21 Changi South Street 1 Singapore 486777
Tel: +65 65420833 | Fax: +65 65426920

Protocol for Culturing MSCs in CelCradle™

Contents

MATERIALS

- MSC Attachment Solution (Biological Industries, 05-752-1) / other coating reagents
- Phosphate Buffer Saline without Mg2+/Ca2+
- Mesenchymal Stem Cells
- Complete media - MSC NutriStem® XF Medium (Biological Industries, 05-200-1A-KT) or other media type
- CelCradle Stage 3000
- CelCradle™ 500A (EscoAster/VaccixCell) or CelCradle™ 500AP
- Celfeeder Pump
- GlucCell Monitoring System (GlucCell Meter + Strips)
- pH Meter
- Long Forceps
- Cell Strainer
- Trypan Blue / Hemotoxylin stain
- Florescent Diacetate (FDA) (1 µg/ml) (ThermoFisher Scientific)
- Propidium Iodide (PI) (1 µg/ml) (ThermoFisher Scientific)
- Hoechst 33342 (1 µg/ml) (ThermoFisher Scientific)
- Dissociating reagents (0.05% Trypsin-EDTA/ Tryple Express/ Accutase/ Accumax)

METHODS

Coating of Carriers (Recommended for media without serum)

a. Bring a single CelCradle bottle (CC-500A or CC-500AP) into BSC Class II hood
b. Coat carriers with 120 ml fibronectin or coating solution (BI, 05-752-1) for 30 min at 37°C
c. Aspirate the coating solution
d. Rinse carriers briefly with 120 ml PBS twice with a swirling motion
e. Proceed with inoculation steps

Note: Follow manufacturer's recommendations for coating of carriers

Seed Preparation

Prepare 5 T-175 flasks with a combined total 2-3 x 10^7 cells for the seeds of one CelCradle bottle.

Inoculation

1. Prepare 2-3 x 10^7 cells, suspended in 120 ml of fresh, pre-warmed culture medium (Ensure pH is between 7.2-7.4 – if required, add 15 mM of HEPES to ensure stability of pH while seeding).
2. Pipette the cells evenly over the top of the carriers.
3. Place the blue vented cap on a sterile petri dish with lid over it to ensure sterility for future use.
4. Cap the CelCradle bottle with a non-vented white cap.
5. Invert the bottle and ensure all carriers fall towards the cap and are submerged in media.
6. While still in the inverted position, swirl well to ensure cells suspension is spread evenly in the packed bed.
7. Transfer the bottle (still in inverted position) into an incubator at 37°C, 5% CO_2 (or at preferred set-points for the cell type being cultured).
8. Allow cells to attach for 3 h. Tilt the bottle slightly and rotate gently every 30 min to allow settled cells to be redistributed in media.
   Note: Perform this step gently as partially attached cells may detach from the carriers with vigorous motion.
9. After 3 h, aseptically remove 10 ml of the cell suspension from the CelCradle bottle.
10. Centrifuge the cell suspension and resuspend the cells in a suitable volume for cell counting to determine the number of cells left unattached. Determine the attachment efficiency.

$$Attachment\ Efficiency\ = \frac{Total\ cells\ seeded\ - Total\ unattached\ cells}{Total\ cells\ seeded} * 100\%$$

11. Proceed to the next section if the attachment rate is higher than 90%

Note: Depending on media, serum and whether coating is used, MSCs are able to achieve an attachment rate of >90% after 2 h. We recommend to end the cell attachment process after 4 h regardless of attachment rate and proceed to culture and expansion phase.

Culture and Expansion

1. With fresh media, top up the CelCradle bottle to 500 ml.
2. Place the bottle into the CelCradle Stage. Set up culture parameters as below and press "Start" to initiate the tiding process.
   i. Up: 1.0 mm/s, Top Holding: 10 s
   ii. Down: 1.0 mm/s, Bottom Holding: 30 s

Perfusion Culture Option (CelCradle™ 500AP)

For the perfusion culture in a CelCradle 500AP, prepare:

a. 1 L perfusion bottle containing 1 L of media (Depending on amount of media used and cell culture duration)
b. Connect media bottle to CelCradle 500AP and pump
c. Set program as follows:
   i. Perfusion volume (1999 ml)
   ii. Day and date to perform perfusion (Everyday starting from the third day)
   iii. Frequency of perfusion e.g: 24 cycles/day

Note: Please refer to the manual for usage of pump.

