

ImageJ plugin for Bayesian analysis of blinking and bleaching

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The explosion of superresolution microscopy research over the last ten years has created a myriad of new techniques¹, all of which have distinct advantages and disadvantages. Localisation microscopy achieves a resolution improvement by taking multiple images, in each of which only a few fluorophores are emitting light². One of the recently developed variations on the localisation technique, Bayesian analysis of blinking and bleaching (3B analysis)³ allows analysis of data with multiple overlapping fluorophores in every image. For those willing to invest in the longer analysis time required by 3B, this offers the possibility of observing cell dynamics at superresolution, as demonstrated in the original paper.

The 3B software is currently supplied as a standalone program which must be compiled and installed before use, which limits its usage to those reasonably familiar with programming. The imaging community has greatly benefited from the existence of standard open source software packages, and ImageJ⁴ in particular has become a standard tool in laboratories which acquire microscopy images.

Here, we present an ImageJ plugin for the 3B software, which includes both the 3B analysis and software for performing a reconstruction. The plugin currently works with 32 bit ImageJ on Windows, and 32 or 64 bit ImageJ on Linux. The reconstructions are themselves ImageJ images and can be manipulated (for example by providing a color map, or computing linescans) in the standard way. This plugin will make 3B analysis accessible to a far wider range of users than before and will aid the exploration of how structures in cells change at the nanoscale in live cells. With a suitable sample and camera, data can be acquired using a standard widefield system³.

A typical workflow is shown in Figure 1. Note that the design of the plugin allows reconstruction to be performed on previous runs. This is important due to the relatively long runtime of 3B analysis and because it allows reconstruction of data produced by the original software. Instructions

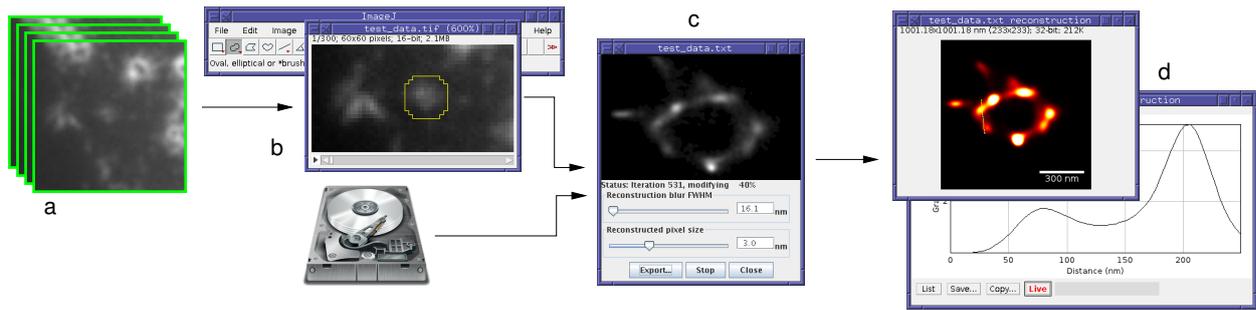


FIG. 1: 3B workflow using ImageJ: (a) load or capture image stack. (b) select area to analyse and run 3B, or load previous run. (c) select reconstruction parameters and export back to ImageJ. (d) Perform analysis of reconstruction using standard ImageJ tools.

for use are provided in the supplementary material, under ‘Help’ in the 3B plugin menu and in the user guide.

It should be noted that in order for the 3B analysis to converge, 200 iterations are typically necessary. Terminating the run before convergence makes it likely that an image will contain artefacts (as with any microscopy method, the experiment and analysis should always be carried out appropriately to minimise the risk of artefacts - guidelines are given in the user guide). The number of iterations is specified in the live 3B analysis window, as is an estimated time to convergence. For example, at a typical pixel size of 100 nm, a $1.5 \times 1.5 \mu\text{m}$ area with 200 frames in the stack would take 6 hours. The time required for analysis increases with the size of the area analysed, the number of fluorophores included in the model, and the number of frames in the stack being analysed.

