Factors implicating the validity and interpretation of mechanobiology studies in simulated microgravity environments

Christine Poon\textsuperscript{1,2}

\textsuperscript{1}School of Biomedical Engineering, Faculty of Engineering, University of Sydney, Sydney, Australia
\textsuperscript{2}School of Biomedical Engineering, Faculty of Engineering & Information Technologies, University of Technology Sydney, Sydney, Australia

Correspondence
Christine Poon, School of Biomedical Engineering, Faculty of Engineering, University of Sydney, Sydney, Australia.
Email: christine.poon@sydney.edu.au

Abstract
Simulated microgravity (s-\(\mu\)g) devices provide unique conditions for elucidating the effects of gravitational unloading on biological processes and are increasingly being applied for mechanobiology studies. However, without proper characterization of the mechanical environment generated by these systems, the interpretation of results is confounded and limited. Furthermore, the cell culture approaches central to s-\(\mu\)g experimentation introduces new factors that can fundamentally affect results, but these are currently not addressed. It is essential to understand the complete culture environment and how constituent conditions can individually and synergistically affect cellular responses in order to correctly interpret results, otherwise outcomes may be misattributed to the effects of microgravity alone. For the benefit of the growing space biology community, this article critically reviews a typical s-\(\mu\)g cell culture environment in terms of three key conditions: fluid-mediated mechanical stimuli, oxygen tension and biochemical. These and the implications of other experimental variables for biological analysis are categorically discussed. A new set of controls is proposed to properly evaluate the respective effects of these conditions in s-\(\mu\)g culture, along with a reporting matrix and potential strategies for addressing the current limitations of simulated microgravity devices as a platform for mechanobiology research.

KEYWORDS
cell culture, experimental methodology, gravitational unloading, mechanobiology, simulated microgravity, space biology

1 | INTRODUCTION

Originally confined to the realm of space biology, simulated microgravity (s-\(\mu\)g) devices are increasingly seen as a novel platform for studying the effects of reduced loading on cellular functions in normal and diseased states, with an overarching aim to facilitate the discovery of novel therapies for both terrestrial and space biomedicine applications. While the systemic effects experienced by astronauts due to lack of gravitational loading during space expeditions are well...
documented, the study of the effects of zero gravity on biological processes and mechanisms has been hindered by the sheer cost and logistical challenges involved in conducting biological studies at the International Space Station, which are magnified for countries without a formal space agency to facilitate the process. On Earth, the few ways of simulating microgravity are limited to free fall in drop towers, intermittent parabolic flight and neutral buoyancy chambers, prompting the development of simulated microgravity devices as a more accessible and cost-efficient alternative platform for studying the cellular effects induced by gravitational unloading as experienced in space.

The main classes of s-μg devices are rotating wall vessel (RWV), 2D and 3D clinostats, random positioning machines (RPMs) and magnetic levitation (Figure 1). RWV, 2D and 3D clinostats operate by counteracting gravitational sedimentation of cells suspended in fluid by gentle rotation typically 60° s⁻¹ or 10 rpm, whereas RPMs redistributing the gravitational vector across multiple dimensions by randomized rotational changes such that at any given time point, the time-averaged gravitational vector within a sample is zero or near-zero. Magnetic levitation devices suspend cells in fluid by applying a strong magnetic field to cells that are typically conjugated to ferromagnetic particles. RPMs represent the most recent evolution of 3D clinostats, where cells are cultured in medium-filled cylindrical pipettes, flasks or other closed vessels that are fixed on or close to the center of rotation of the device. RPMs have been particularly popularized as their randomized time-averaged revectoring modes are thought to provide a more effective proxy of microgravity than repetitive clinostat rotation. Furthermore, their inherent compatibility with standard consumables compared to devices with custom inbuilt chambers, ready translation of standard culture techniques, as well as the relative ease of constructing low-cost devices have led to their growing application.

Since the first clinostat introduced by Julius Sachs in the 19th century, significant developments have culminated in these simulated microgravity devices and there is rapidly growing interest in their application in mechanobiology. Although well established for plant microgravity research, there is the risk that s-μg devices may be applied to study mammalian cell mechanobiology without a thorough understanding of the operating principles and conditions produced by these devices. Few cell biologists would refute the necessity of understanding the mechanical environment for answering mechanobiological questions. However, while simulated microgravity systems have been specifically designed or selected for cell mechanobiology studies, a limitation of recent studies involving RPMs has been that, in some cases, full fluid dynamics characterization was not provided or not reported. Analysis of the fluid kinetics generated within the culture domain during s-μg exposition is essential for assessing and explaining measurable cellular behaviors under these new, mechanically dynamic culture conditions.

Coupled with the sheer variability between systems, treating system conditions as a “black box” has led to contradictory findings within the growing body of simulated microgravity research. For example, s-μg culture is reported to induce apoptosis, reduce endothelial cell migration, inhibit stem cell differentiation and proliferation, as well as suppress metastasis for certain cancer cell lines. Yet in other studies, s-μg culture is reported to reduce apoptosis, enhance stem cell proliferation and differentiation potential, increase endothelial cell migration, promote cancer cell metastasis or yielded a combination of increased stem cell proliferation and reduced differentiation potential. These conflicting results confound our collective understanding and can be attributed to cell and study specificity, where

![Figure 1](image.png)

**Figure 1** The main classes of s-μg devices. RWVs rotate a full cylindrical vessel along a horizontal axis, clinostats rotate samples along one rotational axis, RPMs and 3D clinostats rotate samples on two perpendicular axes and magnetic levitation devices apply strong magnetic fields to suspend cells which typically have been conjugated to ferromagnetic particles. \( \omega \) = angular velocity, \( F_g \) = gravitational force, \( F_m \) = magnetic force, \( r \) = radius
measurable outcomes occur as a summation of responses of a particular cell type to the entire spectrum of physicochemical cues within a culture environment. While s-μg devices do provide more accessible means of studying mechanical unloading on cells and have produced some comparable results with real microgravity culture to date, the effect of other conditions, not just microgravity, must be known and considered in order to correctly understand and explain results.

