IMMUNOHISTOCHEMISTRY

ACCURATE LOCALIZATION OF
TISSUE OR CELLULAR
CONSTITUENTS WITH ANTIBODIES

FUNCTIONAL ROLE OF ANTIBODIES

- Identify the tissue of origin of a metastatic tumour
- Provide data for therapy
- Measure tumour antigen levels

TUMOUR ORIGIN

- CARCINOMA - Pan keratin
- SARCOMA - Vimentin, Desmin
- NEUROENDOCRINE – Synaptophysin
- LYMPHOMA - CD 45

THERAPY

- Hormone Receptors
  - Estrogen and Progesterone
    - Tamoxifen Therapy
  - HER 2-NEU
    - HERCEPTIN
- GIST
  - CD 117
    - Glivex

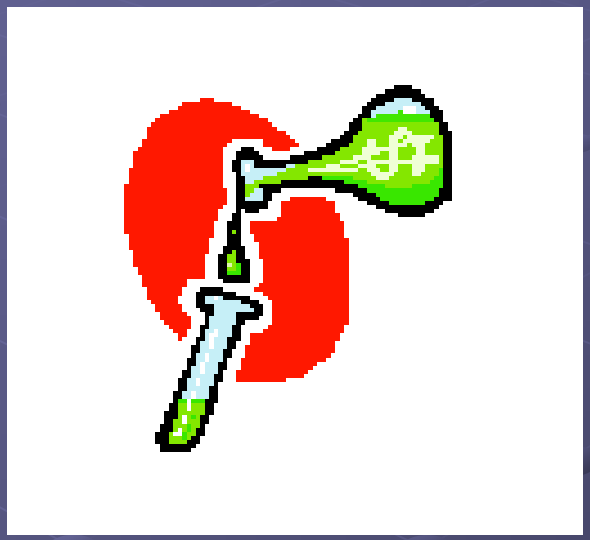
TUMOUR ANTIGEN LEVELS

- CA125 - Ovarian Carcinoma
- CEA - Colon & Gastric Cancer
- PSA,PSAP - Prostate Cancer

Antigen

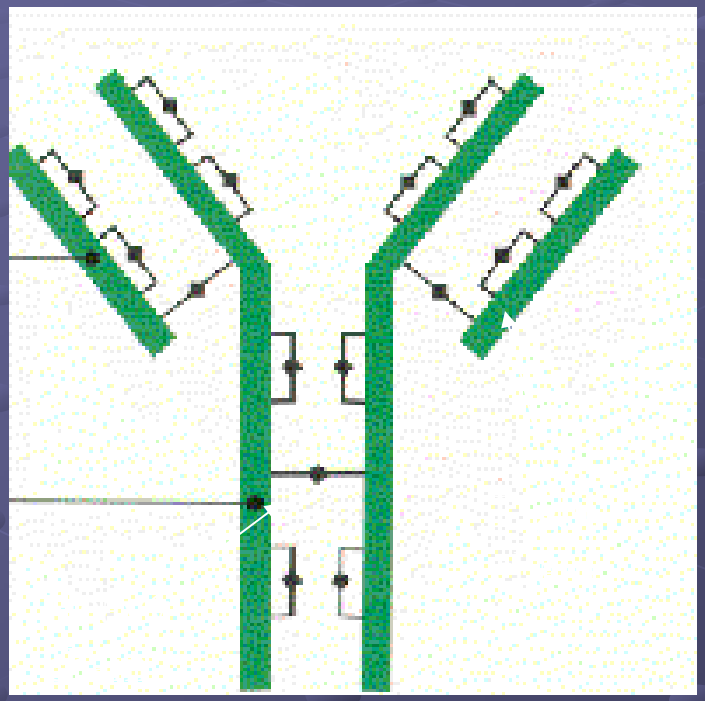
- Substance that can induce a detectable immune response
- Epitope -structural part of antigen.

Antibody



- Immunoglobulins that are produced as a result of the introduction of an antigen.

IgG IMMUNOGLOBULIN MOLECULE

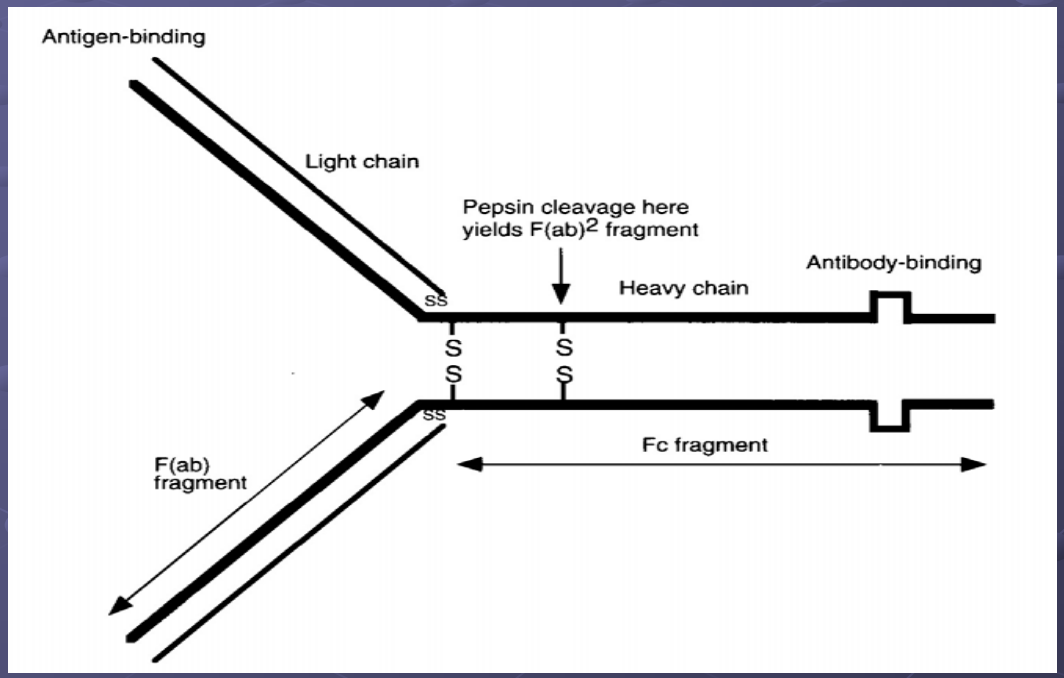


2 Identical Heavy (H) chains
Constant Fragment

2 Identical Light (L) chains
Either kappa or lambda
Variable Fragment

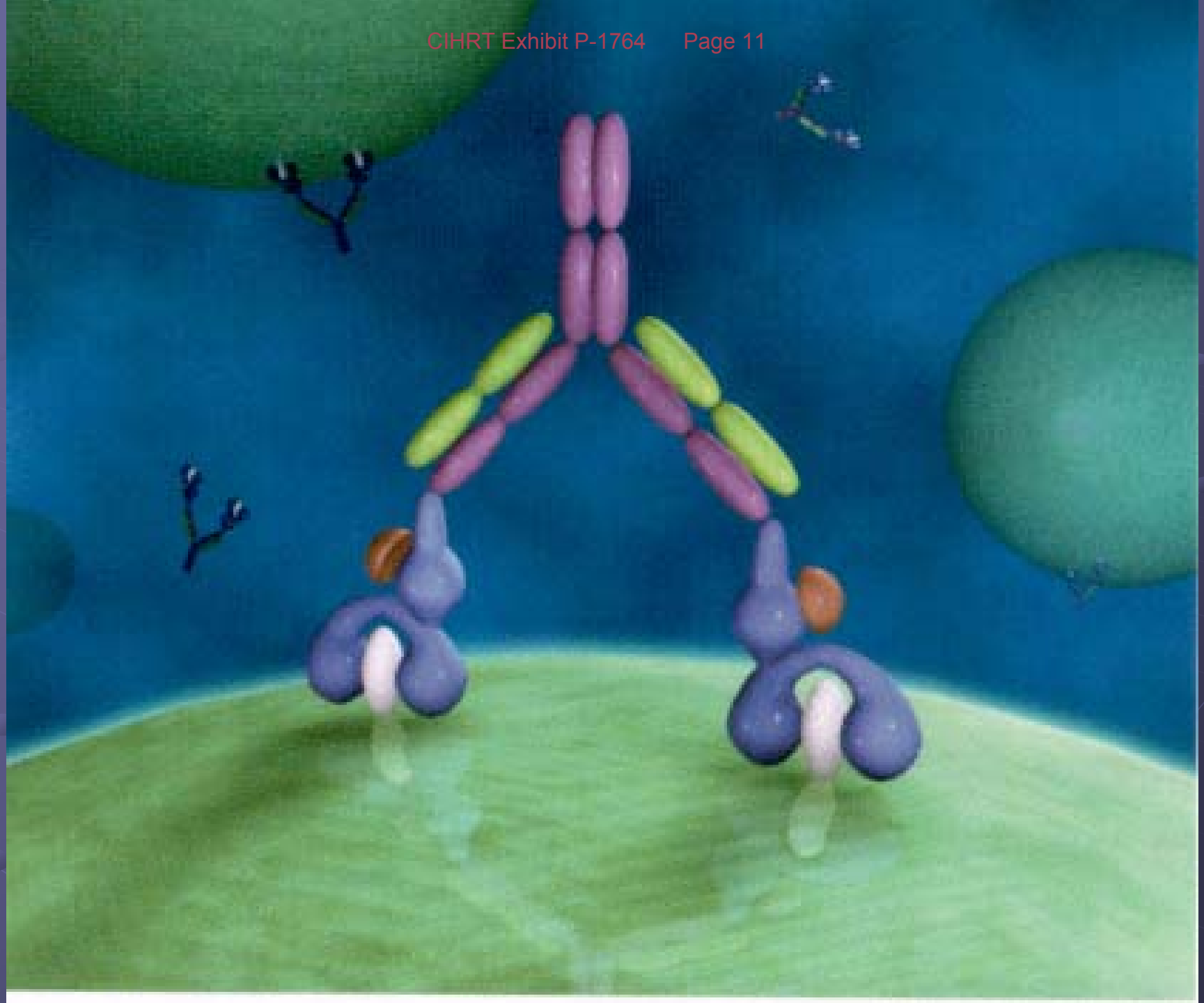
Inter and Intra chain disulfide bonds provide the stability and structure

- Fc portion carries the specific antigenic determinants to which ab raised to that particular IgG can bind



- The primary binds to the antigen in the tissue and then acts as an antigen for a second antibody

- The secondary ab binds to the anitgenic sites on the Fc portion of the primary ab molecule



Polyclonal Antibodies

- Immunochemically dissimilar, react with various epitopes.
- Injected with ag and titre measured.
- Once a high titre is achieved animal bled and serum purified.

Monoclonal Antibodies

- Immunochemically identical,clones of plasma cells directed to one epitope.
- Injected with ag, animal sacrificed. B-lymph fused + myeloma cell = Hybrid Myeloma cell.