Updated versions of the plugin will be made available at <http://3bmicroscopy.com>. We have also created a community site <http://superresolved.com> for people to discuss usage and issues, which we hope will become a generally useful facility to those who use 3B or other superresolution microscopy techniques.

ACKNOWLEDGEMENTS

We would like to thank Jason Rogers, Daniel Matthews and Thomas Iskratsch for beta testing the plugin and Michael Schmid and Curtis Rueden for providing advice and support on ImageJ integration. Work in the GEJ laboratory is supported by the MRC (G1100041); SC is supported by a Royal Society University Research Fellowship.

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ER wrote the ImageJ plugin for 3B, SC and GEJ advised on design for usability. SC and ER wrote the paper, all authors edited the paper.

1. Instructions for using 3B

Analysing some new data

1. Load an image stack

Some test data can be loaded via Plugins > Three B > Load test data

The results should look something like this:



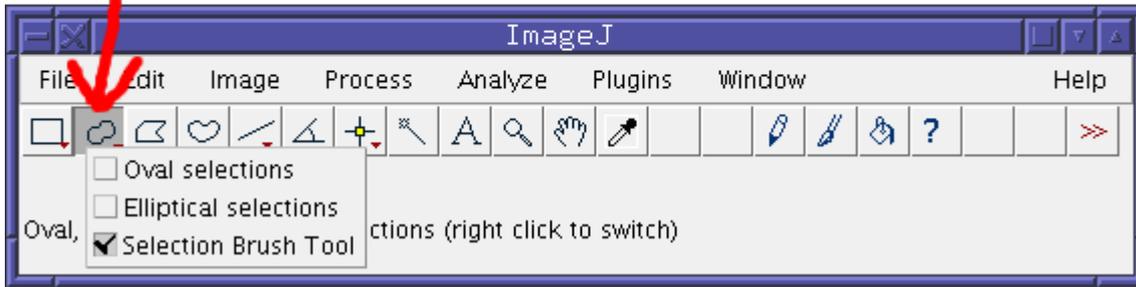
The image must contain significant areas which are not the thing you want to analyse. This is because 3B has to be able to estimate the background level and noise. A good guide is that anything larger than 50×50 will probably be fine.

2. Select a region to analyse

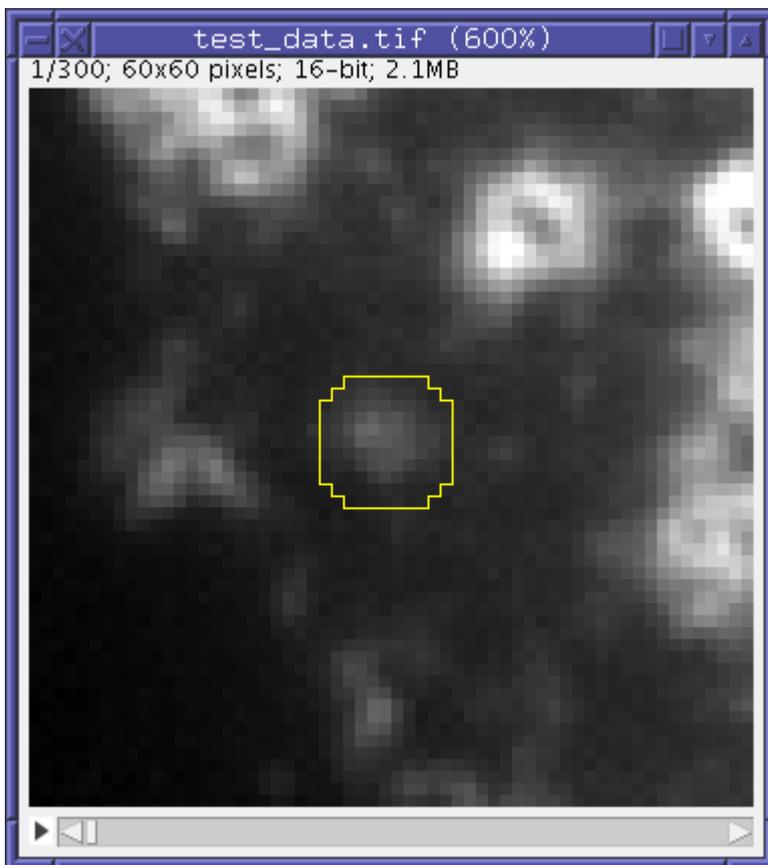
Select a region to analyse. Smaller regions analyse much faster, but you should leave a border of at least one or two pixels between the edge of the region and the pixels of interest. A large region can be cut up into several smaller regions if necessary.

