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Fine structure of the human retina defined by confocal microscopic immunohistochemistry

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ABSTRACT

Introduction: Research in to the pathophysiology of the complex layers of retinal and sub-retinal cells is hampered by inadequate recognition of particular cells and tissues. A comprehensive panel of antibodies recognising retinal tissues is lacking. Our purpose was to determine the value of a panel of antibodies labelling various cells in the human retina.

Method: Five groups of antibodies labelled frozen sections of retinas: (1) protein kinase C-α, Glutamine Synthetase (GS) and ionized calcium-binding adapter molecule 1 (Iba1); (2) Parvalbumin, Calretinin and glial fibrillary acidic protein (GFAP); (3) Thy1, GS and Iba1; (4) Rhodopsin, GS and Iba1; and (5) Brn3a, Rhodopsin and protein kinase C-α. The distribution of these antigens were determined by confocal microscopy and calculated grey value of each antibody in each layer of the retina by Image J.

Results: Different antibodies determined certain retinal layers. Thy 1 is a good determinant of the ganglion cell layer, whilst GS is present in all layers except the photoreceptor layer. Brn3a is specific for the ganglion cell layer whilst parvalbumin marks the ganglion cell layer and the outer plexiform layer. Rhodopsin strongly marks the photoreceptor layer, but this is also marked weakly by GFAP.

Conclusion: The multiple labelling of human retinal cells brings further understanding of the biological characteristics and functions of these cells, and provides a theoretical basis for their possible role in diseases. In the growing field of human retina research, our data may provide a point of reference for future studies of the human retina.

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Human retina; immunohistochemistry; triple labelling; confocal microscopy; cryosection

Introduction

The advanced understanding of the mammalian retina is crucial in the investigation and treatment of ophthalmological disease, and study of the mammalian eye has provided insight [1–3]. Although these studies are informative, human tissues are becoming widely available, providing the opportunity to study the morphology and function of the retina in our own species, which is commonly characterized using cell markers that label specific neurons or glia [4]. Despite similarities in retinal anatomy between humans and other animal species, cell marker immunoreactivity varies between species. For example, antibodies to parvalbumin have been reported to stain AII amacrine cells in both the rat and rabbit retina [5], but in humans, strong immunoreactivity was observed in horizontal cells [6], and calretinin is a suitable marker for AII amacrine cells in the human retina [7]. These data call for a comprehensive study into their potential value in the study of the human eye.

We established five groups of triple-labelled human retinal cells by immunofluorescence staining: (1) protein kinase C-α (PKCα), Glutamine Synthetase and ionized calcium-binding adapter molecule 1 (Iba1); (2) Parvalbumin, Calretinin and glial fibrillary acidic protein (GFAP); (3) Thy1, Glutamine Synthetase and Iba1; (4) Rhodopsin, Glutamine Synthetase and Iba1; and (5) Brn3a, Rhodopsin and PKCα. The purpose of this study was to use confocal microscopy to establish a database of the stereoscopic structure of the human retina by labelling the human retina using multiple antibodies targeted to well-established retinal cell markers commonly used in humans.

Materials and methods

Human donor tissues were collected at the Eye & ENT Hospital of Fudan University immediately after the removal of the cornea for transplantation. This study was approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for medical research involving human subjects.

After the removal of the cornea and the lens, the eyecups containing the retinas were immediately immersed in 4% paraformaldehyde overnight at 4 °C and subsequently transferred to 20% sucrose in

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Table 1. Primary antibodies.

Antibody	Host	Source
Brn3a	Goat	Santa Cruz Biotechnology (sc-31984)
Glutamine Synthetase	Rabbit	Abcam, Cambridge, UK (ab16802)
Glutamine Synthetase	Mouse	Abcam, Cambridge, UK (ab64613)
Anti-PKC alpha	Rabbit	Abcam, Cambridge, UK (ab32376)
Thy1	Mouse	Abcam, Cambridge, UK (ab181469)
Rhodopsin	Mouse	Abcam, Cambridge, UK (ab5417)
Iba1	Goat	Abcam, Cambridge, UK (ab5076)
Calretinin	Rabbit	Abcam, Cambridge, UK (ab702)
Parvalbumin	Mouse	Sigma-Aldrich P3088
GFAP	Goat	Abcam, Cambridge, UK (ab53554)

All antibodies were used at working dilution of 1/200, except Brn3a, at 1/50.

