Non-specific binding of antibodies in immunohistochemistry: Fakes and facts

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Protocols for blocking non-specific antibody (Ab) binding in immunohistochemistry are based on rather contradictory and outdated reports. This prompted us to prove, whether non-specific Ab binding may really lead to unwanted background staining in routinely processed cell and tissue probes. In this study, the probes were fixed and processed according to routine protocols with and without blocking step (goat serum or BSA). Surprisingly, all Ab in probes processed without blocking step did not show any propensity to non-specific binding that might lead to background staining, thus implying that endogenous Fc receptors do not retain their ability to bind Fc portion of Ab after standard fixation. Likewise in routinely fixed probes, we did not found any non-specific Ab binding ascribed to a combination of ionic and hydrophobic interactions. The traditionally used protein blocking step is useless in immunostaining of routinely fixed cell and tissue probes.

Keywords Antibodies - Non-specific immunostaining - Endogenous Fc receptors - Blocking steps

The causes of non-specific background immunostaining might be different, but they have one thing in common: they may complicate the use of immunohistochemistry. Whereas unwanted background staining due to endogenous enzyme activities or endogenous biotin is no more a problem in contemporary immunohistochemistry, nonspecific antibody (Ab) binding leading to unwanted background staining remains subject to considerable debate. Among the possible causes of non-specific binding of Ab, attraction of primary and/or secondary Ab to endogenous Fc receptors (FcRs) was supposed to be the main source of unwanted staining.

FcRs are structures on the surface of certain cells, which bind the Fc region of Ab. They provide an important link between cellular and humoral branches of the immune system by triggering several immune responses including phagocytosis, endocytosis, antibody-dependent cytotoxicity, release of inflammatory mediators, and enhancement of antigen

presentation[1]. The nature of responses depends primarily on the cell type on which these FcRs are expressed. There are several different types of FcRs; they are classified based on the type of immunoglobulins that they recognize[2]. FcRs for immunoglobulins G (IgG) - the most common class of Ab used in immunohistochemistry – are designated Fc-gamma receptors (FcγR). Other FcRs are expressed on multiple cell types and are similar in structure to MHC class I. This receptor is involved in preservation of antibodies and also binds IgG[3].

It was theorized that FcRs can bind Fc region of Ab not only *in vivo* but also in immunohistochemical assays of cell and tissue samples. This concept is picked up over and over again in numerous publications, but we were unable to find out the original resource. Since its inception half a century ago, this concept is being entertained in all reviews and handbooks on immunohistochemistry[4-7]. According to this concept, preincubation with with 5-10% normal serum from the host species of the secondary Ab should prevent non-specific Ab binding to endogenous FcRs. Curiously, this is totally senseless in immunohistochemical assays of human probes, since the vast majority of secondary Ab used in human immunohistopathology come from goat and the goat (host species of the secondary Ab) serum was long ago reported not to bind to FcRs on human cells[8]. Preincubation with solutions containing goat normal serum was also assumed to prevent background staining resulting from a combination of ionic and hydrophobic interactions[5]. Blocking the non-specific background due to FcRs or due to a combination of ionic and hydrophobic interactions is regarded as an obligatory step prior to incubation with primary Ab. See immunohistochemical protocols in all contemporary Ab manufacturers' catalogues (e.g., DIANOVA, ZytoMed, Jackson ImmunoResearch Laboratories Inc., etc.). See also a popular IHC WORLD homepage (http://www.ihcworld.com/) and a homepage of the Ab manufacturer (Dako, Education Guide, Immunohisochemical Staining Methods, http://www.dako.com/de/08002_ihc_staining_methods_5ed.pdf). All Ab manufacturers offer their own ready-to-use blocking solutions; in many cases their formulation is a trade secret.

In spite of the fact that goat serum does not bind to FcRs on human cells[8], goat serum nonetheless remains the most popular in use for FcRs blocking in human immunohistopathology. Some histochemists prefer FcRs blocking with normal swine or rabbit serum[9] without, however, providing any experimental support for their choice. Additionally, more complicated blocking strategies have been reported like employing papain-digested whole fragments of unlabeled secondary Ab enriched with Fc fragments of the same IgG[10]. In theory, the most reasonable approach to prevent the possible non-specific background due to FcRs might be the use of $F(ab')_2$ fragments of Ab instead of the whole IgG

molecule[11], provided that the endogenous FcRs retain their ability to bind Fc portion of IgG Ab after proper fixation, but that is namely the question.

