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Antibodies validated for routinely processed tissues stain frozen sections unpredictably

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ABSTRACT

Background: Antibody validation for tissue staining is required for reproducibility; criteria to ensure validity have been published recently. The majority of these recommendations imply the use of routinely processed (formalin-fixed, paraffin-embedded) tissue. **Materials & methods:** We applied to lightly fixed frozen sections a panel of 126 antibodies validated for formalin-fixed, paraffin-embedded tissue with extended criteria. **Results:** Less than 30% of the antibodies performed as expected with all fixations. 35% preferred one fixation over another, 13% gave nonspecific staining and 23% did not stain at all. **Conclusion:** Individual antibody variability of the paratope's fitness for the fixed antigen may be the cause. Revalidation of established antibody panels is required when they are applied to sections whose fixation and processing are different from the tissue where they were initially validated.

METHOD SUMMARY

Immunostaining on routinely processed tissue, such as that used for human diagnostic purposes, requires a thorough antibody validation. When antibodies validated for such tissue are applied to frozen tissue sections, contrary to expectation, the different type of fixation may change antibody performance in an unpredictable fashion.

KEYWORDS:

antibody validation ● antigen retrieval ● epitope ● fixatives ● frozen sections ● paratope

Over the last 15 years, awareness of the frequent variability and unreliability of data caused by the use of nonstandardized, unvalidated antibodies in various types of immunoassays such as western blotting, immunocytochemistry (ICC) and immunohistochemistry/immunofluorescence (IHC/IF) [1–4], has prompted common guidelines for the correct use of these reagents. An International Working Group for Antibody Validation (IWGAV) convened in 2016 and published a proposal to ensure reproducibility and validity when using antibodies for immunolabeling [5]. Five validation principles and criteria were proposed based on: stain reduction upon genetic ablation of the target ('genetic'), comparison with an antibody-independent method ('orthogonal'), comparison of two different antibodies against the same target ('independent antibody'), comparison with 'tagged protein expression' and mass spectrometry analysis of the immunocaptured proteins ('IMS'). The suitability of each validation method for several applications, including *in situ* staining methods (ICC and IHC) was also reported [5–7].

The IWGAV recommendations are that 'approaches for antibody validation must be carried out in an application- and context-specific manner' [5], suggesting that antibody validation must be independent for extractive (e.g., western blotting, immunoprecipitation) versus *in situ* techniques (ICC, IHC) on the basis of the different modifications that the protein target undergoes in each procedure (see Supplementary Table 1 in [5]). The IWGAV acknowledges the different antigen modifications that occur in ICC versus IHC, but does not make a distinction between organic and crosslinking fixatives in ICC. Furthermore, a large number of publications do not address the question of whether an antibody that is suitable for routinely processed tissue (formalin-fixed, paraffin embedded; FFPE), the most widely available and robust tissue source, may perform equally well on a frozen section or a cell preparation and *vice versa*. It is, however, a known fact that antigen masking and denaturation in FFPE tissue make it harder to find antibodies that work on both types of tissues. A handful of groups [8–10] have addressed the difference in antibody performance in FFPE versus lightly fixed frozen tissue sections, the latter believed at that time to be the 'gold standard' [8]. In those publications, the focus was on the differential antigen retention and optimal staining with each fixation protocol and with the newly introduced antigen retrieval technique, rather than questioning the antibody specificity, which was a given predefined value. A detailed comparison of antibody specificity and performance between western blots, FFPE sections and paraformaldehyde-fixed cultured cells was previously published [11].

We have previously investigated the epitope specificity and binding requirements of a large number of antibodies for human FFPE material by applying them to routinely fixed porcine material [12]. Peptide variation comparison between the two species suggests that FFPE antibodies detect linear epitopes. About 50% did not react, despite complete identity of the epitope in both species. This may have occurred because of the fine requirement of some antibodies for the conformation of specific target side peptide chains not involved in the antigen binding [13,14] or because of nonidentical juxtaposed adjacent proteins in the fixed complex. Of the antibodies reacting with both species, we had evidence of a species-specific negative effect of formalin fixation on BCL6, restricted to one antibody and not to others, while all reacted on acetone-fixed frozen sections [12]. In a separate investigation [15], we found that the epitopes for some antibodies were selectively destroyed by antigen retrieval (AR), possibly because of the conformational nature of the epitope.

These observations suggest that antibodies validated for FFPE sections may bear undisclosed paratope (antigen binding site) variations which may affect sensitivity, validity and usage when applied outside that context. Therefore we investigated a large panel of antibodies, validated for FFPE, for their ability to label lightly fixed (acetone, formalin) frozen tissue sections. We compared routinely processed FFPE sections with acetone-fixed and formalin-fixed frozen sections.

Antibodies raised against 50- to 150-amino acid sequences recognize linear antigenic determinants that are for the most part localized at the surface of the protein target [16,17], with enhanced specificity for the native protein versus nonspecific binders [17]. Thus, by analogy, we expected that FFPE-proof antibodies directed against linear epitopes would work excellently on lightly fixed frozen material, but we found that this is quite often not the case.

Materials & methods

Human specimens

Fully anonymized human surgical specimen leftovers were either snap-frozen in -80°C chilled isopentane (Merck Life Science, Milan, Italy) or fixed overnight at room temperature in buffered 4% formaldehyde (Bio-Optica Milano Spa, Milan, Italy), processed through a graded ethanol gradient, then in xylene and embedded in molten paraffin for sectioning (FFPE).

The study was approved by the Institutional Review Board Comitato Etico Brianza, N. 3204, 'High-dimensional single cell classification of pathology (HDSSCP)', October 2019. Patient consent was obtained or waived according to article 89 of the EU General Data Protection Regulation 2016/679 and decree N.515 of the Italian Privacy Authority (19 December 2018).