Monitoring Cell Growth (Refer to Appendix A)

We recommend daily monitoring of media for the media metabolites such as glucose concentration and pH to determine when to perform media change or supplement with extra glucose to aid optimisation during initial trials.

a. 3 ml media: pH and glucose measurement
b. 3 carriers: live/dead cell staining using FDA, PI, Hoechst stains following standard protocol
c. 10 carriers: harvest using dissociation enzymes to check for live cell count (details in the next section)
d. Perform media change when:
   a. Glucose level falls too low
   b. pH falls below 7.00
   c. Every 3 days (Follow 2D culture protocols if glucose and pH are stable)
f. Harvest cells when cells reach maximum confluence between day 5-7 (Refrain from overgrowing/over-confluency as is the case in T-flasks for MSCs)

Note: Cell growth rate and maximum count is heavily dependent on both cell passage number, media and serum used. Refer to existing literature for information regarding more effective basal media and serum types for MSCs.

Note: Glucose consumption or total cell count can be used as a measurement of confluence. Cells will protrude from the edge of carriers and appear as a healthy web-like configuration when at high confluence (Appendix C).

Cell Harvest

1. Drain culture media into a container using CelCradle strainer.

2. Gently rinse with 500 ml of PBS twice.
3. Drain PBS solution after the two rinses and discard the solution.
4. Add 120 – 150 ml of pre-warmed dissociation enzyme and cover bottle with non-vented white cap.
5. Invert bottle to allow carriers to be soaked in dissociation solution for 15 – 30 min in incubator.
6. Transfer dissociation solution into collection beaker (or discard if little cells is in the solution).
7. Add 120 ml enzyme inhibitor (soybean inhibitor/ serum-containing media) into the bottle, cap the non-vented cap firmly.
   Note: Spent media (containing serum) is sufficient for use as enzyme inhibitor.
8. Invert the bottle. Swirl to allow the carriers to fall downwards towards the cap.
9. Physical agitation:
   a. Method one (refer to appendix B):
      i. In the inverted position, with the flat of the palm, firmly hit the side of the bottle 40 times.
      ii. Rotate the bottle 90 degrees and hit each corner 40 times.
      iii. Repeat the tapping for 1 more bottle revolution. (Total: 4 corners x 2 times)

   b. Method two:
      i. Secure the bottles in the CelShaker and start the cycle time with parameters of:
         - Duration: 3 min, speed 400 rpm
10. Pour/pipette the cell-laden solution into collection beaker.
11. Add 120 ml media into bottle.
12. Repeat steps 8 to 11 three more times but exclude step 11 for the last repeat.
13. Collect all cells by centrifugation and check for cell density and viability.
14. Perform fluorescent staining (Fluorescein diacetate) on harvested carriers to check the harvest efficiency.

Note: If many cells remain on the carriers after harvesting, additional harvesting optimization is required. Refer to the next section for proposed solutions.

Note: Initial washing step with PBS is important to ensure all serum and non-viable cells are washed off. Few cells will be dislodged during this step; most of them should be nonviable cells. This step can increase the harvest viability by removing non-viable cells first.

Note: Sufficient time for enzymatic digestion is critical for a successful cell harvest. Most cells can withstand trypsin-EDTA for above 30 min without altering viability. High cell density will require more dissociation enzymes and time to digest. Accutase (Innovative Cell Technologies, San Diego, CA) can allow more treatment time without harming cells compared with trypsin enzyme.

Note: Dissociation enzymes such as collagenase can be used for more effective harvesting of stem cells and primary cells as these cells produce ECM on the 3D BioNOC™ II carriers.

