nature research

Peer Review Information

Journal: Nature Methods
Manuscript Title: Super-resolution SRS microscopy with A-PoD
Corresponding author name(s): Lingyan Shi

Editorial Notes:

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Lingyan,

Thank you for submitting your manuscript entitled "Super-resolution stimulated Raman scattering (SRS) microscopy". We have given the paper our careful consideration but we regret that we cannot publish it in Nature Methods in its current form.

Among the considerations that arise at this stage are a manuscript's probable interest and immediate practical relevance to a general readership. We do not doubt the technical quality of your work or that it will be of interest to others working in SRS microscopy. However, we do not think that the technical advances presented in the current version of the manuscript will have a sufficiently significant impact on a broader readership to justify publication in Nature Methods.

Our biggest concern was that there was very little in terms of validation of the method compared to ground truth on experimental data. It was not clear to us that the results of the image processing resulted in real and quantitatively correct structures, despite the clear improvements in contrast and resolution. This is an issue that comes up a lot for us with computational methods for improving image resolution.

We would encourage you to expand the paper in this direction to ensure the results are robust and reproducible for biological downstream analysis.

We also were curious whether you could, for example, use the method with fluorescence, where you could generate a super-resolution 'ground truth' image matched to a diffraction limited one. This would also be the first use of this algorithm on fluorescence microscopy, which could broaden the overall appeal of the paper.

Of course, if you added further experiments along these lines, the manuscript should be expanded to a full Article.

Should future experimental data allow you to address our concerns, we would be happy to look at a revised manuscript (unless, of course, something similar has by then been accepted at Nature Methods or appeared elsewhere). This includes submission or publication of a portion of this work somewhere else. In the case of eventual publication, the received date would be that of the revised paper.

If you are interested in submitting a suitably revised manuscript in the future or if you have any questions, please contact me. I am very happy to discuss any of these points.

Thank you for your interest in Nature Methods. I am sorry that on this occasion we cannot be more positive.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Author Rebuttal to Initial Decision

Dear Rita,

Hope all is well.

Thank you so much for giving us constructive feedback on our super resolution SRS imaging manuscript! We have been working on it according to the comments and feedback you and other committee members gave. We significantly improved the manuscript into a full research article instead of a brief communication. Please see our updated manuscript attached to this email.

If Nature Methods is interested in this super resolution SRS manuscript, we will be very excited to submit it as a full research paper for consideration.

Thank you so much.

Looking forward to hearing from you.

Best regards,

Lingyan

Decision Letter, first revision:

Dear Lingyan,

Thank you for your letter asking us to reconsider our decision on your Article, "Super-resolution stimulated Raman scattering (SRS) microscopy". We found the revision greatly improved, and we thank you for your additional efforts.

We are interested in sending your manuscript for review. This is an opportunity for you to resubmit the final versions that are ready for the referees.

When revising your paper:

* address the points listed described below to conform to our open science requirements (not doing so will delay sending the paper out)

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[Redacted] This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope to receive your revised paper within two weeks. If you cannot send it within this time, please let us know. In this event, we will still be happy to reconsider your paper at a later date so long as nothing similar has been accepted for publication at Nature Methods or published elsewhere.

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Please make code and test data available to the referees.

Please include a "Code Availability" subsection in the Online Methods which details how your custom code is made available. Only in rare cases (where code is not central to the main conclusions of the paper) is the statement "available upon request" allowed (and reasons should be specified).

We request that you deposit code in a DOI-minting repository such as Zenodo, Gigantum or Code Ocean and cite the DOI in the Reference list. We also request that you use code versioning and provide a license.

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We look forward to hearing from you soon.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Decision Letter, second revision:

Dear Lingyan,

Please let me begin by apologizing again for our delay in getting feedback from the three reviewers.

Your Article entitled "Super-resolution stimulated Raman scattering (SRS) microscopy" has now been seen by three reviewers, whose comments are attached. While they find your work of potential interest, they have raised serious concerns which in our view are sufficiently important that they preclude publication of the work in Nature Methods, at least in its present form.

As you will see, the reviewers raise concerns about the choice of PSF, the achieved resolution, and the benefits over existing deconvolution approaches, in addition to more detailed concerns about the data. They also raise concerns about the level of novelty, but to our view, these are less important than the concerns about performance.

Should further experimental data allow you to fully address these criticisms we would be willing to look at a revised manuscript (unless, of course, something similar has by then been accepted at Nature Methods or appeared elsewhere). This includes submission or publication of a portion of this work somewhere else. We hope you understand that until we have read the revised paper in its entirety we cannot promise that it will be sent back for peer-review.

If you are interested in revising this manuscript for submission to Nature Methods in the future, please contact me to discuss your appeal before making any revisions. Otherwise, we hope that you find the reviewers' comments helpful when preparing your paper for submission elsewhere.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript, the authors have presented a computational approach to perform fast deconvolution on stimulated Raman scattering microscopy images and received an apparent improvement in spatial resolution. The algorithm, Adam optimization-based Pointillism Deconvolution (A-PoD), has provided a

significant improvement in time consumption and spatial resolution in several biological applications. The claimed spatial resolution has reached below 100 nm, which is by far the highest compared to published literatures. Based on this high spatial resolution, new potentials in biomedical applications were demonstrated.