Other than gravitational revectoring, s-μg culture already presents a novel cellular environment compared to standard techniques in that the culture vessel must be completely filled with culture medium in order to reduce the effects of shear during rotationally-mediated gravitational revectoring. This complete fluid environment (defined here as “full vessel” for ease of reference) affects oxygen diffusion and hydrostatic loading, which fundamentally regulate homeostasis, embryonic development and key cellular processes such as differentiation, migration and proliferation. Rotation of a culture vessel on clinostat, RPM or RWV devices induces fluid shear stresses and improves mass transport, which stimulate cell growth as in bioreactor culture. Furthermore, simulated microgravity devices introduce system-specific artifacts that can affect cellular processes, such as centrifugal forces and vibrations (clinostats, RPMs, RWVs) or magnetic fields (magnetic levitation devices) but are seldom discussed.

Without thorough characterization of the whole culture environment, caution must be taken in interpreting results and solely attributing outcomes to the effects of reduced gravitational loading. Another risk is the premature application or promotion of s-μg-treated stem cells as a therapy for example for spinal cord repair, without first having a definitive correlation between cause and effect. As reproducibility must be established for quality control and regulatory approval of cell-based therapies, understanding the culture environment is a vital first step toward being able to produce desired cellular outcomes reliably and consistently.

Here, for the benefit of the growing space mechanobiology community, the s-μg culture environment is evaluated in terms of three key constituent environments: hydromechanical, oxygen and biochemical (Figure 2). These and their fundamental implications on cellular responses are discussed using the standard T25 culture flask commonly used in s-μg research as an example to illustrate concepts which are applicable to all μg-exposed cell culture environments. A minimum of three experimental controls are proposed to evaluate the respective effects of microgravity and these conditions. Critical experimental design factors and limitations to reproducibility and comparability of s-μg with real microgravity (r-μg) studies are also discussed. Finally, a reporting framework is proposed to encourage more detailed reporting to facilitate comparison between studies and platforms.

2 HYDROMECHANICAL ENVIRONMENT

Cells are sensitive to mechanical stimuli. Therefore, the study of cell mechanobiology necessitates a thorough understanding of the loading conditions that comprise the mechanical environment that cells and tissues of interest are subjected to. Cells cultured in standard static conditions experience atmospheric pressure and hydrostatic pressure from the surrounding culture medium, whereas cells cultured in systems that involve fluid motion (e.g., bioreactors, organ-on-chip and s-μg devices) experience convection and flow-induced shear due to the movement of fluid molecules and particles (e.g., cells)

FIGURE 2 Cellular effects in s-μg studies occur as a sum of responses to hydromechanical, oxygen and biochemical cues in the overall culture environment
against one another. Other than culture substrates, the common environment through which forces (mechanical stimuli) are delivered to cells is the culture medium. In s-μg culture, the generation of microgravity gradients via rotation of the fluid domain adds another layer of mechanical complexity to the overall dynamic fluid environment whereas there is limited to no convection in real microgravity, so it is important to distinguish the effects of flow-mediated mechanical stimuli for comparability.

While flow mechanics can be challenging to characterize experimentally, computational fluid dynamics (CFD) is a well-established engineering discipline that enables reliable mathematical simulation of flow phenomena for design optimisation and analysis of fluid systems. CFD has been increasingly applied by tissue engineers to analyze and evaluate bioreactors and microfluidic devices. CFD characterization of flow mechanics within s-μg systems should likewise be conducted and reported. Wuest et al recently conducted such CFD analysis and found that RPM velocities between 40 and 90 s^-1 causes significant convection within a full standard T25 flask. Fluid shear stresses of approximately 10 mPa and 50 to 300 mPa were determined within the bulk volume and at the walls of the flask respectively, that is, cells would temporarily experience higher shear at the walls of the flask. It is well documented that cells are sensitive to shear, where low stresses within a range of 20 to 200 mPa can affect a range of cell processes and functions, for example, stimulate sodium channel activation, enhance calcium signaling in vascular endothelial cells, induce endothelial stress fiber formation and direct stem cell differentiation toward vascular lineages. There are also shear thresholds that limit the formation of 3D cell aggregates. However, experimental shear stresses can be higher than simulated values due to the presence of air bubbles. Sufficiently high shear can cause shear damage, cell detachment and apoptosis, leading to a lower cell viability or cell counts as commonly observed in s-μg studies. Fluid shear stress-induced cell damage and death depend on the magnitude and duration of shear exposure and the innate resistance of the cell type to shear damage. For example, even short exposure to stresses at and above 5.1 Pa (510 dyn cm^-2) for up to 11.2 seconds significantly reduced the viability of some cancer cell lines, which were shown to be more shear resistant than epithelial cells. Also, parameters including culture medium viscosity, vessel size, geometry, rotational velocities, modes and algorithms determine system-specific loading regimes. As another exemplary CFD study of a s-μg device, Leguy et al simulated a maximum wall shear stress of 8 mPa in a cylindrical vessel sinusoidally rotated at 0.1 to 0.2 Hz. Other than CFD, particle image velocimetry (PIV) may be used to experimentally characterize flow phenomena in s-μg devices. Overall, it is essential to characterize the flow dynamics of each s-μg culture system in order to elucidate the relative contribution of fluid forces and microgravity on cell mechanoresponses, as well as optimize rotational velocities and acceleration to minimize shear.