1× phosphate-buffered saline (PBS) followed by 30% sucrose at 4 °C until they sank to the bottom. The eyecups were cut radially into four slices and then embedded in optimal cutting temperature (OCT) compound (Sakura, Japan). The slices were stored at −80 °C until sectioning. Sections (10–12 µm thick) were cut in the radial plane by a freezing microtome (Leica CM 1950, Leica Microsystems, Germany), mounted onto gelatine-coated slides and stored at −20 °C until use.

For immunohistochemistry, slides were thawed at room temperature and fixed in 4% paraformaldehyde for 10 min. After rinsing in PBS, the sections were treated with 0.3% Triton X-100 for 20 min and then blocked for 30 min in 5% BSA at room temperature. The tissues were

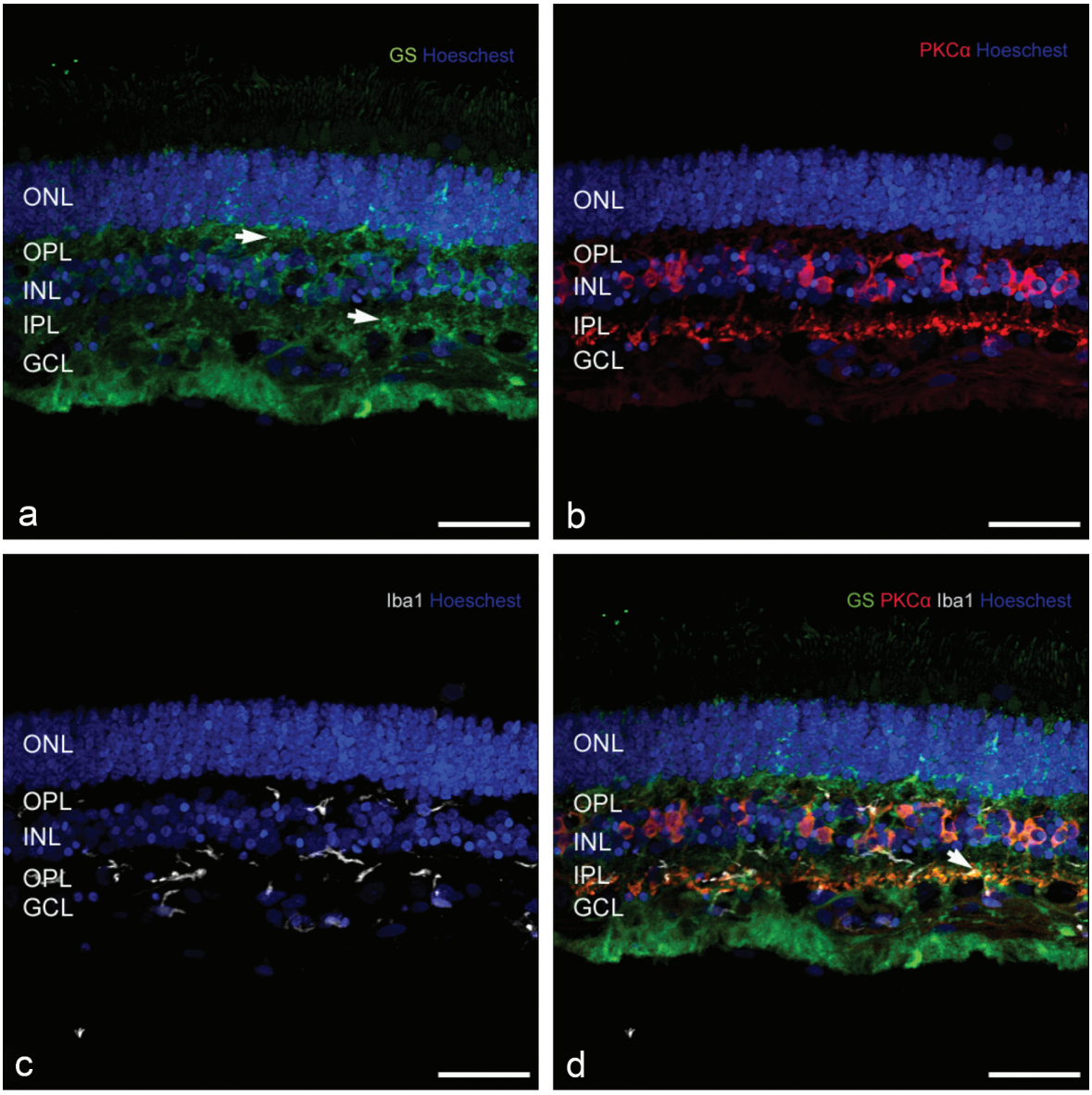

Figure 1. Immunofluorescence labelling in the human retina (×400). red: PKCα; green: GS; grey: Iba1; blue: Hoechst. (a) GS labelling shows Müller cells. The cell body of Müller cells is located in the INL, and the basement membranes extend to the inner boundary membrane. Many side processes are in both plexiform layers (arrows). (b) PKCα labelling shows multiple rod bipolar cells descending to the middle of the IPL where they have prominent terminals, somas are high in the INL adjacent to the OPL. PKCα also had weak positive expression in OPL. (c) Iba1 labelling shows microglia, which are scattered throughout the IPL, GCL, and OPL. (d) Merged images of GS, PKCα, Iba1 and Hoechst labelling. The side processes of Müller cells overlap with the prominent terminals of bipolar cells and microglia (arrows). GS, Glutamine Synthetase; PKCα, protein kinase C-α; Iba1, ionized calcium-binding adapter molecule 1; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; GCL, ganglion cell layer; Scale bar:25 µm.

