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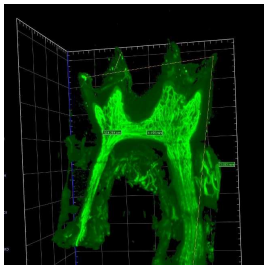
Tooth Clearing with KneeEZ



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Protocol status: Working
We use this protocol and it's working

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Abstract

KneeEZ is a robust method for clearing highly calcified tooth structures. In the Emrick Lab, it can be used to clear extracted tooth samples. In this application, the #D visualization of neuronal endings can be achieved via immunolabeling. Additionally, blood vasculature morphology can be elucidated through lectin labeling. This protocol has been optimized for optical transparency in the tooth in regards to decalcification time.

Attachments

PDF

KneeEZ Protocol for ...

89KB

Materials

Name	Vendor	Catalog Number
32% Paraformaldehyde Aqueous Solution, EM Grade	Electron Microscopy Sciences	15714-S
PBS, Phosphate Buffered Saline, 10X Solution, Fisher BioReagents	Fisher Scientific	BP3991
Lycopersicon Esculentum (Tomato) Lectin (LEL, TL), DyLight 649	Thermo Fisher	L32472
Invitrogen UltraPure 0.5 EDTA, pH 8.0	Thermo Scientific	15575020
Anti-beta III Tubulin antibody - Neuronal Marker	Abcam	#18207
Phosphate Buffer Solution	Sigma Aldrich	P3619
Glycine	Sigma Aldrich	G7126
Heptakis(2,6-di-O-methyl)-B-cyclodextrin	Santa Cruz Biotechnology	CAS 51166-71-3
Nycodenz - Iohexol	Progen	18003
Tetrahydrofuran (THF), anhydrous, 250 ppm BHT added C3H8O FW=72.11	Sigma	#186562
Urea (NH2CONH2)FW=60,06	Sigma	#U5378
ddH2O	MiliQ	
WHEATON liquid scintillation vial with attached cap	Millipore-Sigma	DWK986546
Nalgene vacuum filtration system filter, 0.2 um pore size	Sigma-Aldrich	Z370606
Hu-Friedy #½ DE Hollenback Carver with Regular Handle	Net 32	CVHL1/2

Before start

Solution Preparation:

500 mL of 10% weight/volume EDTA

1. Measure out 268.7 mL of 0.5M EDTA
2. Add appropriate amount of 1X PBS solution to get final volume of solution to 500mL
3. Store at room temperature

Working Phosphate Buffer Solution (0.02M)

1. Add 50 mL of 1M Phosphate Buffer (PB) stock solution to 950 mL of MiliQ ddH2O
2. Store at 4C

EZ View Solution:

1. Obtain a 250 mL glass beaker, stir bar, hot plate, and thermometer
2. Pour 35 mL of 0.02 M PB solution into the beaker and adjust the solution temperature to 37C
3. In order to achieve this temperature change the hotplate heat setting to around 1.2
4. Add the stir bar to the solution at this point. As the solution becomes more viscous, slowly increase the bar's spinning velocity
5. Measure out 52.5g urea and slowly add it to the stirring solution (over the course of 2-3 minutes). Then add 31.25 mg of sodium azide. There will be an immediate temperature drop after adding both solutes. Allow the temperature to equilibrate back to 37C and wait for complete dissolution
6. Weigh 100g of Nycodenz iohexol
7. Vacuum filter and place in container
8. Adjust to final volume of 125 mL with 0.02 PB after solution is clear and filtered. Good at room temperature for up to 2 months

Retro-orbital Injection (Day 1)

1 Anesthetize the mouse via induction at 5%

1.1 Toe pinch to ensure that the mouse is unresponsive

2 Wash samples with PBS (3x60 minutes) with gentle agitation at room temperature

3 Transfer to 10% w/v EDTA with gentle agitation at room temperature

4 Change the solution every two days for a combined total of 6 days

5 Wash decalcified teeth in PBS (3x1 hour) at room temperature with gentle agitation

6 Take the mouse out of the induction chamber and immediately inject 100 uL of Lectin DyLight 647 into both the left and right retro-orbital sinus

