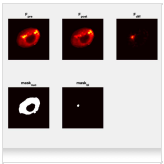

Apr 11, 2021

Fluorescence Recovery after Photobleaching (FRAP) in adult *C. elegans* nuclei

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1 Works for me dx.doi.org/10.17504/protocols.io.bpkymkxw

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ABSTRACT

Protocol for Fluorescence Recovery after Photobleaching (FRAP) in *C. elegans*.
The protocol includes the preparation of agarose pads, efficient mounting of worms, FRAP imaging, and an analysis pipeline.

DOI

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PROTOCOL CITATION

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KEYWORDS

FRAP, C. elegans, TF, Intestine, Nucleus, microscopy, mounting, agarose pad, confocal

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MATERIALS TEXT

- glass slides (Thermo Scientific™ SuperFrost Plus™ Adhesion slides)
- objective slides (high precision, no.1.5H, Marienfeld)
- agarose
- Pasteur pipette
- lab tape
- stereo-microscope
- well slide
- M9 (3.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.5 g NaCl, 1.0 g NH_4Cl. Bring to 1 L with H_2O.)
- levamisole
- silicone glue (picodent twinsil speed)
- SP8 with an HC PL APO 63x 1.3 NA glycerol objective (Leica)

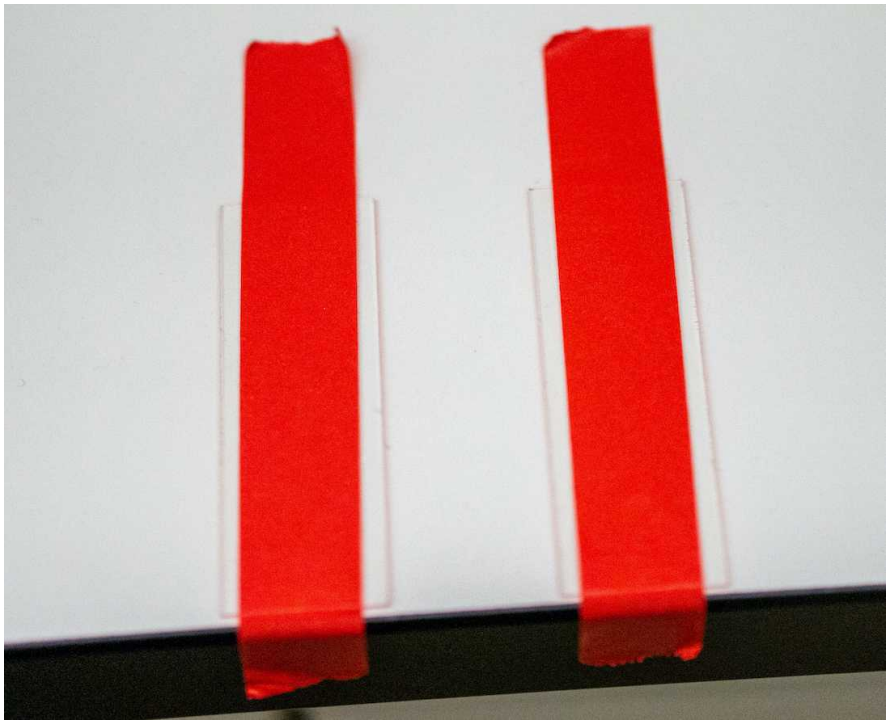
Preparation of agarose pads

1 Dissolve agarose in M9 to create [M]**10 % (w/v)** solution and slowly melt in microwave (lowest setting). Avoid extended boiling to not lose too much liquid through evaporation. Place closed bottle with molten agarose in a **65 °C** water bath.

Different concentrations can be used. High concentrations lead to more immobilization but also more deformation (Kim et al., 2013 Plos One).

Some time in the water bath will help reduce bubbles that may have appeared during melting. However, keeping the bottle too long in the water bath cools the agarose too much making it hard to handle

2 Prepare two glass slides as spacers by taping one layer of lab tape along the slides' long side. Tape them to the table, separated to leave space for one glass slide in between them.



