Workshop in Diagnostic Immunohistochemistry
NordiQC (October 2024)

Immunohistochemical multiplex techniques

Overview, considerations and applications

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Multiplex staining techniques (IHC)

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

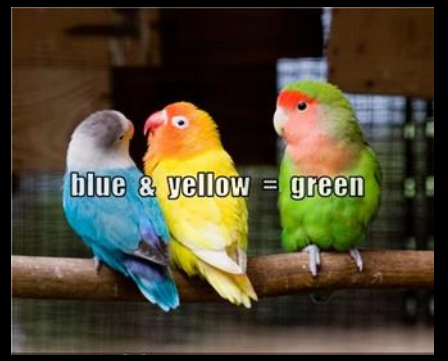
Compared to single color IHC, the main advantage of multiplex IHC is two-fold:

First, it allows for the analysis of multiple parameters simultaneously on a single slide and significantly decreases the amount of tissue required for assessment.

Second, perhaps more importantly, investigating the spatial relationship between multiple cells in cancerous tissue

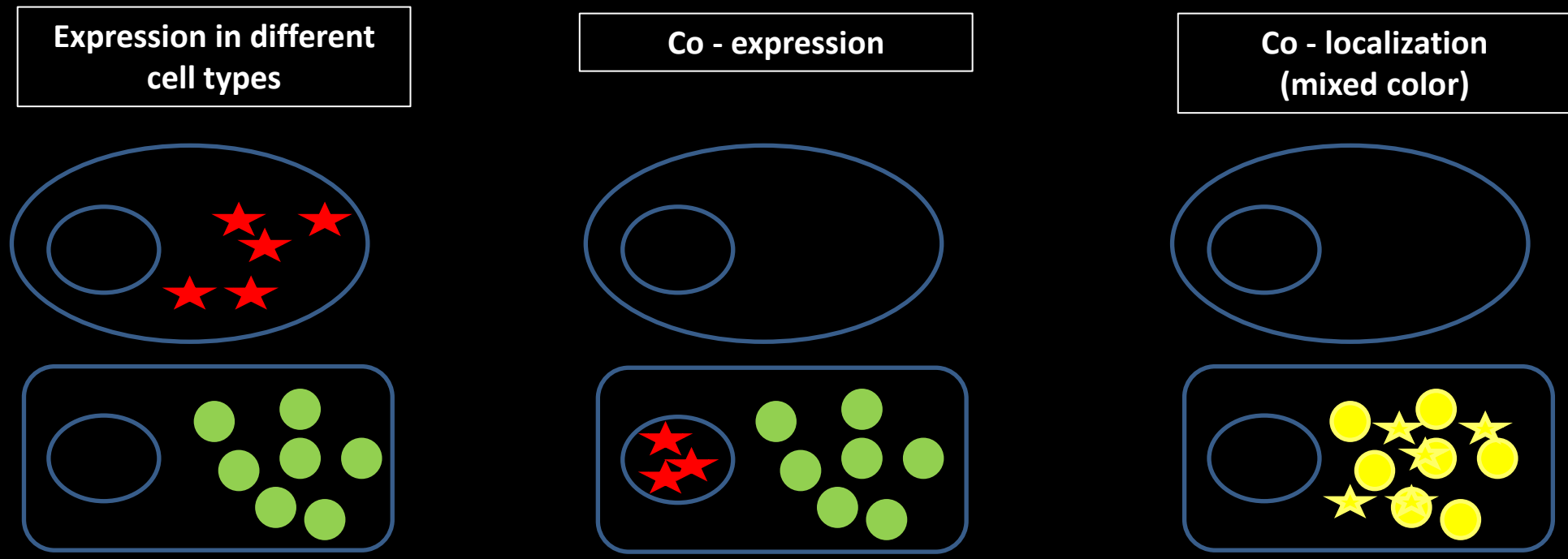
e.g., understanding of the complexity of the tumor microenvironment has led to advances in therapies for patients with cancer (Immunotherapy).

Multiplex staining techniques (IHC)

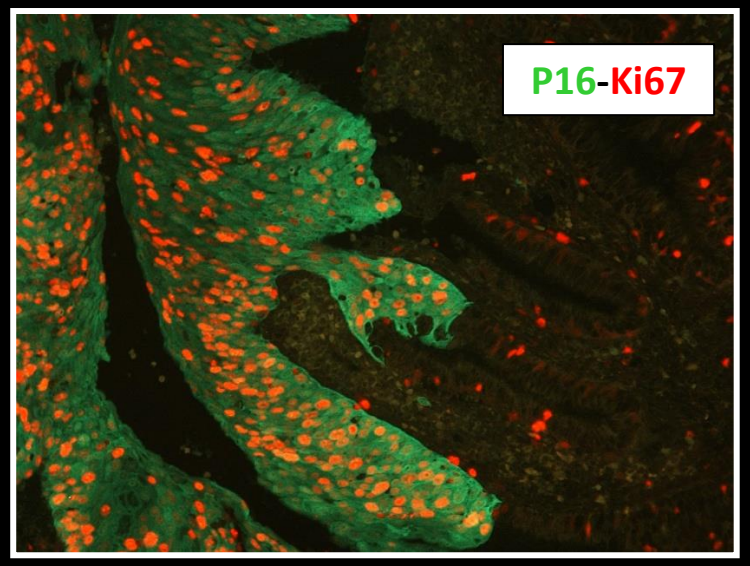


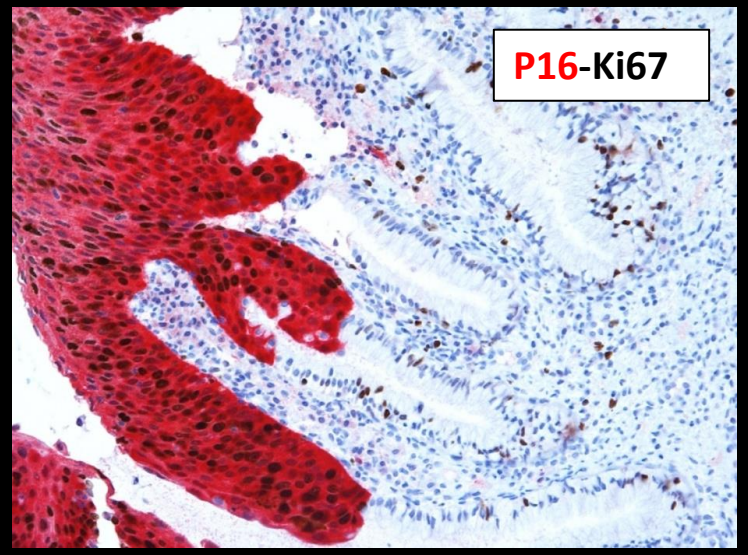
Requirements ("double/multiplex-staining techniques") :

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



Cervix / dysplasia





Multiplex techniques (IHC)

Basic procedures:

- ❑ Cocktails single staining technique (e.g., PAN-CK, AE1/AE3; PIN)
- ❑ Sequential double-staining technique
- ❑ Simultaneous double-staining technique

Advanced procedures

- ❑ Sequential & erasing staining technique (SIMPLE)
- ❑ Multiplex staining technique (Chromogenic and Fluorescent)

Immuno-enzymatic techniques (chromogenic)

Immuno-fluorescence techniques

Challenges or considerations performing multiplex staining techniques:

Which staining technique (double/multiplex) should I use ?

Pre-treatment - do the antigens of interest require the same antigen 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 commercially 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

- iCAPCs - compare individual staining results of the multi labelling technique with <u>optimal</u> single staining procedure (routine)

Multiplex staining techniques (IHC)

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

NO

Immuno-enzymatic DAB based sequential or simultaneous technique

YES

Double/multiplex immunofluorescence technique (simultaneous technique)

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

A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method)

Sequential procedure (Immuno-enzymatic/HRP+AP):

Pre-treatment (Antigen Retrieval)

First primary Ab
Detection with Quanto/Flex+/HRP
Visualization with DAB or Deep Space Black (DAB based)

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.

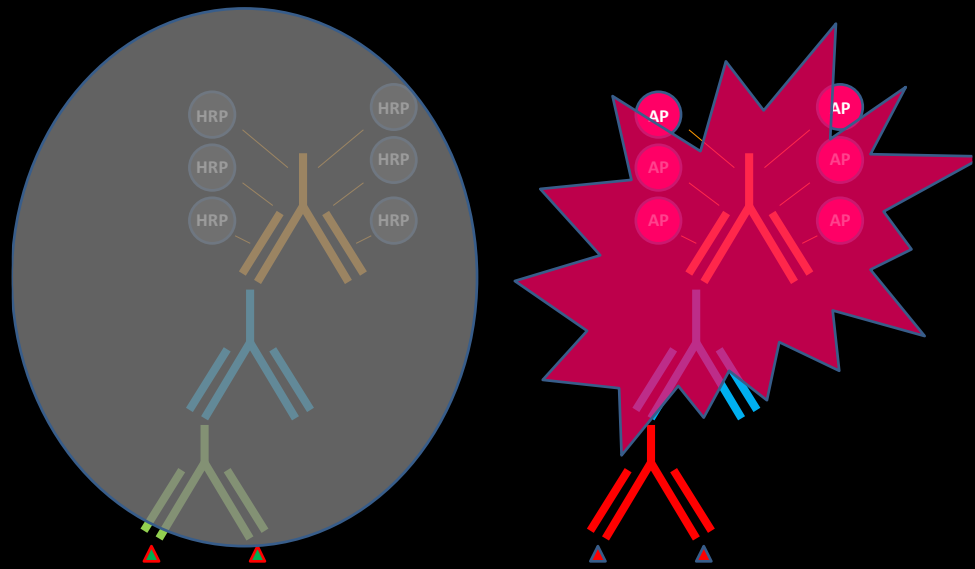
DAB reaction product of the first set of immunoreagents create a barrier that is impermeable for the second set of immunoreagents ~ no cross reactivity

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

Working up a double-immune staining protocol for routine purpose:

- 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 iCAPCs

- 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

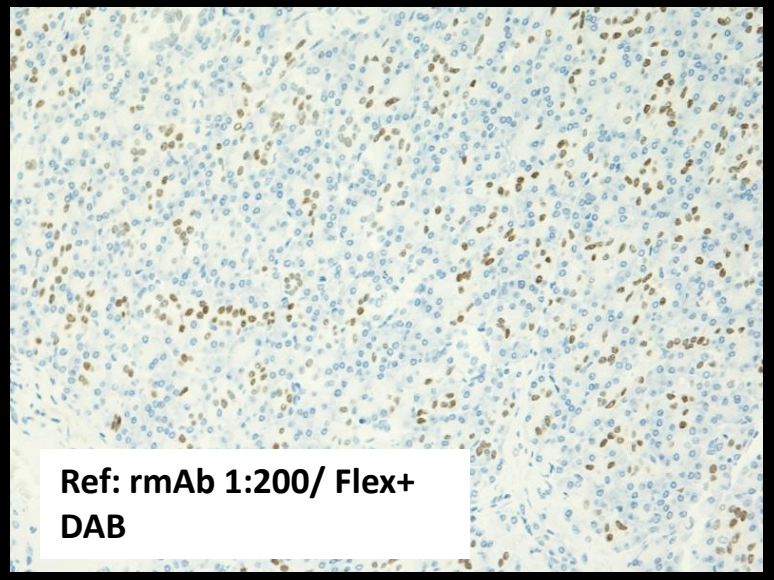
→ Optimized double staining protocol

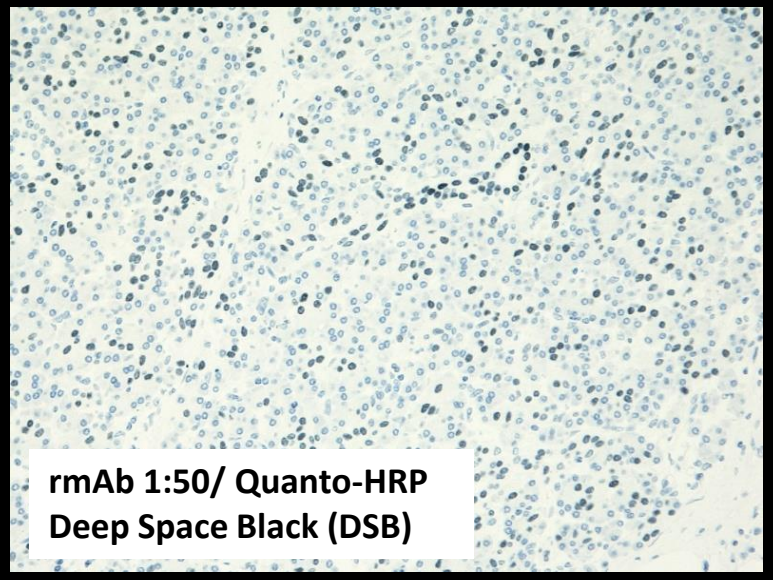
End-result including controls: Optimizing the protocol

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

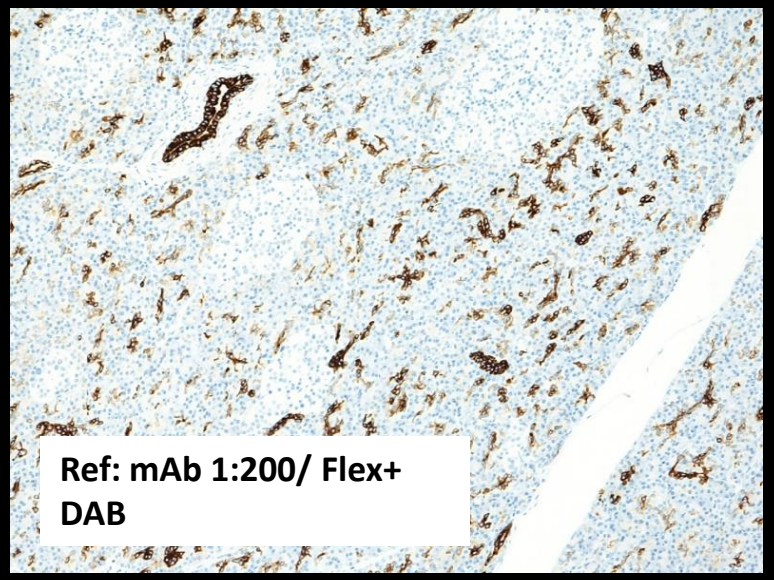
Pancreas

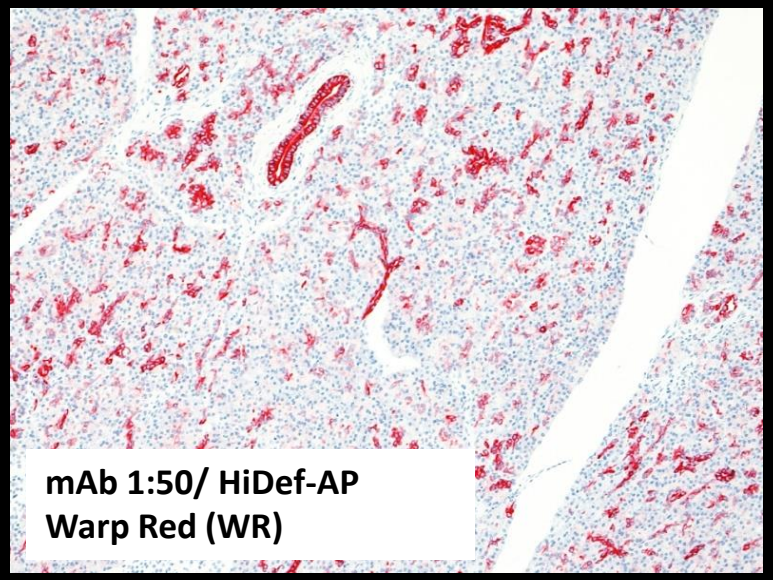
CDX2, EP25 (first seq.)





CK7, OV-TL12/30 (second seq.)

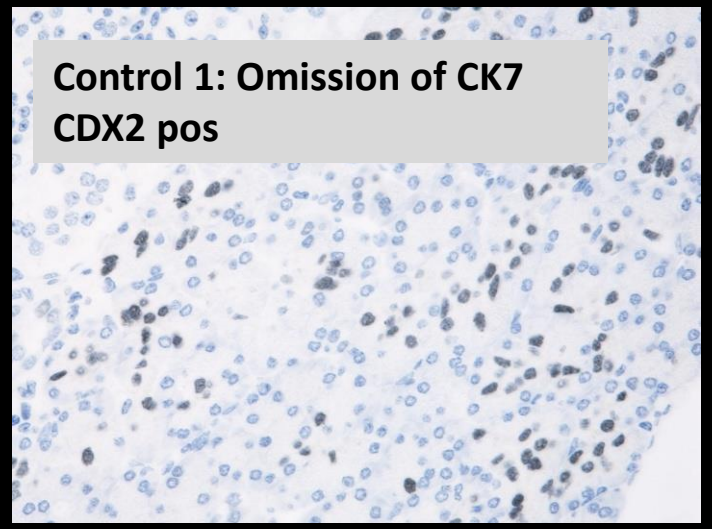


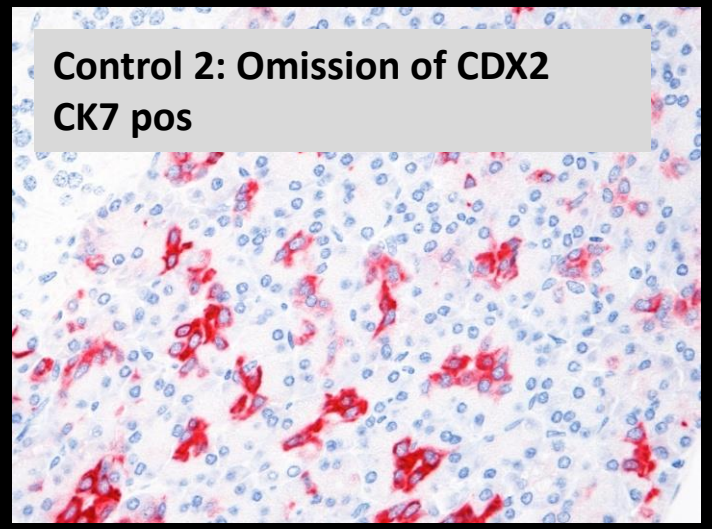


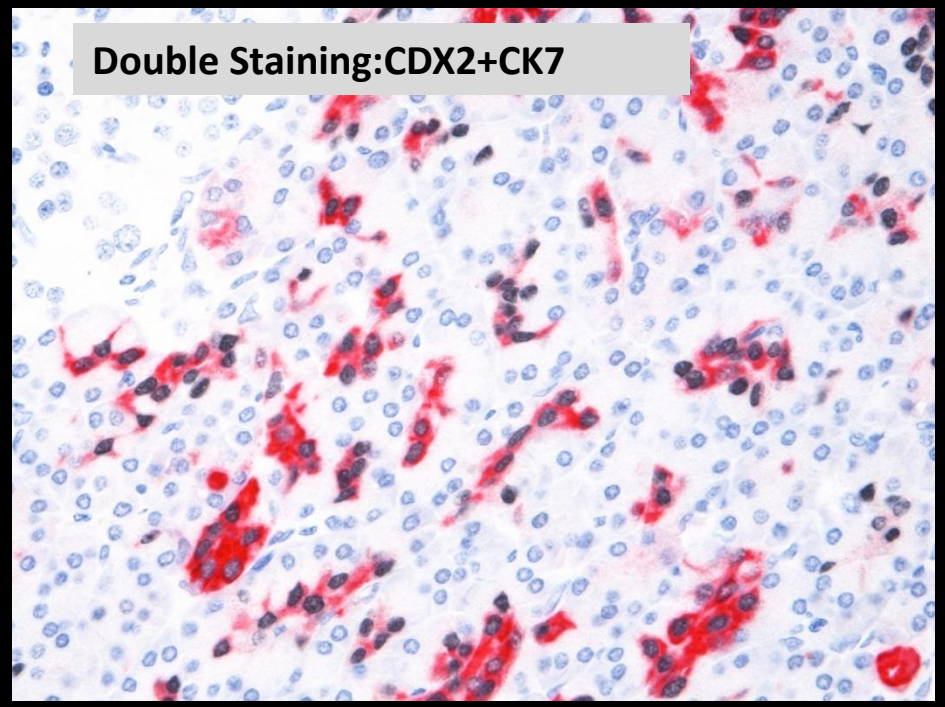
Typical end-result including controls: Optimizing the protocol

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







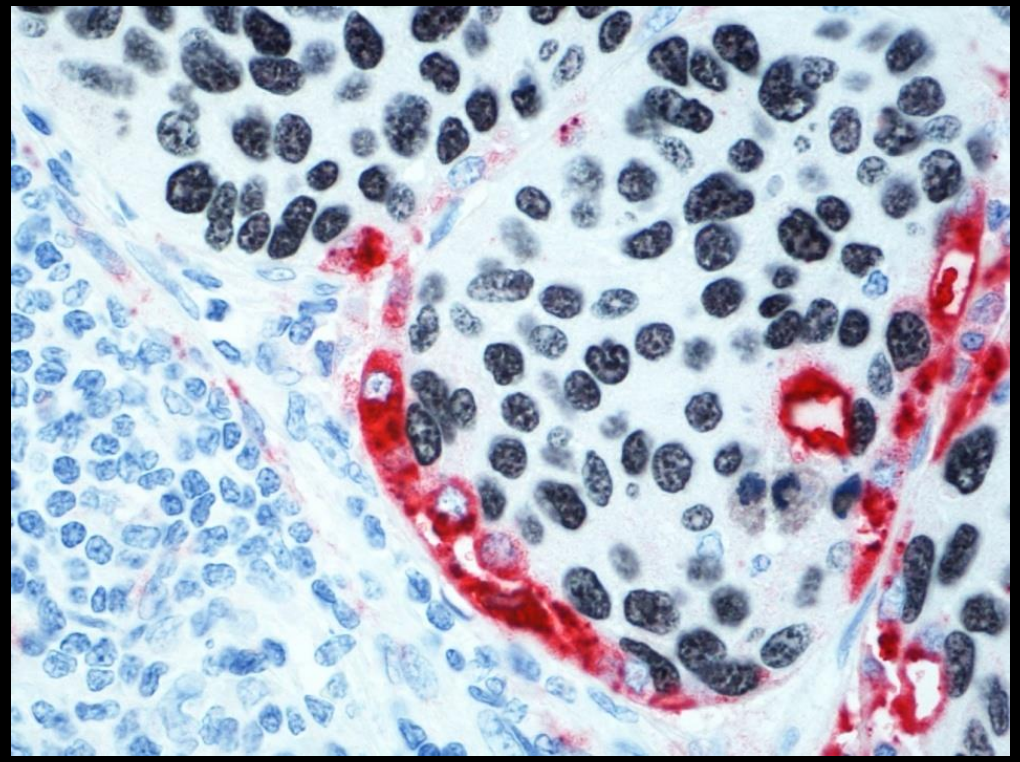
Pancreas

Note : Efficient sheltering capacity of Deep Space Black

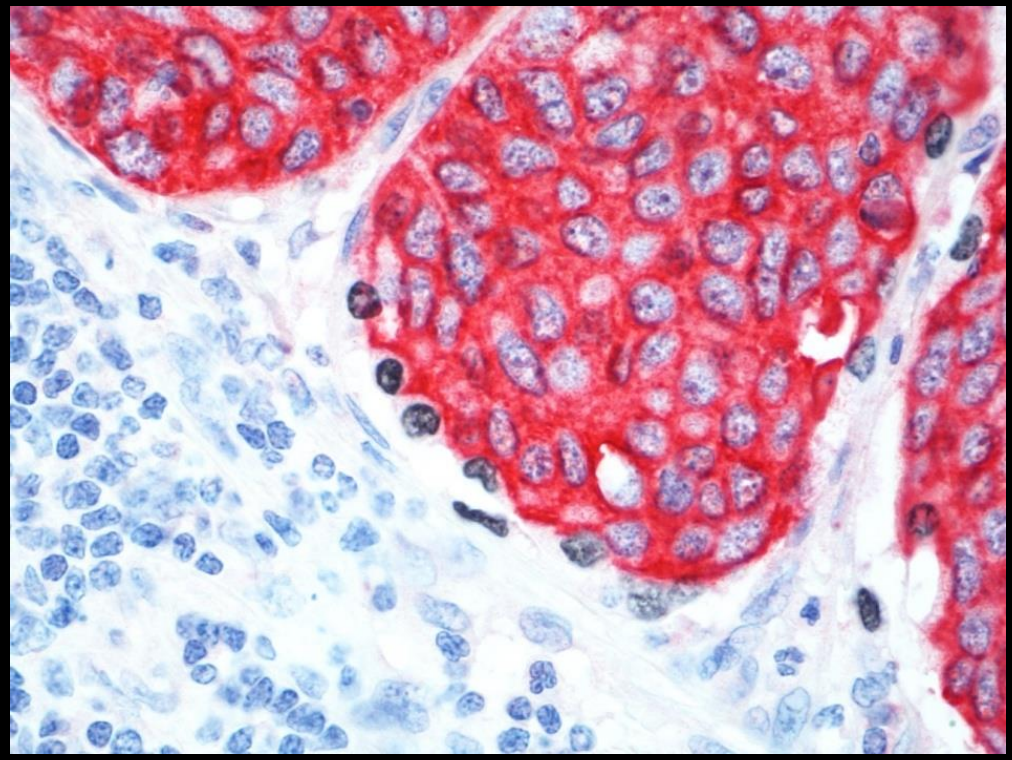
No mixed colors or double colors within controls and in the double staining for CDX2+CK7 (Intercalating ducts of the pancreas are positive for both markers)

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)

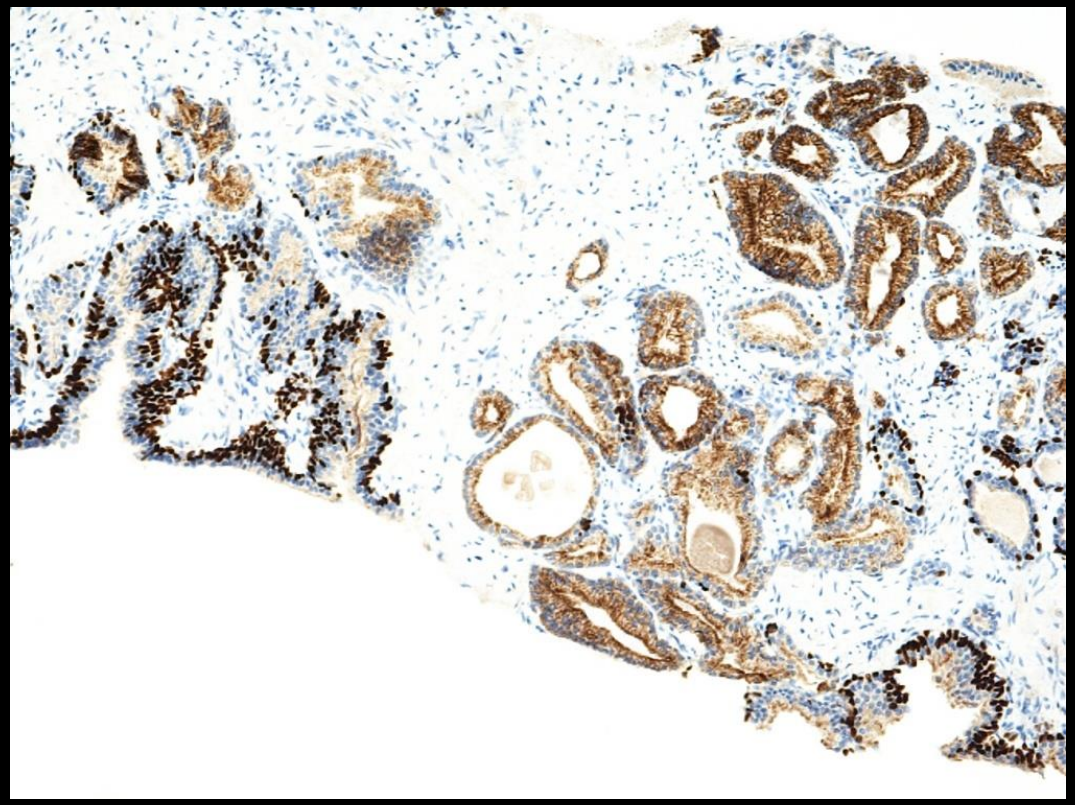


Lung : Squamous cell carcinoma

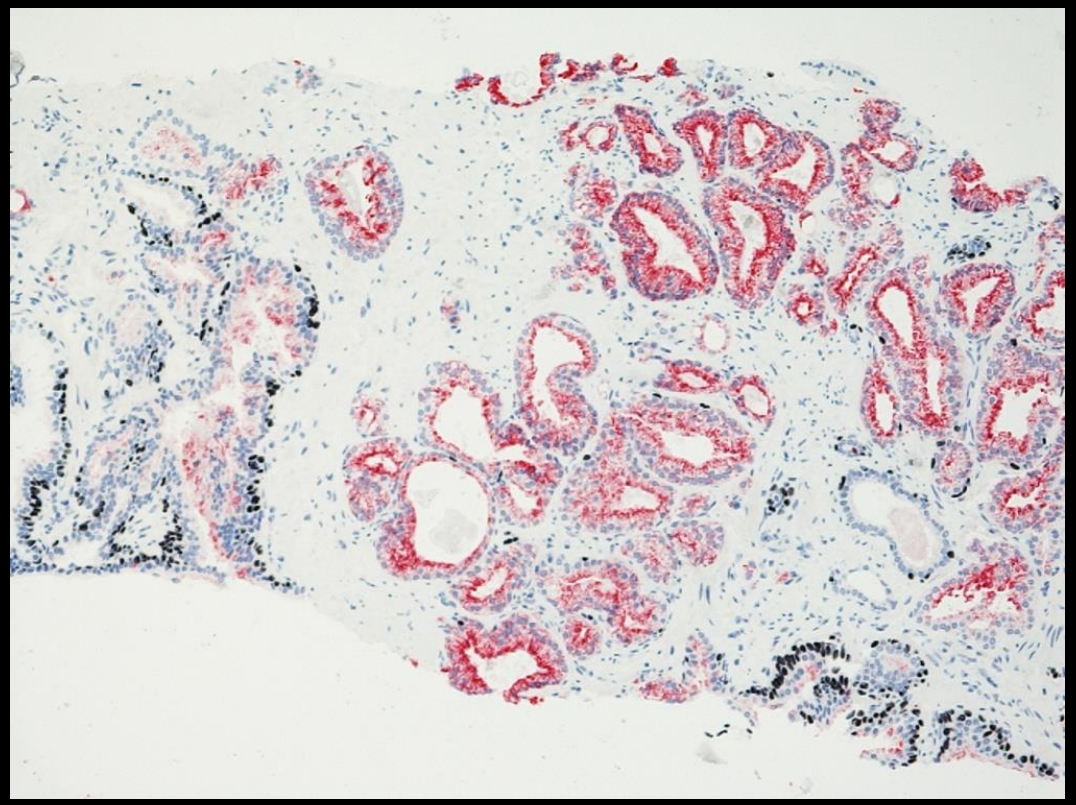
Other useful combinations: P40 + CK5 and/or TTF-1+ Napsin A

Double staining using sequential technique (Immuno-enzymatic)

PIN [cocktail P63 (4A4) + P504s (13H4)] (Mab+Rab)



PIN [P40 (BC28) + P504s (13H4)] / Sequential staining (Mab+Rab)



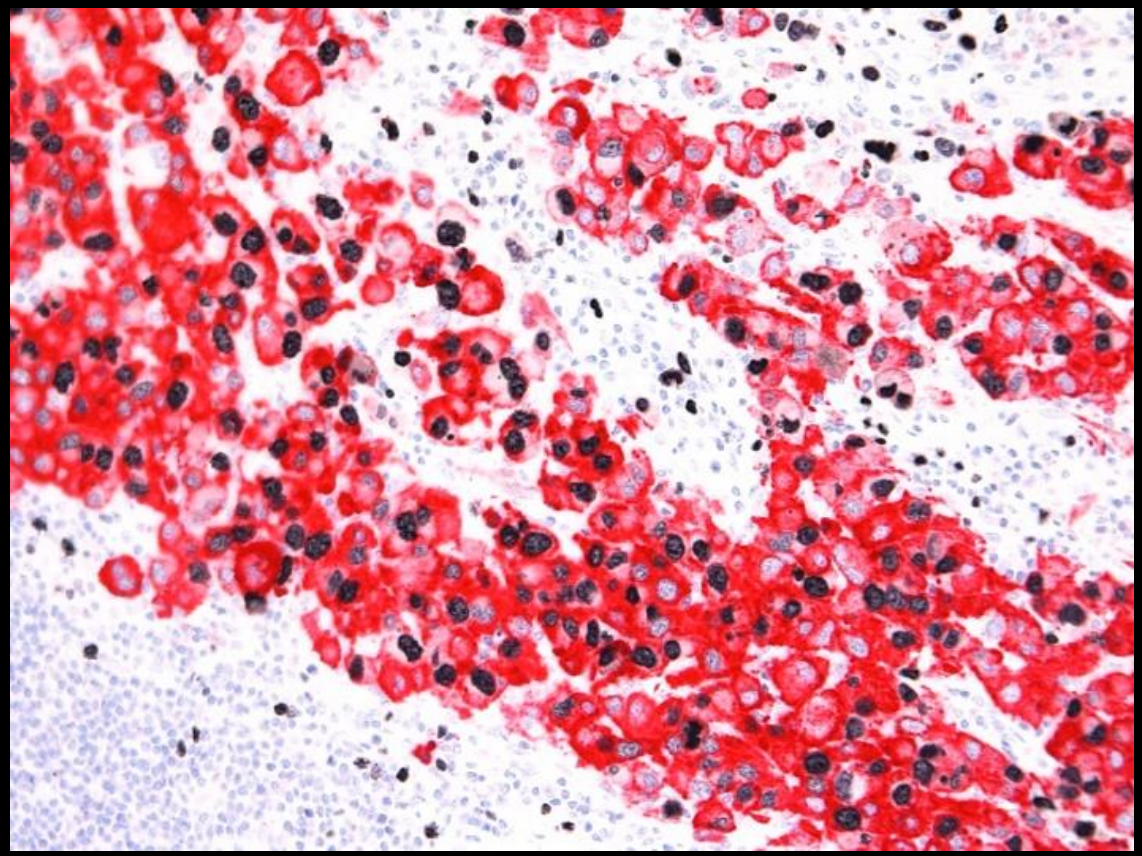
Prostate: Adenocarcinoma

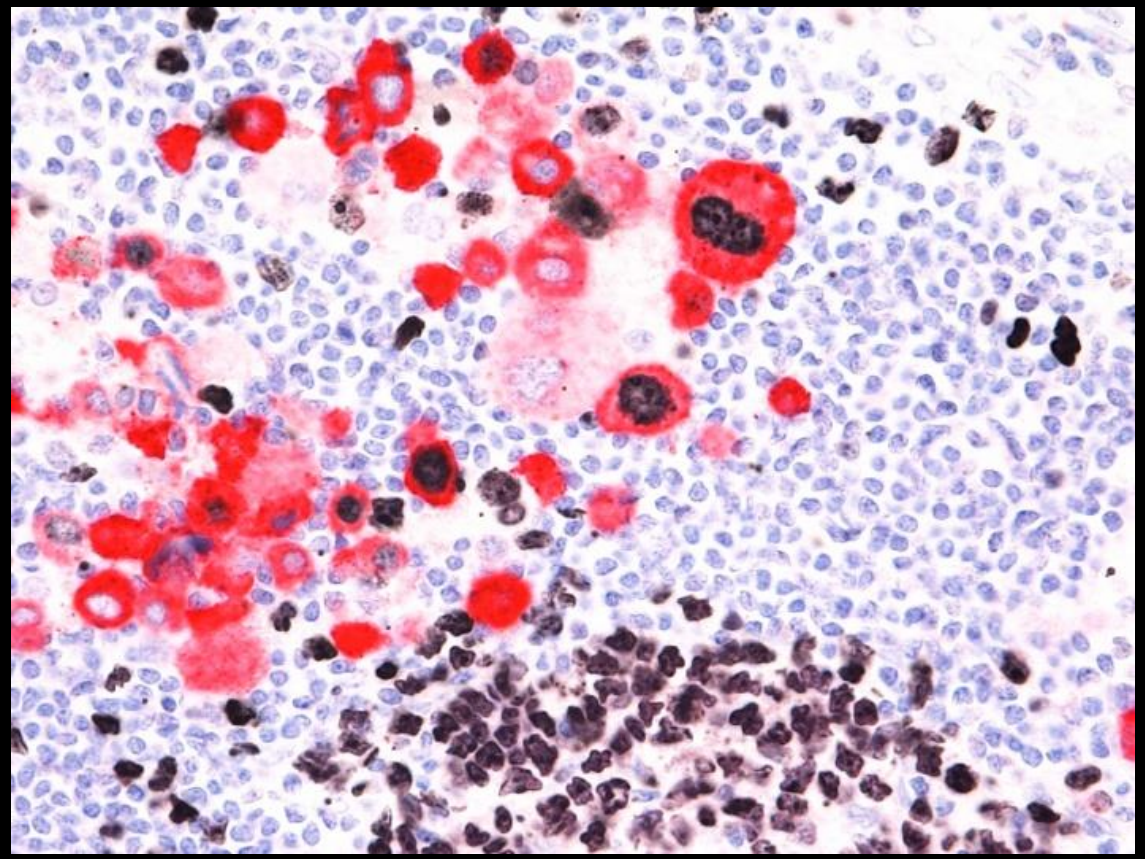
Other useful combinations: : P63 + P504s and/or CK5 (HMw CK) + P504s

Double staining using sequential technique (Immuno-enzymatic)

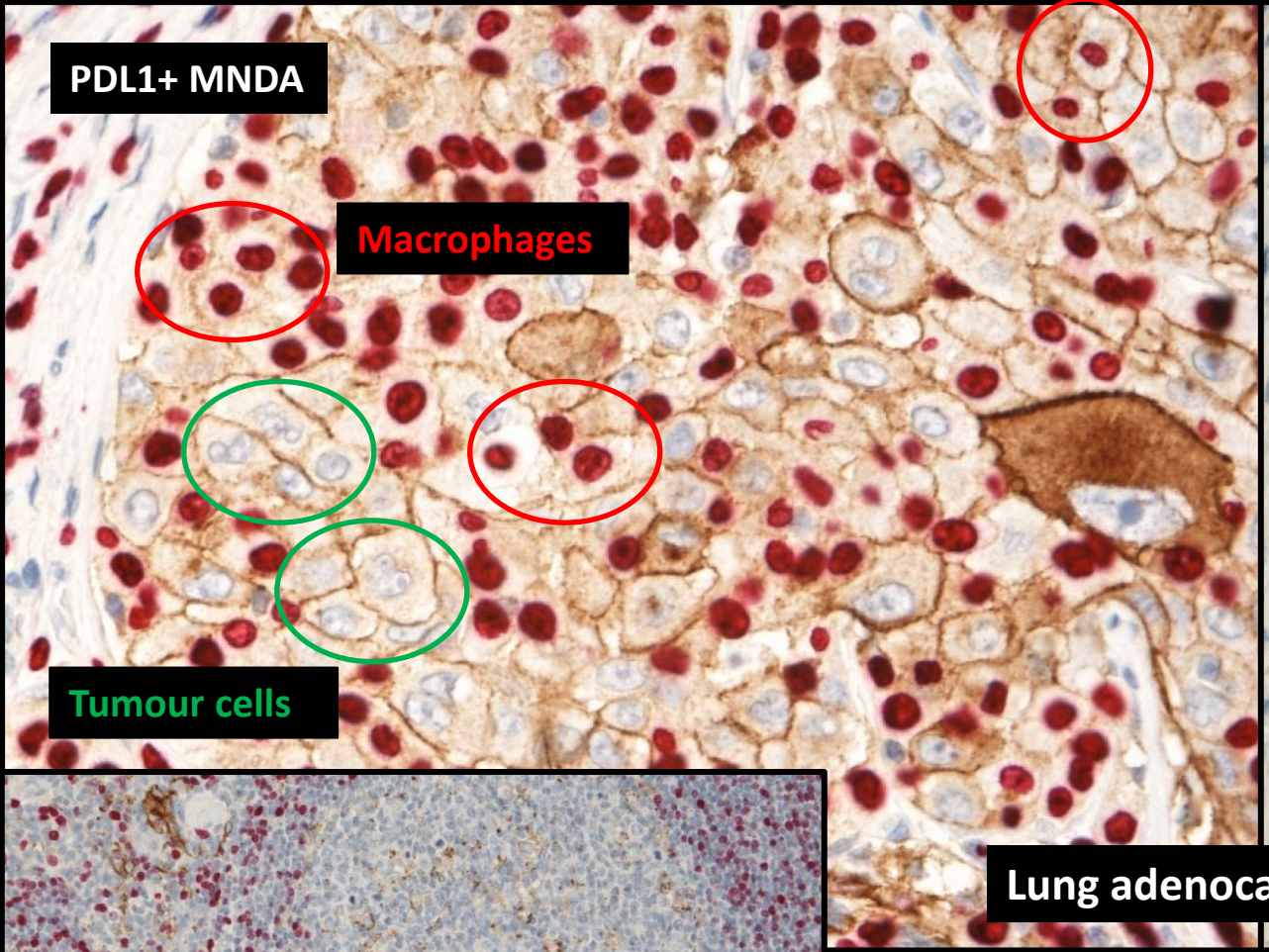
Melanoma (Lymph node)

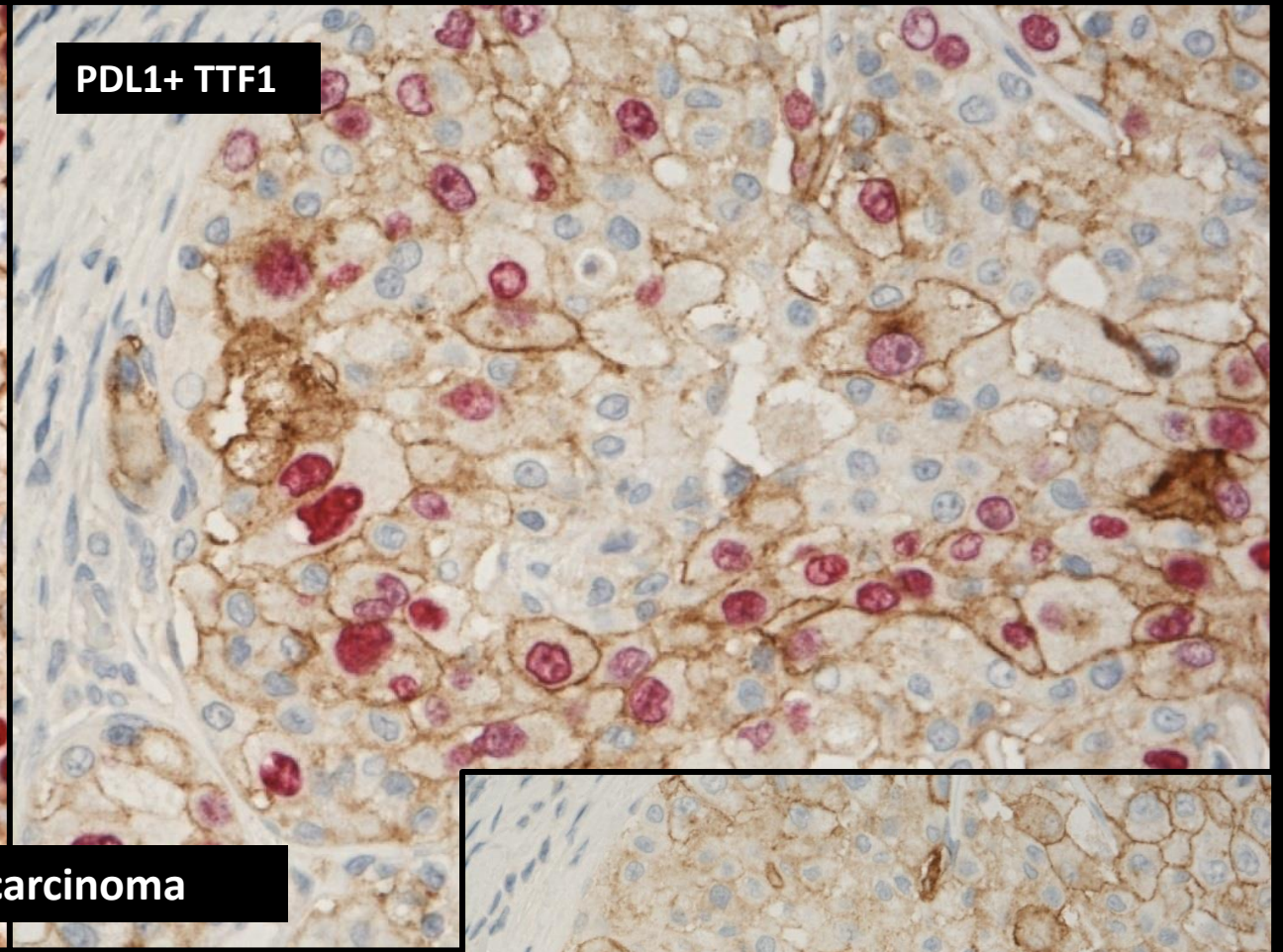
Ki67 , SP6 (1:100) + Melan A, A103 (1:100) (Rab+Mab)



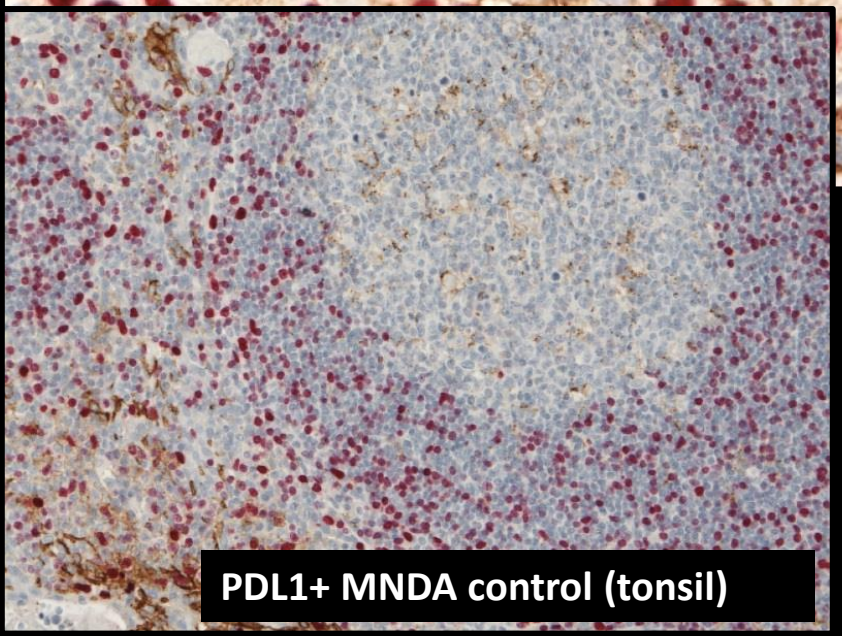


Other useful combinations: Sox-10 + MLA and/or Sox-10 + S100





Lung adenocarcinoma

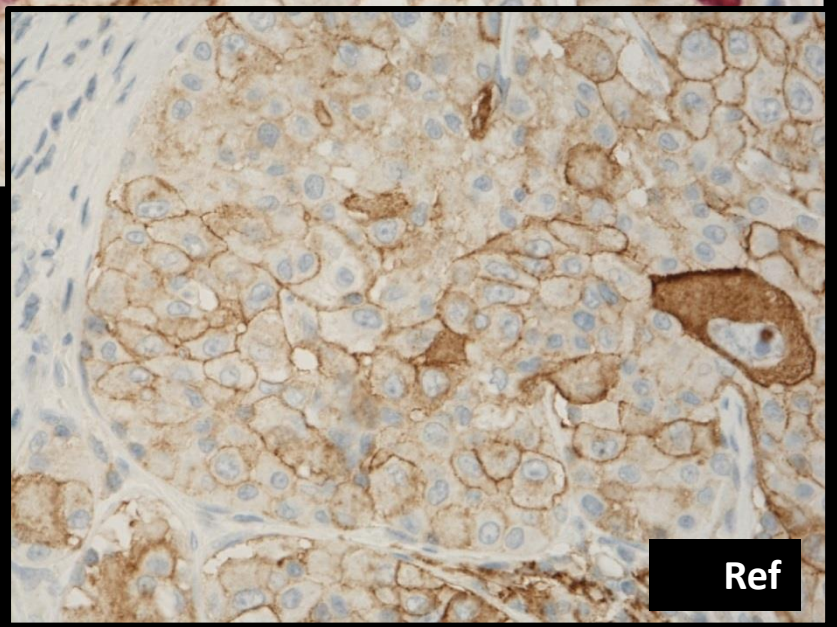


Omnis: HIER Low pH 40`

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



Sequential procedure (Immuno-enzymatic/2xHRP):

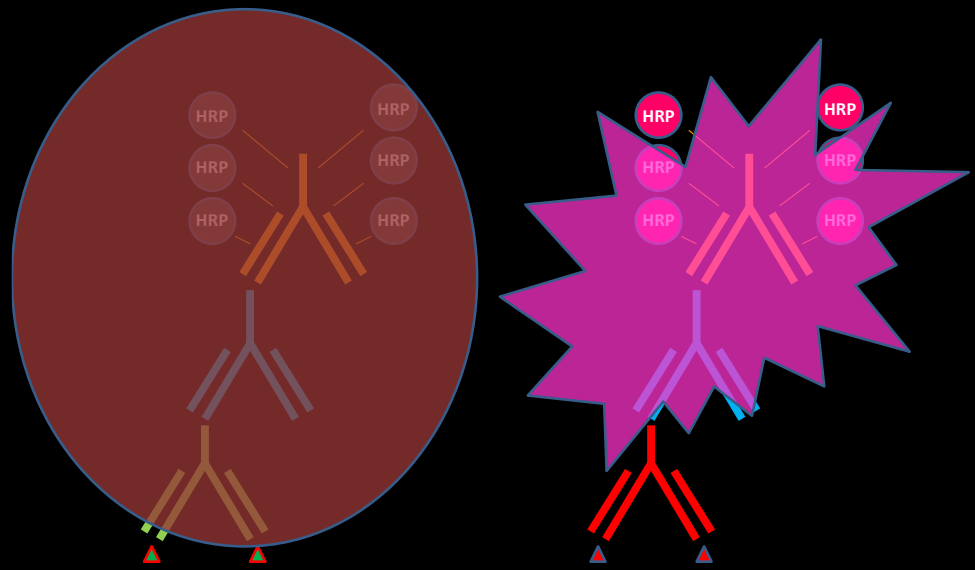
Pre-treatment (Antigen Retrieval)

First primary Ab
Detection with Flex+/HRP (Dako)
Visualization with DAB (Dako).

Second primary Ab (same or different host, Ig-type or subclass)
Detection with Flex+/HRP (Dako)
Visualization with Magenta (Dako)

Counterstain, dehydration and mounting.

Omnis



Detection and visualization reagents (Omnis/Dako)

HRP Magenta Chromogen
Double staining protocol based on 2x Flex with or without linker.

