Technique Talk:
The Basics of Immunohistochemistry

With lesson instructor

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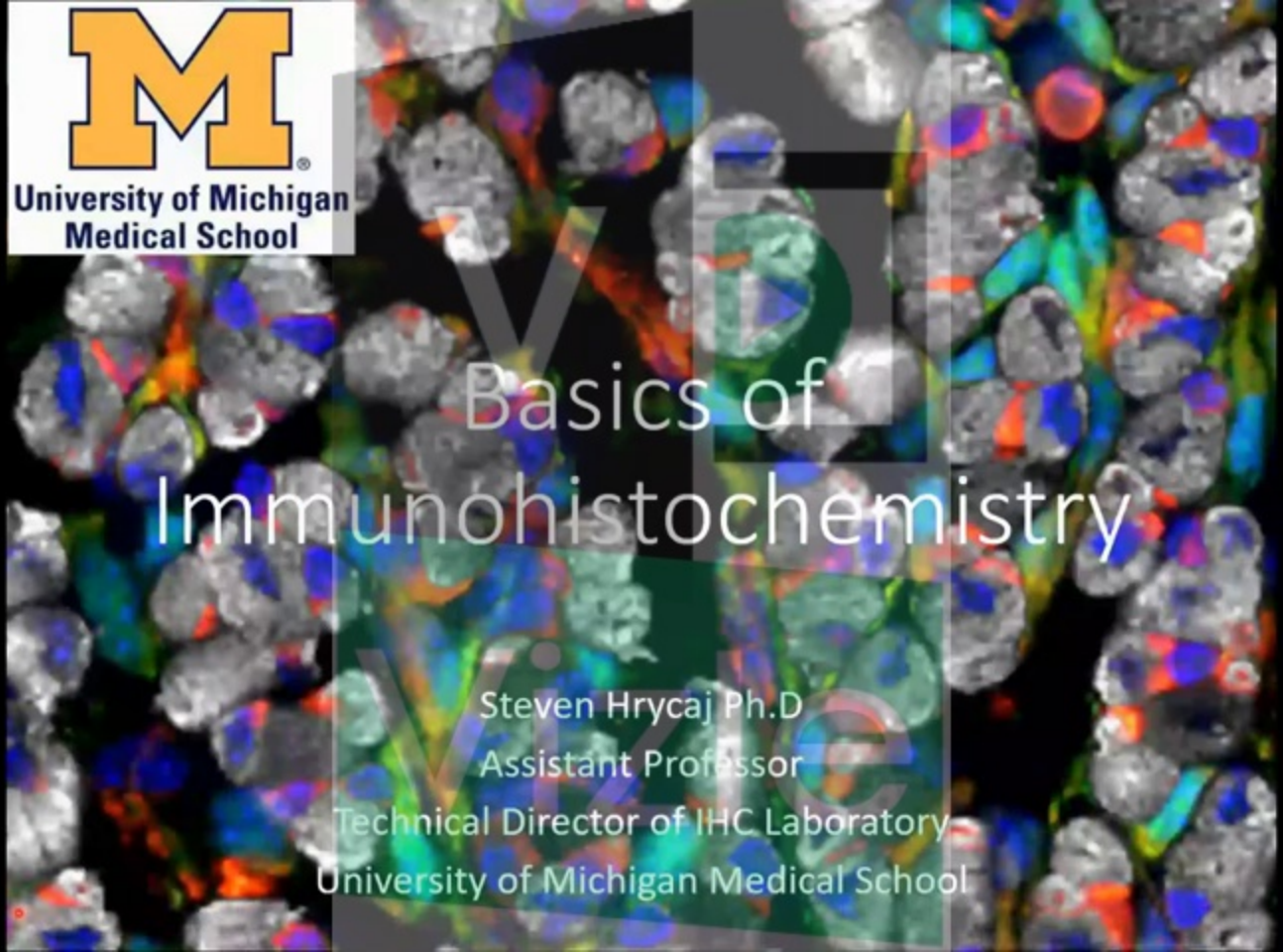
University of Michigan
Medical School
Basics of
Immunohistochemistry
Steven Hrycaj Ph.D
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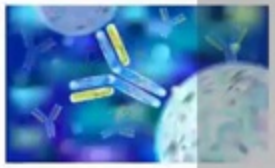
University of Michigan Medical School

What is immunohistochemistry?

Immuno



histo

chemistry

A synergy of 3 scientific disciplines that uses chemical reactions to locate and visualize the interaction between antibodies and their target antigen on sample tissue

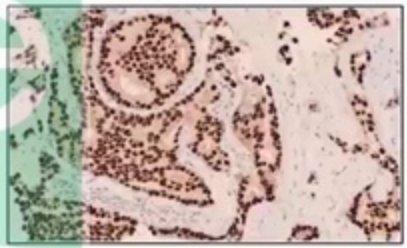
Applications of IHC

- Diagnosis
  - Immunohistochemistry is used to help diagnose diseases, such as cancer, muscle disease, neurodegenerative disorders, etc.
  - Also used to discern between different types of cancer and different cells of origin.

- Prognostic markers in cancer
  - Predicting the likely outcome of an illness
  - Examples: MMR (MLH1, PMS2, MSH2, MSH6)

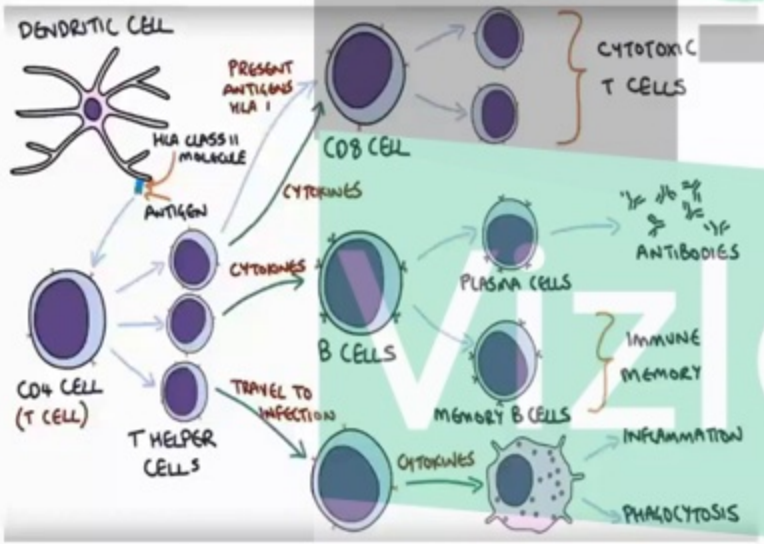
- Prediction of response to therapy
  - Level of expression correlates with response to specific treatments
  - Examples: ER, PR, HER2, PD-L1, CD30

- Research application
  - Proliferation/Apoptosis
  - Cell Signaling
  - Identification of cell types/cytoskeletal structure



The Immune System

- Protects an organism from disease by attacking toxins/foreign substances (antigens) in the body
  - Bacteria/viruses/infectious agents



- Adaptive branch of immune system detects, attacks and remembers specific antigens by using **Antibodies**

