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Frapbot: An Open-Source Application for FRAP Data

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Abstract

We introduce Frapbot, a free-of-charge open source software web application written in R, which provides manual and automated analyses of fluorescence recovery after photobleaching (FRAP) datasets. For automated operation, starting from data tables containing columns of time-dependent intensity values for various regions of interests within the images, a pattern recognition algorithm recognizes the relevant columns and identifies the presence or absence of prebleach values and the time point of photobleaching. Raw data, residuals, normalization, and boxplots indicating the distribution of half times of recovery ($t_{1/2}$) of all uploaded files are visualized instantly in a batch-wise manner using a variety of user-definable fitting options. The fitted results are provided as .zip file, which contains .csv formatted output tables. Alternatively, the user can manually control any of the options described earlier. © 2017 International Society for Advancement of Cytometry

Key terms

fluorescence recovery after photobleaching; Frapbot; web-application; pattern recognition; R language

Molecular live cell imaging techniques that exploit the properties of fluorescent molecules in combination with modern microscope technology are increasingly used to visualize, track, and quantify molecular interactions and mobility in a spatio-temporal manner (1–3). Most fluorescent molecules are prone to photobleaching. The latter results in a time-dependent fluorescence decrease, which among others, depends on the intensity of the excitation light. In the fluorescence recovery after photobleaching (FRAP) technique, this characteristic is exploited to obtain information about the ensemble mobility of fluorescent molecules (4). During FRAP, first fluorescent molecules are irreversibly bleached using a laser pulse of short-duration and high-intensity in a defined region of interest (ROI). Next, the recovery of the fluorescence within the ROI, caused by movement of unbleached fluorescent molecules into the ROI, is monitored using time-lapse microscopy and low intensity laser light. The speed and extent of fluorescence recovery, expressed by the half time of recovery ($t_{1/2}$) and mobile fraction (Mf), can be used as a measure of molecule mobility. When certain experimental conditions are met, the apparent diffusion constant (D_{app}) can be extracted from the FRAP recordings (5,6). In addition, information on the number and relative amount of molecular species with different mobilities and the type of diffusion such as for example anomalous diffusion can be obtained (7,8). To decide which diffusion model describes best a set of FRAP data, repeated curve fitting and data analysis are required. Analysis of a large FRAP data set is therefore time-consuming, and interpretation of data is often challenging. Currently, FRAP data processing and analysis are often performed using commercial software packages. These programs are not generally available to all researchers and restricted to certain operating systems. Moreover, they often provide only a limited set of the functionalities, which are required for correcting, scaling, normalization, and fitting of FRAP data. To address these issues, we here present a platform-independent freeware (Frapbot) that allows automated on- and offline analyses of FRAP datasets. As input, data tables containing columns of intensity-time traces

