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KneEZ Clear: an Effective Tissue Clearing Method for Intact Mouse Joints, including the Knee

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Julia Younis[1], Taeyong Ahn[2], Luis Tovias[1], Azeez Ishola[1], Brendan Lee[1], Joshua Wythe[1], Nele Haelterman[1]

[1]Baylor College of Medicine; [2]University of Virginia

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Nele Haelterman
Baylor College of Medicine

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We use this protocol and it's working

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Abstract

KneEZ Clear is a modified version of the EZ Clear method (**Hsu et al., 2022**), optimized for clearing mouse musculoskeletal tissues. KneEZ Clear works well for the clearing of mouse knee and ankle joints, spines, temporomandibular joints, and teeth.

Critical factors that impact the tissue's optical transparency include fixation and decalcification time and temperature, as well as gradual lipid removal.

This protocol includes injection with fluorescently conjugated lectin to label the vasculature, followed by collection of tissue samples, fixation, tissue delipidation, and refractive index matching (e.g. tissue clearing). While lectin-labeled vasculature serves as a good positive control, it can be omitted when one only wants to look at endogenous fluorescence. In this case, proceed directly to transcardial perfusion of PBS and fixative.

To efficiently label the microvasculature and venous endothelium, we employ a combinatorial approach of retroorbital perfusion followed by transcardial perfusion pre-fixation, followed by an additional pulse of diluted lectin post-fixation. This combination results in superior fluorescent labeling of the venous and capillary vasculature, as well as larger diameter arterial and venous vessels.

Image Attribution

Image of a cleared mouse hindlimb (Expected results, left panel), taken By Taeyong Ahn with a Zeiss Z7 lightsheet fluorescent microscope. Image of Tg(PRG4-BAC-CreER);R26(tdtomato/tdtomato) (expected results, right panel) taken by Jason Kirk with an in-house built mesoSPIM.

Materials

	A	B	C
	Name	**Vendor**	**Product Number**
	Ammonium hydroxide, 28-30% solution in water, ~14.8N, FW=35.05	Fisher	# A669-500
	Sodium hydroxide, 10N in aqueous solution	VWR	# BDH7247-1
	EDTA (ethylenediamine tetraacetic acid) free acid, Ultrapure	VWR	# 97061-404
	Lycopersicon Esculentum (Tomato) Lectin, DyLight 649	Thermo Fisher	# L32472
	Sodium phosphate, dibasic, 98%+ extra pure, anhydrous, Na2HPO4, FW=141.96	Sigma	# 567545
	Sodium phosphate, monobasic, NaH2PO4-H2O, FW=119.98	Sigma	# S0751
	Phosphate Buffered Saline, 10X, pH 7.4, 0.119 M Phosphates, 1.37 M NaCl, 0.027 M KC	Fisher	# BP399-20
	Tetrahydrofuran (THF), anhydrous, 250 ppm BHT added, C3H8O, FW=72.11	Sigma	# 186562
	Triethylamine	Sigma	# 471283-500ML
	Urea, NH2CONH2, FW=60.06	Sigma	# U5378
	Hydrogen peroxide solution	Sigma	# H1009-100ML
	Dimethyl sulfoxide	Sigma	# D8414-1L
	Sodium Azide, Hydrazoic acid sodium salt, NaN3, MW=65.01	Sigma	# S2002
	Nycodenz Iohexol, C19H26I3N3O9, FW=821	ProGen	# 18003

	A	B	C
	Paraformaldehyde (PFA), MW=30.03	Sigma	# P6148
	Agarose	VWR	# 0710
	ddH2O	Milli-Q quality	
	Others		
	Dissection tools (scissors, forceps, and hemostat)	Any	
	pH meter	Any	
	WHEATON liquid scintillation vial with attached cap	Sigma	# DWK986546
	SURFLO Winged Infusion Set 25 G x 3/4" (3 1/2 Tubing)	Terumo Medical Products	# SV*25BLS
	15 mL syringe	Any	
	1 mL syringe	Any	
	27 1/2 G needle	Any	
	Insulin syringe (3/10 mL, 5/16" x 31 G)	Any	
	InfusionONE Syringe Pump / 300 Series / 300-US Just Infusion Single Channel	New Era Pump Systems Inc.	# 300
	Large Syringe Kit:: Model P-SYRKIT-LG, Selection of large syringes plus plumbing supplies	New Era Pump Systems Inc.	# P-SYRKIT_LG
	Refractometer	Atago, PAL-RI 3850 from any vendor	
	Nalgene vacuum filtration system filter, 0.2 um pore size	Z370606 from any vendor	
	Light sheet fluorescence microscope sample holder	Instrument specific	

