Taking the 3Rs to a higher level: replacement and reduction of animal testing in life sciences in space research

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Highlights:
- Tools to replace animal testing in life sciences in space research are gaining *momentum*.
- Simulating tools include microgravity and radiation simulators.
- Modelling tools include primary and stem cells, spheroids and organoids, microphysiological systems and bioprinting.
- Processing, analysis and application tools include systems biology, live-cell, high-content and real-time analysis, high-throughput analysis, artificial intelligence and digital twins.

Abstract:
Human settlements on the Moon, crewed missions to Mars and space tourism will become a reality in the next few decades. Human presence in space, especially for extended periods of time, will therefore steeply increase. However, despite more than 60 years of spaceflight, the mechanisms underlying the effects of the space environment on human physiology are still not fully understood. Animals, ranging in complexity from flies to monkeys, have played a pioneering role in understanding the (patho)physiological outcome of critical environmental factors in space, in particular altered gravity and cosmic radiation. The use of animals in biomedical research is increasingly being criticized because of ethical reasons and limited human relevance. Driven by the 3Rs concept, calling for replacement, reduction and refinement of animal experimentation, major efforts have been focused in the past decades on the development of alternative methods that fully bypass animal testing or so-called new approach methodologies. These new approach methodologies range from simple monolayer cultures of individual primary or stem cells all up to bioprinted 3D organoids and microfluidic chips that recapitulate the complex cellular architecture of organs. Other approaches applied in life sciences in space research contribute to the reduction of animal experimentation. These include methods to mimic space conditions on Earth, such as microgravity and radiation simulators, as well as tools to support the processing, analysis or application of testing results obtained in life sciences in space research, including systems biology, live-cell, high-content and real-time analysis, high-throughput analysis, artificial intelligence and digital twins. The present paper provides an in-depth overview of such methods to replace or reduce animal testing in life sciences in space research.

1. Introduction

"*Space: the final frontier …* ". These epic words used in the prologue of the Star Trek television series and movies illustrate the eagerness of mankind to explore space. In 1961, Yuri Gagarin pioneered spaceflight during a mission that lasted for 108 minutes. The building of space stations, including Salyut, Skylab, Mir and the International Space Station (ISS), offered new opportunities for stays in space up to 6 months at low Earth orbit (LEO) (*i.e.* at an altitude up to 2000 km). Despite more than 6 decades of human spaceflight, however, several aspects related to the effects of space conditions, in particular low gravity and radiation, on human physiology have still not been fully elucidated. Historically, animals have played a pivotal role in the study of the physiological effects of the harsh space environment. In fact, the actual space pioneers were animals. The most famous animal space traveler was the dog named Laika, which was sent into space in 1957 to orbit Earth preceding the first human space traveler by 4 years. Throughout the following decades, various animals have been flown in space, ranging from flies to monkeys (Gray, 1998). At the same time, a number of Earth-based approaches to model space conditions relying on animal experimentation have been introduced. Among those is the hind limb unloading model, which is based on unloading of the hindquarters of rodents using tail suspension or a body harness for several weeks in an attempt to recapitulate some aspects of microgravity (Globus and Morey-Holton, 2016). However, the use of animals in scientific research, including space research, is increasingly being criticized from an ethical perspective. This aligns with the 3Rs concept introduced by William Russell and Rex Burch in 1959, calling for replacement, reduction and refinement of animal experimentation (Russell and Burch, 1959). Furthermore, anatomical and physiological interspecies differences hinder the extrapolation of results obtained from space-related experiments with animals to the human situation, which in turn limits value for translational purposes and applications both in space and on Earth. Constraints related to animal testing could be overcome by testing on human beings. Nevertheless, although valuable, testing on living human beings for life sciences in space research purposes, such as in head-down tilt bed rest, dry immersion and unilateral lower limb suspension approaches in ground-based experiments, does not always provide sufficient information at the mechanistic level (Pandiarajan and Hargens, 2020). In addition, simulation of radiation is not possible because of obvious safety and ethical reasons. New approach methodologies (NAMs) could, at least in part, provide a solution to this ubiquitous issue. A NAM refers to any methodology in biomedical research relying on *in vitro* (*i.e.* human cell culture) or *in silico* (*i.e.* computational) technologies that can be used alone or in combination with other methods (Sewell et al., 2024). In *stricto sensu*, a NAM relates to the replacement component of the 3Rs concept. In a broader sense, however, a NAM could relate to the reduction aspect of the 3Rs principle, such as the use of virtual control groups (Steger-Hartmann et al., 2020) or short-term rodent bioassays linked with omics read-outs (Schmeisser et al., 2023). NAMs have been developed and are being abundantly applied in some areas since many years. This particularly holds true for the toxicology and chemical risk assessment fields, which have witnessed the introduction of several NAMs for basic research as well as for regulatory testing purposes over the past few decades (Schmeisser et al., 2023). The use of NAMs in other biomedical fields is gaining *momentum*. This actually defines the scope of the present paper, in which focus will be put on approaches to fully replace or reduce the use of animals in life sciences in space research. Following a historical synopsis of the use of animals in space research, a state-of-the-art overview will be provided of tools to simulate space conditions on Earth, tools to model human biological and physiological responses to space conditions both in simulated and real-life space environments, and tools supporting the processing, analysis or application of testing results obtained in space research.

2. Animal experimentation in life sciences in space research

A prevailing theory in the 1940s was that humans might not be able to survive a journey in the hostile space environment. Accordingly, animals were launched onboard balloons and rockets to study the effects of space conditions on living organisms. The first travelers were *Drosophila* flies, launched in 1947 onboard a V-2 rocket, to study the mutagenic potential of cosmic radiation in the upper atmosphere (Figure 2). In the US, the next animal was a *Rhesus* monkey named Albert I. This was followed by a series of experiments with non-human primates to address concerns related to the effects of acceleration, deceleration, noise and other factors associated with spaceflight on the mammalian body. The first mouse was flown only in 1950. Since the beginning of the program, animals seemed to be a lesser sacrifice than humans. However, the flights of rockets that carried animals were discontinued in 1952 due to complaints from the public in the US and other countries. This did not eliminate the use of anesthetized hogs and chimpanzees in impact tests on the ground. Meanwhile, in the Soviet Union, rockets launched since 1951 carried nearly 40 dogs before Laika was sent into space (Burgess and Dubbs, 2007). The flight of Sputnik 1 in 1957 and the Cold War fueled the space race and led to human spaceflight despite a considerable lack of understanding of how the space environment affects the human body. The US renewed the launching of non-human primates in 1958, including the chimpanzee Ham, which preceded the suborbital flight of Alan Shepard and Enos that paved the way for John Glenn's orbital flight. In the Soviet Union, the canine program continued until 1966 and demonstrated poor health outcomes of a prolonged stay in space. In 1968, a Soviet spacecraft carrying turtles, flies and other organisms flew around the Moon. In fact, in the 1950s and 1960s, animals were part of the space race, used mostly to improve the development of telemetry systems and to understand spaceflight-induced physiological changes (Burgess and Dubbs, 2007). France was the third nation to join the "Space Club" (Paikowsky, 2017), sending rats, cats, and monkeys to space during the 1960s. Poland launched mice in 1961, yet the flight of Yuri Gagarin 2 days later overwhelmed this achievement. China began launching animals in the mid-1960s, coinciding with the beginning of the biosatellite era. The US built primate biosatellites and participated in the Soviet Cosmos/Bion program along with other nations. These missions have carried monkeys, quail eggs, fish, newts, frogs and other biological material into space (Burgess and Dubbs, 2007). By the end of the 1980s, animal studies provided important information on the effects of spaceflight on multiple systems, including bone and muscle. In 1997, the death of a Bion 11 monkey on the first day after return from space led to the understanding that spaceflight increases the sensitivity to anesthesia and has driven the US National Aeronautics and Space Administration (NASA) to modify the procedures for treating astronauts post-flight (Burgess and Dubbs, 2007). However, this also brought the Cosmos missions to an end due to the increasing pressure by animal right activists.

By 1996, NASA adopted a formal rule, the NASA Principles for Ethical Care and Use of Animals (Dorminey, 2019), which complied with federal and local animal welfare regulations. Multiple institutional animal care committees have been established, including a flight committee. The policies and regulations apply to all vertebrate animals and to some cephalopods, such as octopuses (NASA, F., 2024). Later on, the Commission of the International Committee on Space Research proposed the adoption of an international policy and guidelines for the care and use of animals in space-borne research (Stabekis, 2024). In parallel, research on the ground using analogs, such as rodent hind limb unloading and exposure to simulated cosmic radiation, helped to develop hypotheses that were often tested in space. These models have provided valuable data and complemented spaceflight experiments. Recent examples, involving murine models, include insight into sexual dimorphism in response to ionizing radiation (Burke et al., 2024) and deciphering the role of gut microbiota and fecal metabolites in cardiac remodelling of head-down bed rest (Liu et al., 2023).

The US space shuttle program provided new opportunities, including the flight of the first veterinarian to space in 1993. Research shifted largely to lower-order animals and humans (Burgess and Dubbs, 2007). The space shuttles helped to construct the ISS, which has hosted hundreds of astronauts and many animal species since 2000. NASA, the Japan Aerospace Exploration Agency (JAXA) and the Italian Space Agency have developed specialized rodent habitat systems for the ISS. JAXA also contributed with an aquarium (Murata et al., 2015). The majority of studies involved rodents, with balanced considerations of animal order, sample size and housing space (Betancourt, 2011). Research in animal surrogates on the ISS helped space agencies to reduce the risk of human space exploration in LEO and beyond. This also enabled research and discovery for the benefit of humans on Earth, an application that was not anticipated during the early days of animal experimentation in space. Studies in animals addressed spaceflight-associated bone atrophy, loss of skeletal muscle mass, dysregulated immune system, cardiac dysfunction and changes in the brain. These changes resembled accelerated aging and certain diseases and paved the way for studies aimed at understanding the mechanisms associated with these conditions and for the development of therapeutic strategies (Giulianotti and Low, 2019). A notable example is a series of studies conducted on the ISS using mice by Amgen in collaboration with NASA, which aimed at testing antibodies targeting biological pathways involved in bone loss. These studies contributed to the approval of Denosumab for the treatment of osteoporosis and other bone-related disorders by the US Food and Drug Administration (FDA) in 2010 as well as the approval of Romosozumab for treating osteoporosis in postmenopausal women in 2019 (Spinoff, 2016).

Invertebrates are simpler model organisms that can be maintained in smaller containers or bioreactors and whose use, with a few exceptions, does not require approval by animal care and use committees. Invertebrates have been flown to space to assess the physiological effects of various stressors, adaptation mechanisms and survival. These include fruit flies and other arthropods (Burgess and Dubbs, 2007; Chou et al., 2023; Zhang et al., 2021), nematodes (Beckett et al., 2024; Harada et al., 2016) and tardigrades (Rizzo et al., 2015). The findings from such studies cannot be directly translated to the human body. However, they can provide insight into spaceflight-associated changes or lack thereof in gene transcription (Beckett et al., 2024), signal transduction (Harada et al., 2016), metabolism (Beckett et al., 2024; Rizzo et al., 2015) and even complex functions, such as immune defense (Chou et al., 2023) and sleep (Zhang et al., 2021).

The commercialization and acceleration of spaceflight frequency have enabled a second space age (Mason et al., 2024). Research has hereby shifted to multi-omics, single-cell and precision medicine studies, some of which involving rodents (Cope et al., 2024; da Silveira et al., 2020; Houerbi et al., 2024; Masarapu et al., 2024; Mathyk et al., 2024; Siew et al., 2024). In 2023, the first mammalian embryos have been grown in space. Mice embryos were sent to the ISS as frozen fertilized cells. The embryos were thawed and allowed to grow over 4 days to form blastocysts. Development was similar for these mice compared to ground-based counterparts and artificial gravity control groups (Wakayama et al., 2023). As of 2024, projects onboard the ISS included animal studies ranging from studies on ovarian estrogen production to delivery of bisphosphonate-prostaglandin for the prevention of osteopenia. However, the majority of studies rely on cell cultures, including spheroids and organoids (ISS-National-Laboratory, 2024a).

In 2023, a panel of 75 scientists, overseen by the US National Academies of Sciences, Engineering and Medicine, issued a report calling the US authorities to increase the number of experiments conducted on the ISS. Among the recommendations was the refitting of the retired crew capsule to host rat habitats that would orbit over Earth's poles and allow prolonged and intense exposure to space radiation (Greshko, 2023). In addition, the situation of research onboard the solitary ISS has been changing with the introduction of the Chinese space station

Tiangong and commercial orbital platforms (Mason et al., 2024). Studies involving animals will likely extend to the future, such as for testing genetic editing for combating space-induced cellular harm and as sentinels before sending humans to Mars. The benefit of animal models has been captured by US astronaut Scott Kelly in the context of sample collection from a mouse: "*It's not lost on me that all of the biological processes affecting this mouse are also affecting my own body*" (Kelly, 2018). Yet the species difference and ethics limitations inherent to animal experimentation also translate to studies in space (Eyal and Derendorf, 2019; Low and Giulianotti, 2019) along with habitat volume, weight and costs associated with launching and caring for animals in space. One special aspect of animal experimentation in space is the inability of crew members to treat animals that become sick (Betancourt, 2011; Kelly, 2018). This challenge will be even more impactful in view of uncrewed spaceflights.

3. Simulating tools

3.1. Microgravity simulators

To experience real microgravity conditions, it is necessary to go to outer space (*i.e.* about 6 million kilometres from Earth) or to compensate the gravitational force by a counteracting inertia force. The latter occurs when an object is in free falling conditions, such as on common microgravity platforms like drop towers, sounding rockets, parabolic flights, satellites or the ISS. Since these research opportunities are rare and expensive, ground-based cell culture experiments performed in simulated microgravity conditions are warranted. The selection of the microgravity simulating platform should be done fit-for-purpose (Graf et al., 2024; Herranz et al., 2013). Among the most commonly used microgravity simulators are the clinostat, the random positioning machine (RPM), the NASA-developed rotating wall vessel (RWV) as well as non-rotation-based approaches, such as magnetic levitation (Figure 3). A clinostat is a simple scientific device that continuously rotates culture flasks at a slow constant speed around a single axis (*i.e.* 2D clinostat) or around 2 axes (*i.e.* 3D clinostat) to average the gravity related force on the cell culture close to zero. In a 2D clinostat, the rotational axis is perpendicular to Earth's gravity vector. Assuming the rotational axis along the x-axis in the Earth coordinate system, the acting force on the cells depends on the angle between Earth's gravity vector and the z-axis of the cell culture. During a full rotation with constant speed, the z-axis points to all directions of the yz-plane in the Earth coordinate system for the same amount of time and the average force is close to zero. A 3D clinostat additionally rotates the cell culture around a second rotational axis. This allows to distribute the acting gravitational vector evenly in all directions over time and therefore nullifies the effect of gravity. Since the rotation inevitably leads to acceleration (*i.e.* centrifugation), the culture flask should be placed as near as possible to the rotational axes (Briegleb, 1992). The rotational velocity should hereby be optimized according to the purpose of the experiment (Brungs et al., 2016; Ferranti et al., 2021). Clinostats are commonly used in gravitropism studies, for cell culture experiments (Choi et al., 2021; Nishikawa et al., 2005) and in microbiology research (Allen et al., 2022; Hicks et al., 2023).