APPENDIX A

Staining with Dyes

Live Cell Staining with Fluorescence Dyes

1. Aseptically remove 3 BioNOC™ II carriers from the CelCradle and transfer them to a single well in a 24-well plate.
2. Add 1 ml of culture media to the well. Add dyes at the following final concentrations: 1 µg/ml of Hoescht 33342 (Thermo Fisher, H3570) and 1 µg/ml propidium iodide (PI, Sigma Aldrich P4170).
3. Incubate the carriers for 20-30 min at 37°C, 5% CO_2
4. Add 1 µg/ml fluorescein diacetate (FDA, Thermo Fisher, C34852) into the well and perform imaging with respective filters (Blue for Hoechst 33342, green for FDA and red for PI).

Note: Other types of fluorescence dyes can be used to visualize the cells. Eg. calcein green, acridine orange, Cell tracker etc.

Staining of Fixed Cells with Visible Dyes

1. Aseptically collect 1-2 BioNOC™ II carriers from the CelCradle.
2. Dehydrate and fix the cells in 70% ethanol for 5 min.
3. Discard the ethanol and rinse twice with DI water or PBS.
4. Stain the cells with trypan blue or hematoxylin dye for 5-10 min.
5. Wash off excess dye with DI water.
6. Observe the carriers with cells under bright field microscopy.

Note: Other types of visible dyes may be used. However, fluorescence dyes allow better visualization of cells remaining in carriers as visible dyes do not give a clear view when fewer, sporadically-spaced single cells remain after harvesting.

Harvesting Small Scale for Daily Monitoring

By Dissociation Reagent

Enzymatic reagents for dissociation: We recommend using a more gentle dissociating enzyme compared to trypsin as extended incubation with trypsin will lead to cell damage. Below are several examples of the enzymes used.

1. Transfer three carriers from the CelCradle into a 1.5 ml micro-centrifuge tube.
2. Gently wash the carriers with 1 ml PBS. Remove PBS.
3. Repeat step 2 four more times.
4. Perform enzyme dissociation:
   - i. Trypsin
     - Add 1 ml 0.25% Trypsin-EDTA, incubate at 37°C for 15-30 min.
   - ii. TrypLE Express: (most cell types)
     - Add 1 ml of TrypLE Express, incubate at 37°C for 15-30 min.
   - iii. Accumax/ Accutase: (suitable for stem cells)
     - Add 1 ml Accumax/ Accutase, incubate at room temperature for 15 - 30 min. (Incubation time depends on cell density, we would suggest a study with 15 min, 20 min and 30 min). Accumax is recommended for cells growing in 3D.
   - iv. Collagenase: (suitable for stem cells)
     - Dilute collagenase type I (Thermo Scientific, Cat 17101) to achieve final working

solution of collagenase containing 100 units/ml and 5 mM of $CaCl_2$ dissolved in PBS.

- Add 1 ml of collagenase and incubate for 15 - 30 min. (Please optimize duration of collagenase as required).

5. Transfer the enzyme to a 15 mL tube. Add 1 ml of neutralization media to the carriers.
6. Flick the tube firmly with a pen or metal rod 40 times.
7. Transfer solution to the 15 ml collection tube.
8. Add 1 ml PBS or spent media and pipette up and down to wash out the cells from the carriers and repeat step 5 and 6.
9. Repeat steps 7 for at least 3 more times (total 4 times of collection with PBS/spent media).
10. Centrifuge, discard supernatant and re-suspend cells in lower volumes for counting cells on a hemocytometer. Calculate the average cell number in one carrier.

Note: Depending on the cell confluency, the harvesting procedure may have to be adjusted to be fully optimised.

Glucose Consumption Measurement (With GlucCell or biochemical analyzer)

1. Remove 2 ml of media from CelCradle for glucose measurement using GlucCell meter.
2. Perform the glucose measurement at T_N (Glucose T_N).
3. When fresh media is exchanged, measure control media (Glucose T_0) as baseline.
4. Glucose consumption: Glucose at T_0 – Glucose T_N

pH Monitoring

1. Remove 2 ml of media from CelCradle for pH measurement.
2. Measure media immediately after transfer from CelCradle to ensure pH does not change under room conditions.

