BioLamina
REVOLUTIONIZING CELL CULTURE

INSTRUCTIONS FOR USE 011

BIOSILK 3D SCAFFOLDS

FOR PROLIFERATION AND DIFFERENTIATION OF hPSC

BIOSILK™ IS A COMPLETELY DEFINED BIOMATERIAL THAT CAN SELF-ASSEMBLE INTO DIFFERNT STRUCTURES FOR ROBUST AND RELIABLE 3D CULTURE APPLICATIONS, SUCH AS LONG-TERM ORGANOID CULTURES. BIOSILK IS A BIOMATERIAL MADE OF RECOMBINANT SILK PROTEIN THAT CAN BE EASILY FUNCTIONALIZED WITH BIOLAMININ™ (LAMININ PROTEIN), MAKING IT A 3D CULTURE SYSTEM THAT CAN BE TAILORED FOR A WIDE RANGE OF CELL TYPES. BIOSILK™ 521 IS A PRODUCT WHERE BIOSILK HAS BEEN FUNCTIONALIZED WITH BIOLAMININ 521. BIOLAMININ 521 HAS UNIQUE FUNCTIONAL PROPERTIES, IDEAL FOR INTEGRATION, PROLIFERATION AND SUBSEQUENT LINEAGE-SPECIFIC DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS (hPSCs) IN A 3D FORMAT. THE ADDITION OF BIOLAMININ TO THE BIOSILK ALSO PROMOTES CELL MIGRATION WHICH ALLOWS EFFICIENT MORPHOGENESIS DURING DIFFERENTIATION. THE EXTENDABLE BIOSILK MICROFIBRES ARE NON-IMMUNOGENIC AND BIODEGRADBLE IN VIVO, FACILITATING ITS USE IN CLINICAL APPLICATIONS. THE FOLLOWING PROTOCOL IS FOR ORGANOID FORMATION STARTING FROM AN ATTACHED FOAM FORMAT.

MATERIAL

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 uL to 1000 uL
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

IMPORTANT NOTES

- **Biosilk™ is available as a pure silk solution (Biosilk, BS-0101) or as a pre-mixed solution with Biolaminin™ 521 (Biosilk 521, BS521-0101)**
- **A completely defined biomaterial for robust and reliable 3D culture applications, such as long-term organoid cultures**
- **Biosilk products should be stored at -80°C**
- **Gently thaw the Biosilk products at RT without disturbing the vial**
- **Do not vortex or shake the vial and be careful when mixing to avoid the introduction of air bubbles**
- **The Biosilk products has to be used within 45-50 min after thawing**
- **Re-freezing or long-term storage of thawed Biosilk solution in the fridge is not possible as this will affect product stability and functionality**
- **1 vial of BioSilk or Biosilk 521 is enough material to generate 12-13 foams (24 well format)**
- **The cell suspension should be prepared fresh before mixing into the Biosilk to ensure high cell quality**
- **All steps must be carried out under aseptic conditions. For research use only**

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PROCEDURE IN SUMMARY

The protocol described here is for the expansion and differentiation of hPSCs in Biosilk mixed with Biolaminin 521 (alternatively Biosilk 521 can be used) in a 24-well plate format. Hydrophobic surface culture plates should be used. Prepare a mixture of Biosilk, Biolaminin and cell suspension in medium containing ROCK inhibitor. The mixture should be prepared fresh before the foaming steps (step 1-3 in protocol). For each well, add 22 µL of the mixture to the well center. The 3D scaffold is created by rapid introduction of air bubbles into the mixture by quick pipetting of air, 22 to 25 times using a pipette set at 44 µL. Repeat the procedure until all wells have been prepared. Stabilize the cell-containing foams in a cell incubator at 37°C for 20 min. Gently add 0.7-1 mL pre-warmed cell medium to cover the culture (with ROCKi). Replace the culture medium daily (without ROCK inhibitor). When the desired cell confluence is reached, differentiation can be initiated by the addition of differentiation medium of choice. To generate a floating organoid culture system, the cell-propagated foams can be lifted from the bottom of the well with a cell scraper around day 6-15, depending on the preferred differentiation protocol.

PROTOCOL

FIG 1. ONE-STEP APPROACH FOR BIOSILK FOAM GENERATION

Add the Biosilk-Biolaminin-Cell suspension mixture to the plate and pipette air bubbles to obtain a 3D foam.

Stabilize at 37°C for 20 min.

Add pre-warmed medium to cover the foam. Incubate at 37°C.

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 x10^5 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.*

3. Prepare a Biosilk-Biolaminin-Cell suspension mixture by adding Biolaminin 521 or isoform of choice (25 µL), cell suspension (25 µL), and 10mM ROCK inhibitor (0.27 µL) to the thawed Biosilk solution (250 µL). The final mixture will have a concentration of 10 ug/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 (BS521-0101) 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.

   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:

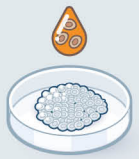
   ! *Insufficient (<22) or excessive (> 25) pipetting for the foam formation will result in an unstable scaffold or low cell viability, respectively.*

   ! *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 uL Biosilk solution to the well and use a pipette, set at 40 uL, 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/µL) for each foam and mix by pipetting an additional 5 times. See protocol described in Fig.2.*

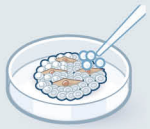
   ! *It takes approximately half to one minute to generate each foam. We recommend to thoroughly plan the procedure for best cell- and foam quality.*

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

Add the Biosilk 521 solution to the plate and pipette air bubbles to obtain a 3D foam.



Add the cell suspension to the Biosilk 521 foam.



Mix by pipetting 5 times. Incubate at 37°C for 20 min.

Add pre-warmed medium to cover the foam. Incubate at 37°C.

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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.

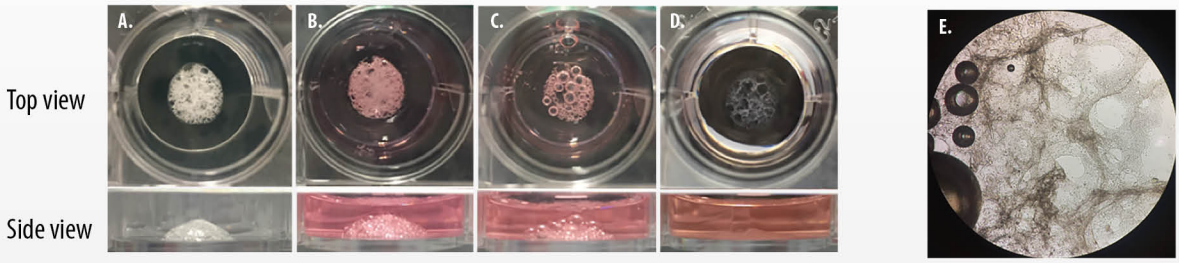
4.5. Remove the plate from the cell incubator. Gently add 0.7-1 mL per well of the pre-warmed medium containing 10 µM ROCK inhibitor, starting dropwise around the foam before slowly filling up to cover the foam.

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.



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 (5x magnification), with seeding density 0.6 x10^5 per foam.

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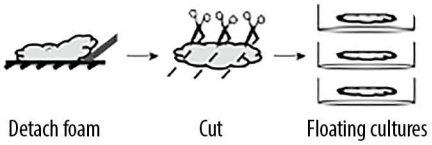
5. Biosilk 521 organoid differentiation

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.



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 midbrain organoid, day 60

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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