Workshop in Diagnostic Immunohistochemistry

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Immunohistochemical double stainings – overview, considerations and applications

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Article

When Tissue Antigens and Antibodies Get Along: Revisiting the Technical Aspects of Immunohistochemistry—The Red, Brown, and Blue Technique

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J. A. Ramos-Vara[1] and M. A. Miller[1]

Table 1. Steps and Variables in an Immunohistochemical Test.

	Steps	Variables
Preanalytical phase	Sample procurement	Delayed fixation, prolonged ischemia, thickness of sample
	Fixation	Cross-linking vs coagulating fixatives, duration
	Decalcification	Type of decalcification solution and duration
	Tissue processing	Paraffin-embedded vs frozen tissues
	Tissue sectioning	Thickness of tissue section, drying temperature and duration, tissue section aging
Analytical phase	Deparaffination	Dewaxing agent
	Antigen retrieval	Detergents, enzymes, HIER
	Blocking nonspecific reactivities	Endogenous enzymes, hydrophobic binding, pigments
	Primary antibody	Monoclonal vs polyclonal, Ag recognition (native vs linear), specificity, species variability
	Detection system	Avidin-biotin vs polymer-based systems, ultrasensitive methods
	Enzyme-substrate-chromogen	Color detection
	Multiplex IHC	Enzyme-substrate combinations
	Counterstain	Contrast between chromogen and counterstain
Postanalytical phase	Control performance	Animal species compatibility, tissue processing
	Interpretation	Pathologist vs automated evaluation
	Report	Percentage of positive cells, positive vs negative threshold, stand-alone test vs ancillary test
		Diagnostic, prognostic, or theranostic test

HIER, heat-induced antigen retrieval; IHC, immunohistocehmistry.

Double/Multiplex staining techniques (IHC)

Multiplex staining can be defined as the detection of two or more antigenic epitopes on one slide

Advantages:

There is a growing need to extract more information from limited tissue samples (less invasive sampling techniques)

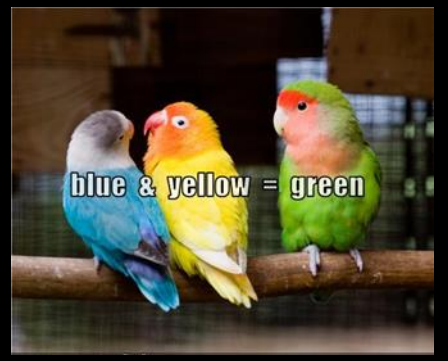
Saves tissue - there is an increasing demand for performing molecular techniques on the "leftovers" (remnants of tissue)

Overcomes some of the problems related to performing serial sections

- **Cellulare structures or small foci of tumor cells may be lost during processing.**

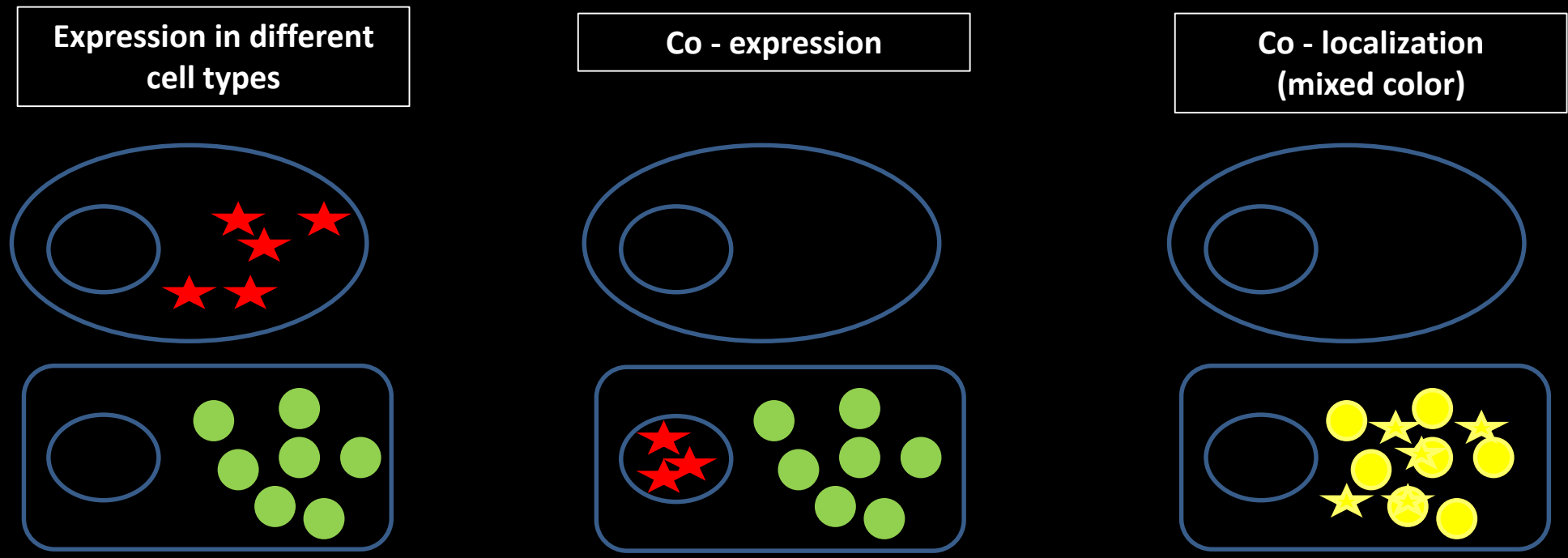
Saves time - microscopy (quick overview)

Multiplex staining techniques (IHC)

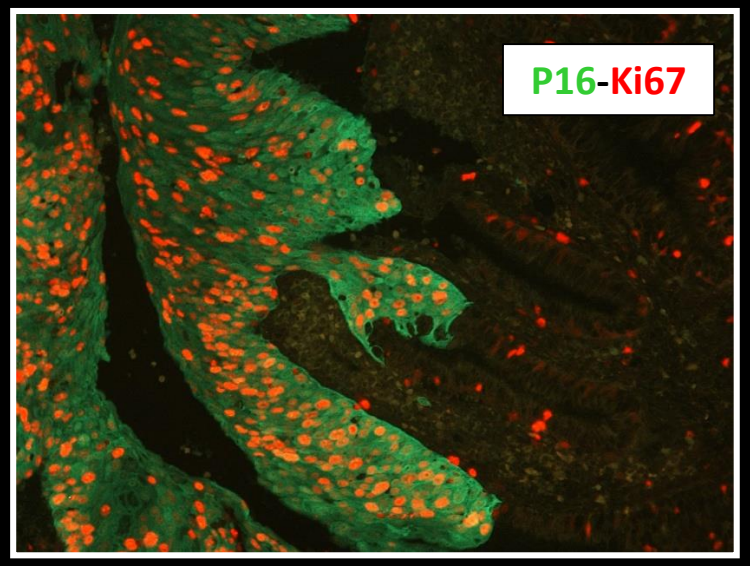


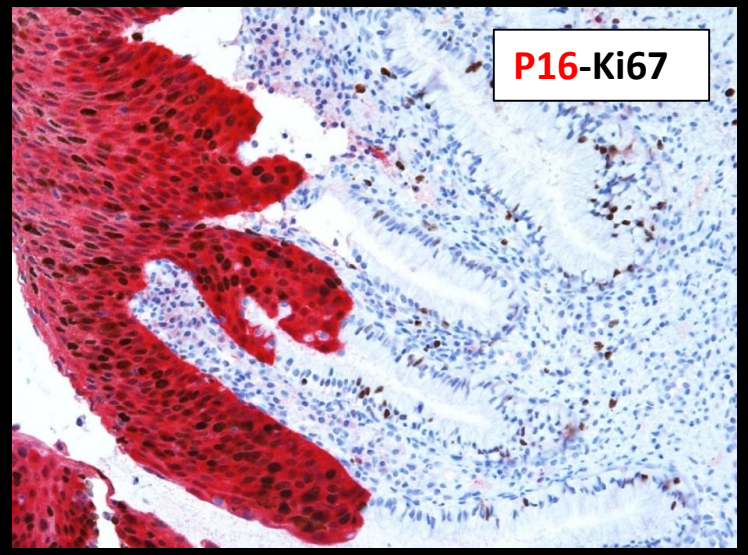
Requirements ("traditional double-staining techniques") :

- ❑ Two different visualization systems showing no cross-reactivity
- ❑ Two different chromogens showing high color contrast and allowing mixed colors at sites of co-localization



Cervix / dysplasia





Double labelling techniques (IHC)

Basic procedures:

- ❑ Cocktails single staining technique (e.g. PAN-CK, AE1/AE3; PIN)
- ❑ Sequential double-staining technique
- ❑ Simultaneous double-staining technique
- ❑ Double (multi) sequential & erasing staining technique (SIMPLE)
- ❑ Virtual double (multi) staining technique

Immuno-enzymatic techniques

Immuno- fluorescence techniques

Challenges or considerations performing multiplex staining techniques:

Which double or multi-staining technique should I use ?

Pre-treatment - do the antigens of interest require the same Epitope retrieval procedure ?

Do primary Abs come in the correct format - important using simultaneous labelling techniques ?

- Are primary Abs made in different host (e.g. mouse, rabbit) ~ If not
- Are primary Abs of different Immunoglobulin type (e.g. IgG, IgM) or subclass (e.g. IgG1, IgG2) ~ If not
- Is one of primary Abs available as a conjugate (e.g FITC, biotin)
- Do the primary Abs have the right specificity and sensitivity

Do the detection systems of choice have the required specificity and sensitivity (e.g. Interspecies cross reactivity) ?

- Are secondary Abs commercial available (app. conjugate) - matching choice of the primary Abs (important using simultaneous techniques)
- Interspecies cross reactivity between primary Abs and secondary Abs (detection system) – pre-absorbed ?
- Interspecies cross reactivity between secondary Abs (secondary Abs made in different host`s) – pre-absorbed ?

Which chromogens /fluorochroms should I use - depending on:

- Type of conjugate (e.g. HRP, AP, Fluorochrom)
- Color compatibility and allowing mixed color of chromogens (spectral differentiation) at sites of co-localization
- Equipment (bright field versus fluorescence microscopy)

How can I inactivate the first set of antibody reagents - important using sequential labelling techniques ?

- Elution of immuno-reagents (Abs) / Heat inactivation
- Take advantage of that DAB, DAB based or metallic deposit can shelter /block for following immunoreagents
- How can I control the efficiency of the inactivating protocol

Controls

- iCAPs - compare individual staining results of the multi labelling technique with optimal single staining procedure (routine)

Multiplex staining techniques (IHC)

Are antigens of interest located in the same cellular compartment (e.g. the cytoplasm) ?

NO

Use a immuno-enzymatic DAB based sequential or a simultaneous technique

YES

Double immunofluorescence technique (simultaneous technique)

Double immuno-enzymatic technique (simultaneous technique / sequential technique)

A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method)

Be critical in selection of antibody pair combinations:

Will the combination provide the information that is needed ?

Will the combination work from a technical point of view ?

Co-localization in CLL ? The DAB deposit (CD23) shields for demonstration of the second antigen of interest (CD5)

Sequential technique (Immuno-enzymatic)

- Preferred method to detect antigenic epitopes in different cell types or in different cellular compartments (e.g. nuclei and cytoplasm)

- The primary antibodies is applied in sequence , detected and visualized individually with two different detection (HRP and/or AP conjugates) & chromogen systems (e.g. DAB and/or Fuchin-Red).

- Each antibody: antigen reaction will yield a specific color of staining that can be easily identified.

- Primary antibodies may be of either the same or different host , Ig-type/subclass (e.g. mouse/IgG1)

- Visualization with DAB, DAB based chromogens or EnzMet (metallic silver) in the first sequence(s) of the detection procedure shields for un-wanted reaction of the following primary antibodies or detection reagents

Not suitable for detection of co-localized signal.

Sequential procedure (Immuno-enzymatic):

Pre-treatment (Antigen Retrieval)

First primary Ab (same or different host, Ig-type or subclass)
Detection with Quanto/Flex+/HRP
Visualization with DAB or Deep Space Black (DAB/Ni ?)

Second primary Ab (same or different host, Ig-type or subclass)
Detection with HiDef/Histo-AP
Visualization with Warp Red (Fuchin-Red)

Counterstain, dehydration and mounting.

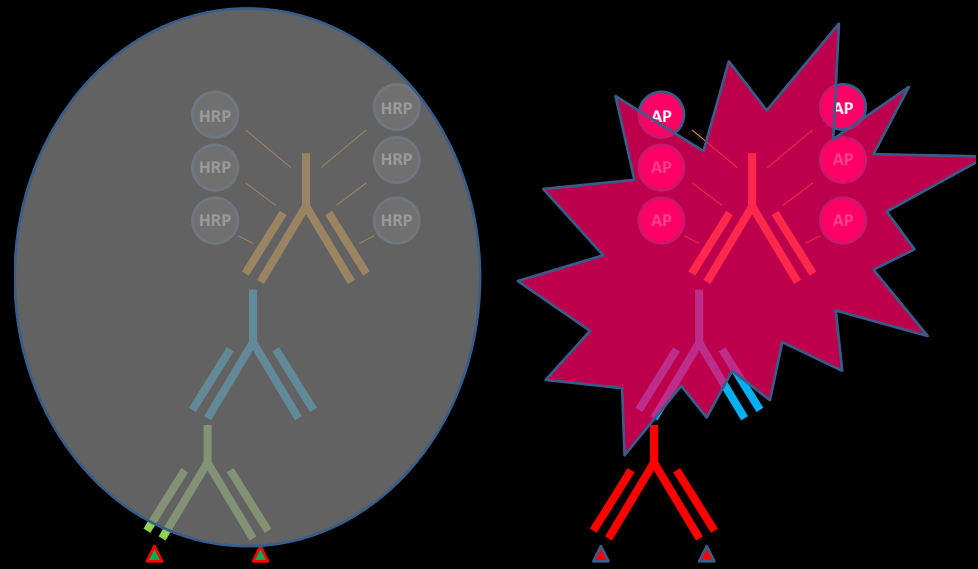
DAB reaction product of the first set of immunoreagents create a barrier that is impermeable for the second set of immunoreagents ~ no cross reactivity

Sternberger LA, Joseph SA. J Histochem Cytochem 27:1424, 1979

Valnes K and Brandtzaeg P . J Histochem Cytochem 1982; 30(6) 518-524.

Metallic deposits (e.g. EnzMET) has the same ability

Inactivation step or blocking procedures not needed



1.Set of Immuno-reagents
Deep Space Black
DAB/Ni ?

2. Set of Immuno-reagents
Warp Red
Fast Red / Fuchin-Red

Optimizing sequential double immuno-staining protocol

Either DAB based (shielding properties) or with blocking procedures between reaction series A & B

Testing:

- Antibody A (dilution series) → Detection A → Chromogen A
- Antibody B (dilution series) → Detection B → Chromogen B

→ Calibrate titre (both primary antibody A & B) according to iCAPs

- Antibody A → Detection A → Chromogen A
- Diluent → Detection B → Chromogen B

→ Control 1 (only reaction for A should be observed / no co-localized signals)

- Diluent → Detection A → Chromogen A
- Antibody B → Detection B → Chromogen B

→ Control 2 (only reaction for B should be observed / no co-localized signals)

- Antibody A → Detection A → Chromogen A
- Antibody B → Detection B → Chromogen B

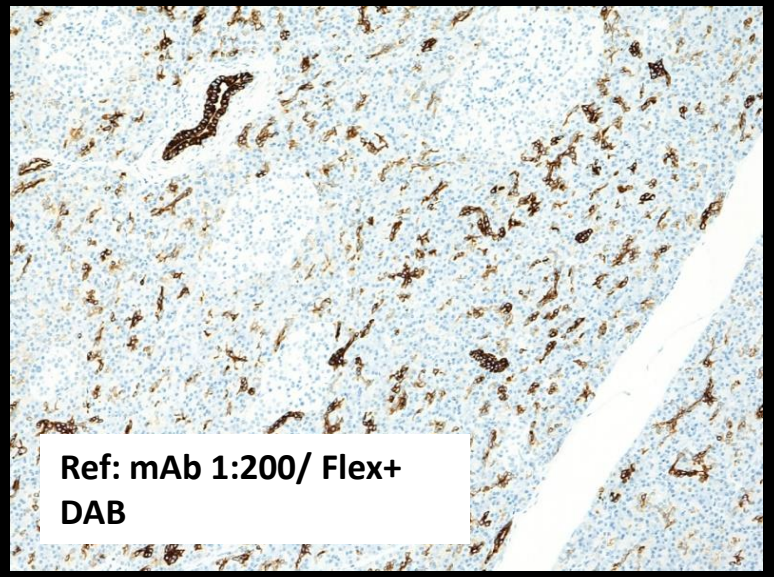
→ Double staining

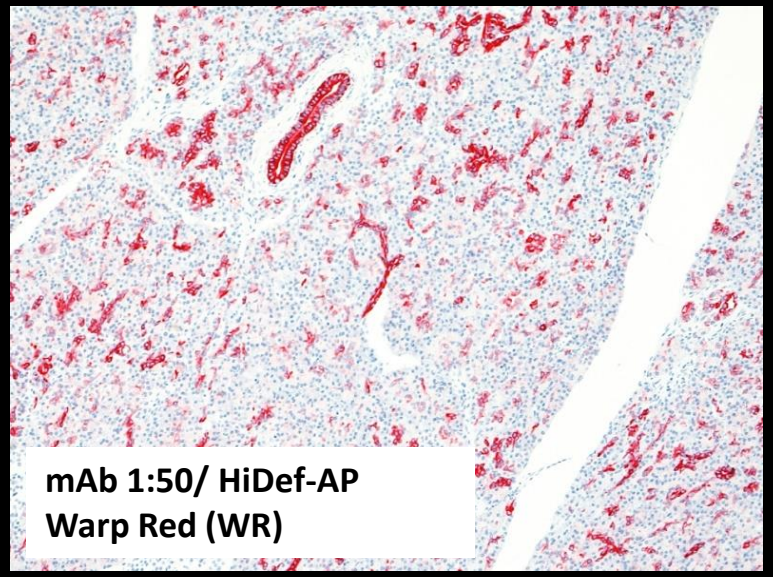
Typical end-result including controls: Optimizing the protocol

CDX2 (EP25) + CK7 (OV-TL 12/30)

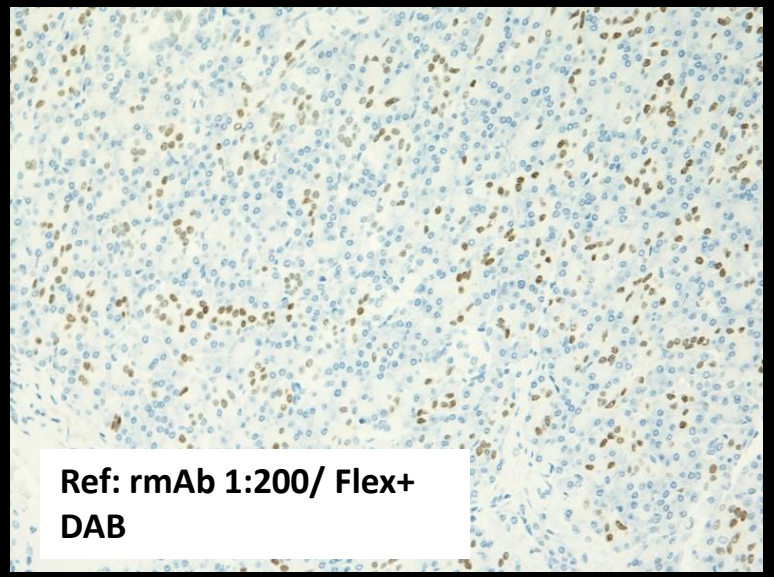
Pancreas

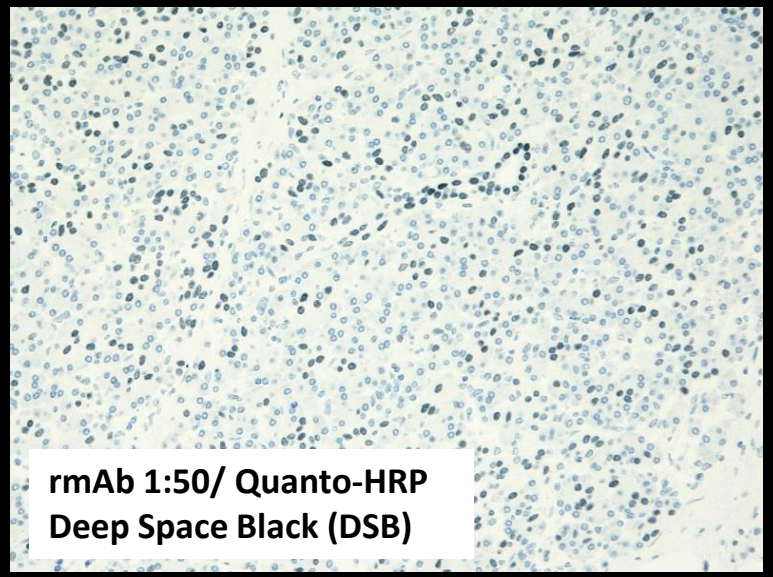
CK7, OV-TL12/30





CDX2, EP25

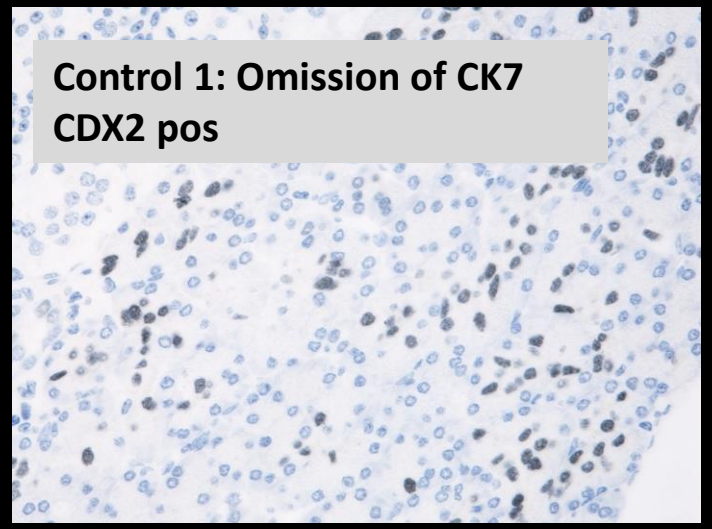


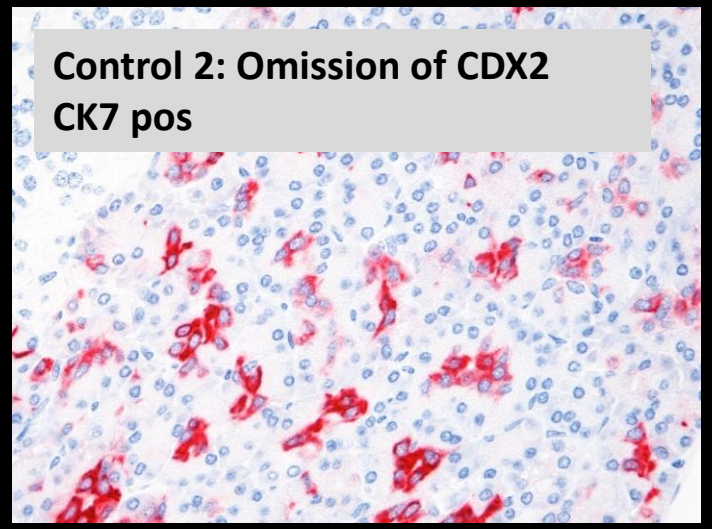


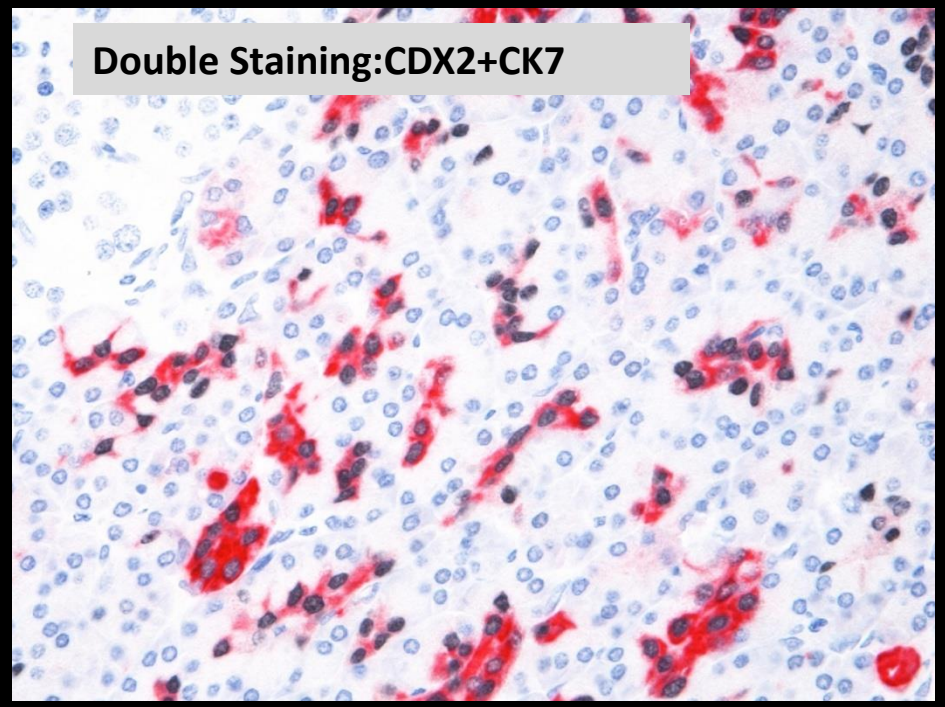
Typical end-result including controls: Optimizing the protocol

Optimal dilutions of CDX2 (EP25 1:50) and CK7 (OV-TL 12/30 1:50)

Sequential staining : Quanto-HRP/ DSB followed by HiDef-AP/WR







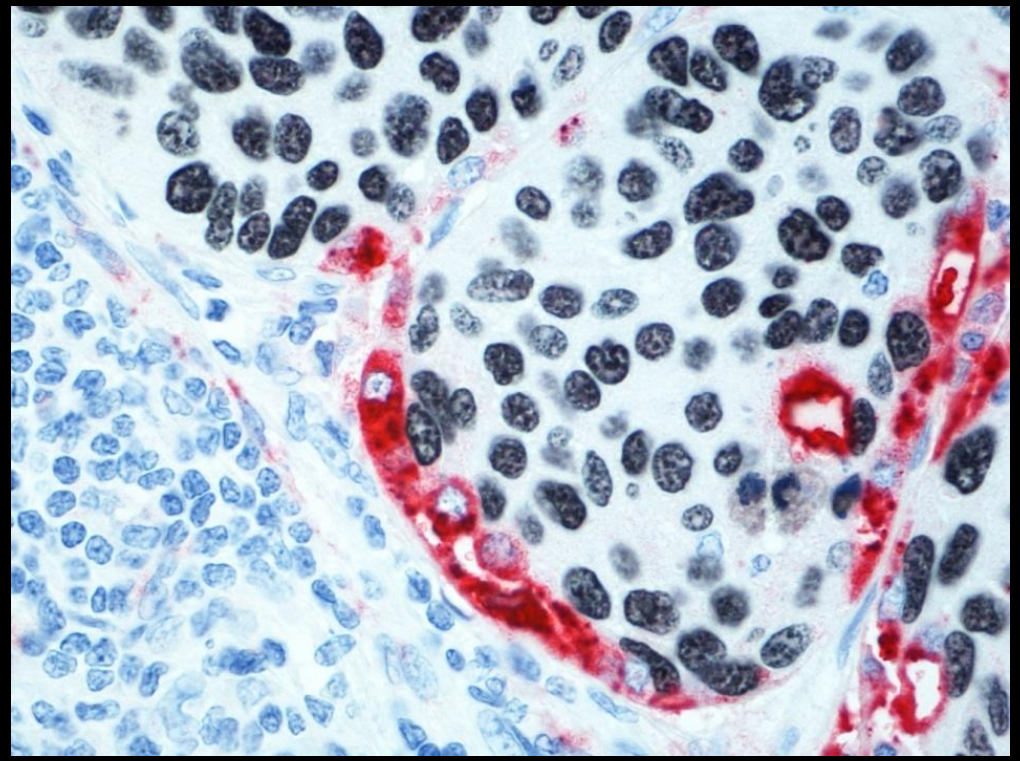
Pancreas

Note : Efficient sheltering capacity of Deep Space Black

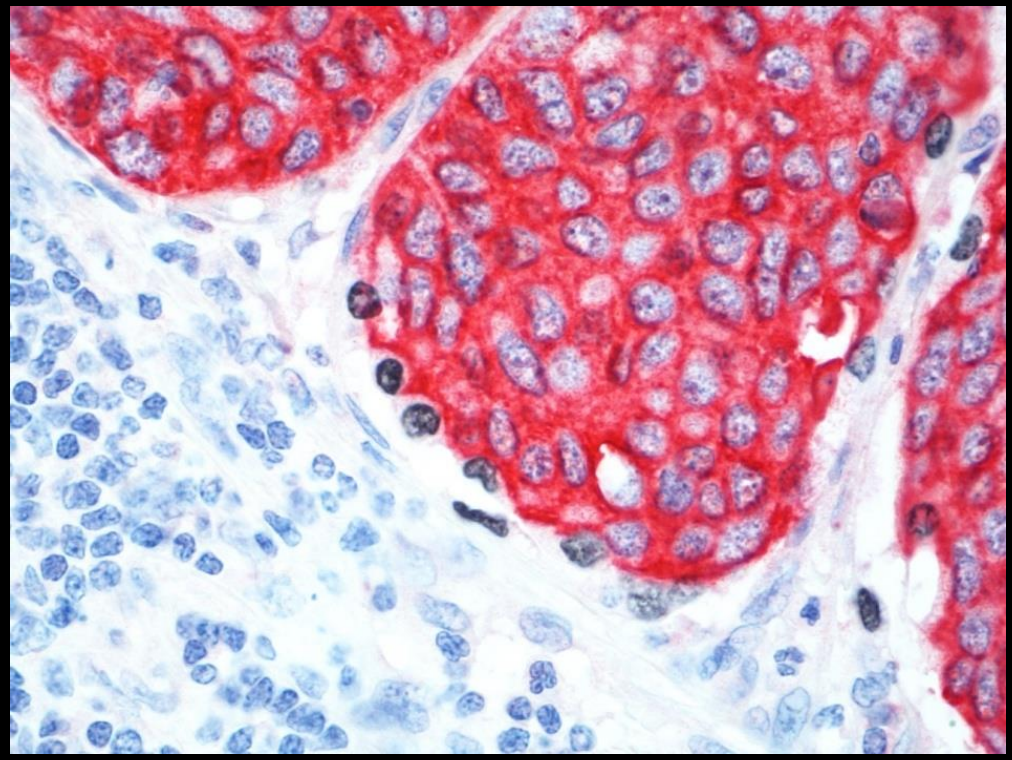
No mixed colors or double colors within controls and in the double staining for CDX2+CK7 (Intercalating ducts of the pancreas are positive for both markers)

Double staining using sequential technique (Immuno-enzymatic)

P40, BC28 (1:25) + Napsin, IP64 (1:100) (Mab+Mab)