There are a couple of advantages of this approach. First, it is computational based, therefore, the barrier to be employed in different labs is minimized. Second, the algorithm itself is not limited to SRS microscopy, it has a potential to be applied in other imaging modalities as well, which provides similar spatial resolution compared to previously published methods like DAOSTORM.

Beside these benefits, there are some concerns on the manuscript as well.

1, The inputs for A-PoD, including the PSF of pump and Stokes beams, are generated by another piece of code, which is idealized. It will be more convincing if the PSF can be measured, for example using fluorescent microbeads, in real experiments and then used as inputs in the algorithm.

2, In the experiment of the standard sample, the authors used 270 microbeads, which are not small enough, considering the raw measurement to be 364 nm in FWHM. The authors should try smaller, such as 100 nm, PS beads. Additionally, the authors need to verify if the microbeads are NIST traceable size standard.

3, The classification and definition of the membrane and the core of lipid droplets are ambiguous. How are they separated in A-PoD images (Fig. 2C)? How the membrane is defined? The phospholipid bilayers are less than 1nm, and the membrane of lipid droplets only has a single layer that is even thinner. This spatial resolution is far below the limitation that this manuscript has claims. It is unclear how such separation is achieved from SRS image without specific labeling being applied.

4, There are differences between A-PoD reconstructed images and other algorithms. The authors should have an explanation on the difference. For example, in Extended Data Fig. 2, the A-PoD result presents discontinuous structures along the mitochondria membrane. In Extended Data Fig. 3 a iii-iv, the A-PoD result obtained structures are larger than claimed resolution and are also different from DAOSTORM.

In summary, more controls and clarification are needed to support the spatial resolution claim of this deconvolution approach and its application in biomedical samples. Based on the data shown in the manuscript, the algorithm provides similar deconvolution results comparable to other published methods in literature.

Reviewer #2:

Remarks to the Author:

Dear Authors,

By reviewing the manuscript entitled "Super-resolution SRS microscopy with A-PoD" I found it relevant and important.

The algorithm used to improve the resolution is not new, it was previously used in fluorescence spectroscopy. However, it is used for the first time for the SRS microscopy.

In the presented analysis, it is not entirely clear why only lipids should be identified by the marker band of 2140 cm-1, CD vibration. This band is related to the presence of D2O in the sample, but why are only lipids produced by biosynthesis?
Another issue is the Mito-Red staining of the mitochondria. How specific is this marker? are we sure that it only stains mitochondria and not lipids?
In a word, does tracing the D-C band carry information about de novo triggered compounds or just a D2O uptake?

Reviewer #3:
Remarks to the Author:
Shi et al presents a superresolution method for SRS imaging based on deconvolution technique, called Adam optimization-based Pointillism Deconvolution (A-PoD). Superresolution images are demonstrated in a variety of samples and compared with the ground truth. Biological application of this technique is also conducted in lipid metabolism in living cells and tissues. the analysis of the size and distribution of the lipid droplets inside living cells are impressive, as this study could only be done by using super-resolution SRS technique with a low excitation power. The deconvolution SRS could be one of the good options in a more economical way.

The authors claim the development of a novel deconvolution algorithm A-PoD in this work, which can only be considered as an improved version of SUPPOSe in literature (Ref 24), as the main idea of A-PoD is identical to SUPPOSe (Ref. 24). The difference lies in step 2 of SUPPOSe: the authors use Adam solver (Ref. 69) instead of the genetic algorithm to minimize the L2 norm. In this regard, A-PoD is a combination of Refs. 24 and 69. The authors also claim that such combination is non-trivial, since the imaging processing speed is greatly improved in A-PoD. Here I have one question:
1.1 Genetic algorithm is chosen in Ref 24, in order to avoid converging to local minima. Of course, gradient decent method, Adam, used in A-Pod is much faster than the genetic algorithm, but the cost is the risk to be trapped in local minima. The authors should investigate such possibility quantitatively, e.g., compare the results obtained by A-Pod and SUPPOSe with the ground truth in terms of L2 norm?

Meanwhile, there are also several technical problems about PSF and resolution as outlined below, which should be fully addressed in the revised manuscript.
1. About the choice of PSF: an accurate PSF plays a key role in a deconvolution algorithm. In this manuscript, the authors have tried the PSF of pump, Stokes and PSF_conv (convolution of the PSFs of pump and Stokes), respectively. Finally, they choose PSF_conv, since the FWHM of the beads in the deconvoluted image matches well with the actual bead size. In this regard, I have two questions:
1.1 The choice of the PSF is incorrect. The correct PSF should be the product of pump and Stokes, i.e., PSF_SRS=PSF_pump*PSF_Stokes, because the SRS signal is proportional the product of pump and Stokes

intensities (Ref. 13 in this manuscript). In addition, it is also improper to choose PSF based on the criteria "FWHM of the beads in the deconvoluted image matches well with the actual bead size", since the former should always be bigger than the latter, due to nonzero PSF size after deconvolution.
1.2 Those PSFs were generated numerically by ImageJ. the authors should compare the simulated PSFs with the measured ones to verify the conclusion. As the actual PSF may be quite different from the simulated one, due to the imperfection of the objective lens and the beam profile in the optical system.

