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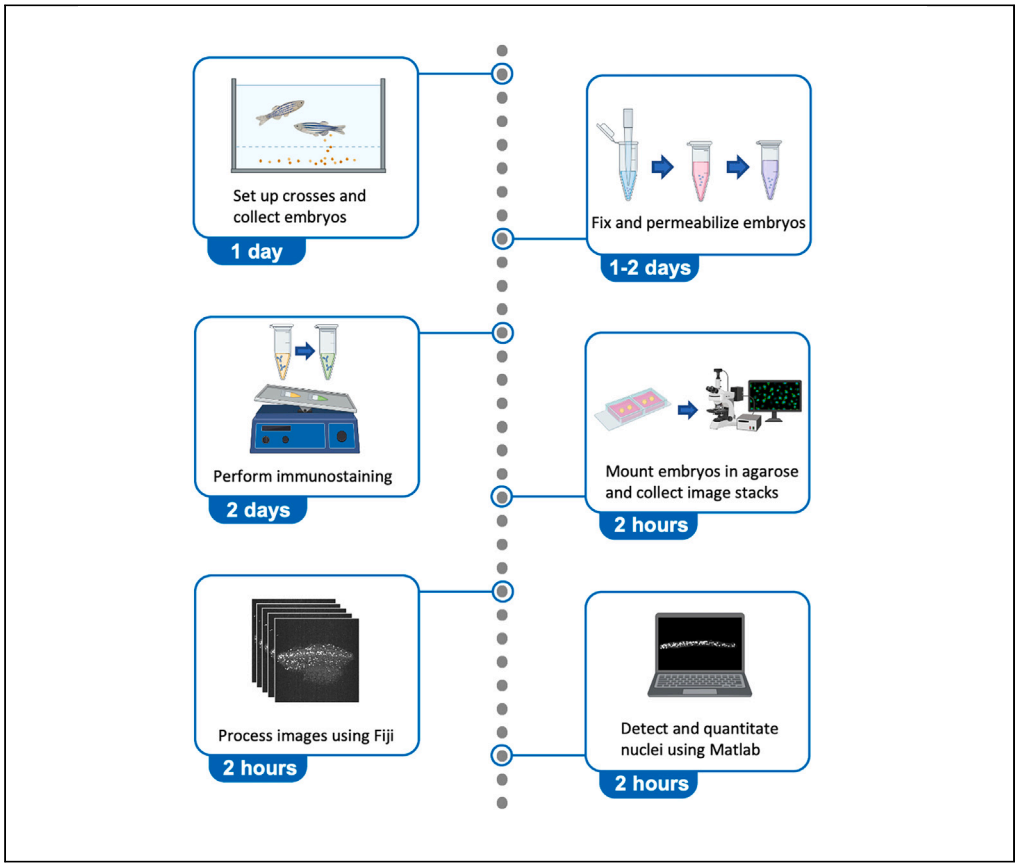
Quantitation of mitotic cells in the neural tube of zebrafish embryos using automated nuclei counting



Here, we provide a protocol to automate the quantification of the number of phospho-histone H3-positive cells in the developing nervous system of zebrafish using a custom MATLAB script to identify labeled nuclei. We describe steps for fixation, immunolabeling, and imaging of zebrafish embryos. We then detail the analysis steps using Fiji and MATLAB. This protocol can be used for fixed, immunolabeled tissue, as shown here, or for live samples, such as cells expressing a histone-GFP fusion protein.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for automatic counting of labeled nuclei

Procedures for immunolabeling, mounting, and imaging

Procedures for image processing and quantitative analysis

Adaptable to other discrete fluorescent objects of interest

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Quantitation of mitotic cells in the neural tube of zebrafish embryos using automated nuclei counting

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SUMMARY

Here, we provide a protocol to automate the quantification of the number of phospho-histone H3-positive cells in the developing nervous system of zebrafish using a custom MATLAB script to identify labeled nuclei. We describe steps for fixation, immunolabeling, and imaging of zebrafish embryos. We then detail the analysis steps using Fiji and MATLAB. This protocol can be used for fixed, immunolabeled tissue, as shown here, or for live samples, such as cells expressing a histone-GFP fusion protein.

