October 15, 2021

Revisions to JoVE submission - JoVE63264

We would like to thank the editorial staff and the reviewers for their comments and suggestions to improve the comprehensiveness of this methods manuscript. Based upon the editorial and reviewers comments, we have included additional references that were previously omitted and expanded on the detail of the methodology presented here. Below, the editorial staffs' and reviewers' comments are in black text while our responses are in blue text. Each addition to the text has been highlighted to distinguish the updated language. The content slated for video production is still highlighted in the text.

——Editorial Responses——

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

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

   The pronouns of "you", "we", "our", and "you" have all been replaced, and sentences have been rephrased to allow continuity in the text.

3. 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. e.g., DeepSee Insight, Grace Biolabs, etc.

   All mentions of commercial language has been removed from the body of the manuscript and only remain in the Table of Materials.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without any brackets.

   The in-line citations have been updated per the request.

5. Please revise the Introduction to also include the following:

   (a) The rationale behind the development and/or use of this technique.

   The rationale behind the development is that there are not many methodologies to assess cutaneous PK of topical drug products with each having advantages and disadvantages.

   (b) The advantages over alternative techniques with applicable references to previous studies.

   Within the first paragraph, we give reference to additional methodologies that one might use to asses the cutaneous PK of topical drug products. Mentioning the advantages and disadvantages for each technique is out of this publications scope. We have provided references to recent studies utilizing each methodology.

   (c) A description of the context of the technique in the wider body of literature.

   As this methodology is a relatively new application of coherent Raman imaging, there is not a large body of literature specifically pertaining to drug quantification within the skin. However, we have added additional references to point the reader to recent advances in this field.

   (d) Information to help readers to determine whether the method is appropriate for their application.

   Additional information has been added to the introduction to reiterate the point that CRI is only limited by sample thickness and the depth to which the laser can reach, which is also a function of the samples refractive index.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly.

Actions within the protocol have been revised to appear in the imperative tense. Several instances outside the protocol (introduction and discussion) have been left to be suggestive rather than definitive based upon the data available internally and published information.

7. 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.

   The protocol has been simplified.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

   (a) 1.1.1 and 1.2: Please specify how the nude mouse bodies and human skin tissue were procured.

   In 1.1.1, the nude mouse bodies are taken from collaborators after they euthanize animals within the colony and then are transported to our lab. This is not a general methodology and each researcher viewing this video/manuscript will need to go about this differently.For the human skin we source our skin from abdominoplasty procedures or from a body donation authority.

   (b) what is Kohler illumination, where is the corresponding setting in the GUI, and how to ensure a correct illumination? What is the polygon and where to find it? Please provide more details. Alternatively, provide a reference to a published protocol, if any, with appropriate citations.

   A reference to what Köhler illumination is has been added to the text. The Köhler illumination allows for optimal sample illumination. There is no GUI needed for this as the researcher is looking through the eyepiece of the microscope. The correct illumination is indicated in the text - when the polygon shape, when looking through the eyepiece, can be seen and touches each side at the same time. A citation to microscope set-up was added for clarity. (1)

   (c) 3.15: please clarify what is meant by 'inside' of the ear relative to skin layers.

   Thank you for this comment. We have clarified to say "anterior portion of the ear" rather than inside.

   (d) 4: The heading 'application of topical formulation' doesn't seem apt for all the steps under it. Please consider splitting the steps into two parts with different headings.

   Yes, the heading is slighly misleading. We have added another section after 4.3 to signify the start of the imaging portion of the experiment "Experimental set-up for drug quantification"

   (e) 4.1: provide details of the specific API used in this study.

   As this was meant to be a general methodology, the formulation and API were not thought to be included. In addition, we work with simple formulations (*i.e.* solvents) and complex formulations such as creams or gels. Therefore it is not possible to single out one API or formulation. The formulation also plays a role in the light scattering and thus drug quantification However, we will place a note to say what we used in this particular example.

   (f) 4.4: please specify the desired beam powers.

   This experimental specific parameters that are not generalizable across studies. These must be figured out *a priori*. The powers used in this study have been added as a note.

   (g) 4.7: what is the condenser and where is it located?

