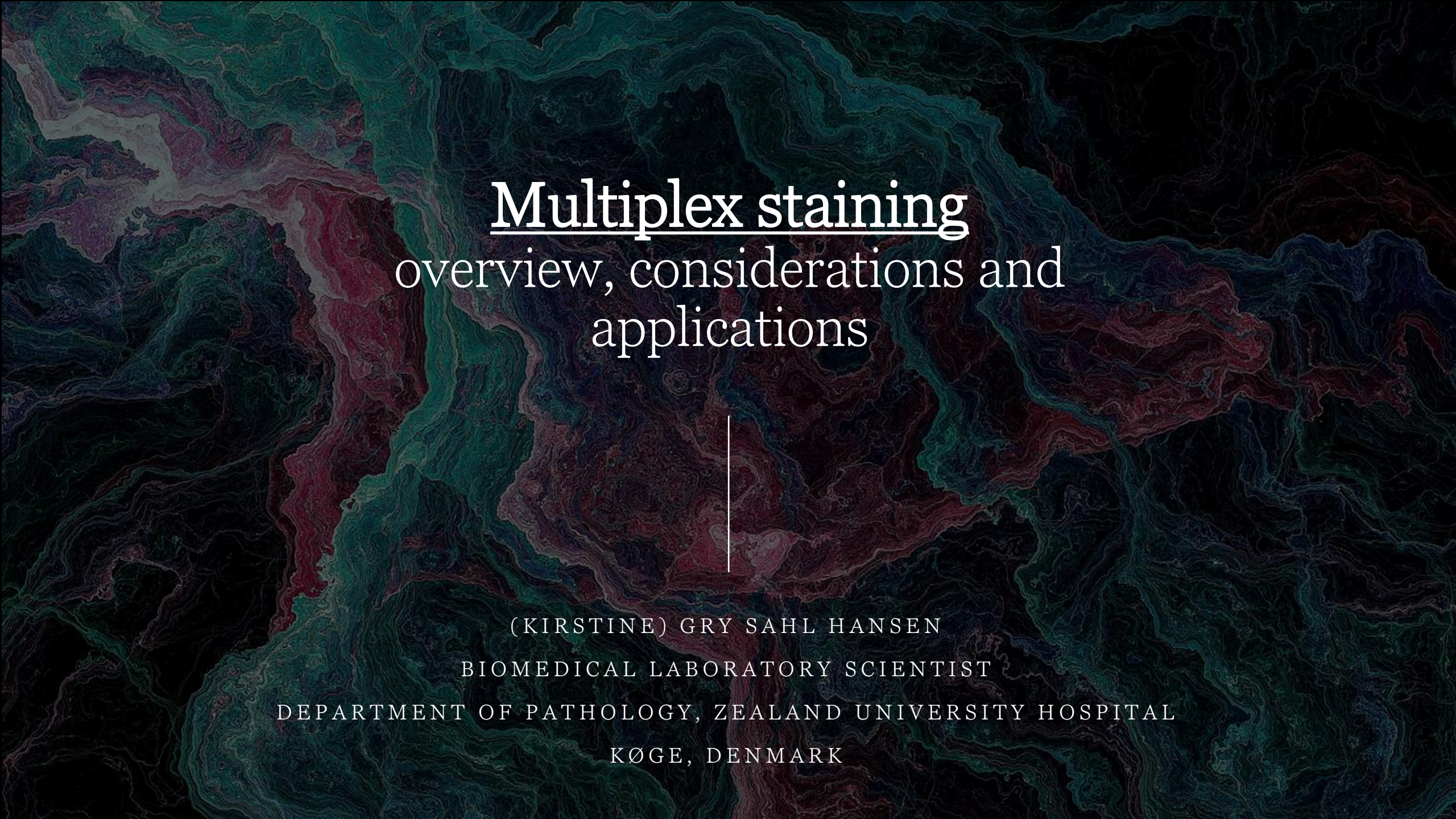




Multiplex staining overview, considerations and applications

(KIRSTINE) GRY SAHL HANSEN

BIOMEDICAL LABORATORY SCIENTIST

DEPARTMENT OF PATHOLOGY, ZEALAND UNIVERSITY HOSPITAL

KØGE, DENMARK

Agenda

- **What is multiplexing?**

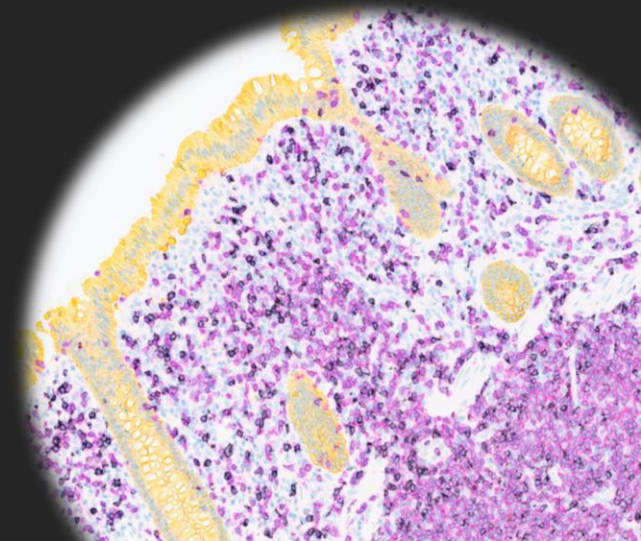
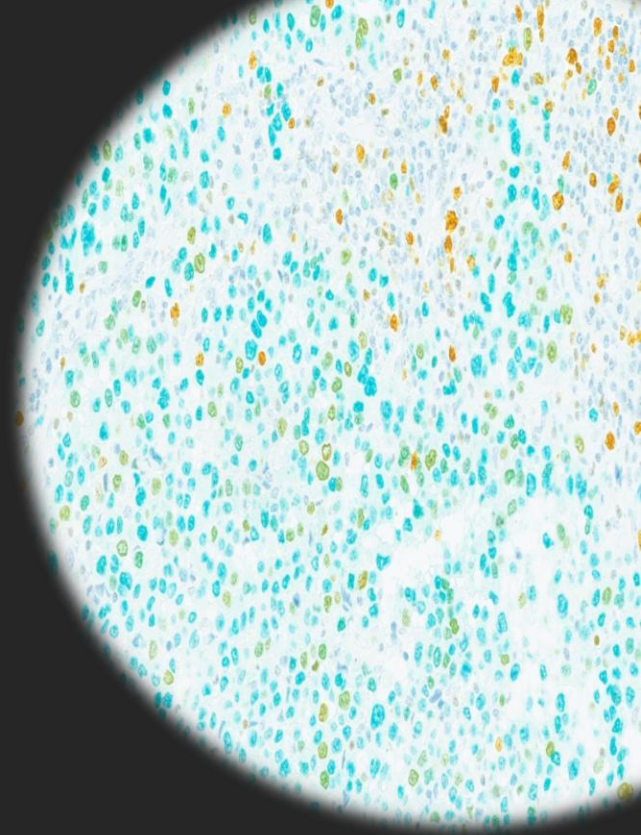
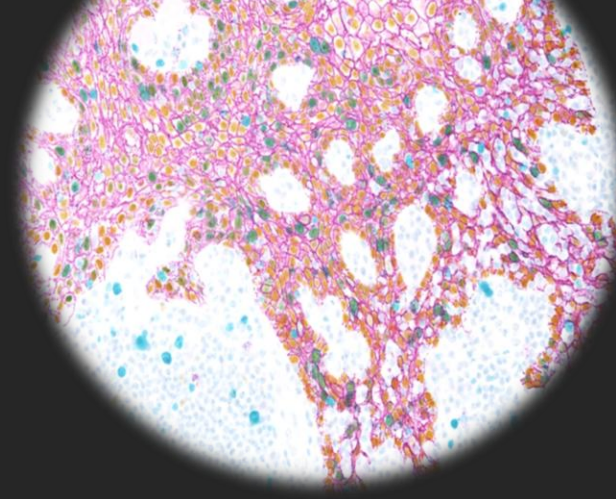
Multiplexing then and now, fluorescence vs. chromogens, translucent chromogens

- **Multiplex staining considerations**

Order of Antibodies, difficult to elute, cross-reactions, color combinations

- **Multiplexing in the future**

From multiplex staining to spatial multiomics

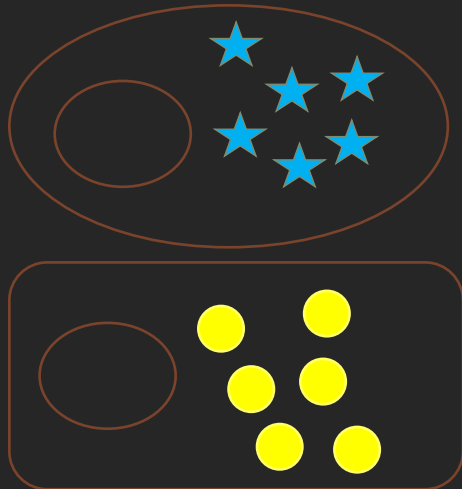


What is multiplexing

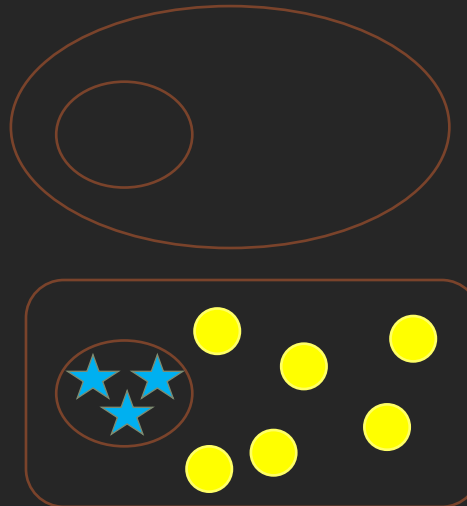
The main advantage of multiplex IHC is:

- Visualization of multiple targets within a single tissue section
- Investigate spatial relationships between multiple cells (e.g. understanding the complexity of tumor microenvironment)

Expression in different
cell types



Co - expression

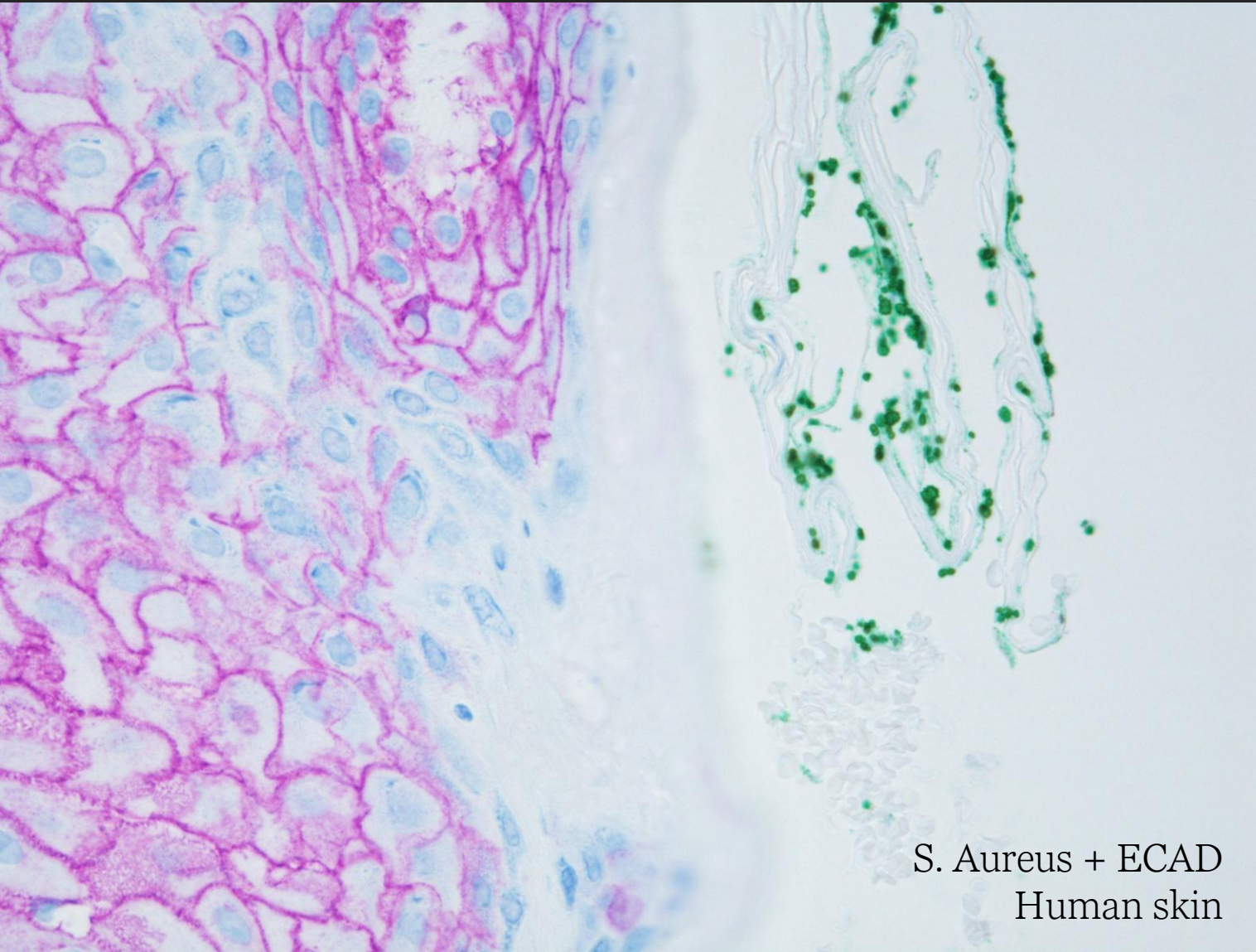


Co - localization
(mixed color)



What is multiplexing

Expression in different cell-types



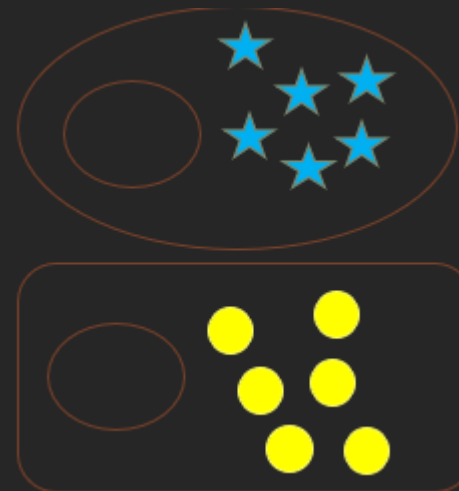
S. Aureus + ECAD
Human skin

S. Aureus, abcam (Green):

Small coccoid bacteria, often arranged in clusters. Granular or punctate staining corresponding to bacterial colonies

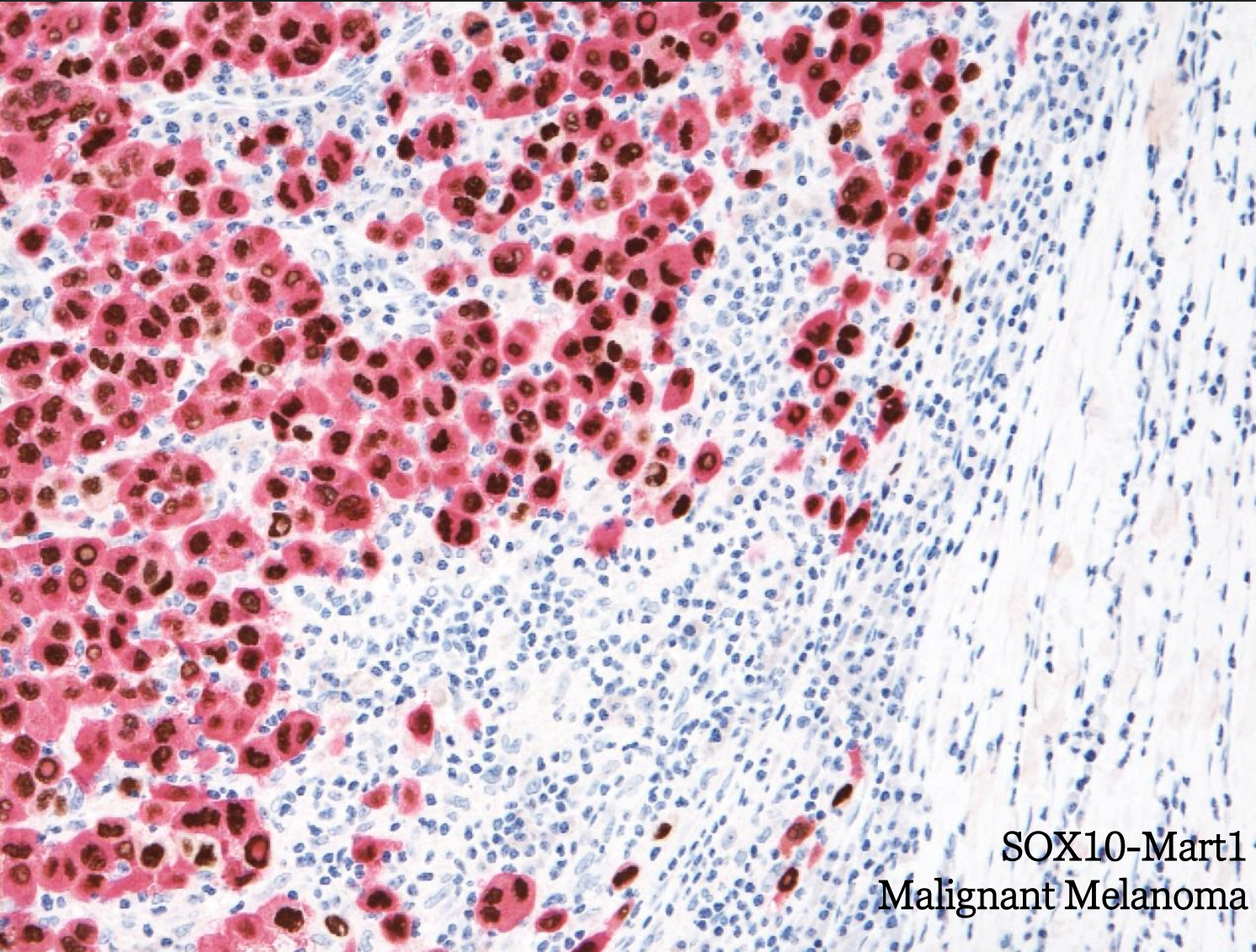
E-Cadherin, Roche (Purple)

Keratinocytes in the epidermis should show clear, distinct membranous staining along cell-to-cell borders. This often produces a characteristic “chicken-wire” or honeycomb-like pattern.



What is multiplexing

Co-expression



SOX10-Mart1
Malignant Melanoma

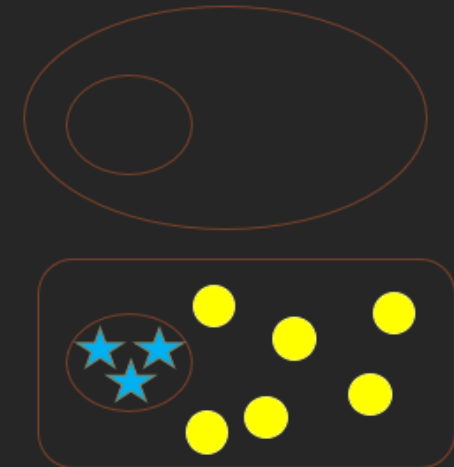
SOX10(DAB):

A moderate to strong, nuclear staining reaction of virtually all melanocytes in the neoplastic cells of the malignant melanoma.

Mart1(Magenta)

A moderate to strong, distinct cytoplasmic staining reaction of the vast majority of neoplastic cells in the malignant melanoma.

