# Immunofluorescent Analysis of Connexin-43 Using Monoclonal Antibodies to Its Extracellular Domain

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Immunofluorescent analysis of connexin-43 was carried out on preparations of fixed and living cultures of rat and human glioma cells, HEK293 cells, and frozen sections of the rat brain with experimental glioma using monoclonal antibodies to recombinant extracellular fragment of connexin-43 (E2 second extracellular loop). These monoclonal antibodies visualized membrane and cytoplasmic pools of connexin-43 in preparations fixed with paraformaldehyde. Incubation of monoclonal antibodies to E2 extracellular loop with living cells led to visualization of only connexin hemichannels on cell membranes. No immunofluorescence characteristic of dimer connexons, organizing the gap junction, was detected. This fact indicates that antibodies to connexin-43 extracellular loop E2, obtained in our study, specifically react with target antigen solely at the stage of connexon presentation on the membrane in the form of hemichannels. These monoclonal antibodies can be used for immunophenotyping and sorting of connexin-43-positive cells *in vitro* and as the guide molecules in addressed delivery of diagnostic preparations and drugs to glioma cells *in vivo*.

**Key Words:** *connexin-43; monoclonal antibodies; glioma; immunofluorescence*

Connexin-43 (Cx43, CXA1_RAT) is a transmembrane protein, the main structural component of cell-cell gap junctions or nexuses between astrocytes in nervous tissue, between cardiomyocytes, and some epithelial cells [18]. Using four transmembrane domains, Cx43 forms hexameric structures (connexons), forming the pore in cell membrane. Phosphorylation and dephosphorylation of C-terminal portions "opens" and "closes" the transmembrane channel [3]. By means of two extracellular fragments (E1 and E2) the Cx43 connexons-hemichannels of the neighboring cells form a proper gap junction, functionally connecting cytoplasm of these cells. The ionic homeostasis and cell volume are supported by these channels; they also promote cell-cell signal transfer, including the signals regulating proliferation, differentiation, apoptosis, adhesion, and migration of embryonic cells during the ontogeny [6,7,16,18].

The involvement of Cx43 in the development of invasive tumors of glia origin attracts special attention [3,7,9,15], but the functions of this protein in the course of glioma cell invasion are not quite clear. It was shown, for example, that the intensity of this protein biosynthesis in gliomas decreases with reduction of their differentiation and that the most "malignant" glioma cells (stages III and IV) have a lesser number of active gap junctions [14,20]. It was reported that hyperexpression of Cx43 as a result of tumor cell transfection with a respective gene inhibited their proliferation [13]. On the other hand, other authors noted an increase of Cx43 level in tumor tissue,

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for example, in neoplastic endotheliocytes [9], and observed a stimulatory effect of Cx43 on invasion of human multiform glioblastoma and its analog in rats (experimental C6 glioma) [3,15].

The tumor suppressive effect of Cx43 is attributed to its interactions with CCN3 soluble inhibitor of proliferation (NOV) [10]. However, later studies, carried out by the same team of scientists, have shown that the expression of Cx43 can be paralleled by more intense production of a CCN3-related protein CCN1 (Cyr61), which (by contrast) stimulates glioma cell proliferation and migration [19]. Presumably, due to this Cx43-positive cells of C6 glioma are characterized by higher capacity to migration than Cx43-negative cells [3].

Opposite changes in Cx43 expression in glioma cells and in the adjacent reactive astrocytes can be responsible for controversies on the Cx43 effect on invasion of poorly differentiated gliomas, as is the case with another astroglial marker, GFAP [5]. Presumably, the Cx43-positive astrocytes, forming heterologous gap junctions with glioma cells, play a certain role in active invasion of glioma [16]. It was shown that Cx43-positive cells are more resistant to oxidative stress and other destructive factors [11]. Hence, to clear out the true role of Cx43 in glioma invasion processes is an important task.