2. About the resolution analysis: the authors claim 52-nm resolution achieved which is misleading. Such resolution is calculated by applying the decorrelation analysis (Ref 25) to the deconvoluted image of 270 nm beads (Fig. 2a). I have three questions:
2.1 I cannot find the decorrelation analysis in Ref 25. Is it the correct Ref? Please provide the details of resolution analysis, e.g., relevant equations.
2.2 For an accurate resolution analysis, the sample size should be smaller than, or at least close to, the resolution. However, in this manuscript, the sample size (270 nm) is much larger than the resolution (52nm). Therefore, such result is not reliable.
2.3 In deconvolution microscope, the final resolution is determined by a lot of factors, e.g., the deconvolution algorithm, the accuracy of PSF and SNRs. In fact, if there's no noise and we have precise PSF, the resolution after deconvolution can be infinitely high, even if we use simple Richardson-Lucy deconvolution algorithm (Ref. 61 in this manuscript). Therefore, it is more meaningful and practical to analyze the resolution by providing SNRs. It would be more convincing if the resolution is analyzed for a series of SNR levels.

Hence, in terms of lackings of the method originality and technical clarities and accuracies in the current work, the reviewer will not recommend for the acceptance of the manuscript for NM.

Author Rebuttal, second revision:

Reviewer #1

Remarks to the Author:

In this manuscript, the authors have presented a computational approach to perform fast deconvolution on stimulated Raman scattering microscopy images and received an apparent improvement in spatial resolution. The algorithm, Adam optimization-based Pointillism Deconvolution (A-PoD), has provided a significant improvement in time consumption and spatial resolution in several biological applications. The claimed spatial resolution has reached below 100 nm, which is by far the highest compared to published literatures. Based on this high spatial resolution, new potentials in biomedical applications were demonstrated. There are a couple of advantages of this approach. First, it is computational based, therefore, the barrier to be employed in different labs is minimized. Second, the algorithm itself is not limited to SRS microscopy, it has a potential to be applied in other imaging modalities as well, which provides similar spatial resolution compared to previously published methods like DAOSTORM. Beside these benefits, there are some concerns on the manuscript as well.

We thank the reviewer for the positive comments and highlights of the key strengths of this study. We have revised our manuscript based on the comments and give our detailed point-by-point response to the comments below.

1. The inputs for A-PoD, including the PSF of pump and Stokes beams, are generated by another piece of code, which is idealized. It will be more convincing if the PSF can be measured, for example using fluorescent microbeads, in real experiments and then used as inputs in the algorithm.

Response:

We thank the reviewer for this constructive suggestion. We agree that using measured PSF as input might work better than using simulated PSF. Therefore, to compare the results using simulated PSF and experimental PSF, we measured the PSF using 100 nm PS beads. From the FWHM of intensity profile of the beads, we measured the size of experimental PSF (Fig. R1, Extended Data Fig. 4a in manuscript). However, using measured PSF did not show any significant difference in the deconvolution results and in the spatial resolution, compared with those by simulated PSF (54 nm vs. 57 nm). Both images did not have any significant artifact or distortion either. In addition, we found the deconvolution result by using the simulated PSF showed the same size of bead image (Fig. R2).

One of the main objectives of this study was to present an algorithm that could significantly increase the resolution for SRS imaging and potentially for other imaging modalities, just as the reviewer commented that "the barrier to be employed in different labs is minimized". We have demonstrated the algorithm on polymer beads and a variety of biological organelles using theoretical PSF, and obtained an enhanced image resolution as well as greatly increased processing speed, which fully validated the capability of this algorithm. Actually, simulated PSF has been previously applied to single molecule localization microscopy (Sage et al., Nat. Methods 2015 and Sage et al., Nat. Methods 2019), and a 3D theoretical model of PSF has also been applied to deconvolution of 3D images (Hayato et al., Sci Rep 2018).

Using simulated PSF for A-PoD allows us to improve the spatial resolutions of already existing/measured images, whose experimental PSFs may not be achievable due to the

change of imaging condition/alignment. This approach will have broader applications in improving the spatial resolutions of imaging while without any significant difference compared with experimentally measured PSF.

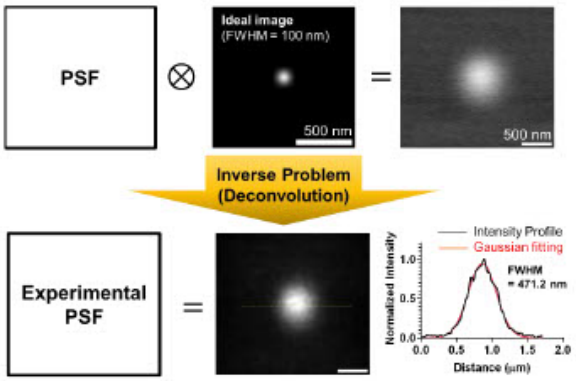

Fig. R1 Measurement of experimental PSF (PSF_{exp}). Experimental PSF was extracted from 100 nm bead image. By deconvolving the measured bead image with artificial 2D Gaussian image having 100 nm FWHM, experimental PSF was calculated. The FWHM of the experimental PSF was 471.2 nm.

