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Protocol for CelCradle™ 500A Operation

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MATERIALS

- Coating Solution (optional)
- PBS without Mg2+/Ca2+
- Cells
- Complete media
- CelCradle Stage 3000
- CelCradle™ 500A (EscoAster/VaccixCell)
- GlucCell Monitoring System (GlucCell Meter + Strips)
- pH Meter
- Long Forceps
- Cell Strainer
- Crystal Violet Dye (CVD)
- 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/Collagenase/Accutase/Accumax)

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METHODS

Coating of Carriers (Optional)

a. Bring a single CelCradle 500A bottle into BSC Class II hood

b. Coat carriers with fibronectin or cell attachment solution for 30min 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 for the seeds for one CelCradle™ 500A bottle.

Cell type	Seeding density (Cells/carrier)
Mesenchymal stem cells	20,000 – 60,000
Chinese hamster ovary cells (CHO)	100,000 – 300,000
HEK293T	100,000 – 500,000
Vero	100,000 – 300,000
Hybridoma OKT 3	100,000 – 300,000
MDCK	60,000 – 120,000

Inoculation

Upright seeding

1. Prepare 400 ml culture medium and 1 – 2 x 10^8 cells seed in 100ml culture medium.
2. Slowly and gently pour 400 ml culture medium into CelCradle-500A bottle. Tilt the bottle to send all the media down into the bellows chamber. Compress the bellows to raise the medium to submerge and wet the matrices.

3. Dispense seeds on the matrix.

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4. Move the bottle into CelCradle Stage.

5. Set up inoculation parameters--- Up: 2.0 mm/s, T_H: 20 s; Down: 2.0 mm/s; B_H: 0 s. and then press “Start” to initiate the immobilization process. (Change “Up” to “Down” by pressing “STOP” key; to set minutes, press and hold either “DELAY” arrow key; to set seconds, press either “DELAY” arrow key in short bursts.)

6. After 3~5 hours, change parameters to Up: 1.0 mm/s, Top Holding: 0 s, Down: 1.0 mm/s, Bottom Holding: 1 min for cell culture (the parameters may vary.)

Reverse seeding

For cells seeded with less than $5x10^7$ cells or for cells that are unable to attach easily (Suited for Mesenchymal Stem Cells)

1. Prepare seed in 120ml of fresh and pre-warmed culture medium (Ensure pH is between 7.2-7.4).
2. Dispense seeds on the pre-coated carriers.

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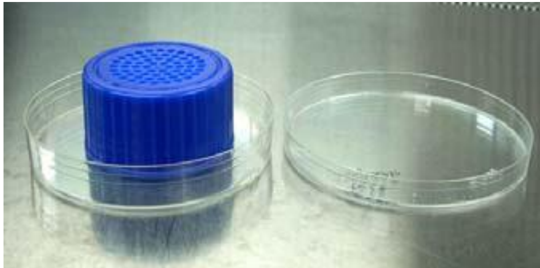
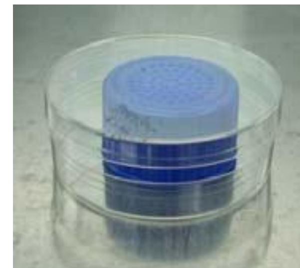
3. Place the blue vented cap in a sterile petri dish and cover the dish to ensure the sterility of the cap for future use.





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 bottle.
7. Move the bottle into incubator.

8. Allow cells to attach for 2-4 h. Swirl and rock bottle gently every 15 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 unattached.

10. Centrifuge and resuspend cells left in the culture media and determine % of attachment
11. Repeat step g – h every hour to check for cell attachment.
12. Stop cell attachment process when the attachment rate is higher than 90%.

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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%.

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: 0 s,
   ii. Down: 1.0 mm/s, Bottom Holding: 1 min

Note: Parameters can be changed accordingly to cell lines characteristics

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 to check glucose, staining and cell count every day, or every 1 day when running initial trials. Prestoblue / MTT/ viability assays for monitoring of cell health on carriers. (Appendix A)

a. 2 ml media: pH and glucose measurement
b. 3 carriers: metabolic activity using PrestoBlue Assay or other viability assays following standard protocol
c. 1 carrier: live/dead cell staining using FDA, PI, Hoechst stains following standard protocol
d. 2 carriers: harvest using trypsin to check for live cell count or by crystal violet dye nuclei count method
e. Change with fresh media when:
   a. Glucose level falls below 1 g/L
   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

Tips: Glucose consumption/ Prestoblue assay 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).

