VolumeFinder: analysis of 3D pointsets in IgorPro

Stephen J. Royle[1] and Thomas R. Honnor[2]

[1]Centre for Mechanochemical Cell Biology, Warwick Medical School, Coventry, UK
[2]Department of Statistics, University of Warwick, Coventry, UK

November 24, 2016

Contents

1 Background

The code in VolumeFinder is used to analyze microtubule organization in mitotic spindles. A full description of the methodology and the application of our code can be found in our paper *Microtubule organization within mitotic spindles revealed by serial block face scanning EM and image analysis.*

The original ideas for mathematical modeling was from TRH. Igor code was written by SJR and the R code was independently written by TRH.

2 Technical notes

This walkthrough was completed on a MacPro (Late 2013) running OS X 10.11.6, using FIJI/ImageJ 2.0.0-rc-43/1.51d and Igor Pro 7.00. Amira 6.0.1 was used for the initial segmentation. The analysis in R was run using RStudio Version 1.0.44.

# 3 Preparing images for analysis

Images from serial block face scanning electon microscopy (SBF-SEM) are captured as TIFFs. In order to use VolumeFinder, the images require segmentation to identify microtubules (MTs). In this workflow, segmentation was done manually using `Amira` (Visualization Sciences Group, FEI). Settings for segmentation were a 5-pixel circle and a value of 2. A 5-pixel circle is defined as a circle of 3-pixel diameter. Other software can be used for manual segmentation, e.g. ImageJ or IMOD; however, the FIJI macro supplied here is written for Amira mesh files.

4   Setup

Place all procedures in `User Procedures` directory which can be found in:

- `/Users/<user>/Documents/WaveMetrics` on Mac

- `C:\Users\<user>Documents\WaveMetrics` on Windows

Place all Amira mesh (`.am`) files to be analyzed in a single directory. These files are named `*.labels.am` by Amira. For the purpose of this document, this directory is called `MT`, and is on the Desktop (Figure 1).
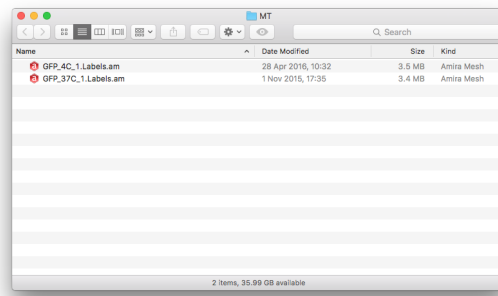
Figure 1:   MT directory containing two amira mesh files for analysis.

Run am2skel.ijm in FIJI. Point to the `MT` directory. This will generate all the files needed for analysis in Igor (Figure 2).
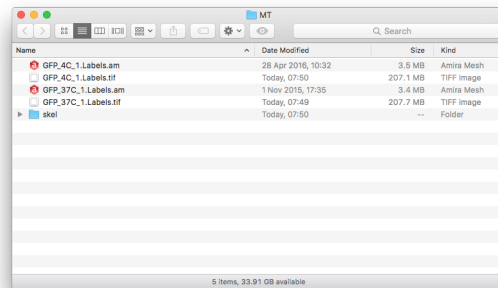

Figure 2:   MT directory after running am2thresh.ijm.

These are:

- TIFF stacks - one for each `.am` file. Binarized to show only MTs. Used for volume analysis

- skeletons - a directory per `.am` file containing single TIFFs (one for each z-slice) of skeletonized views of the binarized TIFF. Stored in directory called `skel`, within `MT`. Used for spatial analysis.