Antibody Structure

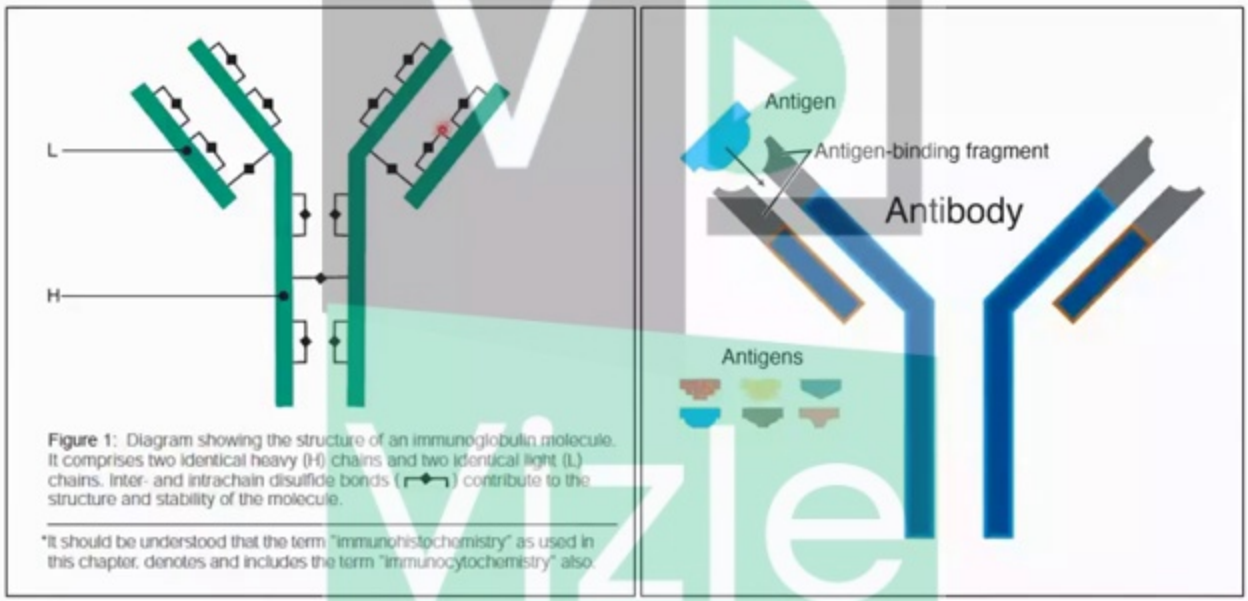
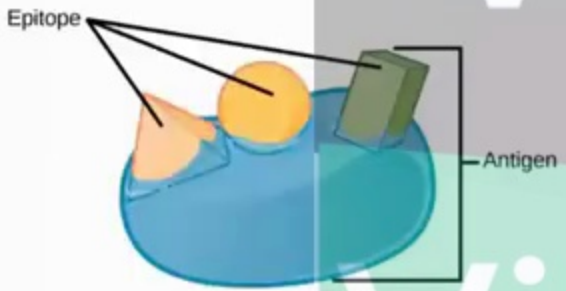


Figure 1: Diagram showing the structure of an immunoglobulin molecule. It comprises two identical heavy (H) chains and two identical light (L) chains. Inter- and intrachain disulfide bonds (⌐¬) contribute to the structure and stability of the molecule.

*It should be understood that the term "immunohistochemistry" as used in this chapter, denotes and includes the term "immunocytochemistry" also.

Antigen/Epitope interaction



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Monoclonal vs Polyclonal

Monoclonal Antibodies

Figure 5: A given clone of monoclonal antibodies reacts with a specific epitope on an antigen.

Polyclonal Antibodies

Figure 4: Schematic diagram of polyclonal antibodies binding to various epitopes on an antigen.

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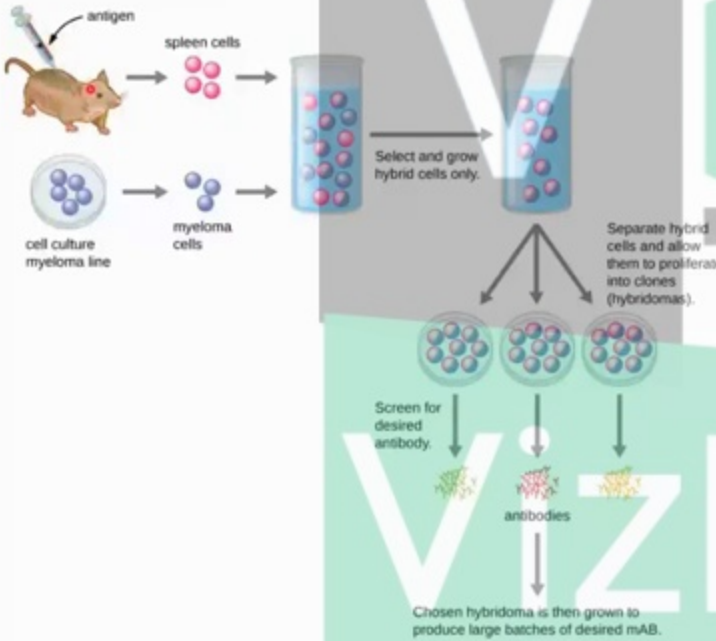
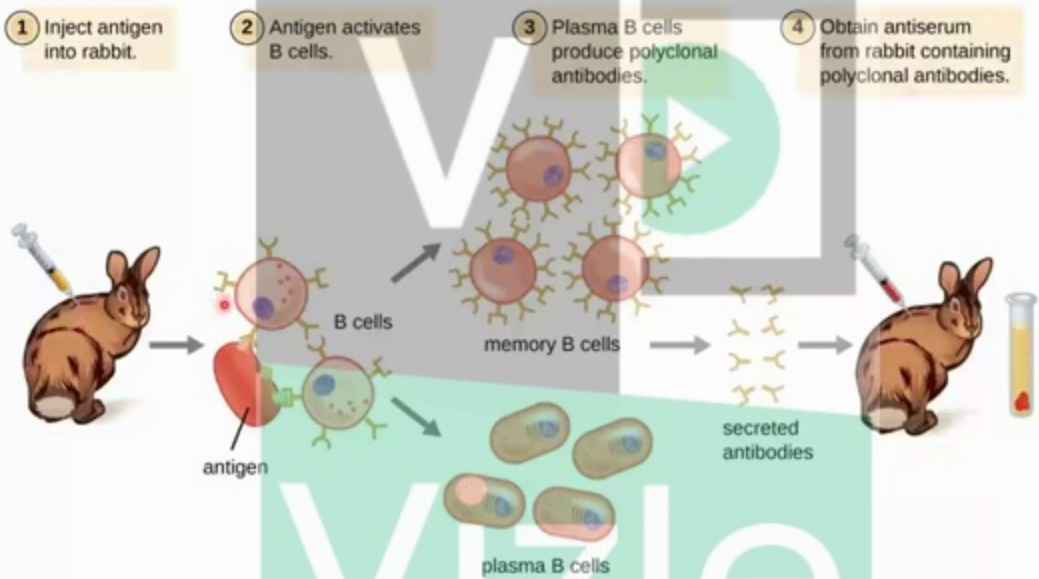
Monoclonal Ab Generation



Advantages:

- Less lot-to-lot variability
- Less cross-reactive
- Reduced non-specific binding
- Lower background staining

Polyclonal Ab Generation



Advantages:

- Antigen-Antibody binding is less affected by changes to antigen conformation from sample preparation and fixation
- Binding to multiple epitopes can enhance signal

Histology

Biopsy

Fixation

Tissue processing

Sectioning

Dehydrate/ Baking

- Sample of tissue is taken from body to determine the presence or extent of disease
- Tissue degradation begins at the time of removal
- Research
  - Dissection of tissue
  - Whole organs can be removed

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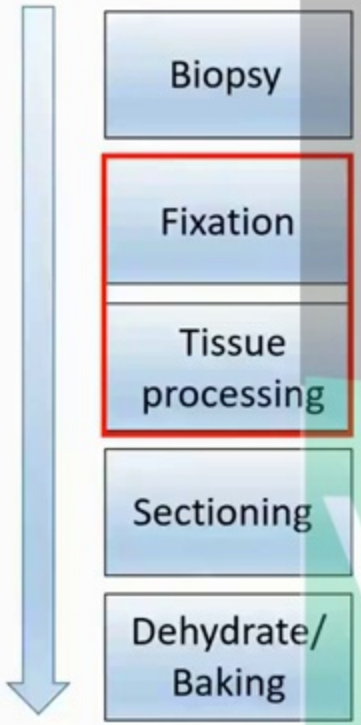
Histology



