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Invited Lecture

New Immunohistochemical Methods for the Visualization of Formalin-Sensitve Antigens in Routinely Processed Paraffin-Embedded Material

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Introduction

Immunohistochemistry is a widely used technique for diagnostic purposes in hematopathology. The vast majority of surface antigens which can be visualized are detected in frozen material. The number of antibodies applicable in formalin-fixed and paraffin-embedded material is still limited.[1,2,3] The same is true for the detection of immunoglobulins which only can be visualized after formalin fixation if B-cells have strong cytoplasmic lg content like plasma cells or plasmacytoid cells. This also hampers the detection of lg-light chain restriction, still one of the most reliable criterion for the identification of B-cell lymphomas.

New techniques for immunohistochemistry were able to increase the staining intensity. The addition of multiple secondary antibodies using indirect peroxidase or alkaline phosphatase methods were shown to led to such an intensification. The introduction of the alkaline-anti-alkaline phosphatase method[4] as well as the ABC method allowed a further intensification. The major downback, however, is lying in the fact that the coupling of secondary antibodies or complexes mostly constituting from polyclonal antisera also lead to an increase of background staining.

However, the detection of cytoplasmatic lg and some inconstant weak surface lg staining on lymphoid cells after formalin fixation indicates that at least some antigens are not completely destroyed by the fixation procedure but may be masked. The introduction of microwave techniques[5,6,7] even more enabled for a more sensitive detection of some antigens which finally leads to the hypothesis that formalin fixation only in part destroys the antigenbinding sites, but additionally leads to an antigen masking. Following this hypothesis the influence of fixation, possibilities for demasking and enhancement techniques of sensitivity have to be discussed. Subsequently for 1. antigen demasking, 2. decrease of spheric hindrances and 3. multiplication of binding sites have to be developed.

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Fixation

In Table 1 and 2 the different fixatives and different fixation times are given in order to define their influence. In Table 3 the principal results indicating the strong influence of fixation for antigen detection are given. PLP used for a limited time of fixation gives the best results concerning antigen preservation. Nearly similar results can be achieved with the use of buffered paraformaldehyde which is the most widely used fixative. However, it has to be kept in mind that formalin also is a highly unstable solution. The pH has frequently to be adjusted when buffered paraformaldehyde is used. Beside this two main factors, e. g. time of fixation and concentration of the fixative, most intensively influence the staining results. Allover buffered paraformaldehyde 4 to 6% is the fixative in our hands which leads to an optimal morphological preservation of the tissue on the one hand and allows for highest antibody dilution on the other hand. Moreover, the reduction of fixation time to 12 to 24h presents another advantage.

Antigen Demasking

A preteatment for the antigen demasking can be performed by the use of detergents (saponin, Triton X-100) or proteases (trypsin).

The use of concentrations of 0.005 to 0.1 % itself only slightly increases the sensitivity for antigen detection.

In recent years microwave treatment during fixation or staining of routinely processed specimens has been shown to shorten times necessary for fixation[8,9] and also increases immunohistochemical stainings.

Table 1 : Fixatives

1	5% phosphate-buffered **formalin**	
2	**SUSA:**	Mixture from solution A and B to a 20 vol. % 35 % formalin and 4 vol. % acetic acid
	Solution A:	4, 5 g sublimate + 0, 5 g NaCl in 70ml destilled water
	Solution B:	2 g trichloracetic acid in 10ml destilled water
3	**PLP:**	prepare fresh, using the following two stock solutions:
	Solution A:	lysine-phosphate-buffer (buffer 0.2M lysine-HCL and 0.1M Na_2OHPO_4 to pH7.4 and dilute in PBS to 0.1M lysine concentration)
	Solution B:	dissolve 8%(W/V) paraformaldehyde in 5.4%(W/V) D-glucose at 60 °C and add a few drops of 40% NaOH to ease dissolving
	to each solution add Na-m-perjodate to a concentration of 0.05M and mix briefly	
4	**ZIF:** 0,5% zincacetate and 0.5% zincchloride in TBS	

Table 2 : Fixative

Fixative	Time	Temperatures	Remarks
5% PBS-formalin	24h 48h 72h	4°C	
PLP	24h 48h	4°C	
SUSA	2h 4h	4°C	subsequently 4% PBS-formalin
ZIF	2h 4h	4°C	subsequently 4% PBS-formalin