Other blocking solutions based on bovine serum albumin (BSA), coldwater-fish gelatin, trypton casein peptone, non-fat dry milk or casein are assumed to prevent non-specific background ascribed primarily to hydrophobic interactions of proteins and to ionic or electrostatic interactions[9, 12, 13]. Casein was claimed to be more effective than normal serum to block hydrophobic background staining[7]. However, casein, BSA and dry milk may contain bovine IgG[14]. Many secondary Ab (i.e. anti-bovine Ig Ab, anti-goat Ig Ab, and anti-sheep Ig Ab) will react strongly with bovine IgG. Therefore, use of BSA, dry milk or casein in the immunohistochemical protocol may significantly increase background and/or reduce antibody titer. Other somewhat more complicated methods thought to reduce Ab binding to tissue proteins, include diluent buffers with a pH different from the pI of the Ab[15]; diluents with low ionic strength (low salt concentration) and addition of non-ionic detergents (e.g., Tween 20, Triton X) or ethylene glycol to the diluent[16]; coincubation of primary antibodies with reduced glutathione, L-cysteine, iodoacetic acid, Ellman's reagent and other thiophilic reagents[17, 18]. The list of recommendations of this kind can be extended, but their practicability is questionable and they are rarely - if any at all - used in praxis.

Furthermore, it was not explicitly documented, whether the non-specific binding of Fc fragments of Ab is a problem equally for frozen and paraffin-embedded tissue sections. On one hand, it was stated (no experimental support provided) that the non-specific staining due to attraction of Fc fragments to FcRs is more common in frozen sections than in routinely aldehyde-fixed paraffin-embedded tissue sections[19, 20]. See also websites: (http://www.ihcworld.com/) and (http://www.dako.com/de/08002_ihc_staining_methods_5ed.pdf). On the other hand, the increased hydrophobicity of proteins after aldehyde fixation and paraffin embedding was claimed to increase the non-specific binding of the Fc portion of IgG Ab[7, 9]. Non-specific staining in paraffin sections was also alleged to happen because of attraction of the Fc portion of IgG Ab to basic groups present in collagen fibers[21].

Taken together, the reports about the possible background immunohistochemical staining due to the non-specific Ab binding in frozen and paraffin-embedded tissue sections and in cytological preparations, are rather contradictory, most of these reports being rather outdated and lacking clear-cut experimental support. This prompted us to prove, whether the commercially available Ab do have a propensity to random non-specific binding in imunolabeling of routinely fixed cell and tissue probes.

RESULTS

In probes that were processed either with or without protein block, we did not observe any differences in immunostaining. Omission of incubation with primary Ab in negative controls also did not led to unwanted background stainings due to anticipated non-specific binding of secondary Ab in probes processed without the protein blocking step, which means that the protein block traditionally used in immunohistochemistry does not influence the quality of immunostaining.

Contrary to the speculative declaration that the unspecific background staining due to endogenous FcRs is more common for frozen sections and cell smears than for paraffin-embedded tissue sections[19, 20], the unspecific background staining has not appeared to be a problem with frozen tissue sections fixed either with formaldehyde (**Fig. 1a-c**) or with acetone, as well as with blood cell smears, cell culture monolayers and cytospins fixed in formaldehyde (not shown).

Likewise, in paraffin sections of formaldehyde-fixed human tissue probes (**Fig. 1d-f**), we have not observed any background staining allegedly ascribed to the increased hydrophobicity of proteins after aldehyde fixation and paraffin embedding[7, 9]. Contrary to the declaration that non-specific staining might happen in paraffin sections because of attraction of the Fc portion of IgG Ab to basic groups present in collagen fibers[21], we did not observed any unspecific background immunostaining in paraffin sections of various collagen-rich tissues like inflammatory bone tissue (**Fig. 1f**), bone tissue seen in bone marrow preparations (**Fig. 2**) or media and adventitia of artery wall (not shown).

In view that FcRs are expressed primarily on monocytes, macrophages, B cells, dendritic cells, neutrophils and platelets[2], we paid special attention to probes where FcRs-bearing cells can be found abundantly - in bone marrow preparations, spleen, tonsils and blood cell smears. Also in these cells, as shown with CD20 immunolabeling of human tonsils (**Fig. 1d**) and with bone marrow preparations immunostained for CD20, CD61 and CD68 (**Fig. 2**), no unwanted background was observed in probes processed with omission of the protein block prior to incubation with primary Ab. Likewise, no unwanted background was found in corresponding negative controls. This allowed us a conclusion that the endogenous FcRs do not retain their ability to bind Fc portion of IgG Ab after fixation routinely used in immunohistochemistry.

