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Protocol for Culturing MSCs in CelCradle™

Contents

MATERIALS

- MSC Attachment Solution (Biological Industries, 05-752-1) / other coating reagents
- Phosphste 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 / peristaltic pump
- Celshaker
- 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)

a. Bring a single CelCradle 500A bottle into BSC Class II hood
b. Coat carriers with fibronectin or cell attachment solution (BI, 05-752-1) for 30 min at 37°C
c. Suction
d. Rinse carrier briefly with 250ml PBS by swirling gently (if required)
e. Continue with inoculation steps
Note: To follow manufacturer's recommendations for coating of carriers

Seed Preparation

Prepare 5 T-175 flasks with 2-3 x 10^7 cells for the seeds for one CelCradle™ 500A bottle.

Inoculation

1. Prepare 2-3 x 10^7 cells seed in 120ml of fresh and pre-warmed culture medium (Ensure pH is between 7.2-7.4 – add 15 mM of HEPES to ensure stability of pH while seeding).
2. Dispense seeds on the pre-coated carriers.
3. Place the blue vented cap in a sterile petri dish and cover the dish to ensure the sterility of the cap for future use.
4. Cap bottle with non-vented white cap.
5. Invert bottle and ensure all carriers fall to the cap and are submerged in media.
6. Swirl well to ensure cells are spread evenly in the bottle.
7. Move the bottle into incubator.
8. Allow cells to attach for 2-4 h. Swirl and rock bottle gently every 30 min to allow cells to be redistributed in media.
9. After 3 h, remove bottle and take 10 ml media to check number of cells that are unattached.
10. Centrifuge and resuspend cells left in the culture media and determine % of attachment
11. Repeat step 7 – 8 every hour to check for cell attachment.
12. Stop cell attachment process when the attachment rate is higher than 90%.

Note: MSCs are able to achieve an attachment rate of >90% after 2 h. We recommend to stop the cell attachment process after 3 h. Attachment rate achieved is approximately 98%.

Duration of attachment (h)	1	2	2.5-3
MSC attachment rate	90	95	>98

Culture and Expansion

1. Top up media in bottle to 500 ml.
2. Move the bottle into CelCradle Stage. Set up inoculation parameters as below and press "Start" to initiate the immobilization process.
   i. Up: 1.0 mm/s, Top Holding: 10 s

ii. Down: 1.0 mm/s, Bottom Holding: 30 s

Perfusion option of cell culture (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™ 500 AP and pump
c. Set program as follows:
    i. Perfusion volume (1999ml)
    ii. Day and date to perform perfusion (Everyday starting from the third day)
    iii. Frequency of perfusion e.g,: 24 cycles/day

Monitoring Cell Growth (Refer to Appendix A)

Monitor daily for residual glucose concentration and the pH of medium to predict the time to change culture media or supplement with extra glucose or sodium bicarbonate.

We recommend checking glucose, staining and cell count every day, or every other day when running initial trials.

a. 2 ml media: pH and glucose measurement
b. 1 carrier: live/dead cell staining using FDA, PI, Hoechst stains following standard protocol
c. 2 carriers: harvest using trypsin to check for live cell count
d. Change with fresh media when:
    a. Glucose level falls below 1 g/L – may pulse in additional glucose to keep glucose at 1.5-2 g/L to ensure enough supplement.
    b. pH goes below 7.00
    c. or every 2-3 days (Follow 2D culturing protocol if glucose and pH are stable)
f. Harvest cells when cells reach maximum confluence between day 5-7 (However, refrain from overgrowing the cells as with all MSCs)

Tips: Glucose consumption or counting total cell number can be used as a measurement of confluence. Alternatively, cells will start appearing as healthy web-like configuration when at high confluence (Appendix B).

Cell Harvest

1. Drain off culture media into a container using CelCradle strainer.
2. Rinse with 500 ml of PBS gently twice.
3. Drain PBS solution after two rinses.
4. Add 120 – 150 ml of pre-warmed dissociation solution and cover bottle with non-vented white cap.
5. Invert bottle upside down to allow carriers to be soaked in dissociation solution for 15 – 30 min in incubator.
6. Remove dissociation solution.