- Fixation step is required to:
  - Preserve cellular components, including soluble and structural proteins
  - To prevent autolysis and/or diffusion of tissue antigens
  - Prevent bacterial decomposition
  - Stabilize and protect the tissue from the deleterious effects of tissue processing

- Two general fixation methods
  - IHC-Paraffin
  - IHC-Frozen

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IHC Paraffin

- Most common sample preparation
- 10% neutral buffered formalin
  - Fixes tissue by forming stable cross-linkages between proteins
  - Fixation time ranges from 4-24 hours
- Dehydrated in alcohol series
- “Cleared” in xylene solutions (removes alcohol)
- Infiltrate tissue with hot paraffin wax (60°C)
- Cool to 20°C to solidify wax into blocks

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Examples of Paraffin Blocks

IHC Paraffin (cont)

- Biggest Pro:
  - Preservation of histology is exponentially better compared to frozen-fixed tissue
- Biggest Con:
  - Intense fixation process generally “masks” antigens
  - Antigen retrieval techniques are required to visualize the vast majority of antigens in paraffin-fixed tissue

IHC Frozen

- Much less laborious compared to paraffin fixation
- Two different methods
  - Snap freeze tissue
    - Liquid nitrogen or Isopentane and dry ice
    - Tissue is fixed post-sectioning (acetone or alcohol)
    - Alcohol/acetone fixation does not create cross-links
    - Antigen retrieval is not required
  - Lightly fix tissue in 4%PFA
    - "Cryoprotect" tissue with submersion into 30% sucrose
    - Freeze into blocks of OCT; store at -80°C

IHC Frozen (cont.)

- Biggest Pro:
  - Preserves enzyme and antigen function
  - No need for antigen retrieval methods
- Biggest Con:
  - Remarkably poor histology compared to paraffin fixation
  - Easier tendency for tissue to fall off slide during IHC protocol

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Histology

- Biopsy
- Fixation
- Tissue processing
- Sectioning
- Dehydrate/ Baking

- Paraffin
  - Performed on microtome at RT
  - 4uM sections
  - Sections can be stored at RT for months
- Frozen
  - Performed in a temperature controlled cryostat (up to -35°C)
  - Sections generally used immediately
  - Can store sections @ -80°C short term (one month maximum)

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Histology

- Biopsy
- Fixation
- Tissue processing
- Sectioning
- Dehydrate/ Baking

- Paraffin only
- Two general methods:
  - Baking
    - Dry oven vertically for 30-60 minutes
      - Removes all water
      - Increases tissue adherence to slide
  - Vacuum dessicator
    - Dessicate slides vertically overnight
      - Removes all water

Chemistry

- Involves two main components:
  - Antibody-Antigen binding
  - Need detection system to visualize

  - Two main types of detections:
    - Enzymatic
      - Horseradish Peroxidase (HRP)
      - Alkaline Phosphatase (AP)
    - Fluorescent
      - FITC/CY3/CY5
  - Basic chemistry is same
  - Reporter molecules differ

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Common detection methods

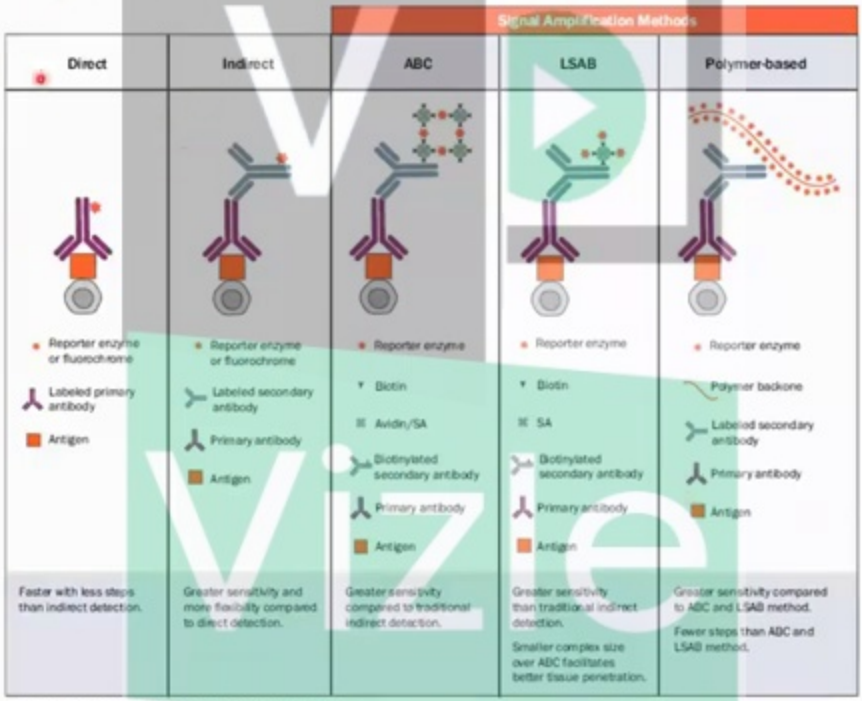
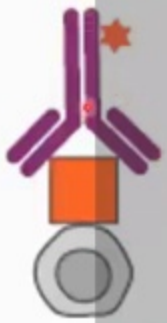


Figure 4. Types and Advantages of Detection Methods

Direct



Reporter enzyme or fluorochrome

Labeled primary antibody

Antigen

- One step staining method
- Labelled Ab's react directly with antigen
- Utilizes only one Ab so procedure is short and quick
- Insensitive due to lack of signal amplification
- Rarely used since advent of indirect methods

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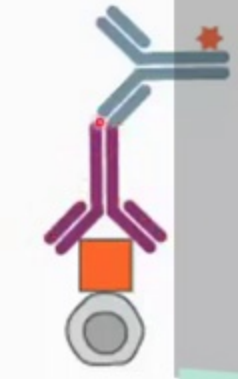
Indirect



Reporter enzyme or fluorochrome

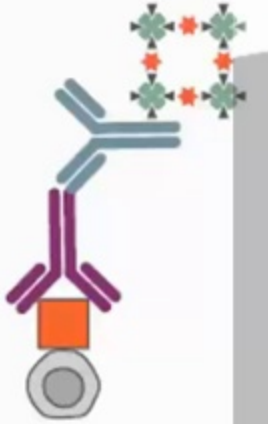
Labeled secondary antibody

Primary antibody

Antigen

- Unlabeled primary Ab reacts with tissue antigen
- Labeled secondary Ab reacts with primary Ab
- More sensitive due to signal amplification through several secondary Ab reactions
- Economy since one secondary Ab can be used with many primary Ab's
- Secondary Ab's are labeled with enzymes (HRP/AP) or fluorescent dyes (FITC/CY3/CY5)

ABC Method



Reporter enzyme

Biotin

Avidin/SA

Biotinylated secondary antibody

Primary antibody

Antigen

- Standard IHC method
  - takes advantage of strong biotin-avidin interaction
- Involves three layers:
  - Unlabeled primary Ab
  - Biotinylated secondary Ab
  - Enzyme-streptavidin or fluorescent dye-streptavidin conjugate
- Allows for signal AMPLIFICATION
- Lots of background can develop if endogenous biotin is not thoroughly quenched (kidney, liver, spleen)

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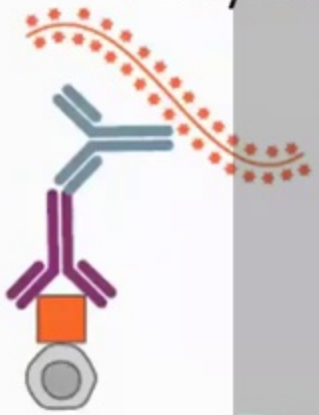
Polymer based approaches



