We are grateful for the great care and expertise that the referees have shared and for their supportive comments. Many of the suggestions from the referees are very reasonable comparisons to other ZMWs and ways to adjust the fabrication protocol. Most of these were already included in our PLOS One paper on this subject[1]. Because the purpose of this JOVE submission is to provide more detail and instruction on fabrication method, we did not repeat all of those comparisons here. We do refer more clearly, as mentioned below, to those points that were published before. Changes in the manuscript noted below appear in red in the resubmitted version.

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have read through the manuscript to check for spelling and grammar issues.

2. Please revise lines 41-43 and 100-101 to avoid textual overlap with previously published work.

We have revised the text in those lines to avoid textual overlap.

3. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE."

We have revised the protocol to only include steps with action items.

4. Please number the figures in the sequence in which you refer to them in the manuscript text.

We have read through the manuscript and ensured that all figures are referred to in sequence. Figure S2 and S3 have been switched to correct this.

5. Figure 2: Please change the time unit "sec" to "s" (i.e., 20 s).

We have changed the time unit from "sec" to "s" in the figures.

6. Table of Materials: Please sort the materials alphabetically by material name.

We have sorted the materials alphabetically within in each section of the materials list.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the presented work K. Chen and co-authors illustrate how nanostructures such as ZMWs can be obtained by colloidal, or nanosphere,
lithography. This is an alternative strategy, with respect to others more expensive and time consuming, to create nanometer-scale masks for waveguide
fabrication. The report describes the approach in detail, with practical considerations for each phase. The method allows thousands of aluminum or gold ZMWs to be made in parallel, with final waveguide diameters and depths of 100-150 nm.

In my opinion the report is well written and very clear to the reader.
I recommend the publication

We thank the reviewer for these comments.

Reviewer #2:
Manuscript Summary:
This manuscript describes a protocol for fabrication of zero mode waveguides (ZMWs) based on colloidal or nanosphere lithography as an alternative to commonly used electron beam lithography or ion beam milling. Fabrication of ZMWs using colloidal or nanosphere lithography is simple and inexpensive. The authors also illustrate an application of the ZMWs for single molecule imaging at higher concentrations.

Minor Concerns:

1. Although fabrication of ZMWs by using the proposed protocol in this manuscript is simple and less expensive, there appears to be a wide distribution of ZMW size. They show 100-150 nm in the figures and in the text in some place and others 100-200 nm. It is not clear if they mean that this encompasses the heterogeneity of hole sizes in a typical preparation. Or is this the range of holes that can be made. The distribution in figure 5e shows a somewhat wider distribution.

We thank the reviewer for catching this inconsistency. There is some variability both within each sample and among samples. 100-200 nm captures most of the distribution of ZMW diameters in Figure 5e, and we have made this consistent in all the figures and text. This range performs well in single molecule fluorescence experiments.

2. Can the ZMW diameter be varied? It would be great to mention directions to make ZMWs with lower or higher than 100 nm in diameter.

We thank the reviewer for this insightful comment. The ZMW diameters can indeed be varied by tuning the melting time in Part 3 of the Protocol. In a previous publication[1], we

characterized the pore size in the waveguide template as melting time was varied (Figure 2e-h in Jamiolkowski et al., 2019). The data show that the pore diameter can be made larger by reducing the melting time, but cannot be made much smaller than 100 nm since pores begin to quickly close past 20-25 sec of annealing. Assuming that users of the method will want the smallest possible pore diameter for maximal fluorescence background reduction, we used the 20 sec melting time in the detailed JoVE protocol. We now refer more clearly in the Discussion to the earlier paper in regard to varying pore size.

3. Line 215 says "3. Bead Annealing for Tuning the Size and Shape of the Colloidal Crystal Template" But there is no information in this section about how to tune the size or shape.

We agree that this section does not provide data on how the pore size varies with melting time. We intend for this JoVE article to provide more details on how to practically implement the method we described in a previous paper[1], so we omit the pore size versus annealing time data. However, we changed the title of the section to “Bead Annealing for Reducing Pore Size in the Colloidal Crystal Template” so that it will not be misleading. We refer to the earlier paper in the Discussion in regard to varying pore size.

