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## **Chemiluminescence-based biosensor for monitoring astronauts' health status during space missions: results from the International Space Station**

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## **Abstract**

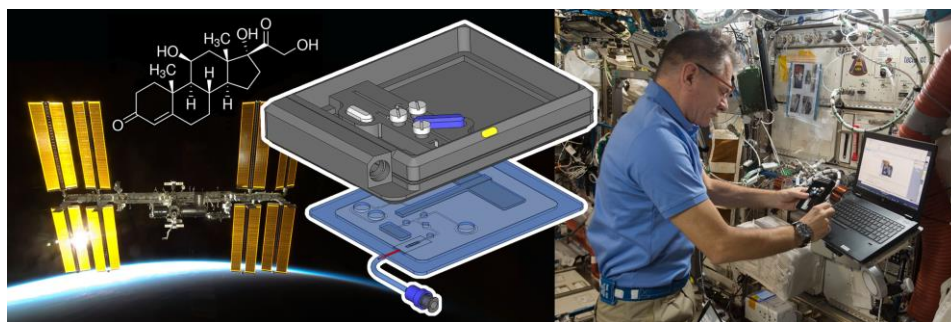
During space missions, real-time monitoring of astronauts' health status is of crucial importance and therefore there is a strong demand for simple analytical devices that astronauts can use to perform clinical chemistry analyses directly onboard.

As part of the "IN SITU Bioanalysis" project, we designed a biosensor for analyzing salivary levels of cortisol in astronauts, a marker of chronic stress. The biosensor is based on the Lateral Flow Immunoassay (LFIA) approach coupled with chemiluminescence (CL) detection and comprised a 3D-printed plastic cartridge containing a sealed fluidic element with the LFIA strip, in which the flow of sample and reagents was activated by pressing buttons on the cartridge and sustained by exploiting capillary forces. For measurement, the photon emission was imaged employing a CL reader based on an ultrasensitive cooled charge-coupled device (CCD) camera.

The payload was designed to operate in microgravity and to withstand mechanical stress, such as take-off vibrations, and onboard depressurization events, while the microfluidics was developed considering alterations of physical phenomena occurring in microgravity, such as bubble formation, surface wettability and liquid evaporation. The biosensor, which was successfully used by the Italian astronaut Paolo Nespoli during the VITA mission (July-December 2017), demonstrated the feasibility of performing sensitive LFIA analysis of salivary cortisol down to 0.4 ng/mL directly onboard the International Space Station. It could be easily adapted for the analysis of other clinical biomarkers, thus enabling the early diagnosis of diseases and the timely activation of appropriate countermeasures.

**Keywords:** Cortisol; Chemiluminescence biosensor; Lateral-flow immunoassay; Space medicine; Point-of-care testing; International Space Station.

## Graphical Abstract



## 1. Introduction

From the first short flights, the duration of space missions has continuously increased and the envisaged future expeditions to Mars will require a journey of several months. As mission destination gets further away, a prompt return to Earth in case of medical emergency will not be possible, so the crew members should be adequately trained and equipped to deal with rapid on-site diagnostics and therapeutic intervention (Cummins et al., 2016; Roda et al., 2018). This aspect is very important considering the risks for astronauts due to exposure to the unnatural space conditions (e.g., microgravity and cosmic radiation), which could for example determine an increased susceptibility to infectious diseases (Mentha et al., 2014; Taylor, 2015).

The International Space Station (ISS) represents a good test bench for the development of technologies suitable for long-term spaceflights. As far as clinical chemical diagnostics is concerned, until now only a few tests have been performed onboard and biological samples are usually send back to Earth for more detailed investigations. Rapid diagnostic tools following the point-of-care testing (POCT) approach are thus required to provide clinically relevant information directly onboard the spacecraft. The design of such devices must consider several factors, such as resistance to mechanical stress (e.g., strong vibrations during launch), peculiar working conditions (e.g., low humidity, microgravity,

high radiation levels) and minimization of space and resources consumption (Nelson, 2011). The stability of reagents exposed for a long time to space conditions is also a relevant issue, which has been addressed by Baquè et al. (2017) who demonstrated a satisfactory stability of free and grafted antibodies in a radiation environment that simulated the conditions of a mission to Mars. In addition, due to the limited knowledge of chemical reactivity and physical phenomena (e.g., liquid behaviour) in microgravity, the development of biosensors for space applications is quite complex and there is a significant risk of unexpected behaviour in space (Roda, 2018).

Until now, only a few technologies have been used onboard the ISS for monitoring astronauts' health status. The commercial i-STAT and Reflotron systems were adapted for space requirements and employed for measuring blood clinical chemistry parameters ([https://humanresearchwiki.jsc.nasa.gov/images/6/62/EMCL\\_RevC\\_2013.pdf](https://humanresearchwiki.jsc.nasa.gov/images/6/62/EMCL_RevC_2013.pdf)). The i-STAT system is based on electrochemical sensors, while the Reflotron employed a dry chemistry approach and reflectance spectroscopy measurement (Markin et al., 1998).