Having performed immunostaining using fluorophore-conjugated Ab, we have also found that omission of the protein blocking step did not led to non-specific background

staining in single and multiple fluorescence immunolabeling with the use of fluorophore-conjugated Ab or streptavidin as demonstrated here with immunofluorescent triple staining of cytokeratin 5, cytokeratin 10 and cytokeratin 14 in adeno-squamouse carcinoma of human mammary gland (**Fig. 3**).

DISCUSSION

During the last decades, the improvements in the reagents and protocols for immunohistopatholgy have led to increasing sensitivity of detection systems widely contributing to elimination of non-specific background immunostaining. However, current protocols for blocking the unwanted background are based on rather contradictory and outdated reports. The concept of attraction of Fc fragments of Ab to FcRs, as well as of non-specific Ab binding due to hydrophobic interactions of proteins or ionic and electrostatic interactions, seems to reflect desperate attempts of immunohistochemists half a century ago to find a plausible explanation for bad immunostainings with home-made Ab that were not always the best quality to that time; antisera could be collected or stored in inappropriate ways or primary Ab could be applied in supra-optimal concentrations. The non-specific background immunostaining may also result from other factors, such as inappropriate immunohistochemical detection method, protracted time of chromogen (e.g., DAB) application, improper fixative, protracted fixation time and interval before fixation. Maintaining of the specimen morphology during fixation is the most important prerequisite for good immunostaining[22]. If the origin is garbage, one cannot expect wonderful results.

The present study was performed on cryosections, cell culture monolayers, blood cell smears and cytospins routinely fixed not longer than 15 min in acetone or alcohol. For paraffin embedding, tissue probes and bone marrow preparations were fixed in 4% buffered formaldehyde for 18-48 hrs (usually 24 hrs) at room temperature. Inspection of possible undesirable effects of protracted fixation or the use of stronger fixation media was beyond the scope of this study, since the influence of fixation strength and the length of fixation on the availability and conformation of antigen epitopes, as well as on the maintaining of the specimen morphology, is generally not known, and often not predictable. To allow proper evaluation and replication of immunohistochemical experiments, Ab in this study were applied strictly according to manufacturers' recommendations. Final concentration of primary Ab was between 1 and 5 µg/ml PBS. Final concentration of secondary Ab was between 5 and 10 µg/ml PBS. Keeping these routinized conditions, we did not observe any

differences in immunostaining in all probes that were processed either with or without protein block.

To summarize, our data allowed us to draw the following conclusions. The endogenous FcRs do not retain their ability to bind Fc portion of IgG Ab after routine fixation in formaldehyde, acetone or alcohol. Likewise, non-specific Ab binding to tissue proteins ascribed to hydrophobic interactions of proteins or ionic and electrostatic interactions does not take place in routinely fixed cell and tissue probes. In contrast to the commonly accepted view, the protein block traditionally used in immunohistochemistry is useless in immunostaining of routinely fixed cell and tissue probes.

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AUTHOR CONTRIBUTIONS

I.B. designed experiments, did research, analyzed and interpreted results, and wrote the manuscript; V.S. did immunohistochemistry; W.B. and M.T. directed the study and analyzed and interpreted results.

METHODS

We performed comparative immunostainings with and without protein blocking step on frozen and paraffin-embedded tissue sections, as well as on cell culture monolayers and cytospins. For paraffin embedding, tissue probes were routinely fixed with 4% formaldehyde in PBS. 4 mμ-thick paraffin tissue sections were deparaffinised with xylene and graded ethanols, and antigen retrieval was achieved by heating the sections in 10mM sodium citrate buffer, pH 6.0, at 95°C x 30 min in a domestic vegetable steamer[23]. Frozen tissue sections, cell monolayers, blood cell smears and cytospins were immunostained after fixation either with 4% formaldehyde, methanol or acetone. The blocking step prior to incubation with primary Ab was performed with either 5-10% goat normal serum or 1% BSA in PBS.