7 Allow the mouse to regain consciousness and wait 5 minutes before beginning perfusion

7.1 This allows for vascular diffusion of lectin

Transcardial Injection and Perfusion (Day 1)

8 100 uL of Lectin DyLight 647 into the left ventricle followed by perfusion

Sample Harvest

9 Extract molars from mouse

9.1 View video example

10 Collect the sample in ice cold PBS

11 Drop-fix teeth in 4C PFA overnight at 4C on the shaker (gently agitation)

Decalcification (Day 2 through Day 8)

12 Wash samples with PBS (3x60 minutes) with gentle agitation at room temperature

13 Transfer to 10% w/v EDTA with gentle agitation at room temperature

13.1 Change the solution every two days for a combined total of 6 days

14 Wash decalcified teeth in PBS (3x1 hour) at room temperature with gentle agitation

Delipidation and RI Matching (Day 8 through Day 12)

15 Place teeth in 50% THF (diluted from 100% with MiliQH2O) at room temperature overnight with gentle agitation (shaker)

15.1 Use glass scintillation vials to avoid plastic degradation and continue to use for future steps

16 Incubate sample in 70% THF for 12 hours

17 Incubate sample in 80% THF for 12 hours

18 Incubate sample in 100% THF for 2x12 hours

18.1 All at room temperature with gentle agitation

19 Wash samples with MiliQH2O (4x1 hour) at room temperature

19.1 After washing, remove as much water in the sample as possible. This is to avoid H2O interaction with the next step (RI Matching)

19.2 Place the teeth in the fume hood for 5 minutes to allow water to dissipate

20 Incubate in EZ Clear solution for 48 hours at room temperature with gentle agitation

20.1 Note 1: EZ Clear solution should be made at least the a couple of days prior to this step to ensure that the EZ Clear solution does not crystalize

20.2 Note 2: after this step, the teeth will become transparent and difficult to visualize. We can incorporate agarose embedding to avoid this issue in the future. For now, marking the cap on the scintillation vial with the amount of teeth in each bottle will ensure that you know the amount of teeth you will need to image

Immunohistochemistry (Day 13 through Day 26)

21 Incubate samples in permeabilization solution (1X PBS + 0.2% Triton X-100 + 20% DMSO + 0.3 M glycine + 0.05% sodium azide) at room temperature for 2 days with gentle agitation

22 Incubate in blocking solution (1X PBS + 0.2% Triton X-100 + 10% DMSO + 6% Donkey/Goat Serum) at room temperature for 2 days

23 Wash samples in PtwH (1X PBS + 0.2% Tween-20) (2x1 hour)

24 Incubate with primary antibody of choice in primary antibody incubation solution (PTwH + 5% DMSO + 3% Donkey/Goat Serum + 1% cyclodextrin) at room temperature for 4 days

24.1 Rabbit anti Beta-Tubulin-III (TUJ1), 1:500

25 Wash samples with PtwH at room temperature and exchange wash solutions 4-5 times until the following day

26 Incubate with secondary antibody in incubation solution (PTwH + 3% Donkey/Goat Serum + 1% cyclodextrin) at room temperature for 4 days

26.1 Goat anti-rabbit 555 (1:1000)

27 Wash in PtwH (3x2 hours) at room temperature

28 Wash with 1X PBS (3x1 hour) at room temperature

29 Wash overnight with 1X PBS at room temperature

30 Incubate in 1X PBS with 0.05% sodium azide for 24 hours at room temperature

2nd RI Matching (Day 27 through Day 28)

31 Wash samples with MiliQH2O (4x1 hour) at room temperature (does this need to happen post IHC, it's not expressly written in the protocol?)

32 After washing, remove as much water in the sample as possible. This is to avoid H2O interaction with the next step (RI matching)

33 Place the teeth in the fume hood for 5 minutes

34 Incubate in EZ clear solution for 48 hours at room temperature with gently agitation

Protocol references

A modified version of the KneeEZ clearing protocol developed by Nele Haelterman's group