In addition to the complex fluid mechanics in s-μg culture, it must be mentioned that the culture medium exerts hydrostatic pressure on the cells due to gravity, which is relevant to any static control and of course, standard cell culture. Hydrostatic pressure (P) is expressed as P = ρgh, where g is the gravitational acceleration constant, and ρ and h are the density and height (depth) of the fluid respectively. This hydrostatic environment is revectored in s-μg culture to dynamic flow regimes, which are governed by Navier Stokes and continuity equations. Other than density, the depth of culture medium directly determines the magnitude of P. For a standard T25 flask, 5 to 7.5 mL of medium is typically added for culturing up to 3 × 10^6 cells at confluency, which translates to a depth between 2 and 3 mm. Assuming the density of culture medium to be 1.007 g cm^-3 at 37°C (as measured for RPMI-1640 medium supplemented with 10% v/v fetal calf serum) and a gravitational constant of 9.81 ms^-2, a 2 to 3 mm thick layer of culture medium would exert 20 to 30 Pa hydrostatic pressure on cells in conventional culture. For a simulated microgravity static control, filling a T25 flask with 50 mL of culture medium produces a fluid depth of 20 mm above the culture surface, which translates to 200 Pa hydrostatic pressure. While this represents a 10-fold increase, daily variations in atmospheric pressure typically occur in the range of hundreds of Pascals, therefore the difference can be considered to be insignificant. However, culture medium depth directly affects a key driver of cellular functions - oxygen tension, which will be discussed in the following section.

## 3 | OXYGEN DIFFUSIVITY & DELIVERY

Oxygen is essential to life and its role as both a metabolic and signaling molecule in modulating many cellular processes and functions is well established. However, oxygen has low solubility in aqueous media and is limited by diffusion distance. The diffusion distance of oxygen is very small in most mammalian tissues, where cells are typically located within 100 μm from its nearest capillary to ensure efficient gas exchange. In contrast, standard cell culture relies on oxygen exchange at the air-liquid interface of the culture medium. After dissolution, oxygen must diffuse through the medium to the cells across depths that vary from several millimeters to over a centimeter depending on the volume of culture medium added to meet cell nutrient demands, as well as the size and geometry of the culture vessel.
FIGURE 3  Comparison of the relative surface areas available for oxygen diffusion between normal flask culture and flasks completely filled with medium as in simulated microgravity culture. In addition to the depth of medium, this area is a limiting factor for oxygen delivery to cells. CSA = cross sectional area

The correlation between culture medium depth and O₂ delivery to cell cultures has been known for decades. However, it is only recently gaining attention as a contributing factor to poor study reproducibility and presents a particularly important consideration for full vessel culture. Oxygen delivery to cells is governed by two overarching equations: (a) Henry's law of solubility, which describes the solubility of a gas in liquid, and (b) Fick's law of diffusion, which describes the flux of a gas in proportion to the thickness of a fluid layer and concentration gradient across a diffusion barrier. How these relations determine the oxygen environment within a culture flask and implications for s-μg studies will be discussed in the following section.

3.1 Oxygen conditions in the culture environment

Desktop RPMs and clinostats are typically designed to operate within a standard incubator, where the ambient oxygen concentration is closer to 18.6% rather than the frequently stated 20% or 20.9% (atmospheric O₂). Unless the gas composition was specifically tailored to suit the purposes of a study, standalone devices with inbuilt incubator chambers can also be expected to maintain O₂ concentrations of approximately 18.6% at 37°C, 5% CO₂ and 100% humidity, that is, an oxygen concentration of 18.6% (or partial pressure of 141 mmHg at sea level) is available for dissolution into the culture medium. This dissolution is governed by Henry's law, \( H = c/p \), which states that the molar concentration of a gas \( (c) \) is directly proportional to the partial pressure \( (p) \) of the gas above the liquid. 1.32 μM O₂ dissolves in pure water per 1 mmHg O₂ partial pressure, however, the presence of salts and solutes lowers O₂ solubility. Approximating culture medium to have the O₂ solubility of blood plasma (1.26 μM O₂ per 1 mmHg at 37°C), culture medium would theoretically contain 177 μM of dissolved O₂ at 141 mmHg O₂ under standard incubator conditions at equilibrium.

3.2 Oxygen diffusion from the incubator to culture medium

In standard flask culture, air from the incubator enters the flask through the lid, where oxygen dissolves into the medium at the air-liquid interface by Henry's law to a concentration of approximately 177 μM. This dissolved oxygen then diffuses through the depth of the medium to cells adhering to the bottom culture surface by Fick’s law of diffusion, \( J = D \Delta C/\Delta x \), where \( J \) is the diffusion flux, \( D \) is the diffusion coefficient of a gas, \( \Delta C \) is the concentration difference on either side of the fluid layer and \( \Delta x \) is the thickness of the diffusion barrier. As direct examples of Fick’s law in action, varying the depth of culture medium was shown to affect the cellular oxygen environment and increasing the depth (volume) of media was shown to directly reduce the rate of oxygen transfer to cells under standard culture conditions. Similarly, increasing the depth of medium in full vessel culture directly reduces oxygen flux. Furthermore, the area available for gas dissolution (air-liquid interface) is limited to the size of the flask opening (Figure 3). Assuming that a standard T25 flask has a horizontal cross-sectional area of 25 cm² and an inner neck diameter of 18 mm, and the lid itself does not limit airflow, completely filling the flask immediately reduces the air-liquid interface to 2.54 cm², that is, it would take 10 times longer for the equivalent amount of oxygen to diffuse into the medium. Secondly, this smaller amount of dissolved oxygen at the flask opening is dispersed through a significantly higher volume and depth of medium, that is, a lower concentration of dissolved oxygen available and slower delivery to cells. Therefore, cells in a static 1 g control group may be experiencing hypoxia (low or inadequate levels of oxygen) or anoxia (no oxygen).

