PhotoBleachingCorrector: Automated photobleaching correction for time lapse fluorescence imaging

Alexander Epstein
Yale University
alexander.epstein96@gmail.com
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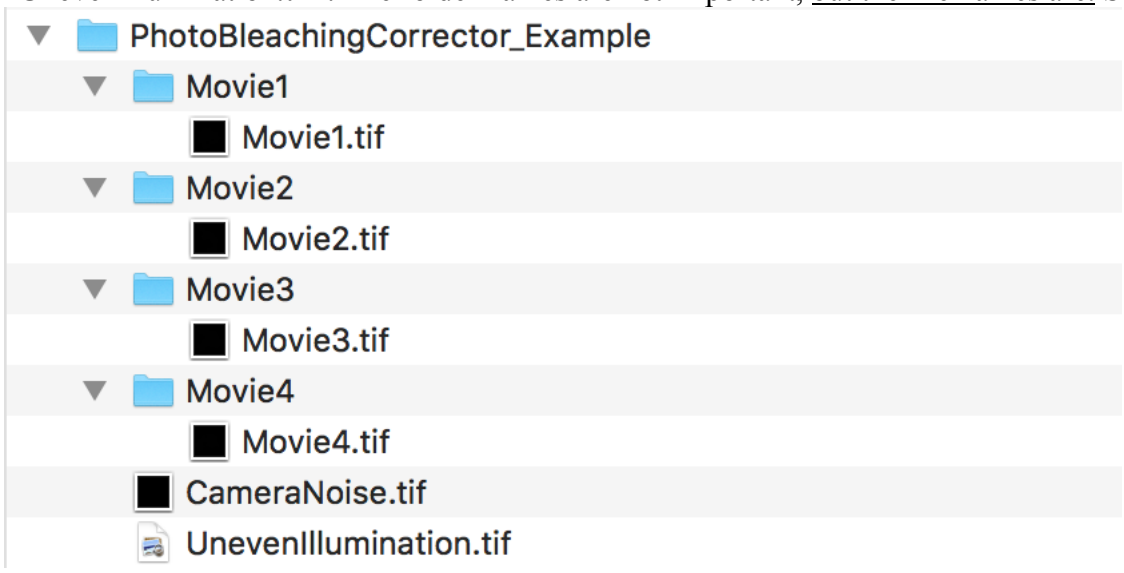
This software was designed to automate photobleaching correction for time lapse movies of Fim1-GFP labeled actin patches in *Schizosaccharomyces pombe* for the PatchTrackingTools plugin (Berro and Pollard, 2014). However, it can correct photobleaching for a wide variety of fluorescence time lapse movies, and need not necessarily be used with PatchTrackingTools.

The software works by tracking the median brightness of all of the pixels that are within cells over time, as for most fluorescent proteins, the median will represent the cytoplasmic fluorescence. It fits a curve to this data, normalizes the brightness curve such that the first frame is 1, and then divides each frame in the movie by the curve. (Epstein et al., 2018, Fig. S4C-H).

Installation: After installing the appropriate version of MATLAB, add all of the .m files (Epstein_PhotobleachingCorrector.m, loadTiffStack.m, and GaussFit2D.m) to your MATLAB folder.

Setting up time lapse movies for processing: Place each of the movies you want to correct in its own individual folder, and then place those individual folders within a parent folder. The parent folder should also contain a camera noise image (one Z-slice), called "CameraNoise.tif" and an uneven illumination correction image (for which the maximum value should be 1), called "UnevenIllumination.tif". The folder names are not important, but the file names are. See below:



Before running the program, make sure to make a copy of your parent folder! You should always have an unprocessed copy of all of your data.

