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Experiment verification test of the Artemis I 'Deep Space Radiation Genomics' experiment☆

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ABSTRACT

When the Artemis missions launch, NASA's Orion spacecraft (and crew as of the Artemis II mission) will be exposed to the deep space radiation environment beyond the protection of Earth's magnetosphere. Hence, it is essential to characterize the effects of space radiation, microgravity, and the combination thereof on cells and organisms, i.e., to quantify any correlations between the deep space radiation environment, genetic variation, and induced genetic changes in cells. To address this, the Artemis I mission will include the Peristaltic Laboratory for Automated Science with Multigenerations (PLASM) hardware containing the Deep Space Radiation Genomics (DSRG) experiment. The scientific aims of DSRG are (*i*) to identify the metabolic and genomic pathways in yeast affected by microgravity, space radiation, and their combination, and (*ii*) to differentiate between gravity and radiation exposure on single-gene deletion/overexpressing strains' ability to thrive in the spaceflight environment. Yeast is used as a model system because 70% of its essential genes have a human homolog, and over half of these homologs can functionally replace their human counterpart. As part of the experiment preparation towards spaceflight, an Experiment Verification Test (EVT) was performed at the Kennedy Space Center to verify that the experiment design, hardware, and approach to automated operations will enable achieving the scientific aims. For the EVT, fluidic systems were assembled, sterilized, loaded, and acceptance-tested, and subsequently integrated with the engineering parts to produce a flight-like PLASM unit. Each fluidic system consisted of (*i*) a Media Bag, (*ii*) four Culture Bags loaded with *Saccharomyces cerevisiae* (two with deletion series and the remaining two with overexpression series), and (*iii*) tubing and check valves. The EVT PLASM unit was put under a temperature profile replicating the anticipated different phases of flight, including handover to launch, spaceflight, and splashdown to handover back to the science team, for a 58-day period. At EVT completion, the rate of activation, cellular growth, RNA integrity, and sample contamination were interrogated. All of the experiment's success criteria were satisfied, encouraging our efforts to perform this investigation on Artemis I. This manuscript thus describes the process of spaceflight experiment design maturation with a focus on the EVT, its results, DSRG's preparation for its planned launch on Artemis I in 2022, and how the PLASM hardware can enable other scientific goals on future Artemis missions and/or the Lunar Orbital Platform – Gateway.

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1. Introduction

NASA's Artemis Program will bring humans back to the surface of the Moon and it will enable controlled biological experiments beyond lower Earth orbit (LEO). While spacecraft in LEO are partly protected from galactic cosmic radiation and solar particle events by Earth's magnetosphere, missions to the Moon and beyond will lack this protection. This poses several scientific questions, including 'What level and kind of damage can we expect to cells and their DNA as a result of these types of radiation?', 'What are the biological processes that improve cells' ability to thrive under conditions of microgravity and space radiation?' and 'Which DNA repair mechanisms are most effective under these conditions?'. With these and other key questions in mind, NASA's Space Biology Program selected four investigations to be included inside the Orion vehicle during the Artemis I mission to "understand how biological systems are affected by a deep space environment in preparation for human missions to the Moon, and eventually Mars" [1]. One of these investigations is Deep Space Radiation Genomics (DSRG), which has the overarching aim of identifying the metabolic and genomic pathways affected by microgravity, space radiation, and the combination thereof. The segregation of results from deep space radiation and microgravity will be enabled via International Space Station (ISS) controls. These observations will then be used to inform pathway-specific and gene-specific approaches designed to ameliorate the detrimental effects of long-term radiation exposure. To achieve this, we will use molecularly barcoded yeast genome-wide knockout (deletion) and overexpression collections, enabling us to interrogate each gene's role in cell survival in this radiation and microgravity environment [2]. Each capsule contained in the PLASM systems (total of 12) contain aliquots derived from the same stock of 6000 deletion mutants or 6000 overexpression strains. Briefly, master pools of these strains are generated by combining 16 independent colonies of each mutant into liquid media at an OD_{600} of 5.0. For this, 200 μl of these mixtures are aliquoted into each gelatin capsule before freeze drying. Based on the fact that one OD_{600} unit of cells contains ~2.7×10^7 cells/ml, each aliquot contains ~5.4×10^6 cells. Dividing this by 6000 (the number of different strains in the mixture) allows us to estimate that each strain in the capsule is represented by, on average, 900 individuals of each strain. This value is consistent with those typically used in on laboratory-based screens [6]. Yeast is used as a model system because 70% of its essential genes have a human homolog, and almost half (47% PMID: 25999509) of these homologs can functionally replace their human counterpart [3]. To meet the demands of the Artemis I mission, the biological material (pools of yeast deletion and overexpression mutants) must be converted into a form suitable for long-term storage at a range of temperature and humidity, and also be impervious to the effects of microgravity. Accordingly, after testing a variety of preconditioning media, we selected the disaccharide trehalose as an additive [5] which would allow one to freeze dry the cells in a manner that would preserve their viability upon rehydration.

