

# Workshop in Diagnostic Immunohistochemistry Aalborg Hospital, September/October 2021

Immunohistochemical double stainings – 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 2 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 ("traditional double-staining techniques") :**

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





# Cervix / dysplasia



# **Multiplex techniques (IHC)**

### **Basic procedures:**

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

Immuno-enzymatic techniques

Immuno- fluorescence techniques



### Challenges or considerations performing multiplex staining techniques:

Which double or multi-staining technique should I use ?

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

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

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

Are secondary Abs 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) ?

Immuno-enzymatic DAB based <u>sequential</u> or simultaneous technique

# YES

**Double immunofluorescence technique (simultaneous technique)** 

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

A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method)

### <u>Sequential procedure (Immuno-enzymatic):</u>

Pre-treatment (Antigen Retrieval)

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

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

Counterstain, dehydration and mounting.

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

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

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

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

Inactivation step or blocking procedures not needed



<u>1.Set of Immuno-reagents</u> 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
- Antibody A → Detection A → Chromogen A
  Diluent → Detection B → Chromogen B
- Diluent → Detection A → Chromogen A
   Antibody B → Detection B → Chromogen B
- Antibody A → Detection A → Chromogen A
   Antibody B → Detection B → Chromogen B

- Control 1 (only reaction for A should be observed / no co-localized signals)
  - Control 2 (only reaction for B should be observed / no co-localized signals)

### Double staining

### Typical end-result including controls: Optimizing the protocol

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

### Pancreas



### CK7, OV-TL12/30

CDX2, EP25

Typical end-result including controls: Optimizing the protocol Optimal dilutions of CDX2 (EP25 1:50) and CK7 (OV-TL 12/30 1:50) Sequential staining : Quanto-HRP/ DSB followed by HiDef-AP/WR





Pancreas

**Note : Efficient sheltering capacity of Deep Space Black** 

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

# **Double staining using sequential technique (Immuno-enzymatic)**

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



Lung : Squamous cell carcinoma

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

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

# **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)



Endothelia cells are "double brown positive" /co-localization Non of the combinations are useful ?

### The order of primary antibodies

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



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

# Be critical selecting antibody pair combinations:



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

# 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.

55 . 1 . 1 . 25

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





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

# **Double Staining-IF (simultaneous technique)**



Note expression of CK 8/18 in all tumor cells

**Colon Adenocarcinoma** 

# Multiplex staining using sequential technique (Immuno-enzymatic)

### <u>Sequential procedure (Three markers):</u>

**Pre-treatment (Antigen Retrieval)** 

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

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

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

Counterstain, dehydration and mounting.



EnzMet (silver) and DAB deposit (after 1. and 2. set of immuno-reagens ) 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)



### <u>Tonsil</u>

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











# Double/Multiple 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

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,<sup>1</sup> 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).

Histochem Cell Biol (2000) 113:19-23	© Springer-Verlag 2000
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.

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

### 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. <u>This</u> <u>time may not necessarily apply to other ovens, antibody</u> <u>combinations or buffers used.</u>

Limitations:

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

Always efficient ?

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

ARTICLE

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

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

### **Control studies**



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  $\beta$ -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  $\mu$ m.

### 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 immunoreagents 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

# Multiplex staining techniques (IHC)

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

Use a immuno-enzymatic DAB based sequential or <u>simultaneous</u> technique

# YES

**Double immunofluorescence technique (simultaneous technique)** 

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

A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (Sequential Immunoperoxidase Labelling and Erasing Method)

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



Primary antibodies <u>must be of different host (e.g., mouse and rabbit) or at least different</u> 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) ?

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

# YES

**Double immunofluorescence technique (simultaneous technique)** 

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

A reversed applications of the primary antibodies (sequential technique)

SIMPLE technique (<u>Sequential Immunoperoxidase Labelling and Erasing Method</u>)

**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 <u>(alternatively - MultiVision kit system for rabbit and mouse primaries including all chromogen reagents by Thermo Fisher Scientific (LabVision)</u>

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

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

**Only for research ?** 

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

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



# **New Chromogens – New opportunities ?**

Tonsil: CD20 (m), immPRESS™ Reagent

(HRP), ImmPACT" VIP (purple).

Tonsil: Cytokeratin AE1/AE3 (m),

NovaRED\*\* (red).

ImmPRESS™ Reagent (HRP), ImmPACT™

# Colorfull chromogens from Vector and Enzo

### Peroxidase Substrates



Prostate: Prostate Specific Antigen (m), ImmPRESS™ Reagent (HRP), ImmPACTT DAB (brown).





### **Alkaline Phosphatase Substrates**



Tonsil: Cytokeratin AE1/AE3 (m), Vector<sup>®</sup> ImmPRESS<sup>™</sup>-AP Reagent, ImmPACT\*\* Vector® Red (magenta)



Tonsil: Cytokeratin AE1/AE3 (m),

Tumor: Cytokeratin (s), VECTASTAIN\*

Elite \* ABC Kit, TMB (blue).

ImmPRESS™ Reagent (HRP),

Vector DAB-Ni (gray-black).

Tonsil: Cytokeratin AE1/AE3 (m), Vector® ImmPRESS™-AP Reagent, Vector\* Blue (blue).











*Enzo* 

Tonsil: LCA (m), immPRESS™ Reagent (HRP), ImmPACT™ AMEC Red (red).



Prostate: Prostate Specific Antigen

(m). ImmPRESS\*\* Reagent (HRP),

ImmPACT"AEC (red).