ANTIBODY CHARACTERISTICS

- AFFINITY
- AVIDITIY

- AFFINITY
  - THE BINDING SITES “FIT” WELL WITH THE ANTIGENIC SITES ON ITS SPECIFIC AG
  - DO NOT WANT THE AB TO BIND TO OTHER AG

- AVIDITY
  - BINDING STRENGTH/STICKINESS
  - DEPENDS UPON THE NUMBER OF “FITTING” SITES BETWEEN THE AG AND THE AB

STAINING METHODS

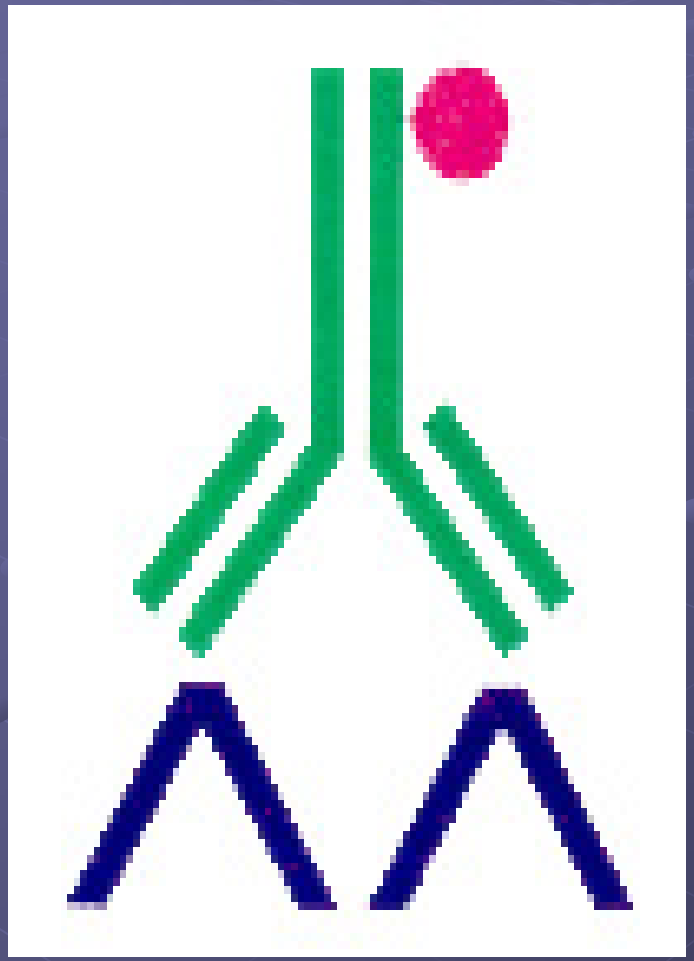
- Direct Method
- Two-Step Indirect Method
- PAP Method
- Avidin and Biotin Method

REQUIREMENTS FOR IHC

- PRESERVATION OF THE ANTIGEN IN THE TISSUE
- SPECIFIC AND SENSITIVE STAINING
- EFFICIENT LABELLING AND DETECTION

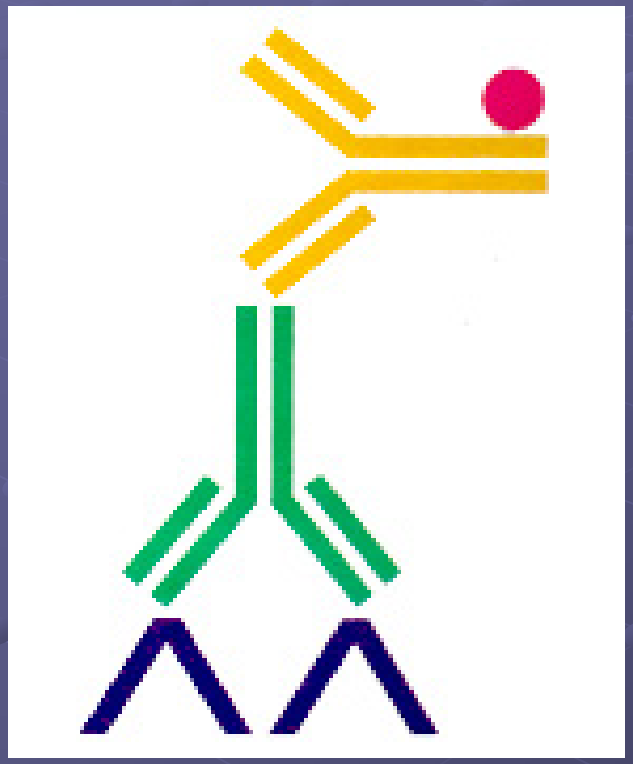
- PRESERVATION IS ACHIEVED BY FIXATION
- WANT THE ANTIGEN MADE INSOLUBLE BUT AVAILABLE FOR DETECTION
- FORMALIN
  - CROSS LINKING FIXATIVE
  - THE LONGER A PIECE OF TISSUE IS LEFT IN FORMALIN THE GREATER THE DEGREE OF CROSS LINKING

DIRECT Method



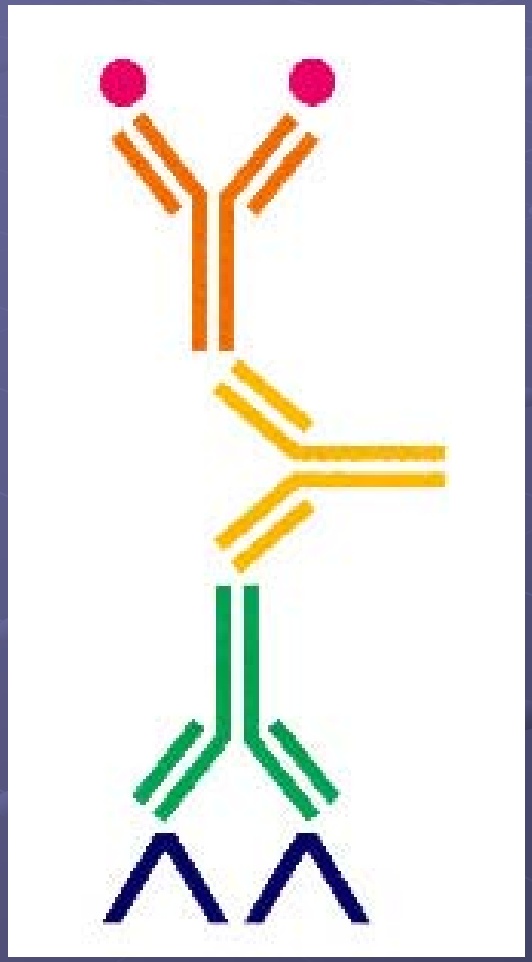
- Enzyme labelled primary antibody reacts with the antigen in the tissue.

TWO-STEP INDIRECT



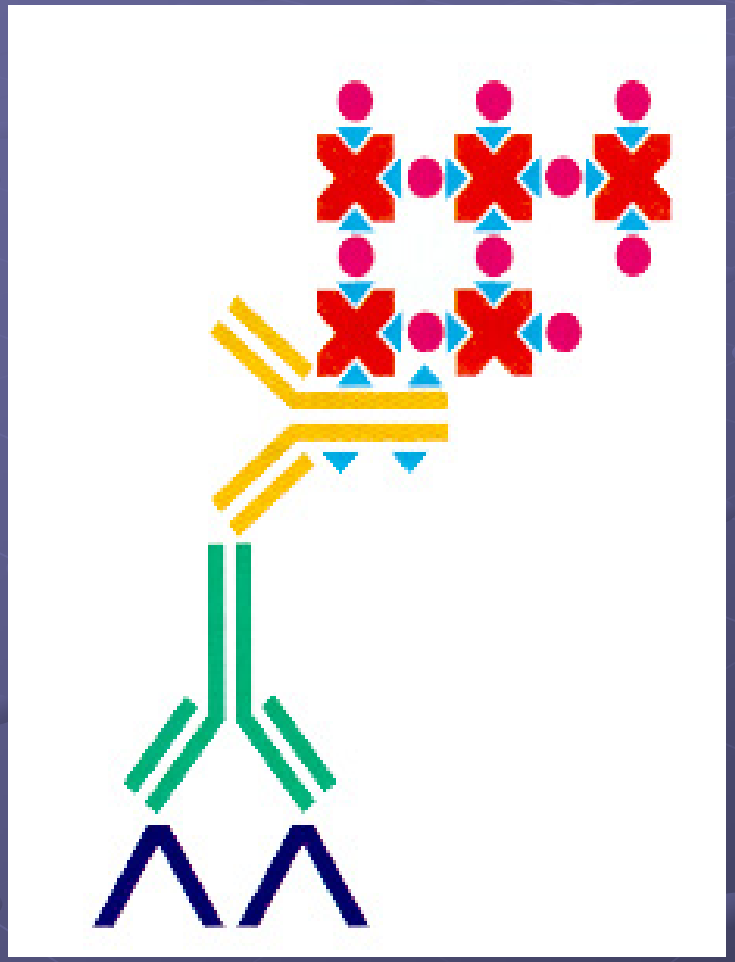
- Unconjugated primary antibody binds to the antigen.
- Enzyme labelled secondary antibody reacts with the primary antibody

PAP Method



- Soluble enzyme immune complex.
- Primary antibody and the animal specific

Avidin-Biotin Method



- Avidin has a strong affinity for biotin.
- Primary antibody is applied, then a biotinylated secondary then the avidin-biotin-enzyme complex.

CONTROLS

- REAGENT
  - Primary
  - Detection System
  - Chromogen
- TISSUE
  - Positive
  - Negative
  - Internal

REAGENT CONTROLS

- Positive and Negative
  - validation of staining technique
  - assessment of handling and fixation
  - standardization of methods and results amongst laboratories
  - education for performance and interpretation

TISSUE CONTROLS

- POSITIVE
  - Processed identically to the specimen
  - Contains the target protein
- NEGATIVE
  - Processed identically to the specimen
  - Does not contain the relevant tissue marker
- INTERNAL
  - Built in control