Artemis I's Orion spacecraft will be launched to LEO by the Space Launch System and will subsequently start its journey to the Moon (trans-Lunar injection), which will require Orion (and our yeast cells) to travel through Earth's radiation belts. If our cells were metabolically active during this radiation belt transit, our results could be confounded (because the cells would be exposed to higher radiation levels during this transit than what is expected beyond Earth's magnetosphere). Accordingly, our experiment requires and features an active controlled start *after* Orion is past the last Van Allen Belt. Furthermore, to ensure the cells will grow in a radiation environment that is not confounded by Earth's magnetosphere, the experiment needs to be activated after Orion is outside of Earth's magnetosheath. These two requirements posed engineering challenges as, for this mission, the biological payloads will have no power, telemetry, or data in general, from Orion. To satisfy the first requirement, we will launch the yeast deletion and overexpression series lyophilized, and the experiment will be activated by rehydration with growth medium past the last Van Allen belt [2]. To achieve this, we developed the Peristaltic Laboratory for Automated Science with Multigenerations (PLASM), an autonomous hardware that can perform this activity while controlling internal temperature and recording environmental data. To satisfy the second requirement, an algorithm (software code) was developed to inform PLASM when it can and cannot start the experiment, ensuring that the cell growth will take place outside Earth's magnetosphere (described elsewhere [4]). For the Artemis I flight, active (time-resolved dose rate) and passive radiation dosimetry will be recorded with NASA-modified and developed, respectively, dosimeters.

Beyond these requirements, however, there are many scientific, engineering, and operational aspects that need to work flawlessly to ensure DSRG's mission will be successful. Hence, a full rehearsal of the experiment was needed to inform whether our experimental design, spaceflight hardware, and concept of operations were ready for flight in Artemis I. In this full rehearsal – called Experiment Verification Test (EVT) – the actual experiment is prepared and performed like it is planned to occur in flight, including the time duration and temperature profile, sample preparation, hardware loading, computer set up (software), cell rehydration, environmental data collection, and subsequent yeast data collection and analyses. This manuscript describes the performance of (section 2) and methods from (section 3) DSRG's EVT. This description may also serve as an example of PLASM's ability to host other types of research in an autonomous fashion, with details of its design and operations to be published separately.

2. EVT performance

PLASM consists of three independent Fluidic Systems, each composed of one Media Bag, tubing, fittings, check valves, and four Culture Bags (see Fig. 1). To ensure the tubing won't loosen during the vibrations of launch or operations, each fitting is fixed in place with respect to the tubing with clamps. The check valves ensure there is no back flow of yeast culture into the tubing or cross-talk between Culture Bags, and their crack pressure ensures growth media goes into all Culture Bags, as described in more detail in Ref. [2]. At launch, each Media Bag will be filled with 32 mL of growth medium, the tubing will be empty, and each Culture Bag will contain a lyophilized yeast deletion or overexpression series, with the Culture Bag mostly free of air. Yeast extract–Peptone–Dextrose (YPD) (Gibco, A13745-01) will be used as the yeast's growth medium, supplemented with Geneticin G418 (Fisher, Cat. No. 507533025) at a 100 μg/mL concentration.

