Cell proliferation assay

The following procedure allows for counting proliferating cells using two Fiji macros:

1. *NucleiSegmentationWithManualCorrection.ijm*, used for segmenting DAPI stained nuclei

2. *CountProliferatingCells.ijm*, to actually count the number of proliferating cells based on immunolabelling.

This pipeline is tailored to cells in culture that have been infected by lentivirus of short-hairpin constructs (directed to target genes) and further immunostained against different proliferation markers, such as ki67 and PH3. A GFP reporter is used to differentiate the infected and non-infected populations, and ki67 and PH3 are counted withing each cell population. The workflow is summarized in Figure 1. The original example image (A) is shown as a composite with GFP in green, Ki67 in red, PH3 in gray and DAPI in blue. The following steps are performed:

Step1. The first macro, *NucleiSegmentationWithManualCorrection.ijm,* extracts and pre-processes the DAPI channel based on local thresholding, in order to reduce the intensity differences between nuclei at different cell stages. The result is shown in (B).

Step 2. Manual correction is used to fine tune the segmentation of the nuclei, and the final binary mask is created (C). This step allows for further separating touching nuclei (red arrowhead), join nuclei that have been divided or delete extra objects (red asterisk).

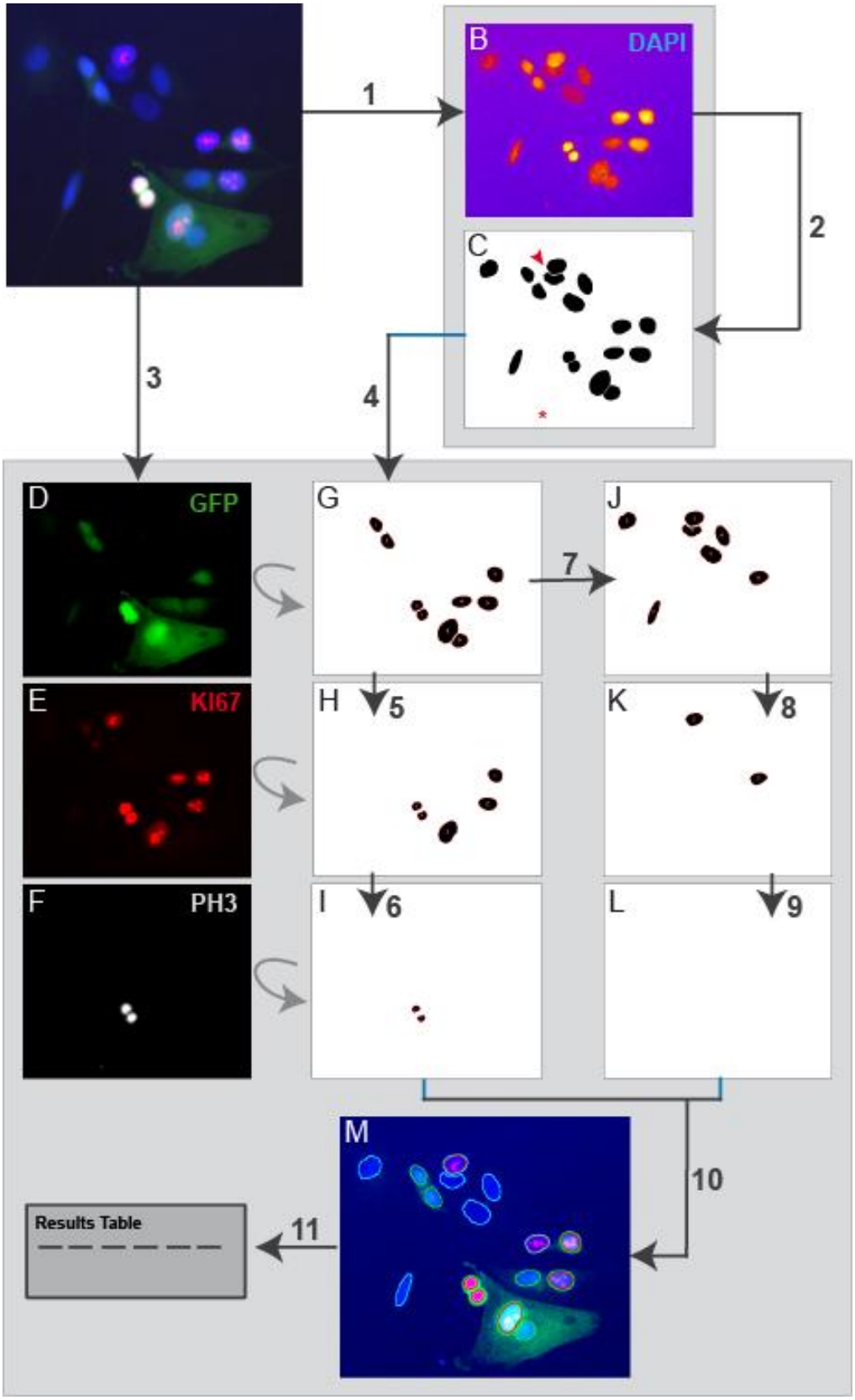
Step 3. The second macro *CountProliferatingCells.ijm,* first separates the channels of the original image: GFP (D), ki67 (E) and PH3 (F); it then creates an extra composite to be used as verification image (similar to A). Finally, the macro prompts the user to select a ROI where the background will be measure for each of the three channels.