For complete details on the use and execution of this protocol, please refer to Biswas et al.[1]

BEFORE YOU BEGIN

1. Download and install FIJI (Image J version 2.9.0). Follow the instructions for your operating system on https://imagej.net/software/fiji/downloads.[2]
2. Download and install MATLAB (current version R2023a) https://www.mathworks.com/products/matlab.html.
3. Download the NoRMCorre package (https://github.com/flatironinstitute/NoRMCorre) and place in the MATLAB search path. This package contains the utility for opening TIFF files.
4. Construct an imaging chamber by using Sugru or Sylgard to build walls on a standard 3 × 1″ microscope slide. This will be used to image immunolabeled zebrafish embryos on an upright confocal or 2-photon microscope fitted with a water immersion objective.
5. Prepare an embryo orienting tool by breaking off the handle of a long cotton swab.
   a. Split the end by pushing a razor blade into one of the ends of the wooden handle by about $1/8^{th}$ inch.
   b. Place a droplet of Crazy Glue or similar adhesive onto the split end.
   c. Push a $^{3}/_{4}$ inch piece of monofilament fishing line into the split created by the razor.
   d. Add more adhesive if needed and let cure.

   Note: The filament can then be used to gently maneuver embryos within agarose droplets for proper orientation before imaging.

Institutional permissions

The zebrafish is a vertebrate species. Research using zebrafish requires permission from an institutional animal care and use committee (IACUC) or its equivalent. All animal procedures were performed in accordance with the Ohio State University animal care committee's regulations.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phospho-histone H3 (Ser10) antibody (1:200 dilution)	Cell Signaling Technology	Cat# 9701; RRID: AB_331535
Alexa Fluor 594 goat anti-rabbit IgG (H + L) (1:500 dilution)	Molecular Probes	Cat# A-11005; RRID: AB_141372
Chemicals, peptides, and recombinant proteins		
16% Paraformaldehyde	Electron Microscopy Sciences	Cat# 15710
Acetone	Sigma-Aldrich	Cat# A4206
PBS	Sigma-Aldrich	Cat# P3813-10PAK
Tween 20	Sigma-Aldrich	Cat# P7949
Low melting point agarose II	VWR	Cat# 0815-25G
Blocking reagent	Roche Diagnostics	Cat# 11096176001
Fetal bovine serum	Gibco	Cat# 16140-071
Experimental models: Organisms/strains		
Zebrafish: WT/TL	Zebrafish International Resource Center (ZIRC)	ZDB-GENO-990623-2
Software and algorithms		
Fiji	http://fiji.sc	RRID: SCR_002285
MATLAB	https://www.mathworks.com/	RRID: SCR_001622

MATERIALS AND EQUIPMENT

60X E3 Embryo Medium

Reagent	Final concentration	Amount
NaCl	300 mM	17.2 g
KCl	10 mM	0.76 g
$CaCl_2$-$2H_2O$	20 mM	2.9 g
$MgSO_4$-$7H_2O$	20 mM	4.9 g
dH_2O	N/A	Add to 1 L

Note: Add water to 1 L and stir until dissolved. Store 60X stock solution in fridge. To make 1X solution, dilute 16.66 mL of 60X stock solution in dH_2O to a total of 1 L. Store 1X solution under non-sterile conditions at 22°C–25°C for 12 months (Westerfield, 1995).[3]

2× PBS

Dissolve two packets of Sigma #P3813-10PAK in 900 mL dH_2O with slow stirring. Add additional dH_2O to a total of 1 L. Store at 4°C for 12 months.

4% Paraformaldehyde

Reagent	Final concentration	Amount
16% paraformaldehyde	4%	10 mL
2× PBS	1×	20 mL
dH_2O	N/A	10 mL

Open glass ampule of 16% paraformaldehyde and transfer 10 mL to 50 mL conical tube. Add 20 mL 2× PBS and 10 mL dH_2O. Store for up to 1 month at 4°C.

⚠ CRITICAL: Use personal protective equipment (PPE), including gloves and surgical mask. Work under a fume hood if possible.

