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

PROOF OF CONCEPT

Protocol for Testing of Tide Motion with BioNOC™ II Carriers

Contents

Introduction

Growing of Cells on BioNOC™ II Carriers with CelXrocker™

The Tide Motion bioreactor achieves mixing, nutrient transport and gas transport by pumping media in and out of a packed bed of the carriers (Figure 1).

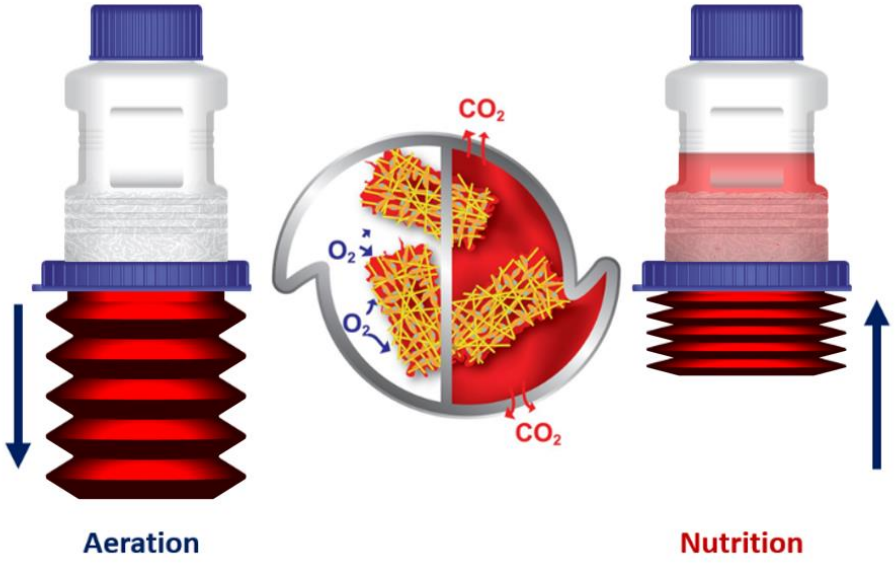


Figure 1: *CelCradle™ operates through the tide motion principle wherein adherent cells attached to BioNOC™ II carriers are alternately exposed to aeration and nutrition phases via the decompression and compression of the bellows holding the culture medium.*

The CelXrocker™ serves as a small-scale system to conduct initial process optimization at lower costs. In this system, BioNOC™ II carriers are seeded with cells and transferred into tissue culture flasks before being placed on a 2-dimensional rocker for the culture period. The rocking motion of the rocker mimics Tide Motion systems by exposing the cells alternately to nutrient and aeration phases as well as providing for the gentle mixing of culture media (Figure 2).

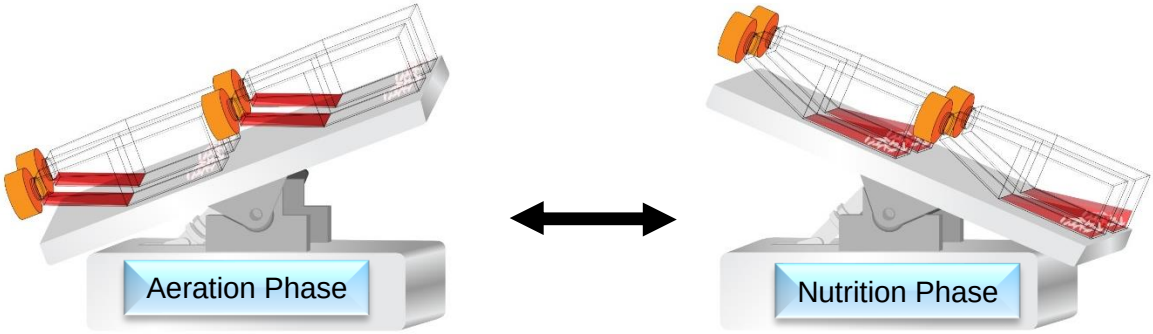


Figure 2: *A schematic diagram showing how the rocking motion of T-flask mimics the Tide Motion principle observed in CelCradle™ bioreactor by alternately exposing carriers to nutrition and oxygen supply on CelXrocker.*

Culturing of Cells in CelXrocker™

Autoclaving of BioNOC™ II carriers

1. Place BioNOC™ II carriers in an autoclavable container
2. Add PBS to carriers. Ensure all carriers are completely submerged.
3. Autoclave at 121°C for 20 min.
4. Store the carriers in PBS until use.

Note: Avoid autoclaving the carriers dry as doing so would damage the surface treatment of the carrier.

Coating of Attachment Factors on Carriers (if required)

1. Aseptically transfer autoclaved carriers from the autoclaved container into a 50 ml tube.
2. Aspirate excess PBS.
3. Coat the carriers as per vendor's recommendation. The carriers should be submerged in the coating agent for the recommended amount of time and temperature.
4. Remove coating solution.
5. Rinse with PBS if needed (coating dependent).
6. Store as XX-coated BioNOC™ II carriers (store at appropriate coating temperature until use).

