All changes made by the authors to the main text of the manuscript are highlighted in purple!

Editorial Comments:

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

We thank the editor for this important note. We would also like to mention that we were surprised to learn that proofreading is not included in the rather high publication cost. At the same time, we have taken the opportunity to carefully check the text for spelling and grammar. We hope that we didn't miss any error.

1. 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 and Reagents.  
   For example: Imaris, Blender, etc.

We have taken this important note from the editor into account and incorporated all commercially used programs into the material and methods part. Furthermore, we have removed all commercial language from the text and inserted general descriptions of the required functions instead (see also marked passages in the manuscript). At the same time, the Blender framework should probably be not considered a commercial product (it is a free access, open source framework), thus we left the Blender name in the text. Moreover, our Python script is very specific to the Blender environment, thus the 3D-to-2D projection process can’t be described in general terms.

1. 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. Please include all safety procedures and use of hoods, etc.

We thank the editor for this helpful hint. We have now reworked the Protocol part into the requested format and we hope that it meets the requirements of the journal. In addition, we have worked on the text in order to depict the individual steps of the protocol more precisely and comprehensibly.

1. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We have decided to completely remove most of the non-directional parts of the protocol from the text in order to guarantee an easier reproducibility of the protocol. We thank the editor for this advice.

1. 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 and a maximum of 4 sentences per step.

The Protocol part has been revised according to the notes of the editor. We have replaced the long text passages with shorter and more concise formulations. Additionally, we now use the NOTE function of the journal to give important hints without affecting the clarity of the protocol.

1. It would help to have example data that can be provided with the article. We cannot film a general protocol so a specific example will allow us to have specific values and parameters to use in the video.

We thank the editor for this important note. For the demonstration of the training process and the classification of the shape data using SOM, we have already uploaded a corresponding example data set to github and referenced it in the text. For the 3D reconstruction of the microscopy data and the calculation of the DFT components, we now also provide sample data sets. The download instructions have been provided at the appropriate sections of the protocol part.

1. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Due to the revision of the protocol part we took the editor's critics to heart and now only marked the most important steps. We hope that this will make the workflow easy to understand and follow. Our current marked protocol length is under 2 pages.

1. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We have checked and revised our protocol with regard to the comprehensibility of the individual steps and the reproducibility of the overall context and hope that we have successfully implemented the editor's suggestions.

1. Please do not highlight the python script steps (4.2, etc.).

During the protocol review process, we realized that the points marked under 4.2., describing Matlab functions to examine the neural network’s topology, would only confuse the reader. We have therefore removed the markings in the corresponding places and converted most of the passages into NOTES.

1. 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].”

We obtained a letter from the copyright handler of Cytometry A by following our Cytometry A editor’s instructions, and we uploaded the approval note as a PDF file to our editorial manager account during the original submission process. The citation of the figures was changed according to the editor’s suggestions.

1. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

We thank the editor for spotting our mistake. We added an Acknowledgements sections to our manuscript and acknowledged colleagues and stated all funding sources for the presented work.

1. Please do not abbreviate journal titles.

We reworked the reference list of the manuscript according to the editor’s suggestion.

**Reviewer 1:**

1. The shape analysis is very effective. Why use SOM? Do you consider to use deep learning method?

We appreciate the reviewer’s feedback about the effectiveness of our shape analysis. During the implementation of our research project we had some discussions about the classification algorithm to be used. We chose SOM because it had distinct advantages for our data set. Due to the 2D overview of clustering, SOM results are very easy to interpret and changes in the hit- and distance-map patterns can be quickly identified by the user. A preliminary tagging of the data by the user to train the algorithm is not necessary for SOM, since SOM belongs to the group of unsupervised learning methods. In addition, such tagging of the dataset by the user may not always be objectively feasible. SOMs have the advantage over other systems that connections that remain hidden to the human observer can be identified by the algorithm. A very good alternative to SOMs are deep learning algorithms, as pointed out by the reviewer. However, deep learning algorithms need a large training dataset in order to function properly. Such large and tagged dataset was not available to us, thus we chose SOM as a valid alternative for our specific problem. Nevertheless, with a larger tagged training dataset, deep learning algorithms can be more efficient and successful than SOM. With the advent of large voluntary image datasets, this goal should be achievable in the not too distant future.

