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3D Visualization of Neural Innervation in Rat Knee Joint Using PEGASOS-based Tissue Clearing

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We use this protocol and it's working

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Abstract

Innervation plays a critical role in osteoarthritis (OA) pain; however, its spatial organization and disease-related alterations remain poorly characterized, largely due to the technical challenges of imaging nerves in intact joints. This protocol outlines a step-by-step workflow for visualizing neurofilament-positive (NF^+) nerve fibers in whole and bisected rat knees using a modified version of the organic solvent-based polyethylene glycol-associated solvent system (PEGASOS) tissue clearing method (Jing et al. 2018). This approach renders rat knee joints of varying thickness optically transparent, enabling high-resolution mapping of joint innervation.

Materials

Reagents:

	A	B	C	D	E
	Name	**Vendor**	**CAS number**	**Catalog Number or Product Code**	**RRID**
	Phosphate buffered solution (10X)	Thermo Fisher		BP39920	
	Paraformaldehyde	Millipore Sigma	30525-89-4	P6148	
	Sodium hydroxide (NaOH)	Millipore Sigma	1310-73-2	S5881	
	Hydrochloric acid (HCL)	Fisher Scientific	7647-01-0	A144-500	
	Immunocal Decalcifier	StatLab		1214	
	Triton X-100	Millipore Sigma	9036-19-5	X100	
	Bovine Serum Albumin (5% TBST)	Boston Bioproducts		IBB-169	
	Normal Goat Serum	Abcam		ab7481	
	Quadrol	Millipore Sigma	102-60-3	122262	
	Ammonium solution	Millipore Sigma	1336-21-6	1.05432	
	Tert-butanol	Millipore Sigma	75-65-0	471712	
	PEG-MMA500	Millipore Sigma	26915-72-0	447943	
	Benzyl Benzoate	Millipore Sigma	120-51-4	B6630	
	Anti-Neurofilament Antibody [NF421 + NFL/736)	Abcam		ab215903	AB_3107022
	Goat anti-mouse Dylight 633	Invitrogen		35512	AB_1307538
	Ammonium solution	Spectrum Chemical	1336-21-6	A-570	

Collection and Fixation of Knee Joints

1 Perform transcardial perfusion using freshly prepared, ice-cold phosphate-buffered solution (PBS), followed by 4% paraformaldehyde (PFA) solution. Use approximately 250–300 mL each of PBS and 4% PFA for a 600 g rat. Indicators of successful perfusion include clear perfusate exiting the right atrium, blanching of the liver, and muscle tremors observed during the initial PFA flush.

2 Dislocate the hindlimb at the hip joint, carefully remove the muscles from the femur and tibia without disturbing the knee joint, excise the fibula, and trim the femur and tibia to the desired length.

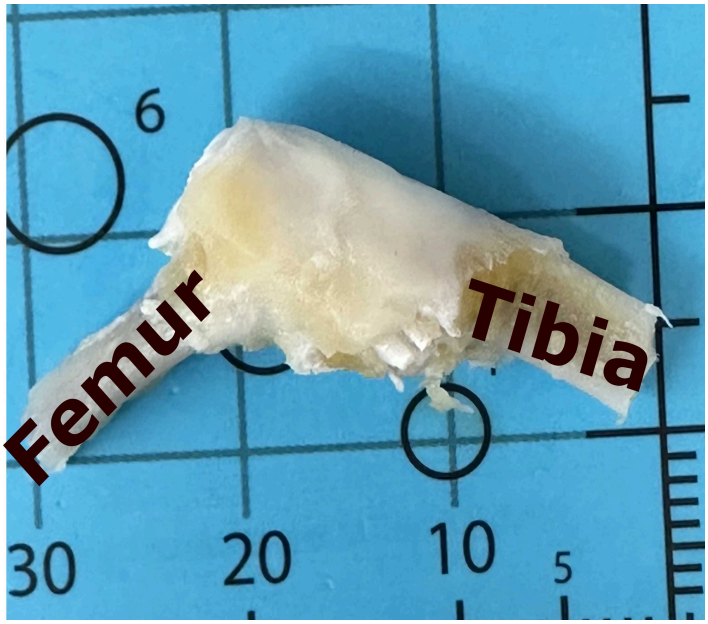

Figure 1. Intact rat knee joint with muscles and fibula removed and bones trimmed approximately at mid-length.

