Spectrasor user manual

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 manual is intended to provide specific information necessary to use the software, for a deeper insight of the theoretical aspects and applications of phasor analysis, other literature should be consulted. In addition, it is recommended for first-time users to also consult the tutorial files provided with the software in order to become familiar with the user interface.

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About the Spectrasor files format

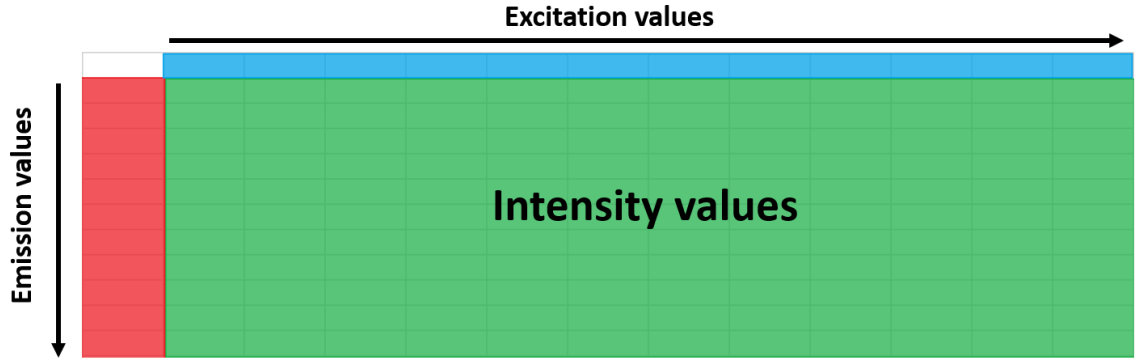
The following list summarizes the Spectrasors file formats used for persistence by saving and loading the user's work.

- **Spectrasor file (*.spr*):** stores a single spectrasor assay. A spectrasor assay contains a list of fluorescence spectra data in both excitation-emission dimensions, together with visual information of the phasor representation (colors, shapes, scales).
- **Spectrasor list file (*.sprl*):** stores a list of spectrasor assays in a single file.
- **Reference point(s) file (*.rfp*):** stores a list of reference points that can be loaded in the fraction unmixing tool.
- **Spectrasor fraction analysis file (*.sprf*):** stores a list of spectrasor assays together with a list of reference points that can be used by the fraction unmixing tool.

Preparing raw data files

Loading raw fluorescence data into Spectrasor is usually the first step needed for the analysis. In order to do that, the raw data must obey certain format and, depending on the spectrofluorometer used, it might be necessary to prepare the raw file before using Spectrasor.

The raw data must be stored as a Comma Separated Value (.csv) file where the first row and the first column contain respectively the excitation and emission values, as illustrated below. The rest of the values will correspond to the fluorescence intensities of the excitation-emission matrix (EEM). The file must not contain any other information additional to the values, which could raise an error while loading.



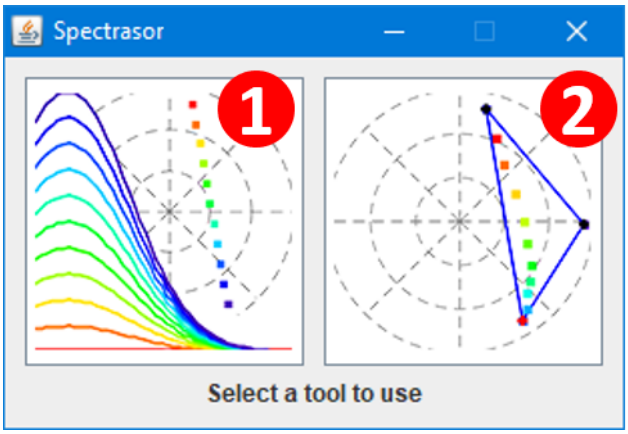
The raw data must have at least two columns or two rows, in which case it will only contain a 1-dimensional spectrum, either being an emission or an excitation spectrum, instead of a full EEM. When loading a raw data file, the user will be asked to indicate the x-axis data units, which can be one of the following:

- wavelength in nanometers.
- wavenumber in cm^{-1}.
- wavenumber in μm^{-1}.

After the data is correctly loaded, the x-axis can be changed at any time.

