# Workshop in Diagnostic Immunohistochemistry
# NordiQC (October 2023)

## Immunohistochemical double/multiplex techniques

## Overview, considerations and applications

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# Double/Multiplex staining techniques (IHC)

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

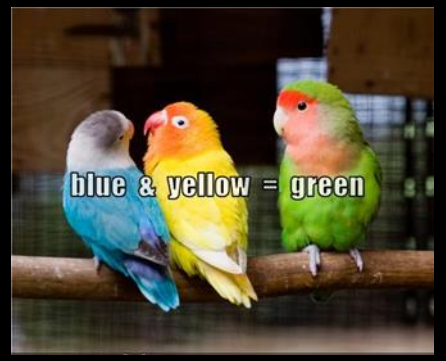
**Compared to single color IHC, the main advantage of multiplex IHC is two-fold:**

**First, it allows for the analysis of multiple parameters simultaneously on a single slide, it 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).**

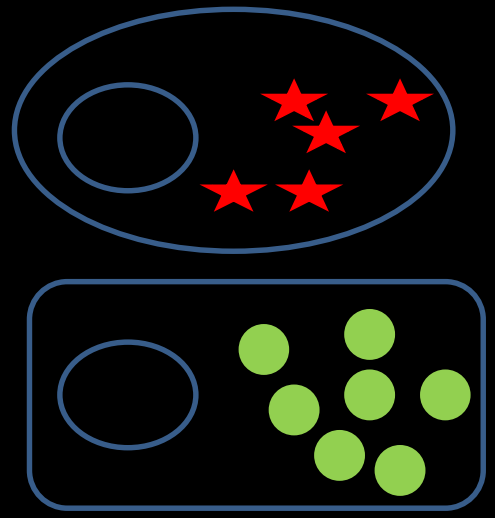
# Multiplex staining techniques (IHC)



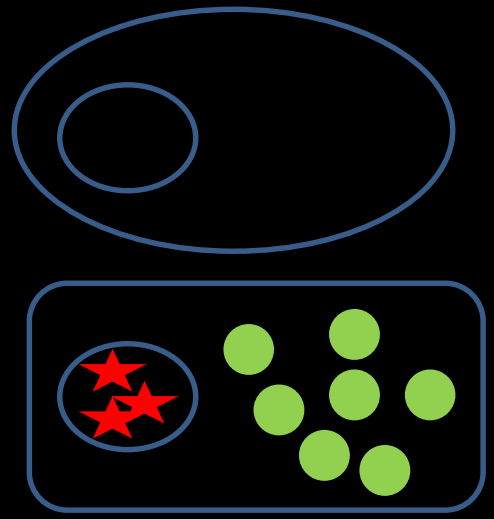

## Requirements ("simple double-staining techniques") :

- ❑ 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

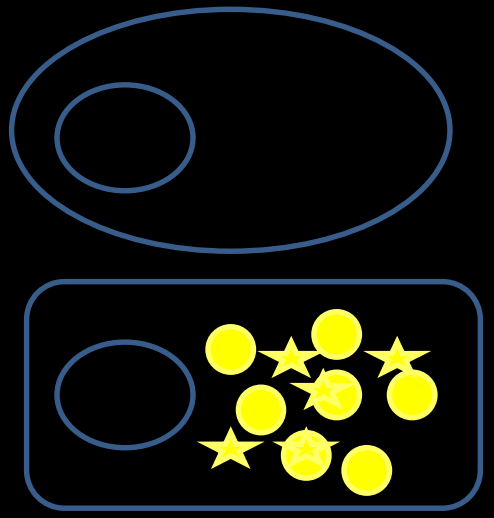
Expression in different cell types



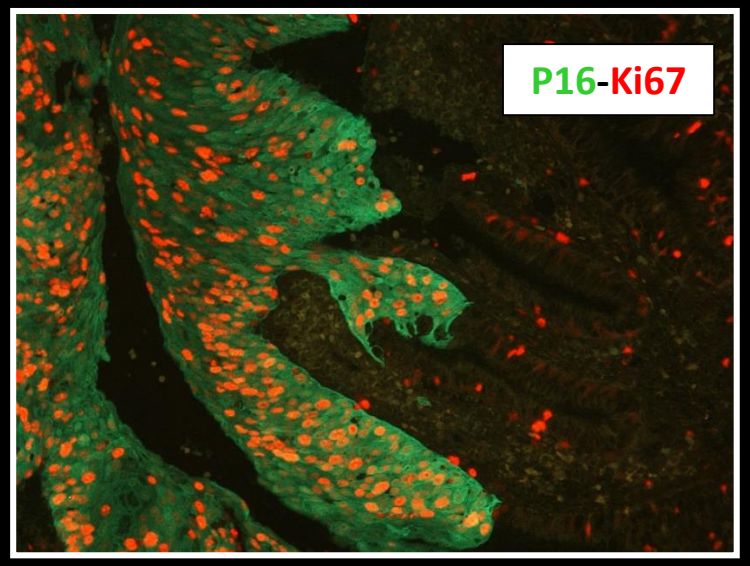
Co - expression

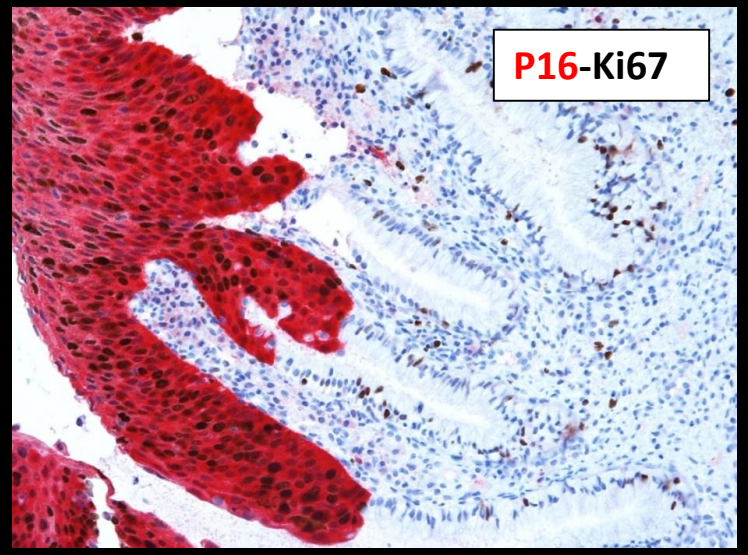


Co - localization (mixed color)



## 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

### A bit more 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.

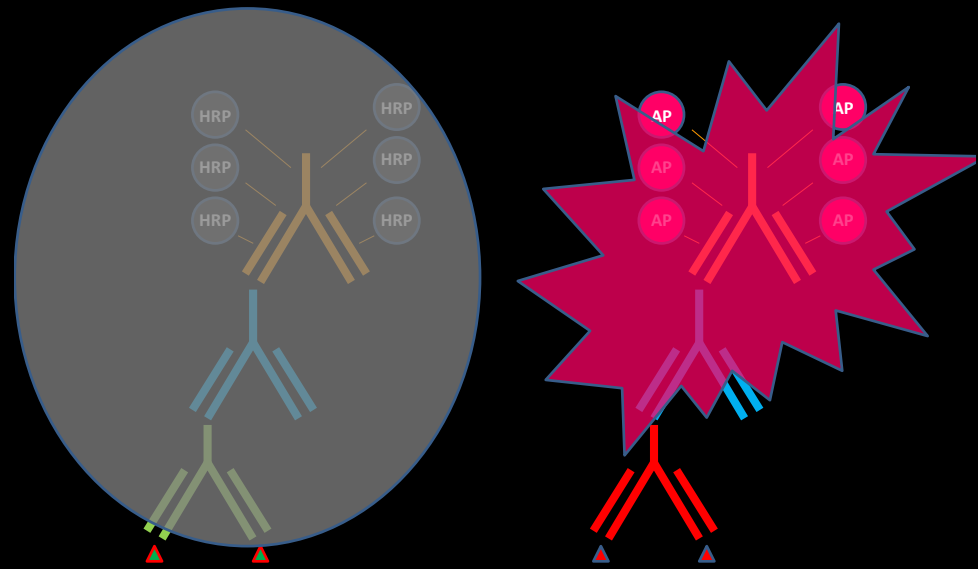
DAB reaction product of the first set of immunoreagents create a barrier that is impermeable for the second set of immunoreagents ~ no cross reactivity

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

**Testing (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

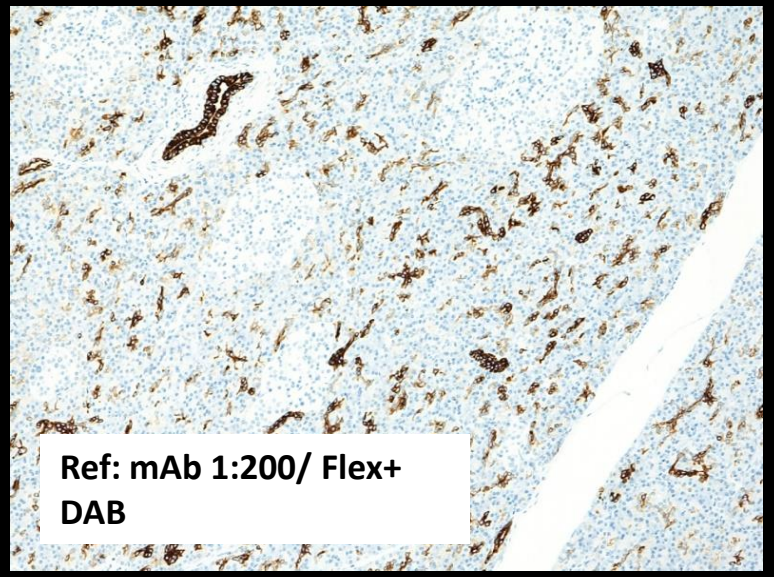
→ Double staining

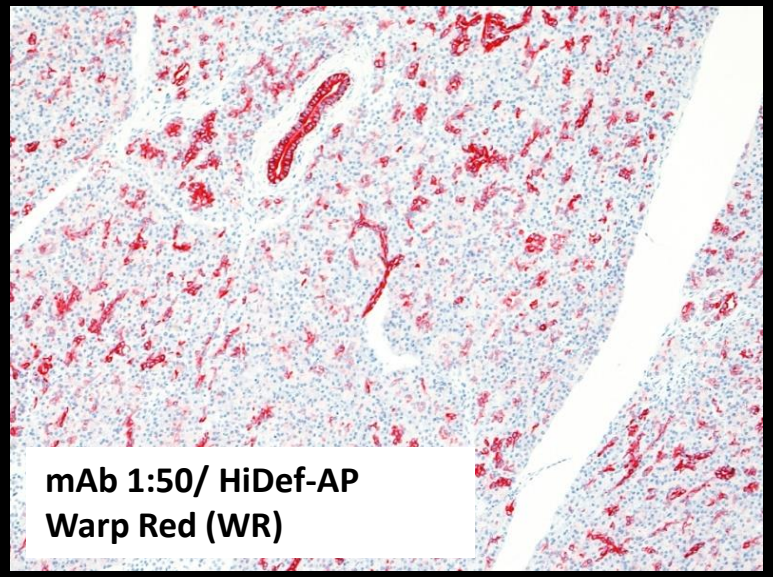
# End-result including controls: Optimizing the protocol

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

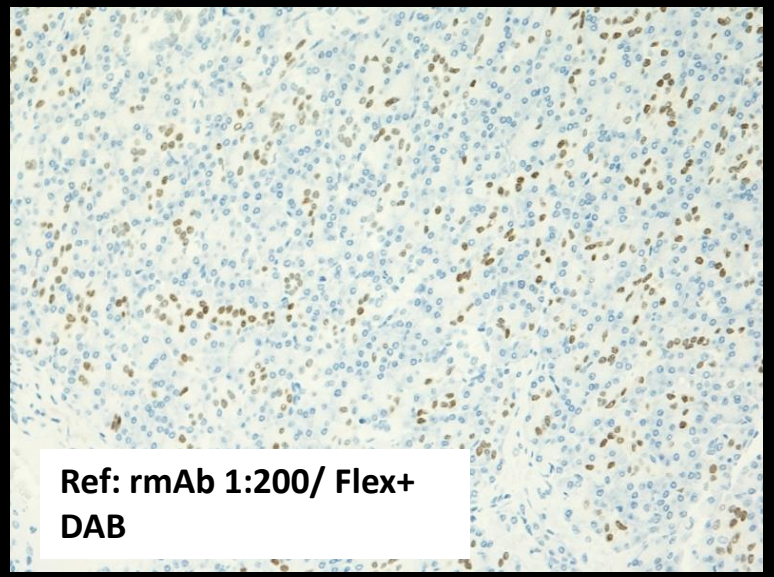
## Pancreas

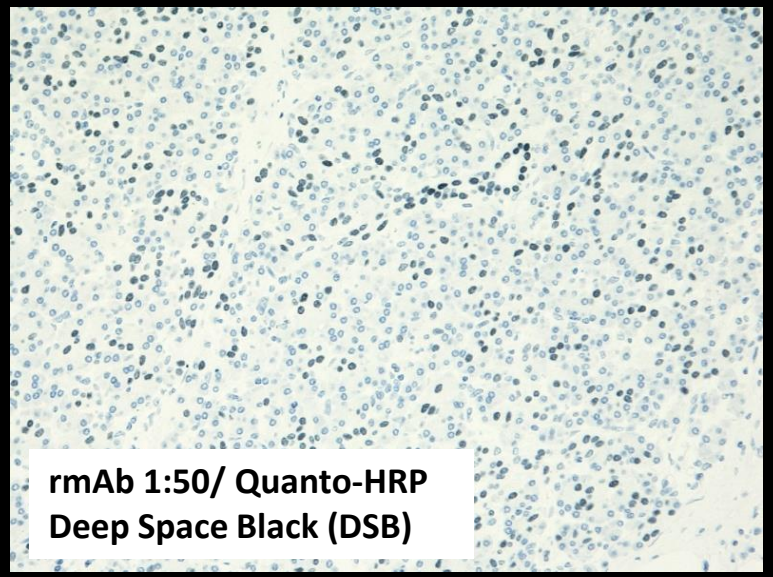
CK7, OV-TL12/30 (second seq.)




CDX2, EP25 (first seq.)

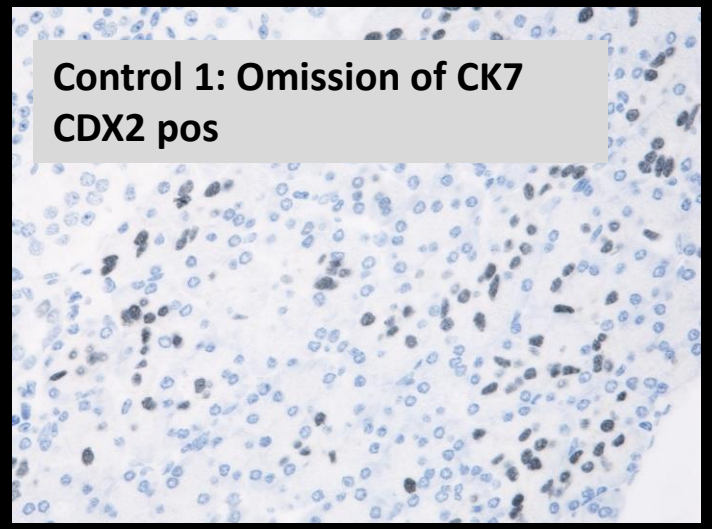


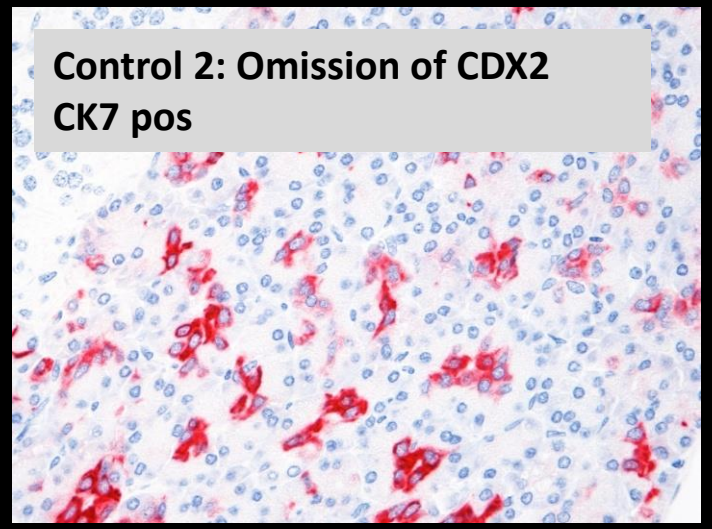



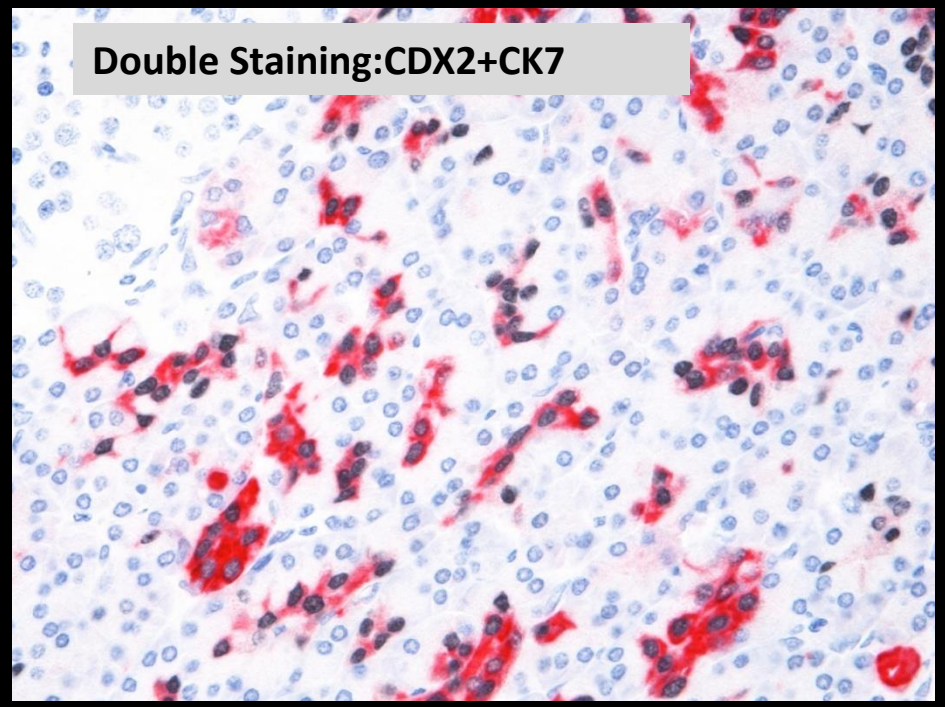
Typical end-result including controls: Optimizing the protocol

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



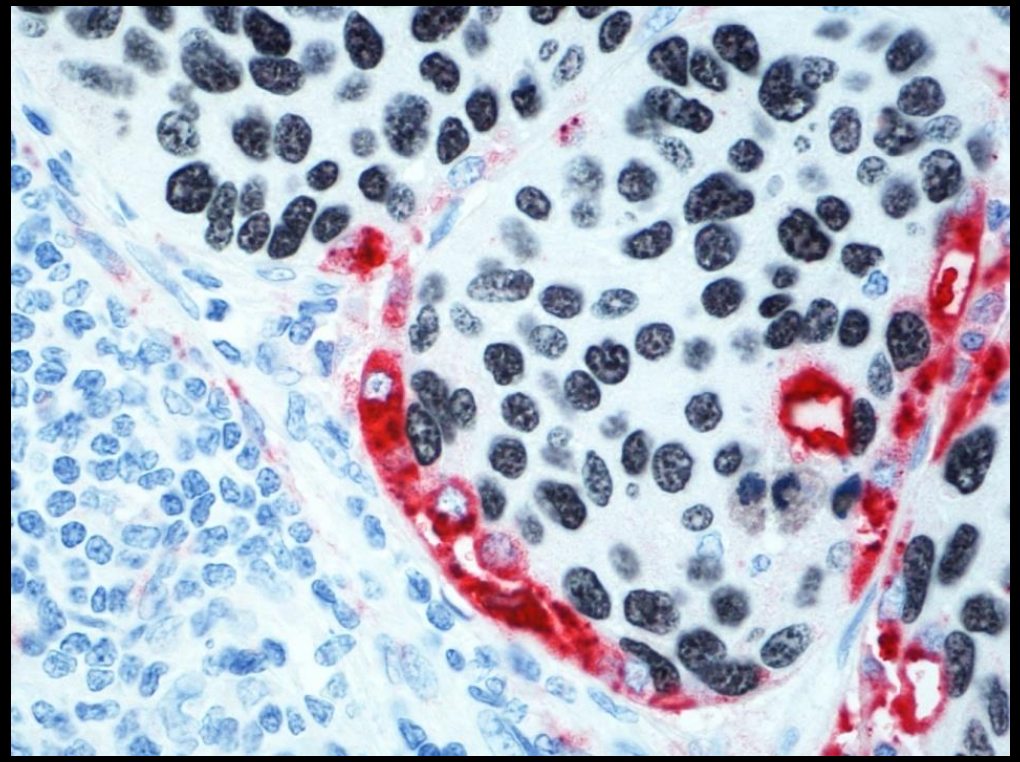

Pancreas

Note : Efficient sheltering capacity of Deep Space Black

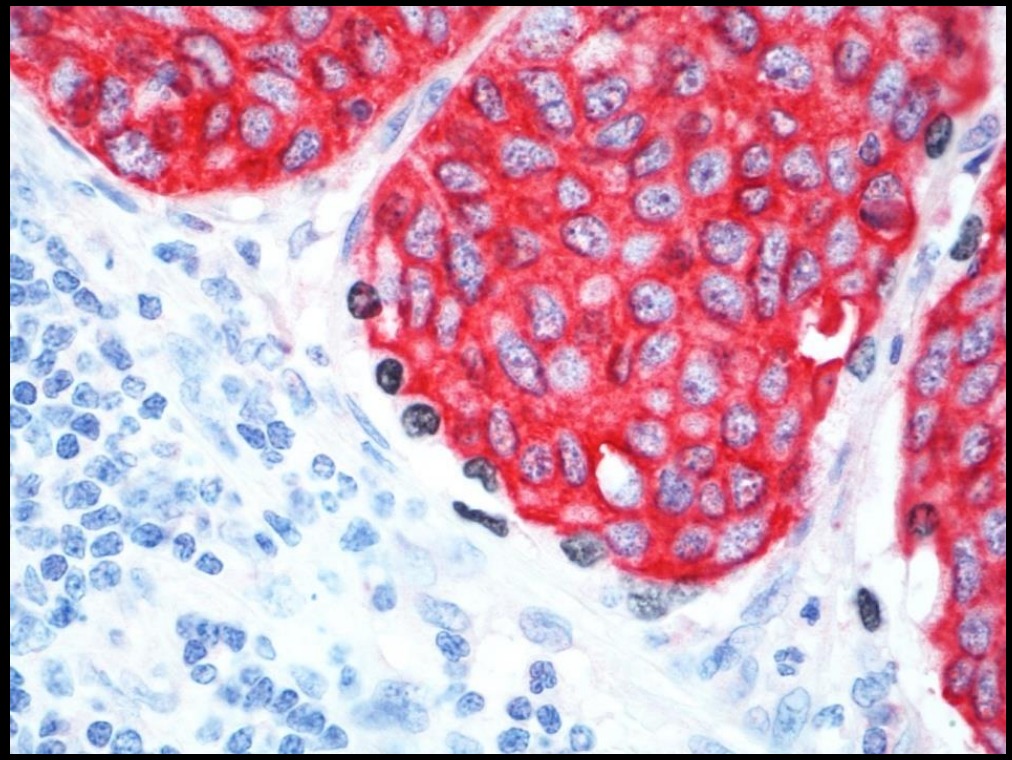
No mixed colors or double colors within controls and in the double staining for CDX2+CK7 (Intercalating ducts of the pancreas are positive for both markers)

# 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

## Sequential procedure (Immuno-enzymatic/2xHRP):

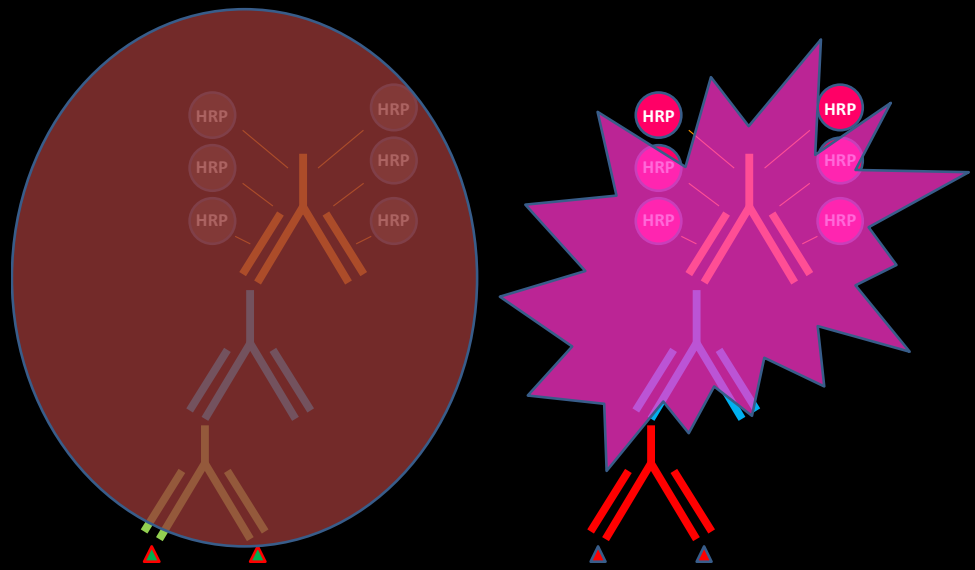
Pre-treatment (Antigen Retrieval)

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.

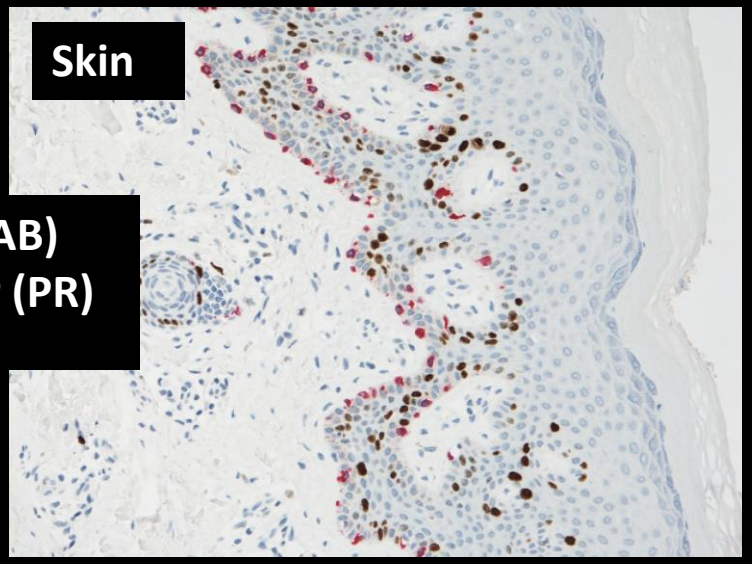
Blocking procedure ($H_2SO_4$) after first set of immuno-reagents (elution)
Additional peroxidase block ($H_2O_2$)

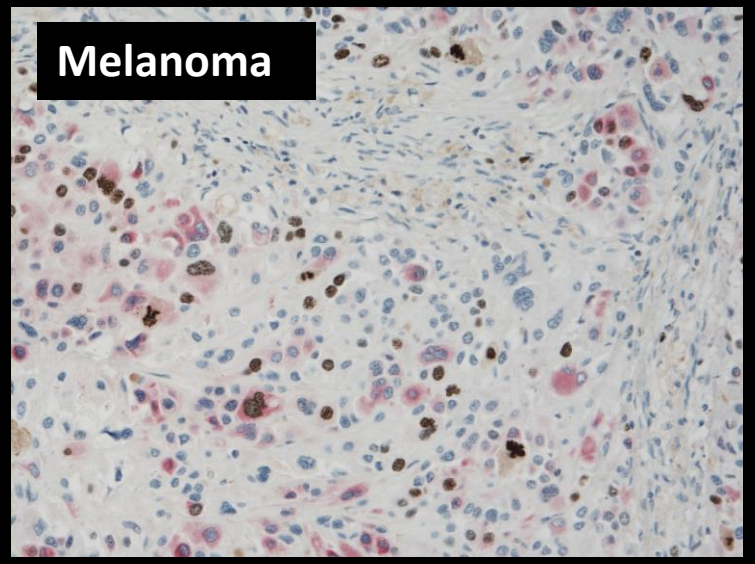
1.Set of Immuno-reagents
Flex+
DAB

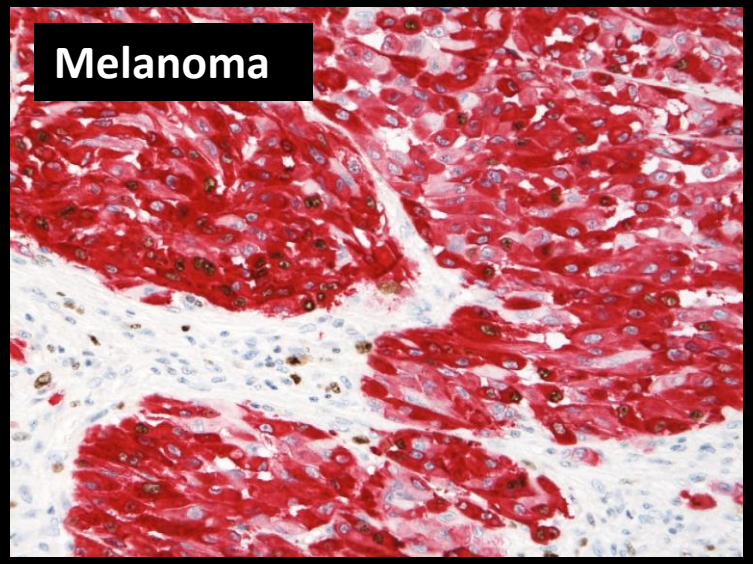
2. Set of Immuno-reagents
Flex+
Magenta

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

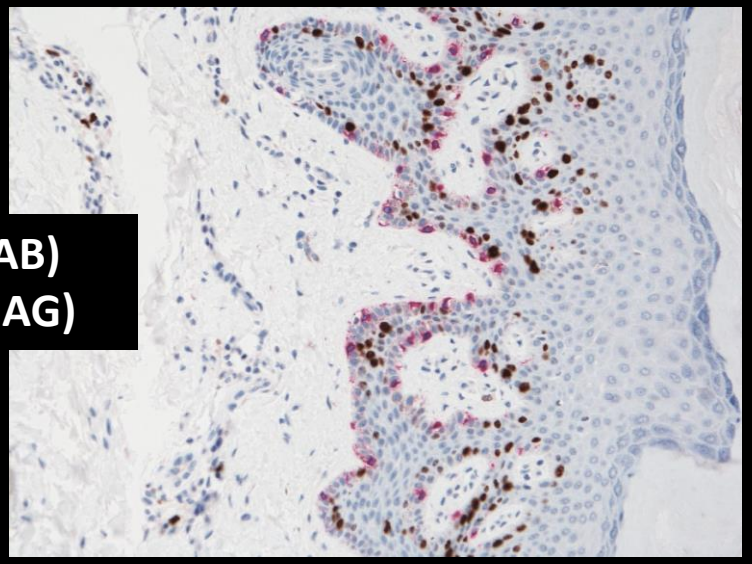
Flex+ (DAB)
Histo-AP (PR)
(incl. Linker)

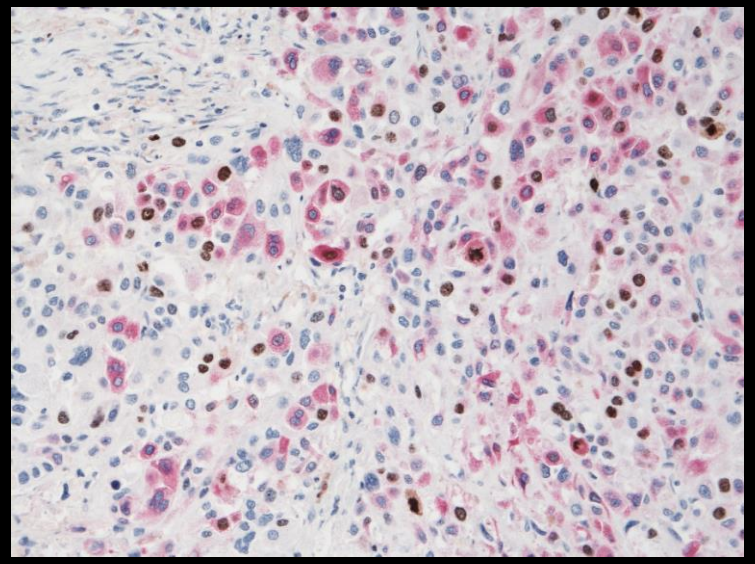


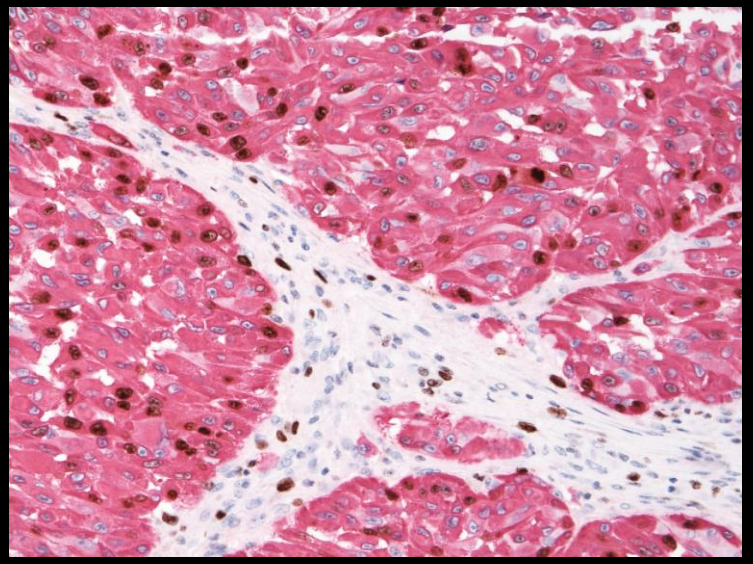




Flex+ (DAB)
Flex+ (MAG)

