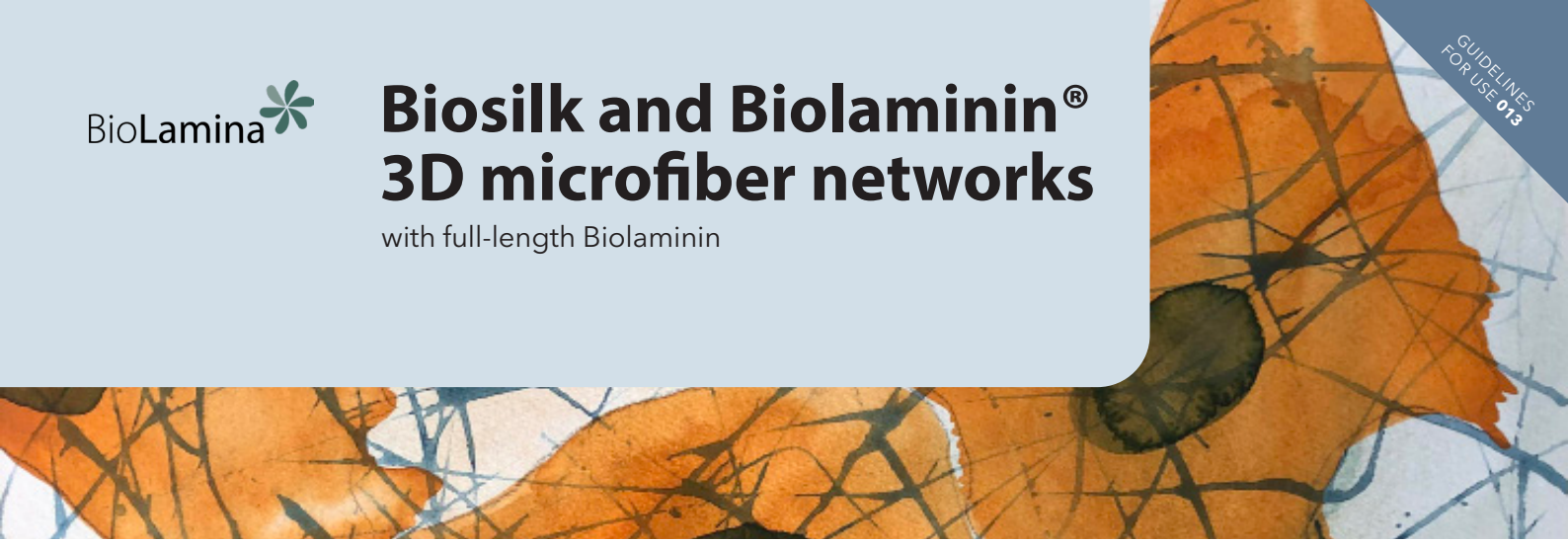
BioLamina

Biosilk and Biolaminin® 3D microfiber networks

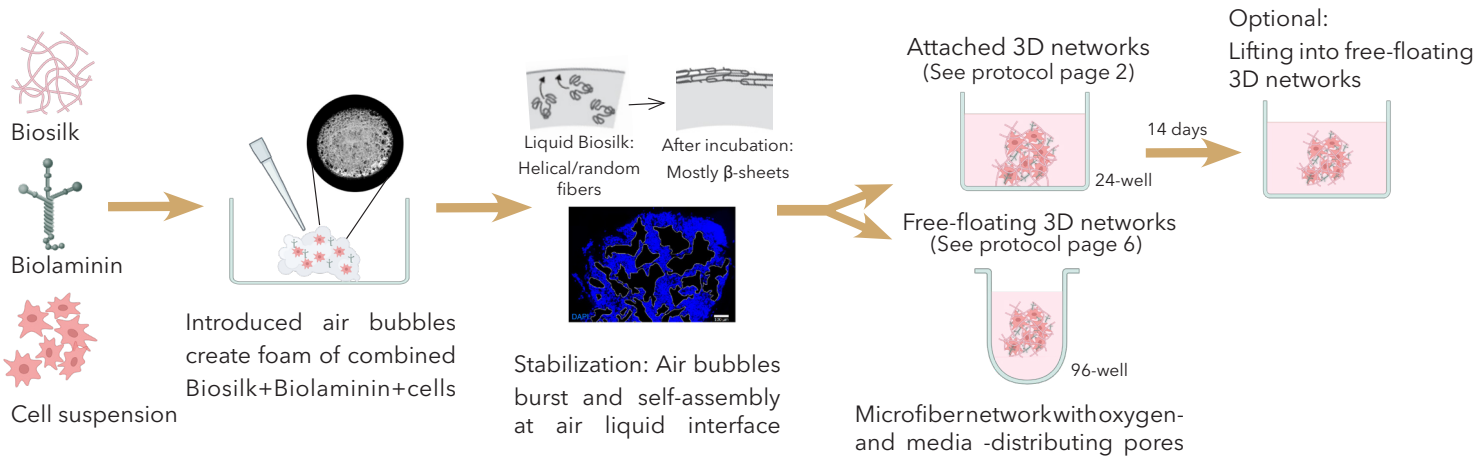
with full-length Biolaminin

GUIDELINES FOR USE 013



Biosilk is a fully defined recombinant biomaterial that self-assembles into microfiber scaffolds, enabling robust and reproducible 3D culture applications, including long term organoid formation. Functionalization with Biolaminin LN allows Biosilk to be tailored to recreate tissue specific microenvironments for various cell types. For human pluripotent stem cells (hPSCs), Biosilk 521(Biosilk functionalized with Biolaminin 521 LN) provides an optimal 3D matrix for cell integration, proliferation, and lineage-specific differentiation. The addition of Biolaminin to the Biosilk scaffold also promotes cell migration which allows efficient morphogenesis during differentiation.

Protocol overview: Create attached or free-floating Biosilk+Biolaminin microfiber networks



Important notes

- Biosilk is available as a recombinant protein (Biosilk, BS-0101) or as a pre-mixed solution with Biolaminin 521 LN (Biosilk 521, BS521-0101).
- Defined biomaterial, together with Biolaminin, forms niche-specific microfiber scaffolds for robust 3D culture applications, such as long-term organoid cultures.
- Thaw gently at RT without moving the vial. Do not vortex or shake the vial, and mix carefully to avoid the introduction of air bubbles.
- Thawed Biosilk has to be used within 45 minutes, or as soon as possible. Re-freezing or long-term fridge storage is not recommended and will result in decreased foaming efficiency.
- All steps must be carried out under aseptic conditions. For research use only.

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Attached Biosilk and Biolaminin organoids (24-well format)

The following protocol is a guideline for blending hPSC with Biosilk 521, establishing attached Biosilk organoids for 3D culture in 24-well plates.

Important notes