7. Add 0.1% trypsin inhibitor (or 10% serum) into 100 ml culture medium.
8. Add the 100 ml solution into the bottle, cap the non-vented cap firmly.
9. Use either or both the following methods to knock out the cells:
    a. Method one:
        i. Tap bottle sharply and steady against your palm for 3 min. Rotate bottle during tapping.
        ii. Invert the bottle. Swirl to wash off the cells from the carriers.
        iii. Perform tapping of bottle. Tap each corner 20-30 times before rotating to next corner.
        iv. Rotate and hit each corner 3 times.
        v. Pour the cell-laden solution into centrifuge bottles.
        vi. Add 100 ml complete media into bottle.
        vii. Repeat bottle tapping and collect cells. Perform washing steps for 4-5 times.
    b. Method two:
        i. Alternatively, secure the bottles in the CelShaker and start the cycle time.
        ii. Duration: 2.5 min, speed 400 rpm
10. Collect all cells by centrifugation and check for cell density and viability.
11. Take carrier samples to check the harvest efficiency.

Tips on harvesting

Initial washing step with PBS is important to ensure all serum and non-viable cells are washed off. Some 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. Cell collection may be done on second run if viability is acceptable.

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 cells 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.

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

APPENDIX A

Staining with Dyes

Live Cell Staining with Fluorescence Dyes

1. Aseptically sample 1-2 BioNOC™ II carrier from CelCradle and transfer to 24 well plates.
2. Add 500 µl of culture media to the well. Add dyes at the following final concentrations: 1 µg/ml of Hoescht 33342 (Thermo Fisher, H3570), 1 uM fluorescein diacetate (FDA, Thermo Fisher, C34852 and 1 µg/ml propidium iodide (PI, Sigma Aldrich P4170) in culture media.
3. Incubate the carriers for 30 min at 37ºC, 5% CO_2 before capturing images at their 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.

Non Fluorescence Stain with Fixed Cells

1. Aseptically collect 1-2 BioNOC™ II carrier from CelCradle.
2. Dehydrate and fix the cells using ethanol dehydration 70% for 5 minutes, and then 99.5% ethanol dehydration for additional 5 minutes.
3. Wash off the ethanol twice, using either DI water or PBS.
4. Stain the cells with hematoxylin, or H&E dye for 5-10 min.
5. Wash off the excess dye with DI water.
6. Observe the carriers with cells under bright field microscopy.

Note: Other types of dyes may be used, eg. Trypan blue.

Use fluorescence dye for staining to obtain better visualization of cells left on carriers post harvesting. Refrain from using coloured dyes.

Harvesting Small Scale for Daily Monitoring

By Dissociation Reagent

Enzymatic reagents for dissociation: Accumax, Trypsin 0.25%, TrypLe Express, Collagenase

1. Transfer three carriers into a 1.5 ml micro-centrifuge tube from CelCradle.
2. Gently wash the carriers with 1ml PBS. Remove PBS.
3. Repeat step 2 four more times.
4. Perform enzyme dissociation:
   i. Trypsin/ TrypLE Express: (most cell types)
      - Add 1 ml 0.25% Trypsin-EDTA, incubate at 37ºC for 10-15 min. Add in 1 ml of neutralization media.
   ii. 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. However, you may use your preferred dissociation agent/method.
   iii. 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. Flick the tube against your fingers/ a metal rod for 10-20 times.
6. Transfer solution to a 15 ml collection tube.
7. Add 1 ml PBS and pipette up and down to wash out the cells from the carriers and repeat step 5 and 6.
8. Repeat steps 7 for at least 3 more times (total 4 times of collection with PBS).
9. Centrifuge, discard supernatant and re-suspend cells in lower volumes for counting cells on a hemocytometer. Calculate the average cell number in one carrier.