User interface

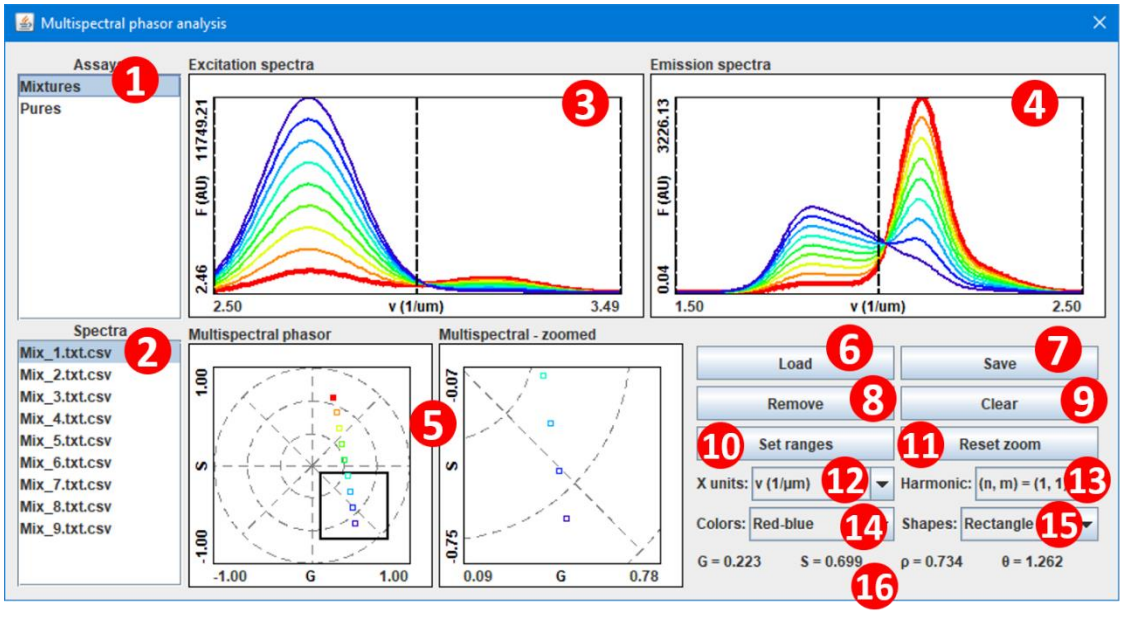
The Spectrasor root window (see below) allows to select which tool the user will use, either the multispectral phasor analysis tool or the fraction unmixing tool.



1. Select to open the multispectral phasor tool.
2. Select to open the fraction unmixing tool.

Multispectral phasor tool

The multispectral phasor tool (below) allows to load raw or pre-saved fluorescence data in order to obtain their phasor plot, configure their visual representation and save/export processed data or images.



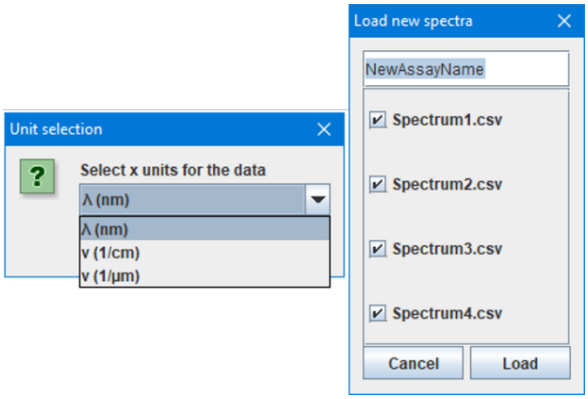
1. A list of the loaded spectrasor assays, which are just a collection of spectra data together with their visual configuration. Multiple selection is allowed for images construction.
2. A list of the spectra included in the selected spectrasor assay.
3. Excitation spectra at the selected emission value. The vertical dotted lines can be moved and they define the range used to calculate the phasors and the selected excitation value used to represent the emission spectra in the graph

to the right. If 1-dimensional emission spectra are loaded, this graph does nothing. The selected spectra are highlighted by thicker curves.

4. Emission spectra at the selected excitation value. The vertical dotted lines can be moved and they define the range used to calculate the phasors and the selected emission value used to represent the excitation spectra in the graph to the left. If 1-dimensional excitation spectra are loaded, this graph does nothing. The selected spectra are highlighted by thicker curves.
5. Phasor plot for the selected assays represented with the full scale (left) or at a zoomed region (right). The zooming region can be defined by drawing a rectangle in the full-scale phasor plot. The phasors for the selected spectra are highlighted by a filled symbol.
6. Load raw data or spectrasor-defined data format. When loading raw data, the user is required to specify the spectra x-axis units (see *Loader dialog* section).
7. Save/export the data, see the *Saving options* section for more information.
8. Remove the selected spectra from the list.
9. Remove all the spectra for the selected assay, therefore removing the assay from the list.
10. Set the spectra x-axis range by specifying the min and max values.
11. Set the phasor plot zoom by specifying the min and max values. If the plot is already zoomed, this button resets to the full scale.
12. Select the x-axis units to display the spectra.
13. Select the 2-dimensional harmonic value to use for phasor calculation. The harmonic is represented as a pair (n, m) where n refers to the excitation part and m to the emission part. If 1-dimensional spectra are used, only the harmonics with a 0 in the missing dimension are valid.
14. Select the color representation for the spectra (and phasor) data in the selected assay. The rainbow options are defined based on the order of the spectrum in the spectra list.
15. Select the shape used to represent the phasors in the phasor plot.
16. Show the phasor coordinate values for the selected spectrum in both cartesian (G, S) and polar (ρ, θ) representations (θ is in radians).