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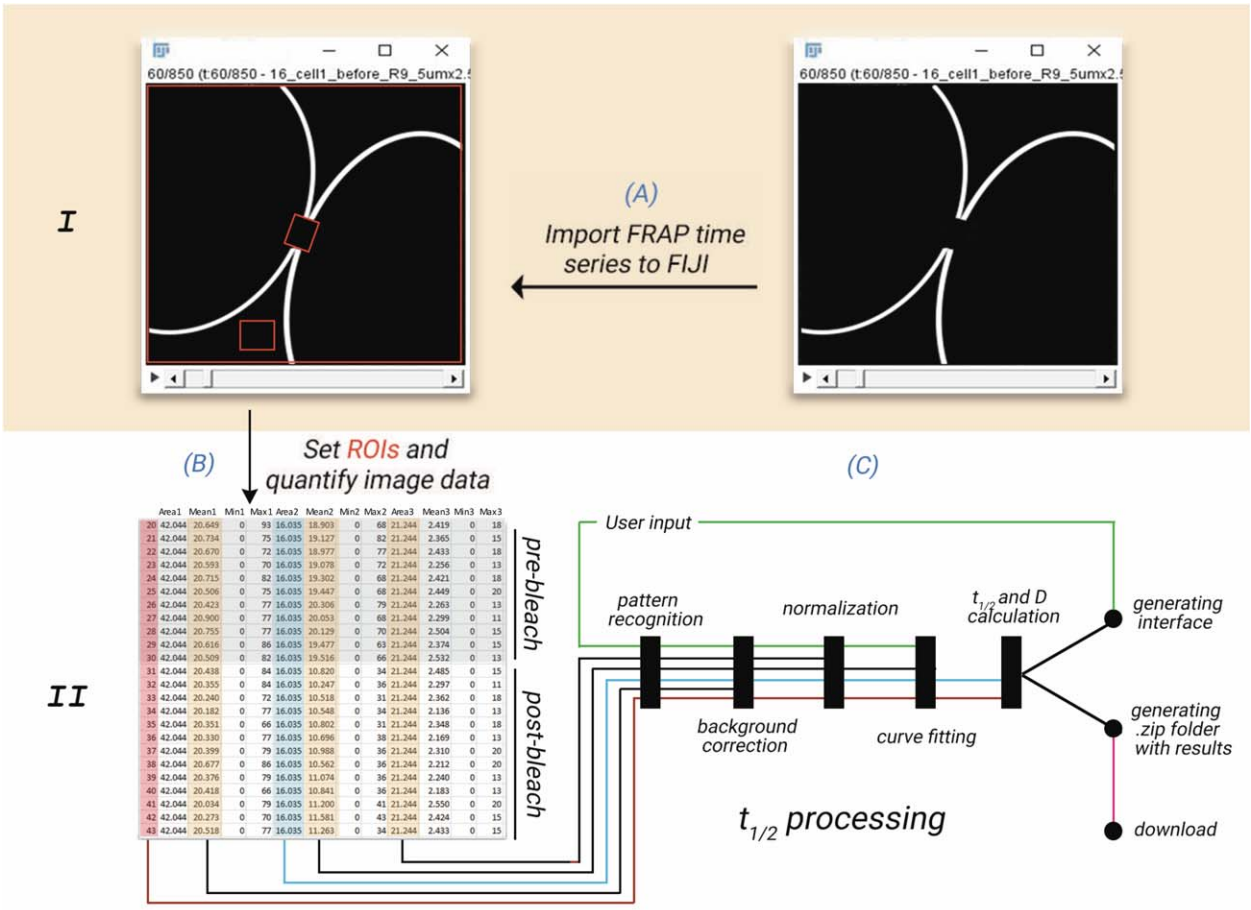

Figure 1. Workflow for Frapbot FRAP analysis. The FRAP raw image series are exported to an image processing program such as Fiji. In Fiji, the regions of interest (ROI) are set (**A**) and their gray values quantified and exported into a table (**B**). Frapbot employs a pattern recognition algorithm over the data table which identifies the columns containing data from the bleach, control and background ROI. If available, the algorithm identifies the bleach time point and adjusts the fitting accordingly. Frapbot then performs normalization and curve fitting to calculate the recovery half time ($t_{1/2}$) (**C**). The calculated values are available via Frapbot's web interface and the collected results are available as download file. The roman numerals refer to pre-Frapbot steps (I) and Frapbot steps (II). [Colour figure can be viewed at wileyonlinelibrary.com]

for various image ROIs, such as bleached region, unbleached reference, and background, are provided. Using a pattern recognition algorithm, Frapbot automatically recognizes the data columns linked to these various ROIs and performs background correction and normalization of the FRAP data. This unsupervised strategy allows an instant overview over the collected data, endorsing a conclusion over the FRAP data quality free from observer bias. Alternatively, Frapbot can be used for supervised processing and analysis of FRAP data by the manual control of individual steps in the algorithm. Results are provided as downloadable files. Taken together, Frapbot provides an integrated online software platform for quantitative FRAP analysis.

Program Overview

Frapbot was developed to analyze time-intensity traces extracted from time-resolved image series. For the extraction of such intensity traces in the form of data tables from microscopy data, Frapbot relies either on FIJI (9,10) or similar solutions. The general workflow is depicted in Figure 1 (for a detailed description and additional information see Supporting Information).

To maximize user accessibility, Frapbot provides a Graphical User Interface (GUI) through which the user can perform all procedures from the data input to manipulation, processing, and finally output (11). Each step can be executed online via the provided Frapbot browser application, compatible with all available Internet browsers or offline by downloading the source code package and performing the analysis in any R programming environment (12,13).

Frapbot's GUI comes along with the following two core sections: the file import and data manipulation section (Fig. 2, left box) and the data output section (Fig. 2, right box).

