Common Misinformation’s in Immunohistochemistry

Tri State Meeting
May 3, 2017

Steven Westra
Reagent Product Specialist
Leica Biosystems

Misinformation in Immunohistochemistry

- Flood of New Markers
- Diagnostic to Predictive
- The More The Better
- Tissue Microarray Hoax
- IHC Quantification

General Comments

- Some antibody’s not new
- New (better) clones to old antigens
- Clones / working conditions: personal experience/preference
- Several excellent vendors, not one endorsed for antibodies
Unique IHC profiles

- HCC: CD34, Collagen IV
- Meningioma: EMA, PR
- Hemangioblastoma: S100, EGFR
- Barrett's esophagus: CDX2
- Hydatidiform mole: p57, FSH (ploidy)
- Hairy cell leukemia: CD20, CD25, TRAcP
- Fibromatosis: β-catenin (nuclear), SMA
- Glomus tumor: SMA (not desmin), +/- SNP

Unique IHC profiles

- Endometrioid CA: vimentin, β-catenin
- PML: SV40, p53
- Monophasic SS: Keratin, bcl2, CD39, β-catenin
- Solitary fibrous tumor: CD34, β-catenin
- PEComas: SMA, melanocytic markers
- Kaposi: HHV8
- Follicular dendirctic cell tumor: CD21, CD35
- Inflammatory MFB tumor: SMA (filamentous), +/-EBV, +/-ALK
- Merkel cell tumor: Ck20+, Synaptophysin+, TTF-
- Carpal Sij+: p16, MIR-1

Summary

- Avoid novel antibodies (except for experimentation) because:
  - Lack of specificity
  - Lack of track record
  - You don’t want to be the Guinea Pig
- Acquire new antibodies only when specificity is verified and reproduced by serious investigators
- Acquire multi-purpose antibodies
Common Misinformations in Immunohistochemistry

- Preanalytic Aspects
- Analytical Factors
- Postanalytic Issues

**WARNING!**

Practicing pathology accelerates aging!
Preanalytical Aspects

- Tissue Factors
  - Fixation
  - Processing
  - Decalcification
  - Storage

Myth:
- Formalin is the best fixative for immunohistochemistry.

Fact:
- The best fixative for immunohistochemistry is no fixative. The next best is a mixture of alcohol and formalin.
Myth:
- A minimum of 6 hours is needed for formalin fixation.

Fact:
- A one hour fixation is quite adequate for small biopsies such as prostate and breast cores.

Myth:
- Overnight tissue processing is superior to rapid microwave technique.

Fact:
- Fixation is the most important factor in IHC; processing is not a determining factor.
Effects of Fixation/Processing

- Change in acidophilic/basophilic properties
- Destruction or masking of antigen epitopes
- Change in morphology
- All of these effects are minimized by optimal formalin fixation

Pre-Analytic

<table>
<thead>
<tr>
<th>What is it</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underfixation</td>
<td>Incomplete fixation, immersion in fixative too short</td>
</tr>
<tr>
<td></td>
<td>- Can result in autolysis and target protein degradation</td>
</tr>
<tr>
<td></td>
<td>- Can produce staining artifacts when dehydrating alcohols are used on under-fixed tissues</td>
</tr>
<tr>
<td>Overfixation</td>
<td>Excessive fixation, immersion in fixative too long</td>
</tr>
<tr>
<td></td>
<td>- Can result in epitope masking</td>
</tr>
<tr>
<td></td>
<td>- Can produce strong nonspecific staining</td>
</tr>
</tbody>
</table>

Essentials for Q/C - Pretest variables

- Proper grossing, fixation and cutting of tissues
- Fixation: **Under fixation** is the biggest obstacle today given the use of antigen retrieval techniques- loss of antigen expression due to under fixation cannot be corrected
- Grossing: assuring that the appropriate regions of a tumor/ specimen are sampled; including benign tissue with tumor in the block for retention of normal internal controls (ER)
**Over-fixed/under-digested tissue:** Tissue morphology looks excellent with weak/no signal and low signal/background ratio due to poor probe accessibility.

*Under-fixed/over-digested tissue:* Poor tissue morphology (tissue appear faded with loss of cell borders), loss of RNA due to protease over-digestion.
Pre-Analytic Processing Reality

- “Over-processing” simply does not occur in any reasonable time frame.
- Poor-processing is usually the result of:
  - Short fixation in formalin
  - Refixation in alcohol
- Under-fixation and processing is the norm
  - Under-fixation is the root cause of all problems
Myth:
- Decalcification adversely affects IHC results.

Fact:
- Decalcification has no ill effects, provided that specimen is adequately fixed first.

Preanalytical Aspects

- Tissue Factors
  - Fixation
  - Processing
  - Decalcification
  - Storage

Myth:
- Unstained recuts of control tissue can be stored indefinitely.

Fact:
- Depending on the antigen, recut slides lose their reactivity anywhere from a few weeks to a few months.
Myth:
- Paraffin blocks lose their antigens with prolonged storage

Fact
- Not if the cut surface is re-covered by molten paraffin.

Preanalytical Factors
- Estimated Preanalytical Variables: 61
- Those With Published Studies: 27
  - Many with conflicting conclusions!

Misinformation in Immunohistochemistry
- Preanalytic Aspects
- Analytical Factors
- Postanalytic Issues
Analytical Aspects

- Immunohistochemistry
  - Antigen Retrieval
  - Primary Antibody
  - Detection System
  - Control Slides

Myth:
- Antigen retrieval should be universally applied to all IHC protocols.