20% Tween 20

Add 20 mL Tween 20 to 80 mL dH_2O and stir with magnet on stir plate until well dissolved. Store at 22°C–25°C indefinitely.

1× PBST

Add 0.5 mL 20% Tween 20 to 100 mL 1X PBS for a final concentration of 0.1% to 1X PBS. Store at 22°C–25°C for up to 1 month.

Blocking Buffer

Reagent	Final concentration	Amount
Roche Blocking Powder	2%	1 g
2× PBS	1×	25 mL
dH_2O	N/A	24.25 mL
20% Tween 20	0.1%	0.25 mL
DMSO	1%	0.5 mL
Fetal bovine serum	10%	Add before use

Dissolve 1 g Roche Blocking Powder in 25 mL 2× PBS and 15 mL dH_2O using vortex and heating (incubate in glass beaker with 60°C–65°C water to help dissolve powder, vortexing every few minutes to help dissolve powder; it is not likely all will go into solution). Add 9.25 mL dH_2O, 0.25 mL 20% Tween 20, 0.5 mL DMSO. Store at 4°C for up to 1 month. Powder that does not go into solution will settle; pipette off of the top when using and avoid pipetting solids. Add 10% fetal calf serum just prior to use.

2% low melting point agarose

Add 0.5 g low melting point agarose to 25 mL 1X PBS in 50 mL conical tube. Place conical tube in glass beaker with enough water to cover agarose solution. Microwave in 20 second intervals to slowly dissolve agarose until completely liquified. Equilibrate in a 40°C water bath for 1 h prior to use. Store in 40°C water bath for up to 1 week.

STEP-BY-STEP METHOD DETAILS

Fixation of zebrafish embryos

Timing: 1–2 days

The first step in this protocol is to collect zebrafish embryos at the desired time points and fix them in paraformaldehyde for further processing. The following steps were performed on embryos at 18 h post fertilization (hpf), but the steps can be adapted for other developmental time points.

1. Set up adult zebrafish mating the day before you want to collect fertilized eggs. Collect embryos from mating pairs and maintain in E3 media at 28.5°C (Westerfield, 1995).[3]
2. Prior to fixation, dechorionate embryos using a fine pair of tweezers. Transfer 25 embryos at 18 hpf for each sample into 1.5 mL centrifuge tubes.

   Note: Sex characteristics are not distinguishable at early stages, but both sexes are typically represented in a clutch of embryos.

3. Add 1 mL of 4% PFA per tube and fix samples for 1–2 h at 22°C–25°C while gently rocking on rocker plate.
4. Remove PFA and rinse embryos 3 × 5 min in 1X PBS. Rinse for 4–24 h in 1X PBS at 4°C while laying tube on its side, or on rocker plate, to further clean the embryos.

   Note: Fixation for longer periods can lead to higher background. Use fresh fixative (less than 1 month old).

⚠ CRITICAL: Use personal protective equipment (PPE), including gloves and surgical mask. Work under a fume hood if possible. PFA is toxic and liquid waste should be collected and discarded according to specific handling protocols.

Permeabilization and immunostaining

Timing: 2 days

Embryos are permeabilized in cold acetone and incubated at -20°C to prepare them for blocking and antibody incubations. Embryos are then rinsed and blocked prior to incubations with primary and secondary antibodies.

5. Prechill a small amount of acetone at -20°C, enough for permeabilizing all samples.
6. Remove PBS from tubes and add 1 mL prechilled acetone.
7. Incubate tubes on their sides at -20°C for 7 min.

Note: For older embryos (48–72 hpf), 8–10 minutes can be used, but timing should be optimized for each age.

8. Quickly remove acetone and wash embryos once quickly in water at 22°C–25°C, dropping the last few drops on top to push floating embryos back down into the tube.

⚠ CRITICAL: Use personal protective equipment (PPE), including gloves and surgical mask. Acetone waste should be collected and discarded according to specific handling protocols.