Core Section I

(1) In core section I of the GUI (Fig. 2, left box), users can select one or multiple FRAP traces by clicking the "Choose

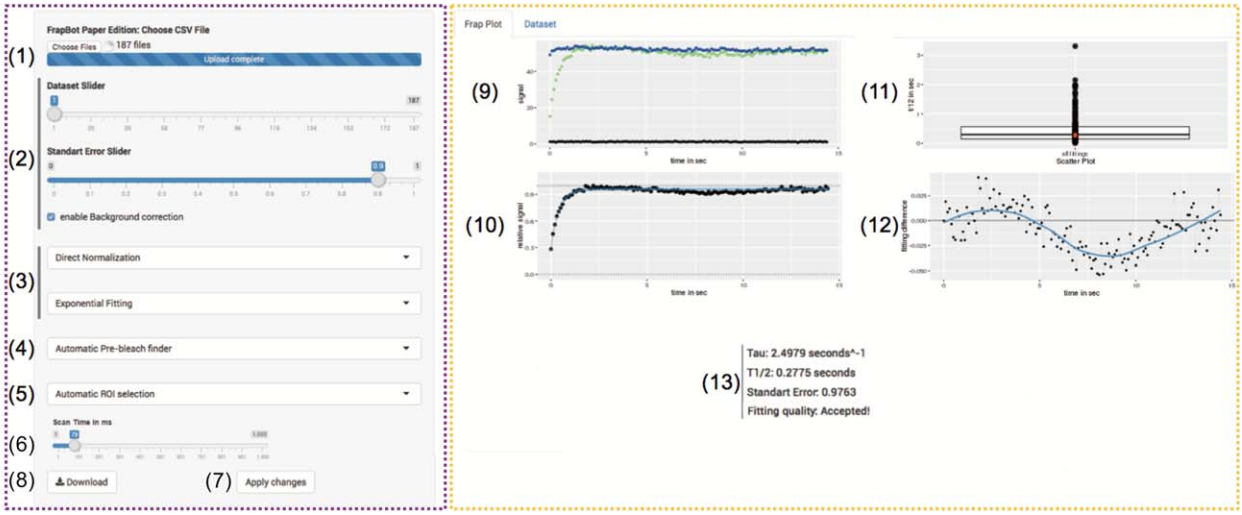

Figure 2. The Frapbot Graphical User Interface with its two core sections, the file import and data manipulation (left box) and the data output section for one selected data-trace (right box). For detailed information see main text and supplemental information. [Colour figure can be viewed at wileyonlinelibrary.com]

files" upload button. In the current version, Frapbot works with .csv and .txt files (14,15). For FRAP analysis, input data tables containing at least time-resolved intensity values from the bleached field (Bleach ROI) and the total fluorescence field (Total ROI) are required next to a certain size (in pixels) of the bleach area. Optionally, the data table can contain an entry for background correction (see point 2) (Background ROI). Alternatively, the user can perform background correction in advance. The input data can be prepared from microscopy image files using an image processing software such as FIJI (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA).

(2) Imported trace sets are fitted in real-time and can be examined separately using the dataset slider. The standard error slider provides a quality filter based on the standard error calculated with residuals of the fitting curve. This feature provides Frapbot with an exclusion algorithm for recovery traces that can be described only poorly by the chosen model. A given standard error can also be used in batch operation to automatically exclude traces from further analysis. If a column with time-dependent background data is provided (see above) Frapbot also provides the option for a vector-based background correction, which means that for each time point, the respective background value is subtracted, in case it was not yet subtracted during the FRAP measurements in the microscope software.

(3) Drop down fields allow data manipulation using the direct or averaged correction. To provide bleach correction, the direct normalization corrects the intensity of each time point based on the Total ROI vector values, while the averaged normalization function reduces the individual outliers by smoothing the Total ROI spline. The exponential and double exponential fitting functions account for one or two diffusing components for each trace. Furthermore, users can perform the curve fitting by using the "custom formula" option, which provides the possibility to enter a user-specified model such as for example anomalous diffusion. All fitting operations are performed with the Levenberg–Marquardt algorithm as stated in Ref. 16.

(4) Frapbot comes along with an implemented automatic prebleach finder, which detects whether the trace contains prebleach values or not. The algorithm is adjusted for each case (prebleach present/not present) to provide the correct model for data analysis. For example, if no prebleach values exist, a model will be employed that does not contain a term for the mobile fraction.

(5) On automatic mode, the automatic ROI recognition algorithm selects the columns containing background, bleach, and total intensity vector out of the provided data set. Alternatively, users can apply a manual selection of columns at any time.