An important problem is to obtain antibodies reacting with extracellular fragments of Cx43. Affinity binding of antibodies to these fragments can inhibit or modulate the assembly of gap junctions, which will make it possible to evaluate their role in regulation of cell processes associated with glioma invasion. Polyclonal antibodies to Cx43 extracellular fragments have been obtained. Inhibitory effect on organization of *in vitro* gap junctions has been demonstrated for them, but these studies were not continued, presumably, because of high heterogeneity of polyclonal antibodies [15].

We carried out an immunofluorescent analysis of Cx43 on preparations of fixed and living rat and human glioma cells and on frozen sections of the rat brain with experimental C6 glioma using monoclonal antibodies to Cx43 recombinant extracellular fragment (E2 second extracellular loop).

## MATERIALS AND METHODS

**Recombinant extracellular fragment design.** The nucleotide sequence of the rat Cx43 second extracellular fragment (E2) (Q173-1208; QWYIYGFSLSAV YTCKRDPCPHQVDCFLSRPTEKTI, 36 amino acid residues, mol. weight 4.28 kDa, pl 7.87) was cloned into pCBDQ and pHPMLQ vectors [8]. These vectors contain the sequences of two domains of human plasma membrane Ca–ATPase hPMCA4b (CBD, 1057-1205 amino acid residues, 17 kDa, and HPML, 166-371 amino acid residues, 22.5 kDa) as high-molecular carriers. Immunogenic activity sufficiently high for obtaining antibodies and high expression of prokaryotic cells were demonstrated for these domains [8,17]. Amplification of Cx43 extracellular loop sequence was carried out with primers: Cx43_173F: 5'-GA TCAGATCTCAGTGGTACAT-CTATGGGT-3'; Cx43_173B: 5'-GATCAAGCTTAGATGGTTTTCTCC GTGGGAG-3' (SibEnzyme), containing recognition sites for BgIII and HindIII restrictases, respectively. The *E. coli* SG13009 cells (Qiagen) were transformed with the resultant HPML-Q Cx43 and CBDO-Cx43 plasmid DNA. Transformed cells were cultured at 37°C in 100 ml LB medium with 50 μg/ml carbenicillin and 25 μg/ml kanamycin. Expression of plasmid DNA was induced by isopropylthiogalactoside (1 mM). Proteins were isolated under denaturing conditions on Ni-NTA agarose according to the protocol of the producer firm (Qiagen). The resultant protein preparations were analyzed by electrophoresis in 12.5% PAAG with 0.1% sodium dodecylsulfate. The protocol for obtaining Cx43 recombinant extracellular fragments has been described in detail previously [1].

**Immunization of animals and obtaining of antibodies.** Purified recombinant proteins were used for immunization of mice (20 μg/animal). Four or five subcutaneous immunization cycles with Freund's complete adjuvant (DiaM) were carried out at 10-12-day intervals. Booster intraperitoneal immunization with purified preparation was carried out after 2 weeks and blood was collected for analysis of antibody level after the next 7 days.

Mice with proven immune response were used to obtain monoclonal antibodies. Isolation of splenocytes and fusion with SP/20 myeloma cells were carried out as described previously [2]. Screening of hybridomas was carried out by IFA with Cx43_173HPML and Cx43_173CBD immobilized on solid phase. Goat antimouse peroxidase conjugated Ab (A 9917, Sigma) diluted 1:20 000 served as second antibodies. The immunoperoxidase reaction was visualized with TMB ready-made solution (Zymed). Positive clones selected by IFA were tested by immunoblotting with the same antigens with subsequent visualization by the ECL-advance kit (GE Healthcare) according to instruction [1]. At the last stage of the study, the hybridoma supernatants were tested in immunohistochemical analysis with rat brain sections in order to select the hybridomas working with native Cx43.

Monoclonal antibodies were purified from ascitic fluid by affinity chromatography on agarose with immobilized G protein (Invitrogen) according to the instruction.