Reporter enzyme

Polymer backone

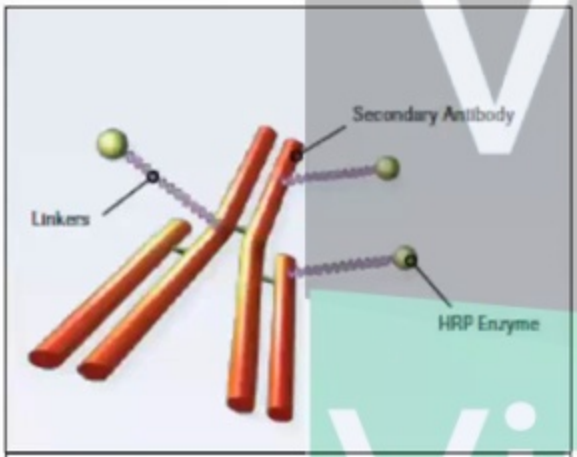
Labeled secondary antibody

Primary antibody

Antigen

- Greater sensitivity compared to indirect and ABC methods
- Fewer steps than ABC method
- No background issues due to lack of biotin
- Large complex size results in steric hindrance and possible incomplete penetration into cells

Immunoperoxidase (IPOX) Lab: *ultra*View Detection Kit



Long arm linkers attach enzymes directly to secondary antibodies

- Small multimer molecule
  - Easily penetrates tissue
  - Minimizes steric hindrance
  - Increases sensitivity
- Biotin-Free chemistry
  - Eliminates endogenous biotin staining
- Less steps than ABC method
  - Faster/cleaner detection
- Ideal for non-sensitive targets
- Majority of Ab's in IPOX lab utilize this detection system

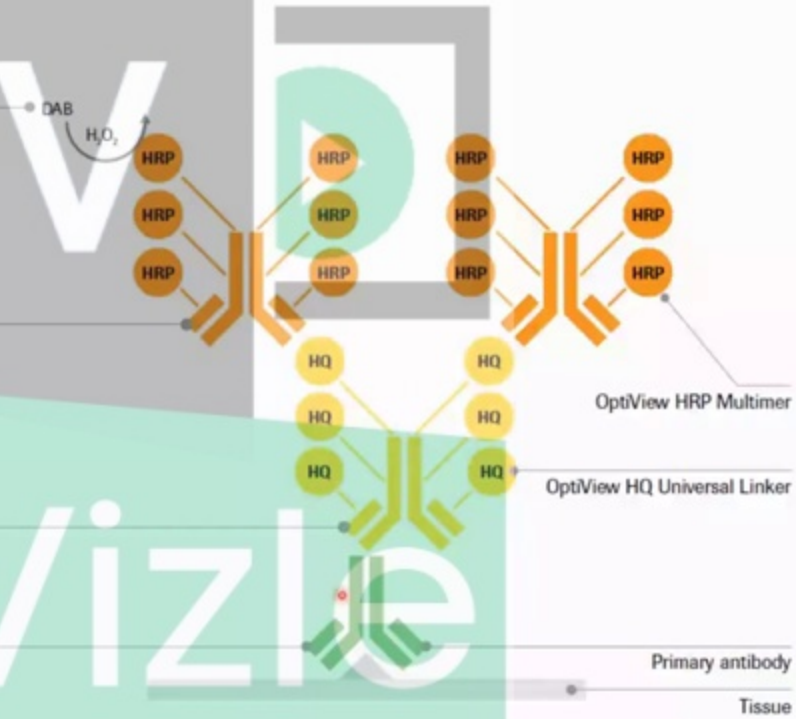
IPOX Lab Detection: OptiView Detection Kit

4. DAB chromogen reacts with HRP and H_2O_2 to generate clean, crisp signal.

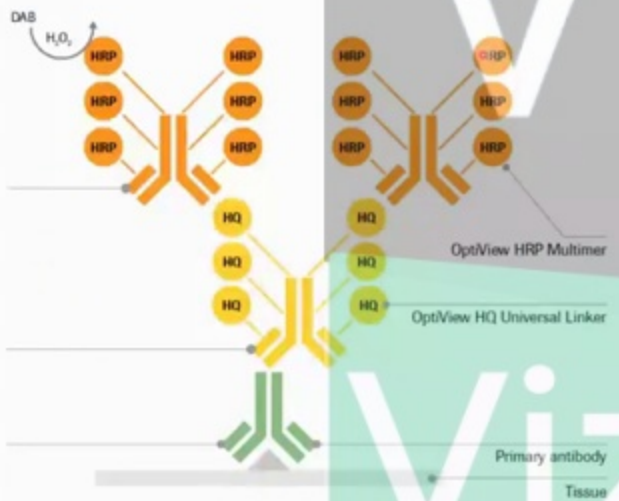
3. OptiView HRP Multimer binds to the HQ haptens. The number of multimer molecules is multiplied in this way, resulting in increased staining intensity without increased background.

2. OptiView HQ Universal Linker, which has numerous non-endogenous HQ haptens, binds the primary antibody.

1. Primary antibody binds to target antigen in tissue.

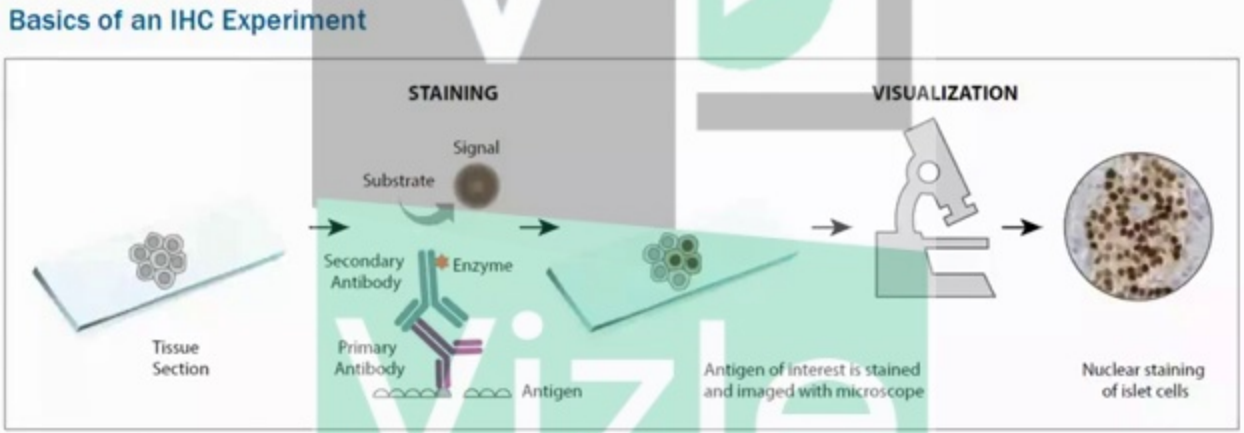


IPOX Lab Detection: OptiView Detection Kit



- Greatly increases sensitivity
  - Increases the number of HRP enzymes at each primary Ab site
  - Allows for visualization of even the lowest expressing antigens
- Enhances stain quality
  - Synthetic non-endogenous hapten-based system eliminates background issue of traditional biotin-based methods
- Most "new" Ab's in IPOX lab are optimized with OptiView detection