(6) Finally, the user can adjust the scan time value (time between images) to account for the frame acquisition frequency. The change of the scan time value leads to a recalculation of all uploaded traces.

(7) Manual data manipulation will be processed after confirming with the provided "Apply changes" button.

(8) Results can be downloaded as .zip file for postprocessing.

Core Section II

At the data output section (Fig. 2, right box), users can follow the effect of data manipulation in real-time or after manual confirmation.

(9) The raw data plot presents data sets of the Bleach ROI (green), Total ROI (blue), and the Background ROI (black) as fluorescence over time. The color code also allows for an assessment by the user that the automated assignment of the traces by the pattern recognition algorithm was performed correctly.

(10) The recovery curve of datasets (black line), normalized to the total fluorescence (dashed line), and the corresponding fitting curve (blue line) are represented as relative fluorescent signal over time for the respective trace.
(11) A boxplot indicates the half-time ($t_{1/2}$) distribution of all uploaded files of an imported dataset. A red-colored data point highlights the $t_{1/2}$ for the displayed plotted trace.
(12) The residuals provide information about the systematic errors of the fitting model.
(13) The summary field includes the recovery time constant (τ), the half time of recovery ($t_{1/2}$), the standard error, and the fitting quality. The "accepted" note appears if the standard error value is above the selected threshold value of the error slider (point 2).

The interactive Frapbot website provides an additional feature section including FRAP tutorials, a link to the published Frapbot article, FAQs, and an update log with information about the Frapbot developmental status.

Experiments

First, we benchmarked Frapbot using a previously published FRAP dataset obtained with inducible human embryonic kidney (HEK293) cells that expressed a monomeric green fluorescent protein (AcGFP1) in the mitochondrial matrix (6). In the original study, the average trace of multiple FRAP measurements ($N = 187$) was manually fitted with a single component exponential model using Origin Pro software, yielding a τ_{mono} value of 0.600 s, corresponding to a $t_{1/2}$ of 0.416 s. Individual analysis of the same FRAP traces by Frapbot (Fig. 3A) and subsequent averaging of the obtained $t_{1/2}$ values, delivered a slightly higher mean halftime ($t_{1/2}$) of 0.463 s (Fig. 3B). This difference might be explained by the larger noise of the individual traces compared to the averaged FRAP curve that influences the quality of the fitting. Frapbot analysis suggests that the $t_{1/2}$ values calculated from the original dataset were not Gaussian distributed. If this is the case, we recommend the use of the median instead of the average for the analysis of similar FRAP data, which provides more robustness against outliers. Consequently, the calculation of the median was implemented in Frapbot (Fig. 2, right box, plot 11).

Second, we applied Frapbot to datasets on mobility measurements of the Chromatin Licensing and DNA Replication Factor 1 (Ctd1), which is involved in the formation of the prereplication complex during G1 phase of a cell cycle. Ctd1 has been shown to be crucial for determining when the selection of the origin of replication (licensing process) occurs to restrict this process exclusively to the G1 phase and thereby maintain the accurate DNA replication during the subsequent S phase (17,18). Thus, a fundamental understanding of the spatiotemporal coordination of Ctd1 to ensure that the right origins are used at the correct point in time is pivotal. Rapsomaniki et al. compared the mobility of Ctd1-GFP ($N = 15$) to nuclear localized GFPnls ($N = 12$) and calculated $t_{1/2}$ values of 0.548 ± 0.06 s for Ctd1-GFP and 0.289 ± 0.5 s for GFPnls (19) that are similar to values of 0.558 ± 0.05 s for Ctd1-GFP and 0.305 ± 0.04 s for GFPnls using double exponential fitting in Frapbot (Supporting Information Fig. S1).

Conclusion

With Frapbot, we have created an open source interactive web application that facilitates state-of-the-art manual or automated analyses of FRAP data using automated pattern recognition of data traces. The reliability of the internal algorithm could be validated in a comparative analysis of published datasets (6). Although the current version of Frapbot