Co-expression



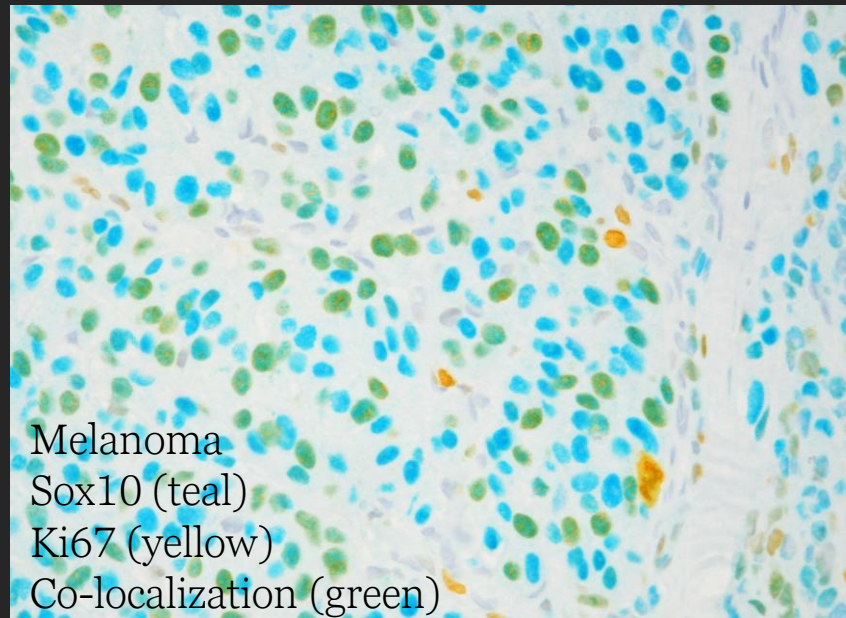
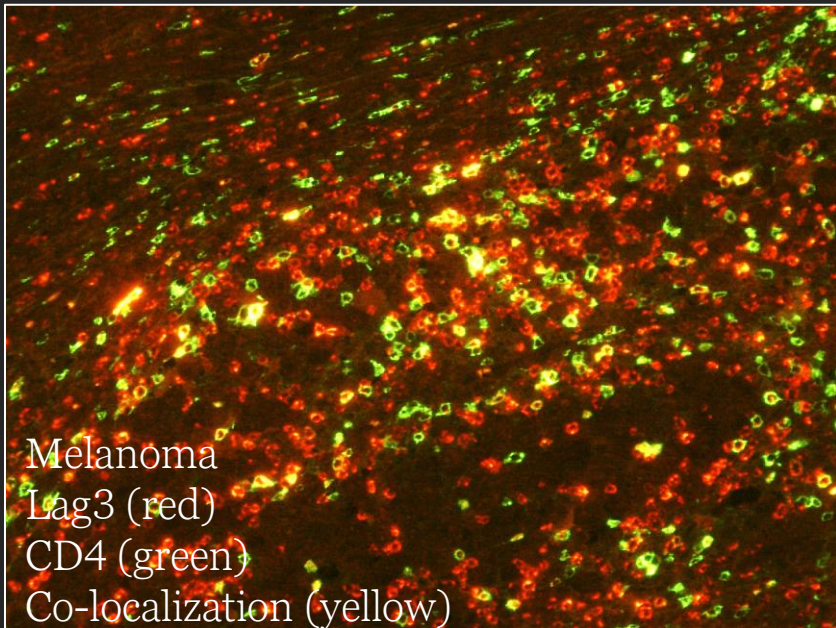
What is multiplexing

Co-localization (mixed color)

This technique is difficult using traditional chromogens

Immunofluorescence used to be the most used method for detection of co-localization (the golden standard)

Ventana Translucent Chromogens allow a color-shift when both colors are present in the same cell and subcellular compartment (nucleus, cytoplasm or cell membrane)



Multiplexing then and now...

Fluorescence is considered the gold standard for multiplexing and generally makes it easier to combine multiple markers.

However, fluorescence has several limitations, including the need for a dark room, more expensive equipment and specialized cameras, as well as signal fading over time.

Chromogenic multiplexing may therefore be a valuable alternative to explore, especially because it allows easier assessment of tissue morphology using conventional bright-field microscopy.

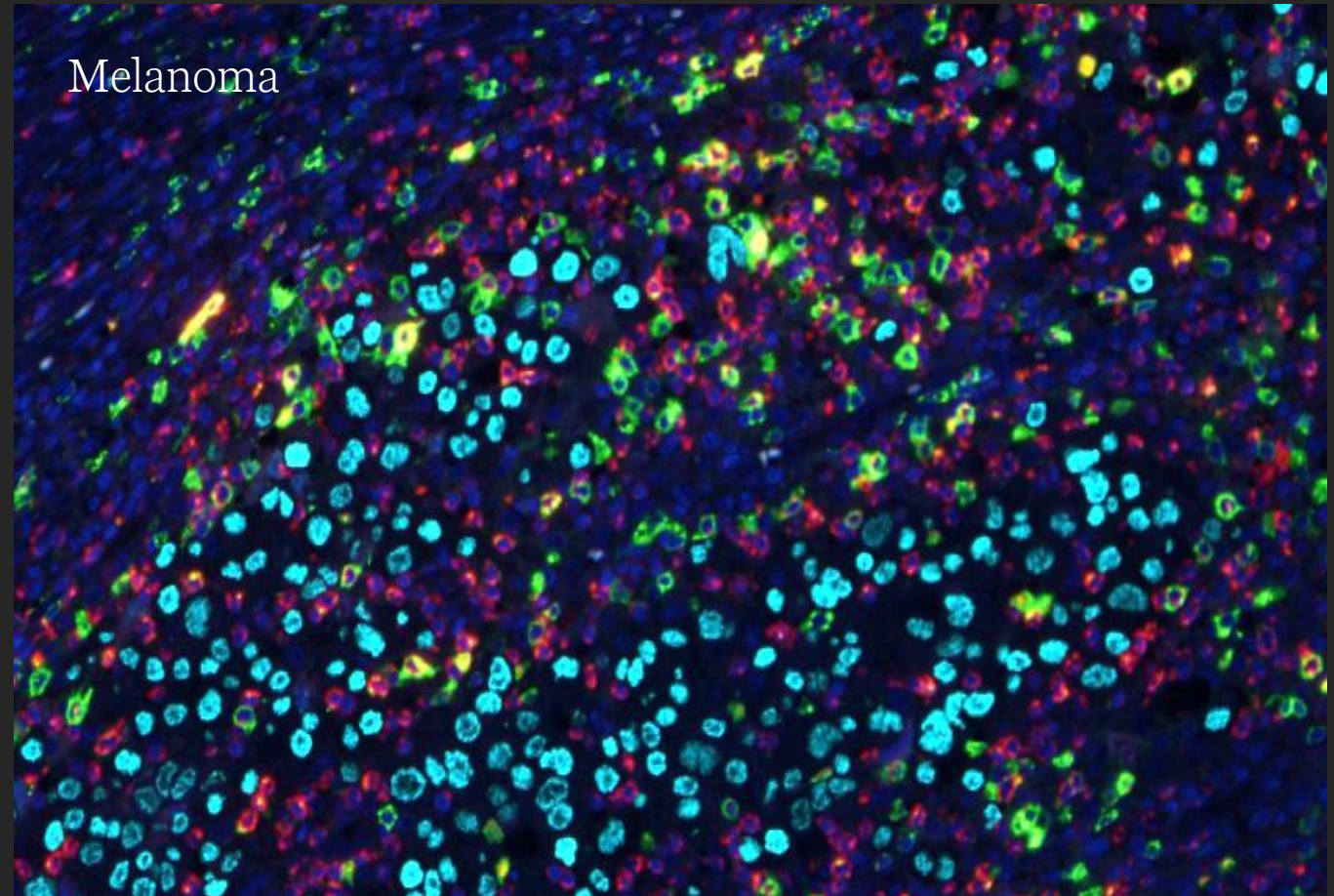
Is it possible to do chromogenic-multiplexing..?
YES!

SOX10, BS7, omnimap@Ms, DCC
-Neu-

LAG3, D2G40, omnimap@Rb, RED
-HD-

CD4, EP204, omnimap@Rb, FAM
DAPI

Co-localization RED and FAM = Yellow



Multiplexing with DAB

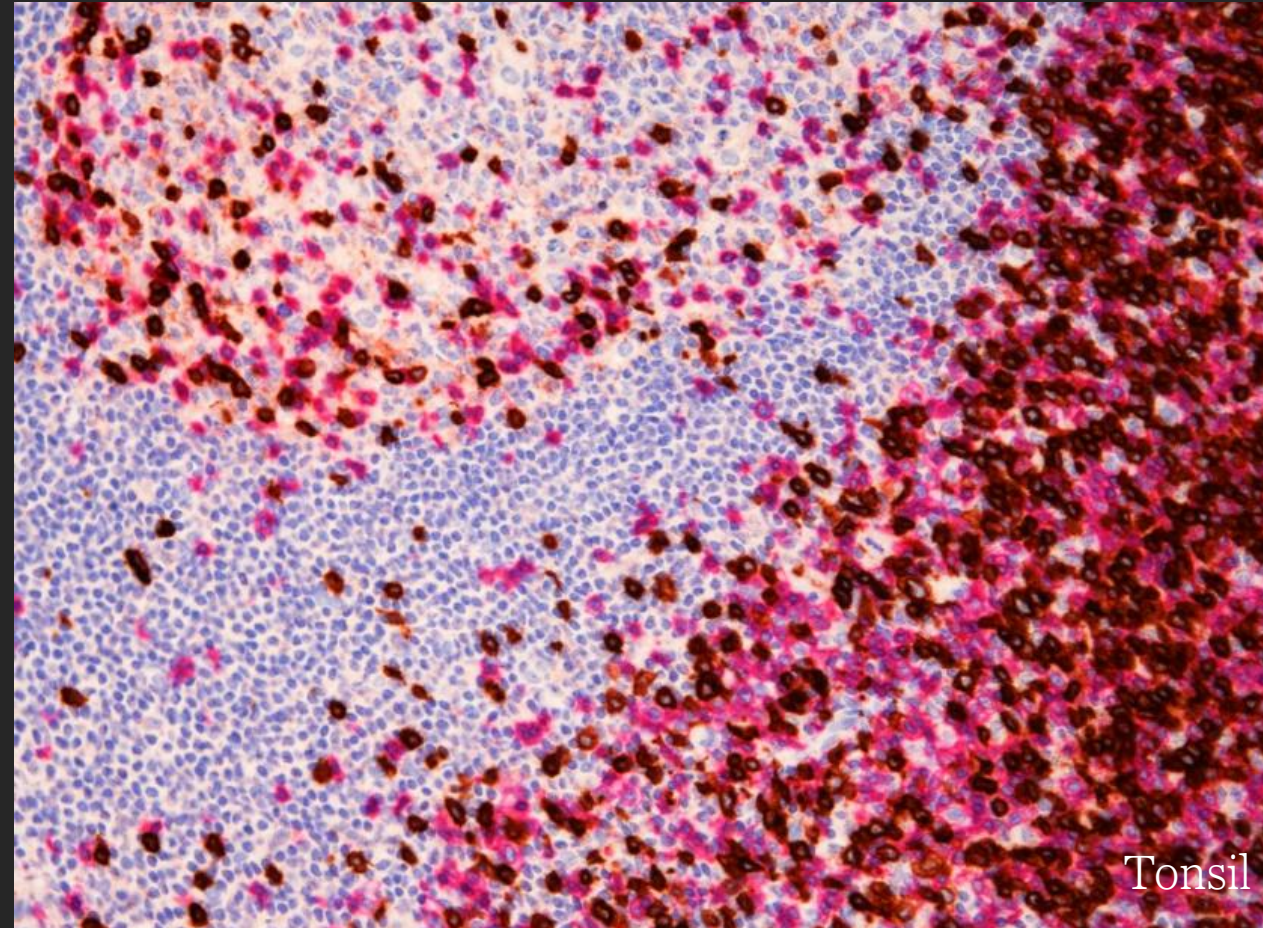
When multiplexing with DAB, cross-reactivity is prevented by the **DAB shielding effect**. The DAB precipitate physically covers the first antibody complex, preventing (cross) reactions between the sequences.

T cells express the T-cell receptor beta protein (TCRB), which includes two constant regions, C1 and C2; each T cell expresses only one.

In healthy individuals, C1 and C2 are equally represented, with roughly 50% expression of each. (polyclonal population)
Malignancy is associated with overrepresentation of either C1 or C2 (monoclonal population)

TCRBC1 (T-Cell Receptor Beta Constant region 1), E6Z3S (DAB)
+

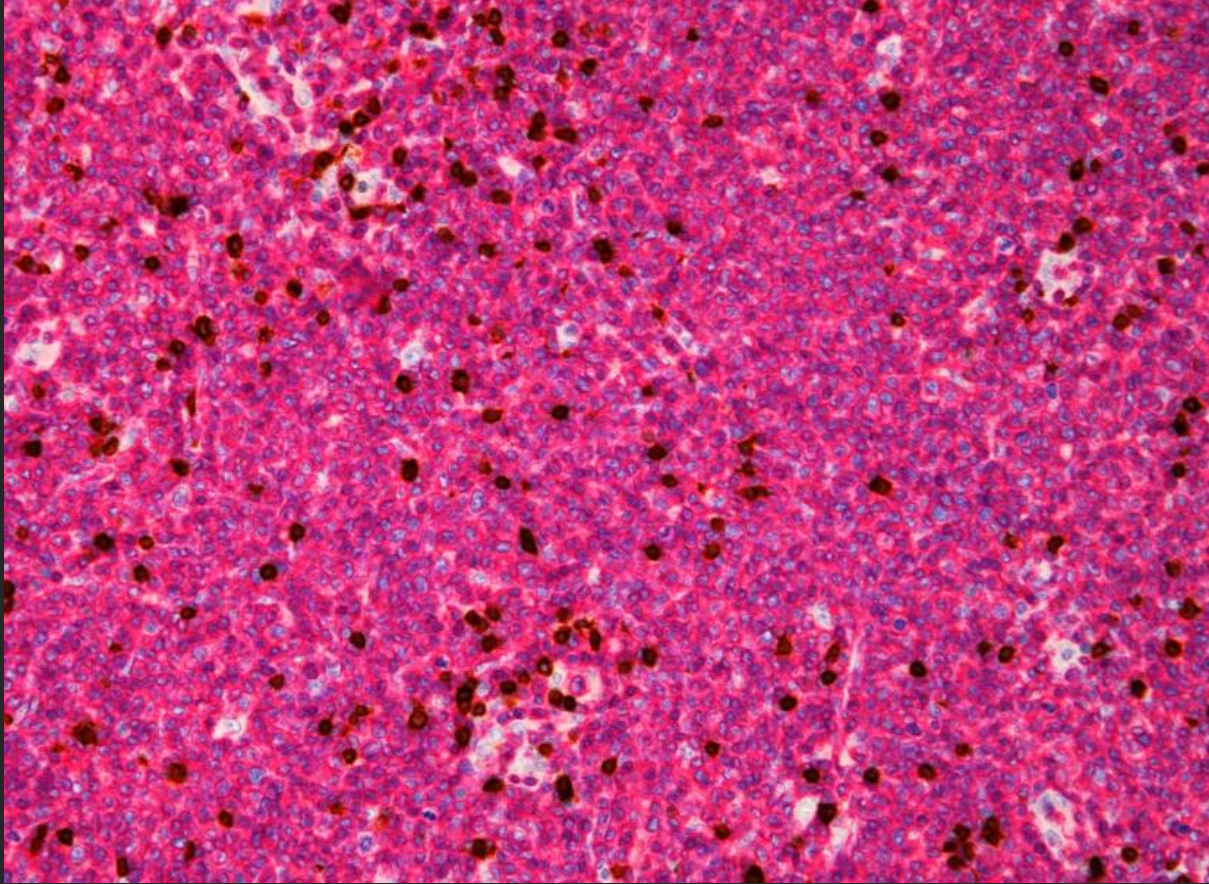
CD3, MRQ39 (magenta) Omnis-platform



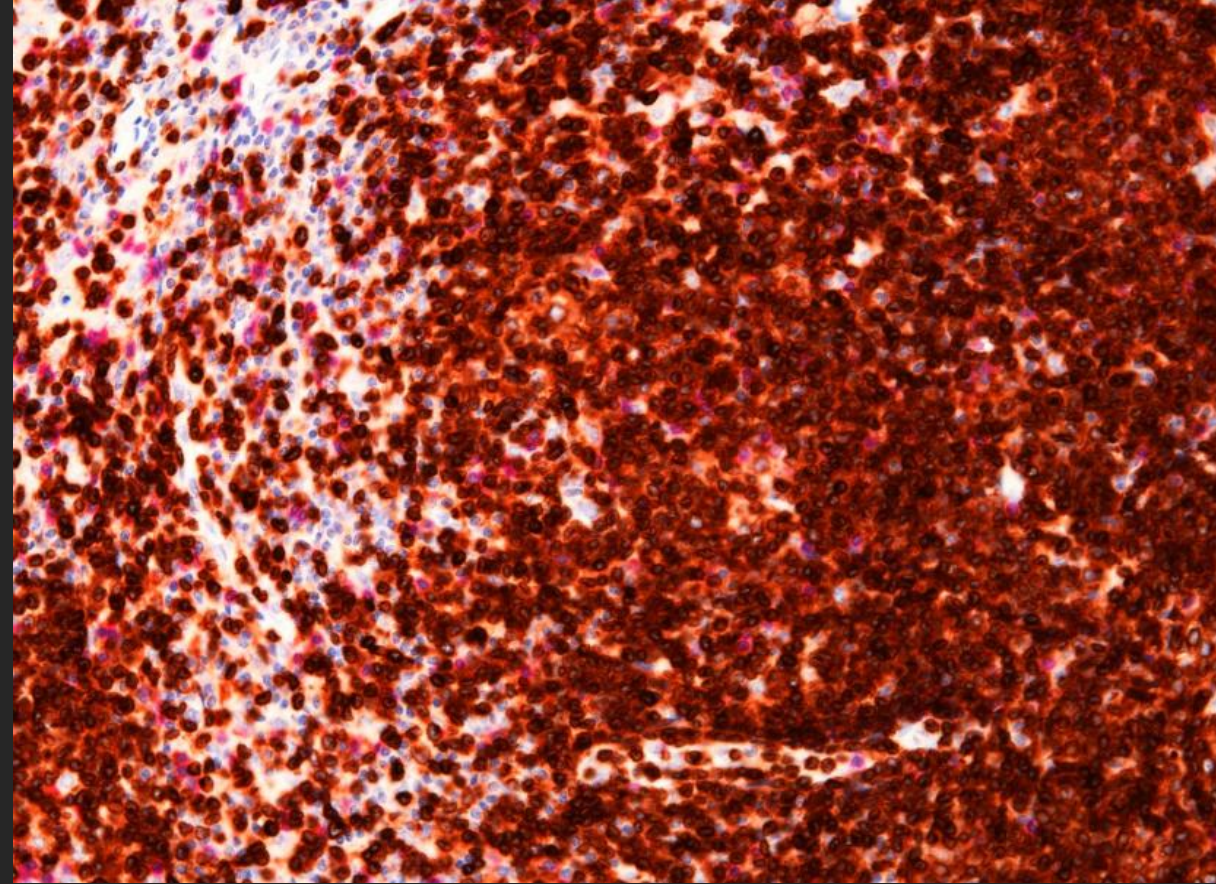
Tonsil

Multiplexing with DAB

*TCRBC1 (T-Cell Receptor Beta Constant region 1), E6Z3S (DAB) +
CD3, MRQ39 (magenta)*



AITL*, TCRBC1-
Clonal expression pattern, associated with malignancy



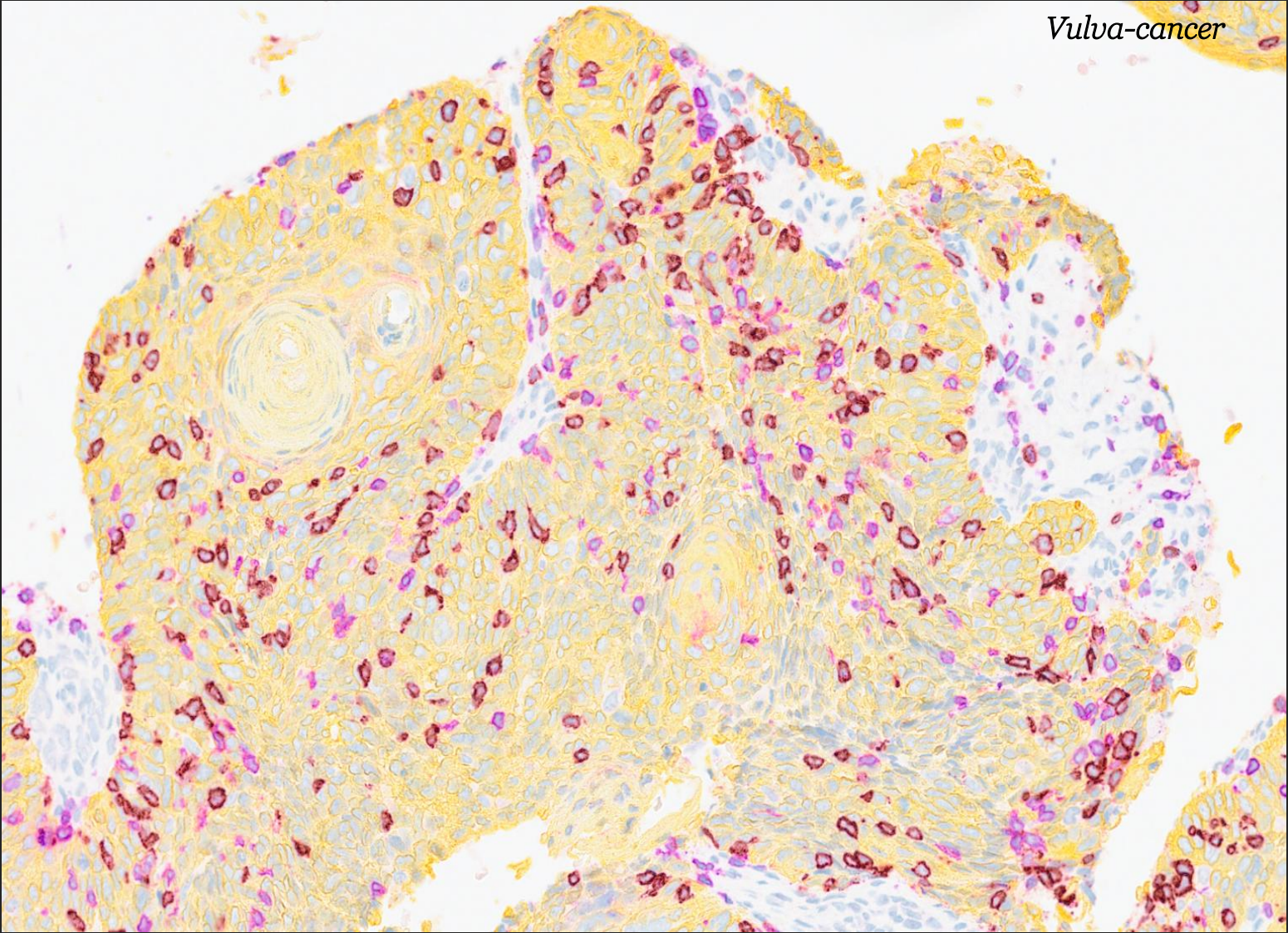
AITL*, TCRBC1+
Clonal expression pattern, associated with malignancy