Step 4. The macro now extracts all the ROIs from the objects generated in step 2 (image C) and loads them onto the *ROI Manager*. A user-defined function named *discardROIUnder()* eliminates from the *ROI Manager* all the selections below a certain intensity threshold, as compared to the ratio between the signal and the background calculated for each ROI in the original image. This function is first used against the GFP channel to keep all the [GFP+] ROIs in the *ROI Manager* and count them. They are then used by the function *createMask()* to generate the [GFP+] binary mask (G).

Step 5. The *discardROIUnder()* function is then used to select the [ki67+] nuclei among the [GFP+] ones. The resulting selections are used to both count and create the [GFP+, Ki67+] mask (H).

Step 6. The *discardROIUnder()* function is now used to select the [PH3+] nuclei among the [GFP+, Ki67+] ones. The resulting selections are used to both count and create the [GFP+, Ki67+, PH3] mask (I).

Figure 1.


B
DAPI
C
D
GFP
E
KI67
F
PH3
G
H
I
J
K
L
M
1
2
3
4
5
6
7
8
9
10
11
Results Table

Step 7. Once the [GFP+] have been screened for proliferation, the [GFP-] mask is created (J) from binary operations between the [GFP+] and the general nuclei masks (C and G). The [GFP-] nuclei are counted.

Step 8. The *discardROIUnder()* function is then used to select the [ki67+] nuclei among the [GFP-] ones. The resulting selections are used to both count and create the [GFP-, Ki67+] mask (K).

Step 9. The *discardROIUnder()* function is finally used to select the [PH3+] nuclei among the [GFP-, Ki67+] ones. The resulting selections are used to both count and create the [GFP-, Ki67+, PH3] mask (L).

Step 10. All the masks are used by the user-defined functions *paintROIs()* and *fillROIs()* to overlay the resulting sets of selections onto the verification image, [GFP+] outlined in green, [GFP-] outlined in cyan, [KI67+] outlined in red and [PH3+] filled in magenta.

Step 11. The macro finally stores the results in a *.xls datasheet and saves it together with the verification image to a folder of choice, using the original image name as part of the files name.

How to use:

1. Open macro *NucleiSegmentationWithManualCorrection.ijm* by drag and drop at the Fiji bar and hit *Run*.
2. When prompted, select a folder to store results.
3. When prompted, select a multichannel image to be analysed.
4. When asked to, on the mask, paint separation lines to further separate touching nuclei. Hit ok. Select “1” to repeat the step or “0” go to the next step.
5. Using shift, select two rois to rejoin. Hit ok. Select “1” to repeat the step or “0” go to the next step.
6. Select one ROI to delete. Hit ok. Select “1” to repeat the step or “0” go to the next step.
7. Check segmentation. Select “1” to go over a second correction round or “0” to accept the segmentation. The mask will be created.
8. Open macro *CountProliferatingCells.ijm* and hit *Run*.
9. When prompted, select a folder to store results.
10. When prompted, select a multichannel image to be analysed.
11. When prompted, select a the mask created with the previous macro.
12. When asked to, on the composite image, select a background ROI.
13. The macro will finish counting and deliver a results table with the counting plus a verification image where ROIs have been overlaid in different colors.