

December
2021
Update

Immunohistochemistry Protocols

EXPERIMENTAL ANIMAL PATHOLOGY LABORATORY, WIMR I, RM. 4018



Protocol Index

AR Cell Signaling 5153	3
AR sc-816 IHC	4
Aromatase IF	5
BMAL1 IHC	6
CD1 α IHC	7
CD3 Dako IF	8
CD8 DAB	9
CD8a DAB	10
CD8a IF	11
CD14 IF	12
CD31 Abcam ab28364 DAB IHC	13
CD31 CellSignaling 77699T DAB IHC	14
CD45 IHC	15
CD45 and Hu Mito IF	16
CD45, ab10559, IF	17
CD45, CS13917, IF	18
CD163 IHC	19
CEBPa IHC	20
Cela1, LS-C293734, IF	21
CL cas 3(9661S)	22
c-Myc, ab32072, IF	23
Coll VII IHC	24
Cox2 IHC	25
Connexin 43 and Hu Mito IF	26
CREB IHC	27
CTT, ab8295 IF	28
Cubilin IHC	29
CXCR4 IF	30
D6 VDR IF	31
Ecad IF	32
ENOS IHC	33
EpCAM ab213500 IHC	34
FoxP3 (Bioss, bs-0269R) IF	35
FoxP3 (Invitrogen, 14-5773-82) IF	36
Galectin 3 DAB	37
GC Globulin DAB	38
GC Globulin and CD44 IF	39
H2AX IF	40
Hu Mitochondria IHC	41
Hu Nuclei IHC	42
Ki67 IHC	43
Ki67 and Hu Mito IF	44
LCA IHC DAB	45

LRP2/Megalin IHC DAB	46
Melan A IHC	47
MLC-2A and Hu Mito IF	48
MLC-2V and Hu Mito IF	49
mTOR IHC	50
NFkB2 IHC.....	51
Nitrotyrosine IHC	52
PD-L1 IHC.....	53
Per1 IHC	54
Per2 IHC	55
Phospho CREB IHC	56
Phospho SMAD IHC	57
Phospho Stat 3 IHC.....	58
Phospho Akt IF	59
Phospho Akt IHC.....	60
Phospho mTOR IHC.....	61
Piezo1,PA5-72974, IF	62
Piezo2,PA5-72975, IF	63
RFP IHC DAB	64
pS6 IHC	65
PTEN IHC	66
SDF1 IHC	67
Stat 3 IHC	68
SUSD2 and PDGFRa costain	69
TRPV4, ab39260, IF	70
TUNEL FFPE	71
VEGFR2 IHC	72
Vimentin and Ecad IF costain.....	73
Vimentin IF.....	74
Vimentin IHC	75
YY1 IHC.....	76

**Rabbit Anti-Human Androgen Receptor (Cell Signaling XP® Rabbit mAb, D6F11, 5153P)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue**

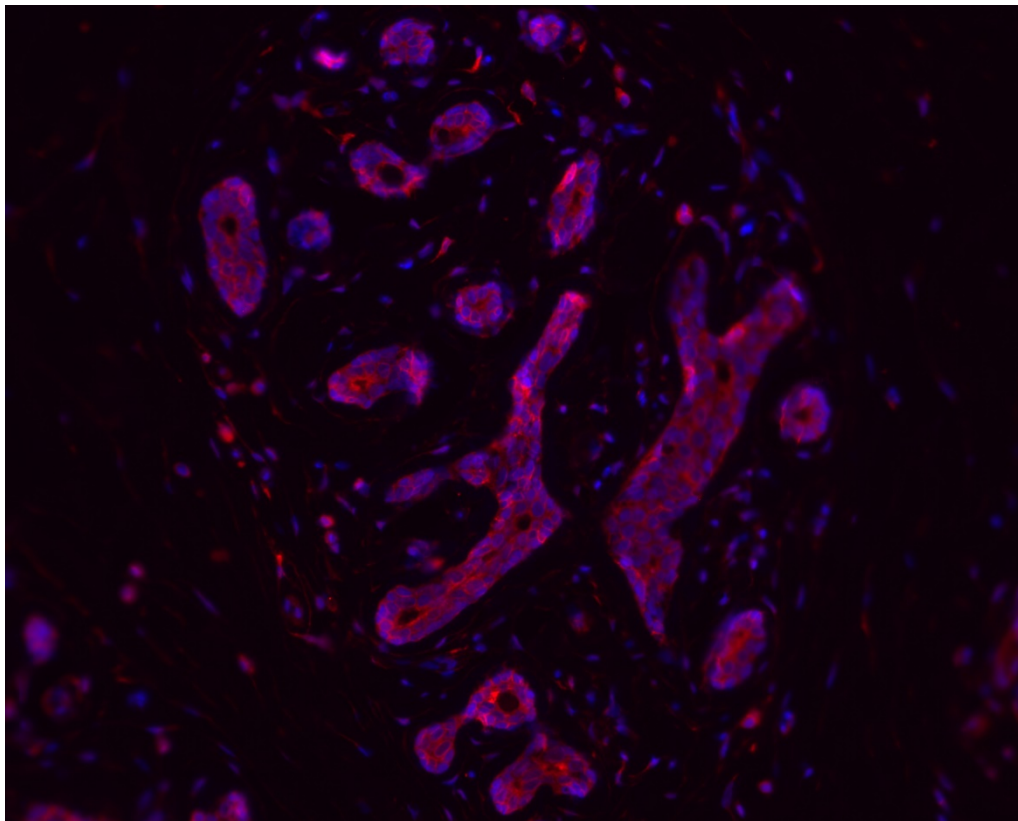
1. Five μm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes \times 3) and hydrated through graded alcohols to deionized water.
4. HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block using 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:400 overnight at 4° C.
11. PBS rinse \times 3.
12. Incubate with Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse in tap H_2O 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit Anti-Human Polyclonal Androgen Receptor (AR (N20) Santa Cruz Biotechnology, Inc., sc-816) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes \times 3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:1600 overnight at 4° C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse in tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit Anti-Aromatase (Abcam, ab18995) Indirect Immunofluorescence on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer, pH 6.0 (10 mM Citric Acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:100 in PBS with 1% goat serum.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (H+L) (Invitrogen) in PBS @ 1:1000 for one half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).