*Angioimmunoblastic T-cell lymphoma

Multiplexing with DAB

CD8-positive T-cells are also CD3-positive, but the CD3 signal is shielded by DAB

STEPS	3Plex
Dewax	70°C 3x8 min
PTT	CC1, 95°C, 48 min
Inhibitor	Inhibitor 8 min
Ab1	CD8 (C8/144B) 1:100RR 37°C, 32 min
Detection 1	Omnimap anti Ms-HRP, 36°C 12 min
Chromogen 1	DAB, 36°C 8 min
Denaturation	No
Ab2	CD3 (Poly) 1:25DD 37°C, 32 min
Detection 2	Omnimap anti Rb-HRP, 36°C 12 min
Chromogen 2	Purple, 36°C, 32 min
Denaturation	Inhibitor 36°C 20 min
Ab3	CKPan 1:200DD 37°C,32 min
Detection 3	Omnimap anti Ms-HRP 36°C 12 min
Chromogen 3	Yellow HRP 36°C 32+16 min
Counterstain	Hematoxylin II 4 min/Bluing 4 min

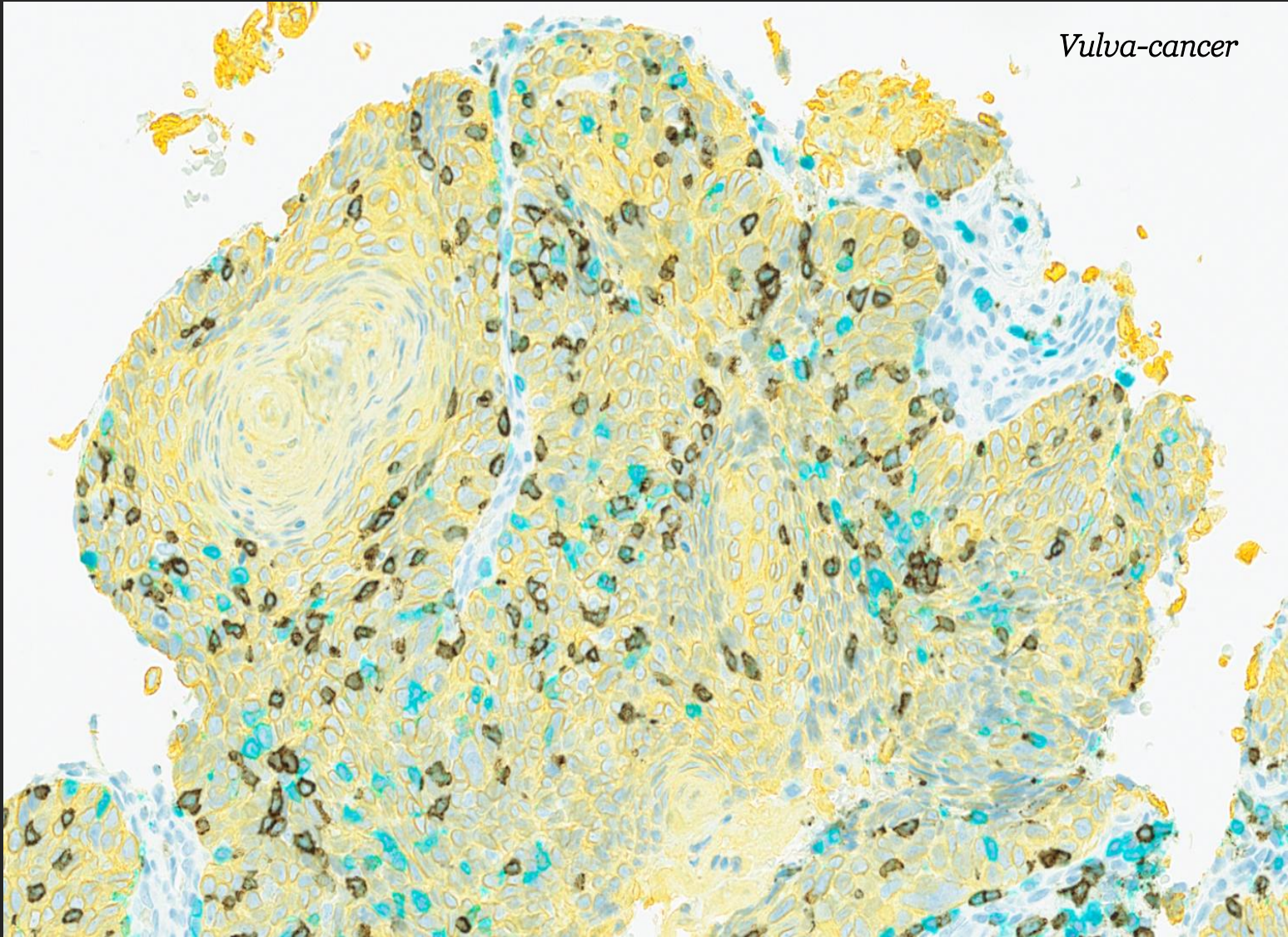


Research purpose

Multiplexing with DAB

CD8-positive T-cells are also CD3-positive, but the CD3 signal is shielded by DAB

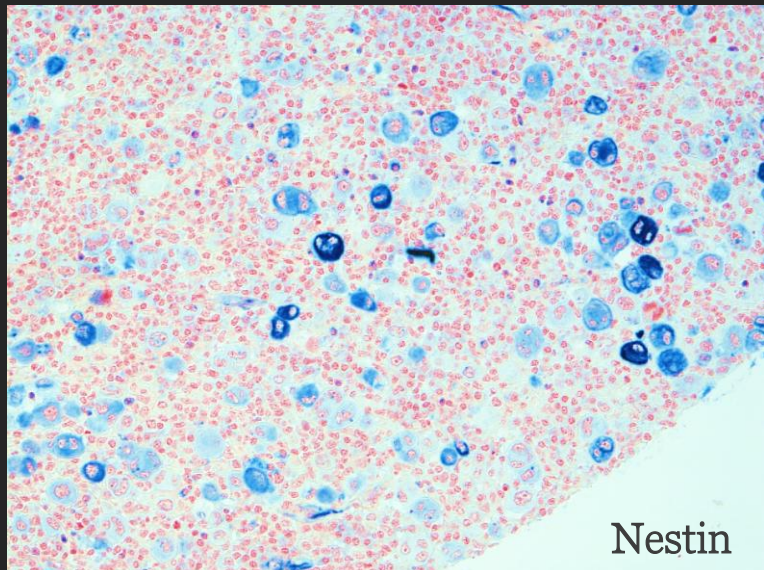
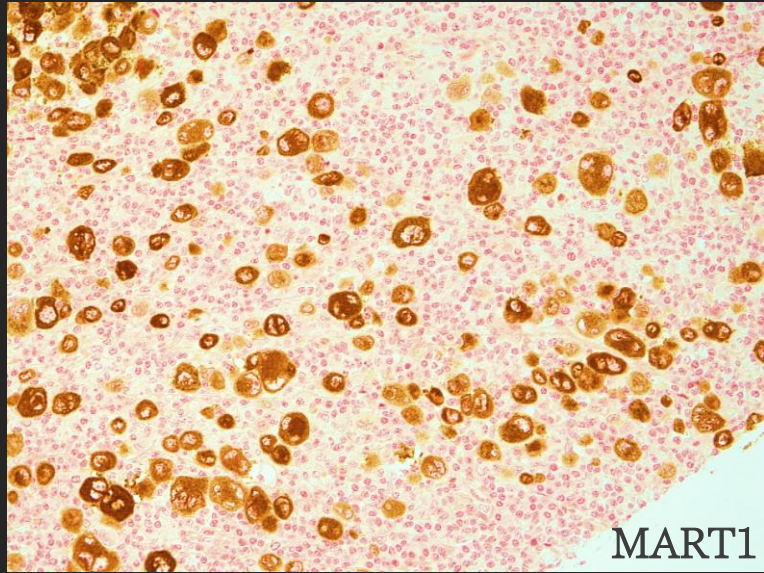
STEPS	3Plex
Dewax	70°C 3x8 min
PTT	CC1, 95°C, 48 min
Inhibitor	Inhibitor 8 min
Ab1	CD8 (C8/144B) 1:100RR 37°C, 32 min
Detection 1	Omnimap anti Ms-HRP, 36°C 12 min
Chromogen 1	DAB, 36°C 8 min
Denaturation	No
Ab2	CD3 (Poly) 1:25DD 37°C, 32 min
Detection 2	Omnimap anti Rb-HRP, 36°C 12 min
Chromogen 2	Teal, 36°C 32+16 min
Denaturation	Inhibitor 36°C 20 min
Ab3	CKPan 1:200DD 37°C,32 min
Detection 3	Omnimap anti Ms-HRP 36°C 12 min
Chromogen 3	Yellow HRP 32+16
Counterstain	Hematoxylin II 4 min/Bluing 4 min



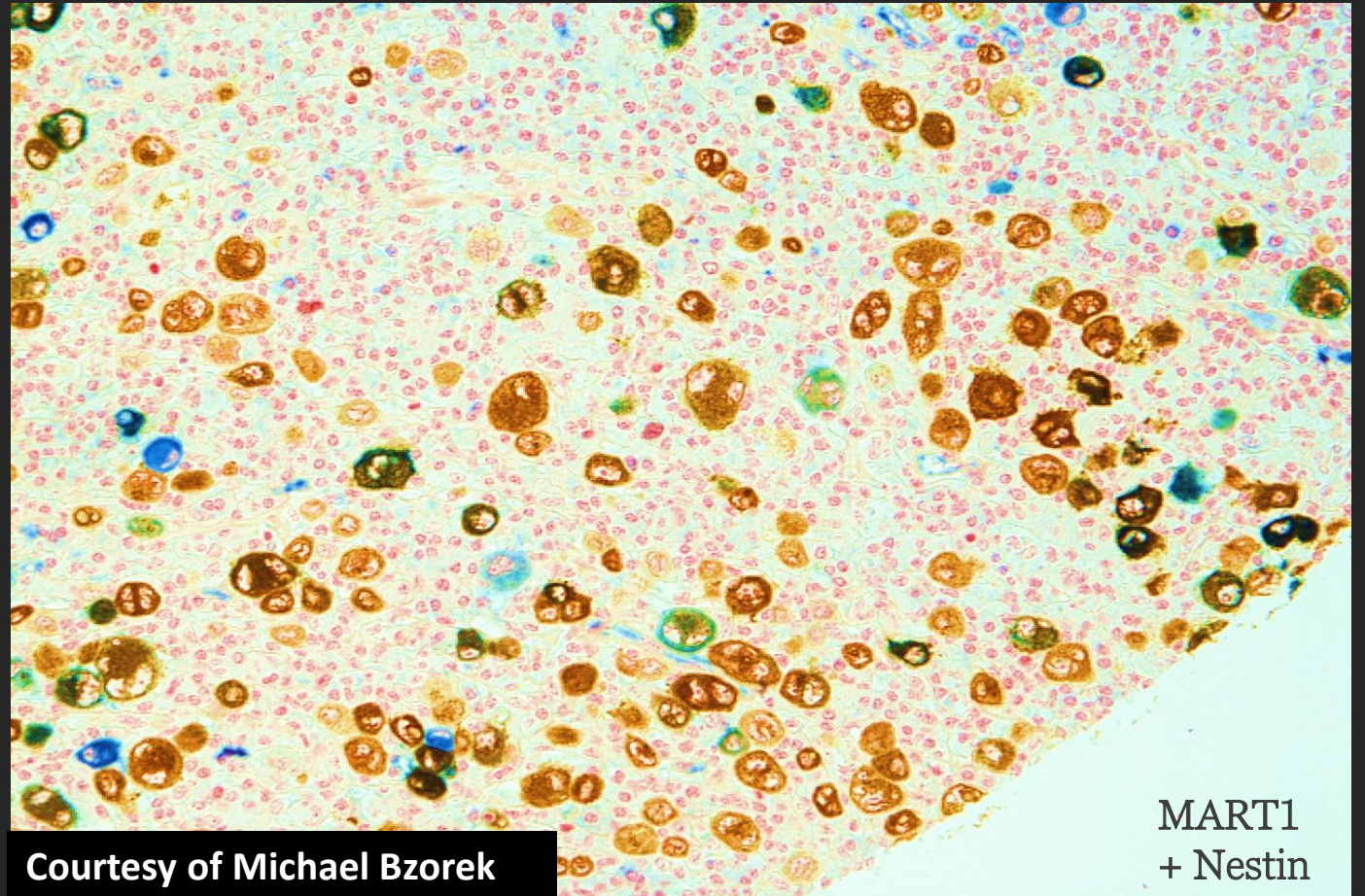
Research purpose

Multiplexing without DAB

Melanoma



Simultaneous procedure: MACH2 Double Staining 1
MART1, EP43 (1:100 RR) Hides Yellow (Enzo)
+
Nestin, 10C2 (1:100 RR) Ferangi Blue (Biocare)



Multiplexing then and now...

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.

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.

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

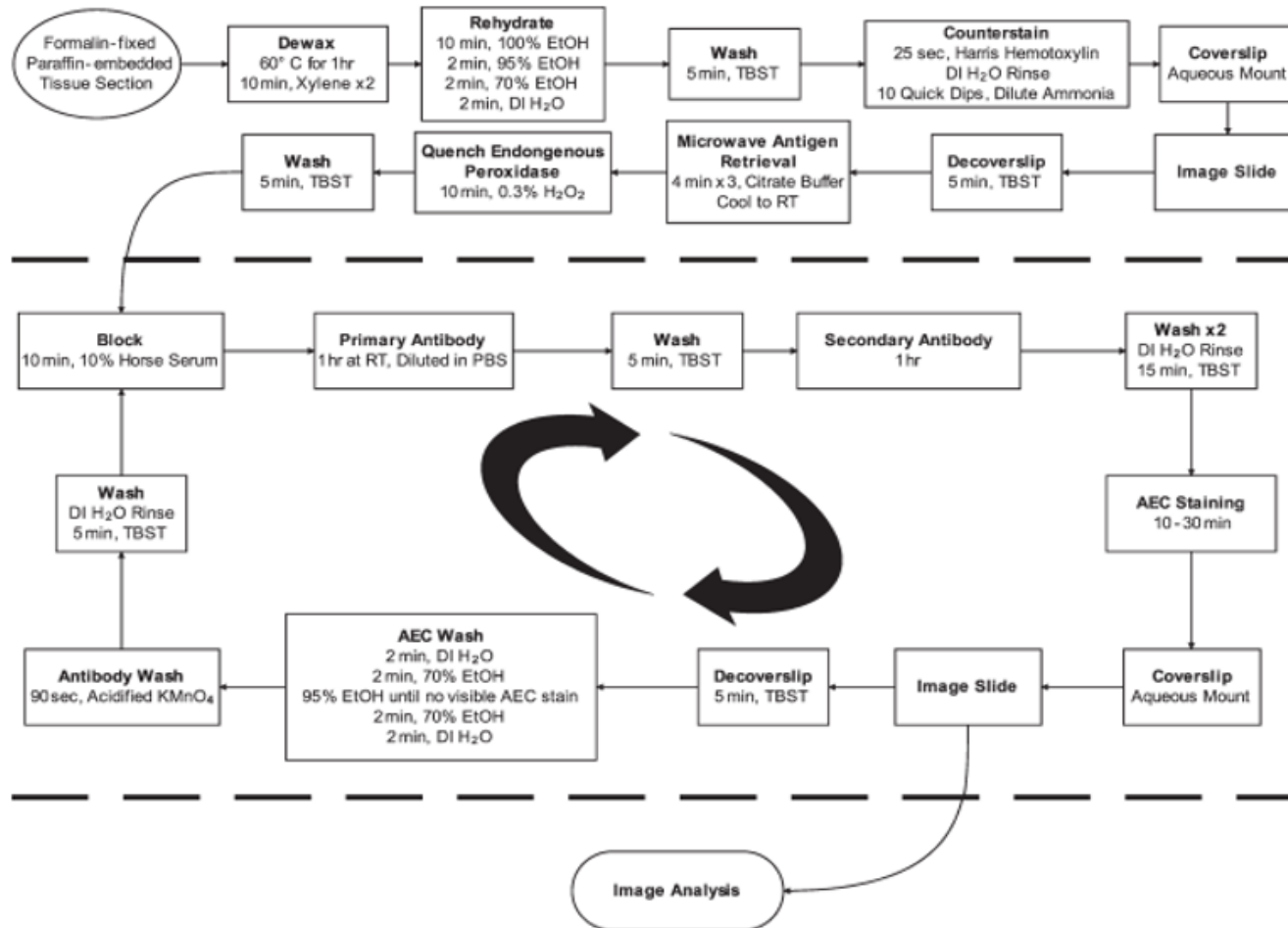


Figure 1 SIMPLE strategy. Formalin-fixed, paraffin-embedded sections are dewaxed, rehydrated, and counterstained before initial probing. Tissue is imaged and then subjected to antigen retrieval, removing the counterstain. Each staining round is conducted using standard immunohistochemical protocols with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

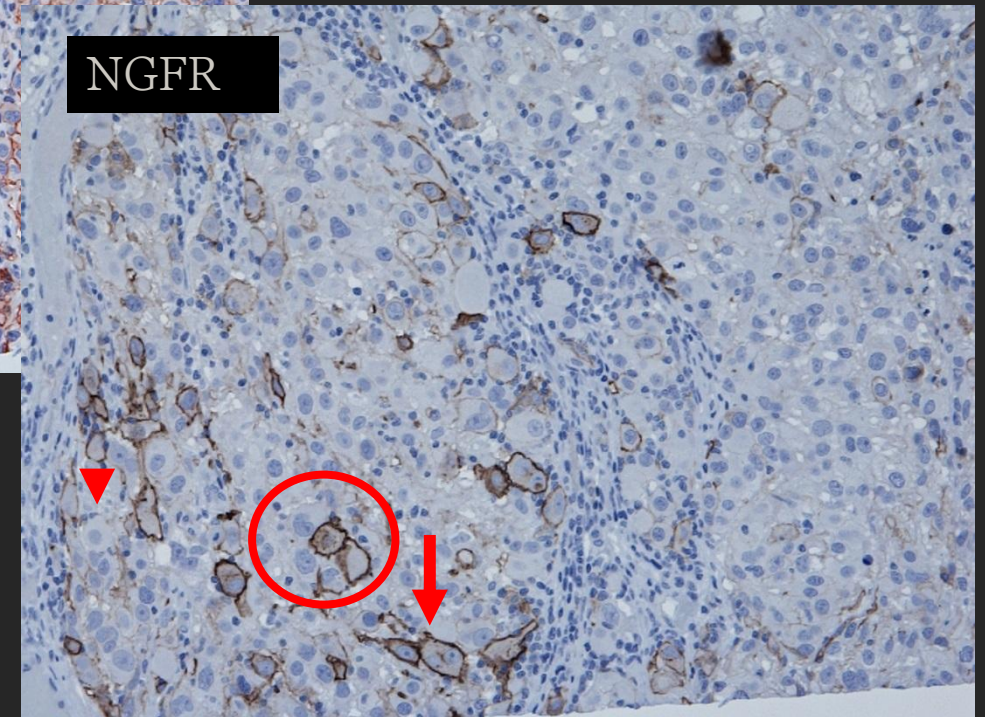
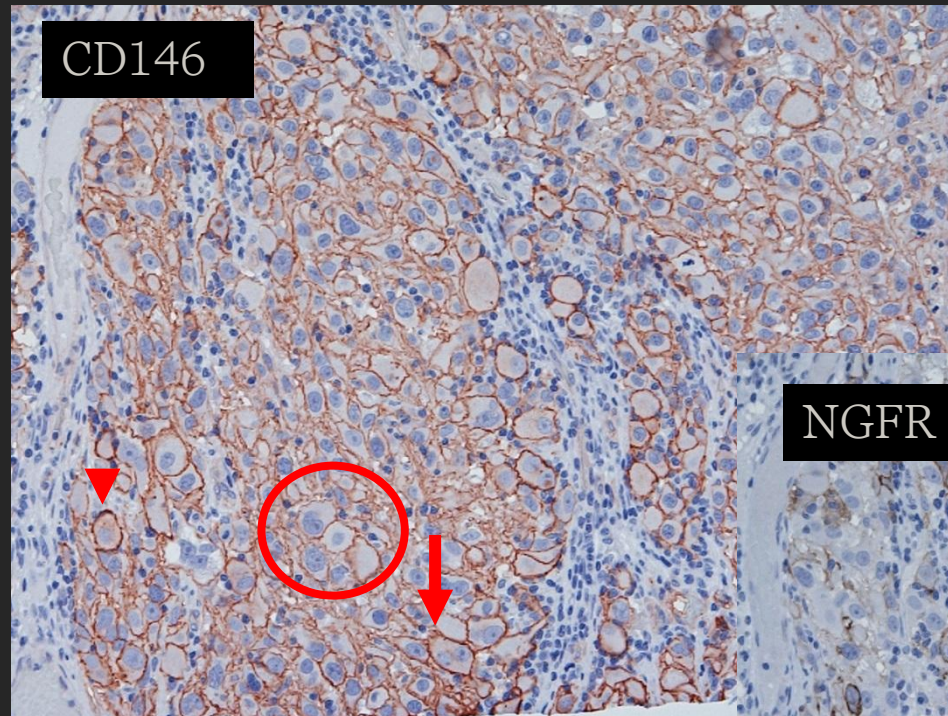
Imaging of slides