4. Line 191 provide the rational for keeping the chamber temperature warmer than room temperature

Rather than a parameter we controlled, the temperature in the chamber was an effect of the hot water evaporation in the closed system. However, the ambient chamber temperature affects the temperature of the ethanol-bead suspension, which can affect the ethanol evaporation rate. The evaporation rate determines how well the beads pack, so we mentioned the temperature we typically see in the chamber when we perform the protocol. However, since the temperature is not explicitly controlled, typically only 1-2 °C higher than room temperature, and not a critical parameter, we de-emphasized the importance of controlling the temperature (lines 213-215).

5. In the fabrication section, the final rinse is with DI water. Even with EBL a final descum etching with subsequent acid wash is necessary to clean up ZMWs. Does rinsing with DI water remove any fluorescent traces? This would be surprising.

We thank the reviewer for this comment. We agree that non-specific fluorescence from insufficiently cleaned slides is a concern for single molecule microscopy, and indeed we do sometimes see nonspecific fluorescent particles during TIRF imaging. We typically bleach these particles with the excitation laser before loading sample into the ZMWs. We are aware that previous papers fabricating ZMWs with electron beam lithography (EBL) use a photoresist stripper[2] or oxygen plasma[3,4] to remove residual resist, but since we are not using electron resist, we did not need to use either cleaning method

after dissolution of the copper posts. However, we did include an oxygen plasma cleaning step after dissolution of the polystyrene template to remove any residual polystyrene (step 4a.4). A previous study using EBL to fabricate gold ZMWs[5] also did not use a descum etching or acid wash step after fabrication and used a simple water and isopropyl alcohol wash after functionalization of the ZMWs with polyethylene glycol (PEG). Based on these considerations, the protocol as described worked well.

6. Line 275 provide the composition of copper and aluminum etchant

We would like to provide the full composition of the copper and aluminum etchant, but the manufacturer of both etchants (Transene) unfortunately does not provide this on their website. However, based on information from a "Copper Etchant 49-1 White Paper" on the Transene website (https://transene.com/wp-content/uploads/Copper-Etch-Article.pdf) and an aluminum etchant type A MSDS from Sigma Aldrich (Product Number 901539), we know the copper etchant is citric acid-based and the aluminum etchant type A (standard aluminum etchant for silicon devices) is phosphoric acid-based. We have indicated this in the Protocol (lines 304-305 and 339-340).

7. Line 572 Typo double "into the"

We thank the reviewer for catching this typo and have fixed it in the manuscript.

Reviewer #3:
Manuscript Summary:
Interesting technique. Generally well described.

Major Concerns:
1. Would be helpful to have a schematic with excitation source and detector (camera?) to show how these SMWs are actually used.

We thank the reviewer for this suggestion and have added an excitation source, objective, and detector to the schematic in Figure 1.

2. Is it possible to have a diagram showing how they compare to the more conventional ones?
In our previous publication[1], we compared the single molecule fluorescence signal to noise ratio from our ZMWs to those of gold ZMWs from another publication[5] using electron beam lithography. Because we wanted to focus on the practical details of implementing the protocol in this article, we did not include this data. We now refer to the earlier paper for this comparison in the Discussion.

3. Do any of the steps produce residues that impact the background?

Yes, the bead annealing step (part 3 of Protocol) increases the likelihood of the polystyrene beads leaving a residue on the glass slide prior to the second metal deposition, which can interfere with metal adherence to the glass. However, we mention in the Discussion that the overnight toluene soak resolves the issue.

4. What is the average number of SMWs that are simultaneously analyzed? How does this impact the statistical signiicance of the results?

Figure 6b shows a typical field of ZMWs in a dual-view TIRF setup with 100x magnification. More than 3000 waveguides are contained in that image. Assuming a conservative estimate of 10% loading based on Poisson statistics, one can obtain a statistically significant sample size of several hundred single molecule traces from a few movies of different spots on the ZMW slide. This was done in Figure 5 of our previous publication[1] introducing the method.

Minor Concerns:
p.2 and 3, not clear to what "their" refers. Avoid use of ambiguous pronouns.

We thank the reviewer for catching this and have removed ambiguous uses of the “their” pronoun in the manuscript.

p.5 Specify time that coverslips can be stored prior to that the last sentenceach step or use.

We thank the reviewer for suggesting this addition. We have added notes for where the protocol can be stopped and how to best the store the templates and ZMW slides for later use.

p.7 Needs to be more clear in the protocol which steps are for gold ZMWs. After reading this section, it was not clear whether the copper deposition step also applies for the gold AMWs and whether the aluminum is also deposited over the copper for the aluminum ZMWs.