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Cell Harvest

1. Drain off culture media into a container using CelCradle strainer.
2. Rinse with 500 ml of PBS gently twice for 10 min each by placing bottle back into CelCradle stage and setting speed to Up rate: 2 mm/sec, down rate: 2 mm/sec, Up holding: 0 sec, down holding: 0 sec
3. Drain PBS solution after two rinses.
4. Add 120 ml of pre-warmed dissociation solution, 0.05% trypsin-EDTA 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 min in incubator.
6. Tap bottle sharply and steady against your palm for 3 min. Rotate bottle during tapping.

7. Add 0.1 mg/ml DNase and 0.1% trypsin inhibitor (or 10% serum) into 100 ml culture medium or HBSS solution.
8. Add the 100 ml solution into the bottle, cap the non-vented cap firmly. Invert the bottle. Swirl to wash off the cells from the carriers.
9. Pour the cell-laden solution into centrifuge bottles.
10. Repeat bottle tapping and collect cells. Repeat steps 6-9 for 4-5 times.
11. Collect all cells by centrifugation and check for cell density and viability.
12. 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.

To ensure complete harvest, gentle agitation of dissociation enzymes can be performed during incubation of enzymes. Invert bottle and place in orbital shaker at 37°C for 15 min at 200 rpm if cells are not able to come off easily.

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.

Addition of 0.5 mg/ml DNase and trypsin inhibitor/ serum will assist in dislodging cells from carriers and ensuring high cell viability.

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APPENDIX A

Staining with Dyes

Live Cell Staining with Fluorescence Dyes

1. Aseptically sample 1-2 BioNOC® II carrier from T- flask 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 T- flask.
2. Dehydrate and fix the cells using ethanol 70% dehydration 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 light microscope with bright field.

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

1. 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 T-flask.
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 II (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).

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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.

2. By CVD (Crystal Violet Dye) Reagent

1. Transfer three carriers into a 1.5 ml micro-centrifuge tube from T-flask.
2. Add 0.5 ml CVD reagent in each micro-centrifuge tube
3. Vortex each micro-centrifuge tube for 60 seconds.
4. Place micro-centrifuge tube into a 37_oC incubator for 2 hour.
5. Vortex several times during incubation
6. Count the nuclei and calculate the average cell number in one carrier.

Note: CVD reagent as supplied by ESCO, catalog no.1400014.
Not suitable for stem cells due to large secretion of ECM.

Cell Proliferation Assay with Prestoblue Assay

1. Transfer 3 carriers from the flask and place 1 carrier in each per well on a 24 well plates. Add blank carriers as control.
2. Add 450 uL of media with 50 µl of Prestoblue into each well.
3. Incubate at 37 °C, 5 % CO_2 for 1 hour.
4. Transfer 100 µl of media to a 96 well plate and read fluorescence values of excitation 560 nm and emission 590 nm with a spectrophotometer or absorbance of 570 nm and OD 600 as reference wavelength.
5. Correct any background values with control.

Note: Refer to manufacturer's recommendation for other cell proliferation assay.

