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Detection of Hypoxia and HIF in Paraffin-Embedded Tumor Tissues

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Abstract

Hypoxia (insufficient O₂ availability) is involved in various biological processes, such as tumorigenesis and inflammation. Hypoxia results in stabilization of hypoxia inducible factors (HIFs) including HIF1 α and HIF2 α . Here we describe a protocol to detect mouse and human tissue hypoxia by using hypoxyprobe and immunohistochemical staining for HIF1 α and HIF2 α .

Keywords

Hypoxia; HIF1 α ; HIF2 α ; Hypoxyprobe; Immunohistochemistry

1 Introduction

Molecular oxygen (O₂) is an essential substrate for mitochondrial ATP production and universal electron receptor for numerous biochemical pathways. Therefore, maintenance of O₂ homeostasis is critical for the survival of most prokaryotic and eukaryotic species [1,2]. O₂ deprivation (hypoxia) triggers complex adaptive responses to match O₂ supply with metabolic and bioenergetic demands. When faced with hypoxia, mammalian cells engage multiple evolutionarily conserved molecular responses, including those coordinated by the hypoxia-inducible factors (HIF) transcriptional regulators. Under normoxic conditions, HIFs are rapidly degraded through an ubiquitin-proteasome pathway, whereas hypoxia results in the stabilization and nuclear translocation of HIF[1,2]. HIFs, including HIF1 α and HIF2 α , transactivate a wide range of genes involved in the regulation of angiogenesis, metabolism, cell survival, and inflammation [3]. There are numerous approaches to detect hypoxia, for example, through directly detecting HIF proteins [4,5], or HIF targets at the mRNA and/or protein levels [6]. Alternatively, hypoxyprobe can be used to detect *in vivo* tissue hypoxia [5,6]. Here, we describe the protocol to detect hypoxia in mouse and human paraffin-embedded tissues by immunohistochemical staining for hypoxyprobe, HIF1 α and HIF2 α (see Note 1).

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2 Materials

2.1 Antibody

Mouse anti-human HIF1 α (HA111, #NB100-296, 1:250; Novus), Mouse anti-human HIF2 α (#NB100-132, 1:5,000; Novus) rabbit anti-mouse HIF1 α (#ab2185, 1: 5,000; Abcam), rabbit anti-mouse HIF2 α (#ab199, 1: 2,000; Abcam), biotinylated goat anti-mouse IgG antibody (#BA9200, 1:500, Vector Laboratories), biotinylated goat anti-rabbit IgG Antibody (#BA1000, 1:500, Vector Laboratories).

2.2 Reagents

Prepare all solutions using ultrapure water and analytical grade reagents. Unless indicated otherwise, prepare and store all reagents at room temperature. Diligently follow waste disposal regulations when disposing waste materials.

1. Hypoxyprobe Kit: 200 mg pimonidazole HCl plus 1.0 ml of 4.3.11.3 mouse MAb (#HP1-200 Kit, Hypoxyprobe, Inc).
2. 4% PFA (Sigma) in 1x PBS.
3. Paraffin.
4. 100% ethanol, 95% ethanol, 70% ethanol.
5. Methanol
6. Xylenes (#247642-4L-CB, Sigma).
7. Normal goat serum (#S-1000, Vector Laboratories).
8. Antigen unmasking solution (#H3300, Vector Laboratories).
9. Hydrogen peroxide solution (H₂O₂) (30% w/w, #H1009, Sigma).
10. VECTASTAIN ABC HRP Kit (Peroxidase, Standard, #PK-4000, Vector Laboratories).
11. DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine (#SK-4100, Vector Laboratories).
12. Hematoxylin (#SH26-500D, Fisher Scientific). 1:5 dilutions with Millipore H₂O.
13. Fisher Chemical™ Permount™ Mounting Medium (#SP15-500, Fisher Scientific).

2.3 Buffers

1. 1x PBS.
2. 1x TT buffer: 25 mM NaCl, 200 mM Tris, pH7.6, 0.05% Tween 20 in Millipore H₂O.

¹HIF1 α and HIF2 α proteins can be accumulated to at least low levels under certain pathological conditions, making hypoxypore the more reliable approach to detect hypoxia.