Paper-based chemistry represents an ideal format for space applications as, contrarily to microfluidic devices made of glass, silicon, and polymers (Hu et al., 2014; Martinez et al., 2010; Nilghaz et al., 2012), there is no need to integrate in the analytical device functional units like pumps (Chin et al., 2007; Hawkins and Weigl, 2010). Indeed, in paper-based analytical devices small fluid volumes are manipulated by exploiting the capillarity phenomenon, which is the dominant force driving fluids behaviour in microgravity (Meseguer et al., 2014). Among paper-based techniques, Lateral Flow Immunoassay (LFIA) is one of the most common approaches in POCT immunodiagnosics (Li et al., 2011; Qin et al., 2012; Posthuma-Trumpie et al., 2009). In LFIAs, the immunoassay is performed on a nitrocellulose membrane on which specific immunoreagents are immobilized in defined positions, while sample and other reagents are transported by the flow driven by capillary forces. Common LFIAs employ antibodies labelled with colloidal

gold, which produce along the strip coloured bands that provide a visual “yes/no” response on the analyte presence. In recent years, quantitative LFIA have been also developed by measuring the colour intensity of bands (Anfossi et al., 2010; Molinelli et al., 2009; Salter et al., 2006) or using alternative labels, such as enzymes (Cho et al., 2006), fluorescent nanoparticles (Li et al., 2010; Xia et al., 2009; Zou et al., 2010), electrochemical labels (Fernandez-Sanchez et al., 2004; Muhammad-Tahir and Alocilja, 2003), and horseradish peroxidase (HRP) enzyme labels detected by chemiluminescence (CL) (Mirasoli et al., 2012a).

Chemiluminescence is particularly suited for the development of biosensors due to its inherent sensitivity and simplicity (no specific sample geometry or excitation source are required) and because the emitted light can be imaged using unexpensive portable charge-coupled-device (CCD) cameras (Mirasoli et al., 2012b; Zangheri et al., 2015a; Zangheri et al., 2015b; Zangheri et al., 2016). Nevertheless, CL detection has never been used in space, and its chemistry should be proved to properly work in microgravity.

In this work we report the development and testing on board the ISS of a technological demonstrator of a CL-LFIA biosensor for the measurement of cortisol in oral fluid, which could be used directly by astronauts during long-term space missions. Cortisol was selected as a model analyte, taking also advantage of the possibility of its measurement in oral fluid, thus enabling non-invasive sampling. However, salivary cortisol levels are quite low (i.e., in the nanomolar range) and require highly sensitive analytical methods, such as those based on CL.

This project, called “IN SITU Bioanalysis”, was financed by the Italian Space Agency (ASI) and performed in collaboration with NASA. Flight experiments have been carried out by the Italian astronaut Paolo Nespoli during his VITA mission (July-December 2017) ([https://www.nasa.gov/mission\\_pages/station/research/experiments/1277.html](https://www.nasa.gov/mission_pages/station/research/experiments/1277.html)).

The biosensor was designed to fulfil the standard requirements of safety and ISS onboard operability requested by NASA. It comprised a disposable 3D-printed plastic cartridge, containing a fluidic element with the LFIA strip and manually activated reagent reservoirs and valves, and a CL reader based on an ultrasensitive cooled CCD camera in a “contact imaging” configuration (Mirasoli et al., 2013; Mirasoli et al., 2012b). The analysis required a simple manual procedure, as the flow of sample and reagents was started by pressing buttons on the cartridge and then sustained by capillary forces. Results could be read directly by the astronaut or, in a telemedicine approach, data were sent to ground personnel for processing and evaluation by medical experts.

## **2. Materials and methods**

### *2.1 Reagents and materials*

Cortisol, polyclonal anti-HRP antibody produced in rabbit, bovine serum albumin (BSA), and Tween-20 were purchased from Sigma Aldrich (St. Louis, MO). Western Lightning Ultra CL substrate for HRP was purchased from Perkin Elmer (Waltham, MA). Polyclonal anti-cortisol antibody produced in rabbit was purchased from Analytical Antibodies (Bologna, Italy). HRP-conjugate cortisol was generously provided by Diametra (Milano, Italy). Phosphate buffered saline (PBS) was prepared as follows: 10 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 2 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 137 mmol L<sup>-1</sup> NaCl, 2.7 mmol L<sup>-1</sup> KCl, pH 7.4. The other reagents were of analytical grade and were employed as received.

Materials for the LFIA strip: glass fiber pad, cellulose pad, nitrocellulose membrane (Hi-flow plus 180 membrane cards), and cellulose adsorbent pad were from Merck Millipore (Billerica, MA); saliva sample pad I (Whatman LF1) and saliva sample pad II (Whatman Standard 14) were provided by GE Healthcare Lifescience (UK).

## *2.2 Preparation of the LFIA strips*

Assay strips for LFIA were prepared following a previously published procedure (Zangheri et al., 2015a). In particular, the rabbit anti-cortisol antibody (T-line) and the rabbit anti-HRP antibody (C-line), at 1:5000 (v/v) and 1:3000 (v/v) dilutions, respectively, were immobilized on nitrocellulose membranes keeping a distance of 4 mm between the two lines. The nitrocellulose membrane was saturated with 1% (w/v) BSA and washed three times with PBS containing 0.05% Tween 20. The membranes were then assembled with sample and adsorbent pads and cut into 5 mm-width sections.

## *2.3 Buffers for cortisol-HRP conjugate working solution*

The composition of conservation buffers tested to achieve long-term stability of cortisol-HRP conjugate working solution is reported in Supplementary material.

## *2.4 LFIA assay format*

The CL-LFIA assay employed a direct competitive format (Figure 1). Sample and HRP-cortisol conjugate loaded in the sample pad migrated along the nitrocellulose membrane by capillarity. Cortisol in the sample competed with HRP-cortisol conjugate for binding to a limited amount of anti-cortisol antibody immobilized on the T-line. The excess of HRP-cortisol conjugate was then captured by anti-HRP antibody immobilized on the C-line. Finally, a CL substrate (luminol/enhancer/oxidant) for HRP was added to the strip and the CL signal was imaged using the CCD camera. According to the competitive assay format, the intensity of the CL signal of the T-line was inversely related to the amount of cortisol in the sample.