Two slides with lab tape

3 Place a clean glass slide between the two spacers. Using a Pasteur pipette, drop a small amount of liquid agarose on the middle, clean glass slide, and immediately place another clean slide on top of the agarose, perpendicular to the other slides to flatten the agarose and gently but firmly press down. The top slide needs to touch the spacer tape to create the thin agarose pad.

4 Carefully remove the two slides forming a cross with the agarose pad in the middle and place them on the bench for a

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quick cool down. Continue preparing agarose pads by placing a new slide between the spacers.

5 Agarose pads can be prepared in advance and stored in an airtight box. Separate layers of pads with clean lab tissue soaked in M9 to prevent drying of the pads. Store the pads still between the two glass slides and only remove one of the slides just before mounting the sample. Slides can be stored for up to 4 weeks at **4 °C** but check for mold formation in the box.

6

Mounting worms on the agarose pad 24m

7 Grow worms to the desired stage, wash off using M9 and collect in an Eppendorf tube. For one 6 cm dish, use 2 ml M9 and collect in a 1.5 ml tube. 5m

8 Let worms settle in the tube. This step can be sped up by placing the tube for a short time at 4°C. Once the worms sunk to the bottom of the tube, remove the supernatant. Depending on the amount of bacteria in the solution, this step can be repeated using fresh M9. 10m

9 Transfer about 40 µl of worms to a well depression slide and add 10 µl of Levamisol (50 mM). Observe worms under a stereomicroscope to confirm the anesthetization of the worms. 5m

If the worms are really dense, the concentration of Levamisol might not be enough to anesthetize the worms.

10 Prepare the agarose pad by cutting off the pad's sides to create a slightly smaller square than the objective slide. Transfer the anesthetized worm onto the agarose pad and gently place a 1.7 µm thick objective slide (high precision, no.1.5H, Marienfeld) on top of the drop. Use lab tissue to remove excess liquid from the space between the pad and the objective slide. 2m

Leaving too much liquid is tricky. Worms will be able to move if there is too much liquid left on the pad. Removing too much will squish the worms. Place the slide under the stereomicroscope while removing liquid using the lab tissue. This way, it is possible to observe if the worms and make sure that the worms stopped moving but are also not broken.

11 Seal the objective slide and agarose pad with two-component silicone glue (picodent twinsil speed). Sealing the slide will prevent the evaporation of M9 during imaging and fix everything in place and avoid any movement of the agarose pad or objective slide. 2m

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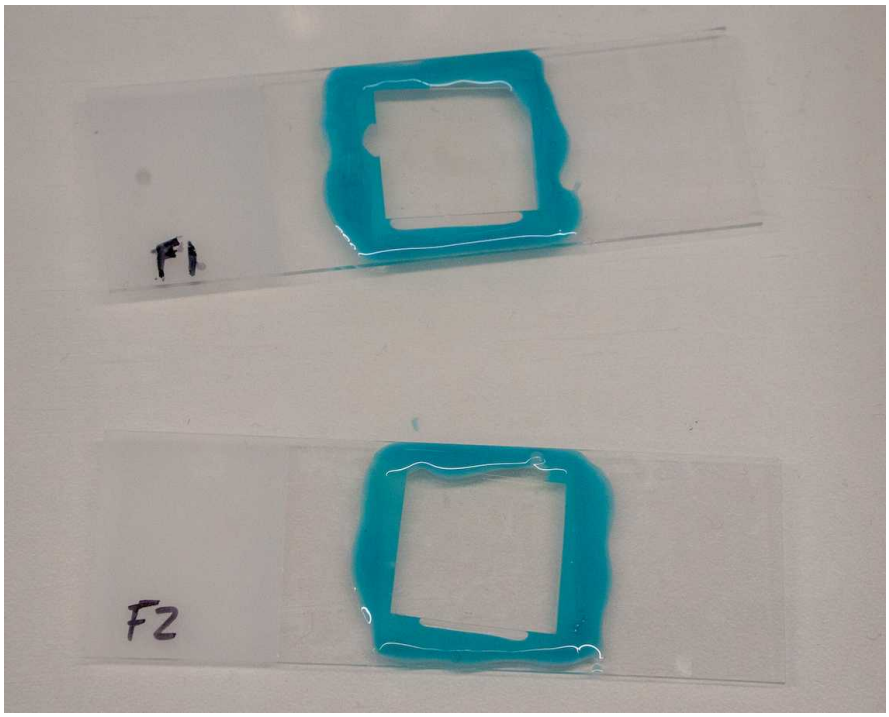