Table: Reagents and Materials used for KneEZ Clear

4% PFA (1 L):

- Weigh and add 40 g of PFA to 250 mL of 1x PBS
- Add 740-1000 µL of 10 N NaOH, swirl bottle to mix
- Incubate the solution at 56°C until PFA is dissolved
- Add stir bar and stir solution continuously

- Increase the total volume to 800 mL with 1x PBS
- pH to 7.4 with HCl or NaOH
- Adjust volume to 1 L with 1x PBS

10% EDTA (1 L):

- Add 60-80 mL of concentrated (28-30%) ammonium hydroxide to 750 mL of 1x PBS
- Add 100 g of EDTA free acid
- Add stir bar and stir solution continuously
- Once EDTA is dissolved, pH to 7.2-7.4 with HCl or ammonium hydroxide
- Adjust volume to 1 L with 1x PBS

EZ View RI Matching Media (125 mL):

Phosphate Buffer Stock Solution (0.1M)

- Add 3.1 g of NaH_2PO4 and 10.9 g of Na_2HPO_4 to 900 mL of ddH_2O.
- Adjust total volume to 1 L with ddH_2O.
- Filter sterilize (<0.45 µm) and store at 4°C.

Phosphate Buffer Working Solution (0.02M)

- Add 100 mL of 0.1 M Phosphate Buffer (PB) stock solution to 400 mL of ddH2O.
- Measure the final solution's pH and adjust to 7.4,
- Store at 4 °C.

EZ View Refractive Index Matching Solution

- Pour 35 mL of 0.02 M PB into a 250 mL beaker and heat to 37 °C. Add stir bar and stir solution continuously.
- Slowly add 52.5 g of urea and 62.5 mg of sodium azide to solution. Wait for all the urea to dissolve.
- Weigh 100 g of Nycodenz iohexol.
- Slowly add ~10 g of Nycodenz iohexol every few minutes, until all 100 g are added.

Note

Nycodenz is slow to dissolve and easily aggregates if added too quickly.
Take care to gradually add small amounts.

- Cover the beaker with aluminum foil and let the solution stir at 37 °C overnight or until the solution is clear.
- Remove the stir bar and adjust the final volume to 125 mL with 0.02 M PB.

- Filter the solution with a ≤0.45 µm vacuum filtration system.
- Measure the refractive index with a refractometer.
- The RI should be between 1.512 and 1.519.

Safety warnings

This protocol needs prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

Tetrahydrofuran is known to degrade plastics and should only be handled in glass containers under a fume hood.

Ethics statement

Procedures involving animal subjects were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were conducted according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine.

Retro-orbital Injection

1 Deeply anesthetize the mouse via inhalation of 4-5% isoflurane to induce anesthesia. Toe pinch to confirm animal is not receptive to painful stimuli.

2 Using an insulin syringe, inject 50 µL of conjugated lectin to the retro-orbital sinus of the right and left eye. Allow the mouse to regain consciousness and mobility, and wait a minimum of 15 minutes before starting the transcardial perfusion procedure to allow lectin to move through the venous and capillary circulation.

Note

Leave the needle in place for a few seconds after injection to prevent the lectin from escaping.

Transcardial Injection and Perfusion

3 Deeply anesthetize the mouse via inhalation of 4-5% isoflurane to induce anesthesia. Toe pinch to confirm animal is not receptive to painful stimuli.

4 On a Styrofoam board, fix the mouse in place by inserting 30-gauge syringes or needles into the paws and board.

5 Spray or wipe the ventral abdominal area of the mouse with 70% ethanol to sterilize the field and prevent fur from entering the chest cavity.