Table 3 : Results

Fixative/Time	Antibody/Concentration		
	CD21 (1 : 1000)	CD23 (1 : 5000)	CD4 (1 : 25)
Formalin 24h	++	+++	++
Formalin 48h	+	++	+
Formalin 72h	+	+	+
PLP 24h	+++	+++	+++
PLP 48h	+++	+++	++
SUSA 2h	+	++	+
SUSA 4h	(+)	(+)	−
ZIF 2h	+++	+++	++
ZIF 4h	++	++	+

Moreover, chaotropic substances as well as heavy metal salt solutions have also been shown to be effective antigen-retrieval agents[6,10]. Table 4 shows a number of available substances which clearly increase staining intensity. This intensification is maximized if these substances are used in combination with microwave treatment.

High concentrations of heavy metal salts or chaotropic substances result in more intensive stainings. However, such high concentrations are also able to destroy the tissue, making the morphological evaluation difficult. Thus, a balance between concentration of heavy metal salts or urea and the time of microwave treatment on the one side and the preservation of morphology on the other side must be found for the different solutions. Moreover, a repeated short microwave treatment is preferable to a single long-time

Table 4 :Intensity of Surface lgM staining, using different concentrations of heavy metal salts and caotropic substances.

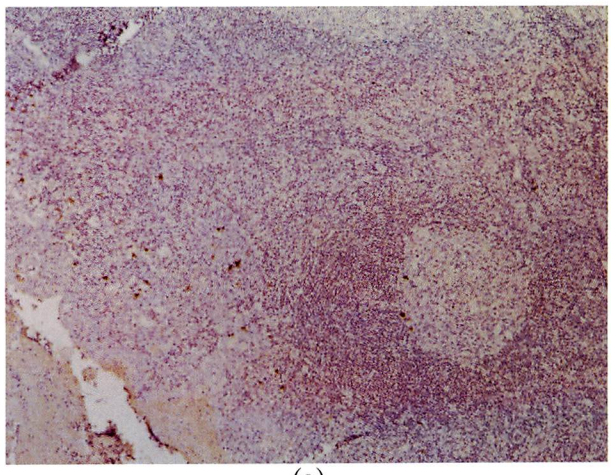
Chemical	Concentration (mM)	pH	Surface lgm
PBS	150	7.4	−
MnCl	1000	5.0	+++*
GnCl	100	5.0	+++
GuCl	1000	5.0	+++*
$CuSO_4$	100	4.0	+
CsCl	100	5.0	+++
ZnCl	100	5.0	+
PbSCN	40		+
PbSCN	160	4.0	+++
Pb-Ac	100	5.0	+
Pb-x	100	7.0	+++
Pb-x	250	7.0	++++
Urea	100	7.0	+
Urea	4000	7.0	++++
Urea	6000	7.0	++++*

*Inferior morphology.
− = Surface(s) lg negative;
+ = slg slightly positive in some cells;
++ = slg slightly positive in all cells;
+++ = slg moderately positive in all cells;
++++ = slg strong positive in all cells,
compared with slg detection in frozen sections.
MnCl = manganese chloride; ZnCl = zinc chloride; $CuSO_4$ = copper sulphate; Gucl= guanidine chlorde; Pb-x = lead-ammonium citrate/acetate complex; Pb-ac = lead acetate iurea (Sigma, Munich, Germany); CsCl = caesium chloride (Roth, Karlsruhe, Germany); PbSCN = lead (ll) thiocyanate (Aldrich, Steinheim, Germany).

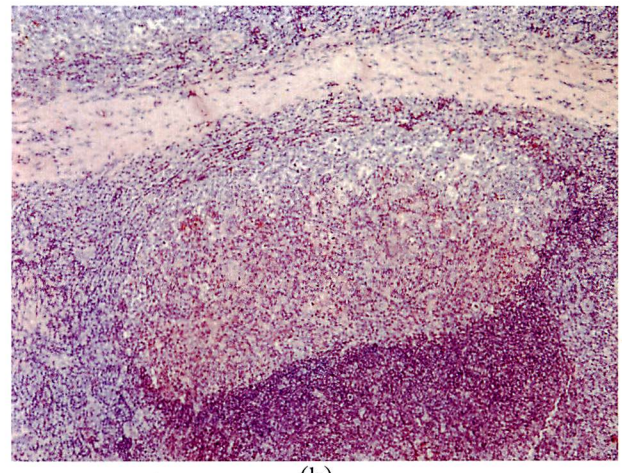
Table 5 :Effccts of single longtime and repeated short-time microwave treatment in respcct of staining intensity of surface lgM and morphology.

