Editor, *Journal of Visualized Experiments* April 12, 2019

Dear Dr. Wu,

We were pleased to hear that the manuscript submitted to *JoVE* was deemed interesting, suitable for publication and, in addition, that it was found to usefully illustrate the process of microarray fabrication by photolithography. We are also grateful for the reviewers and the editorial comments, which helped in clarifying specific sections of the manuscript.

Please find below our response to the reviewers. All changes have been tracked in the revised document, and the information added after review is, in this letter, highlighted in yellow.

Reviewer #1:

"The main weakness is that there are no representative fidelity results for the oligo library preparation, leaving a reader to assume the outcome would resemble previous examples. However, the chemistry used by this group has changed significantly since oligo library fidelity data was presented (light source, 5'-protecting group, presence or absence of capping steps, the 'Express' chemistry, flow cell modifications). This makes it hard to evaluate the functional significance of some of the protocol steps for this application. Existing data suggests that high stepwise efficiencies for coupling and deprotection, are necessary but not sufficient for producing oligo pools that are predominately free of errors, so some further comment on this seems appropriate."

This is a very valid point. The oligonucleotide libraries that we can prepare using microarray photolithography have, up until now, been characterized by either A_{260} absorbance and mass spectrometry[1], which provides a good overview of synthesis efficiency and yield and those characterization methods can routinely be performed after collecting the DNA or RNA library. We have, however, started to study the error rate of microarray fabrication *via* sequencing and are now gathering enough detailed evidence on the sources of synthesis error with the aim to eventually provide a full description of the influence of synthesis parameters and array design on the error-rate of DNA libraries made on high-density arrays. We felt that this investigation was beyond the scope of the present protocol, which was to illustrate the actual process of array fabrication by photolithography, from design preparation and up to collecting the cleaved library. In the past, we have discussed synthetic and optical errors[2], which indeed seem to be predominantly due to coupling or photodeprotection failures, as noted by the reviewer. The various stages of synthesis and array throughput improvements that have been introduced over the years further called for an updated, illustrated overview of our current array fabrication methods. We point out, however, that each individual synthesis upgrade was followed by quality checks –typically by comparing hybridization signals before and after the upgrade– as well as by a direct biological application, for instance with measuring gene expression levels in a population of cells.

Given the present discussion, as well as a further comment by reviewer #1 (*"P1: The abstract says, 'The fabrication protocol is optimized so as to limit the number of synthetic errors…', while l70 notes the historical emphasis on hybridization assays, suggesting to the reader that there will be a non-hybridization quality metric/benchmark forthcoming.*"), we deemed it necessary to inform on an upcoming full study on

the error rate in our current photolithographic synthesis setup. To this end, we have added in the conclusion the following sentence: "The success in digital encoding on DNA and in *de novo* gene assembly depends on sequence fidelity, which translates into the error rate at the synthesis level. Synthetic and optical errors in our current array fabrication protocols will be discussed and reported on elsewhere."

"Abstract: The first sentence of the abstract should be divided into multiple sentences."

We have split the first sentence at "…micrometer-sized mirrors, yielding…" into "…micrometer-sized mirrors. Photolithography yields…"

"Abstract: The phrase, 'mixed-base 25mer' is ambiguous. Is this just an oligo containing multiple nucleotides throughout (ie not a homopolymer) or is it a feature made with mixtures of bases at certain positions?"

The 25mer synthesized according to the described protocol is indeed an oligonucleotide containing all four nucleotide bases within the sequence, and in the corresponding microarray each feature contains a single, well-defined sequence. To alleviate some of the ambiguity, we have modified the corresponding sentence into "template 25mer sequence containing all four bases".

"P1: The abstract says, 'The fabrication protocol is optimized so as to limit the number of synthetic errors…', while l70 notes the historical emphasis on hybridization assays, suggesting to the reader that there will be a non-hybridization quality metric/benchmark forthcoming."

As stated in the response to the first comment, we have indeed started to study, in detail, the error-rate of synthesis during microarray photolithography using sequencing and we intend to describe, in the near future, the nature and sources of error, as well as the influence of various synthetic parameters on the overall sequence fidelity. We believe that the corresponding data warrants its own story, but for clarity, we have indicated, in the conclusion, that this investigation is ongoing and that we will soon report on it.

"P1, l52: 'Stomping grounds' is unusual phrasing and seems out of place."

We have replaced "stomping grounds" with "practical use".

"P5: It may be helpful to generalize aspects of the Expedite protocol to account for variation other users may encounter in the dead volume from unit to unit."

Variations in consumption, pumping and reagent delivery on the Expedite DNA synthesizer are indeed common, requiring occasional checks, tweaks and updates, as well as regular cleaning of the machine and delivery lines. We try to frequently inform on our current Expedite protocols[2-4], but have however found that the protocols do not necessarily translate "pulse-for-pulse" from one DNA synthesizer to another. We do however realize that how the standard Expedite protocols of solid-phase synthesis were adapted to microarray photolithography can be very useful information and, for clarity, we have now added two

representative coupling cycle protocols (for BzNPPOC DNA and NPPOC RNA phosphoramidite coupling) as tables (Tables 1 and 2), referenced in page 5 of the manuscript.