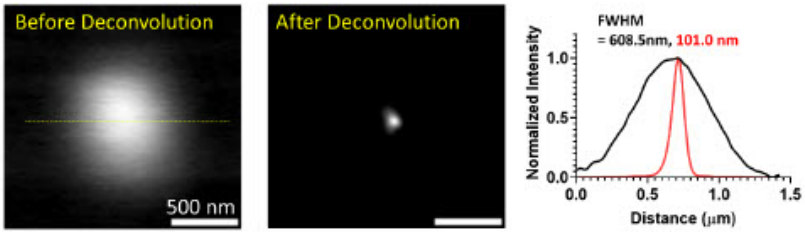

Fig. R2 Deconvolution result of 100 nm bead image with simulated PSF. Using the simulated PSF (PSF_{mul}), the 100 nm bead image was deconvolved. FWHM of image was decreased from 608.5 nm to 101.0 nm.

2. In the experiment of the standard sample, the authors used 270 microbeads, which are not small enough, considering the raw measurement to be 364 nm in FWHM. The authors should try smaller, such as 100 nm, PS beads. Additionally, the authors need to verify if the microbeads are NIST traceable size standard.

Response: Thank you for the good suggestion. We have now measured the PSF using 100 nm NIST traceable beads (Invitrogen, T7279) as suggested (Fig. R1). The results are compared in Fig. R3 (Extended Data Fig. 4 in the revised manuscript), and in the subsection

"Standard sample measurement" in the revision (Results section). Both the 100 nm and 1 μm beads we used are NIST standard traceable beads.

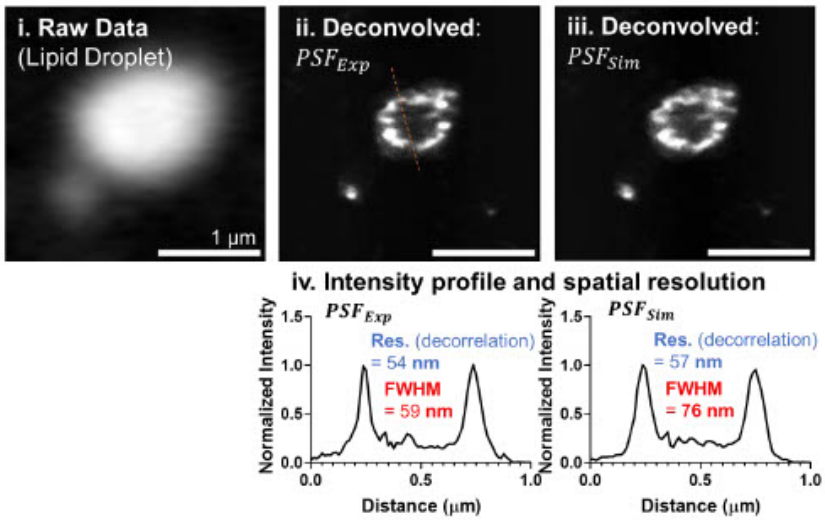

Fig. R3 Comparison of two PSF models. Single LD image was deconvolved using the simulated PSF and experimental PSF. After deconvolution, the raw LD image (in i) was converted to the two images (in ii and iii). From the intensity profiles of the three deconvolved images, membrane thickness was measured. The thinnest part has 59 nm and 76 nm for PSF_{exp} and PSF_{sim}, respectively (in iv). Spatial resolution measured with the decorrelation method was also 57 nm for the three results.

3. The classification and definition of the membrane and the core of lipid droplets are ambiguous. How are they separated in A-PoD images (Fig. 2C)? How the membrane is defined? The phospholipid bilayers are less than 1nm, and the membrane of lipid droplets only has a single layer that is even thinner. This spatial resolution is far below the limitation that this manuscript has claims. It is unclear how such separation is achieved from SRS image without specific labeling being applied.

Response: We agree that the thickness of typical phospholipid bilayers is small (around 4~5 nm, Regan et al., Langmuir. 2019) and the monolayered lipid droplet membrane is even thinner, but the membrane can be detected by SRS based on the -CH vibration signal. A-PoD algorithm calculates the most probable locations of virtual emitters based on the pattern and shape of blur in a raw image, so the precise shape of objects in image is important to find exact locations of virtual emitters. High signal to noise ratio is an important factor to improve spatial resolution of results, while SRS enhances Raman signal significantly (Freudiger et al. Science, 2008). Therefore, the strong signal to noise ratio of SRS lets A-PoD generate super-resolved image lipid membranes with high precision.