All Ab were applied according to manufacturers' recommendations. For immunostainings, we used 45 mouse monoclonal and rabbit polyclonal primary Ab (Table 1). For bright-field microscopy, bound primary Ab were detected with EnVision Horse Radish Peroxidase (HRP) System (DAKO Corporation, Hamburg, Germany) or with AmpliStain™ HRP conjugate (SDT GmbH, Baesweiler, Germany) according to manufacturers' instructions. HRP label was visualized using NovaRed substrate kit (Vector Laboratories, Burlingame, CA, USA). For fluorescence microscopy, we used goat secondary Ab conjugated with Cy3, Alexa Fluor-488, Alexa Fluor-647 or with biotin. The latter was visualized using fluorophore-labeled streptavidin. Secondary system antibodies and other reagents used in this study are presented in Table 2. Single and multiple immunofluorescence labeling were performed according to standard protocols routinely used in immunohistochemistry[23]. Immunostained sections were examined on a Zeiss microscope "Axio Imager Z1". Microscopy images were captured using AxioCam digital microscope cameras and AxioVision image processing (Carl Zeiss Vision, Germany).

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FIGURE LEGENDS

Figure 1 Immunohistochemical staining processed without protein block prior to incubation with primary Ab. Bound primary Ab were detected using DAKO EnVision+ System-HRP (DAKO Corporation, Hamburg, Germany) with NovaRed substrate kit (Vector Laboratories, Burlingame, CA, USA). Nuclei counterstained with Ehrlich hematoxylin. (**a-c**) Immunostaining of human tissue cryosections after routine formaldehyde fixation (3 min by room temperature). (**d-f**) Immunostaining of routinely formaldehyde-fixed paraffin-embedded human tissue sections. (**a**) Immunolabeling of CD34 in capillary endothelium of human kidney. (**b**) Immunolabeling of cytokeratins 8/18/19 in human pancreas carcinoma. (**c**) Immunolocalization of smooth muscle actin in arterial cell wall in human kidney. (**d**) Immunolabeling of CD20 in B lymphocytes in human tonsil. (**e**) Immunolabeling of Glial Fibrillary Acidic Protein in human brain tumor astrocytoma. (**f**) Specific immunolabeling of collagen IV in blood vessel adventitia in inflammatory bone tissue. Note that collagens in connective tissue (collagen I) and in bone (collagen I and V) do not demonstrate any affinity to Fc fragments of either primary or secondary Ab.

Figure 2 Immunodetection of markers of Clusters of Differentiation (CD) in bone marrow preparations. **(a)** Immunolabeling of CD20 in B lymphocytes. **(b)** Immunolabeling of CD61 in megakaryocytes and in thrombocytes. **(c)** Immunolabeling of CD68 in fibroblastic dendritic cell and in monocytes. Bound primary Ab were visualized using AmpliStain™ HRP conjugate (SDT GmbH, Baesweiler, Germany) with NovaRed substrate kit (Vector Laboratories, Burlingame, CA, USA). Nuclei counterstained with Ehrlich hematoxylin. Immunohistochemical staining was performed without protein block prior to incubation with primary Ab. Note in **(a)** the absence of unspecific Ab binding to bone tissue and to hematopoetic cells, in **(b)** the absence of unspecific Ab binding to granulocytes and monocytes and in **(c)** the absence of unspecific Ab binding to granulocytes and megakaryocytes.

Figure 3 Immunofluorescent triple staining of cytokeratin 5, cytokeratin 10 and cytokeratin 14 in adeno-squamouse carcinoma of human mammary gland. Immunolabeling was performed without protein block prior to incubation with primary Ab. (**a**) Immunolocalization of cytokeratin 14 (Alexa 488, green channel). (**b**) Immunolocalization of cytokeratins 10 (Cy3, red channel). (**c**) Immunolocalization of cytokeratin 5 (Alexa 647, pink channel). (**d**) Composite image. Nuclei are counterstained with DAPI.