5 Analysis

Load VFMasterproc.ipf into a new Igor experiment (`.pxp` file). Click Compile if the procedure doesn't auto-compile. This will give several analysis options to the Macros menu (Figure 3).
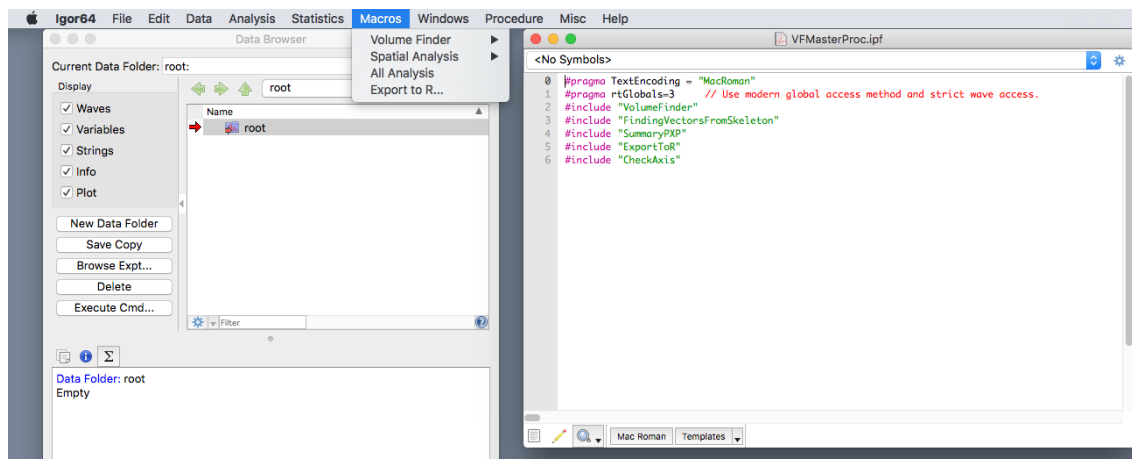
Figure 3: Compiling VFMasterProc.ipf in a new Igor experiment.

Now select one of the following from the Macro menu:

1. Volume Finder

2. Spatial Analysis

3. All Analysis

4. Export to R...

5.1 Volume Finder

This workflow will measure the volume of microtubules in the spindle as a density of the volume in which they are contained. It works on a directory of TIFFs, processing all TIFFs and summarizing them.

To do this, select Macro >Volume Finder >Find Volumes... or execute `VolumeFinder()`from the command line.

Now, point Igor at the directory containing the TIFFs. In our example this is `MT`. This process will import all of the TIFFs and run some calculations.

A summary PDF is created showing the MT volumes, spindle volumes and densities as category plots. A table is also created showing the calculations (Figure 4).

Notes:

- the calculations can take several minutes (be patient)!

- Igor prints in the history how long each file took to process, in seconds.

- Igor converts the values to real world values using a conversion of 12 x 12 x 60 nm. If you are using a different resolution, you will need to edit the line that calls `ScaleIt(xnm,ynm,znm)`.

- this conversion assumes a brush size of 5 px, i.e. circle of 3 px diam, in Amira.
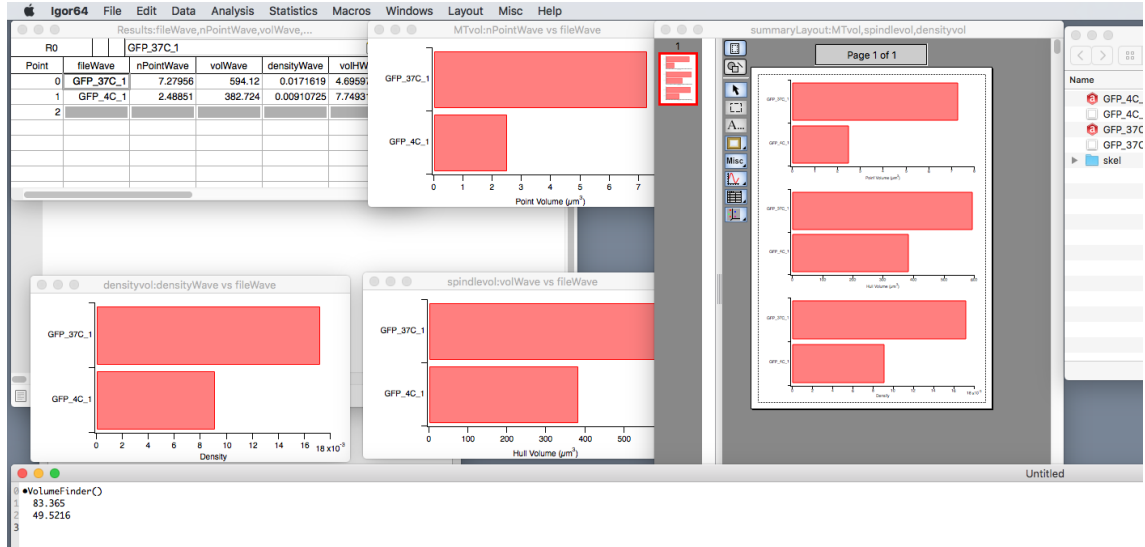
Figure 4: Results of running Volume Finder.

