Lab-on-chip clinorotation system for live-cell microscopy under simulated microgravity

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19 **Abstract**

20 Cells in microgravity are subject to mechanical unloading and changes to the surrounding
21 chemical environment. How these factors jointly influence cellular function is not well
22 understood. We can investigate their role using ground-based analogues to spaceflight, where
23 mechanical unloading is simulated through the time-averaged nullification of gravity. The
24 prevailing method for cellular microgravity simulation is to use fluid-filled containers called
25 clinostats. However, conventional clinostats are not designed for temporally tracking cell
26 response, nor are they able to establish dynamic fluid environments. To address these needs, we
27 developed a Clinorotation Time-lapse Microscopy (CTM) system that accommodates lab-on-
28 chip cell culture devices for visualizing time-dependent alterations to cellular behavior. For the
29 purpose of demonstrating CTM, we present preliminary results showing time-dependent
30 differences in cell area between human mesenchymal stem cells (hMSCs) under modeled
31 microgravity and normal gravity.

32

33 **Keywords:** space biology; clinorotation; clinostat; live-cell; microscopy; stem cell

34

35 **1. Introduction**

36 Cellular specimens in spaceflight exhibit abnormal, time-evolving morphology and
37 cytoarchitecture (e.g. cytoskeleton, focal adhesions, etc.), which may affect certain cell events
38 including replication, differentiation, migration, and signaling [1-3]. These events generally
39 confer broader changes to tissues that can lead to reduced bone mineral density [4,5], muscle
40 atrophy [6,7], back pain [8,9], and other ailments [10]. The success of long-duration human
41 space exploration requires countermeasures that address the fundamental cellular changes

adopted in microgravity and are most effective if they consider the underlying dynamic processes driving these alterations.

The National Aeronautics and Space Administration (NASA), European Space Agency (ESA), and other organizations manage a robust portfolio of research initiatives for space biology, using the International Space Station (ISS) as their flagship facility. However, the ISS is not easily accessible and does not often accommodate continuous monitoring of onboard experiments, thereby limiting the ability to observe time-evolving processes. While ground-based microgravity simulations with conventional clinostats [11-13] are notably less expensive, they also preclude the possibility of real-time cell monitoring. State-of-the-art methods do not easily allow time-dependent investigations to identify the mechanisms of cellular alterations and may consequently lead to an incomplete understanding of how microgravity affects human health.

A brute-force remedy for this latent need is to incorporate a full-scale microscope onto a mega-scale clinorotation platform for ground simulations. Clinorotation was initially developed for studying how plants respond to gravity and is currently the prevailing method for cellular microgravity simulation. It is based on the assumption that a time-averaged nullification of gravity can be achieved by reorienting the gravity vector on biological samples, and that the reorientation is fast enough to ensure that specimens cannot perceive a gravitational bias in any direction. The ESA's clinostat microscope [14] is an example of one mega-scale configuration. Another example was published in 2010 by Pache et. al. [15] and was optimized in 2012 by Toy et. al. [16] to demonstrate how digital holographic microscopy (DHM) with mega-scale clinorotation can monitor cytoskeletal changes in simulated microgravity. Interestingly, these studies showed the first published, same-cell images exhibiting time-dependent lamllipodium

retraction, filopodia extension, and perinuclear actin accumulation under clinorotation compared to static controls.

Even though the clinostat microscope and CR-DHM can be used for time-lapse microscopy, many labs do not have the resources or facility space to incorporate a mega-scale system. Furthermore, mega-scale systems could induce significant mechanical vibrations or impulse loads that may disturb cell cultures. Therefore, we present a clinochip system for Clinorotation Time-lapse Microscopy (CTM) that may also enable long-term, low shear cell culture. While the underlying principles of the clinochip are identical to conventional clinostats, and certainly similar to the mega-scale systems, CTM enables live-cell imaging, without prohibitively large equipment or disruption of culture environments. Importantly, CTM represents a significant step forward in space biology research because it is an affordable, size-manageable system that enables microgravity studies of not only traditional endpoint outcomes, but also dynamic cellular processes.