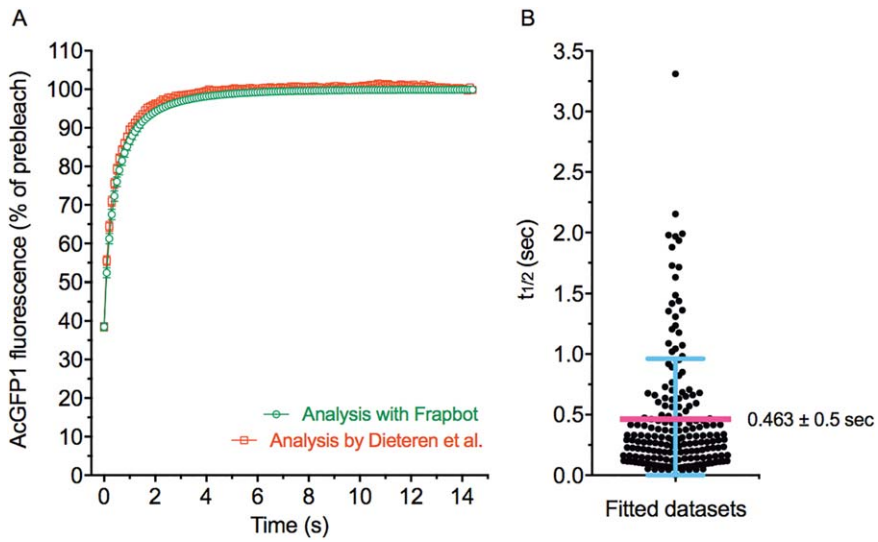

Figure 3. Fluorescence recovery curves (**A**) and $t_{1/2}$ values (**B**) including the mean of AcGFP1 performed in HEK cells. A total of 187 traces of FRAP measurements were averaged first and subsequently fitted manually with a single component exponential model (A, red curve, squares) or first individually analyzed and obtained $t_{1/2}$ values averaged by Frapbot (A, green curve, circles). The plot of $t_{1/2}$ values (B) were generated by Frapbot. The error bars represent the SEM (A) or SD (B). [Colour figure can be viewed at wileyonlinelibrary.com]

provides the most common analysis features, the development will be maintained and extended in upcoming versions. As upcoming updates, we envision a universal acceptance of all data formats, and more configuration options for diffusion modes such as anomalous diffusion. To facilitate a straightforward adaptation of Frapbot, the platform additionally provides interactive explanations and tutorials of various FRAP topics. Furthermore, a detailed manual, a description of the formulas implemented in the Frapbot algorithm, and a general comparison of Frapbot with related software solutions are summarized in the Supporting Information.

Acknowledgments

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Availability

Frapbot is an MIT licensed open source software and works as a browser application and can be accessed independently of any operating system via http://www.frapbot.com/. Source files, Supporting Information, and example data sets can be downloaded from the Frapbot page. The source code is also provided as download for offline analysis and modification.

Conflict of Interest

None declared.

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Supplemental Information

Table of contents

I: Frapbot Start Guide

Summary

Frapbot is an open-source solution for analysis of FRAP data, conceptualized for rapid automated on- and offline read-out and analysis of FRAP traces using state-of-the-art mathematical models for fitting, scaling and normalization. The analysis includes the identification of the specific data columns of each part/region of interest (ROI) of the FRAP trace (by analyzing data values, not column names), the presence or absence of pre-bleach values and the exact bleach time point using pattern recognition algorithms. This automation allows an instant overview over the collected data, endorsing a time-saving first conclusion over the quality of FRAP traces. In addition, Frapbot allows performing the data processing manually. Results are provided as downloadable files.

In this section the procedure on how to evaluate FRAP data with Frapbot is described. This user guide also explains how to generate the required input files for Frapbot by using the open source FIJI software (ImageJ, U. S. National Institutes of Health, Bethesda, USA).

Preparation of data files (.csv or .txt) from microscopy image files using FIJI

(1) Export microscopy image files in a Fiji compatible format (e.g. LSM (Zeiss confocal microscopes) or LIF (Leica confocal microscopes).

(2) In Fiji, open the saved image files and select the complete FRAP image time series (Pre-bleach, bleach and post-bleach sequence), or just the post-bleach sequence if the Pre-bleach quantification is executed separately. The three components are required to calculate the mobile fraction (1).

(3) Select the ROI manager which can be found under the Fiji menue option “Analyze / Tools”.

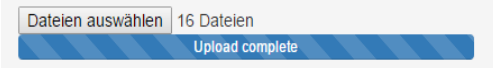
(4) Set a ROI for the total field (Total ROI), bleach region (Bleach ROI) and background region (Background ROI) using the rectangle or circle draw tool and confirm each ROI selection by pushing the button “Add” in the ROI manager menue. The Total ROI is the full image area, which by definition includes the bleach region and background region. The bleach region should be chosen to match the area bleached by the laser and the background area is defined as a not stained area outside the cell. If background is negligible or correction has been done otherwise, the background ROI can be omitted.