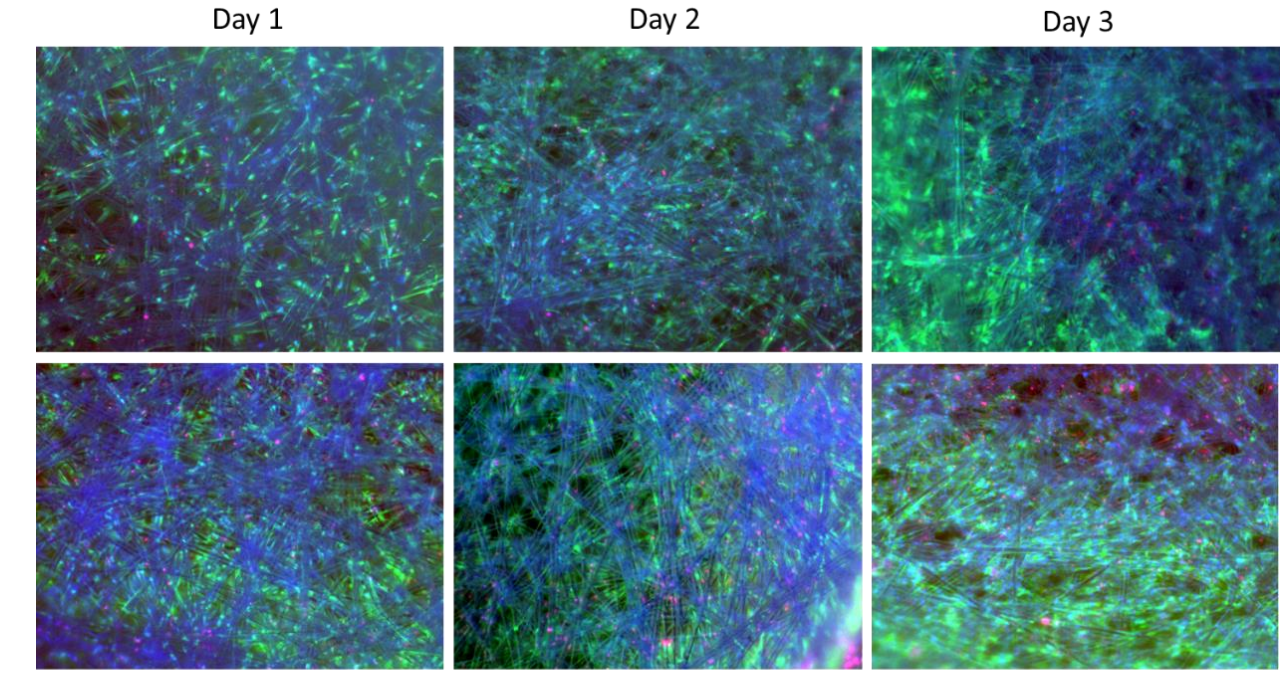
Appendix B

Cell harvest from CelCradle

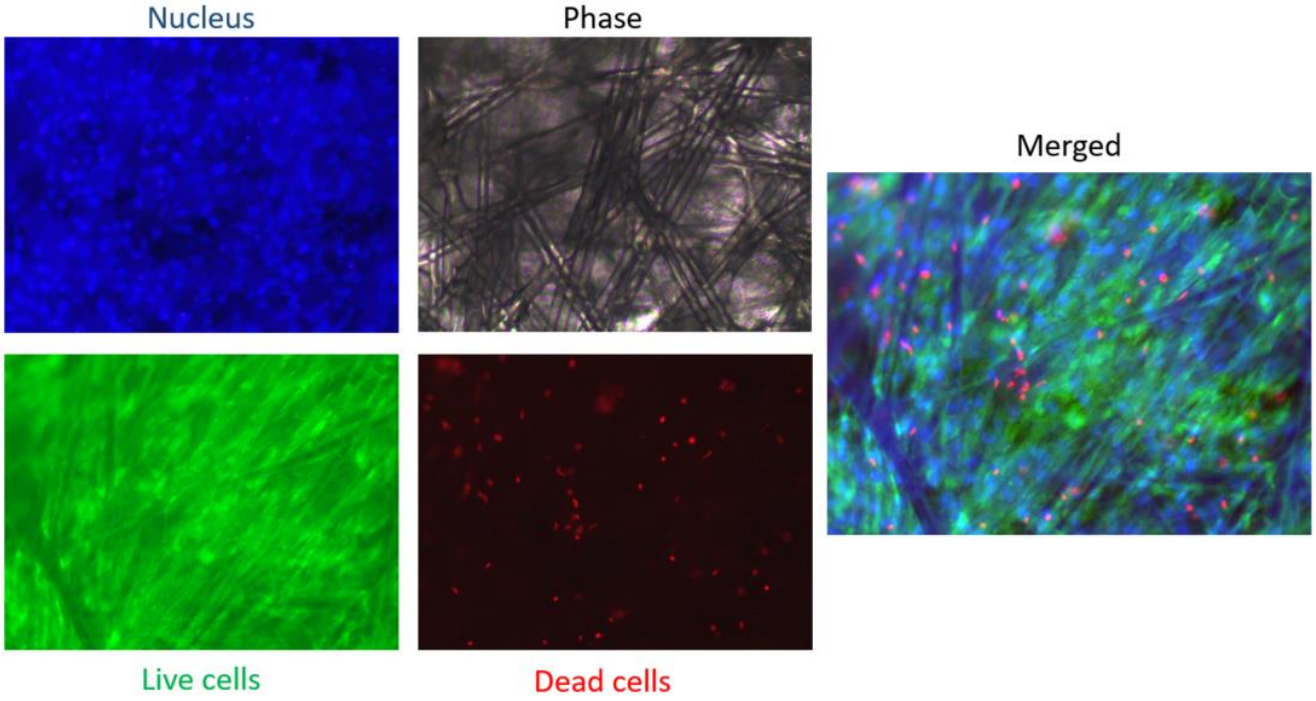
Refer to the embedded video for more information on manual physical agitation (https://www.youtube.com/watch?v=u0GCUHF14Vk).

Appendix C (Results)

Live Cell Staining

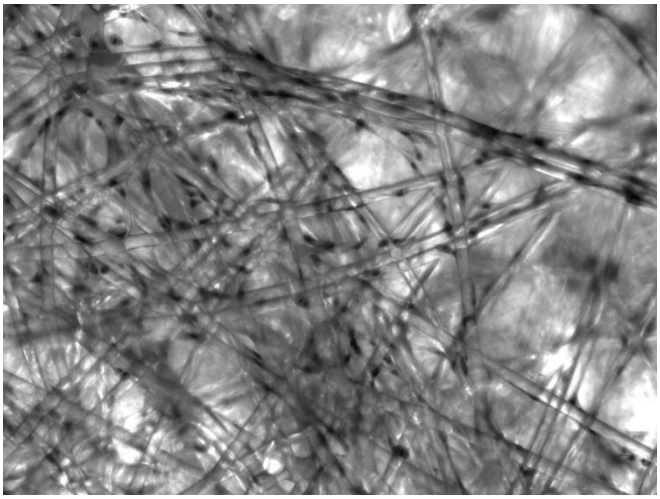


Green: Fluorescein diacetate (cytoplasm), Blue: Hoechst 33342 (nucleus), Red: propidium iodide (dead cells)

