Author's response to reviews

Title: An automated Method for Analysis of Microcirculation Videos for Accurate Assessment of Tissue Perfusion

Authors:

Sumeyra U Demir (sumeyra.demir@signalprocessingtechnologies.com)
Roya Hakimzadeh (contact@signalprocessingtechnologies.com)
Rosalyn S Hobson (rhobson@vcu.edu)
Kevin R Ward (krward@vcu.edu)
Eric V Myer (eric.myer@signalprocessingtechnologies.com)
Kayvan Najarian (knajarian@vcu.edu)

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Author's response to reviews: see over
Response to Reviewer: Can Ince

The authors present a very elegant and promising method for automated microcirculatory image analysis. The detection of microcirculatory density seems very solid, but the detection of flow remains elusive. The following issues need to be clarified/resolved:

A1. Critique: The authors express the capillary density the ratio of the area of capillaries to the entire area of the image. However, what was the diameter threshold applied for separating capillaries from venules? How was the vessel diameter determined?

Response: Vessel diameter is determined using the vessel segmentation algorithm outlined in the Preprocessing, Multi-thresholding, and Segmentation subsection of the Methods section. We used a maximum diameter, P_d, of 13, which corresponds to a vessel diameter of 20 µm at the resolution of the camera used to capture the videos. We feel that this value is below the range of venule diameters and other larger blood vessel structures and focuses on capillaries. This is clarified in the results section. See response to Dr. Dobbe critique B8 below for an explanation of parameter determination.

A2. Critique: Why was capillary density not expressed as the length of capillaries over the area of the image?

Response: It is much easier to form the skeleton of the network of active capillaries and calculate the length of this skeleton to form the density measure, however, since the width (diameter) of capillaries along this network would not stay the same (neither on actual sublingual surface nor on the captured video nor on the processed image), the density measure calculated based on length would be the least reliable measure. This is the observation reported by others in the literature as well. The density measure, on the other hand, since it is incorporating thickness of capillaries into calculation, is less susceptible to this issue. However, since the reviewer has requested the inclusion of length-based density, this measure was calculated and added to the paper.

A3. Critique: How did suboptimal image focus and contrast affect the vessel diameter and area assessment?

Response: This was previously addressed in the Preprocessing, Multi-thresholding, and Segmentation section where we discuss CLAHE and median filtering techniques to overcome suboptimal image focus. This in our opinion is a unique feature of our approach to automation.

A4. Critique: Validate your method in videos in which the proportion of perfused vessels (PPV) is < 1; i.e., some vessels are perfused while others are not.

Response: This study contains cases where PPV = 1 (baseline videos) and PPV < 1 (hemorrhage videos). The stark differences between these two states serves as validation. We have added a notation in the paper to clarify PPV. We have added the definition to the results where we mention the source of the study data.

A5. Critique: Please provide measures of the total capillary density (TCD) and the functional capillary
density (FCD) separately and calculate the PPV.

Response: This was done as recommended. Please see the updated results.

A6. Critique: Since you analyze four sequences of five video frames per time point, please report on the variability (and/or kappa coefficient) of your method within these four analyses. Or perhaps you could analyze five sequential seconds per video clip and then report the variability of your method.

Response: This analysis was added to the paper.

A7. Critique: As the manual analysis with the AVA software is currently the golden standard for microcirculatory image analysis, correlation analysis and Bland-Altman analysis of the MCA-derived TCDs and FCDs versus the AVA-derived TCDs and FCDs would truly validate the MCA method.

The other reviewer, who was also a member of the same team forming AVA and the Bezemer et al paper, strongly believes that the manual analysis with the AVA software is NOT the golden standard and we should not have compared our results with this manually edited AVA results. We are happy that Dr. Ince agrees on this point with us. As shown in the original paper, we have performed statistical analysis (t-test) between these two methods, showing that statistically there is no difference between the two populations. The results of Bland-Altman (Figure 14) are added to the revised version and in our opinion support the equivalency of MCA with manually edited AVA.