   A reference to the microscope set-up has been added in Section 2.6 (1)

   (h) 4.8: how are the specific depths in (1) and XY positions in (2) chosen/determined? Please provide action steps for live-imaging various skin layers of the lipid stack.

   The specific depths, in the case of nude mouse ear skin, are given as examples right after the mention of "specific depths within the skin". We have added a reference to Fig 6. for the reader to refer to when looking for these skin stratifications in nude mouse ear or human skin. The XY positions are chosen when

the exact Z position is unknown. For example, if the goal is to see how the API permeates through the skin, it is feasible to take a z-stack from the skin surface (0 μm) to the subcutaneous fat ($\sim$ 100 μm) with the trade off being the temporal resolution.

(i) 4.9: what are XYZ positions here?

We have added additional text to clarify what is meant by XY postions and XYZ positions.

(j) 4.11: what is the rationale for choosing a specific wavenumber? Is the target wavenumber specific to a given tissue stratification or the API of interest? Please provide more details in the Notes.

This is a valid question. The wavenumber is based upon the molecule that we are targeting. For example, if we are targeting a molecule that has a nitrile stretch then we will set the wavenumber to be 2250 cm $^{-1}$ where as the lipid imaging will target 2850 cm $^{-1}$ as there are abundant CH_2 bonds. Essentially the Raman shift or wavenumber of your drug will be specific to your drug, especially in the fingerprint region ($\sim$ 700-1400 $^{-1}$

(k) 4.11: Please specify what this time delay is. Is it the delay between the pump beam and stokes beam?

The reviewer is correct; the time delay is the delay between the pump and Stokes beams. A time delay stage is an optical component included in the beam path that can be adjusted to ensure that the pump and Stokes beams are overlapped in time. If the time is mismatched, there will be no CARS or SRS signal. An additional citation to Alfonso *et al* was added for reference for a CRS imaging system for biological systems in the introduction. Unfortunately, a tutorial set-up of a CRS table-top system is out of the scope of this manuscript and would require another manuscript for the in-depth coverage of a topic. [(2)]

(l) 4.15: is the post-time-course lipid stack obtained in another image acquisition run? If so, please provide the necessary action steps for filming.

More description has been added to ensure the reader understands that this is another imaging run.

9. Step 5: For software instructions and data analysis, please ensure the inclusion of specific details such as button clicks, numerical settings, screenshots, etc. Please give specific instructions such as "navigate here", "click this", "select this", "check this", "enter this", etc. which are necessary to execute the action item. Please note that software steps without a graphical user interface cannot be filmed.

(a) 5.2: what are lipid images and API channel images? Are they acquired separately or combined at the end of step 4.14?

The lipid images are acquired in the point now labeled 5.8. and have a sentence explicitly stating what these images are.

(b) 5.3.1: how is the lipid image split into CARS and SRS channels? How to add specific image locations to the region of interest manager? As mentioned above, please focus on the "how" question, i.e., how a step is performed by including all the necessary details.

The images are automatically split by checking the "Split Channels" box when the .OIB or .OIR file is loaded into Fiji. A through explanation has been added the text to clarify how one splits the images, and added the specific image locations to the ROI manager.

10. Please insert single-line spacing between individual steps and sub-steps of the Protocol. Please highlight no more than 3 pages of the protocol (including headings and spacings) that identify all the essential action steps to be filmed as a video. Please ensure that the highlighted steps form a cohesive narrative with a logical flow. Please note that we cannot film calculations or equations.

11. Use h for hour, min for minutes, and s for seconds throughout the manuscript. Please include a single space between the numeral and the unit, e.g., 24 h, 30 s, 8 °C, etc.

This has been addressed.

12. All figures: Please use capital letters without brackets as labels for the individual panels.

This has been corrected.

13. Figures 3 and 4: Please increase the font size for the scale bars.

These have been corrected.

14. Figure 4, legend: Please provide a one-line title for the figure. In panels A and B, please specify if the sebaceous glands are from mouse or human.

    Thank you, this has now been corrected.

15. Figure 5: for the x-axis labels, please abbreviate hours as 'h' instead of 'hr'.

    This has been corrected.

16. 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]."

    Figure 6 is an adaptation from a publication from the corresponding author and thus the copyright has been ordered through RightsLink/Elsevier. This will be attached as apart of the re-submission.

17. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

    (a) Any modifications and troubleshooting of the technique

        In the original submission each section was addressed in terms of the troubleshooting to get the system properly working to quantify API within the skin. Modification were also suggest as part of the troubleshooting sequence.

    (b) Any limitations of the technique

        In the original submission, we have mentioned the large size of the data generated, the occlusive nature that is unlike clinical conditions, the dose-duration optimization, and skin thickness preventing optimal imaging.

    (c) The significance with respect to existing methods

        In the last paragraph of the Discussion, we talk about the ability of the approach presented here to "visualize and quantify microscale changes that are otherwise indistinguishable with methodologies such as dermal microdialysis, dermal open-flow microperfusion, tape-stripping, or IVPT studies."

    (d) Any future applications of the technique

        We have now explicitly added a sentence in to talk about the potential of this technique and methodology.

18. Please ensure that the references appear as the following: Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR). For more than 6 authors, list only the first author then et al. Please do not abbreviate journal names.

19. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. And sort the reagents/materials/equipment in Table of Materials alphabetically.

    The Table of equipment and Materials has been corrected.

Reviewer Responses

Reviewer #1: The invited methods article entitled "Visualizing and quantifying pharmaceutical compounds within skin using coherent Raman scattering imaging" by Benjamin A. Kuzma *et al.* describes a coherent Raman scattering imaging methodology to visualize and quantify topical applied pharmaceutical compounds within the human and mouse skin. The manuscript contains very useful information for readers of method journals, especially researchers who make similar measurements on the skin. The manuscript might be improved by incorporating the following comments.

Minor Concerns:

- Page 4, line 106: The author describes that if not used within 24 hours, the skin tissue is frozen as is at -20°C. Is there a possibility that the process of freezing at -20°C as it is may affect the transdermal absorption characteristics via changes in the structure of intercellular lipids?

  This is a outstanding comment - one that warrants question from *every researcher* in the field. Fresh skin is the gold standard to assess topical bioavailability for APIs in topical drug products. However, the ability to procure "fresh" tissue is quite a difficulty. In our current structure, we obtain abdominal skin from elective surgeries and the skin is typically delivered to our lab within 6 h. However, this does not occur that frequently, due to COVID and thus we must resort to using freezing skin upon processing. There is certainly the possibility of the freeze/thaw process that dramatically effects the structure and thus function of the stratum corneum. Frozen skin the type of skin that is utilized throughout studies such as in vitro permeation testing, which investigates the cutaneous pharmacokinetics. There have been conflicting commentaries on whether tissue storage (fresh/frozen) ultimately impacts the bioavailability of the API and how they cutaneous PK profiles are altered from a lack of barrier function.[3;4;5] It is certainly feasible that the skin barrier function is compromised[3;6] while other studies report an *increase* in the barrier function as indicated by a lower drug flux, albeit in preclinical models.[7] However, the majority of these regulatory agencies accept frozen skin in the investigations of percutaneous permeation of compounds[8;9;10]

  Ultimately, we think due to the lack of fresh skin for researchers frozen skin will be utilized with the understanding that it can not fully recapitulate the *in vivo* scenario but comparisons between products can be made when using the same tissue storage conditions. Eventually, there will need to be an investigation into the effect of fresh and frozen skin and how scaling factors can be developed such that frozen skin can be used as an alternative for researchers around the globe for topical/transdermal research as a surrogate for fresh skin. We have done our own independent investigation and did not find a significant difference between fresh and different freezing storage conditions for our compounds tested.

- Page 5, line 122: The author describes a method of scalping the subcutaneous tissue. Isn't it difficult to remove the dermis and subcutaneous tissue cleanly?

  Absolutely, this is fairly difficult. However, after preparing the skin multiple times it becomes more apparent just how much skin must be removed to obtain quality SRS images to quantify drugs; the SRS drug quantification, in our set-up, requires thin samples for light to scattered in the forward direction. This tissue preparation takes a quite of bit of time as rushing through this delicate procedure will lead to cutting the skin all the way through and restarting the tissue preparation process. This, of course, will further delay the start of an experiment. We also thought that this manuscript/video would be a fantastic way to demonstrate how we prepare the skin for these experiments so that others can have reproducible results.