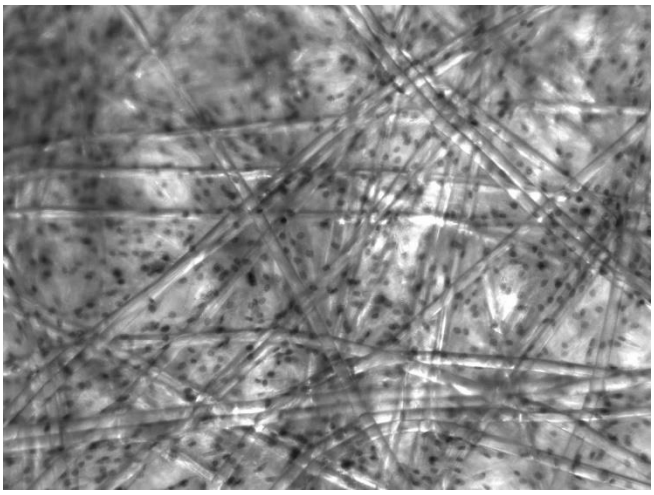


Hemotoxylin Stain

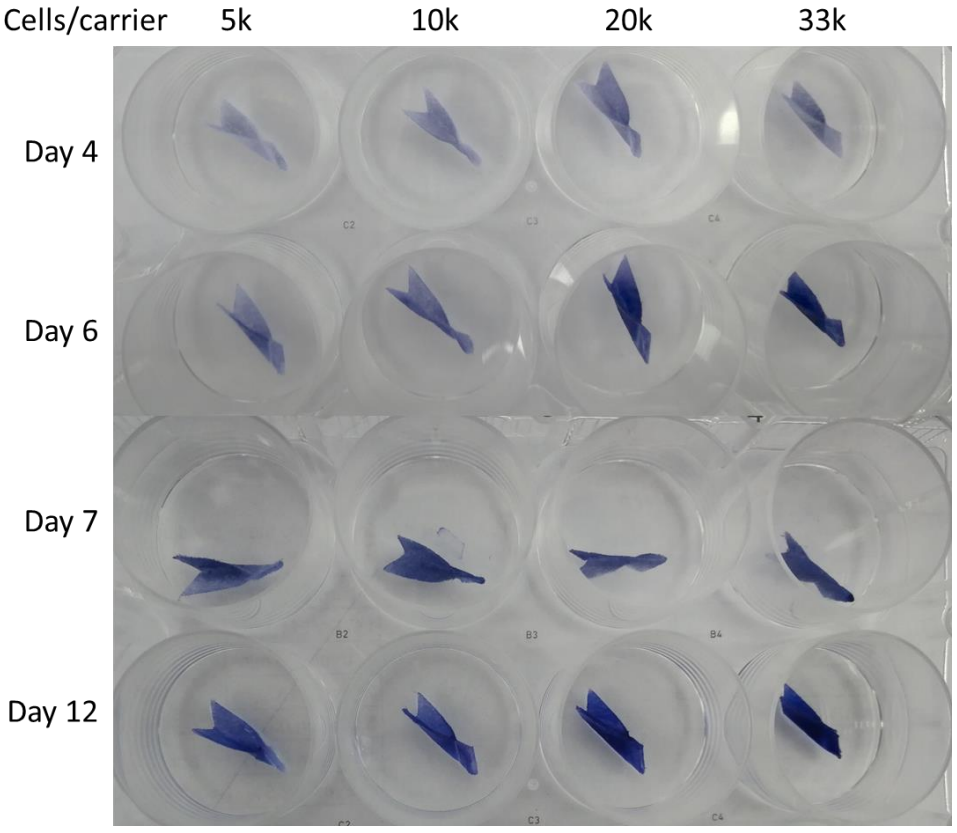
Day 1



Day 3

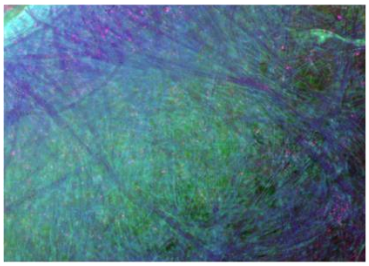


Trypan Blue Stain

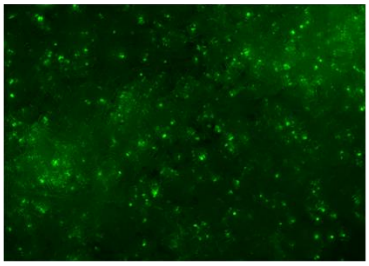