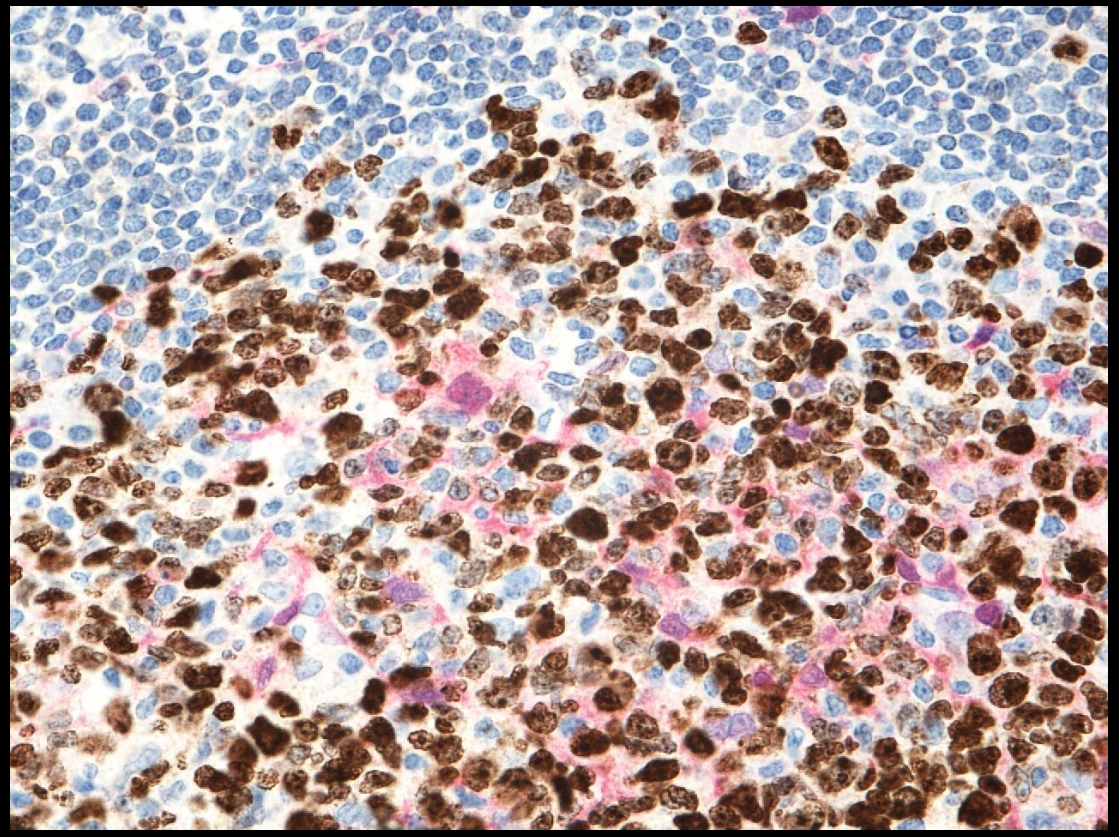




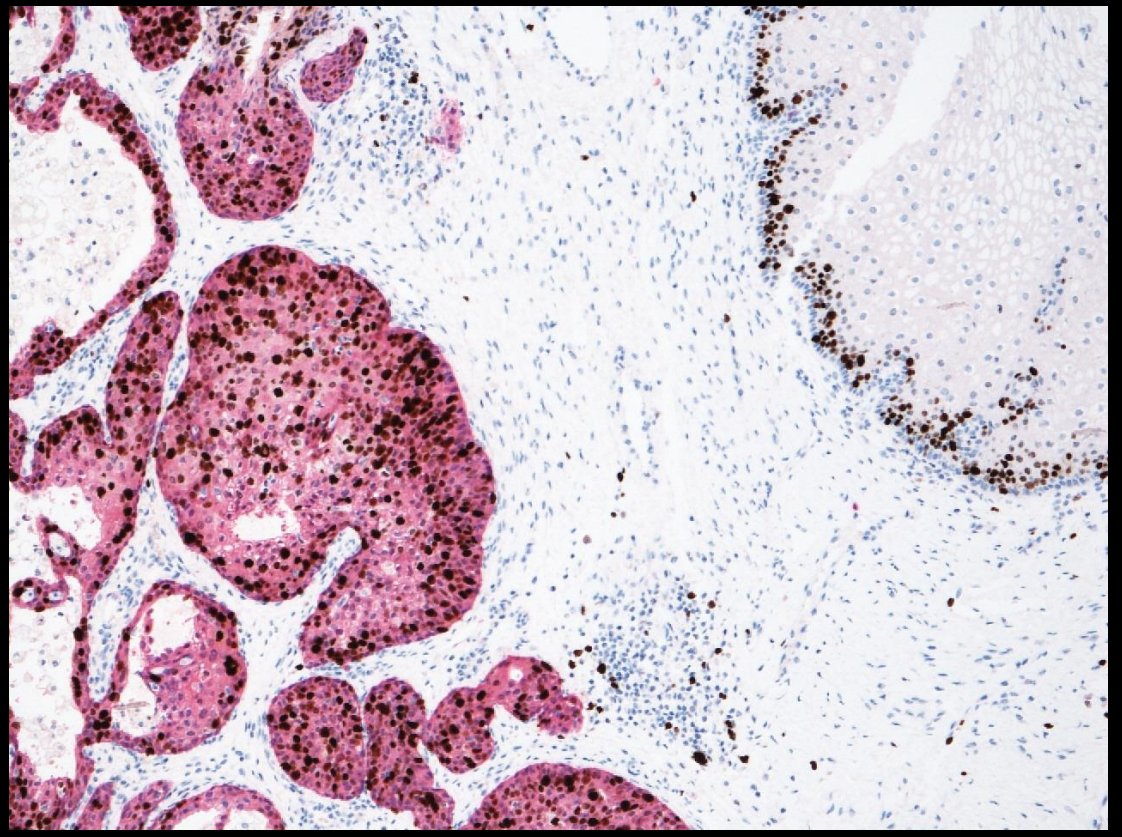


# Ki67 (MIB-1, RTU) and P16 (MXB007, 1:1000)
# HIER High pH (Omnis)

**Tonsil**



**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**

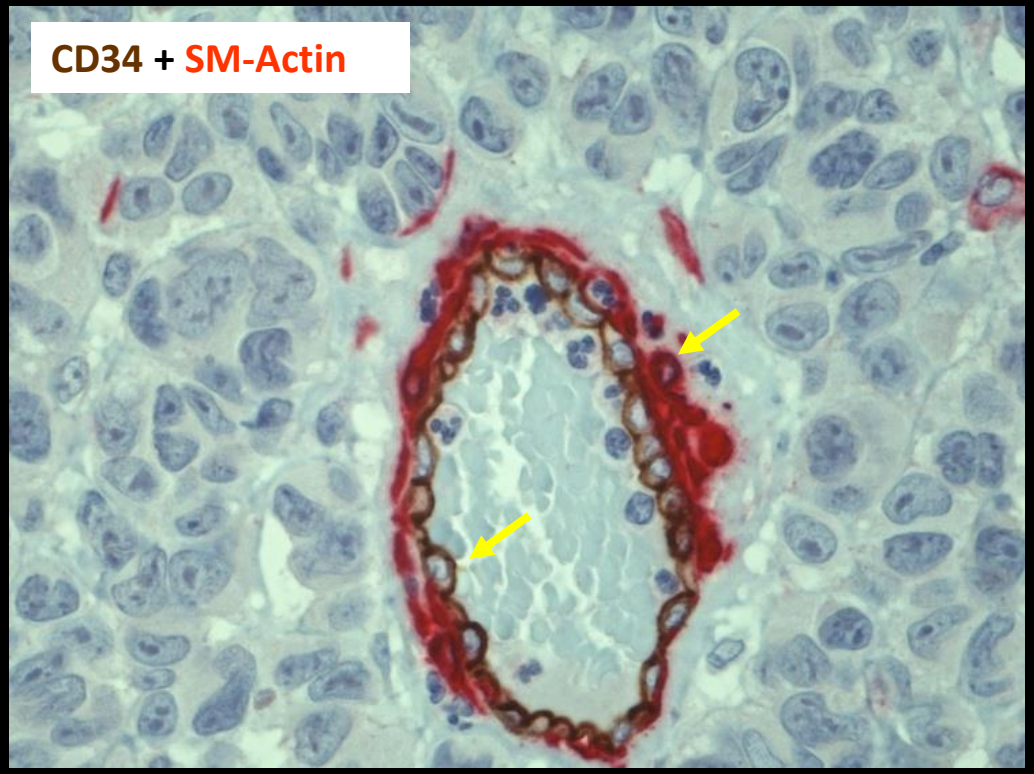
**A reversed applications of the primary antibodies may un-ravel that :**

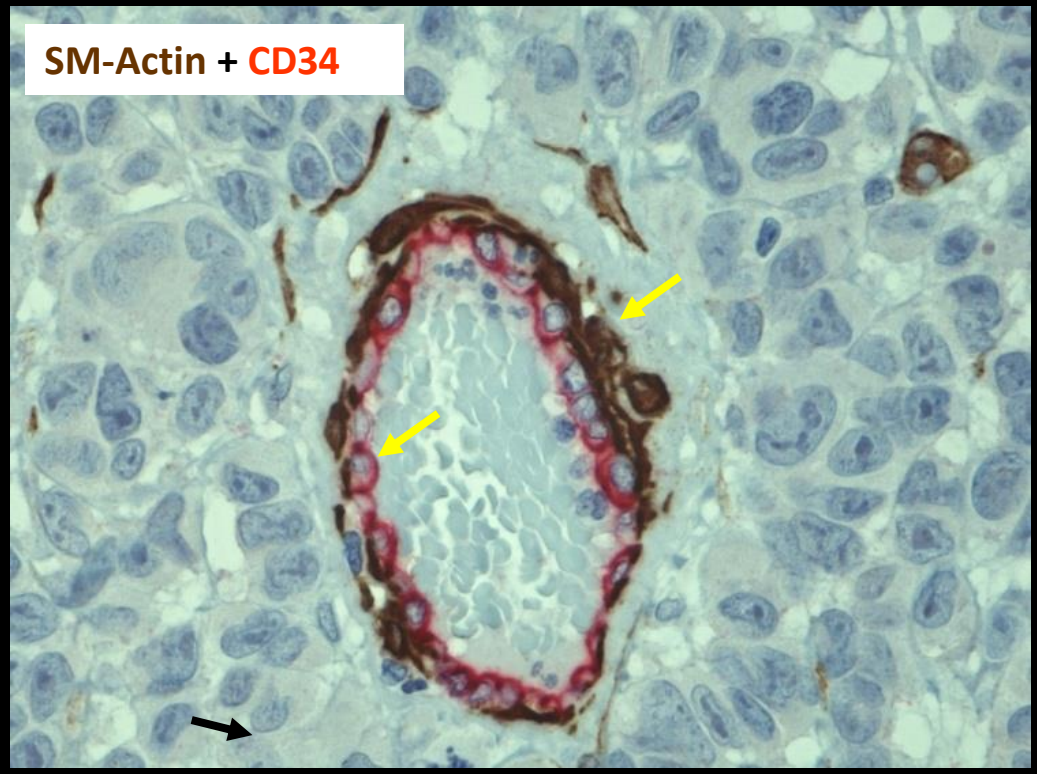
**DAB deposit shields for the second antigen of interest - first and second antigen are in proximity with each other (false negative result)**

**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)



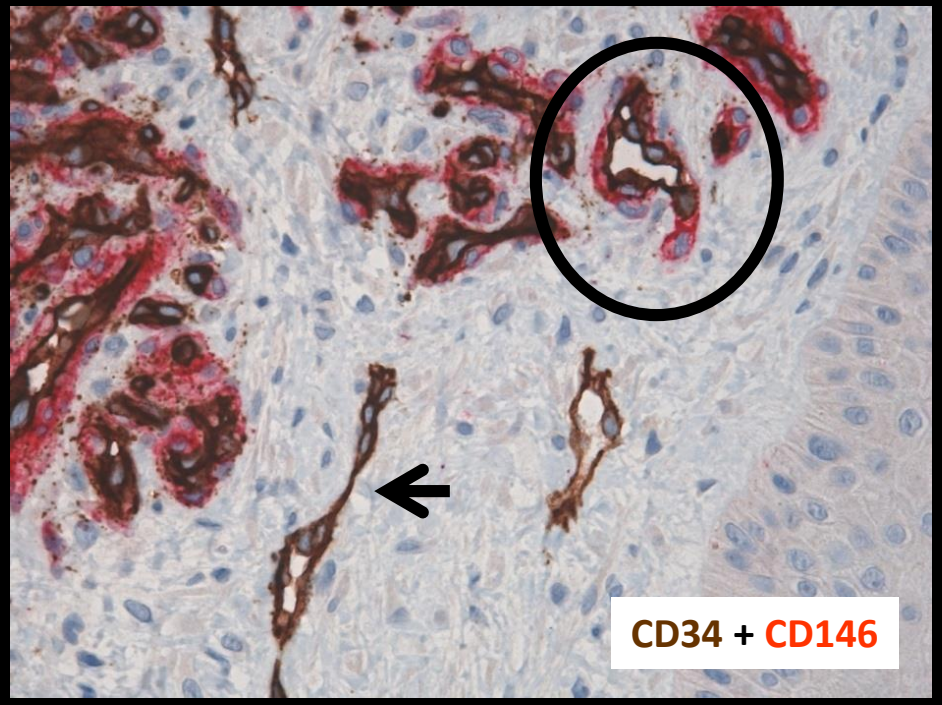




Note: No co-localization of either endothelium or smooth muscles (arrows)

Melanoma

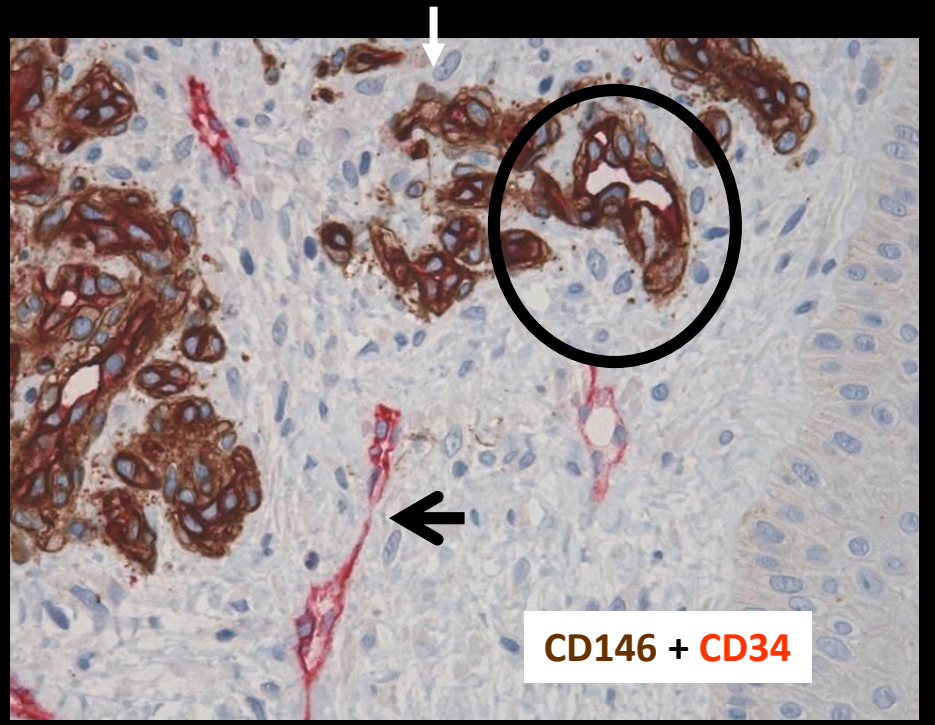
# Double Immuno-staining – reversal stainings

## 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).

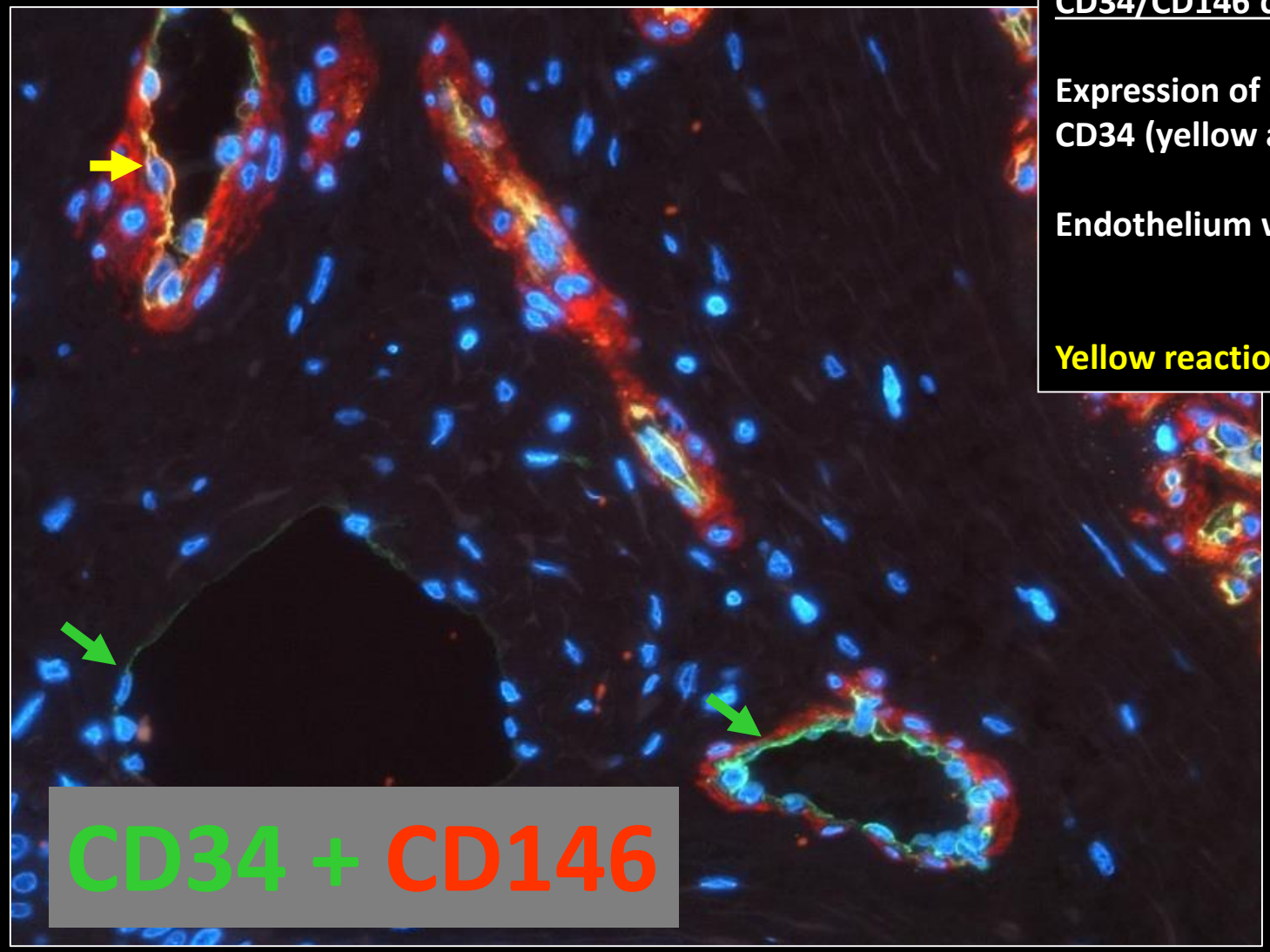



Endothelia cells are “double brown positive” /co-localization

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**

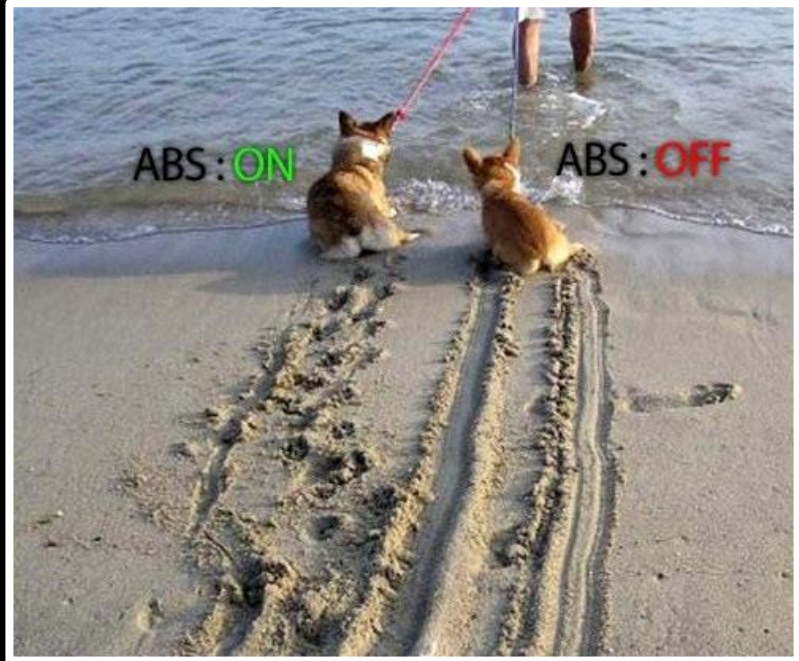
## Hemangioma

# 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.**

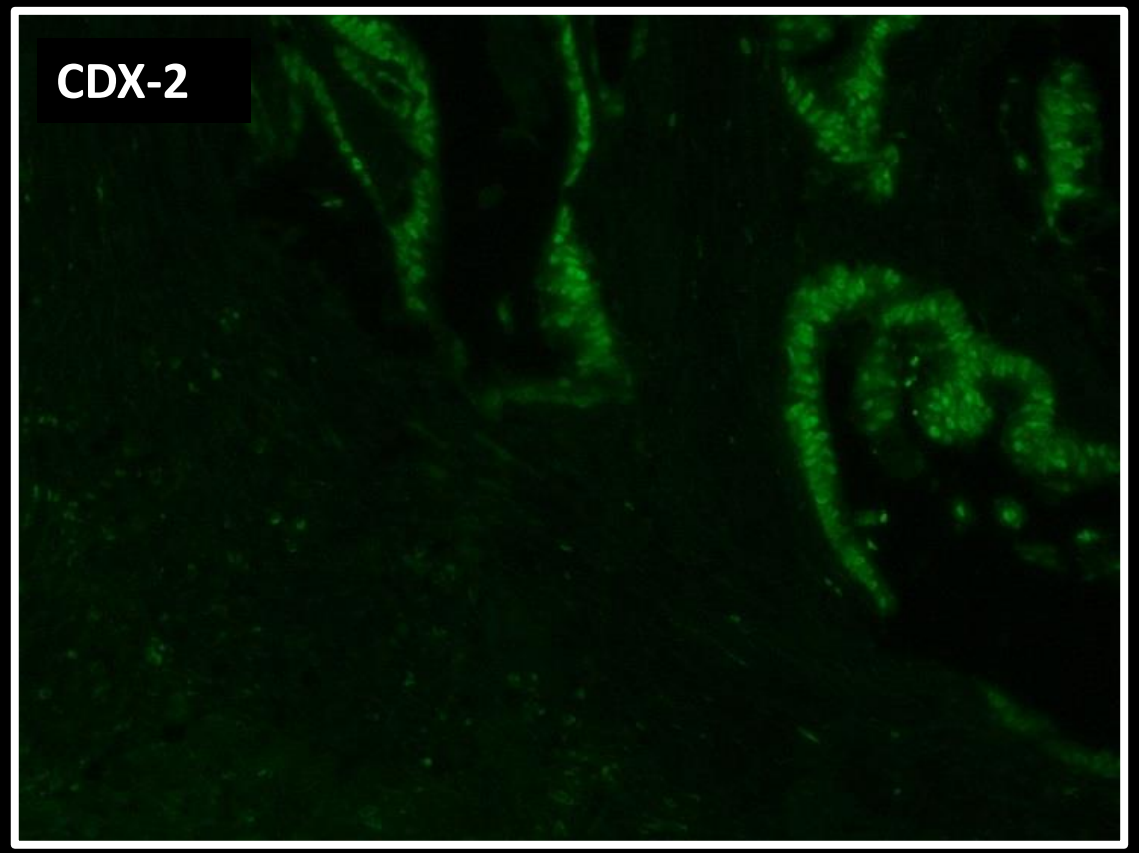
**Tumor cells with infiltrative growth pattern (buddings) show expression of CK 8/18 due to downregulation of CDX2 – arrowheads.**

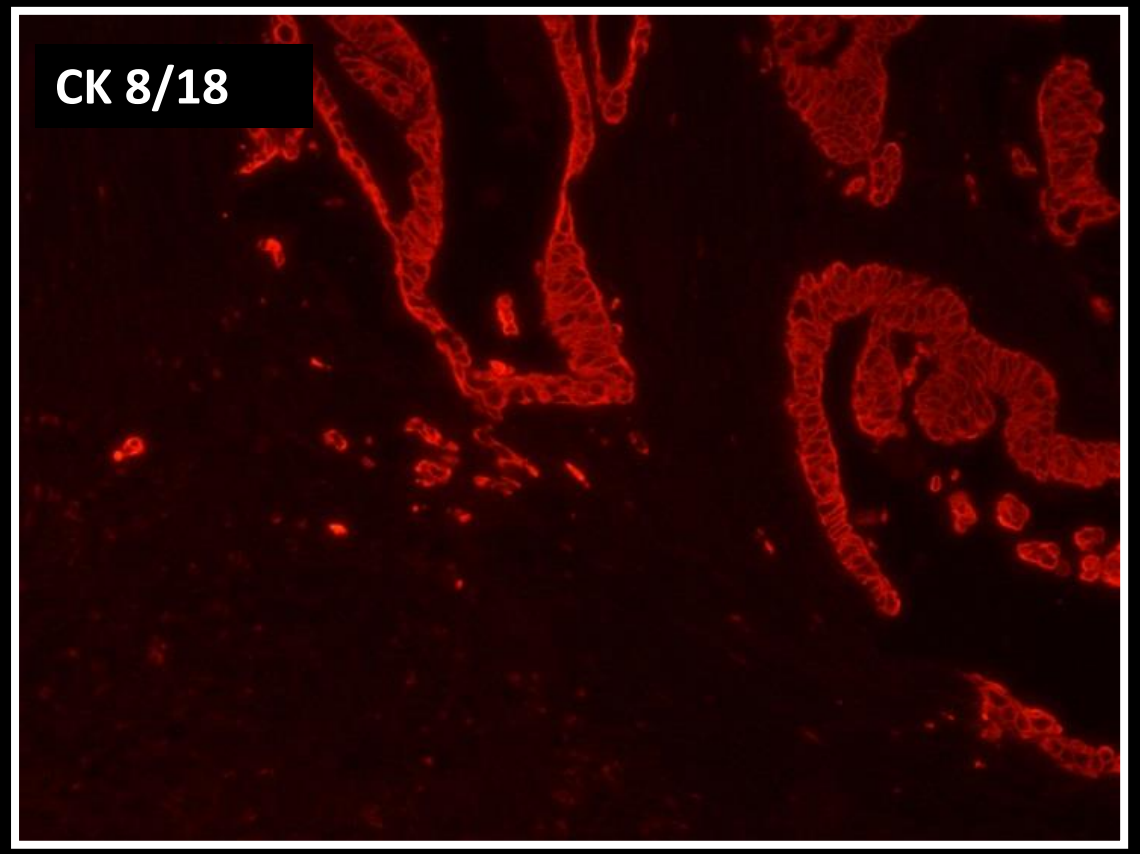



Colon Adenocarcinoma

# 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):

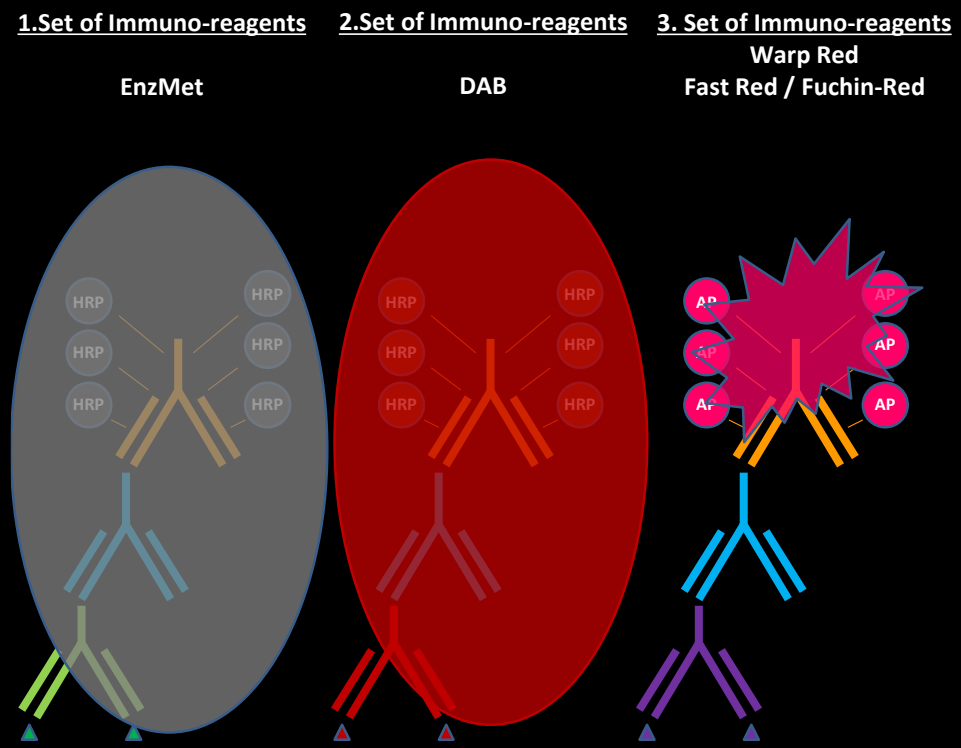
**Pre-treatment (Antigen Retrieval)**

**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).**

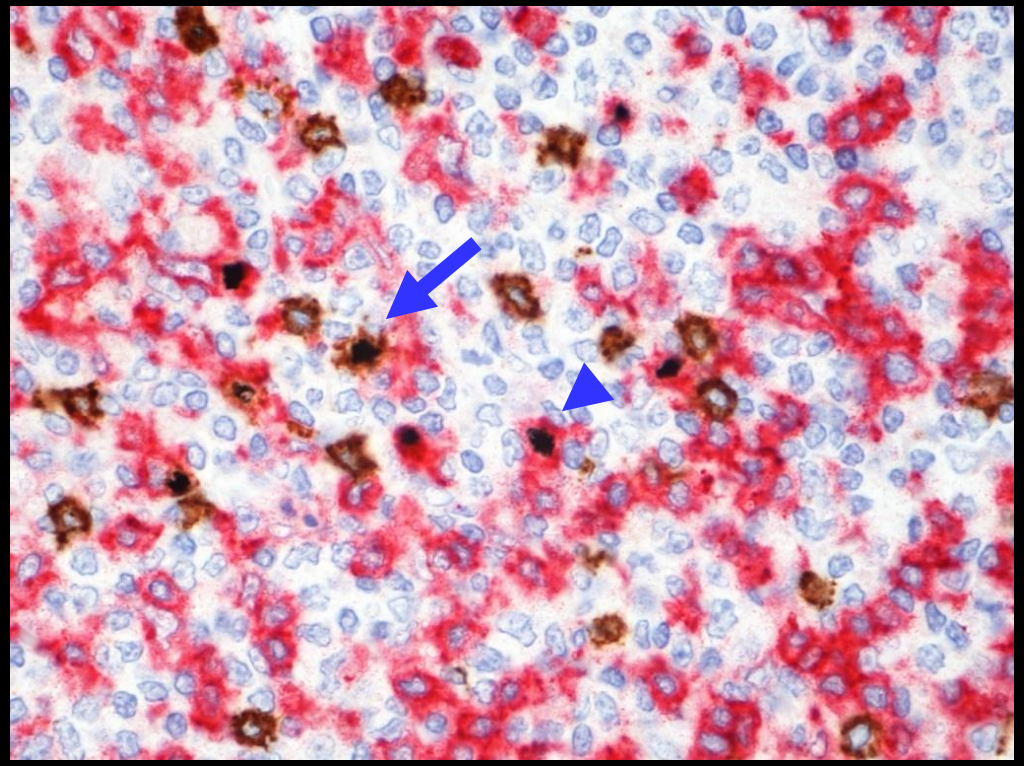
**Counterstain, dehydration and mounting.**




