

## Critical Technical Aspects of Diagnostic Immunohistochemistry

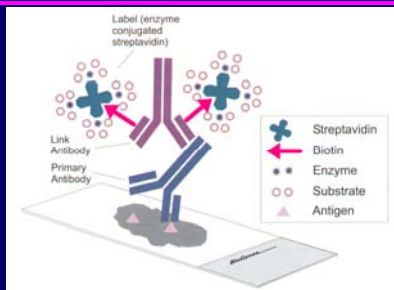
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 ProPath  
 Dallas, Texas



## Technical Aspects of IHC: Overview

1. Immunostain methods (and the endogenous biotin problem)
2. Interpretation of stains
3. Controls and Quality Control
4. Special situations (i.e., no block)
5. Artifacts in IHC
6. Oops.....
7. Miller's Rules of IHC

## IHC: Avidin-Biotin Method

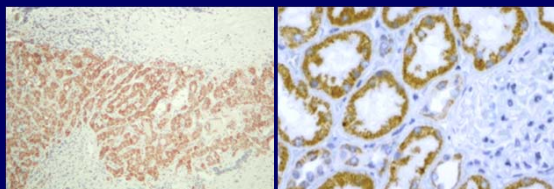


1 Avidin binds 4 Biotin molecules (irreversible)

## The Endogenous Biotin Problem

- Biotin: vitamin - for pyruvate carboxylase (mitochondrial enzyme in Krebs Cycle)
- Liver, kidney, oncocytomas
- Nonspecific binding of avidin or streptavidin detection complex (false pos)
- Artifact enhanced by epitope retrieval (especially with pressure cooker method)
- Varies with antigen retrieval solution used

## Endogenous Biotin: Normal Tissues

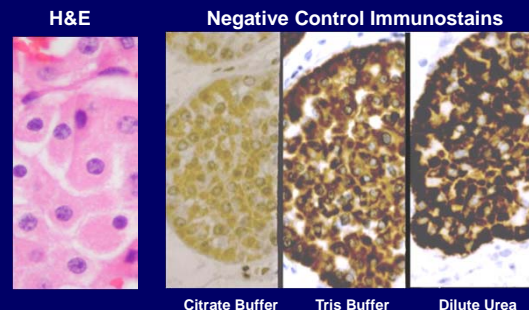


Normal Liver (low)

Normal Kidney (high)

Negative Control Immunostains

## Oncocytoma: Endogenous Biotin



H&E

Negative Control Immunostains

Citrate Buffer

Tris Buffer

Dilute Urea

## The Endogenous Biotin Problem

### Blocking of Endogenous Biotin

- After epitope retrieval step, incubate with dilute avidin solution (binds to all endogenous biotin), then incubate with dilute biotin solution (to saturate all the biotin binding sites on the avidin)
- Then add primary antibody

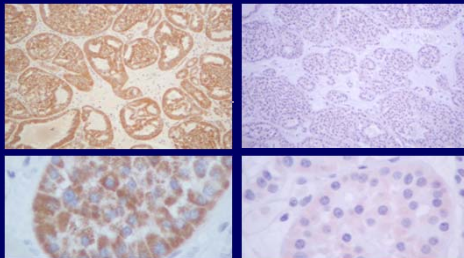
**AVIDIN IS EXPENSIVE!! (\$3400/gm)**

## Blocking of Endogenous Biotin (on the cheap)

- After Antigen Retrieval step, immerse sections in dilute egg white solution for 15 min at RT (2 egg whites in 200 ml DW). Rinse with tap water from a squirt bottle.
- Immerse sections in 0.2% biotin in PBS for 15 min at RT (5% reconstituted non-fat dried milk also works well, since milk is a rich source of biotin).