Running the program: In MATLAB, open and run "Epstein_PhotobleachingCorrector.m". A dialog box will appear with the following options:

- **Number of Z-sections:** Set this to the number of Z-sections in your image stack.
- **Curve fitting model**: This setting controls the type of curve that is used to fit the photobleaching data. We found that photobleaching of GFP-tagged proteins in *S. pombe* is best fit by a double-exponential function ("exp2"), where one term represents GFP bleaching and the other term represents corresponds to bleaching of cytoplasmic autofluorescence. Other options include a single-exponential function ("exp1"), and using the raw data without fitting a curve ("none").
- **Enter 1 to create fake images for patch tracking program**: The PatchTrackingTools program begins by correcting uneven illumination and camera noise, but this program makes such corrections redundant. If using the PatchTrackingTools program, enter 1 here to generate a fake uneven illumination image (all ones) and a fake camera noise image (all zeros) to prevent this.
- **Enter 1 to correct noise and uneven illumination**: If your images are already corrected for camera noise and uneven illumination, set this to 0. In this case, you do not need the "CameraNoise.tif" and "UnevenIllumination.tif" images to be in the parent folder.
- **Enter 1 for automatic segmentation:** Identifies intracellular regions automatically by thresholding, using one and two-term Gaussian fits to an image brightness histogram to determine the threshold (Epstein et al., 2018). If you want to segment some or all images manually (or not at all), set this box to 0. Generally it is recommended to try automatic segmentation first and then switch to manual segmentation should it fail.
- **Enter 1 to overwrite existing segmentations:** Once an image has been segmented previously (either automatically or manually), the segmentation is saved as a .tif stack. If the software is run again, the previous segmentation will be used. If the previous segmentation did not work well, enter 1 here to re-do segmentation.

  Note: if you want to redo segmentation for only some images, open the folders for the images you want to re-segment and delete the file ending in "_CellSegmentation.tif".
- **Enter 1 to save corrected images:** Corrected movies will be saved only if you enter 1 here.
- **Enter 1 to overwrite images:** If set to 0, the corrected image will have the same filename as the original image except with "_Corrected.tif" at the end. However, you enter 1, the corrected movie will be saved with the same filename as the uncorrected movie. The uncorrected movie will be preserved; it will be the image with "_Uncorrected.tif" at the end of its filename. It is recommended to set this to 1 if using the PatchTrackingTools program, since the filename of the time lapse movie is important for this program.
- **Fraction of uneven illumination image to keep:** Poorly illuminated parts of the field bleach at a slower rate than well-illuminated areas, and standard uneven illumination correction processes cannot remedy this. Therefore, this setting is used to crop the least-illuminated areas of your movies. It will crop any part of the field that is dimmer than the

indicated fraction of the maximum value of your uneven illumination image (which is usually 1). For example, if this setting is 1, none of the field will be cropped. If it is 0.5, then any area of the uneven illumination image which has less than 50% of the brightness of the center will be cropped from all time lapse movies.

If you want to crop your image manually to exclude a certain region, you can create a binary image mask using ImageJ. Areas of the field you want to keep should have value 1 while areas you want to crop should have area 0. Save this mask in the parent folder with the filename "imageMask.tif".

Note that if you run all or part of the segmentation process on a parent folder and then change this value, you will need to delete the file "imageMask.tif" in the parent folder in order for your change to take effect.

- **Number of frames to use for segmentation:** If this setting is greater than 1, then several consecutive frames will be segmented (if using automatic segmentation. Only pixels that appear intracellular in a certain fraction of these frames (see Fraction of test frames in which an intracellular pixel must be detected, under advanced settings) will be considered intracellular. Increasing this number generally increases the quality of the segmentation but also increases processing time proportionally. Note that this setting has no effect if using manual segmentation.
- **Number of timepoints at which to perform segmentation:** This setting should be 1 unless your cells move during the time lapse movie, in which case you can increase this number to redo the segmentation at additional timepoints. Increasing this number will. The spacing between segmentations must be less than or equal to than the number of frames to use for segmentation (i.e. if the number of frames to use for segmentation is 10, and there are 60 frames in a movie, segmentation can be performed at no more than 6 timepoints). Increasing this number will increase processing time proportionally.
- **Enter 1 for advanced segmentation settings:** See below.

