

# Immune mapping of the peripheral part of the visual analyzer and optic nerve



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## SUMMARY

**Aim.** To perform immune mapping of the peripheral part of visual analyzer and optic nerve in order to identify potential antigenic targets of autoimmune attack.

**Methods.** Eyes enucleated for terminal painful glaucoma (n = 30) were studied. Immunohistochemistry (IHC) was performed on paraffin-embedded sections of isolated retina and optic nerve using a broad panel of antibodies, i.e., murine monoclonal anti-MBP (myelin basic protein) antibodies, rabbit polyclonal anti-alpha fodrin antibodies, murine monoclonal anti-NSE2 (neuron-specific enolase) antibodies, murine monoclonal anti-GFAP (glial fibrillary acidic protein), and rabbit polyclonal anti-S100 antibodies. IHC reaction was visualized using Mouse and Rabbit Specific HRP/AEC Detection IHC Kit. IHC reaction without primary antibodies included was a negative control. IHC reaction was considered as follows: negative – no specific cellular staining or less than 10% of cells are stained; mild – 10-30% of cells are stained (+); moderate – 30-75% of cells are stained (++); marked – more than 75% of cells are stained (+++); overexpression – 100% of cells intensively express markers. Additionally, staining intensity was considered as mild (+1), moderate (+2), strong (+3) and intense (+4).

**Results.** Immune mapping with a broad panel of monoclonal antibodies identified ocular structures which were stained with IHC markers. Retina was stained with almost all markers of neural differentiation (i.e., antibodies against NSE, GFAP, S100, and  $\alpha$ -fodrin) excepting anti-MBP autoantibodies. IHC reaction intensity in retinal layers and structures varied and depended on markers. Moderate (2+) staining with antibodies against MBP, NSE, GFAP, and S100 and marked (3+) staining with antibodies against alpha-fodrin was detected in the cytoplasm of optic nerve glia.

**Conclusion.** Complete labelling of retina structures was performed. As a result, IHC profiles of retinal neurons, optic nerve axons, interneurons, and microglial cells were described. IHC profiles of retinal layers and optic nerve are useful markers which can be applied in serological diagnostics of various ocular disorders.

**Keywords:** immune mapping, neurospecific proteins, markers, glaucoma, immune diagnostics, retina, optic nerve, autoantibodies, autoantigens.

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Retinal and optic nerve disorders are amongst the leading causes of visual impairment. Their severity and prognosis cannot be assessed by electrophysiological studies only. In hypoxic-ischemic brain injury, neurospecific proteins (NSPs) are commonly used in neurological practice as the markers of pathological processes [1-4].

NSPs are tissue-specific marker proteins of the nervous system. Their histogenetic source is neuroepithelial tis-

sue, i.e., neurons and glia of the nervous system. Currently, more than 30 NSPs are identified, e.g., alpha 2 brain globulin (expressed by oligodendrocytes), S-100, clathrin, tubulin, calmodulin, glial fibrillary acidic protein/GFAP (expressed by astrocytes), neuron-specific enolase/NSE (expressed by neurons), and myelin basic protein/MBP (expressed by oligodendrocytes). The metabolism of neurons and astrocytes determines the susceptibility of these cells

Table 1. The panel of antibodies used

Antibodies	Company	Dilution
Murine monoclonal anti-MBP antibodies	Santa Cruz	1:100
Rabbit polyclonal anti-alpha fodrin antibodies	Abcam	1:100
Murine monoclonal anti-NSE2 antibodies	Leica Biosystems	ready to use
Murine monoclonal anti-GFAP antibodies	BioGenex	1:200
Rabbit polyclonal anti-S100p antibodies	Leica Biosystems	ready to use

Table 2. Immunohistochemistry of neurospecific marker expressed in the retina and optic nerve

