Pupillometry App: User Manual

This User Manual will guide you through the process of using the pupillometry app for the analysis of your data. This app can also be used to analyze any other time-series data, as long as correct input formats are used. This user manual is based on an example dataset which is included in the app. The example dataset can be loaded by a single click in the first tab. To get acquainted with the app it is recommended to replicate the user manual step by step with the example dataset.

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Loading the data

The first step is to load your radius traces of your runs and the corresponding metadata. Example data can be loaded it by clicking on "**load example**". If you use your own data you will need the following:

- One or more .mat files with your pupil radius traces. For each For each recording a separate file is needed
- One single .csv file (can be created with excel, see Figure 1a for example) that contains all the metadata (filenames and their grouping variables, e.g. group / condition / virus etc.)

The .mat files are generated automatically if the standard MATLAB or DeepLabCut pipeline are used. If a different approach is used, the layout of the .mat file should be the following: a single MATLAB matrix with 2 columns (type double), the first indicating frame (or any other time variable) and the second indicating radius (in pixels). The matrix has to be named "R"

For the .csv file, it is crucial, that there is a header for each column and that the entries of the first row (here "filename") correspond to the file names (complete, with file format) of the .mat files uploaded. Other than this, it can have as many or few variables as needed. ***For statistics an Animal variable is required that links each run to a biological replicate.*** Each biological replicate can have one or multiple runs.

R

449x2 double

	1	2
1	4	20.9333
2	6	19.5874
3	8	19.1415
4	10	19.6016
5	12	19.3120
6	14	18.9516
7	16	19.8629
8	18	19.9752
9	20	19.4680
10	22	21.2967
11	24	20.8808
12	26	19.7238
13	28	20.4422
14	30	20.4991
15	32	20.2743
16	34	20.8660
17	36	29.1195

	A	B	C
1	filename	Animal	Virus
2	radii_t1_273.mat	273	V1
3	radii_t2_285.mat	285	V1
4	radii_t3_286.mat	286	V1
5	radii_t4_288.mat	288	V2
6	radii_t5_293.mat	293	V2
7	radii_t6_299.mat	299	V2
8			

Figure 1a: Head of a .mat file as shown in matlab (left) and metadata .csv file (right) as shown in excel.

