Frapid: achieving full automation of FRAP for chemical probe validation

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Abstract: Fluorescence Recovery After Photobleaching (FRAP) is an established method for validating chemical probes against the chromatin reading bromodomains, but so far requires constant human supervision. Here, we present Frapid, an automated open source code implementation of FRAP that fully handles cell identification through fuzzy logic analysis, drug dispensing with a custom-built fluid handler, image acquisition & analysis, and reporting. We successfully tested Frapid on 3 bromodomains as well as on spindlin1 (SPIN1), a methyl lysine binder, for the first time.

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FRAP is an advanced microscopy technique developed to assess the movement of fluorescently-labelled proteins within or between cells [1]. Previously, we and others have extended this technique to validate small molecules that inhibit proteins from binding to fluorescently-labelled proteins within or between cells [1].

Introduction

FRAP is an advanced microscopy technique developed to assess the movement of fluorescently-labelled proteins within or between cells [1]. Previously, we and others have extended this technique to validate small molecules that inhibit proteins from binding to chromatin located in the nucleus of a cell [2–4]. FRAP has been used extensively to characterize inhibitors of acetyl lysine binding domains known as bromodomains. These small molecules, termed chemical probes [5], have substantially increased our understanding of the role of these proteins, and given clues about the druggability of these proteins in a variety of diseases [6] including cancer [7, 8], and inflammation [9]. However, because of its single cell approach, implementing a FRAP assay on a statistically relevant number of cells at several concentrations has so far been laborious, repetitive and time intensive and may consume as much as 8 hours of an operator’s time.

Automated microscope systems and high-content screening platforms have enabled certain experiments to be carried out with minimal supervision. Systems such as the Molecular Devices ImageXpress, Yokogawa Cellvoyager, Perkin Elmer Operetta, Olympus
ScanR and BD Pathway 855 can be instructed to perform pre-programmed tasks such as tile scan and time lapse image acquisitions. However, FRAP is so far not an available automated feature on these commercial systems. In the arena of custom-built systems, the open source Micropilot [10] has indeed given scientists the ability to run automated FRAP assays using training sets to study complex biological questions. Other systems have also capitalized on homebuilt and programmable microscopes to process cells for other advanced optical experiments such as optoporation [11, 12]. Although these systems are allowing scientists to run more advanced experiments without supervision, they lack the ability to deliver small molecules or proteins at predefined timepoints. This capability is crucial for testing compounds in a fully automated system without human intervention. In addition, the Micropilot uses training sets to develop neural networks for identifying cells. This requires users to enter multiple samples of both ‘ideal’ and ‘non-ideal’ images of cells, which requires lengthy procedures.

To address these limitations, we developed Frapid, a system that is capable of automating:

(i) FRAP – (pre-bleach scans, photobleach, and timelapse imaging)
(ii) Scheduled compound delivery via a custom-built fluid handling accessory to transfer small molecules to cells at a pre-instructed time
(iii) Quick identification of cells using fuzzy logic instead of training sets
(iv) Data analysis and distribution of results via email

Materials and methods

The FRAP setup used in Frapid was adopted from [4] and consisted of a Zeiss LSM710 (Carl Zeiss, UK) inverted confocal microscope fitted with a 488 nm argon-ion laser, incubator capable of reaching 37 degrees, 10x 0.45 N.A. and 20x 0.8 N.A. Plan Apochromat objectives, and a motorized stage and objective turret. The system was also equipped with laser autofocus. An appropriate multi-well plate insert was necessary to seat culture plates onto the stage. The system PC’s ran Windows 7 Ultimate 64-bit and had an Intel Core i5-2500 @3.30GHz processor with 32 GB RAM and an integrated video card. The microscope was controlled with ZEN 2009 with Visual Basic 6 macro editing features and MATLAB 2012a (Mathworks, USA) with the fuzzy logic and image processing toolboxes. For editing of Visual Basic macros, a ZEN hardware system key was required. The drivers, Microsoft Excel, and the Visual Basic libraries of the USB to digital I/O interface were also installed following the manufacturer’s manual.

Frapid was tested on 3 bromodomains – Bromodomain-containing protein 4 (BRD4), Cat eye syndrome critical region protein 2 (CECR2), and Bromodomain and PHD Finger Containing (BRPF1) that have been previously published by our lab [4]. These targets were inhibited by chemical probes that were developed and characterized in collaboration with academic and industrial partners, and have been previously validated using manual FRAP. Chemical probes for BRD4, CECR2, and BRPF1 were PFI-1 [13] (Sigma-Aldrich, UK), NVS-1 [14] (Sigma-Aldrich, UK), and OF-1 [15] (Structural Genomics Consortium, UK) respectively. A negative control compound for CECR2, NVS-1C is also available. We also include data that promotes FRAP as an assay for the methyl lysine binder SPIN1 for the first time.