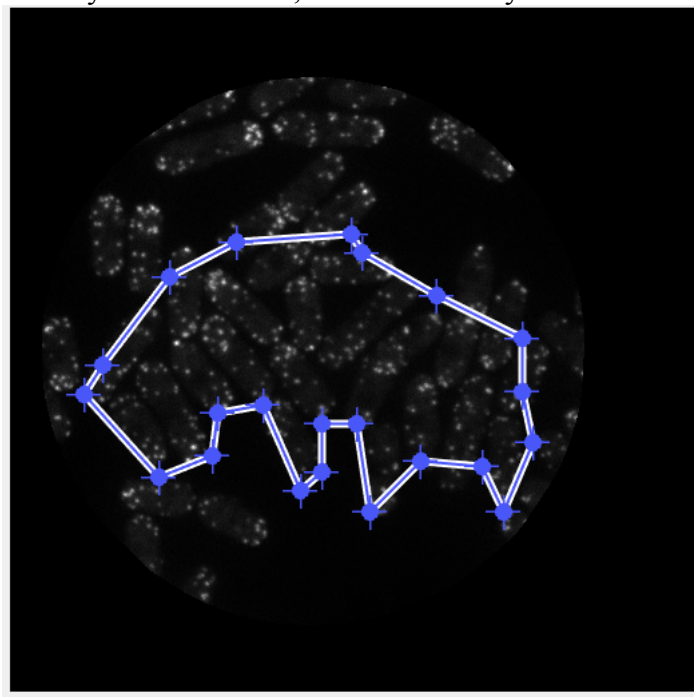
Advanced segmentation settings (optional—change these only if segmentation fails):

- **Segmentation threshold:** This number represents the fraction of pixels that are thought to be intracellular (based on the brightness histogram) at the threshold chosen for segmentation. Changing this number moves the segmentation threshold (vertical line on the pixel brightness histogram). Increasing it moves the line to the right; decreasing it moves the line to the left.
- **Rescaled image width for pixel brightness histogram:** To save computing time, the image is scaled down. Increasing this number will markedly increase the time that it takes to process images, and is generally not recommended.
- **Fraction of test frames in which an intracellular pixel must be detected:** A pixel must appear intracellular in this fraction of segmented frames (see Number of frames to use for segmentation) in order to be marked as intracellular.
- **Bin size for histogram used for segmentation:** Controls the bin size of the pixel brightness histogram. Increase if the histogram does not have enough data points; decrease if there are too few pixels in each data point on the histogram.

The program will now prompt you to select a folder. Be careful to select the parent folder! The program will make changes to the images in the folder you select which may not be easily reversible.

After selecting a folder, if you chose to segment images automatically, the program should run to completion without further input. When it has finished, it will output "All done!" in the command window. Do not close, minimize or deselect MATLAB while the program is running.