Antibody validation

A list with all the primary antibodies validated for FFPE use can be found in Table 1 & Supplementary Table 1. The type of validation of each reagent was listed essentially according to Edfors *et al.* and Uhlén *et al.* [5,18] and modified for tissue staining. The following criteria were used:

- Orthogonal: an antibody is considered validated if it uniquely identifies a cell in the tissue whose high-dimensional phenotype [19,20] corresponds to a cell described by single-cell RNA sequencing (transcriptomics) and bearing a transcript corresponding to the target;
- Independent antibody: either two antibodies directed against two separate epitopes of the same protein and having an identical staining pattern and/or colocalizing by double IF, or one antibody uniquely identifying a cell in the tissue whose high-dimensional phenotype corresponds to a cell whose phenotype is defined by a multidimensional flow cytometry panel, are considered validated;
- Genetic: an antibody is considered validated it its staining or absence of staining corresponds to a genetically engineered ectopic expression or absence of the target.

Two additional published criteria, not included in the IWGAV list, that confer reliability to the staining and are entirely homogeneous to the *in situ* application were considered. These were:

- Peptide microarray: an antibody recognizing its unique peptide target on peptide microarrays [17,21,22] is considered validated;
- Cross-species: an antibody whose reactivity is conserved across genomic target sequence variations in a close mammalian species [12] is considered validated.

We added an additional criterion, 'historic', for any antibody whose widespread use in multiple applications (e.g., CD20) strongly suggests validity, despite lacking one of the criteria listed above. These may be equivalent to the 'level 1' antibodies in Howat *et al.* [2].

Evidence of validation not present in the published literature (either peer-reviewed papers or documentation from producers) was produced in-house. Some antibodies were validated according to multiple criteria. Validated, FFPE-proof antibodies directed against the same protein can be used exchangeably on routinely processed sections, besides obvious variations in host species or isotype [23].

Representative images of the FFPE staining for each antibody can be viewed in published papers [20,24], in [25] or at the Human Protein Atlas website (https://www.proteinatlas.org/).

Frozen tissue fixation

Frozen tissue blocks were sectioned at 5 μm thickness in a Leica CM1850 cryostat (Leica Microsystems GmbH, Wetzlar, Germany) at -20°C. Sections were collected on positively charged slides (Menzel-Glaser Superfrost Plus; Bio-Optica Milano Spa).

Table 1. Primary antibodies, validation and results on sections.

Antibody	Clone	FFPE	Validation	Ac	FA	FA > AR	Classification	Spurious reactivity
AID	MAID-2	Yes	Genetic, independent Ab	Neg	Neg	Neg	Negative	
AXL	C89E7	Yes		+++	+	+/−	Misc	
bcl-2	Bcl-2-100	Yes	Genetic, historic	+++	+++	+++	Conserved	
BCL6	D-8	Yes	Independent Ab	+++	+++	++	Conserved	
Blimp1/PRDM1	6D3	Yes	Independent Ab, historic	†	+/−	+++	FA-blocked	
BMI1	F6	Yes		Neg	Neg	Neg	Negative	
CD10	NCL-CD10-L-270	Yes	Cross-species	++	++	++	Conserved	
CD103	EP206	Yes		+++	neg	+++	FA-blocked	
CD11c	B-6	Yes		Neg	Neg	Neg	Negative	
CD123	7G3	No	Independent Ab	+++	+++	+++	Conserved	
CD123	NCL-L-CD123	Yes	Orthogonal, historic	Neg	Neg	+++	Conformational	
CD133	AC133	Yes	Orthogonal	+++	++	+	Conserved	
CD14	5A3B11B5	Yes	Independent Ab	+/−	†	+++	Conformational	spurious
CD14	poly	Yes	Independent Ab	Neg	Neg	Neg	Negative	
CD141	EPR4051	Yes	Independent Ab	+++	Neg	++	FA-blocked	
CD141	D-3	Yes	Independent Ab	Neg	Neg	+++	Conformational	
CD16	2H7	Yes	Independent Ab	Neg	Neg	Neg	Negative	
CD163	GHI/61	No	Independent Ab	+++	+++	Neg	Conformational	
CD163	RM3/1	No	Independent Ab	+++	Neg	Neg	FA-blocked	
CD163	10D6	Yes	Independent Ab	Neg	Neg	+	Conformational	
CD1A	O10	Yes	Historic	+++	+++	Neg	Conformational	
CD1c	2F4	Yes	Orthogonal, independent Ab	Neg	Neg	Neg	Negative	
CD1c	2D4	Yes	Orthogonal, independent Ab	Neg	Neg	Neg	Negative	
CD1c	UMAB46	Yes	Peptide microarray, orthogonal	Neg	Neg	Neg	Negative	
CD2	B-8	Yes		Neg	Neg	Neg	Negative	
CD20	L26	Yes	Historic	+++	+++	+++	Conserved	
CD206	#685645	Yes		†	+++	+++	FA-dependent	spurious
CD207 langerin	poly	Yes	Historic	Neg	Neg	Neg	Negative	
CD21	2G9 + A3	Yes	Historic	+/−	Neg	+++	FA-blocked	
CD22	SP104	Yes		+++	+++	+++	Conserved	
CD23	UMAB101	Yes		Neg	Neg	++	Conformational	
CD248	B1/35	Yes		+++	Neg	++	FA-blocked	
CD27	EPR8569	Yes		+	+++	+++	Conserved	
CD271	NGFR 5	Yes	Historic	+++	+	++	Conserved	