3 Place the knee joint, with the tibia and femur aligned orthogonally as in Figure 1, in a labeled cassette and post-fix in 4% PFA at 4 °C for 24 hours.

4 Wash the knee joint with 1X PBS to remove residual fixative. Repeat this wash twice, allowing at least a couple of hours between washes or performing them over a few days. *(Note: Collect the waste in a chemical hazard container and dispose of it following Institutional Environmental Health & Safety guidelines.)*

Decalcification

5 Place the knee joints in a 50 mL tube containing approximately 40 mL of Immunocal solution (12–15 times the volume of the sample) and incubate at room temperature in a shaking incubator set to 100 RPM. Replace the Immunocal solution every 2–3 days. *(Note: Complete decalcification of adult rat knees typically requires 7–9 days. Completeness of decalcification can be checked using CT features on an IVIS or with a Faxitron.)*

6 After decalcification, replace the Immunocal solution with distilled water and place the samples in a shaker for at least two hours. Repeat this wash once to ensure thorough removal of residual decalcifying agent.

7 For half-joint preparations, bisect the decalcified knee in the sagittal plane using a sharp razor blade. For whole-joint samples, proceed directly to Step 8.

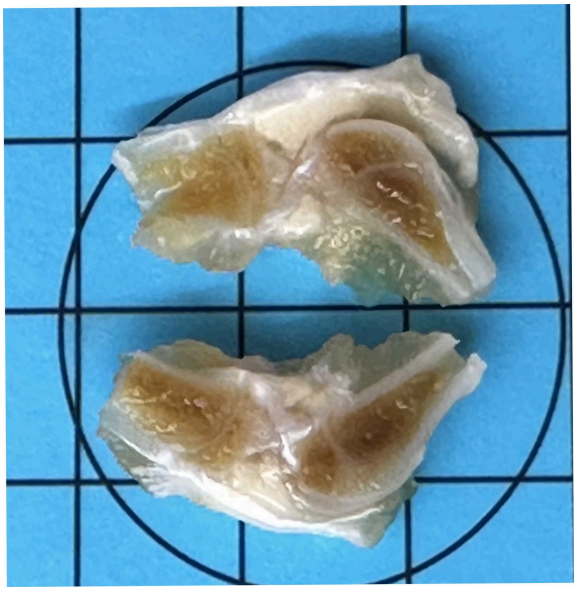

Figure 2. Decalcified rat knee joint bisected in sagittal plane.

Decolorization

8 Place the knee joint samples in Quadrol solution (25% w/v prepared in distilled water). Incubate at 37 °C in a shaker set to 100 RPM for 2 days, replacing the solution daily. Use approximately 20 mL of Quadrol per half knee and 40 mL for a whole knee joint.

9 Place the knee samples in 3% ammonium hydroxide solution and incubate at 37 °C in a shaker set to 100 RPM for 1 day

Immunolabeling

10 Rinse the sample(s) with 1X PBS.

11 Block the sample(s) overnight at 4 °C on a shaker using freshly prepared blocking buffer consisting of 1% Bovine Serum Albumin (BSA), 4% Normal Goat Serum (NGS), and 0.2% Triton X-100 in 1X PBS (PBST). Use approximately 3 mL of blocking buffer per half knee and 5 mL for a whole joint. *(Note: Samples may be transferred to smaller containers such as 5 or 10 mL conical tubes or 20 mL scintillation vials. Ensure the sample is fully immersed.)*

12 Incubate the sample(s) in primary antibody solution containing mouse anti-NF (1:500) diluted in staining buffer (4% Normal Goat Serum in 1X PBST) at 4 °C on a shaker for 7 days. Use approximately 3 mL of antibody solution per half knee and 5 mL for a whole joint.