At this point in time, Frapid has been written specifically for the popular Zeiss LSM series of confocal microscopes equipped with an incubator capable of reaching 37 degrees, a motorized stage and an objective turret. The system also employs laser autofocus. The custom-built fluid handling accessory replaces the microscope condenser, and requires a USB digital I/O interface to control two motors. We used Visual Basic libraries to control the hardware and image acquisition processes. Custom written MATLAB scripts were called by
Visual BASIC for loading image tilescan images, segmenting and identifying cells through fuzzy logic (Fig. 1(b), 1(c)).

Fig. 1. (a) Schematic flowchart of the automated FRAP system, Frapid. Visual Basic (VB) and MATLAB communicate file directories and cell coordinates between each other. VB is the main controller and handles hardware control (fluid pump, stage movement, image acquisition file saving, etc.). On the other hand, MATLAB receives file information from VB and imports images. Then, MATLAB performs image analysis routines, identify cells based on fuzzy logic, analyze FRAP recovery curves, and export to Microsoft Excel for emailing. MATLAB returns control to VB once it has completed the script. (b-c) Each low resolution tile scan image is segmented based on GFP fluorescence to identify individual objects. The area, average intensity, eccentricity, and roundness are extracted from each nucleus and run through MATLAB’s fuzzy logic toolbox to obtain an overall score between 0 and 1. (d-e) Fuzzy logic is highly advantageous over traditional Boolean logic in achieving a higher yield of cells. Due to the relaxed rules of fuzzy logic, a cell (shown as (d)) that is still reasonably near the ideal range of size and intensity, for example, can still be considered for FRAP without outright rejection. (f) Depending on the score, the cell is prioritized and ranked for FRAP, with ‘1’ being scored for the most ideal cell.

Indeed, ever since fuzzy logic was pioneered by Zadeh [16] and later on by Kosko & Isaka [17], fuzzy logic has made its way from commercial appliances to a variety of scientific data analysis algorithms [18–20] to analyze gene and image data. The fuzzy logic routine in Frapid takes inputs of cell size, green fluorescence protein (GFP) fluorescence intensity, eccentricity and roundness to give an overall score between 0 and 1. This score is used to determine if the cell is suitable for FRAP and to rank in which order that cell should be processed (Fig. 1(d)). Fuzzy logic relaxes the traditional and rigid rules of ‘True’ and ‘False’ logic and compensates for cells, such as the cell in Fig. 1(e), that may be on the border of being eliminated based on area (Fig. 1(f)). This has the further advantage of increasing the yield of cells. Fuzzy logic is also quick to program, operate, and does not require thousands of representative images in a training set. This is important as not all users will have the knowledge of programming a neural network or the ability to provide a large sample of images. On the other hand, fuzzy logic quickly allows for new rules to be inserted and tested. Because the code is open-source, users can make further design enhancements.

Technical details of fluid handler

A fluid handler was custom designed and built to allow compounds to be aspirated and dispensed onto cells at the desired time points (Fig. 2). The microscope condenser must be removed in order to install this device (Fig. 3). Briefly, the fluid handler works by retracting
or extending a tube away or towards the plate respectively, in the axial direction of the objective lens. This motion originates from a stepper motor and a rack and pinion gear set. A peristaltic pump (not shown) with directional control aspirates and dispenses the liquid. Lateral motion of the plate is handled by the stage motor. Technical details and assembly instructions of the fluid handler can be obtained from http://www.thesgc.org/frapid.

Fig. 2. Exploded view of fluid handler showing ring mount, main bracket, rack, tube holder, bracket, pinion gear and stepper motor. Technical details and instructions can be obtained from http://www.thesgc.org/frapid.
Fig. 3. The custom-built fluid handler, which replaces the microscope condenser during Frapid. The fluid handler is attached to the microscope arm via the ring mount. A rack and pinion gear converts rotational motion from the stepper motor to linear movement. The rack is also attached to a tube holder that guides the tubing from the retracted position (during lateral stage movement) to the extended position (when aspiring and dispensing fluid into well). Electrical components for controlling the stepper motor can be found at http://www.thesgc.org/frapid.

**Experimental setup and implementation of automated FRAP**

On the day of the FRAP experiment, the plate was observed on an inverted widefield fluorescence microscope (Carl Zeiss, UK) to assess transfection efficiency and viability. Generally, transfection efficiencies of 20% or more were achievable. For cell culture and transfection procedure, please see Appendix.
Each test compound was manually prepared onto the empty side of the plate containing 500 µl HEPES buffered medium in each corresponding well. For example, compounds pipetted into well A4, A5, and A6 will be dosed by the fluid handler onto the cells in wells A1, A2, and A3 respectively. The DMSO concentration was maintained constant throughout the wells at 0.2%. The first test compound well (A4) was manually transferred to its corresponding well (A1) before the plate was incubated for 1 hour at 37 degrees and 5% CO2. The plate was then ready to be transferred to the microscope for a FRAP experiment.