Anti-aromatase IF
@ 200x on human
breast carcinoma

Rabbit Anti- BMAL1 (Novusbio, NB100-2288) Immunohistochemistry on FFPE Murine Cells Embedded in 2% Agar

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer pH 6.0 (10 mM Citric Acid, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:400 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Rabbit) (Cell Signaling #8114S) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

Anti-CD1 α Rabbit Polyclonal (Abcam, ab136922) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five μm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes \times 3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:200 overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH₂O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse in tap H₂O for 10 minutes.
18. DH₂O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit anti-human CD3, (Dako A0452) Immunofluorescence on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut @ 5 μm , mounted on charged slides, and then incubated in an 80 $^{\circ}\text{C}$ oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH_2O .
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris Base, 1mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-CD3 @ 1:50 in PBS with 1% goat serum overnight @ 4 $^{\circ}\text{C}$.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH_2O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rat Anti-Mouse CD8a(ebioscience, 148-080, Clone: 4SM15) Immunohistochemistry on FFPE Mouse Tissue

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer pH 6.0 (10 mM Citric Acid, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:800 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Anti-rat IgG (HRP, Rat) (Vector, MP-7444) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB(Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

Rat Anti-Mouse CD8a (ebioscience, 14-0808, Clone: 4SM15) Immunohistochemistry on FFPE Mouse Tissue

1. Slides were cut at 5 μm , mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80 °C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20) for three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:800 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS $\times 3$.
11. Anti-rat IgG (HRP, Rat, Vector, MP-7444) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS $\times 3$.
13. Stain with DAB (Cell Signaling, #8059S) 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Rabbit Anti-CD8a (Cell Signaling, RabMab, D4W2) Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-CD8a @ 1:800 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 488 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit mAb Anti-CD14(abcam ab133335, EPR3653) Indirect Immunofluorescence on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut at 5 μm , mounted on charged slides, then incubated in an 80 °C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 minutes.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:1000 in PBS with 1% goat serum and 0.1% Triton X-100.
10. Rinse for 5 minutes in PBS $\times 3$.
11. Incubate with Alexa Fluor 647 goat anti-rabbit IgG H&L (Invitrogen) in PBS at 1:500 for one hour at room temperature in the dark.
12. Rinse for 5 minutes in PBS $\times 3$.
13. Rinse in dH₂O 5 minutes
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Anti-CD31 Rabbit Polyclonal (Abcam, ab28364) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue DAB

1. Five μm thick sections were cut from FFPE murine tissue and mounted on charged slides.
2. Place slides in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes \times 3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum, Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:400 overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB substrate (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse in tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

**Rabbit Anti- CD31 (Cell Signaling, #77699T, D8V9E) Immunohistochemistry on FFPE
Murine Tissue DAB**

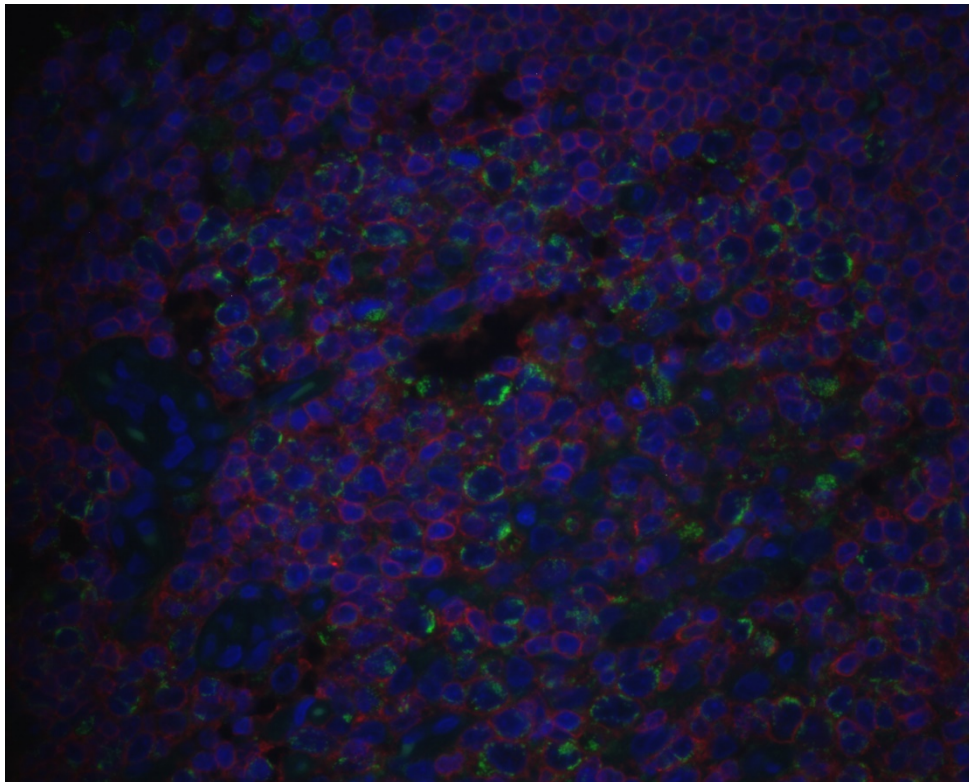
1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer pH 6.0 (10 mM Citric Acid, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:100 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Rabbit) (Cell Signaling #8114S) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