Inoculation

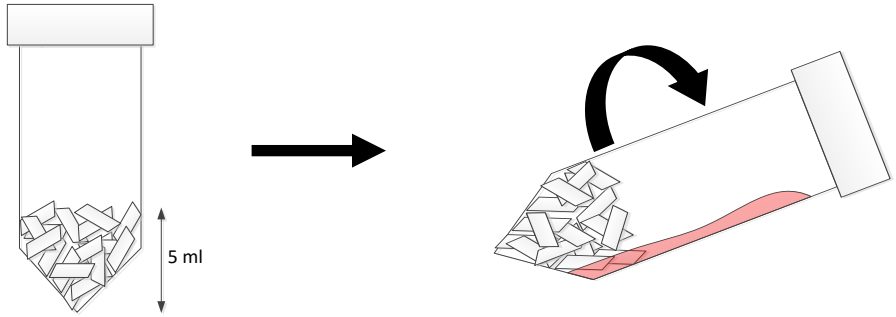
1. Aseptically transfer 30 carriers into 50 ml tube.
2. Inoculate carriers by pipetting required number of cells into 50 ml tube. Refer to Table 1 for optimal cell seeding number per carrier.
3. Add media into 50ml tube until carriers are completely submerged. pH value should be maintained between 7.0 and 7.4 (optimum pH value is 7.2)

Cell type	Seeding density (Cells/carrier)
Chick embryo fibroblasts (CEF)	300,000 – 500,000
A-549	200,000 – 300,000
Chinese hamster ovary cells (CHO)	100,000 – 300,000
HEK293T / PK-15 / IBRS-2	100,000 – 300,000
Vero	100,000 – 300,000
Hybridoma (OKT3)	100,000 – 300,000
MDCK	60,000 – 120,000
Leghorn male hepatoma (LMH)	50,000 – 200,000
MARC-145	50,000
Human mesenchymal stem cells (hMSCs)	20,000 – 60,000
Human diploid cells (WI-38 / MRC-5)	15,000 – 20,000

Table 1: Optimal cell seeding density of various cell types.

Note: A wide variety of cell types can be used to seed onto carriers. Check with Esco personnel for cell types not falling into this category.

4. Gently tilt and rotate the tube 2-3 times to allow uniform mixing of cells with carriers (Refer to diagram below for mixing procedure.)



Note: Avoid tilting the tube pass 90° to minimize cell loss.

5. Incubate the centrifuge tube in upright position for 3 to 5 hours in an incubator (37°C, 5% CO_2) with cap loosened for CO_2 equilibration.
6. At 15 min intervals during the first hour, tighten the cap of the tube, gently tilt and rotate the tube (as described in step 3) to re-suspend cells that may have settled. Loosen the cap and place the tube back in the CO_2 incubator.
7. During the next 2-3 hours, repeat the process (described in step 6) at 30 minute intervals.
8. After 3 hours of incubation, transfer the centrifuge tube to the biosafety cabinet, gently tilt and rotate the tube's contents and sample ~50 µl medium for cell counting. Count suspended cells remaining in the culture medium and determine % of attachment.
9. If a satisfactory attachment rate of 90% or more is achieved (i.e. less than 10% of cells remain in media), proceed to next section. If attachment rate is unsatisfactory, incubate for an additional hour and check attachment rate again. Typically, cell attachment is completed between 3-5 hours.

Note: Please seed at least 3 carriers in a 15 ml falcon tube and 10 carriers in a 50 ml falcon tube to ensure high attachment efficiency. Add enough media to just submerge the carriers. If a falcon tube is not used for seeding, suboptimal attachment rate may be obtained.

Cell Culture

1. Position rocker in CO_2 incubator at 37°C and 5% CO_2. Adjust rocking speed to 4 cycles per min (each cycle consists of a rocking cycle of rocker position from left → right → left).
2. At the end of the attachment period, use a pair of sterile forceps to gently transfer the carriers from the centrifuge tube to a T-75 flask containing 18 ml (0.6 ml of media/carrier) of fresh medium.
3. Centrifuge the media used for inoculation to count the total unattached cells and determine the final attachment efficiency.
4. Place the flask on the rocker prepared in a CO_2 incubator maintained at 37°C and 5% CO_2 (or at preferred set-points for the cell type being cultured).
5. Conduct media change every 2-3 days or according to protocols established in your lab for the particular cell line.

Note: After inoculation, the cells are mildly attached to the carriers. Be gentle while handling the carriers to prevent cells from dislodging.