1 vial of Biosilk+LN or Biosilk 521 is enough material to generate 12-13 foams (24-well format)

Material	Comment
24-well hydrophobic surface plate	Validated surface is Sarstedt cat no 83.3922.500. Not all hydrophobic surfaces for suspension cell culture are suitable for foam attachment
Culture media	Pluripotent culture medium and/or differentiation medium of choice
ROCK inhibitor	Use e.g. Y27632
Pipettes	Pipettes for volumes ranging from 1 µL to 1000 µL
Blade or surgical scissors	For cutting the cell-propagated-foam into the desired size and shape
Cell scraper	For detaching the cell-propagated-foam from well bottom to form a floating organoid
General lab ware for cell culturing	E.g. sterile tubes and pipette tips

Procedure in summary

- Typically 20 000 - 60 000 cells/µL per replicate are needed, for the total volume of 22 µL for each Biosilk/Biolaminin (or Biosilk 521) foam scaffold replicate.
  - o Using Biosilk/Biolaminin: The stock mix is created by adding the cell suspension, Biolaminin LN and Biosilk
  - o Using Biosilk 521: The stock mix is created by adding the cell suspension and Biosilk 521
- Droplets of 22 µL Biosilk/Biolaminin/cell suspension (or Biosilk 521/ cell suspension mix are placed at the bottom of each well. Repeat for all foam scaffold replicates.
- A dense foam scaffold is created by introducing air bubbles by rapid pipetting, up and down into the Biosilk/Biolaminin/cell mix.
- Incubate the cell-containing foams at 37 °C for 20 min to stabilize the structure and add media afterwards.
- When reaching the desired cell confluence, differentiation can be initiated by the addition of differentiation medium of choice.
- Generate a floating organoid culture system by lifting the foam scaffolds from the bottom of the well with a cell scraper around day 6-15

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Protocol

FIG 1. ONE-STEP APPROACH FOR BIOSILK FOAM GENERATION

1 Thaw the Biosilk solution at RT without moving the vial.

Note:

! Do not vortex or shake the vial and avoid the introduction of air bubbles.