9. Remove water and rinse embryos 2 × 5 min washes in PBST at 22°C–25°C while gently rocking on rocker plate.
10. Replace wash solution with 500 μL Blocking Buffer and incubate embryos for at least 1 h at 22°C–25°C on rocker plate.
11. Incubate embryos in anti-phospho-histone H3 primary antibody (diluted 1:200) in Blocking Buffer (200 μL per tube) for 12–24 h at 4°C on rocker plate.

Note: To ensure embryos stay submerged, you can prop the tubes up on their hinges.

12. The next day, rinse embryos 4 × 5 min in PBST at 22°C–25°C on rocker plate.
13. Incubate embryos in secondary antibody (diluted 1:500) in Blocking Buffer (500 μL per tube) for 4 h at 22°C–25°C or for 12–24 h at 4°C on rocker plate.

Note: Embryos should be protected from light during and after they are immunolabeled with the secondary antibody to prevent photobleaching.

14. Rinse embryos for several hours in 5 changes of PBST at 22°C–25°C while gently rocking on rocker plate.

Note: Embryos can be kept in PBS for 12–24 hours at 4°C before imaging without loss of signal.

Mounting embryos in agarose and collecting image stacks

Timing: 1+ days

Embryos are mounted in a row using 2% low melting point agarose in the same orientation to capture regions of interest. The imaging tray is then placed on a confocal or 2-photon microscope and image stacks are collected at 1 µm intervals to capture all the immunolabeled nuclei. Parameters can be changed to collect images from other regions of interest.

15. Equilibrate a 50 mL conical tube containing 25 mL of 2% low melting point agarose in 1x PBS at 40°C before mounting embryos.
16. Select well labeled embryos under fluorescence and place in a small depression slide or similar shallow holding container.
17. Moving quickly, select an embryo from the holding container using a glass Pasteur pipette and quickly drop it into the warm agarose with as little buffer as possible. Remove residual buffer from transfer pipette. Immediately draw up the embryo with the glass pipette and transfer the embryo in a small droplet of liquid agarose onto the glass imaging chamber bottom (Figure 1A).
18. Quickly orient the embryo within the agarose droplet so that the region of interest is facing upward, which is the direction from which the microscope objective will be imaging.
19. Collect image stacks of your region of interest.

Image processing using Fiji/ImageJ

Timing: 1+ days

Image stacks are processed using Fiji/Image J to enhance brightness and contrast and to mask areas that are not included in the analysis (see Methods video S1).

20. Open an image stack in Fiji/ImageJ by selecting "Open" under the File dropdown menu and then selecting the .tif file name of your image stack.
21. In the "Bio-Formats Import Options" window, select "Hyperstack" in the "View stack with" dropdown menu at the top and select "Autoscale" at the bottom left. Click "OK" at the bottom.
22. Adjust Brightness/Contrast of your images by selecting the "Image" dropdown menu and then the "Adjust" and "Brightness/Contrast" options. When you have adjusted the brightness and contrast of the nuclei in your image stack, click "Apply" to retain the settings.
23. To remove noise from your image stack, select the "Process" dropdown menu and then "Filters" and "3D Gaussian Blur". This will blur the pixelated noise within your image. Select a radius that reduces the noise without blurring the image too much. For our images, we used a radius of 1.5 for X, Y and Z sigma. Click "OK" to apply the filter.
24. Make a Z projection of your image stack by selecting the "Image" dropdown menu and then selecting "Stack" and then "Z Project". Select "Max Intensity" to make the Z Projection image (Figure 1B). Click "OK" to continue.

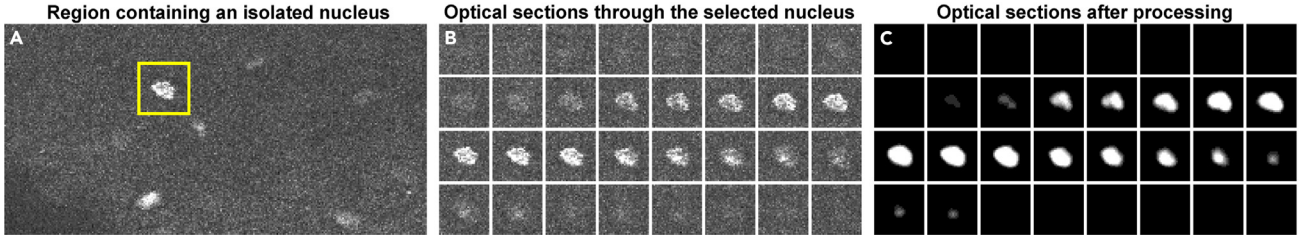

Figure 1. Steps to isolating a well labeled nucleus to use as a template

(A) Shown here is a region of a single optical section from an image stack. An isolated nucleus is shown in the yellow box.