We have now separated the aluminum and gold ZMW protocols into two separate subsections (part 4a and 4b of Protocol).

p.7. It would probably help to describe what "excess" looks like since the metal posts need to stay in place.

We have added more description to the note after the tape pull (lines 272-277).

p. 8. Add caution about proper storage of Chloroform.

We thank the reviewer for catching this omission and have added notes about storage for chloroform, toluene, and the metal etchants.

p.13. Not clear what the " channels" are or what their relationship is to the ZMWs.

We have clarified in the manuscript that the channels are individual ~20 μL flow chambers[6] that can be used for successive single molecule experiments on the same ZMW slide.

Reviewer #4:
Manuscript Summary:
This paper is a simple fabrication method paper, which focuses on fabricating ZMW surface without accessing to specialized nanofabrication facilities. The method described here is simply written. This paper will contribute to widely expand single molecule measurements using ZMW to the lab or research institution including even small group scale. This paper should be published.

We thank the reviewer for these comments.

Reviewer #5:
Manuscript Summary:
The manuscript describes a relatively cheap and ascetic way of fabricating zero-mode waveguides (ZMW), which provide drastic improvement to signal-to-background and signal-to-noise ratios of single-molecule FRET(sm-FRET) experiments. The authors employ nanosphere lithography to achieve down to 100 nm diameter ZMW which are benchmarked using a basic sm-FRET experiment. The distinct feature of the method is using minimal amount of expensive equipment to fabricate sub-diffraction limit ZMW and make the technology available to broader scope of scientists.

Major Concerns:
1. Since the method described is meant for low-resource (in terms of fabrication facility) setting and is in a sense a novel approach for fabrication of ZMW, I feel that the manuscript lacks a concrete and critical discussion on the limitations of such a fabrication method (i.e. resolution, reproducibility, distribution of ZMW sizes and factors affecting it). Looking at the figures it feels like authors have sufficient data to characterize their method better and pin-point some critical parameters and how they affect the quality.

We thank the reviewer for this comment and agree that, as a new method, data should be provided on the distribution of ZMW sizes and reproducibility of ZMW sizes. For this JoVE article, we focus on the practical implementation of the method, and data on the limitations has already been provided in a previous publication[1] introducing the method. Fig. S4 of that paper describes the batch-to-batch variation in ZMW diameters, which is mostly due to differences in melting time during the annealing step (part 3 of the

Protocol). The limit on the resolution of our fabrication method is also due to pores closing in the bead template once the pores reach a threshold size (discussed in lines 558-565). The variation in ZMW sizes within a representative individual slide is provided in Fig. 5E and is comparable to the variation of ZMW sizes within an individual slide fabricated with electron beam lithography in a previous paper using gold ZMWs (Fig. 2 of Kinz-Thompson et al., 2013[5]). Overall, duplicating the characterization published before would be redundant, but we cite that work more clearly now.

2. The proposed protocol contains a very peculiar way of creating controlled humidity environment, through placing a fan above a glass of hot water in a plastic box. Though humidity has been demonstrated by the authors to be a critical parameter affecting the quality of colloidal crystal, reproducing of this particular conditions for achieving right humidity looks tricky. Retrieving or manipulating samples in such setting requires going into the box and disrupting the inside conditions, which makes the method quite operator dependent. Obviously the method has proven to be viable, but it is necessary to describe a way of adapting this protocol to using it in glove box (setting up a humidity glove box can be actually not a very expensive/complicated task) for more reproducible humidity conditions.

We thank the reviewer for this suggestion and would like to mention that we did consider using a humidity-controlled glove box to improve control of the deposition conditions. However, we did not have ready access to a humidity-controlled glove box in our lab and found that it was simple enough and effective to adjust the starting humidity in the plastic chamber on the bench by waiting longer or shorter times for the humidity to rise. We agree with the reviewer that the humidity changes as we go into the box each time to deposit the beads. We acknowledge this in the Results and recommend users only perform 6 depositions at a time. However, even with beads deposited towards the end when the humidity has dropped by about 10%, there is still 1-2 cm^2 of useable bead monolayer area (Fig. 1C) for ZMW templating. Lastly, each deposition takes 10-15 min, so users can quickly improve the depositions by adjusting the starting humidity with each iteration. We do not feel it is necessary to repeat the experiments with a glove box in order to state that as an alternative. We do now mention that a similar atmosphere could also be achieved in a glove box (lines 213-215).