6 Use forceps to lift the skin, then make a lateral incision across the skin of the abdomen using scissors.

7 Cut the skin down the midline of the mouse to the top of the thoracic cavity.

8 With forceps, pull back both flaps of skin to expose the peritoneum.

9 Make a lateral incision across the parietal peritoneum to expose the liver and diaphragm.

10 With small scissors, cut the diaphragm (in a crescent shape, along the ribs, careful to not accidentally puncture the lungs or heart) to expose the thoracic cavity.

11 Make two upward cuts on the left and right side of the rib cage to create a flap that can be lifted to better access the heart. Using a hemostat, grip and secure the flap opening to provide access to the chest cavity.

12 Using an insulin syringe, slowly pierce the left ventricular wall of the heart from the apex of the heart, being careful not to pierce the septum or enter the right ventricle and inject 100 µL
of conjugated lectin to the left ventricle.

Note

Inject at a slow, constant rate so a bolus does not form in the heart. The plunger can be pushed at a slight angle against the syringe wall to better control rate.

Note

Leave the needle in the heart for 1 minute as the lectin circulates.

13 Allow the lectin to circulate for 1-2 minutes.
With small scissors, snip open the right atrium to allow for exsanguination in subsequent steps.

14 Using a syringe pump set to a rate of 4 mL/min connected to a 25-gauge 3/4" winged infusion needle, inject 5 mL of warm (37°C) 1x PBS to the left ventricle to remove the blood. Insert the needle in the puncture made by the insulin syringe (if possible) while stabilizing the heart with blunt forceps. Make sure the needle is angled towards the aorta to prevent lung inflation. When the fluid exiting the mouse is clear of blood, proceed to the next step.

Note

If lectin is not being used, the volume of PBS can increase to 10 mL.

15 Using a syringe pump set to a rate of 4 mL/min connected to a 25-gauge 3/4" winged infusion needle, inject 5 mL of room temperature, freshly prepared 4% PFA to the left

ventricle to fix all tissues. Insert the needle into the puncture hold made in previous steps. Creating a new puncture may cause fluids to leak out.

Note

Muscle contractions and blanching of the liver and mesenteric blood vessels are all signs of successful perfusion.

16 Using a 27.5-guage needle, slowly inject 1 mL of diluted lectin (1:10 dilution in 1x PBS) to the left ventricle. Insert the needle in the puncture hole made in previous steps.

Sample Harvest

16h

17 Remove the skin from the lower half of the mouse. Be careful to not let hairs get on the sample.

18 Place mouse on its abdomen and have its dorsal side face you. With scissors or a razor blade, cut through the femur, close to the hip joint, to remove the hindlimbs from the rest of the body.

Note

To quickly assess for successful vascular labeling, the hindlimb and/or brain can be placed under an epifluorescent microscope. Zoom in to the sample and check that lectin-labeling is present in both large and small diameter vessels.

19 Collect samples in ice-cold 1x PBS.

Note

If the dissection will take longer than 30 minutes, collect samples in ice-cold 4% PFA for better sample preservation.

20 Drop-fix the hindlimb samples in 4% PFA at 4 °C overnight (16:00:00) with gentle agitation

16h

Note

The ratio of fixative to tissue (volume:volume) is essential for adequate fixation. We typically aim for a 20:1 ratio of fixative to tissue. A trimmed hindlimb can be processed in a 50 mL conical tube, but be aware that plastic is often not compatible with the use of downstream
organic solvents or delipidation steps.

Note

Cover samples in foil to prevent bleaching or loss of fluorescent signals for future steps.

Decalcification

5d 6h

21 Wash fixed samples with 1x PBS (3 times for 01:00:00 per wash) under gentle agitation. 3h

22 Transfer samples to 10% EDTA in PBS, and incubate at room temperature, under gentle agitation, for 2 to 5 days, changing the EDTA solution every other day. 5d

Note

Samples from mice under 3 months of age can be decalcified in 2 days, while samples from older mice can require up to 5 days. Confirm that samples are fully decalcified with x-ray before proceeding.

23 Wash decalcified samples with 1x PBS (3 times for 01:00:00) at room temperature under gentle agitation 3h

Methanol Pretreatment and Heme Removal

1d 3h

24 Dehydrate samples with 50% methanol in 1x PBS (x1), 80% methanol (x1), and 100% methanol (x2) for 01:00:00 each step at room temperature with gentle agitation. 4h

25 Bleach samples with 5% H_2O_2 + 20% DMSO in methanol (1 vol 30% H_2O_2 / 1 vol DMSO / 4 vol methanol, ice cold) at 4 °C for 16:00:00 with gentle agitation to remove heme and decolorize tissue. 16h

> Note
>
> If samples are bleached for longer than 16 hours, lectin signal can decrease.