DSRG's EVT took place at the Kennedy Space Center's Space Station Processing Facility between October 19, 2020 and December 22, 2020. Growth media was prepared and was placed at 37 °C for 24 h to check for any contamination. PLASM's tubing, fittings, and check valves were assembled, pressure tested, and sterilized. The tubing assembly was air-pressurized to 0.68 atm (10 psig) and placed in a beaker full of water. No air bubbles emerging from any part of the tubing assembly were observed during a 3-min submersion test, demonstrating successful

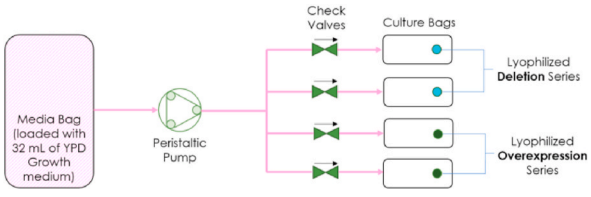

Fig. 1. One of three PLASM fluidic systems, each consisting of one Media Bag (launched with growth medium), one peristaltic pump, and four Culture Bags. Each Culture bag includes a checkvalve to ensure no cross-talk between samples. At launch, each Culture Bag includes a single capsule containing the lyophilized yeast deletion or overexpression series. To activate the experiment, PLASM's pumps will transfer equal amounts of growth medium into the Culture Bags. Updated from Ref. [2].

integrity of the tubing system. Sterilization of the tubing assemblies was performed by flowing and priming them with 70% EtOH – leaving primed for 15 min – and subsequent rinsing with sterile dH_2O, and drying with sterile air. The sterile, gas-permeable Culture Bags were each loaded with a lyophilized yeast deletion or overexpression series capsule, and heat sealed. Each Culture Bag was acceptance tested by air-pressurizing them to 0.68 atm (10 psig) for 10 min, while the pressure was monitored for against an acceptance pressure decay limited to ensure a liquid-tight assembly. The Culture Bags were integrated to the tubing assemblies in groups of four, removing as much air from them as possible prior to assembly, and integrated into PLASM's peristaltic pumps (one per fluidic system). Each of the three sterile Media Bags was attached to a PLASM's warming surface and loaded with 32 ml of growth medium that had passed the 24-hr contamination check, and they had all air removed (see Fig. 2). Each Media Bag was subsequently assembled onto the rest of the respective fluidic system.

With the three fluidic systems fully assembled, each of the 12 yeast deletion or overexpression capsules, inside the Culture Bags, were broken to release the lyophilized cells from their gelatin capsule. This helps ensure each of the ~6000 mutants will be exposed to growth medium, during activation, at the same time (tests with intact gelatin capsule required several hours to fully dissolve, which could result in heterogeneous growth initiation). The Culture Bags were then installed on their PLASM's warming surface, on the opposite side of their corresponding Media Bag, and the three warming surfaces (each with a separate fluidic system). Pumps were then integrated into PLASM. PLASM's internal wiring was installed, a quality control inspection was performed, and then the hardware was closed. PLASM's software was initialized and run to confirm its appropriate functioning.

Following the protocol which will be implemented for the Artemis I flight, PLASM was covered in vacuum panels and placed in a shared payload container together with another of the four Space Biology payloads. This, and a second payload container housing the remaining two Space Biology experiments, were placed inside an environmental test chamber that was programmed to produce a temperature profile as the one expected inside the Orion vehicle. The thermal profile included: handover to launch, spaceflight, and splashdown to handover back to the science team, for a 58-day EVT (October 21, 2020 through December 18, 2020). During this time, PLASM activated the experiment by rehydrating the cells. By the end of the EVT, PLASM was removed from its container and opened. Each of the fluidic systems was removed and the contents of each of the 12 Culture Bags was transferred into a 15 mL conical tube prior to temperature-controlled shipping and analyses. Finally, PLASM's environmental data was retrieved and stored.