Although we could not identify the exact boundary of the membrane, we were able to visualize and distinguish the outer layer membrane and internal space of the lipid droplets. This could not be achieved without A-PoD based SRS, as shown in Fig. 1b. We agree with the reviewer

that the membrane of the lipid droplets could not be clearly defined in our images, we have made a clarification in the revised manuscript, using "the outer layer" rather than "membrane" of LDs (line 202 and 203).

4. There are differences between A-PoD reconstructed images and other algorithms. The authors should have an explanation on the difference. For example, in Extended Data Fig. 2, the A-PoD result presents discontinuous structures along the mitochondria membrane. In Extended Data Fig. 3 a iii-iv, the A-PoD result obtained structures are larger than claimed resolution and are also different from DAOSTORM.

Response: We have added the explanation of such differences in the Discussion. Regarding the apparently discontinuous structure along the mitochondrial membrane, the underlying reason is not exactly clear. It could be due to the raw data itself. Since the algorithm uses discrete virtual emitters and optimizes their distribution, discontinuity may occur during this process. The discontinuous structure is also presented in the literature using the SPIDER algorithm (Hugelier et al., Sci Rep 2016).

In Extended Data Fig. 3, the deconvolution result shows a periodic structure in the neurite. We discussed the difference and quantified the error in the subsection of "STORM image analysis" and Discussion. The difference of the results obtained from A-PoD and STORM is less than 20%. The precision might also be affected by SNR and pixel size, which is a general limitation of most algorithms, not just in A-PoD. In addition, the broad background signal can change the deconvolution result. In a previous paper (Hugelier et al., Sci Rep 2016), the authors also removed low-frequency background using spline. The low-frequency component of background signal may contribute to the differences observed in Extended Data Fig. 3a iii-iv. This is also mentioned in the revision (subsection "STORM image analysis")

Nevertheless, the deconvolution result from averaged widefield image describes the overall shape of mitochondria membrane clearly and the resultant image is sharp enough to resolve these biologically relevant structures (Extended Data Fig. 2).

In summary, more controls and clarification are needed to support the spatial resolution claim of this deconvolution approach and its application in biomedical samples. Based on the data shown in the manuscript, the algorithm provides similar deconvolution results comparable to other published methods in literature.

Response: Following the reviewer's suggestion, additional evidence, explanation, and discussion were added to the revision. Our algorithm yields a resolution better than published methods. As shown in Extended Data Fig. 1c, Richardson-Lucy algorithm cannot converge the size of spot to a single pixel, but A-PoD can. In addition, Richardson-Lucy the A-PoD result of USAF-1951 resolution target also shows a 20% higher resolution than Richardson-Lucy (Extended Data Fig. 8), even though we used theoretical PSF for the A-PoD algorithm. Importantly, our method also greatly enhances the processing speed compared with other algorithms. All these resulted in a substantial major leap forward rather than incremental improvement over the previous methods.

The novelty of the current study includes:

First, the A-PoD method is a development of a new deconvolution method. We compared the difference between A-PoD and Lucy-Richardson, a most generally used algorithm, in Results and Discussion (Extended Fig. 1 and 8). Lucy-Richardson algorithm and other methods have

also been compared in other literatures (Hayato et al, Sci Rep 2018 and Sage et al, Methods 2017). A-PoD method minimizes the ringing artifact near objects, as shown in Extended Fig. 1b and in Discussion (line 364). Although the main equation of image deconvolution (equation in Fig 1.a) shows how to remove blur and improve resolution, because of the artifact, deconvolution microscopy has been considered as a different technique from normal super-resolution microscopy. SUPPOSe shows the theoretical capability, but A-PoD shows the practical capability of super-resolution deconvolution microscopy with much faster speed (2s for processing a mitochondria image, compared to 96 min using SUPPOSe, Extended Data Fig. 2b).

Second, SRS is a third order nonlinear process, different from linear microscopy techniques. The theories of PSF models for fluorescence microscopy have already been established. But, it is not trivial to apply deconvolution for SRS imaging because SRS uses two laser beams (see Discussion, Line 356). For A-PoD deconvolution SRS imaging techniques, we developed a new model of two beam operations, and achieved a spatial resolution of 59 nm, which has not been reported.

In conclusion, our approach shows the novelty and capability of A-PoD deconvolution with SRS microscopy. The deconvolution method can be a solution to improve spatial resolution without any temporal resolution loss. Using A-PoD, we can control artifacts efficiently, and achieve Raman imaging with higher spatial resolution than other Raman imaging techniques.

Reviewer #2

Remarks to the Author:

Dear Authors,

By reviewing the manuscript entitled "Super-resolution SRS microscopy with A-PoD" I found it relevant and important.

Response: Thanks for speaking highly about our work!

The algorithm used to improve the resolution is not new, it was previously used in fluorescence spectroscopy. However, it is used for the first time for the SRS microscopy. In the presented analysis, it is not entirely clear why only lipids should be identified by the marker band of 2140 cm-1, CD vibration. This band is related to the presence of D2O in the sample, but why are only lipids produced by biosynthesis?

