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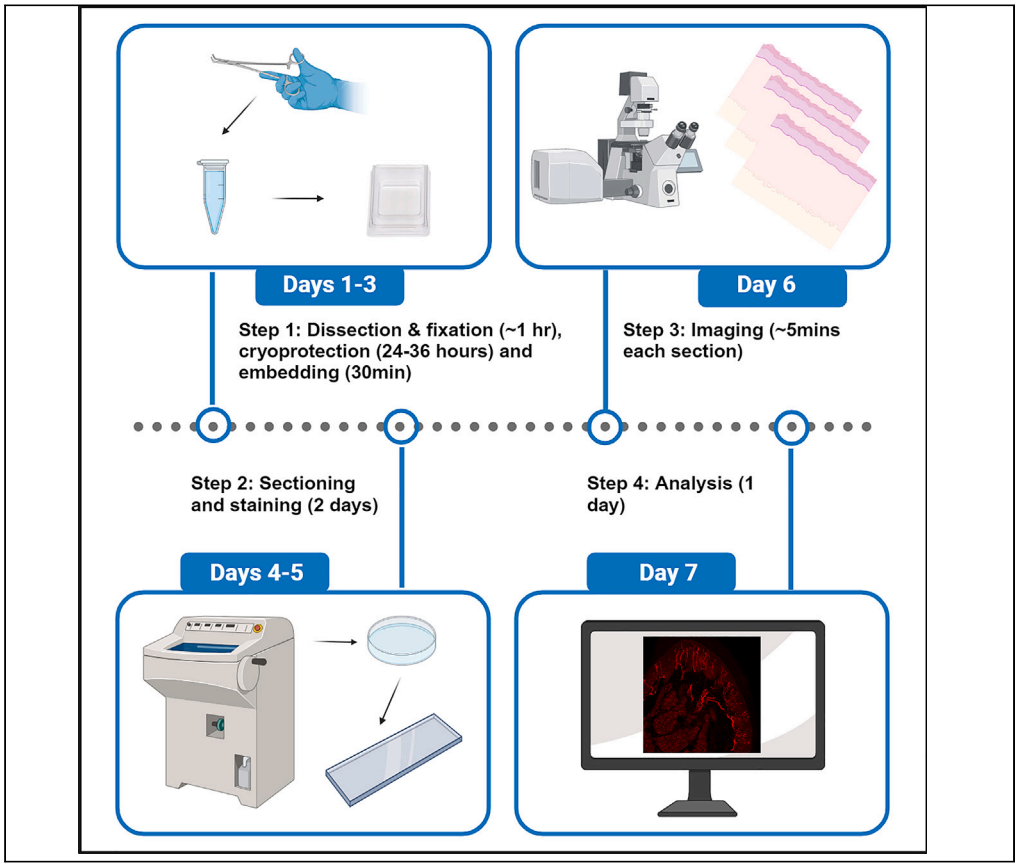
Protocol for semiautomated analysis of sensory neuronal innervation of the mouse footpad skin



Here, we present a protocol for staining of murine skin innervation by either a pan-axonal marker or a genetic tracer of sensory neuron subtypes using floating sections. We also describe steps for using a new MATLAB-based semiautomated routine that facilitates the quantification of innervation density. This protocol can also be applied to other organs, such as the mouse's spinal cord and tongue.

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

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Highlights

Steps for dissection and fixation of the mouse footpad

Steps for staining of footpad floating sections

Quantification of innervation by an algorithm in a user-defined cross-sectional area

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Protocol for semiautomated analysis of sensory neuronal innervation of the mouse footpad skin

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SUMMARY

Here, we present a protocol for staining murine skin innervation by either a pan-axonal marker or a genetic tracer of sensory neuron subtypes using floating sections. We also describe steps for using a new MATLAB-based semiautomated routine that facilitates the quantification of innervation density. This protocol can also be applied to other organs, such as the mouse's spinal cord and tongue. For complete details on the use and execution of this protocol, please refer to Dey et al.[1]

BEFORE YOU BEGIN

Immunofluorescent staining is used to visualize skin innervation in physiological and pathological conditions in rodents and humans.[2–4] This protocol was originally developed to stain and analyze footpad skin innervation of the mouse. However, we also have been applying it successfully to mouse spinal cord and tongue sections. The mouse footpad glabrous skin contains six elevated globular protrusions. To be able to properly compare phenotypes between animals, we only stained the first two protrusions of the footpad. We only imaged the apex of the protrusions where most of the innervation is, and which is the most exposed area to the external environment.