5.2 Spatial Analysis

Spatial analysis is the comparison of MT angles with idealized counterparts generated by ellipsoid theory.

Spatial analysis is run on each dataset. Later, these analyses can be combined and processed using All Analysis.

A schematic diagram to show how the spatial analysis workflow happens is presented in Figure 5.
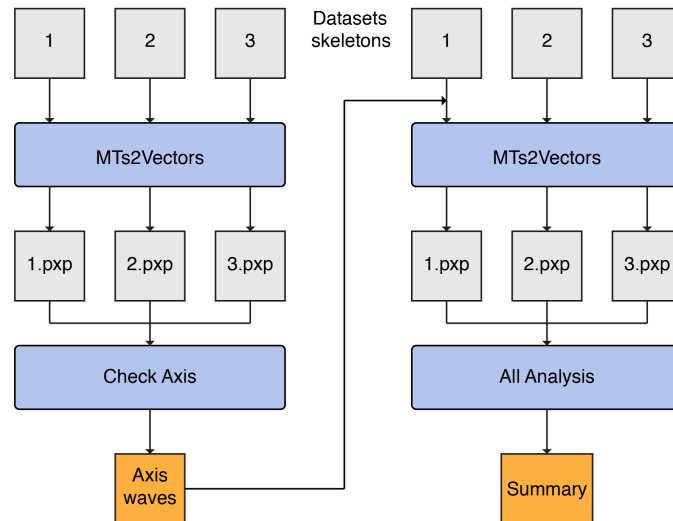

Figure 5: Workflow associated with Spatial Analysis. Typically, the user begins with uncertainty over the location of the spindle poles (left). The output of this analysis can be verified and corrected if necessary using Check Axis. The output of this is four waves which can be used to automatically specify the spindle poles in the second pass through (right).

Spatial Analysis begins with selecting: Macro >Spatial Analysis >MTs2Vectors... or by typing

`MTs2Vectors()` in the command window.

Point Igor at the directory within `MT/skel` that you want to analyse.

In the first instance, you will only have skeletons and will not have spindle axis waves (Figure 6). So select "No" here.
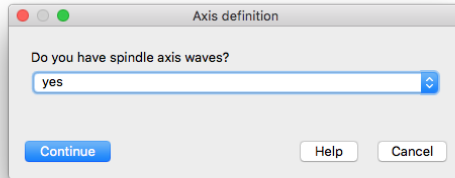

Figure 6: Do you have spindle axis waves? The default is yes. For the first time, you will not have spindle axis waves, so select "No".

You are next asked what the base name is for the skeletons (Figure 7). This should be picked up automatically and should not need changing. For our example, the skeletons have names like `GFP_37C_1.Labels0000-labeled-skeletons.tif` and this tells Igor how to read them.
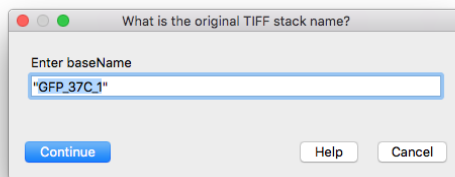
Figure 7: What is the original TIFF stack name? Igor takes a guess at this, but if it is different then change it here.

Next, Igor checks with you what the scaling is of the images. The default is 12 x 12 x 60 nm, but change this if it is different (Figure 8).
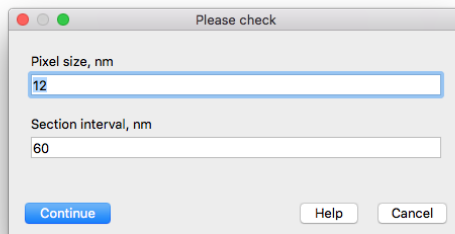

Figure 8: Input voxel size here.