Response: For fluorescence imaging, the theories underlying the PSF models have been already established. But, it is not trivial to apply deconvolution for SRS imaging because SRS utilizes two laser beams. To develop A-PoD deconvolution for SRS images, we created a model of two-laser-beam operation, which is one of the innovative aspects of this study. This aspect is clarified in Discussion of revised manuscript (line 356). Applying this method we achieved a spatial resolution of 59 nm, which is superior to all other recent literatures on super-resolution Raman imaging.

Both lipids and proteins are produced in biosynthesis, but here we focused on lipid changes in cells and tissues. Using DO-SRS, deuterium from D_2O will be incorporated into newly-synthesized biomolecules containing CD bonds, including lipids and proteins (referred to as CD-lipids, CD-proteins). Vibrations of these bonds can be detected by SRS at wavenumbers 2140 cm^{-1} (for CD-lipids) and 2180 cm^{-1} (for CD-protein), respectively. Here, we specifically tuned the wavenumber to 2140 cm^{-1}, and thus the molecules detected were CD-lipids. Please see Ref. 3 for additional details of DO-SRS. For better understanding for the readers, we also added a sentence explaining this in the first paragraph in the manuscript, line 46-51.

Another issue is the Mito-Red staining of the mitochondria. How specific is this marker? are we sure that it only stains mitochondria and not lipids?

Response: In this study, we used a mitochondrial-targeting signal-tagged monomeric RFP (Mito-RFP), not the Mito-Red dye. Mitochondrial membranes are rich in lipids, thus, Mito-RFP signals should be overlapping with lipid signals. We know that Mito-RFP is a mitochondrion-specific marker and have confirmed its mitochondrial localization using immunostaining. Mito-RFP has also been validated in other published literature (e.g., Yazdankhah et al., Autophagy, 2021). We revised the label in the paper using "Mito-RFP", rather than MitoRed to avoid confusion.

In a word, does tracing the D-C band carry information about de novo triggered compounds or just a D2O uptake?

Response: Since we treated cells and fed animals with D_2O-containing water, D_2O will participate the cell metabolic activities and the enzymatic incorporation of deuterium (D) atoms into newly synthesized biomolecules will generate a new chemical bond called carbon-deuterium (C-D) bonds in macromolecules. Within the broad vibrational spectra of C-D bonds, we discover lipid-, protein-, and DNA-specific Raman shifts and develop spectral unmixing methods to obtain C-D signals with macromolecular selectivity. In other words, it is a bioorthogonal chemical imaging that is tracing C-D bonds at 2140 cm-1 is to track newly-synthesized lipids, while tracing C-D bonds at 2180 cm-1 is to track newly-synthesized proteins. see reference #3 for more technical details on DO-SRS imaging platform development.

Reviewer #3

Remarks to the Author:

Shi et al presents a superresolution method for SRS imaging based on deconvolution technique, called Adam optimization-based Pointillism Deconvolution (A-PoD). Superresolution images are demonstrated in a variety of samples and compared with the ground truth. Biological application of this technique is also conducted in lipid metabolism in living cells and tissues. the analysis of the size and distribution of the lipid droplets inside living cells are impressive, as this study could only be done by using super-resolution SRS technique with a low excitation power. The deconvolution SRS could be one of the good options in a more economical way.

Response: We thank the reviewer for the highly positive comments. Our technique not only enables super-resolution SRS but also presents an efficient method to democratize super-resolution SRS for investigating subcellular biochemical processes.

The authors claim the development of a novel deconvolution algorithm A-PoD in this work, which can only be considered as an improved version of SUPPOSe in literature (Ref 24), as the main idea of A-PoD is identical to SUPPOSe (Ref. 24). The difference lies in step 2 of SUPPOSe: the authors use Adam solver (Ref. 69) instead of the genetic algorithm to minimize the L2 norm. In this regard, A-PoD is a combination of Refs. 24 and 69. The authors also claim that such combination is non-trivial, since the imaging processing speed is greatly improved in A-PoD. Here I have one question:

1.1 Genetic algorithm is chosen in Ref 24, in order to avoid converging to local minima. Of course, gradient decent method, Adam, used in A-Pod is much faster than the genetic algorithm, but the cost is the risk to be trapped in local minima. The authors should investigate such possibility quantitatively, e.g., compare the results obtained by A-Pod and SUPPOSe with the ground truth in terms of L2 norm?

Response: Thank you for raising this point. Local minima are an important issue of gradient descent algorithms. Adam was developed to reduce the chance of trapping in local minima, based on a stochastic gradient descent algorithm using penalty parameter. That is why we applied Adam instead of other gradient descent algorithms. Although it might still have a low chance of getting into local minima, genetic algorithms have different problems such as randomness. The results we obtained using Adam solver demonstrated higher precision and shorter processing time compared with the genetic solver that requires at least 96 min for data processing to obtain an image with a reasonable resolution.

Considering the different characteristics of these two optimization algorithms, it is not so straightforward to benchmark and compare their technical performance metrics. We think it is more appropriate to directly examine the ground truth images, including those well-known cellular structures with clear biological relevance. We provided such images and results in Extended Data Fig. 1 and Fig. 8 to demonstrate this point.