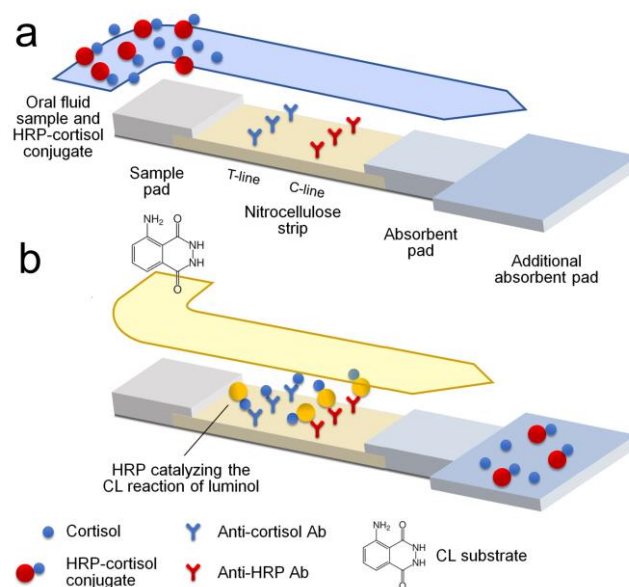


Figure 1.

## 2.5 “IN SITU Bioanalysis” biosensor payload

The “IN SITU Bioanalysis” biosensor payload consisted of three components, namely (a) the disposable LFIA cartridge, (b) the disposable oral fluid sampling equipment (OFSE) and (c) the CL reader and its accessories.

### 2.5.1 LFIA cartridge

The disposable LFIA cartridge consisted of a LFIA fluidic element enclosed in a holder that protects this element and provides the actuators (e.g., buttons and valves) that enable the Astronaut to perform the analysis by a simple manual procedure.

The LFIA fluidic element (Figures 2a and 2d, size 120 x 95 x 3 mm) contains the LFIA nitrocellulose membrane and the reagents necessary for the analysis, as well as the fluidic system for metering the correct amount of oral fluid sample and transferring sample and reagents on the LFIA strip. It is composed by a laser micromachined adhesive polypropylene layer (ca. 190 μm thick) with an engraved fluidic channels network, which is sandwiched between two transparent polypropylene layers (200 μm thick). To facilitate

sealing of the LFIA fluidic element and to increase mechanical strength, a polyester/acrylic adhesive layer was also sandwiched between the polypropylene layers.

The upper polypropylene layer contains a series of embossed elements obtained by vacuum thermoforming, namely:

- a) a 35  $\mu\text{L}$ -volume sample metering chamber;
- b) a sample waste chamber which receives the excess sample after it has filled the sample metering chamber (the chamber contains a stack of absorbing paper sheets – a white sheet over a red one – that provides a visual confirmation of filling the sample metering chamber through a white-to-red colour change);
- c) three pressure-actuated pouches (P1 - P3) that contain the reagents, namely HRP-cortisol conjugate solution (50  $\mu\text{L}$  diluted 1:500 (v/v) in buffer D) in P1 and components A (45  $\mu\text{L}$ ) and B (45  $\mu\text{L}$ ) of the HRP CL substrate in P2 and P3, respectively;
- d) three pressure-actuated elastomeric valves (V1 - V3) that control flows of sample and HRP-cortisol conjugate solution (each valve is a cylindrical cavity filled by polydimethylsiloxane (PDMS, obtained from Dow Corning, Midland, MI));
- e) cavities for the LFIA strip and the additional absorbent pad (because of the high volume of solution loaded in the LFIA strip and the absence of solvent evaporation in the sealed fluidic element, an additional absorbent pad was used to increase absorbing capability of the LFIA strip, thus promoting the complete flow of the added solutions).

The LFIA fluidic element was assembled after inserting all components and filling pouches P1 – P3 with reagents, and then it was thermally sealed along its perimeter to avoid leakage of reagents. A rigid polyetheretherketone (PEEK) tube ( $\varnothing = 0.8$  mm) and a polypropylene support element were glued to the sample injection channel with epoxy glue (Bostik SaldaRapido mixer, UHU Bostik SpA, Milan, Italy) to obtain the sample injection port. Finally, the unidirectional valve for sample loading (Cole-Parmer Instrument

Company, LLC., Vernon Hills, IL) was connected to the sample injection port by a flexible silicone tubing ( $\varnothing = 1.2$  mm).

The LFIA fluidic element holder (Figures 2b and 2e, size 115 x 150 x 25 mm) encapsulates the LFIA fluidic element and acts as the interface between this element and the oral fluid sampling equipment (OFSE), the CL reader and the Astronaut. It was designed using a freely available CAD program (SketchUp Make 2016, Trimble Inc., Sunnyvale, CA) and produced in black acrylonitrile-butadiene-styrene (ABS) copolymer by Fused Deposition Modelling (FDM) 3D printing technology using a Makerbot Replicator 2X printer (Makerbot Industries, New York, NY).

The holder consists of two separate parts. The lower part has a hole that fits the dimension of the LFIA fluidic element and a housing for the unidirectional valve. It also has an aperture for accommodating the fiber optic faceplate of the CL reader, which will be in direct contact with the LFIA fluidic element during the measurement. The upper part contains three nylon screws for the manual actuation of the elastomeric valves and two ABS buttons for delivering sample and reagents to the LFIA strip (the blue button B1 acts on the sample metering chamber and the reagent pouch P1, while the white one B2 operates on the reagent pouches P2 and P3).