(B) The boxed region in A was cropped to a 32 × 32 × 32 volume with the nucleus centered within the volume and no other fluorescent objects present within the volume.

(C) The template was processed by applying a Gaussian Blur (radius = 1.5) and adjusting brightness and contrast.

25. Select the Freehand selection from the ImageJ toolbar. Using a continuous line, encircle the area of interest (Figure 1C).
26. Click on the original window of the image stack and then the "Edit" dropdown menu. Click on the "Selection" option and then select "Restore selection". This should transfer the encircled region of interest from the Z projection to the image stack.
27. Click on the "Edit" dropdown menu again and select "Clear Outside". This will delete the image outside of your encircled region of interest.
28. Save your new image stack as a new file name by clicking on the "File" dropdown menu, clicking on "Save As", and then selecting the "Tiff…" format. Create a new folder and rename all of your new masked files.
29. To generate a template for cross-correlation, identify an isolated, well-labeled nucleus. Crop the nucleus in 3D, such that no other nuclei or fluorescent debris are present within the volume. The presence of extraneous fluorescence within the template will introduce noise into the correlation map. To generate a template nucleus, center the nucleus within a square bounding box, then select the "Image" dropdown menu and then "Crop" (Figure 2A) Then select the "Image/Stack/Tools/Make Substack" command in the Fiji dropdown menu. Select the sections containing the nucleus, leaving at least 2 blank sections above and below the selected nucleus. The nucleus should be centered in XYZ within the volume (Figure 2B). To remove noise, select the "Process" dropdown menu and then, "Filters" and "3D Gaussian Blur" (Figure 2C). Select a radius that reduces the noise without blurring the image too much. For our images, we used a radius of 1.5 for X, Y and Z sigma. Click "OK" to apply the filter.