An RPM consists of 2 gimbals that allow rotating the cell culture around 2 axes with random speed and direction (Wuest et al., 2015). The randomized re-orientation of the cell culture effectively cancels out gravity effects and allows slower rotations (*i.e.* around 10 rpm) compared to clinostats. Consequently, larger cell culture flasks can be used, since the related centrifugal forces are around 0.1g, even several centimeters away from the center of rotation (Wuest et al., 2015).

Clinostats and RPMs allow to mimic long-term microgravity effects in a terrestrial environment. Nevertheless, the gravitational force is permanently present and is only averaged out, which can lead to misinterpretations especially due to short-term gravitational effects. An advantage of the RPM is that experiments can be carried out with 2D and 3D cell cultures to

investigate both gravitational biology and physiological aspects depending on the set-up (Cortes-Sanchez et al., 2023). While clinostats and RPMs average out gravitational effects by rotating the cell culture, RWVs take a different approach. An RWV mimics aspects of microgravity by simulating an infinite free fall in the surrounding cell culture medium. The RWV is a cylindrical bioreactor completely filled with liquid medium in which cells are suspended. The axis of rotation is perpendicular to Earth's gravity vector. Slow rotation velocities facilitate the motion of the whole inner liquid with the same angular rate as the container due to viscous coupling with the container walls. The motion of the whole liquid minimizes shear stress and turbulence that may influence the experiment. In addition, the rotation around the single horizontal axis prohibits sedimentation and the cells or cell clusters stay suspended if the rotation rate is correctly optimized (Schwarz et al., 1992). Cutting-edge RWV systems, like CelVivo's ClinoStar, allow to set the parameters such that objects with different sizes and masses are in simulated free fall condition for quite long time periods. RWV applications range from tissue engineering and cancer research to stem cell biology and space science. By providing a more physiologically relevant cell culture system, the RWV offers insights into how cells and tissues behave in complex environments, advancing both biomedical research and therapeutic development (Ferrarini et al., 2013; Gardner and Herbst-Kralovetz, 2016; Nakazato et al., 2022).

Clinostats, RPM and RWV devices can be used in various set-ups. In view of securing reproducibility of test results, it is of paramount importance to report essential physical and chemical parameters adopted in a specific set-up. This critical set of parameters, known as the Bonn criteria, include angular velocities, the operating mode and highest angular accelerations. Furthermore, properties of the culture vessel, the carrier beads and the composition of the cell culture medium should be reported, including, but not limited to, dimensions of the vessel, density of the media, inoculum density of cells, time of rotation and operating temperature (Hammond and Allen, 2011).

Magnetic levitation is a process where certain materials are repelled by a magnetic field and can be made to float or levitate in a stable position without any support. This occurs due to diamagnetism, a property of materials that causes them to create a weak, opposing magnetic field when exposed to an external magnetic field. Unlike ferromagnetic or paramagnetic materials, which are attracted to magnetic fields, diamagnetic materials are repelled. This repulsion is typically very weak, hence levitation requires very strong magnetic fields. Notable examples of diamagnetic materials are water, copper, graphite and several organic compounds. This allows to levitate biomatter by the influence of strong magnetic fields (*i.e.* > 15 T), which mimics aspects of microgravity (Corydon et al., 2023). This technique is commonly utilized for the exposure of cell cultures (Anil-Inevi et al., 2020), tissues and small organisms (Herranz et al., 2012). Importantly, strong magnetic fields can trigger effects that may lead to misinterpretation of test results with respect to the influence of microgravity (Hemmersbach et al., 2014). An alternative method to levitate is to dope biological samples with magnetic particles, such as magnetic iron oxide, and lift them in the cell culture medium with magnetic fields (Souza et al., 2010).

3.2. Radiation simulators

When travelling into the deep space environment for future crewed exploratory missions to the Moon and beyond, the presence of ionizing radiation has been identified as one of the major obstacles (Chancellor et al., 2014; Durante, 2014). Without Earth's protective atmosphere and magnetosphere, astronauts are bombarded by a complex radiation field created by sporadic solar particle events, containing bursts of energetic light ions originating from the Sun (Mewaldt et al., 2005) and a constantly present background of highly energetic heavy charged particles originating from deep space cosmic events called galactic cosmic rays (Simpson,

1983). The latter include a wide range of charged particles, whereby protons make up approximately 85%, helium ions (*i.e.* alpha particles) account for approximately 14% and the remaining 1% consists of heavier nuclei, high-charge and energy particles (Durante and Cucinotta, 2011). The high-charge and energy particles in galactic cosmic rays are of particular concern for space travel, because their high-charge and energy can lead to dense ionizing tracks as they pass through matter, due to high linear energy transfer values. This feature makes them much more biologically damaging compared to the other particle species present in the galactic cosmic ray spectra (Baeyens et al., 2023; Chancellor et al., 2021; Roobol et al., 2020). Galactic cosmic ray energies can be exceedingly high, often ranging from 100 Mega-electronvolt per nucleon to the Giga-electronvolt per nucleon range. Owing to their high energy, conventional shielding methods are less effective against galactic cosmic rays, because the particles can interact with the shielding material and produce secondary radiation (Durante and Cucinotta, 2011).

Solar particle events, also known as solar flares or solar proton events, are significant releases of energy from the Sun that eject particles into space. Solar particle events are associated with solar flares and are often accompanied by coronal mass ejection. The events vary in intensity, frequency and duration, typically following an 11-year solar cycle with higher probabilities of solar particle events occurring during the periods denominated as solar maxima when increased number of solar spots are observed. The particles ejected during a solar particle event are mostly protons and alpha particles, with energies typically in the range of 10-100 Mega-electronvolt, but can be much higher (Durante and Cucinotta, 2011). During an solar particle event, astronauts are exposed to acute high doses of radiation over a short period of time. This can lead to a higher risk of both short-term and long-term health effects, such as radiation sickness, increased cancer risk, damage to the central nervous system and acute effects on the skin and eyes depending on the dose to which they are exposed. In periods of solar maxima, the fluence of galactic cosmic rays is decreased due to the higher capacity of the heliospheric magnetic fields to block the galactic cosmic rays entering the solar system (Mishra and Mishra, 2016).

Cosmic radiation exposure during space missions varies significantly depending on mission duration, destination, spacecraft shielding, and solar activity. In LEO, such as aboard the ISS, astronauts typically receive a daily dose of approximately 0.3-0.6 millisieverts, primarily due to trapped radiation in the South Atlantic Anomaly and galactic cosmic rays (Cucinotta et al., 2013; NCRP, 2006). For lunar missions, where exposure to galactic cosmic rays and potential solar particle events is more pronounced due to the absence of Earth's magnetospheric shielding, daily doses can increase to 1.0-1.3 millisieverts (Durante and Cucinotta, 2011). Mars missions present an even greater challenge, with estimated daily doses during interplanetary transit ranging from 1.8 to 2.0 millisieverts under current spacecraft shielding configurations (Hassler et al., 2014).

These values illustrate the broad range of radiation exposure astronauts may face, and underscore the importance of mission-specific risk assessment and countermeasure development.The complex field of the space radiation environment is challenging to simulate because of the diverse and high-energy nature of cosmic rays and solar particles. High-charge and energy particles, which are a significant component of galactic cosmic rays, contribute approximately 89% of the total dose equivalent, measured in millisieverts, of the space radiation spectra. These particles, characterized by their high atomic number and energy, pose significant risks to both astronauts and spacecrafts, making accurate simulation and study of their effects critical. High-energy ion beam accelerator facilities are therefore key to studying the biological effects of space-relevant radiation exposure. Highly specialized facilities, such as the NASA Space Radiation Laboratory (NSRL) in the US, the Grand Accelerateur National d'Ions Lourds in France and the GSI Helmholtzzentrum für Schwerionenforschung in

Germany, employ particle accelerators to explore the effects of space radiation on living organisms and instruments. These studies are crucial for developing effective protective measures and for enhancing the understanding of the risks associated with human space exploration. However, uncertainties related to risk estimation remain high (Durante and Cucinotta, 2011; Durante et al., 2007). Strategies are aimed at mimicking the complex field of the space radiation environment using innovative technologies to address one of the contributors to uncertainties in predicting the biological response to space radiation. Of note, some research institutions have taken significant steps to improve the reproduction of galactic cosmic ray spectra by developing advanced galactic cosmic ray simulators (Kim et al., 2015). Thus, the NSRL system can accelerate various ion species at multiple energies with a rapid switching time of less than 2 minutes. This capability allows to approximate a complex mixed space radiation reference field, providing a versatile and realistic testing environment for studying the radiation effects (Huff et al., 2023). At GSI Helmholtzzentrum für Schwerionenforschung in Germany, a hybrid active-passive simulation method is employed, which combines intricate passive beam modulators with actively controlled energy levels in an iron beam. This innovative approach utilizes geometrically complex passive beam modulators to shape the radiation field, along with active adjustments to the energy levels of the iron beam. This method enables a more accurate simulation of the space radiation environment, thereby enhancing the reliability of experimental results (Schuy et al., 2020).

Another contributor to the uncertainties in space health risk prediction is combined exposure to microgravity and ionizing radiation. A research team at the Gunma University Heavy Ion Medical Center in Japan leveraged technology from heavy-ion radiotherapy, such as accelerator and respiratory gating systems, and developed a 3D clinostat and synchronized irradiation system that allows the study of the biological effects of simulated microgravity and heavy ion irradiation as well as their combined effects (Ikeda et al., 2017).

Ground-based facilities offer a simplified yet valuable approach to studying the effects of space radiation. By focusing on specific ion types or radiation qualities, researchers can more easily isolate and analyze the corresponding biological responses. This controlled environment allows for a clearer understanding of the mechanisms underlying radiation-induced damage. Additionally, these facilities often have parallels to particle therapy, enabling findings from space research to be directly translated into clinical applications for cancer patients.

Despite their strengths, ground-based facilities have limitations in fully replicating the complex radiation environment found in deep space. The available simulation platforms cannot accurately reproduce the full spectrum of radiation types, energies and intensities encountered in space. This discrepancy can lead to differences in biological outcomes compared to actual space conditions. Furthermore, the focus on high-dose, high-dose-rate acute exposures in many ground-based experiments may not fully capture the chronic, low-dose rate exposure experienced by astronauts over extended periods.

Another challenge is the limited data on the specific values for many kinds of cells of the human body. Relative biological effectiveness, which accounts for the varying biological effects of different radiation types at the same dose, can significantly influence cellular responses. The lack of comprehensive relative biological effectiveness data for many human cells hinders accurate risk assessment and treatment planning in space radiation environments. Additionally, translating absorbed radiation dose to biological risk is complex. Factors such as gender, age, genetic background and inherent radiation sensitivity, can influence an individual's response to radiation. While absorbed dose is a crucial metric, it does not fully capture the biological impact. The quality factor is introduced to account for the relative biological effectiveness of different radiation types, but it may not fully encompass the complex interactions between radiation and biological systems.

4. Modelling tools

4.1 Primary cells and stem cells

Human primary cells have revolutionized the scientific field, including space research. Human primary cells directly isolated from their tissues of origin are not modified, and show an *in vivo*-like behavior and normal physiology (Table 1). Experiments in actual space and simulated microgravity conditions indicate that altered gravity induces stress in mammalian cells, which activates a complex adaptive response. It is known that culturing cells in microgravity conditions triggers various morphological and molecular changes (Abdelfattah et al., 2024; Graf et al., 2024; Grimm et al., 2020). Human primary cells are used, among others, for drug testing and cytotoxicity investigations, studies of gene function and protein expression, generation of artificial tissues, such as spheroids and organoids, and omics studies in space and on Earth (Abdelfattah et al., 2024; Corydon et al., 2023; Graf et al., 2024). Nevertheless, cell cultures have several disadvantages, including high cost and complexity of the cell culture medium supplemented with or without serum, growth factors and antibiotics. As regards their application in space research, some adherently growing cell types are not suitable for investigation in a scaffold-free modus in an RWV or a rotary cell culture system. Moreover, freshly isolated human primary cells do not provide an unlimited supply because of low growth potential. Characterization of human primary cells is critical, since depending on the tissue of origin, they do not consist of a pure population of cells, which is necessary to generate reproducible results. Cell cultures derived from various donors may differ and do not represent the same features *per se*. This may yield divergent results regarding gene expression changes, protein production, cytokine release or growth behavior. Furthermore, human primary cells can be easily contaminated with bacteria, fungi or mycoplasma. Mycoplasma contamination can remain undetected for a longer time and may be responsible for considerable cellular changes. Mycoplasma tests should therefore be carried out regularly. Human primary cells, stable cell lines and stem cells used for space experiments need to be tested before the actual space mission in order to confirm their suitability to be transported to the ISS and/or their survival in the special environmental conditions of a spaceflight. A technical challenge relates to the design of the hardware that can function fully automatically. Such a bioreactor can endure enhanced physical forces, is connected to fluid storage chambers, performs cell culture medium changes and cell harvesting automatically, and supports cell viability. The container consists of a cell suspension chamber, 2 reserve tanks for cell culture medium and fixative, and a pump for fluid exchange. The selected materials should be durable, non-cytotoxic and not inactivating fixatives. Temperature studies with the selected cell type must be performed to find the lowest and highest temperature for the cells to survive. In addition, material tests with the cells in complete cell culture medium and the selected fixatives should be carried out. Moreover, cell viability must be determined when they encounter the selected hardware materials (Pietsch et al., 2017; Pietsch et al., 2013a).

Several groups have investigated human primary cells derived from the musculoskeletal system, such as primary chondrocytes, primary meniscus fibrochondrocytes, bone cells and skeletal muscle cells, maintained in simulated microgravity conditions (Ecker Cohen et al., 2024; Ma et al., 2024; Steinwerth et al., 2023; Wehland et al., 2020). Chondrocytes are typically grown in suspension in an RWV or cultured adherently in cell culture flasks in an RPM for different time periods (*i.e.* 1-28 days). To avoid sheer stress, the vessels or flasks should be filled completely with cell culture medium devoid of air bubbles. Chondrocytes have demonstrated their suitability for tissue engineering purposes or to study biological processes in microgravity conditions (Steinwerth et al., 2023; Wehland et al., 2020). Primary meniscus fibrochondrocytes obtained from male and female donors seeded onto porous collagen scaffolds to generate 3D meniscus models have been incubated in a rotary cell culture system for 1 week. This *in vitro* setting allowed to gain knowledge about sex disparities in knee

osteoarthritis and for studying sex-specific mechanisms and therapeutic targets (Ma et al., 2024). Several studies examined the effects of chemical or pharmacological compounds as possible countermeasures to treat astronauts' health issues during and after spaceflight. In this respect, it has been demonstrated that amorphous calcium carbonate increases osteogenic differentiation and myotube formation of human bone marrow mesenchymal stem cells and skeletal muscle cells on the ISS. These experiments have been performed as part of 2 ISS missions. The first study was a preliminary experiment, in which stromal murine cells were differentiated into osteoblasts when amorphous calcium carbonate was added to the cell culture medium. The second study was part of Axiom-1's Rakia project mission launched to the ISS in 2022, thereby utilizing organ-on-a-chip methodology with a specially designed autonomous module. Human bone marrow mesenchymal stem cells and skeletal muscle cells were cultured in the presence or absence of amorphous calcium carbonate. Amorphous calcium carbonate supplementation was proposed as beneficial to prevent bone loss and muscle atrophy in space (Ecker Cohen et al., 2024).