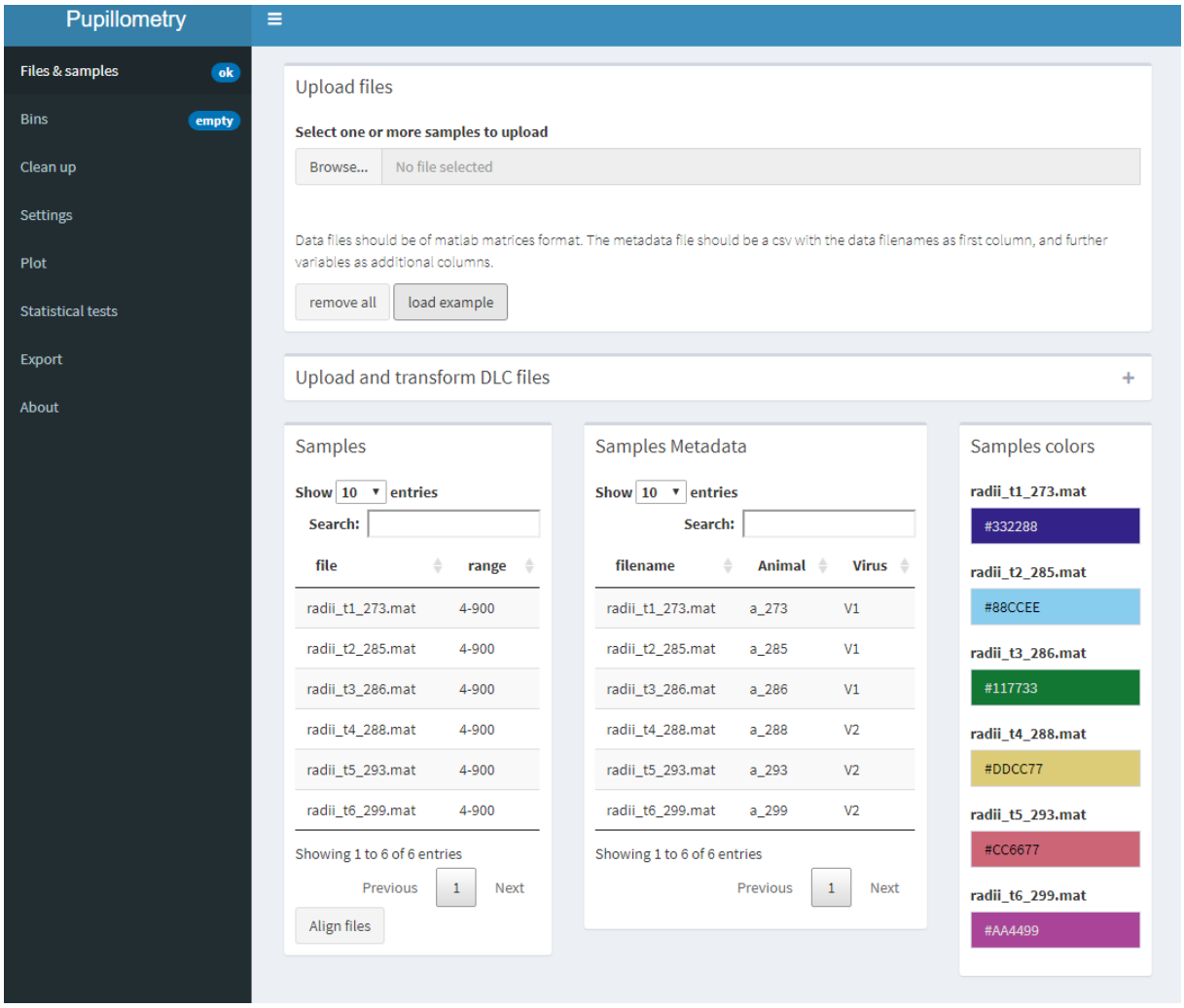

Figure 1b: Example of the Files & samples tab after the example data has been loaded.

Once data and metadata is uploaded the “Files & samples” tab will look as shown in Figure 1b. Following options can be used here:

- **Browse…:** Select files from your computer. Files can be uploaded sequentially or in bulk. To add more data it can be clicked again. The file tab will now show information about samples and metadata that have been loaded. Files can also be imported via drag-and-drop function into the browse bar.
- **(optional remove all:** Unloads all files
- **(optional) load example:** loads example files and metadata provided by us
- **(optional) Sample colors:** allows interactive choice of sample colors for each sample (click on color values to open an selection window)
- **(optional) Align files.** This option aligns the frame range across files in case uploaded files possess inconsistent amounts of frames.

Uploading and transforming DLC data

The pupillometry app includes the option to directly upload DLC .csv exports and transform the point xy-data into pupil radius traces. To access this option expand the “Upload and transform DLC files” sub-tab

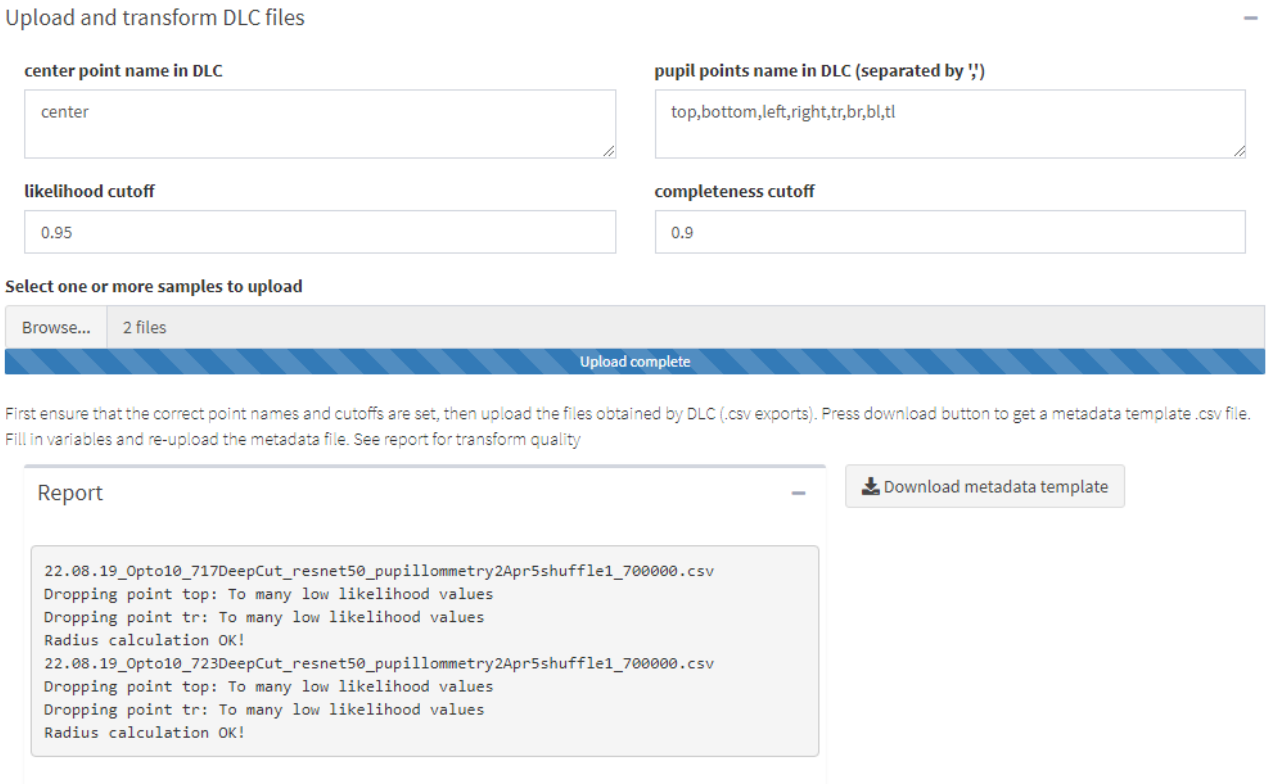

Figure 1c: The Upload and transform DLC files sub-tab after successfully reading and transforming two DLC output .csv files.