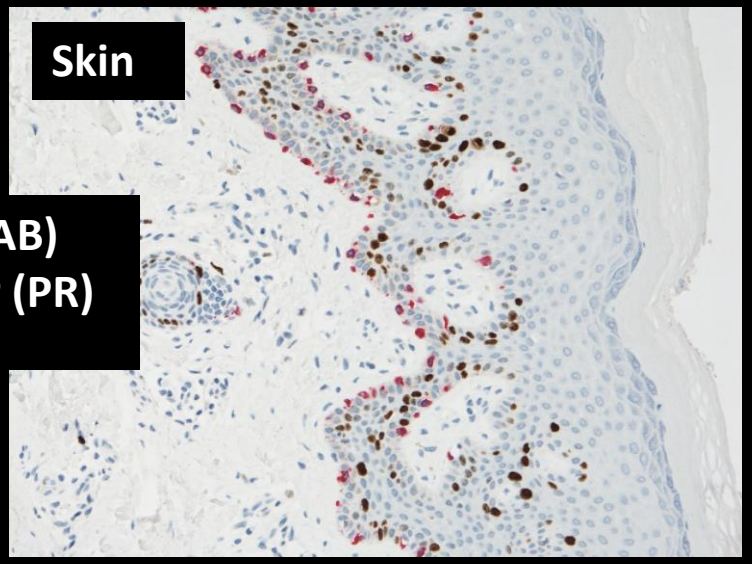
Blocking procedure (H_2SO_4) after first set of immuno-reagents (elution)
Additional peroxidase block (H_2O_2)

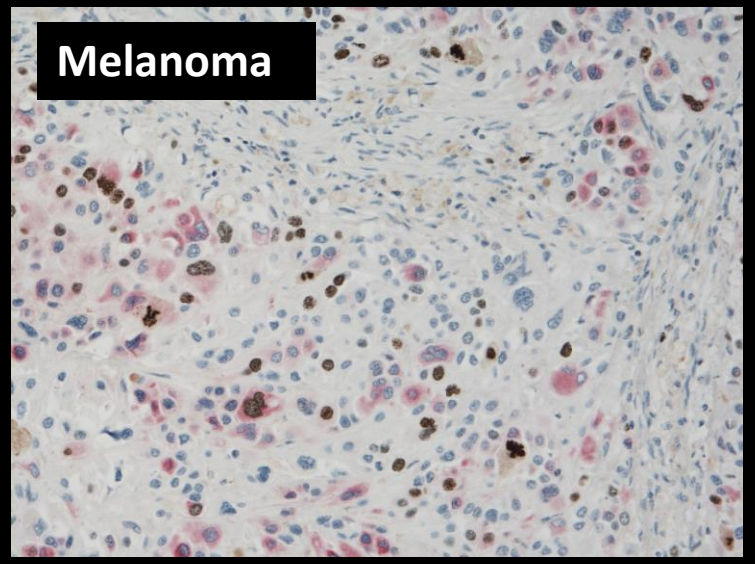
1.Set of Immuno-reagents
Flex+
DAB

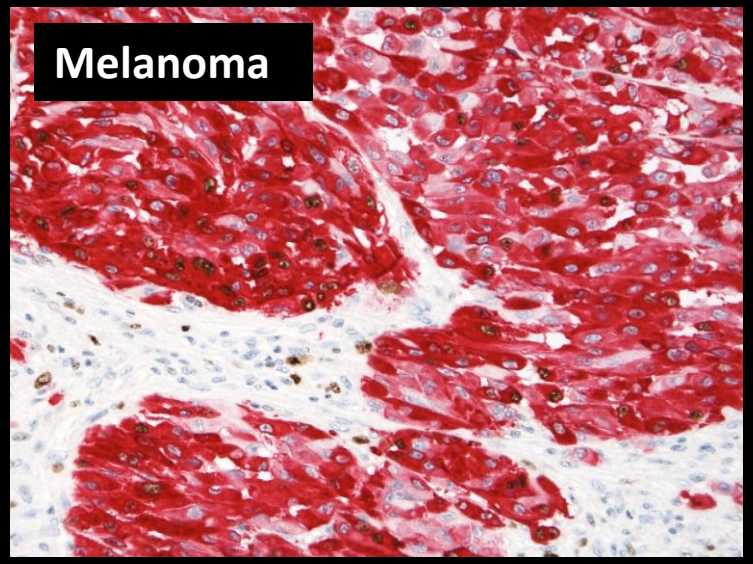
2. Set of Immuno-reagents
Flex+
Magenta

Ki67 (MIB-1, RTU) and MART1 (EP43, 1:30 RR)
HIER High pH (Omnis)

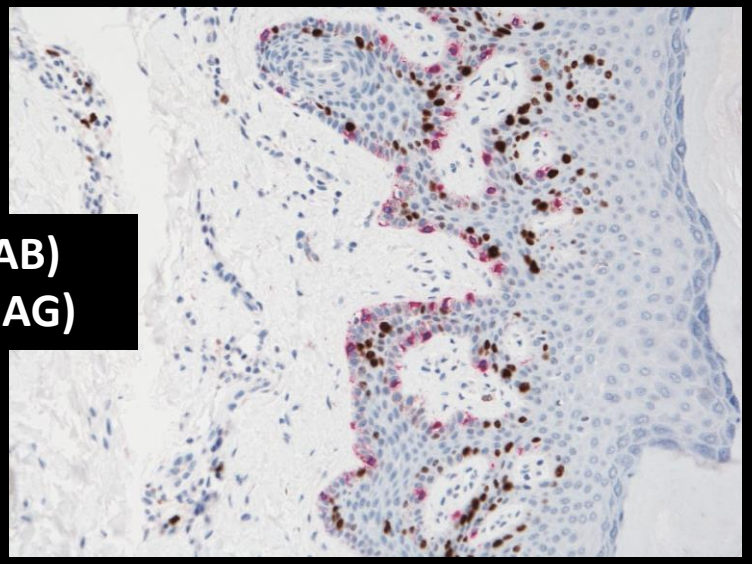
Flex+ (DAB)
Histo-AP (PR)
(incl. Linker)

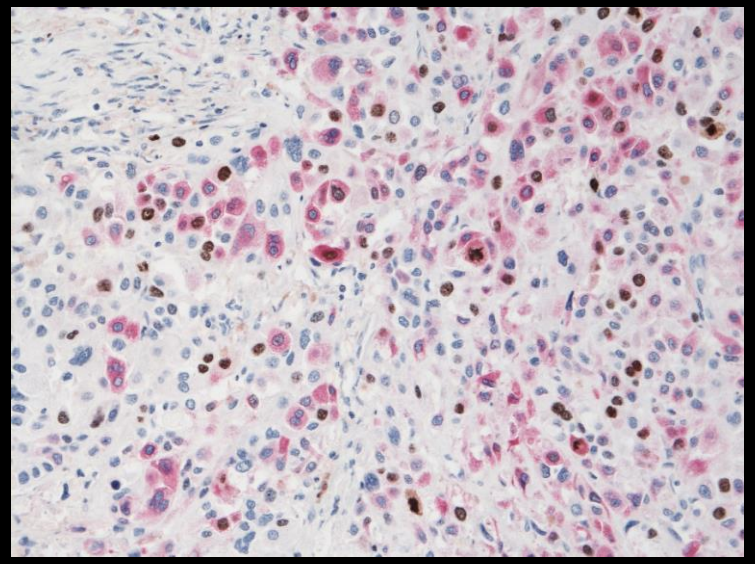


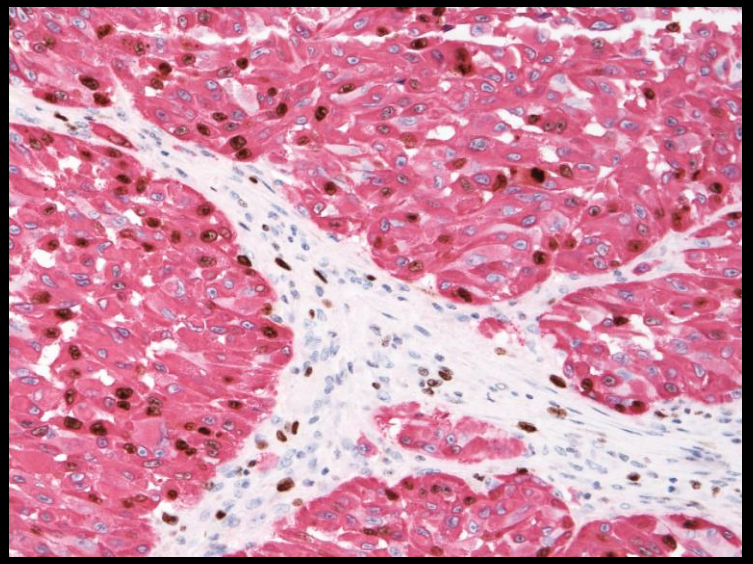




Flex+ (DAB)
Flex+ (MAG)

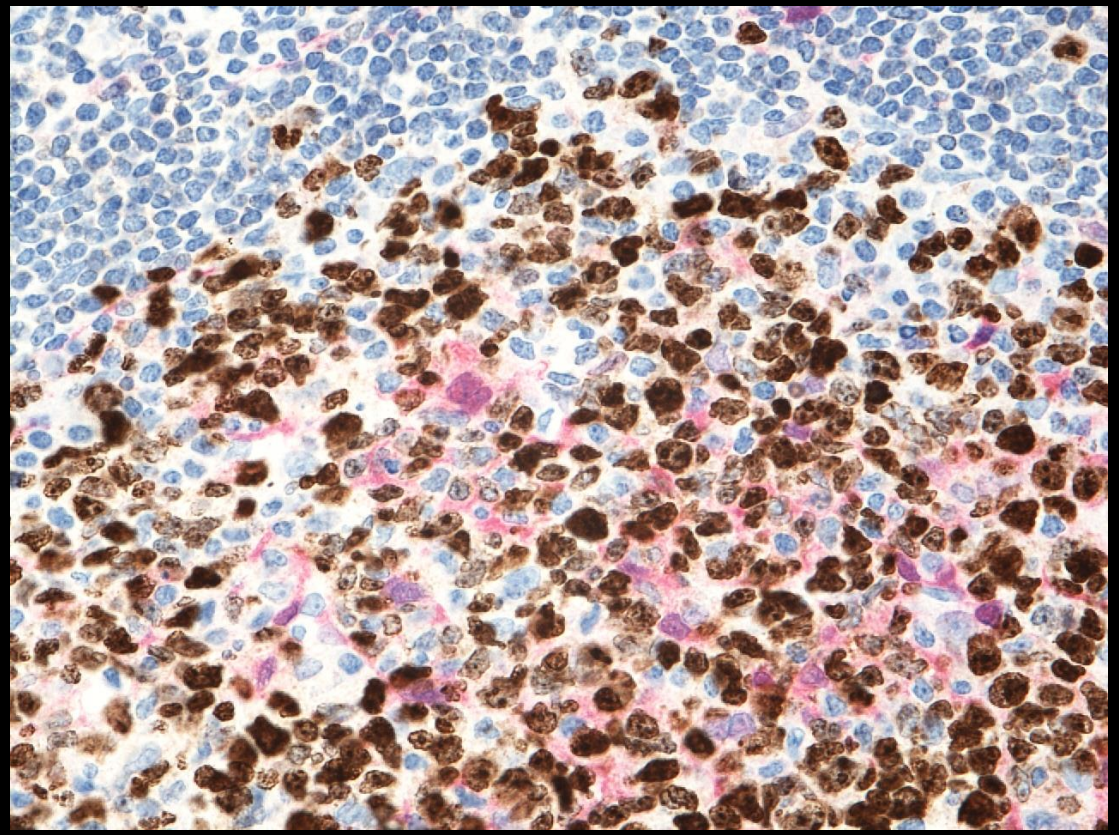




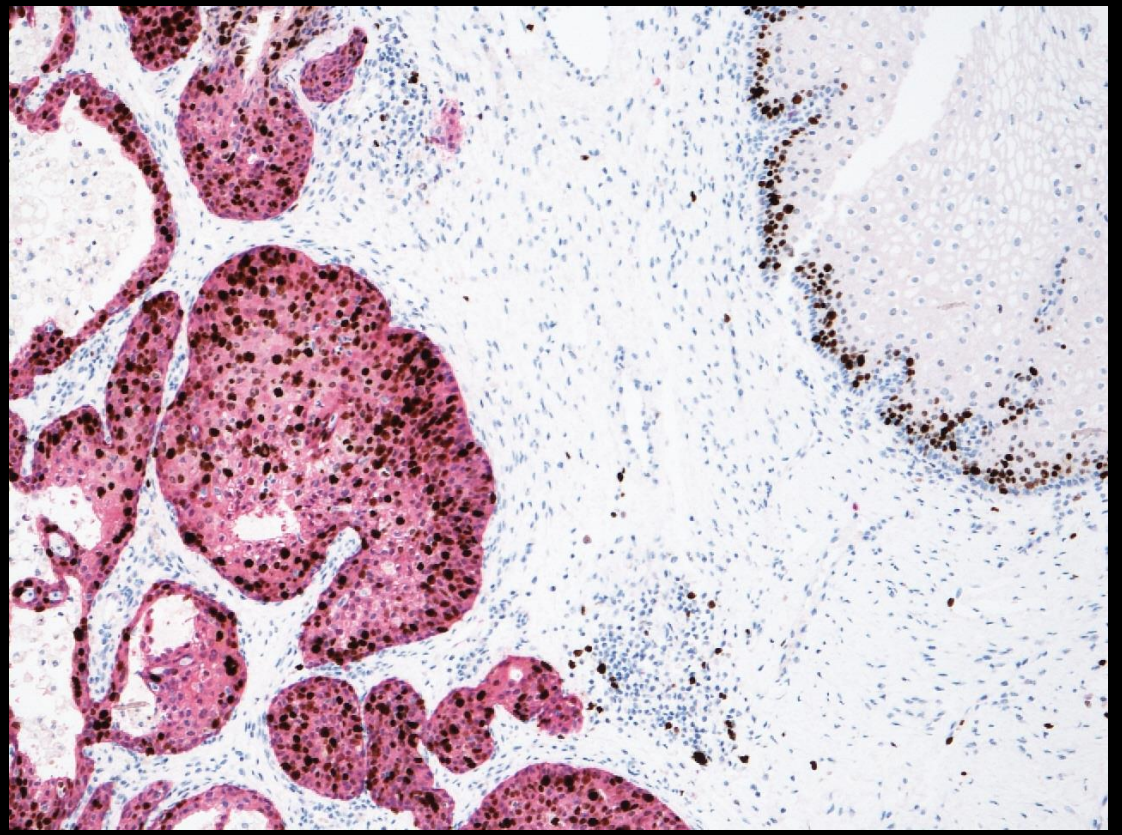


Ki67 (MIB-1, RTU) and P16 (MXB007, 1:1000)
HIER High pH (Omnis)

Tonsil



Uterine Cervix (SCC)



Flex+ (DAB)
Flex+ (MAG)

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

  - If a non-DAB based chromogen is applied and an antibody is difficult to elute (block for unwanted reactions) – apply this antibody in the last sequence

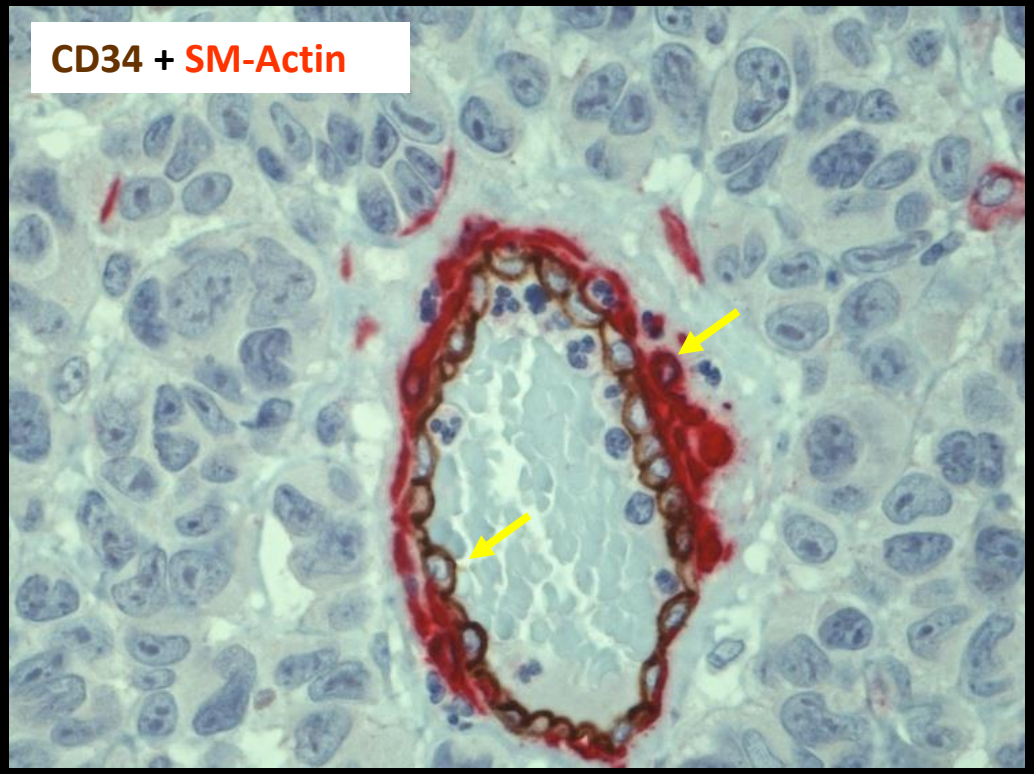
A reversed applications of the primary antibodies may un-ravel that :

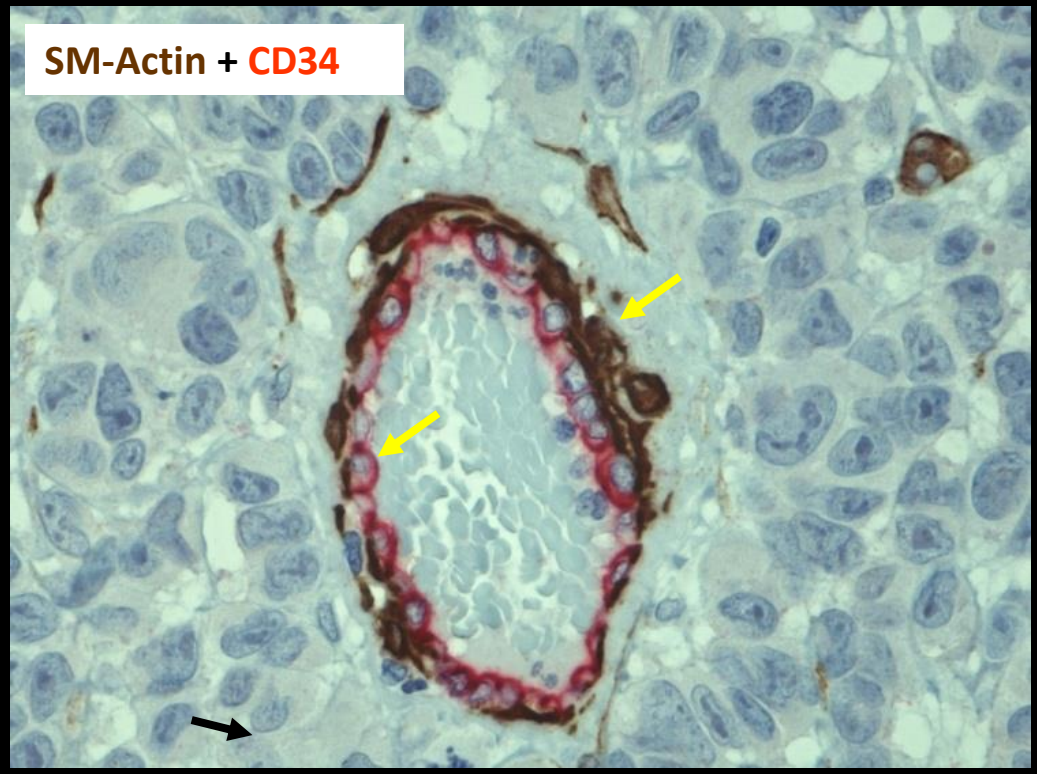
DAB deposit shields for the second antigen of interest - first and second antigen are in close proximity with each other (false negative result)

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)



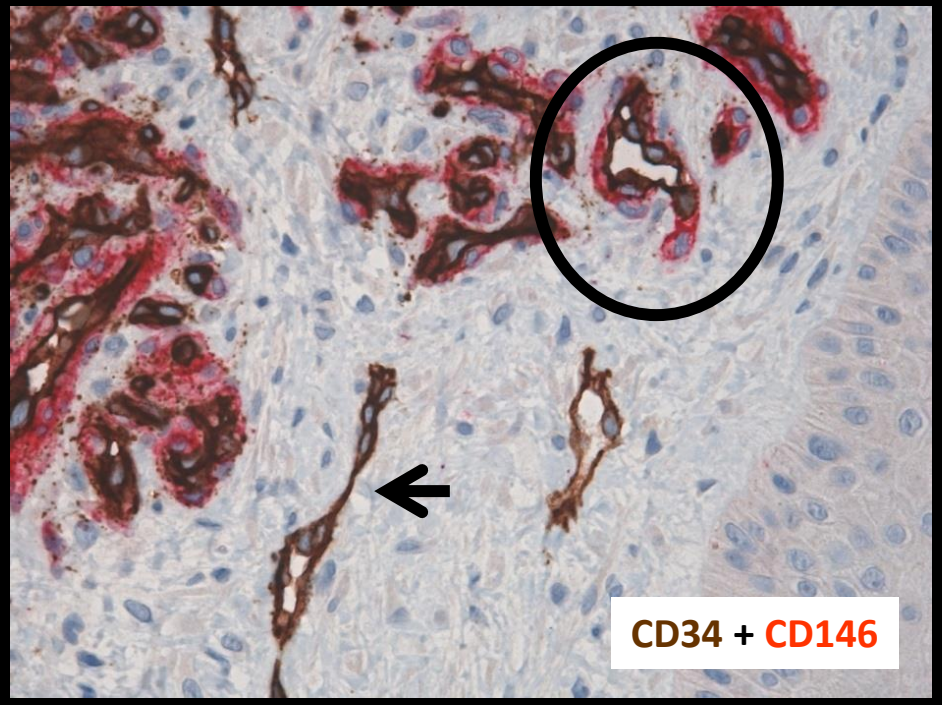


Note: No co-localization of either endothelium or smooth muscles (arrows)

Melanoma

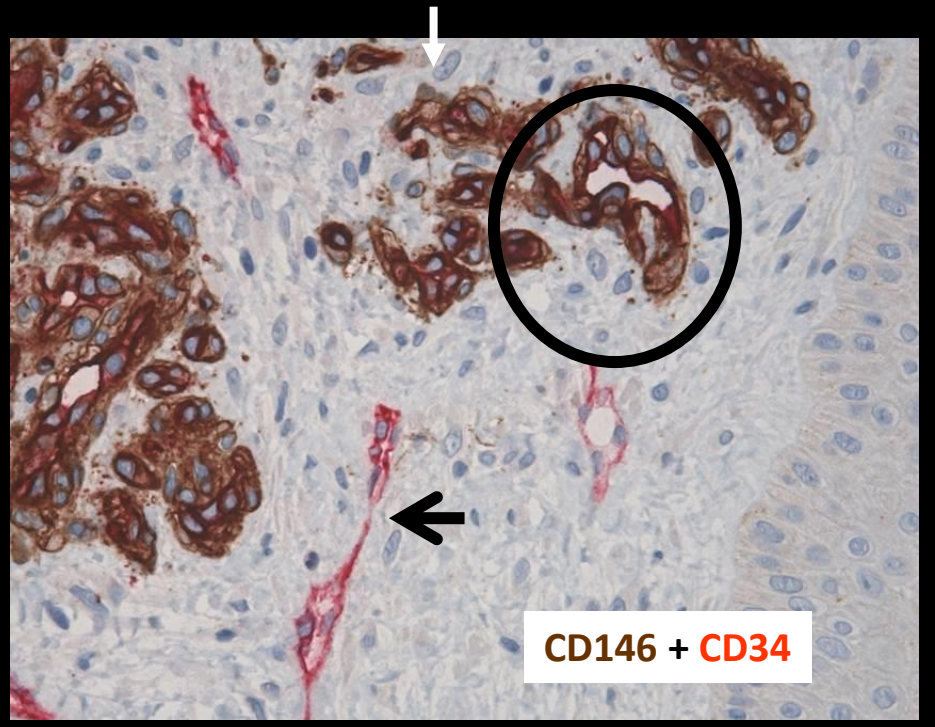
Double Immuno-staining – reversal stainings

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).

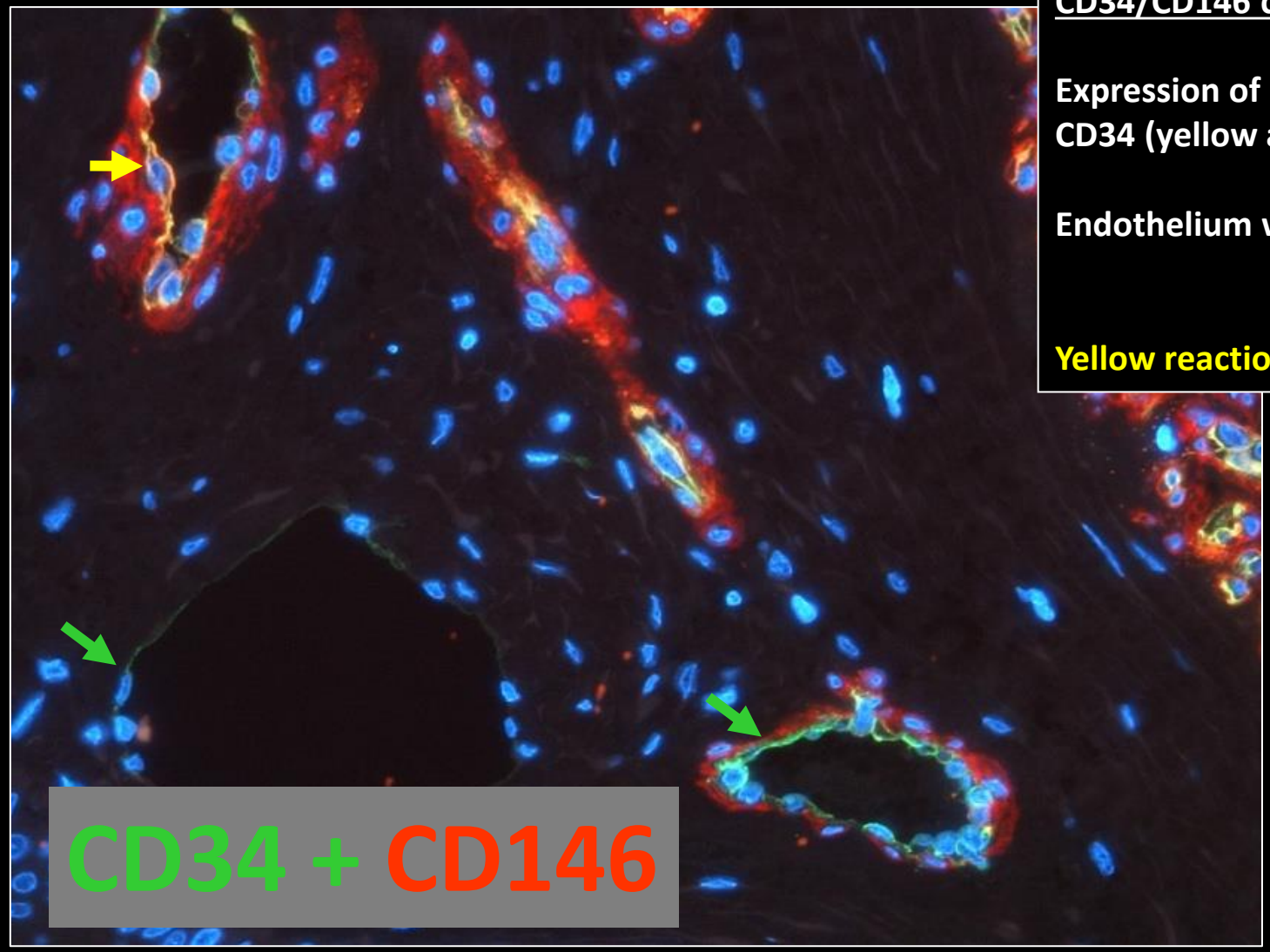


Endothelia cells are “double brown positive” /co-localization

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

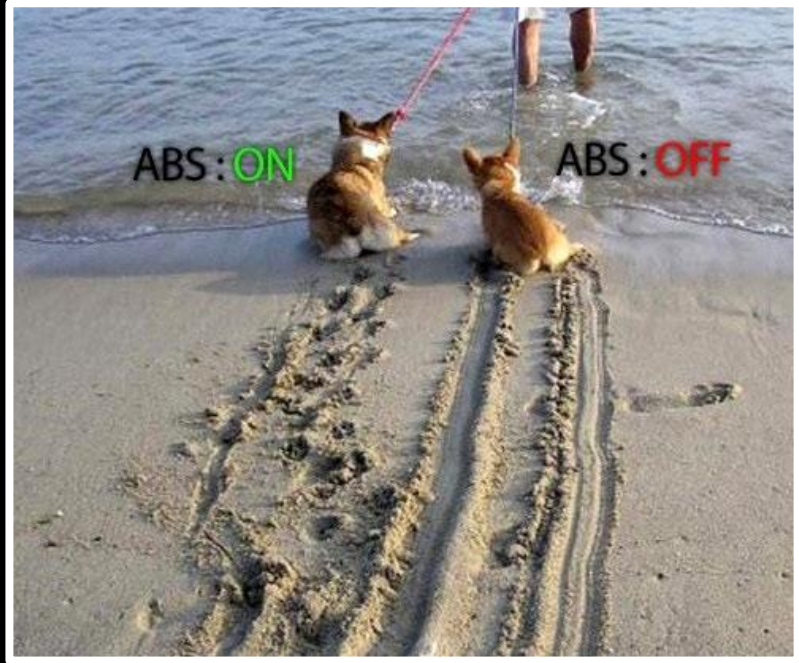
Hemangioma

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.

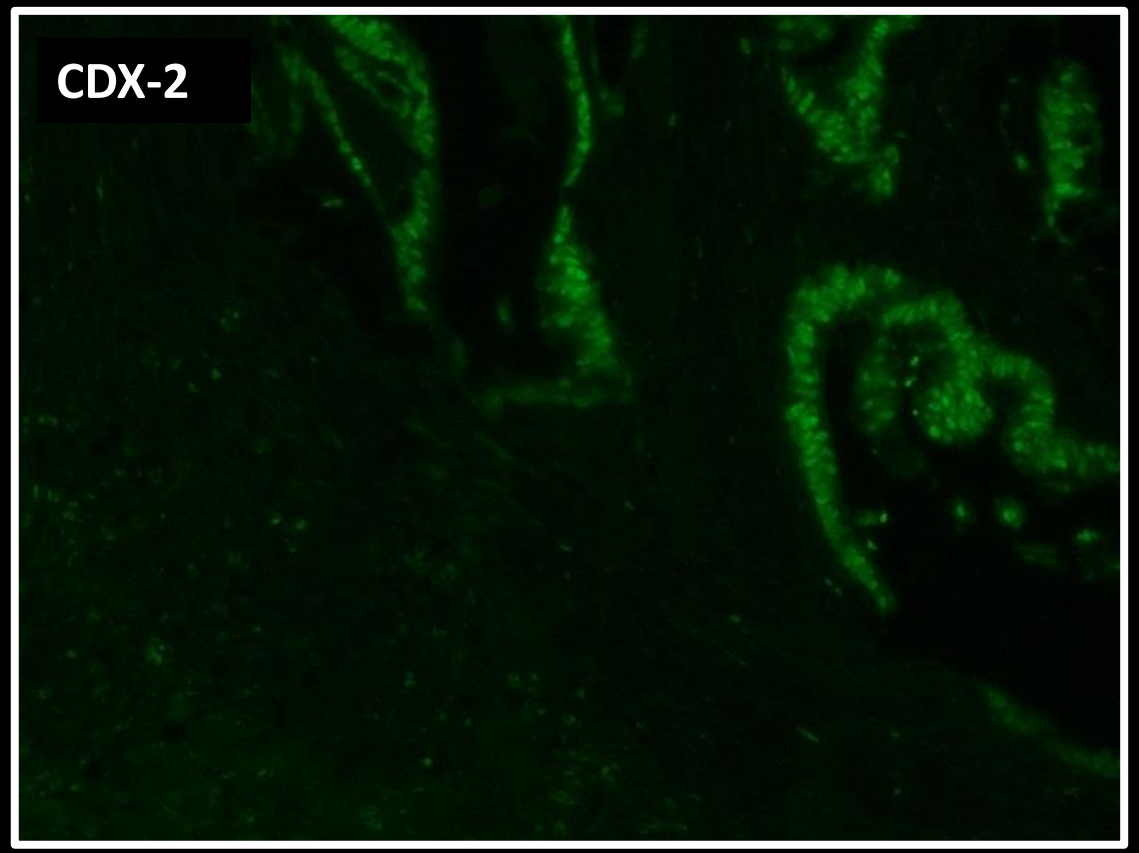
Tumor cells with infiltrative growth pattern (buddings) show expression of CK 8/18 due to downregulation of CDX2 – arrowheads.

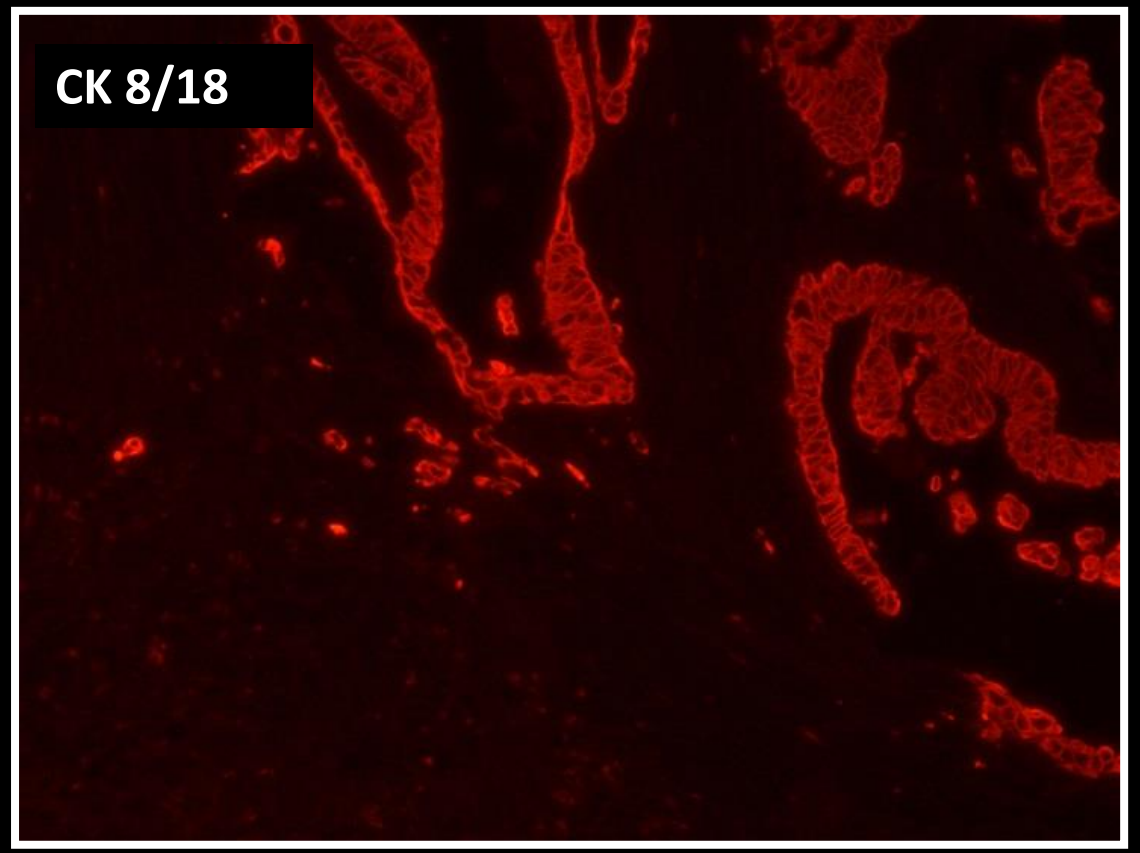


Colon Adenocarcinoma

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

Double Staining-IF (simultaneous technique)





Colon Adenocarcinoma

Note expression of CK 8/18 in all tumor cells

Be critical selecting antibody pair combinations:

Questions to be answered:

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)

Multiplex staining using sequential technique (Immuno-enzymatic)

Sequential procedure (Three markers/2xHRP+AP):

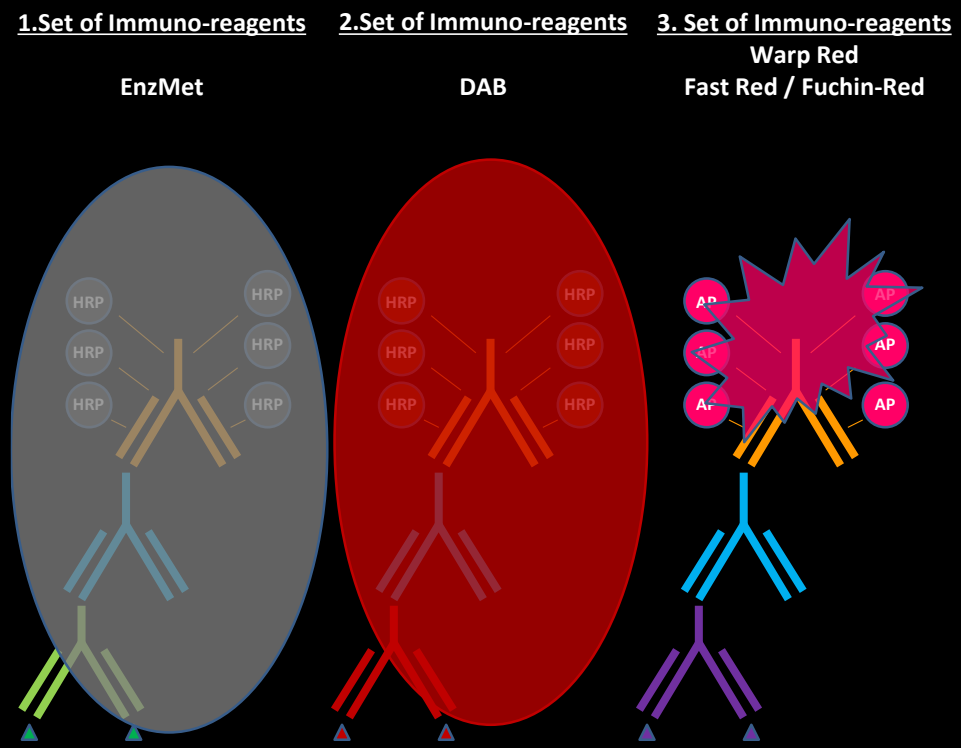
Pre-treatment (Antigen Retrieval)

First primary Ab (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).

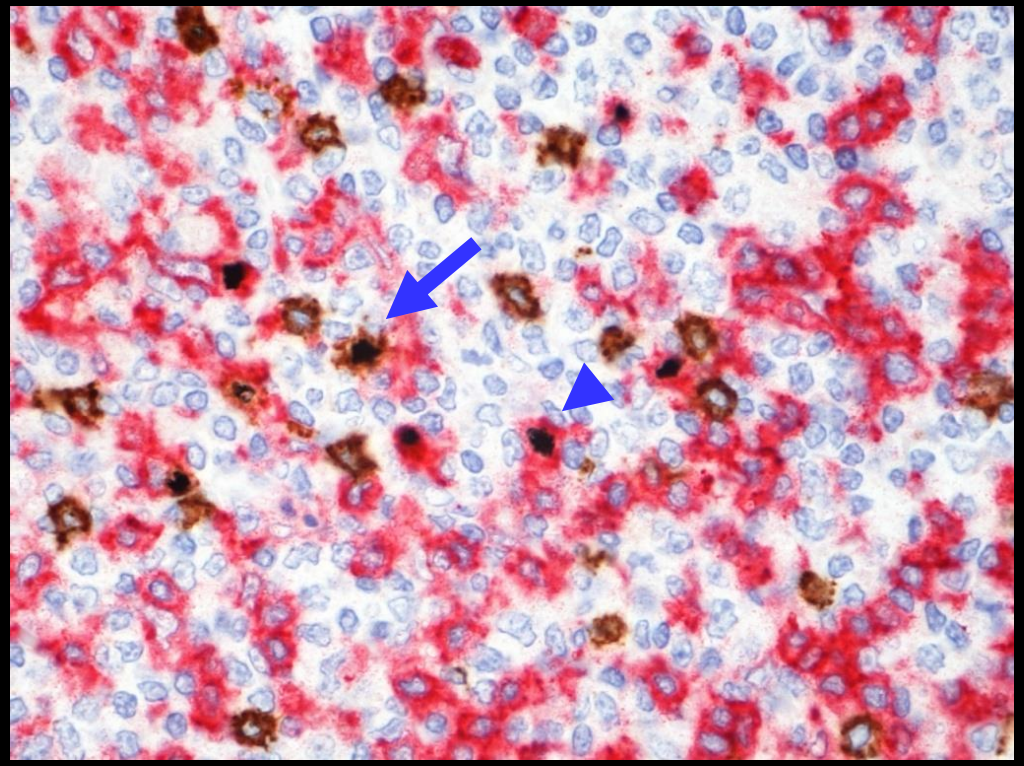
Counterstain, dehydration and mounting.



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

Multiplex staining using sequential technique (Immuno-enzymatic)

FoxP3, 236/E7 + CD8, C8/144B + CD4, EPR6855 (Mab x2 + Rab)



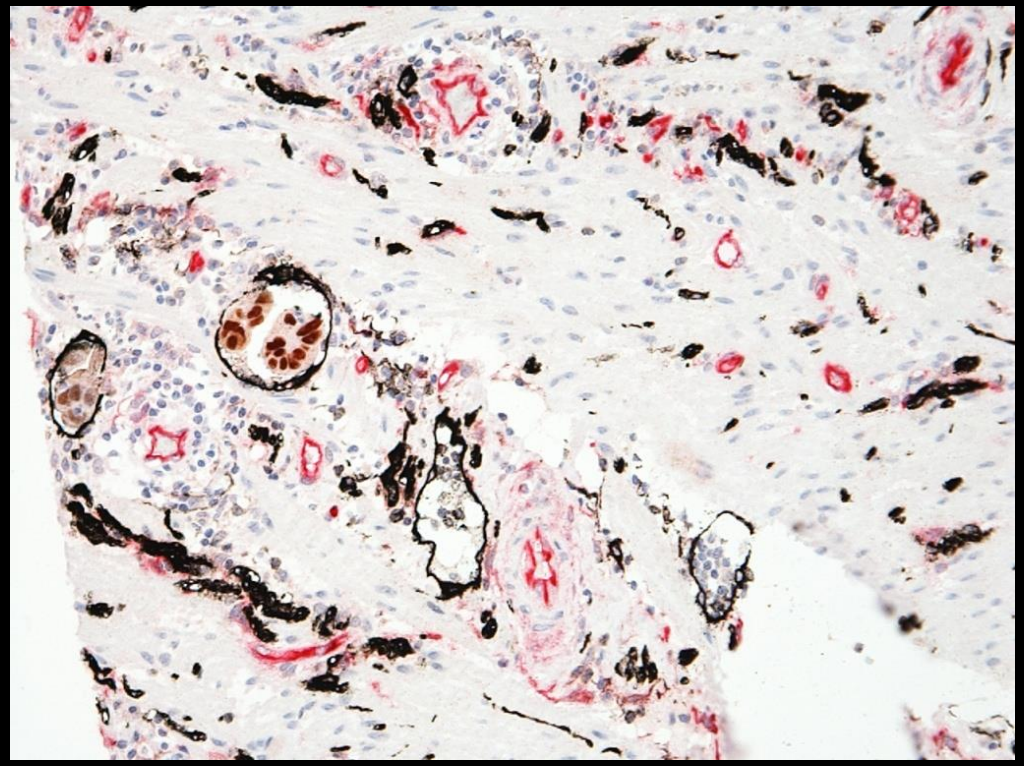
Tonsil

FoxP3 (black nuclear staining)

CD8 (brown membraneous/cytoplasmic staining, arrow)

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

Podo, D2-40 + CDX-2, DAK-CDX2 + CD34, QBEND10 (Mab x3)



Adenocarcinoma colon:

D2-40 (black lymph-endotheliale staining)

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

CD34 (red membraneous endotheliale staining)

Multiplex staining using sequential technique (chromogenic)

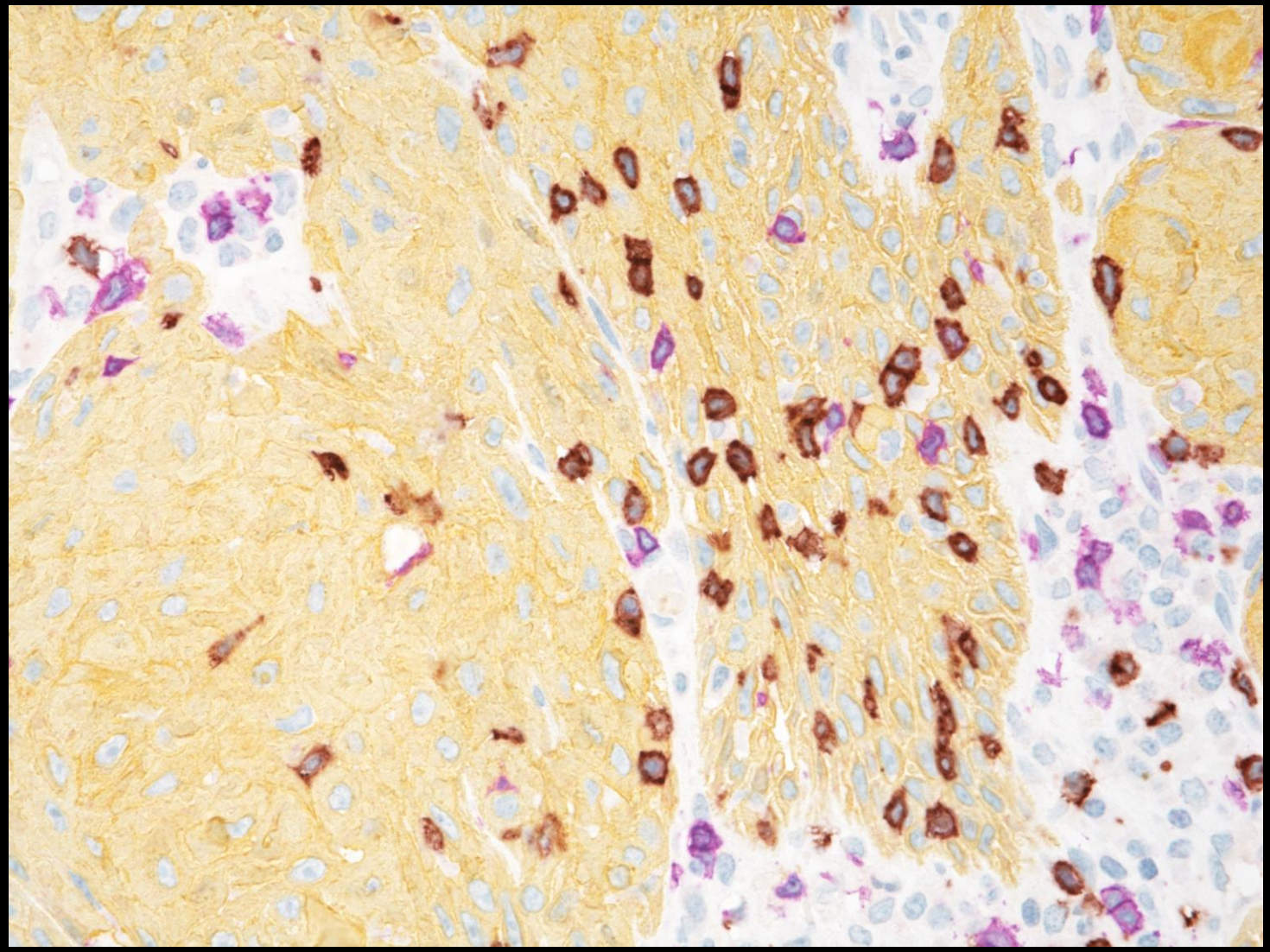
Ventana Discovery

CD8 clone C8/144B (1:100)
OmniMap anti Ms-HRP/DAB

CD3 clone Poly (Dako, 1:25)
OmniMap anti Rb-HRP/Purple

Neutralization (Discovery Inhibitor)

PAN-CK clone BS5 (1:200)
Omnimap anti Ms-HRP/Yellow



Multiplex: Melanoma project

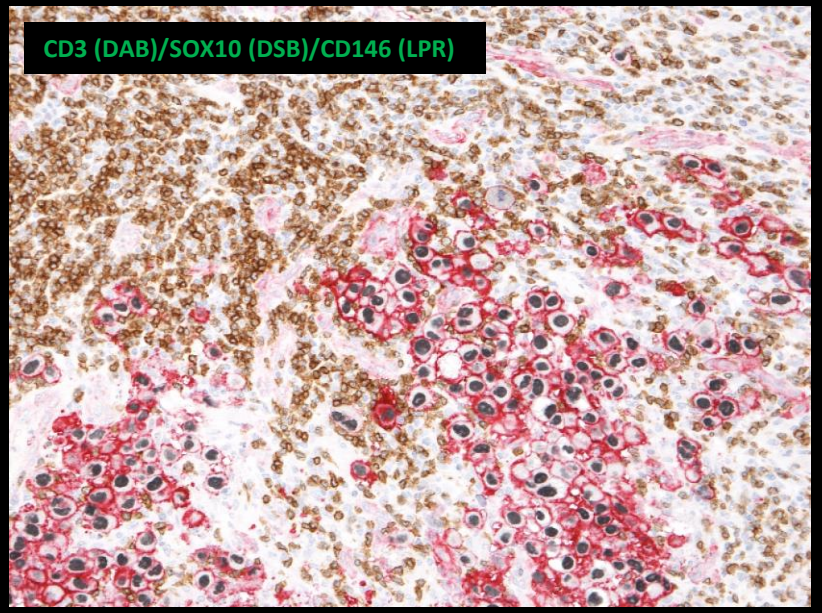
Combinations and color contrast

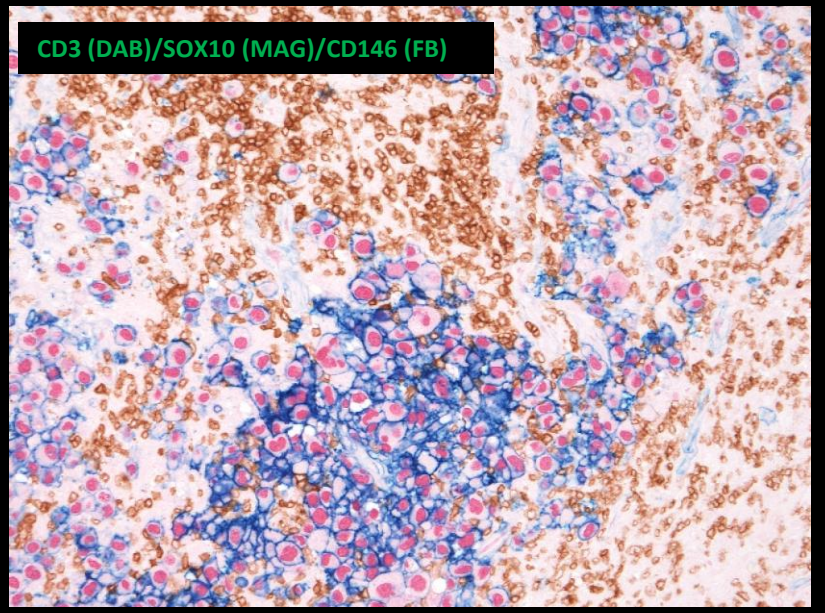
SOX10/CD146/CD3

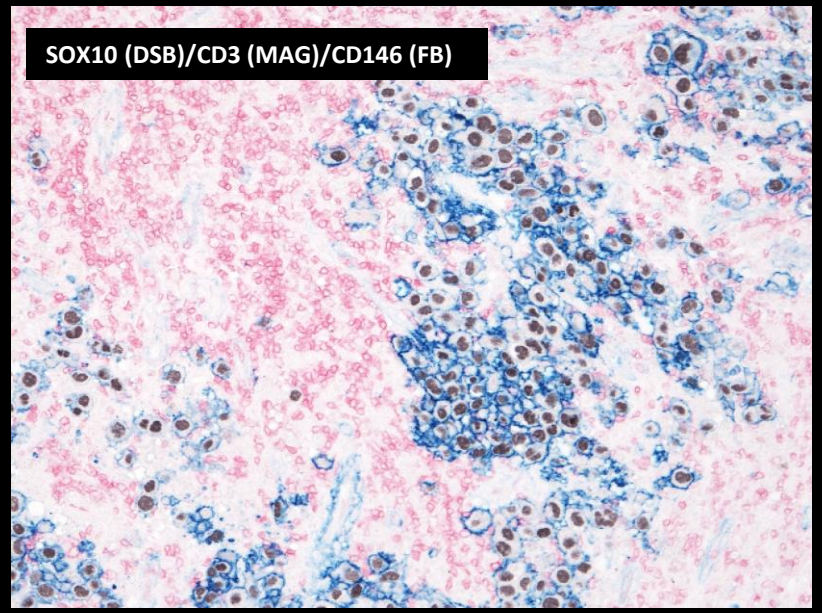
HIER High pH (90°C/60min)

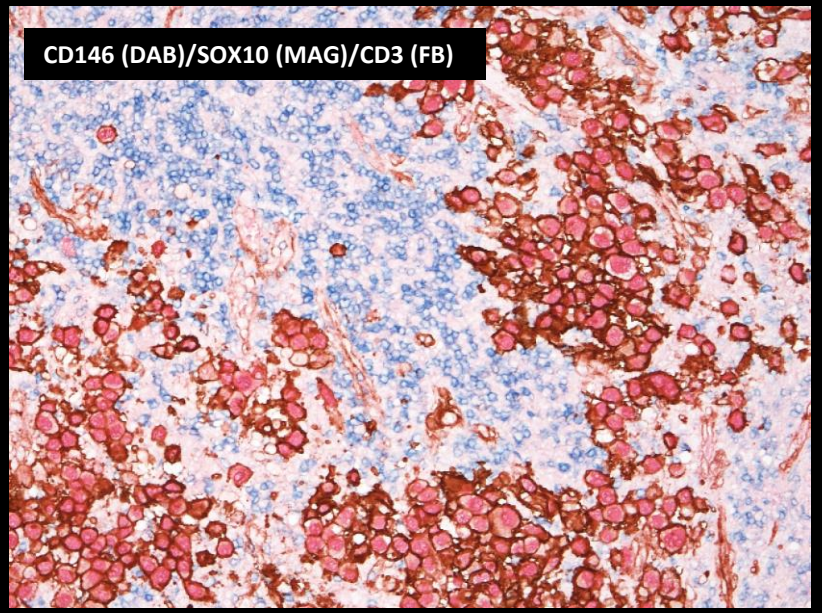
Flex+/MACH2-DS2

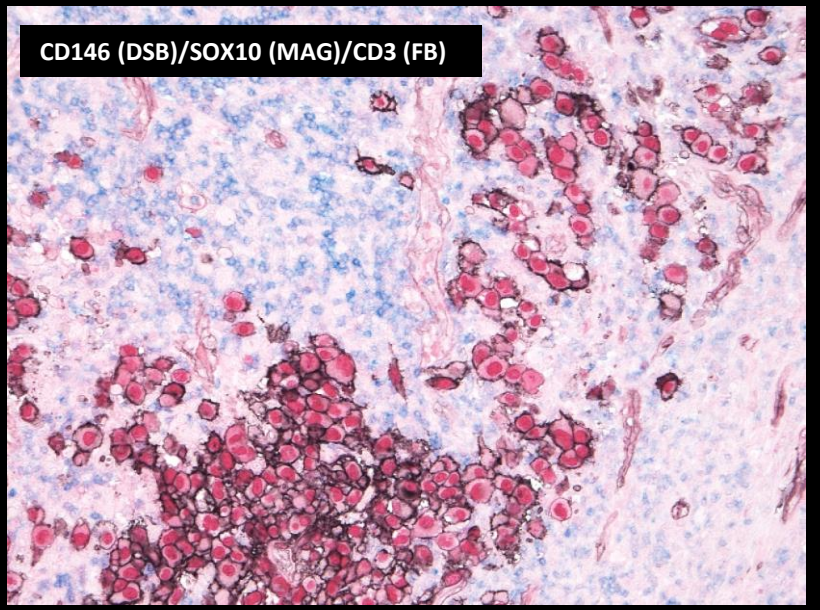
“DAB-based chromogen in the first sequence”











Multiplex staining using sequential technique (Immuno-enzymatic)

Now, what if unexpected color mixing occur (cross reactivity with the prior set(s) of immuno-reagents) ?