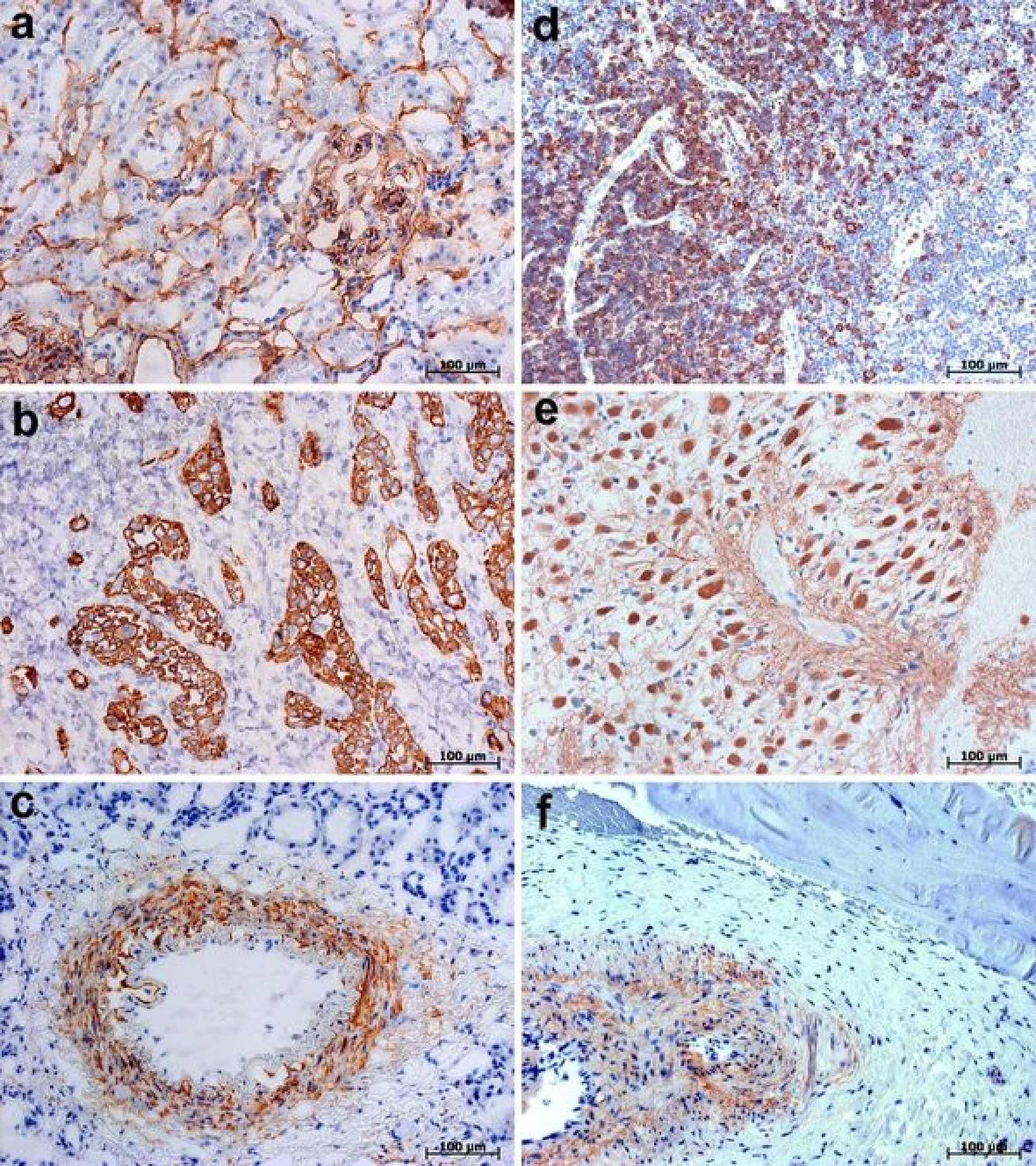
Table 1 Primary antibodies used in this study