Following options are available in the Upload and transform DLC files sub-tab:

- **Center point name in DLC:** The name used for the pupil center point in DLC. If other name than “center” is used this has to be adjusted
- **Pupil points name in DLC (separated by ‘,’): T**he names used for the pupil border points in DLC. If other names than “top,bottom,left,right,tr,br,bl,tl” are used this has to be adjusted. As many points as desired can be used, but a minimum of one has to be present
- **Likelihood cutoff:** Numeric value between 0 and 1 that described the likelihood cutoff used to decide if a points value at a frame is used (if its likelihood is above the value) or if its value is interpolated (if its likelihood is below the value)
- **Likelihood cutoff:** Numeric value between 0 and 1 that describes the completeness required for a point to be included in the radius calculation. If the likelihood value of a point does not pass the likelihood cutoff in more than 100*completeness cutoff % of the frames it will be excluded.
- **Report:** Can be extended to assess import and transformation quality of DLC .csv files. Reports excluded points and files.
- **Download metadata template:** Downloads a .csv template for the metadata. Contains correct entries for filename. Further variables can be added before re-uploading the file.

Selecting bins for statistical analysis

Once all files are loaded, progress to the “Bins” tab. Here you can (optionally) set bins within which you want to run statistical tests. This app is specifically tailored towards comparing baseline vs. response bins and statistical analyses will not be provided if no bins are selected.

There are two options: either one single baseline bin and multiple response bins are entered, or each response bin has its own baseline. In the first case, there will be a comparison of each response to the first baseline, in the second case each response will be compared to its own baseline. We recommend the second option if baseline shifts are present since it allows correcting for them and increases statistical power.