26 Sequentially rehydrate samples with 100% methanol (x2), 80% methanol in 1x PBS (x1), 50% methanol (x1), 1x PBS (x2) for 01:00:00 each step with gentle agitation. 6h

Lipid Removal

27 Place samples in 50% THF (in ddH_2O) with triethylamine, incubate Overnight at room temperature with gentle agitation. 1d

50% THF + triethylamine (20 mL):
10 mL THF, 20 uL triethylamine, adjust volume to 20 mL with ddH_2O

> Note
>
> Be sure to use glass scintillation vials with aluminum-lined caps when performing these steps, as THF degrades plastics.

28 Sequentially incubate samples in 70% THF (x1), 80% THF (x1), and 90% THF (x2), for 12:00:00 each step at room temperature with gentle agitation. 2d

70% THF + triethylamine (20 mL):
14 mL THF, 30 uL triethylamine, adjust volume to 20 mL with ddH_2O

80% THF + triethylamine (20 mL):
16 mL THF, 44.8 uL triethylamine, adjust volume to 20 mL with ddH_2O

90% THF + triethylamine (20 mL):
18 mL THF, 64 uL triethylamine, adjust to 20 mL with ddH_2O

Note

The incubation periods can be extended to up to 24 hours with maintained lectin signal.

29 Remove THF and wash samples with ddH_2O (4 times for 01:00:00) at room temperature with gentle agitation. 4h

Clearing

30 Remove as much H_2O as possible from the sample. 10m

Note

Let samples sit in a fume hood for 10 minutes. Alternatively, a Kimwipe can be used to gently dab the tissue to make this step faster. Avoid Kimwipes if the goal is to image and/or analyze surface vessels or neurons.

31 Incubate the tissue in EZ View solution at room temperature with gentle agitation for 24-48 hours to render the tissue transparent. 2d
Important: Avoid cold temperatures to prevent the urea from crystallizing

Imaging 1d

32 If imaging using a Zeiss Z.1 LSFM system, trim excess muscle from the sample and mount in
1% filtered agarose made in ddH_2O with a sample holder embedded at the top of sample after clearing. For this, you can use a 3 or 5 mL syringe with the top cut off for mounting as shown below.

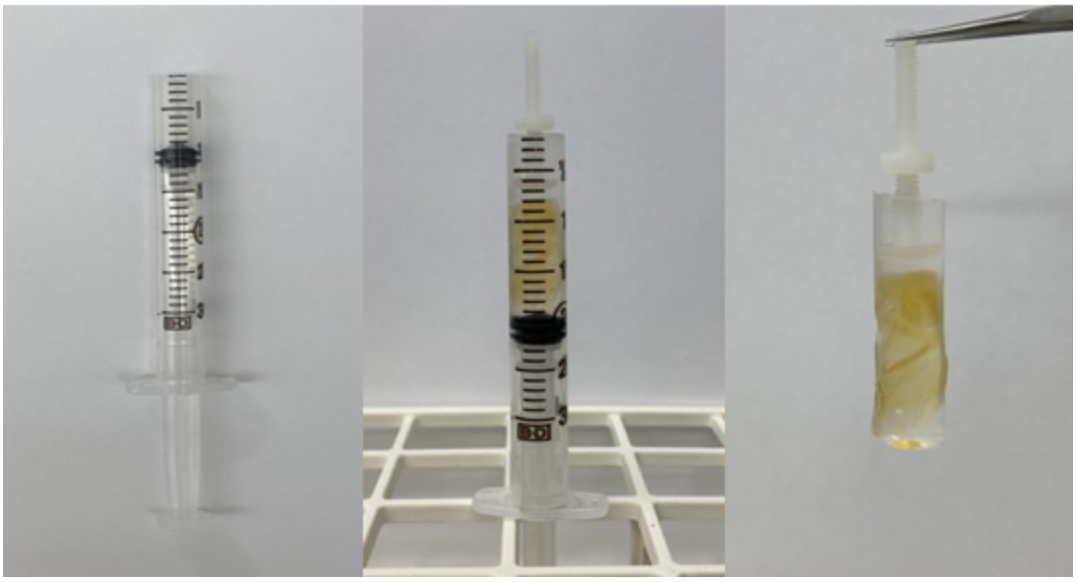


Image displaying cleared mouse hindlimb, embedded in agarose and mounted for imaging on a Zeiss Z1 LSFM