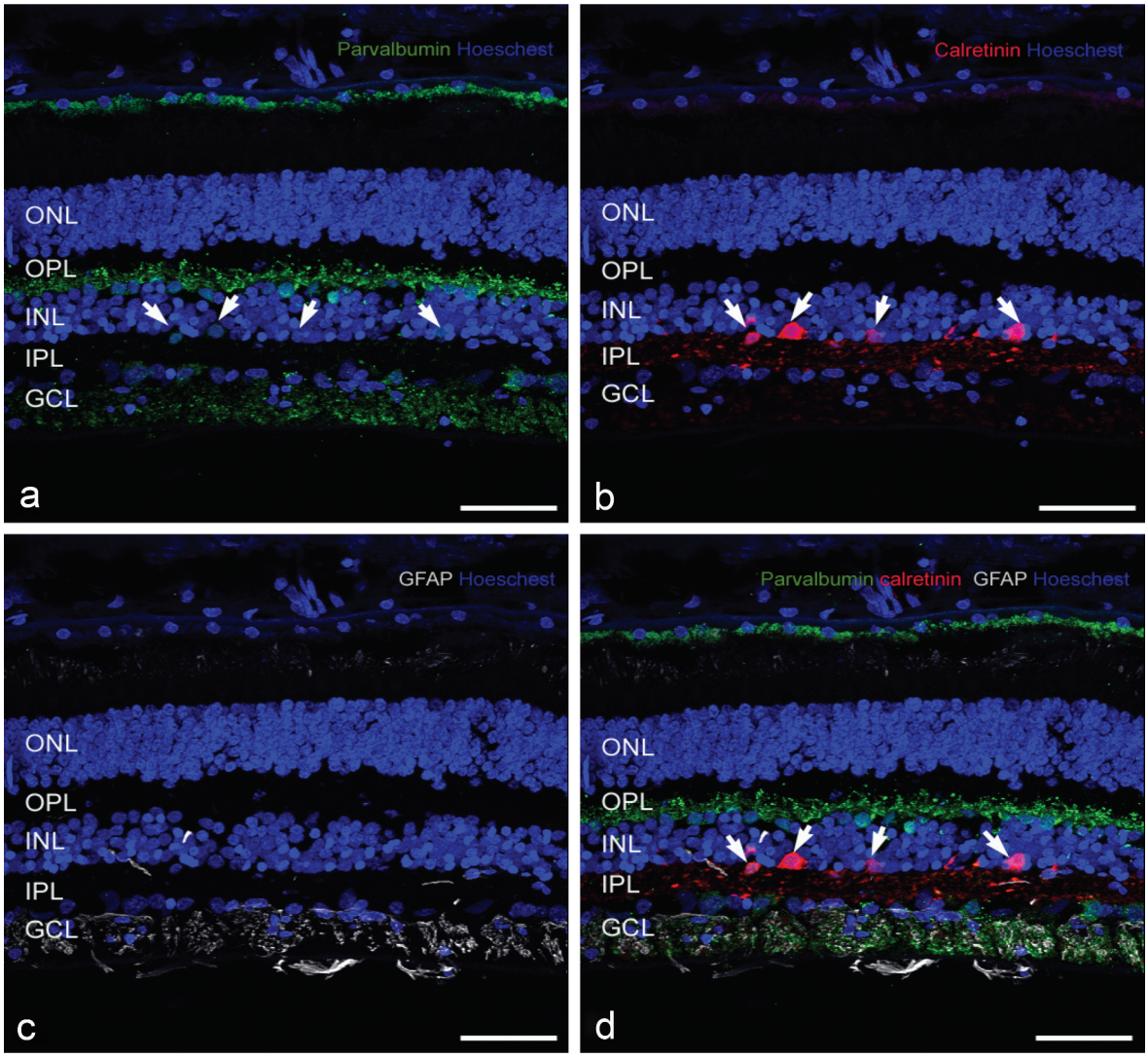

Figure 2. Immunofluorescence labelling in the human retina (×400). red: Calretinin; green: Parvalbumin; grey: GFAP; blue: Hoechst. (a) Parvalbumin immunostaining occurred in horizontal cells, amacrine cells and ganglion cells. Horizontal cell bodies are identifiable; they occupy the proximal layer of the INL, and their processes run distal to the cell bodies to form the OPL. Some lightly stained somas are localized between the horizontal layer and GCL. These cells were co-labelled with Parvalbumin and Calretinin (arrowheads). Additionally, the NFL shows parvalbumin immunoreactivity. (b) Several somas in the amacrine cell layer are well stained with Calretinin (arrowheads); these cells occupy the proximal layer of the INL, and each labelled cell is bistratified with lobules in the IPL. A pattern of weak immunoreactivity was observed in the GCL. (c) GFAP labelling shows astrocytes in the GCL and their processes in the NFL. GFAP also had weak positive expression in the outer section of the photoreceptor. (d) Merged image of Parvalbumin, Calretinin, GFAP and Hoechst staining. GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer; NFL, nerve fibre layer; INL, inner nuclear layer; IPL, inner plexiform layer; Scale bar:25 μm.

rinsed in PBS and then incubated for 24 h at 4 °C in a mixture of PKCα, Glutamine Synthetase and Iba1 or Parvalbumin, Calretinin and GFAP diluted in 3% BSA. Details on the primary antibodies used in this study are described in Table 1. After removing the primary antibodies, the slides were extensively rinsed in 1× PBS, and the specimens were incubated with a mixture of the following secondary antibodies diluted in 3% BSA: donkey anti-mouse IgG conjugated with Alexa Fluor 488 (A32766, Thermo Fisher Scientific, USA), donkey anti-rabbit IgG conjugated with Alexa Fluor 555 (A31572, Thermo Fisher Scientific, USA), and donkey anti-goat IgG conjugated with Alexa Fluor 647 (A32849, Thermo Fisher Scientific, USA). All secondary antibodies were used at a 1:500 dilution. The sections were incubated for 1 h at room temperature. Then, the slides were washed 3 times for 5 min in 1× PBS, and the cell nuclei were counterstained with Hoechst (a popular nuclear counterstain that emits blue fluorescence, H3569, Thermo Fisher Scientific) for 2 min. The slides were washed, coverslipped using antifading reagent (Thermo Fisher Scientific) and sealed with nail polish. Negative controls were prepared in parallel by omitting the primary antibody. Slides were kept at 4 °C until imaging.

Rhodopsin and Thy1 are membrane proteins that do not need to be permeated by Triton X-100. In the rest three groups of staining, we first labelled Thy1 or Rhodopsin, and then labelled the remaining two