Mainly a problem related to use of non-DAB based chromogens

Inactivation of the introduced immuno-reagents (primary Ab and detection systems) :

- ❑ **Elution methods (High salt, extreme pH values and strong oxidizing agents)**
- ❑ **Heat deactivation step using high temperature (97°C-100°C) e.g., in standard Citrate buffer pH6**
  - **Require heat stable chromogens e.g., DAB, VBlue, Vred, LPR and Ventana Translucent Chromogens**
- ❑ **Combination of both ?**

Applied between the individual sequences in the multiplex technique

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

Cross-talk control studies

Demonstrated that:

Using a glycine SDS pH 2 solution was very efficient as "elution method" for the primary Ab (CD20) and superior compared to other "elution" techniques including a intermediate boiling step using citrate buffer pH 6.

Limitations: High affinity antibodies may be difficult to elute – apply problematic antibody last in the sequence

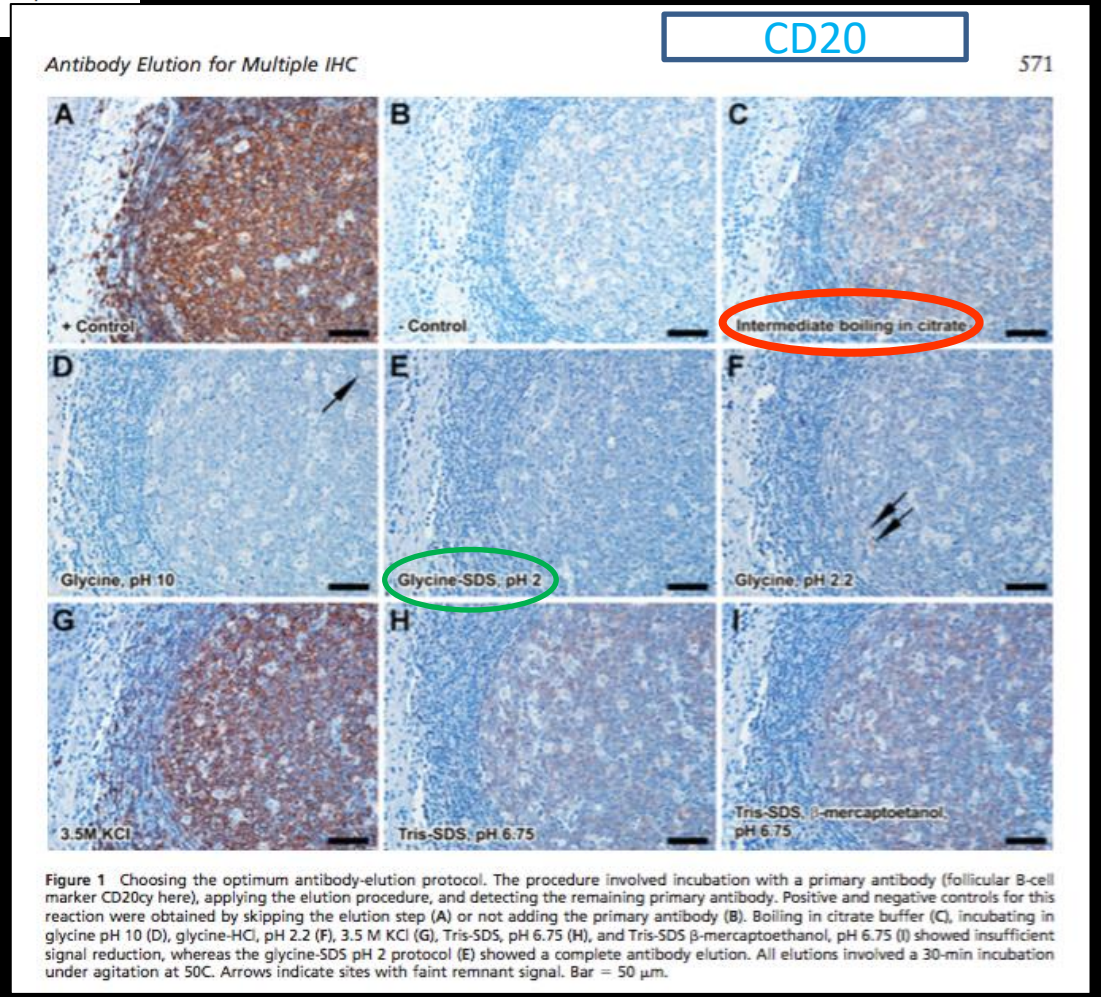
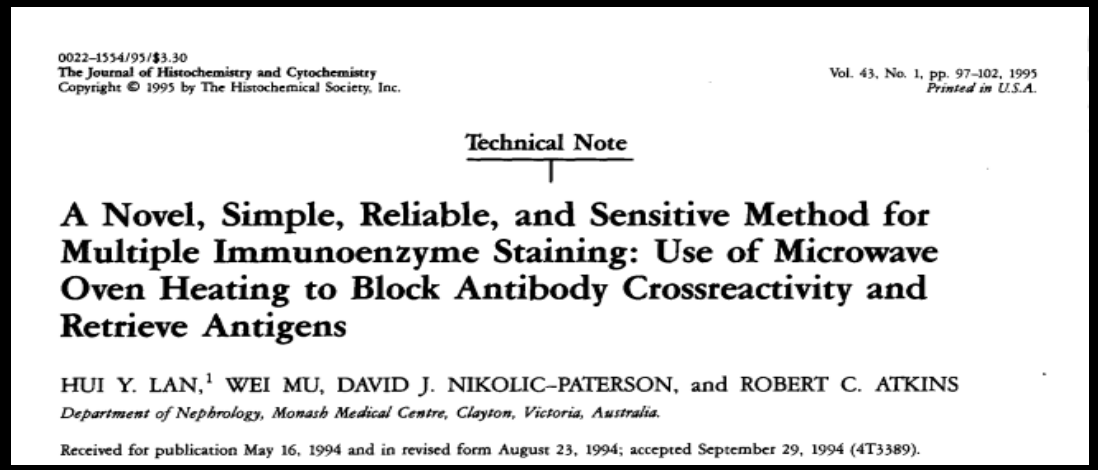


Figure 1 Choosing the optimum antibody-elution protocol. The procedure involved incubation with a primary antibody (follicular B-cell marker CD20cy here), applying the elution procedure, and detecting the remaining primary antibody. Positive and negative controls for this reaction were obtained by skipping 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.



0022-1554/95/$3.30
The Journal of Histochemistry and Cytochemistry
Copyright © 1995 by The Histochemical Society, Inc.
Vol. 43, No. 1, pp. 97–102, 1995
Printed in U.S.A.

Technical Note

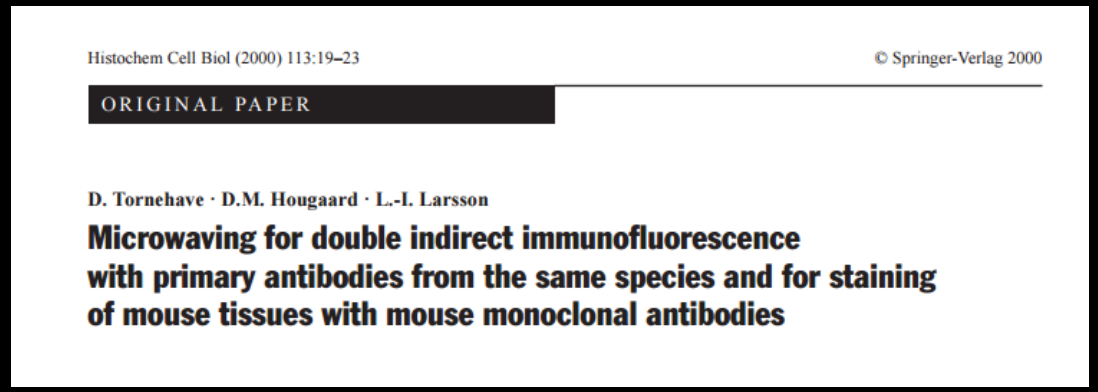
A Novel, Simple, Reliable, and Sensitive Method for Multiple Immunoenzyme Staining: Use of Microwave Oven Heating to Block Antibody Crossreactivity and Retrieve Antigens

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.

Heat deactivation step between sequences in the staining protocol - Limitations:

Require Heat stable chromogens e.g., DAB, VBlue, VRed and LPR (Dako)

For certain antigen/antibody reactions not always efficient - Apply problematic antibody last in the sequences

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 simultaneous technique

YES

Double/Multiplex immunofluorescence technique (simultaneous technique)

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

A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method)

Double enzymatic staining using simultaneous technique

Pre-treatment (Antigen Retrieval)

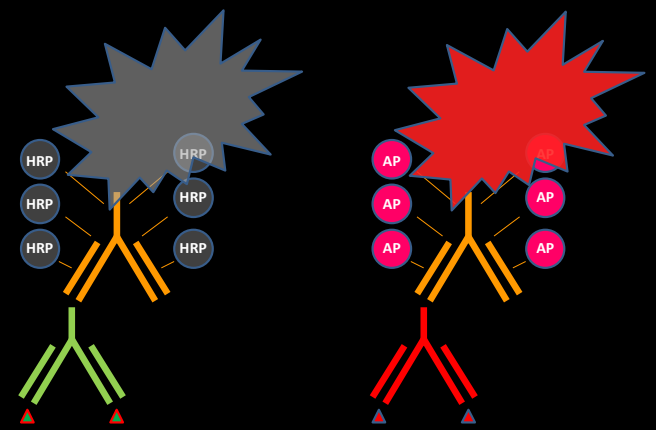
Incubation with mix of primary Abs (Rab+Mab)

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

Incubation with HRP substrate (e.g. , HiDef Yellow, Magenta, DAB or DSB)

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

Counter stain, mounting and microscopy



A 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).

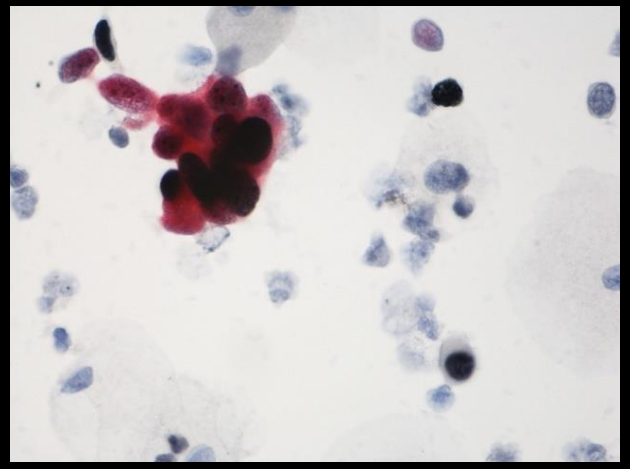
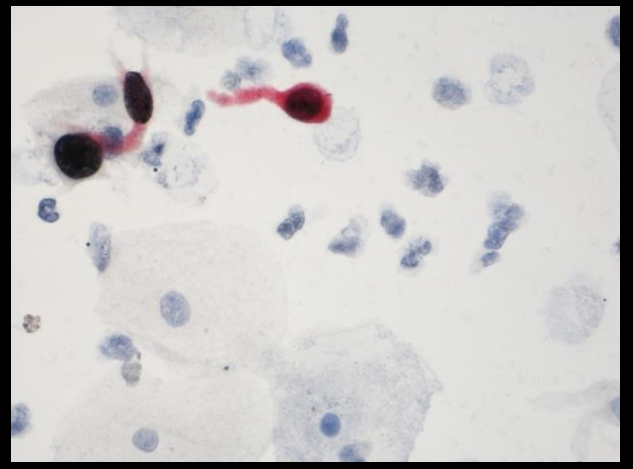
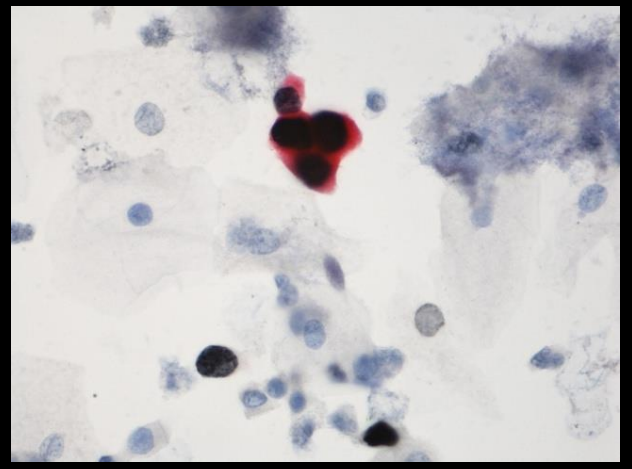
To prevent cross-reaction between secondary antibodies raised against different species, it is recommended to apply second-step antibodies raised in the same host – if not possible, use pre-absorbed secondary antibodies.

Simultaneous double staining : Ki-67, SP6 (1:25) + P16, E6H4 (RTU)

Dysplasia / Cervix (Cytology)

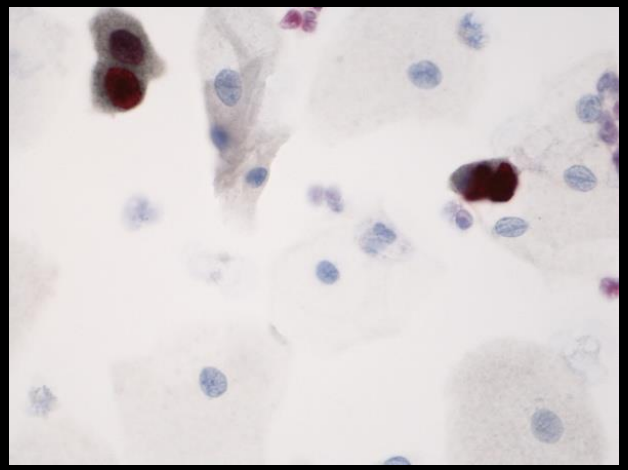
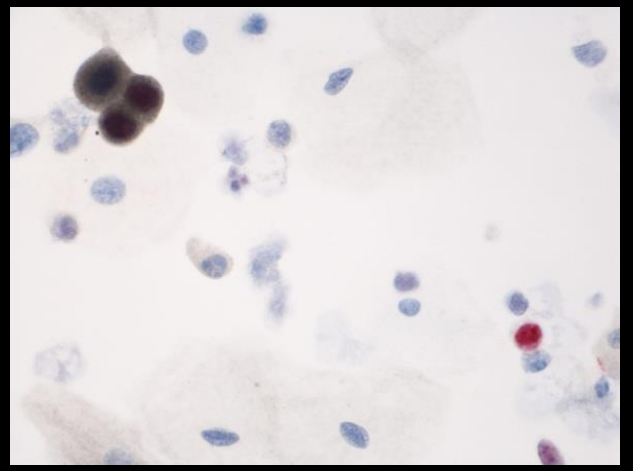
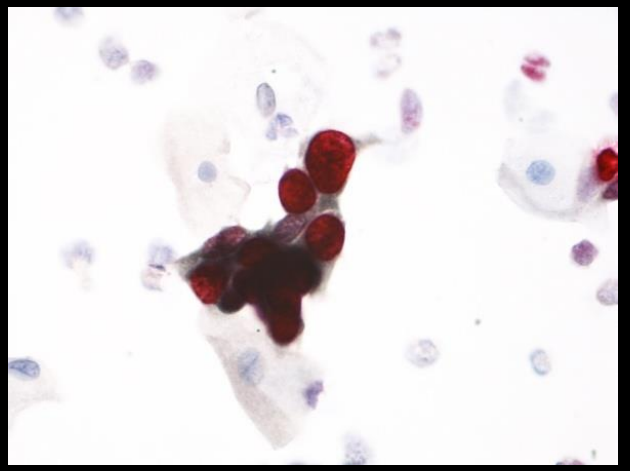
Double Stain 1 (Biocare)

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

Multiplex immunofluorescence technique (simultaneous/sequential technique)

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

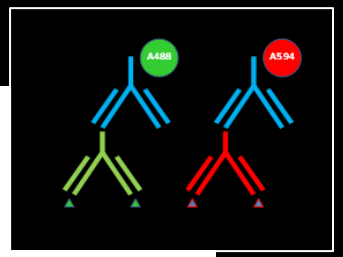
A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method)

Routine methods ?

“The golden standard for demonstration of co-localized antigens”

Immunofluorescence



Journal of Pathology
J Pathol 2000; **191**: 452–461.
DOI: 10.1002/1096-9896(2000)9999:9999<::AID-PATH665>3.0.CO;2-O

Original Paper

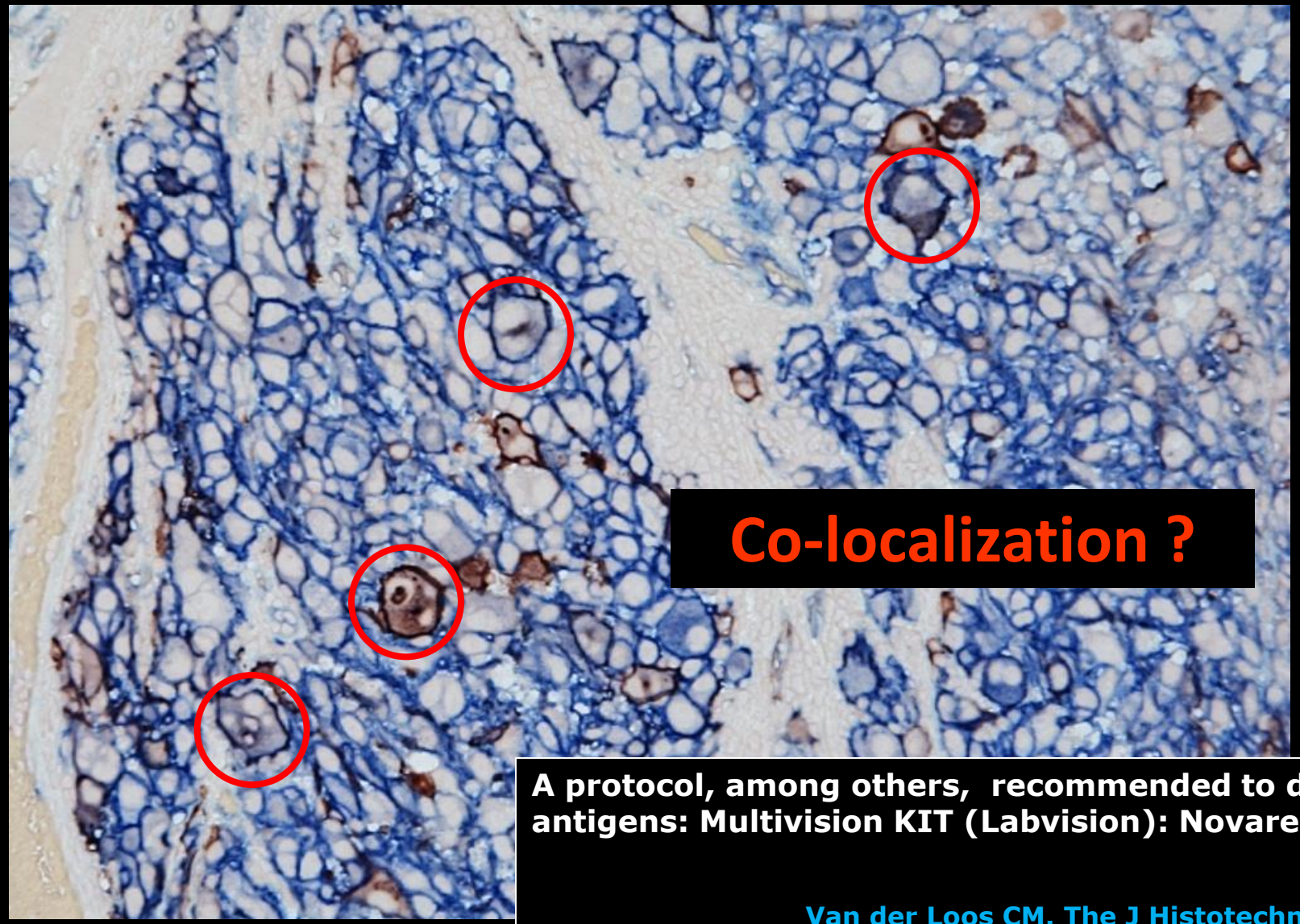
Double immunofluorescence labelling of routinely processed paraffin sections

David Y. Mason*, Kingsley Micklem and Margaret Jones
Leukaemia Research Fund Immunodiagnostics Unit, The Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, Oxford OX3 9DU, UK

Stated that :

- **Double immunoenzymatic labeling of routinely processed human tissues 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.**

NGFR (MRQ21) + CD146 (EPR3208)



A protocol, among others, recommended to detect co-localized antigens: Multivision KIT (Labvision): Novared + Vector Blue

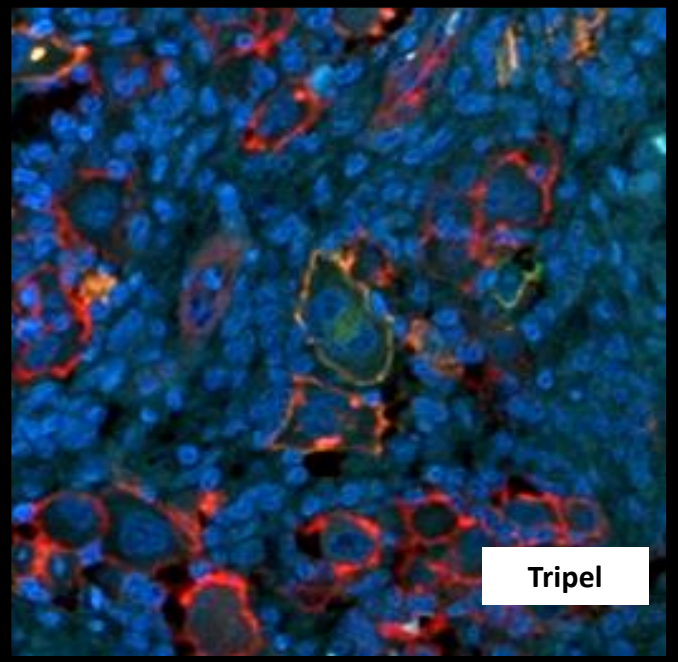
Van der Loos CM. The J Histotechnol 33 (1): 31-40, 2010

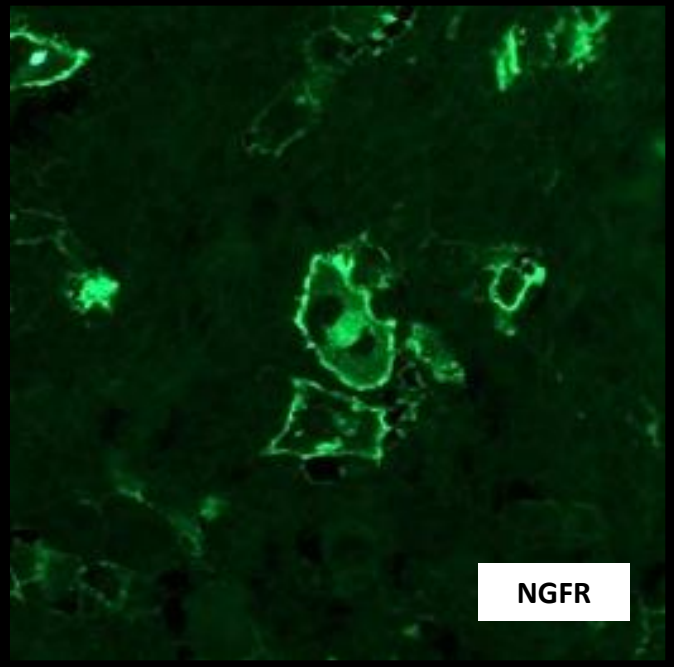
Van der Loos CM et al. J Histotechnol; 31: 119-127, 2008

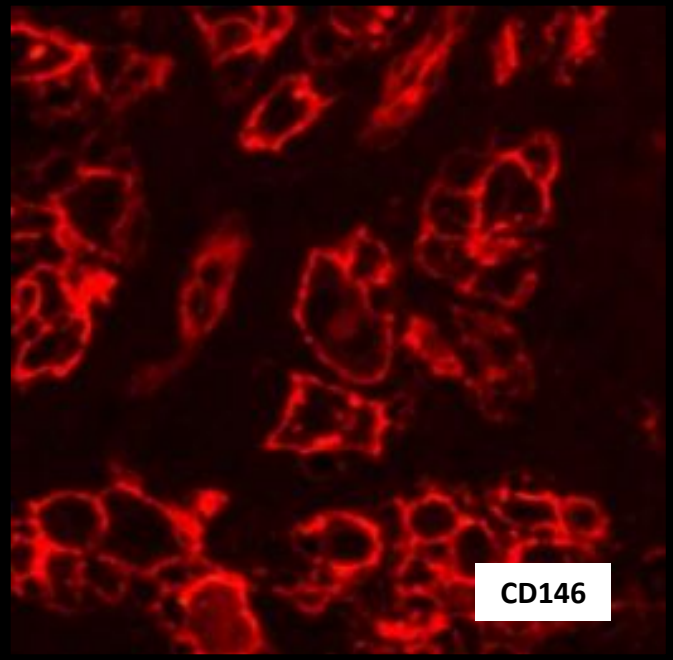
NGFR (MRQ21) + CD146 (EPR3208)

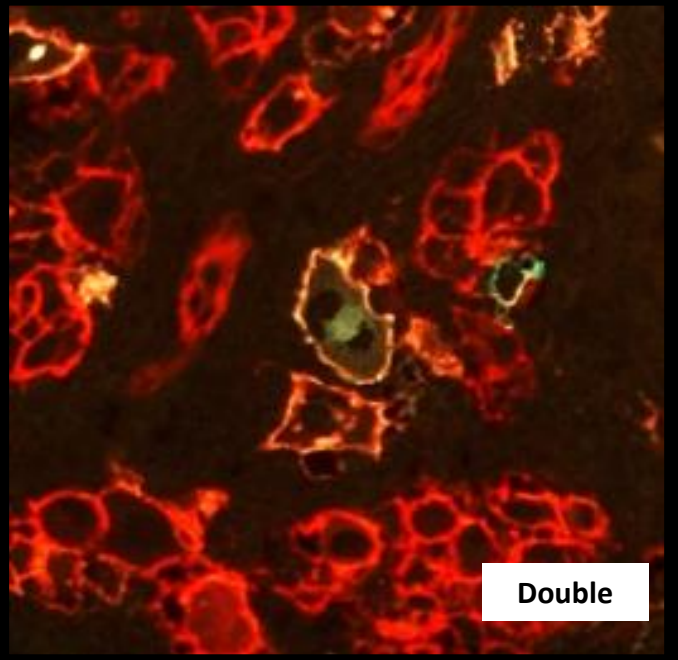
Immuno-fluorescence

Co-localization (yellow reaction product)









Simultaneous procedure (Immuno-Fluorescence):

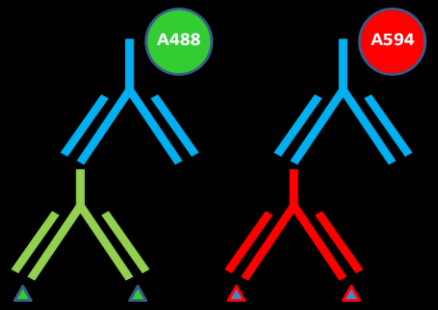
Pre-treatment (Antigen Retrieval)

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

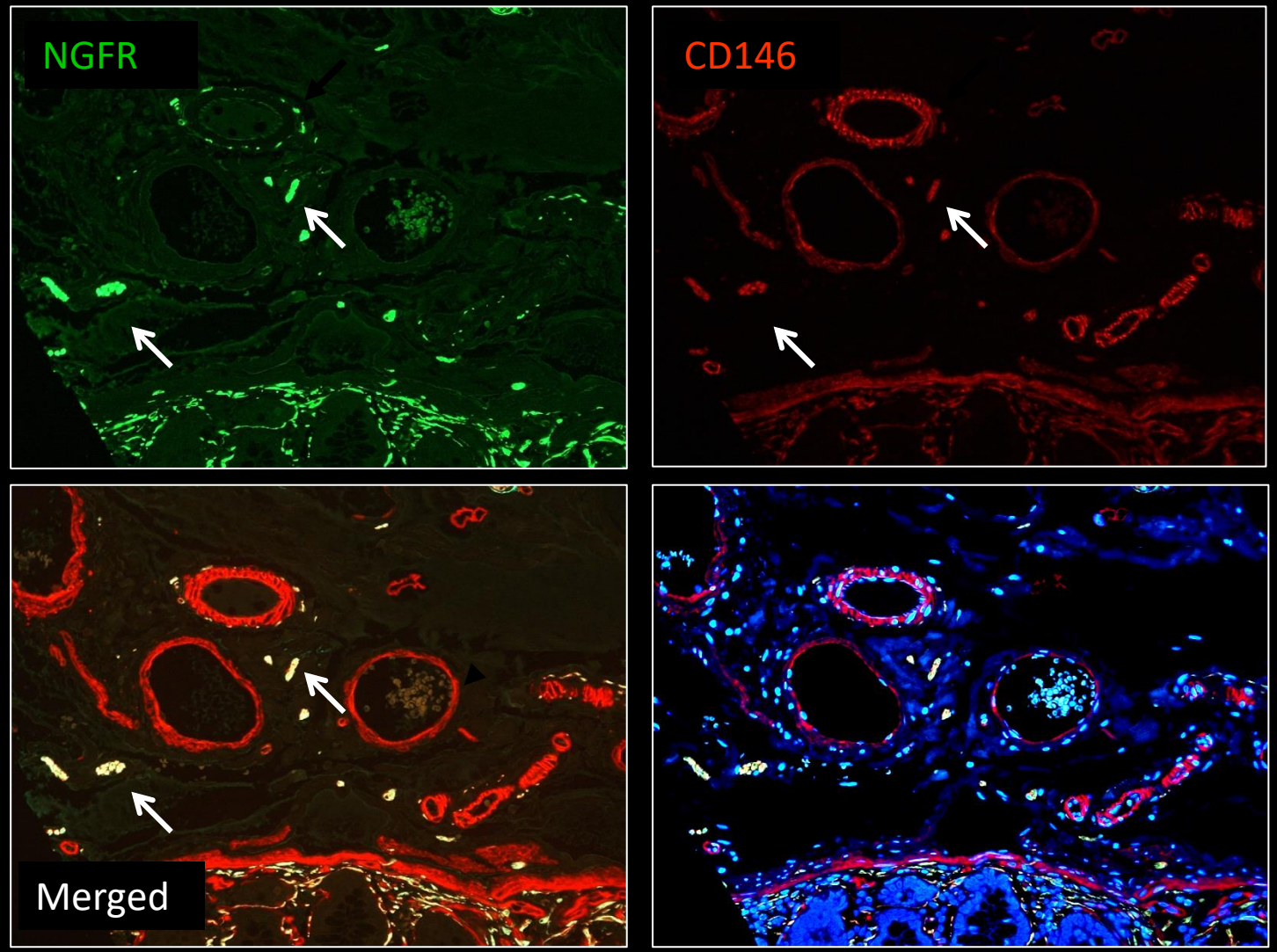
Incubation with Secondary Ab mix (Goat -anti Mab+Rab) (1h).

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

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

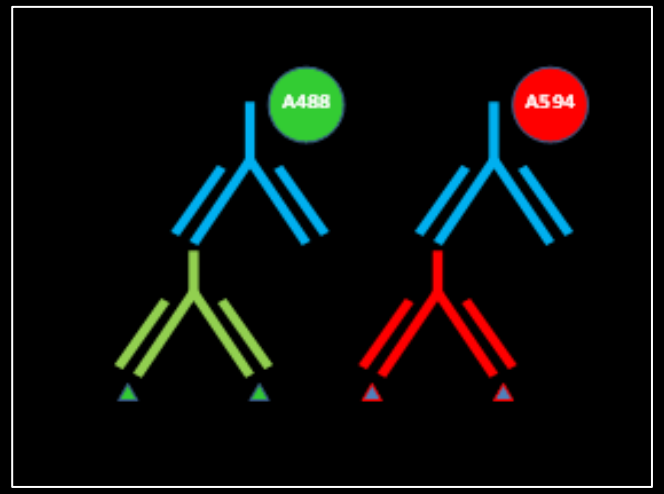


NGFR (Mab, MRQ-21) + CD146 (Rab, EPR3208)



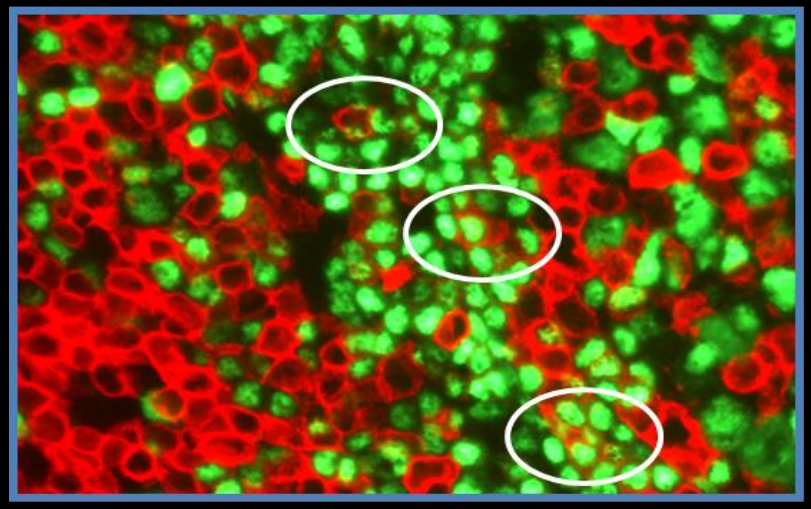
Colon: Co-localization of NGFR and CD146 in peripheral nerves and ganglion cells (yellow reaction product) (arrows).

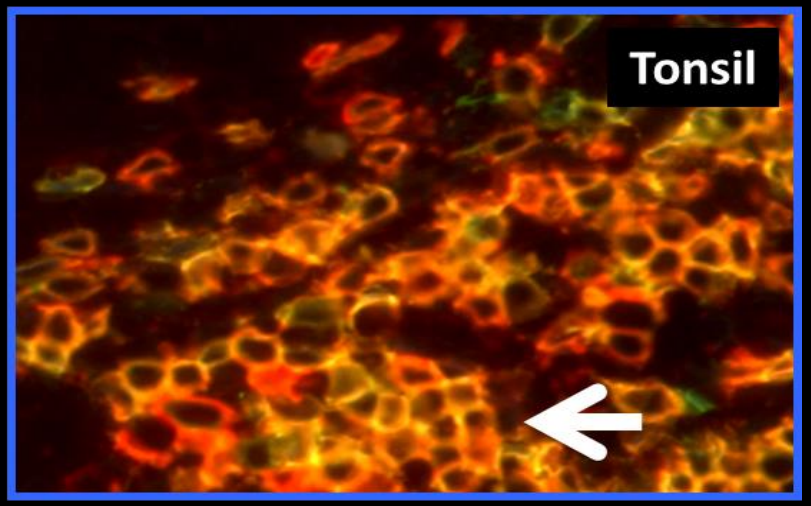
CD146 stains smooth muscles of peri-vascular structures and lamina muscularis mucosa .

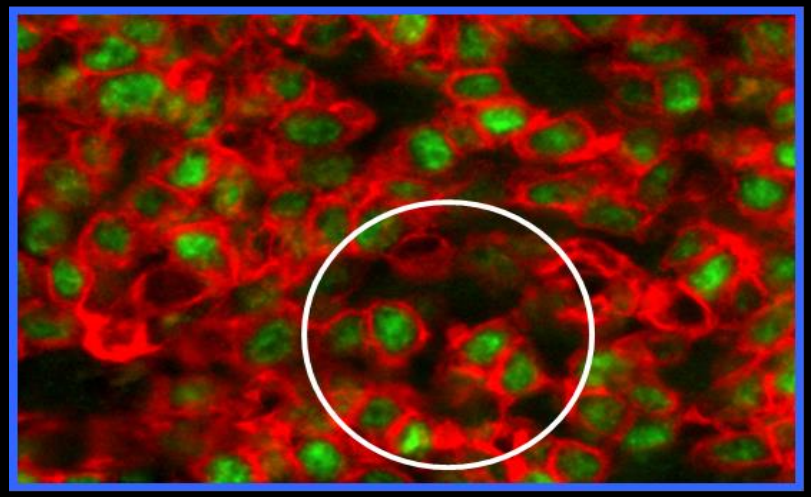


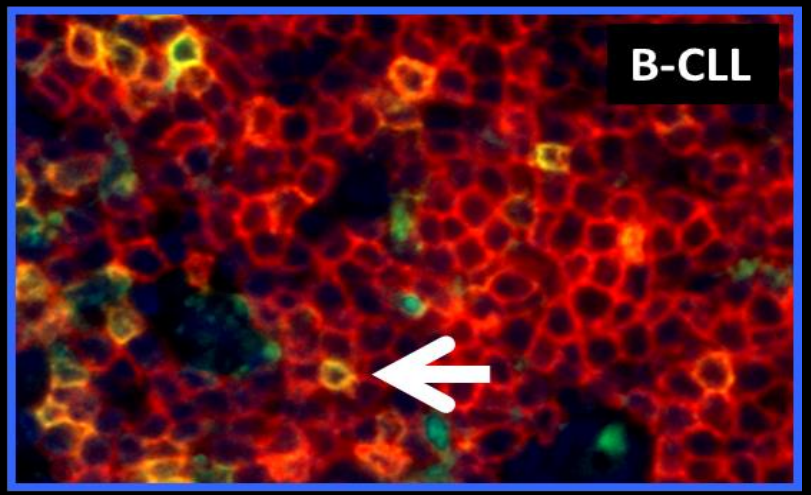
PAX-5, 1EW (TSA amp.) + CD5, SP19

CD3, PS1 + CD5, SP19



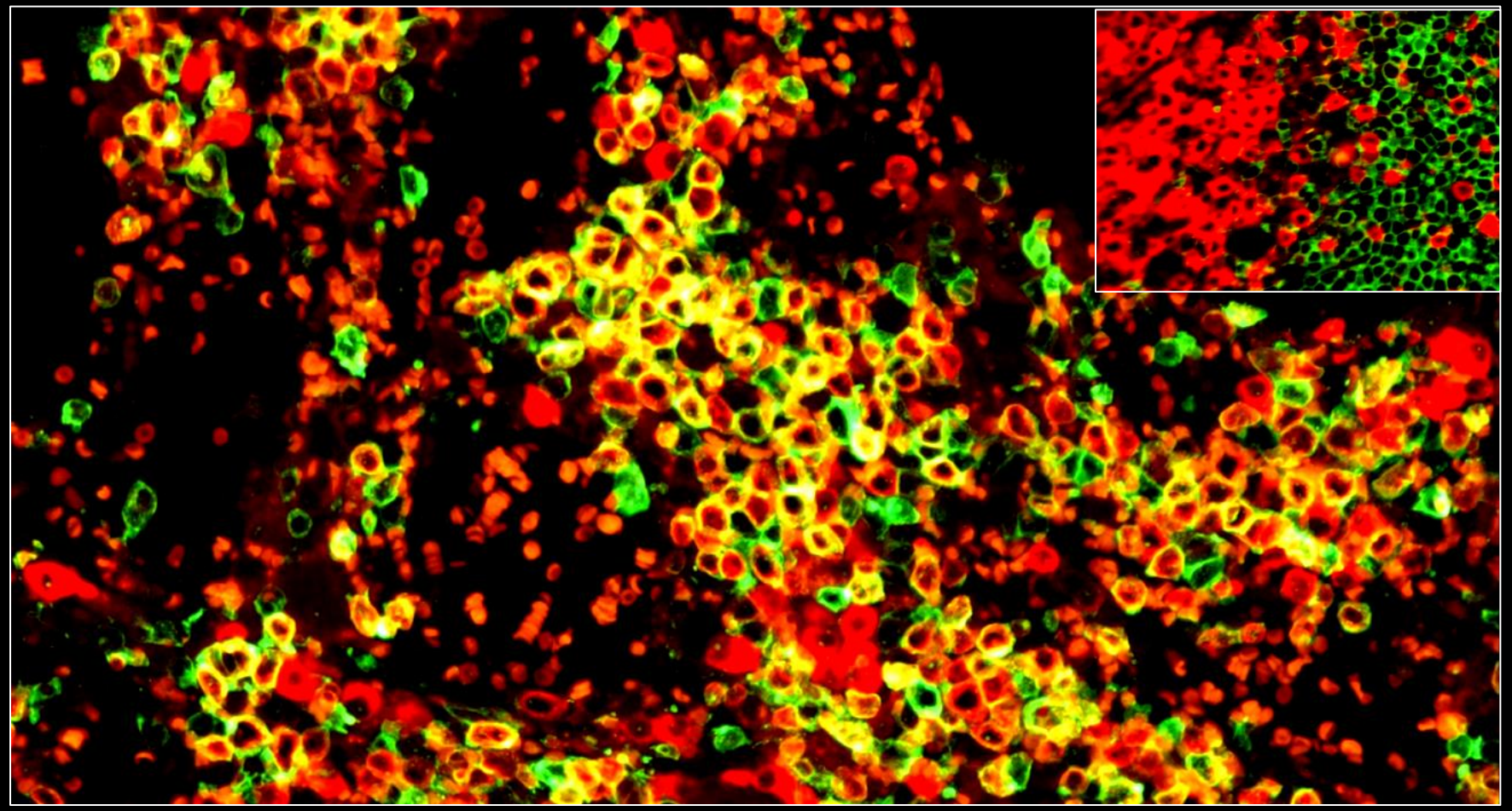






Note : Co-localization of normal T-cells in the tonsil and in the B-CLL (CD3+CD5) and co-expression of the neoplastic B-cell (Pax-5 and CD5) in the B-CLL and normal B-Cells in the mantle zone of the tonsil

CD79a (SP18) + CD7 (LP15)



PT : ALL (B-type)/BMT

Flowcytometry showed aberrant expression of CD7 in the 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

Tonsil (insert)

Fully automated 5-plex fluorescent immunohistochemistry (Ventana Discovery): Using same species antibodies

Principles: TSA-IF (multiplex)

Table 1 Primary antibodies (1°Abs)

Name	Recommended dilution in DAB staining	Species	Clone	Location	Dilution in 5-plex FL	Source
CD20 RUO	1:100	Rabbit	SP32	Membrane	1:140	Spring Bioscience, cat # M3324
FoxP3 RUO	1:100	Rabbit	SP97	Nucleus	1:50	Spring Bioscience, cat # M3974
CD68 RUO	1:100	Rabbit	SP251	Cytoplasm	1:20	Spring Bioscience, cat # M5514
CD3 RUO	1:150	Rabbit	SP162	Membrane	1:300	Spring Bioscience, cat # M4624
CD8 RUO	1:100	Rabbit	SP239	Membrane	1:100	Spring Bioscience, cat # M5394

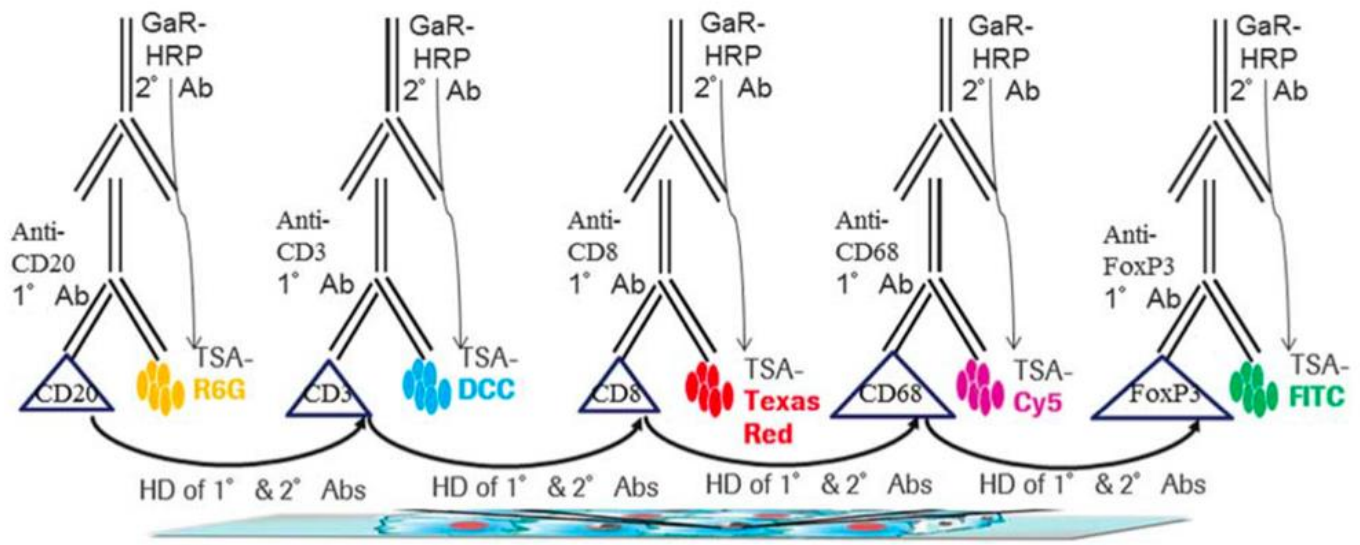


Figure 1 5-Plex IHC detection scheme.