Because no axis waves were defined, the next dialog is populated with default values (Figure 9).

Enter values. A best guess is sufficient for now and can be corrected later. The values are entered as pixels, and are scaled later.

Figure 9: Define spindle pole positions here.

Igor will now run the analysis using the spindle pole definitions that you gave. When it is finished, the option to save the summary PDF (Figure 10) is given and then the experiment itself must be saved. If you are certain of the spindle pole positions, this experiment is now complete.
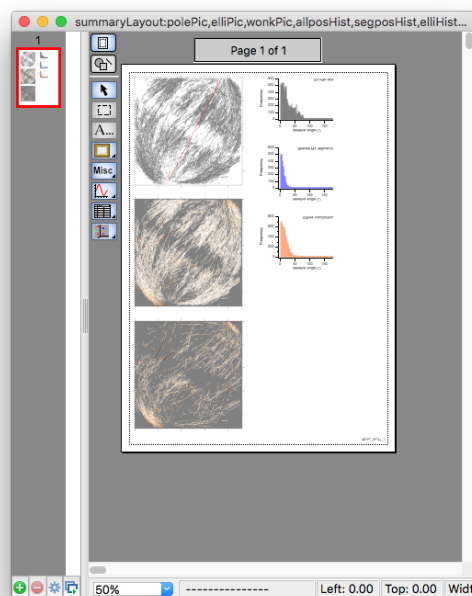
Figure 10: Summary layout is saved as a PDF with the base name specified earlier.

If all the spindle pole positions are defined in axis waves, then the above workflow is much more simple. At the first dialog, select "Yes". Igor will read the spindle pole positions and go through to the analysis, generating the summary PDF.

The axis waves can be defined following verification using Check Axis.

5.2.1 Check axis

You can use Check Axis to verify that the spindle axis is in the right place. It can be difficult to site the spindle poles correctly from SBF-SEM data alone. Using Igor's 3D Gizmo tools, you can look at the positioning of spindle axes for all spindles that you have before committing to the final analysis. This procedure also allows you to output the corrected spindle axis waves to automate the re-analysis.

Select Macro >Spatial Analysis >Check Axis or type `ProcessAll()` in the command window.

Point Igor to the directory where your Igor experiments are saved (in this case this is `MT`). Igor will now load all the `*.pxp` files in the directory.