33 Once agarose solidifies, re-equilibrate in EZ View for an additional 24:00:00 at room temperature with gentle agitation. If using the Zeiss Z1 system, a minimum of 25 mL of EZ View is required for imaging. 1d

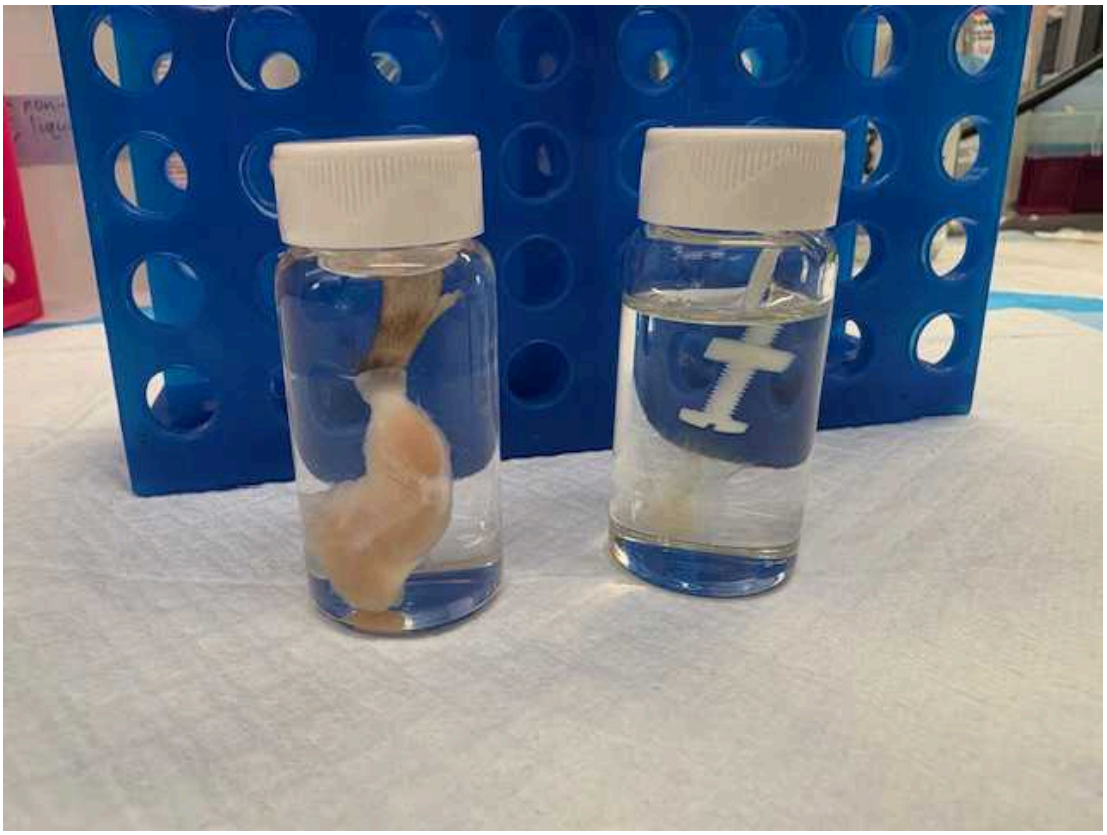
Note

Up to 3 mounted samples can be put in 40-50 mL of EZ View to re-equilibrate. More than 3 samples will make the RI of the EZ View media too low for adequate clearing.

34 Store sample in EZ View solution before, during, and after imaging.

Expected Results

35 Below are two images of KneEZ Cleared adult mouse knee joints (12-14 weeks old):



Left: fixed, uncleared mouse (C57Bl6/J) hindlimb
Right: fixed, KneEZ Cleared mouse (C57Bl6/J) hindlimb, attached to the holder (white) for wholemount imaging.