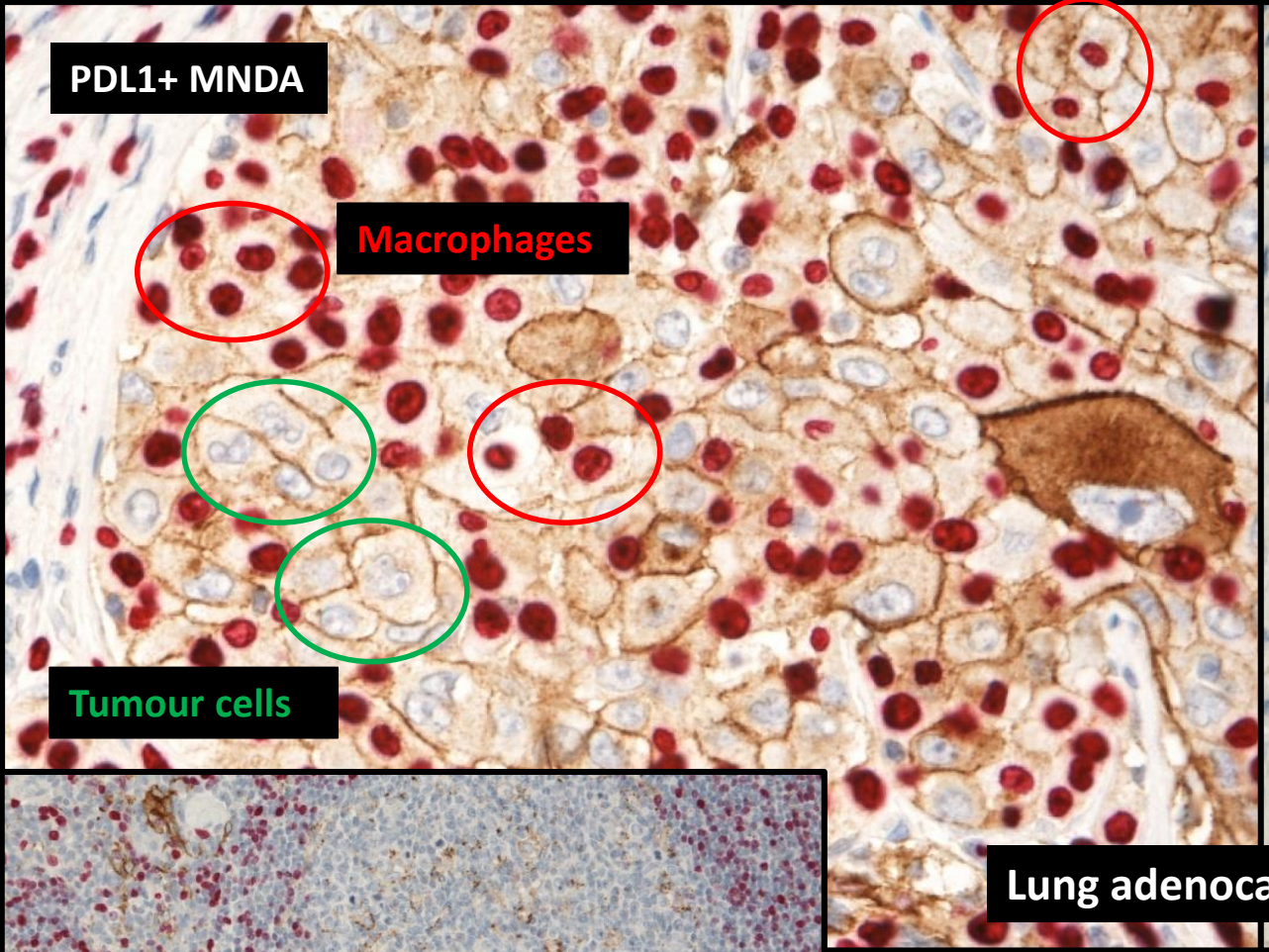


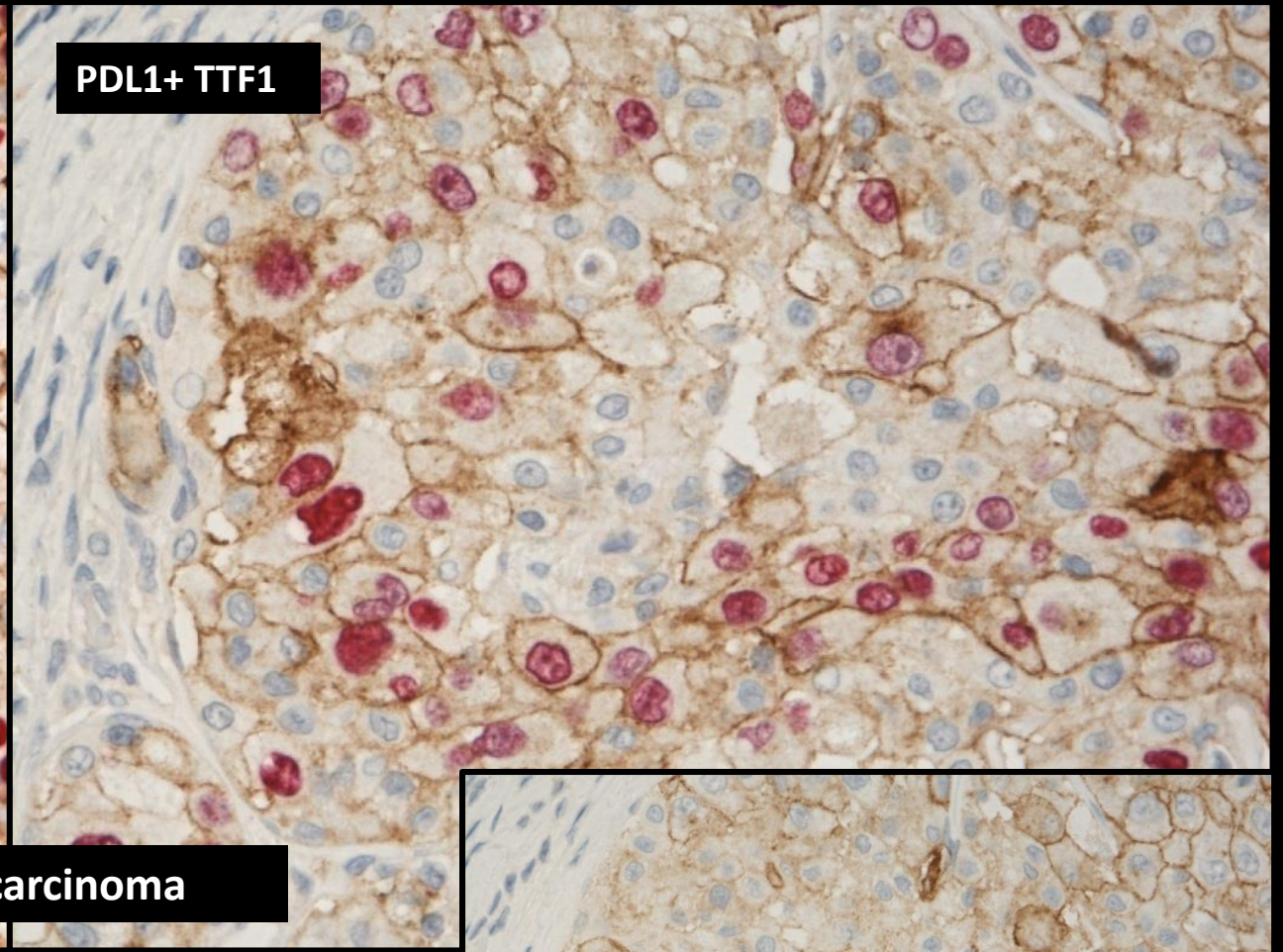
TTF1, SPT24 (1:25) + CK5,XM26 (1:100) (Mab+Mab)



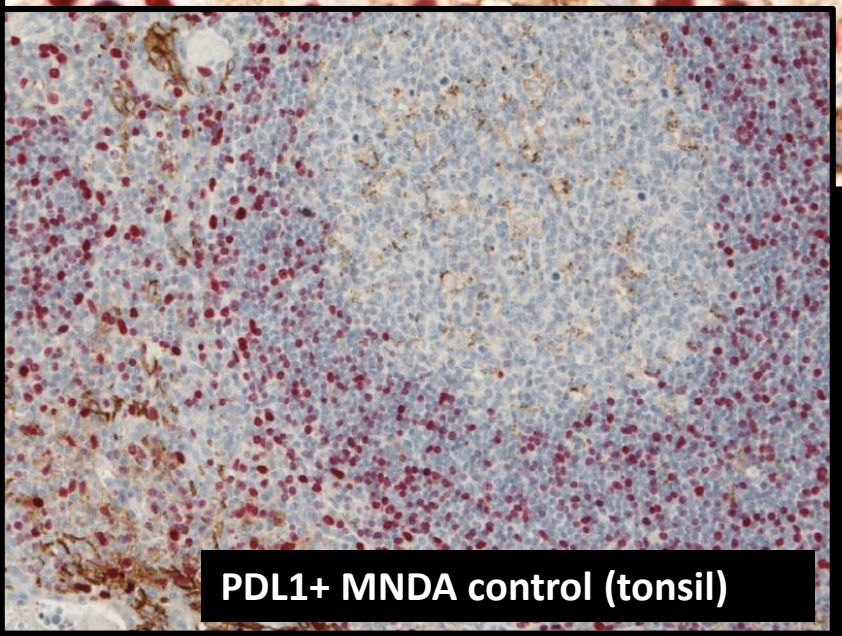
Lung : Squamous cell carcinoma

Other useful combinations: P40 + CK5 and/or TTF-1+ Napsin A





Lung adenocarcinoma

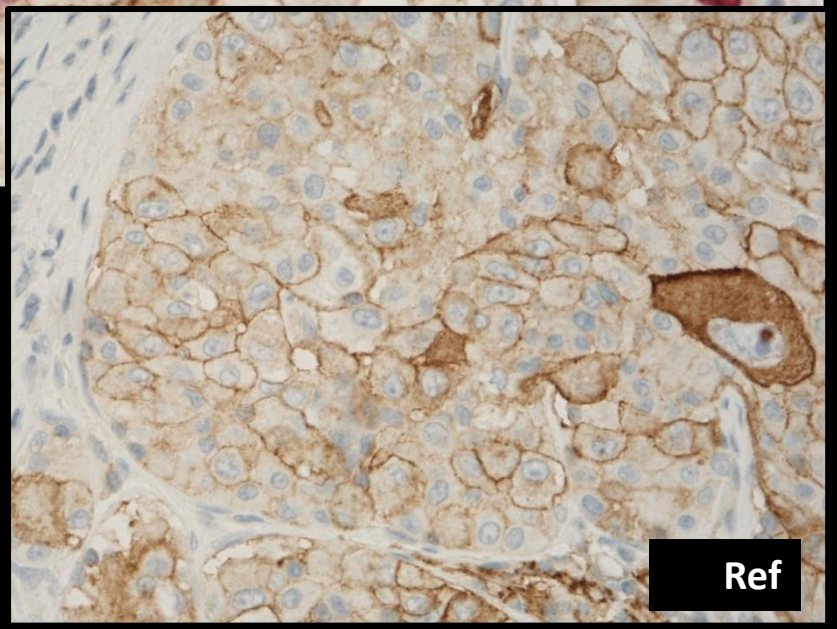


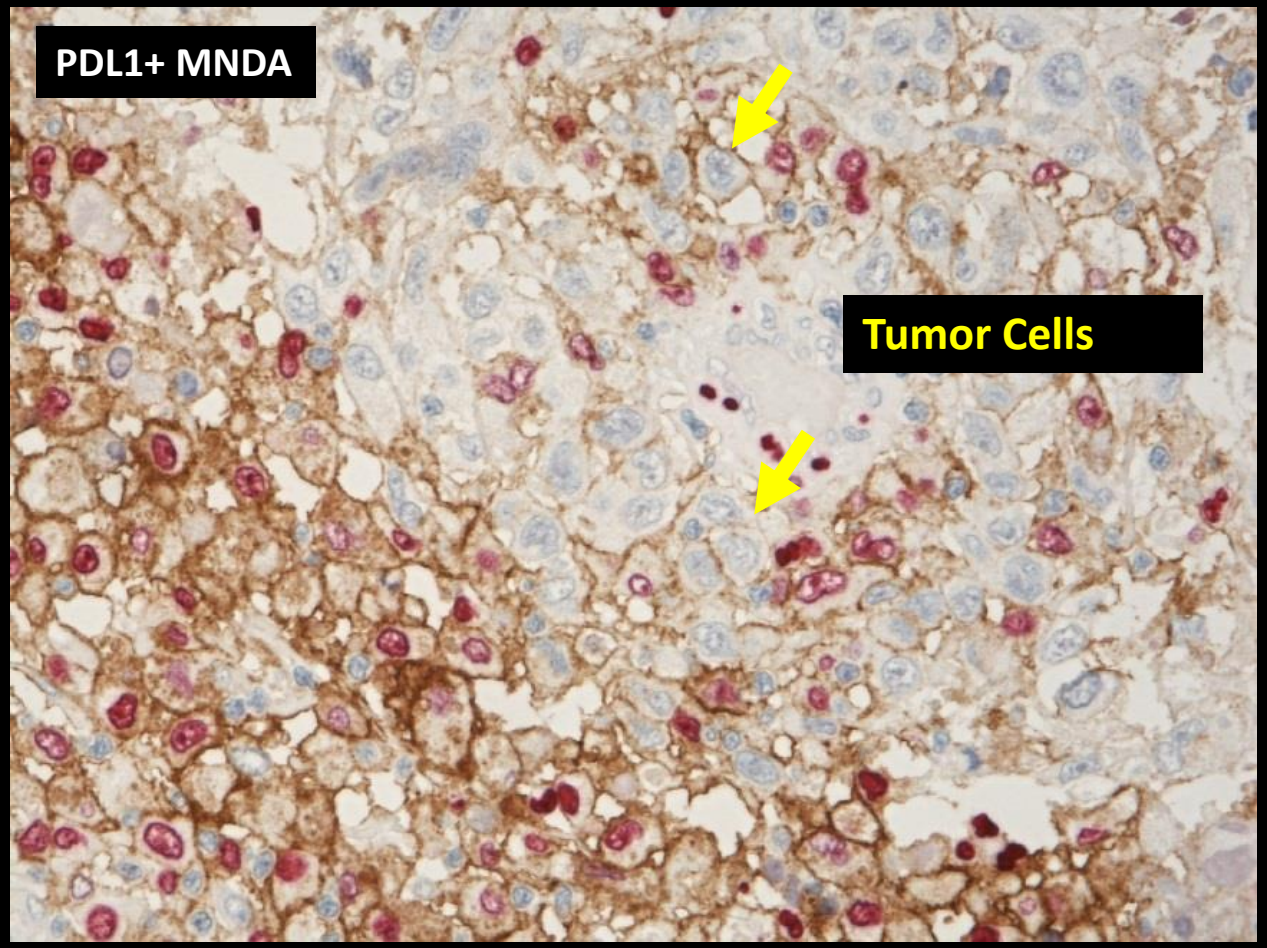
<u>Omnis: HIER Low pH 40`</u>

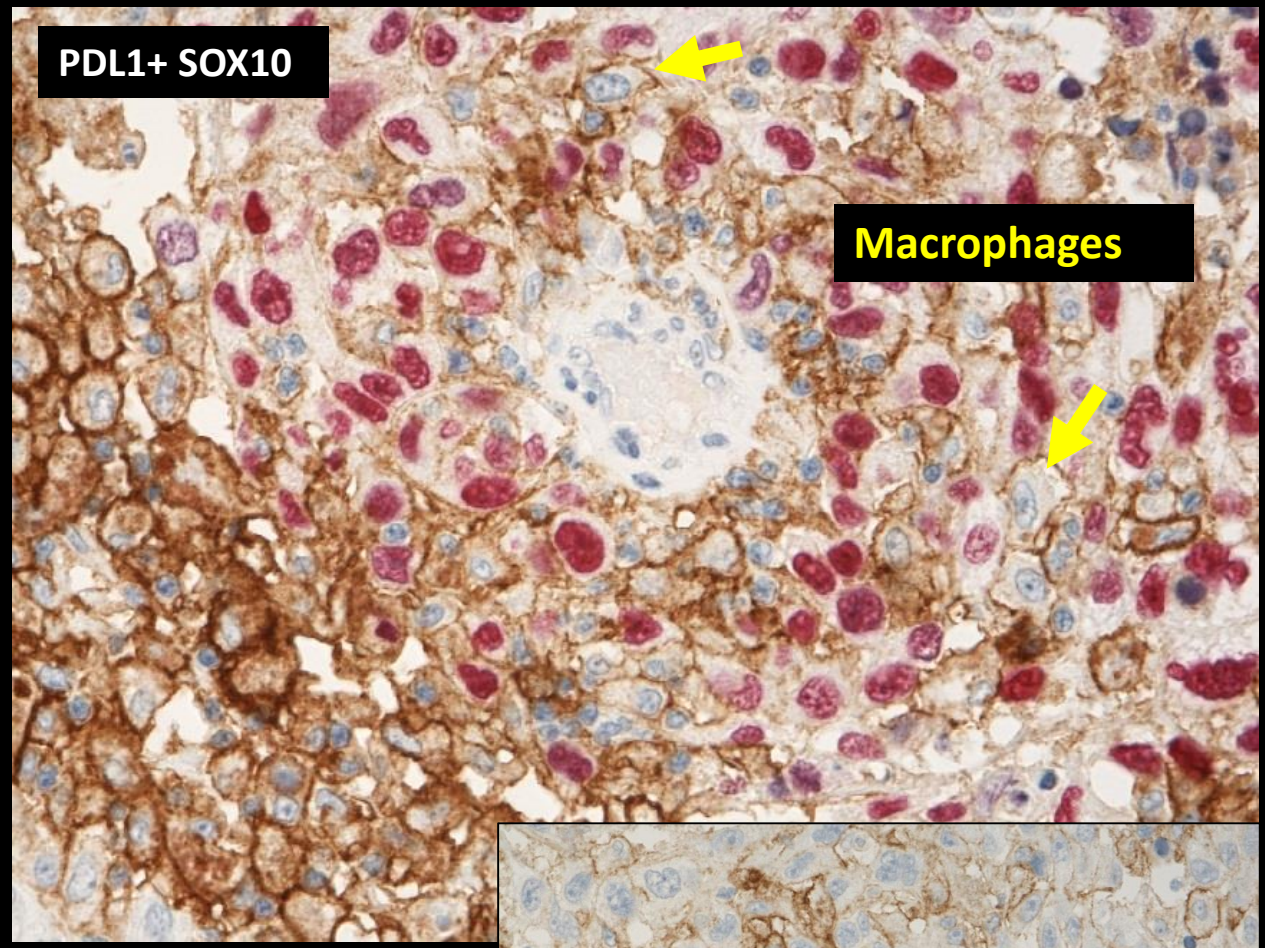
PD-L1, 22C3 (1:20) /Flex+ 40-10-40

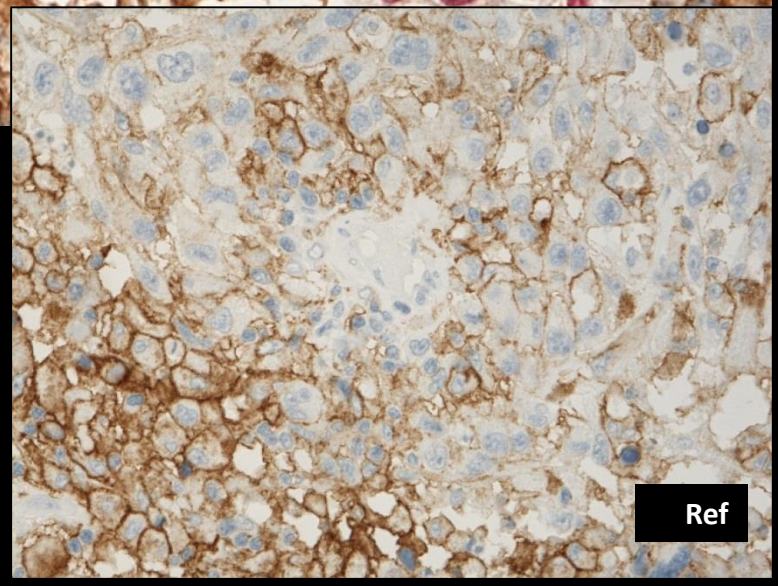
1. MNDA, 253A (1:80) / Histo-AP 30-10-20

2. TTF1, SPT24 (1:200) / Histo-AP 30-10-20









Melanoma

<u>Omnis: HIER Low pH 40`</u>

PD-L1, 22C3 (1:20) /Flex+ 40-10-40

MNDA, 253A (1:80) or SOX10, BS7 (1:200) /Histo-AP 30-10-20

Double staining using sequential technique (Immuno-enzymatic)

The order of primary antibodies

In general:

- Nuclear markers before cytoplasmic or membranous markers
- Membranous markers before cytoplasmic

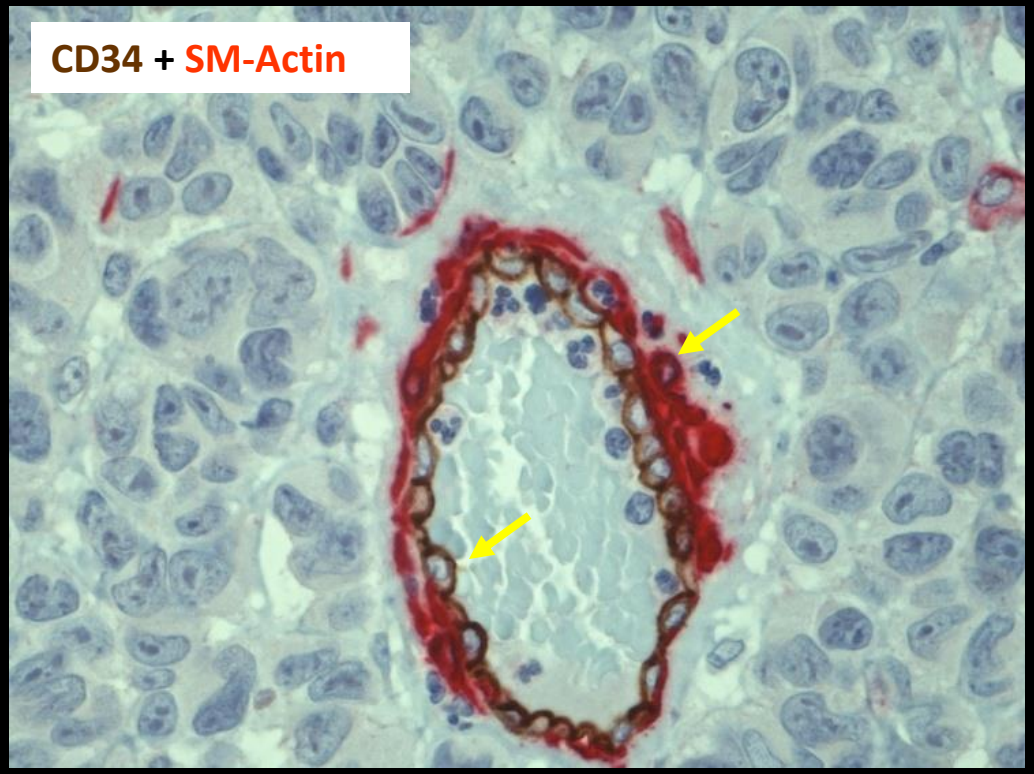
A reversed applications of the primary antibodies may un-ravel that :

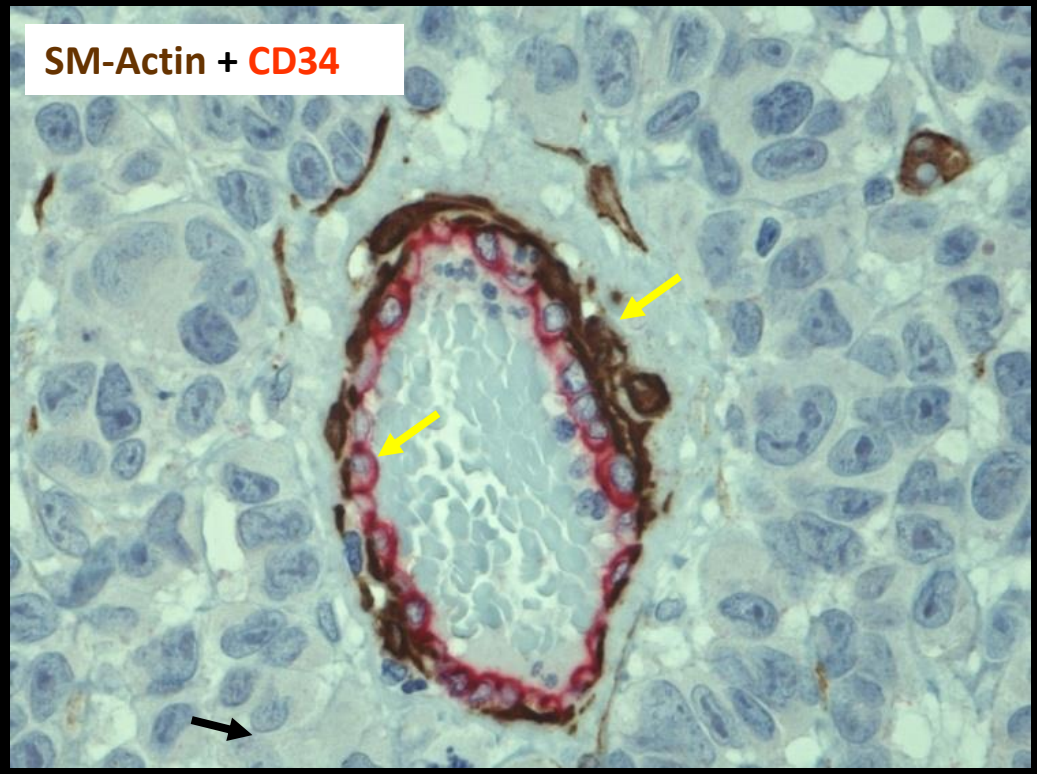
DAB deposit shields for the second antigen of interest - first and second antigen are in close proximity with each other (false negative result)

Co-localized signal is present due to expression of the two antigens of interest in the same cellular compartment

Double staining using sequential technique (Immuno-enzymatic)

CD34 (QBEND 10) + SM-Actin (1A4)



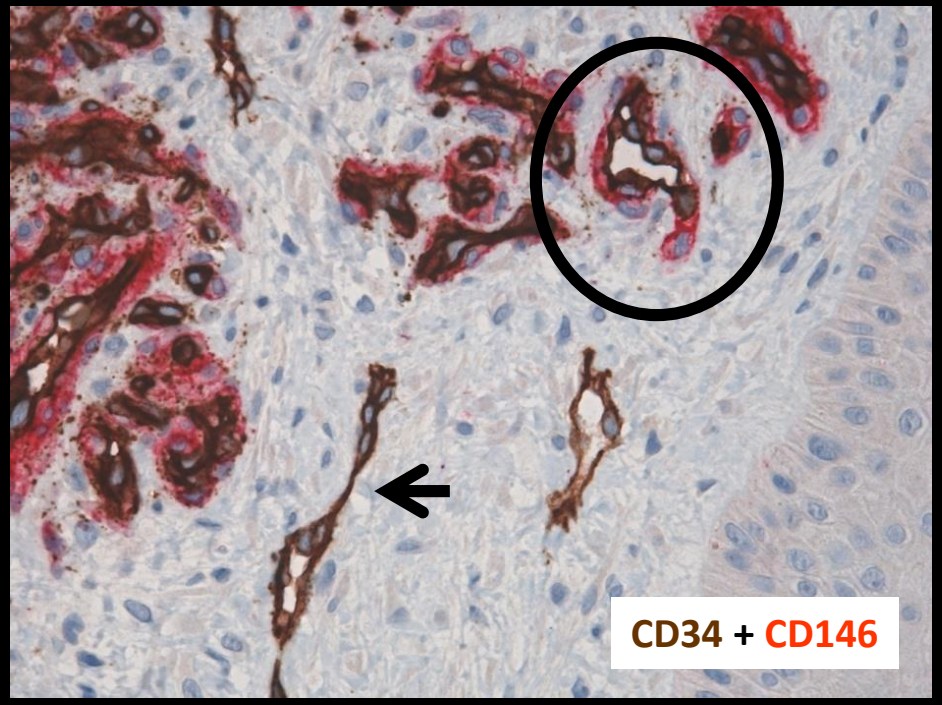


Note: No co-localization of either endothelium or smooth muscles (arrows)

Melanoma

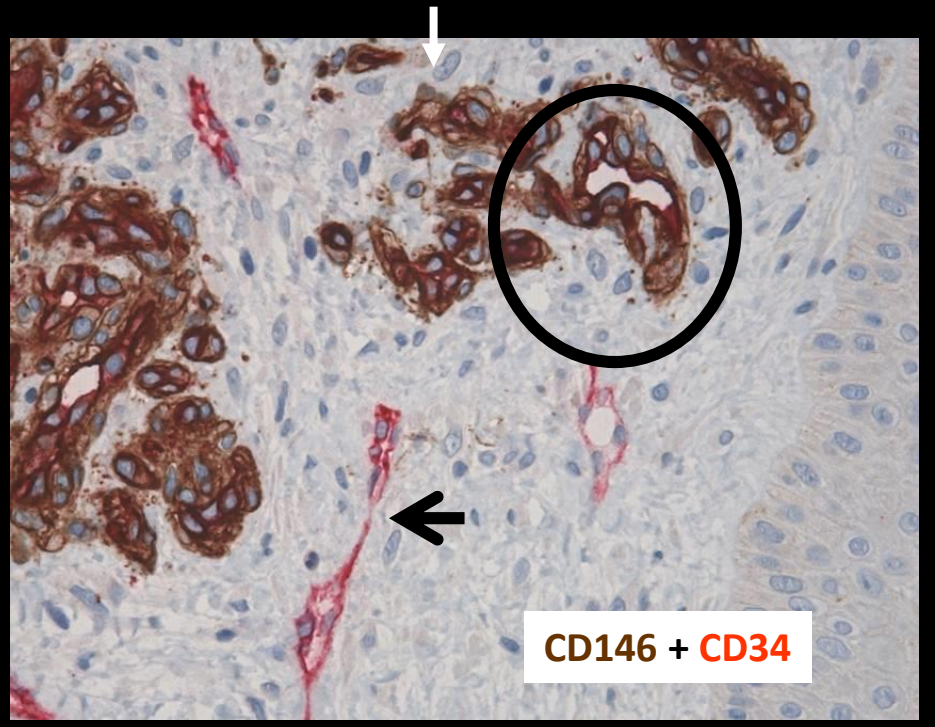
Double Immuno-staining – reversal stainings

CD34 (QBEND 10) + CD146 (EPR3208)



The order of primary antibodies

The brown deposit (DAB) from the first set of immuno-reagents hinder the second set of immuno-reagents (the red subsequent staining – compare the two images).