The LFIA cartridge was assembled by inserting the LFIA fluidic element and the unidirectional valve in the lower part of the holder, then the two parts were glued together using epoxy glue (LOCTITE EA 9394 AERO, Henkel AG & Co. KGaA, Dusseldorf, Germany).

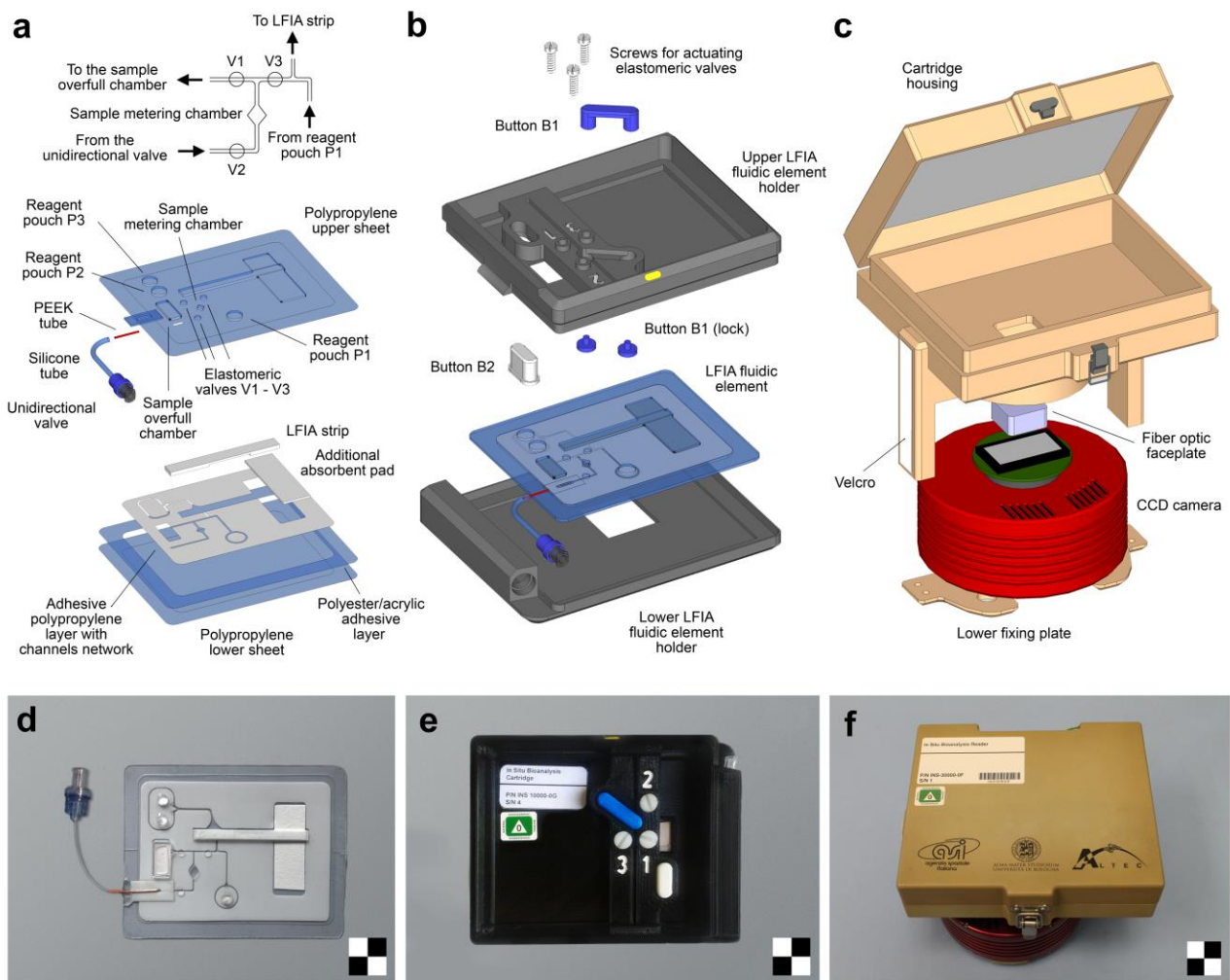


Figure 2.

### 2.5.2 Oral fluid sampling

The OFSE (Figure 5a) comprises a Salivette (Sarstedt AG & Co, Nümbrecht, Germany) with a cotton swab for the non-invasive collection of the oral fluid sample and a needleless polypropylene syringe (Artsana SpA, Como, Italy), which is employed for extracting the oral fluid sample from the cotton swab and for injecting the sample in the LFIA cartridge. The OFSE also includes a small plastic tube with a short cotton swab at its bottom, used in the removal of air bubbles from the sample prior to injection in the LFIA cartridge.

### 2.5.3 CL reader

The CL reader (Figures 2c and 2f, size 175 x 150 x 125 mm) was developed using a commercially available CCD camera (ATIK 11000, ATIK Cameras, New Road, Norwich) equipped with a large format, high resolution monochrome CCD sensor (Kodak KAI 11002, sensor size 37.25 x 25.70 mm) cooled by a two-stage Peltier element to reduce thermal noise.

The CCD camera was modified by replacing its upper part (i.e., the CCD sensor compartment and the optical glass window) with an aluminium cartridge housing assembly, which ensures the correct alignment of the CCD sensor with the LFIA strip during the measurement and avoids interference from ambient light.

