Application of pulsed-excitation fluorescence imager for daylight detection of sparse life in tests in the Atacama Desert

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[1] A daylight fluorescence imager was deployed on an autonomous rover, Zoë, to detect life on the surface and shallow subsurface in regions of the Atacama Desert in Chile during field tests between 2003 and 2005. In situ fluorescent measurements were acquired from naturally fluorescing biomolecules such as chlorophyll and from specific fluorescent probes sprayed on the samples, targeting each of the four biological macromolecule classes: DNA, protein, lipid, and carbohydrate. RGB context images were also acquired. Preparatory reagents were applied to enhance the dye probe penetration and fluorescence intensity of chlorophyll. Fluorescence imager data sets from 257 samples were returned to the Life in the Atacama science team. A variety of visible life forms, such as lichens, were detected, and several of the dye probes produced signals from nonphotosynthetic microorganisms.

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1. Introduction

[2] The search for extraterrestrial life has been guided by the physical characteristics and chemical processes that the scientific community has found associated with terrestrial life. The United States National Aeronautics and Space Administration (NASA) states, for instance, that they focus "on the physics and chemistry of life, using a variety of imaging methods over many spatial scales, and a variety of chemical methods that measure many different chemical parameters at many spatial scales (http://mars.jpl.nasa.gov/technology/lifedetectors/, 18 December 2006)". Some approaches look for chemical signatures (amino acids, organic polymers, metabolic activity, and chemical transformation) by use of mass spectroscopy, optical spectroscopy and other detection methods. Others look for specific biomolecules using antibodies, nucleic acid hybridization probes and enzymatic recognition of the presence of DNA [*Skelley et al.*, 2005; Mars Astrobiology Science and Technology Workshop, 8–10 September 2004, Carnegie Institute of Washington; UREY Project, http://astrobiology.berkeley.edu/projects.htm#UREY]. These methods give high sensitivity for detecting biosignatures in specific small samples. They have limited ability to provide this same sensitivity and specificity for mapping samples collected over extended geographical areas.

[3] Fluorescence detection is a sensitive analytical method that has been used to detect microorganisms in the laboratory and field. Numerous investigators have used the endogenous (intrinsic) fluorescence of chlorophyll, phycobiliprotein, NADH, flavin, tryptophan and other fluorophores for detection [*Shapiro*, 2003]. Also, exogenously (extrinsic) added fluorescent probes, such as DAPI for DNA, are valuable for visualizing microorganisms [*Shapiro*, 2003]. Many other fluorescent probes are available to detect DNA, proteins, lipids, membrane potential changes, and specific targets with DNA probes and fluorescent antibodies. The Molecular Probes (Invitrogen) Handbook (Invitrogen.com) describes many of these probes and their targets.

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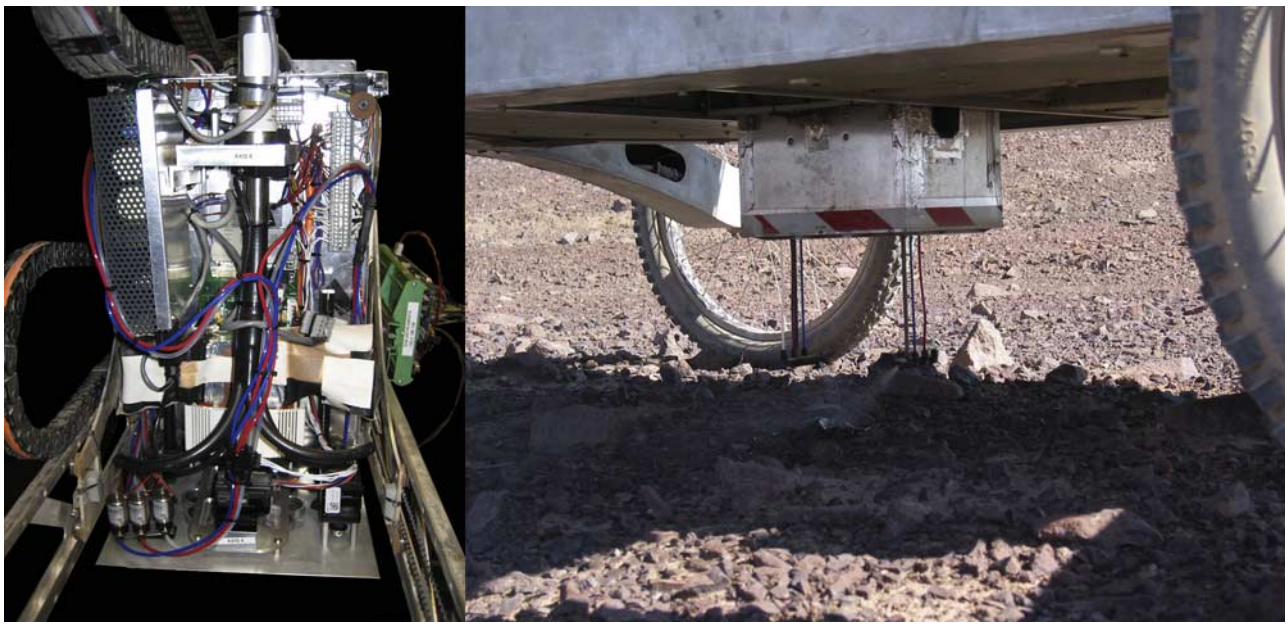

Figure 1. Views of the fluorescence imager: (left) with the cover removed and (right) in the desert applying reagent to a sample. The instrument housing for this proof of concept model was 21 cm × 24 cm × 45 cm.

[4] In 2002 we proposed that microorganisms could be detected in extreme environments with a robot-transported fluorescence imager using multiparameter fluorescence detection of endogenous fluorescence signals together with fluorescence from a panel of exogenously delivered fluorescent probes. Chlorophyll was selected as an endogenous reporter along with extrinsic probes for the general classes of biological macromolecules: DNA, protein, lipid and polysaccharides. We reasoned that the co-localized detection of fluorescence signals from the panel probes would be a strong indication of the presence of life. In some cases it would also be possible to co-localize chlorophyll and visible reflected life images with the extrinsic probe signals. We teamed with robotic engineers and NASA scientists in a 3-year development and testing program to explore this hypothesis.