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)



First sequence: CD146 → detectionsystem → AEC Impact → coverslip

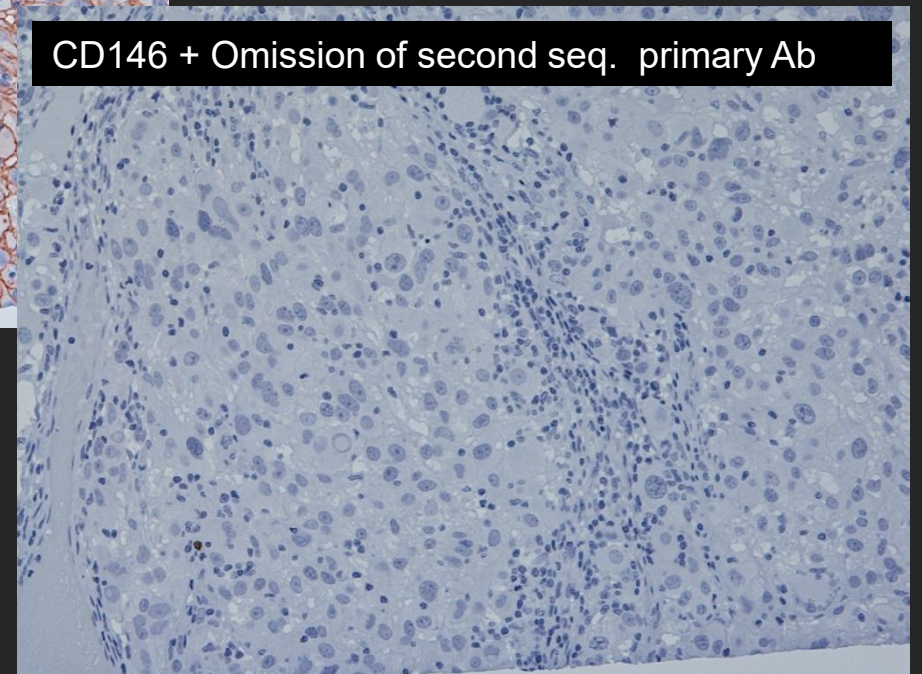
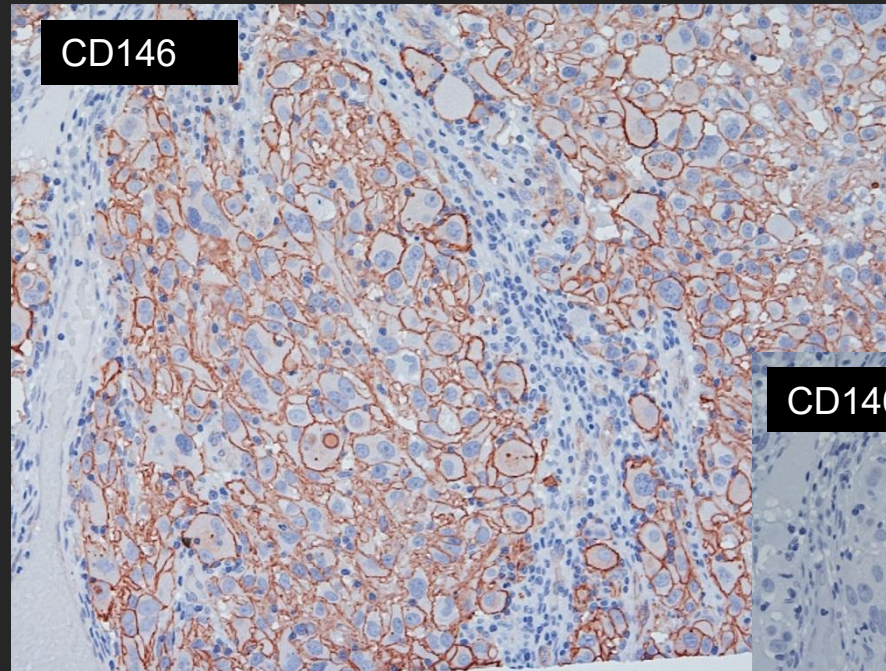
Imaging → de-coverslip → Erasing (Acetone) → Blocking using HIER (Ci)

Second sequence: NGFR → detectionsystem → DAB → coverslip

Courtesy of Michael Bzorek
Super Sensitive (Biogenex)
Impact AEC (Vector Lab)
Melanoma

SIMPLE technique – cross-reactive control

CD146 (EPR3208) + NGFR (MRQ21)

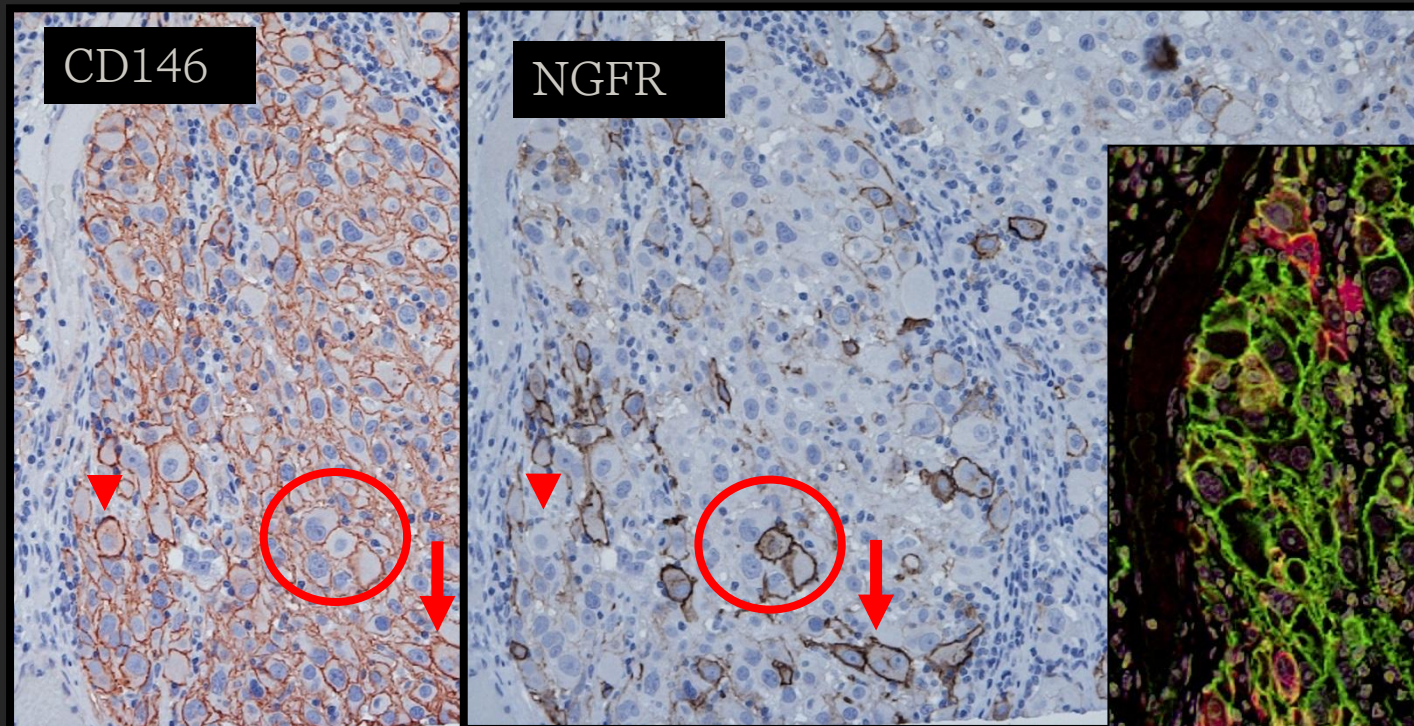


Control experiments:

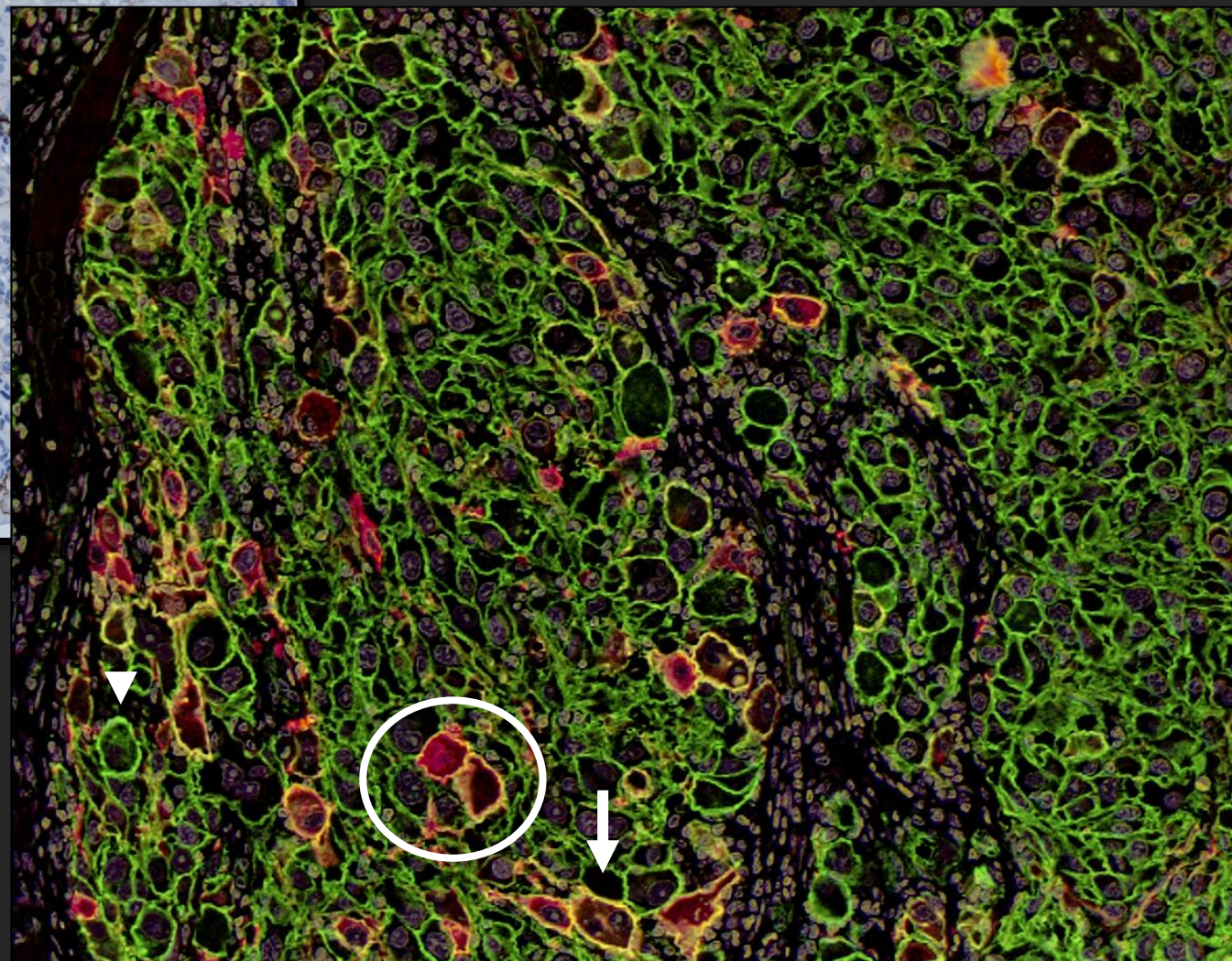
First sequence: CD146 immunostaining => AEC Impact => coverslip

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

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



Photoshop manipulated
“Digital imaging”



Does it look SIMPLE to you?



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

Ventana Discovery:

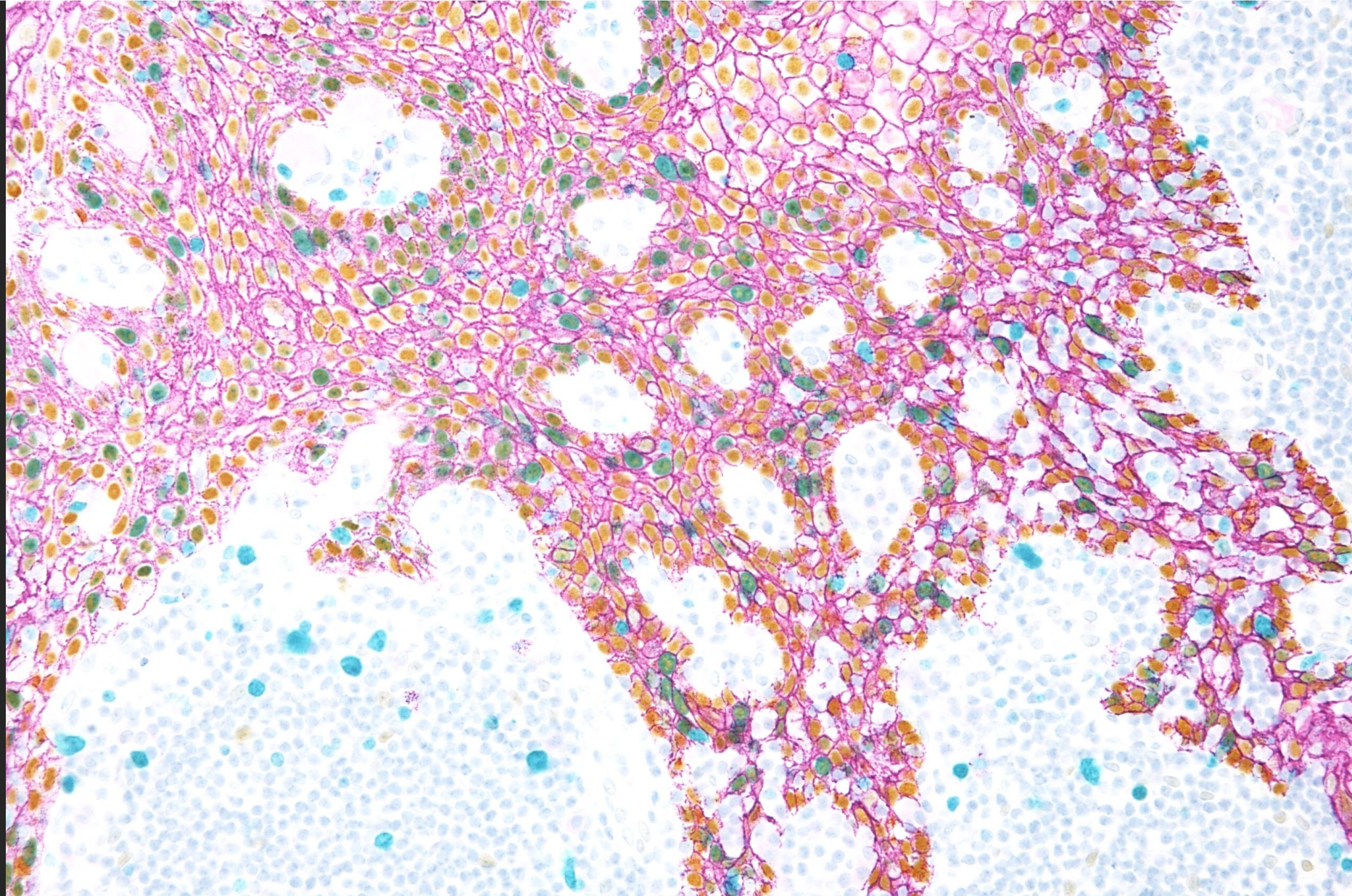
P63 (Disc. Yellow)

Ki67 (Disc. Teal)

ECAD (Disc. Purple)