Each dataset is plotted as a Gizmo – MTs are shown in black and the axis is shown as a red line with P1, C and P2 shown as red, green and blue spheres, respectively (Figure 11). A small panel called CorrectAxis is made to allow you to work with the spindle axis.
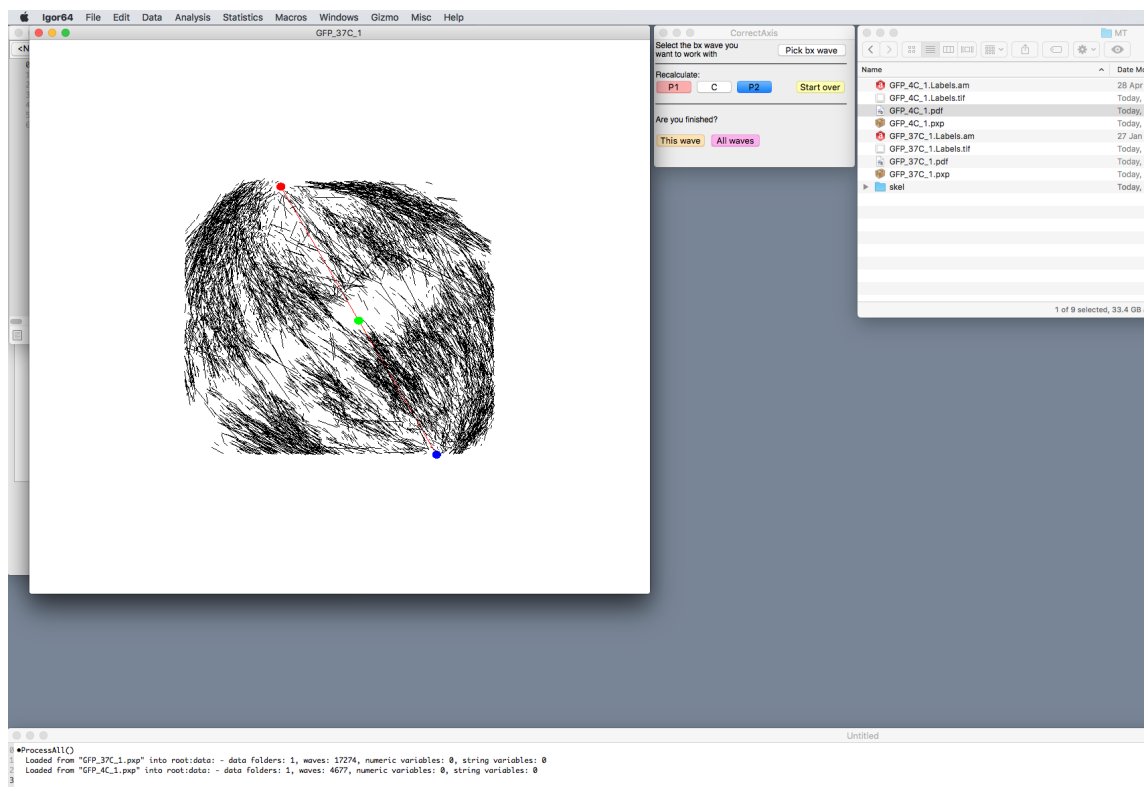
Figure 11: Loading spindles as 3D objects to verify the spindle location.

Select the Gizmo that you would like to work with by selecting Windows >Other Windows >YourSpindleName.

Now click on the "Pick bx wave" button in the CorrectAxis panel, and select the corresponding bx wave.

A table called p1cp2 pops up and allows editing of the spindle axis.

Rotate the spindle and assess the axis definition.

To change any of the points, edit the coordinates directly in the table. Once an edit has been made to either p1, c or p2, the other points can be recalculated by pressing the appropriate button in the panel. For example, if p2 has been changed you might want to position p1, based on p2 and

c; or you may wish to reposition c, based on p1 and p2. A new gray axis is generated to compare with the original data (Figure 12 and 13).
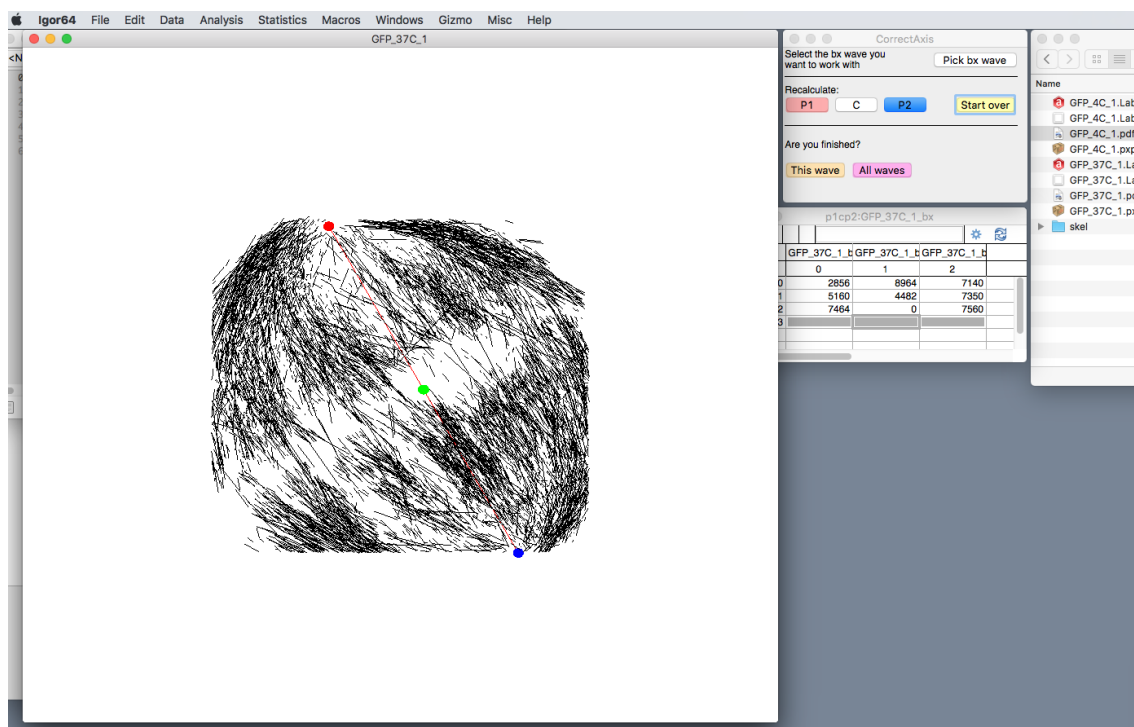
Figure 12: Repositioning the spindle axis. Before repositioning