[5] The detection instrument, called the fluorescence imager (FI), was built and tested in the context of the NASA Astrobiology Science and Technology for Exploring Planets (ASTEP) project, entitled ''Fluorescence Detection of Sparse Life.'' We collaborated with the Carnegie Mellon University (CMU) Robotics Institute in an ASTEP-funded rover mission Life in the Atacama (LITA) to the Atacama Desert in Chile, where the FI was used to study samples on the ground in daylight while the rover traveled across the desert. The Atacama Desert, one of the most arid landscapes on Earth, serves as an analog and test bed for many researchers in the search and detection of life on other planets [see *Cabrol et al.*, 2007; see also, e.g., *McKay et al.*, 2003; D. S. Wettergreen et al., unpublished manuscript, 2007]. The testing scenario had a remote science team, composed of geologists, biologists, instrument and rover specialists, and technology support personnel, sending daily commands to an autonomous rover from Pittsburgh, PA, while engineering and ground truth teams in the desert monitored the instrument and collected the samples for microbiological analysis. In this paper we briefly describe the fluorescent probes and the FI. We then cover the testing of these tools for identifying microorganisms remotely through long-range roving during the 2004 and 2005 LITA field experiments.

2. Materials and Methods

2.1. Fluorescence Imager (FI)

[6] Pulsed light generated from a PerkinElmer FX4400 xenon flashlamp passes through a custom excitation filter wheel and is then coupled into a custom Sunoptic Technologies fiber bundle with four outputs, illuminating the 10 cm × 10 cm field of view (FOV). A modified 12-bit Roper CoolSNAP cooled CCD grayscale camera with a custom emission filter wheel mounted in front of the lens acquires the image (Figure 1). The excitation and emission filters were chosen to match the fluorescent probe specifications. In addition, standard RGB filters (ChromaTechnologies, Brattleboro, VT) were used to digitally construct color images of the FOV using the full spectrum of the flashlamp. The color images were stored in 24-bit PNG format during the 2003 field season and in JPG format during the 2004–2005 field seasons. The fluorescence images were stored as 16-bit TIFF images. The transverse resolution of the FI was measured to be 223 μm. The camera lens is fixed focus. To focus, the rover deploys the FI into optimal focus using an image-processing algorithm. Laboratory tests revealed the system to be better than 100-fold improvement in signal-to-noise ratio relative to use of a conventional light source [*Lanni et al.*, 2007]. The excitation and emission bands that interrogated the probes and chlorophyll are given in Table 1. The DNA and protein probes were deployed in 2004, and the full suite of probes was used in 2005.

Table 1. Fluorescence Excitation and Emission Detection Windows for the Four Probes and Chlorophyll

Probe	Excitation	Emission
Calcofluor (Carbohydrate)	UV (380/30 nm)	Blue (460/50 nm)
SYTO BC (DNA)	Blue (450/50 nm)	Green (510/50 nm)
SYPRO Red (Protein)	Yellow-Green (545/30 nm)	Red-Orange (620/60 nm)
SRE26 (Lipid)	Red (600/80 nm)	Far Red (740/80 nm)
Chlorophyll	Blue (450/50 nm)	Far Red (740/140 nm)
Chlorophyll Check	Yellow-Green (545/30 nm)	Far Red (740/140 nm)

2.2. Image Acquisition Protocol in the Desert

[7] Table 2 presents the final FI protocol of the 2005 field season. To help eliminate the possibility of unusual mineral fluorescence or lighting condition affecting the chlorophyll data, a second chlorophyll fluorescence measurement was introduced during the 2004 field season, which allowed creation of a difference image (see section 3.1). The optical absorption of chlorophyll is significantly less in the green spectrum than in the blue or red spectrum; therefore, we assigned a chlorophyll check channel by exciting the chlorophyll with a yellow-green (545/30 nm) spectrum and acquiring in the same 740/140 nm spectrum.

2.3. Source and Delivery of Probes

[8] Syto BC and SyPro Red dyes were obtained from Molecular Probes, Inc. (now Invitrogen) and Calcofluor White was purchased from Aldrich (cat. no. F3543). SRE-26 was synthesized by warming 5-[4′-dimethylaminophenyl]-2,4-pentadienal (Aldrich) and 1,3-diethyl-2-thiobarbituric acid in methanol and purified by recrystallization from water. The working concentrations of probes applied by spray to soil and rocks were: SYTO BC, 5 μM; SYPRO Red, 4 μM; SRE-26, 2 μM; and Calcofluor White, 10 μM. All were combined as a cocktail in a 5% Pluronic L-44 together with 5% acetic acid solution, as will be discussed below. Reagents were dispersed with a hand held sprayer in 2004 and automatically by the rover in 2005, delivering approximately 3 mL liquid on each FOV.

2.4. Testing the FI and Probes in the Laboratory

[9] Yeast cells, washed and suspended in water (1 mL, 2×10^8 cells/mL), were mixed with the four dyes in Atacama spray solution (5% Pluronic L-44 and 5% acetic acid) and incubated for 10 min at room temperature. Cells were collected on black Isopore membrane (Millipore Corp.) by vacuum filtration, dried and imaged. The cells covered an area slightly over one cm^2. Image sets of the filter were collected using the standard Atacama field protocol. Image intensities were determined from analysis of selected regions of interest on the sample and in control regions not containing stained cells.

3. Results and Discussion

3.1. Overview of the Project

[10] Robotically driven testing of the FI and reagent panel evolved over the three seasons of testing in the Atacama. The first year a crude imager based on fluorescence illumination with high intensity LEDs was constructed. The excitation intensity was not sufficient for daytime imaging and it was decided that nighttime imaging had no future for extensive desert exploration for life. The flash based fluorescence imager was used in the second year along with DNA and protein probes. A significant amount of time in the desert was spent improving software, establishing optimal protocols for imaging and developing protocols for dye delivery. The final imaging protocol is described in Table 2.