Moreover, CTM is compatible with any lab-on-chip device assembled on a standard microscope slide, for example: microcavites for cell culture; chemical gradient generators; cell sorters; and capillary-based separation columns. It can accommodate cells in monolayer, suspension, and 3D constructs. State-of-the-art microfluidic techniques allow us to precisely modulate microscale flow to create complex cell culture environments, a feature that is not always possible with conventional clinostat devices. Specifically, the surge in microfluidics research in the past decade has enabled exciting new capabilities for probing cells in a variety of ways. This technology can easily be leveraged with CTM.

Media exchange between an external reservoir and a rotating "clinochip" platform on CTM is feasible by integrating lab-on-chips with a miniature rotary union for programmable

88 media exchange, continuous media circulation, and chemical infusions. Taken together, the
89 enormous scope of possible microgravity investigations distinguishes clinochips from
90 conventional clinostats. We believe that their affordability, easy implementation, and
91 amenability for live-cell imaging will fully-enable researchers seeking to understand the time-
92 evolution of cellular alterations under microgravity simulation.
93
94 **2. Material and methods**
95 *2.1. CTM system*
96 We fabricated a clinochip system that enables imaging of cells subjected to two-
97 dimensional microgravity simulation and can be operated in parallel with a static chip as a
98 control. The CTM configuration depicted in Fig. 1a uses a stepper motor with a resolution of 200
99 macrosteps per revolution and a two-gear train assembly to transfer rotational motion to a
100 clinochip platform that holds a lab-on-chip device. This rotating platform pivots on a custom-
101 built miniature polyterafluoroethylene (PTFE) rotary joint that allows one rotational degree of
102 freedom about the spin axis. Additionally, the rotary joint is equipped to manage fluid exchange
103 between external fluid reservoirs and devices on the rotating clinochip platform.
104 In brief (refer to Fig. 1b), the rotary joint was fabricated with 19-gauge blunt syringe
105 needle tips that were press-fitted from the rear of CNC-milled PTFE connectors into 1 mm
106 access holes until flush with the microchannel groves on the front. Axially self-aligning
107 neodymium ring magnets (RC86, K&J Magnetics) were pressed into slots at the rear of the
108 connectors and provide substantial clamping force when mating two identical connectors.
109 Commonly used as a material for gaskets, PTFE has some unique properties that also make it
110 suitable for the rotary joint: 1) high compressibility forms a tighter seal at the mating interface;

111 2) hydrophobicity helps to prevent fluid wetting and leakage at the interface; 3) low coefficient
112 of friction allows for easy rotation about the spin axis.
113 Open-loop control is established with LabVIEW (v.10.0, National Instruments) for the
114 stepper motor (HT11-013D, Applied Motion Products), inverted fluorescence microscope (IX81,
115 Olympus Corporation), XY motorized stage (MS-2000, Applied Scientific Instrumentation), and
116 B/W CCD digital camera (ORCA-ER, Hamamatsu Photonics).
117
118 *2.2. Lab-on-chip devices*
119 Live-cell CTM devices were fabricated using a high-frequency corona treater (BD-20AC,
120 Electrotechnic Products) to energetically bond layers of polydimethylsiloxane (Sylgard 184,
121 Dow Corning), i.e. PDMS, at 10:1 ratio of base to curing agent, between 75x25x1 mm glass
122 slides. Geometric features in PDMS were formed by a high-resolution razor cutter (FC8000,
123 Graphtec). To prepare microfluidic devices for experiments, cell culture surfaces, consisting of a
124 200 micron tall by 1 mm wide microchannel constructed from PDMS and glass, were cleaned
125 with 70% ethanol, rinsed in deionized water, and air-dried. Immediately before cell experiments,
126 the microchannel was incubated in ambient for one hour with 15 ug/mL fibronectin (354008, BD
127 Sciences) in phosphate buffer saline (PBS) without Ca++ and Mg++ and then gently rinsed 2-3
128 times with PBS. Fibronectin-treated surfaces were kept hydrated by filling culture cavities with
129 fresh PBS and were sterilized by ultraviolet exposure for 15 minutes prior to cell seeding.
130
131 *2.3. Cell culture experiments*
132 Passage-5 hMSCs were expanded in 6-well plates with hMSC media until confluent.
133 Stem cells were trypsinized, centrifuged, resuspended at 10^5 cells/mL, plated into microchannels,