Heat Deactivation (HD)/ Cross-talk controls important

Influence of HD on Fluorochromes/Epitopes

Detection order	TSA Fluor	Effect of HD on Fluor	1°Ab	Effect of HD on Epitope
1	R6G TSA	Lest Affected	CD20	Most Affected
2	DCC TSA		CD3	
3	Texas Red TSA		CD8	
4	Cy5 TSA		CD68	
5	FITC TSA	Most Affected	FoxP3	Lest Affected

High risk of cross-reactivity due to use of 5x rmAbs

Laboratory Investigation (2017) 97, 873–885

Optimizing sequential tri-plex method on the Discovery (Ventana) :

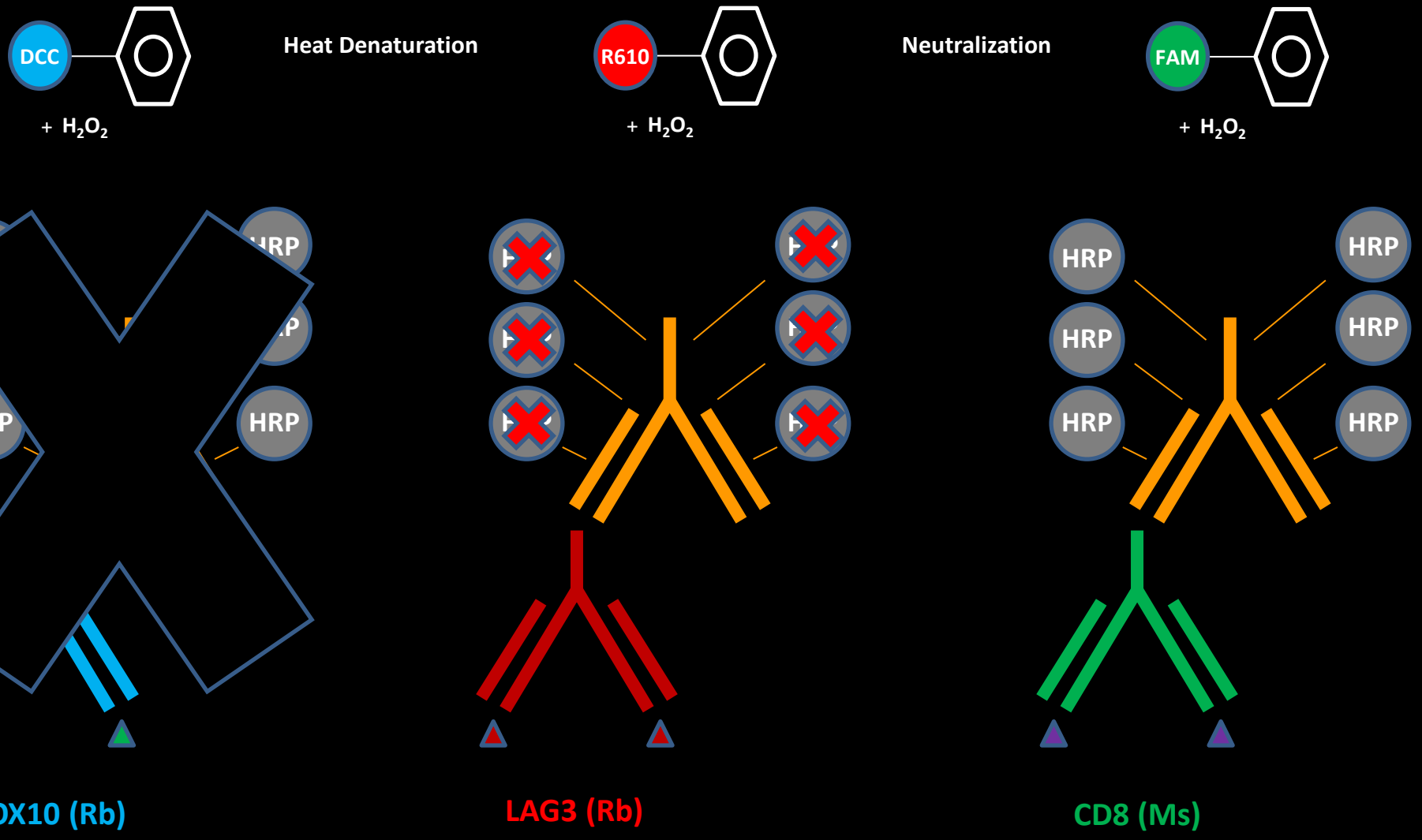
- ❑ SOX10, BS7 (Ms) or SOX10, SP267 (Rb) + LAG3, D2G40 (Rb) → first and second sequence, respectively
- ❑ CD8, C8/144B (Ms) (or all other markers/mouse and rabbit antibodies) → third sequence

Elimination of Cross-reactivity

- ❑ Neutralization step (Disc. Inhibitor) is applied between sequences using different hosts of the primary Abs
  - Eliminates HRP activity of introduced HRP-conjugated detection reagents
  - No false positive reactions with the next seq. of visualization system (TSA-fluorochrome)

- ❑ Heat Denaturation step (CC2/100C/24`) is applied between sequences using same host of the primary Abs
  - Eliminates cross reactivity by elution/denaturing introduced primary Abs and detection systems.
  - No false positive reactions with the next seq. of primary Abs/detection systems

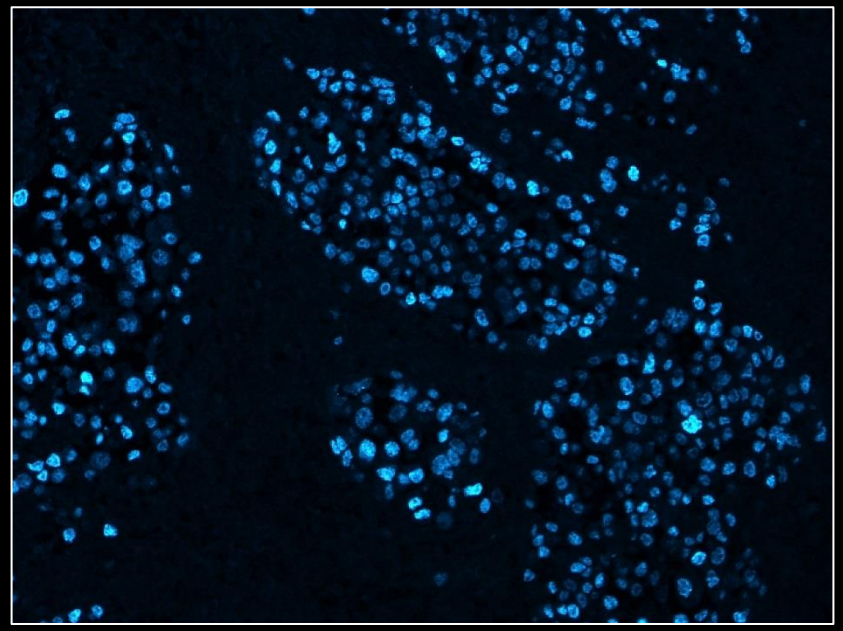
Optimizing a 3-plex method : How it could look like

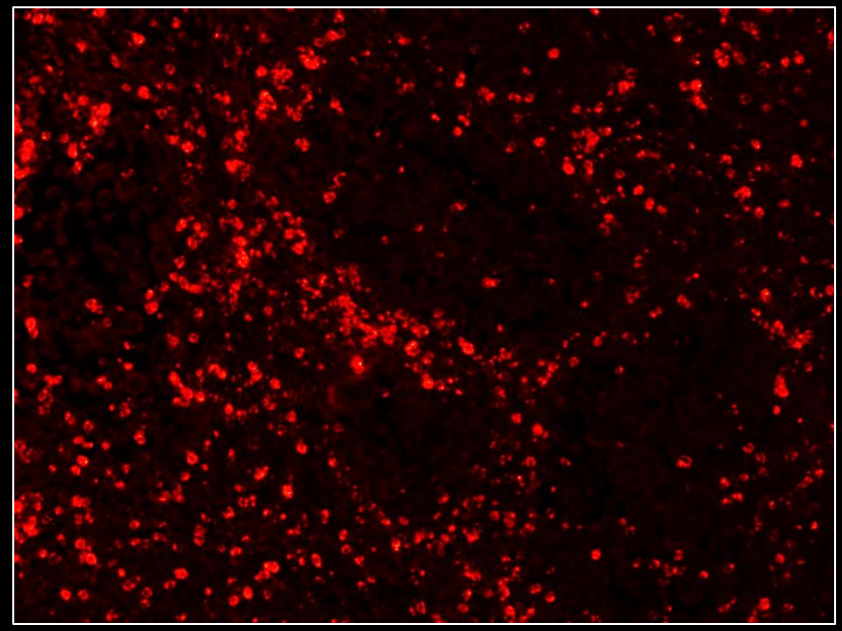


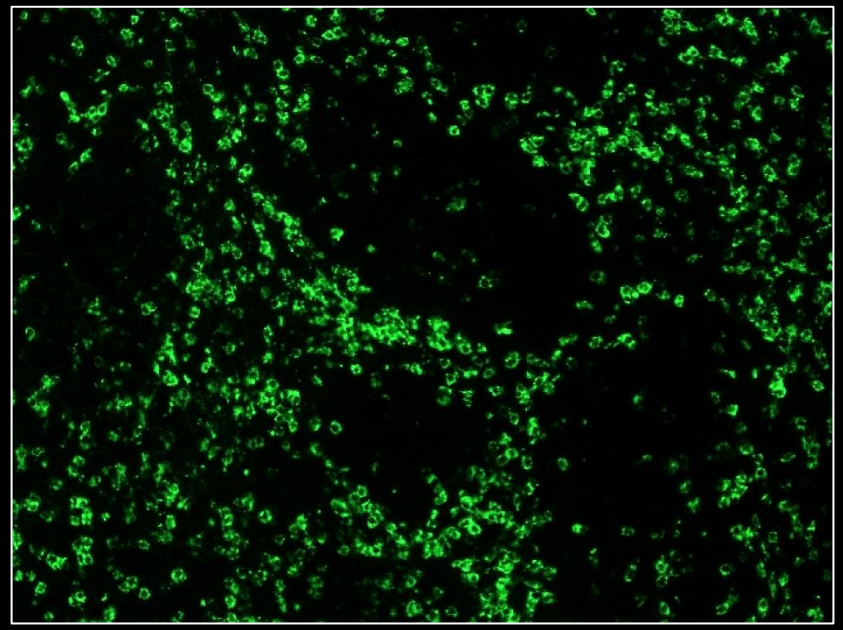
Melanoma

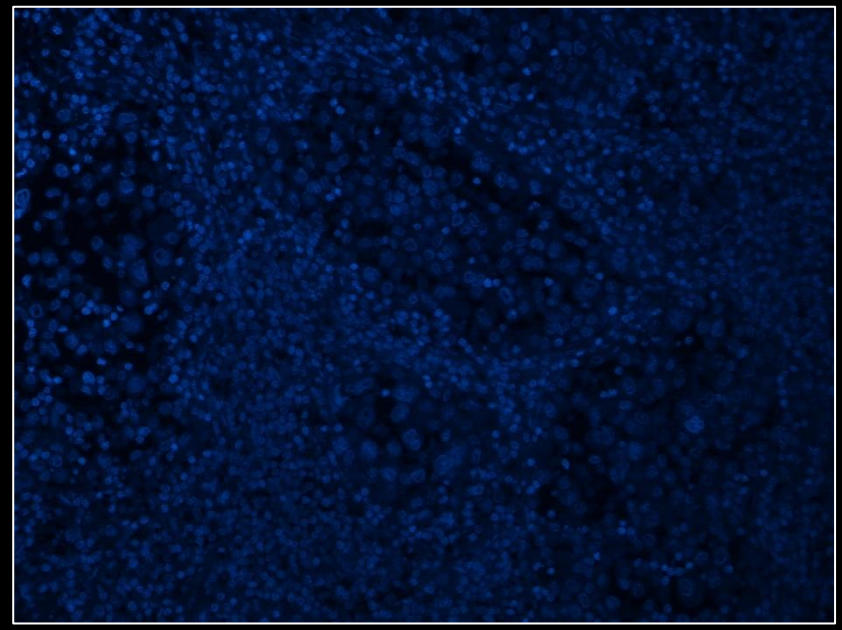
CC1 48`/95C

SOX10, SP267 (DCC) - HD
LAG3, D2G40 (Red610) - N
CD8, C8/144B (FAM)
DAPI







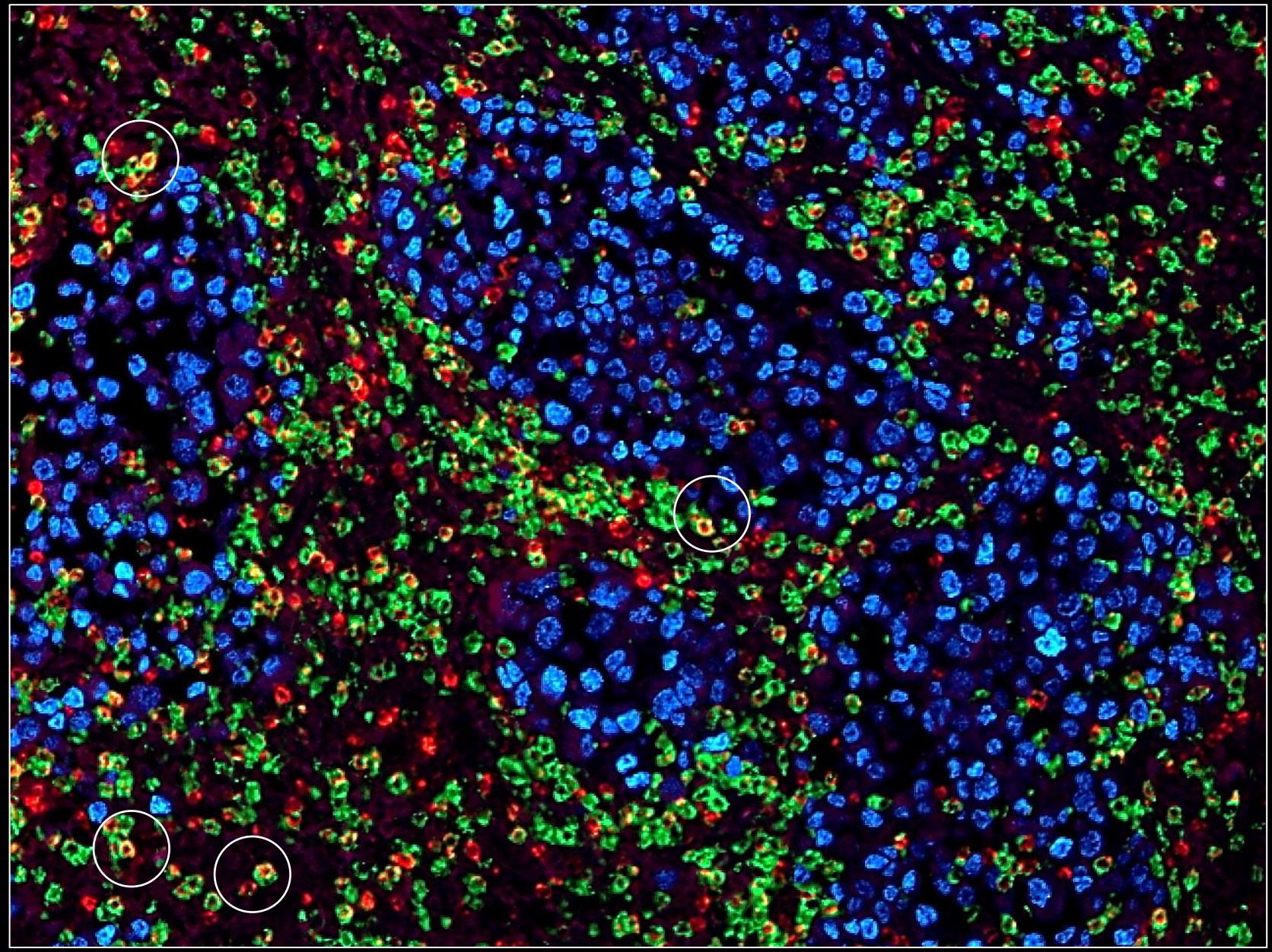


Co-localized signals ?

Melanoma

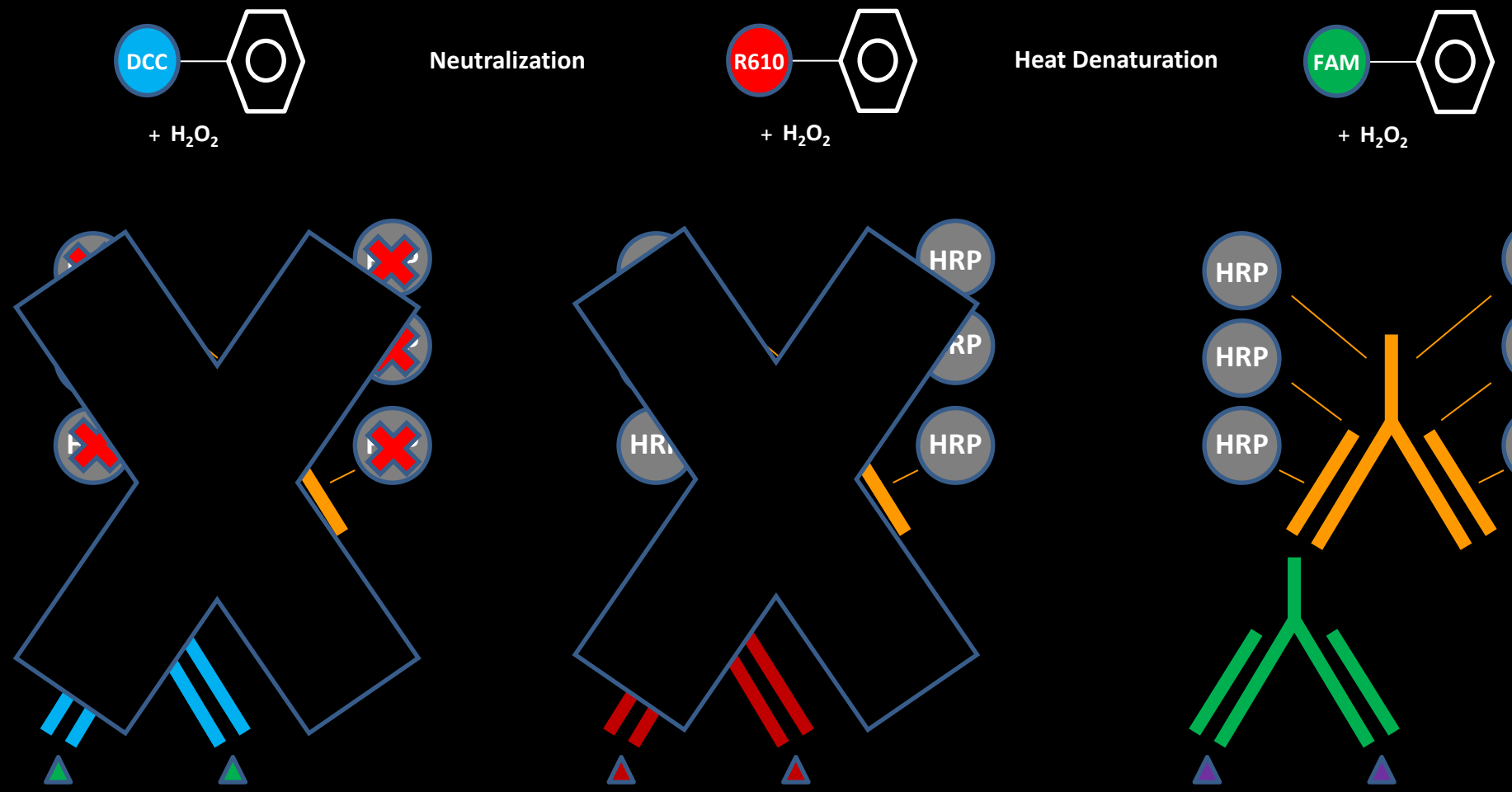
CC1 48`/95C

SOX10, SP267 (DCC) - HD
LAG3, D2G40 (Red610) - N
CD8, C8/144B (FAM)



Co-localized signals

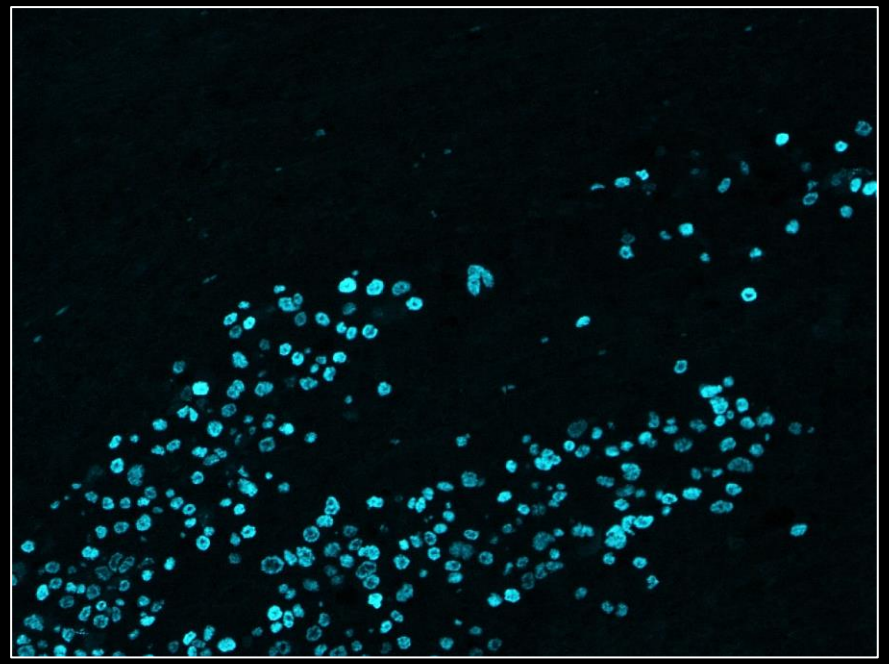
Optimizing a 3-plex method : How it could look like

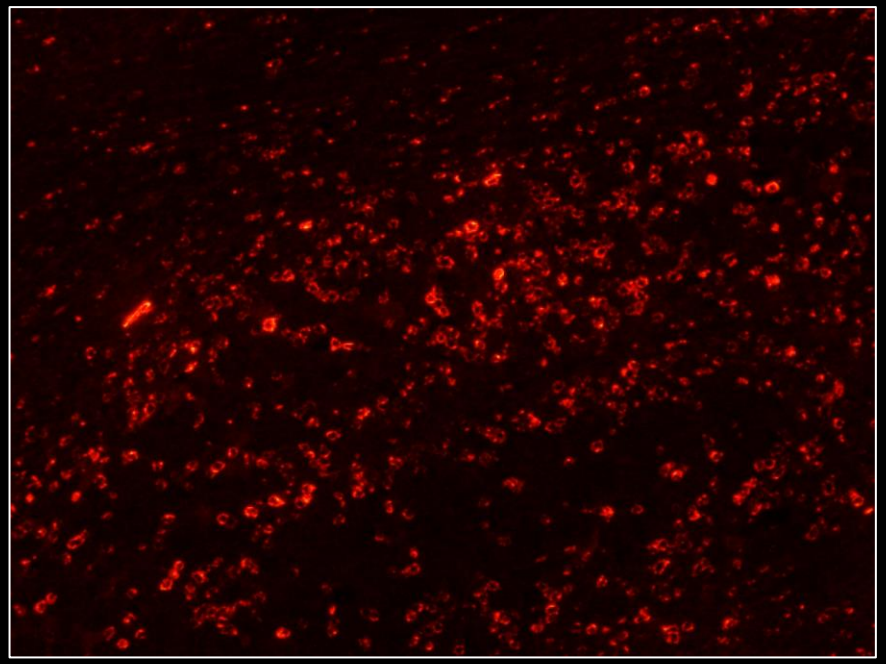


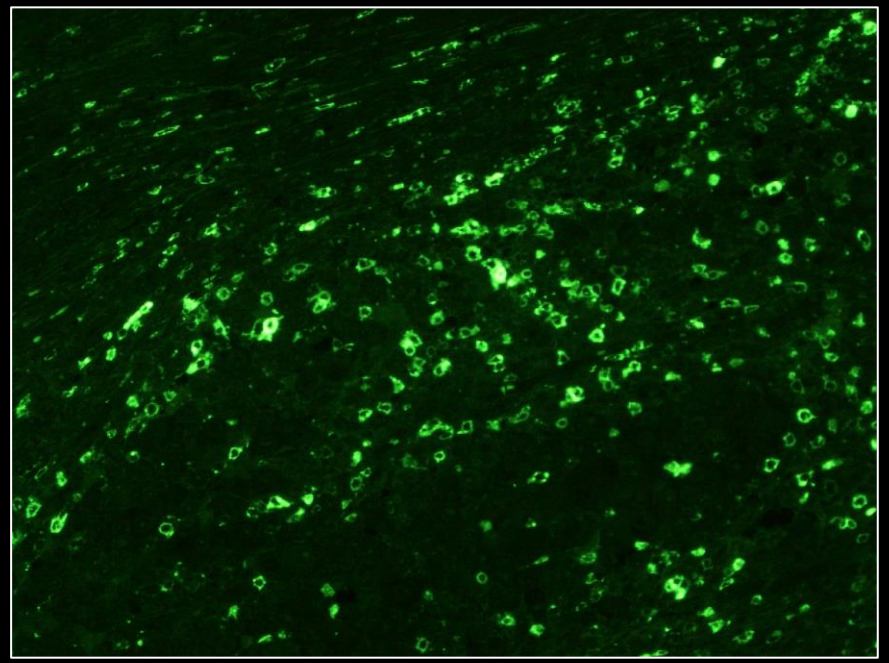
Melanoma

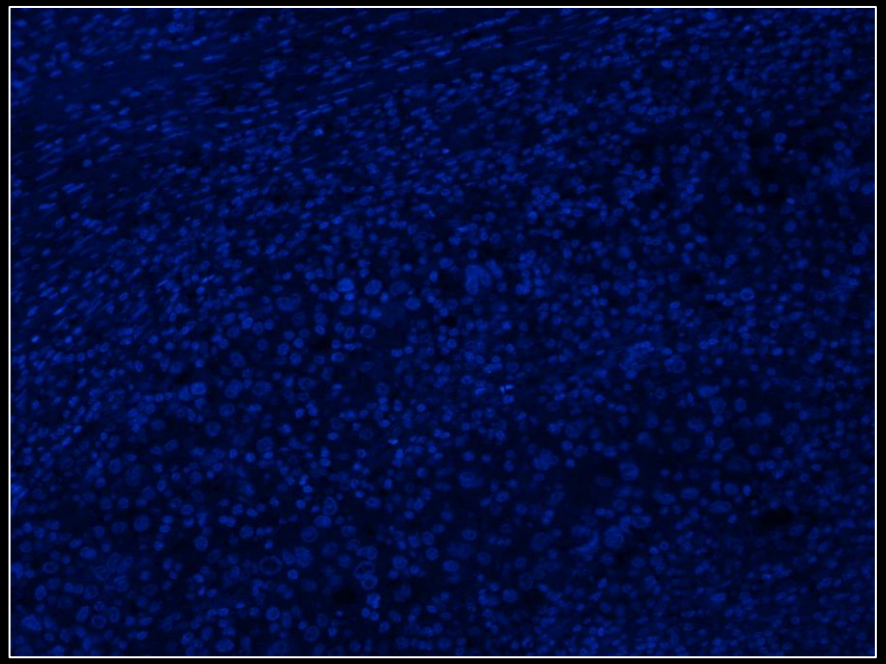
CC1 48`/95C

SOX10, BS7 (DCC) - N
LAG3, D2G40 (Red610) - HD
CD4, EP204 (FAM)
DAPI









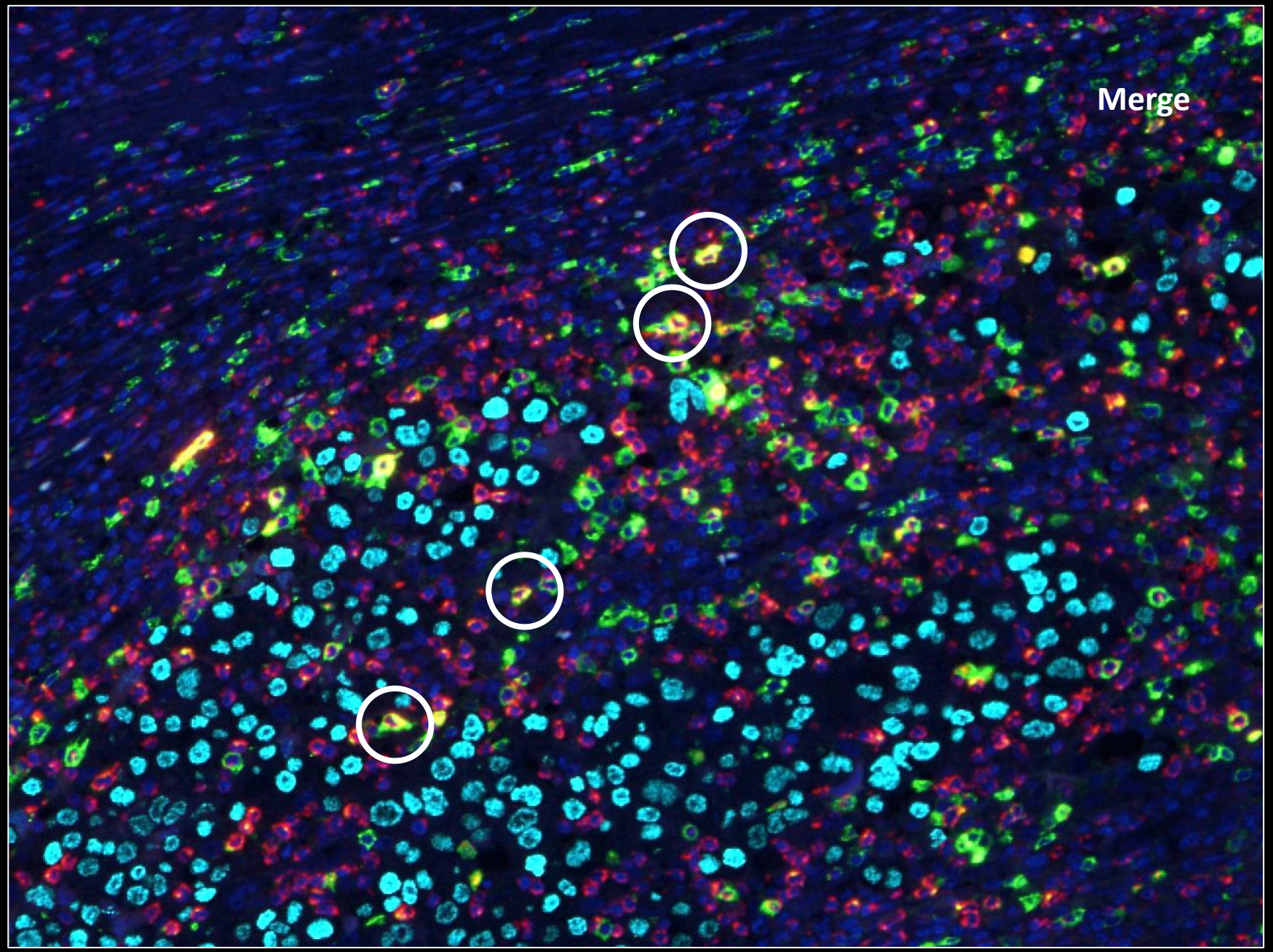
Co-localized signals

Melanoma

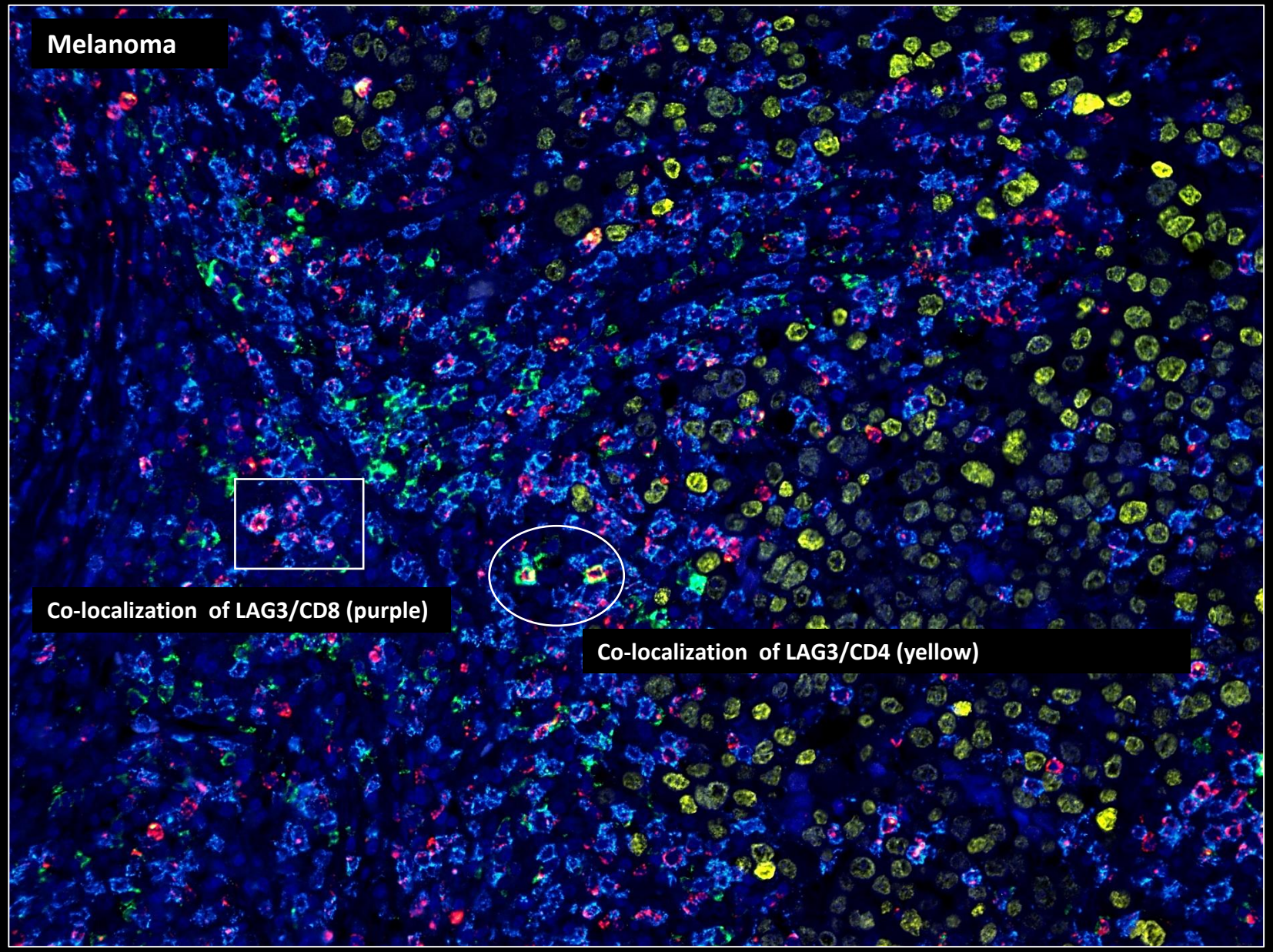
CC1 48`/95C

SOX10, BS7 (DCC) - N
LAG3, D2G40 (Red610) - HD
CD4, EP204 (FAM)
Dapi

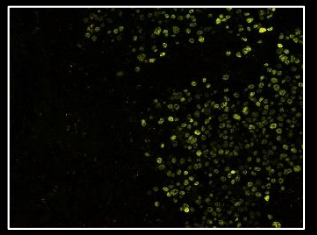
Co-localized signals



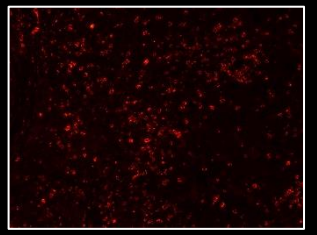
4-Plex: SOX10, BS7 (Ms) + N + LAG3 (Rb) + HD + CD8 (Ms) + N + CD4 (Rb)



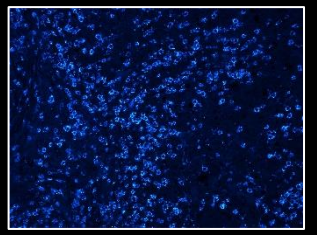
SOX10



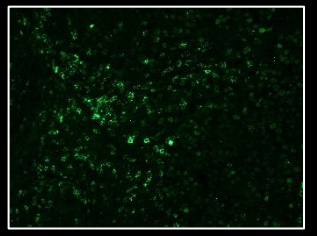
LAG3



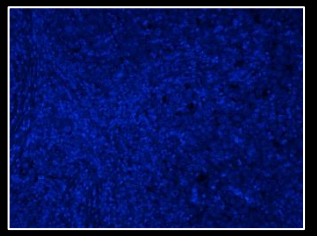
CD8



CD4



DAPI



Optimizing a 3-plex method : Cross-talk controls

Cross-talk controls are important detecting unspecific staining reactions (e.g., cross-reactivity)

Antibody stripping is not always 100% efficient. It is important to empirically determine if previously bound antibodies are removed/denatured appropriately, as to ensure that the signals are not cross-talk

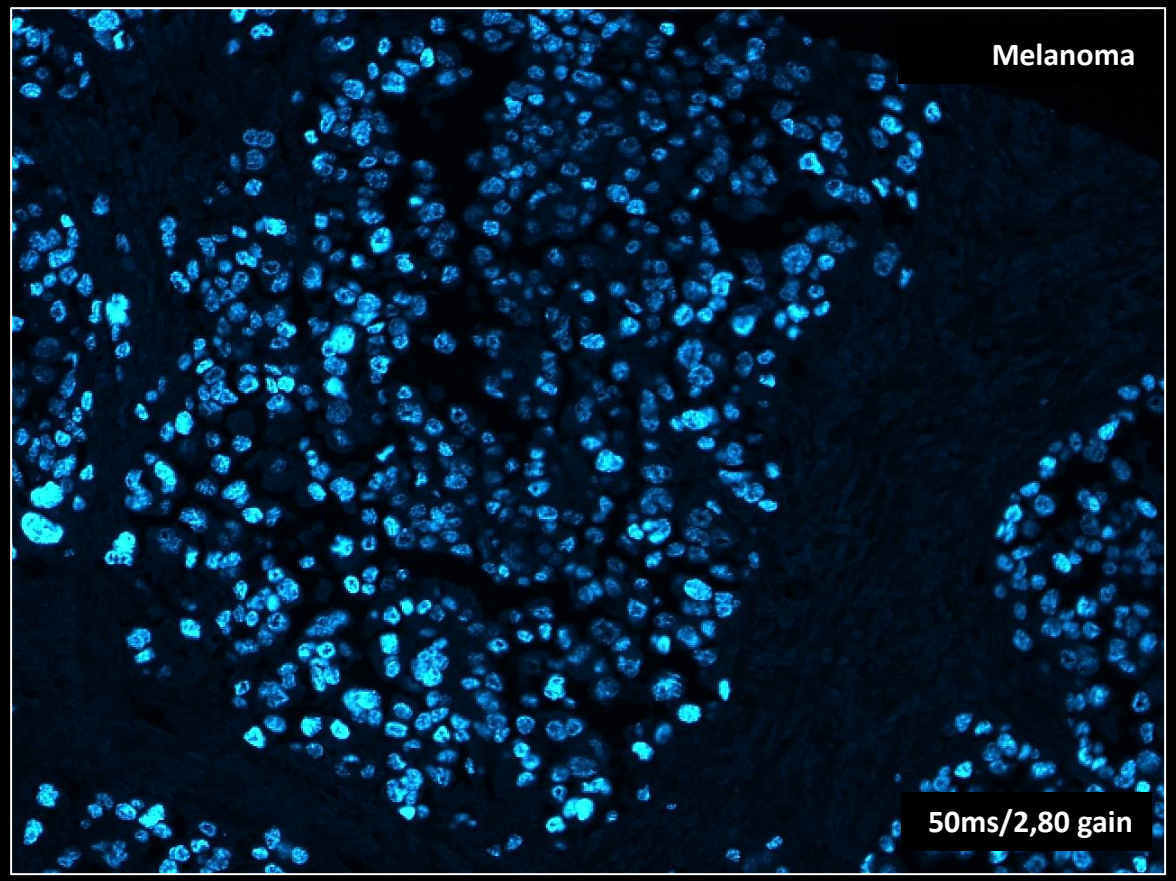
Neutralization control (N) : The neutralization step should eliminate HRP activity of introduced detection systems

Heat deactivation control (HD): The Heat Deactivating step should eliminate cross-reactivity between sequences of introduced immuno-reagents (primary Abs and detection systems)

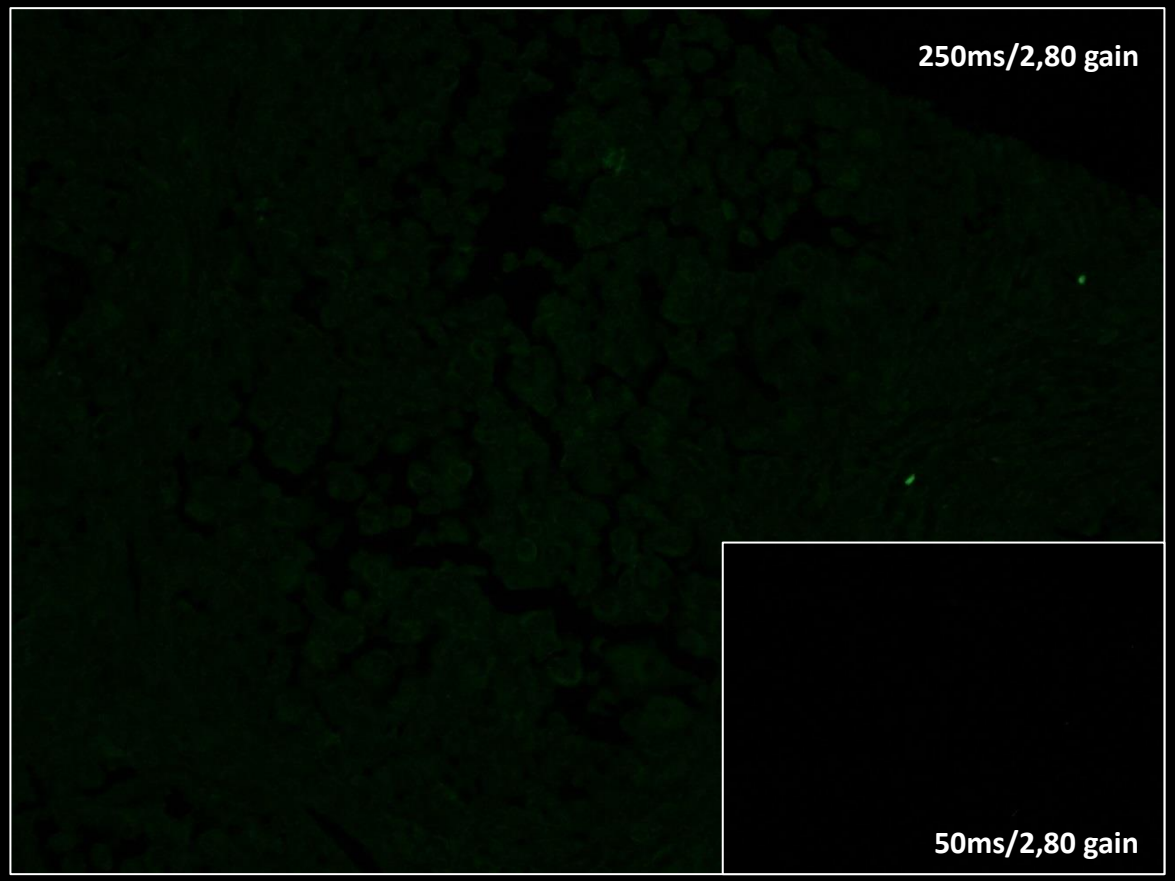
Cross-talk controls: Cross your fingers , otherwise you have a lot of work ahead

Cross-talk controls: Neutralization using Discovery Inhibitor (Ventana)

SOX10, BS7/OmniMap Ms-HRP/DCC

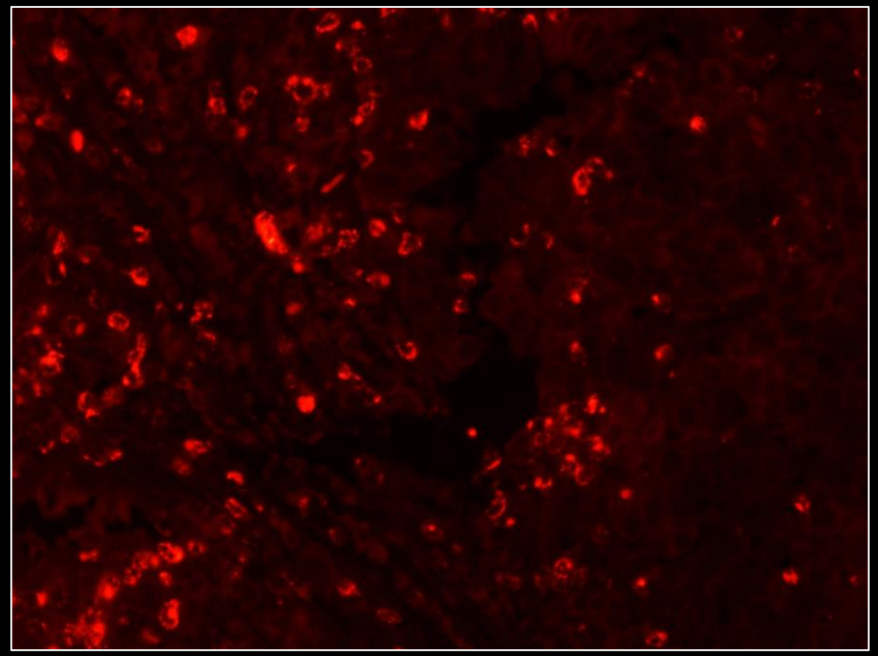


SOX10, BS7/OmniMap Ms-HRP/ Neutralization/ TSA-FAM

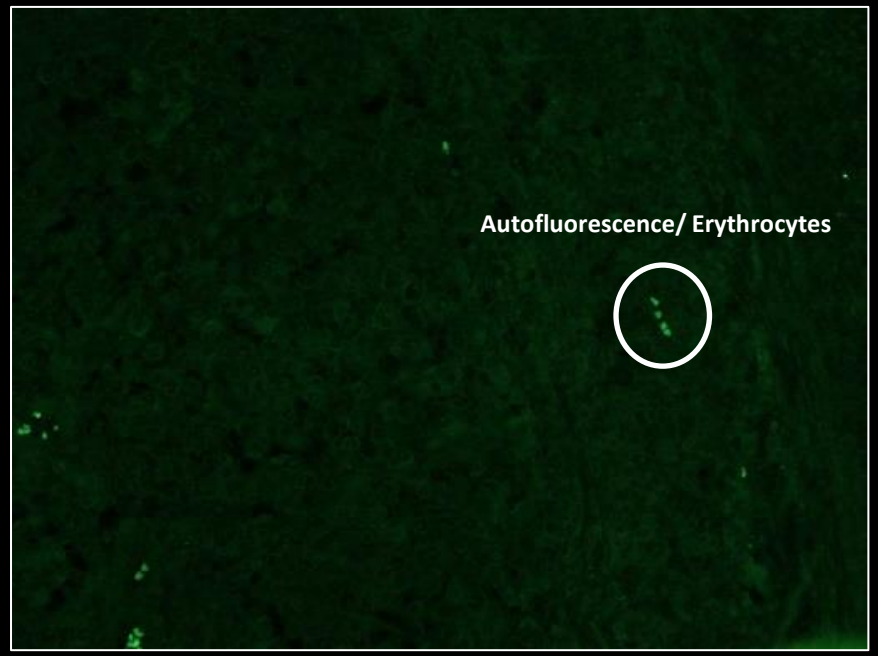


The neutralization step (Discovery Inhibitor) eliminate HRP activity of the detection system. Same effect was obtained using a HD step.

Cross-reactivity: “Drop out control”



Normal staining of LAG3, D2G40



HD control using “drop out”

HD control (Drop out):

LAG3, D2G40 (Rb)

OmniMap anti Rb/HRP

Omission of TSA-Fluorochrome

Heat Denaturation (HD)

Omission of second Ab (Diluent)

OmniMap anti Rb/HRP

TSA-FAM

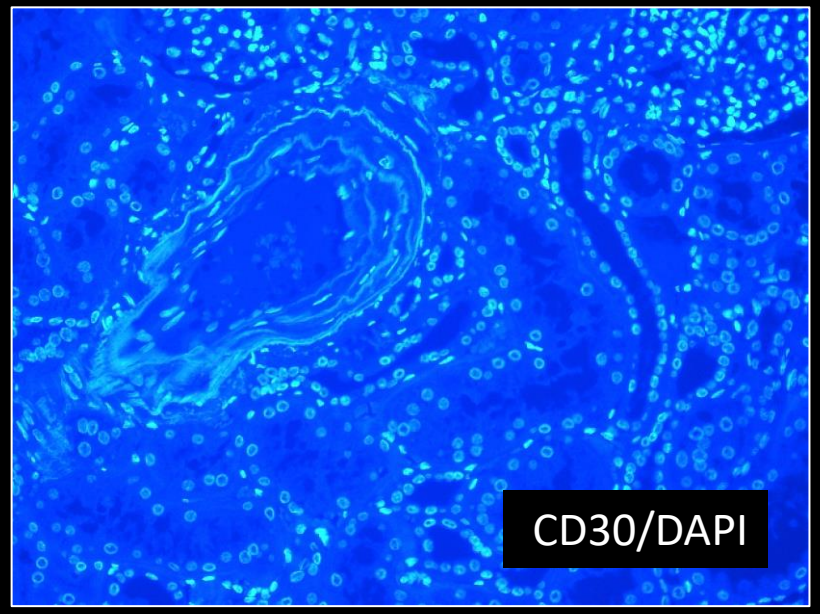
DAPI

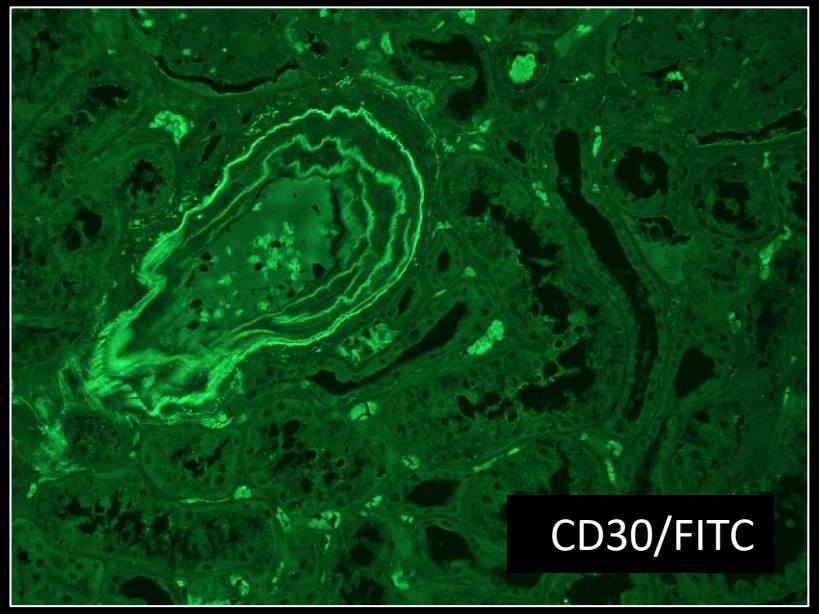
Note: No cytoplasmic/membraneous staining reaction (FAM) of lymphocytes (cross-reactivity with LAG3)

Successful HD step: Efficient elimination of LAG3, D2G40 sequence

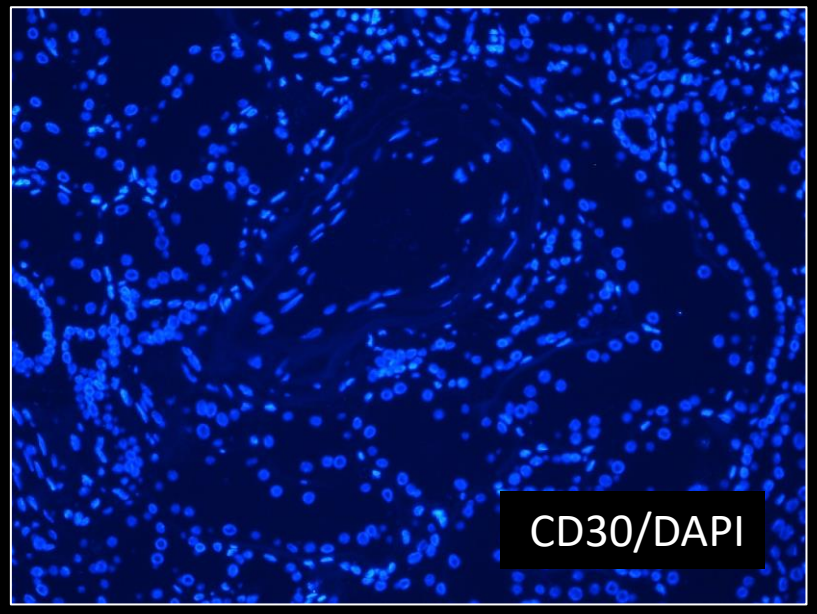
Quenching Autofluorescence

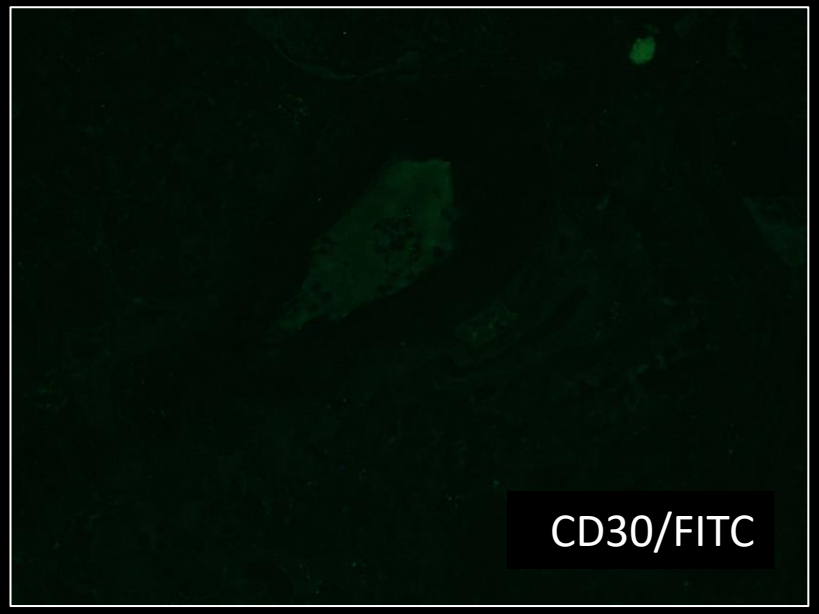
No quenching





Quenching using
TrueView 5` (Vector Lab)

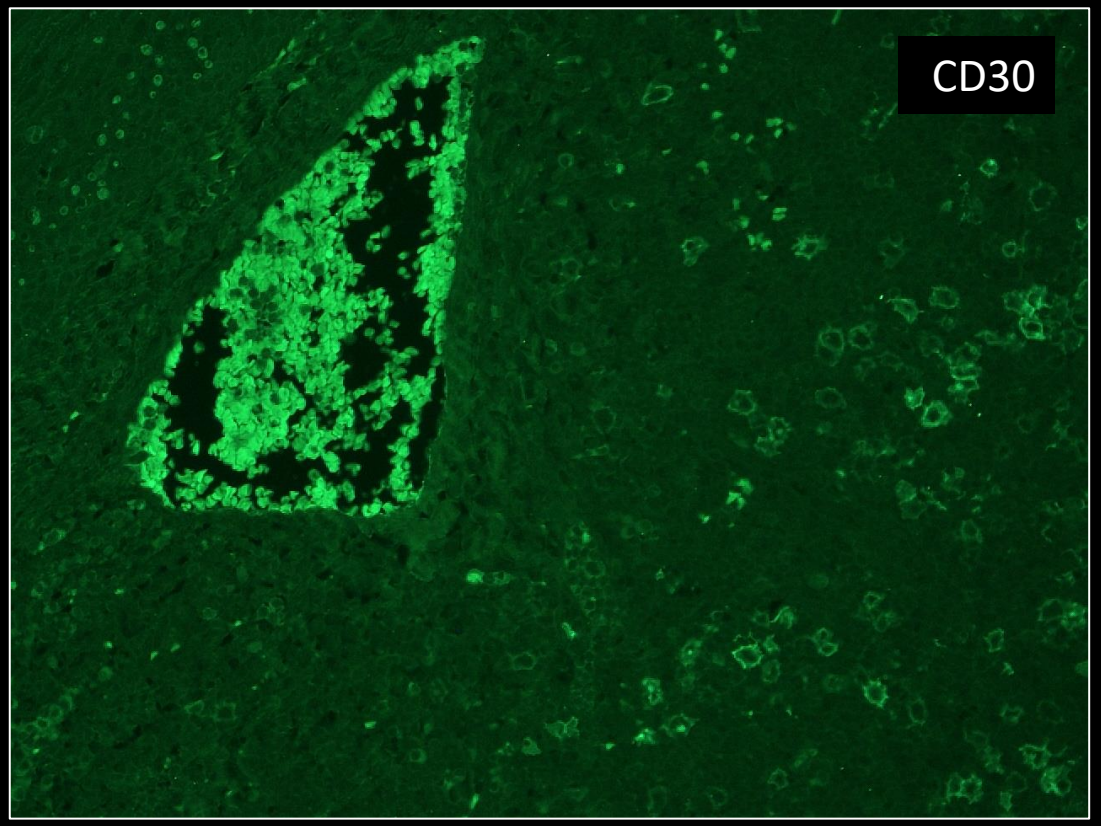




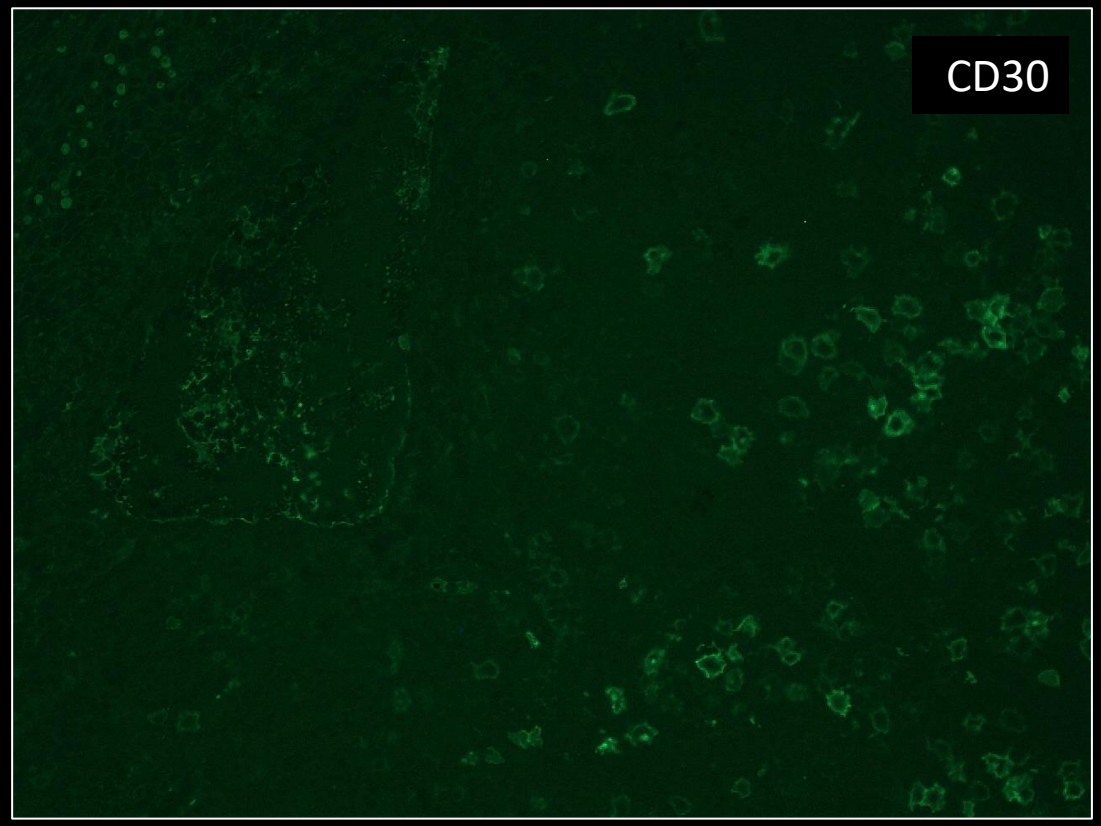
Kidney/ Microscope settings identical

Quenching Autofluorescence

Without TrueView 5`



With TrueView 5` (Vector Lab)