Note: Use Esco CelXrocker™ or a 2D rocker that has a slow and gentle rocking speed of 4-6 cycles per min. If the rocking speed is too fast, cells may detach or have reduced growth. In this case, we suggest growing the cells under static conditions, however, cells may not reach maximum growth conditions due to nutrient insufficiency. If a rocker is not used, add sufficient media to submerge the carriers and leave at static conditions. The optimal condition for testing will be using a 2D rocker that moves leftwards, rightwards and

returns back to the left. Substandard results may be obtained with orbital rockers/ 3D rockers or under static conditions.

Note: The values stated assumes a culture of 30 BioNOC™ II carriers. Amount of media and size of T-flask should be adjusted according to the number of BioNOC™ II carriers used. For example, to culture cells on 50 BioNOC™ II carriers in a single flask, the carriers can be placed in a T-175 flask with 30 ml of culture media. No more than 5 ml of media (10 carriers) in T-25 flask or 18 ml of media (30 carriers) in T-75 flask or 30 ml of media (50 carriers) in T-175 flask of media should be used to avoid wetting of filter vent during rocker; precaution steps should be taken to avoid contamination.

Note: Addition of 0.6 ml/carrier of culture media is recommended for initial testing but can be adjusted based on requirements of different cell types. The addition of 0.6 ml/carrier mimics the amount of media available for each carrier when CelCradle is performed (500ml media per 850 carriers per bottle). However, more media can be provided for each carrier if there is intention to move into perfusion culture system in CelCradle platform.

Note: During initial runs, it is recommended to monitor the growth of cells on the carriers across the culture period. Cell counts could be conducted by harvesting cells from 3 carriers every alternate days. 1 or 2 carriers could also be stained on the same days to observe the growth of cells directly on the carriers (refer to suggested protocols below).

Monitoring of Cell Growth on BioNOC™ II carriers

Cell Harvesting and Counting

1. By Dissociation Reagent
Enzymatic reagents for dissociation: Accumax, Trypsin 0.25%, TrypLe Express, Collagenase

1. Aseptically transfer 3 BioNOC™ II carriers from the T-flask into a 1.5 ml micro-centrifuge tube.
2. Gently rinse the carriers with 1 ml calcium and magnesium free PBS thrice (gently invert the tube 5 times for each wash).
3. Perform enzyme dissociation:
   i. Trypsin/ TrypLE Express: (most cell types)
      - Add 1 ml 0.05 – 0.25% Trypsin-EDTA, incubate at 37^{o}C for 10-15 min.
   ii. Accumax/ Accutase: (suitable for stem cells)
      - Add 1 ml Accumax/ Accutase, incubate at room temperature for 15 to 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 in HBSS.
      - Add 1 ml of collagenase and incubate for 15 - 30 min. (Please optimize duration of collagenase as required).
4. After the incubation period, transfer the enzymes into a 15 ml collection tube.
5. Add neutralization solution or PBS (depending on the enzyme used) into the 1.5 ml tube containing the carriers and flick the tube using the back of a forceps for 40-60 times.
6. Transfer solution to a 15 ml collection tube.
7. Repeat steps 5 and 6 at least 3 more times with 1 ml of PBS or media.
8. Count cells or centrifuge, aspirate supernatant and resuspend cells in lower volume to obtain cell count.
9. Calculate the average cell number in one carrier.
10. Seed cells into well-plates or flasks to check morphology and viability after harvest.

Note: To harvest an entire flask of carriers, harvesting could be done directly in the flask following the above protocol with slight protocol modifications: Add sufficient volume of enzyme to completely submerge the carriers (Add 1.5 to 2 ml of enzyme for every 10 carriers in the flask). The flask may have to be tilted to a corner during the incubation period to completely submerge the carriers.

2. By CVD (Crystal Violet Dye) Reagent

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

1. Aseptically transfer 3 BioNOC™ II carriers from the T-flask into a 1.5 ml micro-centrifuge tube.
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 37ºC incubator for 2 hour.
5. Vortex several times during incubation.
6. Count the nuclei and calculate the average cell number in one carrier.

Cell Staining and Observation

1. Staining with Dyes

1. Aseptically transfer 1-2 BioNOC™ II carriers from the T- flask into a 24 well plate.
2. Dehydrate and fix the cells using ethanol 70% - 100% for 15 minutes.
3. Wash off the ethanol once, 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 colored dyes for post-harvest stains.