Generally, the selection brush is the most useful tool for selecting regions:

Right click here and select the "Selection Brush Tool"



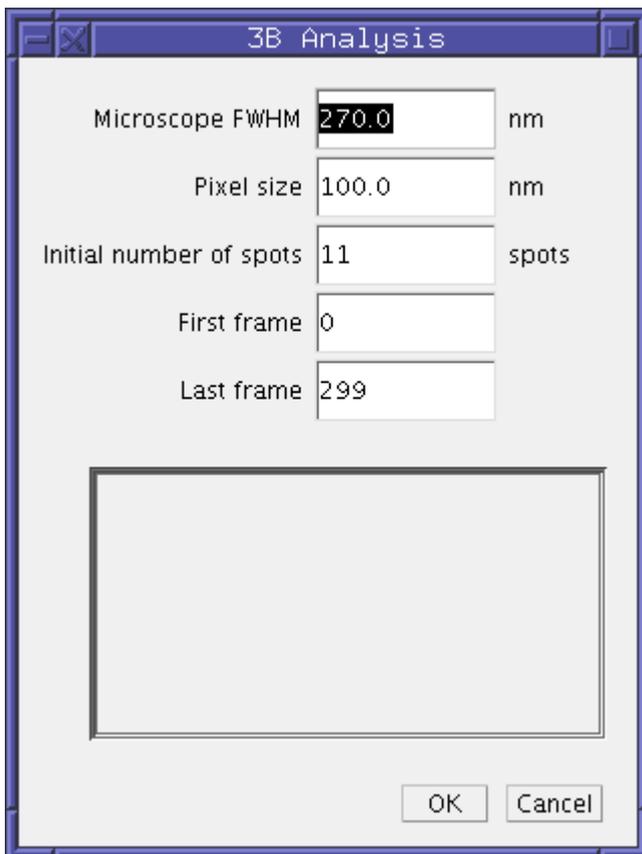
Then paint the area of interest. The results should look like this:



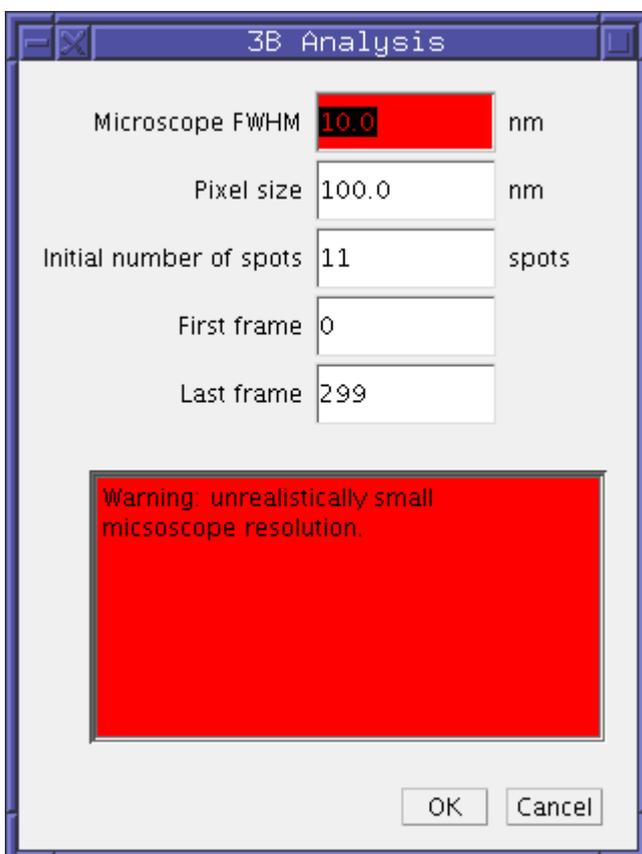
3. Run 3B analysis from the plugin menu

Activate the plugin from the menu `Plugins > Three B > Three B`. If you are reading this tutorial, you almost certainly do not want to select the advanced option. If you go for advanced options, it is easy to create meaningless results, or even crash ImageJ.

Set the pixel size and FWHM of the microscope. You can choose an initial number of spots, but the default value will probably be about right:



If you enter values which are outside the reasonable range of parameters, then the 3B plugin will highlight the bad value and give an error message:

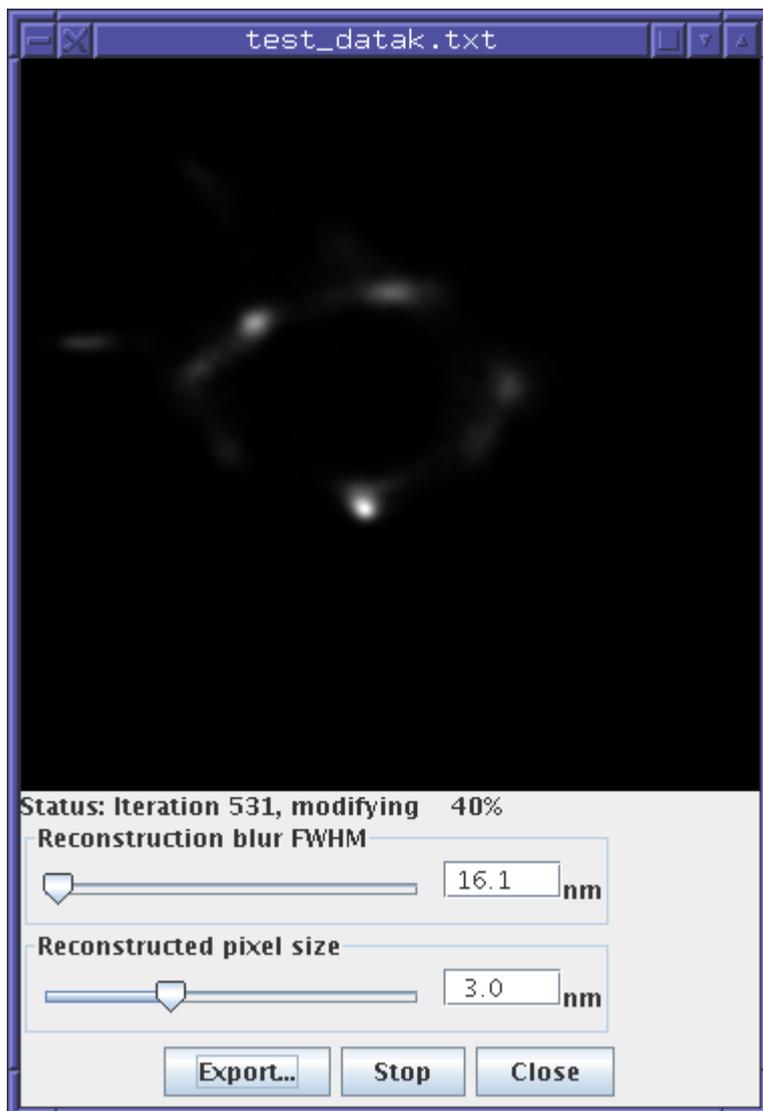


On pressing save, you will be invited to save the raw output of 3B from which a high resolution image can be reconstructed. This file is not an image but it can be loaded by the 3B plugin.

