EasyFRAP-web: a web-based tool for the analysis of fluorescence recovery after photobleaching data

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ABSTRACT

Understanding protein dynamics is crucial in order to elucidate protein function and interactions. Advances in modern microscopy facilitate the exploration of the mobility of fluorescently tagged proteins within living cells. Fluorescence recovery after photobleaching (FRAP) is an increasingly popular functional live-cell imaging technique which enables the study of the dynamic properties of proteins at a single-cell level. As an increasing number of labs generate FRAP datasets, there is a need for fast, interactive and user-friendly applications that analyze the resulting data. Here we present easyFRAP-web, a web application that simplifies the qualitative and quantitative analysis of FRAP datasets. EasyFRAP-web permits quick analysis of FRAP datasets through an intuitive web interface with interconnected analysis steps (experimental data assessment, different types of normalization and estimation of curve-derived quantitative parameters). In addition, easyFRAP-web provides dynamic and interactive data visualization and data and figure export for further analysis after every step. We test easyFRAP-web by analyzing FRAP datasets capturing the mobility of the cell cycle regulator Cdt2 in the presence and absence of DNA damage in cultured cells. We show that easyFRAP-web yields results consistent with previous studies and highlights cell-to-cell heterogeneity in the estimated kinetic parameters. EasyFRAP-web is platform-independent and is freely accessible at: https://easyfrap.vmnet.upatras.gr/.

INTRODUCTION

Cellular processes are governed by the dynamic behavior of proteins within living cells, notably their mobility and interactions. Fluorescence recovery after photobleaching (FRAP) is a live-cell functional imaging technique that allows the exploration of protein dynamic behavior at the single-cell level by exploiting the properties of fluorescent proteins as observed by modern microscopy systems (1–3). FRAP is a photobleaching method: molecules tagged with a fluorescent protein within a defined region of interest (ROI) are illuminated with a high intensity light, resulting in irreversible loss of fluorescence (bleaching step). The fluorescence intensity in the ROI before and after the bleaching step is quantified through time-lapse microscopy. The resulting time-course measurements, referred to as FRAP recovery curves, reflect the rate of fluorescence recovery in the ROI after bleaching and thus provide information on the kinetic behavior of the studied molecules within the living cell (4).

Raw FRAP data consist of single-cell, time-course measurements of fluorescence intensity within specific regions. To fully exploit the experimental measurements and draw robust and biologically relevant conclusions, a number of computational tasks must be carefully executed. Pre-processing of the raw curves aims to eliminate measurement noise and identify and correct experimental artifacts and batch effects, so that FRAP curves from different cells or experiments can be compared (5–7). Quantitative analysis of the data via curve fitting techniques permits the estimation of parameters related to the shape of the recovery curves, namely the mobile fraction of molecules and the time of half maximal recovery of the mobile fraction and can thus provide insight into the underlying kinetics of the protein of interest (1,8). Last, model-based analysis allows for the inference of physical parameters, such as the diffusion coefficient or the association and dissociation rates of the protein of interest (9–17). These approaches, which are based on the development of parametric expressions of the physical pro-

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cesses that are responsible for the underlying kinetics, offer great promise, as they provide parameters which are independent of the experimental set-up. Nonetheless, a number of assumptions and simplifications concerning the underlying processes (e.g. diffusion properties, number of binding components and cell geometry) need to be made, and conflicting kinetic estimates have been reported when different kinetic models fit FRAP curves equally well (1,2), rendering at present this approach non-trivial for the non-expert. Direct comparisons of normalized FRAP curves and curve-derived parameters are therefore used broadly for the analysis of FRAP experiments. As FRAP becomes increasingly popular in biology labs, tools simplifying FRAP analysis for the non-expert find broad applicability.

Software facilitating the analysis of FRAP datasets have previously been developed, such as FrapCalc (https://zenodo.org/record/574203#.WlY8-yOB2Rs), FRAPAnalyzer (11), easyFRAP (8) and Frapbot (18). easyFRAP is a stand-alone tool for quantitative analysis of FRAP data, previously developed by our team, which is broadly used by the FRAP community. To address the need for a quick, interactive and platform-independent solution, here we present easyFRAP-web, an updated version with additional functionalities, implemented as a web application.