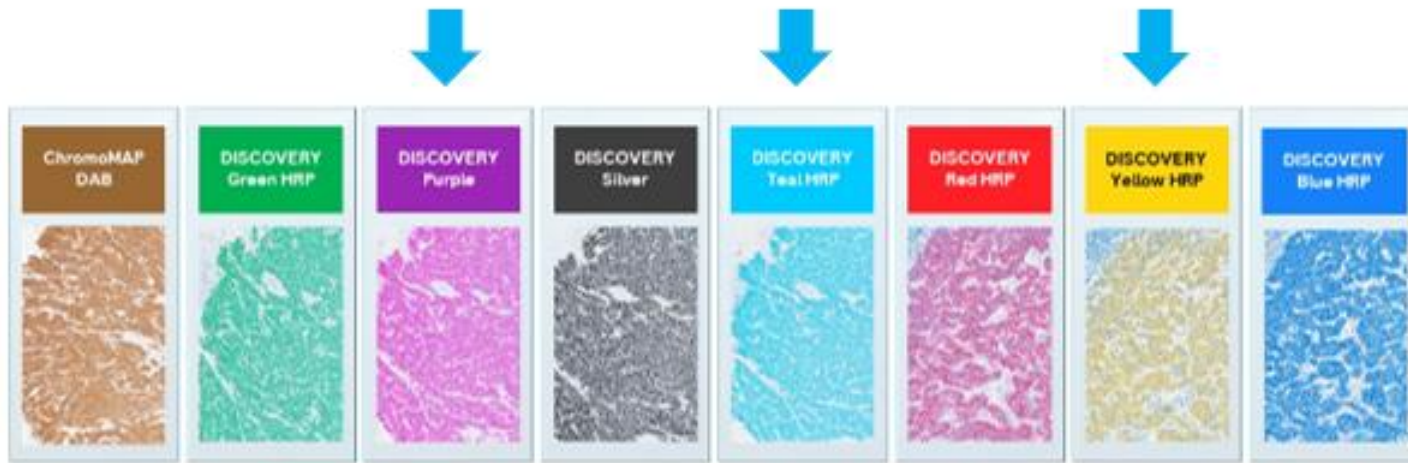
Co-localization

Sequential/HD/Omni-Map

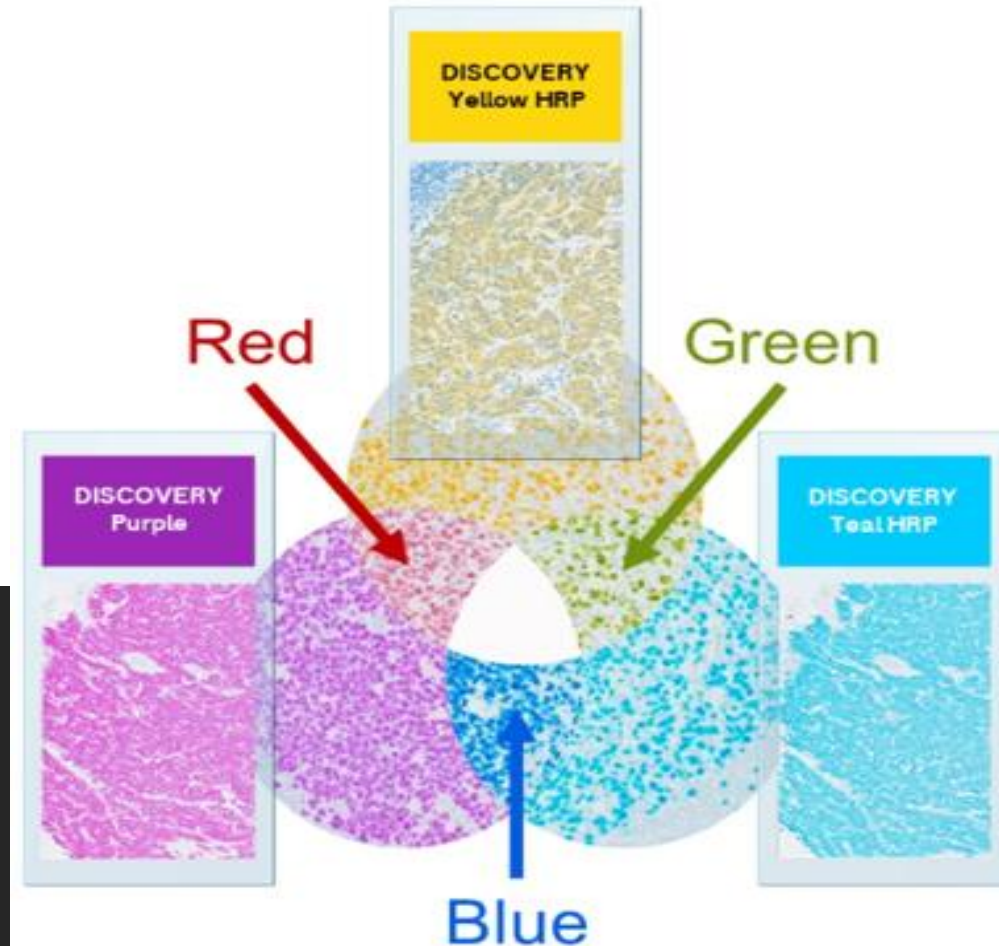


Translucent chromogens from Roche

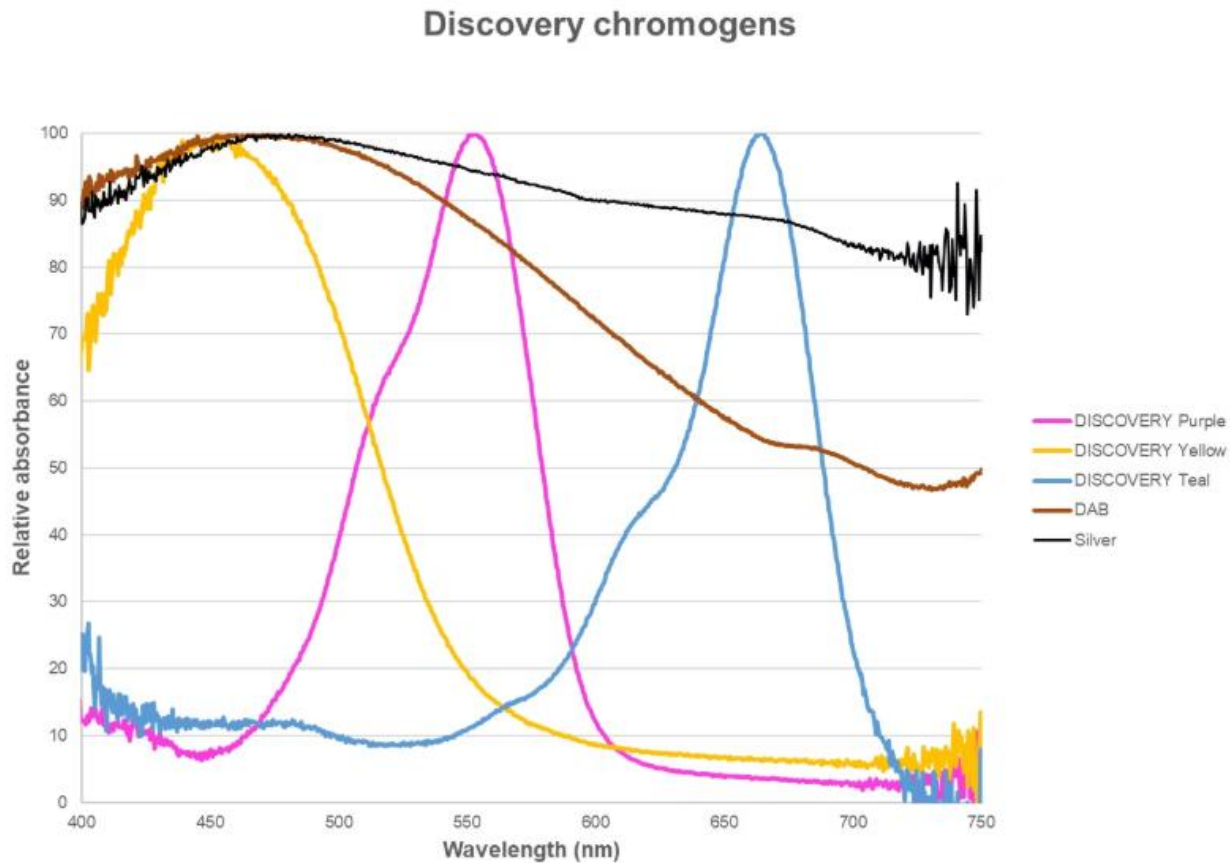
HRP-driven chromogens



Translucent chromogens for demonstration of multiplexed co-localized signals



How does they work..?



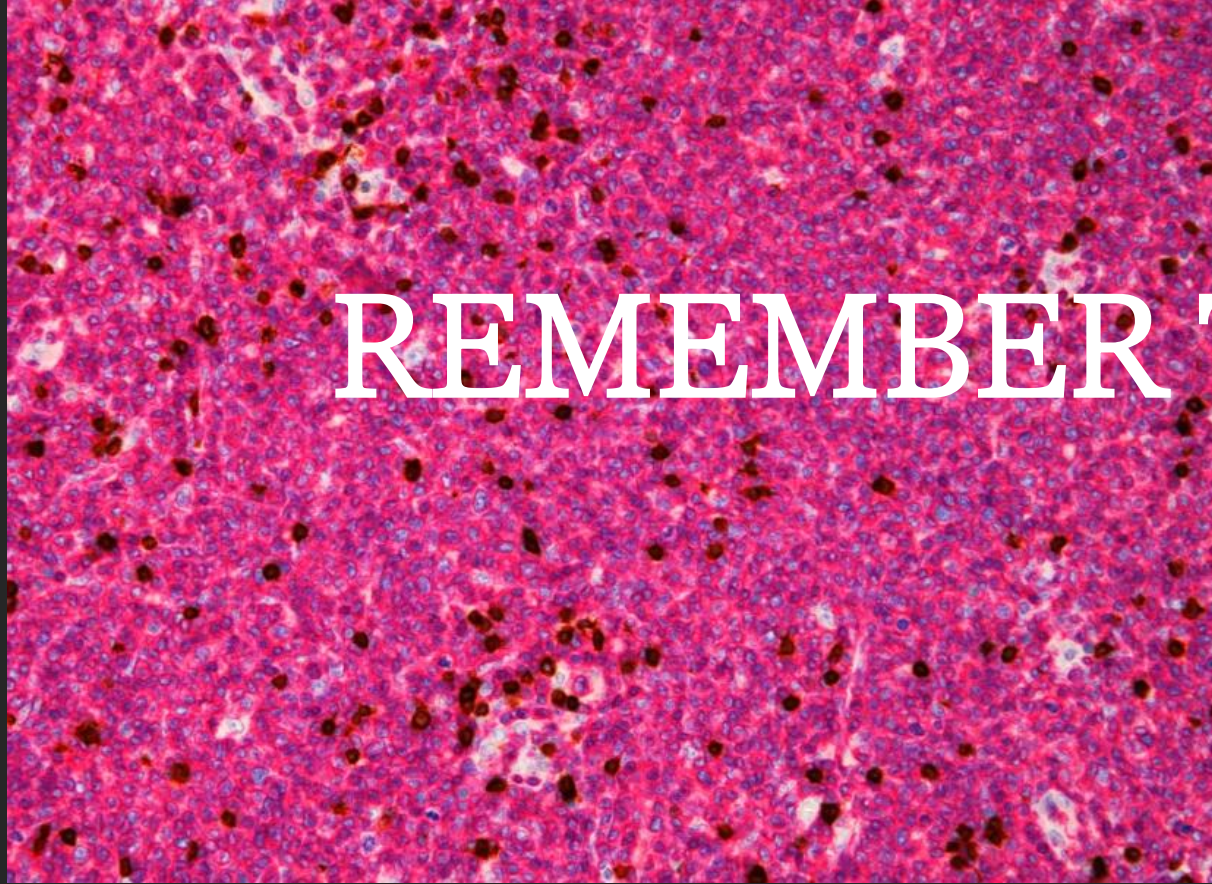
Roche/Ventana Diagnostics

“

Chromogens that have the narrower absorption ranges (Yellow, Purple, 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

Multiplexing with DAB

*TCRBC1 (T-Cell Receptor Beta Constant region 1), E6Z3S (DAB) +
CD3, MRQ39 (magenta)*



AITL*, TCRBC1-
Clonal expression pattern, associated with malignancy



AITL*, TCRBC1+
Clonal expression pattern, associated with malignancy

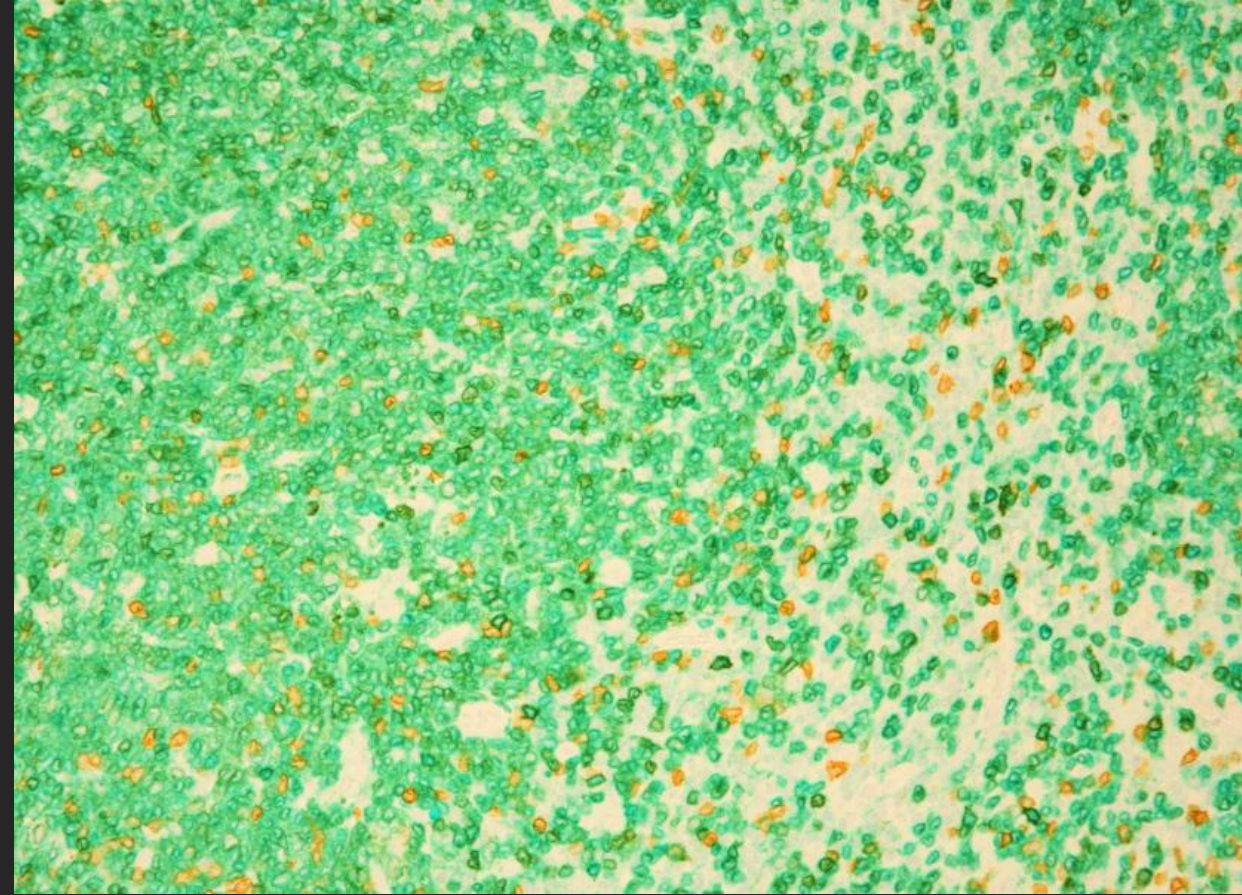
*Angioimmunoblastic T-cell lymphoma

Multiplexing with translucent chromogens

*TCRBC1 (T-Cell Receptor Beta Constant region 1), E6Z3S (Teal) +
CD3, MRQ39 (Yellow)*



AITL*, TCRBC1-
Clonal expression pattern, associated with malignancy



AITL*, TCRBC1+
Clonal expression pattern, associated with malignancy

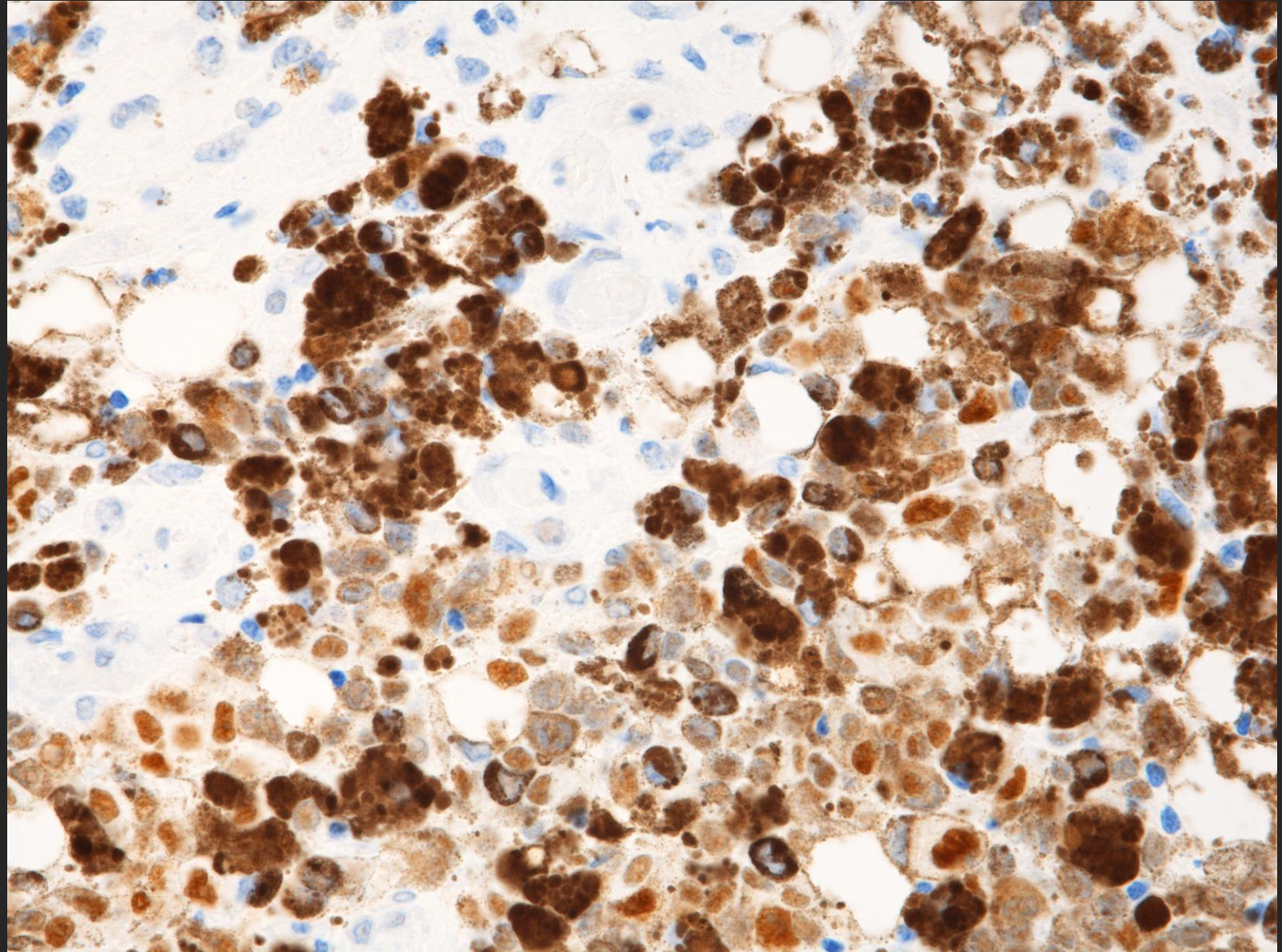
*Angioimmunoblastic T-cell lymphoma

Multiplexing, set up an assay

First, evaluate the expected staining pattern in a single-stain assay.
Normally we use DAB for reference

PRAME, EPR22330 - DAB

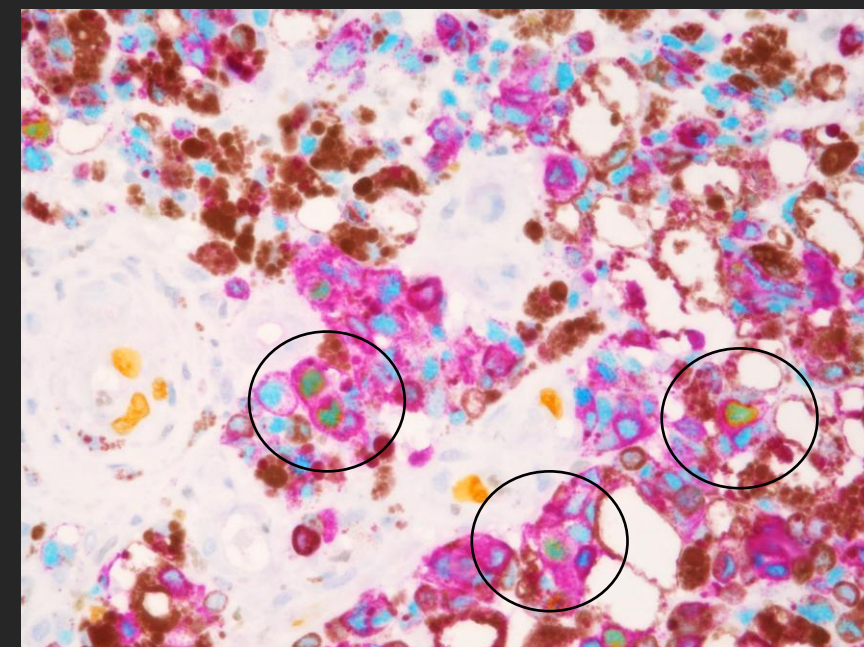
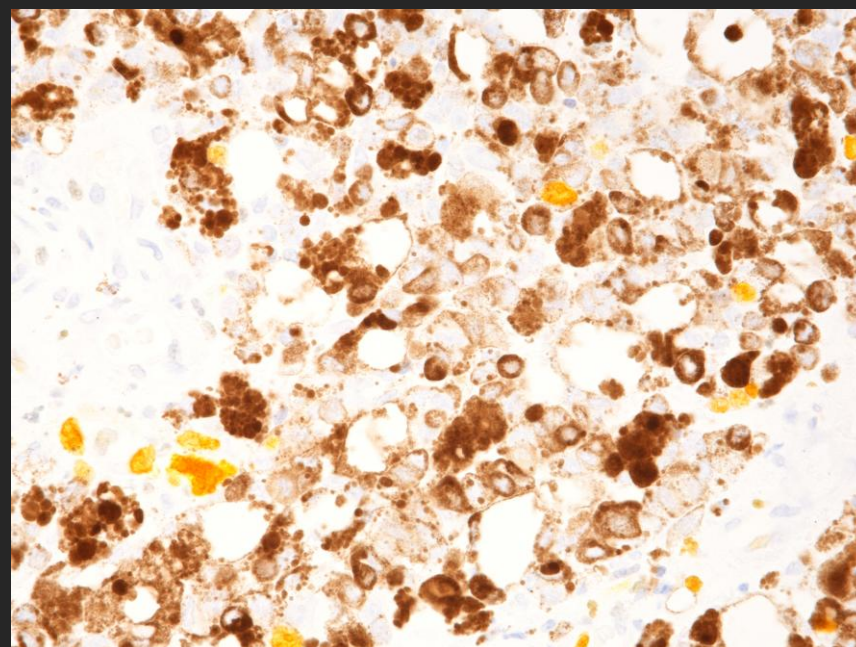
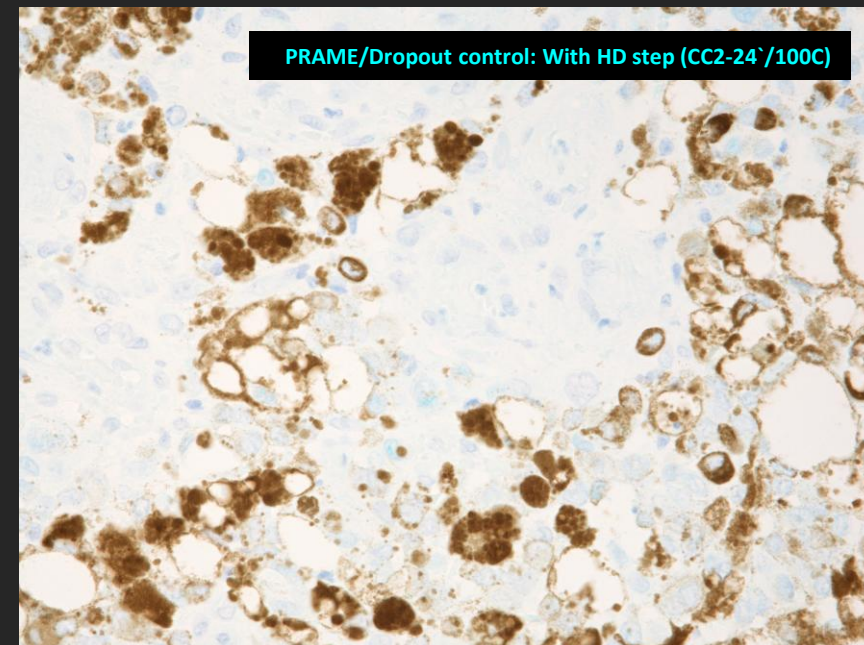
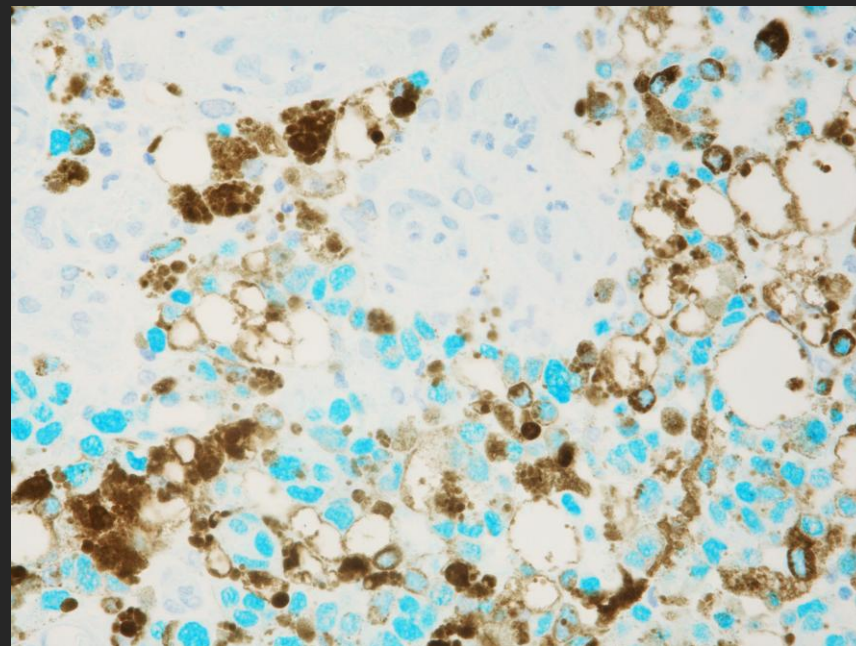
Melanoma