Sarcopenia (*i.e.* the loss of muscle mass and function) is a disorder frequently observed in astronauts. Myostatin expression is involved in both load-free muscle damage and the progression of age-related musculoskeletal disorders. In this context, the efficacy of anti-myostatin antibodies to prevent human satellite cell degeneration induced by simulated microgravity has been evaluated. Anti-myostatin antibodies were applied to satellite cells established from muscle biopsies incubated in an RPM for 3 days. Microgravity induced satellite cell death and changes in myostatin expression levels with group-dependent variations. Anti-myostatin antibody treatment elevated survival and triggered the formation of myotubes, suggesting a role as potential therapeutic strategy to attenuate spaceflight-associated muscle atrophy (Cariati et al., 2022). A recent study examined the effects of clinorotation and *N*-acetylcysteine intervention on activated human primary hepatic stellate cells with focus on proliferation and transcriptome changes. A 24-hour clinostat-exposure reduced proliferation and elevated reactive oxygen species abundance. This proliferative response could be normalized by *N*-acetylcysteine (Fujisawa et al., 2022).

Human macrophages were cultured in cell culture flasks and stored in an incubator inside an aircraft. A rapid transcriptional response to the altered gravity was observed upon parabolic flight, which was specifically manifested in the epigenetic organization of chromatin (Vahlensieck et al., 2022). Another study investigated the effects of short-term and long-term microgravity on the supernatant metabolome of macrophage cultures. Macrophages were grown on makrolon slides and launched with a suborbital rocket, and fixed automatically throughout the flight using a system of chambers. During the CELLBOX-PRIME mission, macrophages on compartmented makrolon slides were placed in automated flight hardware and launched to the ISS, where they were automatically fixed and stabilized, followed by collection of supernatants. Using metabolomics analysis, an increase in amino acid concentration was measured after 5 minutes of altered gravity, yet was inverted after 11 days of microgravity exposure (Thiel et al., 2021).

More than 400 studies using stem cells exposed to microgravity conditions have been published. Stem cells are hereby frequently examined in space or in simulated microgravity conditions to study differentiation, growth behavior and pathways playing a role in astronauts' health and the development of countermeasures to protect astronauts during deep space exploration. Cardiomyocytes derived from human-induced pluripotent stem cells have been incubated in a 2D clinostat installed in an incubator for 2 days (Acharya et al., 2022). Rotation of flasks generates acceleration, exposing the cells to 0.012*g* up to 0.036*g* at the farther distance (Eiermann et al., 2013). It was found that simulated microgravity influences the contractile velocity and function of cardiomyocytes *via* the induction of senescence. Furthermore, a microgravity-controlled axis causing contractile dysfunctions to cardiomyocytes was

demonstrated (Acharya et al., 2022). The transcriptome of adult and neonatal cardiovascular progenitors cultured on the ISS for 1 month has been studied. To carry out an optimal experiment, cell clones were cultured at the Kennedy Space Center ahead of launch to ensure optimal viability *prior* to integration. Live cells were launched aboard SpaceX CRS-11 and flown to the ISS, where they were transferred to the Space Automated Bioproduct Lab. After 30 days, the plate habitats were transferred from Space Automated Bioproduct Lab and packed in NASA-provided stowage for return to Earth. Signaling pathways supporting cell proliferation and survival were induced by microgravity along with transcripts related to cell cycle re-entry, cardiovascular development and oxidative stress. These data indicate that optimally performed short-term microgravity culture benefits life on Earth by activating signature pathways of stemness and survival (Camberos et al., 2021). Long-term simulated microgravity experiments can be performed using an RPM. Cells cultured in different vessels, flasks, slides, 4-well plates or 8-well tissue culture treated microslides or culture flasks can be used for this purpose. Bone marrow stromal cells incubated in an RPM for 8 and 28 days showed reduced osteogenic differentiation and increased energy metabolism. Cytoskeleton re-arrangement and a consequent reduction in osteogenic differentiation potential was observed after 8 days. Thereafter, a remarkable cell plasticity was seen, suggesting that bone marrow stromal cells *in vitro* can adapt to microgravity. Bone marrow stromal cells mineralize the extracellular matrix, but their energy metabolism remains altered (Montagna et al., 2022).
Taken together, culture of primary cells and stem cells in microgravity conditions has provided insight into the effects of microgravity on cellular morphology, altered biological processes, gene and protein expression and growth behavior. Cells maintained in simulated microgravity conditions can experience shear forces, residual accelerations depending on their distance to the center of rotation, and a constant mixture of the cell culture medium. These effects may eventually lead to discrepancies between results from real-life space studies and simulated microgravity experiments, necessitating validation of the results obtained with microgravity simulators in space.

4.2. Spheroids and organoids

Spheroids are 3D cell aggregates formed in the absence of a predefined culture substrate. In contrast, organoids possess the ability to recapitulate organ-specific functions and spatial organization, exhibiting self-organization within a matrix-rich 3D environment (Marsee et al., 2021). These structures can be derived from various cell types, including stem cells, progenitor cells, pluripotent and adult stem cells (Marsee et al., 2021; Yin et al., 2016). Spheroids and organoids differ from 2D cultures by featuring additional cell-cell and cell-matrix interactions that assist in mimicking cellular functions and signaling pathways (Yin et al., 2016). This enhances the physiological relevance, thereby providing insights into human biology, while reducing the need for animal experimentation (Table 1). Platforms for microgravity research using organoids and spheroids include real microgravity exposure through long-term spaceflight research in LEO, parabolic flights and simulated microgravity conditions. Unlike research conducted in LEO and simulated microgravity, parabolic flights are often suboptimal to collect meaningful data on long-term effects of microgravity on biological models.
Exposure of cancer cells to simulated microgravity using an RPM leads to a fraction of the cells remaining adherently growing on the culture flask bottom, while the other fraction forms 3D multicellular spheroids (Grimm et al., 2022; Kopp et al., 2018; Kopp et al., 2016; Kopp et al., 2015; Monti et al., 2021; Pietsch et al., 2013b; Sahana et al., 2023; Sahana et al., 2021; Sahana et al., 2018). *In silico* studies have evaluated the interaction of thyrocytes and thyroid cancer cells, and demonstrated that several interacting proteins are involved in, among others, pathways regulating cell growth and cell membrane structuring (Pietsch et al., 2013b). In this respect, thyroid cancer cells form more and larger spheroids compared to primary thyrocytes

when subjected to simulated microgravity (Kopp et al., 2015). A positive correlation has been established between the actual metastatic microtumor environment and multicellular spheroids with respect to the extracellular matrix, cytoskeleton, morphology, different cellular signaling pathway key proteins and several other components of breast cancer cells (Sahana et al., 2023). The focal adhesion molecules vinculin and beta-catenin are key mediators of breast cancer cells to form multicellular spheroids (Sahana et al., 2021). Monti and group examined human breast epithelial cells and breast cancer cells, and detected apoptosis only after 3 days in breast cancer cells growing in organoid-like structures together with cytoskeletal changes. The early apoptotic response was counteracted by the extracellular signal-regulated kinase and protein kinase B survival pathways occurring in both cell types. However, a significant increase in apoptosis was only detectable in non-adherent breast cancer cells, indicating that loss of adherence and disruption of the cytoskeleton triggered by the RPM can ultimately overcome the pro-survival strategy enacted breast cancer cells (Monti et al., 2021).

Proteomic analyses of breast cancer cells revealed that levels of adherens junction protein E-cadherin are reduced in multicellular spheroids, where proteins of the E-cadherin autodegradation pathway are positively affected and proto-oncogene tyrosine-protein kinase c-Src is detectable. Multicellular spheroid generation can be prevented by inhibition of c-Src, but is promoted by blocking E-cadherin activity. This suggests that the balance of proteins upregulating or downregulating E-cadherin mediates the tendency of breast cancer cells to form multicellular spheroids during RPM exposure (Sahana et al., 2018). Breast cancer cells were maintained for 1 day in an RPM followed by analysis of the interaction of 47 genes. It was found that heme oxygenase 1 and nuclear factor kappa B variants are activated when multicellular spheroids are formed (Kopp et al., 2018). Moreover, breast cancer cells maintained in an RPM for 5 days exhibit duct-like multicellular spheroid formation. *In silico* pathway analysis demonstrated that the corresponding gene products are involved in the organization and regulation of cell shape, in cell tip formation and in membrane-to-membrane docking (Kopp et al., 2016). Simulated microgravity-induced generation of multicellular spheroids was reported for various cancer cell types (Grimm et al., 2022).

A total of 3 spaceflight missions (*i.e.* SimBox, CellBox-1 and CellBox-2) have focused on thyroid cancer spheroid formation in space (Kopp et al., 2016; Ma et al., 2014; Melnik et al., 2021; Pietsch et al., 2013a; Riwaldt et al., 2015a; Riwaldt et al., 2015b). Multicellular spheroid formation after 10 days was visible in an automated culture system during the Shenzhou-8/SimBox space mission (Ma et al., 2014; Pietsch et al., 2013a). The multicellular spheroids formed in space were found to be noticeably larger compared to both RPM experiments and ground controls. Furthermore, the multicellular spheroids formed in space showed higher gene expression of epidermal growth factor and connective tissue growth factor (Pietsch et al., 2013a). Transcriptomics analysis demonstrated gene expression changes for several biological processes, including adhesion to extracellular matrix, proliferation, angiogenesis and signal transduction. The outcome was predominantly antiproliferative (Ma et al., 2014). Proteomics studies of human follicular thyroid carcinoma cells revealed that enhanced extracellular matrix protein production could detain cells from multicellular spheroid formation, while profilin-1 was phosphorylated (Riwaldt et al., 2015b). Caveolin-1 is involved in inhibiting multicellular spheroid formation. Vascular cell adhesion molecule 1-mediated cell-cell adhesion is strengthened, and activated protein kinase C alpha is recruited in *caveolae*, while thyroid cancer cells do not form spheroids (Riwaldt et al., 2015a). The CellBox-2 spaceflight confirmed the results of the SimBox mission. Thyroid cancer cells assembled in multicellular spheroid in space, while extracellular-signal regulated kinase/ nuclear factor kappa B signaling was activated as a major microgravity regulatory pathway (Melnik et al., 2021). In addition, benign primary cells, stable cell lines or stem cells form multicellular spheroids in simulated microgravity conditions (Grimm et al., 2014). As such, 3D growth and scaffold-free formation

of tubular structures and multicellular spheroids of endothelial cells have also been demonstrated during spaceflight and supported the earlier results obtained with endothelial cells after RPM treatment treatment (Krüger et al., 2019b; Pietsch et al., 2017). Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC) were cultured in simulated microgravity conditions and showed elevated sarcomere length, z-disc length, nuclear diameter and nuclear eccentricity (Forghani et al., 2024). These data suggest that RPM treatment enhances the maturation of hiPSC-derived cardiomyocytes with respect to morphology, metabolism and function (Forghani et al., 2024). A subsequent study reported the engineering of microscale progenitor cardiac spheres from hiPSCs maintained in an RPM for 3 days during differentiation to cardiomyocytes (Jha et al., 2016). Enhanced induction, proliferation and viability of cardiac progenitors together with upregulation of the corresponding genes were observed in 3D cultures kept in an RPM. Consequently, a combination of 3D culture and RPM treatment seems suitable to efficiently generate cardiomyocytes (Jha et al., 2016). A recent study conducted aboard the ISS evaluated the use of cryopreservation techniques and a CO_2-independent cell culture medium for 3D cardiac progenitors. This novel cell culture medium composition induces high post-thaw viability and prominent cardiac differentiation in cryopreserved cardiac progenitors (Rampoldi et al., 2021). The development of this CO_2-independent cell culture medium facilitates cardiac organoid culture in space by alleviating the need for a 5% CO_2 atmosphere control aboard the ISS. This enables the analysis of microgravity effects on cellular differentiation and growth in an actual space environment. HiPSCs can also be used to study key aspects of brain development through their use for the generation of human brain organoids, which are capable of generating complex neural network activity *in vitro* (Muotri, 2023). In this context, a rotary cell culture system (RCCS) was used to generate and maintain neural organoids derived from human embryonic stem cells (hESCs) to model human brain development in simulated microgravity conditions (Mattei et al., 2018). Culture in the RCCS supported formation, proliferation of hESC-derived neural organoids as well as their commitment toward glial and neural subtypes. Changes in expression of rostral-caudal neural patterning genes and cortical markers compared to organoids generated in standard conditions were observed (Mattei et al., 2018). A recent study demonstrated the suitability of the RCCS and a microfluidic platform to improve engineering of brain organoids (Saglam-Metiner et al., 2023). Brain organoids generated in both the RCCS and a microfluidic platform revealed high levels of physiological fidelity. These systems are now available as preclinical models to test new therapeutic regimens for neurological disorders on Earth. Recently, the outcome of real-life microgravity on brain organoids was investigated during a 43-day mission aboard the ISS (Sharma et al., 2024). Prefrontal cortex organoids and motor neuron organoids were generated to model both healthy and diseased conditions. The diseased prefrontal cortex organoids were used as a model of Alzheimer's disease, while diseased motor neuron organoids were used to model amyotrophic lateral sclerosis and frontotemporal dementia. Both healthy and diseased brain organoids exhibited significantly higher levels of neurodegeneration in space compared to ground-based controls. Thus, amyloid beta-42, a biomarker for Alzheimer's disease, showed a 2-fold increase in space-based prefrontal cortex organoids compared to terrestrial controls (Sharma et al., 2024). Furthermore, biomarkers, such as phosphorylated tau and Tar DNA-binding protein 43, relevant to amyotrophic lateral sclerosis and frontotemporal dementia, were significantly elevated in space, indicating accelerated neurodegeneration. Concentrations of kallikrein-6, another key marker associated with neurodegenerative processes, were also increased in space-based motor neuron organoids, again underscoring space-induced neuropathology. In the same study, therapeutic responses in space to nanoligomers targeting nuclear factor kappa B and interleukin-6 were tested. These treatments considerably reduced neurodegenerative biomarkers in space, effectively counteracting the effects of microgravity. In fact, in some

cases, biomarker levels in diseased models were reduced below those of healthy organoids on Earth, substantiating the therapeutic potential of these molecules in mitigating space-induced neurodegeneration (Sharma et al., 2024). In a 30-day study aboard the ISS, a high number of differentially expressed genes were identified in both cortical organoids and in dopaminergic organoids. Dopaminergic organoids in LEO showed enrichment of genes associated with neuronal differentiation, synaptic transmission and hypothalamus development, whereas cortical organoids exhibited abundant ciliated cells and glial differentiation. Both organoid types displayed gene enrichment related to Wnt signaling and diencephalon development (Marotta et al., 2023). The SpaceX CRS-16 mission launched neuronal stem cells to the ISS. Neuronal stem cells were observed to proliferate 7 times more in space compared to Earth (Shaka S et al., 2021). Proteomic analysis of the neuronal stem cell secretome and space-flown media revealed a 4-fold increase in stress-related factors (Carpo et al., 2024). An increase in proliferation was also found in a subsequent spaceflight mission on board SpaceX CRS-21 where neuronal stem cell proliferation was higher compared to ground controls. Moreover, the progenitors displayed features of autophagy, including elevated stress proteins levels (Tran et al., 2023).