We have also added the following paragraph in the discussion part: "The DNA synthesizer itself, as well as the reagents and solvents, certainly needs to be as clean as possible in order to achieve the highest yield of oligonucleotide synthesis. However, insoluble material, salts or particles, can accumulate over time in the lines and tubing of the delivery system, leading to a gradual decrease in consumption of reagents and reactants. Where a general cleaning of the synthesizer does not resolve a low output volume, an increase in the number of pulses can be an alternative solution. Particularly useful in the case of low phosphoramidite consumption, the line in the coupling protocol corresponding to the pumping of a mix of phosphoramidite and activator (third line of the coupling subsection in Tables 1 and 2) can be modified, from 6 to 9 pulses without any appreciable negative effect on synthesis quality. Furthermore, the number of pulses of activator needed to bring the amidite/activator mix to the synthesis substrate (currently 6, fourth line in the coupling subsection, see Tables 1 and 2) depends on the DNA synthesizer itself as well as on tubing length in the synthesis cell. This number can be adjusted after replacing the phosphoramidite with a colored solution and counting the number of pulses needed to push the colored mix to the glass substrate for coupling."

"P13, l569: Some comment on the tradeoffs of using the NPPOC-protected cleavable dT amidite vs. a DMT-protected version may be useful here."

One major advantage of using the base-cleavable NPPOC-dT monomer is the ability to selectively deprotect features containing this monomer before the next coupling, using UV light. If, instead, the 5′-DMTr protected counterpart of this base-sensitive unit were to be used, a global acidic treatment would be necessary to remove the DMTr group before continuing with the synthesis, meaning that all features having received this monomer would also couple with the next incoming phosphoramidite. Lack of selectivity with DMTr deprotection then becomes problematic in the synthesis of nucleic acid libraries. In addition, having an NPPOC-only oligonucleotide chemistry entirely removes the need to use an acidic reagent, which thus means that complete oxidation of phosphite triesters before the beginning of the next cycle is not as crucial as for standard solid-phase synthesis, and this translates into higher synthesis efficiency and shorter fabrication time.

"There are places where it can be hard to tell whether the authors are referring to their protocol or the literature at large, leading to oversimplifications. On P12, l528, they suggest array fabrication bypasses capping and there are no oligonucleotide purification strategies, which isn't true even if not explored much on this platform. There are some enzymatic techniques to remove failed sequences which could be employed on libraries which utilize capping, as well as some approaches to selectively capture truncated sequences. Similarly, the wording on P13 suggests the deprotecting strategy they employ is required to producing the library correctly which, while true here, might lead a non-specialist to think there are no other options available (enzymatic cleavage, for example)."

We realize that the terminology used in the discussion part can be seen as indistinctly describing general microarray synthesis protocols and our own *in situ* photolithography setup. Since we naturally refer to our current method for DNA and RNA synthesis on array by maskless photolithography, we clarified some of

the wording in the discussion section. In particular, we added: "Alternative strategies to recover oligonucleotide pools from microarrays without the need for a specific basic treatment exist, are in principle compatible with photolithography and rely on the use of enzymes. For instance, a single deoxyuracil nucleotide can be the target of the uracil-DNA glycosylase (UDG) and excised from the rest of the DNA sequence, or a single RNA unit can be recognized by RNase H type 2 enzymes and the phosphodiester bond 5′ to the RNA cleaved, releasing the 5′ DNA part."

Reviewer #2:

"Figure 2 is too blurred and It is difficult to get any useful information. A high resolution figure and local enlargement if necessary will be helpful."

A low resolution version of Figure 2 was mistakenly uploaded upon initial submission of the manuscript. For submission of the revised manuscript, the high-resolution version of Figure 2 should now be available.

"It is necessary that the nucleic acids libraries cleaved from the slide should be characterized, such as by MS, to prove the successful preparation of the DNA or RNA."

When we introduced our method for base-mediated cleavage of oligonucleotides from *in situ* synthesized arrays, we performed an extensive study of DNA and RNA characterization by LC-MS, each time with indication of the presence of the full-length product[1]. While MS characterization of complex nucleic acid libraries containing thousands of different sequences would be difficult and near-illegible, we routinely quantify the amount of recovered DNA by 260 nm absorbance on a spectrophotometer and have now added a representative spectrum for a recovered DNA library: "Figure 7. Representative absorbance spectrum (220 – 350 nm) of a cleaved, desalted DNA library containing 4000 different sequences, 100-nt in length. A total of 940 ng DNA was isolated from a single array synthesis, corresponding to 30 pmol of DNA total, or 15 pmol per glass substrate."

The next step in library characterization would be sequencing and we have indeed started to sequence our oligonucleotides pools and to learn on the error rate during synthesis. However, we intend to publish a full investigation of the error rate by photolithographic array fabrication elsewhere, as we felt that such a study would be beyond the scope of this protocol.

Finally, we thank the reviewers and editor for pointing out mistakes, grammatical and typographic errors, which have now been corrected.

1 Lietard, J. *et al.* Base-cleavable microarrays for the characterization of DNA and RNA oligonucleotides synthesized in situ by photolithography. *Chem. Commun.* **50** (85), 12903-12906, (2014).

2 Agbavwe, C. *et al.* Efficiency, Error and Yield in Light-Directed Maskless Synthesis of DNA Microarrays. *J. Nanobiotechnol.* **9**, (2011).

3 Sack, M. *et al.* Express photolithographic DNA microarray synthesis with optimized chemistry and high-efficiency photolabile groups. *J. Nanobiotechnol.* **14**, (2016).

4 Holz, K. *et al.* High-Efficiency Reverse (5'-->3') Synthesis of Complex DNA Microarrays. *Sci Rep.* **8** (1), 15099, (2018).