Rat Anti-mouse CD45 (BD Pharmingen, 553076) Immunohistochemistry on FFPE Mouse Tissue

1. Deparaffinize and hydrate sections.
2. Rinse for 5 minutes in dH₂O.
3. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
4. Cool slides for 30 minutes.
5. Block endogenous peroxidase with 3% H₂O₂ in PBS for 20 minutes.
6. Serum block for 30 minutes with 10% goat serum in PBS.
7. Incubate with primary antibody at 1:6400 in PBS with 1% goat serum overnight at 4°C.
8. Rinse for 5 minutes in PBS ×3.
9. Incubate with anti-rat IgG (Vector, MP-7444) for 30 minutes at room temperature.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® DAB (Cell Signaling Technology, 8059S) for 1 minute at room temperature.
12. Rinse in dH₂O.
13. Counterstain for 1 min with Mayer's hematoxylin (Sigma-Aldrich, MHS32).
14. Rinse in tap H₂O for 10 minutes
15. Dehydrate through graded alcohols to xylene.
16. Coverslip with Permount.

Rabbit Anti-CD45 (Cell Signaling, 13917) and Mouse Anti-Human Anti-Mitochondria Antibody, Surface of Intact Mitochondria, Clone 113-1 (Millipore, MAB1273) Dual Immunofluorescence on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-CD45 @ 1:200 and Anti-Human Mitochondria @ 1:1600 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) and Alexafluor goat anti-mouse 488 in PBS, each @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).



Anti human mitochondria(488) and anti CD45(647) on human tonsil(400X)

Rabbit anti-CD45 (Abcam, ab10559) Immunofluorescence on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric Acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-CD45 @ 1:50 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit anti-CD45 (Cell Signaling, 13917, D9M81) Immunofluorescence on Formalin Fixed Paraffin Embedded Human Tissue

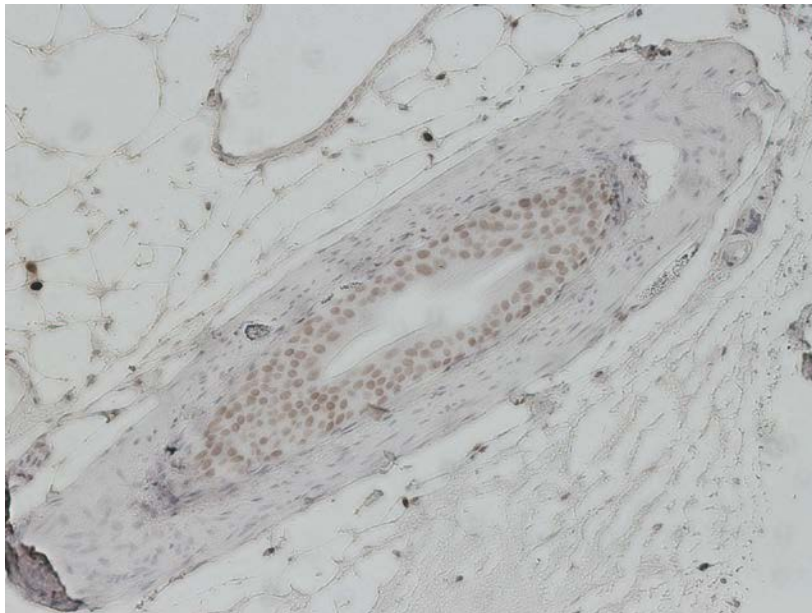
1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric Acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-CD45 @ 1:200 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Mouse Anti-Rat CD 163 Immunohistochemistry on FFPE Sections with Polymeric Secondary

1. Tissue was cut at 5 μm , mounted on charged slides, then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), or Tris-EDTA, pH 9.0(10mM tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 minutes.
7. Rinse in PBS.
8. Block endogenous peroxidase with 3% H₂O₂ in PBS for 20 minutes.
9. Serum block for one hour at room temperature using 10% goat serum in PBS.
10. Avidin block (0.001% avidin in PBS) for 10 min at room temperature.
11. Biotin block (0.001% biotin in PBS) for 10 min at room temperature.
12. Incubate with primary antibody diluted in PBS with 1% goat serum and 0.01% Triton X-100 overnight at 4°C.
13. Rinse for 5 minutes in PBS \times 3.
14. Incubate with SignalStain Boost IHC Detection Reagent, HRP,mouse (Cell Signaling Technologies, Danvers,MA) for 30 minutes at room temperature.
15. Rinse for 5 minutes in PBS \times 3.
16. Stain with DAB (Vector Laboratories, Burlingame, CA).
17. Rinse in tap water.
18. Counterstain 1 min with Mayer's hematoxylin.
19. Rinse in tap H₂O fo 10 minutes.
20. Rinse in dH₂O for 5 minutes.
21. Dehydrate through graded ethanols to xylene.
22. Cover slip with Permount.

Anti-C/EBPa (Cell Signaling, #8178s Rabbit mAb) Immunohistochemistry on FFPE Mouse Tissue

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer pH 9.0 (10 mM Tris Base, 1mM EDTA, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:100 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Rabbit) (Cell Signaling #8114S) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.



C/EBPa IHC, 1:100, pH9 HIER

Rabbit Anti-Cela1 (Lifespan Biosciences, LS-C293734) Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-Cela1 @ 1:200 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 598 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit Anti-Human Cleaved Caspase 3(Cell Signaling, 9661S) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut at 5 μm , mounted on charged slides, then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 minutes.
7. Rinse in PBS.
8. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 20 minutes.
9. Serum block for one hour at room temperature using 10% goat serum in PBS.
10. Incubate with primary antibody at 1:100 in PBS with 1% goat serum and 0.01% Triton X-100 overnight at 4°C.
11. Rinse for 5 minutes in PBS \times 3.
12. Incubate with Signal Stain® Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling, 8114S) for 30 minutes at room temperature.
13. Rinse for 5 minutes in PBS \times 3.
14. Stain with DAB (Cell Signaling, 8059S) for 1 minute at room temperature.
15. Rinse in tap water.
16. Counterstain for 1 minute with Mayer's hematoxylin (Sigma, MHS32).
17. Rinse in tap H₂O for 10 minutes.
18. Rinse in dH₂O for 5 minutes.
19. Dehydrate through graded ethanols to xylene.
20. Cover slip with Permount.

