Dear Editor,

Please find attached the second-round report from the manuscript JoVE54994 entitled “Experimental protocol for detecting cyanobacteria in liquid and solid samples with an antibody microarray chip”, previously submitted to be considered for the JoVE publication.

We have thoroughly reviewed the manuscript to address editorial and reviewers’ comments. We acknowledge the criticism and advice from the reviewers, and we have done the changes to carefully correct and improve all the grammatical errors, typos and oversimplification/generalizations to address his/her main criticisms in order to fully integrate these feedbacks. In addition, we have rewritten several paragraphs of the discussion according to the editorial and reviewer 1’s recommendations. Also, we have reduced the highlighted material following the editorial’s suggestion.

We honestly think that this video may be of interest for researchers using immunological techniques for environmental or biomedical applications. It contains a complete description of the fluorescent sandwich microarray immunoassay (FSMI) from the microarray printing process to the analysis.

Sincerely

Victor Parro.

Answers to the editorial’s comments: Thanks for the exhaustive reading.

1. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Done.

2. Please revise the highlighting of protocol to be 2.75 pages or less. Done.  
  
3. Formatting:  
-“in field” is not latin, and does not need to be italicized. Changed.  
-Please refer to “section” not “step” when referring to sections separated by bold headings (see 3.1, 4.1). Done.  
-Line 480 – Please do not use “excellent”; please use a more objective term. Changed for “high”.  
  
4. Length exceeds 2.75 pg of highlighted material and should be reduced accordingly. We suggest eliminating highlighting of sections 1-3 for continuity. We accepted your suggestion. Thank you for your advice.   
  
5. Grammar:   
-Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance, and some errors are indicated below. This editing should be performed by a native English speaker.  
-Line 35 – “it is described an experimental protocol”. Changed for “To evaluate the risk of water contamination, this article describes an experimental protocol for in field detection of cyanobacterial strains in liquid and solid samples by an antibody microarray chip”.

-Line 39 – “make some aquatic ecosystem to behave”. Changed for “make some aquatic ecosystems behave as true bioreactors”.  
-Line 42 - “damages”. Changed for “Many cyanobacterial strains are toxin producers and few cells are necessary to induce irreparable damages to the environment”.  
-Line 57 – “of 1 h incubation each one”. Changed for “The whole process takes around 3 h, most of it corresponding to 2 1-hr periods of incubation”.  
-Line 67 – “it has been developed a multiplex fluorescent sandwich…”

-Line 70 – “to proportionate tools” Both grammatical errors changed for “a multiplex fluorescent sandwich microarray immunoassay (FSMI) has been developed as a tool to water managers for helping them in…”  
-Line 80 - “that frequently makes difficult to distinguish”. Changed for “they require a certain level of expertise considering that it is often very difficult to distinguish between closely related species2”.  
-Line 87 – “it has been described false positives”. Changed for “false positives have been described”.  
-Line 100 – “quantified”. Changed for “quantify”.  
-Line 112 – “a higher research”. Changed for “further studies”.   
-Line 117 – “hard-working” – not correct use of term. Changed for “very laborious”.   
-Line 120 “ssandwich”. Changed for “sandwich”.  
-Line 140 – “leads to identify”. Changed for “This microarray allows for the identification of potential…”.   
-4.2 – “continuous agitating”. Changed for “continuous agitation”.  
-Line 239 – “homogenates” – wrong word. Changed for “homogenizes”.   
-6.2.1 – “Weight”. Changed for “Weigh”.   
-6.2.2 – “If use a hand-held sonicator”. Changed for “Give at least 5 x 30 sec cycles at 30 kHz stopping 30 sec on ice”.  
-7.1.2 – “avoiding touching”. Changed for “trying to avoid”.  
-7.5.2 – “Disassembly the cassette”. Changed for “Disassemble the cassette”.   
-Line 374 – “proportionates measurements”. Changed for “provides measurements”.  
-Please remove all instances of you or your. Done.   
-Please rewrite the second paragraph of the discussion so that complete sentences are used rather than a long list of sentence fragments. Done.  
-Line 473 – “it is recommended the use of methods”. Changed for “the use of methods to disentangle these cross-reactivity events from the true cognate antigen-antibody reactions by, for example, using deconvolution methods26,27, is highly desirable”.   
-Line 489 – “considering…demonstrate” – incorrect subject/verb pair. Changed for “the fact that CYANOCHIP-FSMI can identify *in situ* cyanobacterial markers, even to associate to different phylotypes or groups, and that CYANOCHIP covers a broad habitats from plankton, benthos and endoliths, demonstrates…”.   
  
6. Additional detail is required: 5.1.1 – What is the composition of the protein printing buffer? Protein printing buffer is a commercial mixture composed of solvents, viscosity enhancers, stabilizers and buffering components to improve sample surface properties during printing. The exact composition is unknown.   
  
7. Branding: 4.4 note, 7.4.1, 8.1 – Alexa 647.

4.1: “Alexa-647” changed for a far-red-fluorescent dye”.

4.3: “Sephadex G-50” changed for “a gel with a fractionation range between 1.5 and 30 kDa trapped into a column”.