Two sealed slides

The silicone glue that dentists use is handy for this step. Nail polish is not as useful for this step as everything is quite wet, and nail polish dries slowly in these circumstances.

12

FRAP imaging

13

Setting up the microscope will depend on different microscope models. Here described is using a commonly used microscope, the scanning confocal microscope (Leica SP8) using an HC PL APO 63x 1.3 NA glycerol objective (Leica).

14 For wGFP, the white light laser is set to 482 nm with 10-15% laser intensity, and the emission detection is set to 488 - 520 nm with a HyD hybrid photodetector and gain of 160%.

15 The scan speed is set to 600 Hz, with bidirectional scanning (phaseX: 29.752) in a frame size of 256 x 256 pixels (Pixel dwell time 0,002425 s). The pinhole is set to 1 AU, and 7x digital zoom is used to zoom in to single intestine nuclei of young adult worms.

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16 For FRAP in the intestine nuclei, 20 pre bleach images are acquired, followed by a point bleach of 700 ms with 100% laser power and subsequent acquisition of recovery images (~500 images) using 10-15% laser power.

FRAP image anaylsis

17 The analysis is performed using a custom-written MATLAB (MathWorks) script and can be downloaded here: https://github.com/ercanlab/2021_Breimann_et_al

MatLab R2018b

Requirements for FRAP Matlab analysis:
The analysis script was developed and tested in Matlab R2018a on Mac OS 10.15.7.

The following Matlab toolboxes are required to run “FRAP_analysis.m”:
- curve_fitting_toolbox
- image_toolbox

The following scripts have to be in the same folder as “FRAP_analysis.m”
- tiffread2.m (by Francois Nedelec)
- struct2.csv.m (by James Slegers)
- matVIS.m (by S. Junek)
- dftregistration.m (by Manuel Guizar-Sicairos, Samuel T. Thurman, and James R. Fienup)
- timesteps.m
- timestepsArray.m

18 ***Select the data for analysis***
The first step after starting the script is to select the input folder with the raw FRAP images and an output folder for the analysis files. A window will pop up and you can navigate to the respective folders (first input, then output).

```
Choose data_tif directory
Choose matlab_results directory
```

Next, you need to select the FRAP dataset you want to analyze. The Leica SP8 creates two image stacks per FRAP experiment, one before the bleach point and one after the breakpoint. First, select the dataset before the bleach and then click on the post-bleach image stack.

```
Load pre-stack
Load post-stack
```

19 ***Manually outline the cell nucleus***
To select only one nucleus for FRAP analysis, you can draw a **ROI**. For that, a window will open with a filtered post bleach image (mean of the first three images) and just start outlining the nucleus by clicking in the image. Once you are