- Page 5, line 127: It is better to specify the orientation of the stratum corneum and dermis (dermis down?) when placed on agarose gel.

  Thank you for the suggestion. We agree and we have clarified this within the text (see below):

  Fresh skin, placed with the dermis in contact with the gel, may be used up to 24-hr without the use of an agarose gel bed, previously described in[11]. Frozen skin should be thawed using the procedure below for optimal results (See Section 3.1.2- 3.1.3).

- Page 7, line 217: I'm worried about moving the skin tissue to 32°C after -20°C. Is there a possibility that the structure of the epidermal cell layer and dermis will be broken and the water that has remained in the

tissue will flow out? Is this temperature condition acceptable in other methods to evaluate pharmaceutical compounds in the skin?

We thank the reviewer for the valid comment. While there is certainly reason to be concerned about the structural integrity of the skin after a freeze thaw cycle, there is minimal damage in the storage of skin at -20 °C than at -80 °C.[3] In addition, there are many regulatory bodies that currently accept skin storage at -20 °C for pharmacology or toxicology studies. Barebero *et al.*, reviews some of the literature available on storage conditions and their impact on the skin's structure. As mentioned in a previous response, there is not really a consensus on the impact of the storage conditions on the permeation kinetics. There is the suggestion that freezing will increase the permeation of compounds, which is reasonable, however side-by-side comparisons of formulations under identical experimental conditions and identical skin storage conditions will yield information pertaining the formulation rather than the skin storage conditions. In addition, the 32 °C temperature is the standard temperature utilized in *in vitro* permeation studies, as that is the average skin temperature.[9]. The below text has been added to the introduction to address these concerns.

Page 4 - Although there have been various commentaries on the suitability and applicability of frozen human skin to accurately recapitulate the *in vivo* permeation kinetics[3;4;5], the use of frozen human skin is an accepted method for the evaluation of API permeation *in vitro*[8;9;10] In this protocol, we demonstrate the visualization of various skin layers in both mouse and human skin while quantifying API concentrations within both lipid-rich and lipid-poor structures.

- Page 7, line 236: It is considered that the light scattering depends on the layer of skin (stratum corneum or dermis on the glass side), and the detection efficiency differs. Could you supplement the description?

  While the layer that is on the glass bottom dish will be readily visible in the CARS signal, it might not be as apparent in the SRS signal, depending on sample thickness. This is because the sample thickness is more important than which side of the skin is down (for human skin). For mouse skin, there is no need to remove skin as it is already adequately thin. However, for human skin there is preparation required. The thinner the skin samples that one can prepare, the better quality images that can be seen in the SRS channel. This is particularly important considering drug quantification is extracted from the SRS channel. If one is unable to visualize the lipid structure in the SRS channel but can in the CARS channel, the sample is too thick. In addition the CARS channel will have a greater signal due to more light being back-scattered. Please see out corrected steps:Page 7.

  If using nude mouse ear, place the inside of the ear with the inside facing toward the glass bottom of a 35 mm, No. 0 Dish (The back of the ear is more prone to imperfections from housing). If using human skin, place with the stratum corneum face down as this will allow drug quantification from superficial layers to the deeper layers.

  NOTE: If human skin is not placed with stratum corneum side faced down, on this inverted microscope, then one will not be able to see past the dermis as there is a fair amount of light scattered and the drug permeating into the stratum corneum cannot be seen.

- Page 8, line 256: The author mentioned that "remove excess formulation". How to remove?

  Thank you for this comment, which was an overlooked detail on our part. The formulation can be removed using either delicate tissue wiper without water or another solvent. In previous experiences, it was found that the way in which the formulation was removed actually increased the permeation of the compound due to the re-solubilized API at the skin surface.[12]. There is also the possibility of removing the formulation with a small (approx. 1 inch) squeegee to wipe away the excess formulation that remains on the skin's surface. We have added the following sentence on Page 8:

  NOTE: The formulation can be removed by using a delicate task wiper or using a small (approx. 1 inch) 3-D printed squeegee in a single direction (*e.g* from north to south).

