

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

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4 Alvin G. Yew<sup>a</sup>, Javier Atencia<sup>b</sup>, Ben Chinn<sup>c</sup>, Adam H. Hsieh<sup>b</sup>

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6 <sup>a</sup>*Corresponding author:*

7 NASA Goddard Space Flight Center

8 Greenbelt, MD 20771, USA

9 [Alvin.G.Yew@nasa.gov](mailto:Alvin.G.Yew@nasa.gov)

10 Phone: 301.286.3734

11

12 <sup>b</sup>Department of Bioengineering

13 University of Maryland

14 College Park, MD 20742, USA

15

16 <sup>c</sup>Department of Electrical Engineering

17 University of Maryland

18 College Park, MD 20742, USA

## 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.

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

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## 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

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

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

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

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

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

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

86       Media exchange between an external reservoir and a rotating “clinochip” platform on  
87 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 polytetrafluoroethylene (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).

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## 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<sup>++</sup> and Mg<sup>++</sup> 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<sup>5</sup> cells/mL, plated into microchannels,

134 and incubated in a microscope-amenable environmental chamber (Precision Plastics) at 37 °C,  
135 50% humidity, and 5% CO<sub>2</sub> for 20 min before microchannels were gently flushed with hMSC  
136 media to remove non-adherent cells. One clinochip and one static chip were placed onto the  
137 CTM system, which was mounted to an XY motorized stage (MS-2000, Applied Scientific  
138 Instrumentation) on an inverted fluorescence microscope (IX81, Olympus Corporation).

139 A group of cells that had been seeded on both the clino- and static chip were randomly  
140 selected for time-lapse microscopy using differential interference contrast (DIC) and phase  
141 contrast. Both chips had similar seeding densities, roughly 5-6 cells in the field of view using a  
142 10X objective, and similar initial morphologies. Before we subjected the clinochip to 60 RPM  
143 clinorotation, we acquired an initial image of both chips at 0 hrs. At each subsequent hour, for  
144 8 hrs, we acquired additional images. Figure 2 shows same-cell images at 0, 1, 4, and 8 hr time  
145 points for 60 and 0 RPM.

146

## 147 **Results**

148 From these timelapse images, we measured time-evolving, same-cell areas using a  
149 custom Matlab algorithm (see Fig 3). Average areas were not different in the first 3 hrs of  
150 clinorotation. After 5 hrs however, cell areas at 0 RPM increased dramatically while cells at  
151 60 RPM showed little change. Significant differences were found at 6-8 hr time points.  
152 Moreover, at each time point, we conducted a visual inspection of other cell groups and found  
153 that morphologies for the randomly selected cells were qualitatively representative of the entire  
154 population in the chip. Although our sample size was small, our preliminary CTM results  
155 demonstrate evidence of substantial changes to hMSC morphology that may affect other  
156 functions important to bone health including differentiation and chemotactic homing.

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

161

## 162 **Discussion**

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

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



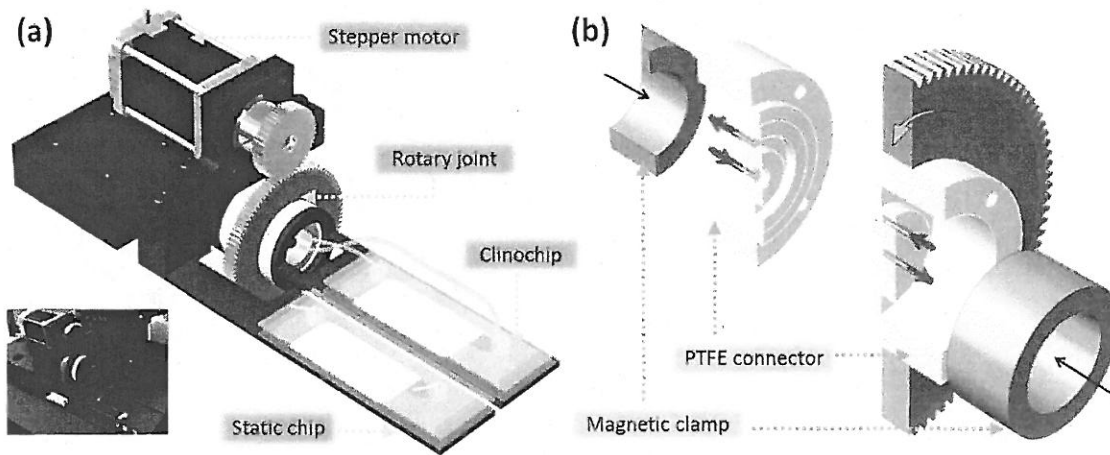
180 more experimental controls to rule out confounding factors than our CTM system. For these  
181 reasons, and for its affordability and versatility, we believe that CTM represents a significant  
182 step forward in space biology research.