13 Wash the sample(s) in PBST over one day. Begin with a quick rinse using 8–10 mL of PBST, followed by incubation in fresh PBST (8–10 mL) at 4 °C on a shaker for 3–4 hours. Repeat this wash cycle twice. *(Note: Samples may be left in PBST overnight at 4 °C on a shaker if needed.)*

14 Incubate the sample(s) in secondary antibody solution containing goat anti-mouse dylight 633 (1:400), diluted in staining buffer, at 4 °C on a shaker for 7 days. *(Note: From this step onward, protect the sample(s) from light to prevent photobleaching.)*

15 Wash the sample(s) in PBST over one day. Begin with a quick rinse using 8–10 mL of PBST, followed by incubation in fresh PBST (8–10 mL) at 4 °C on a shaker for 3–4 hours. Repeat this wash cycle twice. *(Note: Samples may be left in PBST overnight at 4 °C on a shaker if needed.)*

Delipidation and Clearing

16 Place the immunolabeled sample(s) in the gradient delipidation solution and incubate at 37 °C on a shaker set to 100 RPM. *(Note: tert-butanol is solid at room temperature (melting point ~26 °C); melt it by placing the container in a beaker of warm water (~30 °C) before reconstituting it in distilled water.)*

16.1 Incubate sample(s) in 30% tert-butanol with 3% Quadrol for 1 day. Use 15 mL of solution per half knee and 30 mL per whole knee.

16.2 Incubate sample(s) in 50% tert-butanol with 3% Quadrol for 1 day. Use 15 mL of solution per half knee and 30 mL per whole knee.

16.3 Incubate sample(s) in 70% tert-butanol with 3% Quadrol for 1 day. Use 15 mL of solution per half knee and 30 mL per whole knee.

17 Place the sample(s) in a solution of 70% tert-butanol, 30% PEGMMA500, and 3% Quadrol. Incubate at 37 °C on a shaker for 2 days, replacing the solution daily. Use 15 mL per half knee and 30 mL per whole knee. *(Note: After this step, keep samples away from water-containing reagents.)*

18 Place the sample(s) in BB-PEG medium (75% Benzyl Benzoate and 25% PEGMMA500) and incubate at 37 °C for 1 day. Use 15 mL of medium per half knee and 30 mL per whole knee.

19 Replace the BB-PEG medium after 1 day. Use 15 mL for half knee and 30 mL for whole knee.
(Note: Store sample(s) protected from light at room temperature until imaging.)