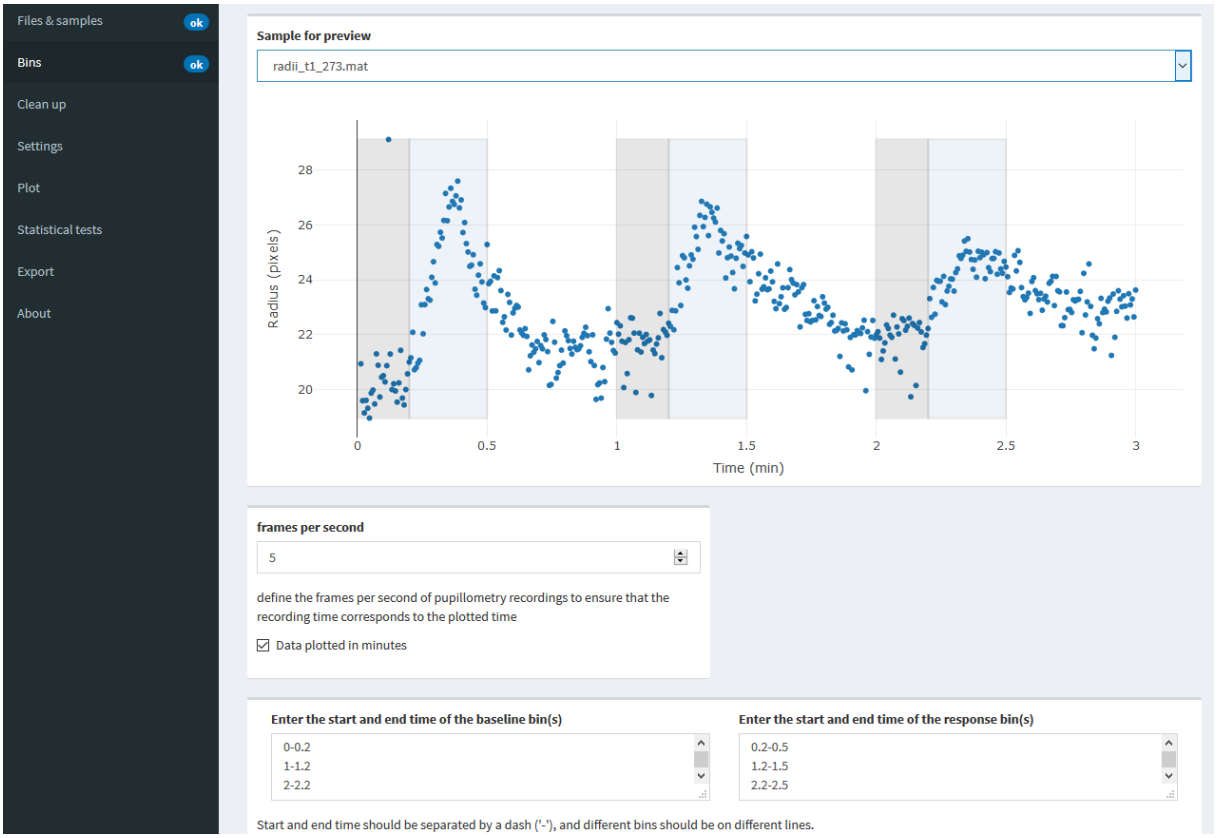

Figure 2: The Bins tab for the example files using 3 baseline bins and 3 response bins. In this example, we used three stimulations, starting at minute 0.2, 1.2 and 2.2. We set the corresponding baseline bins in the 0.2 minutes prior to stimulation and the response bins for a stimulation interval of 0.3 minutes.

Following options are available in the Bins tab:

- **Sample for preview:** Selects the run for which it will interactively plot your selected bins
- **Frames per second:** Adjust the fps to what was used in your measurement. Required for proper time calculation.
- **Data plotted in minutes:** Changes between minute and second representation in all plots

Cleaning up the data (optional)

In case the data is noisy and contains outliers/tracking artefacts, the clean-up tab can help to remove or reduce this problem. If enabled, the window size and outlier-detection limit have to be fine tuned for the data. The same settings are applied to all runs so it is advisable to check all runs for artefacts after outlier removal.

In Figure 3 we demonstrate the outlier removal in our example files. A single outlier value is removed in the radii_t1_273.mat and multiple outliers that are removed in the radii_t2_285.mat when using a window size of 0.3 minutes and a limit of 130% without inducing artefacts in any runs.

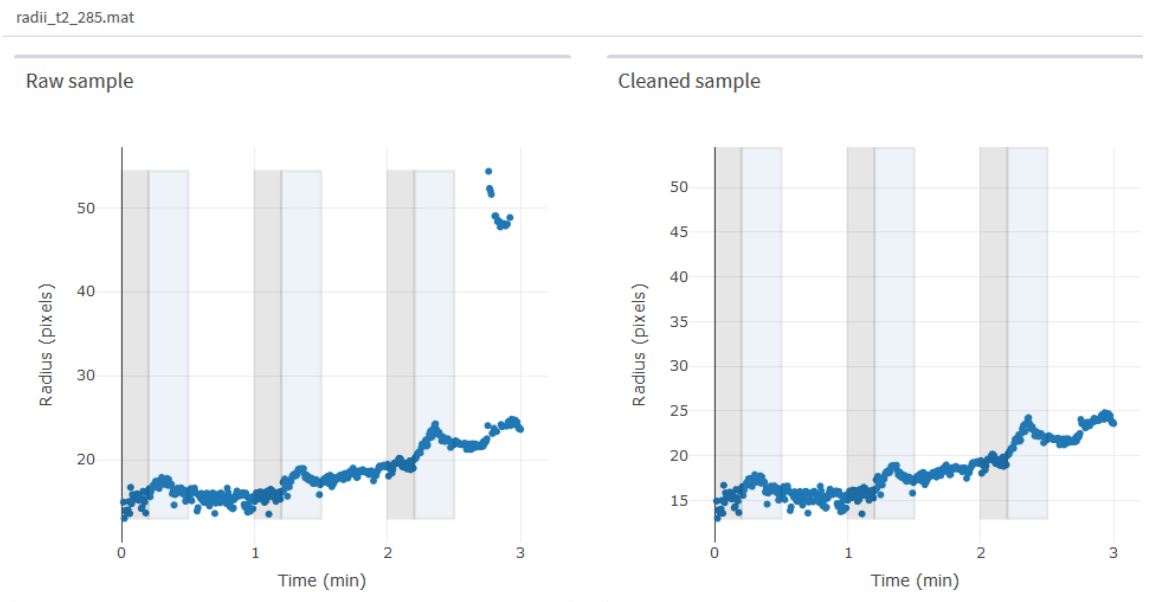

Figure 3: Outlier removal using the data clean up tab. Here we demonstrate outlier removal in two files without introducing any artefacts.

Following options can be found on the sample clean-up tab:

- **Sample for preview:** Plots the selected input run (left) and the cleaned up version (right). It is advised to quickly check all your runs and ensure integrity after clean-up.
- **Clean up data:** Activates the run clean-up with the preset settings. Disabled by default.
- **Outlier-detection window size:** Selects the width of the window within which outliers are detected. Unit will be in minutes or seconds, depending on the selections in the bins tab.
- **Outlier-detection limit:** Sets the limit above or below where a point is considered to be an outlier. The algorithm in the background uses a sliding window and tests for each point if it is below or above a certain percentage of the median value within this window. If an outlier is detected the value will be changed to the average of the previous and next non-outlier data points.

Settings

The “Settings” tab allows customization of how the data is grouped and plotted. This encompasses activation and selection of different normalization methods and personalization of plot properties such as colors, error type etc.