3. The method itself is based on a double lift-off using first the polystyrene mask and then a metal mask generated after first metal evaporation. I was missing a discussion on metal selection for these processes (particularly copper , which is quite a nasty metal and makes this protocol harder to implement in a clean environment) In principle Cr mask should also be possible to use and to be etched away orthogonally to aluminium in Cr etchant. Also on the second lift-off step the authors rather used metal thickness compared to the mask thickness with ratio 2:1 (150 nm metal to 200-300nm pillars) This thickness ratio is borderlining to not resolve the liftoff (usual rule for lift-off is mask: metal 3:1) I would like to ask authors to comment about the metal thickness limitations and whether they experienced any liftoff problems at certain ratios.

4. Figure 1 and Figure 2 contain '3D' projections of the sample, but they are shown without perspective and hence hey look misleading instead of helping to understand the process. Images should either be converted to 2D or perspective should be introduced

We thank the reviewer for these comments. We did originally consider using chromium to create the first metal mask, but chromium etchant, unfortunately, also etches aluminum (https://transene.com/etch-compatibility/). We also tried using gold for the first metal mask, but gold etchant also etched away aluminum. Another gold etchant composed of zinc cyanide and potassium cyanide is indicated as compatible with aluminum on the Transene website, but we avoided the use of cyanide-based etchant due to its toxicity. Besides copper etchant, Ti-Tungsten etchant is another etchant compatible with aluminum, but this would etch the titanium layer used for aluminum adhesion to glass. Lastly, copper etchant is relatively safe to use and is mostly composed of citric acid. Thus, the choices of materials and etchants had a rational basis, and they worked well in our hands.

The reviewer is also correct in pointing out that the aluminum metal cladding thickness did pose some problems for metal post dissolution, which is why we introduced the rubbing step to access the posts for dissolution (steps 4a.7 or 4b.7). We have included a note (lines 300-302) to caution users about the thickness of the aluminum cladding. We also mentioned in the Discussion that the posts are likely pyramidal in shape, which increases the likelihood of the aluminum cladding covering the sides of the posts during deposition. We suggest in the Discussion that users can increase copper height for easier copper post dissolution and should ensure that sample plate holders are not rotating during aluminum deposition. However, we found that with the rub step, dissolution of almost all the copper posts (Fig. 5B) was consistently achievable with 1:3 – 1:2 ratios of aluminum cladding:copper post height (100 nm:300 nm – 150 nm:300 nm).

We also thank the reviewer for pointing out the lack of perspective in Figures 1 and 2 and have changed the cross-sectional views to 2D to reduce confusion.

Minor Concerns:
1) I think that readability of the paper can be dramatically improved if the authors could a) remove redundancy between results and discussions (or reshuffle the text to eliminate the redundancy of certain paragraphs)
b) provide careful linking between protocol-results - discussion. Everywhere in the protocol where the point is expanded to results or discussion - state it. And vice versus - everything that is discussed in results and discussion has to have reference to the particular points of the protocol that are discussed.

We thank the reviewer for this suggestion. We have taken care to read through the manuscript and remove or reshuffle redundant text in the Protocol, Results, and Discussion. We have also added references to the Results and Discussion in the

Protocol and references to the Protocol steps in the Results and Discussion. We believe this has improved readability of the manuscript.

c) Some of the figures contain panels that are not mentioned in text, text mentions Figure 7, where there is no Figure 7. Some of the figures carry the S prefix, where there is no distinct supplementary material attached

We thank the reviewer for catching these errors. For the supplementary figures, JoVE does not have separate sections for supplementary material, but does allow supplementary figures, which are labeled with the S prefix. These figures were included in the submission, and we have referenced all of them in the Results and Discussion.

Though this is a minor concern, I think it should be taken seriously by the authors
2) Authors suggest cleaning of the slide from the polysterine beads after first metal deposition using toluene overnight. Did the authors consider somewhat more harsh , but more efficient removals like RCA1 (could work for copper and Cr, but not for Aluminium). More efficient cleaning between the liftoff steps could provide better resolution and reproducibility

We thank the reviewer for this helpful suggestion and have added this in the Discussion as a possible modification to step 4a.4/4b.4 (lines 593-595).

3) The authors have mentioned dependence of the ZMW size on annealing time. It looks like they have sufficient imaging data to plot some calibration curve like diameter of ZMW vs annealing time. It would be very helpful for people who try to reproduce the protocol.