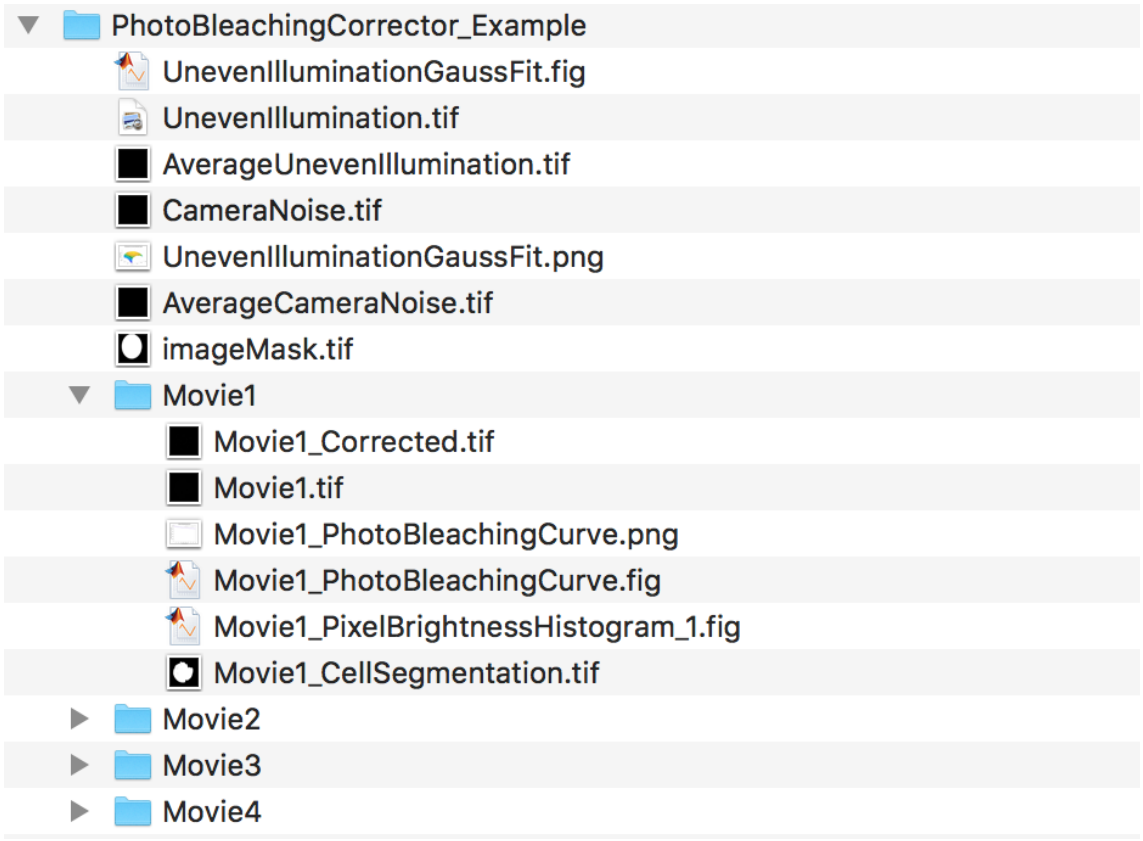
If you did not choose to segment images automatically, the program will ask you for each image whether you want to segment it automatically, manually, or not at all. If you select "Manually", you will have to perform the segmentation yourself. An image should appear at this point within a new figure. Click once in the figure to begin drawing a polygon around a region containing all or mostly cells. Additional clicks define new vertices of the polygon. Close the polygon by clicking again on the first vertex. At this point, you may drag vertices to adjust the polygon. When you are finished, double click anywhere on the image to save your segmentation.



Example of good manual segmentation (before double-clicking).

Note that unlike in many photobleaching correction programs, the segmentation can be very rough. This program only measures the brightness of the *median* intracellular pixel. Any pixels within your polygon that are not intracellular will be much dimmer than the median, and will be ignored if there are not too many of them. Also, it is not important or even desirable to separate individual cells.

After running the program: Your parent folder and individual image folders should now contain several additional files:



Parent folder:

- "ImageMask.tif" is the binary mask used to crop poorly illuminated areas. Black areas (0) will be cropped while white areas (1) will be retained.
- "UnevenIlluminationGaussFit.png" (image) and "UnevenIlluminationGaussFit.fig" (MATLAB figure) show the 2D Gaussian fit to the uneven illumination image that was used to generate the image mask.
- "AverageCameraNoise.tif" and "AverageUnevenIllumination.tif" are the fake camera noise and uneven illumination correction images generated for the PatchTrackingTools software.

Individual image folders:

- If you chose to overwrite existing images, your photobleaching-corrected time lapse movie will have the same name as the original. The original will be saved as a file ending in "_Uncorrected.tif".
- If you did not choose to overwrite existing images, the photobleaching-corrected time lapse movie will have a filename ending in "_Corrected.tif".
- The files ending in "PhotoBleachingCurve.fig" and "PhotoBleachingCurve.png" show best-fit curve to the brightness of the median intracellular pixel over time. The fit equation is given in the top right.
- The file ending in "_CellSegmentation.tif" shows the results of the automated or manual segmentation process. White areas (1) were marked as intracellular; black areas (0) were