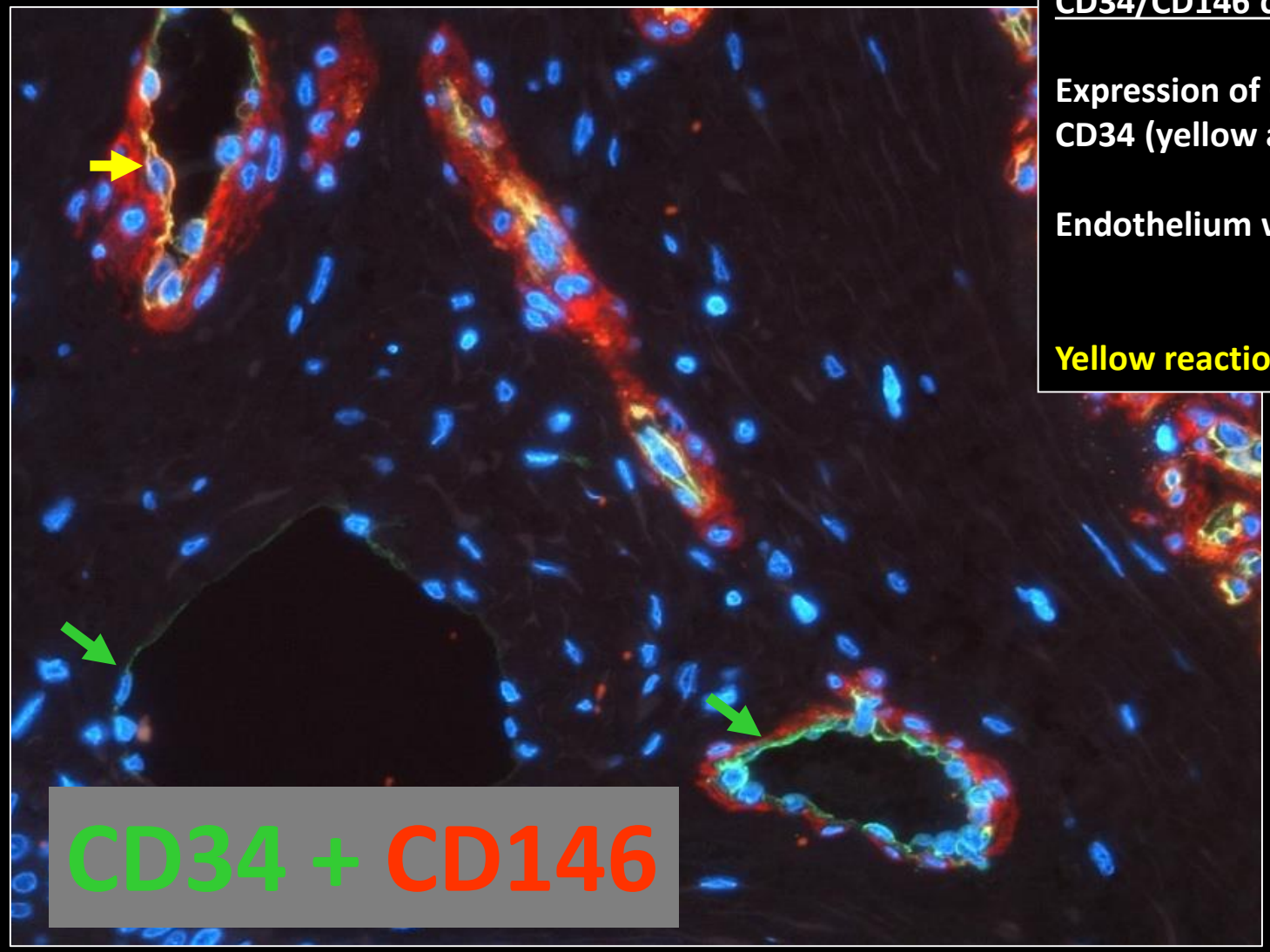


Endothelia cells are “double brown positive” /co-localization

Non of the combinations are useful ?

Hemangioma

Double immunofluorescence staining of vascular structures



CD34/CD146 combination

Expression of CD146 in endothelium and co-localization with CD34 (yellow arrow)

Endothelium without expression of CD146 (green arrow)

Yellow reaction product = co-localized antigens

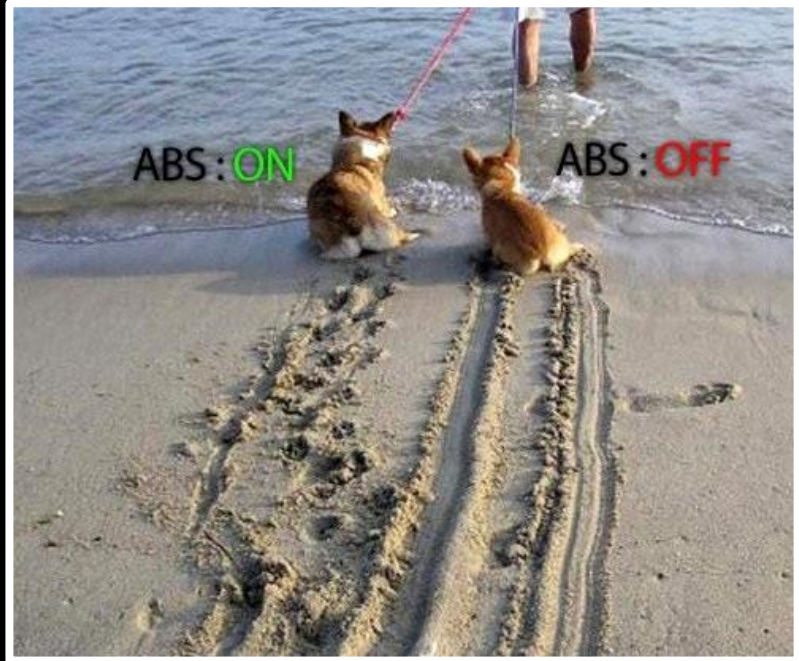
Hemangioma

CDX-2 (DAK-CDX2) + CK 8/18 (5D3)

Problems ?

The DAB deposit may cause problems as it may block for the next set of immuno-reagents - arrows.

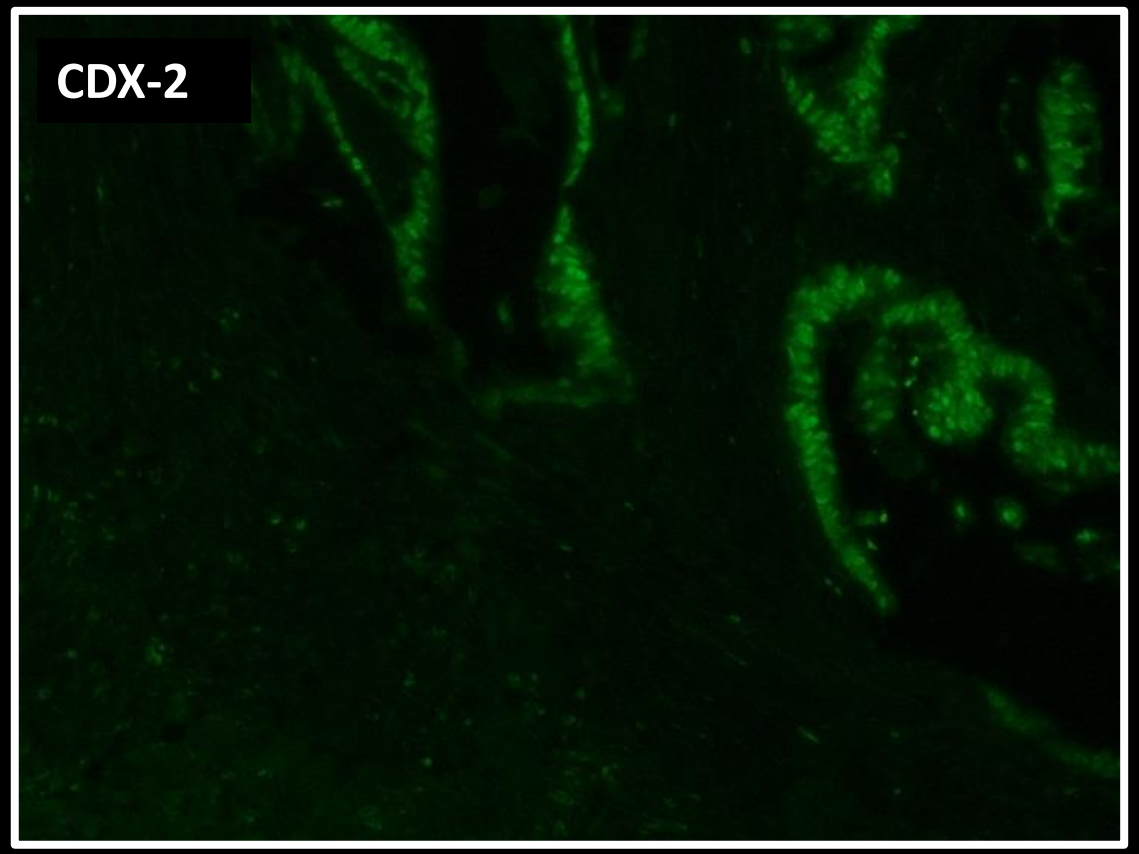
Tumor cells with infiltrative growth pattern (buddings) show expression of CK 8/18 due to downregulation of CDX2 – arrowheads.

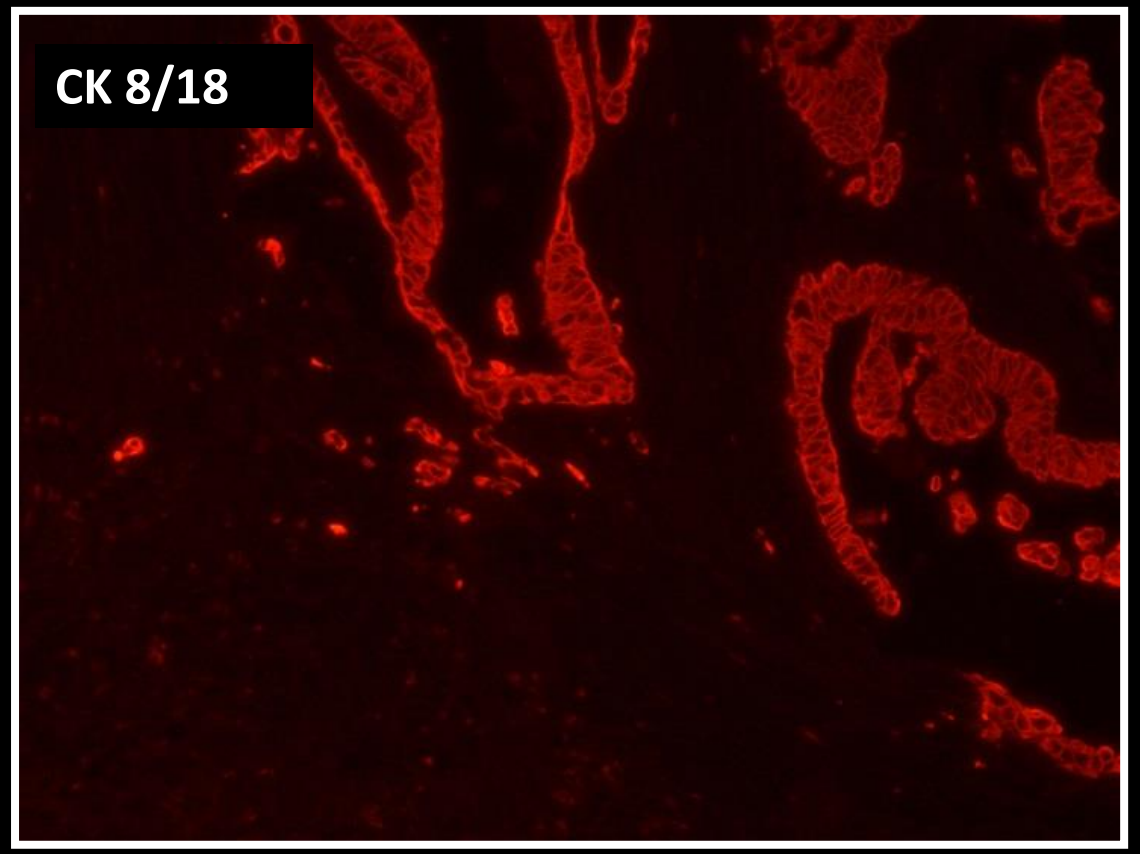


Colon Adenocarcinoma

CDX-2 (EP25) + CK 8/18 (5D3)

Double Staining-IF (simultaneous technique)





Colon Adenocarcinoma

Note expression of CK 8/18 in all tumor cells

Double/Multiple staining using sequential technique (Immuno-enzymatic)

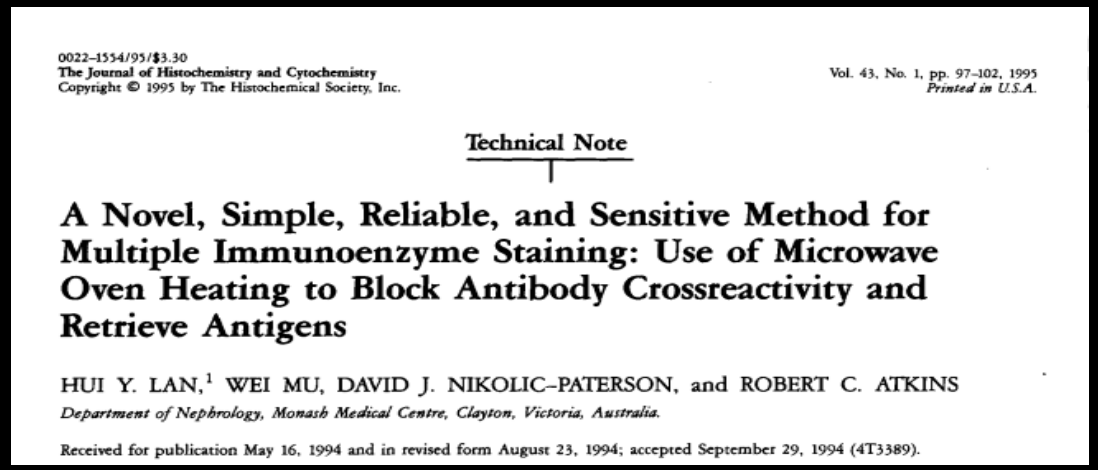
Now, what if unexpected color mixing occur due to cross reactivity with the 1th set of reagents ?

Mainly a problem related to other than DAB or DAB based chromogens

Inactivation of 1th set of immuno-reagents :

- Elution methods (High salt, extreme pH values and strong oxidizing agents)
- Blocking using Heat (97°C-100°C) in standard Citrate buffer pH6

Applied between the 1th and 2nd set of immuno-reagents



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Technical Note

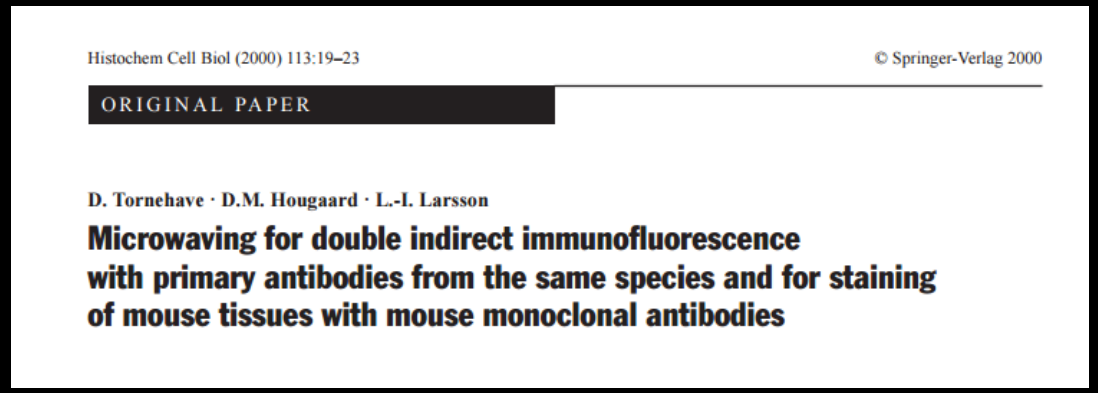
A Novel, Simple, Reliable, and Sensitive Method for Multiple Immunoenzyme Staining: Use of Microwave Oven Heating to Block Antibody Crossreactivity and Retrieve Antigens

HUI Y. LAN,[1] WEI MU, DAVID J. NIKOLIC-PATERSON, and ROBERT C. ATKINS
Department of Nephrology, Monash Medical Centre, Clayton, Victoria, Australia.

Received for publication May 16, 1994 and in revised form August 23, 1994; accepted September 29, 1994 (4T3389).

Blocking buffer: 0.01 M sodium citrate buffer, pH 6.0.

Efficient blocking temperature of 100°C
Efficient blocking time 2x5 min.



Histochem Cell Biol (2000) 113:19–23

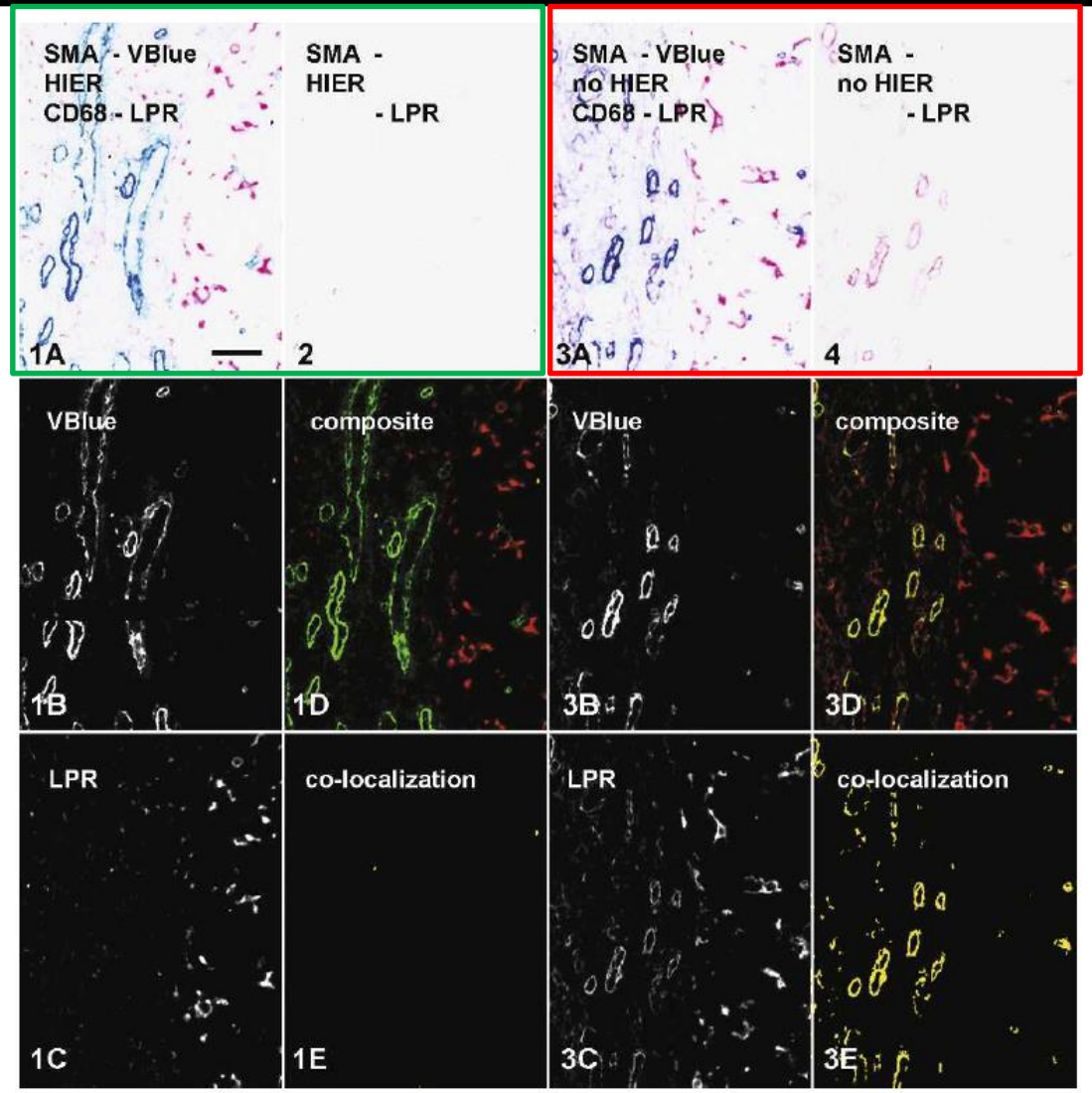
ORIGINAL PAPER

D. Tornehave · D.M. Hougaard · L.-I. Larsson

Microwaving for double indirect immunofluorescence with primary antibodies from the same species and for staining of mouse tissues with mouse monoclonal antibodies

Blocking buffer: 0.01 M sodium citrate buffer, pH 6.0.

Successful double staining of a number of antigens was achieved by a standard 3×5-min microwaving at 780 W. This time may not necessarily apply to other ovens, antibody combinations or buffers used.



Figures 1–4. Figure 1: Microscopic detail of a hyperplastic tonsil (human) showing sequential double AP staining using SMA and CD68 (macrophages) antibodies on an FFPE tissue section. LPRed was applied after the first staining sequence and VBlue after the second staining. (A) Full sequential double AP staining including intervening HIER step. (B) Spectral imaging analysis of (A) demonstrating unmixing of VBlue. (C) Spectral imaging analysis of (A) demonstrating unmixing of LPRed. (D) Spectral imaging analysis of (A) demonstrating a fluorescence-like and pseudo-colored composite (SMA in green and CD68 in red). (E) Spectral imaging analysis of (A) demonstrating the absence of colocalization. Figure 2: Sequential double AP staining including intervening HIER step, but with omission of the first chromogen and second primary antibody, demonstrating no visualization of the first antibody by the second detection system. Figure 3: (A) Full sequential double AP staining without intervening HIER step. (B) Spectral imaging analysis of (A) demonstrating unmixing of VBlue. (C) Spectral imaging analysis of (A) demonstrating unmixing of LPRed. (D) Spectral imaging analysis of (A) demonstrating a fluorescence-like and pseudo-colored composite (SMA in green and CD68 in red). The yellowish mixed color at muscular vessels indicates the presence of false colocalization. (E) Spectral imaging analysis of (A) demonstrating massive false colocalization present at muscular vessels. Note that the blue/purple intermediate color visualizing false colocalization is more distinct in (A) than in Figure 3A, whereas spectral imaging reveals nearly similar amounts of (false) colocalization in (E) and Figure 3E. Figure 4: Sequential double AP staining without HIER step, and with omission of the first chromogen and second primary antibody, demonstrating clear staining of the first antibody by the second detection system. Scale bar = 0.05 mm.

A Generally Applicable Sequential Alkaline Phosphatase Immunohistochemical Double Staining

The J Histotechnol 31:119, 2008.

Chris M. van der Loos and Peter Teeling
Academic Medical Center, Department of Pathology, Amsterdam, The Netherlands

Demonstrarted that:

Sequential double AP staining, without the intervening heat step, clearly shows cross-reactivity between the first and second set of immunoreagents - providing a merge color/reaction product of VBlue or LPRed (purple color).

Limitations:

Require Heat stable chromogens - DAB, VBlue, VRed and LPR (Dako)

Always efficient ?

Volume 57(6): 567–575, 2009
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

Antibody Elution Method for Multiple Immunohistochemistry on Primary Antibodies Raised in the Same Species and of the Same Subtype

Daniel Pirici, Laurentiu Mogoanta, Samir Kumar-Singh, Ionica Pirici, Claudiu Margaritescu, Cristina Simionescu, and Radu Stanescu

Control studies

Demonstrated that:

Elution, using a glycine SDS pH 2 solution, of the antibodies after the first round of immunolabelling was superior in regard of eliminate cross reactivity with the second set of immuno-reagents compared to other methods tested .

This also includes an intermediate HIER step using Citrate pH6

Limitations: High affinity antibodies may be difficult to elute – we have to validate (include proper controls) the efficiency of the chosen blocking procedure optimizing the multiplex procedures

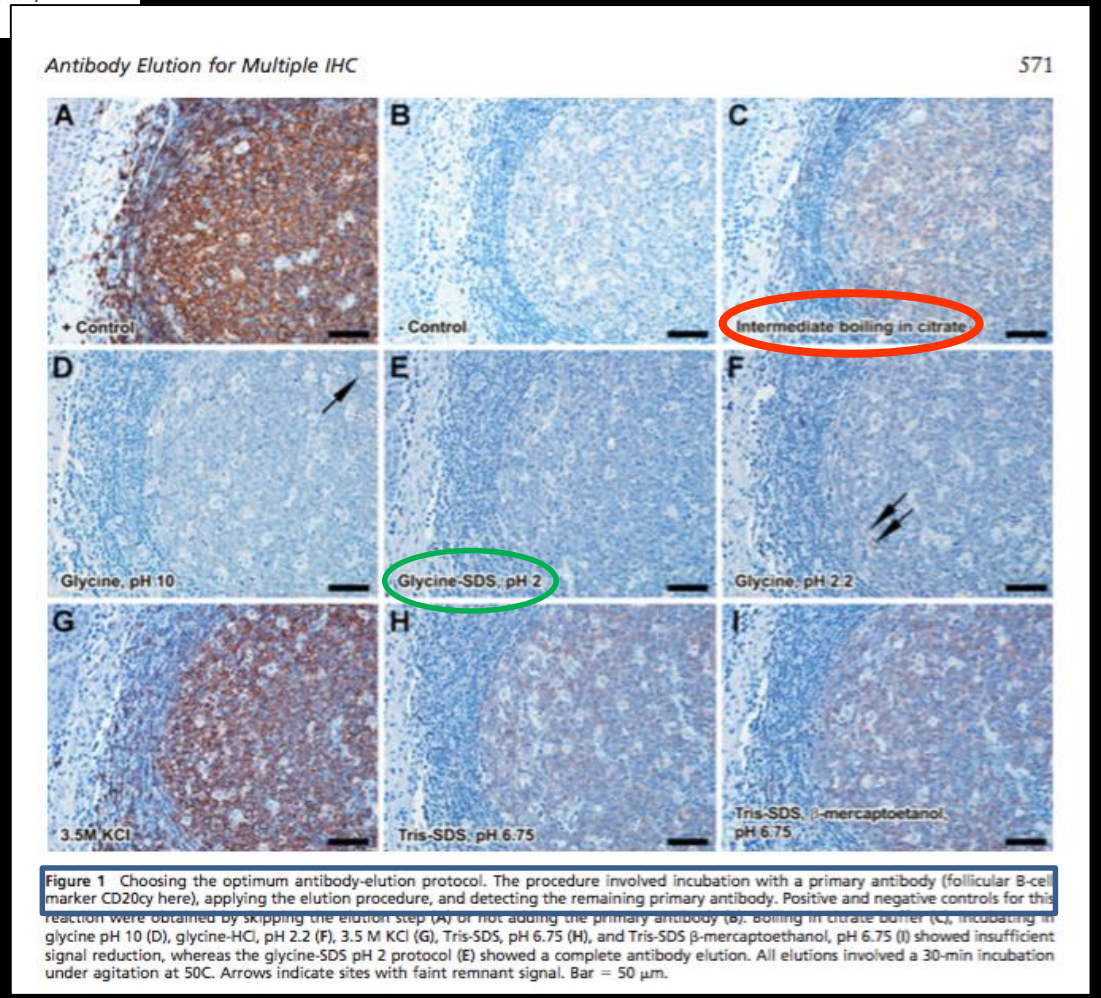


Figure 1 Choosing the optimum antibody-elution protocol. The procedure involved incubation with a primary antibody (follicular B-cell marker CD20cy here), applying the elution procedure, and detecting the remaining primary antibody. Positive and negative controls for this reaction were obtained by skipping the elution step (A) or not adding the primary antibody (B). Boiling in citrate buffer (C), incubating in glycine pH 10 (D), glycine-HCl, pH 2.2 (F), 3.5 M KCl (G), Tris-SDS, pH 6.75 (H), and Tris-SDS β-mercaptoethanol, pH 6.75 (I) showed insufficient signal reduction, whereas the glycine-SDS pH 2 protocol (E) showed a complete antibody elution. All elutions involved a 30-min incubation under agitation at 50C. Arrows indicate sites with faint remnant signal. Bar = 50 μm.

Simultaneous technique (immune enzymatic staining)

- Primary antibody cocktail is applied to the tissue at the same time (simultaneously)

- Primary antibodies must be of different host (e.g. mouse and rabbit) or at least different Ig-types, subclass or one primary Ab with a conjugate (e.g. FITC).

- Reactions are detected with a mix of secondary antibodies conjugated with two different enzymes (e.g. HRP and AP) and applied to the tissue at the same time (simultaneously).

  To prevent cross-reaction between secondary antibodies raised against different species, it is recommended to apply second-step antibodies raised in the same host – if not possible, use pre-absorbed secondary antibodies.

- Each antibody: antigen reaction will yield a specific color of staining that can be easily identified.