[11] Imaging of chlorophyll containing organisms was explored in season 2 because the locations for testing were close (Sites A and B) to the coast where coastal fogs at night provided sufficient moisture to sustain relatively abundant lichens (containing photosynthetic algae or cyanobacteria) on rocks. Obtaining a "differential image" set (image after

Table 2. FI Protocol and Data Product Sizes in the 2005 Atacama Desert Field Expedition

Protocol Step	Biomarker	Filters	Raw Image Format	Full Resoluton Size	Maximum Downlink (JPEG) Size
Dry					
(1)	Morphology	RGB	24-bit color	1 MB	400 KB
(2)	Chlorophyll	450ex, 740em	16-bit intensity	2 MB	400 KB
(3)	Chlorophyll	545ex, 740em	16-bit intensity	2 MB	400 KB
Spray Prep Reagent					
(4)	Spray Coverage	RGB	24-bit color	1 MB	400 KB
(5)	Morphology	RGB	24-bit color	1 MB	400 KB
(6)	Chlorophyll	450ex, 740em	16-bit intensity	2 MB	400 KB
(7)	Chlorophyll	545ex, 740em	16-bit intensity	2 MB	400 KB
(8)	Carbohydrate	350ex, 460em	16-bit intensity	2 MB	400 KB
(9)	DNA	450ex, 510em	16-bit intensity	2 MB	400 KB
(10)	Protein	545ex 620em	16-bit intensity	2 MB	400 KB
(11)	Lipid	665ex 825em	16-bit intensity	2 MB	400 KB
Spray Dye Reagent					
(12)	Spray Coverage	RGB	24-bit color	1 MB	400 KB
(13)	Morphology	RGB	24-bit color	1 MB	400 KB
(14)	Carbohydrate	350ex 460em	16-bit intensity	2 MB	400 KB
(15)	DNA	450ex 510em	16-bit intensity	2 MB	400 KB
(16)	Protein	545ex 620em	16-bit intensity	2 MB	400 KB
(17)	Lipid	665ex 825em	16-bit intensity	2 MB	400 KB
			Total:	29 MB	6.8 MB

moisture applied minus image before moisture applied) was found to be an excellent way to see the appearance of chlorophyll signals (discussed below).

[12] Difference imaging was also used for obtaining the signals from the fluorescent probes. Difference images can be created by subtracting the images obtained with the appropriate filter sets before the fluorescent probe panel was sprayed from the images that were obtained after probe staining. This procedure reduced two additional sources of background; leakage of ambient light through the detection filters and mineral fluorescence from the imaged area. Imaging of fluorescent minerals in the laboratory under simulation of desert imaging conditions validated this procedure. Nevertheless, in the context of desert experimentation, wind generated vibration and rover movement during acquisition of fluorescence images caused blurred images and reduced signal to noise. Digitally produced difference images were significantly degraded. We therefore introduced an interesting and useful method of using human vision to create ''effective difference images'' by rapidly switching the before and after images in front of the viewer. This method was used by the remote scientists in Pittsburgh to ascertain the presence of fluorescent microorganisms in the transmitted images (P. Coppin et al., unpublished manuscript, 2007). Henceforth, this will be referred to as the ''difference image'' method.

[13] The third season in Chile was the longest of the seasons and provided the best testing opportunity. The LITA field team visited three different areas for approximately 7 days each. One coastal area near Sites A and B was studied (Site D). Inland drier Sites E and F were selected for further exploration. There was limited visible evidence of lichens or chlorophyll containing organisms at Sites C, E and F. The lipid and carbohydrate probes were added to the panel and delivery was automated with a sprayer adjacent to the FI. The addition of acetic acid to the Pluronic delivery solution was based on laboratory evidence indicating that this treatment improved penetration of dye probes into microorganisms. However, there was not extensive testing with a large range of microorganisms that possess the various protective structures used to improve survival in desiccated environments. The FI and fluorescent probe detection system and some of the highlights of laboratory and desert testing of the system are provided below.

3.2. Design Considerations and Constraints for the Fluorescence Imager

[14] The LITA approach in the search for life required regional mapping across climatic transects. This necessitated traveling long distances (daily traverses of 3–14 km), with periodic surveys of the local environment along the way. In this way, a larger area, and in theory a greater diversity, of habitats could be investigated versus a targeted sampling scheme [see *Cabrol et al.*, 2007; D. S. Wettergreen et al., unpublished manuscript, 2007]. The rover developed for the project, Zoë, is a four-wheeled vehicle capable of autonomous travel and is powered by a set of lithium-ion batteries that are charged by on-board solar panels (see D. S. Wettergreen et al., unpublished manuscript, 2007). Autonomous sample retrieval was not incorporated into the design. As a result, the FI was required to view and analyze samples directly on the surface of the Atacama Desert during daylight hours. Typical fluorescent dye emission is less than or equal to 10^{-4} of ambient light levels, so the high levels of ambient daylight illumination had to be overcome. Shrouding was considered, but rejected, due to Zoë design considerations. The solution was a novel technique using pulsed light [*Lanni et al.*, 2007]. This short-pulse method has been used in other applications [*Belanger*, 2005; *Belzile et al.*, 2004; *Fay*, 1982; *Ford and Leather*, 1984; *Kim et al.*, 2003; *Li et al.*, 1999; *Ludeker et al.*, 2003; *Norikane and Kurata*, 2001]; for our use, a high-powered flashlamp was utilized as the light source, and a CCD camera's electronic shutter was synchronized to a 25-μs flash. With this system it was possible to carry out daylight fluorescence imaging with shading of the rover itself [*Weinstein et al.*, 2005, 2006]. With an operating assumption that most viable life would likely be found in communities, not individual organisms, we decided to use a 10 cm $\times$ 10 cm FOV for all data collection, sampling at the centimeter to millimeter scale. This provided sufficient resolution for small colonies and at the same time allowed for data collection from a reasonable area to begin a search for life in selected desert location.

[15] The instrument's subsystems power consumption as considered during component selection: The Flash lamp draws 35 W and the CCD and CCD cooling system draws 110W. The motion control system consumes controller 8.6W, X and Z axis motor 10W each, Z axis brake 6W (only on during motion), and filter steppers 4W. Ignoring the power draw for motion due to the short time frame that it is actually occurring, the maximum instantaneous power draw is 145 W. This requires that a Pb-acid or Ni-Cd battery to be at least 1 kg or a Li+ battery to be around 200 g. The total energy consumed by the flash lamp for an image set is 350 mJ or 97 microW-h. During field use the CCD was powered on just before images were acquired. An entire image sequence took approximately 20 min, with a total pause of about 8 min for water and dyes, leaving 12 min of CCD activity. The total energy consumed by the CCD and cooler for an image set is 22 W-h. This requires that, for capturing one image sequence, a Pb-acid or Ni-Cd be at least 500 g or a Li+ battery be at least 125 g.