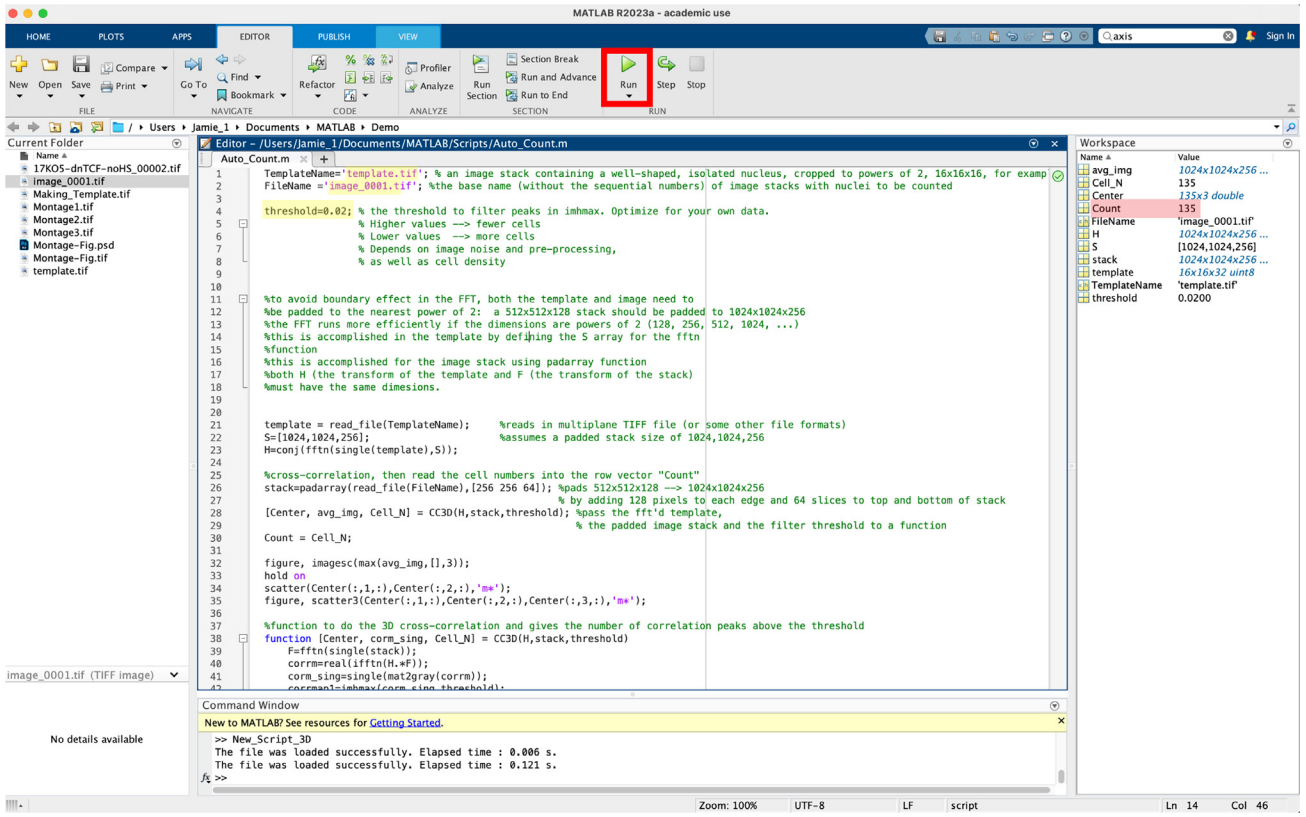

Figure 2. Running the MATLAB script

The script may be run either by clicking the "Run" arrow (red box) or by typing Auto_Count into the Command Window. The only parameters that need to be modified are the filenames and the threshold value (highlighted in yellow) in the Editor Window. Once finished, the number of detected cells will be stored in the "Count" variable (highlighted in red), which can be found in the Workspace (right-hand side of screen).

Quantification of labeled nuclei using MATLAB

Timing: 1+ days

Processed image stacks are analyzed using a custom script written in MATLAB (see Methods video S1).

30. Open MATLAB. Navigate to the folder containing your image stacks and cropped template. Open the Auto_Count.m script in the Editor window. Prior to running the script, parameters such as "threshold" and the filenames of the template and image stack can be edited.
31. Make sure that the Auto_Count.m script is in your MATLAB search path or that it is in the active folder.

```
TemplateName='template.tif'; % an image stack containing a well-shaped, isolated nucleus,
cropped to powers of 2, 16x16x16, for example.

FileName ='image_0001.tif'; %the base name (without the sequential numbers) of image stacks
with nuclei to be counted

threshold=0.02; % the threshold to filter peaks in imhmax. Optimize for your own data.

        % Higher values--> fewer cells

        % Lower values--> more cells

        % Depends on image noise and pre-processing,

        % as well as cell density

%to avoid boundary effect in the FFT, both the template and image need to

%be padded to the nearest power of 2: a 512x512x128 stack should be padded to 1024x1024x256

%the FFT runs more efficiently if the dimensions are powers of 2 (128, 256, 512, 1024, ...)

%this is accomplished in the template by defining the S array for the fftn

%function

%this is accomplished for the image stack using padarray function

%both H (the transform of the template) and F (the transform of the stack)

%must have the same dimensions.

template = read_file(TemplateName);  %reads in multiplane TIFF file (or some other file formats)

S=[1024,1024,256];           %assumes a padded stack size of 1024,1024,256

H=conj(fftn(single(template),S));

%cross-correlation, then read the cell numbers into the row vector "Count"

stack=padarray(read_file(FileName),[256 256 64]); %pads 512x512x128--> 1024x1024x256

                            % by adding 128 pixels to each edge and 64 slices to top and bottom of
stack

[Center, avg_img, Cell_N] = CC3D(H,stack,threshold); %pass the fft'd template,

                              % the padded image stack and the filter threshold to a function

Count = Cell_N;

figure, imagesc(max(avg_img,[],3));

hold on scatter(Center(:,1,:),Center(:,2,:),'m*');

figure, scatter3(Center(:,1,:),Center(:,2,:),Center(:,3,:),'m*');
```

```
%function to do the 3D cross-correlation and gives the number of correlation peaks above the
threshold

function [Center, corm_sing, Cell_N] = CC3D(H,stack,threshold)

  F=fftn(single(stack));

  corrm=real(ifftn(H.*F));

  corm_sing=single(mat2gray(corrm));

  corrmap1=imhmax(corm_sing,threshold);

  corrmap2=imregionalmax(corrmap1);

  masks=bwlabeln(corrmap2);

  objects=regionprops(masks,'centroid');

  Center=cat(1,objects.Centroid);

  Cell_N=length(Center);

end
```