Other than diffusion distance, the actual concentration of oxygen at the cellular level is governed by the complex interplay between the oxygen consumption rate (OCR) of the cells, cell density, media diffusion properties (solute concentration), temperature, geometry of the vessel and position of cells within the culture environment, which determine
the oxygen concentration gradient (ΔC). Higher cell densities and cells with higher OCRs would increase ΔC and oxygen flux (J) according to Fick’s law. Wagner et al\(^8\) compiled a list of experimentally determined OCRs for a number of cell lines, which range from 1 to 120 attamols cell\(^{-1}\) s\(^{-1}\) for a majority of cancer cell lines to 200 to 400 attamols cell\(^{-1}\) s\(^{-1}\) for primary hepatocytes (a high OCR cell type). Based on these OCR values and simplifying Fick’s law by substituting J with cell density-normalized OCR, Place et al\(^8\) calculated the maximum medium thicknesses where the rate of oxygen diffusion exactly matches the OCR of the underlying cell layer. The maximum medium depths to support an equilibrium of oxygen flux and OCR is 12.1 mm for cell lines with an OCR up to 40 attamols cell\(^{-1}\) s\(^{-1}\) and 4.8 cm for a cell type with an OCR of 1.\(^8\) In a standard T25 static control flask, cells will experience a gradient of oxygen concentrations which occur as a function of the distance of a cell from the lid, that is, the location of the air-liquid interface (Figure 3). Here, the oxygen flux profile cannot be assumed to be linear due to the off-center positioning of the air-liquid interface toward one side of the flask while cells are located on a face perpendicular to that of the lid. Real microgravity studies also face similar oxygen diffusion limitations. For s-μg and dynamic control groups, convective mixing from sample rotation improves oxygen dissolution by homogenizing the oxygen concentration more efficiently than by passive diffusion. Hence some effects of s-μg culture are likely due to improved mass transport and oxygen delivery from mixing and must be elucidated. Experimental oxygen concentrations can readily be measured using non-contact probes and sensors such as those currently employed in hypoxia and hyperoxia studies.\(^8\) This practice is recommended.

### 3.3 Physoxia for phenotypic cellular responses

Given that oxygen demand and consumption rate are cell and tissue specific, if follows that s-μg results are partially determined by whether the oxygen conditions within the vessel were hypoxic, physoxic or hyperoxic to the cell or tissue of interest that is, lower, at or higher oxygen respective to physiological levels. For example, apoptosis and cell loss or dysfunction frequently reported in simulated microgravity studies may partially be due to hypoxia or hyperoxia. In the body, oxygen levels range from 0.5% to 14%.\(^9\) Therefore, standard culture at 18% O\(_2\) is generally hyperoxic. Which is suboptimal for certain cell types that functionally require lower oxygen concentrations in the body, for example, hematopoietic\(^9\) and embryonic stem cells.\(^9\) Alternatively, s-μg devices such as the RWV may come with in-built oxygenation systems\(^9\) that may produce conditions that are hyperoxic for cell types of interest. Importantly, lower oxygen diffusion in a full vessel static control may affect cellular responses by which comparisons are made, for example, if control conditions are hypoxic and fluid convection elevates the oxygen concentration to physoxic levels in s-μg, the difference in cellular responses may be overly exaggerated. Therefore, to eliminate oxygen-mediated responses as a variable, the physoxic conditions of a cell type or tissue of interest must be considered and recapitulated.

Physoxic conditions can be provided by first modulating the ambient incubator O\(_2\) concentration as in hypoxia or hyperoxia studies. However, this does not address the issue of low oxygen solubility and the small gas exchange area through a standard flask lid. Methods that can enable more efficient oxygen exchange (with physoxic incubator O\(_2\) levels) include the use of oxygen-permeable closed system culture bags as currently used for monocyte expansion and differentiation,\(^9,96\) custom culture chambers fabricated from oxygen-permeable materials for example, polydimethylsiloxane (PDMS) which has high gas permeability, or PDMS-based microfluidic organ-on-chip devices with a larger surface area to volume ratio. Due to long equilibration times by passive diffusion, culture medium can also be pre-equilibrated to physoxic concentrations before being added to the cells.\(^8\) As oxygen levels in the body occur as a partial pressure of atmospheric oxygen, such concentrations are unlikely to form shearing microbubbles at atmospheric pressure. A combination of both oxygen-permeable materials and medium preoxygenation may be the most effective strategy for reducing the effects of O\(_2\) shock or variability during medium changes.\(^9\)

The ready availability of oxygen-permeable cell culture bags and its closed system environment makes for their ideal translation to s-μg and r-μg research. Ahn et al\(^3\) describe the culture of cells on free-floating polystyrene membranes suspended in custom oxygen-permeable pouches similar to a culture bag, which were mounted on an RPM or cultured statically as a control. This approach addresses the issue of oxygen permeability through standard flasks and provides a comparable neutral buoyancy condition within the control samples. However, how a suspended substrate affects hydrodynamics and the resultant shear forces that act on the membrane/scaffold (where cells are attached) must be characterized. Also, the buoyancy of the membrane will change as cell density increases over time. Nevertheless, oxygen-permeable substrates address the effects of low oxygen permeability in full vessel flasks and are therefore recommended. The other critical gas relevant to cell culture is CO\(_2\) for pH modulation. However, CO\(_2\) has a solubility constant approximately 30x
higher than that of O\textsubscript{2} and buffering agents such as bicarbonate are routinely added to medium. Furthermore, gas permeable substrates would also facilitate CO\textsubscript{2} exchange. Thus, the O\textsubscript{2} environment remains the primarily overlooked factor in cell culture. A comparative study that investigates the effect of different oxygen conditions (physoxic, hypoxic, hyperoxic) while maintaining the same s-\(\mu\)g conditions is recommended to elucidate the specific biological effects of oxygen tension in full vessel simulated microgravity culture.

4 \ | \ CELL SIGNALING AND THE CULTURE ENVIRONMENT

Cells are highly responsive to their environment, where interactions with their physical surroundings including extracellular matrix (ECM) and other cells drive important processes such as cytoskeletal rearrangement, migration, proliferation and differentiation.\textsuperscript{98,99} Current simulated microgravity culture methodologies implicate cell-cell, cell-material and signaling which are intrinsic to the outcomes being measured.