FFPE: yes = working on routinely processed material.
Validation: type and source of validation as detailed in Materials & methods section.
Classification: negative = no staining; conformational = conformational epitope; conserved = staining conserved over fixation types; FA blocked = reactivity blocked by FA fixation; FA dependent = staining depends on FA fixation; negative = no staining; misc = miscellaneous behaviour, mostly reacting with acetone-fixed only, not comprised in the previous classification. Spurious = spurious, non-specific reactivity present. For details of the classification, see text.
†Background, nonspecific staining.
‡Pan epithelial staining.
§Germinal center and dendritic cells staining.
¶Nuclear background staining.
#Endothelium staining.
††Basal tonsil epithelium staining.
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§§Interstitium staining.
¶¶Cytoplasmic positive.
##Pan-nuclear staining.
†††See Figure 2.
Additional data such as species, vendor, RRID number, validation and references can be found in Supplementary Table 1.
Ab: Antibody; Ac: Acetone; AR: Antigen retrieval; FA: Formaldehyde; FFPE: Formalin-fixed, paraffin-embedded.

Table 1. Primary antibodies, validation and results on sections (cont.).

Antibody	Clone	FFPE	Validation	Ac	FA	FA > AR	Classification	Spurious reactivity
CD3	poly	Yes	Independent Ab, cross-species	+	Neg	Neg	FA-blocked	
CD3	CD3-12	Yes	Independent Ab	+++	+/−	++	FA-blocked	
CD3	SP7	Yes	Independent Ab, historic	+++	#	+++	FA-blocked	spurious
CD30	BerH2	Yes	Independent Ab, historic	+++	+	+	Conserved	
CD30	CON6D/ C2	Yes	Independent Ab	+++	+/−	+++	FA-blocked	
CD303	AC144	No	Independent Ab	+++	Neg	Neg	Misc	
CD303	poly	Yes	Independent Ab	†	†	†	Negative	spurious
CD303	124b3.13	Yes	Independent Ab	‡	§	Neg	Negative	spurious
CD30v	E4L4I	Yes	Independent Ab	Neg	Neg	Neg	Negative	
CD31	JC70	Yes	Historic	+	++	+++	Conserved	
CD32abc	B-4	Yes		Neg	¶	†	Negative	spurious
CD34	43A1	Yes	Independent Ab	+++	+++	+/−	Conformational	
CD39	IMG17B5F11	Yes		Neg	Neg	Neg	Negative	
CD4	EPR6855	Yes	Independent Ab	+	+/−	+++	Conformational	
CD4	MT310	No	Independent Ab	+++	Neg	Neg	FA-blocked	
CD45	Hle-1 2D1	No	Independent Ab, historic	+++	+++	Neg	Conformational	
CD45+43	PD7/26 + 35-Z6`	Yes	Independent Ab, historic	+++	+++	+++	Conserved	
CD45R0	UCHL-1	Yes	Independent Ab, historic	+++	++	+++	Conserved	
CD5	CD5/54/F6	Yes	Cross-species, historic	+/−	Neg	+++	FA-blocked	
CD56	123C3.D5	Yes	Historic	#,††	#	#	Negative	spurious
CD64	OTI3D3	Yes	Orthogonal	Neg	‡‡	+++	Conformational	spurious
CD68	KP1	Yes	Orthogonal, independent Ab	+++	++	+++	Conserved	
CD68	PGM1	Yes	Orthogonal, independent Ab	Neg	+/−	+	FA-dependent	
CD69	poly	Yes	Orthogonal	Neg	Neg	Neg	Negative	
CD7	poly	Yes		+	Neg	Neg	Misc	
CD74	UMAB231	Yes	Orthogonal	+/−	Neg	+/−	FA-blocked	
CD79a	JCB117	Yes	Independent Ab, cross-species	++	+	+++	Conserved	
CD79a	HM47/A9	Yes	Independent Ab	+++	+++	+++	Conserved	
CD8	C8/144B	Yes	Independent Ab, historic	+++	††	+++	Conserved	spurious
CD83	F-5	Yes	Independent Ab	Neg	Neg	++	Conformational	
CD86	poly	Yes		Neg	Neg	Neg	Negative	
CD8beta	F5	Yes	Independent Ab	+/−	Neg	++	FA-blocked	
CDKN1B p27	DCS-72.F6	Yes	Orthogonal	+++	+	++	Conserved	spurious
CLEC10A	poly	Yes	Orthogonal	+/−	‡	‡	Negative	spurious