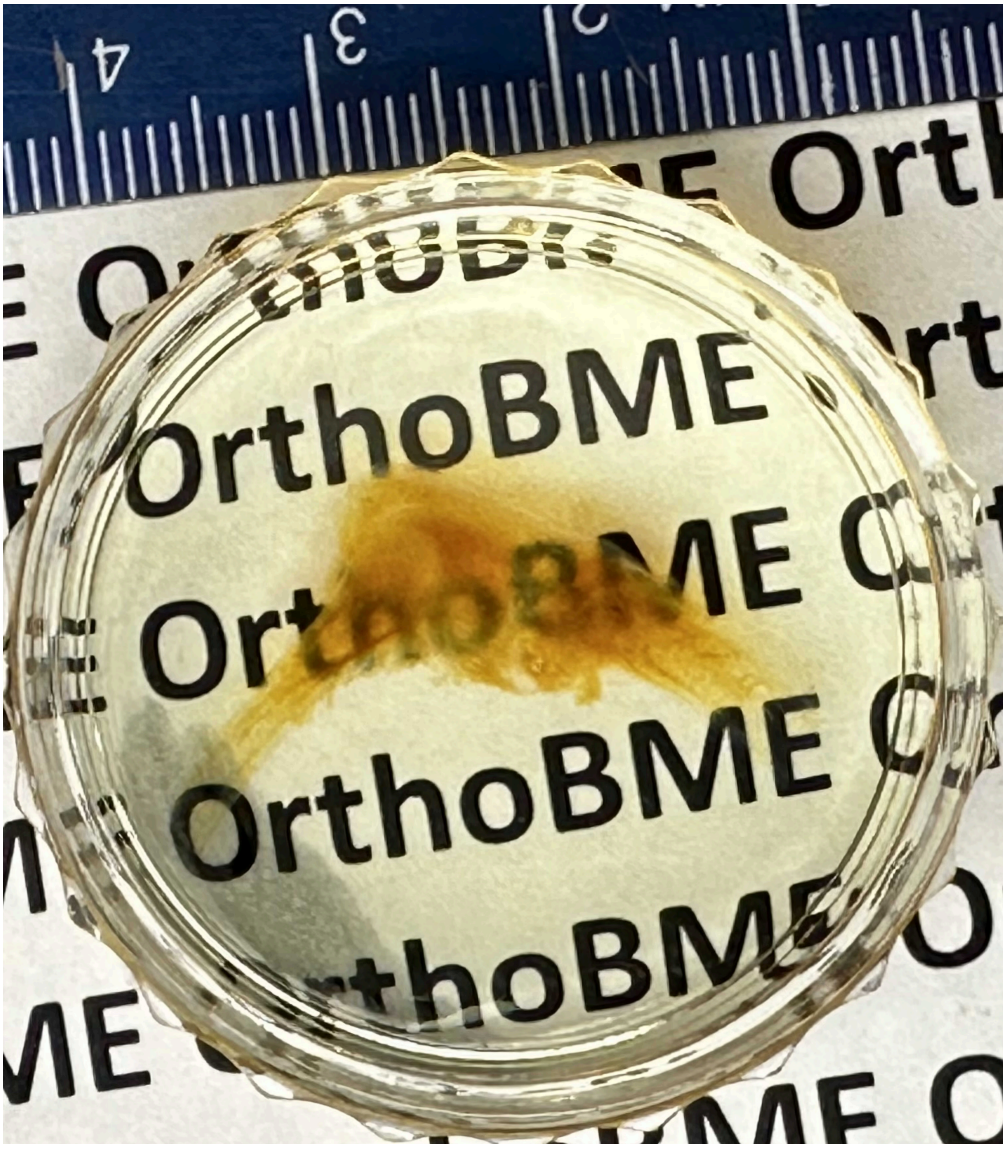

Figure 3. An entire rat knee joint post-clearing.