Immunohistochemical analysis of antibodies was carried out on frozen sections of the rat brain and

preparations of fixed and living rat and human cell cultures. For fixation the monolayer cell cultures were washed in PBS from serum medium, incubated with 4% neutral paraformaldehyde (pH 7.4; 30 min at 4°C), after which they were washed in PBS. A cocktail of monoclonal and polyclonal antibodies in the needed dilution (usually 1-5 μg/ml) was prepared for double staining, incubated with cell preparations overnight, washed, and the samples were incubated with a cocktail of second antibodies (usually a combination of Goat antimouse Alexa Fluor 488 and Goat antirabbit Alexa Fluor 594, Invitrogen), each antibody type diluted 1:1000. Poststaining of cell structures (actin filaments and nuclei) was carried out using Phalloidin-TRITC (Fluka) and DAPI or TOTO 633 (Invitrogen), respectively. All dilutions and washings were carried out with PBS (pH 7.4) containing 0.2% Twin-20 and Triton X-100 and 1% normal goat serum.

Immunocytochemical analysis of living C6 glioma culture was carried out as follows. Monoclonal antibodies to Cx43 extracellular fragment, sterilized by filtration through Millieux filter (0.22 μ; Millipore Bedford MA), were added into the well with living adherent cells in the serum-containing medium to a concentration of 10 μg/ml, incubated for 1 h, after which the preparation was washed in PBS, fixed in 4% paraformaldehyde, and stained as described previously. In the alternative fluorescent visualization variant monoclonal antibodies to Cx43_173, biotin-conjugated using ProtOn kit (Vector Lab.), and streptavidin conjugated with Alexa 532 by means of Alexa Fluor 532 Protein Labeling kit (Invitrogen) were used. Biotin conjugation and conjugation with fluorophore were carried out according to the instruction. Biotin-conjugated antibodies to Cx43 were sterilized by filtration and added into living C6 glioma cell culture to a concentration of 10 μg/ml. After 1-h incubation the culture medium was replaced 2 times and streptavidin-Alexa 532 (1-5 μg/ml) was added. The fluorescence of living cells was recorded using Leica DM 6000 inverted fluorescent microscope.

## RESULTS

Two strains, producing chimerical Cx43 second extracellular fragment (E2; 173-208 amino acid residues) with N-terminal hexahistidine tag and CBD and HPML polypeptides, were created as a result of the insert cloning in pCBDQ and pHPML vectors and subsequent transformation of *E. coli*: Cx43_173CBD: 22.5 kDa, pl 6.02, and Cx43_173HPML: 27.9 kDa, pl 8.36 [1]. Two clones, producing monoclonal antibodies recognizing the native Cx43 E2 fragment, were obtained by subsequent purification of recombinant proteins, immunization of mice, fusion and selection of hybrid cells. Purified from ascitic fluid, these monoclonal antibodies were used for immunohistochemical analysis on sections of the rat brain with experimental C6 glioma. It is known that its development is associated with an intense astroglial reaction around the tumor at all stages of its development [4,5], while GFAP-positive reactive astrocytes can produce Cx43 in high amounts [16].

Scanning laser confocal microscopy with monoclonal antibodies to Cx43_173CBD and polyclonal antibodies to GFAP astroglia marker visualized Cx43 on GFAP-positive reactive astrocytes in the peritumoral roll on the brain sections of a rat with experimental C6 glioma. At low magnification (objective 10.0, 0.40, 1 pixel=1.67 μ) about half of reactive astrocytes acquired a yellow color after coincidence of spectral channels, this corresponding to co-location of GFAP (red fluorescence) and Cx43 (green fluorescence, Fig. 1, *a*). At a greater magnification (objective 63.0, 1.4 OIL, 1 pixel=0.31 μ) characteristic placoid structures of connexons were detected in GFAP-positive astrocytes (Fig. 1, *b*). No fluorescence was seen in the control (without first antibodies).