Prame, EPR20330 (Teal)
Ki67, SP6 (Yellow)
MSA, HMB45 (Purple)

Double positive
(prame+Ki67) = Green

Using Omnimap-
detectionsystem to
avoid cross-reactivity



Melanoma
Co-localization

Multiplexing with translucent chromogens

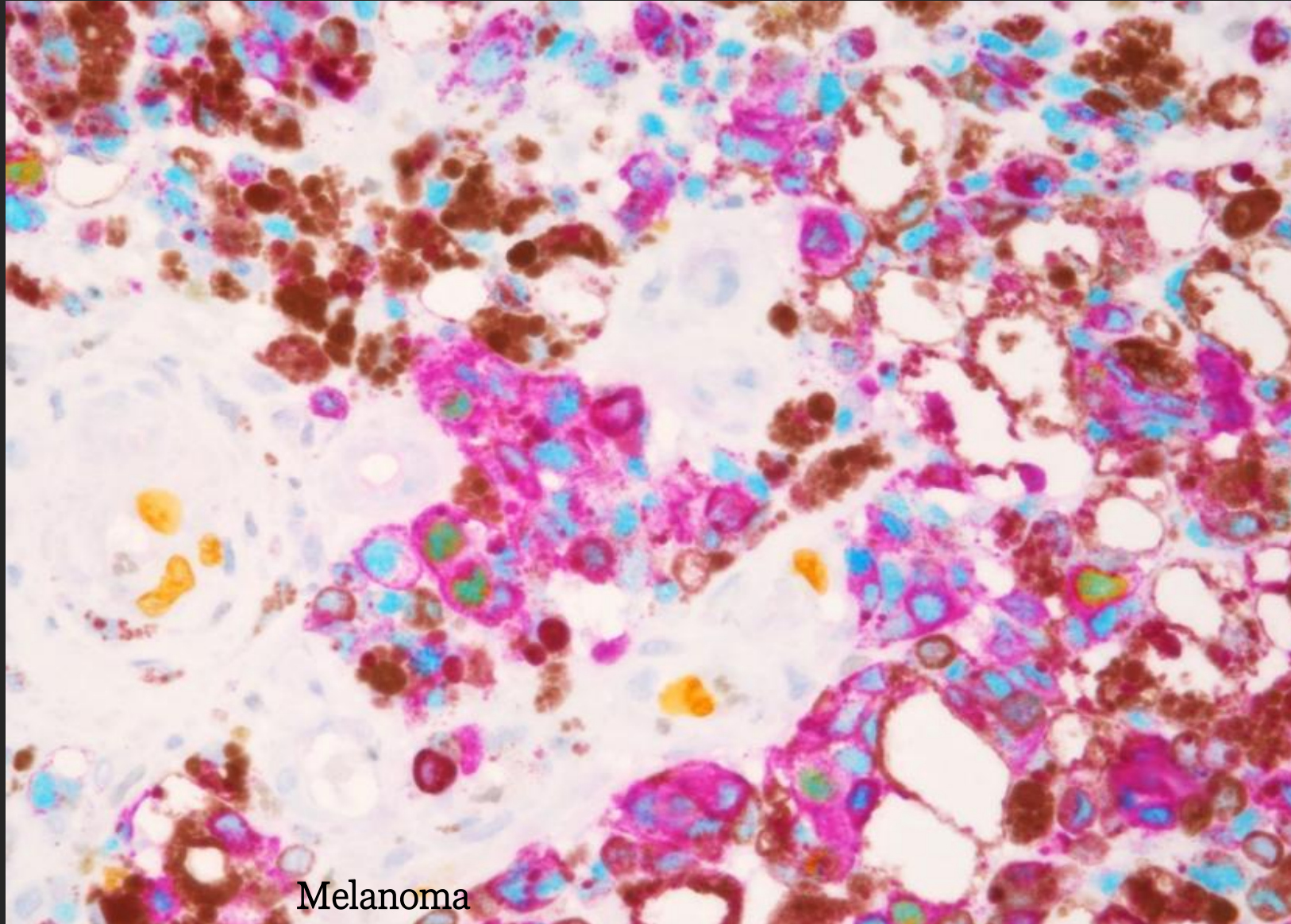
Prame, EPR20330 (Teal)

Ki67, SP6 (Yellow)

MSA, HMB45 (Purple)

Double positive (prame+Ki67) = Green

Using Omnimap-detectionsystem to
avoid cross-reactivity



Multiplexing with translucent chromogens

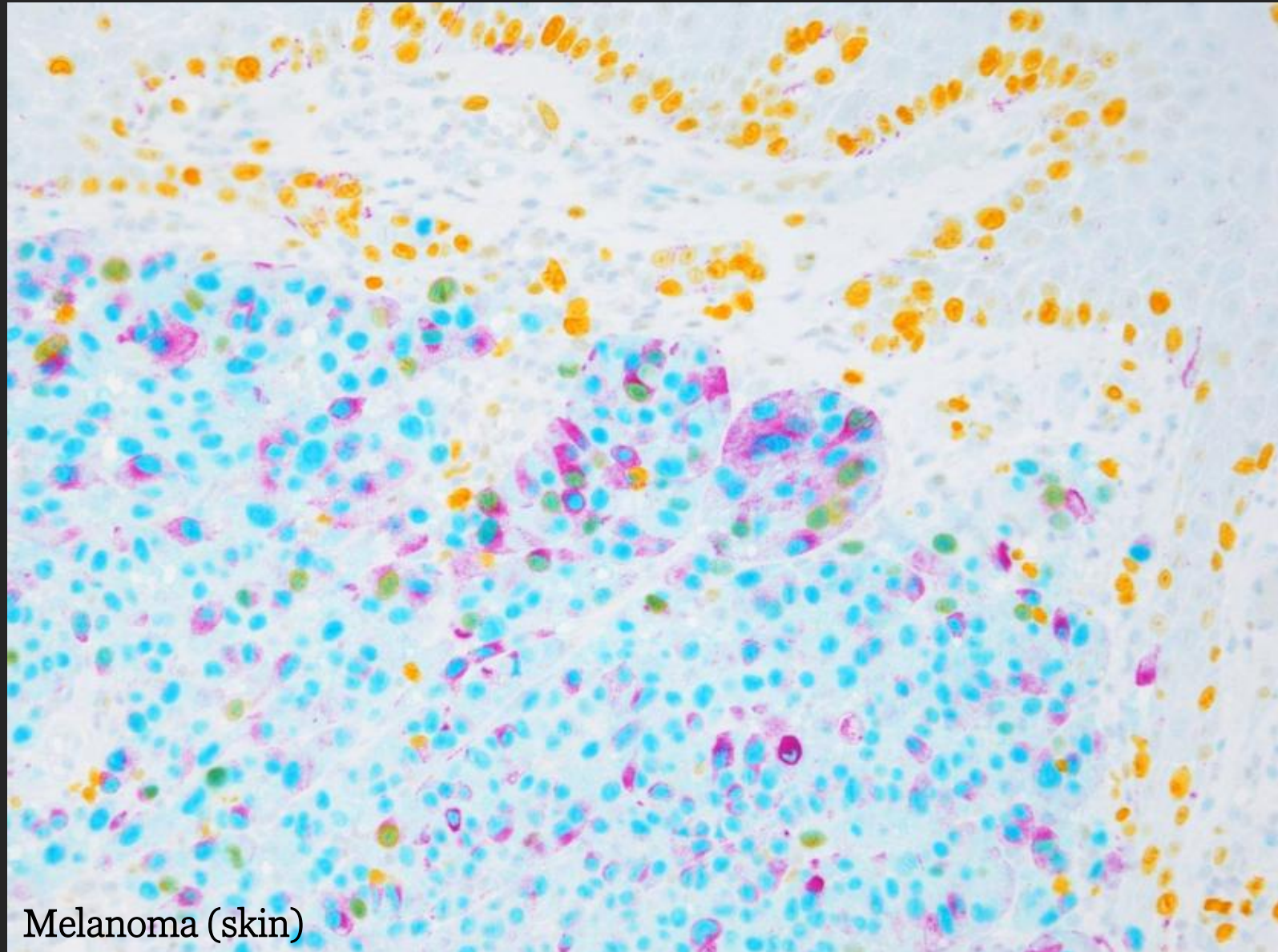
Prame, EPR20330 (Teal)

Ki67, SP6 (Yellow)

MSA, HMB45 (Purple)

Double positive (prame+Ki67) = Green

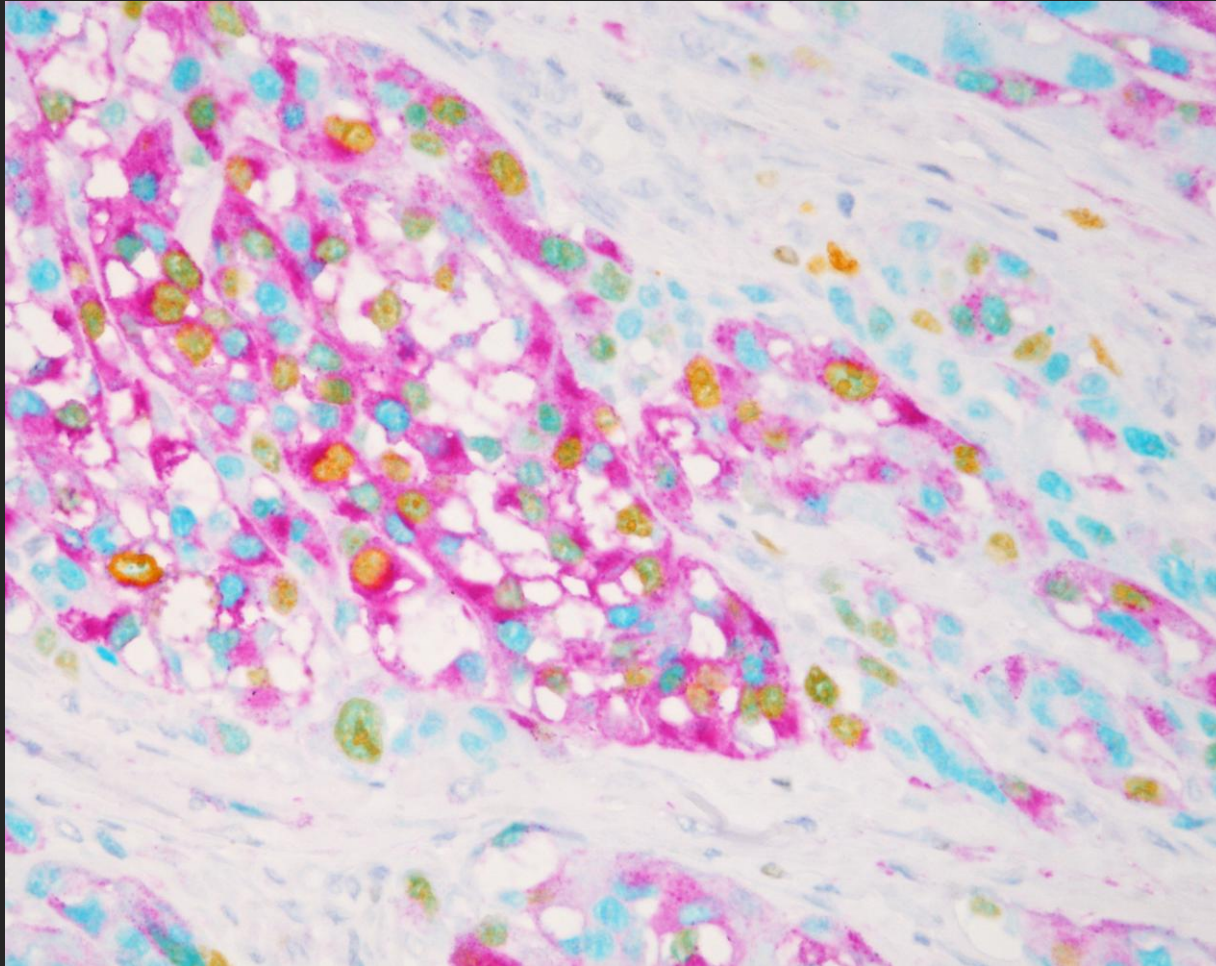
Using Omnimap-detectionsystem to
avoid cross-reactivity



Melanoma (skin)

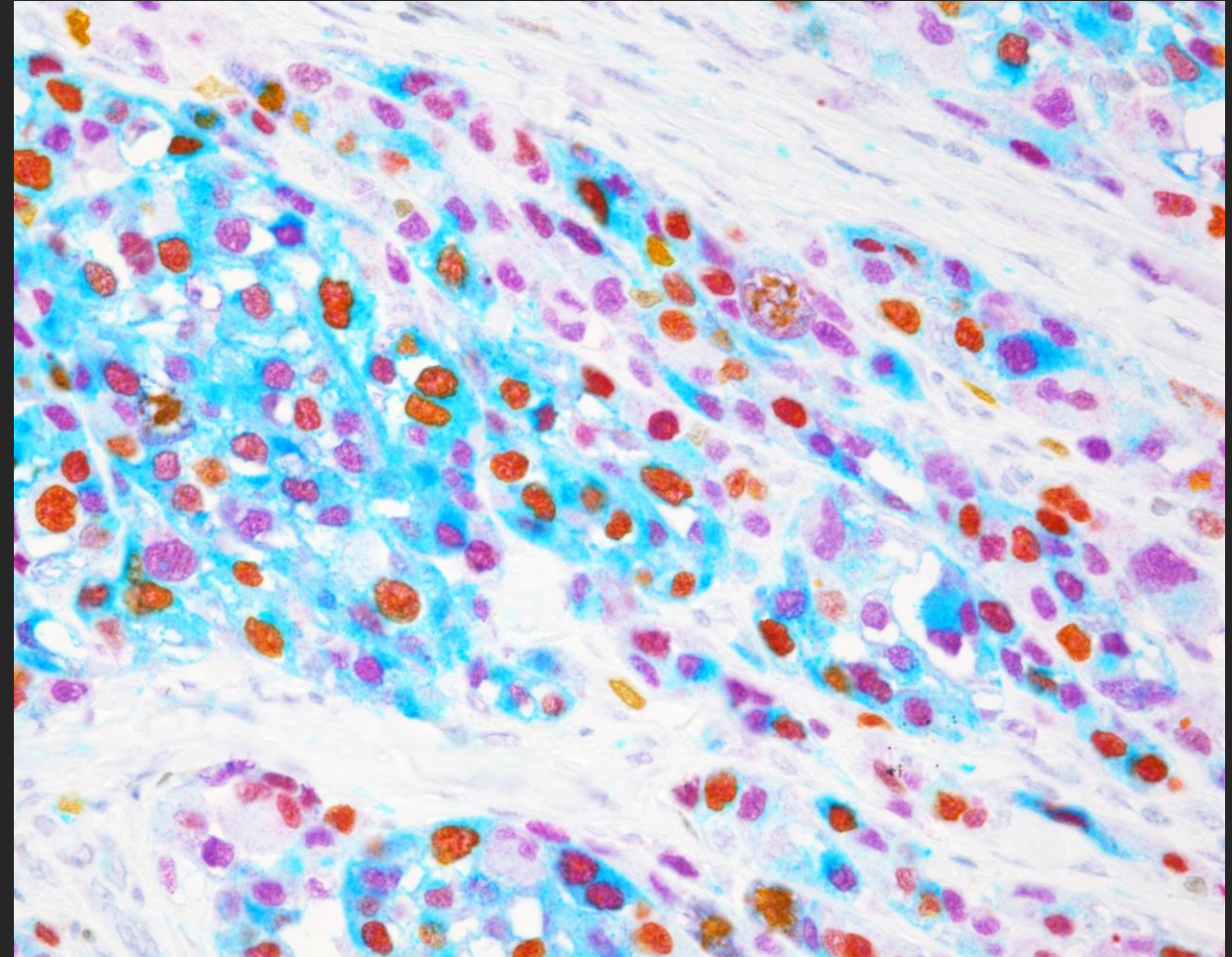
PRAME, rmAb EPR22330
(HQ anti Rb+HQ-HRP/Teal)
+HD
Ki67, rmAb SP6
(OmniMap anti Rb-HRP/Yellow)
+N
MSA, mAb HMB45
(OmniMap anti Ms-HRP/Purple)

Co-Localization: Green (ish)



PRAME, rmAb EPR22330
(HQ anti Rb+HQ-HRP/Purple)
+HD
Ki67, rmAb SP6
(OmniMap anti Rb-HRP/Yellow)
+N
MSA, mAb HMB45
(OmniMap anti Ms-HRP/Teal)

Co-Localization: Orange/Red (ish)



Melanoma

Multiplexing with Ventanas translucent chromogens

Recommendations

- Optimizing single-plex before multiplexing

Proper controls confirming epitope stability and efficient elution of Abs and detection systems

Controls should also include heat and chemical denaturing steps to efficiently strip of the antibody complexes between sequences

- Nuclear-markers before cytoplasmic and membranous markers
- Stain the lowest expressing/sensitive epitope first for optimal target visualization
- Targets requiring unique retrieval, blocking, amplification should be placed later in the staining sequence (those reagents could impact efficient binding of downstream targets)
- For obtaining the most distinct and vibrant hue for each chromogen, use the following order:

DAB-Purple-Red-Yellow-Teal-Green-Silver

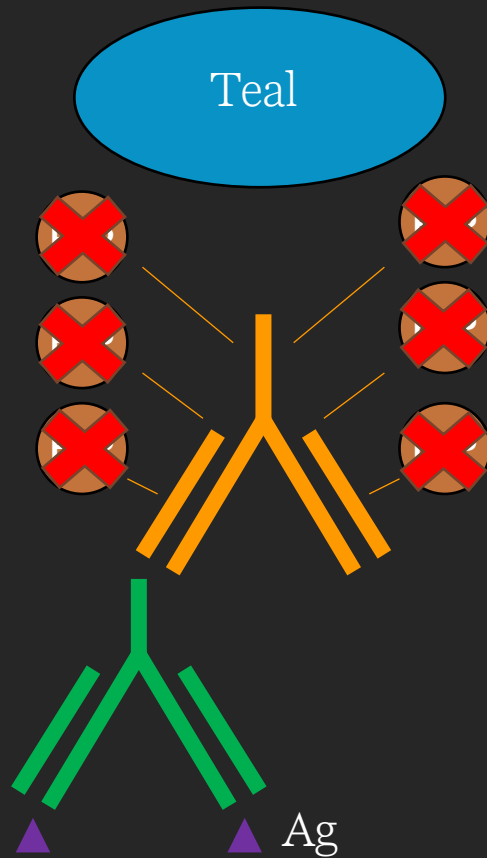
Multiplexing with Ventanas translucent chromogens

Neutralization and heat denaturation

- Avoid any cross-reaction between sequences
- Neutralization with H_2O_2 - quenching HRP-activity (Discovery Inhibitor)
- Heat Denaturation with CC2 (low pH buffer) at high temperature to elute Abs and detectionsystems

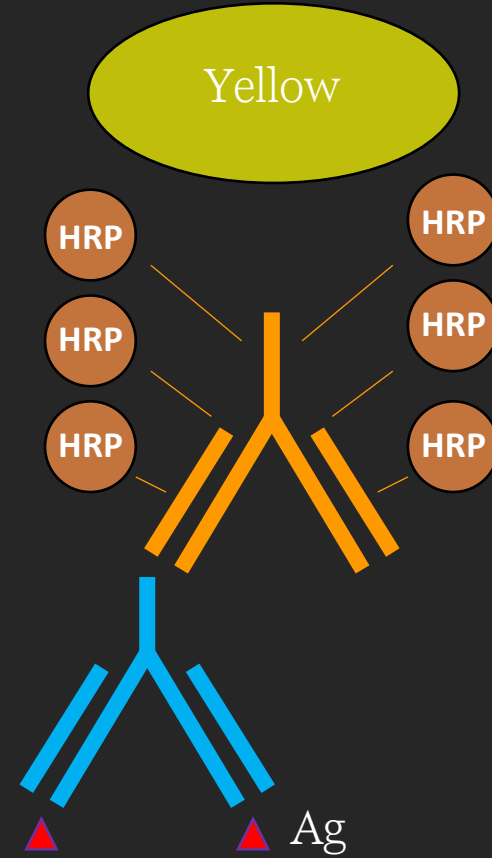
Multiplexing with different species

Neutralization (Discovery inhibitor H_2O_2)