! It will take around 12 min for the frozen Biosilk solution to thaw at ambient temperature. For best result, the thawed solution should be used as soon as possible, latest within 1 hour from removal from -80 °C. The thawed Biosilk solution will gradually turn milky in ambient RT.

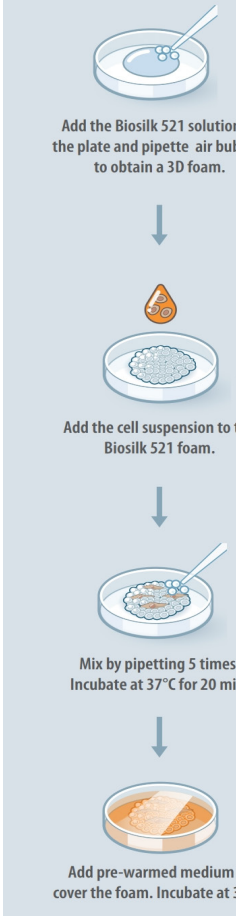
2 Prepare a concentrated single-cell suspension according to "Instruction For Use BL003". For a vial of Biosilk or Biosilk 521, prepare a concentrated cell suspension of a total of 7-14 x105 cells in 25 µL of medium (roughly 20 000- 60 000 cells/µL) supplemented with 10 µM ROCK inhibitor.

Note:

! The cell suspension should be prepared freshly before mixing into the Biosilk-Biolaminin solution to ensure the best cell viability. For best foam stability, prepare the cell suspension for the foam incorporation beforehand or during the Biosilk thawing time.

! Optimize the cell incorporating density for best amplification in the 3D scaffold, as this is cell type-dependent and needs to be adjusted accordingly.

FIG 2. SEQUENTIAL APPROACH TO INCLUDE CELLS AFTER BIOSILK FOAM GENERATION



3 Prepare a Biosilk-Biolaminin-cell suspension mixture by adding Biolaminin 521 or isoform of choice (25 µL), cell suspension (25 µL), and lOmM ROCK inhibitor (0.27 µL) to the thawed Biosilk solution (250 µL). The final mixture will have a concentration of 10 µg/mL Biolaminin and 10 µM ROCK inhibitor. Mix by gently pipetting 3 times without introducing air bubbles. The Biosilk-Biolaminin-cell suspension mixture should be used within 10 min to ensure a high cell viability.

Note:

! If the Biosilk 521 (85521-0101) pre-mixed product is used, the manual addition of Biolaminin can be omitted unless a mix with an additional Biolaminin isoform is desired.

! Be careful when mixing to avoid introduction of air bubbles as this will cause premature fiber formation.

4 3D scaffold formation and maintenance

4.1. Transfer 22 µL of the prepared Biosilk-Biolaminin-cell suspension solution to the center of a well.

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4.2. Use a pipette set at 44 µL, to push air bubbles into the droplet by quick pipetting up and down 22 times, thereby creating a dense foam. Spread out the foam in circular motions with the pipette tip during pipetting to an area covering 0.7-1 cm in diameter. See protocol described in Fig. 1.

Note:

! Insufficient (<22) or excessive(> 25) pipetting for the foam formation will result in an unstable scaffold or low cell viability, respectively.

! If the cells are sensitive to pipetting, an increased cell seeding density could help to increase cell viability. Alternatively, the cells could be mixed in after the foam has been generated. In this case, add 20 µL Biosilk solution to the well and use a pipette, set at 40 µL, to push air bubbles by quickly pipetting up and down 20 times. Add 1-4 µL dense cell suspension (roughly 20 000- 60 000 cells/µLJ for each foam and mix by pipetting an additional 5 times. See protocol described in Fig.2.

! It takes approximately half to one minute to generate each foam. We recommend to thoroughly plan the procedure for best cell- and foam quality.