(5) Quantify each ROI selection via “More / Multi Measure” and set the file format to .csv or .txt. Because of its automated pattern recognition feature, Frapbot does not require an exact order of these ROIs.

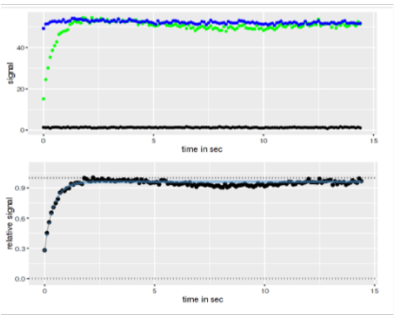
(6) Save the .csv or .txt file via “Results / Options”. The generated .csv or .txt file contains next to the intensity values (grey values) for each time-point also the size (in pixels) of each selected ROI.

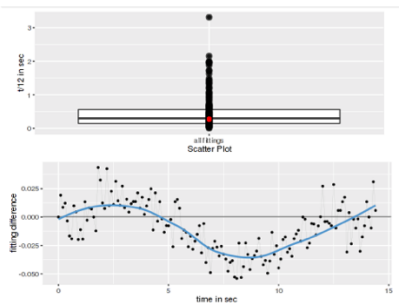
Data analysis using Frapbot

(1) Go to www.frapbot.com and upload a single or multiple .csv or .txt files via the “choose files” button.

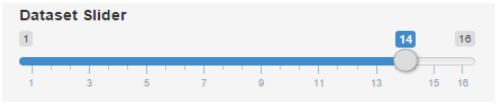


(2) The generated results are immediately shown in the data output section based on the default parameters determined by the automation algorithm. The raw data sets are presented as plots of the Bleach ROI (green), Total ROI (blue) and the Background ROI (black) fluorescence signal over time. Furthermore, a recovery curve of a selected trace (black line), normalized to the median pre-bleach fluorescence (dashed line) and the corresponding fitting curve (blue line) are presented as relative fluorescence over time. These plots refer to an individual FRAP trace of the selected data sets. An additional boxplot indicates the half-time ($t_{1/2}$) distribution of all uploaded traces of a dataset with the red-coloured data point representing the $t_{1/2}$ for the selected trace. The summary field below the plots includes the time constant (τ), the half time of recovery ($t_{1/2}$) and the standard error of a dataset selected with the dataset slider (III). For more information, see II. Definition section (1-6).

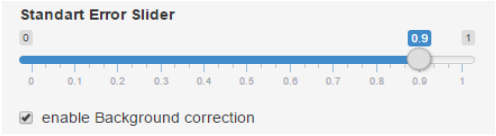




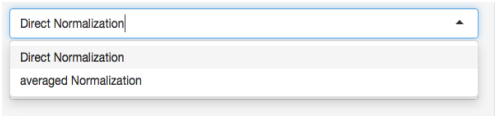
(3) Slide through each sequence with the dataset slider of the uploaded dataset and follow the adaptation of output values at the output section (II) in real-time. The dataset slider appears in the user interface as soon as more than one FRAP trace has been imported.



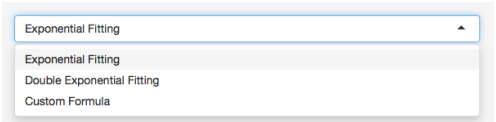
(4) Use the standard error slider to optionally activate the automated data exclusion algorithm for low quality samples based on the standard error of the non-linear fitting. It is possible to disable the background correction in case it was already subtracted during the FRAP measurements in the microscope software. For more information, see II. Definition section (1).



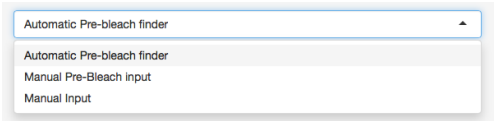
(5) The direct normalization to the total ROI is set as default. Change it to averaged normalization by clicking on the drop-down menu. For background information regarding the appropriate choice of normalization, see II: Definition section (3).



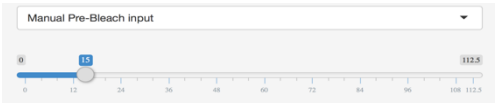
(6) Exponential fitting is set as default. Change it to double exponential fitting or provide an appropriate fitting equation as custom formula by selecting the desired function in the drop-down menu. For background information regarding the appropriate choice of fitting, see II: Definition section (4-6).