The next step was immunofluorescent analysis of purified Cx43 preparations on various cell cultures with monoclonal antibodies. These antibodies exhibited no strict species specificity and detected the native Cx43

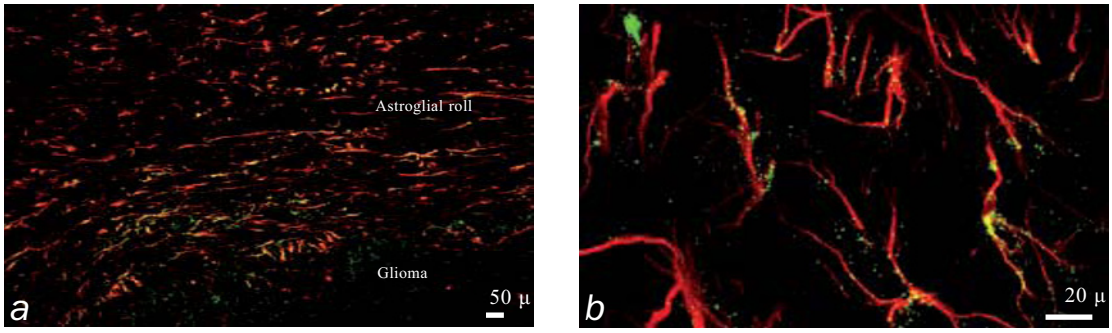


**Fig. 1.** Immunofluorescent visualization of Cx43-positive astrocytes with monoclonal antibodies to Cx43 extracellular fragment E2. Scanning laser confocal microscopy. Green fluorescence: monoclonal antibodies to Cx43+antimouse IgG with Alexa Fluor 488 (Invitrogen); red fluorescence: polyclonal antibodies to GFAP+antirabbit IgG with Alexa Fluor 594 (Invitrogen). *a*) astrocyte roll around glioma (general aspect); *b*) a fragment of astrocyte roll (×60): characteristic placoids of membrane connexons are visualized.

in the rat cell cultures (normal astrocytes, C6 glioma cells) and in human cell cultures (U251 glioblastoma and HEK293 fetal renal epithelium; Fig. 2).

No fluorescence of cell-cell junctions, characteristic of dimer connexons (functional gap junction), was detected in any of the cell preparations. This fact suggested that the resultant antibodies visualized Cx43 at the stage of connexon presentation on the cytolemma in the form of a hemichannel. In fibrillar astrocytes fixed in 4% paraformaldehyde, the antibodies to Cx43 visualized intense placoid granularity in the perinuclear areas, which presumably corresponded to the Cx43 cytoplasmic pool in the endoplasmic reticulum, where the connexons were assembled [18] (Fig. 2, *a*). The membrane placoids were better visualized with monoclonal antibodies to Cx43 in protoplasmic astrocytes (Fig. 2, *b*). Both the membrane and cytoplasmic pools of Cx43 seemed to be visualized in fixed HEK293 and C6 and U251 glioma cells (Fig. 2, *c*-*e*).

The staining was the most intense in the fetal renal epithelial culture, which confirmed the data on high expression of Cx43 during embryogenesis. The fluorescence level was significantly lower in glioma cell cultures, but even in these cultures 5-10% cells were visualized.

Parallel visualization with antibodies to Cx43 E2 and polyclonal antibodies to cadherins and β-catenin (anti-pan-Cadherin, No. 71-7100; anti-β-Catenin, No. 71-2700; Zymed) has shown high expression of adhesion contact protein in the studied cell strains (Fig. 2, *c*, *d*).

Similarly as human U251 glioblastoma, rat C6 glioma is poorly differentiated tumor (stage IV) char-