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CLEC9A	poly	Yes	Orthogonal	Neg	Neg	+	Conformational	
CLEC9A	14N8D7	No	Orthogonal	Neg	Neg	Neg	Negative	
cMAF	poly	Yes		Neg	+/−	+/−	FA-dependent	
cREL	poly	Yes	Orthogonal	+/−	Neg	+	FA-blocked	
CXCL13	poly	Yes	Independent Ab	+++	+	++	Conserved	
CXCR5	#51505	Yes		+++	§§	+++	FA-blocked	spurious
EOMES	poly	Yes		Neg	Neg	Neg	Negative	
FoxP3	236A/E7	Yes	Independent Ab	++	Neg	+++	FA-blocked	
GATA3	poly	Yes		Neg	Neg	Neg	Negative	
GZMB	GRB7	Yes	Historic	Neg	+++	Neg	Misc	
GZMK	GM6C3	No		+++	++	+++	Conserved	
Histone H3	1D8	Yes		+/−	Neg	+++	Conformational	
HLA-DR	L243	No	Orthogonal, independent Ab	+++	+++	Neg	Conformational	
HLA-DR	SPM288	Yes	Orthogonal, independent Ab	+++	+++	+++	Conserved	
ID1	BCH-1/195-14	Yes	Genetic	Neg	Neg	Neg	Negative	
ID2	BCH-3/9-2-8	Yes	Genetic	¶¶	+++	++	FA-dependent	Spurious
IDO	D5J4E	Yes		Neg	Neg	Neg	Negative	
IgD	poly	Yes	Independent Ab	+++	++	+++	Conserved	
IRF4	3E4	Yes	Independent Ab	+/−	+	+/−	Conserved	
IRF4	poly	Yes	Independent Ab, cross-species	+/−	+++	+++	FA-dependent	
IRF8	E-9	Yes	Independent Ab	+/−	+++	+++	FA-dependent	
IRF8	poly	Yes	Independent Ab, cross-species	Neg	+/−	+/	FA-dependent	
kappa	poly	Yes	Independent Ab, orthogonal	+/−	+++	+/−	Conserved	
Ki-67	SP6	Yes	Independent Ab, historic	++	+++	+++	Conserved	
Ki-67	UMAB107	Yes	Independent Ab	+++	+++	+++	Conserved	
Ki-67	MIB3	Yes	Independent Ab, historic	+++	+++	+++	Conserved	
LAG3	11E3	Yes	Independent Ab	++	Neg	Neg	Misc	
lambda	poly	Yes	Independent Ab, orthogonal	+/−	++	+++	Conserved	
LEF1	B-10	Yes		+++	Neg	++	FA-blocked	
LYVE1	poly	Yes		+++	+++	+++	Conserved	
LYZ	poly	Yes	Historic	+/−	+++	+++	FA-dependent	
MX-1	poly	Yes	Orthogonal	Neg	Neg	Neg	Negative	
MYC	EP121	Yes	Independent Ab	+++	Neg	++	FA-blocked	

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Antibody	Clone	FFPE	Validation	Ac	FA	FA > AR	Classification	Spurious reactivity
NFkB1 p50	E-10	Yes	Orthogonal	Neg	Neg	Neg	Negative	
NFkB2 p52	C-5	Yes	Orthogonal	+/−	†	+	FA-blocked	Spurious
OX40	Ber-ACT35	Yes	Genetic, independent Ab	+++	Neg	++	FA-blocked	
Pax5	1H9	Yes	Independent Ab	+++	+/−	+++	Conserved	
PD-L1	pool	Yes	Peptide microarray, historic	+++	‡	++	Conserved	Spurious
PD1 pool	UMAB197 + UMAB199	Yes	Peptide microarray, independent Ab	+++	Neg	++	FA-blocked	
PNAd	MECA-79	Yes	Orthogonal	Neg	++	++	FA-dependent	
Podoplanin	NZ-1.2	Yes	Orthogonal	†††	†††	†††	Conserved	
PU1	G148-74	Yes	Independent Ab, historic	Neg	Neg	Neg	Negative	
PU1pool	B-9 + C-3	Yes	Independent Ab	+/−	Neg	+++	FA-blocked	
RBPJ	3E2	Yes		Neg	+	+++	FA-dependent	
RORC	6F3.1	Yes		Neg	Neg	Neg	Negative	
S100AB	poly	Yes	Independent Ab, historic	Neg	+/−	+	FA-dependent	
SOX9	CL0639	Yes		Neg	Neg	+++	Conformational	
TBET	O4-46	Yes		+/−	+++	+++	FA-dependent	
TCF4	NCI-R159-6	Yes	Orthogonal	+++	++	++	Conserved	
TCF7	poly	Yes		+++	Neg	++	FA-blocked	
TCRd	H-41	Yes	Historic	Neg	Neg	Neg	Negative	
TIM3	poly	Yes	Orthogonal	+++	+	++	Conserved	
TOX1	NAN448A	Yes	Genetic	Neg	++	+++	FA-dependent	
TP53	DO-7	Yes	Independent Ab	Neg	Neg	+++	Conformational	
VISTA	D1L2G + D5L5T	Yes	Orthogonal, independent Ab	++	Neg	+/−	FA-blocked	
vWF	poly	Yes	Cross-species, historic	+++	+++	+++	Conserved	
ZEB1	poly	Yes	Cross-species	+++	##	##	Misc	Spurious
ZEB1	3G6	Yes	Cross-species	†	+++	++	FA-dependent	Spurious

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Additional data such as species, vendor, RRID number, validation and references can be found in Supplementary Table 1.
Ab: Antibody; Ac: Acetone; AR: Antigen retrieval; FA: Formaldehyde; FFPE: Formalin-fixed, paraffin-embedded.

Two fixation methods were chosen. In the first, sections on slides were lifted from the cryostat, placed temporarily in a slide rack, immersed in acetone (Merck) at 4°C for 5 min, the acetone allowed to evaporate, then stored overnight at room temperature in a damp-proof box; the sections were either used the next day or stored wrapped in plastic foil at -20°C for later use.

In the second method, slides with the frozen section attached were immediately immersed in 4% buffered formaldehyde (FA), fixed at room temperature for 18 h (overnight), then washed in Tris-containing buffer for formaldehyde quenching [26]. These sections were stored at 4°C in 50mM Tris-HCl buffer (pH 7.5) containing 0.01% Tween-20 (Merck) and 100 mM sucrose.

Previous published experiments [15] showed that fixation in excess of 30 min is required for tissue stabilization and epitope rescue upon AR, and that 48- or 72-h fixation does not add additional masking compared with overnight fixation; therefore we chose the latter fixation time for all the experiments.

Table 2. Summary table of the staining results, grouped by category.