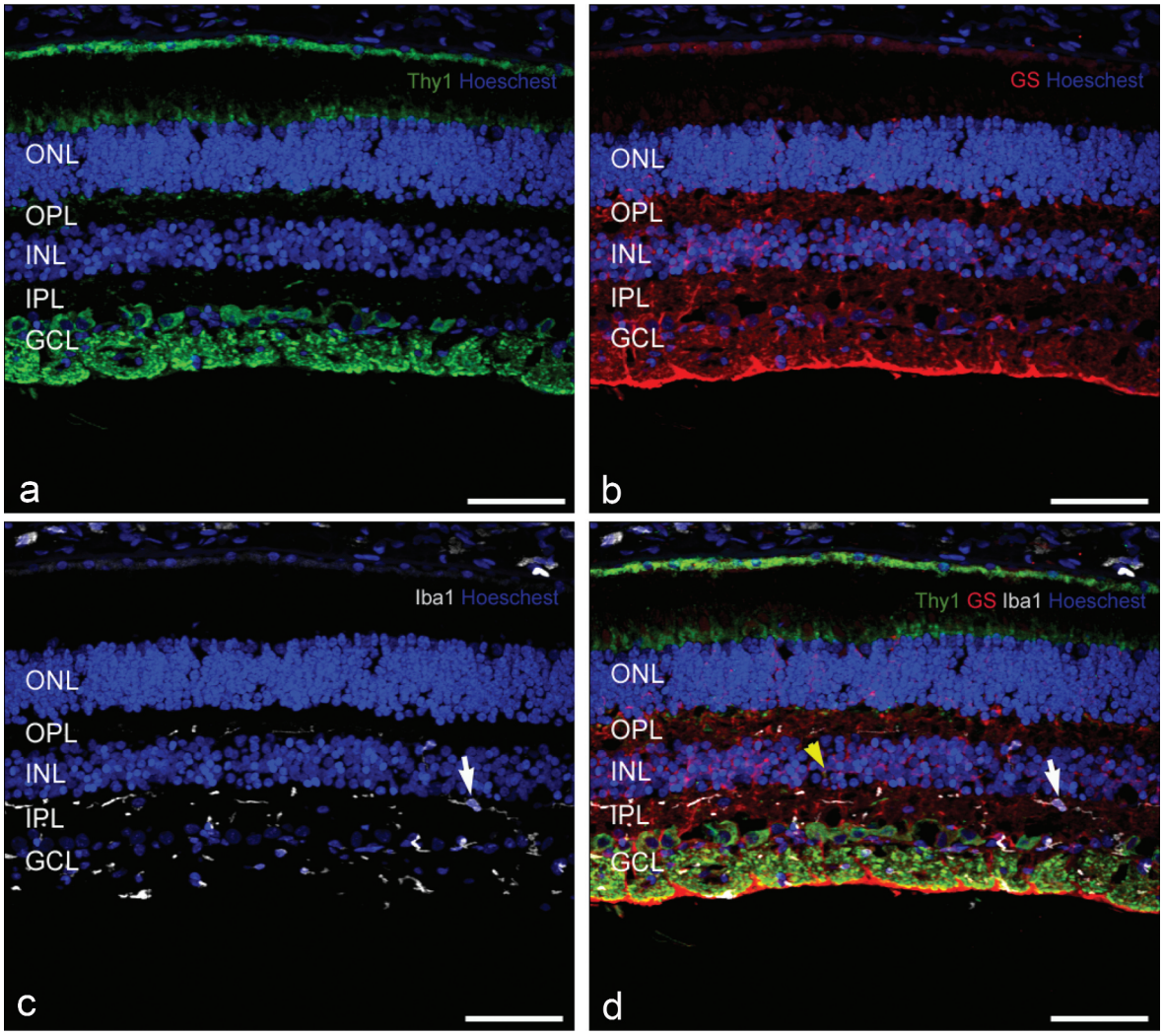

Figure 3. Immunofluorescence labelling in the human retina (×400). red: GS; green: Thy1; grey: Iba1; blue: Hoechst. (a) Thy1 staining labels the cytoplasm and axons of ganglion cells and labels plexiform layers and some cells in the INL and ONL. (b) GS labelling shows Müller cells. (c) Iba1 labelling shows microglia, among which one nucleus is clearly encircled by the cytoplasm (arrow). (d) Merged image of Thy1, GS, Iba1 and Hoechst staining. The endfeet of Müller cells are closer to the vitreous than RGCs. A Müller cell is co-labelled with GS and Thy1 (yellow arrow). GS, Glutamine Synthetase; Iba1, ionized calcium-binding adapter molecule 1; INL, inner nuclear layer; ONL, outer nuclear layer; RGCs, retinal ganglion cells; Scale bar:25 μm.

antibodies. The procedure was basically the same as above.

For confocal microscopy, triple-labelled retinal sections were visualized, and images were captured using a Sp8 Leica microscope. Three-dimensional reconstructions were created by combining a series of 30–40 images in 0.5 mm steps along the Z-axis into one plane. The brightness and contrast of the final figures were equally adjusted by using Adobe Photoshop. The quantitative analysis of immunofluorescence was determined using Image J software.

Results

The human retina is a highly organized tissue with three nuclear layers, the outer nuclear layer (ONL), the inner nuclear layer (INL) and the ganglion cell layer (GCL), and two synaptic layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL) (Figures 1–5). Moreover, the human retina has various cell types. Glutamine Synthetase (Figures 1, 3, and 4) labelled Müller cells; the somas of Müller cells were in the INL, the Müller cell endfeet were at the inner limiting membrane, and the main processes spanned the OPL and IPL. PKCα labelling (Figures 1 and 5) revealed multiple rod bipolar cells descending to the middle of the IPL, where they have prominent terminals, and somas high in the INL adjacent to the OPL. Rhodopsin labelling (Figures 4 and 5) showed segments of rod photoreceptors. Iba1 labelling (Figures 1, 3, and 4) revealed microglia scattered from the retinal ganglion cell (RGC) layer to the OPL. Brn3a (Figure 5) labelled the nuclei of RGCs, while Thy1 (Figure 3) labelled the cytoplasm and axons of ganglion cells. The cytoplasm clearly surrounded the nucleus, and we observed the structure of a mixture of RGC axons. Calretinin (Figure 2),