During automated FRAP, each pre-diluted test compound is pipetted into its corresponding well using the fluid handler before it is processed for FRAP. In the user interface, the user can select the incubation time e.g. the time between compound dosing and FRAP. This is typically 1 hour as optimized and standardized in [4]. The user can also select the number of cells to undergo FRAP in each well. Depending on the kinetics of the bromodomain in question, each cell can typically take between 45 - 90 seconds to process including identification time and timelapse duration. A well of 20 cells typically takes 15 to 30 minutes, but because the total processing time for each well depends on the number of GFP-positive cells present, this can be even higher when transfection efficiency is low. In some cases, the transfection efficiency may be so low that a single tilescan will not yield sufficient cells as specified by the user. Therefore, Frapid will attempt to acquire multiple tilescans (up to 5 per well) in order to reach its target before moving onto the next well. The incubation time and the number of cells are kept constant for all wells in the plate.

Identification and FRAP of suitable cells

To allow Frapid to work more efficiently, a large low-magnification tilescan at 10x is acquired first before zooming in on specific cells at a higher magnification (20x). This method is more effective when transfection efficiencies are generally low where the sole use of a 20x objective would mainly result in dark and empty fields. Each tilescan uses an autofocus based on the reflection of the bottom-side of the plate to find the best focus. Imaging-grade flat-bottom multiwell plates have a consistent thickness across the bottom that allows a fixed offset to work. For this reason, Frapid was not attempted on standard tissue culture plasticware. The next 4 sections will explain in detail how cells are identified, centered, processed for FRAP, and analyzed.

Identification of cells: ZEN tilescan macro

The tilescan macro is intended for quickly finding large numbers of cells over a large area using a 10x objective. Although a 4x objective could be used, a 10x Plan Apo was selected as this gave more sensitivity and resolution for discerning critical features of cells for further analysis. For each bromodomain, the laser power, pinhole, and gain settings were optimized to obtain a suitable signal-to-noise ratio without incurring excess photobleaching. See Table 1 in Appendix for acquisition settings relating to each protein tested. The size of the tilescan can be controlled by the user in the interface, but a 2x2 size is typically sufficient in most cases for obtaining 20 cells.

Custom scripts in MATLAB were written to analyze and select GFP-positive cells that were suitable for FRAP. Cells were identified using image segmentation within MATLAB’s image analysis toolbox. Briefly, the original image was blurred and thresholded using a global threshold level first. Objects that were smaller than 150 pixels or were in contact with the image border were removed. The nucleoli, which appear as dark spots in the nucleus, were identified as critical features for BRD4 and CECR2, and were extracted by subtracting the original and filled versions for each cell. Cells were also avoided if they had bright punctuate spots. In order to identify suitable cells, the primary characteristics selected were area, average intensity, eccentricity, and perimeter-to-area ratio. Selecting cells based on area removed small debris and large clumped cells. The average intensity parameter weeded out cells that would suffer from insufficient signal to noise ratio or saturated due to significantly
more bromodomain expression ([4] shows that this leads to erroneous results). Fitting the bleach region in oblong cells has been a problem so the eccentricity criterion was necessary. Finally, the perimeter-to-area ratio checked for irregularly lobed nuclei which could possibly indicate that the cell was genetically abnormal from the rest of the population. These parameters were calculated for each remaining cell and stored in an array for fuzzy analysis. The actual fuzzy membership rules and ranges were determined empirically and tested on cells in different perturbed conditions e.g. transfected with CECR2 wildtype, CECR2 wildtype with inhibitor, and the catalytic inactivating mutant. The same specimens were used for both manual FRAP and Frapid to rule out differences in physiological conditions. Once the same cells were identified by automated and manual FRAP, Frapid’s membership rules were saved and further tested on other bromodomains.

Each cell was given a weighted score (Fig. 1(f)), which identified the cell’s suitability and rank for FRAP. A rank of ‘1’ indicated that the cell was the most optimal and would be processed first. In addition to the primary characteristics, secondary parameters, such as the object’s x-y coordinates, the major-to-minor axis ratio, and orientation were also stored for later use.