Collectively, these studies show that microgravity-generated 3D multicellular spheroids can be applied as models of tissue and disease development, such as cancer, *in vitro* metastasis and tissue engineering, and to assess therapeutic effects of drug candidates (Grimm et al., 2022). Not only microgravity-generated but also prefabricated 3D cell systems provide important findings for life sciences in space research. Novel high-throughput human organ-on-a-chip systems have been used recently to evaluate blood-brain barrier impairments and astrocyte functions 1-7 days after exposure to 600 Mega-electronvolt per nucleon ^{56}Fe particles and simplified simulated galactic cosmic rays (Verma et al., 2022). More advanced models should shed light on the impact of space radiation in the brain.

4.3. Microphysiological systems

Microfluidic chips are miniaturized devices that allow precise control and manipulation of small fluid volumes, typically in the microliter range or below (Hou et al., 2017; Whitesides, 2006). In recent years, this technology has expanded to accommodate biological applications, including for culturing organoids and engineered microtissue models, where fluid volumes can often exceed the microliter scale, reaching up to milliliters. In such cases, the term millifluidic chips is sometimes used to describe systems handling larger volumes. Collectively, such systems are commonly referred to as microphysiological systems (MPS). MPS not only provide the dynamic micro-environments, such as fluidic flows and biomechanical actuations to the cultured microtissue models, but often allow 3D configurations, such as those containing compartmentalized chambers or extracellular matrices (Bhatia and Ingber, 2014; Ching et al., 2021; Huh et al., 2011; Ingber, 2022; Leung et al., 2022; Low et al., 2021; Rahmani et al., 2022; Vunjak-Novakovic et al., 2021; Zhang et al., 2018). Over time, nearly all organ types have been modeled using these dynamic devices, and the concept has been expanded to include microfluidic systems hosting organoids, sometimes termed organoid-on-chips (Table 1) (Park et al., 2019).

Another unique advantage of microfluidics-based models, including organ-on-chips, compared to the conventional, isolated static cell and tissue cultures is that due to the conveniently designable fluidic connections, it becomes much more feasible to incorporate various biosensing elements into these systems (Ferrari et al., 2020; Fuchs et al., 2021; Zhu et al., 2021). Such a combination is of core value to enhanced usability of the models, in particular towards spaceflight applications, as it allows *in situ*, continual, non-invasive and potentially automated monitoring of microtissue behaviors, which otherwise can only be achieved previously with invasive manual operations. A variety of biosensors have been incorporated

into MPS, such as electrochemical (Aleman et al., 2021; Zhang et al., 2017) or optical (Morales et al., 2016) immunobiosensors for biomarker analyses, electromechanical biosensors for measuring physiological signals (*i.e.* electrophysiology (Hu et al., 2018; Soscia et al., 2020), impedance (Shaughnessey et al., 2022) and force-generation (Rao et al., 2018)) and biosensors for assessing micro-environmental physicochemical parameters, like pH (Zhang et al., 2017) and oxygen tension (Zhang et al., 2017). In addition, diverse imaging methods (Polat et al., 2019; Zhang et al., 2015) and further integration with tissue-clearing (Ondatje et al., 2022), expansion microscopy (Xie et al., 2023) and artificial intelligence (Dai et al., 2023), have been explored.

Offering greater structural and functional relevance than static cell cultures, while also enabling real-time monitoring, organ-on-chip models have made significant progress in space research to better emulate human (patho)physiology at unprecedented levels (Figure 4) (Mu et al., 2022; Yau et al., 2023). In 2016, through partnerships between the National Center for Advancing Translation Sciences (NCATS) at the National Institutes of Health (NIH), NASA and the Center for the Advancement of Science in Space (CASIS), the Tissue Chips in Space initiative was established through the NCATS' Tissue Chips for Drug Screening program to further develop MPS models to be used in LEO biomedical research (Sciences, 2024). This initiative aimed to study how space conditions, such as microgravity, reduced gravity and radiation exposure, present in the ISS US National Laboratory (ISS-NL), affect human health and disease using advanced human-based tissue models (Sciences, 2024). Since 2017, 9 projects have been funded through this initiative, with a collection of 5 spaceflights altogether with 15 MPS payloads successfully completed to date (Table 2) (Mu et al., 2022; Sciences, 2024).

Several human body systems weaken or deteriorate when subjected to a microgravity environment during spaceflight mimicking Earth-bound functional changes. Among those pathophysiological conditions are cardiac dysfunction, sarcopenia, bone density loss, immunosenescence and kidney diseases (Strollo et al., 2018). Outcomes of the Tissue Chips in Space program continuously provide information on several of these conditions. In this respect, experiments with human skeletal muscle tissue chips indicated muscle wasting, changes in contractility function and downregulation of transcripts related to myoblast proliferation and muscle differentiation (Giza et al., 2022; Parafati et al., 2023). In addition, gene classes related to inflammatory pathways were differentially expressed in flight samples cultured from the younger cohort compared to ground controls. These gene expression profiles will provide insights into the underlying pathophysiology of sarcopenia.

Similarly, impaired force-production and increased arrhythmia in spaceflight was observed by using engineered human heart tissues (Conroy, 2024; Mair et al., 2024). Sarcomeres became shorter and more disordered during spaceflight, as well as changes in mitochondrial structure were observed, indicative of mitochondrial dysfunction increase. Gene expression analyses indicated that spaceflight induced changes in the expression of genes and signaling pathways associated with inflammation and heart disorders in addition to expression of genes required for normal heart contraction and mitochondrial function. These findings will provide insights into spaceflight-induced molecular determinants of cardiovascular dysfunction.

To model human post-traumatic osteoarthritis, a cartilage bone synovium MPS was used, where spaceflight-induced upregulation in the release of inflammatory cytokines and tissue glycosaminoglycan-loss was observed (Dwivedi et al., 2024). Metabolomics analyses suggested increased oxidative stress and inflammation under microgravity conditions. In addition, differences in metabolic and bone biomarkers measured in ground control and spaceflight samples were detected.

MPS models of the human kidney proximal tubule allowed for analyses of effects of microgravity on secretion of kidney injury molecule-1, a protein secreted into the urinary filtrate by the proximal tubule (Jones-Isaac et al., 2024; Lidberg et al., 2024). Numerous of

these observations with MPS recapitulate well the spaceflight-induced changes in human physiology based on astronaut multi-omics studies (da Silveira et al., 2020). Cumulatively, these examples illustrate the functionality of the MPS platforms to be used at ISS-NL to study microgravity-induced pathophysiological conditions in humans.

Following the Tissue Chips in Space initiative, the National Science Foundation (NSF) released a collaboration opportunity with CASIS in 2019 to study tissue engineering and mechanobiology in space (Foundation, 2019). This has led to the funding of at least 4 projects focused on modelling cardiovascular and skeletomuscular systems under microgravity (Table 2). To improve the MPS platform technology for utilization as model systems to better understand the effects of space on humans during long-duration spaceflights, NASA further partnered with the NIH, Biomedical Advanced Research and Development Authority (BARDA), and US FDA in 2021 to develop extended-longevity tissue chips for development and testing on Earth with a focus on the generation of systems requiring minimal human intervention. A total of 8 projects were selected, allowing potential spaceflight opportunities depending on the success of the initial phase. This program will adapt 3D tissues and MPS to expand tissue viability and robust function for a minimum of 6 months and fully test and validate these models in response to acute and chronic stressors (Team, 2022).

It is worth noting that, while many ISS-NL experiments involving tissue chips or MPS have already been conducted, publications detailing the data from some of these launches are still forthcoming. This delay is primarily due to the limited sample sizes that can be accommodated on each flight and the engineering challenges that often result in incomplete execution of the originally planned experiments. However, research using Earth-based simulated microgravity technologies, such as RWV bioreactors, RPM devices and clinostats (Nishimura, 2023), has produced a number of concrete results that serve as valuable guidance for future space-based investigations. A recent study developed a continuous-flow lung-on-a-chip model using an RPM, revealing that human lung cancer cell viability decreased under microgravity conditions compared to normogravity in continuous flow (Strods et al., 2024). Interestingly, cell viability in the microgravity-on-chip model was less affected compared to static culture conditions. In another study, an MPS of engineered muscle tissue was used to explore the effects of simulated microgravity (Ren et al., 2024). This research showed significant reductions in contractile force, myofiber size, and the differential expressions of proteins related to muscle contraction, myogenesis regulation, and mitochondrial biogenesis under simulated microgravity.

MPS systems have seen significant growth in their use in space medicine in the past decade. Nonetheless, the current instrumentation demands substantial time, labor, and costs for data-collection and analyses. Improved automation with extended longevity of MPS will facilitate longer experiments in space and the collection of more physiologically-relevant data. In order to utilize MPS for biomedical research at ISS-NL, the key technological improvements in the tissue chip instrumentation systems towards automation and miniaturization were required (Jones-Isaac et al., 2024; Mair et al., 2024; Parafati et al., 2023; Yeung et al., 2020). These technological advances will help to engineer tissue chip platforms towards a smaller footprint and the simplification of systems will result in ease of use and broader accessibility of tissue chips in drug discovery and biomedical research on Earth. To overcome these challenges, NCATS has also recently launched the Miniaturization and Automation of Tissue Chip Systems (MATChS) initiative. This program aims to enhance the supporting instrumentation for tissue chips by applying automation and miniaturization strategies developed through the Tissue Chips in Space program. These advancements are expected to improve operational efficiency and expand the accessibility of tissue chip technology.

4.4 Bioprinting

Bioprinting in 3D is an innovative technology that is transforming the field of tissue engineering (Table 1). Bioprinting on Earth is primarily recognized for its ability to revolutionize tissue engineering by generating functional tissues and organ models. Cells, combined with biomaterials known as bio-inks, are printed in layers to create structures that can be used for various purposes, such as *in vivo* applications like bone regeneration and wound healing (Tripathi et al., 2023). Moreover, with the potential to generate tissues and organs, 3D bioprinting offers solutions to address the organ rejection rates and the severe organ shortage faced by transplant recipients (Lewis et al., 2021). For research purposes, this technology enables the generation of *in vitro* models to mimic 3D tissue models closely resembling the physiological and structural complexity of human tissues. These models foster a deeper understanding of cell interactions, the role of biomolecules and how cells behave in response to their micro-environment (Ali et al., 2024; Tan et al., 2022). Furthermore, the technology allows the creation of disease models that can be used to study disease mechanisms, drug targeting and the development of personalized medicine (Levato et al., 2020; Yi et al., 2019). In addition, by utilizing human cells as a source, bioprinting provides models that are potentially more relevant than animal-based systems, helping to overcome the limitations posed by animal models, such as translatability (Stresser et al., 2024). This growing shift away from animal models is aligned with ethical considerations and the push toward more human-representative *in vitro* systems (eBioMedicine, 2022).

One of the major challenges for tissue engineering on Earth is the need to sustain engineered tissues for long periods of time, particularly due to the difficulty of incorporating vascular networks into these models (Khanna et al., 2022). Moreover, under Earth's gravitational pull, additional support structures are often required to prevent the collapse of tissues under their own weight, especially those requiring low viscosity bio-inks for printing, such as blood vessels, making it difficult to create large or complex tissue models (Rezapour Sarabi et al., 2023). The weightlessness of space enables cells to self-organize into 3D structures, allowing for more complex and functional geometric models to be developed. This includes the generation of cavities, tunnels and voids, which is a critical challenge in Earth-bound tissue engineering (Moroni et al., 2022). The insights derived from space-based experiments therefore hold significant potential to advance tissue engineering practices on Earth by offering novel methodologies to enhance tissue stability and functionality in the development of human-like models, and even complete organs and tissues. Furthermore, the microgravity environment may uncover biological mechanisms obscured by terrestrial gravity, thereby facilitating a more profound understanding of fundamental cellular processes (Van Ombergen et al., 2023). In this context, the mechanisms of metastases formation are not fully understood, but the prevention of metastases is of high importance for the treatment of cancer due the challenges associated with detecting and targeting metastases (Dillekas et al., 2019). In microgravity, multicellular spheroids develop with tumor-like structures, including similar extracellular matrix and cytokine environment, which closely mimic metastasis. The cells in these multicellular spheroids not only undergo changes, but also exhibit distinct behaviors compared to cells grown in flat sheets under normogravity, revealing mechanisms that remain hidden in standard conditions (Krüger et al., 2019a). Accordingly, multicellular spheroids generated in microgravity conditions may represent the closest model for metastasis, which would provide valuable insights into metastasis formation and offer opportunities for testing drugs that inhibit metastasis (Krüger et al., 2019a).

For human space exploration goals, being able to investigate cellular behavior under diverse stressors and environmental variables experienced during a space mission is critical for preparing extended-duration missions, such as a roundtrip to Mars. The human risk model for travelling to space, especially beyond LEO and for long durations, is still not complete

(Antonsen et al., 2023). In view of further destinations, missions become increasingly complex, as crew will be exposed to more extreme space stressors. Fundamental research is still of crucial importance not only to establish a realistic risk model, but also for the development of potential countermeasures. Bioprinting in space therefore lends itself advantageous to study the effects of space stressors on human tissues, as it provides a mechanism of generating representative model constructs (Van Ombergen et al., 2023).

Considering the limitations of conducting life science research in space (Ferranti et al., 2021), bioprinting tissue and organ models on a space platform directly may overcome some of the challenges associated with launching live samples for the study of basic processes in the space environment. By uploading bioinks and mixing them in-flight, researchers can circumvent the need for cell culture medium exchanges and other logistical challenges associated with transporting live samples to space. Bioprinting has already been successfully demonstrated in space. Thus, The NASA and ISS-NL-supported 3DBioFabrication Facility on the ISS, developed by Techshot, now part of Redwire Space, has demonstrated bioprinting of cardiomyocytes and meniscus tissue in-flight (Office, 2023). These bioprinted tissues were returned to Earth for analysis, yet publications on viability and functional relevance of these constructs are as yet lacking. Only one publication is available, providing evidence that 3D bioprinting technology in space is feasible by showcasing successfully bioprinted meniscus tissue. However, there are limitations to the approach that still need to be addressed with additional ground-control studies (Klarmann et al., 2024). The European Space Agency (ESA) is also interested in developing and utilizing a platform or facility that will allow to implement this technique under microgravity. One of the objectives is to develop a versatile platform that combines bioprinting technology with a culturing system, including a centrifuge to simulate different gravitational conditions (Horizon, 2018). This system will enable researchers to perform bioprinting experiments with variable gravity, allowing them to study how cells and tissues respond to altered gravity during spaceflight.

In addition to establishing the mechanisms of fundamental processes for uncovering the risks of space travel, *in situ* biomedical applications making use of 3D bioprinting are also being developed. This is especially relevant for the treatments of wounds, as wound healing processes in microgravity seem to be impaired (Puhl et al., 2022). Implemented on behalf of the German Aerospace Center and developed by OHB System AG in collaboration with scientists at the Technical University Dresden in Germany, a hand-held bioprinter was tested for proof-of-concept on the ISS during the Bioprint FirstAid experiment (Warth et al., 2024). In this proof-of-concept study, hydrogel was directly bioprinted on an astronaut. The objective of the initiative is to develop a system for wound treatment during exploration missions. Although the bio-ink used in this experiment did not contain real cells, follow-up activities are being conducted to establish a system where acute wounds can be patched up in flight using this system (Warth et al., 2024).