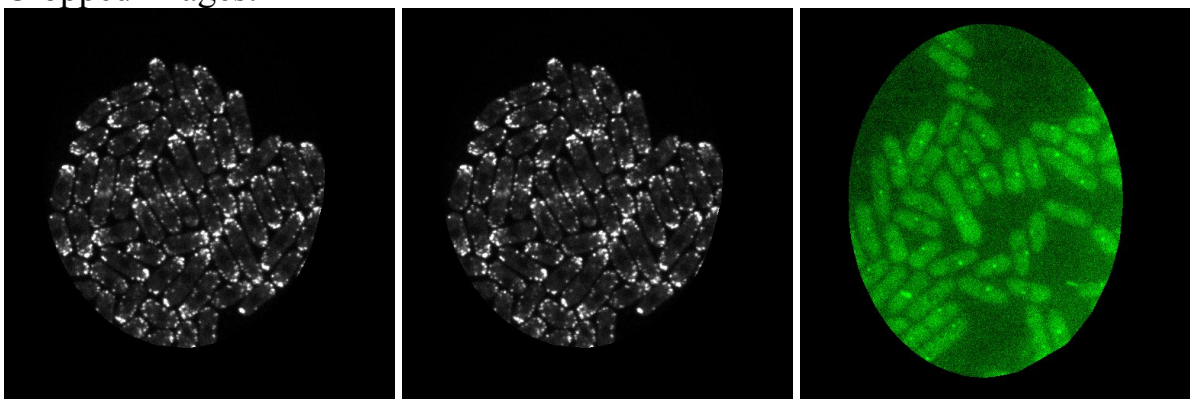
not. The .tif stack should contain one image for each timepoint at which segmentation was performed.
- If automated photobleaching correction was employed, there will be several "PixelBrightnessHistogram" files which show the Gaussian fits used to calculate the threshold for segmentation (Epstein et al., 2018).

Open your corrected image in ImageJ, take a sum projection, and scroll through the frames to see whether the projection appears to have been corrected for photobleaching. A good result means that the intracellular brightness appears uniform throughout.

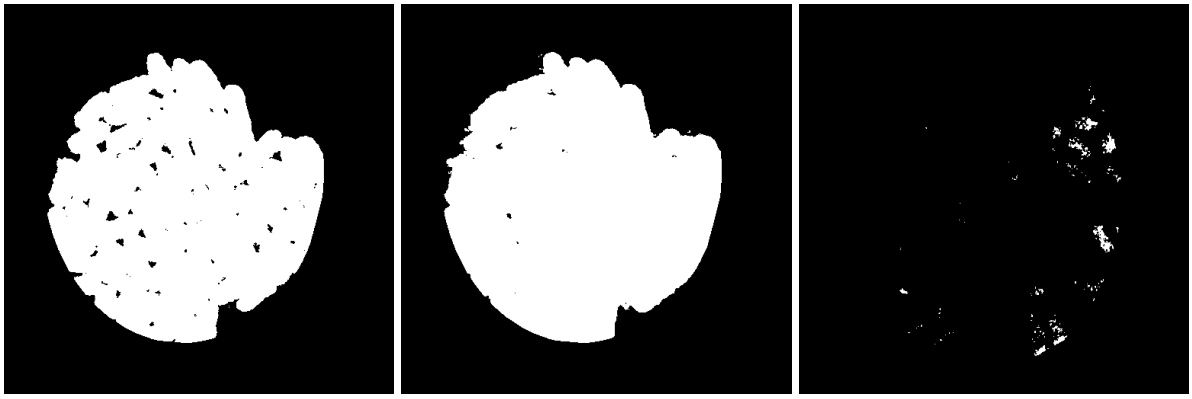
Troubleshooting: Many movies that ultimately can be corrected will not be able to be corrected using the default settings.

- *I received an error code during the segmentation process*.
    - Make sure that all important filenames, such as "CameraNoise.tif" and "UnevenIllumination.tif" are correct.
    - Make sure your folder architecture is correct.
    - "Image type not recognized: could not save."
        - Make sure your image is a 16 or 32-bit .tif file, where brightness values are stored as floating points, integers or unsigned integers.
- *Photobleaching correction fails: the area inside the sample gets dimmer or brighter over time, and/or the photobleaching curve does not fit the data well.*
    - Check the image segmentation by opening the "_CellSegmentation.tif" file. Does the segmentation appear reasonable? The segmentation should include nearly all of the area inside your sample and up to a moderate amount of the area outside your sample. Examples of acceptable and unacceptable segmentation are provided below. If segmentation works poorly, see "Segmentation is poor" sections below.