Staining After Harvesting

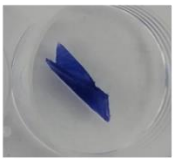
Harvesting (%)	80.3
Viability (%)	97.9



Before harvesting



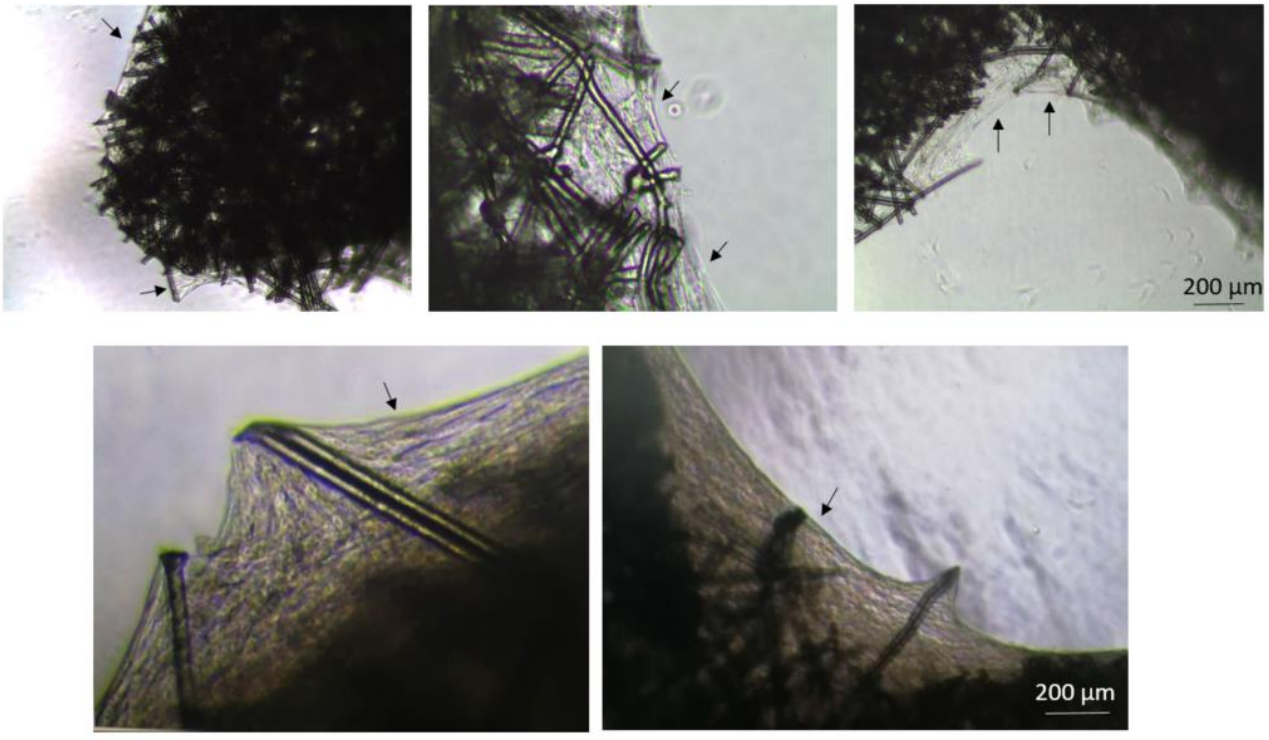
After harvesting