Glucose Consumption Measurement

1. Sample the media from T flask 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.

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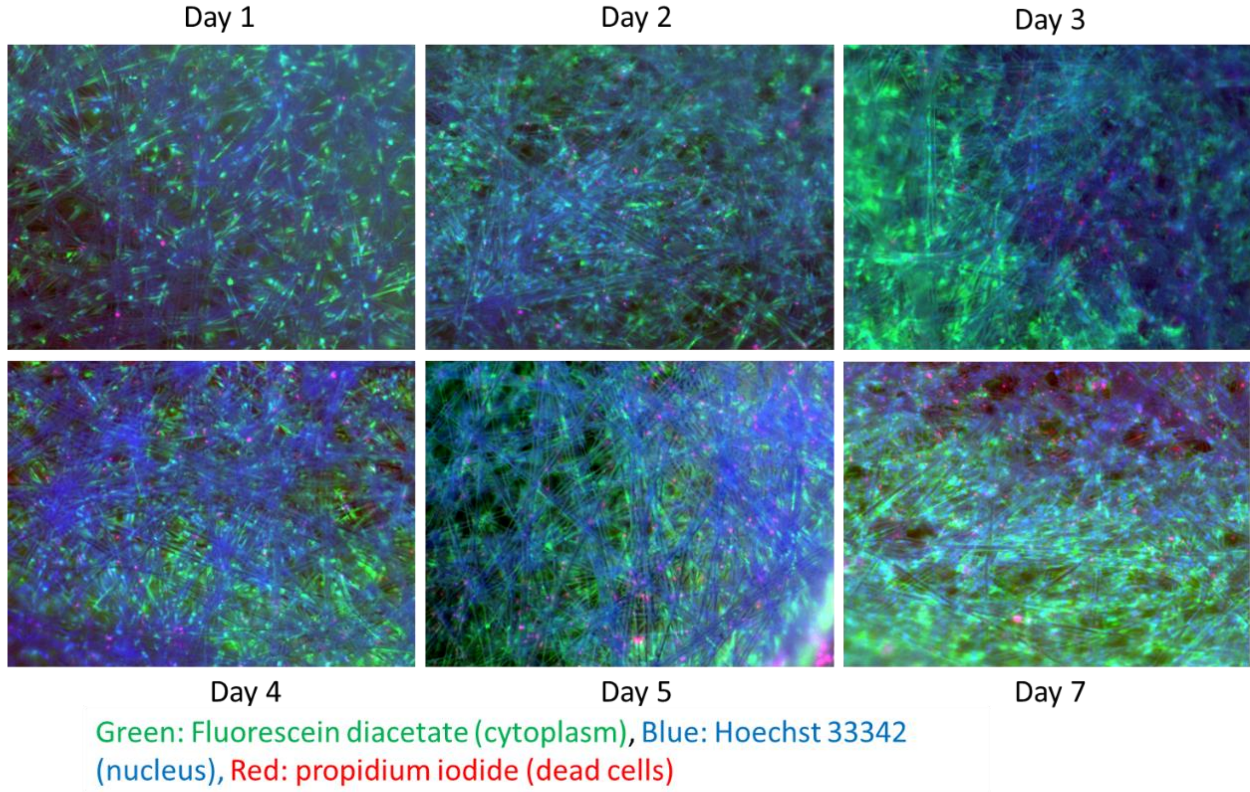
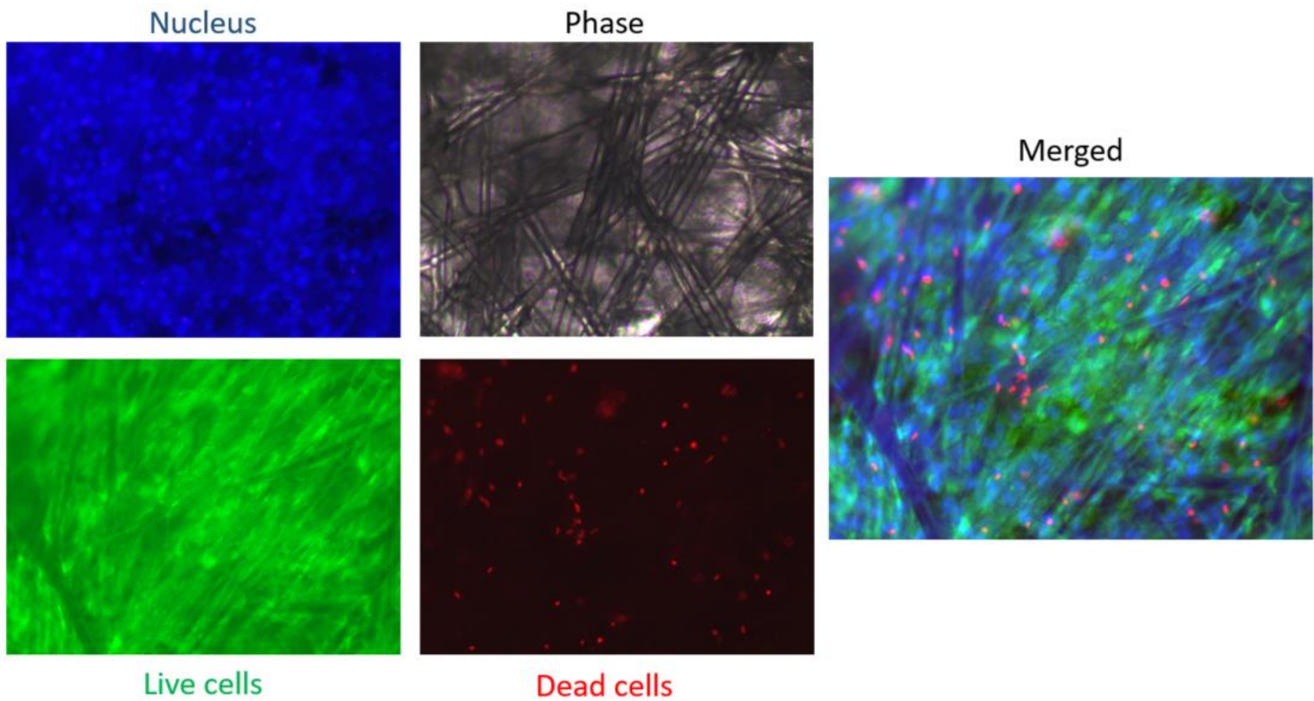
APPENDIX B (RESULTS)