The cartridge housing assembly is composed by the cartridge housing and a lower fixing plate. The cartridge housing, which inner cavity fits the dimensions of the LFIA cartridge, includes a polymethylmethacrylate (PMMA) fiber optic faceplate (size 26 x 26 x 13 mm, Edmund Optics, Barrington, NJ) that conveys the CL signal from the LFIA strip to the CCD sensor, also acting as thermal insulator between the cooled CCD sensor and the LFIA cartridge. The cartridge housing contains a desiccant element which prevents condensation of moisture on the cooled CCD sensor and a layer of padding foam material (ZOTEK F30, Zotefoams plc, Croydon, United Kingdom) inside the lid, which exerted a little pressure on the LFIA cartridge ensuring the close contact between the LFIA cartridge and the fiber optic faceplate even in microgravity. The lower fixing plate mounted on the bottom of the CCD camera and anchored to the lateral “wings” of the cartridge improved CL reader’s robustness.

The CL reader is connected to a laptop via an USB interface. A proprietary software (Artemis Capture, v. 4.5.0.0) provided by the CCD camera producer allowed to control camera settings and acquire CL signals. An AC/DC power adapter for the CL reader and a set of power cables were also included in the “IN SITU Bioanalysis” payload.

## 2.6 Assay procedure

Oral fluid sampling was performed in the morning between 2-4 hours after waking up, and refraining from brushing or flossing teeth, eating, and drinking for at least 60 minutes before sample collection. The Salivette cellulose swab was held in mouth for a few minutes and then placed into the syringe body, which was connected to the plastic tube included in the OFSE. The cellulose swab was gently squeezed until the first bubble-free drops of oral fluid drained from the syringe tip into the plastic tube. The syringe was then connected to the unidirectional valve of the LFIA cartridge.

The analysis was performed on the LFIA cartridge as follows (see top of Figure 2a for details on the fluidics around the sample metering chamber).

- After opening valves V1 and V2 and closing valve V3 by screwing/unscrewing the respective screws, the oral fluid sample was loaded by gently pressing the syringe plunger to fill the sample metering chamber. The injection was stopped when the excess sample reached the sample waste chamber, which was indicated by the colour change inside the chamber.
- Valve V3 was opened, valves V1 and V2 were closed and button B1 was pushed to deliver sample and HRP-cortisol conjugate to the LFIA strip.
- After 1-h incubation time, button B2 was also pushed to deliver to the LFIA strip the HRP CL substrate, then the cartridge was placed into the CL reader.
- After additional 15 min, a series of CL signal acquisitions were performed using pre-programmed signal integration times ranging from 2 to 30 s. The acquired images were stored in the ISS laptop, then transmitted to ground for analysis.

To obtain quantitative information, a freely available image analysis software (ImageJ v. 1.46, National Institutes of Health, Bethesda, MD) was employed for CL signal elaboration. The mean photon emission of the C-line and T-line of the LFIA strip was measured and

corrected for the mean background signal measured in adjacent areas (Figure 3a). The T-line/C-line CL signals ratio was calculated to improve assay reproducibility with respect to environmental and matrix factors that might affect the intensity of the CL signal (e.g., changes in ambient temperature or presence of HRP inhibitors in the sample). Finally, cortisol concentration was calculated by interpolating the T-line/C-line ratio on a calibration curve generated on ground by analysing spiked cortisol-free oral fluid samples using CL cartridges of the same production lot. A four-parameter logistic equation was used to fit the experimental data and obtain calibration curve parameters.

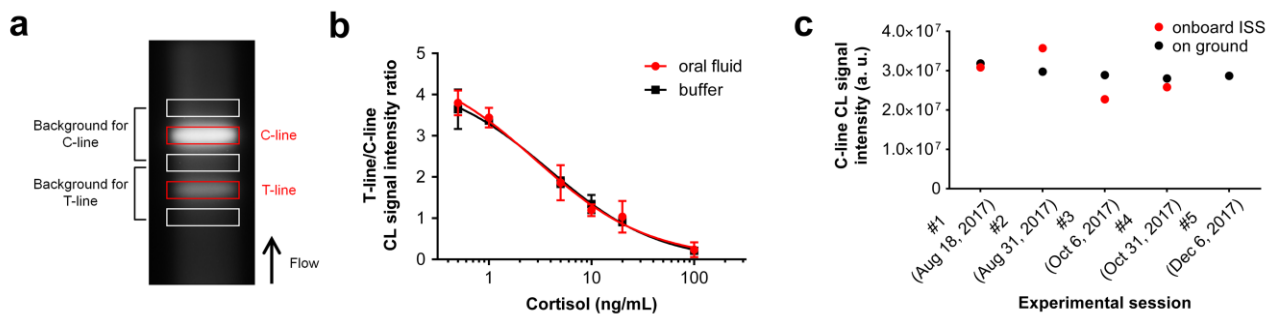


Figure 3

### 3. Results and discussion

In addition to achieving adequate analytical performance, the development of a biosensor for monitoring the health status of the crewmembers during a space mission requires consideration of several crucial technical aspects, such as design of robust instrumentation suitable for microgravity operation, implementation of procedures enabling crewmembers to easily perform the analysis and fulfilment of NASA safety standards aimed at ensuring safety of crewmembers, as well as compatibility with ISS onboard equipment.

