Cyclic Immunofluoresence Staining Protocol (OHSU) V.3

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Cyclic immunofluorescence protocol, including tissue processing and staining, image acquisition and fluorophore bleaching.

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MATERIALS

10mM Citrate Buffer: 2.1 g citrate monohydrate dH2O up to 1000mL 5M NaOH until pH is 6 (approximately 3-4 mL) filtered **Contributed by users Catalog #C-1909**

pH9 Tris/EDTA buffer **Contributed by users Catalog #S2367**

SlowFade™ Gold Antifade Mountant with DAPI **Contributed by users Catalog #S36938**

AF555 NHS Ester MW=1250 **Contributed by users Catalog #A37571**

AF750 NHS Ester MW=1300 **Contributed by users Catalog #A37575**

30% H202 (Make 3% with 1 ml H202 40 ul 5M NaOH 1 ul 10x PBS and 7.96 ml filtered dH20) **Contributed by users Catalog #H1009**

Tissue Preparation

1 For phospho-protein preservation, fix tissues in formalin at 4 degrees C for 12-24 hours. Use a standard histopathological protocol for clearing, dehydration and paraffin processing and embedding. Cut 4-5 um sections of formalin-fixed parrafin-embedded tissues and place on Tanner Adhesive Slides (Mercedes Medical, TNR WHT45AD) 2d

Koei: For phospho-protein preservation, fix tissues in formalin for 12-24 hours immediately after resection. Keep tissues at 4 degrees C from resection through fixation...

2 Bake slides 12-16 hours at 55 degrees C plus 30 minutes at 65 degrees C 16h 30m

Deparafinization

3 Deparaffinize and hydrate sections through xylenes and graded alcohols as follows, using the Sakura Tissue Tek II Manual Slide Staining Set or similar solvent-resistant containers. 43m

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3.1 Xylenes 3 x 5 min
(Change xylene and 100% EtOH when visibly dirty; 1-4x per month, depending on usage)
15m

3.2 100% EtOH 2 x 5 min
(Change xylene and 100% EtOH when visibly dirty; 1-4x per month, depending on usage)
10m

3.3 95% EtOH* 2 x 2 min
*use fresh every time to ensure correct concentration
4m

3.4 70% EtOH* 2 x 2 min
*use fresh every time to ensure correct concentration
4m

3.5 dH2O water* 2 x 5 min
*use fresh every time to ensure correct concentration
10m

Antigen Retreival

4 Perform antigen retreival in a medical (histopathology lab) grade pressure cooker. This protocol is specific to the Biocare Medical Decloaking Chamber Pro or Dako Pascal (Discontinued)
1h

Settings: SP1: 125°C, 30 seconds
SP2: 90°C, 30 seconds
SP limit: 10°C

☒Target Retrieval Solution, pH 9 (10X) **Contributed by users Catalog #S2367**

☒10mM Citrate Buffer: 2.1 g citrate monohydrate dH2O up to 1000mL 5M NaOH until pH is 6 (approximately 3-4 mL) filtered **Contributed by users Catalog #C-1909**

4.1 Fill chamber with 500 ml dH2O

4.2 Fill 1 plastic Coplin jar with 1x Target Retreval Solution (pH9 Tris/EDTA buffer: left over from my olkd protocol from UCSF), prepared with dH2O

4.3 Fill 1 plastic Coplin Jar with 10 mM Citrate buffer, pH6

4.4 Fill 1 Tissue Tek container with dH2O

4.5 Place slides in container with pH 6 buffer and fill in any blank spots with dummy slides to ensure even heating. Place all filled staining containers into pressure cooker chamber. Record the time.

4.6 Close the chamber and hit "start/stop" to begin SP1 -- temperature will rise to 125 degrees and hold for 30 seconds. When chamber beeps, record time and pressure for quality control. (Pressure will be about 15 psi)
15m

4.7 Hit "start/stop" to begin SP2 -- temperature will lower to 90 degrees and hold for 30 seconds. When the chamber beeps again, record the time and pressure (the pressure should be 0 psi).
25m

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4.8 Release pressure by pushing on the knob on the lid and turn off instument.