Rabbit Monoclonal anti-c-Myc (abcam, ab32072, cl Y69) Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer, pH 6.0 (10 mM Citric acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-c-Myc @ 1:200 in PBS with 1% goat serum and 0.1% Triton X-100 overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Anti-Collagen VII Rabbit Polyclonal (Abcam, ab93350) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five μm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes \times 3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) Proteinase K (Dako, S3020) for 10 minutes at room temperature.
5. PBS rinse.
6. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
7. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
8. PBS rinse.
9. Incubate with primary antibody in PBS with 1% goat serum at 1:1600 overnight at 4 °C.
10. PBS rinse \times 3.
11. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
12. PBS rinse \times 3.
13. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
14. DH_2O rinse.
15. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
16. Rinse in tap H_2O for 10 minutes.
17. DH_2O rinse.
18. Dehydrate through graded alcohols to xylene.
19. Cover slip with Permount.

Rabbit Anti- Cox2 (Cell Signaling, #12282) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μm , mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80 °C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O .
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:200 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS $\times 3$.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS $\times 3$.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O .
15. Counterstain for 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Rabbit Anti-Connexin 43 (Sigma, SAB4501173, lot#210874) and Mouse Anti-Human Anti-Mitochondria Antibody, Surface of Intact Mitochondria, Clone 113-1 (Millipore, MAB1273, clone 113-1, lot#3445023) Dual Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue with Human Xenografts

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-Connexin43 @ 1:50 and Anti-Human Mitochondria @ 1:1600 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) and Alexafluor goat anti-mouse 568 in PBS, each @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Anti-CREB (Cell Signaling, #9197 Rabbit mAb (48H2)) Immunohistochemistry on FFPE Mouse Tissue

1. Slides were cut at 5 μ m, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:6400 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS \times 3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS \times 3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Mouse Anti-Cardiac Troponin T (ab8295, 1C11) on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer, pH 6.0 (10 mM Citric Acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-CTT @ 1:1600 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor goat anti-mouse 568 in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Sheep Anti-Cubilin (R&D systems, AF3700, polyclonal) Immunohistochemistry on Formalin Fixed Paraffin Embedded Tissue

***note antibody is supplied in 10µl aliquots to minimize freeze-thaw**

1. Tissue was cut @ 5 µm, mounted on charged slides, then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 20 min.
9. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
10. Primary antibody @ 1:500 in PBS with 1% goat serum and .01% Triton X-100 overnight @ 4°C.
11. Rinse for 5 min. in PBS x 3.
12. Rabbit anti-sheep IgG (H+L) (Invitrogen, 31480) @ 1:500, one hour @ room temperature.
13. Rinse for 5 min. in PBS x 3.
14. DAB (Cell Signaling, 8059S) one minute @ room temperature.
15. Rinse in tap water.
16. Counterstain 1 min with Mayer hematoxylin (Sigma, MHS32).
17. Rinse in tap H₂O 10 min.
18. Rinse in dH₂O 5 min.
19. Dehydrate through graded ethanols to xylene.
20. Cover slip with permount.

Chemokine (C-X-C motif) Receptor 4 Goat Anti-mouse Polyclonal Indirect Immunohistofluorescence on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 μm , mounted on charged slides, then placed in an 80°C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature. Use 10% rabbit serum in PBS.
6. Incubate with primary antibody diluted to 1:50 in PBS with 1% rabbit serum overnight at 4°C.
7. Rinse for 5 minutes in PBS $\times 3$.
8. Incubate with Rabbit anti-goat Alexa Fluor 488 (H+L) (Life Technologies, Grand Island, NY) at 1:500 in PBS for 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS $\times 3$.
10. Rinse in deionized water $\times 3$.
11. Cover slip with ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY).

Positive Control Tissue – Mouse Spleen

Mouse Anti-D6 VDR Dual Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric Acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-D6 VDR in PBS with 1% goat serum and 0.1% Triton X-100 overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor goat anti-mouse 594 (Invitrogen) in PBS @ 1:500 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Monoclonal Rabbit Anti-E-cadherin(24E10) (Cell Signaling, 3195S) Indirect Immunohistofluorescence on FFPE Human Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 μm , mounted on charged slides, then placed in an 80°C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:200 in PBS with 1% goat serum overnight at 4°C.
7. Rinse for 5 minutes in PBS $\times 3$.
8. Incubate with goat anti-rabbit Alexa Fluor 488 (H&L) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS $\times 3$.
10. Rinse in deionized water $\times 3$.
11. Cover slip with Crystal Mount (Biomedex, M03).