Live Cell Staining





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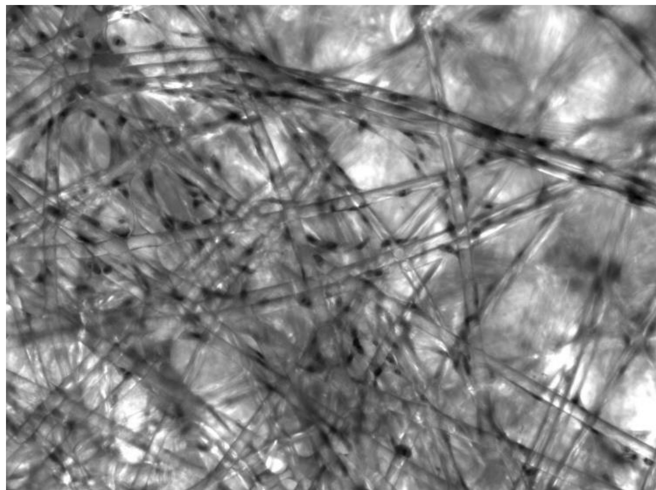
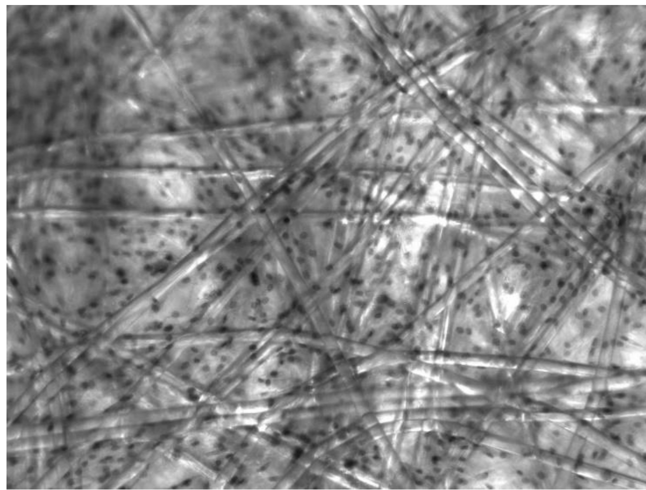
Hemotoxylin Stain on Different Days