MATERIALS AND METHODS

Implementation

Program overview. To tackle the problems of platform dependency and system compatibility, we present easyFRAP-web, a web-based application for the analysis of FRAP raw experimental curves. In easyFRAP-web, a series of interlinked steps facilitate quality assessment, interactive inclusion/exclusion of data, preprocessing/normalization, curve-fitting and curve-based parameter assessment of FRAP datasets (Supplementary Material 1: User Documentation). The first step of the analysis pipeline regards data uploading, where the user can upload FRAP files containing raw FRAP measurements of the bleaching region (ROI1), the total area of fluorescence (ROI2) and a background, non-fluorescent region (ROI3) and the corresponding time-points, from large numbers of single cells. EasyFRAP-web is compatible with all main FRAP data formats (.csv, .txt, .xls, .xlsx), exported by all major FRAP microscope software or ImageJ (19). Upon successfully uploading the data, the Dataset Selection displays a graphical table of the uploaded files (Figure 1A), which allows easy removal or restoration of individual files from the dataset analyzed. At the same time, the recovery curves of the three ROIs as a function of time are plotted for visual inspection (Figure 1B). EasyFRAP-web was designed to support dynamic data visualization, a key element towards interactive data analysis. Functionalities like zoom in/out, drag on selection, save graphs as images at a specific focus, show coordinates on click, synchronization between graphs while zooming and panning are some of the novel features introduced in the new version of easyFRAP. All recovery curves can easily be assessed and noisy or experimentally flawed datasets can easily be identified, allowing users to exclude or restore them at any time from the analysis (see (2) for a discussion on technically assessing FRAP datasets). Additionally, the computational pipeline of easyFRAP-web consists of interconnected steps: every time a change in selection or parameters is made, all sections are instantly triggered and automatic recalculations are propagated to all steps.

The next step concerns the estimation of bleaching depth and gap ratio, two metrics associated with the quality of the data (Figure 1C). Bleaching depth gives an estimation of the degree of fluorescence loss in the bleaching region during the bleach, while gap ratio provides a way to evaluate the amount of total fluorescence remaining in the cell following the bleaching step (for exact definitions see Supplementary Material 2: Manual Appendix). A number of initial fluorescence values can be eliminated from all curves in the dataset, since they often exhibit an exponential decay of fluorescence attributed to acquisition bleaching. After evaluating raw recovery curves and excluding technically flawed data, the user can normalize the raw recovery curves according to the two most common formulas used in the literature: double (6) and full scale (7) (Figure 1D) (details in the Supplementary Material 1). Following background subtraction, FRAP data are scaled to a reference axis from 0 to 1 in order to enable comparison between different curves and different experiments. Double normalization corrects for differences in the starting intensity of ROI1 (bleached area) and for loss in total cellular fluorescence (ROI2) due to the bleaching pulse and to acquisition bleaching. Full scale normalization additionally corrects for differences of the bleaching efficiencies thus making all recovery curves start from 0. Full scale normalization tends to affect the shape of the curves, especially when curves exhibit small bleaching depth values and thus double normalization is usually preferred (2). EasyFRAP-web enhances the quality and the speed of the analysis pipeline with interlinked graphs, as individual normalized curves, mean normalized curves and their standard deviation can be simultaneously assessed with a simple mouse hover. The final step in the analysis regards the estimation of the mobile fraction and the half-maximal recovery time (t-half) via curve fitting, using a single or double term exponential equation (Figure 1E). The data, fitted curve and fitting residuals are visualized in order to evaluate the fit, and goodness-of-fit statistics (R-square) are also provided (see also the Supplementary Material 2). Multiple curves can be fitted individually and all data exported, while the possibility to fit a mean curve is also provided for quick parameter assessment. The user can save the corresponding graphs as images and export the estimated results in a separate .xlsx file after every step for downstream analysis. Lastly, the user can delete the entire dataset from the server.

Implementation details. EasyFRAP-web is open source and the source code of the latest version is freely available on GitHub (see AVAILABILITY). The back-end section of easyFRAP-web is implemented using Microsoft ASP.NET Framework. To offer a simple and effortless navigation experience to users, advanced JavaScript and jQuery techniques and concepts have been developed. EasyFRAP-web has been tested on Linux, OS X (mac OS) and Windows operating systems. To ensure maximum compatibility with the easyFRAP-web interface, the use of Google Chrome (v.56 or later) is recommended. Recent versions of Mi-
Figure 1. The easyFRAP-web graphical user interface. EasyFRAP-web is designed as a single page application consisting of five progressively activated sections to simplify analysis. (A) Data upload panel. Multiple individual FRAP files can be uploaded (left) and subsequently excluded or restored at any time from the file manager (right table). Four different file formats (.csv, .txt, .xlsx, .xls) are supported. (B) Raw data visualization panel. EasyFRAP employs web front-end technologies which allow fully interactive data visualization across the analysis pipeline. Plots consist of dynamic content supporting functionalities like zoom in/out, panning, graph synchronization and pop-up information on click. Upper plot: raw fluorescent intensities of ROI1 of multiple cells. Lower plot: zoom-in of the first time-points of the recovery, allowing better assessment of initial post-bleach recoveries. (C) Bleaching Depth—gap ratio panel. Bleaching depth provides an estimation of the degree of fluorescence loss in ROI1 (the bleaching region) during the bleaching step, while gap ratio indicates the total fluorescence remaining in the cell (ROI2) after the bleaching step. Small values of bleaching depth and gap ratio (typically <0.6) are indicative of insufficient or excessive bleaching respectively. (D) Normalization panel. Raw data are normalized using either double or full-scale normalization. Individual normalized curves (upper graph) as well as the mean normalized curve together with the standard deviation (lower graph) are plotted. (E) Curve fitting panel. EasyFRAP-web performs both individual curve fitting and mean curve fitting and calculates the values of mobile fraction and t-half that serve as indicators of the underlying protein kinetics. At the same time, goodness-of-fit values are also provided (R-square). Results can be exported as .xlsx files for downstream analysis. All graphs can be exported as image files at multiple points during the analysis.
for the analysis of even large datasets and allows for easy experimentation with different parameter values or choices of normalization and fitting functions.

