06/05/2017

DANA Installation Instructions

Link to video: DANA installation

1. Move the DANA folder to a location on your computer that is easily accessible for future use.

Fiji Setup and Plugin Installation

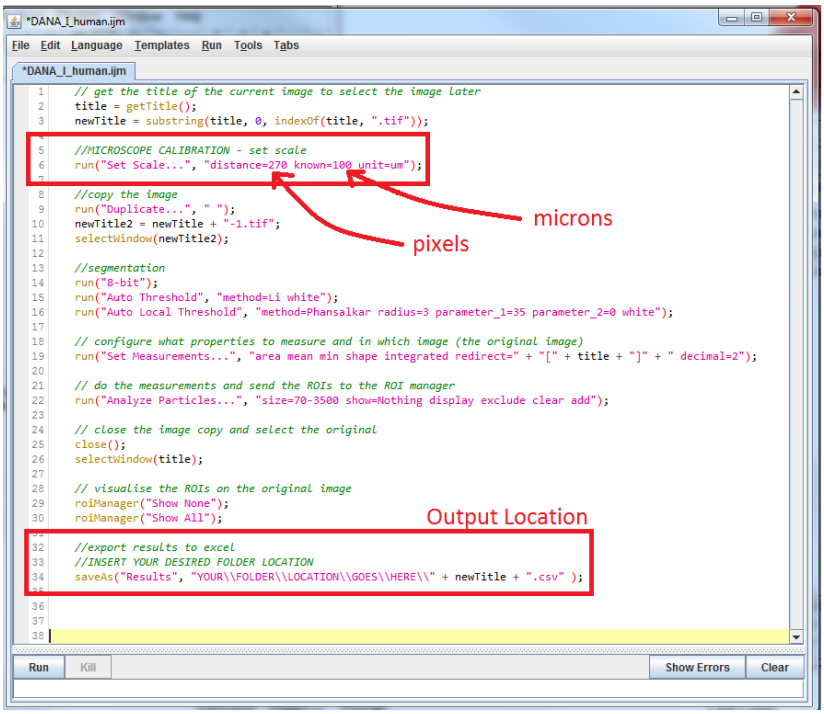
1. Download and install Fiji on your local drive from: https://imagej.net/Fiji/Downloads
2. Open a Fiji window.
3. Within the downloaded DANA folder, navigate to the Fiji Macros folder. Drag the DANA_I.ijm file into the open Fiji window. You will be changing the output location and microscope calibration.
    a. Microscope Calibration:
        i. Insert the proper ratio of pixels to microns specific to your microscope settings.

    *Note: DANA_I_template and DANA_I _batch_template have proper microscope calibration for template images

    b. Output Location:
        i. At the bottom of the file, it asks you to insert your desired folder location. This file path will be where all your initial Fiji output .csv files will be saved for downstream analysis. We recommend selecting the DANA_I_output folder present in the main DANA folder for a simple setup. This location will be referred to as Filepath1 in future steps. Be sure to use '\\' to separate locations within your file path.

    Ex. Filepath1: C:\\Users\\reberya\\Desktop\\DANA\\FIJI_output\\

    *Note: You can adjust the 'analyze particles' function minimum size if desired. DANA_I_template and DANA_I_batch template were increased to 70um^2 to better exclude fragments in the template images.



4. Repeat this process for all .ijm files within the Fiji Plugins folder.
5. Once a proper file path has been inserted, the macros must then be added to Fiji. Navigate to the location where you have installed Fiji on your computer. Then navigate to:

   Ex. Filepath: C:\.....\.....**Fiji**\Fiji.app\plugins\Scripts\Plugins\PUT_YOUR_.ijm_FILES_HERE

   Copy your modified .ijm files and paste them into this folder.
6. Restart Fiji, and verify the macros show up under the 'Plugins' dropdown menu of Fiji. Note: you may have to scroll to the very bottom of the dropdown menu to visualize the macros.

DANA II Environment Setup

1. DANA requires Java version 1.4.0 or later. Download/update java from: https://java.com/en/download/
2. Double click the DANA.exe file to launch DANA.