The practical challenges of space bioprinting, such as managing the delicate process of printing in microgravity conditions and ensuring the stability of printed tissues, remain areas of active research. Future advancements in bioprinting hardware and bio-inks will be essential to realizing the full potential of this technology in space. Nevertheless, 3D bioprinting in microgravity conditions not only holds potential for developing applied biomedical techniques that can be implemented in space to safeguard crew during exploration missions, but also provides a research platform to understand fundamental processes in representative tissue models. Moreover, the future of 3D bioprinting in space holds immense promise for both space exploration and terrestrial medicine. One area of particular interest is the development of organs-on-chips, which combine bioprinted tissues with microfluidic technology to create models that more accurately replicate human physiology. These advanced models allow for continuous perfusion of cells, mimicking the natural flow of nutrients and waste removal seen

in human organs. By coupling 3D bioprinting with organ-on-chip technology, researchers can create highly sophisticated models for drug testing, disease modelling and personalized medicine (Rahmani et al., 2022). In the long term, 3D bioprinting in space could eventually also provide tissues and organs for patients on Earth. As technology advances and the safety of bioprinted tissues and organs is validated, space-based platforms may become an efficient method for producing high-demand tissues for transplant. Furthermore, for space exploration purposes, *in situ* bioprinting could be used to treat injuries, such as burns or wounds during space missions. Having the ability to generate tissues or repair damaged tissues on demand would significantly enhance the safety and sustainability of long-duration missions, reducing the need to return to Earth for medical treatment.

5. Processing, analytical and application tools

5.1 Systems biology

In the new space age, the rapid advancement of technologies has paved the way for groundbreaking discoveries, particularly in understanding the effects of the space environment on human biology. The multitude of experiments conducted to uncover the health risks associated with spaceflight and the subsequent development of countermeasures has generated an enormous amount of data. To effectively manage, analyze and translate this vast dataset into actionable insights, a systems biology approach is critical. This approach integrates various biological data types, such as genomics, transcriptomics, proteomics, metabolomics and other omics disciplines, into a comprehensive framework. By doing so, researchers can capture the complexity of biological systems in space conditions and develop more precise interventions. A key strategy for enabling this approach is the use of multi-omics, which allows for a holistic examination of the biological impact of space environments at multiple molecular levels.

A significant challenge in space biology research is the high cost and limited biological replicates typically associated with space experiments, whether conducted in space or simulated on Earth. However, a number of approaches can overcome these limitations. By demonstrating that results are reproducible across various biological levels, such as DNA, RNA and protein, and across diverse conditions, the inherent variability can be mitigated. This reproducibility can compensate for the low sample sizes, providing robust insights even with fewer biological replicates. An important resource that has facilitated this process is NASA's Open Science Data Repository (OSDR), formerly known as NASA GeneLab (Adamopoulos et al., 2024; Beheshti et al., 2018; Berrios et al., 2021; Overbey et al., 2021). The OSDR serves as a comprehensive, freely accessible hub for space biology data, providing raw and processed multi-omics datasets along with other experimental data types, such as microscopy images, immunoblots, and enzyme-linked immunosorbent assay results. These datasets allow mining previously generated data and produce novel hypotheses, saving both time and resources.

An excellent example of the impact of using publicly available multi-omics data is a study that demonstrated for the first time that the space environment induces systemic mitochondrial dysregulation across multiple organs and circulation. This research mined RNA sequencing, proteomics, methylation and metabolomics data from various tissues of mice flown aboard the ISS and *in vitro* ISS experiments. Through unbiased computational analysis of these data, mitochondrial dysfunction was identified as the primary dysregulated function, leading to downstream effects on immune function, inflammation, lipid metabolism, circadian rhythm and renal function. Importantly, these findings were further validated using physiological data from 69 astronauts, confirming that spaceflight-induced mitochondrial dysfunction impacts human health. This study exemplifies the power of leveraging existing datasets to generate novel insights without the need for additional animal experiments, significantly advancing the field while conserving resources (da Silveira et al., 2020).

More recently, the Space Omics and Medical Atlas (SOMA) collection of papers in *Nature* further underscored the power of multi-omics approaches in space biology (Jones et al., 2024; Mason et al., 2024; McDonald et al., 2024; Overbey et al., 2024). The work around the Inspiration4 mission, the first all-civilian commercial spaceflight, showcased how diverse omics techniques can yield high-impact discoveries from actual human spaceflight data (Mason et al., 2024). The Inspiration4 mission generated a wealth of data, including single-nucleotide RNA-sequencing, single-cell assay for transposase-accessible chromatin with sequencing, proteomics, metabolomics and metatranscriptomics, which were combined with biometric measurements from wearable devices that monitored heart rate, sleep patterns, and ocular health (Jones et al., 2024). A significant result came from spatial single-nucleotide RNA sequencing of skin biopsies from the Inspiration4 astronauts, revealing that spaceflight induced localized inflammation in the skin driven by Kirsten rat sarcoma virus activation (Park et al., 2024). The precision offered by this spatial transcriptomics approach allowed researchers to map specific regions of the skin where inflammatory markers were elevated, potentially linking these findings to the skin issues experienced during space missions.

Additionally, the multi-omics analysis of microbial exchanges during spaceflight has provided critical insights into astronaut health. Metatranscriptomics revealed significant host-microbe exchange among the crew members of the Inspiration4 mission, highlighting the implications for infectious disease transmission in long-duration space missions. These findings underscore the importance of monitoring microbial dynamics in confined environments, such as space capsules, and will inform strategies to mitigate health risks during future space exploration, including missions to Mars (Tierney et al., 2024).

Beyond understanding biological mechanisms, multi-omics approaches have also been instrumental in developing potential countermeasures for space-related health risks. The role of spaceflight-associated microRNAs in cardiovascular health risks has been explored, thereby identifying microRNAs as potential therapeutic targets in a human 3D microvessel cell culture (McDonald et al., 2024). MicroRNAs, a class of non-coding RNAs that regulate gene expression, have emerged as critical regulators of biological processes (Ambros, 2004). A spaceflight-associated microRNA signature was identified using OSDR data, which set the foundation for subsequent studies (Malkani et al., 2020). Further analysis using archived murine tissues from spaceflight and simulated experiments confirmed that these microRNAs play a role in spaceflight-induced biological changes. Importantly, this work led to the discovery that inhibiting specific microRNAs could mitigate the damage caused by galactic cosmic radiation in a 3D human microvessel tissue model, simulating the radiation dose astronauts would experience during a Mars mission (Malkani et al., 2020; McDonald et al., 2024; Wuu et al., 2020).

These examples illustrate the power of combining multi-omics analyses with advanced computational tools and human tissue models to develop novel countermeasures for space travel without relying on animal models. By repurposing publicly available data, leveraging *in silico* bio-informatics techniques, and employing human-relevant experimental systems, researchers can push the boundaries of space biology research, advancing both understanding of spaceflight-induced health risks and the development of strategies to mitigate them.

5.2. Live-cell, high-content and real-time analysis

For decades, real-time analysis of living samples has been essential to understand how organisms respond to microgravity. This approach complements studies relying on post-flight analysis of fixed specimens. Exposure to altered gravity can induce a wide range of cellular changes. Mammalian cells exposed to microgravity often exhibit distinct morphological alterations (Vorselen et al., 2014). The application and feasibility of confocal laser scanning microscopy in microgravity research were first demonstrated in 1992 (Goede et al., 1992).

Early live imaging studies were conducted on graviperception of ciliates during sounding rocket missions (Hemmersbach-Krause et al., 1993). The German Aerospace Center developed the slow rotating centrifuge microscope NIZEMI ("Niedergeschwindigkeits-Zentrifugenmikroskop"), a versatile instrument for terrestrial hypergravity and space microgravity research in biology and materials science (Friedrich et al., 1996). The NIZEMI microscope was flown on the space shuttle flight STS-65, the second flight of the International Microgravity Laboratory in 1994. Researchers have recently determined the thresholds at which various organisms respond to microgravity and hypergravity up to 1.5g (Hemmersbach et al., 2005; Hemmersbach et al., 1996). During a mission of the space shuttle Columbia, the gravitaxis of the unicellular flagellate *Euglena gracilis* was studied under accelerations ranging from 0*g* to 1.5*g* using the NIZEMI microscope (Häder et al., 1996). Following these early microscopic studies performed during a spaceflight, a lab-on-chip clinorotation time-lapse microscopy system was developed to visualize cellular behavior under simulated microgravity in 2014 (Yew et al., 2014). Human endothelial cells, chondrocytes and cancer cells have been studied in short-term microgravity conditions provided by parabolic flights (Aleshcheva et al., 2015; Grosse et al., 2012; Ulbrich et al., 2011). A common result was that the different cell types exhibited early cytoskeletal modifications upon the first parabola. For a long time, microscopic studies were only possible after flight on fixed cells. The availability of an airworthy confocal laser scanning microscope was therefore desirable. A few years later, it became possible to study living cells during a parabolic flight or a TEXUS sounding rocket flight. These experiments have revealed morphological changes in thyroid and breast cancer cells, including alterations in the actin and microtubule cytoskeleton, as observed using the Fluorescence Microscope Analysis System in Space (FLUMIAS) spinning disc microscope. The compact FLUMIAS system, designed for rapid live-cell imaging in real microgravity conditions, has enabled the direct observation of cytoskeletal changes in cancer cells expressing LifeAct-green fluorescent protein or mCherry-tubulin marker proteins (Corydon et al., 2016; Nassef et al., 2019). FLUMIAS was developed for a variety of cell types. Furthermore, sounding rocket flights of the FLUMIAS spinning disc microscope included experiments with human immune cells. Cytoskeletal rearrangements within these cells were imaged during the flight (Thiel et al., 2019b). Imaging during long-term exposure to microgravity on the ISS was verified using a FLUMIAS-DEA technology demonstrator (Thiel et al., 2019a). The German Space Agency has recently enhanced the FLUMIAS system for high-resolution, live-cell imaging on the ISS (Figure 5). This allows researchers to study biological responses to gravity levels ranging from 0*g* to 1*g*, including life-support conditions. FLUMIAS aims to elucidate how organisms, ranging from bacteria to humans, adapt to altered gravity. The key areas to investigate include gravity signaling and sensing thresholds in living organisms, underlying cellular and molecular mechanisms of adaptation, and timescales of adaptation for cells, organs and whole organisms. Live-cell imaging in space is crucial for observing dynamic processes and signaling pathways affected by microgravity. These include highly adaptable structures like the cytoskeleton and membrane systems in cells. FLUMIAS allows not only for time-lapse observations but also 3D reconstruction of gravity-modified cellular structures by capturing images from multiple focal planes.

The FLUMIAS facility is a 120 kg experiment platform designed for installation in ESA's Columbus module. Its centerpiece is a high-resolution fluorescence structured illumination microscope mounted on a centrifuge (Table 3). An adjacent storage magazine can hold up to 6 experiment blocks. While the facility itself remains on the ISS, the experimental blocks are interchangeable and brought to the station for each mission. Each experimental block contains a scientific sample on a movable stage, a microscope objective and a life support system. The only required crew interaction is the insertion of experimental blocks into the magazine, while all other operations are controlled from Earth. The FLUMIAS facility offers 2 types of

experimental blocks, namely a mammalian and a plant experimental block. The primary difference is the perpendicular orientation of observation with respect to the centrifugation gravity vector. Mammalian experimental blocks include a life-support system with multiple features, while plant experimental blocks incorporate a greenhouse. Each experimental block provides a separate environment for experiments, allowing for highly customized culture conditions, such as temperature, media exchange, stimulation and staining. For microscopic observations, living specimens are cultivated in either a 1-channel or 4-channel microslide with a glass or polymer bottom connected to the life-support system and integrated into the experimental blocks. FLUMIAS caters to a broad spectrum of research areas, including plant biology, microbiology (*i.e.* fungi and bacteria), cancer research and human health fields, like immunobiology, muscle maintenance and studies of neuronal/glial alterations and metabolic differences. The adaptable experimental blocks even allow applications in material science.

The German Aerospace Center's Institute of Aerospace Medicine's project "Live Assessment of Astrocytic Reactivity under Space Conditions" utilizes FLUMIAS to investigate how astrocytes adapt to microgravity. Astrocytes can become reactive, exhibiting increased metabolic activity, morphological changes, migration, neuro-inflammation pathway activation and altered gene expression. FLUMIAS enables live visualization of these reactive features, (re)adaptation processes and the sensitivity thresholds of astrocytes to changes in gravity. This project will investigate cytoskeletal dynamics and mitochondrial activity in order to gain insights into the early stages of reactive astrogliosis.

Before launching a space mission, rigorous ground-testing is crucial to verify experimental parameters. In any altered gravity experiment, controls are essential for validating scientific results. Unlike standard laboratory work, each environmental variation and experimental parameter must be thoroughly verified on Earth due to the high cost and rarity of spaceflight missions. Optimal reliability and a well-established interaction between biological and technical systems are paramount for ensuring meaningful scientific outcomes. The FLUMIAS science reference model, operated by the Institute of Aerospace Medicine at the German Aerospace Center, is a fluorescent structured illumination microscope used to verify and control essential microscopic parameters in preparation for experiments. A fully functional FLUMIAS engineering model with centrifugation capabilities will also be available at the German Aerospace Center for flight readiness testing and the development of imaging procedures. FLUMIAS is a project run by the German Aerospace Center developed by Airbus Friedrichshafen based on an innovative microscope by TILL I.D. in Germany. FLUMIAS will be transferred to ESA as a national contribution, with commissioning expected to be completed by the end of 2026. The research program is organized through ESA and the German Aerospace Center. A total of 11 experiments have been selected for the initial missions. Another possibility to investigate cells in space on the ISS is the Japanese experiment module Kibo, a versatile platform for various scientific activities, including microscopy. High-content analysis and imaging on the ISS became possible in 2020 with the arrival of the CSU-W1 confocal scanner unit. This unit is a key component of the Chiyoda Corporation's COSMIC confocal microscope system. JAXA astronaut Satoshi Furukawa performed microscope operations in the ISS Kibo module, studying samples for the cell gravisensing space biology study. The confocal space microscope is a JAXA facility that captures high-resolution fluorescence images of biological samples in space. It employs spatial filtering techniques to reduce out-of-focus blur and glare, enabling detailed imaging of specimens with varying thicknesses. The COSMIC device allows real-time visualization of fundamental cellular and tissue structures as well as cellular functions. Other teams have conducted real-time imaging experiments in space. The Chinese TZ-1 cargo spacecraft carried automated cell culture equipment and live-cell imaging techniques to study the effects of microgravity on mouse embryonic stem cell proliferation and differentiation. During a 15-day mission, bright field and fluorescent images

of cell growth were captured in microgravity. Real-time image data were analyzed on Earth (Lei et al., 2018). High-content microscopy systems suitable for the ISS are under development. High-content analysis of hundreds of live cells expressing green fluorescent protein-fused 53BP1 proteins enabled precise quantification of DNA damage movement after X-ray exposure (Georgescu et al., 2015). These studies suggest that DNA double-strand breaks migrate to specific nuclear domains for repair in mammalian cells. High-content microscopy of millions of cells in 15 mouse strains confirmed the existence of these repair domains (Penninckx et al., 2019) and revealed their genetic modulation in mice (Cekanaviciute et al., 2023). Similar studies in a large human cohort using high-content imaging of blood samples identified a correlation between baseline DNA damage and radiation toxicity (Pariset et al., 2020).