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

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

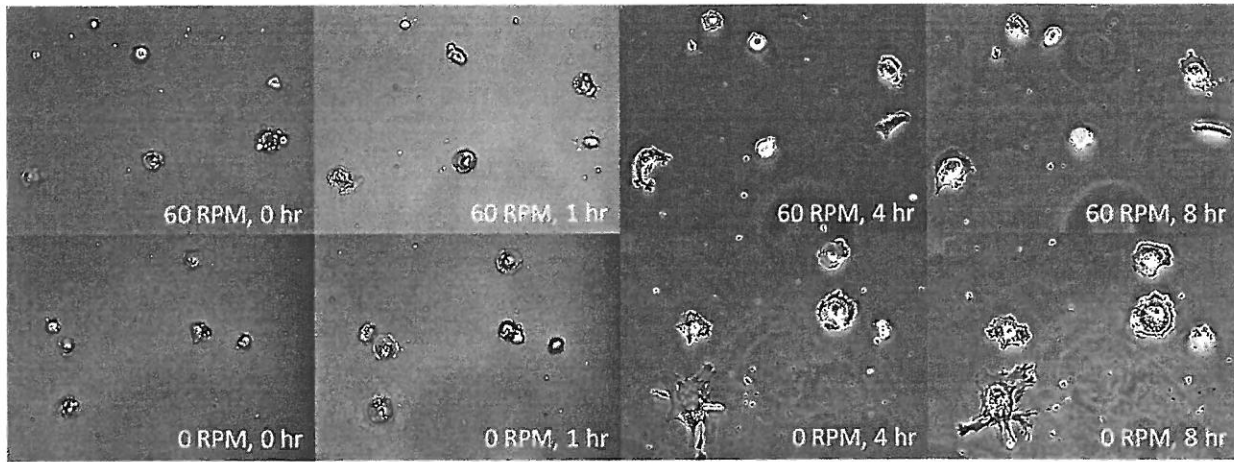
199         While CTM is a powerful tool for space biologists, the design that we've presented can  
200 only be used to simulate microgravity in 2D, i.e. one axis of rotation. Although this is not  
201 considered a major hurdle in microgravity research, as other investigators still use 2D clinostats,  
202 3D microgravity simulation through random positioning machines may be a superior model for

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



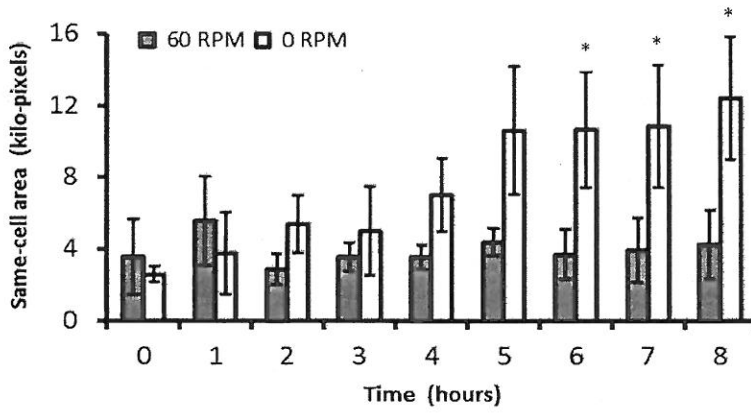
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**Figure 1.** Microscope stage-amenable, Clinorotation Time-lapse Microscopy (CTM) system enables live-cell imaging of cells. (a) CTM components include a clinochip for simulated microgravity and static chip for a 1-g static control. (b) exploded computer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



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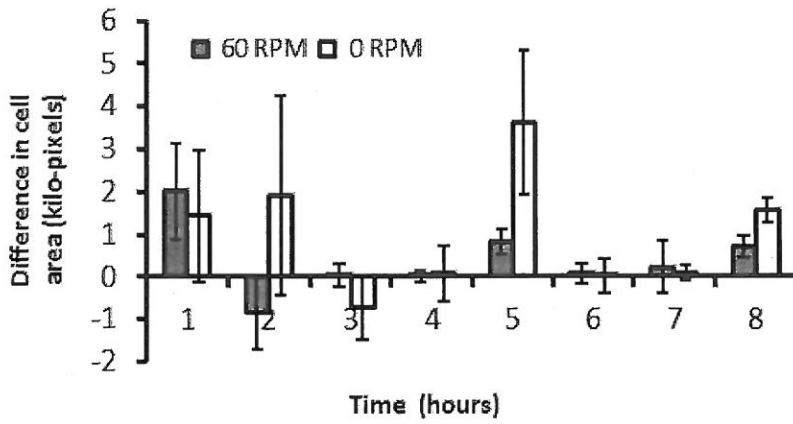
222 **Figure 3.** Mean values of same-cell areas ( $n=3$ ) and 1 S.D. error bars. From calculated cell areas

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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