Microwave setting	Time*	PbSCN	Urea
1	1	+	+
1	5	+	++
1	10	++	+++
3	1	++	++++
3	5	+++	++++*
3	10	+++	++++*

*Time in minutes operating from boiling. (which was usually reached after 5 min of irradiation at microwave setting 3).
+ = slg slightly positive in some cells;
++ = slg slightly positive in all cells;
++ = slg moderatly positiv in all Cells;
++++ = slg strong positive in all cells,
*Inferior morphology.



(a)



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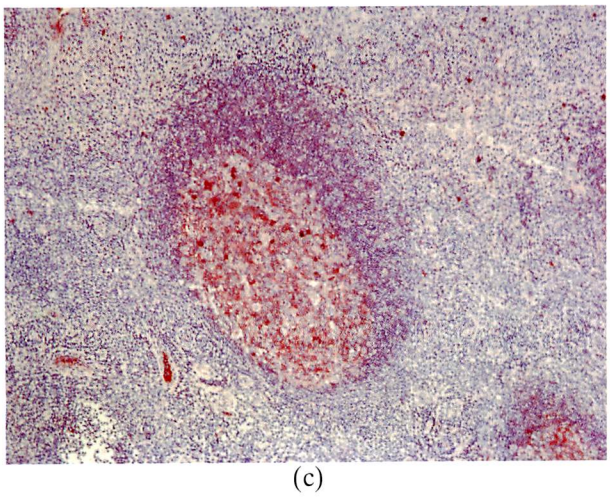

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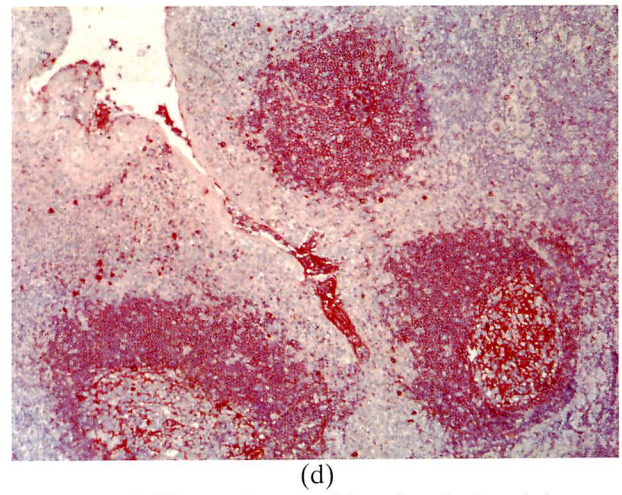
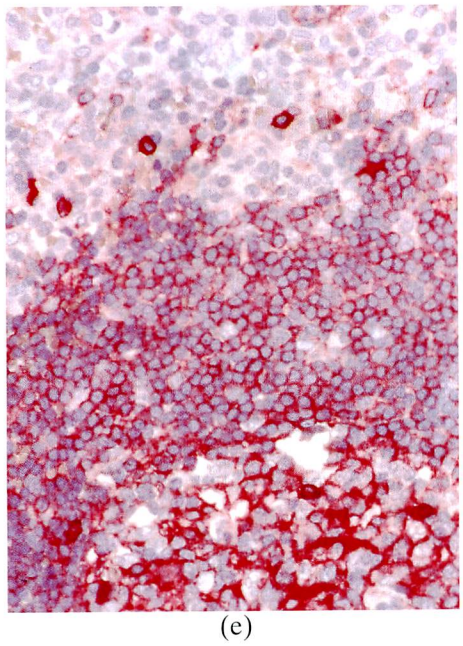


(d)



(e)

Fig. 1 a-e: Different immunohistochemical staining techniques are shown by the use of a monoclonal anti-lgM antibody;

a: indirect peroxidase,
b: APAAP,
c: ABC-method,
d: microwave pretreatment combined with ABC-method (low power),
e: the same method as d (high power). The follicular mantel cells show an intensive surface staining additionally to some surrounding plasma cells and FDC.

treatment (Table 5).

The results also show that a combination of pretreatment with detergents or proteases and microwave pretreatment leads to highest staining intensities.