If you make a mistake, the axis wave can be reset to the original values using the "Start over" button. When you have finished with this spindle, press "This wave" button and repeat with the next spindle.

Once all spindles and their axes have been verified, click "All waves" button. This will prompt to save the axis wave files, save them in a new directory `MT/points`.

The axis waves can also be defined manually by saving appropriately named waves into the correct directory, but this is not recommended.

Once the axes have all been defined, you can go back to MTs2Vectors and reload the skeletons, using the spindle axis waves in `MT/points`.

Notes:

- the first file selection dialog that opens after selecting "Yes" to having spindle waves, is to specify the directory containing these waves. The second file selection dialog is now to specify the skeleton directory.

- two other options are available from Macros >Spatial Analysis. These are Remake Report and Redo Ellipse Comparison. Both options allow the user to enter the workflow at a later point and circumvent reloading the skeletons (which is a lengthy process).

- the spindle axis waves are named: labelWave.ibw,r_cWave.ibw,r_p1Wave.ibw,r_p2Wave.ibw. All are needed for automated load and analysis.
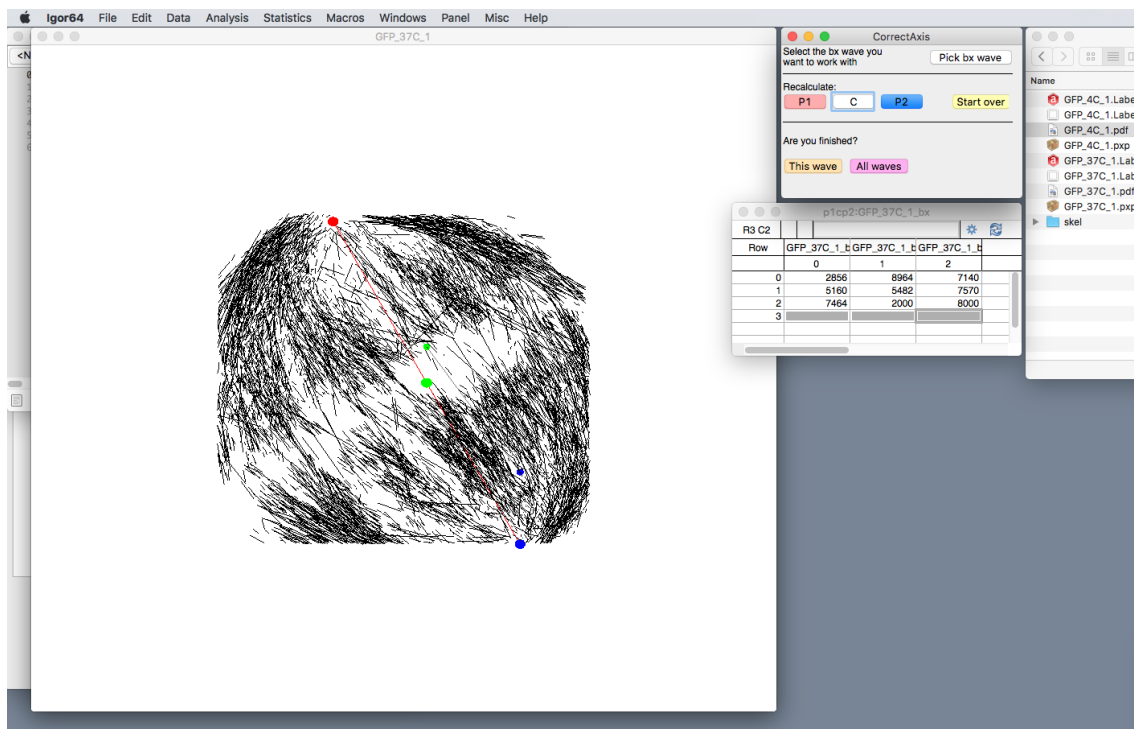
Figure 13: Repositioning the spindle axis. After repositioning, in this case y and z coordinates of p2 were altered and the c point was recalculated.