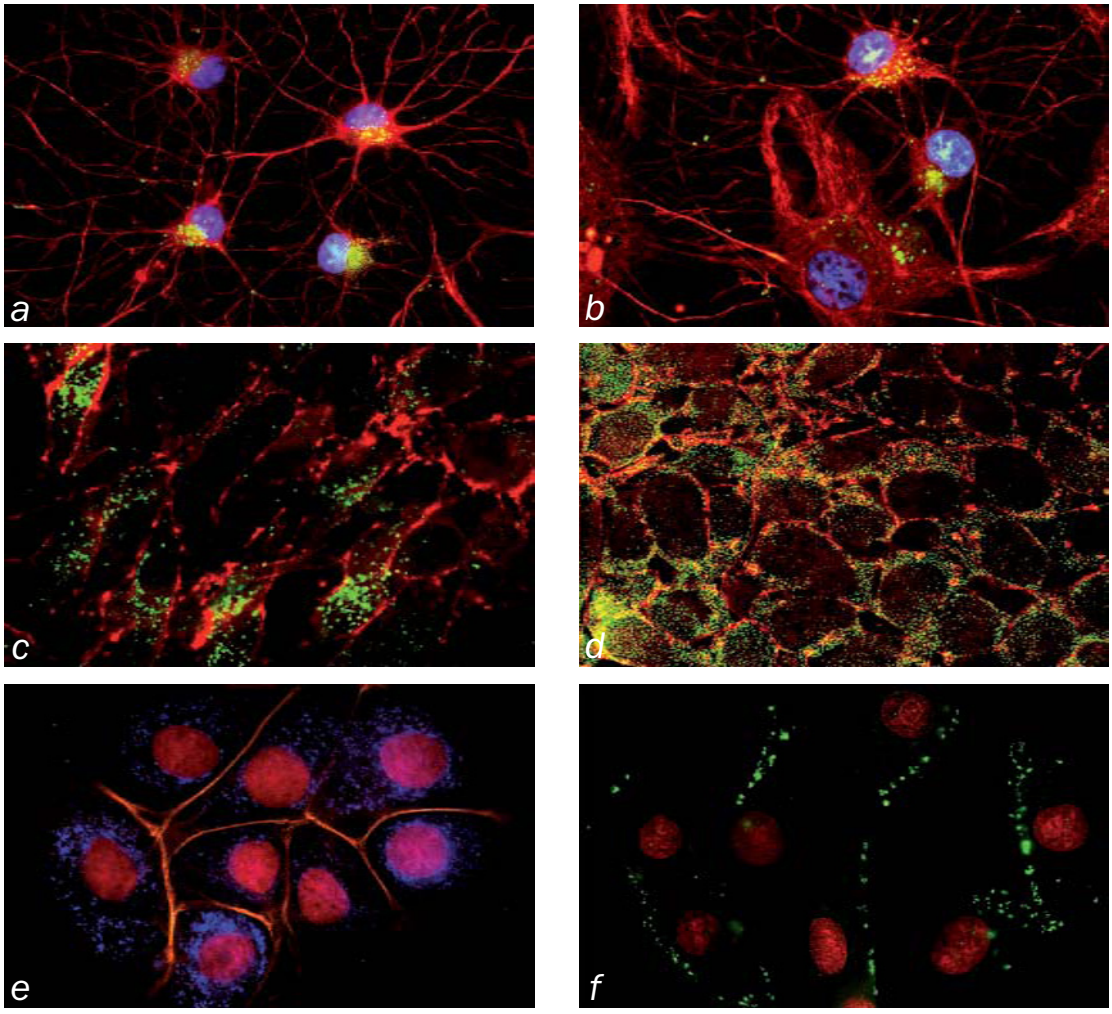


**Fig. 2.** Immunofluorescent analysis of Cx43 in cell cultures using antibodies to E2 extracellular fragment (×1000). *a*, *b*: astrocyte culture (*a*: fibrillar; *b*: protoplasmic) from neonatal rat brain: red fluorescence: polyclonal antibodies to GFAP; green fluorescence: monoclonal antibodies to Cx43; cell nuclei: DAPI; *c*) rat C6 glioma: red fluorescence: β-catenin; green fluorescence: monoclonal antibodies to Cx43; *d*) fetal renal epithelium (HEK293): red area: pan-cadherins; green area: monoclonal antibodies to Cx43; *e*) human U251 glioblastoma: blue fluorescence: monoclonal antibodies to Cx43 and anti-mouse IgG with Alexa Fluor$^{R}$ 350; orange-red fluorescence: actin filaments stained with Phalloidin TRITC; cell nuclei: TOTO 633. *a*-*e*: cultures fixed in paraformaldehyde; *f*) Cx43 visualization in living C6 glioma culture (green fluorescence), cell nuclei poststained with TOTO 633.