32. In the Editor window, assign the name of your cropped template to the variable, *TemplateName*, in the Auto_Count script. For example, TemplateName = 'template.tif'; Assign the name of the file to. Be analyzed to the variable, *FileName*. For example, 'image_0000' or 'wild_type': FileName = 'wild_type.tif'; or FileName = 'image_0001.tif';
33. Run the script by typing Auto_Count at the MATLAB command line or by clicking the "Run" button in the Editor tab toolbar. The number of cells detected is governed by the threshold parameter. Increasing the threshold increases the stringency of the peak detection (reduces the number of identified cells). The threshold will need to be adjusted to each new dataset and use (Figure 3).
34. The number of cells detected in each stack will be stored in the variable, Count, which will appear in the Workspace window on the right-hand side of the MATLAB environment (Figure 3).

EXPECTED OUTCOMES

With this approach, users should generate a total count of fluorescently labeled objects in a 3D image stack, as well as the objects' *x*, *y*, *z* coordinates. These represent the positions of each object that closely matches the shape and intensity profile of the template.

LIMITATIONS

Our protocol detects high contrast, circular or ovoid objects in 3D image stacks. Noisy images or weakly-labeled objects will degrade the performance of this approach and would require careful tuning of the threshold applied to the correlation map in order to exclude background peaks. While this correlation-based approach could, in principle, be used to identify instances of more complex objects, this straight-forward implementation is best suited for objects with approximate spherical symmetry. In addition, for best performance, objects should be clearly separated to yield clear peaks in the correlation maps. For example, densely labeled samples, such as DAPI-stained samples, could pose a challenge to this approach. In addition, tuning of the threshold parameter will also influence how cross-correlation deals with mitotic profiles. Phospho-Histone H3 will label condensed chromosomes in metaphase or anaphase, for example. These profiles will generate correlation peaks, but will be weaker than for fully ovoid nuclei. A lower threshold would correctly count anaphase or telophase chromatin as two cells, while a higher threshold may exclude these objects.