We thank the reviewer for this suggestion. We have done this in a previous publication introducing the method[1] (Figure 2 of Jamiolkowski et al., 2019). We focus on the practical implementation of the method in this JoVE article and refer to the earlier publication for this data.

4)The authors offer using the protocol for making both gold and aluminium ZMW, but the parts of the protocol that describe it are written out in a confusing manner since for gold ZMW pillars should be made out of aluminium. Therefore I suggest to split the gold ZMW in a separate small subprotocol.

We have separated the aluminum and gold ZMW protocols into two separate subsections (part 4a and 4b of Protocol).

5) It would be also worth mentioning nanoimprint lithography as a method for fabrication of ZMWs.

We thank the reviewer for bringing this method to our attention and have added it to the Introduction (lines 98-103).

Reviewer #6:

Manuscript Summary:

The authors describe a protocol for a unique and convenient fabrication of zero-mode wave guides via nanosphere lithography which circumvents the expense and time associated with traditional e-beam lithography or FIB approaches. While the fabrication protocol is detailed, the manuscript is lacking in details on how to test the fabricated ZMWs in a single-molecule experiment as no protocol was provided for testing ZMWs post fabrication. Further, it is unclear why devices fabricated via this approach have a dramatically lower maximal concentration (~200 times lower than expected) compared to devices fabricated by standard approaches. Given the understandable desire to make ZMWs more accessible to the broader biochemistry community with a less expensive fabrication scheme, it is important to add a discussion about the limitations of ZMWs fabricated via this approach. In revised form that addresses the comments below, this manuscript would be acceptable for publication.

Page Line Comment

- 1 39 The focus on single-molecule enzymology in the abstract and manuscript is a bit narrow and ignores the many labs that are using ZMWs to study other systems. The focus instead should be on studying weak-affinity interactions (e.g. enzyme-substrates, protein-ligand interactions, protein-protein interactions). The authors may consider reviewing and citing the following comprehensive and up-to-date review of ZMWs:

Crouch, Garrison & Han, Donghoon & Bohn, Paul. (2018). Zero-mode waveguide nanophotonic structures for single molecule characterization. Journal of Physics D: Applied Physics. 51. 193001. 10.1088/1361-6463/aab8be

We thank the reviewer for bringing this review article to our attention and have added this reference and discussion of other applications of ZMW technology to the Introduction (lines 90-93).

- 2 166 Does the fabrication of ZMWs via nanosphere lithography need to be accomplished inside a clean-room or can all steps be performed in general lab space? This should be made clear in the steps of protocol. Clean-room access itself can be limiting for production of ZMWs.

We thank the reviewer for this question. None of the steps need to be completed in a clean room. Some institutions may already have electron beam or thermal evaporation systems installed in clean rooms, but this protocol does not assume access to a clean room. We have added a note indicating this in the Protocol (line 134).

- 8 366 Details about how the authors performed the single-molecule FRET experiments

for testing ZMWs need to be included. For example, which buffers were used; what excitation source(s) were used; what was the frame rate of collection; which lasers, objectives, and detector were used? These details are necessary for the reproducibility of the experiments and validation of successful fabrication.

We thank the reviewer for catching this omission. We have added more details on the imaging protocol to the Results section (lines 397-422) as well as reference to the similar smFRET imaging protocol we used in our previous paper[1] introducing the fabrication method.

- 8 376 The fact that signal-to-noise deteriorating at 500 nM with the use of FRET inside ZMWs is concerning. FRET increases the effective concentration limit of ZMW and, in fact, Goldschen-Ohm et al., has shown that FRET in ZMWs works ups to 1 mM. The authors need to comment on why the signal-to-noise is much lower in ZMWs fabricated via nanosphere lithography compared to traditional approaches (e.g. e-beam lithography, FIB) that can get access to single digit uM.

M. P. Goldschen-Ohm, D. S. White, V. A. Klenchin, B. Chanda, R. H. Goldsmith, Angew. Chem. Int. Ed. 2017, 56, 2399.

8 378 What is the highest concentration of a freely diffusing dye (no-FRET) that the authors can still detect inside the ZMWs? Typical ZMWs have a detection limit around 1-10 μM.

We thank the reviewer for bringing this article to our attention. In our previous publication[1] introducing our method, we included single molecule data on freely diffusing dye at 0 nM, 1 nM, 10 nM, 50 nM, 500 nM, and 1 μM concentrations, consistent with the 1-10 μM detection limit that was observed for freely diffusing dye in Goldschen-Ohm et al., 2017. This detection limit was also consistent with gold ZMW data from Kinz-Thompson et al., 2013[5], who fabricated the ZMWs with electron beam lithography.