1. Sequence (Ms)

Neutralization
(eliminates HRP activity)



2. Sequence (Rb)

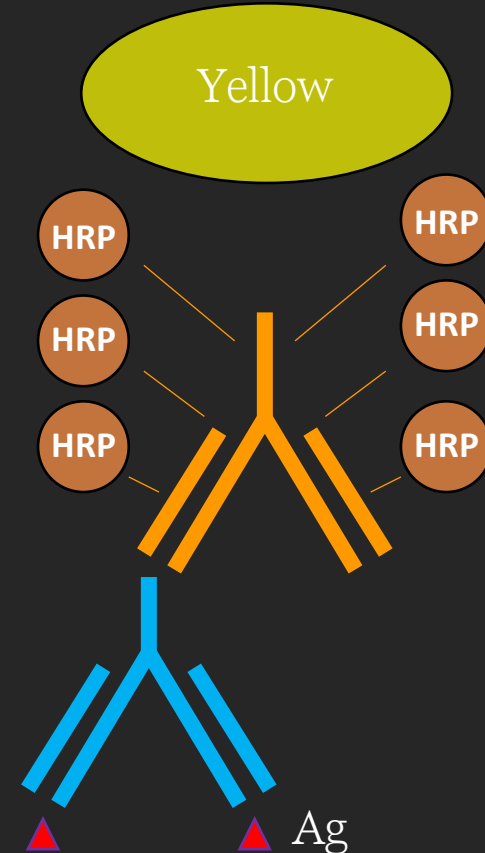
Multiplexing with same species

Heat Denaturation (CC2, 100°C, 24 min)



1. Sequence (Ms)

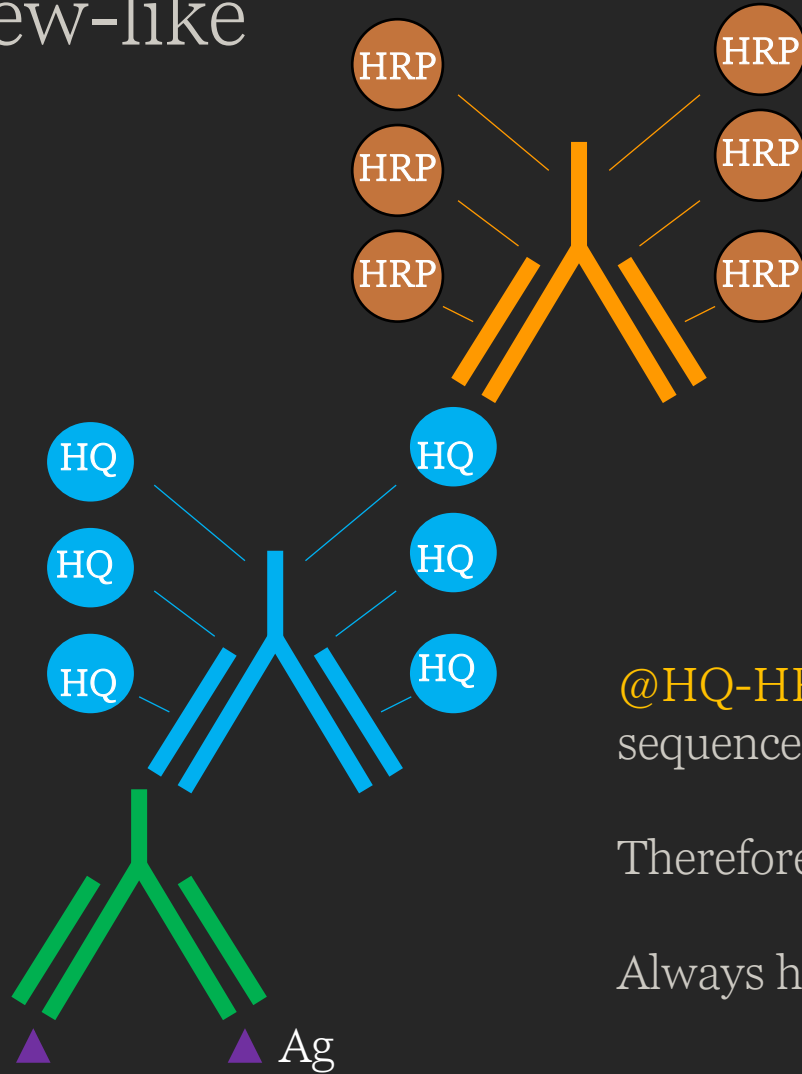
Heat Denaturation



2. Sequence (Ms)

Multiplexing with HQ-linker

“Optiview-like”

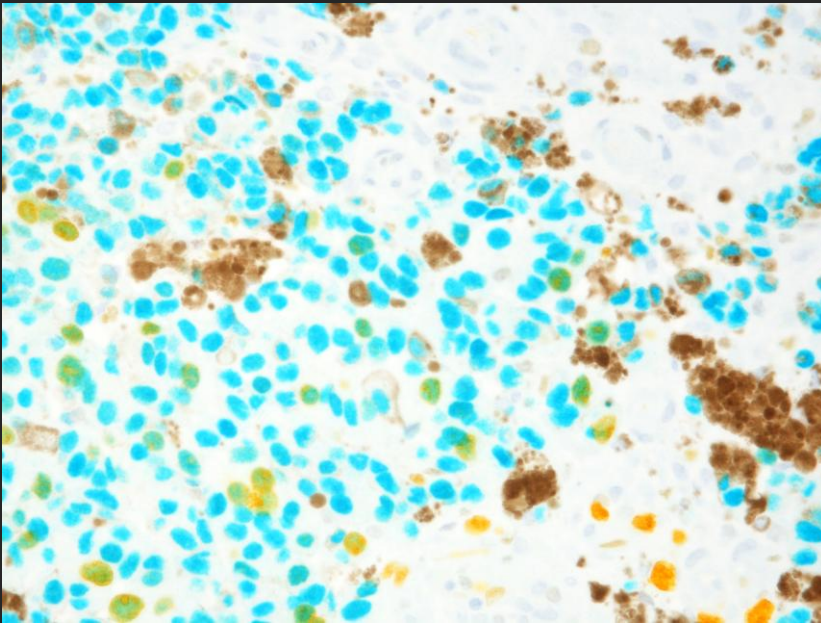


@HQ-HRP is made in mouse and might cause some problems, if second sequence involving mouse-ab.

Therefore always include Drop-out controls, especially when using HQ.

Always heat denaturate between sequences when using HQ.

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

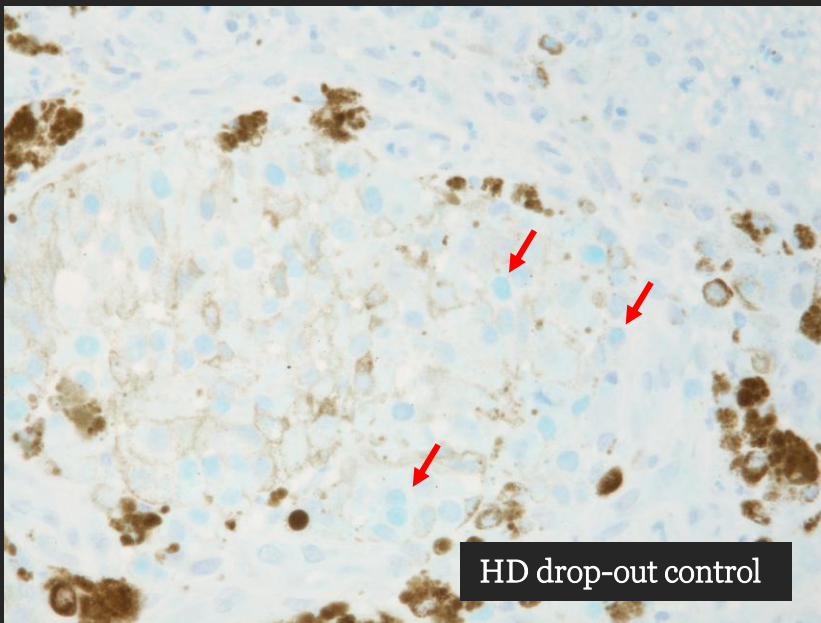
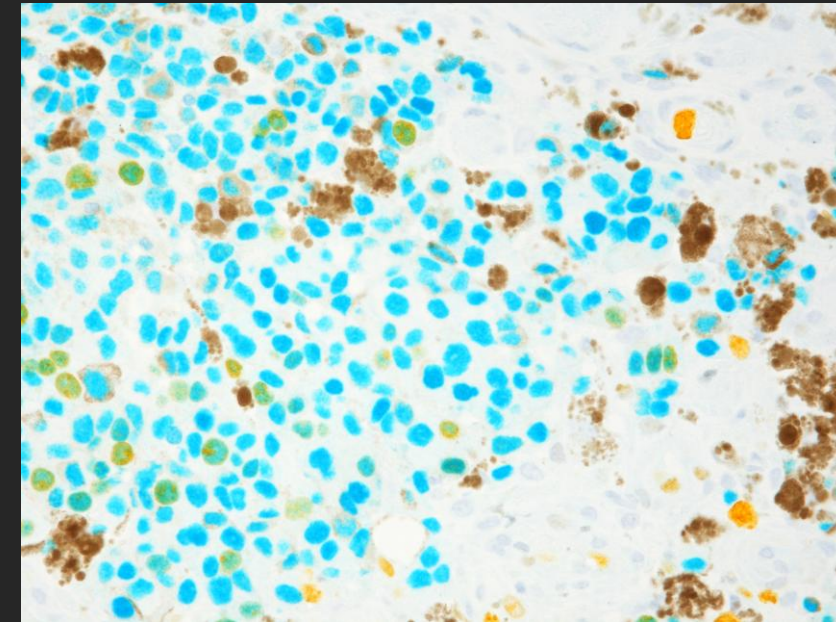


Importantly:

When mouse-based HQ-HRP is used in the first staining sequence, cross-reactivity with OmniMap anti Ms-HRP in second sequence may occur.

Appropriate drop-out controls should therefore be included.

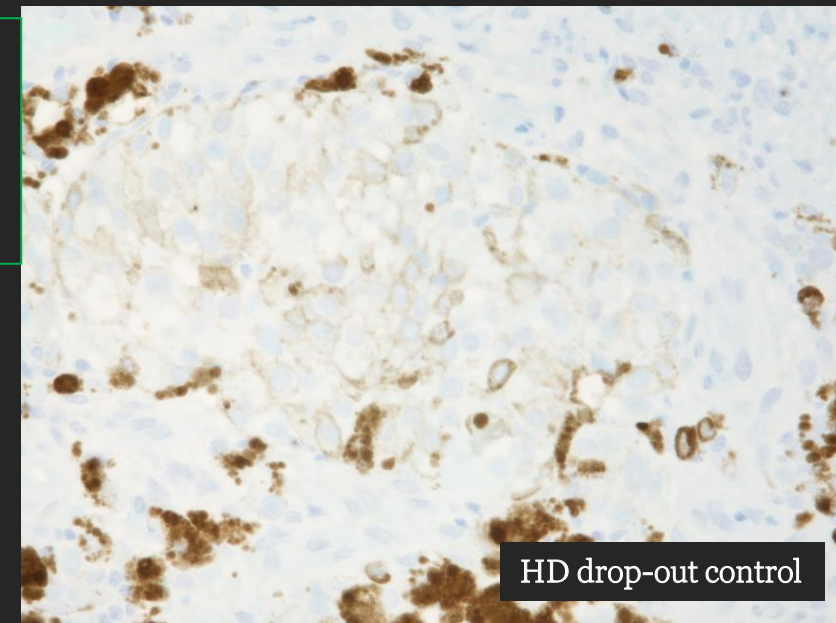
SOX10 (SP267), anti Rb-HQ+HQ-HRP, Teal
+ HD (CC2-24'/100°C)
Ki67 (SP6), OmniMap anti Rb-HRP, Yellow



HD drop-out control

SOX10, SP267
anti Rb-HQ/HQ-HRP
No chromogen
+HD (CC2-24'/100C)
No primary Ab
Anti Rb-OmniMap-HRP
Teal

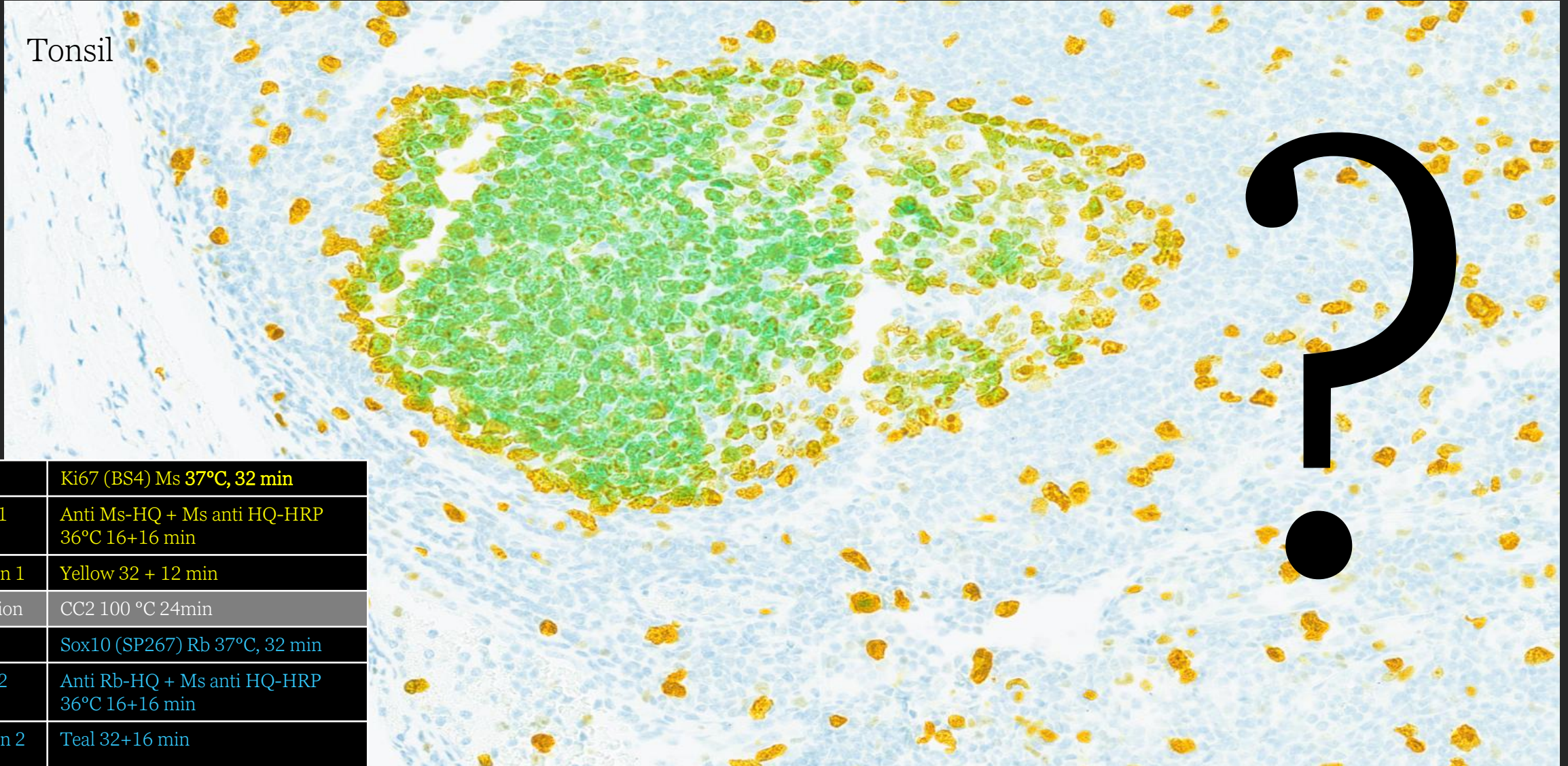
SOX10, SP267
anti Rb-HQ/HQ-HRP
No chromogen
+HD (CC2-24'/100C)
No primary Ab
Anti Ms-OmniMap-HRP
Teal



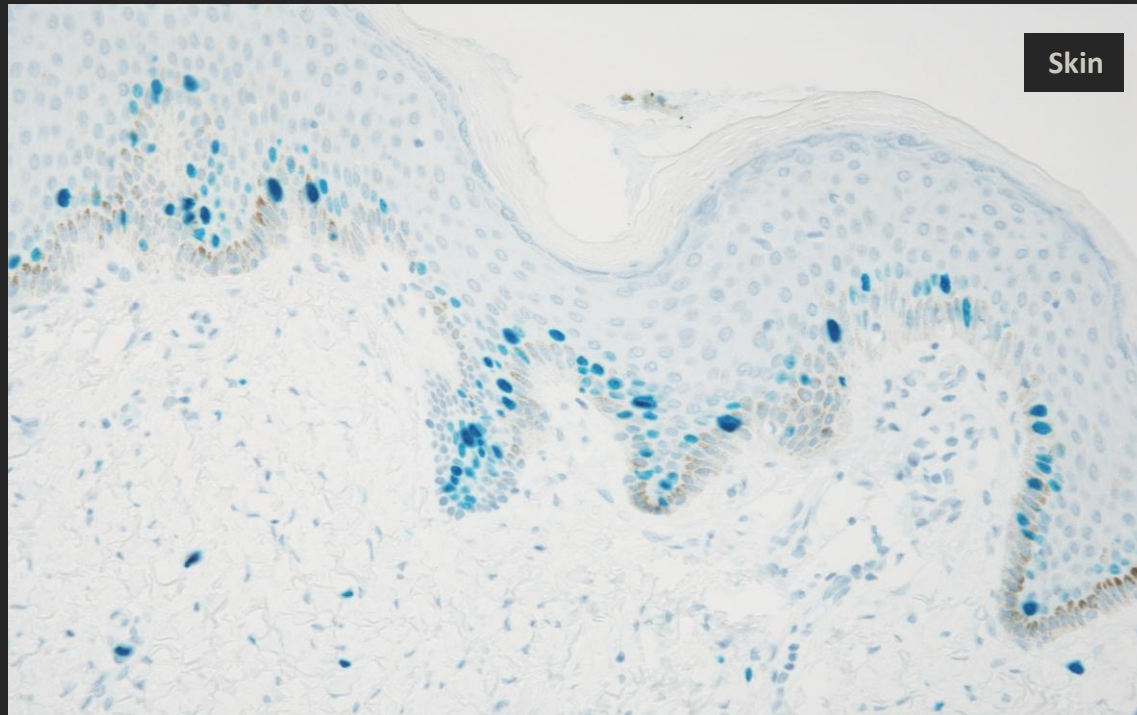
HD drop-out control

Ki67-Sox10

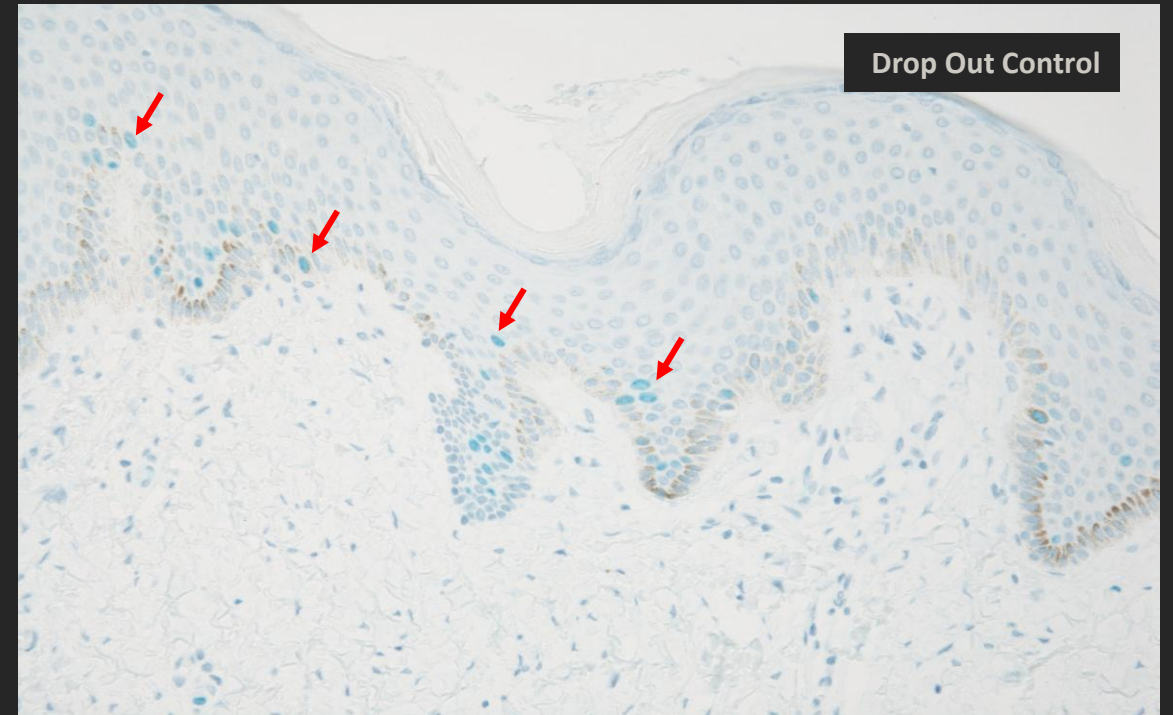
Demonstration of co-localized signals?