3. Blocking buffer: 1x TT buffer containing 4% BSA and 2% goat serum.

2.4 Others

1. Tissue-Tek manual slide staining set
2. Coverslips
3. Microtome
4. PAP pen.

3 Methods

3.1 Hypoxyprobe injection (see Note 2)

1. Prepare hypoxyprobe solutions (100 mg/ml) in 0.9% saline (see Note 3).
2. Inject hypoxyprobe solution intraperitoneally into mice at 60 mg/kg.
3. 1.5 to 2 h after injection, harvest tissue for fixation and processing (see below)

3.2 Tissue fixation and processing

1. Dissect tissue of interest from animal and place immediately into cold PBS.
2. Fix tissue in 4% PFA overnight at 4°C with gentle shaking.
3. Transfer tissue into a plastic cassette, trim the tissue with a new razor blade if necessary.
4. Wash tissue in PBS 2 times with 30 min each time.
5. Tissue dehydration in 70% ethanol for 1 h. Shake if possible.
6. Tissue dehydration in 95% ethanol 2 times with 1h each. Shake if possible.
7. Tissue dehydration in 100% ethanol for 1 h (see Note 4).
8. Tissue dehydration in new 100% ethanol 3–4 times with 1 h each and gentle shaking.
9. Transfer tissue to xylenes and soak 2 times with 1 h each.
10. Remove xylenes as much as possible, and place tissue in paraffin solution 3 times with 1 h each (see Note 5).
11. Embed tissue with fresh paraffin solution. Orient if necessary. Be sure to place the cutting face downward (bottom of the base mold).
12. Cool the block for 20 min. Remove the molds, clean the blocks.
13. Paraffin-embedded tissues are sectioned at 5 μ m using a microtome.

²All animal protocols must be reviewed and approved by the institutional animal care and use committee.

³We do a further 10-fold dilution from stock before each injection.

⁴The tissue can be stored in 100% ethanol at –20°C before further processing.

⁵Make sure the paraffin temperature doesn't exceed 58°C.

3.3 Immunohistochemistry (carry out all procedures at room temperature unless otherwise specified)

1. Prewarm the slides in a dry oven at 55 °C for 20 min.
2. Deparaffinize the slides in xylenes 2 times with 20 min each.
3. Rehydrate the slides: 100 % ethanol for 2 min, 2 times; 95% ethanol for 2 min, 2 times; 70% ethanol for 2 min; tap water for 5 min.
4. Antigen unmasking. We use commercial antigen unmasking solution and follow the the manufacture's instructions.
5. Block endogenous peroxidase activity: incubate the slides in 0.6% H₂O₂ for 15 min (see Note 6).
6. Wash slides in TT buffer for 3 times with 5 min each.
7. Remove extra buffer from the slides and draw a barrier around the section with a PAP pen (see Note 7).
8. Lay slides flat in a humidified chamber, add blocking buffer to cover the sections, and incubate for 1h.
9. Tip off the blocking buffer, and incubate the slides with primary antibody diluted in blocking buffer overnight at 4 °C.
10. Wash the slides with TT buffer for 3 times with 5 min each (see Note 8).
11. Incubate the slides with secondary antibody diluted in blocking buffer for 1 h.
12. While the secondary antibody is incubating, prepare the ABC-HRP reagent following the kit instructions (see Note 9).
13. Wash the slides with TT buffer 3 times with 5 min each.
14. Incubate the slides with ABC-HRP for 30 min.
15. Wash the slides with TT buffer for 3 times with 5 min each.
16. Prepare the DAB substrate following the kit instructions.
17. Wash the slides once with tap water.
18. Lay slides flat on a paper towel or bench paper, apply DAB substrate solution to cover the sample area. Let the reaction run 0.5 –10 min away from direct light. Monitor the staining under microscope. DAB substrate incubation time should be determined empirically for optimal staining.
19. Rinse the slides in tap water 3 times with 5 min each.
20. Counterstain the slides with hematoxylin solution for 0.5–1 min.

⁶Dilute 30% H₂O₂ in methanol to make 0.6% H₂O₂ solution.

⁷Be careful not to let the tissue sections dry out. Keep the slides in TT buffer before and after drawing barrier.

⁸Do not cross contaminate the samples, i.e. wash negative control or different antibodies separately.

⁹The mixed ABC reagents must sit for 30–40 min before adding to the slides.

21. Rinse the slides in tap water for 3 times with 5 min each.
22. Dehydrate the slides: 70% ethanol for 1min, 95% ethanol for 1 min, 100% ethanol 2 times with 1 min each, xylene 2 times with 5 min each.
23. Mount the slides.
24. See the following figure for representative IHC staining of hypoxyprom (A), human HIF1 α (B), mouse HIF1 α (C) and HIF2 α (D). Please refer to the reference 4 for human HIF2 α IHC staining.

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Figure.

(A) Immunohistochemical staining for hypoxypromer in pancreata from 2-month-old p48-Cre; Lox-Stop-Lox-Kras^{G12D} mice with areas of pancreatic intraepithelial neoplasia (PanIN). (B) Immunohistochemical staining for human HIF1α from human pancreatic tissue with pancreatic ductal adenocarcinoma. (C) Immunohistochemical staining for mouse HIF1α from 2-month-old p48-Cre; Lox-Stop-Lox-Kras^{G12D} mice with areas of PanINs. (D) Immunohistochemical staining of mouse HIF2α from 2-month-old p48-Cre; Lox-Stop-Lox-Kras^{G12D} mice with areas of PanINs. Scale bar: 50 μm.