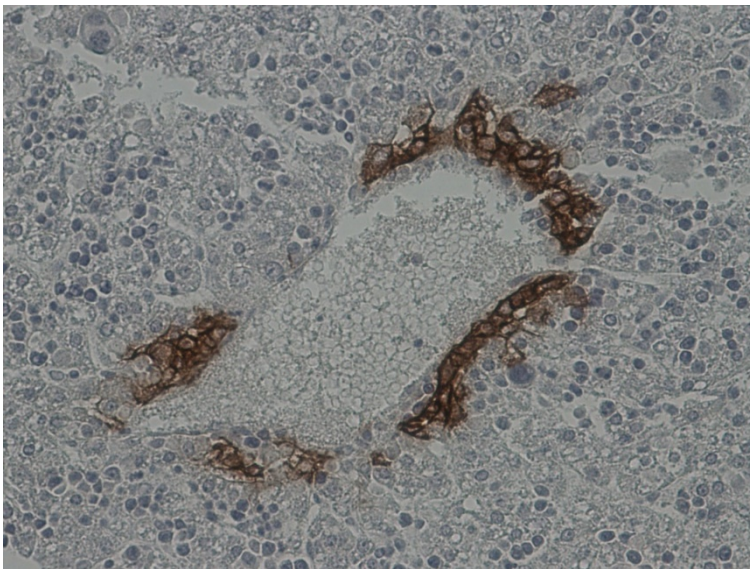
Rabbit Anti-human eNOS (Thermo, PA1-037) Indirect Immunohistochemistry on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 μm , mounted on charged slides, then placed in an 80 $^{\circ}\text{C}$ oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. PBS rinse.
6. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
7. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
8. Incubate with primary antibody diluted to 1:100 in PBS with 1% goat serum and 0.01% triton X-100 overnight at 4 $^{\circ}\text{C}$.
9. Rinse for 5 minutes in PBS $\times 3$.
10. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit)(Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
11. Rinse for 5 minutes in PBS $\times 3$.
12. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
13. dH_2O rinse.
14. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
15. Rinse with tap H_2O for 10 minutes.
16. dH_2O rinse.
17. Dehydrate through graded alcohols to xylene.
18. Cover slip with Permount.

Rabbit Anti-EpCAM (EPR20532) (abcam, ab213500) Immunohistochemistry on FFPE Murine Tissue

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer pH 6.0 (10 mM Citric Acid, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:1600 in PBS with 1% goat serum and 0.01% Triton X-100 overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Rabbit) (Cell Signaling #8114S) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

See positive staining below



EpCAM(ab213500) @ 1:1600, pH 6.0 citrate HIER, fetal mouse liver, 200X

Polyclonal Rabbit Anti-FoxP3 (Bioss, bs-0269R) Indirect Immunohistofluorescence on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 μm , mounted on charged slides, then placed in an 80 °C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:800 in PBS with 1% goat serum overnight at 4°C.
7. Rinse for 5 minutes in PBS $\times 3$.
8. Incubate with goat anti-rabbit Alexa Fluor 594 H&L (Life Technologies, Grand Island, NY) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS $\times 3$.
10. Rinse in deionized water $\times 3$.
11. Cover slip with ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY).

Positive Control Tissue – Mouse Spleen

Rat Anti-FoxP3 (Invitrogen, 14-5773-82, FJK-16S, Mab) Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-FoxP3 @ 1:200 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 564 goat anti-rat (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

**Mouse Anti-Galectin 3 mAb(Thermo Scientific, MA1-940, Clone A3A12)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue**

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes \times 3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS] for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:200 overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent(HRP, Mouse)(Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit Anti-GC Globulin (Invitrogen, PA5-79301, polyclonal) Immunohistochemistry on Formalin Fixed Paraffin Embedded Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 20 min.
9. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
10. Primary antibody @ 1:50 in PBS with 1% goat serum and .01% Triton X-100 overnight @ 4°C.
11. Rinse for 5 min. in PBS x 3.
12. Signal Stain® Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling, 8114S) 30 minutes @ room temperature.
13. Rinse for 5 min. in PBS x 3.
14. DAB (Cell Signaling, 8059S) one minute @ room temperature.
15. Rinse in tap water.
16. Counterstain 1 min with Mayer hematoxylin (Sigma, MHS32).
17. Rinse in tap H₂O 10 min.
18. Rinse in dH₂O 5 min.
19. Dehydrate through graded ethanols to xylene.
20. Cover slip with permount.

Rabbit Anti-GC Globulin (Invitrogen, PA5-79301, polyclonal) and Rat Anti-CD44 (eBioscience, 14-0441-82, mAb) Immunofluorescence on Formalin Fixed Paraffin Embedded Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Let slides cool to room temperature.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-GC Globulin @ 1:50 and anti-CD44 @ 1:100 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Goat anti-rabbit IgG Alexafluor 594 (Invitrogen, A-11012) @ 1:200 and Goat anti-rat IgG Alexafluor 647 (Invitrogen, A-21247) @ 1:500 for 1 hour at room temperature.
12. Rinse for 5 min. in PBS x 3.
13. Place slides in dH₂O.
14. Cover slip with Prolong Gold with DAPI (Invitrogen, P36931).

Rabbit anti-Phospho-Histone H2A.X Indirect Immunofluorescence Immunohistochemistry on Formalin Fixed Paraffin Sections

1. Deparaffinize and hydrate sections.
2. Rinse for 5 minutes in dH₂O.
3. Serum block for one hour at room temperature using 10% goat serum in PBS.
4. Rinse with PBS.
5. Incubate with primary antibody diluted 1:480 in PBS with 1% goat serum overnight at 4°C.
6. Rinse for 5 minutes in PBS ×3.
7. Incubate with goat anti-rabbit Alexa Fluor 488 at 1:400 in PBS 30 minutes at room temperature in the dark.
8. Rinse for 5 minutes in PBS ×3.
9. Cover slip with Crystal Mount with propidium iodide at 1 µl/ml.

Note: Keep IF stained slides in the dark, at 4°C, to prevent fading of the fluorophore!