Structure	IHC markers				
	MBP	NSE	GFAP	S100	$\alpha$ -fodrin
Retina:					
Photoreceptors:	–	+	–	+	+ / –
outer segments (1)	–	3+ 4+	–	4+	–
inner segments (2)	–	3+ 4+	–	4+	3+
External limiting membrane (3)	–	4+	4+	3+	4+
Outer nuclear cell layer (4) 1st neuron	–	3+	3+	3+	3+
Outer plexiform layer (5)	–	4+	3+	3+	4+
Inner nuclear cell layer (6) 2nd neuron	–	3+	3+	3+	3+
Inner plexiform layer (7)	–	4+	3+	3+	4+
Ganglion cell layer (8) 3rd neuron	–	3+	3+	4+	3+
Retinal nerve fiber layer (9)	–	3+	3+	3+	1+
Inner limiting membrane (10)	–	2+	–	3+	3+
Optic nerve (11)	2+	2+	2+	2+	3+

to the hypoxic or ischemic injury [5]. Astrocytes are considered to be more resistant to hypoxic cell injury [6, 7]. Glial mitochondria are characterized by the greater resistance to hypoxic injury as compared with neural mitochondria, i.e., hypoxia-mediated inhibition of mitochondrial respiratory chain activity in glial cells is much less than in neural cells [8-10]

Several authors reported the data on NSP levels in the serum of ophthalmic disorders patients. Western blot is regarded as a tool for serological screening and is used to detect even traces of specific autoantibodies against NSP in various disorders. Western blot enables to assess a broad spectrum of many autoantigens simultaneously and to identify subtle changes of their repertoire and the repertoire of complementary autoantibodies.

Most recent studies evaluate just one or several autoantibodies which are specifically associated with an ocular disorder. In glaucoma, elevated serum levels of autoantibodies against glutathione S-transferase [11], phosphatidylserine [12], NSE [13, 14], glycosaminoglycan [15], rhodopsin [16], alpha-fodrin [17], tumor necrosis factor/TNF [18, 19],

and gamma-synuclein [20] were revealed. Many researchers face difficulties in interpreting findings due to the lack of data on antigen profile of the peripheral part of visual analyzer and optic nerve. Few studies in this field cannot cover the whole spectrum of serological markers [21-24]. In addition to elevated or reduced (as compared with controls) serum levels of autoantibodies, the expression of complementary autoantigens in the optic nerve and/or retinal neurons may serve as a direct evidence of an antigen pathogenic role only. Considering this, we made an attempt to address this issue and to perform a study.

## AIM

To perform immune mapping of the peripheral part of visual analyzer and optic nerve in order to identify potential antigenic targets of autoimmune attack.

## MATERIALS AND METHODS

Postmortem eye donation presents difficulties, so the objects of morphological studies were eyes enucleated for terminal painful glaucoma (n = 30). Immune mapping was

performed in the isolated retina and optic nerve in several steps.

The first step was mandatory morphological study of enucleated eyes to verify the diagnosis and to specify morphological abnormalities. The block of excised tissues was fixed with 10% neutral formalin for one day. Macro examination was performed following fixation. Blocks containing relevant tissue fragments were embedded in paraffin wax using standard protocol. Ten 4-5 micron sections of the tissue were cut from each block and stained with hematoxylin and eosin. Specimen examination and photorecording was performed using microscope OPTON (Carl Zeiss Meditec, Jena, Germany) with TV-camera at 40×, 125× and 400× magnifications.

The second step was immunohistochemistry (IHC) performed on paraffin-embedded sections for standard morphological study (see Table 1).

Paraffin-embedded sections were deparaffinated and rehydrated using standard technique. Antigen retrieval was performed by sections heating on water bath in preheated citrate buffer (95–99°C) for 45 min. Specimens were cooled at room temperature for 15-20 min and transferred into phosphate buffer for 5 min. To block endogenous peroxidase, sections were incubated in the dark with 3% peroxide (prepared on distilled water) for 20 min and washed in phosphate buffer for 5 min. To block non-specific antigen binding, sections were incubated with 1% bovine serum albumin for 15 min. Incubation with primary antibodies was performed at 4°C for 40 min. Following this procedure, were washed twice for 5 min in phosphate buffer.

