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Labeling and DAB oxidation of EdU-treated HEK293T cells with Cy5 azide and Fe-TAML azide for light and transmission electron microscopy

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We use this protocol and it's working.

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Disclaimer

This protocol is provided for informational purposes only and should be performed by individuals trained in laboratory techniques and safety procedures. The authors and publishers of this protocol do not assume any responsibility for accidents or damage resulting from the use of this protocol. Users are encouraged to consult additional references and relevant safety data sheets for specific reagents and procedures employed in this protocol.

Abstract

This protocol outlines the click chemistry labeling of EdU-pulsed DNA with Fe-TAML azide to catalyze DAB oxidation by hydrogen peroxide to generate localized osmiophilic precipitate detectable by light and electron microscopy.

Materials

Click Buffer

	A	B
	0.1M NaCl	0.29 g
	50 mM HEPES pH 7.4	0.595 g
	0.1% Saponin	50 mg
	Double Distilled Water	50 mL
	1N Sodium Hydroxide	1.25 mL

Before start

Prior to implementing this protocol, ensure all materials and reagents are prepared according to the specified concentrations and conditions. Adhere strictly to the indicated time frames and temperatures during each step to achieve optimal results. Perform all procedures in a designated laboratory space equipped with appropriate safety measures and waste disposal systems.

1 HEK293T cells were plated onto MatTek dishes containing 35mm glass bottom No. 0 coverslips coated with poly-d-lysine.

2 The next day, 10 µM EdU is added to the cells and incubated for 12:00:00 12h

3 Cells are fixed with 2% glutaraldehyde (16220, Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (18851, Ted Pella), pH 7.4 containing 2 mM $CaCl_2$ for 00:05:00 minutes at 37 °C and then at 4 °C for 00:55:00 . 1h

4 Remove fixative and wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ (5 x 1 min) at 4 °C

4.1 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (1/5) 1m

4.2 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (2/5) 1m

4.3 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (3/5) 1m

4.4 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (145) 1m

4.5 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (5/5) 1m

5 Wash cells with PBS pH 7.4 (2 x 1 min) at room temperature.

5.1 Wash cells with PBS pH 7.4 for 00:01:00 at room temperature. (1/2) 1m

5.2 Wash cells with PBS pH 7.4 for 00:01:00 at room temperature. (2/2) 1m

6 Rinse cells (2 x 1 min) with filtered 1% BSA in PBS pH 7.4 at room temperature.

6.1 Rinse cells for 00:01:00 with filtered 1% BSA in PBS pH 7.4 at room temperature. (1/2) 1m

6.2 Rinse cells for 00:01:00 with filtered 1% BSA in PBS pH 7.4 at room temperature. (2/2) 1m

7 Carry out the Click reaction of the cells at room temperature and protect them from light. Use a mixture of 1.0 µM Cy5 azide and 28 µM Fe-TAML azide solution freshly prepared from 900 µl click buffer, 10.0 µl CuSO4 (100 mM in water), and the reaction initiated with 100 μl of freshly prepared aqueous sodium ascorbate (100 mM).

	A	B
	10 mM Cy5 azide	1.0 µl
	28 µM Fe-TAML azide	1.0 µl
	Click buffer (see materials)	900 µl
	100 mM CuSO4	10.0 µl
	100 mM aqueous sodium ascorbate	100 µl

8 After 30 minutes, a second 100 µl aliquot of newly prepared aqueous sodium ascorbate (100 mM) is added for another 00:30:00 incubation. 30m

9 Quickly quick rinse cells twice with filtered 1% BSA in PBS pH 7.4 at room temperature.

10 Wash cells with PBS pH 7.4 (5 x 1 min) at room temperature.

10.1 Wash cells with PBS pH 7.4 for 00:01:00 at room temperature. (1/5) 1m

10.2 Wash cells with PBS pH 7.4 for 00:01:00 at room temperature. (2/5) 1m

10.3 Wash cells with PBS pH 7.4 for 00:01:00 at room temperature. (3/5) 1m

10.4 Wash cells with PBS pH 7.4 for 00:01:00 at room temperature. (4/5) 1m

10.5 Wash cells with PBS pH 7.4 for 00:01:00 at room temperature. (5/5) 1m

11 Collect fluorescence imaging of the labeled cells with Cy5 azide.

12 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 (5 x 1 min).

12.1 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (1/5) 1m

12.2 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (2/5) 1m

12.3 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (3/5) 1m

12.4 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (4/5) 1m

12.5 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (5/5) 1m

13 5.4 mg of 3,3'- Diaminobenzidine (DAB) (D8001-10G, Sigma-Aldrich) is dissolved in 1.0 ml of 0.1 N HCl and 9.0 ml of 50 mM Bicine 100 mM NaCl pH 8.3 was added with 10 µl H_2O_2 (final, 40 mM from 30% stock) to the DAB solution.

14 Add the DAB/H2O2 solution to the cells by a 0.22µm Millex 33mm PES sterile filter (SLGSR33RS, Sigma-Aldrich) at room temperature. Reaction time is between 00:15:00 and 00:30:00 . 45m

15 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 (3 x 1 min).

15.1 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (1/3) 1m

15.2 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (2/3) 1m

15.3 Wash cells with 100 mM NaCl 50 mM Na·Bicine pH 8.3 for 00:01:00 . (3/3) 1m

16 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ (5 x 1 min) at 4 °C .

16.1 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (1/5) 1m

16.2 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (2/5) 1m

16.3 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (3/5) 1m

16.4 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (4/5) 1m

16.5 Wash cells with 0.1 M sodium cacodylate buffer pH 7.4 containing 2 mM $CaCl_2$ for 00:01:00 at 4 °C . (5/5) 1m

17 Post fix cells with 1% osmium tetroxide (19150, Electron Microscopy Sciences) containing 0.8% potassium ferrocyanide and 2 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer pH 7.4 for 00:30:00 at 4 °C . 30m

18 Wash cells (5 x 2 min) with ddH_2O at 4 °C .

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

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

19 Dehydrate cells with an ice-cold graded dehydration ethanol series of 20%, 50%, 70%, 90%, 100% (anhydrous) for 00:01:00 each and 3 x 100% (anhydrous) at room temperature for 00:01:00 each. 2m

20 Infiltrate cells with one-part Durcupan ACM epoxy resin (44610, Sigma-Aldrich) to one-part anhydrous ethanol for 00:30:00 , 2 times with 100% Durcupan resin for 01:00:00 each, a final change of Durcupan resin and immediately placed in a vacuum oven at 60 °C for 48:00:00 to harden. 2d 1h 30m

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.