Meanwhile, there are also several technical problems about PSF and resolution as outlined below, which should be fully addressed in the revised manuscript.

1. About the choice of PSF: an accurate PSF plays a key role in a deconvolution algorithm. In this manuscript, the authors have tried the PSF of pump, Stokes and PSF_conv (convolution of the PSFs of pump and Stokes), respectively. Finally, they choose PSF_conv, since the FWHM of the beads in the deconvoluted image matches well with the actual bead size. In this regard, I have two questions:

1.1 The choice of the PSF is incorrect. The correct PSF should be the product of pump and Stokes, i.e., PSF_SRS=PSF_pump*PSF_Stokes, because the SRS signal is proportional the product of pump and Stokes intensities (Ref. 13 in this manuscript). In addition, it is also improper to choose PSF based on the criteria "FWHM of the beads in the deconvoluted image matches well with the actual bead size", since the former should always be bigger than the latter, due to nonzero PSF size after deconvolution.

Response: We appreciate the suggestion. We have changed the results by using the PSF = PSF_{pump}*PSF_{Stokes} in the revised manuscript and Extended Data Fig 4. We also compared the deconvolution results and their resolutions by using experimental PSF, PSF of the product of PSF_{pump} and PSF_{Stokes}, and PSF of the convolution of PSF_{pump} and PSF_{Stokes}, respectively, in Fig. R4 below. These three deconvolution results using different PSFs are close to each other because of the noise level in the image. Theoretically, as the reviewer pointed out in the comment 2.3, SNR is important to improve deconvolution precision. If the PSFs have close sizes, the SNR effect can become more dominant than the exact PSF size.

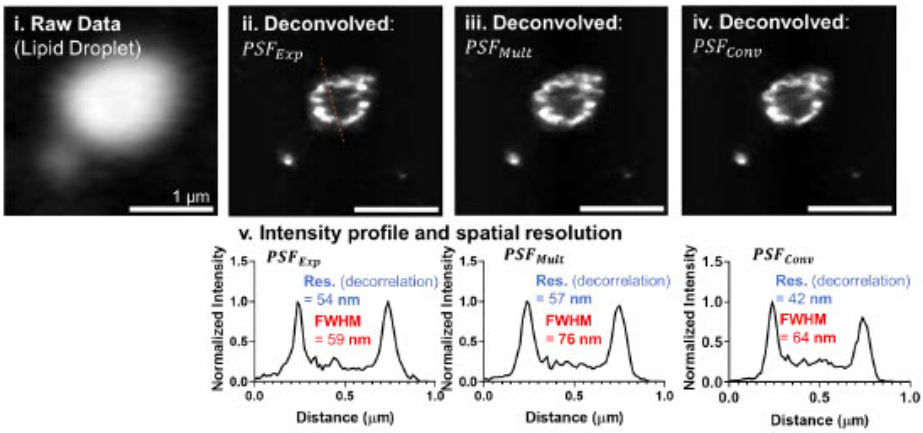

Fig. R4 Comparison of three PSF models. Single LD image was deconvolved using the two simulated PSFs and experimental PSF. After deconvolution, the raw LD image (in i) was converted to the three images (in ii, iii, and iv). From the intensity profiles of the three deconvolved images, membrane thickness was measured. The thinnest part has 59 nm, 76 nm, and 64 nm for PSF_{exp}, PSF_{mult}, and PSF_{conv}, respectively (in v). Spatial resolution measured with the decorrelation method was also less than 57 nm for the three results.

1.2 Those PSFs were generated numerically by ImageJ. the authors should compare the simulated PSFs with the measured ones to verify the conclusion. As the actual PSF may be quite different from the simulated one, due to the imperfection of the objective lens and the beam profile in the optical system.

Response: Thanks for the comment. Yes, the theoretical PSF is imperfect. This issue was also brought up by Reviewer 1. Now, we have measured the PSF using 100 nm beads and revised the manuscript with new experimental data (Extended Data Fig. 4). Fig R4 shows similar results obtained from the simulated PSF and experimental PSF. In addition, as explained in response to Reviewer's comments, simulated PSF has been previously applied in multiple studies (Sage et al., Nat. Methods 2015; Sage et al., Nat. Methods 2019; Hayato et al., Sci Rep 2018), and using simulated PSF allows us to improve the spatial resolutions of already existing/measured images, whose experimental PSFs may not be achievable due to the change of imaging condition/alignment. This approach will have broader applications in improving the spatial resolutions of imaging while without any significant difference compared with experimentally measured PSF.

2. About the resolution analysis: the authors claim 52-nm resolution achieved which is misleading. Such resolution is calculated by applying the decorrelation analysis (Ref 25) to the deconvoluted image of 270 nm beads (Fig. 2a). I have three questions:
2.1 I cannot find the decorrelation analysis in Ref 25. Is it the correct Ref? Please provide the details of resolution analysis, e.g., relevant equations.