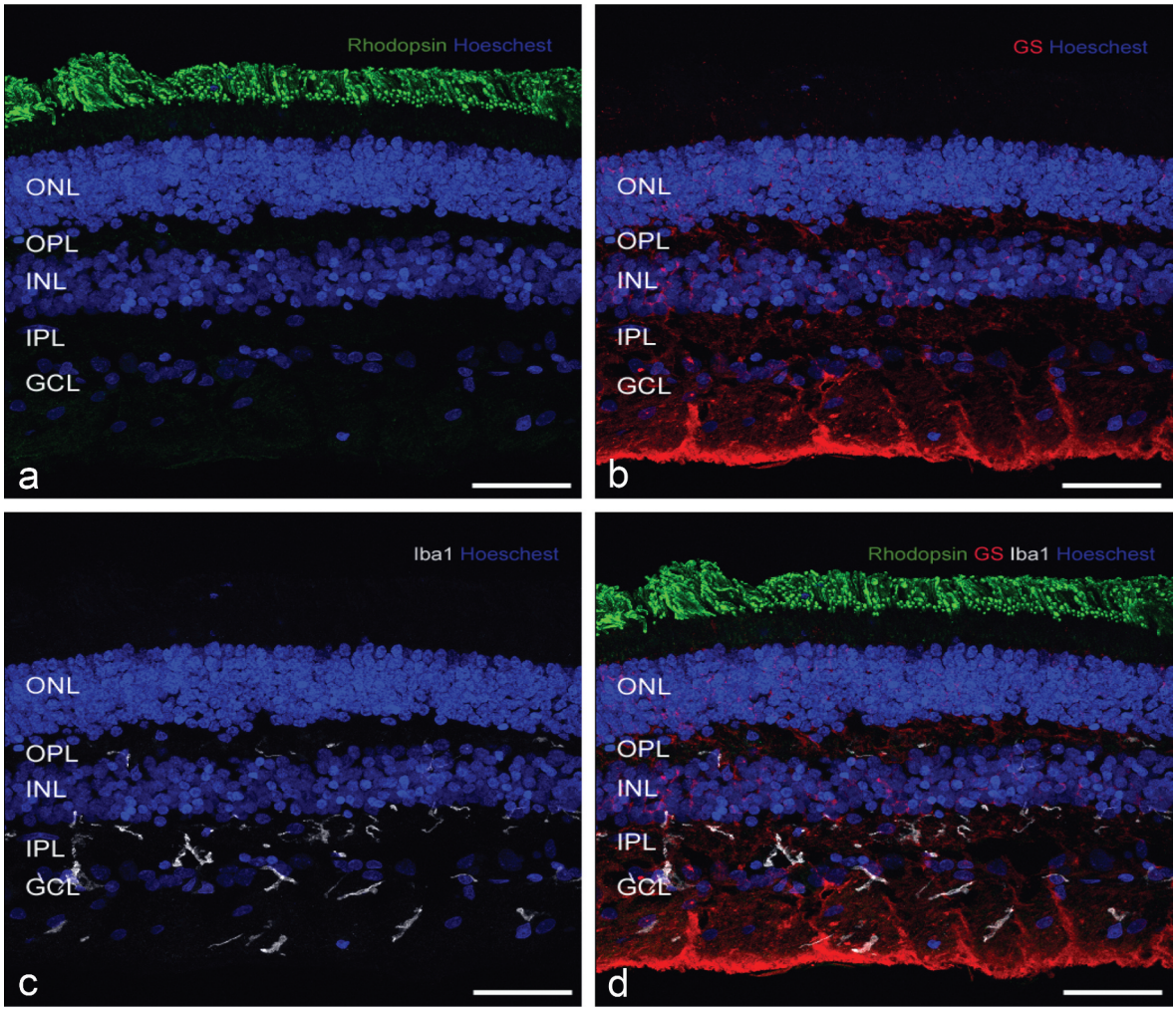

Figure 4. Immunofluorescence labelling in the human retina (×400). red: GS; green: Rhodopsin; grey: Iba1; blue: Hoechst. (a) Rhodopsin labelling shows segments of the rod photoreceptors. (b) GS labelling shows Müller cells. (c) Iba1 labelling shows microglia. (d) Merged image of Rhodopsin, GS, Iba1 and Hoechst staining. GS does not overlap with Rhodopsin. GS, Glutamine Synthetase; Iba1, ionized calcium-binding adapter molecule 1; Scale bar: 25 μm.