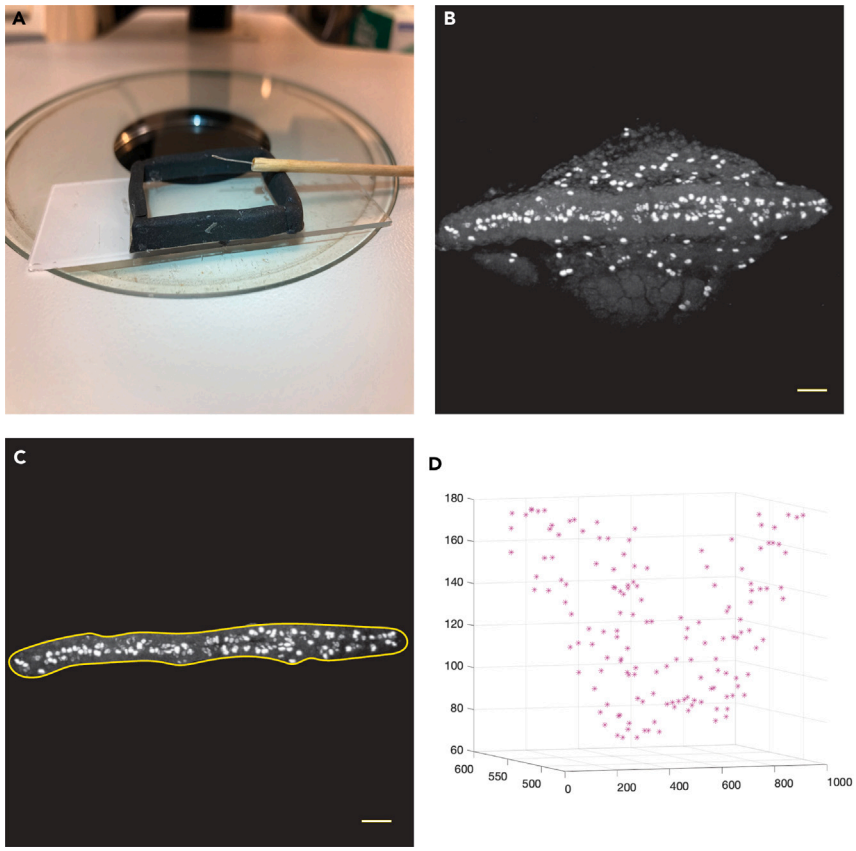

Figure 3. Imaging chamber, steps of image processing and final 3D graph showing nuclei counted within region of interest

(A) Shown here is one example of an imaging chamber, made using Sugru to create walls to hold buffer on a standard 3 inch by 1 inch slide. The embryo orienting tool was made using monofilament fishing line and the handle of a long cotton swab.

(B) A maximum intensity projection of a 128 image stack of phospho-histone H3-immunopositive cells in an 18 hpf embryo was made using Fiji/ImageJ image processing program. Scale bar, 50 μm.

(C) The freehand selection tool was used to select the nuclei located within the neural tube and to exclude labeled cells on the surface of the yolk. The selection was applied to the image stack and the "Clear Outside" function was selected to remove the surrounding nuclei. Scale bar, 50 μm.

(D) Running the MATLAB Script "Auto_count.m" on the processed image stack yields a total count of labeled nuclei found only within the neural tube throughout the 128 images. Scale bar, 50 μm.

TROUBLESHOOTING

Problem 1

The fluorescent signal is weak, making the nuclei hard to detect.

Potential solution

- Make sure 4% paraformaldehyde is not too old. We routinely use fixative for up to one month, but if problems persist, make fresh paraformaldehyde solution for fixation (see Step 3 in fixation of zebrafish embryos section).
- Don't let embryos sit in PBST solution too long after rinsing out secondary antibody. If embryos will be imaged the following day, place in PBS instead of PBST for 12–24 h (see step 14 in permeabilization and immunostaining section).
- Primary anti-phospho-histone H3 antibody can lose efficacy over time, so a new aliquot of antibody might solve the problem of weak signal (see step 11 in permeabilization and immunostaining section).

Problem 2

There are too many or too few detected nuclei.

Potential solution

- Adjust the threshold parameter in the MATLAB script. Raise the threshold (more stringent filtering) to exclude lower correlation peaks and lower the number of cells; lower the threshold (less stringent filtering) to increase the number of detected cells (see step 33 in quantification of labeled nuclei using MATLAB section).
- Filter the image stacks and template using Gaussian Blur and/or adjust image contrast. The Gaussian Blur suppresses noise and should reduce spurious peaks. Similarly, flattening the image background will also suppress noise in the correlation map (see steps 22–23 in image processing using Fiji/ImageJ section).

Problem 3

Nuclei are detected along edges of the correlation map.

Potential solution

- Increase the padding on the edges of the image stack by increasing the values in the padarray and in S (dimensions of original image stack plus padding; see step 31 in quantification of labeled nuclei using MATLAB section):
  - stack = padarray(read_file(FileName),[256 256 64]);
  - S = [2048 2048 256];

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, James Jontes (jontes.1@osu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study generated new code, which is provided within this manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102596.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

M.R.E. designed and performed research, processed and analyzed data of this project, and wrote the manuscript. J.D.J. conceived and wrote the MATLAB script, secured funding, and critically revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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