Double enzymatic staining using simultaneous technique

Pre-treatment (Antigen Retrieval)

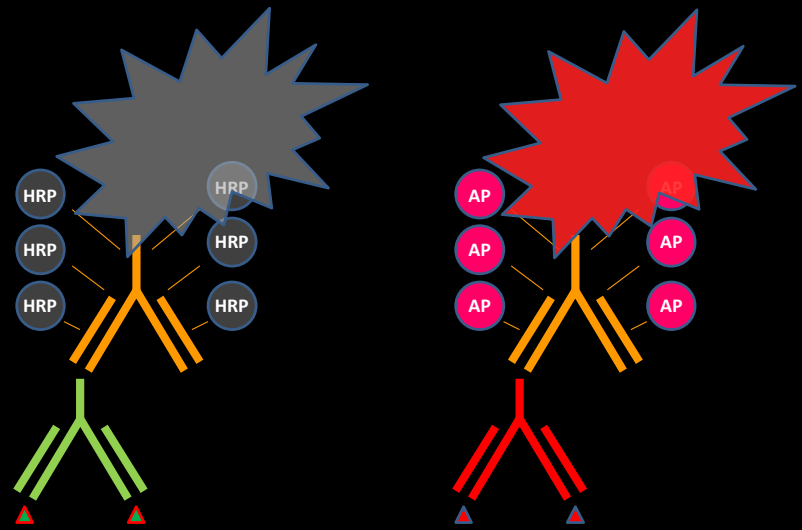
Incubation with mix of primary Abs (Rab+Mab)

Incubation with polymer/multimer mix (anti -Mab/HRP + anti -Rab/AP)

Incubation with HRP substrate (e.g. DAB or DSB)

Incubation with AP substrate (e.g. Fast Red or Warp Red)

Counter stain, mounting and microscopy



Commercial kits (Naestved Lab):

Lab Vision™ MultiVision Polymer Detection System: anti-Mouse-AP and anti-Rabbit-HRP (TL-012-MARH)

Lab Vision™ MultiVision Polymer Detection System: anti-Mouse-HRP and (TL-012-MHRA)

Biocare MACH2 Double Stain 1: anti-Mouse-AP and anti-Rabbit-HRP (MRCT523)

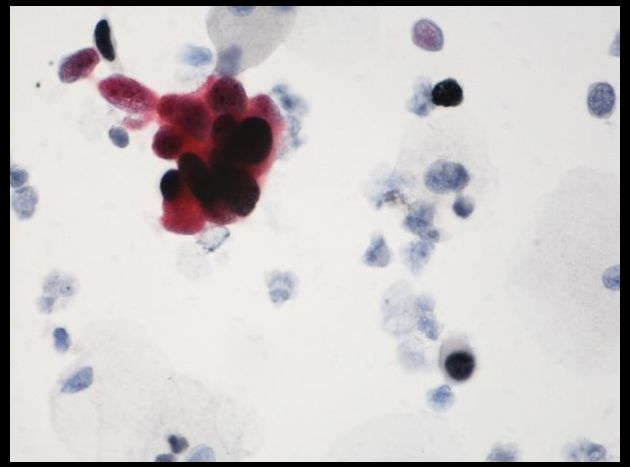
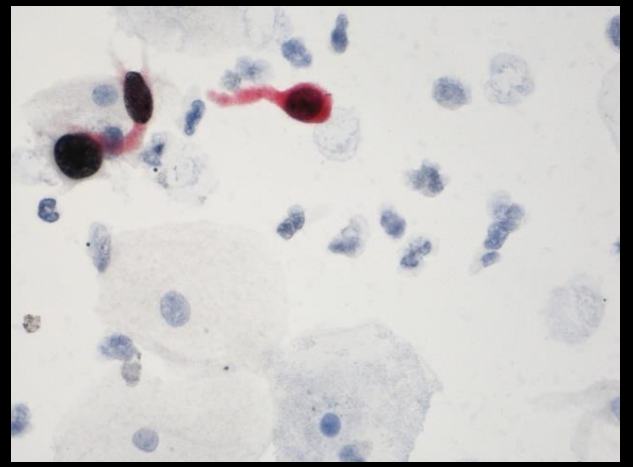
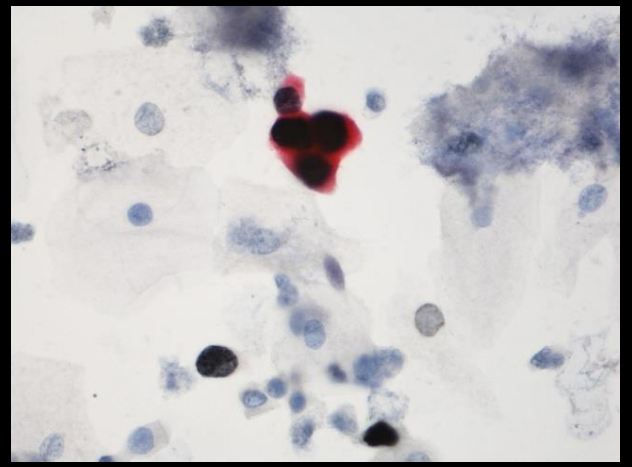
Biocare MACH2 Double Stain 2: anti-Mouse-HRP and anti-Rabbit-AP (MRCT525)

MACH2 Double Staining : Ki-67, SP6 (1:25) + P16, E6H4 (RTU,Ventana)

Dysplasia / Cervix (Cytology)

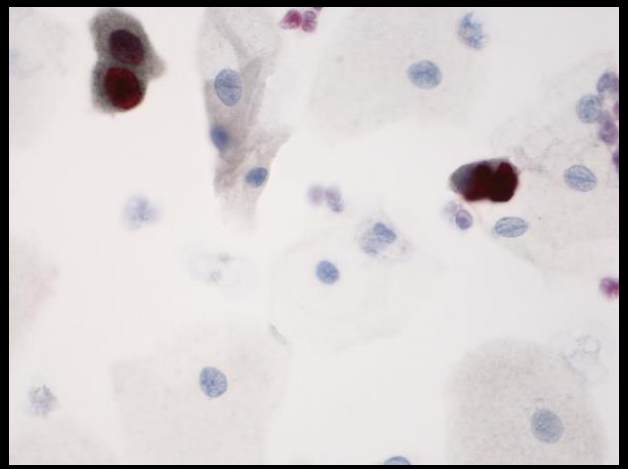
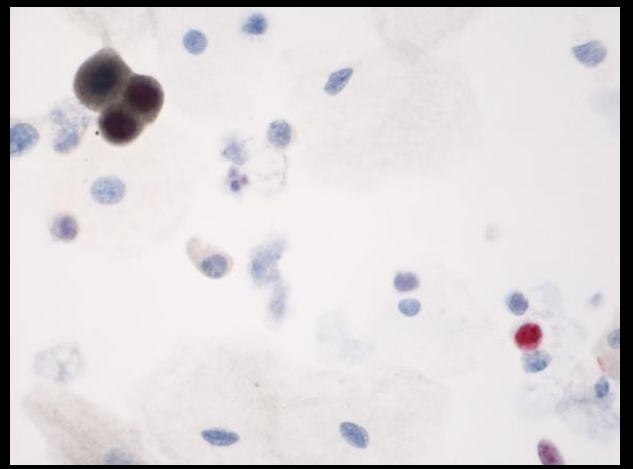
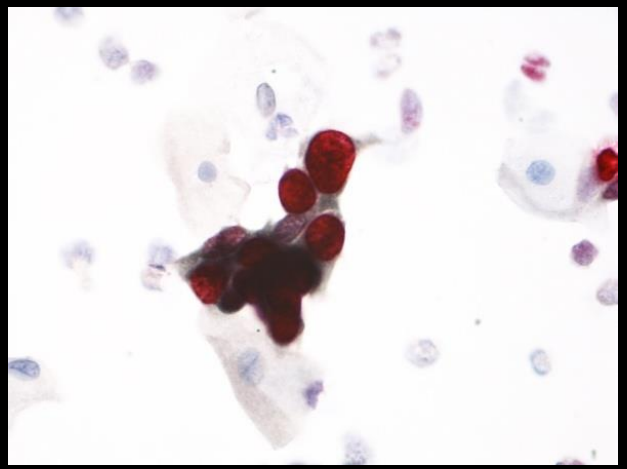
Double Stain 1 (Biocare)

Anti-Rab/HRP
Anti-Mab/AP

Double Stain 2 (Biocare)

Anti-Rab/AP
Anti-Mab/HRP

Cytological specimens fixed in NBF 30` → 96 alk. 10` → Wash buffer → HIER/TE 20`

Multiplex staining techniques (IHC)

Are antigens of interest located in the same cellular compartment (e.g. the cytoplasm) ?

NO

Use a immuno-enzymatic DAB based sequential or a simultaneous technique

YES

Double immunofluorescence technique (simultaneous technique)

Double immuno-enzymatic technique (simultaneous technique / sequential technique)

A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method)

Routine methods ?

Co-localized signal using double immuno-enzymatic techniques ?

Only a few chromogen combinations fulfill the criteria of a good visual contrast between the basic colors and a good contrasting mixed color at sites of co-localized antigens:

- **Red–blue combination, composed of HRP activity visualized with amino-ethyl carbazole (AEC) and alkaline phosphatase (AP) activity visualized with naphthol- AS-MX-phosphate/Fast Blue BB**

- **Vector NovaRed (Vector Laboratories) and Vector Blue (VBlue) for HRP and AP activities, respectively (alternatively - MultiVision kit system for rabbit and mouse primaries including all chromogen reagents by Thermo Fisher Scientific (LabVision))**

- **Red–blue combination, composed of alkaline phosphatase (AP) activity visualized with VBlue and AP activity visualized with Liquid Permanent Red (Dako) – sequential technique with intermediary HIER step to block first set of immuno-reagents**

- **Red–turquoise color combination composed of AP and beta-galactosidase activities, respectively. (Compared with most HRP and AP reaction products, beta-galactosidase activity visualized by X-gal/ferro-ferri cyanide is relatively insensitive/inefficient and also quite diffusely localized - unprecise)**

Only for research ?

Van der Loos CM. The J Histotechnol 33 (1): 31-40, 2010

Van der Loos CM et al. J Histotechnol; 31: 119-127, 2008

J Pathol. 2000 Aug;191(4):452-61.

Double immunofluorescence labelling of routinely processed paraffin sections.

Mason DY, Micklem K, Jones M.

Argued that double immuno-enzymatic labelling of routinely processed tissue are :

- ❑ Time-consuming
- ❑ Prone to background staining
- ❑ Rarely suitable for detecting two antigens present at the same site - since one label tends to obscure the other.

Demonstrated that double immunofluorescence labelling :

- ❑ Is more rapid than enzyme-based techniques
- ❑ Avoids the problems of interpreting two antigens present at the same site
- ❑ Only minimal tissue autofluorescence was observed.

The double immunoflourescence procedure may represent the optimal technical approach for demonstration of co-localized antigens in routinely processed tissue samples.

NGFR (MRQ21) + CD146 (EPR3208)

NGFR (MRQ21) + CD146 (EPR3208)

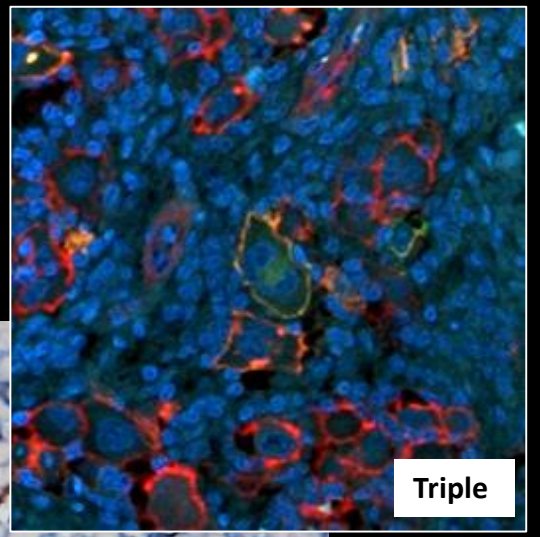
Double immuno-fluorescence technique

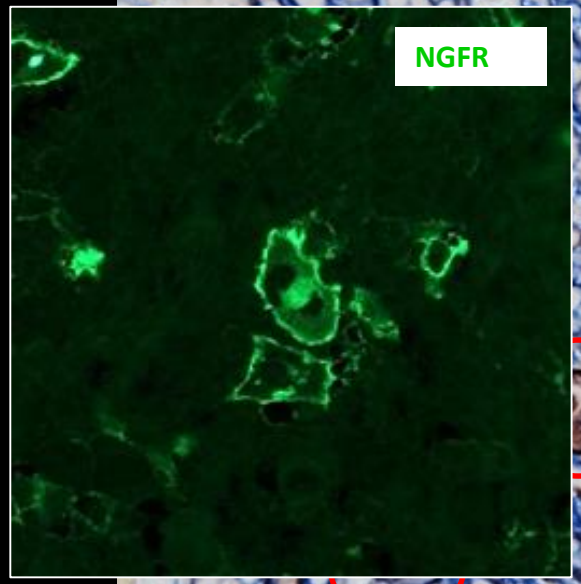
Melanoma

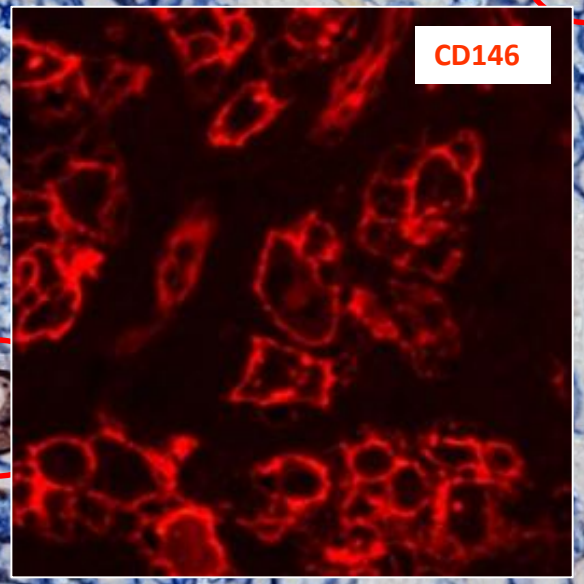
Melanoma

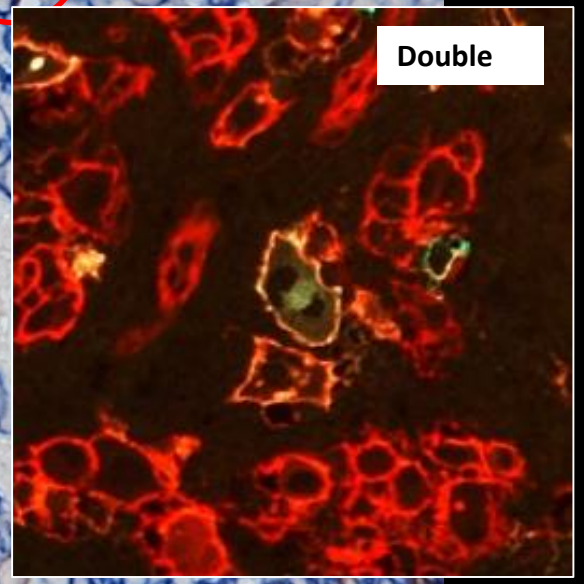
Thermo/ LabVision – Multivision KIT

Co-localization ?

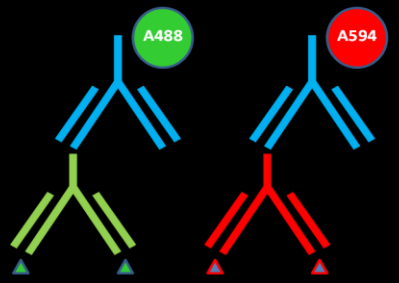








Note: Co-expression in tumour cells from melanoma (yellow colour).

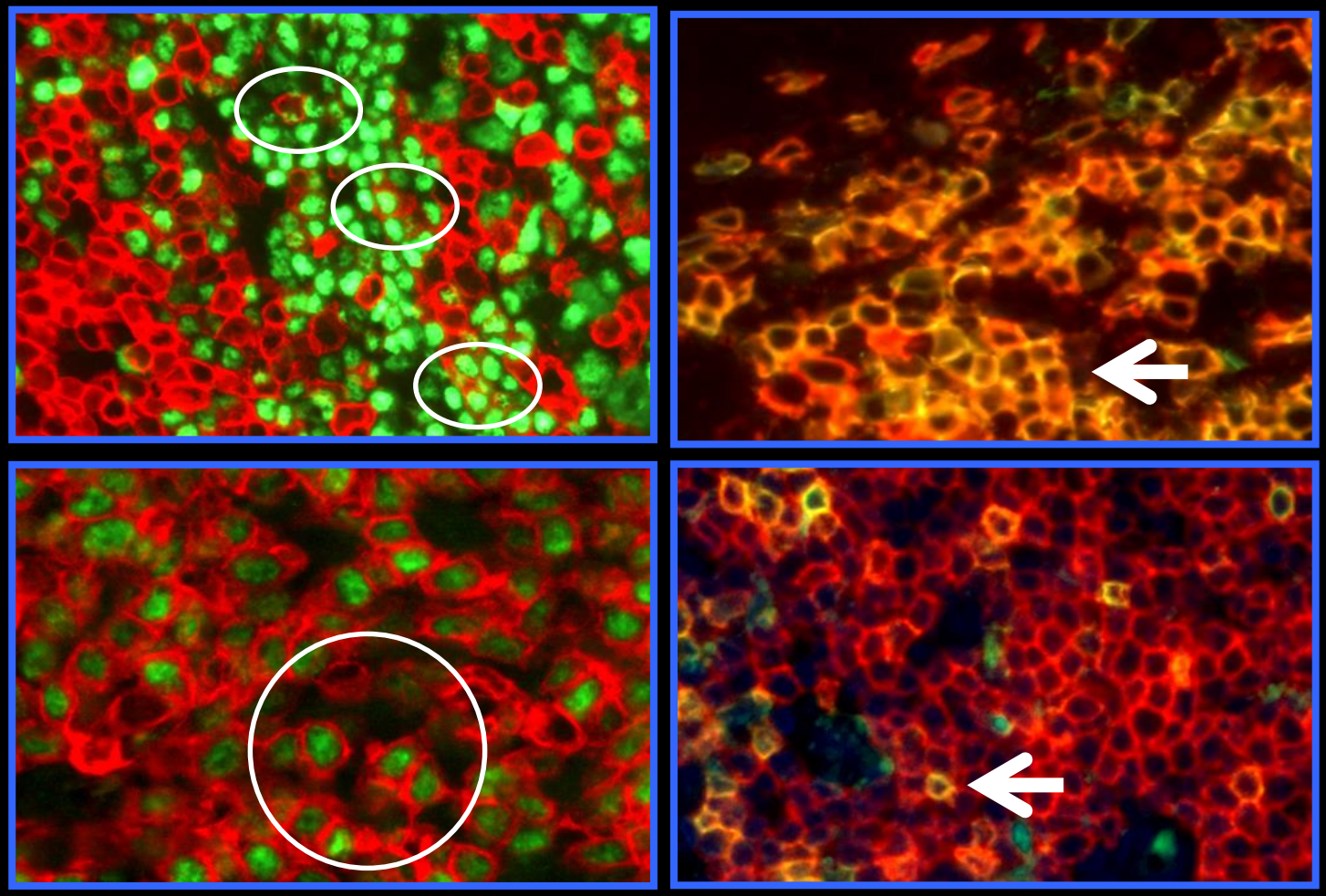


PAX-5, 1EW (TSA amp.) + CD5, SP19

CD3, PS1 + CD5, SP19

Tonsil

B-CLL



Note : Co-localization of normal T-cells in the tonsil and in the B-CLL (CD3+CD5) and co-expression of the neoplastic B-cell (Pax-5 and CD5) in the B-CLL and normal B-Cells in the mantle zone of the tonsil

Volume 57(10): 899–905, 2009
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

SIMPLE: A Sequential Immunoperoxidase Labeling and Erasing Method

George Glass, Jason A. Papin, and James W. Mandell

Department of Biomedical Engineering, (GG,JAP) and Department of Pathology (Neuropathology) (JWM), University of Virginia, Charlottesville, Virginia

Table 1 Comparison of SIMPLE with existing multiprobe immunolabeling methods

	SIMPLE	Traditional multi-chromagen IHC	Multiplex-immunostain chip	Multicolor IF
Maximum labels per section	5+	2–3	50	3
Use on paraffin-embedded archival tissue	+	+	+	−/+
Ability to overcome autofluorescence/photobleaching	+	+	+	−
Colocalization within a single cellular compartment	+	−	−	+
Compatible with primary antibodies from same species	+	−	+	−

SIMPLE is compared with traditional two- or three-color multichromagen immunohistochemistry (IHC), the multiplex immunostain chip method (Furuya et al. 2004), and multicolor immunofluorescence (IF) methods.

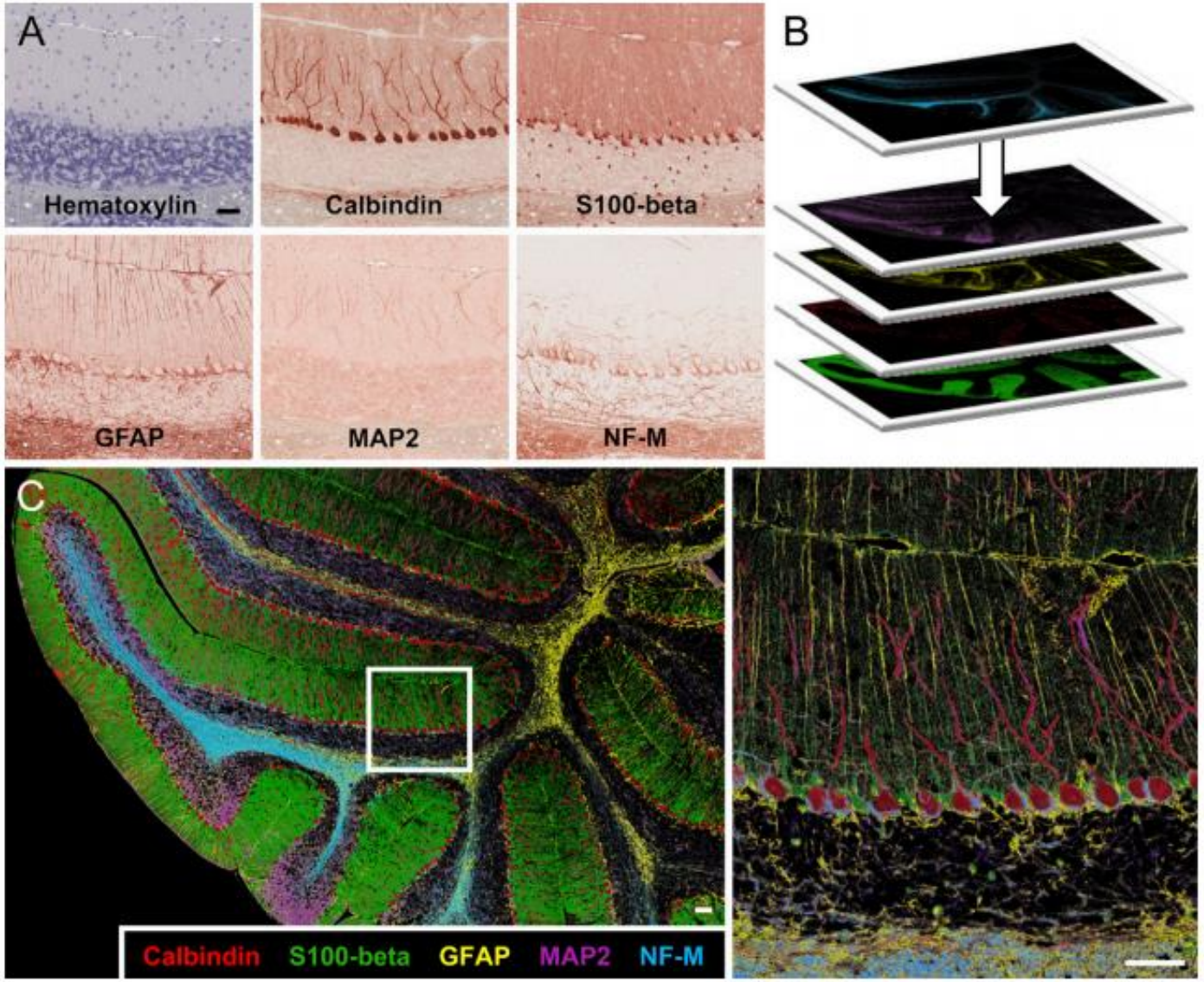


Figure 3 Simultaneous visualization of five antigens in mouse cerebellum. **(A)** Adult mouse brain was counterstained with hemotoxylin, then sequentially probed with polyclonal antibodies to calbindin, S100-β, and GFAP, and monoclonal antibodies to MAP2 (AP18) and neurofilament (NF-M) 2H3. **(B)** The images were individually pseudocolored and overlaid. **(C)** The small boxed area in the left panel is shown magnified at right. The resultant image reveals the morphology of different cell types and fine details of interactions of Purkinje cells, Bergmann glia, astrocytes, and basket cell terminals that would not be obvious with single or dual labeling. Bar = 50 μm.

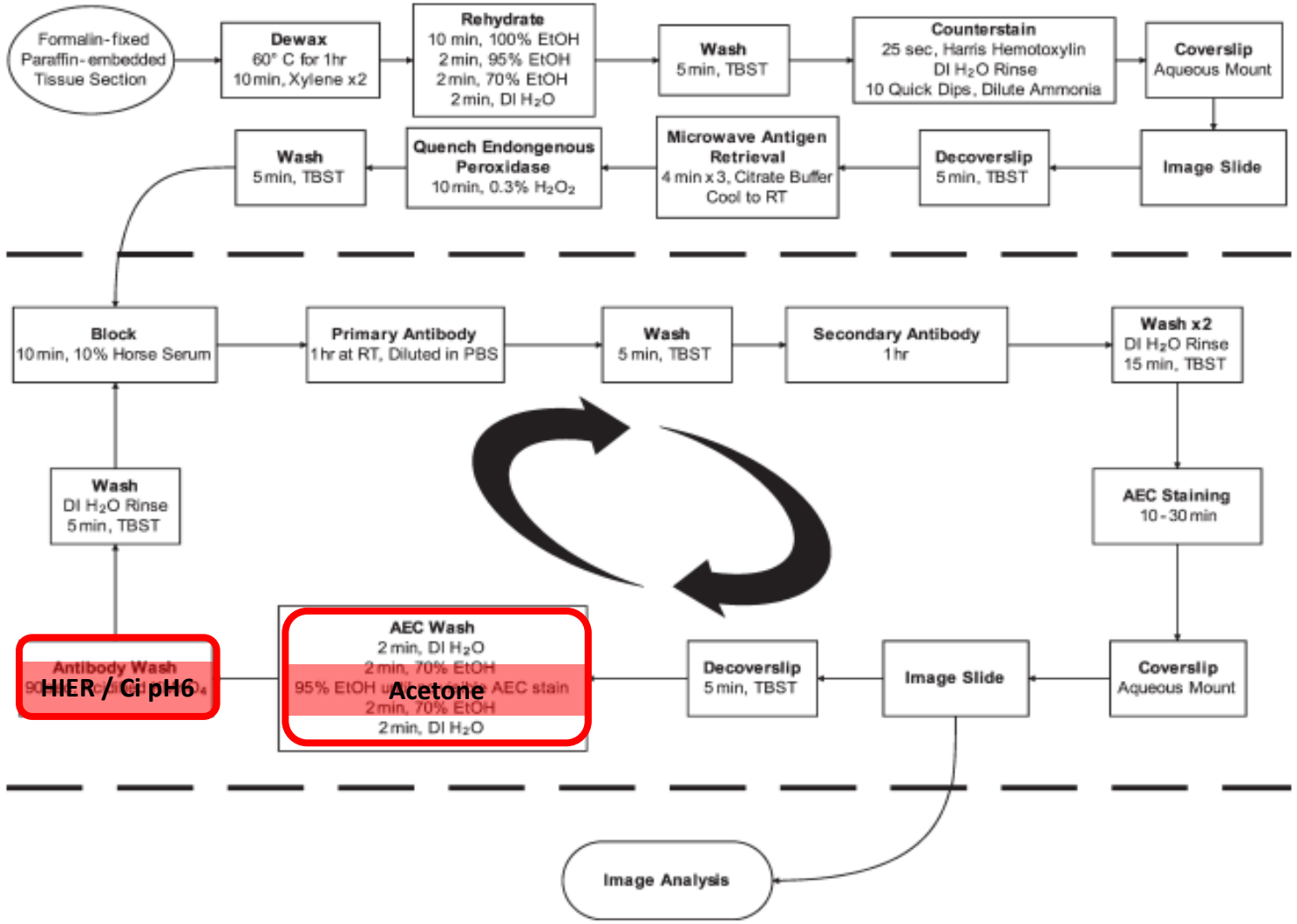


Figure 1 SIMPLE strategy. Formalin-fixed, paraffin-embedded sections are dewaxed, rehydrated, and counterstained before initial probing. Tissue is imaged and then subjected to antigen retrieval, removing the counterstain. Each staining round is conducted using standard immunohistochemical protocols with the alcohol-soluble red peroxidase substrate 3-amino-9-ethylcarbazole (AEC). After each round of staining, the tissue is imaged and then stripped of AEC precipitate in ethanol. Antibody is then eluted in acidified permanganate, and the tissue is subjected to the next round of staining.

Requirements

Chromogen that can be erased (AEC)

Aqueous mounting

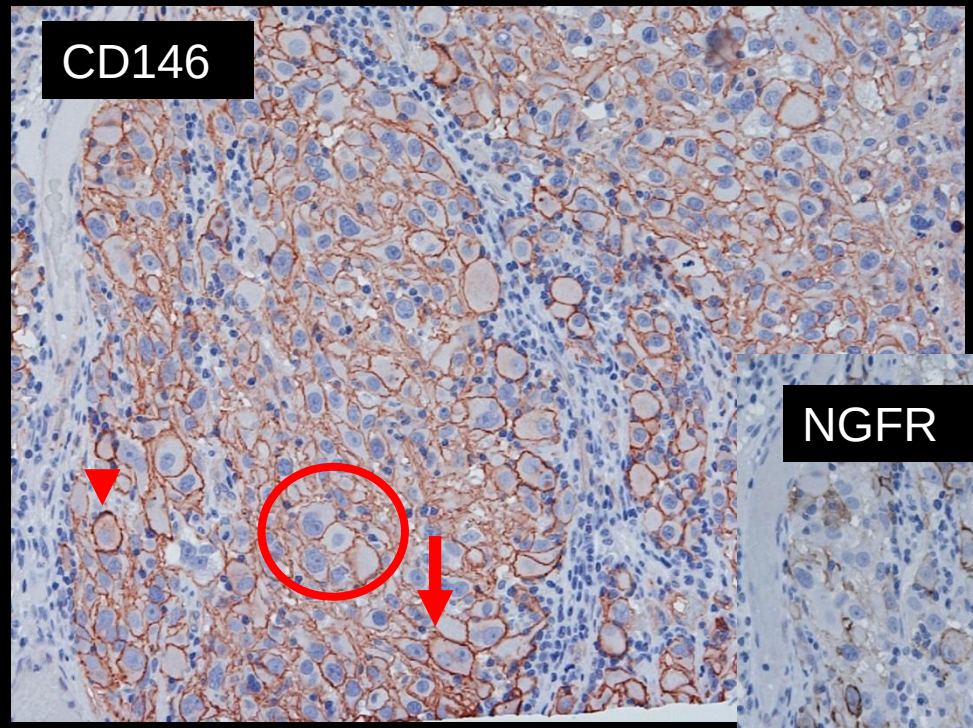
Imaging of slides

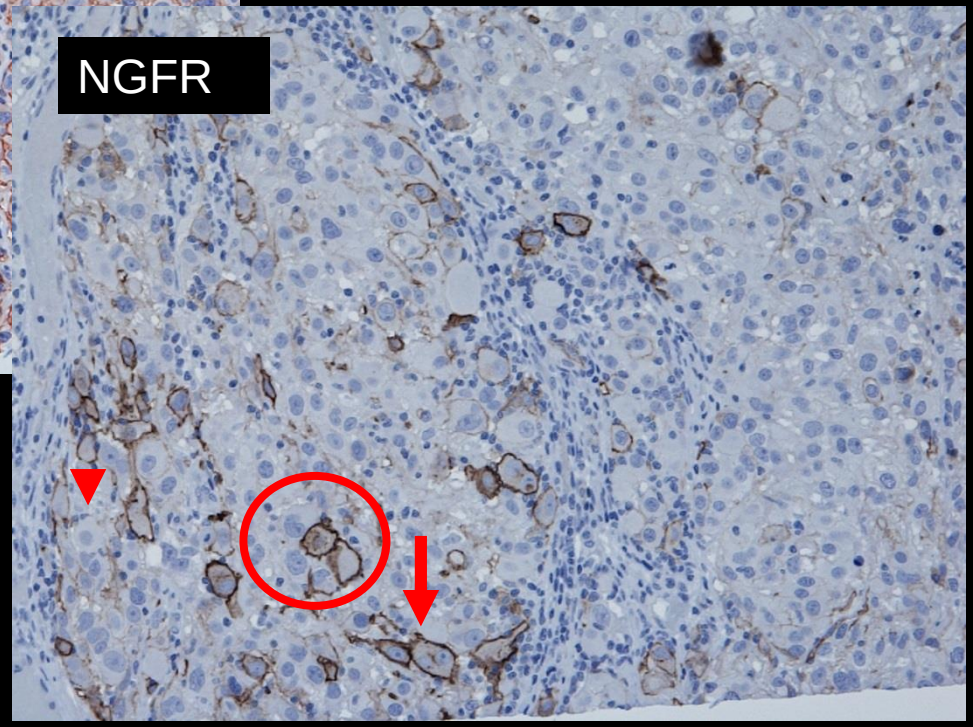
Removal of coverslip

AEC wash reagent that is gentle to detection of the following epitopes of interest

Efficient blocking procedure for the immuno-reagents applied

SIMPLE technique: CD146 (EPR3208) + NGFR (MRQ21)





Super Sensitive (Biogenex)
Impact AEC (Vector Lab)