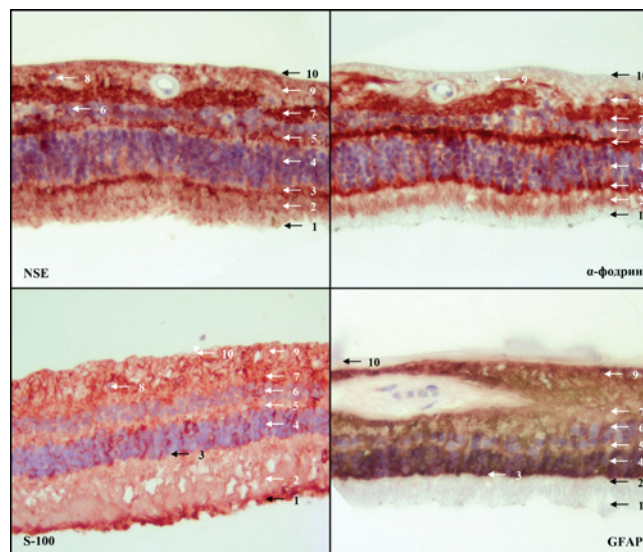
To block non-specific antigen binding, sections were incubated with 1% bovine serum albumin for 15 min. Incubation with primary antibodies was performed at 4°C for 40 min. Following this procedure, were washed twice for 5 min in phosphate buffer.

Incubation with secondary antibodies (LSABTM+ Kit, DAKO, Denmark) was performed at room temperature for 20 min, then sections were washed twice for 5 min. Incubation with streptavidin-labeled antibodies (LSABTM+ Kit, DAKO, Denmark) was performed at room temperature for 20 min, then sections were washed thrice for 5 min. IHC reaction was visualized using Mouse and Rabbit Specific HRP/AEC Detection IHC Kit (Abcam, Cambridge, UK). Reaction was performed for 5-10 min. Slides were stained with Mayer's hematoxylin and mounted into factory water-based medium.

IHC reaction without primary antibodies included was a negative control.

Staining results were studied using Axiolab E re Microscope (Carl Zeiss Meditec, Jena, Germany) at 10×, 20× and 40× magnifications. The localization of IHC staining in a cell (nucleus, cytoplasm, membrane) for each marker and the specific structure/layer fixated by the antibodies were registered.

IHC reaction was considered as follows: negative —



**Fig. 1.** IHC profile of the retina

1 — outer segments of photoreceptors, 2 — inner segments of photoreceptors, 3 — external limiting membrane, 4 — outer nuclear cell layer, 5 — outer plexiform layer, 6 — inner nuclear cell layer, 7 — inner plexiform layer, 8 — ganglion cell layer, 9 — retinal nerve fiber layer, 10 — inner limiting membrane.

no specific cellular staining or less than 10% of cells are stained; mild — 10-30% of cells are stained (+); moderate — 30-75% of cells are stained (++); marked — more than 75% of cells are stained (+++); overexpression — 100% of cells intensively express markers. Additionally, staining intensity was considered as mild (+1), moderate (+2), strong (+3) and intense (+4).

## RESULTS

Immune mapping with a broad panel of monoclonal antibodies identified ocular structures stained with IHC markers (see Fig. 1). Staining location and intensity were similar in all retina samples due to the same (terminal) stage of the disease (see Table 2).

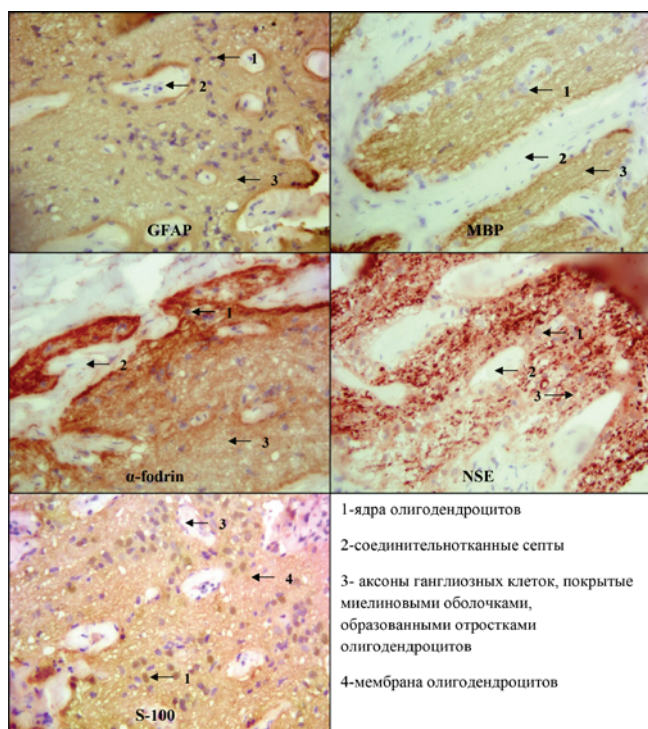
It was demonstrated that retina as a main peripheral photoreceptive part of visual analyzer was stained with antibodies against almost all markers of neural differentiation (i.e., NSE, GFAP, and S100 excepting MBP) as well as with antibodies against α-fodrin. IHC reaction intensity in retinal layers and structures varied and depended on markers.

