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Opponent's opinion on the dissertation of Mgr. David Vondrášek "Morphological and mechanical properties of fibrous tissue localized in congenital talipes equinovarus"

The knowledge of molecular biology has made it possible to accurately classify a number of diseases diagnostically, but only indirectly, based on the presence of indirect markers - i.e. gene expression or the presence of various DNA variants. The actual phenotype of cells or tissues - i.e. the actual property of the material - has long been neglected. Therefore, I greatly appreciate the pioneering methodological approach to find a way to biophysically characterize such a difficult-to-analyze specimen.

During clinical examination, changes in the "stiffness" of the ligament are observed, but such definitions are very vague from a physical point of view, and the question arises as to what underlies this "stiffness". The amount of material or the change in its structure and physical properties? To find an answer, the author has chosen the path of using very non-mainstream techniques, namely two-photon microscopy, polarization microscopy and atomic force microscopy, and in addition he has imaged the specimens correlatively, based on the premise that by correlatively combining the images, and additional meta-information can be extracted. The work is straightforward, well written, almost textbook didactic, and it is evident throughout that it is clearly leading the reader to the goal. I appreciate the great care taken in the preparation of the native specimen to preserve its biophysical properties as much as possible.

Despite the strong positives, it is somewhat difficult to understand from the results what is seen in the microscopic images - how the sample is spatially oriented for imaging on the coverslip, how the long axis of section is oriented on coverslip. For example, in the polarization image (Figure 11), the orientation of the fiber bundles is approximately diagonal across the slide (is this the case of a FOV, or is this occurring throughout the sample? Shouldn't the measured angle be normalized to this direction and not to the x-axis? Also, in Figure 10, where the "top-down view" is mentioned, the depth encoding is visible, but why is it lower in the lower part of the image? Also, it is hard to imagine the orientation of Figure 7. Finally, it is not clear how the proportion of lipids assessed by THG varies in different parts of the sample. In all these cases, the presence of a simple cartoon-like illustration showing the whole sample, its section plane, the direction of view of the microscope with its field of view would be of fundamental benefit.

In addition to the actual microscopic techniques and their image analysis, great care has been taken in this work to create correlative microscopy, a very difficult task. However, the interpretation of the results

as well as the discussion make only very limited use of this advantage, e.g. by marginal remarks in the discussion. Simple image correlation or more complex methods of quantitative multimodal image analysis are not used at all. What is the relationship between local orientation, frequency of crimp patterns, and collagen, lipid, and mechanical properties?

Despite these shortcomings, the work is of a very high standard, and the results obtained and methodological approaches described are valuable for the field of mechanobiology, for understanding the pathophysiology of the disease, and potentially (in the future) for diagnosis. In addition to this thesis, David Vondrasek is author/co-author of five WOS-indexed papers, which clearly demonstrates his ability to work as a scientist, and I am happy to recommend this thesis for defense. I enclose the following questions:

1. Do you consider the change in the biophysical properties of the analyzed tissue to be a primary cause of disease (i.e., due to genetic changes in the connective tissue proteins) or a secondary phenomenon (e.g., intrauterine compression or other factors cause compression of the tissues, thereby changing their physical properties)?

2. You use 120 um thick sections. AFM only indents units of micrometers and does not produce a completely physiological type of mechanical stress on a given tissue. With tensile testing, you mention complications due to the nature of the sample. What method of mechano-phenotyping would you use? With AFM, have you observed any differences in various parts of the sample?

3. The samples or fields of view may differ in the proportion of lipids, which the author noted. Given that lipids affect mechanical properties, would it be appropriate, for example, to compare the mechanical properties of only those samples with comparable lipid fractions to reduce the variability?

4. SHG signal of collagen: you write that the orientation and distribution can be analyzed quantitatively, which is a great advantage over fluorescence microscopy. What are the factors that can interfere with quantification? The age of the slide, the thickness, the way the slide was cut?

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