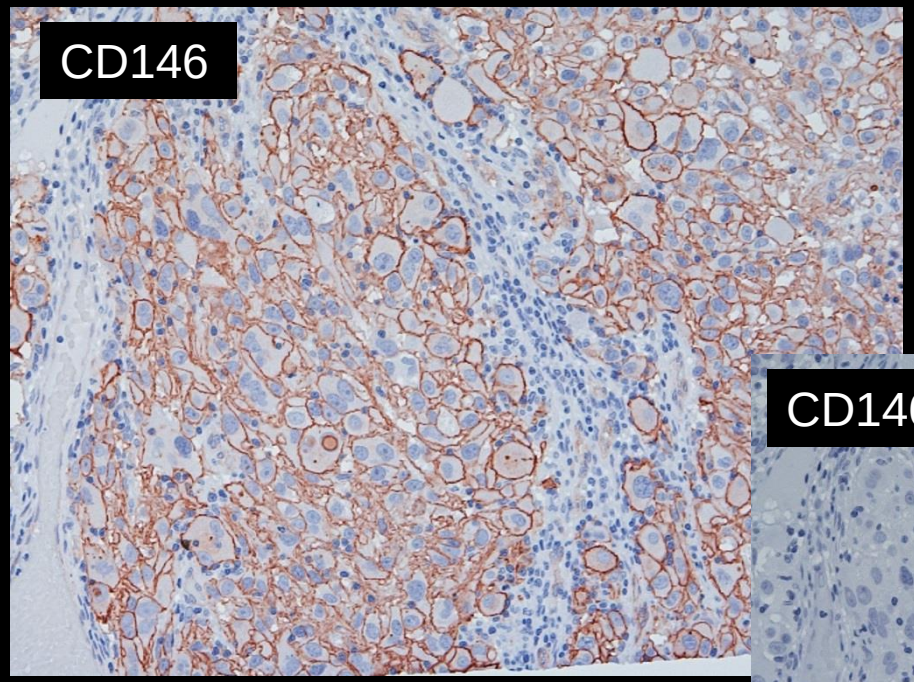
First sequence: CD146 immunostaning => AEC Impact => coverslip

Imaging => de- coverslip => Erasing (Acetone) => Blocking using HIER (Ci)

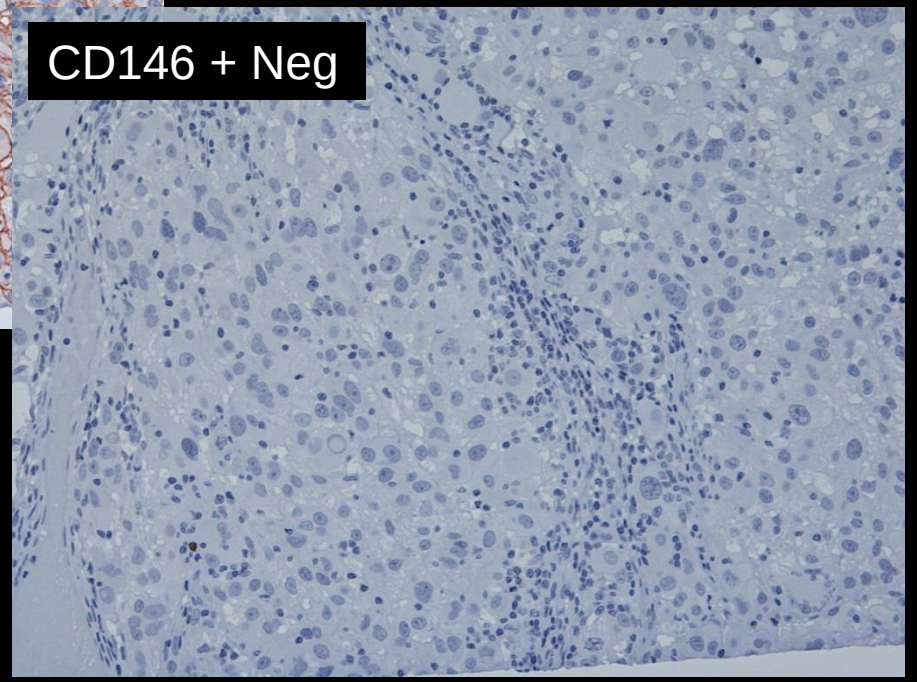
Second sequence: NGFR immunostaning => DAB => coverslip

Melanoma

SIMPLE technique: CD146 (EPR3208) + Neg



Control



Control experiments:

First sequence: CD146 immunostaning => AEC Impact => coverslip

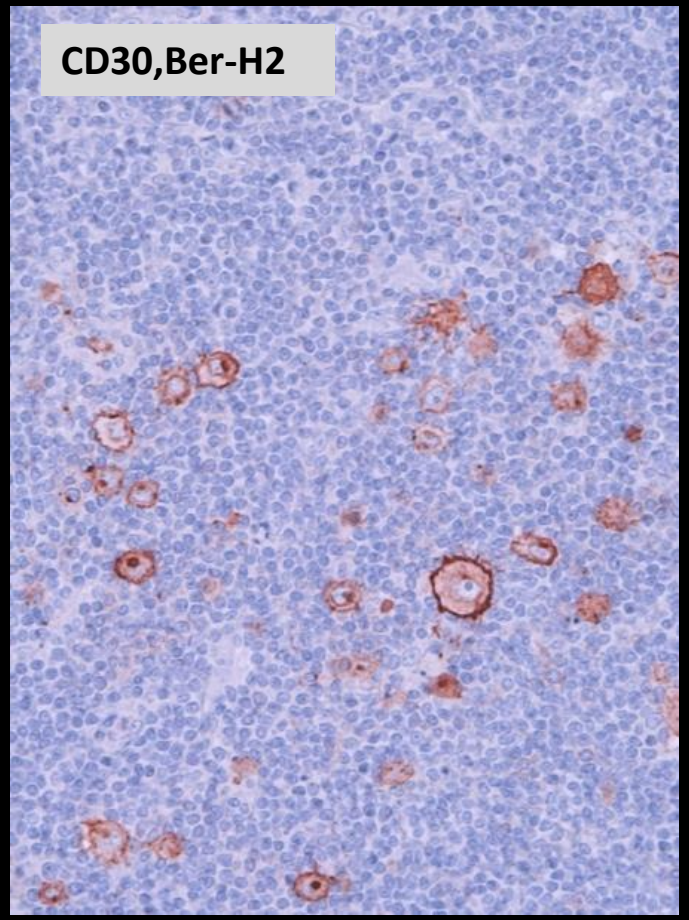
Imaging => decoverslip => Erasing (Acetone) => Blocking using HIER (Ci)

Second sequence: Omission of primary Ab => immunostaning => DAB => coverslip

Melanoma

Simple-Technique

Staining: CD30 (first cycle) → CD15 (second cycle)



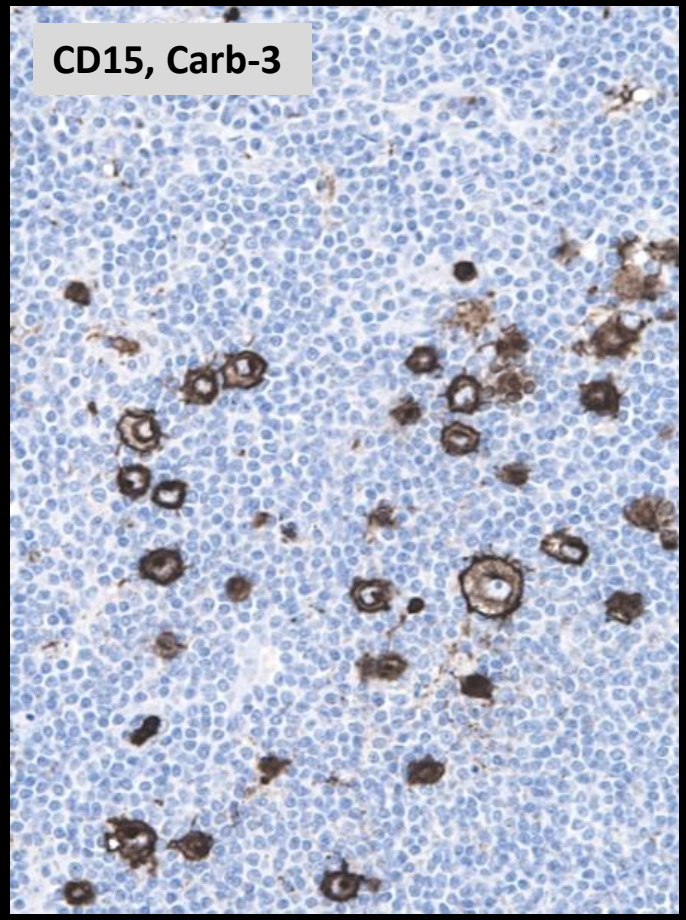
AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

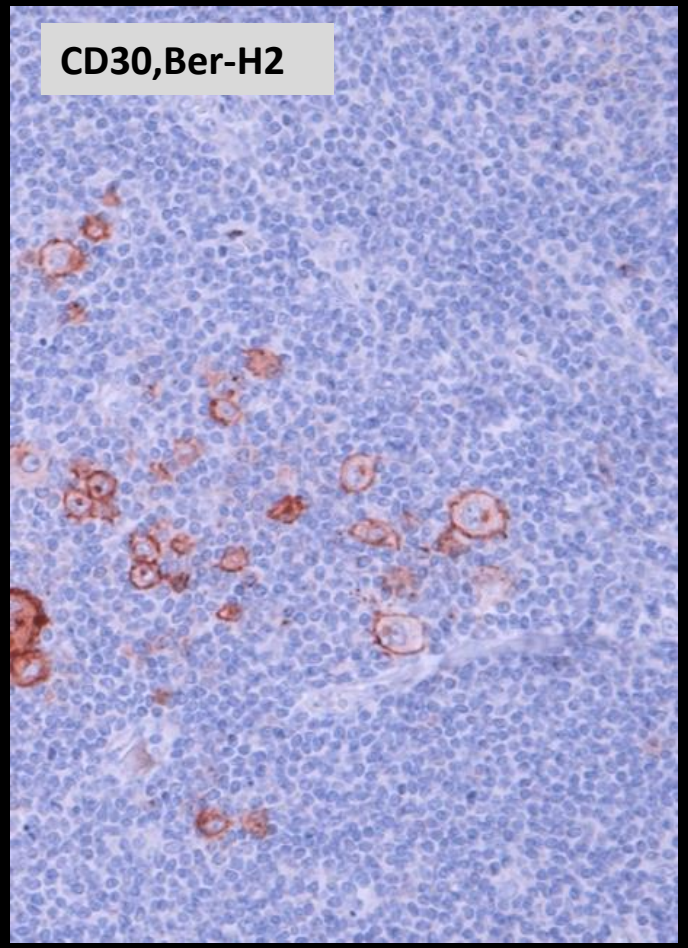
Cycle (immunostaining) repeated with CD15 /DAB



Hodgkin Lymphoma

Simple-Technique

Control staining: CD30 (first cycle) → Omission of CD15 (second cycle)



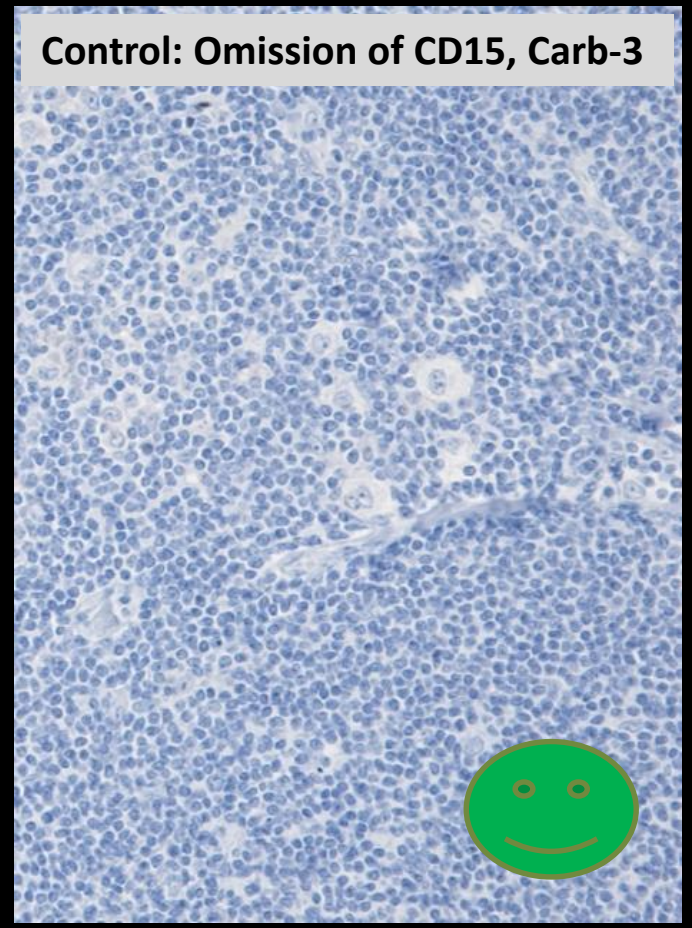
AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

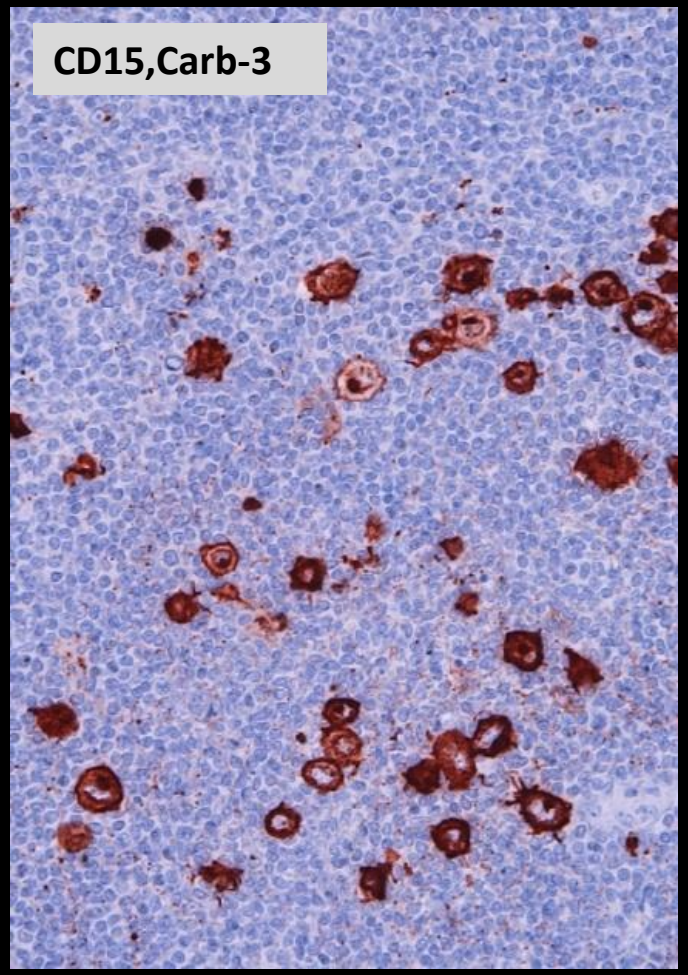
Cycle (immunostaining) repeated without CD15 /DAB



Hodgkin Lymphoma

Simple-Technique

Staining: CD15 (first cycle) → CD30 (second cycle)



AEC Erasing and Blocking (Elution / Denaturation)

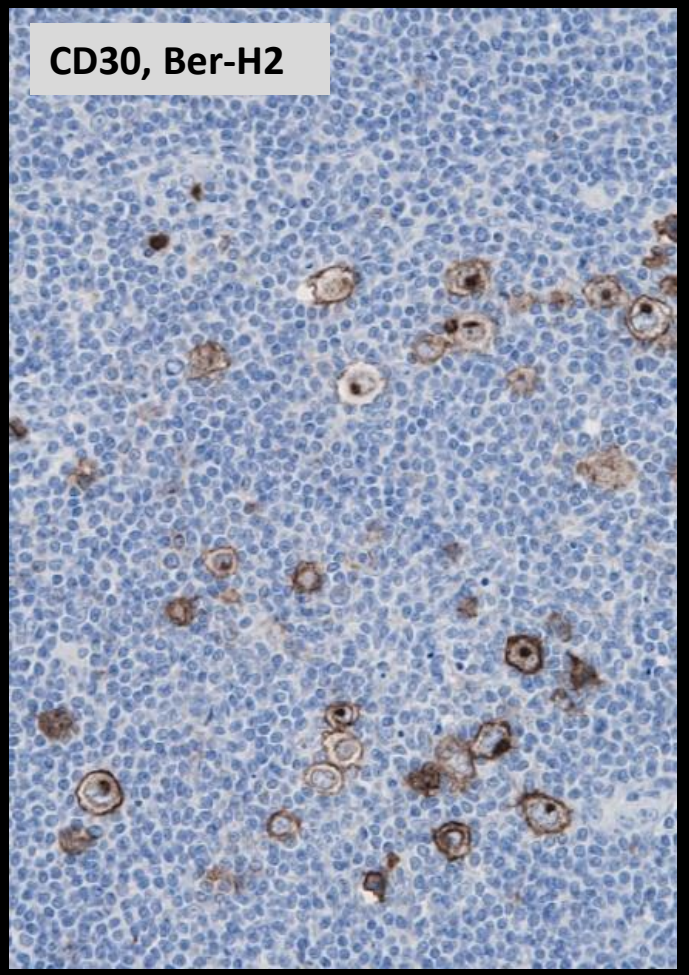
De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

Cycle (immunostaining) repeated with CD30 /DAB

Stainings looks OK, but ……

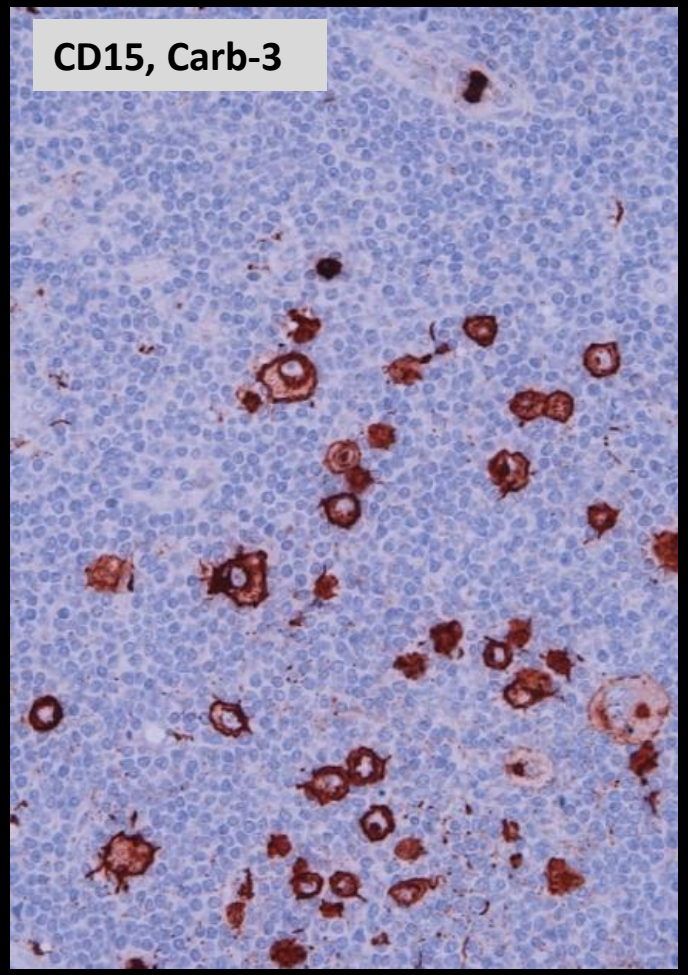


Hodgkin Lymphoma

Simple-Technique

Control staining: CD15 (first cycle) → Omission of CD30 (second cycle)

CD15, Carb-3



AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

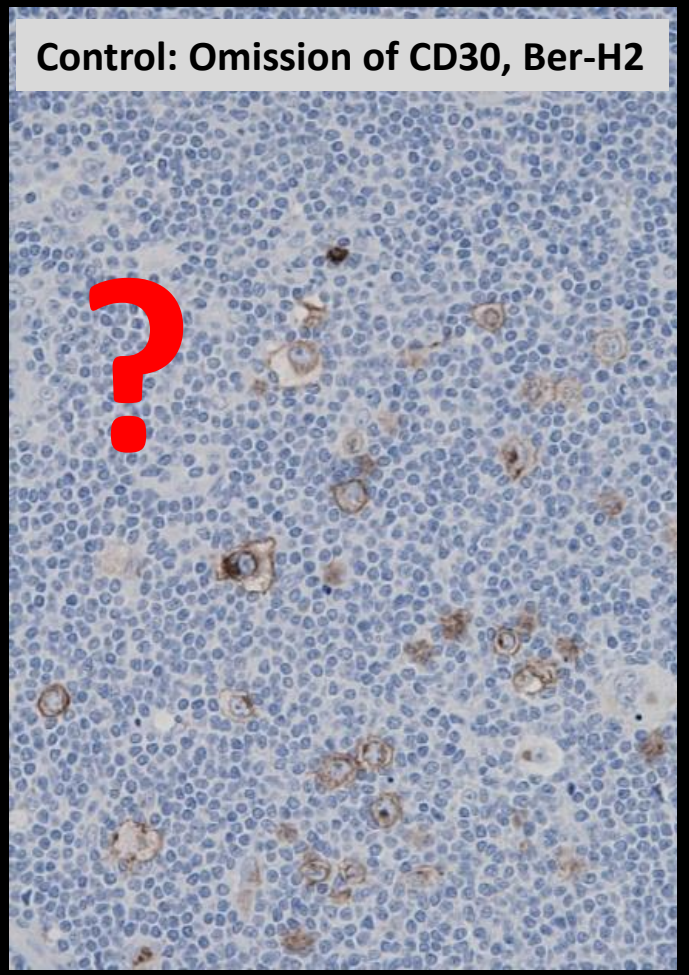
Blocking with Ci pH 6 (99°C/30`)

Cycle (immunostaining) repeated without CD30 /DAB

Problem with the blocking procedure:

High affinity Abs ?
Antigen density ?
Inefficient blocking procedure ?

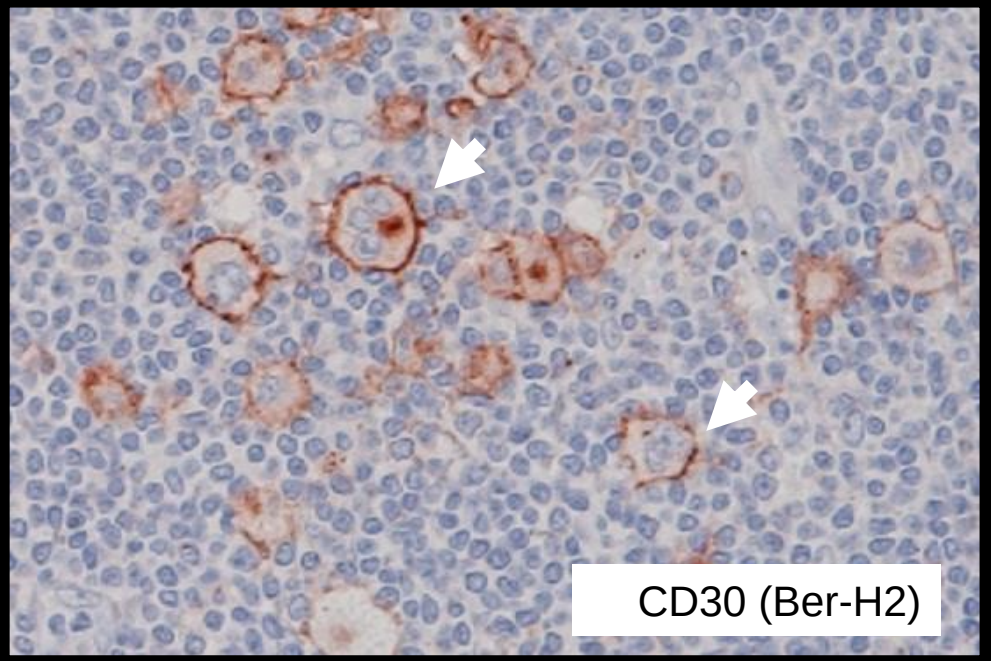
Control: Omission of CD30, Ber-H2

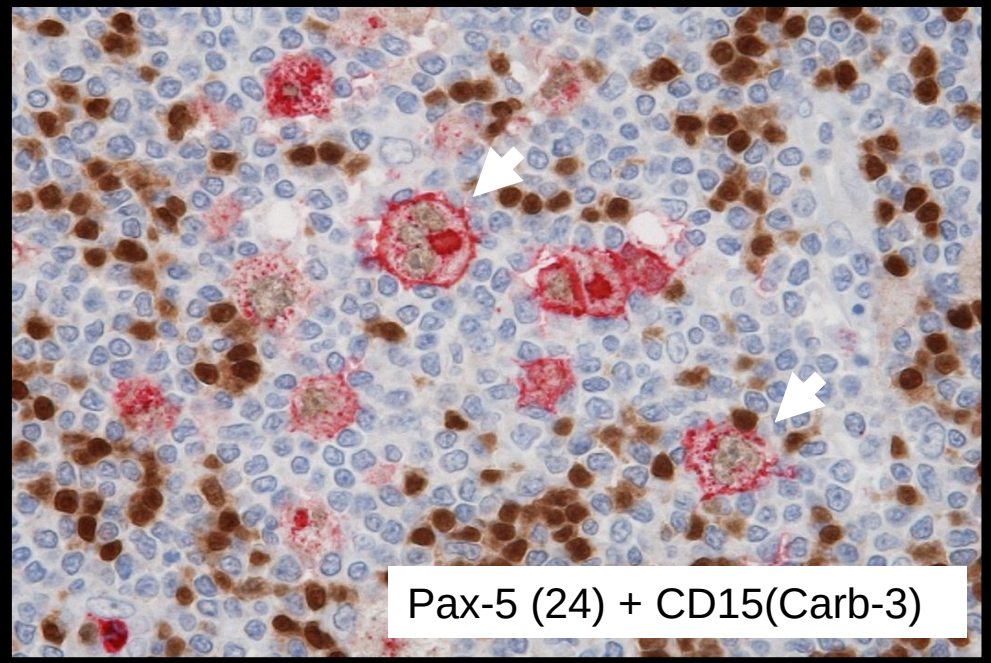


Hodgkin Lymphoma

Combining SIMPLE technique with sequential double immune enzymatic method

CD30 (first cycle) → combined with standard sequential staining for Pax-5 and CD15





Erasing & blocking (heat) first set of immune reagents followed by "normal" sequential technique

Note: Co-localization of CD30 and CD15 in Hodgkin cells; Co-expression of Pax-5 (weak) and CD15 (CD30) in Hodgkin cells. Strong staining of normal B-lymphocytes with Pax-5.

Can we make the Simple technique even more simple ?

Do we have to use a sequential technique ?

Is it possible to avoid the blocking step (HIER in Ci) ?

Simultaneous Immunoperoxidase/phosphatase Labelling and Erasing Method

SIMPLE-Technique

SIMPLE-Technique (simultaneous procedure)

Dewax and Pretreatment (Antigen Retrieval)

Incubation with a mix of primary antibody reagents

Rabbit & Mouse monoclonal antibodies

Incubation with Dual-labelling Detection reagents

MultiVision (Mouse-HRP og Rabbit-AP) or MACH2 Double Staining 1 or 2

Incubation with HRP / Chromogen AEC Impact

Hematoxylin (one quick dip) / Mounting (hydrophilic) / Imaging / De-coverslip / Water or buffer

Erasing: Acetone followed by app. buffer

Incubation with AP / Chromogen Permanent Red / LBV-Blue /Warp Red

Mounting (hydrophilic PR or hydrophobic Warp Red / LBV-Blue) / Imaging

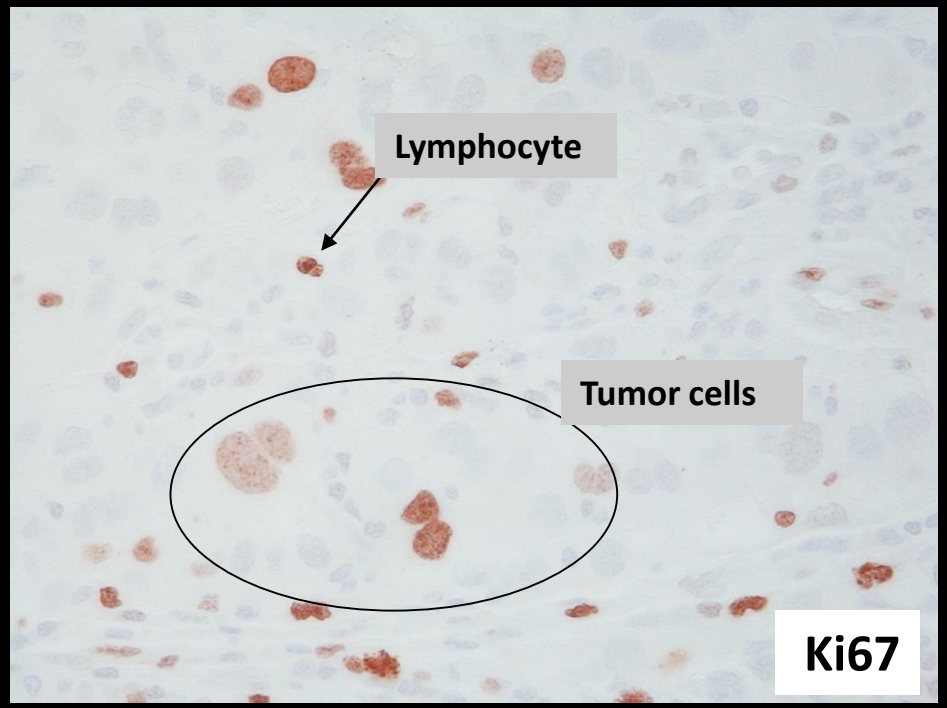
Blocking procedure not needed

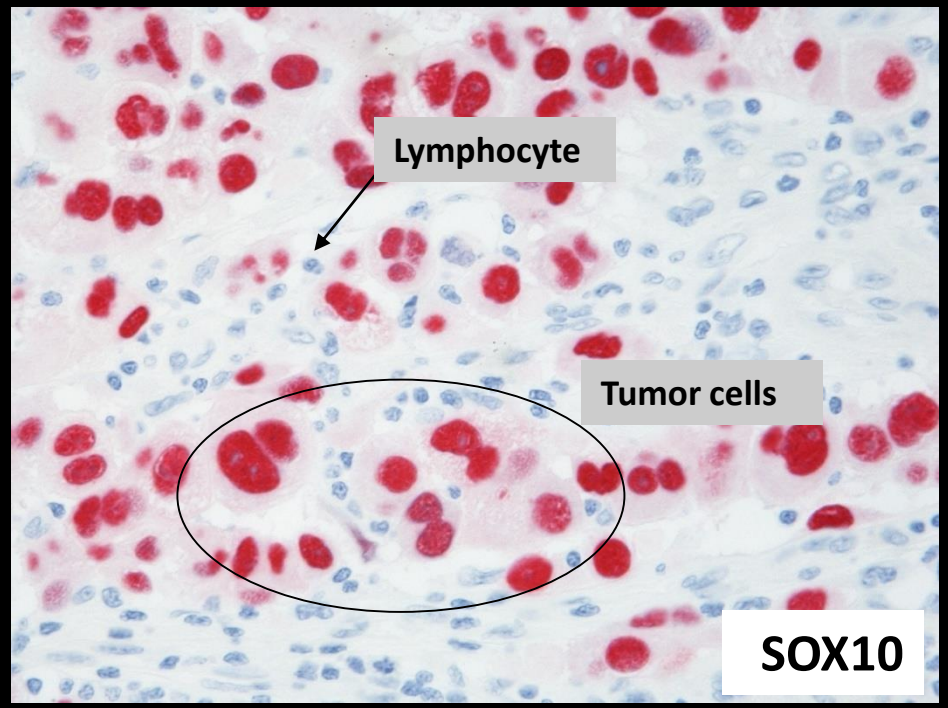
SIMPLE-Technique (simultaneous proc.): MACH2 Double Staining 1