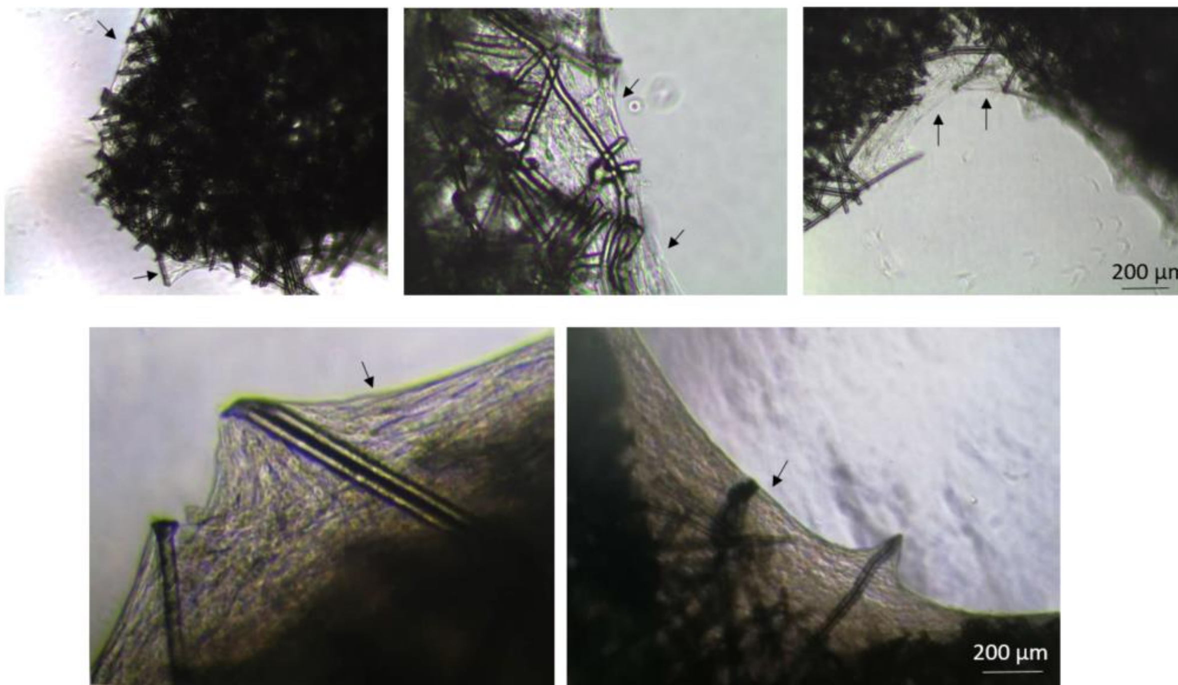
Day 1



Day 3



Cells at High Confluence (MSCs)