Reviewer #2: The paper by Kuzma et al describes the protocol of Coherent Raman scattering Imaging (CRI) in skin studies. The proposed methodology reveals an opportunity for quantification of skin components over time for estimates of micro- and macro-scale bioavailability and, potentially, bioequivalence. The paper is well-written and contains full description of the proposed protocols as well as full description of all required materials and equipment. The theme of the study is quite important and researchers in different fields are interested in pushing of Raman spectroscopic studies into clinical/cosmetological/biological applications. Thus, creation of video guide for the analysis of skin tissues with Raman spectroscopy techniques is demanded topic.

- In my opinion in Discussion section it is important to mention possible in vivo application of the proposed CRI technique. Thus, may the authors provide some information about laser safety (maximum permissible exposure) during possible in vivo skin examination?

  Thank you for this insightful comment. Indeed, conducting experiments on the benchtop can only go so far and directing a large power to an *ex vivo* sample is certainly not feasible. The maximum permit power allowed for *in vivo* usage is XX based upon XX equation, per our calculations. With this is in mind, the translation from benchtop to bedside is currently an active area of research in our group with these clinical studies slated to begin shortly. We have added the following statement into the discussion section:

  To be added

- Is there some specific purpose to highlight some text in yellow?

  The purpose of the highlighting the text in yellow was the convention provided by the journal to denote which sections should be video tapped. There was no significance to the reviewer.

- Maybe it is proper to denote what means Lambdapump and Lambdastokes in equation 1?

  Thank you. We have now indicated what each variable is in Equation 1.

- Size bars in figures 3 and 4 are rather small, it is better to increase the bars.

  Agreed. Thank you for this observation. We have updated these images with scale bars that are more legible.

References

[1] Sanderson, J. Fundamentals of microscopy. *Current Protocols in Mouse Biology*, 10(2):e76, 2020.

[2] Alfonso-García, A., Mittal, R., Lee, E.S., Potma, E.O. Biological imaging with coherent Raman scattering microscopy: a tutorial. *Journal of Biomedical Optics*, 19(7):071407, 2014.

[3] Nielsen, J.B., Plasencia, I., Sørensen, J.A., Bagatolli, L. Storage conditions of skin affect tissue structure and subsequent in vitro percutaneous penetration. *Skin Pharmacology and Physiology*, 24(2):93–102, 2011.

[4] Barbero, A.M., Frasch, H.F. Effect of frozen human epidermis storage duration and cryoprotectant on barrier function using two model compounds. *Skin Pharmacology and Physiology*, 29(1):31–40, 2016.

[5] Babu, R., *et al.* The influence of various methods of cold storage of skin on the permeation of melatonin and nimesulide. *Journal of Controlled Release*, 86(1):49–57, 2003.

[6] Kemppainen, B., Riley, R., Pace, J., Hoerr, F. Effects of skin storage conditions and concentration of applied dose on [3H] T-2 toxin penetration through excised human and monkey skin. *Food and Chemical Toxicology*, 24(3):221–227, 1986.

[7] Sintov, A.C., Greenberg, I. Comparative percutaneous permeation study using caffeine-loaded microemulsion showing low reliability of the frozen/thawed skin models. *International Journal of Pharmaceutics*, 471(1-2):516–524, 2014.

[8] Skelly, J.P., *et al.* FDA and AAPS report of the workshop on principles and practices of in vitro percutaneous penetration studies: relevance to bioavailability and bioequivalence. *Pharmaceutical Research*, 4(3):265–267, 1987.

[9] OECD. *Guidance Document for the Conduct of Skin Absorption Studies.* 2004.

[10] OECD. *Test No. 428: Skin Absorption: In Vitro Method.* 2004.

[11] Osseiran, S., Austin, L.A., Cannon, T.M., Yan, C., Langenau, D.M., Evans, C.L. Longitudinal monitoring of cancer cell subpopulations in monolayers, 3D spheroids, and xenografts using the photoconvertible dye DiR. *Scientific Reports*, 9(1):1–10, 2019.

[12] Kuzma, B.A., Senemar, S., Ramezanli, T., Ghosh, P., Raney, S.G., Stagni, G. Effect of formulation wipe-off time on topical bioavailability of metronidazole using dermal microdialysis. In *AAPS Pre-Clinical Development - Chemical Entities*, pages 532–535. American Association of Pharmaceutical Scientists, 2018.