Reactivity	Acetone	FA	FA > AR	Abs (n)	%	Note
Conserved	Pos	Pos	Pos	35	27.8	Reactivity preserved on all fixations
FA-blocked	Pos	Neg	Pos	25	19.8	Inactivated by FA fixation (rescued by AR)
FA-dependent	Neg	Pos	Pos	14	11.1	Requires FA fixation
Conformational	Pos Neg	Pos Neg	Neg Pos	17	13.5	Heat-induced appearance/disappearance
Misc	Pos/neg	Neg/pos	Neg	6	4.8	Mostly positive in acetone-fixed only
Neg	Neg	Neg	Neg	29	23.0	No reactivity
Total				126		

Abs: Antibodies; AR: Antigen retrieval; FA: Formaldehyde; Neg: Negative; Pos: Positive.

Frozen sections were dipped for 5 min in 2.5% horse serum in Tris-buffered saline (TBS; Vector Laboratories, CA, USA) before use for Fc receptor blocking.

Antigen retrieval

AR was performed by placing the sections in an 800-ml glass container filled with the retrieval solutions (10 mM EDTA in Tris buffer, pH 8; Merck) and irradiating in a household microwave oven at full power for 8 min, followed by intermittent electromagnetic radiation to maintain constant boiling for the set time and cooling the sections to about 50°C before use. We found that 20 min of AR on FA-fixed frozen sections did not produce any additional retrieval compared with 1 min or 5 min (Supplementary Figure 1A) but negatively affected the integrity of the formalin-fixed frozen section (Supplementary Figure 1B); therefore a protocol comprising 8 min preheating and 1 min AR was used throughout.

Immunolabeling

Sections were processed for indirect IHC or IF labeling as previously described in detail [20,24], by staining serial frozen sections once without stripping and reprobing.

Briefly, the sections were incubated for 30 min with optimally diluted primary antibodies (see Supplementary Table 1) in combinations of up to four (which allowed cross-control for staining conditions) [23] , washed and counterstained with specific distinct fluorochrome-tagged secondary antibodies [24]. The slides, counterstained with DAPI and mounted, were scanned on an S60 Hamamatsu scanner (Nikon, Florence, Italy) at 20 × magnification [24]. The optimal exposure time for each antibody–fluorochrome combination was set in advance on FFPE and validated over multiple samples in a multiplex experiment on routinely processed material ([19] and data not shown). Quadruple simultaneous IF staining guarantees internal control for effective staining. Dubious results because of sparse cell representation or very weak staining across the treatments were confirmed in single-stain IHC. The vast majority of the tests were done on tonsil sections, which constitute the optimal positive control for a panel in which antileukocyte antibodies are over-represented. Acetone-fixed, formalin-fixed and formalin-fixed AR-treated frozen sections were all processed simultaneously.

Results were scored semiquantitatively (neg, +/−, +, ++, +++) and qualitative staining features (diffuse, nonspecific staining, etc.) were annotated.

Preparation of immunofluorescent & single-color images

Single .ndpi images for each case were saved as .tiff files and autofluorescence was subtracted [24]. Grayscale images were inverted, brightness and contrast adjusted with the default ImageJ function (NIH, MD, USA) [27], simultaneously processing a stack of three experimental sets (acetone, FA, FA followed by AR [FA>AR]) before producing individual images.

Images of DAB-stained sections lightly counterstained with hematoxylin were deconvoluted using an ImageJ plug-in [28] and the DAB-only image used for iconography.

A collection of original TIFF images for the antibody staining is available in the Supplementary material.

Results & discussion

In order to test the validity of staining on lightly fixed tissues for antibodies used on routinely processed material (FFPE), we revisited the validation criteria for each antibody according to extended criteria, including the ones suggested by the IWGAV (Table 1).

Once the validity of the antibody panel was revised, we grouped the antibodies into six classes based on their staining performance (Figure 1 & Table 2).

The overwhelming majority of antibodies chosen (118/126) were selected for being reactive with AR-treated FFPE sections, yet 29 (22.8%) did not stain any type of lightly fixed sections.

Fourteen antibodies – negative or weakly positive on acetone-fixed sections – required formalin fixation for detection (11.1%) (labeled 'FA-dependent' in Figure 1 & Tables 1 & 2) and AR was able to enhance the staining in only three of them.