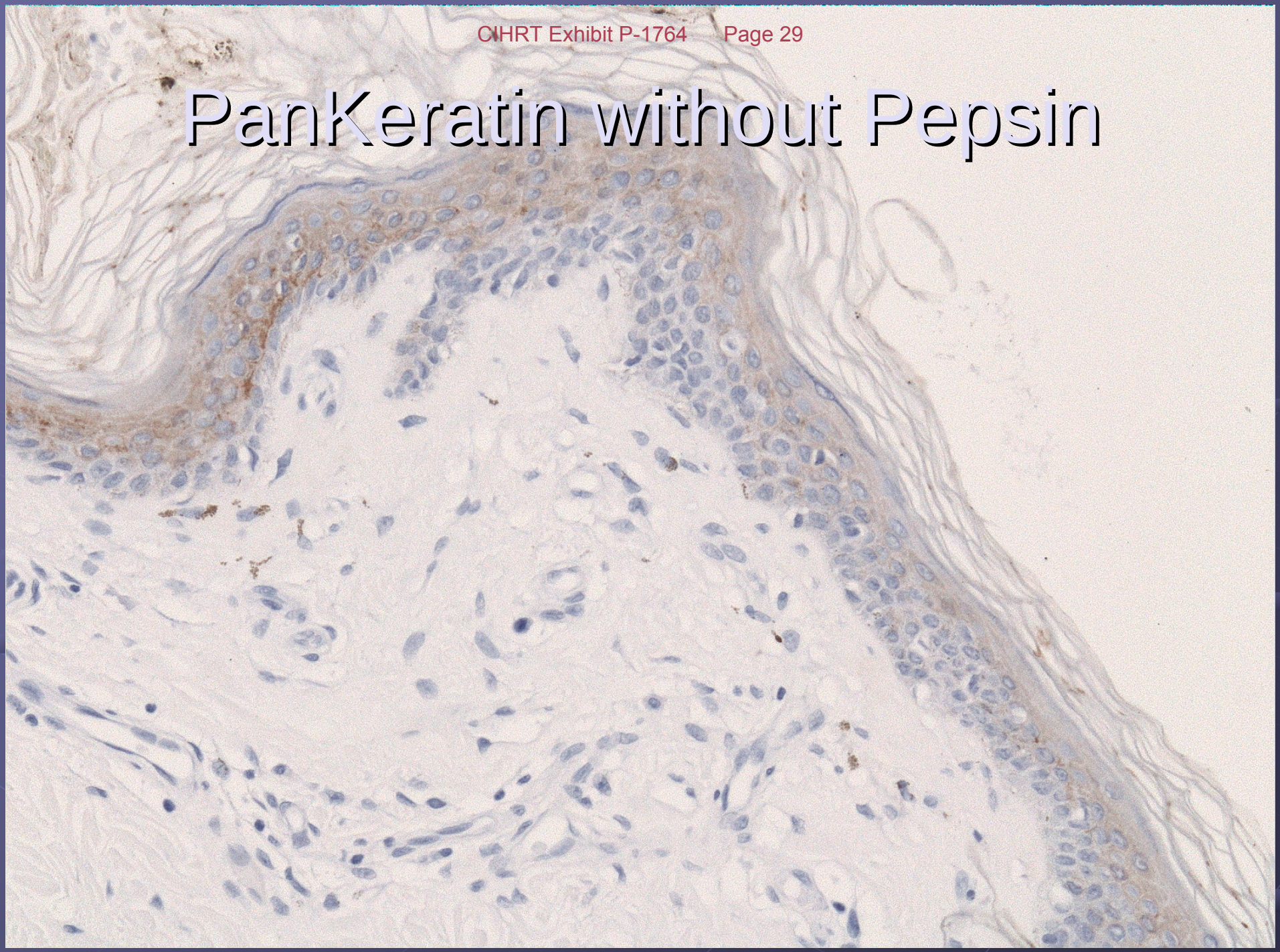
PRETREATMENTS

- Proteolytic digestion
- HIER aka Heat Induced Epitope Retrieval

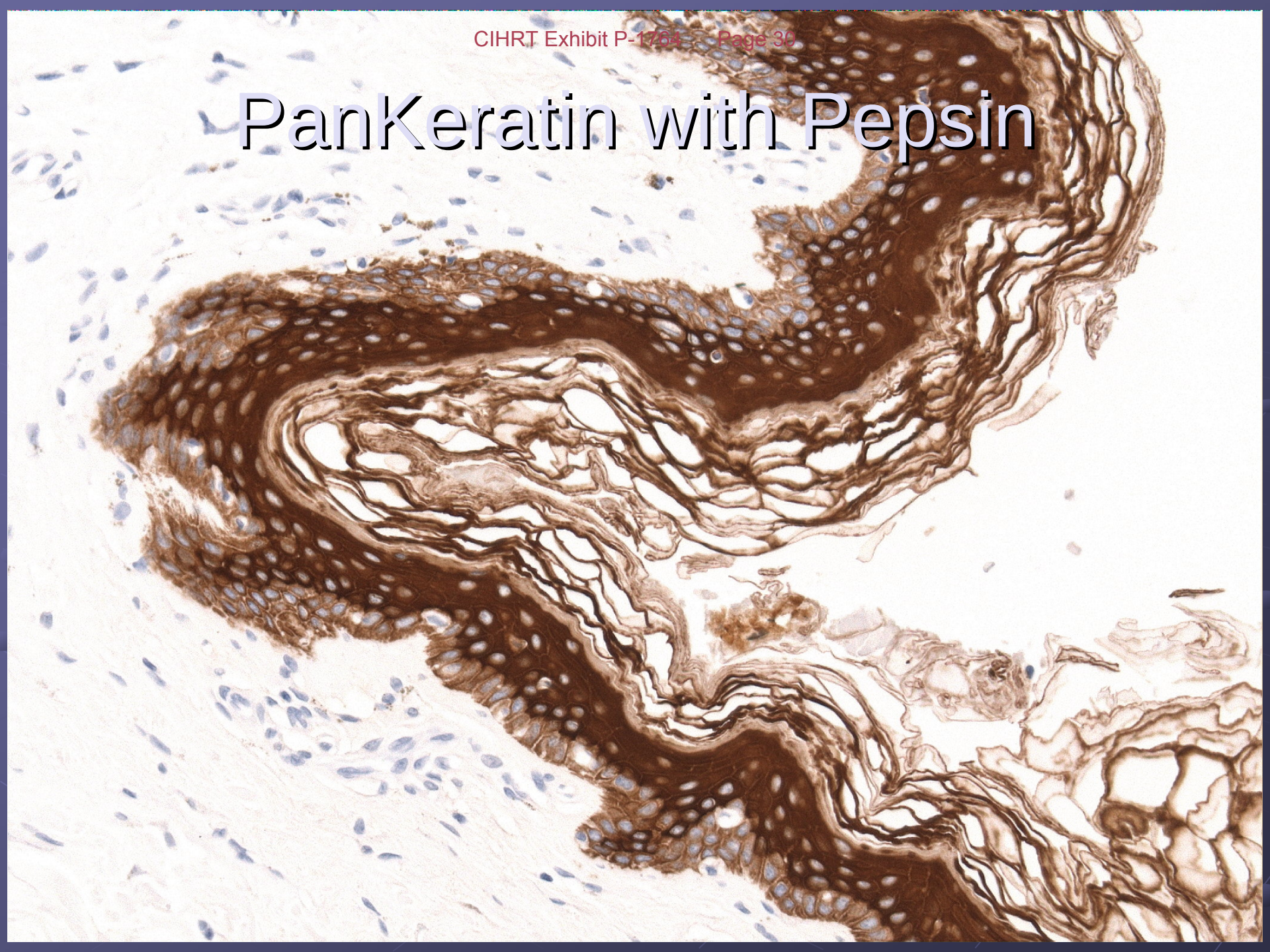
Proteolytic Digestion

- Pepsin
- Proteinase K
  - Formalin fixes by forming cross-linking methylene bridges
  - digestion compensates for the impermeable nature of the non-coagulant fixative
  - the enzyme etches the tissue allowing the epitope to be exposed.

PanKeratin without Pepsin



PanKeratin with Pepsin



HIER

- Heating provides the energy to rupture the hydroxyl bonds and releases tissue-bound calcium ions which break the fixative bond permanently exposing the epitope.

- Efficiency of HIER is a function of time, temperature, pH and the chemical composition of the buffer.

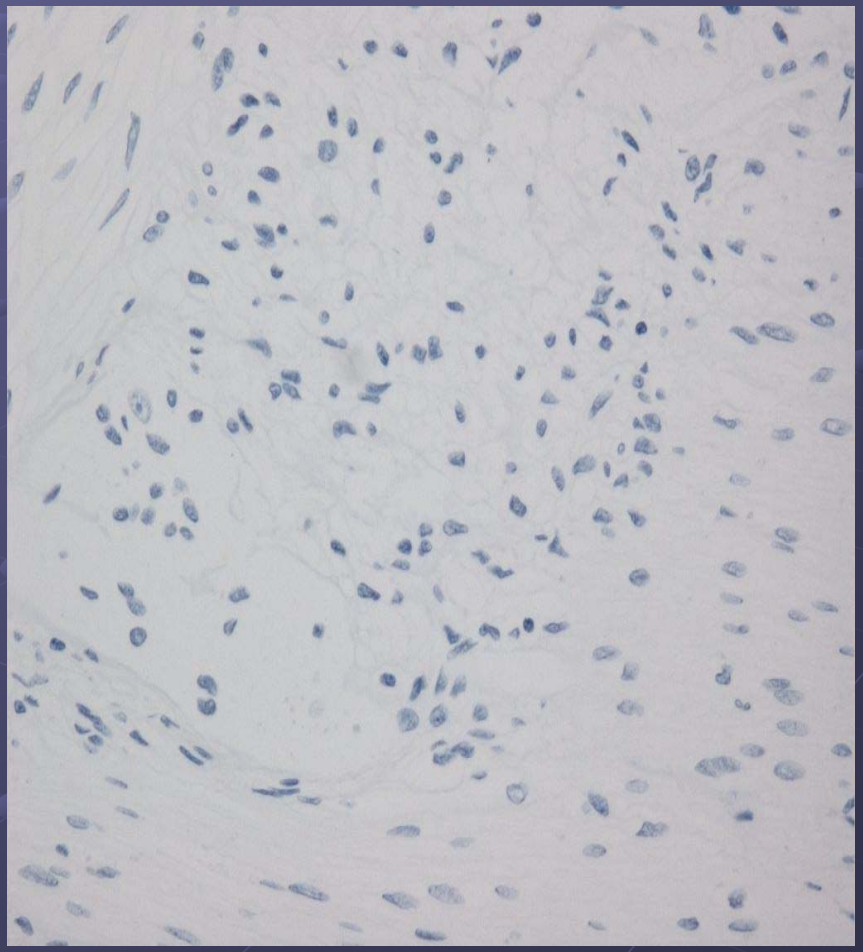
PGP 9.5

- Pretreatment

  Pepsin

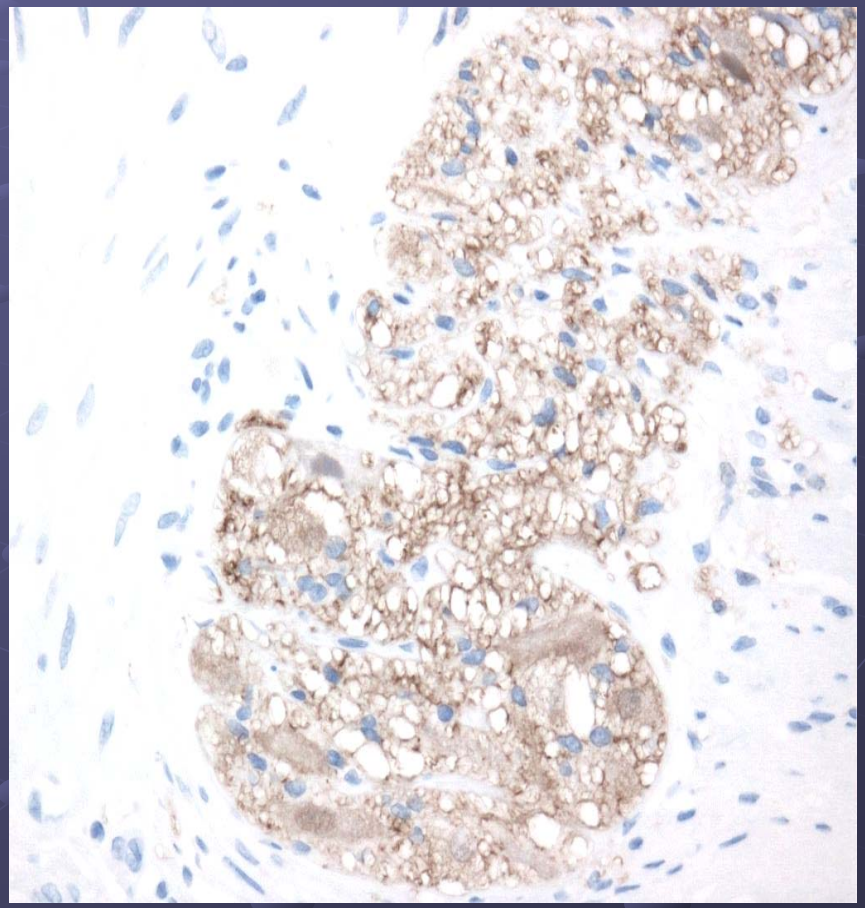
  37º C 10 min.