4.1 \ | \ Cell-cell and cell-material interactions

The adhesion of cells to ECM and other cells is essential for their communication and function\textsuperscript{100} and disrupted cellular contact is implicated in many diseases.\textsuperscript{101} Therefore, there is a question of whether rotationally suspending detached cells constitute an appropriate biological model in s-\(\mu\)g studies. In the body and conventional 1-g culture, cells readily attach to ECM, one another or substrate surfaces. In the absence of any attachment surface, cells in suspension simply divide and attach to each other, just as how cells are artificially coaxed to aggregate into spheroids in low-attachment plates. While appropriate for hematopoietic and embryonic stem cell culture, detached cultures and spheroids do not model cell-ECM interactions for a vast majority of tissues in the body, nor cell-substrate interactions for comparability with a static sedimented and adhered control. It would be of interest to investigate whether cell-material interactions affect outcomes in s-\(\mu\)g studies. Indeed, there is the immediate opportunity to compare the effects of substrate-attached (free-floating) cultures with unattached suspension on cell behaviors in s-\(\mu\)g. For example, the pre-attachment of cells to microbead carriers prior to s-\(\mu\)g culture has been investigated\textsuperscript{102} and found to increase viability and reduce cell loss.\textsuperscript{23} Another way of modeling the effects of adhesion would be to culture cells on 3D scaffolds or as 3D tissue constructs prior to exposure to s-\(\mu\)g, which would emulate substrate interactions that cells would experience in standard culture and provide a more realistic approximation of cells and tissues within the body in space.

4.2 \ | \ The effect of culture medium volume on autocrine & paracrine signaling

The significantly higher volume of culture medium used in full vessel simulated microgravity culture not only affects pericellular oxygen levels - cells secrete autocrine and paracrine factors that govern and regulate their functions. Autocrine signaling is important for all cells and particularly for cancer cells, where oncogene expression can upregulate the production of self-sustaining autocrine growth and survival factors for many cancers.\textsuperscript{103} Where the culture medium volume to cell number ratio is too high, these secreted factors may become overly diluted and fall below the concentration thresholds required for signaling growth or survival. Cells then cannot sense or sustain a high enough concentration of these self-signaling factors so behave atypically, de-differentiate, not proliferate and/or die.\textsuperscript{104}

Again, using the T25 flask as an example: assume that cells are cultured to confluency in 5 mL of medium as per conventional culture. These cells secrete a certain quantity of autocrine signaling molecules which are dispersed into the 5 mL of medium. In a full vessel static control, the same quantity of factors are diffused into 50 mL of culture medium, which equates to a 10-fold dilution in factors/mL/cell. This can significantly reduce autocrine signaling and may be another factor behind apoptosis and lower cell numbers typically reported in simulated microgravity studies. For example, RWV (full vessel) culture of intestinal epithelial cells was shown to disrupt intestinal epithelial barrier integrity\textsuperscript{105} and increased culture medium volume was found to reduce osteoblast fusion and mineralization (bone formation).\textsuperscript{106} In paracrine studies, cell-conditioned medium extracted from s-\(\mu\)g culture was reported to have a cross-pathogenic effect on endothelial cell and smooth muscle cell proliferation and migration compared to normal gravity-conditioned medium.\textsuperscript{107} Real microgravity was found to enhance vascular endothelial growth factor (VEGF) expression in thyroid carcinoma spheroid
cultures, but both VEGF secretion and VEGF expression decreased in s-μg (RPM) culture, where differences may be attributed to hypoxia. Reduced autocrine signaling due to full vessel dilution may be an underlying factor whether for as-is culture or conditioned medium in paracrine s-μg studies. Fluid convection in simulated microgravity culture may additionally reduce the immediate concentration of autocrine factors in the pericellular environment compared to passive diffusion in static culture (Figure 4).

In standard culture, cell seeding density is considered in terms of cell number per unit area (cm²). However, as cells are kept suspended in s-μg culture, seeding density must be considered in terms of cells/volume of medium. A recommended density of $0.7 \times 10^6$ cells per 3 mL medium for a T25 flask equates to $0.2 \times 10^5$ cells mL⁻¹ or $1 \times 10^6$ cells/flask. Even so, the effect of the culture medium volume to cell density ratio on autocrine signaling is currently unknown even for standard culture where medium volume is variable and warrants further consideration for full vessel culture. Concentrations of autocrine factors can be readily measured and normalized by cell number; this is recommended for preliminary optimisation of culture medium volume to cell density ratios. The optimal cell seeding density to culture medium volume ratio may exceed cell numbers at confluence for 2D culture, or confluence may be rapidly reached with a high seeding density. For example, $1 \times 10^6$ cells seeded into a static T25 control flask will sediment and rapidly reach confluency ($2.5-3 \times 10^6$ cells). In this case, the static control must be revisited, which will be discussed in Section 5.4. An exception would be for scaffold or microbead cultures, where all experimental groups would have the same seeding density and culture medium volume.

5 | EXPERIMENTAL DESIGN

In any scientific experiment, the methodology by which the study was conducted can influence outcomes. Biological assays involving cells that are sensitive to their environment are no exception, where differences in culture protocol can lead to variable results for the same cell type. However, culture protocols vary between s-μg studies depending on the system, cell type and research question. The effect of this and other key experimental parameters that can affect the outcome and interpretation of simulated microgravity experiments are discussed in the following section.

5.1 | Cell culture protocol

The concept of microgravity is approximated by keeping cells suspended in a state of free fall (perceived near-weightlessness), which is currently achieved by seeding cells into a full vessel then subjecting them to rotation such that the rotational velocity of the fluid counteracts sedimentation due to gravity. Unless pre-attached to scaffolds or microbead carriers with a similar density to culture medium, cells seeded into a s-μg culture vessel are typically individual and free-floating, then proliferate in suspension into multicellular clusters or spheroids. Cell cultures must be unattached to the walls of the vessel to undergo simulated functional weightlessness, where optimal constant rotational motion of the culture fluid causes rotation of a cell or cell cluster around its own axis such that it remains stationary with respect to the surrounding liquid phase. Please also see van Loon’s explanation in. While this concept readily applies for clinostats and RWVs, RPMs generate complex and chaotic flow regimes where the time-averaged gravitational vector is zero or near-zero. Regardless of the operating principle, it is necessary to make a distinction between free-floating and cell cultures that are attached to the culture vessel. Attached cells experience deformations caused by inertial shear
TABLE 1 Effect of time between cell seeding and s-μg culture on observable growth patterns