IHC Basics/Basic Work Flow



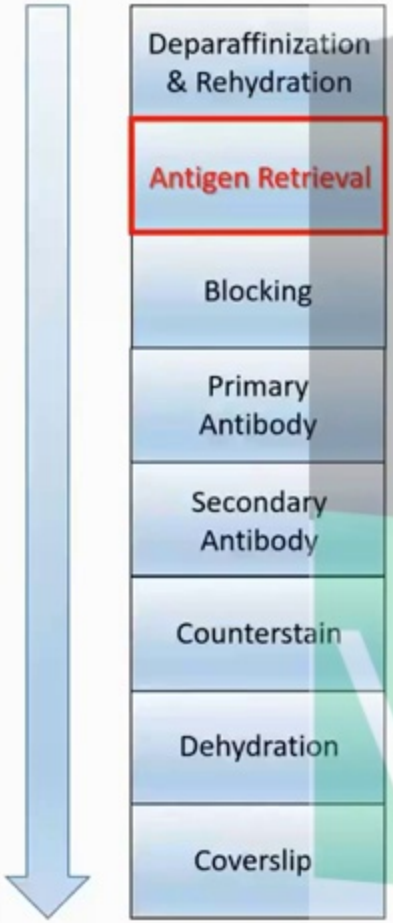
Basic IHC Protocol

- Deparaffinization & Rehydration
- Antigen Retrieval
- Blocking
- Primary Antibody
- Secondary Antibody
- Counterstain
- Dehydration
- Coverslip

Deparaffinization & Rehydration

- Deparaffinization:
  - 2 changes of Xylene (5 min each)
  - Paraffin must be completely removed before staining
    - Insufficient removal leads to spotty uneven background staining
- Rehydration:
  - Dip slides in graded EtOH series
    - 100% → 90% → 70% → 50% → 30% EtOH (3 min each)
  - Dip slides in ddH_2O (5 min)

Basic IHC Protocol



Antigen Retrieval

- Cross links produced during paraffin fixation can block Ab access to target antigens
- Approximately 85% of antigens fixed in formalin require some type of antigen retrieval to optimize IHC results
- Antigen retrieval cleaves protein-protein cross-links produced during formalin fixation
- Two main antigen retrieval methods:
  - PIER: Proteolytic-induced Enzyme Retrieval
  - HIER: Heat-induced Enzyme Retrieval
- Not required for frozen-fixed tissue

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PIER: Proteolytic-induced Enzyme Retrieval

- Exposes epitopes by enzymatic digestion of protein-protein cross-links
- Suitable for retrieving more difficult epitopes
- Harsher method that can damage tissue morphology
- Used less frequently compared to HIER

Enzyme	Working Concentration	Digestion Concentration
Trypsin	0.05% to 0.1%	37°C (10 to 40 min)*
Proteinase K	20 µg/mL	37°C (20 min)
Pepsin	0.4%	37°C (30 to 180 min)

Table 3. Digestion Enzymes for PIER.

HIER: Heat-induced Enzyme Retrieval

- Exposes antigens using high-temperature heat-induced cleavage of the protein–protein cross-links produced during fixation
  - Heating at high temperatures (100°C) for a shorter duration (10–20 minutes) is better than using lower temperatures for a longer time
- Compared with PIER:
  - More gentle experimental conditions; allows for more control over experimental parameters
  - HIER better preserves tissue morphology.
- Various methods of heat delivery are acceptable:
  - Pressure cooker
  - Water bath
  - Microwave
  - Rice cooker

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HIER: Heat-induced Enzyme Retrieval

- Several different pH buffers are used for antigen retrieval:
  - 0.01M Sodium Citrate Solution (pH 6.0)
  - 0.5M Tris-EDTA (pH 8.0-9.0)
- Correct retrieval buffer needs to be experimentally determined

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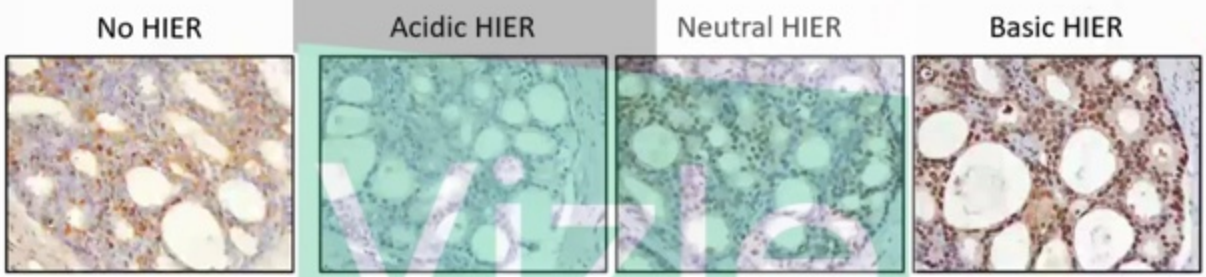
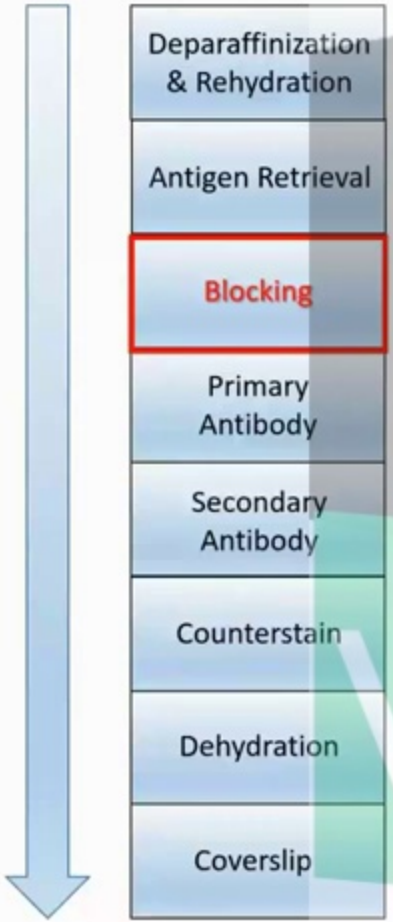
HIER: Heat-induced Enzyme Retrieval

- Several different pH buffers are used for antigen retrieval:
  - 0.01M Sodium Citrate Solution (pH 6.0)
  - 0.5M Tris-EDTA (pH 8.0-9.0)
- Correct retrieval buffer needs to be experimentally determined



p27/Kip1 in immersion-fixed paraffin-embedded human prostate cancer tissue following incubation of sections for 10 minutes at 95 °C in the specified antigen retrieval solution.

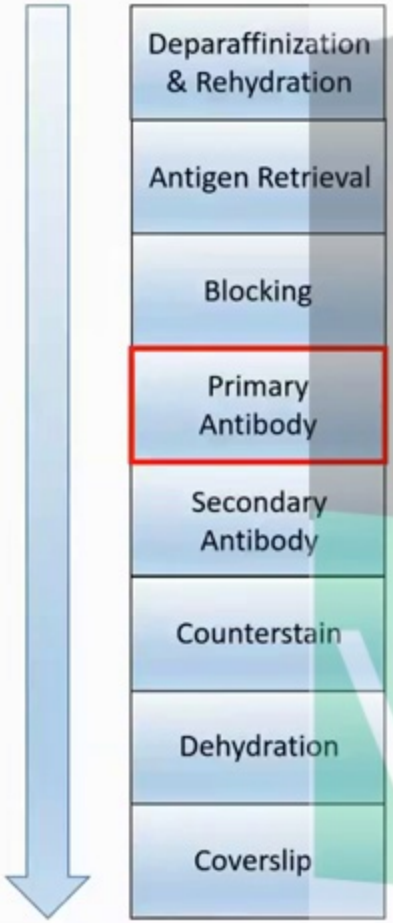


Basic IHC Protocol

Blocking

- Blocking with sera or a protein blocking reagent prevents non-specific binding of antibodies to tissue or to Fc receptors
- Absence of blocking step will result in "blushy" background developing due to non-specific binding of primary Ab
- Serum is most common blocking agent
  - contains Ab's that bind to non-specific sites.
  - Using serum matching the species of the secondary antibody is recommended.
- Block non-specific Ab binding in clinic:
  - Casein
  - 0.5M NaCl