4.4 note: “Alexa-647” changed for “the fluorescent dye”.

7.4.1: The sentence with Alexa-647 changed for “Add 50 µl of an antibody mixture containing the 17 anti-cyanobacterial strain antibodies labeled each of them with the fluorochrome in TBSTRR, 1 % (w/v) BSA”.

8.1: The sentence with Alexa-647 changed for “Scan the slide for fluorescence at the maximum emission fluorescence peak for the far-red-fluorescent dye in a scanner for fluorescence”.  
  
8. Discussion: Please discuss the significance with respect to alternative methods; please also discuss the limitations of the protocol.

Answers to the reviewer 1’s comments: Thanks for the exhaustive reading.

**Reviewer #1:**

*Major Concerns:*  
Many details in the protocol are missing and should be explained clearly. Otherwise, nobody would be able to repeat successfuly the experiment.  
Following information are missing in the protocol:  
1. Preparation of immunogens:  
The authors have to explain the key point that grown bacteria cells have to be inactivated before immunization of animals. Done. We have added a note at the end of the section 1 to explain the reason why we inactivate cells before animal immunization.

1.1. Information about growth medium is missing. Done. Culture medium and growth conditions for each cyanobacteria are listed in Table 1.

Quantification method for determination of cell concentrations is missing. Done.  
1.2 The Quantification method for determination of cell concentrations is missing. Done. These two steps rewritten.   
  
2. Production of polyclonal antibodies: the authors have to explain the screening method to find rabbits that produce the polyclonal antibodies of interest. Which titer should be found in serum? The screening method is normally performed by a specialized company. They supply results of ELISA experiments carried out with each rabbit serum, providing the serum titer. This titer give us information if there is a positive humoral response in the animal. A serum titer between 1:2000 and 1:10000 is recommended by providers to work with.   
Each antibody have to be characterized (sensitivity and selectivity) before usage in microarray experiments!. We do not consider that one of the objectives of the present paper is to describe how the sensitivity and selectivity of antibodies is performed. These protocols are highly described in the literature (e.g. Blanco et al., 2015) and they are easy to follow for a new user.   
  
3. Antibody purification: Besides protein concentration the activity of antibodies should be tested. Wrong treatment could reduce the activity of antibodies. Please give an advice. Yes, it is very important to test the activity of antibodies before labeling. We always perform it by ELISA, but we considered that extending current manuscript in the description of the standard immunological methods was out of the scope. Anyhow, we mention it in the new version.

4. ok  
  
5.1.1)The term protein printing buffer have to be explained in detail because this is the main point for microarray production! We use a commercial buffer. See the answer to editorial comments.   
  
5.1.2. ditto. OK.  
  
Note: please give the advice that low binding plates should be used!. Done.  
  
Discussion: It is not the case that the analysis can be performed rapidly and simply in the field. The total analysis from sample incubation to data processing takes at least 4 h and needs a large set of equipment and many manual process steps. Please, write your statements in a more realistic way. Ok. We are agree with your comment. We include this observation in the discussion.   
Please explain, why cyanobacteria with the same immunogen pattern should be found on other planets. My opinion is that antibodies are very selective and only the set of antigens used for immunization can be found in a sample. We are agree with you up to a point because we can only study life in other planets as we know it on Earth. Then, we must based our research on terrestrial life. Also, we only need a signal in the CYANOCHIP to detect something related with cyanobacteria markers. We understand a pattern as several signals in the CYANOCHIP that are consistent in different places. However, we only look for single positive signals in space exploration.   
Please explain in detail the process for quantitative analysis of cyanobacteria by the CYANOCHIP used for early warning systems. For such tasks, the CYANOCHIP has to be calibrated with standard concentration of cyanobacteria. Working ranges, sensitivity, selectivity and precision has to be determined.

We consider CYANOCHIP a qualitative biosensor for multiple detection and classification of cyanobacteria. We determine the working dilution for each antibody in a titration curve by FSMI. And we also test the sensitivity and specificity for each antibody as described in Blanco *et al*.22.

The authors have to explain in detail which experiments can be done in future using the CYANOCHIP and which scientific conclusion can be made from such experiments.

The conclusion paragraph has been modified and extended so that it contains the reviewer request.

*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A

Answers to the reviewer 2:

**Reviewer #2:**  
*Manuscript Summary:*  
A microarray for the detection of cyanobacteria was presented. A multiplexed system for the monitoring of at least 17 cyanobaterial strains was developed. A protocol including sample preparation is given. The total process needs about 3 hours.  
  
*Major Concerns:*  
It is not clear, how the cyanobacteria should be designated unambiguously.

These cyanobacteria are from the most common genera found in European water bodies.

Are the strains obtained from a collection or from natural samples?. All the strains, with the exception of K17, belong to the Antonio Quesada’s group from Autonoma University (Madrid, Spain). Antibody against *Planktothrix rubescens* was generated from a natural sample of this cyanobacteria monospecific bloom from Vilasouto reservoir (northern Spain).   
  
*Minor Concerns:*  
Some typos should be removed by careful revision, eg. Fig. "Multitanalyte". Thanks. Done.  
  
*Additional Comments to Authors:*  
N/A