Mouse Monoclonal Anti-Human Anti-Mitochondria Antibody, surface of intact mitochondria, clone 113-1 (Millipore, MAB1273) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five μm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Place slides in an 80°C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes $\times 3$) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 9.0 Tris-EDTA solution (10 mM tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:1600 overnight at 4°C .
11. PBS rinse $\times 3$.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Mouse, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse $\times 3$.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Anti-Human Nuclei Mouse Monoclonal (Chemicon, MAB4383) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five μm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Place slides in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes \times 3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:800 overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Mouse, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Ki67 Mouse Monoclonal (VP-K452, Vector Laboratories, Inc.) Immunohistochemistry on Formalin Fixed Paraffin Embedded Tissue

1. Tissue was fixed for 24 hours in 10% neutral buffered formalin, dehydrated through graded ethyl alcohols, paraffin infiltrated, and paraffin embedded.
2. Tissue sections were cut at 5 μm and mounted on slides.
3. Slides were deparaffinized in xylene and hydrated through graded ethyl alcohols to water.
4. Antigen retrieval was performed in a pH 6.0 citrate buffer (10mM citric acid, 0.05% Tween 20) for 3 minutes in a Biocare decloaker (Biocare, Concord, CA).
5. Slides were washed with PBS three times.
6. Nonspecific binding was blocked with 10% goat serum in PBS for one hour and endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS for 10 minutes.
7. The slides were then incubated with primary antibody (Ki67 Mouse Monoclonal, VP-K452, Vector Laboratories, Inc.) at a 1:800 dilution in PBS with 1% goat serum overnight at 4 °C.
8. After washing with PBS three times, the sections were incubated with Signal Stain® Boost IHC Detection Reagent (HRP, Mouse, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
9. Following three washes in PBS, slides were developed with Incubate with Signal Stain® DAB Substrate Kit (Cell Signaling Technology, Beverly, MA), counterstained with Mayer's hematoxylin, dehydrated and cover slipped with Permount.

All reagents and chemicals are from Sigma, St. Louis, MO unless otherwise noted.

Rabbit Anti-Ki67/MK167 (Novus bio, NB600-1252, SP6, lot#N671) and Mouse Anti-Human Anti-Mitochondria Antibody, Surface of Intact Mitochondria, Clone 113-1 (Millipore, MAB1273, clone 113-1, lot#3445023) Dual Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue with Human Xenografts

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-Ki67 @ 1:400 and Anti-Human Mitochondria @ 1:1600 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) and Alexafluor goat anti-mouse 568 in PBS, each @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

**Mouse Anti-Human Leukocyte Common Antigen (Biocare Medical, CM 016)
Immunohistochemistry on FFPE Human Cells**

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:100 in Van Gogh Yellow (Biocare Medical) overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Mouse) (Cell Signaling #8125s) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Vector, SK-4100) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

Rabbit Anti-LRP2/Megalin (Abcam, ab76969, polyclonal) Immunohistochemistry on Formalin Fixed Paraffin Embedded Tissue

***note antibody is supplied in 10 μ l aliquots to minimize freeze-thaw**

1. Tissue was cut @ 5 μ m, mounted on charged slides, then incubated in an 80 $^{\circ}$ C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 20 min.
9. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
10. Primary antibody @ 1:1000 in PBS with 1% goat serum and .01% Triton X-100 overnight @ 4 $^{\circ}$ C.
11. Rinse for 5 min. in PBS x 3.
12. Signal Stain[®] Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling, 8114S) 30 minutes @ room temperature.
13. Rinse for 5 min. in PBS x 3.
14. DAB (Cell Signaling, 8059S) one minute @ room temperature.
15. Rinse in tap water.
16. Counterstain 1 min with Mayer hematoxylin (Sigma, MHS32).
17. Rinse in tap H₂O 10 min.
18. Rinse in dH₂O 5 min.
19. Dehydrate through graded ethanols to xylene.
20. Cover slip with permount.

Anti-Melan A Rabbit Polyclonal (Abcam, ab118440) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five μm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Place slides in an 80°C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes $\times 3$) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:800 overnight at 4°C .
11. PBS rinse $\times 3$.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse $\times 3$.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with H_2O 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Mouse Anti-MLC-2A (Synaptic Systems, 311 011, lot# 1-42 IgG2b) and Mouse Anti-Human Anti-Mitochondria Antibody, Surface of Intact Mitochondria, Clone 113-1 (Millipore, MAB1273, lot#3445023, clone 113-1) Dual Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue with Human Xenografts

1. Tissue was cut @ 5 μm , mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. MLC-2A @ 1:100 and Anti-Human Mitochondria @ 1:1600 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor goat anti-mouse IgG2b 594(Invitrogen) and Alexafluor goat anti-mouse IgG1 647 in PBS, each @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit Anti-MLC-2V (Proteintech, 10916-1-AP, no lot#) and Mouse Anti-Human Anti-Mitochondria Antibody, Surface of Intact Mitochondria, Clone 113-1 (Millipore, MAB1273, clone 113-1, lot#3445023) Dual Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue with Human Xenografts

1. Tissue was cut @ 5 μm , mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-MLC-2V @ 1:400 and Anti-Human Mitochondria @ 1:1600 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) and Alexafluor goat anti-mouse 568 in PBS, each @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit Anti-human mTOR (Cell Signaling, #2972) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μ m, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS \times 3.
11. Incubate with Signal Stain® Boost IHC Reagent(HRP, Rabbit, Cell Signaling #8114S) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS \times 3.
13. Stain with DAB(Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

**Anti-NFκB2 Rabbit anti-mouse Polyclonal (Lifespan Biosciences, Inc., LS-B11757)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue**

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Place slides in an 80° C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER(heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:400 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) 30 minutes at room temperature.
13. PBS rinse ×3.
14. DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH₂O rinse.
16. Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse with tap H₂O for 10 minutes.
18. dH₂O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Anti-Nitrotyrosine (abcam, ab42789) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80 $^{\circ}$ C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes \times 3) and hydrated through graded alcohols to deionized water.
4. HIER(heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block [10% goat serum (Sigma, St. Louis, MO) in PBS] 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.01% Triton X-100(Sigma, St. Louis, MO) at 1:50 overnight at 4 $^{\circ}$ C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Positive control tissue – mouse liver