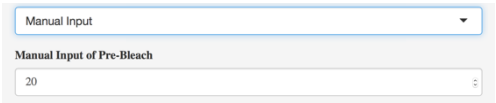
(7) Automatic Pre-Bleach Finder is set as default.



Change it to Manual Pre-Bleach Input to select the range of the pre-bleach sequence manually by clicking on the drop-down menu.

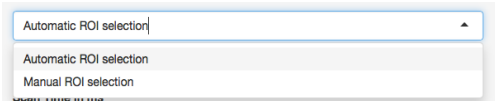


Select for the Manual Input to provide the median of the pre-bleached sequence, if it was calculated elsewhere.

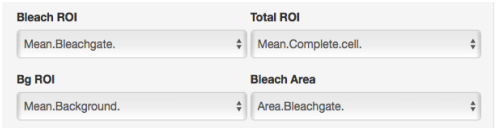


For background information regarding the appropriate choice of pre-bleach option, see II: Definition section (7).

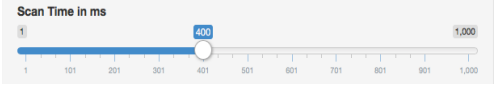
(8) The automatic region of interested (ROI) selection is set as default. Additionally, an option for a manual selection mode is provided.



For Bleach-, Total or Background ROI select for the Mean values and for the Bleach area select for the size value of the Bleach ROI. The bleach area is required for the determination of the apparent Diffusion coefficient (D).



(9) Adjust the scan interval by using the slider. The selected slider value (here 400 ms) is set to account for time between one to the next image in a given sequence.



(10) Confirm manual changes by clicking the “Apply changes” button. Plots in the Frapbot data output section will be adjusted immediately. Conclude the analysis process by pressing the download button. Generated results are provided as .csv file for further processing.

II: Definitions

The Frapbot internal algorithm is based on vector mathematics, therefore all following steps can be assumed to cover vectors of all data time points.

(1) The background signal is subtracted from both the signals of the total and the bleach area. The signals of these ROI areas are defined as Total, Bleach and Background. Thus, the variables bTotal [1] and bBleach [2] correspond to the background-subtracted ROI mean signal.

$$1)\ bTotal\ =\ Total - Background$$

$$2)\ bBleach =\ Bleach - Background$$

(2) In the bleach correction step, the median of the Total area (bTotal) is defined as 100 % [4] (2). The corresponding bleach correlation vector is then calculated for each vector point [5]. Finally, the bBleach is divided by the correlation vector to account for the process of fluorophore bleaching [6].

$$4)\ medControl = median(bTotal)$$

$$5)\ correlationVector = \frac{bTotal}{medControl}$$

$$6)\ cbBleach = \frac{bBleach}{correlationVector}$$

(3) Normalization: All cbBleach vector values are then normalized to the median of the pre-bleach values (which is consequently set to 100%) [7] to remove systematic error among experiments (3,4).

$$7)\ ncbBleach = \frac{cbBleach}{median(prebleach)}$$

(4) For the fitting process (5,6), the first post-bleach ncbBleach value and the "plateau" values have to be determined. The first post bleach value is further defined as C [8] and the ending values defined as B [9].

$$8)\ C = firstPostBleachValue$$

9) B = median (last 5% of values of ncbBleach)

(5) The fitting process is based on the Levenberg-Marquard algorithm (7-9). Frapbot offers multiple fitting options based on the specific experiment. In this example, the most basic and

best known FRAP equation is used describing one/two freely diffusive components and an immobile fraction which results in an incomplete recovery of the values to the starting intensity. Tau represents the dependent variable:

$$10.1)\ fitting = B(1 - e^{-tau*t}) + C$$

$$10.2)\ fitting = A(1 - e^{-tau1*t}) + B(1 - e^{-tau2*t}) + C$$

(6) Tau is then used to calculate the half time ($t_{1/2}$) [11]. Furthermore, the apparent diffusion constant (D) can be calculated [12]. Here we provide the formula for circular bleach areas. The equation shown below is a derivation of the Soumpasis Diffusion equation

($D = 0.224 * r^2 / t_{1/2}$)(10).