Basic IHC Protocol



Primary Antibody

- Most Primary Ab's are produced in mouse or rabbit
- Added to tissue in blocking solution
  - Ab dilution needs to be experimentally determined
- Incubation times vary
  - 37°C for 30 minutes
  - RT for 1 hour
  - 4°C overnight

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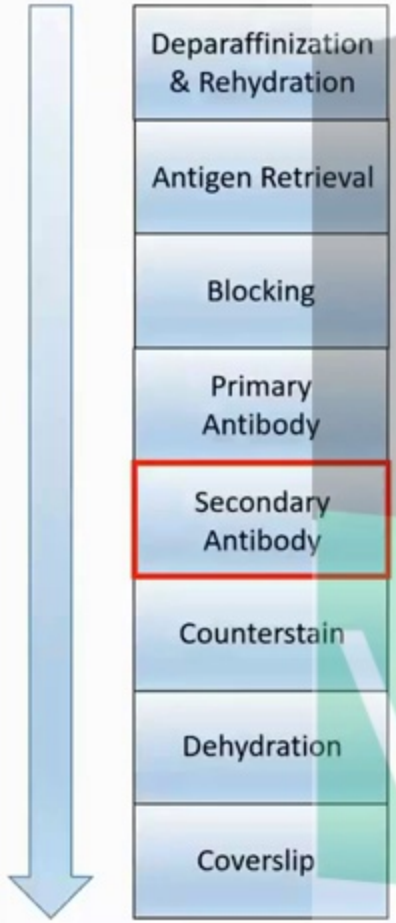
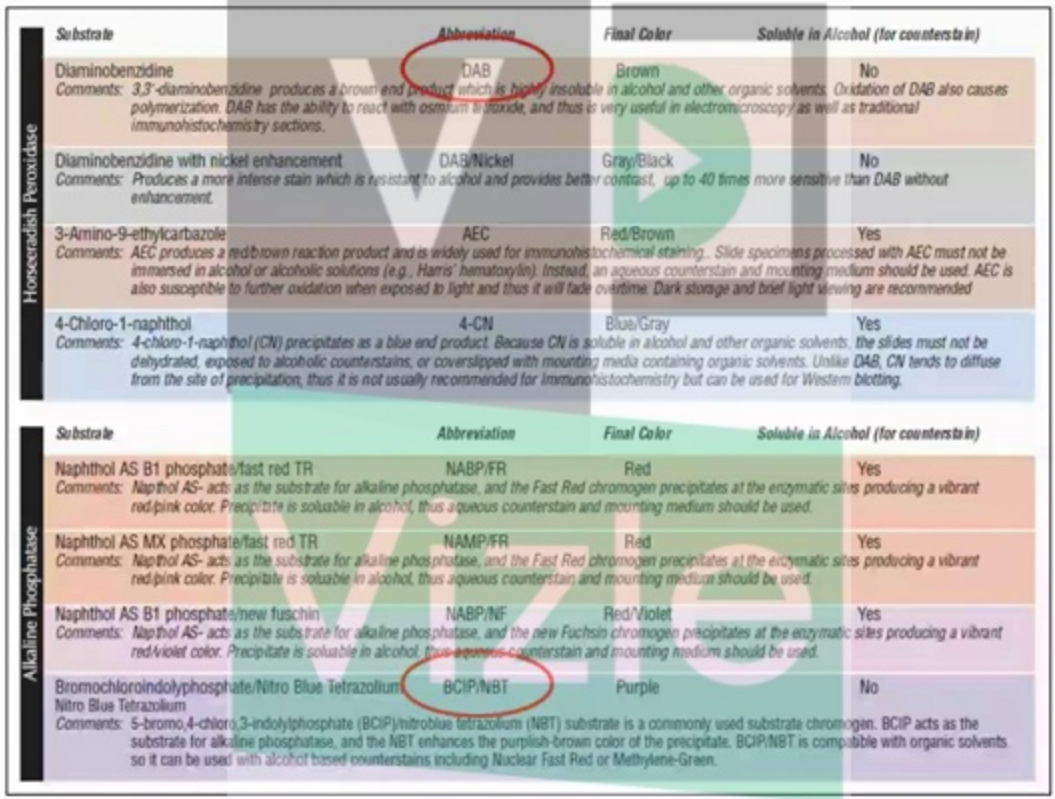


Basic IHC Protocol

Secondary Antibody

- Directed against animal primary Ab was produced in
  - Secondary Ab's are generally produced in donkey or goat
- Added to tissue in blocking solution
  - Usually 1:500 – 1:1000 dilution
  - Incubate @RT for 1-2 hours
- Secondary Ab's are conjugated to allow for detection
  - Enzymatic (HRP/AP)
  - Fluorophores

HRP/AP enzymatic detection options



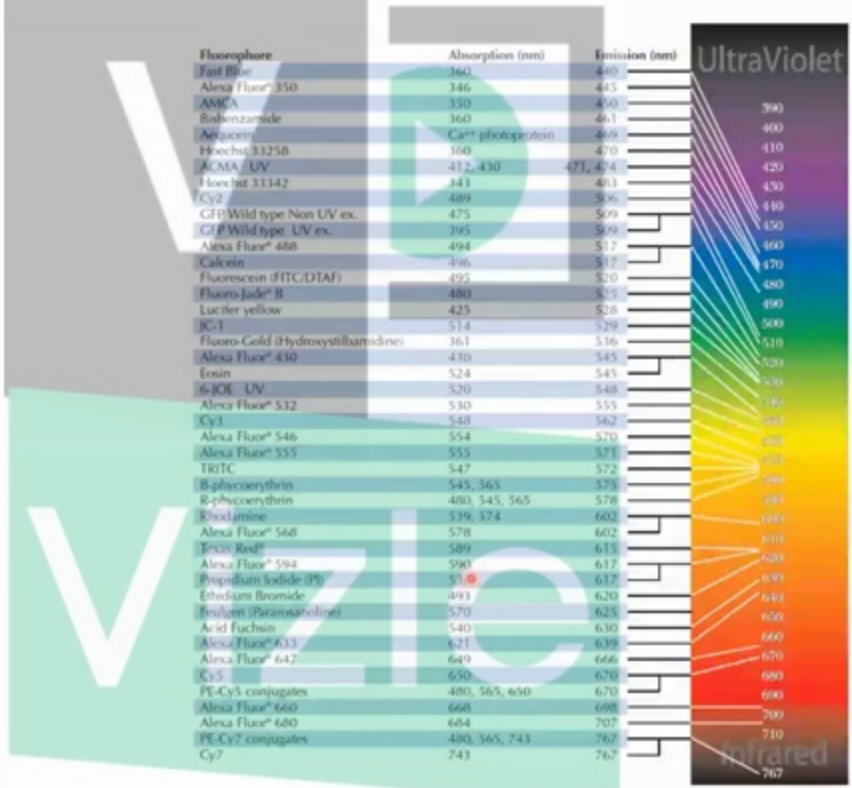
Horseradish Peroxidase

Substrate	Abbreviation	Final Color	Soluble in Alcohol (for counterstain)
Diaminobenzidine	DAB	Brown	No
Comments: 3,3'-diaminobenzidine produces a brown end product which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerization. DAB has the ability to react with osmium tetroxide, and thus is very useful in electronmicroscopy as well as traditional immunohistochemistry sections.			
Diaminobenzidine with nickel enhancement	DAB/Nickel	Gray/Black	No
Comments: Produces a more intense stain which is resistant to alcohol and provides better contrast, up to 40 times more sensitive than DAB without enhancement.			
3-Amino-9-ethylcarbazole	AEC	Red/Brown	Yes
Comments: AEC produces a red/brown reaction product and is widely used for immunohistochemical staining. Slide specimens processed with AEC must not be immersed in alcohol or alcoholic solutions (e.g., Harris' hematoxylin). Instead, an aqueous counterstain and mounting medium should be used. AEC is also susceptible to further oxidation when exposed to light and thus it will fade overtime. Dark storage and brief light viewing are recommended			
4-Chloro-1-naphthol	4-CN	Blue/Gray	Yes
Comments: 4-chloro-1-naphthol (CN) precipitates as a blue end product. Because CN is soluble in alcohol and other organic solvents, the slides must not be dehydrated, exposed to alcoholic counterstains, or coverslipped with mounting media containing organic solvents. Unlike DAB, CN tends to diffuse from the site of precipitation, thus it is not usually recommended for Immunohistochemistry but can be used for Western blotting.			