2. Live Cell Staining with Fluorescence Dyes

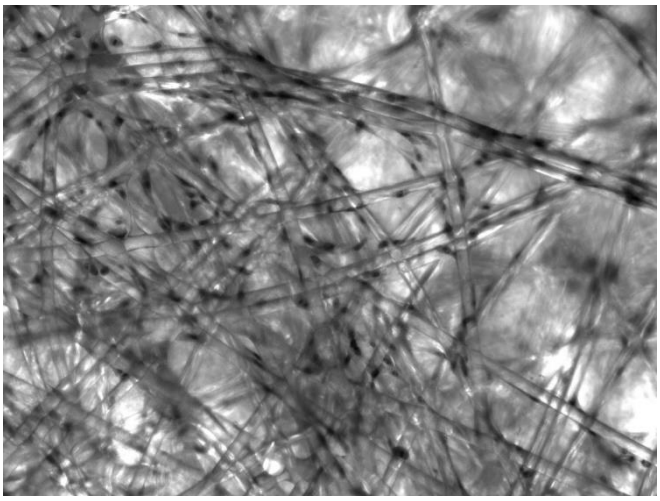
1. Aseptically transfer 1-2 BioNOC™ II carriers from the T- flask into a 24 well plate.
2. Add 500 µl of culture media to the well. Add dyes at the following final concentrations: 1 µg/ml of Hoescht 33342 (Thermo Fisher, H3570), 1 uM calcein green (Thermo Fisher, C34852 and 1 µg/ml PI (propidium iodide, 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 calcein green and red for PI).

Note: Other fluorescence dyes can be used to visualize the cells. Eg. fluorescein diacetate, Cell tracker etc.

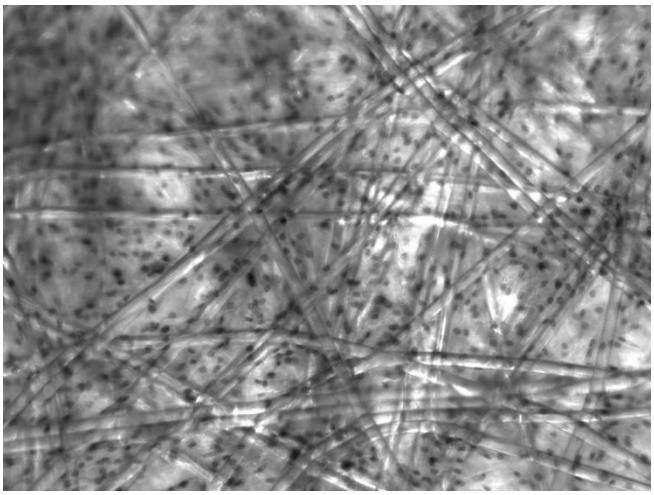
Carrier Staining Examples

1. Hematoxylin staining of fixed human Mesenchymal Stem Cells

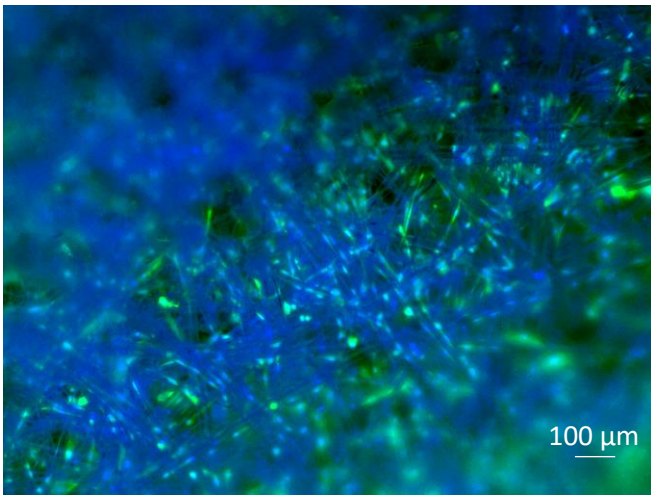
Day 1

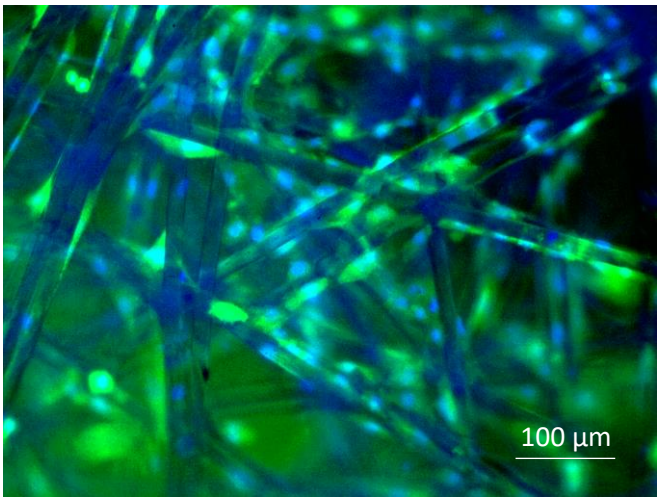


Day 3



2. Fluorescence staining of live human Mesenchymal Stem Cells





Green: Calcein green for cytoplasm, blue: Hoechst 33342 for nucleus, red: propidium iodide for dead cells (none or little observed)