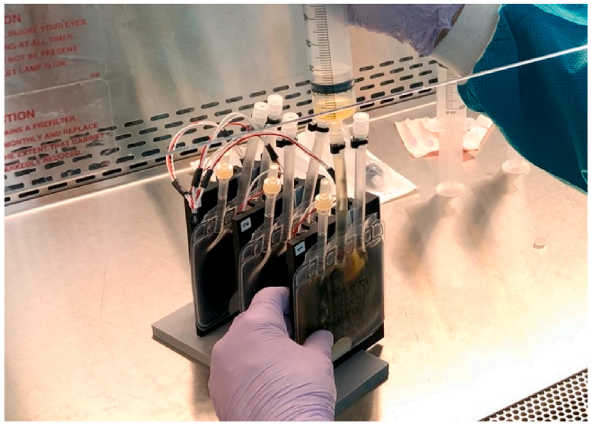

Fig. 2. A Media Bag being loaded with 32 ml of growth medium. Two Media Bags (each mated to an independent fluidic system) can be seen behind, each attached to a PLASM warming surface. The rest of PLASM's internal components are not shown.

3. EVT sample processing

Yeast pools were collected from the EVT samples as described in Ref. [6] and aliquots at 50.0 OD/ml were thawed and diluted to an OD of 10.0 in YPD. Diluted yeast were mixed 1:1 with 40% sterile trehalose dissolved in YPD. Size 3 (~300 µl min volume) gelatin capsules were obtained from Well.ca. For each gelatin capsule, the smaller, lower halves were separated from the top halves and placed in 150 mm disposable petri dishes for UV irradiation in a Spectroline UV cabinet for 5 min. 50 µl of yeast pool in 20% trehalose at an OD of 5.0 was pipetted into the smaller half of each capsule and the larger half of the capsule was replaced and gently locked into place. Individual, filled capsules were then placed inside microcentrifuge tubes. To allow for gas exchange, a hole was made in the capped lid with a heated 18-gauge needle. Samples in microfuge tubes were freeze dried in a Labconco FreeZone device at −70 °C at a vacuum for 48 h. Each batch of capsules was tested by rehydrating each capsule in media for 6 h at room temperature, then subjected to viability using a spot assay as described for Fig. 5. Specifically, to verify viability, rehydrated capsules were placed in 500 µl of autoclaved 18 Ohm water for 6 h at room temperature. Serial two-fold dilutions, starting at an OD of 0.5, were prepared in a flat bottom 96-well plate and 2 µl pipetted onto a YPD plate and incubated for 48 h at 30 °C (Fig. 3).

4. EVT results

The PLASM hardware and yeast samples were used to quantify our performance against the success criteria (Table 1) which was agreed upon by NASA and DSRG's implementation and science teams. This table describes the range of outcomes for six aspects of the experiment based on the post-flight data analyses. EVT results are presented here and discussed under each of the table's six criteria.

As shown in Fig. 4, when PLASM was opened at the end of EVT, (*i*) all 12 Culture Bags were observed to be properly loaded with growth medium, (*ii*) each showed yeast growth which manifested as collections of turbid regions within the bags (see circle using four Culture Bags as example), and (*iii*) the Media Bags were empty (one shown with an arrow in Fig. 4). With 12 out of 12 Culture Bags activated, an 'excellent' score was achieved for the 'Experiment Activation' category.

To quantify cell growth at the end of the experiment, each sample was diluted in YPD media 1:100 (stepwise 1:10 dilutions) and the OD_{600} measured with the Eppendorf BioPhotometer using YPD media as a blank. For all 12 samples, the final OD_{600} ranged from 7.6 to 14.8. Given that the criteria to score 'excellent', 'cell growth' was to have OD > 5 in 9–12 Culture Bags, this was achieved with the EVT (Table 2).

DNA quantification was carried out by counting strain-specific barcodes by sequencing (Bar-seq [7]). First, 1.0 OD_{600} of each sample was pelleted and genomic DNA extracted using a Zymo YeaStar genomic DNA kit (Cat #D2002) according to the manufacturer's protocol. After

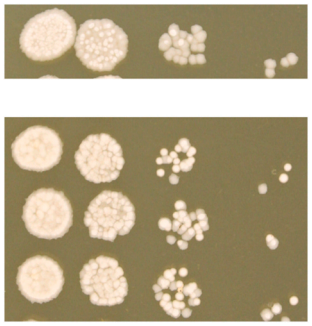

Fig. 3. Spot dilution to test viability of freeze-dried pools. Top panel control pool stored at 4 °C for 48 h. Bottom panel 2 µl of three independent freeze-dried samples.