and incubated in a microscope-amenable environmental chamber (Precision Plastics) at 37 °C, 50% humidity, and 5% CO_2 for 20 min before microchannels were gently flushed with hMSC media to remove non-adherent cells. One clinochip and one static chip were placed onto the CTM system, which was mounted to an XY motorized stage (MS-2000, Applied Scientific Instrumentation) on an inverted fluorescence microscope (IX81, Olympus Corporation).

A group of cells that had been seeded on both the clino- and static chip were randomly selected for time-lapse microscopy using differential interference contrast (DIC) and phase contrast. Both chips had similar seeding densities, roughly 5-6 cells in the field of view using a 10X objective, and similar initial morphologies. Before we subjected the clinochip to 60 RPM clinorotation, we acquired an initial image of both chips at 0 hrs. At each subsequent hour, for 8 hrs, we acquired additional images. Figure 2 shows same-cell images at 0, 1, 4, and 8 hr time points for 60 and 0 RPM.

Results

From these timelapse images, we measured time-evolving, same-cell areas using a custom Matlab algorithm (see Fig 3). Average areas were not different in the first 3 hrs of clinorotation. After 5 hrs however, cell areas at 0 RPM increased dramatically while cells at 60 RPM showed little change. Significant differences were found at 6-8 hr time points. Moreover, at each time point, we conducted a visual inspection of other cell groups and found that morphologies for the randomly selected cells were qualitatively representative of the entire population in the chip. Although our sample size was small, our preliminary CTM results demonstrate evidence of substantial changes to hMSC morphology that may affect other functions important to bone health including differentiation and chemotactic homing.

We also took measurements for the absolute difference of same-cell areas between each time point and the previous point, as shown in Fig. 4. While much variability exists in the data, specimens at 0 RPM were measured at approximately 70% higher average difference when compared with 60 RPM.

Discussion

The goal of this paper was to present a way to improve on state-of-the-art clinorotation devices. Since particle physics in conventional clinostats is impossible to accurately control in experiments, cells can be subjected to mechanical forces and chemical gradients that might not be physiological. Additionally, adherent cells in these clinostats need to be seeded on microcarrier beads that have limited surface area for proliferation, which prohibits long-term culture. Moreover, the constant movement of cells through culture media makes dynamic bioassays, which are important for a more holistic understanding of cellular response, generally unattainable. Finally, without the ability to manipulate culture conditions, for example, by modulating the chemical microenvironment, conventional clinostats can only offer a narrow range of possible science investigations.

In conjunction with lab-on-chip technologies, the CTM methods described in this paper addresses these issues and may enable a wide range of live-cell, time-dependent investigations in simulated microgravity. As a whole, CTM allowed us to identify the time-evolution of cell response in simulated microgravity without the limitation of only being able to obtain images at static time-points that are usually the extent of the capabilities afforded by conventional clinostat devices. Using static time points would limit the ability to understand how the time-dosage of microgravity affects cells, introduces more variability in experimental data, and may require

more experimental controls to rule out confounding factors than our CTM system. For these reasons, and for its affordability and versatility, we believe that CTM represents a significant step forward in space biology research.

Our preliminary experiments examine early spreading in hMSCs, when cells are only loosely attached and could mimic how daughter cells in mitosis may behave in microgravity. We hypothesize that microgravity-induced morphological alterations may also affect lineage commitment and may be responsible for the markedly lower rates of differentiation observed in stem cells flown in space [17]. This hypothesis warrants further study, but agrees with previously published work showing that simulated microgravity disrupts hMSC function by enhancing adipogenesis and reducing osteoblastogenesis [18,19]. In future work, we will use CTM to understand how microgravity may affect early attachment by fluorescently tagging cytoskeletal elements and correlating cell morphology with long-term rates of proliferation.