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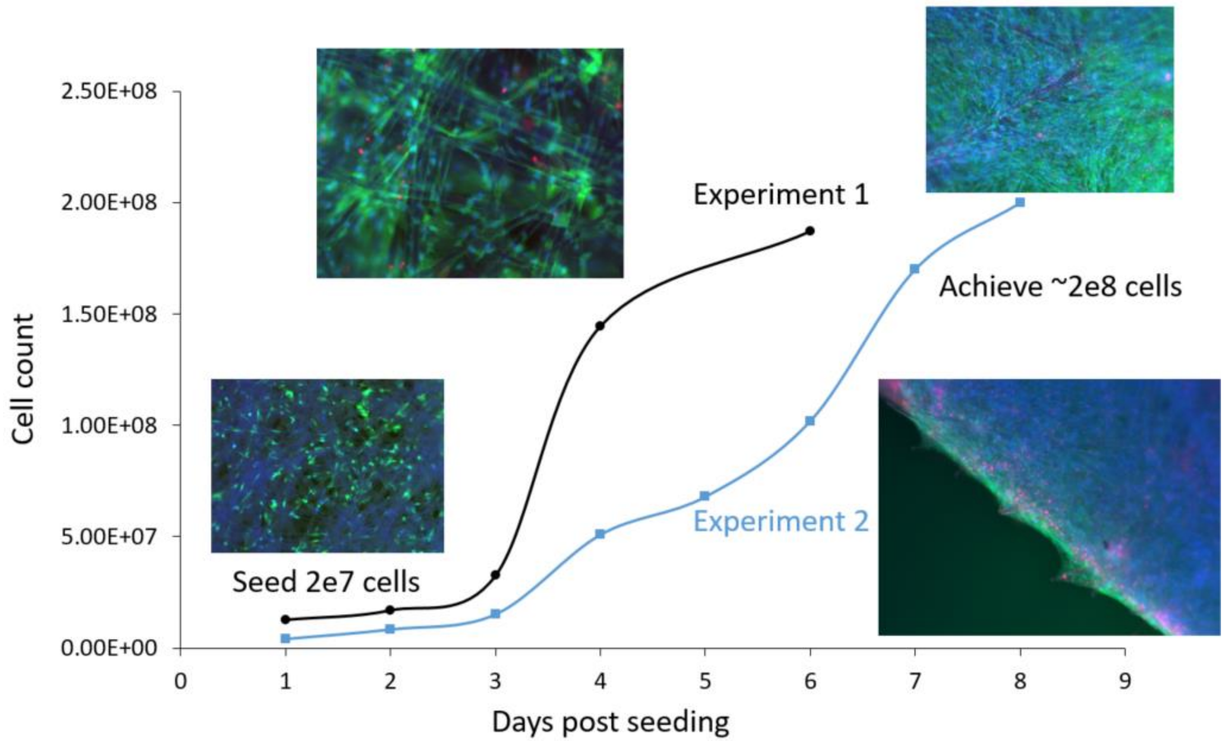
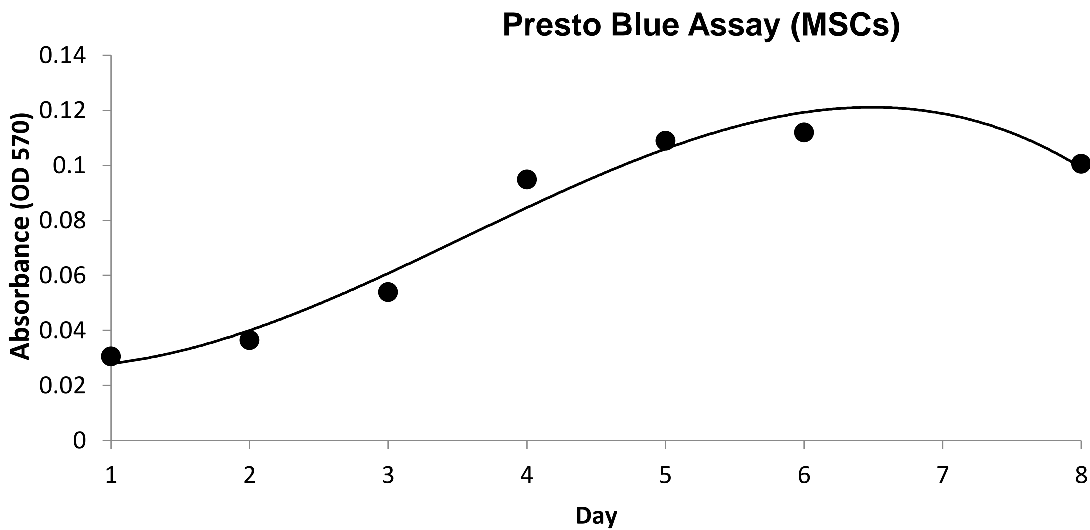
Cell Growth (MSCs)



