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14 October 2019

Phillip Steindel, Ph.D.
Review Editor
JoVE
1 Alewife Center
Cambridge MA 02140

Dear Dr. Steindel,

I have submitted the accompanying revised manuscript and figures, "Clarifying and Imaging *Candida albicans* Biofilms", for your review.

All text changes in the revision have been marked in red font.

The pages appended to this letter detail the changes that were made in response to your editorial instructions, and our responses to the many helpful comments of the four reviewers.

We changed the title of the manuscript to the suggestion of Reviewer 4.

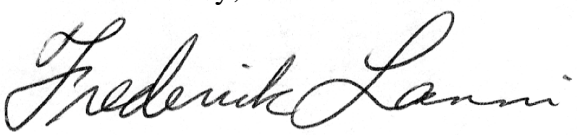
The biggest text change was removing the end notes from the four protocols, and rewriting this information into the Discussion.

The figure panels originally submitted have been combined into single figures (Figs. 1-5) as per your instructions. In my opinion, this is less satisfactory than separate panels. I will be happy to provide the individual panels, if your graphic artist agrees with me.

The content we suggest for videography runs from protocol step (2.3) through step (4.7), text lines 216-311. That is slightly over 2 pages in the revised manuscript.

Thank you for your patience, and your consideration of our research for your journal.

Sincerely,



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Changes in v9 document from v8:

version identifier: REVISION / v9e / 14oct2019
line numbering turned on.
Text changes are marked in red font throughout.

Changes in response to Editorial Comments:

General:
Changed to single line spacing in all paragraphs.
Changed to Calibri 12-point font throughout.
Corrected all instances of "um" to "μm".
Content for videography: Protocol steps (2.3) through (4.7).
Text lines 216-311, mainly manuscript pages 5-6.

Introduction:
wording changes: line 80
line 152

Protocols:
Removed all end notes (Notes) from protocols 1-4. The information in the Notes now appears as new text in the Discussion - paragraphs 2-10.

line 220: changed wording: extend time period when processing thicker specimens.

Representative results:
lines 332-336: Figure 1 legend has been revised.
lines 339-348: Figure 2 legend has been revised. Sentence on schematic diagram has been added.
Other figure legends have minor revisions.

Discussion:
line 407: added text

lines 410-539: Inserted text from 4 sets of end notes removed from the individual protocols. These notes were revised to constitute Discussion paragraphs 2-10. New text is in red font. References to these paragraphs were inserted into the protocols.

lines 564-565: changed wording: biofilm adhesion and hyphal entanglement appear to provide the surface anchoring needed for hyphae to efficiently invade a substratum.

lines 569-571: added text.

Figures:
Removed embedded figures from text.
Figure 1: Dropped Figure 1(a) and combined the remaining two panels at the same scale. Updated the legend.
Figure 2: Added schematic panel to Figure 2 in response to Reviewer #2, and updated legend.
Figure 3: Combined 3a and 3b, and updated legend.

Figure 4: Combined 4a, 4b, and 4c, and updated the legend.
Figure 5: Added axial scale color bar, updated legend.

Acknowledgements: updated

References:
lines 584-690: Put citations in standard format.
Citations 19-27 were re-ordered and renumbered.

Table of Materials: updated

Changes in response to Reviewer comments:

We thank the four Reviewers for their comments, suggestions, and corrections.

Reviewer #1:
corrected all instances of 'hyphus' to 'hypha'.

Reviewer #2:
The biggest disadvantage of our approach is that it isn't applicable to living specimens. Because that is apparent, we don't see the need to state it. We do state that the method is intended for fixed specimens in the Short Abstract, in the Abstract, and twice in the Introduction! We also didn't really include a paragraph on advantages - such as not having to cut serial sections, and register images afterward. Again, that is apparent, therefore no need to state explicitly. In the Introduction (lines 140-162), we specifically address some intrinsic difficulties of our approach.

The reviewer correctly points out the need for specialty objectives. We have updated our discussion of long working distance immersion microscope objectives in the text (see lines 490-539). In the original manuscript, we described two Zeiss objectives used in our work - one an obsolete model (40x 0.85NA), and one a modern multi-immersion lens (25x 0.8NA) that is readily obtainable. To address this point further, we tested a modern Nikon long working distance multi-immersion objective (10x 0.5NA) which can be directly immersed in methyl salicylate and other clarifying solvents. Information on this objective is included in the revised text. (lines 503-507)

Reviewer #2 line items:
1. We wrote the first paragraph to summarize relevant common knowledge. Citations start immediately in 2nd paragraph.

2. micron symbol corrected throughout.

3. lines 152 and 555: added reference to use of butanol (n = 1.399) to adjust refractive index.

4. Yes, we agree. Glutaraldehyde definitely increases broadband autofluorescence (and also strongly attenuates dTomato fluorescence). That has not been a problem in most of our work, but particularly affects quantitative imaging with GFP and RFP tags. We feel that this is widely known, and therefore didn't address the problem in the original text. We have added a note on glutaraldehyde in the protocol (2.2) - lines 213-214.

5. We've always used formalin or formaldehyde at 4% for mammalian cell work, where its action is more or less instantaneous, as seen by live-cell microscopy. To limit osmotic effects and autofluorescence, we keep the fixative concentration as low as possible. We have used formalin in the 4-10% concentration range with no noticeable difference in results.

6. The suggested diagram has been added as a panel in Figure 2.

7. line 301: defined "INA".

8. lines 317-318: Changes made as suggested. References to Fig. 2 and Fig. 3 have been made explicit.

9. line 327: changed terminology to 'lateral budding yeast' as suggested.

10. line 332: added title/caption to Figure 1.

11. Figure 2: references to the figure panels have been corrected.

12. Figure 2 legend: Yes, direct water immersion = dipping objective, as opposed to indirect water immersion through a coverglass.

13. Figures 2 and 4c graphic errors corrected.

14. Figure 4 legend: DAY185 is the wild-type strain used as a control in Woolford, *et al* (cited as #14). Its origin can be traced back from that paper to a 2000 paper by Davis, *et al*. Since that earlier paper is not directly related to the current manuscript, we believe that citing Woolford *et al* is sufficient here.

15. Figure 5 legend: The color scale depth range was added to the legend as text.

16. lines 544-545: The term 'visual immersion refractometry' has been replaced with 'By darkfield illumination and visual inspection'.

Reviewer #3:
The reviewer is correct - The images in Figure 2 do not appear as sharp as in the high-resolution figures (Figs. 4 and 5). In the 63x water-immersion image, that is due to the refraction of light by the cells in the biofilm. The MS immersion image was enlarged from 40x to match the magnification of the water-immersion image. That involved some interpolation which 'softened' its appearance. However, these images show clearly the main benefit of clarification.

Reviewer #4:
Changed title of article as suggested: Clarifying and Imaging *Candida albicans* Biofilms

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