May 24, 2024

Labeling of microtubules using mouse anti-β-tubulin primary monoclonal antibody with secondary Fe-TAML-peg4-Cy5-goat anti-mouse IgG conjugate and oxidation of DAB with H2O2 for light and transmission electron microscopy

DOI
https://dx.doi.org/10.17504/protocols.io.261ge5dpdg47/v1

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.261ge5dpdg47/v1

External link: https://www.biorxiv.org/content/10.1101/2023.08.25.554352v2

Protocol Citation: Mason Mackey, Mark Ellisman, Stephen Adams 2024. Labeling of microtubules using mouse anti-β-tubulin primary monoclonal antibody with secondary Fe-TAML-peg4-Cy5-goat anti-mouse IgG conjugate and oxidation of DAB with H2O2 for light and transmission electron microscopy. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261ge5dpdg47/v1

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

Created: May 17, 2024

Last Modified: May 24, 2024

Protocol Integer ID: 100230

Keywords: TEM, DAB oxidation, oxidation reaction, Correlated Light and Electron Microscopy, Fe-TAML, small molecule peroxidase , tubulin primary monoclonal antibody with secondary fe, labeling of microtubule, microtubule, antibody, primary monoclonal antibody, tubulin, transmission electron microscopy this protocol, oxidation of dab, transmission electron microscopy, using mouse

Funders Acknowledgements:
R01
Grant ID: GM138780 (MHE)
R01
Grant ID: GM086197 (SRA/DB),
R35
Grant ID: GM128859 (JTN)
NSF
Grant ID: 2014862 (MHE)
R01
Grant ID: AG081037 (MHE)

Abstract

This protocol details the labeling of microtubules using mouse anti-β-tubulin primary monoclonal antibody with secondary Fe-TAML-peg4-Cy5-goat anti-mouse IgG conjugate and oxidation of DAB with H_2O_2 for light and transmission electron microscopy.

Materials

CSB buffer:

	A	B
	Pipes buffer	10 mM
	NaCl	150 mM
	EGTA	5 mM
	MgCl2	5 mM
	Glucose monohydrate, pH 6.8	5 mM

- Anti-β-Tubulin antibody, Mouse monoclonal, clone TUB 2.1 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T5201
- 3,3′-Diaminobenzidine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8001- 10G
- Durcupan™ ACM **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**44610
- Fe-TAML-peg4-Cy5-goat anti-mouse IgG
- BSA **Sigma Catalog #A8022-100G**

Labeling of microtubules

2d 17h 59m

1 Culture HEK293T cells on MatTek plates containing poly-D-lysine coated glass bottom No.0 coverslips in DMEM supplement with 10% fetal bovine serum.

2 Rinse the cells (x3) with cytoskeleton stabilizing buffer, at 37 °C and fixed with 4% paraformaldehyde (19202, Electron Microscopy Sciences) and 0.05% glutaraldehyde (16220, Electron Microscopy Sciences) in CSB at 37 °C for 00:05:00 and for 00:25:00 at 4 °C. 30m

CSB buffer:

	A	B
	Pipes buffer	10 mM
	NaCl	150 mM
	EGTA	5 mM
	MgCl2	5 mM
	Glucose monohydrate, pH 6.8	5 mM

3 After fixation, first wash the cells with CSB (5 × 1 min) at 4 °C.

3.1 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (1/5) 1m

3.2 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (2/5) 1m

3.3 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (3/5) 1m

3.4 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (4/5) 1m

3.5 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (5/5) 1m

4 Treat with 0.1% saponin and 0.05% glycine in CSB for 20 mins at 4 °C while on a rocker.

5 Wash the cells in CSB buffer with 0.05% glycine (3 × 1 min) at 4°C.

5.1 Wash the cells in CSB buffer with 0.05% glycine 00:01:00 at 4 °C. (1/3) 1m

5.2 Wash the cells in CSB buffer with 0.05% glycine 00:01:00 at 4 °C. (2/3) 1m

5.3 Wash the cells in CSB buffer with 0.05% glycine 00:01:00 at 4 °C. (3/3) 1m

6 Block the cells with 1% BSA (A8022-100G, Sigma), 1% normal goat serum (NGS) and 0.05% glycine in CSB for 00:20:00 at 4 °C. 20m

7 Incubate the cells with primary mouse monoclonal antibody to β-tubulin (300-fold dilution, clone Tub2.1, T5201, Sigma) for 03:00:00 at 4 °C in 1% BSA, 1% NGS and 0.05% glycine in CSB buffer. 3h

8 Remove primary antibody and wash with 1% BSA, 1% NGS and 0.05% glycine in CSB (5 x 3 min) at 4°C.

8.1 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (1/5) 3m

8.2 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (2/5) 3m

8.3 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (3/5) 3m

8.4 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C . (4/5) 3m

8.5 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C . (5/5) 3m

9 Incubate the cells with secondary Fe-TAML-peg4-Cy5-goat anti-mouse IgG conjugate in 1% BSA (0.15 ml diluted to 1ml) in 1% NGS and 0.05% glycine in CSB for Overnight at 4 °C . [Adams et al., 2023]. 8h