Ki67, SP6 (1:25) + Sox-10, BC34 (1:20)

AEC (Impact) - Erasing (Acetone) - Warp Red

Melanoma





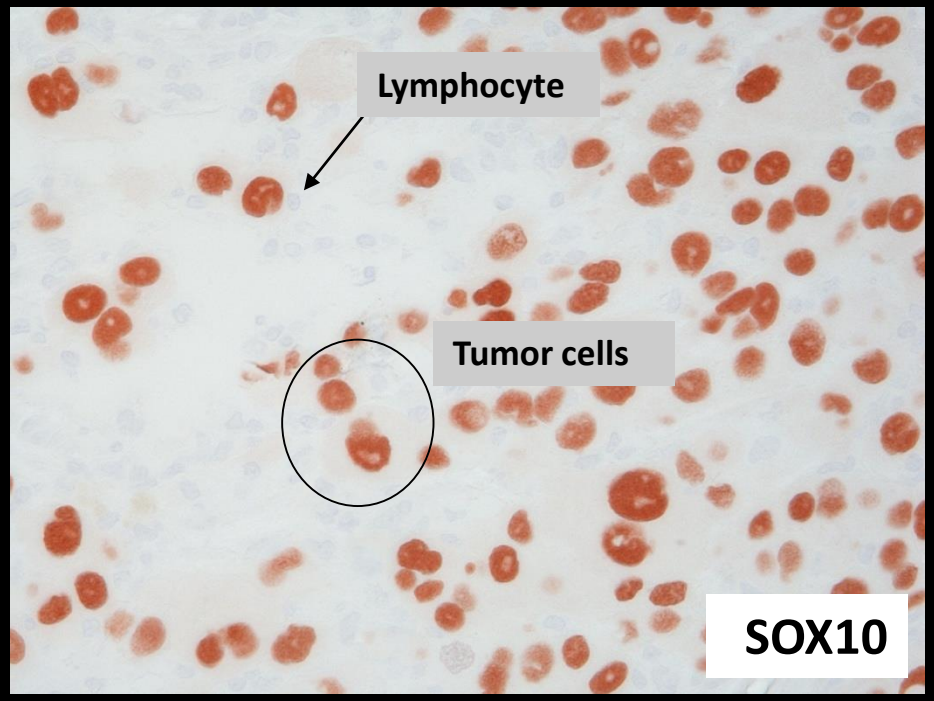
AEC (Impact) - (1dip Hematoxylin / coverslip-Aqueous/ Imaging / de-coverslip / Erasing-Acetone) - Warp Red

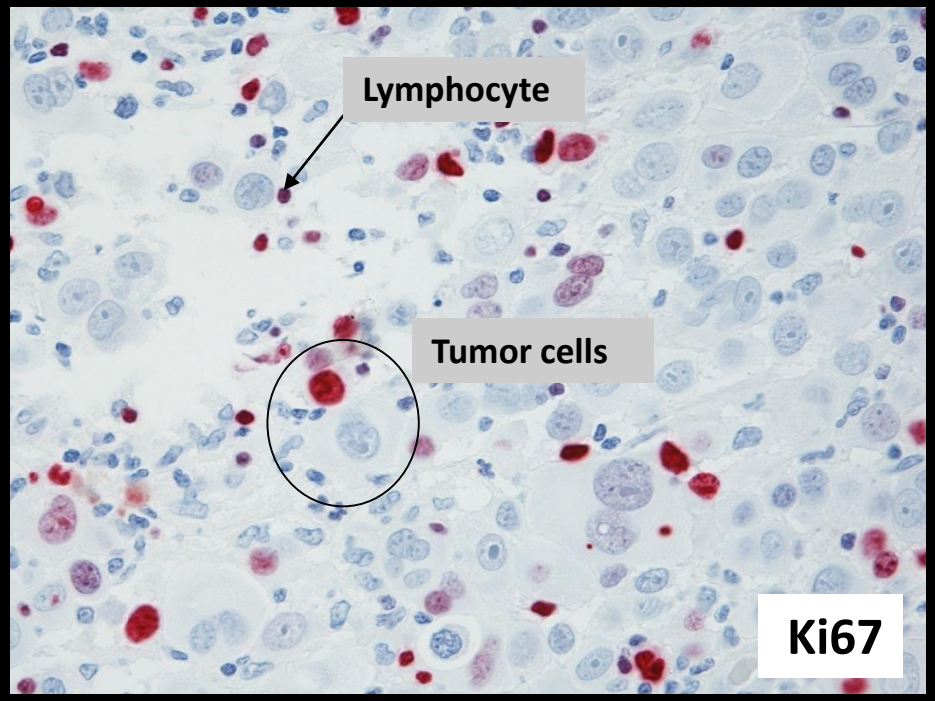
SIMPLE-Technique (simultaneous proc.): MACH2 Double Staining 1

Sox-10, BC34 (1:20) + Ki67, SP6 (1:25)

AEC (Impact) - Erasing (Acetone) - Warp Red

Melanoma

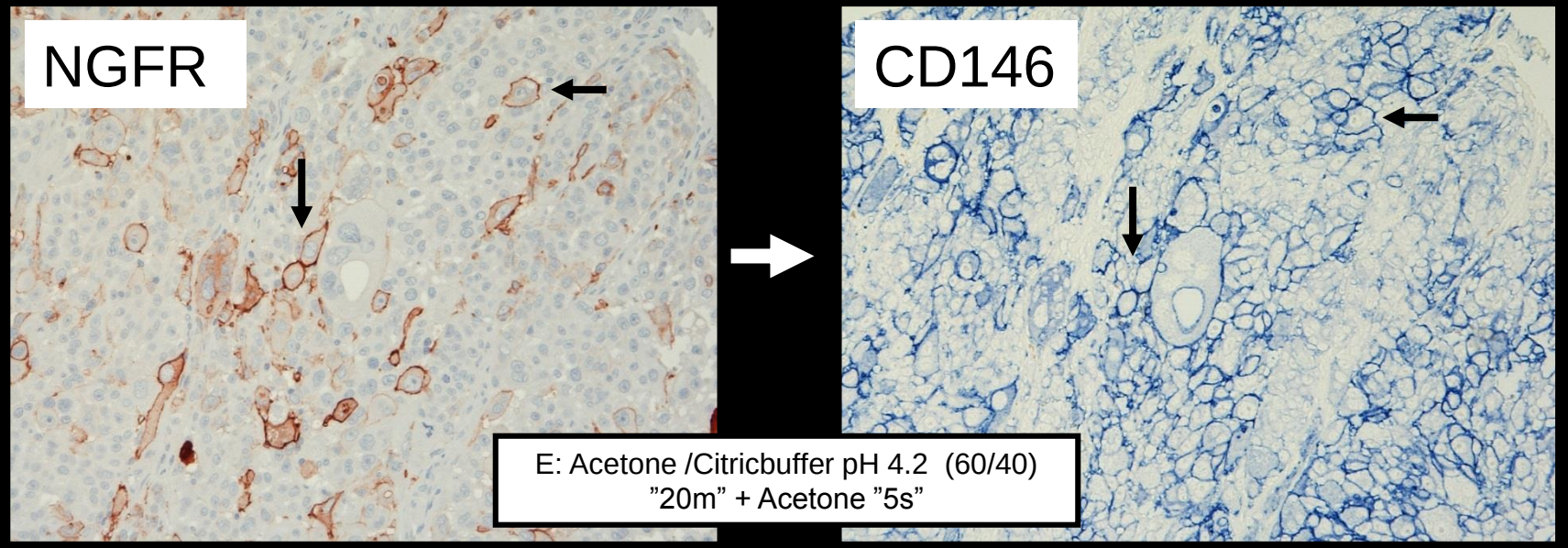


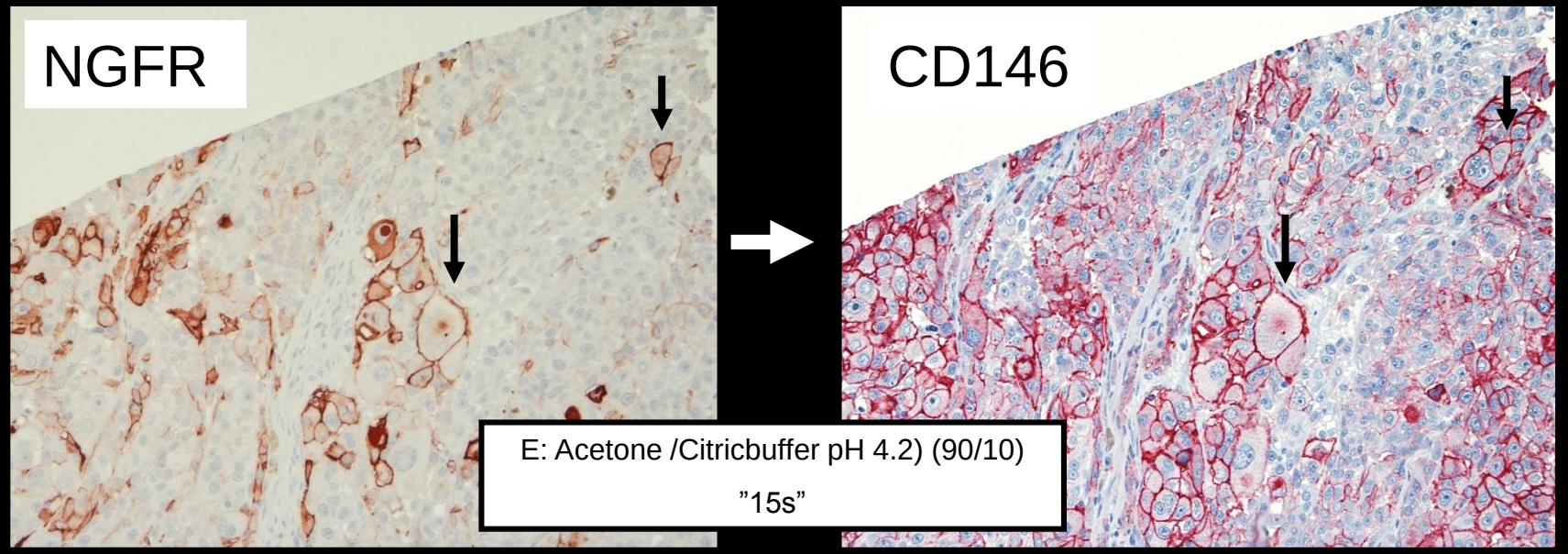


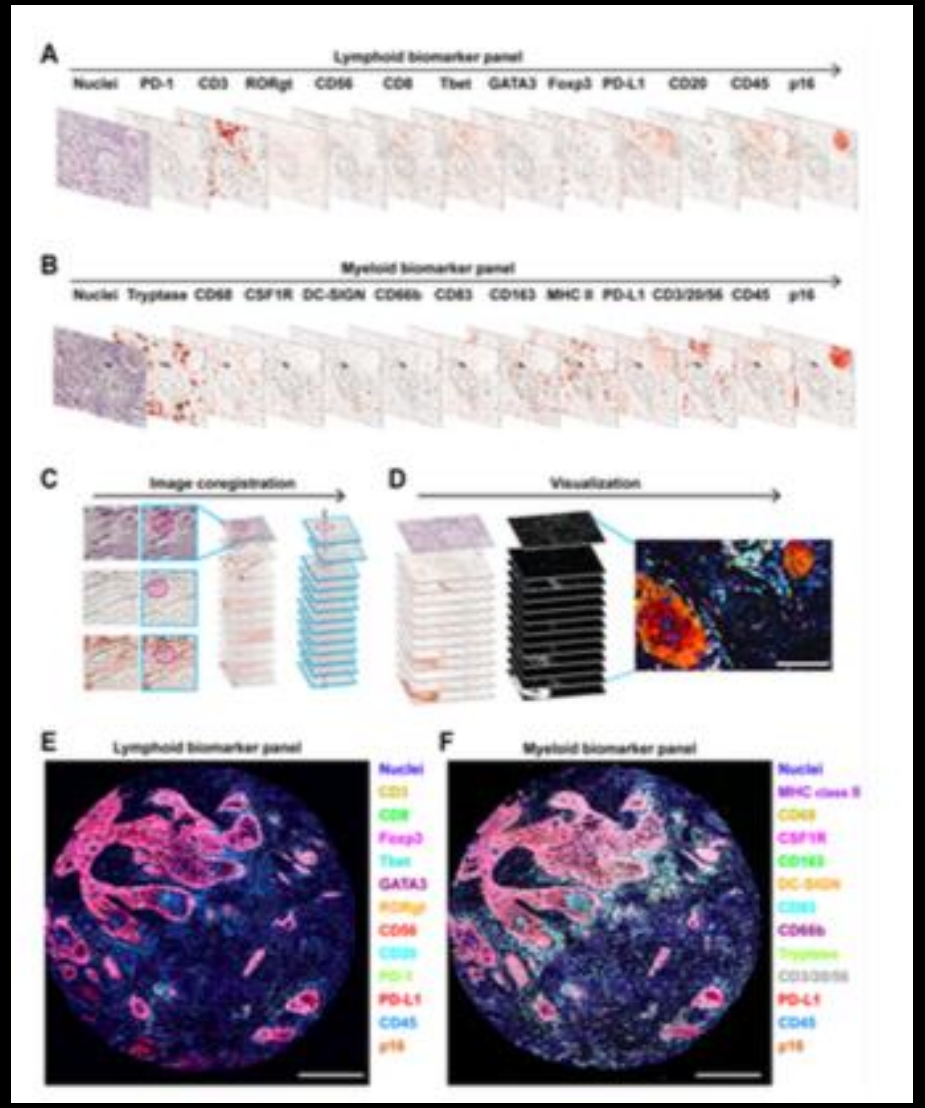
AEC (Impact) - (1dip Hematoxylin / coverslip-Aqueous/ Imaging / de-coverslip / Erasing-Acetone) - Warp Red

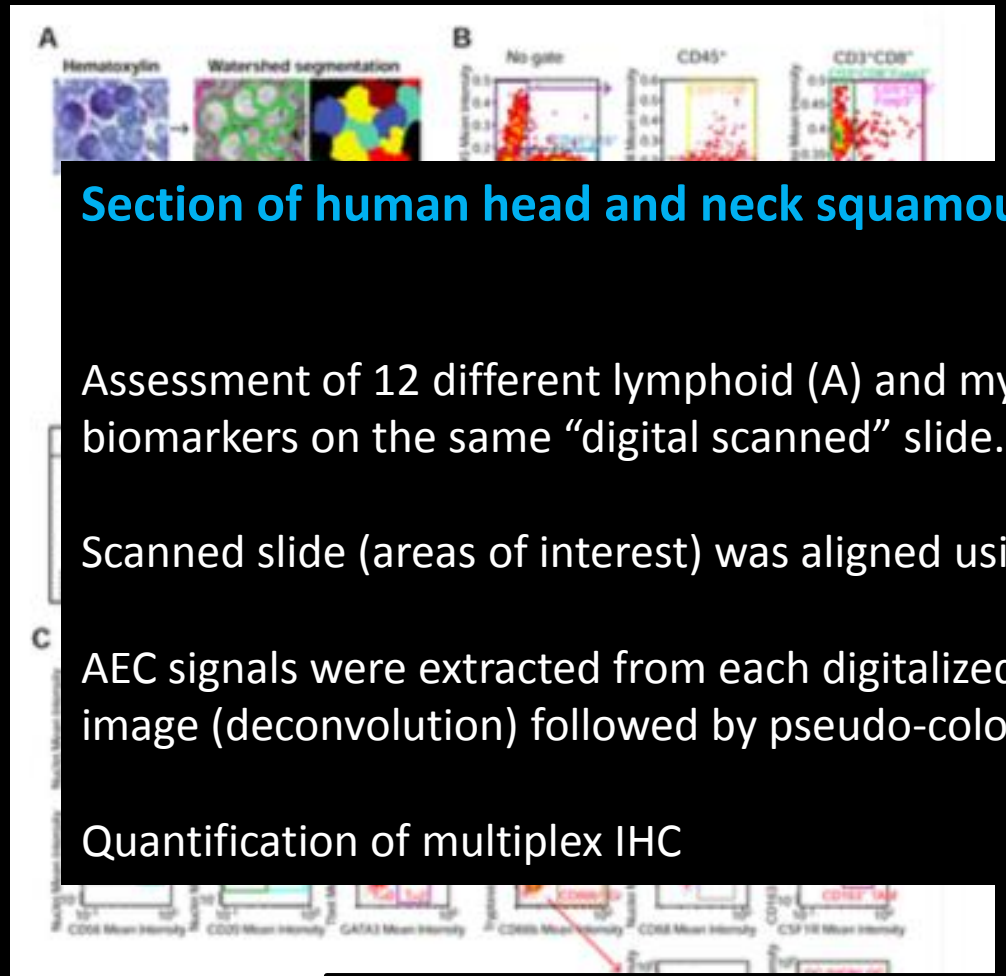
NGFR (MRQ21) + CD146 (EPR3208)

SIMPLE-Technique (simultaneous proc.)









Section of human head and neck squamous cell carcinoma

Assessment of 12 different lymphoid (A) and myeloid (B) biomarkers on the same “digital scanned” slide.

Scanned slide (areas of interest) was aligned using CellProfiler

AEC signals were extracted from each digitalized single marker image (deconvolution) followed by pseudo-coloration

Quantification of multiplex IHC

Published in final edited form as:

Cell Rep. 2017 April 04; 19(1): 203–217. doi:10.1016/j.celrep.2017.03.037.

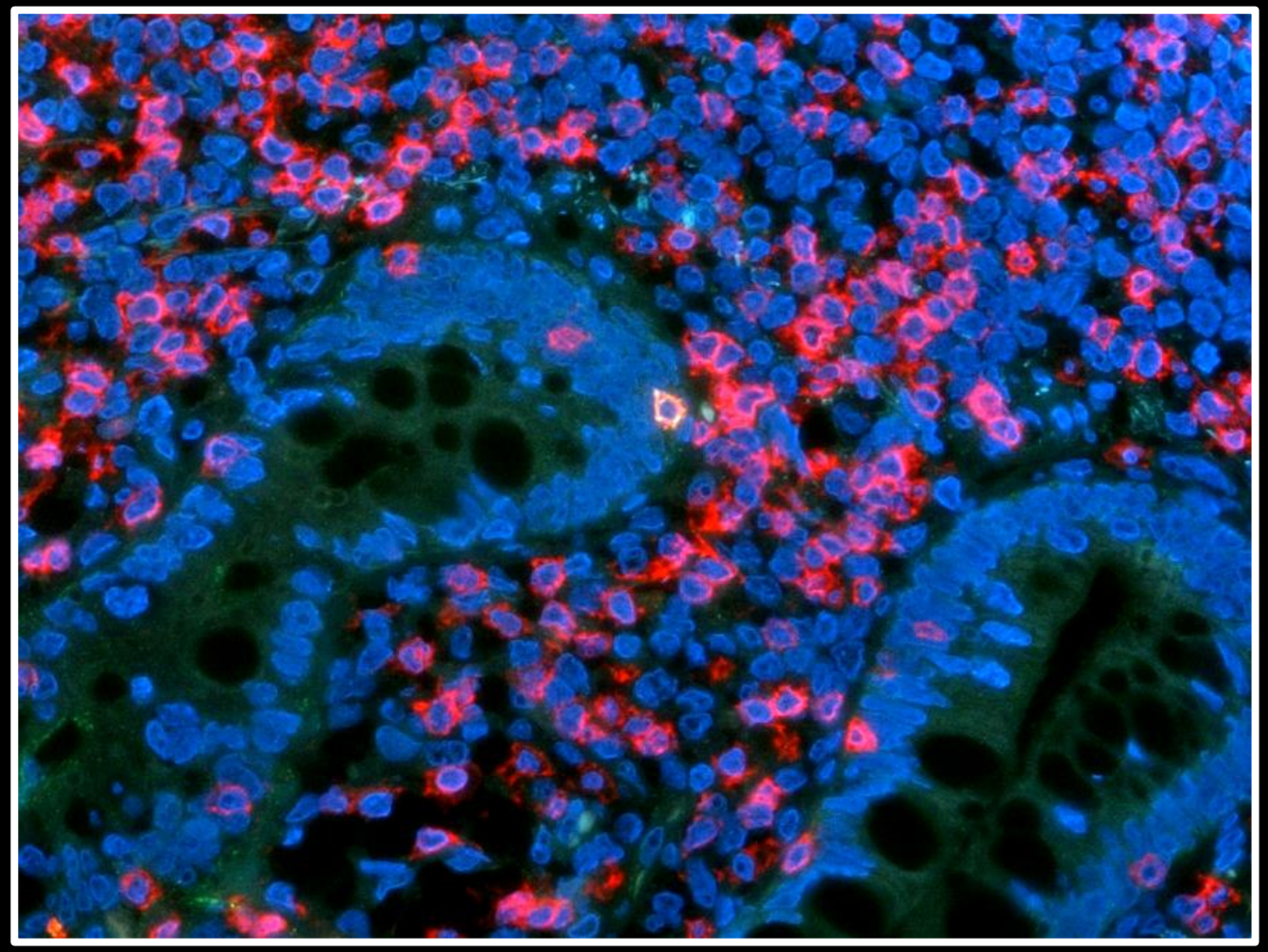
Quantitative multiplex immunohistochemistry reveals myeloid-inflamed tumor-immune complexity associated with poor prognosis

The Future: Simple and multiplex (TSA) techniques for routine purpose ?

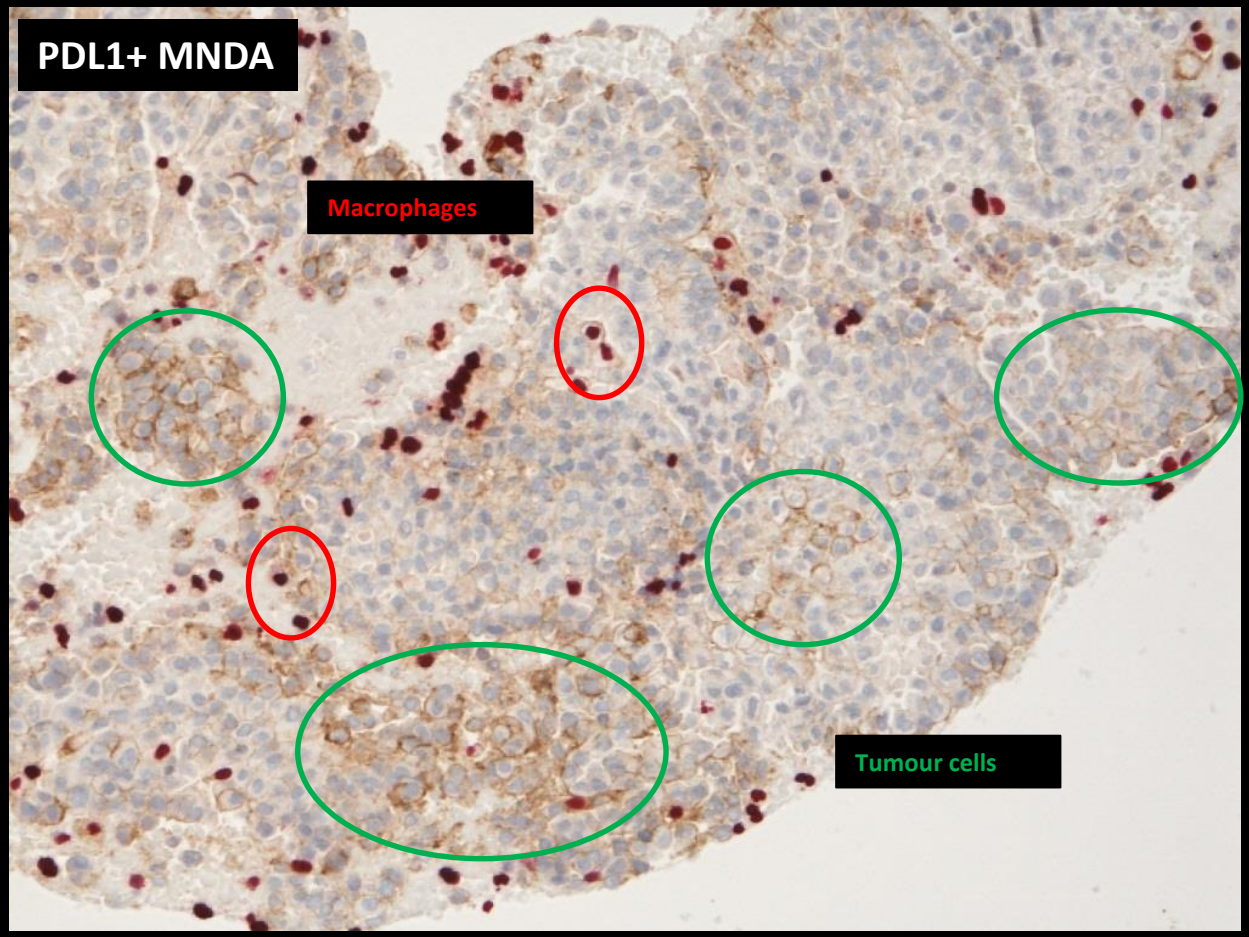
Double immuno-staining - The basal fundament for a technical "optimal" performance:

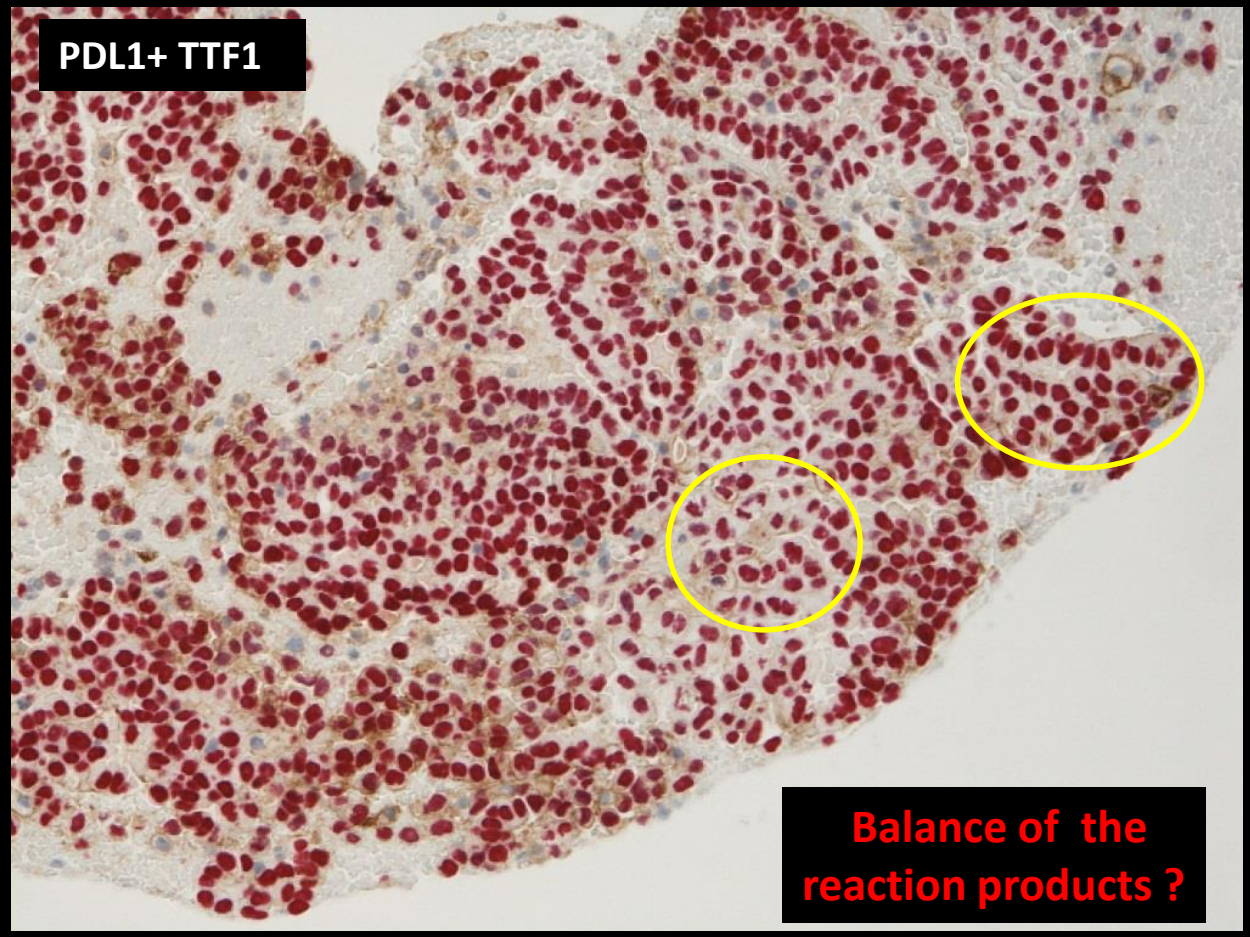
- **Appropriate tissue fixation and processing**
  - Garbage in – Garbage out
- **Appropriate and efficient epitope retrieval**
  - Do the antigenic epitopes of interest require the same pre-treatment ?
- **Appropriate choice of antibody pairs / clones and dilutions**
  - Do the primary antibody pairs provide app. sensitivity & specificity depending on the chosen detection method ?
  - Is primary antibody pairs available in app. format depending on the chosen detection method ?
- **Robust, specific & sensitive double immuno - detection and visualization systems**
  - Sequential or simultaneous double immuno-staining techniques ?
  - For the routine: Is a double immuno - detection and visualization system (with appropriate sensitivity) always available ?
- **Appropriate choice of control material**
  - Calibrating the primary antibody concentrations carefully according to Critical Staining Quality Indicators (iCAPs)

Thank you for your attention



TCR γ (Mab,3.20) /TSA amp. + CD3 (Rab,SP19)