Before performing any experiment with mice, an institutional permission for animal study must be obtained. In our work, animals were bred, housed and the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Weizmann Institute of Science.

KEY RESOURCES TABLE

REAGENT or RESOURCES	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Tuj1 (1:1,000)	Abcam	Ab 18207; RRID: AB_444319
Rabbit anti-GFP(1:1,000)	Abcam	Ab6556 ; RRID: AB_305564
Alexa Fluor 647 anti-rabbit (1:500)	JAC	711605152; RRID: AB_2492288
Chemicals, peptides, and recombinant proteins		
DAPI-fluoromount	Southern Biotech	0100-20
Albumin (bovine)	MP Biomedicals	0216006980
Normal donkey serum	Abcam	AB-ab7475-25
Paraformaldehyde	EMS	15710
OCT	Sakura Tissue-Tek	4583

(*Continued on next page*)

Continued		
REAGENT or RESOURCES	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Kif2a plox (any age/sex adult mice starting from P10)	Dey et al.[1]	
B6.129S1-Mrgprdtm1Mjz/Mmnc (any age/sex adult mice starting from P10)	The Mutant Mouse Resource and Research Center – University of North Carolina	36772
ICR (any age/sex adult mice starting from P10)	Envigo	1006
Software and algorithm		
Fiji		github.com/fiji/fiji
MATLAB	MathWorks, Inc.	
Innervation index of the sensory terminal	MATLAB file exchange	https://www.mathworks.com/matlabcentral/fileexchange/133877-innervation-index-of-the-sensory-neuron-terminal
Other		
Disposable cryomold	Bar Naor, Israel	BN62534-25S
Cryostat	Leica	CM1950

MATERIALS AND EQUIPMENT

Blocking solution		
Reagent	Final concentration	Amount
Goat Serum	20%	800 µL
Triton-X 10%	0.04%	160 µL
Albumin (Bovine)	10%	400 mg
PBS	-	~3 mL
Total		4 mL

It is advisable to make the solution fresh for each batch of staining. The Triton-X 10% stock can be stored at 4°C for 3 months.

STEP-BY-STEP METHOD DETAILS

Dissection of the footpad and fixation

Timing: ~1 h

This part describes the dissection and fixation procedure of the mouse footpad staining (Figure 1).

1. Create an incision from the side of the ankle.
   a. using thin scissors carefully cut around the glabrous skin
   b. with the help of a pair of fine forceps, peel off the skin from the footpad.
   c. Remove all the muscles and tendons underneath the skin.
2. Quickly transfer the skin into a 1.5 mL tube containing 500 µL of 4% paraformaldehyde.
3. Fix for 30 min on ice (for glabrous skin, which is ~1 mm thick) and wash thrice in PBS.

Cryoprotection and embedding

Timing: 24–36 h in sucrose solution, 30 min for embedding

This part describes the cryoprotection and embedding part, which is important for optimal staining.

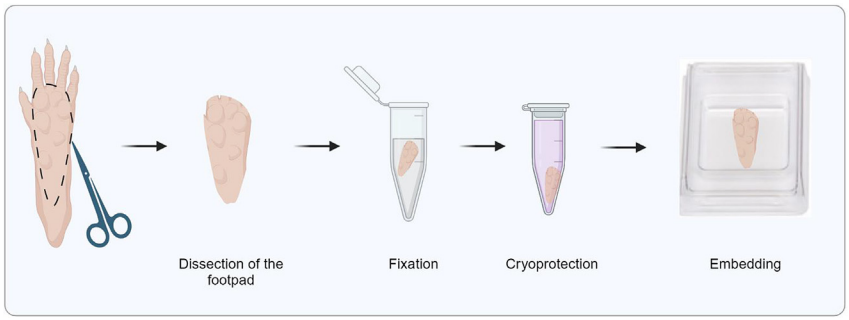

Figure 1. Workflow of dissecting, fixation and cryoprotection of the mouse footpad skin
Created using biorender.com.