Identifying bleach region and preparing for FRAP: ZEN macro imagescan

Each nucleus that is positively identified by the fuzzy set is imaged at a magnification of 20x for further analysis and, subsequently, FRAP. The laser power and pinhole were empirically set so that the gain required was between 600 and 800 for sub-saturation [4]. See Table 2 in appendix for complete listing of protein-specific acquisition parameters. Images at 3 different gain settings of each cell were acquired, with the brightest image below saturation being used for further analysis. This was to take into account the slight differences in average intensity and had the added advantage for increased throughput. From the best of the 3 images, a custom MATLAB script identified the region of interest, which was to be located away from dark nucleoli and saturated foci. To do this, the script iteratively places a movable mask that represents the bleach region around the nuclei, and analyzes the average intensity within the region. The most ideal position of the mask was considered to be where the average intensity is highest (without exceeding saturation) and has a homogenous set of pixel intensity values. Although users can select different bleach region sizes, the data here was obtained with a bleach region of 70 pixels (approximately 4.9 µm) in diameter and corresponds to literature from [4]. The distance between the optimal position of the bleach region and the current position of the stage is calculated. Using this distance, the stage repositions the cell so that the bleach region is in the center of the image. Thus, downstream analysis of the bleach region is simplified.

Processing cell for FRAP: ZEN macro FRAP

Bleaching was performed with a modified protocol from reference [4] and includes a 405nm and 488nm argon-ion laser tightly focused through a 20x 0.8 N.A. Plan Apochromat objective. Briefly, 5 pre-bleach frames were acquired at low power before bleaching at 100% power of both lasers. The duration of the subsequent timelapse is bromodomain dependent and should allow sufficient time for near or complete recovery. As some bromodomain containing proteins e.g. CECR2 have a relatively long recovery time, the duration of the timelapse was as long as 1 minute. Photobleaching during timelapse was observed, which had a detrimental impact on further analysis. To solve this, images were acquired with a 2 second interval rather than continuously to reduce the exposure of cells to the laser. For all bromodomains and SPIN1, a ‘line step’ of 2 was used, which scans every 2nd row of pixels to increase the scanning speed. This is to accommodate the fast recovery times of e.g. BRPF1 and SPIN1 (halftime ~1 second). See Table 3 in Appendix for complete listing of protein-specific acquisition parameters.
Analyzing recovery curves

Analysis of the recovery curves was done by a custom script in MATLAB, which analyzed all cells within a well as a batch. First, the nucleus in the first frame of the timelapse series was thresholded using a global threshold limit and segmented. Objects smaller than 10,000 pixels or in contact with the image border were removed. The resulting mask was used for all subsequent frames. To identify the background, the images were inverted, thresholded, and segmented. Since the bleach region was at the center of the image, the mask was generated in real time based on the user-defined bleach region diameter. These three regions were then analyzed at each timepoint (t): 1) bleach region, $(F(t)_{ROI})$ 2) the entire nucleus for photobleaching correction $(F(t)_{total})$, and 3) the region surrounding the nucleus for background subtraction $(F(t)_{BG})$. The relative fluorescence intensity of the bleach region of each nucleus was calculated at each timepoint using [Eq. (1)] described by Phair et al. [21], where $F(p)$ is the mean intensity during the first 5 prebleach scans:

$$F(t)_{norm} = \frac{F(t)_{ROI} - F(t)_{BG}}{F(t)_{total} - F(t)_{BG}} \times \frac{F(p)_{total} - F(p)_{BG}}{F(p)_{ROI} - F(p)_{BG}}$$  

Once the normalized fluorescence intensity was calculated for each timepoint, it was curve-fitted using the double exponential curve, [Eq. (2)]:

$$y = y_0 + A_1(1 - \exp(-x / t_1)) + A_2(1 - \exp(-x / t_2))$$

The curve fitting toolbox in MATLAB obtained the parameters of $y_0$, $A_1$, and $A_2$ and calculated the level of fluorescence at the half recovery point from [Eq. (3)]:

$$y_{1/2} = y_0 + \frac{A_1 + A_2}{2}$$

where $y_0$ and $y_{1/2}$ are the fluorescence intensities immediately after photobleaching and at half recovery respectively for that nucleus. The half time of recovery was then obtained using MATLAB’s equation solver. The halftimes obtained from all cells are automatically written into an Excel spreadsheet (Fig. 4), which also performs a quality control check and rejects any curve fits with a plateau of more than 1.1 and an R-square value of less than 0.97. Users have the option to reinstate rejected cells or remove other cells, which will cause the average and standard deviation to update accordingly. In Fig. 4, cell 7 is automatically given a verdict of ‘0’ since it does not meet the goodness of curve fitting and plateau criteria. While cell 8 meets the $R^2$ value criteria, the plateau of curve fit is above 1.1, thus, giving an unusually high recovery time. Both cells are excluded from the average and standard deviation calculation. After the final well was analyzed, Frapid alerted the user via email with the attached data file.
Fig. 4. Data presented in spreadsheet format with average and standard deviation half times for each well. Results displayed in an Excel spreadsheet detailing the a) verdict, b) cell number, c) recovery halftime, d) R² value for goodness of curve fit, e) the plateau from curve fitting, f) normalized recovery plot showing data points (blue) and curve fit (red), g) image of cell during pre-bleach, h) image of cell immediately after bleach, i) segmentation mask of cell, j) automated pre-calculation for determining the population standard deviation, label l. Specifically, this is the square of the difference between the individual cell’s recovery half time and the population average recovery half time, k) average recovery half time of cells with verdict ‘1’, l) standard deviation of individual half time values of cells with verdict ‘1’, m) the position of the well represented by a two digit number that designates the column and row numbers respectively. Cells 7 and 8 were rejected due to their R² value and/or plateau of curve fit. Users can manually change the verdict of any cell, causing the average and standard deviation to update automatically.

**Results**

Cells were transfected with plasmids encoding for GFP fused with BRD4, CECR2, BRPF1 or SPIN1 as described in the methods section. With the exception of SPIN1, an inhibitor for each was also tested at various concentrations along with a DMSO control. In each experiment we also tested a DMSO-treated catalytically inactive mutant, which denoted the 100% displacement off chromatin.

Cells transfected with bromodomains generally had different appearances. For example, BRD4 always localized to the nucleus and the nucleoli were observable as dark spots. SPIN1 also localized only to the nucleus but no accumulation in nucleoli were observed. In contrast,
CECR2 and BRPF1 could only be observed to localize in the nucleus in a small population of low expressing cells, while the remaining transfected cells had GFP fluorescence in the cytoplasm. Here, image segmentation and fuzzy logic were able to quickly identify cells that were ideal and even close-to-ideal for FRAP. Analyzing a large tilescan containing ~200 cells took less than 5 seconds.

To optimize fuzzy parameters, automated and manual FRAP were performed on samples containing CECR2 WT, CECR2 WT + inhibitor, or the catalytically inactivated mutant. Both methods identified the same cells for FRAP analysis. As shown in Fig. 5, the half recovery times for all 3 samples showed a similar trend.

![Graph showing comparison of half recovery times](image)

Fig. 5. Comparison of half recovery times on the same cells selected by Frapid and manual FRAP. Samples included either CECR2 WT, CECR2 WT with inhibitor, or the catalytically inactivating mutant (N140A). Frapid identified the same cells as in manual FRAP producing half recovery times that were similar, suggesting that empirically determined fuzzy parameters were optimal. Error bars are standard error of mean.

The optimized fuzzy parameters were used to acquire data on other bromodomains. During FRAP of BRD4, the half recovery time was approximately 1.8 seconds at 100% inhibition and the wildtype was just under 4 seconds (Fig. 6(b)). For CECR2, the recovery time for the wildtype (WT) and mutant were approximately 11 and 6 seconds respectively (Fig. 7). The probe NVS-1 was used as a known test compound which reduced recovery times in a dose dependent manner. The inactive version, NVS-1C, did not have any significant impact on the recovery time. BRPF1 had the fastest half recovery times of the 3 bromodomains tested with less than 0.5 seconds at 100% inhibition whereas the WT was approximately 1.8 seconds (Fig. 8).
Fig. 6. (a) Montage comparing fluorescence recovery for BRD4 in cells transfected with either BRD4 WT, N140F / N443F mutant, or BRD4 WT + PFI-1. White circle represents bleach region. (b) Bar chart of average recovery times for each condition tested. ** and * denote p<0.0005 and p<0.005 respectively as determined by Wilcoxon Rank-sum test. Error bars are standard deviation and each group represents 20 cells. (c) Average recovery curves of more than 20 cells for each condition. Scale bar represents 5µm.
Fig. 7. CECR2. a) Montage of U2OS cells expressing green fluorescent protein fused to wild-type CECR2, wild-type CECR2 treated with NVS-1, or N140A mutant. White circle represents bleach region. b) Bar chart comparing half recovery time between wildtype, N140A mutant, or wildtype treated with NVS-1. * represents p<0.005 determined by Wilcoxon Rank-Sum test. Error bars are standard deviation and each group represents 20 cells. c) Fluorescence recovery curves over time comparing wildtype, N140A mutant, or wildtype treated with NVS-1 or the inactive version, NVS-1C. Each curve is represented by 20 cells. Scale bar represents 5µm.
Validation of Frapid

We compared half recovery times from Frapid with independently obtained data using manual FRAP for all 3 bromodomain wildtypes and their corresponding mutants. As shown in Fig. 9, the half recovery times produced by Frapid and manual FRAP are comparable and show similar trends.
FRAP has been well established as an assay to validate probes against readers such as bromodomains and members of the malignant brain tumor (MBT) family [22]. We decided to extend this technique further by testing and automating FRAP on Tudor domains such as SPIN1.