Antibodies	Source	Dilution	Tissues/Cells*
IgA (alpha), (rabbit Ab)	DAKO	1/2000	1, 2, 3
IgG (gamma), (rabbit Ab)	DAKO	1/1000	1, 2, 3
IgM (my, μ), (rabbit Ab)	DAKO	1/1000	1, 2, 3
Bcl2 (mouse Ab)	DAKO	1/100	2, 4, 5, 6, 7
α Smooth Muscle Actin (mouse Ab)	DAKO	1/50	4, 5, 6, 7, 8, 9
α Smooth Muscle Actin (rabbit Ab)	AbCam	1/200	4, 5, 6, 7, 8, 9
ApoE (rabbit Ab)	Santa Cruz	1/100	10
CD3 (mouse Ab	Novocastra	1/200	2, 23
CD10 (mouse Ab)	Novocastra	1/50	5
CD20 (mouse Ab	DAKO	1/500	2, 3, 10
CD32 (mouse Ab)	AbCam	1/1000	3, 23
CD34 (mouse Ab)	Novocastra	1/50	1, 9, 25
CD61 (mouse Ab)	Novocastra	1/100	10
CD68 (mouse Ab)	DAKO	1/200	2, 3, 10
CD117, c-Kit (rabbit Ab)	DAKO	1/100	4, 5
Cytokeratins 5 (rabbit Ab)	Medac	1/100	4, 5, 6, 7, 8, 24
Cytokeratin 5/6 (mouse Ab)	DAKO	1/50	4, 5, 6, 7, 8, 24
Cytokeratin 7 (mouse Ab)	DAKO	1/200	4, 5, 6, 7, 8, 24
Cytokeratin 10 (mouse Ab)	DAKO	1/50	4, 5, 6, 7, 13
Cytokeratin 14 (mouse Ab)	Jackson ImmunoRes	1/500	4, 5, 6, 7, 8, 24
Cytokeratin 18 (mouse Ab)	Sigma	1/50	4, 5, 6, 7, 8, 24
Cytokeratin 8/18 (mouse Ab)	Zytomed	1/50	4, 5, 6, 7, 8, 24
Cytokeratin AE1/AE3 (mouse Ab)	DAKO	1/50	2, 4, 5, 6, 7, 13
Cytokeratins 8/18/19 (mouse Ab)	Immunotech	1/100	4, 5, 6, 7, 8
Collagen IV (mouse Ab)	DAKO	1/20	5, 11
Desmin (mouse Ab)	DAKO	1/200	12, 25
E-Cadherin (mouse Ab)	DAKO	1/50	1, 4, 5
Calcitonin (rabbit Ab)	DAKO	1:500	15
Calponin (mouse Ab)	DAKO	1/50	9, 16, 17
EMA (mouse Ab)	DAKO	1/50	1
Estrogen Receptors (rabbit Ab)	Thermo	1/200	4, 5
GFAP (mouse Ab)	DAKO	1/100	18
GFP (rabbit Ab)	AbCam	1/500	19
HMB45 (mouse Ab)	DAKO	1/50	13
Kappa Light Chains (rabbit Ab)	DAKO	1/8000	2, 10
Lambda Light Chains (rabbit Ab)	DAKO	1/8000	2, 10
Ki67 (rabbit Ab)	Thermo	1/200	2, 4, 5, 6 , 7, 24
MIB1 (mouse Ab)	DAKO	1/20	2, 4, 5, 6 , 7, 24
Myf-4 (mouse Ab)	Zytomed	1:50	20
nNOS (rabbit Ab)	Transduction Lab.		16, 17, 21, 22
eNOS (rabbit Ab)	Transduction Lab.		9, 16, 17, 21, 22
P53 (mouse Ab)	DAKO	1/50	5, 7
p63 (mouse Ab)	DAKO	1/200	4, 5, 6, 7
S100 (rabbit Ab)	DAKO	1/2000	2, 5, 7, 10
Vimentin (mouse Ab)	DAKO	1/200	15
Vimentin (rabbit Ab)	AbCam	1/1000	5, 9

***Probes immunostained in this study: Human kidney (1), Human tonsil (2), Human lymph nodes (3), Human mammary gland (4), human breast tumors (5), Human salivary gland (6), Human salivary gland tumors (7), Human lacrimary gland (8), Human aorta (9), Human bone marrow (10), Human bone tissue (11), Gastrointestinal tissue (12), Skin (13), Neonatal rat cardiomyocytes (14), Human thyroid gland (15), Human muscle tissue (16), Rat muscle tissue (17), Human brain astrocytoma (18), Mouse heart (19), Human rabdomyosarcoma (20), Human pancreas (21), Rat pancreas (22), Human blood cell smears and cytospins (23), Cell cultures of human adenoid cystic carcinoma (24) and of neonatal rat cardiomyocytes (25)**