4. Transfer the skin into a 1.5 mL tube filled with 25% sucrose solution (1.5 mL) and keep it in a revolver rotator (10 rpm) at 4°C until the tissue sinks to the bottom,

   Note: Usually the tissue sinks to the bottom after around 30–40 h.

   ⚠ CRITICAL: The revolver rotator helps with proper mixing of liquid and penetration of sucrose into the tissue.

5. Once the tissue sinks to the bottom, remove it from sucrose and embed it with OCT in a small mold (25 × 20 × 5 mm) on dry ice in the correct orientation (Figure 2).
6. Wrap the mold in aluminum foil, label it and place it at −80°C.

Cryosectioning and staining

Timing: 2 consecutive working days

This part describes various steps for obtaining slices from a cryostat and optimal staining of the slices from footpad (Figure 3).

7. Remove the blocks from 80°C and transfer to −20°C for at least 30 min before cutting.
8. Remove the OCT block from the mold and secure it on the sample holder.
9. In the cryostat, set the section thickness to 30–50 μm.

   Note: We generally use 30 μm sections in our staining. Thinner sections are more permeable for antibody, whereas thicker sections would be used for staining a nerve structure expected to be heavily branched.

10. Cut a section from the tissue and let it roll up until deposited on the top of the block.
11. Carefully pick up one side of the roll with a pair of forceps and transfer it to a 35 mm dish with 1 mL of PBS.
12. Let the OCT dissolve until the section is afloat on the PBS surface.
13. Follow up with the additional sections as needed collecting them in the same 35 mm dish making sure they do not overlap.

    Note: If sequential staining needs to be done, sections have to be arranged consecutively in a 24- or 48-well plate.

14. Wash thrice with PBS for 2 min each so that all the OCT from the solution is eliminated.
15. Block in the blocking solution for 3–4 h.

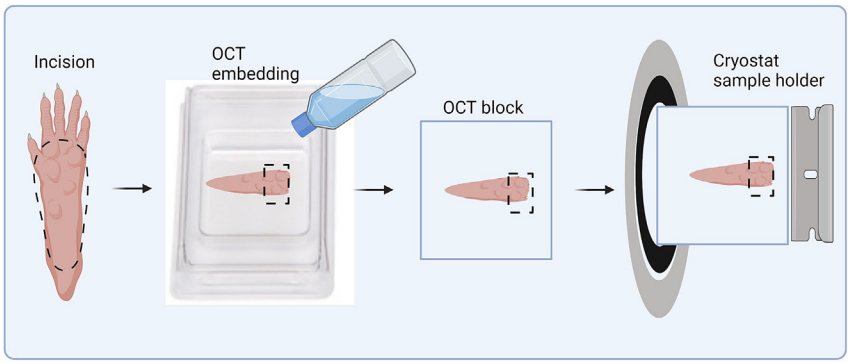

Figure 2. Orientation of the footpad in various steps of staining

16. Remove blocking solution
    a. add primary antibody (antiTuj1/anti-GFP 1:1000) in blocking solution to the plate,
    b. incubate for 16–20 h at 4°C with gentle agitation.
17. Wash thrice in PBS for 2 min each.
18. Add secondary antibody (donkey anti-rabbit 647 1:500) in blocking solution or 1x PBS + 10% serum,
    a. cover the plate with aluminum foil.
    b. incubate at 24°C for 3 h.
19. For mounting, first, clip the end of a 1 mL-pipette tip.
    a. fill it with the blocking solution several times so that the inside of the tip is coated.
20. Collect the sections from the plate and carefully deposit them on a slide; use more than one slide if necessary. Apply gentle liquid (PBS) jet with a pipette to straighten a section if it folds on itself.
21. Once the sections settle flat on the slide, remove as much of residual liquid as possible with smaller pipette tips and tissue paper.
22. After the section attach to the slide completely, rehydrate the section by dipping the slide quickly in PBS.
23. Mount with mounting media containing DAPI and coverslip.