Multiplex staining considerations (KI67)



Ab 1	Ki67 (SP6) Rb
Detection 1	Anti Rb-Omnimap-HRP 36°C 12 min
Chromogen 1	Teal 32+16 min



Ab 1	Ki67 (SP6) Rb
Detection 1	Anti Rb-Omnimap-HRP 36°C 12 min
Chromogen 1	%
Denaturation	CC2 100 °C 24min
Ab 2	%
Detection 2	Anti Rb-Omnimap-HRP
Chromogen 2	Teal 32+16 min

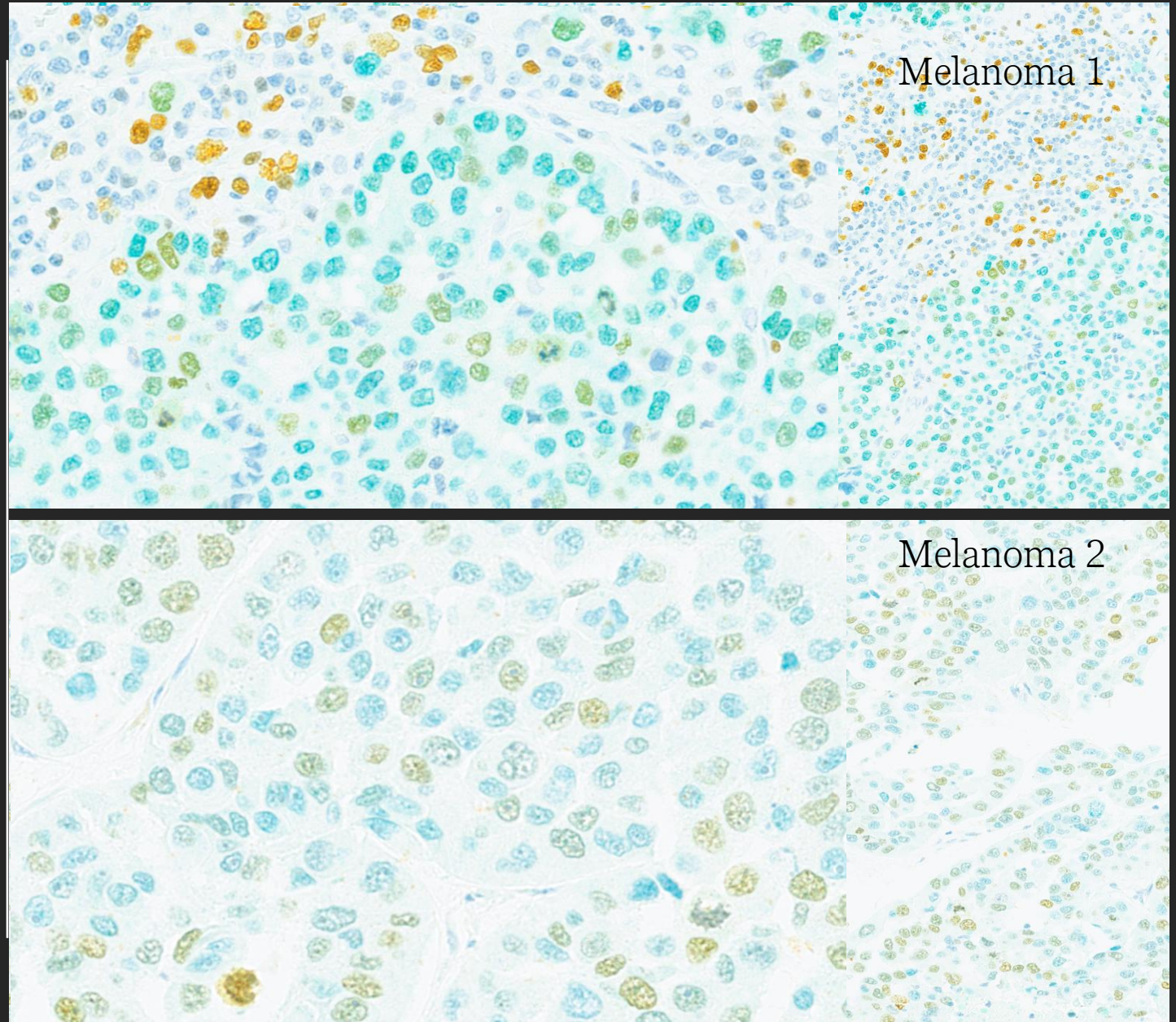
Difficult to elute Ki67, why Ki67 is placed last in the 2-plex

Courtesy of Michael Bzorek

CALIBRATION

Ab1	Sox10 (SP267) Rb 37°C, 32 min
Detection 1	HQ@Rb, HRP@HQ, 36°C 12 + 12 min
Chromogen 1	Teal, 36°C 32+16 min
Denaturation	CC2°C 100 24min
Ab2	Ki67 Ms 37°C, 32 min
Detection 2	Omnimap@Ms-HRP, 36°C 12 min
Chromogen 2	Yellow, 36°C 48 min
Counterstain	Hematoxylin II 4 min/Bluing 4 min

OBS When testing:
Cases can show a different staining-pattern eg. weak or strong.
What is the best way to calibrate the staining?
Too strong staining makes it difficult to elute 1.step. Too weak staining causes troubles seeing the green mixed color.



Multiplex staining considerations

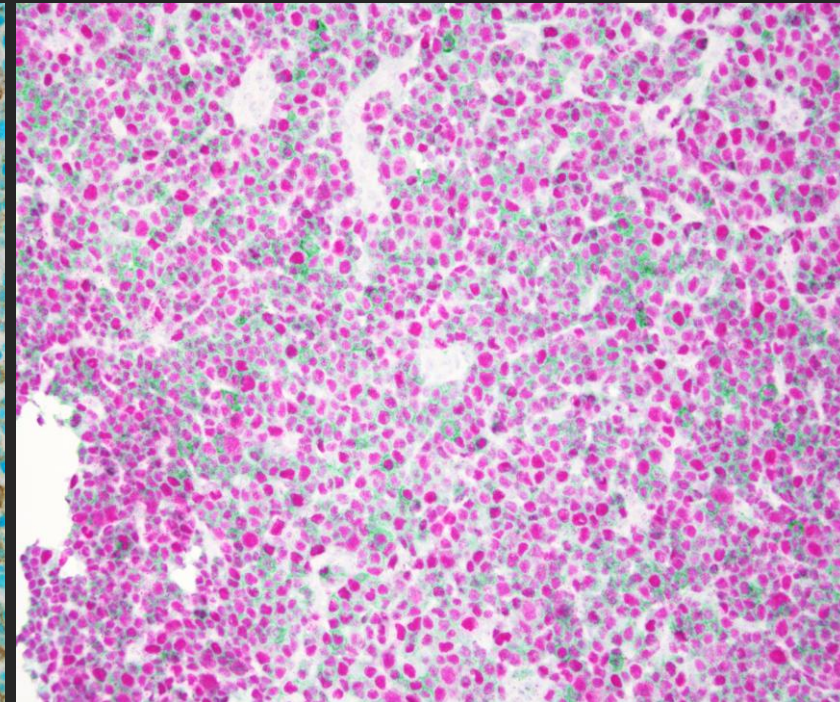
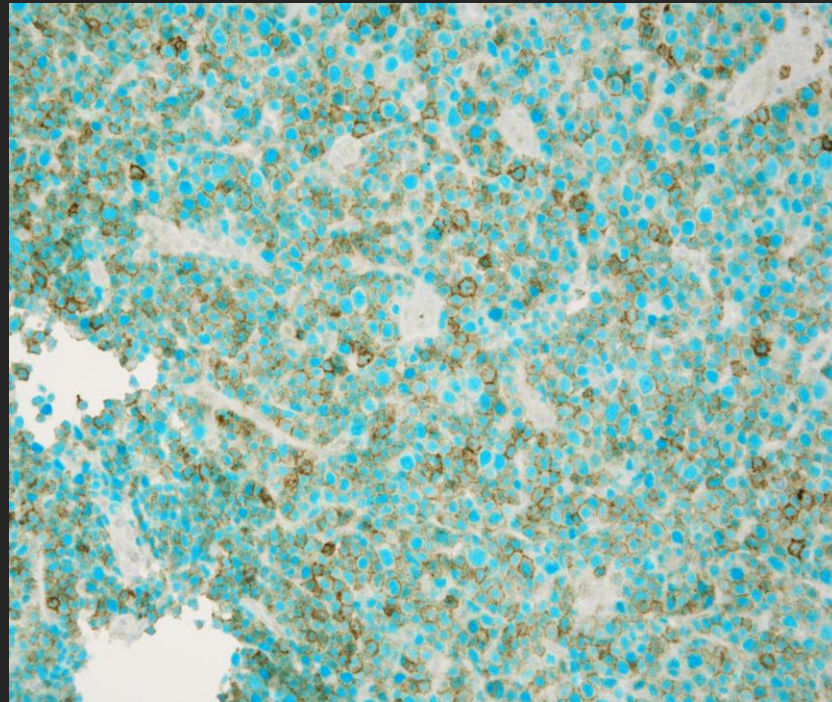
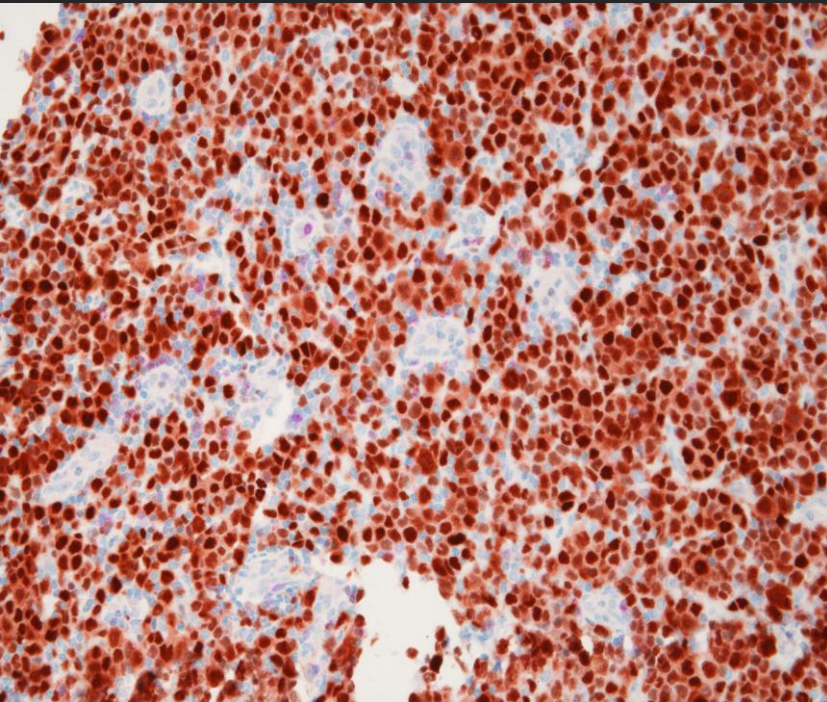
TOX, nuclear (DAB) +
CCR4, membrane (Purple)

The combination provided difficulties due to TOX expression (bleeding) in the cytoplasm of the positive CCR4 cells.

CCR4, membrane (DAB) +
TOX, nuclear (Teal)

Provided the expected reaction pattern but in cases with strong CCR4 staining (DAB), TOX (Teal) could not enter the nuclei of the cells

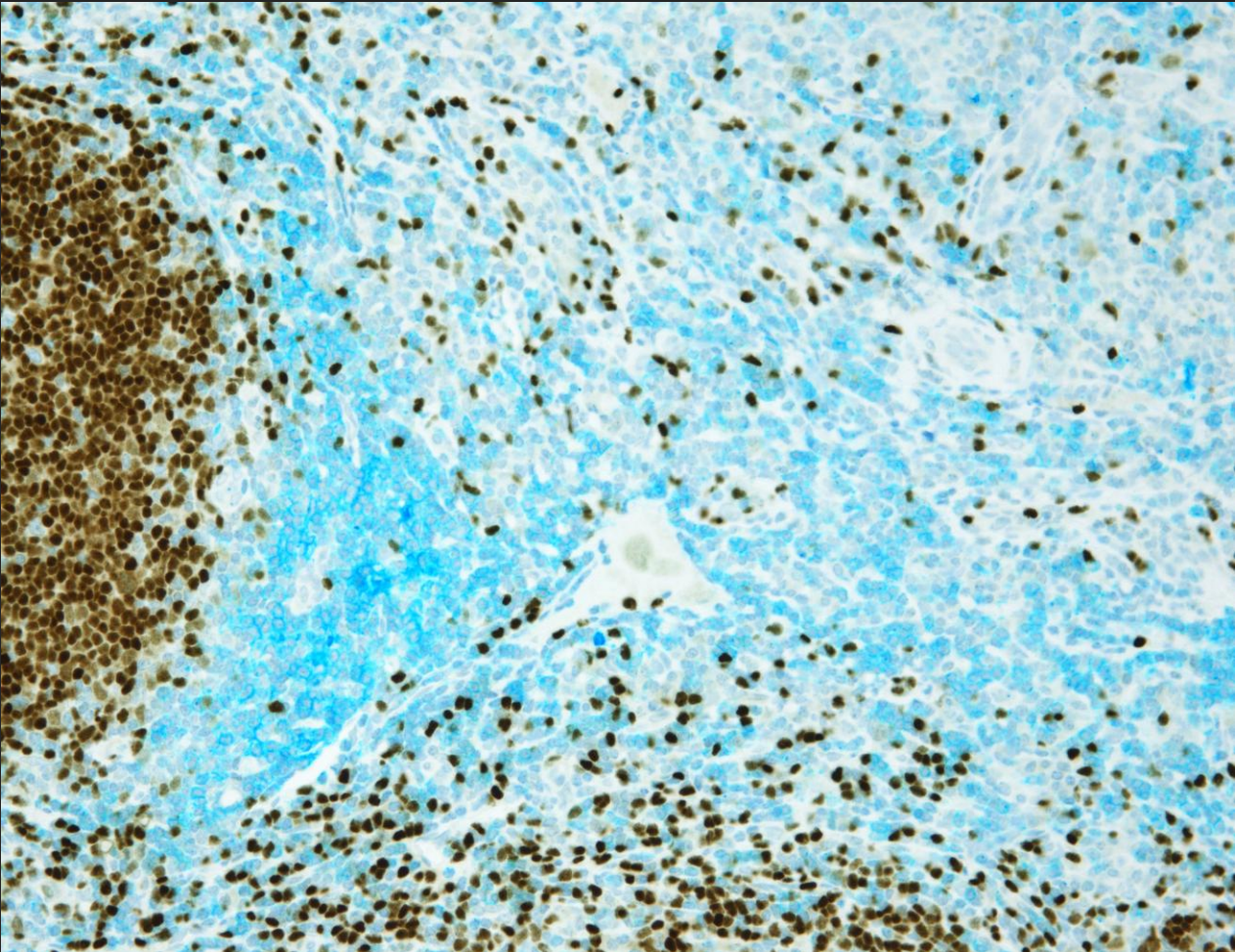
CCR4, membrane (Green) +
TOX, nuclear (Purple)



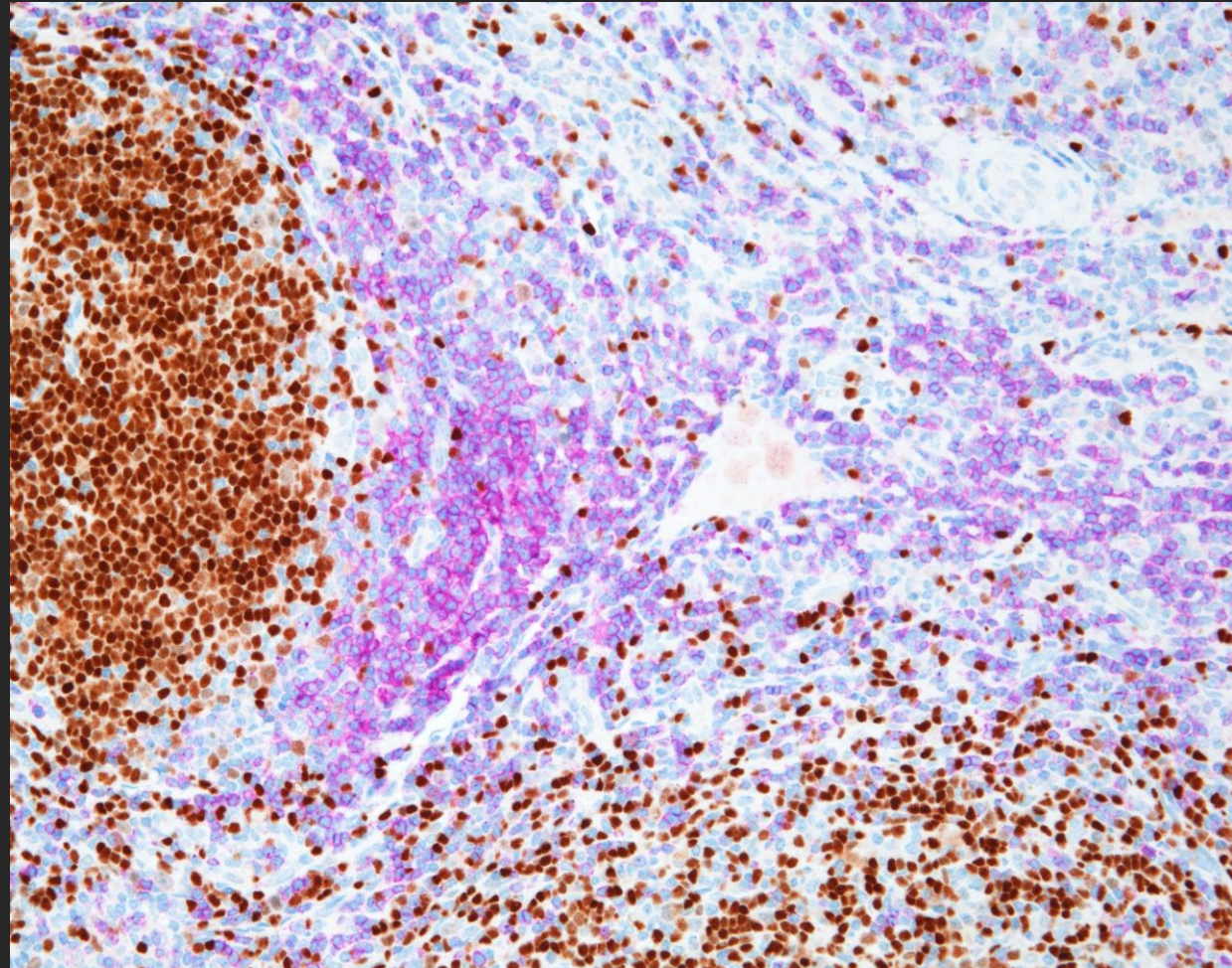
Multiplex staining considerations

Contrast

PAX5, nuclear (DAB)
CCR7, membrane (Teal)



PAX5, nuclear (DAB)
CCR7, membrane (Purple)



The future of multiplexing: from IHC to spatial multiomics

Platform Comparison

What each technology adds as multiplexing moves toward spatial multiomics

Platform	Core method	Protein	RNA	Spatial / biological output
DISCOVERY ULTRA	Automated IHC / ISH	Multiplex IHC; chromogenic or IF (up to 8 proteins)	ISH; RNAscope/BaseScope-compatible	Morphology-preserving multiplex tissue staining
COMET	Sequential IF + cyclic imaging	Up to 24 proteins	Up to 12 RNAscope targets	Same-section RNA + protein; subcellular imaging
PhenoCycler-Fusion 2.0	Barcode-based cyclic IF	100+ biomarkers	Protein-focused platform	Whole-slide, single-cell spatial phenotyping
Hyperion XTi	Imaging Mass Cytometry	40+ simultaneous markers	Specialized multiomic workflows possible	High-dimensional spatial proteomics; no spectral overlap
Xenium / CosMx	Spatial molecular imaging	Xenium: up to 27; CosMx: 64+	Xenium: 100s; CosMx: >19,000 RNAs	Single-cell / subcellular transcriptome + protein context
GeoMx DSP	Digital Spatial Profiling (ROI/AOI)	Very high-plex; 1,200+ protein targets available	Whole transcriptome: 18,000+ genes	Compartment-/ROI-level molecular profiling; pathway discovery

Multiplexing is evolving from “more stains on one slide” toward integrated protein + RNA + morphology + spatial context.

Keypoints

Always include appropriate Drop-out controls to identify potential cross-reactivity and sequence-dependent artefacts.

Vendor recommendations are starting points — not final answers.

Optimize and test empirically. What works in one setup may not work in another.

Drop-out Controls

Do not rely solely on vendor protocols. Use their recommendations as guidance, but validate the assay on your own tissue, platform and workflow.

Let the staining result guide you. Multiplex assays often require repeated testing and adjustment.

Remember Drop-out controls

Our (the labs) responsibility is to provide pathologists and researchers with reliable, well-validated results they can trust.

The background of the slide is a dense, microscopic view of tissue. It features numerous small, rounded cells with prominent, dark blue nuclei. Interspersed among these are smaller, more irregular cells in shades of green and orange. The overall texture is granular and complex, typical of a histological section.

Thank you for your attention