| Time between cell seeding and s-μg culture                                      | Outcome                                                                 |
|-------------------------------------------------------------------------------|-------------------------------------------------------------------------|
| Cells are cultured statically for 24 hours to allow complete adhesion         | Cells are mostly attached to the flask and experience wall-shear          |
|       or cultured to confluency prior to s-μg culture                           | stresses. Few detached cells may form spheroids                         |
| Cells seeded then allowed to attach to the flask for a short amount of time    | Not all cells are attached to the flask. Attached cells experience wall   |
|                                                                                   | shear, unattached cells proliferate in suspension and form spheroids     |
| Cells are seeded into a flask or vessel then immediately loaded on a s-μg      | Some cell attachment. The majority of cells remain suspended and form     |
|       device                                                                      | spheroids                                                               |

and rotation-mediated fluid convection at the boundary of the fluid domain, as well as by changes in g-vector direction regardless of whether the time average approaches zero.113 There are also the chronic effects of scalar gravity and cell attachment as covered in Section 4.1. Confusion regarding the distinction between free-floating (non-attached to the vessel) and attached samples can lead to improper design of s-μg cell culture studies and limit the interpretation of results. As an example, a recent study by Svejgaard et al investigated cancer cells that were allowed to establish in differently sized flasks for 24 hours prior to exposure to s-μg on an RPM and clinostat, that is, the cells were attached to the flask.57 Such an approach may be suitable for modeling cancer metastasis into fluid environments under simulated microgravity, however, attached cultures are ideally subjected to s-μg de novo for example, on microbeads or a scaffold. Studying the effect of simulated microgravity on vessel-attached cultures is a bifurcation of s-μg culture technique from the original premise of keeping cells suspended21 and therefore must be clearly reported rather than simply referred to as the “flask method” as in.114 Furthermore, variations in culture protocol can lead to different results and so it is essential to also report these parameters clearly. The potential outcomes of differences in time between cell seeding and s-μg culture are summarized and compared in Table 1.

In the current exploratory stage of the field, there is the opportunity and need to investigate a plethora of effects. For example, it would be of interest to compare the effects of partial rotation for example, 90° vertical to upside down position on an attached culture vs full RPM rotation to better deduce cell mechanoresponses to changing g-vectors with a non-zero time average. As introduced in Section 4.1, an immediate progression is the translation of 3D cultures to more accurately model tissue-like cell-cell and cell-material interactions in s-μg studies, which will necessitate additional steps within the experimental workflow. It is expected that as 3D tissue engineering and s-μg techniques become more established, there will be a convergence of these two platforms and eventually, consensus on standardized platform-specific culture methodologies.

5.2 Sample geometry & position on s-μg devices

Current s-μg devices generate a range of partial gravity between 0 and 0.9 g where the magnitude depends on the rotational velocity as well as the size, geometry and position of the sample with respect to the center of rotation.115 However, multiple flasks are often simultaneously loaded on an RPM or clinostat, where not all samples can be placed on the center of rotation. Also, flasks can be positioned in any orientation. Positional differences introduce spatial gradient effects across the entire experimental group as well as within individual flasks. The greater the distance of any sample point from the center of rotation, the higher the effects of residual centrifugal acceleration in clinostat rotation6 and mixed (centrifugal and centripetal) acceleration in RPM operation.7,113 Therefore, it is advisable to place samples on or close to the center of rotation and minimize the size of the sample (the distance of any point from the center of rotation).113 In the case of RWV culture, this can be done by reducing the size and radius of the vessel. For RPM or clinostat studies, the most optimal orientation of a T25 flask would be with the largest faces parallel and central to the primary rotational axis (Figure 1) and least optimal orientation is where the largest face is perpendicular to the rotational axis. It is anticipated that custom chambers fabricated with oxygen permeable materials, for example, cell culture bags or PDMS devices will replace standard flasks in s-μg culture. Regardless, CFD and mathematical modeling are recommended to calculate, optimize and justify the maximum number of samples that can be simultaneously loaded on a RPM or clinostat without generating significant spatial effects according to respective rotational modes. Otherwise, samples with mirrored positions with respect to the primary rotational axis (and which therefore experience the same range of microgravity) can be compared within an experimental set.
5.3 | Duration of simulated microgravity exposure

The duration that cells were exposed to simulated microgravity is an important experimental variable, which can range from 1 hour to over 14 days. There is increasing evidence that simulated microgravity exerts temporal effects due to cellular adaptation. For example, prolonged exposure to simulated microgravity (between 2 and 14 days) was shown to reduce dendritic T cell activation whereas short term exposition (<72 hours) increased dendritic cell maturation markers. Extended s-μg exposition of neuronal cells was shown to increase their recovery time in readapting to normal gravitational conditions and s-μg culture time was shown to determine the differentiation pathways of mesenchymal stem cells. The biological effects of microgravity exposure duration time are important to establish in concert with rationalized study durations that more accurately model the effects of prolonged spaceflight.

5.4 | What constitutes a meaningful control?

Simulated microgravity systems deliver a unique combination of fluid convection and gravitational revectoring effects which exert fluid shear and suspend cells respectively. This distinct mechanical combination has been found to be an effective means of generating spheroids and raises the question of what constitutes a meaningful control. Moreover, fluid convection improves mass transport and nutrient delivery to cells. However, most researchers use a static full vessel control which only models homogenous hydrostatic loading and so does not provide comparable hydromechanical and diffusion kinetics. To account for fluid mixing effects, a shaken control (eg, by placing a flask on a simple shaker or rocking plate) can be used to better deduce the effects of simulated microgravity from fluid convection. For a dynamic clinorotation control, rotation around a vertical axis (as opposed to horizontal) will allow the modeling of shear and convection with gravity. A possible dynamic control could also be set up to investigate and compare the effects of partial rotation (non-zero g-vector) as mentioned in Section 5.1.