Glucose Consumption Measurement (With GlucCell or other bioanalyzer)

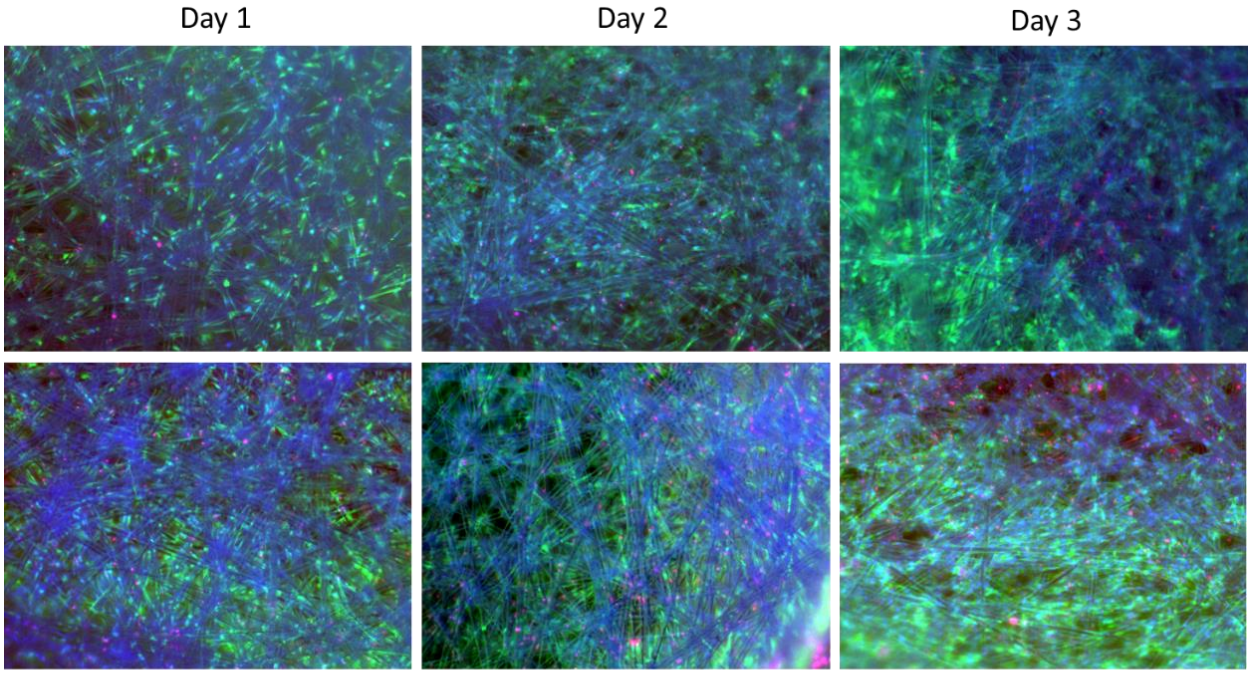
1. Remove 2 ml of media from CelCradle for glucose measurement using GlucCell meter.
2. Perform the glucose measurement at T_N (Glucose T_N).
3. When fresh media is exchanged, measure control media (Glucose T_0) as baseline.
4. Glucose consumption: Glucose at T_0 – Glucose T_N