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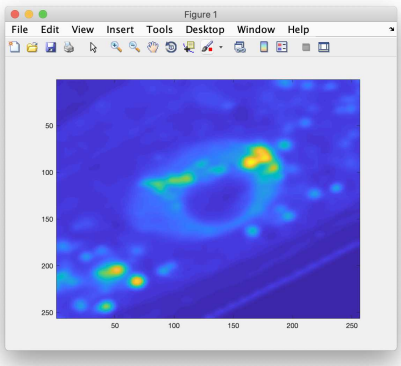
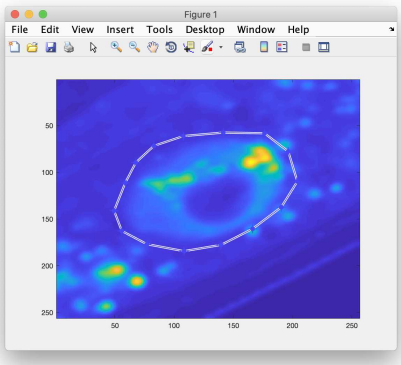
satisfied with the outline, double click in the middle of the selected ROI to accept it.



C. elegans intestine nucleus

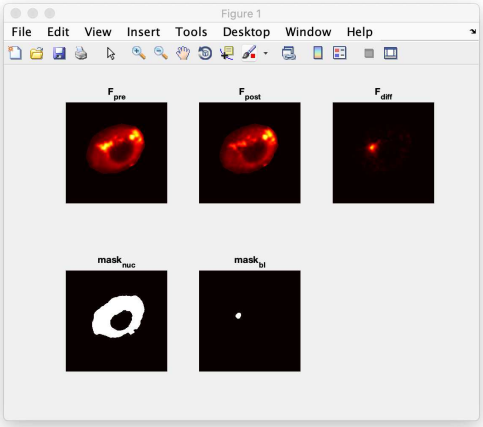


ROI selected nucleus

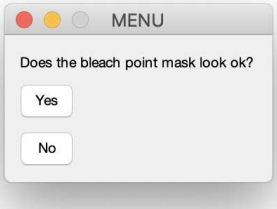
20 *Automatically detect the bleach point*

The next step automatically detects the bleach point by automated thresholding (Otsu's Method) of an image of the difference of the mean pre-bleach images and the mean of the first five post-bleach images.
A window will appear with the pre-bleach ($\mathbf{F_{pre}}$) and post-bleach ($\mathbf{F_{post}}$) images and the difference between the two images ($\mathbf{F_{diff}}$). The lower row depicts the selected mask ($\mathbf{mask_{nuc}}$) from the previous step (thresholded) and the mask for the bleach point ($\mathbf{mask_{bl}}$) based on $\mathbf{F_{diff}}$ above.

At this point, there is the option to change the threshold for the bleach point selection. Simply press **No** in the second window and write a value between 0-1in the Command Window(the starting point is 0.6), and press enter. If you are happy with the bleach point detection, press **Yes**. The displayed overview image is saved as [XX_mask.tif] to the previously selected output folder.



Display of nuclear and bleach point mask



Window to accept the bleach point

21 *Inspect the results*

The following steps are automatically executed and will save the results to the previously selected output folder:

The file [XX_bleaching_correction.tif] shows the correction for the **acquisition bleaching**. Acquisition bleaching is detected in the mean intensity of the whole nucleus region of interest in the post-bleach images. This decrease in intensity is fitted with a monoexponential decay and used to correct the acquisition bleaching during fluorescence