Quenching autofluorescence also seems to reduce the specific signal (time in TrueView needs to be adjusted)

Tonsil/ Microscope settings identical

Multiplex using chromogens

Co-localized signal

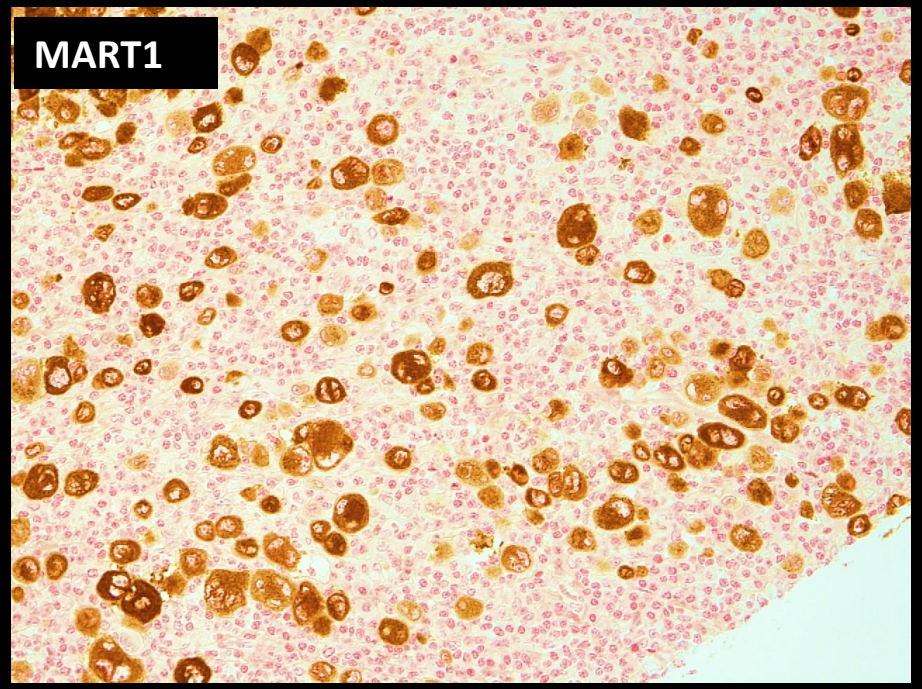
Simple technique

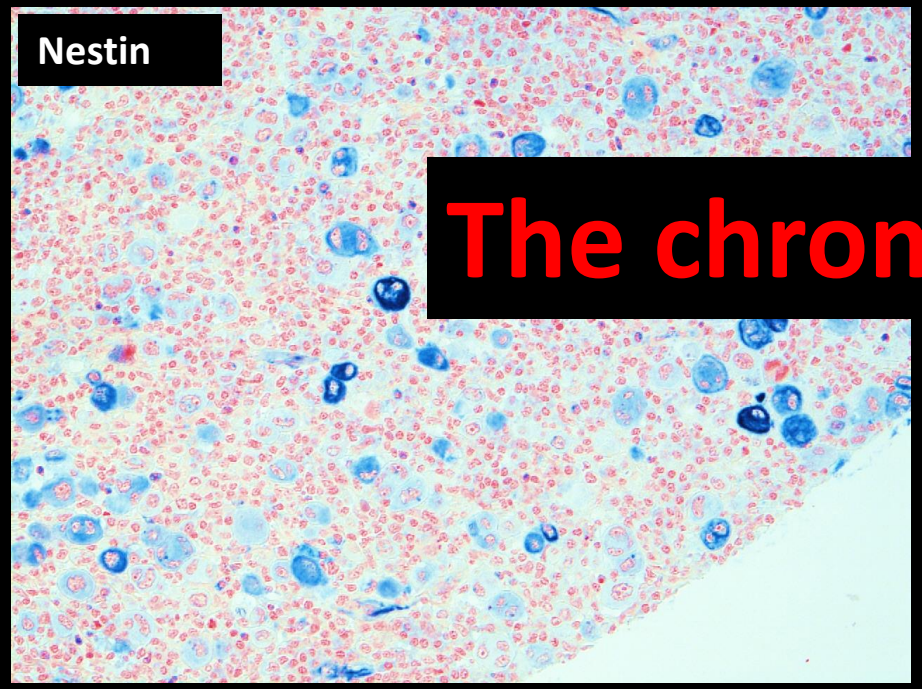
Use of new translucent chromogens

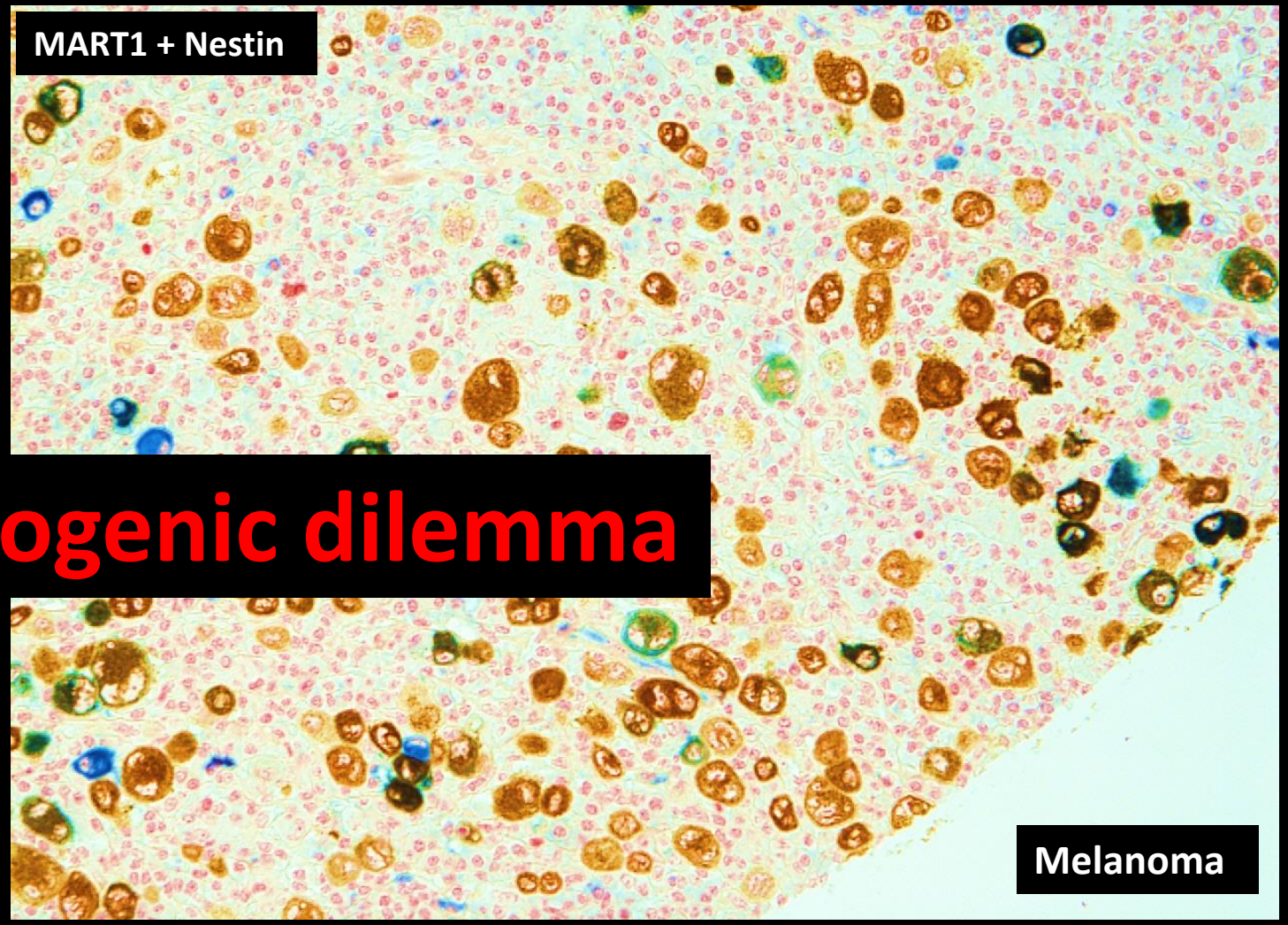
Simultaneous procedure: MACH2 Double Staining 1

MART1, EP43 (1:100 RR) + Nestin, 10C2 (1:100 RR)

Hidef Yellow (Enzo) – Ferangi Blue (Biocare)







The chromogenic dilemma

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Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

SIMPLE: A Sequential Immunoperoxidase Labeling and Erasing Method

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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.

Simple technique : A relative new method which cannot only be used to demonstrate antigens located in different cellular compartments but also can be used to demonstrate co-localized antigen in the same cellular compartment.

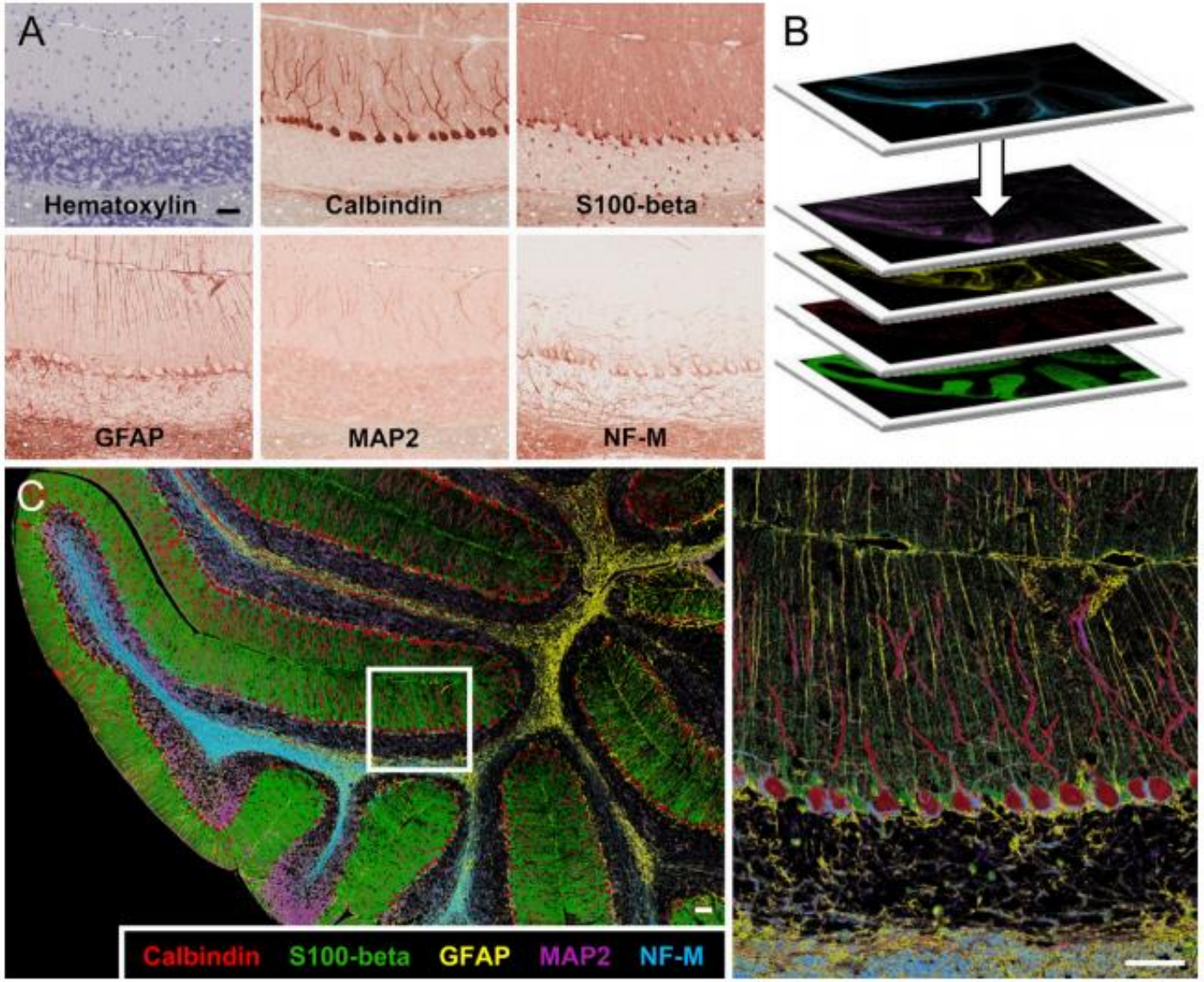


Figure 3 Simultaneous visualization of five antigens in mouse cerebellum. **(A)** Adult mouse brain was counterstained with hemotoxylin, then sequentially probed with polyclonal antibodies to calbindin, S100-β, and GFAP, and monoclonal antibodies to MAP2 (AP18) and neurofilament (NF-M) 2H3. **(B)** The 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.

Pseudo-colored and overlaid

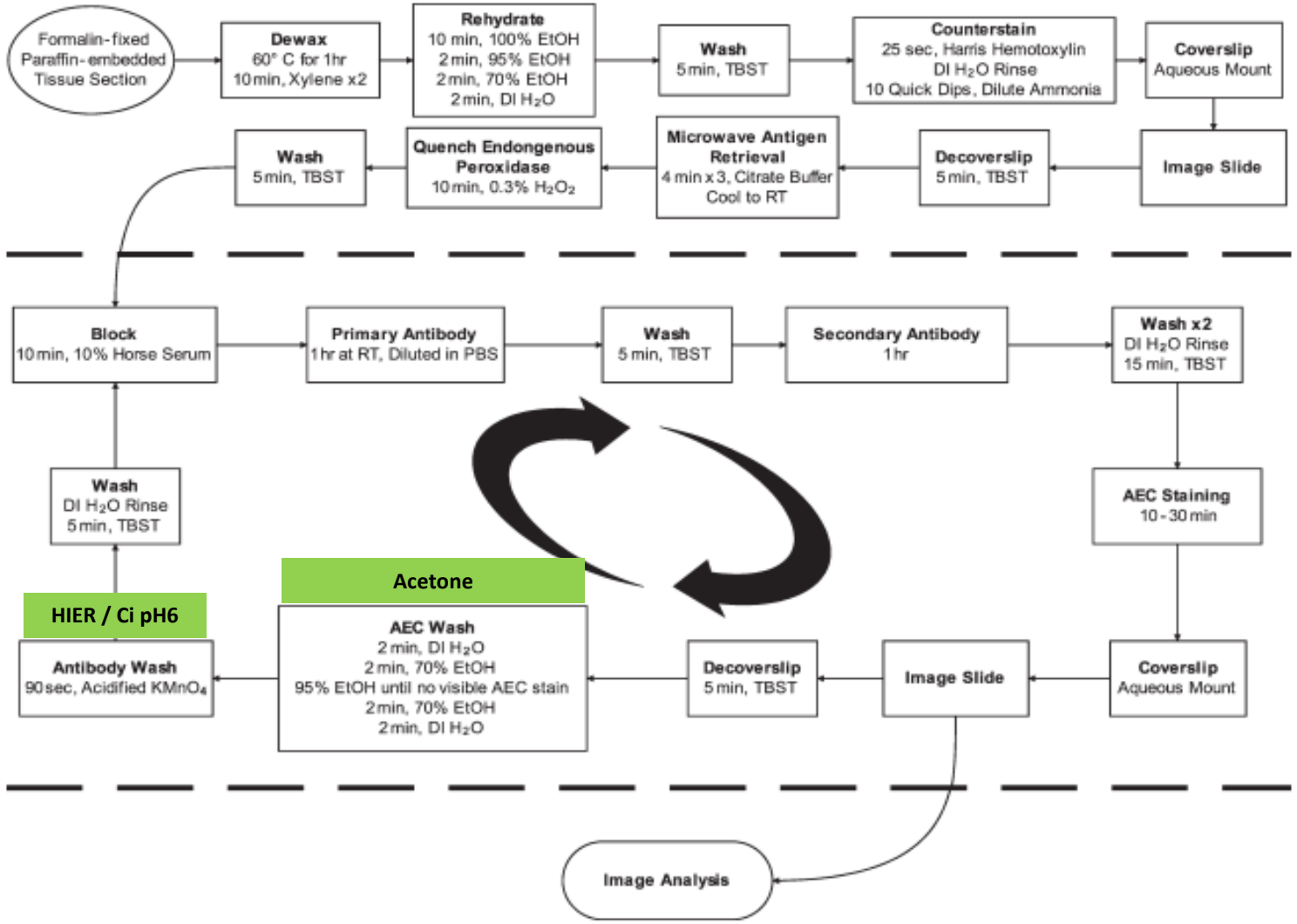


Figure 1 SIMPLE strategy. Formalin-fixed, paraffin-embedded sections are dewaxed, rehydrated, and counterstained before initial probing. Tissue is imaged and then subjected to antigen retrieval, removing the counterstain. Each staining round is conducted using standard immunohistochemical protocols with 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

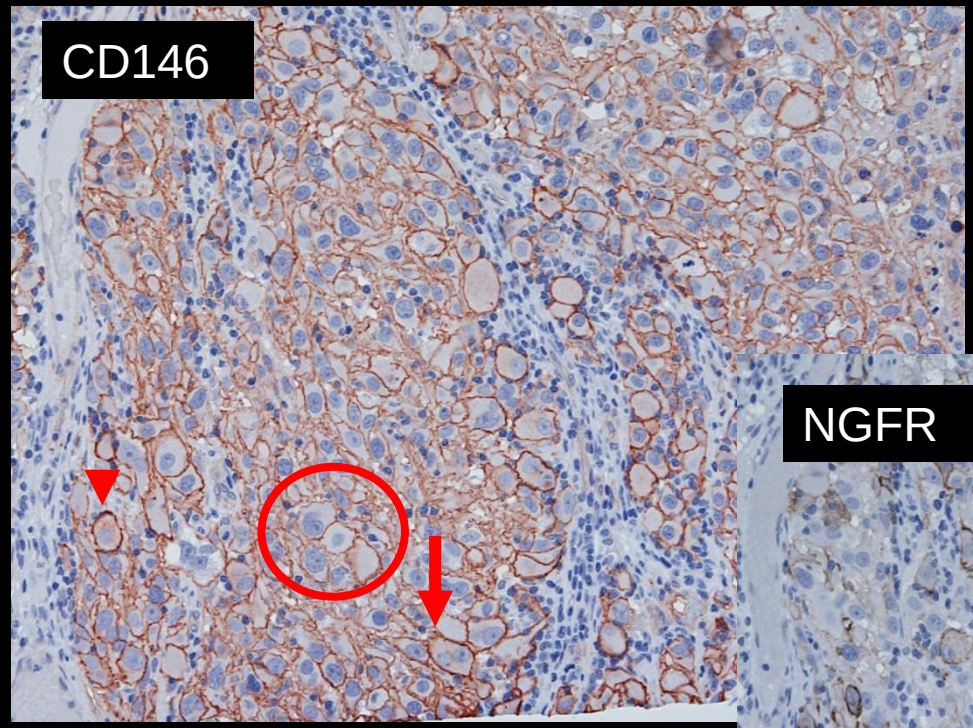
Imaging of slides

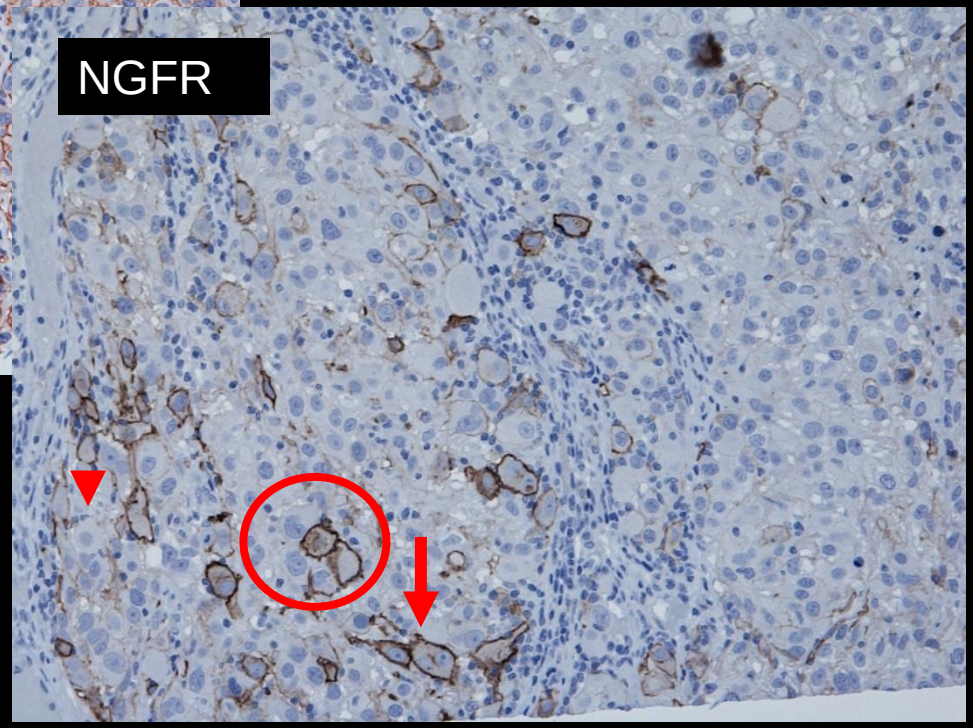
Removal of coverslip

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

Efficient blocking/elution procedure for the immuno-reagents applied

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





Super Sensitive (Biogenex)
Impact AEC (Vector Lab)

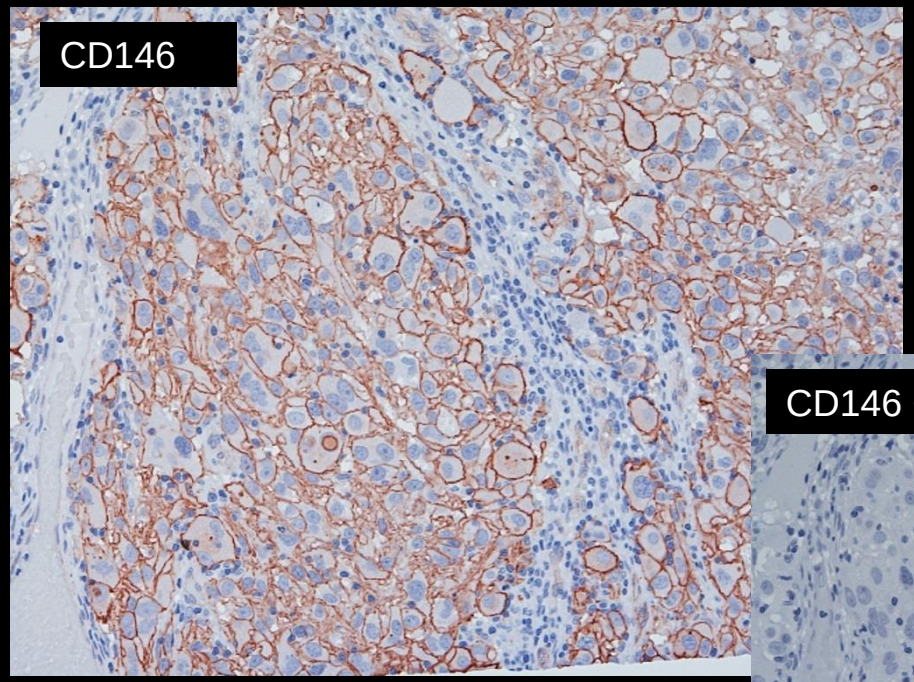
First sequence: CD146 immunostaning => AEC Impact => coverslip

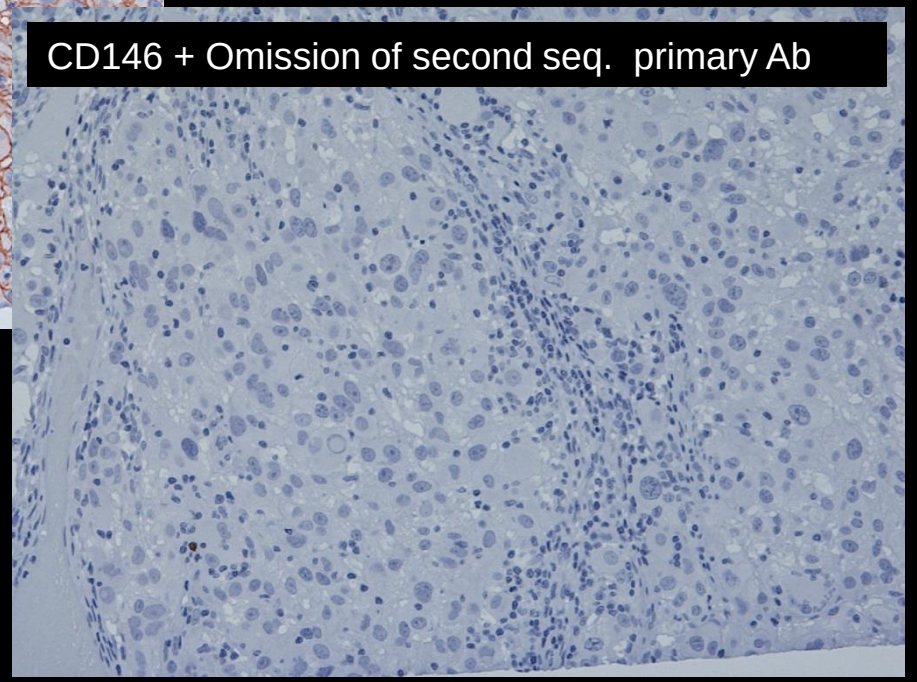
Imaging => de- coverslip => Erasing (Acetone) => Blocking using HIER (Ci)

Second sequence: NGFR immunostaning => DAB => coverslip

Melanoma

SIMPLE technique: Cross-reactivity control





Control experiments:

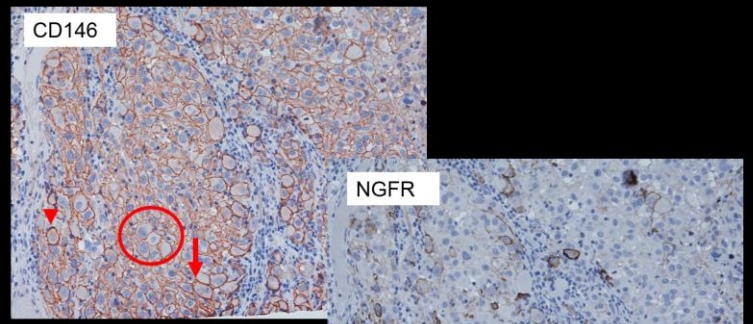
First sequence: CD146 immunostaining => AEC Impact => coverslip

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

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

Melanoma

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



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

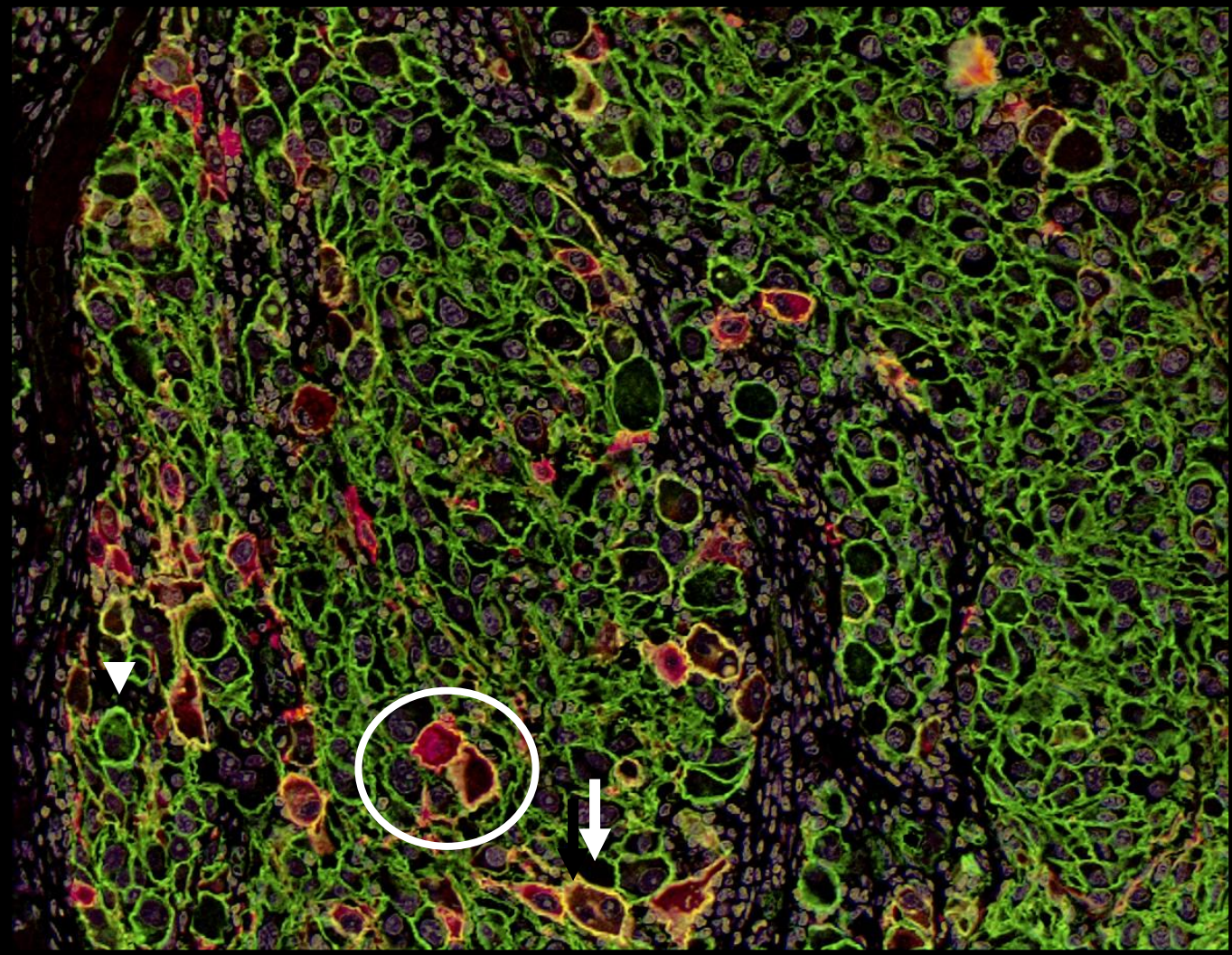
Imaging => de- coverslip => Erasing (Acetone) => Blocking using HIER (Ci)

Second sequence: NGFR immunostaning => DAB => coverslip

Melanoma

Photo Shop manipulated

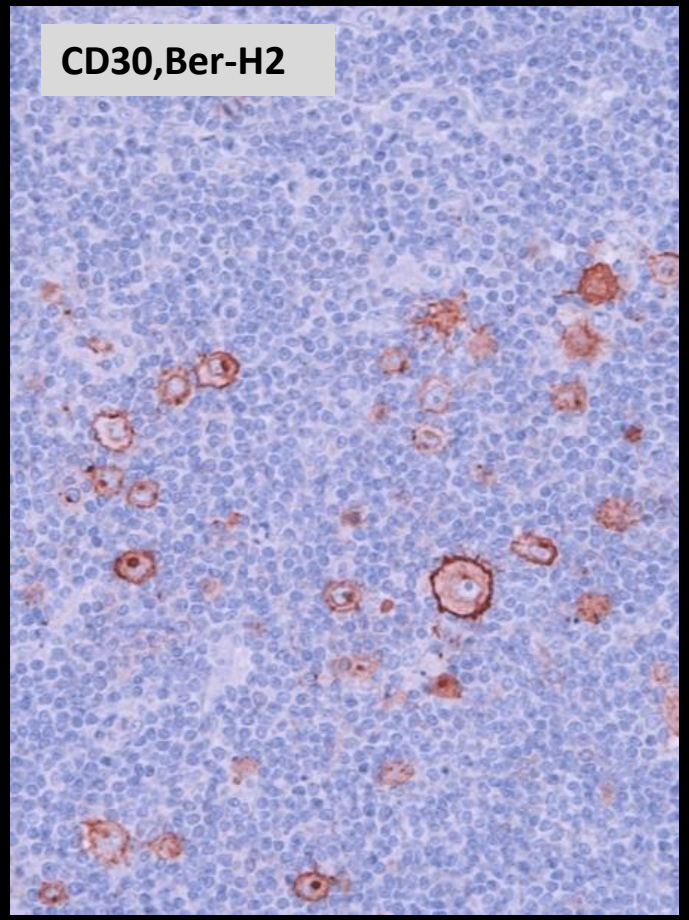
“Digital imaging”



CD146 NGFR Co-Localized

Simple-Technique

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



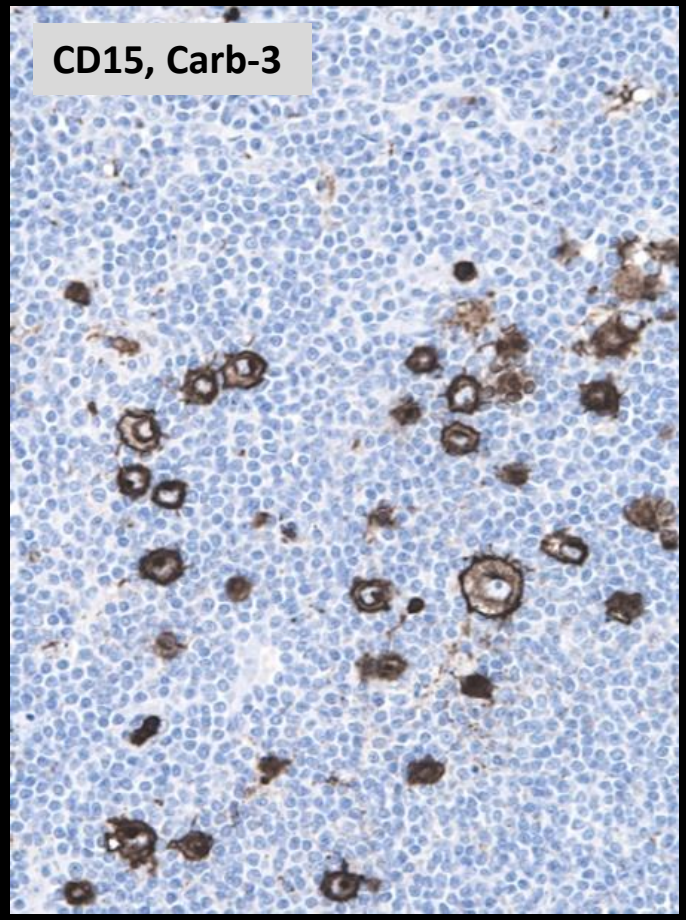
AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

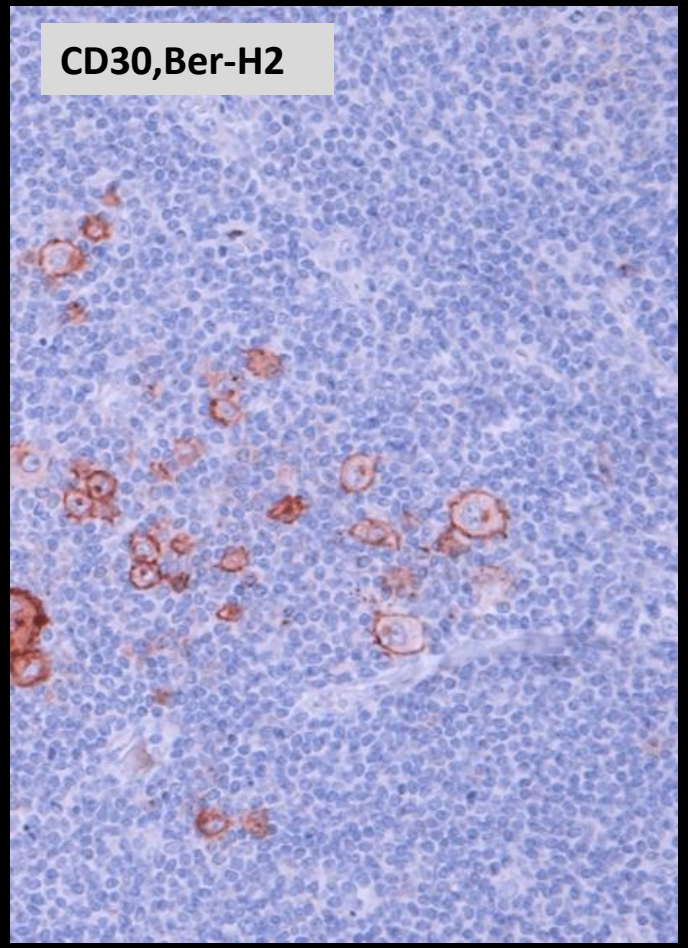
Second sequence repeated with CD15 /DAB



Hodgkin Lymphoma

Simple-Technique

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



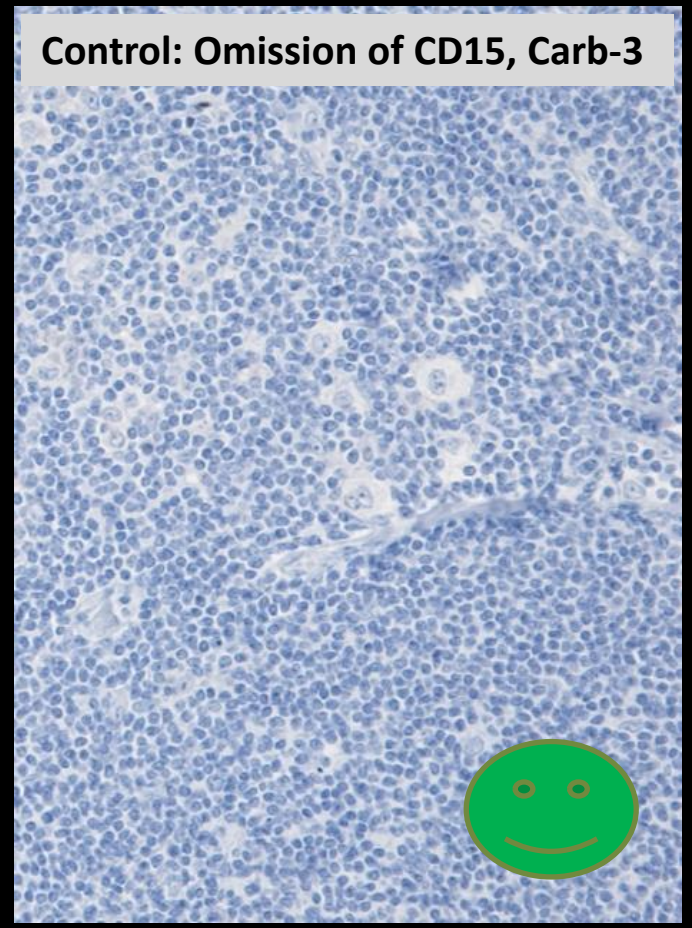
AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

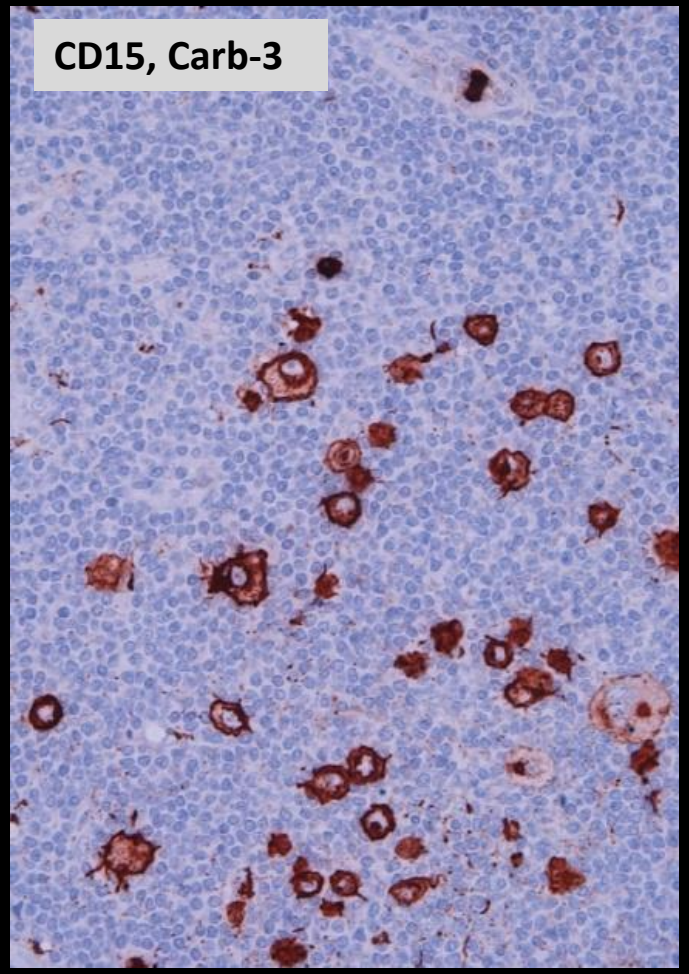
Second sequence repeated without CD15 /DAB



Hodgkin Lymphoma

Simple-Technique

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



Hodgkin Lymphoma

AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

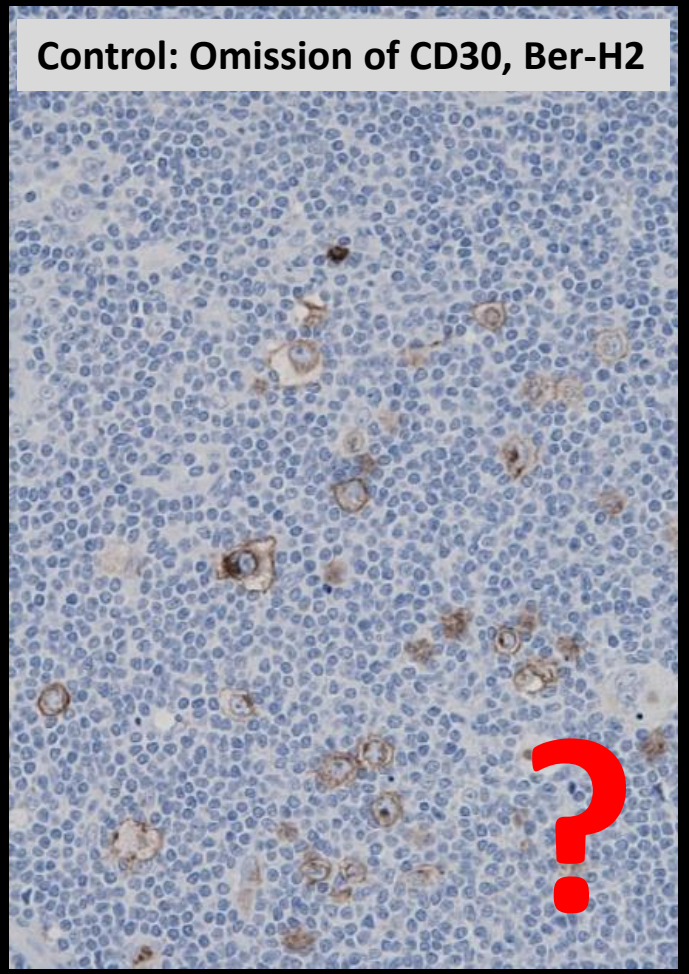
AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

Second sequence repeated without CD30 /DAB

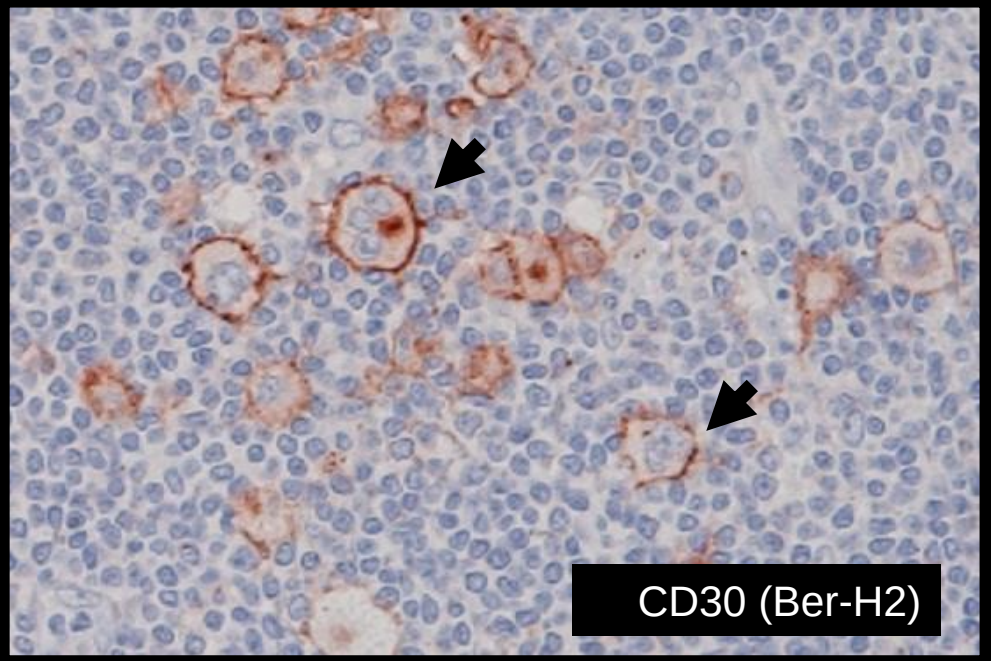
Problem with the blocking procedure:

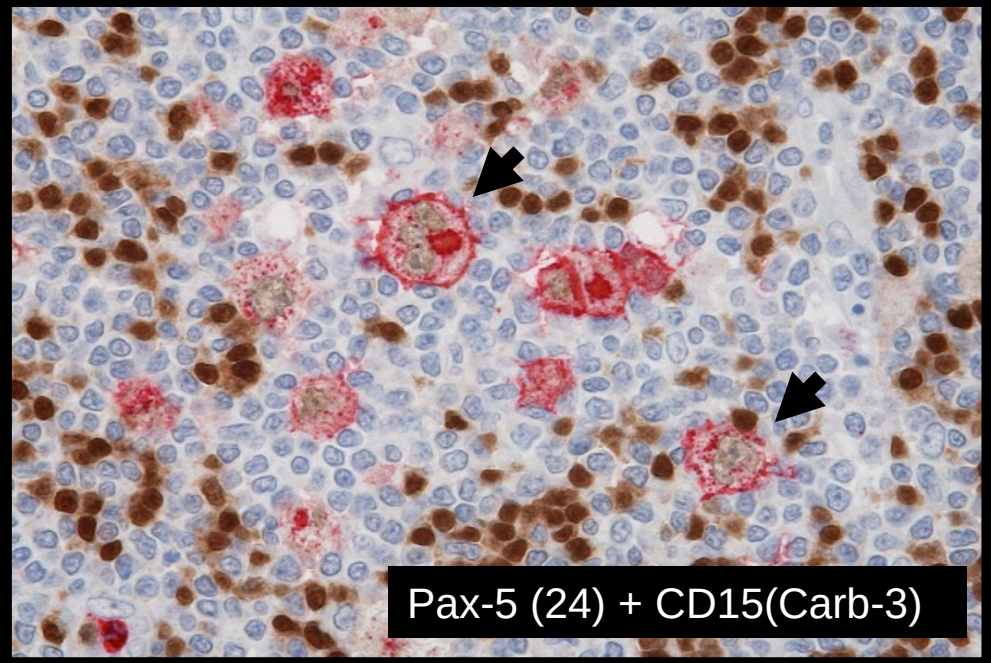
High affinity Abs ?
Antigen density ?
Inefficient blocking procedure ?