Regardless of the class of s-μg device, the ideal experimental groups are a full vessel static control, dynamic full vessel control and s-μg under physoxia. Until physoxic culture becomes common practice, there is the question of whether a full vessel static 1 g control can provide a suitable baseline of cellular functions for comparison with s-μg results. Although cell lines have been well characterized under standard culture, the effects of full vessel culture have not been established and so any differences in cell characteristics compared to standard culture need to be known. Therefore, three controls are proposed for simulated microgravity experiments performed under standard incubator conditions: (a) conventional static culture, (b) static full vessel and (c) dynamic full vessel. Together, these would allow for more holistic evaluation of the respective effects of oxygen tension, fluid mixing (mass transport, shear) and gravity on cellular responses (Figure 5). Should the optimal seeding density exceed cell numbers at confluency in a static control T25 flask as discussed in Section 4.2, the same cell seeding density to culture medium volume ratio can be achieved in a static control using a larger flask as long as medium depths are kept constant. The concept of these controls can be readily adapted for pipettes and culture bags.
There is also the challenge of outcome dependency in control design. For example, what would be a suitable static control if s-μg spheroid formation is both an aim and binary outcome that actively depends on suspension via rotation? Here, no spheroids would in a full vessel static equivalent (flask or RWV chamber) due to cell sedimentation, so perhaps another established means of producing spheroids for example, low adhesion round-bottom well plates would provide more meaningful comparison. The equivalent cell density to culture medium volume ratio and time course required to culture spheroids of comparable sizes must then be considered. Furthermore, introduction of 3D culture techniques for example, microbead or scaffold-based constructs which will necessitate a revision of the static control.

Given the importance of scientific controls for experimental validity and the reliability of results, there must be no ambiguity regarding control group conditions. In the case of a static control, the volume of culture medium used must be reported. Otherwise, a “static control” could be misinterpreted as (or actually refer to) a static control flask cultured with a standard volume of medium. An example of this is,121 where it is unclear whether the static control is full vessel or standard culture. Here, it cannot be assumed that oxygen diffusion through the silicon membrane of a RWV produces the same oxygen levels as by passive diffusion of incubator air into a flask as the diffusion pathways are different. Good experimental practice would be to use the same type of vessel for all groups. While standard (no-treatment) cultures are an acceptable control for determining the effect of one experimental variable like for example, drug dosage response, a s-μg + full vessel group cannot be compared against a standard culture control alone. Finally, comparison of s-μg studies with real-μg (r-μg) studies that occur in space require consideration of an additional variable - ionizing radiation, which necessitates further controls and will be discussed in more detail in Section 6.

5.5 Reporting framework

The need for clearer reporting practices regarding the use of s-μg practices has previously been suggested.6,122 This is necessary not just for compliance with the Bonn Criteria for Good Laboratory Practice, but also to facilitate more ready comparison between studies and avoid potential misinterpretation of methodology. Therefore, it would be helpful for a tabulated summary of experimental parameters and values to be explicitly reported in simulated microgravity studies, which at the minimum could contain information as in Table 2. For real microgravity studies, basic culture conditions such as vessel dimensions, cell density, medium volume and duration of microgravity exposure should likewise be summarized.

Finally, given the growing prevalence of peer-reviewed scientific video protocols for example, JoVE, published studies should ideally be supplemented by at least a short video of the experimental setup in operation to show sample conditions for transparency as videos are more informative and less prone to selective reporting than photographs. A risk of not doing so would allow say, studies to claim that simulated microgravity exposure causes adverse cell events, for example, high cell death and/or low cell viability, without mentioning the presence of shearing air bubbles within the culture vessel which

**Table 2** Sample reporting matrix for simulated microgravity studies

| Parameter                      | Value                                                                 |
|--------------------------------|----------------------------------------------------------------------|
| System(s) and manufacturer     | RPM (Dutch Space, Leiden, NL)                                       |
| Rotational velocity & mode     | 10 rpm, 10 rpm, sinusoidal                                          |
| Culture vessel                 | T25 flask (Corning)                                                  |
| Cell line(s)                   | C6 glioma (ATCC CCL-107)                                            |
| Cell seeding density           | 0.2 × 10⁵ cells mL⁻¹                                                  |
| Culture media                  | DMEM +10% v/v FCS (Sigma Aldrich)                                   |
| Volume of medium               | 50 mL                                                                |
| Temperature                    | 37°C                                                                  |
| Control (s)                    | Static full vessel, dynamic full vessel                              |
| Culture technique              | Microbead attachment                                                 |
| Physoxic condition             | Yes: 25-30 mmHg O₂                                                   |
| Range of microgravity          | 0.2-0.5 g                                                            |
| Microgravity exposure duration | 3 days                                                               |
would be the main underlying cause of the significant cell death and loss observed and reported. Videos also facilitate the communication of more complex concepts related to s-μg device operation.

6 | COMPARABILITY OF S-μG STUDIES WITH CONDITIONS IN SPACE

A primary goal of simulated microgravity research is to elucidate the mechanisms behind known deleterious health effects caused by prolonged exposure to gravitational unloading in space, such as loss of bone mineral density,123 immune dysfunction,124 muscle atrophy,125 neurological deterioration,126-128 oedema and visual impairment.129 As previously introduced, a number of studies have reported comparable biological results between ground-based s-μg with real microgravity culture despite differences between the hydromechanical environments, which is possible if the combined effects of the culture conditions discussed so far are sufficiently similar. Likewise, any variations in these conditions would lead to differences between results. A key difference between ground based and real microgravity experiments is the high level of ionizing radiation in space, which poses significant acute and chronic health risks, including cancer, tissue degeneration, central nervous system (CNS) damage and radiation sickness.130-134

Cosmic radiation remains one of the greatest challenges to prolonged space expeditions. Radiation doses as low as 10 to 50 mSv already increase the risk of developing cancer.135 In space, astronauts are routinely exposed to cumulative radiation doses between 1 and 2 mSv per day,136 which could amount to whole-body radiation doses of over 1 Sv for a 3 year mission.137 These high levels of cosmic radiation damage DNA and are well known to drive abnormal cellular functions in cultured cells,131 where their effects have been the subject of great interest in radiation biology research.138 Cosmic radiation can affect any cell in space, from in vitro cultures to whole organisms, where radiation may directly or indirectly contribute to the development of any microgravity-associated conditions including bone loss139 and immune suppression by T-cell and macrophage deactivation.140