Imaging

Timing: 4–6 min for each section depending on the microscope

The following part describes various imaging parameters for obtaining images of the stained slices from the footpad.

24. Check with epifluorescence microscope if the nerve terminals can be observed in the dermis and epidermis region of the skin.
25. If yes, take z-stack images in the confocal with 1 μm step size.
26. We took images on a confocal microscope (Olympus FV100) using Fluoview (FV10- ASW 4.1).
27. The magnification that we used in the images provided is 20x. However, depending on the goal of the experiment, 40–60x can also be used.
28. We used 80 μm pinhole for 20x magnification. To reduce background and prevent bleaching of samples we used minimal laser intensity (1%–2%).
29. All the images were taken with the same settings within one experiment. Looking at the image histograms while taking the images and tweaking image settings is a good practice.
30. We avoided staining with green fluorophores to avoid autofluorescence.
31. Import images in Fiji and save them as 16-bit tiff files.
32. Save all the similar files in a single folder,

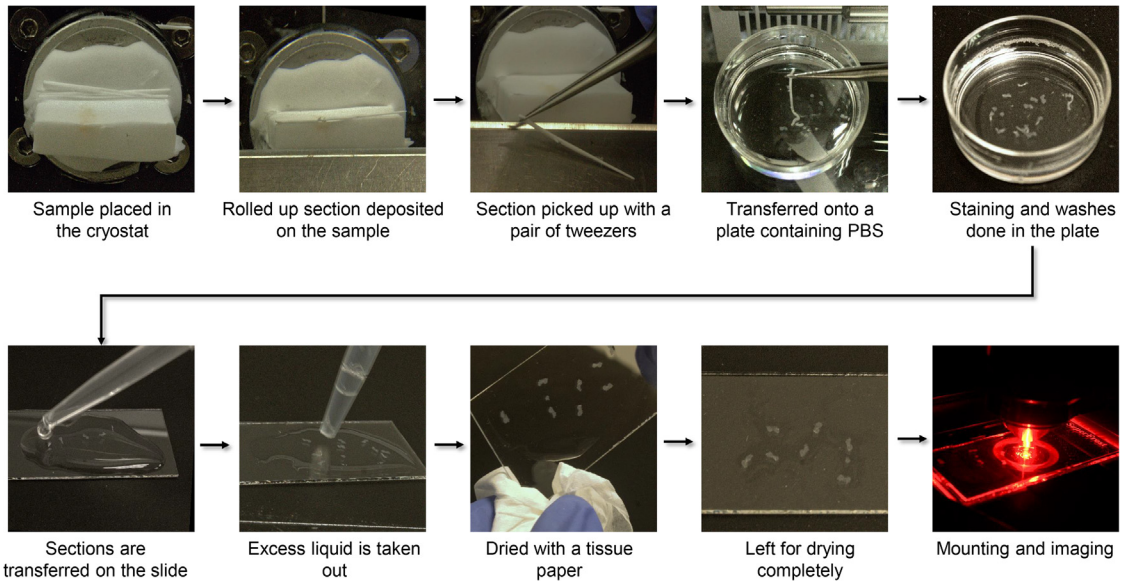

Figure 3. Workflow of cryosectioning and staining of the footpad skin

Note: For example, if you have 10 images from WT mouse 1, create a folder called WT1 and save all 10 images into it, and similarly save pictures from other mice in folders named WT2, KO1, KO2 etc.

33. For each set of images, go to Image>Adjust>threshold and choose a lower threshold manually that best represents the innervation pattern.

Note: There might be some noise spillover under certain threshold settings required to include all the innervation in the analysis, but if the noise is up to 5% it can be overlooked (Figure 5).

Analysis

This step describes the flow of the analysis to be done using MATLAB (Figure 4, Supplementary file Code S1).

Note: Install MATLAB and install "Image processing toolbox".