**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)



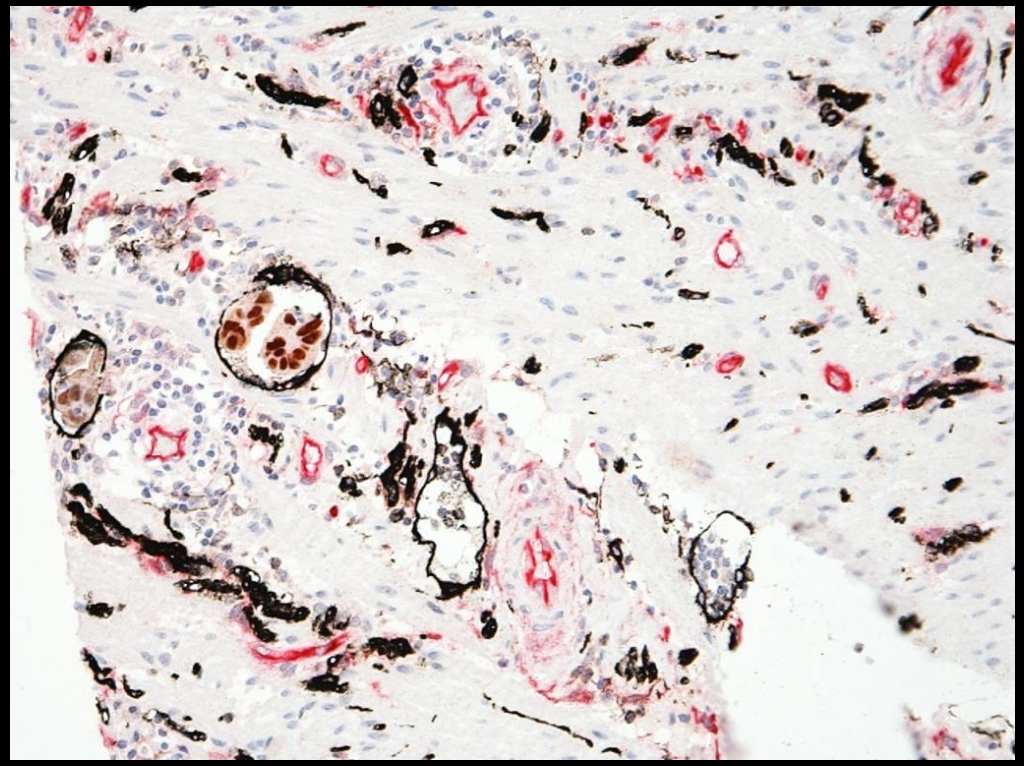
## Tonsil

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 (Melanoma)

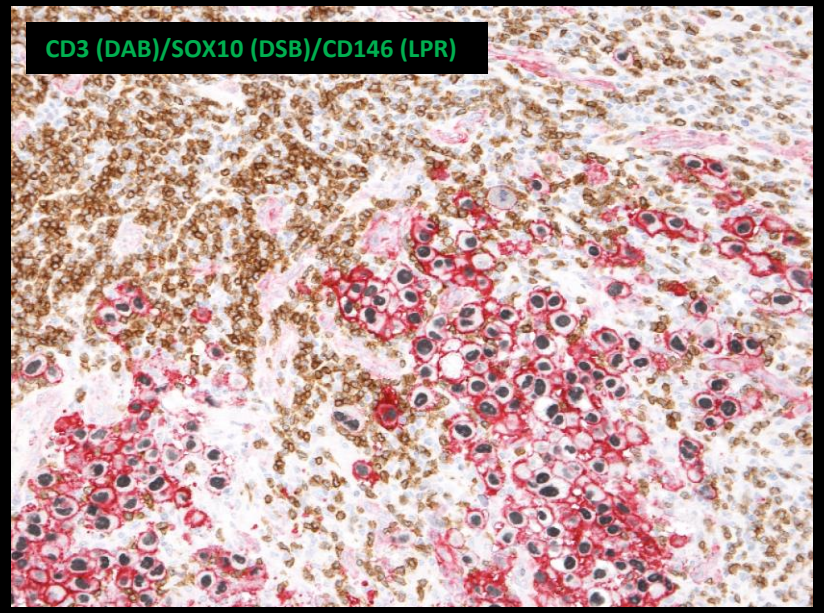
## Combinations and color contrast

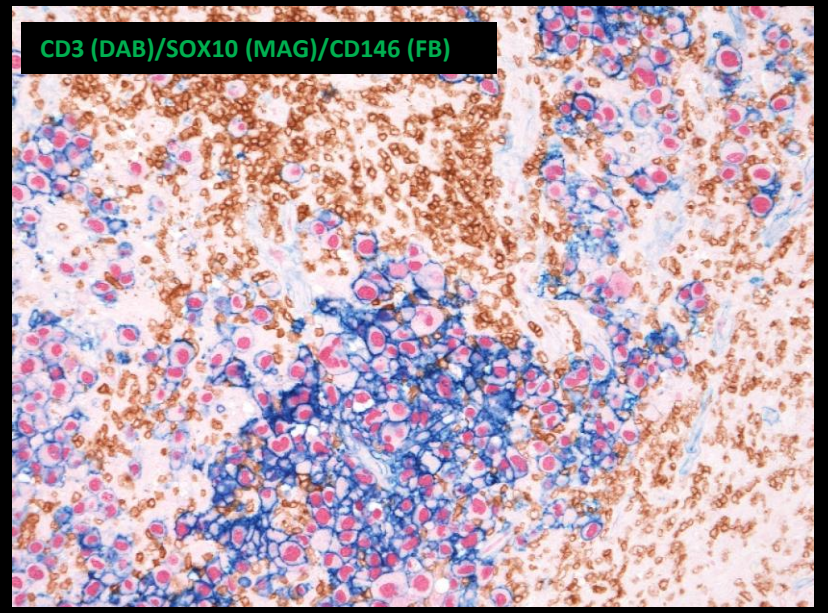
**SOX10/CD146/CD3**

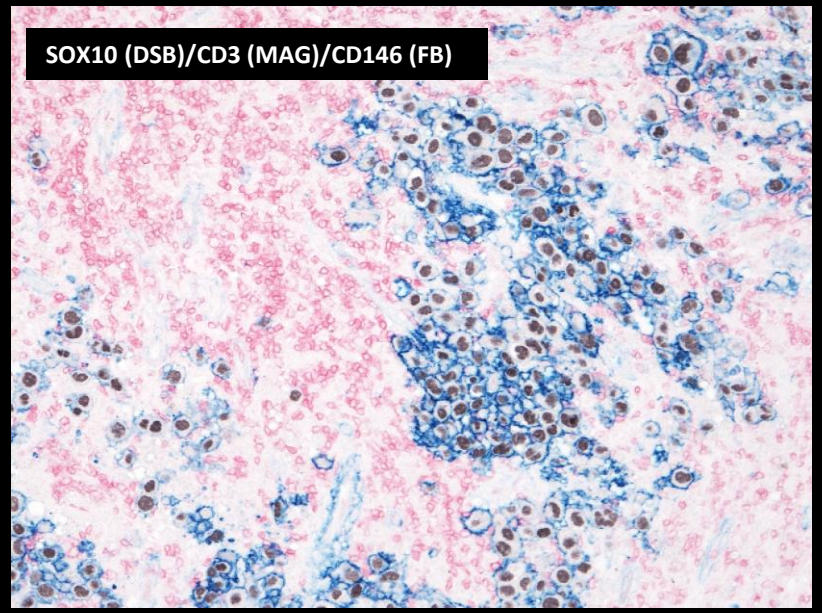
**HIER High pH (90°C/60min)**

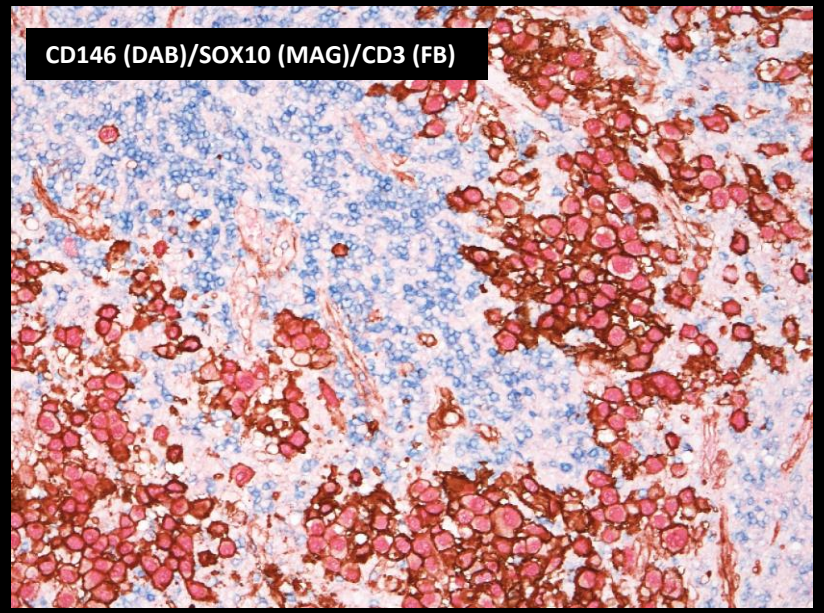
**Flex+/MACH2-DS2**

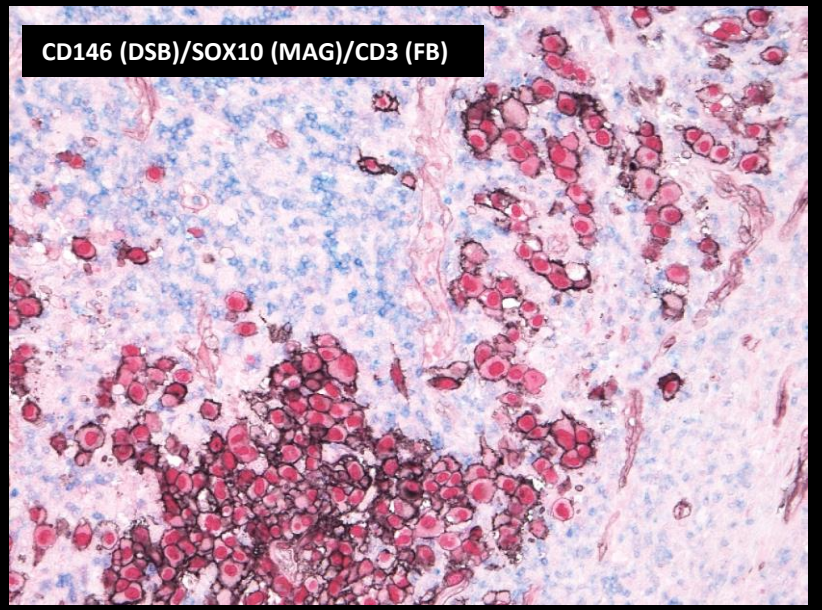
**Always DAB-based chromogen in the first sequence**



# Multiplex staining using sequential technique (Immuno-enzymatic)

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

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

## Inactivation of the prior set of immuno-reagents :

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

Applied between the individual sequences in the multiplex technique

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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:

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

This also includes an intermediate HIER step using Citrate pH6

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

CD20

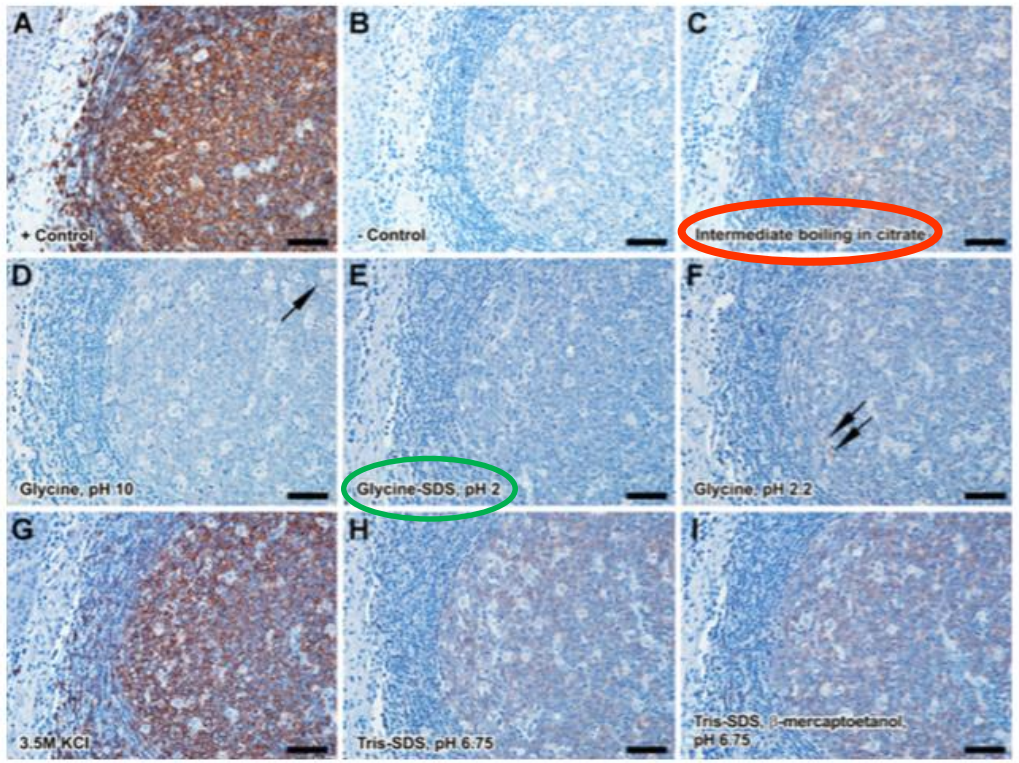
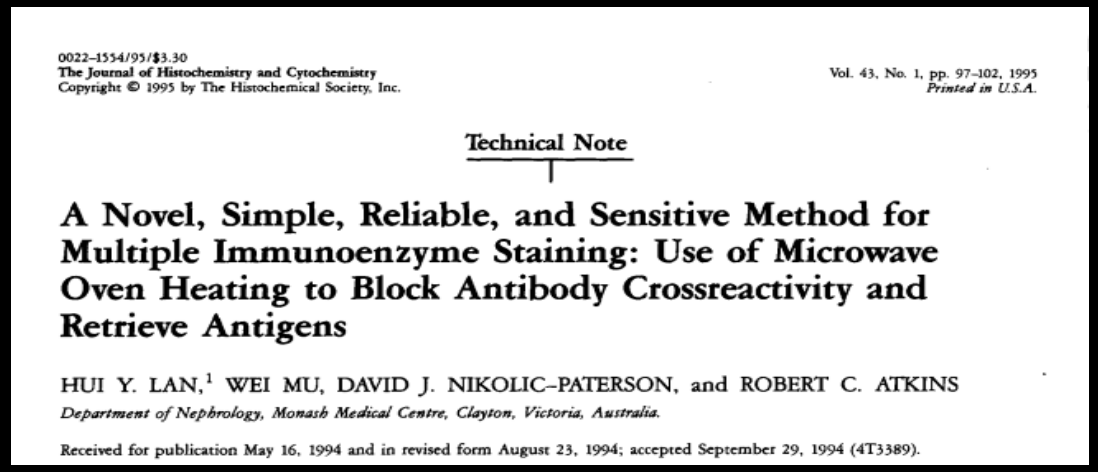



**Figure 1** Choosing the optimum antibody-elution protocol. The procedure involved incubation with a primary antibody (follicular B-cell marker CD20cy here), applying the elution procedure, and detecting the remaining primary antibody. Positive and negative controls for this reaction were obtained by 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
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Vol. 43, No. 1, pp. 97–102, 1995
Printed in U.S.A.

Technical Note

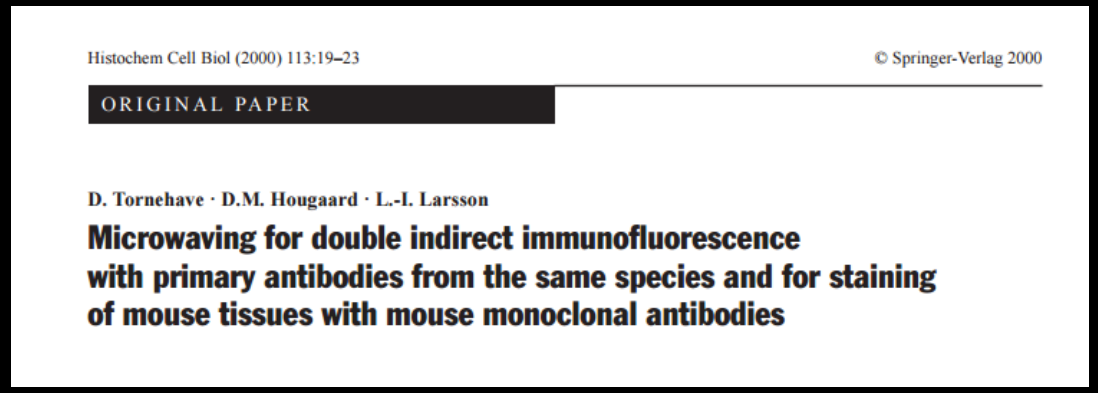
A Novel, Simple, Reliable, and Sensitive Method for Multiple Immunoenzyme Staining: Use of Microwave Oven Heating to Block Antibody Crossreactivity and Retrieve Antigens

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 - DAB, VBlue, VRed and LPR (Dako)**

**For certain antigen/antibody reaction not always efficient**

# 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)

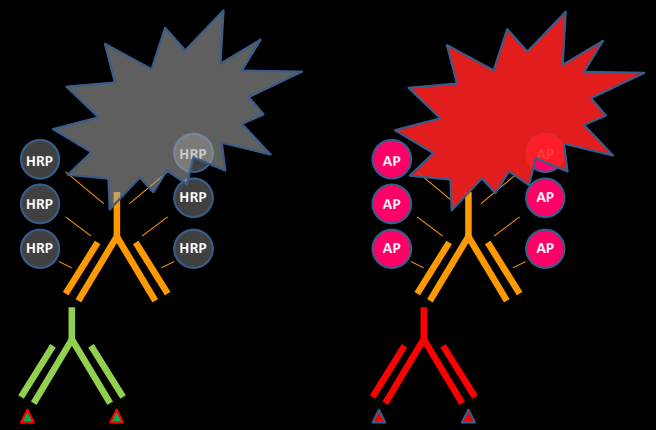
Incubation with mix of primary Abs (Rab+Mab)

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.

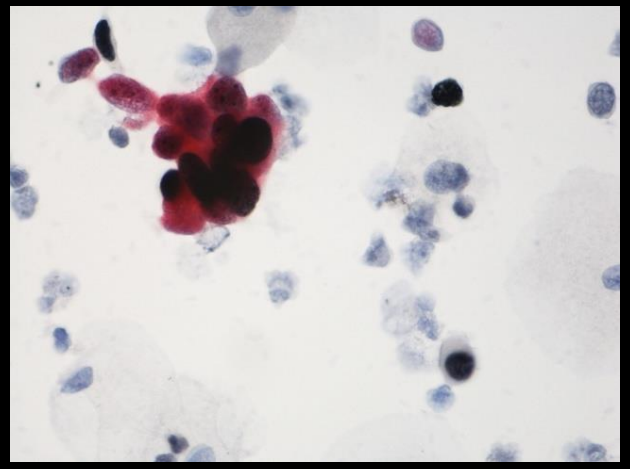
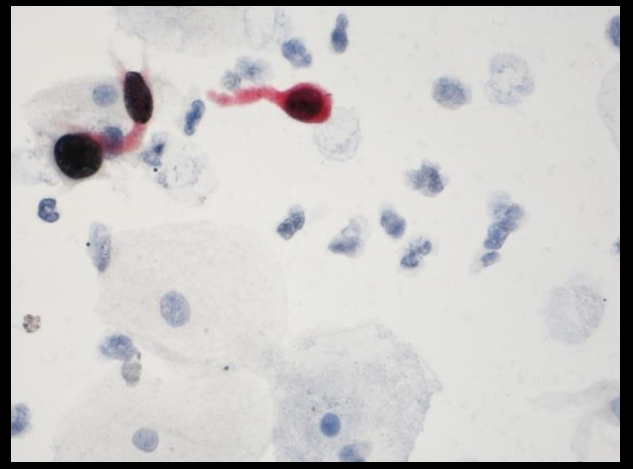
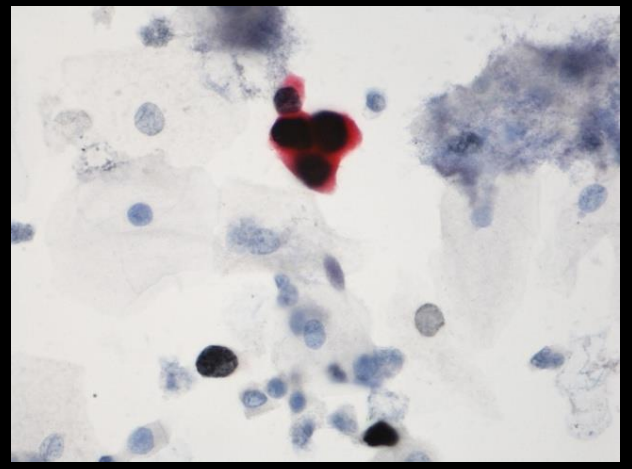
Each antibody: antigen reaction will yield a specific color of staining that can be easily identified.

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

**Dysplasia / Cervix (Cytology)**

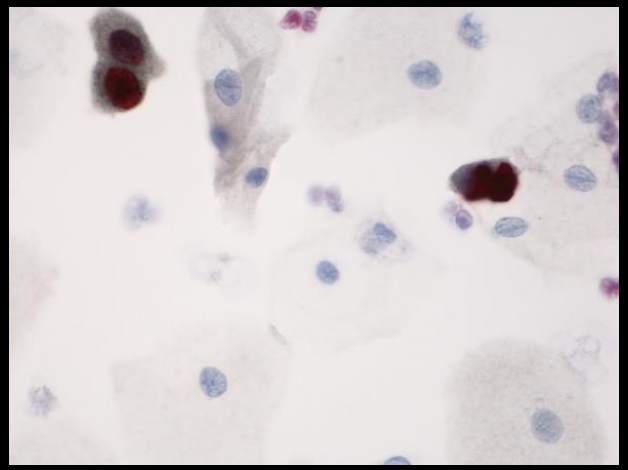
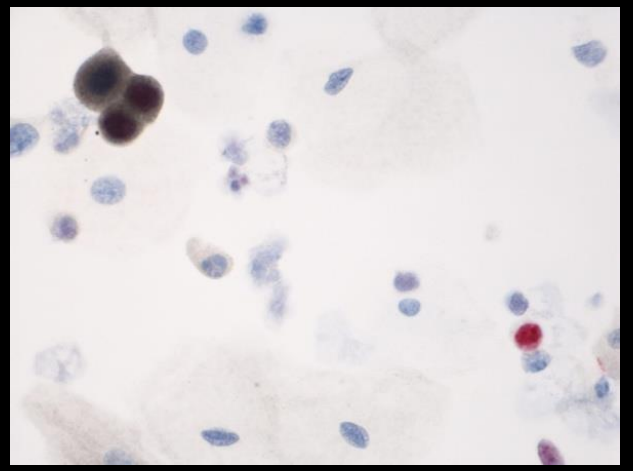
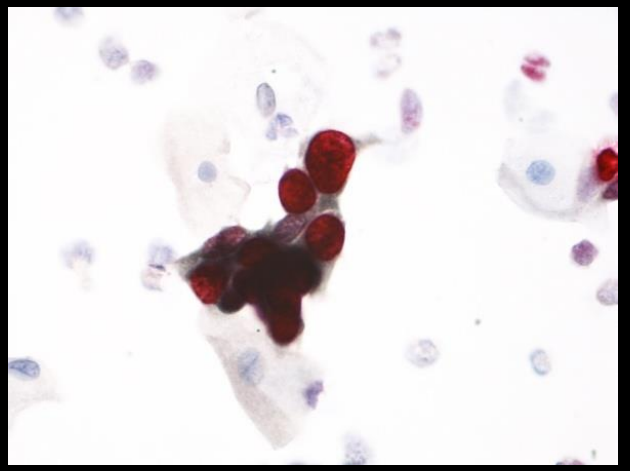
**Double Stain 1 (Biocare)**

**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**

**Double/Multiplex immunofluorescence technique (simultaneous/sequential technique)**

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

**A reversed applications of the primary antibodies (sequential technique)**

**SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method )**

**Routine methods ?**

# Co-localized signal using double immuno-enzymatic techniques ?

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

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

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

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

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

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)

NGFR (MRQ21) + CD146 (EPR3208)

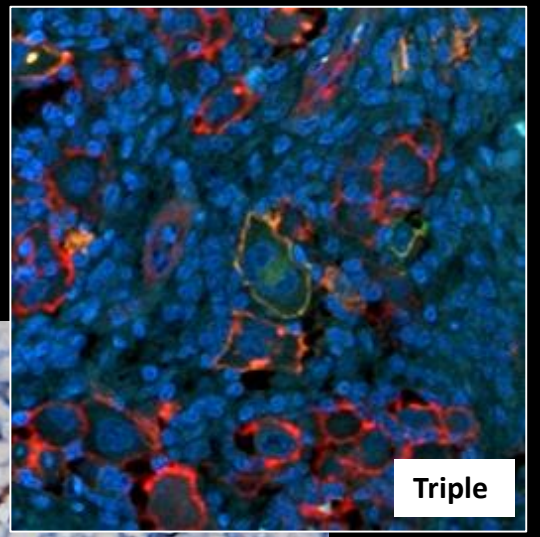
Double immuno-fluorescence technique

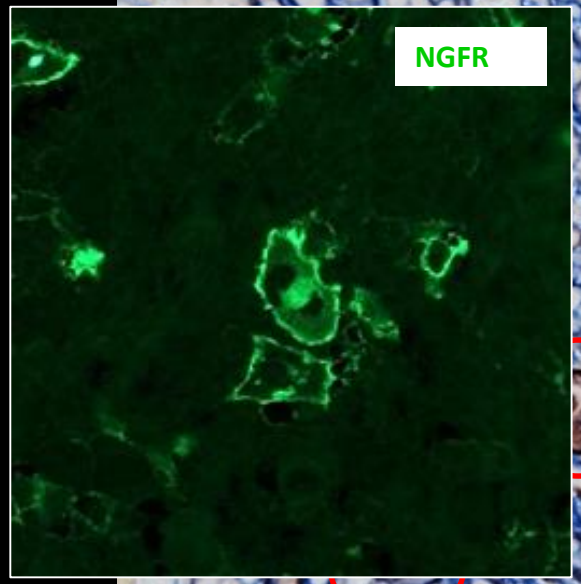
Melanoma

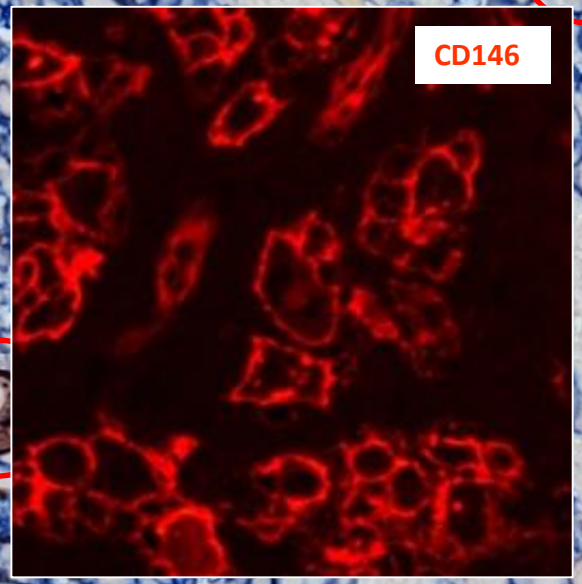
Melanoma

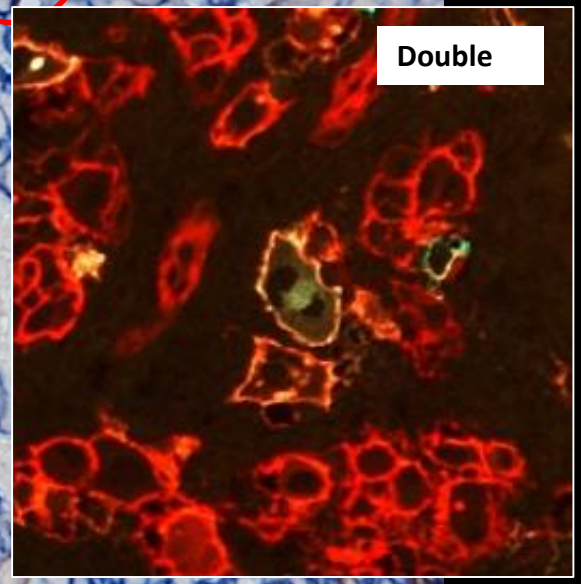
Thermo/ LabVision – Multivision KIT

Co-localization ?




