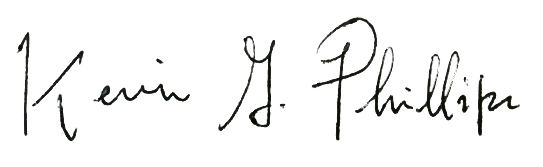
Dear Michelle,

We thank the reviewers for their constructive comments. We have incorporated their remarks into our revised manuscript. Below you will find that we have responded to each of the reviewers in bold italics. Thank you for the careful administration of our manuscript through the review process. We look forward to addressing any further editorial concerns or questions the reviewers might have.

Best Regards,



Kevin Phillips

Department of Biomedical Engineering

School of Medicine

Oregon Health & Science University

Portland, OR 97239.

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Dear Dr. Phillips,

Your manuscript JoVE50988R2 'Quantitative optical microscopy: measurement of cellular biophysical features with a standard optical microscope' has been peer-reviewed and the following comments need to be addressed.

*Please use the "track changes" function in word as you revise your manuscript text to address these comments. When you have revised your submission, please upload the revised document along with an additional word document with individual responses to each of the editorial and peer review comments below. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.*

**Editorial comments:**

\* All of your previous revisions have been incorporated in to the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.

\* Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

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

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

The authors' use a commercially available optical microscope to obtain quantitative information about the mass, volume and density of biological cells. While they use the non-interferometric quantitative phase microscopy (NIQPM) to study the mass and density information, they use Hilbert Transform Differential Interference Microscopy (HT-DIC)to obtain cell volume. Although, both NIQPM and HTDIC has certain limitations on non-biological samples, the authors' use of these techniques in fixed cellular specimens abrogates the limitations. Most importantly, the authors' understand these limitations and have clearly stated them in the article. The article is well written and it would be of relevance to the JoVE audience. I recommend the publication of this manuscript with minor revisions.

***We thank the reviewer for these kind words.***

*Major Concerns:*

None

*Minor Concerns:*

a)It would be easier if authors' can include a discussion of how they accurately obtained the NA of the condenser lens to vary from 0.1 and 0.9. Since most of the commercial microscopes have an adjustable aperture stop which is not graded, it would be useful if the authors' can explain this step in detail.

***Our instrument possesses a readout of the NA of the condenser lens. We have updated our manuscript to reflect that one must be able to know the value of the NA using either a graded stop or an electronic source in the microscope interface.***

b)It is not clear why the authors' need to use a narrow band color filter for bright field imaging. If this is done to satisfy certain spectral conditions for NIQPM, the authors' should clearly indicate the wavelength of this color filter that was used.

***We are fixing the value of the refractive increment, which is a wavelength dependent quantity, to its value in the green. We have updated our manuscript to reflect this.***

c)It is better to explain clearly the pseudo DIC image. Although it is phase map obtained from bright field, it is not clear why it is called pseudo DIC. Also, it is not clear why this is important to have a true DIC image.

***Comparison of the pseudo DIC to the true DIC is the only check one can perform on the validity of the reconstructed phase map in the absence of a priori information concerning the sample. The procedure also ensures that the correct focal plane is used to center the phase calculation. We have updated our manuscript to reflect the importance of the pseudo DIC image. Thank you for this crucial suggestion.***

*Additional Comments to Authors:*

None

**Reviewer #2:**

*Manuscript Summary:*

With the detail information and codes being included, this protocol provides a very useful method to characterize cellular parameters using regular light microscopy. Therefore, it is suitable to publish in JoVE. However, the authors need to slightly revise the manuscript to improve the readability before publish.

***Thank you for your kind words.***

*Major Concerns:*

There is no Major concern.

*Minor Concerns:*

The title of the manuscript makes the protocol a degenerate method. However, the protocol is written to fit to a specific brand of microscope with the specific software that controls the microscope. The Title doesn't need to be change but every places in the protocol that is involved with specific terms or formats associated to the special brand of microscope needs to be modified to make it more general to the users. Also, the attached MATLAB codes need to be specified to be useful to the output format from the microscope software.