Cropped images:



Binary segmentations:



Good segmentation Acceptable segmentation Poor segmentation

  - Do your cells move significantly during the course of imaging? If so, you may need to increase the number of timepoints at which segmentation is performed. You may need to decrease the number of frames to use for segmentation in order to allow this.
  - Do you have enough median brightness data points to fit a curve? If this is the issue the PhotoBleachingCurve file should reveal that the fit function does not match your data well. If you have only a small number of frames, try using "none" for your fit equation.
  - It is possible that the rate of photobleaching is not constant, for example if the laser power/exposure time is not consistent between your images. Again, if this is the issue the PhotoBleachingCurve file should reveal that the fit function does not match your data well. Try taking new images or use "none" for your fit equation.
- *Segmentation is poor: too much of my image is marked as intracellular.*
  - Under "advanced settings", try increasing the segmentation threshold.
  - Under "advanced settings", try increasing the "fraction of test frames in which an intracellular pixel must be detected".
  - Look at the histogram file and increase/decrease the bin size as needed under "advanced settings".
  - If all else fails, perform segmentation manually. It is possible that your image is too dim
- *Segmentation is poor: too little of my image is marked as intracellular.*
  - Under "advanced settings", try decreasing the segmentation threshold.
  - Under "advanced settings", try decreasing the "fraction of test frames in which an intracellular pixel must be detected".
  - Look at the histogram file and increase/decrease the bin size as needed under "advanced settings".
  - If all else fails, perform segmentation manually.
  - Sometimes this will happen if the entire field is full of cells. In this case, select "No segmentation".
- *The segmentation appears noisy.*
  - Generally this should not affect your photobleaching curve too much, but try increasing the "number of frames to use for segmentation". Under advanced settings, try increasing or decreasing the "fraction of test frames in which an intracellular pixel must be detected".

- *Parts of my sample near the edges of the frame get brighter over time in the corrected time lapse movie.*
    - Poorly-illuminated areas bleach more slowly than well-illuminated ones. Since the program assumes that all areas of an image bleach at the same rate, poorly-illuminated parts can be overcorrected. Try lowering the "Fraction of uneven illumination image to keep" setting to crop out these areas. Make sure to delete the "imageMask.tif" file so that your new setting will apply.
- *The background area outside my sample gets brighter over time in the corrected time lapse movie.* Unfortunately, photobleaching decreases the signal-to-noise ratio of your images, making the sample dimmer relative to the background. When correcting for photobleaching, the sample brightness is held constant, so the background becomes brighter over time. This is an inescapable side effect of photobleaching correction. If the background is so bright as to mask your sample, then you should look for ways to minimize photobleaching during the imaging process.
- *The photobleaching curve appears flat, or seems to spike up and down at random without displaying a downward trend.*
    - Make sure that the movie you are trying to correct has not already been corrected for photobleaching. This is especially possible if you chose to overwrite images.
- *The program runs extremely slowly or does not finish*.
    - If it has not gotten past uneven illumination correction and created the "imageMask.tif" file, try making your own "imageMask.tif" file manually using ImageJ.
    - Under Advanced Settings, try decreasing the "Rescaled image width for pixel brightness histogram".
    - Try decreasing the "number of frames to use for segmentation" and the "number of timepoints at which to perform segmentation".

References

Berro, J., and T.D. Pollard. 2014. Local and global analysis of endocytic patch dynamics in fission yeast using a new "temporal superresolution" realignment method. *Mol Biol Cell*. 25:3501-3514.

Epstein, A.E., S.V. Espinoza Sanchez, and T.D. Pollard. 2018. Phosphorylation of Arp2 is not essential for Arp2/3 complex activity in fission yeast. *bioRxiv*.