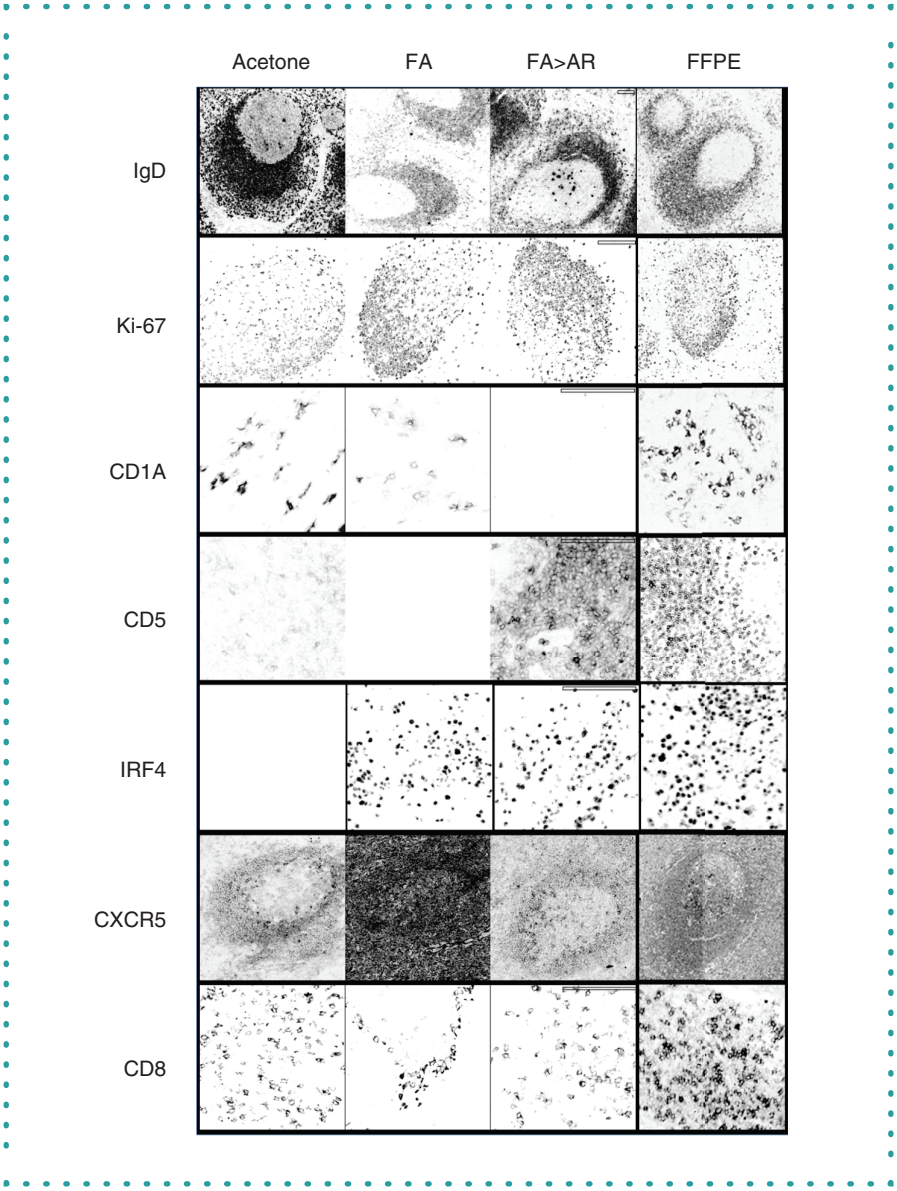

Figure 1. Examples of antibody staining on lightly-fixed frozen tonsil tissue.
From left to right columns, representative examples of staining obtained on acetone, FA, FA followed by AR (FA>AR) and routinely processed tissue (FFPE), respectively. From top to bottom: IgD (goat poly) and Ki-67 (UMAB107) are conserved in intensity across the fixations, the former reduced after FA ('conserved' group). CD1A (O10) staining is lost upon heating (AR) ('conformational' group). CD5 (CD5/54/F6) staining is lost upon FA and regained after AR ('FA blocked' group). IRF4 (goat poly) requires FA to be detected ('FA-dependent' group). CXCR5: FA causes a diffuse, nonspecific staining for CXCR5 (#51505) (spurious staining in 'FA blocked' group). Lymphocyte CD8 (C8/144B) staining is maintained and specific, except upon FA, where it is greatly reduced, while a nonspecific strong staining of the basal epithelial layer is produced (spurious staining in 'conserved' group). Scale bar = 50 μm.
AR: Antigen retrieval; FA: Formaldehyde; FFPE: Formalin-fixed, paraffin-embedded.

FA fixation did reduce epitope detectability in a quarter of the cases and abolished the reactivity in as many as 24 cases (19.8%) ('FA-blocked' in Figure 1 & Tables 1 & 2); in all these instances, AR rescued the optimal reactivity.

The epitope composition of the 'FA-blocked' antibodies was available for five antibodies only and short enough to be informative in one (CD3-12; ERPPPVPNPDYEPC). This epitope contained no lysine, but did contain four other amino acids bound by formaldehyde (arginine, asparagine, aspartic acid and tyrosine) [29] and was sensitive to FA fixation on frozen sections.

In order to define any time-dependent kinetics by which the epitopes might be masked, seven antibodies from the 'FA-blocked' group were selected for the greatest differential between plainly FA-fixed and AR-treated and were tested on sections fixed for increasing times (10 min and 0.5, 3, 6 and 18 h). All but one (CD163/RM3/1) did stain the sections fixed in FA for 10 min. but the increase in fixation time progressively reduced or abolished the tissue stainability (Figure 3).

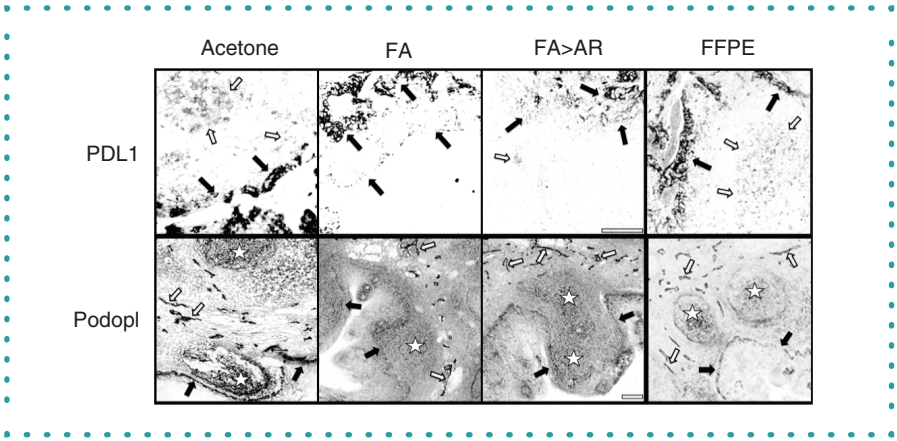

Figure 2. Examples of differential antibody staining depending on tissue fixation. PDL1: macrophage (empty arrows) and epithelial cell staining (black arrows) for PDL1 (cocktail of rabbit MAbs) is maximal in acetone-fixed section, equivalent to FFPE sections. Macrophage stain is all but gone in FA-fixed, but partially retrieved after AR. Epithelial staining is preserved in FA-fixed and a basal layer staining is added; this latter is lost upon AR. Podoplanin (NZ-1.2) produces a bright staining of the basal epithelium (black arrows), endothelial cells (empty arrows) and follicular dendritic (star) cells in acetone-fixed sections. Upon FA fixation, endothelial cells remain strong, while the other structures are weaker. Scale bar = 50 μm.
AR: Antigen retrieval; FA: Formaldehyde; FFPE: Formalin-fixed, paraffin-embedded; MAb: Monoclonal antibody.