### Photoreceptors

Outer segments of photoreceptors were intensively stained with antibodies against NSE (3+ or 4+) and S100 (4+) but not with antibodies against GFAP and α-fodrin. Inner segments of photoreceptors were intensively stained with antibodies against S100 (4+), NSE (3+ or 4+), and α-fodrin (3+) but not with antibodies against GFAP and MBP.

### External limiting membrane

External limiting membrane was intensively stained



**Fig. 2.** IHC profile of the optic nerve

with antibodies against NSE (4+), GFAP (4+), S100 (3+), and  $\alpha$ -fodrin (4+).

#### Outer and inner nuclear cell layers

Outer and inner nuclear cell layers were stained with the antibodies against the markers of neural differentiation, i.e., NSE (3+), GFAP (3+), S100 (3+), and  $\alpha$ -fodrin (3+) but not with antibodies against MBP.

#### Outer and inner plexiform layer

Outer and inner plexiform layer were stained with antibodies against NSE (4+) and  $\alpha$ -fodrin (4+) more intensively as compared with nuclear cell layers. The intensity of IHC reaction with antibodies against GFAP and S100 was similar to that of nuclear layers.

#### Ganglion cell layer

Ganglion cells were intensively stained with antibodies against S100 (4+) and less intensively with antibodies against NSE (3+), GFAP (3+), and  $\alpha$ -fodrin (3+). IHC reaction with antibodies against MBP was negative.

#### Nerve fiber layer

Nerve fiber layer was intensively stained with antibodies against NSE (3+), GFAP (3+), and S100 (3+) and mildly with antibodies against  $\alpha$ -fodrin (1+). IHC reaction with antibodies against MBP was negative.

#### Inner limiting membrane

Inner limiting membrane was stained with antibodies

against S100 (3+), NSE (2+), and  $\alpha$ -fodrin (3+) but not with antibodies against MBP and GFAP.

#### Optic nerve

The cytoplasm of the processes of optic nerve glial elements were moderately (+2) stained with antibodies against MBP, NSE, GFAP, and S100 and more intensively with antibodies against  $\alpha$ -fodrin (3+).

#### DISCUSSION

It is well-known that human retina consists of three hierarchical cell layers, i.e., outer nuclear cell layer comprised of photoreceptor nuclei (1st neuron), inner nuclear cell layer comprised of bipolar cells (2nd neuron), and ganglion cell layer (3rd neuron). It is obvious that IHC profile of the first neuron is basically different from these of the second and third ones (see Fig. 1). Rods and cones stem from the tissues of neural differentiation. This can be demonstrated by positive IHC reaction with antibodies against NSE and S100. However, IHC reactions with antibodies against GFAP and MBP are negative. Moreover, IHC profiles of outer and inner segments of photoreceptors are different as well. These segments essentially differ in their functionality. Evolutionally, outer segments of photoreceptors are adapted to perform photochemical processes by their structure. Inner segments are responsible for cellular energy metabolism. Morphologically, inner segments of rods and cones are rich in mitochondria which functioning requires cytoskeleton integrity. This can be accounted for the expression of  $\alpha$ -fodrin, organ-specific protein of the cytoskeleton, in the inner segments of photoreceptors and the lack of its expression in the outer segments.

Bipolar cells localized in the inner nuclear cell layer are second neurons [25]. Each bipolar cell connects with several photoreceptors via the dendrites.