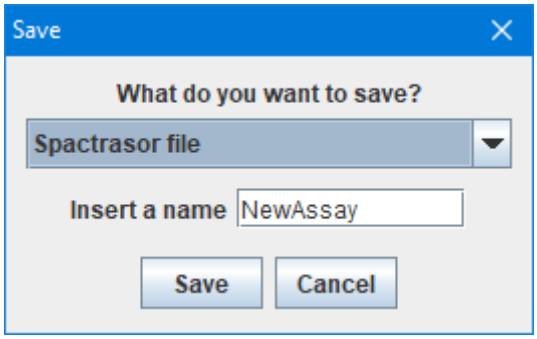
Loader dialog

When loading raw data to create a new spectrasor assay, it is possible to open multiple files at once and a dialog box will prompt asking the name for the assay, which spectra are included in the assay, and the units for the x-axis, as illustrated below.



Saving options

The multispectral phasor tool has several saving options for different purposes. When the save button is pressed, a dialog box (below) will prompt asking what kind of file the user wants to save/export.

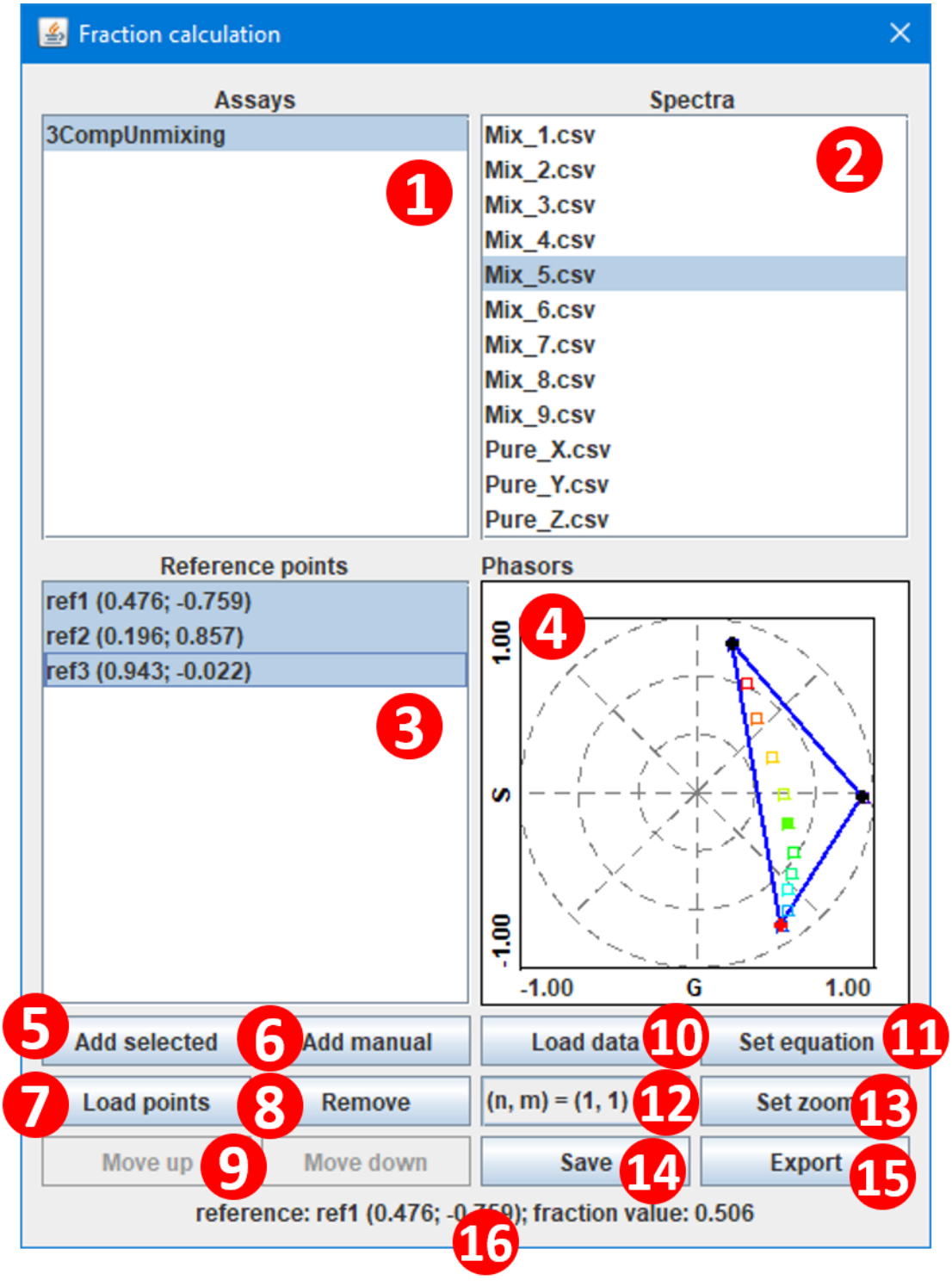


The options available to choose are:

- **Spectrasor file:** save the selected assay as a spectrasor file (.***spr***) that can be loaded in the future. This file will contain all the spectra of the assay together with other visual configurations. If more than one assay is selected, then the file will be a spectrasor list file (.***sprl***) that will contain all the assays in a single file. This file types are also necessary to use the fraction unmixing tool (see the *Unmixing tool* section).
- **Excel file:** export the phasor data to an excel file. The excel file will contain information on the excitation and emission full range as well as the excitation and emission value selected when data was saved. It will also include the phasor coordinates (both cartesian and polar) for each spectrum in the assay and at each harmonic. Note that when either n or m in the harmonic is 0, the phasor is calculated using a 1-dimensional spectrum, which corresponds to the excitation/emission spectrum at the selected emission/excitation value. For example, using the harmonic (0, 1) will gives the spectral phasor for the fluorescence emission spectrum at the selected excitation value (i.e., the middle vertical dotted line in the excitation spectrum graph). If more than one assay is selected, then the same information is exported for each assay as different sheets on the excel file.
- **Images:** save the phasor plot as an image (.***png***). If the zoom option is used, two images will be saved, the zoomed one and other containing the full scale with the zoomed region highlighted. If more than one assay is selected the images will contain all the phasors together in a single plot, each with its own user-defined visual settings. Note that the symbol for the phasors in the images saved will be filled symbol instead of open symbol.
- **All at once:** perform data save/export for the previous 3 options at once.

Unmixing tool

The fraction unmixing tool (below) allows to set and solve a system of linear equations for spectrasor assays. To do this, two steps are needed: set the points to use as reference (corresponds to the phasors of the components whose fractional contribution will be calculated in the unmixing procedure); and set the system of equations to use for the unmixing procedure (see the next section for more information).



1. A list of the loaded spectrasor assays, which are just a collection of spectra data together with their visual configuration.
