**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author reply: Thanks a lot for this editorial comment. We already did it.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Author reply: We did this.

3. Figure 1: Please explain the left and right panels in the figure legend.

Author reply: We added the corresponding information.

4. Figure 2: Please label the different parts, if possible.

Author reply: Thanks a lot for this comment. We believe that the current version is clear enough.

5. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Author reply: We added the information.

6. Please revise lines 59-65, 76-78, 257-259, 282-284 to avoid previously published text.

Author reply: We have done this.

7. Please provide an institutional email address for each author.

Author reply: We added the information.

8. Keywords: Please provide at least 6 keywords or phrases.

Author reply: Yes, we added two more keywords.

9. Abstract: Please expand the Abstract to briefly describe the advantages, limitations, and applications of the technique.

Author reply: We added the information.

10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: EKSPLA, Sigma-Aldrich Co., LLC, Nanjing Genscript Biotechnology Co. Ltd, etc.

Author reply: We corrected the commercial language.

11. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Author reply: We did this.

12. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Author reply: We have revised the text.

13. 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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Author reply: We have corrected the phrased as required.

14. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Author reply: Thanks a lot. We have tried our best to polish the protocol on calculating the Fresnel coefficients describes. We believe that the current version is concise and to the point.

15. Lines 73-215: Please note that calculations are not appropriate for filming; therefore I suggest unhighlighting these steps.

Author reply: We agree with comment and unhighlighted these steps.

16. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to publishbed material specifying how to perform the protocol action. See examples below.

17. Line 220: Please list an approximate volume and concentration of the solution to prepare.

Author reply: We have added the concentration.

18. Line 226: Please describe how this is done. What are the substrates?

Author reply: We added the necessary information.   
19. Lines 229-231: Please describe how to prepare the PHEMA films using a spin-coater and specify the film thickness as well as the solution concentration and spin speed used in this step.

Author reply: We did this.  
20. Line 233: How many films are produced?

Author reply: One film was prepared on one substrate. And we repeated the experiment for at least 4 or 5 times.  
21. Line 238: What volume of Na2CO3 solution is used?

Author reply: The solution volume used was 3 L.   
22. Line 241: Is the SF product removed from the solution in step 2.1 and placed in a container with deionized water? Please clarify. What volume of deionized water is used?

Author reply: Yes, it is. We have clarified it.

23. Line 246: What volume of LiBr solution is used?

Author reply: 1 g SF was solved in ~4ml LiBr solution.   
24. Line 249: How often is the deionized water changed during dialysis?

Author reply: The deionized water was changed 3 times every day.   
25. Line 288: Please describe how to perform the SFG measurement. For instance, please revise the paragraph in lines 55-72 to a stepwise protocol showing how the measurement is done.

Author reply: In the SFG experiment, we only need to adjust the optical path. It is difficult to present how to optimize the optical path by adjusting the rotary knobs. We sincerely hope the editor can understand this.   
26. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

Author reply: We added the necessary information in the discussion part.

“To investigate the structural information from a molecular level, SFG has its inherent advantages, saying, monolayer or sub-monolayer sensitivity and interfacial selectivity, which can be applied to study various interfaces, such as the solid/solid, solid/liquid, solid/gas, liquid/gas, liquid/liquid interfaces. Although the equipment maintenance and the optical alignment are still time-consuming, the payback is significant in that the detailed molecular-level information at the surfaces and interfaces can be obtained.”

27. References: Please do not abbreviate journal titles.  
Author reply: We have corrected them.

**Reviewers' comments:**  
**Reviewer #1:**  
Manuscript Summary:  
**General Comment:** In this manuscript, the authors demonstrate an experimental methodology to selectively detect the buried interface, which could be potentially useful to avoid the interference by the 2 interfaces of a thin film. Instead of the conventional method of using with different incident angles to selectively detect the interface in SFG field, in this journal, the authors fixed the incidental angle, and calculated Fresnel coefficient of different polarizations as a function of thickness, then choose the suitable thickness to selectively detect the substrate/film interface or film/air interface.  
I think it is a good paper to be published in JoVE after answer the follow questions and minor corrections in the manuscript.  
**Author Reply:** We thank the reviewer for such comments. The interference effect can lead to difficulty to analyze the SFG spectra, which is the reason why we introduced a methodology to solve it.

**Major Concerns:**  
**Comment 1:** Selectively detecting the 2 interfaces (film/substrate and film/air) by using 2 different incidental angles is quite well-known in the SFG field - for example, in the paper of Langmuir, 2015, 31 (45), pp 12401-12407; the authors used incident angles (with respect to the surface normal of the sapphire prism face) of 42°, 16°, and 2° to probe the Polymer/air, Polymer/H2O (and Polymer/D2O), and Polymer/sapphire interfaces, respectively. Since the refractive index of mediums are different, thus the calculated incident angles are also different and could be used to distinguish the interfaces/surfaces. How did the author compare their methodology of using 2 thicknesses to differentiate the 2 interfaces vs. the conventional method of using 2 different incidental angles to differentiate the 2 interfaces? What is the advantage of the authors method?