Using DANA

DANA is designed to first process images from a single sample in Fiji (DANA_I) and then pool and process the output from these images in DANA_II. A single sample is defined as all of the images associated with a particular subject/organism. DANA_I can be run sequentially, one image at a time, or as a batch, where a folder of images is selected and the DANA_I rapidly loops through all the images within that folder. If you are sure your images contain less than 7 overlapping cells, then the batch processing version of DANA_I should be used. The more overlapping cells present in a sample's images, the harder it is for DANA to accurately quantify NETosis. DANA_I exports data on the images to a user specified folder from which DANA_II draws from. **If this folder contains .csv files from multiple samples, then these .csv files will be processed together by DANA_II.**

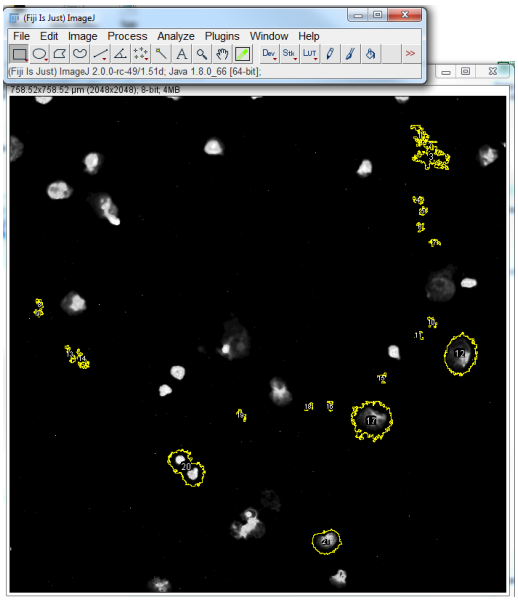
A sample set of images is available within the 'Template Images' folder of the main DANA folder. DANA_I_template and DANA_II's default parameters have been optimized to analyze these images. The Optional Parameter 'IO' can be used. This corresponds to neutrophils treated with ionomycin. The Summary.csv file provides information comparing between groups – in this case, ionomycin treated and untreated neutrophils.

Link to video: DANA Operation

To use DANA:

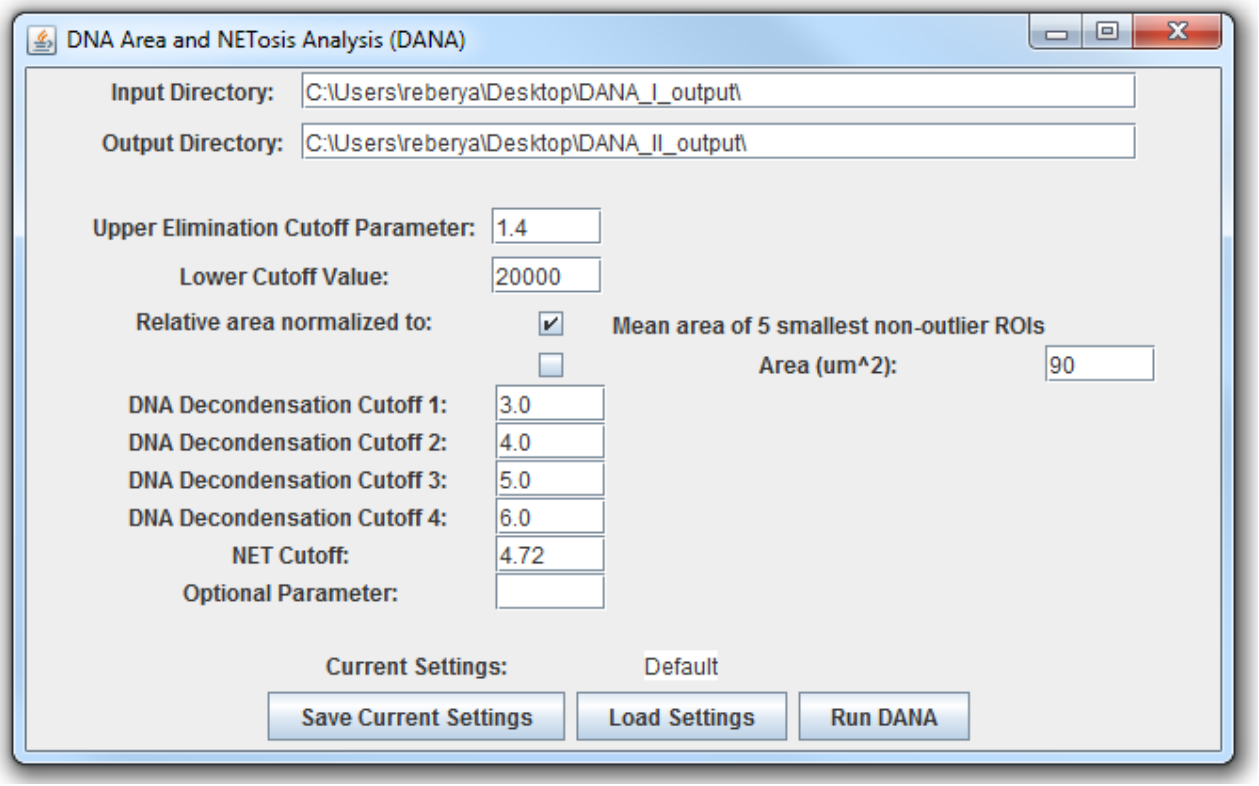
1. Open Fiji.
   a. If using batch processing, select the DANA_I_batch macro and then choose the folder containing your images of interest
   b. If using DANA on a per image basis, open all images from a sample in Fiji and select DANA_I from the Plugins menu.
      i. After running the macro on a single image, verify by eye that the image:

1. Does not contain more than 7 ROIs (yellow outlines) containing multiple cells. Checking/unchecking the 'Show all' checkbox within the ROI Manager window facilitates this process.
2. Accurately depicts the cells shown in the image. Rarely, when an image has an atypical exposure, DANA_I presents ROIs that are not representative of the cells in the image. An example is shown below.



   ii. If the image does contain 7+ ROIs with multiple cells or does not accurately represent the cells shown in the image, delete that .csv file from your output folder (located at Filepath1).
   iii. Repeat this process for all images in a single sample.

2. Run DANA_II by double clicking DANA_II.exe
   a. Basic setup
      i. Change the 'Input Directory' field to Filepath1.
      ii. Change the 'Output Directory' field to a location of your choice. We recommend selecting the DANA_output folder present in the main DANA folder for a simple setup. This location will be referred to as Filepath2.
      iii. Click the 'Save Current Settings' button and save these settings as 'default' to the DANA_settings folder present in the main DANA folder.
   b. If samples are human neutrophils and were prepared as outlined in the main text, proceed using the default settings. Alternatively, proceed to DANA_optimization and input the parameters (i.e. lower cutoff value, upper elimination cutoff parameter, NET cutoff) generated through this process.
   c. Click 'Run DANA'. Your results should now be present in the output directory specified in step 4 of 'DANA II Environment Setup' (Filepath2).



Input Directory: (Filepath1) The location DANA_II pulls image specific .csv files from. Be sure to use '\\' to separate folders. Note: this should also be the output location of DANA_I.

Output Directory: (Filepath2) The location DANA_II exports output files to. Be sure to use '\\' to separate folders. This is where the final data will be located.

Upper Elimination Cutoff Parameter: Used to determine upper bound of raw integrated density from which multiples are excluded. Utilized in the formula: $Upper\ Cutoff = \bar{x} + (value * SD)$

Lower Cutoff Value: Minimum raw integrated density value below which ROIs are excluded. Adjusted for consistently dim or condensed samples. This value should be lowered if DANA_II is incorrectly eliminating cells and labelling them as fragments.

Mean area of 5 smallest non-outlier ROIs: Computes relative area as a function of the areas of the 5 smallest ROIs with areas above the lower cutoff value. Recommended setting.

Area (um^2): Computes relative area as a function of a user-defined area.

DNA Decondensation Cutoffs: Relative area thresholds. DANA_II counts the number of cells in an image with relative areas above these thresholds. The default values are used for optimizing a NET cutoff.