pH Monitoring

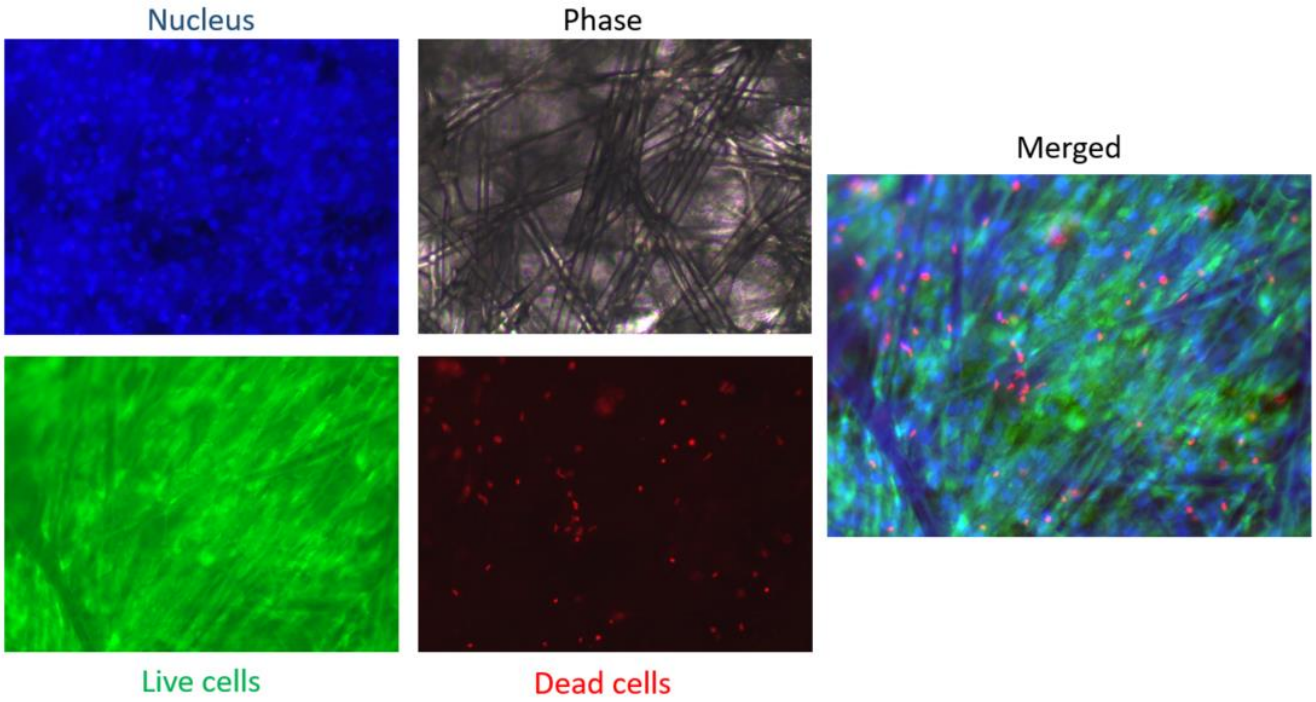
1. Remove 2 ml of media from CelCradle for pH measurement.
2. Measure media immediately after transfer from CelCradle to ensure pH does not change under room conditions.

APPENDIX B (RESULTS)

Live Cell Staining

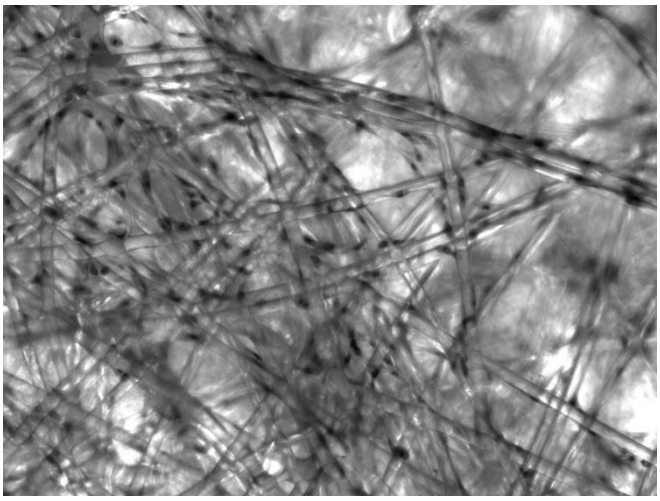


Green: Fluorescein diacetate (cytoplasm), Blue: Hoechst 33342 (nucleus), Red: propidium iodide (dead cells)