3.3. Selection of Site for Atacama Desert Testing

[16] The LITA field studies began in 2003 in an area labeled Site A near Salar Grande. Basic techniques and technologies were tested at this time with the first rover, Hyperion. Details about science and rover operations are described by *Cabrol et al.* [2007] and D. S. Wettergreen et al. (unpublished manuscript, 2007). In 2004, Zoë, a new rover designed for the LITA project objectives and the Atacama terrain, was introduced together with a full suite of on-board instruments, including the FI, and two sites, labeled Site B and Site C, were investigated. Site B, near the coastal range, was classified as a ''wet'' location relative to the more arid site C in the arid interior of the Atacama. The 2005 field studies had Zoë investigate another coastal region, Site D, and two other interior regions, Sites E and F [*Piatek et al.*, 2007; *Cabrol et al.*, 2007].

3.4. Imaging Protocol in the Desert

[17] The FI is a sophisticated instrument capable of programmable acquisition of reflected light and fluores-

cence signals from a 100 cm^2 area. It is capable of 60 excitation/emission filter position combinations for visible and fluorescence images. Typically, the remote science team chose to limit the detection to two basic modes of operation during the science operations: Chlorophyll FI (C-FI: RGB and chlorophyll fluorescence) and Full FI (F-FI: RGB, chlorophyll fluorescence, and dye probe fluorescence). Variations and combinations of these modes produced a number of different sample data products. These include a single FOV of either C-FI or F-FI, three side-by-side FOVs of F-FI which could be mosaiced together (see Figure 6), a five-position set of C-FI or F-FI FOVs for greater coverage of a locale, and C-FI or F-FI FOV sets of a plow trench, acquired before and after the plow operation and positioned in the center and on the margins of the trench. During the 2005 field season, Science on the Fly periodic sampling was introduced [*Smith et al.*, 2007]. With that procedure, every C-FI of the transect up to the remote science team's defined limit was followed up with an F-FI if on-board image processing indicated a positive signal. From the five sites visited in the two field seasons, FI data sets from 257 samples were returned to the LITA science team, comprising a total of 2609 image requests. The FI protocol called for RGB, chlorophyll fluorescence detection, and then assaying with the dye probes and associated fluorescence imaging. The final Yr 2005 Protocols for use of the imager in the desert are provided in the Materials and Methods section and in Table 2.

[18] Science-on-the-fly periodic sampling was introduced in the 2005 field season [*Smith et al.*, 2007]. This capability enabled the remote science team to define the spacing and number of C-FI image sets that the FI would acquire during a given transect. On board processing would examine the chlorophyll fluorescence images and when it detected a positive signal, further examination would follow by dispensing dye and acquiring a F-FI image set.

3.5. Fluorescence Image Data Handling and Analysis

[19] A FI data product included RGB and fluorescence images. White light illumination was used to obtain standard RGB color images. For this imaging mode, standard emission filters for red (R, 630 nm center wavelength/60 nm bandwidth), green (G, 535/40), and blue (B, 470/40) were used. R, G, and B images were captured sequentially and scaled afterward to produce a color image. Fluorescence images were constructed by acquiring a 50-step sequence of a no-flash background image followed by a flash image, using a 25-μs exposure. We established an ambient light correction as follows. The corrected fluorescence image was produced by summing the background and flash images followed by a subtraction of the summed background images from the summed flash images.

[20] Data acquired in the field was returned to the science team once daily and was limited to a packet of 150 MB to conform to the project requirement of Mars-relevant data return. The full resolution data of the FI samples in one day (22 MB per sample) alone would easily exceed this limit. Methods were considered for data compression to render more samples while preserving resolution for analysis.

[21] The first data reduction approach used conversion of fluorescence images from the 16-bit TIFF format to 8-bit JPEG contrast-stretched images thus eliminating the full depth of the data. Since image quality decreased and quantitative analysis was not possible, this approach was quickly rejected by the science team. A method of transmitting subsampled (thumbnail) 16-bit fluorescence images was then used. The science team would analyze these images and selectively request full resolution images on the following day. Thumbnail images were difficult to interpret and the delay in receiving full resolution images was frustrating to the science team.

[22] The final data reduction approach was to equalize the contrast-stretch images values for each fluorescent channel and save a full sized 8-bit JPEG image. The scaling factor used for the conversion was saved in the metadata to preserve the approximate intensity. JPEG compression was chosen for its ability with typical fluorescence images, its consistency with other image data acquired on Zoë, and the compatibly with the web-based data presentation created for the remote science team. Web-based tools were created to easily display each data set and the associated metadata.

[23] This method significantly reduced the data volume. In the 2005 field season, although more images were acquired per data set, the volume of data was no more than 6.8MB (see Table 2). The combined data reduction method and web-based tools was an efficient method for sample interpretation (see P. Coppin et al., unpublished manuscript, 2007) and enabled the remote science team to concentrate on developing Zoë's next days traverse.

3.6. Choice of Fluorescent Probes

[24] We aimed to create a panel of bright fluorescent dyes for DNA, protein, lipid, and carbohydrate that bound specifically to their target, fluoresced only when bound to the target, were sufficiently water soluble for delivery, and could be detected independently with optical excitation and emission filters. We also attempted to develop a delivery medium, water or water with mild surfactant that would allow the reagents to be accessible to their target molecules in desert microorganisms. The probe panel and delivery media were to be tested in the laboratory and in the Atacama Desert.

[25] Nucleic acid detection: SYTO dyes (Molecular Probes-Invitrogen) bind to DNA with over a thousand-fold increase in fluorescence intensity. Various SYTO dyes have shown selective staining of different types of bacteria. A mixture of SYTO dyes, SYTO BC, which labels most strains of live bacteria with similar fluorescence intensities was used in laboratory and field staining of this project.

[26] Protein detection: SYPRO Red has been used to detect proteins in electrophoresis gels and to determine the protein content of bacterial cells. Although this dye requires protein denaturation with sodium dodecyl sulfate (SDS) for quantitative staining, SYPRO Red can stain live bacteria effectively with a fluorescence enhancement of over 100-fold.