For biosensor development, we selected LFIA as it is a simple and well-established format for POCT, which operation is not expected to be negatively affected by microgravity. Materials and channels size and geometry of the LFIA fluidic element were selected to

avoid the most common problems encountered in fluids handling in space, such as bubbles formation, solutions mixing and surface wetting impairment. As concerns the HRP-catalyzed CL reaction exploited for sensitive detection, one important concern might be the potential effect of microgravity on the enzymatic reaction. Indeed, controversial results have been reported about changes of enzyme catalysis kinetics in microgravity due to alterations of the enzyme active site arrangement (Maccarrone et al., 2001, Ranaldi et al., 2003). Moreover, the role played by HRP in the CL reaction involves a complex catalytic cycle (Campomanes et al., 2015) that also depends on the presence of enhancers and co-enhancers in the CL substrate (Marzocchi et al., 2008). Since a reliable simulation of the HRP CL reaction in microgravity cannot be performed on ground, we could not rule out the possibility of an unexpected behavior affecting the CL readout.

### *3.1 Optimization of the immunoassay*

The LFIA procedures and materials were optimized for the quantification of cortisol in oral fluid in the relevant physiological range (i.e., 0.6 - 10 ng/mL) and for easy implementation in the LFIA cartridge (e.g., by reducing the analytical steps).

#### *3.1.1 Anti-cortisol antibody*

Four different anti-cortisol antibodies were immobilized at different concentrations on LFIA nitrocellulose membranes, then tested by analysing cortisol-free oral fluid samples and cortisol-spiked (1 and 10 ng mL<sup>-1</sup>) oral fluid samples. Results obtained with the antibody providing the best performance (highest signal in the absence of the cortisol and clear discrimination between the different cortisol concentrations) are shown in Supplementary material. Basing on such findings, the 1:5000 (v/v) dilution of such antibody was employed for subsequent experiments. The affinity constant of the used antibody is in the order of 10<sup>12</sup> M<sup>-1</sup>.



### *3.1.2 Saturation agent*

Treatment of the nitrocellulose strip with a suitable saturation agent after antibodies deposition is fundamental to reduce nonspecific adsorption of the immunoreagents, thus improving the signal/background ratio (Anfossi et al, 2010). Different saturation agents (i.e., BSA, animal gelatin, non-fat dry milk and soy milk) were tested in the analysis of cortisol-free oral fluid samples and the best results were obtained by incubating the nitrocellulose strip for 5 min at room temperature with a 1% (w/v) BSA solution in PBS buffer.

### *3.1.3 Optimization of HRP-cortisol conjugate concentration*

The optimal HRP-cortisol conjugate (labeled analyte) concentration was assessed by comparing the dynamic ranges of calibration curves generated by analysing cortisol-free oral fluid samples spiked with known amounts of cortisol using 1:250, 1:500 and 1:1000 (v/v) HRP-cortisol conjugate dilutions in buffer D. The obtained dynamic ranges were 1.5 - 50 ng/mL for dilution 1:250 (v/v), 0.2 - 50 ng/mL for dilution 1:500 (v/v) and 0.5 - 33 ng/mL for dilution 1:1000 (v/v). Therefore, the 1:500 dilution was chosen as that providing the best dynamic range LOD and sensitivity (slope) for the detection of cortisol in the physiological concentration range.

### *3.1.4 LFIA strip materials*

Concerning LFIA strip materials, particular attention was devoted to the sample pad choice, since, rather than being a passive element, it can play an important role in improving the performance LFIA assays of complex matrices by performing a sort of sample pre-treatment (e.g., by filtering out particulate matter or disrupting matrix components such as salivary mucins). We compared different commercial sample pads by

analysing cortisol-free oral fluid samples. As shown in Supplementary material, the highest signal/background ratio was obtained using the saliva pad II, which was thus selected for assay development. Its better performance was probably due to the ability to block substances present in the oral fluid, whose absorption on the nitrocellulose membrane altered reagents migration along the nitrocellulose strip and caused a diffused non-specific signal. To further improve sample migration and minimize sample-to-sample variation (which is expected important for oral fluid) the sample pad was presaturated with a 3% (w/v) BSA solution containing 0.1% (v/v) Tween 20.

### *3.1.5 Shelf-life of reagents: stability of HRP-cortisol conjugate*

Considering that the expected duration of the VITA mission was about 6 months, the LFIA cartridges should be stable at least this period when stored at 4°C. While antibodies immobilized on the nitrocellulose strip and commercial HRP CL substrates are known to be stable for long periods, the composition of diluted HRP-cortisol conjugate working solutions needed to be optimized to ensure long-term conservation. Different conservation buffers (A – E, see Supplementary material) were evaluated using an accelerated stability test. Cortisol-HRP conjugate solutions at three concentration levels, namely 1:250, 1:500 and 1:1000 (v/v) dilutions, were stored at 37°C for up to 4 weeks. In such conditions, each week of conservation roughly corresponded to 1 year of storage at 4°C (Deshpande, 1996). The solutions were periodically assayed to assess reduction of the antibody binding ability and/or HRP label activity by analysing a cortisol-free oral fluid sample and comparing the results with those obtained using a freshly prepared cortisol-HRP conjugate solution in the same buffer.

As shown in Supplementary material, only buffers D and E provided a satisfactory stability of cortisol-HRP conjugate (up to 3 weeks at 37°C) at all concentration levels. Buffer D was selected since it gave more reproducible results over the storage period.

### *3.1.6 Washing*

Since oral fluid contains substances that interfere with the CL reaction, a washing step is usually required in CL-LFIA assays prior to CL measurement (Zangheri et al., 2015a). However, to eliminate this step we evaluated the possibility to increase the amount of CL substrate loaded onto the LFIA strip and exploit its flow also for washing. Indeed, as shown in Supplementary materials, comparison of the results obtained by analysing cortisol-free oral fluid samples using analytical protocols either including or not a washing step showed no significant differences between the CL signals. Therefore, no washing step was implemented in the assay, thus simplifying the layout of the LFIA cartridge and reducing the steps of the analytical protocol.