The WT and mutant half recovery times were 1.3 and 0.9 seconds respectively (Fig. 10(c)). The assay window was small, so to create more methyl binding sites for SPIN1 and, thus, a larger separation between WT and mutant recovery times, we incubated cells overnight with IOX1, a pan-demethylase inhibitor [23]. We performed a titration of IOX1 between 12.5µM to 200µM and observed that a significant increase in recovery time could be achieved at 100µM or above. Significant toxicity was not seen at any concentration. When IOX1 was used at 100µM, the WT half recovery time had increased from 1.3 to 1.7 seconds (Fig. 10(b)). Frapid is therefore also extendable to test inhibitors of other chromatin binding proteins.
Fig. 10. SPIN1. a) - Montage of U2OS cells expressing green fluorescent protein fused to wild-type SPIN1, wild-type SPIN1 treated with IOX1, or F141A mutant. White circle represents bleach region. b) - bar chart comparing half recovery time between F141A mutant, wildtype, or wildtype treated with IOX1. * represents p<0.005 determined by Wilcoxon Rank-Sum test. Error bars are standard deviation and each group represents 20 cells. c) – Fluorescence recovery curves over time comparing wildtype, F141A mutant, or wildtype treated with IOX1. Each curve is represented by 20 cells. Scale bar represents 5μm.

**Discussion**

The half recovery times obtained in this present study coincide with data generated from previous studies [2–4] and validate the proposed automated system. The observed subtle inconsistencies between Frapid and manual FRAP are possibly due to differences in objective lens sensitivities (20x air objective vs 40x oil immersion objective). However, these do not significantly affect the assay window nor prevent Frapid from identifying potent inhibitors. FRAP has already been implemented for other bromodomains [2–4] such as TRIM24, SMARCA2, and CREBBP. Thus, with automation, more chemical probes can be validated against these targets as well in order to understand their biological role in cells. New data from the M-methyllysine-binding protein SPIN1 suggests that FRAP and its automation can be applied to a wider range of epigenetic targets beyond bromodomains.

The single-cell nature of manual FRAP is a limitation in obtaining detailed dose response curves without significant time investment. As recovery times are sufficiently rapid, simultaneous imaging of multiple cells is not possible without compromising time resolution. Therefore, each cell must be monitored till completion before advancing to the next cell. Automation of FRAP can allow more elaborate experiments with more conditions and replicates to occur without constant human supervision.

One limitation of the proposed automated system is its lower sensitivity to light. Low expressing bromodomains will be more difficult to detect with the 10x and 20x air objectives despite using high numerical aperture versions. Increasing laser power, pinhole size, and gain...
all have disadvantages in terms of photobleaching, lateral resolution and noise respectively, which may have an impact on the reported half recovery time accuracy.

Frapid has been designed for use on a Zeiss confocal platform due to the ease of creating custom macros. Other microscope manufacturers do exist, however, the ability to develop scripts at the time of this project was less practical. The art of developing home-built systems has become popular due to the cost savings and customizability. The custom-designed fluid handler and MATLAB scripts could be transferred to such systems. The Visual Basic scripts that control hardware, however, may need to be rewritten in another language such as C++. Here, we present an open-access method complete with documentation to allow readers to apply the basic framework and redeploy Frapid on a homebuilt system.

Although Frapid was initially designed for use to validate small molecule inhibitors against chromatin readers, the algorithms for controlling stage movement, cell identification, and laser photobleaching can be implemented for other applications. As shown by Dushek et al. and Goehring et al. [24, 25], FRAP is already an established method for assessing membrane protein mobility in cells. The movement of Ras, proteins involved in signal transmission within a cell, was studied by Goodwin et al. [26] using FRAP. Finally, the intracellular communication via gap junctions between primary human tenocytes was modelled with FRAP [27]. The authors showed that these gap junctions could also be blocked using an inhibitor which has clinical relevance to human tendon repair. Such experiments could be similarly automated and implemented by Frapid to increase robustness of data or range of perturbed conditions.

Frapid is not confined to photobleaching experiments. It can be reprogrammed to steer a multiphoton laser to induce DNA damage and examine the role of bromodomains in DNA repair, as done by Gong and colleagues [28]. Other chromatin modifiers can also be studied with Frapid as well. In a similar way to Gong et al., Young et al. [29] showed that the tudor domain JMJD2B was recruited to double stranded DNA breaks, which could be monitored over time by confocal microscopy. Since the recruitment time is of the order of tens-of-seconds up to a minute, the advantage of automated FRAP is that multiple cells can be monitored simultaneously in a cycle without a significant detriment to temporal resolution.