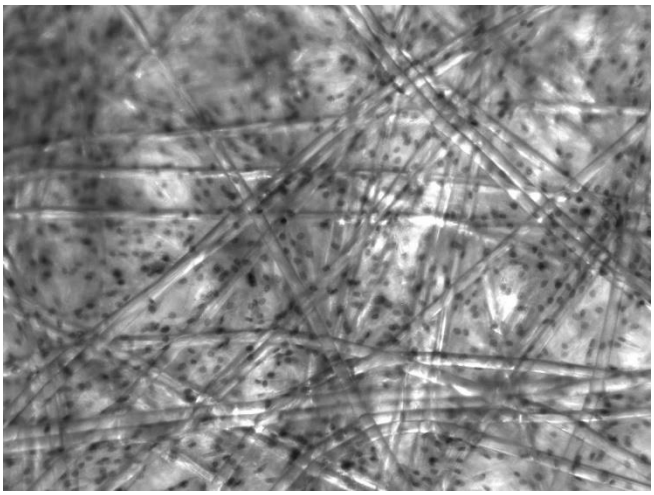


Hemotoxylin stain on different days

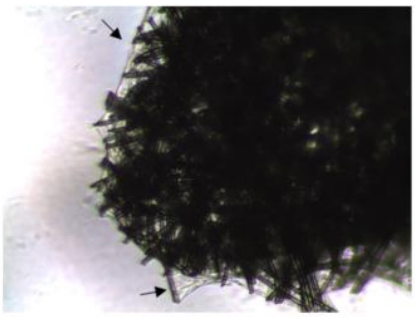
Day 1

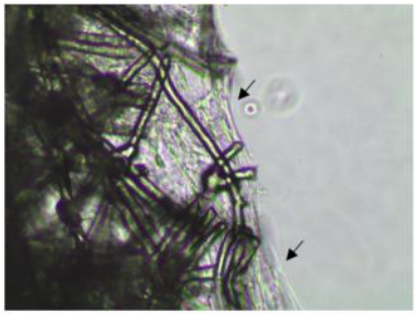


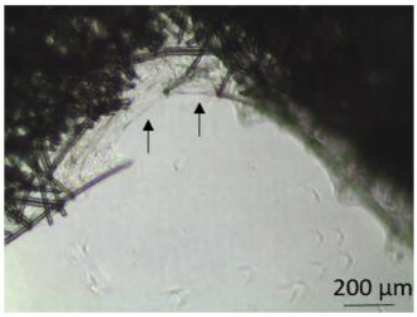
Day 3