several somas in the amacrine cell layer were well stained and occupied the proximal layer of the INL, and each labelled cell was bistratified with lobules in the IPL. A pattern of weak immunoreactivity was observed in the GCL. Parvalbumin immunostaining (Figure 2) occurred in horizontal cells, amacrine cells and ganglion cells. Horizontal cell bodies were identifiable and occupied the proximal layer of the INL, and their processes ran distal to the cell bodies to form the OPL. Some lightly stained somas were localized between the horizontal layer and GCL. These cells were co-labelled by Parvalbumin and Calretinin. GFAP labelling (Figure 2) showed astrocytes in the GCL and their processes in the nerve fibre layer, surrounding the blood vessels of the superficial plexus. Based on the five sets of immunofluorescence images, The grey value of each antibody in each layer of the retina (from Rhodopsin layer to RGC layer) was calculated by Image J. According to the grey value, we summarized the distribution of each antibody in each layer of the retina, and the results are shown in Table 2.

Discussion

The major landmarks of the retina, two synaptic layers sandwiched between three nuclear layers, are obvious, even in unstained sections [8]. However, the somas of retinal cells are indistinguishable, and the details of their structures cannot be distinguished among other cells [9]. To uncover the details of the retinal structure, we need to stain different cell types separately. Furthermore, it is very useful to observe several cell types at once so that the number and position of one cell type relative to another can be determined. Immunofluorescence staining of retinal cryosections is the method used to identify and quantify specific cell populations [10]. Confocal microscopy is a suitable tool to observe immunofluorescence staining because it permits the simultaneous acquisition of three or more different labels in addition to providing improved resolution and 3D visualization. However, only small fractions of tissue can be analysed using confocal microscopy. Therefore, any field changes may easily be missed, representing a notably limitation of pathological states.