4.9 Remove the lid and, going quickly to retain heat, use forceps to remove the slides one at a time from pH6 buffer, dip them once brielfy in the hot water in the Tissue Tek container, and place them in the pH9 buffer. Put the lid back on the chamber and leave the slides for 15 minutes. 15m

4.10 Rinse slides in 2 changes of dH2O to cool to room temperature. Post antigen retreval, do not allow the slides to dry at any time. 30s

4.11 Wash slides 1 x 5 min in PBS. 5m

Quenching

5 30m

Quench in 3% H2O2 for 30 minutes (also reduces tissue autofluorescence)

⊠30% H202 **Contributed by users Catalog #H1009**

5.1 Prepare 10 ml quenching solution per slide (make fresh each time?).
i. 7.96 mL MilliQ H2O
ii. 1 mL 10x PBS
iii. 40 µl 5M NaOH
iv. 1 mL 30% H2O2 - add last, right before using

Koei: 30% Store condition?

5.2 Add 10 mL quenching solution to each compartment of the plastic chamber (Item # ?).

5.3 Take slides out of PBS, tap off excessl, and place face down in quenching solution.

5.4 Turn on lamp and position right above slides, with chamber lid off.

5.5 Quench for 30 minutes to fully quench Cy2/AF488 signal. 30m

5.6 After 30 minutes, remove slides with forceps and rinse 3 x 2 min in 1x PBS in a Coplin jar. 6m

Blocking

6 Block for 30 minutes with 10% NGS and 1% BSA in 1x PBS. For smaller tissues, cover with 50 ul of blocking buffer (no coverslip required if buffer completely covers tissue). For larger tissues, use 75-100 ul of blocking buffer and use a plastic coverlsip to cover and evenly spread the buffer over the tissue. 30m

Koei: Ragents and materials
1. Bovine Serum albumin (Sigma, A7906).
2. Phosphate buffered saline, 10x solution (Fisher, BP399).
3. Normal goat, rabbit serum (Vector lab, S-1000).
4. Plastic cover film (Grace Bio-Lab).

6.1 Prepare blocking solution: 10% NGS and 1% BSA in 1x PBS and apply to tissues. Cover with plastic coverslip is necessary (IHC World, IW-2601). 5m

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6.2 Incubate tisssues at room temperature in a humidity chamber for 30 minutes.

Staining

7 Stain tissue with direct-labelled primary antibodies conjugated to Alexa-Fluor dyes (AF488, AF555, AF647 and AF750)

7.1 Prepare primary antibodies diluted in 5% NGS, 1% BSA in 1x PBS. Use 25-100 µl depending on size of tissue

7.2 Apply primary antibody, cover with plastic coverslip, and incubate for 2 hours at room temp in humidity chamber, protected from light. (OR incubate overnight at 4°C for stronger staining) 16m

7.3 Soak briefly in PBS to removeplastic coverslip. Then, transfer slide to new Coplin jar filled with PBS.

7.4 Wash 3 x 5 min in PBS, protecting from light. 15m

7.5 Mount in Slowfade Gold DAPI mounting media (Life Technologies, S36938). Use compressed air to blow dust off coverslip before mounting. Use ~15µl mounting media for small coverslips and ~30µl for large coverslips. Carefully drain/blot off excess mounting media.

Koei:
1. Cover glass #1.5 thickness, 24x30 mm and 24x50 mm (Corning Life Sciences, 2980-243 and 2980-245)
2. Antifade Mountant with DAPI (Thermo, S36938)

Repeat 1h 28m

8 After mounting, image section on microscope/scanner. Then, repeat Quenching (step 5) and Staining (Step 7), followed by imaging.

8.1 Perform full slide fluorescent scanning with the Zeiss AxioScan.Z1 or similar instument.

8.2 After a successful scan is obtained, remove coverslips by immersing slides in PBS for 5-30 minutes. Allow coverslip to slide off naturally, without manually pushing or pulling it. 30m

8.3 Transfer to a new Coplin jar filled with PBS. Wash 3 x 2 minutes in PBS to remove residual mounting media. 6m

8.4 Perform 30 minutes of quenching, as in step 5. 36m

8.5 Apply primary antibodies as in step 7. 16m

8.6 Repeat for desired number of rounds.

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