No staining observed



PGP 9.5

- Pretreatment
  - HIER
  - Citrate Buffer pH 6.0
  - 3 min @ 115 ° C

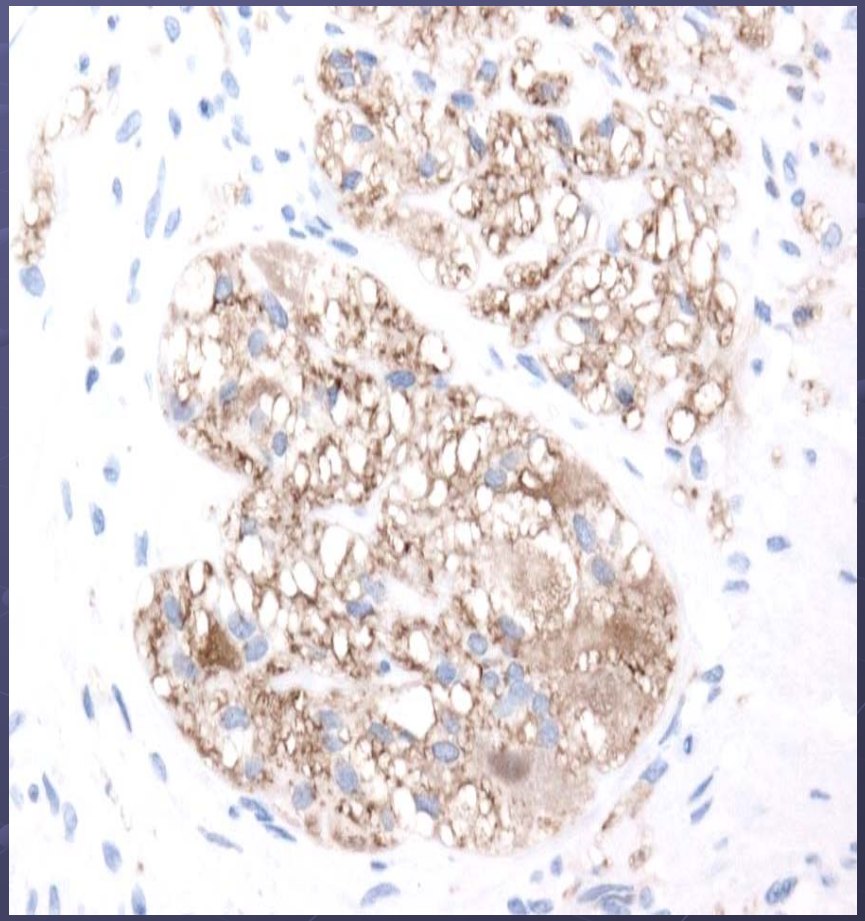


PGP 9.5

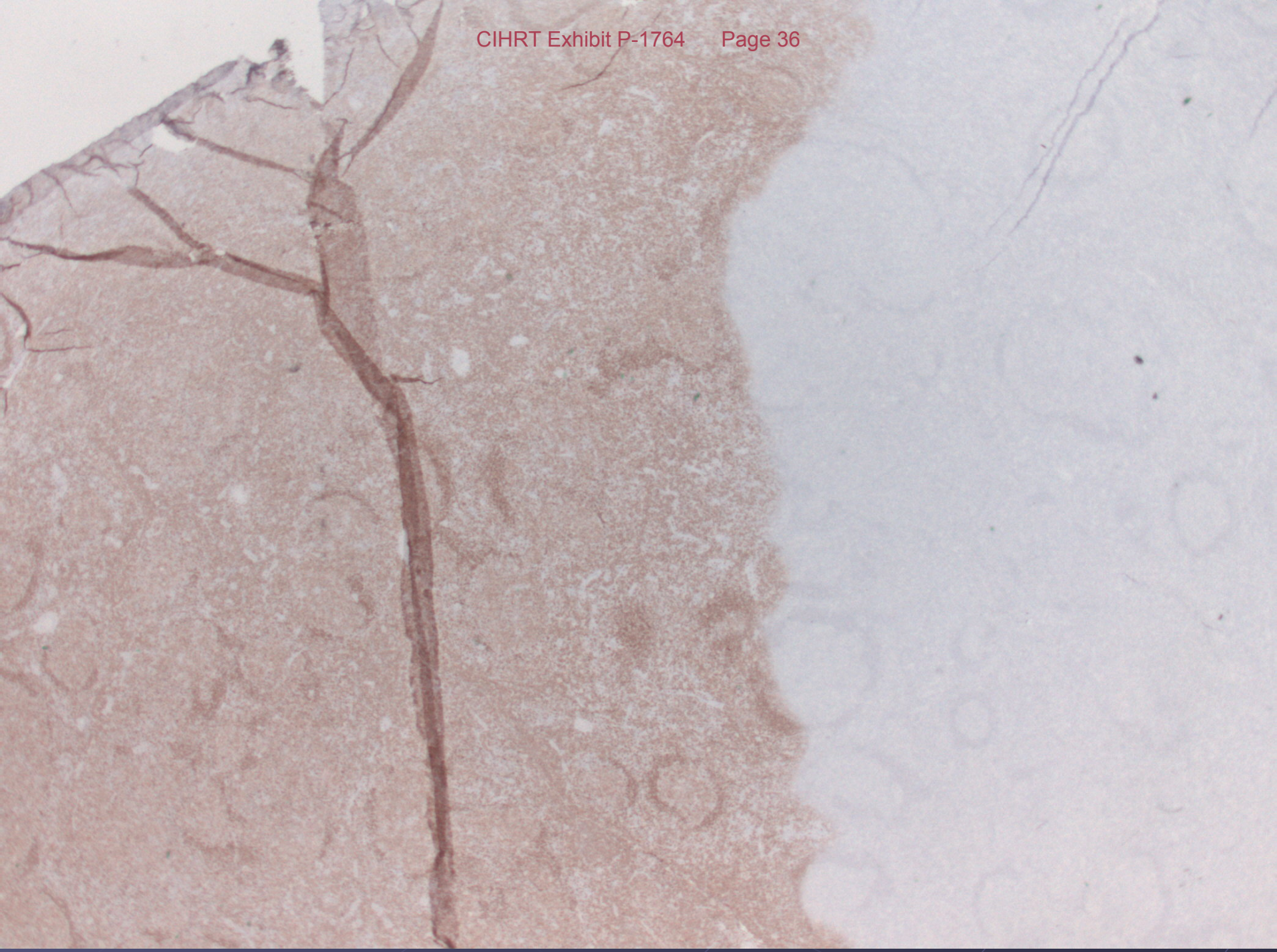
- Pretreatment
  - HIER
  - Tris-HCl pH 9.0
  - 3 min @ 115 ° C