4.3. Repeat step 4.1 to 4.2 to create the desired number of foams. The solution is sufficient for creating 12–13 foams.

4.4. Place the lidded plate with the cell-containing foams in an incubator at 37°C for 20 min. During this time, the Biosilk product polymerizes and the 3D structure is stabilized.

4.5. Remove the plate from the cell incubator. Gently add 0.7–1 mL per well of the pre-warmed medium containing 10 µM ROCK inhibitor, starting dropwise around the foam before slowly filling up to cover the foam.

4.6. Place the plate back into the incubator.

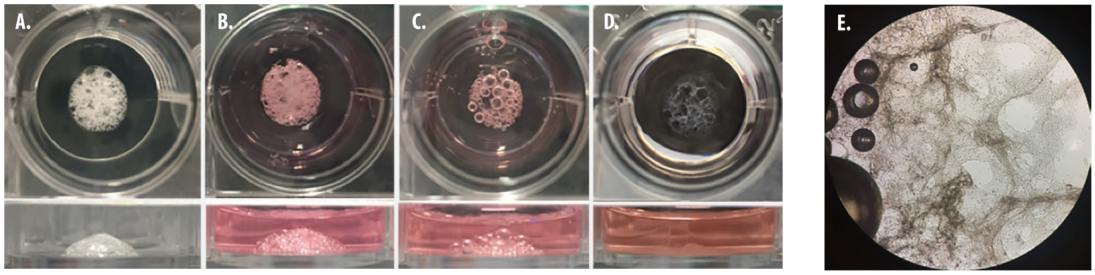
4.7. Feed the cells daily or at appropriate frequency with fresh culture media without ROCK inhibitor.

Foam morphology attached to the cell culture plate

Representative pictures of the Biosilk 521 foam taken from above and from the side of the cell culture well, day 0 to day 3 after formation. Note that the initial small bubbles merge into larger bubbles that disperse after a few days, resulting in a 3D scaffold with cells integrated between the microfibers.

Top view

Side view



A. Day 0. Biosilk 521 foam without medium after 20 min stabilization at 37°C.

B. Day 0. Biosilk 521 foam with a medium.

C. Day 1. The small bubbles start to merge into bigger bubbles.

D. Day 3. The bubbles have dispersed and the Biosilk 3D scaffold has formed with cells organized between the microfibers (approx. 10 cell layer thick).

E. Day 4. A microscope image example of human embryonic stem cells (hESC) cultured in Biosilk 521 (5× magnification), with seeding density 0.6 × 10^5per foam.

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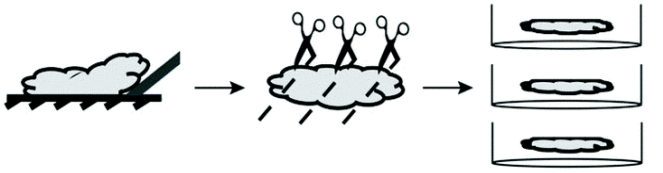
5 Biosilk 521 organoid differentiation

5.1. Pluripotent stem cells seeded in Biosilk-Biolaminin foam generally need to be cultured in medium supporting pluripotency for 3 to 4 days with daily feeding to reach desired confluence before switching to differentiation medium. Depending on lineage differentiation and protocol used, culture for 1 to 2 weeks in suitable medium with appropriate feeding frequency is needed.

5.2. When cells have reached the desired confluency within the microfibrillar network, manually detach the foam from the bottom of the well using a cell scraper or a pipette tip. Cut the foam structure into 2–4 pieces (approx. 2 mm thick) using a blade or a pair of small scissors and transfer to new low-attachment culture plates for culture as free-floating entities.



Detach foam Cut Floating cultures

Note:

! Before the foam can be detached from the culture surface for free-floating organoid culture, the cells first have to be amplified in the attached foam. It usually takes 1 to 2 weeks to generate desired confluency.

! Embedding the organoid in Matrigel is not needed to maintain the organoid shape and cell phenotype. If embedding is preferred, a xenofree and defined material is recommended (e.g. HyStem™ available from Merck).

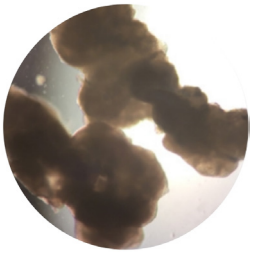
! If using another cell type than hPSCs, culture with appropriate culture medium and feeding frequency before detaching the foam for free-floating organoid culture.