34. Download the innervation program from the following link (https://www.mathworks.com/matlabcentral/fileexchange/133877-innervation-index-of-the-sensory-neuron-terminal), extract it from the zipped folder (attached here as a supplementary file Code S1).
35. Create a folder "ANALYSIS" which should contain WT1, WT2, KO1, KO2…… and the program called "innervationindex".
36. Click on the program, a dialog box will appear.
37. Two methods are incorporated into the program to differentiate between the foreground and background. One, the OTSU method, which works if the signal to noise ratio is very close to 1, or two, thresholding.
38. Choose the appropriate method from the drop-down menu.
39. Note that for skin innervation staining, it is recommended to use the thresholding method.
40. Insert the chosen threshold value from step 5 of "Imaging".
41. Select a method for drawing ROI, "freehand" or "segmented line".
42. Press on "load image" and browse in your computer for one of the folders that you want to analyze, for example, WT1 and press ok.

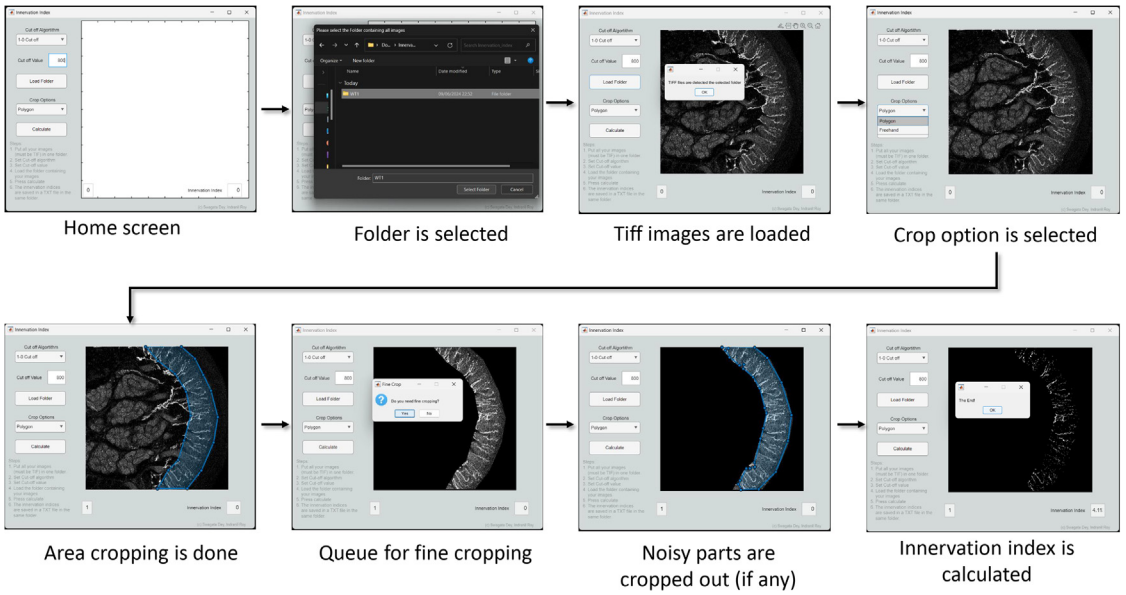

Figure 4. Workflow of the "innervation index" MATLAB program

43. The first image from the folder will appear inside the dialog box.
44. Crop the images
45. Note that there are two cropping steps in the program.
46. In the first crop step, the area has to be selected by which the total innervation has to be normalized.
47. In the second crop, the background area is carefully trimmed to remove the regions that were stained but should not be incorporated in the quantification (finecrop).

    Note: For example, the dermal papilla, sweat gland or the unwanted stains towards the outside of the epidermis.

    Note: If fine cropping is not required, there is the option to skip it.

48. After cropping all the images in the folder, the innervation index will be calculated based on the chosen area and threshold value and shown in the window.

    Note: The innervation index is calculated as total innervation/total area*100.

49. In the folder, there will be two result files: one .csv where one can find only the innervation index of the corresponding files and another .mat file where one can find many different intermediate parameters, such as area as area, total innervation as ones etc.

EXPECTED OUTCOMES

Here we have described a method for staining and quantification of the skin innervation in the mouse (Figures 5 and 6). Although we have only referred to the footpad skin, the same approach can be readily adapted for skin from other parts and other organs such as the tongue, spinal cord, etc. For the footpad skin, the innervation is highly variable between different sections of the same animal. Therefore, it is recommended to quantify multiple sections to detect a consistent trend. We generally start by cutting at least 25 and imaging 12 sections. The innervation index value that we routinely get is around 4–10.