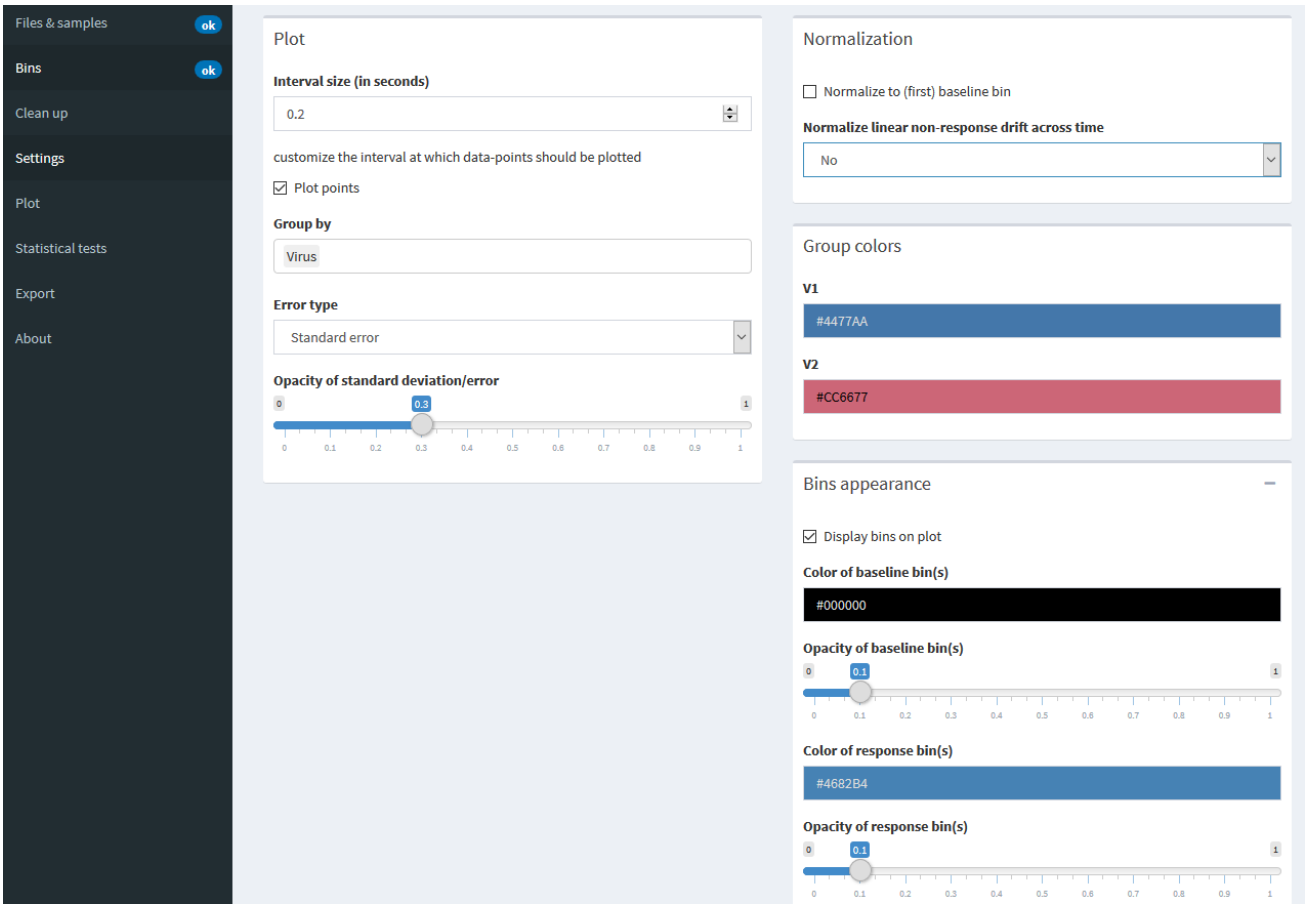

Figure 4: The settings tab with our example files

Following options are available:

- **Interval size:** Changes the interval in which the data is aggregated and plotted (e.g. 1 second, it would average the data in 1 second bins and plot this value)
- **Plot points:** Enables or disables points in your graphs. If disabled only lines will be plotted
- **Group by:** Selects variables by which files will be grouped for plotting. Multiple variables can be selected and a combined variable for these will be generated automatically.
- **Error type:** Selects the error type for which error intervals of group will be plotted. Options are standard deviation, standard error and 95% confidence interval
- **Normalize to first baseline bin:** If selected data will be normalized to % compared to first baseline (average). This can help reduce variability if large baseline differences exist between animals.
- **Normalize linear non-response drift across time:** This option can be enabled if there is a linear drift within the runs where the pupil radius increases or decreases due to any dependent effects. Not recommended unless strong effects are present. Select global drift to

adjust for average global drift (across all runs) or run-specific drift to remove drifts within each run.

- **Group colors:** Displays the current groups that will be plotted, and enables interactive color selection for each (click on color).
- **Bins appearance:** Contains multiple settings that allow customization of bins.

