**Supplementary data and figures for “Antibodies validated for routinely processed tissue unpredictably stain frozen tissue sections.”**

Supplementary material and methods.

Supplementary Material and Methods for Supplementary figure 1

Frozen sections were immediately fixed in 4% buffered formaldehyde (FA) at RT for 18h (overnight), then washed in Tris-containing buffer for formaldehyde quenching [1]. Antigen retrieval (AR) was performed by placing the sections in a 800 ml glass container filled with the retrieval solutions (10 mM EDTA in Tris-buffer pH 8, Merck), irradiate in a household microwave oven at full speed for 8 min, followed by intermittent electromagnetic radiation to maintain constant boiling for 1min., 5 min. or 20 min., and cooling the sections to about 50Co before use. Subsequently the sections were immunostained in indirect immunofluorescence [2] with antibodies against Ki-67, Vimentin and KRT 19 counterstained with DAPI, mounted and images obtained with the S60 fluorescent scanner. To obtain a quantitative estimate of image intensity (Supplemental Fig 1A), autofluorescence background was subtracted and the submaximal fluorescence intensity calculated [3]. Fluorescence intensity for each antibody at 1, 5 and 20 minutes was expressed as percentage increase over the control. To evaluate the effect of AR on section preservation (Supplemental Fig. 1B), sections treated as above were scanned for the DAPI channel and the images were inverted with ImageJ without any further modification.

Supplementary Material and Methods for Supplementary figure 2

Cell culture, Immunostaining and Microscopy

MCF10A non transformed immortalized breast epithelium cells were grown in DMEM 1 Ham’s F12 Medium (1:1) containing 5% FBS, 2 mM glutamine, 50 ng/ml penicillin/streptomycin (all from Lonza, Switzerland), cholera toxin (Sigma-Aldrich, MO), 10 µg/ml insulin (Roche, Switzer- land), 100 µg/ml hydrocortisone (Sigma-Aldrich), and 20 ng/ ml EGF (PeproTech, NJ) at 378Cin 5%CO2. Cells were grown on glass coverslips coated with 0.5% gelatin (wt/vol) in PBS. Exponentially growing cells were fixed in 4% paraformaldehyde (wt/vol) at room temperature (RT) for 10 min (standard conditions), 4h and for an overnight (18 h) to detect effects of crosslinking duration on immune-stainability. Fixed MCF10A cells were washed and permeabilized for 10 min in a permeabilization buffer containing 0.1% Triton X- 100 (vol/vol) in PBS. Coverslips were then immersed for 30 min in a blocking solution, 5% BSA (wt/vol) in PBS, then incubated for 1 h at RT with rabbit anti 53BP1 primary antibody (ab36823, Abcam) diluted 1 to 100 in blocking solution. After washes cells were incubated with Alexa 647-conjugated donkey anti rabbit secondary antibody (Jackson ImmunoResearch). Washed coverslips were then stained with DAPI (ThermoFisher) and mounted in Mowiol-containing mounting media for widefield fluorescence microscopy analysis. Images were acquired with a Nikon Ti2 inverted microscope equipped with an Orca Flash 3 camera (Hamamatsu) employing a 20x 0.75 NA objective. Confocal stacks were acquired to eliminate out-of-focus signals employing an A1R confocal scanhead with a 60x oil immersion 1.4 NA objective.

Acquired images have been processed by the open-source ImageJ software (W. Rasband, NIH,<http://imagej.nih.gov/ij>). Widefield images were corrected by background subtraction using the Rolling-Ball algorithm included in the program. Intensity has been measured by thresholding whole cells and reporting the mean-pixel value. Nucleus vs Cytoplasm Ratio has been calculated measuring respectively the mean pixel values on a nuclear and cytoplasmic Region Of Interest for each cell. Maximum Z projection have been produced in ImageJ to provide a representation of the whole cell volume.

Supplementary figure 1

Effect of Antigen retrieval on immunostainability and preservation of fixed frozen sections.

A: Percentage increase in specific fluorescence over the control for antibodies against Ki-67, vimentin (VIM) and keratin 19 (KRT19) after 1, 5 and 20 minutes of Antigen Retrieval.

B: Changes in tissue preservation (tonsil) in control sections or antigen retrieval-treated sections for 1, 5 or 20 minutes. Note loss of nuclear details and partial detachment, most evident in the 20 minutes treated section. Scale bar = 100 µm

Supplementary figure 2

Effect of extended crosslinking on antibody penetration in single cultured cells (MCF10A, non-transformed immortalized breast epithelium). Cells were immunostained for detection of 53BP1 nuclear protein. A minor fraction of the protein is also present in the cytoplasm.

A: Widefield fluorescence imaging of fixed cells at the indicated time-points (10 min, 4h, 18h). Pixel size: 330 nm.

B: Confocal Maximum Projection of two cells at 10 min and 18 h. A cell representing the most extreme time dependent changes in the detected-antigen distribution have been selected. Pixel size: 70 nm

C: Quantification of the effect on signal intensity and distribution: signal intensity progressively decreased over time (lower graph). A completely altered and artifactual localization was detected with accumulation of the antibody in the cytoplasm and lowering of the nuclear/cytoplasmic ratio (upper graph).

Supplementary TABLE 1

List of primary antibodies.

Abbreviations:

Species: Rb= rabbit; RbMab= rabbit monoclonal ab; g1, g2a, g2b, g3= mouse isotypes.

FFPE: yes= working on routinely processed material.

RRID: Resources Identification Portal identifier (<https://scicrunch.org/resources>)

Supplementary dataset

The folder contains individual antibody staining (~100) on frozen tonsil sections, fixed with acetone, 4% formaldehyde or antigen retrieved (AR) following formalin fixation. Each image is a stack of 3 TIFF unmodified fluorescence images on serial sections. Fixation and antibody name can be found in each image title. The collection is a compressed .zip file.

The dataset can be downloaded at the Mendeley repository:

Cattoretti, Giorgio; Bolognesi, Maddalena M; Bosisio, Francesca M; Faretta, Mario; Mascadri, Francesco (2020), “Antibodies validated for routinely processed tissue unpredictably stain frozen tissue sections.”, Mendeley Data, V1, doi: 10.17632/j88c2ftpsr.1

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2. Bolognesi MM, Manzoni M, Scalia CR *et al.* Multiplex Staining by Sequential Immunostaining and Antibody Removal on Routine Tissue Sections. *Journal of Histochemistry & Cytochemistry*, 65(8), 431-444 (2017).

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