### *3.1.7 Method performance*

The analytical performance of the method was evaluated by producing calibration curves through the analysis of spiked cortisol-free oral fluid samples using LFIA cartridges. Comparison with calibration curves obtained in PBS indicated a negligible matrix effect (Figure 3b). The limit of detection (LOD) of the assay, defined as the cortisol concentration corresponding to the T-line/C-line CL signal ratio of the blank minus three times its standard deviation, was 0.2 ng/mL. Assay reproducibility was evaluated on three repeated calibration curves by calculating the relative coefficient of variation (CV%) associated to the T-line/C-line CL signal ratio for each concentration. The assay demonstrated a good reproducibility, with CV% values below 15% for each point. Indeed, Assay accuracy was also evaluated by analysing 10 saliva samples and comparing the results with those obtained using a commercial ELISA kit for salivary cortisol (Cortisol saliva ELISA kit, obtained from Diametra). The results (see Supplementary materials) showed a good

correlation between the reference immunoassay method, with recovery values comprised between 84% and 112%.

In addition, LFIA cartridges were stored at 4°C and periodically tested for 6 months by generating calibration curves. As reported in Table 1, the parameters of the calibration curve did not significantly change over time, thus demonstrating no loss of analytical performance. Calibration curves were also independent from the orientation of the LFIA cartridge with respect to the Earth's gravity, which supported the suitability of the device for operation in a microgravity environment.

### **3.2 Flight payload design**

#### *3.2.1 Design of the LFIA cartridge*

The LFIA cartridge, which was designed following an approach similar to that used in Sciutto et al 2018, is intended to deliver appropriate volumes of sample and reagents to the LFIA strip and to enable the Astronaut to perform the assay by a sequence of simple manual operations. Reagents were preloaded into the cartridge, so that volume of each reagent pouch was adjusted to provide the correct amount, taking also into account the dead volume of the flow channels. Instead, to control the amount of oral fluid sample, the LFIA fluidic element inside the LFIA cartridge comprises a 35- $\mu$ L sample metering chamber connected to the unidirectional valve (excess sample loaded in the cartridge was discharged in the sample overflow chamber). The flow of the oral fluid sample is controlled by the elastomeric valves, which can be closed (or opened) by hand screwing (or unscrewing) the corresponding nylon screw. No valves are required to regulate reagents flow because the adhesive polypropylene layer reagent seals the reagent pouches and, once the correspondent button is pressed, the reagent can flow in only one direction. Delivery of sample and reagents was obtained by pressing the cartridge buttons, thus

squeezing reagent pouches or sample metering chamber and transferring the fluids on the LFIA strip (oral fluid and reagents have to be delivered in pairs, thus each button acted on two fluid reservoirs).

### *3.2.2 Safety tests*

For flight payload qualification, Safety Data Package and Declared Material List documents were prepared and a three-level NASA Safety Review was successfully passed. Compatibility with the ISS environment was ensured at all stages to face PIRN (Preliminary Interface Revision Notice) evolution on NASA side and to negotiate with ASI/NASA the most simple, effective and convenient payload configuration.

The LFIA cartridge required a preliminary evaluation by NASA toxicologists. Due to the relatively low toxicity and the small volume (less than 150  $\mu\text{L}$ ) of chemical reagents, the LFIA cartridge was rated as a “marginal hazard” and a single containment level was requested. This requirement was fulfilled by thermal sealing of the LFIA fluidic element and use of an unidirectional valve for sample loading. The LFIA cartridge was also tested for resistance to temperature changes and ability to withstand sudden depressurization events. The latter test was performed in an ultra-high vacuum chamber, in which a series of LFIA fluidic elements were exposed to vacuum (down to  $10^{-4}$  bar) for 30 minutes. The elements were then inspected to confirm the absence of any leakage of reagents.

The CL reader was subjected to a variety of test, e.g., for radiated emissions, radiated susceptibility, generation of magnetic fields and acoustic emissions (the tests were performed at the facilities of G.S.D. S.r.l., Pisa, Italy). The fulfillment of other requirements was verified by numerical simulations. For example, Figure 4a shows the stress on the CL reader aluminium alloy (Anticorodal 6082) structure during launch, which has been used to estimate its maximum deformation, while the results of thermal analysis, indicating the

temperature of the external surfaces of the CL reader during operation, are reported in Figure 4b.