Plotting the data

The data will be plotted in the “Plot” tab. It contains an interactive plot and an exportable plot. The interactive plot is a convenient and fast way to visualize your data, whereas the exportable plot (collapsed by default) will offer you customization and export of a high quality pdf file.

Interactive plot:

If groups are enabled the interactive plot will produce a ribbon plot for grouped values, otherwise a line-plot for individual runs. The colors will correspond to the selected colors in the “Settings” (grouped plot) or Files & samples (individual line plots) tab. Hovering over the plot will indicate runs/groups and x-/ y-axes.

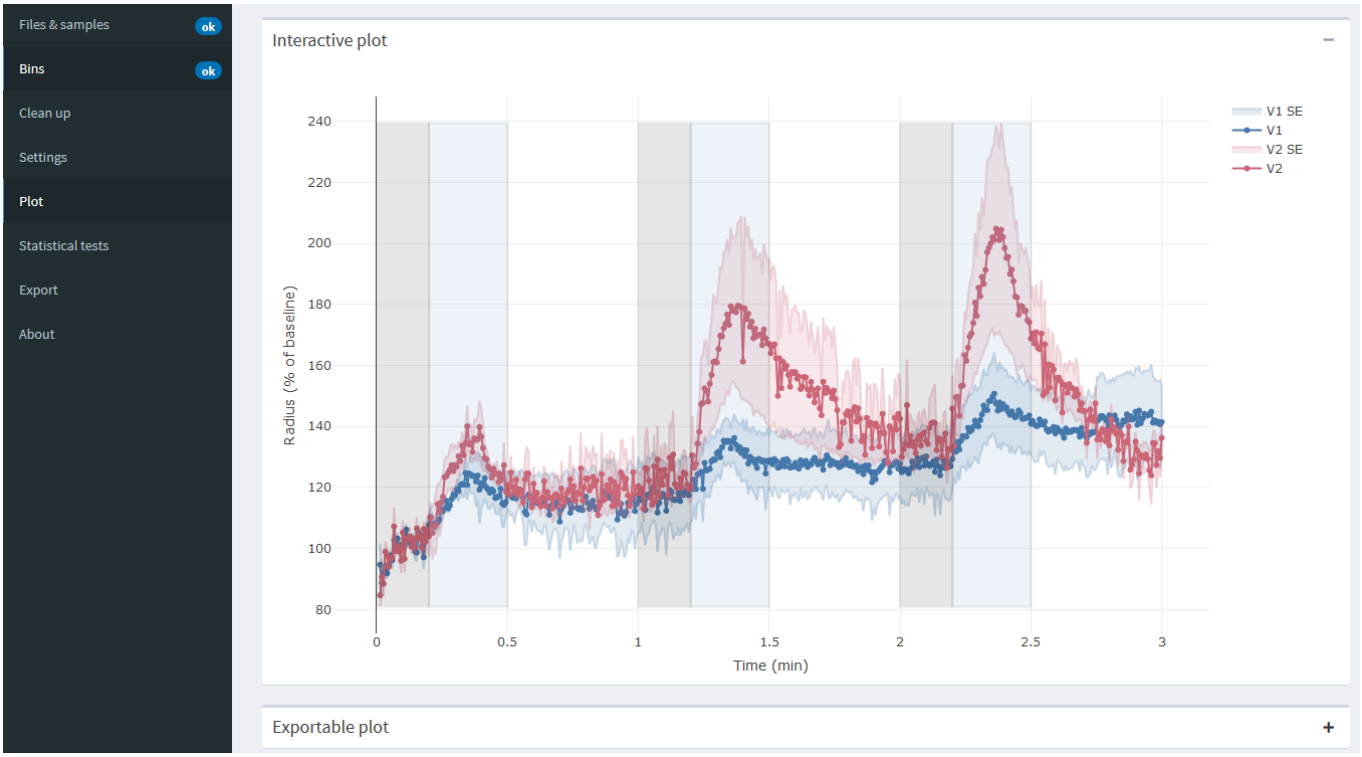

Figure 5a: Interactive plot.

Exportable plot:

The exportable plot can be enabled by pressing the “+” on the right side. By default, it will have predetermined plot title, legend and axis labels with the ability to customize any of them. It will also enable customization of x-axis and y-axis dimensions. Click the “Download” button to export the final plot as pdf (Figure 5b).

Figure 5b: Exportable plot with fully customized title, legend and axes.

Statistical tests

This tab will perform statistical tests on the data. It uses linear mixed effect models (R package "lme4" using the lmer() function) to test for response effects and effects of other variables. It is possible to perform relatively simple analyses with little user input, but also allows testing more complicated models for users proficient with linear mixed effect models.