Rabbit Anti- PD-L1 (Cell Signaling, #13684) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μ m, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80 °C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20) for three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS \times 3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS \times 3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

**Rabbit Anti- Per1 (Novusbio, NBP2-24589) Immunohistochemistry on FFPE Murine Cells
Embedded in 2% Agar**

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer pH 6.0 (10 mM Citric Acid, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:25 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Rabbit) (Cell Signaling #8114S) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

**Rabbit Anti- Per2 (Novusbio, NB100-125) Immunohistochemistry on FFPE Murine Cells
Embedded in 2% Agar**

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer pH 9.0 (10 mM Tris base, 1mM EDTA, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:200 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Rabbit) (Cell Signaling #8114S) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

**Anti-Phospho-CREB (Cell Signaling, #9198 Rabbit mAb(Ser133) (87G3))
Immunohistochemistry on FFPE Mouse Tissue**

1. Slides were cut at 5 μm , mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:800 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS $\times 3$.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS $\times 3$.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain for 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

**Anti-SMAD3 (Cell Signaling, #52903 Rabbit mAb (phospho 423+425) (EP823Y))
Immunohistochemistry on FFPE Mouse Tissue**

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer pH 9.0 (10 mM Tris Base, 1mM EDTA, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:100 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Signal Stain® Boost IHC Reagent (HRP, Rabbit) (Cell Signaling #8114S) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

Rabbit Anti-mouse Phospho-Stat 3(Tyr705)(D3A7) (Cell Signaling, #9145 XP Rabbit mAb) Immunohistochemistry on FFPE Canine Tissue

1. Slides were cut at 5 μm , mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in Tris-EDTA buffer pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20), three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:400 in PBS with 1% goat serum and 0.1% Triton X-100 overnight at 4°C.
10. Rinse for 5 minutes in PBS $\times 3$.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS $\times 3$.
13. Stain with DAB (Cell Signaling, #8059S) 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

**Anti-Phospho-Akt (Ser473)(D9E) Rabbit Monoclonal (Cell Signaling, 4060S)
Immunofluorescence on Formalin Fixed Paraffin Embedded Mouse Tissue**

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Place slides in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes \times 3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Circle sections with Liquid Blocker Pap Pen (Newcomer Supply).
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS, Sigma, G6767 for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:100 overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Alexa Fluor Goat anti-rabbit 594 (H+L) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. dH₂O rinse.
15. Cover slip with Prolong Gold Antifade Reagent with DAPI, (Life Technologies, P36931)

**Anti-Phospho-Akt (Ser473)(D9E) Rabbit Monoclonal (Cell Signaling, 4060S)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue**

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80°C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes \times 3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:100 overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

**Rabbit Monoclonal Anti-human Phospho-mTOR(Ser2448)(49F9) (Cell Signaling, #2976)
Immunohistochemistry on FFPE Human Tissue**

1. Slides were cut at 5 μm , mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS $\times 3$.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS $\times 3$.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Rabbit Anti-Piezo1 (Thermo-Fisher,PA5-72974) Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric Acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-Piezo1 @ 1:50 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit Anti-Piezo2 (Thermo-Fisher,PA5-72975) Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-Piezo2 @ 1:200 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 598 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit Anti-RFP (600-401-379, Rockland Inc.) Immunohistochemistry on FFPE Tissue

1. Slides were cut @ 5 μ m, mounted on charged slides, and dried @ room temperature overnight.
2. Slides placed in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in Citrate buffer pH 6.0 (10 mM Citric Acid, 0.05% tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 min.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 min.
8. Serum block for 60 min. @ room temperature. Use 10% goat serum in PBS.
9. Primary antibody @ 1:1600 in PBS with 1% goat serum and 0.1% Triton X-100 overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. ImmPRESS goat anti-rabbit IgG (Vector, MP-7451) 30 minutes @ room temperature.
12. Rinse for 5 min. in PBS x 3.
13. DAB (Cell Signaling, #8059S) one minute @ room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O 10 min.
17. Rinse in dH₂O 5 min.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with permount.

**Rabbit Anti-Phospho-S6 Ribosomal Protein (Ser235/236) (Cell Signaling, #2211)
Immunohistochemistry on FFPE Human Tissue**

1. Slides were cut at 5 μm , mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4°C.
10. Rinse for 5 minutes in PBS $\times 3$.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS $\times 3$.
13. DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Anti-PTEN (D4.3) Rabbit Monoclonal (Cell Signaling, 9188) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80° C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes \times 3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:100 overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Polyclonal Rabbit Anti-SDF1 (Bioss, bs-4938R) Indirect Immunohistofluorescence on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 μm , mounted on charged slides, then placed in an 80°C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature. Use 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:200 in PBS with 1% goat serum overnight at 4°C.
7. Rinse for 5 minutes in PBS X 3.
8. Goat anti-rabbit Alexa Fluor 594 (H&L, Life Technologies, Grand Island, NY) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS X 3.
10. Rinse in deionized water X 3.
11. Cover slip with ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY).