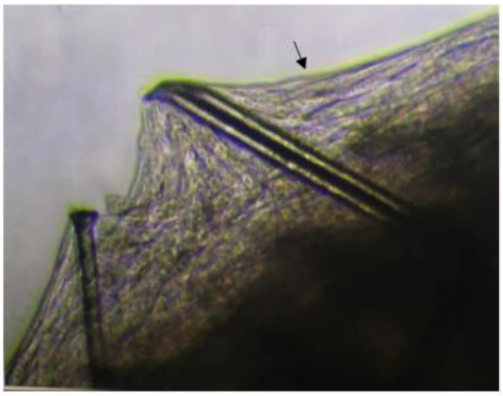


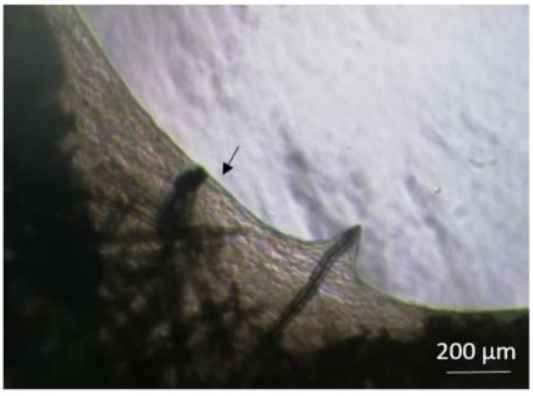
Cells at High Confluence



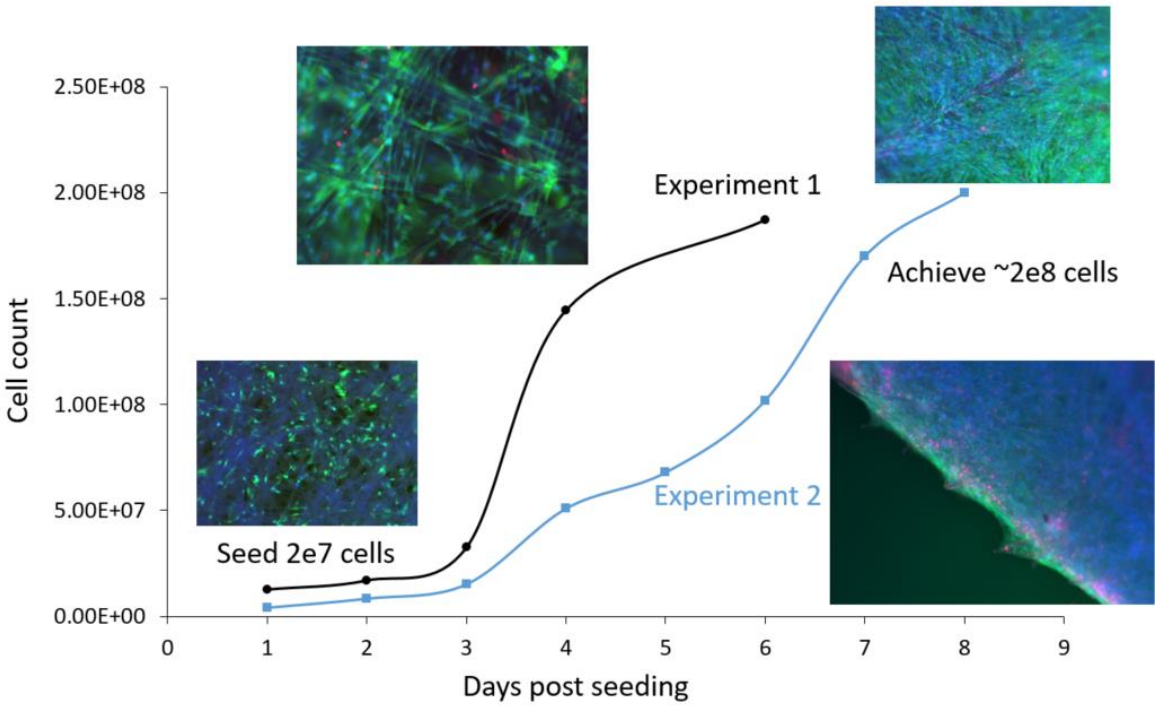




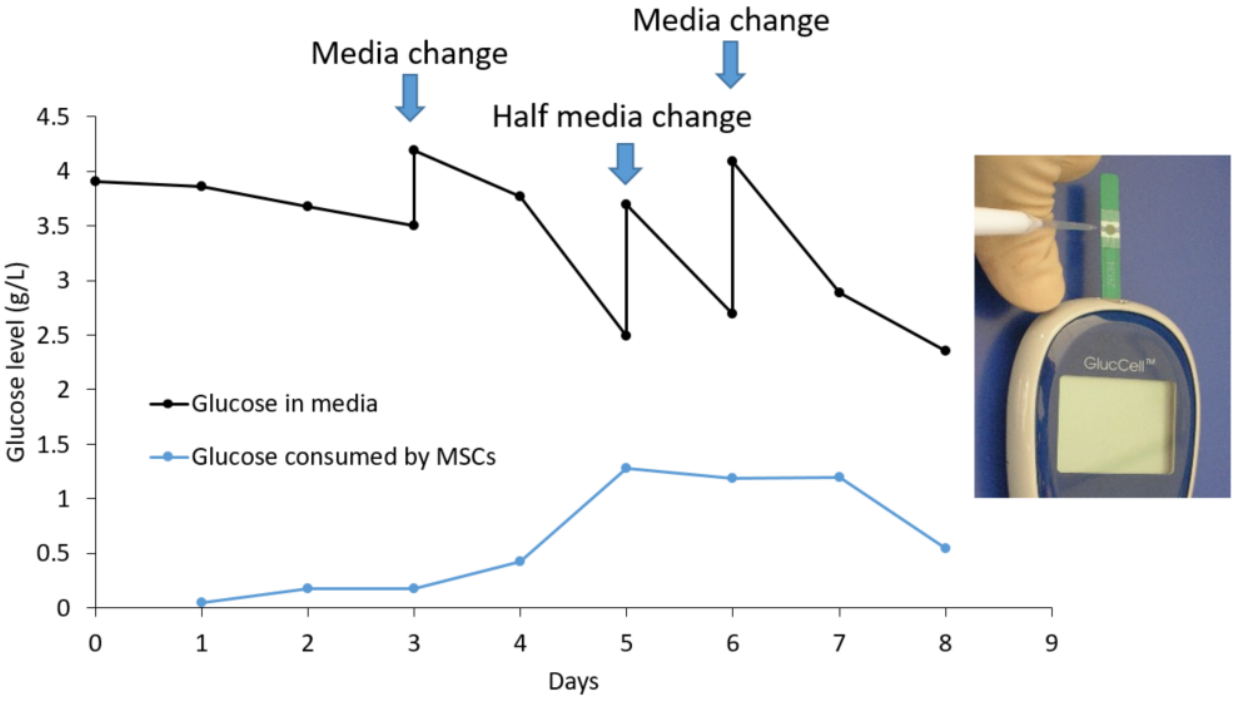




Cell Growth



Glucose Consumption Level



pH Monitoring