Figure 1 a to e indicates the different staining results using different immunhistochemical methods for the detection of lgM always at the same antibody dilution. Figure 2 a and b gives an example for surface lg-light chain restriction in a chronic lymphocytic leukemia using a detergent and additional microwave treatment.

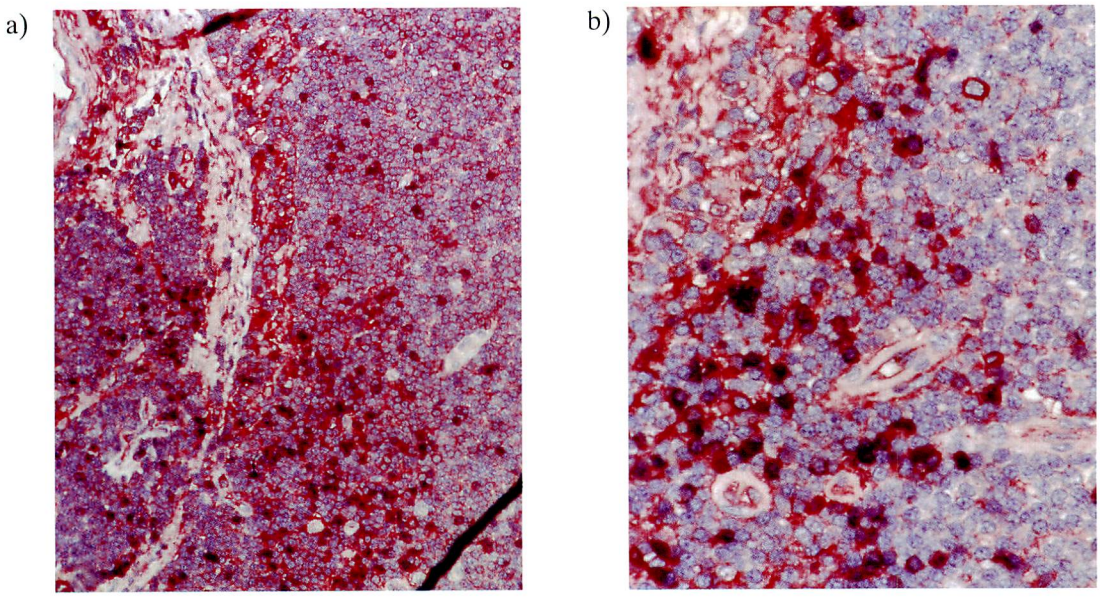


Fig. 2: Light chain *k*- (a) and *λ* - (b) staining shows a restriction to *k* with surface Ig-staining in a case of a B-chronic lymphocytic leukemia.

Multiplication of antibody-binding sites

As mentioned above different immunohistochemical techniques may allow for an increase of the staining intensity due to the addition of enzyme-linked secondary antibodies. However, such an addition of bridging antibodies is limited due to the fact that this paralleled by an increase of the background staining.

A different approach is used by a method which allows for a covalent deposition of biotin molecules[11,12]. Combining such a detection system with the above mentioned pretreatment procedures a 100- to 10,000-fold increase of stainig sensitivity of the immunohistochemical reaction can be achieved. This technique was recently described as the ImmunoMax method[13] which now allows the detection of primarily formalin sensitive antigens.

In principle, after blocking endogenous peroxidase, the section is incubated with a primary antibody to which a secondary biotinylated rabbit anti-mouse or mouse anti-rabbit antibody is bound. With the use of an anti-biotin bridging antibody the ABC peroxidase complex is coupled. Under the influence of H_2O_2 the biotinylated tyramine complex is then covalently coupled to tyrosine (Table 6). The availability of the deposition of a high amount of biotin molecules leads thus to an increase of 30- to 200-fold amplification on its own (Abb. 1).

The use of this ImmunoMax method today allows the detection of a number of leukocyte surface molecules, e. g. CD1a, CD2, CD3, CD4, CD5, CD7, CD26 (Figure 3 and 4) in formalin-fixed tissue.

Abb 1: ABC and ImmunoMax Methood:
Following the ABC peroxidase complex, under the influence of H_2O_2, the biotinylated tyramine, symbolized by the small triangles, couples covalently to tyrosin. The high amounts of biotin molecules lead to an increased binding of ABC-complexes again.