[27] Lipid detection: Nile Blue and Nile Red dyes partition into lipid domains. Nile Red gives a large fluorescence enhancement, yielding better distinction between the lipid and nonlipid regions. We designed a novel styryl dye, SRE-26, with a structure similar to many membrane potential indicators. This new dye is highly fluorescent in lipid environments where it exhibits a >50-fold fluorescence enhancement over its fluorescence in water. SRE-26 is uncharged for greater lipid solubility, but it maintains

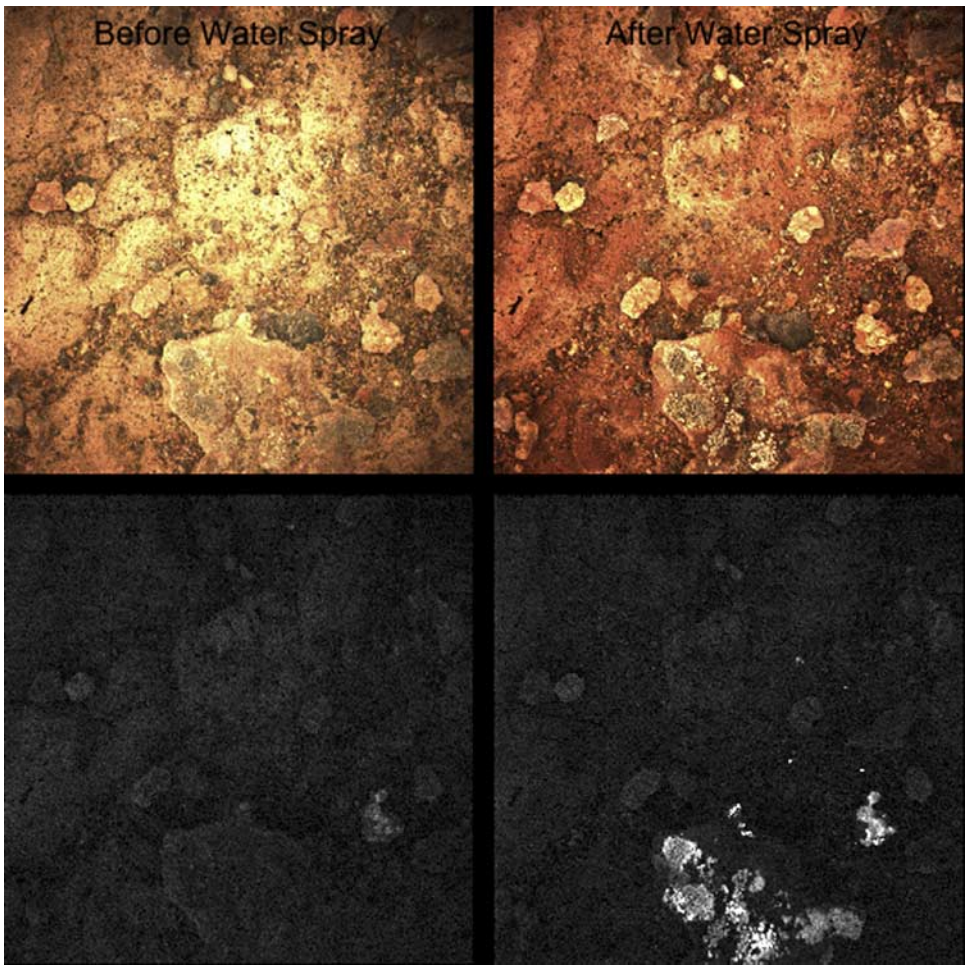

Figure 2. The effect of water on (top) visual identification and on (bottom) chlorophyll emission of desert lichens on rocks from Site B Locale 1. Images on the left are without added water; images on the right are after water application.

significant modest water solubility. The far-red fluorescence of this dye is well separated from most other probes used in this study.

[28] Polysaccharide detection: Biofilms are consortia of microbial colonies bound together by extracellular polymeric substances (EPS), consisting mainly of polysaccharides and proteins. Polysaccharides, such as EPS and chitin of fungal cell walls can be stained with Calcofluor White M2R. The mechanism of fluorescence enhancement on binding of Calcofluor White to its target is not entirely understood. Although the detection sensitivity of EPS may be lower than preferred, the 10-fold enhancement on binding appears to be adequate for this project.

[29] Addition of the mild nonionic Pluronic surfactant to aqueous solutions containing the dye panel served to keep the probes in solution. Additional benefits of the detergent are discussed elsewhere in this manuscript. Pluronics, a series of nonionic triblock polymer surfactants composed of ethylene oxide-propylene oxide-ethylene oxide domains, are nontoxic and are available with a range of surfactant properties.

3.7. Laboratory Testing of Imager and Fluorescent Probe Panel

[30] Yeast cells stained with all four probes in the panel washed and placed on a black substrate showed strong fluorescence with appropriate filter sets on a fluorescence imaging microscope at high power. The cell density in this experiment was 10^6 cells/mm^2. The same sample imaged with the FI in the laboratory under the same conditions used in the Atacama field study also produced strong fluorescence. The signal to background values for the lipid, protein, DNA, and polysaccharide probes were 27, 10, 200, and 230, respectively. Cells at this density form a thin biofilms that is readily detectable. Microorganisms that have protective coatings that cannot be penetrated by probe may not be detectable. Imaged cells without stain gave signals 1% or less than the stained cells.