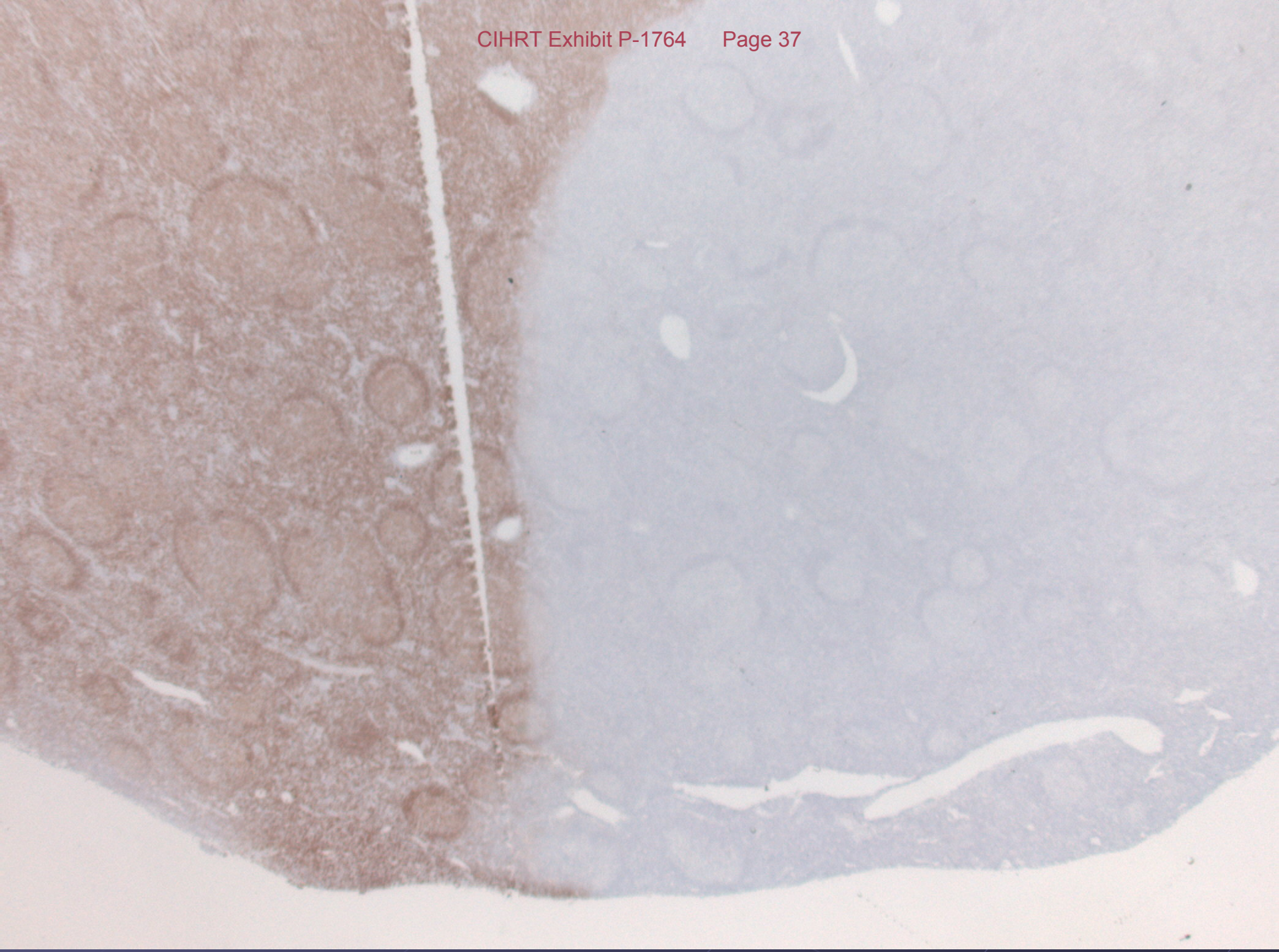
End Product intensified

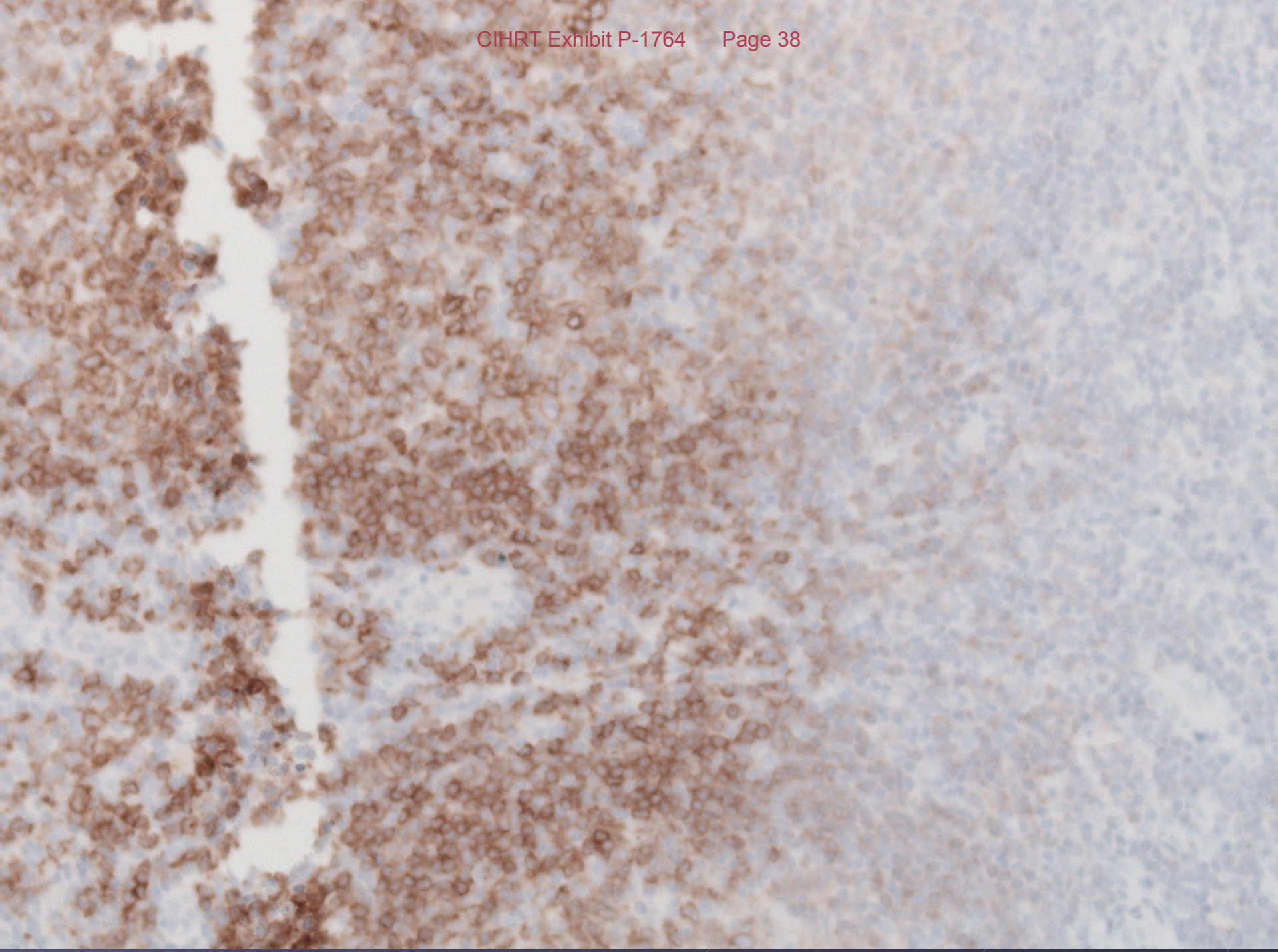
High signal/low noise

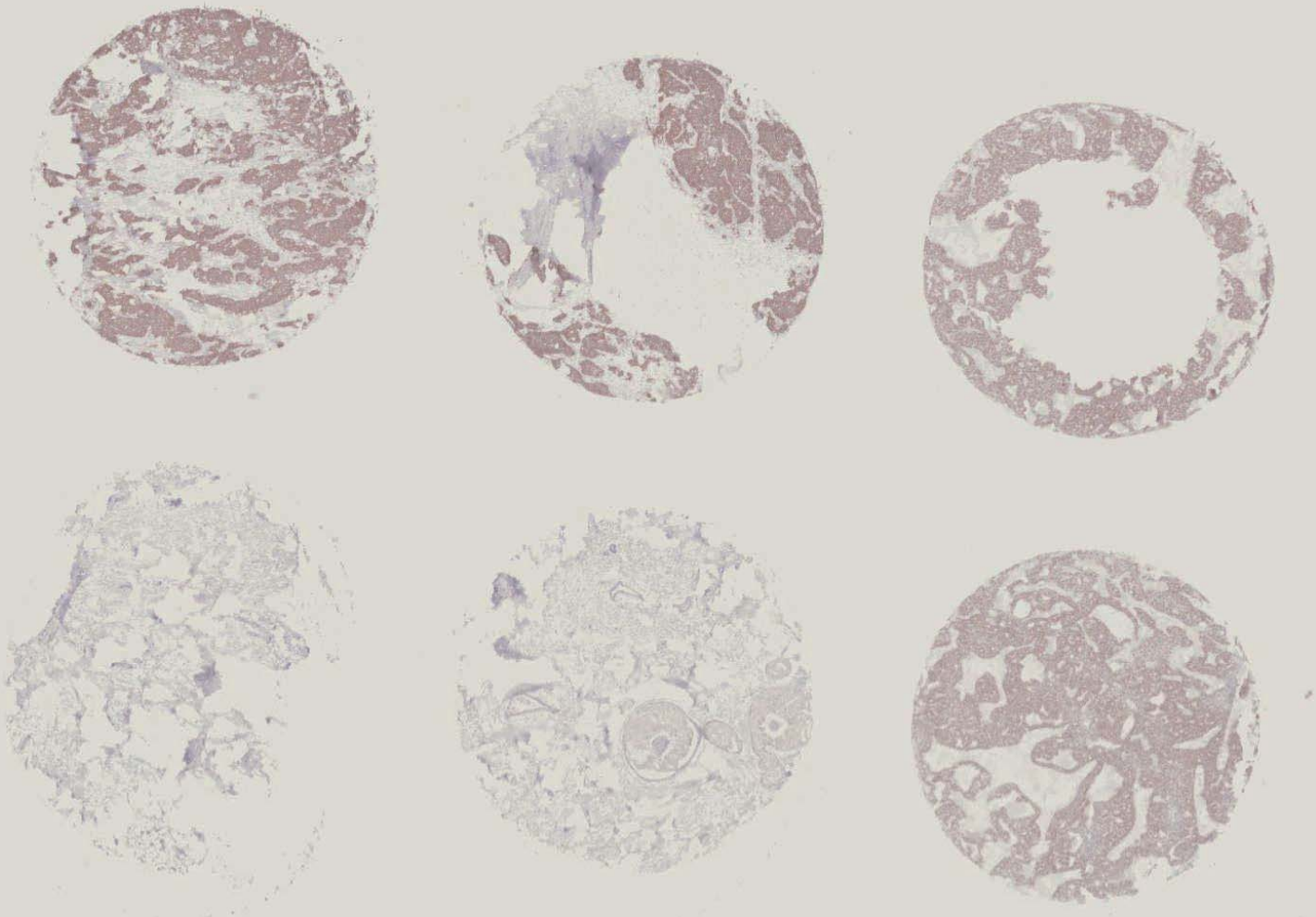


SECTION QUALITY





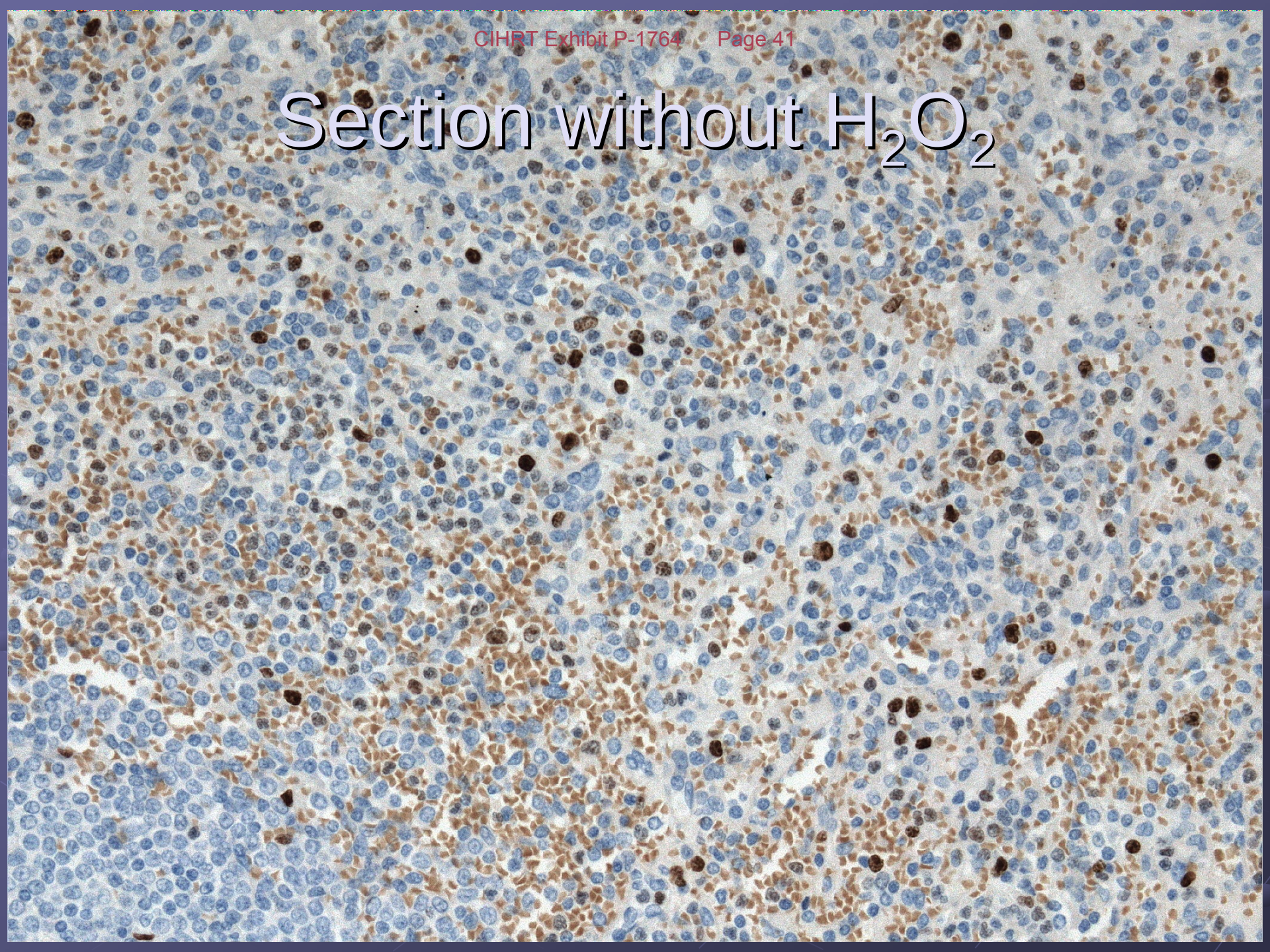




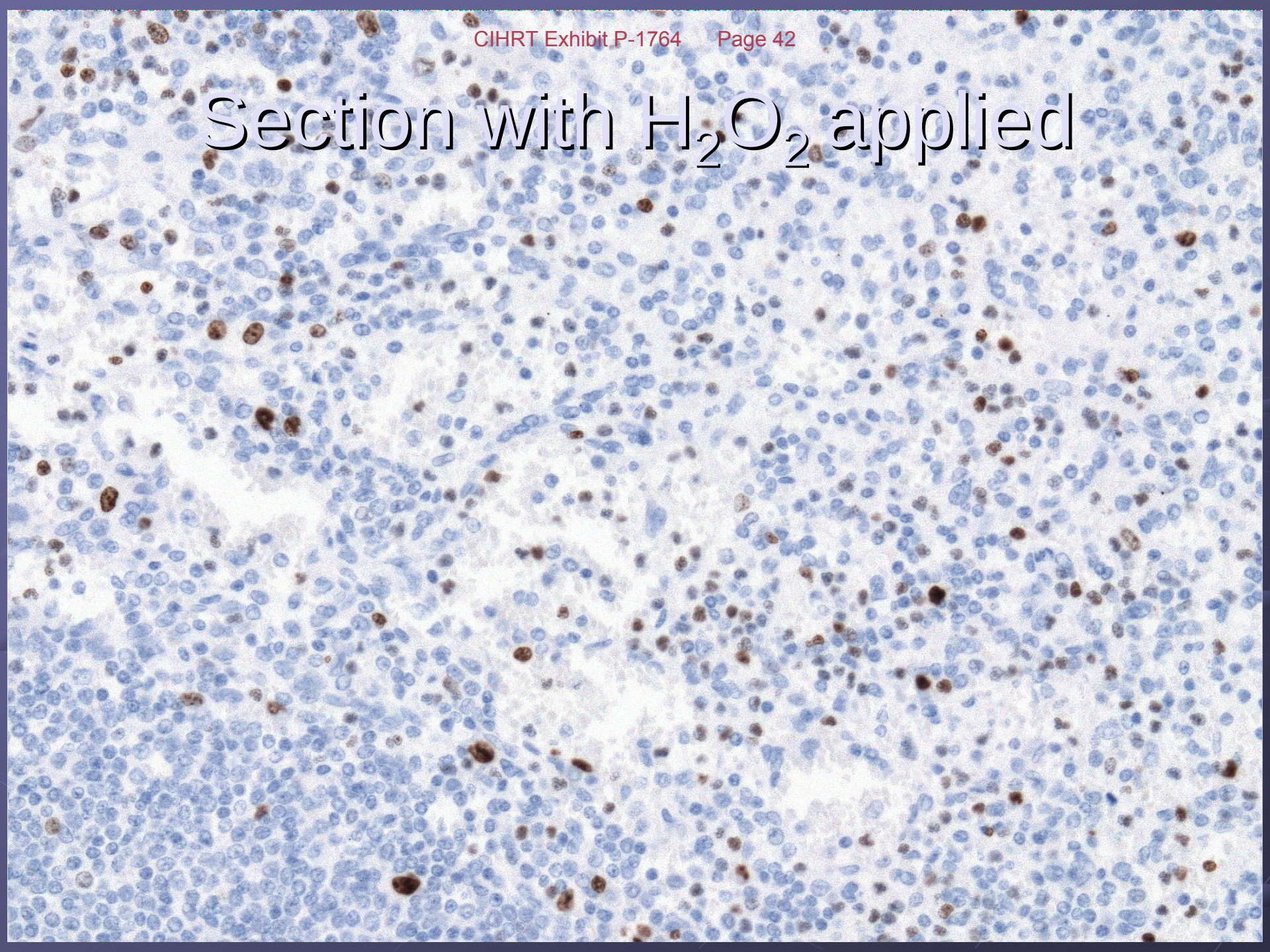
BACKGROUND STAINING

- ENDOGENOUS PEROXIDASE ACTIVITY
- ENDOGENOUS AVIDIN-BINDING ACTIVITY