A8. Critique: The authors describe in their paper that “…there are currently no tools for the real-time analysis of the videos produced by the imaging systems.” However, such a study has been published in which a modified algorithms of the AVA software for fast (<30 sec) and fully automated TVD assessment (Bezemer et al., Med Biol Eng Comp 2011; 49: 1269-1278) was presented. This study validated the software by reanalyzing 325 SDF video clips from a study in intensive care patients and found correlation with manually-obtained TVDs, both in videos in which PPV=1 and in videos in which PPV<1. The authors should discuss the advantage of the method they present over that of the method developed by Bezemer et al. In that study by Bezemer et al a method was presented for microvascular perfusion assessment based on temporal pixel contrast analysis which was tested in video simulations and a true SDF video clip. It was found that the method was limited by high cell densities and velocities, which severely impeded the applicability of this method in real SDF images.

Response: In our opinion, Bezemer et al, as presented in the paper discussed above, is merely a very general concept that does not introduce significant novelty over AVA. Specifically, none of the “block diagrams” described for this method such as “contrast analysis”, “contrast thresholding” or “centerline contrast” is described in any level of mathematical details to allow independent assessment and comparison of this method to our method. The improvements described for this method over the AVA seems minimal and some thresholds selected for parameters (e.g. the value of 2.00 for contract score) were not clearly justified. More importantly, there is no implementation of this algorithm which can be used to compare our method (or any other method) with. Dr. Ince, who is also a co-author of Bezemer paper, states that this method’s performance “…was limited by high cell densities and velocities, which severely impeded the applicability of this method in real SDF images…” We believe this is due to the fact that many factors and thresholds in this method were set to fixed numbers that need adjustment from one video to another (just as in AVA). This is exactly the point we are making when we claim that the method described in our paper might be the only algorithm that is fully automated. Since the technique described by Bezemer is not commercially available, a performance comparison is not possible at this
time. Perhaps such a comparison can be done in the future. We feel however, that the results reported here will be valuable for study and consideration by themselves.

We have provided a short discussion of these issues in the discussion section of the paper.

A9. Critique: In the present paper the authors employ somewhat similar methodology for (binary) flow detection; i.e., calculating the sum of the pixel intensity differences between frames for twenty consecutive frames. However, the authors did not validate (or even evaluate) this method. The fundamental problem with such analysis methods is that in vessels without flow as well as in vessels with very high cell densities and/or velocities, pixel intensity fluctuations will be relatively low. Do the authors account for this and if so, how? Furthermore, low image focus and contrast further reduce the pixel intensity fluctuations as a result of red blood cell flow. This should also be acknowledged in the paper.

Response: Please see B8 and B15 below for part of the explanation. The method we chose is in reality validated in the results in their comparison to manually edited AVA and through the visual inspection and machine learning algorithms developed for the automation process. We now mention this limitation in the paper.

A10 Critique: Based on the above comment, please provide measures of the TCD and the FCD separately.

Response: As mentioned earlier, we have now provided separate measures of TCD and FCD.
Response to Reviewer: Iwan Dobbe

B1. Critique: The proposed method is compared with a commercially available package (AVA), which uses a semi-automatic method for analyzing microvascular networks. The authors compare their method with AVA in two ways: 1) just after AVA’s automatic analysis, 2) after manual editing. The former comparison is not realistic because AVA only yields acceptable results “after” editing.

Response: We respectfully disagree with the reviewer. This analysis was included to demonstrate just how much editing is required. It forms the basis for the desire for a completely automated setting. Without this comparison, it is difficult to show just how much work is required along with the challenges of developing a fully automated system.

B2. Critique: In addition, the paper claims that the automatic part of AVA analysis yields a lot of false positives without elucidating on the many AVA settings that they have used to get these false positives (especially AVA’s filtering parameter, “sigma” is of influence here). Different AVA settings probably yield much better results than shown by the figures in the manuscript.

Response: We utilized the AVA software as demonstrated by the manufacturer and its tutorial. Each video was processed and analyzed identically. Subjecting each video to different AVA filtering can be argued as not providing a fair comparison between edited AVA and the MCA technique described in this paper. The fact that the edited AVA and MCA FCD determinations were comparable, demonstrate the AVA methodology we used (again according to the tutorial provided by the manufacturer of AVA) is suitable. We have added a statement in our methodology section indicating that we followed the manufacturer’s instructions.