## Egg whites as source of avidin

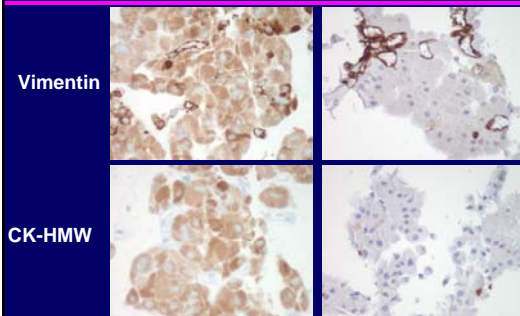
Kidney tissue, negative control sections



No endogenous biotin block

Endogenous biotin block, egg whites

## Endogenous Biotin: It's still out there!

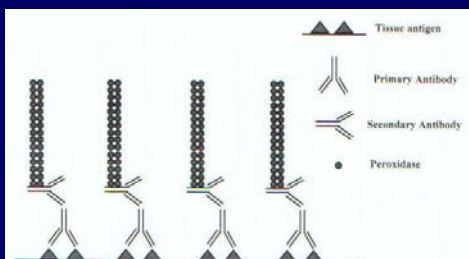


Original Lab

Repeat stains

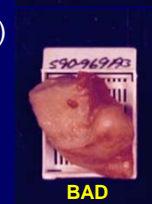
## Easier solution to the biotin problem

- Use a polymer-based method

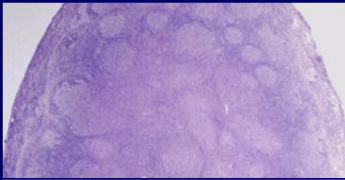


## IHC: FIXATION

- GARBAGE IN - GARBAGE OUT
- Cut sections thinly (2 mm)
- If H&E's don't look good, the IHC won't look good either



## Lymph Node - Poor Fixation



H&E Stain



CD20  
Immunostain

Underfixed

## IHC: Fixatives

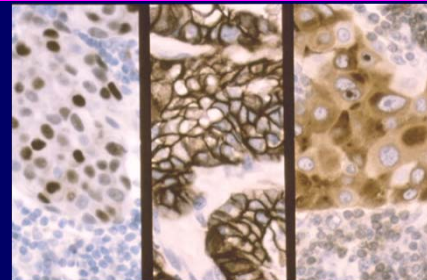
- Neutral Buffered formalin is the best all-around fixative
- Do not let tissues languish in formalin
- For prolonged storage: 70% ethanol works well

## IHC: Interpretation of Stains

### TRUE POSITIVE STAINING

- Important to know expected pattern of immunoreactivity (membranous vs. cytoplasmic vs. nuclear)
- Cell to cell heterogeneity critical

## IHC: True-Positive Staining



ER  
nuclear

HER-2  
membranous

GCDFP-15  
cytoplasmic

## IHC: Interpretation of Stains

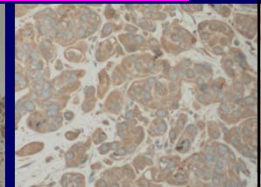
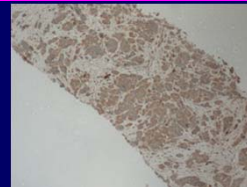
### FALSE POSITIVE STAINING

- Uniform cytoplasmic "blush" that lacks cell-to-cell heterogeneity ("spray-painted")
- Can be very intense, and localized to tumor cells with clean background
- Often due to inappropriate primary Ab titer (or use of "predilute ready-to-use" Ab)

## False Positive Immunostaining

Secondary to inappropriate primary Ab titer

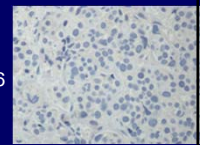
69M  
LN Bx  
CD56  
automated  
predilute



Pos  
Control  
Original  
Lab  
Carcinoid



Rpt  
CD56



## IHC: Controls

### Negative Reagent Control Slide

- A slide where primary Ab is omitted and replaced with something else
- As of July 2012, no longer required by CAP if using polymer-based method
- Required if Avidin-Biotin method used – best to have 1 NRC slide for each different type of antigen retrieval

## IHC: Tissue Controls

### Positive Tissue Controls (PTC)

Tissues expected to be positive

### Negative Tissue Controls (NTC)

Tissues expected to be negative

**Internal PTC and NTC:** Within patient tissue

**External PTC and NTC:** Non-patient tissue – should be mounted on same slide as patient

Close attention must be paid to BOTH External AND Internal PTC and NTC !