Apply problematic antibody in the last sequences

Combining SIMPLE technique with sequential double immune enzymatic method





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

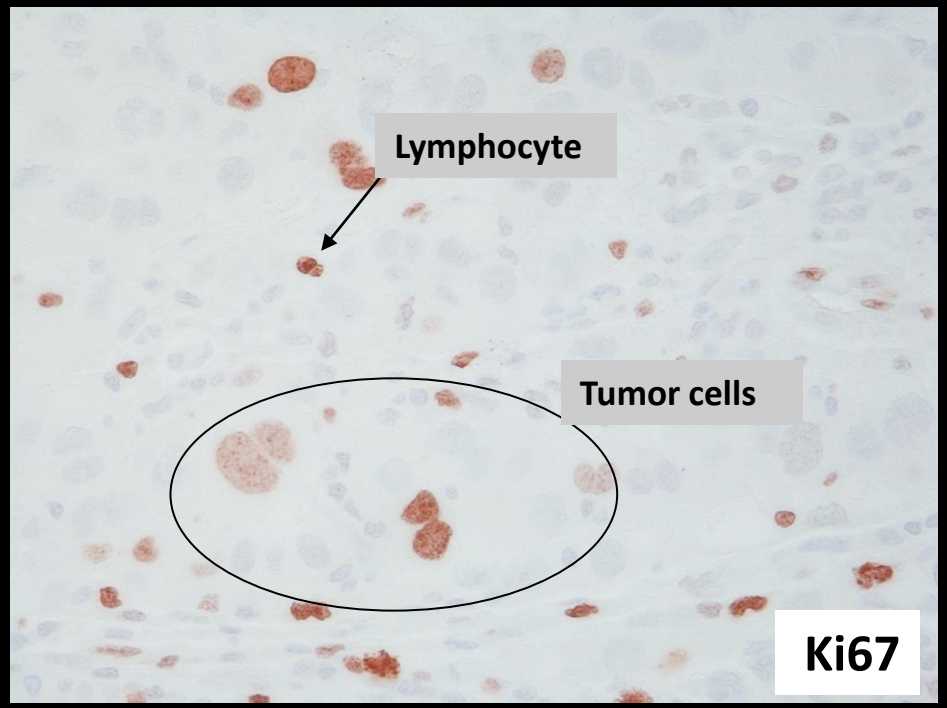
Blocking procedure not needed

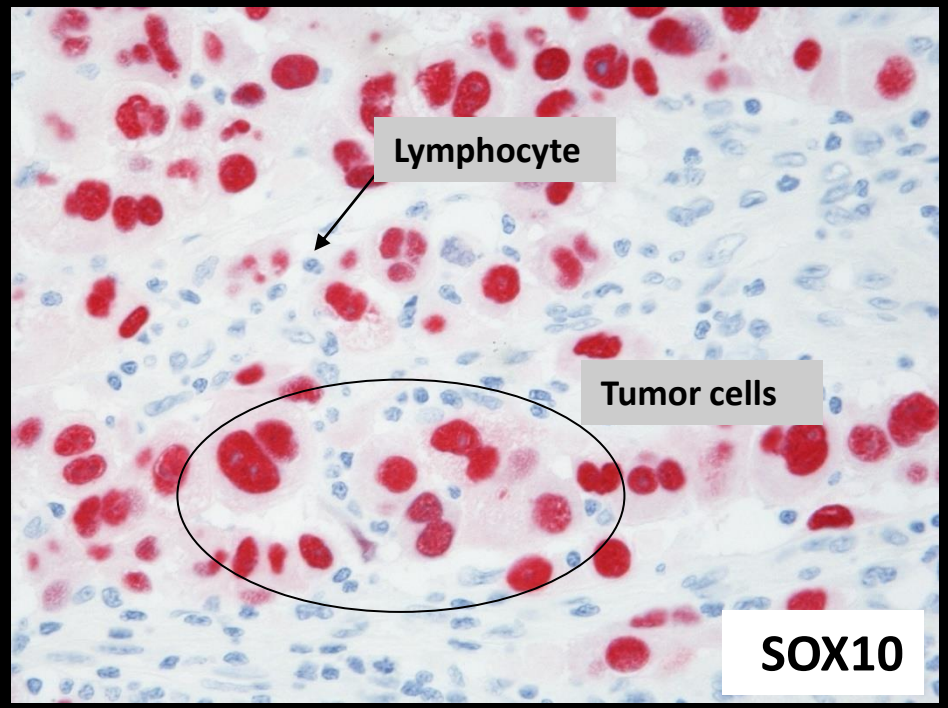
SIMPLE-Technique (simultaneous proc.): MACH2 Double Staining 1

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

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

Melanoma





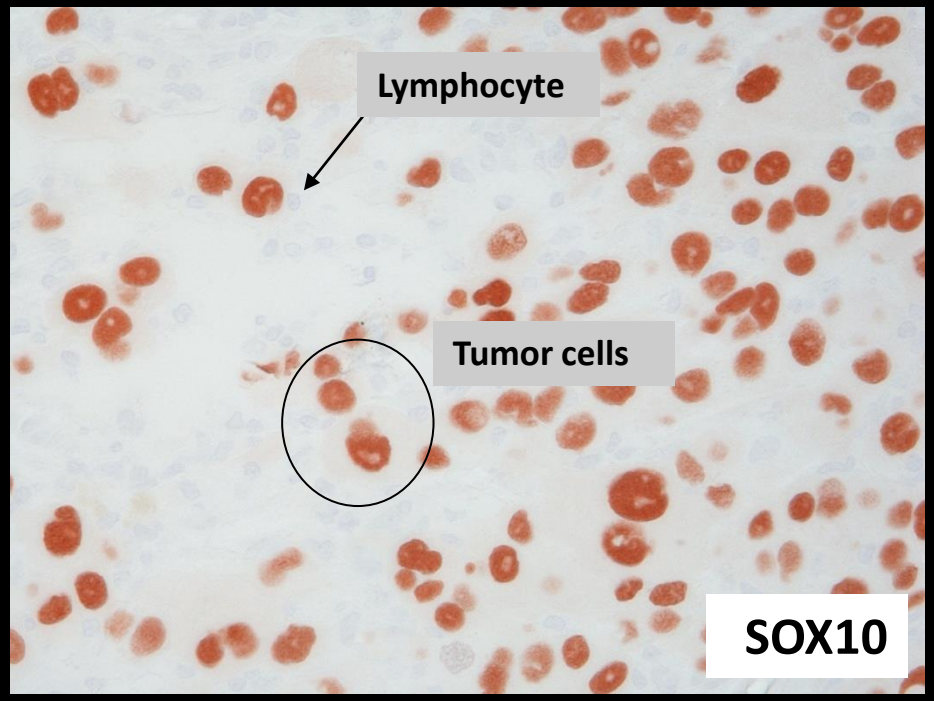
AEC (Impact) - (1dip Hematoxylin / coverslip-Aqueous/ Imaging / de-coverslip / Erasing-Acetone) - Warp Red

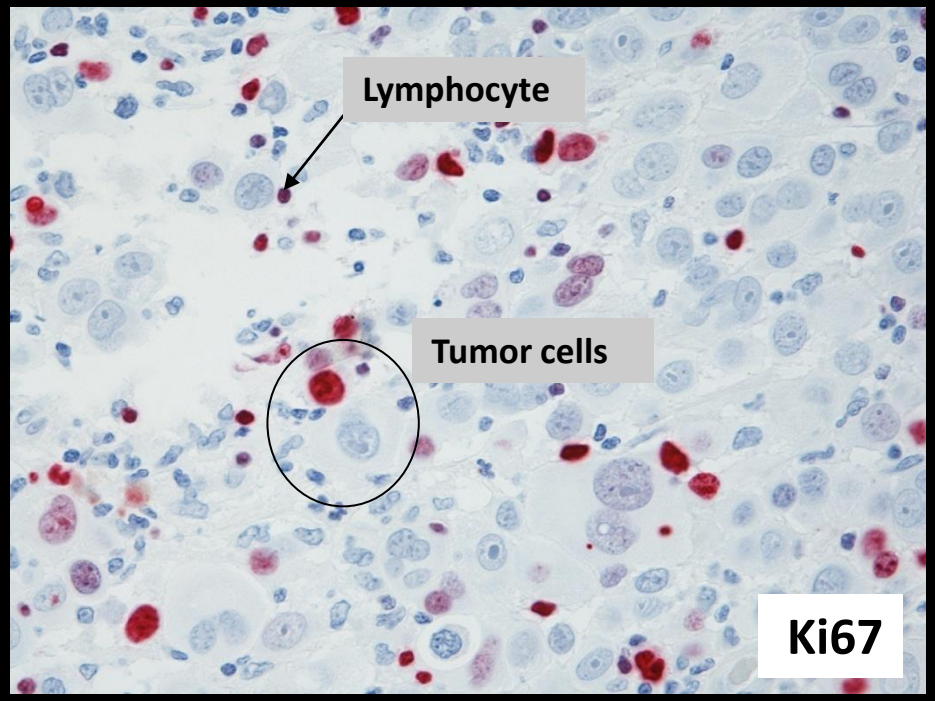
SIMPLE-Technique (simultaneous proc.): MACH2 Double Staining 2

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

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

Melanoma





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

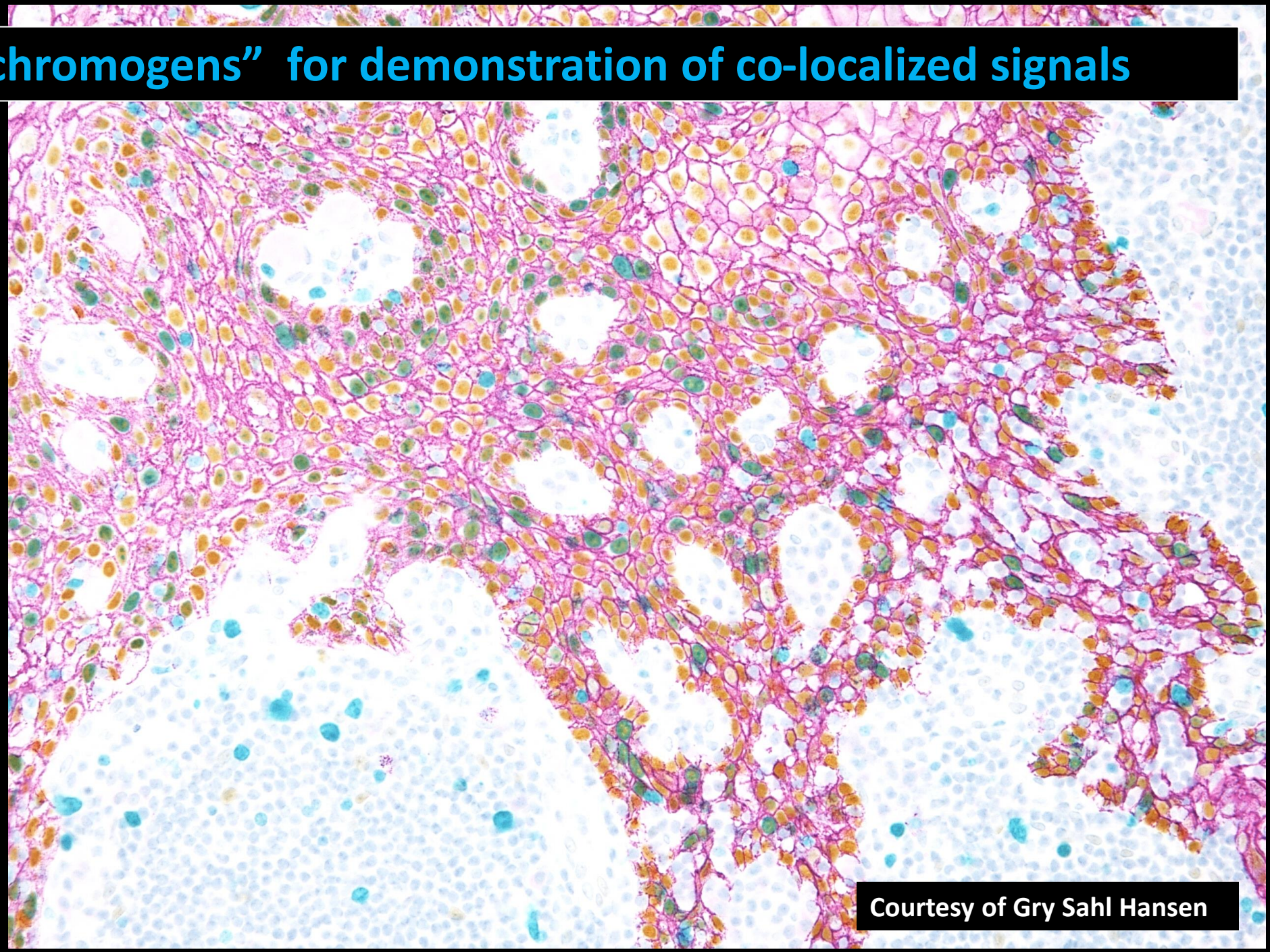
New “translucent chromogens” for demonstration of co-localized signals

Ventana Discovery:

P63 (Disc. Yellow)
Ki67 (Disc. Teal)
ECAD (Disc. Purple)

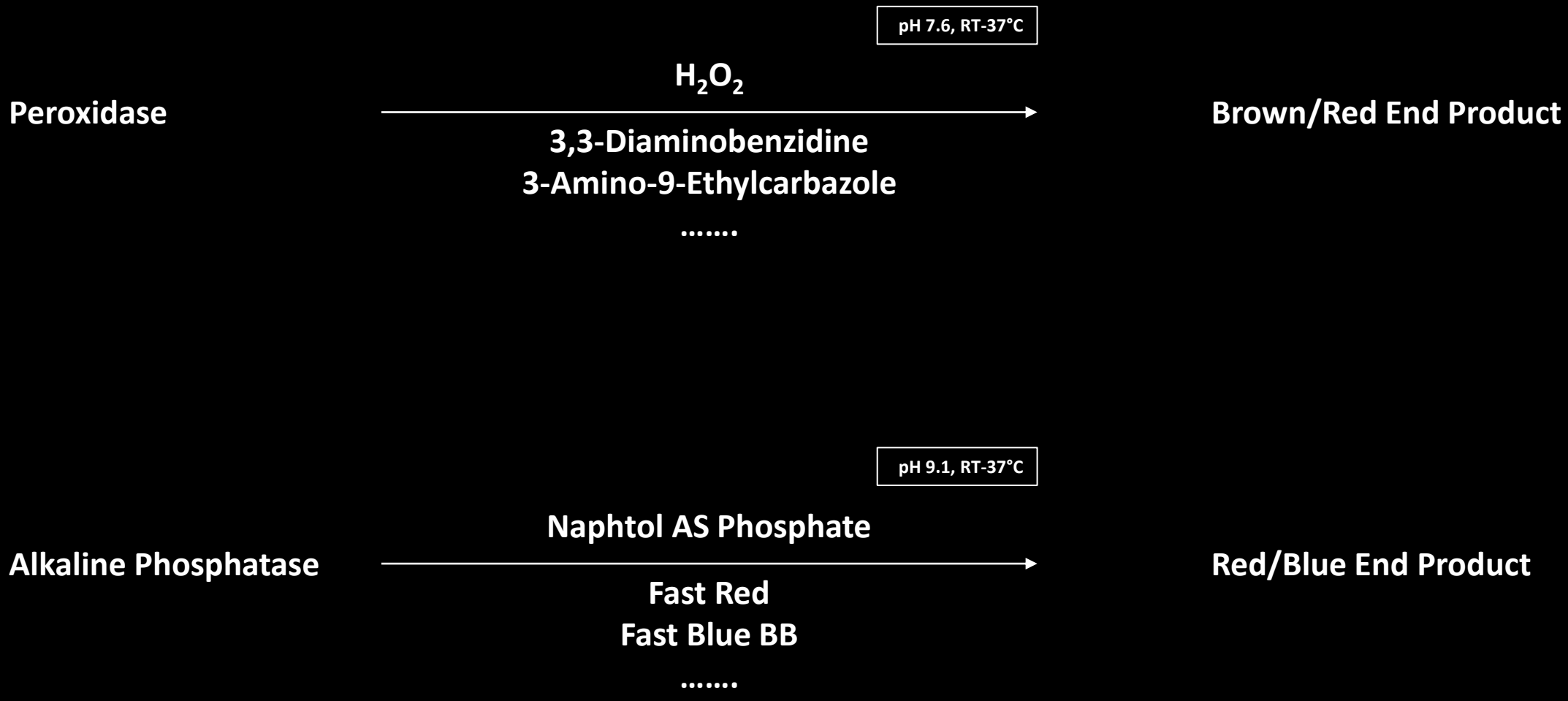
Co-localization

Sequential/HD/Omni-Map



Courtesy of Gry Sahl Hansen

Basic enzyme histochemistry for commonly used chromogens in immunohistochemistry



Tissue Research and Early Development, Ventana Medical Systems, Inc., Tucson, AZ, USA

Covalently deposited dyes: a new chromogen paradigm that facilitates analysis of multiple biomarkers *in situ*

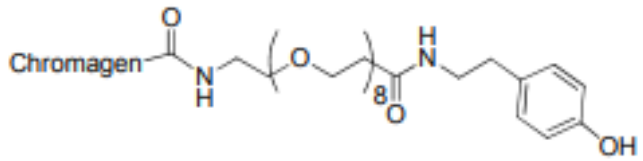
William A Day, Mark R Lefever, Robert L Ochs, Anne Pedata, Lauren J Behman, Julia Ashworth-Sharpe, Donald D Johnson, Eric J May, James G Grille, Esteban A Roberts, Jerry W Kosmeder and Larry E Morrison

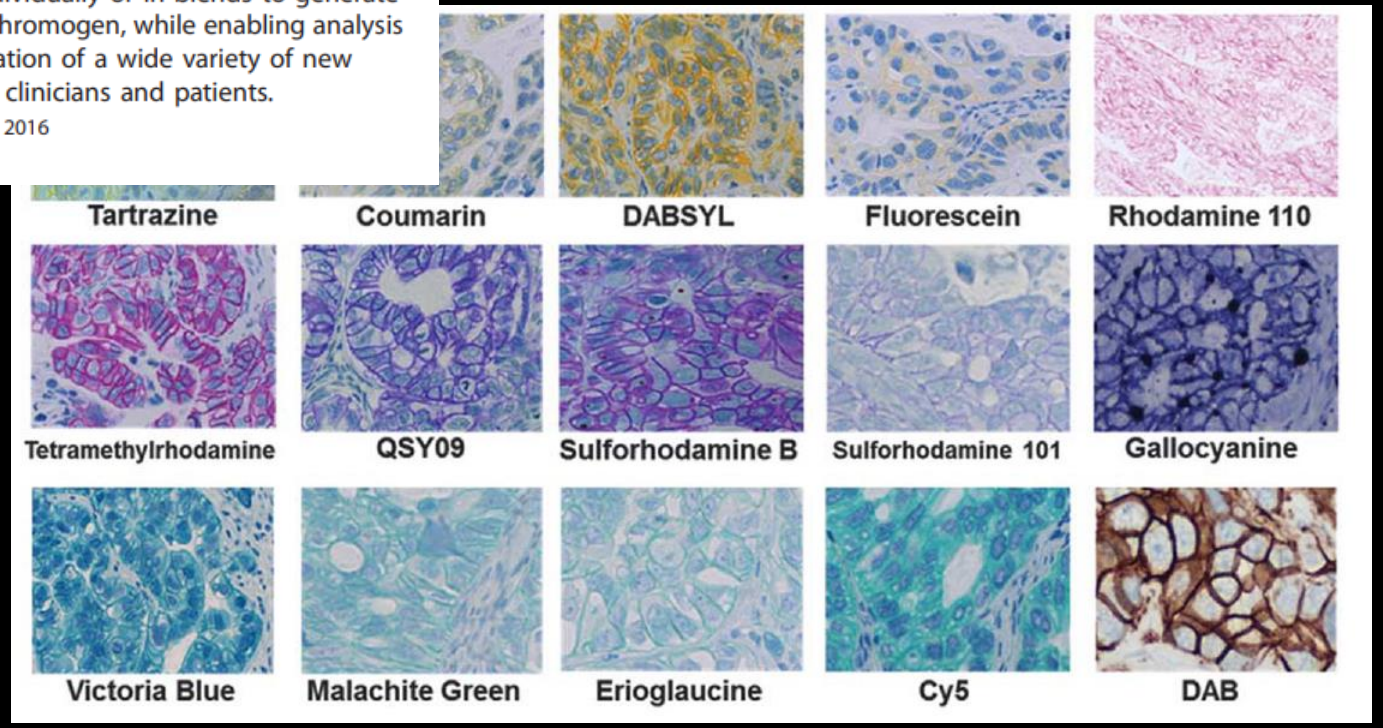
Multiplexed analysis of multiple biomarkers in a tissue sample requires use of reporter dyes with specific spectral properties that enable discrimination of signals. Conventional chromogens with broad absorbance spectra, widely used in immunohistochemistry (IHC), offer limited utility for multiplexed detection. Many dyes with narrow absorbance spectra, eg rhodamines, fluoresceins, and cyanines, potentially useful for multiplexed detection are well-characterized; however, generation of a chromogenic reagent useful for IHC analysis has not been demonstrated. Studies reported herein demonstrate utility of tyramine-chemistry for synthesis of a wide variety of new chromogenic dye conjugates useful for multiplexed *in situ* analysis using conventional light microscopes. The dyes, useful individually or in blends to generate new colors, provide signal sensitivity and dynamic range similar to conventional DAB chromogen, while enabling analysis of co-localized biomarkers. It is anticipated that this new paradigm will enable generation of a wide variety of new chromogens, useful for both research and clinical biomarker analysis that will benefit clinicians and patients.

Laboratory Investigation (2017) **97,** 104–113; doi:10.1038/labinvest.2016.115; published online 21 November 2016

Demonstrate that conjugation of tyramine to dyes can produce several spectrally distinct chromogens, and these chromogens can be effectively integrated into multiplexed brightfield in situ assays.

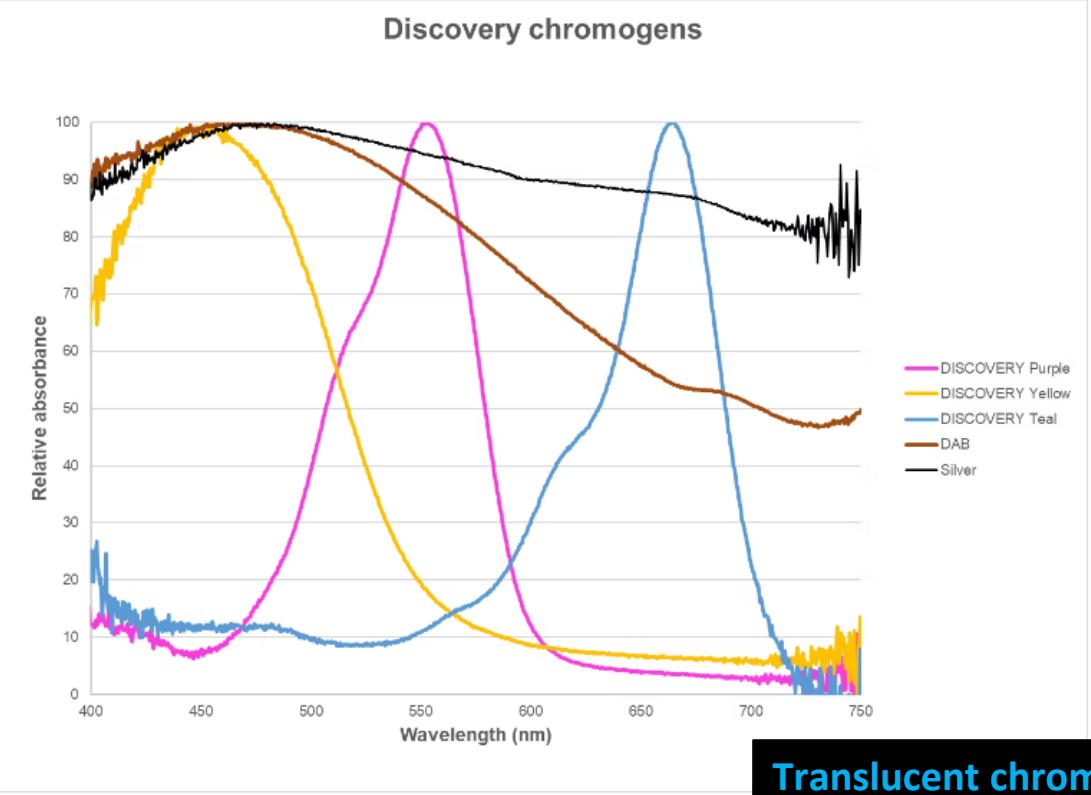
Dye conjugated to Tyramine/Tyramide





Roche/Ventana Diagnostics

How does using translucent chromogens for co-localization experiments result in a color change?



Traditional chromogens such as DAB and Fast Red-based chromogens have very broad absorption spectra (see Figure 1 - Chromogen Spectra). Chromogens that have the narrower absorption ranges (Purple, Yellow, Teal) can take on translucent qualities. This is because narrow absorption chromogens leave more absorption spectra for other dyes to occupy when they are deposited in the same physical space and then mixing of colors is observed.

Figure 1 - Chromogen Spectra

The following dyes can exhibit translucent properties as they contain only one primary color from the (cyan, yellow, magenta, black) color space: Discovery Purple (magenta), Discovery AP Yellow (yellow), Discovery Teal (cyan).

Translucent chromogens

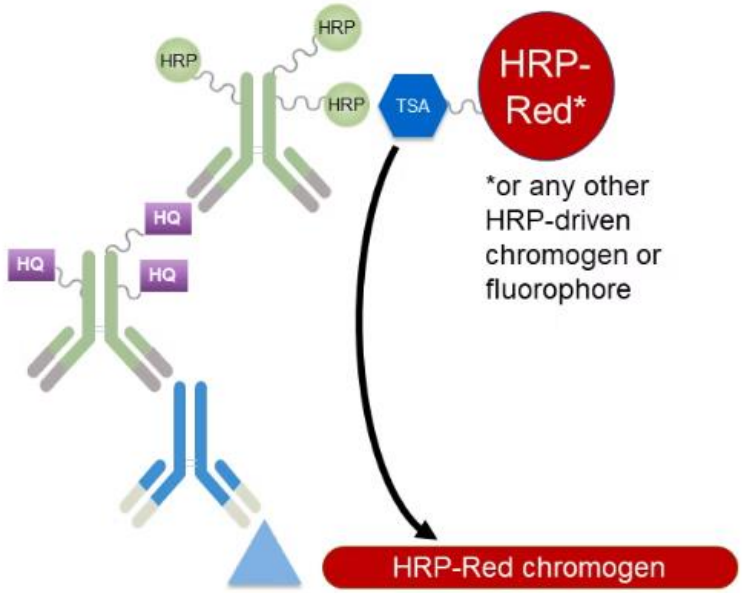
Tyramine conjugated dyes has a relative narrow absorbance spectra making these “modified dyes” suitable for multiplexing and for co-localized signals - “each dye only occupy a smaller fraction of the visible spectrum and leaving more space for other dyes”.

New “translucent chromogens” for demonstration of co-localized signals

These new chromogens are based on dyes conjugated to Tyramide, and thus, visible and displaying basic colors before any immunohistochemical process has even been performed (e.g., introduction of HRP reagents).

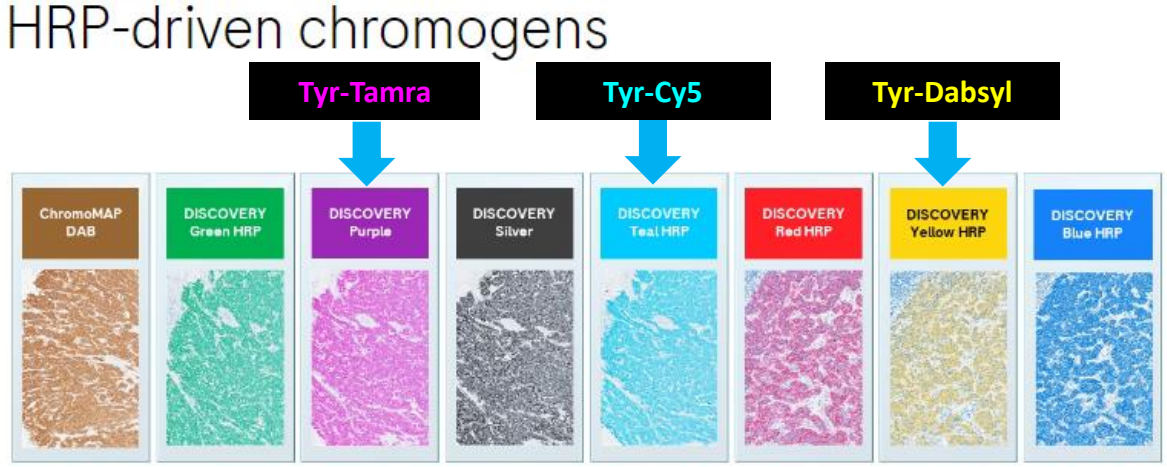
Standard IHC chromogens as DAB are more or less invisible and first visible after the enzymatic process has taken place - creating a deposit in close vicinity to the immunohistochemical reactions (e.g., HRP catalyzed).

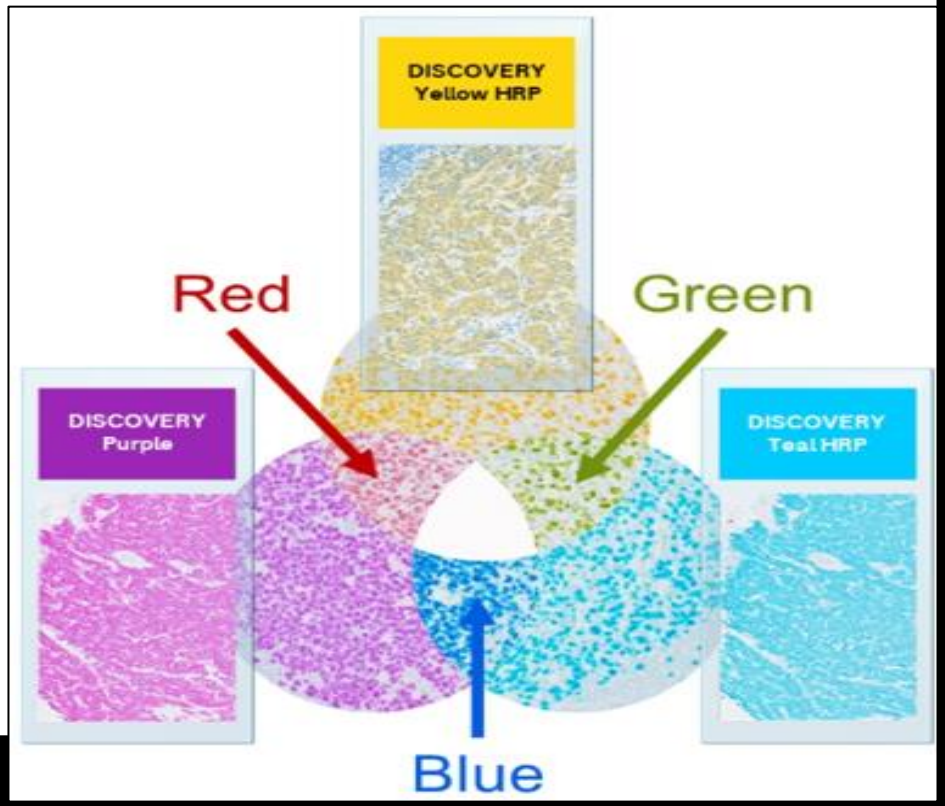
Translucent chromogens for demonstration of co-localized antigens



Tyramide chemistry is a powerful technique that can be used to stain or even amplify the signal of immunohistochemistry (IHC) staining. One major advantage is that the dye forms a covalent bond with proteins in the tissue for greater stability.

Like traditional IHC, the primary and secondary (HRP) antibodies are incubated with the tissue sample to bind to the antigen of interest. The chromogenic dye is designed with a tyramine group that becomes reactive after interaction with HRP in the presence of hydrogen peroxide to form a highly reactive intermediate. Similarly, tyrosine residues in the endogenous proteins nearby will become activated after contact with the HRP, and then condense with the dye intermediate to form a covalent bond and local deposition of the chromogenic (or fluorescent) dye. Finally, the sample is counterstained with a nuclear stain, such as DAPI or hematoxylin, to visualize the cellular structures. The result is a highly specific and sensitive signal.





Roche/Ventana Diagnostics

PRAME and HMB45 for discriminating benign from malignant melanocytic lesions

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Diagnostic utility of combining PRAME and HMB-45 stains in primary melanocytic tumors

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ABSTRACT

Background: Pathologists face ongoing challenges distinguishing between benign and malignant melanocytic tumors. PRAME (PReferentially expressed Antigen in Melanoma) has a demonstrated value distinguishing between these types of lesions. However, the sensitivity of single immunohistochemistry is variable. HMB-45 is another valuable marker, but on its own, has a limited ability in setting of primary melanocytic tumors. This study sought to evaluate the diagnostic potential of a dual panel combining PRAME and HMB-45 in the assessment of primary melanocytic tumors.

Methods: 259 tumors, of which 141 were benign nevi, 31 dysplastic nevi (either low- or high grade dysplasia), and further 87 malignant melanomas, were retrieved from the department's archives and assessed by two experienced dermatopathologists. New sections were stained with PRAME and HMB-45, respectively. For PRAME, a nuclear, and for HMB-45, a cytoplasmic staining, was considered positive and scored as described in the literature on a scale from 0 to 4+. Only dermal component was assessed on HMB-45 stain.

Results: PRAME was diffusely expressed in only 1 benign nevus, with focal expression in further 28 compared to 22 diffusely and 103 focally HMB-45-positive benign nevi. 5 high-grade dysplastic nevi showed diffuse PRAME expression in epidermal component, with varying degree of positivity in adjacent dermal compartment, and further 8 dysplastic nevi showed only focal expression. HMB-45 was diffusely expressed in only 2, with focal expression in 23, and no apparent positivity in remaining 6 dysplastic nevi. In invasive melanoma group, PRAME stained >75 % cells in 64/87 tumors, however, 10/87 melanomas were completely negative. HMB-45 was captured diffusely in 49/87 melanomas, 32 showed patchy expression, and 6 tumors were blank negative. Diffuse 4+ PRAME positivity showed superior sensitivity and specificity of 73,6 % and 96,5 %, respectively, compared to HMB-45, 56,3 % and 86,0 %, respectively. No nevi showed double 4+ positivity, however, the sensitivity for double positivity was only 49.4 %.

Conclusion: Our results confirm the superiority of PRAME over HMB-45 in the differential diagnosis of melanocytic tumors. However, combined staining can significantly increase specificity, rendering a benign diagnosis more unlikely in a double 4+ diffuse positivity setting.

Add on Ki67 ?

HMB45 staining in benign nevi:

Positive in Junctional nevi
Positive in compound nevi (epidermal part) – dermal part negative
Negative in Intradermal nevi

HMB staining in atypical nevi:

Might also be positive in the dermal part of the lesion

Many "uncommen" subtypes of nevi (e.g., Blue, Spitz and Spitzoid nevi) are positive for HMB45

Melanoma

Primary melanoma of the skin are positive (>95%)
Desmoplastic malignant melanoma on positive in app. 30% of the cases
Metastatic melanoma app. 60-80% positive for HMB45

Multiplex staining using the “new translucent chromogens”:

PRAME, EPR20330 or SOX10, SP267 (rmAbs)

Ki67, SP6 (rmAb)

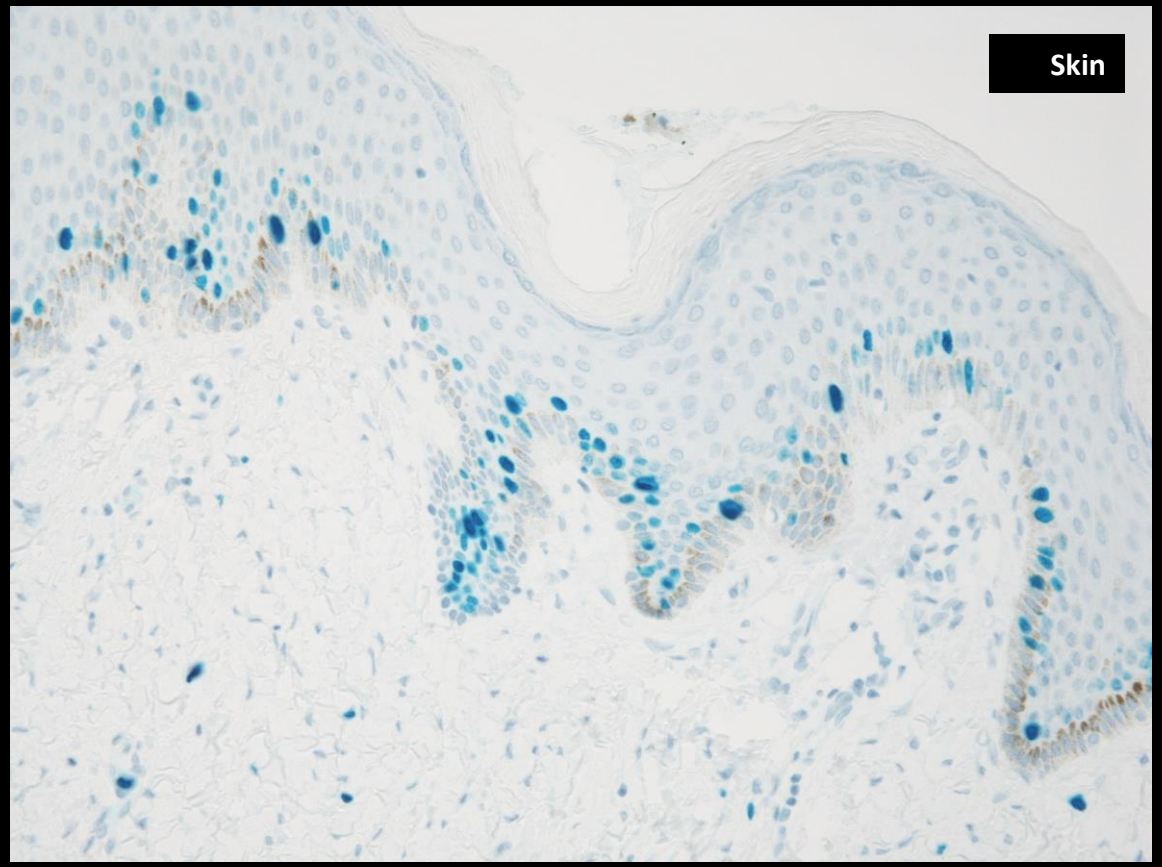
MLA,BS52 or MSA, HMB45 (mAb)

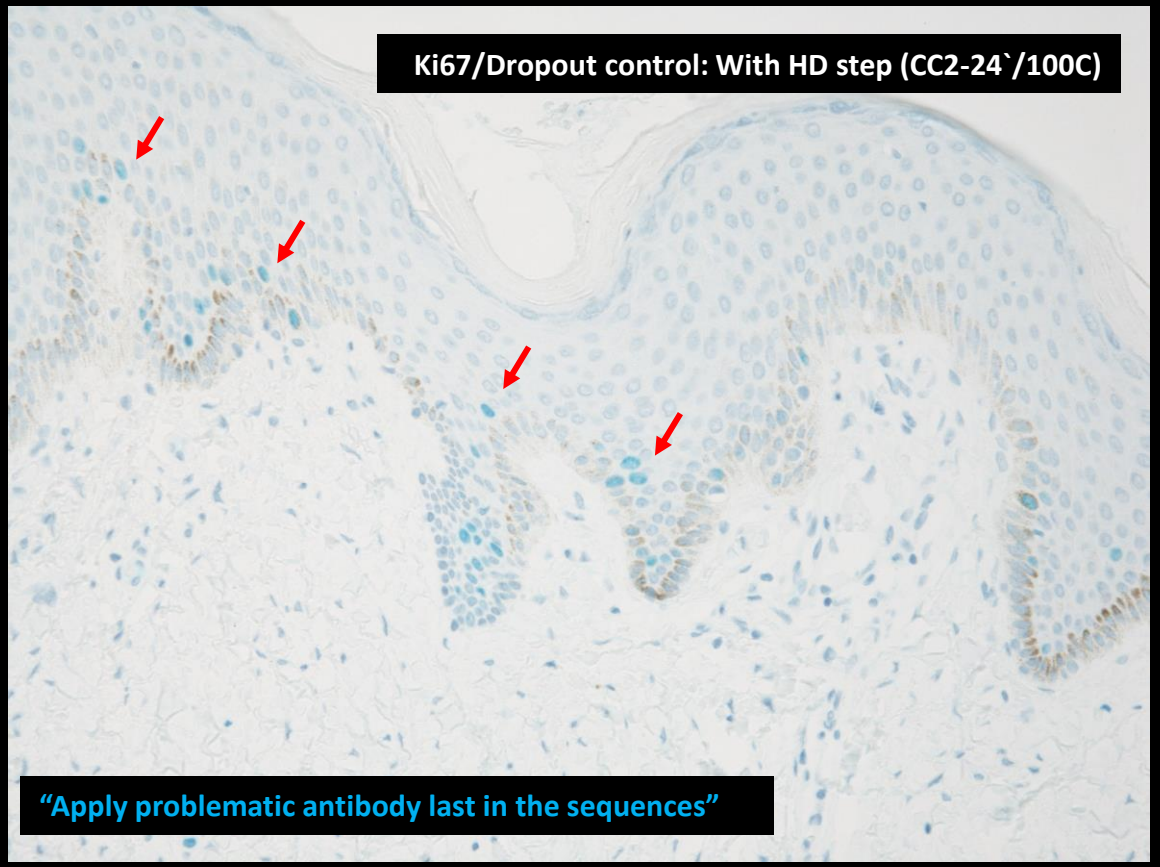
Other marker that might be useful: P16 (often loss in malignant melanomas)

Clinical use/purpose

Discriminating benign melanocytic lesions (e.g., nevi/atypical nevi) from malignant melanoma

Ki67, SP6 (rmAb/OmniMap-HRP/Teal) + HD drop-out control



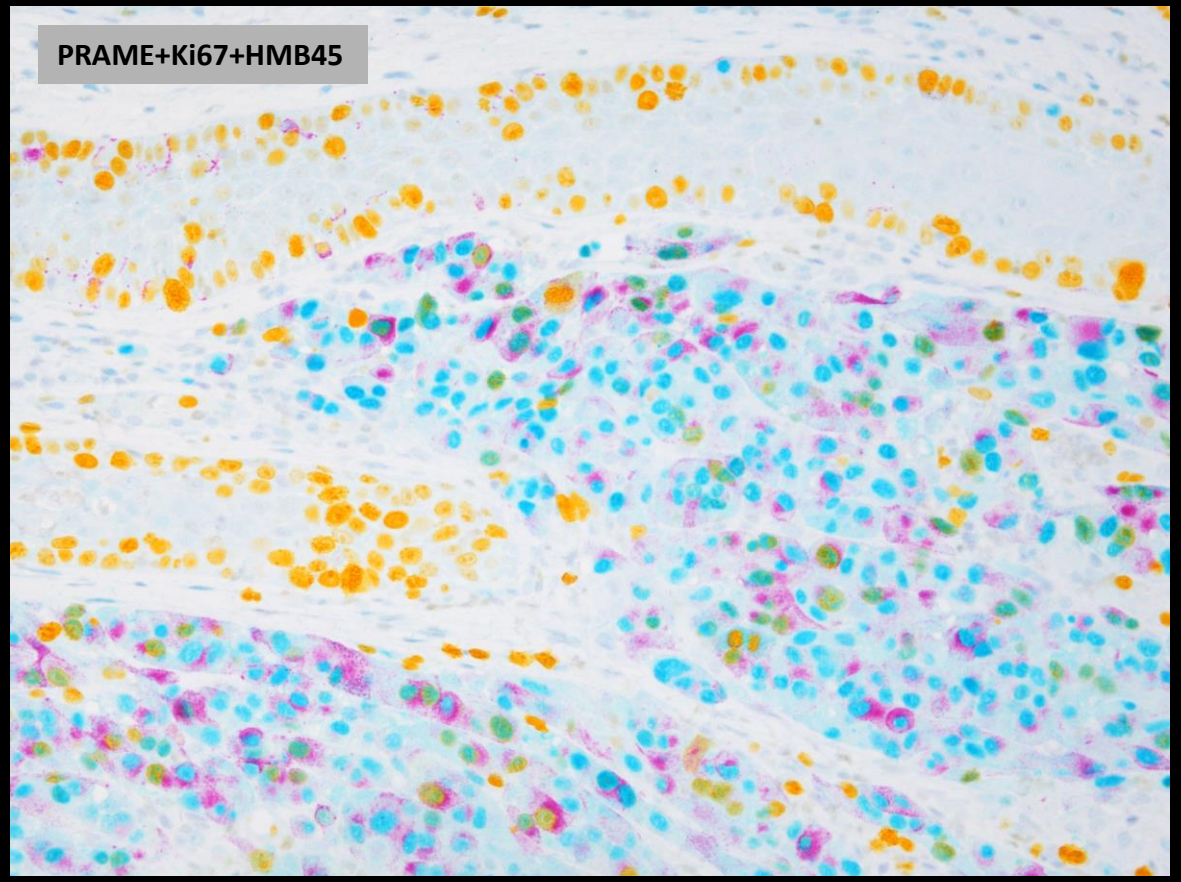


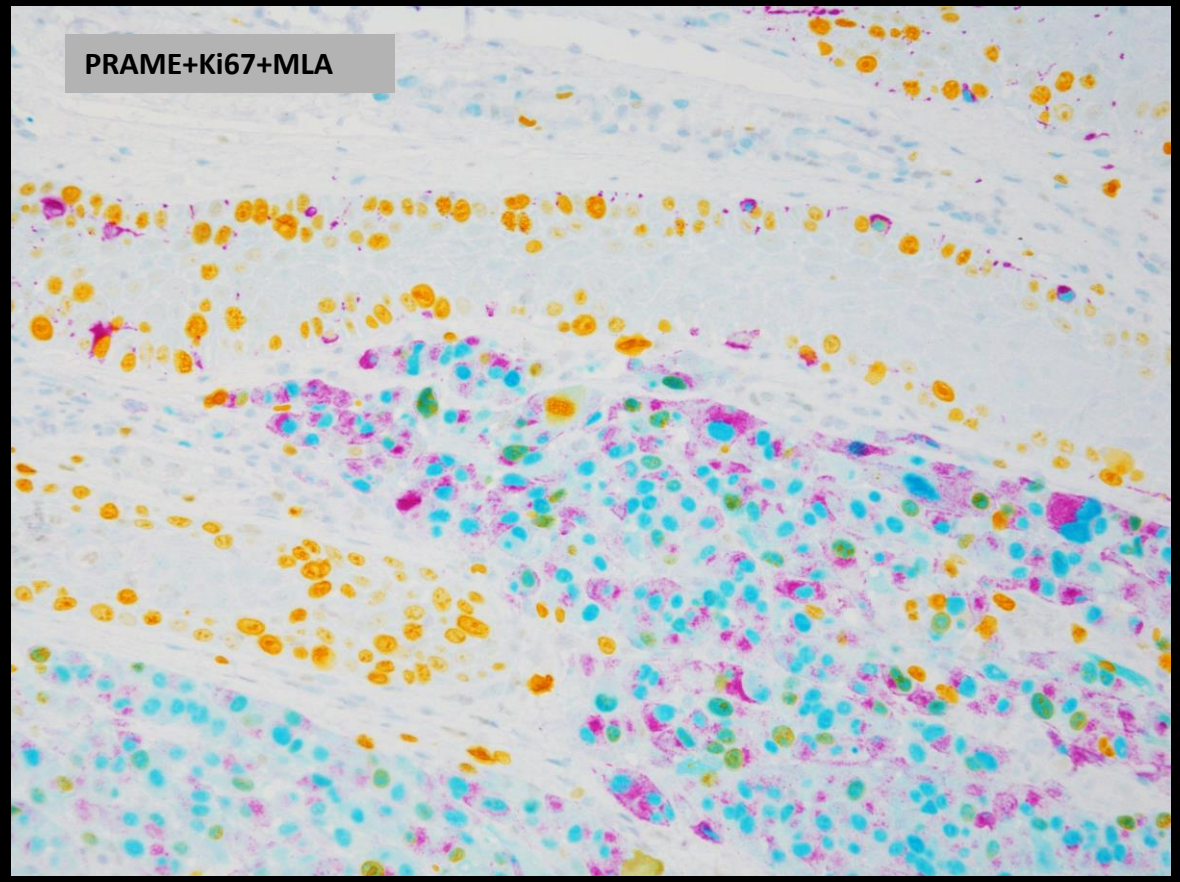
SOX10, SP267 (rmAb) + Ki67, SP6 (rmAb/OmniMap-HRP/Teal)

PRAME, EPR20330 (rmAb) + Ki67, SP6 (rmAb/OmniMap-HRP/Teal) + MSA, HMB45 or MLA, BS52 (mAb`s)

"PRAME, EPR20330 (rmAb) + MSA, HMB45 or MLA, BS52 (mAb) + Ki67, SP6 (rmAb/OmniMap-HRP/Teal)" – not tested

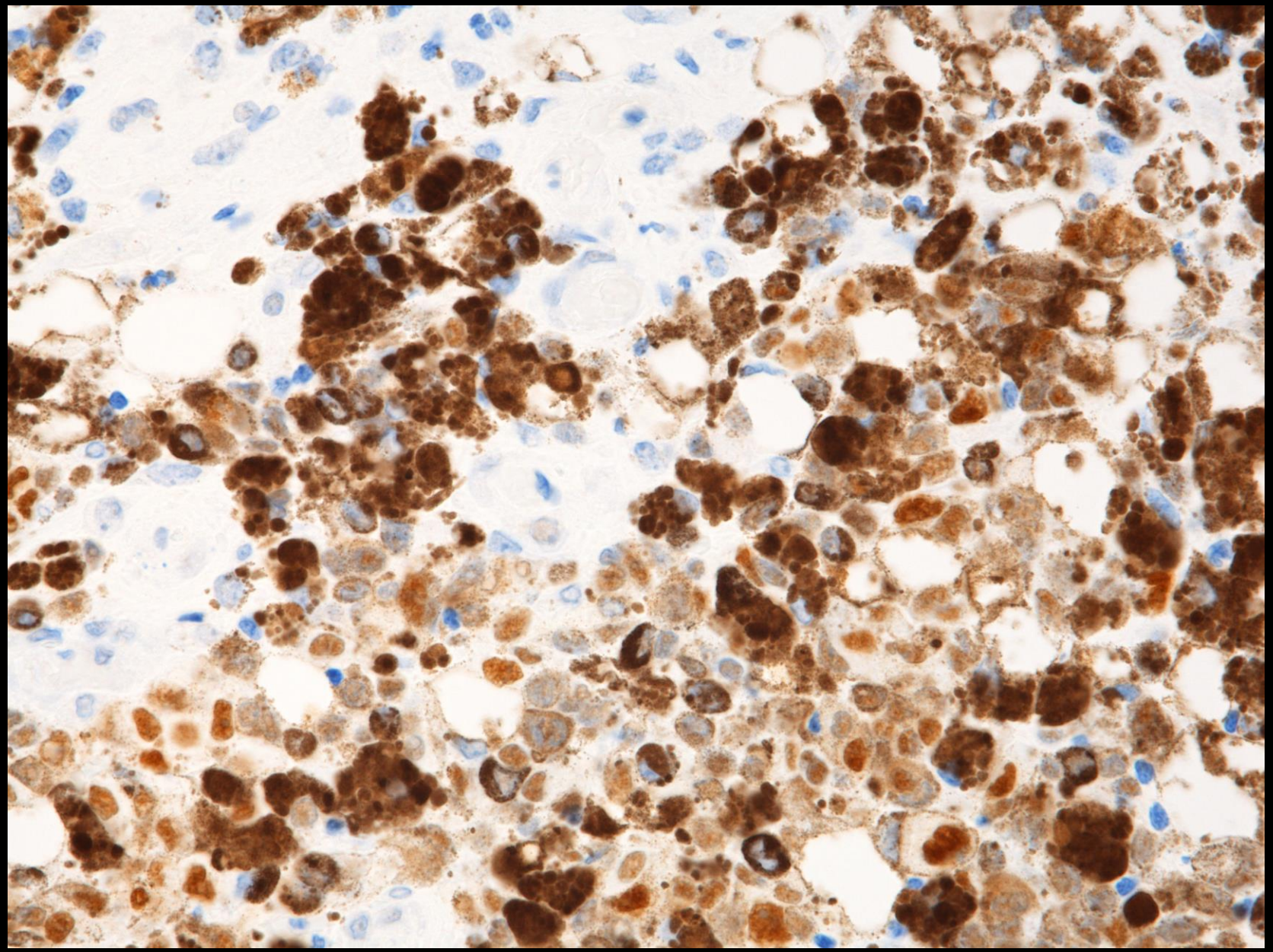
Skin: malignant melanoma





PRAME, rmAb EPR22330 (OmniMap-HRP/Teal) → HD → Ki67, rmAb SP6(OmniMap-HRP/Yellow) → N → MLA, mAb BS52 or MSA, HMB45 (OmniMap-HRP/Purple)

PRAME, rmAb EPR22330 (H24`/Flex+/DAB)



Melanoma

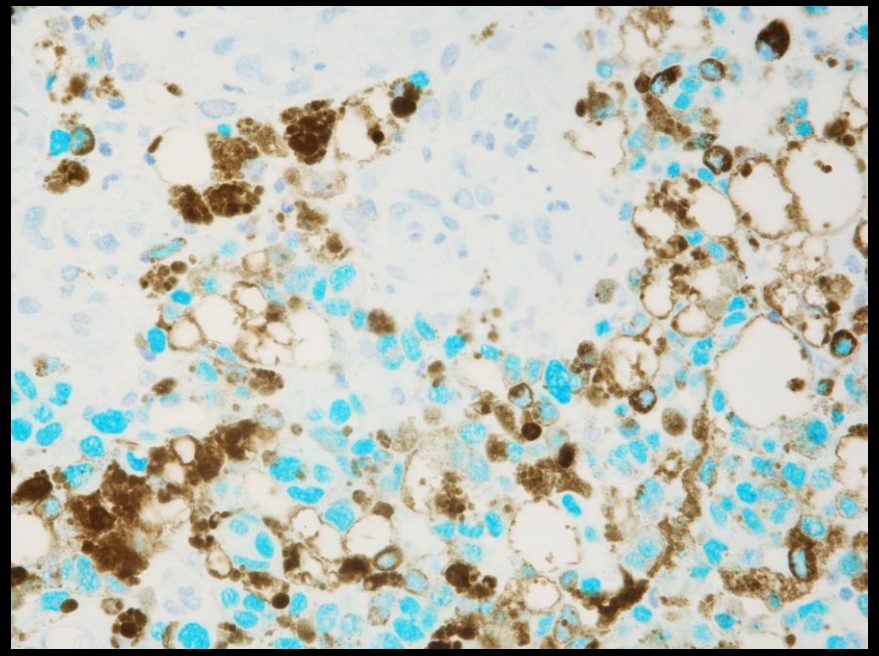
PRAME, rmAb EPR22330
(OmniMap-HRP/Teal)