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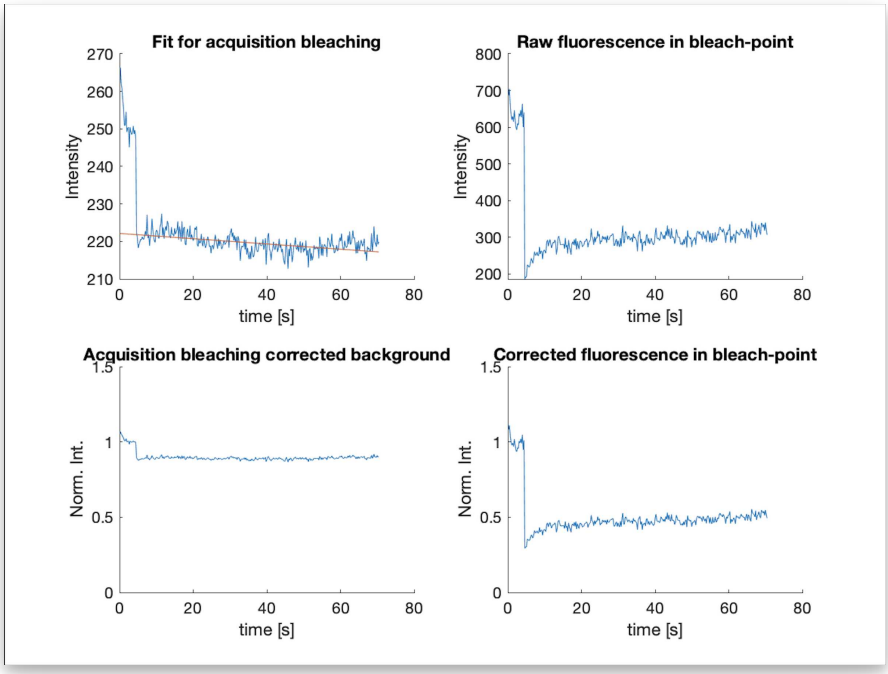
recovery. To correct for differences in initial intensity and extent of photobleaching, such that different datasets could be directly compared, each acquisition bleaching corrected curve is then normalized to an initial value of 1.



Plots for acquisition bleaching correction

22 The fitted and **normalized recovery curve** is saved as [XX_recovery.tif] to the output folder. The graph displays the normalized fluorescence in the whole nucleus (red) and the bleach point recovery (black) fitted with a monoexponential function with nonlinear least-squares-based fitting. The immobile ($\mathbf{f_{im}}$) and mobile fractions ($\mathbf{f_{mo}}$) are displayed in the image. The recovery time constant (**?**) and t-half ($\mathbf{t_{0.5}}$) values from the fit of the curve. The fitting of the curve can only be changed directly in the MATLAB script (Section 7).

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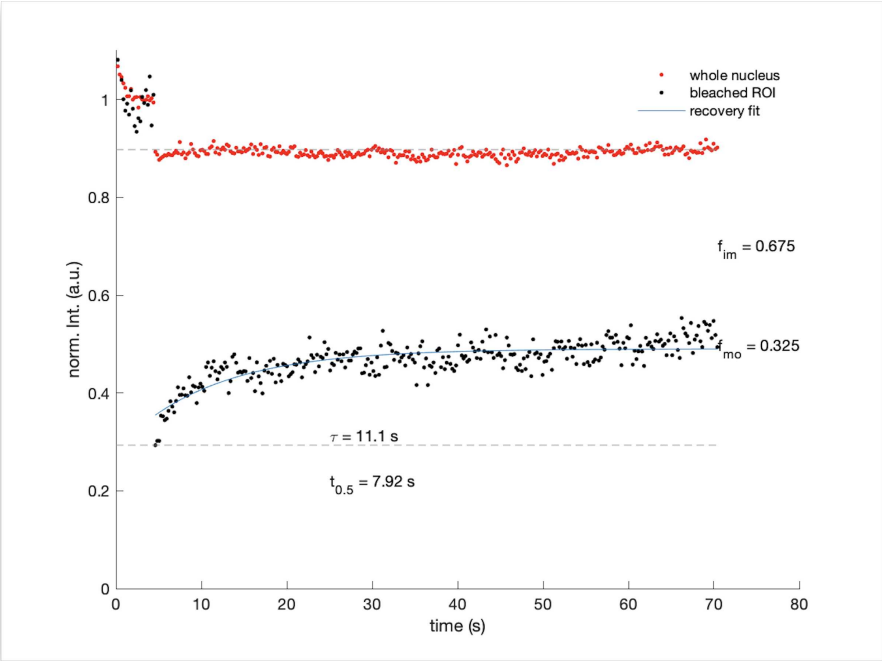

Normalized recovery curve

23 To check how well the fit describes the observation, a set of goodness of fit values is saved in the [XX_gof.csv] file in the results folder. It contains different statistics:

- The sum of squares due to error (**SSE**) (values closer to 0 are good)
- **R-square** (values closer to 1 are good)
- Degrees of Freedom (**DFE**)
- **Adjusted R-square** (values closer to 1 are good)
- Root mean squared error (**RMSE**) (values closer to 0 are good)

These values can be used to select the best fit or filter data.