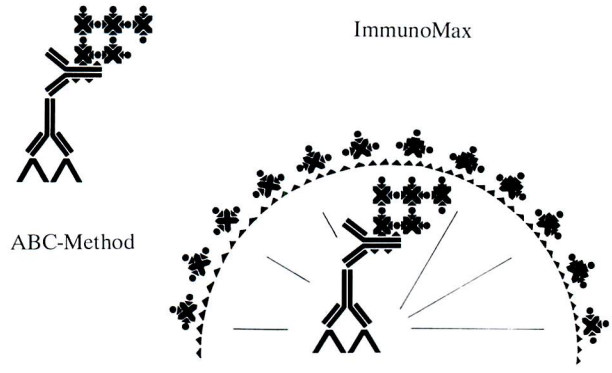


Table 6 : Flow chart of the ImmunoMax Method.

Steps	ImmunoMax Method
1.	Ag retrieval steps
2.	H_2O_2 blocking, rabbit/mouse normal serum
3.	primary Ab
4.	secondary biotinylated rabbit anti-mouse or mouse anti-rabbit
5.	optional: antibiotin-bridging
6.	ABC peroxidase + H_2O_2 + biotinylated tyramine
7.	ABC peroxidase/alkaline phosphatase
8.	color development

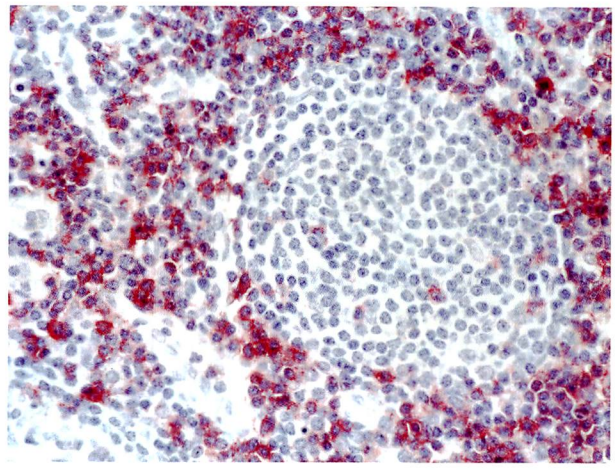
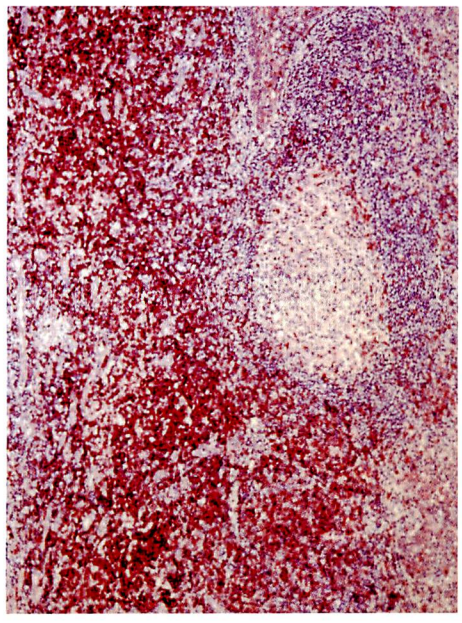


Fig. 3. Staining for CD7 in a human tonsil after formalin fixation.

a)



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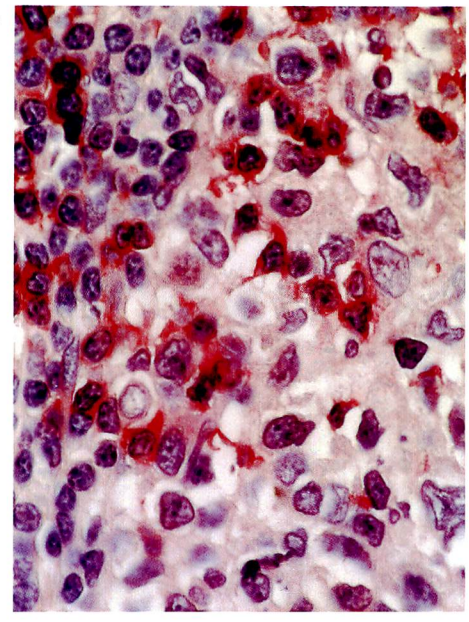

Fig. 4. CD5 staining in a formalin-fixed and paraffin embedded human tonsil(a+b). Recognize the well-preserved morphology of lymphoid cells within the border beween follicular mantle and germinal center(b).

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