We acknowledge that the signal to noise of the acceptor dye from FRET excitation is not as high as that observed in Goldschen-Ohm et al., 2017. This could be due to differences in experimental setup. Goldschen-Ohm et al., 2016[7] suggested that the FRET efficiency between the fluorescent cyclic nucleotide and DyLight650 on the cyclic nucleotide binding domain was near 100%, while the apparent FRET efficiency between our oligo bound Cy3 and Cy5 was around 50%. In addition, the donor dye rather than the acceptor dye was freely diffusing in solution in Goldschen-Ohm et al. In contrast, our experiment had the acceptor dye diffusing in solution. Since Goldschen-Ohm et al. varied the concentration of the donor dye, the acceptor dye trace from FRET was likely less affected by higher concentrations. Another previous study (Zhao et al., 2014)[8] measuring FRET in EBL-fabricated ZMWs also reported issues with nonspecific binding of the diffusing acceptor labeled protein to the ZMWs, making FRET difficult to resolve at diffusing acceptor concentrations higher than 1 μM. We have added discussion on

the FRET concentration limit to the text (lines 598-612).

9 431 In Figure 6 (or elsewhere), could the authors provide the average spacing between ZMWs?

We thank the reviewer for catching this omission. The distance between the centers of adjacent ZMWs is set by the hexagonal close packing of the 1 μm beads ($\frac{2}{\sqrt{3}} * 500\ nm = 557\ nm$). The average waveguide spacing of 559 nm measured in the high-resolution AFM image in Figure 5C is consistent with the packing geometry. We have added this to the Results (lines 389-392).

11 492 Thorough cleaning of the cover-glasses prior to ZMW fabrication is indeed critical for successful metal deposition. However, the proposed method herein uses a crude cleaning procedure (KOH, organic washes) compared to the more commonly used cleaning methods, such as SC-1, piranha, or oxygen plasma. Could the authors comment on how this cleaning protocol was established and why more robust procedures were not used?

We thank the reviewer for this question. In a previous JoVE article detailing a cleaning protocol for TIRF smFRET experiments[6], the glass slides were cleaned with organic washes and piranha and then used directly for imaging. We based the cleaning procedure in part 1 of the Protocol on the organic wash steps in Chandradoss et al. and found that sonication with KOH and organic solvents sufficiently cleaned the glass coverslip surfaces to obtain well-spread monolayer bead templates during part 2 of the Protocol. Since the glass slides still go through many subsequent treatments for ZMW fabrication after bead deposition, we saved additional cleaning for later steps, including plasma oxygen cleaning in step 4a.4/4b.4. We have also added in the manuscript that additional cleaning of the ZMW with aluminum compatible acid wash or Piranha can be used before passivation (lines 595-597).

References

1 Jamiolkowski, R. M. *et al.* Nanoaperture fabrication via colloidal lithography for single molecule fluorescence analysis. *PLOS ONE.* **14** (10), e0222964, (2019).

2 Foquet, M. *et al.* Improved fabrication of zero-mode waveguides for single-molecule detection. *Journal of Applied Physics.* **103** (3), 034301, (2008).

3 Levene, M. J. *et al.* Zero-mode waveguides for single-molecule analysis at high concentrations. *Science.* **299** (5607), 682-686, (2003).

4 Miyake, T. *et al.* Real-Time Imaging of Single-Molecule Fluorescence with a Zero-Mode Waveguide for the Analysis of Protein–Protein Interaction. *Analytical Chemistry.* **80** (15), 6018-6022, (2008).

5 Kinz-Thompson, C. D. *et al.* Robustly Passivated, Gold Nanoaperture Arrays for Single-Molecule Fluorescence Microscopy. *ACS Nano.* **7** (9), 8158-8166, (2013).

6 Chandradoss, S. D. *et al.* Surface passivation for single-molecule protein studies. *J Vis Exp.* 10.3791/50549 (86), (2014).

7 Goldschen-Ohm, M. P. *et al.* Structure and dynamics underlying elementary ligand binding events in human pacemaking channels. *eLife.* **5** e20797, (2016).
8 Zhao, Y. *et al.* Dark-Field Illumination on Zero-Mode Waveguide/Microfluidic Hybrid Chip Reveals T4 Replisomal Protein Interactions. *Nano Letters.* **14** (4), 1952-1960, (2014).