2. A list of the spectra included in the selected spectrasor assay.
3. A list of the reference points included. These points are referred to the specific harmonic selected.
4. Phasor plot for the selected assays. A zoomed scale can be defined by drawing a rectangle on the image. Selected reference points are represented by a black or red cross symbol. If more than one reference is selected, then the first one in the selection is drawn in red, the others in black and blue lines joining the points are included. The order in where the lines joining the points are drawn is defined by the order of the points in the list (this will be important for more than 3 selected points, where a different drawing order leads to a different outcome). The phasors for the selected spectra are highlighted by a filled symbol.
5. Add the current selected spectrum as a reference point. A dialog will prompt asking the user to give a name to the point. Using this option will, as well, add the selected spectrum as a reference point for each harmonic. These is the preferred way (but not the only) to add reference points.
6. Add a reference point by manually defining the (G, S) coordinates. A dialog will prompt asking the user to give a name to the point. Using this option will add the point only for the current selected harmonic.
7. Load previously saved reference points (see the saving option for more information).
8. Remove the selected reference points from the list.
9. Reorder the reference points by moving up or down the selected one. If more than one point is selected this option is unavailable.
10. Load the spectrasor assay data to use. This includes spectrasor files (.***spr***) or spectrasor list files (.***sprl***) previously generated using the multispectral phasor tool or a spectrasor fraction analysis file (.***sprf***) previously saved by this tool.
11. Set the system of linear equations to use in the unmixing procedure (see the next section for more information). A number of components (equations) will be asked.
12. Select the harmonic to represent in the phasor plot and to add/modify its reference points list.
13. Set the phasor plot zoom by specifying the min and max values. If the plot is already zoomed, this button resets to the full scale.
14. Save the data in one of several options: save the phasor plot as an image, including the selected reference points and lines joining them; save a reference points file (.***rfp***) with all the selected reference points or with one point as an average of all the points in the list; save the whole project as a spectrasor fraction analysis file (.***sprf***) for future usage.
15. Export the results of the fraction unmixing calculation to an excel file. The excel will include the (G, S) coordinates of all the loaded spectrum, a description of the system of equations used and the fraction calculated for the selected reference points. If more than one assay is selected, then the same information is exported for each assay as different sheets on the excel file.