## IHC: Controls

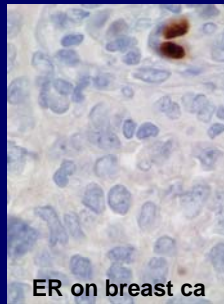
Don't forget about internal controls!!

### Internal positive controls:

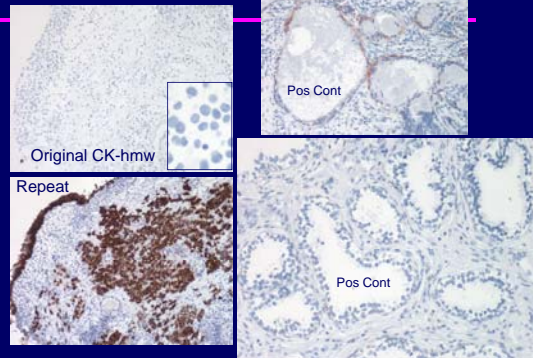
Look for reactivity in cells that should be positive

### Internal negative controls:

Make sure expected negative cells are indeed negative



## Control issues – CK-HMW stains



## Elements of Successful Quality Control in IHC

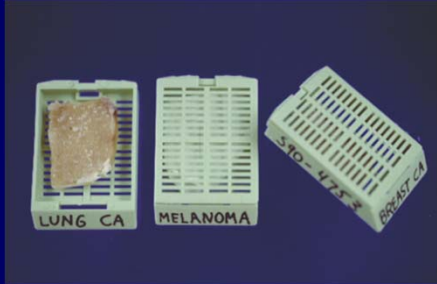
- Must be continuous & ongoing
  - Importance of good controls
  - Evaluate all Abs before & during use
  - Determine optimal titers of all Abs
- MANUFACTURER'S TITERS ARE GUIDELINES ONLY!**
- THERE IS NO SUCH THING AS A "PREDILUTE READY-TO-USE Ab!"**

## IHC Quality Control

### MULTITUMOR SANDWICH BLOCKS

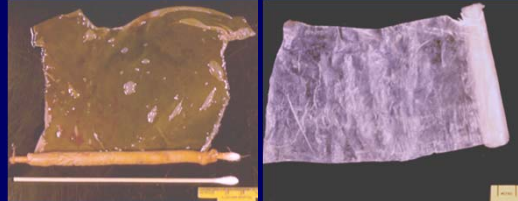


## MTSB: Collecting Tissue



## MTSB: Collecting Amnion

Used for separating rows of tumors



## Multitumor blocks



Strips in mold on hot plate



Transfer to Cold Plate



Add paraffin and cool

## Multitumor Blocks



## Multitumor Blocks



## Multitumor Blocks



1 "loaf": 4-6 blocks – 3000-4000 sections

## Multitumor Blocks



## MTSB: Key of tumors

MULTITUMOR SANDWICH BLOCK #396-15

KEY TO SLIDES

SLIDING TUMORS: Next to the center of the sandwich block, there is a color cassette marked with red ink, which defines the "T" axis. On an adjacent side of the block, there is a cassette of the block period with green ink, which defines the "S" axis. The tumor on the corner of the block closest to the green and red-marked cassettes is designated the "T0" specimen. Tumors cut to the right of the "T0" axis are marked with the "T" axis. Tumors cut to the left of the "T0" axis are marked with the "T" axis. Tumors cut above the "T0" axis are marked with the "S" axis. Tumors cut below the "T0" axis are marked with the "S" axis.