Alkaline Phosphatase

Substrate	Abbreviation	Final Color	Soluble in Alcohol (for counterstain)
Naphthol AS B1 phosphate/fast red TR	NABP/FR	Red	Yes
Comments: Napthol AS- acts as the substrate for alkaline phosphatase, and the Fast Red chromogen precipitates at the enzymatic sites producing a vibrant red/pink color. Precipitate is soluable in alcohol, thus aqueous counterstain and mounting medium should be used.			
Naphthol AS MX phosphate/fast red TR	NAMP/FR	Red	Yes
Comments: Napthol AS- acts as the substrate for alkaline phosphatase, and the Fast Red chromogen precipitates at the enzymatic sites producing a vibrant red/pink color. Precipitate is soluable in alcohol, thus aqueous counterstain and mounting medium should be used.			
Naphthol AS B1 phosphate/new fuschin	NABP/NF	Red/Violet	Yes
Comments: Napthol AS- acts as the substrate for alkaline phosphatase, and the new Fuchsin chromogen precipitates at the enzymatic sites producing a vibrant red/violet color. Precipitate is soluable in alcohol, thus aqueous counterstain and mounting medium should be used.			
Bromochloroindolyphosphate/Nitro Blue Tetrazolium	BCIP/NBT	Purple	No
Nitro Blue Tetrazolium			
Comments: 5-bromo,4-chloro,3-indolylphosphate (BCIP)/nitroblue tetrazolium (NBT) substrate is a commonly used substrate chromogen. BCIP acts as the substrate for alkaline phosphatase, and the NBT enhances the purplish-brown color of the precipitate. BCIP/NBT is compatible with organic solvents so it can be used with alcohol based counterstains including Nuclear Fast Red or Methylene-Green.			

Fluorescent detection options



Fluorophore	Absorption (nm)	Emission (nm)
Fast Blue	360	440
Alexa Fluor® 350	346	445
AMCA	350	450
Bisbenzamide	360	461
Aequorin	Ca^{++} photoprotein	469
Hoechst 33258	360	470
ACMA, UV	412, 430	471, 474
Hoechst 33342	343	483
Cy2	489	506
GFP Wild type Non UV ex.	475	509
GFP Wild type UV ex.	395	509
Alexa Fluor® 488	494	517
Calcein	496	517
Fluorescein (FITC/DTAF)	495	520
Fluoro-Jade® B	480	525
Lucifer yellow	425	528
JC-1	514	529
Fluoro-Gold (Hydroxystilbamidine)	361	536
Alexa Fluor® 430	430	545
Eosin	524	545
6-JOE, UV	520	548
Alexa Fluor® 532	530	555
Cy3	548	562
Alexa Fluor® 546	554	570
Alexa Fluor® 555	555	571
TRITC	547	572
B-phycoerythrin	545, 565	575
R-phycoerythrin	480, 545, 565	578
Rhodamine	539, 574	602
Alexa Fluor® 568	578	602
Texas Red®	589	615
Alexa Fluor® 594	590	617
Propidium Iodide (PI)	536	617
Ethidium Bromide	493	620
Perylene/Pararosanilines	570	625
Acid Fuchsin	540	630
Alexa Fluor® 633	621	639
Alexa Fluor® 647	649	666
Cy5	650	670
PE-Cy5 conjugates	480, 565, 650	670
Alexa Fluor® 660	668	698
Alexa Fluor® 680	684	707
PE-Cy7 conjugates	480, 565, 743	767
Cy7	743	767

Basic IHC Protocol

- Deparaffinization & Rehydration
- Antigen Retrieval
- Blocking
- Primary Antibody
- Secondary Antibody
- **Counterstain**
- Dehydration
- Coverslip

Counterstain

- After IHC staining, a second stain is usually applied to provide overall morphology and cellular context
  - Hematoxylin
  - Methyl Green
  - Nuclear Fast Red
  - DAPI (for fluorescence)

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Basic IHC Protocol

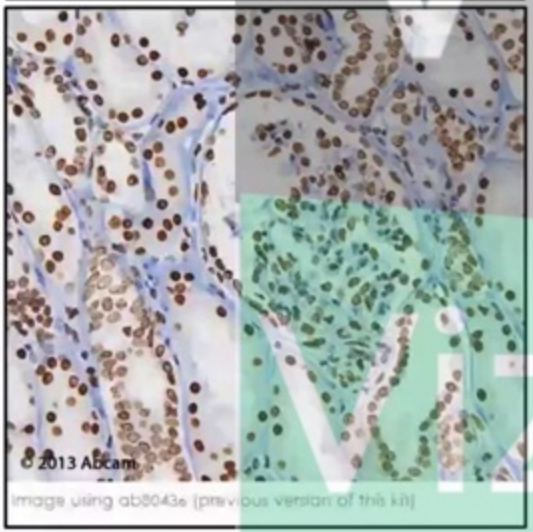
- Deparaffinization & Rehydration
- Antigen Retrieval
- Blocking
- Primary Antibody
- Secondary Antibody
- Counterstain
- **Dehydration**
- Coverslip

Dehydration

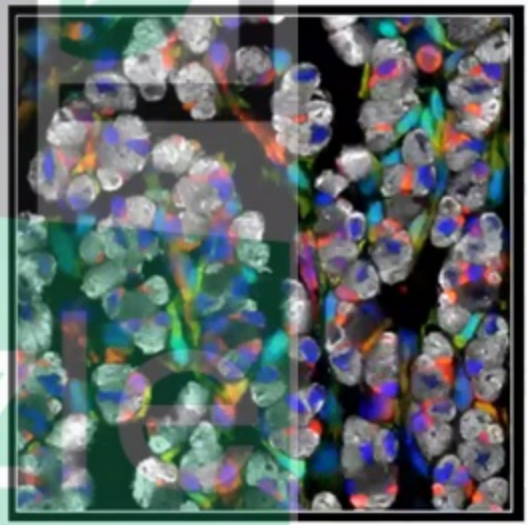
- After counterstaining, slides are dehydrated and cleared in xylene
  - Prepares slides/sections for coverslipping
- Dip slides in graded EtOH series:
  - 30% → 50% → 70% → 90% → 100% EtOH
  - 3-5 minutes each
- Dip slides in two changes of xylene
  - 5 minutes each