Table 2 Secondary antibodies and other reagents

Antibodies	Source	Dilution	Label
Goat Normal Serum	Jackson ImmunoRes	1/100	w/o
Mouse Normal Serum	Jackson ImmunoRes	1/100	w/o
Bovine serum albumin	Biomol	1%	w/o
Goat anti−mouse IgG Ab	Invitrogen	1/200	Alexa Fluor 488
Goat anti−mouse IgG Ab	Invitrogen	1/200	Alexa Fluor 555
Goat anti−mouse IgG Ab	Invitrogen	1/100	Alexa Fluor 647
Goat anti−rabbit IgG Ab	Invitrogen	1/100	Alexa Fluor 647
Biotin-SP-AffiniPure Fab Fragment Goat Anti-Mouse	Jackson ImmunoRes	2-10 µg/ml	Biotin
Biotin-conjugated anti-mouse IgG3	BD Pharmingen	1/25	Biotin
Streptavidin	Jackson ImmunoRes	1/200	Cy3
Anti-mouse EnVision^{+} System-HRP	DAKO Corporation	ready-to-use	HRP
Anti-rabbit EnVision^{+} System-HRP	DAKO Corporation	ready-to-use	HRP
AmpliStain™ anti-Mouse 1-Step HRP	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
AmpliStain™ anti-Rabbit 1-Step HRP	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
4',6-diamidino-2-phenylindole (DAPI, nuclear counterstaining)	Sigma	5 µg/ml	w/o
Vector® *NovaRED*™ Substrate Kit	Vector Laboratories, Burlingame, CA, USA	ready-to-use	w/o
VECTASHIELD® Mounting Medium	Vector Laboratories, Burlingame, CA, USA	ready-to-use	w/o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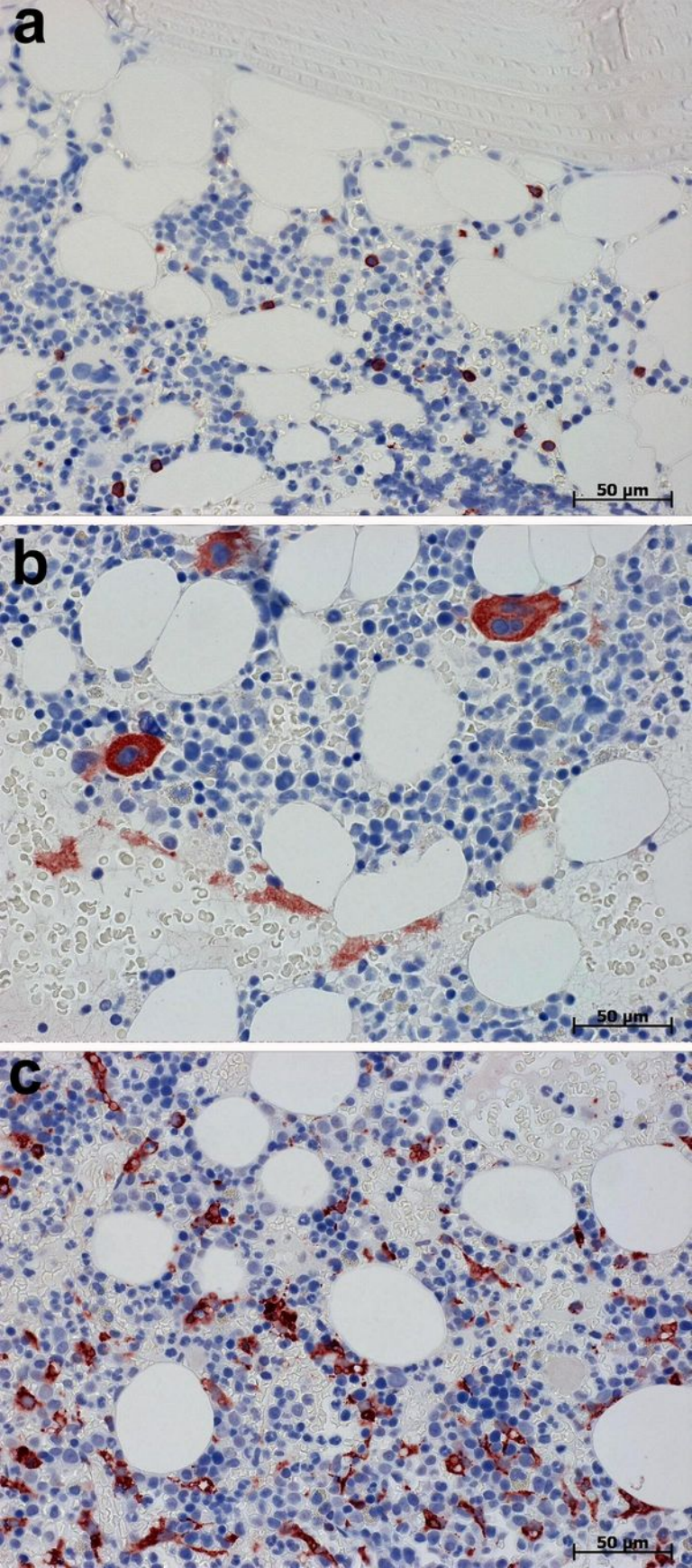
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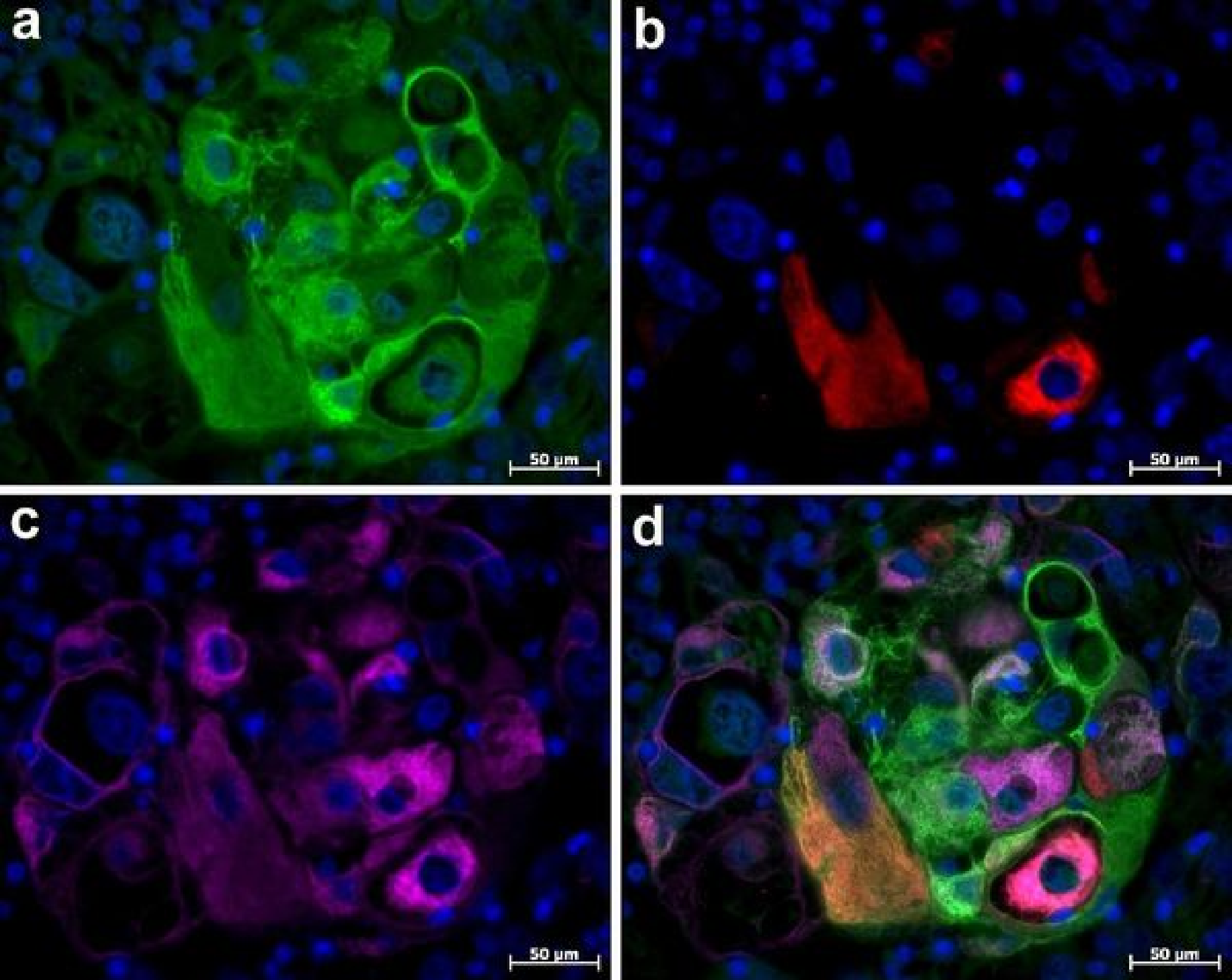
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