MS01 Breast Ca	MS02 Prostate Adenoca
MS03 Breast Ca	MS03 Prostate Adenoca
MS04 Colon Adenoca (green)	MS04 Papillary Ca Thyroid (green)
MS05 Breast Ca	MS05 Pilo Adenoma Thyroid (green)
MS06 Breast Ca	MS06 Lung Adenocarcinoma Ca
MS07 Breast Ca	MS07 Lung Adenocarcinoma Ca
MS08 Gastrointestinal Pancre	MS08 Gastrointestinal Ca Lung (green)
MS09 Meninge	MS09 Lung Adenoca
MS10 Glioma (Pilo) Breast Ca (green)	MS10 Glioma (Pilo) Breast Ca (green)
MS11 Breast Melanoma (green)	MS11 Meninge (green)
MS12 Gastrointestinal Ca (green)	MS12 Glioma (Pilo) Breast Ca (green)
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## Multitumor Block Sections

- Easy to make using your own tissue – 1 block can serve as pos control for MANY different Abs
- 1 "loaf" good for 3000-4000 slides
- Continuous monitor of quality control – contain multiple external PTC and NTC to demonstrate sensitivity and specificity.
- Best if mounted on same slide



## Workup of a New Antibody

- What is the best antigen retrieval method?
- What is the optimal titer?
  - Day 1: Select a titer, run a slide with no retrieval, several with proteases of choice, several in AR solutions of choice.
  - Day 2: Using the optimal antigen retrieval method, run a set of 4 to 6 serial two-fold dilutions of the primary antibody.

## Workup of New Antibody

### Use of Multitumor Sandwich Blocks

- Many different tumor of all types
- Wide range of expected pos AND expected neg tumors (often with different antigen densities)
- Perfect for determining optimal titers of primary antibodies to maximize true-positive staining and minimize false-positive staining











## IHC: Titering of Primary Ab

- Must evaluate BOTH known positive cases and known negative cases
- Serial 2-fold dilutions convenient (e.g., 1:100, 1:200, 1:400, etc.)
- Readily allows detection of contaminated antibodies from vendors (e.g., CD7 Ab contaminated with CK-LMW)



## Importance of Proper Titrers: Calcitonin

Ab Titer	Medullary Ca	Prostate Ca's
1:2000		
1:4000		
1:8000		
1:16,000		

## Lame excuses for using Predilutes

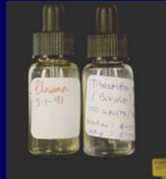
- "We don't have time to do that: we're really busy."
- "My sales rep says it's unnecessary."
- "They're so convenient."
- "Doing titers is confusing."
- "We've been doing fine in the past without titering."
- "We use an automated stainer so it's not necessary."

## IHC on Cytologic Specimens

### Rule #1

## GET A CELL BLOCK!!!

- Use of Thrombin +/- Plasma to assist in obtaining cell block material



## Cytopathology Made Simple

Rodney T. Miller, M.D.

Get a good cell block and do immunostains on it if you can't figure it out on the H&E.