Response: Thank you for pointing out an error in the reference number. We apologize for it. The correct reference is Ref. 27 (Descloux et al., Nature methods. 2019). The method was developed to quantify spatial resolution of localization microscopy. It calculates the correlation of normalized intensity in Fourier domain with circular filtering. Because the main process of this deconvolution method is the localization of virtual molecules, it is proper to be used for quantifying spatial resolution.

2.2 For an accurate resolution analysis, the sample size should be smaller than, or at least close to, the resolution. However, in this manuscript, the sample size (270 nm) is much larger than the resolution (52 nm). Therefore, such result is not reliable.

Response: Thanks for the comment. This was also brought up by Reviewer #1. In our revised manuscript, we have measured the PSF using 100 nm beads and analyzed images of lipid droplet (Extended Data Fig. 4b in the revision, and Fig. R4). From the deconvolution result, we measured the resolution to be 54 nm using decorrelation method and the thickness of lipid membrane 59 nm.

2.3 In deconvolution microscope, the final resolution is determined by a lot of factors, e.g., the deconvolution algorithm, the accuracy of PSF and SNRs. In fact, if there's no noise and we have precise PSF, the resolution after deconvolution can be infinitely high, even if we use simple Richardson-Lucy deconvolution algorithm (Ref. 61 in this manuscript). Therefore, it is more meaningful and practical to analyze the resolution by providing SNRs. It would be more convincing if the resolution is analyzed for a series of SNR levels.

Response: The objective of this study is to achieve a higher resolution (up to 59 nm now) by using A-PoD based SRS images in a practical imaging condition. Our results clearly support the main conclusion that A-PoD brings SRS imaging to the realm of super-resolution in a normal SRS imaging condition. The method to quantify and improve resolution by denoising SRS images will be a good direction for our next stage study.

Hence, in terms of lackings of the method originality and technical clarities and accuracies in the current work, the reviewer will not recommend for the acceptance of the manuscript for NM.

Response: Here, we respectfully disagree with the comments about originality. As we clarified in the response to reviewer #1's comments and in the Discussion of the manuscript revision, we demonstrated for the first time a new method for super-resolved SRS imaging, which opens new opportunities to investigate biomolecular and biochemical processes at an unprecedented finer scale. Due to the high speed, A-PoD is much more applicable than

SUPPOSe, and the approach of deconvolution is also new. Theoretical background of deconvolution SRS and A-PoD has also important meaning for wider application.

Regarding the technical clarities and accuracies, we have addressed them in the revised manuscript. For example, we imaged 100 nm beads in addition to 270 nm beads (in Fig. 2, and Fig. R2), which gives more accurate measurements of the PSF and the resolution. We also used a new PSF model ($PSF = PSF_{pump} * PSF_{Stokes}$) to better clarify the technical advances. Results from the experimental PSF and simulated PSF were compared and showed no significant difference, which validates the use of simulated PSF in our study. Therefore, we believe our current work and results are important to the field.

Decision Letter, third revision:

Dear Lingyan,

My apologies for the delay considering your appeal on your Article, "Super-resolution stimulated Raman scattering (SRS) microscopy". After careful consideration we have decided that we are willing to consider a revised version of your manuscript that is updated as you've described.

When revising your paper:

* include a point-by-point response to our referees and to any editorial suggestions

* please underline/highlight any additions to the text or areas with other significant changes to facilitate review of the revised manuscript

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We look forward to hearing from you soon.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Decision Letter, fourth revision:

Dear Lingyan,

Thank you for submitting your revised manuscript "Super-resolution SRS microscopy with A-PoD" (NMETH-A47324E). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Methods, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

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Thank you again for your interest in Nature Methods. Please do not hesitate to contact me if you have any questions. We will be in touch again soon.

Sincerely,
Rita

Rita Strack, Ph.D.
Senior Editor
Nature Methods

Reviewer #1 (Remarks to the Author):

The authors have thoroughly addressed my major concerns on the manuscript. Although whether the manuscript provides a novel deconvolution algorithm or just an improvement over the previous methods is a debatable opinion, there is no doubt that the A-PoD method has pushed the spatial resolution of SRS imaging to a new level.

Reviewer #2 (Remarks to the Author):

The authors significantly improved the work, performed additional measurements and analyzes, corrected the manuscript and fully and convincingly answered the questions,
The work can be accepted in this form.

Final Decision Letter:

Dear Lingyan,

I am pleased to inform you that your Article, "Super-resolution SRS microscopy with A-PoD", has now been accepted for publication in Nature Methods. Your paper is tentatively scheduled for publication in our April print issue, and will be published online prior to that. The received and accepted dates will be June 2, 2022 and Jan 17, 2023. This note is intended to let you know what to expect from us over the next month or so, and to let you know where to address any further questions.

Acceptance is conditional on the data in the manuscript not being published elsewhere, or announced in the print or electronic media, until the embargo/publication date. These restrictions are not intended to deter you from presenting your data at academic meetings and conferences, but any enquiries from the media about papers not yet scheduled for publication should be referred to us.

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Best regards,
Rita

Rita Strack, Ph.D.
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