On pressing OK, the 3B plugin will start analysing the data.

4. Viewing the data

The 3B plugin will bring up its control panel:

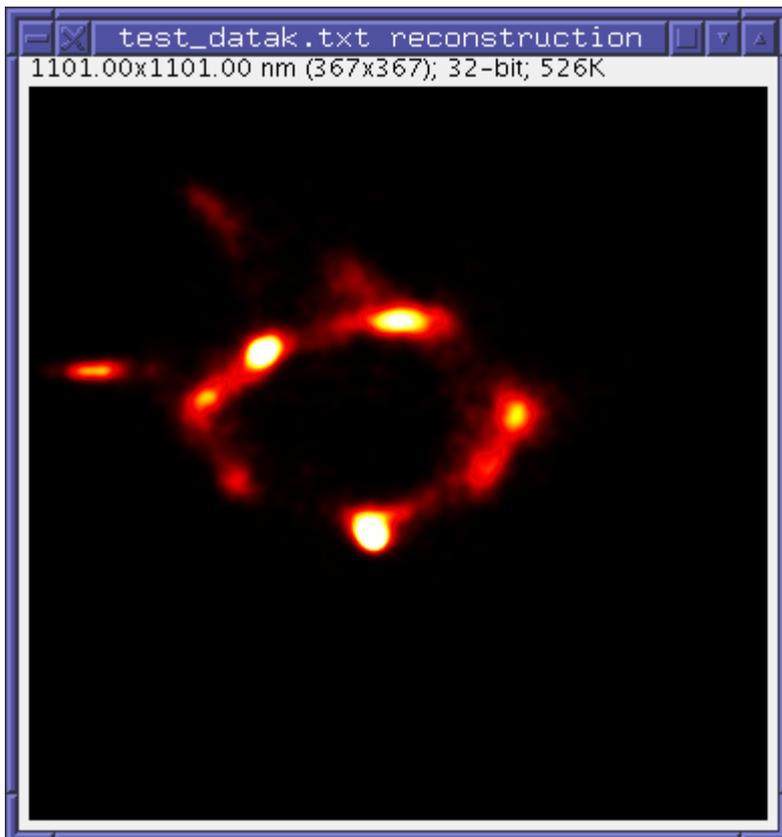


The control panel shows a live view of the reconstruction as it is running, and shows you which iteration the system is on. The control panel allows you to set the pixel size and FWHM of the reconstructed image. A larger number of iterations will allow you to use a smaller FWHM.

The 3B analysis will run for 1200 iterations which is almost certainly more than enough. The 3B run can be stopped when the reconstruction does not improve further. You do not need to save the data once it is stopped, since the output file is written continuously during the analysis.

5. Saving images, applying colourmaps, etc

At any stage during a 3B run or afterwards, you can press the "Export..." button to get an ImageJ image. Once you have this image, a colourmap can be added (for example Image > Lookup Tables > Fire) and the brightness and contrast can be adjusted (Image > Adjust > Brightness/Contrast) to enhance the clarity of the results. For convenience, the 3B plugin provides the glow/hot colourmap (shown here) under Plugins > Three B > Glow LUT You also may wish to add a scalebar (Analyze > Tools > Scale bar...). The image will already be calibrated to the correct pixel size.



2. Loading an old analysis

Load an old analysis using Plugins > Three B > Open 3B run. Some sample data has been provided with the plugin and can be loaded with Plugins > Three B > Open test data results. The view dialog is almost identical to the control panel for running 3B, and can be used in exactly the same way. For further instructions, see [1.4 Viewing the data](#). Note that you have to specify the pixel size when you load data from a file. This is because 3B uses the FWHM in pixels (not nm), so it does not make use of or record the pixel size in nm.