Large ganglion (multipolar) cells localized in the ganglion cell layer are the bodies of third neurons. Ganglion cell usually connects with several bipolar cells.

The second neuron and the third neuron have the same IHC profiles as they express neurospecific markers, i.e., NSE and S100. Their anatomic and functional integrity is maintained if cytoskeleton is intact. The latter is determined by GFAP and  $\alpha$ -fodrin. The second neuron and the third neuron of the visual analyzer, likewise the first one, do not express MBP.

Axons of ganglion cells form the retinal nerve fiber layer and optic nerve. These axons are lacking in myelin sheath thus providing retinal transparency. Their IHC profile is characterized by NSE, S100, and GFAP expression and the lack of MBP expression.

Hierarchically, visual pathway (i.e., photoreceptors, bipolar and ganglion cells) includes two types of interneurons. Horizontal cells are localized in the outer plexiform layer while amacrine cells are localized in the inner plexiform layer. Horizontal cells communicate with each other



and bipolar cells through the synapses and provide a feedback to photoreceptor as well. Amacrine cells that are rich in neurotransmitters communicate with each other and ganglion cells through the synapses and provide a feedback to bipolar cells.

IHC profiles of the outer and inner plexiform layers are identical. The expression of NSE, S100, and GFAP by their interneurons demonstrates that these cells belong to highly differentiated neural tissues. The structure of interneurons is determined by  $\alpha$ -fodrin, a protein which controls cytoskeleton integrity, therefore, they express  $\alpha$ -fodrin extensively. The axons of these cells have no myelin sheath (IHC reaction with antibodies against MBP is negative).

It is well-known that neuroglia builds up retinal «skeleton» (or architecture). These cells stretch radially across the thickness of the retina and form structural limitations, i.e., external and inner limiting membranes. Neuroglia provides support, buffering and trophic functions. The four major types of glial cells are Muller cells, astrocytes, oligodendrocytes, and microglia. Large glial Muller cells which occupy extracellular space pass radially through the retinal layers, from the photoreceptors to the vitreous membrane. Muller cell bodies sit in the inner nuclear layer and project thick and thin processes in either direction to the outer limiting membrane and to the inner limiting membrane. Muller cells are the principal glial cells of the retina. They have a range of functions including support, buffering and trophic ones. These cells maintain the stability of neuronal functions in CNS. Muller cells are integrated in neural network thus modulating specific neuronal activity.

Microglia is limited with proximal retina up to the inner plexiform layer inclusive. Principal functions of microglia are to stimulate macrophages and to promote phagocytosis of degenerating retinal neurons.

Astrocytes are typically localized in retinal nerve fiber layer, however, they can be found in other layers as well. These cells are closely adjacent to retinal vessels and ganglion cell axons. They are vital to maintain the integrity of blood-brain and blood-aqueous barriers. Astrocytes contain glycogen and are able to supply neurons with glucose.

Close associations between microglia and astroglia under normal and pathological conditions are established.

It is well-known that in primates radial glia is immunoreactive to GFAP in the most active stage of neuronal migration. Mitotic activity of postnatal radial glia is temporarily arrested, and these cells act as a scaffold for migrating neurons. Stable differentiated supporting cells are required to maintain the architecture and functions of such a complex structure the retina is. In eyes with terminal glaucoma, radial glia localized in retinal nerve fiber layer is MBP-immunonegative and demonstrates poor staining with antibodies against  $\alpha$ -fodrin.

It was demonstrated that glial cells of the optic nerve (i.e., visual pathway) moderately expressed MBP, NSE, S100, and GFAP and intensively expressed  $\alpha$ -fodrin. 70% of nuclei and membranes of optic nerve oligodendrocytes were fixated with antibodies against S100. Myelinated axons of ganglion cells which form optic nerve were stained with antibodies against MBP (see Fig. 2).

Clinical application of our findings is that retinal structures were completely characterized since IHC profiles of visual neurons, axons, interneurons, and microglia were specified. Their IHC profiles in terminal glaucoma eyes indicate that these markers can be applied into serologic immunodiagnostics of retinal and optic nerve disorders. They can be also used to assess the integrity of blood-aqueous barrier. The perspectives of accurate and detailed interpretation of serological findings are revealed as well.

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