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

# New Chromogens for light microscopy (co-localized signals)

**Diagnostic Biosystems, Enzo, Vector laboratories, Ventana …….**

## Double enzymatic staining using simultaneous technique

Pre-treatment (Antigen Retrieval)

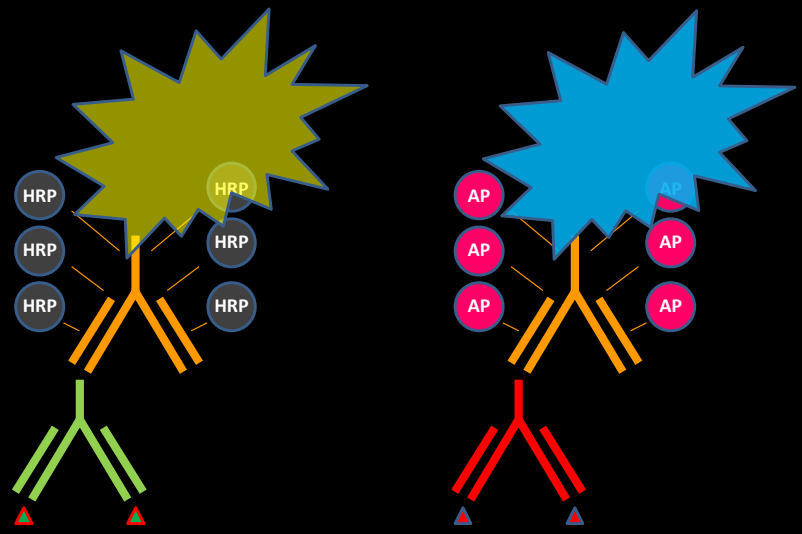
Incubation with mix of primary Abs (Rab+Mab)

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

Incubation with HRP substrate (e.g., Hi-Def Yellow/Enzo)

Incubation with AP substrate (e.g., Ferangi Blue/Biocare)

Counter stain, mounting and microscopy




## Commercial detection kits (Naestved Lab):

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

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

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

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

Omnis

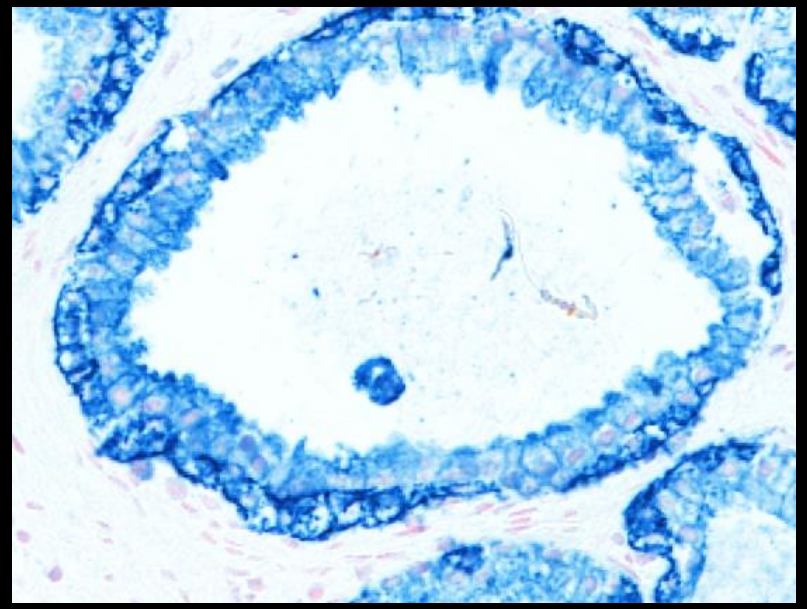
# Simultaneous procedure: MACH2 Double Staining 1 (Omnis)

**CK8, EP17 (1:500) + CK-PAN, AE1/AE3 (RTU)**

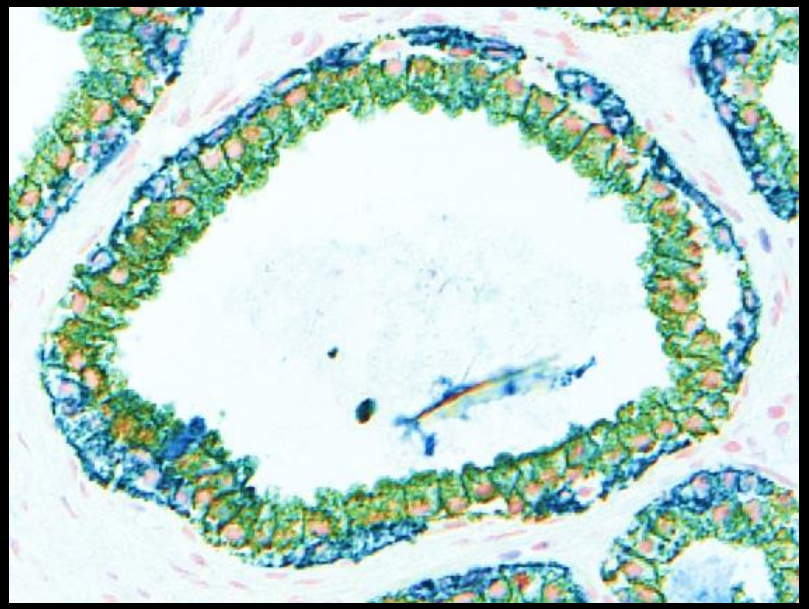
**Hidef Yellow (Enzo) – Ferangi Blue (Biocare)**

**CK8 (without FB)**


**CK-Pan (without HY)**



**CK8+CK-Pan**



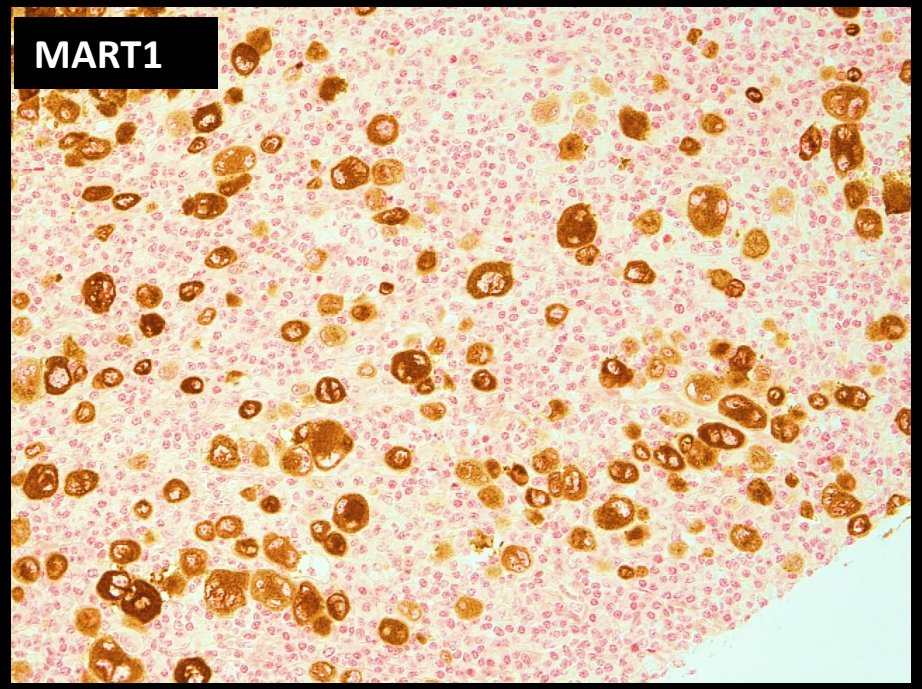
**Counter Stain:  Nuclear Fast Red**

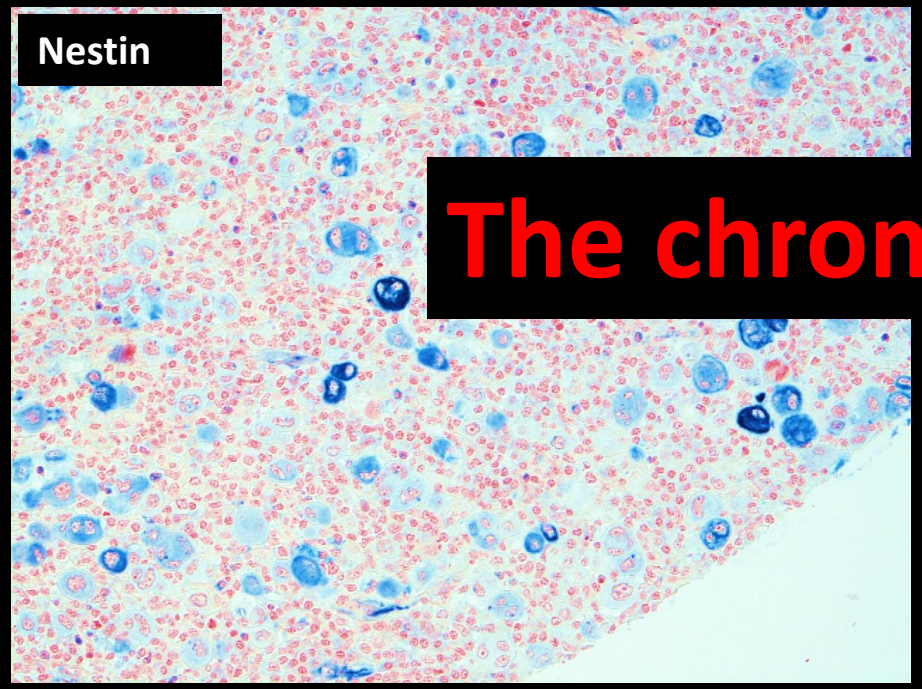
**Images enhanced: Modified saturation (.pptx)**

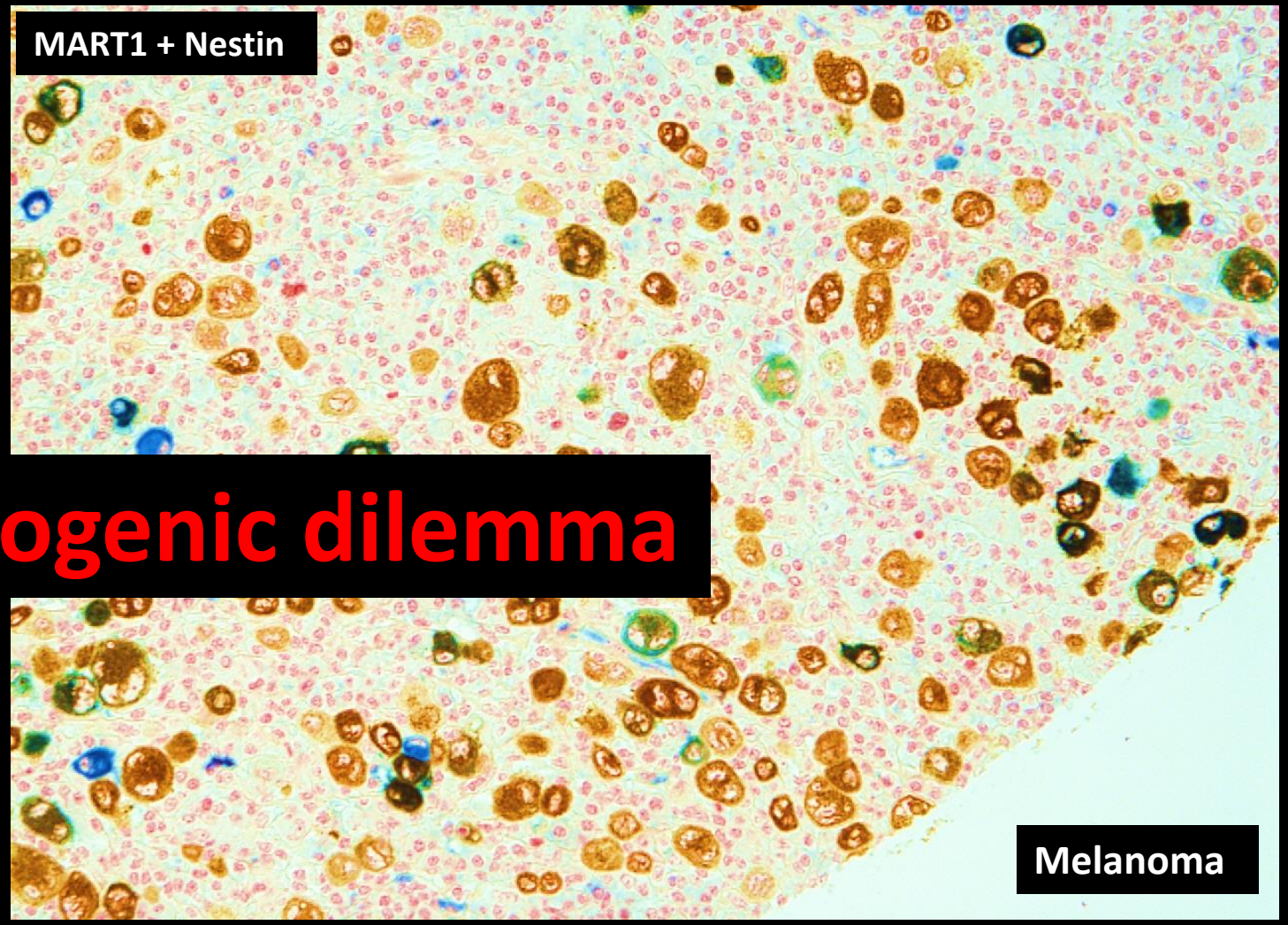
# Simultaneous procedure: MACH2 Double Staining 1

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

**Hidef Yellow (Enzo) – Ferangi Blue (Biocare)**




## The chromogenic dilemma

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

ARTICLE

# SIMPLE: A Sequential Immunoperoxidase Labeling and Erasing Method

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

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

**Table 1** Comparison of SIMPLE with existing multiprobe immunolabeling methods

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

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

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.

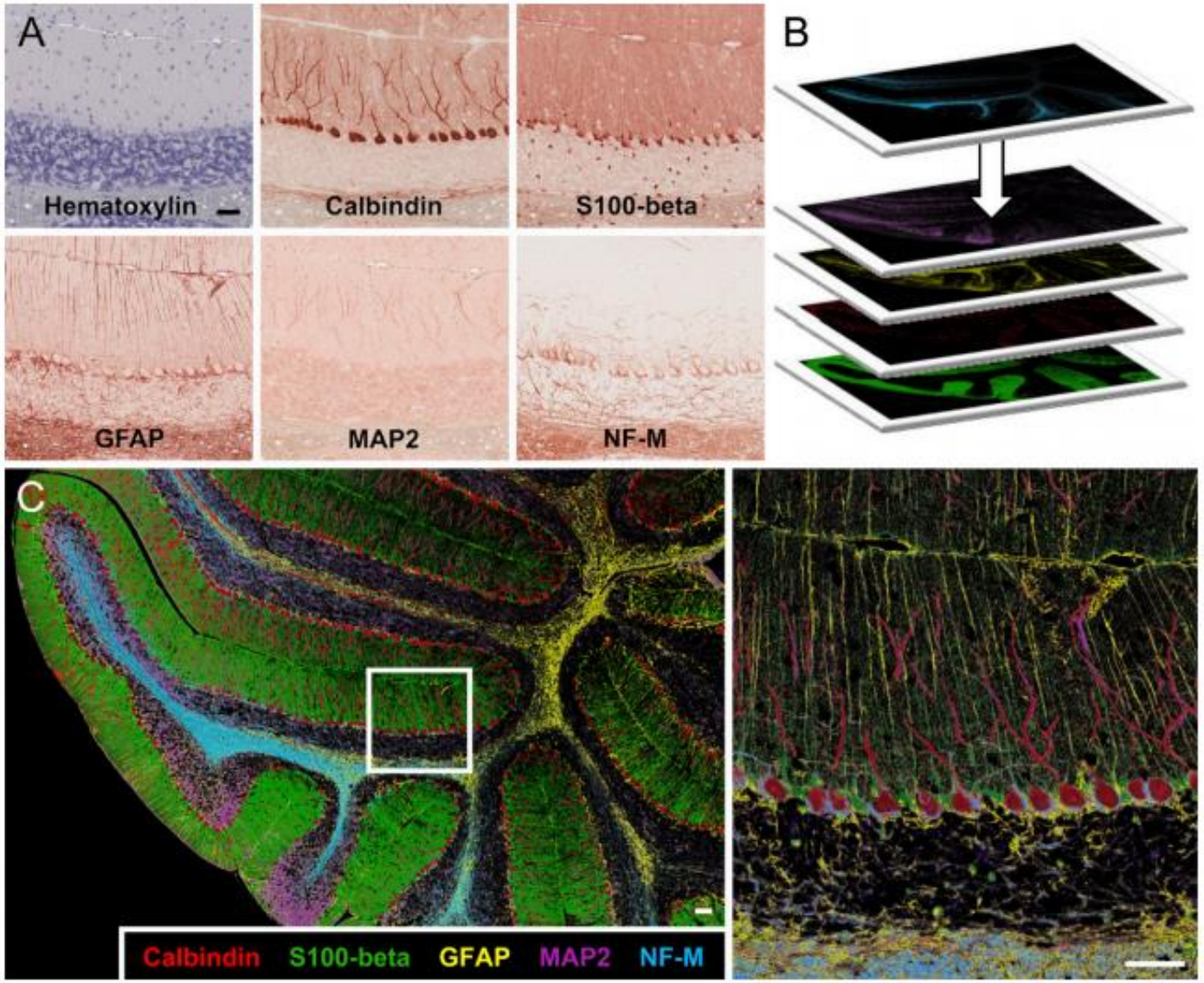



**Figure 3** Simultaneous visualization of five antigens in mouse cerebellum. **(A)** Adult mouse brain was counterstained with hemotoxylin, then sequentially probed with polyclonal antibodies to calbindin, S100-β, and GFAP, and monoclonal antibodies to MAP2 (AP18) and neurofilament (NF-M) 2H3. **(B)**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

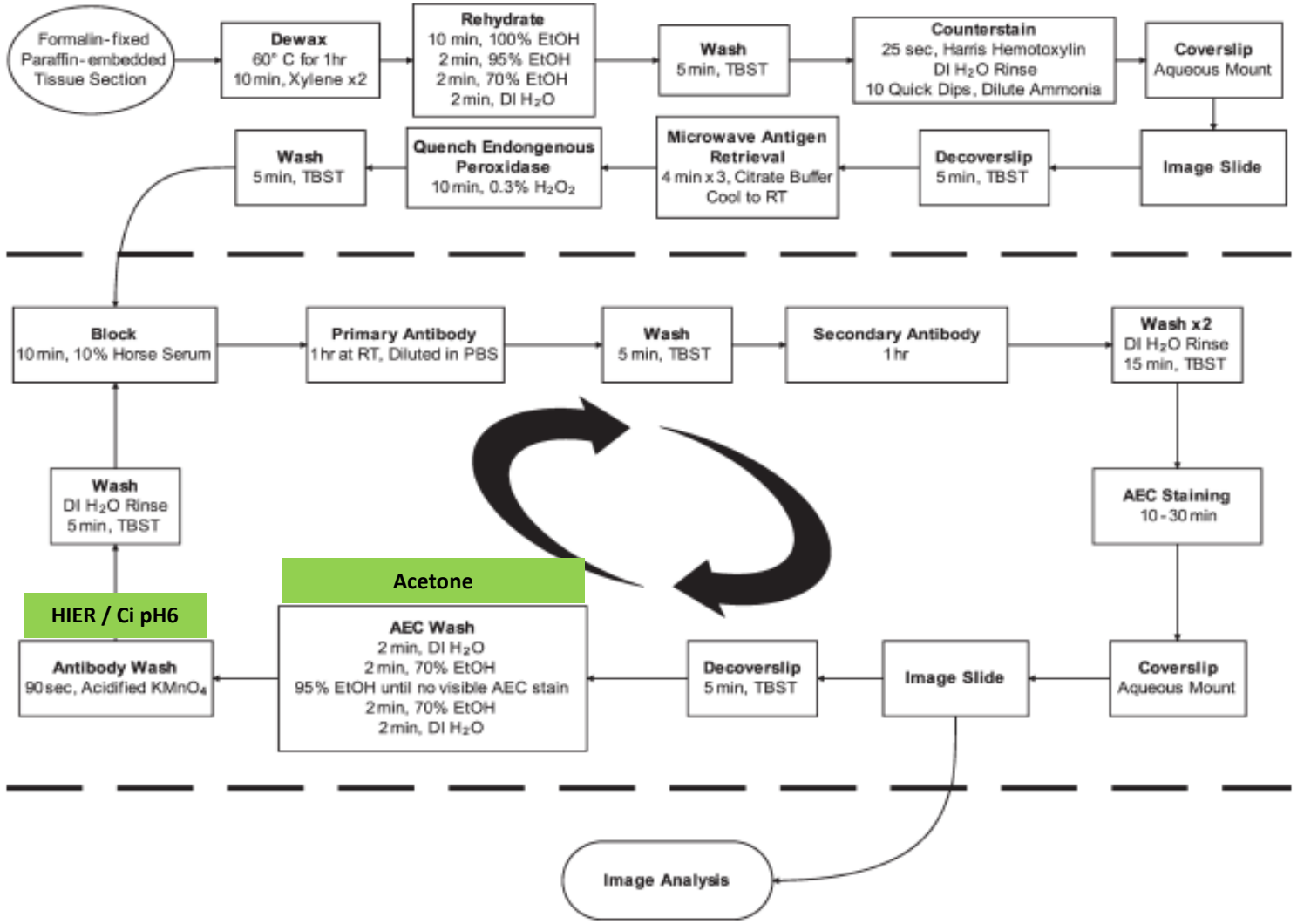



**Figure 1** SIMPLE strategy. Formalin-fixed, paraffin-embedded sections are dewaxed, rehydrated, and counterstained before initial probing. Tissue is imaged and then subjected to antigen retrieval, removing the counterstain. Each staining round is conducted using standard immunohistochemical protocols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

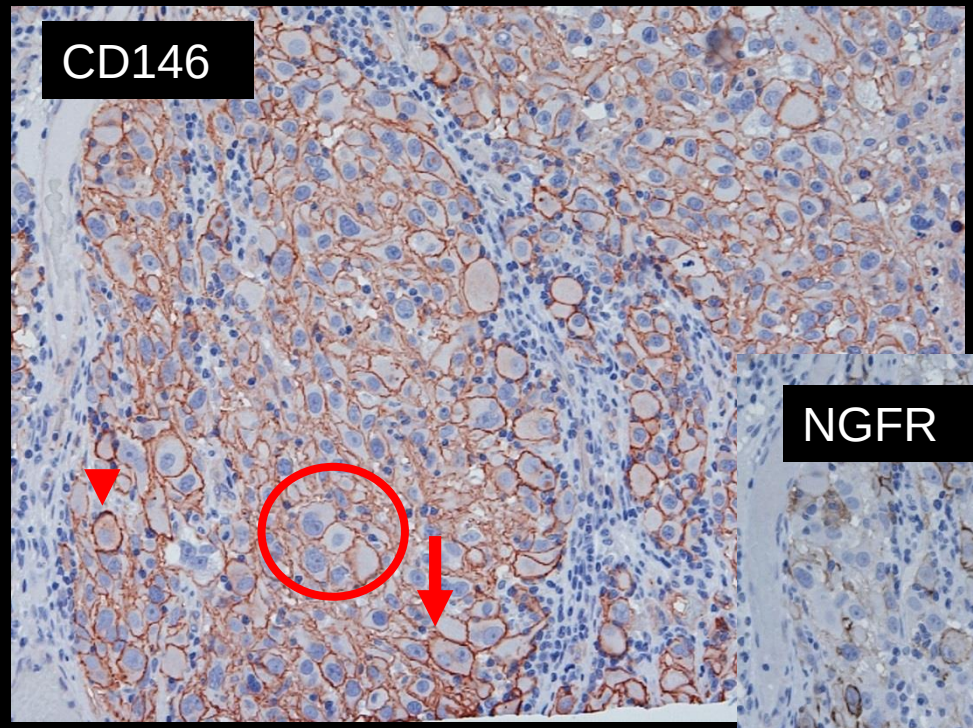
**Imaging of slides**

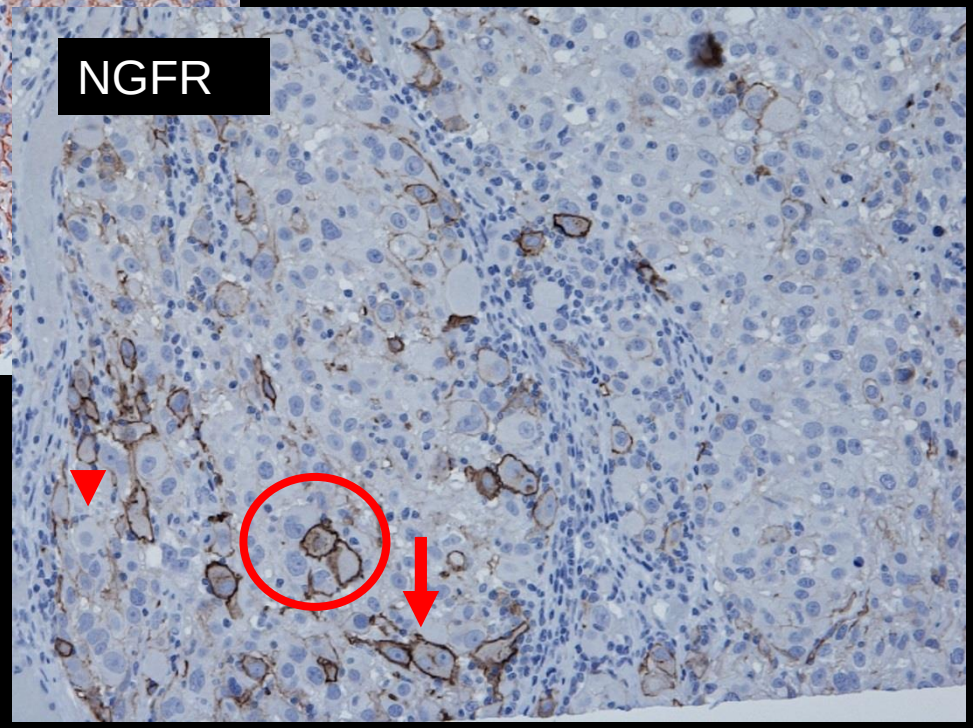
**Removal of coverslip**

**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)

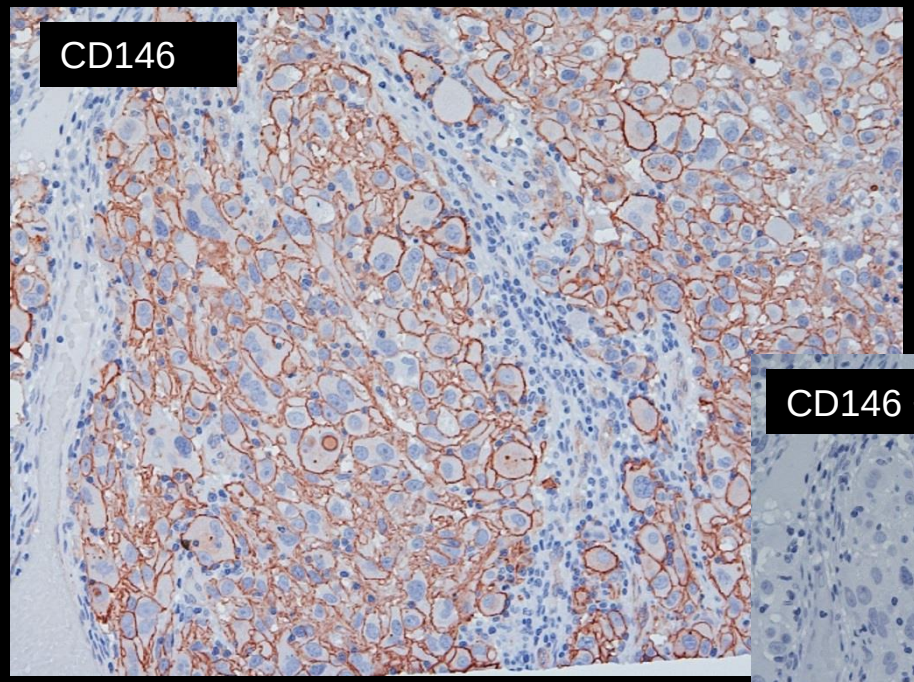
First sequence: CD146  immunostaning => AEC Impact => coverslip

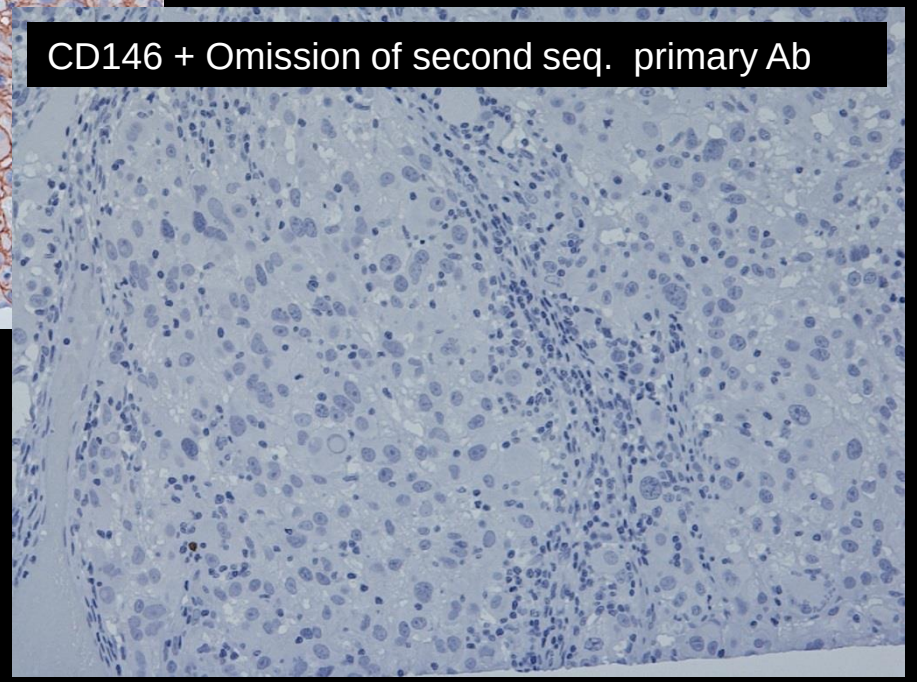
Imaging => de- coverslip => Erasing (Acetone) => Blocking using HIER (Ci)

Second sequence: NGFR immunostaning => DAB => coverslip

Melanoma

# SIMPLE technique: Cross-reactivity control




**Control experiments:**

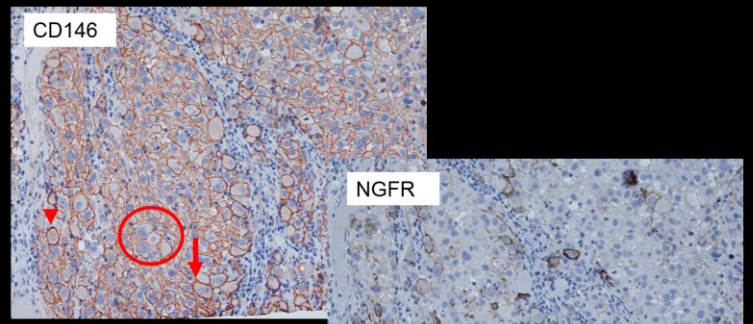
**First sequence: CD146 immunostaining => AEC Impact => coverslip**

**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

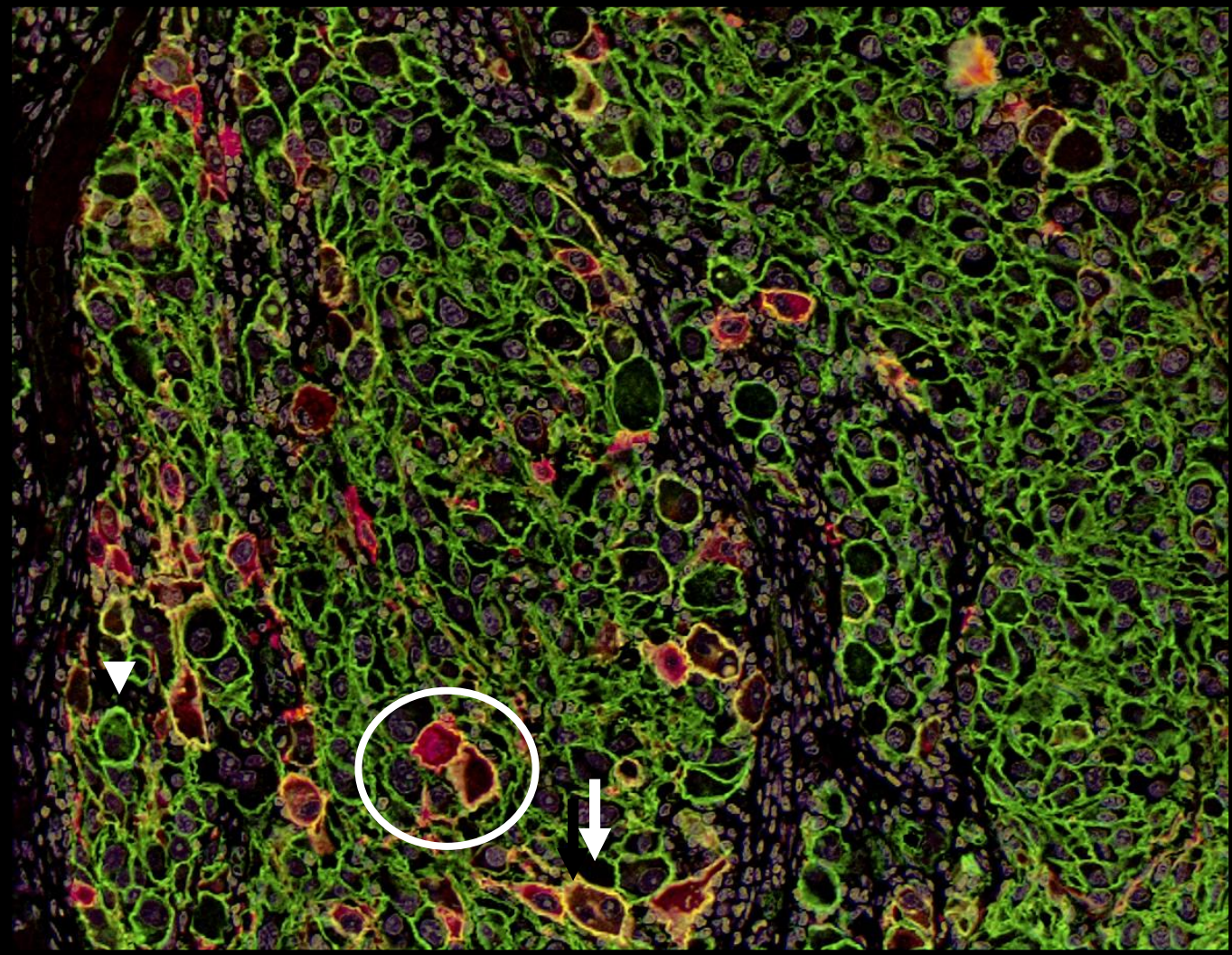
Imaging => de- coverslip => Erasing (Acetone) => Blocking using HIER (Ci)

