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# HISTOLOGY ANALYSIS

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## *Protocol for manual myofiber CSA measurement in FIJI/ImageJ*

1. Open FIJI/ImageJ.
2. Import the .czi image file using the Bio-Formats Importer.
3. During import, open all available sections or series so you can review the full set of images.
4. Select the Split Channels option so that FIJI opens each channel separately.
5. After import, keep only the images corresponding to channel 2, C=2, because this channel provides the clearest visualization of myofiber boundaries for cross-sectional area measurement.
6. Review all available sections from channel 2 and compare them carefully.
7. Choose the most suitable section for analysis by considering the following criteria:
  - fibers appear relatively uniform and consistent in shape
  - the section is not too oblique, so fibers do not appear artificially elongated
  - cell boundaries are clearly visible
  - the image is in adequate focus
  - the section shows minimal tearing, folding, blur, or other artifacts
  - the section contains enough intact fibers for measurement.
8. Exclude sections that show excessive distortion, major tearing, severe blur, unclear fiber boundaries, or many incomplete fibers.
9. Once you identify the best section, set the image scale so that all measurements are reported in  $\mu\text{m}^2$  rather than pixel units.
10. Use the image calibration or known pixel size to define the scale correctly.
11. Open Analyze > Set Scale and confirm that the unit is set to  $\mu\text{m}$  so that area measurements are recorded in  $\mu\text{m}^2$ .
12. Open Brightness/Contrast and click Auto to improve visualization of the fiber borders.

13. Use brightness and contrast adjustment only to improve visibility during manual selection, and do not use it to alter the image in a way that changes the measurement itself.
14. Divide the selected image conceptually into four quadrants so you can sample fibers from across the entire section.
15. In each quadrant, manually select 25 fibers, for a total of 100 fibers per image.
16. Include only fibers that meet the following criteria:
  - the full cross-section is visible
  - the boundary is clearly defined
  - the fiber is not cut off by the image edge
  - the fiber is not merged with a neighboring fiber, the fiber is not severely distorted
17. Exclude fibers that are incomplete, torn, blurred, merged, heavily distorted, or impossible to trace reliably.
18. Use a consistent selection strategy across all quadrants and avoid choosing only unusually small or unusually large fibers.
19. Outline each selected fiber manually using the appropriate selection tool in FIJI/ImageJ.
20. After outlining each fiber, add the selection to the ROI Manager.
21. Continue this process until you collect all 100 ROIs in the ROI Manager.
22. Check the ROI list to confirm that all selected fibers have been included.
23. Once all ROIs have been collected, measure all selected fibers at the same time using the Measure function from the ROI Manager.
24. Record the cross-sectional area values in the Results window.
25. Save the Results table and export the CSA data file for downstream statistical analysis.

#### *Downstream myofiber CSA data analysis in R*

26. Open RStudio.
27. Load the required R packages for data import, data organization, statistical analysis, and figure generation.
28. Import the exported CSA data files from FIJI/ImageJ.
29. Add experimental condition labels to each dataset, such as treatment group, infection status, and timepoint.
30. Combine all datasets into one dataframe.
31. Convert grouping variables into factors.
32. Check that all samples are assigned to the correct groups.
33. Treat each sample as the true statistical unit, because multiple fibers measured from the same sample are not independent biological replicates.

34. Summarize the fiber-level CSA values for each sample.
35. For each sample, calculate the number of measured fibers, mean CSA, median CSA, and standard deviation of CSA.
36. Use sample-level mean CSA for statistical testing.
37. Use individual fiber-level CSA values only for visualization of CSA distributions.
38. Create analysis subsets based on the comparisons of interest.
39. For two-group comparisons within one condition, compare sample-level mean CSA between groups.
40. Use Welch's t-test for mean comparisons when appropriate.
41. Use Wilcoxon rank-sum tests as non-parametric supporting comparisons when appropriate.
42. For comparisons involving two experimental factors, fit a linear model using sample-level mean CSA as the response variable.
43. Include the two main factors and their interaction term in the model.
44. Use the model to test for main effects and interaction effects.
45. Check model assumptions using residual normality tests and homogeneity of variance tests.
46. Use ANOVA to evaluate the significance of the model terms.
47. Use estimated marginal means and pairwise comparisons when group-level follow-up comparisons are needed.
48. Generate boxplots of sample-level mean CSA for the main comparisons.
49. Overlay individual sample points on the boxplots.
50. Generate density plots, frequency plots, or histograms to show fiber-level CSA distributions.
51. Interpret fiber-level plots as descriptive visualizations only.
52. Report CSA values in  $\mu\text{m}^2$ .
53. Report statistical results based on sample-level mean CSA, not individual fiber-level values.
54. Save the processed data, statistical outputs, and figures for reporting.