Section without H_2O_2



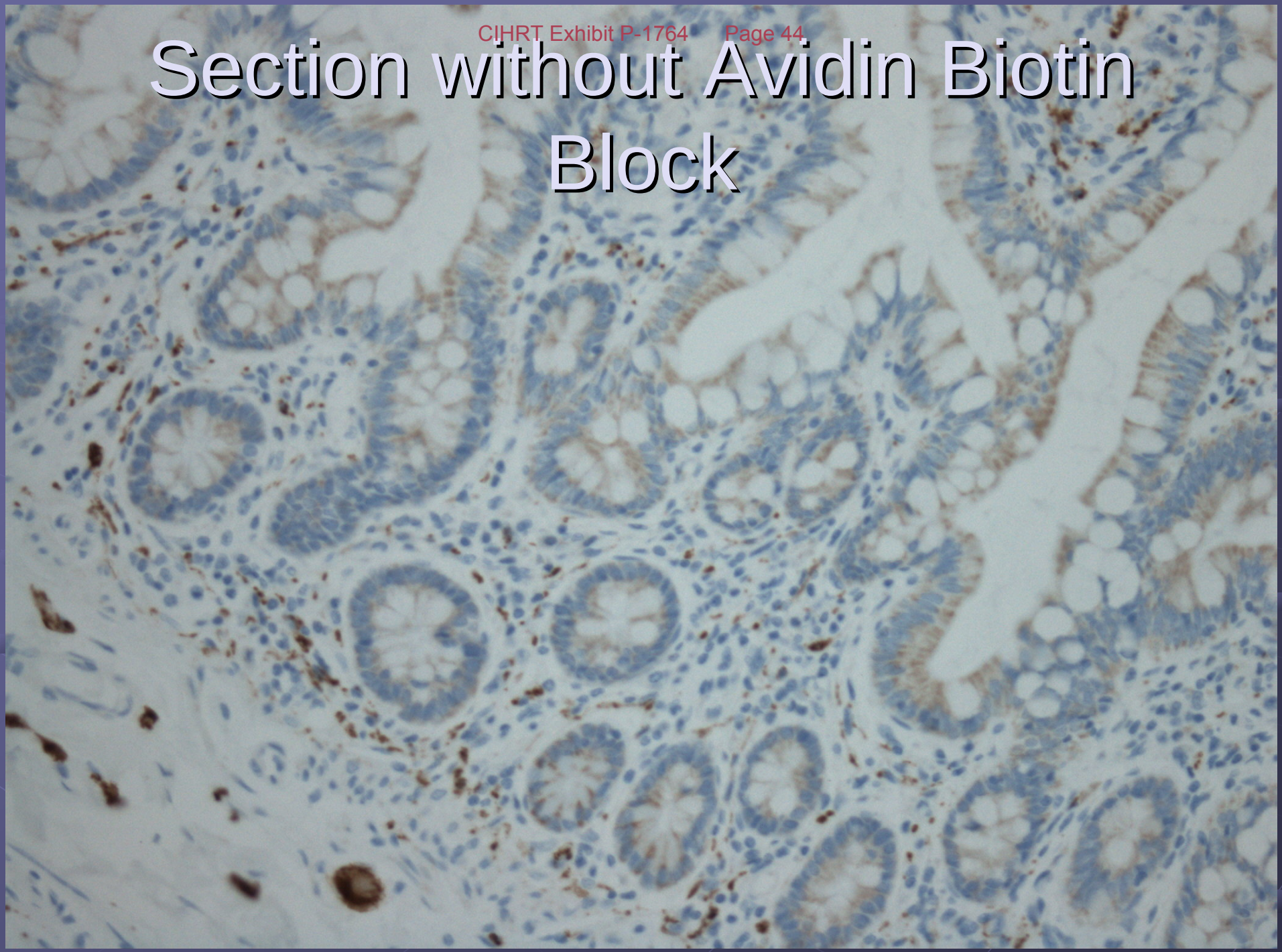
Section with H_2O_2 applied



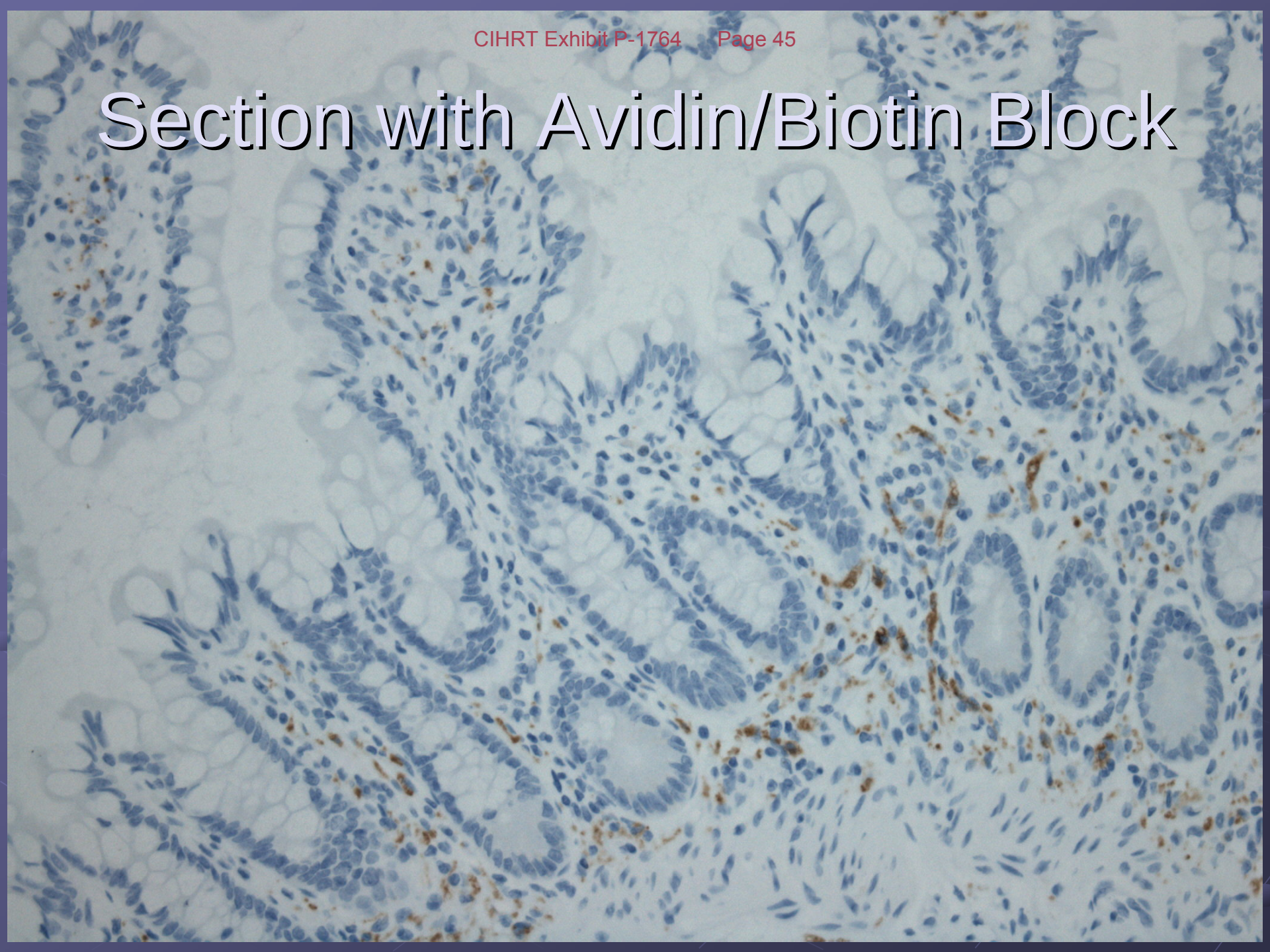
ENDOGENOUS AVIDIN-BINDING ACTIVITY

- Biotin is a vitamin and coenzyme.
- Biotin binds avidin or streptavidin specifically.
- Non-specific staining resembles a diffuse,cytoplasmic pattern.
- Eliminate with avidin-biotin blocking.