B3. Critique: In addition, the authors claim that AVA has no possibility to add vessels by drawing them manually. This is an incorrect statement, because recent AVA versions do have that functionality. A vessel’s centerline can be drawn and the vessel width can be set interactively.

Response: We utilized the only version of AVA available to us. We have inquired several times to the company making AVA as to newer versions but have never received a reply. This may be due in part that we have attempted to partner with the Microvision (makers of the microrcirculatory camera used in this devices) but they have declined partnership in developing new software. We can, therefore, only report results using what is available.

B4. Critique: I believe the question to answer in this manuscript should be how well the automatic analysis performs compared to the golden standard, which is probably manual analysis (with/without AVA).

Response: We believe edited AVA is the current “gold” standard. Dr. Ince (the other reviewer) agrees. We believe our analysis for this initial paper, is thus suitable.

B5. Critique: And, how fast the new method is compared to this golden standard? Bezemer et al., Med Biol Eng Comp, 8, 2011, also describes a fully automatic method for vascular analysis. Regarding to speed, it is better to compare the proposed method with other fully automatic methods like that of Bezemer.
Response: The technology described is Bezemer, to our knowledge is not a commercially available product and we have no access to it. In addition, our study was completed prior to the publication of Bezemer et al. Given this, and the fact that Dr. Dobbe is a co-author on the Bezemer paper and has a developmental history with AVA, we do not think this critique is fair.

**B6. Critique: Define the term “active vessel” (better: perfused vessels?)**

Response: We do use both terms in the paper. The Bezemer paper uses “perfused vessels” consistently to mean what we call “active vessels.”

**B7. Critique: What are the “machine learning algorithms” that you refer to?**

Response: The machine learning methods, which are used in the method, are mentioned in the manuscript. As discussed in the manuscript, the use of machine learning will compensate for variations due to differences in factors such as lighting, pressure, video quality and specific machine/camera used for imaging.

**B8. Critique: Many parameters affect the results of your algorithm? An overview of these parameters and their parameter values would be convenient. Please show why the chosen parameter values are optimal.**

Response: The parameters as well as how these parameters are empirically optimized using data are explained in the methods section. Specifically, a series of training videos were used as the training set in which we change the values of these parameters over a reasonable range and choose the values that give the parameters providing the best segmentation results. In this study, “the best segmentation result” was visually evaluated. This process is referred to in almost all image processing literature as empirical optimization of parameters. This explanation was added to the manuscript.

**B9. Critique: You use the term (near) real-time analysis. The MCA algorithm still needs 20s to analyze a video sequence. In my perception that is not even “near” real time.**

Response: This appears to be simply a difference in opinion. We believe it is fair to consider our analysis near-real time since real time would denote no delay in results reporting after signal acquisition.

**B10. Critique: You use the term images are “added”, while referring to binary images. These are actually “OR’ed” in you method (as is correctly written in Fig. 2)**

Response: We have corrected the term in the paper, using “unioned” instead of “added.”

**B11. Critique: In your conclusions, you refer to future work. That is not a conclusion.**

Response: We respectfully disagree. The conclusion is a simple summary of the study with additional statements of what future efforts will concentrate on.

**B12. Critique: Regarding Fig. 1 and related text: What kind of morphological operations are used?**

Response: We have denoted the additional morphological operations in the text. The figure, in our
opinion, does not require elaboration as it is a simple flow chart.

B13. Critique: Why is the video sequence split into 5-frame segments, instead of “one” longer segment for segmentation? Does it actually improve something? Please explain in the text.

Response: Averaging more than five frames results in “over-averaging” phenomena that would eliminate some of the important features important for segmentation. Experimentally, our results indicate that the 5-frame approach provides the best results. This explanation was added to the manuscript.

B14. Critique: Why is region growing necessary? What type of region growing is used?

Response: This has been explained in the paper in the methods section as a means to overcome apparent disconnection of vessels.

B15. Critique: Flow, in this method, is assessed in a binary way: flow or no-flow. It seems to me that blood “velocity” is an important parameter determining oxygen delivery. For example, a network with an extremely low flow will probably perform equally worse as a no-flow network. But the proposed method judge one type as “flowing” the other as “not flowing”. Please explain why binary assessment is adequate.