3.8. RGB and Chlorophyll Detection

[31] Testing in the laboratory and in the desert indicated that phototrophs, such as lichens and cyanobacteria, in a low moisture state do not exhibit high levels of chlorophyll fluorescence [*Demmig-Adams et al.*, 1990; *Seddon and Cheshire*, 2001; *Proctor and Smirnoff*, 2000; *Sass et al.*,

Table 3. A Comparison of FI Biosignature Ratings[a]

Sample Label	Morphology	Chlorophyll	DNA	Protein	Lipid
B02_001_055_FI_-08	2	2	2	1	
B02_001_055_FI_00	1	2	Bd	1	
B02_001_055_FI_08	0	2	2	1	
B02_002_070_FI_-08	0	1	1	Bd	
B02_002_070_FI_00	0	2	0	1	
B02_002_070_FI_08	2	2	1	1	
B02_003_075_FI	0	1	0	0	
B03_003_101_FI	1	2	0	Bd	
B03_004_106_FI	1	1			
B03_005_111_FI	1	2	0	Bd	
B03_006_094_FI	1	2	0	1	
B04_007_023_FI	0	0	0	0	
B04_008_030_FI	0	0	1	1	
B04_010_037_FI	0	0	1	1	
B05_014_042_FI	0	0	2	2	
B05_019_ RUNOUT_FI	2	2	2	1	
B06_019_013-014_FI	1	2	1	2	
B07_024_013_ FI_RUNOUT	0	0	1	2	
C09_025_112_FI	0	0	2	2	
C10_026_219_FI	0	0	2	2	
C10_029_202_FI	0	0	2	2	
C10_030_126_FI	0	0	1	2	
C11_034_314_FI	0	1	2	2	
C11_034_316_FI_C	0	0	2	2	
C12_034_517_FI	0	1	2	2	
C12_034_519_FI_C	0	0	1	2	
C12_036_452_FI	0	2	2	2	
C13_038_555_FI	0	1	2	2	
C14_040_564_FI	0	0	2	2	
D03_050_127_FI	0	0	1	1	2
D04_110_212_FI	2	2	2	2	2
D04_120_222_FI	0	0	0	2	2
D05_140_276_FI	0	0	0	2	2
D05_170_292_FI	0	0	0	2	2
D05_190_296_FI	0	0	0	2	2
D06_210_328_ TRN/09618	2	2	2	2	2
D06_210_329_FI	0	0	0	2	2
E09_340_024_FI	0	0	0	1	0
E10_440_115_FI	0	0	0	2	2
F14_720_024_FI	0	0	0	0	2

[a]Each biosignature was ranked 2 (positive), 1 (ambiguous), or 0 (negative). Sample label nomenclature is as follows. The first letter indicates the site of exploration and the day of imaging. The second number group is the locale of testing. The remaining numbers pertain to image bookkeeping. The carbohydrate probe gave a positive signal at only one sample area: D03_050_127_FI. Bad data are indicated by "bd" and means that the imager failed to acquired an image because of a software or hardware failure or there was significant wind-driven rover movement during the image acquisition that caused blurring during difference imaging. Blank spaces indicate data images not taken.

2002]. Hydration of cellular macromolecules is essential to function and is known to affect plant photosystem fluorescence as well [*Vertucci et al.*, 1985]. We found that the application of water 2 min before image acquisition increased the fluorescence significantly and enhanced the visualization of the surface in the RGB images (Figure 2). When imaging photosynthetic bacteria one might expect phycobiliprotein fluorescence in the DNA and protein channels of the FI. We observed no fluorescence or only weak fluorescence in these channels before addition of the dye probes. This suggests that these accessory proteins, if present, are tightly coupled to the chlorophyll centers.

3.9. Fluorescence Signals From Panel of Four Extrinsic Fluorescent Probes

[32] Images obtained before incubation with the probe panel were later observed by differential imaging at the Remote Site in Pittsburgh. Differential imaging of stained soil/rock indicated signals in some imaged locales and not others. We propose that the difference imaging that showed no signal from any of the four dyes provided evidence that the dyes interacting with soil/rocks did not produce signals that would be mistaken for microorganism generated signals. We suspect that pooling, concentrating and drying of probes in pockets and fractures of rocks occasionally produced fluorescent signals that are known to appear when certain fluorescent dyes form precipitates. Generally, however, the emission of fluorochromes such as the DNA, protein and lipid dyes used in this project is known to be quenched upon aggregation.

[33] We found at times that the emission levels of the dye probes were much lower than expected with lichens that gave strong chlorophyll signals (see additional discussion below), suggesting that the dye probes were not binding their respective target biomolecules. This may be due to the inability of certain aqueous probes to penetrate the cell protective structures of the microorganisms. Adding a dilute Pluronic surfactant to the dye solution may increase the ability of the fluorescent probes to reach their targets in some laboratory testing. The Pluronic's direct effect on the microbiota is unknown, but surfactants tend to "soften" or break up protective coatings, as *Laouar et al.* [1992] reported in yeast cells. The protocol was further modified in 2004 to apply 5% acetic acid in water to the sample before acquiring the predye fluorescence images (in 2005, the acetic acid was incorporated directly into the prep reagent). Acetic acid has limited biological toxicity and readily evaporates from the substrate after application. It is commonly used in histological preparations of plant and fungal tissues (e.g., Carnoy's and FAA fixatives) [*Jensen*, 1962] and causes transient swelling of tissues [*Lambert et al.*, 2003]. To be consistent, the acetic acid was added to the dye solution, as well. We believe that the dye penetration problem provides a challenge for future research using fluorescent probes to quantify desert microorganism.

3.10. RGB, Chlorophyll, and Dye Probe Fluorescence at Test Sites

[34] Table 3 summarizes the wide variability in RGB, chlorophyll and dye probe images for different sites in the LITA field study. In this table we used a numerical system that represents a "human-derived" estimate of the probability that microorganism are present in the area imaged. A "2" in the RGB image means that several knowledgeable scientists at the Remote Site in Pittsburgh judged that there were obvious particles or groups of lichen or microorganism present. A "1" means that they felt that there were "probably" microorganism present and a "0" that there were none visible in the image. A similar scale was used for the difference fluorescence images for chlorophyll and each of the probes in the dye panel. Signal intensity was part of the consideration for the