5.3. Feed the free-floating organoid cultures at appropriate frequency until further analysis or desired applications.

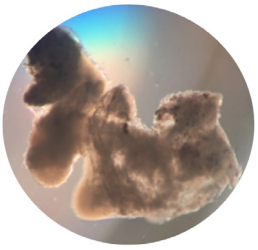
Free floating organoid morphology

Representative pictures (5x magnification) of hESC-derived neural organoids cultures in Biosilk mixed with Biolaminin 111. The cells spontaneously reorganize within the free-formed Biosilk biomaterial network and the cellular constructs grow in size. The organoid structures are well perfused giving the cells within the organoid good exposure to medium nutrition, enabling long-term cultures with high cell viability.

Cerebral organoids, day 40



Ventral mid brain organoid, day 60



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Free-floating Biosilk and Biolaminin organoids (96-well format)

The following protocol outlines how to combine hPSCs with Biosilk 521 to generate free-floating 3D organoids in 96-well plates.

Important notes

1 vial of Biosilk+LN or Biosilk 521 is enough material to generate 40-42 organoids (96-well format)

Material	Comment
96-well cultivation plate	U-shaped ultra-low (ULA) attachment plate eg. NunclonTM SpheraTM 96-well plate (#174925, Thermofisher), CellCarrier Spheroid (#605530, Perkin-Elmer)
Culture medium and solutions for making single cell suspensions	5 e.g. Essential 8 medium (Gibco) or mTeSR Plus supplemented with ROCK inhibitor, 10 µM
Biosilk	Stock solution 3 mg/mL (Biosilk, BioLamina)
- Biosilk 521	- Biosilk pre-combined with LN521 (#BS521-0101)
- Biosilk/ Biolaminin	- Biosilk combined with any other LN-isoform by the researcher (#BS-0101)
Cells	hPSC cell line of choice
Piptettes	- A 10-100 µL pipette, for adding the Biosilk suspension and cell medium to stock mix - A single or multi-pipette set (of max 100 µl) at 30 µL, for the formation of the Biosilk/Biolaminin foam - A 1-10 µL pipette, for adding the cell suspension to the stock mix and aliquoting of the Biosilk solution
General labware	Sterile 1.5 mL tubes and sterile pipette tips

Summary of protocol

- The amount of Biosilk (or Biosilk 521) and cells for each replicateis calculated for 2 mg/mL Biosilk and typically 10 000-60 000 cells per replicate are needed, for the total volume of 10 µL for each Biosilk/Biolaminin (or Biosilk 521) foam scaffold replicate.
  - o Using Biosilk/Biolaminin: The stock mix is created by adding the cell suspension, Biolaminin and Biosilk
  - o Using Biosilk 521: The stock mix is created by adding the cell suspension and Biosilk 521
- Droplets of 10 µL Biosilk/Biolaminin/cell suspension (or Biosilk 521/ cell suspension mix are placed at the bottom of each well. Repeat for all foam scaffold replicates.
- A dense foam scaffold is created by introducing air bubbles by rapid pipetting, up and down into the Biosilk/ Biolaminin/cell.
- Media is added to each well to induce floating of the cell-containing Biosilk/ Biolaminin (or Biosilk 521) foam scaffolds.
- Floating Biosilk/Biolaminin/cell-foam scaffolds (or Biosilk 521/cell-foam scaffolds) are transferred into new wells using a spoon for easy continuation and media exchange of the floating Biosilk/Biolaminin organoid culture.

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Protocol

1 Preparation of plate and media

1.1. Place a U-shaped ultralow (ULA) attachment plate in the tissue culture hood (laminar flow cabinet) and prepare the pipettes needed.

1.2. Pre-warm cell culture medium for the cell splitting step.

2 Preparation of a concentrated cell solution

2.1. Prepare a concentrated single cell solution (>10 000 cells/µL) diluted in warm media supplemented with 10 µM Rocki (typically 10 000-60 000 cell per foam scaffold replicate are needed).

For cell number and cell suspension volume: See table 3.2.1.

3 Preparation of the Biosilk/Biolaminin/cell mix solution late and media

Note:
Do not shake, flip or vortex the tube with Biosilk solution

3.1. Bring the vial of Biosilk from -80°C to RT and thaw with the tube standing still. When thawed and in solution (usually takes 12-15 min) immediately proceed to step 4.