Examples of chromogenic and fluorescent IHC

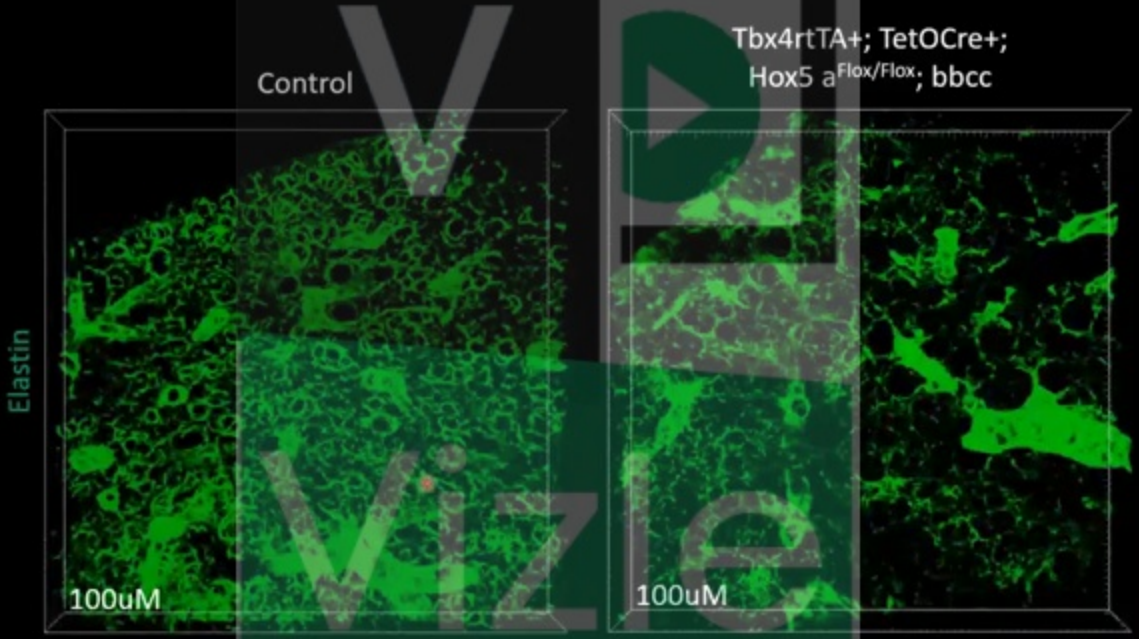
HRP-DAB IHC



Hoxa11eGFP/Tw2/My32/DAPI
Immunofluorescence



3D analysis of *Hox5* conditional triple mutants reveals a discontinuous elastin network (Dox from P0-P7)

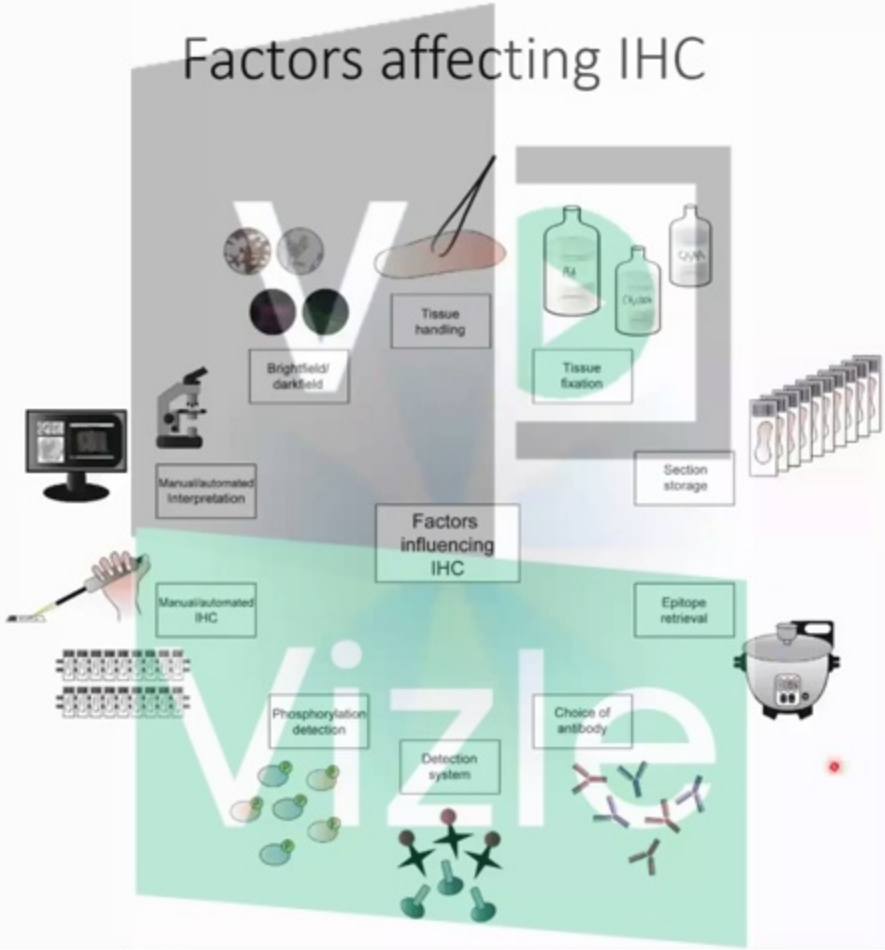


P7

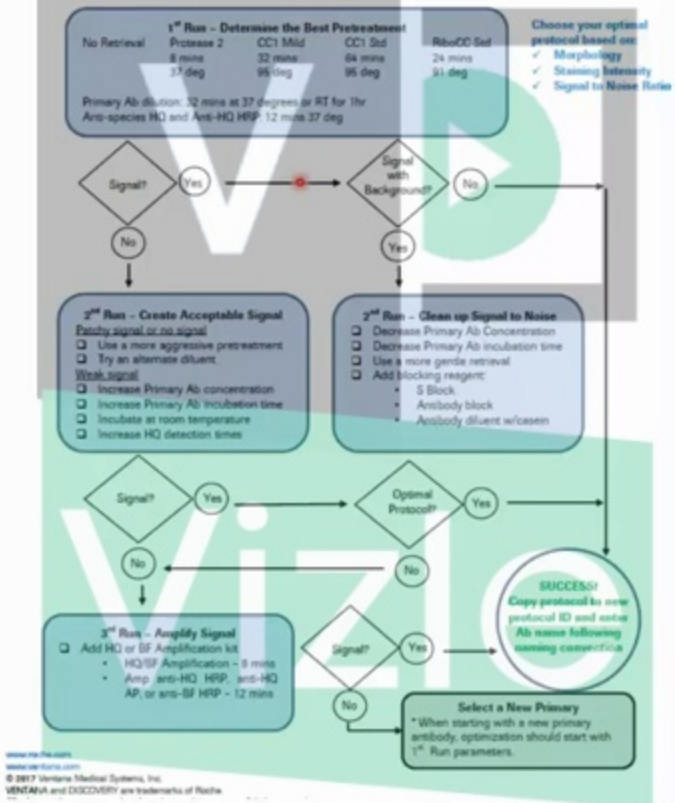
3D analysis of *Hox5* conditional triple mutants reveals a discontinuous elastin network (Dox from P0-P7)

P7

Factors affecting IHC



General optimization strategy



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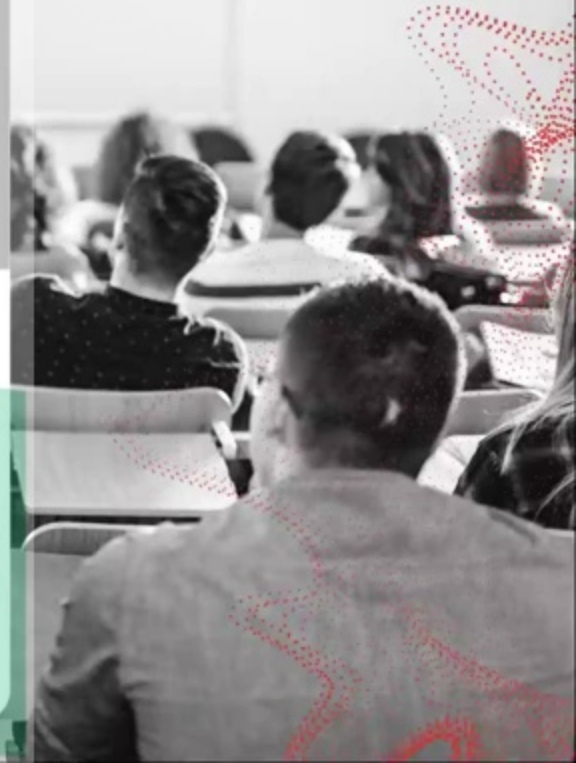
Questions???

Question & Answer



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