Section without Avidin Biotin Block



Section with Avidin/Biotin Block

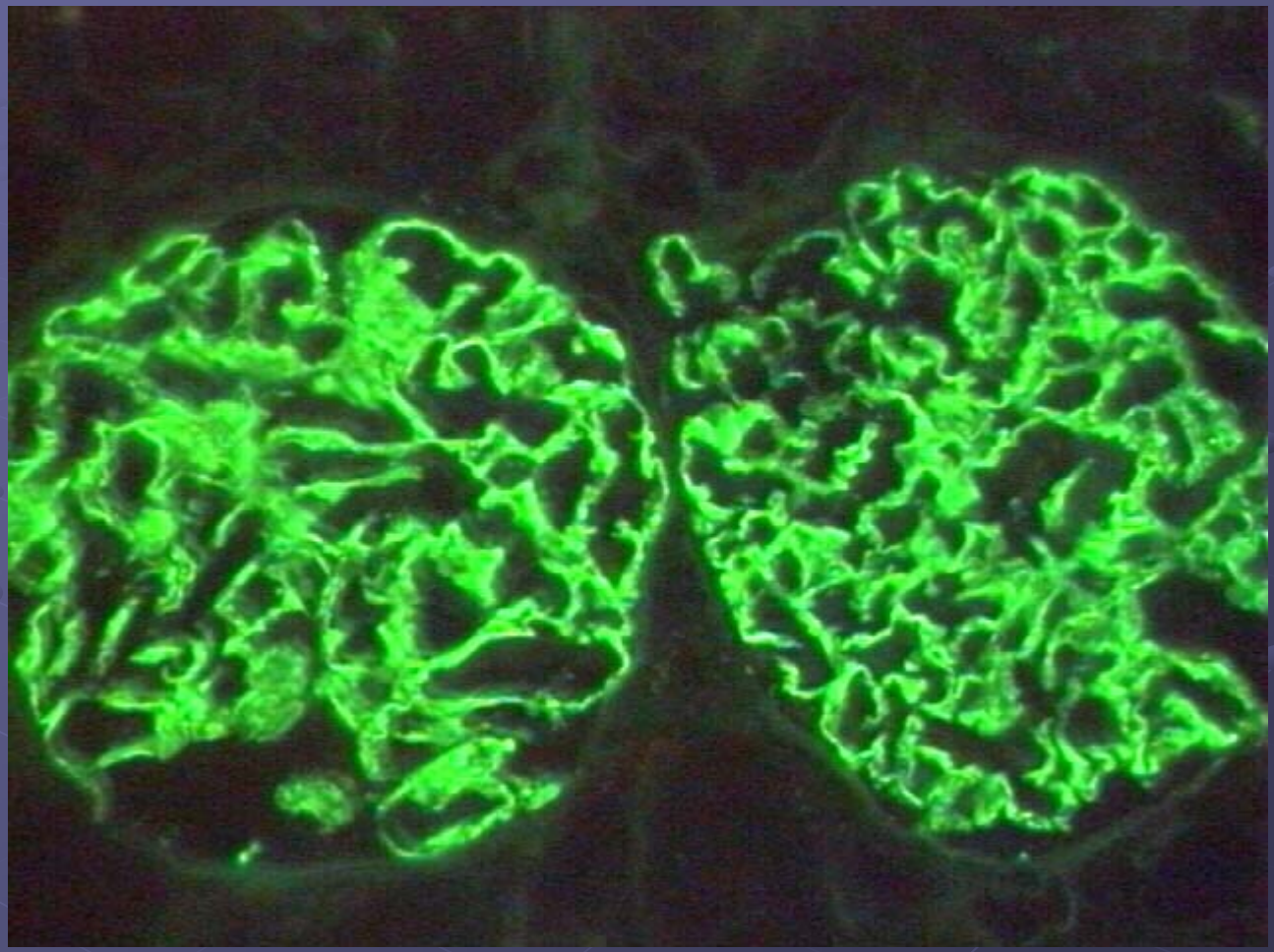


CHROMOGENS

- IMMUNOFLUORESCENCE
- IMMUNOENZYMATIC

IMMUNOFLUORESCENCE

- FITC
- TEXAS RED
- DAPI



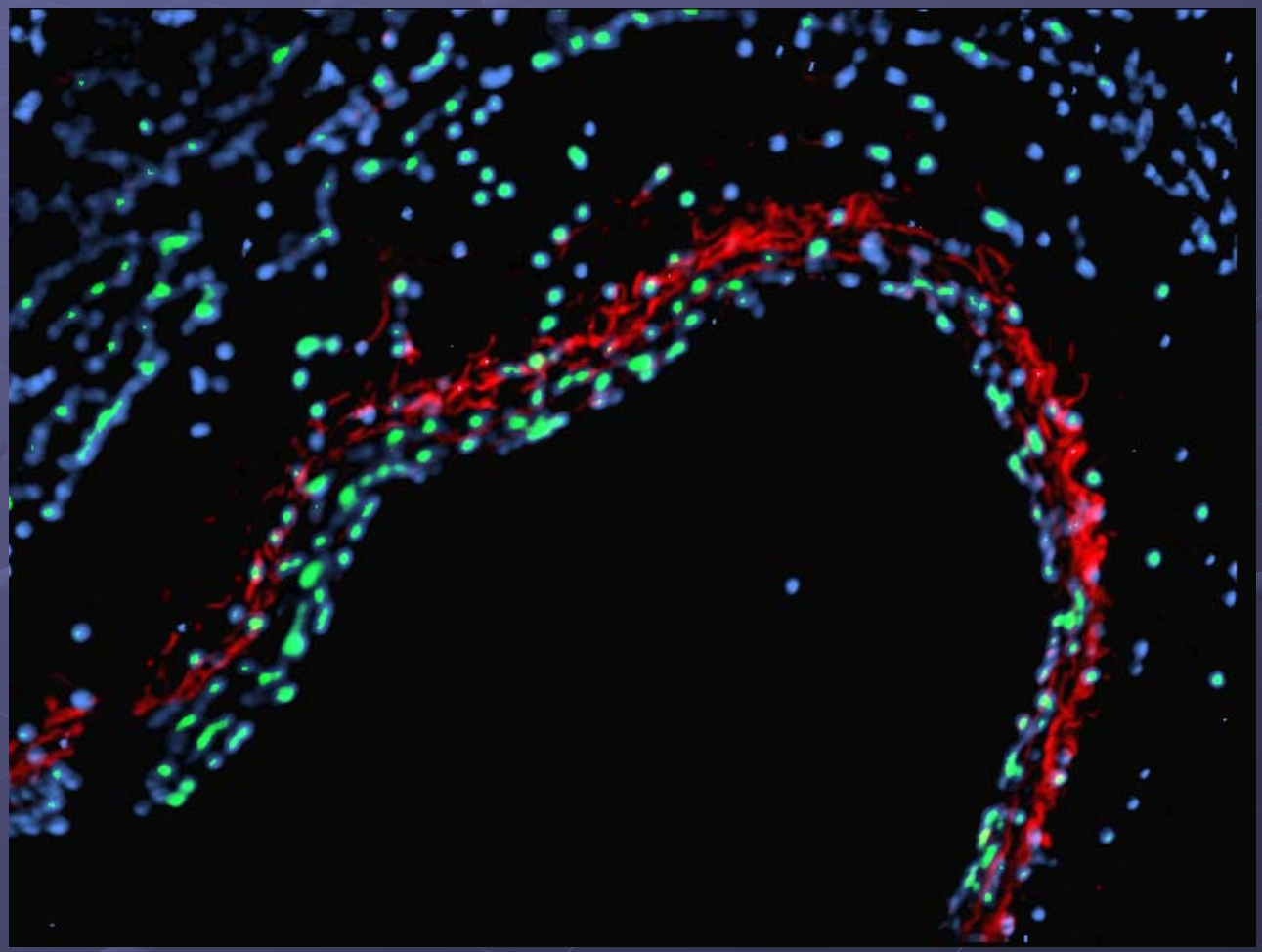
Triple Fluorescent Staining

DAPI-
nuclear

FITC-
TUNEL

Texas Red-
vWF

400x
Rat lung



IMMUNOENZYMATIC STAINING

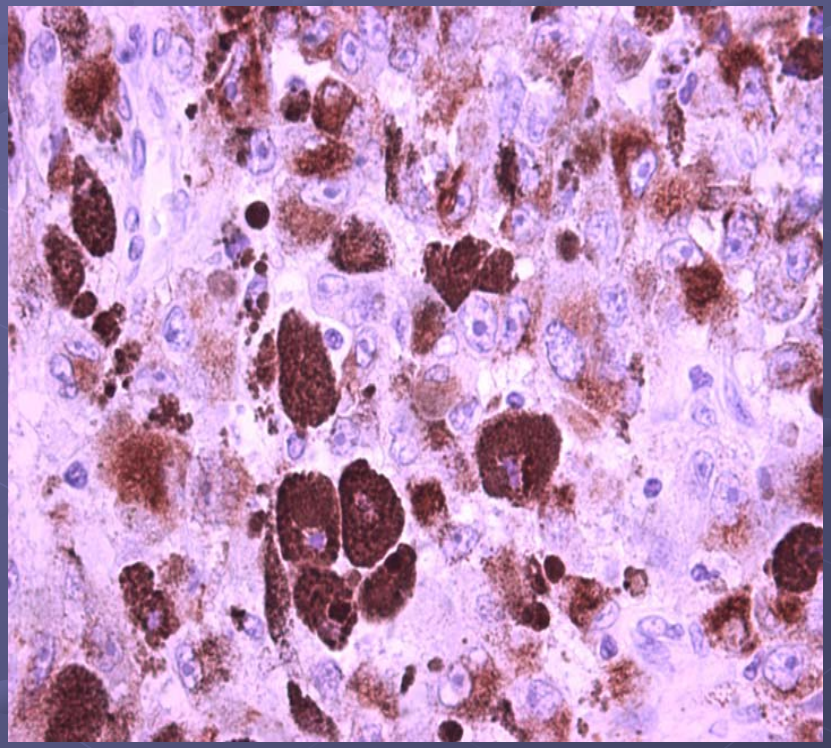
- Allows the visualization of cell components
- Enzyme-substrate reactions convert colourless chromogens into coloured end products which precipitates at the site of the antigen that is localized.
- Reaction is insoluble upon oxidation.

