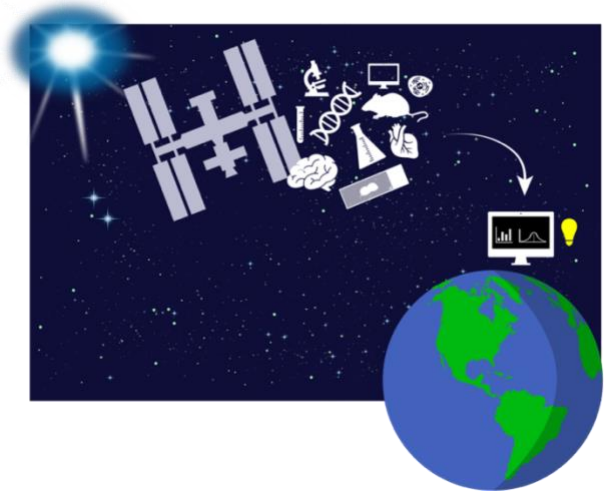
TOPICAL: TAKING SPACE BIOLOGY TO THE NEXT ERA BY ENSURING STANDARDIZATION, APPLICABILITY OF *IN SITU* TISSUE ANALYSIS AND COMPUTATIONAL PREDICTIONS

Graphical abstract



- Standardization of omics experiments is required
- Single-cell RNA-sequencing and spatially-resolved transcriptomics experiments are needed to extract more information from biological specimens exposed to spaceflight
- Artificial Intelligence will reduce the time required by astronauts to physically work on scientific experiments

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Introduction

A new space era has begun with plans to send humans to the Moon, Mars and on deep space missions. In the coming years and decades, astronauts and civilians will seek to travel in space for increasingly long periods of time. However, the effects of long-term space travel and living in extraterrestrial human settlements on biological systems remain obscure. Hence, to resolve this, performance of biological experiments on specimens of upcoming space missions will become essential to understand the systemic, tissue-specific and cell-specific physiological adaptations required for living in the space within each species. In addition, *we must be able to compare as much biological information as possible across different space missions*. This involves the understanding of biological systems at the scale of their functional unit, the cell, and in its own spatial location, which is known to determine its molecular identity (Asp et al., 2019). Cutting-edge technologies like single-cell RNA-sequencing (scRNA-seq) and spatially-resolved transcriptomics provide the opportunity to reach such a fine resolution. Single-cell RNA-sequencing techniques have been developing rapidly in the past decade to study the transcriptomes of thousands of different cells from different tissues during development, health and disease tissues (Wilbrey-Clark et al., 2020). However, scRNA-seq does not preserve the spatial information of the samples which is crucial to understand cell-cell interactions (Armingol et al., 2021). Spatially-resolved transcriptomics approaches have been developed to overcome this limitation and gain a deeper understanding of the spatial gene expression patterns in different tissues. Among these, the Spatial Transcriptomics (ST) technology (Ståhl et al., 2016; Salmén et al., 2018), selected as Method of the Year by Nature Methods in 2020 (Marx, 2021) and among the Top 10 Inventions in 2020 by The Scientist magazine, allows to integrate the histological information of tissue sections with their gene expression content. Moreover, it has shown its applications in plant tissue sections as well (Giacomello et al., 2017; Giacomello & Lundeberg, 2018) suggesting its potential universal application across species.

Despite the disruptive power of scRNA-seq and ST, the biological information that can be extracted employing these new types of technologies is highly dependent on the *quality of the input material and the mitigation of potential confounding factors*. This is why the *standardization of sample handling procedures, from collection to data analysis, including freezing, conservation and transport, as well as recording metadata* information *is essential* to compare results across space missions.

Challenges in Space Missions and Sources of Variation

Processing samples on the International Space Station (ISS) requires adaptation of protocols used on ground to the space flight environment. A spaceship has finite space, limited crew-time and the experiments are subjected to microgravity and ionizing radiation in a closed environment. The samples flown for space life sciences research are precious and provide valuable insights. ***Preservation*** of the sample is a quintessential step for biological research and is crucial in space missions owing to the duration of storage. Conducting life science experiments on the ISS is challenging despite the recent advances in technology. Collection of good quality tissue is difficult in the space environment due to limited resources and availability of labor. The practices used on ground for fixing specimens may not be the best procedures to be replicated on board and need to be adapted to the specific environment in the space station.