Although s-μg and real-μg studies are increasingly being compared for validation,6,141 cosmic radiation must be emulated in ground based studies for more meaningful comparison and to better study the potentially synergistic biological effects of ionizing radiation and microgravity. For example, Shanmugajaran et al found potentially additive effects of low doses of radiation (0.1-0.5 Gy) and s-μg on osteoclast fusion whereas the synergy was offset by higher radiation doses above 0.5 Gy.142 An extensive review conducted by Morena-Villanueva et al143 found conflicting reports where the majority of ground-based studies showed that s-μg culture increased cell sensitivity to radiation and decreased DNA repair, whereas in-space studies suggested that microgravity in spaceflight has no effect on DNA repair responses.144 The overall biological effects of radiation occur as a balance between the dose, individual susceptibility and intrinsic cellular mechanisms for DNA self-repair. Therefore, a range of effects may be produced, further contributing to results variability. It is anticipated that more combinative studies will be carried out and that next-generation s-μg systems have inbuilt capabilities that allow the effects of ionizing radiation on cell cultures to be simultaneously studied. Ikeda et al report such an RPM, where samples are exposed to carbon-ion irradiation when in the horizontal position.145 The need for new metrics to assess the overlapping effects cosmic radiation and microgravity has been raised by Yata-gai et al,146 who have also proposed a helpful framework to classify the spectrum of biological effects that can be produced.147

Overall, whenever s-μg results are being compared to real microgravity studies in space, equivalent doses of cosmic radiation must be modeled in ground-based s-μg studies in order to delineate any synergistic and relative biological effects. Two additional controls are required in addition to the three previously proposed in Section 5.4: (a) static full vessel + radiation and (b) dynamic full vessel + radiation. Another consideration for the comparability of s-μg and r-μg studies is the lack of convection in zero gravity and different ambient vibrations encountered aboard spacecraft compared to ground-based facilities. Finally, the duration of s-μg exposure in ground-based studies must be comparable to the length of r-μg studies to allow for direct comparison.

7 | CONCLUSIONS AND PERSPECTIVES

Simulated microgravity devices indeed provide a unique platform for investigating the biological effects of mechanical unloading. However, rapidly growing interest in applying s-μg devices for mechanobiology research raises the risk of a new technological platform being adopted without critical evaluation of how the overall culture conditions produced by these systems can holistically affect cellular functions. Without a thorough understanding of these novel s-μg culture
environments, results may be misinterpreted as only being due to the effects of microgravity and thus lead to potentially oversimplified and/or incorrect conclusions. Before entering the race to make new discoveries based on gravitational unloading, we must recall one of the core tenets of tissue engineering and cell biology - that cells are highly sensitive to environmental cues. Therefore, it is necessary to know, understand and control these cues in order to interpret and correlate cell responses. Here, the s-\(\mu\)g culture environment was distilled into three key constituent conditions: hydromechanical, oxygen and biochemical. The significance of these conditions and how each may contribute to outcomes observed in s-\(\mu\)g studies were examined and critical experimental design factors were discussed. Given that measurable outcomes represent a balance of cellular responses to all conditions within a culture environment, three controls were proposed to evaluate the respective effects of these conditions and simulated microgravity: (a) a standard static culture representing known baseline characteristics of the cell type of interest, (b) a static full vessel control to determine the effects of increased culture medium volume, and (c) a dynamic full vessel control to compare the effects of fluid convection and hydromechanical stimuli. If physoxic conditions are maintained and cell density is optimized, a standard culture control is unnecessary. Comparison of simulated microgravity studies with real microgravity culture in space necessitates additional s-\(\mu\)g controls to model the effects of ionizing radiation, while new s-\(\mu\)g approaches and culture techniques may require revision of control groups.

In addition to rigorous control design and selection, more stringent reporting of experimental conditions (rotational speed, volume of culture medium, oxygen concentration, cell seeding density, range of microgravity generated, and duration of microgravity exposure) is required to allow for more meaningful comparison between systems and studies. A clear, tabulated reporting summary matrix was suggested to encourage this practice as mandated by Good Laboratory Practice. Provision of a supplementary video that clearly shows the experimental setup and device operation was also suggested to enhance reporting. In the future, standardization of platform specific (clinostat, RPM, RWV) culture protocols will be necessary for reproducibility, particularly for potential therapeutic application of s-\(\mu\)g cultured stem cells, which additionally requires the development of quality control screening protocols and regulatory frameworks.

Finally, as 3D tissue culture and microgravity techniques become more established, it is expected that there will be a convergence of the two platforms which will see a shift from “reductionist” flask-based studies to 3D cultures in real and simulated microgravity to better model tissue effects as experienced in space. Overall, s-\(\mu\)g culture represents a new frontier for mechanobiology. With proper characterization and considered application, s-\(\mu\)g devices can serve to further our understanding of space conditions on the human body, advance our knowledge of fundamental cellular processes in health and disease and potentially facilitate some ground-breaking discoveries.

ACKNOWLEDGEMENTS
The author would like to thank Dr. Joris Goudsmits for fruitful discussion and his generous time in reviewing this work.

PEER REVIEW INFORMATION
Engineering Reports thanks Paola Divieti Pajevic, Magda Gioia, and other anonymous reviewers for their contribution to the peer review of this work.

PEER REVIEW
The peer review history for this article is available at https://publons.com/publon/10.1002/eng2.12242.

CONFLICT OF INTEREST
There are no conflicts of interest to declare.

ORCID
Christine Poon https://orcid.org/0000-0002-3825-1193

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**How to cite this article:** Poon C. Factors implicating the validity and interpretation of mechanobiology studies in simulated microgravity environments. *Engineering Reports*. 2020;2:e12242. [https://doi.org/10.1002/eng2.12242](https://doi.org/10.1002/eng2.12242)