Conclusion

Frapid consists of custom-written MATLAB and Visual Basic scripts written for a Zeiss confocal microscope to achieve full automation of compound screening in FRAP. These scripts allowed the system to dose small molecule inhibitors onto cells at the right timepoint, identify suitable cells for FRAP, photobleach, acquire timelapse data, and analyse data without human intervention. The system we present here is capable of completing a FRAP experiment in a similar duration but without any human intervention.

Cells will inherently display a continuous distribution of fluorescence intensities and morphologies. As shown by Philpott et al. (2014), FRAP can give dubious results on cells that are at the extremes. Fuzzy logic allows easy implementation of rules to identify suitable cells.

It is important to note that the overarching goal of Frapid is to minimize human oversight rather than significantly reduce experimental duration. Due to the inherent single-cell nature of both manual and automated FRAP, a bottleneck occurs during acquisition of timelapse data when no other processes can be performed. Alternatively, enhancement of the output rate can be achieved by shortening the 30-second / datapoint laser autofocusing routine via a Definite Focus system. This, however, is an expensive accessory and is not normally found on most systems.

Electronic information

Software availability. The open source code, step-by-step manual and documentation are available as supplementary software at http://www.thesgc.org/frapid.
Appendix

Cell culture

U2OS cells (American Type Culture Collection, USA) were cultured in McCoy’s 5A medium (Sigma Aldrich, UK) supplemented with 10% fetal calf serum (Life technologies, UK), and 1% GlutaMAX (Life technologies, UK) in T75 or T25 flasks (Corning, UK). Cells were passaged before reaching confluence using Dulbecco’s phosphate buffered saline (Sigma Aldrich, UK) and TrypLE (Life technologies, UK).

BRPF1 cloning

The plasmids encoding for BRD4 and CECR2 fused with GFP were from reference [4]. The SPIN1 construct was a gift from Roland Schuele’s lab at Freiburg University.

The N708F single point mutation was introduced into BRPF1 cDNA using 15 cycles QuikChange II PCR protocol (Agilent Technologies). Gateway entry clone containing three copies of the wild type or mutated BRPF1 (Uniprot ID P55201) bromodomain was constructed by multiple ligation independent cloning (LIC) protocol [30]. Briefly, pDONR221 vector (Invitrogen) was linearized by PCR using primers 221-F (5’-AAGAAAAAGGAGTGCTTTTCTTGTACAAAGTTGCCATTATAAAGAA) and 221-R (5’-ATGGATTGGAATGCTTTTTTTGTACAAAGTTGCCATTATAAAAA) encoding LIC sites and nuclear localisation signal (NLS). Three copies of the BRPF1 bromodomain spanning amino acids from E627 to G740 were PCR amplified using primers encoding LIC extensions specific for each repeat and purified using Qiagen PCR purification kit. Primers BRPF1-F1 (5’-CTACTTCAATCCAGAGATGCAGCTGACTCCTTTT) and BRPF1-R1 (5’- GCCAACATCCTAGCGGAGGTTCGATTTCTGCTTGG) were used to amplify the first copy, BRPF1-F2 (5’-GGAAGGTTGAGGAGATGGTGGACCATTTTCTGCTTGG) and BRPF1-R2 (5’-GGAGGAAGATTGTTGATGGAGGACCCGCCATTTTTTCTGCTTGG) to amplify the second copy and BRPF1-F3 (5’- TCCTTCACCATCAGCAGTCCAACCTTTTCGGGAGATGCAGCTGACTTGG) and BRPF1-R3 (5’-ACCTTTGCATTTTCTTGGGGGACCATTTTTTCTGCTTGG) to amplify the third copy of the bromodomain. Fragment encoding first and third repeats of the bromodomain were treated with T4 DNA polymerase and dCTP and linearized pDONR221 vector and second repeat of the bromodomain were treated with T4 DNA polymerase and dGTP. Treated fragments were combined and directly transformed into E.coli MACH1 strain. Clones containing the correct insert were identified by colony PCR and confirmed by sequencing. The GFP tagged triple BRPF1 bromodomain constructs was constructed by Gateway LR recombination reaction between pcDNA™6.2/N-EmGFP-DEST (Invitrogen, cat # V356-20) and an entry clone encoding for a wild type or mutated triple bromodomain constructs.