- The End

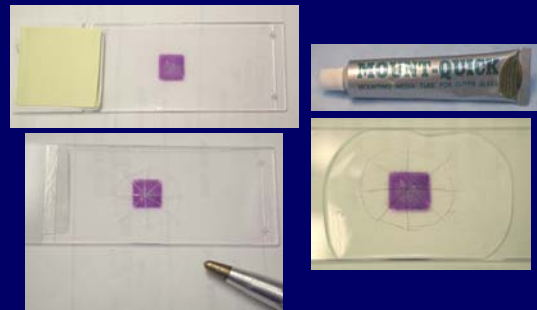
## IHC on Cytologic Specimens

### Rule #2

Use NON-ADHESIVE slides to make your smears

Allows use of tissue transfer techniques

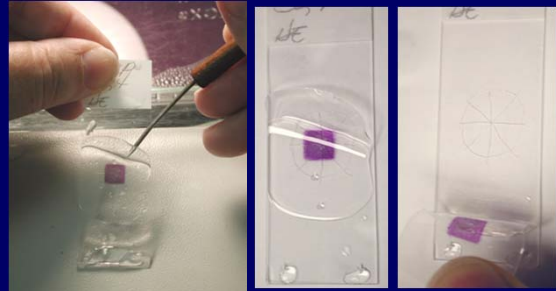
## Tissue Transfer IHC: Preparation



### Tissue Transfer IHC: Soak & Peel



### Tissue Transfer IHC: Peeling



### Tissue Transfer IHC



### What if it's an adhesive slide? Try Tissue Protection IHC

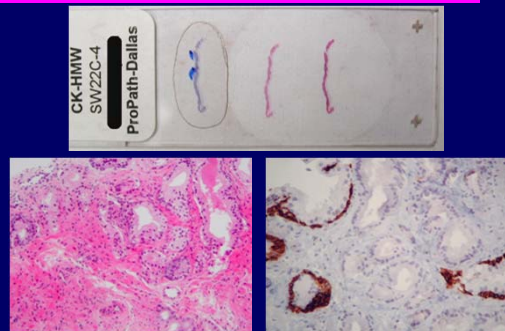


### Tissue Protection IHC: Prostate bx



Appearance of slide after Antigen Retrieval

### Tissue Protection IHC: Prostate bx

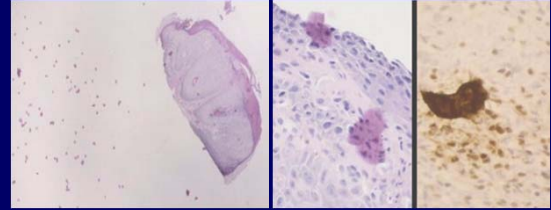




## IHC: Artifacts

- Endogenous biotin
- "Desquamartifact"
- Bubble Artifact
- Drying Artifact
- Trapping Artifact
- Edge Artifact
- "Bleeding" Artifact
- Nuclear Artifact
- Bad Titer Artifact
- Poor Fixation
- Microbial Contam.
- B-5 Artifact
- Pencil Artifact
- Waterbath Artifact
- Checkerboard Artifact
- "CK Juice" Artifact

## IHC: Desquamartifact



Low power

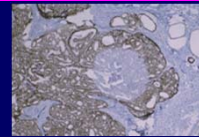
High power

CK AE1/AE3

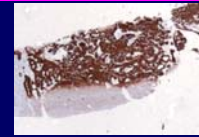
## Desquamartifact Remedy



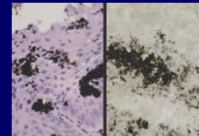
## IHC: Artifacts



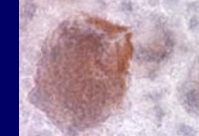
Bubble artifact



Drying artifact



Graphite Pencil artifact

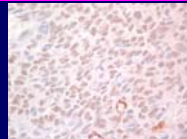


Trapping artifact, FNA smear

## IHC: Artifacts



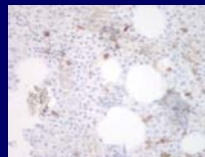
Bleeding artifact



Nuclear artifact, SMA

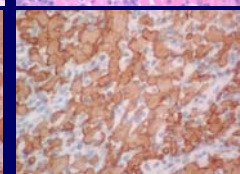
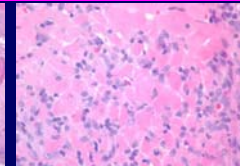
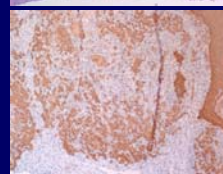