Second sequence: NGFR immunostaning => DAB => coverslip

Melanoma

**Photo Shop manipulated**

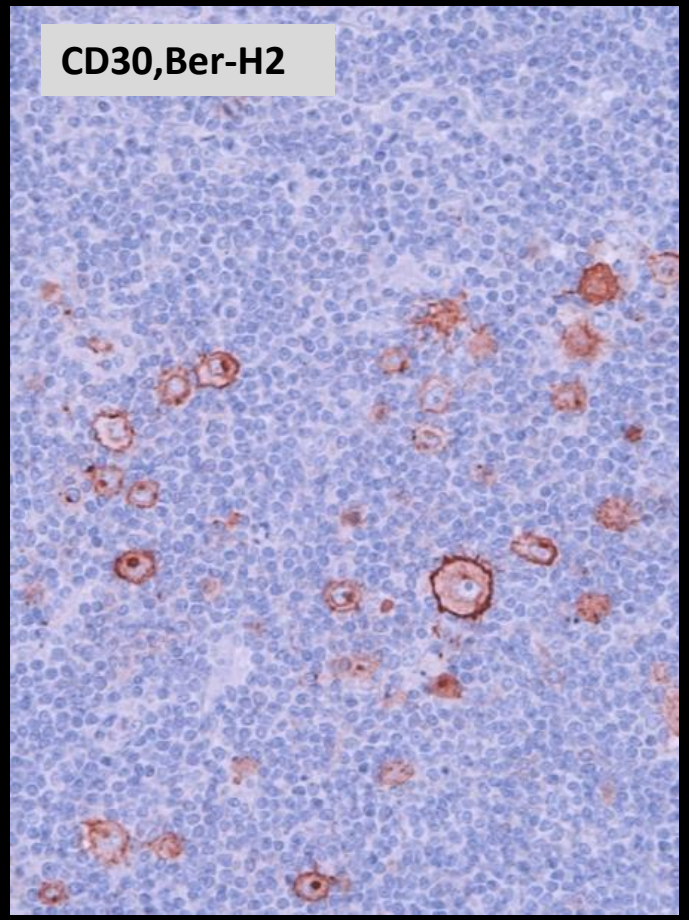
**“Digital imaging”**



CD146 NGFR Co-exp

# Simple-Technique

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



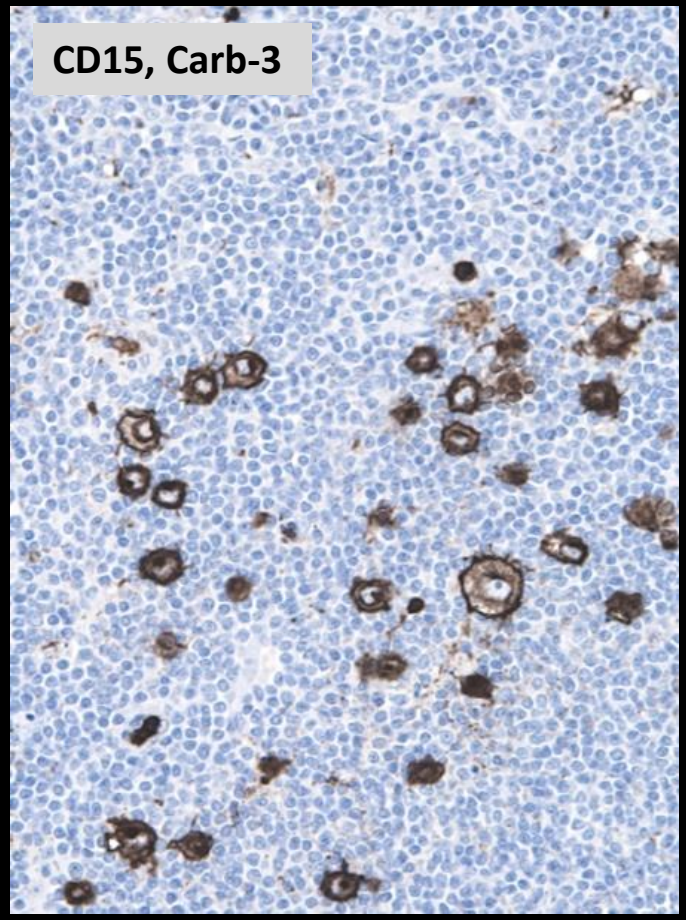

**AEC Erasing and Blocking (Elution / Denaturation)**

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

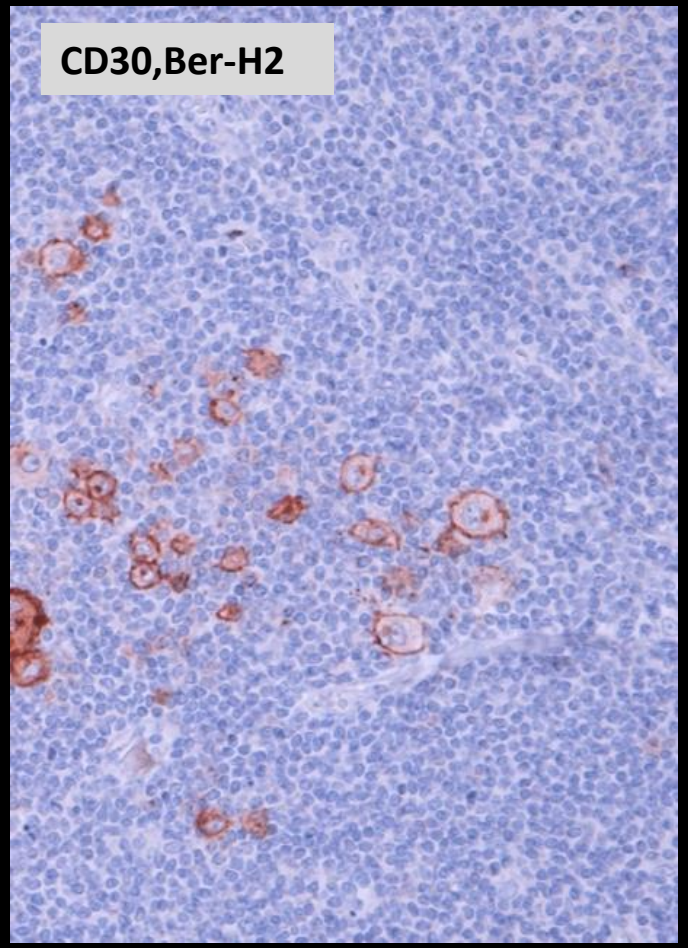
Second sequence repeated with CD15 /DAB




Hodgkin Lymphoma

# Simple-Technique

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



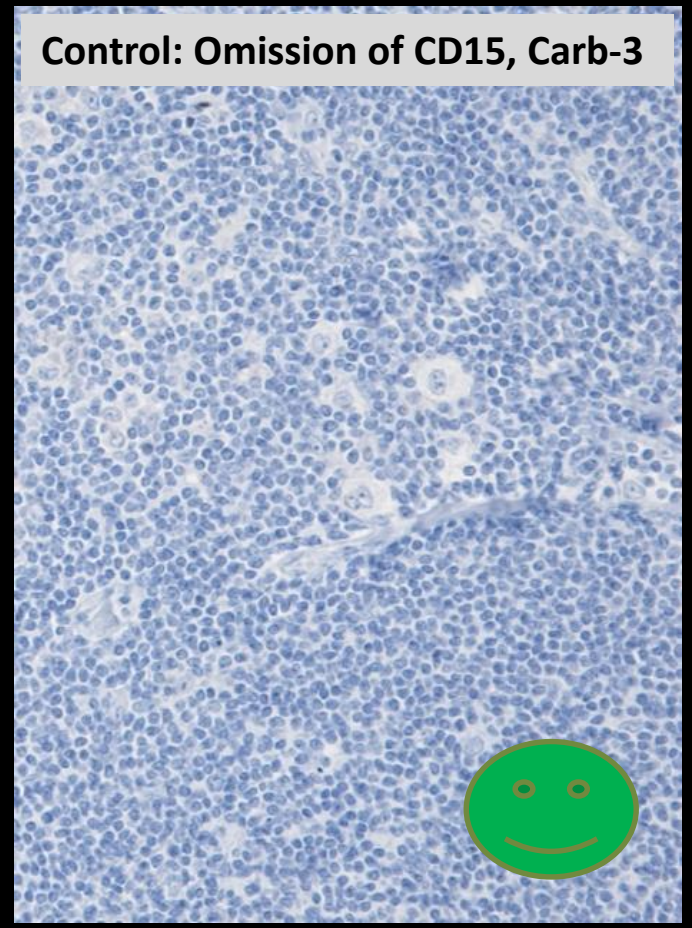

**AEC Erasing and Blocking (Elution / Denaturation)**

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

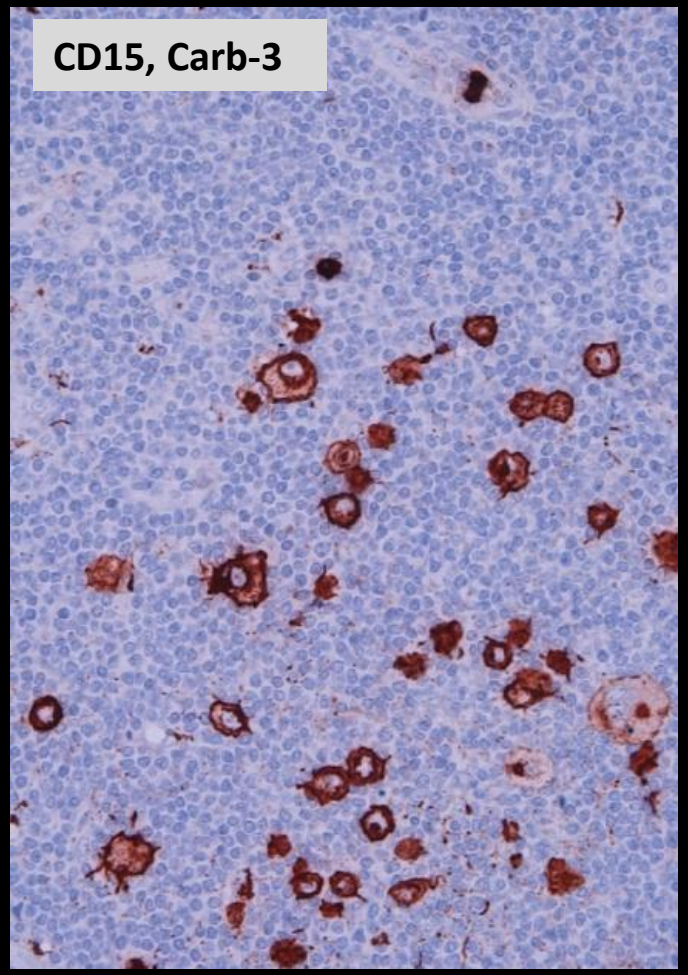
Second sequence repeated without CD15 /DAB




Hodgkin Lymphoma

# Simple-Technique

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




**AEC Erasing and Blocking (Elution / Denaturation)**

De-coverslip (buffer)

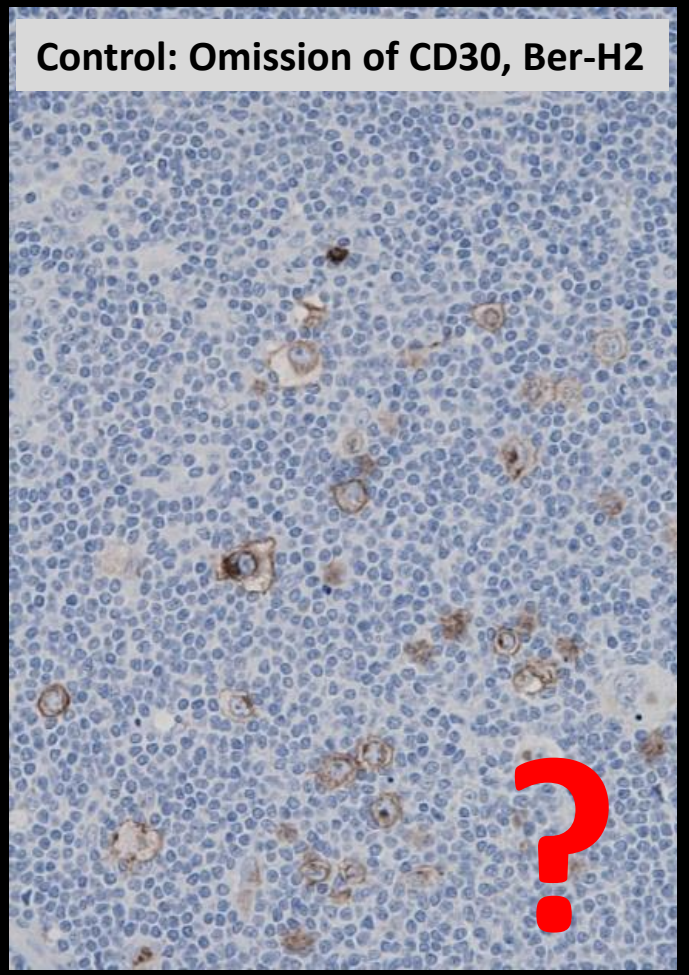
AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

Second sequence repeated without CD30 /DAB

**Problem with the blocking procedure:**

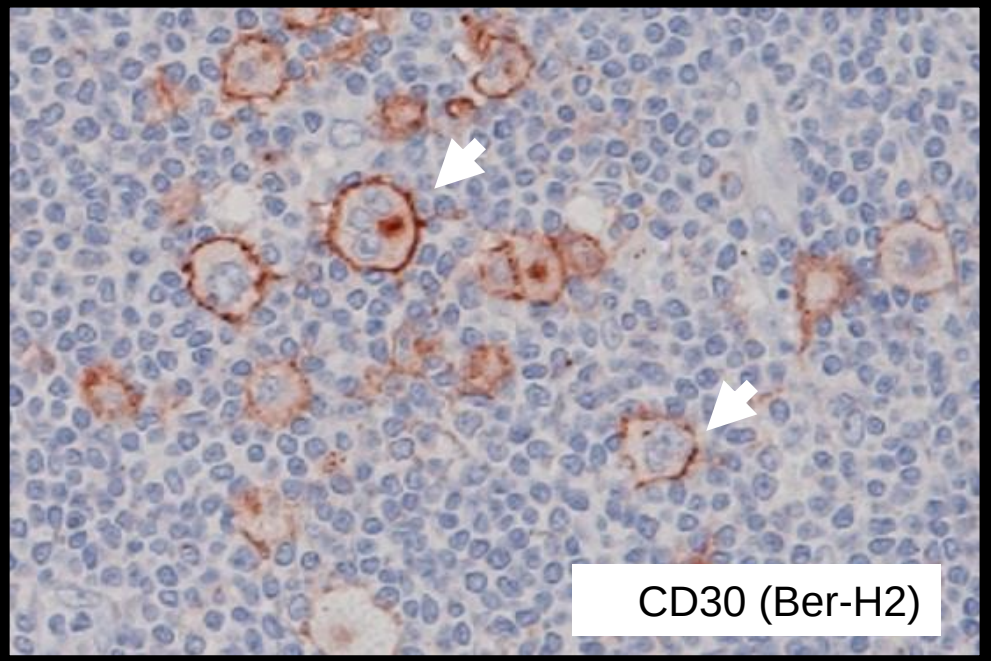
High affinity Abs ?
Antigen density ?
Inefficient blocking procedure ?

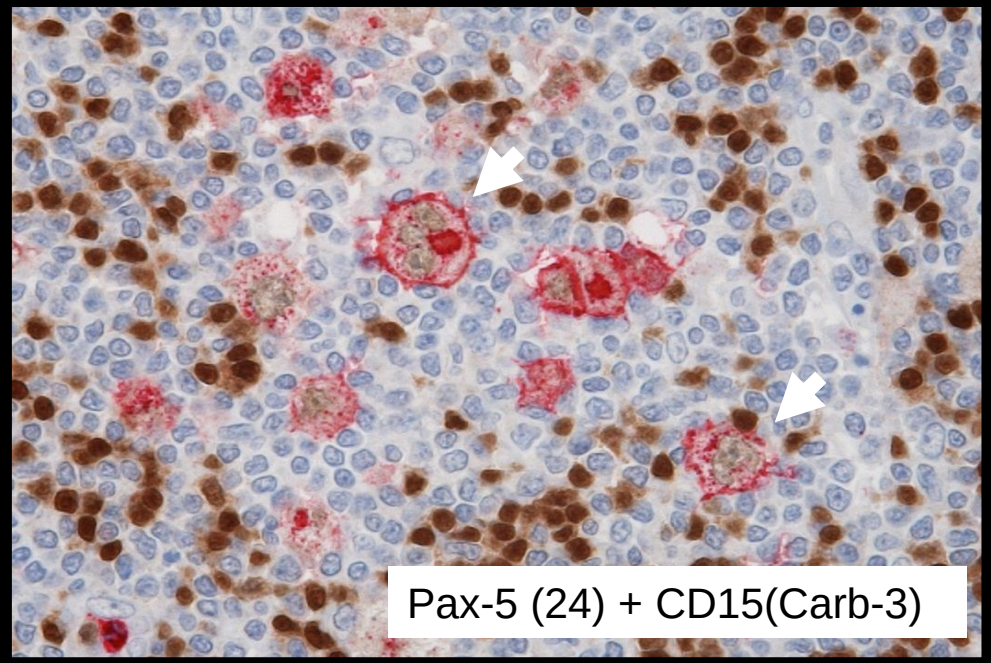



Hodgkin Lymphoma

# Combining SIMPLE technique with sequential double immune enzymatic method

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




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

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


## Multiplex Immunofluorescence : 3 or 4 markers

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 (and CD4)

# Fully automated 5-plex fluorescent immunohistochemistry with tyramide signal amplification and 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 |

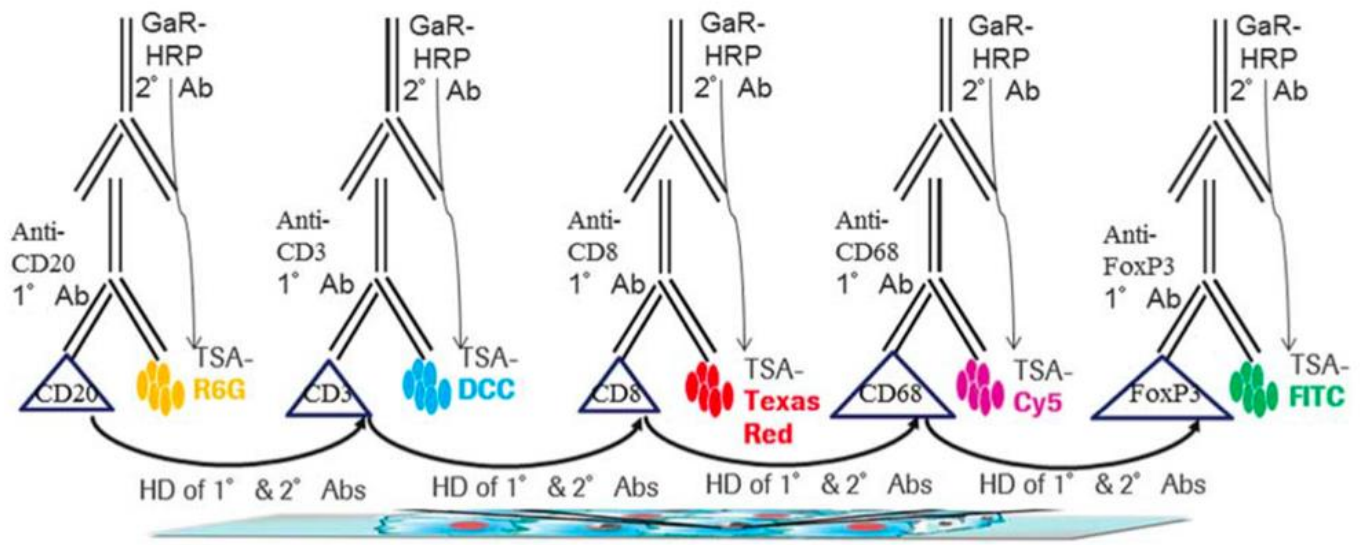



**Figure 1** 5-Plex IHC detection scheme.

**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 |

Laboratory Investigation (2017) 97, 873–885

## Optimizing a 3-plex method :

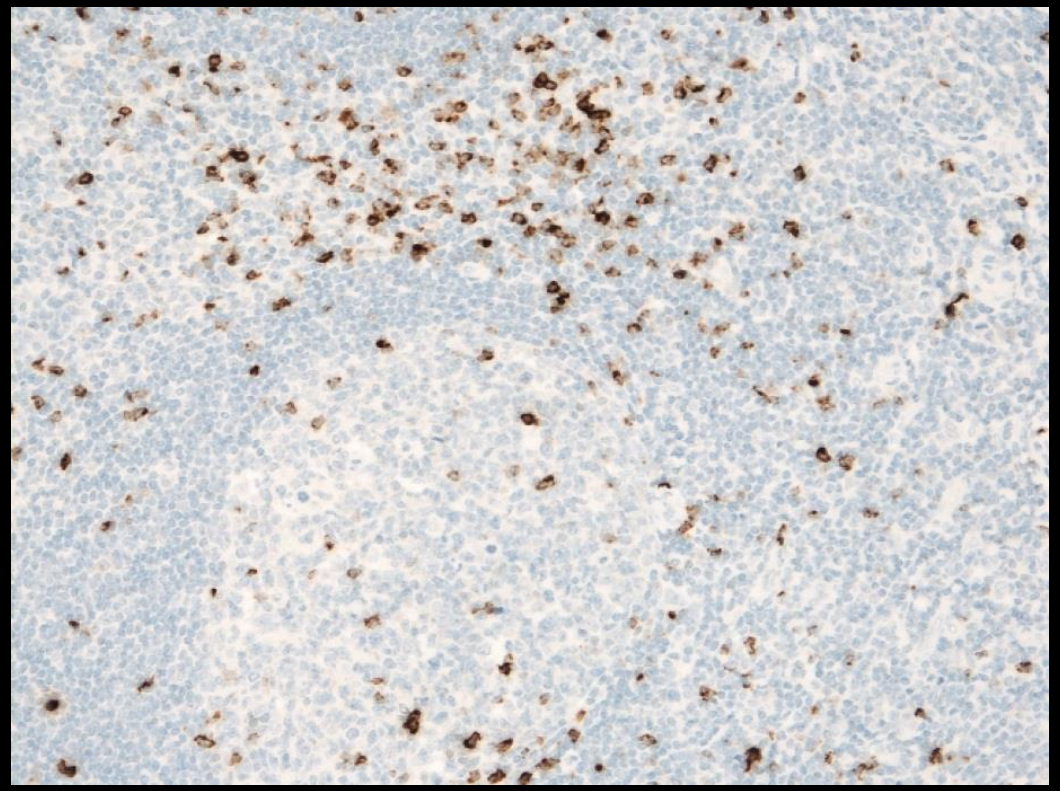
- SOX10, BS7 (Ms) or SOX10, SP267 (Rb) + LAG3, D2G40 (Rb) → first and second sequence, respectively
- CD8, C8/144B (or all other markers e.g., CD4) → third sequence
    - Neutralization step is applied between sequences using different host`s of the primary Abs
        - Eliminates HRP activity of introduced immunoreagents
    - Heat Denaturation step is applied between sequences using same host of the primary Abs e.g., mAb`s in both first and second sequence
        - Eliminates cross reactivity between introduced immunoreagents.

## Cross-talk Controls:

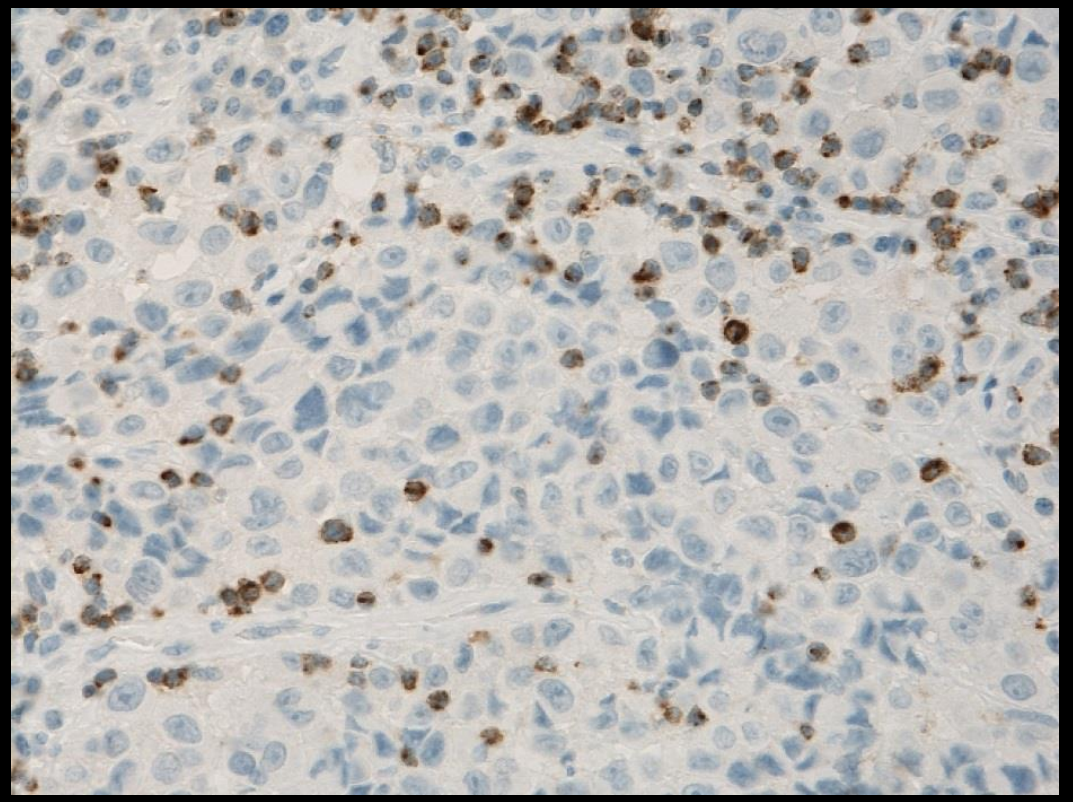
- Neutralization (N) control of HRP activity (should be completely eliminated)
    - Applied between sequences using different host`s of the primary Abs e.g., SOX10, BS7 (Ms) and LAG3, D2G40 (Rb) - detection systems do not cross-react (e.g., use Goat anti-Ms/HRP in the first and Goat anti-Rb/HRP in the second sequence).
- Heat Denaturation (HD) control of cross-reactivity between introduced immunoreagents (no reactions should be seen).
    - Applied between sequences using same host of the primary Abs e.g., mAb`s in both first and second sequence.

# LAG3, D2G40 (1:50): CC1 48` (95°C)/ Gt-anti-Rabbit (HQ) + anti-HQ + DAB (Ventana Discovery)

**Tonsil**

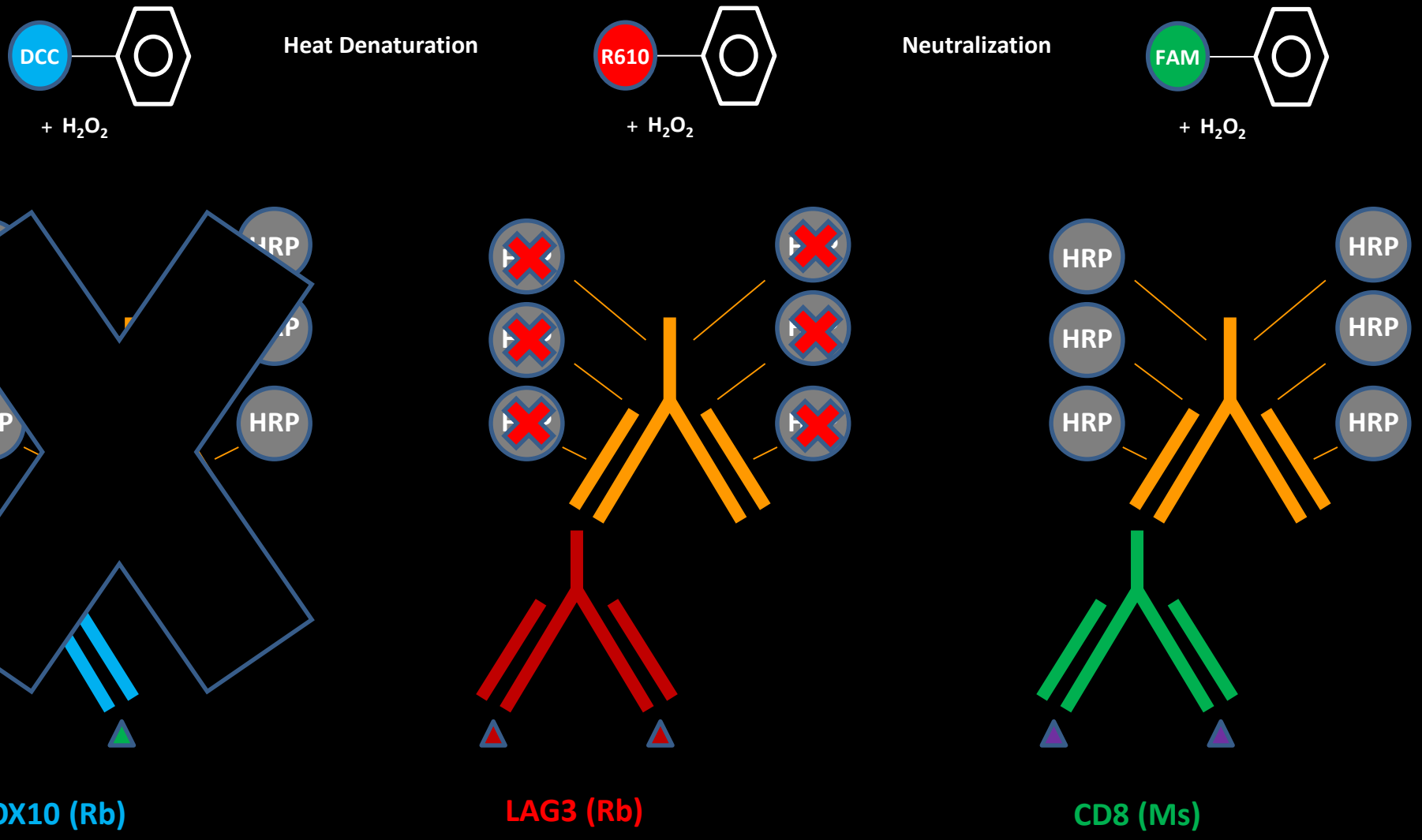


**Melanoma**



LAG3 ~ Marker of exhausted T-cells

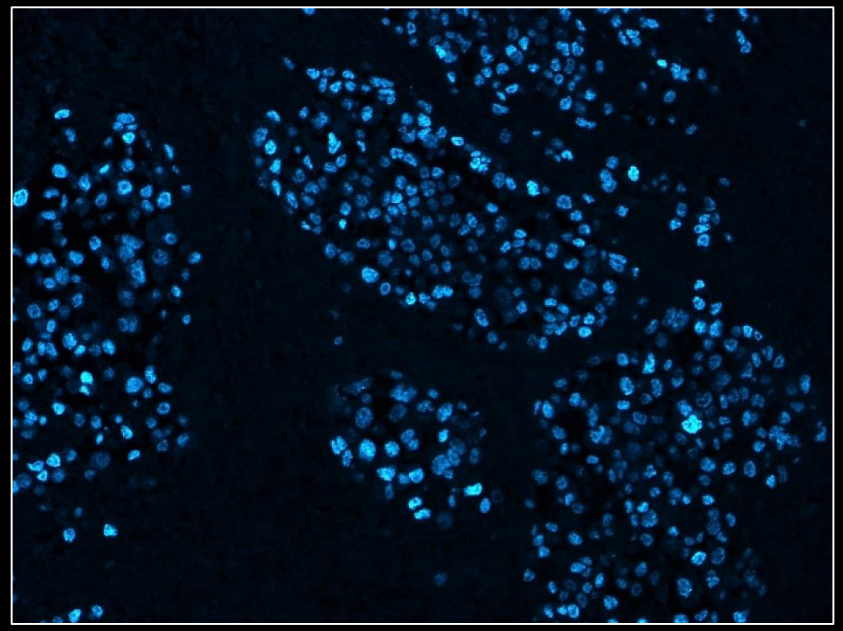
# Optimizing a 3-plex method : How it could look like