The ISS has limited facilities and resources that allow for animal euthanasia, general dissection, and sample processing. Implementation of life science experiments require that crew members are protected from potentially hazardous materials and that experiments are shielded from possible contamination. Sealed work areas dedicated to life sciences on the ISS provide bioisolation (Bonting et al, 1988) and have imposed safety requirements for all potentially toxic substances. Limitations in euthanasia methods and sample preservation are largely due to quantity restrictions, incompatible chemical composition, and containment standards that are insignificant in Earth-based wet labs. Primary animal euthanasia methods on the ISS includes intraperitoneal injection of Ketamine/Xylazine (Beheshti et al, 2019) or Euthasol (Choi et al, 2020), followed by the secondary method of exsanguination and bilateral thoracotomy. Unlike common practices utilized on Earth, the overdose of carbon dioxide or isoflurane are not primary euthanasia methods due to containment and exposure restrictions. Gloveboxes on the ISS provide containment and are conditioned for euthanasia, sample collection, and sample preservation. Immediately after euthanasia, sample collection occurs with dedicated equipment and supplies. All chemical preservation and fixation kits (**Table 1**) are prepared on Earth with pre-labeled and pre-aliquoted chemicals in vials or containment bags. Crew members do not prepare preservative or fixative solutions on the ISS. This necessitates the prepared chemicals to maintain efficacy from the time of preparation until sample collection, which can be weeks or months depending on the experiment duration. This is a limiting factor in the types of preservation methods that can be utilized on the ISS. Accommodations are made for temperature and light-sensitive properties, but chemicals otherwise require minimal activation and hardware.

Table 1: Tissue Fixation Techniques on the ISS.

Tissue Fixation	Properties
RNALater	a. Non toxic b. Protects cellular RNA c. Stabilizes tissue sample d. Eliminates the requirement of immediate freezing of samples
Slow freeze (-80°C freezers or colder)	a. Good for cellular preservation b. Requires specialized equipment c. Formation of ice crystal artifacts
10% NBF washed with PBS stored in 70 % ethanol	a. Less toxic b. Low cost c. Penetrates tissue relatively fast d. Random cross linking of proteins e. chemical modification of nucleic acids
4% PFA transfer to PBS containing 0.1% sodium azide	a. Preservation of morphology b. Limited use for RNA extraction

Rapid freeze to -185°C	a. Rapid freezing through conduction b. Good for cellular preservation c. Requires Specialized equipment d. High Cost

Rapid Freeze hardware, installed on the ISS in 2019, rapidly freezes biological samples through conduction by providing a -185°C interface. The Glovebox Freezer and the Cryo Chiller freeze multiple samples over a short period of time and maintain a consistent freeze rate from sample to sample. Prior to installation of the Rapid Freeze hardware on the ISS, cryogenic methods were limited to slow freeze in -80°C freezers, which caused partially degraded transcripts and inhibited identification of some of the molecular pathways altered by spaceflight. The Rapid Freeze hardware now provides freezing rates comparable to immersion in liquid nitrogen.

Crew members are trained in limited sample collection procedures and perform no analysis on the ISS, therefore, tissues and carcasses are preserved for return to Earth at refrigerated, frozen, or ambient temperatures. If animals are returned to Earth alive, euthanasia and sample collection procedures are performed by the spaceflight investigators. Biospecimens not utilized by the spaceflight investigators are preserved by the NASA Biospecimen Sharing Program and ingested into the biorepository, NASA Biological Institutional Scientific Collection (NBISC). NBISC identifies, documents, and provides the samples to the public community of researchers. This is the critical step in allowing non-spaceflight investigators access to the rare tissue samples for further analysis.

Data analysis challenges in spatially resolved transcriptomics

Fulfilling the potential of spatial transcriptomics will require continued improvements on both the protocols and the computational methods for analyzing these dataset sets.
Transcriptomics assays are currently the most common omics assays performed in space biology, with more than 250 transcriptomics spaceflight and corresponding analogue experiments stored at the NASA GeneLab repository (Berrios et al., 2021). In space transcriptomics, sample preservation methods can greatly affect gene expression as discussed elsewhere (Rutter et al., 2020; Lai Polo et al., 2020), and this is influenced by sample collection and sample preservation methods. Processing of live animals on Earth has shown changes in RNA due to reentry conditions, acclimation and circadian rhythm. In addition, mRNA degradation can also occur, affecting the quality of the reads sequenced reads and therefore the alignment rate. The use of a snap-freezing preservation method, and appropriate controls have shown to alleviate, but not solve, this problem. Importantly, these issues are expected to affect single-cell and spatial transcriptomics assays.

Newest versions of commonly used scRNA-seq software tools include methods for the analysis of spatial data, allowing to operate on gene count matrices, images and spatial coordinates. These tools include methods for quality control and preprocessing, normalization, dimensionality reduction, clustering, visualization in spatial coordinates, and finding spatially variable and spatial marker genes. Despite these advances, challenges exist associated with metadata standards and data processing, including the development of standardized metrics and benchmarks, reliable

cell segmentation, cell-type identification, integration of expression patterns to anatomical features, finding complex patterns, integration of data from different specimens, time points or perturbations and technologies (Atta and Fan, 2021).