*Editorial Comment: For script writing purposes we ask that you leave the protocol as it is - written for your specific set up as you will demonstrate it in the video. However, if you would like to add more details as requested, you may add additional information about adapting the protocol to other systems elsewhere in the manuscript (such as the discussion) or in the protocol, but excluded from filming (not highlighted).*

***While the protocol is written for our setup, we have updated the discussion, as suggested, to emphasize that the procedure could work on any system in which there is control over the NA of illumination, the z-position of the sample and/or the objective lens.***

**Reviewer #3:**

*Manuscript Summary:*

This paper describes the details of how to apply two microscopy techniques for quantitative measurement of optical density and determination of particle volume. The theory is not dealt with in detail, but references to the literature and previous work by the authors are given for validation. Guidance is given on how best to collect the z-stacks of images that form the raw data for the reconstructions as well as detailed instructions for running the software (written in Matlab and included as supplementary material). Although perhaps not everyone has access to the 0.1 micrometer computer controlled stepping, required to generate the z-stack, in an off-the-shelf microscope, the technique is interesting even if one doesn't have that and the software is valuable. I have a few comments.

*Comments:*

1) Section 2.2. and 3.1. It would be helpful for users that don't have Slidebook in front of them, if the statements that say the Aperture slide bar is moved to the furthest left or right positions, could have an explanation of what that meant. Is furthest right the most closed down aperture and the left the widest open aperture or vice versa or something else?

***We have updated the protocol as suggested.***

2) Section 5.4 and section 6.4. What are the criteria for deciding which image is "best focus"? What if two images look about equally in focus? I think this issue may be dealt with as part of the expected results (one does fitting the known phase maps or by comparing computed DIC with observed DIC images), but if so then a notation should be made noting that best focus was determined as described in the Representative Results. Also, other than defining parameters, a description of what section 5.4 does -- perhaps "alignment and rotation of images for Hilbert transform" would be helpful.

***All “best focus” means is that the sample is in focus enough to be able to drag the boxes around the feature of interest. We have updated the protocol to clarify this point. We have also updated the HTDIC protocol to explain what it does using the reviewers suggested wording. Thank you for the suggestion.***

*Minor Concerns:*

p.4, paragraph 2, line 7. I don't understand the use of "principle" here. Do the authors mean "principal". Is that word even necessary?

Section 5.4. No parentheses are needed around "(where the DIC image of the sample is in best focus)". This also applies in Section 6.4.

Section 5.5. A word is missing in "Position the box the feature of interest". Perhaps "over" should be added between "box" and "the".

Section 5.11. What is "Figure 500"? The same question applies to Figure 600, 700 and 800 in a subsequent item.

***We have corrected the grammar. Also, the figures are indeed produced by the program. We have clarified this point.***

*Editorial Comment: I was also confused when I first read the manuscript as to the figure numbering. Please specify with a brief statement that these figures are the ones produced by the programs. While this will be evident in the video, it will be helpful if it is clarified in the text as well.*

Also you want to find the "borders" of the cell, not the "boarders" of the cell. This issue recurs in Section 6.8 and in the Discussion and maybe elsewhere, so a search and replace should be done.

Equations in the discussion should have the symbols defined. I suppose one can figure out from the numbers and units and text following the equations what the symbols are, but it would be better to be explicit.

Discussion, paragraph 2, line 2. "possess" does not agree with "density map". Either density map needs to be plural or possess should be possesses.

***We have corrected the grammar. Glad someone caught it!***

**Reviewer #4:**

*Manuscript Summary:*

This manuscript details a potentially useful optically-based techniquee for characterizing fixed cells on slides.

*Major Concerns:*

None.

*Minor Concerns:*

None.

*Additional Comments to Authors:*

The authors have done a good job in revising the manuscript in response to the previous round of reviews. I believe that this version of the protocol is ready to proceed to the video production stage.

***Thank you!***