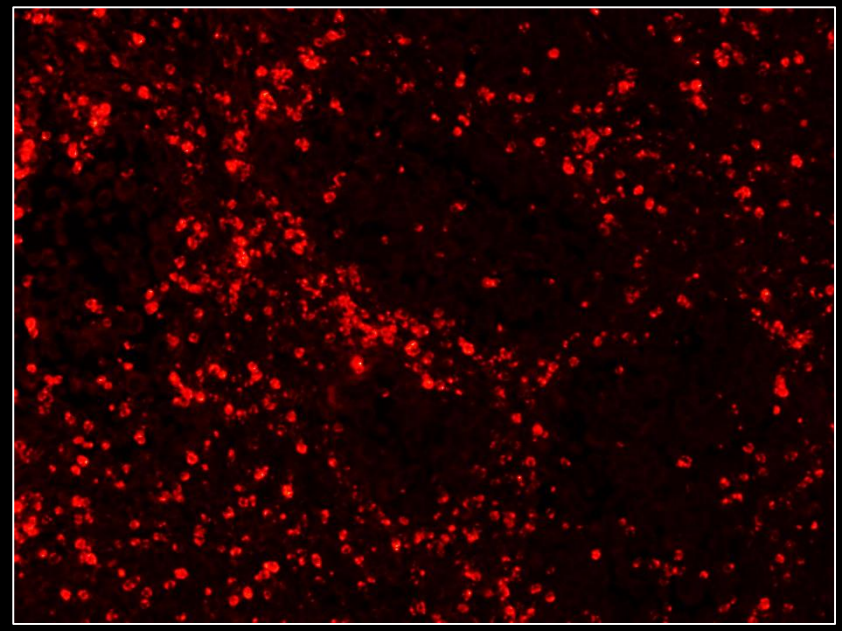


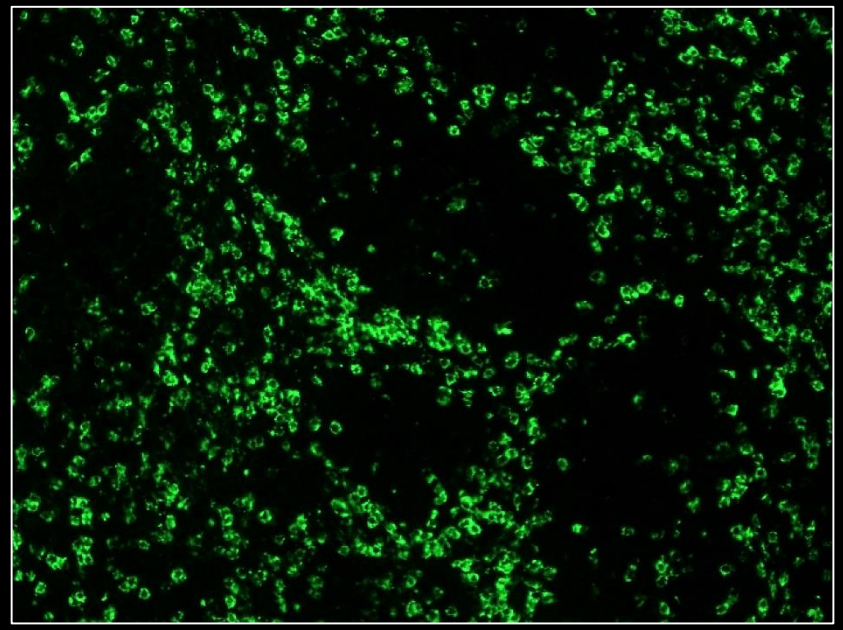
Melanoma

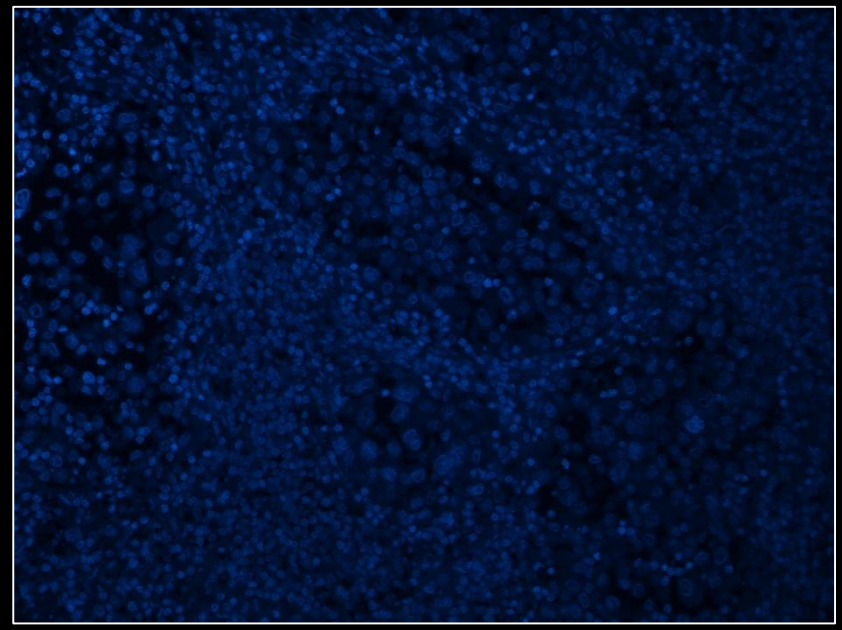
**CC1 48`/95C**

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







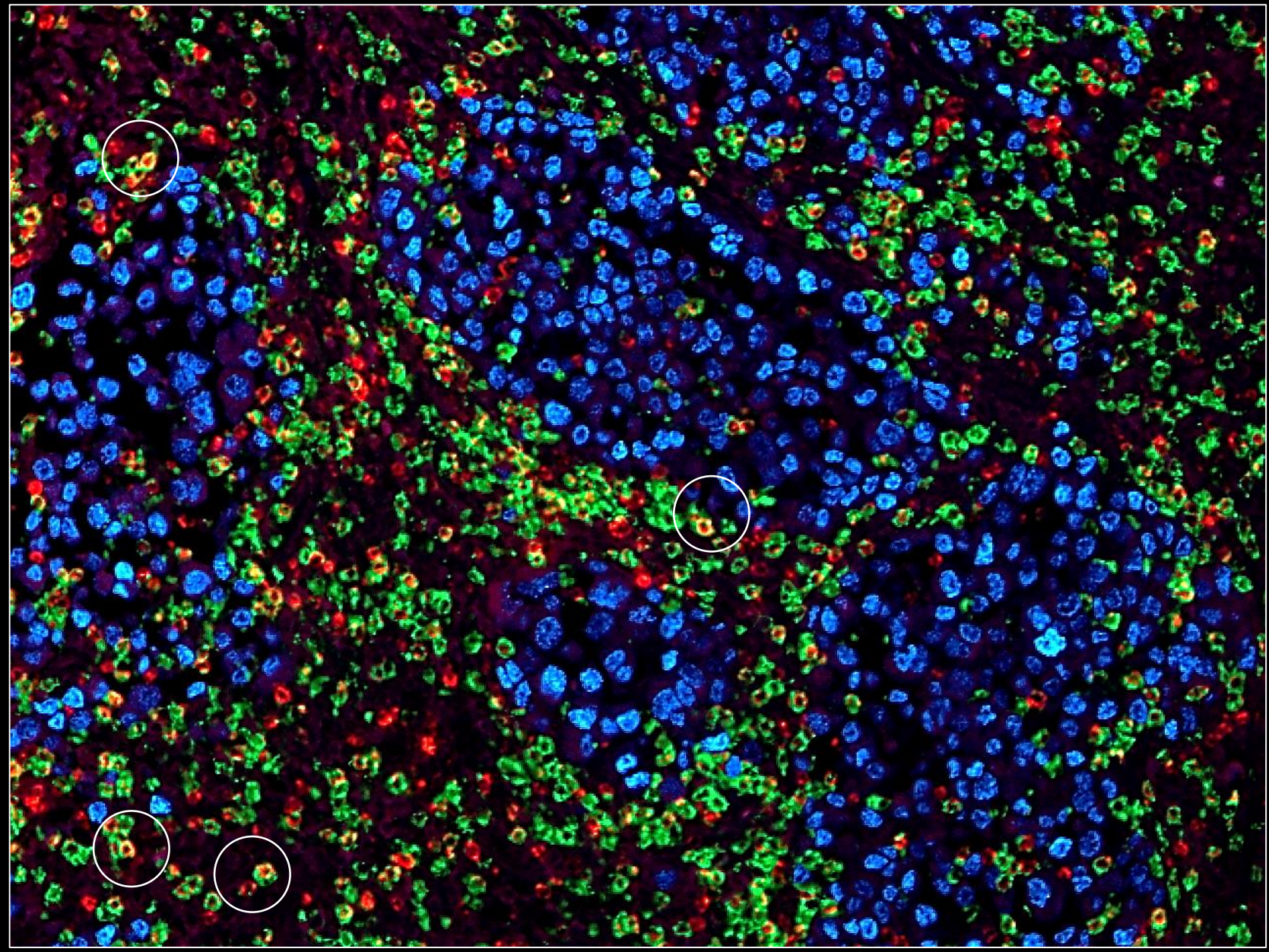


**Co-localized signals ?**

Melanoma

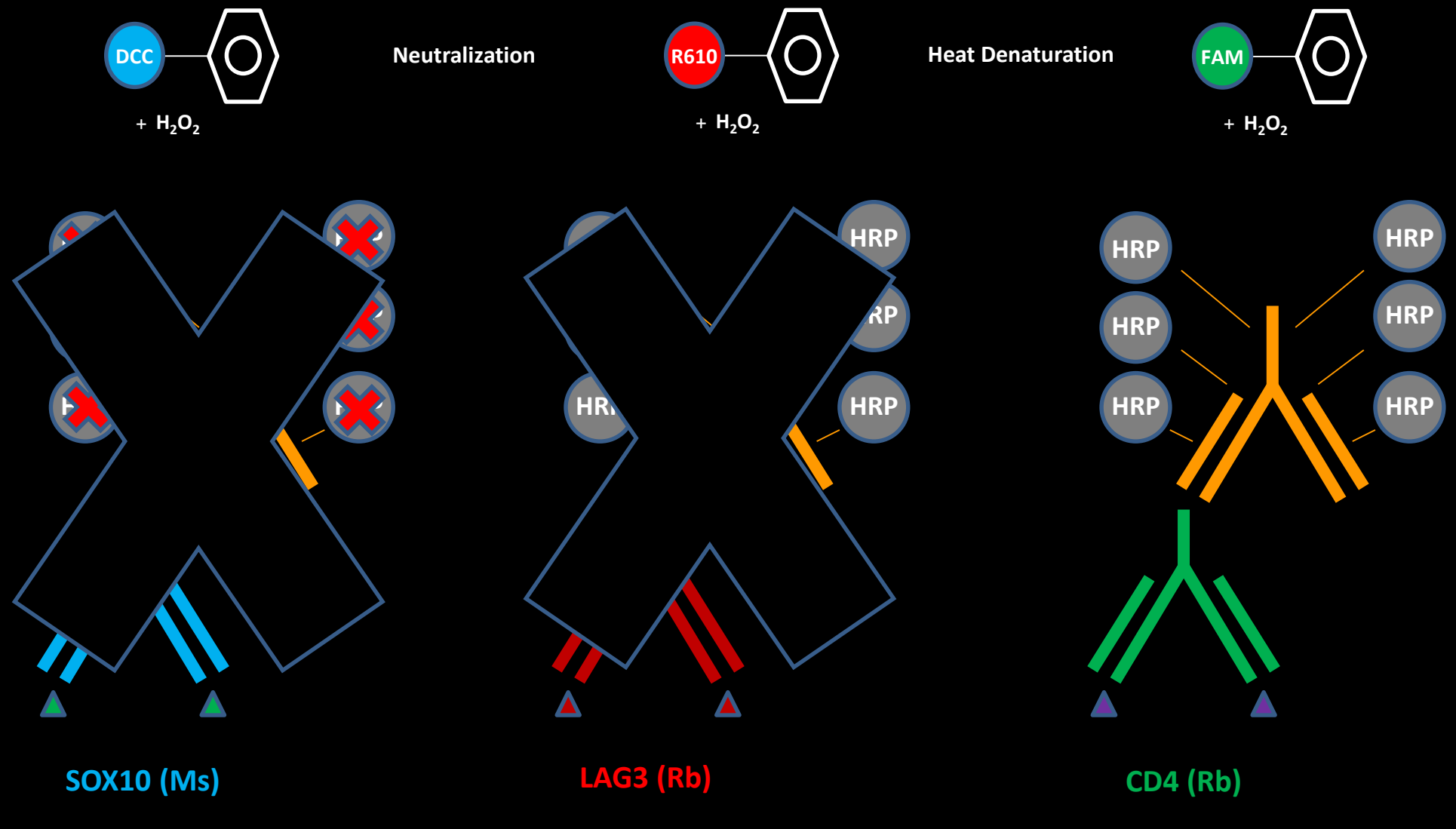
**CC1 48`/95C**

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



**Co-localized signals**

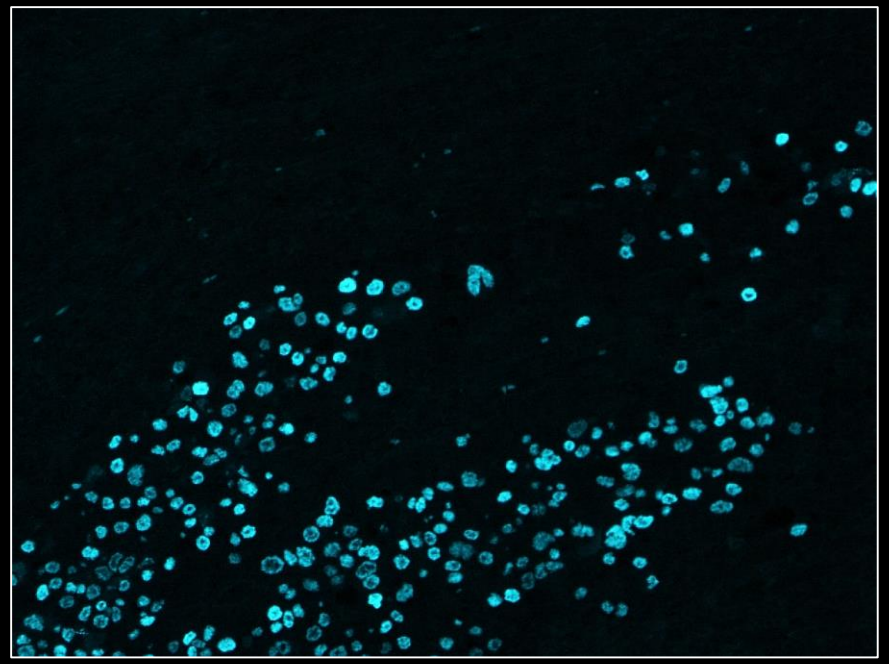
# Optimizing a 3-plex method : How it could look like

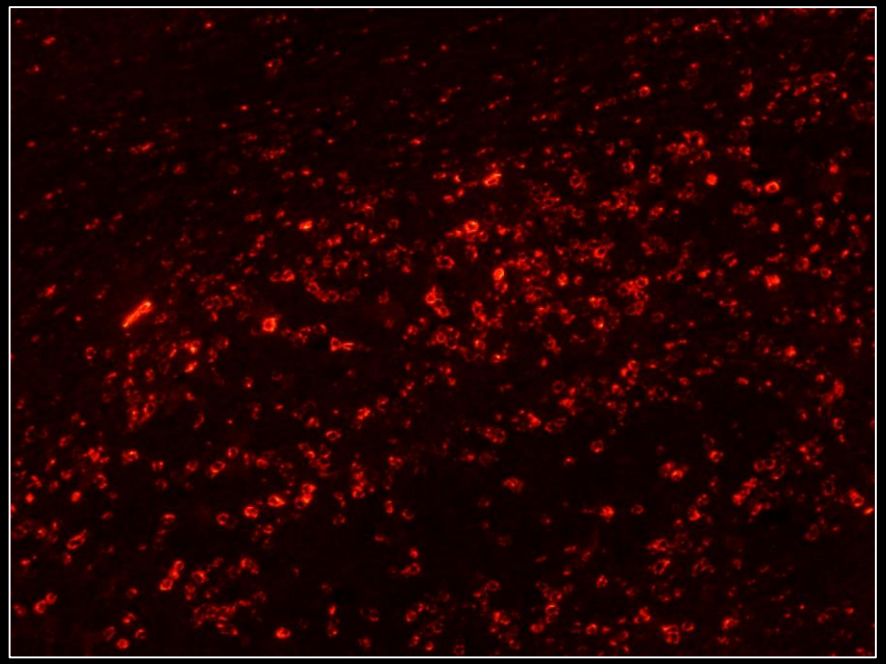


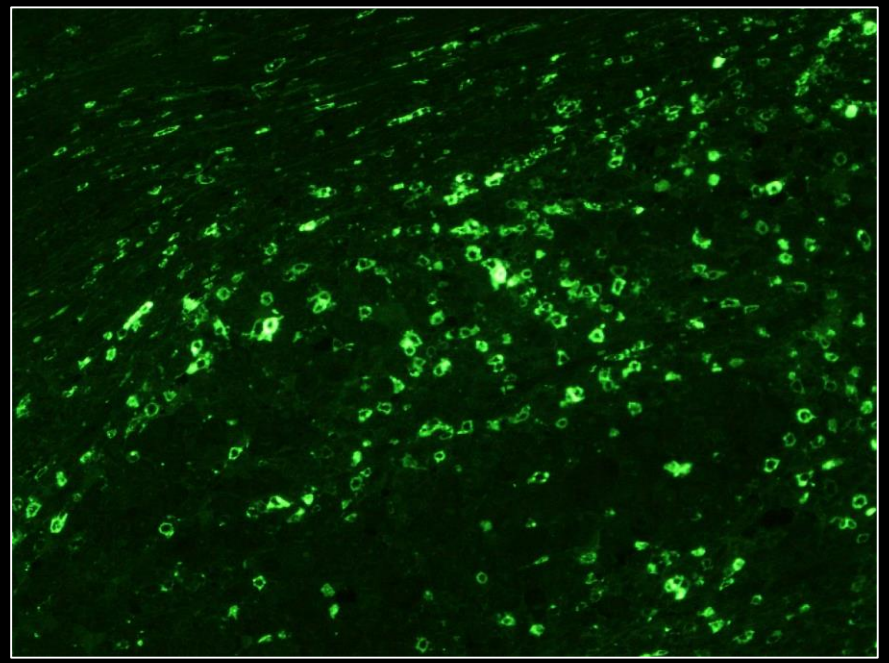
Melanoma

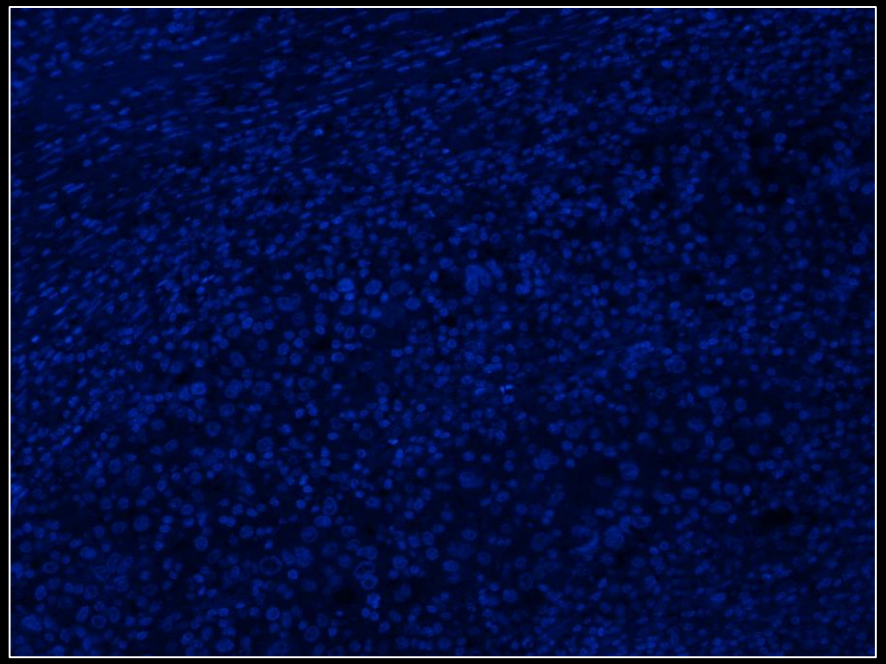
**CC1 48`/95C**

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









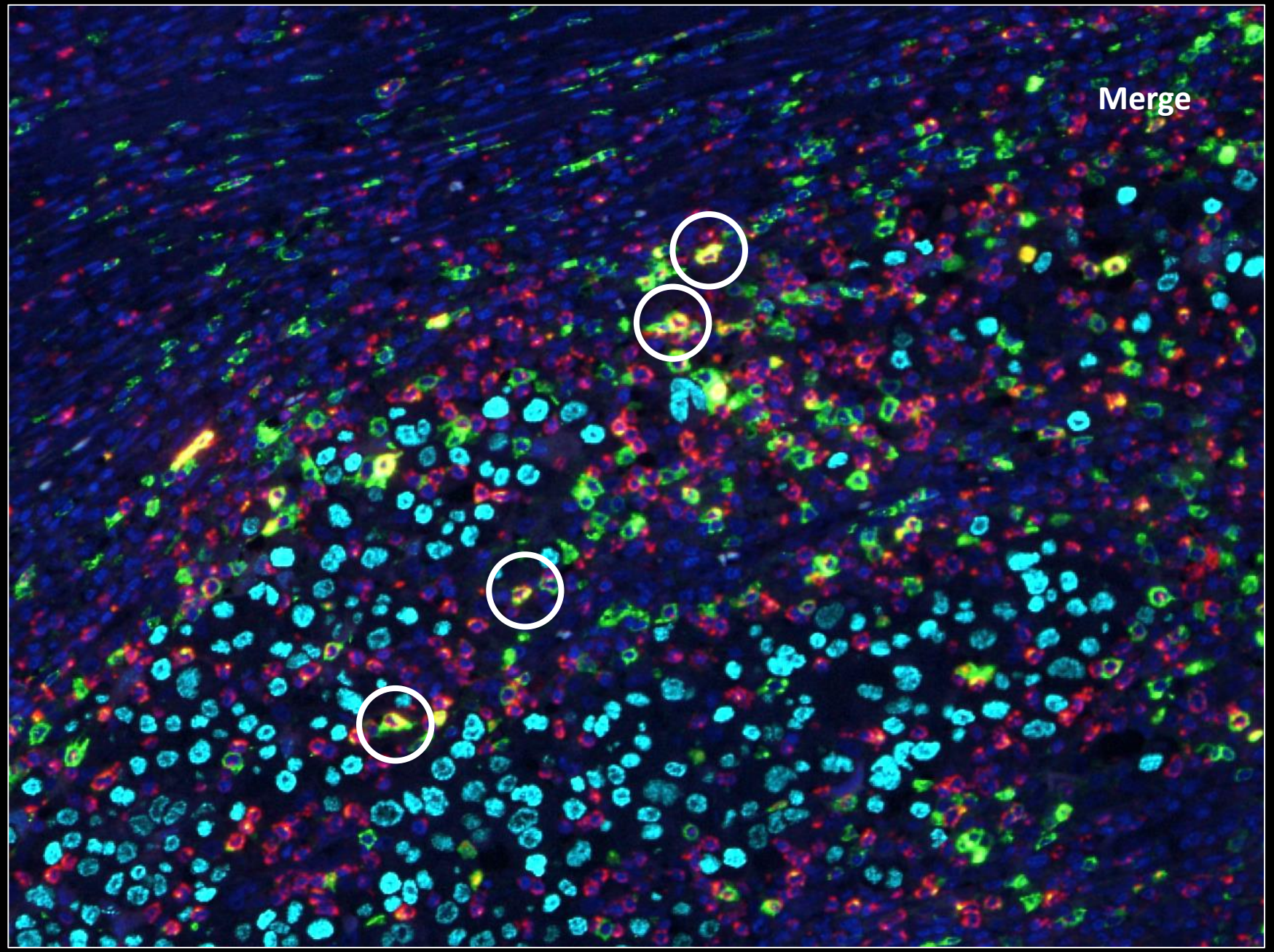
**Co-localized signals**

Melanoma

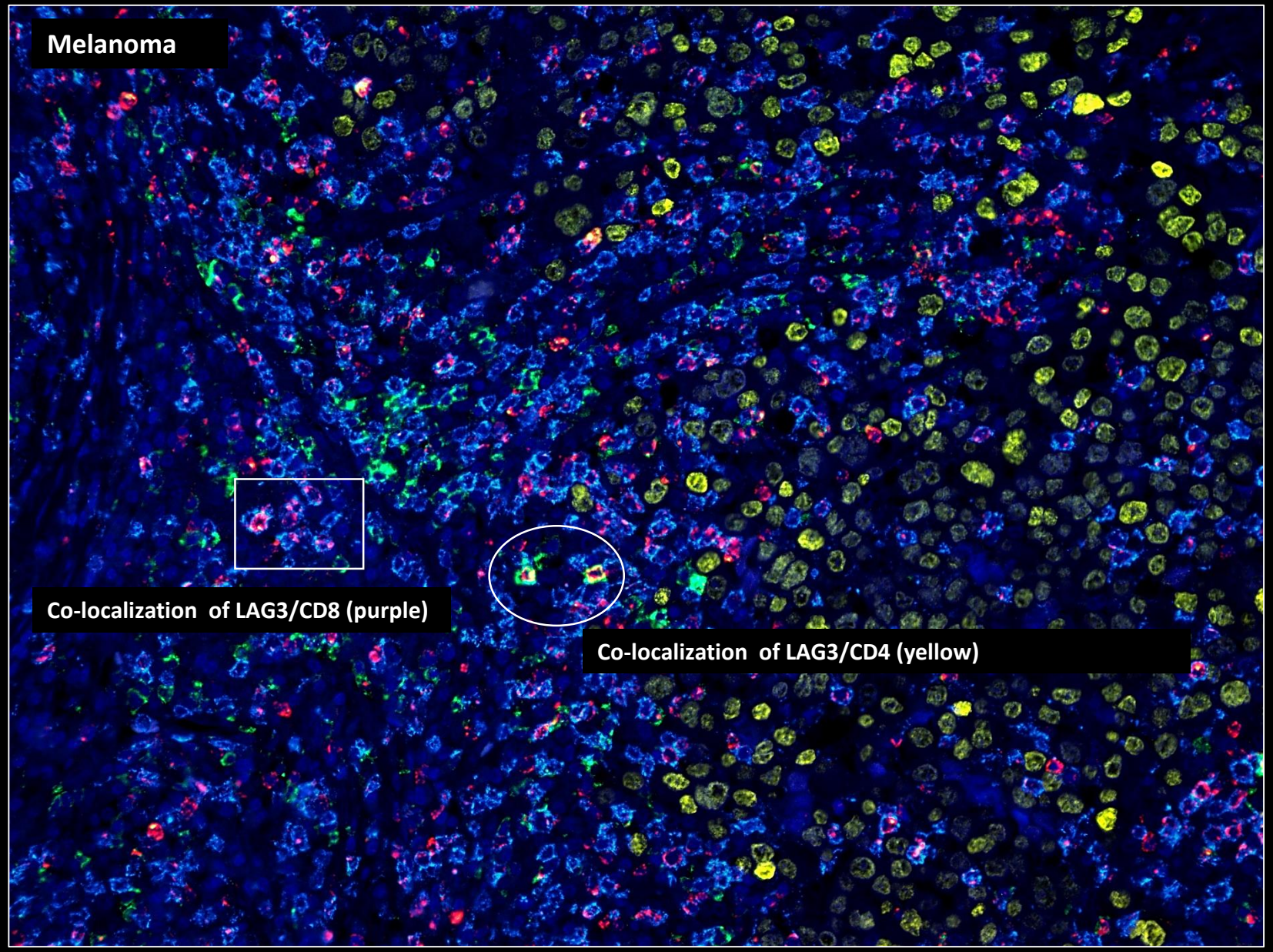
**CC1 48`/95C**

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

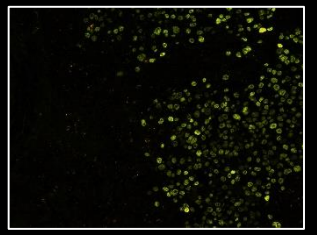
**Co-localized signals**



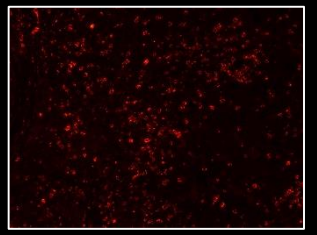
# 4-Plex: SOX10, BS7 (Ms) + N + LAG3 (Rb) + HD + CD8 (Ms) + N + CD4 (Rb)



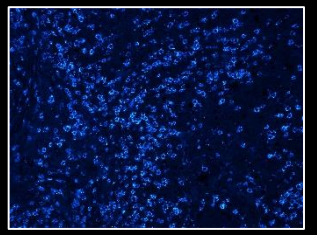

SOX10



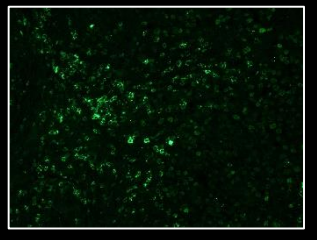
LAG3



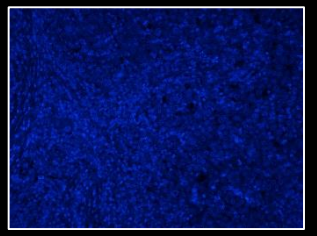
CD8



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

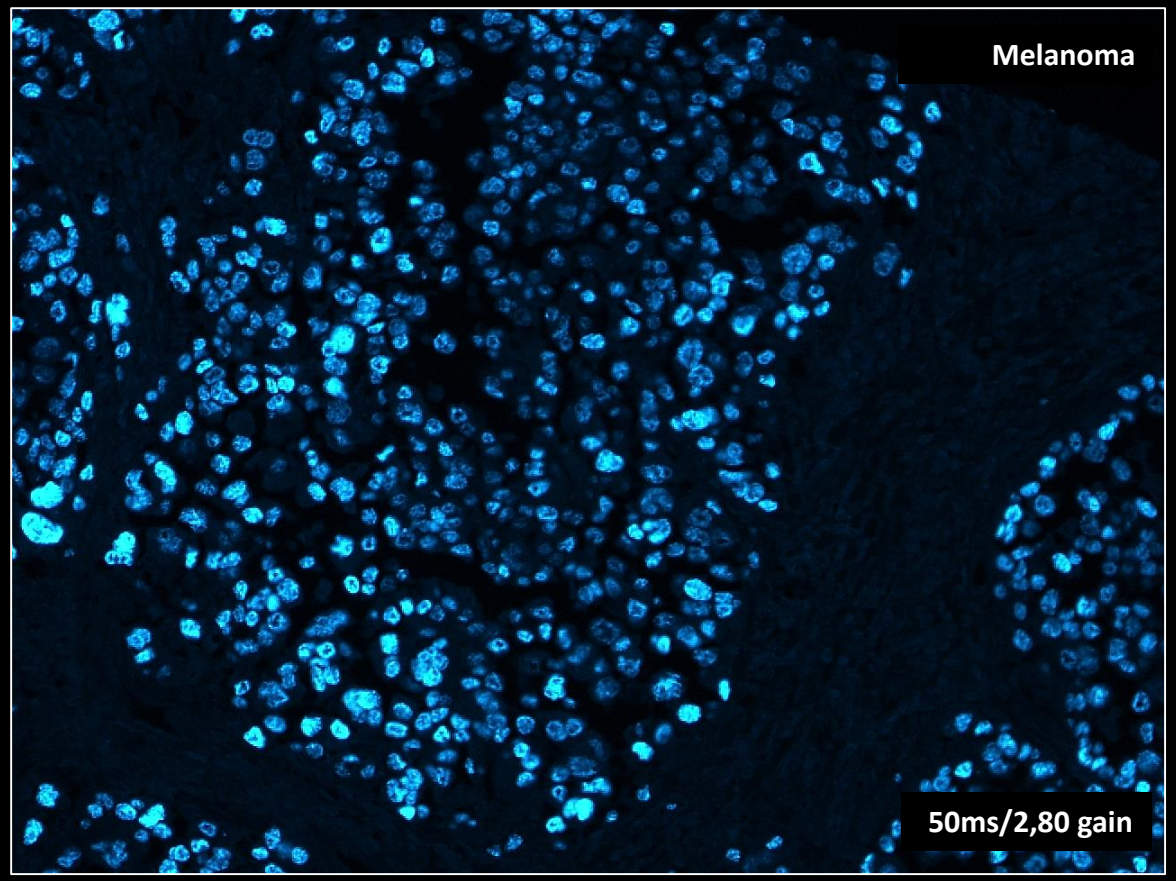
Neutralization control (N) : The neutralization step should eliminate HRP activity of introduced detection systems

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

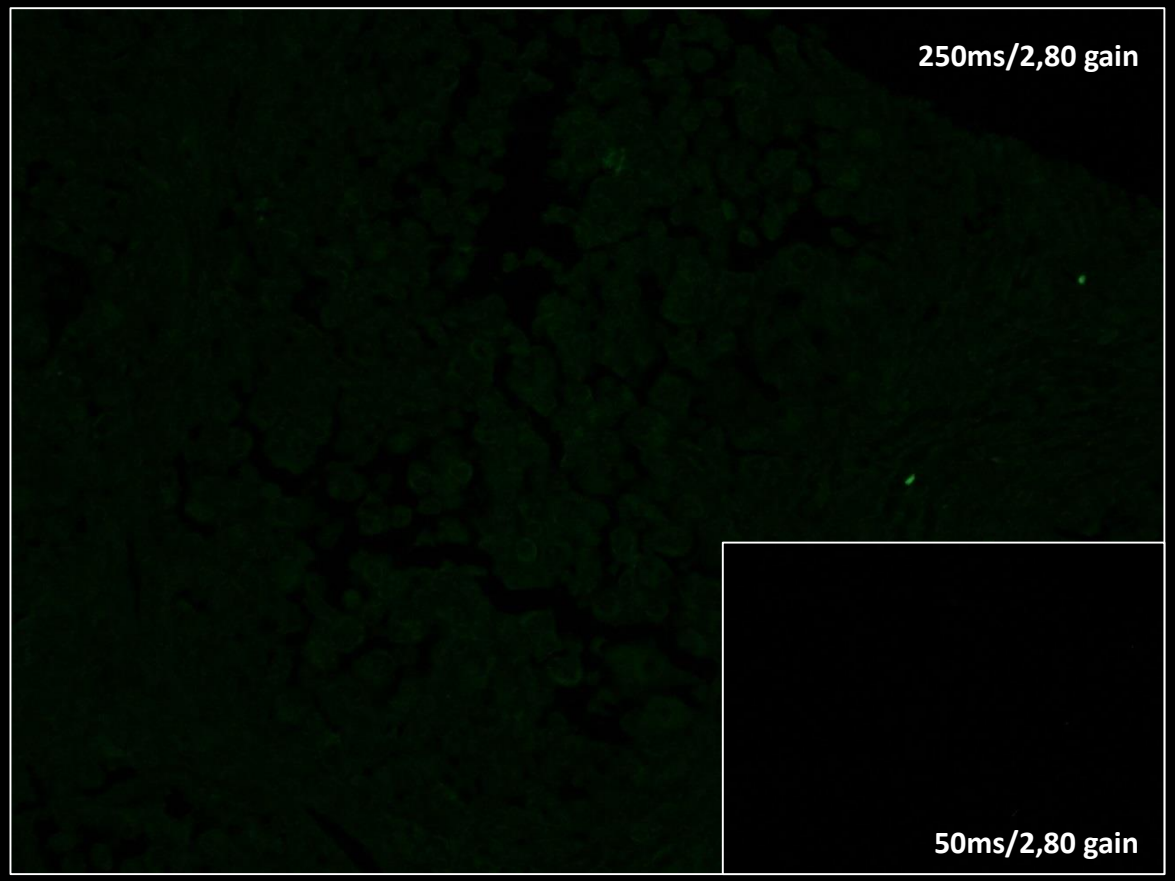
Cross-talk controls: Cross your fingers , otherwise you have a lot of work ahead

# Cross-talk controls: Neutralization using Discovery Inhibitor

SOX10, BS7/OmniMap Ms-HRP/DCC

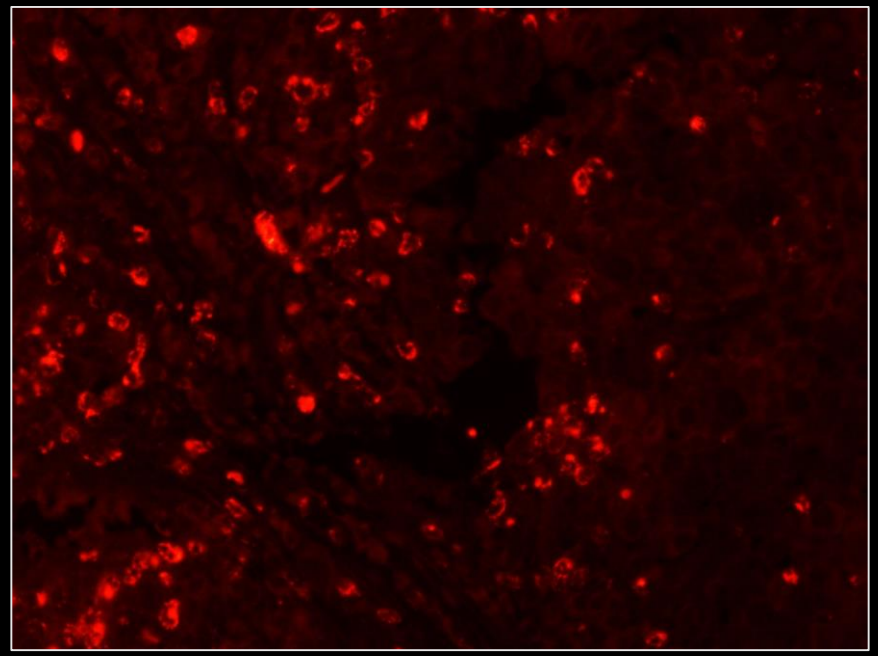



SOX10, BS7/OmniMap Ms-HRP/ Neutralization/ TSA-FAM

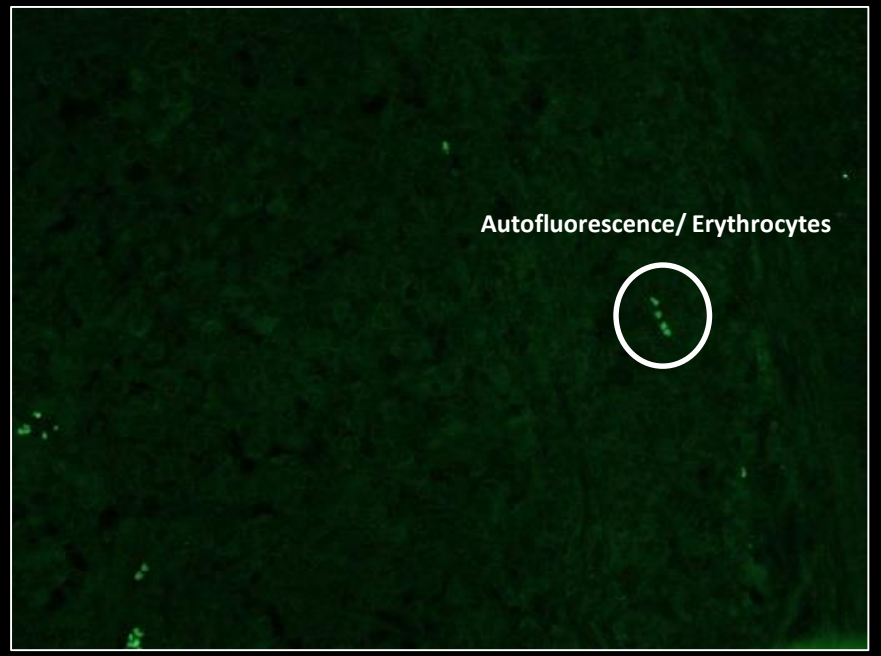



The neutralization step (Discovery Inhibitor) eliminate HRP activity of the detection system. Same effect was obtained using a HD step.