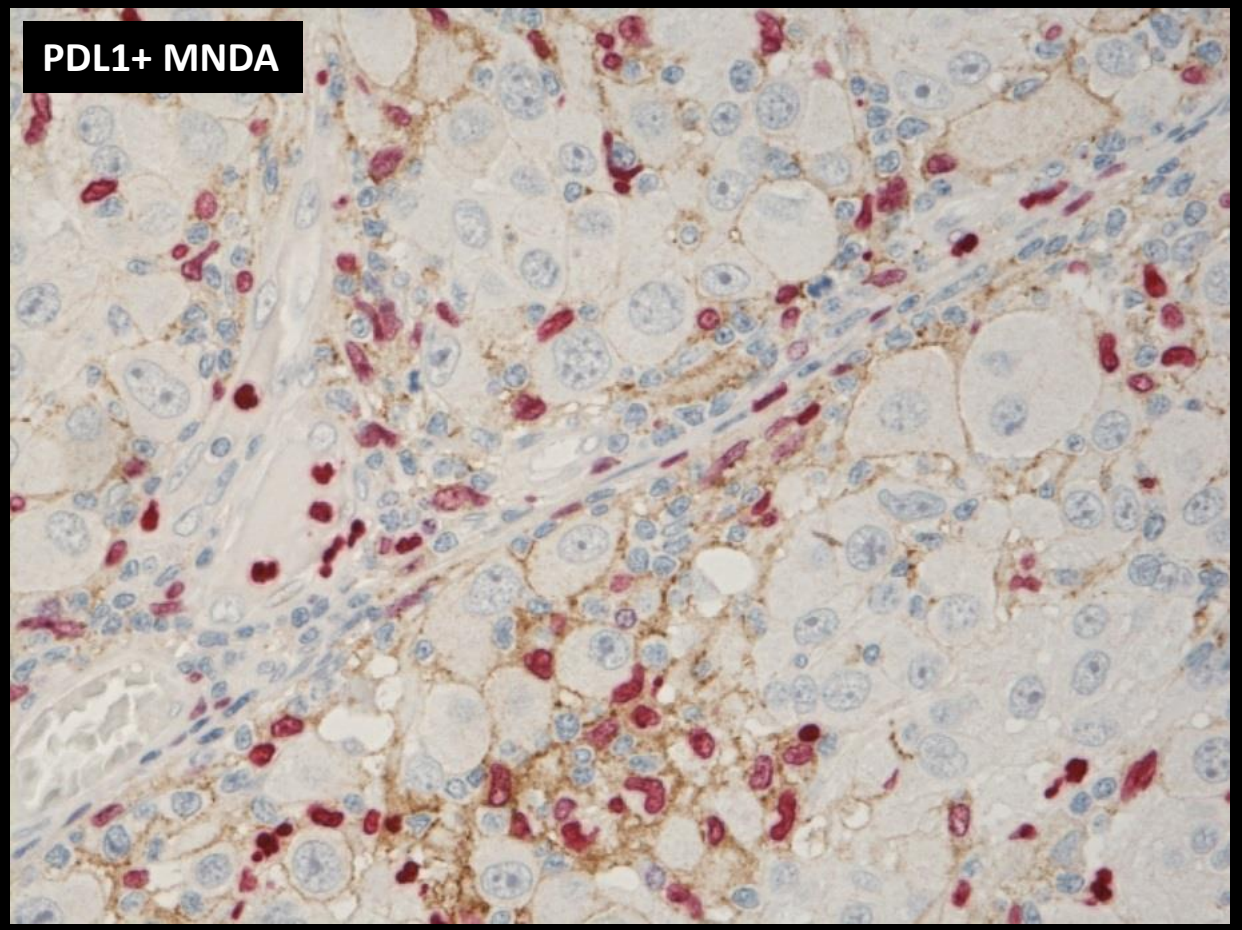


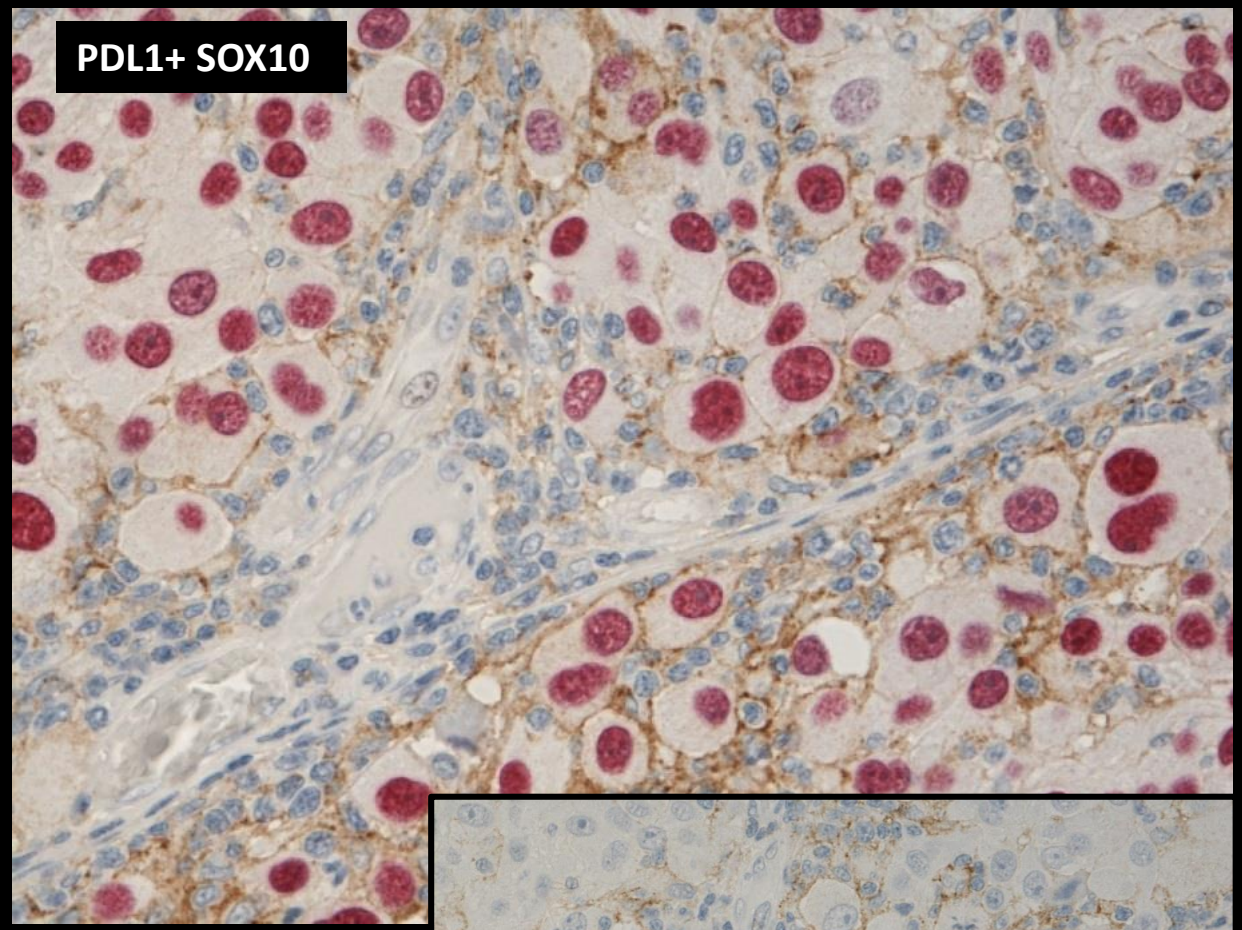
Lung adenocarcinoma

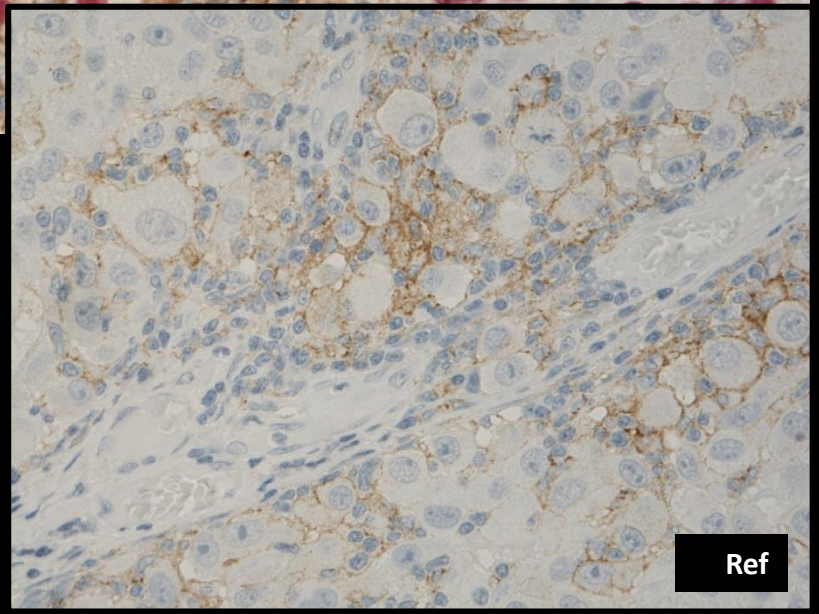
Omnis: HIER Low pH 40`

PD-L1, 22C3 (1:20) /Flex+ 40-10-40

MNDA, 253A (1:80) or TTF1, SPT24 (1:200) /Histo-AP 30-10-20







Melanoma

<u>Omnis: HIER Low pH 40`</u>

PD-L1, 22C3 (1:20) /Flex+ 40-10-40

MNDA, 253A (1:80) or SOX10, BS7 (1:200) /Histo-AP 30-10-20

Triple staining using sequential technique (Immuno-enzymatic)

LAB Næstved

Sequential procedure (Immuno-emzymatic):

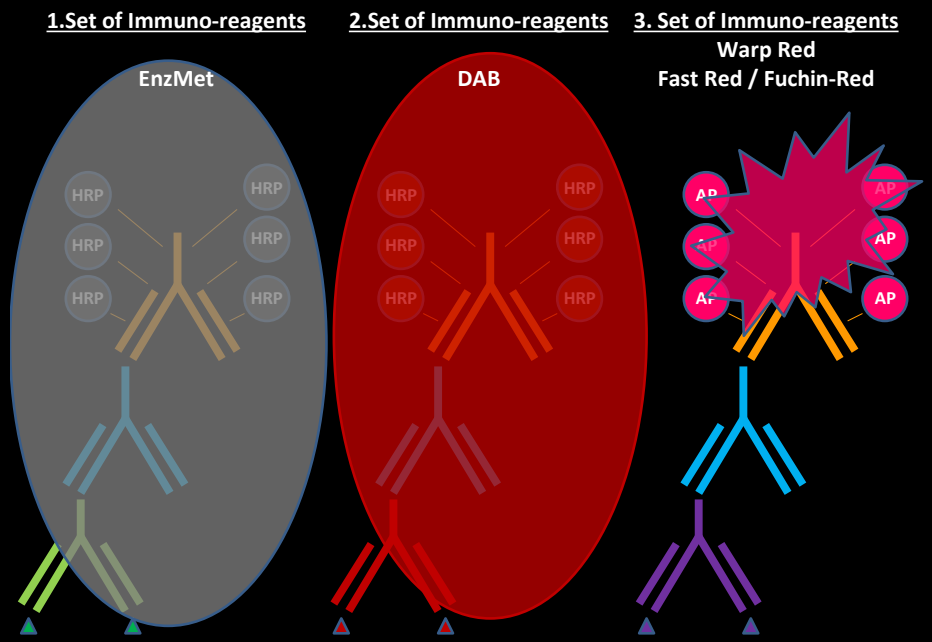
Pre-treatment (Antigen Retrieval)

First primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Quanto/HRP (10+10 min).
Visualization with EnZMet (5 min).

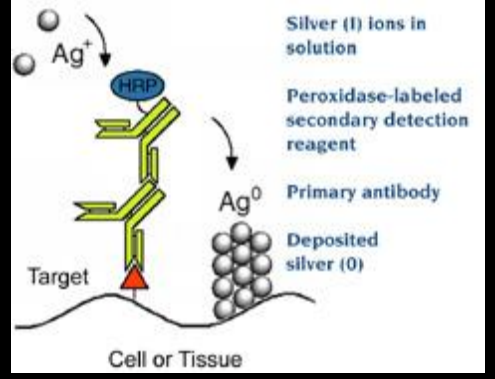
Second primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Quanto/HRP (10+10 min).
Visualization with DAB (7min).

Third primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Hi-Def /AP (10+10 min).
Visualization with Warp Red (Fuchin-Red) (7min).

Counterstain, dehydration and mounting.



EnzMet (silver) and DAB deposit (after 1. and 2. set of immuno-reagens) shelter / shields for un-specific reaction of the following primary antibodies and/or detection reagents.

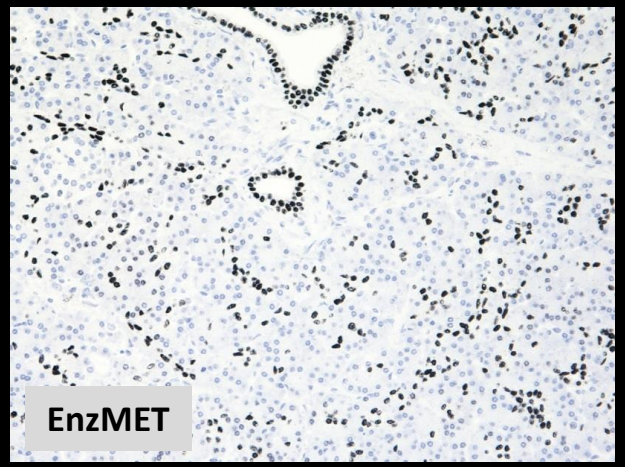


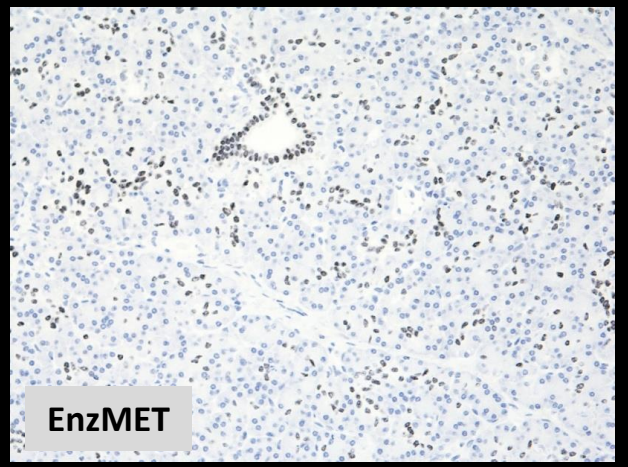
EnzMET versus DAB

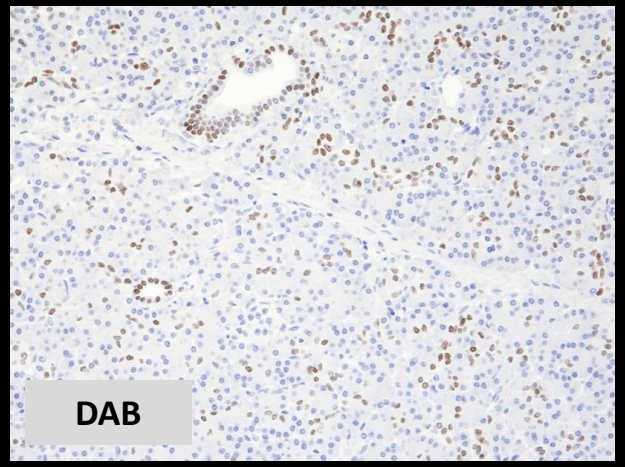
CDX-2, DAK-CDX2

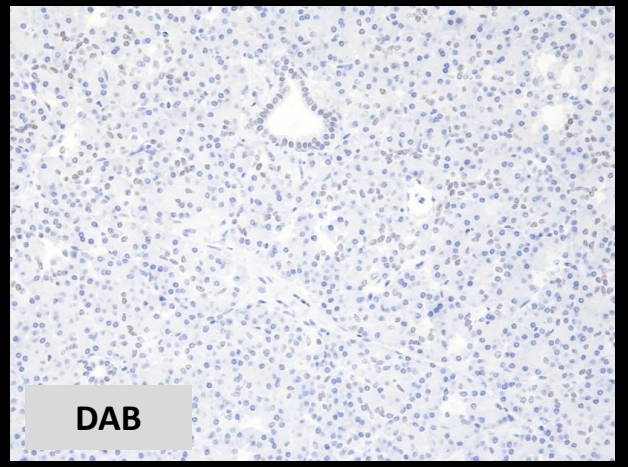
Pancreas

1:25 **1:100**



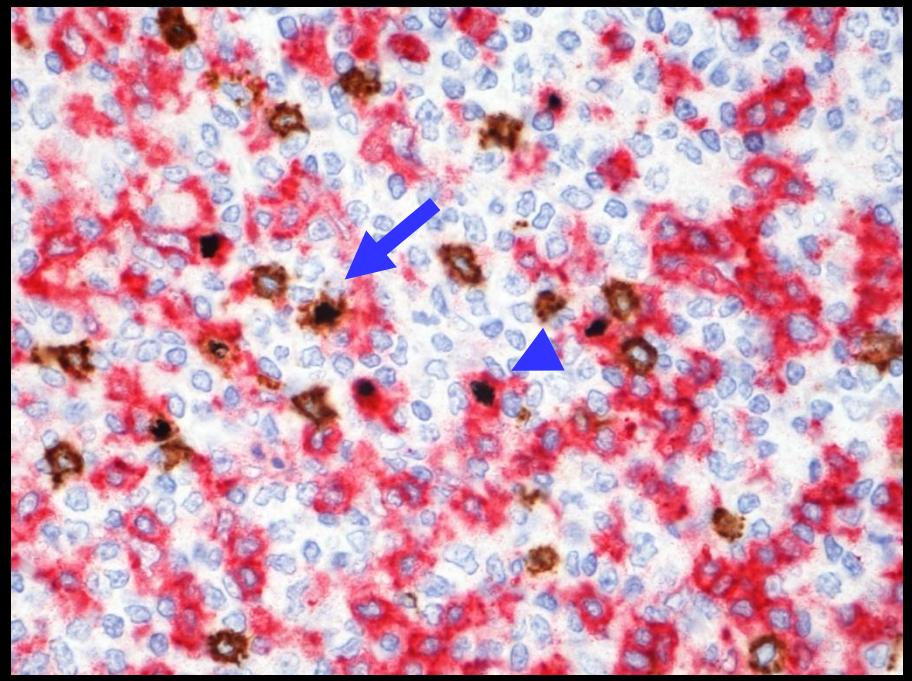


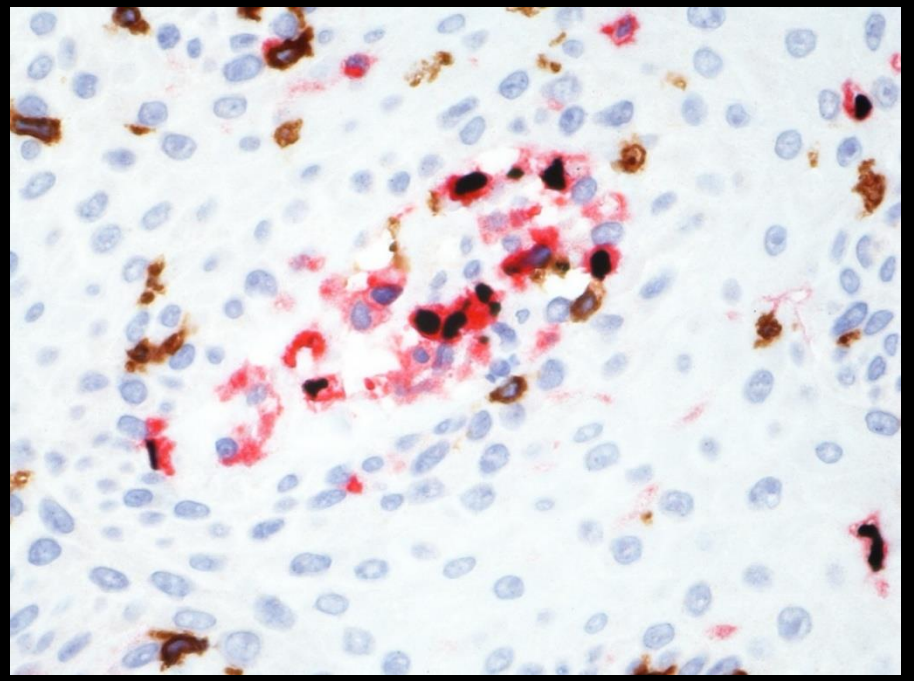




Triple staining using sequential technique (Immuno-enzymatic)

FoxP3 (236/E7) + CD8 (C8/144B) + CD4 (EPR6855) (Mab+Mab+Rab)





Tonsil

FoxP3 (black nuclear staining)

CD8 (brown membraneous/cytoplasmic staining, arrow)

CD4 (red membraneous/cytoplasmic staining, arrow-head)

Skin

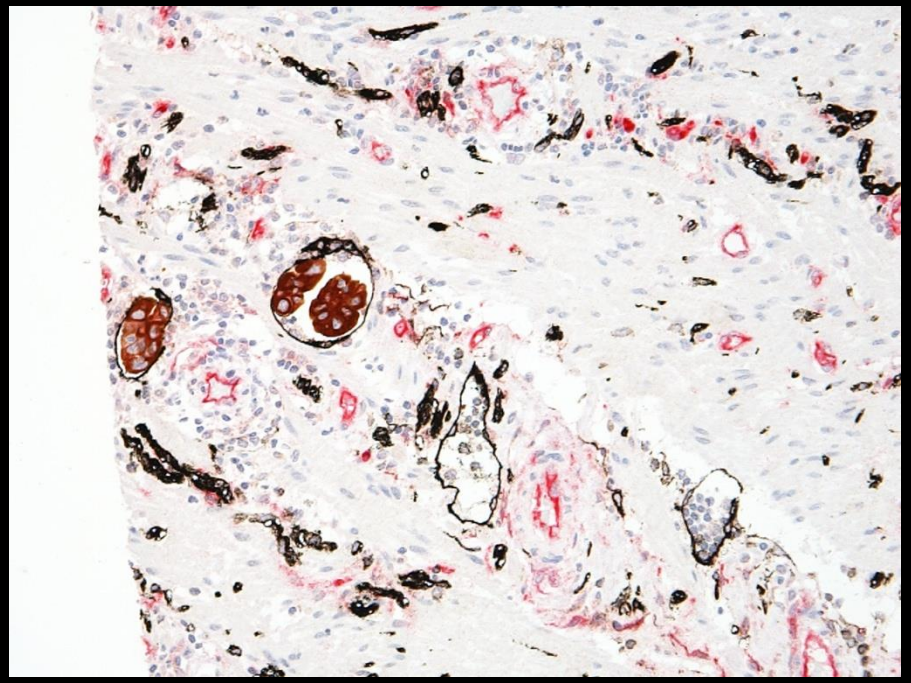
FoxP3 (black nuclear staining)

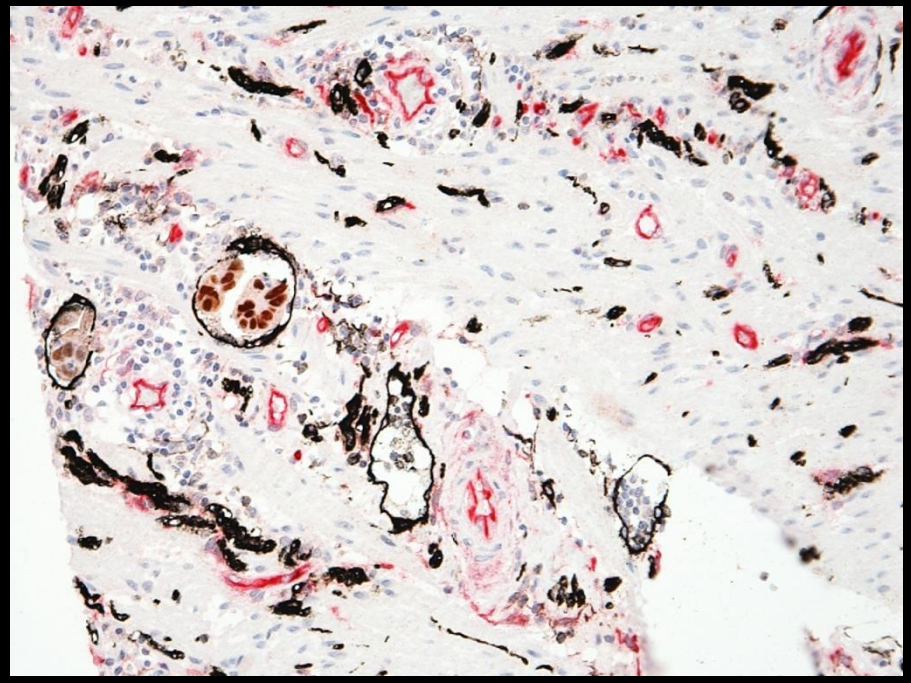
CD8 (brown membraneous/cytoplasmic staining, arrow)

CD4 (red membraneous/cytoplasmic staining, arrow-head)

Triple staining using sequential technique (Immuno-enzymatic)

Podoplanin (D2-40) + CDX-2(DAK-CDX2) or CK20 (K_s20.8) + CD34 (QBEND10) / (Mab x3)





Adenocarcinoma colon:

D2-40 (black lymph-endotheliale staining)

CK20 (brown cytoplasmic staining of the tumor cells)

CD34 (red membraneous endotheliale staining)

Adenocarcinoma colon:

D2-40 (black lymph-endotheliale staining)

Cdx-2 (brown nuclear staining of the tumor cells)

CD34 (red membraneous endotheliale staining)

Simultaneous procedure (Immuno-Fluorescence):

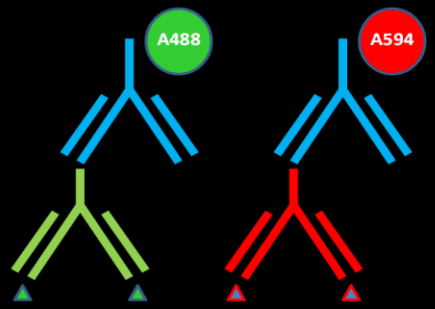
Pre-treatment (Antigen Retrieval)

Incubation with Primary Ab mix (e.g different host/ Mab+Rab) (1h).

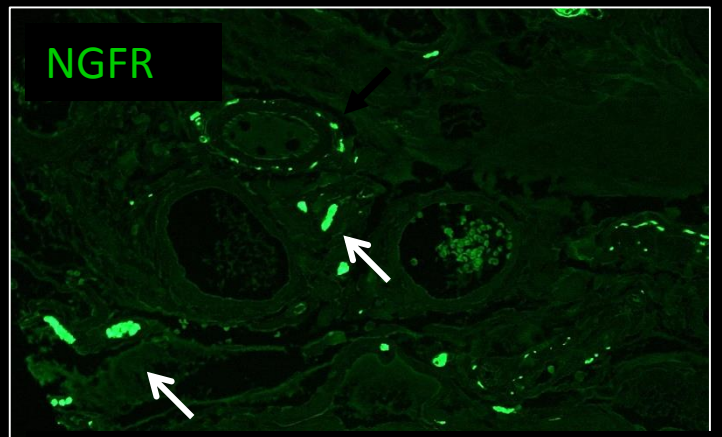
Incubation with Secondary Ab mix (Goat -anti Mab+Rab) (1h).

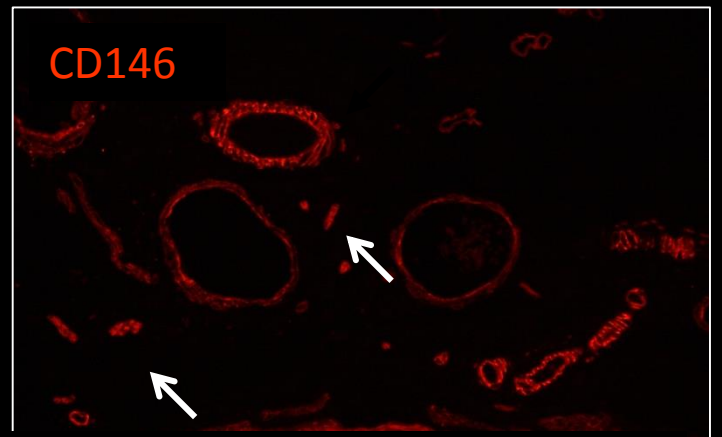
Alexa Fluor 488 anti mouse Ig + Alexa Fluor 594 anti rabbit Ig

Dehydration + air-drying + coverslipping with Vectashield (+ DAPI)



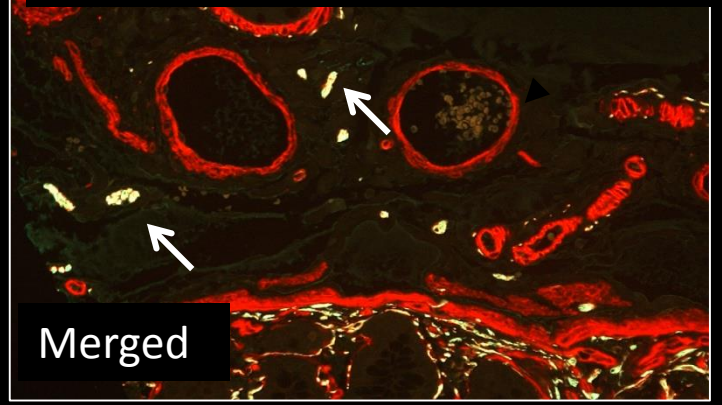
NGFR (Mab, MRQ-21) + CD146 (Rab, EPR3208)

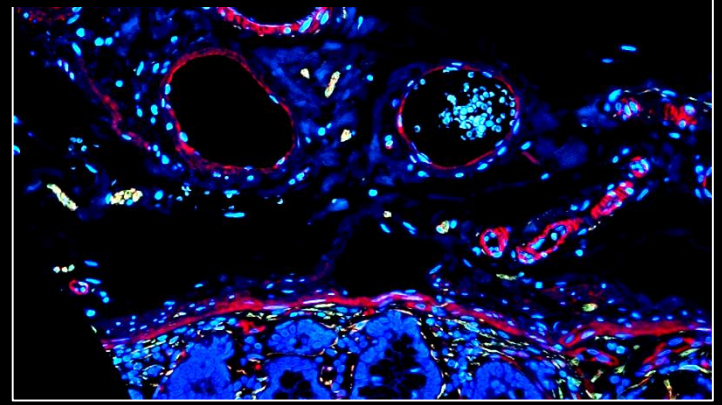




Colon: Co-localization of NGFR and CD146 in peripheral nerves and ganglion cells (yellow reaction product) (arrows).

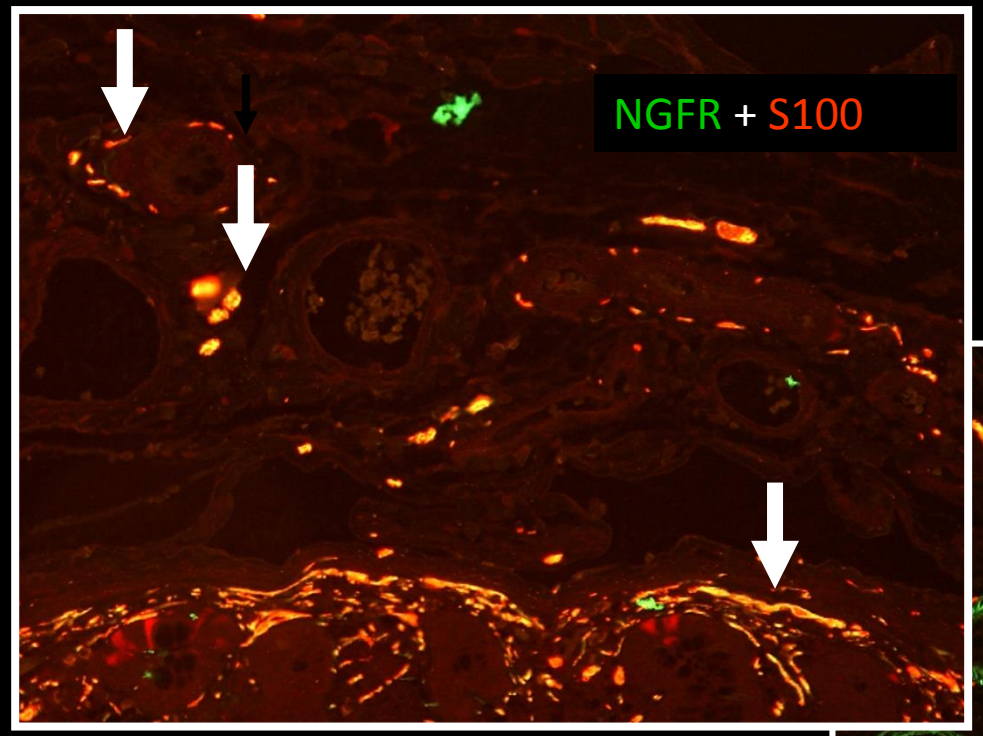
CD146 stains smooth muscles of peri-vascular structures lamina muscularis mucosa .