Cell Viability/Proliferation Assay



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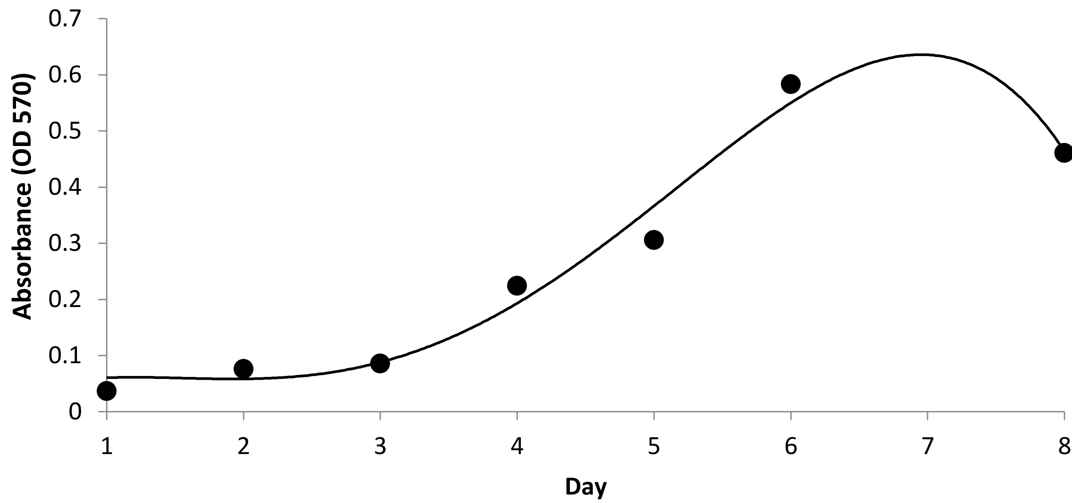
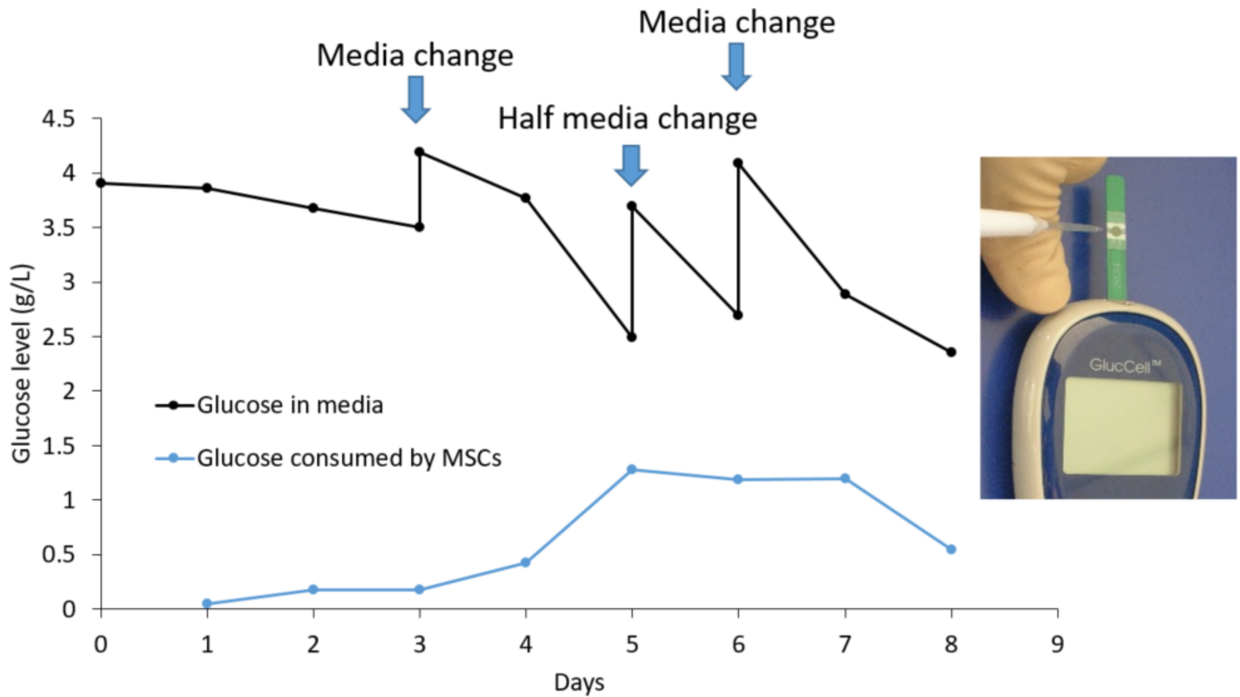
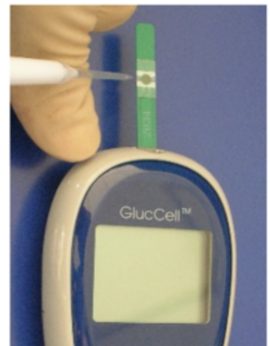


Glucose Consumption Level (MSCs)





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