5.3. High-throughput screening

The idea of high-throughput screening (HTS) in its most reduced form is simply the efficient testing of large numbers of potential solutions to a given challenge. In practice, HTS often comprises a broad array of testing methodologies, diverse instrumentation, advanced computational algorithms and complex automation. In this context, the pharmaceutical industry has the recurring challenge of identifying novel modulators of cellular targets, such as enzymes that are important drug targets for disease. Instead of synthesizing a small number of compounds and testing them one-by-one for interactions with the target enzyme, HTS methodologies can be employed where hundreds of thousands and even millions of compounds are tested against the target enzyme in timespans of days to a few weeks. Thus, these HTS methods allow for the testing of a vast amount of chemical space that would otherwise not be feasible to explore (Achyuthan and Whitten, 2007; An and Tolliday, 2010; Blay et al., 2020; Inglese et al., 2007; Sundberg, 2000).

HTS methodologies can be applied to challenges related to space and space-based travel. Indeed, various HTS approaches have been suggested for space-based radiation prophylactic and countermeasure discovery (Putt et al., 2022). However, traditional HTS methodologies that use standardized microtiter plates with 384-wells or 1536-wells per plate (Auld et al., 2020), which work well for biochemical and simple cell line-based testing, may not be amenable to the more advanced simulating and modelling tools currently being developed for space research. Several of these new space research tools are various MPS that are designed specifically for microgravity and radiation studies (Mu et al., 2022). These MPS employ complex cell mixtures to produce 3D cultures and organoids along with engineered substrates to generate the desired necessary physiology (Leung et al., 2022). In general, these systems can better recapitulate the actual human condition as compared to 2D cultures in standard microtiter plates. While these MPS are certainly far more efficient than conventional bioreactors, due to their need for fluid reservoirs, tubing, fluid pumps, regulating systems and other chip-external components, the vast majority of developed MPS are still bulky and not suitable for HTS applications (Donoghue et al., 2021; Jones-Isaac et al., 2024; Wikswo et al., 2013; Yeung et al., 2020).

Efforts have been spent on miniaturizing a number of these MPS and some have even been utilized on the ISS. One example is a kidney-on-a-chip system that originally required more than 30 meters of tubing, 4 syringe pumps and 2 incubators that occupied as much as 1350L of volume. Following optimization, this system occupied only 45 L and has been used on the ISS multiple times (Jones-Isaac et al., 2024). While this is certainly a marked improvement over the original design, the current system is not able to process the hundreds or thousands of samples that would be necessary to truly be considered “high-throughput”. Therefore, to fully unleash the potential of HTS in space research, MPS must be designed with testing throughput in mind, preferably with some sort of industry-standard form factors that are compatible with high-throughput data collection equipment. Additionally, these systems must be amenable to

space-specific testing, such as overall form factors that are conducive for continuous low-dose radiation exposure or transport and testing in the ISS for microgravity experiments. Several groups have endeavored to increase the throughput of MPS, including the use of a parallel perfused array of 12 individual bioreactors in the same footprint of a standard 96-well microtiter plate (Domansky et al., 2010) and an improved system with 12 independent microfluidic systems (Phan et al., 2017). Higher throughput systems, containing 40 modules with 3-channels or 96 modules with 2-channels, also have been developed (Wevers et al., 2016).

More recently, advanced organ-on-a-chip platforms have been described, including a system that can simultaneously test 96 different chip devices in a standard 96-well microtiter plate format (Azizgolshani et al., 2021). This system incorporates integrated electrical sensors, oxygen sensors and transparent optical access to the cells, which allows for potential high-content screening applications, such as image-based analysis (Boutros et al., 2015). Additionally, cells can be easily removed through the microchannels, which can then be analyzed *via* any number of techniques, including transcriptomics, proteomics, metabolomics and lipidomics.

With MPS that are now approaching reasonable throughput numbers for true HTS applications (Song and Jeong, 2024), these systems need to be further adapted to the unique challenges of space research, such as microgravity studies and radiation experiments. To test the impact of continuous microgravity on cells and tissues in MPS, these systems need to be able to reach and perform their functions in low earth orbit, such as on the ISS (Mu et al., 2022; Song and Jeong, 2024). For launch, systems must be as compact and light weight as possible along with being able to withstand the elevated gravity forces experienced during transport (Dasch, 2005). Once on the ISS, the system should carry out its functions in the most automated fashion possible. While some sensors and other measurement technologies can be incorporated directly into HTS MPS (Azizgolshani et al., 2021), the more advanced omics analyses will still need to be performed on Earth. Therefore, these systems also must be able to safely collect and store samples for testing.

Adapting HTS MPS for probing the effects of simulated space-based radiation on Earth is a slightly simpler task. However, systems need to be designed for physical compatibility with radiation sources that best mimic space radiation, such as the NASA Space Radiation Laboratory at Brookhaven National Laboratory (Norbury et al., 2016). While it is not expected that the levels of radiation present in these studies would negatively impact the majority of materials present in most MPS (Seguchi and Morita, 1999), the impact and stability of the materials after prolonged radiation exposure should be verified *prior* to any large scale HTS applications. Otherwise, the hardware for HTS MPS for Earth-based space radiation is similar to most other applications (Mu et al., 2022; Tavakol et al., 2024; Tavakol et al., 2023).

With HTS-amenable MPS built for long-term low-dose radiation exposure, assays and radiation-specific readouts can be specifically tailored for the desired study. In particular, assays and read-outs could be utilized to further understand the physiological changes of various cell and tissue types when exposed to various types of radiation using omics technologies. Additionally, these systems could be used to identify prophylactic radioprotective agents or radiation treatments. Small molecules, biologics and even cell-based therapies could be studied in these MPS using many HTS-amenable assay types. Thus, binding to a cellular receptor of interest can be determined via displacement of a fluorescent probe, even in complex 3D cell cultures (Stoddart et al., 2016). DNA breaks can be detected through phosphorylation of Ser139 of histone H2AX to gamma-H2AX (Kuo and Yang, 2008) or through a bimolecular fluorescence complementation assay (Kodama and Hu, 2012) that measures the interactions of Mediator of DNA damage checkpoint protein 1 with gamma-H2AX (Jungmichel and Stucki, 2010). Redistribution of fluorescently labeled proteins to

different cellular compartments can be identified by high-content screening (Boutros et al., 2015). Changes in a specific gene expression level can be quantitated using a bioluminescent or fluorescent reporter gene (Solberg and Krauss, 2013). The number of cells undergoing cell death can be determined *via* a plethora of different assays (Mery et al., 2017).

MPS have only recently reached the technological readiness for true HTS applications and these HTS-amenable systems must be optimized to overcome the unique challenges posed by space research. As these systems mature, HTS will undoubtedly help solve some of the pressing issues in space research such as better understanding of the physiological changes induced by prolonged exposure in microgravity and the identification of efficacious prophylactic radioprotectors for chronic exposure to space radiation that will enable longer space voyages outside the protective magnetosphere of Earth.

5.4. Artificial intelligence and digital twins

A significant challenge in space biology is the scarcity of data from animals flown in space. Although all experimental data from animals are findable and accessible under the OSDR (Berrios et al., 2021; Sanders et al., 2024), rodent research missions on the ISS typically involve only a few dozen animals per mission. As a result, data are sparse and fragmented across separate experiments. Recently, this limitation was highlighted during a NASA initiative to produce a large, standardized, artificial intelligence-ready dataset for space biology research. Benchmark datasets were generated across all NASA Science Divisions to provide the scientific community with relevant datasets for testing new artificial intelligence algorithms. To overcome this data limitation, a 2-step process was employed to generate a larger synthetic dataset. First, Gaussian noise was added to the transcript counts, increasing the sample size from 112 to 6832, while preserving biological variability. Next, a Wasserstein generative adversarial network with gradient penalty (Viñas et al., 2021) was applied to produce more realistic synthetic data that mimicked the original RNA sequencing data's distribution. Such an approach created a robust, diverse and artificial intelligence-ready dataset that is now publicly available for benchmarking (NASA, 2024). This example demonstrates the potential of artificial intelligence in generating synthetic datasets that maintain biological complexity and relevance. A similar methodology could be applied to anonymize existing astronaut cohorts. In this regard, the Avatar method has successfully generated synthetic datasets for 2 large patient cohorts, including the AIDS clinical trial (*i.e.* 2139 patients and 26 variables) and a Wisconsin Breast Cancer Diagnosis (prediction dataset (*i.e.* 683 observations and 10 variables) (Guillaudeux et al., 2023). The Avatar method preserved the signal quality comparable to generative adversarial networks, while allowing for additional privacy metrics. By training an Avatar or generative adversarial network on private biological information from astronaut cohorts, it may be possible in the future to create synthetic datasets that can be shared with the scientific community, ensuring individual privacy, while providing access to valuable knowledge.

Another popular approach for generating synthetic datasets relate to variational auto-encoders. Variational auto-encoders encode data into a latent representation, simplifying data into their core features and then decode it back, making it particularly suited for dimensionality reduction and the generation of new data. They were initially introduced in biology to generate drug-like molecules for specific biological targets (Lim et al., 2018). In contrast, generative adversarial networks are adept at modelling nonlinear behaviors, outperforming mechanistic models in predicting intricate biological processes (Tsialiamanis et al., 2021). More generally, synthetic datasets preserve data types, covariates and the embedded biological information. Ideally, analyzing synthetic data will lead to the same biological conclusions as analyzing real data, making it indistinguishable from the original data. However, all 3 approaches (*i.e.* generative adversarial networks, Avatar and variational auto-encoders) still face challenges in predicting

biological responses that are not part of their training dataset. A potential solution lies in using conditional models, which generate data based on specific conditions, such as age, sex, genetic, phenotypes or strain. Unlike traditional models, conditional models incorporate these covariates directly into the data generation process, allowing them to simulate new scenarios by leveraging the relationships between conditions and the data structure, even beyond the original training scope. For example, using sex as a condition, if data from brain cells of male and female mice in ground control samples are available, but only female mice brain cells in flight samples, the effects of spaceflight on the brain cells of a young male mouse can be simulated, even without having ever observed it. This approach also allows for testing interventions and "what-if" scenarios by simulating perturbations that have not been observed (Wu and Koelzer, 2024). Such *in silico* trials are being explored to complement costly, time-consuming and under-powered clinical trials (Katsoulakis et al., 2024). Conditional models are integral to the broader artificial intelligence field of digital twin technology (Figure 6).

By definition, a digital twin mimics the real counterpart in real-time, offering valuable insights for monitoring, optimization and remote interventions. The concept of digital twins was first used by NASA in the 1960s during the Apollo 13 mission, which suffered an explosion in the oxygen tanks critically damaging the main engine and oxygen supplies and led to the famous line "*Houston, we've had a problem*". A ground replica was used to simulate the spacecrafts leading to the safe return of the crew (Nat.Comput.Sci.Edit., 2024). Digital twins are now being leveraged for healthcare (Erol et al., 2020) and have been discussed in the context of precision space health (Scott et al., 2023), but a full human replica remains too complex, leading the biology community to focus on more modest biological systems for digital twins, such as organoid data or specific tissues, like cardiac tissue (Corral-Acero et al., 2020).

Digital twins can generate large artificial patient cohorts, as seen with simulated tumors that create millions of distinct patients, enabling deep phenotyping and personalized treatment planning in cancer therapy (Stahlberg et al., 2022). Digital twins can also benefit from transfer learning (Xu et al., 2019), where a specific task is learned from one given dataset and then refined to another similar task. In the context of space exploration, synthetic datasets could be reinforced by incorporating larger, relevant epidemiological cohorts, such as the Million Person Study of low-dose radiation health effects, designed to evaluate risks among US nuclear workers and veterans (Boice et al., 2022). Another valuable cohort is the Japanese atomic bomb survivors, who were exposed to an acute high-dose of low-linear energy transfer radiation (Preston et al., 2007). Bed rest studies, which mimic the impact of microgravity on bone loss and muscle atrophy, are other cohorts that could add relevant features for space exploration. Each cohort offers comparable human characteristics to conditions in space, while adding various co-variate, such as chronic low-dose radiation for MPS, multi-generational data and a different ethnicity exposed to acute low-linear energy transfer radiation for the A-bomb cohort or pseudo-microgravity for the bed rest studies, making them valuable additions to space biology research.

Another promising tool is the knowledge graph. One can think of a knowledge graph as a database of databases, allowing the seamless integration of very distinct types of data and allowing the rapid integration of new information. Thus, the Scalable Precision Medicine Oriented Knowledge Engine model is a knowledge graph currently containing 43 million nodes (*i.e.* 26 types) and 165 million edges (*i.e.* 91 types), integrating data from over 60 foundational biomedical databases (Morris et al., 2023). Applied to NASA GeneLab transcriptomic data, it helped infer human-like physiological changes in space-flown mice. More specifically, statistical tests evaluated the differential distribution of node ranks between ground controls and space samples of murine RNA sequencing data (Nelson et al., 2021). Interestingly, nodes such as space motion sickness (symptom), regulation of blood vessel diameter (biological process), taste receptor complex (cellular component), vitamin D, metabolism (pathway) and

sympathetic nervous system (anatomy), all scored among the top 5% of nodes. These top-ranking nodes in this analysis reflect known spaceflight effects in humans, validating the Scalable Precision Medicine Oriented Knowledge Engine approach. These results also illustrate how animal data, often dismissed due to their inbred, non-diverse nature, can effectively translate to human physiological responses, providing valuable insights even across special differences.

6. Conclusions and perspectives

Space exploration is no longer science fiction, but a scientific fact. Indeed, the ongoing Artemis program of the NASA intends to take humans back to the Moon in the next few years (Artemis, 2024), whereas crewed missions to Mars are planned in the course of the next few decades. Concomitantly, private companies, like SpaceX (SpaceX, 2024) and Virgin Galactic (Virgin-Galactic, 2024), are laying the foundation for space tourism. In addition, as the ISS is slated to be decommissioned by 2030, companies, such as VAST Space (Vast-Space, 2024) and Axiom Space (Axiom-Space, 2024), will offer commercial manufacturing and research opportunities at the ISS to civilian astronauts. As a result, human presence in space is expected to steeply increase in near future. However, although more than 600 astronauts have been sent into space to conduct exploration missions thus far (Wikipedia, 2024), the mechanisms driving the effects of the space environment on human physiology are still not fully understood. NAMs are particularly well-positioned to fill such mechanistic knowledge gaps and, simultaneously, to bypass the use of animal experimentation. NAMs have gained considerable attention in the last few years in a regulatory context, especially when used for safety testing of chemical compounds. In this respect, the US FDA Modernization Act 2.0 allows the use of alternatives to animal testing, including organoids, MPS and *in silico* tools, to seek exemptions for assessing drug safety and effectiveness during the preclinical phase (Consolidated Appropriations Act, 2023). Although not explicitly named as such, NAMs have been used for many years in life sciences in space research. However, as the result of progressive interdisciplinary collaboration and a rapid succession of technological innovations, novel NAMs have been introduced at high pace over the past decade. At present, NAMs are mainly used in the life sciences in space research to model human biological and physiological responses to space conditions both in simulated and real-life space environments (*i.e.* replacement NAMs). These range from simple monolayer cultures of individual primary or stem cells all up to bioprinted 3D organoids and microfluidic chips that recapitulate the complex cellular architecture of organs (Table 1). Out of these NAMs, MPS models arguably provide unparalleled advantages in emulating human health and disease states due to their ability to recreate precise physiological conditions at the micro-scale, reflecting human-specific tissue responses more accurately than some of the other NAMs (Low et al., 2021). In space research, MPS models offer exceptional utility by enabling high-fidelity simulations of human organ systems under microgravity and radiation conditions, critical for understanding space-induced physiological changes and advancing countermeasures for long-duration missions (Mu et al., 2022). Notable examples of MPS-enabled space health applications include the investigation of muscular disorders that require dynamic configurations to accurately emulate their pathophysiology, such as skeletal muscle wasting (Giza et al., 2022; Parafati et al., 2023) and cardiac arrythmia (Mair et al., 2024). Additionally, inflammatory and related complex conditions, like post-traumatic osteoarthritis (Dwivedi et al., 2024), are effectively recapitulated on these sophisticated, high-content *in vitro* platforms, which provide essential built-in interactive microenvironments. Other approaches used in parallel to these NAMs do not fully replace animal testing *per se*, but rather contribute to the reduction of animal experimentation (*i.e.* reduction NAMs). These include tools to mimic space conditions on Earth, such as microgravity and radiation simulators, as well as tools to support the processing,

analysis or application of testing results obtained in life sciences in space research, including systems biology, live-cell, high-content and real-time analysis, high-throughput analysis, artificial intelligence and digital twins.