NET cutoff: A relative area threshold. Any non-excluded ROI with a relative area above this threshold is labelled as a NET.

Optional Parameter: A string of text which DANA_II uses to separate images within a single sample. DANA_II searches for this string in the file name and provides information on this subgroup in the summary file. For example, if half of a sample is treated with drug ABC and these image file names contain the string 'ABC', then DANA_II will provide information on the ABC subgroup, the untreated subgroup, and both groups combined in the Summary.csv file.

DANA Optimization

Link to video: DANA Optimization

1. Choose 10-15 images that are representative of your image population, drawing from 1-3 samples. These images will be used to optimize DANA's parameters. Open these images in Fiji.
2. Open the Optimization.xlsx spreadsheet available in the DANA main folder.
3. For each image, count:
    a. The total number of cells (not including overlapping cells or cells touching the edge of the image)
    b. The total number of NETs – not including overlapping cells or cells touching the edge of the image
    c. The total number of multiples – overlapping cells not touching the edge of the image
        i. Ex. Two cells touching one another would be 1 multiple; 3 cells touching one another would be 1 multiple. Note: You can run DANA_I to help determine which cells are grouped into a single ROI. This well facilitate an accurate upper elimination cutoff parameter.

    Place these values in the 'Image Counts' tab of the Optimization.xlsx spreadsheet

Upper Cutoff Parameter and Lower Cutoff Value Optimization

1. For the images chosen in step 1 of DANA optimization, run DANA_1 on these images. Exclude images with 7+ multiples, or images not accurately represented (see image above) by deleting its corresponding .csv file in the output folder located at Filepath1.
2. Open DANA_II, in the 'Upper Elimination Cutoff' text field enter '1.2', and run DANA. Using the 'Mean area of 5 smallest non-outlier ROIs' is recommended.
3. Open the newly generated summary.csv file and look in cell B14 to see if the Lower Cutoff Parameter needs to be changed. DANA will indicate "YES" if it detects more than 20 total fragments within the sample. When the lower cutoff value is set too high, dimmer ROIs in the images will be labeled as fragments. Therefore, if 'YES':
    a. Open the images listed beneath 'YES' in FIJI and run DANA_I.
    b. Open the .csv files for the corresponding images.
    c. Look at all the ROIs labeled as fragments.
        i. If you agree with this classification, note the lower cutoff parameter and proceed to step 4.
        ii. If DANA has mislabeled cells as fragments, determine the Raw Integrated Density for those cells and set the Lower Cutoff Parameter beneath this value. Repeat step 2-3 until you are satisfied with DANA's fragment classification.
4. Open the output .csv files for each image and copy the 'Multiples' value (Q17) into the corresponding image's cell in the 'Multiples' column of the 'Upper Cutoff Optimization' tab.
5. Repeat step 2 and 4 using the cutoffs 1.5, 1.8 and 2.1 with the current subset of images.
6. After completing this process for all 15 images with cutoffs of 1.2, 1.5, 1.8, and 2.1, select an upper cutoff that provides the lowest average absolute difference. If there is a tie, you may select a value in between these two upper cutoffs.

NET Cutoff Optimization

1. Using the newly generated upper cutoff value and lower cutoff parameter, or the default cutoff values, run DANA_II on the 15 selected images.
2. For each image, record the number of cells with relative areas above 3.00, 4.00, 5.00, and 6.00 (Q23-Q26) in the 'NET Cutoff Optimization' tab. Then change the DNA decondensation cutoff parameters from [3.0, 4.0, 5.0, 6.0] to [7.0, 8.0, 10.0, and 12.0] and repeat this process.
3. Fill in the 'Best Cutoff' column, selecting the cutoff parameter that most provides the closest number of NETs to that found by eye.
4. The generated NET cutoff value will propagate in M19 after the Best Cutoff column is complete.
5. Save a copy of your newly generated settings, which should include:
    a. Selected Input/output directory
    b. Upper Elimination Cutoff Value
    c. What the relative area is normalized to
    d. NET Cutoff