Transfection and preparation of cells

The plasmids encoding for BRD4 and CECR2 fused with GFP were from reference [4]. Cells were reverse transfected 24 hours before a FRAP experiment. For each FRAP condition, 0.2 µg of DNA and 0.5 µl of Lipofectamine 2000 (Life Technologies, UK) were each diluted in 50 µl of OptiMEM (Life Technologies, UK) and then thoroughly mixed before incubating for 20 minutes at room temperature. During the incubation period, cells were trypsinized, resuspended in antibiotics-free culture medium and counted. For each FRAP condition, a lipofectamine-plasmid complex was mixed with 150,000 cells and seeded into a well of a 24-
well optical grade flat-bottom plate (Thistle Scientific, UK). Only the left half of the plate was seeded starting with the WT DMSO control at the upper left corner (A1). The plate was incubated at 37 degrees and 5% CO₂ for 6 hours, where the medium was later changed to fresh McCoy’s 5A medium with HEPES supplemented with 10% foetal calf serum (Life technologies, UK), and 1% GlutaMAX (Life technologies, UK) and containing SAHA at 2.5 µM before incubating the plate overnight at 37 degrees and 5% CO₂. 500 µl of this medium was also added to the corresponding compound wells on the right of the plate in advance to allow the temperature and pH to reach equilibrium.

The microscope incubator should be switched on and left overnight to reach 37 degrees Celsius prior to the FRAP experiment. For added temperature stability, several bottles of water were placed inside the incubator to increase the heat latency.

**Frapid configuration profiles**

Table 1. Acquisition parameters for Tilescan

| Protein name | BRD4 | CECR2 | BRPF1 | SPIN1B |
|--------------|------|-------|-------|--------|
| Configuration name | tile BRD4 | tile cecr2 | tile BRPF | tile SPIN1 |
| Detector gain | 700 | 760 | 720 | 740 |
| offset | 0 | 0 | −20 | 0 |
| Channel 1 bandpass | 500-550 | 500-550 | 500-550 | 500-550 |
| dichroic filter | 488 nm | 488 nm | 488 nm | 488 nm |
| resolution | 1024x1024 | 1024x1024 | 1024x1024 | 1024x1024 |
| average | 2 | 2 | 2 | 2 |
| zoom | 1 | 1 | 1 | 1 |
| bidirectional | yes | yes | yes | yes |
| laser wavelength (nm) | 488 | 488 | 488 | 488 |
| power (%) | 1.5 | 2 | 1 | 1 |
| pinhole (A.U.) | 52 | 185.1 | 83.2 | 83.2 |

Table 2. Acquisition parameters for Imagescan

| Protein name | BRD4 | CECR2 | BRPF1 | SPIN1B |
|--------------|------|-------|-------|--------|
| Configuration name | Image BRD4 1, 2, 3 | Image cecr2 1, 2, 3 | Image BRPF 1, 2, 3 | Image SPIN1 1, 2, 3 |
| Detector gain | 630, 700, 770 | 650, 700, 750 | 700, 750, 800 | 700, 750, 800 |
| offset | 0 | 0 | −10 | −10 |
| Channel 1 bandpass | 500-550 | 500-550 | 500-550 | 500-550 |
| dichroic filter | 488 nm | 488 nm | 488 nm | 488 nm |
| resolution | 512x512 | 512x512 | 512x512 | 512x512 |
| average | 1 | 1 | 1 | 1 |
| zoom | 6 | 6 | 6 | 6 |
| bidirectional | no | no | no | no |
| laser wavelength (nm) | 488 | 488 | 488 | 488 |
| power (%) | 0.4 | 1.1 | 0.5 | 0.5 |
| pinhole (A.U.) | 133.1 | 186.1 | 83.2 | 83.2 |
Table 3. Acquisition parameters for FRAP

| Configuration name | BRD4  | CECR2  | BRPF1  | SPIN1B |
|--------------------|-------|--------|--------|--------|
| Detector gain      | 1, 2, 3 | 1, 2, 3 | 1, 2, 3 | 1, 2, 3 |
| Offset             | 0     | 0      | −10    | −10    |
| Channel 1 bandpass | 500-550 | 500-550 | 500-550 | 500-550 |
| Resolution         | 512x512 | 512x512 | 512x512 | 512x512 |
| Average            | 1     | 1      | 1      | 1      |
| Zoom               | 12    | 12     | 12     | 12     |
| Bidirectional      | yes   | yes    | yes    | yes    |
| Laser wavelength (nm) | 488  | 488    | 488    | 488    |
| Power (%)          | 0.4   | 1.1    | 0.5    | 0.5    |
| Pinhole (A.U.)     | 133.1 | 186.1  | 83.2   | 83.2   |
| Bleaching wavelength (nm) | 488  | 488    | 488    | 488    |
| Bleaching power (%) | 100  | 100    | 100    | 100    |
| Prebleach scans    | 5     | 5      | 5      | 5      |
| No. of timelapse frames | 240  | 30     | 75     | 50     |
| Time lapse interval (s) | 0    | 2      | 0      | 0      |

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