- the summary PDF is made showing three plots: 1) a Z-projection of all MTs along with the spindle axis in red, 2) MTs colored according to the angle difference, and 3) MTs with greater than 20°; and three histograms: 1) comparison of angles relative to the spindle axis (gray), 2) angles relative to MTs within 80 nm of each MT (blue), 3) angle difference compared to the idealized spindle (orange).

- for 3D display of the original data, add the wave `vec3d` to a new Gizmo as a Path.

- for 3D display of the modeled data, add the wave `elli3dre` to a new Gizmo as a Path.

5.3   All Analysis

In order to combine all Igor experiments, select Macro >All Analysis or type `AllAnalysis()` in the command window. Point Igor to the directory containing all Igor Experiments (in this case this is `MT`), Igor will ignore other files in the directory. Igor makes some calculations and generates 3 PDFs: histograms of angle differences from the idealized data (histE.pdf), histograms of angle differences compared to nearest neighbors (histS.pdf), a summary of the data (report.pdf) showing mean, median and standard deviations of the distributions shown in histELayout. Save the PDFs and this experiment (Figure 14).

5.4   Export to R

In order to combine all Igor experiments, select Macro >Export to R... or type `LoadAndExportToR()` in the command window.
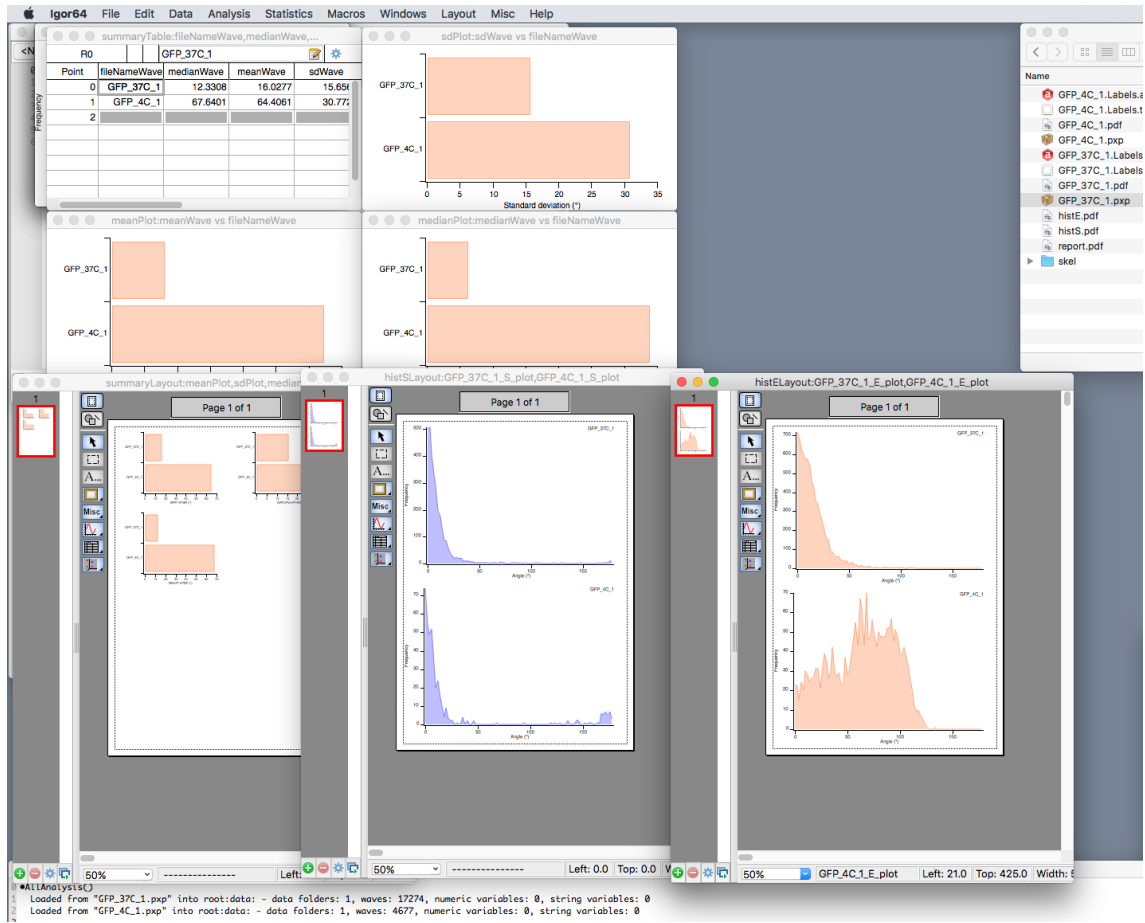
Figure 14: Outputs from All Analysis.