**Author Reply:** We thank the reviewer for raising such a good question. We carefully read this paper, Langmuir, 2015, 31 (45), pp 12401-12407. The methodology by adjusting incident angles is an alternative way in this case. We cannot say which one (theirs and ours) is better since both are feasible and useful. For ours, to detect two single interfaces respectively, two samples have to be prepared with the appropriate thicknesses. For theirs, one sample with the chosen thickness should be enough. However, changing the incident angles will definitely change the optical path. The authors (Langmuir, 2015, 31 (45), pp 12401-12407) have to play with it. We sincerely hope the reviewer can be satisfied with our reply.

**Comment 2:** In the 2nd paragraph of discussion portion, the authors discussed the results of 2 different conformation of below and above the critical concentration. Isn't it better to support authors results by providing SFG spectra evidence of below this threshold concentration (ordered secondary structure) and above the threshold concentration (overlapped secondary structure)?

**Author Reply:** We thank the reviewer for raising such a positive comment. We added the corresponding SFG spectra.



**Figure 7.** Normalized chiral (*psp*) SFG spectra in the amide I (Panel A) and N-H (Panel B) ranges for the PS/SF solution (90 mg/mL) interface before and after adding methanol. The dots are experimental data and the solid lines are the fitted curves. Spectra have been offset for clarity.



**Figure 8.** Normalized chiral (*psp*) SFG spectra in the amide I (Panel A) and N-H (Panel B) ranges for the PS/SF solution (1 mg/mL) interface before and after adding methanol. The dots are experimental data and the solid lines are the fitted curves (blue). Spectra have been offset for clarity.

**Comment 3:** In the 3rd paragraph of discussion portion, instead of just explanation, shall the authors prove the experiment data / spectra evidence to prove the addition of of Ca2+ concentration effect on the water SFG vibrational signals?

**Author Reply:** Yes, according to this comment, we added the corresponding SFG spectra.



**Figure 9.** Achiral (*ssp*, A) and chiral (*spp*, B) SFG spectra for the duplex oligonucleotide-anchored lipid bilayer in contact with the Ca2+ solutions with different concentrations (from 0.6 mM to 6 mM). The data points were approximately fitted by using the Lorentz equation. The change of the integrated area for the water vibrational signals as a function of the Ca2+ concentration was presented (*ssp,* C; *spp*, D). All the spectra have been normalized and offset for clarity.

**Minor Concerns:**

**Comment 1:** How did the authors get the peak position labeled in Figure 5 and 6? It would be better for the authors fit the spectra with Lorentzian equation and then get the accurate peak positions.

**Author Reply:** We thank the reviewer for raising such a good comment. We fitted the spectra with Lorentz equation and the results are shown in Figure 5 and 6.

**Reviewer #2:**  
Manuscript Summary:  
**General Comment:** This manuscript describe the overall protocol of the analysis of the sum-frequency spectra using regional formulation, preparation of the samples on the specific prism, and the analysis of the results. I therefore would recommend acceptance in the JOVE if the authors can fully address my suggestions described below, which would improve the quality of the manuscript.

**Author Reply:** We thank the reviewer for the positive comment. SFG is a powerful tool to study the molecular-level structures at the surfaces/interfaces. Here, we provided the protocol by summarizing our recent experimental results.

Minor Concerns:  
**Comment 1:** It would be better to show the spectral fitting procedures.

**Author Reply:** Yes, according to this comment, the spectral fitting procedures were added in the protocol section.

**Lorentz Equation**

The Lorentz equation was used to fit the SFG spectra to extract the vibrational information such as the intensity, half width at half maximum (HWHM) for a specific vibrational mode.

(25)

where represents the intensity of the *qth* vibrational mode, represents the resonant frequency, denotes the half width at half maximum (HWHM) and represents the scanning frequency of the incident IR beam.

**Reviewer #3:**  
Manuscript Summary:  
**General Comment:** Clear and concise summary.

**Author Reply:** We thank the reviewer for this positive comment.

Major Concerns:  
**Comment 1**: Context of the work is only briefly introduced. A more comprehensive background, with particular emphasis to the SFG technique, its uniqueness and usefulness in the investigation of material interface properties should be given. More specific information can be added as to what "fundamental science from the molecular level" can be understood. Also, it is not clear how the actual samples were selected and what is the specific aim of the study.

**Author Reply:** We added contents in light of the reviewer’s comment, “Currently, a broad range of scientific issues related to surfaces and interfaces are being investigated using SFG, especially for complex interfaces with respect to polymers and biomacromolecules, such as the chain structures and structural relaxation at the buried polymer interfaces, the protein secondary structures and the interfacial water structures9-26.”

Minor Concerns:  
**Comment 1:** More consistency e.g. sometimes authors refer to "prism" other times to "substrate" (e.g. point 3.1.6). More clarity as to what is the "bottom medium" in these measurements (e.g. point 2 and Fig.3).

**Author Reply:** We thank the reviewer for this constructive comment. The prism and the substrate are the same thing. We believe that the audiences can understand this.

To clarify the “bottom medium”, we added one note in a bracket, “(the bottom medium can be air, liquid or solid.)” at point 2 and the legend of Fig.3.