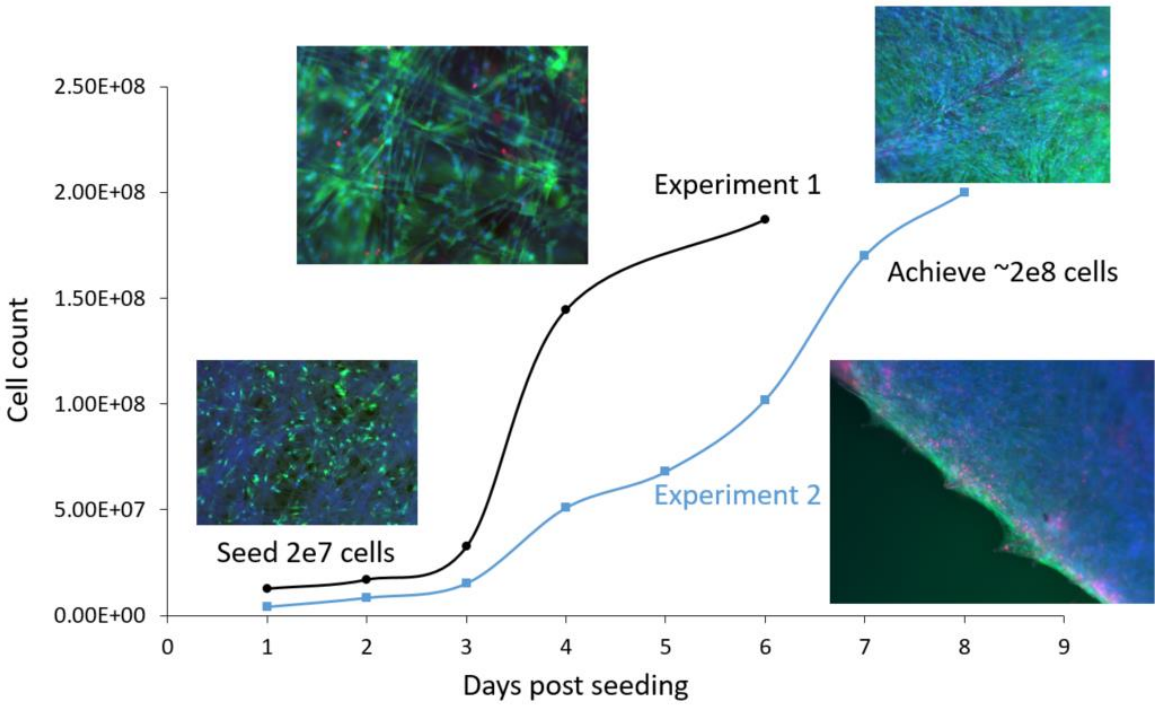
Before harvesting

After harvesting

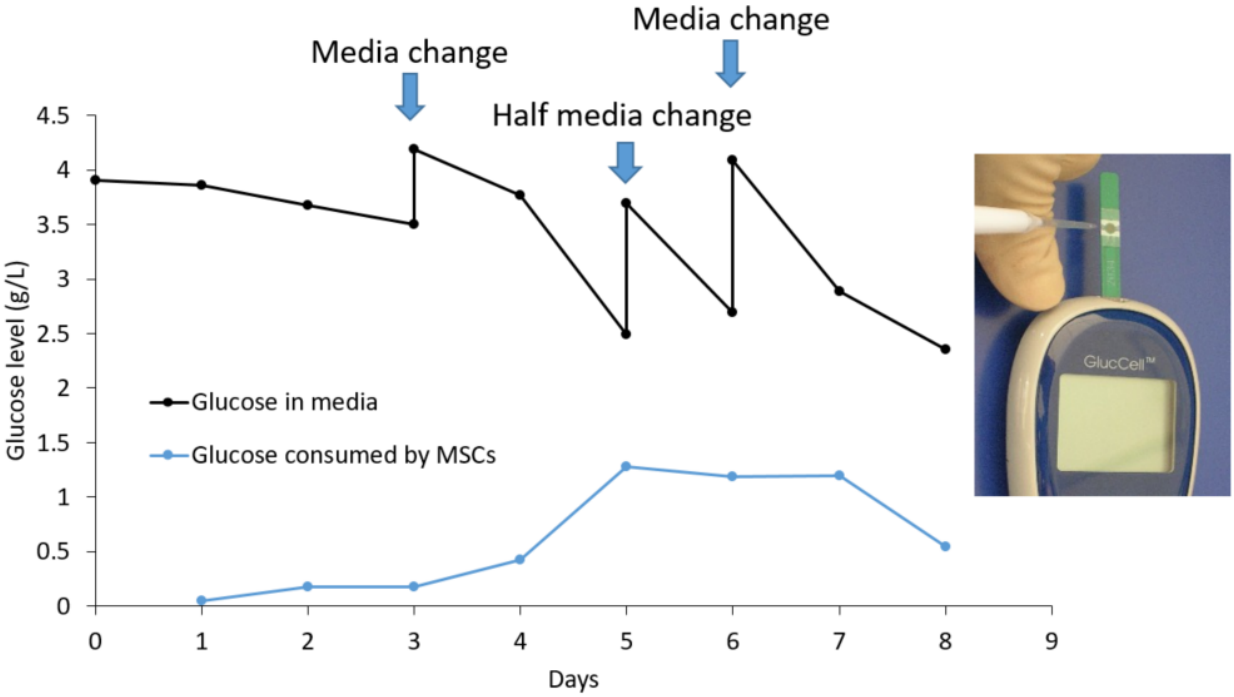
Cells at High Confluence



Cell Growth



Glucose Consumption Level



pH Monitoring