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3D-topography of cell nuclei in a vertebrate retina—A confocal and two-photon microscopic study

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ABSTRACT

We demonstrate methods to simultaneously acquire and evaluate the pattern of cell nuclei in the three cell layers of the vertebrate retina as an aspect of its functional morphology. 3D-position, shape and quantity of fluorescence-labelled cell nuclei are measured using laser scanning microscopy at several retinal locations, the pros and cons of single and two-photon excitation are compared. Subsequently topographies of all discriminable morphotypes are calculated via linear interpolation of local countings. In addition derived maps are calculated correlating density- and layer thickness-distributions to demonstrate the potential of 3D-morphometry in the retina. In the European anchovy *Engraulis encrasicolus* L. (Engraulididae, Teleostei) the angular density of all involved cell types varies considerably with the location in the hemispherical coordinate system. All cells belonging to the photopic system show a density peak in the ventro-temporal quadrant, suggesting acute vision in the frontal binocular visual field. A second, less pronounced maximum is found nasally.

1. Introduction

The vertebrate retina is a combined two-dimensional light sensor and imaging computer with several parallel detection channels and signalling pathways, transducing electromagnetic irradiation patterns from the environment into filtered neuroelectric excitation patterns within the animals' central nervous system. Retinal functions like acuity, sensitivity or contrast mechanisms rest on geometrically well ordered structures, a principle that legitimates functional morphology as one practicable way to learn about animal vision (see e.g. Ali, 1975; Lythgoe, 1979; Archer et al., 1999). Besides histology and fine-structure this is also true for the analysis of the three-dimensional arrangement of retinal cell nuclei. Whereas single cell somata hardly can be circumscribed due to tight packing, and dendrites as well as axonal terminals cannot be assigned to their cells in the cable mess of the plexiform layers without sophisticated staining techniques, the cell nuclei are neatly separated in the retina and easily analyzable with light microscopic resolution. Radially (i.e. normal to the retinal surface) they are sorted in three distinct nuclear layers (outer and inner nuclear layer, layer of G-cell nuclei) divided by two plexiform layers (Borwein, 1981; Dowling, 1987). Within a single nuclear layer they are separated from their neighbours by interspaces containing somatic cytoplasm and to some degree they are classifiable according to position, size, shape and internal structure. Thus the cell nuclei can be drawn on as proxies for the cells to analyze their sterical arrangement, the local densities and ratios of different cell types and their global density (and ratio) distributions.

Most studies dealing with the acquisition, analysis and interpretation of cell densities and/or density distributions (topography) of the vertebrate retina concentrate on cell types near the vitreal or scleral surface of retinal wholemounts. *Ganglion cell* distribution is generally determined by Nissl-staining (for teleosts see e.g. Collin and Pettigrew, 1988a,c; Cook and Becker, 1991; Douglas et al., 2002) and/or retrograde labelling via the optic nerve. Fluorescence labelling of G-cells is reported only in few topographic studies yet (teleosts: Douglas et al., 2002; pig: Garcia et al., 2005; human: Sjöstrand et al., 1994). *Cone photoreceptor* density was either investigated using wholemounts on the basis of DIC (human: Curcio et al., 1987; elasmobranchs: Litherland and Collin, 2008) or IHC (e.g. Kryger et al., 1998; Schivitz et al., 2008 for higher vertebrates) or using conventionally stained tangential sections (for teleosts see e.g.: O'Connell, 1963; Beaudet et al., 1997; Reckel et al., 2001; Miyazaki et al., 2002; Heß, 2009). Determination of *rod photoreceptor* densities is a difficult venture and can be achieved via counting nuclei (Zaunreiter et al., 1991) or inner segments in case of rod

Abbreviations: A, amacrine cell; B, bipolar cell; C, cone photoreceptor; CLSM, confocal laser scanning microscopy; DIC, differential interference contrast; G, ganglion cell; GL, ganglion cell layer; H, horizontal cell; IHC, immunohistochemistry; INL, inner nuclear layer; IPL, inner plexiform layer; IR, infra-red; ONL, outer nuclear layer; PE, pigment epithelium; R, rod photoreceptor; SNR, signal-to-noise-ratio; 2PM, two-photon microscopy.

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monolayers (Steinberg et al., 1973; Andrade da Costa and Hokoç, 2000; Litherland and Collin, 2008). The topographic distribution of rods has been demonstrated in few studies by mapping (e.g. Curcio et al., 1990; Chandler et al., 1999; Zhang et al., 2004) or by transects (Østerberg, 1935; Calderone et al., 2003). Despite the importance of *second order neurons* as determinants of visual performance only few quantitative and/or topographic studies on horizontal, bipolar and amacrine cells have been realized (e.g. O'Connell, 1963; Euler and Wässle, 1995; Wässle et al., 2000; Zhang et al., 2004).

To meet the 3D nature of the retina and to largely utilize the information potential of its complex nuclear pattern we looked for a resource-saving method to simultaneously record all cell types of a retinal fragment (within the microscopic field-of-view) and to get an overall picture of differentiated cell topographies and correlations by interpolation maps from multiple local measurements. Methodical difficulties arise from (1) the great imaging depth required for wholemounts or (2) alternatively the great effort for radial sectioning, (3) the dense packing of cell nuclei especially in the ONL, (4) the partially uncertain morphological classification of INL and GL-nuclei and (5) the dimension gap between nuclear size (defining resolution) and retinal size (defining tissue area/volume required to be investigated).