Checkerboard artifact 100x

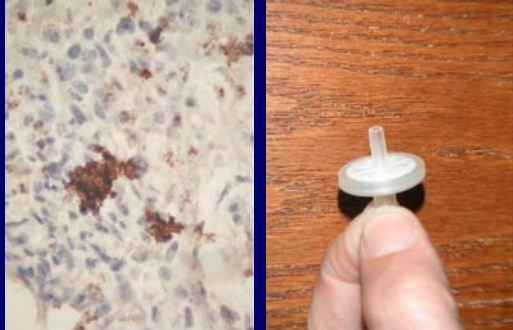


Checkerboard artifact 400x

## IHC: Artifacts – "CK Juice"



## IHC: Junk Artifact



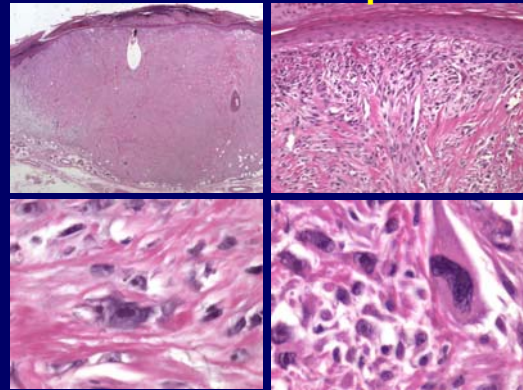
## OOPS.....

84 year old female underwent shave biopsy of a 1 cm nodule on the arm. H&E showed malignant spindle cell tumor. Bx sent to UFMC, reported as **spindle cell melanoma** based on pos S100 and neg keratin.

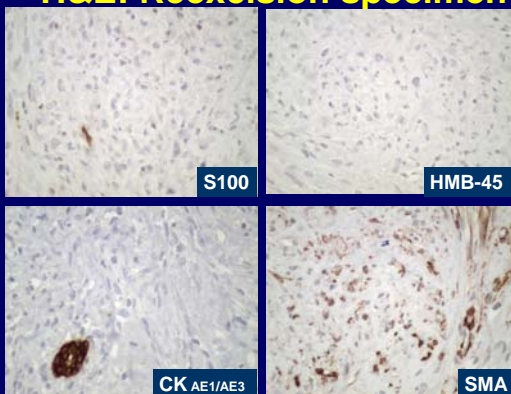
## OOPS.....

Wide local excision subsequently performed, and immunostains repeated at ULCH. Now it is neg for both S100 and keratin. Sent to ProPath for workup – S100 and keratin (and some other stains) neg. DX: **AFX**

## H&E: Reexcision specimen



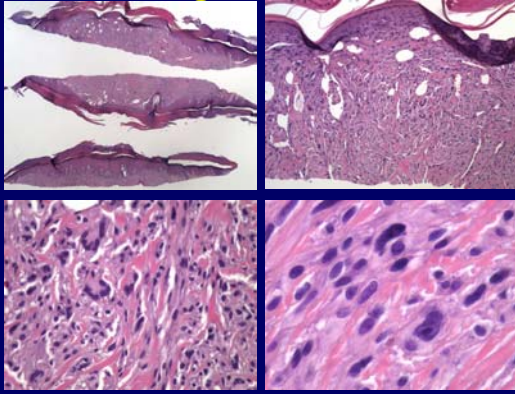
## H&E: Reexcision specimen



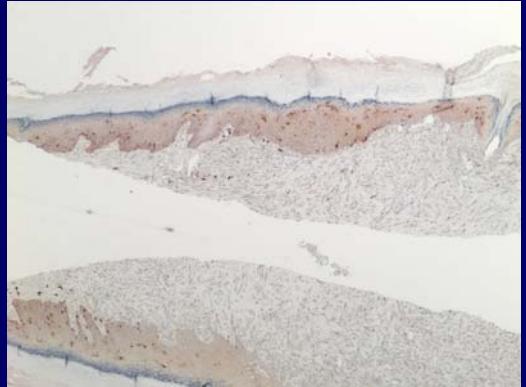
## OOPS.....

Surgeon, who trained at UFMC, refuses to believe that UFMC may have made a mistake. Original slides from the UFMC obtained for review.