Similar data have been obtained on cells grown as single layers and fixed for various amounts of time, whereby past the 2 h of FA fixation, a nuclear antigen was increasingly difficult to detect, requiring antigen retrieval to regain access to the target protein (Supplementary Figure 2). In addition, nonspecific cytoplasmic antibody binding is elicited with increased fixation time.

A group of 17 antibodies (13.5%) had immunoreactivity either selectively elicited by the AR treatment (12 antibodies) or abolished by the heat treatment. We defined those as 'conformational' because of the possible disruption (or creation) of an epitope by altering the conformation of one or more protein loops generating the epitope (Figure 1 & Tables 1 & 2).

A group of six antibodies (4.8%) that we named 'miscellaneous' (Tables 1 & 2) behaved differently than the previous groups and were composed mostly of antibodies reacting with acetone-fixed sections only. One antibody (GZMB) selectively labeled FA-fixed frozen sections.

The remaining antibodies (35/126; 27.8%) had conserved reactivity across the three preparations: acetone fix, FA fix, FA>AR ('conserved'; Figure 1 & Tables 1 & 2).

Whenever the protein target was present in multiple cell types of diverse origin, distinct cell types responded differently to the fixation protocols. As an example, podoplanin (Figure 2) labels three cell types (endothelium, basal epithelium and follicular dendritic cells) but only the endothelial cells were stained with equivalent intensity across the three fixation protocols. Analogously, PDL1 macrophage staining is affected by fixation more than the epithelial staining (Figure 2). For some proteins (e.g., LYZ, S100AB), a loss of the target was noticed from sections in acetone-fixed tissue.

Spurious reactivity, due either to nonspecific background staining or to discrete unrelated targets (Figure 1) was detected in 17 instances (13.4%), 10 of them (7.8%) in FA-fixed sections.

In 19 instances, the same protein was tested with multiple independent antibodies; they all showed variations in staining pattern, except two which had identical staining (Table 1).

A minority of antibodies validated for use on FFPE material can successfully stain frozen sections, either formalin- or acetone-fixed. The most likely reason for such unexpected results may lie in the subtle requirement of the paratope for an unique epitope binding environment, composed not just of the epitope, but also of the neighboring residues on the protein sequence [13,14] or of adjacent unrelated proteins and their posttranslational modifications [30]. This epitope microenvironment is affected by the fixation method and, when applicable, by further denaturation provided by high heat.

We used a panel of antibodies, a third of which had an 'independent antibody staining' type of validation; thus we had more than one antibody for the same protein, staining the FFPE tissue in an identical fashion. Differently from previous published work [8–10], the use of multiple antibodies for the same target (35% of the panel) has disclosed a remarkably heterogeneous staining ability across the experimental conditions, suggesting that the source of variance in these cases is variability of the paratope rather than the epitope. Alternatively, neighboring protein loops might make the epitope inaccessible for one but not another equivalent antibody.

For the first time, we analyzed the effect of FA fixation time of fresh/frozen tissue with a panel of antibodies selected for being affected by the fixative ('FA-blocked'). The progressive masking – for some antibodies almost immediate, for others over several hours – may be caused by two mechanisms. The first is a generic 'sieve' effect by which a progressive amount of generic steric hindrance prevents the antibody from penetrating the tissue and reaching its target; however, this effect would affect equally all antibodies identical