1. Line 242, why not use the inline function "selforgmap" provided by Matlab?

We appreciate the reviewers suggestion about the Matlab inline function. We actually already use this function in the provided Matlab script (line 72) to initiate the SOM. We provided this Matlab script for inexperienced users to simplify the task of training an SOM. However, the utilization of the above-mentioned inline command is indeed sufficient for more experienced users.

1. Line 253, why 2,000 iterations? What is the effect of setting other values of maximum iteration number?

The SOM starts to learn to distinguish between the various feature vector spaces only after the proper number of iterations. At the same time, too many iterations will result in “over-learning”, i.e., where the number of highly responsive artificial neurons will drop, thus the SOM would lose its functionality. We tested various numbers of iterations and found that after 2000 iterations the shape-based SOM learning pattern has stabilized. We recommend using 2000 iterations for systems similar to ours.

1. From 303, there are too many question marks in the equation. Can you check them?

In the final pdf version generated by JoVE at the end of the original submission process we cannot see any question marks in the Equation section and everything looks fine. However, we reassessed this part to make sure not to overlook any mistakes.

1. The results are quite good. One paper may help the readers understand your paper, and may be mentioned, see "Multiple sclerosis identification based on fractional Fourier entropy and a modified Jaya algorithm"

We appreciate the reviewer’s feedback about our proposed methodology and we now include a reference to the paper that the reviewer mentioned.

**Reviewer 2:**

**Major Concerns:**

1. In its current version the protocol appears not to be very user friendly. Firstly it requires 4 different softwares, 2 of which are commercial. Secondly, the delivered scripts should still be edited by the user if required (step 2.3 and 4.1.5 of the protocol). The protocol would be easier to use if the scripts are integrated together. Ideally the user would run Imaris for the surfaces and then run a custom Matlab GUI that invisibly calls the python and fiji scripts before running the SOM (with options for train and test). This may not be feasible, but at least the Blender and Fiji scripts should be integrated together. Also any of the user-defined parameters should be changeable in the GUI instead of within the scripts. In this way the users of the protocol do not need to know how to use 4 different programs and get used to reading scripts.

We thank the reviewer for the suggestion of integrating the script together and adding a GUI to customize the parameters for the software. We plan to work on the integration of Imaris and Matlab together with the Python script and the Fiji code and hope to make this available at a not too distant future time point. We already implemented a GUI for the Blender part to allow the user to change parameters without having to edit the script. We adjusted the Protocol accordingly.

1. The introduction to the protocol as well as the steps from 1 - 4.2.2.3 are not easy to follow. It could be written more to the point. The introductory text can be integrated in the bullet points and the different steps need to have conclusive titles. Thus the protocol could be followed step by step.

We followed the reviewer’s suggestion and transformed the introduction part of the protocol into bullet points. We also reworked the protocol and used a more concise language. Moreover, we transformed several steps into Notes and we hope that the protocol can be now be followed more easily.

1. The projections of the cells are obtained in Blender (Step 2). The authors advice to use at least 6 different looking angles for the projections and more if the shape is complex. How can the user know how many projections should be made? Is there a way to check that there is no under-sampling of the shape?

We tested various numbers of rotation and found that for the precise description of microglia six rotation were satisfactory. We would not recommend using a lower value for microglia (or other, similarly complex shapes), in order to avoid loss of information. The number of rotations needed may vary for each cell type depending on the size and complexity of the shape of the cell. A higher number of rotations will lead to a more precise transformation of the 3D shape into 2D. A simple way to check the necessary amount of rotations is depicted in Figure 5. If the DFT components’ amplitude does not change with an increased number of rotations, we assume that we don’t gain any further information about the shape by having more rotations.

1. To obtain the perimeter of the projections of the cells, the authors use the active contour algorithm in Fiji (step 3). Since the data is already segmented and appears to be a binary (only zeros and ones) 8-bit image, couldn't the authors make use of an easier and less computationally expensive method, like an edge-detector (Sobel filter)?