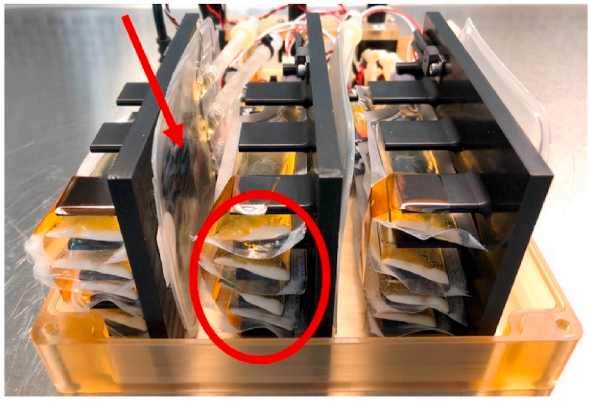

Fig. 4. Open PLASM at end of EVT. The arrow shows one of the Media Bags depleted, as PLASM transferred their contents into their respective four Culture Bags. The circle shows yeast grown inside four Culture Bags.

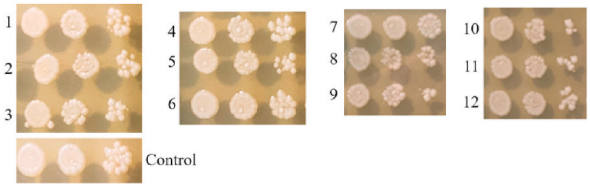

Fig. 5. Growth observed on YPD agar plates. BY4743 (the parent of the deletion and overexpression collections) was used as control, and dilution series of it and the 12 EVT samples (numbered from one through 12) were spotted on YPD agar media. No contamination was observed.

Table 1
DSRG success criteria table.

Criteria	Excellent	Acceptable	Unacceptable
Experiment Activation	9-12 CBs are successfully activated	5-8 CBs are successfully activated	0-4 CBs are successfully activated
Cell Growth	OD > 5 in 9–12 CBs	OD > 5 in 5–8 CBs	OD > 5 in 0–4 CBs
DNA Barcode Sequencing	DNA barcode counts in 9–12 CBs	DNA barcode counts in 5–8 CBs	DNA barcode counts in 0–4 CBs
RNA Integrity Number (RIN)	RIN ≥7 in 9–12 CBs	RIN ≥5 in 9–12 CBs	RIN <5 in 9–12 CBs
RNA Mass	>0.5 μg from 9 to 12 CBs	0.05–0.5 μg from 9 to 12 CBs	<0.05 μg from 9 to 12 CBs
Sample Contamination	No contamination in any culture	<10% of colonies are not *S. cerevisiae*	>50% colonies are not *S. cerevisiae*

CB = Culture Bag.

Table 2
DSRG EVT results.

Sample	Cell growth (OD_{600})	DNA barcoding (average counts/strain)	Total RNA mass (μg)	RIN
1	14	873	1.52	7.3
2	13	1100	1.24	7.1
3	14.8	1124	1.77	8.4
4	14.6	1079	1.28	7.8
5	11.7	853	1.8	7.2
6	14.7	1026	2	8.1
7	10.8	1136	2.11	8.6
8	14.5	1017	1.42	7.9
9	7.6	1024	1.83	7.8
10	13.1	1116	2.1	7.7
11	15.5	1019	2.22	7.6

DNA extraction, two independent 20 μl PCR reactions, one for the uptag and the other for the downtag, were set up with the following cycling conditions: 98 °C for 3 min, 25 cycles of 98 °C for 10 s, 59 °C for 30 s and 72 °C for 15 s, and final 72 °C for 5 min with 4 °C hold. This barcode amplification was performed by using two sets of common primers. These amplicons were then pooled in equal amounts and subjected to an additional five cycles of PCR to attach the tagmentation sites and unique molecular identifiers (PCR2) for Bar-seq. For detailed explanation and primer sequences see Refs. [6,7].

All 12 samples were then pooled, purified using SPRI beads and loaded onto an Illumina NEXT-SEQ 550 flow cell. 1X75 single-end reads were obtained, and mapped to the reference sequence of strain barcodes. Our in-house quality control pipeline requires a minimum of 500 counts/strain and the EVT samples ranged between 853 and 1136 counts/strain. Thus, the 'DNA barcode sequencing' was scored as 'excellent'.