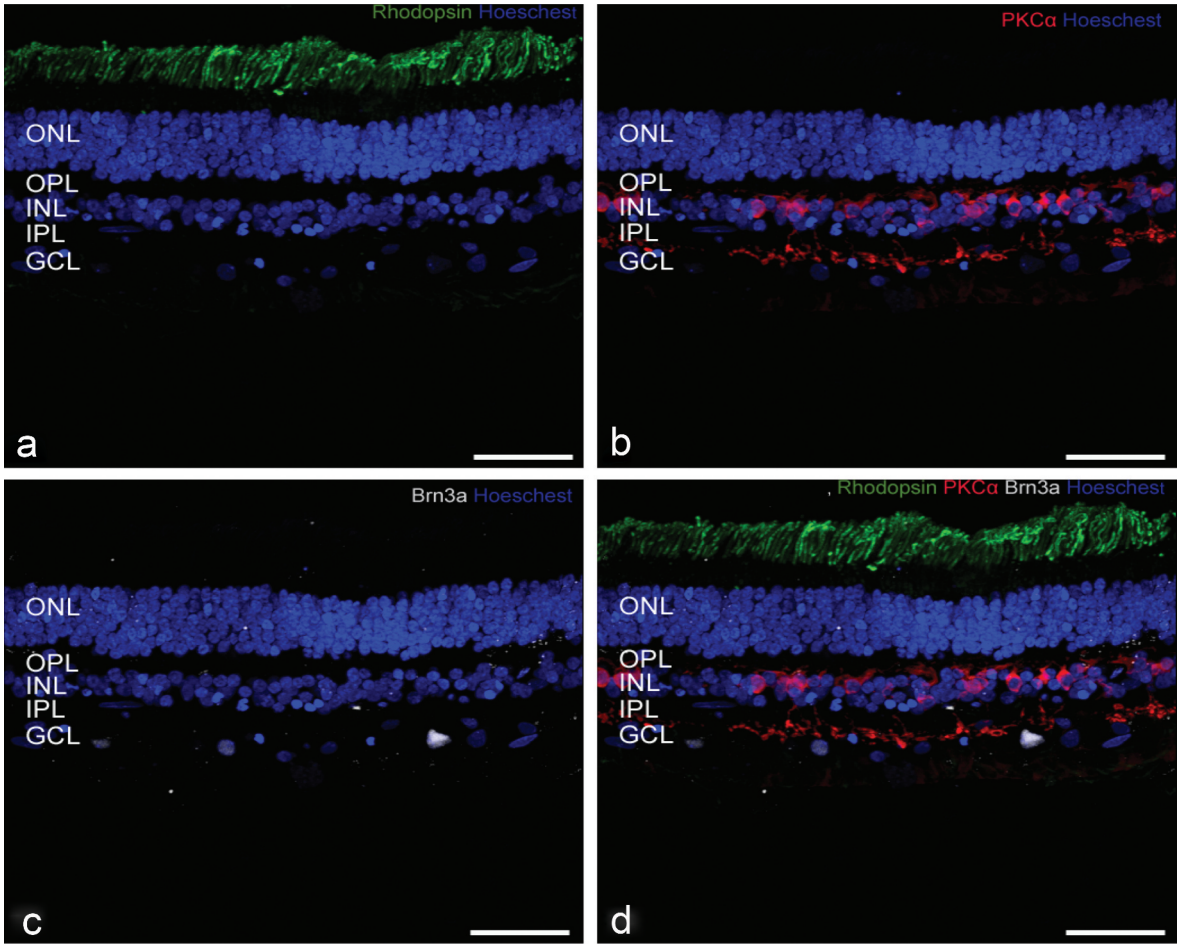

Figure 5. Immunofluorescence labelling in the human retina (×400). red: PKCα; green: Rhodopsin; grey: Brn3a; blue: Hoechst. (a) Rhodopsin labelling shows segments of the rod photoreceptors. (b) PKCα labelling shows multiple bipolar rods. (c) Brn3a labelling shows the nuclei of RGCs. (d) Merged image of Rhodopsin, PKCα, Brn3a and Hoechst staining. PKCα, protein kinase C-α; RGCs, retinal ganglion cells; Scale bar: 25 μm.

Table 2. Summary of the distribution of the antibodies and tissue reactivity.

Retinal layers	Thy1	GS	Brn3a	GFAP	Iba1	PKCα	Calretinin	Parvalbumin	Rhodopsin
Ganglion cell layer	+++	+++	+++	+++	+++	-	+	++	-
Inner plexiform layer	+	+++	-	+	+++	+++	++	-	-
Inner nuclear layer	+	+++	-	+	+	+++	+++	+	-
Outer plexiform layer	+	+++	-	-	+++	+	-	+++	-
Outer nuclear layer	+	+++	-	-	-	-	-	-	-
Photoreceptor layer	-	-	-	+	-	-	-	-	+++

+,weak immunoreactivity, the grey value is between 40–66; ++, moderate immunoreactivity, the grey value is between 66–75; +++,strong immunoreactivity, the grey value is >75; -,negative. Gray value is used to represent the fluorescence intensity. Gray value can be derived using Image J software. First, define 'set measurements' and then select 'Measure' from the analyse menu. GS = glutamine synthetase, GFAP = glial fibrillary acidic protein, Iba1 = ionized calcium-binding adapter molecule 1, PKCa = protein kinase C-α.

In this study, we labelled all cell types of the neuroretina. Each cell subtype had a different marker. We chose commonly used antibodies [4,11,12], such as Glutamine Synthetase, a specific marker of Müller cells [13], and PKCα, bipolar rod-specific marker [14]. Other antibodies can be used to label several cell types, such as parvalbumin, which is strongly expressed in horizontal cells and weakly expressed in amacrine cells and RGCs [11]. In this study, the results are basically consistent with previous reports. It has been reported that a few bipolar cells express calretinin [2], but in this study, we did not observe this result. Thy1, a cell surface glycoprotein expressed in mature RGCs [15]. Thy1 and Glutamine