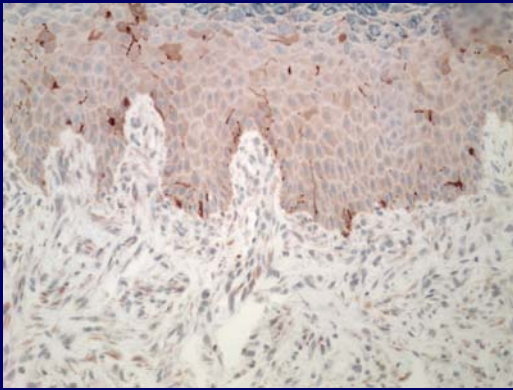
**H&E: Original shave bx**



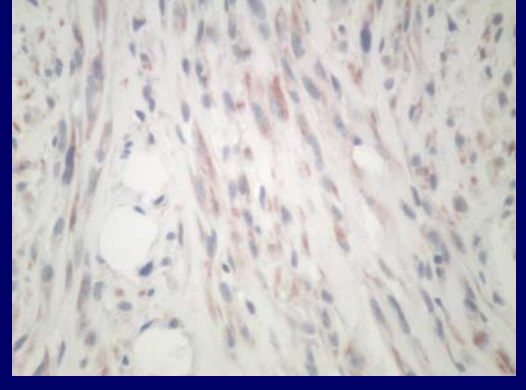
**S100: Original shave bx**



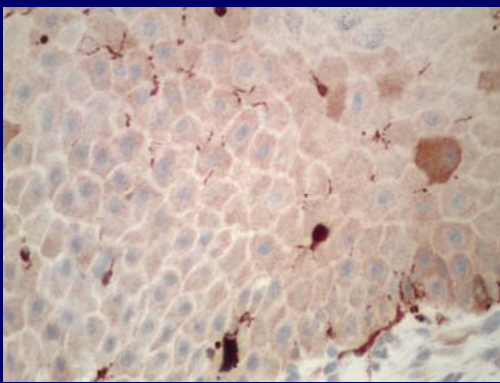
**S100: Original shave bx**



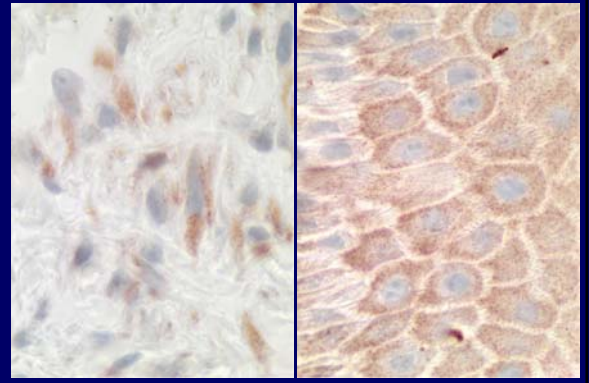
**S100: Original shave bx**



**S100: Original shave bx**



**S100: Original shave bx**



## Mistakes that were made

1. Positive control tissue not on same slide (lab uses “batch” positive controls)
2. No negative control slide with case
3. Lack of attention to expected pattern of reactivity (S100: nuclear and cytoplasmic)
4. Lack of recognition of characteristics of true positive and false positive stains
5. Lack of attention to internal positive and negative controls

## Miller's Rules of IHC: 1

- Generate a logical DDx, and use PANELS
- Treat your tissue right (Garbage in.....)
- IHC is not perfect, and tumors do not read textbooks. Correlate with H&E
- Make multitumor blocks and use them (and mount on same slides with patient tissue)
- Determine your own optimal titers

**(PREDILUTE = BAD)**

## Miller's Rules of IHC: 2

- Know spectrum and expected pattern of reactivity of markers used, know true positive & false positive stains, and don't ignore controls (internal and external)
- Block endogenous biotin if using avidin-biotin-based methods
- For cytologic IHC, GET A CELL BLOCK, and put smears on NON-ADHESIVE slides
- DON'T RUN A STAGNANT LAB

## Immunohistochemistry