EasyFRAP-web was tested using data from FRAP analysis of the cell cycle regulator Cdt2 (20,21) tagged with eGFP, on a Leica SP5 confocal microscope equipped with FRAP booster. MCF7 cells transiently transfected with eGFP-Cdt2 were locally irradiated through a micropore filter with UV-C (254 nm), incubated for 15 min (to permit steady-state recruitment to damaged sites) and analyzed by FRAP in parallel to control, non-irradiated cells (Figure 2A). Raw data were imported as .csv files to easyFRAP-web. After raw data visualization and double normalization (Figure 2B), the curves were fitted using a double exponential equation and the mobile fraction and t-half of all curves were computed. In control cells, the mobile fraction was estimated at 0.98 ± 0.02 (mean ± standard deviation) and the t-half at 0.27 ± 0.06 s, while treated cells exhibited a lower mobile fraction (0.85 ± 0.07) and a higher t-half (1.4 ± 0.64 s) (Figure 2C). We thus conclude that, consistent with prior analyses (22), eGFP-Cdt2 exhibits long-term interactions with chromatin in locally UV-C irradiated cells, evidenced by a slower fluorescence recovery and larger mobile fraction compared to control cells. Comparison of the distributions of the quantitative parameters shows a high degree of variability for eGFP-Cdt2 in locally UV-C irradiated cells, indicative of cell-to-cell heterogeneity (Figure 2D). This is consistent with the behavior of other cell cycle regulators, such as Cdt1, PCNA and the MCM family of proteins (9,22,23).

EasyFRAP-web estimated parameters are in agreement with other available tools (see Supplementary Material 3: Comparison Table S2) while it provides a number of advantages (Supplementary Material 3: Comparison Table S1). Notably, the platform independency of easyFRAP-web simplifies access: easyFRAP-web only requires access to the internet and a web-browser, in contrast to earlier programs requiring installation of dedicated, and in some cases commercial, software. The dynamic and interactive visualization of FRAP curves linked with the possibility to interactively add/remove datasets from the analysis, features unique to easyFRAP-web, facilitate rapid assessment of all curves and simplifies identification and exclusion of noisy or problematic data. Dynamic linking of all analysis steps further speeds up analysis. In addition, the intuitive analysis steps, together with the tips, prompts and detailed error messages provided, simplify analysis for the non-expert.

CONCLUSION
EasyFRAP-web is a platform-independent application for qualitative and quantitative analysis of FRAP data. The user can easily analyze FRAP datasets through an intuitive web interface with interlinked analysis steps (e.g. experimental data assessment, normalization and estimation of quantitative parameters) that enable real-time update every time an option is altered. In addition, easyFRAP-web permits data and figure export for further analysis. We demonstrated the accuracy of easyFRAP-web by analyzing a dataset of eGFP-Cdt2 transfected cells in the presence or absence of UV-C irradiation treatment. As FRAP be-
comes widespread in biology laboratories, easyFRAP-web with its additional functionalities aims to significantly simplify analysis of FRAP datasets.

**DATA AVAILABILITY**

**Project name**: easyFRAP-web  
**Project home page**: https://easyfrap.vmnet.upatras.gr/  
**Archived version**: https://github.com/gkoulouras/easyFRAP-web  
**Programming language**: Microsoft ASP.NET, R, JavaScript  
**Operating system(s)**: Platform-independent  
**Web browser**: Google Chrome (v.56 or later)  
**License**: GNU General Public License (GPLv3)

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**Author contributions**: G.K. designed and implemented the web application; M.A.R., N.N.G. and A.P. helped design and tested the web application; G.K., M.A.R., A.P. and N.N.G. wrote the manuscript; A.P. performed FRAP experiments; A.P., M.A.R., N.N.G., G.K. analyzed the data. S.T. and Z.L. designed the study, supervised implementation and proofread the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY TABLE**

| Experiment | T-half (sec) | Mobile fraction | R-square   | N   |
|------------|-------------|-----------------|------------|-----|
| no UV      | 0.27 (±0.06)| 0.98 (±0.02)    | 0.94 (±0.02) | 20  |
| loc UV     | 1.4 (±0.64)| 0.85 (±0.07)    | 0.97 (±0.05) | 20  |

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