3.2. Using Biosilk/Biolaminin: Prepare the Biosilk/cell mix: transfer 250 µL Biosilk solution from the concentrated stock vial to a new sterile 1.5 mL tube. Gently add a 37.5 µL Biolaminin isoform at a final concentration of 0.01 mg/mL and prepared cell suspension (see 2.1. or 3.2.1.) to the Biosilk solution to a final concentration of 2 mg/mL Biosilk. Mix slowly (no vortexing or shaking) without introducing any air bubbles.

Using Biosilk 521: Prepare the Biosilk 521/cell mix: transfer 250 µL Biosilk 521 solution from the concentrate stock vial to a new sterile 1.5 mL tube. Gently add prepared cell suspension (see 2.1. or 3.2.1.) to the Biosilk solution to a final concentration of 2 mg/mL Biosilk.

3.2.1. Example calculation

Using Biosilk / Biolaminin

Biosilk stock	**3 mg/ mL**
Volume (µL) of Biosilk (Final concentration: 2mg/mL)	250
Volume (µL) of Biolaminin (Final concentration: 0.01 mg/mL)	37.5
Volume (µL) of cell suspension (containing 375 000 cells)	87.5
Total volume (µL) Biosilk/Biolaminin/ cell suspension	375
Scaffold replicates	37

Using Biosilk 521

Biosilk 521 stock	**3 mg/ mL**
Volume (µL) of Biosilk 521 (Final concentration: 2 mg/ml)	250
Volume (µL) of cell suspension (containing 375 000 cells)	125
Total Volume (µL) Biosilk 521/ cell suspension	375
Scaffold replicates	37

3.3. Immediately proceed with step 4 below

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4 Preparation

4.1. Transfer 10 µL of the prepared Biosilk/Biolaminin/cell (or Biosilk 521/cell) solution to the center of each culture well (Fig.3A). Do not touch the well walls with the pipette tip (see Fig.3A)

4.2. Use either a single or multi-pipette (of max 100 µL) and set it to 30 µL. Air bubbles are pushed into the droplets by quickly pipetting up and down for 15 seconds with speed 2-3 strokes/second (Fig.3B)., thereby creating a dense foam scaffold (see Fig.3C).

4.3. Place the plate with the cell-containing Biosilk/ Biolaminin (or Biosilk 521) foam scaffold in an incubator at 37°C for at 10-15 min. During this time the stabilization of the 3D structure occurs (Fig.3D).

4.4. Add 150 µL pre-warmed media with the tip angled at the side of the culture wells, with enough force to make the cell-containing Biosilk/ Biolaminin (or Biosilk 521) foam scaffold loosen from the bottom (Fig.3E). If the foam scaffolds are stuck at the bottom, flush the well with one more stroke to make the foam scaffold lift (Fig.3F).

5 Transfer scaffolds

5.1. Add pre-warmed media to new wells on a F-bottom cell culture plate. To facilitate medium changes, a larger plate format (e.g. 48-well) can be used.

5.2. After 1 day of cultivation (Fig.3G) use a small sterile metal spoon, for the transfer of the floating foam scaffolds to the new wells (see Fig.3H). If required, multiple cell-containing Biosilk/Biolaminin– (or Biosilk 521) foam scaffolds can be pooled into 1 well.