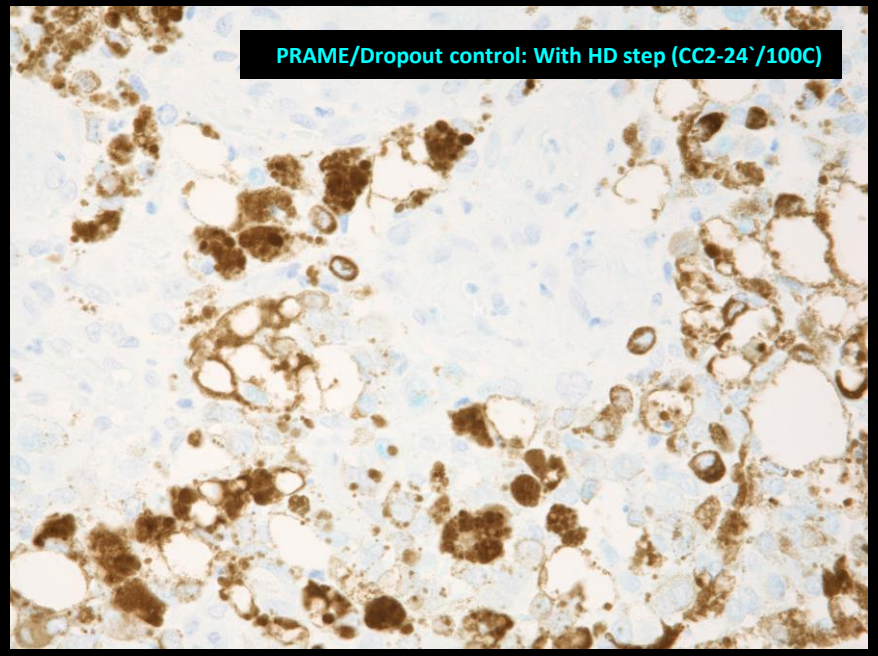
+HD

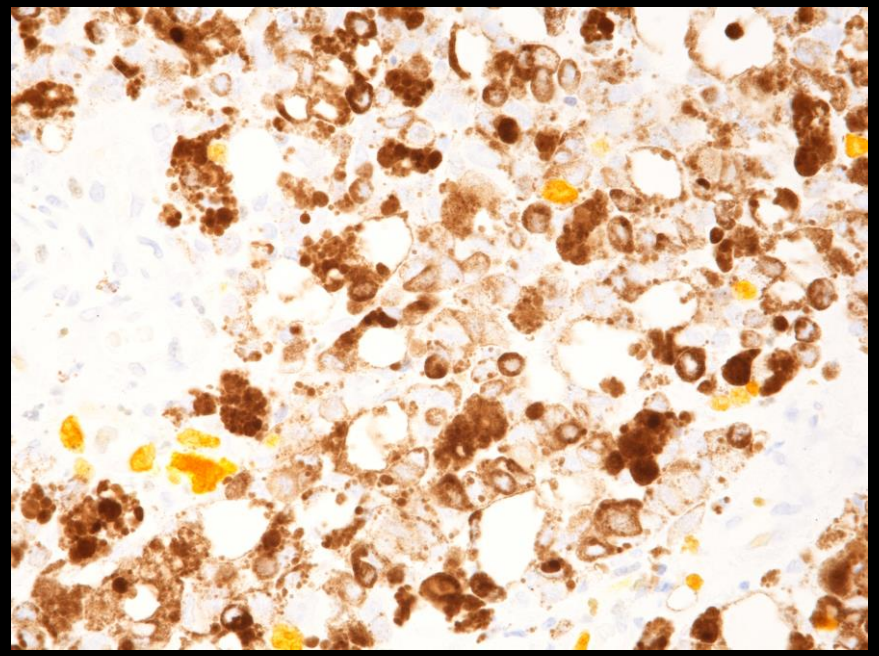
Ki67, rmAb SP6
(OmniMap-HRP/Yellow)

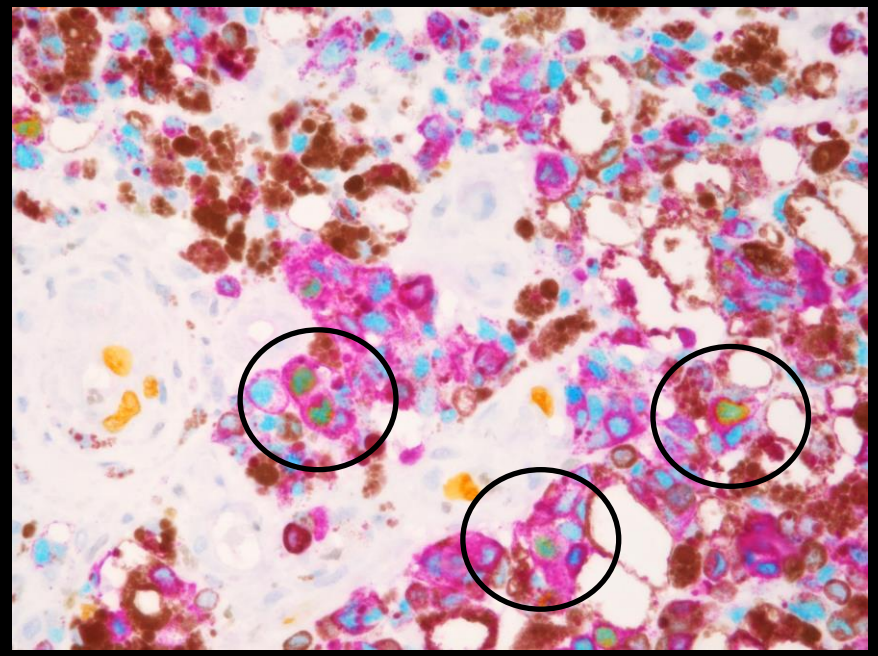
+N

MSA, mAb HMB45
(OmniMap-HRP/Purple)









Melanoma

Co-localization

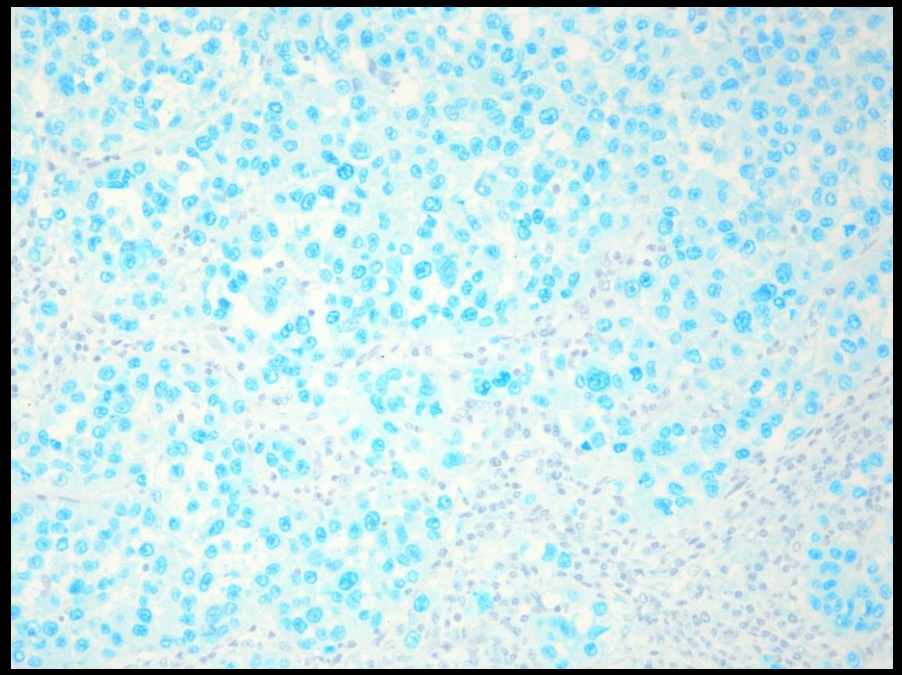
PRAME, rmAb EPR22330
(anti-HQ-HRP/Teal)

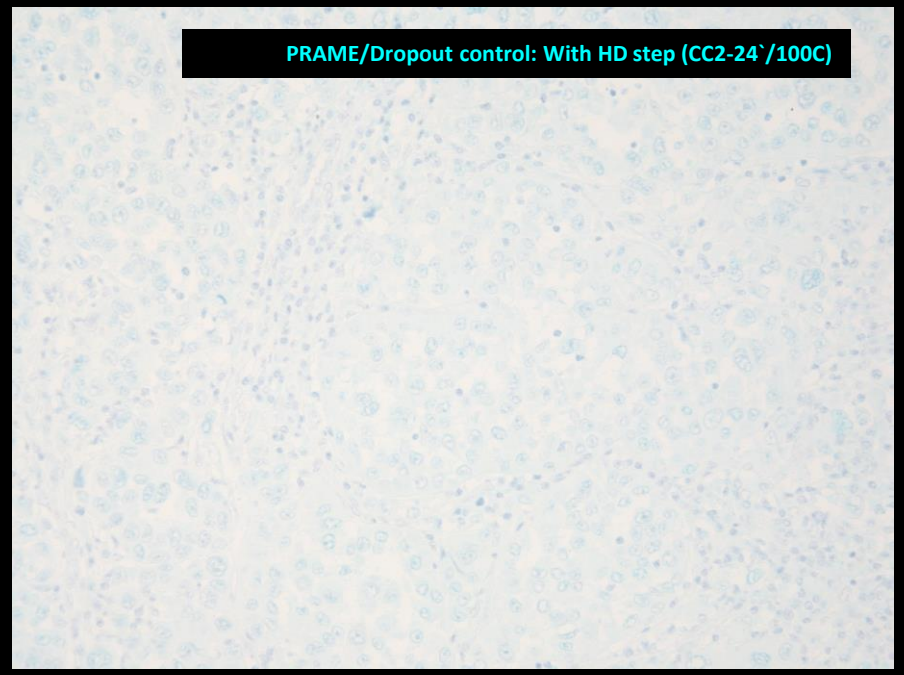
+HD

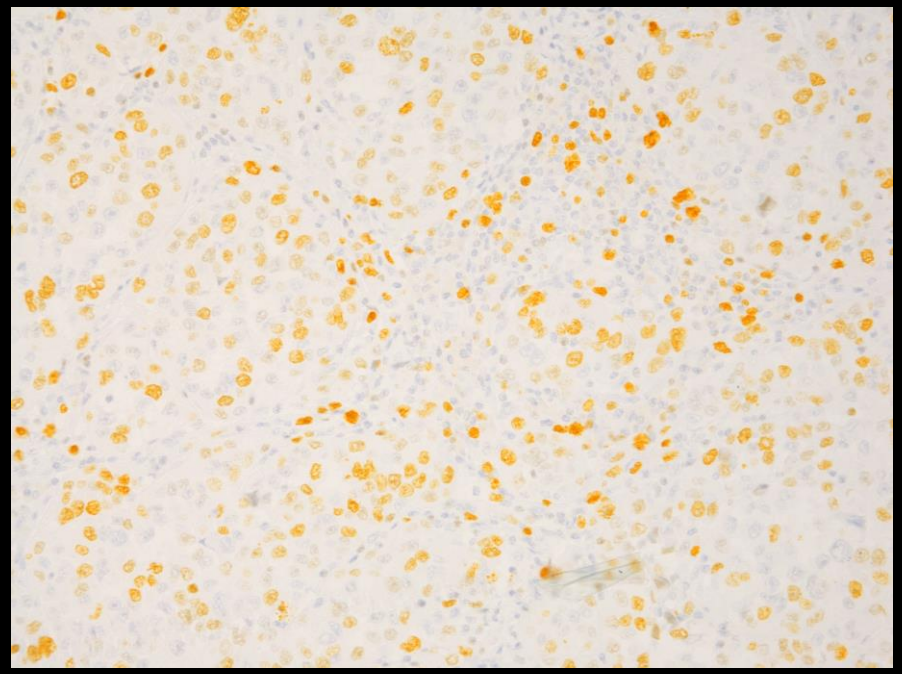
Ki67, rmAb SP6
(OmniMap-HRP/Yellow)

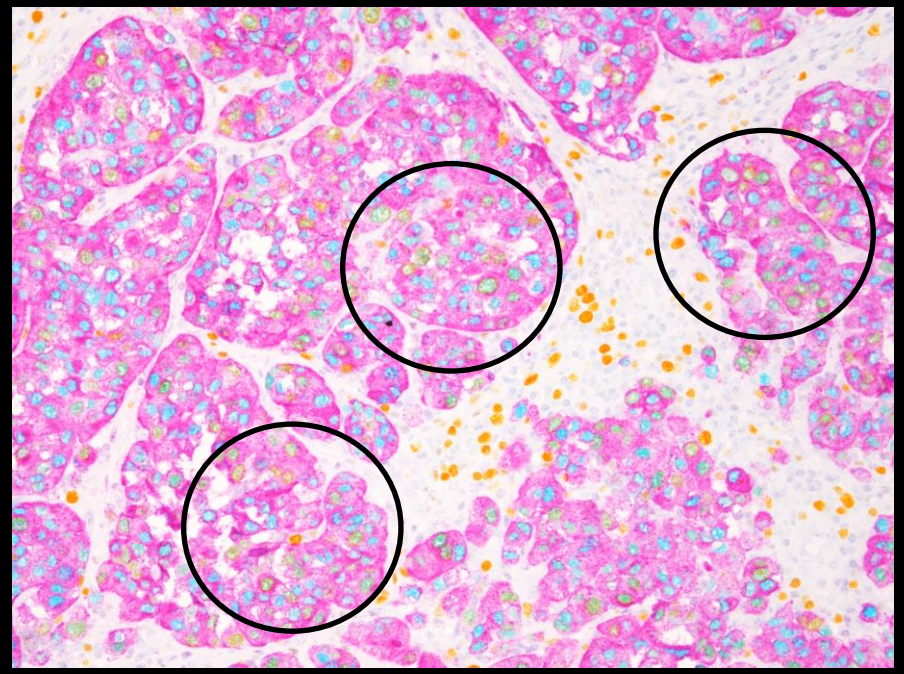
+N

MSA, mAb HMB45
(OmniMap-HRP/Purple)









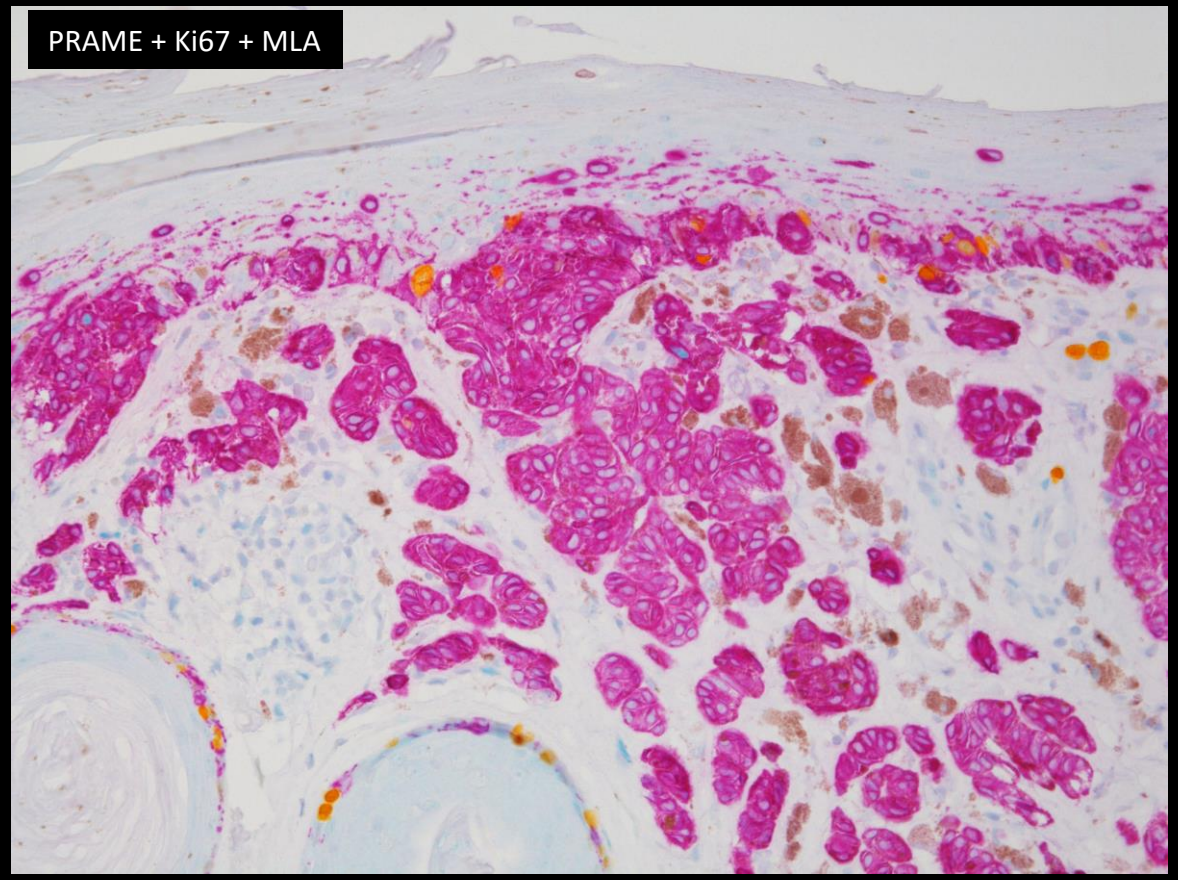
Melanoma

Co-localization

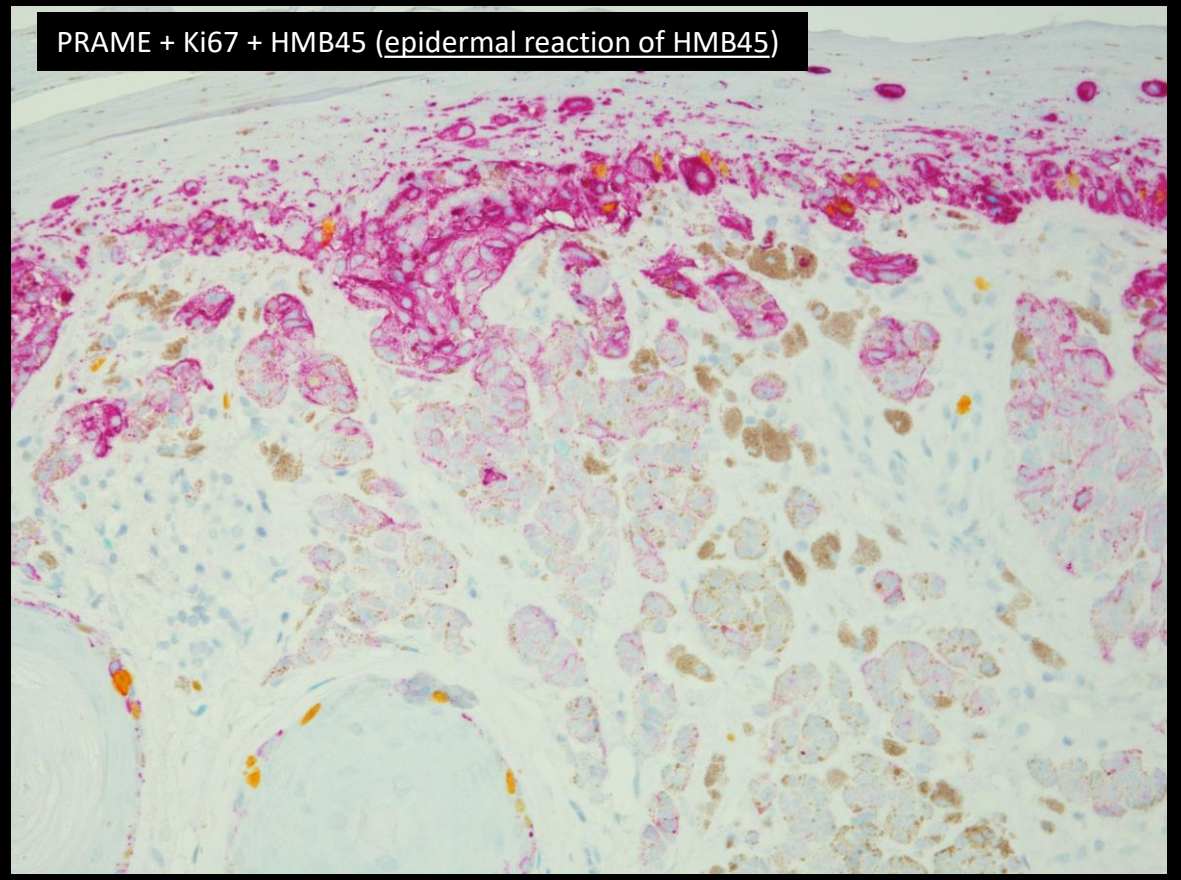
Compound Nevi, “Atypical proliferations”

“Benign melanocytic lesion of the skin”

PRAME + Ki67 + MLA



PRAME + Ki67 + HMB45 (epidermal reaction of HMB45)

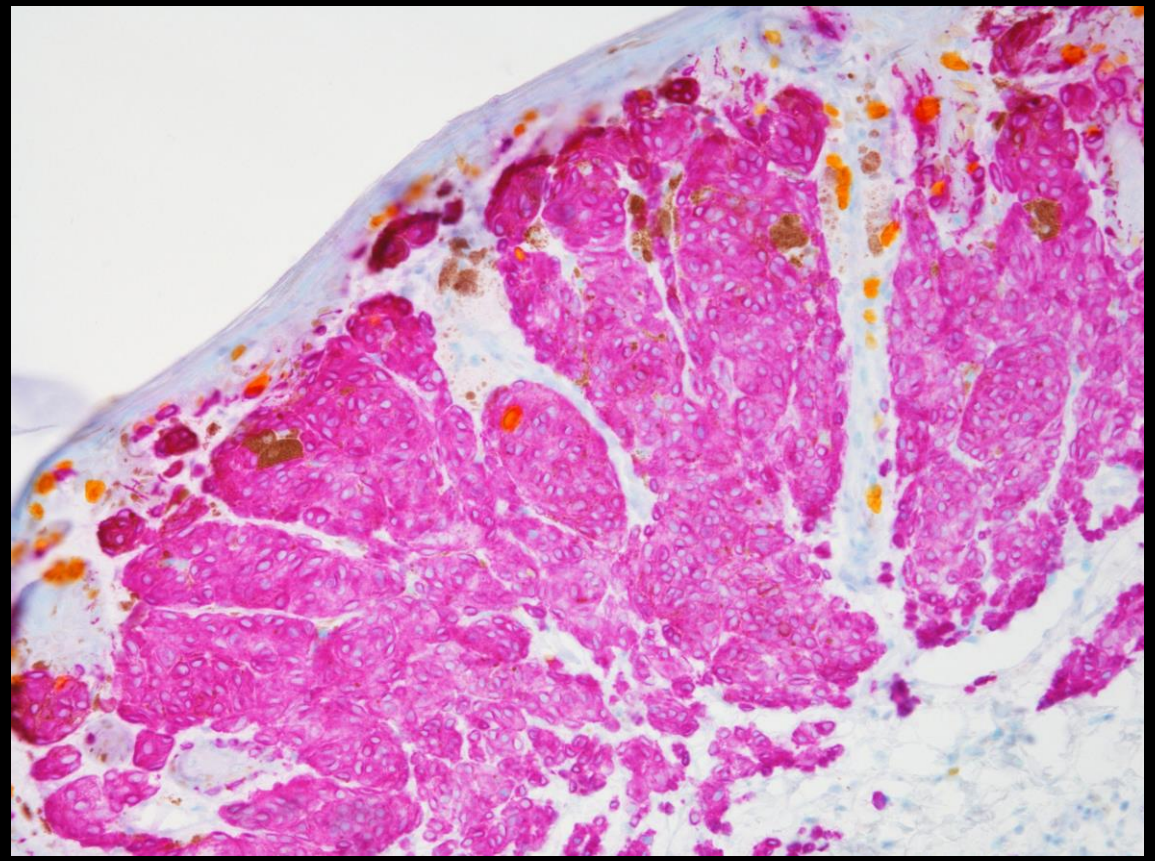


PRAME, rmAb EPR22330 (OmniMap-HRP/Teal) ➡ HD ➡ Ki67, rmAb SP6(OmniMap-HRP/Yellow) ➡ N ➡ MLA, mAb BS52 or MSA, mAb HMB45 (OmniMap-HRP/Purple)

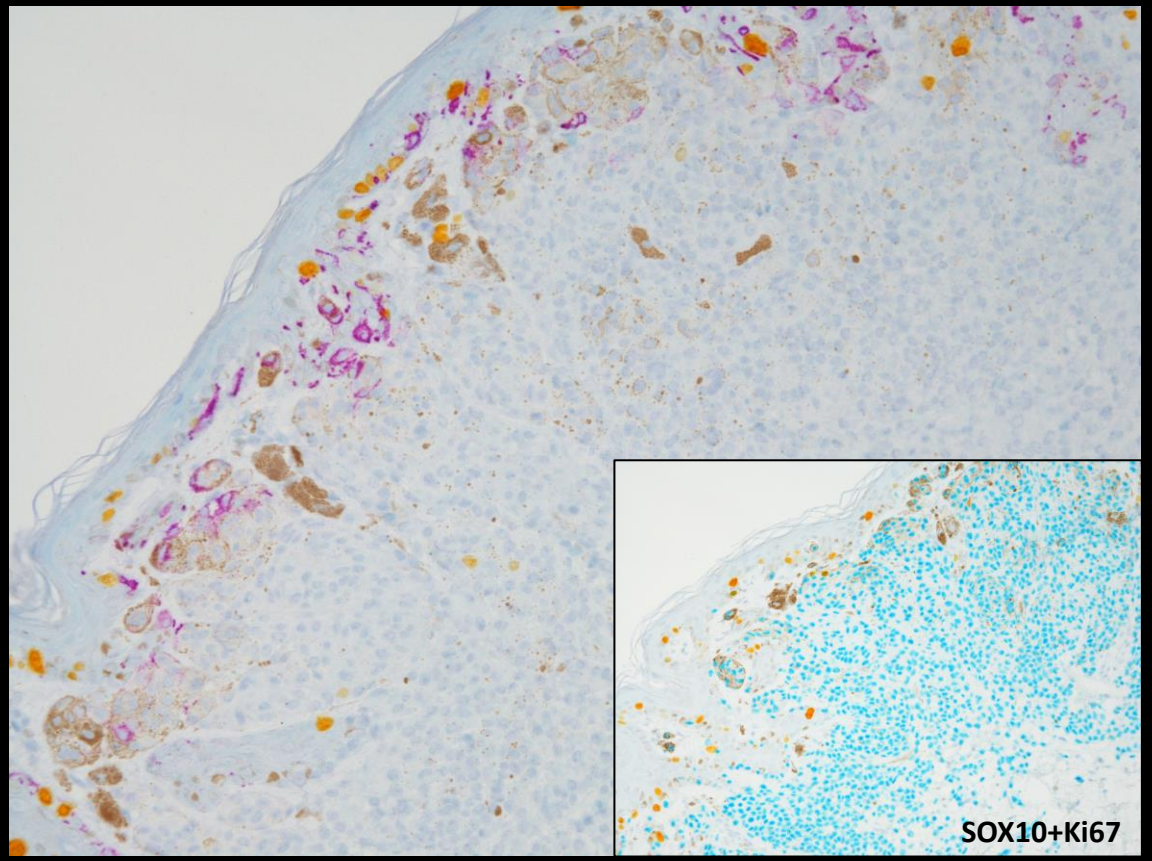
Compound Nevi

"Benign melanocytic lesion of the skin"

PRAME + Ki67 + MLA



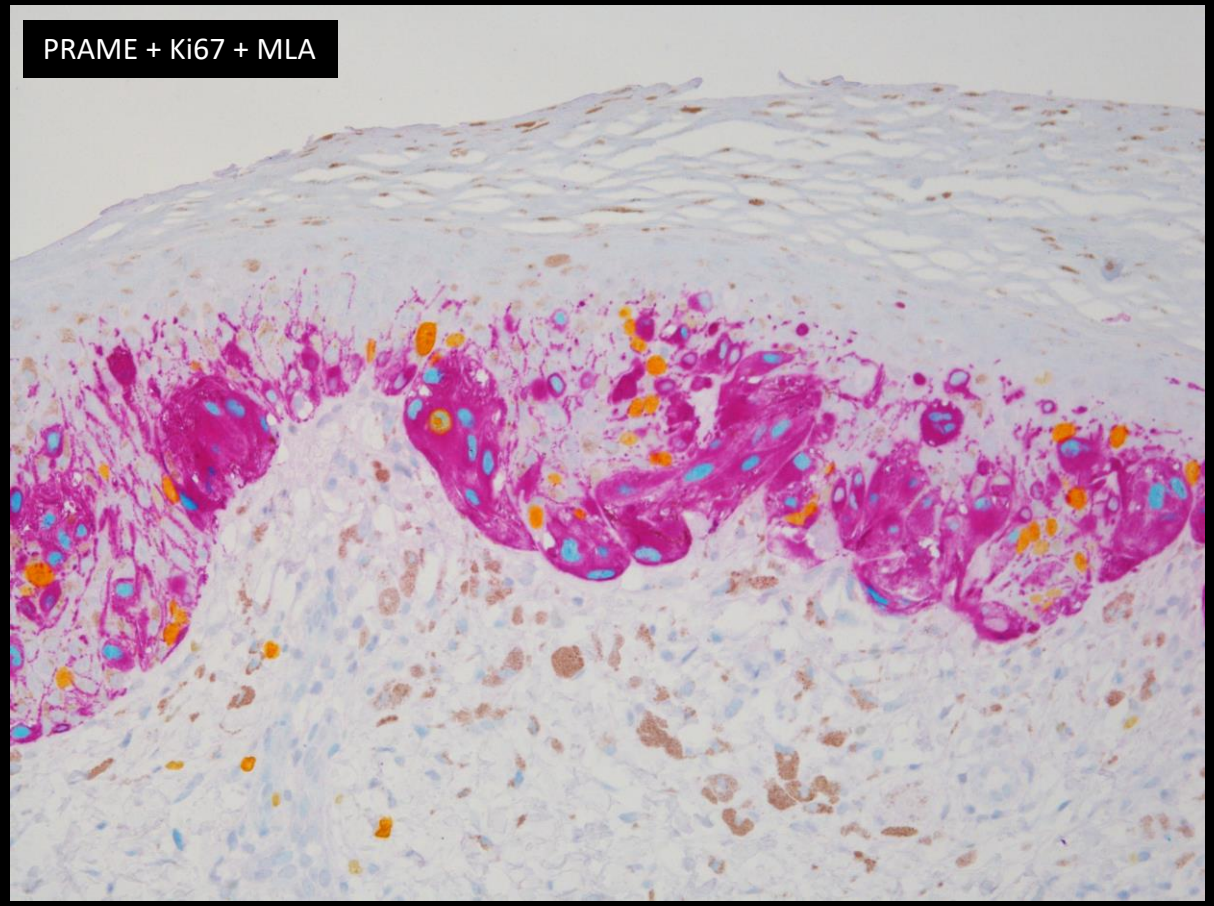
PRAME + Ki67 + HMB45 (epidermal reaction of HMB45)

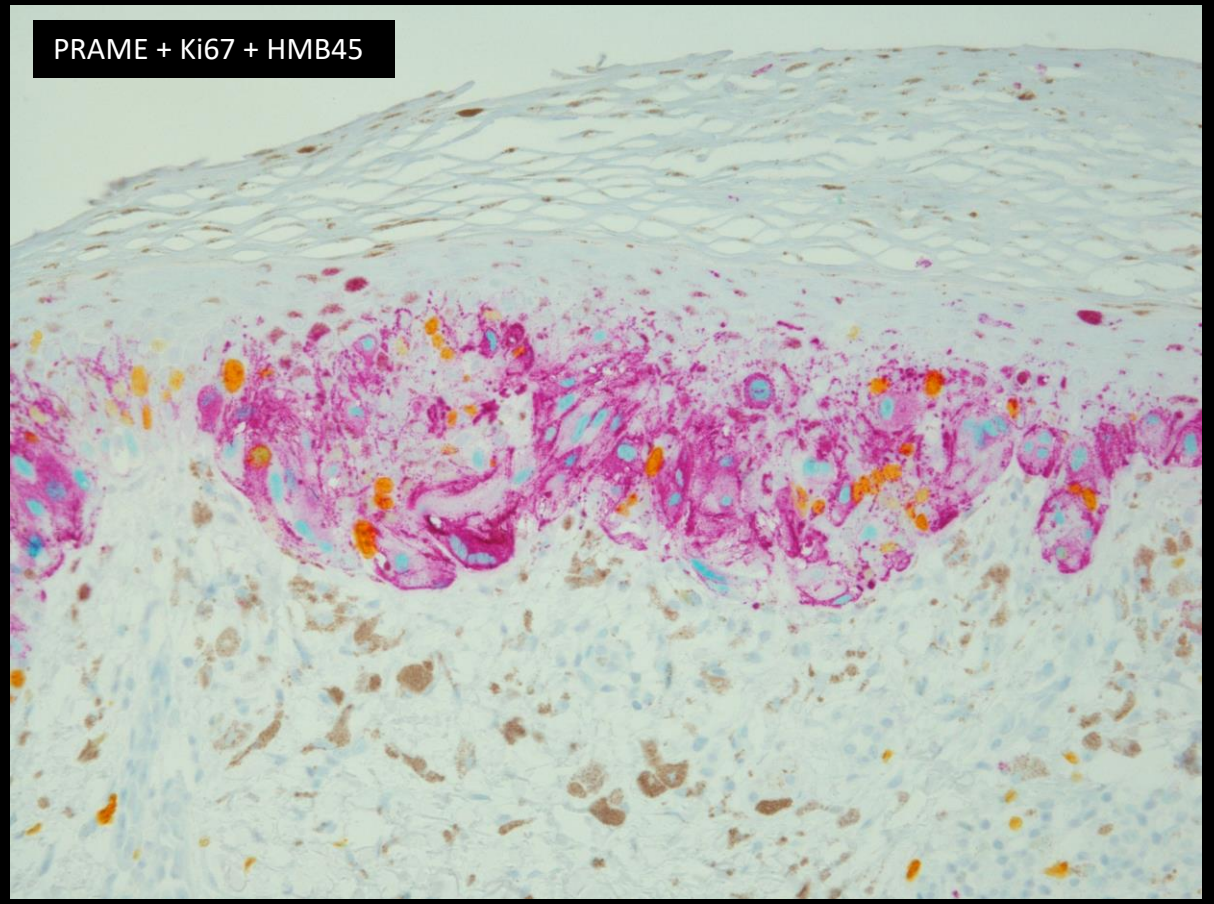


PRAME, rmAb EPR22330 (OmniMap-HRP/Teal) → HD → Ki67, rmAb SP6(OmniMap-HRP/Yellow) → N → MLA, mAb BS52 or MSA, mAb HMB45 (OmniMap-HRP/Purple)

Challenging Melanocytic lesion (PRAME positive)

Junctional nevi, Spitzoid morphology, Atypical proliferations

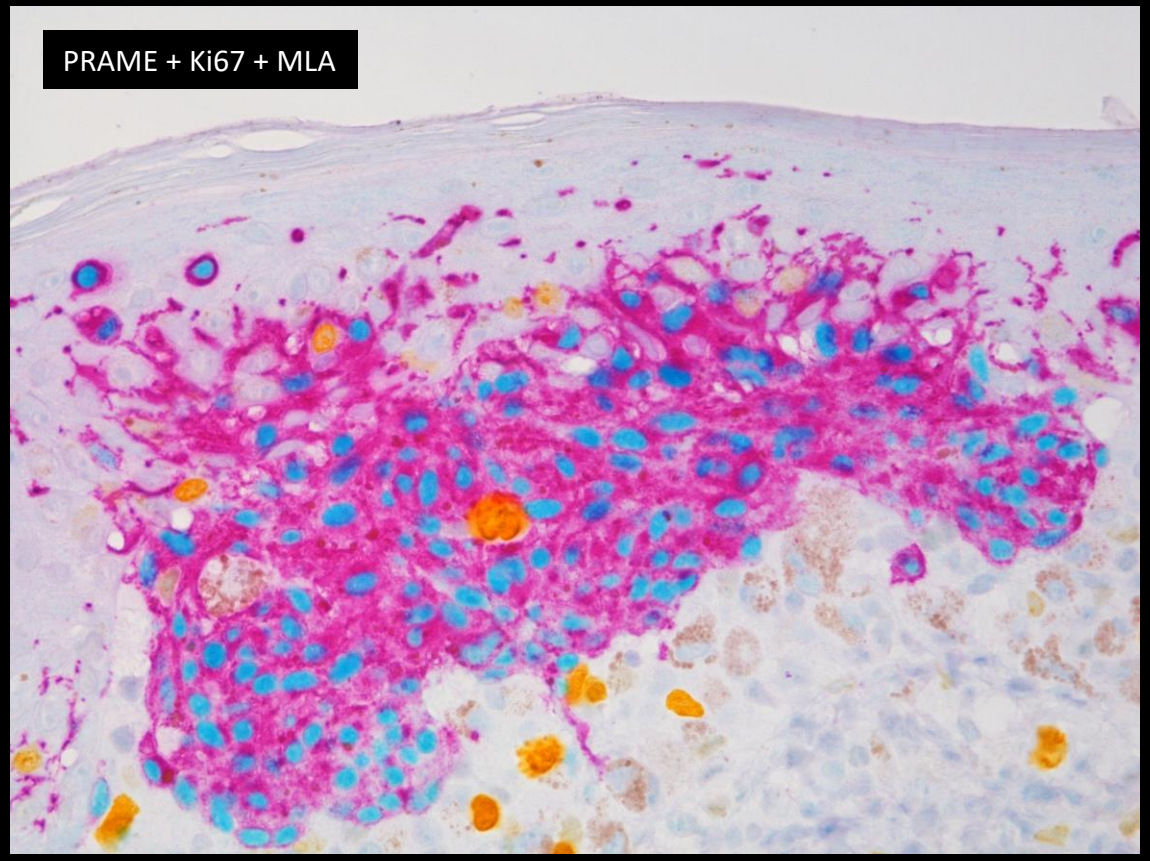


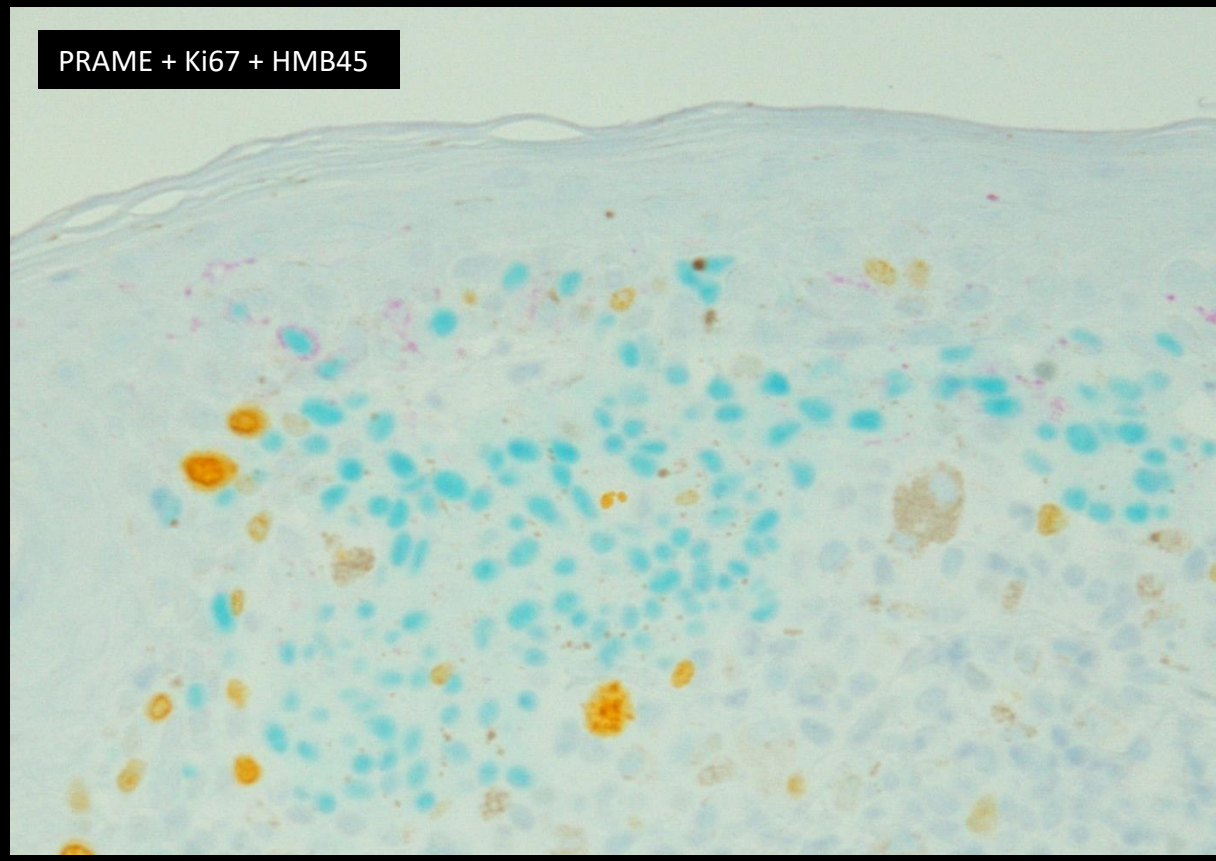


PRAME, rmAb EPR22330 (OmniMap-HRP/Teal) → HD → Ki67, rmAb SP6(OmniMap-HRP/Yellow) → N → MLA, mAb BS52 or MSA, mAb HMB45 (OmniMap-HRP/Purple)

Challenging Melanocytic lesion (PRAME positive)

Nevi, Atypical proliferations



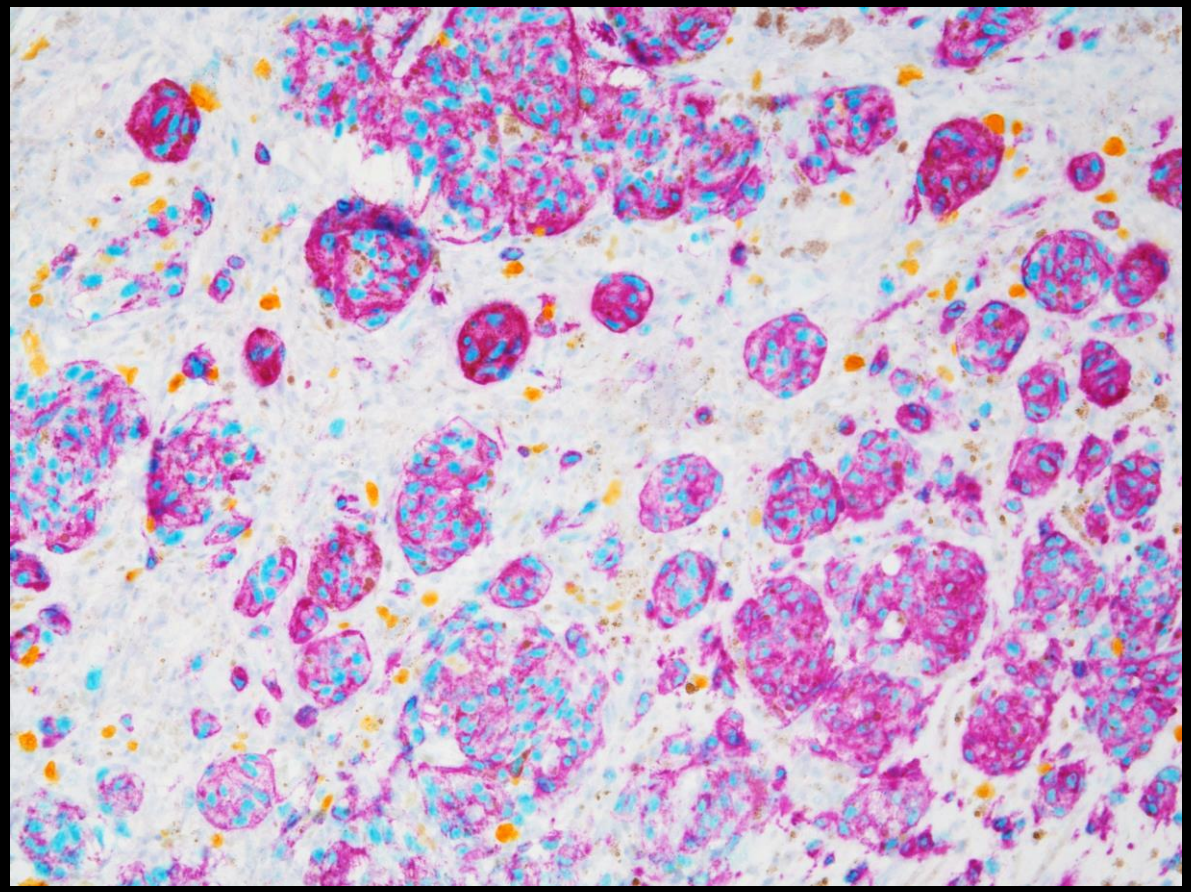


PRAME, rmAb EPR22330 (OmniMap-HRP/Teal) ➡ HD ➡ Ki67, rmAb SP6(OmniMap-HRP/Yellow) ➡ N ➡ MLA, mAb BS52 or MSA, mAb HMB45 (OmniMap-HRP/Purple)

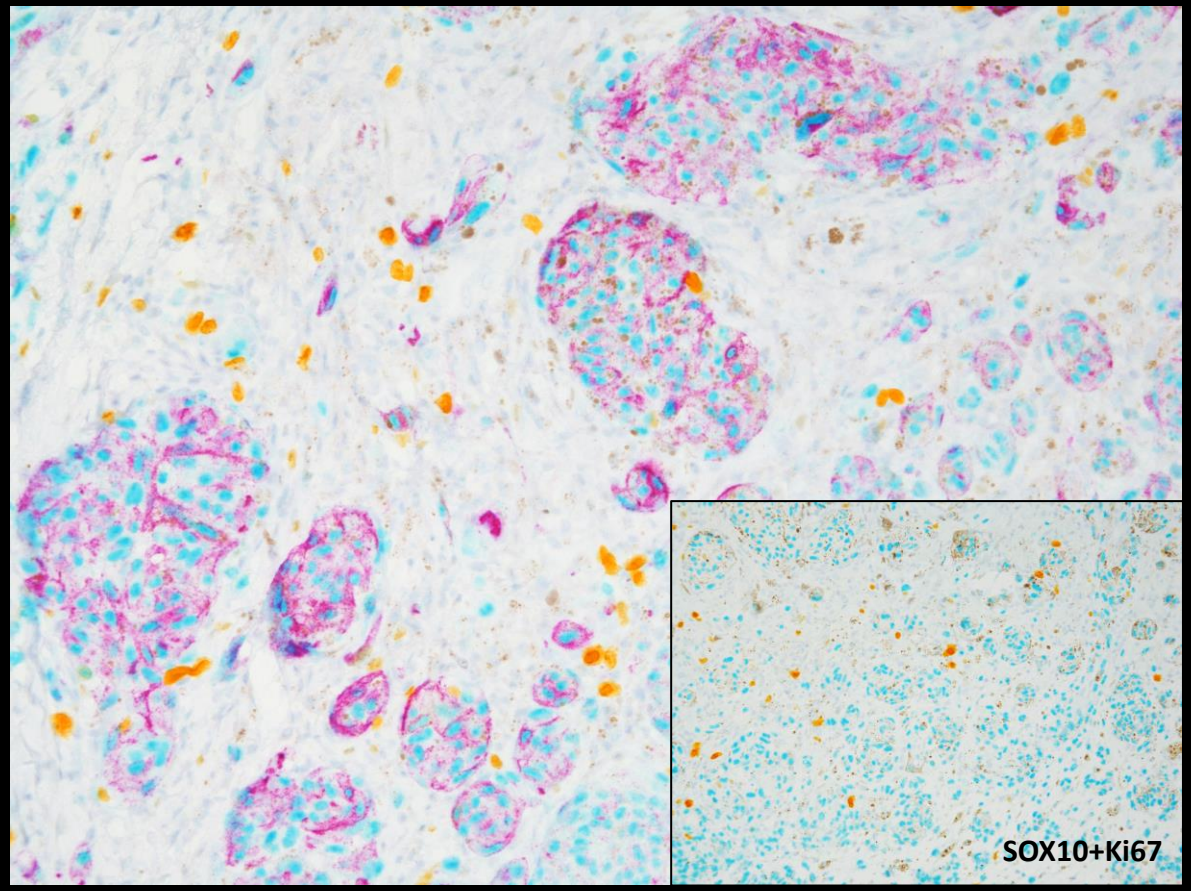
Challenging Melanocytic lesion (PRAME positive)

Blue Nevi

PRAME + Ki67 + MLA



PRAME + Ki67 + HMB45



PRAME, rmAb EPR22330 (OmniMap-HRP/Teal) → HD → Ki67, rmAb SP6(OmniMap-HRP/Yellow) → N → MLA, mAb BS52 or MSA, mAb HMB45 (OmniMap-HRP/Purple)

Considerations using PRAME with Ki67 to demonstrate “co-localized” nuclear staining

Discriminating benign melanocytic lesions from primary malignant melanomas

- Approximately 10-20% of primary malignant melanomas are negative for PRAME
- Only 35% of desmoplastic melanomas are positive for PRAME.
- PRAME can be expressed in malignant tumors of various sites/lineage and should be considered in the diagnostic work-up of malignant neoplasms of unknown origin

- 25-30% of benign or atypical Spitzoid lesions are positive for PRAME
- 10-15% of benign melanocytic tumors show positivity (often focal in a minority of nuclei)

Selected references

Lezano et al.: AJSP. 2018(11):1456-1465

Raghavan et al.: J Cutan Pathol. 2020 47(12):1123-1131

Cassalia et al.: Int. J. Mol. Sci.2024, 25(3), 1532

Googe et al.: Am J Dermatopath. 2021, 43(11):794-800

→ SOX10 in combination with Ki67 might be a better choice

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Immunohistochemical double nuclear staining for cell-specific automated quantification of the proliferation index – A promising diagnostic aid for melanocytic lesions

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ARTICLE INFO

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Melanocytic lesions
Proliferation index
Ki67
Multiplex immunohistochemistry
Digital pathology
Digital image analysis

ABSTRACT

Aims: Pathologists often use immunohistochemical staining of the proliferation marker Ki67 in their diagnostic assessment of melanocytic lesions. However, the interpretation of Ki67 can be challenging. We propose a new workflow to improve the diagnostic utility of the Ki67-index. In this workflow, Ki67 is combined with the melanocytic tumour-cell marker SOX10 in a Ki67/SOX10 double nuclear stain. The Ki67-index is then quantified automatically using digital image analysis (DIA). The aim of this study was to optimise and test three different multiplexing methods for Ki67/SOX10 double nuclear staining.

Methods: Multiplex immunofluorescence (mIF), multiplex immunohistochemistry (mIHC), and multiplexed immunohistochemical consecutive staining on single slide (MICSSS) were optimised for Ki67/SOX10 double nuclear staining. DIA applications were designed for automated quantification of the Ki67-index. The methods were tested on a pilot case-control cohort of benign and malignant melanocytic lesions (n = 23).

Results: Using the Ki67/SOX10 double nuclear stain, malignant melanocytic lesions could be completely distinguished from benign lesions by the Ki67-index. The Ki67-index cut-offs were 1.8% (mIF) and 1.5% (mIHC and MICSSS). The AUC of the automatically quantified Ki67-index based on double nuclear staining was 1.0 (95% CI: 1.0;1.0), whereas the AUC of conventional Ki67 single-stains was 0.87 (95% CI: 0.71;1.00).

Conclusions: The novel Ki67/SOX10 double nuclear stain highly improved the diagnostic precision of Ki67 interpretation. Both mIHC and mIF were useful methods for Ki67/SOX10 double nuclear staining, whereas the MICSSS method had challenges in the current setting. The Ki67/SOX10 double nuclear stain shows potential as a valuable diagnostic aid for melanocytic lesions.

Demonstrated that:

Digitalized Ki67/SOX10 double nuclear stain highly improved precision of Ki67 interpretation - discriminating benign from malignant melanocytic lesions of the skin.

Highlights

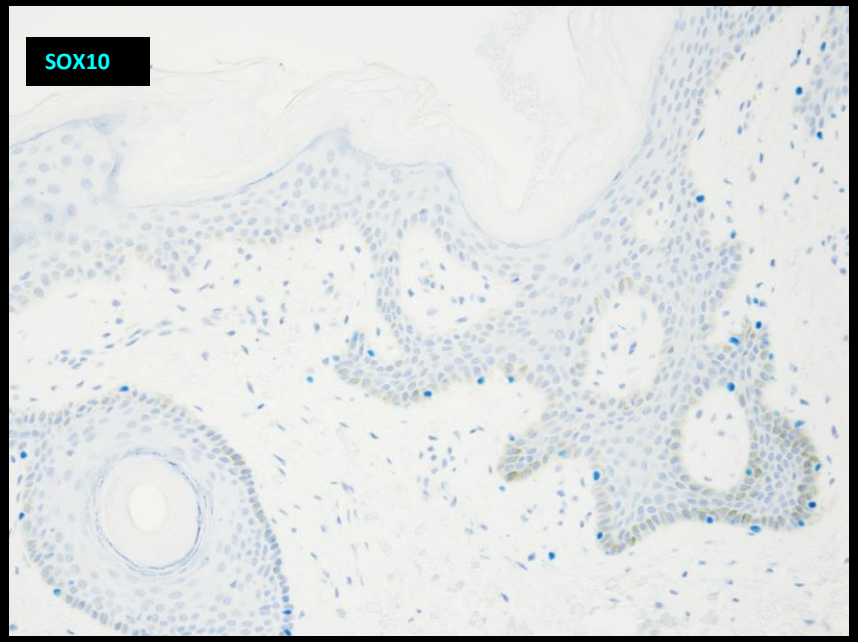
- The proliferation marker Ki67 is elevated in malignant melanocytic lesions.
- Unprecise quantification limits the diagnostic utility of the Ki67-index.
- Multiplex immunohistochemistry enables cell specific Ki67 assessment.
- Digital image analysis enables precise and reproducible quantification.
- The novel Ki67/SOX10 double nuclear stain improved the diagnostic precision of the Ki67-index.