Response: We would completely agree that blood velocity/flow is an important component in determining oxygen delivery. While we would like to develop future computational methods of flow analysis, the current binary approach provides a means to determine FCD and this was the purpose of this study. FCD has been demonstrated to provide important information regarding tissue perfusion. While future efforts may concentrate on developing improved flow metrics, this should not detract from the current study. We have some additional statements in the discussion indicating the importance of future efforts in regards to flow metrics.

B16. Critique: Regarding Fig. 2 and related text:
Many images are shown but the difference between many is hardly noticeable. You might as well simplify the explanation by only showing a few (3?) threshold-related columns.

The figure is meant to provide an illustration of the overall complexity of the approach.

B17. Critique: Regarding Fig. 3 and related text:
This figure and the text is completely unclear to me. What is the angle of a vessel? Do you mean the orientation in the field of view? Or, the curvature of a vessel? I assume that the two curved lines represent a single vessel? Why is it curved that way? Why do all “Nearest background pixels to 5*5 neighborhood, N24” are all at the same position? This seems very unlikely to me. Please improve the figure and the text.

Response: We have clarified the image and explanation for figure 3. The process is used to find the centerline pixels in valid vessels and then reconstruct the vessel using the found diameter. The image may have lead the reader to believe that all 24 neighbors had the same nearest background, but what it is meant to show (and has been clarified) is that only one nearest background pixel of the 24 is used for the remaining calculations: the one which yields the greatest distance to the candidate’s nearest background pixel. The angle and contrast ratio serve only to validate the candidate pixel and are not
meant to be applied to the final vessel. The curvature of the vessel walls in the image is only meant to show that vessels change diameter and that the process is robust enough to account for such changes.

**B18. Critique: Regarding Fig. 4-8 and related text:**
The contrast of these images seem “very” low to me. Is it possible to analyze (and compare) results based on images with this blurriness at all? The point-spread function seems determined by scattering of the tissue, instead of the hardware optics. This hampers finding the actual width of vessels since it is widened by this point-spread. The FCD in terms of area taken by functional vessels divided by total image area will be biased due to this point-spread.

Response: It is possible to analyze these using both AVA and MCA. This is the point of our approach. Our approach takes into account the differences in quality of the images in its automation. This is more problematic with AVA resulting in the need for more editing. This is different from the cases in the Bezemer paper where the analysis seems to have been done on very high quality images. Our ultimate desire here is to provide a tool allowing for more widespread use of microcirculatory imaging and decision-making.

**B19. Critique: The intermediate results of initial (fully automatic) AVA analysis largely depend on the filtering parameter “sigma”. Changing this parameter will totally change the intermediate results. You should first optimize this sigma for this application, before making comparisons.**

Response: As mentioned earlier, we utilized the AVA software as instructed by the manufacture’s tutorial. Optimization of every image defeats the purpose of automation. In addition, “optimization” in this regard is subjective.

**B20. Critique: Regarding Fig. 9 and Table 2 and related text:**
It is evident that intermediate results are not useful to draw conclusions. AVA is a semiautomatic method. The results after automatic analysis should be edited by hand to obtain meaningful results. It is therefore not realistic to use/show these intermediate results in any comparison. However, fully automatic analysis is of interest. You should focus on how your method compares to manual analysis (either with/without AVA), which I guess is the golden standard, and how much faster it is compared to existing methods (such as manual analysis, the semi-automatic method of Dobbe et al, 2008 (AVA), or fully automatic using the method of Bezemer et al., 2011).

Response: Again, as we have previously responded, we respectfully disagree. Reporting the unedited AVA results provides the reader a frame of references regarding true automation as well as the amount of editing that is required. This sets up the context for desiring better automation. Also as we have pointed out, no comparison with Bezemer et al is possible given it is not commercially available.

**B21. Critique: Regarding Fig. 10-11 and Tables 1, 3, and related text:**
The scales are left unexplained. Is there a statistically significant difference between the proposed method and AVA?

Response: We have now labeled the scales in the figures. We have now also included Bland-Altman analyses regarding a comparison between edited AVA and the MCA approach.