Point Igor to the directory containing all Igor Experiments (in this case this is MT), Igor will ignore other files in the directory.

Igor loads each Igor Experiment and saves two text files (csv) per experiment in the same directory, MT(Figure 15).

The first file, *_labels.txt has a list of the vector object names in a single column for reference. The other txt file is a csv with 6 columns of the xyz coordinates of the start and end of each vector per line.
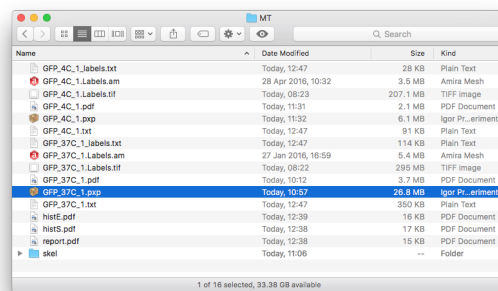
Figure 15: MT directory showing the outputs of LoadAndExportToR().

6 Analysis using R

As an alternative to `MTs2Vectors`, we wrote an R-based analysis tool. You may wish to run the analysis in R if you are more familiar with this software. Perhaps you wish to do further processing in R, or maybe you simply want to verify the results from the Igor analysis.

6.1 Mitotic spindle modelling

The R markdown file Mitotic_spindle_modelling.Rmd can be run in RStudio. This analysis requires the xyz coordinates of the start and end points of all lines generated from the skeletons. These coordinates can be output in csv format using `LoadAndExportToR()` as described above. Alternatively, you may want to use some other non-Igor workflow to get the coordinates and run the analysis in R. Details of how to setup the coordinates for analysis are given here. If the files containing the coordinates are organized as described, the markdown file can be simply loaded into RStudio and when the user clicks **knit** the full analysis is performed and outputs go into designated folders in the directory (Figure 16).
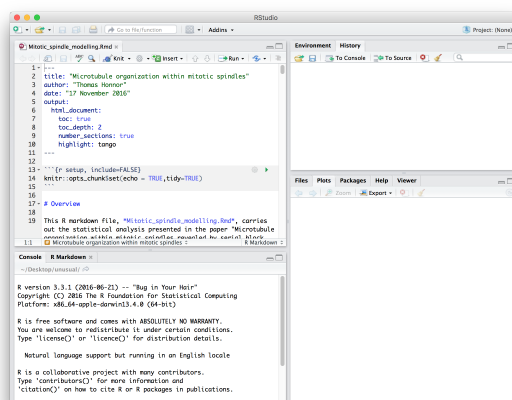
Figure 16: RStudio showing Mitotic_spindle_modelling.Rmd. Once the files are in place, all you have to do is click knit.