The reviewer correctly states that an edge detecting algorithm such as the Sobel filter can be successfully applied on binarized datasets. However, most of the edge-finder algorithms have difficulties if the shape of the object has holes in it. This can be sorted out if a filter is applied before or after the Sobel filter, in order to get rid of the “false” edges (holes in the shape) that were found. We found the Snake algorithm simpler in this regard, because it starts from the outside of the cell and is able to detect the outline and invaginations of the cell membrane in a reliable manner even if the shape has hole(s) in it, without the need for an additional filter.

1. In the 'representative results' section (Fig. 3b and c) it is unclear whether testing the trained-SOM was done on cells that were included in the training dataset. If so, no real conclusions can be made on the performance of the SOM from the results on test-data. The use of the word 'subset' (l. 355, 356, 363 and 462) implies that the test-set are part of the training dataset although it could also mean that they are another part of the entire dataset. It needs to be clearly stated in the text on which data the SOM is trained and tested and what is shown in the figures.

We thank the reviewer for spotting this imprecise wording on our behalf. Different datasets were used for the training and for the later classification with the trained SOM. We changed the text accordingly to clarify that the cells which were tested on the SOM were not included in the initial training dataset of the SOM.

1. The results of the time-dependent cell shape analysis show the changes over time of the DFT of the cell shapes of 2 types of cells (Fig.4). Since the protocol is explicitly useful for data without time component, does a trained SOM classify these cells at single time-points to the groups they belong to (resp. 'Mobile' and 'Interacting')? That would confirm that the proposed method is able to distinguish between cell types in single time points.

Indeed the trained SOM was in our case capable of classifying the cells from single time points into the group that the medical expert appointed. We agree with the reviewer that our method is able to distinguish cells in single time points.

1. In the discussion (l. 459-461) the authors mention that the method is very fast which argues for 'superiority', but no timing nor any comparison to other methods is given.

We appreciate the reviewer comment about the missing performance characterization. Regarding the timing aspect, we can provide an example: the medical expert who classified the cells manually needed approximately 4 weeks for his analysis of the dataset, whereas our workflow needed only about 1 day, including all steps. We added a corresponding section to the discussion.

1. The videos have no voice-over that explains what is being done. Addition of an explanatory voice could be useful to follow better what is being done.

The videos were only provided by us to clarify our approach to the reviewers, as suggested by the editor. These videos will be re-shot professionally, using the voice-over of an actor provided by JoVE.

**Minor Concerns:**

1. The introduction could be more explanatory, for example an explanation of SOM could be added as well as an explanation of the 20 DFT components that are talked of later on.

Following the reviewers suggestion we added explanatory parts in the introduction for DFT and SOM.

1. In the list of equipment Matlab & Machine Learning Toolbox are missing

We thank the reviewer for spotting our mistake. We added the entries accordingly in the Materials and Methods section.

1. In l.351/352 the reference to Figure 2a-d appears incorrect: a-b are examples of cell forms (as observed by expert) but c-e are results of SOM

We thank the reviewer for spotting our mistake. We corrected the figure legend according to the reviewer’s suggestion.

1. In l.259, protocol part 2.1.5, the authors speak of a Python script where the paragraph describes the Matlab part of the protocol.

We once again thank the reviewer for spotting our error. We fixed this issue in the text.

1. -7.

We are sorry about the inconvenience caused by the faulty video “Video\_Blender\_fromBlender\_Workflow.flv”, it has now been replaced with an updated video using the GUI-based Python script built into Blender “AutoRotate\_v2.0.blend”. We also recreated the video corresponding to the GUI-based Python script that we newly added to this work (“Video\_Blender\_fromPython\_Workflow.flv” The rest of the videos seem to play flawlessly in VLC. Please also note that these videos were only provided to clarify our approach to the reviewers (as suggested by the editor). We do not plan to publish these videos, rather they will be shot professionally including proper voice-over by the JoVE staff. At this stage of the revision we are not referring to the videos anymore in the manuscript. Nevertheless we also uploaded the updated versions of the videos for the Reviewers’ and Editor’s convenience.