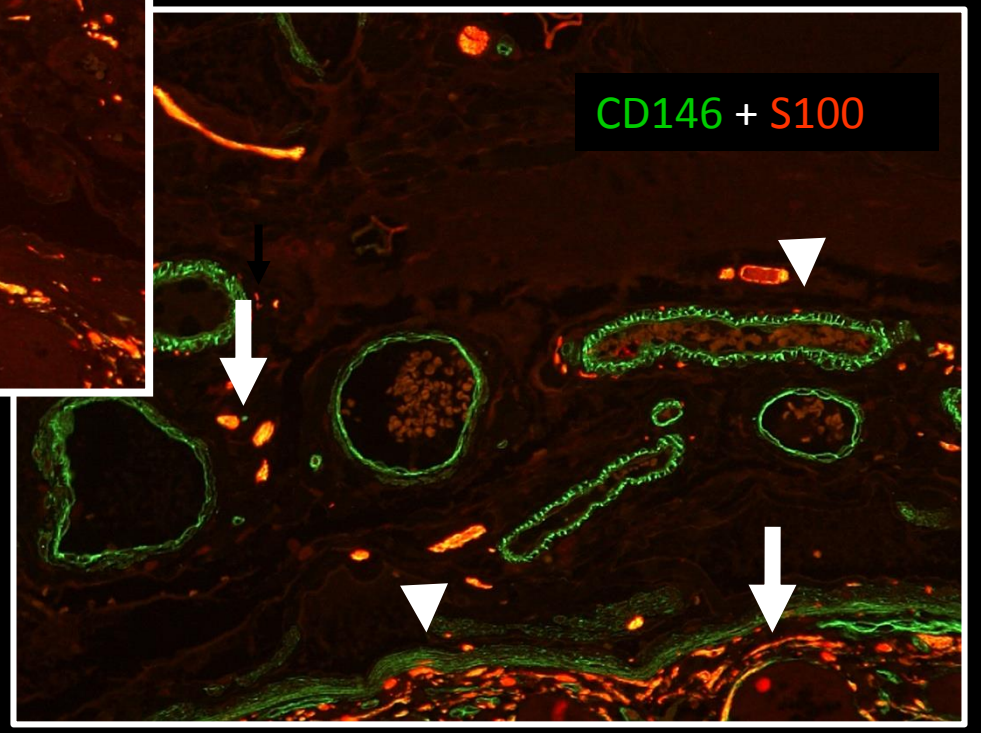


S100(Rab, poly) + CD146 (Mab, N1238) / NGFR (Mab, MRQ-21)

Bowell



Note: Co-localization of NGFR and S100 in peripheral nerves and ganglion cells (arrows) (yellow colour) .



Note: Co-localization of CD146 and S100 in peripheral nerves and ganglion cells (arrows).

Also, CD146 stains peri-vascular structures and smooth muscles of lamina muscularis mucosa (arrowheads).

Double immuno-fluorescence staining using simultaneous technique

LAB Næstved

Simultaneous procedure using TSA amplification:

Exactly the same method as the "normal" double immufluorescence procedure except for introducing HRP in the secondary Ab mix and a final amplification step using Alexa Fluor 488 conj. Tyramide reagent.

Mechanism of Tyramide amplification:

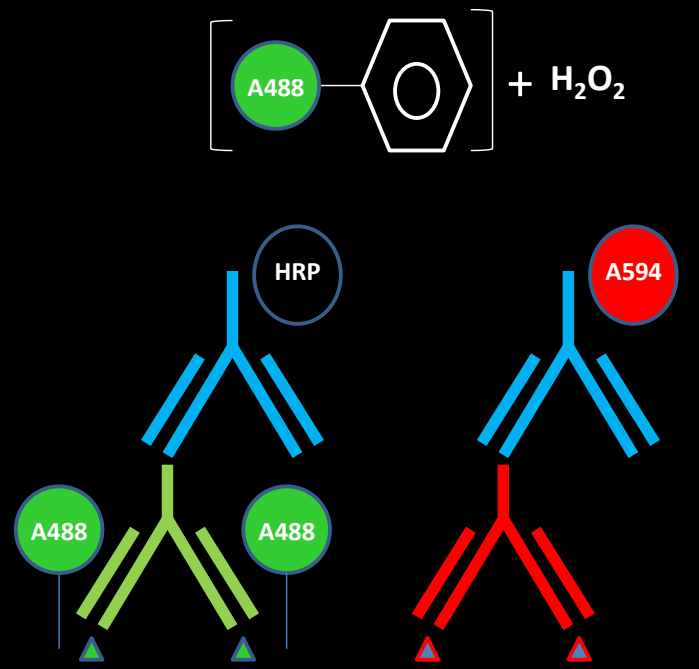
-Introducing HRP
-Incubation with A488 conjugated Tyramide and H_2O_2

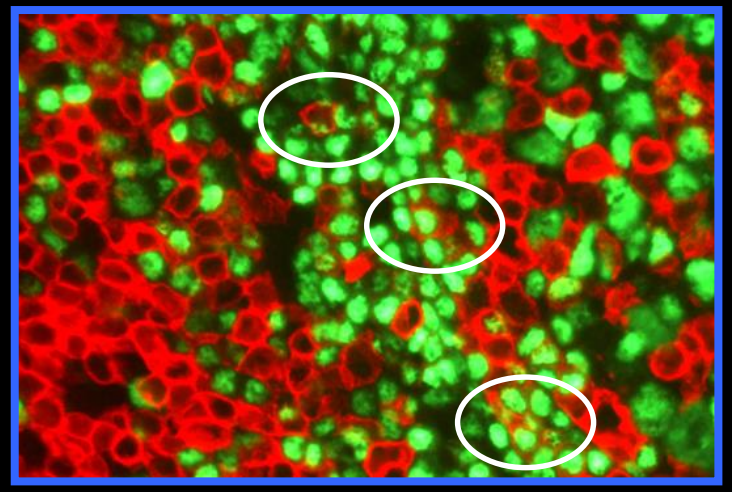
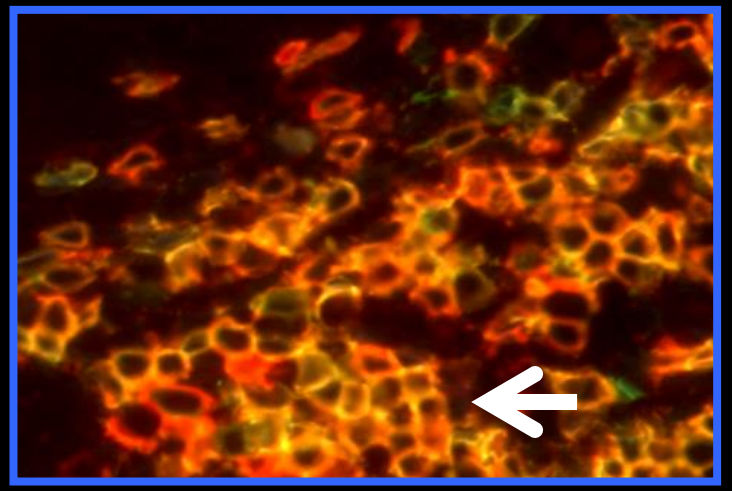
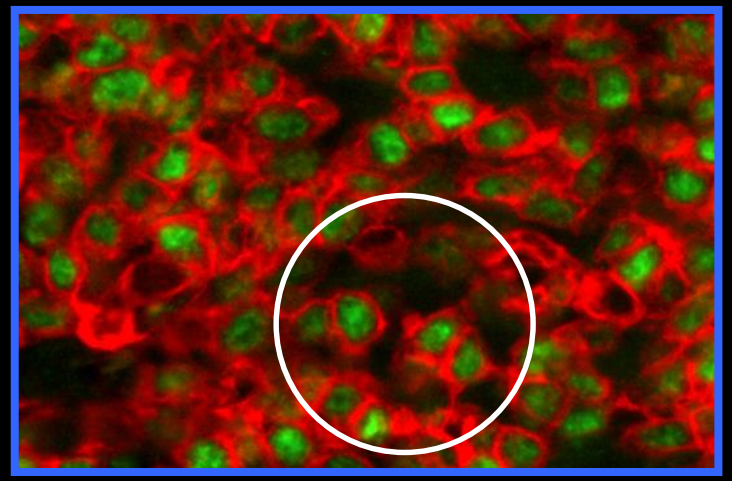
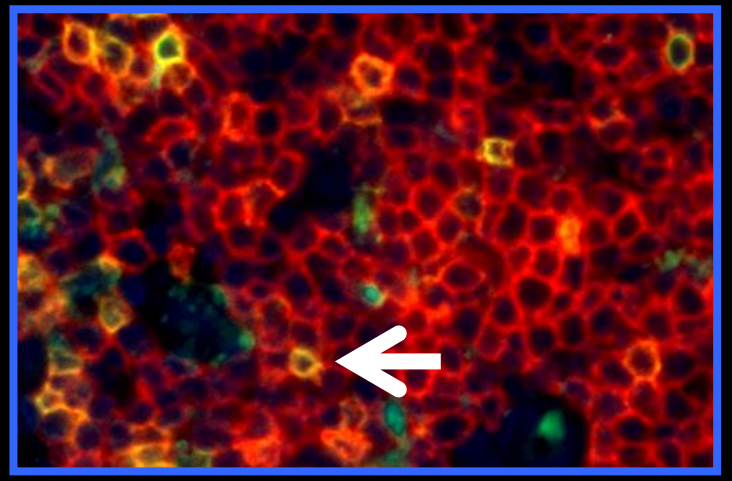
Tyramide , phenolic compound, converted into an short-lived extremely reactive intermediate

Intermediates covalently binds to electron rich regions of adjacent proteins (esp. tyrosine) – rapidly

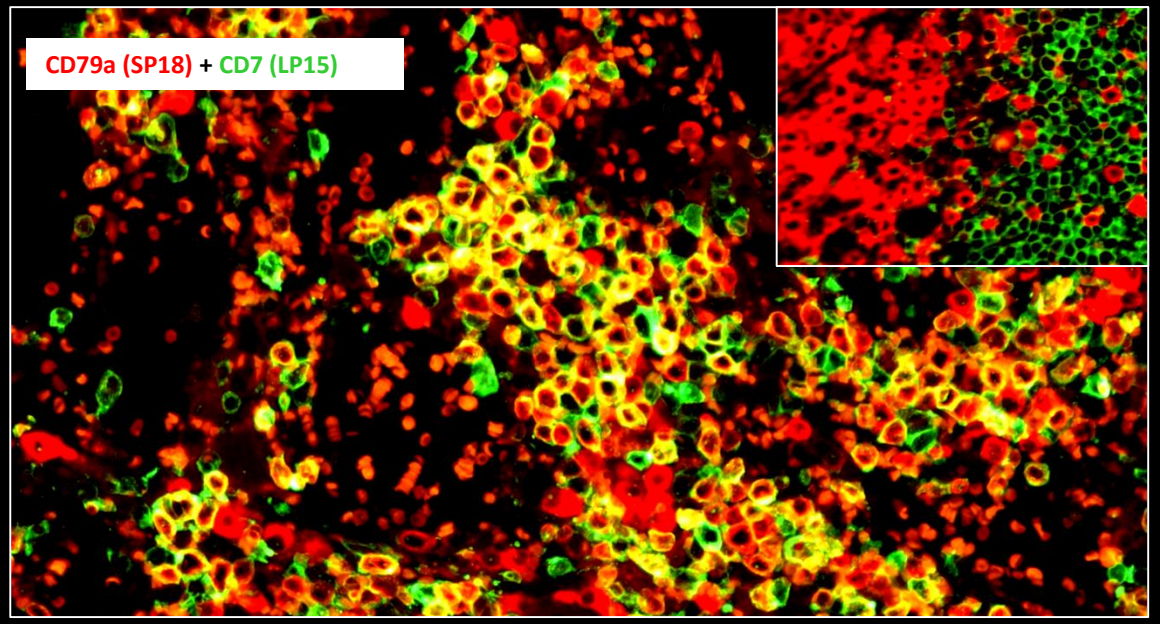
- Deposit of A488 in close vicinity of Ab/Ag reactions

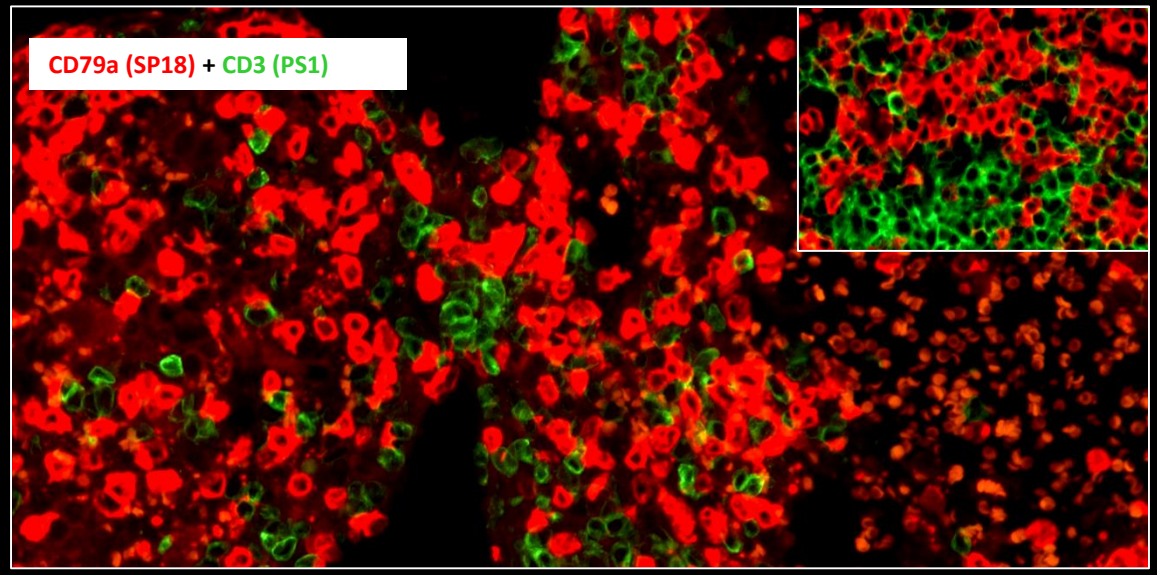
-Visualization of A488 deposit under fluorescence microscopy



	PAX-5, 1EW (TSA amp.) + CD5, SP19	CD3, PS1 + CD5, SP19
Tonsil		
B-CLL		

Note : Co-localization of normal T-cells in the tonsil and in the B-CLL (CD3+CD5) and co-expression of the neoplastic B-cell (Pax-5 and CD5) in the B-CLL and normal B-Cells in the mantle zone of the tonsil





PT : ALL (B-type)

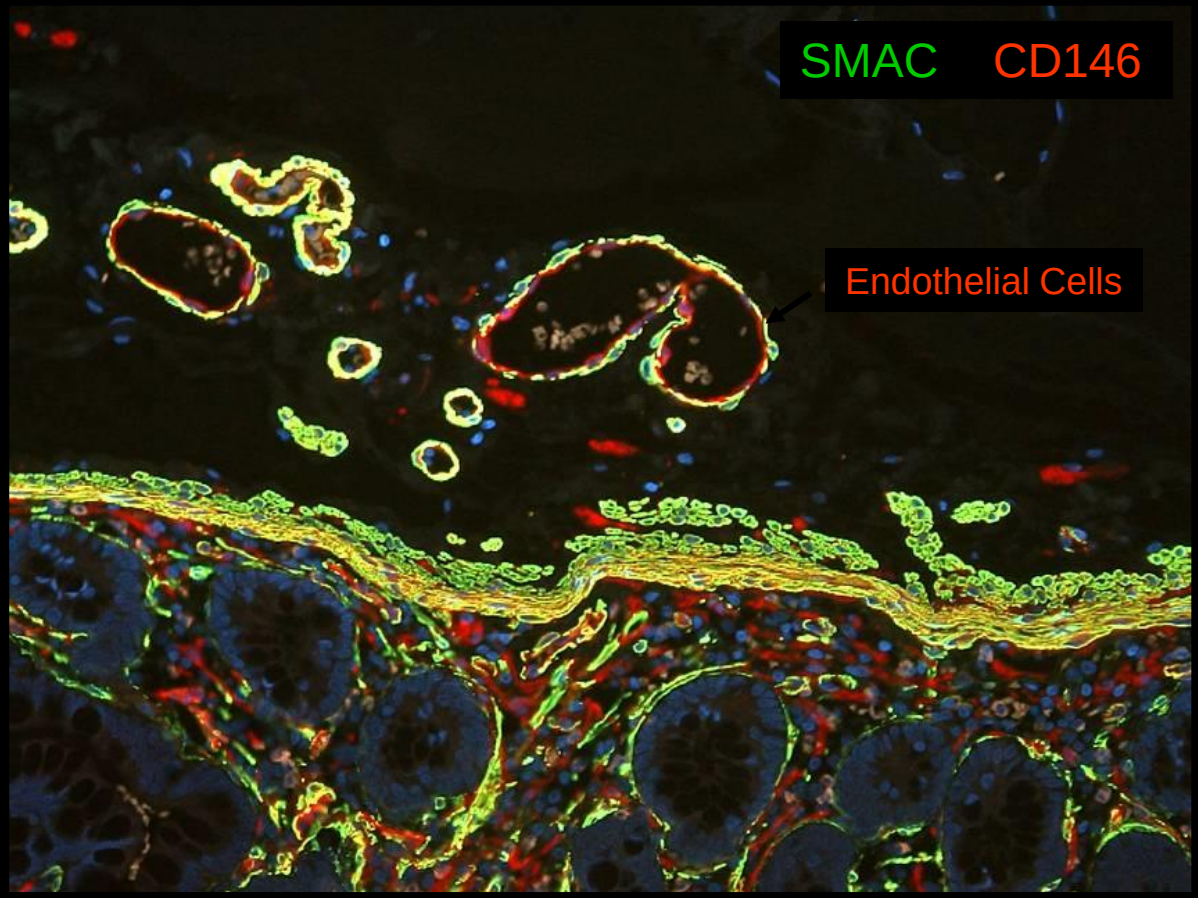
Flowcytometry showed aberrant expression of CD7 in neoplastic B-cells

Note: Aberrant co-localization between CD79a (B-cell marker) and CD7 (T-cell marker) of the neoplastic B-cells in the ALL

The reaction pattern between CD79 and CD3 showed expression of the respective markers in different cell types (neoplastic B-cells and T-cells).

Tonsil (insert`s)

Normal expression of the B-cell marker (CD79a) and the T-cell markers (CD7 or CD3). No co-localized signals



Note: Co-expression of CD146 and SM-actin (SMAC) in peri-vascular structures.

Note: Cells of Nervous system - Red

Some final remarks to double immunofluorescence labeling using simultaneous technique:

Control of cross-reactivity between secondary Abs and primary Abs:

Test on tissue specimens in which two antigens is expressed in different cells and easy to differentiate (e.g. SMA and CD68)

Incubate with primary Ab-X followed by detection with secondary antibody anti-Y
= Negative

Incubate with primary Ab-Y followed by detection with secondary antibody anti-X
= Negative

Omission of primary Ab-X followed by detection with secondary antibody cocktails (anti-X and anti-Y)
= Only antigen Y should display a positive signal without any signs of co-localized signal

Omission of primary Ab-Y followed by detection with secondary antibody cocktails (anti-X and anti-Y)
= Only antigen X should display a positive signal without any signs of co-localized signal

Drawbacks of immunofluorescence techniques:

Fading of fluorescence signal upon storage

Quenching of fluorescence signal at excitation (fluorescence microscopy)

Auto fluorescence cause by formaldehyde fixation (especially connective tissue – collagen fibers)

PD-L1

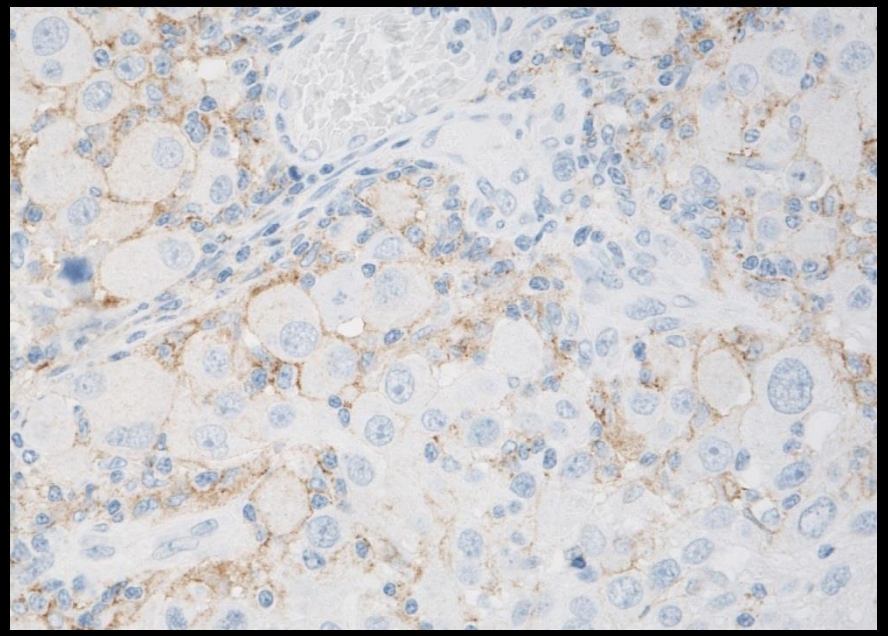
Omnis: HIER Low pH 40` ; PD-L1, 22C3 (1:20) /Flex+ 40-10-40

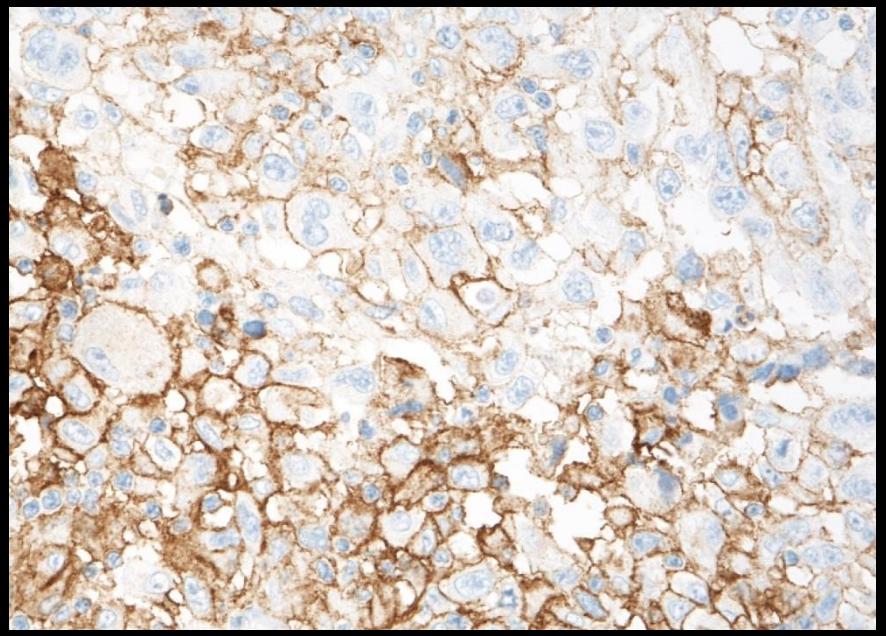
Removal of coverslip, rehydration and staining of SOX10

PD-L1 + SOX10

Omnis: HIER low pH 10`; SOX10 (BS7, 1:200 BS) 30`; Histo-AP/PR

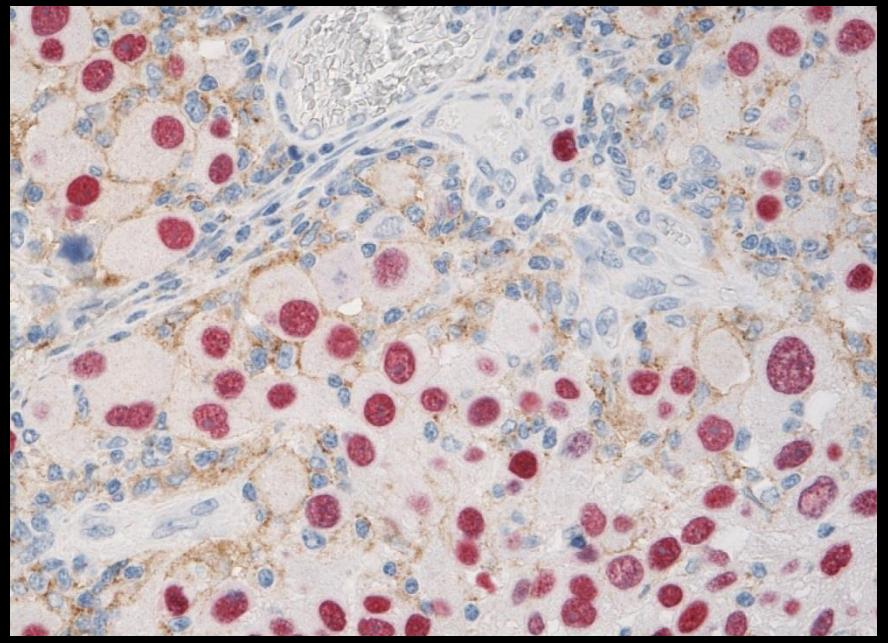
In principal, Flex+-HRP can be used

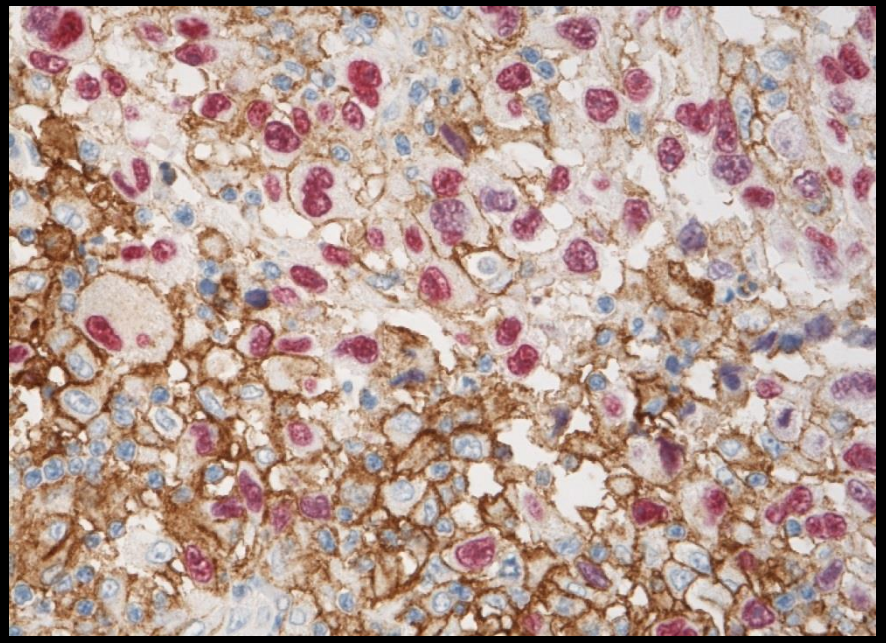




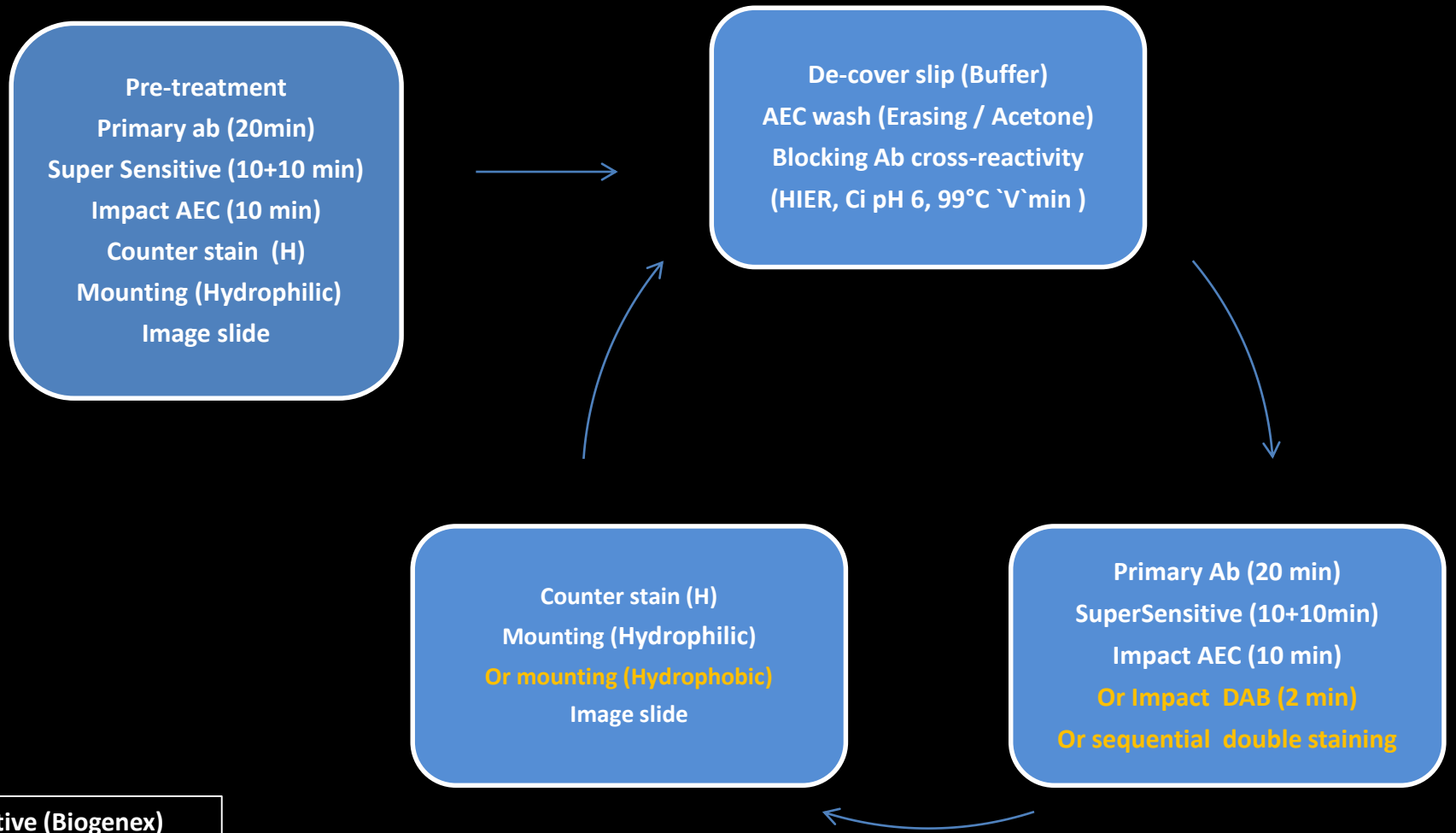
Melanoma (case 1)

Melanoma (case 2)





Simple technique (modified)



Super Sensitive (Biogenex)
Impact AEC (Vector Lab)