Studying hMSCs is particularly useful because they are important for maintaining bone health and play an integral role in bone fracture healing. Normal cell functions are hypothesized to be adversely affected in spaceflight and may partially explain the decreased bone health and generally poor quality of fracture healing in animal models flown in space. The incomplete understanding of hMSC behavior, as related to bone health in space, may jeopardize the success of future, long-duration manned missions; however, CTM provides a way to improve our understanding.

While CTM is a powerful tool for space biologists, the design that we've presented can only be used to simulate microgravity in 2D, i.e. one axis of rotation. Although this is not considered a major hurdle in microgravity research, as other investigators still use 2D clinostats, 3D microgravity simulation through random positioning machines may be a superior model for

microgravity. In order to achieve 3D clinorotation on a microscope stage-amenable platform, clinochip devices would need to be significantly reduced in size. Also, a completely new type of rotary joint would need to be designed to accommodate the additional axis of rotation. These design limitations can also be considered for future work.

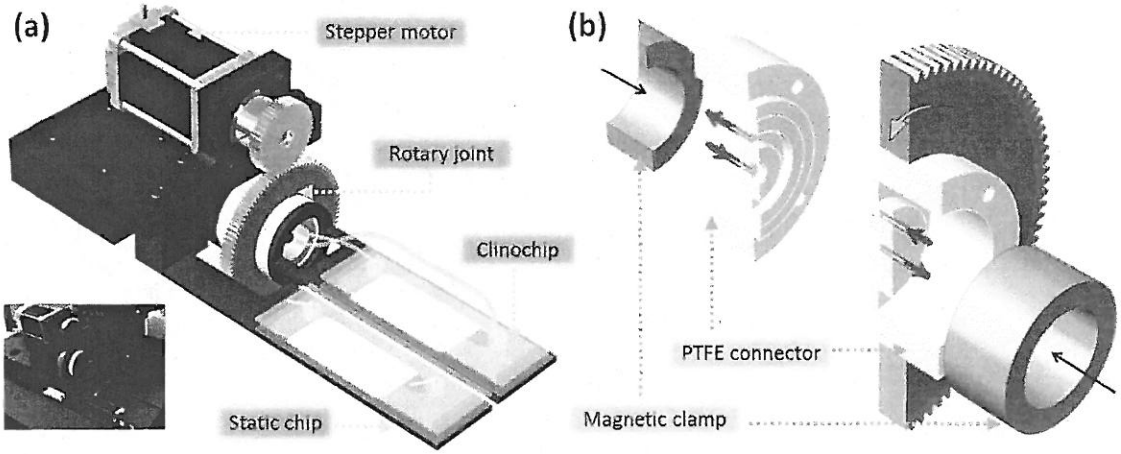


Figure 1. Microscope stage-amenable, Clinorotation Time-lapse Microscopy (CTM) system enables live-cell imaging of cells. (a) CTM components include a clinochip for simulated microgravity and static chip for a 1-g static control. (b) exploded computer model of rotary union designed to allow media perfusion into clinochips for long-term cell culture.