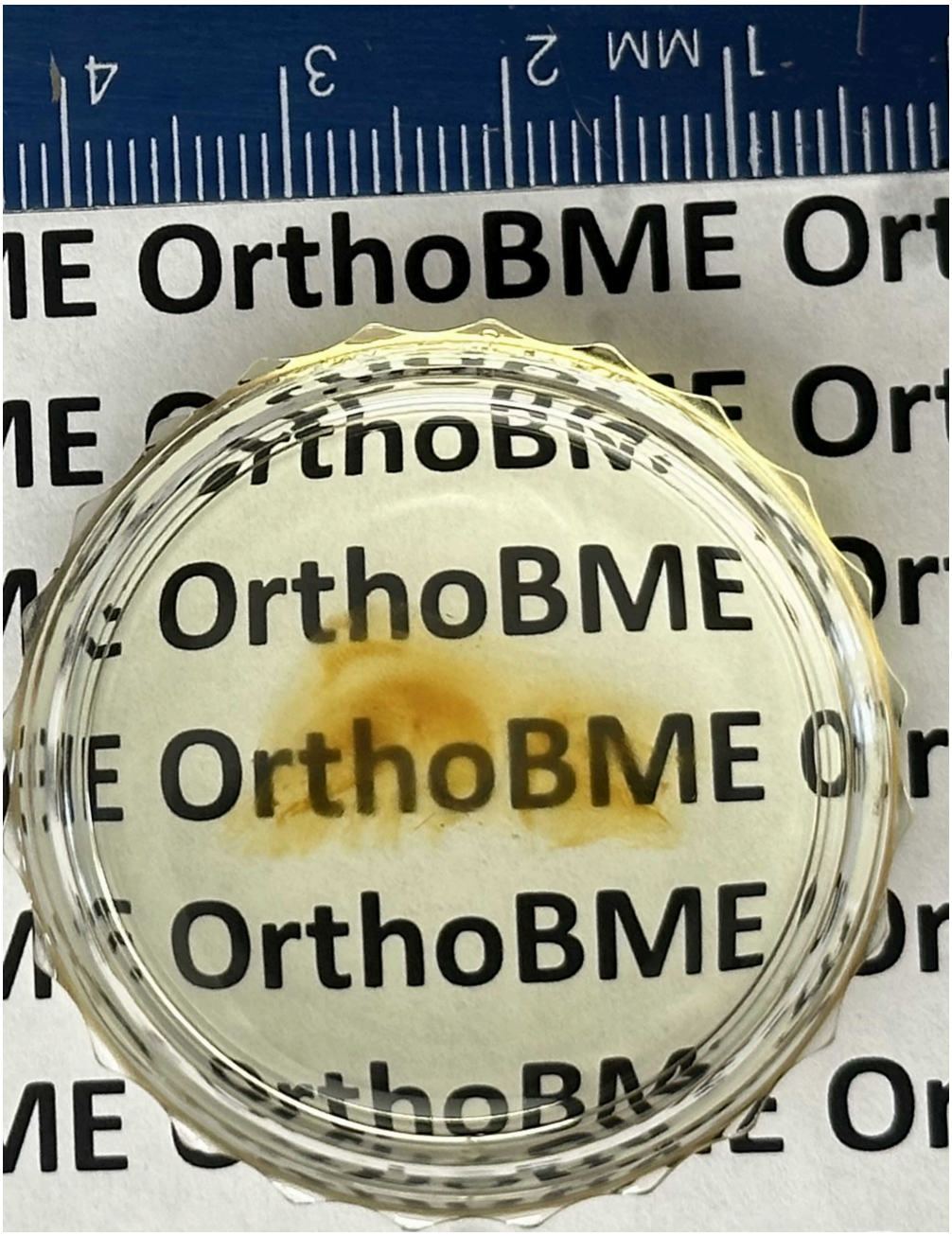

Figure 4. A bisected (in sagittal plane) rat knee post-clearing.

20 Cleared sample(s) can be imaged using confocal and lightsheet microscopy techniques to visualize nerves.

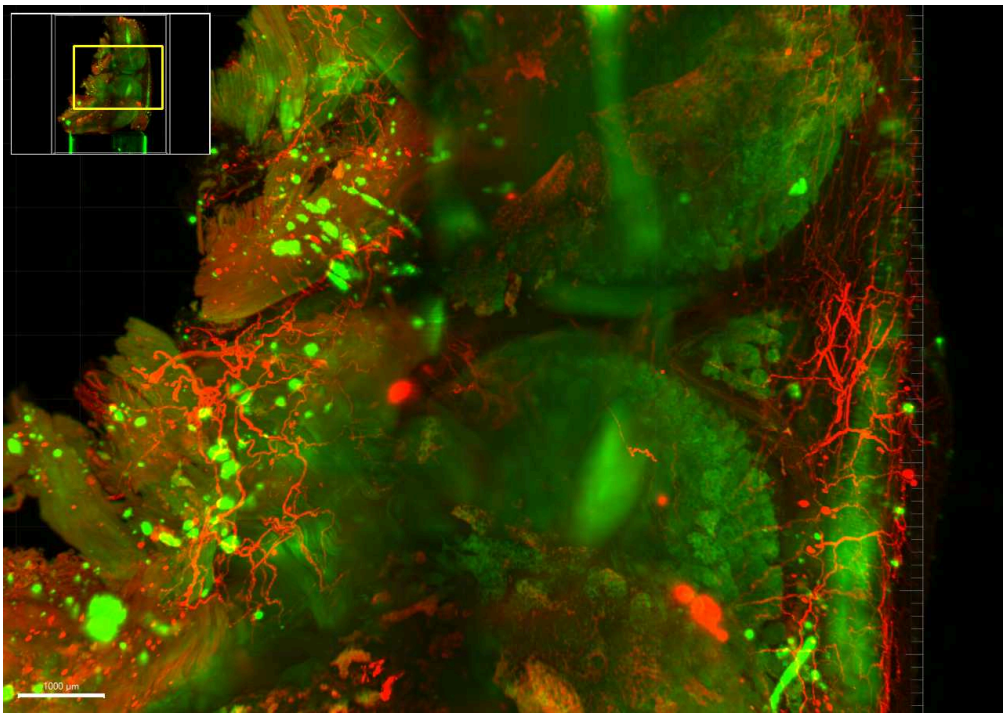

Figure 5. An example of a PEGASOS-cleared rat knee (bisected in a sagittal plane) immunolabeled with neurofilament antibody (red). Green represents autofluorescence. The image was acquired using a MesoSPIM Lightsheet microscope.

Protocol references

1. Jing, D. *et al.* Tissue clearing of both hard and soft tissue organs with the PEGASOS method. *Cell Res.* **28**, 803–818 (2018).

Acknowledgements

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