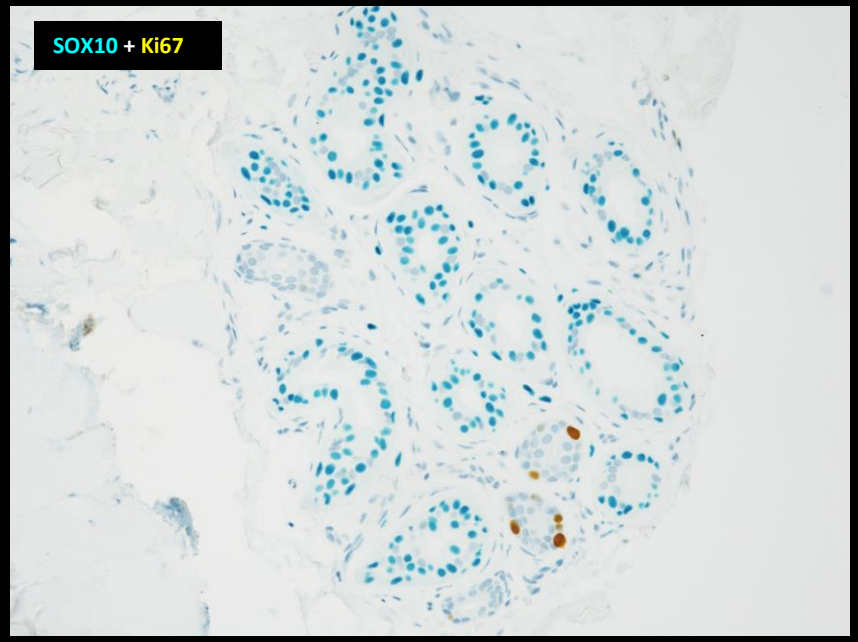
SOX10, SP267 (anti-HQ-HRP/Teal)

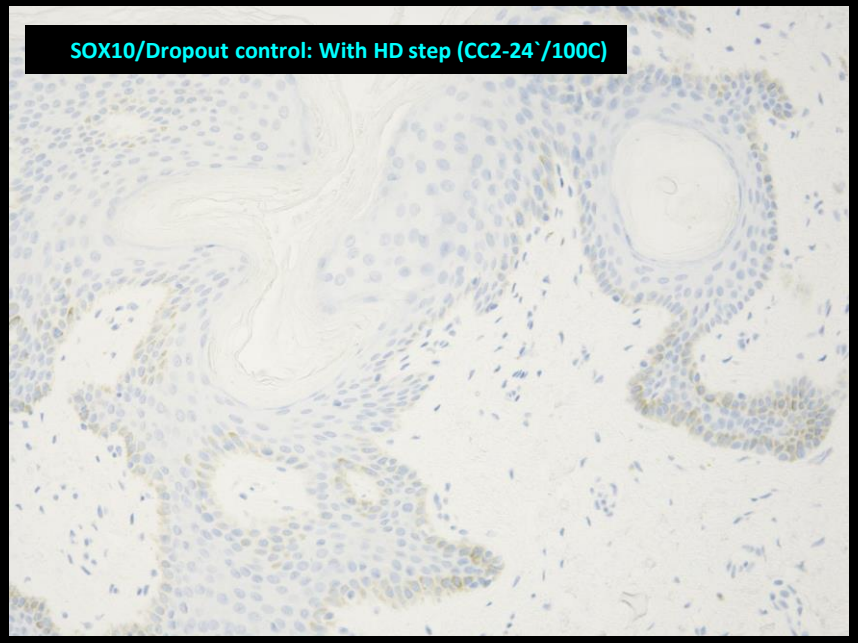
+HD (CC2-24`/100C)

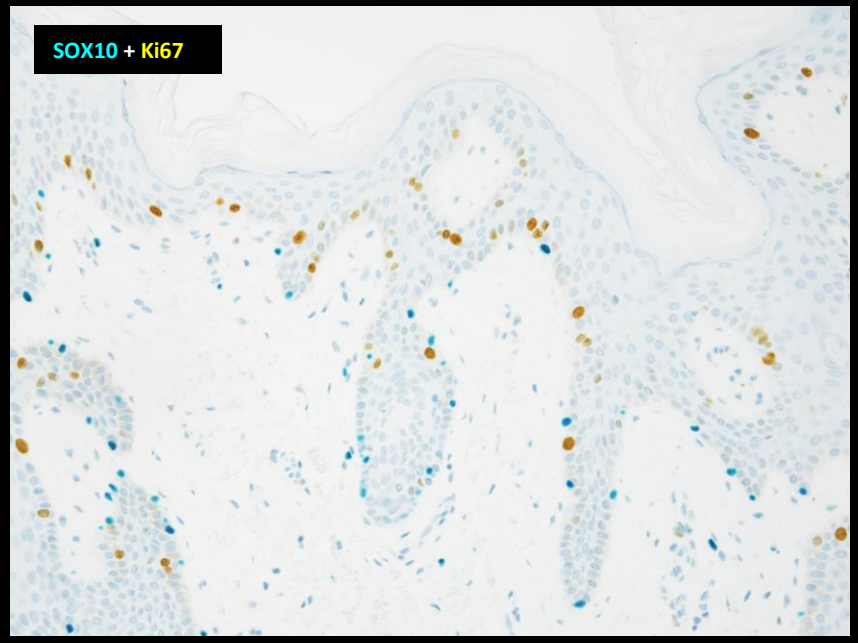
Ki67, SP6 (OmniMap-HRP/Yellow)

Normal Skin
(including sweat glands)









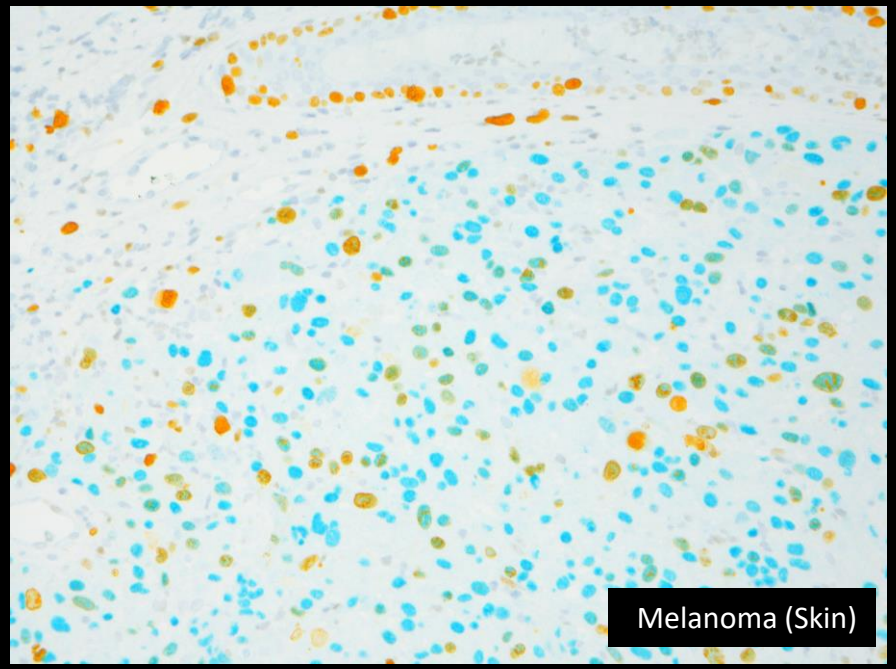
DB staining

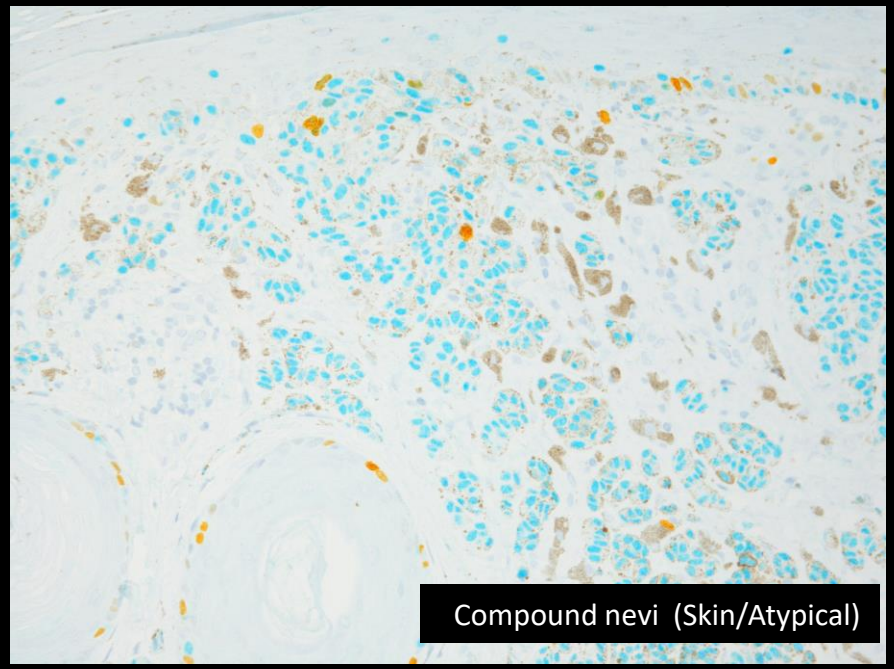
SOX10, SP267 (anti Rb-HQ/ Ms anti-HQ-HRP/Teal)

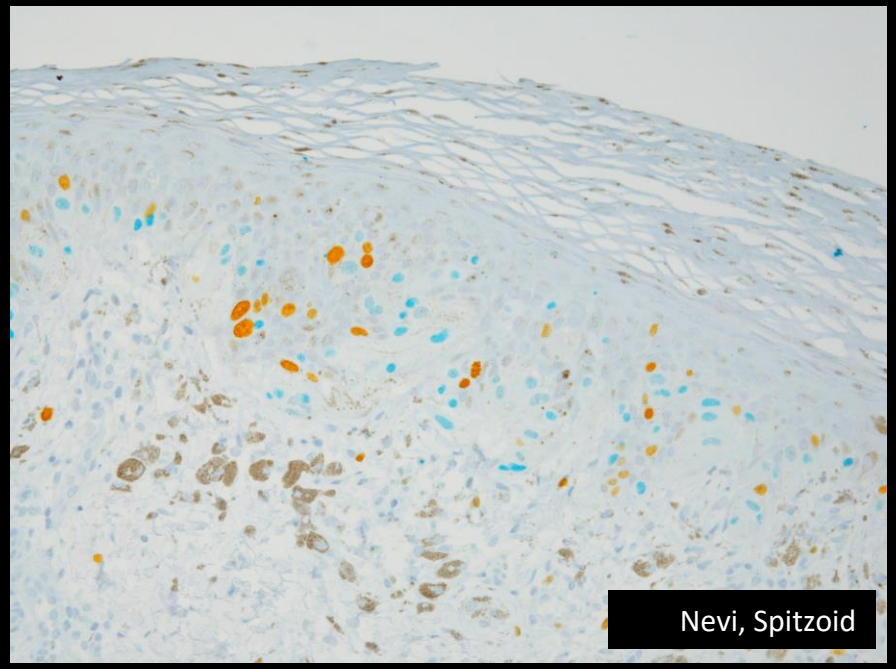
+HD (CC2-24`/100C)

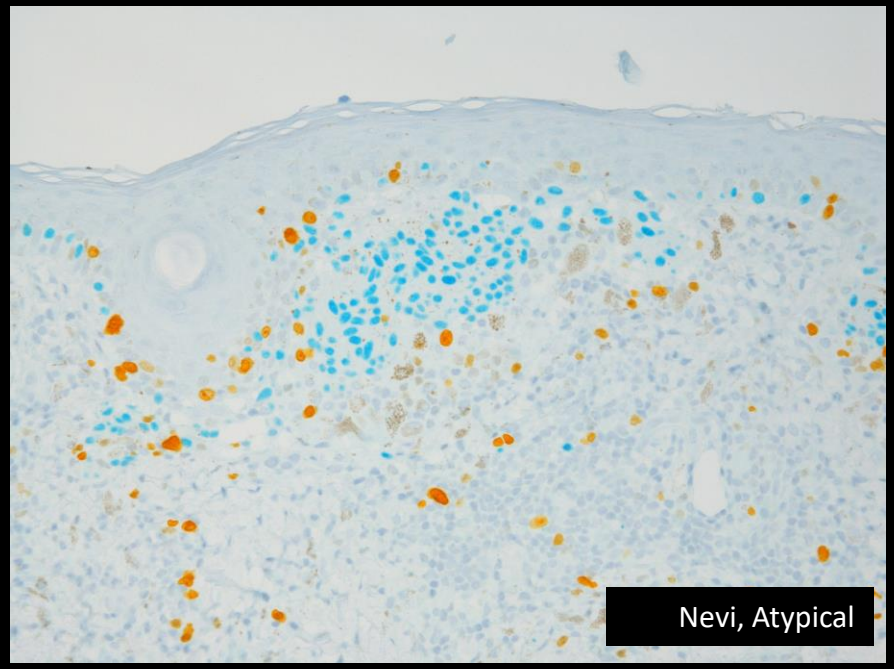
Ki67, SP6 (OmniMap anti Rb-HRP/Yellow)

Only the melanoma display co-localized signals



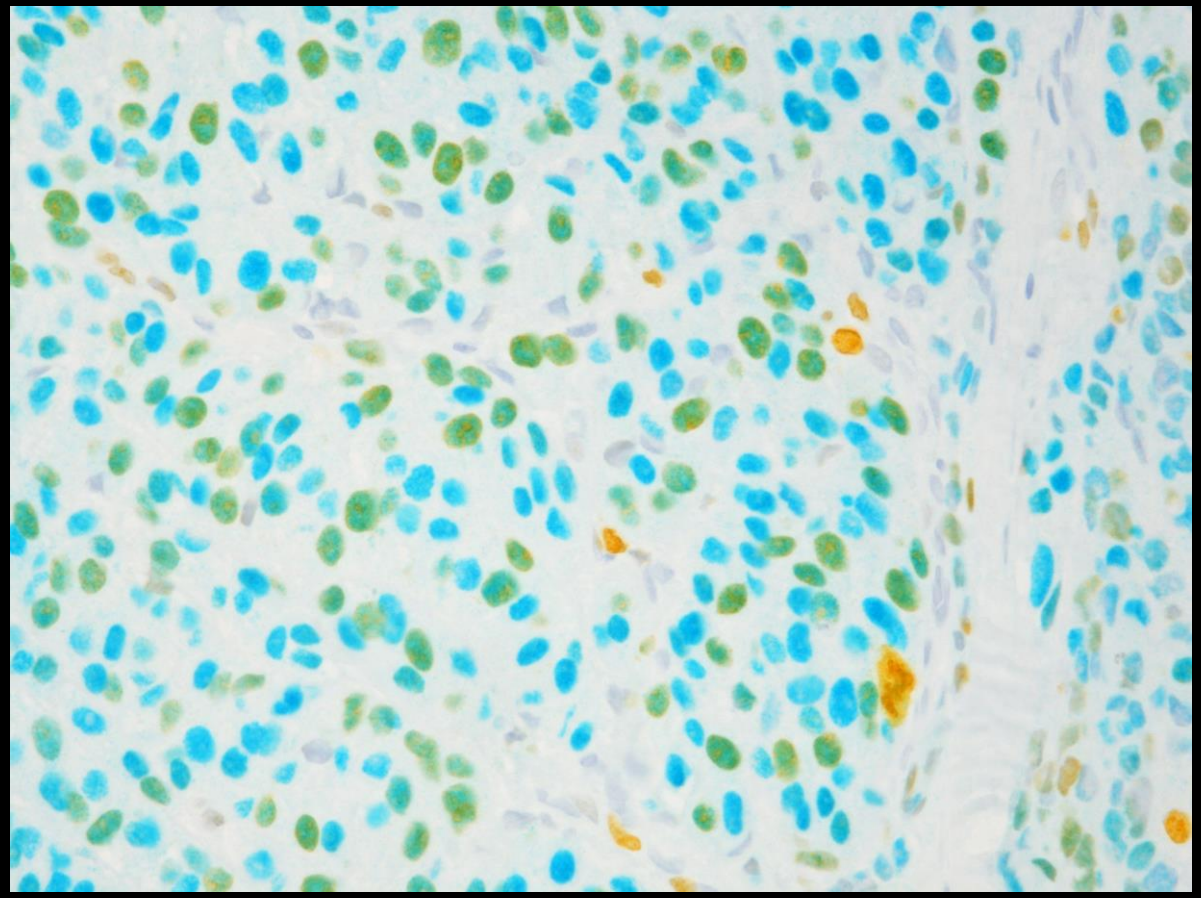






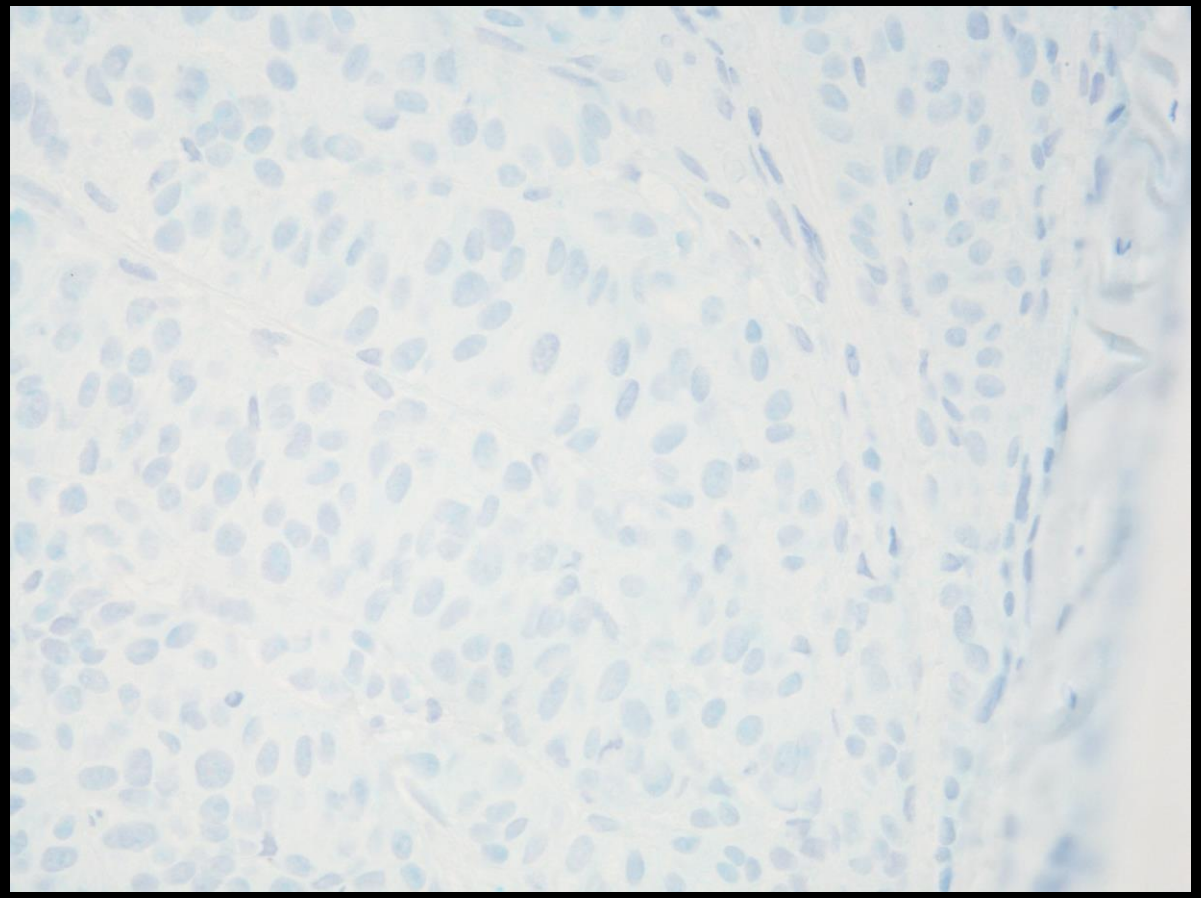
DB staining

SOX10, SP267 (anti Rb-HQ/ Ms anti-HQ-HRP/Teal)
+HD (CC2-24`/100C)
Ki67, SP6 (OmniMap anti Rb-HRP/Yellow)



HD drop-out control (cross-reactivity/SOX10)

SOX10, SP267 (anti Rb-HQ/anti-HQ-HRP) – No chromogen (Teal)
+HD (CC2-24`/100C)
OmniMap anti Rb-HRP/Teal – No Primary Ab (Ki67, SP6)



Melanoma

Melanoma

Which combination would you use for demonstration of co-localized signals ?

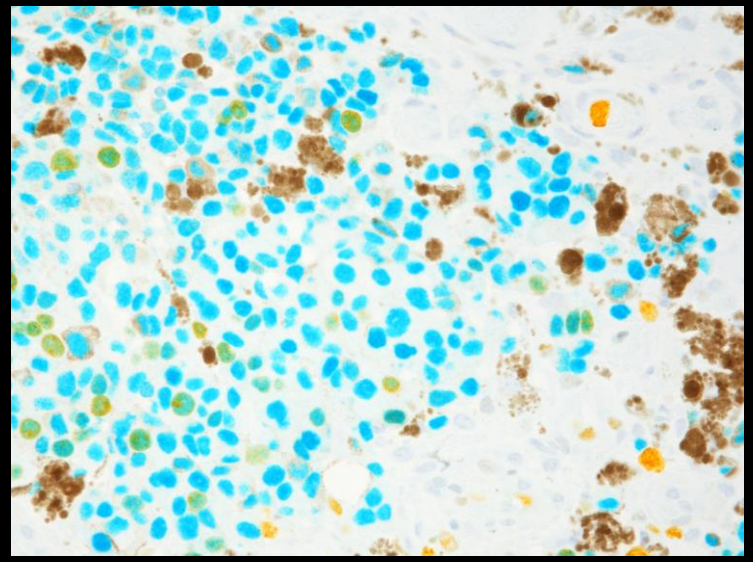
Why ?

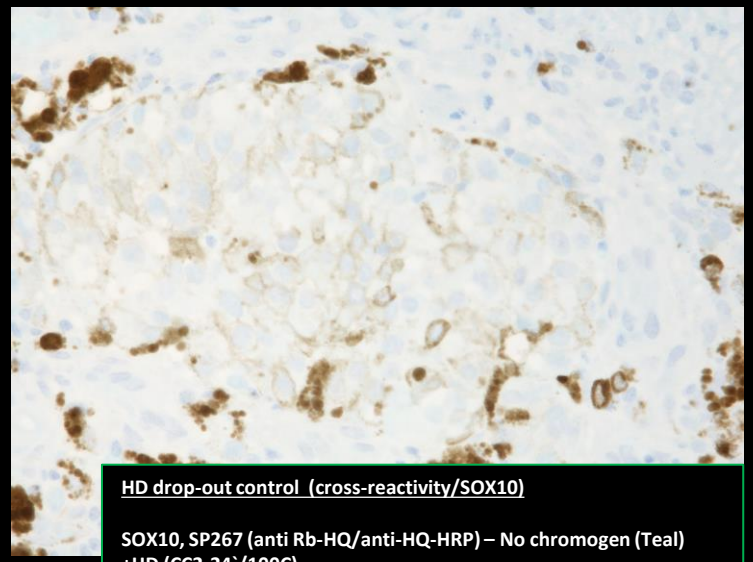
HD drop-out controls

Importantly:
Using anti Ms-HQ /anti Rb-HQ + Ms anti-HQ-HRP as detection system might cause problems, and thus, should include drop-out controls due to cross-reactivity between Ms-anti-HQ applied in first sequence and OmniMap anti Ms-HRP in following sequence

DB staining

SOX10, SP267 (anti Rb-HQ/Ms anti-HQ-HRP/Teal)
+HD (CC2-24`/100C)
Ki67, SP6 (OmniMap anti Rb-HRP/Yellow)



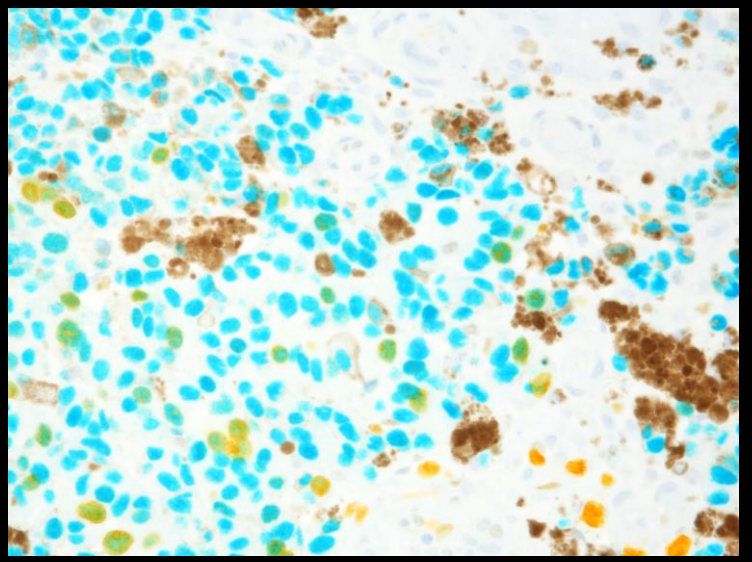


HD drop-out control (cross-reactivity/SOX10)

SOX10, SP267 (anti Rb-HQ/anti-HQ-HRP) – No chromogen (Teal)
+HD (CC2-24`/100C)
OmniMap anti Rb-HRP/Teal – No primary Ab (Ki67,SP6)

DB staining

SOX10, SP267 (anti Rb-HQ/Ms anti-HQ-HRP/Teal)
+HD (CC2-24`/100C)
Ki67, BS4 (OmniMap anti Ms-HRP/Yellow)



HD drop-out control (cross-reactivity/SOX10)

SOX10, SP267 (anti Rb-HQ/Ms anti-HQ-HRP) – No chromogen (Teal)
+HD (CC2-24`/100C)
OmniMap anti Ms-HRP/Teal – No Primary Ab (Ki67, BS4)

PRAME, rmAb EPR22330
(anti-HQ-HRP/Teal)

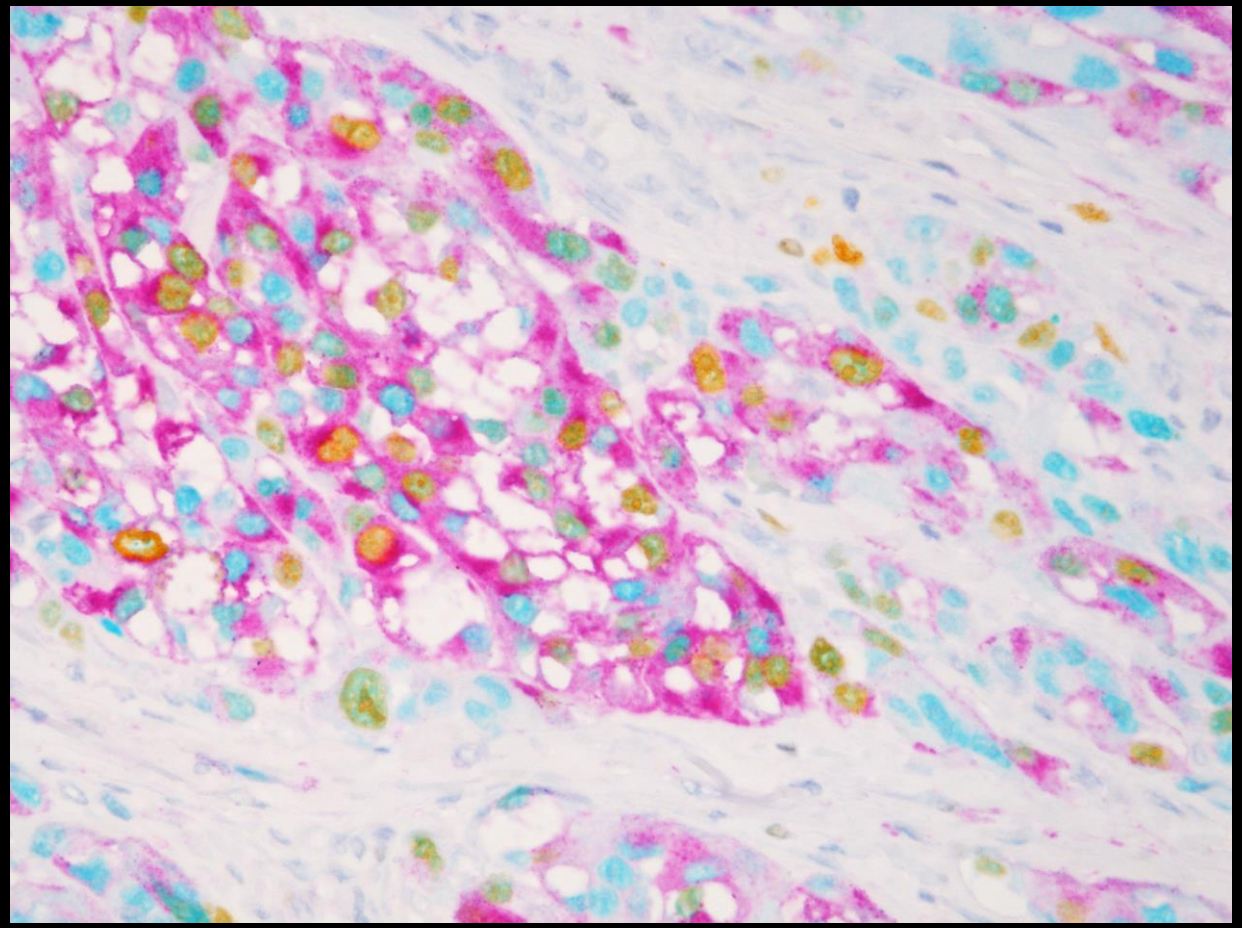
+HD

Ki67, rmAb SP6
(OmniMap-HRP/Yellow)

+N

MSA, mAb HMB45
(OmniMap-HRP/Purple)

Co-Localization: Green (ish)



PRAME, rmAb EPR22330
(anti-HQ-HRP/Purple)

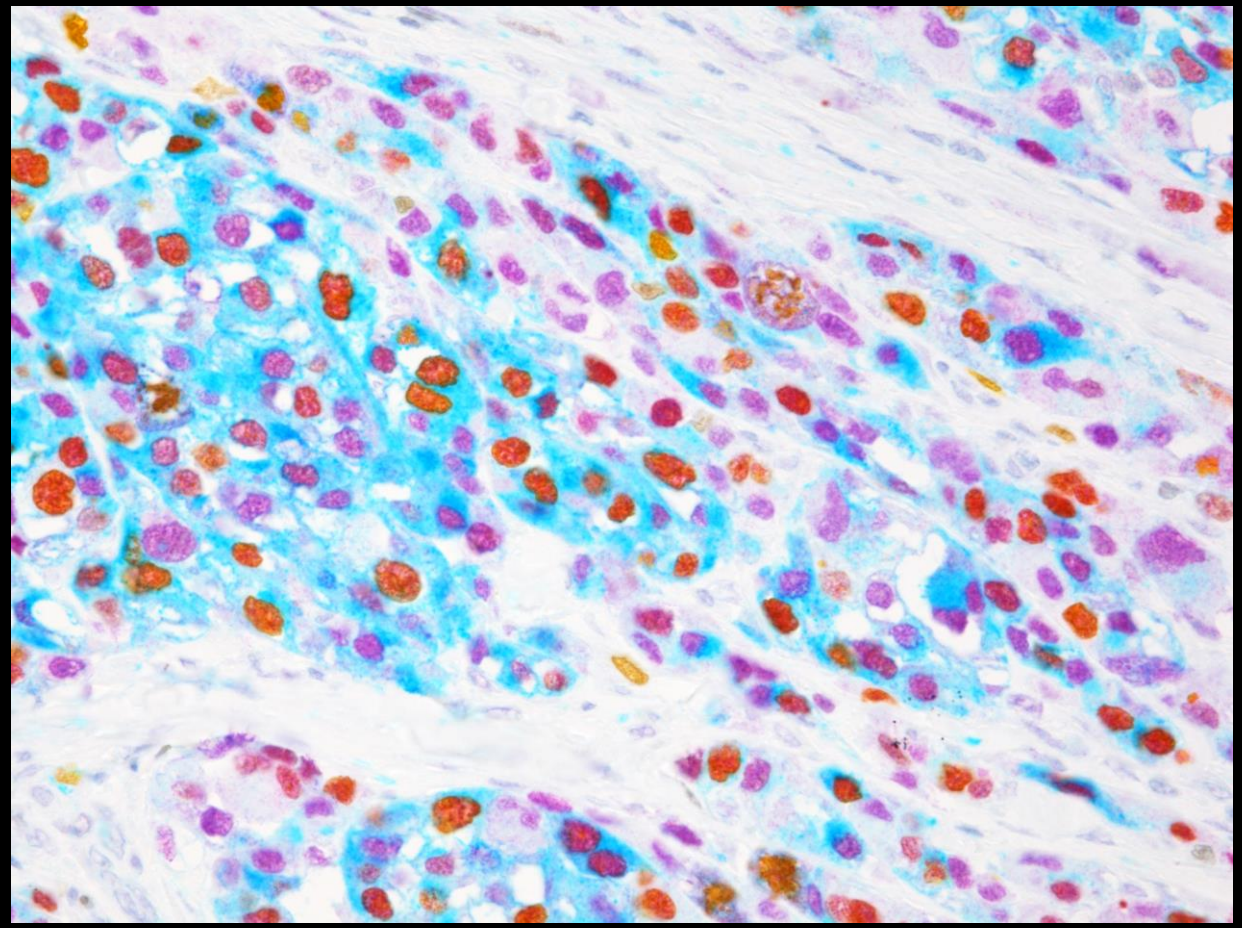
+HD

Ki67, rmAb SP6
(OmniMap-HRP/Yellow)

+N

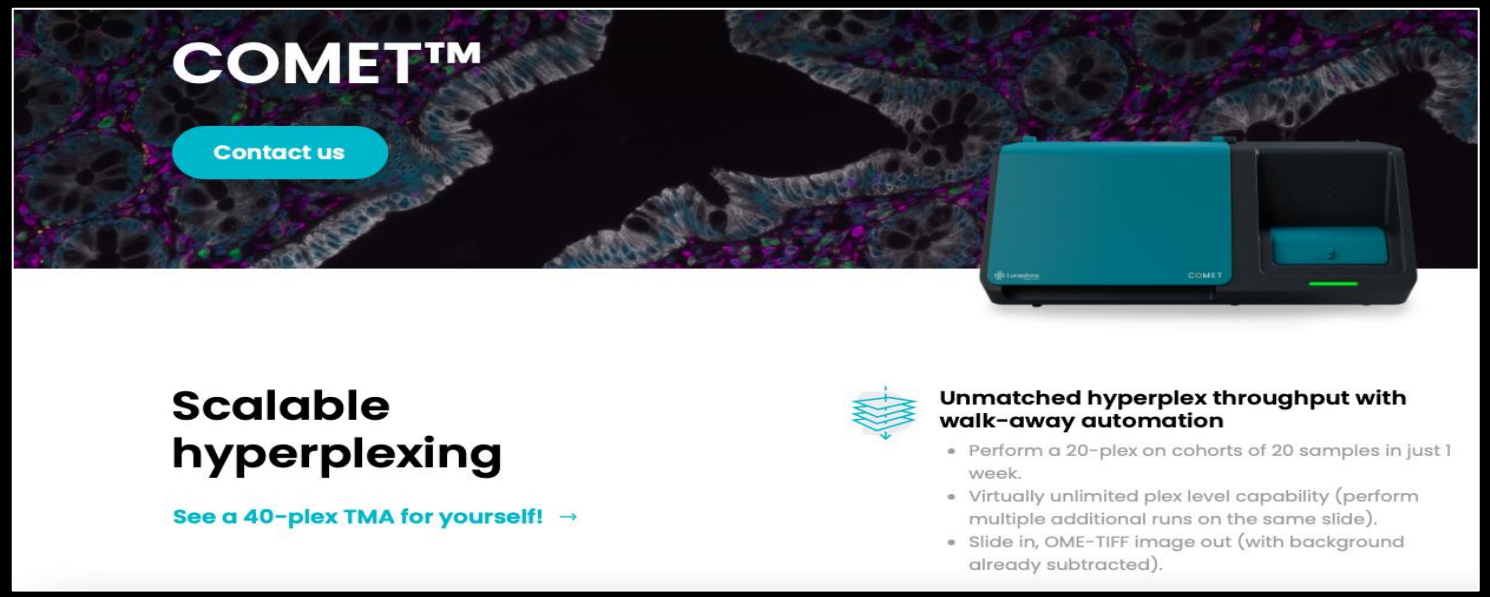
MSA, mAb HMB45
(OmniMap-HRP/Teal)

Co-Localization:Orange/Red (ish)



Melanoma

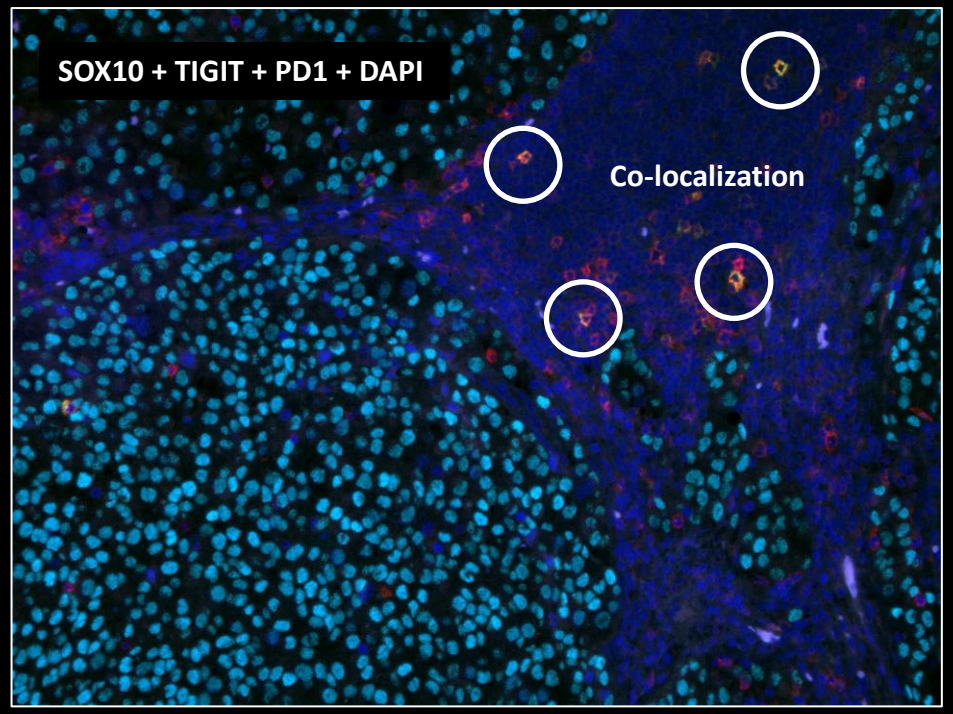
The future is already here: Advanced techniques (End-to-end solutions)

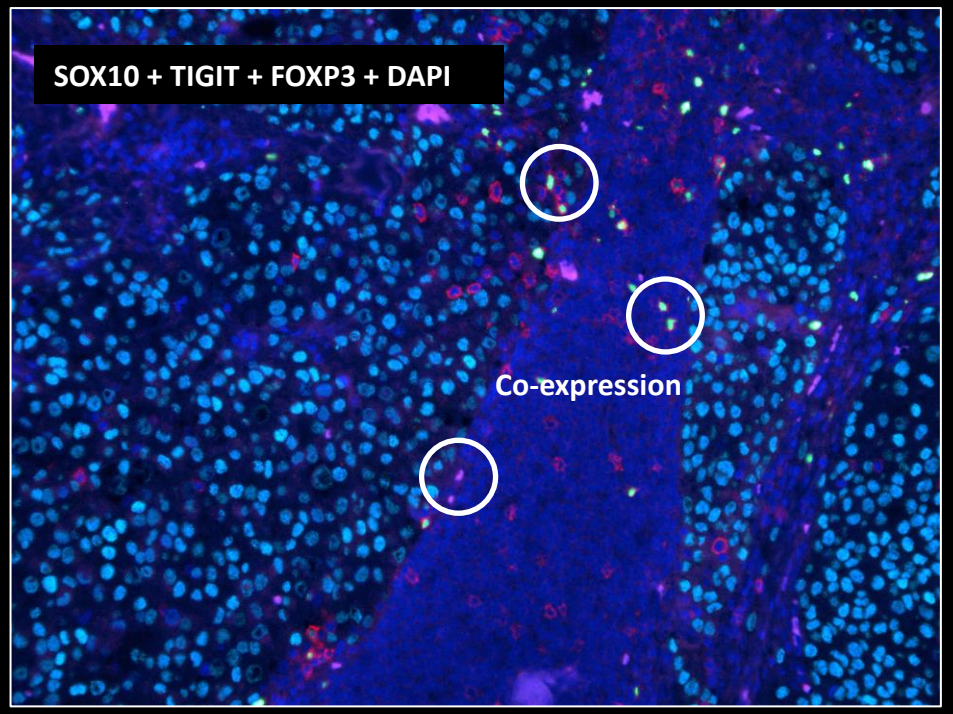


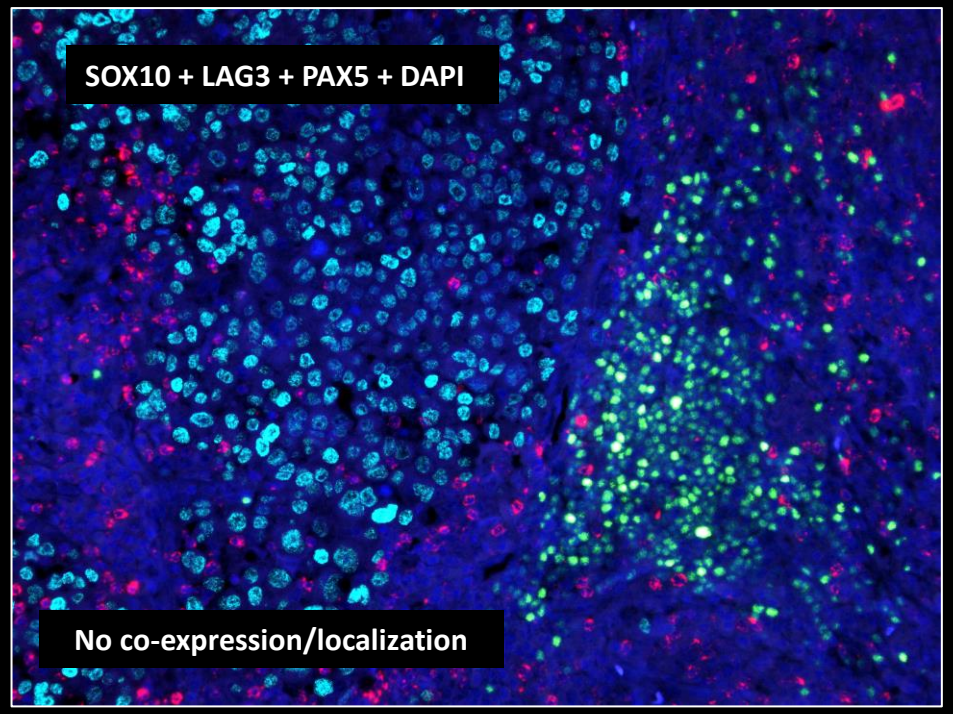
Lunaphore: COMET

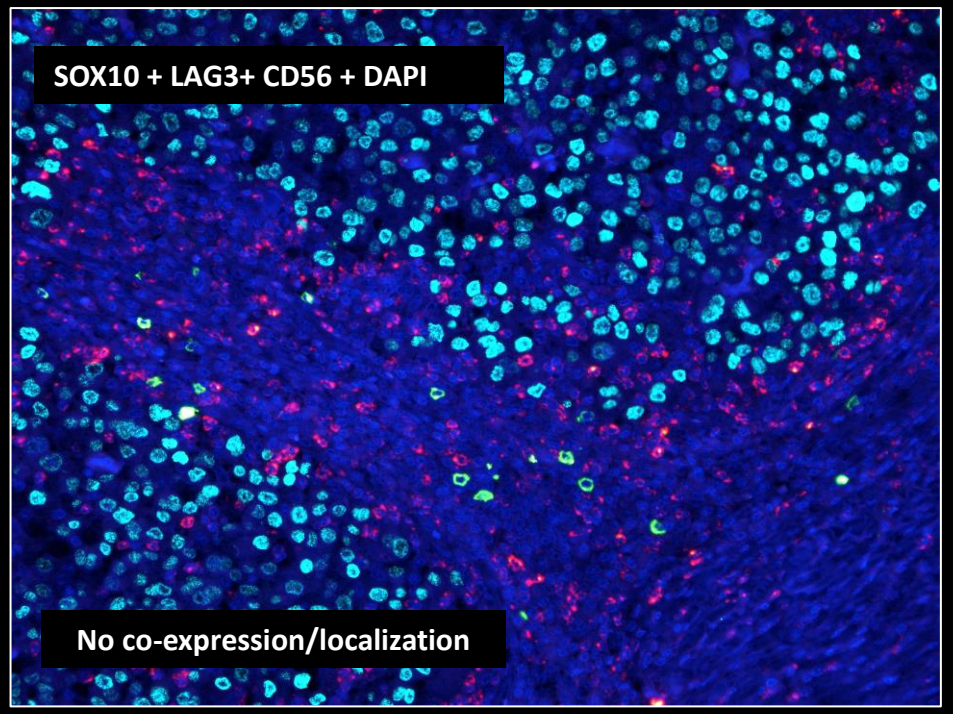
Akoya Bioscience: Phenocycler (Codex)

Thank you for your attention









Multiplex Immunofluorescence

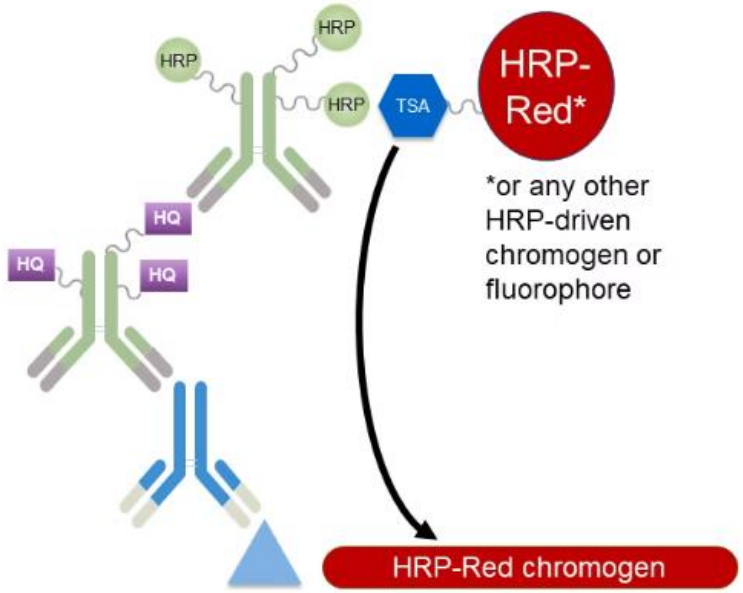
SOX10, BS7 or SP267 (DCC/Sp. Aqua) + LAG3 or TIGIT (R610/TxR)

✶ CD3/CD4/CD8/CD56/FOXP3/PD1/PAX5 (FAM/FITC)

Exemplified by the combination(s):

SOX10/LAG3/CD8 (or CD4)

Roche/Ventana Diagnostics

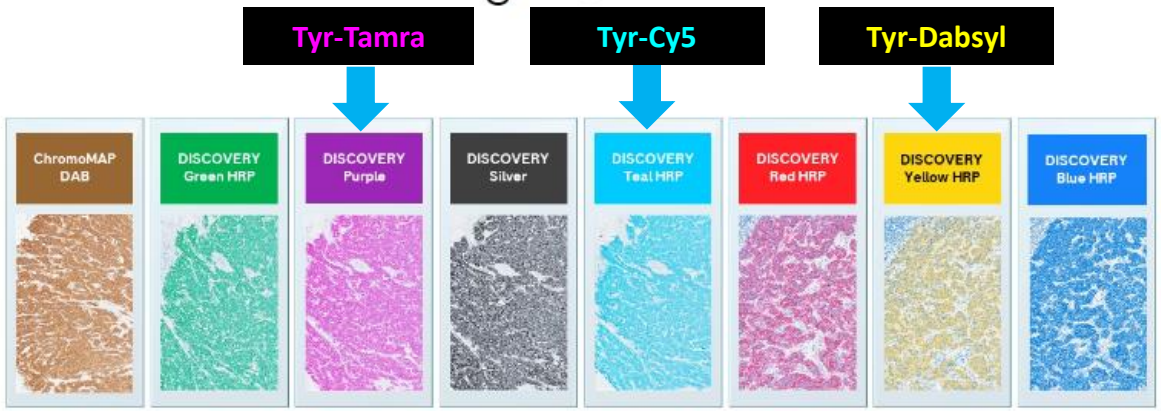


Tyramide chemistry is a powerful technique that can be used to stain or even amplify the signal of immunohistochemistry (IHC) staining. One major advantage is that the dye forms a covalent bond with proteins in the tissue for greater stability.

Like traditional IHC, the primary and secondary (HRP) antibodies are incubated with the tissue sample to bind to the antigen of interest. The chromogenic dye is designed with a tyramine group that becomes reactive after interaction with HRP in the presence of hydrogen peroxide to form a highly reactive intermediate. Similarly, tyrosine residues in the endogenous proteins nearby will become activated after contact with the HRP, and then condense with the dye intermediate to form a covalent bond and local deposition of the chromogenic (or fluorescent) dye. Finally, the sample is counterstained with a nuclear stain, such as DAPI or hematoxylin, to visualize the cellular structures. The result is a highly specific and sensitive signal.

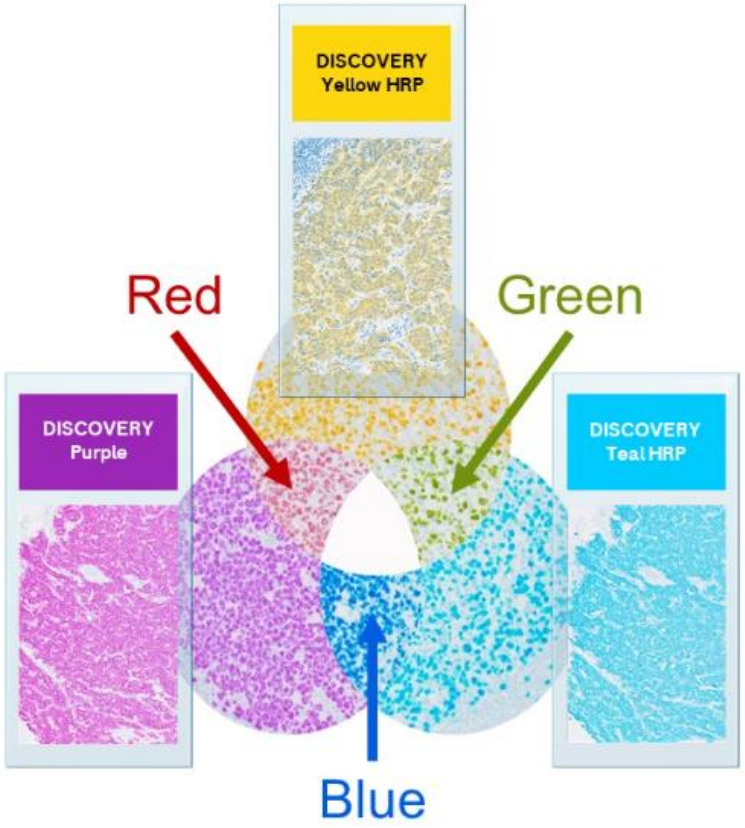
Translucent chromogens for demonstration of multiplexed co-localized signals

HRP-driven chromogens



AP-driven chromogens

Translucent chromogens enable multiplexed IHC co-localization.



If the biomarkers of interest are present in the same sub-cellular compartment (nucleus, cytoplasm or cell membrane) this overlapping expression of targets is a key consideration when selecting chromogen options for IHC multiplexing. When designing a chromogenic IHC assay that includes overlapping target biomarker expression, opaque colors such as DAB and Silver should not be used.

Traditionally, analysis of overlapping targets has been accomplished using immunofluorescence, but the availability of new Ventana translucent chromogens has provided an alternative method - in brightfield! Translucent chromogens allow a color shift when both colors are present in the same cell and sub-cellular compartment.

Availability of translucent chromogens such as Purple, Yellow, and Teal has opened up the ability to visualize overlapping targets in brightfield IHC or ISH multiplexed assays.

Visit our Multiplexing Resources page for more in-depth information on performing multiplexed IHC.

Roche/Ventana Diagnostics