Positive Control Tissue – Mouse Spleen

Mouse Anti-Stat 3(124H6) (Cell Signaling, #9139 Mouse mAb) Immunohistochemistry on FFPE Canine Tissue

1. Slides were cut at 5 μ m, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80°C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH₂O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20) for three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H₂O₂ in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:600 in PBS with 1% goat serum and 0.1% Triton X-100 overnight at 4°C.
10. Rinse for 5 minutes in PBS \times 3.
11. Incubate with Signal Stain® Boost IHC Reagent(HRP, Mouse) (Cell Signaling #8125S) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS \times 3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH₂O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H₂O for 10 minutes.
17. Rinse in dH₂O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

**Rabbit Anti-SUSD2 (LS Bio, LSA8474) and Mouse Anti-PDGFR alpha (Novus, NBP2-46357)
Dual Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue with
Human Xenografts**

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in RNAscope Target Retrieval, (Advanced Cell Diagnostics, 322001), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-SUSD2 @ 1:400 and Anti-PDGFRa @ 1:20 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 647 goat anti-rabbit (Invitrogen) and Alexafluor goat anti-mouse 568 in PBS, each @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Rabbit Anti-TRPV4 (abcam, ab39260) Immunofluorescence on Formalin Fixed Paraffin Embedded Murine Tissue

1. Tissue was cut @ 5 μ m, mounted on charged slides, and then incubated in an 80° C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 min. in dH₂O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric Acid, 0.05% tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 min.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Anti-TRPV4 @ 1:25 in PBS with 1% goat serum overnight @ 4°C.
10. Rinse for 5 min. in PBS x 3.
11. Alexafluor 598 goat anti-rabbit (Invitrogen) in PBS @ 1:1000 for one-half hour at room temperature, in the dark.
12. Rinse for 5 min. in PBS x 3.
13. Rinse in dH₂O 5 min.
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

TUNEL Immunohistochemistry on FFPE Sections

(*In situ* Cell Death Detection Kit,POD, #11684817910, Roche Diagnostics, Penzberg, Germany)

1. Sections were cut at 5 μm and mounted on slides. Slides were placed in oven at 80°C for 20 minutes to melt paraffin. Slides were then deparaffinized in three changes of xylenes, five minutes each, then hydrated through graded ethanols to deionized water.
2. Wash in PBS, pH 7.4, for 30 minutes at room temperature.
3. Block endogenous peroxidase with 3% H_2O_2 in PBS for 10 minutes at room temperature.
4. Wash in PBS for 5 minutes.
5. Incubate in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 8 minutes.
6. Incubate positive control sections in 30 U/ml DNase grade 1 in PBS 10 minutes at room temperature (50 μl /slide).
7. Prepare TUNEL reaction mixture and keep on ice:
 - a. Remove 100 μl Label Solution (vial 2) for negative controls.
 - b. Add total volume (50 μl) of Enzyme Solution to the remaining 450 μl label solution in vial 2 for 500 μl TUNEL reaction mixture.
 - c. Mix well.
8. PBS 5 minutes $\times 2$.
9. Add 50 μl TUNEL reaction mixture to samples:
 - a. Use 50 μl per slide Label Solution for negative controls.
 - b. Incubate for 1 hour at 37 °C in the dark.
10. Wash in PBS for 5 minutes $\times 3$.
11. Incubate with 50 μl /slide Converter-POD(vial 3) for 30 minutes at 37 °C.
12. Wash in PBS for 5 minutes $\times 3$.
13. Stain with DAB until developed (DAB substrate kit for peroxidase, Vector Laboratories, Inc., Burlingame, CA)
14. Wash in dH₂O for 10 minutes.
15. Counterstain with Mayer's hematoxylin for 30 seconds.
16. Tap water rinse for 10 minutes.
17. Wash in dH₂O 10 minutes.
18. Cover slip with Permount.

**Anti-VEGF Receptor 2(55B11) Rabbit Monoclonal (Cell Signaling, #2479)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue**

1. Five μm thick sections were cut from FFPE murine tissue and mounted on charged slides.
2. Place slides in an 80°C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes $\times 3$) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 9.0 EDTA-Tris solution (10mM Tris Base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:1200 overnight at 4°C .
11. PBS rinse $\times 3$.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse $\times 3$.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Mouse Anti-Vimentin (Dako, M0725, clone V9) and Monoclonal Rabbit Anti-E-cadherin(24E10) (Cell Signaling, 3195S) Indirect Immunohistofluorescence Costaining on FFPE Human Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 μm , mounted on charged slides, then placed in an 80°C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric acid, 1 mM EDTA, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with anti-vimentin antibody diluted to 1:6400 and anti-Ecadherin diluted to 1:200 in PBS with 1% goat serum overnight at 4°C.
7. Rinse for 5 minutes in PBS $\times 3$.
8. Goat anti-mouse Alexa Fluor 555 (H+L) and Goat anti-rabbit Alexa Fluor 488 (H+L) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS $\times 3$.
10. Rinse in deionized water $\times 3$.
11. Cover slip with Crystal Mount (Biomedex, M03).

Mouse Anti-Vimentin (Dako, M0725, clone V9) Indirect Immunohistofluorescence on FFPE Human Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 μm , mounted on charged slides, then placed in an 80°C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric acid, 1 mM EDTA, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker chamber (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:6400 in PBS with 1% goat serum overnight at 4°C.
7. Rinse for 5 minutes in PBS $\times 3$.
8. Incubate with goat anti-mouse Alexa Fluor 555 (H&L) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS $\times 3$.
10. Rinse in deionized water $\times 3$.
11. Cover slip with Crystal Mount (Biomedex, M03).

**Anti-Vimentin (D21H3) XP® Rabbit Monoclonal (Cell Signaling, 5741)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue**

1. Five μm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80°C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes $\times 3$) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 9.0 Tris-EDTA solution (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:400 overnight at 4°C .
11. PBS rinse $\times 3$.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) 30 minutes at room temperature.
13. PBS rinse $\times 3$.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Anti-YY1 Rabbit Polyclonal (Santa Cruz, sc-1703) Immunohistochemistry on Formalin Fixed Paraffin Embedded Canine Tissue

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Place slides in an 80° C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes \times 3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) or pH 9.0 Tris-EDTA solution (10mM Tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at overnight at 4 °C.
11. PBS rinse \times 3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse \times 3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.