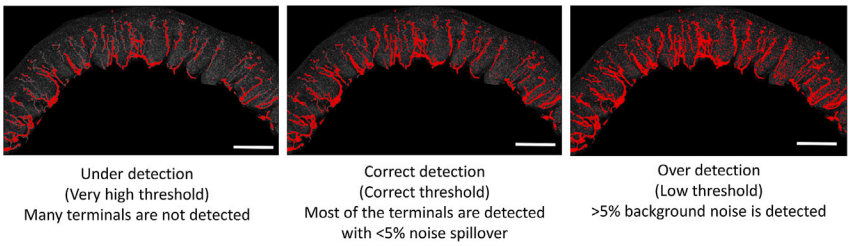

Figure 5. Optimal thresholding methods for the best results
Scale bar – 50 μm.

QUANTIFICATION AND STATISTICAL ANALYSIS

We analyze at least 12 sections from one animal and 5–6 pairs of WT vs. KO or untreated vs. treated animals. Then we compare them using T-test.

LIMITATIONS

Skin harbors numerous immunoglobulin-producing cells. Thus, using primary antibodies of the mouse origin tend to result in a very noisy staining. Even when using non-mouse antibodies, due to the excess noise in the images, it is sometimes difficult to define a threshold that includes all the innervation and completely cuts off the background noise. The innervation pattern varies significantly even inside a single sample, so many sections has to be processed before making a decision about a phenotype.

TROUBLESHOOTING

Problem 1

Very noisy staining, hardly any nerve terminal visible (Steps 2, 3, 7, 15, 16).

Potential solution

- Use EM grade paraformaldehyde for fixation.
- Fix in ice for exactly 30 min. Overfixation or underfixation can cause noisy staining.
- When possible, avoid primary antibodies of mouse origin.
- Transfer the OCT embedded tissue −80°C to −20°C at least 20–30 min before cutting.
- Make a fresh 10% Triton-X100 solution every few months.

Problem 2

The program does not open the images in the dialog box (Step 36).

Potential solution

- Install Image processing toolbox in MATLAB.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Avraham Yaron.

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Swagata Dey.

Materials availability

No new materials have been associated with this protocol.

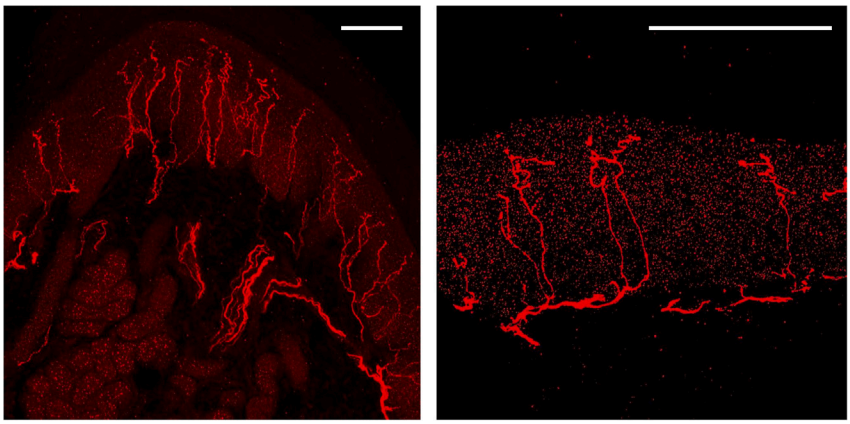

Figure 6. Expected outcome of the staining with mouse footpad with GFP antibody where Mrgprd mouse expresses endogenous GFP

(A) 20X, (B) 60X. Scale bar – 50 μm.

Data and code availability

The innervation index code associated with this protocol is available in MATLAB file exchange https://www.mathworks.com/matlabcentral/fileexchange/133877-innervation-index-of-the-sensory-neuron-terminal.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.D. performed the work, generated the figures, and wrote the paper, A.K. edited the paper, and A.Y. supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103312.

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