While all these challenges exist also for ST data, some are more specific to space biology due to the inherent problems explained in previous sections related to data sample collection and processing. In space data, *true* morphological and transcriptional variability occurring due to an underlying phenomenon in microgravity, radiation and confinement should not be confused with artifacts created by sample collection, preservation, and transportation. Therefore, software tools aiming to remove confounding factors, improve transcript quantification, and reconstruct morphology and architecture of damaged tissue using image analysis techniques will be essential. Specifically, reconstruction of deformed images using image registration techniques, and the development of computational tools to elucidate space-driven modification of tissue organization will be necessary.
Metadata standards for ST data will involve dealing with image, coordinates data and gene expression count matrices. Metadata could be stored in MAGE-TAB format with few modifications, as done currently in ArrayExpress for the experiment type "spatial-transcriptomics by high-throughput sequencing". Beyond data storage, where to submit the data will influence its application by users and the broad research community. While Space Biology repositories should be the first choice, atlases which perform data processing need to be tailored to challenges above. Submitting data to non-space focused atlases on the other side could facilitate data integration with other conditions, technologies, and species.

Recommendations
In both the present and future, we shall see the development of a sustainable need for access to microgravity in order to push further life sciences research in space. To achieve this goal, we need:

- **to ensure that sample processing is carried out using both standards and metadata normalization of spaceflight "omics" experiments, especially to employ techniques like scRNA-seq and ST**. We envision the creation of protocols for storage and, when compatible, dissection of biological samples onboard of the ISS as well as protocols for processing, dissection and storage of any biological samples returned to Earth compatible with the above-mentioned technologies.

- **to have Standard Operating Protocols approved for use by scientists planning and conducting life sciences research on board the space stations in order to follow a common route and thus ensuring consistent data being generated that will be suitable for comparative analysis**. Such standard work practices will become pivotal in understanding the effects of spaceflight on the biology of life and preparing the international community toward developing safe and effective crewed space exploration beyond low earth orbit. In doing so, this will enable two important concepts or ideas: (1) space for Earth applications, and (2) space for space applications. Both concepts will ensure that low earth orbit stations will provide a rich proving ground for the technologies that need to go further and deeper into space such as the moon (Artemis program)

and Mars. **The goal of omics experiment standardization is also shared by the International Standards of Space Omics Processing (ISSOP) consortium, which gathers international omics and space biology experts around the world to contribute to this mission**. Moreover, looking at the current boom in private astronauts and space tourism as exemplified by Virgin Galactic, Blue Origin and SpaceX, trained scientists for standardized procedures is also of interest to the life sciences community in space. We should all care about understanding how to expand the pool of people qualified to fly and how to care for them medically in orbit. Future state of the art labs in space will need to drive provision of profitable research services and/or development of biomedical manufacturing applications.

- **to have a state-of-the-art lab capable of conducting spatial omics in orbit**. This will allow for *in situ* analysis onboard the Station rather than the gather and return samples model of the ISS today. In future, **automated** high-throughput platforms and machine learning algorithms for spatial omics are expected to reduce crew time in the laboratory. This will speed the return of research results, move at the pace of commercialization and ensure that what we measure in onboard samples has not been confounded by the re-entry process.

- **to expand the use of Artificial Intelligence and Machine Learning modules.** These can be applied to analyze data on Earth but also be prepared and sent as payloads to low-earth orbit to facilitate and assist in the analysis and interpretation of data obtained on site from biological payloads sent on the same or subsequent missions. We envision that applications such as these will significantly reduce the time required by astronauts to physically work on scientific experiments. Computer assisted machine learning applications will therefore minimize errors, enhance capacity and increase efficiency in conducting state-of-the-art life sciences research in low-earth orbit. Moreover, data downlink is a significant spaceflight challenge that is amplified by distance from Earth. The execution of automated analyses in-flight can reduce data dimensionality, enabling investigators to receive meaningful data without the significant delays that downlink of raw data can cause. The raw data can always be eventually returned via hardware for validation, but performing analyses in space would allow for investigators to receive useful results faster, which could even have an impact on how they wish the experiment to proceed. For example, meaningful phenotypic information can be extracted from in-flight videos of model organisms via automated ML algorithms, but even with compression, the large file sizes mean that the bandwidth requirements for downlinking these videos is very high. Performing the analyses in orbit and returning the phenotypic parameters extracted from the videos is significantly more practical due to the reduction in file size. The same concept applies to omics pipelines, whereby processed data has significantly reduced downlink requirements compared to unprocessed data.

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