10 Then wash the cells (5 × 1 min) with CSB at 4°C.

10.1 Wash the cells for 00:01:00 with CSB at 4 °C . (1/5) 1m

10.2 Wash the cells for 00:01:00 with CSB at 4 °C . (2/5) 1m

10.3 Wash the cells for 00:01:00 with CSB at 4 °C . (3/5) 1m

10.4 Wash the cells for 00:01:00 with CSB at 4 °C . (4/5) 1m

10.5 Wash the cells for 00:01:00 with CSB at 4 °C . (5/5) 1m

11 Fix the cells with 2% glutaraldehyde in CSB for 00:20:00 at 4 °C . 20m

12 Wash the cells (5 × 1 min) with CSB at 4°C.

12.1 Wash the cells for 00:01:00 with CSB at 4 °C . (1/5) 1m

12.2 Wash the cells for 00:01:00 with CSB at 4 °C . (2/5) 1m

12.3 Wash the cells for 00:01:00 with CSB at 4 °C . (3/5) 1m

12.4 Wash the cells for 00:01:00 with CSB at 4 °C . (4/5) 1m

12.5 Wash the cells for 00:01:00 with CSB at 4 °C . (5/5) 1m

13 Image the cells for fluorescence labeling.

14 Dissolve and add 5.4 mg of 3,3′- Diaminobenzidine (DAB) (D8001-10G, Sigma-Aldrich) in 1.0 mL of [M] 0.1 Mass Percent HCl and 9.0 mL of [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 with 10 µL H_2O_2 (final, [M] 40 millimolar (mM) from 30% stock) to the DAB solution.

15 Wash the cells (2 × 2 min) with 50 mM Bicine 100 mM NaCl pH 8.3 at 4°C.

15.1 Wash the cells for 00:02:00 with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 at 4 °C . (1/2) 2m

15.2 Wash the cells for 00:02:00 with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 at 4 °C . (2/2) 2m

16 Add the DAB/H_2O_2 solution to the cells by a 0.22µm Millex 33mm PES sterile filter (SLGSR33RS, Sigma-Aldrich) at Room temperature . Reaction time is 01:00:00 - 01:30:00 . 1h 30m

17 Remove the DAB solution, and wash the cells with 50 mM Bicine 100mM NaCl pH 8.3 (2 × 2 min) on ice.

17.1 Wash the cells with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 for 00:02:00 On ice . (1/2) 2m

17.2 Wash the cells with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 for 00:02:00 On ice . (2/2) 2m

18 Wash the cells with 0.1 M sodium cacodylate buffer pH 7.4 (3 × 2 min) on ice.

18.1 Wash the cells with [M] 0.1 Mass Percent sodium cacodylate buffer pH 7.4 for 00:02:00 . (1/3) 2m

18.2 Wash the cells with [M] 0.1 Mass Percent sodium cacodylate buffer pH 7.4 for 00:02:00 . (2/3) 2m

18.3 Wash the cells with [M] 0.1 Mass Percent sodium cacodylate buffer pH 7.4 for 00:02:00 . (3/3) 2m

19 Do a final primary fixation with 2% glutaraldehyde in [M] 2 millimolar (mM) $CaCl_2$ [M] 0.1 Mass Percent sodium cacodylate pH 7.4 for 00:30:00 at 4 °C . 30m

20 Remove the fixative and wash the cells (5 × 2 min) with 0.1 M sodium cacodylate (18851, Ted Pella) pH 7.4 at 4°C.

20.1 Wash the cells for 00:02:00 with [M] 0.1 Mass Percent sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C . (1/5) 2m

20.2 Wash the cells for 00:02:00 with [M] 0.1 Mass Percent sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C . (2/5) 2m

20.3 Wash the cells for 00:02:00 with [M] 0.1 Mass Percent sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C. (3/5) 2m

20.4 Wash the cells for 00:02:00 with [M] 0.1 Mass Percent sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C. (4/5) 2m

20.5 Wash the cells for 00:02:00 with [M] 0.1 Mass Percent sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C. (5/5) 2m

21 All cells are post-fixed with 1% osmium tetroxide (19150, Electron Microscopy Sciences) containing 0.8% potassium ferrocyanide, [M] 2 millimolar (mM) $CaCl_2$ and in [M] 0.1 Mass Percent sodium cacodylate pH 7.4 for 00:30:00 at 4 °C. 30m

22 Wash the cells (5 × 2 min) with ddH_2O at 4°C.

22.1 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (1/5) 2m

22.2 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (2/5) 2m

22.3 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (3/5) 2m

22.4 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (4/5) 2m

22.5 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (5/5) 2m

23 Dehydrate the cells by an ice-cold graded dehydration ethanol series of 20%, 50%, 70%, 90%, 100% (anhydrous) for 00:01:00 each and 3 × 100% (anhydrous) at Room temperature for 00:01:00 each. 2m

24 Infiltrate the cells with one-part Durcupan ACM epoxy resin (44610, Sigma-Aldrich) to one-part anhydrous ethanol (1:1) for 00:30:00 . 30m

25 Infiltrate the cells 3 times with 100% Durcupan resin for 01:00:00 each. 1h

26 Do a final change of Durcupan resin and immediately place cells in a vacuum oven at 60 °C for 48:00:00 to harden. 2d

Protocol references

Adams, Stephen R., et al. "Fe-TAMLs as a new class of small molecule peroxidase probes for correlated light and electron microscopy." *bioRxiv* (2023): 2023-08.