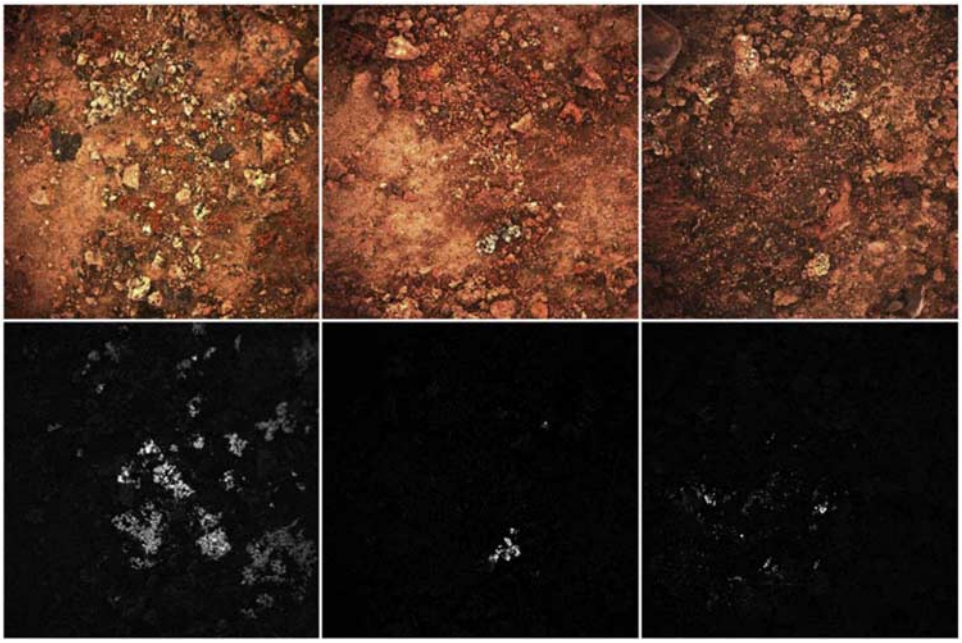

Figure 3. Assorted lichen fields: (a) Site B Locale 19, (b) Site D Locale 210, and (c) Site D Locale 260. (top) RGB images; (bottom) chlorophyll fluorescence images.

fluorescence images. Overlap of the fluorescence signal with a fluorescence or RGB signal in the same imaged field further strengthened a positive rating. The size of the particles and total coverage by fluorescence were not considerations.

[35] In Table 3, a number of the areas imaged showed signals from one or more of the fluorescent probes but seldom did all of the probes in the panel produce signals that overlapped. Accessibility of the biological targets to the probes may explain this variation. It is also expected that different organisms will contain different relative amounts of carbohydrate, protein, lipid and nucleic acid. This would affect relative intensities and, if one of the signals fell below a background threshold established by the remote team, the corresponding probe image would be classified as negative.

[36] Many lichens exhibiting strong visible morphology exhibit no probe fluorescence or just a few of the probes presented weak signals. Figure 4, for example, demonstrates a lichen sample that showed appreciable dye fluorescence only from the lipid probe (a surface probe). Some samples produced signal both from probes that would require penetration (DNA), as well as from surface probes (protein). Other samples showed significant signal only from the surface (protein and lipid) probes (see Figure 5). As shown in Figure 6, a sample from Site C Locale 25 selectively exhibited both DNA and protein probe fluorescence in the light colored veins of a rock.

[37] We believe that the testing illustrated in Table 3 and Figures 3–6 demonstrates the potential for robot-carried imaging and fluorescent probes to investigate the presence of microorganisms in arid regions. However, clearly improvements need to be made in the delivery system and probes that will allow better access to target molecules in the cells and increased sensitivity while avoiding artifacts of fluorescence background.

4. Conclusions

[38] One of the most significant accomplishments of the FI development was the ability to successfully image fluorescence in daylight. As long as direct sunlight was not illuminating the sample, weak fluorescence signals could be detected and useful data collected. This enabled Zoë to travel and sample throughout the daylight hours as the remote science team instructed in the uploaded plan. The only significant limitation was operations at the beginning and end of each day, when low-angle sunlight could interfere with data collection. The use of dyes that fluoresce only when bound to biomolecules was also critically important to the success of these studies. The difference imaging methods adopted by the remote team were important for timely data analysis that facilitated for planning of the next day's search for life program on the desert.

[39] Field and laboratory testing provided significant data demonstrating the utility of water and softeners as a preimaging agent in the desert. It is probable that the low moisture levels of the Atacama cause much of the biota to switch into a semi-desiccated state and/or a moisture protective mode during the majority of the daylight hours. In such a case chlorophyll fluorescence is decreased and dye probes would have difficulty reaching intracellular target biomolecules. This is evident in Table 3 and Figures 4–6 that not all of the four probes are equally efficient at staining and that the differences appear to depend on the micoorganism in the sample area. In contrast, by applying the prep reagent, chlorophyll fluorescence levels increased signifi-

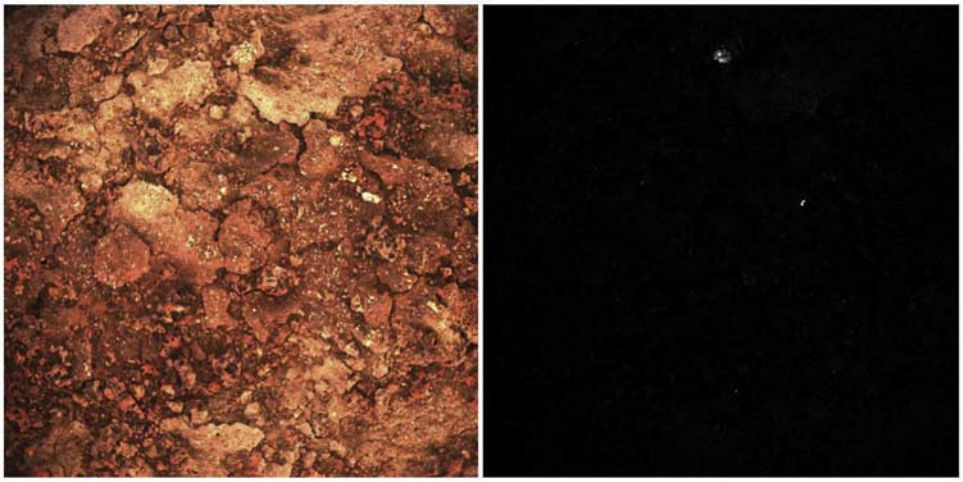

Figure 4. (left) A sample at Site D Locale 200 with two lichens visible in the FOV. (right) The only dye probe with appreciable fluorescence was from the lipid probe.

cantly. The specific protective mechanisms of the bacteria and other desert microorganisms are not completely understood, however, and continued research will be necessary for more complete success.