**Co-localized signal:** 

# Fluorescent dyes ? **Choice of chromogen ?**

**Courtesy: Ole Nielsen, Dept. of Pathology, Odense University Hospital, Denmark** 

**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 kits (Naestved Lab):**

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

Lab Vision<sup>™</sup> 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)





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)



**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)



Nestin

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- $\beta$ , 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  $\mu$ m.



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

### Requirements

Chromogen that can be erased (AEC)

### **Aqueous mounting**

Imaging of slides

### **Removal of coverslip**

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

Efficient blocking procedure for the immunoreagents applied

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



First sequence: CD146 immunostaning => AEC Impact => coverslip Imaging => de- coverslip => Erasing (Acetone) => Blocking using HIER (Ci)

Melanoma

# SIMPLE technique: CD146 (EPR3208) + Neg



**Control experiments:** 

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

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

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

Melanoma

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



• • • • • • •

Melanoma

# Photo Shop manipulated

"Digital imaging"



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



AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

Cycle (immunostaining) repeated with CD15 /DAB



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



AEC Erasing and Blocking (Elution / Denaturation)

**De-coverslip (buffer)** 

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

Cycle (immunostaining) repeated <u>without</u> CD15 /DAB



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



AEC Erasing and Blocking (Elution / Denaturation)

**De-coverslip (buffer)** 

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

Cycle (immunostaining) repeated with CD30 /DAB

Staining looks OK, but .....



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



AEC Erasing and Blocking (Elution / Denaturation)

De-coverslip (buffer)

AEC Wash (Erasing) / Acetone

Blocking with Ci pH 6 (99°C/30`)

Cycle (immunostaining) repeated without CD30 /DAB

Problem with the blocking procedure:

High affinity Abs ? Antigen density ? Inefficient blocking procedure ?



### Combining SIMPLE technique with sequential double immune enzymatic method

CD30 (first cycle)  $\rightarrow$  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.



# Simple techniques and number of markers ?



### Section of human head and neck squamous cell carcinoma

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

Scanned slide (areas of interest) was aligned using CellProfiler

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

### Quantification of multiplex IHC





# HHS Public Access Author manuscript

Cell Rep. Author manuscript; available in PMC 2017 August 21.

Published in final edited form as: *Cell Rep.* 2017 April 04; 19(1): 203–217. doi:10.1016/j.celrep.2017.03.037.

Quantitative multiplex immunohistochemistry reveals myeloidinflamed tumor-immune complexity associated with poor prognosis Can we make the Simple technique even more simple ?

# Do we have to use a sequential technique ?

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

**Simultaneous** Immunoperoxidase/phosphatase Labelling and Erasing Method

**SIMPLE-Technique** 

# **SIMPLE-Technique (simultaneous procedure)**

**Dewax and Pretreatment (Antigen Retrieval)** 

**Incubation with a mix of primary antibody reagents** 

Rabbit & Mouse monoclonal antibodies

**Incubation with Dual-labelling Detection reagents** 

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

Incubation with HRP / Chromogen AEC Impact

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

**Erasing: Acetone followed by app. buffer** 

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

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

Blocking procedure not needed

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

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

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

### Melanoma



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

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

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

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

### Melanoma



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

# **The Future: Advanced multiplex techniques**

### Hyperion Imaging System Workflow

### Imaging Mass Cytometry (Fluidigm)







Design

Stain panels using IHC-validated antibodies conjugated to metal tags.

Image tissues (FFPE and frozen) or fixed cells with metalconjugated antibodies.

biomarkers using precise laser-directed protein capture and detection with CyTOF technology.





Analyze using post-analytical imaging and secondary analysis software tools.



### **Digital Spatial Profiling (Nanostrings)**







CODEX ANTIBODIES CODEX REPORTERS CODEX BARCODES **3 MAIN COMPONENTS OF** шш\_\_\_\_ CODEX TECHNOLOGY CODEX conjugated antibodies Fluorophore conjugated Activated oligonucleotides for building antibody panels onucleotides for visualization for easy conjugation of custom CODEX antibodies of CODEX antibodies CODEX ANTIBODY PANEL TISSUE STAIN SINGLE-STEP STAINING TO 大大大大大大大大 PRESERVE SAMPLE INTEGRITY -大大大大大大大大 大大大大大大大大 大大大大大大大大 REVEAL IMAG REMOVE ... -.-> \*\*\* 1. 1. 1. 1. 大大大 大大 **ドドド** 大大 REPEAT

1

AUTOMATED PROCESS OF IMAGING BIOMARKERS USING THE CODEX INSTRUMENT AND A COMPANION MICROSCOPE

Codex (Akoya Bioscience)

# Thank you for your attention



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





### PT: ALL (B-type)

Flowcytometry showed aberrant expression of CD7 in neoplastic B-cells

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

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

### Tonsil (insert`s)

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

### Simultaneous procedure (Immuno-Fluorescence):

Pre-treatment (Antigen Retrieval)

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

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

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

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



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



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

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





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

# Image: Constrained and the second and the s

Bowell

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



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

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

### **Double immuno-fluorescence staining using simultaneous technique**

### LAB Næstved

Simultaneous procedure using TSA amplification:

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

Mechanism of Tyramide amplification:

-Introducing HRP -Incubation with A488 conjugated Tyramide and H<sub>2</sub>O<sub>2</sub>

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

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

Deposit of A488 in close vicinity of Ab/Ag reactions

-Visualization of A488 deposit under fluorescence microscopy





# CD79a (SP18) + CD3 (PS1)

### PT: ALL (B-type)

Flowcytometry showed aberrant expression of CD7 in neoplastic B-cells

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

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

### Tonsil (insert`s)

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



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

Note: Cells of Nervous system - Red



# Tonsil

# **B-CLL**

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

**CD3**, **PS1** + **CD5**, **SP19** 



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