16. Display a summary of the fraction value obtained for the selected reference point and the selected spectrum. If more than one reference point is selected, then the first one in the selection will be shown.

Setting the system of equations

In the spectral phasors' context, achieving the unmixing of signals is reduced to a linear algebra problem, where the mixed case is broken down into a linear combination of "pure" components contribution. Understanding phasors as vectors, the problem is reduced to solve a system of linear equations satisfying:

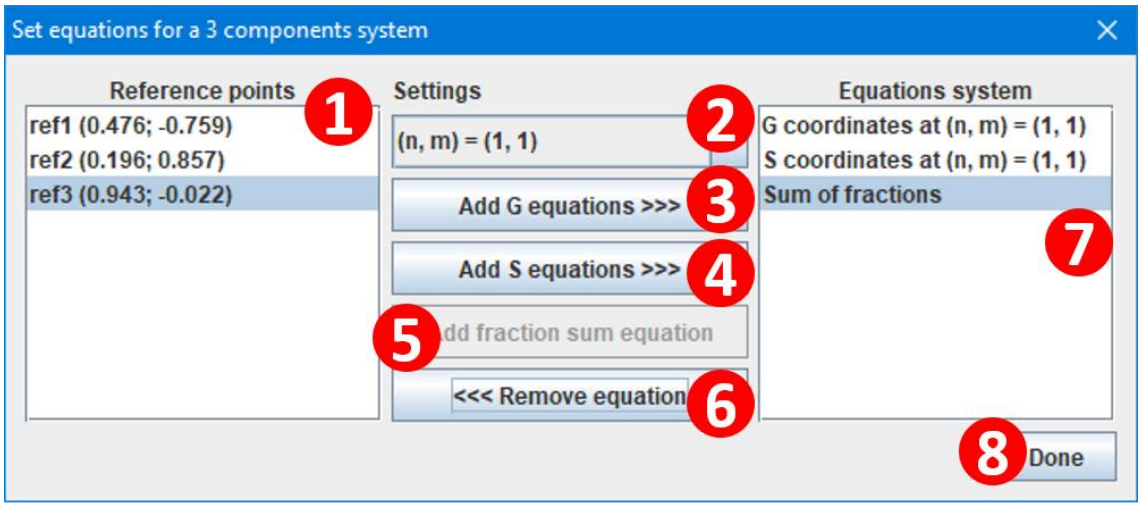
$$\sum_{i=1}^{N} f_i \boldsymbol{V}_{i:H} = \boldsymbol{V}_{m:H}$$

Where $\boldsymbol{V}_{i:H}$ is the phasor for the "pure" component i at the harmonic H, f_i is the fractional contribution of each $\boldsymbol{V}_{i:H}$, $\boldsymbol{V}_{m:H}$ is the mixed phasor at the harmonic H and N is the number of "pure" components.

Due to the fact than the phasor has two coordinates, the vector equation above contains two linear equations for each harmonic H: the G coordinate equation and the S coordinate equation. In addition, is possible to include a constriction that all fractions must add to one:

$$\sum_{i=1}^{N} f_i = 1$$

Therefore, the problem consists on finding f_i by knowing the $\boldsymbol{V}_{i:H}$ and $\boldsymbol{V}_{m:H}$. To do that is necessary to construct a system of N equations. The *Set equations* dialog (below) allows to do this.



1. A list of the reference points included. These points are referred to the specific harmonic selected. The order of the points is very important because it defines the order of the N components, no matter the reference name. If equations

from different harmonics are added, is assumed that the reference points order is the same. Different order for different harmonics will give unwanted results.

2. Select the harmonic to show references and add equations.
3. Add the G coordinate equation to the system. This is possible if the number of reference points at the selected harmonic match the number of components of the system.
4. Add the S coordinate equation to the system. This is possible if the number of reference points at the selected harmonic match the number of components of the system.
5. Add the sum of fractions constraint as an equation to the system.
6. Remove the selected equation from the system.
7. A list of the equations added to the system.
8. Return to the unmixing tool with a system of equations defined. This will be possible if the number of equations in the system match the number of components.