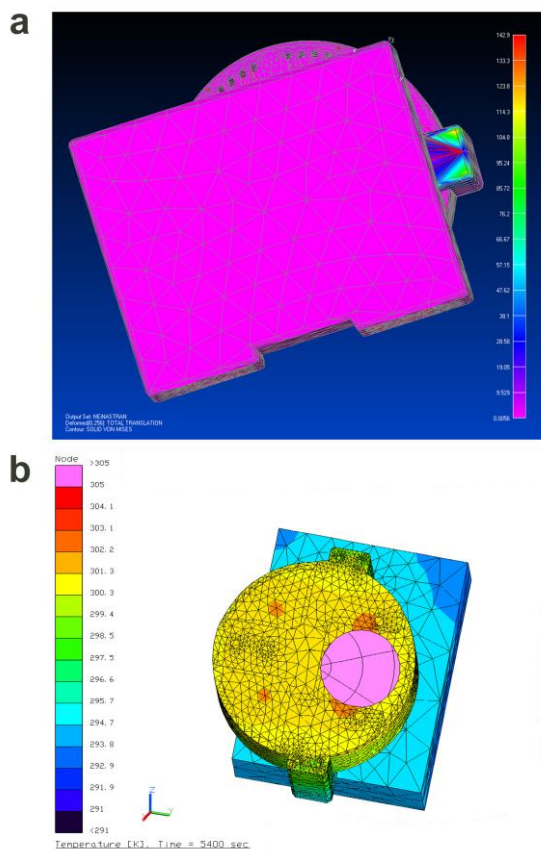


Figure 4

### 3.3 Onboard experiments

#### 3.3.1 CL-LFIA results

The “IN SITU Bioanalysis” hardware (OFSE, CL reader and accessories, Figure 5b) was delivered to the ISS with the SpaceX CRS-11 flight (June 3, 2017), while LFIA cartridges were shipped to NASA Kennedy Space Centre in Cape Canaveral, Florida at controlled temperature (4°C) and loaded onboard the SpaceX-12 Dragon capsule two days before launch (August 14, 2017). After docking to the ISS, the LFIA cartridges were cold stowed onboard until use.

The Italian astronaut Paolo Nespole performed five experimental sessions in the period July – December 2017. In addition, oral fluid samples were collected before and after the space mission and analyzed in our laboratory as reference samples from the same

subject. All the flight experiments gave information about the oral fluid cortisol concentration except the last one, in which no CL signal was observed. This has been tentatively attributed to a detachment of the adhesive polypropylene layer inside the LFIA fluidic element or to a defective cartridge (indeed, LFIA cartridges cannot be subjected to a complete functional test after production). For all the experimental sessions, the parallel tests carried out on ground using a twinned LFIA cartridge stored at 4°C in our laboratory were successful. As the results are concerned, in all cases the oral fluid cortisol levels measured in flight were comparable to those obtained in pre- and post-flight analyses.



Figure 5

### 3.3.2 Chemiluminescent detection

As stated above, the behavior in space of the HRP catalyzed CL reaction based on the luminol/oxidant system employing p-coumaric acid as enhancer was investigated. CL images acquired during ISS experiments (see for example Figure 3a) were superimposable to those obtained in ground experiments, considering both spatial distribution and intensity of the CL signal. We investigated in more detail the possible effect of microgravity on the HRP-catalyzed CL reaction by comparing the intensity of the C-line measured onboard the ISS with that obtained in parallel on ground experiments (Figure 3c). The C-line average intensity for the on ground experiments was  $(32 \pm 3) \times 10^6$  RLU (CV% = 9.5%), while that of the experiments performed onboard the ISS was  $(29 \pm 6) \times 10^6$  RLU (CV% = 20%). Although the intensity of the CL signals measured in the ISS

experiments showed a higher variability, no systematic difference in the intensity compared to on ground values was evident.

## **Conclusions**

The developed device demonstrated the feasibility of performing sensitive immunological clinical chemistry analyses directly onboard the ISS and, for the first time, suitability of enzyme-catalyzed CL reactions for ultrasensitive detection in microgravity. Indeed, the HRP CL reaction was not affected by weightless conditions and no significant adverse events (e.g., bubble formation, alterations in solution mixing and surface wetting) were observed in device operation. The cortisol concentrations measured onboard the ISS were consistent with the expected cortisol levels in oral fluid.

The demonstration of the suitability of quantitative CL-LFIA for space applications paves the way for future developments in biosensing in space. Being CL a simple but extremely sensitive detection principle, multiplex CL-LFIA assays could be developed for detecting panels of biomarkers at nanomolar range, thus enabling an early disease diagnosis (e.g., cardiac failure or bone metabolism impairment) or a general health status check. Nevertheless, the experiments showed that further device improvement is possible. For example, to reduce onboard storage requirements, a reusable cartridge with disposable LFIA fluidic elements would be convenient, since only the LFIA fluidic elements need cold storage. Moreover, cartridge miniaturization and/or assay procedure automation could be envisaged exploiting lab-on-a-chip technologies, while CMOS smartphone cameras might be used as an alternative to CCD cameras.

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## **Figure captions**

Figure 1. Principle of the competitive CL LFIA assay for cortisol: (a) loading of oral fluid sample and HRP-cortisol conjugate and (b) loading of the HRP CL substrate.

Figure 2. Schematic drawings and images of (a,d) the LFIA fluidic element, (b,e) the LFIA cartridge and (c,f) the CL reader. A detailed view of the fluidics of the LFIA fluidic element around the sample metering chamber is shown on top of panel (a). Scale checkerboards are 2x2 cm.

Figure 3. (a) CL image of the LFIA strip showing the areas used for the evaluation of the CL signals of T-line and C-line and of the background signal; (b) comparison of calibration curves obtained in the optimized experimental conditions by analyzing cortisol standards in oral fluid and PBS; (c) comparison of the C-line CL signal intensities measured in the experiments performed onboard the ISS and in the parallel tests carried out on ground using twinned LFIA cartridges.

Figure 4. Results of numerical simulations performed on the CL reader model to evaluate (a) tolerance to launch loads and (b) temperatures of the external surfaces during operation.

Figure 5. (a) The oral fluid sample equipment and (b) the “IN SITU Bioanalysis” hardware at the NASA Kennedy Space Centre (Cape Canaveral, FL), ready for loading onboard the SpaceX-11 Dragon capsule; (c) the Italian astronaut Paolo Nespoli performing the experiment onboard the ISS (images courtesy of NASA).