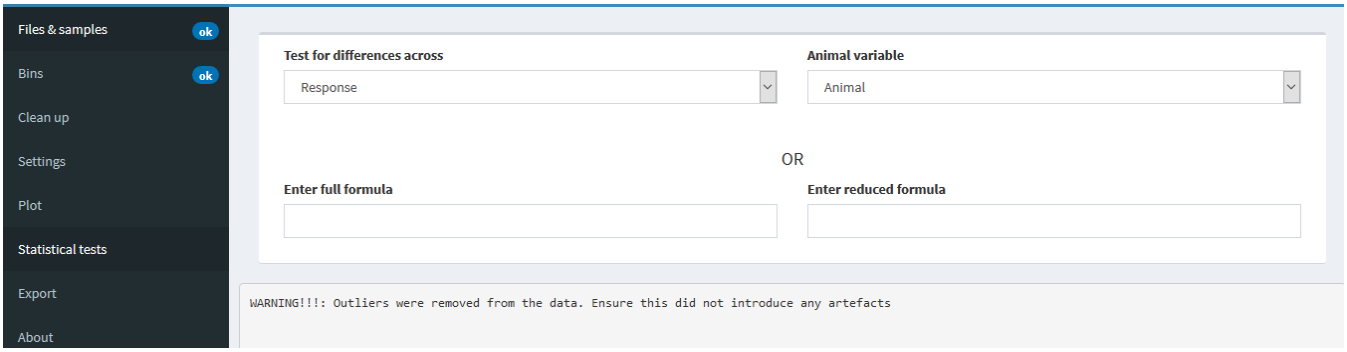

Figure 6a: Default setup of the statistical test tab with the example data. Here the app will test for a response vs baseline effects.

Basic analysis:

By default, the basic analysis will be performed without any user input (Figure 6a). ***Ensure that the correct animal variable is selected***; otherwise, the test might do something unintended. With the default settings, this will tests if pupil radius reacts to the stimulation by comparing the average pupil radius of baseline bins to the average pupil radius of response bins.

More specifically, it tests for a response effect in your data by adjusting for animal specific differences (e.g. adjust for animal specific differences in pupil radius). If multiple baseline – response bin pairs are tested it will further adjust for time specific changes (e.g. global shift in baseline, for example due to anesthesia effects).

Following metrics are of interest for the basic analysis report (here with the example data)

```
Fixed effects:
            Estimate Std. Error      df t value Pr(>|t|)
(Intercept)  17.3537     2.3299  5.4561   7.448 0.000467 ***
Response1     3.9667     0.5571 27.0000   7.120 1.18e-07 ***
Time2         3.4439     0.6824 27.0000   5.047 2.68e-05 ***
Time3         5.6418     0.6824 27.0000   8.268 7.09e-09 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

The following interpretations can be drawn:

- the response effect (baseline vs response) is significant with an average increase of 3.96 ±0.55 pixels and a with a p-value of $1.18 * 10^{-7}$
- There is a time dependent shift in baseline pupil radius where both the 2nd and 3rd bin-pairs have different pupil radius from the first.

Extended analysis:

The app contains extended analyses that can be performed with minimal user input. By selecting a different variable from the "Test for differences across" a comparison similar to a 2-way ANOVA for Variable * Response can be performed. ***Ensure that the correct animal variable is selected***; otherwise, the test might do something unintended.

In the following example we test whether pupil radius reacts to the stimulation in our example dataset, pupil radius depends on the virus injected and if our pupil response to stimulation depends on the injected virus. This type of analysis is fully supported by the app. Simply select the corresponding variable in the "Test for differences across" selection (Figure 6b).

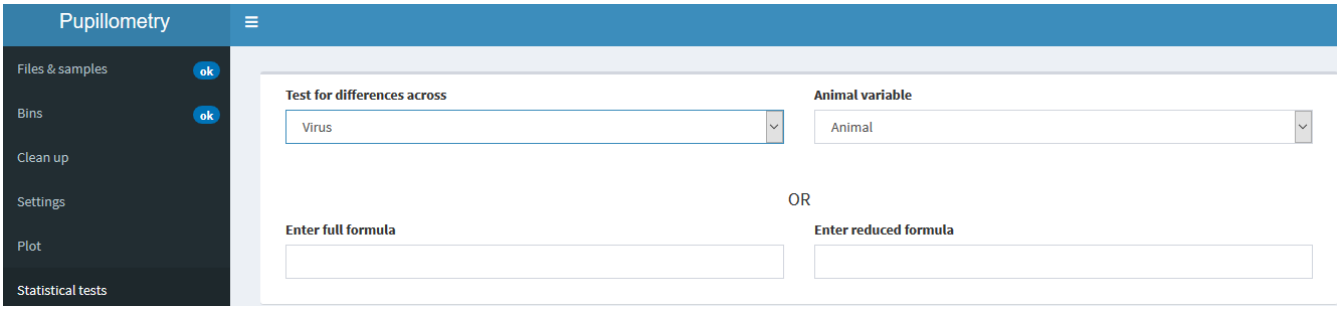

Figure 6b: Extended analysis of the example data. Here we will test a Virus * Response type of interaction.

This tests for a Response * Virus effect in the present data by blocking for animal specific differences (e.g. adjust for animal specific differences in pupil radius). If multiple baseline – response bin pairs are tested it will further adjust for time specific changes (e.g. global shift in baseline).

Following metrics are of interest for the extended analysis:

```
Fixed effects:
                 Estimate Std. Error      df t value Pr(>|t|)
(Intercept)       18.5352     3.6179  4.1673   5.123 0.006149 **
VirusV2           -2.3631     5.0904  4.0833  -0.464 0.666159
Response1          2.7552     0.7292 26.0000   3.778 0.000832 ***
Time2              3.4439     0.6315 26.0000   5.453 1.02e-05 ***
Time3              5.6418     0.6315 26.0000   8.933 2.10e-09 ***
VirusV2:Response1  2.4231     1.0313 26.0000   2.350 0.026669 *
```

The following interpretations can be drawn:

- There is no difference between Virus V1 and Virus V2 (p = 0.66)
- the response effect (baseline vs response) is significant with an average increase of 2.75 ±0.72 pixels and a with a p-value of 0.0008
- There is a time dependent shift in baseline pupil radius where both the 2nd and 3rd bin-pairs have different pupil radii from the first.

There is an interaction between Virus and Stimulation. The stimulation of Virus V2 animals leads to an on average 2.42 ± 1.03 pixel larger response than the stimulation of Virus V1 animals. This effect is significant with a p-value of 0.026

Customizable analysis:

To test more complex analyses, the app allows entering full and reduced models for any conceivable model using any number of variables. We recommend this option for users accustomed with linear mixed effect models and more specifically the lmer() function of the lme4 package for R. The formula syntax for lmer applies.

In our example data, we use a model to correct for time effects within individual animals (not global shift, but the shift within single animals). The reason for such an analysis might be that individual animals react differently to anesthesia effects, so the time dependent increase in the pupil radius is animal specific. If we want to model this using lmer our random term would change from (1|Animal) to (1|Animal/Time) (Figure 6c). Therefore, we nest the baseline-response pairs within individual Animals and determine a different Intercept for all of them.

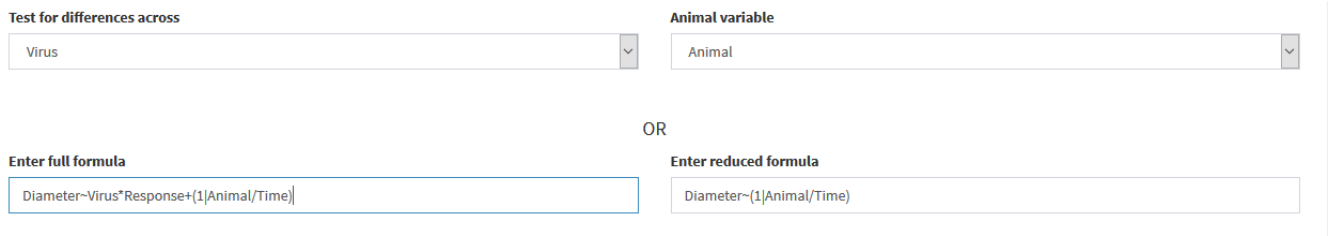

Figure 6c: Customized analysis of the example data. Here we will test a Virus * Response type of interaction by adjusting for time depended effects nested within animals.

Following metrics are of interest for the report of the extended analysis:

```
Fixed effects:
                 Estimate Std. Error      df t value Pr(>|t|)
(Intercept)       21.5946     3.5927  4.0238   6.011 0.003785 **
VirusV2           -2.3939     5.0808  4.0238  -0.471 0.661933
Response1          2.7244     0.3908 16.0000   6.971 3.15e-06 ***
VirusV2:Response1  2.4264     0.5527 16.0000   4.390 0.000457 ***
---
```

The following interpretations can be drawn:

- There is no difference between Virus V1 and Virus V2 (p = 0.66)
- The response effect (baseline vs response) is significant with an average increase of 2.72 ±0.39 pixels and a with a p-value of $3.15*10^{-6}$
- There is a time dependent shift in baseline pupil radius where both the 2nd and 3rd bin-pairs have different pupil radii from the first.
- There is an interaction between Virus and Stimulation. The stimulation of Virus V2 animals leads to an on average 2.42 ± 0.55 pixel larger response than the stimulation of Virus V1 animals. This effect is significant with a p-value of 0.000457

When comparing the results to the ones obtained by the extended analysis, we increase the power of our analysis since nesting baseline-response pairs within animals decreases the overall variability of our measurements.

Exporting the data

The final tab allows viewing and export the data used by the app. Through the "**Choose a Dataset**" the data to view and/or export can be selected. The "**Download**" button will export a .csv file of the selected dataset. Following datasets are available:

- **Raw data:** The raw data that was loaded by the pupillometry app before any normalizations.
- **Normalized data:** The data after normalization that was used for plotting / statistics.
- **Bin Results:** The final values that were tested with the linear model.
- **Test Results:** exportable version of the statistical results