The RNA Integrity Number (RIN) is an indicator of the quality of the RNA. To score 'excellent' in the 'RIN' and 'RNA Mass' categories, the EVT-produced samples needed to have a RIN ≥7 and > 0.5 μg of total RNA per sample, in nine or more Culture Bags. To assess this, 2.0 OD_{600} of each sample was centrifuged and the pellet drop-frozen in liquid nitrogen. Total RNA was purified using a glass bead-TRIzol extraction. Samples were resuspended in water and analyzed by Qubit fluorimeter and using an Agilent Bioanalyzer with high-sensitivity RNA microfluidic chip. The 12 EVT samples ranged from 7.1 to 8.6 RIN and 1.24 and 2.22 μg of total RNA, yielding an 'excellent' score in both categories.

Finally, two parallel approaches were taken to determine if any of the 12 samples were contaminated: a spot assay and microscopic visualization. First, we transferred 100 μl of cells at $OD_{600} = 1$ to a well of a 96-well plate, and three 5-fold serial dilutions were prepared in media; then 5 μl from each well were spotted onto a YPD agar plate and grown for three days at 30 °C. No contamination was detected after 72 h, and the viability of the cultures was comparable to the control sample (Fig. 5). For microscopic visualization, an EVOS phase contrast microscope with a 40X objective was used to assess 5 μl drops of each culture, which showed no evidence of non-*Saccharomyces* cells (Fig. 6).

5. Conclusions

According to the criteria established for this EVT, DSRG's experiment design and PLASM succeeded, achieving 'excellent' scores in all of the experiment's categories. Satisfying these criteria indicates: (*i*) the readiness of this payload for cislunar spaceflight on Artemis I, and (*ii*) the suitability of PLASM to enable temperature-controlled, autonomous

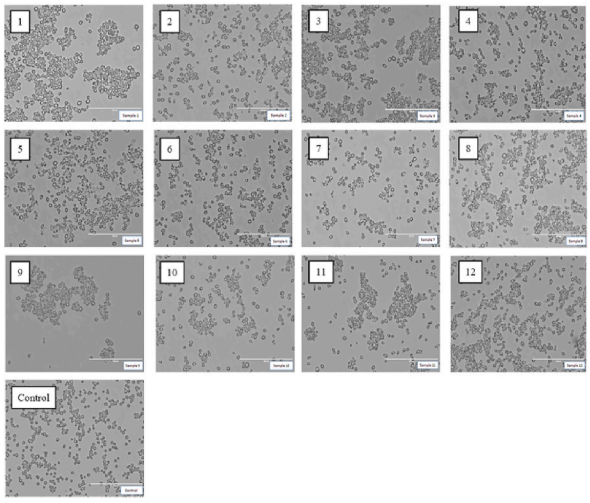

Fig. 6. No contamination was observed in either of the 12 EVT samples via phase contrast microscopy. Scale bar = 100 μm.

scientific research.

It is important to highlight the flexibility of this experimental system, both in terms of the flight environments as well as the biological materials. PLASM may be used not only on future Orion missions, but also on other platforms such as high-altitude balloons, suborbital spacecraft, free-flying LEO missions, private space stations, and Lunar Orbital Platform – Gateway, many of which may have less crew time available than the International Space Station (ISS) making its autonomous capabilities an enabling advantage. A future follow-on flight of PLASM is scheduled for ISS to provide supplemental data, specifically allowing us to separate the effects of microgravity from radiation exposure because the ISS studies are carried out under the Van Allen belts. The Deep Space Radiation Genomics project has been designed to perform genome-wide studies on the effect of environmental stresses (such as space radiation, microgravity, etc.) on the physiology of living cells in space and leverage the power of the deletion library of *S. cerevisiae* to identify individual genes—or pathways—affected. In the near term, this information will be crucial for informing downstream studies focusing on the stress response of higher organism and mammalian cells. In parallel, PLASM may be adapted for other single cell studies in space, including high-value microbial populations and mixed metagenomic samples.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: KKV is a co-founder and has an equity interest in LiquiGlide Inc. KKV acknowledges a board position with LiquiGlide Inc.

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