DPY-27_gof

sse	rsquare	dfe	adjrsquare	rmse
0.17386	0.64402	297	0.64162	0.024195

24 For further analysis and averaging of different experiments, the normalized values for the FRAP curve, and the tau value and percent of the immobile and mobile fractions are saved to the file [XX_pyan.txt]. The data structure is as follows: the first value is the tau-value, then the mobile fraction and the immobile fraction. From the 4th value on, the normalized FRAP recovery values are listed.

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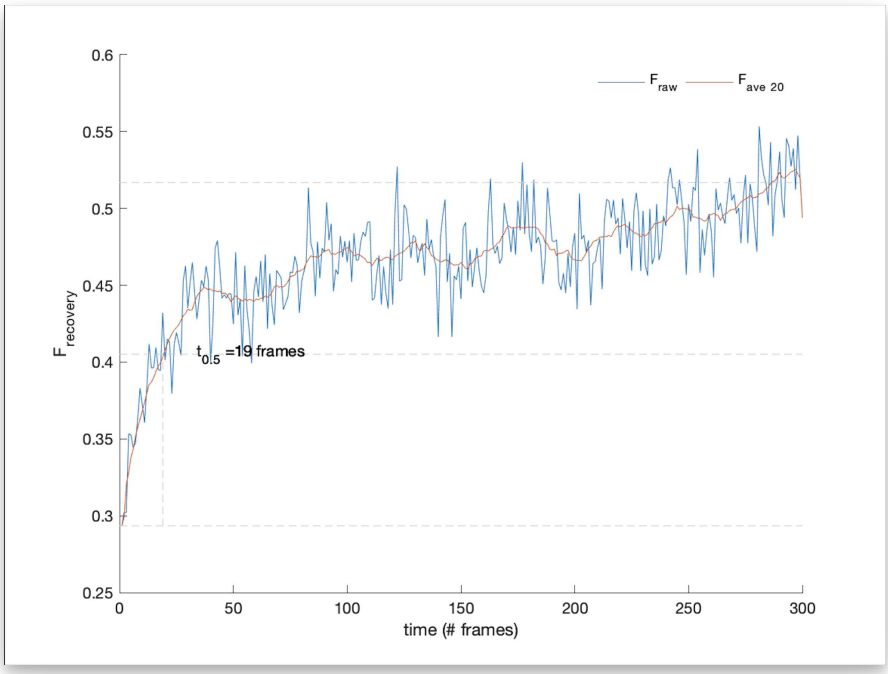
DPY-27_pyan.txt

```
11.105604
0.325330
0.674670
1.081505
1.109774
1.040203
1.000913
0.977532
0.991800
0.969363
1.018980
0.981383
0.945939
0.934449
0.962033
0.955650
1.005550
0.993130
1.019749
0.989532
1.047338
0.947250
1.009947
0.293376
0.302209
0.302165
0.353501
0.352501
0.344462
0.347372
```

25 The t-half value is calculated by two different approaches.
Firstly, using the fit of the recovery curve the fluorescence intensity at the half-maximum timepoint of the fit is calculated and saved as [XX_t_half_value_from_fit.txt].
Secondly, the more direct way is to calculate the fluorescence intensity at the half-maximum timepoint without using the fit. A visual representation of this can be found in the image [XX _thalf_no_fit.tif] and the estimated value in [XX t_half_value_no_fit.txt]



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