# 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

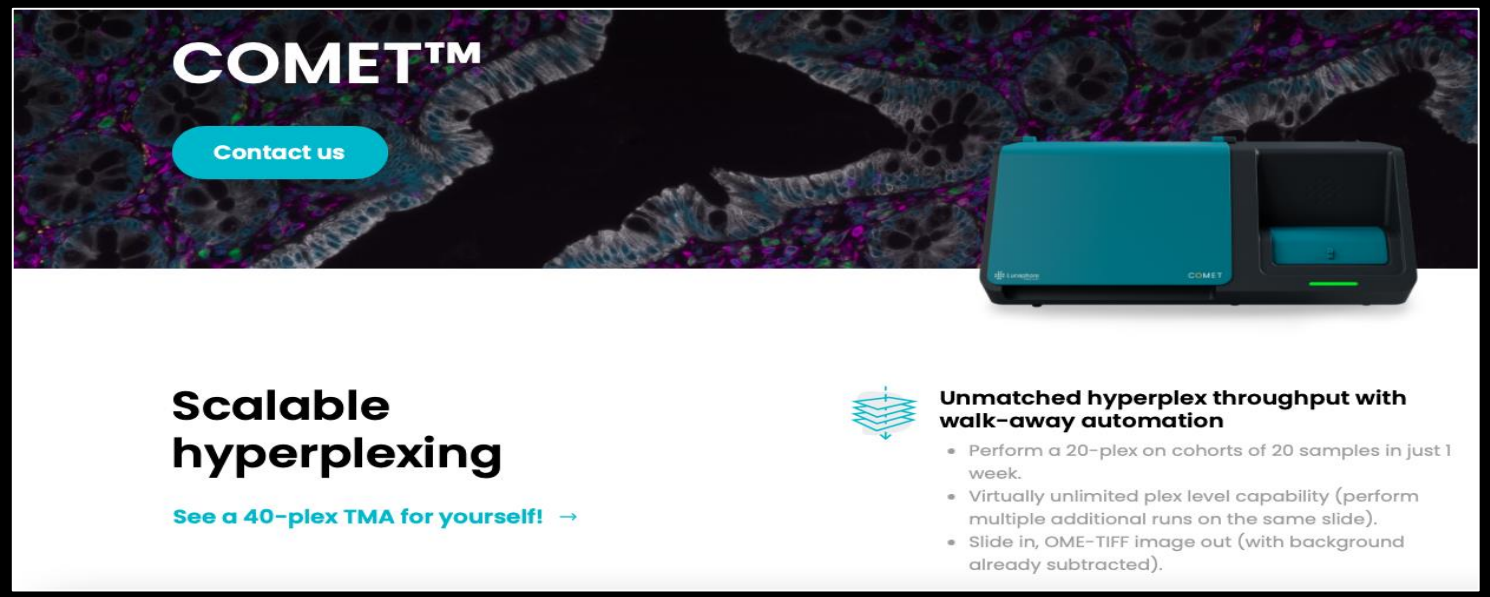
TSA-FAM

DAPI

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

Successful HD step: Efficient elimination of LAG3, D2G40 sequence

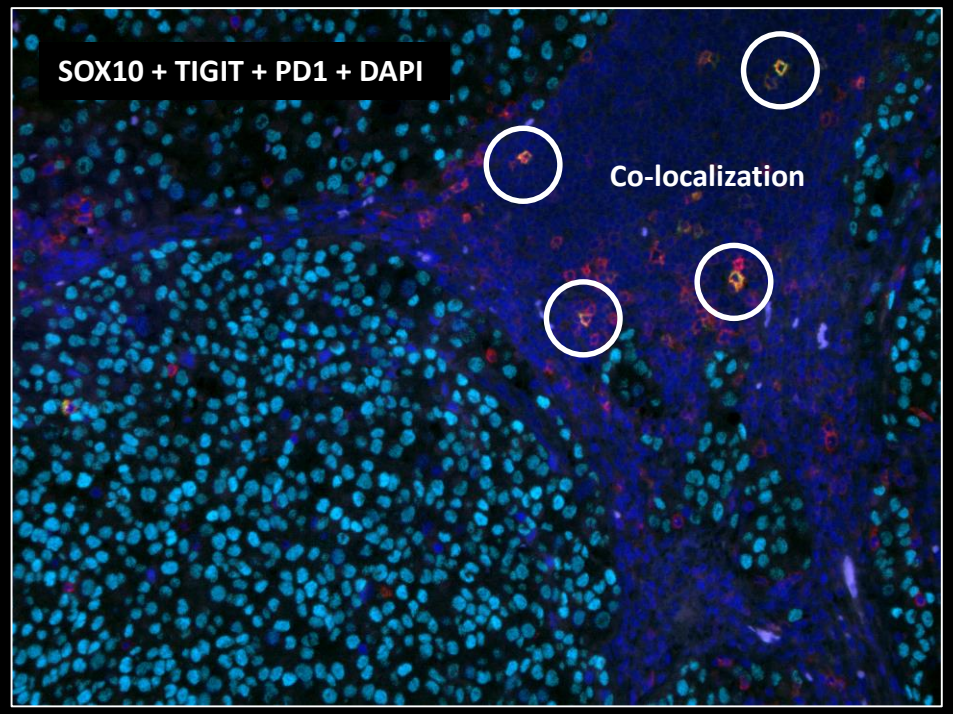
# The future is already here: Advanced techniques (End-to-end solutions)

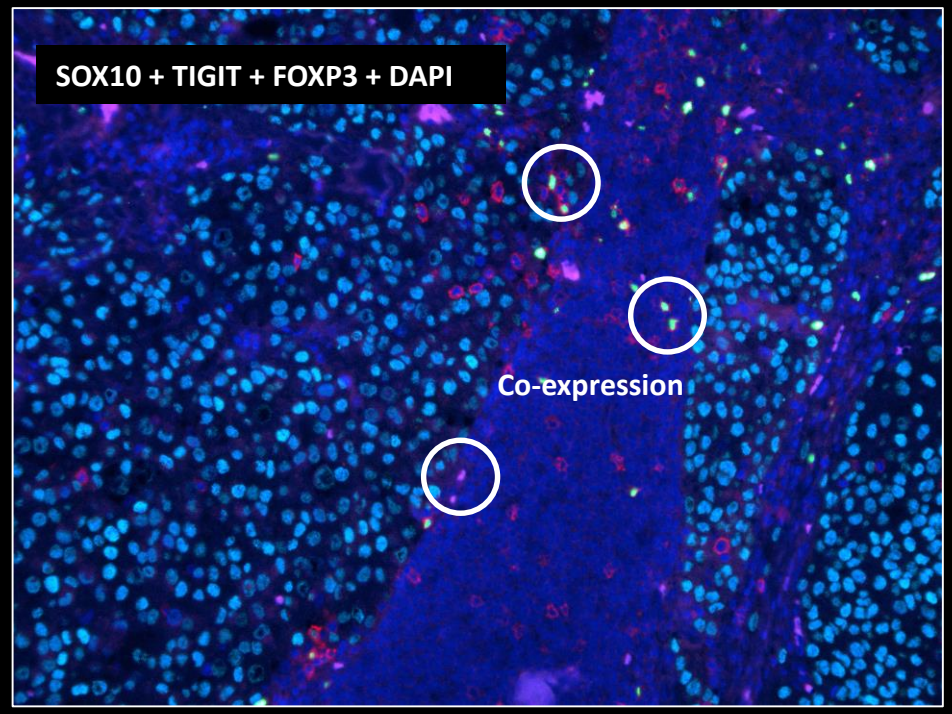


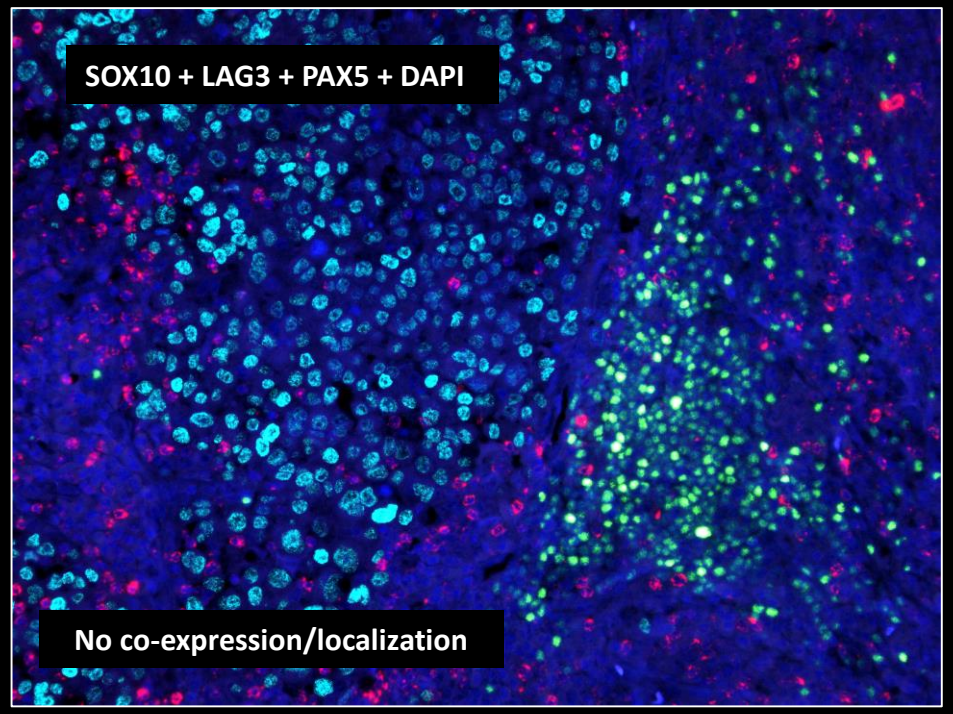

Lunaphore: COMET

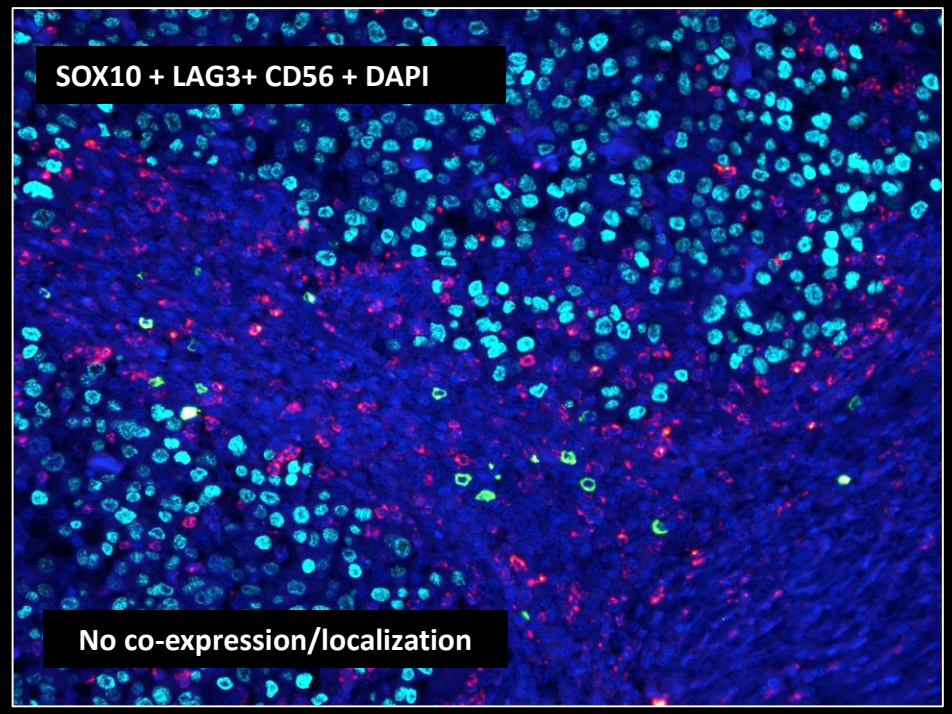
Akoya Bioscience: Phenocycler (Codex)

Thank you for your attention



## 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

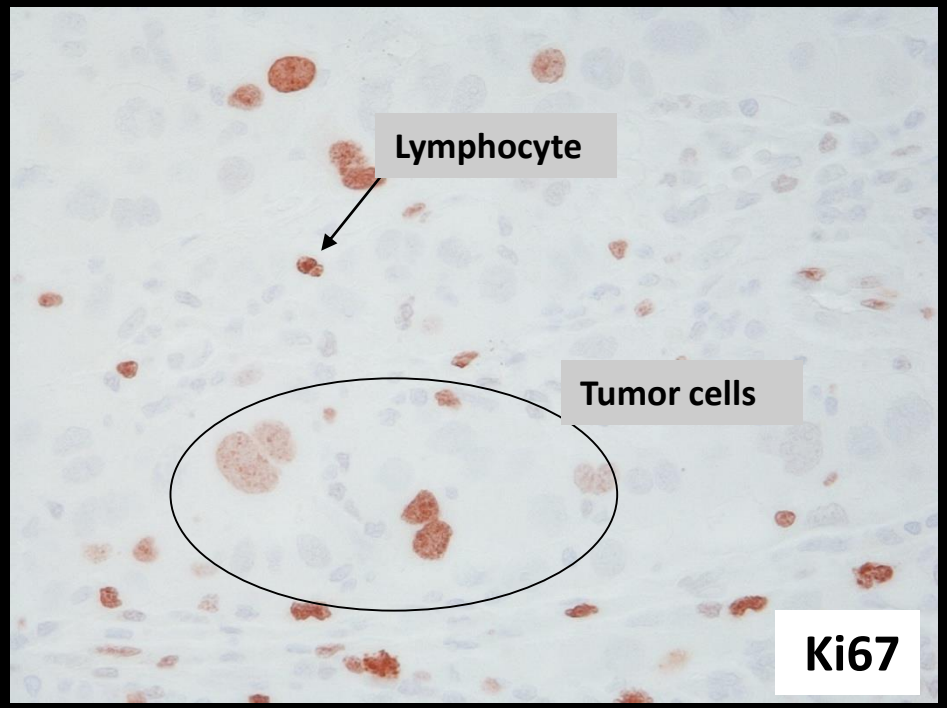
**Blocking procedure not needed**

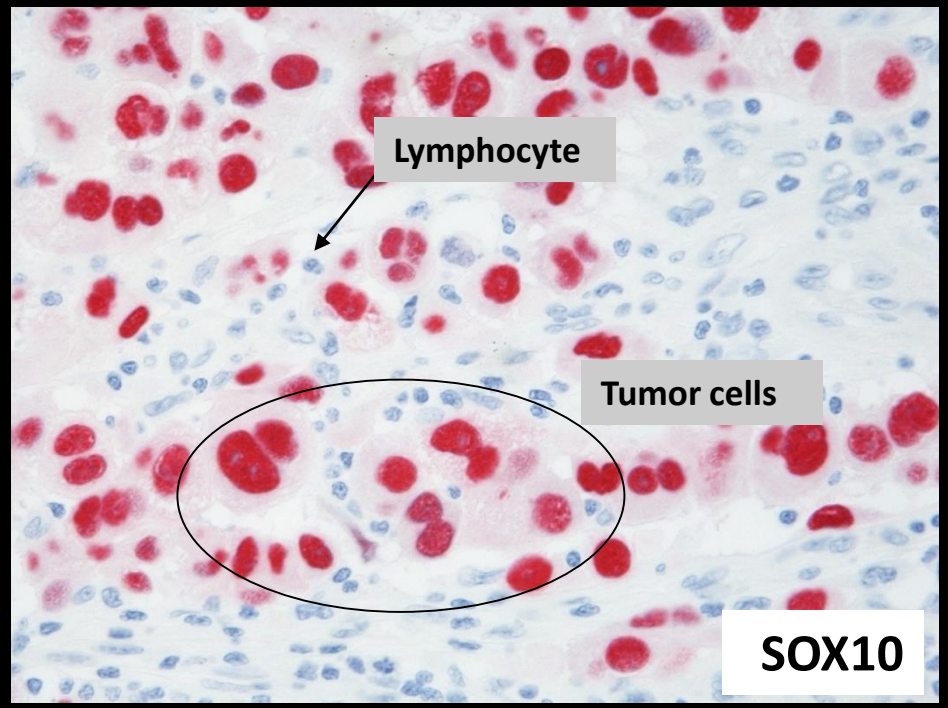
# SIMPLE-Technique (simultaneous proc.): MACH2 Double Staining 1

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

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

## Melanoma



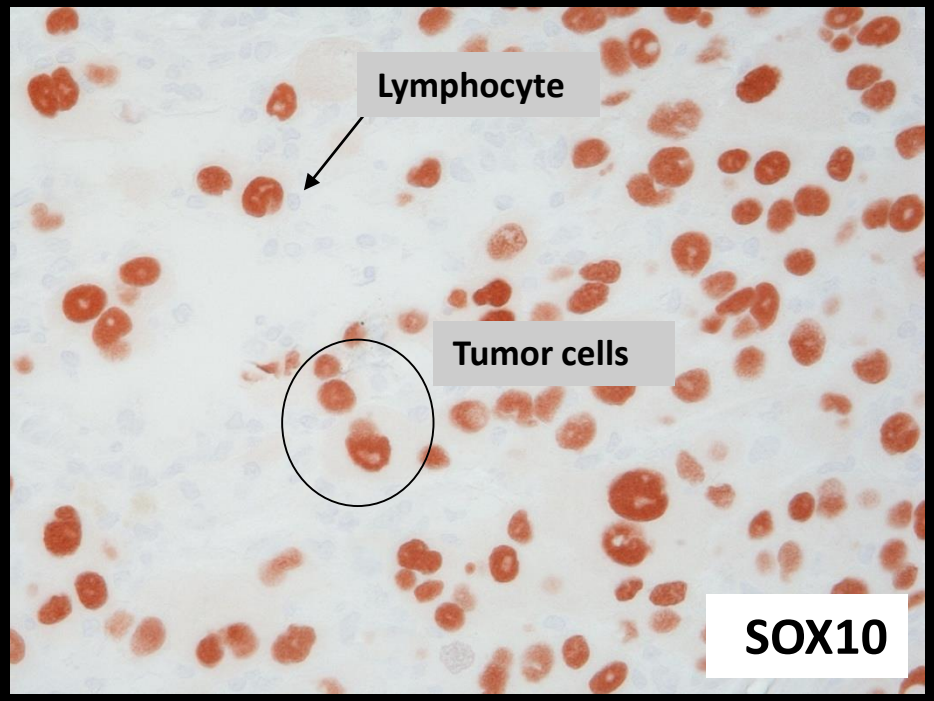

**AEC (Impact) - (1dip Hematoxylin / coverslip-Aqueous/ Imaging / de-coverslip / Erasing-Acetone) - Warp Red**

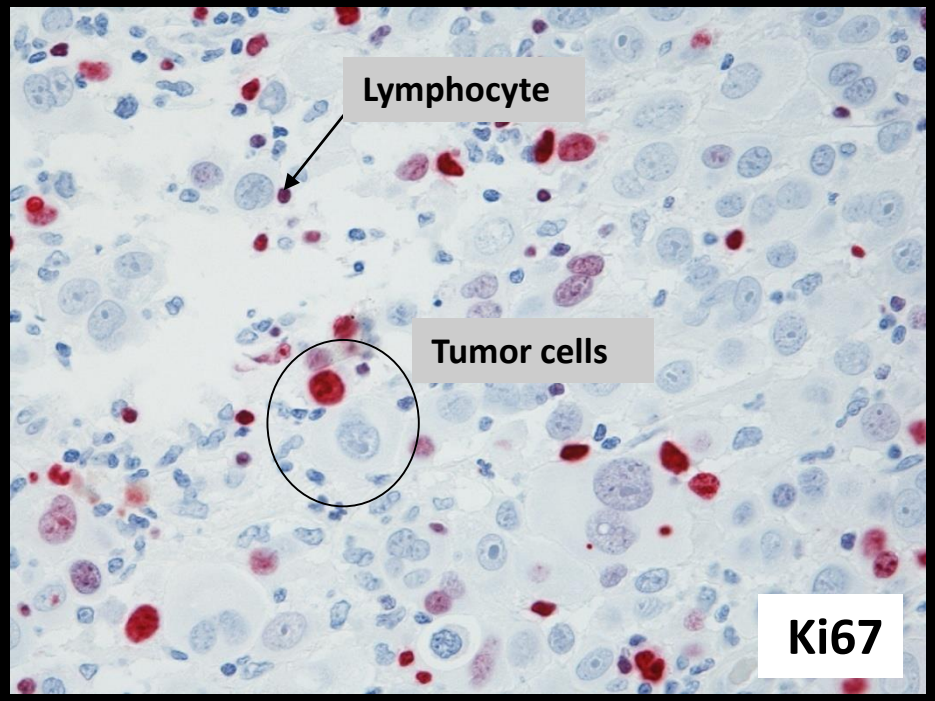
# SIMPLE-Technique (simultaneous proc.): MACH2 Double Staining 2

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

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

## Melanoma

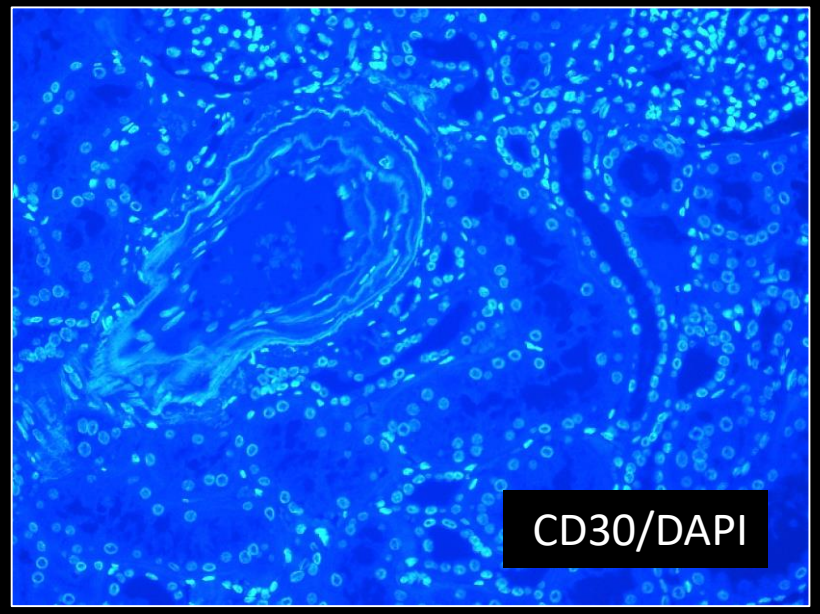


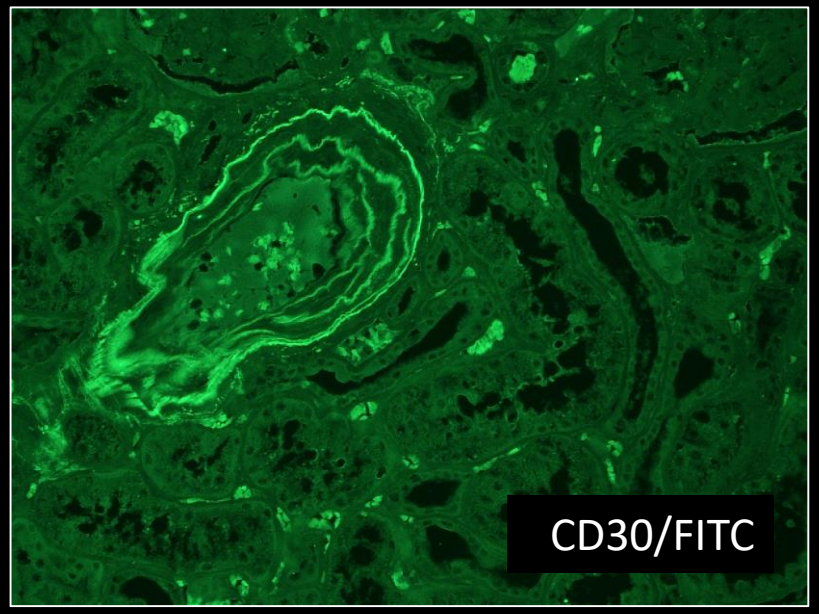




**AEC (Impact) - (1dip Hematoxylin / coverslip-Aqueous/ Imaging / de-coverslip / Erasing-Acetone) - Warp Red**

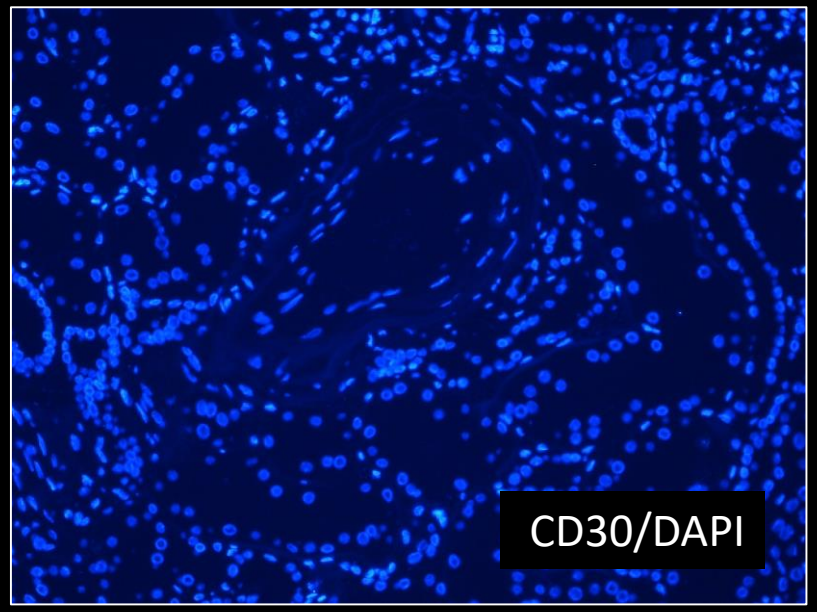
## Quenching Autofluorescence

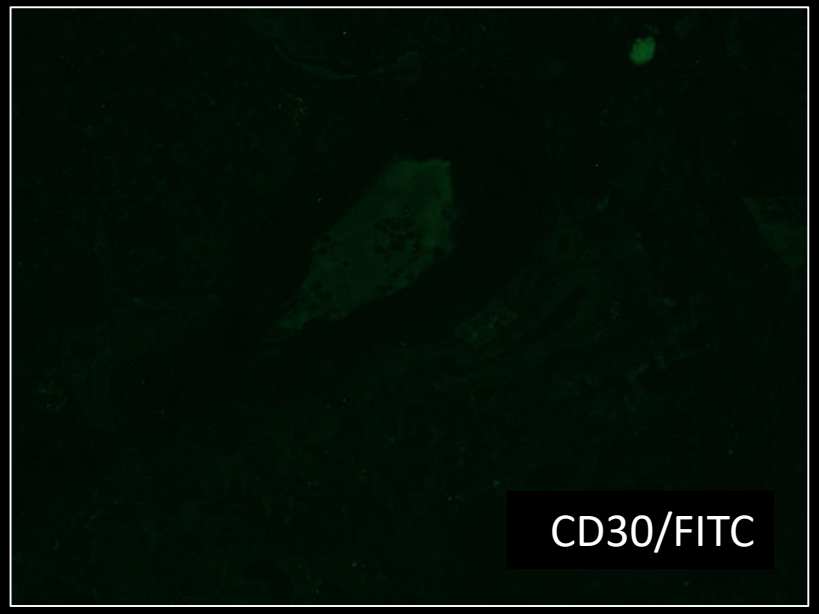
Without TrueView 5`




With TrueView 5`
(Vector Lab)