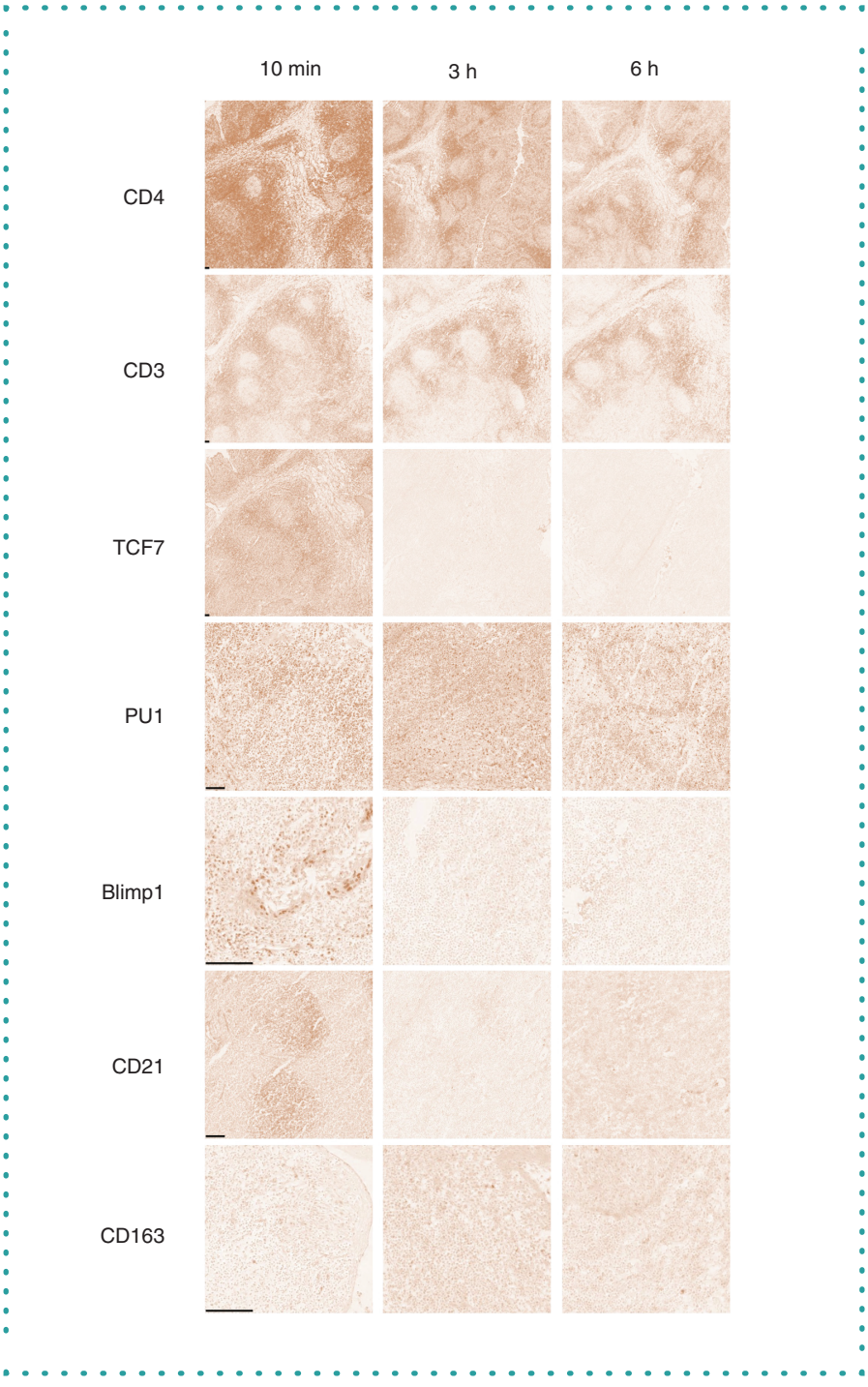

Figure 3. Time-dependent immunostainability of formaldehyde-fixed frozen sections. Frozen tonsil sections were FA-fixed for the time shown on top and stained by indirect immunohistochemistry with DAB. The deconvoluted DAB color is shown. Scale bar = 100 μm.
Antibody clones are: CD4 (MT310); CD3 (rat CD3-12); TCF7 (goat poly); PU1 (B-9 + C-3); Blimp1 (rat 6D3); CD21 (2G9 + A3); CD163 (RM3/1).

in molecular weight and protein characteristics, and this is not the case. The other, more probable, cause is a target-specific progressive change of the epitope microenvironment, affecting to a varying extent each antibody in a paratope-specific fashion. The biochemistry of this time-dependent effect is largely unknown and may depend on protein structure accessibility [31] and formaldehyde-induced transient adducts [32].

The recommendation to limit the use of a particular antibody to the technique for which it has been validated (e.g., western blot, immunoprecipitation, IHC) [5,18] may now be made in a more detailed fashion also for immunological *in situ* techniques, distinguishing

frozen sections from FFPE material and, within frozen sections, whether acetone or FA is used and whether AR is required. Particularly worrisome for staining reliability across fixations is the presence, albeit small, of unrelated discrete reactivity, for example CD8 on epithelia (this report and [33,34]) or the effect of treatment-associated variability on epitopes shared across diverse cell types (e.g., podoplanin, PDL1) (Figure 2).

A bias for this study is the choice of antibodies, enriched for non-IgG1, nonrabbit antibodies (42%), while mouse IgG1 and rabbit monoclonal or polyclonal antibodies are the most frequently available. The selection is the consequence of the multiplexing staining strategy [23] and may result in greater antibody diversification by selecting a variety of host species recognizing the same protein in lightly fixed specimens. More antibodies raised in the same species, of the same isotype and directed against nonoverlapping or partially overlapping epitopes are needed to better investigate our findings.

Collectively, these data suggest that antibody binding may be very dependent on tissue fixation and processing in a subtle and unexpected fashion, these processes affecting binding and specificity for the target. Revalidation is necessary when antibodies are applied to a substrate different from the one on which they have been previously validated.

Future perspective

Lightly fixed material is amenable to various *in situ* hybridization and *in situ* sequencing techniques, which today are combined with antibody-mediated *in situ* proteomics. The efficiency of such multi-omics techniques is much higher on frozen sections than on FFPE material; thus a reconsideration of antibody validation and staining on such material provides a timely reminder of the complexity of the *in situ* antibody staining techniques.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0149

Author contributions

G Cattoretti and M Bolognesi equally designed the experiments. M Bolognesi and M Faretta devised the image analysis algorithms and performed visual and digital image analysis as well as bioinformatic evaluation. F Mascadri and L Furia performed the immunostaining experiments. F Bosisio provided essential reagents. M Bolognesi, F Bosisio and G Cattoretti wrote the manuscript. All authors read and approved the final manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study has been approved by the Institutional Review Board Comitato Etico Brianza, N. 3204, 'High-dimensional single cell classification of pathology (HDSSCP)', October 2019. Patients consent was obtained or waived according to article 89 of the EU General Data Protection Regulation 2016/679 and decree N.515 of the Italian Privacy Authority, 19 December 2018.

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