[40] The FI appears useful in detecting lichens, small plants and other visible life forms larger than about 1 mm within its FOV. RGB morphology correlated well spatially with fluorescence signals from endogenous chromophores of photosynthetic organisms. Additional benefit of the FI, however, was encountered in more arid regions where microbial but not morphologically distinct lichen life exists. There, a biologist would have trouble detecting bacterial hotspots, and detection would likely occur only after sample return and extensive laboratory analysis. The FI has shown that it is capable of detecting microorganisms if they are on the surface at a sufficient density (approximately a million per square millimeter) and if they permit entry of aqueous fluorescent probes.

[41] The goal of the FI in the larger LITA project was to collect a variety of biosignatures. The ability to acquire fluorescence data in daylight provided the LITA team a tool to potentially map life over large regions as the rover traveled throughout each day. The results of this aspect of the field study are reported by *Warren-Rhodes et al.* [2007a,

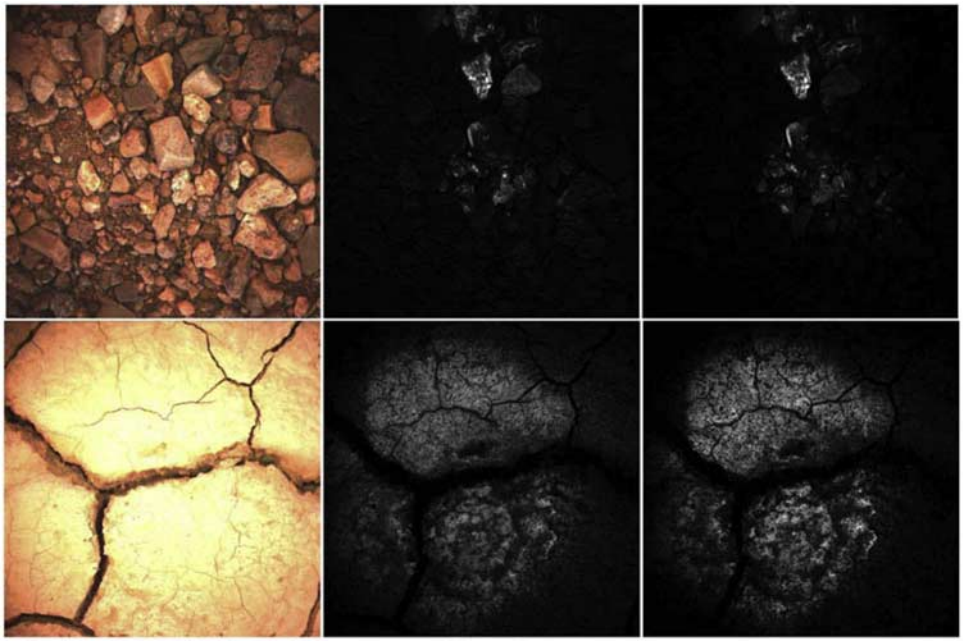

Figure 5. Examples of fluorescence only from the surface dye probes (no chlorophyll or other dye probes): (left) RGB, (middle) protein probe fluorescence, and (right) lipid probe fluorescence. The top row is Site F Locale 830; the bottom row is within the mud flat at Site E Locale 670.

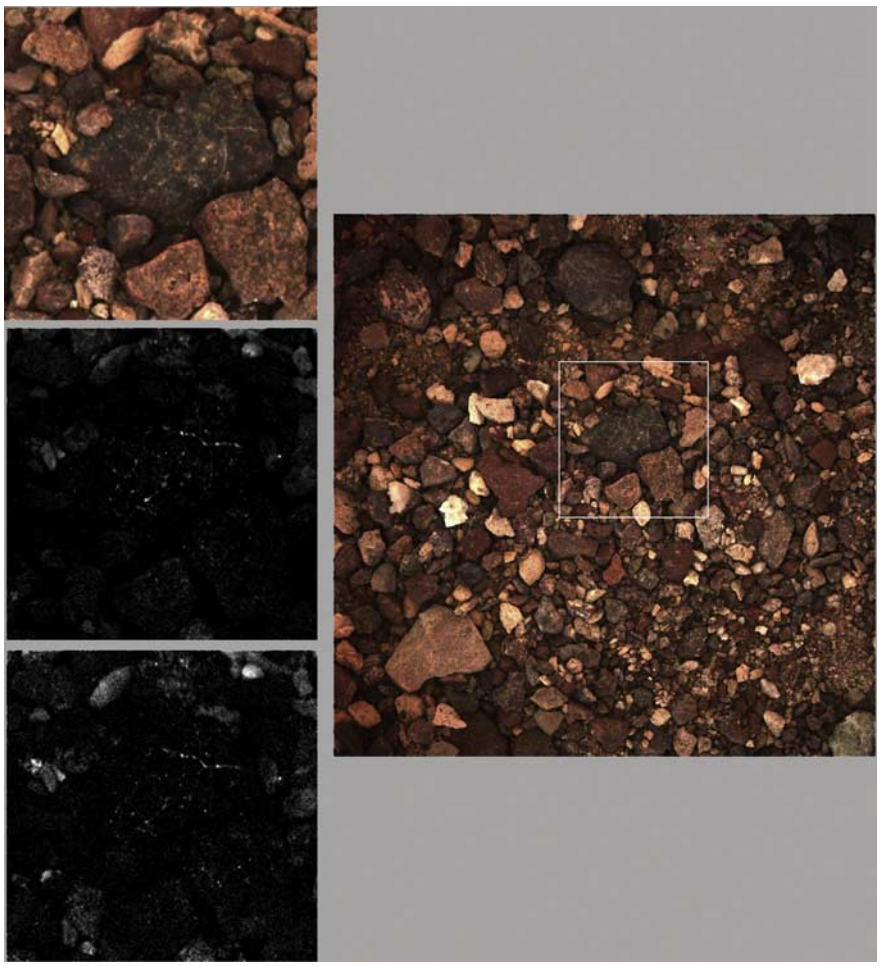

Figure 6. Site C Locale 25. Viewing the rock in the center of the FOV (right large image; top left image), the protein probe fluorescence (middle left), and DNA probe fluorescence (bottom left) were selectively seen in the light-colored veins in the rock. No chlorophyll fluorescence was present in this sample.

2007b]. More research is required to perfect the dye probes and probe penetration for optimal use.

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