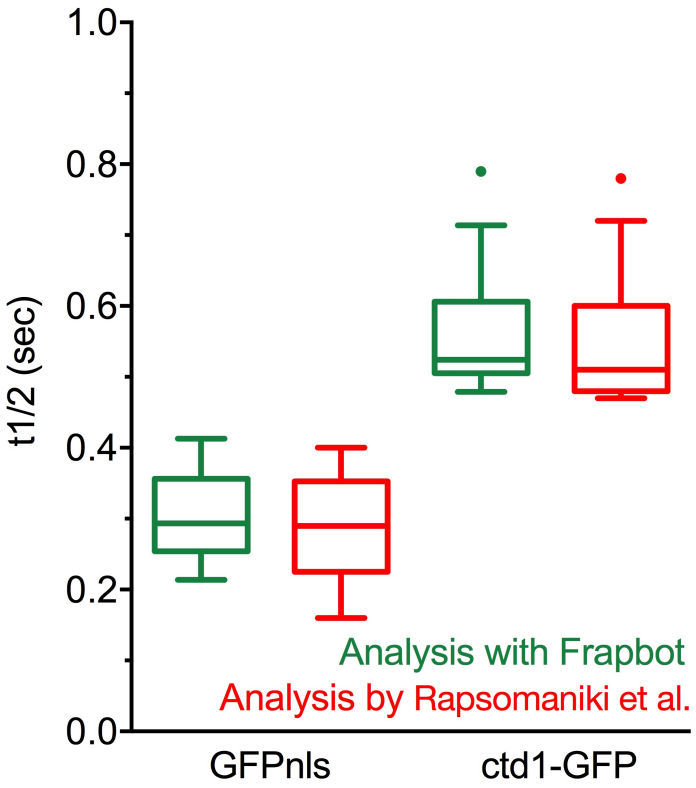
$$11)\ t\,{}^{1}\!/_{2} = \frac{\ln(2)}{tau}$$

$$12)\ D = 0.24 * area * \frac{1.94}{t\,{}^{1}\!/_{2}}$$

(7) Automated Pre-Bleach time-point finder: To find the time-point of the bleaching step, two vectors are created containing the raw data of the Bleach ROI. The numerical values of the second vector are shifted by 1 row. Those two vectors are then subtracted from each other, to form a third vector, containing information about the difference in intensity from $t_{(n)}$ to $t_{(n+1)}$. The maximum value of this third vector is extracted and compared to a threshold of 30% of the corresponding $t_{(n-1)}$ value. If that maximum value surpasses the 30% threshold, that point is set as bleach-time point. All following calculations are then based on that time-point, and the median of the pre-bleach defined as 100%. The fitting procedure contains only the retained post-bleach values.

(8) The calculated values with additional information are then displayed in Frapbot and can be either copied out or exported per download.

III: Experimental validation



Supplemental Fig. 1: Comparison of $t_{1/2}$ values for GFPnls (left plot) and Ctd1-GFP (right plot) analysed by Frapbot and by Rapsomaniki et al. using easyFRAP (11). For the datasets, double exponential fittings were used. GFPnls (left): n = 12, Frapbot: $t_{1/2}$ = 0.305 +/- 0.04 s, EasyFrap: $t_{1/2}$ = 0.289 +/- 0.5 s. Ctd1-GFP (right): n = 15, Frapbot: $t_{1/2}$ = 0.558 +/- 0.05 s, EasyFrap: $t_{1/2}$ = 0.548 +/- 0.06 s. The slight differences can be explained by the smaller tolerance level the Frapbot fitting algorithms accepts which results in more fitting iterations.

III: Advantages of Frapbot over related software

Although there are other software solutions for FRAP data analysis available next to Frapbot, it facilitates the analysis of FRAP data through a series of unique features:

1) Frapbot is an open-source solution and thereby provides unlimited access.

2) Frapbot is available as an online application for all current internet browsers and thereby independent of any operating system but also as source code for offline use in R programming environments provided for all available operating systems.

3) Columns containing the relevant data entries are automatically identified based on pattern recognition of numerical data. This is also the case for the bleach time point.

4) The self-written automation algorithms allow the instantaneous analysis and quality assessment of FRAP traces.

5) Frapbot allows simultaneous handling of large datasets consisting of many FRAP traces with automated features to exclude poorly fittable traces from the analysis.

6) Data read-out and analysis functions are also available for manual execution and control (transparency during data analysis).

7) Frapbot works with multiple data formats (.csv, .txt) (12,13).

8) The program provides a simple and intuitive Graphical User Interface as single screen mode (14).

9) Frapbot provides the option for the download of all calculated parameters.

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