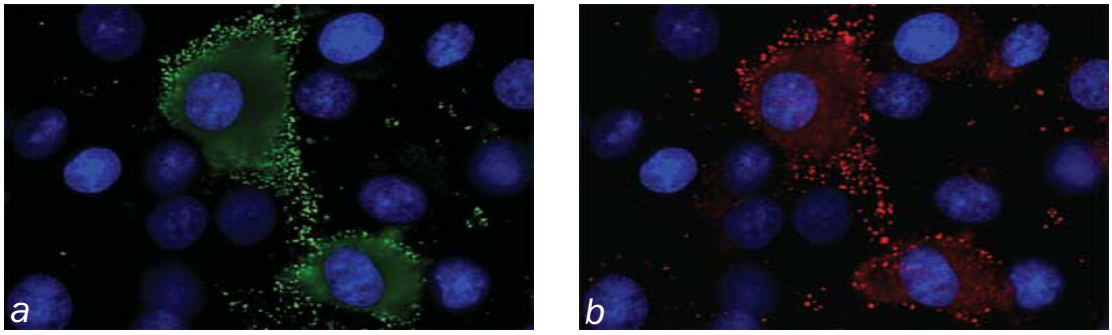


**Fig. 3.** Immunofluorescence of Cx43 in C6 glioma culture with antibodies to E2 extracellular fragment (*a*) and commercial antibodies to Cx43 (*b*; ×1000).

acterized (according to published data) by low expression of Cx43 and other gap junction proteins [14,20]. However, immunofluorescent analysis with antibodies to E2 fragment has shown the presence of Cx43-positive cells. Hence, our study did not confirm the data of some authors on complete absence of gap junction protein synthesis in "malignant" gliomas. This is in line with the results of a later study, which has detected Cx43 expression in C6 glioma cells and derived its Cx43-positive strain [3]. Let us note, that the expression of Cx43, detected by immunofluorescence, does not prove the formation of functional gap junctions in glioma cells. This fact has to be proven by other approaches, for example S. Goldberg's method with cytoplasmic fluorescent stain transfer [12].

In order to verify the results of Cx43 visualization in glioma cells, we carried out a comparative analysis of antibodies to E2 extracellular fragment with commercial antibodies to Cx43 (anti-Cx43, polyclonal No. 71-0700, Zymed). Two-color immunofluorescent visualization on paraformaldehyde-fixed C6 glioma preparation was carried out for this purpose. The results have indicated almost complete coincidence of immunofluorescence induced by our monoclonal antibodies to Cx43 E2 and commercial anti-Cx43 antibodies (Fig. 3). Some differences in the immunofluorescence location can be attributed to specificity of the antibodies used, as commercial antibodies were obtained by immunization with Cx43 C-terminal cytoplasmic fragment.

It was important to clear out whether the antibodies to Cx43 extracellular fragment, obtained in our study, would react with connexons in living cells. We used two alternative approaches to solve this problem. One consisted in incubation of living C6 glioma cells with monoclonal antibodies to Cx43 E2 with subsequent fixation and visualization by fluorescent-labeled anti-species antibodies. The other was carried out on nonfixed living cells using a system of biotin-conjugated antibodies to Cx43 and streptavidin with fluorescent label.

Placoid structures were visualized on living glioma cell membranes as a result of the first and second experiments (Fig. 2, *f*). No perinuclear staining, characteristic of fixed cell preparations, was seen. These data suggested that the antibodies obtained in our study recognized the native conformation of Cx43 extracellular fragment in living C6 glioma cells.

Hence, monoclonal antibodies to Cx43 second extracellular loop (E2, Q173-1208) have been obtained and characterized. These antibodies recognize Cx43 in paraformaldehyde-fixed rat and human cell preparations and in living cells expressing Cx43. These antibodies can be used for *in vitro* studies, for example, for controlling the efficiency of Cx43 cell transfection and in other experiments investigating the gap junction functions. The antibodies are fit for immunoperoxidase and immunofluorescent immunochemical analysis technologies. We can expect that they will be used in flow cytofluorometry and for isolation of Cx43-positive cells by a cell sorter. In addition, antibodies actively reacting with living glioma cells can be tried as vectors for target transport of bioactive compounds and genetic material to glioma cells in experimental *in vitro* and *in vivo* studies.

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