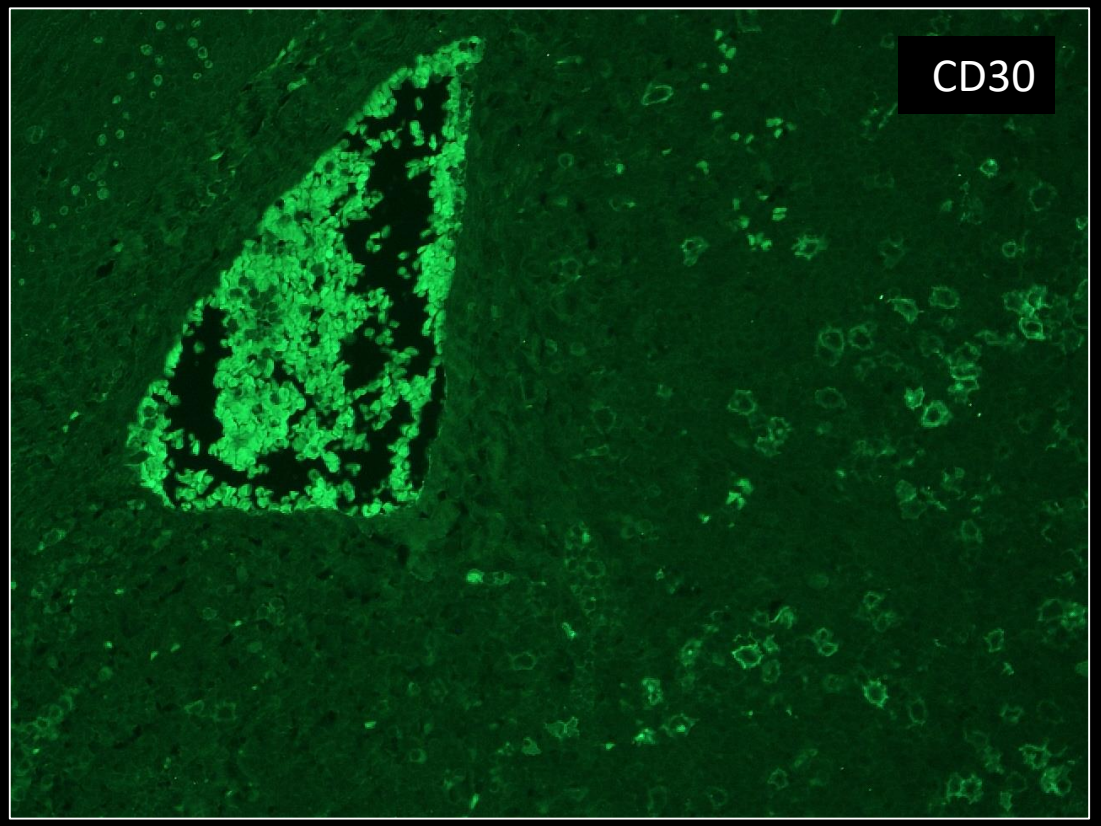


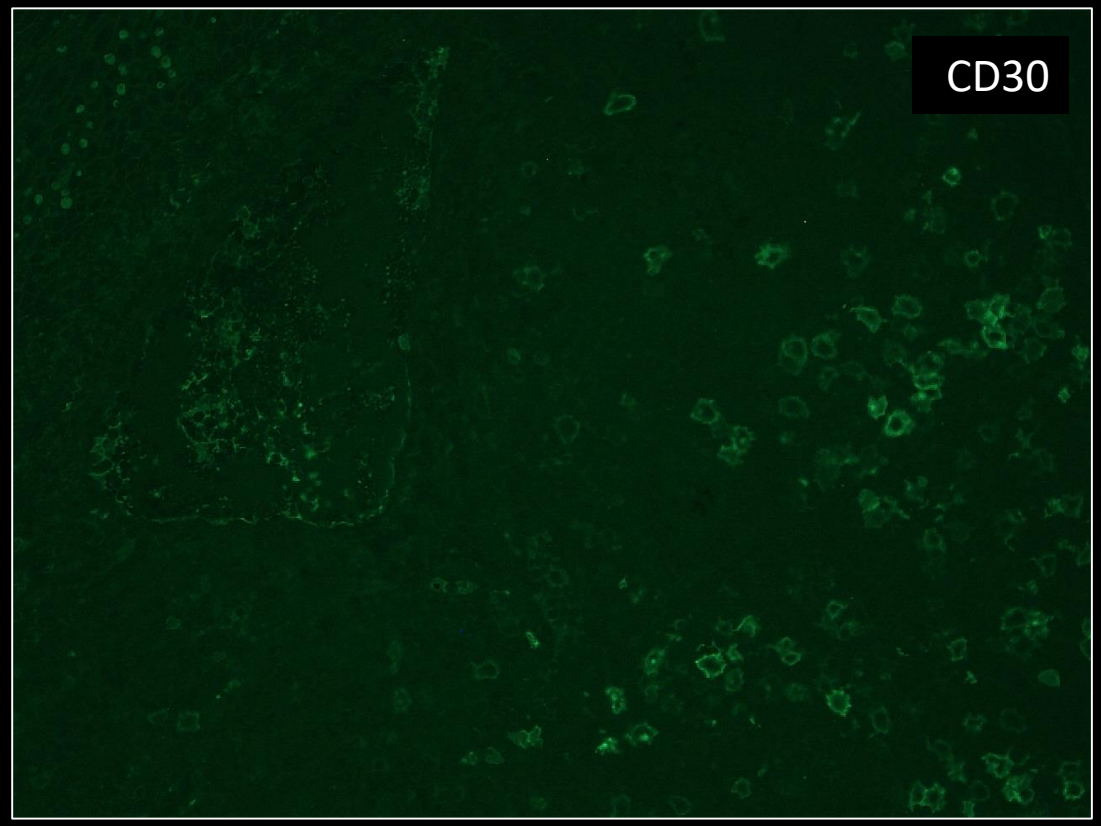

Kidney/ Microscope settings identical

# 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**

# 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 crosstalk**

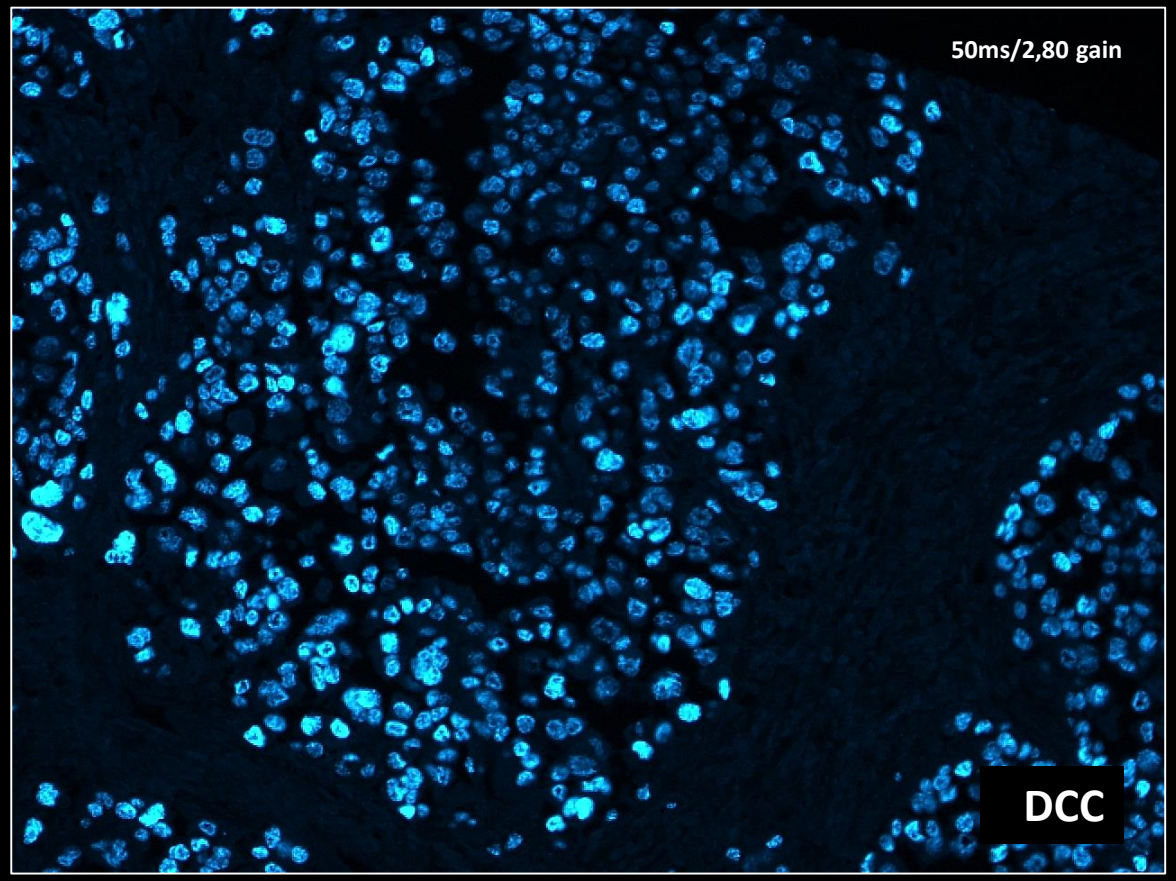
**First sequence: SOX10, BS7 → OmniMap anti-Ms - HRP → TSA/DCC (Sp. Aqua) → N (incl. N control of HRP activity)**

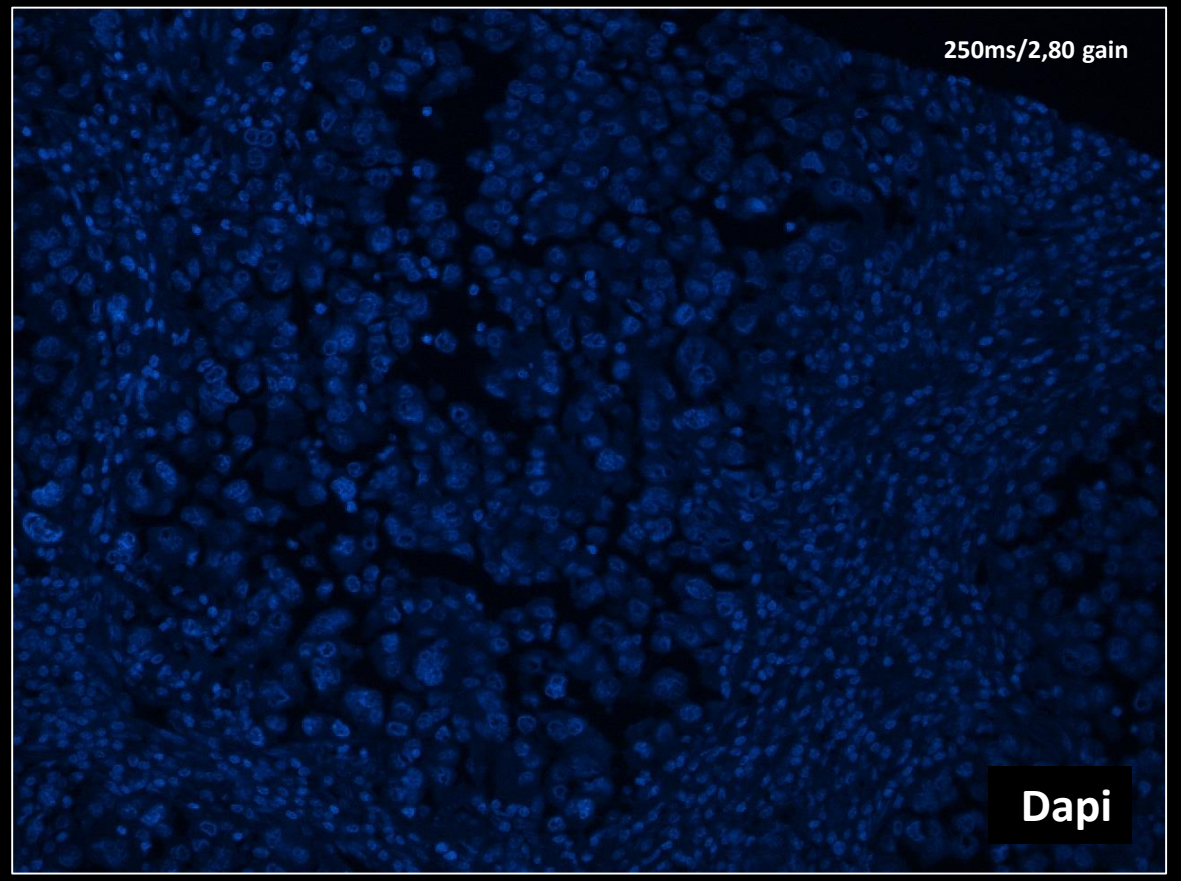
**Second sequence: LAG3, D2G40 → OmniMap anti-Rb HRP → TSA/R610 (TxR) → HD (incl. HD controls for SOX10 and LAG3)**

**Third sequence: CD4, EP204 (or Mouse Ab) → OmniMap anti-Rb/HRP (or OmniMap anti-Ms/HRP) → TSA/FAM (FITC)**

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

SOX10, BS7/OmniMap Ms-HRP/DCC

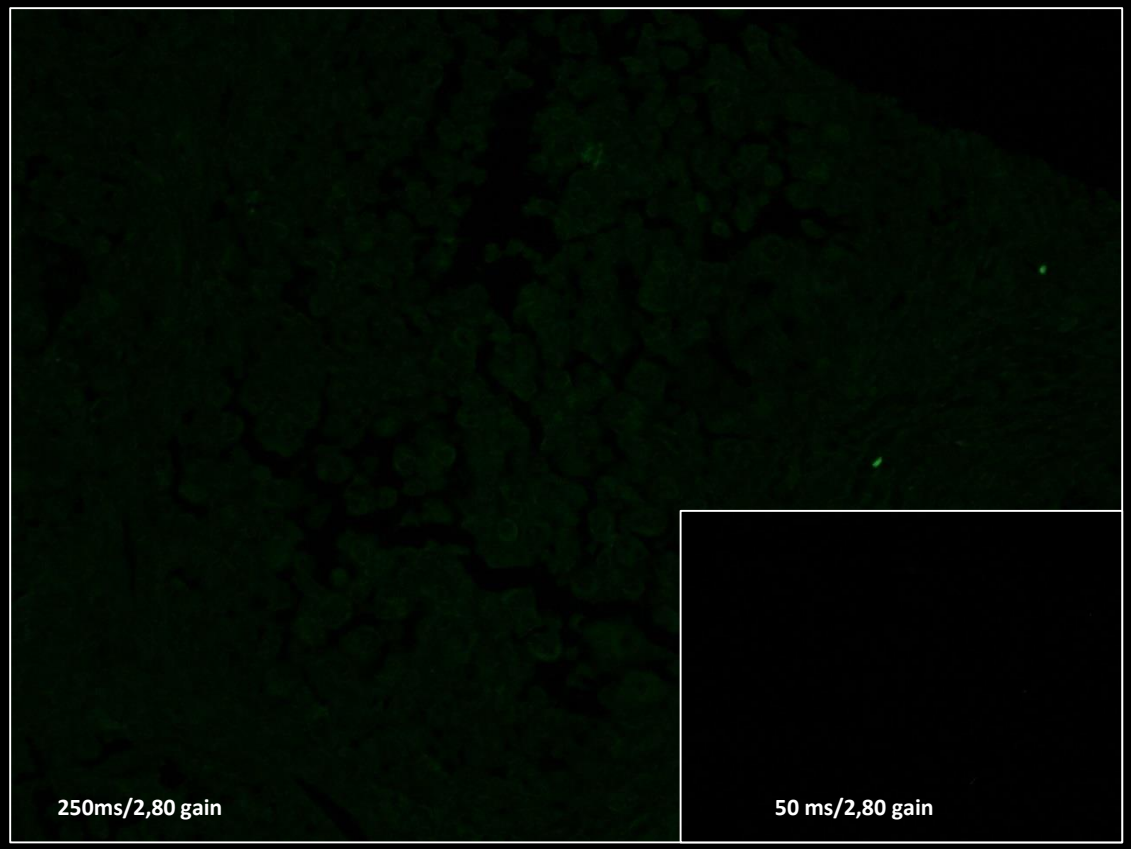


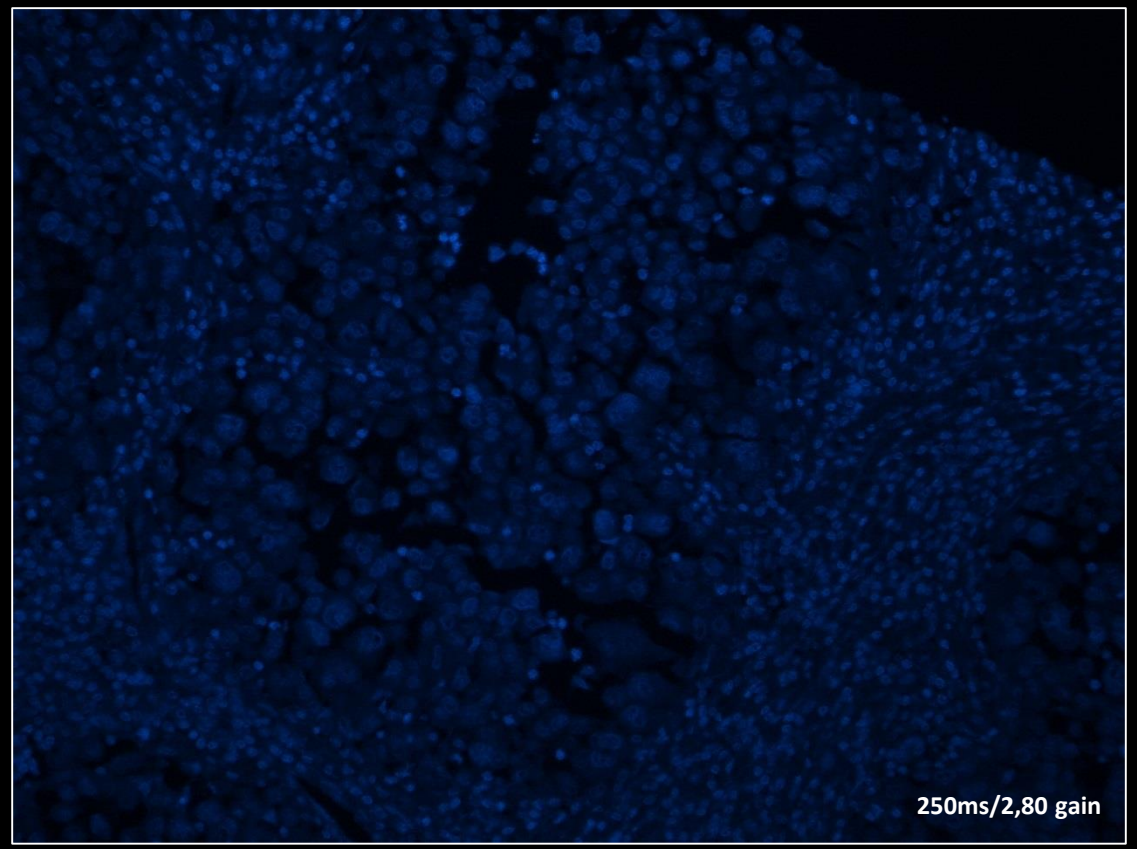




**Melanoma**

HIER: CC1 (48`/95°C)

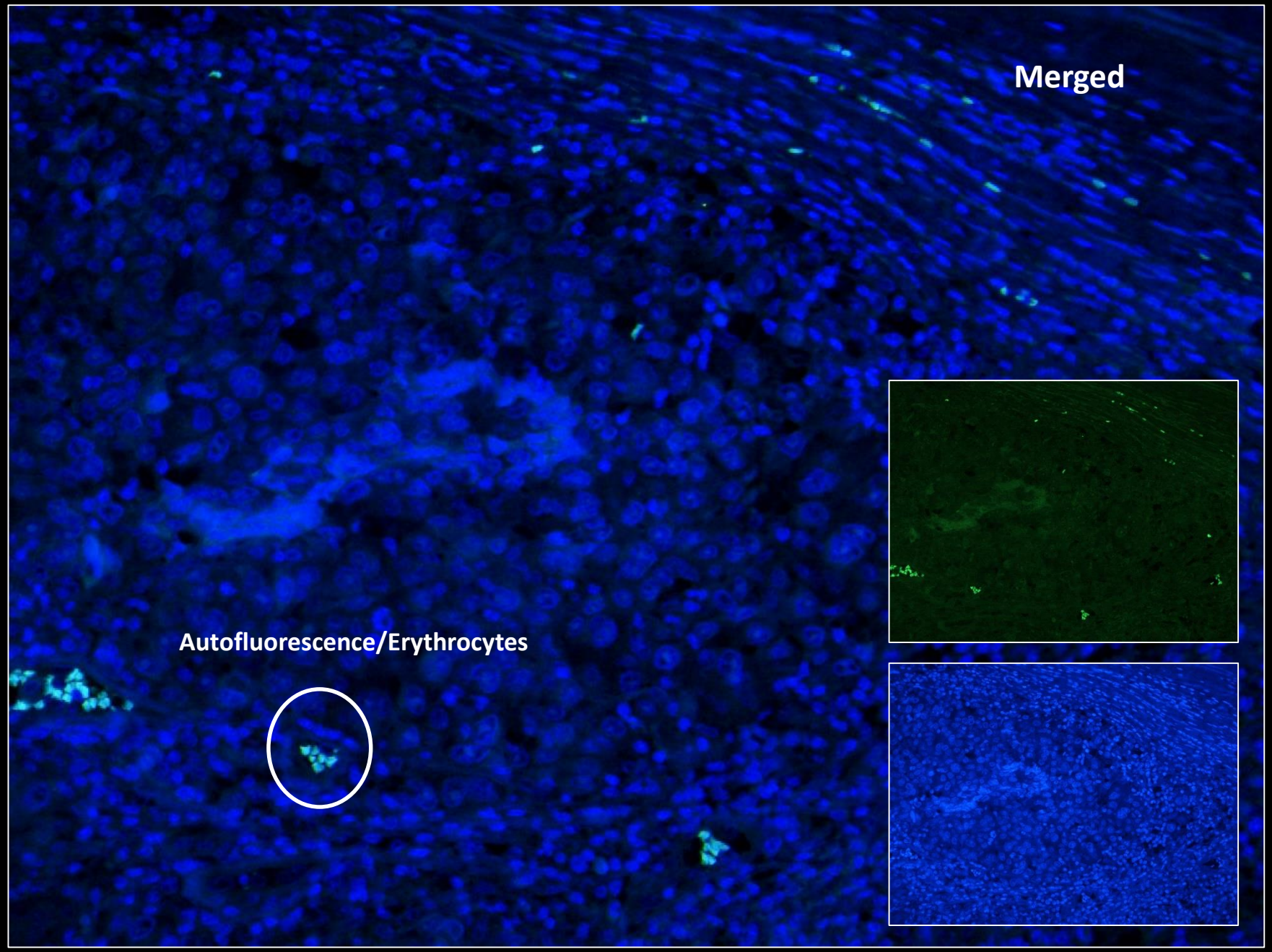
SOX10, BS7/OmniMap Ms-HRP/ Neutralization/ TSA-FAM: “Eliminate HRP activity of the detection system”




Melanoma

HIER: CC1 (48`/95°C)




## HD control (Drop out):

SOX10, BS7 (Ms)

OmniMap anti Ms/HRP

Omission of TSA-Fluorochrome

Heat Denaturation (HD)

Omission of second Ab (Diluent)

OmniMap anti Ms/HRP

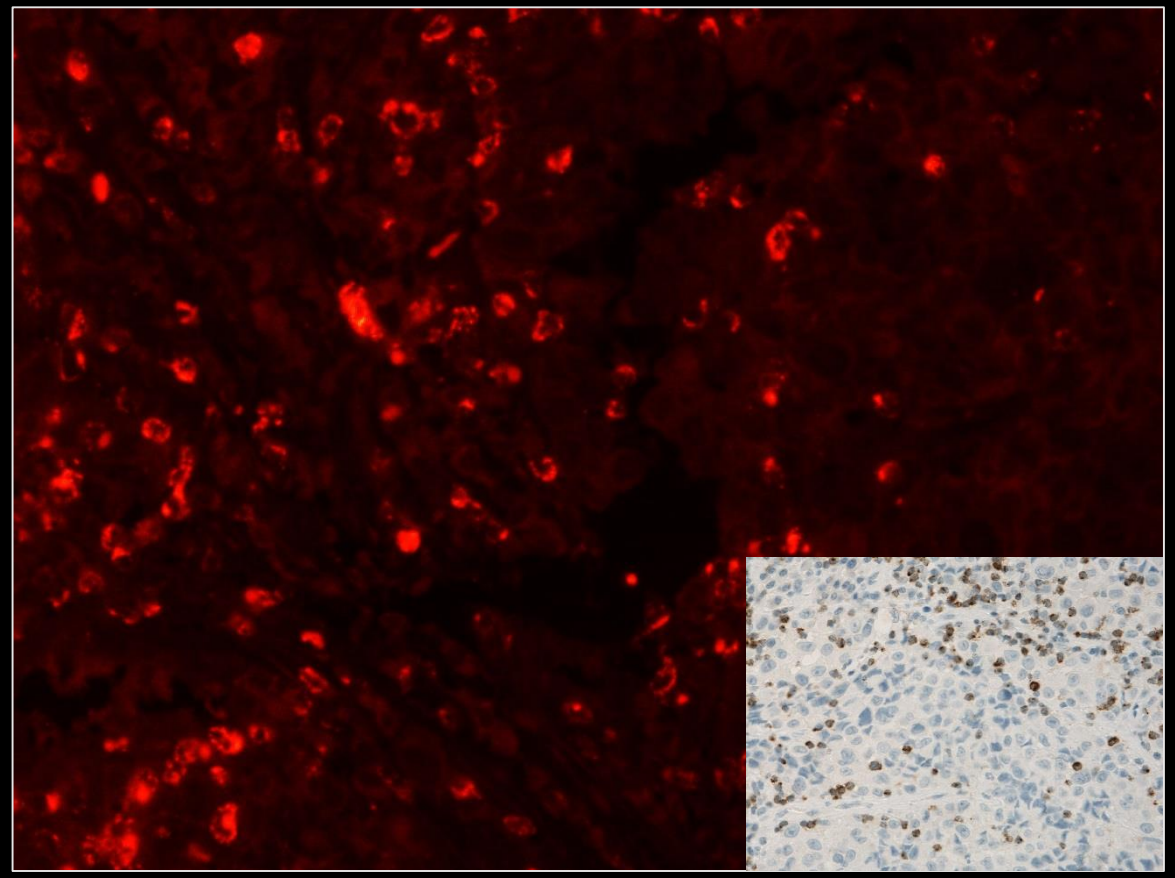
TSA-FAM

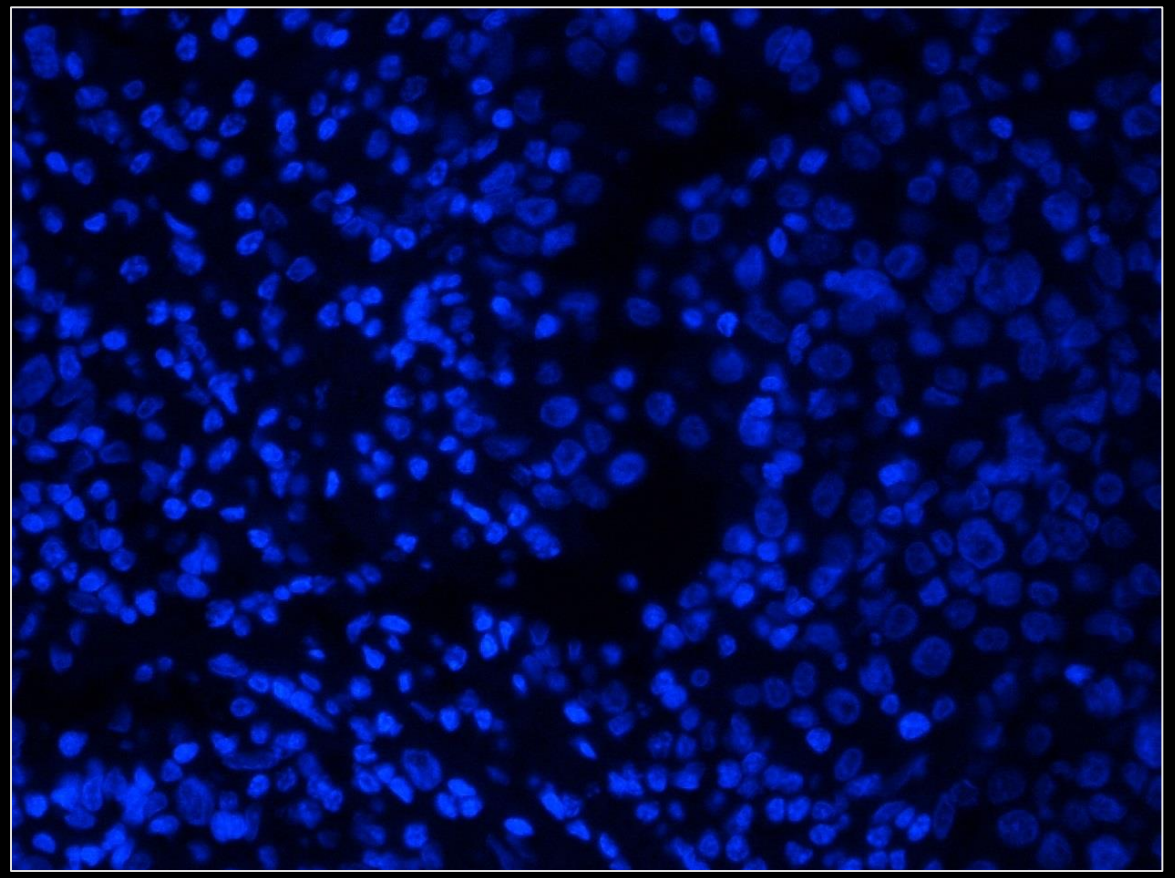
DAPI

Note: No nuclear staining reaction (FAM) of the neoplastic cells in the melanoma (cross-reactivity with SOX10)

Successful HD step: Efficient elimination of SOX10, BS7 sequence

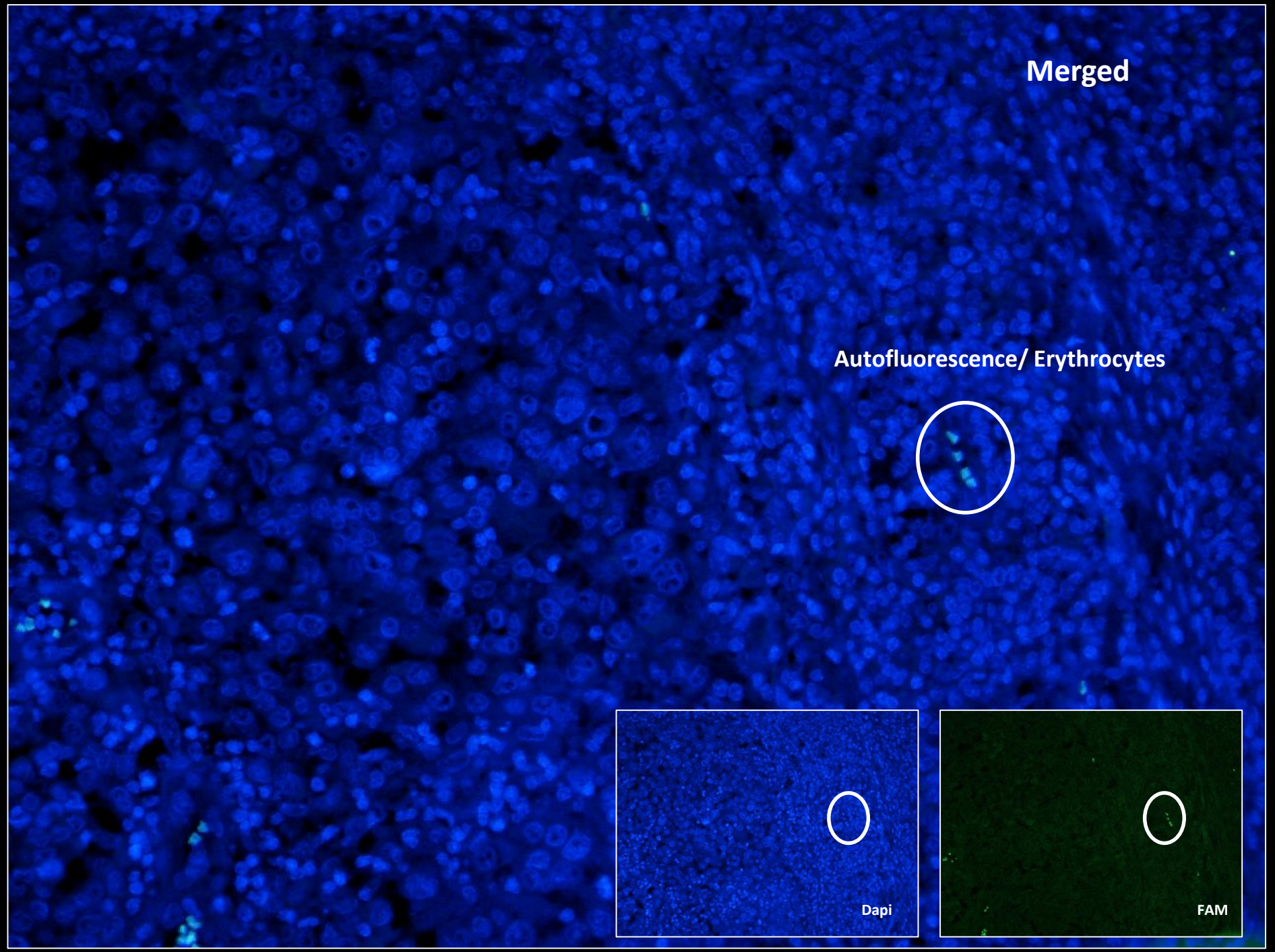
LAG3, D2G40/OmniMap Rb-HRP/Red 610





Melanoma

HIER: CC1 (48`/95°C)




## 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