It should be stressed that research performed with tools to replace or reduce animal testing in the life sciences in space field is not only relevant for space applications as such, but is equally of paramount importance for terrestrial medicine. In this context, spaceflight-induced physiological changes closely mirror some age-related disease states, except that these changes often manifest clinically relevant markers more rapidly in space, occurring over weeks or months compared to years and decades on Earth (Capri et al., 2023). This accelerated onset and progression of aging provides an opportunity to model diseases in space and to gain insights into the mechanisms controlling age-related dysfunction at a faster pace. Similarly, the identification and testing of therapeutic targets could be accelerated. The development of potential therapeutics targeting aging-related dysfunctions is notoriously difficult. Microgravity conditions could provide unique opportunities for conducting clinical trials for therapeutics targeting aging dysfunction and aging-related pathologies and chronic diseases, such as cancer, neurodegeneration, heart disease and osteoarthritis. MPS in particular have emerged as powerful tools for biomedical research and are assigned essential roles in drug discovery, regulatory approval, safety and efficacy assessment and precision medicine studies. The Tissue Chips in Space 2.0 program recently announced by NCATS in partnership with the National Institute of Aging and CASIS will develop complex organ MPS to allow better modelling of the whole organism to study the effects of microgravity conditions on human body. This program will provide insights on human pathophysiology, especially aging-related functional decline and age-related diseases and will allow for better understanding of the complexity and heterogeneity of aging and associated pathologies. This will lead to better characterization of the hallmarks of aging, such as epigenetic alterations, changes in telomere length, shift in multi-omic profiles, post-translational modifications and dysbiosis. The outcomes of this program are anticipated to contribute to the development of senescence biomarkers and identification of therapeutic targets and treatments (ISS-National-Laboratory, 2024b).

Overall, the growing use of tools to replace or reduce animal testing in life sciences in space research parallels the increasing criticism on the use of animals for scientific testing purposes in general, which calls for more radical implementation of the 3Rs concept. While full replacement of animal experimentation by human-centered and *in vivo*-relevant NAMs is the ultimate goal, efforts should also be further focused on reduction and refinement. In this context, the development and application of virtual control groups, based on historical control data combined with cutting-edge artificial intelligence and advanced statistical methods, is currently being explored in regulatory toxicology studies (Steger-Hartmann et al., 2020), and also holds promise for reduction of animal use in the biomedical area, including life sciences in space research. Refinement measures aimed at minimizing distress and improving welfare include the use of anesthetics and analgesics, environmental enrichments and humane endpoints amongst others (Russell and Burch, 1959). In fact, several additional Rs have been proposed since the establishment of the 3Rs concept 65 years ago, such as Rs designed to enhance scientific value (*e.g.* robustness, registration and reporting) (Strech and Dirnagl, 2019). This has recently evolved to a 12Rs framework encompassing 3 domains each with their own Rs, including animal welfare (*e.g.* reduction and replacement), social values (*e.g.* responsibility and regulation) and scientific integrity (*e.g.* reproducibility and relevance). This 12Rs framework was introduced as a tool to promote, facilitate and harmonize the ethical conduct in the use of animals for scientific purposes across the world (Brink and Lewis, 2023). The life sciences in space research field will undoubtedly witness the implementation of this 12Rs framework in the next few years aligned with the booming use of NAMs. This will save

the life of a considerable number of laboratory animals, while simultaneously contributing to safe space exploration by mankind.

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Figures and Table legends

Fig. 1 *3Rs principles and new approach methodologies*. The 3Rs principles call for replacement, reduction and refinement of animal experimentation, and form the conceptual basis for new approach methodologies (NAMs).

Fig. 2 *Overview of the history of animal experimentation in life sciences in space research.* The upper part of the timeline represents milestones with regard to animals in space in the former Soviet Union, while the lower part of the timeline depicts milestones of the US unless mentioned differently (created with BioRender.com).

Fig. 3 *Overview of microgravity simulators.* (a) Desktop random positioning (RPM) machine. (b) Incubator RPM (iRPM) containing a miniaturized CO_2 incubator mounted on the inner rotating gimbal (top). The principle forces acting on the cells inside the culture flasks are shown below. The monolayer is exposed to high shear forces due to the motion and weight of the liquid column. Suspended cells or aggregates are displaced inside the culture medium and experience simulated microgravity as described by the mathematical equation in which the sum of all vectors equals close to zero over time. The motion creates a turbulent flow inside the culture flask. (c) 2D clinostat (top). Simulated microgravity is achieved by keeping small cells in motionless suspension (bottom). (d) NASA-developed rotating wall vessel (RWV) and (e) Clinostar (top). The RWV and clinostar devices share the same working principle by creating an infinite free fall (bottom). The yellow arrows indicate the direction of cell culture rotation. Advantages and disadvantages for each of the microgravity simulators are presented.

Fig. 4 (a) *Astronaut Christina Koch operating kidney chips at the ISS-NL*. Reproduced with permission from reference (Yeung et al., 2020) (copyright 2020, John Wiley and Sons). (b) *Overview of tissue chips for recapitulating multiple tissue level physiological functions*. Microchannels and in-chip microstructures are labeled in red and blue. Multiple tissues can be incorporated into one chip (adapted with permission from reference (Mu et al., 2022) with updates).

Fig. 5 *FLUMIAS development roadmap.* Steps in the technical development of the FLUMIAS-ISS microscope are illustrated, which will enable (fluorescent) live-cell imaging on the ISS from 2026 onwards. The first version of the microscope, which relied on spinning-disc technology, was used for a parabolic flight (PF, green circles) and has been in service since 2014. A modified model has been used on TEXUS sounding rockets since 2015 (yellow circles). In 2018, a first demonstrator version of FLUMIAS with the new light-sheet technology was on the ISS. It proved that the new microscope technology works and that high-resolution fluorescence microscopy is possible on the ISS. Ultimately, FLUMIAS-ISS (red circles) will combine the microscope with a life support system and a centrifuge to change the gravity conditions (CDR, critical design review).

Fig. 6 *Architecture of synthetic data generation approaches.* (a) Conditional generative adversarial network in which a generator (G) samples from a latent space of random data (z) and a distribution of labels (y) to create a synthetic sample, which the discriminator (D) then determines as real or fake as compared to real samples drawn from a training set (X). (b) After training the network, it may be deployed to generate new synthetic samples of the same dimension (p) that are conditioned on the labels (y) (b). (c) Conditional variational auto-encoder in which the encoder (E) samples data from a training set of real data (X) and their corresponding labels (y) to create a compact encoding (Z), which the decoder (D) then decodes into synthetic data. (d) Deploying the auto-encoder after it has been trained to create new synthetic samples by sampling from the latent space (Z) and conditioned on labels (y). (e) The avatar method, which encapsulates both the training and deployment in a single pass. Each of the n samples (yellow circles) are drawn from a dataset (X) and projected into a multi-dimensional compact representation. The k-nearest neighbors to each sample are identified, and a synthetic sample is randomly generated within the space of the k nearest neighbors.

Table 1: *Comparison of in vitro models and approaches.* Comparison of primary cells/stem cells, organoids/spheroids, microphysiological systems and bioprinting for life sciences in space research for multiple criteria.

Table 2: *Representative efforts on tissue chips for space exploration in the ISS-NL.* Adapted with permission from reference (Mu et al., 2022) and expanded (BARDA, Biomedical Advanced Research and Development Authority; FDA, Food and Drug Administration; NASA, National Aeronautics and Space Administration; NCATS, National Center for Advancing Translational Sciences; NIBIB, National Institute for Biomedical Imaging and Bioengineering; NIH, National Institutes of Health; NSF, National Science Foundation).

Table 3: *Features of the FLUMIAS-ISS microscope* (EB, experiment block; fps: frames per second; LSS, life support system; SIM, structured illumination microscope).

Figures

Figure 1

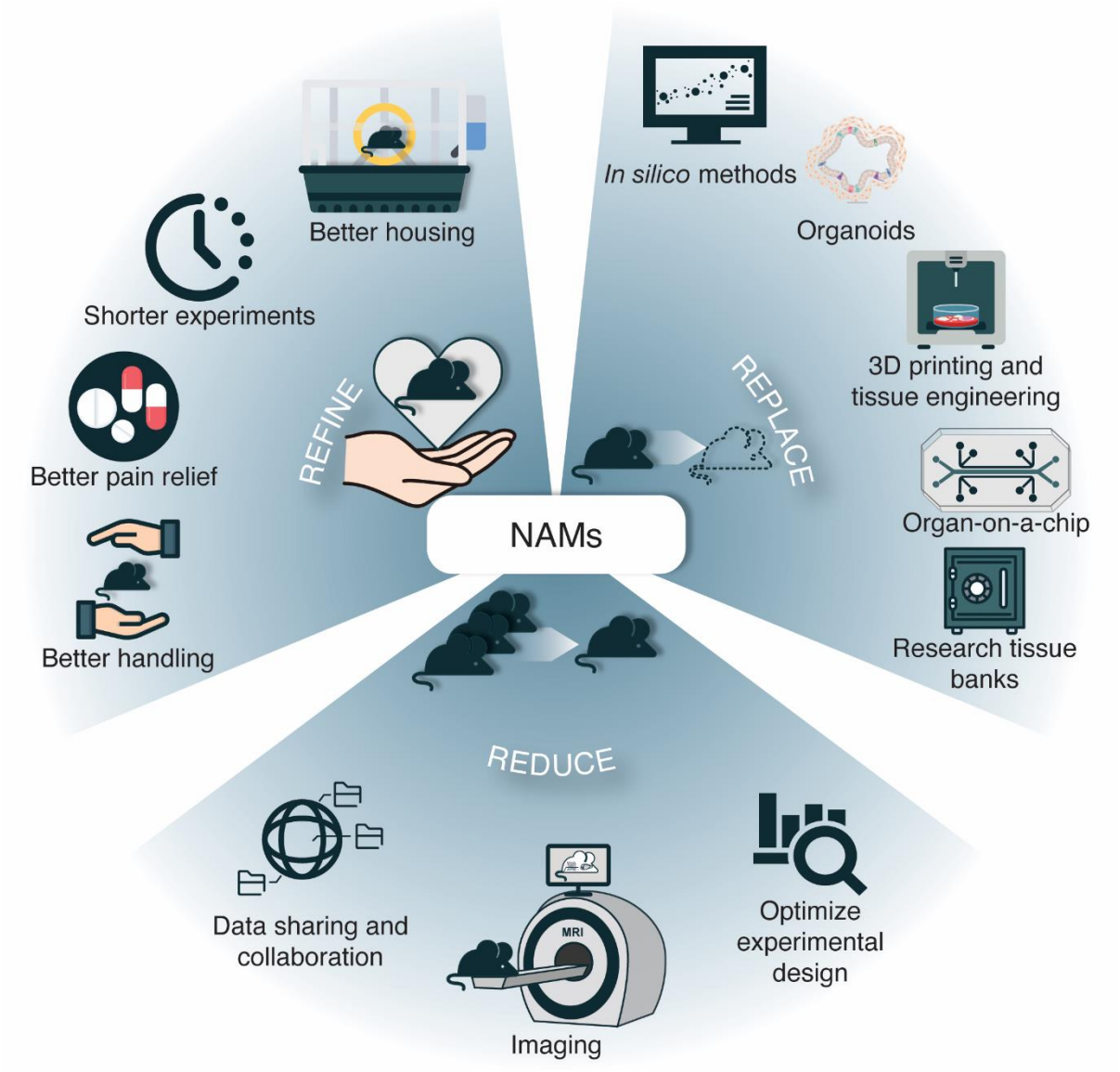
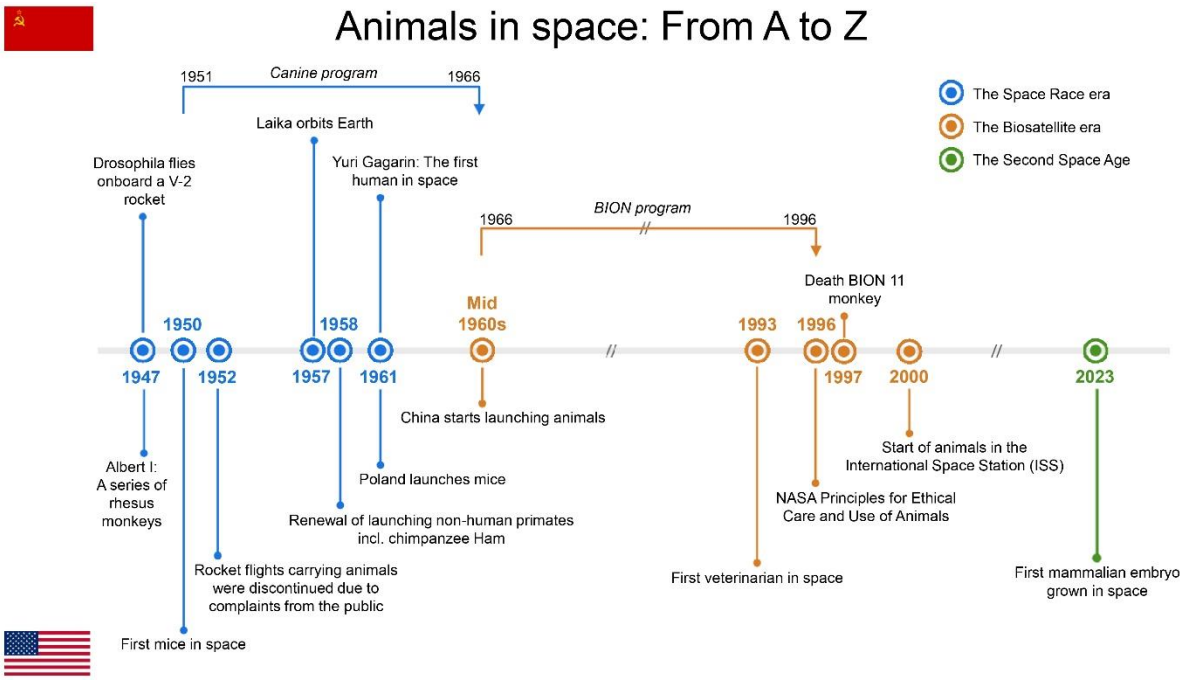
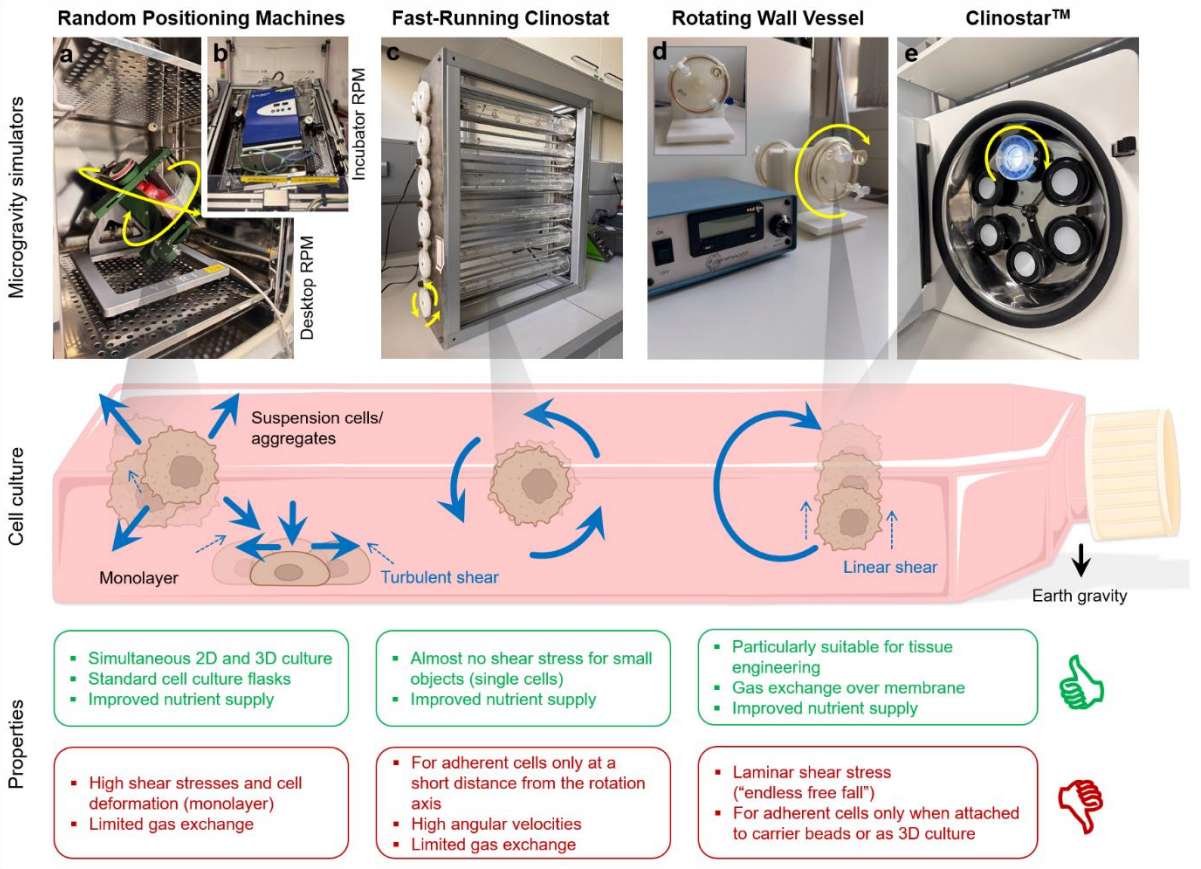