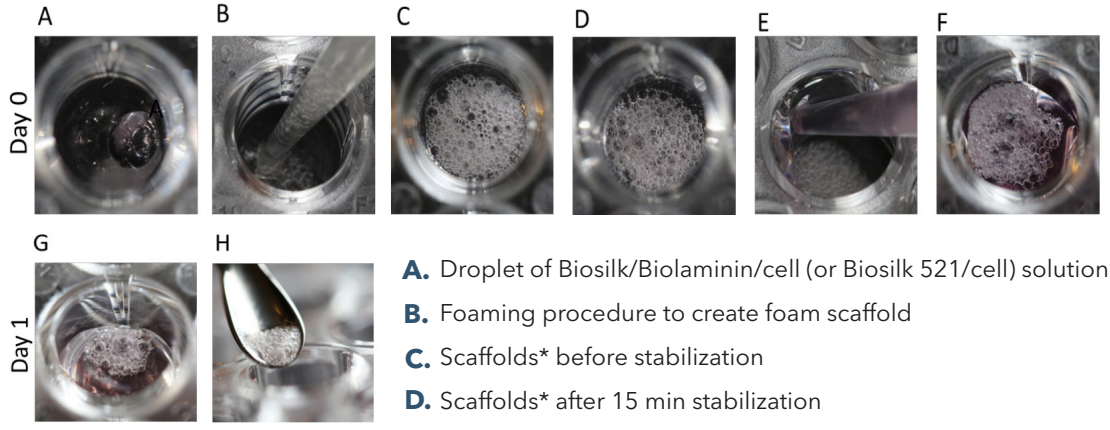
6 Culture

6.1. One day after seeding, spirate the medium and wash once with warm PBS. This step will help to remove the air bubbles to transfer the foam into a silk network.

6.2. Add fresh medium for culture.

FIGURE 3

Generation of Biosilk/Biolaminin/cell (or Biosilk 521/cell) scaffolds



A. Droplet of Biosilk/Biolaminin/cell (or Biosilk 521/cell) solution in culture medium in 96-well

B. Foaming procedure to create foam scaffold

C. Scaffolds* before stabilization

D. Scaffolds* after 15 min stabilization

E. Addition of culture medium (flush wall to lift scaffold)

F. Scaffolds* floating in culture medium

G. Scaffolds* with 10 000 hPSCs after one day in culture

H. Transfer of scaffolds* with wet sterile metal spoon

Scaffold = Biosilk/Biolaminin/cell (or Biosilk 521/cell) scaffold

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Trouble shooting

1. The 3D foam does not shape properly or is very flat.
   - Make sure a hydrophobic culture plate is being used (e.g. Sarstedt 83.3922.500). If the plate is not hydrophobic, the Biosilk solution will be difficult to pipette and the foam will become flat.

2. The stabilized 3D foam detaches from the bottom.
   - Make sure a hydrophobic culture plate is being used (e.g. Sarstedt 83.3922.500).
   - The generated foam diameter is too small. A too small and thick scaffold will bear more "lifting force" when the medium is added. Make sure that the foam covers a diameter of 0.7-1 cm.
   - The medium should be added gently. Slowly add the media dropwise around the foam before gently letting it cover the foam.

3. The stabilized 3D foam partially disperses while adding medium.
   - Make sure to pipette enough to generate a stable foam. Insufficient (<22) pipetting will result in an unstable scaffold.
   - The Biosilk solution has been standing for too long before use or was roughly handled. Thaw the Biosilk solution without moving the vial and use the solution as soon as possible, within 1 hour (including thawing time). Never centrifuge the Biosilk solution, not even if it turns milky.
   - The ratio of the total added Biolaminin + cell suspension volume to the Biosilk volume used has been higher than 1/5. If the ratio is exceeded, the Biosilk will be too diluted to be able to generate a stable scaffold. The total added Biolaminin volume should not exceed 1/10 of the Biosilk volume. If the cells will be mixed in after foam generation, do not use more than 4µL of cell suspension to 20 µL of Biosilk or Biosilk 521 solution.
   - The bubbles of the 3D scaffold are too big and has made the foam fragile. During foam formation, try to generate small evenly distributed bubbles.
   - Ensure that the temperature for stabilization is 37°C. Seed the foams in the outer wells of the plate or increase the stabilization time to 25 min.

4. The cells do not amplify properly or grow unevenly in the Biosilk scaffold.
   - The pure cell suspension or the Biosilk-Biolaminin-cell suspension mixture has been standing too long before use. The Biosilk-Biolaminin-cell suspension mixture should be used immediately after preparation (within 10 min) to ensure high cell viability. Work in a way so that the concentrated cell suspension is ready as soon as the Biosilk solution is ready to be used. Supplement both the Biosilk-Biolaminin-cell suspension mix and the cell culture medium with a final concentration of 10 µM ROCK inhibitor for the first 24h.
   - The cell suspension or the Biosilk-Biolaminin-cell suspension mixture was handled roughly. Excessive (> 25) pipetting during the foam formation will result in low cell viability. Use cells that are of good quality and that are in a rapid expansion phase (not over-confluent cells).

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