Fact:
- The use of antigen retrieval depends on the fixative and the primary antibody.

Myth:
- Antigen retrieval corrects poor or inadequate fixation.

Fact:
- Antigen retrieval only corrects OVER fixation not UNDER fixation.
Optimize retrieval methods

The same retrieval technique is used for all primaries on the assumption that there is a successful universal HIER method.

Antigen Retrieval

- Not all antibodies need antigen retrieval
- All antibodies are different
- Each antibody has to be accessed on an individual basis to determine the best antigen retrieval
- Even if an antibody does not require AR its dilution performance is often enhanced

Myth:
- Post fixation of slides corrects under fixation for optimal IHC results.

Fact:
- Post fixation does not salvage antigen loss caused by initial delayed or inadequate fixation.
Analytical Aspects

- Immunohistochemistry
  - Antigen Retrieval
  - Primary Antibody
  - Detection System
  - Control Slides

Primary Antibodies

- Monoclonal
  - Mouse
  - Rabbit

- Polyclonal
  - Rabbit
  - Goat
  - Others

Myth:
- Rabbit monoclonals are more sensitive than mouse monoclonals.

Fact:
- Monoclonals are monoclonals; larger animals do not yield more sensitive reagents.
Analytical Aspects

- Immunohistochemistry
  - Antigen Retrieval
  - Primary Antibody
  - Detection System
  - Control Slides

Myth:
- More sensitive detection systems are preferable.

Fact:
- There is a trade off between higher sensitivity and higher quality IHC.
IHC Controls

- Known positive: A known positive tissue
- Known Negative: A known negative tissue
- Antibody Control: Replacement of antibody

Question:
What are the best positive and negative controls?

Answer:
The best positive and negative controls are internal controls.

Antibody selection
Before Optimizing Antibodies

- Determine appropriate control tissue - Is the antigen present?
  - Read the specification sheet for that antibody
  - Call the manufacturer for information about antibody
  - Look up Journal articles
Myth:
- Antibody control is substitution of primary antibody with a buffer solution.

Fact:
- Antibody control is replacement of the primary antibody by an antibody with a different specificity.

Misinformation in Immunohistochemistry

- Preanalytic Aspects
- Analytical Factors
- Postanalytic Issues

Myth:
- IHC stains gradually lose their intensity in storage.

Fact:
- Not if DAB is used as the chromogen.
Marker flood: how useful are they?
UM/JHS Lab, 2010

Potentially useful by literature 179
Commercially available 112
Tested in our laboratory 76
Selected for routine use 31

The More, the Better?
- Diagnostic IHC by buckshot approach
- Diagnostic IHC by pinpoint aiming
IHC: Potential Abuses

 Unnecessary utilization
 Irrational large panels

Why large IHC panels are used:
- Lack of cell specific markers
- Subjectivity of panel selection
- Experience of the observer
- ? Financial incentive (never)

IHC of Tissue Microarrays

To save time and money
It is not representative
Sausage/Multi Tumor Block/Tissue Microarray

- Contain anywhere from 10 to 80 pieces of tissue in one block
- Time consuming to make, but will save time in the long run
- Include both strong, weak and if possible intermediate expression of antigens
- A skin punch biopsy needle is used to cut out representative tissue
  - 2 to 6 mm depending on personal preference, the size of the original sample in the block and the number of pieces in the block

Optimization: Titrations & Controls

Tissue Control

4 multi-tissue control blocks for approximately 180/220 markers
- Block 1: Appendix, hepatocyte, tonsil, pancreas
- Block 2: Brain, striated muscle, skin, melanoma
- Block 3: Lung, prostate, placenta, thyroid
- Block 4: Thymus, bone marrow, Hodgkin Lymphoma, tonsil

NordiQC
Diagnostic to Predictive

- Diagnostic IHC has been practiced as an art.
- Predictive IHC has to be used as a science.

IHC for predictive markers requires:

Standardization!

Standardization!

Standardization!

Standardization!
Where are we now with standardization?

<table>
<thead>
<tr>
<th>Tissue Handling</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Fixation</td>
<td>NO</td>
</tr>
<tr>
<td>Tissue Processing</td>
<td>NO</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Y/N</td>
</tr>
<tr>
<td>Image Analysis</td>
<td>YES</td>
</tr>
</tbody>
</table>

Antibody Diluent Matters

- Monoclonal antibodies are more susceptible
- Phosphate buffered saline (PBS) based diluents was always recommended
- Studies showed that it decreases reactivity of most of the monoclonal the antibodies
- Special antibody diluents are now commercially available
Peroxidase Blocking

• Some antibodies (PAX-5) can be affected by the peroxidase blocking step.
• Denaturing the epitope of recognition
• Simply switch these step so the antibody is incubated before the $H_2O_2$ block.

IHC Quantification

- Without standardization of pre-analytical factors the results are unreliable.
- Are there biological or clinical reasons to quantify IHC?

Conclusion

- Avoid novel antibodies except for experimentation
  - Lack of track record, don’t want to be the guinea pig
  - Lack of specificity
- Acquire new antibodies when specificity established and verified by experienced pathologists
- Acquire multipurpose antibodies
- Economic volume justification
- Cocktail as needed
Thank you