell density is ≤20% and R^2 ≥0.975 is achieved on three separate depositing trials. Once this is achieved, training is complete.