For the 3D-investigation of nuclear patterns in appropriate resolution it appears favourable to use optical sectioning microscopy of fluorescence-labelled nuclei in combination with computer aided evaluation of digital 3D-image data. The following advantages are obvious: (1) cell nuclei are easily accessible to morphometric studies, because they can be stained quantitatively and with a brilliant signal-to-noise-ratio via specific fluorescent dyes. (2) Optical sectioning microscopy is widely accessible for the acquisition of 3D-data and provides well aligned image stacks of relatively thick retinal fragments with a well-defined voxel size and small out-of-focus-blur required for three-dimensional counting of nuclei. (3) Software for the display and analysis of 3D-fluorescence-data is easily available or easy to programme.

In the phase of method optimization it is essential to find advantageous combinations of (1) the imaging principle (confocal or two-photon microscopy), (2) spectrally appropriate fluorescence dyes, (3) low-effort preparations methods (wholemount or slices), (4) the dimensions of the scanning window and (5) the density of measuring sites across the retina. In a first step the procedure should provide a set of digital 3D-data stacks containing nuclear images of all three layers with good signal-to-noise-ratio, in a resolution that allows the clear separation of densely packed rod nuclei and some classification of INL nuclei, with retinal coordinates as precise as possible and optimized for correct acquisition of topographic gradients with minimal total data volume. In a second step the display and evaluation of raw-data (3D arrays of grayscale values) and meta-data (nuclear positions, shapes, countings, maps) has to be effected that in addition opens up new possibilities for correlative analysis.

In this study, the methods were tested and adapted to the spatial dimensions and to the resolution required for the retina of the European anchovy *Engraulis encrasicolus*. This teleost species was chosen due to its extraordinary outer retinal structure characterized by an unusual fine-structure and arrangement of cone photoreceptors enabling polarization contrast vision (O'Connell, 1963; Fineran et al., 1976; Novales-Flamarique and Harosi, 2002; Heß et al., 2002, 2006). About 90% of the retinal area contains two cone types (long and short cones) alternately lined up in long chains ("polycones") with their outer segment lamellae arranged radially and orthogonally between neighbours. Another peculiarity is the fact that the anchovy's pigment epithelium is strongly geared with the neuroretina and cannot be detached without causing damage, thus DIC techniques cannot be applied for cell countings. Based on the nuclear distribution patterns obtained here, conclusions can be drawn about the visual field, some visual capabilities and neuronal wiring of European anchovies.

2. Materials and methods

2.1. Animals and fixation

Animals (European anchovy, *E. encrasicolus* (Linnaeus, 1758)) of about 12 cm standard length were obtained from fishermen (Rovinj, Croatia, North-Adriatic) arriving in the dawn two hours after taking their nocturnal catch aboard. The eyes (diameter about 7 mm) were enucleated from the head immediately, cornea, lens and vitreous removed and the remaining eye cups immersed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) and stored at 4 °C for several days.

2.2. Tissue preparation and fluorescence labelling

For fluorescence microscopy flat retina *wholemounts* were prepared by washing the eye cups in phosphate buffer, removing the sclera and cutting a number of incisions in the peripheral retina normal to the margin (Figs. 2A and 3A). The tissue was mounted on glass slides, G-cell layer up, cell nuclei stained with To-Pro-3 (molecular probes, 1:500) or DAPI (molecular probes, 1:1000) for several hours in the dark at room temperature, washed with buffer, embedded in anti-fading reagent (VectaShield, Vector Laboratories) and sealed under a cover slip with nail polish. To avoid squeezing, the cover slips were not applied directly onto the tissue, but spaced about 450 μm from the glass slide by stacks of 3 cover slip fragments on each side. The same stains were applied to *vibratome sections*: after embedding retinal fragments in 4% agarose, 75 μm sections were cut with a Leica VT1000S vibratome radially (i.e. planes normal to the retinal surface). Vibratome sections were mounted between glass slides and cover slips without spacers.

2.3. Confocal microscopy

In a first step a contiguous area of about 1250 μm × 2000 μm of the G-cell layer was scanned in the ventro-temporal quadrant of a DAPI-stained retinal wholemount (Fig. 1C). For this purpose slightly overlapping stacks (256 μm × 256 μm, pixel-size 0.5 μm, optical slice spacing 1.0 μm) were acquired with an upright confocal microscope (Leica SP5 AOBS) using a Leica HCX APO L U-V-I 40x long distance water dipping objective (NA = 0.8) and the 405 nm diode laser line. Brightest point projections (*XY* plane, Fig. 1A) of each stack were stitched horizontally to get a single widefield fluorescence picture of the G-cell layer near the area ventrotemporalis. Subsequently every nucleus (except some elongate nuclei, most likely from endothelial cells) in a rectangular field of 1330 μm × 1030 μm was tagged manually in Photoshop with a circular black dot to get clear and uniform abstractions (Fig. 1B) for the following automatic counting and mapping in IDL (Interactive Data Language 7.0, Research Systems, Inc.). The number of cell nuclei was calculated in any chosen area by dividing the total number of black pixels by the area of a single dot (69 pixels). To get a clue about an appropriate counting window size for a smooth and still correct display of cell-density gradients, square areas of 50, 70.7 and 100 μm edge length (2500, 5000 and 10000 μm^2) were tested in different ways: with the help of simple self-programmed IDL algorithms 1D-line profiles (Fig. 1D) and 2D-contour maps (Fig. 1E–J) were generated. The cell-density topography of the testing area was displayed either using "gliding" counting windows centred at every pixel of Fig. 1B subsequently (Fig. 1H), or by interpolating the density values of fixed neighbouring squares covering the testing area completely (Fig. 1E–G) or partially (Fig. 1I and J).