Synthetase were co-localized, indicating that Müller cells have the characteristics of neurons and may be transformed into neurons under certain conditions. Through the multiple labelling of human retinal cells, we can further understand the biological characteristics and functions of these cells, as well as provide clues and a theoretical basis for their possible role in diseases.

To perform triple-labelling immunohistochemistry, first, three primary antibodies must be obtained from three different hosts. Then, we tested each primary antibody individually to find the appropriate concentration. If all three primary antibodies labelled the same location, such as membrane protein or cytoplasmic protein, when incubating the primary antibody, a mixture of the primary antibodies could be made to add the antibodies at the same time. If three primary antibodies did not label the same location, we used the membrane protein antibody first and then the other antibodies. The antibody concentrations used were generally in accordance with the instructions. For most primary antibodies, 1:200 was the appropriate dilution. However, if some antibodies were incubated for a long time, such as Brn3a, the concentration (1:50) may need to be increased.

The study of human retinal substructures should provide insight into ophthalmic pathophysiology [16]. Our data represents an advance in biomedical science as it provides a reference point for the use of combinations of certain antibodies to study retinal structures using confocal microscopy.

Summary table

What is known about this subject:

- Single-antibody labelling of the human retina provides some limited information regarding the structure and composition of the various tissues.

What this paper adds:

- The panel of antibodies can differentiate different retinal layers.
- The morphology and location of different cells and the relationship between these cells provide clues and a theoretical basis for their possible role in diseases.

Disclosure statement

No potential conflict of interest was reported by the authors.

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