ENZYMATIC MARKERS

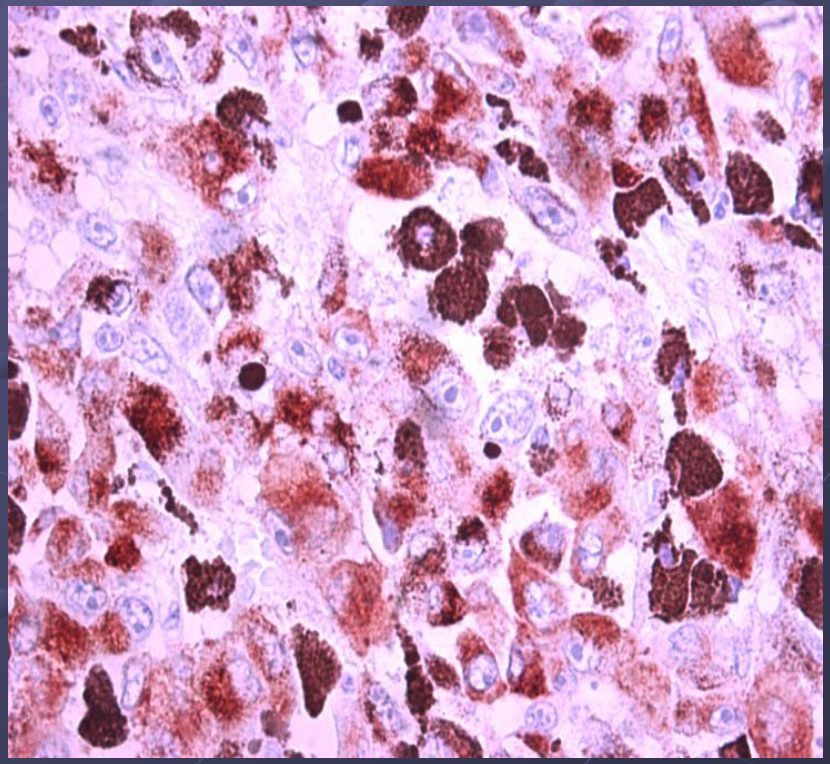
- DAB
  - Brown end product which is highly insoluble in alcohol and other organic solvents.
- NOVA Red
  - Red end product which is insoluble in alcohol and other organic solvents.

HMB45

DAB

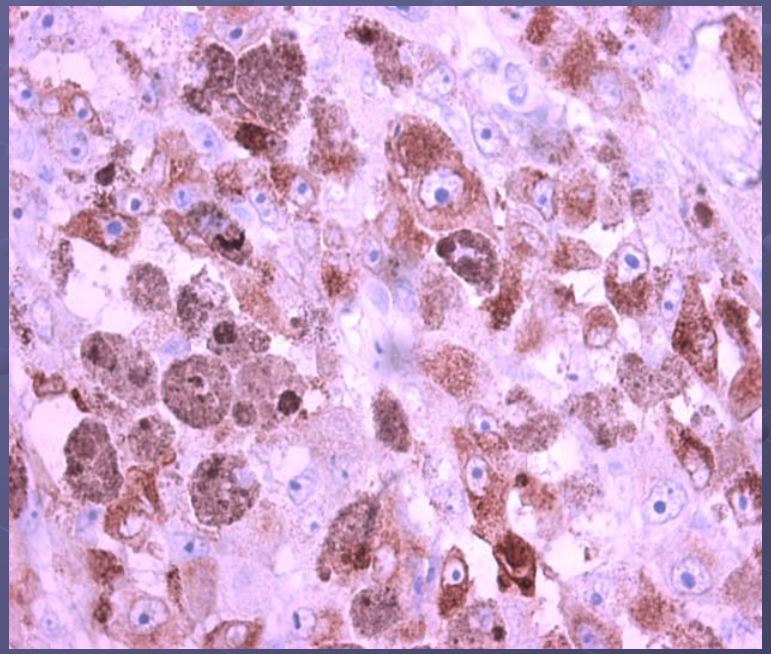


NovaRED

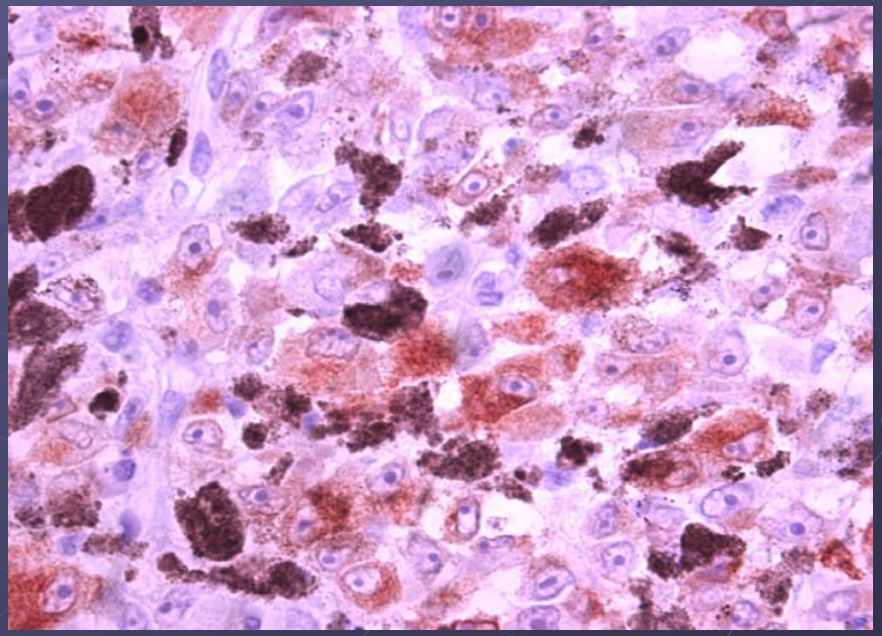


MART-1

DAB



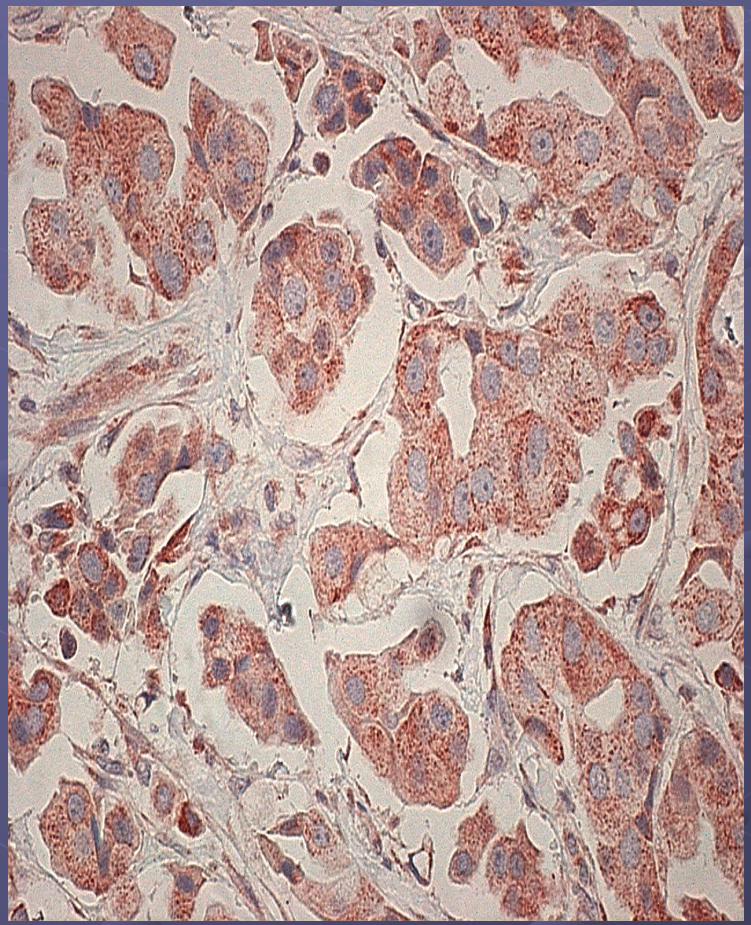
NovaRED



SPECIMENS

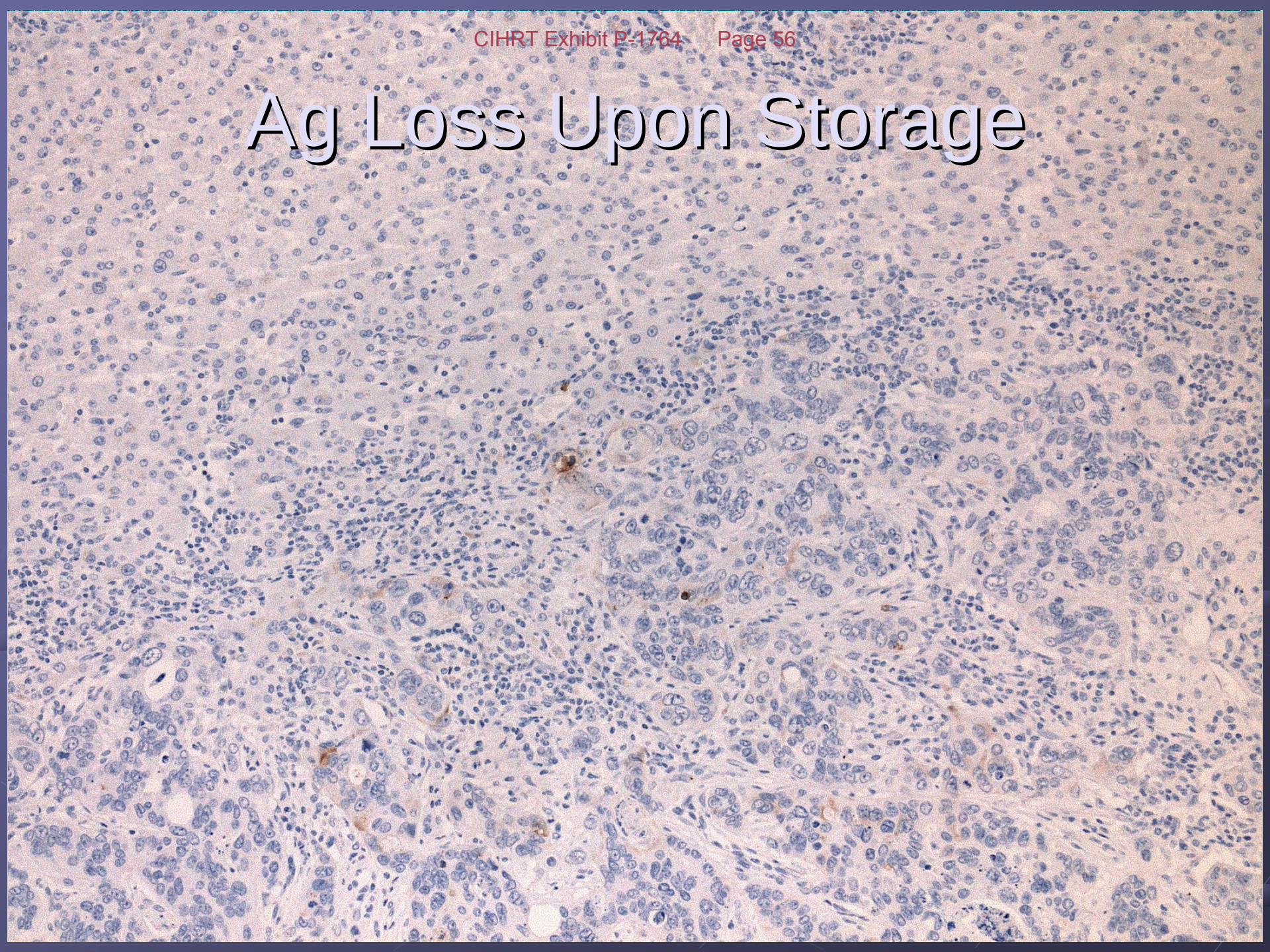
- **F**ormalin fixed, paraffin embedded
  - pretreatments
- **F**rozen specimens
  - acetone fixed (preserves immunoreactive sites)
- **B**lood Smears
  - acetone fixed
- **C**ytospins
  - alcohol fixed (preserves chromatin & nuclear changes)

Factors affecting the Quality of Immunostaining



- Antibody titre
- Antibody Dilution
- Incubation Time
- Incubation Temperature
- Pretreatments
- Buffers, pH

Ag Loss Upon Storage



Freshly Cut Section