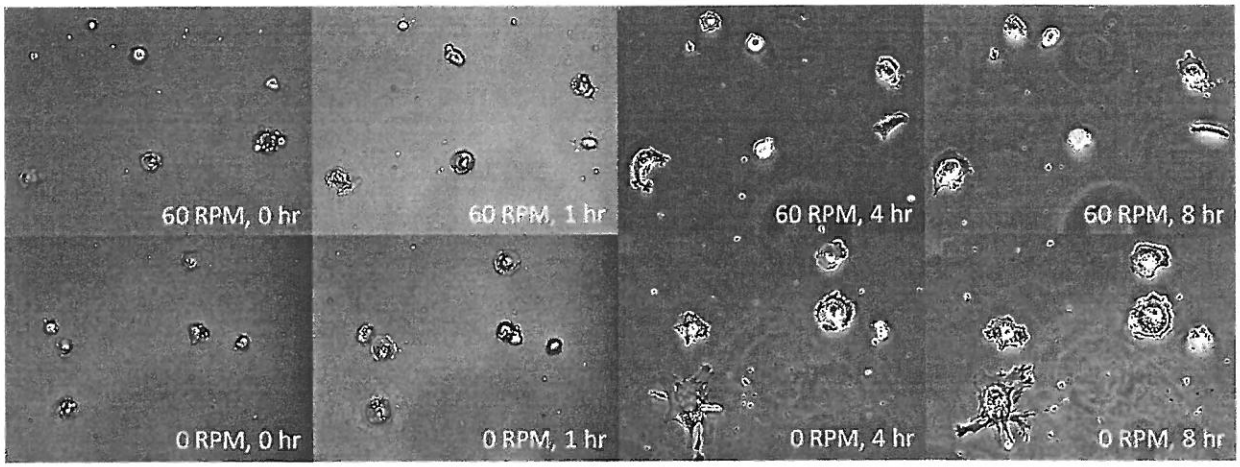
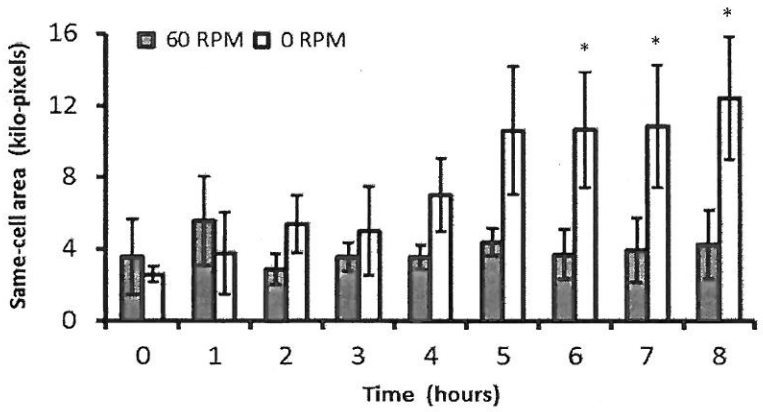


Figure 2. Time-evolution of early spreading in hMSCs imaged under DIC and phase contrast at 60 RPM clinorotation and at 0 RPM static control. Cells at 0 RPM were more spread at 4-8 hrs compared to 60 RPM.



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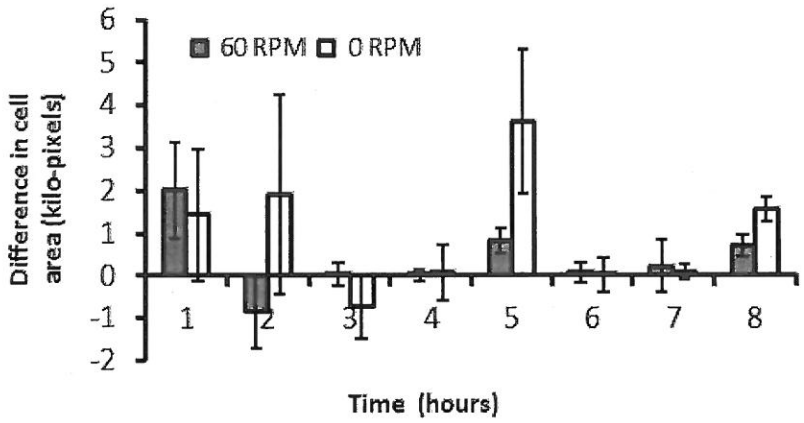
222 **Figure 3.** Mean values of same-cell areas (n=3) and 1 S.D. error bars. From calculated cell areas

223 at 8 hrs (based on images from Fig 2), cells with the three median values were digitally-tagged.

224 To eliminate outliers in cell behavior, only the tagged cells were then used to calculate areas at

225 all remaining time points and used for comparison of means. * p<0.05 difference in cell area

226 between the 60 and 0 RPM chips.



227

228 **Figure 4.** Difference in cell area between current time point and previous time point (n=3) and
229 1 S.D. error bars. To eliminate outliers in cell behavior, only the 3 median values of difference
230 were used for analysis. Specimens at 0 RPM averaged 70% higher differences when compared
231 with 60 RPM.

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