Figure 2


Animals in space: From A to Z
1951
Canine program
1966
Laika orbits Earth
Drosophila flies onboard a V-2 rocket
Yuri Gagarin: The first human in space
1966
BION program
1996
The Space Race era
The Biosatellite era
The Second Space Age
Death BION 11 monkey
Mid 1960s
1950
1958
1993
1996
1947
1952
1957
1961
1997
2000
2023
China starts launching animals
Albert I: A series of rhesus monkeys
Poland launches mice
Start of animals in the International Space Station (ISS)
Renewal of launching non-human primates incl. chimpanzee Ham
NASA Principles for Ethical Care and Use of Animals
Rocket flights carrying animals were discontinued due to complaints from the public
First veterinarian in space
First mammalian embryo grown in space
First mice in space

2592 Figure 3



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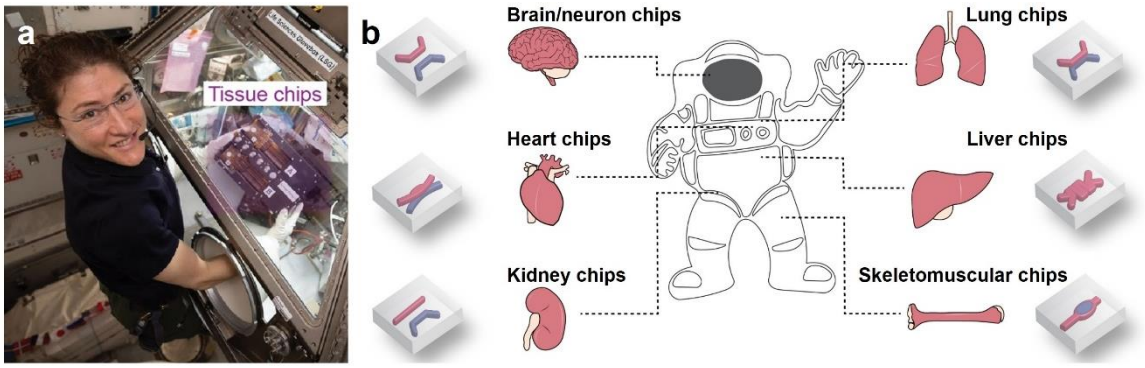

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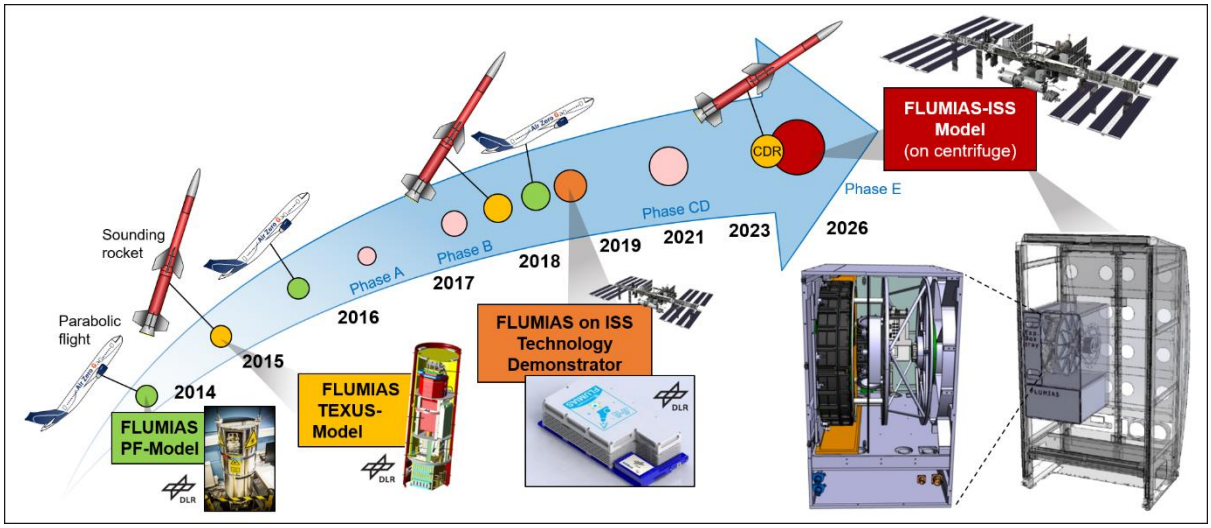

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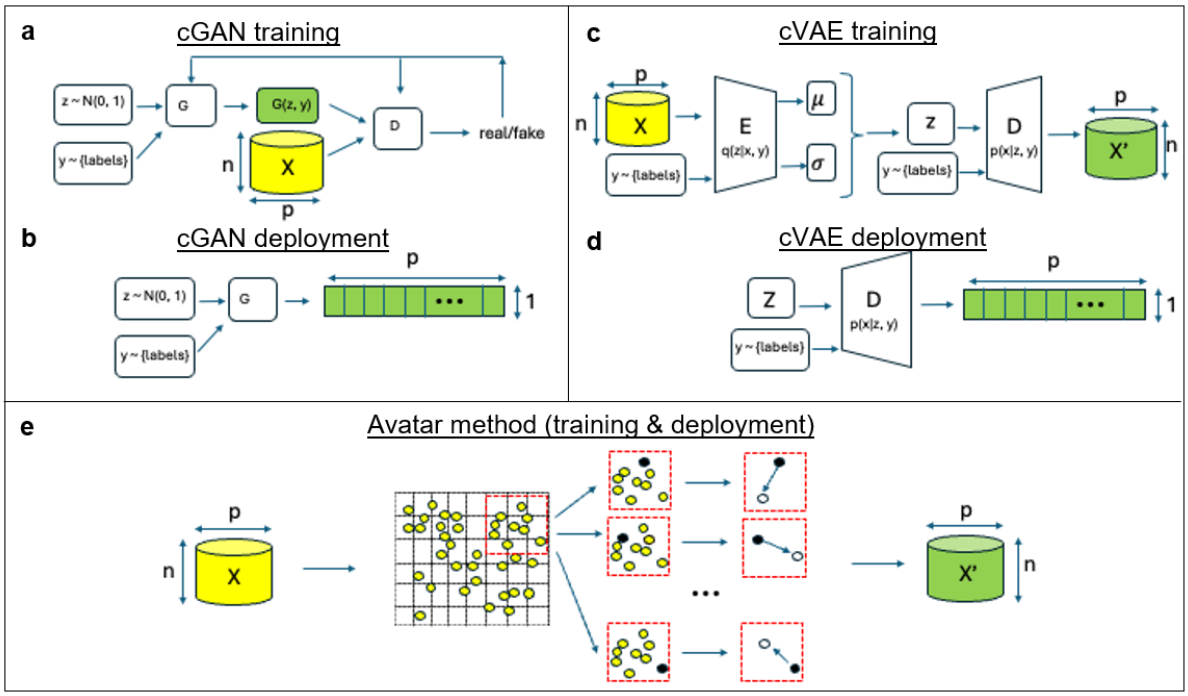
Figure 6


a
cGAN training
z ~ N(0, 1)
G
G(z, y)
D
real/fake
y ~ {labels}
n
X
p
b
cGAN deployment
z ~ N(0, 1)
G
p
1
y ~ {labels}
c
cVAE training
p
n
X
E
q(z|x, y)
μ
σ
z
D
p(x|z, y)
p
X'
n
y ~ {labels}
d
cVAE deployment
Z
D
p(x|z, y)
p
1
y ~ {labels}
e
Avatar method (training & deployment)
p
n
X
p
X'
n

2616 # Tables

2617 Table 1

	Primary cells/stem cells	Spheroids/organoids	Microphysiological systems	Bioprinting
Accessibility				
Cost				
Complexity	Isolated static 2D model	3D model with cell-cell and cell-matrix interactions	2D and 3D models with dynamic micro-environment	Printed 3D model composed of cells and biomaterials
Reproducibility	Limited by variability among donors	Limited by variability among donors and spontaneous self-organization	Using the same device/brand good reproducibility	High potential for reproducibility as soon as standards are developed
Advantages	*In vivo*-like behavior and normal physiology	Mimic cellular functions and signaling pathways due to various cell types	Mimics dynamic physiological environment	Generating tissues/organs in space
	Cost-effective	Organoids: recapitulate organ-specific functions	Real-time, non-invasive monitoring with biosensors	In space: self-organize into 3D structures
	Personalized approach possible	Higher variability and stability than 2D	Creation of 3D configurations possible	Control of cell placement and tissue structure
Limitations	Challenges in making and changing the medium	Challenges in making and changing the medium	Engineering challenges	On earth: additional support structures required to prevent collapsing
	Limited lifespan and proliferative capacity	Difficult to uniform size and composition	Requires specialized equipment	Requires advanced bioprinting equipment
	Characterization of cells needed	Only a fraction forms spheroids	Limited sample size	Still in development
	No organized tissue architecture	Difficulty in monitoring in space	High amount of data produced	

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2620 Table 2

Targeted tissues and cells	Physiological pathways and diseases	Launch dates	Funding agencies
Brain, liver, and gut	Brain-liver-gut axis, aging	> 2025 if selected for flights	NASA NIH BARDA FDA
Brain	Neurotoxic stress		
Neurovasculature	Chronic inflammation and neurodegeneration		
Vessel	Atherosclerosis		
Multiple tissues	Human tissues to stressors		
Multiple tissues	Repair after hypoxia		
Heart, vascular tissues	Response to radiation exposure		
Kidney	Acute/chronic exposure to drugs and toxins		
Bone	Bone loss	2024-2025	NSF
Vessel	Arterial pressure myography		
Skeletal muscle	Sarcopenia		
Heart	*Streptococcus pneumoniae* cardiotoxicity		
Skeletal muscle	Muscle wasting	December 2020, July 2022, November 2022	NCATS
Heart	Cardiomyopathy	December 2020, March 2023	
Intestine	Bacterial infection	March 2020	
Cartilage-bone-synovium	Musculoskeletal disease	May 2019, December 2020	
Lung	Lung host defense	May 2019	
Blood-brain barrier	Microgravity effects	May 2019, December 2021	
Stem cells	Immunological senescence	Dec 2018, July 2022	
Kidney	Proximal and distal tubule functions	May 2019, June 2021	
Heart	Cardiac dysfunction	March 2020	NCATS NIBIB

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2622 Table 3

Parameter	Case	Value [Unit]
g-level	Set by centrifuge velocity	0 to 1 in steps of 0.01 [*g*]
Laser excitation	SIM, 1 wavelength at a time	405 / 488 / 561 / 640 [nm]
lateral resolution	SIM, 40x / 20x / 10x	350 / 700 / 1000 [nm]
	Brightfield, 40x / 20x / 10x	600 / 1000 / 1400 [nm]
Axial resolution	SIM, 40x / 20x / 10x	1.6 / 5 / 15 [µm]
	Brightfield, 40x / 20x / 10x	1 / 3 / 9 [µm]
Power in sample plane	Raw / brightfield mode	10 / 1 in steps of 0.1 [mW]
Number of raw per SIM	SIM is calculated from raw images	7 [# frames]
Temporal resolution	Raw / SIM / brightfield	21 / 4 / 10 [fps]
Exposure time per frame	Raw / brightfield	40 / 40 [ms]
FoV	Raw, SIM and Brightfield, 40x / 20x / 10x	435x305 / 870x610 / 1740x1220 [μm^2]
Sample temperature	Mammalian EB / Plant EB	25 to 40 / 18 to 25 both +/-0.5 [°C]
Volume of 4 tanks in LSS	Individually controlled via valves	100 / 5 / 5 / 5 [ml]
Number of slide channels	Polymer or glass bottom	1 or 4 [# channels]
Axial range	SIM 40x / 20x / 10x	20 / 200 / 1000 [µm]

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