Spectrasor brief tutorial

Spectrasor is an open-source and multi-platform Java software developed for the analysis of fluorescence data by using a (multi)spectral phasor approach. This tutorial is intended to offer an initial guideline for using the software, for theoretical aspects and applications of phasor analysis consult other literature. For a better understanding of the software's user interface consult the user manual provided.

This tutorial is divided in two parts, one related to the Multispectral phasors tool and the other related to the Fraction unmixing tool. All the raw files provided for illustrations are mathematically constructed using gaussians equations and they do not represent real or experimental data.

Part I: Multispectral phasors tool

This is a very simple tool, with the main purpose of load and prepare the data, set the visual configuration for the phasors and save for future usages, or export the data as excel or images.

Before opening Spectrasor, take a look at the *SingleGaussian.csv* file provided and notice its format. This is a full excitation-emission matrix, where the first column contains the emission wavenumber values (in μm^{-1}) and the first row contains the excitation wavenumber values (also in μm^{-1}). For more information on the raw data to be loaded by the Spectrasor software, consult its user manual.

1. Open Spectrasor and go to the Multispectral phasors tool (the one in the left).

2. Click on "Load" and browse to the *SingleGaussian.csv* file location and open it (note that the File Type selected in the browser should be Comma Separated Value). A dialog window will open asking for the x-axis units, select wavenumber in μm^{-1} and click "Ok". A loader window will open with the list of spectra selected (only one) and requesting a name for the new assay. Give it a name and click "Load".
   The raw data is correctly loaded and you can now visualize the excitation/emission spectra as well as the phasor plot. Because there is only one spectrum loaded there will be only one dot in the phasor plot.

3. Change the visual representation of the phasor to your preferred shape and color by using the "Shapes" and "Colors" selectors (the option "Red-Blue" or "Blue-Red" means changing the colors along the loaded spectra list following a rainbow pattern).

4. Change the position of the vertical lines in the spectrum graphs and notice the changes in the phasor position. The vertical lines at both ends of the spectrum represents the range used to calculate the phasor, therefore, changing the range will change the phasor coordinates. The middle vertical line represents the selected excitation/emission value from where the emission/excitation

graph is obtained. Because the data comes from is a simple perfect gaussian, changing the middle line will only change the scale of the graphs but not its shape.

5. Click on “Load” and browse to the *TwoGaussians.csv* file. This data is also a full excitation-emission matrix, but it is obtained by combining two different gaussians, therefore, its shape is not perfect. Select wavelength this time and give it a name.

6. With the new assay selected, move the middle vertical lines and notice the difference in the graphs’ shape.

7. Change the x-axis units to μm^{-1} and select both assays using the Ctrl key. Move the middle vertical line until both graphs are at a similar scale and notice the difference between them. This difference is also reflected in the phasors’ coordinates.

8. Select the first assay and change the harmonic value to (0, 1). This means to construct the phasor only for the emission part at the selected excitation value. Change the middle line in the excitation graph and notice that the phasor does not change. This is due to the fact that this data is a perfect gaussian and changing the excitation will only change the emission graph scale, and the phasor do not depend on this.

9. Do the same for the second loaded assay. Notice that in this case, there is a slight change on the phasor position when the excitation value changes. This occurs because in this constructed data the shape of the emission graph is not the same at each excitation value.
   Note: *for a multispectral phasor case (when the harmonic does not contain a 0) the selected excitation/emission values are not important because the full matrix is used.*

10. Select both assays and click “Save”. Select “All at once” and click “Save”. Browse to the location you want to save for each case. A spectrasor assay list file (.***sprl***), an excel file and an image will be saved.

Part II: Unmixing tool.

In this part, you will perform two unmixing procedures: one for a three components system and one for four components system. Before using the unmixing tool, the data needs to be prepared using the multispectral phasors tool. If you like, you can skip to the unmixing process and use the provided spectrasor assay list files (.***sprl***).

Preparing the data

1. Open Spectrasor and go to the Multispectral phasors tool.

2. Click on “Load” and browse to the provided *3ComponentsUnmixing* folder. Select all the raw data files named *Mix_#.csv* (9 files), select wavenumber in μm^{-1}, name it “Mixed” and open it.

3. Repeat the previous step and open the 3 *Pure_#.csv* files and name the assay “Pure”.

4. Select both assays and click on “Save”. Select Spectrasor file and save the file in your preferred location.

5. Click “Clear” the remove the loaded assays.

6. Repeats the steps from 2 – 5 but using the data in the *4ComponentsUnmixing* provided folder. You should have now 4 “pure” spectra and 1 “mixed” spectrum.

Three components unmixing

1. Open the Unmixing tool in Spectrasor.

2. Clock “Load” and browse to the spectrasor list file (.***sprl***) created in the previous section (or open the provided file if you decided to skip the previous section). Remember to change the File Type to Spectrasor list file.

3. Select the “Pure” assay and the “Pure_X” spectrum. Click “Add from selection” to add a reference point. Name it as “X”.

4. Repeat the previous step with the “Pure_Y” and “Pure_Z” spectra and name them as “Y” and “Z” respectively.

5. Select the “Mixed” assay to see the phasors. Select all three references to see the blue triangle.

6. Click on “Set equations” to set the system to solve. Select 3 components and click “Ok”.

7. In the opened dialog select the (1, 1) harmonic (selected by default) and click “Add G equations”, “Add S equations” and “Add fraction sum equation”. This is the typical 3-component system of equations that uses only one harmonic. Click “Done”.

8. Change the selection to “Mix_5” spectrum and the X reference point and notice the text printed in the bottom. The fraction should be around 50%. Change the reference point and notice the change in the value.

9. Select all references, click “Save” and select “Yes” to save an image only. Click “Export” to save an excel file with the unmixing results.

10. Explore the excel file and get familiar with the exported data.

Four components unmixing

1. Open the Unmixing tool in Spectrasor and follow steps 2 – 4 from previous section including a fourth reference point from the “Pure_W” spectrum and name it as “W”. Make sure to add the reference in the order: W, X, Y and Z.

2. Select the “Mixed” assay and the four reference points to see the phasor plot.

3. Change to the (1, 2) harmonic and repeat the previous step to compare.

4. Make sure that the reference points have the same order in the four multispectral harmonics, click “Set equations” and select 4 components.

5. Change to the harmonic (1, 1) and click on “Add G equations”, then repeat the same for the (1, 2), (2, 1) and (2, 2) harmonics. Your system should have 4 G coordinates equations, one for each harmonic. Click “Done”.

6. Select the “Mixed” spectrum and the X reference point. Its fraction should be around 20%.

7. Select the four reference points and click “Export” to save an excel file with the unmixing results.

8. Click again on “Set equations” to change the system of equations and select 4 components.

9. Repeat step 5 but this time adding the S equations for each harmonic.

10. Select the four reference points and click “Export” to save an excel file with the unmixing results. Compare with the previous excel, results should be the same.

11. Repeat the procedure with your own system of equations and compare.